CHAPTER 2

Transcriptional profiling of human skin-resident Langerhans cells and CD1a$^+$ dermal dendritic cells: differential activation states suggest distinct functions

Saskia J.A.M. Santegoets
Susan Gibbs
Kim Kroeze
Rieneke van de Ven
Rik J. Scheper
Carl A. Borrebaeck
Tanja D. de Gruijl
Malin Lindstedt

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Abstract

In human skin, two main populations of dendritic cells (DC) can be discriminated; dermal DC (DDC) and epidermal Langerhans cells (LC). Although extensively studied, most of the knowledge on DDC and LC phenotype and function is obtained from studying DDC and LC cultured *in vitro* or DDC and LC migrated from skin explants. These studies have left the exact relationship between steady-state human LC and DDC unclear: in particular whether CD1a$^+$ DDC represent migrated LC or whether they constitute a separate subset. In order to gain further insight in the kinship between skin-resident CD1a+ DDC and LC, we analyzed CD1a+ DDC and LC, isolated from steady-state skin samples, by high density microarray analysis. Results show that the CD1a+ DDC specifically express markers associated with DDC phenotype, such as the macrophage mannose receptor (MMR), DC-SIGN, the scavenger receptor CD36, coagulation factor XIIIa (FXIIIa) and chemokine receptor CCR5, whereas LC specifically express Langerin, membrane ATPase (CD39) and CCR6, all hallmarks of the LC lineage. In addition, under steady state conditions both DC subsets display a strikingly different activation status, indicative of distinct functional properties. CD1a+ DDC exhibit a more activated pro-inflammatory, migratory and T cell stimulatory profile as compared to LC, whereas LC mainly express molecules involved in cell adhesion and DC retention in the epidermis. In conclusion, transcriptional profiling is consistent with the notion that CD1a+ DDC and LC represent two distinct DC subsets, and that under steady state conditions CD1a+ DDC and LC represent opposites of the DC activation spectrum.

Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) with the unique ability to initiate and maintain primary immune responses. As key sensors of danger, immature DC reside in peripheral tissues, such as the skin and mucosal sites. DC are specialized in antigen capture and constantly sample the environment for antigens, local inflammation and pathogens (1-4). Upon recognition of danger signals, immature DC undergo a process called DC maturation, resulting in the inhibition of antigen uptake, as well as in a switch in chemokine receptor expression and subsequent migration of the DC to the secondary lymphoid organs, where they present the antigen to T cells. In the human skin, two main populations of DC can be discriminated, Langerhans cells (LC), which can be found in the epidermis (5,6) and dermal DC (DDC), which are located in the dermis. *In vitro* differentiation studies from CD34$^+$ precursor cells suggest that DDC and LC originate from a common myeloid DC precursor and have several features in common. These include the expression of high levels of MHC class I and class II molecules, co-stimulatory and adhesion molecules, as well as the expression of certain leukocyte/myeloid markers such as CD45RO, CD13 and CD33 and a lack of CD3, CD19, CD20, CD16 and CD56 lineage markers as reviewed by Larregina and co-workers (7). However, both DC subsets also exhibit specific “DDC” and “LC” characteristics. LC are characterized by the expression of the C-type lectin Langerin, which is responsible for the formation of Birbeck granules, a typical hallmark for the LC lineage (8,9). In addition, LC express E-cadherin (10), membrane adenosine triphosphatase (ATPase) and CCR6 (11), whereas DDC do not. On the other hand, DDC can be distinguished from LC by the expression of certain C-type lectins, such as

macrophage mannose receptor (MMR) and DC-SIGN (12,13), as well as by the expression the scavenger receptor CD36 (14) and the expression of coagulation factor XIIIa (FXIIIa) (15,16). Furthermore, DDC can also express the monocyte/macrophage marker CD14 (5,6,17).

Although it has been described that both DDC and LC are professional APC capable of inducing primary immune responses *in vitro* and *in vivo*, functional differences between the DC subsets have also been reported. The observed difference in C-type lectin expression indicates that both DC subsets may recognize and react to different spectra of pathogens (18). Besides that, *in vitro* generated DDC have been described to more efficiently drive the differentiation of naïve B cells into IgM-secreting plasma cells, while LC have been described to be more potent *in vitro* stimulators of cytotoxic T cells (19), as well as more potent inducers of Th1 responses due to their inability to produce IL-10 upon CD40 ligation (6). However, compared to the abundant *in vitro* data, data clarifying *in vivo* functions of human LC are scarce. Recently, immunogenic properties of LC *in vivo* have been questioned based on murine studies. Rather than directly activating immune effector cells, LC were reported to function as transporters of antigen, carrying antigen from skin to lymph nodes (LN) and transferring the antigenic cargo to LN-resident DC for actual antigen presentation and CTL priming (20-22).

It must be emphasized that most of our current knowledge on DDC and LC phenotype and function was obtained through the study of DDC and LC cultured *in vitro* from CD34+ HPC or blood-derived monocytes (5,19,23,24). Analyzing DC migrated from skin explants or directly isolated from epidermal and dermal cell suspensions revealed that the skin DC population is quite heterogeneous. Based on the expression of CD1a and CD14 different DC populations could be identified: besides epidermal LC ($CD1a^{high}$/$Langerin^+$/Birbeck $granule^+$), three distinct DDC subsets could be discerned, i.e. $CD1a^+$/$CD14^-$ DDC, $CD1a^-$/$CD14^+$ DDC and $CD1a^-$/$CD14^-$ DDC (25,26). Although extensively studied, the relationship between human LC and the various DDC subsets remains unclear and controversial, with both $CD14^+$-to-$CD1a^+$ and $CD1a^+$-to-$CD14^+$ (trans-) differentiation events having been reported (27,28). Larregina and co-workers recently defined $CD1a^+$ skin-emigrated DC as LC and $CD1a^-$/$CD14^-$ skin-emigrated DC as DDC (7,29), whereas Angel and co-workers suggested $CD1a^+$ DDC to be distinct from migrating LC, as concluded from intermediate levels of CD1a and the absence of Langerin expression (26). Notably, activated LC have been demonstrated to down-regulate CD1a and Langerin, making it difficult to distinguish skin migratory LC from skin-resident DDC (14,30).

Microarray technology has made it possible to study the expression levels of thousands of genes in parallel, with only relatively small amounts of material. By performing global transcriptional profiling of skin-derived $CD1a^+$ DDC and $CD1a^+$ LC, using high-density microarray analysis and extracting the differentially expressed genes, we aimed to elucidate whether $CD1a^+$ DDC and $CD1a^+$ LC, obtained from resting, non-inflamed skin, are two truly separate DC subsets or whether they represent a functional continuum of one subset. Besides that, we also studied transcript levels of genes that might be related to their *in vivo* function.

Our data demonstrate a remarkable difference in maturation status under steady state conditions between CD1a+ DDC and LC, with $CD1a^+$ DDC displaying a more activated pro-inflammatory and

migratory profile, and LC exhibiting a more quiescent profile, expressing genes involved in cell adhesion and DC retention in the epidermis. Based on the obtained gene expression profiles they nevertheless adhere to the previously proposed classic definitions of the DDC and LC subset phenotypes, indicating that CD1a$^+$ DDC and LC are two distinct DC subsets and that CD1a$^+$ DDC thus do not merely represent one end of the functional continuum of LC.

Materials and Methods

Isolation of Dermal Dendritic Cells and Langerhans Cells from Skin.

Human skin specimens were obtained from healthy donors undergoing corrective breast or abdominal plastic surgery after informed consent. 3-mm thick slices of skin containing both the epidermis and the dermis were cut by use of a dermatome. Slices of skin were cut in pieces of 1 cm2 and incubated with 2.4 U/ml Dispase II (Roche Diagnostics, Mannheim, Germany) for 30 – 60 minutes at 37ºC. The epidermis and dermis were separated with tweezers and washed with PBS. To isolate LC, the epidermal sheets were incubated with PBS with 0,05 % trypsin (Invitrogen Life Technologies, Carlsbad, CA) for 10 minutes at 37ºC, and single-cell suspension was prepared by pushing the tissue through 100-µm pore nylon cell strainers (Falcon) with a plunger of a 2-ml syringe. Epidermal cell suspension were enriched for LC by density centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway) and CD1a-guided magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). To isolate dermal DC, the dermis was incubated with PBS containing 0.48 U/ml Dispase and 6 mg/ml Collagenase A (Boehringer Mannheim, Mannheim, Germany) at 37ºC for 2 hours, after which single cell suspension was prepared by pushing through 100-µm pore nylon cell strainers with a plunger of a 2-ml syringe. Cell suspension was enriched for DDC by CD1a-guided magnetic cell sorting (MACS).

Antibodies and Flow Cytometry.

PE- or FITC-labeled Abs directed against human CD83, Langerin (Immunotech, Marseille, France), CD1a, CD86 and DC-SIGN (all from BD Biosciences, Mountain view, CA) were used for flow cytometric analysis. Antibody staining was performed in PBS supplemented with 0.1% BSA and 0.02% natrium-azide for 30 minutes at 4ºC. Stained cells were analyzed on a FACSCalibur (BD Biosciences) using Cell Quest software.

Preparation of cRNA and gene chip hybridization.

RNA isolation and gene chip hybridization was performed as described (31). Briefly, cell pellets of skin isolated DDC and LC, from 3 different donors, were dissolved in TRIzol Reagent (Life Technologies) and stored at –20ºC. After chloroform extraction, total RNA was precipitated in isopropanol, rinsed with 70% ethanol, lyophilized, and dissolved in 10 µl of distilled water. Fragmentation, hybridization, and scanning of the Human Genome U133 Plus 2.0 Arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). The preparation of labeled cRNA was performed according to the Two-cycle Eukaryotic Target Labeling assay protocol, using the GeneChip Expression 3' amplification two-cycle labeling and control reagents kit (Affymetrix).

Briefly, cDNA was generated from total RNA (20–150 ng), using SuperScript II (Invitrogen Life Technologies) and a T7-oligo(dT) promoter primer (Affymetrix). After a second-strand cDNA synthesis, cDNA was converted to cRNA by an in vitro transcription reaction (Ambion MEGAscript T7 kit. Foster City, CA). Thereafter, the cRNA was purified using RNeasy Mini kit (Qiagen, Hilden, Germany), and the yield was controlled with a spectrophotometer. A second cycle of cDNA synthesis was performed, followed by the same cleanup as above and a second in vitro transcription reaction cycle with biotin-labeled ribonucleotides and T7 RNA polymerase. Labeled cRNA was purified, using RNeasy Mini kit (Qiagen), quality controlled with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and denatured at 94°C before hybridization. The samples were hybridized to the Human Genome U133 Plus 2.0 Array at 45°C for 16 h by rotation (60 rpm) in an oven. The arrays were then washed, stained with streptavidin-PE (Invitrogen Molecular Probes), washed again, and scanned with a GeneArray Scanner (Affymetrix).

Microarray data analysis.

The fluorescence intensity was analyzed, using the GeneChip Operating Software (GCOS) 1.1 (Affymetrix), and scaled to a target value of 100. Further data analysis was performed with GeneSpring 7.1 software (Agilent Technologies). For clustering, the samples were normalized per gene, which makes the median value for each gene across the samples equal to 1. A gene and condition tree clustering was performed on the LC and DC samples to distinguish replicate similarities. The tree clustering algorithm, based on Pearson correlation, was used on genes denoted P (present) in either DDC or LC (three replicates) with a signal intensity above 200, to eliminate borderline expression, and displaying a fold change in mean expression level of ±2 between the two populations, giving a total of 1480 genes. The expression of selected genes were significantly different ($p<0.05$) as determined by 1-way ANOVA.

Results and Discussion

Phenotyping CD1a$^+$ DDC and LC by flow cytometry.

Dermal and epidermal cell suspensions were analyzed for the presence of DC by flow cytometry. As shown in figure 1*A*, CD1a$^+$ DC could be detected in both dermal (top left panel) and epidermal cell suspensions (bottom left panel). Next, DC were isolated from the dermal and epidermal suspensions on the basis of the pan-skin DC marker CD1a. Purity of CD1a$^+$ dermis-derived DC (hereafter referred to as CD1a$^+$ DDC) and CD1a$^+$ epidermis-derived LC (hereafter referred to as LC) were more than 90%. As shown in Figure 1*A*, CD1a$^+$ DDC exhibit DDC characteristics, expressing intermediate levels of CD1a and no Langerin (top right panel), whereas skin LC exhibit LC characteristics, expressing high levels of CD1a and Langerin (lower right panel). The isolated LC displayed an immature phenotype, as indicated by the absence of CD83 expression, whereas the CD1a$^+$ DDC expressed CD83, indicative of mature phenotype (Figure 1*A* and *B*). Importantly, this differential expression of CD83 was also observed for CD1a$^+$ cells in freshly prepared dermal and epidermal cell suspensions (see Figure 1*A*), suggesting that CD83 expression was not induced by the isolation procedure. In

addition, both DC subsets exhibit similar levels of the co-stimulatory and adhesion molecules CD86 and HLA-DR (Figure 1*C*).

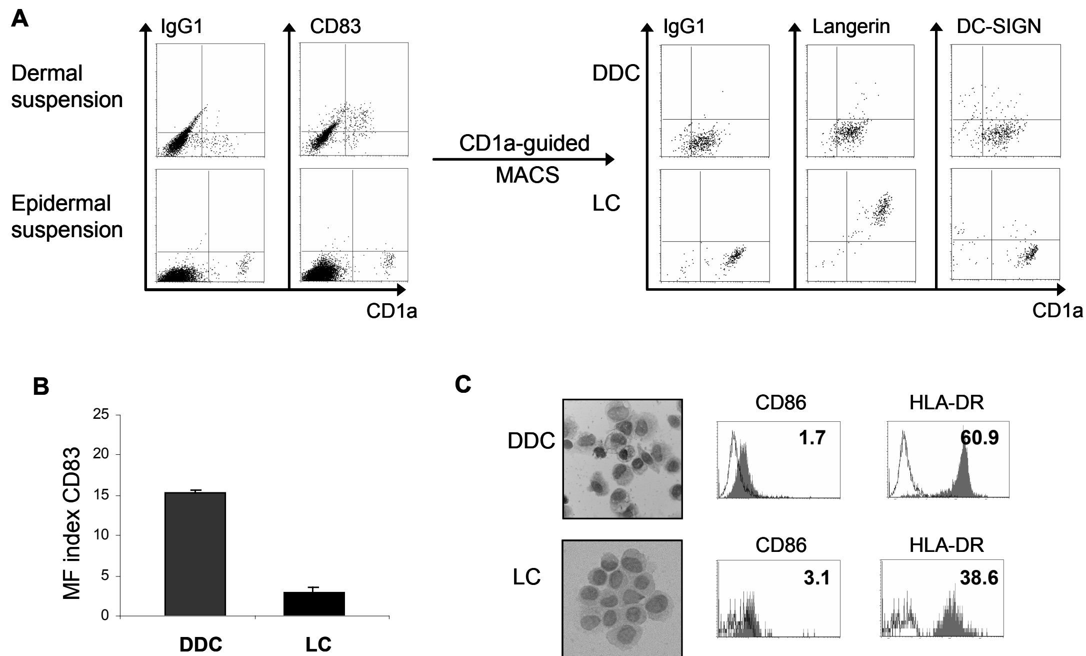

Figure 1. Phenotype and Morphology of skin-derived CD1a+ Dermal Dendritic Cells (DDC) and Langerhans Cells (LC). Skin DDC and LC obtained from healthy human skin specimens were analyzed by flow cytometry for the expression of CD1a and CD83 prior to CD1a-guided magnetic cell sorting (*A; left panel*) and for the expression of CD1a, Langerin and DC-SIGN (*A; right panel*), CD83 (*B*) and CD86 and HLA-DR (*C*) after CD1a-guided magnetic cell sorting. (*A*) Skin DDC expressed intermediate levels of CD1a and no Langerin (top panel), whereas skin LC expressed high levels of CD1a and Langerin (bottom panel). (*B*) CD83 expression of skin DDC and LC is depicted as the mean of mean fluorescence intensity (MFI) ± SEM of three independent experiments. (C) Photographs of both skin DDC and LC were taken from cytocentrifuge preparations (cytospins, 400x magnification). Flowcytometric analysis of MACS-isolated CD1a+ DDC and LC. *Open histograms*, isotype- matched controls; *closed histograms*, the marker as indicated above. Mean fluorescence indices are listed in the upper right corner. Data shown are representative of 3 independent experiments.

Phenotyping CD1a+ DDC and LC by transcriptional analysis.

To make an extensive transcriptional comparison of CD1a+ DDC and LC, RNA isolated from each DC subset was hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays containing >54,000 probe sets and covering 38,500 human genes. mRNA expression profiles of CD1a+ DDC and LC were assessed from 3 individual donors. To determine whether the isolated CD1a+ DDC and LC indeed represented true DDC and LC, both DC subsets were first subjected to a global transcriptional analysis and markers associated with DDC and LC biology were extracted. For each subset, the intensity signals for selected marker genes that were expressed (denoted *present*) and had an intensity level of >200 were assessed. As shown in Table I, the transcriptional patterns of CD1a+ DDC and LC fully support previous reports on DDC and LC phenotype definitions (32,33). CD1a+ DDC express the C-type lectins MMR and DC-SIGN, the scavenger receptor CD36, coagulation FXIIIa and the chemokine receptor CCR5, but do not express markers associated with LC phenotype such as Langerin, membrane ATPase (CD39) and CCR6, while skin LC express the C-type lectin Langerin, membrane ATPase and the chemokine receptor CCR6 (Table I), but do not express MMR, DC-SIGN,

CD36 or FXIIIa (Table I). Based on this panel of DC subset-defining markers (Table I), we thus conclude that CD1a$^+$ DDC adhere to the previously proposed definition of the DDC phenotype and are not likely to represent migratory LC, as also described by others (26). In particular the observation that CD1a+ DDC express DDC-defining markers associated with an immature phenotype such as DC-SIGN and MMR, argues against the possibility of migrating LC adopting a skin DDC transcriptional profile under the influence of environmental (i.e. dermal) factors, as *"de novo"* expression of such a marker is not consistent with the degree of maturation that might be expected in migrating LC.

Table I. Differential characteristics of CD1a$^+$ dermal DC (DDC) and Langerhans cells (LC).

Gene	Literature		mRNA expression profile	
	DDC*	LC*	CD1a$^+$ DDC†	LC†
CD1a	+	+	++	+++
CD205, DEC-205	+	+	+	++
CD206, MMR	+	-	++	-
CD207, Langerin	-	+	-	+++
CD209, DC-SIGN	+	-	+	-
CD36	+	-	+	-
Factor XIIIa	+	-	++	-
CCR5	+	-	++	-
CCR6	-	+	-	+
ATPase	-	+	-	+
E-cadherin	-	+	++	+++

* Expression described in literature; reviewed by Larregina *et al*. and Valladeau *et al.* (32,33). Values given: - is absent, + is present.
† Determined by high density microarray analysis. Signal intensity levels: - <200, + 200 - 500, ++ 500-5000, +++ >5000

LC display a non-immunogenic, non-migratory phenotype under steady-state conditions.

In order to gain insight into functional differences between CD1a$^+$ DDC and LC, genes that were differentially expressed (2-fold change in expression level, $p<0.05$) were further clustered into groups, as illustrated by a heat map in Figure 2. Seven groups of differentially expressed genes are presented according to function: chemokines/chemokine receptors, interleukins/interleukin receptors, TNF/TNF receptor family, adhesion, immune responses, endo/exocytosis and "others". In addition, to further characterize both DC subsets, a panel of genes was selected based on their specific known function in DC biology (Table II A to G). This comparative analysis revealed that LC, residing in the epidermis under steady state conditions, exhibit a non-stimulatory phenotype. As illustrated by Figure 2, LC display a non-migratory profile, expressing molecules involved in cell adhesion such as E-cadherin, ICAM-3 and epidermal surface antigen (ESA), or involved in DC retention such as junctional adhesion molecules (JAM-A) and CD47, but lacking expression of molecules involved in DC migration such as CCR7 (Table II.B), as also described by others (10,34-37). Indeed, ligation of CD47, also known as integrin-associated protein, has been demonstrated to regulate LC maturation and migration, resulting in the suppression of LC function, inhibition of T cell priming and the subsequent inhibition of the establishment of an immune response (38). Yu and co-workers suggested that the firm adhesion between LC and keratinocytes, resulting in suppression of DC migration (38), might be CD47-mediated. Indeed, it has been shown that CD47-expressing cells can firmly adhere to other CD47-expressing cells without the need for interaction with CD47 ligands such as SIRP-1α or thrombospondin-1 (39).

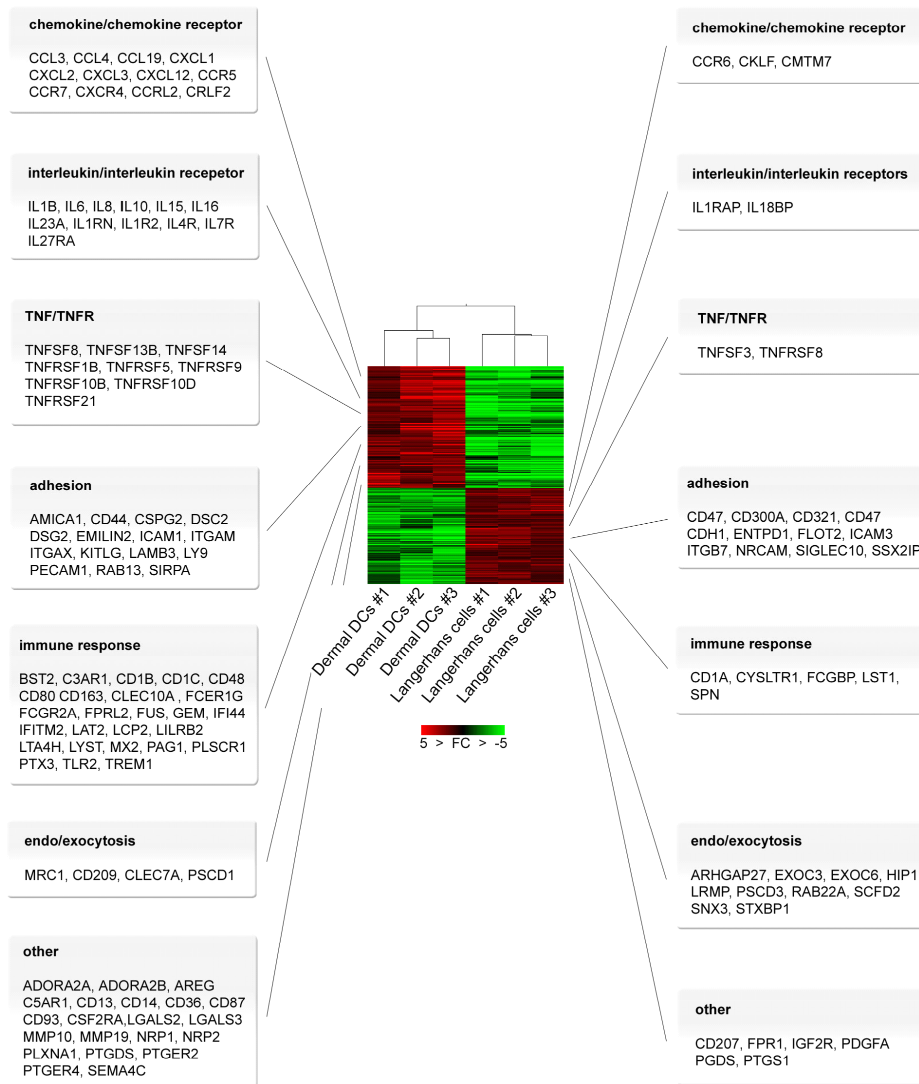

Figure 2. Hierarchical clustering of differentially expressed genes in skin DDC and skin LC. Subset-selective transcriptional profiles in freshly isolated skin CD1a+ DDC (n=3, left columns) and LC (n=3, right columns) were identified by filtering (described in *Materials and Methods*) and sorted according to their gene ontogeny into various groups: chemokines/chemokine receptors, interleukins/interleukin receptors, TNF/TNF receptors, adhesion, immune responses, endo/exocytosis and others. Color changes, within a row, indicate expression levels relative to the median of the sample population. Because the samples are normalized to a median value of 1, the color bar range of 5 (red) to -5 (green) represents high and low expression levels, respectively.

Furthermore, these data support previously described findings that LC trafficking is not only controlled at the level of chemokine/chemokine receptor expression, but also at the level of cell adhesion (32,40). In addition, LC express relatively low levels of co-stimulatory and adhesion molecules such as CD80, CD40 and CD54 (Table II.A), or pro-inflammatory cytokines and cytokine receptors (Table II.C), and do not express T cell stimulation molecules such as 4-1BB and CD30L, indicating that under steady state conditions LC are poor T cell stimulators (Table II.E). The LC's non-T cell stimulatory profile is further illustrated by the expression of CD43 (also known as leukosialin), a glycoprotein that is only expressed on immature dendritic cells and implicated in the inhibition of non-specific T cell contacts (41,42). In addition, under steady state conditions LC also do not exhibit proper

B cell stimulatory capacity, as illustrated by the absence of B cell activating factor (BAFF), a TNF family member, which is known to play an important role as a co-stimulator of B lymphocyte proliferation and function (43).

Table II. Relative expression levels of genes associated with DC biology

Category	Gene	Skin DDC*	Skin LC	UniGene ID
A. Co-stimulatory, adhesion molecules and integrins	CD13	++	-	Hs.1239
	CD14	+	-	Hs.75627
	CD1a	++	+++	Hs.1309
	CD1b	++	+	Hs.1310
	CD1c	+++	++	Hs.1311
	CD11b	++	-	Hs.172631
	CD11c	+	-	Hs.385521
	CD31	++	-	Hs.78146
	CD40	++	+	Hs.504816
	CD54	++	+	Hs.386467
	CD80	+	-	Hs.838
	CD86	++	++	Hs.27954
	CD83	+++	+++	Hs.79197
B. Chemokines and chemokine receptors	CCL3, MIP-1α	+++	-	Hs.73817
	CCL4, MIP-1β	+++	++	Hs.75703
	CCL19, MIP-3β	+	-	Hs.50002
	CCL20, MIP-3α	+++	++	Hs.75498
	CCL22, MDC	+++	+++	Hs.97203
	CXCL1, Gro-α	++	-	Hs.789
	CXCL12, SDF-α	+	-	Hs.436042
	CXCL2, MIP-2α	+++	-	Hs.75765
	CXCL3, MIP-2β	+++	-	Hs.89690
	CCR5	++	-	Hs.511796
	CCR6	-	+	Hs.46468
	CCR7	+++	-	Hs.1652
	CXCR4	+++	++	Hs.421986
	C3aR1	++	-	Hs.155935
	C5aR1	++	-	Hs.2161
	FPRL2	++	+	Hs.445466
C. Cytokines and cytokine receptors	IL-1α	++	++	Hs.1722
	IL-1β	+++	+++	Hs.126256
	IL-6	++	-	Hs.512234
	IL-8	+++	+++	Hs.624
	IL-10	++	-	Hs.193717
	IL-15	++	-	Hs.168132
	IL-16	+	-	Hs.170359
	IL-18	+	+	Hs.83077
	IFN-β1	+	-	Hs.93177
	IL-23p19	++	+	Hs.98309
	IL-1 R1	+++	+++	Hs.82112
	IL-1 R2	++	++	Hs.25333
	IL-1 R3	+	++	Hs.143527
	IL-1 RN	++	-	Hs.81134
	IL-4 R	++	+	Hs.75545
	IL-7 R	++	-	Hs.362807
	IL-10 Rα	++	++	Hs.327
	IL-13 Rα1	++	++	Hs.285115
	IL-18 BP	-	++	Hs.325978
	IL-18 R1	+	+	Hs.159301
	IL-18 Rβ	+	-	Hs.158315
	IFN-γ R1	++	++	Hs.180866
	IFN-γ R2	++	++	Hs.409200
	GM-CSFR	+	-	Hs.520937

[*] Signal intensity levels:- <200, + 200 - 500, ++ 500-5000, +++ >5000)

Table II (continued)

Category	Gene	Skin DDC*	Skin LC	UniGene ID
D. C-type lectins, Fc- and scavenger receptors	Dec-205	+	++	Hs.153563
	MMR	++	-	Hs.75182
	Langerin	-	+++	Hs.199731
	DC-LAMP	+++	++	Hs.10887
	DC-SIGN	+	-	Hs.278694
	Dectin-1	+++	++	Hs.161786
	C-type lectin 13	+++	+	Hs.54403
	Selectin E	+	-	Hs.89546
	E-cadherin	++	+++	Hs.194657
	CD36	+	-	Hs.443120
	CD163	++	-	Hs.74076
	FcεR1 α chain	+++	+++	Hs.897
	FcεR1 γ chain	++	+	Hs.433300
	FcγR2a	++	+	Hs.352642
	FcγR2b	++	++	Hs.126384
	FCGRT	+	++	Hs.111903
E. TNF/TNFR family	CD40	++	+	Hs.504816
	TNF-α	++	++	Hs.241570
	TNF-RI,	+	+	Hs.159
	TNF-R2	++	++	Hs.256278
	CD30	-	+	Hs.1314
	4-1BB	++	-	Hs.193418
	APRIL	-	+	Hs.54673
	BAFF	++	-	Hs.270737
	CD30L	+	-	Hs.177136
	TRAIL-R2	++	+	Hs.51233
	TRAIL-R4	+	-	Hs.129844
	Lymphotoxin B R	+	++	Hs.376208
F. Transcription factors	Id2	+++	++	Hs.180919
	REL	++	++	Hs.44313
	Ikaros	+	-	Hs.435949
	IRF4	++	+	Hs.127686
	STAT1	++	++	Hs.21486
	STAT3	++	++	Hs.421342
	STAT4	++	-	Hs.80642
	STAT5a	++	+	Hs.437058
	STAT5b	+	-	Hs.434992
	STAT6	+	++	Hs.437475
G. Immune-modulatory factors	IL-10	++	-	Hs.193717
	PGE2 R2 (EP2)	+	-	Hs.2090
	PGE2 R3 (EP3)	++	+	Hs.527970
	PGE2 R4 (EP4)	+++	++	Hs.199248
	INDO, IDO	++	-	Hs.840

* Signal intensity levels:- <200, + 200 - 500, ++ 500-5000, +++ >5000)

CD1a$^+$ DDC display an activated and migratory phenotype under steady-state conditions.

In stark contrast to LC, CD1a$^+$ DDC exhibit an activated phenotype, expressing higher levels of co-stimulatory and adhesion molecules (e.g. CD54, CD80, CD86, CD40; see Table II), pro-inflammatory cytokines (like IL-1β, IL6, IL-8, IL-15 and IL-16), cytokine receptors (IL-1R, IL-4R, IL-7R and IL-27Ra; see Table II.C), prostaglandin E2 receptors EP2 and EP4, and TNF family members, capable of providing T and B cell stimulation such as 4-1BB, CD30L and BAFF (Table II.E). This activated phenotype is also illustrated by the observation that CD1a$^+$ DDC express chemokines that are involved in directing leukocyte migration, such as monocyte-derived chemokine (MDC; CCL22), macrophage inflammatory protein (MIP)-1 α (also known as CCL3), MIP-1β (CCL4), Gro-α (CXCL1) and SDF-α (CXCL12). In addition, CD1a$^+$ DDC also expressed chemokine receptors that are involved in lymph node homing such as CCR7 and CXCR4 (Table II.B).

The more activated immuno-stimulatory phenotype of CD1a$^+$ DDC as compared to LC, was also confirmed by the expression CD44, C3aR1 and C5aR1. CD44 has been described to be up-regulated on skin DDC and LC upon activation, and to be involved in emigration from the skin and in adhesion to the T cell zones of the lymph nodes, thereby showing its importance for the ability of DC to induce primary T cell responses within the LN (44). As shown in Figure 2, CD44 is significantly up-regulated on CD1a$^+$ DDC compared to skin LC, indicating that indeed CD1a$^+$ DDC exhibit a more activated phenotype capable of T cell binding in the LN. This more activated phenotype was further illustrated by the observation that CD1a$^+$ DDC, but not LC, expressed the complement receptors C3aR1 and C5aR1, both of which are involved in DC homing to inflammatory sites. This is in line with findings from others, demonstrating that freshly isolated CD83$^+$ skin DC expressed both C3aR1 and C5aR1 (45), whereas C5aR1 was not observed in the majority of immature skin LC (46). Moreover, the previously reported observation that C5aR1 was expressed on a small number of skin LC that were located in the proximity of the basal membrane and exhibited a more activated and migratory phenotype, further supports the apparent association between complement receptor expression and maturation induction (46).

Mature CD1a$^+$ DDC express immunosuppressive factors: maintenance of tolerance in the steady state.

The finding that even in the absence of apparent danger signals CD1a$^+$ DDC display a fully mature and T cell stimulatory phenotype may seem counterintuitive. It should however be noted that although CD1a$^+$ DDC exhibit an activated and migratory phenotype under steady state conditions, they also display immunosuppressive features. As demonstrated in Table II.G, under steady state conditions CD1a$^+$ DDC express relatively high levels of IL-10 and indoleamine 2,3-dioxygenase (IDO) transcripts. The anti-inflammatory cytokine IL-10 has been described to negatively regulate the immune response either via the induction of T cell tolerance (47) or the development of regulatory T cells (48,49) and IDO has been described to convey immunosuppressive effects by degrading the essential amino acid tryptophan, thereby down-regulating T cell functions (50,51). The observation that the activated CD1a$^+$ DDC also display immunosuppressive features under steady-state conditions indicates that the activated phenotype does not automatically equal T cell activation, and that without additional danger signals CD1a$^+$ DDC may be mainly involved in the maintenance of tolerance. In keeping with this notion we previously described CD1a$^+$ DC in skin-draining LN to all display a CD83$^+$ mature phenotype, even in the steady state (52).

In conclusion, this extensive comparative analysis of CD1a$^+$ DDC and LC by means of transcriptional profiling was the first of its kind, and generated valuable information about the *in vivo* phenotype and function of both DC subsets. The obtained gene expression profiles clearly demonstrate that the two isolated DC populations adhere to the previously proposed definitions of the DDC and LC phenotype, suggesting them to constitute separate subsets. In addition, CD1a$^+$ DDC and LC show a remarkable difference in functional maturation status under steady state conditions, indicating that LC are quiescent and sedentary whereas CD1a$^+$ DDC are continuously carrying antigen to lymph nodes and capable of stimulating T cells, as also described by recently by Angel *et al* in

humans and Kissenpfennig *et al* in mice (26,53). The observed expression of transcripts of such suppressive factors as IL-10 and IDO suggests that such T cell stimulation by mature CD1a$^+$ DDC in the steady state may result in the induction of tolerance.

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