

Transcriptional profiling of interstitial dendritic cells and Langerhans cells derived
from the human CD34+ dendritic precursor cell line MUTZ-3

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Abstract

In man, two main populations of dendritic cells (DC) can be discriminated, i.e. interstitial DC (IDC) and Langerhans cells (LC), which are located in connective tissues and the epidermis, respectively. *In vitro*, IDC and LC can be cultured from peripheral blood-derived monocytes or from blood or bone marrow-derived CD34+ hematopoietic progenitor cells. We showed recently that the human CD34+ acute myeloid leukemia (AML) cell line MUTZ-3 is able to differentiate into both CD1a+/DC-SIGN+ IDC and CD1a+/ Langerin+/Birbeck granule-expressing LC. In this study, we set out to analyze the MUTZ-3-derived IDC and LC by high density microarray analysis. Through transcriptional profiling we were able to confirm our previous findings from membrane marker analyses, i.e. that MUTZ-3 IDC and LC exhibit true IDC and LC characteristics, thereby confirming the validity of MUTZ-3 as a myeloid DC and LC model. Moreover, comparative analysis revealed novel transcripts which were found specifically over-expressed in MUTZ-derived IDC as compared to LC (or vice versa) concomitantly with their respective physiological counterparts, i.e. skin-derived CD1a⁺ Dermal DC (DDC) and LC. We conclude that MUTZ-3 derived IDC and LC represent a readily available, physiologically relevant and well defined model to investigate DDC and LC biology *in vitro*.

Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) with the unique ability to initiate and maintain primary immune responses. In the human body, two main populations of DC can be discriminated, Langerhans cells (LC), which can be found in the epidermis and other epithelia (1,2) and dermal or interstitial DC (DDC or IDC), which can be found in the dermis and in connective tissues throughout the body. *In vitro*, IDC and LC can be cultured from peripheral blood-derived CD14+ monocytes or from blood- or bone marrow-derived CD34+ hematopoietic progenitor cells (HPC) (1,3-7). Although both DC subsets originate from a common myeloid DC precursor and share several features, such as the expression of high levels of MHC class I and class II molecules, co-stimulatory and adhesion molecules, of certain myeloid markers such as CD13 and CD33, and a lack of CD3, CD19, CD20, CD16 and CD56 lineage markers (8), both DC subsets also exhibit specific "IDC/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, the expression of E-cadherin, membrane adenosine triphosphatase (ATPase) and chemokine receptor-6 (CCR6) (9-12). On the other hand, IDC/DDC can be characterized by the expression of the C-type lectins macrophage mannose receptor (MMR) and DC-SIGN, as well as by the expression of the scavenger receptor CD36, the coagulation factor XIIIa (FXIIIa) and the monocyte/macrophage marker CD14 (1,13-18).

Although it has been described that both IDC/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 (19). Besides that, *in vitro*-generated IDC/DDC have been described to more efficiently drive the differentiation of naïve B cells into IgM-secreting plasma cells (2), while LC have been described to be more potent *in vitro*

stimulators of cytotoxic T cells, as well as more potent inducers of Th1 responses due to their inability to produce IL-10 upon CD40 ligation (2,20).

The development of *in vitro* DC differentiation protocols has facilitated DC biology studies tremendously. Yet, generating sufficient numbers of DC for these studies is often hampered by scarcity of DC precursors and inter- and intra-donor variability in DC differentiation. To circumvent these problems, it would be favorable to culture DC from an unlimited and readily available source. We recently showed that the human CD34⁺ acute myeloid leukemia (AML) cell line MUTZ-3 can differentiate into functional DC in a cytokine-dependent manner (21,22). Indeed, upon stimulation with GM-CSF, TNF- α and IL-4 or TGF- β and subsequent maturation, fully mature, immuno-stimulatory DC-SIGN⁺ Interstitial DC (IDC) or Langerin⁺/Birbeck granule⁺ Langerhans cell (LC), exhibiting CTL priming as well as lymph node-homing capabilities, could be generated (Santegoets *et al*, submitted).

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 transcriptional profiling of MUTZ-3 IDC and LC, using high-density microarray analysis, we aimed to determine whether MUTZ-3-derived IDC and LC exhibit true IDC and LC characteristics. Extensive transcriptional analysis was performed to identify novel subset-specific transcripts and obtain additional clues to functional differences between IDC and LC.

Materials and Methods

Cell lines.

The human CD34⁺ acute myeloid leukemia cell line MUTZ-3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) was cultured in MEM- α medium containing ribonucleotides and deoxyribonucleotides (Life Technologies, Paisley, UK) supplemented with 20 % Fetal calf serum (Perbio, Helsingborg, Sweden), 100 I.E./ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 μ g/ml streptomycin sulphate (Radiumfarma-Fisiopharma, Naples, Italy), 2.0 mM L-glutamine (Invitrogen, Breda, The Netherlands), 0.01 mM 2-mercapoethanol (Merck, Darmstadt, Germany) and 10 % 5637-conditioned medium (CM) (21,23).

***In vitro* generation of MUTZ-3-derived interstitial and Langerhans cells.**

MUTZ-3 IDC and MUTZ-3 LC were generated as described (21,24). Briefly, MUTZ-3 progenitors were cultured in 12 well tissue culture plates at a concentration of 1×10^5 /ml in the presence of 100 ng/ml GM-CSF (Schering-Plough, Madison, N.J), 20 ng/ml IL-4 (R&D systems, Minneapolis, MN) and 2.5 ng/ml TNF- α (Strathmann Biotec, Hamburg, Germany) for 7 days for MUTZ-3 IDC differentiation and in the presence of 100 ng/ml GM-CSF, 10 ng/ml TGF- β (BioVision, Mountain View, CA) and 2.5 ng/ml TNF- α for 10 days for MUTZ-3 LC differentiation. Every 3 days new cytokines were added.

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% sodium-azide for 30 minutes at 4°C. 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 (25). Briefly, cell pellets (2 - 5x10E6 cells/pellet) MUTZ-3-derived IDC and LC, from 3 independent experiments, were dissolved in TRIzol Reagent (Invitrogen Life Technologies) and stored at -20°C. After chloroform extraction, total RNA was precipitated in isopropanol, rinsed with 70% ethanol, lyophilized, and dissolved in 10 µl of distilled water. Prior to gene chip hybridization, all RNA samples were quality controlled with Agilent 2100 Bioanalyzer. All RNA samples fulfilled requirements of minimum yield of 250 ng per sample and minimum RNA integrity number (RIN) of 8.0. 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 each subset, the intensity signals for selected marker genes that were expressed (denoted *present*) and had an intensity level of >200 were assessed.

Results and Discussion

Phenotyping of MUTZ-3-derived IDC and LC by flow cytometry.

IDC and LC were generated from the MUTZ-3 cell line as described previously (22). Upon stimulation with GM-CSF, TNF- α and IL-4, MUTZ-3 progenitors acquired an IDC phenotype, expressing CD1a, DC-SIGN but lacking Langerin (figure 1, top panel), whereas MUTZ-3 progenitors cultured in the presence of GM-CSF, TNF- α and TGF- β acquired an LC phenotype, expressing CD1a, Langerin, and CCR6 but lacking DC-SIGN (figure 1, lower panel). Furthermore, MUTZ-3 IDC and MUTZ-3 LC exhibited an immature phenotype, expressing only low/intermediate levels of the co-stimulatory molecule CD86 and virtually no CD83.

Phenotyping of MUTZ-3-derived IDC and LC by transcriptional analysis.

To make an extensive transcriptional comparison of MUTZ-3-derived IDC and LC, RNA isolated from both DC subsets was hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays containing more than 54,000 probe sets and covering 38,500 human genes. mRNA expression profiles of MUTZ-3 IDC and LC were assessed from 3 individual cultures. To determine whether the MUTZ-3 IDC and LC indeed represent true IDC and LC, both DC subsets were first subjected to a global transcriptional analysis and 15 markers associated with IDC 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.

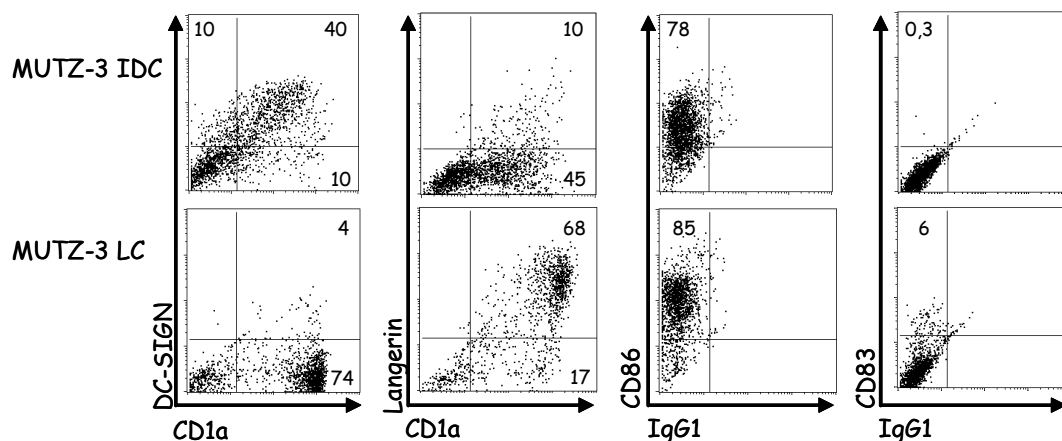


Fig. 1 Phenotype of MUTZ-3-derived Interstitial Dendritic Cells (IDC) and Langerhans Cells (LC). MUTZ-3 progenitors cultured in the presence of GM-CSF, TNF- α and IL-4 or TGF- β develop into CD1a+/DC-SIGN+ IDC (*top panel*) or CD1a+/Langerin+ LC (*bottom panel*) respectively, expressing intermediate levels of the co-stimulatory molecule CD86 and no CD83. Data shown for MUTZ-3 IDC and LC phenotype is representative of 4 independent experiments. Percentage-positive cells are depicted in the quadrants.

As shown in Table I and Figure 1 and as found for skin-derived DDC and LC (Santegoets *et al*, manuscript in preparation), MUTZ-3-derived LC express higher levels of CD1a transcripts and protein as compared to MUTZ-3 IDC. In addition, MUTZ-3 IDC specifically express markers associated with IDC phenotype, such as the C-type lectin DC-SIGN and the coagulation FXIIIa. On the other hand,

MUTZ-3 LC specifically express markers associated with LC phenotype, such as the membrane ATPase and the chemokine receptor CCR6. In addition, mutually exclusive expression of DC-SIGN and CCR6 could also be confirmed at the protein level by MUTZ-3 IDC and LC, respectively (Figure 1). In contrast to earlier reports for skin DDC and LC, MUTZ-3 LC express transcripts of IDC-associated markers such as MMR, CD36, CCR5 and CD1b and MUTZ-3 IDC express transcripts of LC-associated markers such as Langerin. Yet, expression levels of MMR and CD1b transcripts were much higher in MUTZ-3 IDC as compared to MUTZ-3 LC, indicating that the relative expression levels followed the same pattern as described for skin DDC and LC (Table I). Similarly, Langerin transcript levels were much lower in MUTZ-3 IDC compared to MUTZ-3 LC, and moreover translated into clear differences in membrane protein expression (Figure 1). Of note, membrane expression of E-cadherin on MUTZ-3 LC, determined by flow cytometry and previously reported (22), could not be confirmed by microarray analysis, indicating that caution should be observed in interpretation of transcriptional analysis in relation to actual protein expression profiles. Nevertheless, analyzing similarity between MUTZ-3 IDC and LC and their primary counterparts at the transcriptional level for all 15 IDC/LC defining markers demonstrated that in 9 of 15 IDC/LC-defining markers the relative expression between the two MUTZ-3 DC subsets followed the same pattern as reported for their primary counterparts (Table I).

Table I. Typical DDC and LC characteristics of MUTZ-3 derived IDC and LC.

Gene	mRNA expression profile		Literature	
	MUTZ-3 IDC [†]	MUTZ-3 LC [†]	IDC [†]	LC [†]
CD1a [‡]	++	+++	+	++
CD1b	++	+	+	-
CD1c	+++	++	+	+/-
CD1d	-	-	+	-
CD206/MMR	+++	++	+	-
CD207/Langerin [‡]	++	+++	-	+
CD209/DC-SIGN [‡]	+	-	+	-
CD36	++	++	+	-
E-cadherin	-	-	-	+
ATPase	-	+	-	+
FXIIIa	+	-	+	-
CCR2	-	-	+	-
CCR5	+	+	+	-
CCR6 [‡]	-	++	-	+
CD64/FcγRI	-	-	+	-

^{*} Analyzed by using high density microarray; Signal intensity levels: - <200, + 200 - 500, ++ 500-5000, +++ >5000

[†] From literature; expression on human CD34-derived IDC and LC, CD14⁺ monocyte-derived IDC or skin-derived IDC and LC, reviewed by Larregina *et al.* (8) and Valladeau *et al.* (26).

[‡] (Differences in) expression confirmed at the protein level by flow cytometry

Transcriptional profiles are consistent with a quiescent, non-activated state of the immature MUTZ-3 IDC and LC.

To fully characterize both DC subsets and find clues for functional differences between the two subsets, MUTZ-3 IDC and LC were analyzed for the expression of a panel of genes that were selected on the basis of their known association with DC biology (Table II). As listed in Table II, the immature (i.e. non-activated) state of the MUTZ-3 IDC and LC subsets was generally confirmed through

transcriptional analysis with a relatively non-stimulatory, non-migratory phenotype, as illustrated by the expression of a relatively limited number of transcripts encoding inflammatory cytokines, chemokines and chemokine receptors (Table IIB and C). This observation is in keeping with protein data, showing no or only low level of production of pro-inflammatory chemokines like CCL20 and CCL5 by MUTZ-3 IDC and LC (data not shown) and by their inability to produce pro-inflammatory cytokines like IL-12 and IL-15 (Santegoets *et al*, submitted). In addition, transcript levels for T cell stimulatory molecules such as CD30L or 4-1BBL were generally low or absent in both immature MUTZ-3 IDC and LC, confirming their resting state (Table II, or not listed). Surprisingly, relatively high levels of CD83 transcripts in both DC subsets seemed at odds with this observation, but surface expression of CD83 was found (virtually) absent by flow cytometric analysis (figure 1). However, there were more notable exceptions: a higher level of maturation in the LC was suggested by a more consistent expression of various TNF(R) family members and a higher expression level of CD86 transcripts (Table II), the latter of which was confirmed at the protein level (figure 1) and translated into stronger activation of allogeneic T cells by MUTZ-3 LC than by IDC in a mixed leukocyte reaction (22). Follow-up analyses will include activated MUTZ-3 IDC and LC, matured under different conditions. These studies should reveal further up-regulation of transcripts involved in T cell stimulation, of which we have previously reported both mature MUTZ-3 DC and LC to be fully capable (22,24). Interestingly, and different from skin-derived DC (Santegoets *et al*, submitted), immature MUTZ-3 IDC and LC do express BAFF (B cell activating factor) and APRIL (a proliferation-inducing ligand), TNF family members that are known to play a role as co-stimulators of B lymphocyte proliferation and function (27,28)

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

Category	Gene	MUTZ-3 IDC	MUTZ-3 LC	UniGene ID
A. Co-stimulatory, adhesion molecules and integrins	CD13	++	++	Hs.1239
	CD14	-	+	Hs.75627
	CD31	+	+	Hs.78146
	CD40	+	+	Hs.504816
	CD54	++	++	Hs.386467
	CD80	+	+	Hs.838
	CD86	-	++	Hs.27954
	CD83	++	++	Hs.79197
B. Chemokines and their receptors	CCL3, MIP-1 α	+	-	Hs.73817
	CCL4, MIP-1 β	+	-	Hs.75703
	CCL19, MIP-3 β	-	-	Hs.50002
	CCL20, MIP-3 α	-	-	Hs.75498
	CCL22, MDC	-	++	Hs.97203
	CXCL1, Gro- α	+	-	Hs.789
	CXCL12, SDF- α	-	-	Hs.436042
	CXCL2, MIP-2 α	-	-	Hs.75765
	CXCL3, MIP-2 β	-	-	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 their receptors	IL-1 α	-	-	Hs.1722
	IL-1 β	++	++	Hs.126256
	IL-6	-	-	Hs.512234

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

Table II (continued)

Category	Gene	MUTZ-3 IDC	MUTZ-3 LC	UniGene ID	
C. Cytokines and their receptors (continued)	IL-8	+++	++	Hs.624	
	IL-10	-	-	Hs.193717	
	IL-15	-	-	Hs.168132	
	IL-16	+	+	Hs.170359	
	IL-18	-	+	Hs.83077	
	IFN- β 1	-	-	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 R α	++	++	Hs.327	
	IL-13 R α 1	++	++	Hs.285115	
	IL-18 BP	++	++	Hs.325978	
	IL-18 R1	+	+	Hs.159301	
	IL-18 R β	+	++	Hs.158315	
	IFN- γ R1	+	++	Hs.180866	
	IFN- γ R2	++	++	Hs.409200	
D. C-type lectins and scavenger receptors	GM-CSFR	++	++	Hs.520937	
	CD205, Dec-205	+	+	Hs.153563	
	CD206, MMR	+++	++	Hs.75182	
	CD207, Langerin	++	+++	Hs.199731	
	CD208, DC-LAMP	+++	++	Hs.10887	
	CD209, 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	
	CD40	+	+	Hs.504816	
	TNF- α	+	++	Hs.241570	
	TNF-R1,	++	++	Hs.159	
	TNF-R2	+	+	Hs.256278	
	CD30	-	+	Hs.1314	
	4-1BB	-	++	Hs.193418	
	E. TNF/TNF receptor family	APRIL	++	++	Hs.54673
		BAFF	++	++	Hs.270737
CD30L		-	-	Hs.177136	
TRAIL-R2		++	++	Hs.51233	
TRAIL-R4		+	+	Hs.129844	
Lymphotoxin B R		++	+++	Hs.376208	

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

Differentially expressed transcripts between the MUTZ-3 IDC and LC subsets.

Notable differences in expression of transcripts between the subsets, that may point to differential functions and deserve further attention, are 1) low-level expression of the pro-inflammatory chemokines CCL3, CCL4, and CXCL1 in the IDC that is completely lacking in the LC, 2) specific and relatively high level of expression of CCL22 (Macrophage-derived chemokine, MDC) by LC, previously also reported for skin-derived and histiocytotic LC (29) and indicative of an ability to attract Th2 and regulatory T cells (30,31), and 3) the presence of the classic T cell-associated markers CD30 and 4-1BB specifically on LC. Particularly noticeable is the relatively high level of 4-1BB expression, which was also found on murine DC and shown to induce DC maturation upon cross-linking, resulting in IL-6 and IL-12 release and increased stimulation of T cells (32).

In order to identify novel subset-specific transcripts and find additional clues for the utility of MUTZ-3 IDC and LC in the study of differential functions between the two DC subsets, we compared over-expression of transcripts between MUTZ-3 IDC and LC with data obtained from an equivalent microarray analysis performed with their physiological *in vivo* counterparts: i.e. freshly isolated LC and CD1a⁺ DDC from human skin (Santegoets *et al*, submitted). In total, 12 genes were found to be significantly up-regulated in terms of transcript levels in MUTZ-3 IDC as compared to undifferentiated MUTZ-3 progenitors (demonstrating association with differentiated DC) and simultaneously significantly higher in MUTZ-3 IDC than in MUTZ-3 LC, as well as in skin-derived DDC in comparison to skin-derived LC. In a similar analysis for MUTZ-3 LC, 21 genes were found significantly up-regulated as compared to undifferentiated MUTZ-3 progenitors and MUTZ-3 IDC and simultaneously significantly higher in skin-derived LC in comparison to skin-derived DDC. The relatively low number of subset-specific markers that were identified through this analysis may be due to pre-existent expression levels of other subset-specific transcripts at the MUTZ-3 progenitor stage that did not significantly change during IDC or LC differentiation and were therefore omitted from the final selection. Follow-up analyses are therefore needed to ascertain IDC/LC-subset association of transcript expression independent of differentiation and/or maturation state. Nevertheless, the specific expression of classic DDC/LC subset-defining markers, such as FXIIIa and DC-SIGN for IDC/DDC and Langerin and CCR6 for LC, could be confirmed through this analysis, indicative of its validity. In addition, specific expression of transcripts from several novel genes, previously associated with cell biological processes such as growth control, apoptosis, differentiation, migration, signaling, and the recognition and binding of Gram-negative bacteria and of antibodies, were identified for each of the IDC/DDC and LC subsets. MUTZ-3 derived IDC/LC provide an ideal tool for the study of the possible role of such genes in IDC/DDC or LC biology, since in contrast to their physiological skin counterparts, they provide sufficient numbers of cells for e.g. knock-down RNA interference studies and subsequent extensive functional and phenotypic analyses.

In summary, transcriptional analysis demonstrated that in general MUTZ-3-derived IDC and LC adhere to the proposed definitions of the IDC and LC phenotype, and thus phenotypically resemble their primary counterparts. Differentially expressed transcripts between these DC subsets (validated by the similarly differential expression in their cutaneous counterparts) are attractive candidates for further study to delineate functional differences. By providing unlimited numbers of standardized IDC/DDC and LC, the MUTZ-3 cell line constitutes an attractive and relevant tool for this purpose. In addition, in order to determine the validity of the MUTZ-3 cell line as a model for primary myeloid human IDC and LC differentiation, we are currently in the process of performing comparative transcriptional analysis between MUTZ-3 progenitors and MUTZ-3-derived IDC and LC.

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