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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

Aarti Nayyar,*† Wojciech Dawicki,*† Hui Huang,* Meiping Lu,* Xiaobei Zhang,* and John R. Gordon†

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.v. 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: 000-000.

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, eosinophils 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 transduced 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

*Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada; †Division of Respiratory, Critical Care and Sleep Medicine, Department of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

†Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada; †Division of Respiratory, Critical Care and Sleep Medicine, Department of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

A.N. and W.D. equally share first authorship of this article.

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Address correspondence and reprint requests to Dr. John R. Gordon, Division of Respiratory, Critical Care and Sleep Medicine, Room 3610, Royal University Hospital, 103 Hospital Drive (Box 120 R.U.H.), Saskatoon, SK S7N 0W8, Canada. E-mail address: john.gordon@usask.ca

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; DC10, IL-10–differentiated DC; HDM, house dust mite allergen; r-DC, IL-10–differentiated DC (transtracheally injected).
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 immuno-inflammatory 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 mAbs (MP-1α, 2M1, and CD40L) (CCL3) and anti-mouse CD11b (M1/70) (3B, and 2D7); FITC-labeled anti-mouse CD11b (clone M1/70), CD14 (clone mouse rC5-3), 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, 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/microcyte Abs (clone CL:A3-1) (Serotec, 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 7.5 ng/ml GM-CSF alone (to generate immature cells) or with 50 ng/ml of either IL-10 (to generate tolerogenic cells; DC10s) or, for some experiments, TNF (to generate immunostimulotis 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 (OVA-DC10) 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 Abs 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-3β, respectively, as the specific ligands, as noted (18). Our DC10s express modestly to markedly lower levels of CD40, CD54, CD80, CD86, and MHCII relative to mature DC-TNF (7); avidly phagocytosed FITC-dextran and were responsive to MIP-1α, but not to MIP-3β; and expressed substantially higher levels of TGF-β, IL-10 than did immature DCs (Supplemental Fig. 1). They were not appreciably contaminated with monocytes (CD14), neutrophils (CD16/32), macrophages (F4/80), B cells (CD19) or T cells (CD3), as determined by FACS (i.e., isotype control-level staining only).

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 (ODbiopt) 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 cyto centrifuge 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. Lower lymphocyte subsets were obtained (ICN Pharmaceuticals, Aurora, IL), as per standard protocols for leukocytes and the supplier’s recommendation (12, 19). The cells were then washed 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 × 106 allergen-pulsed immature DCs, DC10s, or DC-TNFs, administered intratracheally (t.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 overlying their trachea was reflected surgically, and the wound was stapled closed after the DC or saline injection. AHR was measured weekly 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 increase the efficiency 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 × 106 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 individuals, suggesting that our challenge dose was relevant in the context of 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; DasyTech USA, Amberst, 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%/Tve1). This parameter accurately reflects bronchiolar constriction, as opposed to alveolar constriction or airway occlusion (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
hoc 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 $\leq 0.05$.

**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 ($p = 0.01$; 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 eosinophil 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-immune DC– or saline-treated asthmatic animals were rich in eosinophil infiltrates, whereas OVA-DC10 treatment reduced eosinophilia by $59.8 \pm 3.6\%$ ($p < 0.01$, 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-immune 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, $279 \pm 117$ pg/ml; DC10-treated, $94 \pm 23$ pg/ml; IFN: asthmatic, $873 \pm 385$ pg/ml; DC10-treated, $124 \pm 51$ pg/ml), indicating that we had not simply induced Th2 $\rightarrow$ 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 $\sim 3$ 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 ($p \leq 0.001$, 13 wk).
IgE panel represents the background signal in the serum of normal mice (norm.). The experiments in (A) Asthma was induced, and the mice were treated with saline (asthma) or OVA-pulsed immature DCs, DC10s, (OVA-DC10), or HDL-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, \( p < 0.05 \)), IL-5, and IL-13 (both, \( p < 0.001 \); right panel). * or ** \( p < 0.05 \) 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, \( p < 0.01 \); 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 (\( n = 5 \) animals per group). * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) versus immature DC recipient mice.

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 DC10 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. (\( p < 0.01 \) 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 (\( p < 0.01 \); Fig. 3B). Intriguingly, the levels of IL-10 and TGF-\( \beta \) in the airways of the t.t. and i.p. DC10 recipient mice were upregulated, and substantially so (>5-fold) for TGF-\( \beta \) (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,
respectively, whereas the corresponding TGF-β levels were 95 and 338 pg/ml, respectively.

A critical factor related to therapeutic induction of tolerance is that the treated individual be able to resist spurious allergen challenges without reverting to the asthma phenotype. To assess this, we induced tolerance in OVA-asthmatic mice with DC10s 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 ≥ 5 animals per 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 ≥ 5 animals per group).
pulmonary Th2 responses, but is somewhat less effective in re-
asthma phenotype, as determined 4 wk posttreatment in a model
sensitized animals and that DC10s can substantially reverse the
associated with TGF-

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

Others have shown that TGF- (10) or IL-10– (10) differentiated DCS
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 associ-
ated with TGF-β–differentiated DCS (10), or with CD8α+ DCS
(12), is not fully allergen specific, whereas DC10-induced tol-
erance is driven by cognate processes (7, 11). We found that i.v.
delivery of DC10 was completely ineffective in inducing toler-
ance 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,
especially abrogating it within 3 wk, although this normali-
zation 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 dis-
tinct mechanisms. One is IgE/mast cell dependent (30, 31), and
another is eosinophil/IL-5 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 ex-
trinsic 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 1-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 obser-
vations), 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 re-
sponsible for amelioration of AHR. We (H. Huang, W. Davwicki,
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 con-
sistent with the argument that the DC10’s IL-10 secretion may
initiate normalization of AHR. Our DC10 tracking data also indi-
cate that the numbers of treatment DC10s decline in the lungs 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 obser-
vations), 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 upregu-
lates regulatory T cell function and thereby dampens AHR in a
rat model of asthma, and that this suppression of bronchial re-
sponsiveness 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 em-
ploy 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
metric relationship exists between the numbers of Ag-presenting DCs and the ensuing immune response (41). Our DC10s were CCR7lo, whereas at 4–8 mo this effect had increased to ≥80%. Similarly, at 3 wk the eosinophil responses to allergen challenge were ~60% below those at the time of treatment, and at 8 mo posttreatment this had increased to ~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 ~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 sensitivity 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+; 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+ TGF-β 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 α4β7 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 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 μg/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 μg/ml ragweed) (21, 22). Moreover, as noted, our preliminary titrations had indicated that aerosol challenge with 100 μg/ml of nebulized OVA induces ~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


