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Regulatory dendritic cell expression of MHCII and IL-10 are jointly requisite for induction of tolerance in a murine model of OVA-asthma

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Abstract

Background: Allergen-presenting dendritic cells differentiated with IL-10 (DC10) reverse the asthma phenotype in mice by converting their Th2 cells to regulatory T cells (Tregs). DC10 express elevated levels of IL-10, but substantially reduced levels of MHCII and costimulatory molecules, so the relationships between these factors with each other and tolerogenicity have not been clearly elucidated.

Methods: We assessed the roles of these inputs in DC10 reversal of OVA-associated asthma-like disease by treating affected mice with OVA-pulsed DC10 generated from wild-type or IL-10-sufficient MHCII^{-/-} or CD80/CD86^{-/-} mice, or with MHCII-intact IL-10-silenced DC10.

Results: IL-10 silencing did not discernibly affect the cells' immunobiology (e.g., costimulatory molecules, chemokines), but it eliminated IL-10 secretion and the cell's abilities to induce tolerance, as determined by assessments of airway hyper-responsiveness, eosinophilia, and Th2 responses to recall OVA challenge. MHCII^{-/-} DC10 expressed normal levels of IL-10, but, nevertheless, were unable to induce allergen tolerance in asthma phenotype mice, while tolerance induced by CD80/CD86^{-/-} DC10 was attenuated but not eliminated. We also assessed the induction of multiple Treg cell markers (e.g., ICOS, PD-1, GITR) on pulmonary CD25⁺ Foxp3⁺ cells in the treated mice. Wild-type DC10 treatments upregulated expression of each marker, while neither IL-10-silenced nor MHCII^{-/-} DC10 did so, and the CD80/86^{-/-} DC10 induced an intermediate Treg cell activation phenotype.

Conclusion: Both IL-10 and MCHII expression by DC10 are requisite, but not sufficient for tolerance induction, suggesting that DC10 and Th2 effector T cells must be brought together in a cognate fashion in order for their IL-10 to induce tolerance.

As professional antigen-presenting cells (APCs), dendritic cells have an important role in the control of the adaptive immune responses. Activation of T cells by APCs requires three signaling events, one elicited by T-cell receptor (TCR) recognition of the major histocompatibility complex (MHC)/peptide complex

presented by the APC, another by the APCs' costimulatory molecules (e.g., CD80, CD86), and the third by a T cell-polarizing signal that emanates from the stimulated APCs (e.g., IL-12 or IL-10). Depending on the nature of these three signals, the dendritic cells can induce either effector T-cell (Teff cell) or regulatory T-cell (Treg) responses (1). It is thought that dendritic cells that express reduced levels of MHCII and costimulatory molecules thereby present antigen in an inherently inefficient manner, leading to T-cell unresponsiveness or anergy (2), but tolerogenic dendritic cells can also upregulate

Abbreviations

AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; DC10, IL-10-differentiated dendritic cells; Teff cell, effector T cell; Treg cell, regulatory T cell; siRNA, small interfering RNA.

the activity of CD4⁺CD25⁺Foxp3⁺ Treg cells (3). IL-10 has been implicated in the induction and maintenance of T-cell tolerance (4). It can reduce expression of MHC class II and costimulatory molecules on dendritic cells and thereby induce anergy (2), but IL-10-exposed dendritic cells can express IL-10 themselves and thereby augment Treg cell responses (5).

There have been numerous reports of IL-10-treated or IL-10-exposed dendritic cells influencing tolerance in mouse models and in people (6). Instillation of allergen-presenting DC10 into the airways of systemically sensitized mice reduces the establishment of the asthma phenotype following airway allergen challenge and that is at least in part dependent on the dendritic cell's IL-10 expression (7). However, this leaves open the question of whether the IL-10 that is secreted by dendritic cells operates in the context of cognate dendritic cell–Th2 Teff cell interactions or whether it may exert its tolerogenic influence through bystander effects. For example, infusion of IL-10 into the airway (8) and airway epithelial transfection with an IL-10-expressing adenovirus (9) both reportedly can dampen allergen-driven Th2 responses. Treatment of asthma phenotype mice with DC10 (10–12) or with IL-10 lentivirus-transfected dendritic cells (13) can fully reverse even well-entrenched disease. Similarly, DC10 generated from asthmatic individuals can induce autologous Th2 cell tolerance *ex vivo*, and they also do so by fostering the outgrowth of CD25⁺Foxp3⁺ Treg cells from within the donor's Th2 Teff cell populations. These Treg cells express the activation markers lymphocyte activation gene 3 (LAG3) and cytotoxic T-lymphocyte antigen-4 (CTLA4) (11). In mouse models, DC10 induce Th2 Teff cells to transdifferentiate into Treg cells, and these activated Treg cells also express an array of activation markers, including LAG3, CTLA4, inducible costimulatory molecule (ICOS), programmed cell death-1 (PD-1), and glucocorticoid-induced TNF receptor-related protein (GITR) (10). Despite our knowledge regarding the capacity of DC10 to induce such tolerance, the contributions to tolerance outcomes of each of the three antigen-presenting signals offered by these cells and their relationship(s) to one another have not been rigorously investigated.

Herein, we critically assessed the roles that the MHC–Ag–TCR complex, CD80/CD86 (i.e., costimulatory molecules), and IL-10 have in DC10-induced asthma-like disease tolerance. We generated DC10 from the bone marrow of H-2Ia^{b-/-} (i.e., MHCII^{-/-}) or CD80/86^{-/-} mice and silenced IL-10 expression in wild-type DC10 using IL-10 small interfering RNA (siRNA), used these cells to treat OVA-asthma phenotype mice, and then characterized their impact on the asthma phenotype and on pulmonary Treg cell activation.

Materials and methods

Mice

Wild-type CD45.2⁺ and congenic CD45.1⁺ female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA), while CD80/86 and H-2Ia^b gene knock-out (KO) mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, MN). All mice

were treated according to the guidelines of the Canadian Council on Animal Care.

Generation of DC10 and establishment of asthma phenotype in mice: ELISA, airway hyper-responsiveness, and chemotaxis assays

The generation of DC10 has been described previously (10, 12, 14). Briefly, bone marrow cells were seeded in RPMI-1640 supplemented with 1% antibiotics/antimycotics, 50 μ M 2-mercaptoethanol, and 10% heat-inactivated FCS containing 20 ng/ml GM-CSF. On day 10, the nonadherent (dendritic) cells were resuspended in complete medium supplemented with 7.5 ng/ml GM-CSF alone (to generate immature cells; DC-GMCSF) or with 50 ng/ml of either IL-10 (to generate tolerogenic cells; DC10) or TNF (to generate immunostimulatory DC-TNF). On day 13, the cells from each culture were pulsed for 2 h at 37°C with 50 μ g/ml stock commercial OVA (Grade V, Sigma Chemical Co, Mississauga, ON; OVA–DC10) that was not specifically endotoxin depleted and then the cells were washed.

To induce the asthma phenotype, mice were sensitized with two i.p. injections (week 0 and 2) of 2 μ g of OVA/alum, followed by 20-min exposures on days 30, 32, and 34 to nebulized aerosols of 1% OVA in saline (15). Two weeks after the last aerosol exposure, the animals were treated with 1×10^6 DC given i.p. and then assessed for airway hyper-responsiveness (AHR) 4 weeks thereafter. One day later, the animals were exposed for 20 min to nebulized aerosols of 1% OVA, and 48 h later, they were killed to assess their airway recall responses to the OVA challenge. Cytochrome slides of airway cells obtained by bronchoalveolar lavage (BAL) were stained with Wright's solution (16) to generate differential cell counts, while the BAL fluids were aliquoted and stored at –80°C prior to assay for cytokines. Single-cell suspensions of lung parenchymal cells were generated by enzymatic dispersal of the tissues (15), and lung CD4⁺ T cells were isolated by magnetic sorting (12), as noted. All samples from each animal were processed and assayed independently. We employed standard capture ELISAs to assess IL-4 (clone 30340), IL-5 (clone TRFK5), IL-9 (CI 222604), IL-13 (Clone 38213), IL-10 (clone JES052A5), IL-12 (Clone 48110), and IFN γ (Clone 37801/37875), with matched capture and detection antibody pairs and recombinant cytokine standards from R&D Systems (Minneapolis, MN). Cell culture supernatants and BAL fluids were not diluted, but plasma samples were diluted 1 : 10 in PBS. The detection limits for our cytokine ELISAs are routinely 5–10 pg/ml. For the IgE ELISA, IgE in the plasma was captured using goat anti-mouse IgE and the OVA-specific antibody therein detected by the use of biotinylated OVA (17); these data are expressed as relative IgE optical density (OD₅₉₅) units.

Measurement of AHR

Airway hyper-responsiveness was assessed in conscious animals by head-out, whole-body plethysmography, as noted in detail (14, 16). Briefly, air was supplied to the body compartment of a plethysmograph via a small animal ventilator, and changes

in the airflow through the body compartment were monitored. Doubling doses of nebulized methacholine (0.5–20 mg/ml) were delivered to the head compartment, and bronchoconstriction data were gathered as running 1-s means of the airflow at the 50% point in the expiratory cycle (Flow@50%TVe1). This parameter accurately reflects bronchiolar constriction, as opposed to alveolar constriction or airway occlusion, as noted (17, 18).

Tracking of DC10 *in vivo*

Allergen-loaded CD45.1⁺ congenic B6 mouse DC10 were injected i.p. into asthma phenotype CD45.2⁺ C57BL/6 mice (5×10^6 cells/mouse), as above. After 2, 7, 14, and 21 days, we collected the lungs, spleens, cervical lymph nodes (Cer. LN), and mediastinal (i.e., lung-draining) LN from each animal and generated single-cell suspensions by either mechanical (spleens, lymph nodes) or enzymatic (lungs) dispersal of the tissues (16). In addition, we performed BAL on the recipients to recover airway cells. Each cell population was analyzed by flow cytometry (Beckman Coulter Epics XL, and FlowJo v.8 software).

Silencing of IL-10 expression by DC10

The IL-10 siRNA sequences employed were designed using online software (Qiagen, Valencia, CA); BLAST searches were carried out to ensure that the sequences would not target other transcripts. The target sequence of the IL-10 siRNA was 5'-CA GGGATCTTAGCTAACGGAA-3'. The sense and antisense alignments for the siRNA were 5'-GGGAUCUUAGCUA ACGGAAtt-3' and 3'-gtCCCUAGAAUCGAUUGCCUU-5', respectively. The siRNAs were transfected into DC10 using a commercial kit (RNAi human/mouse starter kit, Qiagen), according to the supplier's protocol. A nonsilencing random nucleotide control siRNA of no known specificity was transfected into wild-type DC10 as an irrelevant siRNA control. All ribonucleotides were synthesized and annealed by the supplier (Qiagen). Modified Boyden chamber microchemotaxis assays were used to assess the responsiveness of the cells to the CCR5 and CCR7 ligands CCL3 and CCL19, respectively, as noted previously (19).

Quantitative RT-PCR

The specific PCR primers were as follows: IL-10 sense, 5'-AA GCCTTATCGGAAATGATCCA-3'; IL-10 antisense, 5'-GCT CCACTGCCTTGCTCTTATT-3'; β -actin sense, 5'-AGAGG GAAATCGTGCCTGAC-3'; and β -actin antisense, 5'-CAAT AGTGATGACCTGGCCGT-3'. The conditions employed for the qRT-PCR reactions were as follows: cDNA synthesis, 50°C for 30 min; PCR, 1 cycle at 95°C for 5 min, 35 cycles at 95°C for 15 s; and 60°C for 1 min. All PCR products were resolved in 1% agarose gels with ethidium bromide staining.

Activation markers of Treg cells

Dendritic cells differentiated with IL-10 generated from wild-type CD80/86^{-/-} or MHCII^{-/-} mice, IL-10-silenced DC10

from wild-type mice (1×10^6 cells/mouse), or saline were injected i.p. into asthma phenotype mice, as above. At 3 weeks post-treatment, we purified T cells from the lungs and lung-draining lymph nodes using nylon wool columns and stained them with FITC-CD4, PE-cy5-Foxp3, and PE-labeled antibodies against the indicated Treg cell markers. The CD4⁺ cells were gated and then Foxp3⁺PE⁺ cells were assessed for the expression of CD25 (clone 3C7), ICOS (clone 15F9), PD-1 (clone J43), GITR (clone DTA-1), LAG3 (clone C9B7W), and CTLA-4 (clone UC10-4B9) by flow cytometry using antibodies purchased from eBioscience (San Diego, CA).

Statistical analysis

All data were analyzed with the aid of a commercial software program (GraphPad Prism 5.0, LaJolla, CA). Multigroup comparisons were assessed by either one-way ANOVA with Tukey's *post hoc* testing or by Wilcoxon signed rank tests (FACS analyses). Values of $P < 0.05$ were considered significant.

Results

DC10 delivered i.p. migrate to the lungs and draining lymph nodes

We have reported previously that the onset of tolerance in DC10-treated asthma phenotype mice is progressive, such that AHR is moderately diminished in mice at 2 week after treatment and disappears entirely within 3 week of treatment, while the airway Th2 responses to recall allergen challenge (i.e., eosinophilia, Th2 cytokines) lag behind somewhat (12, 19). We questioned whether there were temporal relationships between the onset of this tolerance and trafficking of the DC10 within the recipients. Others have reported that leukocytes within the peritoneum traffic via the diaphragmatic lymphatics to the thoracic duct and thereby into the peripheral circulation, although materials in the pleural cavity move to the mediastinal lymph node (20). We previously noted as unpublished observations our attempts to track DC10 that had been labeled with the fluorescent dye DiI, wherein we were unable to unequivocally conclude that the fluorescent cells detected in the target organs were intact DiI-stained DC10, as opposed to phagocytic cells that had engulfed effete DiI-stained DC10 or cells that had nonspecifically acquired the DiI stain (21). Herein, we employed the cell surface CD45.1 as a definitive marker of intact DC10. Thus, we injected asthma phenotype CD45.2⁺ B6 mice i.p. with DC10 generated from the bone marrow of CD45.1⁺ congenic B6 mice and used FACS to detect the CD45.1⁺ cells in an array of organs across 3 weeks (Fig. 1). At 2 days post-transplant, a substantial proportion of the cells recovered from the airways (BAL) and lung parenchyma (Lung par.) were CD45.1⁺ (i.e., the injected DC10), with modest numbers also appearing in the lung-draining mediastinal lymph nodes (Med. LN), while few DC10 were detectable in the spleens or cervical lymph nodes (Cer. LN). At 1 week, the proportions of DC10 in the lungs and mediastinal lymph nodes peaked, and some DC10 were evident at this time in the spleens, but

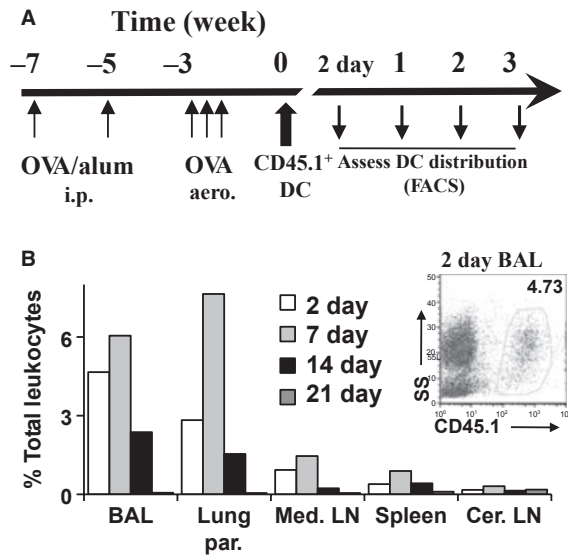


Figure 1 IL-10-differentiated dendritic cells that are delivered intraperitoneally to asthma phenotype mice migrate to the lungs and draining lymph nodes. IL-10-differentiated dendritic cells (DC10) were generated from the bone marrow of CD45.1⁺ B6 mice and injected into asthma phenotype congenic CD45.2⁺ B6 recipient mice as indicated in the Materials and methods section. After 2, 7, 14, or 21 days, the mice were killed and the proportions of CD45.1⁺ cells among total cells recovered from their airways (BAL), lung parenchyma (Lung par.), mediastinal lymph nodes (Med. LN), spleens, and cervical lymph nodes (Cer. LN) were assessed by flow cytometry. One representative experiment of two is shown ($n = 5$ mice/group).

not in the cervical lymph nodes. When we assessed the absolute numbers of CD45.1⁺ DC10 in these systems at this point in time, we found 0.45×10^5 DC10 in the BAL, 7.64×10^5 DC10 in the parenchyma of the lungs, 0.44×10^5 DC10 in the mediastinal lymph nodes, and 7.12×10^5 DC10 in the spleens of our animals. Thus, while there was a predilection for the DC10 to traffic to the lungs, an equal proportion of the cells had found their way into the spleen at this time. The numbers of DC10 were markedly reduced within each site at 2 week post-treatment, and by 3 week, DC10 were no longer discernible in either the peripheral organs or the lymph nodes. We found negligible numbers of marker-positive cells in the blood, bone marrow, or liver at any time (data not shown). These data indicate that DC10 that are delivered intraperitoneally increasingly accumulate in the asthma target organ, the lungs, and the lung-draining lymph nodes across 1 week after passive transfer and that they continue to be present at 2 weeks after treatment. We have reported previously that DC10 treatment is associated with activation of pulmonary Treg cells beginning at 1 week after treatment (10), although significant effects of DC10 on AHR are first discernible in asthma phenotype mice at 2 week (12, 19). This suggests that the DC10 treatment may well engage their target Th2 Teff cells *in situ* in the lungs or lung-draining lymph nodes.

IL-10 secretion is essential to DC10-induced tolerance in established asthma

As a prelude to assessing whether IL-10, MHCII, or co-stimulatory molecule-compromised DC10 can induce tolerance in our model, we first confirmed that DC10, but not specific allergen-presenting control GM-CSF-differentiated immature (DC-GMCSF) or TNF-differentiated immunostimulatory (DC-TNF) dendritic cells, can do so. We induced the asthma phenotype and treated the animals with the various dendritic cells as indicated in the Materials and Methods section and as depicted schematically in Fig. 2A. We confirmed that at 4 week after DC10 treatment, the airways of the mice were tolerant of allergen aerosol challenge, as determined by assessments of airway eosinophilia (Fig. 2B), mixed Th1/Th2 airway cytokine responses (Fig. 2C,D), and development of lung parenchymal T-cell Th2 cytokine responses (Fig. 2E). On the other hand, mice treated with DC-TNF developed asthma-phenotype airway eosinophilia, airway Th2 cytokine responses (Fig. 2B,C), and AHR (data not shown), while mice treated with DC-GMCSF displayed a robust AHR, airway eosinophilia, and lung Th2 cytokine responses (data not shown), and lung parenchymal CD4⁺ T-cell Th2 cytokine responses (Fig. 2E).

As noted, IL-10 expression by DC10 has been reported to play a role in preventing localization of allergic disease to the lungs in systemically sensitized mice (7), but we wished to confirm whether IL-10 also plays a role in DC10-mediated reversal of the asthma phenotype in our model. To test this, we first generated DC10 in which IL-10 expression had been silenced using siRNA approaches and then used these cells to treat the mice. We assessed the efficiency of our DC10 transfection protocols using a fluorescent control siRNA construct and found that at 48 h after transfection, essentially all DC10 in our cultures were siRNA positive (Fig. 3A). We then optimized the dose of IL-10-specific siRNA required to negate IL-10 secretion. Transfection of DC10 with 40 nmol siRNA essentially eliminated IL-10 secretion by our DC10, even after they had been stimulated overnight with LPS (1 μ g/ml; Fig. 3B), and brought DC10 IL-10 mRNA levels to background (Fig. 3C), while transfection with 40 nmol scrambled nonsense siRNA had no discernible impact on IL-10 expression (Fig. 3B). We also evaluated the potential toxicity of the siRNA and transfection reagents for our DC10 by measuring their viability at 24 h after transfection, as determined using annexin V and propidium iodine (PI) staining to assess apoptosis and necrosis, respectively (Fig. 3D). The results confirmed that neither reagent affected DC10 viability. We also assessed the abilities of the control siRNA and IL-10 siRNA-transfected cells to phagocytose FITC-dextran, as a surrogate antigen, and to respond to CCL3 and CCL19, as ligands for the tissue inflammatory chemokine receptor CCR5 and the lymph node-homing chemokine receptor CCR7, respectively. IL-10-silencing had no impact on the DC10's phagocytic activities or chemotactic responses to dendritic cell-relevant chemokines and had no discernible impact on the cell's expression of MHCII,

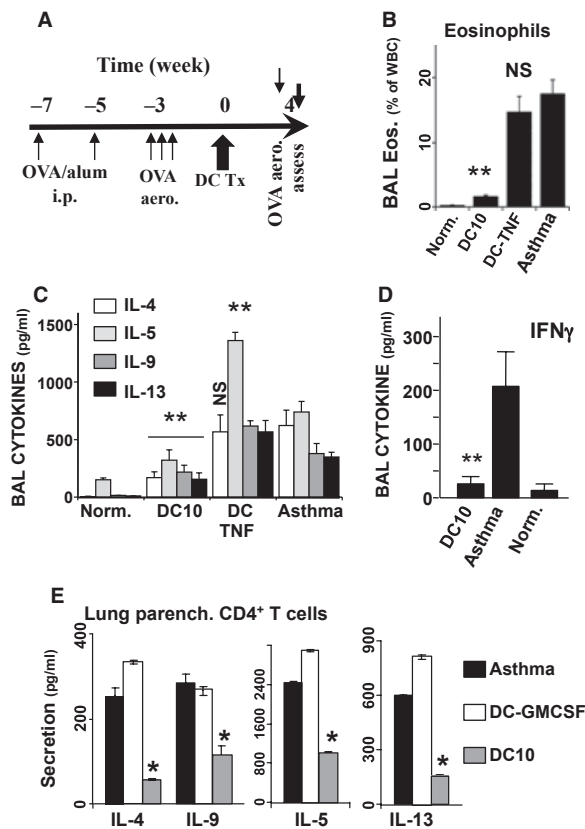


Figure 2 DC10, but not control immature or immunostimulatory DC, reverse airway hyper-responsiveness and induce Th2 tolerance in a mouse model of asthma. An asthma phenotype was induced in B6 mice by systemic sensitization with OVA–alum and repeated airway challenge with 1% OVA aerosols. Two weeks after the last aerosol exposure, the animals were treated with 1×10^6 OVA-presenting immature dendritic cells (DC-GMCSF) or cells that had been differentiated in the presence of IL-10 (DC10) or TNF (immunostimulatory DC; DC-TNF), and 27 days later, the mice were assessed for airway hyper-responsiveness (AHR). On day 28, they were challenged again with 1% OVA aerosols and 2 days later harvested for assessments of airway (bronchoalveolar lavage; BAL fluid) eosinophils and cytokines. The animal's lung parenchymal T cells were purified by CD4 MACS, cultured for 2 days, and then the culture supernatants were assayed for Th2 cytokines, as depicted. (A) Schematic of the experimental protocol. (B) BAL eosinophilia. (C, D) BAL Th1 and Th2 cytokines levels. (E) Lung parenchymal T-cell Th2 cytokine expression. Airway eosinophil and mixed Th1/Th2 cytokine responses were significantly dampened by the DC10, but not the control dendritic cell treatments. These data are representative of three experiments. NS, *, **, $P > 0.05$, or ≤ 0.05 and 0.01 , respectively, relative to the responses of the asthma phenotype mice. Results are expressed as the mean $\pm$ SEM.

CD11c, CD40, CD54, CD80, or CD86, as determined by flow cytometry (data not shown). These data confirmed that silencing IL-10 expression by DC10 did not significantly alter their expression of these antigen presentation-associated markers or parameters.

We next evaluated the impact of IL-10 silencing on the therapeutic effectiveness of DC10 in our mouse model. We treated asthma phenotype B6 mice with saline or 1×10^6 wild-type, IL-10-silenced, or scrambled nonsense siRNA-transfected DC10 and 25 days later assessed the treated animal's AHR to methacholine, as well as that of normal healthy control mice (Fig. 4A). The airways of the saline-treated asthma phenotype animals were fully hyper-responsive to methacholine, while those of wild-type DC10- and negative control siRNA-transfected DC10-treated animals were normoresponsive. On the other hand, the IL-10-silenced DC10 were completely ineffective in reducing AHR in these animals. One day after assessing their AHR, we challenged the animals with aerosolized allergen (1% OVA, 20 min), and 2 days later, we killed the animals and examined their airways for evidence of Th2 recall responses to the allergen challenge, looking at BAL fluid eosinophils and Th2 cytokines (IL-4, IL-5, IL-9 and IL-13). The wild-type and scrambled nonsense siRNA-transfected DC10 treatments brought each of these parameters to near baseline, while IL-10 silencing again negated the tolerogenic activities of the DC10 (Fig. 4B,C). Taken together, these data indicate that IL-10 expression by DC10 is essential for their tolerogenic activities in our model.

Induction of tolerance by DC10 is also critically dependent on MHCII–TCR interactions

As suggested above, in principle, the IL-10 that is secreted by DC10 could affect tolerance through bystander effects alone, without a need for cognate T cell–APC interactions. Infusion of recombinant IL-10 into the airway is known to reduce Th2 immunoinflammatory responses in experimental asthma (8). Thus, we next assessed whether DC10 that do not express a MHCII molecule (i.e., cannot engage T cells through their TCR) or CD80 and CD86 are compromised in their abilities to induce tolerance. For this and the next experiment, we generated DC10 from the bone marrow of MHCII^{−/−} and CD80/CD86 double-knockout mice, incubated them with specific allergen *in vitro*, and then used them to treat asthma phenotype mice, as above. We assessed whether these DC10 secreted IL-10 at levels observed in wild-type cells and found no differences in IL-10 expression between the three populations of cells. The wild-type DC10 released 347 ± 138 pg/ml over 48 h, while the MHCII^{−/−} DC10 secreted 241 ± 44 pg/ml IL-10 and the CD80/CD86^{−/−} DC10 secreted 249 ± 55 pg/ml of IL-10 (for both knockout DC10 populations, $P \geq 0.05$ vs wild-type DC10).

When we assessed the AHR of the mice at ≈ 4 week post-treatment, we found that the recipients of the MHCII^{−/−} DC10 still presented with an asthma phenotype AHR ($P \geq 0.05$ vs saline-treated asthma phenotype mice), while the animals that had been treated with wild-type DC10 were normoresponsive (Fig. 5A). This loss of the ability to affect AHR applied also to the airway eosinophil and IL-4, IL-5, IL-9, and IL-13 responses to recall allergen challenge (Fig. 5B,C). In each case, there were no statistically significant differences in the responses of the saline- and

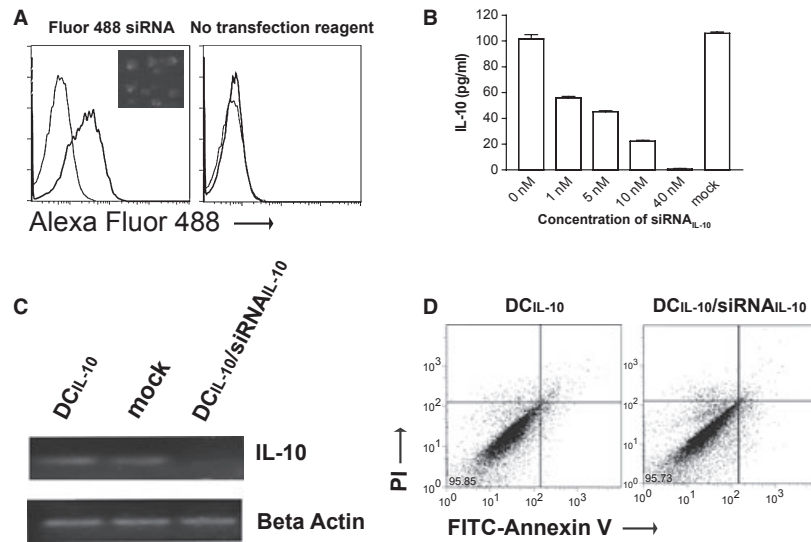


Figure 3 Optimization of IL-10 silencing in DC10 by transfection with IL-10-specific siRNA. (A) DC10 cells were transfected with Alexa Fluor 488-labeled control siRNA, and the efficiency of transfection was evaluated by flow cytometry and fluorescence microscopy 48 h later. (B) IL-10 secretion by DC10 that were transfected with the indicated levels of IL-10-specific siRNA or with 40 nmol scrambled nonsense control siRNA was assessed by ELISA 48 h after

transfection. (C) Messenger RNA harvested from untreated DC10 or DC10 treated with 40 nmol IL-10-specific (siRNA) or nonsense control (Scram) siRNA was submitted for qRT-PCR analysis of IL-10 mRNA expression, and the PCR products were visualized in ethidium bromide-stained agarose gels. (D) Assessments of the impact of IL-10 silencing on DC10 apoptosis and necrosis, as determined by flow cytometry using annexin V and propidium iodide (PI) staining.

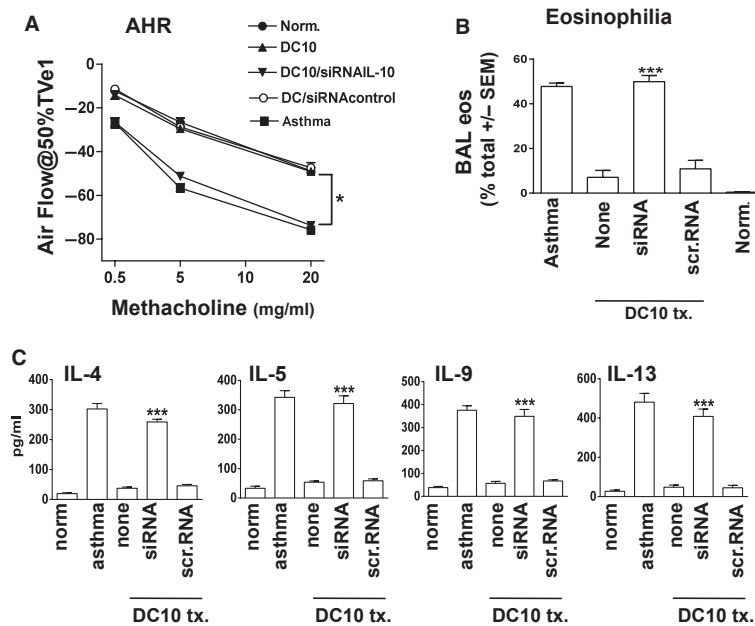


Figure 4 Silencing of IL-10 secretion by DC10 eliminates their therapeutic effectiveness in a mouse model of asthma. An asthma phenotype was induced in B6 mice as in Fig. 2, after which the animals were treated with saline (asthma) or 1×10^6 allergen-presenting otherwise-untreated DC10 (none), IL-10-silenced DC10 (DC10/siRNA), or nonsense control scrambled siRNA-transfected DC10 (DC10/scr.RNA). Healthy normal mice (norm.) were included as a control group. (A) After 27 days, the animal's AHR to

aerosolized methacholine was assessed as noted in Fig. 2. The following day, the animals were challenged with nebulized aerosols of 1% OVA, and 48 h later, we assessed their airway (B) eosinophil and (C) Th2 cytokine (IL-4, IL-5, IL-9, and IL-13) responses to the recall allergen challenge, as determined from assessments of BAL fluids. The graphs depict the pooled data from three experiments. *, ***, $P < 0.05$, or 0.001 vs the DC10-treated group animals ($n = 5$ mice/group). Results are expressed as the mean $\pm$ SEM.

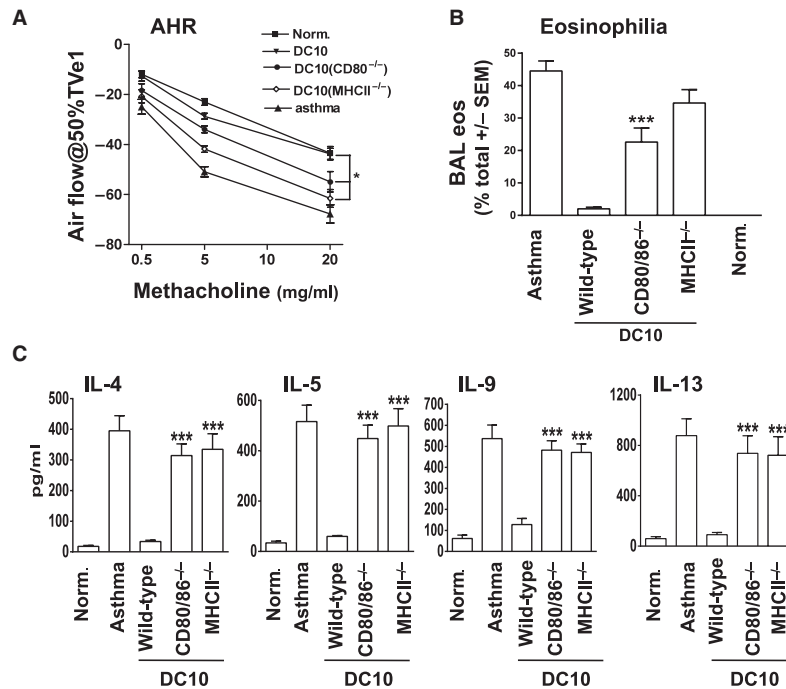


Figure 5 Successful induction of disease tolerance is dependent on both MHCII and costimulatory molecule expression by the treatment DC10. An asthma phenotype was induced in B6 mice as noted in Fig. 2, after which the animals were treated with saline (asthma), or 1×10^6 wild-type DC10, or DC10 generated from CD80/CD86^{-/-} or MHCII^{-/-} mice. Healthy normal mice were included as a control group. (A) After 27 days, the animal's AHR to aerosolized methacholine

was assessed as noted in Fig. 2. The following day, the animals were challenged with nebulized aerosols of 1% OVA, and 48 h later, we assessed their airway (B) eosinophil and (C) Th2 cytokine (IL-4, IL-5, IL-9, and IL-13) responses to the recall allergen challenge. The graphs depict the pooled data from three experiments. *, ***, $P < 0.05$, or 0.001 vs the DC10-treated group animals ($n = 5$ mice/group). Results are expressed as the mean $\pm$ SEM.

MHCII^{-/-} DC10-treated asthmatic mice ($P \geq 0.05$), while asthma phenotype mice that had been treated with wild-type OVA-presenting DC10 displayed a tolerant phenotype.

Interestingly, the AHR of the mice treated with the CD80/86^{-/-} DC10 fell midway between that of the asthma phenotype and the normal mice (Fig. 5A), while their recall airway eosinophil and Th2 cytokine responses to OVA aerosol challenge were unaffected by the CD80/86^{-/-} DC10 treatment (Fig. 5B, C). Indeed, these costimulation-attenuated DC10 were no different than MHCII^{-/-} DC10 in terms of their abilities to affect the airway Th2 cytokine response (Fig. 5C). These data suggest that IL-10 expression alone is insufficient to render DC10 tolerogenic and that they must engage their target Th2 Teff cells in a cognate (i.e., MHCII-dependent) fashion in order for their IL-10 to induce a Th2 to Treg cell phenotype switch. Moreover, optimal phenotype switching also required participation of CD80 and/or CD86, likely because of their contributions to high-avidity immunological synapse formation.

IL-10 expression and MHCII expression by DC10 are essential for activation of Treg cells in DC10-treated asthmatic mice

We have recently reported that DC10 treatments lead to conversion of Th2 Teff cells into CD4⁺CD25⁺Foxp3⁺ Treg cells

in the lungs of treated mice (10) and that these Treg cells display augmented levels of the activation markers ICOS, PD-1, GITR, LAG-3, and CTLA-4 (10). We thus sought to determine whether the altered antigen-presenting skills of our various compromised DC10 also translated into a reduced ability to activate pulmonary Treg cells in our treated mice. We treated asthma phenotype animals with saline, or with wild-type, IL-10-silenced, MHCII^{-/-} or CD80/CD86^{-/-} DC10, and 3 weeks later, we purified T cells from the lungs of these mice using nylon wool columns and assessed their expression of each of these markers by FACS. We obtained $\approx 3 \times 10^6$ T cells from the lungs and lung-draining lymph nodes of each mouse, irrespective of the treatment group. Furthermore, consistent with previous reports that induction of tolerance does not lead to changes in the numbers of CD25⁺Foxp3⁺ cells in these compartments in rodent models of asthma, but rather to heightened Treg cell activation (e.g., ref. 10, 12), we saw no differences in the levels of these cells in our animals (Fig. 6, upper panel). However, it was readily apparent that the OVA-presenting DC10 upregulated expression of each marker as we had observed previously, while the pulmonary CD4⁺ T cells of the IL-10-silenced or MHCII^{-/-} DC10-treated mice did not upregulate these markers above the levels seen in asthma phenotype mice (Fig. 6). Interestingly, the CD80/CD86^{-/-} DC10 induced an intermediate level of Treg cell activation relative to

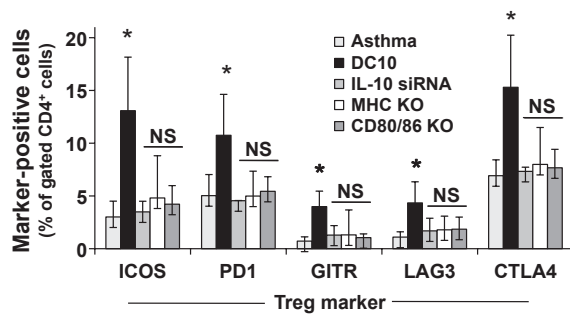


Figure 6 DC10 upregulate activation marker expression on CD4⁺Foxp3⁺ Treg cells in asthma phenotype mice in an IL-10-, MHCII-, and costimulatory molecule-dependent fashion. DC10 generated as in Figure 4 were used to treat asthma phenotype B6 mice. At 3 week post-treatment, we purified T cells from the lungs and lung-draining lymph nodes of the treated mice and stained them with fluorochrome-labeled antibodies against CD4, Foxp3, and the Treg cell activation markers CD25, ICOS, PD-1, GITR, LAG3, or CTLA-4 (upper panel). The proportions of Foxp3-positive cells that expressed each marker are depicted graphically. One representative experiment of two is shown. NS and * indicated not statistically significant and $P \leq 0.05$, respectively, vs asthma phenotype cells. Results are expressed as the mean $\pm$ SD.

that seen with wild-type, IL-10-silenced or MHCII^{-/-} DC10 (Fig. 6). This experiment confirmed that both IL-10 expression and an ability to engage target Th2 cells in a cognate fashion are critical to induction of tolerance by DC10 in this model, but also that the costimulatory molecules CD80 and/or CD86 are also involved in this process.

Discussion

In this study, we assessed the importance of IL-10 and MHCII expression by cognate allergen-presenting DC10 on their induction of immunologic tolerance in asthma phenotype mice, but also the role(s) of costimulatory molecule expression by these cells. Specifically, we used DC10 that were deficient in MHCII or CD80 and CD86, or IL-10-silenced DC10, and found in each case that the deficiency reduced the tolerogenic activity of these cells as determined by assessments of AHR and Th2 immunoinflammatory responses to recall allergen challenge. IL-10 silencing did not discernibly affect the viability of the DC10, their expression of MHCII or costimulatory molecules, or their phagocytic or chemotactic activities, indicating that it was indeed their IL-10 that was the critical effector molecule for these DC10. On the other hand, MHCII^{-/-} DC10, which expressed wild-type DC10 levels of IL-10, were also incapable of inducing tolerance, while IL-10-sufficient CD80/CD86^{-/-} DC10 were suboptimal in their abilities to induce tolerance.

IL-10 is a key immunomodulatory cytokine, with both immunosuppressive and immunostimulatory effects on T cells having been reported (22). It can directly inhibit T-cell growth (2) and induce long-lasting antigen-specific

T-cell anergy (23). IL-10 knockout mice do not develop AHR after allergen sensitization and challenge despite a significant pulmonary inflammatory response that includes increased airway eosinophilia (24), while IL-10 infusion into the airways of asthma phenotype mice dampens their responses to recall allergen challenge (8). It had been reported that, unlike wild-type DC10, DC10 that are generated from the bone marrow of IL-10 knockout mice are unable to prevent the development of the asthma phenotype in a mouse model (7). Whether the IL-10 released by these DC is critical for reversal of the asthma phenotype in animals with well-established disease (10, 12, 19) had not been assessed, nor had it been determined whether cognate T-cell engagement by the IL-10-expressing DC10 is critical to that tolerance. Our data indicate that the dendritic cell indeed must engage target Th2 Teff cells in a cognate manner in order for the IL-10 they secrete to productively affect the latter cells. It has been reported that expression of IL-10 by the host's cells, presumptively their Treg cells, is also critical for the induction of tolerance in asthmatic mice, even when the treatment dendritic cells have been engineered to express exceptional levels of IL-10 (13). We have shown previously that DC10 induce the host's effector Th2 cells to transdifferentiate into a CD25⁺Foxp3⁺ Treg cell phenotype and that IL-10 expression by these Treg cells drives their suppression of Th2 responses, at least *in vitro* (10). IL-10-silenced DC10 did not induce tolerance despite the fact that the recipient's Treg cells would be fully capable of secreting IL-10, suggesting that the lack of tolerance was attributable to a failure to induce or activate Treg cells by the IL-10-compromised DC10. In keeping with this, our data confirm that while wild-type DC10 treatments led to robust activation of the host's Treg cells, as determined by expression of ICOS-1, PD-1, GITR, LAG-3, and CTLA4, IL-10-silenced DC10 induced no such Treg cell activation. We had documented previously that DC10-induced Treg cells upregulate each of these activation markers (10–12, 19). Thus, our data confirm in another way the importance of IL-10 expression by DC10 in the development of Treg cells.

As suggested above, the fact that IL-10-intact, but MHCII^{-/-} DC10 were also unable to induce tolerance clearly indicates that DC10 must be engaged in cognate interactions with T cells in order for their IL-10 to have a significant impact. This is of substantial significance in the therapeutic setting, because it indicates that tolerance induced by DC10 will be exquisitely antigen specific and therefore not lead to broad immunosuppression. Notwithstanding, while Treg cell activation occurs via TCR signaling and is therefore antigen specific, activated Treg cells can and do nonspecifically suppress dendritic cells and T cells in their immediate environment (25). Thus, while an allergen-activated Treg cells could potentially suppress a response to an irrelevant antigen delivered simultaneously into its immediate environment, in general it would not efficiently suppress responses to challenges delivered outside the context of specific allergen exposure. Our previous observations that DC10 must present cognate allergen to intimately engage Th2 cells (as determined by fluorescence resonance energy transfer [FRET])

assays) from asthmatic people or asthma phenotype mice and induce tolerance therein (11, 12, 19) fit well with our present data. Our data also has implications for therapeutic use of these cells in the context of polyallergic individuals. In principle, each allergen to which an individual is sensitive would need to be individually targeted by DC10, although it is open to speculation as to whether one could treat sensitivity to two independent allergens by loading dendritic cells with both. Others have reported that human CD14⁺ monocyte-derived dendritic cells can present multiple antigens (26).

The dendritic cell's costimulatory molecules provide important signals that can independently influence the outcomes of antigen presentation, such that these cells can have either immunosuppressive or immunostimulatory effects on T cells (27). It is well recognized that dendritic cells require both MHC peptide-TCR complex and costimulatory molecule engagement to optimally activate T cells (28, 29), but there is increasing evidence that some costimulatory molecules are integral to the induction of tolerance by regulatory dendritic cells. Thus, CD40L/CD154 blockade can induce long-lived, antigen-specific tolerance (30), while the CD80/CD86 ligand CTLA-4 attenuates T-cell activation and fosters peripheral tolerance (31). The late-acting costimulatory molecules CD80 and CD86 are important for full induction of Teff cell activation (32), and our data suggest that these molecules are also required for successful activation of Treg cells. Dendritic cells have the capacity to acquire and present antigens from dying cells (33), such that it might be argued that in our model, DC10 or parts thereof (e.g., exosomes) are taken up by the recipient's APC and that it is those endogenous APCs that induce tolerance. The observed requirement for MHCII, CD80/86, and IL-10 expression by the DC10 treatment indicates that these cells must directly interact with T cells to promote tolerance and that their therapeutic effects

are not realized by transfer of their ingested allergens to the recipient's dendritic cells or other APCs.

Taken together, our observations confirm that DC10 intimately engage pulmonary Teff cells to promote their conversion to Treg cells and show that MHCII and CD80/86 engagement, combined with the DC10's secretion of IL-10, are each integral to the mechanisms that mediate DC10-induced tolerance. Thus, this tolerance does not arise because of a lack of Teff cell stimulation, but rather because these cells are engaged and activated by the tolerogenic APC in precisely the manner that induces Treg cell differentiation.

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Author contributions

H.H., W.D., and J.R.G. designed the research, analyzed the results, and contributed to the writing of the manuscript; M.L. established the initial model for tracking cell trafficking and performed the DC-GMCSF and DC-TNF experiments; A.N. did the lung parenchymal T-cell experiment; X.Z. set up the asthma mouse model, harvested BAL, and did differential counts of BAL cells, while H.H. performed all other experiments.

Conflicts of interest

None of the authors have perceived or real conflicts of interest.

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