Dendritic cells contribute to the development of atopy by an insufficiency in IL-12 production

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Background: IL-12 is a crucial factor in the development and course of allergic diseases. By virtue of their IL-12 production, dendritic cells (DCs) are potent inducers of T_H1 responses. However, distinct subsets of DCs have also been shown to induce T_H2 differentiation.
Objective: We hypothesized that DCs from atopic and nonatopic individuals might differ in their propensity to skew T-cell responses to either the T_H1 type or the T_H2 type. To this end, we investigated the cytokine patterns produced by DCs from atopic and nonatopic individuals, and we attempted to clarify whether this could be due to different DC lineages or, alternatively, to different microenvironmental factors.
Methods: DCs were generated from lymphocyte-depleted PBMCs from atopic and nonatopic donors and fully matured with monocyte-conditioned medium. Production of IL-4, IL-5, IL-10, IL-12, and IL-13 in response to CD40 ligation was measured with ELISA. DC subsets were identified in PBMCs from freshly drawn blood by 3-color flow cytometry.
Results: Compared with DCs from healthy donors, monocyte-derived DCs from atopic patients produced less bioactive IL-12 and IL-10. DC production of IL-4, IL-13, and IL-5 was not detected. Relatively more $CD123^+$ DCs, corresponding to T_H2-inducing "DC2s," were found in PBMCs from atopic patients.
Conclusion: The data suggest that in addition to the described abnormalities in the patients' T-cell populations, DCs might also critically contribute to the atopic/allergic T_H1 outcome in the patient and thus to the disease. (J Allergy Clin Immunol 2002;109:89-95.)

Key words: *Dendritic cells, T_H1/T_H2, IL-12, IL-10, atopy, CD123, IL-4, IL-5, IL-13*

Allergic diseases such as atopic dermatitis and allergic asthma are characterized by an imbalance in the T_H1/T_H2 cell system, a T_H2 cell cytokine pattern being favored.[1] T_H2 cells are characterized by their production of IL-4, IL-5, IL-10 and IL-13 and thus induce IgE-dependent, immediate-type hypersensitivity reactions and eosinophilia. In atopic individuals, T_H2 cells are preferentially generated during sensitization against an allergen.

Abbreviations used
DC: Dendritic cell
MCM: Monocyte-conditioned medium

IL-12 is primarily produced by antigen-presenting cells—in particular, by dendritic cells (DCs). It is a key cytokine for the differentiation of naive $CD4^+$ cells toward a T_H1-cytokine pattern.[2] Several in vivo studies have documented its crucial role in the development and course of atopic disorders. Hamid et al[3] performed intradermal grass pollen tests in patients after 4 years of specific immunotherapy, measured the late response, and performed biopsies 24 hours later. In comparison with grass-pollen allergic subjects without immunotherapy, a significant increase in allergen-induced IL-12 mRNA could be found in the biopsy specimens of the treatment group. This correlated positively with IFN-γ and negatively with IL-4 mRNA levels. A recent study by the same group[4] showed that IL-5 production from PBMCs and from T cells collected from bronchoalveolar lavage fluid in allergic asthmatic subjects could be inhibited by IL-12 and IFN-γ, indicating that human T cells obtained from bronchoalveolar lavage fluid during the asthmatic late response are still susceptible to IL-12. These data raised hopes of the utilization of IL-12 as a potential therapeutic tool.

DCs are potent antigen-presenting cells specialized to initiate primary immune responses in naive T and B lymphocytes,[5] and they play a key role in determining the types of these responses.[6,7] They have repeatedly been shown to produce IL-12 in large amounts when stimulated by (1) bacteria or bacterial products,[8,9] (2) virus,[10] or (3) ligation of their CD40 and/or MHC class II molecules.[8,11] Furthermore, recent studies[12-14] have shown that the capacity of DCs to induce IL-12 production critically depends on their state of maturation and that IL-12 is predominantly produced at the onset of this process. This aspect is important because it is with respect to the mature, T cell–activating DCs that IL-12 would presumably be most relevant for the generation of specific T_H1 immunity. On the other hand, there is accumulating evidence that distinct populations of DCs make markedly less IL-12 and can therefore skew T-cell responses to a T_H2 pattern.[7]

Accordingly, the present study aimed at comparatively investigating the patterns of those cytokines that are relevant to the T_H1/T_H2 decision-making and that are

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produced by DCs from atopic and nonatopic individuals—most importantly, IL-12. We also attempted to clarify whether this could be due to different DC lineages or, alternatively, to different microenvironmental factors.

PATIENTS, MATERIALS, AND METHODS

Those in the study population were recruited from patients of the allergy unit and the clinical staff. Consent was given by all subjects.

Atopy was defined as allergic rhinoconjunctivitis (due to pollen, house dust mites, animal dander, and so forth) or atopic dermatitis. As a screening procedure, skin prick testing with a panel of routine inhalative allergens was performed through use of commercially available extracts (Alk Abello, Copenhagen, Denmark); these included tree mix, grass mix, mugwort, horse epithelia, dog epithelia, cat epithelia, cow epithelia, and house dust mite. All skin prick tests were carried out on the volar surface of the forearm. Normal saline solution and histamine hydrochloride (10 mg/mL) were used as negative and positive controls, respectively. Wheals with diameters measuring at least one half that of histamine and 3 mm or larger were regarded as positive, according to European Academy of Allergology and Clinical Immunology guidelines.[15] All sites were read after 20 minutes.

Total serum IgE was determined through use of the standard RAST technique (AutoCAP system, Pharmacia, Uppsala, Sweden). Results exceeding 100 kU/L were considered clearly elevated.

Media and reagents

The cell culture medium used throughout was RPMI-1640 supplemented with 0.1% gentamycin (all from PAA, Linz, Austria), 1% autologous plasma, and 200 mmol/L glutamine (Sebak GmbH, Suben, Austria), as described elsewhere.[16] Medium for CD40 ligand–transfected cells (TBA cells) was RPMI-1640 supplemented with 10% FCS, 0.1% gentamycin, 200 mmol/L glutamine, 1% MEM (100×) Non-essential Amino Acids (GIBCO BRL, Paisley, Scotland), and 1% HEPES (Biochrom KG, Berlin, Germany).

Generation of DCs

DCs were generated from lymphocyte-depleted PBMCs according to established standard procedures.[16] Blood cells were from freshly drawn heparinized blood. Briefly, an initial 7-day "priming culture" was carried out in the presence of GM-CSF (800 U/mL) and IL-4 (1000 U/mL). GM-CSF was from Novartis (Basel, Switzerland; Leukomax; specific activity, 1.1×10^6 U/mg), and IL-4 was from Genzyme (Cambridge, Mass; specific activity 5×10^7 U/mg). Cells were fed with fresh medium every other day. Populations of immature DCs were split in half on day 7 and cultured for 3 more days ("differentiation culture") in the presence or absence of 25% to 33% v/v of monocyte-conditioned medium (MCM).[16] Because the quality of MCM with regard to its capacity to render DCs terminally mature is somewhat variable from donor to donor, we supplemented it with TNF-α (10 ng/mL) and PGE2 (Prostin E, Pharmacia-Upjohn, Uppsala, Sweden; 1μg/mL).[17] This ensured a stable and reproducible quality as monitored by the high levels of CD83 expression. TNF-α was generously provided by Dr G. R. Adolf (Bender, Vienna, Austria; specific activity, 6×10^7 U/mg). GM-CSF and IL-4 were still present during this period. On day 10, cells were collected and immature DCs (ie, those cultured without MCM) and mature DCs (ie, those cultured with MCM) were analyzed for IL-4, IL-5, IL-10, IL-12, and IL-13 production.

Stimuli to induce and modulate cytokine production in DCs

Murine myeloma cells transfected with the human CD154/CD40-ligand molecule (P3xTBA7 cells) were used to ligate the CD40 molecule on the surface of DCs.[18] Wild-type cells served as negative controls (P3x63Ag8.653-WT). These cells were a kind gift of Dr R. A. Kroczek, Robert-Koch-Institut, Berlin, Germany. Maximal IL-12 release by DCs was achieved at a ratio of 1 viable transfectant cell to 2 DCs, as described elsewhere.[12]

Determination of cytokine production

Immature or mature DCs were washed out (3 ×) of cytokine-containing culture media. They were counted under the hemocytometer and analyzed for CD83 expression by flow cytometry; 1×10^6 DCs per milliliter were plated into 24-well or 48-well multiwell tissue culture plates in total volumes of 1 mL and 0.5 mL culture medium, respectively. FITC-conjugated anti-CD83 was from Beckman-Coulter-Immunotech, Marseille, France. Supernatants were taken at 48 hours and stored at –80°C until analysis with ELISA. For determination of IL-12, we used a sandwich ELISA from BD-Pharmingen (San Diego, Calif) containing capture mAb 20C2 that specifically recognizes the p70 heterodimer but not the free p40 chains.[19] Detection limits were 20 pg/mL of IL-12. IL-10, IL-4, and IL-5 were measured with commercial ELISAs (BioSource-Medgenix, Fleurus, Belgium). The IL-13 ELISA was also purchased from BD-Pharmingen.

Identification of DC subsets

PBMCs isolated from freshly drawn blood were analyzed through use of 3-color FACS with mAbs from BD Immunocytometry Systems (San Diego, Calif) ("DC bundle"). $CD123^+$ DCs were identified from their lack of lineage markers and their high levels of HLA-DR. Monocytes, lymphocytes, and natural killer cells were excluded by their lineage markers with help of an antibody cocktail containing anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-56. Basophilic granulocytes were identified by their distinct $CD123^+$/HLA-DR^- phenotype.

Statistical evaluation

Levels of IL-4, IL-5, IL-10, IL-12, and IL-13 in DCs of atopic and nonatopic donors were compared through use of the Mann-Whitney *U* test. Because values were not distributed in a Gaussian fashion (as determined through use of the Kolmogorov-SmirnovTest), this test was appropriate. *P* values less than .05 were considered statistically significant. Data are presented as "box-and-whisker" plots. The line inside each box indicates the median value. The box delimits the interquartile range—ie, one fourth of all of the values lie under the lower end of the box, and one fourth of all of the values lie above the upper end. The so-called "whiskers" end at the minimum and maximum values.

RESULTS

Characteristics of the study population

A total of 33 subjects were recruited (16 women and 17 men; mean age, 31.4 years; age range, 20-49 years). Of these, 22 were classified as atopic; the other 11 served as healthy controls. The 2 groups did not differ significantly with regard to age or sex. Every patient with a diagnosis of atopic dermatitis also showed a sensitization to 1 or more inhalative allergens. Total IgE levels were equally distributed between the various atopic diseases.

General properties of monocyte-derived DCs of atopic patients

DC yields after the 10-day culture were equal for patients and healthy controls. The immature and mature DCs were also indistinguishable with respect to morphology under phase-contrast microscopy. Finally, in

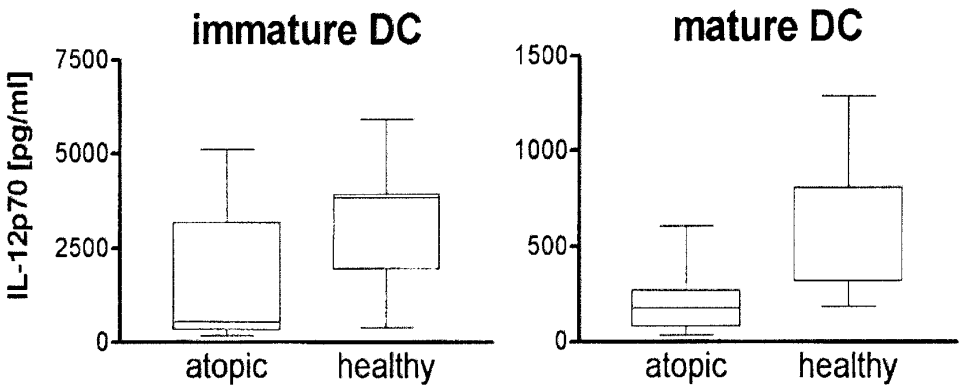

FIG 1. Monocyte-derived DCs from atopic patients produce less bioactive IL-12. Box-and whisker plots of IL-12 secreted by immature *(left panel)* and mature *(right panel)* DCs from patients (n = 19) and healthy donors (n = 10) are presented. The medians are indicated by the lines inside the boxes; the median for "mature DC/healthy" is at the very top of its box. Each box delimits the inner 50% of all values. The so-called "whiskers" end at the minimum and maximum values. Note that scaling of the y-axis is different in the 2 panels. Differences in IL-12 secretion between DCs from healthy and atopic donors were statistically significant ($P < .05$ for immature DCs and $P < .005$ for mature DCs).

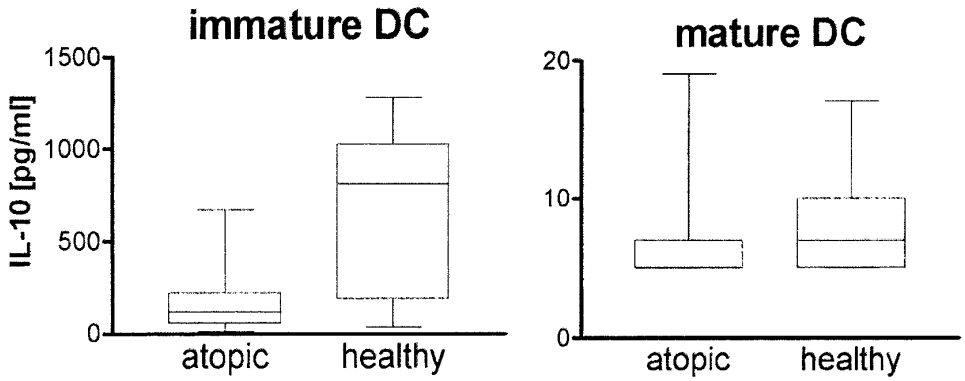

FIG 2. Monocyte-derived DCs from atopic patients produce less bioactive IL-10. Box-and whisker plots of IL-10 produced by immature *(left panel)* and mature *(right panel)* DCs from atopic patients (n = 19) and healthy controls (n = 11) are presented. The median for "mature DC/atopic" is at the very bottom of the box. Note that scaling of the y-axis is different in the 2 panels, emphasizing that mature DCs make much less IL-10 than immature DCs. Differences in IL-10 secretion between DCs from healthy and atopic donors were statistically significant for immature DCs ($P < .05$); for mature DCs, they did not reach statistical significance.

agreement with Bellinghausen et al,[20] no phenotypic differences became apparent with regard to the expression of CD86 and CD83, which were routinely monitored for assessment of DC maturation.

Monocyte-derived DCs of atopic patients produce less bioactive IL-12

Immature and mature DCs from atopic subjects and normal controls were compared for the ability to secrete IL-12 p70 heterodimer in response to CD40 ligation. Unstimulated DCs—ie, in the absence of CD40 ligand-expressing transfectants—secreted no detectable amounts of IL-12 in most cases. ELISA data showed that immature DCs produced substantially more IL-12 than mature DCs, as described previously for healthy donors[12,13] but not for atopic patients, in whom the differences were also highly significant ($P < .0001$). Importantly, both immature and mature DCs from atopic patients secreted significantly less IL-12 than their counterparts obtained from healthy donors (Fig 1). For immature cells, the median amount of IL-12 produced was 544 pg/mL for atopic patients (n = 19); this compared with 3880 pg/mL for healthy controls (n = 10; $P = .034$). For mature DCs, the corresponding values were 195 pg/mL for atopic patients and 811 pg/mL for healthy controls ($P = .0011$).

Monocyte-derived DCs of atopic patients produce less IL-10

DCs from both controls and atopic patients secreted measurable amounts of IL-10 only in response to CD40 ligation. As was the case with IL-12, IL-10 was made in much higher quantities by immature DCs than by terminally mature cells. Surprisingly, DCs from patients (n = 19) secreted less IL-10 than DCs from controls (n = 11). The values were as follows: for immature DCs, 145 pg/mL in patients versus 791 pg/mL in controls ($P = .017$); for mature DCs, 5 pg/mL in patients versus 7 pg/mL in healthy controls (the difference not being significant; Fig 2).

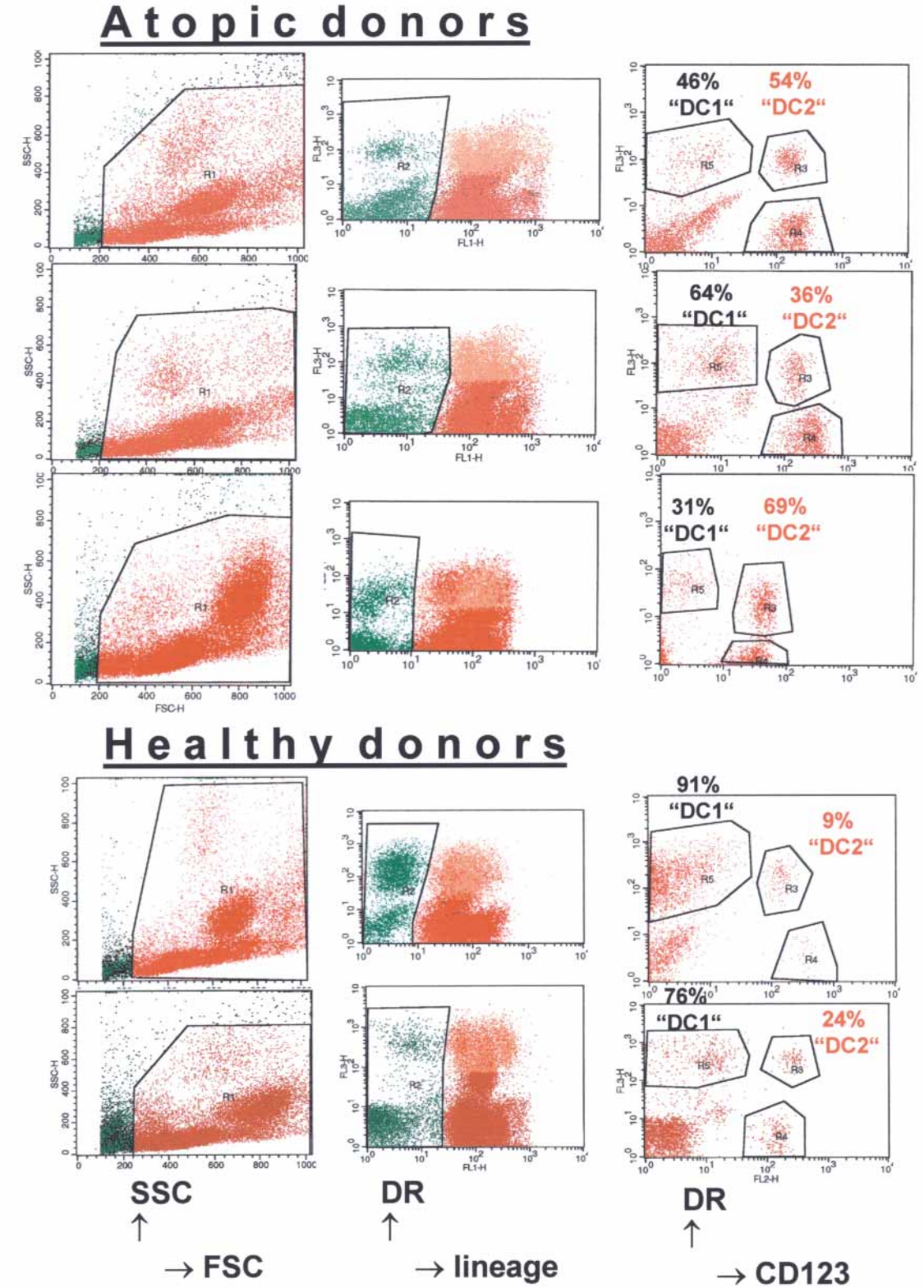

FIG 3. CD123 expression on DCs from fresh blood. PBMCs were obtained by Lymphoprep centrifugation and stained with a cocktail of antibodies. Examples from 3 atopic patients *(top rows)* and 2 healthy control subjects *(bottom rows)* are presented. In a first step, viable cells were selected for further analysis on the basis of light scattering properties *(left panels; green dots)*. In a second step, cells expressing lineage markers (ie, T cells, B cells, NK cells, and monocytes) were excluded from further analysis *(middle panels; red dots)*. Among the remaining lineage-negative cells, DCs were defined as HLA-DR$^+$ cells in a third step *(right panels)*. DCs were further subdivided according to their expression of CD123 (x-axes of right panels). HLA-DR$^+$ cells (ie, all DCs) were set equal to 100%, and the percentages of CD123$^-$ ("DC1") and CD123$^+$ (atopy-promoting "DC2") DCs are indicated. Note that the relative proportions of CD123$^+$ DCs appear larger in the atopic patients.

Monocyte-derived DCs do not produce IL-4, IL-5, or IL-13

Immature and mature DCs from both atopic and healthy donors were compared for the ability to secrete cytokines that drive T-helper responses toward a T_H2 pattern. Of IL-4, IL-5, and IL-13, none could be detected in supernatants of DCs either in response to CD40 ligation or in the absence of such a stimulus.

Atopic patients have relatively more circulating "DC2s" than "DC1s"

PBMC populations were analyzed for the presence of DCs by 3-color flow cytometry. There were no significant differences in the absolute numbers of DCs per microliter of blood. Similarly, the absolute numbers of DC subtypes (ie, $CD123^+$ ["DC2s"] and $CD123^-$ ["DC1s"]) appeared not to be grossly different. When we compared the proportions of DC subsets, more "DC1s" than "DC2s" were present in most samples; this was true for both patients and normal controls. However, samples from atopic donors contained proportionally even fewer "DC1s" (Fig 3). Whereas healthy controls had approximately 3 times more "DC1s," atopic patients had only twice as many ($CD123^-$:$CD123^+$ ratio = 3.11 ± 2.87, range = 1.07-11.40, n = 12 for healthy controls; $CD123^-$:$CD123^+$ ratio = 2.16 ± 1.20, range = 0.44-3.56, n = 17 for atopic patients). These values were not significant, however.

DISCUSSION

Role of DCs in the development of atopic disease

Several scenarios are conceivable in the decision-making for the differing immune responses seen in atopic and nonatopic subjects; dysregulation in the production of IL-4 and dysregulation in the susceptibility to IL-4 of T cells have been described.[21] On the other hand, it can be assumed that DCs from atopic and nonatopic patients differ with regard to their preferences to induce T_H1 or T_H2 immune responses. Kalinski et al[22] have shown that IL-12–deficient DCs from normal donors promote a T_H2 cytokine pattern. Another report[23] described a reduced IL-12 and IFN-γ release in whole blood cultures from patients with allergic asthma. DCs were not specifically looked at in that study. Here, we specifically investigated monocyte-derived DCs from atopic individuals, and our data indicate that these DCs produce less IL-12 than their counterparts from healthy controls. This, in turn, could lead to a relative imbalance in the T_H1/T_H2 system, favoring the induction of a T_H2 cytokine pattern. In keeping with this hypothesis, a recently published study[20] has demonstrated an enhanced production of IL-4, IL-5, and IL-10 in T-helper cells from atopic donors after stimulation with allergen-pulsed autologous DCs.

Bellinghausen et al[20] measured IL-12 p70 secreted by DCs that had been fed allergens during the time of maturation but in the absence of a T cell–related stimulus. No differences between healthy and atopic subjects became evident. We have tested the IL-12 secretion of DCs in response to CD40 ligation, a stimulus that mimics T-cell contact and that might be relevant for the in vivo situation in which an allergen-laden DC arrives in the lymph node and "decides" whether to induce a T_H1 or a T_H2 allergen-specific T-cell response. Importantly, in response to this T cell–related stimulus, DCs of atopic patients make less IL-12. Thus it appears that the data of the 2 studies do not contradict each other but rather complement each other.

Constitutive IL-12 production by functionally different subpopulations of DCs

One explanation for the reduced IL-12 production by DCs of atopic patients might be an altered balance of different DC subsets. Functionally different DC lineages, each with a high or low IL-12–producing capacity, have been described recently. In situ, expression of the IL-3 receptor α chain (CD123) was identified as a specific marker of a subclass of DCs in lymphoid organs.[24] These $CD123^+$ cells were shown to make little IL-12 and to skew T-helper cells toward a T_H2 pattern.[25] We therefore wondered whether atopic patients might have constitutively higher proportions of $CD123^+$ DCs ("DC2s") than healthy controls. Although our data did not reach statistical significance, they support the presumption that indeed, in atopic individuals, the relatively higher numbers of "DC2s" that present the allergen to the T cells might lead to the development of a T_H2 cytokine pattern.

An alternative or additional explanation for the diminished IL-12 production by DCs might be a genetic one. The p40 component of IL-12 is located on chromosome 5,[26] which is also linked to total serum IgE concentrations[27] and bronchial hyperresponsiveness.[28] This has not been addressed experimentally hitherto.

Influence of the local microenvironment on the IL-12 production of DCs

Myeloid DCs have been viewed as T_H1-promoting antigen-presenting cells in several studies.[29] In a recent report[30] it was shown that neither a T_H1– nor a T_H2–inducing capacity is an intrinsic feature of myeloid DCs; rather, both can be aquired by immature DCs in response to signals from the local microenvironment. IFN-γ was found to stably induce polarized DCs with enhanced IL-12–producing capacity. A lack of IFN-γ in the early phase of maturation would thus favor a T_H2 response. Another explanation would be a preponderance of IL-4 at this time. In the light of recent data this seems less likely, however. It has been shown[12,31,32] that though IL-4 acts as an inhibitor of LPS-induced IL-12 production, it potently enhances the production of bioactive IL-12 p70 in CD40 ligand–stimulated DCs. T_H2 cells and mast cells have been described as main sources of IL-4. We show here that neither immature nor mature DCs secrete IL-4; this is irrespective of their origin from healthy donors or atopic patients and irrespective of their stimulation by ligation of CD40.

IL-10 and DC-derived IL-12

IL-10 can inhibit proinflammatory cytokines such as IL-12.[12] We therefore expected an enhanced secretion of IL-10 in the patient group, as previously shown for monocytes.[33] Surprisingly, DCs from patients produced not more but less IL-10. This emphasizes the profound cellular changes that occur when a monocyte develops into a DC. Our data are in line with the findings of Van der Pouw Kraan et al,[23] who observed only little IL-10 secretion after stimulation of whole blood cultures with *Staphylococcus aureus* Cowan I strain. In our experiments, IL-10 production by mature DCs was low and did not significantly differ for healthy controls and atopic individuals. It is the very mature DC, however, that induces and skews the T-cell response in the lymph node. Therefore, it seems unlikely that DC-derived IL-10 contributes to the observed diminished production of IL-12 or the development of T_H2 responses.

Clinical relevance

Our data suggest that DCs might be a feasible target for immunotherapies. One might envisage scenarios wherein autologous allergen-pulsed DCs are administered to atopic patients to elicit potent T_H1 responses that would counteract the prevailing T_H2 responses. Recent in vitro data show that this might induce IFN-γ without affecting IL-5 production in T cells of atopic patients.[34] Moreover, our findings support encouraging results from murine models indicating that antigen-induced airway hyperresponsiveness can be blocked by IL-12.[35,36] Local or systemic administration of IL-12 could thus provide a novel method of immunotherapy. It is important to note, however, that great care must be taken when IL-12 and/or DCs are targeted as a therapeutic tool, inasmuch as cytokines (eg, the novel IL-23[37]) and cells other than those implied by the "T_H2 hypothesis" are involved in the regulation of allergic inflammation. Variable cytokine levels might be due to variable allergen exposure or to different stages of the allergic march. The combination and proportions of cytokines and their receptors might thus be more decisive than their absolute levels. Moreover, according to the "connectionist theory" of Hyland,[38] allergic inflammation is explained not by linear causality but by alterations of multiple circuits within a network. It remains to be determined how the goal of a global understanding of atopy can best be achieved.

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