Distinct Molecular Signature of Human Skin Langerhans Cells Denotes Critical Differences in Cutaneous Dendritic Cell Immune Regulation

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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 (DC)" 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 tumor necrosis factor-α (TNF-α) confirmed the unique molecular signature of LCs. Although LCs conformed to the phenotype of professional APC, inflammatory signaling activated primarily genes associated with cellular metabolism and mitochondrial activation (e.g., *CYB561* and *MRPS35*), cell membrane re-organization, and antigen acquisition and degradation (*CAV1* and *PSMD14*; $P<0.05$–$P<0.0001$). Conversely, TNF-α induced classical activation in DDCs with early downregulation of surface receptors (mannose receptor-1 (*MRC1*) and C-type lectins), and subsequent upregulation of cytokines, chemokines (*IL1a*, *IL1b*, and *CCL18*), and matrix metalloproteinases (*MMP1*, *MMP3*, and *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.

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INTRODUCTION

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 (Sagebiel, 1972; Kubo *et al.*, 2009) and facilitate antigen uptake with surface receptors including C-type lectin, Langerin (Valladeau *et al.*, 2000) and CD205 (Santegoets *et al.*, 2008; Flacher *et al.*, 2010). As a result of antigen uptake, LCs can stimulate efficient primary and secondary immune responses to viral antigens, including influenza (Klechevsky *et al.*, 2008, 2010), 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 human immunodeficiency virus by LCs results in efficient degradation of viral particles, and thus induces protection against human immunodeficiency virus infection (de Witte *et al.*, 2007). LC maturation is likely to be critical for efficient induction of T-cell responses (Grabbe *et al.*, 1992; Banchereau *et al.*, 2003; van der Vlist *et al.*, 2011; Polak *et al.*, 2012). The maturation signals come from LC cross-talk with surrounding tissue, e.g., tissue-derived pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α; Ratzinger *et al.*, 2004; Berthier-Vergnes *et al.*, 2005; Polak *et al.*, 2012), thymic stromal lymphopoietin (Ebner *et al.*, 2007; Nakajima *et al.*, 2012), and tumor growth factor-β (Geissmann *et al.*, 1999; Bauer *et al.*, 2012), danger-associated molecular patterns

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Abbreviations: APC, antigen-presenting cell; CCL, chemokine C-C motif ligand; DDC, dermal dendritic cell; EBV, Epstein–Barr virus; LC, Langerhans cell; MMP, matrix metalloproteinase; RMA, robust multichip average; TNF-α, tumor necrosis factor-α

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(Kool *et al.*, 2011) and recognition of pathogen-derived signals, including pathogen-associated molecular patterns (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 (Zhao *et al.*, 2003; Ritter *et al.*, 2004; Bennett *et al.*, 2005; Kaplan *et al.*, 2005; Noordegraaf *et al.*, 2010; van der Aar *et al.*, 2013). Recently, along with others, we have demonstrated that the direct interactions within the immunological synapse are critically important for human LCs' capacity to stimulate CD8 T lymphocytes (van der Aar *et al.*, 2011; Banchereau *et al.*, 2012; Polak *et al.*, 2012) 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 (Zaba *et al.*, 2007; Teunissen *et al.*, 2012), immediately after 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 show distinctively different transcriptional profiles of these two skin-derived antigen-presenting cells (APCs), and denote the key role of protein metabolism and antigen processing in LC biology.

RESULTS

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 and 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*, and *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*, and *CD86*; Supplementary Figure S1a–c online). Using a well-defined

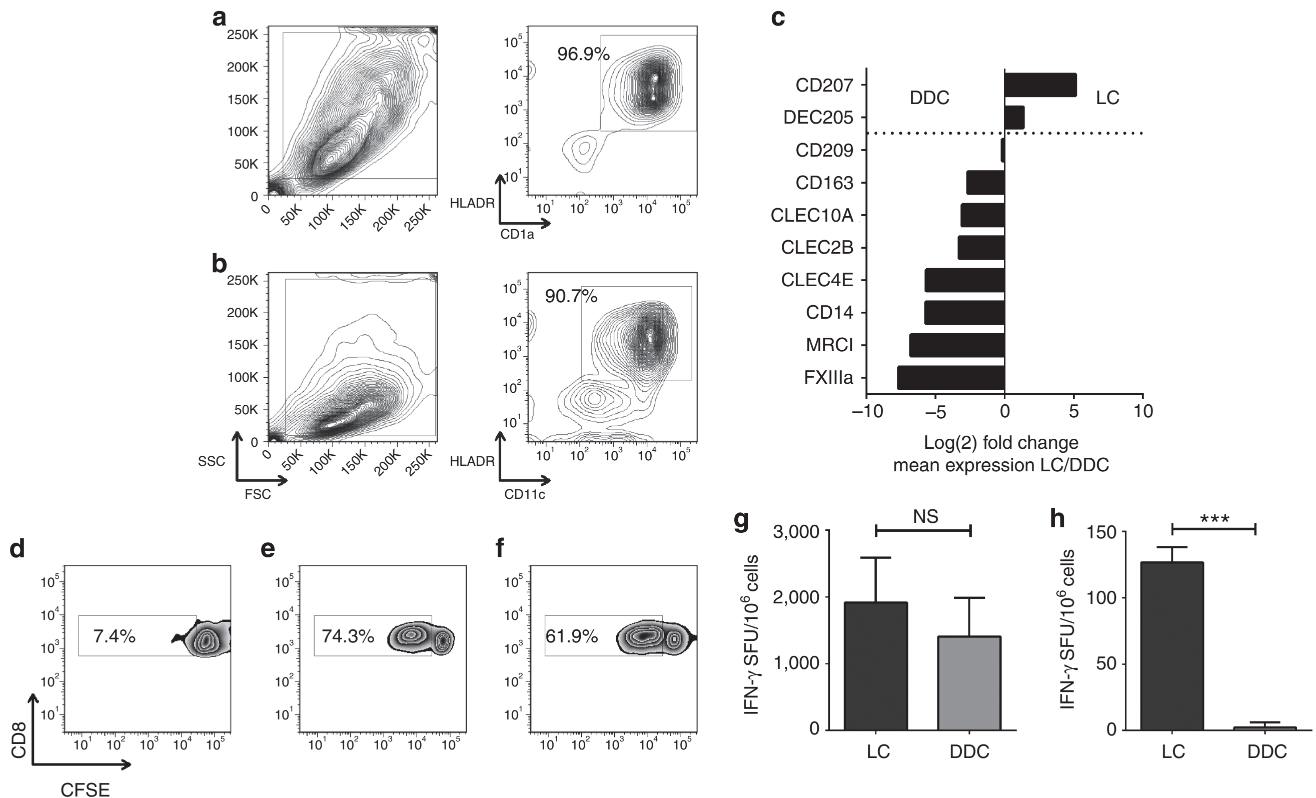

Figure 1. Skin migratory CD1a+ Langerhans cells (LCs) and CD11c+ dermal dendritic cells (DDCs) are professional antigen-presenting cells. (**a**, **b**) Flow cytometric staining of human skin isolated, bead-purified migratory (**a**) LC and (**b**) DDC. Representative example. (**c**) $Log_2(x)$ expression of DC markers in unstimulated LCs and DDCs, microarray analysis ($n=3$ independent skin donors, in duplicate). (**d–f**) CFSE dilution assay of Epstein–Barr virus (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; ***$P<0.005$). CFSE, carboxyfluorescein succinimidyl ester; FSC, forward scatter; NS, not significant; SFU, spot forming units; SSC, side scatter.

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 1g). 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).

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 analyzed the transcriptome of CD1a+ LCs and CD11c+ DDCs over a 24-hour time course of stimulation with an epidermal pro-inflammatory cytokine, TNF-α. Multidimentional scaling analysis (Figure 2a) and sample-to-sample clustering (BioLayout *Express*^{3D}, Figure 2b) of the transcriptomes of CD1a+ LCs and CD11c+DDCs indicate that the gene expression profile of LCs are quite distinct from DDC. In addition, 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 online).

Characterization 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 $\log_2(x)$ robust multichip average (RMA)-normalized expression level between the cell types; Supplementary Table S1 online). 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 online). Both LC and DDC were sensitive to TNF-α signaling. However, although 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

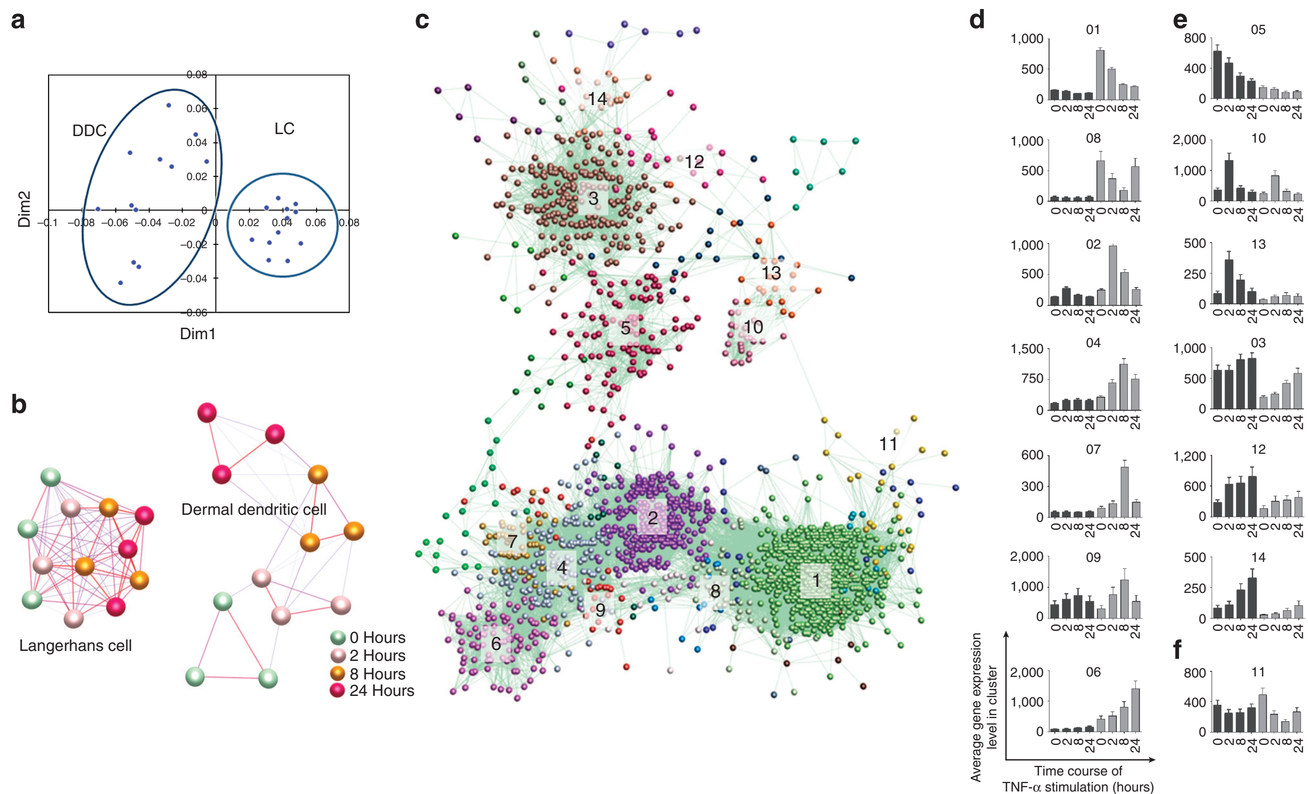

Figure 2. CD1a+ Langerhans cell (LC) and CD11c+ dermal dendritic cells (DDCs) show distinctively different pattern of gene expression. Visual representation of whole transcriptome analysis of CD1a+ LCs and CD11c+ DDCs ($n=3$ independent skin donors, time course (0, 2, 8, and 24 hours) of stimulation with tumor necrosis factor-α (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 two-dimensional scaling. (**b**) Sample-to-sample clustering of CD1a+ LCs and CD11c+ DDCs expression profiles (BioLayout *Express*^{3D}, correlation coefficient (r)=0.96, Markov clustering algorithm (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 *Express*^{3D}, $r=0.85$; MCL=1.7) of 2,334 probesets differentially regulated by TNF-α. Lines (edges) represent the similarity between transcripts; circles (nodes) represent genes. (**d**–**f**) Mean (±SEM) expression profiles for clusters 1–14, LC (black bars) and DDC (grey bar).

typically associated with immune responses, involved in cytokine–cytokine receptor interactions, chemokine signaling pathways, Toll-like receptor signaling pathway, and Fc-γ receptor-mediated phagocytosis (Supplementary Table S2 online). 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 signaling (*ABCA1*, *GEM*, *IRAK3*, *KL*, *NDRG1*, and *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 overexpressed in migratory LC were involved in cytoskeleton reorganization and membrane re-modeling (*ACTB*, *CNN*, *DSP*, *ANK3*, *PFN1*, *SYNPO*, and *PLEK2*), endocytosis and intracellular transport (*AP1B1*, *AP2S1*, *SH3KBP1*, *SNX4*, and *SNX7*), proteolysis (*FBXO2*, *PSMC3*, *UCHL3*, *USP46*, and *TRIM32*), and mitochondrial activity (*ACOT1*, *ACOT7*, *ACOX3*, *CYB561*, *NDUFB7*, and *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 online).

Migratory skin DCs and trypsinized 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 trypsinization protocol (Santegoets *et al.*, 2008; Allen *et al.*, 2010; Hutter *et al.*, 2012; Harman *et al.*, 2013; Supplementary Table S3 online). Comparison of the genes differentially expressed in either LCs or DDCs revealed the same pattern of expression in both the migratory and trypsinized models (Supplementary Figure 2 online). Furthermore, gene set enrichment analysis (Subramanian *et al.*, 2005) of T0 LCs ($n=18$ data sets), confirmed that migratory LC genes of interest were also significantly enriched in trypsinized LCs, enrichment score = 0.55, $P<0.02$ and enrichment score = 0.42, $P<0.0019$, enrichment score = 0.52, $P<0.01$ (Supplementary Table S3 1, 2, and 3 online) respectively, as compared with DDC. Similarly, gene expression in migratory DDC populations were replicated in trypsinized DDCs, (enrichment score = 0.78, $P<0.05$) in comparison with trypsinized LCs. As seen in migratory LCs, DAVID functional gene classification and functional annotation of genes of increased expression in trypsinized LCs were involved in cell-to-cell adhesion, mitochondrial function, and metabolism, and trypsinized DDCs reflected migratory DDCs showing overexpressed genes involved in receptor-mediated pathogen uptake, cytokine signaling, and immune responses (Supplementary Figure S2 online; Supplementary Table S4 online). However, some differences between migratory and trypsinized cells were evident. As predicted, in comparison with trypsinized cells, migratory LCs showed reduced (but not absent) expression of cell adhesion molecules, increased proteasome activity, and upregulation 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 trypsinized DDCs showed very closed resemblance. Upon migration, they further upregulated and extended their scavenging and pro-inflammatory characteristics.

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 Express^{3D} (Pearson coefficient cutoff $r=0.85$, Markov clustering algorithm 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-normalized gene expression levels in comparison with unstimulated cells (Bayesian estimation of temporal regulation cutoff threshold $P=0.05$, Aryee *et al.*, 2009). The resultant gene transcription network diagram was highly organized 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 and d; Supplementary Figure S2 online, Supplementary Table S5 online). The dynamics of gene expression in these clusters recapitulate the typical myeloid DC activation pattern (Banchereau *et al.*, 2003). Upon activation, DDCs downregulated surface cell receptors, associated with antigen capture, and reduced phagocytosis (clusters 01 and 08, including *CD163*, C-type lectin receptors *CLEC1A*, *CLEC4G*, *CLEC5A*, *CLEC7A*, *CXCL2*, and *CXCR7* and FC-γ receptors *FCGR2A*, *FCGR2B*, and *FCGR3A*), whereas expression of genes involved in transcription, intracellular signaling, and cytokine synthesis peaked at 2 hours (cluster 02; e.g., *CREM*, *RPL28*, *TNF*, *IL7*, *IL1RN*, *CXCR4*, *CCL3*, *CCL4*, *DYRK-3*, *RIPK1*, *MAPK2K3*, *ZNF36*,

and *ZNF295*) followed by an upregulation of genes involved in synthesis and secretion of a wide range of cytokines and chemokines, matrix metalloproteinases (MMPs, known to be involved in rearrangement of extracellular matrix), and regulators of cell migration between 8 and 24 hours (clusters 04, 06, 07, and 09; e.g., *IL1A*, *IL23A*, *MMP1*, *MMP7*, *MMP9*, *MMP14*, *CCL1*, *CCL2*, *CCL17*, *CCL18*, *CCL24*, and *CXCL6*, Figure 2c and d; Supplementary Figure S3 online; Supplementary Table S5 online).

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 and 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 downregulated the catabolism of carbohydrates and fatty acids (cluster 05), and increased expression of proteins involved in endocytosis, intracellular transport, and signaling, protein degradation, including genes coding for proteasome assembly units and protein degradation enzymes (clusters 03, 12, and 14). TNF-α signaling also altered LC mitochondrial function, inducing high expression levels of mitochondrial ribosomal protein expression (for individual gene profiles, see Supplementary Figure S4 online). 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 upregulated in LCs following TNF-α, including *IL15* and *CCL22* (cluster 03; Figure 3a and c; Supplementary Figure S4 online; Supplementary Table S5 online). However, among the genes upregulated in LCs during stimulation with TNF-α, and positioned separately from the core myeloid DC clusters (clusters 03, 12, and 14, Figure 2c and e), we identified several genes primarily associated with cytoskeleton organization, but which have also been reported in immune responses or immune signaling, including *SNX 11* (sorting nexin 11; cluster 12) and *SYNPO* (synaptopodin; cluster 14). Cluster 14 genes, such as *CAV1*, link processes of antigen acquisition and regulation of immune responses, including genes involved in endocytosis, cytoskeleton reorganization, and regulation of signal transduction (*PTPRK*).

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

Caveolin-1 dependency of LC cross-presenting function

LC-associated molecular networks clearly indicated a relationship between TNF-α signaling and induction of genes grouped in clusters 03, 12, and 14. To confirm their dependence on TNF-α, in contrast to immune-related gene expression, we validated the TNF-α induced upregulation of *CAV1* and *PMSD14* in LCs versus DDCs by quantitative PCR (Figure 3a–d) in cells isolated from three independent skin donors. In contrast, *CCL18* was uniquely upregulated in DDC (Figure 3e and f). The gene expression pattern assessed by quantitative PCR validated the microarray data (Figure 3b, d, and f). Although proteasome function is indisputably associated with antigen processing and presentation, we were interested to test whether the increased expression of *CAV1* in LC might contribute to their superior cross-presenting facility. Caveolin-1 function is specifically inhibited by filipin III (Yan *et al.*, 2004; Sato *et al.*, 2012). 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 ($0.1–1\,\mu g\,ml^{-1}$; 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 recognized 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 (Zhao *et al.*, 2003; Ritter *et al.*, 2004; Bennett *et al.*, 2005; Kaplan *et al.*, 2005; Noordegraaf *et al.*, 2010). Previous work to characterize 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 (Klechevsky *et al.*, 2008; Flacher *et al.*, 2010; van der Aar *et al.*, 2011; Banchereau *et al.*, 2012; Polak *et al.*, 2012), and others the converse (de Witte *et al.*, 2007; Santegoets *et al.*, 2008; Lundberg *et al.*, 2013; van der Aar *et al.*, 2013). Along with others, we have previously demonstrated that LCs are superior activators of CD8 T cells, due to differential signaling via CD70 and their exceptional efficiency in cross-presenting protein antigens to CD8 T cells (van der Aar *et al.*, 2011; Polak *et al.*, 2012;) 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 (Ratzinger *et al.*, 2004; Munz *et al.*, 2005; Banchereau *et al.*, 2012; Polak *et al.*, 2012). In this study, we aimed to undertake a comprehensive analysis, combining transcriptomic assessment with functional readout to characterize primary cutaneous DC function.

The data in this study show 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

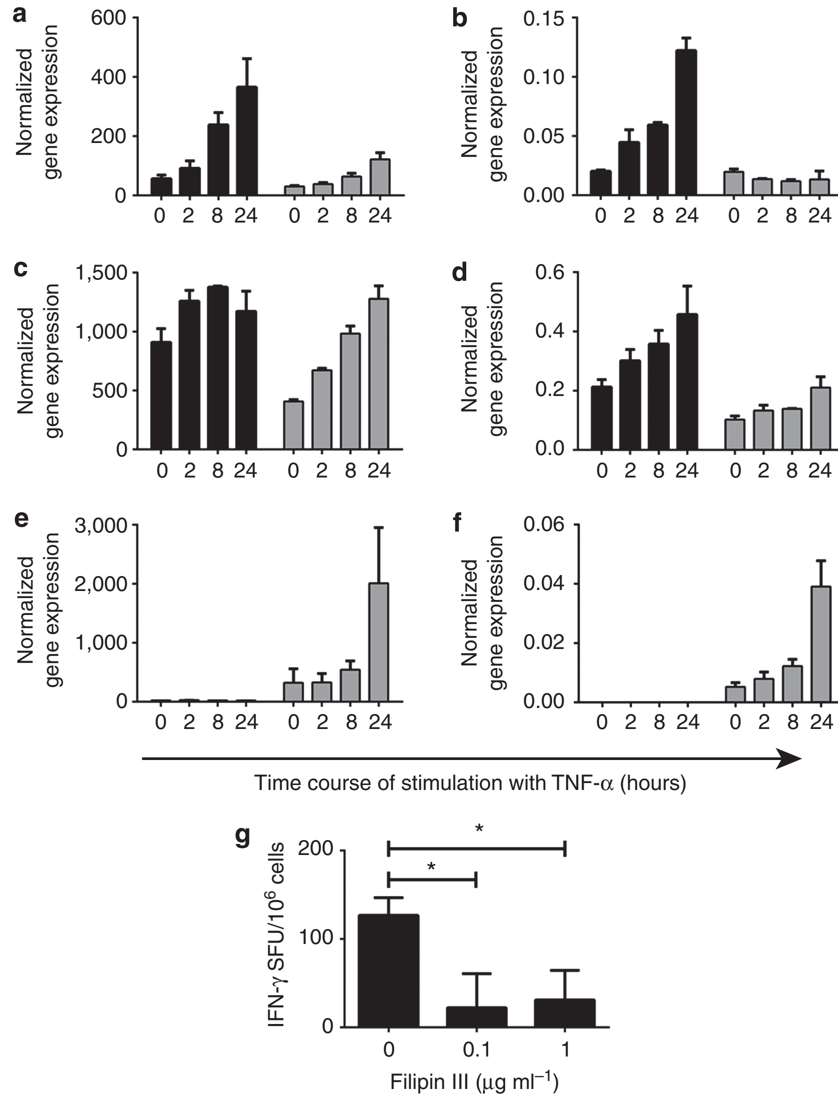

Figure 3. Genes organizing structure of biological membranes are important for Langerhans cell (LC) function. (**a**–**f**) Gene expression of *CAV1* (**a**, **b**), *PMSD14* (**c**, **d**), and *CCL18* (**e**, **f**) in LC (black bars) or dermal dendritic cells (DDCs; grey bars) assessed by microarrays (**a**, **c**, **e**; robust multichip average (RMA) normalized) and quantitative PCR (qPCR; **b**, **d**, **f**; expression normalized to house-keeping gene *YWHAZ* (2^{-dCT}) at various time points following stimulation with tumor necrosis factor-α (TNF-α)). (**g**) IFN-γ ELISpot assay of cross-presentation to Epstein–Barr virus (EBV)–specific CD8+ by LC treated with or without caveolin-1 inhibitor (filipin III). $n=3$ independent skin donors. *$P<0.05$. SFU, spot forming units.

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 (Hutter *et al.*, 2012; Harman *et al.*, 2013; Hume *et al.*, 2013; Lundberg *et al.*, 2013). Direct comparison of the whole transcriptomes of migratory cells and cells isolated rapidly by trypsinization 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. Although 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 trypsinized cells as well as the evident changes on migratory cells induced by TNF-α, justifies the analysis of these data to explore the immunological signaling in cutaneous DCs. However, we acknowledge that differences in the kinetics of molecular signals induced by TNF-α are likely to exist *in situ* as compared with *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 keratinocytes (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). Similarly, 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 overactivation 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 utilize mechanisms preventing the invasion of the micro-organisms, 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 because of 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 modeled 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, and 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 study provide 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 that may reflect a key evolutionary need for different functional roles related to tissue compartmentalization. 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

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 the manufacturer's protocol (epidermal cells: CD1a+, dermal cells: CD11c+, Miltenyi Biotec, Bisley, 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 (25 ng ml^{-1}, Miltenyi Biotec) 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, Manchester, 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).

Genechip microarray data analysis

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

Comparison of skin DC transcriptomes. Unfiltered RMA-normalized microarray data were analyzed using multidimensional scaling. The proximity matrix was created on the basis of Euclidean distance dissimilarities calculation (XLstat, Addinsoft, New York, NY) for 2, 3, and 4 dimensions. In addition, a sample-to-sample Pearson correlation matrix was calculated using BioLayout *Express*^{3D} and the resultant graph of relationships of $r > 0.96$ was visualized. For comparison of migratory with enzymatically digested skin, DCs raw data (.cel) files from human skin LCs and DDCs data sets available in GEO database (http://www.ncbi.nlm.nih.gov/geo/; Supplementary Table S3 online) were used and processed as previously described (Mabbott *et al.*, 2010). Owing to the pronounced study-related batch effect only LCs and DDCs from GSE23618 were compared directly with our migratory cell data set (Hierarchical Clustering Explorer, University of Maryland), whereas GSE16395 and GSE35340 LCs were assayed for the presence of identified gene signatures using gene set enrichment analysis (Subramanian *et al.*, 2005).

Statistical identification of differentially expressed genes and network analysis. To identify genes regulated by exposure of skin DCs to TNF-α, a cutoff threshold 0.05 of Bayesian estimation of temporal regulation (Aryee *et al.*, 2009) for genes showing ⩾1.5-fold difference between the maximum gene expression level and time 0 control in $\log_2(x)$ RMA-normalized gene expression levels was applied for both cell types. Using network analysis tool BioLayout *Express*^{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 $r \geqslant 0.85$. In this

context, nodes represent individual probesets (genes per 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 Markov clustering algorithm within the BioLayout *Express*^{3D} tool with an Markov clustering algorithm inflation value (which controls the granularity of clustering) set to 1.7, as reported previously (Theocharidis *et al.*, 2009).

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-analysis of variance for each cluster separately (GraphPad Prism, La Jolla, CA) and *P*-values assessed with Bonferroni correction.

Validation of gene expression differences by quantitative PCR. The expression of chosen genes was validated with quantitative PCR, using the TaqMan gene expression assays for target genes: YWHAZ (HS03044281_g1), CAV1 (Hs00971716_m1), PSMD14 (Hs01113429_m1), CCL18 (Hs00268113_m1) (Applied Biosystems, Life Technologies, Paisley, UK) in cells isolated from three independent skin donors. RNA extraction (RNeasy micro kit, Qiagen) and reverse transcription (NanoScript kit; Primer Design, Southampton, UK) were carried out accordingly to the manufacturer's protocol. For details, see Supplementary Materials and Methods online.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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