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Tolerogenic Dendritic Cells Induce CD4⁺CD25^{hi}Foxp3⁺ Regulatory T Cell Differentiation from CD4⁺CD25^{-lo}Foxp3⁻ Effector T Cells

Hui Huang,* Wojciech Dawicki,[†] Xiaobei Zhang,* Jennifer Town,* and John R. Gordon*[†]

IL-10–differentiated dendritic cells (DC10) induce allergen tolerance in asthmatic mice, during which their lung Th2 effector T cells (Teffs) are displaced by activated CD4⁺CD25^{hi}Foxp3⁺ T cells. Intestinal DCs promote oral tolerance by inducing Ag-naïve T cells to differentiate into CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), but whether DCs can induce Teffs to differentiate into Tregs remains uncertain. In this study, we addressed this question in OVA-asthmatic mice that were treated with DC10. OVA-presenting DC10 treatment maximally activated lung Tregs in these animals at 3 wk posttreatment, as determined by upregulation of activation markers (ICOS, programmed cell death-1, glucocorticoid-induced TNFR-related protein, LAG3, and CTLA-4) and in functional assays. This in vitro regulatory activity was ≥90% reduced by treatment with anti-IL-10 but not anti-TGF-β Abs. In parallel cultures, OVA- but not house dust mite (HDM)-presenting DC10 induced ≈43% of CFSE-labeled CD25^{-lo}Foxp3⁻ Teffs from asthmatic OVA-TCR transgenic mice to differentiate into tolerogenic CD25^{hi}Foxp3⁺ Tregs. We recapitulated this in vivo using OVA-asthmatic mice that were coinjected with OVA- or HDM-presenting DC10 (i.p.) and CFSE-labeled CD4⁺CD25^{-lo}Foxp3⁻ Teffs (i.v.) from the lungs of asthmatic DO11.10 mice. From ≈7 to 21% of the activated (i.e., dividing) DO11.10 Teffs that were recovered from the lungs, lung-draining lymph nodes, or spleens of the OVA-DC10 recipients had differentiated into CD4⁺CD25^{hi}Foxp3⁺ Tregs, whereas no CFSE-positive Tregs were recovered from the HDM-DC10-treated animals. These data indicate that DC10 treatments induce tolerance at least in part by inducing Teffs to differentiate into CD4⁺CD25^{hi}Foxp3⁺ Tregs. *The Journal of Immunology*, 2010, 185: 000–000.

Multiple laboratories have reported on the tolerogenic activities of IL-10–differentiated dendritic cells (DC10) in mouse models or ex vivo with human T cells (1–8). Thus, DC10 can protect against the development of OVA-induced asthma (4) or reverse the asthmatic phenotype in OVA (9) or house dust mite (HDM) allergen-sensitized mice, reducing airway hyperresponsiveness (AHR) to methacholine, eosinophilia, and Th2 responses to allergen challenge and circulating levels of allergen-specific IgE and IgG1. DC10-mediated asthma tolerance is allergen-specific (4, 8) and IL-10–dependent (4). In our hands, tolerance is first discernible at 2 wk following i.p. delivery of allergen-presenting DC10, and by 3 wk, the asthmatic animal's AHR disappears entirely. The Th2 reactivity of pulmonary T cells wanes progressively from 2 wk forward, such that by 8 mo, their responsiveness to recall allergen challenge in vivo is

near background (10). Four DC10 treatments at 2-wk intervals bring the asthma phenotype to near background within 8 wk (10). Studies employing DCs that have been transfected with an IL-10–expressing lentivirus, and which thus express exceptionally high levels of IL-10, indicated that endogenous IL-10 expression (e.g., by T cells) is also critical to asthma tolerance in that model (11). In inducing a robust asthma tolerance, treatments with these virally transfected DCs increase the numbers of CD4⁺CD25⁺Foxp3⁺ cells present in the lung-draining (mediastinal) lymph nodes (11). This occurs also in the lungs and mediastinal lymph nodes of DC10-treated HDM-asthmatic mice, and adoptive transfer of pulmonary CD4⁺ T cells from these mice into asthmatic recipients induces full HDM tolerance in the recipients (M. Lu, W. Dawicki, X. Zhang, H. Huang, and J.R. Gordon, submitted for publication).

Intestinal DCs that present innocuous environmental (e.g., commensal bacterial) Ags to naïve T cells in the mesenteric lymph nodes induce their differentiation to CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), and evidence indicates that expression of TGF-β is central to this process (12). Naïve T cells can be readily converted to a Treg phenotype by culture with either CTLA-4-Ig (13) or TGF-β (14–16), but there has been no hard data to date regarding the conversion of Ag-experienced (i.e., effector) T cells (Teffs). It has been reported that tuberculin purified protein derivative (PPD)-specific IFN-γ–producing CD4⁺ cell lines from PPD-sensitive donors become anergized poststimulation with immobilized anti-CD3 and begin to express high levels of Foxp3 mRNA (17), but Foxp3 expression by itself does not confer activated regulatory cell status on CD4⁺CD25⁺ T cells (18) (M. Lu et al., submitted for publication). Coculture of specific allergen-presenting DC10 from atopic asthmatic donors with autologous peripheral blood Th2 phenotype cells also induces allergen-

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; DC10, IL-10–differentiated dendritic cells; GITR, glucocorticoid-induced TNFR-related protein; HDM, house dust mite; MFI, median fluorescence intensity; Norm, normal; PD-1, programmed cell death-1; PPD, purified protein derivative; Teff, effector T cell; Treg, regulatory T cell.

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tolerance associated with the outgrowth of activated CD4⁺CD25⁺Foxp3⁺ Tregs (8). Nevertheless, whereas DC10 induce asthma tolerance through activation of Tregs, it is not known whether activated Teffs differentiate into Tregs in treated subjects. In this study, we examined the mechanisms by which DC10 induce Tregs in asthmatic mice. Our data indicate that DC10 do activate Tregs in the lungs of asthmatic mice and that these cells do differentiate from CD4⁺CD25⁻Foxp3⁻ Teffs.

Materials and Methods

Reagents and mice

PE-conjugated anti-mouse CD25, ICOS, programmed cell death-1 (PD-1), glucocorticoid-induced TNFR-related protein (GITR), LAG3, and CTLA-4 Ab and a mouse regulatory T cell staining kit were purchased from eBioscience (San Diego, CA). Mouse rGM-CSF and IL-10 and matched capture and detection Ab pairs and protein standards for our ELISAs were obtained from R&D Systems (Minneapolis, MN). Anti-mouse CD4 MACS beads and mouse CD4⁺CD25⁺ regulatory T cell isolation kits were purchased from Miltenyi Biotec (Auburn, CA). The lipid dye DiI was purchased from Molecular Probes (Carlsbad, CA). The sources of all other reagents have been reported previously (8, 19). Female BALB/c and C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Saint Constant, Quebec, Canada). DO11.10 OVA-specific TCR-transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice that expressed GFP under the control of the Foxp3 promoter were kindly provided through Dr. S. Rudensky (University of Washington, Seattle, WA) and bred in our institutional animal care unit. All mice were treated in accord with the guidelines of the Canadian Council for Animal Care.

Generation of tolerogenic DCs

Bone marrow DCs were generated largely as reported previously (20, 21), and differentiated to tolerogenic or immunostimulatory phenotypes by further culture with 50 ng/ml recombinant murine IL-10 (DC10) or 1 µg/ml *Escherichia coli* serotype 0127:B8 LPS (DC-LPS), respectively. The cells were pulsed with 50 µg/ml OVA or irrelevant control HDM allergen for 2 h, then washed extensively before use. In our hands, DC10 express low levels of costimulatory molecules (e.g., CD40, CD80) and MHC class II compared with mature DCs and secrete significantly higher levels of IL-10, but little TGF-β, relative to immature DCs (8, 10).

Establishment of the asthma mouse model and DC10 treatments

OVA-specific asthma was induced in BALB/c, C57BL/6, or OVA-TCR-transgenic DO11.10 or GFP-Foxp3-transgenic mice by sensitization with OVA-alum and airway exposure to nebulized aerosols of 1% OVA, as noted in detail previously (19, 22). The asthmatic mice were treated i.p. with 10⁶ allergen-presenting DC10 2 wk after their last airway exposure to allergen, also as noted previously (10).

Assessments of Treg activities

We used *in vitro* and *in vivo* approaches to assess the regulatory activities of CD4⁺CD25^{hi} T cells recovered from the enzymatically dispersed lung tissues (22) of asthmatic mice that had been treated with OVA-presenting DC10 1, 2, 3, or 4 wk earlier. The putative Tregs were purified by positive-selection magnetic sorting from these or untreated normal mice and assessed in functional assays for regulatory activities.

Flow cytometry for expression of activation markers. We purified T cells from the lungs of asthmatic GFP-Foxp3-transgenic mice that had been treated 3 wk earlier with 1 × 10⁶ OVA- or HDM-presenting DC10 (i.p.). The cells were stained with PE-Cy5-CD4 and PE-labeled isotype control or specific Ab against CD25, ICOS, PD-1, GITR, LAG3, or CTLA-4 (intracellular staining), then the CD4⁺ cells were gated and GFP⁺ (i.e., Foxp3⁺) cells were analyzed by FACS.

***In vitro* assessments.** CD4⁺ Th2 Teffs for the assays were magnetically sorted from the lungs of untreated asthmatic donor mice. In preliminary experiments done in 96-well plates, we titrated the numbers of Th2 cells and irradiated (3000 rad) OVA-pulsed DC-LPS required to optimally stimulate Th2 cell proliferation and Th2 cytokine secretion and the impact of similarly irradiated putative Tregs on this response. Thus, for the experiments reported in this study, we cocultured Th2 cells (10⁵ cells/well) with half-maximal numbers of OVA-presenting DC-LPS (3.7 × 10³

cells/well) and putative Tregs (10⁵ cells/well). In some assays, we added neutralizing anti-IL-10 or anti-TGF-β Ab (each 10 µg/ml) to the cultures. After 2 d, the CD4⁺ Th2 cells' proliferative and cytokine (IL-4, -5, -9, and -13 and IFN-γ) responses were assessed using standard [³H]thymidine uptake and ELISA assays, respectively.

***In vivo* assessments.** We injected 10⁶, 5 × 10⁵, 2.5 × 10⁵, or 1.25 × 10⁵ Treg i.v. into untreated asthmatic recipient mice and 4 wk later assessed the AHR of the mice. The following day, we challenged them for 20 min with aerosols of 1% nebulized OVA, and 2 d later, we euthanized them to assess their pulmonary immunoinflammatory responses as noted in detail (22). We did differential cell counts on their bronchoalveolar lavage (BAL) leukocytes and assessed the BAL fluid levels of IL-4, -5, -9, and -13.

Measurement of AHR

AHR was assessed in conscious animals by head-out, whole-body plethysmography, as noted in detail (19, 22). Briefly, air was supplied to the body compartment of a plethysmograph via a small animal ventilator, and changes in the airflow through the body compartment were monitored. Doubling doses of nebulized methacholine (0.75–25 mg/ml) were delivered to the head compartment, and bronchoconstriction data were gathered as running 1 s means of the airflow at the 50% point in the expiratory cycle (Flow@50%Tve1). This parameter accurately reflects bronchiolar constriction, as opposed to alveolar constriction or airway occlusion (23, 24).

ELISA for airway Th2 cytokines

Our capture cytokine ELISA protocols have been reported in detail previously (19, 22). BAL fluids were not diluted for the assays. Cytokine levels are presented as picograms per milliliter based on recombinant protein standard curves; all assays were sensitive to 5–10 pg/ml.

Assays for conversion of CD4⁺CD25⁻Foxp3⁻ Teffs to CD4⁺CD25^{hi} T cells

We assessed whether Tregs could differentiate from CD4⁺CD25⁻Foxp3⁻ Teffs both *in vitro* and *in vivo* using CD25⁻Foxp3⁻ cells purified by negative selection from the pulmonary CD4⁺ cells of asthmatic OVA-specific TCR-transgenic DO11.10 mice. These cells were stained with CFSE (2 µM; 20 min at 37°C).

***In vitro* assay.** We cocultured Teffs (10⁵/well) with OVA- or HDM-presenting (i.e., nonspecific allergen) DC10 or OVA-presenting immunostimulatory DC-LPS (3 × 10⁴/well) in culture medium that was supplemented with IL-2 (10 U/ml) as a growth factor. After 5 d, cells were fixed and permeabilized, then stained with PE-Cy5-labeled anti-Foxp3 Ab. For the FACS analysis, we gated on the CFSE-positive cells and assessed both proliferation and Foxp3 expression. We also assessed the *in vitro* regulatory activities of the cells arising in these cultures as noted above.

***In vivo* assay.** We injected 5 × 10⁶ CFSE-stained Teffs from asthmatic DO11.10 donors i.v. into asthmatic BALB/c recipient mice and at the same time treated the recipients with 1 × 10⁶ OVA- or HDM-presenting DC10 i.p. Two weeks later, single-cell suspensions generated from the lungs, mediastinal lymph nodes, and spleens of the recipients were stained with PE-Cy5 Foxp3 and PE-CD25 mAb and analyzed by FACS, wherein we gated on the activated (i.e., dividing) CFSE⁺ cells.

Statistics

All data are expressed as the mean ± SEM. Multigroup comparisons were assessed by ANOVA with post hoc Fisher's least significant difference testing, whereas AHR to methacholine was assessed by linear regression analyses. Significance was established at *p* ≤ 0.05.

Results

Pulmonary CD4⁺CD25^{hi}Foxp3⁺ cells become activated following tolerogenic DC treatment of asthmatic mice

Treatment of asthmatic mice with DC10 broadly ameliorates airway Th2 and eosinophil responses to allergen challenge within 4 wk of treatment (10). In both OVA and HDM models, tolerogenic DC treatments augment the regulatory activities of CD4⁺ T cells at this time (M. Lu et al., submitted for publication) (11). However, when we assessed the numbers of CD4⁺CD25^{hi}Foxp3⁺ cells in the lungs or lung draining (i.e., mediastinal) lymph nodes of saline- or DC10-treated OVA-asthmatic mice at 4 wk following

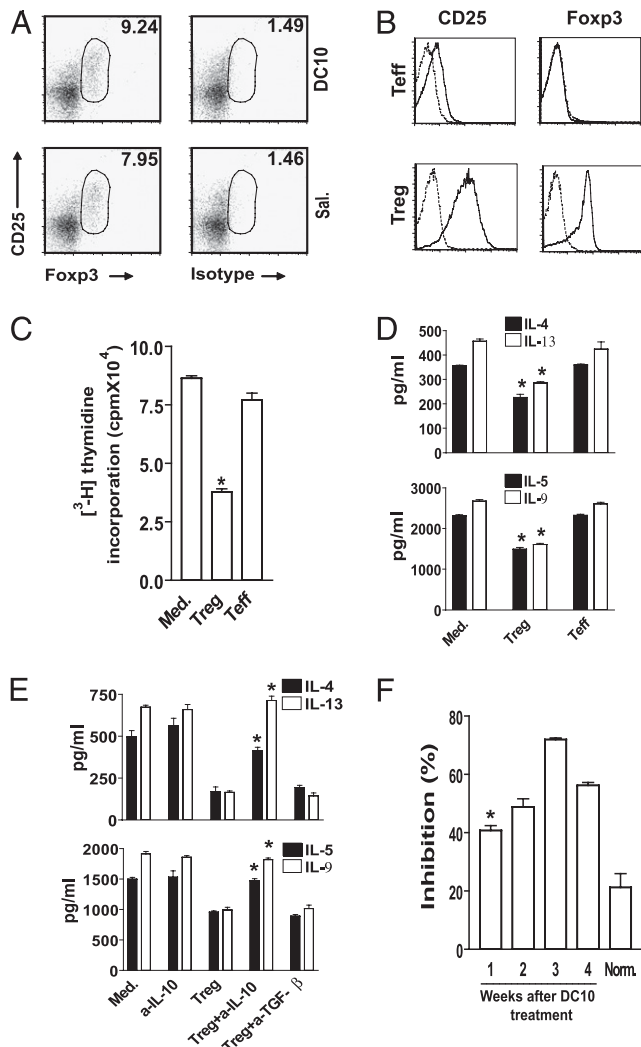


FIGURE 1. DC10 treatment of asthmatic mice increases the regulatory activity of their pulmonary $CD4^+CD25^{hi}$ cells. Asthmatic BALB/c mice were treated with 1×10^6 specific allergen-presenting DC10, and, at varying times thereafter, we magnetically sorted $CD4^+CD25^{hi}$ cells from their lungs. We assessed the abilities of these cells (10^5 cells/well) to regulate proliferation and cytokine expression by allergen-presenting immunostimulatory DC (DC-LPS)-activated Th2 Teffs (3.7×10^4 and 10^5 cells/well, respectively) from asthmatic donor mice. **A**, The lung $CD4^+$ T cells from DC10 treatment and saline control group mice were analyzed by FACS for expression of CD25 and Foxp3. **B**, FACS analysis of CD25 and Foxp3 expression by freshly purified Teffs and Tregs. **C**, $CD4^+$ Th2 cells from untreated asthmatic mice and irradiated DC-LPS were cultured alone (medium), with purified irradiated pulmonary $CD4^+CD25^{hi}$ (Treg), or with $CD4^+CD25^{-/lo}$ cells (Teff) from asthmatic mice that had been treated 4 wk earlier with DC10. After 48 h, we assessed the asthmatic Th2 cell proliferative responses by [^3H]thymidine uptake assay as well as the levels of IL-4, -5, -9, and -13 in the culture supernatants by ELISA. $*p < 0.05$ versus Teff group; $n = 3$ (**D**). **E**, We also added 10 $\mu g/ml$ of neutralizing anti-IL-10 to the Th2 effector/DC-LPS cultures (a-IL-10) or the Treg/Th2 effector/DC-LPS cocultures (Treg + a-IL-10), or anti-TGF- β to the Treg/Th2 effector/DC-LPS cultures (Treg + a-TGF- β), to assess the contributions of these mediators to the putative $CD4^+CD25^{hi}$ T cell regulatory functions. $*p < 0.05$ versus the Treg group; $n = 3$. **F**, To determine whether the kinetics of Treg activation in the lungs of the DC10-treated mice matched the in vivo observation of greater tolerance at 4 wk (versus 2 or 3 wk) posttreatment, we harvested $CD4^+CD25^{hi}$ cells from mice at 1, 2, 3, or 4 wk after DC10 treatment and assessed their regulatory activities. The putative regulatory cells we isolated from DC10-treated asthmatic mice were $\approx 70\%$ $CD25^{hi}Foxp3^+$, whereas the $CD4^+CD25^{-/lo}$ cells were $< 5\%$ positive for these markers. These cells displayed significantly higher

DC10 delivery, we found no differences in the proportions of these cells in the two groups (Fig. 1A). Thus, $CD4^+CD25^{hi}Foxp3^+$ cells comprised a mean of $7.07 \pm 0.88\%$ and $8.24 \pm 1.41\%$ of the pulmonary $CD4^+$ T cells in the saline- and DC10-treated animals, respectively, and they comprised 2.62 and 2.54% of the mediastinal lymph node $CD4^+$ T cells in these respective groups. Others have similarly reported equivalent proportions of $CD4^+CD25^{hi}Foxp3^+$ cells in tolerant and nontolerant animals in other model systems (18).

We used a Treg magnetic-sorting kit to purify these cells and Teffs from the lungs of asthmatic mice that had been treated 4 wk earlier with OVA-presenting DC10. The Tregs expressed high levels of both CD25 and Foxp3, whereas the magnetic column Teff flow-through population expressed low levels of CD25 and no discernible Foxp3. The median fluorescence intensity (MFI) of CD25 on the Teffs was 5.41 (isotype control MFI, 2.51), whereas the MFI of CD25 on the Tregs was 15.8 (isotype control MFI, 3.34; Fig. 1B). We next assessed the respective abilities of the purified Tregs and Teffs from DC10-treated asthmatic mice to suppress the activation of pulmonary Teffs from untreated asthmatic donors by OVA-presenting immunostimulatory DCs (DC-LPS). The $CD4^+CD25^{hi}$ T cells from the lungs of the DC10-treated mice reduced proliferation of these Teffs by $53.8 \pm 11.8\%$ (Fig. 1C) and reduced their expression of Th2 cytokines by 35–40%, whereas the Teffs from DC10-treated mice had no discernible impact in these assays (Fig. 1D). To determine whether the regulatory activity of these cells was dependent on their expression of IL-10 or TGF- β , we also tested the impact on this Th2-proliferative response of neutralizing Abs. Anti-TGF- β Ab had no effect on T cell proliferation, but the anti-IL-10 Abs eliminated the tolerogenic activities of the $CD4^+CD25^{hi}Foxp3^+$ cells from the DC10-treated asthmatic mice (Fig. 1E). The anti-IL-10 Abs had no impact on the Th2 cytokine responses of Teffs from asthmatic mice (Fig. 1E, a-IL-10), suggesting that these cells were not the source of the IL-10 in the Treg/Teff cocultures.

We next examined the kinetics of pulmonary Treg induction after DC10 treatment, purifying $CD4^+CD25^{hi}Foxp3^+$ cells from the lungs of normal mice or asthmatic mice that had been treated with DC10 1–4 wk earlier. We had previously found that DiI-stained DC10 traffic from the peritoneal cavities of recipient mice to the airways and lung-draining (mediastinal) lymph nodes (M. Lu, H. Huang, and J.R. Gordon, unpublished observations). Maximal accumulation of these cells in the mediastinal nodes occurs at 3 wk postimplantation, when up to 15% of the cells recovered from the nodes were signal-positive (H. Huang and J.R. Gordon, unpublished observation). In the current study, we found that $CD4^+CD25^{hi}$ T cells from the lungs of normal mice had modest regulatory activity in our in vitro assay, whereas pulmonary $CD4^+CD25^{hi}$ T cells from the DC10-treated asthma phenotype mice were significantly more active (Fig. 1F). At 1 wk after DC10 delivery, there was a ≈ 2 -fold increase in regulatory activity relative to the $CD4^+CD25^{hi}$ T cells from normal mice, and this activity continued to increase to a maximum ($\approx 72\%$ inhibition) at 3

regulatory activity relative to $CD25^{hi}Foxp3^+$ cells of normal (Norm) mice ($p < 0.05$; $n = 5$), inasmuch as they, but not $CD4^+CD25^{-/lo}$ cells, reduced expression of each Th2 cytokine in the cultures. Anti-IL-10 Abs eliminated the regulatory activity of the $CD25^{hi}Foxp3^+$ cells, whereas the anti-TGF- β Abs had no impact. Peak activation of these regulatory cells in DC10-treated mice occurred at 3 wk posttreatment. The data in A, B, and E are representative of three experiments, whereas those in C, D, and F are representative of two experiments. $*p < 0.05$.

wk. Interestingly, despite the fact that tolerance in asthmatic mice is progressive well beyond 4 wk (9), the activity of the purified 4-wk Tregs was diminished relative to that of 3-wk Tregs (Fig. 1F).

To further assess the activation of Tregs in our model, we used asthmatic mice that expressed GFP under the control of the Foxp3 promoter as a means of easily tracking Tregs. Three weeks after treating these mice with OVA- or irrelevant allergen (i.e., HDM)-presenting DC10 or OVA-presenting GM-CSF-differentiated DC (DC-OVA; as a negative control population), we examined the expression of a panel of regulatory cell surface markers (i.e., ICOS, PD-1, GITR, and LAG3) as well as intracellular CTLA-4 on Tregs recovered from their lungs. We found that the OVA-pulsed DC10 treatments led to increased expression of a number of these (Fig. 2). Thus, the proportions of Foxp3⁺ cells expressing ICOS, PD-1, GITR, LAG3, and CTLA4 were increased ~2–10-fold relative to the analogous cells from HDM-presenting DC10-treated or DC-OVA-treated (i.e., nontolerant) asthmatic mice. In keeping with our observation of no differences in the numbers of CD4⁺CD25^{hi} cells in normal, asthmatic, or tolerant mice (Fig. 1A), we did not observe any differences in CD25 expression in these mice.

Passive transfer of tolerance with DC10-induced pulmonary CD4⁺CD25^{hi}Foxp3⁺ cells

We next sought confirmation that the CD4⁺CD25^{hi}Foxp3⁺ cells from the lungs of DC10-treated mice could operate as regulatory cells *in vivo*. We titrated the numbers of Tregs required to transfer tolerance, giving asthmatic mice either saline or $1.25\text{--}10 \times 10^5$ CD4⁺CD25^{hi} cells (i.v.) from mice treated 3 wk earlier with DC10. Given our demonstration that Tregs from asthmatic animals possess only ~30% of the regulatory activity of DC10-activated Tregs, we did not also titrate these cells or Tregs from the lungs of control DC-treated asthmatic mice. We assessed AHR in the recipients weekly thereafter, then administered a recall allergen challenge (20 min of nebulized 1% OVA aerosol) on day 28 and sacrificed the animals 2 d later. We assessed airway eosinophilic inflammation and Th2 cytokine responses, as determined by BAL fluid IL-4, -5, -9, and -13 levels. The AHR of the animals given 10^6 DC10-induced Tregs was fully normalized by 21 d post-transfer (Supplemental Fig. 1) and remained so at 4 wk (Fig. 3A). When we transferred 5×10^5 3 wk Tregs, AHR was not discernibly affected at 2 or 3 wk (Supplemental Fig. 1), but by 4 wk, it was significantly reduced ($p < 0.05$ versus saline-treated asthmatic mice). Lower numbers of Tregs were without effect

on AHR, at least as of 4 wk posttransfer (Fig. 3A). Passive transfer of Tregs also reduced airway eosinophilia and Th2 cytokine levels in a dose-dependent manner. Specifically, either 5×10^5 or 10^6 cells reduced the eosinophil responses to background, whereas 2.5×10^5 or fewer cells were without effect (Fig. 3B; $p > 0.05$ versus saline-treated asthmatic mice). In accord with this, Th2 responses were also amenable to Treg tolerance, such that delivery of 10^6 cells reduced the Th2 response to near background and 5×10^5 Tregs reduced them very markedly ($p < 0.05$, versus saline-treated asthmatic animals), whereas transfer of fewer cells was without discernible effect (Fig. 3C).

For a more finely tuned *in vivo* confirmation of the kinetics for DC10-driven pulmonary Treg induction, we transferred a limiting number (5×10^5) of pulmonary Tregs from mice treated 1–4 wk earlier with DC10 and again assessed their impact on AHR and on Th2 cytokine and eosinophilic inflammatory responses to airway recall allergen challenge (Supplemental Fig. 2). When used at these limiting numbers, only the cells from animals treated 3 wk earlier with DC10 were sufficiently activated to alter AHR or eosinophilia (for both, $p < 0.05$ versus saline-treated asthmatic recipients) in the asthmatic recipients. The airway Th2 responses were slightly more amenable to regulation, such that 5×10^5 Tregs from mice treated with DC10 either 3 or 4 wk earlier significantly affected airway cytokine levels ($p < 0.05$ for each cytokine versus saline-treated asthmatic recipients). The 1- and 2-wk cells were ineffective in reducing airway Th2 cytokine expression ($p > 0.05$ for each cytokine).

DC10 induce the differentiation of CD4⁺CD25^{−/lo}Foxp3[−] Teffs into CD4⁺CD25^{hi}Foxp3⁺ Tregs

It has been reported that naive T cells are readily converted to a regulatory phenotype when costimulated with CTLA-4-Ig (13) or TGF- β (14–16). Our DC10 do not express significant levels of TGF- β relative to either immature or TNF-treated *in vitro*-differentiated DCs (10), but our data indicate that they are clearly tolerogenic in asthmatic animals, wherein they appear to fully reverse Th2 T cell responses. Thus, we questioned whether they induce CD25^{−/lo}Foxp3[−] Teffs to differentiate into Tregs. It has been shown that Teff cell lines generated from tuberculin PPD-challenge skin sites can be anergized *ex vivo* and that Foxp3 expression is upregulated in concert with this induction of anergy (17), but, as far as we are aware, there have been no reports demonstrating that Tregs can differentiate from Ag-experienced

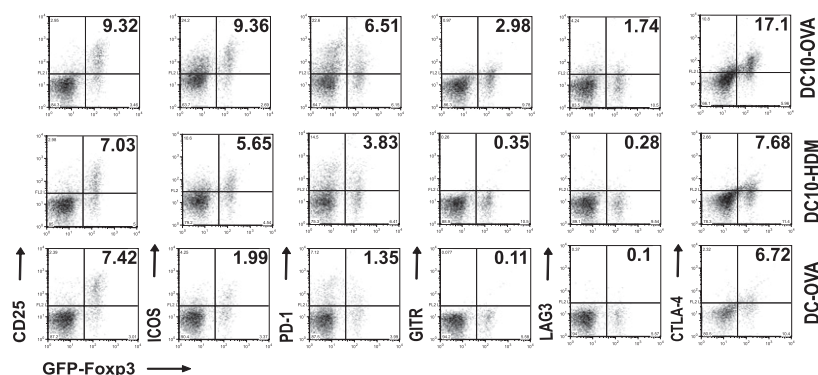


FIGURE 2. CD4⁺CD25^{hi} cells from the lungs of DC10-treated asthmatic animals express augmented levels of Treg activation markers. OVA-asthma was induced in GFP-Foxp3-transgenic mice as noted for BALB/c mice in Fig. 1. These mice were then treated with either OVA- or irrelevant allergen (HDM)-presenting DC10 (DC10-OVA or DC10-HDM, respectively) or nontolerogenic DC-OVA, then at 3 wk posttreatment, we purified T cells from the lungs of each mouse and stained them with PE-Cy5-CD4 and PE-labeled Abs against the Treg activation markers ICOS, PD-1, GITR, LAG3 or CTLA-4. It is readily apparent that the OVA-presenting DC10 upregulated expression of ICOS, PD1, GITR, and LAG3 on pulmonary CD4⁺CD25^{hi} cells relative to their expression on CD4⁺CD25^{hi} cells from the lungs of HDM-presenting DC10-treated or DC-OVA-treated mice. One representative experiment of three is shown.

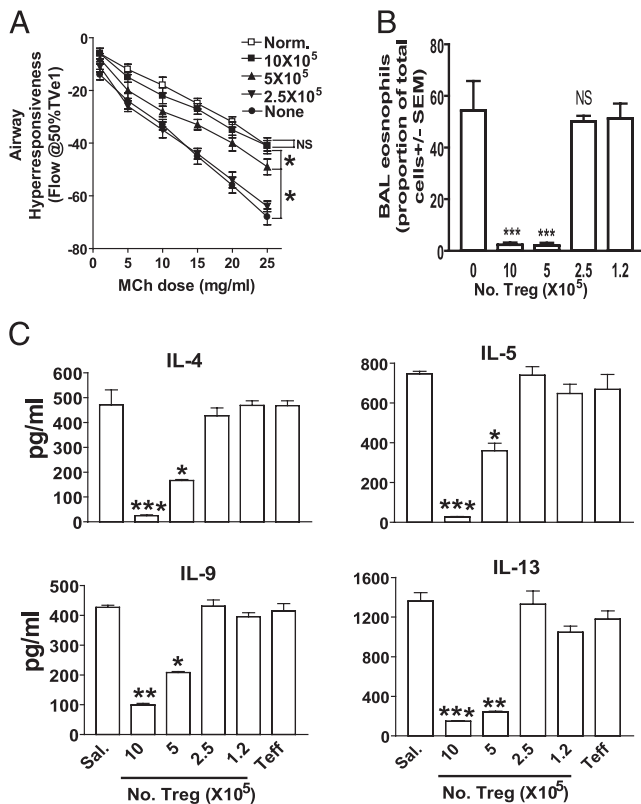


FIGURE 3. Passive transfer of CD4⁺CD25^{hi} Tregs from DC10-treated animals reverses the asthma phenotype in asthmatic recipient mice. CD4⁺CD25^{hi} cells were purified from the lungs of asthmatic mice ($n = 5$ /group) treated 3 wk earlier with DC10, as in Fig. 1. We passively transferred 10^6 , 5×10^5 , 2.5×10^5 , or 1.2×10^5 of these Tregs into asthmatic recipients (i.v.), and 25 d later, assessed their airway hyperresponsiveness (AHR) to aerosolized methacholine (A). The following day, we challenged the animals for 20 min with nebulized aerosols of 1% OVA, and 48 h later, assessed their pulmonary immunoinflammatory responses. The 10×10^5 Treg treatments nearly normalized AHR (relative to the normal saline controls) at 4 wk posttreatment. The AHR in the 5×10^5 Treg-treated mice was significantly different relative to the asthma phenotype mice ($p < 0.05$). Eosinophil (B) and Th2 (C) cytokine responses to the recall allergen challenge. We observed reductions in each asthma parameter assessed based on the numbers of Tregs passively transferred, including each Th2 cytokine, but transfer of $\leq 2.5 \times 10^5$ Tregs had no discernible impact on the asthma phenotype of the recipients. One representative experiment of two is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Teffs. To test this directly, we set up an in vitro culture system in which CD4⁺CD25^{-lo}Foxp3⁻ Teffs purified from the lungs of asthmatic OVA-TCR-transgenic DO11.10 mice were stained with CFSE and cocultured with specific (OVA) or irrelevant allergen (HDM)-presenting DC10 or OVA-presenting immunostimulatory cells (DC-LPS). After 5 d, we analyzed the CFSE⁺ cells from these cultures to assess their proliferation (CFSE dilution) and expression of Foxp3 (Fig. 4A), but we also used magnetic sorting to purify the CD25^{hi} cells that were induced in these cultures and titrated their regulatory activities (Fig. 4B). In the cultures containing irrelevant allergen-presenting DC10, we observed little if any proliferation of the Teffs and no expression of Foxp3 by the CFSE-labeled cells, and this makes sense based on the known allergen specificity of DC10-induced tolerance (4, 8). The DC-LPS strongly induced Teff cell proliferation but not Foxp3 expression, but the bulk of the CD4⁺CD25^{-lo}Foxp3⁻ cells in the OVA-presenting DC10 cocultures had proliferated, and ~45% of them expressed Foxp3 at high levels (Fig. 4A). When we

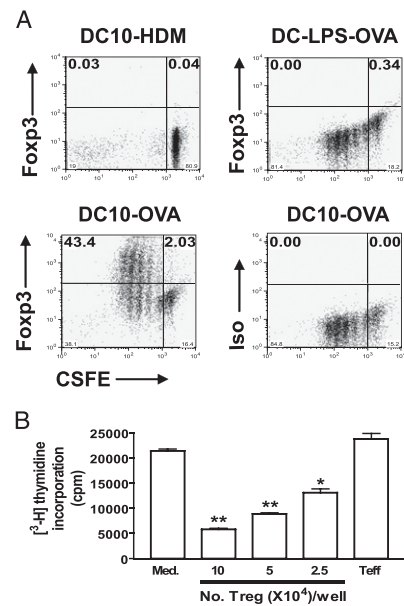


FIGURE 4. Specific, but not irrelevant, allergen-presenting DC10 induce the differentiation of CD4⁺CD25^{-lo}Foxp3⁻ Teffs from asthmatic donors into CD4⁺Foxp3⁺ Tregs in vitro. OVA-asthma was induced in OVA-TCR-transgenic DO11.10 mice as noted above, then we purified CD4⁺CD25^{-lo}Foxp3⁻ Teffs from the lungs of these mice as in Fig. 1. A, The Teffs were stained with CFSE and cocultured with specific (OVA)- or irrelevant (HDM) allergen-presenting DC10 or OVA-presenting DC-LPS (10^5 Teffs + 3×10^4 DC/well); $10 \mu\text{g/ml}$ IL-2 was added to the cultures as a growth factor. Five days later, the cells in the cultures were analyzed by FACS for Teff proliferation (CFSE-dilution) and Foxp3 expression. B, To confirm that the induced CD4⁺CD25^{hi}Foxp3⁺ were functional as regulatory cells, we used magnetic sorting to purify the CD25^{hi} T cells from 5-d cocultures of asthmatic DO11.10 mouse pulmonary Teffs and OVA-DC10 or OVA-DC-LPS. These induced DO11.10 CD4⁺CD25^{hi} Tregs (or control Teffs) were then irradiated (3000 rad) and added to cultures of OVA-presenting DC-LPS + Th2 Teffs from asthmatic C57BL/6 mice. Th2 Teff proliferative responses were assessed as in Fig. 1. One representative experiment of two is shown. * $p < 0.05$; ** $p < 0.01$ versus the Teff group ($n = 4$).

magnetically sorted the induced CD25^{hi} cells back out of these cultures and titrated their activity in vitro, we found them to be highly effective in dampening DC-LPS-induced Teff cell proliferation (Fig. 4B; $p \leq 0.05$ versus CD25^{hi} T cells sorted from DC-LPS/Teff cell cultures). These data confirmed that DC10 are fully capable of efficiently inducing CD4⁺CD25^{-lo}Foxp3⁻ Teffs from asthmatic mice to differentiate into functional regulatory cells in vitro and that this response is fully dependent on cognate allergen presentation.

To confirm the in vivo relevance of this observation, we also assessed whether delivery of DC10 to asthmatic mice would similarly induce Teffs to differentiate into CD25^{hi}Foxp3⁺ Tregs. We magnetically purified CD4⁺CD25^{-lo}Foxp3⁻ Teffs from OVA-asthmatic DO11.10 mice, labeled them with CFSE, and injected them i.v. into asthmatic recipients. At the same time, we injected the recipients i.p. with either OVA- or HDM-presenting wild-type DC10. Two weeks later, we generated single-cell suspensions from the lungs, mediastinal lymph nodes, and spleens of the treated mice, stained the cells with anti-Foxp3 and anti-CD25 Abs, and analyzed the cells by FACS, gating on the dividing (i.e., activated) CFSE⁺ cells (Fig. 5). We found negligible numbers of CFSE⁺CD25^{hi}Foxp3⁺ T cells in the lungs (0.21%), lung-draining lymph nodes (0.64%), or spleens (0.43%) of the animals we had treated with HDM-presenting DC10, but there were significant numbers of activated CFSE⁺CD25^{hi}Foxp3⁺ cells in the asthmatic mice that had

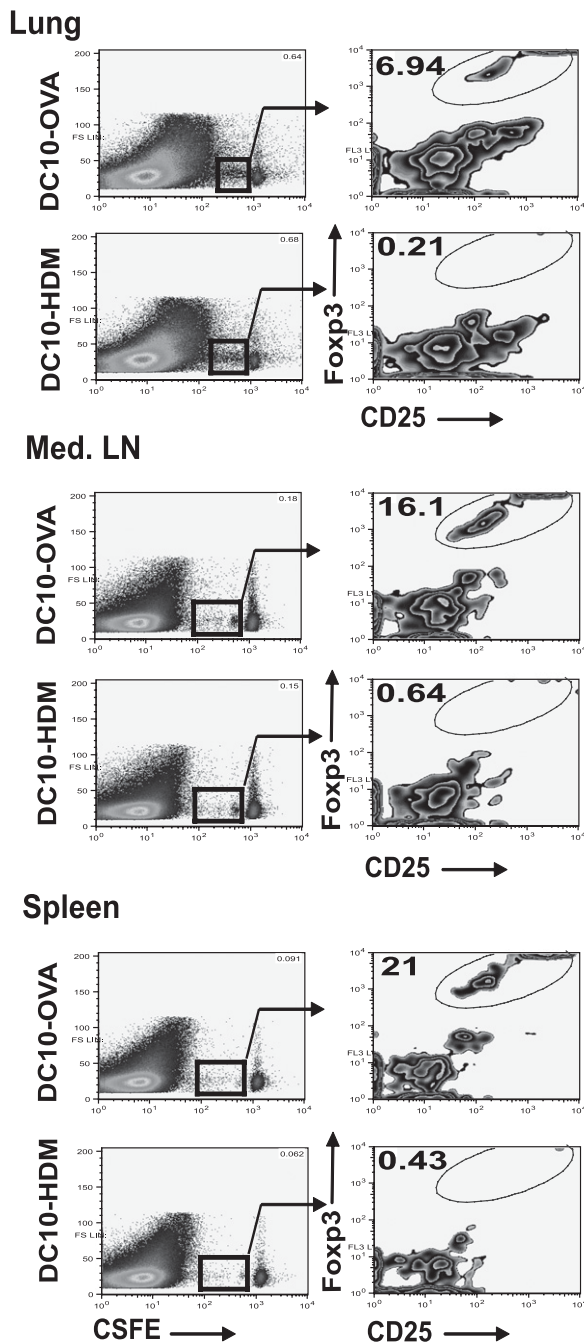


FIGURE 5. DC10-induced differentiation of $CD4^+CD25^{hi}Foxp3^+$ Tregs from $CD4^+CD25^{-/-}Foxp3^-$ Teffs in vivo. To confirm the in vivo relevance of DC10-driven conversion of $CD4^+CD25^{-/-}Foxp3^-$ Teffs into regulatory cells, we injected (i.v.) 5×10^6 CFSE-labeled $CD4^+CD25^{-/-}Foxp3^-$ T cells from OVA-asthmatic DO11.10 mice and 10^6 OVA- or HDM-presenting DC10 from wild-type BALB/c mice (i.p.) into asthmatic BALB/c recipients. After 2 wk, we prepared single-cell suspensions from the lungs (upper panels), mediastinal lymph nodes (middle panels), and spleens (lower panels), stained the cells for Foxp3 and CD25, and gated on the activated (i.e., dividing) CFSE⁺ cells (left panels, boxed cells) to assess the expression of these markers by FACS. There was no conversion of CFSE-labeled $CD4^+CD25^{-/-}Foxp3^-$ Teffs into $CD25^{hi}Foxp3^+$ Tregs in the mice treated with HDM-presenting DC10, whereas there was substantial differentiation of Tregs from the Teffs in the OVA-presenting DC10-treated animals. One representative experiment of two is shown.

been treated with specific allergen-presenting DC10. We found that 7, 16, and 21% of the proliferating CFSE⁺ cells recovered from the lungs, mediastinal lymph nodes, and spleens, respectively, were

now $CD25^{hi}Foxp3^+$ (Fig. 5). Taken together, our in vitro and in vivo data indicated that during DC10-mediated induction of allergen-tolerance in asthmatic mice, $CD4^+CD25^{-/-}Foxp3^-$ Teffs do differentiate into $CD4^+CD25^{hi}Foxp3^+$ Tregs.

Discussion

Multiple laboratories have reported that $CD4^+$ cells take on regulatory activities with tolerance induction in asthma (M. Lu et al., submitted for publication) (8, 11, 18), but the mechanisms by which this occurs had not been defined. We documented in this study that in inducing tolerance in asthmatic mice, DC10 also induce $CD4^+CD25^{hi}Foxp3^+$ Tregs. The kinetics with which these cells were activated in the lung correlated very well with the acquisition of asthma tolerance in mice models (M. Lu et al., submitted for publication) (10). Nevertheless, whereas peak Treg activation occurred at 3 wk after the DC10 treatment, we have found that tolerance induced by a single DC10 treatment is progressive over many months in our mouse model (10). This suggests that alternate mechanisms supplant the pulmonary Treg-dependent tolerance that sets in over the first few weeks following DC10 treatment, perhaps in the context of infectious processes that incorporate regulatory DCs and/or alternate Treg populations (25). For example, Tregs can induce myeloid DCs to adopt a regulatory phenotype (26, 27), whereas DCs can reciprocally express substantial control over Treg populations (28).

It had been shown previously that gut lamina propria or mesenteric lymph node, but not splenic, DCs can induce naive T cells to differentiate to a regulatory phenotype without need for exogenous input (i.e., TGF- β) (29), and this would be appropriate in a compartment routinely presented with commensal (i.e., nonpathogenic) bacteria (30). In the lungs, which are also under constant exposure to innocuous foreign Ags (e.g., pollens), pulmonary DCs that present such Ags to naive T cells express a semimature phenotype and IL-10 and thereby induce Treg responses that prevent development of pathogenic (e.g., allergic) responses (31). The fact that we generated Teff pools from the lungs of fully symptomatic asthmatic mice indicates that these cells would have been educated Teffs as opposed to naive T cells. Thus, our data showing that DC10 induced the differentiation of these Teffs into $CD4^+CD25^{hi}Foxp3^+$ Tregs provides our first clear documentation that Teffs are amenable to such a phenotypic change. We have shown previously that specific allergen-presenting DC10 generated from $CD14^+$ monocytes of asthmatic individuals similarly induce the outgrowth of $CD25^+Foxp3^+LAG3^+CTLA-4^+$ Tregs from autologous Th2 Teff populations. Those Tregs subsequently suppressed autologous Teff responses in a contact-dependent manner (8), as reportedly occurs with Tregs in other model systems (32, 33). However, in the mouse model we employed in this study, our DC10-induced regulatory activity was dependent on expression of IL-10 by the T cells, a characteristic consistent with a Tr1-like phenotype regulatory cell. Others have reported that IL-10-treated DCs induce anergy among Ag-specific T cells in part via CTLA-4 (34). Nevertheless, IL-10 expression by both the treatment DC10 (4) and endogenous host cells (11) has been implicated in tolerance induction in asthmatic mice.

Interestingly, although ectopic expression of Foxp3 alone is reportedly sufficient to turn Teffs into Tregs (35–38), there were equivalent numbers of $CD4^+CD25^{hi}Foxp3^+$ cells in the lungs of asthmatic mice irrespective of whether they were fully asthmatic or their pulmonary $CD4^+CD25^{hi}Foxp3^+$ cells were activated and expressed a regulatory phenotype. And, although DC10 induce Th2 Teffs from atopic asthmatic individuals to take on an activated Treg phenotype (i.e., $CD4^+CD25^+Foxp3^+LAG3^+CTLA-4^+$ IL-10-secreting T cells), the cells in these cultures do not express

increased levels of Foxp3 relative to those in cultures containing immunostimulatory DC-activated Tregs (8). A similar observation has been made with CD4⁺CD25^{hi}Foxp3⁺ cells from asthmatic rats versus those rendered allergen-tolerant by chronic airway exposure to allergen (18). This provides further evidence that Foxp3 expression by itself is not sufficient for optimal induction of a regulatory phenotype in CD4⁺CD25^{hi} cells. The fact that the numbers of pulmonary CD4⁺CD25^{hi}Foxp3⁺ cells were equivalent in our asthmatic and tolerant animals, despite the observation that sizable numbers of Tregs had ostensibly converted to activated Tregs in the latter group, suggests that Treg homeostatic control mechanisms (39) were operative in their lungs. It is recognized that DCs and Treg populations control one another in a reciprocal homeostatic fashion (28), and this raises the question of whether the tolerogenic DCs we introduced into asthmatic animals may exercise homeostatic control over lung Treg numbers. Perhaps the CD4⁺CD25^{hi}Foxp3⁺ cells that were present in the asthmatic lung prior to tolerance induction represent a subpopulation of cells that are uniquely susceptible to apoptotic (40) or other control mechanisms.

Tregs play important roles in maintaining the balance between protective and pathogenic immune responses (18, 41). To date, both naturally occurring thymic CD4⁺CD25⁺ Tregs and inducible Tregs have been recognized, with the latter cells including IL-10- and TGF- β -secreting Tr1 and Th3 cells, respectively (42). Naturally occurring Tregs constitute 1–5% of the CD4⁺ T cells in healthy adult mice and humans, but regulatory cells with similar surface markers and functions can also be induced in the periphery. These cells can be isolated from mice and humans based on their high-level expression of CD25 (the IL-2R α -chain). Other markers that were originally thought to be specific for naturally occurring Tregs include CTLA-4 and GITR but, like CD25, they are also expressed by activated T cells. Our data indicated that DC10 increased the expression of ICOS, PD-1, GITR, LAG3, and CTLA-4 with the acquisition of the regulatory phenotype, and this fits well with observations by others (43).

In summary, our data support the observation that these cells induced CD4⁺CD25^{lo}Foxp3⁺ Tregs to differentiate into CD4⁺CD25^{hi}Foxp3⁺ Tregs. We did not assess whether they also directly activated or induced proliferation of pre-existing CD4⁺CD25^{hi}Foxp3⁺ T cells, but these cells can indeed proliferate strongly and particularly so under the influence of IL-2 (44). We also did not rigorously investigate the cellular interactions between the DC10 and Tregs in asthmatic animals, but we know that these populations do engage one another intimately and in an Ag-specific fashion (M. Lu et al., submitted for publication) (8). These will be important issues to address in the future.

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Disclosures

The authors have no financial conflicts of interest.

References

- Müller, G., A. Müller, T. Tüting, K. Steinbrink, J. Saloga, C. Szalaga, J. Knop, and A. H. Enk. 2002. Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells in vivo. *J. Invest. Dermatol.* 119: 836–841.
- Bellinghausen, I., U. Brand, K. Steinbrink, A. H. Enk, J. Knop, and J. Saloga. 2001. Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J. Allergy Clin. Immunol.* 108: 242–249.
- Steinbrink, K., M. Wölfl, H. Jonuleit, J. Knop, and A. H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* 159: 4772–4780.
- Koya, T., H. Matsuda, K. Takeda, S. Matsubara, N. Miyahara, A. Balhorn, A. Dakhama, and E. W. Gelfand. 2007. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *J. Allergy Clin. Immunol.* 119: 1241–1250.
- Lau, A. W., S. Biester, R. J. Cornall, and J. V. Forrester. 2008. Lipopolysaccharide-activated IL-10-secreting dendritic cells suppress experimental autoimmune uveoretinitis by MHCII-dependent activation of CD62L-expressing regulatory T cells. *J. Immunol.* 180: 3889–3899.
- Wakkach, A., N. Fournier, V. Brun, J. P. Breittmayer, F. Cottrez, and H. Groux. 2003. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18: 605–617.
- Perona-Wright, G., S. M. Anderton, S. E. Howie, and D. Gray. 2007. IL-10 permits transient activation of dendritic cells to tolerize T cells and protect from central nervous system autoimmune disease. *Int. Immunol.* 19: 1123–1134.
- Li, X., A. Yang, H. Huang, X. Zhang, J. Town, B. Davis, D. W. Cockcroft, and J. R. Gordon. 2010. Induction of type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. *Am. J. Respir. Cell Mol. Biol.* 42: 190–199.
- Nayyar, N., X. Zhang, F. Li, and J. R. Gordon. 2004. Therapeutic modulation of severe allergic lung disease using bone marrow-derived dendritic cells. *Clin. Invest. Med.* 27: 123A (Abstr.).
- Nayyar, A. 2009. Tolerogenic dendritic cell-induced tolerance induction in a mouse model of asthma. Doctoral dissertation, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
- Henry, E., C. J. Desmet, V. Garzé, L. Fiévez, D. Bedoret, C. Heirman, P. Faisca, F. J. Jaspar, P. Gosset, A. P. A. Jacquet, et al. 2008. Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma. *J. Immunol.* 181: 7230–7242.
- Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol.* 3: 331–341.
- Razmara, M., B. Hilliard, A. K. Ziarani, Y. H. Chen, and M. L. Tykocinski. 2008. CTLA-4 x Ig converts naive CD4+CD25- T cells into CD4+CD25+ regulatory T cells. *Int. Immunol.* 20: 471–483.
- Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
- Pyzik, M., and C. A. Piccirillo. 2007. TGF- β 1 modulates Foxp3 expression and regulatory activity in distinct CD4+ T cell subsets. *J. Leukoc. Biol.* 82: 335–346.
- Siewert, C., U. Lauer, S. Cording, T. Bopp, E. Schmitt, A. Hamann, and J. Huehn. 2008. Experience-driven development: effector/memory-like alpha β Foxp3+ regulatory T cells originate from both naive T cells and naturally occurring naive-like regulatory T cells. *J. Immunol.* 180: 146–155.
- Vukmanovic-Stejic, M., E. Agius, N. Booth, P. J. Dunne, K. E. Lacy, J. R. Reed, T. O. Sobande, S. Kissane, M. Salmon, M. H. Rustin, and A. N. Akbar. 2008. The kinetics of CD4+Foxp3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo. *J. Clin. Invest.* 118: 3639–3650.
- Strickland, D. H., P. A. Stumbles, G. R. Zosky, L. S. Subrata, J. A. Thomas, D. J. Turner, P. D. Sly, and P. G. Holt. 2006. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J. Exp. Med.* 203: 2649–2660.
- Gordon, J. R., F. Li, A. Nayyar, J. Xiang, and X. Zhang. 2005. CD8 α +, but not CD8 α -, dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J. Immunol.* 175: 1516–1522.
- Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
- Lutz, M. B., N. A. Kukutsch, M. Menges, S. Rössner, and G. Schuler. 2000. Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy in vitro. *Eur. J. Immunol.* 30: 1048–1052.
- Schneider, A. M., F. Li, X. Zhang, and J. R. Gordon. 2001. Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease following sensitization with limiting doses of ovalbumin-alum. *Cell. Immunol.* 212: 101–109.
- Vijayaraghavan, R., M. Schaper, R. Thompson, M. F. Stock, and Y. Alarie. 1993. Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract. *Arch. Toxicol.* 67: 478–490.
- Vijayaraghavan, R., M. Schaper, R. Thompson, M. F. Stock, L. A. Boylstein, J. E. Luo, and Y. Alarie. 1994. Computer assisted recognition and quantitation of the effects of airborne chemicals acting at different areas of the respiratory tract in mice. *Arch. Toxicol.* 68: 490–499.
- Mahnke, K., T. S. Johnson, S. Ring, and A. H. Enk. 2007. Tolerogenic dendritic cells and regulatory T cells: a two-way relationship. *J. Dermatol. Sci.* 46: 159–167.
- Misra, N., J. Bayry, S. Lacroix-Desmazes, M. D. Kazatchkine, and S. V. Kaveri. 2004. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J. Immunol.* 172: 4676–4680.
- Houot, R., I. Perrot, E. Garcia, I. Durand, and S. Lebecque. 2006. Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. *J. Immunol.* 176: 5293–5298.
- Darrasse-Jeze, G., S. Deroubaix, H. Mouquet, G. D. Victoria, T. Eisenreich, K. H. Yao, R. F. Masilamani, M. L. Dustin, A. Rudensky, K. Liu, and M. C. Nussenzweig. 2009. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J. Exp. Med.* 206: 1853–1862.

29. Yamazaki, S., A. J. Bonito, R. Spisek, M. Dhodapkar, K. Inaba, and R. M. Steinman. 2007. Dendritic cells are specialized accessory cells along with TGF- β for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors. *Blood* 110: 4293–4302.
30. Coombes, J. L., and F. Powrie. 2008. Dendritic cells in intestinal immune regulation. *Nat. Rev. Immunol.* 8: 435–446.
31. Akbari, O., R. H. DeKruyff, and D. T. Umetsu. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2: 725–731.
32. Akdis, C. A., and M. Akdis. 2009. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J. Allergy Clin. Immunol.* 123: 735–746, quiz 747–748.
33. Stephens, G. L., J. Andersson, and E. M. Shevach. 2007. Distinct subsets of FoxP3+ regulatory T cells participate in the control of immune responses. *J. Immunol.* 178: 6901–6911.
34. Steinbrink, K., E. Graulich, S. Kubsch, J. Knop, and A. H. Enk. 2002. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 99: 2468–2476.
35. Randolph, D. A., and C. G. Fathman. 2006. Cd4+CD25+ regulatory T cells and their therapeutic potential. *Annu. Rev. Med.* 57: 381–402.
36. Toda, A., and C. A. Piccirillo. 2006. Development and function of naturally occurring CD4+CD25+ regulatory T cells. *J. Leukoc. Biol.* 80: 458–470.
37. 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.
38. Stock, P., R. H. DeKruyff, and D. T. Umetsu. 2006. Inhibition of the allergic response by regulatory T cells. *Curr. Opin. Allergy Clin. Immunol.* 6: 12–16.
39. Almeida, A. R., B. Rocha, A. A. Freitas, and C. Tanchot. 2005. Homeostasis of T cell numbers: from thymus production to peripheral compartmentalization and the indexation of regulatory T cells. *Semin. Immunol.* 17: 239–249.
40. Yolcu, E. S., S. Ash, A. Kaminitz, Y. Sagiv, N. Askenasy, and S. Yarkoni. 2008. Apoptosis as a mechanism of T-regulatory cell homeostasis and suppression. *Immunol. Cell Biol.* 86: 650–658.
41. 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. Köhl, 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.
42. Xystrakis, E., Z. Urry, and C. M. Hawrylowicz. 2007. Regulatory T cell therapy as individualized medicine for asthma and allergy. *Curr. Opin. Allergy Clin. Immunol.* 7: 535–541.
43. Piccirillo, C. A., and A. M. Thornton. 2004. Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. *Trends Immunol.* 25: 374–380.
44. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive Foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626–635.

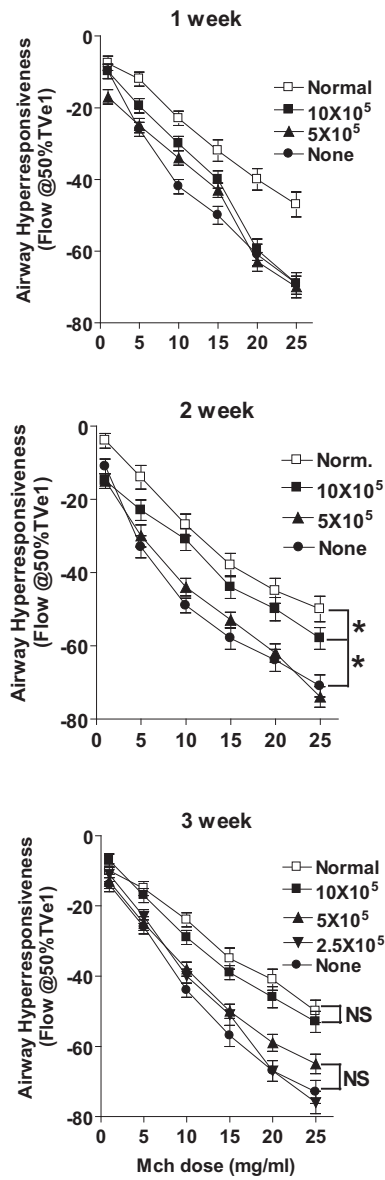
Supplemental Figure Captions

Supplemental Figure S1. Kinetics with which airway hyperresponsiveness is reduced in asthmatic animals given varying numbers of CD4⁺CD25^{hi} Treg from DC10-treated animals.

Varying numbers of CD4⁺CD25^{hi} cells from the lungs of asthmatic mice treated 3 week earlier with DC10 were passively transferred into asthmatic recipients (n=5) as in Fig. 3. At 1, 2, and 3 wk post-transfer, the animals' airway hyperresponsiveness (AHR) to aerosolized methacholine was assessed by head-out whole-body plethysmography. Transfer of 10⁶ Treg nearly normalized AHR (relative to the normal saline controls) at 3 wk post-treatment, while transfer of $\leq 5 \times 10^5$ Treg had no significant impact on the AHR of the recipients at 1, 2 or 3 wk. One representative experiment of two is shown. NS, not significantly different; *, p<0.05 versus its partnered group.

Supplemental Figure S2. *In vivo* confirmation of the induction kinetics for the regulatory activities of pulmonary Treg from DC10-treated asthmatic mice. In order to confirm *in vivo* the activation of the CD4⁺CD25^{hi}Foxp3⁺ cells induced by DC10 treatment of asthmatic mice, we passively transferred limiting numbers of these Treg into asthmatic recipients (5×10⁵ per mouse), then assessed their asthma phenotype as in Figure 3. **(A)** AHR of the recipients to methacholine as determined at day 25 after treatment. At 48 hour after recall allergen challenge we assessed **(B)** eosinophilic airway inflammation and **(C)** BAL IL-4, -5, -9 and -13 levels. When used at these limiting numbers, it was readily apparent that the pulmonary Treg were maximally activated at 3-week post DC10-treatment. (*, p<0.05; **, p<0.01; ***, p<0.001, versus the saline-treated asthmatic mice [ALD]). One representative experiment of two is shown.

Huang et al, Fig. S1



Huang et al, Fig.S2

