Fehres CM¹, Bruijns SCM¹, Sotthewes BN¹, Kalay H¹, Schaffer L², Read SR², de Gruijl TD³, Garcia-Vallejo, JJ¹ and van Kooyk Y¹*

¹Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands
²DNA Array Core Facility, The Scripps Research Institute, La Jolla, CA, USA
³Department of Medical Oncology, VU University Medical Center
Amsterdam,
The Netherlands
*Corresponding author

Submitted
Chapter 2

Phenotypic and functional characterization of human skin antigen presenting cell subsets
Abstract

Cutaneous antigen presenting cells (APCs) are critical for the induction and regulation of skin immune responses. The human skin contains phenotypically and functionally distinct APCs subsets that are present at two separated locations. While CD1a\textsuperscript{high} LCs form a dense network in the epidermis, the CD14\textsuperscript{+} and CD1a\textsuperscript{+} dermal dendritic cells (dDCs) reside in the dermal compartment. A better understanding of the biology of human skin APC subsets is necessary for the improvement of vaccine strategies that use the skin as administration route. In particular, progress in the characterization of uptake and activatory receptors will certainly improve APC-targeting strategies in vaccination. Here we performed a detailed analysis of the expression and function of glycan-binding and pattern-recognition receptors in skin APC subsets. The results demonstrate that under steady state conditions human CD1a\textsuperscript{+} dDCs were phenotypically most mature as measured by the expression of CD83 and CD86, whereas the CD14\textsuperscript{+} dDCs showed a higher expression of the CLRs DC-SIGN, mannose receptor and DCIR and had potent antigen uptake capacity. Furthermore, steady state LCs showed superior antigen cross-presentation as compared to the dDC subsets. Our results also demonstrate that the TLR3 ligand polyribosinic-polyribocytidylic acid (pI:C) was the most potent stimulator of cytokine production by both LCs and dDCs. These studies warrant further exploration of human CD1a\textsuperscript{+} dDCs and LCs as target cells for cancer vaccination to induce anti-tumor immune responses.
Dendritic cells (DCs) are a heterogeneous population of antigen-presenting cells (APCs) that are essential in the induction of adaptive immune responses. Monocyte-derived DCs (moDCs) have been classically used as an in vitro model for human DCs [1]. However, moDCs do not completely resemble steady state tissue resident DCs and are mainly characterized by an inflammatory profile that is hardly found in vivo [2]. Besides, the variety of DC subpopulations described in different human tissues makes it difficult for this in vitro model to fit all possible DC subtypes [3-5]. Because of limitations in the availability of viable APCs from human tissues, still relatively little is known about the functional and phenotypic specialization of the human APC network under steady state conditions and their transition and response towards inflammatory conditions. Amongst all organs, the skin is of particular interest, especially for its potential applications as application route for antigen-specific immunotherapy against cancer[6].

Recent studies have reported functional specializations of the APC subsets found in human skin. At least 3 distinct populations of APCs have been characterized in steady state human skin: epidermal Langerhans cells (LCs) that are characterized by high expression of CD1a, EpCAM, and langerin; and HLA-DR⁺ dermal DCs (dDCs), which can be further subdivided based on the expression of CD14 and CD1a [7]. Human LCs have been described to preferentially induce the differentiation of CD4⁺ T cells to a T helper 2 profile and to induce CD8⁺ T cells responses [8]. Human CD1a⁺ dDCs are phenotypically more mature than CD14⁺ dDCs, respond rapidly to CCL19/CCL21 by migrating to the lymph nodes and showed CD4⁺ and CD8⁺ T cell stimulating capacity [9]. In contrast, unstimulated, steady state CD14⁺ dDCs have been described to secrete IL-10 and induce regulatory T cells (Tregs) and follicular T helper cells (Tfh) [8;10]. Moreover, steady state, CD14⁺ dDCs show a poor ability to stimulate allogeneic T cell proliferation [8;11] and to migrate to lymph nodes [12]. Besides the CD14⁺ and CD1a⁺ dDCs, a minor population of HLA-DR⁺CD1a⁺CD14⁻ double negative (DN) dDCs exists that includes, amongst others, CD141⁺ dDCs [13]. These cells are homologous to murine tissue CD103⁺ and splenic CD8⁺ DCs and are superior in cross-presentation of soluble antigens [12]. Variable expression of CD141 is also found on CD14⁺ dDCs, however, these cells lack the features of CD141 hi dDCs and induce Tregs via the secretion of IL-10 [10]. In addition to DCs, the human dermis also contains a network of tissue-resident CD14⁺ dermal macrophages, which are not able to migrate from the skin [12].

Thus, skin-resident APC subsets play an important role in the polarization of T cell responses and the maintenance of peripheral tolerance via the induction of Tregs. The ability of cutaneous APCs to induce specific T cell responses can be influenced by maturation signals that these cells receive at the time of antigen recognition [8].
Under inflammatory conditions, such as in psoriasis or atopic dermatitis, skin APC numbers, and in particular CD1a⁺ dDCs, are increased, as well as their maturation status [14]. On the other hand, intradermal administration of the anti-inflammatory cytokine IL-10 increased the migration of a CD14⁺CD141⁺ dDC subset that expressed low levels of activatory co-stimulatory molecules, high expression of PD-L1, induced the differentiation of Tregs and, consequently, mediated a poor expansion of CD4⁺ and CD8⁺ T cells [15]. We have previously reported that skin APC subsets hardly mature upon intradermal vaccination with TLR ligands at concentrations that induce the maturation of moDCs in vitro, suggesting that the skin microenvironment favors the maintenance of an anti-inflammatory milieu that may be part of global peripheral tolerance mechanisms [16]. Although several studies have contributed to a better understanding of human skin-resident DC subsets, still questions remain with regard to their detailed phenotypical and functional properties in steady state and their response to inflammatory cues. In this study, we aimed to characterize the glycan-binding receptor and TLR-associated phenotype and related functional properties of human LCs and dDCs under steady state and analyzed how they are affected by inflammatory conditions.

**Material and methods**

**Reagents**
rhGM-CSF and rhIL-4 were obtained from Biosource (Camarillo, CA) and used at concentrations of 262.5 U/ml and 112.5 U/ml, respectively. Polyinosinic:polycytidylic acid (pI:C) was used at a concentration of 20 ug/ml (Invivogen) and LPS (Sigma-Aldrich) at 20 ng/ml. These concentrations were based on optimal maturational effects on moDCs cultured in vitro as previously reported [17].

**Isolation of LCs and dDCs**
Primary human LCs and dDCs were isolated from human skin explants obtained within 24 h after cosmetic surgery from healthy donors and with informed consent (Bergman Clinics, Bilthoven, The Netherlands). Shortly, 5-mm thick slices of skin, containing the epidermis and dermis, were cut using a dermatome. The slices were incubated in Dispase II (1 mg/ml, Roche Diagnostics) in IMDM supplemented with 10% FCS (BioWhittaker), 50 U/ml penicillin (Lonza), 50 μg/ml streptomycin (Lonza) and 10 μg/ml gentamycin (Lonza) overnight at 4°C followed by the mechanical separation of dermis and epidermis using tweezers. The epidermis was washed in PBS, cut into small pieces and incubated in PBS containing DNase I (200 U/ml, Roche Diagnostics) and trypsin (0.05%, Invitrogen) for 30 min at 37°C. After incubation, a single cell suspension was generated using 100 μm nylon cell strainers (BD Falcon) and cells were layered on a Ficoll gradient. An average of 1x10⁴ LCs per cm² of tissue with a purity higher than 90% were obtained and characterized as CD1a⁺ langerin⁺
Phenotypic and functional characterization of human skin antigen presenting cell subsets

Phenotypic and functional characterization of human skin antigen presenting cell subsets by flow cytometry as described below. After separation from the epidermis, the dermis was cut in small pieces and incubated in PBS containing collagenase (6 mg/ml, Roche) and dispase II (1 mg/ml) for 2 h at 37°C. A single cell population was generated using 100 μm nylon cell strainers. After obtaining single cells populations, LC and dDC cell suspensions were cultured in IMDM supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μg/ml gentamycin. When indicated, isolated dDCs and LCs were MACS-sorted using CD1a and HLA-DR microbeads (MACS, Miltenyi Biotec, Germany) or DC subsets were FACS-sorted using a MoFlo cell sorter (Beckman Coulter) and fluorescent antibodies directed against HLA-DR (mlgG2a, clone L203), CD1a (mlgG1, clone HI149) and CD14 (mlgG2b, mMoP9; all from BD, San Jose, CA).

Phenotypic analysis of isolated cells
Phenotypic analysis of isolated skin APCs was performed by flow cytometry. Cells were washed in PBS supplemented with 1 % BSA and 0.02% NaN₃ and incubated for 30 min at 4°C in the presence of appropriate dilutions of fluorochrome-conjugated mAbs to CD1a, CD14, CD70 (mIgG3, clone Ki-24), CD86 (mIgG1, clone 2331), HLA-DR (BD, San Jose, CA), HLA-ABC (mlgG2a, clone W6/32; ImmunoTools, Friesoythe, Germany) or CD83 (mlgG2b, clone HB15e; Beckman Coulter Immunotech), or corresponding isotype-matched control mAbs (BD, San Jose, CA). The cells were subsequently analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR, USA).

Cytokine ELISA
The levels of IL-1β, IL-6, IL-8, IL-10 and TNF-α in the biopsy-conditioned supernatants were quantified using standard sandwich ELISA antibody pairs from Biosource following manufacturer’s instructions.

Real-Time PCR
FACS-sorted human skin APC subsets were pooled from at least 4 skin donors to obtain sufficient cell numbers. Cells were lysed and mRNA was isolated using an mRNA Capture kit (Roche). cDNA was synthesized using the Reverse Transcription System kit (Promega) following manufacturer’s guidelines. Oligonucleotides were designed using the Primer Express 2.0 software (Applied Biosystems) and synthesized by Invitrogen Life Technologies (Invitrogen). Real-Time PCR analysis was performed as previously described using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems) [18]. GAPDH was used as an endogenous reference gene.

Glycogene microarray analysis
Skin APC subsets were purified using MACS beads after 3 days of cell migration using CD19 and HLA-DR beads for the dDC sample and CD1a for the LC samples. CD19⁺ B cells were depleted from the dDC samples prior to RNA isolation. Analysis of gene
expression was conducted using a custom gene microarray as previously described [19]. Briefly, RNA was extracted using the Qiagen RNeasy Mini kit and used to probe the GLYCOv4 oligonucleotide array, a custom Affymetrix GeneChip (Affymetrix, Santa Clara, CA, USA) designed for the Consortium for Functional Glycomics with approximately 1260 human probe-IDs related to glycosylation-related genes. Total RNA sample quality was checked with an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA from each preparation was labeled using the MessageAmp II-Biotin Enhanced Amplification kit (Ambion Inc., Austin, TX, USA). Hybridization and scanning of the GLYCOv4 chip were performed according to the Affymetrix recommended protocols [20]. Raw data files for each of the experiments performed are available at the Consortium for Functional Glycomics website (www.functionalglycomics.org/fg).

**Peptide synthesis**
Peptides were produced by solid phase peptide synthesis using Fmoc chemistry with an automated peptide synthesizer (Protein Technologies, USA). Upon cleavage, peptides were purified by preparative HPLC to a yield higher than 95 %. Peptide sequence was confirmed using electrospray mass spectrometry.

**Internalization of fluorescently labeled OVA**
Skin APC subsets were cultured in a 96-well round bottom plate and indicated concentrations of Alexa Fluor 549-conjugated OVA were added to the wells. After 2 h of incubation, cells were washed extensively and internalization of OVA was directly measured using the FACS Calibur and FlowJo software. In order to discriminate internalization of OVA by the CD1a+ and CD14+ dDCs, the subsets were stained using antibodies for CD1a and CD14.

**Antigen presentation to a human CD8+ T cell clone specific for GP100**
A CD8+ T cell clone specific for GP100,280-288 was generated and cultured as previously described [21]. 2x10⁴ MACS-isolated CD14+ dDCs, CD1a+ dDCs or LCs were cultured in a 96-well round bottom plate. A 25 aa long GP100 peptide containing the immunodominant GP100 epitope GP100,280-288 or the 9 aa long immunodominant epitope was added to the isolated skin APC subsets at indicated concentrations. After 2 h of incubation, cells were extensively washed and GP100-specific CD8+ T cells (100,000/well) were added to the wells. After 24 h, supernatants were taken and IFN-γ levels were measured by sandwich ELISA using specific antibody pairs from Biosource.

**Statistical analysis**
Results were analyzed using one-way or two-way ANOVA followed by Bonferroni Multiple Comparison test using GraphPad Prism software (GraphPad Software, San Diego, CA). Results were considered to be significantly different when p<0.05.
Phenotypic and functional characterization of human skin antigen presenting cell subsets

Results

Dermal DCs and LCs have distinct patterns of CLRs and cytokine expression

The human skin can anatomically be divided into two main compartments: the epidermis, which contains a dense network of keratinocytes and LCs; and the dermis, which is mainly composed of connective tissue with a few scattered fibroblasts, dDCs and macrophages [22]. In order to investigate the expression of genes encoding for glycan-binding receptors (C-type lectins, siglecs and galectins) in APCs present in the dermis and epidermis, we isolated APCs from both skin layers and performed a microarray-based gene expression analysis. Prior to analysis we verified the purity of the cells by flow cytometry, as shown in Fig. 1A. As expected, detailed analysis of the differentially expressed genes amongst both populations confirmed the exclusive expression of langerin in LCs and DC-SIGN in dDCs (Fig. 1B). Although several genes, such as galectin-1, -3 and -9, MGL and dectin-1 were highly abundant on both APC subpopulations, a number of glycan-binding receptors defined a cell-specific expression signatures as described in Table 1. Thus, next to langerin, Siglec-5 and L-SIGN were specific to LCs, whereas dDCs expressed higher levels of CLEC4A (also known as DCIR), Macrophage mannose receptor, AGR2, CLEC1A, COLEC12, MDL1, Galectin 2 and Siglec 7 (Table 1). Most of these glycan-binding proteins have been involved in antigen uptake, illustrating that dDC have a more specialized

Figure 1. Gene expression analysis on isolated human skin DC populations. A. Enzymatically isolated LCs and dDCs were purified using CD1a and HLA-DR MACS beads, respectively. Cell purity was verified using flow cytometry. Data is shown for one representative LC and dDC sample. N=4. B. Microarray gene array analyses on isolated LCs and dDC samples. Data are shown for 3 independent LCs and dDC samples.
Characterization of human skin APC subsets in steady state

To further characterize the different types of APCs we performed enzymatic digestions and analyzed the resulting single cell suspensions by flow cytometry. Based on forward and side scatter properties and the expression of HLA-DR, CD1a and CD14, we characterized a CD1a⁺CD14⁻ subset, a CD14⁻CD1a⁺ subset and a double negative (DN) subset present in the dermis and the HLA-DR⁺CD1ahigh LCs in the epidermis (Fig. 2A). We first focused on the expression of activation and maturation markers and found that LCs expressed low levels of CD86 and CD83 and intermediate levels of MHC-I and MHC-II after isolation (Fig. 2B). In contrast, the dDC subsets displayed higher expression levels of these molecules, specially the CD1a⁺ dDCs. In steady state, CD1a⁺ dDCs seemed activated, based on their high levels of CD86 and CD83 as compared to other dDCs. We speculated that the enzymatic treatment of the dermis and epidermis to isolate the different APC subsets might have reduced

Table 1. Gene expression analysis of glycan-binding receptors expressed by human LCs and dDCs. Average and total scores of the genes that were highly expressed are depicted for the dDCs samples (left column) and LC samples (right column). Genes exclusively expressed by dDCs or LCs are depicted in blue or red, respectively.

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function in recognizing a broad repertoire of antigens for internalization than LCs. Based on these data, we can conclude that the APC subsets found in the epidermis and dermis of human skin share a significant amount of glycan-binding receptor genes, but have also unique CLR genes on LC and dDCs illustrating that these cells may differ in their antigen recognition and uptake function.

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the expression of surface markers, so we sorted the abovementioned subpopulations and repeated the analysis using quantitative RT-PCR. As shown in Fig. 2C, transcripts for CD86 and CD83 were highly abundant in CD1a+ dDCs, hardly present in DN dDCs and, in contrast to the data obtained by flow cytometry, LCs had a high expression of CD83. In summary, a side by side comparison revealed that the CD1a+ dDCs expresses the highest levels of maturation markers under steady state conditions as compared to DN DCs, CD14+ dDCs and LCs, which did express MHC-I and –II, but varying amounts of the co-stimulatory molecules CD86 and CD83.

Figure 2. The CD1a+ dDC subset is the phenotypically most mature human skin APC subset under steady conditions. A. Subset distribution in percentages of CD1a+ and CD14+ dDCs (upper panel) or CD1a+ LCs (lower panel) after gating on isolated HLA-DR+ cells. Dot plots of a representative experiment are shown. N=8. B. Surface expression of MHC class I and II, CD86 and CD83 was measured by flow cytometry on isolated HLA-DR+CD14+ dDCs, HLA-DR+CD1a+ dDCs and HLA-DR+CD1ahigh LCs. Grey histograms depict matching isotype controls. Histograms of a representative experiment are shown. N= 3. C. Relative mRNA levels of CD83 and CD86 compared to the housekeeping gene GAPDH are shown present in FACS-sorted, steady state DN, CD14+ or CD1a+ dDCs and LCs. Due to low cell numbers of each subset after sorting, the subsets contain combined cells of at least 4 skin donors. N=2, each measured in duplicate.
**Human skin APC subsets show a restricted expression pattern of CLRs and TLRs**

In order to confirm the data obtained by microarray analysis, we further explored the expression of a selected set of CLRs and TLRs by quantitative RT-PCR. We performed the analysis on highly purified, FACS-sorted APC subsets, which enabled us to separate the dermal DCs in the three main subsets found under steady state conditions. CD14⁺ dDCs expressed high levels of transcripts for DC-SIGN, mannose receptor (MR) and DCIR, whereas CD1a⁺ dDCs predominantly expressed MGL (Fig. 3A). As expected, langerin transcripts were found only in human LCs, as shown by the microarray data (Fig. 3A and Table 1). Dectin-1 transcripts were found in all four human DC subsets. Transcripts for all TLRs, except TLR6 and TLR9, were expressed in all steady state skin APC subsets (Fig. 3B). Interestingly, we could only detect minor differences that were not significant in TLR expression levels throughout the cell types investigated. Human LCs did not express TLR5 and showed low levels of TLR2, which is in line with previous findings [17;23] (Fig. 3B). Altogether, based on the expression of CD14, CD1a, CLRs, TLRs and co-stimulatory molecules 4 main population of human skin DCs can be operationally described: the relatively mature CD1a⁺ dDCs expressing MGL, high co-stimulatory molecules, the CD14⁺ dDCs expressing DC-SIGN, DCIR and MR and CD86, the epidermal LCs expressing langerin and low co-stimulatory molecules and the DN dDC subset. The latter does not show specific markers, probably because this subset is most heterogeneous.

**Isolated dDCs are unresponsive to TLR ligands**

In order to investigate whether TLR ligands activate freshly isolated dDC subsets we cultured the epidermal and dermal cell suspensions for 24 h in vitro in the presence of the TLR3 ligand pI:C, the TLR4 ligand LPS, or the cytokines GM-CSF and IL-4 (GM/4). Intradermal ex vivo injection of a combination of these two cytokines was previously shown to induce DC maturation [16]. No significant changes in the frequency of CD14⁺ dDCs, CD1a⁺ dDCs or LCs were observed after 24 h of culture with GM/4, pI:C, LPS or medium as compared to untreated cells directly upon isolation (Fig. 4A). These findings indicate that the skin APC subsets did not alter their subset characteristics or loss viability during the 24 h of in vitro culture despite of the different treatments. Next, we analyzed the expression of the maturation markers CD83, CD86 and CD70, as well as the expression of MHC-I and -II on the skin DC subsets. As shown in Fig. 4B, the 24 h of culture in the presence of TLR ligands or GM/4 hardly affected the maturation status of any of the APC subsets. The CD1a⁺ dDCs showed the highest expression of co-stimulatory molecules, as previously observed under steady state conditions (Fig. 3). LCs also displayed a certain level of spontaneous maturation after 24 h of culture in medium (Fig. 4B), but as in the case of CD1a⁺ dDCs, the addition of GM/4, LPS or pI:C did not further enhance the maturation status. Nor were the CD14⁺ dDCs affected in their maturation status after 24 h of culture. Together, these data provide evidence that isolated skin DC subsets are not responsive to maturation signals ex vivo.
Figure 3. Expression of CLRs and TLRs by highly purified, FACS-sorted human skin APC subsets. mRNA expression of various CLRs (A) or TLRs (B) was examined on FACS-sorted human skin APC subsets using RT-PCR analyses. Due to low cell numbers of each subset after sorting, the distinct subsets contain combined cells of at least 4 skin donors. N=2, each measured in duplicate.
Isolated dDCs and LCs respond to TLR ligands by the production of pro-inflammatory cytokines and IL-10

Although the dDC subsets and LCs did not mature phenotypically upon stimulation with the TLR ligands or GM/4, we also investigated the cytokine profile of these cells after 24 h of culture in the presence of IMDM, GM/4, pi:C or LPS. We compared the cytokine production of total dDCs and LCs and did not discriminate between the CD1a+ and CD14+ dDCs, since the separation of the dDC subsets by MACS or sorting affected the dDCs substantially and hampered the cytokine production. In contrast to the lack of upregulation of co-stimulatory molecules observed in Fig. 4, the dDCs

Figure 4. Human skin APC subsets do not mature in vitro upon inflammatory conditions. A. Percentages of CD14+ dDCs, CD1a+ dDCs and LCs directly after isolation or after 24 h of culture of epidermal and dermal suspensions in medium (IMDM), GM-CSF and IL-4 (GM/4), pi:C or LPS. N=3 B. Surface expression of molecules associated with DC maturation, CD86, CD83 and CD70 and molecules association with T cell activation, MHC class I and II measured directly after isolation of the skin DC subsets or after 24 h of culture of epidermal or dermal suspensions in the presence of indicated reagents. N=3.
responded to the TLR ligands with a significant increase in the secretion of IL-6, IL-1β, and IL-10 (Fig. 5, upper panel). Additionally, secretion of TNF-α by the dDCs was enhanced after stimulation with pl:C (Fig. 5). In contrast, LCs responded to pl:C by a significant increase in the secretion of IL-6, IL-8 and TNF-α as compared to medium alone, whereas LPS only induced significant changes in the secretion of IL-10 and IL-8 (Fig. 5, lower panel). Overall, human LCs produced less cytokines compared to dDCs.

**CD14+ dDCs have potent antigen internalization capacities, whereas LCs are most potent in antigen cross-presentation and CD8+ T cell activation**

To further characterize the functional properties of the isolated skin APCs, we analyzed the capacity of the cells to internalize and subsequently process antigens for presentation to CD8+ T cells. In particular DCs have shown to be superior in cross-presentation compared to the other APCs, an important mechanism to induce virus- or tumor-specific CD8+ T cells [2]. We exposed cells to fluorescently-labeled OVA (ovalbumin) and measured binding/uptake of OVA after 2 h using flow cytometry. The CD14+ dDCs showed a significantly higher signal as compared to the CD1a+ dDCs and LCs (Fig. 6A). To determine whether the internalization of antigen also facilitated cross-presentation by the skin APC subsets, we allowed internalization of a 25 aa long GP100 peptide, which requires processing for loading on MHC-I molecules. The APC subsets were incubated with the 25 aa long GP100 peptide for 2 h and subsequently co-cultured with a CD8+ T cell clone specific for GP100280-288, an immunodominant epitope present in the 25 aa long GP100 peptide. As expected based on the maturation profile and antigen internalization data, CD1a+ dDCs

![Figure 5. dDCs and LCs secrete cytokines after culture in the presence of GM/4 or the TLR ligands pl:C and LPS.](image-url)
showed a significantly higher dose-dependent activation of the CD8+ T cell clone compared to the CD14- dDCs, as measured by the production of IFN-γ (Fig. 6B). Unexpectedly, steady state human LCs were the most potent inducers of CD8+ T cell activation after cross-presentation (Fig. 6B). Although steady state CD14- dDC were very efficient at capturing antigen (Fig. 6A), their cross-presentation capacity was rather poor (Fig. 6B). To examine if LCs were truly superior in cross-presentation or whether the expression levels of MHC class I on these cells had any influence, we incubated the isolated skin APC subsets with the immunodominant peptide GP100280-288, which does not need processing and can be directly loaded on MHC class I molecules, and co-cultured the cells with the CD8+ T cell clone. This resulted in a significant higher activation of the T cell clone by the LCs compared to the CD1a+ and CD14- dDCs, providing evidence that LCs have the highest ability to induce effector-memory CD8+ T cell activation under steady state conditions (Fig. 6C). In

Figure 6. Antigen internalization and CD8+ T cell stimulatory capacities of steady state isolated human skin APC subsets. A. Internalization of fluorescently labeled OVA by the isolated skin APC subsets after 2 h of incubation as measured by flow cytometry. A representative experiment is shown (N=3). ***p<0.001 measured by the two-way ANOVA followed by the Bonferroni multiple comparison test. B. MACS-isolated human skin APC subsets were incubated with a 25 aa long GP100 peptide for 2 h, washed and co-cultured with HLA-A2+ GP100-specific CD8+ T cells. After 24 h, IFN-γ levels were analyzed in the supernatants as measure for T cell activation using ELISA. A representative experiment is shown. N=3, each experiment performed in triplicate. *p<0.05 and ***p<0.001 as measured by the two-way ANOVA followed by the Bonferroni multiple comparison test. C. MACS-isolated human skin DC subsets were pulsed with the 9 aa long GP100 peptide for 2 h, washed, and co-cultured with the HLA-A2+ GP100-specific CD8+ T cell clone. IFN-γ production by the T cells was measured using ELISA. N=3, each measured in triplicate. *p<0.05 as measured by the one-way ANOVA followed by the Bonferroni multiple comparison test.
conclusion, although the CD14+ dDC subset internalized protein antigen most efficiently, the human LCs showed the highest capacity to cross-present peptide antigens and to activate effector CD8+ T cells.

Discussion

Here we have shown that operationally distinct human APC subsets in the skin (based on CD14 and CD1a expression) in steady state (i.e. isolated ex vivo) display differential expression of CLRs and TLRs and cross-presenting ability. In addition, we have shown that the CD1a+ dDCs are the most mature human skin DC subset under steady state conditions based on the expression of co-stimulatory molecules and production of cytokines, in keeping with our earlier findings [24]. However, human LCs showed a superior ability to cross-present antigenic peptides, without the need for TLR-mediated activation, based on the highest activation of antigen-specific effector-memory CD8+ T cells. Gene expression profiling showed that human dDC and LCs have different CLR signatures. We have found exclusive expression of DC-SIGN, DCIR, macrophage mannose receptor and CLEC1A on steady state dDCs. Most of these CLRs have a role in antigen internalization [25-27], illustrating that dDC have a more specialized function in recognizing a broad repertoire of antigens for internalization than LCs.

We directly isolated human APC subsets using trypsin and collagenase instead of allowing the migration of the subsets. This method let us to analyze the phenotype and function of the subsets under steady state conditions. Spontaneous migration of skin APCs has been shown to already induce maturation and a reduction in antigen uptake capacities [28], thereby affecting both phenotype and function of the cells. Moreover, the migration protocol for skin APCs could potentially favor the migration of a particular subset, while non-migratory or slowly migrating cells could be excluded from analysis. Recently, it has been demonstrated that the pattern of gene expression by LCs and CD11c+ dDCs was retained when migratory skin subsets were compared to trypsinized subsets, despite the fact that phenotypic immunological maturation, i.e. increased expression of co-stimulatory molecules, was observed in the migratory skin subsets [29]. Understanding of the phenotype and function of both steady state and migratory skin APCs is necessary for the generation of targeted cutaneous vaccination strategies that induce immune stimulation, but need to prevent immune regulation. In addition, it has been shown that LCs induce activation and proliferation of skin-resident T regs under steady state conditions, whereas they activated and induced the proliferation of effector-memory T cells and limited T reg activation in the presence of foreign pathogens [30]. This indicate that LCs maintain tolerance in steady state healthy skin, but can activate protective skin-resident memory T cells upon infectious challenge. Therefore, studying skin APC subsets both in steady state and upon inflammatory
stimulation is essential to design effective DC-targeting vaccination strategies. In line with previously published work using migratory or in vitro generated LCs [8;31-33], we here show that steady state LCs are superior in the cross-presentation of soluble peptides to effector-memory CD8+ T cells compared to dDCs. Although LCs were superior in the activation of CD8+ T cells, they produced significantly less pro-inflammatory cytokines (Fig. 5) and were phenotypically less mature compared to steady state CD1a+ dDCs (Fig. 2c). It has been suggested that LC maturation is tightly regulated by interactions with the microenvironment to prevent overt and harmful inflammatory responses, which may lead to disruption of the skin barrier and entry of infectious and harmful pathogens [29]. Moreover, we used memory T cell clones in our studies which are less dependent on co-stimulatory molecules and cytokines as compared to naïve T cells [27], which might also explain why steady state human LCs were superior in activating the Gp100-specific CD8+ T cell clone despite their low expression of co-stimulatory molecules and cytokines. Indeed in previous experiments, using separately migrated and cytokine-activated LCs and dDCs, we showed superior antigen-specific priming of naïve CD8+ T cells predominantly by CD1a+ dDCs in keeping with their phenotypically more mature phenotype [24]. The method of isolation might also affect the in vitro phenotype and function of LCs. Here, we showed that enzymatically isolated LCs expressed low levels of maturation markers and did hardly secrete pro-inflammatory cytokines after TLR stimulation. However, migratory LCs responded to TLR3 stimulation by the production of TNF-α, IL-6 and an upregulation of CD86 and CD70 (data not shown). Based on the high expression of multiple CLRs by the CD14+ dDCs (Fig. 3A) and their homology to murine CD11b+ macrophages [12], we assumed that these cells would internalize antigens efficiently. Indeed, the data presented in Fig. 6A demonstrated that CD14+ dDCs internalized significantly more OVA compared to CD1a+ dDCs. A possible explanation could be that CD14+ dDCs are more efficient in receptor-mediated antigen internalization, facilitated through the highly expressed CLRs DC-SIGN and MR, which is in line with previous findings were we have shown that the migrated CD14+ dDCs internalized more Dextran-FITC compared to the CD1a+ dDCs [11]. In addition, DC-SIGN-mediated internalization of glycan-modified liposomes by CD14+ dDCs resulted in enhanced cross-presentation compared to the CD1a+ dDCs, providing evidence that the antigen formulation and mode of internalization have an influence on antigen cross-presentation ([33] and unpublished data, CM Fehres).

A lot of research has focused on the comparison between LCs and total dDCs, without making a distinction between the CD14+ and CD1a+ dDC subsets, showing that LCs are phenotypically and functionally more mature than dDCs [24;34;35]. We previously performed a genome-wide analysis between isolated steady state LCs and CD1a+ dDCs and reported superior activation of CD1a+ dDC in keeping with our findings in the present study, similarly showing CD1a+ dDCs to be more mature compared to LCs under steady state conditions, as measured by maturation markers.
and pro-inflammatory cytokine production. Since CD1a+ dDCs represent a large proportion of relatively mature dDCs with high expression levels of CCR7 suggesting a high migratory potential to draining lymph nodes [12], more research should be aimed at further investigating these cells in DC-targeted vaccination protocols to induce (anti-tumor) immune responses. Importantly, in this study we additionally analyzed the distinct CD14+ and CD1a+ subsets of dDCs with emphasis on CLR and TLR expression patterns. We recently demonstrated that the unique expression of langerin on LC and DC-SIGN on CD14+ dDCs, can be used to enhance receptor mediated antigen uptake and cross-presentation in these cells[33]. Importantly, we have demonstrated that the size and or multivalency of the targeting structure is of crucial importance to facilitate targeting through DC-SIGN or langerin and cross-presentation by the CD14+ dDCs and LCs, respectively[33].

We have found that the isolated LC and dDC subsets were not sensitive to in vitro TLR stimulation at concentrations where moDCs responded vigorously showing an upregulation of co-stimulatory molecules (Fig. 4B). Rather, we found that culturing both freshly isolated LCs and dDCs overnight in medium without activating cytokines or TLR ligands was already enough the induce an upregulation in the expression of co-stimulatory molecules similar to the upregulation seen when TLR ligands were added to the cultures. No changes were observed in the percentages of LCs, CD14+ dDCs or CD1a+ dDCs after overnight culture with cytokines or TLR ligands, suggesting that the subset distribution under these in vitro culture conditions was stable. This relative unresponsiveness of dDCs and LCs to TLR ligands might be induced by factors still present after isolation from the skin and is in line with our previous findings in the migrated cutaneous DC subsets after intradermal administration of TLR ligands [16]. In both studies we observed modest activating effects of TLR3 engagement by pI:C.

Altogether, our data clearly showed that the 3 main populations of APCs present in the steady state human skin each express a different set of TLRs, CLRs, co-stimulatory molecules and display a distinct CD8+ T cell cross-presenting capacity. Steady state CD14+ dDCs are less mature cells with a high capacity to internalize antigens, whereas the LCs were superior in the activation of CD8+ effector-memory T cell responses. Based on these results, LCs and CD1a+ dDCs seems suitable targets for targeted anti-tumor immunotherapy in the presence of TLR3 ligands.
Chapter 2

References

Phenotypic and functional characterization of human skin antigen presenting cell subsets


