

Helper T-cell responses elicited by Der p 1-pulsed dendritic cells and recombinant IL-12 in atopic and healthy subjects

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Background: Environmental allergens, such as *Der-matophagoides pteronyssinus* group 1 antigen (Der p 1), induce T_{H2}-type responses in atopic patients, whereas healthy individuals have T_{H1}-type responses to the same antigens. Because of their efficient synthesis of IL-12, dendritic cells (DCs) are potent inducers of T_{H1}-type immune responses.

Objective: We sought to determine whether DCs would skew allergen-specific T_{H2}-type responses from atopic individuals. **Methods:** Purified CD4⁺ T cells from healthy donors or atopic individuals were cultured in the absence or presence of recombinant (r)IL-12 with DCs derived from PBMCs and pulsed with Der p 1. Supernatants of DC-T cell cocultures were assayed by ELISA for IL-5 and IFN- γ .

Results: A T_{H1}-type response developed in purified CD4⁺ T cells from healthy donors in response to Der p 1-pulsed DCs, as indicated by high levels of IFN- γ in culture supernatants. In contrast, CD4⁺ T cells from atopic donors displayed a T_{H2}-type profile characterized by high levels of IL-5 and low levels of IFN- γ . The addition of rIL-12 (10 ng/mL) to DC-T cell cocultures resulted in the induction of IFN- γ secretion by Der p 1-specific CD4⁺ T cells from atopic patients, whereas their production of IL-5 was not inhibited. Using flow cytometry after intracytoplasmic staining, we found that IFN- γ and IL-5 were secreted by distinct CD4⁺ T-cell subpopulations.

Conclusion: The cytokine profile of Der p 1-specific T_{H2}-like cells from atopic individuals is maintained when the allergen is presented by DCs, even in the presence of exogenous rIL-12. (J Allergy Clin Immunol 2000;105:346-52.)

Key words: Der p 1, dendritic cell, IL-12, atopy, T_{H2} cell

T-cell responses of atopic patients to environmental allergens are characterized by a T_{H2} pattern, and several studies suggest that successful specific immunotherapy is associated with a shift in the cytokine profile of allergen-reactive CD4⁺ T cells.¹⁻³ Among the factors that might promote a T_{H1}-type instead of a T_{H2}-type response, IL-12 was found to be instrumental in many systems.⁴ However, it is still unclear whether IL-12 might skew the activ-

Abbreviations used

DC: Dendritic cell

PE: Phycoerythrin

PMA: Phorbol 12-myristate 13-acetate

ities of highly polarized human T_{H2} cells.⁵⁻⁷ Dendritic cells (DCs) secrete high levels of IL-12, especially on maturation, which can be induced by exposure to bacterial LPS.⁸ We therefore hypothesized that CD4⁺ T cells isolated from allergic donors would display a T_{H1}-type phenotype when activated in vitro with immature or mature DCs pulsed with Der p 1. Because we observed that even mature DCs induced the production of high levels of IL-5 by allergen-specific T cells, we further determined the effects of the addition of exogenous IL-12 on the Der p 1-specific T-cell responses elicited by DCs.

METHODS

Culture medium and reagents

Culture medium consisted of RPMI-1640 (GIBCO, Paisly, Scotland) supplemented with 2 mmol/L L-glutamine (GIBCO), gentamicin (20 μ g/mL), 50 μ mol/L 2-mercaptoethanol, 1% nonessential amino acids (GIBCO), and 10% FCS (Bio-Whittaker Europe) or 10% human serum for DC-T cell cocultures. Recombinant IL-4 (24 $\times$ 10⁶ IU/mg) and recombinant GM-CSF (LEUCOMAX, 16 $\times$ 10³ IU/mg) were kindly provided by Schering-Plough (Kenilworth, NJ). Purified Der p 1 was purchased from Lofarma Allergeni (Milan, Italy). LPS from *Escherichia coli* (0128:B12) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemicals (Bornem, Belgium). Calcium ionophore A23187 was purchased from Calbiochem-Novabiochem (Nottingham, UK). Recombinant human IL-12 was purchased from R&D Systems Europe Ltd (Abingdon, UK).

Endotoxin contamination in Der p 1 samples was controlled by 2 passages on polymyxin B gel (Bio-Rad Laboratories, Nazareth EKE, Belgium) to ensure that the endotoxin content of the final dilutions used was less than 10 pg/mL, as determined by a limulus amoebocyte lysate assay (QCL-1000, Bio-Whittaker Europe).

Subjects

A group of subjects with clinical mild intermittent asthma attending the clinic of Allergology and Respiratory Diseases in the Centre Hospitalier Universitaire Saint Pierre of Brussels were asked to participate in the study. Their treatment required β_2 -agonists alone to avoid downregulation of peripheral blood T lymphocytes induced by inhaled corticosteroids.⁹ Bronchodilators were stopped 12 hours before each blood sampling. Allergy to mite allergen was demonstrated in each patient by a skin prick test for the Der-

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matophagoides pteronyssinus extract (ref 0315, Stallergenes SA, Paris, France) producing immediate response of a greater than 5-mm wheal compared with that produced by the negative control. For comparison, we also studied heparinized blood from healthy volunteers.

Generation of DCs from peripheral blood of atopic and healthy donors

DCs were generated from PBMCs, as described by Romani et al.¹⁰ Briefly, PBMCs from healthy volunteers were isolated by density centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway), resuspended in culture medium, and allowed to adhere onto 6-well plates. After 2 hours at 37°C, nonadherent cells were removed, and adherent cells were cultured in 3 mL of medium containing GM-CSF (800 U/mL) and IL-4 (500 U/mL). Every other day, 300 μ L of medium was removed and replaced by the same volume of fresh medium containing 2400 U of GM-CSF and 1500 U of IL-4. After 6 days of culture, nonadherent cells corresponding to the DC-enriched fraction were harvested, washed, and used for subsequent experiments. As we have previously reported,⁸ the DC-enriched fraction obtained according to this protocol routinely contains more than 90% of DCs, as assessed by morphology and FACS analysis.

Flow cytometric analysis

For immunophenotyping, cells were washed in PBS supplemented with 0.5% BSA and 10 mmol/L Na₂CO₃ and incubated for 30 minutes at 4°C with one of the following murine mAbs: phycoerythrin (PE)-conjugated anti-HLA-DR IgG2a mAb (Becton Dickinson, Mountain View, Calif); PE-conjugated anti-CD86 (B7-2) IgG2b mAb (Pharmingen, San Diego, Calif); PE-conjugated anti-CD80 (B7-1) IgG1 mAb (Becton Dickinson); PE-conjugated anti-CD83 IgG2b mAb (Imunotech, Marseille, France); and PE-conjugated anti-CD40 IgG1 mAb (Biosource International, Camarillo, Calif). As controls, cells were stained with corresponding isotype-matched control mAbs. Analysis was done by using a FACSCalibur flow cytometer (Becton Dickinson).

Cytokine determination

ELISA kits were purchased from Biosource Europe (Nivelles, Belgium) for quantification of IL-12 (p40), which detects both the heterodimeric (p70) and homodimeric (p40-p40) forms of human IL-12. IL-12 (p70) production was measured by using an ELISA kit provided by Genzyme (Leuven, Belgium). IFN- γ and IL-5 concentrations were measured by using 2-site sandwich ELISA with antibodies from Chromogenix (Mölnådal, Sweden) and Pharmingen, respectively.

DC-T cell cocultures

Responder cells were CD4⁺ T lymphocytes purified from the PBMCs of allergic and healthy donors by means of negative selection with a cocktail of hapten-conjugated antibodies (including anti-CD8, anti-CD11b, anti-CD16, anti-CD19, anti-CD39, and anti-CD56 antibodies), MACS microbeads coupled to anti-hapten mAbs, and a MACS column (Palo Alto, Calif). CD4⁺ T cells were more than 90% pure, as assessed by FACS analysis. Stimulator cells were syngeneic DCs that had been pulsed with 5 μ g/mL Der p 1 for 3 hours. For their maturation, DCs were stimulated with 1 μ g/mL LPS overnight. Purified CD4⁺ T cells were cocultured with autologous DCs at a ratio 10:1 for 5 days. Recombinant (r)IL-12 (10 ng/mL) was added at the beginning of the cultures.

Intracytoplasmic cytokine staining

For the detection of intracytoplasmic cytokines in CD4⁺ T cells, 2×10^6 /mL PBMCs isolated from an allergic donor were first stim-

TABLE I. IL-12 production by DCs

	IL-12 p70 (pg/mL)		IL-12 p40 (pg/mL)	
	Without LPS	With LPS	Without LPS	With LPS
Allergic patients				
1	2	700	<40	21,762
2	5	46	47	21,440
3	<2	189	64	38,388
4	4	63	<40	10,444
5	<2	5	<40	6,736
6	<2	36	220	12,176
Control subjects				
1	<2	41	<40	9396
2	32	494	224	21,995
3	18	200	207	24,843
4	<10	78	206	23,289
5	73	69	73	23,882

DCs from allergic (n = 6) and healthy (n = 5) donors were incubated overnight in medium alone (immature) or with 1 μ g/mL LPS (mature). IL-12 p40 and p70 production in the culture supernatants was measured by using the ELISA technique.

ulated by Der p 1 (5 μ g/mL) for 3 days. Cells were then incubated in fresh medium for 3 days before their short stimulation with PMA (10 ng/mL) and calcium ionophore (100 ng/mL) for 5 hours. Bouffeldin A (Sigma; 10 μ g/mL) was added into the cultures for the last 3 hours to prevent cytokine secretion. Cells were then washed in PBS and fixed with 80 μ L of solution A from Fix and Perm cell permeabilization kit (Imtec diagnostics NV, Antwerpen, Belgium) for 20 minutes. After washing in PBS, PBMCs were permeabilized in 80 μ L of solution B from the kit and stained simultaneously with the following antibodies: peridinin chlorophyll α -protein—conjugated anti-CD4 IgG1 mAb; FITC-conjugated anti-IFN- γ IgG2b mAb; and PE-conjugated anti-IL-5 IgG2a mAb (Becton-Dickinson). As controls, cells were stained with corresponding isotype-matched control mAbs. Triple-stained PBMCs were analyzed by using a FACSCalibur flow cytometer (Becton Dickinson).

Statistical analysis

Data were compared by using unpaired (Mann-Whitney) or paired (Wilcoxon) nonparametric tests.

RESULTS

Phenotypic similarity of DCs from allergic patients and healthy donors

Immature DCs were generated from PBMCs of patients allergic to Der p 1 or healthy individuals by a 6-day culture in the presence of IL-4 and GM-CSF, as previously described.¹⁰ Mature DCs were obtained by a further incubation with 1 μ g/mL LPS for 24 hours.⁸ We first compared the production of IL-12 p40 and IL-12 p70 (the heterodimeric bioactive form of IL-12) by DCs from healthy control subjects and allergic patients. We observed that significant levels of IL-12 p70 were only detected in supernatants of mature DCs obtained after LPS exposure. As shown in Table I, IL-12 levels in supernatants of DCs were not significantly different in allergic individuals versus control subjects. Indeed, mature DCs from allergic donors secreted 1849 ± 4684 pg/mL IL-12 p40 compared with 2068 ± 2859 pg/mL IL-12 p40 pro-

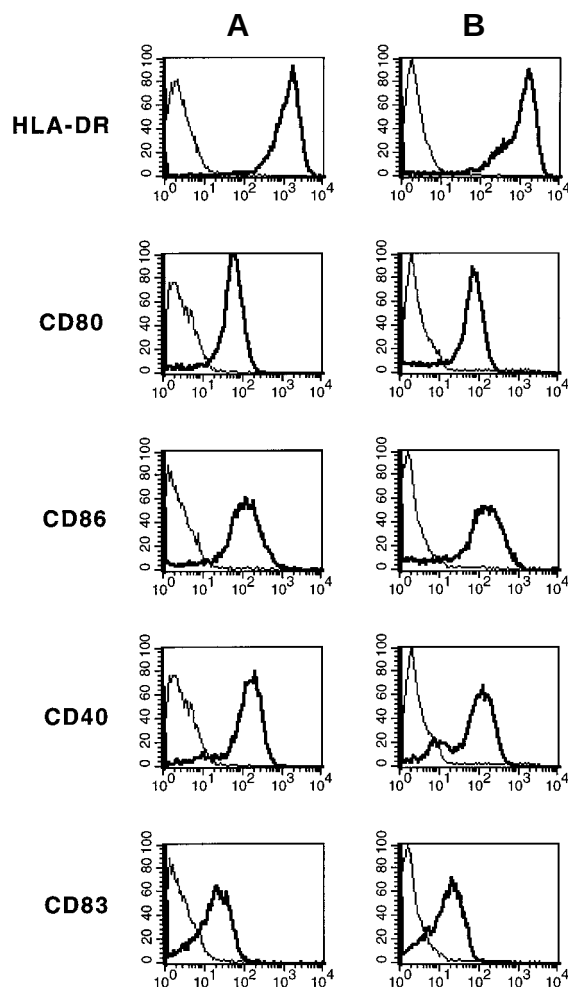


FIG 1. Mature DCs from healthy donors (**A**) and allergic patients (**B**) express similarly high levels of HLA-DR, CD80, CD86, CD40, and CD83 molecules. DCs were generated as described in the "Methods" section, incubated overnight with 1 μ g/mL LPS, and then analyzed by means of flow cytometry. One of 3 representative experiments is shown. Thick lines, FACS profiles after staining with anti-HLA-DR, anti-CD80, anti-CD86, anti-CD40, and anti-CD83 mAb; thin lines, FACS profiles after staining with the isotypic controls.

duced by DCs from control subjects under the same conditions ($P > .3$) and 173 ± 109 pg/mL IL-12 p70 compared with 176 ± 84 pg/mL IL-12 p70 ($P > .4$).

In parallel, we analyzed the surface expression of HLA-DR, CD80, CD86, CD40, and CD83 molecules on mature DCs from allergic and healthy donors. As shown in Fig 1, which is representative of 3 independent experiments on different donors, DCs from healthy donors (Fig 1, A) and allergic patients (Fig 1, B) both expressed similarly high levels of those molecules, which are known to be critical for the antigen-presenting cell function of DCs.¹¹

Therefore in contrast with a previous study,¹² we did not find phenotypic differences between DCs generated from peripheral monocytes of allergic patients and control DCs.

Production of IL-5 and IFN- γ by CD4⁺ T cells stimulated by Der p 1-pulsed DCs

In this series of experiments, CD4⁺ T cells purified from PBMCs of 6 allergic patients and 5 healthy donors were cocultured with autologous DCs pulsed with Der p 1 at a DC/T cell ratio of 1:10. When Der p 1 was presented by immature DCs, only low levels of cytokines were detected in supernatants of cultures prepared with cells of healthy control subjects. Although higher amounts of IL-5 were found in supernatants of cultures prepared with cells from allergic donors (Fig 2), there was no significant difference between the 2 groups ($P > .05$). When Der p 1-pulsed DCs were rendered mature by LPS exposure before being seeded in coculture, CD4⁺ T cells from healthy individuals produced high levels of IFN- γ and low levels of IL-5, which corresponds to a T_{H1}-type response. In comparison with healthy control subjects, CD4⁺ T cells from atopic patients produced significantly lower levels of IFN- γ under the same conditions ($P = .05$), whereas their secretion of IL-5 was much higher ($P = .004$; Fig 2). Interestingly, when immature and mature DCs were compared in atopic individuals, mature DCs were significantly more efficient for the induction of IL-5 production ($P = .03$) but not IFN- γ production (Fig 2). Taken together, these data indicate that Der p 1-specific CD4⁺ T cells from healthy donors display a T_{H1}-type phenotype and require mature DCs to be stimulated, whereas Der p 1-specific CD4⁺ T cells from allergic patients maintain their T_{H2} phenotype whatever the type of DC (ie, immature vs mature) used to present the allergen.

Upregulation of IFN- γ production both in healthy individuals and allergic donors after addition of exogenous rIL-12 to DC-T cell cocultures

We next sought to modify the profile of cytokines secreted by Der p 1-specific T cells by adding high amounts (10 ng/mL) of rIL-12 to cocultures of CD4⁺ T cells with Der p 1-pulsed mature DCs. As shown in Fig 3, the addition of rIL-12 significantly upregulated the production of IFN- γ by Der p 1-specific T cells both in allergic individuals ($P = .03$) and healthy control subjects ($P = .06$). On the other hand, rIL-12 did not affect the hyperproduction of IL-5 by cells of allergic patients, with IL-5 levels in DC-T cell cocultures being 1082 ± 294 pg/mL (mean $\pm$ SEM) and 1036 ± 255 pg/mL in the presence or absence of rIL-12, respectively ($P > .05$).

Production of IFN- γ and IL-5 by distinct T-helper populations on stimulation with Der p 1 and IL-12

Intracytoplasmic staining for IFN- γ and IL-5 was used to determine whether those cytokines were produced by the same subset of Der p 1-specific CD4⁺ T cells in allergic individuals on stimulation in the presence of rIL-12.

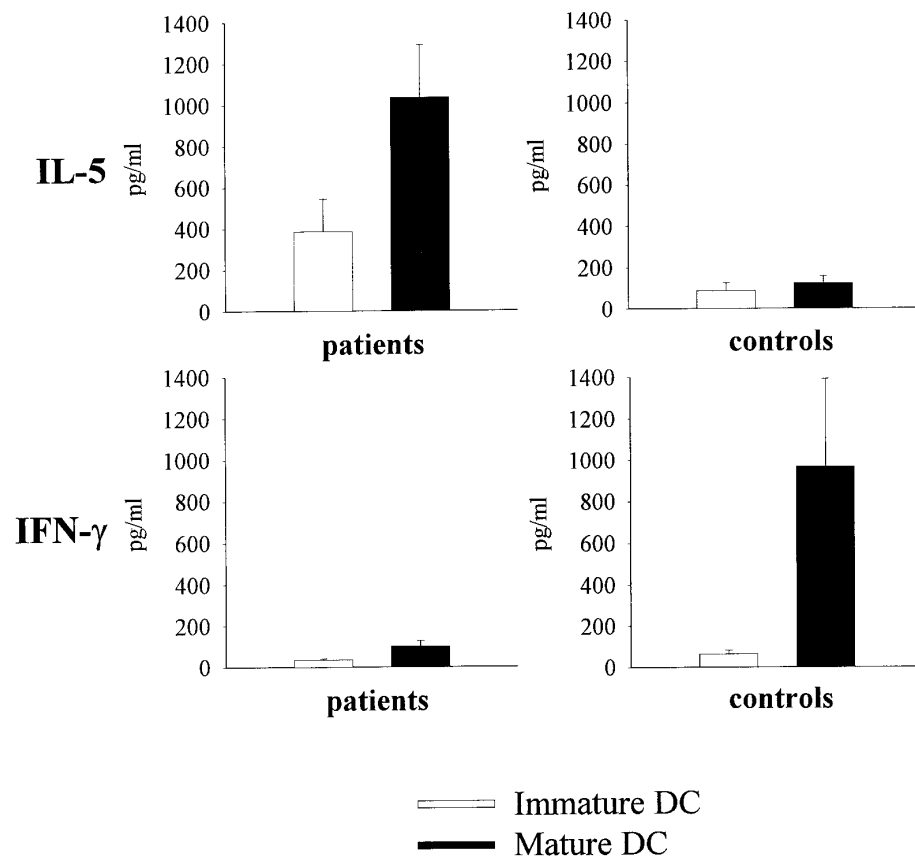


FIG 2. Der p 1-specific CD4⁺ T cells from allergic donors maintain their T_{H2} phenotype whatever the type of DCs used to present the allergen. CD4⁺ T cells purified from 6 allergic patients and 5 healthy donors were cocultured with autologous DCs pulsed with Der p 1 at a DC/T cell ratio of 1:10 for 5 days. Cytokine levels of cytokines in culture supernatants were determined by using ELISA. White bars, Immature DCs; black bars, mature DCs.

In a first set of experiments, we used PBMCs from an allergic donor cultured for 3 days in medium alone or containing Der p 1 in the absence or presence of rIL-12. After a resting period of 3 days in medium alone, cells were briefly stimulated with PMA and ionomycin in the presence of Brefeldine A according to established protocols¹³⁻¹⁵ and then stained for intracytoplasmic CD4, IFN-γ, and IL-5. In the absence of Der p 1, significant numbers of IFN-γ⁺ CD4⁺ T cells were detected, whereas only very few CD4⁺ T cells were positive for IL-5. The effect of rIL-12 in the absence of Der p 1 was to increase the frequency of IFN-γ⁺ CD4⁺ T cells, which most likely represent preactivated T cells of irrelevant specificity (Fig 4, A). Der p 1 stimulation in the absence of rIL-12 did not significantly modify the frequency of IFN-γ⁺ CD4⁺ T cells, which remained at the background level, whereas a clear induction of IL-5-secreting CD4⁺ T cells was observed. When rIL-12 was added during the first incubation period, the frequency of IFN-γ-secreting cells was higher in the presence than in the absence of Der p 1, indicating the appearance of IFN-γ⁺ CD4⁺ T cells secreting IFN-γ in this condition, which is in agreement with the data obtained by using the ELISA technique

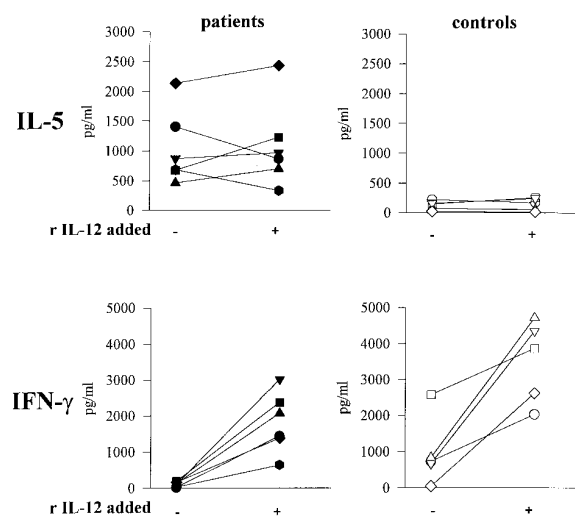


FIG 3. Effects of addition of exogenous rIL-12 to DC-T cell cocultures. rIL-12 (10 ng/mL) was added to cocultures of CD4⁺ T cells with Der p 1-pulsed mature DCs. Cytokine levels in culture supernatants were determined by using ELISA after 5 days of culture. Filled symbols, Allergic patients (n = 6); open symbols, control subjects (n = 5).

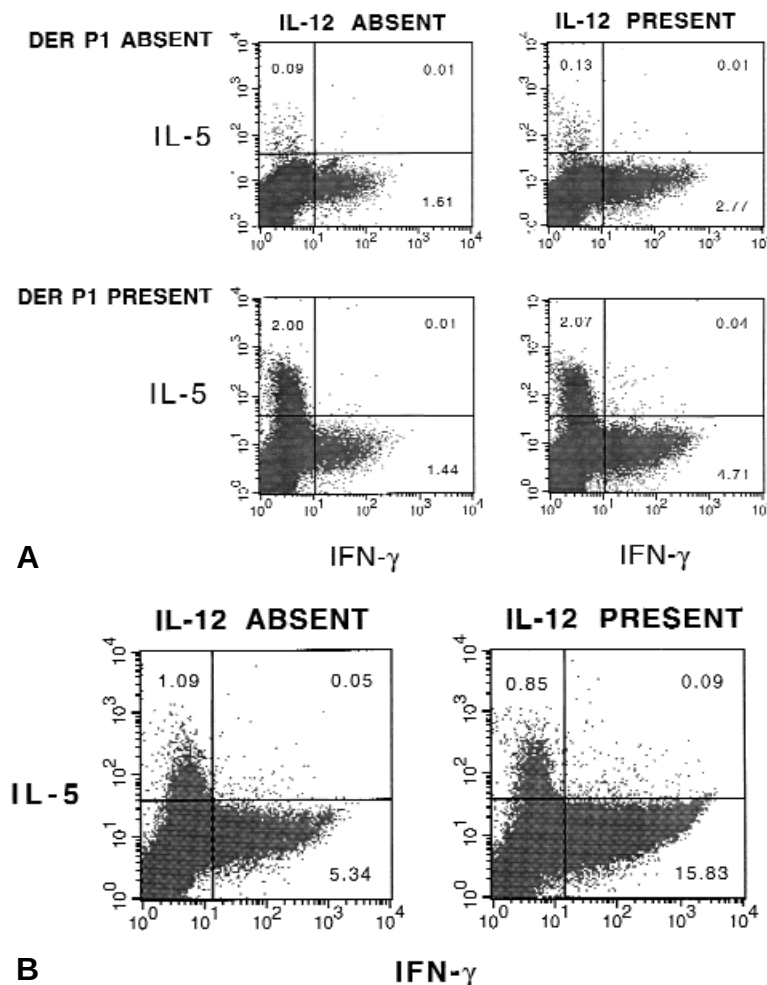


FIG 4. IFN- γ and IL-5 are produced by distinct T helper cell populations on stimulation with Der p 1 and IL-12. **A**, PBMCs from one representative allergic patient were stimulated or not with Der p 1 (5 μ g/mL) for 3 days in the absence or presence of rIL-12. **B**, Purified CD4⁺ T cells were cultured for 7 days with Der p 1-pulsed DCs in the absence or presence of rIL-12. After a resting period of 3 days in fresh medium, cells were incubated with PMA (10 ng/mL) and ionomycin (100 ng/mL) for 5 hours. Brefeldine A was added into the cultures 3 hours before staining for intracytoplasmic CD4, IL-5, and IFN- γ . Plots were obtained after gating on CD4⁺ T cells.

(data not shown). As clearly apparent in Fig 4, A, these IFN- γ ⁺ T cells were negative for IL-5, indicating that Der p 1-specific T cells secreting IFN- γ and Der p 1-specific T cells secreting IL-5 belong to distinct subsets. The same conclusion was reached when we applied this intracytoplasmic cytokine-staining protocol after stimulation of purified CD4⁺ T cells with Der p 1-pulsed mature DCs in the absence or presence of IL-12. As shown in Fig 4, B, IFN- γ and IL-5 were secreted by distinct T-cell subsets under this condition as well.

DISCUSSION

Dendritic cells from the respiratory mucosa are essential for presenting inhaled antigens to previously primed T_{H2} cells and are critical for the subsequent development of asthma.^{16,17} It even seems that those airway antigen-presenting cells possess the inherent property to initiate T_{H2} responses.¹⁸ Our results first show that DCs generat-

ed from human peripheral monocytes are also potent antigen-presenting cells for CD4⁺ T-cell stimulation with the aeroallergen Der p 1, confirming recent data obtained by Sung et al¹⁹ using Der p 2 allergen. When Der p 1 was presented by mature DCs to CD4⁺ T cells from nonallergic donors, high amounts of IFN- γ and low levels of IL-5 were measured in the culture supernatants, which reflects a T_{H1}-type phenotype. On the other hand, very low levels of IFN- γ but high amounts of IL-5 were secreted by Der p 1-specific T cells from allergic donors when stimulated by immature DCs pulsed with Der p 1. This IL-5 production was further increased after activation with fully mature DCs, whereas IFN- γ secretion was not enhanced (Fig 2). Presentation of Der p 1 by mature DCs to allergen-specific T helper cells isolated from atopic patients thus results in an exacerbation of their T_{H2} cytokine profile. Although 2 distinct subsets of human DCs have been recently described as myeloid monocyte-derived DC1, inducing T_{H1} differentiation, and DC2,

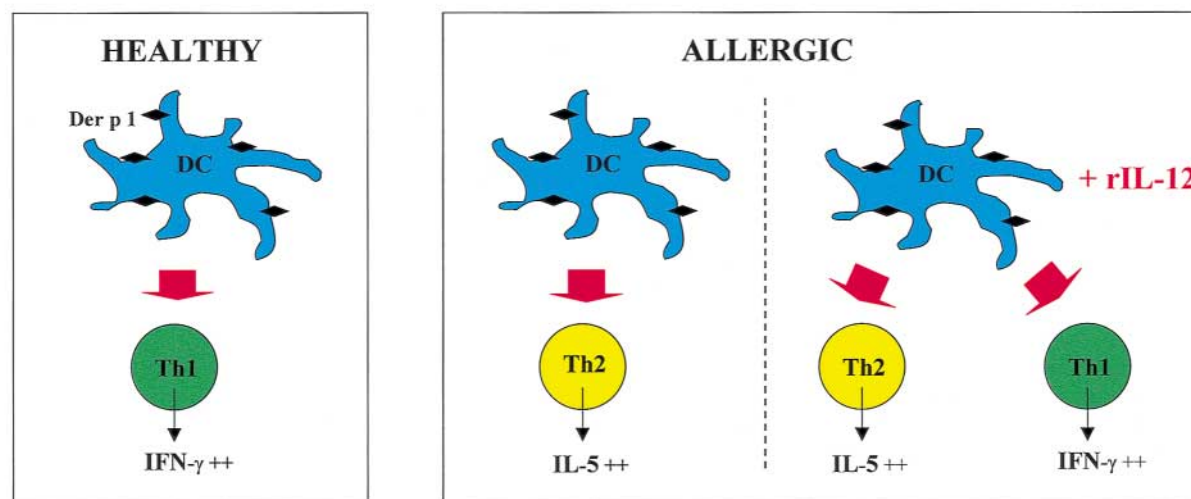


FIG 5. Helper T-cell response is induced by Der p 1-pulsed DCs in healthy versus allergic individuals.

derived from plasmacytoid cells inducing T_{H2} differentiation,²⁰ our observation correlates much more with the concept developed by Kapsenberg et al²¹ that myeloid DCs are flexible and can adopt T_{H1} - or T_{H2} -polarized effector functions.²¹

IL-12 is a major inducer of T_{H1} responses in mice and human subjects.⁴ Moreover, there is some evidence that human T_{H2} -like clones can be induced to secrete IFN- γ in the presence of exogenous rIL-12⁵ and that rIL-12 may be therapeutically beneficial in correcting the T_{H1}/T_{H2} imbalance in allergic asthma.²²⁻²⁷ However, the direct effect of rIL-12 on freshly isolated T_{H2} cells from allergic individuals remains unclear.^{6,7,28,29} Indeed, the reversibility of the cytokine profile of committed T_{H2} cells seems to depend on a number of factors, including the microenvironment in which they develop.³⁰⁻³² Our data show that rIL-12 does not significantly affect the production of IL-5 by Der p 1-specific $CD4^+$ T cells from atopic patients but induces IFN- γ production by another subset of Der p 1-specific $CD4^+$ T cells. Our interpretation is that rIL-12 is unable to induce IFN- γ by polarized memory, Der p 1-specific, T_{H2} -type cells, perhaps because these cells have lost the expression of functional IL-12 receptors.³² We suggest, as shown in Fig 5, that the other T-cell subset that produces IFN- γ in the presence of rIL-12 could correspond to recently activated Der p 1-specific $CD4^+$ T cells present in the peripheral blood of allergic patients.

The main conclusion of this study is that antigen-presenting cells known to promote T_{H1} responses, such as mature DCs, do not affect the overproduction of IL-5 by Der p 1-specific $CD4^+$ T cells from allergic donors, even in the presence of a potent T_{H1} -polarizing factor, such as rIL-12. Although it remains possible that increasing IFN- γ production in atopic patients might be beneficial by decreasing IL-13 synthesis,³³ our data suggest that immune deviation strategies with DCs and exogenous cytokines might be limited by the fixed phenotype of allergen-specific T_{H2} cells.

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