

The atopic skin-like microenvironment modulates the T cell-polarising cytokine production of myeloid DCs, as determined by laser scanning cytometry

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ABSTRACT

Because it is not known exactly when or where myeloid dendritic cells (mDCs) acquire their atopic dermatitis (AD)-specific T cell-polarising ability in patients with this condition, we used laser scanning cytometry (LSC) to determine whether isolated peripheral blood mDCs from AD patients differed from cells from controls in their cytokine expression profiles *de novo* and after stimulation with *Staphylococcus* enterotoxin B (SEB) and thymic stromal lymphopoietin (TSLP), which represents an AD-like microenvironment. Unstimulated mDCs from AD patients showed pluripotent T cell-polarising capacity, and the surrounding skin microenvironment was essential for the distinctive, disease-specific activity of mDCs (Th2-Th22 bias). We also emphasise that LSC is an attractive technique to study the effect of new DC-targeted therapeutic modalities in AD.

KEYWORDS: atopic dermatitis, laser scanning cytometry, myeloid dendritic cells

Background

A dysregulated skin barrier and altered innate and adaptive immune functions play critical roles in the pathogenesis of atopic dermatitis (AD), and myeloid dendritic cells (mDCs) are a vital link between these systems (1, 2). In AD, mDCs are actively involved in the initiation and maintenance of skin inflammation, and the enhanced migration of these cells coincides with the eruption of skin lesions (3). The tissue microenvironment tightly controls mDC activation, which initiates the differentiation and lineage commitment of several skin-infiltrating Th subpopulations (Th2, Th1, and Th22 cells in the chronic phase of AD) (4-6). Characteristic features of the skin microenvironment include skin colonisation with superantigen-producing *Staphylococcus aureus*, which correlates with the severity of the disease, and the presence of thymic stromal lymphopoietin (TSLP), which is strongly overproduced by keratinocytes in lesional and non-lesional skin due to skin barrier dysfunction (7-11).

Question addressed

Although skin-infiltrating Th cell subpopulations and skin DCs are well characterised in the context of AD, it is not known exactly when or where mDCs acquire their ability to polarise Th cells towards the characteristic atopic subtypes. Because recent data indicate that blood CD1c⁺ DCs are considered precursors of tissue mDCs (12), we aimed to investigate whether isolated peripheral blood mDCs from AD patients and healthy controls differ in their cytokine expression profiles *de novo* and to determine how the T cell-polarising capacity of these mDCs is altered after stimulation with *Staphylococcus enterotoxin B* (SEB) and TSLP, which is an AD skin-specific tissue microenvironment.

Experimental design

Myeloid DCs were cultured in the absence (unstimulated) or presence (stimulated) of SEB and TSLP. In addition to detecting the activation of mDCs (DAPI, CD83, CD86 staining), our aim was to analyse combinations of mDC-derived cytokines/chemokines that have well-characterised roles in the lineage commitment of the different Th cell subtypes, i.e., IL-12

indicates Th1 polarisation; IL-2, IL-4, and CCL17 indicate Th2 polarisation (13, 14); IL-6 and TNF α indicate Th22 polarisation (15, 16); TGF β 1, IL-23, and IL-6 indicate Th17 polarisation (17); and TGF β 1 and IL-10 indicate Treg polarisation (18).

See supporting information for details (Appendix S1).

Results

Under unstimulated conditions, mDCs from atopic patients produced higher levels of IL-2, CCL17, IL-6 and TNF α (Table S1, Figure 1a/Box1), and the alterations in CCL17 and TNF α expression were statistically significant. In the cases of IL-12p40/p70 (Table S1, Figure 1a/Box2), IL-10, TGF β 1 and IL-23p19 (Table S1, Figure 1a/Box3), no differences between the two examined unstimulated groups were observed.

After stimulation with SEB and TSLP, we observed 3 different patterns of cytokine production by mDCs from AD patients compared to controls. First, the levels of previously elevated cytokines (IL-2 and CCL17, which indicate Th2 polarisation; IL-6 and TNF α , which indicate Th22 polarisation) further increased, and the differences became statistically significant, except that for CCL17, when atopic mDCs were compared to control cells (Table S1, Figure 2a-d, f/Box1). Second, the IL-12p40/p70-producing capacity (Th1 polarisation) of mDCs from atopic donors significantly decreased relative to that of control cells (Table S1, Figure 2e, f/Box2). Third, the expression levels of IL-10, TGF β 1 and IL-23p19 by stimulated mDCs did not differ between healthy individuals and AD patients (Table S1, Figure 2f/Box3). IL-4 was not expressed by either of the mDC populations under any culture conditions (not shown).

A greater population of larger, more active cells with decondensed nuclei was generally observed in AD patients than in healthy individuals, and these cells were identified by low DAPI intensities (Figure 1b and e). In general, the observed changes in the cytokine profiles could be assigned only to the larger cells with decondensed chromatin (Figure 1d, dark grey population). After stimulation, further chromatin decondensation was observed in both examined groups (Figure 1c and f). Despite the fact that these nuclear changes most likely reflect increased transcription, mDCs derived from patients and controls all remained in the G0/G1 phase of the cell cycle (Figure 1g).

Discussion

First, the results of this laser scanning cytometry-aided study support the hypothesis that unstimulated, circulating mDCs from AD patients seem to have the ability to produce all Th cell-polarising cytokines, similar to control cells, although atopic mDCs showed biased elevation of cytokines for Th2 (IL-2, CCL17) and Th22 (IL-6, TNF α) polarisation relative to non-atopic control cells.

Second, our present study indicates that mDCs from AD patients and healthy controls respond differently to the specific, AD skin-like tissue microenvironment. When mDCs were cultured in the presence of SEB and TSLP, which we used to create a model of an AD tissue environment, the biased alterations in the levels of Th2- and Th22-polarising cytokines became statistically significant in cells from atopic patients relative to those from healthy controls. Fujita et al. also confirmed the pluripotent Th cell (Th1, Th2, Th17, Th22)-polarising capacity of freshly isolated skin-derived resident and inflammatory DCs, and they emphasised that the recruitment of disease-specific Th subsets is determined primarily by the microenvironment associated with chronic inflammation in AD (14). In an analogous experimental system, Reefer et al. demonstrated that in DC-T cell co-cultures after TSLP, allergen or SEB stimulation, Th cells from AD patients produce Th2-type cytokines, although that study did not investigate Th22 cytokine expression (19). In our experiments, we also observed that unstimulated mDCs had a similar capacity to polarise Th1 cells in atopic and non-atopic individuals; however, this ability of atopic mDCs became impaired after stimulation. Consistent with the results of our study, Lebre et al. found that CD1c⁺ DCs exhibit aberrant functions in AD patients because these cells could not induce a Th1 immune response, even in the presence of a strong Th1 stimulus (20).

Due to the prevalent initiator but complex nature of mDCs in the skin's immune system, many therapeutic approaches target these cells via modifications of their function or the surrounding tissue microenvironment, i.e., topical treatment of AD with calcineurin inhibitors and corticosteroids (21, 22). Therefore, it is important to develop methods that are suitable for the investigation of the direct effects of newly developed therapies on DCs. Consequently, we want to emphasise a third point: that LSC is a useful technique to monitor mDC functions *in vitro*.

Conclusions

Because of the low number of mDCs in the peripheral blood and the limited blood sample volume that is available, especially in childhood, it is challenging to directly investigate the function of these cells from patients. To our knowledge, we are the first to directly determine the intracellular cytokine profile and activation of circulating mDCs in AD patients using multiparametric laser scanning cytometry (LSC). Measurements utilising this slide-based technique allow for the analysis of specimens with low cell numbers (10^5 or less) but provide results with statistical relevance.

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Conflict of interests

The authors state that there are no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Materials and methods.

Table S1. Data on patients, activation and maturation markers and cytokines.

Figure 1. Intracellular cytokine production by unstimulated mDCs from AD patients and healthy controls and nuclear morphology of unstimulated and stimulated cells

(a) Laser scanning cytometric analysis of the expression of intracellular cytokine/chemokine combinations in unstimulated mDCs from atopic and non-atopic donors. The cytokines are grouped based on their known T cell-polarising features and on the 3 different patterns of changes in their intracellular expression levels. Box 1: IL-2, CCL7, IL-6 and TNF α , which induce Th2 and Th22 polarisation; Box 2: IL-12p40/p70, which induces Th1 polarisation; and Box 3: IL-10, TGF β 1 and IL-23p19, which induce Th17 and Treg polarisation. (b, c) Analysis of nuclear morphology based on the fluorescent intensity of DAPI in unstimulated and stimulated cells from healthy donors and atopic patients (e, f). (d, g) Determination of the DNA content by calculating the integral of fluorescence over the nucleus (DNA index, DI). The DNA content of each cell was normalised to the average DNA content of G0/G1 phase cells, and the extent of DNA condensation was estimated using an accepted LSC method that was based on the brightest pixel intensity of the nuclear DAPI fluorescence. mDC activation did not require cell division: the DI of unstimulated, small cells (light grey, g1 gate, in upper left direction in panel d) was 1 (indicated in panel g); and the DI of unstimulated, large mDCs (dark grey, g2 gate, in lower right direction in panel d) was 1.21 (shown in panel g). There were no mDCs in G2/M phase (DI=2). The data are presented as averages with their SEM values for eight healthy controls (n=8 for HC) and eleven patients (n=11 for AD). Significance was calculated using a one-tailed Student's t-test for two independent populations according to the following criteria: p<0.05 for * and p<0.025 for **. One- and two-parameter histogram plots were selected from the records of representative patients and healthy controls.

Figure 2. Intracellular cytokine production by SEB- and TSLP-stimulated mDCs from AD patients and healthy controls

(a-e) Scattergrams of the examined cytokines/chemokines based on their cellular fluorescence intensities in stimulated samples for AD patients relative to the intensities in cells from healthy controls. The protein content of a cell, on the ordinate, was estimated based on the integral intensity of that protein's label, which is the sum of the fluorescence pixel intensities for the given event. The protein aggregate of a cell, on the abscissa, approximates the highest cytokine concentration within a cell and was

estimated based on the highest fluorescence pixel intensity value for an event. (f) Column graph of the different intracellular cytokines/chemokines after SEB and TSLP stimulation in AD patients and healthy individuals. (a-d, f/Box1) Expression of the Th2-polarising cytokines IL-2 and CCL17 and the Th22-priming cytokines IL-6 and TNF α . (e, f/Box2) Expression of the Th1-inducing cytokine IL-12p40/p70. (f/Box3) Expression of IL-10, TGF β 1 and IL23p19. The details of the statistical calculations and the sample size are specified in the legend of Fig. 1. Two parameter plots were selected from the records for a representative patient and healthy control.

Figure 1.

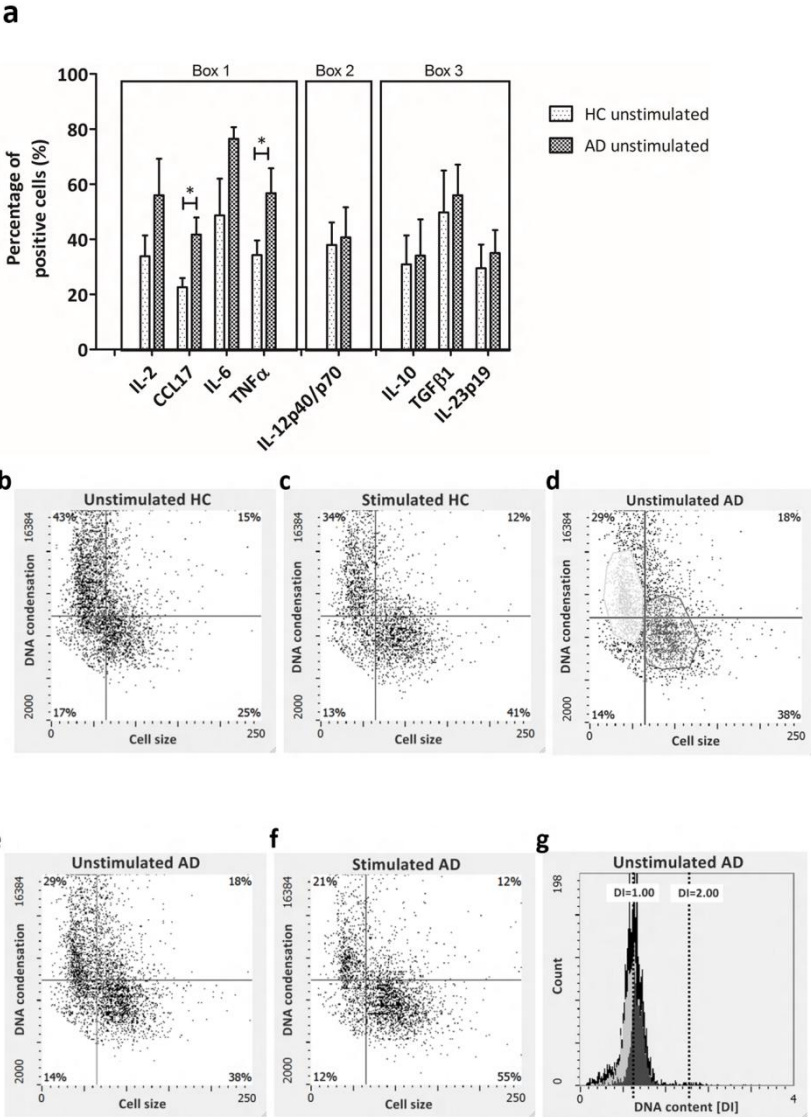
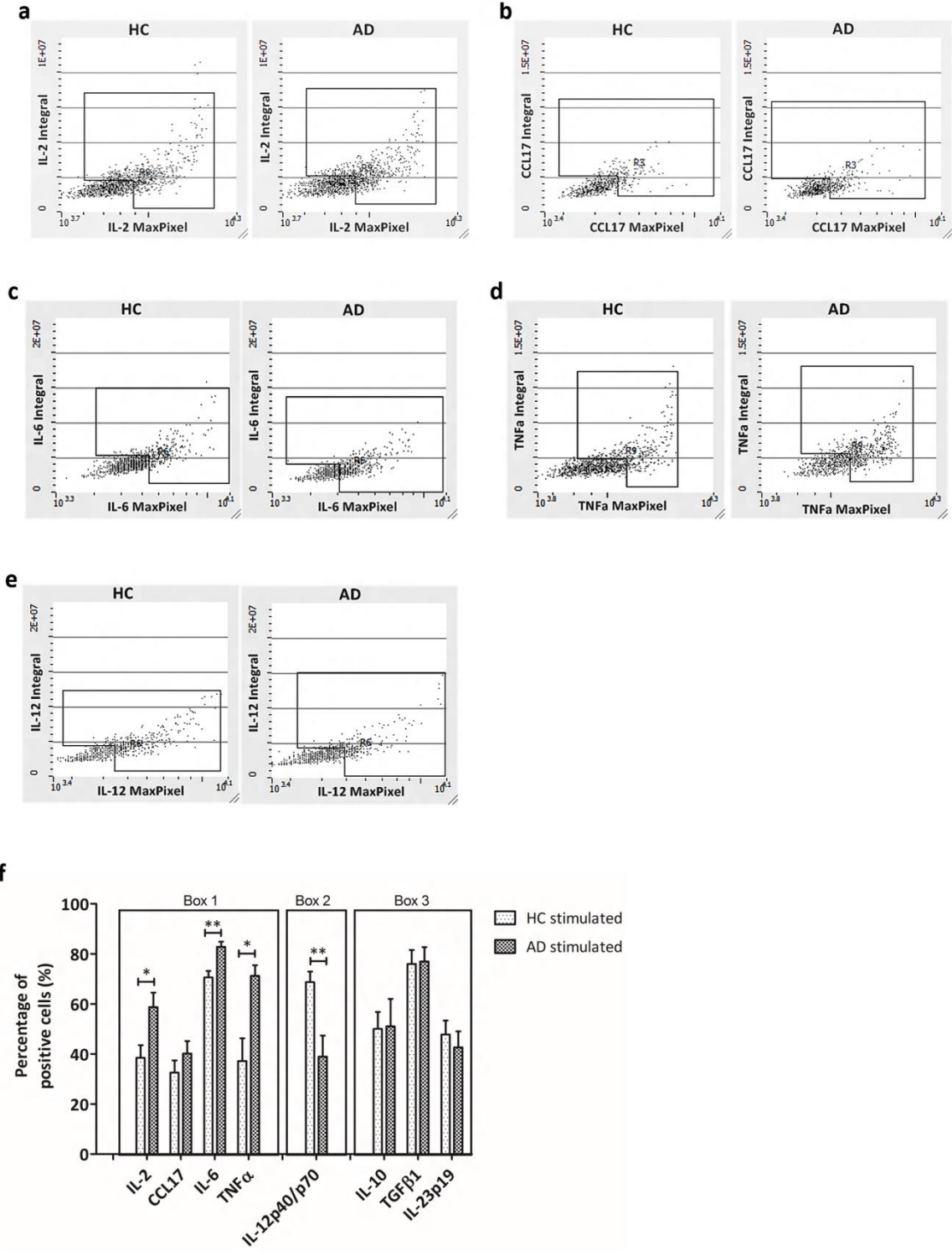


Figure 2.



Supporting information

Appendix S1.

MATERIALS AND METHODS

Patients

Eleven patients with AD and 8 age-matched healthy controls were enrolled in this study (Table S1). All patients fulfilled the diagnostic criteria established by Hanifin and Rajka (1). Informed consent was obtained from all of the participants according to the principles of the Declaration of Helsinki, and the study was approved by the local Ethics Committee of the University of Debrecen, Hungary. The activity of the disease was determined using the subjective SCORAD index, and the sensitivity of the patients was assessed based on the total serum IgE level (Table s1). In all patients, Hyper IgE Syndrome (HIES) was excluded according to the HIES clinical scoring system (2). Patients who were enrolled in this study had severe skin symptoms and had not been treated with oral glucocorticosteroids or other systemic immunomodulatory agents for at least four weeks. They also had not used antihistamines or topical corticosteroids for at least five days prior to blood sampling.

Cell isolation and cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained using Ficoll Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation from the peripheral blood of AD patients and healthy controls. mDCs were isolated from PBMCs using the CD1c (BDCA-1)⁺ Dendritic Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, after the depletion of CD19⁺ B cells, CD1c (BDCA-1)⁺ cells were positively selected. Purified mDCs were then cultured in RPMI-1640 medium (Miltenyi Biotec GmbH) supplemented with 10% FBS (Lonza Group Ltd, Basel, Switzerland) and antibiotics (PAA Laboratories GmbH, Pasching, Austria) for 48 hours in the presence (stimulated) or absence (unstimulated) of 30 ng/ml TSLP (eBioscience Inc., San Diego, CA, USA) and 100 ng/ml SEB (Sigma-Aldrich CO, St Louis, MO, USA).

Cell surface marker and intracytoplasmic cytokine staining

We analysed mDC-derived cytokines/chemokines that have well-characterised roles in the lineage commitment of the different Th cell subtypes, i.e., IL-12, which indicates Th1 polarisation; IL-2, IL-4 and CCL17, which indicate Th2 polarisation (3, 4); IL-6 and TNF α , which indicate Th22 polarisation (5, 6); TGF β 1, IL-23 and IL-6, which indicate Th17 polarisation (7); and TGF β 1 and IL-10, which indicate Treg polarisation (8). Cytokine production by unstimulated and stimulated cells was analysed after 6 hours of incubation with 3 μ g/ml brefeldin A solution to inhibit cytokine secretion. After incubation, the cells were harvested, washed with FACS buffer (phosphate-buffered saline containing 1% bovine serum albumin) and aliquoted into tubes. The cells were first stained with the cell surface marker-specific antibodies anti-CD11c-APC (L161, BioLegend, San Diego, CA, USA), anti-CD83-FITC (HB15e, BD, Franklin Lakes, NJ, USA) and anti-CD86-PE (IT2.2, BioLegend) for 30 minutes in the dark at 4°C. The stained cells were then washed with FACS buffer and fixed by adding 100 μ l of IC Fixation Buffer (eBioscience) for 20 minutes in the dark at room temperature (RT). Then, the cells were washed with 1x Permeabilisation Buffer (eBioscience), and after centrifugation, the pellet was resuspended in 100 μ l of 1x Permeabilisation Buffer and stained with various combinations of the following intracellular cytokine-specific antibodies: anti-IL-10-PE (JES3-19F1), anti-IL-2-PerCP-Cy5.5 (MQ1-17H12), anti-IL-4-PerCP-Cy5.5 (MP4-25D2), anti-IL-6-FITC (MAb11), anti-TNF α -PerCP-Cy5.5 (MQ2-13A5) (BioLegend), anti-IL-23p19-PE (23dcdp, eBioscience), anti-IL-12(p40/p70)-FITC (C11.5, BD), anti-TGF β 1-CFS (9016) and anti-CCL17/TARC-PE (54015) (R&D Systems, Minneapolis, MN USA). Then, the cells were incubated in the dark at RT for 20 minutes and washed first with 1x Permeabilisation Buffer and subsequently with FACS buffer. After centrifugation, the pellet was resuspended in 30 μ l of FACS buffer and loaded into the channels of an ibidi μ -Slide VI (ibidi GmbH, Martinstried, Germany). Before storage, the channels were filled with 150 μ l of FACS buffer, and, prior to measurement, 3 μ g/ml DAPI (Sigma-Aldrich) was added for 20 minutes at RT. Because we did not observe any significant changes in the fluorescence intensities of the stained cells for any of the antibodies in the presence or absence of an Fc receptor blocker (FcR Blocking Reagent, Miltenyi Biotec GmbH), unstained cells served as a negative control.

Laser scanning cytometry

To acquire and analyse multiparametric cytometry data from a limited number of cells, an iCys Research Imaging Cytometer and the accompanying software were utilised (iNovator Application Development Toolkit and iBrowser Data Integration Software, Compucyte Corporation, Westwood, MA, USA). Cells in an ibidi slide were mounted onto a computer-controlled, stepper motor-driven stage. The following lasers were used in multi-track mode to separately excite the fluorophore-conjugated antibodies: a 405-nm violet diode laser for DAPI; a 488-nm argon-ion laser for FITC, CFS, PE and PerCP-Cy5.5; and a 633-nm HeNe gas laser for APC dyes. Emission from an area of $0.06125 \mu\text{m}^2$ (sampling area of one pixel of $0.25 \mu\text{m} \times 0.245 \mu\text{m}$ size) of the sample was collected through an Olympus 40x objective (0.75 NA) using photomultiplier tubes (PMT). With the appropriate emission filters, each PMT monitored a specific fluorescence channel; for example, DAPI was detected at 463 ± 20 nm, FITC and CFS were detected at 530 ± 15 nm, PE was detected at 580 ± 15 nm, and PerCP-Cy5.5 and APC were detected at 675 ± 25 nm. Cellular events were segmented independent of the cytokine fluorescence when the sum of the scatter signal from the membranes and the DAPI signal from the nuclei was higher than a pre-set threshold value. The fluorescence intensities of all fluorochromes and the other cellular properties inside the contour line were measured and presented as imaging and cytometric parameters. The following parameters were analysed: Integral, which is the sum of the pixel intensities for a given event that provides information about the cellular level of the labelled protein; Max pixel, which is the highest pixel value for an event that provides information about the highest concentration of the label in a cell; Area, which is the area bounded by the contour line in square micrometres; and cell location. From these parameters, data regarding cytokine production, cell and nuclear size alterations, cell cycle phase (according to the DNA content) and DNA condensation could be assessed. To calculate the DNA index (DI), which indicates how many times larger the DNA content of a cell is than the DNA content of a G0/G1 diploid cell, G0/G1 phase cells were assumed to have a DI equal to 1. The level of DNA condensation was estimated using the Max pixel of the nuclear DAPI fluorescence (9). To exclude non-relevant events, a multiple-step gating strategy was developed. First, cell clusters and debris were removed based on cell area and CD1c positivity, and this filtering was confirmed by the visual identification of cellular events after relocation. The differentiation stage of the mDCs was then determined according to the level of nuclear condensation. Finally, data on the

cytokine expression and membrane markers of at least 1000 gated cells were collected and analysed on a cell-by-cell and a cell-population basis using statistical software (GraphPad Prism and Microsoft Excel). A compensation matrix was generated according to the iCys manual and BD's recommendations using polystyrene microparticles from the BD CompBeads Set Anti-Mouse Ig, κ (BD Biosciences).

Statistics

The data were normally distributed, significant differences were identified using an unpaired Student's t-test with GraphPad Prism version 5 (GraphPad, San Diego, CA, USA), and the data are presented as the means \pm SEM. P values less than 0.05 (*) or 0.025 (**) were considered statistically significant.

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Table S1. Data on patients, activation and maturation markers and cytokines.

	Healthy controls (n=8)		Atopic dermatitis patients (n=11)		P value	
Age (years)	24±3.71		19.8 ±5.44		NS	
Gender						
Female	5 (62.5%)		5 (45.4%)		n/a	
Male	3 (37.5%)		6 (54.6%)		n/a	
Development of the disease						
Before age 2	None		4 (36.4%)		n/a	
After age 2	None		7 (63.6%)		n/a	
SCORAD	None		52±9		n/a	
Serum total IgE (KU/L) (Ref.: >87 KU/L – >9 years)	-		2002.2±1984.5		n/a	
LDH (U/L) (Ref.: 135-220 U/L)	<220 U/L		480.3±138.1		n/a	
Eosinophil count (G/L) (Ref.: 0.01-0.6 G/L)	<0.6 G/L		1.16±1.4		n/a	
Asthma, allergic rhinitis	None		7 (63.6%)		n/a	
Maturation and activation markers						
	HC	AD		HC	AD	
	Unstimulated	Unstimulated	P value	Stimulated	Stimulated	P value
CD86 (%positive cells)	63.5±4.7	36.5±8.3*	NS	72.3±3.5	70.5±3.0*	*0.0153
CD83 (%)	42.5±3.9	46.5±9.8**	NS	48.3±11.4	75.8±2.6**	**0.0286
IL-2 (%)	33.8±7.6	55.9±13.3	NS	38.5±5.0	58.7±5.8	0.0192
CCL17 (%)	22.6±3.3	41.7±6.2	0.0265	32.5±4.9	40.2±4.9	NS
IL-6 (%)	48.6±13.3	76.5±4.2	NS	70.6±2.6	82.8±2.1	0.0053
TNFα (%)	34.3±5.3	56.8±9.0	0.0489	37.2±9.1	71.2±4.2	0.0136
IL-12p40/p70 (%)	37.9±8.2	40.7±10.9	NS	68.7±4.3	38.9±8.4	0.0080
IL-10 (%)	30.9±10.5	34.1±13.2	NS	50.0±6.7	51.0±10.9	NS
TGFβ1 (%)	49.7±15.2	55.9±11.1	NS	75.9±5.5	76.9±5.7	NS
IL-23p19 (%)	29.5±8.6	35.0±8.3	NS	47.8±5.6	42.7±6.4	NS

n/a: not applicable