

Impaired IL-10–dependent Induction of Tolerogenic Dendritic Cells by CD4⁺CD25^{hi}CD127^{lo/-} Natural Regulatory T Cells in Human Allergic Asthma

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Rationale: Tolerogenic dendritic cells and natural regulatory T cells have been implicated in the process of infectious tolerance in human allergic asthma. However, the significance of the influence of natural regulatory T cells on tolerogenic dendritic cells in the disease has not been investigated.

Objectives: We aimed to characterize the mechanism of induction of the tolerogenic phenotype in circulating blood dendritic cells by allergic asthmatic natural regulatory T cells.

Methods: The study was performed in a cohort of 21 subjects with allergic asthma, 21 healthy control subjects, and 21 subjects with nonallergic asthma. We cultured blood dendritic cells with natural regulatory T cells to study the induction of tolerogenic dendritic cells. Flow cytometry and proliferation assays were employed to analyze phenotype and function of dendritic cells as well as IL-10 production from natural regulatory T cells.

Measurements and Main Results: Dendritic cells cultured with natural regulatory T cells up-regulated IL-10, down-regulated costimulatory molecules, and stimulated the proliferation of CD4⁺CD25⁻ effector T cells less potently. Allergic asthmatic natural regulatory T cells were significantly less efficient in inducing this tolerogenic phenotype of dendritic cells compared with healthy control and nonallergic asthmatic counterparts. Furthermore, this defective function of natural regulatory T cells was associated with their decreased IL-10 expression, disease severity, and could be reversed by oral corticosteroid therapy.

Conclusions: These results provided the first evidences of impaired induction of tolerogenic dendritic cells mediated by natural regulatory T cells in human allergic asthma.

Keywords: asthma; natural regulatory T cells; dendritic cells; IL-10; infectious tolerance

Although it has been well established that inflammatory cascades in allergic asthma propagate from the activation of innate immune cells, such as mast cells, macrophages, and dendritic cells, to the induction of helper T-cell type 2 (Th2)–biased CD4⁺ T cells and IgE-producing B cells of the adaptive immune system, less is known about cellular subsets that orchestrate the immune tolerogenic network in the disease. Emerging evidence has suggested a role for regulatory T cells as well as tolerogenic dendritic cells in regulating inflammatory responses in allergic asthma (1, 2).

Regulatory T cells are T cells that possess suppressive activities against cytokine production and proliferation of effec-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Evidence suggests that tolerogenic dendritic cells can augment beneficial natural regulatory T-cell function in allergic asthma, but the existence of a converse mechanism by which natural regulatory T cells mediate the induction of tolerogenic dendritic cells has not been explored.

What This Study Adds to the Field

Induction of tolerogenic dendritic cells by human allergic asthmatic natural regulatory T cells is impaired, due in part to reduced IL-10 expression, a defect that can be ameliorated by oral corticosteroids.

tor T cells. Several subtypes of regulatory T cells with distinct phenotypes (3), cytokine production profiles (4), and mechanisms of immune suppression have been described (5). Among these are CD4⁺CD25^{hi}CD127^{lo/-} natural regulatory T cells and adaptive regulatory T cells, such as type 1 regulatory T cells and helper T type 3 regulatory cells. Suppressive effects of type 1 regulatory T cells and helper T type 3 regulatory cells are dependent on the production of soluble inhibitory cytokines, IL-10 and transforming growth factor- β , respectively; whereas natural regulatory T cells are reported to express both soluble IL-10 and membrane-bound transforming growth factor- β and can also exert their immunoregulatory activities via contact-dependent mechanisms (5). Even though multiple subsets of regulatory T cells might be involved in the regulation of inflammation in allergic asthma (6), natural regulatory T cells are the most studied subset. Decreased frequency and diminished suppressive activity of pulmonary natural regulatory T cells have been documented in studies of human allergic asthma (7). Furthermore, murine data also suggested that natural regulatory T-cell-mediated suppression reversed airway hyperresponsiveness, inflammation, and remodeling (8, 9). From these pioneering works, strategies to induce the function or number of natural regulatory T cells have been attempted, many of which focus on the use of a unique subtype of tolerogenic dendritic cells (10).

In contrast to conventional dendritic cells, tolerogenic dendritic cells are characterized by their reduced expression of costimulatory molecules, in particular CD40, CD80, and HLA-DR, and elevated expression of the immunosuppressive cytokine IL-10 (11). They can inhibit the proliferation of effector T cells as well as induce several subtypes of regulatory T cells (12, 13). Human tolerogenic monocyte-derived dendritic cells elicited allergen tolerance by inducing natural regulatory T cells (14). Similarly, murine studies also showed that ectopic expression or induction of IL-10 in dendritic cells suppressed experimentally induced asthma by enhancing the frequency of allergen-specific natural regulatory T cells (15, 16). Collectively, these observa-

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tions pointed to the potential involvement of tolerogenic dendritic cells and natural regulatory T cells in the process of infectious tolerance (17) in allergic asthma.

Even though most studies have been focused on the induction of regulatory T cells by tolerogenic dendritic cells in human asthma or experimental models of the disease, natural regulatory T cells have been shown to be involved in phenotypic and functional regulation of antigen-presenting cells (18). Interaction between these innate immune cells and natural regulatory T cells directed them toward a tolerogenic state of activation (19, 20). In addition, depletion of natural regulatory T cells led to augmentation of dendritic cell-mediated airway inflammation in an experimental model of asthma, suggesting a critical role of natural regulatory T cells in phenotypic and functional maintenance of dendritic cells (21). To date, the significance of the influence of natural regulatory T cells in the induction of tolerogenic dendritic cells in human allergic asthma has not been investigated. Thus, we used an *in vitro* cell culture system to evaluate the induction of the tolerogenic phenotype in blood dendritic cells by natural regulatory T cells. Our data provided the first line of evidence of impaired induction of tolerogenic dendritic cells mediated by natural regulatory T cells in human allergic asthma.

METHODS

Human Subjects

The study was approved by the Stanford University (Stanford, CA) Administrative Panel on Human Subjects in Medical Research. The study population included 21 subjects with allergic asthma, 21 healthy control subjects, and 21 subjects with nonallergic asthma. All subjects signed informed consent forms before participating in the study. Asthma diagnosis was established by evidence of episodic and partially reversible airflow obstruction or airway hyperresponsiveness and exclusion of alternative diagnoses (NHLBI Expert Panel Report-3, 2007). All patients had been monitored for at least 6 months by a board-certified asthma, allergy, and immunology specialist at Stanford to ensure the diagnosis was correct. Subjects with allergic asthma were distinguished from subjects with nonallergic asthma on the basis of history of allergic symptoms, elevated blood IgE levels (>50 IU/ml), and positive skin tests to allergens. Spirometry was performed by a fully qualified respiratory therapist and the study coordinator, both of whom had more than 15 years of experience in asthma studies. Patients with an FEV₁ less than 60% were considered to have severe asthma. Those in the range of 60 to 80% were considered to have moderate asthma and those with an FEV₁ greater than 80% were considered to have mild asthma. Comprehensive clinical data were collected at each patient visit including history, disease severity, medication status, common allergens, IgE level, FEV₁, and FEV₁/FVC ratio (Table 1). Healthy control subjects were defined as nonsmoking subjects greater than 17 years of age with a total serum IgE of less than 25 IU/ml, negative skin testing (compared with positive histamine control), and no evidence of lung disease or allergic symptoms. In addition, there was no evidence of obstructive or restrictive lung disease for healthy control subjects on spirometry testing.

Cell Isolation

Cell populations of interest were isolated from up to 450 ml of whole blood. CD4⁺ T cells were purified with CD4⁺ rosette kits (Stemcell Technologies, Vancouver, BC, Canada). The CD4⁺ T-cell fraction was stained with CD25 (clone 4E3; BD Biosciences, San Jose, CA) and CD127 (clone SB199; BioLegend, San Diego, CA) antibodies and sorted for CD4⁺CD25^{hi}CD127^{lo/-} natural regulatory T cells and CD4⁺CD25⁻ effector T cells. Cells were rested in complete medium after purification for 2 hours before undergoing downstream assays. The purity of natural regulatory T-cell and effector T-cell fractions was confirmed by forkhead box P3 (Foxp3) staining (clone 206D; BioLegend) to be more than 95 and 99%, respectively. Approximately 50% of blood volume was used to purify circulating dendritic cells. CD3⁺ T cells were first depleted from

peripheral blood mononuclear cells with rosette kits (Stemcell Technologies) to reduce bulk cell numbers for downstream sorting. This cell fraction was stained with lineage cocktail and DR antibodies to identify blood dendritic cells. Dendritic cells were sorted on the basis of their Lin⁻DR⁺ phenotype. In addition, dendritic cells from mixed cell cultures were reisolated by depleting CD3⁺ T cells with anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To avoid cell aggregates, magnetic sorting medium was supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline plus 0.5% fetal bovine serum, as per the manufacturer's recommendations. All procedures were performed according to the manufacturer's standard protocols.

Cell Cultures

Co-cultures were set up at a 1:1 ratio of natural regulatory T cells to dendritic cells. Other cell ratios (1:0.5 and 1:2 ratio of natural regulatory T cells to dendritic cells) were also evaluated. Natural regulatory T cells (0.25×10^6) and the appropriate number of dendritic cells were used for each well. Cells were cultured at 10^6 cells/ml in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin (complete medium) for 30 hours in the presence of a 50-ng/ml concentration of anti-CD3 antibodies (clone HIT3; BD Biosciences). Other time points (20 and 40 h) were also evaluated. For neutralization experiments, neutralizing antibodies against IL-10 (mIgG2b, clone 23738; R&D Systems, Minneapolis, MN) and isotype control (Sigma, St. Louis, MO) were used at 5 μ g/ml. All assays were performed in triplicate.

Cell Stimulation Assays

For IL-10 detection, 0.25×10^6 natural regulatory T cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml; Sigma) and ionomycin (1 μ g/ml; Sigma) for 5 hours in the presence of brefeldin A (diluted 1 $\times$ solution was added in the last 2.5 h; BioLegend). For tumor necrosis factor (TNF)- α and IL-10 detection, dendritic cells reisolated from mixed cell cultures were stimulated with LPS (50 ng/ml) for 24 hours in the presence of brefeldin A. For proliferation assays, dendritic cells and allogeneic healthy control effector T cells were resuspended at 10^6 cells/ml in complete medium and co-cultured at a 1:1 ratio. Anti-CD3 antibodies (clone HIT3; BD Biosciences) were diluted to 2 μ g/ml. Each cell or antibody solution was plated at 50 μ l/well in 96-well U-bottom plates. Additional medium was added to the wells so that the final volume in each well reached 200 μ l. Cells were pulsed with 1 μ Ci of ³H per well on Day 6 and harvested on Day 7 with a cell harvester. ³H incorporation was determined with a Wallac 1450 MicroBeta TriLux liquid scintillation counter. All assays were performed in triplicate.

Flow Cytometry

Surface staining for stimulatory molecules (CD40, CD80, and DR) and intracellular cytokine staining (IL-10 and TNF- α) were performed as per the standardized protocol from BD Biosciences. Data were acquired with a FACSCalibur (BD Biosciences). Detection of phosphorylated STAT3 (signal transducer and activator of transcription-3) by dendritic cells in response to recombinant IL-10 and phosphorylated Akt by natural regulatory T cells in response to anti-CD3 stimulation were performed according to the phospho-flow cytometry protocol I and phospho-CBA assays from BD Biosciences, respectively. Briefly, dendritic cells were stimulated with IL-10 (50 ng/ml) in the presence or absence of various doses of neutralizing IL-10 antibody at 37°C for 10 minutes. Natural regulatory T cells were stimulated with anti-CD3 antibodies (50 ng/ml) at 37°C for 10 minutes. For dendritic cells, stimulated cells were then fixed with 1% paraformaldehyde for 10 minutes at 37°C and subsequently permeabilized in ice-cold methanol for 10 minutes before being subjected to phosphorylated STAT3 staining. For natural regulatory T cells, stimulated cells were lysed and cell lysates were subsequently analyzed for expression of phosphorylated Akt and total Akt according to the manufacturers' protocols. The data acquisition threshold was set on the forward scatter channel to exclude dead cells and debris with very low size. Compensation of flow cytometric data as performed electronically with FlowJo (Tree Star, Ashland, OR) for standardization.

TABLE 1. SUBJECT DEMOGRAPHICS

Patient	Age (yr)	Asthma Duration (yr)	Severity	Prednisone	Other Medications	IgE (IU/ml)	Allergic Status	FEV ₁ (% predicted)	FEV ₁ /FVC (%)
AA1	56	15	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	245	Tree, mold	52	63
AA2	35	32	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	173	Cat	53	72
AA3	37	34	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	2,342	Tree, dust mite	56	69
AA4	21	15	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	1,274	Dog	57	73
AA5	18	17	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	2,314	Dog	58	75
AA6	24	18	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	1,083	Weed, grass	55	67
AA7	32	19	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	1,872	Tree, mold	51	74
AA8	34	29	Moderate	10 mg daily	Fluticasone, salmeterol 250 mg twice daily	376	Cat, mold	60	76
AA9	18	13	Moderate	None	Fluticasone, salmeterol 250 mg twice daily	756	Grass, dust mite	76	81
AA10	19	15	Moderate	None	Fluticasone, salmeterol 250 mg twice daily	788	Weed, grass	73	82
AA11	21	15	Moderate	None	Fluticasone, salmeterol 250 mg twice daily	298	Grass, dust mite	79	76
AA12	18	17	Moderate	None	Fluticasone, salmeterol 250 mg twice daily	1,305	Tree, dust mite	67	81
AA13	22	5	Mild	None	Albuterol puffs, 2 every wk	275	Grass	92	80
AA14	21	6	Mild	None	Albuterol puffs, 2 every wk	373	Mold	85	82
AA15	29	25	Mild	None	Albuterol puffs, 2 every wk	205	Grass, mold	90	80
AA16	29	2	Mild	None	Albuterol puffs, 2 every wk	254	Grass	91	81
AA17	25	18	Mild	None	Albuterol puffs, 2 every wk	190	Tree	84	80
AA18	21	12	Mild	None	Albuterol puffs, 2 every wk	342	Weed, dog	87	81
AA19	19	14	Mild	None	Albuterol puffs, 2 every wk	298	Grass, dust mite	93	86
AA20	27	19	Mild	None	Albuterol puffs, 2 every wk	569	Grass, dog	88	80
AA21	26	11	Mild	None	Albuterol puffs, 2 every wk	304	Dust mite, dog	86	82
26.29 ± 1.97		16.71 ± 1.77						74.05 ± 3.41	77.9 ± 1.1
NA1	18	3	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	51	67
NA2	28	23	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	53	72
NA3	47	19	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	55	69
NA4	22	9	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	58	73
NA5	34	2	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	56	71
NA6	18	3	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	54	77
NA7	18	12	Severe	20 mg daily	Fluticasone, salmeterol 500 mg twice daily	Less than 50	None	59	72
NA8	27	22	Moderate	None	Fluticasone, albuterol puffs, 1 every wk	Less than 50	None	61	76
NA9	38	32	Moderate	None	Fluticasone, albuterol puffs, 1 every wk	Less than 50	None	69	76
NA10	23	13	Moderate	None	Albuterol puffs, 2 every wk	Less than 50	None	73	77
NA11	27	14	Moderate	None	Albuterol puffs, 2 every wk	Less than 50	None	76	76
NA12	35	18	Moderate	None	Albuterol puffs, 2 every wk	Less than 50	None	79	75
NA13	25	5	Mild	None	Albuterol puffs, 2 every wk	Less than 50	None	95	80
NA14	19	n/a	Mild	None	Albuterol puffs, 1 every wk	Less than 50	None	91	86
NA15	21	3	Mild	None	Albuterol puffs, 1 every wk	Less than 50	None	88	81
NA16	25	13	Mild	None	Albuterol puffs, 1 every wk	Less than 50	None	95	82
NA17	18	3	Mild	None	Albuterol puffs, 2 every wk	Less than 50	None	94	85
NA18	24	2	Mild	None	Albuterol puffs, 2 every wk	Less than 50	None	91	81
NA19	29	6	Mild	None	Albuterol puffs, 1 every wk	Less than 50	None	89	82
NA20	31	5	Mild	None	Albuterol puffs, 1 every wk	Less than 50	None	95	80
NA21	24	4	Mild	None	Albuterol puffs, 1 every wk	Less than 50	None	91	80
26.24 ± 1.64		10.55 ± 1.92						74.9 ± 3.7	77.24 ± 1.08

Definition of abbreviations: AA = allergic asthma; NA = nonallergic asthma; n/a = not available.

Statistical Analysis

All statistical procedures were performed with Prism software (Graph-Pad, San Diego, CA). Data were tested for normality (Kolmogorov-Smirnov test) and variance equality (Bartlett’s test) before being subjected to appropriate statistical tests. Differences with *P* < 0.05 were considered statistically significant.

RESULTS

Healthy Control Natural Regulatory T Cells Induce Tolerogenic Dendritic Cells via an IL-10–dependent Mechanism

It has previously been established that activated effector T cells express both CD25 and CD127 and could be distinguished from natural regulatory T cells, which express high levels of CD25 and little to no CD127 (22, 23). Therefore, to avoid contamination of activated effector T cells, CD25 and CD127 antibodies were used for our sorting strategy (see Figure E1A in the online supplement). Natural regulatory T cells were defined as CD4⁺CD25^{hi}CD127^{lo/–} cells whereas effector T cells were defined as CD4⁺CD25[–]CD127[–] cells (Figure E1A). Purified natural regulatory T cells indeed showed more than 95% purity, indicated by the high expression of Foxp3, their cardinal marker, compared

with effector T cells (Figure E1B). The influence of natural regulatory T cells on the dendritic cell phenotype was evaluated by comparing the percentage change in expression of CD40, CD80, DR, TNF-α, and IL-10 in purified dendritic cells rested in complete medium alone with those that been cultured with autologous natural regulatory T cells. Healthy control natural regulatory T cells and dendritic cells were co-cultured at 2:1, 1:1, and 1:2 ratios for various amounts of time (20, 30, and 40 h) (see Figure E2 in the online supplement). In healthy control subjects, natural regulatory T-cell–primed dendritic cells showed decreased expression of CD40, CD80, and DR (Figure 1A). Furthermore, dendritic cells were negatively isolated from co-cultures with anti-CD3 microbeads and subsequently stimulated with LPS to analyze their cytokine production. To avoid aggregation of dendritic cells and natural regulatory T cells into cell clusters, isolation medium was supplemented with 5 mM EDTA, a calcium-chelating reagent used to prevent cell clumping. Analysis of reisolated dendritic cells by staining with anti-CD3 antibody and examination of cell morphology showed more than 99% purity by this method (see Figure E3 in the online supplement). LPS stimulation of dendritic cells reisolated from cultures with natural regulatory T cells showed a marked reduction in TNF-α production and enhanced expression of IL-10 (Figure

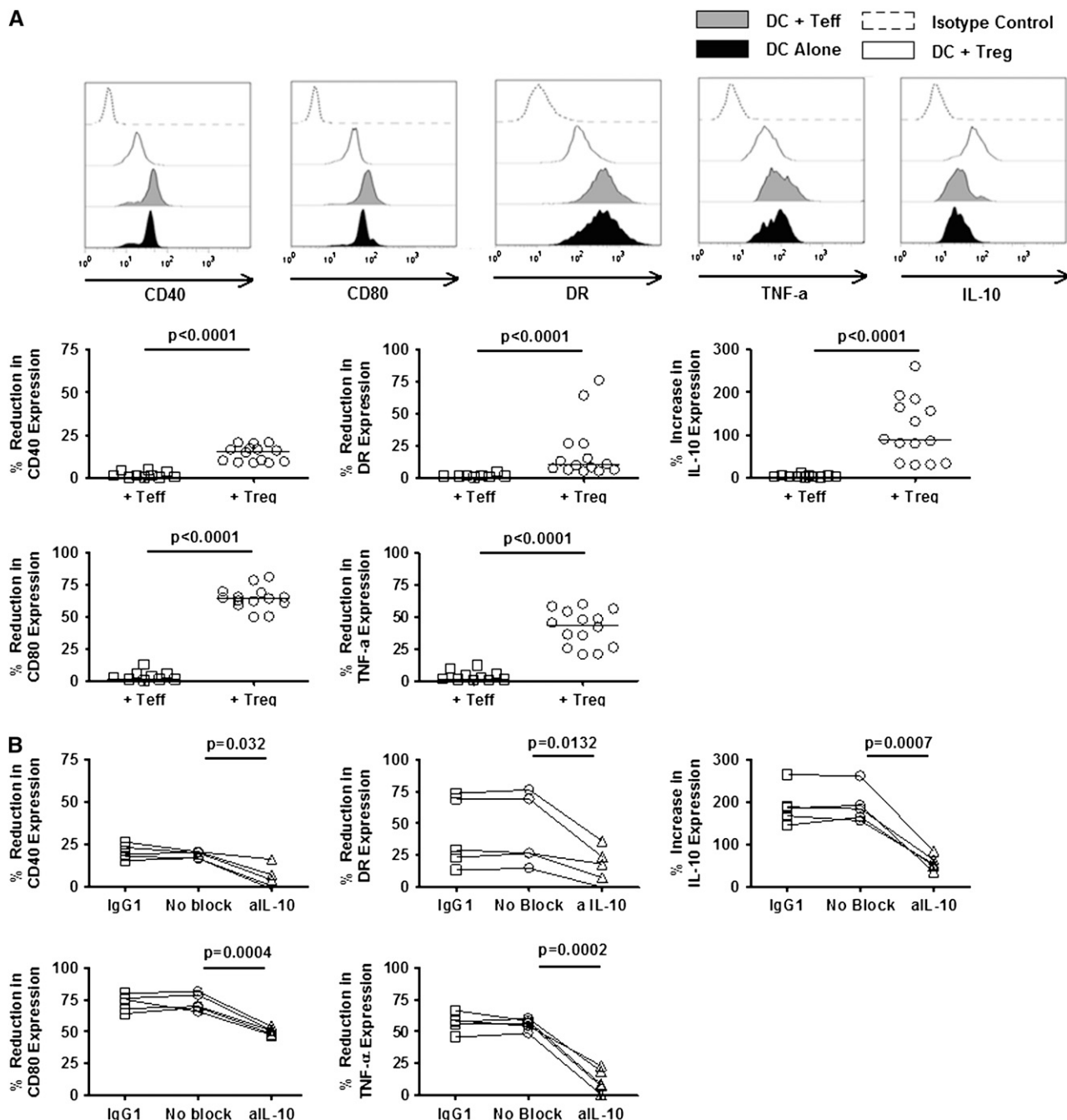


Figure 1. Modulation of phenotype of healthy control dendritic cells cultured with various autologous T-cell subsets. (A) *Top*: Representative fluorescence-activated cell-sorting plots of expression of CD40, CD80, DR, tumor necrosis factor (TNF)- α , and IL-10 in healthy control dendritic cells (DCs) in the presence of autologous natural regulatory T cells (Treg)/effector T cells (Teff) or in cultures with complete medium alone. *Dashed line plots* represent isotype control staining. *Bottom*: Percentage changes in expression of CD40, CD80, DR, TNF- α , and IL-10 in DCs in the presence of natural regulatory T cells or effector T cells ($n = 14$ for each group). (B) Effects of blocking IL-10 antibody (aIL-10) on phenotypic alteration of healthy control DCs cultured with autologous natural regulatory T cells ($n = 5$ for each group). Negative control experiments were performed with low-endotoxin IgG1 antibody. *Dot-plot graphs with horizontal bars* represent median values. Wilcoxon tests were used for statistical analyses of DR expression on DCs in (B) because the data were not normally distributed. Paired *t* tests were used for statistical analyses of the rest of the data with normal distribution in (A) and (B).

1A). Titration experiments showed that optimal effects of natural regulatory T cells on the phenotype of dendritic cells occur at a 1:1 cell ratio after 30 hours, which was used for subsequent experiments. Under these conditions, healthy control natural regulatory T cells markedly suppressed the expression of CD40, CD80, DR, and TNF- α and up-regulated IL-10 production by autologous dendritic cells (Figure 1A). Interestingly, parallel control co-cultures,

in which natural regulatory T cells were replaced by effector T cells, did not induce significant changes in dendritic cell phenotype (Figure 1A). Therefore, these results suggested that the ability to induce the tolerogenic phenotype of dendritic cells was specific to the natural regulatory T-cell subset.

Several lines of evidence have suggested that IL-10 was critical for induction of the tolerogenic phenotype of antigen-

presenting cells (14, 15). To investigate whether IL-10 was involved in the induction of tolerogenic dendritic cells by natural regulatory T cells, we used blocking antibodies against IL-10 in our co-culture system with healthy control cells. We first examined the efficacy of neutralizing antibodies against IL-10 by analyzing the ability of this reagent to block IL-10-mediated phosphorylation of STAT3 in dendritic cells *in vitro*. We found that neutralizing antibodies at 5 μ g/ml completely inhibited STAT3 activation in dendritic cells that were stimulated with recombinant IL-10 at 50 ng/ml (see Figure E4A in the online supplement). This concentration of blocking antibodies was used for subsequent experiments. Introduction of IL-10-blocking antibodies into co-cultures suppressed the induction of tolerogenic dendritic cells, which was not seen in parallel cultures with isotype control antibodies (Figure 1B). Thus, IL-10 was necessary for natural regulatory T-cell-mediated induction of tolerogenic dendritic cells.

Allergic Asthmatic Natural Regulatory T Cells Are Less Efficient in Inducing Tolerogenic Dendritic Cells

Similar assays were subsequently performed with allergic asthmatic and nonallergic asthmatic cells. Frequencies of circulating natural regulatory T cells, their expression of Foxp3 (measured by mean fluorescence intensity), and the purity of isolated natural regulatory T cells were not significantly different among all subject groups (Figures E1A and E1B). Dendritic cells from all subject groups also exhibited no significant differences in the expression of DR, CD40, CD80, IL-10, and TNF- α in cultures without natural regulatory T cells (Figure 2A). However, in co-culture assays, we found that allergic asthmatic natural regulatory T cells were significantly less able to induce the tolerogenic phenotype of autologous dendritic cells than healthy control counterparts (Figure 2B; and see Figure E5 in the online supplement). Furthermore, dendritic cells were reisolated from co-cultures and assayed for their ability to stimulate allogeneic effector T-cell proliferation. The purity of reisolated dendritic cells (>99%) was similar among all subject groups (Figure E3). Their stimulatory potential was evaluated by comparing the change in ^{3} H incorporation between assays using purified dendritic cells cultured in complete medium alone and those using natural regulatory T-cell-primed dendritic cells. The same source of allogeneic healthy control effector T cells was used in each assay to control for individual variation in lymphoproliferative potential. ^{3} H incorporation assays showed that natural regulatory T-cell-primed dendritic cells from subjects with allergic asthma were significantly more stimulatory than healthy control dendritic cells (Figure 2B and Figure E4B). Furthermore, analysis of nonallergic asthmatic cells did not show significant differences compared with healthy control subjects, suggesting that this functional defect was specific to allergic asthmatic but not nonallergic asthmatic cells (Figures 2A and 2B).

To investigate whether this phenomenon resulted from intrinsic defects in allergic asthmatic natural regulatory T cells, we cultured dendritic cells from healthy control subjects (or subjects with allergic asthma) with allogeneic allergic asthmatic (or healthy control) natural regulatory T cells. Our analysis showed that healthy control dendritic cells cultured with allogeneic allergic asthmatic natural regulatory T cells exhibited a suboptimal tolerogenic phenotype compared with those cultured with autologous healthy control natural regulatory T cells (Figure 3). However, allergic asthmatic dendritic cells cultured with allogeneic healthy control natural regulatory T cells exhibited a similar tolerogenic phenotype compared with healthy control natural regulatory T-cell-primed healthy control dendritic cells (Figure

3). Collectively, these results suggested that the defective induction of tolerogenic dendritic cells in subjects with allergic asthma was specifically associated with a potential functional defect in allergic asthmatic natural regulatory T cells, but not dendritic cells.

Suboptimal Induction of Tolerogenic Dendritic Cells by Natural Regulatory T Cells in Subjects with Allergic Asthma Is Associated with Their Reduced Expression of IL-10

To investigate whether IL-10 was associated with the functional defect of allergic asthmatic natural regulatory T cells, we first analyzed IL-10 expression and secretion by purified natural regulatory T cells in all subject groups by fluorescence-activated cell sorting and ELISA. PMA- and ionomycin-stimulated allergic asthmatic natural regulatory T cells showed a significant decrease in intracellular IL-10 expression, measured as total mean fluorescence intensity, percentage of IL-10⁺ cells, and mean fluorescence intensity of IL-10⁺ cells, compared with healthy control subjects and subjects with nonallergic asthma (Figure 4A). Similarly, IL-10 secretion by allergic asthmatic natural regulatory T cells, measured by ELISA, was significantly lower compared with those of healthy control subjects and subjects with nonallergic asthma (Figure 4A). Because natural regulatory T cells in co-cultures with dendritic cells were stimulated with anti-CD3 antibody, we also examined their IL-10 production under this condition. We first used Akt activation as the readout for natural regulatory T cell responsiveness to anti-CD3 stimulation (24). Natural regulatory T cells from all subject groups expressed similar levels of total Akt and phosphorylated Akt in response to anti-CD3 stimulation (Figure E4C). However, IL-10 production in response to this stimulation was significantly lower in allergic asthmatic natural regulatory T cells compared with healthy control and nonallergic asthmatic counterparts (Figure E4D). These results suggested that whereas natural regulatory T cells among all subject groups are equally responsive to anti-CD3 stimulation, their defective IL-10 production was specific to subjects with allergic asthma. Furthermore, correlation analysis showed a significant positive correlation between the ability of natural regulatory T cells to induce tolerogenic dendritic cells and their expression of secreted IL-10 (Figure 4B). Altogether, these results suggested that suboptimal induction of tolerogenic dendritic cells by allergic asthmatic natural regulatory T cells was associated with their diminished expression of IL-10.

Expression of IL-10 by Natural Regulatory T Cells and Their Ability to Induce Tolerogenic Dendritic Cells Are Correlated with Asthma Severity and Can Be Modulated with Oral Corticosteroids

Correlation analysis of *in vitro* cellular function and clinical parameters of allergic asthma was performed. The ability of natural regulatory T cells to suppress CD40, DR, and TNF- α expression by dendritic cells in subjects with severe and moderate allergic asthma was significantly reduced compared with those with mild disease (Figure 5A). In addition, the ability of natural regulatory T cells to suppress IL-10 production and CD80 expression by dendritic cells in subjects with severe allergic asthma was significantly lower than in subjects with mild and mild/moderate disease, respectively (Figure 5A). Furthermore, expression of IL-10 in natural regulatory T cells from subjects with severe and moderate allergic asthma was also significantly lower than in those with mild disease (Figure 5B). Other clinical parameters such as subject age, asthma duration, and IgE level were not significantly correlated with any parameters of *in vitro* cellular function (data not shown).

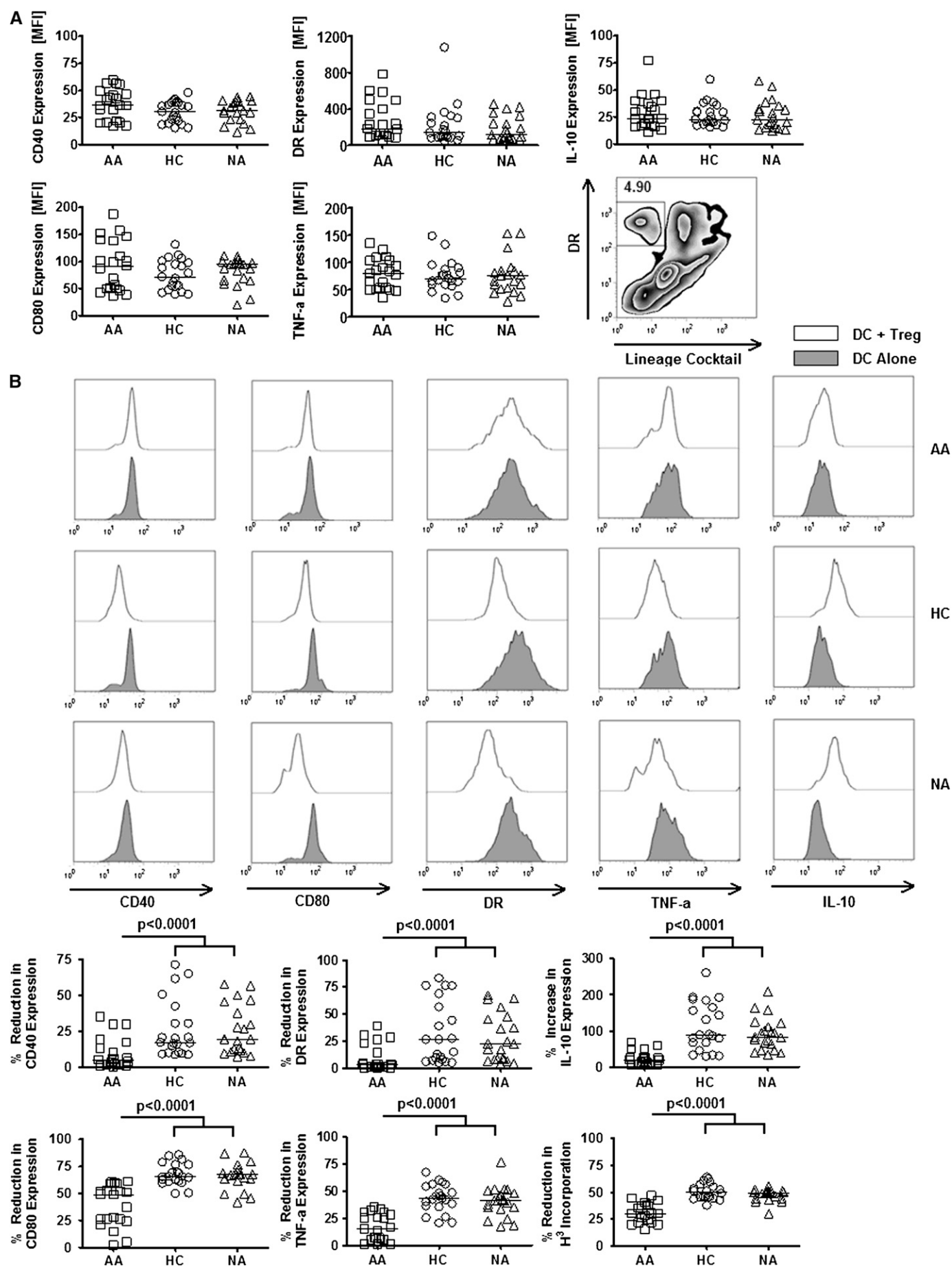


Figure 2. Phenotypic and functional alteration in dendritic cells (DCs) cultured with autologous natural regulatory T cells (Treg) from subjects with allergic asthma, healthy control subjects, and subjects with nonallergic asthma. (A) *Bottom right:* Representative fluorescence-activated cell-sorting (FACS) plot of sorting strategy for circulating dendritic cells. Costaining with lineage cocktail antibodies and DR identified DR⁺Lineage⁻ dendritic cells from CD3 T-cell-depleted fraction. *Top and bottom left:* Expression of CD40, CD80, DR, tumor necrosis factor (TNF)- α , and IL-10 in purified dendritic cells among the three subject groups, in cultures without natural regulatory T cells (n = 21 for each group). (B) *Top:* Representative FACS plots of expression of CD40, CD80, DR, TNF- α , and IL-10 in dendritic cells in the presence or absence of autologous natural regulatory T cells from representative subjects with allergic asthma (AA), healthy control subjects (HC), and subjects with nonallergic asthma (NA). *Bottom:* Percentage changes in expression of CD40, CD80, DR, TNF- α , and IL-10 in purified dendritic cells cultured with autologous natural regulatory T cells among the three subject groups (n = 21 for each group). Percentage change in ³H incorporation in proliferation assays, using purified dendritic cells or natural regulatory T cell-cultured dendritic cells to stimulate allogeneic effector T-cell proliferation, was used to assess the stimulatory potential of dendritic cells. A similar source of allogeneic effector T cells was used for all assays. *Dot-plot graphs with horizontal bars* represent median values. Kruskal-Wallis tests were used for statistical analysis of CD80 on dendritic cells in (A) and percentage changes in CD40, CD80, and DR expression on dendritic cells were used in (B) because the data had unequal variances. Analysis of variance was used for statistical analyses of the rest of the data with normal distribution and equal variances. MFI = mean fluorescence intensity.

Cellular subsets used in the analyses described previously were derived from subjects who were undergoing a 7-day corticosteroid withdrawal procedure immediately before blood was drawn. Therefore, to evaluate the effects of corticosteroids on peripheral blood natural regulatory T-cell function, we organized a second blood draw from six subjects with moderate to severe allergic asthma (AA1 to AA5, and AA7) who were continuously on high-dose oral corticosteroids for 1 week before blood was drawn (Table 1). *In vitro* functional assays of natural regulatory T cells were performed to compare their ability to induce tolerogenic dendritic cells. We found that natural regulatory T cells from subjects who were receiving uninterrupted oral corticosteroid therapy showed a significant increase in their ability to induce tolerogenic dendritic cells compared with those derived from the same subjects undergoing an oral corticosteroid withdrawal procedure (Figure 6A). Addition of exogenous IL-10

to cultures of natural regulatory T cells and dendritic cells from subjects who were undergoing oral corticosteroid withdrawal also enhanced the induction of tolerogenic dendritic cells (Figure 6A). Concurrently, natural regulatory T cells from subjects receiving uninterrupted oral corticosteroid therapy showed a significant increase in IL-10 production (Figure 6B). Altogether, this evidence suggested that oral corticosteroid therapy enhanced natural regulatory T-cell expression of IL-10 and their ability to induce tolerogenic dendritic cells.

DISCUSSION

We have shown here that defective *in vitro* interaction between natural regulatory T cells and dendritic cells from subjects with allergic asthma led to the failure to induce the tolerogenic phenotype of dendritic cells. In our study, natural regulatory

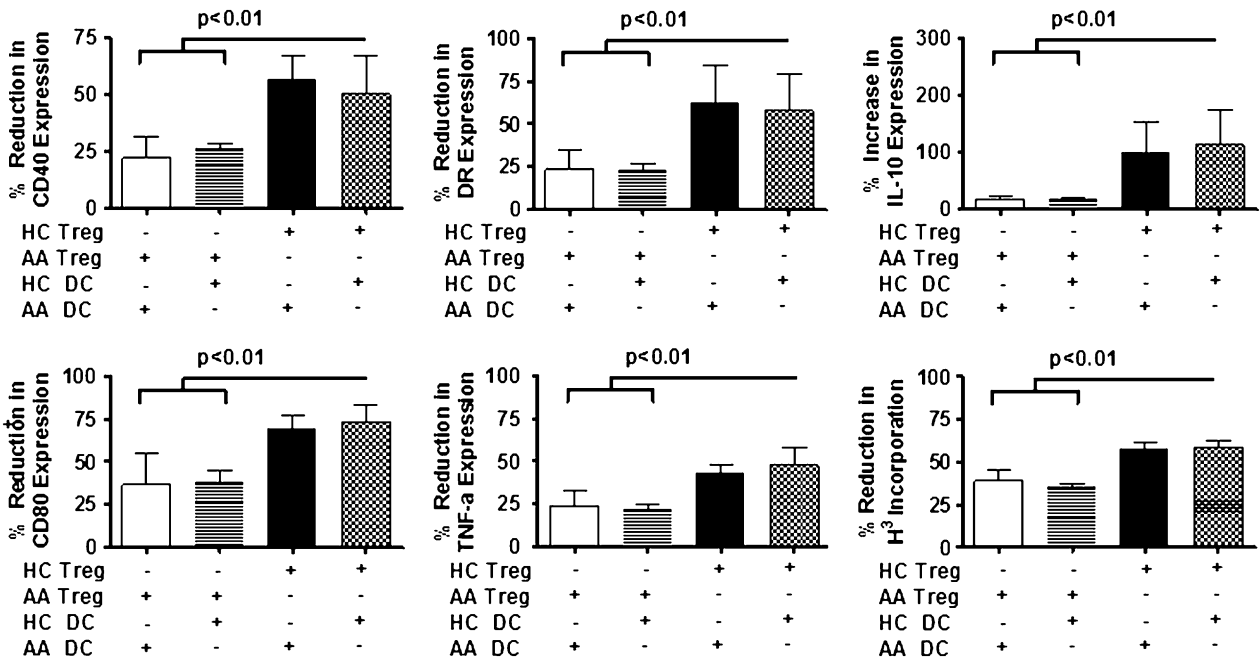


Figure 3. Phenotypic and functional alteration in dendritic cells cultured with allogeneic natural regulatory T cells from subjects with allergic asthma (AA) and healthy control subjects (HC). Percentage changes in expression of CD40, CD80, DR, tumor necrosis factor (TNF)- α , and IL-10 and stimulatory potential (³H incorporation assay) of purified dendritic cells (DC) cultured with allogeneic natural regulatory T cells (Treg) between subjects with allergic asthma and healthy control subjects (n = 7 for each group). *Bar graphs* with mean values and standard deviations. Wilcoxon tests were used for statistical analyses of CD80, TNF- α , and IL-10 expression on dendritic cells because the data were not normally distributed. Paired *t* tests were used for statistical analyses of the rest of the data with normal distribution.

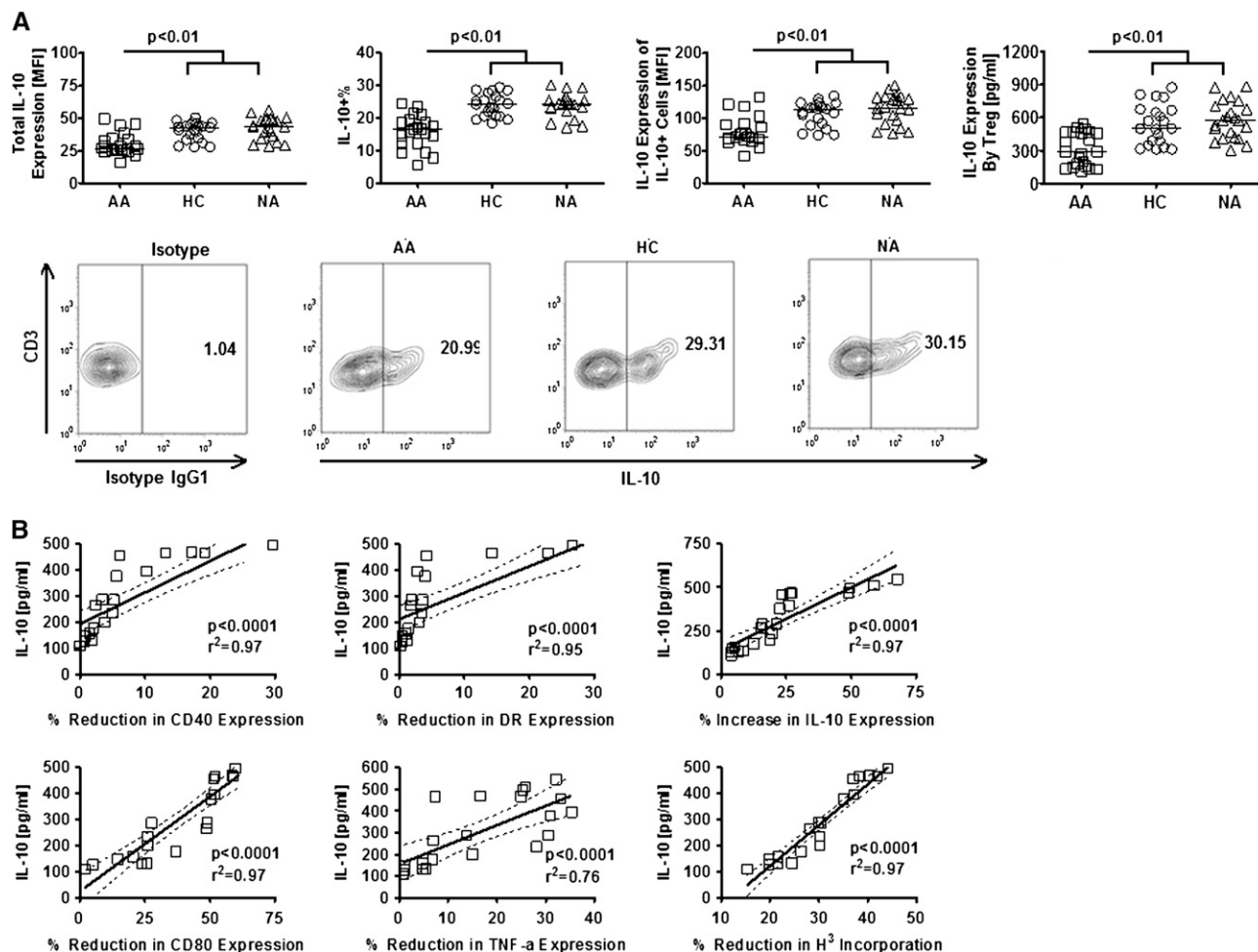


Figure 4. Expression of IL-10 in natural regulatory T cells from subjects with allergic asthma (AA), healthy control subjects (HC), and subjects with nonallergic asthma (NA). (A) Top left and middle: Intracellular expression of IL-10 by phorbol 12-myristate 13-acetate (PMA)- and ionomycin-stimulated natural regulatory T cells from the three subject groups (n = 21 for each group). Data represent total IL-10 mean fluorescence intensity (MFI), percentage of IL-10⁺ cells, and IL-10 MFI of IL-10⁺ cells. Top right: IL-10 secretion by PMA- and ionomycin-stimulated natural regulatory T cells from the three subject groups (n = 21 for each group). Bottom: Fluorescence-activated cell-sorting plots of IL-10 expression by PMA- and ionomycin-stimulated natural regulatory T cells from representative subjects with allergic asthma, healthy control subjects, and subjects with nonallergic asthma. Gating threshold was defined using negative isotype control. (B) Correlation of IL-10 expression in PMA- and ionomycin-stimulated natural regulatory T cells and different parameters of phenotypic and functional alteration in dendritic cells from subjects with allergic asthma (n = 21 for each graph). Solid lines represent linear regression lines. Dashed curves represent 95% confidence intervals. Dot-plot graphs with horizontal bars represent median values. Analysis of variance was used for statistical analyses of all data with normal distribution and equal variances in (A). Pearson tests were used for correlation analyses of normally distributed data in (B). TNF-α = tumor necrosis factor-α.

T-cell-induced tolerogenic dendritic cells exhibited lower expression of HLA-DR and costimulatory molecules (CD40 and CD80), down-regulated expression of the proinflammatory cytokine TNF-α, and up-regulated expression of the immunosuppressive cytokine IL-10. These dendritic cells are also less able to stimulate effector T-cell proliferation than dendritic cells that had not been exposed to natural regulatory T cells. These characteristics were consistent with the previously reported phenotype and function of tolerogenic dendritic cells (11, 25). It is worth noting that because of the nature of the *in vitro* system, it is not clear how the microenvironment at sites of allergic inflammation would affect the interaction between natural regulatory T cells and dendritic cells. It is likely that in these local environments, functional interference by other inflammatory mediators might also contribute to the impairment of natural regulatory T-cell activity with respect to the induction of tolerogenic dendritic cells in subjects with allergic asthma (26, 27). Alternatively, compensation for the defective function of natural regulatory T cells

might occur via activities of other antiinflammatory or tolerogenic cell subsets, such as adaptive regulatory T cells (28). Therefore, a more extensive functional analysis of pulmonary natural regulatory T cells as well as other adaptive regulatory T cells with respect to the induction of tolerogenic dendritic cells would yield further insights about the potential presence of several dynamic immunomodulatory mechanisms at sites of allergic inflammation *in vivo*.

The impaired interaction between natural regulatory T cells and dendritic cells reported here was associated with defective natural regulatory T-cell function in subjects with allergic asthma, but not dendritic cells, as healthy control regulatory T cells efficiently induced the tolerogenic phenotype in allergic asthmatic dendritic cells. In contrast to previous studies, which demonstrated that impaired natural regulatory T-cell function was limited to only a subset of subjects with allergic asthma (29–31), our study used a more stringent gating method (22, 23) for flow sorting to avoid contamination of CD25⁺CD127^{low} natural

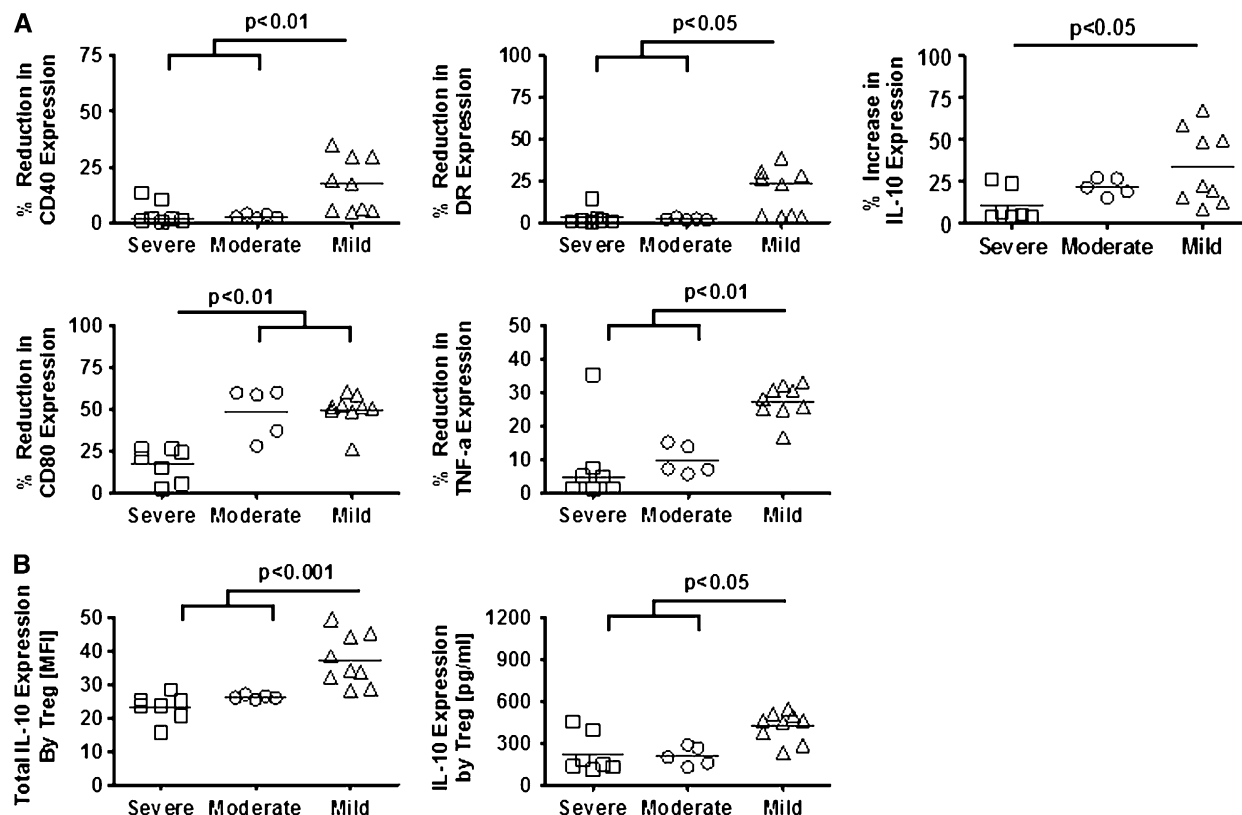


Figure 5. Correlation between *in vitro* cellular functions and the severity of asthma. (A) Phenotypic alteration in natural regulatory T-cell (Treg)-primed dendritic cells from subjects with allergic asthma of various degrees of severity ($n = 7$ for subjects with severe asthma, $n = 5$ for subjects with moderate asthma, $n = 9$ for subjects with mild asthma). (B) IL-10 expression in phorbol 12-myristate 13-acetate- and ionomycin-stimulated natural regulatory T cells from subjects with allergic asthma with various degrees of severity ($n = 7$ for subjects with severe asthma, $n = 5$ for subjects with moderate asthma, $n = 9$ for subjects with mild asthma). Dot-plot graphs with horizontal bars represent median values. The Kruskal-Wallis test was used for statistical analyses of data with small sample sizes. MFI = mean fluorescence intensity.

regulatory T cells with CD25⁺CD127^{hi} activated effector T cells, which are present at particularly high frequency in the peripheral blood of subjects with severe allergic asthma. This difference in methodology could perhaps explain why those studies did not find a consistent decrease in natural regulatory T-cell function in allergic asthma. In addition, our data also suggested that defective natural regulatory T-cell-mediated induction of tolerogenic dendritic cells is specific to the allergic subtype of asthma but not the nonallergic subtype. Our data did not rule out the possibility that natural regulatory T cells exhibit distinct suboptimal regulatory function in other allergic and pulmonary diseases. A possible explanation is that each disease possesses a signature inflammatory environment. Thus, the effects of the systemic and local cytokine milieu of each disease on natural regulatory T-cell function might well be different.

IL-10 has been suggested to be an important antiinflammatory mediator in allergic asthma via its suppressive activities against activation of Th2-biased CD4⁺ T cells and airway hyperreactivity (32, 33). Here we showed that IL-10 indeed mediated the induction of tolerogenic dendritic cells by natural regulatory T cells. Blocking IL-10 led to a suboptimal induction of tolerogenic dendritic cells in healthy control subjects. In addition, defective induction of tolerogenic dendritic cells in allergic asthma was associated with reduced expression of IL-10 by natural regulatory T cells. Exogenous IL-10 was able to rescue the defective induction of tolerogenic dendritic cells by natural regulatory T cells in allergic asthma. These results are consistent with the reported role of IL-10 in inducing the tolerogenic phenotype of

antigen-presenting cells (16, 34). Furthermore, we showed that oral corticosteroid therapy could up-regulate IL-10 expression by natural regulatory T cells and concurrently enhance their ability to induce tolerogenic phenotype of dendritic cells, consistent with previous reports on the induction of IL-10 expression by corticosteroids (35, 36). Interestingly, we did not observe modulatory effects of β -agonists on IL-10 expression of natural regulatory T cells as demonstrated by Peek and colleagues (36). This discrepancy might be due to the fact that in their *in vitro* study, β -agonists could directly act on natural regulatory T cells in cell cultures whereas in our *in vivo* study, these drugs, which are designed to have low systemic absorption so that their main sites of action are at the airway, did not directly act on circulating regulatory T cells.

Data presented here did not rule out the possibility that impaired induction of tolerogenic dendritic cells is due to an intrinsic defect in allergic asthmatic natural regulatory T cells. However, given several documented defects in the function of regulatory T cells in allergic asthma (37–39), it is likely that this functional impairment might be secondary to other immunological abnormalities in the disease, for several reasons. First, the reduced IL-10-dependent functional modulation of tolerogenic dendritic cells by natural regulatory T cells from subjects with allergic asthma might result from the absence of IL-10-producing natural regulatory T cells from the peripheral blood of subjects with allergic asthma, possibly via retention or active recruitment of these cells to local tissues (i.e., lymph nodes or airway) to suppress allergic inflammation. Second, prednisone, an immuno-

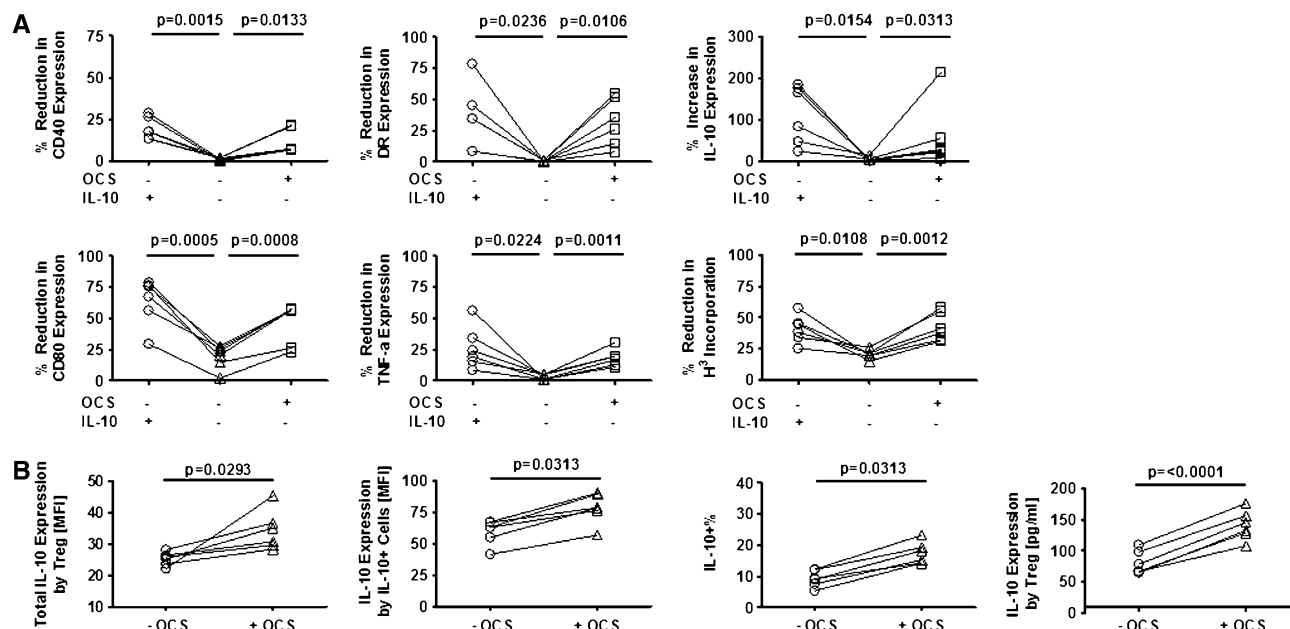


Figure 6. Modulation of phenotype of natural regulatory T-cell (Treg)-primed dendritic cells from subjects with allergic asthma and function of natural regulatory T cells by oral corticosteroids (OCS) and exogenous IL-10. (A) Phenotypic and functional alteration in natural regulatory T-cell-primed dendritic cells among subjects with allergic asthma undergoing an OCS withdrawal procedure and subjects receiving uninterrupted oral corticosteroid therapy ($n = 6$ for each group). Recombinant IL-10 was also introduced to natural regulatory T-cell-dendritic cell co-cultures to assess the effects of exogenous IL-10 on phenotype and function of dendritic cells. (B) IL-10 expression in phorbol 12-myristate 13-acetate (PMA)- and ionomycin-stimulated natural regulatory T cells among subjects with allergic asthma undergoing OCS withdrawal and uninterrupted OCS therapy ($n = 6$ for each group). Paired t tests were used for statistical analyses of IL-10 expression by PMA- and ionomycin-stimulated natural regulatory T cells because they were normally distributed. Wilcoxon tests were used for statistical analyses of the rest of the data because they were not normally distributed. MFI = mean fluorescence intensity; TNF- α = tumor necrosis factor- α .

suppressive reagent that effectively reduced the predominant Th2 cytokine environment in allergic asthma, could correct the impaired natural regulatory T-cell function in the disease. Even though corticosteroids might act to reverse the defective function of an intracellular master regulator of natural regulatory T-cell function such as Foxp3 (40), leading to subsequent modulation of many of their immunosuppressive features, corticosteroids might also reduce the severity of inflammation in allergic asthma, leading to less pronounced effects of these mediators on natural regulatory T-cell function. Thus, functional defects of natural regulatory T cells might be secondary to the inflammatory nature of allergic asthma. Alternatively, mobilization of regulatory T cells from the circulation to local tissues by steroids might also be a potential explanation for the reduced expression of IL-10 by circulating natural regulatory T cells from subjects with allergic asthma. Mechanistic studies of how corticosteroids influence natural regulatory T-cell function in allergic asthma might provide novel insights to determine whether these cells are intrinsically defective in the disease.

To date, it is not known whether infectious tolerance or direct suppression is the major mechanism of immune regulation of natural regulatory T cells (17, 41). Given the paucity of natural regulatory T cells, it is plausible to suggest that infectious tolerance might be important in the amplification of immunosuppressive signals by inducing tolerogenic phenotype in other cellular subsets. Our study provides evidence of defective IL-10-dependent induction of tolerogenic dendritic cells mediated by natural regulatory T cells in allergic asthma. We have also demonstrated the effectiveness of corticosteroids in modulating this functional impairment. Thus, natural regulatory T-cell-mediated induction of tolerogenic dendritic cells represents both a potential deregulated step in infectious

tolerance mechanisms in allergic asthma and a promising target for therapeutic intervention to effectively manage clinical outcomes of the disease.

Conflict of Interest Statement: K.D.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; C.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.C.N. received \$100,000 in institutional grants as the principal investigator for an asthma clinical study from AstraZeneca and received \$100,001 in institutional grants as the principal investigator for an asthma clinical study from Genentech.

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References

1. Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J Allergy Clin Immunol* 2009;123:735-746.
2. Lambrecht BN, Hammad H. Lung dendritic cells: targets for therapy in allergic disease. *Handb Exp Pharmacol* 2009;188:99-114.
3. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive Foxp3⁺ regulatory T cells: more of the same or a division of labor? *Immunity* 2009;30:626-635.
4. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3⁺ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* 2009;10:689-695.
5. Sojka DK, Huang YH, Fowell DJ. Mechanisms of regulatory T-cell suppression: a diverse arsenal for a moving target. *Immunology* 2008;124:13-22.
6. Ryanna K, Stratigou V, Safinia N, Hawrylowicz C. Regulatory T cells in bronchial asthma. *Allergy* 2009;64:335-347.
7. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, Griese M, Krauss-Etschmann S. Quantitative and functional impair-

- ment of pulmonary CD4⁺CD25^{hi} regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007;119:1258–1266.
8. Burchell JT, Wikstrom ME, Stumbles PA, Sly PD, Turner DJ. Attenuation of allergen-induced airway hyperresponsiveness is mediated by airway regulatory T cells. *Am J Physiol Lung Cell Mol Physiol* 2009;296:307–319.
9. Kearley J, Robinson DS, Lloyd CM. CD4⁺CD25⁺ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J Allergy Clin Immunol* 2008;122:617–624.
10. Belkaid Y, Oldenhove G. Tuning microenvironments: induction of regulatory T cells by dendritic cells. *Immunity* 2008;29:362–371.
11. Adorini L, Penna G. Induction of tolerogenic dendritic cells by vitamin D receptor agonists. *Handb Exp Pharmacol* 2009;188:251–273.
12. Wu K, Bi Y, Sun K, Xia J, Wang Y, Wang C. Suppression of allergic inflammation by allergen-DNA-modified dendritic cells depends on the induction of Foxp3⁺ regulatory T cells. *Scand J Immunol* 2008;67:140–151.
13. Cools N, Van Tendeloo VF, Smits EL, Lenjou M, Nijs G, Van Bockstaele DR, Berneman ZN, Ponsaerts P. Immunosuppression induced by immature dendritic cells is mediated by TGF- β /IL-10 double-positive CD4⁺ regulatory T cells. *J Cell Mol Med* 2008;12:690–700.
14. Li X, Yang A, Huang H, Zhang X, Town J, Davis B, Cockcroft DW, Gordon JR. Induction of Th2 cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. *Am J Respir Cell Mol Biol* (In press)
15. Henry E, Desmet CJ, Garz   V, Fi  vez L, Bedoret D, Heirman C, Faisca P, Jasp  r FJ, Gosset P, Jacquet AP, et al. Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma. *J Immunol* 2008;181:7230–7242.
16. Fujita S, Yamashita N, Ishii Y, Sato Y, Sato K, Eizumi K, Fukaya T, Nozawa R, Takamoto Y, Yamashita N, et al. Regulatory dendritic cells protect against allergic airway inflammation in a murine asthmatic model. *J Allergy Clin Immunol* 2008;121:95–104.
17. Honey K, Cobbold SP, Waldmann H. CD40 ligand blockade induces CD4⁺ T cell tolerance and linked suppression. *J Immunol* 1999;163:4805–4810.
18. Mahnke K, Ring S, Bedke T, Karakhanova S, Enk AH. Interaction of regulatory T cells with antigen-presenting cells in health and disease. *Chem Immunol Allergy* 2008;94:29–39.
19. Taams LS, van Amelsfort JM, Tiemessen MM, Jacobs KM, de Jong EC, Akbar AN, Bijlsma JW, Lafeber FP. Modulation of monocyte/macrophage function by human CD4⁺CD25⁺ regulatory T cells. *Hum Immunol* 2005;66:222–230.
20. Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS. CD4⁺CD25⁺Foxp3⁺ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc Natl Acad Sci USA* 2007;104:19446–19451.
21. Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, Sproles AA, Shah JS, K  hl J, Belkaid Y, et al. CD4⁺CD25⁺ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 2005;202:1549–1561.
22. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med* 2006;203:1701–1711.
23. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006;203:1693–1700.
24. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol* 2003;3:317–330.
25. Buckland M, Lombardi G. Aspirin and the induction of tolerance by dendritic cells. *Handb Exp Pharmacol* 2009;188:197–213.
26. Lin YL, Shieh CC, Wang JY. The functional insufficiency of human CD4⁺CD25^{high} T-regulatory cells in allergic asthma is subjected to TNF- α modulation. *Allergy* 2008;63:67–74.
27. Duan W, So T, Croft M. Antagonism of airway tolerance by endotoxin/lipopolysaccharide through promoting OX40L and suppressing antigen-specific Foxp3⁺ T regulatory cells. *J Immunol* 2008;181:8650–8659.
28. Robinson DS. Regulatory T cells and asthma. *Clin Exp Allergy* 2009;39:1314–1323.
29. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergies during birch pollen season. *Clin Exp Allergy* 2004;34:1364–1372.
30. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, Carr VA, Robinson DS. Relation of CD4⁺CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;363:608–615.
31. Bellinghausen I, Klostermann B, Knop J, Saloga J. Human CD4⁺CD25⁺ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 2003;111:862–868.
32. Xystrakis E, Urry Z, Hawrylowicz CM. Regulatory T cell therapy as individualized medicine for asthma and allergy. *Curr Opin Allergy Clin Immunol* 2007;7:535–541.
33. Ogawa Y, Duru EA, Ameredes BT. Role of IL-10 in the resolution of airway inflammation. *Curr Mol Med* 2008;8:437–445.
34. Steinbrink K, W  lfel M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 1997;159:4772–4780.
35. Dao Nguyen X, Robinson DS. Fluticasone propionate increases CD4CD25 T regulatory cell suppression of allergen-stimulated CD4CD25 T cells by an IL-10-dependent mechanism. *J Allergy Clin Immunol* 2004;114:296–301.
36. Peek EJ, Richards DF, Faith A, Lavender P, Lee TH, Corrigan CJ, Hawrylowicz CM. Interleukin-10-secreting “regulatory” T cells induced by glucocorticoids and β_2 -agonists. *Am J Respir Cell Mol Biol* 2005;33:105–111.
37. Nguyen KD, Vanichsarn C, Fohner A, Nadeau KC. Selective deregulation in chemokine signaling pathways of CD4⁺CD25^{hi}CD127^{lo/-} regulatory T cells in human allergic asthma. *J Allergy Clin Immunol* 2009;123:933–939.e10.
38. Nguyen KD, Fohner A, Booker JD, Dong C, Krensky AM, Nadeau KC. XCL1 enhances regulatory activities of CD4⁺CD25^{high}CD127^{low/-} T cells in human allergic asthma. *J Immunol* 2008;181:5386–5395.
39. Nguyen KD, Vanichsarn C, Nadeau KC. Increased cytotoxicity of CD4⁺ invariant NKT cells against CD4⁺CD25^{hi}CD127^{lo/-} regulatory T cells in allergic asthma. *Eur J Immunol* 2008;38:2034–2045.
40. Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, R  ckert B, Mantel PY, Menz G, Akdis CA, Blaser K, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* 2004;114:1425–1433.
41. Becker C, Stoll S, Bopp T, Schmitt E, Jonuleit H. Regulatory T cells: present facts and future hopes. *Med Microbiol Immunol (Berl)* 2006;195:113–124.

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**Impaired IL-10-dependent Induction of Tolerogenic Dendritic Cells by
CD4⁺CD25^{hi}CD127^{lo/-} Natural Regulatory T Cells In Human Allergic Asthma**

Khoa D. Nguyen, Christopher Vanichsarn, and Kari C. Nadeau.

ONLINE DATA SUPPLEMENT

SUPPLEMENTARY FIGURE LEGENDS

SUPPLEMENTARY FIGURE 1. Distribution and phenotype of circulating natural regulatory T cells from allergic asthmatic, healthy control, and non-allergic asthmatic subjects. **A.** *(Left)* Frequency of circulating natural regulatory T cells among 3 subject groups (n=21 for each group). *(Right)* Representative FACS plot of sorting strategy for circulating natural regulatory T cells from purified CD4⁺ T cells. Co-staining with CD127 and CD25 identified the natural regulatory T cell subset. Gatings for CD4⁺CD25⁺CD127^{lo/-} natural regulatory T cells and CD4⁺CD25⁻ effector T cells were established based on negative thresholds from staining with isotype controls. **B.** *(Left)* Expression of Foxp3 in purified natural regulatory T cells among 3 subject groups (n=21 for each group). *(Right)* FACS plots of Foxp3 and CD25 expression in purified natural regulatory T cells and effector T cells from representative allergic asthmatic (AA), healthy control (HC), and non-allergic asthmatic (NA) subjects. Dot-plot graphs with horizontal bars represented median values.

SUPPLEMENTARY FIGURE 2. Titration of cell ratios and incubation times for co-cultures of natural regulatory T cells and dendritic cells from allergic asthmatic, healthy control, and non-allergic asthmatic subjects. Percentage changes in expression of CD40, CD80, DR, TNF- α , and IL-10 in purified dendritic cells cultured with autologous natural regulatory T cells from allergic asthmatics (**A**), healthy controls (**B**), and non-allergic asthmatics (**C**). Data represent cultures of natural regulatory T cells and dendritic cells at 2:1, 1:1, and 1:2 ratios. Cells were analyzed after being cultured for 20, 30, and 40 hours (n=3 experiments with similar results).

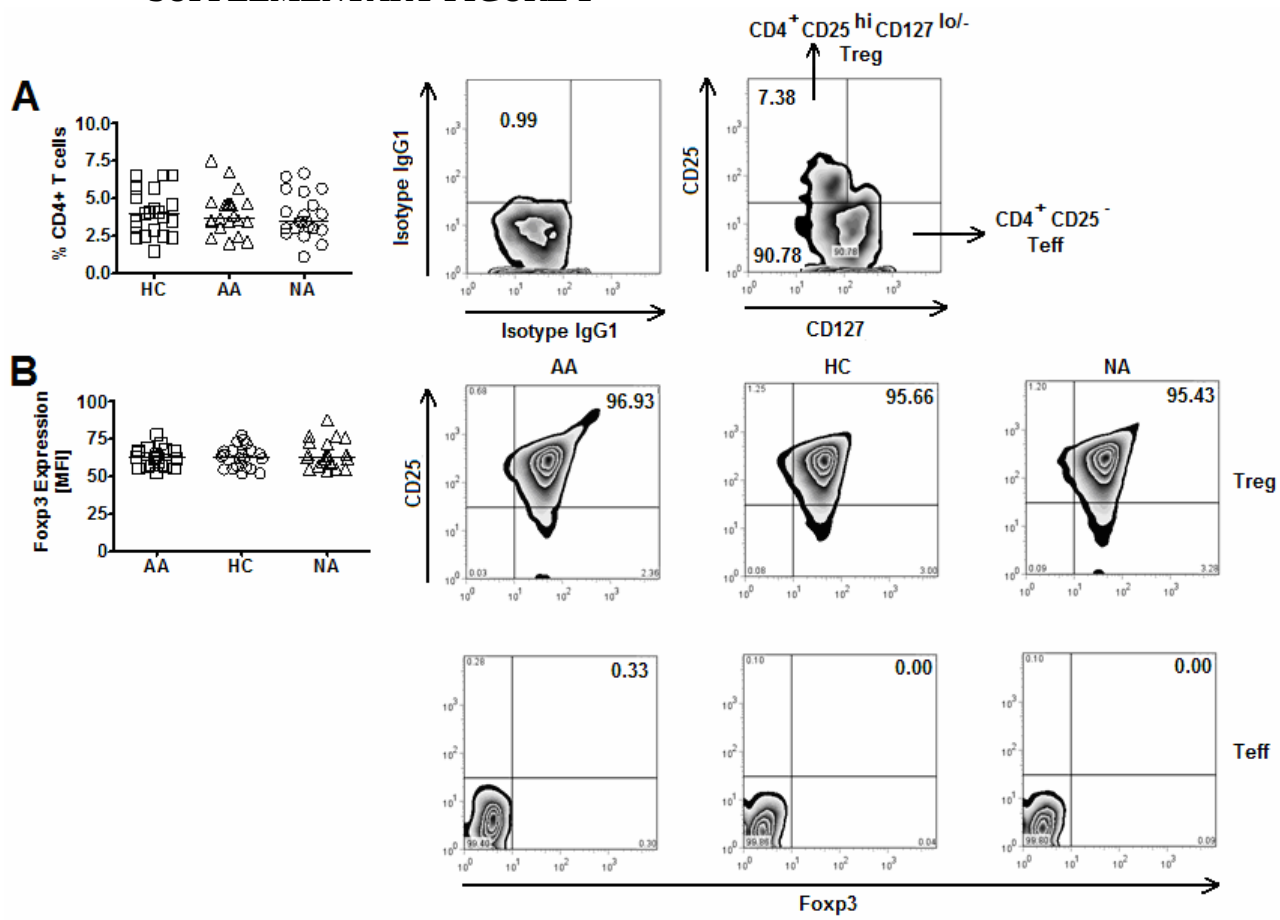
SUPPLEMENTARY FIGURE 3. Re-isolation of natural regulatory T cells and dendritic cells from allergic asthmatic, healthy control, and non-allergic asthmatic subjects after co-cultures. Representative FACS plots of natural regulatory T cells and dendritic cells in co-cultures (1st row) and after re-isolation with CD3 microbeads (2nd and 3rd rows). Purity of re-isolated natural regulatory T cells and dendritic cells from representative allergic asthmatic, healthy control, and non-allergic asthmatic subjects were evaluated by cell morphology and CD3 staining.

SUPPLEMENTARY FIGURE 4. A. Inhibition of IL-10 activity *in vitro* by neutralizing antibody against IL-10 (n=5). Efficacy of neutralizing antibody was tested by its ability to inhibit recombinant IL-10 mediated activation of STAT3 in purified dendritic cells. Data represented expression of phosphorylated STAT3 (phospho-STAT3) in unstimulated dendritic cells or stimulated dendritic cells from representative healthy controls in the presence of different concentrations of neutralizing antibodies against IL-10. Media with recombinant IL-10 was pre-incubated with neutralizing antibody against IL-10 for 0.5 hours before being added to cell cultures. **B.** Proliferation of effector T cells measured by H³ incorporation in stimulation assays with purified dendritic cells or natural regulatory T cell-primed dendritic cells. **C.** Comparison of responses to anti-CD3 stimulation by natural regulatory T cells among 3 subject groups (n=21 each). Responses to anti-CD3 stimulation were measured by anti-CD3 antibody-mediated activation of AKT in natural regulatory T cells. Data represented expression of phosphorylated AKT (phospho-AKT) in response to anti-CD3 stimulation and total AKT by natural regulatory T cells. **D.** IL-10 secretion by natural regulatory T cells from 3 subject groups in response to anti-CD3 stimulation (n=21 for each group). Paired t-tests were used for statistical analysis of the

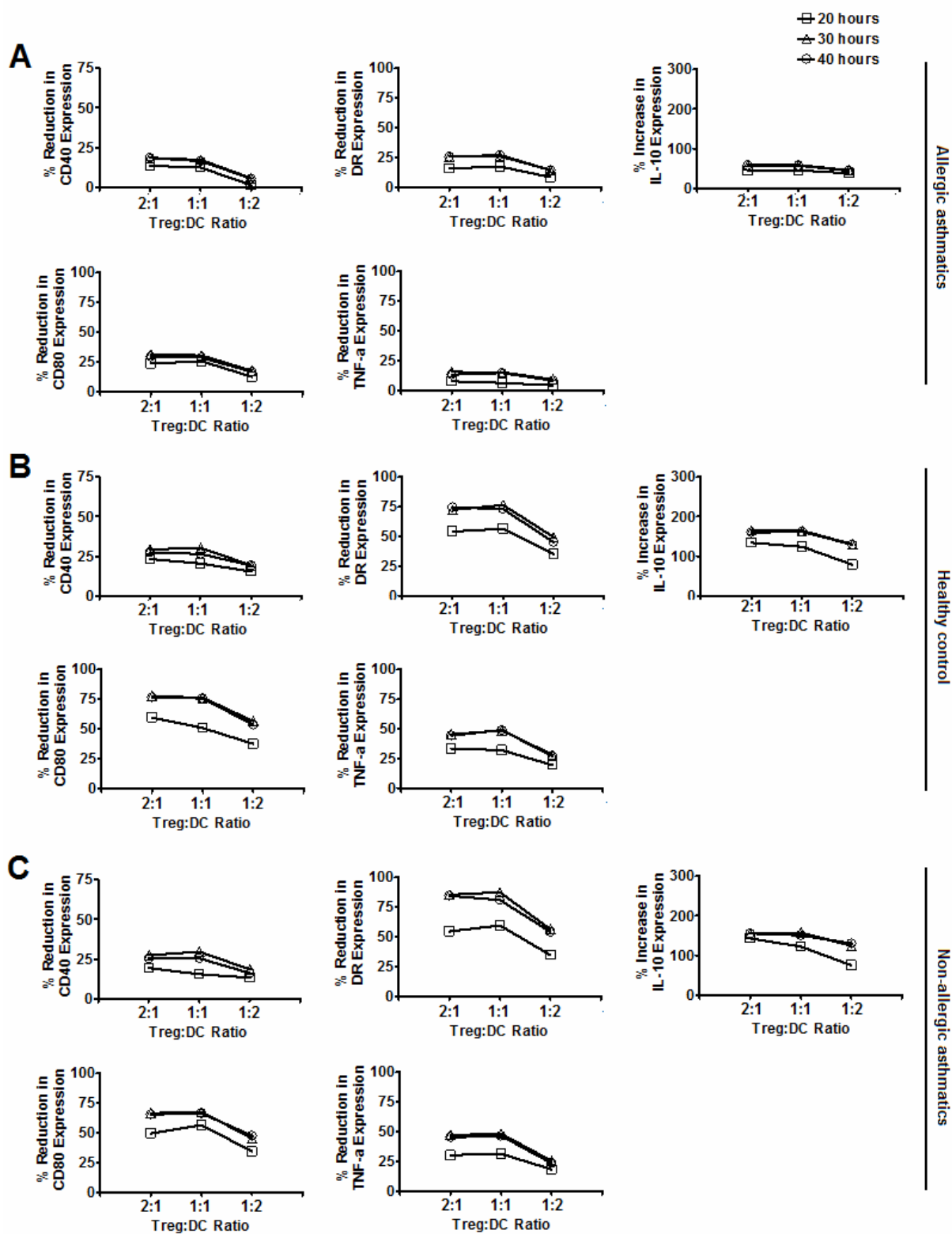
data with normal distribution in A. ANOVA were used for statistical analysis of the data with normal distribution and equal variances in C and D. Dot-plot graphs with horizontal bars represented median values.

SUPPLEMENTARY FIGURE 5. Expression of CD40, CD80, DR, TNF-a, and IL-10 in Treg-primed dendritic cells among 3 subject groups (n=21 for each group). ANOVA was used for statistical analysis of the data with normal distribution and equal variances.

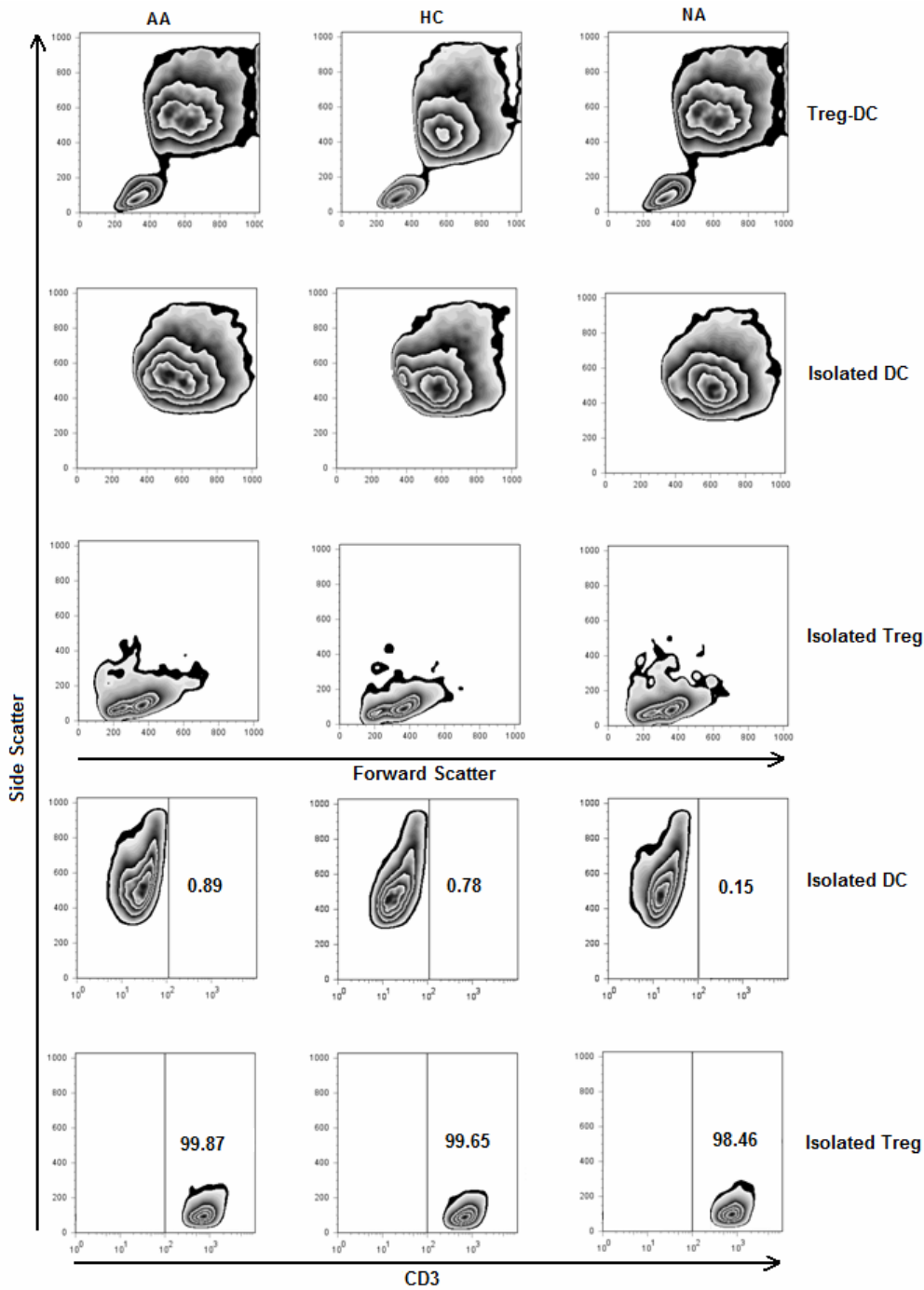
SUPPLEMENTARY FIGURE 1



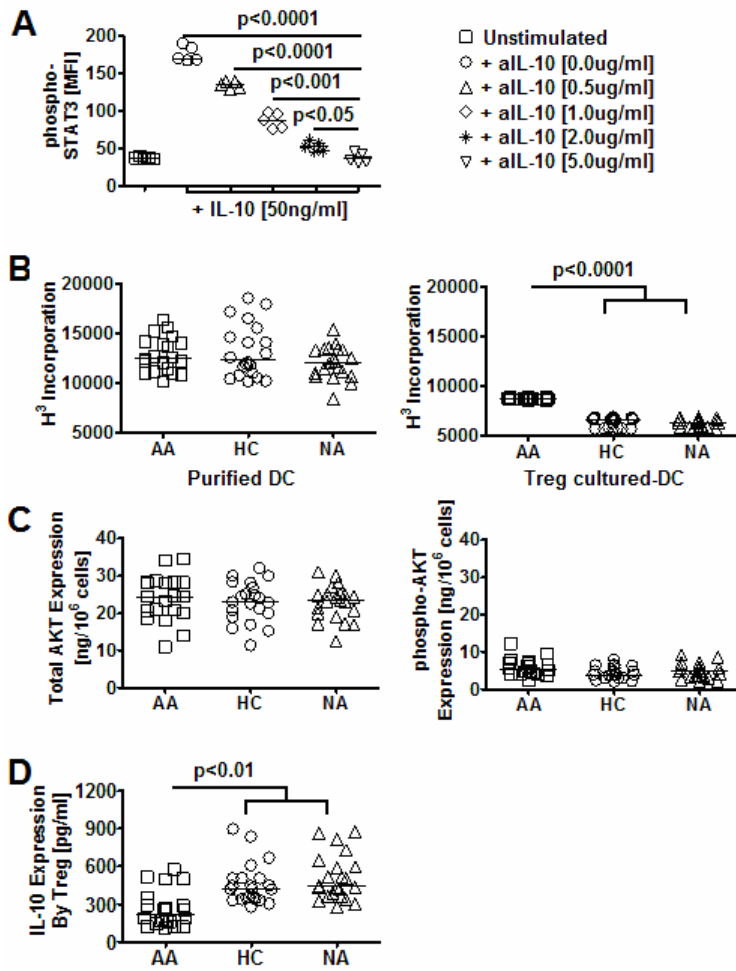
SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4



SUPPLEMENTARY FIGURE 5

