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Induction of Prolonged Asthma Tolerance by IL-10–Differentiated Dendritic Cells: Differential Impact on Airway Hyperresponsiveness and the Th2 Immunoinflammatory Response

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IL-10–differentiated dendritic cells (DC10s) can prevent allergen sensitization and reverse the asthma phenotype in mice with established disease. However, little is known about the time-frames over which this tolerance is effective. We report that at 2 wk after i.p. or transtracheal delivery of 1 × 10⁶ OVA-, but not house dust mite–presenting, DC10s to OVA-asthmatic mice, significant diminution of airway hyperresponsiveness (AHR) was first apparent, whereas AHR was abrogated between 3 and 10 wk posttreatment. At 13 wk, AHR returned to pretreatment levels but could again be reversed by DC10 retreatment. The impact of a single DC10 treatment on airway eosinophil and Th2 cytokine responses to recall OVA challenge, and on OVA-specific IgE/IgG1 responses, was substantial at 3 wk posttreatment, but progressively increased thereafter, such that at 8 mo, airway eosinophil and Th2 responses to recall allergen challenge remained ∼85–95% suppressed relative to saline-treated asthmatic mice. Four biweekly DC10 treatments, whether transtracheal or i.p., reduced all asthma parameters to near background by 8 wk, whereas s.c. DC10 treatments did not affect AHR but did reduce the airway Th2 responses (i.e. DC10 had no discernible effects). Repeated challenge of the DC10-treated mice with aerosolized OVA (100 μg/ml) did not reverse tolerance, but treatment with the indoleamine-2,3-dioxygenase antagonist 1-methyltryptophan or neutralizing anti–IL-10R from days 12 to 21 after DC10 therapy partially reversed tolerance (Th2 cytokine responses, but not AHR). These findings indicate that DC10-induced Th2 tolerance in asthmatic animals is long lived, but that DC10s employ distinct mechanisms to affect AHR versus Th2 immunoinflammatory parameters. The Journal of Immunology, 2012, 189: 72–79.

Asthma is characterized by intermittent and reversible airway obstruction, airway hyperresponsiveness (AHR), and eosinophilic airway inflammation, driven by underlying Th2 immune responses (1). Conventional treatments have not addressed the immunologic basis of this disease but rather are primarily symptomatic in nature, targeting either the AHR (e.g., bronchodilators) or inflammatory responses (e.g., steroids) (2). Numerous reports have clearly indicated that immunologic tolerance can be induced in multiple disease models through use of regulatory dendritic cells (DCs) (3–7) that are either generated by differential culture conditions or genetically modified. The abilities of such tolerogenic DCs to prevent experimental Ag sensitization have been documented in model systems ranging from cancer (8) to allergic diseases (9). TGF–β–differentiated DCs have been reported to ∼50% reduce the localization of allergic disease to the airways in animals that are sensitized systemically to an allergen, but this was not an allergen-specific effect (10), and IL-10–differentiated DCs (DC10s) are similarly effective in blocking asthma sensitization (11). Quiescent allergen-presenting, splenic CD8α+ DCs can ∼50% reverse established asthmatic responses in a mouse model of asthma, including AHR, eosinophilias and Th2 responses, but systemic allergen-specific IgE/IgG1 responses are relatively resistant to tolerization with such DCs (12). In contrast, we have reported that specific allergen-presenting DC10s are highly effective in reversing AHR and airway Th2 recall responses to allergen challenge within 3–4 wk of treating house dust mite allergen (HDM)-asthmatic animals (7). This reversal of Th2 effector T cell responses can be recapitulated with CD14+ monocyte-derived DC10s that induce allergen tolerance ex vivo among autologous T cells of asthmatic individuals. This tolerance is associated with the differentiation of CD4+CD25+ Foxp3+ regulatory T cells that suppress Th2 responses (5). We have further shown that DC10s induce T effector cells from asthmatic animals to alter their phenotype, adapting a bona fide regulatory T cell phenotype (13). Although DCs that are retrovirus transfected to express very high levels of IL-10 can induce a long-lasting and robust tolerance (14), significant ethical issues would arise with using such cells clinically. The tolerance induced by regulatory DCs that are differentiated ex vivo with IL-10 is seemingly robust (7, 11, 13), but we have little information regarding
the life span of that tolerance or its durability under allergen rechallenge conditions.

In our study, we explored these issues using OVA-presenting DC10s in a mouse model of OVA-asthma. Specifically, we followed the clinical course of tolerance over ~8 mo in DC10-treated animals, assessed the impact of the anatomic route by which DC10s are given, as well as the impact of multiple DC10 treatments on the asthma phenotype. We show that DC10s can provoke a prolonged and highly effective allergen-specific tolerization of the asthmatic Th2 immunoinflammatory responses in the treated animals, although targeting of AHR is only transiently effective.

Materials and Methods

Animals, reagents, and materials

Female BALB/c mice were obtained from our institutional Animal Resource Centre; all animal treatments were in accord with the guidelines of the Canadian Council on Animal Care. The reagents/materials used and their sources have largely been reported (7, 12, 13). Additional reagents and their sources include the following: 1-methyltryptophan (1-MT) and placebo slow-release polymer pellets (release rate, 10 mg/day; Innovative Research of America, Sarasota, FL); neutralizing anti–IL-10R Abs (obtained through H. Tabel (University of Saskatchewan, Saskatoon, SK, Canada) (15); mouse anti–MIP-1α (CCL3) and MIP-3B (CCL19) (R & D Systems, Minneapolis, MN); FITC-labeled anti-mouse CD11b (clone M1/70), CD14 (clone mouse mC5-3), CD16/CD32 (clone 2.4G2), CD19 (clone 1D3), and CD45RB (clone 16A); and FITC-labeled isotype control rat IgG2b, IgG2a, and IgG1, hamster IgG1, IgG2, and IgM, mouse IgG2a, and IgG2b Abs (PharMingen Canada, Mississauga, ON, Canada); FITC-labeled anti-CD205 (clone MCA949) and F4/80 anti-macrophage/monocyte Abs (clone CL-3.13) (Serotech, Oxford, U.K.).

Generation of DCs

BM-derived DCs were generated largely as noted (16). Briefly, bone marrow cells were seeded in RPMI 1640 supplemented with 1% antibiotics/antimycotics, 50 μM 2-ME, and 10% heat-inactivated FCS (complete medium) containing 20 ng/ml GM-CSF. The culture medium was replaced on days 6 and 8 with fresh GM-CSF–containing medium, and on day 10 the nonadherent DCs were resuspended in complete medium supplemented with 5 ng/ml GM-CSF to generate immature (i.e., isotype control-level staining only) or with 50 μg/ml of either IL-10 (to generate tolerogenic cells; DC10s) or, for some experiments, TNF to generate immunostimulatory DC-TNFs. As noted previously (16), the removal of the adherent cells at day 10 and transfer of the nonadherent cells into low-dose GM-CSF substantially increases the purity of DCs in our cultures (to 90–95% DCs). On day 13, a portion of the cells from each culture were pulsed for 2 h at 37°C with 50 μg/ml OVA (DC-OVA10) or, as a negative control for Ag specificity (7), house dust mite allergen (HDM-DC10); then the cells were washed.

DC characterization

The cells were assessed by FACS for expression of the noted markers using a FACSscan (Becton Dickinson, Mountain View, CA), with isotype-matched control Ab for all populations. To assess phagocytosis, cells were incubated for 30 min at either 4°C or 37°C with FITC-dextran (100 μg/ml), then washed with ice-cold PBS, fixed in 0.5% paraformaldehyde, and analyzed by FACS (17). CCR5 and CCR7 expression was determined using microchemotaxis assays with MIP-1α and MIP-3B, respectively, as the specific ligands, as noted (18). Our DC10s express modestly to markedly lower levels of CD40, CD54, CD80, and CD86, and MHCII relative to mature specific ligands, as noted (18). Our DC10s express modestly to markedly lower levels of CD40, CD54, CD80, and CD86, and MHCII relative to mature specific ligands, as noted (18).

ELISA

Our ELISA protocols have been reported in detail previously (5, 7, 12, 19). Cell culture supernatants and bronchoalveolar lavage (BAL) fluids were not diluted, but plasma samples were diluted 1:10 in PBS with Tween 20. The detection limits for our cytokine ELISAs are routinely 5–10 pg/ml. Positive controls for our assays included samples from saline-treated asthmatic mice, whereas the negative control samples were from healthy normal mice as well as, for all ELISAs, an assay control of BAL medium alone. For the IgE ELISA, IgE in the plasma was captured using goat anti-mouse IgE and the OVA-specific Ab therein detected by use of biotinylated OVA (19); these data are expressed as relative IgE OD (OD595) units.

Asthma model and animal samples

We have previously reported on our model of OVA-induced asthma (12, 19). Briefly, 6- to 10-wk-old BALB/c mice were sensitized with two i.p. injections (days 0 and 14) of 2 μg OVA/alum, followed by 20-min exposures (day 30, 32, and 34) to nebulized aerosols of 1% OVA in saline. This process reliably induces a severe asthma phenotype, with high-airway Th2 (IL-4, -5, -9, and -13) and circulating IgE and IgG1 responses to OVA, ±60% airway eosinophilia, and AHR. Blood smears and cytocentrifuge slides of airway cells obtained by BAL were stained with Wright’s solution (20). Lung tissue single-cell suspensions were prepared by enzymatic dispersal with collagenase and hyaluronidase (12, 19) 1 d after in vivo allergen challenge, after which the mononuclear cells were purified by density gradient centrifugation (Pharmingen, San Diego, CA, USA) and cultured for 48–72 h in complete medium (2 × 10⁶ cells/ml) without additional exogenous OVA. All samples from each animal were processed, aliquoted, stored at −80°C, and assayed independently.

DC treatments

Two weeks after their last sensitizing exposure to OVA aerosols, the asthmatic mice were given saline, or 1 × 10⁵ allergen-pulsed immature DCs, DC10s, or DC-TNFs, administered transtracheally (i.t.), i.p., s.c., or i.v., in 20–100 μl saline (n = 5). For the t.t. injections, the mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and the skin overlaying their trachea was reflexed surgically, and the wound was sutured closed after the DC or saline injection. AHR was measured weekly at weeks 4, 6, and 8, and in most experiments the mice were exposed to OVA aerosol (20 min, 1% OVA) on day 21, then sacrificed on day 22 for assessment of their asthma phenotype responses. For testing the long-term effects of a single DC10 treatment, plasma for OVA-specific Ab analyses was obtained as noted; at 8 mo, the mice were again exposed to OVA aerosols, and then sacrificed 1 d later. To determine whether multiple DC10 treatments would improve the efficacy of tolerance induction, the mice were treated with DC10 (i.p.) as above, but then again at 2, 4, and 6 wk; they were exposed to OVA aerosols 24 h prior to harvest at week 8. To determine whether repeated allergen challenge would reverse tolerance, DC10-treated asthmatic mice were given 1 × 10⁵ DC10s (i.p.) on weeks 0, 2, and 4 to induce tolerance, then challenged for 10 min with a physiologically relevant dose of nebulized OVA (100 μg/ml) on days 42, 46, and 50, and harvested on day 54 for full assessment of their asthma parameters; AHR was assessed on days 45, 49, and 53. In preliminary experiments, we determined that a 10-min challenge of asthmatic mice with 100 μg/ml OVA induces an airway eosinophil response that is ~81% of maximal. Moreover, it has been reported previously that cumulative doses of 15–20 ng house dust mite extract (21) or 5 μg/ml ragweed (22) are sufficient to induce both immediate and late-phase bronchoconstriction responses in asthmatic invididuals, suggesting that our challenge dose was relevant in the context of the human disease. In experiments to assess the role of indoleamine-2,3-dioxygenase (IDO) in tolerance, slow-release (10 mg/day) tablets of 1-MT or placebo were implanted s.c. on day 12 after DC10 treatment. In some experiments, the DC10-treated asthmatic mice were given anti–IL-10R Ab (5 mg/kg body weight) (15) on days 12, 15, 18, and 21 posttreatment, and assessed first for AHR and then for their asthma phenotype, as above.

Airway hyperresponsiveness

AHR was assessed in conscious animals by head-out, whole-body plethysmography, as noted in detail (12, 19). Briefly, air was supplied to the head and body compartments of a plethysmograph via a small-animal ventilator, and changes in the airflow through the body compartment were monitored using a flow sensor linked to a computer-driven real-time data acquisition/analysis system (DasyLab 5.5; Dasytec USA, Amherst, NH). Doubling doses of nebulized methacholine aerosols (0.75–25 mg/ml) were delivered to the head compartment of the plethysmograph, and bronchoconstriction data were gathered as running 1 s means of the airflow at the 50% point in the expiratory cycle (Flow@50%TV1). This parameter accurately reflects bronchiolar constriction, as opposed to alveolar constriction or airway exclusion (23), and has been shown to correlate well with invasive measurements of AHR (24, 25).

Statistics

All data were expressed as the mean ± SEM. Group statistics were assessed by one-way ANOVA, with Tukey’s multiple comparison post
Results
DC10 treatment induces a robust, but transient, reversal of AHR in asthmatic animals

Tolerogenic DCs can induce a seemingly robust blockade of AHR, whether the DCs are used prophylactically (10, 11) or therapeutically (7, 12), but none of these studies have examined the durability of that tolerance over prolonged time-frames. In our study, we first delivered $1 \times 10^6$ OVA-presenting immature DCs (OVA-Imm DC), DC10s (OVA-DC10), DC-TNFs (OVA-DC-TNF), or saline (asthma) (protocol, Fig. 1A) administered t.t. into asthmatic mice and then assessed their AHR over time. None of the treatments significantly affected AHR at 1 wk, but by week 2 the OVA-DC10 group began to show signs of improvement ($p = 0.03$, versus saline-treated asthmatic mice), and by day 21 their AHR was fully normalized ($p \leq 0.01$; Fig. 1B). The airways of the OVA-asthmatic animals that were treated with OVA-presenting immature DCs or DC-TNFs remained fully hyperresponsive (each, $p < 0.05$, versus saline-treated asthmatic mice). Delivery of OVA-DC10s i.p. reversed AHR with identical kinetics, whereas s.c. injection of DC10s had no impact on AHR (Supplemental Fig. 2). The airways of the OVA-DC10-treated mice remained fully normalized at 8 wk, but, interestingly, at 10 wk the effects of the DC10s on AHR began to wane and by week 13 the airways of the DC10-treated mice were fully hyperresponsive again (Fig. 1B). A second treatment with $1 \times 10^6$ OVA-DC10s at week 14 again fully abolished AHR within 3 wk (data not shown).

Allergen-specific asthmatic Th2 tolerance induced by DC10 treatments is prolonged and progressive

OVA-DC10 treatment also affected the pulmonary eosinophilia and Th2 responses of the asthmatic mice, as determined at 3 wk after treatment, although not as much as it had affected AHR. The animals were treated as above, or, as a control for allergen specificity of DC10, they were treated with HDM-presenting DC10s. All animals were challenged with OVA aerosol 1 d before sacrifice to reactivate their pulmonary Th2 responses (protocol, Fig. 2A).

The airways of OVA-immature DC– or saline-treated asthmatic animals were rich in eosinophil infiltrates, whereas OVA-DC10 treatment reduced eosinophilia by 59.8 ± 3.6% ($p < 0.01$, versus asthmatic mice); the irrelevant allergen control HDM-DC10 treatments had no significant effect on the eosinophil response ($p > 0.05$, versus asthmatic mice; Fig. 2B, left panel). The circulating levels of OVA-specific IgE were also significantly reduced in the OVA-DC10– ($p \leq 0.05$, versus asthmatic mice), but not in the OVA-immature DC– or HDM-DC10-treated mice (for both, $p > 0.05$ versus asthmatic mice; Fig. 2B, middle panel). IgG1, but not IgG2a, levels were also significantly reduced by the OVA-DC10 treatment (Supplemental Fig. 3A), as were BAL fluid levels of Th2 cytokines (Fig. 2B, right panel). Thus, IL-4, IL-5, IL-9, and IL-13 levels were each reduced ($p \leq 0.01$ or 0.05, versus asthmatic animals). The airway levels of IL-12 and IFN-γ were also reduced by the OVA-DC10 treatment (IL-12: asthmatic, 279 ± 117 pg/ml; DC10-treated, 94 ± 23 pg/ml; IFN: asthmatic, 873 ± 385 pg/ml; DC10-treated, 124 ± 51 pg/ml), indicating that we had not simply induced Th2 to Th1 immune deviation. HDM-DC10s did not alter the airway Th2 cytokine responses of the OVA-asthmatic animals (data not shown), confirming that tolerance induced by DC10 is allergen specific (7).

Our observation that DC10 treatment ablated AHR for only ~3 mo led us to question whether it would have similarly short-term effects on asthmatic Th2 immunoinflammatory disease. Thus, we treated OVA-asthmatic mice with saline (asthmatic) or OVA-presenting DC10s or immature DCs, as above, and we followed the treatment effects for 8 mo, using the animals’ serum IgE levels as a noninvasive measure of tolerance (protocol, Fig. 2A). One day before sacrifice, we challenged the mice with concentrated (10 mg/ml) aerosols of OVA to invoke airway recall responses. As might be expected for animals that had not been exposed to allergens for a prolonged period of time, the circulating levels of OVA-specific IgE in the saline-treated asthmatic mice waned across the 8 mo of the experiment ($p \leq 0.01$), but nevertheless remained elevated relative to normal control mice or DC10-treated asthmatic animals. IgE levels of the DC10-treated mice were markedly lower than those of the saline-treated asthmatic mice at all times, approaching background within 4 mo of treatment (99% suppressed) and remaining suppressed at 8 mo ($p \leq 0.001$).

FIGURE 1. Regulatory DC-induced tolerization of AHR is progressive but transient in asthmatic mice. (A) Asthma was induced in BALB/c mice, as described in Materials and Methods. At 2 wk later, the asthmatic animals were given saline (white triangles) or $1 \times 10^6$ OVA-pulsed immature DCs (gray circles), tolerogenic DC10s (OVA-DC10; black squares), or TNF-activated DCs (OVA-DC-TNF; gray diamonds) and (B) their AHR was assessed by head-out whole-body plethysmography at the indicated times. Healthy negative control mice (norm. [control]; white squares) were also assessed. From 14 to 21 d after cell transfer, the OVA-DC10 recipients normalized bronchial responsiveness to methacholine, whereas the asthmatic, immature DC–, and DC-TNF–treated animals remained hyperresponsive. At 8 wk the DC10-treated animals retained their tolerance, but by wk 10 they began to regain their hyperresponsiveness to methacholine, and by 13 wk they had fully reverted to the bronchial responsiveness of asthmatic mice. The depicted experiments were repeated three times ($n \geq 5$ animals per group). *$p \leq 0.05$, **$p \leq 0.01$. 

homic testing (Sigma Stat Version 2.0, SPSS, Chicago, IL). Data not normally distributed were log transformed for analysis. Significance was assigned when $p$ values were $\leq 0.05$. 

Materials and Methods

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FIGURE 2. Asthmatic Th2 tolerance induced by treatment with DC10s is progressive and long lasting. (A) Asthma was induced, and the mice were treated with saline (asthma) or OVA-pulsed immature DCs, DC10s, (OVA-DC10), or HDM-pulsed DC10s (HDM-DC10). After 3 (B) or 32 (C) wk, the animals were exposed to aerosolized 1% OVA for 20 min, then sacrificed 1 d later and processed as described in Materials and Methods. (B) At the 3-wk time-point, the OVA-DC10s, but not HDM-DC10s, reduced the airway eosinophil responses (left panel) and the circulating levels of OVA-specific IgE (middle panel) and also ameliorated the BAL levels of IL-4, IL-9 (both, $p \leq 0.05$), IL-5, and IL-13 (both, $p \leq 0.001$; right panel). * or ** $p \leq 0.05$ or 0.001, respectively, versus immature DC-treated control mice. (C) Mice were treated with DCs or saline, and serum OVA-specific IgE levels were assessed at 2, 4, and 8 mo. The OVA-specific IgE levels declined somewhat across the 8 mo in the saline-treated asthmatic mice, as well as the immature DC- and DC10-treated mice, but by 4 mo the IgE responses had declined to near baseline in the DC10 treatment group and remained stable thereafter (left panel). The BAL Th2 cytokine levels were also reduced significantly by the DC10s, but not the immature DC treatment (for each cytokine, $p \leq 0.01$; middle panel), and this outcome held also for the expression of these cytokines by the lung parenchymal lymphocytes (right panel). The dashed line across the lower portion of the IgE panel represents the background signal in the serum of normal mice (norm.). The experiments in (B) were repeated 5 times, whereas the experiments in (C) were repeated twice ($n \geq 5$ animals per group). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus immature DC recipient mice. versus levels in either the saline- or the immature DC-treated mice; (Fig 2C, left panel). The airway Th2 recall responses were also markedly affected by the DC10 treatment; the BAL fluid levels of IL-4, IL-5, IL-9, and IL-13 were 86–96% lower (each, $p \leq 0.001$) than those of immature DC- or saline-treated asthmatic animals (Fig. 2C, middle panel). This trend was mirrored also by the in vitro Th2 cytokine responses of the animals’ lung tissue mononuclear cells to the recall allergen challenge (Fig. 2C, right panel). The responses of the cells from the saline-treated asthmatic animals were significantly lower than those of the OVA-presenting immature DC-treated mice (for each cytokine, $p \leq 0.001$), whereas the lung T cell IL-4, IL-5, IL-9, and IL-13 responses of the DC10-treated asthmatic animals were reduced by 91–99% relative to the immature DC-treated mice (for each cytokine, $p \leq 0.05$). A single DC10 treatment also protected the lungs from eosinophilic inflammatory responses to allergen challenge for at least 8 mo (Supplemental Fig. 3B). At 24 h after recall allergen challenge, the airway eosinophilia of the OVA-DC10 treated mice was ~80% lower than that of OVA-presenting immature DC-treated mice ($p \leq 0.01$).

Repeated delivery of tolerogenic DCs augments tolerance in asthma

Although a single treatment with specific allergen-presenting DC10s brought the asthmatic phenotype close to background over time, we wished to know whether we could boost the speed of tolerance onset by repeated delivery of DC10 and whether we could identify more clinically relevant treatment routes. Thus, we gave asthmatic mice saline or $1 \times 10^6$ OVA-presenting DC10s four times at biweekly intervals, using t.t., i.p., s.c., or i.v. delivery routes (protocol, Fig. 3A). We assessed AHR at 8 wk, prior to challenging the mice with OVA aerosols, and sacrificed them 1 d later, as above. The bronchial responsiveness of the t.t. and i.p. DC10 recipients was fully normalized at 8 wk, but the AHR of the asthmatic s.c. (Fig. 3B) and i.v. (data not shown) DC10 recipients was not discernibly affected by four DC10 treatments. Repeated t.t. DC10 treatments reduced the IgE (Fig. 3C, left panel) and IgG1 responses by 90–93% at 8 wk (Supplemental Fig. 4), whereas the OVA-specific IgG2a and IgA responses were reduced by 87% and >98%, respectively (data not shown). The i.p. delivery of DC10s similarly reduced the OVA-specific IgE (Fig. 3C, left panel), IgG1, and IgG2a responses by 84%, 94%, and 87%, respectively. DC10 given by the s.c. route did not significantly impact the IgE response (Fig. 3C; $p > 0.05$). Again, i.v. delivery of DC10 was completely ineffective (data not shown).

The airway Th2 cytokine responses were markedly reduced in the animals repeatedly given DC10s, whether t.t. or i.p. ($p \leq 0.01$ or 0.001, respectively; Fig. 3C, right panel). Of interest, s.c., but not i.v., delivery of DC did significantly reduce airway Th2 cytokine responses ($p \leq 0.01$; Fig. 3B). Intriguingly, the levels of IL-10 and TGF-β in the airways of the t.t. and i.p. DC10 recipient mice were upregulated, and substantially so (>5-fold) for TGF-β (Supplemental Fig. 4), whereas this did not occur in the s.c. DC treatment group. For example, BAL IL-10 levels in the saline- and i.p. DC10-treated asthmatic mice were 141 and 197 pg/ml.
We had previously noted that, in vitro, IDO plays a role in CD8+ DC-induced asthmatic T cell tolerance (12), and others have reported that IL-10 expression by DC10s (11) is important to their tolerogenic functions. We therefore assessed whether these molecules also have roles in the effector phases of DC10-induced tolerance, administering either neutralizing anti–IL-10R Abs or the IDO antagonist 1-MT (via s.c. slow-release pellets) to groups of DC10-treated asthmatic animals from day 12, just before the effects of DC10 treatment were first discernible, to the time of sacrifice (day 21) (Fig. 5). The anti–IL-10R Ab treatments had no significant impact on bronchial responsiveness (dose of methacholine provoking a 20% fall in pulmonary airflow: anti–IL-10R, 1.57 mg/ml; isotype control Ab, 1.58 mg/ml) or the OVA-specific IgE response (Fig. 5A, right panel). In contrast, neutralizing the IL-10R did significantly affect DC10-dependent dampening of the eosinophil recall response, as well as the IL-5 and IL-13 responses (Fig. 5A; each, $p \leq 0.01$, or $***p \leq 0.001$ versus their relevant saline-treated asthmatic control group).

**Roles for IL-10 and IDO in DC10-mediated allergen tolerance**

We had previously noted that, in vitro, IDO plays a role in CD8α+ DC-induced asthmatic T cell tolerance (12), and others have reported that IL-10 expression by DC10s (11) is important to their tolerance outcome. (A) Asthma was induced, and the animals were treated with DC10s or an equal volume of saline via the same route as their matched DC recipients. All animals were given a recall allergen challenge with aerosolized OVA 24 h before sacrifice. (B) AHR of the mice in the t.t., i.p., and s.c. treatment groups at week 8 (the i.v. treatments had no impact on any of the asthma parameters assessed; data not shown). As expected, the AHR of both the t.t. and the i.p. DC10 recipients remained normalized at 8 wk, whereas the AHR of the s.c. DC10 treatment mice was not significantly different from that of the saline-treated asthmatic animals ($p \geq 0.05$). (C) Repeated t.t. or i.p. treatments brought the serum OVA-specific IgE levels down to near baseline (left panel), whereas the s.c. treatment was not nearly as effective. Of interest, all three routes of DC10 delivery were effective in reversing the Th2 cytokine response to recall allergen challenge (right panel). The background IgE signal detected in healthy control mice (norm.) is indicated with a dashed line. The experiments depicted were repeated three times ($n \geq 5$ animals/group). $*p \leq 0.05$, $**p \leq 0.01$, or $***p \leq 0.001$ versus their relevant saline-treated asthmatic control group.

**FIGURE 3.** Repeated administration of DC10s can augment T and B cell allergen tolerance in asthma, whereas the route of delivery determines the tolerance outcome. (A) Asthma was induced, and the animals were treated with DC10s or an equal volume of saline via the same route as their matched DC recipients. All animals were given a recall allergen challenge with aerosolized OVA 24 h before sacrifice. (B) AHR of the mice in the t.t., i.p., and s.c. treatment groups at week 8 (the i.v. treatments had no impact on any of the asthma parameters assessed; data not shown). As expected, the AHR of both the t.t. and the i.p. DC10 recipients remained normalized at 8 wk, whereas the AHR of the s.c. DC10 treatment mice was not significantly different from that of the saline-treated asthmatic animals ($p \geq 0.05$). (C) Repeated t.t. or i.p. treatments brought the serum OVA-specific IgE levels down to near baseline (left panel), whereas the s.c. treatment was not nearly as effective. Of interest, all three routes of DC10 delivery were effective in reversing the Th2 cytokine response to recall allergen challenge (right panel). The background IgE signal detected in healthy control mice (norm.) is indicated with a dashed line. The experiments depicted were repeated three times ($n \geq 5$ animals/group). $*p \leq 0.05$, $**p \leq 0.01$, or $***p \leq 0.001$ versus their relevant saline-treated asthmatic control group.

**FIGURE 4.** Tolerance induced by DC10s is not broken by repeated rechallenge with physiologic levels of aerosolized allergen. (A) Experimental protocol, wherein asthma was induced and then the animals were treated three times with DC10s or saline (i.p.). At week 8, we began a series of three recall allergen (100 μg/ml OVA: DC10/OVA) or saline (DC10/sal.) challenges, each followed 3 d later by an assessment of AHR; asthmatic control mice (asthma) received saline in place of both the DC10 treatment and the allergen recall challenge. On day 54, we sacrificed the mice and analyzed their asthma phenotype, as above. (B) Three recall challenges with OVA did not significantly affect the AHR of the mice (upper left panel), their airway Th2 cytokine, IFN-γ (upper right panel), or eosinophil (lower left panel) responses, or the ex vivo expression of Th2 cytokines or IFN-γ by lung T cells (lower right panel)—in each case relative to saline-challenged DC10-treated asthmatic animals. The experiments depicted were repeated three times ($n \geq 5$ animals per group).
FIGURE 5. Roles for IL-10 and IDO in the effector phase of DC10-induced tolerance. Asthma was induced, and the animals were treated one time (i.p.) with DC10 or saline, and assessed 3 wk later for AHR and asthma phenotype, as in Fig. 2A. At day 12 posttreatment, we began giving the mice either anti–IL-10R Ab (5 mg/kg, day 12, 15, 18, and 21) or the competitive IDO antagonist 1-MT or placebo slow-release capsules (s.c.) (A) The anti–IL-10R Ab treatments significantly affected the airway IL-5, IL-13, and eosinophil responses to allergen challenge but did not significantly affect AHR (p > 0.05), IL-9 levels (p = 0.056), or the IL-4 response (data not shown), relative to isotype control Ab-treated mice. (B) Antagonizing IDO had no significant impact on AHR, but did significantly affect the IL-5, IL-9, and IL-13 responses to recall allergen challenge, and also affected the circulating OVA-specific IgG1, but not IgE, levels. The experiments depicted were repeated three times (n ≥ 5 animals per group), *p ≤ 0.05, **p ≤ 0.01 versus their relevant saline-treated asthmatic control group animals. each p < 0.01 or 0.001 versus placebo treatment). The 1-MT–associated IL-4 response did not achieve statistical significance relative to that of placebo-treated animals (p > 0.05).

Discussion
Our data demonstrate that delivery of specific allergen-presenting DC10s administered either directly into the airways or i.p. can be highly effective in reducing the Th2 asthma phenotype in experimental animals for up to 8 mo, but that AHR is reduced to background for only ~3 mo. On the contrary, we had reported previously that airway delivery of quiescent OVA-presenting splenic CD8α+ DCs modestly (~50%) reverses AHR, eosinophilia, and pulmonary Th2 responses, but is somewhat less effective in reducing the allergen-specific IgE and IgG1 responses (12). Others have shown that TGF-β (10), IL-10 (10)– (10) differentiated DCs can blunt development of the asthma phenotype in systemically sensitized animals and that DC10s can substantially reverse the asthma phenotype, as determined 4 wk posttreatment in a model of established house dust mite asthma (7, 11). Tolerance associated with TGF-β–differeniated DCs (10), or with CD8α+ DCs (12), is not fully allergen specific, whereas DC10-induced tolerance is driven by cognate processes (7, 11). We found that i.v. delivery of DC10 was completely ineffective in inducing tolerance in asthmatic mice, and this agrees with other findings (26–28), although unlike cells delivered via the tail vein, IL-10 transgene-expressing DCs do prolong graft survival if injected into the portal vein (28).

AHR, or hyperirritability of bronchial smooth muscle, is the hallmark of asthma (29), such that its amelioration would be an ideal outcome of asthma therapeutics, and this was one of the more remarkable findings in DC10-induced tolerance. We found that the OVA–DC10 treatments were effective in reducing AHR, essentially abrogating it within 3 wk, although this normalization lasted only 3 mo. We are not aware of any reports of transient corrections of AHR with regulatory DCs, and at this time we have no direct experimental evidence relating to the mechanisms mediating this effect. Evidence from other reports indicates that AHR can be related functionally to multiple distinct mechanisms. One is IgE/mast cell dependent (30, 31), and another is eosinophil/Th2 dependent and mast cell independent (32), and we have reported that AHR can develop in the absence of any evidence of IgE or Th2 responses when animals are sensitized with very low doses of allergen (19). This latter model (19) may reflect some of the processes that regulate “intrinsic” versus extrinsic asthma etiology and pathogenesis (33). In the current study, reversal of AHR began prior to observable effects on IgE levels, or IL-5 and eosinophil responses, and this clearly indicates that a dampening of smooth muscle responses can be implemented even in the face of substantial classical Th2 allergen sensitivity. It has been shown that expression of an IL-10 transgene within the airways similarly suppresses AHR (34) and also that IL-10 can block smooth muscle cell activation by inflammatory stimuli in vitro or in vivo, at least in part by directly inhibiting NF-κB degradation and NF-κB nuclear translocation (35). We have found in our laboratory that DC10s, delivered i.p. rapidly migrate to the airways (M. Lu, H. Huang, and J.R. Gordon, unpublished observations), and we reported in this article and elsewhere that human and mouse DC10s secrete elevated levels of IL-10 relative to control DCs (5, 7, 13). Thus, IL-10 released locally in the lungs by the treatment DC10s could potentially have been in part responsible for amelioration of AHR. We (H. Huang, W. Dawicki, and J.R. Gordon, unpublished observations) and others (3, 11) have found that knockdown or knockout of IL-10 expression by DC10 eliminates their abilities to affect AHR, and that is consistent with the argument that the DC10’s IL-10 secretion may initiate normalization of AHR. Our DC10 tracking data also indicate that the numbers of treatment DC10s decline in the lung at the same time as these cells begin to appear in the lung-draining lymph nodes (M. Lu, H. Huang, and J.R. Gordon, unpublished observations), and this suggests that either an alternate factor must regulate AHR at this time or that an alternate source of IL-10 must be or become positioned in the lungs, at least temporarily. Others have reported that chronic allergen exposure upregulates regulatory T cell function and thereby dampens AHR in a rat model of asthma, and that this suppression of bronchial responsiveness requires continual allergen challenge; interrupting the chronic challenge process leads to loss of tolerance and a return of AHR (36). However, in our model AHR returns to the animals despite a continuation of Th2 tolerance, and we have reported that DC10 treatments drive the conversion of Th2 effector T cells in the lungs into CD4+CD25+Foxp3+ regulatory T cells that employ IL-10 as a primary tolerogenic mechanism (13). In concert with this, we have observed conversion of the tolerant animals’ endogenous lung DCs into regulatory cells that also secrete IL-10.
been reported that CCR7+ bone marrow-derived DCs that are “tolerogenic” DCs in a mouse model of multiple sclerosis is as had been reported previously (26). Intravenous delivery of our hands four biweekly DC10 treatments brought allergen sen-

sitivity may well wane below levels required to control AHR at 10–12 wk after a single DC10 treatment.

Our DC10 treatments also progressively ameliorated the recipients’ allergen-specific Th2 responses. At 3 wk posttreatment, the IgE response was \( \sim 48\% \) lower than at the time of treatment, whereas at 4–8 mo this effect had increased to \( \geq 80\% \). Similarly, at 3 wk the eosinophil responses to allergen challenge were \( \sim 60\% \) below those at the time of treatment, and at 8 mo posttreatment this had increased to \( \sim 90\% \) suppression, and the suppression of the Th2 cytokine response was similarly progressive. As noted above, we have shown that DC10s activate regulatory T cells in the lungs of asthmatic mice, with regulatory T cells recovered at 3 wk after DC10 treatment having significantly more activity than those recovered at earlier times (13). We do know, however, that these regulatory T cells subsequently convert the animals’ endogenous lung DCs to a regulatory phenotype (C. Li, W. Dawicki, H. Huang and J.R. Gordon, unpublished observations), and this feed-forward cascade of events might be anticipated to progressively improve the asthma phenotype. Others have reported, of course, that regulatory T cells have a significant impact on DCs, inducing them to take on a regulatory phenotype (36–39). The time-frames required for a single DC10 treatment to achieve full effect on the asthma phenotype were still quite prolonged relative to what might otherwise be desired, although allergen-specific immunotherapy protocols can require significantly more time yet to reduce allergic symptomatology by \( \sim 75\% \) (e.g., 2 y) (40). As such, the observation that multiple DC10 treatments much more rapidly reduced disease severity was important—in our hands four biweekly DC10 treatments brought allergen sen-

tivity to near background within 8 wk. It is interesting then that DC10s that were delivered i.v. had no discernible impact on asthma, as had been reported previously (26). Intravenous delivery of “tolerogenic” DCs in a mouse model of multiple sclerosis is also ineffective in reversing disease, whereas s.c.-delivered cells were effective in that model (27).

It is intriguing that the cells delivered s.c. in our model were only partially effective in reversing the asthma phenotype. It has been reported that CCR7\(^+\) bone marrow-derived DCs that are injected s.c. (i.e., in the footpad) efficiently migrate to the draining lymph nodes of mice and there trigger immune responses, whereas CCR7\(^-\) DCs do not do so efficiently (41). It was noted that a geometric relationship exists between the numbers of Ag-presenting DCs and the resulting T cell response, wherein decreasing by half the numbers of DCs in the draining nodes reduces by 4-fold the ensuing immune response (41). Our DC10s were CCR7\(^{hi}\), such that it could have been they were not able to readily traffic from the skin to the draining lymph nodes and therefore were not as efficient as DC10s that do efficiently reach the lung-draining lymph nodes (i.e., t.t. and i.p. DC10s), as noted above. Certainly, the s.c.-delivered DC10s had little impact on AHR, and that would be consistent with a need for these cells, or the regulatory T cells they induce (13), to reside within the lungs to affect bronchial reactivity. One could speculate that regulatory T cells induced in one compartment (e.g., skin-draining lymph nodes) may not as efficiently traffic to alternate compartments (e.g., the lungs), such that they may not have been present in sufficient numbers to impact AHR. Thus, for example, CD103\(^{hi}\) TGF-\( \beta \) and retinoic acid-expressing DCs from the intestine induce regulatory T cell responses to innocuous gut symbionts, and these induced regulatory T cells express CCR9 and CD4\( \beta \) integrin, such that they recirculate back to the gut but not other compartments (42). Whether other types of regulatory DCs induce regulatory T cells that recirculate to yet other compartments is an interesting question, the answer to which could have ramifications for compartment-specific applications (e.g., the CNS in multiple sclerosis) of DC immunotherapies.

Our data suggest that both IL-10 and IDO play a role in DC10-dependent reversal of the asthmatic Th2 phenotype, but not in reversal of AHR, at least during the early effector phases of tolerance. Others have reported that IL-10 expression by IL-10–differentiated DCs is required to prevent development of the asthma phenotype in systemically sensitized mice (11) and that IL-10 expression by host cells, presumed to be regulatory T cells, is required for induction of tolerance in asthmatic mice that have been treated with IL-10–lentivirus-transfected DCs (14); the latter authors did not assess tolerization of AHR (14). In addition, we have reported that neutralization of IL-10 prevents DC10-induced regulatory T cells from suppressing effector T cell responses in vitro in our OVA-asthma system (13). It is important to keep in mind that other cells that express Th2 cytokines (e.g., mast cells) can also be regulated by IL-10 (43). Indeed, mast cell-derived IL-10 may well play regulatory roles in asthma and tolerance (44). Although we have no experimental insights into why the anti–IL-10R treatments reduced airway IL-5 and -9 levels and eosinophil responses to allergen recall challenge in our model, but not the IL-4 or IgE responses or AHR, others have also reported that administration of anti–IL-10 during the resolution phase of the asthmatic response attenuates the natural resolution of airway eosinophil responses to recall allergen challenge without affecting AHR (45). Given the linkage between IL-4 and B cell IgE production (46), it would not be unexpected that these two parameters might run in parallel, as we had observed.

In our hands, i.p. DC10 treatments led to a state of tolerance sufficiently robust that three allergen challenges across 11 d did not lead to a return of the asthma phenotype, as determined by assessments of AHR, airway eosinophilia and Th2 cytokine responses, and lung-draining lymph node Th2 responses. Others had demonstrated previously that tolerance induced by treatment of asthmatic mice with DCs transfected with an IL-10–lentivirus construct, such that the cells expressed very high levels of IL-10 relative to our DC10, is also robust (14), although retrovirus-transfected DCs would likely not be employed clinically. The concentration of OVA with which we challenged our mice (100 \( \mu \)g/ml) was higher than levels reported previously to induce immediate and late-phase bronchoconstriction responses in allergen-challenged asthmatic individuals (i.e., 15–20 ng HDM or 5 \( \mu \)g/ml ragweed) (21, 22). Moreover, as noted, our preliminary titrations had indicated that aerosol challenge with 100 \( \mu \)g/ml of nebulized OVA induces \( \sim 80\% \) maximal airway eosinophil responses in asthmatic mice.

In conclusion, we have found that IL-10–differentiated DCs differentially impact the AHR and Th2 immunoinflammatory components of the asthma phenotype. As has been reported multiple times, such regulatory cells induce a normalization—in our hands, abrogation—of AHR within 3 wk of treatment; however, although this effect is relatively prolonged, it is nevertheless transient. Within 10–12 wk of the DC10 treatment, AHR returns to pretreatment levels, although it can again be reduced to baseline by retreatment of the asthmatic animals. In contrast, amelioration of the Th2 immunoinflammatory response is progressive over at least 8 mo, when the Th2 phenotype has largely waned. Repeating the
regulatory DC treatments augments the speed of tolerance onset and the depth of the tolerance, which is resistant to reversal by repeated allergen rechallenge. Finally, our data confirm that IL-10 and IDO are involved in the effector phases of this tolerance.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure S1. Characterization of bone marrow DC differentiated as either immature or tolerogenic populations. Bone marrow cells from BALB/c mice were differentiated to a DC phenotype as noted in the Materials and Methods section. The cells cultured with GM-CSF alone were designated as immature cells (Imm), versus those differentiated in the presence of IL-10 (DC10) or, as a relative control, TNF (DCTNF). We assessed the phagocytic activity of the cells by uptake of FITC-dextran, wherein the DC were incubated with 100 μg/ml FITC-dex for 30 min at either 4 or 37°C, washed, then assessed by FACS for fluorescence (left panels). At 37°C, but not at 4°C, both immature DC and DC10 were avidly phagocytic (Mean fluorescent intensities [MFI], 14 and 10, respectively), while neither were so at 4°C (MFI, ≤1.5). (Middle panel) Assessment of CCR5 and CCR7 expression by the differentiated DC populations. The responses of immature DC, DC10 or DCTNF to CCL3 (a CCR5 ligand) and CCL19 (a CCR7 ligand) were assessed using microchemotaxis assays, as indicated in material and methods. The immature and DC10 were highly responsive to CCL3 and modestly responsive to CCL19, while the DCTNF were highly responsive to CCL19, but not CCL3. In addition, we assessed IL-10 and TGFβ secretion by the immature DC and DC10, and show here the release by these cells of both cytokines over 48 h in culture, as determined by ELISA. **, p≤0.001 versus immature DC or DC10 (chemotaxis) or versus immature DC (TGFβ, IL-10 assays). These experiments were repeated three times.

Supplemental Figure S2. Impact of the route by which a single DC10 treatment is administered on airway hyperresponsiveness in asthmatic mice. Asthma was induced, and the animals treated with DC10 or saline as in the Materials and Methods, with the exception that the DC10 were given either t.t., i.p., s.c., or i.v. Depicted are the AHR values of the mice in the t.t., i.p. and s.c. treatment groups at wk 4 (the i.v. treatments had no impact on any of the asthma parameters assessed; data not shown). The AHR of both the t.t. and i.p. DC10 recipients was normalized, while that of the s.c. DC10-treatment mice was not statistically significantly different from the saline-treated asthmatic animals (p≥0.05). N.S., not significantly different versus saline-challenged DC10-treated asthmatic animals. The experiments depicted were repeated 3 times (n≥5 animals/group).
Supplemental Figure S3. Impact of DC10 treatments on the early IgG1 and IgG2a responses and 8-month eosinophil responses. Asthma was induced in BALB/c mice as in the Materials and Methods, and 2 wk later the mice were treated i.p. with saline (asthma), or 1x10^6 OVA-pulsed immature DC (Imm. DC) or DC10 (OVA-DC10), or house dust mite (HDM)-presenting DC10 (HDM-DC10). (A) On dy 21, we assessed the animal’s OVA-specific plasma IgG1 and IgG2a antibodies by ELISA. As a reference, the OVA-specific IgG1 and IgG2a antibody levels in normal mice are at background. (B) At 8 months the animals were exposed one time to an aerosol of 1% OVA, and then sacrificed 24 h later and processed in the Materials and Methods. The airway eosinophil response to allergen recall challenge was determined from differential counts of BAL cells. *, **, p≤0.01 or 0.001 versus Imm. DC recipient mice. The experiments in panel A were repeated 5 times, while the experiments in panel B were repeated twice (n≥5 animals/group).

Supplemental Figure S4. Repeated intraperitoneal or transtracheal administration of DC10 augments T and B cell allergen tolerance in asthma. Asthma was induced, and the animals treated with DC10 or saline as indicated in the Materials and Methods, with the exception that the DC10 were administered biweekly a total of four times (i.e., wk 0, 2, 4, and 6), and they were given either t.t., i.p., s.c., or i.v. At 8 wk, after the animal’s AHR was assessed, the mice were challenged with OVA aerosols, then sacrificed 24 h later. The BAL eosinophil (left panel) and TGFβ (middle panel) levels were assessed as noted. The i.p. and t.t. routes of DC10 delivery were particularly effective in reducing airway eosinophilia, and they were also associated with markedly increased levels of airway TGFβ expression. (right panel) Each of the t.t., i.p., and s.c. treatment modalities brought the serum OVA-specific IgG1 levels down to near baseline in the 8 wk time-frame of the experiment. The i.v. route of DC10 administration had no impact on any of the asthma parameters assessed (data not shown). *, p≤0.01 or **, p≤0.001, versus their relevant saline-treated asthmatic control group. The experiments depicted were repeated 3 times (n≥5 animals/group).