Distinct molecular signature of human skin Langerhans cells denotes critical differences in cutaneous dendritic cell immune regulation.

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**Abbreviations:**

ANOVA, analysis of variance; APC, antigen presenting cell; CCL, chemokine C-C motif ligand; DAMP, danger associated molecular pattern; DDC, dermal dendritic cell; EBV, Epstein-Barr Virus; HIV, Human Immunodeficiency Virus; HLA, Human Leukocyte Antigen; IL, interleukin; LC, Langerhans cell; MCL, Markov Clustering Algorithm; MDS, Multidimensional Scaling; MMP, matrix metalloproteinase; PAMP, pathogen-associated molecular pattern; qPCR, quantitative polymerase chain reaction; RMA, robust multichip average; TGF-β, tumour growth factor-β; TLR, toll-like receptor; TNF-α, tumour necrosis factor-α
Abstract:

Langerhans cells (LCs) are professional antigen presenting cells (APCs) residing in the epidermis. Despite their high potential to activate T lymphocytes, current understanding of human LC biology is limited. Genome-wide comparison of the transcriptional profiles of human skin migratory CD1a+ LCs and CD11c+ dermal dendritic cells (DDCs) demonstrated significant differences between these ‘dendritic cell’ types, including preferential expression of 625 genes (p<0.05) in LC and 914 genes (p<0.05) in DDC. Analysis of the temporal regulation of molecular networks activated after stimulation with TNF-α confirmed the unique molecular signature of LCs. Although LCs conformed to the phenotype of professional APC, inflammatory signalling activated primarily genes associated with cellular metabolism and mitochondrial activation (e.g. CYB561, MRPS35), cell membrane re-organisation and antigen acquisition and degradation (CAV1, PSMD14) (p<0.05 - p<0.0001). Conversely, TNF-α induced classical activation in DDCs with early down-regulation of surface receptors (MRC1, C-type lectins), and subsequent up-regulation of cytokines, chemokines (IL1a, IL1b, CCL18) and matrix metalloproteinases (MMP1, MMP3, MMP9), (p<0.05 - p<0.0001). Functional interference of caveolin abrogated LCs superior ability to cross-present antigens to CD8+ T lymphocytes, highlighting the importance of these networks to biological function. Taken together these observations support the idea of distinct biological roles of cutaneous DC types.

Keywords:

Langerhans Cell, TNF-α, caveolin, cross-presentation, transcriptome
Skin-resident dendritic cells (DCs), including epidermal Langerhans cells (LCs), orchestrate cutaneous immune responses while helping to maintain tissue homeostasis (Banchereau et al., 2003; Polak et al., 2012; Seneschal et al., 2012a). LCs are located in the epidermis and are able to use their dendrites to reorganize upper epidermal tight junctions, allowing constant sampling of the tissue and environment (Kubo et al., 2009). They can acquire particulate antigens via phagocytosis and endocytosis (Kubo et al., 2009; Sagebiel, 1972) and facilitate antigen uptake with surface receptors including C-type lectin, Langerin (Valladeau et al., 2000) and CD205 (Flacher et al., 2010; Santegoets et al., 2008). As a result of antigen uptake, LCs can stimulate efficient primary and secondary immune responses to viral antigens, including influenza (Klechevsky et al., 2010; Klechevsky et al., 2008), Epstein-Barr Virus (EBV): (Polak et al., 2012), measles (van der Vlist et al., 2011), mycobacteria (Hunger et al., 2004) and fungi (de Jong et al., 2010). However, LC interactions with pathogens are not limited to activation of antigen-specific T lymphocytes, as Langerin-mediated uptake of HIV by LCs results in efficient degradation of viral particles, and thus induces protection against HIV infection (de Witte et al., 2007). LC maturation is likely to be critical for efficient induction of T cell responses (Banchereau et al., 2003; Grabbe et al., 1992; Polak et al., 2012; van der Vlist et al., 2011). The maturation signals come from LC cross-talk with surrounding tissue e.g. tissue-derived pro-inflammatory cytokines, including TNF-α (Berthier-Vergnes et al., 2005; Polak et al., 2012; Ratzinger et al., 2004), TSLP (Ebner et al., 2007; Nakajima et al., 2012) and TGF-β (Bauer et al., 2012; Geissmann et al., 1999), damage associated molecular patterns (DAMPs) (Kool et al., 2011) and recognition of pathogen-derived signals, including pathogen-associated molecular patterns (PAMPs) (Peiser et al., 2008; Tang et al., 2010).
LCs anatomical location in the outermost part of the skin and mucosal tissue combined with their classical DC capacity for antigen capture, processing and presentation make a strong case for them acting as the primary gatekeepers against infection and other exogenous pro-inflammatory stimuli. However, the immunostimulatory role of LCs, as compared with dermal DCs (DDCs) in cutaneous immunity has been much debated (Bennett et al., 2005; Kaplan et al., 2005; Noordegraaf et al., 2010; Ritter et al., 2004; van der Aar et al., 2013; Zhao et al., 2003). Recently, we and others have demonstrated that the direct interactions within the immunological synapse are critically important for human LCs’ capacity to stimulate CD8 T lymphocytes (Banchereau et al., 2012; Polak et al., 2012; van der Aar et al., 2011, {Banchereau, 2012 #1049} To better understand the molecular mechanisms regulating LC function, we undertook microarray analysis of gene expression changes in two subsets of migratory DCs isolated directly from human skin: CD1a+ epidermal LCs and CD11c+ DDCs (Teunissen et al., 2012; Zaba et al., 2007), immediately post isolation and over a time course stimulation in culture with TNF-α, an epidermal pro-inflammatory cytokine. The results of transcription network analysis, validated by functional assays, clearly demonstrate distinctively different transcriptional profiles of these two skin-derived APCs, and denote the key role of protein metabolism and antigen processing in LC biology.

Results

1. Molecular and functional analysis of migratory CD1a+ epidermal LCs and CD11c+ dermal DDCs.
LCs and DDCs isolated by migration over 48 hours from human skin (termed “migratory”) were 85%-96% CD1a+/HLA-DR+ (LC) and 82-90% CD11c+/HLA-DR+ (DDC) as assessed with flow cytometry (Figure 1 a,b). Expression of markers classically associated with LC (CD207 – langerin, CD205/DEC205) and DDC (FXIIIa, and mannose receptor-1 (MRC1), CD14, CD163, CD209 and C-type lectins: CLEC10A, CLEC2B, CLEC4E) in unstimulated cells was confirmed by microarrays (Figure 1c). As expected, all skin DCs exhibited the molecular signature of a professional APC, including high expression levels of genes involved in antigen presentation to T cells: HLA class I and II, β2 microglobulin (B2M) and class II HLA transactivator (CIITA), and co-stimulatory molecules (CD40, CD80, CD86) (Supplementary Figure S1 a-c). Using a well-defined HLA-A2 EBV epitope, we confirmed that both mature LCs and DDCs pulsed with specific peptide efficiently induced A2 EBV-specific CD8+ cell proliferation (Figure 1d-f) and activation (Figure 1 g). By fusing the same epitope into a 39 amino acid long peptide, requiring antigen processing for presentation on HLA-A2, we confirmed that only LCs showed significant ability to cross-present antigens as we have reported previously (Polak et al., 2012) (Figure 1h).

2. LC and DDC transcriptomes are distinctively different.

To investigate the molecular mechanisms underpinning the functional superiority of LC to cross-present antigens during maturation and pro-inflammatory conditions, we analysed the transcriptome of CD1a+ LCs and CD11c+ DDCs over a 24 hour time course of stimulation with an epidermal pro-inflammatory cytokine, TNF-α. Multidimensional scaling (MDS) analysis (Figure 2a) and sample-to-sample clustering (BioLayout Express3D, Figure 2b) of the transcriptomes of CD1a+ LCs and CD11c+ DDCs indicate that the gene expression
profile of LCs are quite distinct from DDC. Additionally, over 24 hour stimulation with TNF-α, while CD11c+ DDC showed clear evidence of an ongoing adaptation of transcription throughout the time period, LCs displayed only minor changes to their transcriptional profiles (Figure 2b, Supplementary Table S1).

3. Characterisation of the biological processes in migratory LCs and DDCs.

Genome-wide transcriptome comparison of unstimulated LCs and DDCs identified 969 probesets (625 genes) preferentially expressed in LC, and 1,648 probesets (914 genes) preferentially expressed in DDC (1.5 fold difference in log₂(x) RMA normalised expression level between the cell types; Supplementary Table S1). To determine the biological processes specific to CD1a+LCs and CD11c+ DDCs, these lists of genes were submitted to DAVID bioinformatic database analysis (Huang da et al., 2009a, b), (Supplementary Table S2). Both LC and DDC were sensitive to TNF-α signalling. However, while DDCs overexpressed TNFRSF10A, TNFRSF10B, TNFRSF10D, TNFESF14 and TNFRSF21, LC highly expressed two isoforms of TNFRSF11 (A and B) and TNFRSF8. The biological function of migratory LCs was strikingly different to DDCs, as predicted by DAVID functional gene classification and functional annotation. Migratory DDC expressed multiple genes typically associated with immune responses, involved in cytokine-cytokine receptor interactions, chemokine signalling pathways, Toll-like receptor signalling pathway and Fc-γ receptor mediated phagocytosis (Supplementary Table S2). DDC expressed a broad spectrum of receptors, including pathogen recognition associated cell surface receptors (Figure 1c). Many of the DDC-overexpressed genes involved in signal transduction were directly involved in immune signalling (ABCA1, GEM, IRAK3, KL, NDRG1, PYCARD). The DDC effector genes included
abundant immune mediators (including complement proteins and low levels of cytokines and chemokines). In contrast, the majority of genes over-expressed in migratory LC were involved in cytoskeleton reorganisation and membrane re-modelling (ACTB, CNN, DSP, ANK3, PFN1, SYNPO, PLEK2), endocytosis and intracellular transport (AP1B1, AP2S1, SH3KBP1, SNX4, SNX7), proteolysis (FBXO2, PSMC3, UCHL3, USP46, TRIM32) and mitochondrial activity (ACOT1, ACOT7, ACOX3, CYB561, NDUFB7, NQO1). Biological pathways identified in LC were involved primarily in cell metabolism (KEGG pathways annotation). LC preferentially expressed a small number of receptors, including CD207 (langerin), lipoprotein receptors (LSR and LDLR), and only a handful of genes primarily involved in immune processes (CCL22, CD70, CLU, COTL1, HLA-DQA, and TAPBL) (The full list of genes preferentially expressed in LCs and DDCs: Supplementary Table S2).

4. Migratory skin DCs and trypsinised skin DCs demonstrate strongly matching transcriptomes.

To confirm the relevance of these findings to the in vivo situation we also challenged our model system against whole transcriptome data from DCs rapidly isolated from skin using a trypsinisation protocol (Allen et al., 2010; Harman et al., 2013; Hutter et al., 2012; Santegoets et al., 2008) [Supplementary Table S3]. Comparison of the genes differentially expressed in either LCs or DDCs revealed the same pattern of expression in both the migratory and trypsinised models (Supplementary Figure 2). Furthermore Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) of T0 LCs (n=18 datasets), confirmed that migratory LC genes of interest (GOI) were also significantly enriched in trypsinised LCs, ES=0.55, p<0.02 and ES=0.42, p<0.0019, ES=0.52, p<0.01 [Supplementary Table S3 1,2,3] respectively, as compared with DDC. Similarly, gene expression in migratory DDC populations were
replicated in trypsinised DDCs, (ES=0.78, p<0.05) in comparison with trypsinised LCs. As seen in migratory LCs, DAVID functional gene classification and functional annotation of genes of increased expression in trypsinised LCs were involved in cell-to-cell adhesion, mitochondrial function and metabolism, and trypsinised DDCs reflected migratory DDCs showing overexpressed genes involved in receptor-mediated pathogen uptake, cytokine signalling and immune responses (Supplementary Figure S2, Supplementary Table S4). However, some differences between migratory and trypsinised cells were evident. As predicted, in comparison to trypsinised cells, migratory LCs showed reduced (but not absent) expression of cell adhesion molecules, increased proteasome activity and up-regulation of the expression of co-stimulatory molecules. This suggests, that despite acquiring a T cell activatory phenotype and immunological maturation, the overall genetic profile underpinning their biology remained similar. Migratory and trypsinised DDCs showed very closed resemblance, upon migration they further upregulated and extended their scavenging and pro-inflammatory characteristics.

5. Activation of human skin APCs with the pro-inflammatory cytokine TNF-α results in temporal coordination of immune-related gene transcription in DDCs, but not in LCs.

To investigate transcriptional events during activation of skin LCs and DDCs, we reconstructed networks of co-regulated genes in BioLayout Express3D (Pearson coefficient cut-off $r=0.85$, MCL inflation value $=1.7$) over a time course of stimulation with TNF-α for 2,334 probesets showing >1.5 fold difference in log2(x) RMA normalised gene expression levels in comparison with un-stimulated cells (Bayesian Estimation of Temporal Regulation
cut-off threshold $p=0.05$, (Aryee et al., 2009)). The resultant gene transcription network diagram was highly organised and comprised of two main loosely connected network structures, representing genes expressed in a cell type-specific manner: the top part of the diagram contained genes preferentially expressed in LC, bottom part in DDC (Figure 2c-f).

More than 90% of transcripts were expressed differentially in LCs and DDCs, including 1,859 genes regulated by TNF-α selectively in DDCs and 306 genes selectively in LC. The separate grouping of TNF-α regulatory networks in DDCs and LCs supported a dramatic functional and temporal discordance between these two cell types (Figure 2c). To understand the biological events during the temporal regulation with TNF-α, the gene network was clustered using Markov Clustering Algorithm (inflation value=1.7). This identified 13 clusters of genes expressed preferentially in DDCs, 12 clusters grouping genes expressed preferentially in LC, and 6 small clusters containing genes regulated in the same manner in both cell types (Figure 2). The analysis of biological processes enriched in the genes grouped within 14 largest clusters (containing 1,887 transcripts) demonstrated, that seven of them (clusters 01, 02, 04, 06-09) contained genes predominantly involved in inflammatory responses that were highly expressed in DDCs in contrast to LCs (Figure 2c,d, Supplementary Figure S2, Supplementary Table S5). The dynamics of gene expression in these clusters recapitulate the typical myeloid DC activation pattern (Banchereau et al., 2003). Upon activation, DDCs down-regulated surface cell receptors, associated with antigen capture, and reduced phagocytosis (cluster 01, 08, including CD163, C-type lectin receptors CLEC1A, CLEC4G, CLEC5A, CLEC7A, CXCL2, CXCR7 and FC-γ receptors FCGR2A, FCGR2B, FCGR3A ), while expression of genes involved in transcription, intracellular signalling and cytokine synthesis peaked at 2h (cluster 02; e.g. CREM, RPL28, TNF, IL7, IL1RN, CXCR4, CCL3, CCL4, DYRK-3, RIPK1, MAPK2K3, ZNF36, ZNF295) followed by an up-regulation of genes involved in synthesis and secretion of a wide range of cytokines.
and chemokines, matrix metalloproteinases (known to be involved in rearrangement of extracellular matrix) and regulators of cell migration between 8 and 24h (clusters 04, 06, 07, 09; e.g. *IL1A, IL23A, MMP1, MMP7, MMP9, MMP14, CCL1, CCL2, CCL17, CCL18, CCL24, CXCL6*, Figure 2c,d, Supplementary Figure S3, Supplementary Table S5).

In contrast, LCs showed low levels of DDC gene cluster expression and the changes induced by stimulation with TNF-α were minimal. The six biggest LC-associated clusters (03, 05, 10, 12-14, Figure 2c,e) grouped genes relatively highly expressed in un-stimulated cells, and activation with TNF-α had a proportionally lesser effect on gene expression changes in LCs as compared with gene changes induced in DDCs. During the stimulation with TNF-α LC down-regulated the catabolism of carbohydrates and fatty acids (cluster 05), and increased expression of proteins involved in endocytosis, intracellular transport and signalling, protein degradation, including genes coding for proteasome assembly units and protein degradation enzymes (cluster 03,12,14). TNF-α signalling also altered LC mitochondrial function, inducing high expression levels of mitochondrial ribosomal protein expression (for individual gene profiles see Supplementary Figure S4). Interestingly, genes involved in mitosis could also be identified in all LC-associated clusters, supporting the hypothesis of a self-repopulating ability previously postulated for LCs (Hemmerling *et al.*, 2011; Kanitakis *et al.*, 2011). Perhaps surprisingly, only a limited number of genes typically associated with immune responses were preferentially up-regulated in LCs following TNF-α, including *IL15*, and *CCL22* (cluster 03; Figure 3a,c, Supplementary Figure S4, Supplementary Table S5). However, amongst the genes up-regulated in LCs during stimulation with TNF-α, and positioned separately from the core myeloid DC clusters (clusters 03, 12, and 14, Figure 2c,e), we identified several genes primarily associated with cytoskeleton organisation, but which have also been reported in immune responses or immune signalling, including *SNX 11*.
(sorting nexin 11; cluster 12) and SYNPO (synaptopodin; cluster 14). Cluster 14 genes, such as \textit{CAV1}, link processes of antigen acquisition and regulation of immune responses, including genes involved in endocytosis, cytoskeleton reorganisation and regulation of signal transduction (\textit{PTPRK}).

Genes regulated in the same manner in LCs and DDCs were less frequent (243 transcripts), and grouped in 6 smaller clusters, including cluster 11 (Figure 2c,f). These included genes associated with nucleus, RNA processing and transcription, cytoskeleton, cobalamin biosynthesis, hemopoiesis and leukocyte development. The interactive 3D map of skin migratory DC transcriptome is available at http://www.macrophages.com/LC_vs_DC.

6. **Caveolin-1 dependency of LC cross-presenting function.**

LC-associated molecular networks clearly indicated a relationship between TNF-\(\alpha\) signalling and induction of genes grouped in clusters 03, 12 and 14. To confirm their dependence on TNF-\(\alpha\), in contrast to immune-related gene expression, we validated the TNF-\(\alpha\) induced up-regulation of \textit{CAV1} and \textit{PMSD14} in LCs versus DDCs by qPCR (Figure 3a-d) in cells isolated from three independent skin donors. In contrast, \textit{CCL18} was uniquely up-regulated in DDC (Figure 3e-f). The gene expression pattern assessed by qPCR validated the microarray data (Figure 3 b,d,f). Whilst proteasome function is indisputably associated with antigen processing and presentation, we were interested to test whether the increased expression of \textit{CAV1} in LC might contribute to their superior cross-presenting facility. Caveolin-1 function is specifically inhibited by filipin III (Sato et al., 2012; Yan et al., 2004). We titrated filipin
III concentrations against LC and DDC viability in overnight cell culture and confirmed that this molecule is non-toxic at the concentrations used (0.1-1 μg/ml; data not shown). Using HLA-matched DCs pulsed with proGLC, inhibition of caveolin with filipin III completely abrogated the ability of LCs to cross-present antigens to EBV-specific CD8+ T lymphocytes (Figure 3g).

Discussion

LCs have long been recognised as key sentinels in human cutaneous immunity, but recent experimental murine models demonstrating their apparent redundancy in cutaneous immune responses has called their role into question (Bennett et al., 2005; Kaplan et al., 2005; Noordegraaf et al., 2010; Ritter et al., 2004; Zhao et al., 2003). Previous work to characterise the relative roles of human cutaneous DCs in the skin have shown conflicting results, with some groups reporting that LCs are the key APC inducing skin immunity (Banchereau et al., 2012; Flacher et al., 2010; Klechevsky et al., 2008; Polak et al., 2012; van der Aar et al., 2011), and others the converse (de Witte et al., 2007; Lundberg et al., 2013; Santegoets et al., 2008; van der Aar et al., 2013). We and others have previously demonstrated that LCs are superior activators of CD8 T cells, due to differential signalling via CD70 and their exceptional efficiency in cross-presenting protein antigens to CD8 T cells (Polak et al., 2012; van der Aar et al., 2011) as well as IL-15 secretion directly into the immune synapse (Banchereau et al., 2012). However, in contrast to DCs derived from the dermis, LCs produce fewer typical inflammatory mediators, including low levels of IL-1β and IL-12p70 (Banchereau et al., 2012; Munz et al., 2005; Polak et al., 2012; Ratzinger et al., 2004). In this
study we aimed to undertake a comprehensive analysis, combining transcriptomic assessment with functional readout to characterise primary cutaneous DC function.

The data in this study shows that transcriptomically, phenotypically and functionally, both cutaneous DC populations are professional APCs, as highlighted by high HLA-class I and II expression and the ability to activate T lymphocytes. In addition, these data help to explain the apparently conflicting findings about LC function by different research groups: as can be seen following stimulation with TNF-α, read-outs of inflammatory mediators or ‘activation-status’ are likely to show lower levels in LCs, whereas functional assessments demonstrate enhanced function. The distinctiveness of LC molecular networks indicates that not all tissue DC types are biologically equal, and their biology is adapted to the specific requirements of the local tissue microenvironment (Harman et al., 2013; Hume et al., 2013; Hutter et al., 2012; Lundberg et al., 2013). Direct comparison of the whole transcriptomes of migratory cells and cells isolated rapidly by trypsinisation indicates, that despite phenotypic immunological maturation migratory cells retain the pattern of the gene expression in steady-state, in particular high expression of genes involved in cell metabolism, protein catabolism, and cytoskeleton rearrangement in LCs as compared with DDCs, as well as pronounced difference in expression of genes involved in inflammatory responses between LCs and DDCs. Whilst neither isolation technique perfectly reflects the in vivo situation, and inevitably migration induced specific phenotypic features including increased expression of co-stimulatory molecules, we feel that the replication of the same gene expression profiles in both migratory and trypsinised cells as well as the evident changes on migratory cells induced by TNF-α, justifies the analysis of these data to explore the immunological signalling in cutaneous dendritic cells. However, we acknowledge that differences in the kinetics of molecular signals induced by TNF-α are likely to exist in situ as compared to in vitro. We
would predict that these will be especially important in relation to DC cross-talk with tissue structural cells, e.g. maturation signal provided by E-cadherin:E-cadherin between LC and KC (Mayumi et al., 2013). The dichotomy between molecular networks of human LCs and DDCs, recapitulating differences in their biology, may reflect a different origin of these cell types, as currently suggested by (Chorro and Geissmann, 2010; Hoeffel et al., 2012). Likewise, it is possible that the distinctiveness of LC molecular networks is a direct result of interactions between LCs, structural cells of the epidermis, and the symbiotic microbiota during tissue resident differentiation from ‘LC stem cells’ (Merad et al., 2008; Sere et al., 2012). In this environment, careful regulation to prevent over-activation and harmful inflammatory responses under pro-inflammatory conditions would be critical, particularly because uncontrolled inflammation may lead to disruption of the skin barrier and permit entry of infectious and noxious agents into the body. Indeed, LC can utilise mechanisms preventing the invasion of the microorganisms, limit the presentation of bacterial antigens, and maintain tissue homeostasis inducing regulatory T cells in the steady-state (de Witte et al., 2007; Seneschal et al., 2012b; van der Aar et al., 2013). The increased mitochondrial activation, indicating a higher metabolic rate, as shown here might be an adaptation specific to the epidermal microenvironment which is low in nutrient and oxygen due to the lack of vasculature. Such a potentiated metabolism would also benefit LC in hydrolysis of a variety of macromolecules from pathogenic organisms, including bacterial cell walls and fungi. As modelled here in skin migratory cells, in the inflammatory conditions, e.g. when in situ LCs are exposed to pro-inflammatory cytokines, like TNF-α, the enhanced endocytosis, proteasomal degradation and intracellular transport (clusters 03,12,14), alongside the decreased metabolism of macromolecules (cluster 05), would result in increased antigen presentation and activation of adaptive immune responses.
In conclusion, LCs are APCs with all the appropriate machinery for this purpose, but in contrast to DDCs are highly efficient at presentation and cross-presentation of antigen, and the data in this current study provides evidence that this is mediated by key differences in gene expression which regulates antigen uptake and processing. Furthermore, the relative constancy of the LC molecular network following activation by TNF-α, suggests a more differentiated cell type which may reflect a key evolutionary need for different functional roles related to tissue compartmentalisation. In addition, our findings support the idea that LCs represent an attractive proposition for targeted immunological intervention. Topical or micro-needle vaccine delivery may be expected to preferentially target LCs, thereby promoting a strong CD8+ immune response, as indeed was demonstrated for transcutaneous influence vaccination (Combadiere et al., 2010). The ability of LCs both to prime naive CD8 T cells (Banchereau et al., 2012) and to potently activate memory CD8 T cell responses (Polak et al., 2012; Seneschal et al., 2012b; van der Aar et al., 2013) renders these cells suitable as targets for induction of both systemic and skin homing immune responses.

Materials and Methods

1. Isolation and culture of human skin migratory DCs

Skin specimens and blood samples were acquired from healthy individuals after obtaining informed written consent with approval by the Southampton and South West Hampshire Research Ethics Committee in adherence to Helsinki Guidelines. Primary cutaneous DCs were isolated as described previously (Polak et al., 2012). Migratory epidermal and dermal DCs were purified with magnetic beads according to manufacturer’s protocol (epidermal
cells: CD1a+, dermal cells: CD11c+, Milenyi Biotec, UK). Cells were assayed for yield and cell viability, and unstimulated cells (time 0, 250,000/cell type/donor) were harvested immediately. For analysis of changes in gene expression upon activation, DCs were stimulated with TNF-α (25ng/ml, Miltenyi Biotec, UK) for 2, 8 and 24 hours (250,000 cells/cell type/donor/time point). Harvested cells were cryopreserved at -80°C in RLT buffer (Qiagen, UK) + 1% β-mercaptoethanol. DC pulsing with EBV-derived peptides, EBV-peptide specific T cell expansion and ELISpot assays were performed as described previously (Polak et al., 2012).

2. Genechip microarray data analysis

RNA was isolated using RNeasy mini kits (Qiagen, UK) as per the manufacturer’s protocol. RNA concentration and integrity was determined with an Agilent Bioanalyser. All the samples had a RIN of 7.0 or above and were taken forward for labelling. Gene expression analysis was carried out using the Human Genome U-219 Affymetrix platform by ARK-Genomics Centre, The Roslin Institute, Edinburgh. Expression data were normalised using the Robust Multichip Average (RMA) package within the Affymetrix expression console package and annotated. After an initial QC check, the data was taken forward for analysis. Microarray data GEO accession number: GSE49475.

a. Comparison of skin DC transcriptomes
Unfiltered RMA-normalised microarray data were analysed using multidimensional scaling (MDS). The proximity matrix was created on the basis of Euclidean distance dissimilarities calculation (XLstat, Addinsoft) for 2, 3 and 4 dimensions. In addition, a sample-to-sample Pearson correlation matrix was calculated using BioLayout Express\textsuperscript{3D} and the resultant graph of relationships of \( r > 0.96 \) was visualised. For comparison of migratory with enzymatically digested skin DCs raw data (.cel) files from human skin LCs and DDCs datasets available in GEO database (http://www.ncbi.nlm.nih.gov/geo/, Supplementary Table S3) were used and processed as previously described (Mabbott et al., 2010). Due to the pronounced study-related batch effect only LCs and DDCs from GSE23618 were compared directly with our migratory cell dataset (Hierarchical Clustering Explorer, University of Maryland, USA), while GSE16395 and GSE35340 LCs were assayed for the presence of identified gene signatures using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005).

b. Statistical identification of differentially expressed genes and network analysis

To identify genes regulated by exposure of skin DCs to TNF-\( \alpha \), a cut-off threshold 0.05 of Bayesian Estimation of Temporal Regulation (Aryee et al., 2009) for genes showing \( \geq 1.5 \) fold difference between the maximum gene expression level and time 0 control in \( \log_2(x) \) RMA normalised gene expression levels was applied for both cell types. Using network analysis tool BioLayout Express\textsuperscript{3D}, a transcript-to-transcript correlation matrix was calculated for 2,334 probesets fulfilling the criteria above, where each column of data was derived from a different sample (donor/cell type/condition) and each row of data represents an individual probeset (Freeman et al., 2007). A non-directional network graph of the data was generated.
for a Pearson correlation coefficient of $r \geq 0.85$. In this context, nodes represent individual probesets (genes/transcripts) and the edges between them Pearson correlation coefficients between individual probesets above the threshold value. The network graph was then clustered into groups of genes sharing similar profiles using the MCL algorithm within the BioLayout Express$^\text{3D}$ tool with an MCL inflation value (which controls the granularity of clustering) set to 1.7, as reported previously (Theocharidis et al., 2009).

c. Cluster annotation and analysis of gene expression profiles

Gene set enrichment analysis was performed using the ‘‘functional annotation clustering’’ tool, (similarity threshold 0.5, multiple linkage threshold 0.5, EASE:1.0 and Benjamini correction) from DAVID (Huang da et al., 2009a, b) web-based analysis tool and confirmed by detailed direct analysis using Gene Expression Atlas (http://www.ebi.ac.uk/gxa/). Average gene expression profiles for LC and DDCs (four time points, in triplicate) were compared with two-way repeated ANOVA for each cluster separately (Graph Prism, USA) and p-values assessed with Bonferroni correction.

d. Validation of gene expression differences by qPCR

The expression of chosen genes was validated with qPCR, using the TaqMan gene expression assays for target genes: YWHAZ (HS03044281_g1), CAV1 (Hs00971716_m1), PSMD14 (Hs01113429_m1), CCL18 (Hs00268113_m1) (Applied Biosystems, UK) in cells isolated from three independent skin donors. RNA extraction (RNeasy micro kit, Qiagen, UK) and
reverse transcription (NanoScript™ kit; Primer Design, UK) were carried out accordingly to manufacturers protocol. For details see Supplementary material: Materials and Methods.

**Conflict of interests:** The authors declare no conflict of interests.

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References:


presentation or cross-priming, than do dermal-interstitial or monocyte-derived dendritic cells.


Figure legends.

Figure 1. Skin migratory CD1a+ LCs and CD11c+ DDCs are professional antigen presenting cells.

(a, b) Flow cytometric staining of human skin isolated, bead-purified migratory (a) LC and (b) DDC. Representative example.

(c) Log$_2$(x) expression of DC markers in un-stimulated LCs and DDCs, microarray analysis (n=3 independent skin donors, in duplicate).

(d-f) CFSE dilution assay of EBV-specific HLA matched CD8+ T cell line stimulated by EBV-peptide (d) or with EBV-peptide pulsed LCs (e) or DDCs (f).

(g) IFN-γ ELISpot assay of EBV-specific CD8+ activation by 9 amino acid EBV-peptide pulsed LCs (black bar) or DDCs (grey bar). (n=3).

(h) IFN-γ ELISpot assay of 39 amino acid EBV-specific CD8+ activation by EBV-long peptide pulsed LCs (black bar) or DDCs (grey bar). (n=3, unpaired T test: ns= not significant, ***: p<0.005).

Figure 2. CD1a+ LC and CD11c+ DDCs show distinctively different pattern of gene expression.

Visual representation of whole transcriptome analysis of CD1a+ LCs and CD11c+ DDCs (n=3 independent skin donors, time course (0,2,8,24h) of stimulation with TNF-α).
(a,b) Sample-to-sample clustering. (a) Multidimensional scaling analysis, LC (right quadrants) and DDC samples (left quadrants) as indicated. Kruskal’s stress 0.099 for 2-dimensional scaling. (b) Sample-to-sample clustering of CD1a+ LCs and CD11c+ DDCs expression profiles (BioLayout Express3D, correlation coefficient (r) = 0.96, MCL=2.2). Lines (edges) represent the similarity between samples. Circles (nodes) represent transcriptomes measured at different time points.

(c) Transcript-to-transcript clustering. (BioLayout Express3D, r= 0.85; MCL = 1.7) of 2,334 probesets differentially regulated by TNF-α. Lines (edges) represent the similarity between transcripts, circles (nodes) represent genes.

(d,e,f) Mean (± SEM) expression profiles for clusters 1-14, LC (black bars) and DDC (grey bar).

Figure 3. Genes organising structure of biological membranes are important for LC function.

(a-f) Gene expression of CAV1 (a,b), PMSD14 (c,d) and CCL18 (e,f) in LC (black bars) or DDCs (grey bars) assessed by microarrays (a,c,e; RMA normalised) and qPCR (b,d,f; expression normalised to house-keeping gene YWHAZ (2^−dCT) at various time points following stimulation with TNF-α.

(g) IFN-γ ELISpot assay of cross-presentation to EBV-specific CD8+ by LC treated with or without caveolin-1 inhibitor (filipin III). n=3 independent skin donors * p<0.05.
Figure 1. Skin migratory CD1a+ LCs and CD11c+ DDCs are professional antigen presenting cells.
Figure 2. CD1a+ LC and CD11c+ DDCs show distinctively different pattern of gene expression.
Figure 3. Genes organising structure of biological membranes are important for LC function.