Initiation and regulation of specific immune responses by keratinocytes and dendritic cells. Role of cytokines and chemokines linking innate and specific immunity
Lebre, M.C.G.M.

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CHAPTER 6

Reduced production of bioactive IL-12 and IFN-α by peripheral blood dendritic cells in atopic dermatitis patients

M. Cristina Lebre\textsuperscript{1,2}, Folkert A. Blok\textsuperscript{2}, Toni M.M. van Capel\textsuperscript{1}, Jan D. Bos\textsuperscript{2}, Edward F. Kno\textsuperscript{1}, Martien L. Kapsenberg\textsuperscript{1,2} and Esther C. de Jong\textsuperscript{1,2}

\textsuperscript{1}Department of Cell Biology and Histology, \textsuperscript{2}Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
\textsuperscript{3}Department of Dermatology and Allergology, University Medical Centre Utrecht, Utrecht, The Netherlands

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disorder associated with a generalized bias of Th2 cells producing the cytokines IL-4, IL-5 and IL-13. As the Th1/Th2 balance depends on signals provided by dendritic cells (DC) we questioned whether circulating DC in AD patients, compared to DC from healthy controls, differ in their phenotype and function. Purified BDCA1+ myeloid DC from AD patients showed a selective and dramatic reduction of IL-12p70 release, a cytokine that promotes the development of Th1 cells. Message (m)RNA analysis indicated that the decreased ability of BDCA1+ DC to produce IL-12p70 is likely to be due to a defective expression of both IL-12p35 and IL-12p40 subunits. Accordingly, even after maturation in the presence of an extremely potent Th1 stimulus (double stranded (ds)RNA plus IFN-γ), BDCA1+ DC from AD patients induced considerably less IFN-γ-producing Th cells, compared to BDCA1+ DC from healthy controls. This reduced capacity of BDCA1+ DC could neither be attributed to a deficiency in the expression of class II MHC or costimulatory molecules nor to a reduced capacity of these cells to induce T cell proliferation. Moreover, analysis of peripheral blood BDCA4+ plasmacytoid DC from AD patients demonstrated that BDCA4+ DC produce significantly lower levels of IFN-α, which is essential in natural immunity to virus and bacterial infections. The defective IL-12 and IFN-α production by DC may contribute to the maintenance of the allergic state in AD patients, as well as their increased susceptibility to skin infections with pathogens that require protective IFN-α and Th1 cell responses.

INTRODUCTION

Atopic dermatitis (AD) is an eczematous skin disease that is characterized by elevated levels of total and antigen-specific IgE and IgG4, by increased numbers of blood eosinophils, increased numbers of Langerhans cells (LC) and T cells in lesional skin. AD has been associated with the activation of peripheral blood T cells that secrete T helper (Th) type 2 (Th2) cytokines (IL-4, IL-5 and IL-13) favoring the differentiation of eosinophilic granulocytes and supporting the production of high amounts of IgE. In addition, a biphasic model for AD skin has been proposed, in this model a shift from a Th2 type response in the initiation phase to a Th1/Th0 response in the late and chronic phase (when IFN-γ production predominates) is observed. A major issue of current study is the definition of molecular and histophysiological mechanisms underlying the preferential development of Th2 cells in AD, taking into account
BDCA1+ DC in AD patients produce reduced levels of IL-12p70

that the development of a particular Th response is mediated by signals derived from antigen presenting cells (APC).

Dendritic cells (DC) are professional APC that have a central role in the initiation and regulation of immune responses both in lymphoid and non-lymphoid tissues. There is however considerable intra- and inter-tissue variation in the phenotype, morphology, function, and tissue localization of different DC populations. Human blood DC have been recently divided into five distinct subsets, CD1b/c⁺, CD16⁺, BDCA3⁺, CD123⁺, and CD34⁺ DC. In particular, the so-called myeloid DC (mDC) which are CD1c⁺(BDCA1)CD11c⁺CD45RO⁺CD123low (IL-3R α-chain) have the ability to produce IL-12 in response to bacterial compounds or CD40L, and require GM-CSF for survival. Conversely, plasmacytoid DC (PDC) are BDCA4⁺CD11c⁺CD45RA⁺CD123high and require the presence of IL-3 for survival. Upon herpes or influenza virus infection, PDC, but not the mDC, produce high amounts of type I IFNs, IFN-α and IFN-β. These cells are of major importance during viral infections, when they are massively recruited to the site of infection and serve as an important source of antiviral type I IFNs. Host defense molecules such as type I IFNs play a vital role in innate resistance to a wide variety of infectious agents through the induction of direct, cell-autonomous resistance to viral and microbial pathogens. In addition, type I IFNs have been increasingly recognized as essential early warning molecules signaling the presence of pathogens, providing a pivotal function at the interface between innate and adaptive responses.

Although it has been described that IFN-α therapy decreases IL-4-mediated IgE synthesis in AD patients it remains to be established whether IFN-α production levels in AD patients are similar or not compared to healthy controls.

The production of IL-12 by DC, triggered by their exposure to microbial products or their interaction with activated T cells, is known to play a critical role in the induction of Th1-mediated immune responses. Bioactive IL-12 is a heterodimeric cytokine composed of two subunits, p35 and p40, encoded by different genes. Both subunits must be expressed in the same cell to generate the bioactive form, p70, of the cytokine. The notion that dysregulated IL-12 levels may be important in allergy is supported by several studies of human asthmatic and AD patients. It was demonstrated that the number of IL-12p40-expressing cells in bronchial biopsy specimens from allergic asthmatics was significantly less than that found in the lungs of normal control subjects. Moreover, it was shown that bioactive IL-12 production in whole blood cultures of patients with allergic asthma and in monocytes of
patients AD is significantly lower compared to control subjects, and is associated with decreased IFN-γ production by Th cells. These studies may indicate an aberrant function of APC in allergic diseases, but to date no studies have been performed with the most relevant APC, the DC.

Dysfunction of DC subsets in AD patients could be associated with aberrant expression of cytokines/cell surface molecules that in turn may be responsible for the preferential development of Th2 cells observed in this skin disease. To gain insight into the in vivo characteristics of DC, we investigated whether peripheral blood DC, the stage in the DC life cycle that immediately precedes recruitment into the skin or into other non-lymphoid or lymphoid tissues, are different in AD patients compared to healthy, nonatopic subjects. Here we show that BDCA1 myeloid DC from AD patients expressed significant lower levels of IL-12 and TNF-α, in response to double-stranded RNA, poly I:C plus IFN-γ, and induced the development of T cells with reduced capacity to produce IFN-γ. In addition, BDCA4 plasmacytoid DC from AD patients produced lower levels of IFN-α in response to the bacterial product Staphylococcus aureus Cowain strain I (SAC). These data suggest that in AD, blood DC may also critically contribute to the development of the atopic phenotype by producing reduced levels of the Th1-associated cytokines, IL-12 and IFN-α.

MATERIAL AND METHODS

Subjects
Peripheral heparinized blood samples were obtained from 17 patients with AD (see table I for characteristics), who were diagnosed on the basis of the Millennium criteria for AD. None of the patients studied had been treated with systemic glucocorticoids and no topical corticosteroids were used for 1-2 weeks before their blood donation. As control group 16 healthy subjects aged-matched (total serum IgE <50 kU/L) and with no history of AD, allergic rhinitis, or asthma were studied. Donation of blood and skin biopsies by patients and healthy controls followed approval by the ethical committee of the Academic Medical Center (Amsterdam, The Netherlands). All patients and normal subjects gave written informed consent.
Isolation and culture of BDCA1+ and BDCA4+ DC

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation with Lymphoprep (Nycomed, Torshov, Norway). Myeloid DC, BDCA1+ and plasmacytoid, BDCA4+ DC were isolated from PBMC by magnetic cell sorting (MACS) using a high gradient MACS device (Mini-MACS®; Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, CD19+ B cell-depleted PBMC were incubated with BDCA1-PE monoclonal antibody (mAb) and BDCA4-PE mAb followed by anti-PE magnetic beads (Miltenyi Biotec). To analyze the purity of the obtained BDCA1+ and BDCA4+ cell populations, the cells were incubated with a cocktail of FITC-conjugated mAb specific for CD3, CD14, CD16, CD19, CD20, CD34 and CD56 receptors (lineage, lin; BD Bioscience). The purity of each cell population was >90%. Purified BDCA1+ and BDCA4+ DC were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Paisley, U.K.) containing 10% FCS (HyClone, Logan, UT). While BDCA1+ DC were cultured in medium supplement with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands), BDCA4+ DC were cultured in the presence of IL-3 (10 ng/ml; Strathmann Biotech, Hannover, Germany).

Table I. Characteristics of patients with AD

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Stimulation of BDCA1+ and BDCA4+ DC

BDCA1+ and BDCA4+ DC were stimulated (2x10^4 cells/200 μl; 96-well plates, Costar, Cambridge, MA) by fixed Staphylococcus aureus Cowan strain I (SAC, 75 μg/ml; Calbiochem, San Diego, CA), synthetic double-stranded (ds)RNA polyriboinosinic
polyribocytidylic acid (poly I:C, 20 μg/ml; Sigma-Aldrich, St. Louis, MA), CD40L-transfected J558 cell line (2x10⁵ cells; a gift of Dr. P. Lanc, University of Birmingham, Birmingham, U.K.) alone or in combination with rhIFN-γ (1000 U/ml; a gift of Dr. P.H. van der Meide, U-CyTech, Utrecht, The Netherlands). Supernatants were harvested after 24 h. The levels of IL-12p70, IL-12p40, IFN-α, TNF-α, IL-6 and IL-10 were measured by ELISA (see below).

Analysis of cell surface molecules expression by FACS
To analyze the phenotype of circulating DC, freshly isolated or matured BDCA1⁺ DC were incubated with FITC-conjugated mAb against CD86 (mouse IgG1; BD Pharmingen, San Diego, CA), allophycocyanin (APC)-conjugated anti-CD83 mAb (mouse IgG2b; Caltag Laboratories, Barlingame, CA) and PerCP-conjugated anti-HLA-DR mAb (mouse IgG2b; BD Bioscience, San Jose, CA). As controls, cells were stained with corresponding isotype-matched control mAb (values of their mean fluorescence intensity (MFI), were subtracted to each value of the appropriate fluorochrome-conjugated mAb, delta (Δ)MFI). Stained cells were analyzed using a four-color cytometer (FACSCalibur, Becton Dickinson).

Isolation of CD4⁺CD45RA⁻CD45RO⁺ naive Th cells (ThN)
ThN were isolated from peripheral blood leukocytes with the negative selection human CD4⁺/CD45RO⁺ column kit (R&D Systems, Minneapolis, MN). This method yielded highly purified (>98%) CD4⁺CD45RA⁻CD45RO⁺ ThN as assessed by flow cytometry (data not shown).

Analysis of proliferation of T cells cocultured with BDCA1⁺ DC in a mixed lymphocyte reaction (MLR)
Poly I:C plus IFN-γ-matured BDCA1⁺ myeloid DC where tested for their ability to stimulate allogeneic ThN in a MLR. ThN (2.5x10⁵ cells/200 μl) were cocultured in 96-well flat-bottom culture plates with different concentrations of matured DC. After 5 days, cell proliferation was assessed by the incorporation of [³H]thymidine ([³H]-TdR, Radiochemical Centre, Amersham, Little Chalfont, U.K.) after a pulse with 13 KBq/well during the last 16 h, as measured by liquid scintillation spectroscopy.
BDCA1+ DC in AD patients produce reduced levels of IL-12p70

Induction of Th1 or Th2 cell responses by mature BDCA1+ DC

ThN (8×10^3 cells/200 μl) were cocultured in 96-well flat-bottom culture plates with allogeneic BDCA1+ DC (2.0×10^4 cells/200 μl), matured for 24 h in the presence of poly I:C plus IFN-γ, which were coated with the superantigen Staphylococcus aureus enterotoxin B (SEB, 100 pg/ml; Sigma-Aldrich). On day 5, IL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were further expanded. After 14 days, resting memory Th cells were harvested, washed, and restimulated for 6 h with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich) in order to analyze Th cell cytokine production profile at the single cell basis. The cells were fixed in paraformaldehyde (2%; Sigma-Aldrich), permeabilized with saponin (0.5%; Sigma-Aldrich), and labeled with FITC-coupled IFN-γ mAb (Becton Dickinson) and PE-coupled IL-4 mAb (Becton Dickinson). The cells were evaluated by FACSScan (Becton Dickinson).

Generation of monocyte-derived DC and their stimulation

Immature monocyte-derived DC (mo-DC) were generated from monocytes in cultures performed in IMDM (Life Technologies) containing 1% FCS (HyClone), rhuGM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; PBH), as described. Immature DC were stimulated (2×10^4 cells/200 μl; 96-well plates, Costar) by fixed SAC, poly I:C or CD40L-transfected J558 cell line alone or in combination with rhuIFN-γ, as stated above. Supernatants were harvested after 24 h and the levels of IL-12p70 were measured by ELISA (see below).

Quantification of IL-12 p35, p40 and β2-microglobuline mRNA levels by real-time PCR

BDCA1+ DC (4.0×10^5) were stimulated for 6h in the presence or in the absence of the following stimuli: poly I:C plus IFN-γ or SAC. After lysing the cells total RNA was purified by using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacture’s instructions. cDNA was generated using the First strand cDNA synthesis kit for RT-PCR (MBI Fermentas, St Leon-Rot, Germany). To anneal the primer to the RNA, 9 μl of total RNA, 1 μl oligo(dT)18 and 1 μl D(N), were added. This mix was then heated for 5 minutes at 94°C. Quantification of IL-12p35 and IL-12p50, and as a control β2-microglobulin (β2μ) transcripts, real-time PCR was performed in a LightCycler (Roche Diagnostics,
Mannheim, Germany) based on specific primers and general SYBR green fluorescence detection. The primer sequences were the following: β2m sense: 5'-AAGATTCAAGTTTACTCACGTC-3'; β2μ anti-sense: 5'-TGATGCTGCTTACATGTCTCG-3' (melting temperature (Tm) 62°C); IL-12p35 sense: 5'-CTTCACCACCTCCAAAAACCT-3'; IL-12p35 anti-sense: 5'-AGCTCATCCTCTATCAATAGT-3' (Tm 60°C); IL-12p40 sense: 5'-ATTGAGGTCATGGTGGATGC-3'; IL-12p40 anti-sense: 5'-AATGCTGGCATTTTTGCGGC-3', resulting in the amplification of PCR products of 294 bp (β2μ), 532 bp (p35) and 297 bp (p40). IL-12 mRNA (p35 or p40) levels were expressed as the absolute number of copies normalized against β2μ mRNA. This was achieved by generating standard curves from serial dilutions of standards. These standards consisted in PCR products that included the IL-12p35 or IL-12p40 amplicon and that were purified following standard procedures.

Evaluation of cytokine production by ELISA

Determination of IL-12p40 and IL-12p70 concentrations in culture supernatants was performed by specific solid-phase sandwich ELISA as previously described. Pairs of specific monoclonal antibodies (mAbs) and recombinant cytokine standards were obtained from BioSource International (Camarillo, CA) for determinations of IL-6, TNF-α and IFN-α, and from BD Pharmingen for IL-10 determination. The detection limits of these ELISA are as follows: IL-6, 20 pg/ml, TNF-α, 20 pg/ml, IFN-α, 100 pg/ml, IL-12p70, 3 pg/ml, IL-12p40, 100 pg/ml.

Immunohistochemical analysis of BDCA1+ and BDCA4+ DC in skin sections

For immunohistochemical staining, tissues specimens were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), cryopreserved in liquid N2 and stored at -20°C. Acetone-fixed cryosections (6 μm) were prepared and incubated with specific mouse mAbs against BDCA1 (IgG2a, Miltenyi Biotec) or BDCA4 (IgG1, Miltenyi Biotec) Ags for 1 h at 37°C after blocking endogenous peroxidase activity with 0.3% H2O2 and 0.1% sodium azide (NaN3), and after preincubation (15 min) with 10% normal goat serum (Dakopatts). An IgG1 or IgG2a isotype mAbs were used as negative controls. After incubation with biotinylated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) for 30 min at room temperature, skin sections
BDCA1+ DC in AD patients produce reduced levels of IL-12p70

were rinsed with PBS and incubated for 30 min with streptavidin horseradish peroxidase-conjugated (Strep-HRP, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands). After washing, 3-aminole-9-ethylcarbazole (AEC, Sigma-Aldrich) as used as chromogen. With this procedure BDCA1+ and BDCA4+ DC stained red. Other cellular elements were counterstained with hematoxylin (Cellpath, Newton Powys, UK). Then the sections were dried and mounted with glycerol/gelatin.

For double immunostainings slides were incubated with 0.3% H2O2 and 0.1% NaN3 followed by a preincubation (15 min) with 10% normal goat serum (Dakopatts). Then the sections were incubated for 1 h with the primary mAb against BDCA1 (IgG2a, Miltenyi Biotec) followed by incubation with FITC-coupled goat F(ab')2 anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). Then the sections were incubated with rabbit anti-FITC (Dakopatts) followed by incubation with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Dakopatts). After blocking with normal mouse serum (10%, Dakopatts), the biotinylated primary mAb against BDCA4 (IgG2a, Miltenyi Biotec) was added to the sections followed by an incubation with Strep-HRP (CLB). AP staining was developed using naphtol AS-MX phosphate (20 mg, Sigma-Aldrich) plus levamisole (15 mg, Sigma-Aldrich) plus Fast blue BB salt (1 mg, Sigma-Aldrich) in 100 mM Tris-HCl buffer, pH 8.5. With this procedure BDCA1+ DC stained blue. AEC staining was performed as stated above. With this procedure BDCA4+ DC stained red. Slides were analyzed with a wide field upright microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands).

Statistical analysis

Data are expressed as mean ± SEM. Data were analyzed for statistical significance with the GraphPad InStat® software (version 3.00; GraphPad InStat, Inc., San Diego, CA). The nonparametric unpaired Student t test was used to compare variables between two groups. A P value <0.05 was considered as the level of significance.
RESULTS

BDCA1+ and BDCA4+ DC frequencies in peripheral blood from AD patients do not differ from healthy controls

To study the mechanisms underlying decreased cell-mediated immunity and IL-12 production in patients with AD, we examined the frequencies of BDCA1+ and BDCA4+ DC in these patients and compared to DC from healthy controls. To this aim, PBMC from healthy controls (n=16) and AD patients (n=16) were analyzed by flow cytometry after staining with mAbs against BDCA1 or BDCA4 Ags and lineage (Lin; CD3, CD14, CD16, CD19, CD20, CD34 and CD56) markers. Flow cytometry analysis of PBMC from AD patients demonstrated no significant differences in the frequency of both BDCA1+ and BDCA4+ DC from healthy controls compared to DC from AD patients (Fig. 1A and B).

**Figure 1.** Frequencies of BDCA1+ and BDCA4+ DC in PBMC from healthy controls and AD patients. PBMC were isolated from healthy controls or from AD patients and analyzed by FACS after two-color staining with a combination of FITC-labeled mAbs against lineage markers (Lin) and PE-labeled BDCA1 or BDCA4. (A) An example of the frequency of BDCA1+ and BDCA4+ DC in one healthy donor and in one AD patient is shown. The numbers indicate the percentage of cells in the respective quadrant. (B) Frequency (%) of BDCA1 and BDCA4 in PBMC from healthy controls (n=16) and AD patients (n=16). Results are expressed as mean±SEM. Data were analyzed for statistical significance using a two-tailed Student’s t-test. *P<0.05, **P<0.01, ***P<0.001 compared to healthy controls.
BDCA1+ DC in AD patients produce reduced levels of IL-12p70

**Figure 2.** Cytokine production by isolated BDCA1+ and BDCA4+ DC from healthy controls and AD patients after *in vitro* stimulation with poly I:C+IFN-γ or SAC, respectively. BDCA1+ and BDCA4+ DC were isolated from PBMC by indirect magnetic labeling with PE-conjugated primary mAbs and anti-PE mAb-conjugated microbeads followed by enrichment of labeled cells by MACS. After 24 h stimulation the contents of IL-12p70, IL-12p40, TNF-α, IL-6, IL-10 or IFN-α were analyzed by specific ELISAs. (A) Purified BDCA1+ DC stimulated with poly I:C+IFN-γ from AD patients show reduced capacity to produce IL-12p70 and TNF-α, compared to healthy controls. (B) Purified BDCA4+ DC from AD patients show reduced capacity to produce IFN-α compared to healthy controls. Results represent the mean ± SEM of cytokine concentrations contained in the culture supernatants obtained from different healthy controls and AD patients BDCA1, n=10; BDCA4, n=16. Data were analyzed for statistical significance using a two-tailed Student’s *t*-test, *p<0.05, **p<0.01, ***p<0.001 compared to healthy controls.

**AD patients show impaired IL-12p70 and IFN-α production by BDCA1+ and BDCA4+ DC, respectively**

AD is a skin disease associated with the preferential development of Th2.1,2 As the development of a particular Th response is dependent on DC-derived signals24 we questioned whether the Th2 phenotype observed in AD patients may result from a deficiency in the production of Th1-associated cytokines by peripheral blood DC. To this aim, purified BDCA1+ (Fig. 2A) and BDCA4+ (Fig. 2B) DC were stimulated with poly I:C plus IFN-γ or with SAC, stimuli that optimally induce IL-12p70 or IFN-α respectively. After 24 h, the cytokines present in culture supernatants were analyzed by specific ELISAs. Poly I:C plus IFN-γ-stimulated BDCA1+ DC from AD patients show selectively and significantly (*P<0.001) lower levels of IL-12p70 and TNF-α compared to BDCA1+ DC derived from healthy controls. The production of IL-6, IL-10 and IL-12p40 by BDCA1+ DC from AD patients was not significantly different from healthy controls (Fig. 2A). While the levels of IFN-α production by SAC-stimulated BDCA4+ DC from AD patients were selectively and
significantly ($P<0.05$) low compared to healthy controls, the levels of TNF-$\alpha$, IL-6 and IL-10 production were similar (Fig. 2B). SAC-stimulated BDCA4$^+$ DC did not produce detectable levels of IL-12p70 (data not shown).

**BDCA1$^+$ DC from AD patient express similar levels of HLA-DR, CD83 and CD86 compared to healthy controls**

Purified BDCA1$^+$ DC from healthy controls and AD patients were stimulated *in vitro* with poly I:C, or SAC, or CD40L, and HLA-DR, CD83 and CD86 expression was measured after 24 h by FACS. Freshly isolated BDCA1$^+$ DC were used as control. Fig. 3 shows that irrespective of the stimuli tested, BDCA1$^+$ DC upregulated the expression of HLA-DR, CD83 and CD86 and there were no significant differences between DC derived from healthy controls and AD patients. Addition of IFN-$\gamma$ to the BDCA1$^+$ DC cultures did not change the expression of these mature phenotype markers studied (data not shown). Freshly isolated BDCA4$^+$ DC from both healthy controls and AD patients also show similar levels of HLA-DR, CD83 and CD86 expression, albeit low in this DC subset (data not show).

![Figure 3](image-url). Freshly isolated and activated BDCA1$^+$ DC from AD patients express similar levels of HLA-DR, CD83 and CD86 compared to healthy controls. Freshly isolated or cultured (24 h after exposure to poly I:C, or SAC, or CD40L-transfected J558 cells) BDCA1$^+$ DC were stained with mAbs to HLA-DR, CD83 or CD86, and analyzed by FACS. ΔMFI represents the difference between the various staining and the isotype control. Results represent the mean ± SEM of ΔMFI from different healthy controls ($n=6$) and AD patients ($n=6$). Data were analyzed for statistical significance using a two-tailed Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to healthy controls.
Poly I:C-matured BDCA1⁺ DC from AD patients exhibit reduced capacity to induce IFN-γ-producing T cells compared to healthy controls, albeit their similar capacity to support naive Th cell proliferation

To evaluate the consequences of a reduced capacity to produce IL-12p70 by BDCA1⁺ DC from AD patients, we subsequently studied the capacity of mature BDCA1⁺ DC to stimulate naive Th cells in allogeneic MLR, and to induce a particular Th1/Th2 secretion profile. Fig. 4A shows that poly I:C-matured BDCA1⁺ DC from AD patients were as effective as BDCA1⁺ DC from healthy controls in inducing naive Th cell proliferation. These results are in line with the similar expression of mature phenotype markers by BDCA1⁺ DC from both healthy controls and AD patients.

To test whether poly I:C plus IFN-γ-matured BDCA1⁺ DC from AD patients have a different capacity to bias the development of Th1 or Th2 cells, mature BDCA1⁺ DC were cocultured with naive Th cells in the presence of superantigen (SEB). After 14 days, the percentage of cells producing IL-4 and/or IFN-γ was evaluated by stimulation of effector T cells with PMA/ionomycin and analysis of intracellular cytokine expression at the single cell basis by FACS (Fig. 4B). Although the priming of BDCA1⁺ DC from both healthy controls and AD patients with poly I:C plus IFN-γ result in DC that biased for the development of Th1 cells, the percentage of IFN-γ-producing Th cells induced by mature BDCA1⁺ DC from AD patients was lower. In addition, a higher percentage of IL-4-producing Th cells induced by these cells was observed. Fig. 4B shows a representative experiment out of ten with similar results. These findings indicate that BDCA1⁺ DC from AD patients have a reduced capacity to induce Th1 responses compared to healthy controls, in accordance with their strongly reduced IL-12p70 production.

BDCA1⁺ DC and monocyte-derived DC (Mo-DC) from AD patients produce low levels of IL-12p70 in response to different stimuli

To analyze whether the reduced capacity of BDCA1⁺ DC to produce IL-12p70 was not a consequence of the particular stimulus tested, BDCA1⁺ DC were stimulated with poly I:C, or SAC, or CD40L in the absence or in the presence of IFN-γ. In addition, we verified whether the reduced capacity of BDCA1⁺ DC from AD patients to produce IL-12p70 was also observed in Mo-DC, generated from peripheral blood monocytes. Irrespective to the stimuli used both BDCA1⁺ DC and Mo-DC from AD patients produced significantly lower levels of
Figure 4. Poly I:C+IFN-γ-matured BDCA1+ DC from healthy controls and AD patients exhibit similar capacity to support naive Th cell proliferation (A). BDCA1+ DC were cultured for 24 h in the presence of poly I:C+IFN-γ. Then matured BDCA1+ DC were cultured at different numbers with allogeneic naïve CD45RA CD4 Th cells. After 5 days, the cells were pulsed during the last 16 h with [3H]thymidine ([3H]-Tdr). Mature BDCA1+ DC from healthy controls (○, open circles), mature BDCA1+ DC from AD patients (●, close circles). Data are expressed as the mean ± SEM of counts per minute (cpm) from different healthy controls (n=6) and AD patients (n=6). Data were analyzed for statistical significance using a two-tailed Student’s t-test. *p<0.05, **p<0.01, ***p<0.001 compared to healthy controls. Poly I:C+IFN-γ-matured BDCA1+ DC from an AD patient have reduced capacity to induce IFN-γ-producing Th cells compared to healthy control (B). Matured BDCA1+ DC were loaded with SEB and cocultured with naïve Th cells. After 14 days, resting memory Th cells were stimulated for 6 h with PMA+ionomycin in the presence of brefeldin A. The expression of IL-4 and IFN-γ was assessed by intracellular staining. Results are expressed as the percentage of cells in each population. A representative experiment out of ten with similar results is shown.

IL-12p70 compared to healthy controls (Fig. 5 shows a representative experiment out of six with different AD patients and controls). These results suggest that decreased IL-12p70 in AD patients may be due to an intrinsic deficiency of APC to produce this Th1-promoting cytokine.
BDCA1+ DC in AD patients produce reduced levels of IL-12p70

**Figure 5.** Reduced capacity of BDCA1+ DC and Mo-DC to produce IL-12p70 is independent of the type of stimuli. Purified BDCA1 DC and Mo-DC from both healthy controls and AD patients were stimulated for 24 h with poly I:C, or SAC or CD40L-transfected J558 cells in the absence or in the presence of IFN-γ. IL-12p70 levels were measured by ELISA in culture supernatants. Data are mean±SEM of a representative experiment out of six with similar results. Data were analyzed for statistical significance using a two-tailed Student’s t-test, *P<0.05, **P<0.01, ***P<0.001 compared to healthy controls.

**IL-12 p35 and p40 gene expression are defective in stimulated BDCA1+ DC from AD patients**

Since the secretion of bioactive IL-12p70 requires the production of both IL-12 subunits: p35 and p40, we investigate the mechanisms of IL-12p70 deficiency observed in BDCA1 DC from AD patients by analyzing the mRNA expression of these subunits by quantitative real-time PCR. Fig. 6 shows a representative experiment out of five with similar results. Both p35 and p40 mRNA expression by poly I:C plus IFN-γ-stimulated BDCA1+ DC from the AD patient was lower compared to the healthy control. BDCA1+ DC stimulation with SAC resulted in the selective induction of p40 in the absence of p35 induction. Also in this case p40 expression of stimulated BDCA1+ DC was lower compared to the healthy control.

**Figure 6.** Reduced expression of IL-12(p35) and IL-12(p40) mRNA levels in BDCA1+ DC from AD patients. BDCA1+ DC from healthy controls or AD patients were incubated in the absence or in the presence of poly I:C+IFN-γ or SAC. After 6 h, mRNA was extracted, reverse transcribed, and amplified by quantitative real-time PCR using specific primers for IL-12(p35), IL-12(p40), or β2-microglobulin (β2µ). IL-12(p35) and IL-12(p40) mRNA levels were normalized against β2µ mRNA levels. Data show one representative experiment out of six with similar results.
BDCA1+ and BDCA4+ DC are present in elevated numbers in lesional skin of AD patients

Since AD is characterized by the accumulation of DC in the dermis, we analyzed by immunohistochemistry whether BDCA1+ and BDCA4+ DC are present in non-lesional and lesional skin of AD patients and compared to normal healthy controls. BDCA1 (Fig. 7A) and BDCA4 (Fig. 7D) staining in normal healthy skin (five separate samples) was detected in the upper part of dermis close to the border epidermis/dermis. BDCA1+ DC were also detected in the epidermis (Fig. 7A, arrows) while BDCA4+ DC were absent. In non-lesional skin from three different AD patients both BDCA1+ (Fig. 7B) and BDCA4+ cells (Fig. 7E) were observed in the dermis. However, BDCA1+ DC were absent in the epidermis of non-lesional AD skin. Lesional skin of AD patients is characterized by the thickness of the epidermis and a massive infiltration of activated CD4+ T cells and APC namely DC, monocytes and macrophages. Indeed, elevated numbers of BDCA1+ DC (Fig. 7C) are observed in the epidermis and dermis of lesional AD skin. BDCA4+ DC (Fig. 7F) were also present in the epidermis (arrow) at elevated numbers and in the dermis. Isotype control stainings (Fig. 7G) gave a negative staining.

Since both BDCA1+ and BDCA4+ DC are expressed in lesional epidermis of AD patients we confirmed the specificity of BDCA1 and BDCA4 mAbs by performing double stainings. Figure 8 shows single positive BDCA1 (blue cells) and BDCA4 (red cells) DC present in epidermis of lesional AD skin, suggesting that BDCA1 and BDCA Ags are not co-expressed. Further double stainings are currently being performed using antibodies against BDCA1 and 4 antigens in combination with antibodies against the myeloid DC marker CD11c and the PDC marker CD123 (IL-3Rα).
Figure 7. Immunohistochemical analysis of BDCA1⁺ and BDCA4⁺ DC in skin. Cryosections of human skin from healthy controls (n=5) (A, D, G), non-lesional (n=3) (B, E) and lesional (n=3) (C, F, H) AD patients were stained (red) with specific mAbs against BDCA1 (A, B, C) or BDCA4 (D, E, F), and counterstained with hematoxylin (blue). BDCA1⁺ and BDCA4⁺ DC were present in the dermis of healthy controls (A and B, respectively) and BDCA1⁺ DC were also present in the epidermis (arrows). BDCA1⁺ (B) and BDCA4⁺ DC (E) were also present in the dermis of non-lesional AD skin whereas BDCA1⁺ DC were absent in the epidermis. An increased number of BDCA1⁺ DC is observed in the epidermis and dermis of lesional AD skin (C). BDCA4⁺ DC accumulated in the epidermis in lesional AD skin (F, arrow) although some cells were also present in the dermis in contact with infiltrated cells. Isotype control antibody gave negative staining (G). Original magnification 10x.

DISCUSSION

The present study shows that both BDCA1⁺ myeloid DC and BDCA4⁺ plasmacytoid DC (PDC) from AD patients exhibit aberrant functions compared to healthy controls. BDCA1⁺ DC from AD patients are characterized by a significant decreased capacity to produce bioactive IL-12p70 in response to various stimuli as a result of a defective IL-12p35 and IL-12p40 mRNA gene expression. Consistent with their reduced capacity to produce IL-12, BDCA1⁺ DC from AD patients induce the development of Th cells with a decreased
frequency of IFN-γ-positive cells. Moreover, BDCA4⁺ DC-derived IFN-α in AD patients is lower compared to healthy controls.

These aberrant functions observed in the two blood DC subsets in AD patients could be caused by a decrease or increased frequencies of specific cell types. In fact, it has been demonstrated that patients suffering from systemic lupus erythematosus, myeloid leukemia, Sézary syndrome or human immunodeficiency virus (HIV) exhibit reduced numbers of peripheral blood DC. There are contradictory reports concerning the frequencies of peripheral blood DC in atopic patients compared to healthy controls. In one report patients with atopy (asthma and AD) have a slightly enhanced percentage of PDC, while the percentage of CD11c⁺ myeloid DC did not differ. In the other report the frequency of Lin⁻HLA-DR⁻CD123⁺ PDC in patients suffering from AD or allergic rhinoconjunctivitis was not statistically significantly different, whereas the frequency of Lin⁻HLA-DR⁻CD123⁺ myeloid DC was significantly reduced compared to healthy subjects. In addition, Upham et al. demonstrated that the number of myeloid DC is not different between normal controls and atopic asthmatics in the absence of relevant allergen challenge. These discrepancies are likely to be due to the differential characterization of atopic patients and differential characterization of both myeloid and PDC subsets. In addition, from these data it is not clear which myeloid subpopulation present in peripheral blood (CD16⁺, or BDCA1⁺ or BDCA3⁺ DC) is responsible for the increased or decreased cell frequency in atopic patients. Our study, which defines two blood DC subsets, demonstrates that the frequencies of both BDCA1⁺ and BDCA4⁺ DC present in blood from AD patients did not differ from healthy controls. However, it remains to be established whether the frequencies of the other two CD11c⁺ DC
present in peripheral blood, CD16⁺ and BDCA3⁻ DC, are different in AD patients compared to healthy controls.

IL-12 is a proinflammatory cytokine that is produced by APC, such as macrophages, B and DC cells. The biologically functional form of IL-12 is a 70-kDa heterodimer (IL-12p70), which consists of disulfide-bounded 40-kDa (p40) and 35-kDa (p35) subunits, which plays a key role in the induction of cellular immunity by promoting the proliferation of NK and T cells and the differentiation towards Th1 cells. AD patients are characterized by a biased polarization towards Th2 cells. In addition, the response to mycobacterial antigens, although dominated by IFN-γ, is less polarized towards Th1 cells as compared to healthy controls. Previously it has been shown that APC from atopic patients e.g., monocytes and monocyte-derived DC, produced less bioactive IL-12 compared to healthy controls. Here we show for the first time that BDCA1⁺ DC present in peripheral blood of AD patients have a decreased capacity to produce bioactive IL-12 in response to various stimuli compared to healthy controls. Moreover, poly I:C plus IFN-γ (a potent Th1-inducing stimulus) induced BDCA1⁺ DC maturation that subsequently induce the development of Th cells with low capacity to express IFN-γ compared to healthy controls. These findings are of particular importance since DC are the most potent APC in the initiation of specific immune responses. In addition, our results indicate that the polarization towards Th2 observed in AD patients may be due to a deficient IL-12 production by BDCA1⁺ DC but not due to a reduced number of these circulating DC in peripheral blood neither due to a decreased capacity of these cells to induce proliferation of T cells.

Analysis of the IL-12p35 and p40 gene expression (mRNA) suggested that the reduced IL-12p70 production observed in AD patients might be due to a decreased mRNA expression of both subunits. In addition, although IL-12p40 gene expression in AD BDCA1⁺ DC was defective, the protein synthesis of this subunit was similar compared to healthy controls, suggesting the existence of posttranscriptional regulation.

PDC are the main producers of type I IFNs, IFN-α and IFN-β, innate immune molecules that are crucial after bacterial and viral challenge. The in vivo effects of type I IFNs are associated with promoting an antiviral and antibacterial state, including a broad spectrum of cellular targets. For instance, by producing high levels of IFN-α response to viruses, PDC (BDCA4⁺) may protect myeloid DC (BDCA1⁻) from the cytopathic effect of the virus and may exert an adjuvant effect on antibody responses. The disease AD is complicated by recurrent infections of skin lesions by bacterial, viral, and fungal pathogens. About 30% of
patients with AD have bacterial or viral infections in the skin. The decreased secretion of IFN-α by AD BDCA4+ DC, and BDCA1+ DC-derived IL-12 and TNF-α, may have serious consequences on the induction of antibacterial and antiviral immune response and may account for the susceptibility of patients suffering from AD to skin infections with bacteria and viruses.

Our results established a clear difference in the function of myeloid circulating DC from AD patients compared with their normal counterparts, however is still unknown whether other atopic disease, such as atopic asthma may also display similar abnormalities, or whether these differences are associated with the inflammatory status of the skin. Our preliminary data show that BDCA1+ DC from atopic asthma patients have a reduced capacity to produce bioactive IL-12, and in contrast to BDCA1+ DC from psoriasis patients which release similar levels of IL-12p70 compared to healthy controls (data not shown).

In situ immunohistochemical studies revealed that BDCA1+ and BDCA4+ DC are present in the skin under steady-state conditions (healthy controls) and the number of both DC subsets is increased in both dermis and epidermis in lesional AD skin. These data suggest that these subsets may contribute to the cutaneous inflammation observed in these patients.

A recent study indicated that skin epithelial cells, keratinocytes may provide the initial trigger of the allergic immune cascade in AD. Epithelial-derived thymic stromal lymphopoietin (TSLP) not only potently activates DC, but also endows DC with the ability to polarize naive T cells to produce Th2 cytokines. Moreover, TSLP is highly expressed by keratinocytes from patients with both acute and chronic AD in situ. It is, therefore, tempting to speculate that TSLP skin concentrations in AD may reach the bone-marrow and peripheral blood and prime DC progenitors and precursors, respectively. Moreover, it is unclear whether TSLP can trigger the development of AD or this molecule plays a role in the amplification of the disease since TSLP is mainly detected in lesional AD skin. However, it remains to be determined whether TSLP may reduce the production of IL-12 or IFN-α by stimulated BDCA1+ or BDCA4+ DC, respectively, and whether the treatment of AD patients (e.g. glucocorticoids, tacrolimus) may restore the capacity of BDCA1+ and BDCA4+ DC to produce IL-12 or IFN-α, respectively.

In summary, in AD patients, both circulating DC subsets exhibit qualitative, but not quantitative, intrinsic abnormalities. Our observations may be relevant to the increased susceptibility of AD patients to intracellular pathogens and viruses but also may have
important implications for the development of new strategies for the treatment of this chronic skin disease.

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Chapter 6


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