

Effects of antiasthmatic agents on the functions of peripheral blood monocyte-derived dendritic cells from atopic patients

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Background: Dendritic cells (DCs), the antigen-presenting cells in the airway, play a critical role in asthma. Nevertheless, there is little information on the effects of antiasthmatic agents on DCs. **Objectives:** The purpose of the present study was to determine the effects of representative antiasthmatic agents, including cysteinyl leukotriene (cysLT) 1 receptor antagonists, corticosteroid, and tacrolimus, on DCs in inducing allergy.

Methods: Human peripheral blood monocyte-derived DCs (MoDCs) generated from atopic and healthy subjects were pulsed with *Dermatophagoides farinae* allergen in the presence of medium alone, pranlukast, montelukast, dexamethasone, or tacrolimus. The mRNA expressions of cysLT receptor, cysLTs producing enzymes, and various surface markers on MoDCs, as well as the concentrations of cysLTs, IL-10, and IL-12 in cultured supernatants, were determined. MoDCs were also cocultured *in vitro* with autologous CD4⁺ T cells, and IL-5 and IFN- γ production was measured.

Results: MoDCs of atopic patients expressed mRNAs of cysLT1 receptor and cysLT-producing enzymes, and allergen pulsing significantly increased cysLT production. MoDCs of atopic patients showed a T_H2-favoring phenotype and induced T_H2-skewed cytokine production from autologous CD4⁺ T cells. Dexamethasone and tacrolimus inhibited allergen-pulsed MoDC-induced cytokine production by autologous CD4⁺ T cells without and with IL-10 inhibition, respectively. CysLT1 receptor antagonists had no effect on MoDC functions.

Conclusion: Our results indicate that MoDCs of atopic patients induce a T_H2 reaction. Corticosteroid and tacrolimus, but not cysLT1 receptor antagonists, inhibit T_H2 reactions, and this effect is probably mediated through different pathways. (*J Allergy Clin Immunol* 2004;114:538-44.)

Key words: Monocyte-derived dendritic cell, cysteinyl leukotriene, cytokine, cysteinyl leukotriene 1 receptor antagonist, dexamethasone, tacrolimus

Abbreviations used

cysLT: Cysteinyl leukotriene
DC: Dendritic cell
FLAP: 5-LO activating protein
5-LO: 5-Lipoxygenase
LTC₄S: Leukotriene C₄ synthase
MoDC: Monocyte-derived dendritic cell
PE: Phycoerythrin

Dendritic cells (DCs) are the major antigen-presenting cells involved in the induction of the primary immune response.¹ Immature DCs are extremely efficient in capturing antigens in peripheral tissues and carrying them to the T-cell area of draining lymph nodes, wherein mature DCs prime naive T cells to differentiate into T_H1/T_H2 cells. Among the factors that determine T_H1/T_H2 differentiation, cytokine environment during antigen presentation is the most important factor.² Bacterial components, such as LPS, induce the production of IL-12 and IL-18 by DCs. These cytokines are critical for the development of polarized T_H1 responses. On the other hand, little is known about the factors involved in initiating a T_H2 response.

Recent studies used *in vitro* generated DCs from human peripheral blood monocytes, wherein they are cultured in the presence of GM-CSF and IL-4.³ It has been reported that these peripheral blood monocyte-derived DCs (MoDCs) from atopic patients induce autologous CD4⁺ cells to produce T_H2 cytokines.^{4,5}

DCs are critically involved in the pathogenesis of bronchial asthma, a T_H2-mediated respiratory disease. Interestingly, DCs express histamine receptors,⁶ β_2 -adrenergic receptors,⁷ and cysLT1 receptors,⁸ which could be targets for antiasthmatic drugs. Nevertheless, the effects of antiasthmatic drugs on the functions of human DCs are yet to be determined. We hypothesized that DCs from atopic patients might show skewed T_H1/T_H2 differentiation toward T_H2 cells compared with those from healthy subjects and that antiasthmatic drugs might alter DC functions. To verify this hypothesis, we compared the cytokine profile in DCs and in autologous CD4⁺ T cells cocultured with DCs in atopic patients and healthy subjects. We also evaluated the effects of various antiasthmatic drugs on MoDCs and autologous CD4⁺ T cells cocultured with MoDCs.

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METHODS

Subjects

The present study included 10 nonatopic control donors (male/female ratio, 5:5; age, 29.1 ± 2.2 years [mean $\pm$ SD]) and 14 atopic patients (male/female ratio, 7:7; age, 27.5 ± 2.7 years). The atopic patients had at least one allergic disease, including bronchial asthma, atopic dermatitis, or allergic rhinitis, and were sensitized to *Dermatophagoides farinae* as confirmed by the presence of serum *D farinae*-specific IgE antibodies (RAST class ≥ 3). None of the subjects had ever smoked or had other pulmonary or bronchial diseases for at least 2 weeks before blood collection. None of the atopic patients was receiving any treatment at least 8 weeks before the study. The present study was approved by the Ethics Committee of Nagasaki University Hospital, and written informed consent was obtained from each subject.

Generation of peripheral blood MoDCs

DCs were generated from peripheral blood monocytes, as described previously.³ In brief, PBMCs were isolated from heparinized blood by using Ficoll-Paque Plus (Amersham Biosciences Corp, Piscataway, NJ) density gradient centrifugation. In the next step, PBMCs were incubated at 1×10^7 cells per well in RPMI-1640 (Invitrogen Corp, Carlsbad, Calif) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% FBS (Invitrogen), hereafter referred as cRPMI, for 45 minutes in a 6-well plate (Becton Dickinson Labware, Franklin Lakes, NJ) supplemented with FBS at 37°C. After removal of nonadherent cells by washing the dishes 3 times with warm RPMI-1640 medium, adherent cells were cultured in 3 mL of cRPMI containing GM-CSF (800 U/mL; R&D Systems, Inc, McKinley Place, Neb) and IL-4 (500 U/mL, R&D Systems, Inc). After 7-day culture, nonadherent cells corresponding to the DC-enriched fraction were harvested, washed, and used for subsequent experiments. The DC-enriched fraction contained more than 90% DCs, as confirmed by morphology and FACS analysis, as described previously.⁹ On day 7, immature DCs were pulsed with 10 μ g/mL *D farinae* allergen extract (LSL Inc, Tokyo, Japan) in the presence or absence of pranlukast (10^{-6} mol/L; ONO Pharmaceutical Co, Osaka, Japan), montelukast (10^{-6} mol/L, Merck, NJ), dexamethasone (10^{-6} mol/L; Sigma Chemical Co, St Louis, Mo), or tacrolimus (10^{-6} mol/L; Fujisawa Pharmaceutical Co, Osaka, Japan). Each drug concentration was chosen on the basis of previous studies and reported physiologic concentrations.^{8,10-12} Cell viability was measured by means of trypan blue staining before and after the addition of drugs, and all cultures were found to contain more than 95% viable cells. The LPS concentration in the *D farinae* allergen extract was less than 0.96 EU/mg *D farinae* (Limulus Amebocyte Lysate Test; E-Toxate; Sigma-Aldrich, St Louis, Mo). On day 9, the supernatants were collected, and mature DCs were washed 4 times and used for DC-T-cell cocultures, FACS analysis, and RT-PCR. In a preliminary experiment MoDCs were cultured with *D farinae* allergen extract in the absence (medium) or presence of solvents (ethanol or ethanol and NaOH), and the results of all experiments showed no significant differences between groups.

DC-T-cell cocultures

On day 9 of DC culture, PBMCs were isolated from the same donor by means of density gradient centrifugation, as described above, and filtered through a human CD4 subset mini-column kit (R&D Systems, Inc) to isolate CD4⁺ T cells. The purity of recovered cells ranged from 88% to 95%. CD4⁺ T cells were cocultured with autologous DCs at a ratio of 10:1 for 5 days. On day 14, supernatants were collected.

Quantitative determination of IL-12, IL-10, TNF- α , IL-5, IFN- γ , and cysteinyl leukotrienes

The concentrations of IL-12, IL-10, and TNF- α in the cultured supernatants on day 9 were measured by means of ELISA (R&D Systems, Inc), and the concentrations of cysteinyl leukotrienes (cysLTs) in the same supernatants were measured by using the enzyme immunoassay method (Cayman Chemical Co, Ann Arbor, Mich), according to the protocol provided by the respective manufacturers. The detection limits were 5.0 pg/mL, 3.9 pg/mL, 4.4 pg/mL, and 13 pg/mL for IL-12, IL-10, TNF- α , and cysLTs, respectively. The concentrations of IL-5 and IFN- γ in supernatants on day 14 were measured by means of ELISA (R&D Systems, Inc), and the detection limits were 3.0 pg/mL and 8 pg/mL, respectively.

RT-PCR

Total RNA was isolated from DCs on day 9 by using TRIzol Reagent (Life Technologies Inc, Rockville, Md) with the method provided by the supplier. cDNA was synthesized from 1 μ g of total RNA by using a SuperScript One-Step RT-PCR System with Platinum Tag DNA Polymerase (Invitrogen Life Technologies, Inc) and amplified by using 200 ng of cDNA with primers complementary to the published sequences of human β -actin, CysLT1 receptor, leukotriene C₄ synthase (LTC₄S), 5-lipoxygenase (5-LO), and 5-lipoxygenase activating protein (FLAP; Sigma Genosys, Hokkaido, Japan). Thirty-five cycles were performed (15 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, and 1 minute at 72°C for extension). After amplification, portions of the PCR products were electrophoresed on 1% agarose gel and visualized with ethidium bromide. Controls in which the reverse transcription step was omitted confirmed that the PCR products reflected mRNA levels rather than contaminating genomic DNA.

Surface marker expression by flow cytometry

On day 9, DCs were incubated with saturating concentrations of fluorochrome-conjugated mAbs at 4°C for 30 minutes and then washed twice in CellWASH (Becton Biosciences, San Jose, Calif). Data were collected with a FACSCalibur (Becton Biosciences), and data analysis was performed with Cellquest software (Becton Biosciences). The following fluorochrome-conjugated mouse mAbs against human cell-surface proteins were used for DC surface marker staining: FITC-conjugated anti-HLA-DR; phycoerythrin (PE)-conjugated anti-CD83; PE-conjugated anti-CD123; and PE-conjugated anti-CD11c. All antibodies were obtained from BD PharMingen (San Diego, Calif). Data were presented as mean fluorescence intensity.

Statistical analysis

Results are expressed as means $\pm$ SEM. Differences between groups were examined for statistical significance by using the Mann-Whitney test. A *P* value of less than .05 denoted the presence of a statistically significant difference.

RESULTS

Human MoDCs express cysLT1 receptor and produce cysLTs

RT-PCR demonstrated that MoDCs expressed mRNAs for cysLT1 receptor, 5-LO, FLAP, and LTC₄S (Fig 1, A). cysLT production from immature DCs of healthy subjects was not different from that of atopic subjects (Fig 1, B). Compared with immature DCs, cysLT production from D

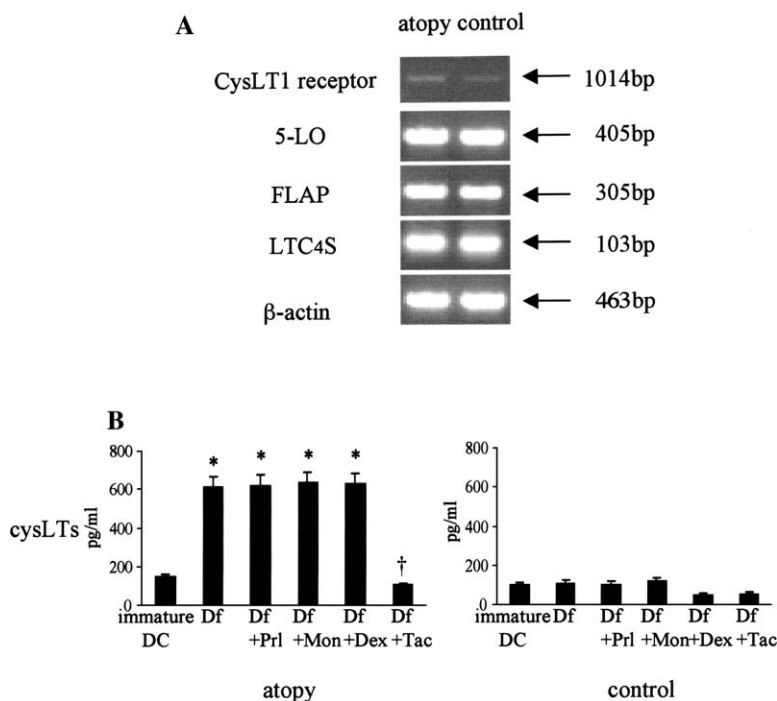


FIG 1. Human MoDCs express CysLT1 receptor, 5-LO, FLAP, and LTC₄S mRNA and produce cysLTs. **A**, The expression levels of CysLT1 receptor, 5-LO, FLAP, LTC₄S, and β-actin mRNAs were determined in human MoDCs by means of RT-PCR. The result is a representative gel of RT-PCR analysis from 3 independent experiments. **B**, Comparison of cysLT production by MoDCs from atopic patients and healthy donors. Data represent means ± SEM. * $P < .05$ versus immature DCs; † $P < .05$ versus *D farinae* allergen-pulsed MoDCs. Df, *Dermatophagoides farinae*; Prl, pranlukast; Mon, montelukast; Dex, dexamethasone; Tac, tacrolimus.

farinae allergen-pulsed mature DCs were significantly higher in the atopic group, whereas it did not change in the healthy group (Fig 1, B). Tacrolimus significantly inhibited cysLT production from *D farinae* allergen-pulsed mature DCs, whereas pranlukast, montelukast, and dexamethasone failed to inhibit cysLT production in the atopic group (Fig 1, B). Considered together, these results indicate that MoDCs of atopic patients express cysLT-producing enzymes and cysLT1 receptor and produce cysLTs after allergen pulse.

Cytokine production by MoDCs

IL-12 production in all groups of MoDCs was less than the detection limit of ELISA. Production of IL-10 and TNF-α by immature DCs was similar in the healthy and atopic groups (Fig 2). *D farinae* allergen pulsing significantly increased IL-10 production by mature DCs in the atopic group, but not in the healthy group, compared with immature DCs. Tacrolimus, but not pranlukast, montelukast, and dexamethasone, inhibited IL-10 production from *D farinae* allergen-pulsed mature DCs in both groups. *D farinae* allergen pulsing significantly increased TNF-α production by mature DCs in both the atopic and healthy groups. Tacrolimus and dexamethasone, but not pranlukast and montelukast, significantly inhibited TNF-α production by *D farinae* allergen-pulsed mature DCs in both groups.

Cytokine production by CD4⁺ T cells

Production of IL-5 and IFN-γ by CD4⁺ T cells cocultured with immature DCs was not significantly different between the healthy and atopic groups (Fig 3). IL-5 production by CD4⁺ T cells cocultured with *D farinae* allergen-pulsed DCs was significantly higher in the atopic group compared with cocultures with immature DCs. However, no such change was noted in the healthy group. In the atopic group dexamethasone and tacrolimus, but not pranlukast and montelukast, significantly inhibited IL-5 production by CD4⁺ T cells cocultured with *D farinae* allergen-pulsed mature DCs. In both groups IFN-γ production by CD4⁺ T cells cocultured with *D farinae* allergen-pulsed DCs was significantly higher than in cocultures of immature DCs. Dexamethasone and tacrolimus, but not pranlukast and montelukast, significantly inhibited IFN-γ production from CD4⁺ T cells cocultured with *D farinae* allergen-pulsed mature DCs.

Expression of surface markers on MoDCs

In comparison with healthy subjects, HLA-DR expression was significantly downregulated in the atopic group (Fig 4). However, there were no significant differences between the atopic and healthy groups with regard to the expression levels of CD83, CD11c, and CD123, irrespective of *D farinae* allergen pulse. Tacrolimus significantly inhibited CD83 expression in both groups.

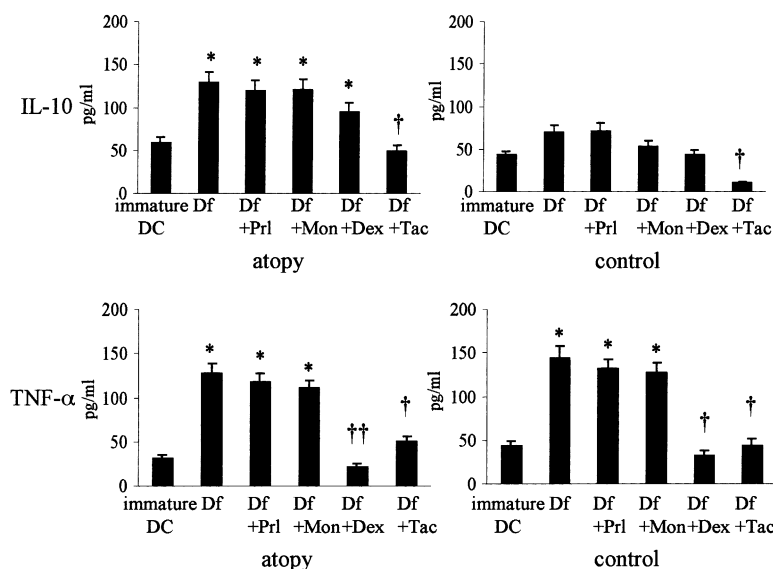


FIG 2. Comparison of cytokine production by MoDCs from atopic patients and healthy donors. Data represent means $\pm$ SEM. * $P < .05$ versus immature DCs, † $P < .05$ versus *D farinae* allergen-pulsed MoDCs, †† $P < .01$ versus *D farinae* allergen-pulsed MoDCs. Df, *Dermatophagoides farinae*; Prl, pranlukast; Mon, montelukast; Dex, dexamethasone; Tac, tacrolimus.

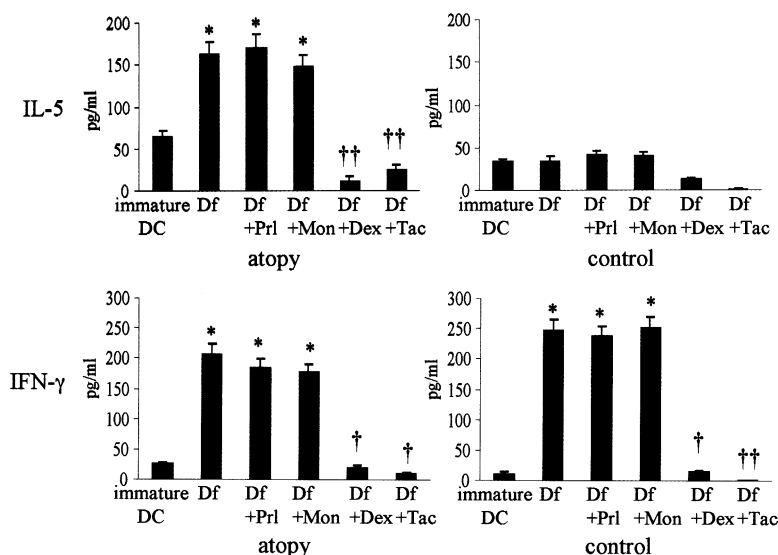


FIG 3. Comparison of cytokine production by CD4⁺ T cells from atopic patients and healthy donors after stimulation with autologous MoDCs. Data represent means $\pm$ SEM. * $P < .05$ versus coculture with immature DCs, † $P < .05$ versus coculture with *D farinae* allergen-pulsed MoDCs, †† $P < .01$ versus coculture with *D farinae* allergen-pulsed MoDCs. Df, *Dermatophagoides farinae*; Prl, pranlukast; Mon, montelukast; Dex, dexamethasone; Tac, tacrolimus.

Pranlukast, montelukast, and dexamethasone failed to alter surface marker expression on MoDCs.

DISCUSSION

The major findings of the present study are as follows. First, MoDCs from *D farinae* allergen-sensitized subjects

expressed cysLT1 receptor, 5-LO, FLAP, and LTC₄S mRNAs, and *D farinae* allergen pulsing significantly increased cysLT production. Second, MoDCs of atopic subjects showed T_H2-favored phenotype and induced T_H2-skewed cytokine production from autologous CD4⁺ T cells. Third, dexamethasone and tacrolimus inhibited allergen-pulsed MoDC-induced cytokine production by autologous CD4⁺ T cells through a different pathway.

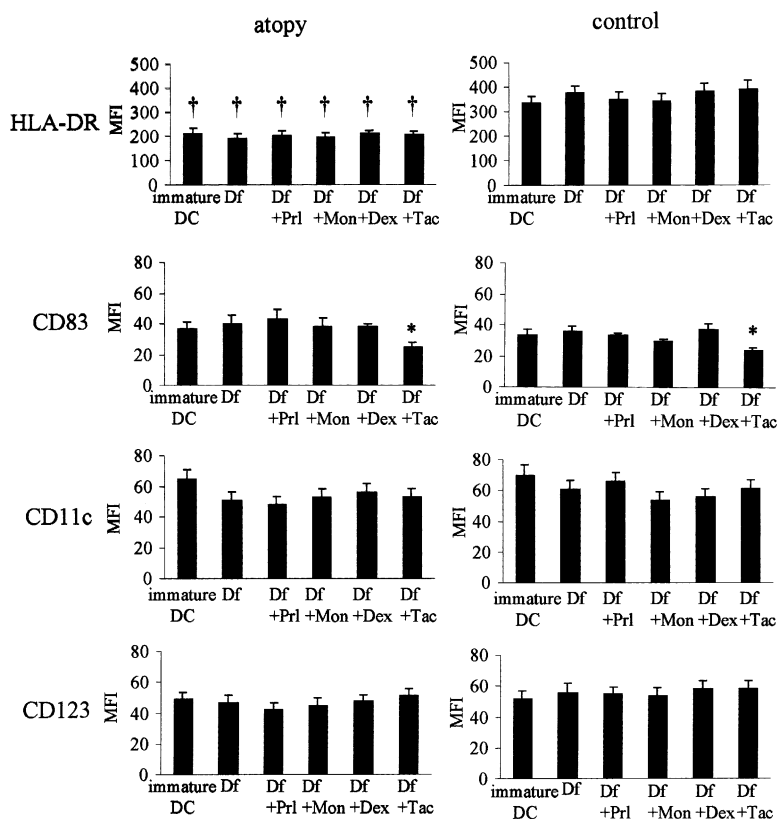


FIG 4. Surface expression of HLA-DR, CD83, CD11c, and CD123 on MoDCs of atopic patients (n = 6) and control subjects (n = 6). MoDCs (day 9) were examined for surface expression of the indicated molecules. Data shown are mean $\pm$ SEM values of mean fluorescence intensity. †P < .05 versus healthy group. *P < .05 versus *D farinae* allergen-pulsed MoDCs. Df, *Dermatophagoides farinae*; Prl, pranlukast; Mon, montelukast; Dex, dexamethasone; Tac, tacrolimus.

Finally, cysLT1 receptor antagonists did not alter MoDC functions.

In the present study MoDCs of atopic patients produced a significant amount of IL-10, but not IL-12, after allergen pulsing compared with those of healthy donors. IL-12 is a critical cytokine for the development of a polarized T_H1 response.¹³ IL-10 suppresses IL-12 production by DCs and induces a polarized T_H2 response.¹⁴ Thus increased IL-10 production by DCs might be the likely reason for the induction of a T_H2 response in atopic patients. It was also reported that the T_H2-favored immune response observed in atopic patients is not due to diminished IL-12 production by MoDCs.⁴ The concentrations of IL-12 produced by MoDCs were less than the detection limit in all groups of MoDCs in the present study, and thus it is unlikely that induction of a T_H2 response was due to a reduction of IL-12. Steinbrink et al¹⁵ reported that IL-10 inhibited the development of fully mature DCs and that DCs precultured with IL-10 induced a state of alloantigen-specific anergy in CD4⁺ T cells. These anergic T cells revealed a reduced production of T_H1 cytokines. In the present study inhibition of MoDC maturation by IL-10 might play a role in the induction of a T_H2 response.

In the present study allergen-exposed DCs promoted a T_H1 response in healthy subjects, whereas those of atopic subjects promoted a T_H2 response, which could cause and maintain allergic inflammation. One limitation of the present study was that helper T cells were not separated into naive or memory components. Thus it remains unclear whether MoDCs affected naive T-cell differentiation or the already memory T cells. In this regard Bellinghausen et al⁴ showed that allergen-pulsed MoDCs with TNF- α , IL-1 β , and prostaglandin E₂ successfully induced T_H2 cytokine production from both naive and allergen-specific memory helper T cells of atopic donors but not healthy donors.

The effects of corticosteroid and tacrolimus on DCs and autologous CD4⁺ T cells are controversial.^{11,16} In the present study only tacrolimus inhibited IL-10 production by *D farinae* allergen-pulsed MoDCs. Dexamethasone and tacrolimus significantly inhibited IL-5 and IFN- γ production by autologous CD4⁺ T cells in atopic patients. On the basis of the role of IL-10 in the development of immune deviation, tacrolimus might inhibit T_H2 response at least partially through inhibition of IL-10 production from DCs. Because dexamethasone does not use this pathway, it is suggested that a different mechanism of

action is involved in the inhibition of the T_H2 response between tacrolimus and corticosteroid. In contrast to IL-10, both dexamethasone and tacrolimus inhibited TNF- α production by MoDCs. TNF- α promotes DC maturation^{17,18} and DC migration from nonlymphoid tissues to primary lymphoid organ.¹⁹ Thus dexamethasone and tacrolimus used a common pathway in inhibiting the T_H2 response, wherein reduction of TNF- α production from MoDCs might result in inhibition of DC maturation and migration. The expression of CD83, a DC-specific costimulatory marker, is enhanced through DCs maturation. In the present study tacrolimus inhibited CD83 expression, and thus it might influence MoDC maturation. Dexamethasone did not inhibit CD83 expression, and thus in terms of surface marker expression on MoDCs, tacrolimus and dexamethasone are considered to have different effects on MoDCs. In the present study, although MoDCs were extensively washed before coculture with T cells, one cannot exclude a possible carryover effect of antiasthmatic agents on cytokine release by T cells on the basis of intracellular uptake and subsequent release in addition to the direct effect of these agents on MoDCs.^{20,21} It is noteworthy that tacrolimus directly downregulated the stimulatory capacity of human epidermal Langerhans cells in skin mixed lymphocyte reaction.¹² Thus we believe that tacrolimus inhibited the production of cytokines from autologous T cells at least in part through a direct effect on MoDCs.

The reason for the differences in DC phenotype between the atopic group and the healthy group is not clear at present. The number of DCs that express FC ϵ RI is significantly higher in asthmatic patients than in control subjects,²² and MoDCs of asthmatic subjects express high-affinity FC ϵ RI,²³ which renders the allergen uptake more efficient and thus enhances the activation of allergen-specific T_H cells. Another possible reason is the low HLA-DR expression in atopic patients observed in the present study. Low antigen doses and low avidity interactions between T cells and DCs could favor the development of T_H2 responses.²⁴ It is possible that the low HLA-DR expression in atopic patients participates in the T_H2 response.

CysLTs represent the primary mediators of the early asthmatic response. Langerhans cells of normal human epidermis express 5-LO, FLAP, and LTC₄S mRNAs,²⁵ and DCs generated from CD34⁺ hematopoietic progenitors and those in lymphoid organs also express 5-LO and FLAP.²⁶ To our knowledge, the present study is the first to show that human MoDCs express mRNAs for the cysLT1 receptor and produce cysLTs after allergen pulse. We previously reported that cysLTs could influence murine bone marrow-derived DCs in developing T_H2 -dominant allergic airway inflammation⁸ and that pranlukast, a cysLT1 receptor antagonist, attenuates allergen-specific TNF- α production and nuclear factor κ B nuclear translocation in peripheral blood monocytes of atopic asthmatic patients.¹⁰ On the basis of these reports, we originally expected that cysLT1 receptor antagonists inhibit the human MoDC cytokine production. Unexpectedly,

cysLT1 receptor antagonists inhibited cytokine production in neither DCs nor T lymphocytes. In this regard Figueroa et al²⁷ reported that cysLT1 receptor mRNA and protein were expressed in only 20% of human T cell-free PBMCs. In agreement with their findings, our results showed only sparse expression of cysLTR1 mRNA in MoDCs, probably explaining the lack of MoDC response to cysLTR1 antagonist. This finding is in marked contrast to murine bone marrow-derived DCs, which express abundant cysLT1 receptor and respond well to cysLTR1 antagonist.⁸ Interestingly, Robbiani et al²⁸ reported that multidrug resistance-associated protein 1 regulates DC migration to lymph nodes, apparently by transporting LTC₄. It will be an interesting issue for future research to determine whether cysLT1 receptor antagonists modulate DC mobilization from peripheral tissue to lymph nodes, as well as to investigate the effects of other antiasthmatic drugs on DCs.

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