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Comparison of Induced Versus Natural Regulatory T Cells of the Same TCR Specificity for Induction of Tolerance to an Environmental Antigen

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

Recent evidence shows that natural CD25⁺Foxp3⁺ regulatory T cells (nTreg) and induced CD25⁺Foxp3⁺ regulatory T cells (iTreg) both contribute to tolerance in mouse models of colitis and asthma, but there is little evidence regarding their relative contributions to this tolerance. We compared the abilities of nTreg and iTreg, both from OVA-TCR-transgenic OTII mice, to mediate tolerance in OVA-asthmatic C57BL/6 mice. The iTreg were differentiated from Th2 effector T cells by exposure to IL-10-differentiated dendritic cells (DC10) in vitro or in vivo, whereas we purified nTreg from allergen-naïve mice and exposed them to DC10 before use. Each Treg population was subsequently repurified and tested for its therapeutic efficacy in vitro and in vivo. DC10 engaged the nTreg in a cognate fashion in Forster (or fluorescence) resonance energy transfer assays, and these nTreg reduced in vitro OVA-asthmatic Th2 effector T cell responses by 41–56%, whereas the comparator iTreg reduced these responses by 72–86%. Neutralization of IL-10, but not TGF- β , eliminated the suppressive activities of iTreg but not nTreg. Delivery of 5×10^5 purified nTreg reduced allergen challenge-induced airway IL-4 ($p \leq 0.03$) and IL-5 ($p \leq 0.001$) responses of asthmatic recipients by $\leq 23\%$ but did not affect airway hyperresponsiveness or IgE levels, whereas equal numbers of iTreg of identical TCR specificity reduced all airway responses to allergen challenge by 82–96% ($p \leq 0.001$) and fully normalized airway hyperresponsiveness. These data confirm that allergen-specific iTreg and nTreg have active roles in asthma tolerance and that iTreg are substantially more tolerogenic in this setting. *The Journal of Immunology*, 2013, 191: 000–000.

The extent to which naturally occurring CD25⁺Foxp3⁺ regulatory T cells (nTreg) and induced CD25⁺Foxp3⁺ regulatory T cells (iTreg) have nonredundant versus complementary roles in immune tolerance is not clear (1). For example, iTreg alone reportedly can induce tolerance in a chronic model of pulmonary inflammation (2), but in a mouse model of colitis both nTreg and iTreg are required to induce full disease tolerance (3). The observations regarding largely disparate TCR β -chain specificities within these two populations notwithstanding, it is clear that, at least in some settings, deployment of both nTreg and iTreg is required for the full expression of tolerance (3). This contrasts with the popular notion that the *raison d'être* for nTreg is to prevent the development of autoimmune disease (4), whereas that of iTreg is to reduce the activation of T cells directed against innocuous environmental Ags (1). Additional observations

that suggest a greater degree of complexity in the roles of nTreg and iTreg, at least in the context of environmental Ags, include the report that depletion of CD25⁺ T cells in naïve wild-type mice leads to augmented asthma severity following subsequent allergen sensitization (5, 6). Moreover, passive transfer of CD25⁺Foxp3⁺ T cells from naïve mice into asthmatic recipient animals reduces their asthma severity if the transfers are performed within 3–4 wk of asthma induction (7).

The recognition that regulatory T cells (Treg) are potently immunosuppressive has galvanized efforts to use these cells therapeutically (8, 9). Passive transfer of Treg that populate the periphery was shown to suppress pathology in, for example, experimental models of autoimmune disease (10, 11). One unanswered question regarding clinical application of this approach is which population of Treg (i.e., nTreg versus iTreg) would be best used in any specific setting (12, 13). There has been speculation that, overall, iTreg would be therapeutically superior to nTreg in settings other than autoimmunity because iTreg would be expected to target Ag-specific T cells with greater efficiency; however, there is a lack of compelling evidence to support this proposal. It was recently reported that, in mouse models of human skin transplantation, iTreg, but not nTreg, treatment led to modestly reduced expression of two of three inflammatory markers in one model, although no such effect was observed in a second model, and transplant rejection was not assessed (14). This leaves open the question of just how comparable nTreg and iTreg truly are in terms of their abilities to ameliorate pathology.

In this study, we compared the abilities of nTreg and iTreg of the same Ag specificity to suppress allergic Th2 responses in vitro and to alter the asthma phenotype in a mouse model of firmly entrenched OVA-asthma. In both cases, the Treg were derived from OVA TCR-transgenic OTII mice and were exposed either in vitro

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Abbreviations used in this article: AHR, airway hyperresponsiveness; DC10, IL-10-differentiated dendritic cell; DC-LPS, LPS-activated immunostimulatory OVA-presenting dendritic cell; FRET, Forster (or fluorescence) resonance energy transfer; GfTR, glucocorticoid-induced TNF-like receptor; HDM, house dust mite; iTreg, induced CD25⁺Foxp3⁺ regulatory T cell; LAG3, lymphocyte-activation gene 3; MHCII, MHC class II; nTreg, naturally occurring CD25⁺Foxp3⁺ regulatory T cell; PD-1, programmed death-1; PD-L1, programmed death ligand-1; Teff, effector T cell; Treg, regulatory T cell.

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or in vivo to OVA-presenting IL-10-differentiated dendritic cells (DC10). We reported previously that such DC10 can efficiently induce Th2 effector T cells (Teff) isolated from the lungs of asthmatic mice to differentiate into CD4⁺CD25⁺Foxp3⁺ iTreg, as well as that 5×10^5 unfractionated pulmonary Treg (i.e., iTreg plus nTreg) recovered from asthmatic mice rendered allergen-tolerant by treatment with DC10 can, in turn, reduce the asthma phenotype by ~50% in passive-transfer recipients (15). In this study, we show that passive transfer of 5×10^5 purified iTreg from DC10-treated asthmatic OTII mice can efficiently suppress all aspects of the asthma phenotype, whereas equal numbers of sorted nTreg that are recovered from DC10-tolerized asthmatic OTII recipient mice have only a modest impact in this model.

Materials and Methods

Mice and the asthma model

Female 6–8-wk-old C57BL/6 (B6) mice were purchased from Charles River Laboratories (Wilmington, MA). B6.CD45.1 congenic and OTII OVA-specific TCR-transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Foxp3-GFP knock-in mice were kindly provided through Dr. A.Y. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY). Double-positive GFP-Foxp3/B6.CD45.1 mice were generated by crossing GFP-Foxp3 and B6.CD45.1 mice. Triple-positive GFP-Foxp3/B6.CD45.1/OTII mice were generated by crossing double-positive GFP-Foxp3/B6.CD45.1 and OTII mice. The GFP-Foxp3/B6.CD45.2/OTII mice were generated by crossing GFP-Foxp3 and OTII mice. The B6.CD45.1/OTII mice were generated by crossing B6.CD45.1 and OTII mice. The phenotypes of offspring mice were confirmed by flow cytometry analyses in which peripheral blood leukocytes from all mice were stained with anti-mouse CD45.1 and/or Vβ5.1,5.2 TCR Abs.

Asthma was induced in the mice by two i.p. injections (days 0 and 14) of 2 mg OVA/alum, followed by 20-min exposures (days 28, 30, and 32) to nebulized aerosols of 1% OVA in saline (16). DC10 and Treg treatments were initiated 2 wk later (day 46). All mice were bred and housed in our institutional animal care unit and used in accordance with the guidelines of the Canadian Council for Animal Care.

Reagents

PE-conjugated anti-mouse CD25 (clone 3C7), ICOS (clone 15F9), programmed death-1 (PD-1; clone J43), glucocorticoid-induced TNF-like receptor (GITR; clone DTA-1), CTLA-4 (clone UC10-4B9), and lymphocyte-activation gene 3 (LAG3; clone C9B7W) Abs and mouse Treg staining kits were purchased from eBioscience (San Diego, CA). Recombinant mouse IL-4, IL-5, IL-13, IL-17, and IFN-γ and matched capture and detection Ab pairs for our ELISA and neutralizing anti-TGF-β Ab were obtained from R&D Systems (Minneapolis, MN). The neutralizing anti-IL-10 Ab was a gift from Sigma-Aldrich (Mississauga, ON, Canada). Anti-mouse CD4 MACS beads and mouse CD4⁺CD25⁺ Treg isolation kits were purchased from Miltenyi Biotec (Auburn, CA). Bead-free mouse CD4⁺CD25⁺ Treg isolation kits were purchased from Invitrogen (Burlington, ON, Canada). The lipid dye DiI was purchased from Molecular Probes. The sources of all other reagents were reported previously (15, 17, 18).

Forster (or fluorescence) resonance energy transfer assay of cognate DC10–nTreg interactions

To assess whether our DC10 productively engage nTreg, we used time-resolved Forster (or fluorescence) resonance energy transfer (FRET) assays, as noted (19, 20). FRET occurs when paired fluorophores are sufficiently close (≤ 8 nm) that energy released from one dye (DiO; excitation and emission wavelengths, 484 and 501 nm, respectively) can excite the partner dye (DiI; emission wavelength, 565 nm) to fluoresce. In our assays, LPS-activated immunostimulatory OVA-presenting dendritic cells (DC-LPS) or DC10 that had been pulsed with specific or irrelevant allergen were labeled with DiO, whereas nTreg were labeled with DiI, and then the cells were cocultured for ~6 h in 96-well plates (each, 2×10^5 cells/well) in a fluorescent microplate reader (NovoStar; BMG). The cultures were exposed to light of 484 nm wavelength, and their emission of fluorescence at 565 nm wavelength was assessed. The data are presented as relative fluorescence units.

Generation and comparison of in vitro-induced iTreg and nTreg

CD4⁺CD25⁺Foxp3⁺ Teff were purified from the lungs of asthmatic mice, whereas CD4⁺CD25⁺Foxp3⁺ Treg were purified from naive OTII mice by negative- and positive-selection magnetic sorting, respectively; we previously characterized these Teff as CD4⁺CD25^{lo}CD44^{hi}CD69⁺CD62L^{lo}Foxp3⁺ (21). The purified Teff and Treg (10^5 cells/well) were cocultured with DC10 (3×10^4 cells/well) in U-bottom 96-well plates for 5 d and then the CD4⁺CD25⁺ iTreg and DC10-exposed CD4⁺CD25⁺ nTreg were positively selected from their respective cultures by magnetic sorting. Both populations of cells were stained with PE-labeled Ab against the Treg markers ICOS, PD-1, GITR, LAG3, neuropilin-1, Helios, and CTLA-4 to determine their relative expression. The 5-d-cultured iTreg and nTreg (10^5 cells/well) were also cocultured for 48 h with DC-LPS (3.7×10^3 cells/well) and Th2 Teff (10^5 cells/well) from asthmatic mice (15). The cell cultures were pulsed with [³H]thymidine during the last 18 h, and thymidine incorporation was determined by liquid scintillation counting. The levels of the indicated cytokines were assessed in the cultural supernatants by ELISA. In some assays, we added neutralizing anti-IL-10 or anti-TGF-β Ab (each, 10 μg/ml) to the cultures. In one set of experiments, we generated iTreg by culturing CD4⁺ from naive OTII mice with plate-bound anti-CD3 (1 μg/ml) and rIL-2 (0.2 ng/ml) and rTGF-β (5 ng/ml) for 5 d, after which the iTreg were purified back out of the cultures by magnetic sorting and assessed for suppressor activity relative to DC10-induced Treg.

Generation and comparison of in vivo-induced Treg and nTreg

FACS analysis. CD4⁺CD25⁺Foxp3⁺ Teff and CD4⁺CD25⁺Foxp3⁺ Treg were purified from asthmatic GFP-Foxp3/B6.CD45.1/OTII and naive GFP-Foxp3/B6.CD45.2/OTII mice, respectively. Both Teff and Treg (5×10^6 cell each) were injected i.v. into asthmatic B6.CD45.2 recipient mice, which were also treated i.p. at the time of passive transfer with 1×10^6 B6.CD45.2⁺ DC10. CD45.1 and GFP were used as markers for transplanted CD45.1⁺ Teff that had converted to an iTreg phenotype (i.e., CD45.1⁺GFP⁺ cells), as well as markers for the transplanted nTreg or their progeny (i.e., CD45.1⁺GFP⁺ cells). One week after the injection, CD4⁺ T cells were purified from the spleens of the DC10-treated recipients and stained with PE-Cy5-CD45.1 and PE-labeled Ab against the indicated Treg markers and analyzed by flow cytometry. CD45.1⁺GFP⁺ (iTreg) and CD45.1⁺GFP⁺ (nTreg) were gated and evaluated for the expression of CTLA-4, ICOS, PD-1, GITR, and LAG3.

Assessment of Treg activities. CD4⁺CD25⁺Foxp3⁺ Teff and CD4⁺CD25⁺Foxp3⁺ nTreg were selected from asthmatic and naive B6.CD45.1/OTII mice, respectively, using magnetic sorting, and then injected i.v. into different groups of asthmatic B6.CD45.2 recipient mice (5×10^6 cell/mouse) that were simultaneously treated i.p. with 10^6 OVA-presenting CD45.2⁺ DC10. CD45 was used as a marker for the donor-origin (i.e., CD45.1⁺) Teff and nTreg versus the endogenous CD45.1⁺ Treg of the recipients. One week later, CD4⁺CD25⁺ Treg were purified from each group of mice by magnetic sorting and labeled for further sorting with PE-CD45.1 Ab and anti-PE paramagnetic beads. The iTreg and nTreg were purified from their respective populations of mice, and their abilities to inhibit Th2 cell activation were assayed as above.

In vivo comparison of iTreg and nTreg. We injected (i.v.) 5×10^5 in vitro-induced iTreg and nTreg, generated as described (15), into otherwise untreated asthmatic recipient mice.

Airway hyperresponsiveness. Four weeks later we assessed the airway responses of the mice to doubling doses of nebulized methacholine (0.5–20 mg/ml) using head-out, whole-body plethysmography (19, 22). This parameter, gathered as running 1-s means of the airflow at the 50% point in the expiratory cycle (Flow@50%Tve1), accurately reflects bronchiolar versus alveolar constriction or airway occlusion (23, 24) and accurately correlates with invasive measurements of airway hyperresponsiveness (AHR) (25, 26).

Airway immunoinflammatory response. The day after AHR was assessed, the mice were given a recall allergen challenge (1% nebulized OVA for 20 min); 2 d later, we euthanized them to assess airway eosinophil numbers and IL-4, IL-5, IL-13, IFN-γ, and IL-17 levels, as well as their relative levels of serum OVA-specific IgE and IgG1, as described (22).

Statistical analysis

All data are presented as mean $\pm$ SEM. Multi-group comparisons were assessed by one-way ANOVA with Tukey post hoc testing, whereas AHR to methacholine was assessed by linear regression analyses. The *p* values < 0.05 were considered significant.

Results

Cognate, but not irrelevant, allergen-presenting DC10 effectively engage nTreg in vitro

We showed previously by FRET analysis and other approaches that, in both mouse and human systems, DC10 efficiently engage Teff in an allergen-specific fashion (19, 20) and promote their conversion into allergen-specific iTreg both in vitro and in vivo (15). We took advantage of this latter observation to generate OVA-specific iTreg for the current study. Because nTreg require cognate activation via their TCR for induction of their suppressive effects (27), we first assessed whether OVA-presenting DC10 engage freshly purified nTreg in a productive manner, as determined by FRET analysis. We labeled irrelevant allergen (house dust mite [HDM])- and OVA-presenting DC10 or DC-LPS with the lipophilic FRET donor dye DiI and stained pulmonary $CD4^+CD25^+Foxp3^+$ nTreg from healthy OVA TCR-transgenic OTII mice with the FRET partner dye DiO. The labeled dendritic cells were cocultured with the stained nTreg, whereas negative controls included DiO-stained T cells or DiI-stained dendritic cells alone. We found that the OVA-presenting DC10 and DC-LPS both intimately engaged the OTII nTreg over a number of hours, albeit with somewhat different kinetics, whereas no FRET signals above background were detected in the HDM-DC10/nTreg cultures (Fig. 1). We reported previously that human DC10, which express reduced levels of MHC class II (MHCII) and costimulatory molecules relative to immunostimulatory dendritic cells, induce long-lasting immunological synapse formation with autologous Teff, although with less intense FRET signals than do immunostimulatory dendritic cells (20). Thus, these data indicated that OTII nTreg engage in a cognate fashion with OVA-presenting DC10.

Treg induced by coculture with DC10 are better than DC10-stimulated nTreg at inhibiting Th2 cell responses in vitro

To directly compare the suppressive activities of iTreg and nTreg, we purified $CD4^+CD25^{lo}Foxp3^+$ Teff from the lungs of asthmatic OTII mice and purified nTreg from allergen-naïve OTII mice. As noted above, we previously characterized these Teff as $CD4^+CD25^{lo}CD44^{hi}CD69^+CD62L^{lo}Foxp3^+$ (21). We cocultured each

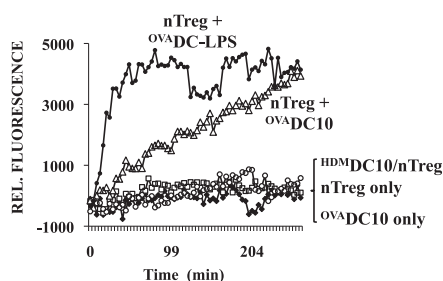


FIGURE 1. DC10 intimately engage nTreg in an allergen-specific fashion. Irrelevant allergen (HDM)- and OVA-pulsed DC10 or DC-LPS were stained with the FRET donor dye DiO, whereas lung $CD4^+CD25^+Foxp3^+$ T cells purified from healthy OVA TCR-transgenic OTII mice (nTreg) were stained with the lipophilic FRET partner dye DiI. The OVA- or HDM-presenting DC10 or DC-LPS were cocultured with the OTII nTreg, whereas negative controls included DiO-stained T cells or DiI-stained dendritic cells alone. The cells were exposed to fluorescent light (488 nm; the DiO excitation wavelength) and assessed for FRET energy release at 565 nm wavelength (the DiI emission wavelength) over ~5 h. The data are expressed as relative fluorescence units. OVA-presenting DC10 induced strong FRET responses among the OTII nTreg, albeit with somewhat delayed kinetics relative to OVA-presenting DC-LPS, whereas HDM-presenting DC10 induced no significant FRET signals. One representative experiment of two is shown ($n = 3$).

of these T cell populations with OVA-presenting DC10 for 5 d, isolated the iTreg and DC10-exposed nTreg from their respective cultures, and then assessed their relative abilities to suppress proliferation and Th2 cytokine expression in cocultures of OVA-presenting DC-LPS and Th2 Teff from OVA-asthmatic mice. The Th2 Teff proliferated strongly and secreted IL-4 and IL-5 in response to the DC-LPS triggering (Fig. 2A, 2B; Med. group); adding additional Teff to these cultures did not alter that response. In contrast, adding DC10-exposed nTreg to the cultures reduced each of these Th2 responses by ~41–56%, whereas adding iTreg had a substantially greater suppressive effect (81–86%) on all three parameters. Because IL-10 and TGF- β are two important inhibitory cytokines secreted by Treg (28), we also assessed their relevance to the suppressive abilities of our iTreg and nTreg, adding specific neutralizing Ab to the cocultures to determine their impact on Th2 cell proliferation and Th2 cytokine secretion (Fig. 2C, 2D). Neither anti-IL-10 nor anti-TGF- β Ab discernibly affected the suppression associated with the nTreg, whereas neutralization of IL-10 in the iTreg cocultures abrogated their suppressive effects. Anti-TGF- β had no impact on the iTreg activities. We also compared the relative efficacy with which 5×10^4 DC10- or equal numbers of anti-CD3/TGF- β -induced Treg (29) suppressed DC-LPS-triggered Teff proliferative responses and found that the DC10 iTreg were modestly more effective than were the alternatively induced Treg (in two experiments, 5×10^4 DC10-induced Treg suppressed Teff responses by $27 \pm 8.16\%$ and $31 \pm 6.0\%$, whereas anti-CD3/TGF- β -induced Treg suppressed these responses by $23 \pm 2.64\%$ and $8.16 \pm 7.35\%$). These data indicated that DC10-induced iTreg have a substantially greater contribution to the regulation of Th2 responses in vitro than do nTreg that are of identical Ag specificity and that have seen similar exposure to allergen-presenting DC10. It also indicated that these iTreg used IL-10 as their primary tolerogenic mediator, as was reported previously for peripherally induced $CD4^+CD25^+Foxp3^+$ Treg (30, 31).

DC10-induced activation of nTreg and iTreg

As noted previously, nTreg induce tolerance in a contact-dependent fashion, using cell surface molecules, as opposed to secreted mediators, as primary tolerogenic effectors (4, 27). To determine whether our DC10-induced iTreg and nTreg differentially expressed Treg-associated cell surface markers, we assessed their expression of ICOS, PD-1, GITR, LAG3, CTLA-4, neuropilin-1, and Helios by FACS. We found that our Treg that were induced in vitro from Teff purified from the lungs of asthma-phenotype mice expressed modestly to markedly higher levels of ICOS and CTLA-4 than did the nTreg but largely equivalent levels of PD-1, GITR, LAG3, and neuropilin-1 and lower levels of Helios (Fig. 3B). As an assessment of the relevance of these in vitro findings to Treg that might mediate tolerance responses in vivo, we also assessed the expression of ICOS, PD-1, GITR, LAG3, and CTLA-4 on iTreg and nTreg that were induced or stimulated in vivo. To generate the iTreg, we transferred $CD4^+CD25^{lo}Foxp3^+$ Teff from asthmatic $CD45.1^+$ GFP-Foxp3 OTII donor mice (Fig. 3B) into asthmatic B6 ($CD45.2^+$) mice that we simultaneously treated i.p. with 1×10^6 OVA-presenting DC10. To obtain the nTreg, we purified $CD4^+CD25^+Foxp3^+$ nTreg from allergen-naïve $CD45.2^+$ GFP-Foxp3 OTII donors (Fig. 3B) and cotransferred these into the DC10-treated Teff recipient mice. One week after the DC10 treatment, the animals' $CD4^+$ T cells were sorted magnetically and submitted for FACS analysis, gating on the $CD45.1^+GFP^+$ (iTreg) and $CD45.1^-GFP^+$ (nTreg) $CD4^+$ T cells (Fig. 3C). It was readily apparent that in vivo-induced iTreg expressed substantially higher levels of PD-1, LAG3, and CTLA-4, as well as modestly elevated

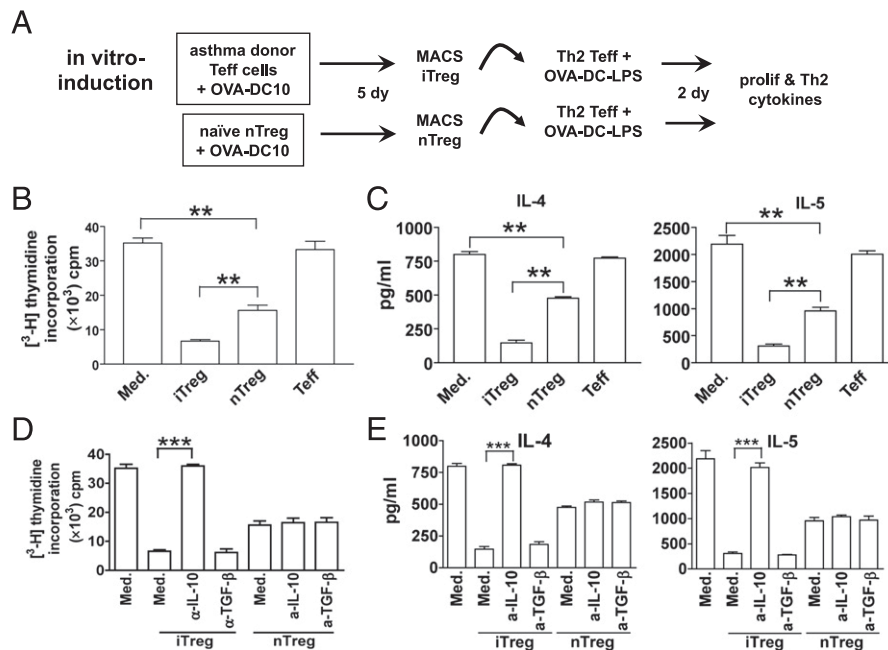


FIGURE 2. OVA TCR-transgenic Treg induced in vitro by DC10 treatment inhibit asthmatic T effector cell activation more efficiently than do nTreg of identical Ag specificity. $\text{CD4}^+\text{CD25}^{\text{lo}}\text{Foxp3}^-$ Teff were purified from the lungs of asthmatic OVA TCR-transgenic OTII mice, whereas $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ nTreg were purified from allergen-naïve OTII mice. OVA-presenting DC10 were cocultured for 5 d with these Teff or nTreg and then the DC10-induced Treg (iTreg) and nTreg were positively selected from their respective cultures by magnetic sorting. **(A)** Schematic diagram of the experimental protocols used in these experiments. The abilities of each of these Treg populations (1×10^5 cells/well) to inhibit proliferation **(B)** and Th2 cytokine (IL-4, IL-5) secretion **(C)** by DC-LPS-activated Teff (3×10^4 and 1×10^5 cells/well, respectively) freshly purified from the lungs of OVA-asthmatic C57BL/6 (B6) mice were assessed in vitro, as noted in *Materials and Methods*. To assess the contributions of IL-10 and TGF- β to the suppressive activities of these Treg, we also assessed the impact of neutralizing Ab (each, 10 $\mu\text{g/ml}$) on DC-LPS-induced proliferation **(D)** and Th2 cytokine secretion **(E)**. One representative experiment of three is shown. ** $p < 0.01$, *** $p < 0.001$.

levels of ICOS and GITR, than did the nTreg. Nevertheless, the mean fluorescence intensity for each of these markers on the nTreg was significantly elevated relative to isotype control-stained cells. Similarly, both the nTreg and the iTreg expressed higher levels of each marker than did transferred Teff from asthmatic mice (Supplemental Fig. 1).

Having seen differences in the expression of regulatory markers by in vitro- and in vivo-differentiated Treg, we next compared their activities. We generated cells for these assays in vivo, as above, with the exception that the Teff and nTreg were originally derived from asthmatic and naïve CD45.1^+ OTII donor mice, respectively. Each Treg population was transferred into its own group of DC10-cotreated asthmatic B6 recipient mice (5×10^6 T cells/mouse); 1 wk later, the $\text{CD4}^+\text{CD25}^+\text{CD45.1}^+$ iTreg and $\text{CD4}^+\text{CD25}^+\text{CD45.1}^+$ nTreg were purified from the respective recipients by magnetic sorting (Fig. 4A). We assessed the activities of these Treg by adding them into cocultures of Teff from OVA-asthmatic B6 mice and OVA-presenting DC-LPS (Fig. 4B, 4C). Control cultures were not supplemented with Treg (Med.) or were supplemented with additional $\text{CD4}^+\text{CD25}^-\text{Foxp3}^-$ Teff purified from asthmatic donor B6 mice. We found that the in vivo-stimulated nTreg reduced DC-LPS-induced Teff proliferation in this assay by $45.7 \pm 7.1\%$, whereas the same numbers of iTreg of the same TCR specificity reduced proliferation by $73 \pm 5.5\%$ ($p \leq 0.01$). When we measured the levels of IL-4, IL-5, and IL-13 in these cultures we observed a similar outcome: the nTreg reduced the levels of these three Th2 mediators by $40.5 \pm 6.5\%$, $52.2 \pm 13.6\%$, and $55 \pm 3.2\%$, respectively, whereas the iTreg reduced their expression by $72.7 \pm 8.5\%$, $82.7 \pm 10.3\%$, and $89.3 \pm 1.9\%$, respectively (for each cytokine, $p \leq 0.01$). Thus, consistent with the data from our in vitro-induced iTreg, in vivo-

induced iTreg were substantially more effective than were nTreg at blocking Th2 activation in vitro.

DC10-induced Treg more efficiently ameliorate the asthma phenotype than do DC10-stimulated nTreg of the same Ag specificity

We reported previously that passive transfer of 5×10^5 $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ cells (i.e., unfractionated Treg) from DC10-tolerized asthmatic mice reverses the asthma phenotype by $\sim 50\%$ of asthmatic recipients; 2.5×10^5 of these Treg are completely ineffective in this model, whereas 1×10^6 Treg fully corrects the phenotype (15). In this study, we directly compared the in vivo regulatory activities of iTreg and nTreg from OVA TCR-transgenic OTII mice. Although we know that iTreg readily populate the lungs of DC10-treated asthmatic mice (15), we first confirmed by FACS that nTreg and iTreg that have been delivered i.v. can migrate to the lungs and lung-draining lymph nodes. Two days after injecting 5×10^6 CFSE-labeled nTreg or iTreg into different asthmatic recipient mice, 1.50 and 0.29% of the cells present in their lungs and lung-draining lymph nodes were nTreg and iTreg, respectively. To assess their respective regulatory activities, we next used half-maximal numbers (i.e., 5×10^5) of purified DC10-induced Treg or equal numbers of DC10-exposed nTreg, both generated in vitro as above, to treat asthmatic mice at day 46 after initiation of sensitization; 4 wk later we examined the impact of the treatments on the animals' asthma phenotype. We assessed their AHR to methacholine using head-out whole-body plethysmography to determine the airflow rates at the 50% point in the expiratory cycle (15, 18, 19). The AHR of the animals given 5×10^5 DC10-induced iTreg was fully normalized ($p > 0.05$ versus normal mice), whereas the same number of nTreg had no

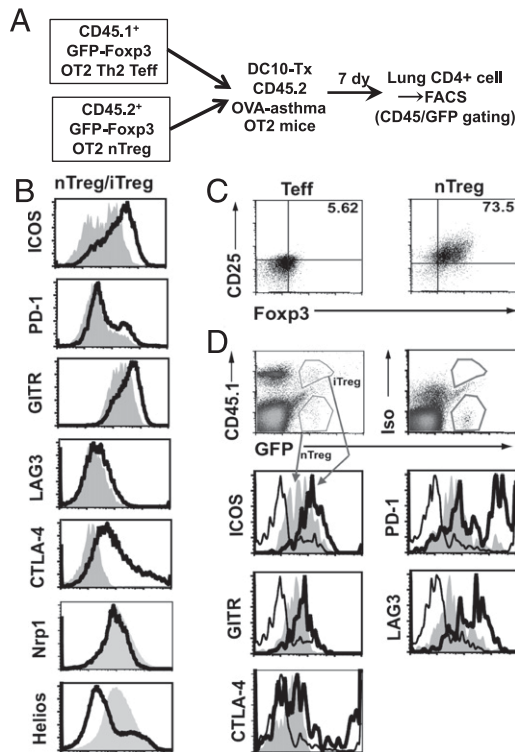


FIGURE 3. DC10-induced Treg express substantially higher levels of PD-1, LAG3, and CTLA-4 than do nTreg. In vitro- and in vivo-induced iTreg and nTreg were assessed for expression of the Treg-associated cell surface markers ICOS, PD-1, GITR, LAG3, neuropilin-1 (Nrp1), Helios, and/or CTLA-4, as noted in *Materials and Methods*. **(A)** Schematic diagram of the experimental protocol for in vivo induction and purification of DC10-induced, Teff-derived iTreg and DC10-exposed nTreg. **(B)** iTreg (black line) and nTreg (gray fill) were generated in vitro and sorted from their respective cultures, as in Fig. 2, and then submitted for FACS analysis of the indicated markers. **(C)** Analogous populations of Treg were also generated in vivo by transferring (i.v.) 5×10^6 CD4⁺CD25^{lo}Foxp3⁺ Teff that had been purified from asthmatic CD45.1⁺ GFP-Foxp3 OTII donor mice (left panel) and CD4⁺CD25^{lo}Foxp3⁺ nTreg purified from allergen-naïve CD45.2⁺ GFP-Foxp3 OTII donor mice (right panel) into the same DC10-treated asthmatic CD45.2⁺ B6 recipients. **(D)** One week after the DC10 treatment, the animal's CD4⁺ T cells were magnetically sorted and stained with specific or isotype-control Ab for CD45.1 (upper panels), as well as the indicated markers, with gating on GFP and CD45 to discern the iTreg and nTreg populations. One representative experiment of three is depicted for each panel.

discernible effect on the asthmatic AHR (Fig. 5A). We then administered a recall allergen challenge to the animals and 2 d later assessed their airway eosinophil and Th2 cytokine (IL-4, IL-5, IL-13) responses. Across three experiments, passive transfer of nTreg reduced IL-4 levels by $8.8 \pm 1.8\%$ ($p \leq 0.03$, versus saline-treated asthmatic mice), IL-5 levels by $22.9 \pm 6\%$ ($p \leq 0.001$, versus saline-treated asthmatic mice), and bronchoalveolar lavage fluid eosinophil numbers by $16.2 \pm 5.1\%$ ($p = 0.07$, versus saline-treated asthmatic mice) (Fig. 5B). Transfer of iTreg reduced the numbers of airway eosinophils and IL-4, IL-5, and IL-13 levels to near background ($p \leq 0.001$, versus nTreg-treated asthmatic mice). In addition, the iTreg treatment markedly reduced the levels of OVA-specific IgE and IgG1 Ab ($p \leq 0.001$ and 0.01 , respectively, versus nTreg-treated asthmatic mice), whereas nTreg had no discernible impact ($p > 0.05$) on the IgE or IgG1 Ab responses. Finally, because Treg require engagement via their TCR to be activated, but once activated sup-

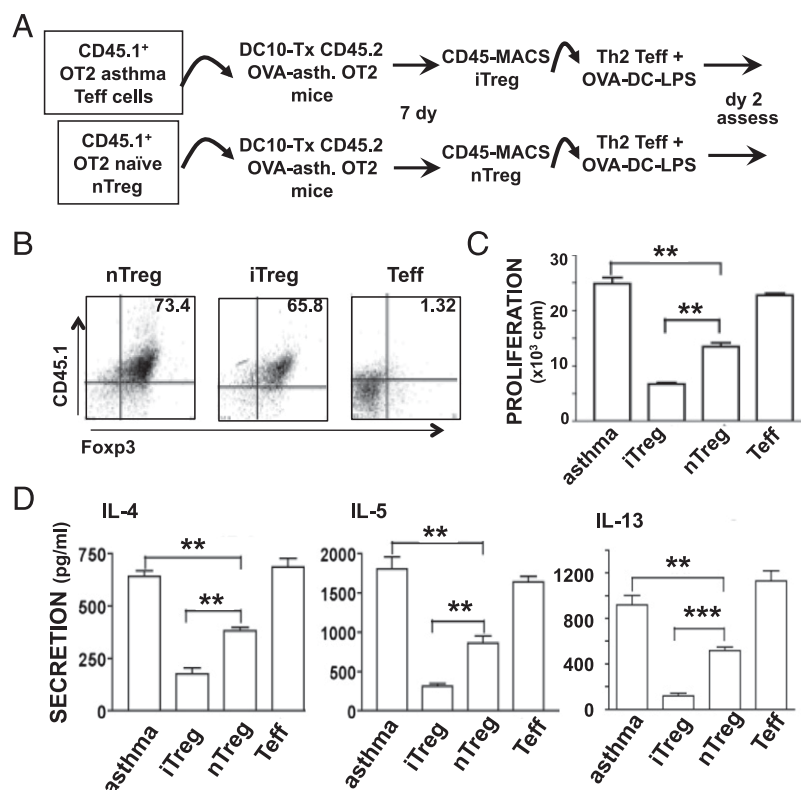
press Teff nonspecifically (32), it was not unexpected that the modest IFN- γ and IL-17 responses that we observed in our asthmatic mice (33) were also suppressed in the iTreg- and nTreg-treated animals. The airways of our saline-treated asthmatic mice contained $180 \pm 39.2\%$ and 151 ± 15.7 pg/ml of IFN- γ and IL-17, respectively; iTreg treatments reduced the IFN- γ and IL-17 responses by $73 \pm 9\%$ and $22.7 \pm 21\%$, respectively, whereas the nTreg treatments reduced them by $13 \pm 10\%$ and $8.8 \pm 12\%$, respectively.

Discussion

We demonstrated in this study that allergen-presenting DC10 engage nTreg in a cognate fashion, as determined using FRET assays. These DC10-stimulated nTreg suppressed the in vitro proliferation and Th2 cytokine responses of immunostimulatory dendritic cell-activated Th2 Teff by 41–56%, although DC10-induced iTreg were substantially more tolerogenic in this in vitro system. The regulatory activities of these iTreg, but not nTreg, were completely blocked by anti-IL-10 Ab, and they also expressed higher levels of immunoregulatory molecules (e.g., PD-1, CTLA-4), as well as significant amounts of neuropilin-1 and Helios. We further found that transferring 5×10^5 purified DC10-exposed nTreg only modestly reduced the asthma phenotype in recipient mice, whereas equal numbers of purified DC10-induced iTreg were highly effective in reducing asthmatic responses. As we observed previously, asthmatic B cell (i.e., OVA-specific IgE and IgG1) responses take substantially longer to wane than do Th2 responses following tolerogenic treatments (16).

It has been proposed that nTreg and iTreg serve distinct functions: nTreg primarily target autoimmune responses (4), whereas iTreg modulate responses to exogenous Ags (1). One piece of evidence that supports this is that depletion of nTreg in naïve mice leads to an autoimmune syndrome (34); however, it was since reported that similar depletion of CD4⁺CD25^{lo}Foxp3⁺ T cells in naïve mice also leads to augmented asthma severity following subsequent allergen sensitization (5, 6). As noted above, it also was shown that passive transfer of nTreg from naïve mice into asthmatic recipient animals relatively early after asthma induction reduces their asthma phenotype (7). This suggests that, although there may well be some divergence of function between iTreg and nTreg, the story may be more complex than a simple dichotomy between endogenous versus exogenous Ag dedication for the two cell types. For example, recent evidence indicates that both iTreg and nTreg play requisite roles in the establishment of full disease tolerance in a mouse model of colitis (3), although it is evident that there are situations in which one Treg population alone can mediate tolerance. Thus, also as noted above, iTreg are sufficient to induce tolerance in a mouse model of pulmonary inflammation (2). It was reported that the TCR repertoires of the iTreg and nTreg that are effective in the above-noted colitis model overlap by only 3–15% (3); this is taken as further evidence that these two cells target distinct sets of immunologic processes. However, many Ags feature multiple epitopes, some of which may be immunodominant and others less so, whereas many environmental challenges (e.g., microorganisms, allergens) are complex and present the immune system with numerous, likely multi-epitope, Ags, and each would require a unique TCR. Thus, the reported restricted overlap in TCR β -chain specificities of iTreg and nTreg (3) notwithstanding, there could be ample room for iTreg and nTreg with disparate TCR specificities to target the same disease complex. The evidence that we present in this article confirms that iTreg and nTreg of identical TCR specificity are able to target anti-OVA responses of Th2 cells from OVA-asthmatic wild-type mice both in vitro and in vivo, although they do so with substantially

FIGURE 4. Treg induced in vivo by DC10 treatment of asthmatic mice have greater inhibitory activity in vitro than do nTreg of equivalent Ag specificity. **(A)** Schematic diagram of the experimental protocol used. **(B)** CD4⁺CD25⁺Foxp3⁺CD45.1⁺ iTreg and nTreg were induced in vivo and purified by CD45-based magnetic sorting. These cells, together with control populations of Teff from asthmatic B6 mice, were assessed for their abilities to antagonize proliferation **(C)** and IL-4, IL-5, and IL-13 secretion **(D)** by DC-LPS-activated Teff (3×10^4 and 1×10^5 cells/well, respectively) freshly purified from the lungs of OVA-asthmatic B6 recipient mice. One representative experiment of three is shown. $**p < 0.01$, $***p < 0.001$.



different efficacy. In our model system, the iTreg were much more effective in suppressing asthmatic Th2 responses than were nTreg of identical TCR specificity (i.e., OVA₃₂₃₋₃₃₉) (35).

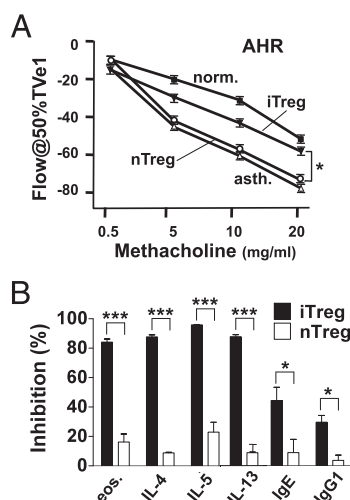


FIGURE 5. Passive transfer of DC10-induced OTII Treg, but not nTreg of identical Ag specificity, efficiently ameliorates the asthma phenotype in recipient mice. We injected 5×10^5 iTreg or nTreg (i.v.), generated in vitro as in Fig. 2, into otherwise untreated asthmatic recipient mice ($n = 5$) and assessed the impact of the treatments on the animals' asthma status 4 wk later. **(A)** At 4 wk, we assessed AHR to methacholine, as determined by the airflow rate at the 50% point in the expiratory cycle (Flow@50%TVe1). **(B)** The following day, we challenged the mice for 20 min with an aerosol of 1% nebulized OVA; 2 d later, we assessed the extent to which the iTreg versus nTreg treatments suppressed the asthmatic airway eosinophilia; bronchoalveolar lavage fluid IL-4, IL-5, and IL-13 recall responses; and the serum levels of OVA-specific IgE and IgG1. One representative experiment of three is shown. $*p < 0.05$, $***p < 0.001$.

Natural Treg require TCR signaling via APCs to be activated (27), and our FRET data indicate that cognate, but not irrelevant, allergen-presenting tolerogenic DC10 intimately engaged the nTreg. However, an unanswered question is whether these naturally tolerogenic DC10 would have activated the nTreg with which they were cultured in the same way as immunostimulatory dendritic cells might have. Irrespective of whether the DC10 did so, in our in vitro study the nTreg were added into cocultures of LPS-activated OVA-presenting dendritic cells, and these also would be expected to activate the nTreg via TCR signaling (27). In principle, that could explain our data that suggest that nTreg were apparently qualitatively better at inducing tolerance in our in vitro assays than they were in vivo. In our model, the iTreg would have recently differentiated from Teff under the influence of treated DC10 and their activation status would have been relatively high at the time of passive transfer (15), which might have contributed to their tolerogenic efficacy.

Our nTreg productively engaged cognate, but not irrelevant, allergen-presenting DC10, indicating that MHCII at least was critical to nTreg engagement. We also reported recently that both MHCII and CD80/CD86 are critical to DC10-induced tolerance (33), suggesting that both TCR and CTLA4 expression could have contributed to these cognate DC10–nTreg interactions. In addition, we found that DC10 express high levels of programmed death ligand-1 (PD-L1) (C. Li, Y. Ma, W. Dawicki, and J.R. Gordon, unpublished observations), which similarly could well engage the PD-1 expressed by our nTreg in this study. Peripherally induced CD4⁺CD25⁺Foxp3⁺ Treg were reported to use IL-10 as their primary tolerogenic molecule (30, 31), and this is consistent with our report that bulk CD25⁺Foxp3⁺ Treg from the lungs of DC10-treated asthmatic mice similarly use IL-10 as their primary effector molecule (15). Although iTreg can also include a population of CD25[−] cells that use IL-10 to suppress T cell responses (36, 37), based on our FACS analysis, our magnetically sorted iTreg were predominantly CD25⁺Foxp3⁺ cells (data not shown).

FACS analysis of our Treg confirmed that the iTreg also strongly expressed the CD4-related MHCII-binding iTreg marker LAG3, which is associated with both cell-intrinsic and -extrinsic signaling (38), as well as PD-1 and CTLA4, which provide inhibitory signals via the CD28/B7 pathway (39). LAG3 on iTreg can provide inhibitory signals to immature dendritic cells on engaging their MCHII molecules (38), and this fits well with other observations in our laboratory that the endogenous lung dendritic cells of DC10-treated mice take on a regulatory phenotype several weeks after DC10 treatment of asthmatic mice (C. Li, H. Huang, W. Dawicki, and J.R. Gordon, unpublished observations), in concert with the appearance of highly activated Treg in the lungs (15). The augmented expression of PD-1 by the in vivo-induced iTreg is consistent with PD-1 playing an important role in the formation and activities of these cells (40). PD-1 expression by our iTreg also fits well with the fact that these cells were induced by exposure to DC10, which express low levels of MHCII, CD40, CD80, and CD86 (16, 20) and, thereby, induce attenuated TCR stimulation relative to mature allergen-presenting dendritic cells (20). It was reported that low-level TCR stimulation augments PD-1-dependent negative signaling in T cells (41). Moreover, we know that our DC10 express high levels of PD-L1 (Y. Ma, C. Li, S.A. Gordon, W. Dawicki, and J.R. Gordon, unpublished observation), and PD-L1/PD-1 signaling is important in the induction of iTreg (40). CTLA-4 can play a dual function in T cells, where it is inducible and controls Teff homeostasis (42), as well as in Treg, within which it is constitutively expressed (4). Other investigators (43) reported the upregulation of CTLA4 and GITR expression on Treg, presumably iTreg, in the context of hookworm infections, whereas vasoactive intestinal peptide-induced Treg similarly express augmented levels of CTLA4 (44). It is interesting that the in vivo-induced Treg in our study expressed higher levels of PD-1 than did the in vitro-induced cells. This could be due to the increased levels of eosinophilic inflammation that would be found in the asthmatic lung relative to the in vitro cultures, inasmuch as inflammation was reported to increase PD-1 expression by CD4⁺ T cells (45). The combined presence in vivo of both DC10 and the above-noted DC10-induced endogenous lung regulatory dendritic cells might have contributed further to this discrepant PD-1 expression, given that tolerogenic dendritic cells reportedly drive PD-1 expression by CD4⁺ T cells (46). Finally, although Helios and neuropilin-1 were initially reported as markers that distinguish nTreg and iTreg (47, 48), subsequent reports indicated that both can be expressed by iTreg. Helios expression by iTreg is reportedly dependent on interactions between the APC and the Treg being induced (49), whereas neuropilin-1 expression is apparently confined to iTreg that develop in the context of chronic inflammation (e.g., chronic asthma) (50). In both cases this fits well with our data, whereby DC10 induce Treg that drive immunologic tolerance in a mouse model of chronic asthma.

It was reported that passive transfer of 5×10^5 wild-type nTreg is sufficient to offset otherwise inevitable lethality in Foxp3-deficient mice (3). This number of nTreg was also sufficient to ameliorate the asthma phenotype in an OVA/alum model not unlike our own, but only if the cells were transferred early in the induction protocol; nTreg transfer after entrenchment of the phenotype (i.e., day 46) had no discernible impact on the disease (7). Interestingly, our laboratory routinely treats fully asthmatic mice with DC10 (16, 19) or Treg (15) at precisely this time (i.e., day 46), and we see substantial to full reversal of the phenotype within 3–4 wk. The nTreg used by Kearley et al. (7) were unmanipulated, whereas ours had been exposed to tolerogenic DC10, which might explain the differences in our day-46 treatment outcomes. We showed in this study that our OVA-presenting DC10 engaged OTII

mouse nTreg in a cognate fashion and, thus, could have stimulated the regulatory activities of these cells. Previously, we titrated the numbers of unfractionated CD4⁺CD25⁺Foxp3⁺ Treg from the lungs of DC10-treated asthmatic mice that are required to passively transfer tolerance to asthmatic recipients and found that 1×10^6 cells ablated the asthma phenotype in the recipients, whereas 5×10^5 or 2.5×10^5 Treg reduced the asthma phenotype by ~50% or had little, if any, impact on the phenotype, respectively (15). Thus, it seems feasible that our transference of limiting numbers (i.e., 5×10^5) of Treg was important in clearly distinguishing the disparate activities of iTreg versus nTreg in our asthma model. Our data indicated that Treg are able to gain access to the asthmatic lung, and this is consistent with other reports that functional nTreg are recruited into and reside within the lungs of both healthy and asthmatic mice (51). Moreover, other investigators (52) reported similar recoveries of CFSE-labeled iTreg from the lungs and lung-draining lymph nodes of asthmatic recipients injected i.v. with these cells. Nevertheless, we cannot rule out that the nTreg that we transferred might have been less efficient in engaging allergen-specific Teff in the lungs in comparison to iTreg. These caveats notwithstanding, our data indicate that DC10-induced Treg are distinctly superior to nTreg of identical Ag specificity in their abilities to antagonize the asthma phenotype in a mouse model of fully entrenched disease.

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Disclosures

The authors have no financial conflicts of interest.

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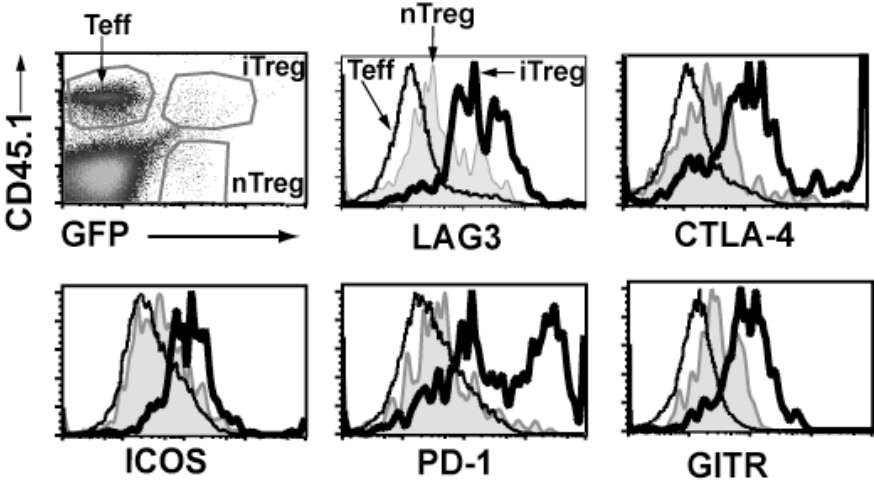
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SUPPLEMENTARY FIGURE CAPTIONS

Supplementary Figure S1. Comparison of the expression levels of ICOS, PD-1, GITR, LAG3 and CTLA-4 by Teff, iTreg and nTreg cells. CD4⁺CD25⁻Foxp3⁻ Teff and CD4⁺CD25⁺Foxp3⁺ Treg cells were purified from asthmatic GFP-Foxp3/CD45.1 and naïve GFP-Foxp3/CD45.2 OT2 mice, respectively. Both T cell populations (5x10⁶ cells/recipient) were injected *i.v.* into asthmatic B6 (CD45.2) mice which were then treated *i.p.* with 1x10⁶ OVA-presenting B6 DC10. One wk later CD4⁺ T cells were purified from the recipients' spleens, and the cells stained with PE-cy5-CD45.1 and PE-labeled antibodies against the indicated Treg cell markers. CD45.1⁺GFP⁺ (iTreg), CD45.1⁻GFP⁺ (nTreg) and control CD45.1⁺GFP⁻ (Teff) cells were gated and their marker expression was analyzed by flow cytometry. One representative experiment of two is shown.

Supplementary Figure S2. Intravenously injected iTreg and nTreg are both able to immigrate into the lungs of recipient mice. DC10-induced Treg and DC10-exposed nTreg were purified as in Fig. 2, labeled with CFSE, and then 5x10⁶ CFSE-labeled nTreg or iTreg were injected *i.v.* into different asthmatic recipient mice. Two days later an array of tissues were harvested from the recipients and assessed for the numbers of CD4⁺ CFSE-labeled cells by FACS. Depicted are the FACS scatterplots for both Treg populations and for cells recovered from the lungs of negative control mice that were given *i.v.* PBS injections alone. Both Treg populations could be detected in the lungs of the recipients, and at levels proportionate to those found in the spleens

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