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J Immunol 2005; 174:2396-2403; doi: 10.4049/jimmunol.174.4.2396
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Compartmental Imbalance and Aberrant Immune Function of Blood CD123⁺ (Plasmacytoid) and CD11c⁺ (Myeloid) Dendritic Cells in Atopic Dermatitis

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Atopic dermatitis (AD) is a pruritic, chronically relapsing skin disease in which Th2 cells play a crucial role in cutaneous and extracutaneous immune reactions. In humans, CD11c⁺CD123⁻ myeloid dendritic cells (mDC) and CD11c⁻CD123⁺ plasmacytoid DC (pDC) orchestrate the decision-making process in innate and acquired immunity. Since the number and function of these blood dendritic cell (DC) subsets reportedly reflect the host immune status, we studied the involvement of the DC subsets in the pathogenesis of AD. Patients with AD had an increased DC number and a low mDC:pDC ratio with pDC outnumbering mDC in the peripheral blood compared with normal subjects and psoriasis patients (a Th1 disease model group). The mDC:pDC ratio was correlated with the total serum IgE level, the ratio of IFN-γ-producing blood cells:IL-4-producing blood cells, and the disease severity. In vitro allogeneic stimulation of naive CD4⁺ cells with atopic DC showed that the ability of pDC for Th1 induction was superior or comparable to that of mDC. In skin lesions, pDC infiltration was in close association with blood vessels expressing peripheral neural addressins. Therefore, compartmental imbalance and aberrant immune function of the blood DC subsets may deviate the Th1/Th2 differentiation and thus induce protracted allergic responses in AD. The Journal of Immunology, 2005, 174: 2396–2403.

Materials and Methods

Patients

In total, 51 AD patients, 10 psoriasis vulgaris patients, and 17 nonallergic healthy individuals were enrolled in this study. The AD patients were diagnosed on the basis of the criteria of Hanifin and Rajka (14) and included 34 men and 17 women ranging in age from 12 to 45 years (mean ± SD, 25 ± 7 years). The severity of the skin lesions was assessed according to the scoring index of atopic dermatitis (SCORAD) method (minimum point, 0; maximum point, 103) (17) and ranged from 12 to 84 (mean ± SD, 49 ± 22). The total serum IgE level varied from 64 to 70,400 IU/ml (mean ± SD, 10,017 ± 14,120). Since all patients, except one (64 IU/ml), showed total serum IgE levels above normal levels, it was suggested that the AD group represented an extrinsic type of AD (18). The patients had received treatment with a topical corticosteroid (5–10 g/week) and/or tacrolimus (<5 g/week) ointments, and, if necessary, with oral antihistamines. Psoriasis was chosen as a Th1 disease control (19). The psoriasis patients were diagnosed on the basis of the clinical and histological features and included 8 men and 2 women ranging in age from 32 to 43 years (mean ± SD, 32 ± 4 years). The disease severity in the patients was evaluated as “mild” by the psoriasis area and severity index score (minimum point, 0; maximum point, 97) (20) and ranged from 30 to 40. The patients were treated with a topical corticosteroid (5–10 g/week) and/or calcipotriene ointment (<10 g/week). None of the patients had ever received treatment with a topical corticosteroid (5–10 g/week) and/or tacrolimus (<5 g/week) ointments, and, if necessary, with oral antihistamines.
taken oral corticosteroids. The normal nonatopic individuals with normal levels of total serum IgE (37 ± 25 IU/ml; range, 0–189) included 11 men and 6 women ranging in age from 23 to 40 years (mean ± SD, 30 ± 5 years). Blood samples were obtained from the patients after discontinuation of the treatment for at least 3 days. Informed consent was obtained from all of the participants, and the study was approved by the ethical committee of the Hamamatsu University School of Medicine.

**Antibodies**

A mixture of FITC-conjugated anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56 (anti-lin) mAbs, PE-conjugated anti-CD11c and anti-CD123 mAbs, PerCP- and FITC-conjugated anti-HLA-DR mAb, and PE-conjugated anti-CD45RA mAb were obtained from BD Biosciences. A FITC-conjugated mAb against chemokine receptor CCR4 was a kind gift from Kyowa Hakko Kogyo (Tokyo, Japan). FITC- and PE-conjugated mAbs against CXCRI3, IFN-γ, and IL-4 were purchased from BD Pharmingen. A purified rat anti-mouse FNAld carbohydrate epitope (CD62L ligand) mAb was obtained from BD Pharmingen.

**Enumeration of Blood DC**

Sensitive detection of DC was achieved by exclusion of cells positive for CD3, CD14, CD16, CD19, CD20, or CD56. Briefly, peripheral blood was drawn into 0.2% EDTA-containing Vacutainer tubes, and aliquots (100 μl) were subjected to three-color flow cytometry. Cells were incubated with a mixture of anti-lin mAbs, a PerCP-conjugated anti-HLA-DR mAb, and either a PE-conjugated anti-CD11c mAb to detect mDC or a PE-conjugated anti-CD123 mAb to detect pDC in the dark at room temperature for 30 min, followed by the addition of lysis buffer to remove the RBC. After washing once with PBS containing 0.5% FCS, 0.1% sodium azide, and 1% paraformaldehyde, >5 × 10^5 cells/sample were analyzed on a FACSCalibur flow cytometer (BD Biosciences). The results were analyzed using the FlowJo software (TreeStar). The number of DC was expressed as the percentage of the total leukocytes. The ratio of mDC:pDC was calculated as follows: mDC/pDC = absolute number of mDC × 100/absolute number of pDC.

**Intracytoplasmic cytokine staining**

PBMC were obtained by Ficoll-Hypaque centrifugation and cultured at 10^6 cells/ml in 24-well culture plates in complete RPMI 1640 containing 5% autologous plasma and antibiotics in the presence of 1 μg/ml PMA and 0.1 μg/ml calcium ionophore for 24 h. One microliter of GolgiPlug (Cytotox/ Cytoperm Plus kit; BD Pharmingen) was added to each well during the last 6 h of culture according to the manufacturer’s protocol. The cells were then reacted with PBS containing 0.1% saponin, and stained with FITC-conjugated anti-human IFN-γ mAb and PE-conjugated anti-human IL-4 mAb for 30 min. Flow cytometric analysis was performed as above. The ratio of Th1:Th2 was expressed as the number of intracytoplasmic IFN-γ^+^ cells × 100/number of intracytoplasmic IL-4^+^ cells.

**Enrichment of mDC and pDC**

mDC and pDC were enriched from PBMC using BDCA-1 and BDCA-2 cell isolation kits (Miltenyi Biotec), respectively, according to the manufacturer’s protocols. Both mDC and pDC were estimated to represent >80% of the cells in the enriched fractions by flow cytometric analysis.

**Enrichment of naive CD4^+^ CD45RO^-^ T cells**

PBMC from normal individuals were subjected to negative selection using a CD4^+^ double sort kit and CD45RO Microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. The purity was >98% and the cells were used as naive CD4^+^ lymphocytes.

**In vitro cytokine production of MLR-stimulated CD4^+^ cells**

DC fractions from AD patients or normal individuals were treated with 50 μg/ml mitomycin C for 45 min and washed extensively. Allogeneic naive CD4^+^ lymphocytes (5 × 10^5 cells/ml) from normal individuals were cocultured with either mDC or pDC (3 × 10^5 cells/ml) in 24-well plates for 4 days, followed by expansion induced by the addition of 20 U/ml IL-2 for 8 days. The expanded cells were then restimulated with PMA (1 μg/ml) and ionomycin (0.1 μg/ml) for 12 h and subjected to measurements of either the IL-4 and IFN-γ concentrations or the surface expression of CXCRI3 and CCR4 using the corresponding fluoresceinated mAbs. The disparity of MHC class I between DC and CD4^+^ cell donors was always confirmed by the Terasaki method at each MLR.

**Multicolor immunofluorescent staining of skin biopsies**

Punch-biopsy skin specimens were snap frozen in tissue-embedding medium and processed for triple immunofluorescent staining. Skin sections were incubated with a mixture of rat anti-PNAd, PE-conjugated anti-CD123 mAb, and either FITC-conjugated anti-HLA-DR or FITC-conjugated anti-CD45RA mAbs and then reacted with 7-amino-4-methylcoumarin-3-acetic acid-conjugated F(ab’)_2 of goat anti-rat IgG (Jackson ImmunoResearch Laboratory). Each procedure was performed in the dark at room temperature for 30 min, followed by extensive washing. The specimens were observed under a fluorescence microscope with filters for excitation at 360, 490, and 540 nm. Negative controls were obtained by omission of the primary Abs.

**Statistical analysis**

Statistical significance (p < 0.05) was analyzed by the Student t test and Mann-Whitney U test. Associations between the mDC:pDC ratio and allergic parameters were assessed by Spearman’s rank test as a nonparametric analysis, with the allergic parameters as dependent variables.

**Results**

**Enumeration of blood DC subsets in AD patients, psoriasis patients, and normal individuals**

The absolute number of blood DC and the DC population among the total leukocytes were 34.0 ± 22/ml and 0.53 ± 0.29% in AD patients, 14.1 ± 7.5/ml and 0.24 ± 0.12% in psoriasis patients, and 17.8 ± 9.0/ml and 0.36 ± 0.18% in normal subjects, respectively. Therefore, the blood DC levels in AD patients were significantly higher than those in psoriasis patients (p < 0.008) and normal individuals (p < 0.0002).

The percentages of mDC and pDC in peripheral leukocytes are depicted in Fig. 1. A–C, mDC represented 0.49 ± 0.49% of the total leukocytes (absolute number, 25.9 ± 17.3/μl) in AD patients and this value was slightly, but significantly, higher than the 0.29 ± 0.16% (absolute number, 14.5 ± 7.9/μl, p < 0.02) in normal subjects. pDC represented 0.15 ± 0.20% (absolute number, 8.2 ± 6.3/μl) of the total leukocytes in AD patients and this value was >2-fold higher than the 0.065 ± 0.045% (absolute number, 3.3 ± 2.2/μl) in normal individuals (p < 0.001). The distribution of blood DC was comparable between psoriasis patients and normal individuals and thus significantly lower than that in AD patients.

The pDC dominance in AD patients over psoriasis patients and normal individuals was clearly demonstrated by direct comparison of the mDC:pDC ratios among the study groups (Fig. 1D). The mDC:pDC ratio of 3.7 ± 2.0 in AD patients was significantly lower than the 7.5 ± 7.4 in normal individuals (p < 0.003, Mann-Whitney U test) and 7.7 ± 7.0 in psoriasis patients (p < 0.003). In addition, the ratios in normal individuals and psoriasis patients were highly variable, ranging from 1.5 to >20, whereas those in AD patients were distributed in a relatively narrow range (0.7–10.5). Therefore, the low mDC:pDC ratio in AD patients was associated with a relative increase in the number of pDC over mDC among the DC subsets in the peripheral blood.

**Correlation between the mDC:pDC ratio and the serum IgE level in AD**

The core parameters representing Th2 responses in allergic disorders are overproduction of IgE and eosinophilia (21). Therefore, the correlation between the mDC:pDC ratio and the serum IgE level or eosinophil number in the circulation was examined. As shown in Fig. 2A, the mDC:pDC ratio was inversely correlated with the total serum IgE level (r = 40, r = −0.40, p < 0.015, Spearman’s rank test). In contrast, there was no correlation between either the percentage or number of blood eosinophils and the mDC:pDC ratio (data not shown).
Correlations between the mDC:pDC ratio and the cytokine profiles of CD4+ lymphocytes

Since the DC type involved in the interaction with naive T cells is a critical factor in the determination of Th cell polarization (4), it is possible that the mDC:pDC balance influences the distribution of Th1 and Th2 cells in the peripheral blood. Therefore, we analyzed the production pattern of IFN-γ and IL-4 by T cells after polyclonal stimulation to examine the relationship between the Th1:Th2 ratio and the mDC:pDC ratio. As illustrated in Fig. 2B, the ratio of the number of IFN-γ+ cells (Th1 cells):number of IL-4+ cells (Th2 cells) was weakly but significantly correlated with the mDC:pDC ratio (n = 19, r = 0.462, p < 0.04, Spearman’s rank test). These data suggest that the Th2 shift, as envisaged by the elevated level of serum total IgE, was determined in part by the low mDC:pDC ratio.

Correlation between the mDC:pDC ratio and clinical severity

Although Th1 cells have been reported to provoke chronic skin lesions, Th2 dominance is a characteristic feature throughout the course of AD (15, 22, 23). This suggests that the severity of AD reflects, in part, the degree of peripheral Th2 tilting, which would be under the influence of the balance among DC subsets in the circulation. SCORAD was selected to define the severity of AD since critical elements for AD evaluation are included in this scoring method. Fig. 2C shows a significant inverse correlation between the SCORAD index and the mDC:pDC ratio (n = 25, r = −0.497, p < 0.02, Spearman’s rank test).

Therefore, these data suggest that the distribution of the blood DC subsets dictated the allergic status measured by the core immunological and clinical parameters in AD.

In vitro Th1/Th2 polarization of naive CD4+ T cells induced by blood DC

It is widely accepted that mDC and pDC lead naive CD4+ T cells to Th1 and Th2 differentiation, respectively (4, 24). To investigate whether this notion was true for AD, IL-4 and IFN-γ production by CD4+ T cells was examined in MLR in which CD4+ CD45RO+ T cells were stimulated with allogeneic DC subpopulations and subsequently expanded by polyclonal stimuli.

We showed in Fig. 3A four representative FACS analyses of purified pDC and mDC in AD and normal subjects to validate the physical separation method. As shown in Fig. 3A, the contaminating cells were <20% of the total cells and more than one-half of them were negative for HLA-DR. The HLA-DR+ cells were CD4+, CD8-, or CD14-, suggesting residual B cells. Moreover, there was no numerical DC difference due to adjustments. These
FIGURE 3. In vitro Th1/Th2 polarization of naive T cells by blood DC. Naive CD4⁺ T cells were stimulated in MLR with mDC or pDC from AD patients and normal individuals, followed by expansion with IL-2. A, Representative analyses of purified mDC and pDC in a normal (left) and an AD subject (right). Numbers indicate percentage among mononuclear cells. B, IL-4 and IFN-γ concentrations in the supernatants of cultures generated with mDC-enriched (□) or pDC-enriched (■) fractions from three healthy individuals (N#1–3) and three AD patients (AD #1–3). Vertical bars indicate the SD. C, Percentages of CD4⁺ cells containing IL-4 but not IFN-γ (○) and IFN-γ but not IL-4 (●) among the total cultured cells generated with mDC or pDC fractions from normal subjects and AD patients.
data essentially excluded the effect of contaminating cells on Th differentiation between AD and normal subjects.

Although the concentrations of IL-4 and IFN-γ ranged widely in cultures generated with DC, pDC were always superior to mDC in the induction of IL-4 production by CD4⁺ T cells irrespective of the DC source (Fig. 3B). IFN-γ production was more pronounced in cultures with mDC than in those with pDC when the DC were derived from normal individuals. These results confirm the consensus that mDC and pDC guide Th1 and Th2 cytokine productions, respectively, in normal subjects. In contrast, DC from AD patients acted on naive T cells in the opposite way to normal DC for IFN-γ production since the cytokine levels were higher in cultures containing pDC than mDC.

To ascertain the polarity of T cell differentiation at the single-cell level, we performed intracytoplasmic staining for IFN-γ and IL-4 in CD4⁺ T cells under the same culture conditions as above. As shown in Fig. 3C, with normal DC, the frequencies of IL-4-producing cells were higher in three of four experiments and those of IFN-γ-producing cells were always lower under the pDC-rich than the mDC-rich condition. This again confirms the contributions of mDC to Th1 and of pDC to Th2 differentiation in normal subjects. In contrast, although the percentages of IL-4-producing cells were essentially higher with pDC than mDC, IFN-γ production was more pronounced with pDC than mDC in AD patients. These results suggest that the DC subsets in AD were functionally aberrant in the induction of Th1/Th2 differentiation.

CXCR3 and CCR4 expressions of naive CD4⁺ T cells induced by blood DC

Recent findings have indicated that Th1 differentiation is associated with the expression of distinctive chemokine receptors, in that

FIGURE 4. Percentages of CD4⁺ cells expressing CCR4 but not CXCR3 (□) and those expressing CXCR3 but not CCR4 (■). Naive CD4⁺ T cells were stimulated with mDC or pDC from normal subjects (N) and AD patients as in Fig. 3.

FIGURE 5. pDC in AD lesional skin. Cells coexpressing CD123 (A) and CD45RA (B, merged C) and those coexpressing CD123 (D) and HLA-DR (E, merged F) have infiltrated around PNAd-expressing vessels (G) in the mid dermis. Arrows indicate pDC. CD123 is weakly positive in a vascular lumen (+). Triple immunofluorescence staining; original magnification, ×400.)
Th1 cells are CXCR3+/CCR4− while Th2 cells bear CCR4 but not CXCR3 (25, 26). Therefore, we analyzed the capacity of DC for inducing Th1/Th2-related chemokine receptor expression in naive CD4+ T cells expanded in MLR plus polyclonal stimuli. In cultures containing DC from normal subjects, pDC and mDC preferentially generated CXCR3+/CCR4− cells and CXCR3+/CCR4+ cells, respectively (Fig. 4). In contrast, in cultures containing DC from AD patients, the induction of both CXCR3+/CCR4− and CXCR3+/CCR4+ populations was comparable between mDC and pDC, except for one experiment in which pDC were inferior to mDC in CCR4+ cell induction. These results further confirm the functional difference between DC from AD patients and normal subjects in the promotion of Th1- and Th2-related chemokine receptor expression.

Infiltration of pDC in relation to PNAd expression in AD skin lesions

The preferential accumulation of pDC at inflammatory sites in allergic and cancer patients raises the possibility that the maturation and function of pDC take place on site (6, 27). Immunohistochemistry of AD skin lesions (n = 3) disclosed that CD45RA+/CD123+HLA-DR+ DC (identified as pDC) infiltration was in close association with vessels that strongly expressed PNAd, ligands for CD62L (L-selectin), (28) in the mid and deep dermis (Fig. 5). In contrast, pDC did not infiltrate psoriatic lesions (n = 3) despite occasional vascular expression of PNAd in the lower dermis (data not shown). There was no positive staining for pDC or PNAd in normal skin (n = 4). CD11c−HLA-DR− DC mainly resided abundantly in the upper dermis lesions of AD and psoriasis patients, consistent with previous studies (29, 30). Since Langerhans cells and dermal DC express CD11c, this population seemed to contain these resident DC and mDC. CD11c+ cells were present in the epidermis but hardly detectable in the dermis of normal subjects. These findings suggest that pDC migrated into the inflammatory sites of AD through interaction with PNAd+ vascular endothelium.

Discussion

The present results revealed aberrant profiles in the distribution and function of the DC subsets in the blood and lesional skin of AD patients compared with normal individuals and psoriasis patients (a Th1 disease model group). The blood DC distribution was not conform to the consensus that mDC and pDC in normal individuals facilitate Th1-related and Th2-related immune responses, respectively (4). While inducing Th2 differentiation of naïve T cells, atopic pDC were superior or comparable to mDC in IFN-γ production and CXCR3 expression with the resultant participation of this cell type in Th1 differentiation. This indicates that the functional differences between mDC and pDC were indistinct, and rather that the immunological effects of these DC subsets overlapped. An emerging concept is that acquisition of DC functions is not totally predetermined but rather plastic, such that the microenvironment regulates the magnitudes, types, and directions of the functional development of DC (50). Therefore, it is possible that immune dysfunctions of DC, as observed in AD, are not only inherent but also instructed under the influence of the in vivo conditions favoring atopic allergy. Recent reports have demonstrated that IL-18 derived from blood monocytes and keratinocytes in AD promotes Th1 induction by pDC through IL-18R expression (45, 48). Neither atopic nor normal pDC released detected levels of IL-12p40 in culture supernatants by cytokine beads assay or expressed IL-12p35 mRNA by RT-PCR, indicating that IL-12 was not critical for induction of cells with IFN-γ production (data not shown). As a result of the development of pDC subsets and DC in AD, the interaction of naïve T cells and DC goes in the unfavorable direction, leading to excess Th2 tilting, as exemplified by the high serum IgE level and low blood Th1:Th2 ratio. In contrast, there was no correlation between the eosinophil number and the DC differentiation status in AD. This might reflect the fact that eosinophil navigation is directed by local production of chemokines such as RANTES and eotaxin and/or vascular expression of cell adhesion molecules rather than eosinopoiesis by Th2 cytokines (51–53).

Critical immune parameters have been demonstrated to reflect the disease severity in AD (15). We found that the blood mDC: pDC ratio was significantly correlated with the disease severity, as estimated by SCORAD. Since the essential ingredients for AD assessment are included in this scoring method, it is suggested that DC are directly involved in the disease progression, as has been reported for HIV infection (39, 41, 54) and cancer (33, 55). In accordance with these findings, immunohistochemical staining showed infiltration of pDC in close association with dermal vessels expressing PNAd in the lesional skin of AD. The interaction between the L-selectin (CD62L) highly expressed on pDC and the L-selectin ligand PNAd on endothelial venules may be responsible for the pDC infiltration. Mucosal inflammation following Ag challenge in patients with a nasal allergy contains pDC in the context of the PNAd-positive vasculature (6). The absence of pDC infiltration in psoriasis despite the occasional vascular expression of PNAd suggests that chemotactic factors are also critical for the migration of pDC into AD lesions (45). Epithelial-derived thymic stromal lymphopoietin potently activates mDC to induce Th2 development (56). Therefore, these data indicate that DC are crucial participants in the establishment of lesions related to atopic allergy.
Although Th2 dominance is a hallmark of AD, both Th1 and Th2 cells reside in skin lesions. Immunological examination of atopic patch test lesions as a model of AD revealed a biphasic pattern with IL-4 production at the early phase and the emergence of IFN-γ at late time points (57). In accordance with these findings, the initiation of AD is driven by Th2 cell activation, whereas the chronic inflammatory response is dominated by a Th1-type response (58). Therefore, although Th2 cells are essential players in the allergic responses throughout the course, the participation of Th1 cells with a late onset prolongs the kinetics and contributes to the protracted inflammation of AD. In ordinary Th1-type inflammation, migration of pDC may promote the development of Th2 responses, thereby sustaining skin inflammation and the protracted inflammation of AD. In ordinary Th1-type inflammatory skin lesions seem to enhance both Th1 and Th2 responses as predicted by the in vitro ability of DC subsets to augment both Th1 and Th2 differentiation, thereby sustaining skin inflammation in AD.

Acknowledgment
The technical assistance of K. Sugaya is appreciated.

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