

Induction of Type 2 T Helper Cell Allergen Tolerance by IL-10-Differentiated Regulatory Dendritic Cells

Xiuling Li^{1,2}, Aimei Yang, Hui Huang¹, Xiaobei Zhang¹, Jennifer Town¹, Beth Davis³, Don W. Cockcroft³, and John R. Gordon^{1,3}

¹Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada; ²National Vaccine and Serum Institute, Beijing, China; and ³Division of Respiriology, Critical Care, and Sleep Medicine, Royal University Hospital, Saskatoon, Canada

In mouse models of asthma, therapeutic use of allergen-presenting IL-10-differentiated dendritic cells (DCs) can abrogate airway hyper-responsiveness, and reduce other asthma-related responses to near background. Analogous human DCs can suppress human T cell responses *in vitro*, but the operative mechanisms are poorly defined. We investigated the ability of IL-10-treated human DCs to induce tolerance among autologous T cells of subjects with asthma and the mechanisms by which they do this. CD14⁺ monocyte-derived DCs were differentiated in the presence of IL-10 (DC10) *ex vivo* from 11 donors with asthma and 4 control donors, and characterized for relevant markers. They were pulsed with specific or irrelevant allergen, and cultured with autologous peripheral blood CD4⁺ T cells, either alone or together with autologous immunostimulatory DCs (DC-TNF), and the impact of this treatment on the T-cell responses was assessed for each donor. The DC10 expressed reduced levels of some relevant markers (CD40, CD80, human leukocyte antigen-DR) and stimulatory cytokines (IL-6 and IL-12), but augmented levels of Ig-like transcript-2/CD85j and IL-10 relative to DC-TNF. In cocultures, they dampened DC-TNF-driven T helper (Th) type 2 cell proliferation and cytokine (IL-4, -5, and -13) secretion. They also drove the development from atopic CD4⁺CD25^{lo}Foxp3^{lo} cells of a population of IL-10-secreting CD25⁺Foxp3⁺LAG-3⁺CTLA-4⁺ regulatory T cells (Tregs). These Tregs suppressed stimulatory DC-induced autologous Th2 cell proliferation and cytokine secretion in a contact-dependent manner. Our data indicate that IL-10-treated human DCs induce Th2 cell allergen tolerance *ex vivo* by driving the differentiation of Tregs.

Keywords: asthma; tolerance; dendritic cell; regulatory T cell; IL-10

Atopic diseases, such as rhinitis, asthma, and atopic eczema, are dominated by T helper (Th) type 2 immune responses leading to accumulation of eosinophils and IgE production, with sensitization of tissue mast cells (1). Current data suggest that naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs), and perhaps inducible populations of regulatory cells, are involved in the prevention of allergic immune responses in allergen-challenged, healthy, individuals without atopy (2). Although there is no generalized defect in CD4⁺CD25⁺ Tregs within subjects with atopy, such as might be seen in immunosuppressed (e.g., IPEX) individuals (3), the abilities of CD4⁺CD25⁺ Tregs from individuals with atopy to down-regulate allergen-specific Th2 responses is reportedly diminished compared with similar cells from individuals without atopy (4). Successful outcomes in allergen-

CLINICAL RELEVANCE

This report details studies in which we show that human IL-10-differentiated monocyte-derived dendritic cells (DCs; DC10) express regulatory cell markers (e.g., Ig-like transcript 2, IL-10), and can block activation of autologous subject T cells by immunostimulatory DCs (DC-TNF). The DC10 induce the outgrowth from peripheral blood CD4⁺ T cells of individuals with asthma of a population of IL-10-expressing CD25⁺Foxp3⁺LAG-3⁺CTLA-4⁺ regulatory T cells (Tregs), which can subsequently themselves antagonize DC-TNF activation of autologous type 2 T helper cells in a contact-dependent manner. These data suggest that DCs such as these might be useful for dampening allergen-specific responses in individuals with atopy by inducing Treg responses.

specific immunotherapy are reportedly related to increases in peripheral T-cell tolerance associated with IL-10-producing CD4⁺CD25⁺ T cells (5). Indeed, it has been suggested that the balance between allergen-specific Tregs and Th2 cells may be decisive in the development of allergy (2).

Peripheral dendritic cells (DCs) avidly phagocytose all antigens in their environment and present these to T cells, but it is the context in which they acquire these antigens that largely determines the outcome for the T cell (e.g., Th1 differentiation). Signaling through their inflammatory cytokine-, chemokine-, or pathogen-associated molecular pattern receptors up-regulates expression of costimulatory molecules (e.g., CD40, CD80, CD86) and Th1-predisposing cytokine (e.g., IL-12) expression. On the other hand, presentation by quiescent cells, with their modest capacity for costimulation and low expression of IL-12 leads to abortive T-cell activation (6, 7). Thus, innocuous foreign antigens or self-antigens are normally presented in a tolerogenic context (8). The mechanism(s) by which this process is subverted in atopy-prone individuals is complex and not entirely clear, but involves genetic and environmental factors (9).

IL-10 is a potent anti-inflammatory and immunoregulatory cytokine produced by many cells, including some tumor cells. It was reported some time ago that DCs purified from the interior of IL-10-expressing melanoma tumors are tolerogenic (10). Similarly, DCs that are differentiated in the presence of IL-10 take on a 'regulatory' phenotype, whereby they can induce T cell anergy *in vitro* (11). We reported previously that allergen-presenting, otherwise quiescent, splenic CD8 α ⁺ DCs from mice can partially ameliorate the asthma phenotype in mice with severe asthma (12), and it has been shown that transforming growth factor (TGF)- β (13) or IL-10- (14) differentiated DCs can dampen pulmonary allergen sensitivity in mice. We have found that treatment of mice with asthma with such IL-10-skewed DC (DC10) can induce full normalization of airway hyperresponsiveness within 3 weeks, and progressive waning of Th2 responses (cytokines, eosinophilia, IgE) to near background over the next

(Received in original form January 17, 2009 and in final form March 18, 2009)

This work was supported by grants from the Canadian Institutes for Health Research (J.R.G.).

Correspondence and requests for reprints should be addressed to John R. Gordon, Ph.D., Rm 3610, Royal University Hospital, 103 Hospital Drive, Box 120 R.U.H., Saskatoon, SK, Canada S7N 0W8. E-mail: john.gordon@usask.ca

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 42, pp 190–199, 2010

Originally Published in Press as DOI: 10.1165/rcmb.2009-0023OC on April 16, 2009

Internet address: www.atsjournals.org

2–5 weeks. Furthermore, DC10 treatment of asthma phenotype mice leads to significant activation of CD4⁺CD25⁺Foxp3⁺ Tregs, as opposed to simple Th2 cell anergy (M. Lu, X.Z., H.H., and J.R.G., unpublished data). This induction of tolerance is allergen specific and critically dependent on DC expression of IL-10 (14). Herein, we investigate the ability of analogous IL-10-treated human DCs to similarly induce tolerance among Th2 cells of subjects with asthma *ex vivo*, and whether this was associated with augmented Treg activity.

MATERIALS AND METHODS

Donors

A total of 11 donors with atopy signed informed consent forms and provided 50–60 ml of heparinized blood, as per our institutional medical ethics review panel, and in accord with the related Declaration of Helsinki. Anthropomorphic and relevant medical data for each subject are provided in Table 1. None of our donors were smokers, none were on corticosteroids (inhaled or otherwise), and none used (or did rarely use) salbutamol (per required need). All subjects that had asthma were mildly or very mildly asthmatic (FEV₁ > 70% of predicted values while off medication), and each had developed asthma in their childhood. All subjects were tested out of their allergen season (i.e., January–March) or, where relevant (cat allergen), were not exposed to allergen over a 2-week period before their blood was drawn. The four control donors, half of whom were males, were healthy, nonsmoking individuals without atopy or asthma, and were 21–57 years of age. These control donors were used to establish protocols (e.g., DC differentiation) and as a source of stimulatory cells for mixed lymphocyte responses.

Reagents and Materials

The supplies used and their commercial sources were as follows: RPMI 1640, FCS, and 1% antibiotics/antimycotics (Invitrogen Life Technologies, Burlington, ON); FITC-labeled dextran (40 kD) and prostaglandin E2 (Sigma-Aldrich, Mississauga, ON); Lymphocyte Separation Medium density gradient medium (Valeant Pharmaceuticals, Aliso Viejo, CA); Immulon-4 ELISA plates (Dynatech Laboratories, Chantilly, VA); matched capture and biotinylated detection antibody pairs and recombinant protein ELISA standards for human IL-2, -4, -5, -10, and -13, IFN-γ, and TGF-β (R&D Systems, Minneapolis, MN); recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, -4, -6, -10, and -12 and TNF, and FITC- or phycoerythrin (PE)-conjugated monoclonal anti-human CD4, CD14, CD25, CD40, CD54, CD80, CD83, Ig-like transcript-2 (ILT2)/CD85j, CD86, and human leukocyte antigen (HLA)-DR, and isotype control mouse IgG1, IgG2a antibodies (BD Pharmingen, Mississauga, ON); horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA); ABTS (2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonic

acid]diammonium salt) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD); anti-CD14 paramagnetic microbeads, CD4 (negative selection), and CD4⁺CD25⁺ Treg magnetic-activated cell sorting (MACS) isolation kits (Miltenyi Biotec, Auburn, CA); “U” bottom 96-well and flat-bottom 24-well Transwell tissue culture plates (Corning Costar; Fisher Scientific, Nepean, ON); [³H]-thymidine (Perkin-Elmer, Waltham, MA); and human 180 gene dendritic and antigen-presenting cell-specific arrays (SAB Biosciences, Frederick, MD). All allergens were purchased from Omega Labs (Mississauga, ON), including standardized five-grass mix (40,000 protein nitrogen units [PNU]/ml), birch (40,000 PNU/ml), cat (10,000 PNU/ml), dog (10,000 PNU/ml), and horse (10,000 PNU/ml).

Generation of DCs

Peripheral blood mononuclear cells were purified from donors with atopy by density gradient centrifugation, and the CD14⁺ monocytes isolated by positive selection magnetic sorting. The monocytes were differentiated into immature DCs by culture for 4 days at 37°C in RPMI 1640 supplemented with 1% antibiotics/antimycotics, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FCS (complete medium), and containing 200 ng/ml rhIL-4 and 100 ng/ml rhGM-CSF. The immature cells (DC-GM-CSF) were then cultured for another 2 days in this medium, but to which was added a cytokine cocktail containing rhIL-1β, rhIL-6 (each 10 ng/ml), TNF (20 ng/ml), and prostaglandin E2 (500 ng/ml), either alone (mature DCs [DC-TNF]) or with rhIL-10 (50 ng/ml; tolerogenic DCs [DC10]) (15). On Day 6, the cultured DCs were pulsed for 2 hours with the subject’s specific allergen (50 μg/ml), as determined previously by skin prick testing and clinical diagnosis, and then washed extensively. In a preliminary experiment, we had assessed the impact of the allergen-loading time on the abilities of DC10 versus DC-TNF to induce autologous T-cell proliferative responses. The proliferative responses (³H-thymidine uptake) by autologous T cells exposed to DC-TNF that had been pulsed with allergen for 2 versus 18 hours was 26,082 (±3,012) versus 26,087 (±5,210) cpm, respectively, whereas the analogous values for T cells exposed to DC10 were 13,910 (±2,752) versus 12,002 (±1,369) cpm, respectively. CD14⁺ mononuclear cells (used for purification of CD4⁺ T cells) and DCs were either used directly or cryopreserved in 90% FCS/10% DMSO for later use.

DC Characterization

DCs were stained for FACS using fluorochrome-conjugated antibodies specific for CD14, CD40, CD54, CD80, CD83, ILT2/CD85j, CD86, or HLA-DR (FACScan; Becton Dickinson, Mountain View, CA). Control cells were stained with isotype-matched irrelevant specificity antibodies. To assess phagocytosis, the cells were incubated for 30 minutes at 37°C with FITC-dextran (100 μg/ml) in PBS, then washed with ice-cold PBS, fixed in 0.5% paraformaldehyde, and analyzed by FACS. IL-10 expression was determined using quantitative RT-PCR (qRT-PCR) and ELISA. IL-6, IL-12, and TGF-β secretion over 72 hours by otherwise untreated DCs was assessed by ELISA.

To further assess the impact of the various DC differentiation regimes on the phenotype of these cells, we also ran RNA from each population on commercial human dendritic and antigen-presenting cell-specific gene arrays, according to the supplier’s protocol. Briefly, 3 μg of total RNA was labeled with biotinylated dUTP in reverse transcription and linear polymerase replication reactions; then the denatured cDNA probe was hybridized to the arrays overnight at 60°C. The signals were visualized on X-ray film using a chemiluminescent substrate. Image analysis was completed using SAB Biosciences dedicated Web-based integrated software (GEArray Expression Analysis suite; <http://geasuite.sabiosciences.com>).

Purification of T Cells

CD4⁺ T cells were obtained from peripheral blood mononuclear cells by negative selection, and the CD25⁺ T cells purified by positive selection magnetic sorting (CD4⁺CD25⁺ cells comprised the column flow-through population). The cells so purified were greater than or equal to 98% CD4⁺, greater than or equal to 95% CD4⁺CD25⁺, and greater than or equal to 98% CD4⁺CD25⁺, respectively, as determined by FACS (data not shown).

TABLE 1. ANTHROPOMETRIC AND MEDICAL DATA FOR THE STUDY SUBJECTS

Subject with Atopy	Gender	Age (Yr)	Height (In)	Diagnosis	FEV ₁ (% Pred)	Allergen Used	Other Known Sensitivities
1	F	39	63	AR	76	Birch	Mixed tree, nuts
2	M	61	66	AA	86	Grass	Trees
3	M	31	70	AA	71	Cat	Grass
4	M	25	73	AA	89	Grass	HDM, ragweed
5	M	27	71	AA	84	Grass	Weeds, trees, horse
6	M	45	68	AA	87	Cat	Grass
7	M	22	72	AR/AA	98	Grass	Cat
8	F	40	65	AA	109	Cat	None
9	F	21	68	AA	85	Cat	Dog, horse, alternaria
10	F	27	67	AA	85	Cat	Horse
11	M	21	69	AA	88	HDM	Cat, grass, weeds

Definition of abbreviations: AA, atopic asthma; AR, allergic rhinitis; HDM, house dust mite.

Proliferation Assays

Mixed lymphocyte reactions were used initially to assess the relative immunostimulatory activities of our DC10. For these reactions, the indicated numbers of DC10 or DC-TNF were cultured with constant numbers of allogeneic peripheral blood T cells from control donors without atopy (2×10^5 T cells/well). As with all our proliferation assays, after 72 hours, we added 1 μ Ci of ^{3} H-methylthymidine to the wells overnight and harvested the cellular DNA onto glass fiber filters using routine methods. The proliferative responses were assessed by measuring the levels of ^{3} H-methylthymidine incorporation into cellular DNA using standard liquid scintillation counting.

To assess the tolerogenic activity of our DCs, 2×10^5 CD4⁺ T cells were cocultured in 200 μ l of complete medium with titrated numbers of autologous irradiated (3,000 rads), specific allergen-pulsed IL-10-differentiated DCs (DC10), with or without 2×10^4 irradiated immunostimulatory (i.e., mature) allergen-presenting DCs (DC-TNF). In preliminary experiments, we titrated the number of DC-TNF required for maximal induction of Th2 cell proliferation for five of our donors. For the experiments reported herein, we used half-maximal numbers of DC-TNF in part as a control against the possibility that addition of equal numbers of DC10 could suppress the Th2 response simply by virtue of overcrowding.

To assess the functions of putative Tregs generated *ex vivo*, we MACS-purified CD4⁺, CD4⁺CD25⁻, or CD4⁺CD25⁺ T cells from primary cultures of DC10 and autologous atopic CD4⁺ T cells, and cocultured 1×10^5 of these with 2×10^5 freshly purified autologous T cells and 2×10^4 irradiated autologous DC-TNF. To assess the importance of cell-cell contact in Treg inhibition of Th2 responses, we used 24-well plates with 0.4- μ m polycarbonate filter Transwell inserts. We placed 2×10^6 CD4⁺ Th2 cells and 1×10^5 irradiated DC-TNF in the lower chamber of each well, and 1×10^6 irradiated putative regulatory CD4⁺ or CD4⁺CD25⁺ T cells in the upper chamber. In control wells, the latter T cells were added to the lower chambers. All assays were run in triplicate for 72 hours at 37°C. In some assays, the culture supernatants were removed from the wells for cytokine detection, and fresh medium containing ^{3} H-methylthymidine (1 μ Ci/well) was added to the cells.

ELISA Quantification of Cytokine Production

Our ELISA protocols have been reported in detail previously (12, 16, 17). We used commercial matched capture and detection antibody pairs and recombinant protein standards for IL-4, -5, -6, -10, -12, -13, TGF- β , and IFN- γ (R&D Systems). The detection limit for all cytokines was 4–10 pg/ml.

qRT-PCR

Total RNA was isolated from MACS-purified CD4⁺ T cells or cultured DCs using RNeasy Mini kits (Qiagen, Mississauga, ON), according to the supplier's instructions. qRT-PCR was performed using a Brilliant qRT-PCR Master Mix kit (Stratagene Cloning Systems, La Jolla, CA). The sequences for the primers employed were as follows: Foxp3—forward, 5'-ATC TAC CAC TGG TTC ACA CGC AT-3' and reverse, 5'-CTC CAC CCG CAC AAA GCA C-3'; lymphocyte activation gene-3 (LAG-3 /CD223)—forward, 5'-TTG GAG CAG CAG TGT ACT TCA CA-3', and reverse, 5'-CTA AGG CAG AAA ATC GTC TTG GT-3'; cytotoxic T lymphocyte antigen-4 (CTLA-4)—forward, 5'-CCA GTG GAA ATC AAG TGA ACC TC-3', and reverse, 5'-GCA TTT TGC TCA AAG AAA CAG C-3'; IL-10—forward, 5'-GAA CCA AGA CCC AGA CAT CAA G-3', and reverse, 5'-GGC ATT CTT CAC CTG CTC CAC-3'; GAPDH—forward, 5'-TGC CTC CTG CAC CAC CAA C-3', and reverse, 5'-GGC CAT CCA CAG TCT TCT GG-3'.

Intracellular Ca²⁺ Flux Assay of Antigen-Specific DC-T Cell Interactions

To assess whether allergen-specific interactions occurred between DC10 and autologous T cells, we assessed antigen presentation-induced T-cell intracellular Ca²⁺ flux. DC-TNF or DC10 that had been pulsed with the donor's specific allergen or an irrelevant allergen were cultured for 3 hours with autologous T cells that had been labeled with the Ca²⁺ indicator dye, Fluo-4 AM (1×10^5 DC and 2×10^5 T cells). We tracked intracellular Ca²⁺ flux using a microplate spectrofluorimeter (NovoStar Optima; BMG Labtech, Cary, NC), and expressed the data as relative fluorescence units.

Forster (or Fluorescence) Resonance Energy Transfer Assay of DC-T Cell Interactions

To document, in another way, whether immunologically relevant interactions occurred between our DCs and T cells, we employed time-resolved Forster (or fluorescence) resonance energy transfer (FRET) assays. FRET occurs when paired fluorophores are sufficiently close (≤ 80 Å) that energy released from one (in our case, the lipophilic dye, DiO; excitation and emission wavelengths, 484 and 501 nm, respectively) can excite its partner dye (DiI; emission wavelength, 565 nm) to fluoresce at a distinct, alternate wavelength. Only if the DC and T-cell membranes are sufficiently intimately engaged physically will FRET occur. In our assays, DC_{TNF} or DC_{IL-10} that had been pulsed with allergen were labeled with DiO, whereas autologous T cells were labeled with DiI, then the cells were cocultured in 96-well plates (each 2×10^5 cells/well) and assessed across approximately 8 hours in a fluorescent microplate reader (NovoStar, BMG). The FRET data are presented as relative fluorescence units.

Statistical Analysis

A Student's *t*-test was used to test the statistical significance of parametric data, and Wilcoxon signed rank assays were used for nonparametric data. The results were considered significant if *P* was less than or equal to 0.05.

RESULTS

IL-10-Differentiated DCs Express a Tolerogenic Phenotype

Phenotypically immature CD14⁺ monocyte-derived DCs were differentiated from all donors (Table 1) in the absence (DC-TNF) or presence (DC10) of IL-10. DC-TNF have been reported previously to be highly immunostimulatory, whereas DC10 express a regulatory phenotype (11). Our DC10 expressed reduced levels of costimulatory and maturation markers relative to the DC-TNF (Figure 1A). Specifically, we observed significantly reduced cell surface expression of CD86, HLA-DR, and CD54 on the DC10, with modest reductions in CD40 and CD80, but significantly higher levels of ILT2/CD85j. The DC10 did appear to be somewhat less mature than the DC-TNF by other measures as well, in as much as they expressed lower levels of CD83 (Figure 1A) and much more avidly phagocytosed FITC-dextran (Figure 1B).

As noted above, the production of inflammatory or regulatory cytokines by DCs during antigen presentation also plays an important role in T-cell differentiation (6). Thus, we investigated the influence of the IL-10 treatments on IL-6, -10, and -12 production by our DCs. Maturation of DCs in the presence of IL-10 strongly inhibited their expression of IL-6 and IL-12 in comparison with the control DC-TNF, whereas IL-10 expression was significantly up-regulated ($P \leq 0.01$ versus DC-TNF for each parameter). In six of six subjects, we observed no significant differences in TGF β release by DC-TNF and DC10 (188 ± 64 and 169 ± 48 pg/ml, respectively). Furthermore, the DC10 were poor inducers of mixed lymphocyte reactions when cocultured with T cells from other donors (Figure 1B; $P \leq 0.01$ versus DC-TNF). When we compared the phenotype of DC10 from the one donor without atopy or asthma (Donor 1, Table 1) with that of the DC10 from the donors with atopy and asthma, or, for that matter, other control donors without atopy or asthma, we observed no discernible differences, as assessed using the parameters noted in Figure 1.

We also used cDNA arrays to assess the expression of a battery of DC/antigen-presenting cell markers by our cells, and found that only a modest number of markers were differentially expressed (i.e., $\geq$ twofold differences) by immature CD14⁺-monocyte-derived DCs (DC-GM-CSF), DC-TNF, and DC10. The DC-TNF expressed more IFN- α 1, RelB, CCL5, -16, -19, -21, and CXCR1, and less inducible T cell co-stimulatory molecule (ICOS), HLA-DM alpha (HLA-DMA), HLA-DM beta (HLA-DMB), MAC1, and CCR1, -3, and -5 than the

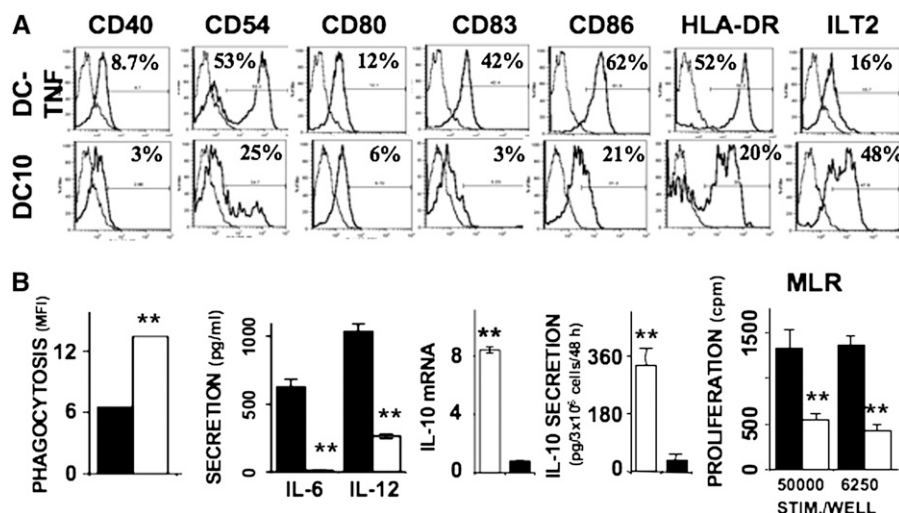


Figure 1. Characterization of IL-10-differentiated monocyte-derived dendritic cells (DCs). DCs were differentiated from CD14⁺ monocytes of donors with asthma by culture in recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) and rhIL-4 for 4 days, then matured for 48 hours by culture in IL-1 β , IL-6, TNF- α , and prostaglandin E₂, with (DC10) or without (DC-TNF) IL-10. (A) FACS characterization of DC10 or DC-TNF using specific antibodies for CD40, CD54, CD80, CD83, CD86, human leukocyte antigen (HLA)-DR, and ILT2/CD85j (heavy lines) or isotype control antibodies (light lines). (B) The phagocytic activity of the cells was assessed by uptake of FITC-dextran (100 μ g/ml) over 30 minutes at 37°C, as determined by FACS (left panel). The secretion of IL-6 and IL-12p70 by DC-TNF or DC10 over 48 hours was determined by ELISA, whereas expression of IL-10

was determined by quantitative RT (qRT)-PCR and ELISA for mRNA and protein (pg/3 $\times$ 10⁶ cells/48 h), respectively (middle three panels). The abilities of the DCs to induce allogeneic T-cell proliferation was assessed in mixed lymphocyte reactions (MLR; right panel), as noted in MATERIALS AND METHODS. The depicted data are from one individual, and are representative of data from five such donors. Closed bars, DC-TNF; open bars, DC10. ** $p \leq 0.01$ compared with DC-TNF.

immature cells (Figure 2). The DC10 expressed more IFN- α 1 than the immature cells (Figure 2), and much more IL-2 (Table E5 in the online supplement) than the DC-TNF, but, in many respects, appeared to have a phenotype that fell between the immature and TNF-induced cells. The only array markers that differentiated the DC10 and DC-TNF were CCL21 and CXCR1, which were expressed at higher levels in the mature cells. Both populations expressed lower levels of ICOS, HLA-DMA, HLA-DMB, and MAC1 than the immature cells, and higher levels of IFN- α 1. The data for the remaining array markers indicated that the three populations expressed these at more or less equivalent levels (as shown in Tables E1–E5).

IL-10-Differentiated DCs Interact with Autologous T Cells in an Allergen-Specific Fashion

If regulatory DCs were to be used clinically, it would be important to know whether they interact with T cells in an allergen-restricted fashion. We used two assays to confirm this. For the FRET assay depicted in Figure 3A, we cocultured FRET acceptor dye (DiI)-stained DC10 with FRET donor dye (DiO)-stained autologous Th2 cells from a subject allergic to grass. The DC10 were loaded to present either the specific (grass) or an irrelevant (in this case, ovalbumin) allergen. The specific allergen-presenting DC10 that interacted with autologous Th2 cells emitted a strong signal for at least 8 hours, whereas the ovalbumin-presenting DC10 emitted only weak signals (i.e., did not productively engage autologous T cells). To confirm these DC10–Th2 cell interactions in another way, we used intracellular Ca²⁺ flux assays (Figure 3B). We cocultured Fluo-4AM-stained T cells with autologous immunostimulatory DC-TNF or DC10 that had been pulsed with specific or irrelevant (non-specific) allergen. The specific allergen-presenting DC-TNF strongly induced Ca²⁺ flux in the T cells relative to cells cultured without any DC. The specific allergen-presenting DC10 also induced Ca²⁺ flux in the T cells, whereas irrelevant allergen-presenting DC10 induced no discernible Ca²⁺ responses (nonspecific; Figure 3B). These data suggest that, although the DC10 did not signal as strongly into the T cells as did DC-TNF, they nevertheless did signal in an allergen-specific fashion. We also confirmed the relative strength of specific allergen-presenting DC-TNF and DC10 interactions with au-

tologous T cells using FRET assays (Figure E1), and found similar results. That is, DC-TNF, which we have shown express higher levels of the antigen presentation molecules, HLA-DR, CD54, and CD86, engaged these T cells in a quantitatively stronger fashion than did DC10. Culture of DC10 (or Th2 cells; data not shown) alone produced no detectable signal.

Allergen-Presenting, IL-10-Differentiated DCs Display a Regulatory Phenotype

Our DC10 expressed IL-10 and ILT2, and were not stimulatory in mixed lymphocyte reaction cultures, suggesting that they could be tolerogenic (11). We first examined the abilities of the allergen-presenting DC-TNF and DC10 to stimulate proliferation of autologous T cells, titrating both populations onto constant numbers of T cells (Figure 4A). The DC-TNF strongly drove CD4⁺ T cell proliferative responses, whereas relatively weak responses were observed with the DC10. Although these data are consistent with the DC10 being regulatory, we could also have obtained these results if the DC10 were simply inefficient in presenting allergen to T cells. Thus, we next tested the abilities of specific allergen-presenting DC10 to inhibit the activation of T cells induced by autologous allergen-presenting DC-TNF, using Th2 cell proliferation and IL-4, -5, and -13 expression as readouts (Figures 4B and 4C). As above, culture of DC-TNF with Th2 cells alone (0 DC10/well) induced strong proliferative responses that were accompanied by marked expression of Th2 cytokines. However, adding DC10 to these cultures inhibited DC-TNF-induced CD4⁺ T cell-proliferative responses in a dose-dependent fashion, such that we observed 30–50% inhibition at DC10:T cell ratios of 1:4 to 1:16 (Figure 4B). This was a consistent observation with DC10 from numerous donors with atopy ($n = 8$). We also observed reduced expression of IL-4, -5, and -13 by Th2 cells when DC10, but not DC-TNF, were present (Figure 4C; $n = 6$).

As a measure of how well such tolerogenic effects of DC10 might translate across from one allergen to another, we also compared the responses of five donors allergic to cats to those of four donors allergic to grass. We assessed expression of IL-10 by their DCs, and the abilities of these cells to suppress autologous T-cell proliferative and Th2 cytokine responses to specific allergen-presenting DC-TNF challenge (Figure 5). In short, we

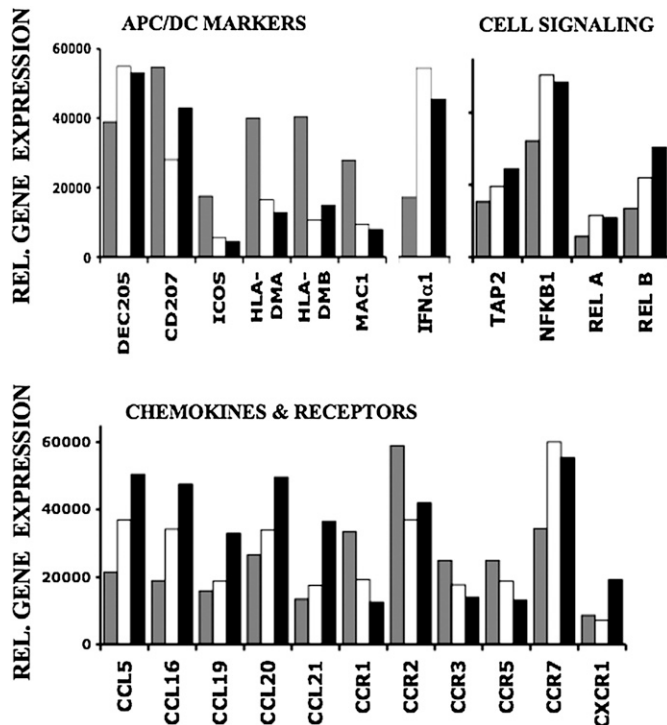


Figure 2. Expression analysis of selected dendritic and antigen-presenting cell markers in human monocyte-derived DCs. Immature DCs were harvested from Day 4 cultures of bone marrow cells in GM-CSF and IL-4 (gray bars), as noted in the materials and methods, while putatively regulatory DC10 (open bars) and immunostimulatory DC-TNF (closed bars) were further differentiated from immature cells, as in Figure 1. RNA was purified from each population and subjected to cDNA array analysis of 180 dendritic and antigen-presenting, cell-relevant markers. Depicted are those markers with differences in signal of 2 or greater, in genes expressed as DC surface markers or IFN- α 1, intracellular signaling markers, and a number of DC-relevant chemokines and their receptors. The bulk of the remaining genes within the arrays were not differentially expressed, with the exception of IL-2, -16, and -18 (see online supplement). APC, antigen-presenting cells; DEC, DC-specific endocytic receptor; HLA-DM, human leukocyte antigen-DM (alpha or beta); ICOS, inducible T cell co-stimulatory molecule; MAC1, macrophage antigen-1; REL, reticuloendotheliosis viral oncogene homolog (A or B); TAP2, transporter 2 ATP-binding cassette.

found no significant differences in the abilities of DC10 from these two groups of people to express IL-10 or to suppress immunostimulatory DC-induced T-cell proliferation. Similarly, DC10 from both individuals allergic to cats and those allergic to grass more or less equally suppressed the Th2 responses of autologous peripheral blood T cells (Figure 5, right panel).

Coculture of T Cells from Donors with Atopy with Allergen-Presenting DC10 Activates CD4⁺ Cells with a Regulatory Phenotype

We wished to assess whether our tolerogenic DCs induced differentiation of Tregs from within the T cell pool of the donors with asthma. To do this, for five of our donors with atopy, we cocultured their DC10 or DC-TNF with autologous T cells for 7 days, then repurified the T cells by magnetic sorting, and expanded them in the presence of IL-2 for another 7 days before assessing their expression of a panel of Treg markers. In general, there were few CD4⁺CD25⁺Foxp3⁺ cells among the T cells that had been cultured without DCs (two typical subjects shown in Figure 6A, left panel), but coculture of donor T cells with ei-

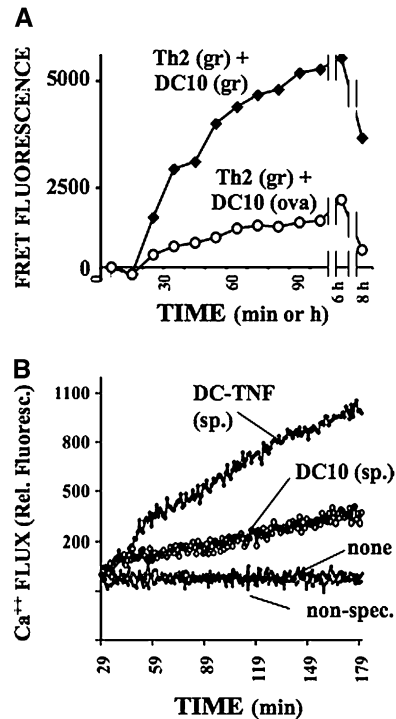


Figure 3. DC10 interact in an allergen-specific fashion with autologous T cells. DC10 and DC-TNF were generated as in Figure 1 from peripheral blood monocytes of eight individuals and pulsed with allergen. Autologous CD4⁺ T cells were similarly purified from these individuals by MACS. (A) T cells from a donor with asthma who is grass allergic were stained with the FRET donor dye, DiO. Their DCs were loaded either with specific allergen (gr, grass) or an irrelevant allergen (ova, ovalbumin), washed, and stained with the FRET partner dye, Dil. The DC10 (2×10^4 cells/well) were cultured with the T cells (2×10^5 cells/well) for 8 hours, during which the FRET emissions were monitored continuously as

noted in MATERIALS AND METHODS. (B) The CD4⁺ T cells of three individuals (2×10^5 cells/well) were loaded with the Ca²⁺ indicator dye, Fluo4-AM, and either cultured alone (none) or exposed for 3 hours in intracellular Ca²⁺ flux assays to specific (sp.) or irrelevant (nonspecific [non-spec.]) allergen-pulsed autologous DC10 or DC-TNF (2×10^4 cells/well). For graphic simplicity, the results of the irrelevant allergen-presenting DC-TNF plus T cells are not depicted. Coculture of specific allergen-presenting DC10 with autologous T cells induced substantial FRET signals, whereas irrelevant allergen-presenting DC10 induced only weak signals. Similarly, specific allergen-presenting DC-TNF induced stronger responses than the specific allergen-presenting DC10. No signals were observed with DC or T cells cultured alone. Th2, type 2 T helper cell.

ther DC10 or DC-TNF equally augmented the outgrowth of CD4⁺CD25⁺Foxp3⁺ T cells. We examined expression of Treg activation markers on these T cells, and found augmented expression of LAG-3 (~twofold increases), CTLA-4 (~11-fold increases), and IL-10 (~sixfold increases) by the CD4⁺ T cells from the DC10 cocultures relative to those from the DC-TNF cocultures (Figure 6B).

We examined the ability of the putative Tregs from these long-term cocultures to suppress the activation of fresh autologous donor T cells (Th2) by allergen-presenting DC-TNF (Treg:Th2 cell ratio, 1:2; Figure 7). The DC-TNF induced strong proliferative responses by the donor Th2 cells, and the addition of CD4⁺ cells from long-term DC-TNF-T cell cocultures did not alter this response. On the other hand, CD4⁺ cells from long-term DC10-T cell cocultures reduced the proliferative responses by approximately 50% ($P \leq 0.01$ versus the DC-TNF Th2 cells). We also assessed Th2 cytokine secretion in these cultures, and found that the CD4⁺ T cells from the DC10-T cell cocultures reduced IL-5 and -13 release by 50–60% ($P \leq 0.01$ versus the DC-TNF-T cells alone; Figure 7B). Interestingly, the IL-4 response proved more refractory to such regulation.

Characterization of DC10-Induced Tregs

We had found expression of both IL-10 and CTLA-4 on CD4⁺ cells from the long-term cultures that possessed this regulatory

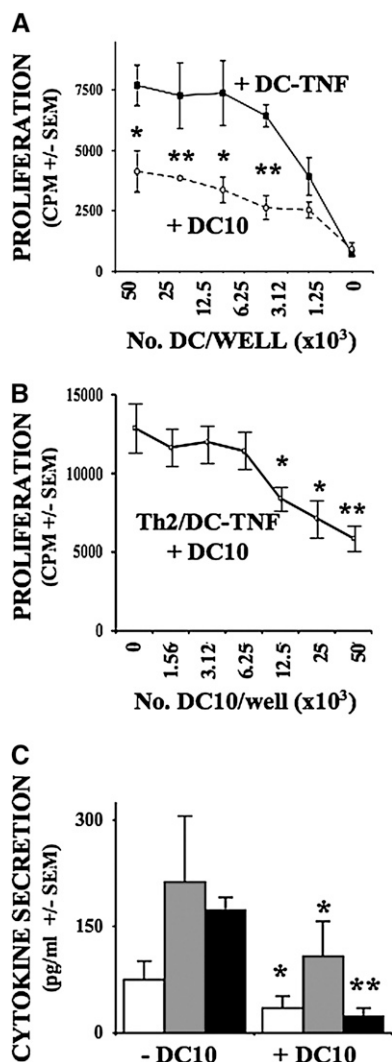


Figure 4. DC10 weakly stimulate autologous T cells, but suppress their activation by allergen-presenting immunostimulatory DC-TNF. DC10 and DC-TNF were generated as in Figure 1 from peripheral blood monocytes of eight individuals and pulsed with allergen. Autologous CD4⁺ T cells were purified from these individuals as in Figure 3. (A) Proliferative responses of T cells (2×10^5 cells/well) that were stimulated for 3 days with the indicated numbers of irradiated autologous allergen-pulsed DC-TNF or DC10. (B) Proliferative and (C) Th2 cytokine responses of 2×10^5 CD4⁺ T cells after coculture for 72 hours with irradiated autologous DC-TNF (2×10^4 cells/well) plus the indicated numbers of (B) or 2.5×10^4 (C) irradiated autologous DC10 ($n = 5$). Open bars, IL-4; gray bars, IL-5; closed bars, IL-13. Cell proliferation was assessed by ³H-thymidine incorporation and cytokine expression by ELISA. DC10 were poorly stimulatory for autologous Th2 cells, and suppressed the stimulatory DC-induced Th2 cell proliferation and cytokine expression. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with DC-TNF.

activity. Thus, in principle, these cells could operate in a contact-dependent or -independent manner, and this is what we addressed in our next experiment. We purified and irradiated CD4⁺ cells from the long-term DC10–Th2 cocultures, and placed them in the top chamber of Transwell plates with allergen-presenting DC-TNF and Th2 cells in the bottom chamber (negative contact), and, 3 days later, assessed the Th2 cell–proliferative responses (Figure

8A). In control wells, we added the regulatory cells from the long-term cultures directly into the lower chambers with the DC-TNF and Th2 cells (positive contact). We observed greater than 30% inhibition of the proliferative responses of the Th2 cells only when the regulatory cells were added directly to the Th2 cells, which indicated that this regulatory activity was cell contact-dependent.

To evaluate which subpopulation of the CD4⁺ T cells from the long-term coculture carried the regulatory activity, we MACS fractionated the CD4⁺ T cells from long-term DC10 and DC-TNF cocultures based on expression of CD25, and irradiated these cells. We added the CD4⁺CD25[−] or CD4⁺CD25⁺ cells from both types of long-term culture to autologous DC-TNF-stimulated Th2 cells (Treg:Th2 ratio, 1:2), and assessed the Th2 cell–proliferative responses (Figure 8B). We found that the CD4⁺CD25⁺ T cells from DC10 cocultures more than 50% suppressed Th2 responses driven by immunostimulatory DC. Neither the CD25[−] nor CD25⁺ cells from the long-term DC-TNF cocultures displayed discernible suppressive activities in these assays, nor did the CD4⁺CD25[−] cells purified from the DC10 cocultures. Again, DC10 from our donors with atopy but not asthma were just as capable of driving differentiation of such CD4⁺CD25⁺ Tregs in this system as were DC10 from the donors with atopy and asthma.

DISCUSSION

The present results demonstrate that allergen-presenting, IL-10-differentiated DCs (DC10) from subjects with atopy are able to suppress autologous Th2 responses driven by immunostimulatory DCs (DC-TNF). The DC10 expressed elevated levels of IFN α 1, IL-2, ILT2, and IL-10, reduced levels of costimulatory molecules (CD40, CD54, CD80, CD83, CD86, and HLA-DR) and stimulatory cytokines (IL-6, IL-12), and equivalent levels of DC-specific endocytic receptor-205 (DEC205) and CCR7, relative to mature DCs. These DC10 displayed direct tolerogenic activities for autologous Th2 cells, but they also induced the differentiation or outgrowth of a population of activated CD4⁺CD25⁺ Tregs, which themselves subsequently induced tolerance in a contact-dependent manner.

It has been reported by others that human monocyte-derived DCs that are differentiated in the presence of IL-10 can reduce allogeneic T-cell proliferation and IL-2/IFN- γ expression *ex vivo* (11). These alloantigen-induced anergic T cells express reduced levels of CD25, but could subsequently suppress syngeneic T-cell proliferation and IL-2/IFN- γ expression via a CTLA-4-dependent pathway (15). On the other hand, glucocorticoid-differentiated human DCs express glucocorticoid-induced leucine zipper, which is important in the induction of IL-10-producing CD25⁺Foxp3⁺CTLA-4⁺ Treg

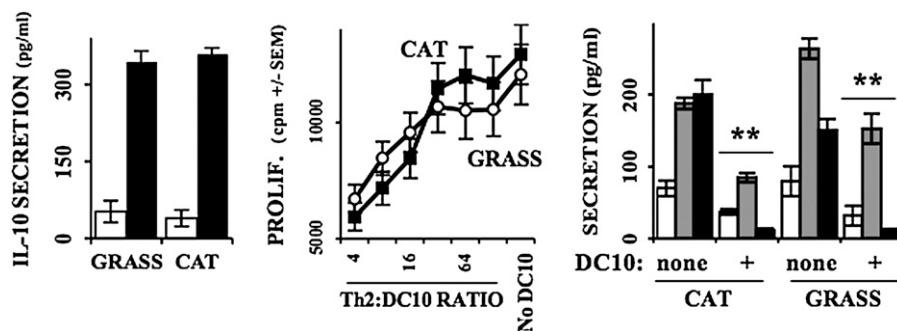


Figure 5. DC10 generated from CD14⁺ monocytes of subjects with grass- and cat-related asthma are equally able to suppress immunostimulatory DC-TNF-driven autologous T-cell activation. DC10 were generated and autologous T cells purified from five individuals with cat-related asthma and four with grass-related asthma, as in Figure 3. (Left panel: open bars, DC-TNF; closed bars, DC10) Secretion of IL-10 by the DC10 was assessed as in Figure 1. (Middle and right panels) The abilities of these DC10 to suppress DC-TNF-induced autologous T-cell proliferative and Th2 cytokine secretion

responses were assessed as in Figure 4. The DC10 from individuals with either type of asthma secreted equivalent levels of IL-10 in culture, and were equally capable of suppressing autologous Th2 cell proliferative and cytokine secretion responses. Open bars, IL-4; gray bars, IL-5; closed bars, IL-13. ** $P \leq 0.01$ compared with DC-TNF.

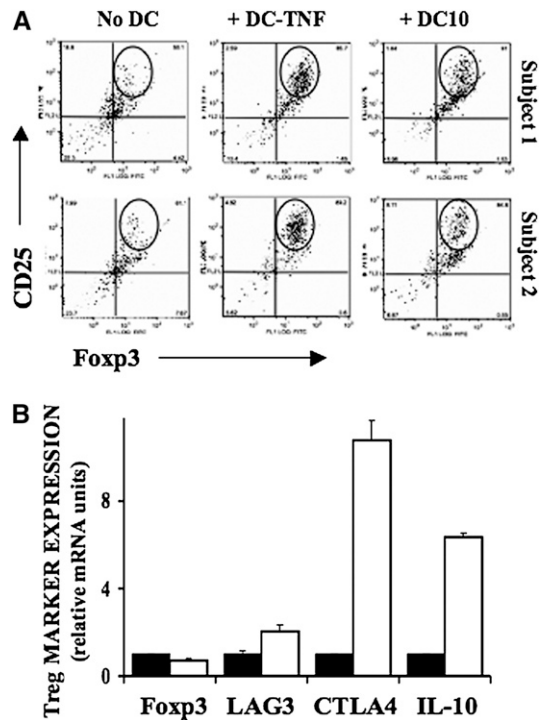


Figure 6. DC10-induced expression of regulatory T cell (Treg) markers on peripheral blood Th2 cells. We cultured CD4⁺ T cells from donors with asthma with irradiated autologous specific allergen-presenting DC10 or DC-TNF for 7 d, then expanded the T cells with IL-2 for 7 d, and finally MACS-purified the CD4⁺ cells from these 2 wk cultures. (A) We stained the T cells for CD4, CD25, and Fxp3, gated on the CD4⁺ cells, and assessed the proportions of the gated cells that were CD25⁺Fxp3⁺ double-positive using three-color FACS. There were no differences in the proportions of CD25⁺Fxp3⁺ cells among the gated cells that had been cultured with either DC-TNF or DC10, but there were few double-positive cells among control T cells that were not cultured with DCs (no DC). The data depicted are from two representative subjects. (B) We used qRT-PCR to assess expression of Fxp3, lymphocyte activation gene (LAG)-3, cytotoxic T-lymphocyte antigen (CTLA)-4, and IL-10 in the CD4⁺ cells recovered from the long-term DC-TNF (closed bars) or DC10 (open bars) cultures. The levels of Fxp3 mRNA in the two populations were equivalent, but there was significant up-regulation of each of the other markers. These data are from three individuals.

(18). But DCs that are differentiated with mycophenolic acid, an immunosuppressant used in transplantation medicine, are phenotypically similar to those generated in our cultures, and can inhibit proliferation of autologous T cells (19). Others have reported that TNF-differentiated human DCs (i.e., equivalent to our DC-TNF) maintain Fxp3 expression by Treg in culture, as we observed herein, but, interestingly, that they can also convert CD25⁺ cells to become functional Tregs (20). We observed no such regulatory population arising in our short- or more long-term DC-TNF/Th2 cell cultures. Immature monocyte-derived human DCs (generated by culture with GM-CSF and IL-4 alone) have been shown to inhibit antigen-specific responses when injected into healthy individuals (21), and we have reported that allergen-pulsed, quiescent murine CD8 α ⁺ splenic DCs can induce asthma tolerance after transfer into the airways of asthma-phenotype mice (12).

The immunostimulatory DC-TNF we generated expressed high levels of major histocompatibility complex (MHC) class II (HLA-DR), costimulatory molecules (e.g., CD40, CD54, CD86), and IL-12. As such, they were ideally equipped to activate T cells (22, 23), whereas the DC10 expressed reduced levels of each of

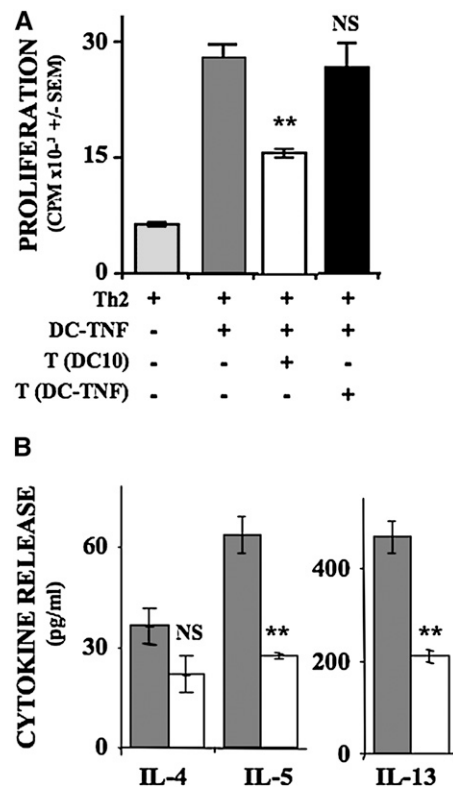
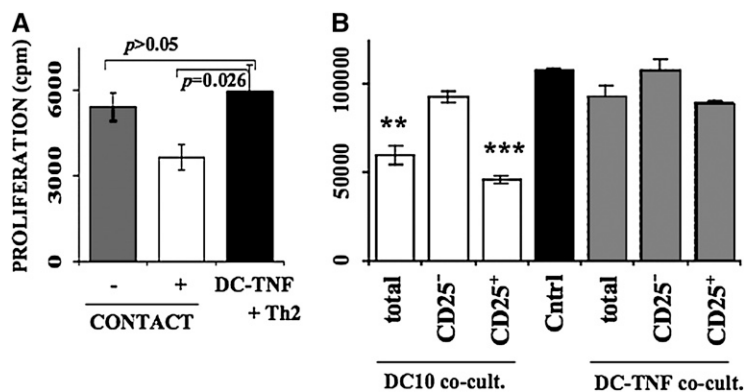


Figure 7. DC10-induced Tregs suppress DC-TNF-driven Th2 cell activation. Peripheral blood CD4⁺ T cells (Th2; 2×10^5 cells/well) were MACS-purified from donors with asthma and cultured for 72 hours with (+) or without (-) freshly generated, irradiated, autologous, allergen-presenting DC-TNF (2×10^4 cells/well). To some of these cultures we added 1.25×10^4 irradiated putative regulatory cells purified from long-term cocultures of DC10 [T(DC10)] or DC-TNF [T(DC-TNF)] with autologous CD4⁺ T cells (as in Figure 6). After 72 hours the proliferative and Th2 cytokine responses of the Th2 cells were assessed as in Figure 4. The T cells purified from the long-term DC10–Th2 cell cocultures were able to suppress DC-TNF-induced Th2 cell proliferation and Th2 cytokine expression, whereas the analogous cells from long-term DC-TNF–Th2 cell cocultures carried no such suppressive activities. These data are representative of those from four individuals. ** $P \leq 0.01$ compared with DC-TNF plus Th2. NS, not significant.

these, but also higher levels of IL-10 and the inhibitory receptor ILT2/CD85j. The precise mechanisms by which IL-10 is able to inhibit the functions of DCs have not been fully elucidated. However, because it was shown that DCs purified from the interior of IL-10-expressing melanoma tumors are tolerogenic (10), numerous studies have highlighted the ability of IL-10 to render differentiating DCs tolerogenic (14, 24–26). In addition, we have found, in a mouse model of house dust mite asthma, that house dust mite-presenting DC10 are highly tolerogenic (M. Lu, X.Z., H.H., and J.R.G., unpublished data). Similar to what we noted herein, IL-10 has been reported to up-regulate expression of the inhibitory receptor, ILT2/CD85j, on monocyte-derived DCs generated from patients with systemic lupus erythematosus (27). ILT2 has an important role in the regulation of natural killer (NK) cells and T lymphocytes (28, 29), and it has been suggested that ligation of ILT2 on DCs by self-HLA molecules may importantly affect the DC's tolerogenic functions (29). Expression of IFN- β 1 by DC10 paralleled that of IL-10. IL-10 and IFN- β 1 are both class 2 α -helical cytokines, and thus share some activities that affect T cells and DCs (e.g., repression of antiapoptotic functions, antiproliferative effects [30]), but their



discernible regulatory activity, whereas the CD25⁺ cells from the DC10/Th2 cocultures carried all of the regulatory activity from these cultures. ** $P \leq 0.01$ and *** $P \leq 0.001$ relative to DC-TNF/Th2 control wells. The results are representative of three separate experiments.

role(s) in DC10-induced tolerance induction, if any, remains unexplored. DC-TNF certainly expressed reduced levels of both of these cytokines. The tolerogenic functions of our DC10 could also be related, in part, to their reduced expression of Langerin/CD207. CD207 increases the efficiency of antigen presentation by MHCII and MHCI molecules, suggesting that reduced Langerin expression could contribute to compromised T-cell activation (31). Although functional immaturity of DCs that are presenting antigen to T cells can lead to T cell anergy or tolerance, our DC10 developed at least some features in common with mature immunostimulatory DC-TNF, including reduced expression of ICOS (32), HLA-DM (33), and MAC1, and increased expression of RelB (34) and CCR7. Interestingly, it has been reported that CCR7 expression by semimature DCs is critical to tolerance induction *in vivo* by allowing these cells to immigrate into lymph nodes, where they productively interact with specific T cells (35).

Our data confirm that the DC10-autologous T-cell interactions were allergen specific, in as much as these cells engaged one another for prolonged periods of time in a manner sufficiently intimate to emit FRET signals. FRET occurs when paired fluorophores are within less than or equal to 80 Å (~8 nm) of each other, such that energy released from the FRET donor (on the T cells) can excite its FRET partner dye on the DCs to fluoresce. Only if the DC and T-cell membranes are sufficiently so engaged physically will FRET occur. Immunologic synapses bring T cells and antigen-presenting cells only to within 15 nm of one another (36), but during such synapse formation, the antigen-presenting cells also fully transfer to the T cells biologically relevant patches of plasma membrane containing peptide-MHC complexes and costimulatory molecules (37, 38). This suggests that the DC10 did, indeed, engage these Th2 cells productively. The reduced levels of DC10-T cell engagement relative to that observed with immunostimulatory DC could be explained by the lower expression of accessory molecules by the DC10, in as much as these are critical components of optimized immunological synapse formation. The allergen specificity was confirmed in the intracellular Ca^{2+} flux assays, wherein only specific allergen-presenting DC10 induced signaling in the T cells. The lack of signal with the irrelevant allergen-presenting DC10, which would have expressed identical levels of HLA-DR, CD54, CD86, and IL-10, indicates that these, by themselves, were not involved in initiating the DC10-Th2 cell interactions. Rather that the MHCII must be loaded with allergen peptides that would be recognized by the Th2 cell T-cell receptors, and thereby induce the formation of stable immunologic synapses. These would allow exchange of membranes between the two partner cells (37, 38). If such an exchange did not occur, then the FRET fluorophores would not have otherwise been able to engage one another. The

Figure 8. Characterization of DC10-induced Tregs. (A) Irradiated Tregs, induced and purified as in Figure 7, were tested to determine whether their suppression of autologous Th2 cell proliferation was a contact-dependent process. The DC10-induced Tregs were added to the upper (negative contact) or lower (positive contact) chambers of 24-well Transwell plates and Th2 cells, as well as allergen-presenting DC-TNF to the lower chambers. A separate set of control wells contained no Tregs (DC-TNF + Th2). The data indicate that DC10-induced Treg activity was contact dependent. (B) The T cells that were purified from the long-term DC10 and DC-TNF cocultures were used as is (total), or MACS-fractionated into CD25⁻ and CD25⁺ subpopulations, each of which was tested for its ability to suppress DC-TNF-induced Th2 cell proliferative responses, as in Figure 7. The T cells from the long-term DC-TNF/Th2 cocultures showed no

augmented Ca^{2+} flux and FRET signals observed with specific allergen-presenting DC-TNF suggests that the augmented levels of HLA-DR, accessory antigen presentation molecules, and potentially also CD207 on these cells (31), were indeed functionally relevant in immunological synapse formation.

Although the numbers of CD4⁺CD25⁺Foxp3⁺ cells in our long-term cultures were not differentially affected by the presence of DC10 versus DC-TNF, we did observe increased expression of the Treg activation markers, LAG-3/CD233 and CTLA-4, and augmented IL-10 in the CD4⁺ T cells that arose under the influence of our tolerogenic DC10. Others have similarly reported that tolerance does not necessarily lead to increases in the numbers of CD4⁺CD25⁺Foxp3⁺ cells, but does increase their expression of these markers (39). LAG-3, an MHCII-binding, CD4-related T-cell surface molecule, has a negative regulatory influence when present such that activated Treg retain LAG-3. Thus, CD4⁺CD25⁺ T cells from LAG-3^{-/-} mice exhibit reduced regulatory activity, whereas ectopic expression of LAG-3 confers suppressor activity on CD4⁺ T cells (40). CTLA-4 is a member of the CD28-B7 Ig superfamily of immune regulatory molecules, but, unlike CD28, it has negative regulatory influences on T-cell activation. It has been suggested that CTLA-4 may preferentially dampen T-cell responses rather early in their genesis (41), and this would perhaps fit well with the time frames of our studies described here. The DC10 that we generated also expressed increased levels of IL-2 relative to DC-TNF, and this could potentially have contributed to their abilities to activate and expand the Tregs in our cocultures. IL-2 has been shown to be an important growth factor for Treg, particularly when tolerogenic DCs are present (20, 42). It has been well established that IL-10 plays important roles in modulation of allergic diseases. For example, IL-10 expression correlates with positive outcomes in specific immunotherapy subjects (43). IL-10 and IFN- α , both of which were expressed by our DC10, together strongly induce the differentiation of type 1 regulatory T (Tr1) cells from CD4⁺ T cells (44), and Tr1 cells are thought to be important to positive clinical outcomes in specific immunotherapy for allergic diseases (45). Tr1 expression of both IL-10 and TGF- β is reportedly important in their tolerogenic functions (45), and, like naturally occurring CD4⁺CD25⁺ Tregs, Tr1 cells express CD25, but unlike Tregs, they also express repressor of GATA-3 (45). However, we did not assess whether our Tregs expressed either TGF- β or repressor of GATA-3, so we cannot identify these activities with Tr1 cells. It has been shown, however, that Tr1 cells can prevent Th2 sensitization and IgE production if adoptively transferred before experimental sensitization (46).

In summary, we provide evidence suggesting that human monocyte-derived, IL-10-treated DCs expressed a regulatory

DC phenotype. They inhibited allergen-specific Th2 immune responses by autologous T cells, and also induced outgrowth of a population of IL-10-expressing CD25⁺Foxp3⁺LAG-3⁺CTLA-4⁺ Tregs *ex vivo*. These data open the question of whether DCs such as these might be useful for dampening allergen-specific responses in individuals with atopy, and whether they could potentially do so via induction of Treg responses.

Conflict of Interest Statement: X.L., A.Y., H.H., X.Z., J.T., B.D., and J.R.G. do not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.W.C. has acted as a consultant for Pharmaxis (2005–2008) for AUD\$14,000 and served on the advisory boards of Methapharm (2004–present), AstraZeneca Canada (2004–present), and Merck (2004–present) all for \$0. He has participated as a guest speaker for GlaxoSmithKline, AstraZeneca, Merck, Pharmaxis, and BI Canada for a token honoraria of less than CAN\$1000 per session. He has also received several industry-sponsored grants from Genetech, Inc. for \$26,000 per patient (approximately 10), Boehringer-Ingelheim for \$11,500 per subject (approximately 10), AllerGen (NCE Centres of Excellence), CIC establishment award (2007–2008) for \$70,000 and (2006–2007) for \$50,000, MedImmune (2006–2007) for \$13,000, Topogen (AllerGen CIC & Industry partnership) (2005–2006) for \$20,000–\$30,000, IVAX Research (AllerGen CIC & Industry partnership) (2005–6) for \$21,000 per subject (approximately 3), AllerGen NCE CIC IVAX Collaboration (2006) for \$60,291, and AllerGen Establishment Award (2005–2006) for \$50,000.

References

- Galli SJ, Lenz CS. Allergy. In: Paul WE, editor. *Fundamental immunology*. Philadelphia: Lippincott Raven; 1999. pp. 1127–1174.
- Umetu DT, Dekruyff RH. Immune dysregulation in asthma. *Curr Opin Immunol* 2006;18:727–732.
- Le Bras S, Geha RS. IPEX and the role of Foxp3 in the development and function of human Tregs. *J Clin Invest* 2006;116:1473–1475.
- Robinson DS. Regulation: the art of control? Regulatory T cells and asthma and allergy. *Thorax* 2004;59:640–643.
- Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;111:1255–1261.
- Reid SD, Penna G, Adorini L. The control of T cell responses by dendritic cell subsets. *Curr Opin Immunol* 2000;12:114–121.
- Holt PG. Pulmonary dendritic cells in local immunity to inert and pathogenic antigens in the respiratory tract. *Proc Am Thorac Soc* 2005;2:116–120.
- Holt PG. The role of airway dendritic cell populations in regulation of T-cell responses to inhaled antigens: atopic asthma as a paradigm. *J Aerosol Med* 2002;15:161–168.
- Holt PG. The role of genetic and environmental factors in the development of T-cell mediated allergic disease in early life. *Paediatr Respir Rev* 2004;5(Suppl A):S27–S30.
- Enk AH, Jonuleit H, Saloga J, Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer* 1997;73:309–316.
- Steinbrink K, Jonuleit H, Muller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8⁺ T cells resulting in a failure to lyse tumor cells. *Blood* 1999;93:1634–1642.
- Gordon JR, Li F, Nayyar A, Xiang J, Zhang X. CD8 alpha⁺, but not CD8 alpha⁻, dendritic cells tolerate Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. *J Immunol* 2005;175:1516–1522.
- Zhang-Hoover J, Finn P, Stein-Streilein J. Modulation of ovalbumin-induced airway inflammation and hyperreactivity by tolerogenic APC. *J Immunol* 2005;175:7117–7124.
- Koya T, Matsuda H, Takeda K, Matsubara S, Miyahara N, Balhorn A, Dakhama A, Gelfand EW. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *J Allergy Clin Immunol* 2007;119:1241–1250.
- Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4⁺ and CD8⁺ anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 2002;99:2468–2476.
- Gordon JR, Swystun VA, Li F, Zhang X, Davis BE, Hull P, Cockcroft DW. Regular salbutamol use increases CXCL8 responses in asthma: relationship to the eosinophil response. *Eur Respir J* 2003;22:118–126.
- Schneider AM, Li F, Zhang X, Gordon JR. Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease following sensitization with limiting doses of ovalbumin-alum. *Cell Immunol* 2001;212:101–109.
- Hamdi H, Godot V, Maillot MC, Prejean MV, Cohen N, Krzysiek R, Lemoine FM, Zou W, Emilie D. Induction of antigen-specific regulatory T lymphocytes by human dendritic cells expressing the glucocorticoid-induced leucine zipper. *Blood* 2007;110:211–219.
- Lagaraine C, Hoarau C, Chabot V, Velge-Roussel F, Lebranchu Y. Mycophenolic acid-treated human dendritic cells have a mature migratory phenotype and inhibit allogeneic responses via direct and indirect pathways. *Int Immunol* 2005;17:351–363.
- Banerjee DK, Dhodapkar MV, Matayeva E, Steinman RM, Dhodapkar KM. Expansion of Foxp3^{high} regulatory T cells by human dendritic cells (DCs) *in vitro* and after injection of cytokine-matured DCs in myeloma patients. *Blood* 2006;108:2655–2661.
- Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* 2001;193:233–238.
- Abbas AK. The control of T cell activation vs. tolerance. *Autoimmun Rev* 2003;2:115–118.
- Clatza A, Bonifaz LC, Vignali DA, Moreno J. CD40-induced aggregation of MHC class II and CD80 on the cell surface leads to an early enhancement in antigen presentation. *J Immunol* 2003;171:6478–6487.
- Bellinghausen I, Brand U, Steinbrink K, Enk AH, Knop J, Saloga J. Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. *J Allergy Clin Immunol* 2001;108:242–249.
- Steinbrink K, Wolf M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 1997;159:4772–4780.
- Yang S, Li W, Liu W, Gao C, Zhou B, Li S, Li Y, Kong Y. IL-10 gene modified dendritic cells induced antigen-specific tolerance in experimental autoimmune myocarditis. *Clin Immunol* 2006;121:63–73.
- Figuerola-Vega N, Galindo-Rodriguez G, Bajana S, Portales-Perez D, Abud-Mendoza C, Sanchez-Torres C, Gonzalez-Amaro R. Phenotypic analysis of IL-10-treated, monocyte-derived dendritic cells in patients with systemic lupus erythematosus. *Scand J Immunol* 2006;64:668–676.
- Morel E, Bellon T. HLA class I molecules regulate IFN-gamma production induced in NK cells by target cells, viral products, or immature dendritic cells through the inhibitory receptor ILT2/CD85j. *J Immunol* 2008;181:2368–2381.
- Young NT, Waller EC, Patel R, Roghanian A, Austyn JM, Trowsdale J. The inhibitory receptor LILRB1 modulates the differentiation and regulatory potential of human dendritic cells. *Blood* 2008;111:3090–3096.
- Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* 2004;22:929–979.
- Idoyaga J, Cheong C, Suda K, Suda N, Kim JY, Lee H, Park CG, Steinman RM. Cutting edge: Langerin/CD207 receptor on dendritic cells mediates efficient antigen presentation on MHC I and II products *in vivo*. *J Immunol* 2008;180:3647–3650.
- Wang F, Zhu W, Liu T, Sun Z, Ju S, Ju S, Yu G, Xie W, Deng Z, Lu B, et al. The expression analysis of ICOS-I on activated T cells and immature dendritic cells as well as malignant B cells and grave's-disease-derived thyroid tissues by two novel mAbs against human ICOS-I. *Tissue Antigens* 2007;69:62–72.
- Hornell TM, Burster T, Jahnsen FL, Pashine A, Ochoa MT, Harding JJ, Macaubas C, Lee AW, Modlin RL, Mellins ED. Human dendritic cell expression of HLA-DO is subset specific and regulated by maturation. *J Immunol* 2006;176:3536–3547.
- Neumann M, Fries H, Scheicher C, Keikavoussi P, Kolb-Maurer A, Brocker E, Serfling E, Kampgen E. Differential expression of Rel/NF-kB and octamer factors is a hallmark of the generation and maturation of dendritic cells. *Blood* 2000;95:277–285.
- Worbs T, Forster R. A key role for CCR7 in establishing central and peripheral tolerance. *Trends Immunol* 2007;28:274–280.
- Tibaldi EV, Sallia R, Reinherz EL. CD2 molecules redistribute to the uropod during T cell scanning: implications for cellular activation and immune surveillance. *Proc Natl Acad Sci USA* 2002;99:7582–7587.
- Hao S, Yuan J, Xu S, Munegowda MA, Deng Y, Gordon J, Xing Z, Xiang J. Antigen specificity acquisition of adoptive CD4⁺ regulatory T cells via acquired peptide-MHC class I complexes. *J Immunol* 2008;181:2428–2437.
- Hwang I, Huang JF, Kishimoto H, Brunmark A, Peterson PA, Jackson MR, Surh CD, Cai Z, Sprent J. T cells can use either T cell receptor or

- CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. *J Exp Med* 2000;191:1137–1148.
39. Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, Sly PD, Holt PG. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4⁺CD25⁺ regulatory T cells. *J Exp Med* 2006;203:2649–2660.
 40. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, *et al.* Role of LAG-3 in regulatory T cells. *Immunity* 2004;21:503–513.
 41. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev* 2008;224:166–182.
 42. Yamazaki S, Patel M, Harper A, Bonito A, Fukuyama H, Pack M, Tarbell KV, Talmor M, Ravetch JV, Inaba K, *et al.* Effective expansion of alloantigen-specific Foxp3⁺CD25⁺CD4⁺ regulatory T cells by dendritic cells during the mixed leukocyte reaction. *Proc Natl Acad Sci USA* 2006;103:2758–2763.
 43. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, Staple SQ, Aalberse RC, Till SJ, Durham SR. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* 2004;172:3252–3259.
 44. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN- α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* 2001;166:5530–5539.
 45. Battaglia M, Gregori S, Bacchetta R, Roncarolo MG. Tr1 cells: from discovery to their clinical application. *Semin Immunol* 2006;18:120–127.
 46. Cottrez F, Groux H. Specialization in tolerance: innate CD4⁺CD25⁺ versus acquired Tr1 and Th3 regulatory t cells. *Transplantation* 2004; 77(1, Suppl)S12–S15.

Induction of Type 2 T Helper Cell Allergen Tolerance by IL-10–Differentiated Regulatory Dendritic Cells

Xiuling Li^{1,2}, Aimei Yang, Hui Huang¹, Xiaobei Zhang¹, Jennifer Town¹, Beth Davis³,
Don W. Cockcroft³, and John R. Gordon^{1,3}

¹Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada;

²National Vaccine and Serum Institute, Beijing, China; and ³Division of Respiriology, Critical
Care and Sleep Medicine, Royal University Hospital, Saskatoon, Canada

Online Data Supplement

SUPPLEMENTARY DATA TABLES E1 - E5

Table E1. Array expression analysis data for pertinent intracellular signaling intermediates in immature (DC-GMCSF), tolerogenic (DC10) and mature (DC-TNF) monocyte-derived dendritic cells.

Symbol	RELATIVE mRNA EXPRESSION (spot density)			DIFFERENTIAL EXPRESSION ^A
	DC-GMCSF	DC10	DC-TNF	
GUSB	48815	47096	50043	-
ACPP	19471	23350	27498	-
ADAM19	51729	52614	46383	-
ADAMDEC1	11960	12755	23012	R-M
ADAR	60447	59221	57801	-
AIM2	16277	15440	18917	-
ARHGDIB	53989	27412	26733	-
ATF4	15649	17006	19399	-
ATM	28762	16970	28374	-
B3GALT2	5395	9128	8503	-
ICOSLG	8493	11026	7957	-
PPIA	61703	60900	58664	-
BASP1	32345	54470	43415	-
BTG1	61740	63578	61605	-
PPIA	61041	59183	56523	-
CCT6A	20242	17268	11902	-
CDC42	55032	51993	46710	-
CDKN1A	44829	53084	53551	-
CDW52	62798	50255	44249	-
CEBPA	48929	20610	17398	I-R, I-M
CFLAR	61791	54270	55864	-
CHD4	58003	47528	48845	-

RELATIVE mRNA EXPRESSION (spot density)				
Symbol	DC-GMCSF	DC10	DC-TNF	DIFFERENTIAL EXPRESSION ^A
CLECSF8	19278	11509	9497	I-M
CLECSF12	63776	56516	60524	-
CLECSF6	64395	55091	54437	-
C1QL1	47188	47815	57496	-
CSF1R	63525	58077	59924	-
CST3	63403	59822	60779	-
CST7	63547	59221	61853	-
DCP1B	17655	19184	14114	-
TM7SF4	59866	59968	51886	-
DCTN2	22650	14686	9016	-
EBI3/IL-27a	10326	8177	13079	-
ECGF1	25289	25105	27271	-
F13A1	47141	11822	8824	I-R, I-M
FSCN3	14850	10934	11347	-
G1P2	7364	10160	4320	R-M
G1P3	10237	10658	8086	-
GBP2	29710	26765	20767	-
GBP3	43671	42640	31117	-
GPX4	29193	38286	26045	-
IFI16	39344	11819	14294	I-R, I-M
IFI27	58228	48144	55476	-
IFI30	21342	15585	15312	-
IFI35	13692	5279	9250	I-R
IFI44	17446	5573	3908	I-R, I-M
IFIT1	46806	40122	49867	-
IFIT2	8212	7535	2155	I-M
IFIT3	3978	4828	1768	R-M

RELATIVE mRNA EXPRESSION (spot density)				
Symbol	DC-GMCSF	DC10	DC-TNF	DIFFERENTIAL EXPRESSION ^A
IFITM3	12564	20197	36913	I-M
IGSF6	54724	30775	21270	I-M
ISG20	51000	50212	58217	-
ITGAM	27900	9389	7917	I-R, I-M
ITGB2	25826	18616	13697	-
LILRB4	14555	10876	8043	-
LIPA	60348	46269	45034	-
LOC286272	10461	9328	9318	-
LRP1	7405	9223	12766	-
MAP4K3	8685	10788	6897	-
MARCKS	61188	62242	61609	-
MGC27165	16495	24533	33860	I-M
MARCKSL1	18587	37642	27879	I-R
ARID5A	7185	14843	26351	I-R, I-M
MT2A	18970	33307	47508	I-M
MX1	26686	29952	30529	-
NR3C2	17624	13063	10287	-
PFN1	59742	60827	58762	-
PIM2	16578	20520	20183	-
PLAUR	29098	22069	18141	-
PNRC1	47774	46741	53860	-
POR	55232	57501	58437	-
PRG1	52905	57242	54298	-
PRKRA	13189	13931	10084	-
PSCDBP	29774	51382	43381	-
RAC1	58256	59569	59191	-
RNASE6	43060	17550	16572	I-R, I-M
S100A4	57204	56099	58646	-

RELATIVE mRNA EXPRESSION (spot density)				
Symbol	DC-GMCSF	DC10	DC-TNF	DIFFERENTIAL EXPRESSION ^A
S100B	17166	13516	6672	I-M, R-M
SOD2	24445	37776	22095	-
STK4	33885	54180	56360	-
TNFSF11	5997	7384	6851	-
TNFSF14	30317	40126	51573	-
TNFSF4	2655	10168	32176	I-R, I-M
TNFSF9	3612	12042	24769	I-R, I-M
TRA1	21954	32436	25035	-
VCL	53169	52855	55615	-
ZNF398	7123	10688	9922	-
^B ACT β	58113	54696	58260	-
^B GAPDH	61081	59796	59648	-
^B β 2M	60347	61334	60613	-

^A Differential expression is defined as a ≥ 2 -fold difference in signal between DC-GMCSF (immature; I) and DC10 (regulatory; R) or DC-TNF (mature; M) cells. Thus, for example, I-R indicates significant differences in expression between the DC-GMCSF and the DC10.

^B ACT β , GAPDH and β 2M are included as control house-keeping gene products.

Table E2. Array expression analysis data for cluster of differentiation (CD) antigens in monocyte-derived dendritic cells.

Symbol	RELATIVE mRNA EXPRESSION (spot density)			DIFFERENTIAL EXPRESSION ^A
	DC-GMCSF	DC10	DC-TNF	
CD1A	62780	58379	50460	-
CD1B	64111	50140	48504	-
CD1C	62592	25310	30360	-
CD1D	11413	9821	10380	-
CD36	62912	54775	59871	-
CD44	62590	57785	60485	-
CD47	20898	18538	18696	-
CD58	56770	53656	58179	-
CD68	63883	56859	54826	-
CD74	64115	61014	60873	-
LY75	38927	54959	53076	-
FAS	19154	26211	27143	-
^B ACT β	58113	54696	58260	-
^B GAPDH	61081	59796	59648	-
^B β 2M	60347	61334	60613	-

^A Differential expression is defined as a ≥ 2 -fold difference in signal between DC-GMCSF (immature; I) and DC10 (regulatory; R) or DC-TNF (mature; M) cells. Thus, for example, I-R indicates significant differences in expression between the DC-GMCSF and the DC10.

^B ACT β , GAPDH and β 2M are included as control house-keeping gene products.

Table E3. Array expression analysis of toll-like receptors (TLR) in monocyte-derived human dendritic cells.

Symbol	RELATIVE EXPRESSION (spot density)			DIFFERENTIAL EXPRESSION ^A
	DC-GMCSF	DC10	DC-TNF	
TLR1	24198	11034	15358	I-R
TLR2	60290	55354	53891	-
TLR3	12333	12517	4823	I-M, R-M
TLR4	24838	26439	21330	-
TLR5	4570	12465	9998	I-R, I-M
TLR6	9521	8703	12465	
TLR9	3495	5701	11207	I-M, R-M
^B ACT β	58113	54696	58260	-
^B GAPDH	61081	59796	59648	-
^B β 2M	60347	61334	60613	-

^A Differential expression is defined as a ≥ 2 -fold difference in signal between DC-GMCSF (immature; I) and DC10 (regulatory; R) or DC-TNF (mature; M) cells. Thus, for example, I-R indicates significant differences in expression between the DC-GMCSF and the DC10.

^B ACT β , GAPDH and β 2M are included as control house-keeping gene products.

Table E4. Array expression analysis of immunoglobulin receptors in monocyte-derived human dendritic cells.

Symbol	RELATIVE EXPRESSION (spot density)			DIFFERENTIAL EXPRESSION ^A
	DC-GMCSF	DC10	DC-TNF	
F _C αR	15922	6082	5669	I-R, I-M
F _C εR1A	63300	45379	51948	-
F _C εR1G	63641	60169	61541	-
F _C εR2	63027	58814	54400	-
F _C γR1A	28621	32804	47593	-
F _C γR2B	48033	23530	43928	-
F _C γR3B	61463	49301	32888	I-M
F _C αR	15922	6082	5669	I-R, I-M
^B ACTβ	58113	54696	58260	-
^B GAPDH	61081	59796	59648	-
^B β2M	60347	61334	60613	-

^A Differential expression is defined as a ≥ 2 -fold difference in signal between DC-GMCSF (immature; I) and DC10 (regulatory; R) or DC-TNF (mature; M) cells. Thus, for example, I-R indicates significant differences in expression between the DC-GMCSF and the DC10.

^B ACTβ, GAPDH and β2M are included as control house-keeping gene products.

Table E5. Array analysis of cytokine expression by monocyte-derived human dendritic cells.

Symbol	RELATIVE EXPRESSION (spot density)			DIFFERENTIAL EXPRESSION ^A
	DC-GMCSF	DC10	DC-TNF	
IFN β 1	9058	11188	5665	R-M
IFN γ	52093	56243	58427	-
IFN γ R1	16092	8190	4819	I-R, I-M
IL1 α	7058	5088	3918	I-M
IL1 β	60286	61032	58981	-
IL2	7411	13877	3605	I-M, R-M
IL4	7645	14320	23348	I-R, I-M
IL15	13428	6513	7390	I-R
IL17	5444	3334	3873	-
MIF	48266	60870	60220	-
TNF	26547	27700	34778	-
^B ACT β	58113	54696	58260	-
^B GAPDH	61081	59796	59648	-
^B β 2M	60347	61334	60613	-

^A Differential expression is defined as a ≥ 2 -fold difference in signal between DC-GMCSF (immature; I) and DC10 (regulatory; R) or DC-TNF (mature; M) cells. Thus, for example, R-M indicates significant differences in expression between the DC10 and DC-TNF.

^B ACT β , GAPDH and β 2M are included as control house-keeping gene products.

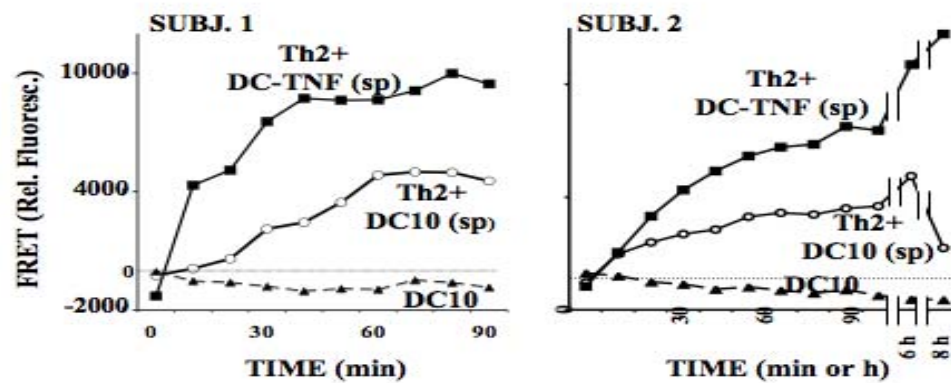


Figure E1. DC10 develop prolonged, intimate contact with autologous T cells. DC10 and DC-TNF were generated as in Fig. 1 from peripheral blood monocytes of two subjects and pulsed with allergen, while autologous CD4⁺ T cells were purified by MACS. The T cells were stained with the FRET donor dye DiO and the dendritic cells with the partner FRET dye DiI. The DC10 were either cultured alone (DC10) or with T cells (2×10^5 cells/well) for up to 8 h, while control wells contained DiI-stained immunostimulatory DC-TNF and DiO-stained Th2 cells.