Induction of anergic allergen-specific suppressor T cells using tolerogenic dendritic cells derived from children with allergies to house dust mites

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Background: Dendritic cells (DCs) regulate the immune response to allergens in the lung; they induce either effector or regulatory T cells, which promote or suppress, respectively, the development of allergy. IL-10 is a potent immunosuppressive cytokine that induces type 1 regulatory (Tr1) T cells.

Objective: To generate allergen-specific Tr1 cells *in vitro* from children with allergy.

Methods: Monocyte-derived DCs from children with allergy to house dust mites (HDM) were generated by incubating the cells with IL-10 and pulsing them with Der p 2, a major HDM allergen, or by pulsing them with Der p 2 and incubating them with IL-10 during their last 2 days of differentiation.

Results: Der p 2–specific T-cell proliferation and T_H2 cytokine production were significantly reduced when T cells from patients with allergy to HDM were activated with autologous Der p 2–pulsed DCs that had been differentiated or incubated with IL-10. T-cell lines generated with Der p 2–pulsed DCs that were differentiated with IL-10 were hyporesponsive to reactivation with Der p 2 and able to suppress Der p 2–specific T_H2 effector cells.

Conclusion: Dendritic cells differentiated in the presence of IL-10 and pulsed with allergen gave rise to a population of tolerogenic DCs that induced allergen-specific Tr1 cells. This finding represents an important step forward to the prospective clinical application of tolerogenic DCs to modulate allergen-specific T-cell responses. (J Allergy Clin Immunol 2010;125:727-36.)

Key words: *Allergy, dendritic cells, Der p 2, house dust mites, IL-10, T_H2 cells, Tr1 cells*

Abbreviations used

DC:	Dendritic cell
DC-10:	Dendritic cell differentiated in the presence of IL-10
Dp2-DC:	Dendritic cell pulsed for 2 days with Der p 2
HDM:	House dust mite
IL-10–DC:	Dendritic cell cultured in the presence of IL-10 for the 2 last days of differentiation
iDC:	Immature dendritic cell
LPS-DC:	Dendritic cells matured with LPS
LPS-Dp2-DC:	Dendritic cells pulsed for 2 days with Der p 2 and activated with LPS
Dp2-IL-10-DC:	Dendritic cells pulsed for 2 days with Der p 2 in the presence of IL-10
Dp2-DC-10:	Dendritic cells differentiated in the presence of IL-10 and pulsed for 2 days with Der p 2
ILT:	Immunoglobulin-like transcript
mDC:	Mature dendritic cell
MFI:	Mean fluorescence intensity
rh:	Recombinant human
SI:	Stimulation index
Tr1:	Type 1 regulatory T
Treg:	Regulatory T

Allergy to house dust mites (HDMs) is one of the most common forms of allergy, affecting about 15% of individuals in Western countries. Clinical characteristics of the patients include rhinoconjunctivitis, atopic dermatitis, and asthma. The immunological hallmark is a T_H2-skewed response that activates cellular events (eosinophilia, B-cell isotype switching to IgE production, mast cell degranulation, and so forth) in target tissues. Several allergens are involved in the immune responses to HDM; *Dermatophagoides pteronyssinus* allergen (Der p) 2 is one of the most common. About 80% of HDM-sensitive subjects have IgE antibodies specific for Der p 2.[1,2]

Defects in the balance between effector T_H2, T_H1, and regulatory T (Treg) cells are believed to underlie allergic reactions. Several Treg-cell subsets are defective in patients with allergy, including naturally occurring $CD4^+CD25^+$forkhead box protein (FOXP) 3^+ Treg cells[3,4] and adaptive type 1 regulatory T cells (Tr1).[4,5] Adaptive Tr1 cells, mainly through the production of IL-10, suppress not only T_H2 cells but also most effector cells involved in allergic inflammation, including eosinophils, mast cells, and basophils.[5,6] The balance between Tr1 and T_H2 cell compartments has an important role in the development of allergic responses. Allergen-specific effector T_H2 and suppressor Tr1 cells exist in healthy individuals and individuals with allergy. However, healthy individuals have an increased number of allergen-specific Tr1 cells and a

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TABLE I. Demographic and clinical description of the study population

Patients' characteristics	Patients* (n = 52)
Sex (girls/boys)	24/28
Age (y)	8.4 ± 3.4†
Range	2.8-14.4
Family history of asthma and/or allergy	34/52 (65.4)
Main symptom of house dust mite allergy	22/52 (42.3)
Asthma	25/52 (48.0)
Rhinoconjunctivitis	5/52 (9.6)
Atopic dermatitis	
Concurrent symptoms of allergy	25/52 (48.0)
Duration of symptoms (y)	3.7 ± 2.6
Respiratory infections	6/52 (11.5)‡
Occasional use of symptom relievers	30/52 (57.7)§

Note. Values given as number of patients/number of total patients recruited (%).
*All patients were defined allergic on the basis of the presence of both a history of allergic symptoms after allergen exposure and of specific IgE and/or skin prick tests positive to HDM.
†Data are expressed as means ± SDs unless differently stated.
‡Five patients had upper respiratory infections.
§Patients with asthma occasionally (< once/wk) used inhaled salbutamol for cough or asthma. Nobody used corticosteroid *per os*.

decreased number of allergen-specific T_H2 cells compared with subjects with allergy,[7,8] indicating that regulatory mechanisms mediated by Tr1 cells are defective in patients with allergy. Because individuals with allergy appear to have a defect in development of allergen-specific Tr1 cells, tolerance might be re-established and patients with allergy cured through induction and/or expansion of allergen-specific Tr1 cells. An important issue would be then how to induce and/or expand allergen-specific Tr1 cells in patients with allergy. So far, the long-term solution for the treatment of allergy is allergen-specific immunotherapy that, when successful, is associated with the induction of peripheral tolerance involving Tr1 cells that secrete the IL-10 and TGF-β.[9-13] However, immunotherapy has a number of limitations that must be considered, including the risk of severe systemic side effects.[14,15]

Several protocols have been developed to generate Tr1 cells *in vitro*[16]; although IL-10 is required for Tr1-cell induction, it is not sufficient for the *in vitro* differentiation of human Tr1 cells in the absence of antigen-presenting cells.[17] Tr1 cells can be induced by dendritic cells (DCs) that are at an immature stage of differentiation,[18,19] as well as by a specialized subset of DCs, termed *tolerogenic DCs*.[16,20] Immature DCs (iDCs), differentiated *in vitro* and incubated with IL-10, have reduced allostimulatory capacity and induce anergic, antigen-specific T cells.[21] Furthermore, DCs matured in the presence of exogenous IL-10 induce antigen-specific, anergic T cells,[22] which suppress T-cell proliferation through a cell–cell contact mechanism.[23] We recently identified and characterized a new population of tolerogenic DCs, termed *DC-10s*, that induce antigen–specific Tr1 cells with a single stimulation (Gregori et al, submitted July 2009).

In the current study, we compared the ability of DCs incubated with IL-10 (IL-10-DCs) and DC-10s to modulate allergen-specific T-cell responses and to prime T cells to become allergen-specific Tr1 cells *in vitro*. We demonstrate that although both DC subsets are powerful modulators of Der p 2–specific T_H2 responses, only DC-10s are able to induce allergen-specific anergic T cells with suppressive activity.

METHODS

Study subjects

The study included 52 children with histories of allergic symptoms (asthma, rhinoconjunctivitis, or atopic dermatitis) after exposure to HDM (HDM patients). All patients had positive results to skin prick tests (≥3 mm) and/or increased levels of IgE specific (CAP/FEIA; Phadia & Upjohn, Uppsala, Sweden) to an extract of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (ALK-Abelló, Hørsholm, Denmark); the patients had negative results in tests for allergies to cow's milk, egg, grass, birch, *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium*, *Olea* tree, and cat and dog dander. The demographic and clinical characteristics of the patients are reported in Table I. Ten age-matched healthy children (5 boys and 5 girls) without a personal and family history of allergy were included as controls (mean, 11.7 ± 4.1 years; range, 3.5-17.3 years). The study was approved by the local medical ethics committee (Policlinico di Tor Vergata, Tor Vergata University, Rome, Italy), and informed consent was obtained from the parents.

Cells preparation

PBMCs were purified by gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden).

DC differentiation

$CD14^+$ monocytes were isolated from PBMCs by positive selection using anti-CD14–conjugated magnetic Microbeads (MACS; Miltenyi Biotec, Belgish Gladbach, Germany) according to the manufacturer's instructions. The negative fraction ($CD14^-$) was collected and cryopreserved for later use. DCs were differentiated by culturing $CD14^+$ cells with recombinant human (rh) GM-CSF (100 ng/mL; R&D Systems, Minneapolis, Minn) and rhIL-4 (10 ng/mL; R&D Systems) for 5 days in RPMI 1640 containing 5% pooled AB human serum (BioWhittaker, Verniers, Belgium), 100 U/mL penicillin/ streptomycin (Bristol-Myers Squibb, New York, NY), and 2 mM/L L-glutamine (GIBCO BRL, Gaithersburg, Md). After 5 days, extractive purified endotoxin-free Der p 2 (1 μg/mL; Lofarma, Milan, Italy) was added, and DCs were cultured for an additional 2 days (Dp2-DCs). The optimal concentration of Der p 2 was defined from a dose-response curve (0.1 to 10 μg/mL). In parallel, DCs were left unstimulated (iDCs) or maturated with LPS from *Escherichia coli* (1 μg/mL; Sigma Chemicals, St Louis, Mo; LPS-DCs). In some experiments, Dp2-DCs were simultaneously activated with LPS (1 μg/mL; LPS-Dp2-DCs). Dp2-DCs incubated with IL-10 (Dp2–IL-10–DCs) were obtained by pulsing DCs with Der p 2 (1 μg/mL) in the presence of rhIL-10 (40 ng/mL; BD Biosciences, San Jose, Calif) for 2 days. Alternatively, DC-10s pulsed with Der p 2 (Dp2-DC-10s) were obtained by culturing $CD14^+$ cells with rhGM-CSF (100 ng/mL) and rhIL-4 (10 ng/mL) in the presence of rhIL-10 (10 ng/mL) for 7 days and pulsing them with Der p 2 (1 μg/mL) during the last 2 days. The purity and maturation state of DCs were routinely checked by flow-cytometric analysis to determine expression of CD14, CD1a, CD80, CD83, CD86, and HLA-DR (BD Biosciences).

T-cell differentiation

DCs (1×10^5) were cultured with 1×10^6 autologous $CD14^-$ cells in 1 mL X-vivo 15 medium (BioWhittaker), supplemented with 5% pooled AB human serum (BioWhittaker) and 100 U/mL penicillin/ streptomycin (Bristol-Myers Squibb). After 7 days of culture, rhIL-2 (20 U/mL; Chiron, Amsterdam, Holland) was added, and cells were expanded for an additional 7 days. At day 14, T-cell lines were collected and tested for their ability to respond to Der p 2.

Der p 2–specific T-cell proliferative responses

To analyze Der p 2–specific T-cell proliferation, autologous $CD14^-$ cells (5×10^4/well) were cultured in parallel with iDCs, Dp2-DCs, Dp2-IL-10-DCs, and Dp2–DC-10s (5×10^3/well at a 10:1 ratio of T cells:DCs) in 96-well,

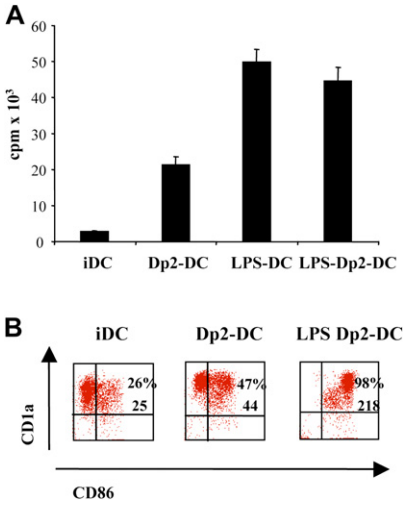

FIG 1. Der p 2 induces partial maturation of DCs, and Der p 2–pulsed DCs promote allergen-specific T-cell proliferation. **A,** $CD14^-$ cells from patients with HDM allergy were stimulated with autologous iDCs, DCs pulsed with Der p 2 (1 μg/mL; Dp2-DCs), DCs matured with LPS (1 μg/mL; LPS-DCs), or DCs pulsed with Der p 2 and matured with LPS (LPS-Dp2-DCs) at a ratio of 10:1. Proliferative responses were evaluated after 5 days of culture. One representative patient of the 3 analyzed is shown. **B,** CD86 expression on iDCs, Dp2-DCs, or LPS-Dp2-DCs was evaluated by flow cytometry analysis. *Numbers* represent the percentages of positive cells and the MFI. Data from 1 representative patient of 8 patients tested are presented.

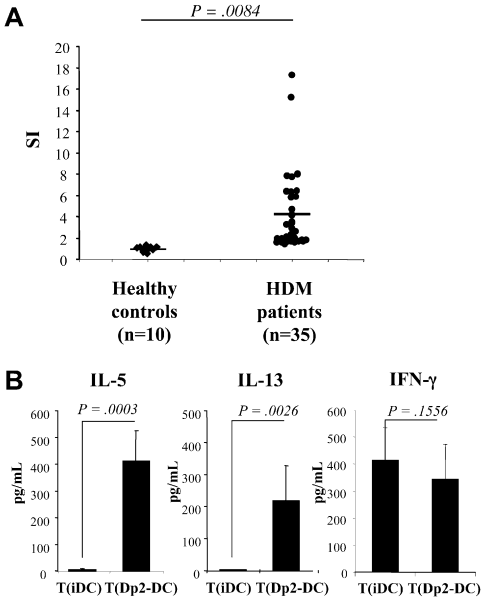

FIG 2. Dp2-DCs induce a T_H2-cell response in patients who are allergic to HDM (HDM patients) but not in healthy controls. **A,** $CD14^-$ cells from HDM patients and controls were stimulated with autologous Dp2-DCs at a ratio of 10:1. Results are expressed as SI. The *horizontal bars* indicate the means. A responder was defined any patient displaying a SI greater than or equal to the mean SI + 2 SDs, obtained by stimulating T cells of healthy controls with autologous Dp2-DCs. The cutoff value was set at 1.4. **B,** Production of IL-5 (n = 20), IL-13 (n = 18), and IFN-γ (n = 7; means ± SEMs) by T(iDC) and T(Dp2-DC) cells. Culture supernatants were collected after 5 days, and cytokine production was determined by the Bioplex Protein Array system.

round-bottom microplates, in triplicate, in a final volume of 200 μL medium. After 5 days of culture, T-cell proliferation was assessed by overnight incorporation of [^{3}H] thymidine (1 μCi/well; Amersham International, Amersham, United Kingdom). A responder was defined as any patient that had a stimulation index (SI) greater than or equal to the mean SI + 2 SDs obtained by stimulating T cells of healthy controls with autologous Dp2-DCs. The cutoff was set at 1.4 (1.0 ± 2 * (0.2) = 1.4).

To analyze the allergen-specific proliferative capacity of T(Dp2-DC), T(Dp2-IL-10-DC), or T (Dp2–DC-10) cell lines, cells (5×10^4/well) were cultured alone or with autologous monocytes (5×10^4/well at a 1:1 ratio of T cells:monocytes) in the absence or presence of Der p 2 (1 μg/mL) and Herpes Simplex Virus 1 (HSV1) (0.5 μg/mL) in 96-well, round-bottom plates in a final volume of 200 μL medium. In addition, T-cell lines (5×10^4/well) were stimulated with immobilized anti-CD3 mAb (10 μg/mL; Orthoclone [OKT3]; Jansen-Cilag, Raritan, NJ) and 12-0-tetradodecanoyl-phorbol-13-acetate (10 ng/mL; Sigma) in complete medium. After 2 days of culture, T-cell proliferation was assessed from overnight incorporation of [^{3}H] thymidine (1 μCi/well).

To test the suppressive capacity of T(Dp2–DC-10s), T(Dp2-DC) cells (5×10^4/well) were stimulated with autologous monocytes (5×10^4/well at a 1:1 ratio of T cells:monocytes) pulsed with Der p 2 (1 μg/mL) in the absence or in the presence of T(Dp2–DC-10s) (1:1 ratio) in 96-well, round-bottom plates, in a final volume of 200 μL medium. In some cultures, neutralizing antibodies against IL-10 receptor (R) (30 μg/mL; 3F9; BD Biosciences) and anti–TGF-β (50 μg/mL; 1D11; R&D Systems) were added. After 3 days of culture, supernatants were collected for cytokine detection.

Cytokine production assays

Levels of IL-4, IL-5, IL-10, IL-13, TNF-α, and IFN-γ production by cocultured DCs and T cells were measured from supernatants by the Bioplex Protein Array system according to the manufacturer's instructions (BioRad, Hercules, Calif). To measure production of IL-10, TNF-α, and IL-12 by DCs, cells were left unstimulated or activated with 50 ng/mL rhIFN-γ (R&D Systems) and 20 ng/mL LPS (Sigma); supernatants were collected 48 hours later, and the cytokines were measured by ELISA (BD Biosciences).

Statistical analysis

Results are presented as means ± SDs unless differently stated. The Student *t* test was used to compare the mean values of cell proliferation and cytokine production between patients and controls and between different cell culture conditions. A *P* value <.05 was considered statistically significant.

RESULTS

Der p 2–pulsed DCs promote T_H2-cell responses in HDM patients

Stimulation of T cells from HDM patients with autologous Dp2-DCs resulted in Der p 2–specific T-cell proliferation (Fig 1, *A*). Surprisingly, T cells stimulated with autologous DCs activated with LPS and pulsed with Der p 2 for 2 days (LPS-Dp2-DCs) displayed a proliferative response comparable to that obtained with DC activated with LPS for 2 days (LPS-DCs), indicating that activation with LPS completely masked the ability of DCs pulsed with Der p 2 to elicit an allergen-specific T-cell response. The ability of Der p 2–pulsed DCs to elicit an allergen-specific T-cell response paralleled the increase in CD86 expression in terms of percentage and of mean fluorescence intensity (MFI; 43% ± 14%, MFI 35.5 ± 18 vs 33% ± 14%, MFI 27 ± 13 in Dp2-DCs and iDCs, respectively; n = 8, Fig 1, *B*). These results indicate that pulsing with Der p 2 induced a partial maturation of DCs that promoted induction of allergen-specific T-cell responses *in vitro*. As expected, Der p 2–pulsed DCs promoted proliferation

of T cells from HDM patients but not from aged-matched healthy controls (SI values of 4.2 ± 3.7 and 1.0 ± 0.2, respectively; $P = .0084$; Fig 2, *A*). Among children with allergy, Der p 2–specific T-cell proliferation was observed in 35 of 46 patients (76.1%).

T cells from HDM patients stimulated with Dp2-DCs secreted significantly greater amounts of IL-5 (410 ± 110 pg/mL, $P = .0003$, in 20 of 26 patients with allergy) and of IL-13 (220 ± 110 pg/mL, $P = .0026$ in 18 of 26 patients) compared with cells activated with iDCs (6 ± 3 pg/mL and 3 ± 1 pg/mL, respectively; Fig 2, *B*). Importantly, T cells stimulated with Dp2-DCs secreted similar levels of IFN-γ as T cells primed with iDCs (416 ± 119 pg/mL vs 344 ± 125 pg/mL; n = 7). Thus, Der p 2–pulsed DCs promoted the proliferation of allergen-specific T cells with a T_H2 cytokine production profile without skewing cytokine production toward the T_H1 profile.

Differentiation and characterization of tolerogenic DCs

We compared the phenotype and function of DCs cultured in the presence of IL-10 for the 2 last days of differentiation (IL-10–DCs) and DC-10s; IL-10–DCs were $CD14^-CD1a^+$ and expressed lower levels of HLA-DR, CD80, CD83, and CD86 compared with mature DCs (mDCs), whereas DC-10s were $CD14^+CD1a^-$ and displayed a myeloid mature phenotype based on their expression of HLA-DR, CD80, CD83, and CD86 (Fig 3, *A*). Therefore, incubation of DCs with IL-10 during the last 2 days of differentiation results in a population of DCs that is phenotypically different from that obtained by differentiation of DCs in the presence of exogenous IL-10.

IL-10–DCs and DC-10s spontaneously secreted comparably high levels of IL-10 and no IL-12 (Fig 3, *B*). However, IL-10–DCs secreted larger amounts of TNF-α compared with DC-10s. Upon activation with LPS and IFN-γ, although IL-10–DCs and DC-10s secreted high levels of IL-10, IL-10–DCs produced significantly greater amounts of IL-12 and TNF-α compared with DC-10s (Fig 3, *B*). These results indicate that activated DC-10s maintain their ability to secrete IL-10 but produce less IL-12 and TNF-α than IL-10–DCs.

Naive $CD4^+$ T cells stimulated with allogeneic IL-10–DCs or DC-10s had a significantly lower proliferative response compared with naive $CD4^+$ T cells primed with mDCs: 68% ± 17% (n = 6; $P = .006$) and 85% ± 8% (n = 6; $P = .001$) average percent reduction, respectively (Fig 3, *C*). As expected, iDCs also poorly stimulated allogeneic naive $CD4^+$ T cells, with 64% ± 19% ($P = .017$; n = 6) less proliferation. Interestingly, the stimulatory capacity of DC-10s was significantly less than that of IL-10–DCs (n = 6; $P = .038$; Fig 3, *C*). Similarly, IFN-γ production by naive $CD4^+$ T cells stimulated with allogeneic IL-10–DCs or DC-10s was lower compared with production by naive $CD4^+$ T cells primed with mDCs (data not shown). Overall, these findings indicate that even though IL-10–DCs and DC-10s differ in phenotype and cytokine production, both cell types displayed low stimulatory capacity.

Modulation of the Der p 2–specific T-cell response by tolerogenic DCs

T cells from HDM patients were stimulated with Der p 2–pulsed IL-10–DCs (Dp2–IL-10–DCs; n = 16) or with Der p 2–pulsed DC-10s (Dp2–DC-10s; n = 14); T cells stimulated with Dp2-DCs were used as controls. In cells from all but 1 patient, Dp2–IL-10–DCs and Dp2–DC-10s promoted significantly lower proliferative responses compared with that elicited by Dp2-DCs (69% ± 20%, n = 16, $P = .00023$, and 65% ± 21% less proliferation, respectively, n = 14, $P = .0038$; Fig 4, *A*). T cells stimulated with Dp2–IL-10–DCs (T[Dp2–IL-10–DCs]) secreted significantly less IL-5 (220 ± 140 pg/mL, mean ± SE; n = 7; $P = .04$) and IL-13 (50 ± 30 pg/mL; n = 5; P = not statistically significant [NS]) compared with control T cells (T[Dp2-DCs]; 340 ± 180 pg/mL, n = 7, and 115 ± 60 pg/mL, n = 5, respectively; Fig 4, *B*). These data were confirmed by the reduced ratio of IL-5 to IFN-γ: 1.1 ± 0.4 for T(Dp2–IL-10–DC) cells and 2.5 ± 1.4 for T(Dp2-DC) cells, respectively (n = 7).

Similarly, T cells primed with Dp2–DC-10s (T[Dp2–DC-10s]) secreted significantly less IL-5 (98 ± 37 pg/mL; n = 13; $P = .015$) and less IL-13 (27 ± 8 pg/mL; n = 13; $P = .075$, NS) compared with T(Dp2-DC) cells (390 ± 119 pg/mL and 258 ± 160 pg/mL, respectively; Fig 4, *B*). The ratio of IL-5 to IFN-γ was also significantly reduced in T(Dp2–DC-10) cells compared with T(Dp2-DCs) cells (7.1 ± 4.3 vs 49.4 ± 30.2, respectively; n = 13; $P = .05$). Taken together, these results indicate that autologous Der p 2–pulsed IL-10–DCs and DC-10s inhibited allergen-specific T-cell proliferation and IL-5 and IL-13 production.

Der p 2–pulsed DC-10s induce T-cell anergy

The ability of Dp2–IL-10–DCs and Dp2–DC-10s to modulate allergen-specific T-cell responses prompted a study of whether they would be able to generate allergen-specific suppressor T cells. The low number of $CD14^+$ cells routinely obtained from a single blood sample of pediatric patients prevented comparison of Dp2-DCs that had been incubated with IL-10 and Dp2–DC-10s in generating allergen-specific regulatory T cells; numbers of $CD14^+$ cells were not sufficient to differentiate the DCs required to perform repetitive stimulation with IL-10.[23] We therefore investigated only the ability of DC-10s to induce Tr1 cells, because a single stimulation of $CD4^+$ T cells with allogeneic DC-10s is sufficient to induce allospecific Tr1 cells (S. Gregori, submitted July 2009).

House dust mite patients' T cells, primed with Dp2–DC-10s, were hyporesponsive to reactivation with autologous monocytes that had been pulsed with Der p 2 (counts per minute [cpm], 9633.6 ± 920.6 in T[Dp2-DC] cells vs 4061.5 ± 577.1 in T[Dp2–DC-10] cells; $P = .0026$). In particular, in 13 of 16 patients tested, we observed an average reduction of proliferation of 63.2% ± 24.4% in T(Dp2–DC-10) cells compared with proliferation of T(Dp2-DC) cells ($P = .0013$; Fig 5, *A*). T(Dp2–DC-10) cell lines secreted significantly lower amounts of IL-5 (378 ± 289 pg/mL; n = 15; $P = .05$) and IL-13 (263 ± 166 pg/mL; n = 15; $P = .003$) compared with T(Dp2-DC) cell lines (917 ± 766 pg/mL and 546 ± 234 pg/mL, respectively), with a reduction of the ratio of IL-5 to IFN-γ (7.7 ± 5.9 vs 33.0 ± 23.2 in T[Dp2–DC-10s] and T[Dp2-DCs], respectively). Conversely, allergen-induced TNF-α and IFN-γ production was comparable between the 2 cell lines (Fig 5, *B*), indicating that stimulation with Dp2–DC-10s does not prevent T-cell activation. Importantly, on restimulation with HSV1, T(Dp2–DC-10) and T(Dp2-DC) cell lines displayed similar proliferative capacity (Fig. 5, *C*). Moreover, on polyclonal stimulation, T(Dp2–DC-10) and T(Dp2-DC) cell lines secreted comparable amounts

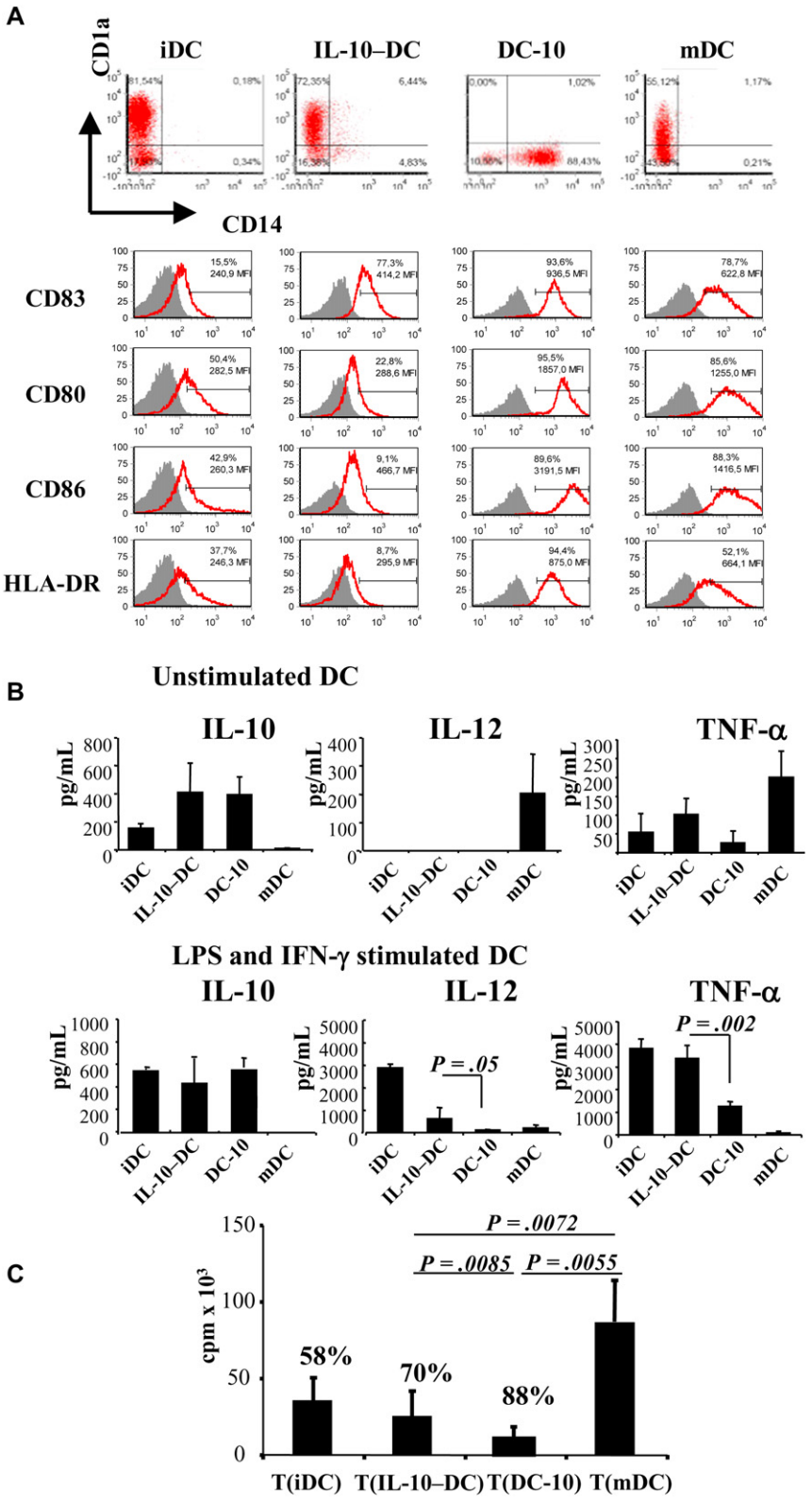

FIG 3. IL-10–DCs and DC-10s are phenotypically and functionally different. **A,** Monocyte-derived DCs were differentiated by incubation with IL-4 and GM-CSF for 5 days and then incubated with IL-10 (40 ng/mL) for an additional 2 days (IL-10–DCs), or with IL-4 and GM-CSF in the presence of IL-10 for 7 days (10 ng/mL; DC-10s). As a control, DCs were cultured for an additional 2 days with (mDCs) or without LPS (iDCs). Expression levels of CD14, CD1a, HLA DR, CD80, CD83 and CD86 were evaluated by flow cytometry. Data from 1 representative donor of 4 tested are presented. **B,** iDCs, IL-10–DCs, DC-10s, and mDCs were cultured alone or activated with IFN-γ (50 ng/mL) and LPS (1 μg/mL). Culture supernatants were collected after 48 hours, and cytokine levels were determined by ELISA in 4 independent experiments. **C,** Naive $CD4^+$ T cells were stimulated with allogenic iDCs, DC-10s, IL-10–DCs, and mDCs at a ratio of 10:1. Proliferative responses were evaluated 4 days after culture. *Numbers* represent the percentage of inhibition of proliferation of T cells primed with IL-10–DCs or DC-10s compared with proliferation of T cells stimulated with mDCs. Results from 1 representative donor of 6 tested are shown.

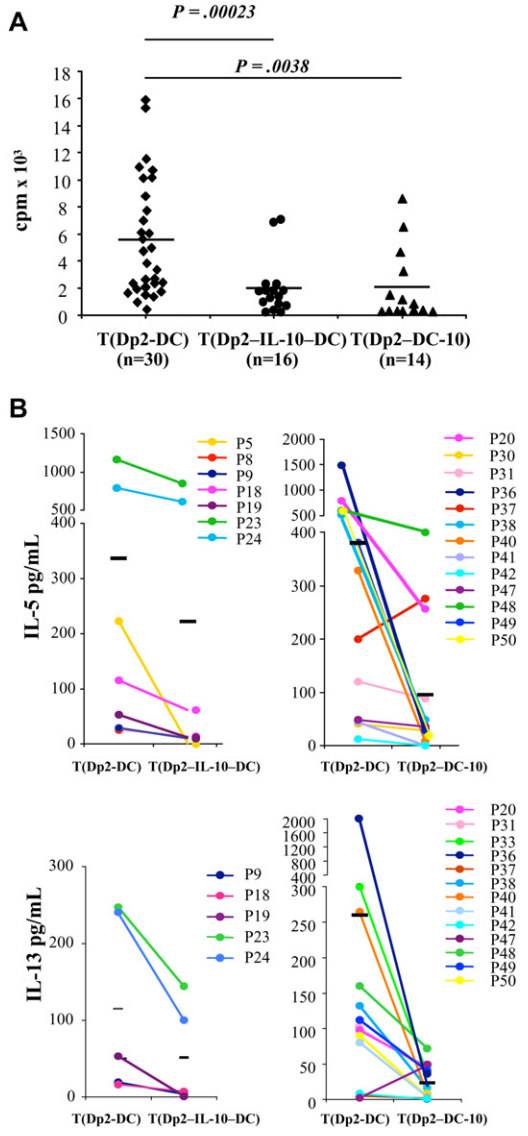

FIG 4. Dp2–IL-10–DCs and Dp2–DC-10s inhibit Der p 2–specific T-cell responses. T cells from HDM patients were stimulated with autologous Dp2-DCs (T[Dp2-DCs]), IL-10–Dp2–DCs (T[Dp2–IL-10–DCs]), or Dp2–DC-10s (T[Dp2–DC-10s]) at a ratio of 10:1. **A,** Proliferative responses were evaluated after 5 days of culture. *Dots* represent each individual patient, and *bars* represent the means of cpm. **B,** Cytokine production was determined in T-DC cell culture supernatants by the Bioplex Protein Array system. Results relative to T(Dp2–IL-10–DCs) and T(Dp2–DC-10s) are shown in the *left and right panels*, respectively, for each patient. *Horizontal bars* represent the mean cytokine production values. IL-5 production by T(Dp2–IL-10–DCs) versus T(Dp2-DCs), $P = .04$; by T(Dp2–DC-10s) versus T(Dp2-DCs), $P = .015$. IL-13 production, P = NS.

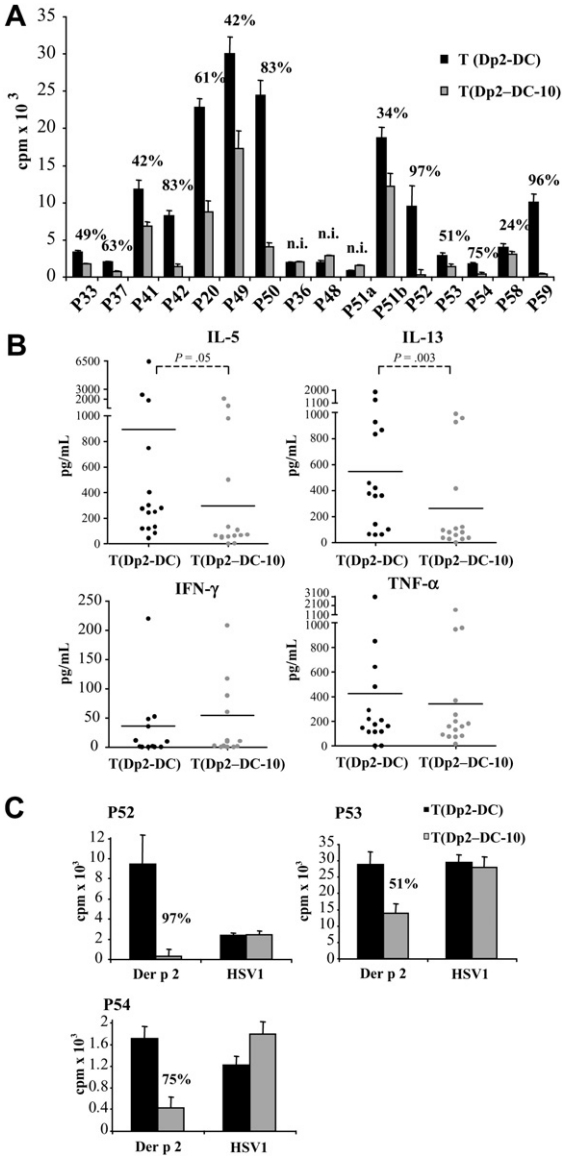

FIG 5. Dp2–DC-10s induce allergen-specific T-cell anergy. **A,** $CD14^-$ cells from HDM patients were stimulated with autologous Dp2-DCs (T[Dp2-DCs]) or Dp2–DC-10s (T[Dp2–DC-10s]) for 14 days. After stimulation, T-cell lines were collected and tested for their ability to proliferate in response to autologous $CD14^+$ cells pulsed with Der p 2 (1 μg/mL) at a ratio of 1:1. Two days later, proliferation was evaluated. Proliferative responses of 16 HDM experiments are shown. Means of triplicates ± SDs and percentages of anergic cells are presented for each patient; *n.i.*, anergy not induced. Average allergen-induced proliferation of T(Dp2–DC-10s) versus T(Dp2-DCs), $P = .0026$ (n = 16). **B,** Levels of IL-5, IL-13, IFN-γ, and TNF-α in culture supernatants were determined by the Bioplex Protein Array system. The cytokine profile of each patient is shown, and the *horizontal bars* represent the mean values. T(Dp2–DC-10s) versus T(Dp2-DCs), IL-5 production, $P = .05$; IL-13 production, $P = .003$; IFN-γ production, P = NS; and TNF-α production, P = NS; n = 15. **C,** Induction of T-cell anergy by Dp2-DCs is allergen-specific. The proliferative responses of cells from 3 patients, in which anergy was induced, are shown on restimulation of T(Dp2–DC-10s) with either Der p 2 (1 μg/mL) or HSV1 (0.5 μg/mL) in the presence of autologous monocytes.

of IL-5 and IL-13 (data not shown), showing that Dp2–DC-10s promote anergy of allergen-specific T cells. Thus, activation of T cells from HDM patients with autologous Dp2–DC-10s promotes the induction of anergic, Der p 2–specific T cells that secrete low amounts of allergen-specific T_H2 cytokines.

Der p 2–pulsed DC-10s induce suppressor T cells

Anergic T cells generated with Dp2–DC-10s suppressed IL-5 and IL-13 production by Der p 2–specific T(Dp2-DC) cell lines; average reductions in IL-5 and IL-13 secretion were 62.3% ± 29.5% (n = 5; $P = .08$) and 50.5% ± 25.3% (n = 5; $P = .02$), respectively (Fig 6, *A*). Although the levels of IFN-γ in culture supernatants were very low, no differences were

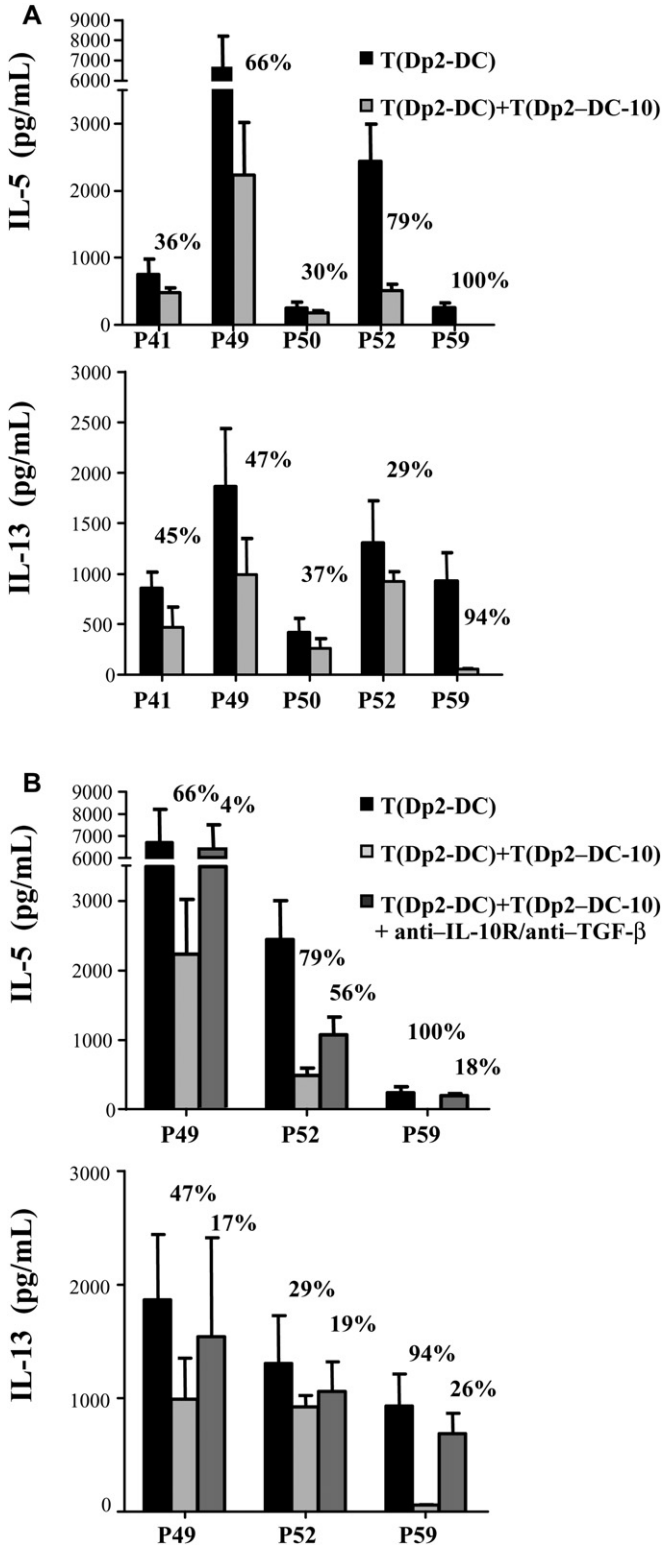

FIG 6. Dp2–DC-10s induce allergen-specific Tr1 cells. $CD14^-$ cells from HDM patients were stimulated with autologous Dp2-DCs (T[Dp2-DCs]) or Dp2–DC-10s (T[Dp2–DC-10s]) for 14 days. T(Dp2-DC)–cell lines were cultured with autologous $CD14^+$ pulsed with Der p 2 (1 μg/mL) in the presence or absence of autologous T(Dp2–DC-10) cell lines at a ratio of 1:1. **A,** Culture supernatants were collected after 3 days of culture; levels of IL-5 and IL-13 were measured by using the Bioplex Protein Array system. *Numbers* represent the percentage suppression of cytokine production in T(Dp2-DCs) cocultured in the presence of T(Dp2–DC-10s) compared with T(Dp2-DCs) alone (IL-5, $P = .08$; IL-13, $P = .02$; n = 5). **B,** T(Dp2–DC-10) cells suppress cytokine production of T(Dp2-DC) cells partially via IL-10 and TGF-β. T(Dp2–DC-10) cell lines were tested for their ability to suppress cytokine production by allergen-specific T cells in response to autologous $CD14^+$ cells pulsed with Der p 2 (1 μg/mL) in the absence or presence of mAbs against IL-10R (30 μg/mL) and TGF-β (50 μg/mL). Levels of IL-5 and IL-13 were measured in culture supernatants after 3 days of culture. *Numbers* represent the suppression rate of cytokine production in T(Dp2-DC) cells cocultured with T(Dp2–DC-10) cells, with or without mAbs against IL-10R and TGF-β, compared with T(Dp2-DC) cells alone (reversion of IL-5, $P = NS$; reversion of IL-13, $P = .05$; n = 3).

observed between T(Dp2-DC) and T(Dp2-DC) + T(Dp2–DC-10) cultures (20.0 ± 12.3 and 28.2 ± 18.6; n = 5, respectively; $P = NS$). To investigate whether anergic, allergen-specific T cells generated with Dp2–DC-10s are functionally equivalent to Tr1 cells, we performed suppression assays in the presence of neutralizing mAbs against IL-10R and TGF-β. In 3 patients tested, the suppression, mediated by T(Dp2–DC-10) cells, was partially reverted by the addition of the mAbs, with average reversion values of 74% ± 27% (n = 3; $P = NS$) and 79% ± 5% (n = 3; $P = .05$) in IL-5 and IL-13 secretion, respectively (Fig 6, *B*). Therefore, Dp2–DC-10s are able to generate a population of anergic, allergen-specific T cells that are functionally equivalent to Tr1 cells.

DISCUSSION

In the current study, we found that monocyte-derived DCs pulsed with Der p 2 are efficient in promoting Der p 2–specific T_H2 response in T cells from HDM patients but not healthy controls. In addition, we demonstrated that IL-10 efficiently modulates the effector functions of Der p 2–pulsed DCs: either incubation of Dp2-DCs with IL-10 or differentiation of Dp2-DCs in the presence of IL-10 resulted in a population of DCs that efficiently inhibited Der p 2–specific T_H2 cell proliferation and cytokine production. Importantly, Dp2–DC-10s induced anergic, allergen-specific T cells that were functionally equivalent to Tr1 cells.

The ability of allergen-pulsed DCs to induce T_H2-cell response has been previously demonstrated using autologous Der p 1–pulsed DCs that were shown to stimulate T cells from patients with allergy to secrete IL-4.[24] However, this is the first evidence that autologous Der p 2–pulsed DCs efficiently stimulate a Der p 2–specific T_H2-cell response in HDM patients but not in healthy individuals. The frequency of T-cell response in our cohort (76.1%) is consistent with the prevalence of IgE reactivity to Der p 2 described in the literature.[1,2] Moreover, our data are in line with previous reports demonstrating that Der p 2 stimulation of total PBMCs from patients with HDM allergy, but not from healthy controls, promptly induced allergen-specific proliferation of T_H2 cells.[25,26]

Substantial amounts of IL-5 and IL-13 were detected after 5 days of T-cell stimulation with autologous, Der p 2–pulsed DCs. Nevertheless, in some patients, IL-5 and IL-4 were undetectable. The lack of IL-5 and IL-4 production in some patients with allergy, already reported,[27-29] could be a result of the low frequency of Der p 2–specific T_H2-cell clones in the peripheral blood of HDM patients.[30] IL-5–producing and IL-4–producing T cells accumulate and infiltrate the inflamed bronchial mucosa of

patients with allergy.[30] The discrepancy between IL-5 (detected in 20 of 26 patients with allergy) and IL-4 production (detected in 3 of 26 patients) could be also explained by the fact that the peak of IL-4 production occurs before that of IL-5 and then rapidly declines.[31-33] Indeed, IL-4 is a growth factor for B and T_H2 cells, which might result in consumption of IL-4 in the majority of the cell cultures from patients with allergy. Alternatively, the phase of disease, acute versus chronic, might justify a differential involvement of IL-4 and IL-5, respectively, in the allergic inflammatory response.[34]

Exposure of DCs to LPS or cross-linking of CD40 promotes DC maturation and optimizes antigen presentation.[35] However, activation of Der p 2–pulsed DCs with LPS did not improve the specific Der p 2–presenting capacity of DCs beyond the effects of LPS alone. On the other hand, Der p 2 pulsing *per se* induced partial maturation of DCs, demonstrated by their selective upregulation of CD86 expression and inability to promote Der p 2–specific responses. This finding is supported by a recent report that identified a novel mechanism of Der p 2–mediated allergenicity; Der p 2, by mimicking Toll-like receptor 4 activation, promotes an immune response that is skewed toward a T_H2 cytokine profile.[36]

It has been proposed that the type of costimulatory molecules expressed by DCs shapes the cytokine expression profile of T_H cells: CD86 is more important than CD80 in promoting T_H2 responses.[37] mAbs against CD86, but not CD80, inhibit allergic reactions and T-cell proliferation.[38,39] In addition, the production of IL-5 and IL-13 by allergen-specific T cells from patients with asthma depends on CD86 in the periphery and on CD80 in inflamed tissues.[40]

DCs, either incubated with IL-10 during the last 2 days of differentiation or differentiated in the presence of IL-10, were able to modulate T-cell responses, despite their different phenotypes and cytokine production profiles. Both cell types inhibited allergen-specific T-cell proliferation and markedly reduced the ability of T cells to secrete IL-5 and IL-13. The ability of IL-10[6,7,41-43] and of DCs that were incubated with IL-10[44,45] to inhibit the T_H2 response *in vitro* has been extensively demonstrated. Adoptive transfer of bone marrow–derived DCs, cultured in the presence of IL-10, to sensitized mice decreased airway hyperresponsiveness, inflammation, and T_H2 cytokine production.[46] These effects were associated with IL-10 production by autologous DCs.[46] In patients, IL-5 and IL-13 secretion by T cells, activated with IL-10–exposed DCs or DC-10s, was reduced without skewing the cytokine profile toward T_H1; modification of DC function by IL-10 therefore might attenuate the allergic response without increasing the risk of T_H1-mediated diseases. Therapeutic strategies for controlling T_H2-immune responses based on upregulation of a T_H1-mediated response are not ideal because they have the potential of inducing autoimmunity and the risk of exacerbating allergic immune responses. Moreover, TNF-α production by anergic T cells was preserved (data not shown), indicating that innate immunity was not affected, but the tolerogenic effect of DC-10s was directed mainly toward T_H2 effector cells.

The inhibitory influence of IL-10 on the antigen-presenting function of DCs is mediated by several phenotypic and functional alterations, including downregulation of MHC class II and costimulatory molecules and inhibition of variety of proinflammatory cytokines.[47-53] We showed that incubation of Der p 2–pulsed DCs with IL-10 significantly downregulated CD86 expression. Nevertheless, we did not observe a correlation between CD86 downregulation and the reduction of allergen-specific T-cell proliferation, indicating that the poor stimulatory capacity of IL-10–exposed Dp2-DCs does not result from inhibition of costimulation. In addition, although Dp2–DC-10s displayed a mature phenotype, they maintained their ability to endocytose antigens (data not shown) and downmodulate allergen-specific T-cell proliferation. Taken together, these data indicate that poor stimulatory capacities of IL-10–exposed Dp2-DCs and Dp2–DC-10s do not result from lack of costimulation. The capacity of DCs to inhibit T-cell proliferation and promote T-cell anergy and Treg-cell differentiation is not merely associated with a reduced expression of costimulatory molecules,[54,55] but it is associated with the expression of tolerogenic molecules, including inhibitory molecules such as ICOS-ligand (L)[42,56] and immunoglobulin-like transcript (ILT)–3 and ILT4.[57]

We recently identified and characterized a population of tolerogenic DCs, termed DC-10s, that are present *in vivo* and can be differentiated *in vitro* in the presence of IL-10. DC-10s are $CD14^+CD16^+CD11c^+CD11b^+$, secrete high levels of IL-10 but not IL-12, display a mature myeloid phenotype ($CD83^+CD80^+CD86^+$), and express tolerogenic molecules such as ILT4 and HLA-G antigen. A single stimulation of allogeneic $CD4^+$ T cells with DC-10s induces a population of anergic T cells that contains as much as 15% IL-10–secreting T cells that have suppressive activity (Gregori et al, submitted July 2009). We now demonstrate that DC-10s pulsed with Der p 2 are powerful inducers of allergen-specific anergic Tr1 cells that secrete low amounts of T_H2 cytokine and suppress effector T_H2 cells. Tr1 cells have a pivotal role in modulating allergen-specific T-cell responses,[7,8] and their induction is required to re-establish tolerance in patients with allergy.[9-13,58] It has been demonstrated that allergen-specific Tr1 cells are present in atopic and nonatopic patients,[7] and we showed that DC-10s are present in peripheral blood (Gregori et al, submitted July 2009). Therefore, it would be interesting to investigate the role and impact of DC-10s in promoting allergen-specific Tr1 cells in atopic versus nonatopic patients to define better the requirements for the induction of allergen-specific Tr1 cells.

Collectively, our results demonstrate that Der p 2–pulsed DCs promote an allergen-specific T_H2 response *in vitro* and that this response can be modulated by IL-10. Importantly, we show that antigen-pulsed DC-10s are a novel and interesting tool to be used to generate antigen-specific Tr1 cells *in vitro*. Further investigation of these cells might lead to safe and effective DC-10–based therapies for patients with allergies.

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Clinical implications: The use of tolerogenic DCs to modulate type 2 immune responses and induce allergen-specific suppressor T cells reveals therapeutic and prophylactic interventions for allergic diseases.

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