Myeloid but not plasmacytoid blood DCs possess Th1 polarizing and Th1/Th17 recruiting capacity in psoriasis

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Abstract

Psoriasis is a common inflammatory skin disease and dendritic cells (DCs) play crucial role in the development of skin inflammation. Although the characteristics of skin DCs in psoriasis are well defined, less is known about their peripheral blood precursors. Our aim was to characterize the phenotypic features as well as the cytokine and chemokine production of $CD1c^+$ myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in the blood samples of psoriatic patients. Blood DCs were isolated by using a magnetic separation kit, and their intracytoplasmic cytokine production and CD83/CD86 maturation/activation marker expression were investigated by 8-colour flow cytometry. In $CD1c^+$ mDCs the intracellular productions of Th1, Th2, Th17, Th22 and Treg polarizing cytokines were examined simultaneously, whereas in pDCs the amounts of IFNα as well as IL-12, IL-23 and IL-6 were investigated. The chemokine production of both DC populations was investigated by flow-cytometry and ELISA. According to our results psoriatic $CD1c^+$ mDCs were in a premature state since their CD83/CD86 maturation/activation marker expression, IL-12 cytokine, CXCL9 and CCL20 chemokine production was significantly higher compared to control cells. On the other hand, blood pDCs neither produced any of the investigated cytokines and chemokines nor expressed CD83/CD86 maturation/activation markers. Our results indicate that in psoriasis not only skin but also blood mDCs perform Th1 polarizing and Th1/Th17 recruiting capacity, while pDCs function only in the skin milieu.

Keywords: psoriasis, dendritic cell, cell surface marker, T cell polarizing cytokine, chemokine

Abbreviations: mDCs/pDCs/DDCs, myeloid/ plasmacytoid/ dermal dendritic cells; IL, interleukin; IFNα, interferon alpha; TNFα, tumor necrosis factor alpha; iNOS, inducible nitric oxide synthase; PASI, Psoriasis Area and Severity index; PBMCs, peripheral blood mononuclear cells; FSC/SSC forward and side light scattering ; RT, room temperature;

1. **Introduction**

Psoriasis is a common chronic inflammatory skin disease affecting approximately 2% of the population worldwide [1-3]. Both activated dendritic cells (DCs) and T cells play a crucial role in the pathogenesis of this disease, since their abnormal activation and a Th1/Th17 cytokine environment lead to the typical exaggerated proliferation of keratinocytes, which is characteristic to psoriasis [4].

Skin DCs contribute both to the initiation and to the maintenance of the disease [4]. Plasmacytoid DCs (pDCs), which are currently considered to promote inflammation after being activated by LL-37/self-DNA complexes, are the main sources of IFNα [5], which induce the IFNα dependent maturation of myeloid DCs (mDCs) [6]. A 30-fold increase in the number of CD11c$^+$ mDCs was detected in the dermal compartment of psoriatic skin lesions compared to uninvolved or normal skin [7], and these cells are responsible for further sustenance and amplification of the T cell mediated inflammatory processes [8-10]. In psoriasis the mDCs can be divided into two distinct populations: CD11c$^+$ CD1c$^-$ inflammatory DCs, also known as TIP-DCs and the CD11c$^+$ CD1c$^+$ resident DCs, also known as dermal DCs (DDCs) [11]. TIP-DCs, beyond their TNFα and iNOS production [7,12-14], together with the so called "background" CD1c$^+$ DDCs, are important sources of Th17 activating cytokine IL-23 [10,11]. Moreover CD1c$^+$ DDCs are potential producers of the Th1 polarizing cytokine IL-12 [7,14,15].

In addition to their T cell polarizing cytokine production DCs are important sources of chemokines. Psoriatic skin exhibits increased expression of several chemokines [16-18], and skin DCs were found to produce CXCL1, CXCL8, CXCL9, CXCL10 as well as CCL20 chemokines [15]. CXCL1, CXCL9 and CXCL10 are chemoattractants for Th1 lymphocytes, CXCL8 recruits neutrophils, while CCL20 attracts Th17 lymphocytes, DCs and monocytes

[19]. The precise contribution of skin DC subsets to the chemokine secretion is not clearly defined, but they are considered to be released by Langerhans cells, DDCs, TIP-DCs and also by pDCs [15,16,19,20].

Taken together, skin DCs are important in the development of psoriatic skin inflammation. At the same time their blood precursors and their phenotypic and functional characteristics in psoriasis are scarcely explored. Since it has emerged that peripheral blood pre-DCs can serve as precursors of not just DDCs, but also of TIP-DCs [21,22] [17,23] , the relevance of their characterization is essential.

We aimed to examine the characteristic features of psoriatic blood CD1c^{+} mDCs and pDCs focusing on their activation and maturation stages, as well as on their cytokine and chemokine release. Our results indicate that not only skin but also peripheral blood mDCs derived from psoriatic patients are able to produce disease-specific Th1/ Th17 cytokines and chemokines, while pDCs seem to function only in the skin milieu.

2. Materials and methods

2.1. Patients and controls

Peripheral blood was obtained from patients with psoriasis (n=21, 13 males, 8 females, mean age: 51.5±13.8 years,) and from healthy controls (n=17) matching in gender and age. Randomly selected patients took part in the CD1c$^+$ mDC and pDC investigations or in ELISA measurements, since the number of the extractable cells from one patient limited the number of experiments/patients. 12 patients /9 controls were recruited to the cytokine examination of CD1c$^+$ mDCs, 5 patients/ 4 controls to the pDCs' investigations together with the chemokine investigations of CD1c$^+$ mDCs, while 4 patients/4 controls were involved in the ELISA experiments. All patients who took part in this study had moderate-to-severe psoriasis, based on Psoriasis Area and Severity index (PASI) calculation (mean PASI: 23.9±10.1) and had received neither systemic treatment nor phototherapy for their psoriasis at least 4 weeks prior to blood sampling. Informed consent was obtained from all participants according to the Declaration of Helsinki principles. The local Ethics Committee of the University of Debrecen, Hungary, approved the study.

2.2. Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque Plus (GE Healthcare Bio-Science AB, Uppsala, Sweden) gradient centrifugation from the peripheral blood of both psoriatic patients and healthy controls. Blood DCs containing both CD1c$^+$ myeloid DCs and pDCs were isolated from PBMCs using the Blood Dendritic Cell Isolation Kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. For ELISA examinations CD1c$^+$ DCs were separated using a CD1c$^+$ Blood DC isolation kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Purified blood

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DCs were cultured in RPMI 1640 (Miltenyi Biotech GmbH) medium supplemented with antibiotics (PAA Laboratories GmbH, Pasching, Austria) and 10% FBS (Lonza Group Ltd, Basel, Switzerland) for 6 hours. For chemokine ELISA experiments supernatants of purified CD1c$^+$ mDCs were collected after 24h culturing. 1x 10^5 dendritic cells were cultured in 200 μl cell culture medium in a well of a 96 well-plate with flat bottom. Since our aim was to detect the characteristics of DCs in physiological conditions, moreover we could detect prominent cytokine and chemokine production even without stimulation, no further stimulations were applied during the examinations.

2.3. Cell surface marker and intracytoplasmic cytokine staining

Cytokine/chemokine production of the cells was analysed after 6 hours of incubation with 3μg/ml Brefeldin A (eBioscience Inc, San Diego, CA, USA) solution for the inhibition of their secretion. After incubation, the cells were harvested, washed in phosphate-buffered saline (PBS) with 5% FBS and 0.5mM EDTA, and then resuspended in FACS buffer (PBS containing 1% bovine serum albumin) and aliquoted into tubes (one for the negative control, four for the staining). Cells were first stained with the following fluorescent dye-conjugated monoclonal antibodies against the following cell surface markers: anti-CD1c-APC-Cy7 and anti-CD11c-APC or anti-CD11c-FITC to identify CD1c$^+$/CD11c$^+$ mDCs, anti-CD303-PerCP-Cy5.5 and anti-CD304-Pacific Blue to identify pDCs, and anti-CD86-PE or anti-CD83-PerCP-Cy5.5 to examine the maturation or activation state. Cells were stained for 30 minutes in the dark at 4°C. Stained cells were washed with FACS buffer and fixed by Intracellular (IC) fixation buffer (eBioscience, San Diego, CA, USA) for 20 minutes in the dark at room temperature (RT). Then cells were washed with 1x Permeabilization Buffer (eBioscience) and after centrifugation, the pellet was resuspended in 100μl 1x Permeabilization Buffer and

stained with assorted combinations of the following intracellular cytokine/chemokine specific antibodies: PE labelled human monoclonal antibodies against TGFβ, IL-23p19, IFN-α, CCL17, CXCL1, CXCL10; FITC labelled antibodies against IL-6, IL-12, IL-2, CXCL8; PerCP-Cy5.5 labelled IL-10, IL-4, TNFα and APC labelled anti-human CXCL9 and CCL20. All the antibodies are from Biolegend, San Diego, CA, USA, except for TGFβ, CCL17, CXCL1, CCL20 which are from R&D Systems, Minneapolis, MN, USA. Then the cells were incubated in the dark at RT for 20 minutes and washed first with 1x Permeabilization Buffer and subsequently with FACS Buffer. After centrifugation, the pellet was resuspended in 200 μl FACS buffer and kept in 4°C until measurement. Unstained cells served as negative control.

2.4. Flow cytometry

Flow cytometric measurements were carried out on fixed cells on a Beckman Coulter Navios cytometer (Beckman Coulter) applying eight colour flow cytometric protocol. Typically 100,000–500,000 cells were acquired, depending on the yield of cell isolation. Forward (FSC) and side light scattering (SSC) were used to detect cellular objects and to exclude debris and clustered cells. Dendritic cell subtypes were gated on the basis of the following backbone markers' expression: CD11c, CD1c, CD304, CD303. Expression of cytokines/chemokines and activation markers were investigated in the gated pDC or CD1c^{+} mDC populations. List mode data (LMD) files were analyzed using Navios software v. 1.1 and Kaluza^{TM} software version 1.2 (Beckman Coulter). Histograms were plotted on 'logicle' axes in Kaluza software.

2.5. Identification of the secreted chemokines by ELISA

The exact amount of the psoriasis related CXCL8, CXCL9, CXCL10 and CCL20 chemokines was also analysed using CXCL8/IL8, CXCL9/MIG, CXCL10/IP10 and CCL20/MIP-3α Quantikine ELISA Kits (R&D Systems), all according to the manufacturer's instructions. The supernatants of $CD1c^+$ mDCS originated from 4 patients and 4 controls were investigated. The detection limits in Quantikine ELISAs are: CXCL1- 10 pg/ml, CXCL8- 3.5 pg/ml, CXCL9- 3.84pg/ml, CXCL10- 1.67 pg/ml. CCL20- 0.47pg/ml.

2.6. Statistical analysis

To assess the distribution of the data, the Kolmogorov–Smirnov test was used. Because of their normal distribution, we determined mean ± standard error of mean (SEM) values. We used the independent t-test for statistical comparison of two or more experimental data, respectively. Differences were considered to be significant when $p < 0.05$. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Analysis of correlations was performed by Spearman r test. SPSS ver. 18.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

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3. Results

3.1. Psoriatic blood CD1c$^+$ mDCs exhibit premature state and produce IL-12

From the peripheral blood of patients with psoriasis and healthy volunteers CD1c$^+$ mDCs and pDCs were separated parallel by using Blood DC isolation kit. To examine the phenotypic features and the cytokine/chemokine production of the CD1c$^+$ mDCs, CD1c$^+$/CD11c$^+$ DCs were gated from this DC "cocktail".

The CD83 maturation marker appeared significantly more frequently on the surface of psoriatic blood CD1c$^+$ mDCs compared to control cells (74.9 % vs. 30.42 %, respectively, p=0.0103 Figure 1.A). Most of the cells in both groups expressed CD86 activation marker, but significantly increased fluorescence intensity could be detected in the psoriatic group (CD86 MFI 3.68 vs. 3.28 respectively, p=0.0445 Figure 1.B).

In psoriatic blood CD1c$^+$ mDCs Th1 (IL-12), Th2 (IL-2, CCL17), Th17 (TGFβ, IL-23, IL-6), Th22 (IL-6 and TNFα), and Treg (TGFβ and IL-10) polarizing cytokines were simultaneously detected by flow-cytometry using an 8-color staining method. Significantly higher percentage of psoriatic CD1c$^+$ mDCs produced IL-12 compared to control cells (68.9%. vs. 12.8%, respectively, p=0.013 Figure 2.A.). Not only the number of IL-12 positive cells, but also the amount of IL-12 in these cells was significantly higher (IL-12 MFI: 1.01 vs. 0.75 in psoriatic CD1c$^+$ mDCs vs. control cells, p=0.008, Figure 2. B.). Furthermore the mean fluorescence intensity of IL-12 correlated significantly with the clinical severity index of patients (PASI) (Spearman test, r=0,6110; p=0.0494; Figure 2.C), and the number of IL-12 producing cells and PASI also showed some, but not significant correlation (data not shown).

3.2. Psoriatic blood pDCs are inactive and exhibit an immature phenotype

To investigate the maturation/activation status and the cytokine production of blood pDCs, CD11c$^-$/CD1c$^-$/ CD303$^+$ (BDCA2$^+$) /CD304$^+$ (BDCA4$^+$) cells were gated from the "DC cocktail" obtained after using Blood DC isolation kit.

Blood pDCs exhibited an inactive phenotype, since they could not express either CD83 maturation or CD86 activation markers reflecting the maturation/activation status of the cells. When examining the function of these cells we focused on the detection of IL-12, IL-23 and IL-6, as psoriasis-related cytokines and also on IFNα, the main cytokine produced by pDCs in psoriatic skin. None of these cytokines could be detected in psoriatic blood pDCs.

3.3. Psoriatic blood CD1c$^+$ mDCs, but not pDCs produce Th1/Th17 recruiting chemokines

3.3.1. Investigation of intracytoplasmic chemokine production by flow-cytometry

The chemokine production potential of both psoriatic blood CD1c$^+$ mDCs and pDCs was examined first by flow-cytometry. Chemokines, published earlier to be produced by psoriatic skin DCs [15], were investigated: CXCL1, CXCL8, CXCL9 and CXCL10 as Th1, and CCL20 as Th17 recruiting chemokines.

Although the frequency of chemokine producing DCs were nearly similar in patients and controls (Figure 3. A.), our investigations revealed that psoriatic blood CD1c$^+$ mDCs were able to produce CXCL9 and CCL20 in a significantly higher (CXCL9 MFIs: 3.83 vs. 1.72, p<0.0001, CCL20 MFIs: 3.14 vs. 1.36, p=0.0275 in patient and control groups respectively, Figure 3. B.), whereas CXCL8 and CXCL10 in a slightly higher amount (CXCL8 MFIs: 2.85 vs. 2.56; CXCL10 MFIs: 0.85 vs. 0.78; neither are significant, Figure 3.B.) compared to healthy control cells. The CXCL1 chemokine production was almost equal in the two mDC

groups (CXCL1 MFIs: 1.05 vs. 1.01, not significant). The chemokine production of psoriatic blood pDCs was negligible compared to CD1c$^+$ mDCs (Figure 3.B.) and was similar to control cells.

3.3.2. Measurement of released chemokines by ELISA

Since mDCs were found to produce different Th1/Th17 recruiting chemokines, their release was also investigated by ELISA from the supernatant of CD1c$^+$ mDCs.

Psoriatic blood CD1c$^+$ mDCs were able to release CCL20 in a significantly higher and CXCL8, CXCL10 in a markedly higher amount compared to control CD1c$^+$ mDCs (CCL20 mean concentration: 47.2 pg/ml vs. 4.9 pg/ml, p=0.0042, CXCL8 mean concentration: 29.9 ng/ml vs.14.01ng/ml, not significant; CXCL10 mean concentration: 92.07 pg/ml vs. 25.2 pg/ml, not significant, Figure 4). Interestingly CXCL9 could not be detected at a notable level in the supernatant of either psoriatic or control CD1c$^+$ mDCs, in contrast to the results of the flow-cytometry investigations (data not shown).

4. Discussion

The contribution of distinct skin DC subsets to the pathogenesis of psoriasis was well explored in the last decade [14], while the role of blood DCs is less understood. Since CD1c$^+$ blood mDCs and pDCs are potential precursors of skin DCs, in this study we focused on the examination of the phenotypic characteristics and cytokine/chemokine production of these poorly investigated cells in psoriatic patients [21,22]. Investigating blood precursor DCs can

give information on whether DCs in psoriasis acquire their specific characteristics exclusively in the skin microenvironment or if they are already influenced even in the blood by some disease-specific factors. These questions can be answered by examining DCs directly separated from peripheral blood, instead of applying DCs that have been differentiated *in vitro* from monocytes, and although this method results in fewer cells from the same amount of blood, their investigation can give more information on the physiological condition.

In our experiment we simultaneously investigated the expression of CD83/CD86 maturation and activation markers and the production of Th1, Th2, Th17, Th22 and Treg polarizing cytokines in circulating CD1c$^+$ mDCs. Although blood DCs generally circulate in an inactive form, according to our results psoriatic blood CD1c$^+$ mDCs are in a pre-mature state, since they expressed CD83 and CD86 markers in a significantly higher amount. Furthermore they produced significantly more Th1 polarizing cytokine IL-12, which correlated with the severity of the disease. In psoriasis IL-12, which is considered to be one of the key cytokines, is produced by skin mDCs [7,10,11,14], but our results indicate that their peripheral blood precursors are also potential sources of this cytokine. Although it is well known that psoriatic skin DCs express CD83 and CD86 DC maturation/activation markers [24] and release IL-12 and IL-23 cytokines [7,10,11,14,15], data regarding the phenotype and cytokine production of their peripheral blood precursors are hardly available [25], our results may fill the niche.

The pre-activated status of psoriatic CD1c$^+$ mDCs was confirmed not only by their cytokine production but also by their chemokine secretion, since both CXCL9 and CCL20 were produced in a significantly, while CXCL8 and CXCL10 in a markedly higher amounts compared to control cells. These results suggest that blood CD1c$^+$ mDCs in psoriatic patients have an increased potential to produce Th1/Th17 recruiting chemokines. Our results are

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consistent with the observations of Fujita et al., [15], since they detected a similar Th1/Th17 recruiting chemokine production in psoriatic skin DCs.

In contrast to our observations on CD1c^{+} mDCs, investigations on the activation/ maturation status and on the cytokine/chemokine production of blood pDCs revealed that neither psoriasis related cytokines nor a significant amount of chemokines could be produced by these cells. They also exhibited an inactive phenotype, since the expression of CD83 maturation and CD86 activation markers could not be detected on their surface. The hypothesis suggested by Nestle et al. could be strengthened by our results, that despite the crucial role of skin pDCs in the initiation of psoriasis, their peripheral blood precursors circulate in resting state and became activated locally in the psoriatic skin milieu [5].

Taken together, the characterization of cell surface markers and cytokine/chemokine production of blood precursor DCs in psoriatic patients indicate that while pDCs exhibit a resting phenotype, CD1c^{+} mDCs are in a pre-mature stage, and are able to produce disease-specific mediators even in the peripheral blood. The lack of maturation/activation markers and the functional inactivity of pDCs suggest that their activation is performed only in the skin milieu. In contrast, the pre-mature status of psoriatic CD1c^{+} mDCs proven by their phenotypic and functional properties suggests that their maturation is markedly influenced by the microenvironment even in the blood and not only in the skin milieu, although this proinflammatory blood milieu is probably the consequence of the primarily occurring skin inflammation. The bloodstream of psoriatic patients suffering from severe skin lesions contains a unique set of cytokines, such as TNF-α, IFNγ, IL-6, IL-8, IL-12 and IL-18 [26]. Among them TNF-α and IL-6, together with IFN-α may allow blood pre-mDCs to reach this pre-mature state [3], and these pre-activated cells can be further activated easily and rapidly after entering the skin leading to a vicious circle. Novel biological agents (*e.g.* anti-TNF-α

therapy) can have a doubly beneficial impact by modifying the effect of these cytokines both in the skin and in the blood of the patients, thus influencing the maturation and the function of the pre-DCs in the blood.

5. Conclusion

In this study we have examined the characteristic features of psoriatic blood $CD1c^+$ mDCs and pDCs focusing on their activation and maturation stages, as well as on their cytokine and chemokine release. Our results indicate that not only skin but also peripheral blood $CD1c^+$ mDCs derived from psoriatic patients are able to produce disease-specific Th1/ Th17 cytokines and chemokines, while pDCs seem to function only in the skin milieu.

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The authors declare no conflicts of interest.

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Figure 1. Phenotypic characterization of blood mDCs in psoriasis. A. The CD83 maturation marker appeared significantly more frequently on the surface of psoriatic blood mDCs compared to control cells. **B.** Significantly increased CD86 fluorescence intensity could be detected in the psoriatic group. Columns represent the mean ± SEM of 12 patients and 9 controls. * p<0. 05, ** p<0.01

Figure 2. Cytokine production of blood mDCs in psoriasis. A. The percentage of IL-12 producing mDCs was significantly higher in psoriasis compared to controls. Percentages of cytokine producing cells are described using mean ± SEM; **B.** Not only the number of IL-12 positive cells, but also the amount of IL-12 produced by these cells was significantly higher (IL-12 MFI: 1.01 vs. 0.75 in psoriatic mDCs vs. control cells, p=0.008). **C.** The mean fluorescence intensity of IL-12 in mDCs significantly correlated with PASI. (Spearman test, r=0.6110; p=0.0494).

patients n=12; controls n=9; **p<0.01; *** p<0.001

Figure 3. Chemokine production of blood mDCs and pDCs investigated by flow-cytometry. A. The frequency of chemokine producing DCs were nearly similar in patients and controls. **B.** Blood CD1c$^+$ mDCs produced CXCL9 and CCL20 in a significantly higher amount. The chemokine production of pDCs was negligible compared to mDCs. Columns represent the mean ± SEM of five patients and four controls.

* p<0.05, *** p<0.001

Figure 4. Chemokine production of blood CD1c+ mDCs investigated by ELISA.

Psoriatic CD1c$^+$ mDCs produced CCL20 in a significantly and CXCL8 and CXCL10 in a markedly higher amount than control DCs. Columns represent the mean ± SEM of four patients and four controls. ** p<0.01