Convergent evolution of monocyte differentiation in adult skin instructs Langerhans cell identity

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Summary.

Langerhans cells (LCs) are distinct among phagocytes; functioning both as embryo-derived, tissue-resident macrophages in skin innervation and repair, and as migrating professional antigen-presenting cells, a capability classically assigned to dendritic cells (DCs). Here, we demonstrate that both intrinsic and extrinsic factors imprint this dual identity. Using ablation of embryo-derived LCs in murine adult skin and tracking differentiation of incoming monocyte-derived replacements, we found intrinsic intra-epidermal heterogeneity. We observed that ontogenically distinct monocytes give rise to LCs. Within the epidermis, Jagged-dependent activation of Notch signaling, likely within the hair follicle niche, provided an initial site of LC commitment prior to metabolic adaptation and survival of monocyte-derived LCs. In human skin, embryo-derived LCs in newborns retained transcriptional evidence of their macrophage origin, but this was superseded by DC-like immune modules after post-natal expansion. Thus, adaptation to adult skin niches replicates conditioning of LC at birth, permitting repair of the embryo-derived LC network.

Introduction

Langerhans cells (LCs) are a specialized and highly conserved population of mononuclear phagocytes that reside in the outer epidermis of the skin. Initially defined as prototypic dendritic cells (DCs) due to their potential to migrate to draining lymph nodes (LNs) and initiate T cell immunity (1), subsequent fate mapping studies supported a common origin with tissue macrophages in other organs (2). As such, LCs are the only resident macrophage population that acquires the DC-like ability to migrate out of the tissue (3). Unlike DCs, however, LCs depend on colony stimulating factor 1 receptor (CSF1R) signaling for survival (4), and perform more macrophage-like functions via interaction with peripheral nerves (5) and promotion of angiogenesis during wound healing (6). But the signals that control this functional dichotomy within the spatial context of intact skin remain poorly defined.

Tissue-resident macrophage (TRM) identity is imprinted on fetal and adult monocyte precursors by the local anatomical niche wherein instructive signals permit convergent differentiation and survival of resident cells irrespective of ontogeny (7, 8). Environmental signals determine TRM identity via epigenetic regulation of specific transcription factor networks (9, 10), controlled by the transcription factor Zeb2 (11, 12). These niches have been carefully delineated in the lungs and liver where interaction with local epithelia supports differentiation of TRM populations (13, 14). Implicit in these models is the concept of a single niche that provides that provides a physical scaffold, trophic factors to support maintenance of the network, and the signals to imprint a TRM identity specific to that site (15). However, we questioned whether this model would also apply to LCs in the skin wherein monocytes exiting the blood must traverse the dermis and cross a basement membrane to (re)populate the LC network.

Murine CX3CR1^{+} fetal macrophage precursors enter the developing skin and differentiate into LC-like cells (2, 16), but don't mature into bona fide embryo-derived (e)LCs until after birth (17, 18). By contrast, human eLCs differentiate within the epidermis before birth and histologically resemble adult cells by an estimated gestational age of 18 weeks (19, 20). Entry of LC precursors into the BMP7- and TGFβ-rich environment of the epidermis results in activation of a Runx3- and Id2-dependent program of differentiation (2, 3, 21, 22), defined by expression of the c-type lectin Langerin (CD207) and high expression of cell adhesion molecules including EpCAM and E-cadherin.

Once resident, eLCs depend on IL34 for survival (23, 24) and the network is maintained throughout life via local proliferation of mature LCs (25–27), independent of the adult circulation. In both mice and humans, eLCs are sparse at birth but undergo a proliferative burst within the first week of exposure to the external environment in mice (18), and within 2 years of age in humans (19), but we do not know if or how this post-natal transition may shape eLC identity and function.

Pathological destruction of the eLC network during graft-versus-host disease (GVHD) results in replacement of eLCs with donor bone marrow (BM)-derived LCs (28, 29). We and others have shown that acute inflammation and destruction of eLCs in murine models of GVHD or UV irradiation triggers influx of monocytes to the epidermis (4, 30–32). By tracking monocyte differentiation, we demonstrated that epidermal monocytes undergo differentiation to $EpCAM^{+}CD207^{neg}$ precursors, which become long-lived monocyte-derived (m)LCs that are transcriptionally similar to the cells they replace, are radio-resistant, and acquire DC-like functions of migration to LNs and priming of T cells (32). These data suggested that migration into the epidermal environment was sufficient to instruct differentiation of short-lived monocytes into long-lived LCs. However, lineage tracing studies have revealed heterogeneity within classical $Ly6C^{+}$ monocytes such that both granulocyte-macrophage progenitors (GMPs) and monocyte-dendritic cell progenitors (MDPs) can give rise to classical monocyte populations (33, 34) which can be distinguished by expression of the surface markers CD177 and CD319 respectively, and which differentially seed TRM populations across the body (35). These data suggest that intrinsic factors determined by monocyte ontogeny may also shape tissue macrophage differentiation.

Here, we sought to determine how intrinsic and extrinsic factors combine to direct monocyte differentiation in the epidermis and whether the local skin environment plays an instructive or permissive role in this process. Utilizing our model of LC replacement, we have determined the process by which distinct cellular niches in the skin epidermis permit differentiation and survival of long-lived resident LCs. We demonstrate that a combination of BM monocyte ontogeny and environmental signals provided by the adult hair follicle niche instruct programs of LC development towards DC-like cells that replicate post-natal LC maturation in human skin. Together, these data reveal mechanisms of convergent adaptation to the epidermal niche that imprints the distinct LC identity in the skin.

Results.

Single cell transcriptomics reveals monocyte-derived cell heterogeneity in the inflamed epidermis.

To determine the molecular pathways that resulted in successful tissue residency and differentiation of mLCs in adult skin, we exploited our murine model of minor h-antigen mismatched hematopoietic stem cell transplantation (HSCT), in which allogeneic T cells destroy resident eLCs (26, 31). In this model, mLCs subsequently replace the eLC network (32). We carried out single-cell RNA-sequencing (scRNA-seq) on sorted donor $CD11b^{+}MHCII^{+}$ cells isolated from the epidermis 3 weeks post-BMT with male antigen-specific Matahari (Mh) T cells (post-BMT+T cells) (36) (Figures 1A, S1A). Analysis at this time point allowed us to map the spectrum of $CD11b^{hi}$ monocytes, $CD11b^{int}EpCAM^{+}CD207^{neg}$ LC precursors and $CD11b^{int}EpCAM^{+}CD207^{+}$ LCs we have previously defined in the epidermis (32). Dimensionality reduction and clustering of the cells demonstrated unexpected heterogeneity within donor BM-derived cells, including several transcriptionally diverse clusters of cells that surrounded a central collection of still distinct but more convergent clusters (Figure 1B). We used a parametric bootstrapping method sc-SHC (single-cell significance of hierarchical clustering) to demonstrate that the data, particularly the central clusters, were not overfit and thus likely to be biologically relevant (Figure S2A) (37).

Identification of the differentially expressed genes that defined the clusters (Figures 1C, S1B) revealed 3 populations of mLCs: resident (res) mLCs (*Cd207*, *Epcam*, *Mfge8*); cycling mLCs (*Top2a*, *Mki67*; which also retained weakened expression of the res mLC signature); and cells that appeared to be poised for migration out of the epidermis, which we have termed migrating (mig) mLCs (*Cd83*, *Nr4a3*, *Ccr7*) in order to reflect a similar term used for these cells in human LC datasets (38–40). Consistent with these human LC data, mig mLCs downregulated genes associated with LC identity (*Cd207*, *Epcam*) and instead expressed a generic monocyte-derived DC signature (Figures 1C, S1B) that showed the highest enrichment score for human migrating LCs across all clusters (Figure 1D) (38). These data suggest that some mLCs are constitutively primed for migration, as observed in human steady state eLCs (39). We also identified a classical monocyte cluster (Mono) which expressed *Plac8*, *Lyz2*, *Ly6c2*, *Tmbs10*, *Chil3*; Figure S1B), likely to have recently arrived in the

epidermis. Interestingly, these cells resembled monocytes recruited to skin wound sites, early in the healing process (41). Two additional small populations of cells shared monocyte/neutrophil activation markers and signs of recent oxidative stress (S100a$^+$ mono and Hmox1$^+$ mono) (Figure 1C). The central overlapping clusters expressed genes associated with monocyte-derived macrophages in other tissues: the central cluster that shared most similarity to classical monocytes were defined as interferon-stimulated gene monocytes (ISG mono; *Isg15*, *Ifit2*, *Ifit3*) as these genes were also evident in the classical monocyte cluster; conversely, we observed a cluster of *Mrc1* (*Cd206*)- and *Arg1*-expressing monocyte derived macrophage-like cells (Mrc1$^+$ mac) that resemble those found in the dermis (Figures 1C, S1B) (42). These cells appeared to have differentiated along a default macrophage pathway to express canonical tissue macrophage genes and shared some similarity to the converting macrophages recently identified in the pleural cavity after nematode infection (43). Intriguingly, we also detected a separate cluster of cells that were identified by their upregulation of *Ccl17*, *Mgl2*, *Dcstamp*, and *Itgax*, genes linked to monocyte-derived DCs. (Figures 1C, S1B). Given the lack of clarity into the identity of these cells, we labelled them “converting monocyte-derived cells” (MC), in reference to a similar transitional population of cells recently described (43). Flow cytometry data validated the heterogeneous cell fates, showing loss of monocytes over time with expansion of mLCs and CD206$^+$ macrophages (Figures 1E, F). Analysis of eLCs co-isolated from the GVHD skin at the 3 weeks revealed 3 clusters of cells that resembled those identified in human skin (39): clusters 1 and 2 were defined as resident eLCs while the third cluster mirrored *Ccl22*$^+$*Nr4a3*$^+$ migrating mLCs (Figure S2B).

To better understand the path by which monocytes became mLCs, we inferred the trajectory of monocyte development towards finite cell states using Slingshot and RNA velocity (Figures 1G and H) (44, 45). These analyses revealed S100a$^+$ monocytes as one of the endpoint cell states, illustrated by expression of *Clec4d* (Figure 1G). This Slingshot-derived pathway passes mainly via Mrc1$^+$ macrophages, while RNA velocity also suggested that some of this may be due to direct differentiation of incoming monocytes via the *Hmox1*$^+$ monocyte cluster (Figure 1H). A separate pathway towards migratory mLC was inferred by steadily increasing expression of *Ccl22* (Figure 1G); this distinct route is likely driven by the down regulation of *Cd207*, which is not present in migratory mLC (40). By comparison, upregulation of *Epcam* defined monocytes destined to become

resident mLCs (Figure 1G). While differentiation towards resident mLC populations was clearly defined as an endpoint (Figure 1H), the direction of differentiation predicted by RNA velocity displayed some uncertainty within Mrc1^{+} macs and ISG monocytes that was resolved once cells enter the MC cluster, congruent with expression of *Epcam* (Figure 1G). This apparent lack of commitment was illustrated by the short latent time and relative increase in unspliced versus spliced transcripts within ISG monos and Mrc1^{+} macs compared to committed mLC populations (Figure S2C). Focusing on differentiation towards resident mLCs, progenitor marker gene analysis demonstrated loss of monocyte-specific genes (*Ly6c2*, *Plac8*) and acquisition of LC-defining genes (*Cd207*, *Epcam*, *Mfge8*), those associated with cell adhesion (*Cldn1*) and production of non-inflammatory lipid mediators (*Ptgs1*, *Ltc4s*, *Lpar3*, *Hpgds*) (Figure S2D).

Thus, specification of a mLC fate in the adult epidermis occurs *in situ* in the skin, but it is likely that not all monocytes receive these signals, and some undergo a default macrophage differentiation. Rather than discrete points of cell fate decisions, our combined analyses identify a continuum of gene expression across the central clusters which converge to program mLC development in some cells.

Monocyte ontogeny determines mLC repopulation.

Heterogeneity in epidermal monocyte fate may be explained by either intrinsic bias within incoming Ly6C^{hi} monocytes and/or extrinsic programming within a specified tissue niche. To test the first possibility, we investigated the epidermal monocyte population in more detail. Reclustering of the classical monocyte cluster revealed three clusters of cells that were also evident within the parental CD11b^{+}MHCII^{+} cell dataset (Figure 2A, B). Enrichment of gene signatures for GMP-derived monocytes (GMP-Mos) and MDP-derived monocytes (MDP-Mos) (35) and expression of the defining genes *Cd177* and *Slamf7* suggested that cluster 3 represented GMP-Mos, whereas cluster 2 were MDP-Mos (Figure 2C, D). This identification was supported by direct comparison between clusters 2 and 3; cluster 2 cells expressed higher levels of *Sell* (encoding CD62L) and classical monocyte genes (*Plac8*, *Ly6a2*), whereas cluster 3 expressed *C1q* genes and *H2-Aa*, associated with MDP-Mos (Figure 2E). To better understand the macrophage/DC potential within monocyte subsets, we compared expression of a defined panel of genes associated with each cell type: cluster 3 (GMP-

Mos) appeared more macrophage-like with higher expression of the glutathione reductase (*Gsr*) and *Cx3cr1*; cluster 2 was distinguished by increased, but differential, expression of *Id2*, *Mgl2*, and *Batf3*, suggesting a closer relationship with DC-like cells (Figures 2F, S3A). Notably, *Mgl2* and *Ccl17*, markers of MDP-Mo progeny in the lung (35), were expressed within MDP-Mo clusters (Figures 2F, S3A) and also within the MC cluster from our scRNA-seq dataset (Figure S1B). Cluster 1 was the dominant population that appeared to bridge clusters 2 and 3, contained the bulk of differentiated cells when considered in the context of the complete dataset (Figure 2B), and had downregulated genes expressed by MDP-Mo/cluster 2 while acquiring expression of GMP-Mo-associated genes such as *Sell* (Figure 2F), but notably maintained high expression of the LC-defining transcription factor Id2. Therefore, it was possible that these cells represented a mix of monocytes differentiating from clusters 2 and 3.

To test whether different monocytic precursors were intrinsically biased towards becoming mLC, we first sorted GMP, MDP or total Ly6C^{high} monocytes from murine BM (gating strategy in Figure S3B) and cultured these cells with GM-CSF, TGFβ and IL34 to promote generation of CD24^{+}EpCAM^{+} mLC-like cells (21, 32, 46). MDPs, but not GMPs, generated mLCs *in vitro* (Figures S3C, D). Notably, however total Ly6C^{high} monocytes were consistently superior at generating CD24^{+}EpCAM^{+} mLC-like cells (Figures S3C, D). Therefore, we exploited recently-described Ms4a3$^{Cre/+}$:R26$^{LSL\text{-}TdTomato}$:Cx3cr1$^{GFP/+}$ lineage reporter mice to track the fate of GMP-derived monocytes *in vivo* (35). These mice permitted tracing of GMP progeny via *Ms4a3*-dependent tdTomato (tdTom) expression (47), with GFP labeling of BM and blood monocytes, but not LCs (48). BM cells from these mice demonstrated the expected expression pattern of fluorescent proteins (Figure 2G). Injection of BM from female Ms4a3$^{Cre/+}$:R26$^{LSL\text{-}TdTomato}$:Cx3cr1$^{GFP/+}$ mice into male C57BL/6 recipients resulted in the clear expansion of both tdTom^{+}GFP^{+} and tdTom^{neg}GFP^{+} CD11b^{high} donor cells in the epidermis 3 weeks post-transplant (Figures 2H, I), supporting our scRNA-seq data showing the recruitment of both GMP-derived (tdTom^{+}) and MDP-derived (tdTom^{neg}) monocytes. Some of these cells had already begun to downregulate expression of Cx3cr1/GFP, consistent with the loss of *Cx3Cr1* in our cluster 1 cells (Figure 2F), before becoming CX3C1^{neg} LCs (Figure 2H). However, the contribution of GMP-Mos was more substantial than predicted, representing 85.5%±1.9 (SEM, n=6) of the total population of donor CD11b^{high} cells

(Figure 2I). We were precluded from using congenic hosts in these experiments due to rejection of the donor BM from our CD45.1/B6 recipients and Ms4a3-tdTom^{neg} LCs contained both MDP-Mo-derived mLCs and residual eLCs at this time point. To distinguish the resident and recruited (donor) LC populations, we took advantage of an observation that eLC expressed lower levels of CD45 than mLCs (Figures S3E, F). The majority of Ms4a3-tdTom^{neg} LCs were host eLCs at this time point but there was a clear contribution of Ms4a3-tdTom^{neg} donor mLCs to this population (Figures 3J, S3G). Thus, despite the relatively infrequency of MDP-derived monocytes in the CD11b^{high} population, both GMP- and MDP-derived cells gave rise to committed EpCAM^{+}CD24^{+} mLCs, which had downregulated expression of Cx3Cr1 (Figures 2H, J). Together, these data suggest that damage to the eLC network initiates recruitment of both GMP-Mo and MDP-Mo, which expand via a common intermediary to re-establish the nascent mLC network.

Differentiating monocytes lose *Zeb2*-regulated macrophage identity to become mLCs independent of Ahr signaling.

Analysis of the transcription factors that showed the closest correlation with the Slingshot trajectory from monocytes to resident mLCs revealed that the predicted differentiation trajectory was dominated by loss of the tissue macrophage-specifying factor Zeb2 (Figure 3A). Expression of *Zeb2* was mutually exclusive to *Epcam*$^{+}$ cells (Figures 3B, C), suggesting that, unlike other resident macrophage populations, *Zeb2* expression is suppressed during specification of mLCs. Epidermal monocytes also downregulated expression of *Klf6*, linked to pro-inflammatory gene expression and therefore consistent with emergence of quiescent LCs (49), as well as the transcription factors *Fos* and *Stat1*. Notably, *Zeb2* was highly negatively correlated with expression of the LC-defining transcription factors *Id2* and the aryl hydrocarbon receptor (*Ahr*) that are also upregulated by eLCs *in utero* (Figures 3A-C) (2). Mirroring DCs that migrate out of the skin, mLCs poised for migration were defined by upregulation of *Irf4*, *Rel* and *Nr4a3* (Figure S4A) (38, 40, 50, 51).

Previous work has shown that monocyte expression of Ahr biases differentiation towards moDCs rather than moMacs (52). Ahr is not required for eLC development from pre-macrophages *in utero* (Figures S4B,C) (53), but we questioned whether Ahr signaling could be important for monocyte differentiation to mLCs in adult skin. Supporting this, *Ahr* was expressed by a small

number of cells in the MC cluster, potentially indicating those cells were en route to becoming mLCs (Figures 3D, E). To test whether Ahr signaling was required for monocyte differentiation *in vitro*, we generated mLCs in the presence or absence of the Ahr inhibitor StemRegenin1 (SR1) or the agonist 6-Formylindolo[3,2-b]carbazole (FICZ) (52). EpCAM^{+} cells were more sensitive to FICZ, which activated higher expression of *Ahr,* and the cytochrome P450 enzyme *Cyp1b1,* which is directly regulated by Ahr, compared to EpCAM^{neg} cells (Figure 3F). However this activation of Ahr signaling did not result in an increase in the frequency of EpCAM^{+} mLC-like cells, probably due to Ahr ligands already present in culture media, such as tryptophan (Figures 3G and S4D) (54). By contrast, inhibition of Ahr signaling ablated differentiation of EpCAM^{+} cells, demonstrating a requirement for mLC development *in vitro* (Figure 3G and S4D). Guided by these data, we tested the requirement of mLCs on Ahr *in vivo*. Exploiting the expression of ID2 and Langerin by LCs, we used Id2^{BFP} (Figure S4E,F) (55) reporter mice to generate competitive chimeras in which irradiated Langerin^{GFP}.B6 males received a 1:1 mix of BM from female Ahr-replete (Ahr$^{+/+}$.Id2^{BFP}.B6) reporter mice or Ahr-deficient (Ahr$^{-/-}$.B6) donors with Matahari T cells (Figure 3H). Three weeks later, the epidermis was analyzed for presence of mLCs and precursor populations. Consistent with the requirement for Ahr in CD4^{+} T cells, Ahr-deficient BM cells did not contribute to repopulating splenic CD4^{+} T cells in chimeras (Figure 3I) (53). We observed a slight bias towards Ahr-competent CD11b^{+} cells in the spleen across experiments (Figure 3I) suggesting a systemic disadvantage towards loss of Ahr signaling, however this ratio of Ahr-deficient to Ahr replete cells was maintained and not decreased within epidermal CD11b^{+} cells or their descendants (Figure 3I). Therefore, these data suggested that Ahr signaling was not required for mLC differentiation *in vivo*. From these data, we proposed that loss of Zeb2 is a critical step for differentiation of mLCs, and, while Ahr signaling was required for monocyte differentiation *in vitro*, regulation by Ahr did not determine a mLC fate within adult skin.

A distinct follicular keratinocyte niche imprints mLC fate.

To understand the signals regulating the transition from loss of a *Zeb2*-linked macrophage program to commitment to a LC identity, we next sought to define the mLC niche *in vivo*. Imaging of the skin after BMT with T cells revealed abundant MHCII^{+} cells in the inflamed dermis 3 weeks post-transplant with localization of CD11b^{+}MHCII^{+} cells at the upper hair follicle epidermis, an anatomical

site previously associated with monocyte recruitment to the epidermis (Figures 4A, B) (56). Therefore, to define where and how monocytes differentiated within potential epidermal niches, we performed scRNA-seq on $CD45^{neg}$ keratinocytes (Figure S5A) and $CD11b^{+}MHCII^{+}$ cells sorted from the same epidermal samples 3 weeks post-BMT with T cells and integrated the data with our existing $CD11b^{+}MHCII^{+}$ epidermal dataset. Clustering of $CD45^{neg}$ cells followed by differential expression testing identified a set of cluster-specific markers that corresponded with cluster markers of a previously published mouse scRNA-seq dataset (57). The comparison indicated clusters of interfollicular epidermis-derived basal cells (*Krt14*^{high}*Krt5*^{high}) and terminally-differentiated epidermal cells of the stratum spinosum (*Krt10*^{high}), as well as a cluster that combined *Krt79*^{high}*Krt17*^{high} cells of the upper hair follicle with a small sub-cluster of *Mgst1*$^{+}$ cells that were likely to come from the sebaceous gland (Figures 4C, D, S5B). Two other clusters were identified as cycling cells (*Mki67*) and putative *Cdc20*$^{+}$ stem cells.

To predict which keratinocytes could support differentiation of epidermal monocytes, we analyzed expression of factors known to be required for monocyte or mLC survival (*Csf1*, *Il34*, *Bmp7*) and residency (*Tgfb1* and *2*, *Epcam*) (3). While *Csf1* was not expressed by epidermal keratinocytes, *Il34*, which also binds the CSF1 receptor, was localized to *Krt10*^{high} terminally-differentiated cells, consistent with its role as a survival factor for the mature LC network within the interfollicular epidermis (Figure 4E). We could only detect low levels of *Tgfb1* and *2* and *Bmp7* transcripts, although notably, *Tgfb1* was abundantly expressed across our epidermal myeloid cell dataset, consistent with its cell-autonomous function (Figure S5C) (58). By contrast, the TGFβ -activating *integrin beta-8* (but not *beta-6*) was specifically expressed by *Krt79*^{high}*Krt17*^{high} upper hair follicle cells as previously shown (Figure S5D (59)). We also detected highly specific restriction of *Epcam* by upper hair follicle cells (Fig 4E). Expression of the cell adhesion molecule EpCAM, is associated with residency of monocytes within the alveolar space and differentiation to alveolar macrophages (14), and EpCAM expression demarcates isthmus region epithelial cells of the upper hair follicle which express CCL2 (56). Therefore, we postulated that this localized area may provide a niche for recruited monocyte-derived $EpCAM^{+}$ LC precursors. Indeed, $CD11b^{+}$ monocytes were located at EpCAM-rich sites within the hair follicle (Figure 4F). Protein analysis demonstrated high levels of EpCAM on follicular epithelium, which transiently decreased at the peak of T cell-mediated pathology

in this model (Figures 4G, S5E) (31, 32), suggesting loss of adhesion to this niche could contribute to the bottleneck in mLC differentiation that we observed in our previous study (32).

Using the LIANA framework (60), we predicted potential interactions between *Krt79*^{high}*Krt17*^{high} follicular cluster and clusters that lay along the increasing EpCAM expression axis (monocytes, ISG mono, MCs and resident mLCs). This analysis validated that follicular keratinocytes were most likely to signal towards differentiating monocytes (Figure 4H), rather than established mLCs resident within the IL34-rich interfollicular cells. Assessment of key receptor-ligand interactions identified several potential interactions via *Apoe* from follicular epithelial cells (Figure 4I), consistent with the need for monocytes to adapt to the lipid-rich epidermal environment (61). Of potential recruitment pathways, the Cxcl14-Cxcr4 axis was predominantly directed to monocytes, in agreement with previous work showing CXCL14-mediated recruitment of human monocytes to the epidermis before differentiation to mLCs (62). In addition, follicular epithelial cells were the exclusive source of *Jagged-1* and *-2* (*Jag1*, *Jag2*) ligands, capable of initiating Notch signaling in monocytes and, to a lesser extent, MCs (Figures 4I, S5F). We detected expression of Jag-1 and 2 proteins on EpCAM^{+} keratinocytes, but this was not altered in the context of immune pathology (Figures 4J, S5G).

To determine whether Jagged signaling was required for differentiation of mLCs *in vivo* we adapted a protocol previously used to define the role of Notch signaling in the differentiation of monocyte-derived Kupffer cells (13). 12 days after transplant, mice received Jag-2 blocking antibodies every 2 days and epidermal myeloid cells were analyzed 3 weeks post-transplant. The frequency of CD11b^{high} monocytes entering the epidermis, and EpCAM^{+} precursors was unaffected by Jagged blockade (Figure 4K). However, we observed a variable but distinct trend towards a reduction in the generation of mLCs in the absence of Jagged signaling. Our findings therefore reveal a precise and spatially restricted hair follicle niche that permits not only recruitment, but potentially commitment of monocytes to resident mLCs in the adult epidermis via the activation of Notch signaling. This niche is separate from the interfollicular epidermis which provides the IL34 required for maintenance of the differentiated LC network.

Differentiating monocytes metabolically adapt to the epidermal environment.

Our data supported a scenario in which mLC differentiation was dependent on interactions at distinct epidermal sites: a hair follicle niche to recruit and potentially instruct monocyte differentiation, and an interfollicular niche providing IL-34 for survival. Metabolic adaptation of macrophages to utilize fatty acid oxidation pathways is essential for long-term survival as quiescent TRM (63, 64). Therefore, we postulated that only differentiated resident mLC would show evidence of metabolic adaptation to the epidermal environment. To test this, we analyzed metabolic pathway usage across cell clusters using COMPASS (65), which identifies cellular metabolic states using scRNA-seq data and flux balance analysis (Figure S6A). These data showed that, despite being present in the epidermis during the same 3-week time-line post-BMT, mLC metabolism was dominated by fatty acid oxidation, while MCs expressed higher levels of pathways linked to amino acid metabolism, suggesting active cellular processes. Differentiated mLCs also displayed a markedly different metabolic signature compared to Mrc1^{+} macrophages, despite co-localization in the epidermis. Use of SCENIC (Single Cell Regulatory Network Inference and Clustering) (66) to infer transcription factor-target gene groups (regulons) that were more highly active in resident mLCs than other populations revealed that those regulons enriched within resident mLC were dominated by transcription factors known to be upregulated in response to hypoxia and a lipid rich environment (*Zeb1* or *Rxra* and *Srebf2*, respectively) (Figure S6B) (67-69) These data suggested that responsiveness to the epidermal environment informs mLC development, enabling metabolic adaptation of mLC to the lipid-rich epidermal environment.

Notch signaling is sufficient to program mLC differentiation.

Our data suggested a working model in which recruitment of monocytes to the upper follicular epidermis initiated a molecular cascade that resulted in loss of Zeb2 but niche-dependent activation of Notch, resulting in the differentiation of CD207^{+}EpCAM^{+} *Ahr*-expressing mLCs. To define mechanistic pathways leading to a mLC fate in the adult skin, we first tested the impact of Notch signaling on monocytes. Provision of Jag1, but not the Notch ligand Delta-like ligand 4 (DLL4), was sufficient to enhance differentiation of monocytes towards EpCAM^{+} mLC-like cells *in vitro* in the absence of TGFβ and IL34 (Figure 5A). We noted that DLL4 appeared to inhibit mLC development in these cultures (Figure 5A). While Notch signaling did not augment mLC frequencies beyond that

induced by TGFβ and IL-34, we observed selection of a mLC fate at the expense of other default monocyte-derived macrophage- or DC-like cells indicated by expression of CD11c and CD206/CD64 (Figure 5A, B).

Therefore, to determine the effect of Notch signaling combined with other environmental signals in the skin, we cultured monocytes with GM-CSF/TGFβ/IL-34 with or without Jag1, the Ahr agonist FICZ, or both, and compared the transcriptional changes within sorted $CD11b^{low}EPCAM^{+}$ mLC-like cells (Figures 5C and S7A). Cells were clustered by stimulation with considerable overlap between groups (Figures 5D, E). Activation of Ahr signaling did not markedly distinguish clusters along principal component (PC) 1 and over-laid the impact of Jag1, likely due to the dominant activation of the Ahr-responsive gene *Cyp1a1* (Figure S7B and Data file S1). However, provision of Jag1 signaling alone led to a marked transcriptional separation, suggesting fundamental reprogramming of $EpCAM^{+}$ cells in this group. Comparison to our *in vivo* gene signatures demonstrated that Notch signaling was sufficient to program mLCs that expressed both LC-defining transcription factors (*Id2*, *Ahr*) and genes upregulated within the skin environment (*Epcam*, *Cd207*, *Cldn1*, *Mfge8*) (Figure 5F; highlighted box). Moreover, direct comparison of our scRNA-seq gene signatures defining *in vivo* epidermal cell populations within our bulk RNA-seq dataset demonstrated that Jag1 signaling prescribed resident mLC identity (Figure 5G; highlighted box). The additional activation of Ahr signaling pushed cells towards a cycling mLC phenotype (Figure 5G). Thus, Notch signaling is sufficient to restrict the differentiation potential of monocyte-derived cells, directing them away from a default macrophage program towards a mLC fate.

Post-natal maturation of eLCs in human skin induces expression of a DC-like immune gene program that mirrors mLC development.

Our data demonstrated that monocytes adopted a distinct pathway of differentiation in murine adult skin, characterized by loss of Zeb2, to become mLCs. We therefore questioned whether such a transition away from a classical macrophage identity occurred in humans. Post-natal maturation of intestinal macrophages has been linked to the acquisition of immune functions (70). We posited that a similar maturation process occurred in human skin after birth and was linked to LC specification. To address this, we analyzed a collection of samples taken from newborn babies (<28

days), infants (1 month – 1year) and children by bulk RNA-seq (2-15 years) (Figures 6A, S8A, Table S3). As previously demonstrated in mice and humans (18, 19), we observed marked expansion in eLC numbers in the transition from newborns to infants (Figure 6B). Visualization of the RNA-seq data as a co-expression network revealed a central gene program that is high in newborns and encodes basic macrophage functions including protein transport, RNA processing, and cadherin binding (Cluster 1,2, and 4, Figure 6C and Data file S2 Figure S8). Specifically, there was a significant increase in transcriptional activity associated with induced immune activation from newborns to infants and children (Cluster 5, Figures 6C, D) that was also enriched for biological processes such as antigen processing and presentation (Figure 6E and Data file S2), suggesting that more DC-like functions were activated once the skin environment and LC network has fully matured. To determine how post-natal maturation of eLCs in human skin compared to mLC differentiation in adult murine skin, we analyzed expression of the key factors associated with mLC development. Proliferation of eLCs in infant skin resulted in a marked loss of *ZEB2* expression with an increase in *RUNX3*, with a trend suggesting concomitant upregulation of *AHR* and increase in *EPCAM* in some individuals (Figure 6F). In summary, we demonstrate that monocytes recruited to the epidermis after immune pathology adopt a distinct pathway of differentiation to mLCs that mirrors post-natal program in human skin.

Discussion

Whether LCs are macrophages or DCs has long been debated (71) and both designations are still routinely applied in the literature, despite fate mapping studies demonstrating the embryonic macrophage origin for these cells (2, 4, 16, 72). Here, we have begun to resolve this discussion, demonstrating that differentiation within the epidermal environment drives a shift from more macrophage-like to DC-like cells. Within the skin, localization and signaling within a specialized hair follicle niche is correlated with loss of the Zeb2, the regulator of TRM identity at other barrier sites, and Notch-dependent expression of the LC-defining transcription factors Id2 and Ahr, to generate long-lived mLCs (2, 21). This adaptation to adult skin mirrors post-natal maturation of eLCs which is characterized by the appearance of a specific gene program associated with DC-like immune functions. Thus, specification of LCs within the skin environment drives evolution of a distinct population of TRMs, which are the only resident macrophages that can migrate to draining LNs and prime T cell immunity.

There is a growing awareness of the heterogeneity within Ly6C$^+$ monocytes, that represent a pool of cells derived from either GMPs or MDPs (33–35). By exploiting the Ms4a3-reporter mouse, we have tracked the fate of these ontogenically distinct monocytes in the epidermis. GMP-Mos are more common in the blood and have been shown to preferentially seed the lungs upon viral infection (35). Consistent with this, most monocytes in the epidermis were Ms4a3-labeled GMP-Mos. However, we show that MDP-Mos also contribute to the epidermal pool and that, both GMP-Mo and MDP-Mo appear to differentiate via a common intermediary to become mLCs. Further studies are needed to determine whether these intrinsic differences may be linked to the LC populations identified in healthy human skin (39), and potentially translate to differential LC function.

Evidence for the macrophage origin of embryonic LCs comes from fate-mapping studies in which labelled yolk sac (2, 72) and fetal liver (16) macrophage/monocytes gave rise to the nascent LC network in the developing embryo. Moreover, use of the macrophage-restricting gene *Mafb* to lineage trace myeloid cells labelled all LCs in the healthy adult skin (73), and generation of LCs from CD34$^+$ stem cells requires suppression of the transcription factor Klf4 associated with differentiation of moDCs (74). But, LCs express high levels of CD24 and Zbtb46, considered markers of conventional DCs (73). Moreover, activation and migration initiates the expression of a convergent

transcriptional program shared with DC populations leaving the skin (40, 75, 76). Migrating LCs upregulate an IRF4-dependent gene program that is also evident in moDC (38, 50). We believe our data begin to reconcile this dichotomy. Zeb2 is a critical regulator of cell fate that specifies TRM identity across barrier sites (11, 12) and we show that only those cells that have lost *Zeb2* expression upregulate *EpCAM* to become mLCs. Key Zeb2 regulatory elements control function in embryonic and HSC-derived macrophages (77) and molecular cross-talk with Id2 has been shown to determine cDC2 specification (78). We speculate that a similar process may permit expression of Id2 in differentiating mLCs and initiating the shift from more macrophage-like to DC-like cells. Further studies are needed to dissect the potential molecular interactions between *Zeb2* and *Epcam* and/or other LC-defining genes.

To determine the extrinsic signals regulating mLC development, we characterized the epidermal niche and identified a spatially restricted area of the follicular epidermis at which homotypic binding of $EpCAM^+$ MCs provides access to local Notch signals. The epidermis is composed of layers of stratified epithelial cells, which are interspersed in non-glabrous skin with follicular structures that support the cycles of hair growth. Within follicular epithelial cells, tightly demarcated areas of CCL2 expression at the upper follicular isthmus are associated with recruitment of monocytes to the epidermis (56). In addition, spatially distinct sites of integrin expression regulate activation of latent TGFβ, with αvβ8 required for accumulation of LCs around the isthumus region and αvβ6 needed to establish the mature LC network in the interfollicular epidermis (59). $MHCII^+CD11b^+$ cells accumulated around hair follicles upon induction of inflammation in LC-depleted bone marrow (BM) chimeras, and LC repopulation was impaired in mice lacking hair follicles (56). But whether the hair follicle site provides a differentiation niche, or merely serves as a point of entry for monocytes into the epidermis was not known. Our data suggest that the follicular niche is not only a site of recruitment but also provides instructive signals via Notch signaling for differentiation to mLCs before they relocate within the keratinocytes of the differentiated epidermis. This finding may explain the inefficiency with which recruited epidermal monocytes were predicted to become long-lived mLCs (32), since LC precursors will compete for Notch signals within a spatially restricted hair follicle niche. Our findings reflect the importance of Notch for differentiation of monocyte-derived Kupffer cells in the liver (13), hinting at shared exploitation of Notch pathways across tissue

macrophage niches, although we identified Jagged as the ligand rather than delta-like ligand DLL4 in the liver (13). However, we suggest that the physical restraints in the epidermis and separation from the circulation impose a two-niche model whereby hair follicle keratinocytes instruct LC molecular identity, but the differentiated intra-follicular keratinocytes provide the scaffold and trophic factors that support survival and permit adaptation to the lipid-rich epidermal environment.

Notch signaling has previously been linked to human mLC development in vitro (22, 79, 80), while a recent study that employed scRNA-seq of human LCs revealed the presence of 2 eLC populations in the skin, linking Notch signaling to expansion of $EpCAM^{neg}$ cells (39). By contrast, our data suggest that Notch signaling restricts murine monocyte differentiation into mLCs at the expense of other fates. It is possible that Notch signaling promotes a more DC-like program in mLCs. Indeed, Notch2 signaling has been shown to promote differentiation and function of cDC2s in a variety of tissues (81–83). Furthermore, Notch signaling in monocytes has been shown to suppress a macrophage fate in favor of a DC fate and is required for differentiation to monocyte-derived $CD207^{+}CD1a^{+}$ cells characteristic of LC histiocytosis (84). Additionally, activation of Notch in human $CD1c^{+}$ DCs is sufficient to promote differentiation to LC-like cells that contain Birbeck granules (85).

Ahr is an evolutionarily conserved cytosolic sensor that functions as a ligand-dependent transcription factor to control cell fate decisions in gut immune cells (86) and direct monocytes towards a DC-like rather than a macrophage fate (52). The role of Ahr signaling in LCs remains unclear; eLC begin to express Ahr upon differentiation *in utero* (2), but our data suggest that expression increases post-birth. The epidermis of Ahr-deficient mice is replete with LCs (53), albeit a less activated population, probably due to the absence of dendritic epidermal T cells (DETC) and reduced GM-CSF production in the skin of these mice (87). Moreover, mice fed with chow deficient in Ahr dietary ligands had normal numbers of LCs (88), but these cells did not migrate to draining LNs. In contrast, LC-specific deletion of Ahr led to a reduction in epidermal LCs (89). We tested the role of Ahr signaling for the differentiation of mLCs and found that blockade of Ahr prevented monocyte differentiation *in vitro*, supporting a previous study using $CD34^{+}$ precursors (90). However, use of competitive chimeras demonstrated that Ahr-deficient monocytes could become mLCs *in vivo*. We observed that canonical (*Cyp1b1*) Ahr signaling was active in mLCs *in vitro* but not *in vivo*, suggesting activation of alternative pathways within the epidermal environment.

Murine and human eLCs undergo a burst of proliferation after birth (18, 19), but whether expansion of eLC was associated with maturation of the network, as has been demonstrated for the LCs of the oral mucosa (91), was unknown. To address this question, we assembled a RNA-seq dataset from eLCs sorted from newborn children, up to 1 year-old infants and older children. These data revealed the documented increase in LC density after the first 28 days of life (19) and demonstrated that this increase was associated with a marked change in gene expression whereby the macrophage-associated genes *MAFB* and *ZEB2* were downregulated, while we observed trends towards an increase in *AHR* and *EPCAM*. Notably, this transition was accompanied by the expression of gene modules associated with enhanced immune and DC-like functions. These findings support our proposed concept of gene regulatory networks defining LC function whereby interaction between Ahr and Irf4 activates expression of immunogenic function and migration to LNs (76). The signals that trigger eLC proliferation in the skin are not known. While skin commensal bacteria per se are not required for LC development and survival (92), it is possible that the increase in microbiota diversity during the first year of life could play an important role in conditioning the LC niche, although further experiments are required to test this hypothesis.

In conclusion, convergent evolution of monocytes within adult skin imposes expression of these gene programs to mirror post-natal conditioning and maintain this distinct population of DC-like cells in the epidermis.

Materials and Methods

Study design

The aim of this study was to define intrinsic and extrinsic factors that shaped repopulation of epidermal LCs with monocytes in the inflamed skin. We used an in vivo model of LC replacement and measured changes to cell populations by combining single cell RNA sequencing with flow cytometry and confocal microscopy. Sample sizes were based on previous experiments, and the availability of genetically engineered donors. No outliers were excluded, and the number of replicates and independent experiments is given in each figure. The nature of our model means that we transplant female donor BM into male recipients and therefore we are restricted by the sex of the mice used. However, for in vitro BM cultures, both males and females are used. There was no randomization and blinding was not required for these experiments since we use objective readouts such as flow cytometry. Recipients were co-housed were possible.

Mice

C57BL/6 (B6) were purchased from Charles River UK. Langerin.DTR^{GFP} were originally provided by Adrian Kissenpfennig and Bernard Malissen (Centre d'Immunologie de Marseille-Luminy, CNRS, Marseille, France) (93), T cell receptor (TCR)-transgenic anti-HY Matahari were provided by Jian Chai (Imperial College London, London, UK) (36) and CD45.1 mice were bred in-house at University College London (UCL) Biological Services Unit. ID2^{BFP} reporter mice were a kind gift from Andrew McKenzie (University of Cambridge). The pBAD-mTagBFP2 plasmid was a gift from Vladislav Verkhusha (Albert Einstein College of Medicine, New York, USA) (Addgene plasmid no. 34632 ; http://n2t.net/addgene:34632 ; RRID:Addgene_34632) (55, 94). All procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 and were approved by the Ethics and Welfare Committee of the Comparative Biology Unit (Hampstead Campus, UCL, London, UK).

Human samples

Human skin samples were collected with written consent from donors with approval by the South East Coast - Brighton & Sussex Research Ethics Committee in adherence to Helsinki Guidelines (ethical approvals: REC approval: 16/LO/0999). Donor information is listed in Table S3.

Bone marrow (BM) transplants

Recipient male CD45.2 C57BL/6 mice were lethally irradiated (10.4Gy of total body irradiation, split into two doses over a two-day period) and reconstituted 4 hours following the second dose with 5 x 10^6 female CD45.1 C57BL/6 bone marrow cells, 2 x 10^6 CD4 T cells, with 1 x 10^6 CD8 Matahari T cells administered by intravenous injection through the tail vein. CD4 and CD8 donor T cells were isolated from spleen and lymph node single cell suspensions by magnetic activation cell sorting (MACS; Miltenyi) using CD4 (L3T4) and CD8a (Ly-2) microbeads (Miltenyi) according to manufacturer's instructions. In some experiments, BM from $ID2^{BFP}$ C57BL/6 mice was used to track donor LC. In some experiments, $Langerin.DTR^{GFP}$ male mice were used as recipients to track host LCs. To lineage trace monocyte-derived cells, BM from $Ms4a3^{Cre/+}R26^{LSL\text{-}TdTomato}$:$Cx3cr1^{GFP/+}$ female mice was used as donor cells. In some experiments, BM from $Cxcr4^{CreERT2}R26^{LSL\text{-}TdTomato}$ (Cxcr4tm1.1(cre/ERT2)Stum, kindly donated to R.G. by Ralf Stumm (Jena, Germany)) was used to track donor LC. To activate recombination mice received 3 doses of 0.12 mg tamoxifen per gram of body weight for 3 consecutive days in 100 µl volume.

Mixed chimera experiments

BM from $AHR^{-/-}$ mice was a gift from Brigitta Stockinger (Francis Crick Institute, London, UK) (95). Lethally irradiated male $Langerin.DTR^{GFP}$ C57BL/6 mice received a 50:50 mix of BM from AHR KO and $ID2^{BFP}$ female mice, with CD4 T cells and CD8 Matahari T cells. Three weeks following transplant, epidermis and spleens were processed and analyzed for chimerism by flow cytometry.

In vivo antibody treatment experiments

Lethally irradiated male CD45.2 C57BL/6 mice received BM from female CD45.1 C57BL/6 mice, with CD4 T cells and CD8 Matahari T cells (BMT+T cells). Mice received intra-peritoneal injections of 250µg anti-Jagged 2 (Clone: HJM2-1; BioXCell 1.25mg/ml) or anti-IgG isotype control (polyclonal;

BioXCell 1.25mg/ml) antibodies on day 12, 14, 16 and 19 post-BMT+T cells. Epidermal cells were analyzed by flow cytometry on day 20 post-BMT+T cells.

Tissue processing

Mouse skin. Epidermal single cell suspensions were generated as described (32, 96). Dorsal and ventral sides of the ear pinna were split using forceps. These were floated on Dispase II (2.5mg/ml; Roche), in HBSS and 2% FCS for 1 hour at 37˚C or overnight at 4˚C, followed by mechanical dissociation of the epidermal layer by mincing with scalpels. Cells were passed sequentially through 70- and 40μm cell strainers in 1mM EDTA, 1% FCS, PBS solution.

Human skin. Fat and lower dermis was cut away and discarded before dispase (2 U/ml, Gibco, UK) digestion for 20h at 4°C. Epidermal sheets were digested in Liberase^{TM} (13 U/ml, Roche, UK), for 1.5h at 37°C.

Bone marrow cells. BM single cell suspensions were prepared from femurs and tibias of donors using a mortar and pestle. Red blood cells were lysed in 1ml of ammonium chloride (ACK buffer) for 1 min at room temperature. Cells were washed and resuspended in complete RPMI (RPMI supplemented with 10% FCS, 1% L-glutamine, 1% Penicillin-streptomycin) until used.

In vitro cultures

GMP and MDP cultures

GMP, MDP and Ly6C^{hi} monocytes from whole BM were FACS isolated and seeded in 96-well tissue-culture treated flat-bottom plates. Cells were cultured in complete RPMI and supplemented with recombinant GM-CSF (Peprotech; 20ng/ml), TGFβ (R&D systems; 5ng/ml) and IL34 (R&D systems; 8μg/ml). The medium was partially replaced on day 2 of culture and completely replaced on day 3, and cells were harvested on day 6.

Monocyte cultures

Monocytes were isolated from whole BM by MACS using Monocyte Isolation Kit (BM; Miltenyi, 130-100-629) as per manufacturer's instructions. Monocytes were resuspended in complete RPMI and plated at $5x10^5$ cells per well in tissue-culture treated 24-well plates. Cells were cultured as described above

Monocyte co-cultures

OP9, OP9-Jag1 and OP9-DL4 cell lines were gifted by Victor Tybulewicz (Francis Crick Institute, London, UK) and were cultured in RPMI supplemented with 10% FCS, 1% L-glutamine, 1% Penicillin-streptomycin, MEM NEAA, sodium pyruvate, HEPES buffer and ß-mercaptoethanol. OP9 cells were seeded into tissue-culture treated 24-well plates at 2×10^4 cells per well and incubated overnight at 37˚C. The next day, following monocyte isolation, cells were counted, and 1×10^5 monocytes were seeded on to OP9 cells. Cells were cultured as above.

Flow cytometry and cell sorting

Mouse. Cells were distributed into 96-well V-bottom plates or FACS tubes and incubated in 2.4G2 hybridoma supernatant for 10 min at 4˚C to block Fc receptors. Cells were washed with FACS buffer (2% FCS, 2mM EDTA, PBS) before adding antibody cocktails that were prepared in a total volume of 50µl per test in Brilliant Stain Buffer (BD Biosciences) and FACS buffer. Cells were incubated with antibodies for 30 mins on ice then washed with FACS buffer. Viability was assessed either by staining cells with Fixable Viability Dye eFluor680 (eBiosciences) or Propidium iodide (PI), for fixed or unfixed cells respectively. For intracellular staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) for 30 mins on ice. Cells were subsequently washed in permeabilization buffer before adding antibody cocktails that were prepared in a total volume of 50µl per test in permeabilization buffer. Cells were incubated with antibodies for 30 mins on ice then washed with permeabilization buffer. Antibodies used are listed in Table S1

Human. Antibodies used for cell staining were pre-titrated and used at optimal concentrations. For FACS purification, LCs were stained for CD207 (anti-CD207 PeVio700), CD1a (anti-CD1a VioBlue) and HLA-DR (anti-HLA-DR VioGreen), Miltenyi Biotech, UK) (Table S1).

When required, cells were acquired on a BD Fortessa analyzer equipped with BD FACSDiva software; or sorted into either complete RPMI or RLT lysis buffer (Qiagen) or Trizol using a BD Aria III.

Immunofluorescence imaging

Skin biopsies were embedded in OCT compound (Leica). 10 mm sections were cut using a cryostat (Leica) and stored at -20°C. Tissue was blocked for 2 hours at room temperature with 5% BSA (Sigma Aldrich), 5% Donkey Serum (Merck) in 0.01% PBSTween-20 (PBST). Sections were incubated with primary antibodies as listed in Table S2. Antibodies were detected using donkey Cy2- or Cy5-conjugated secondary Fab fragment antibodies (Jackson Laboratories) and nuclei stained using Hoechst 33342 (1:1000, Sigma Aldrich), and mounted using Prolong Gold anti-fade mounting media. Images were acquired on a Leica SP8 confocal microscope and subsequently analyzed using NIH ImageJ software.

Generation of single-cell RNA sequencing data

Single cell suspensions from murine epidermis were stained for FACS as described above. Donor or host $CD11b^{+}MHCII^{+}$ cells and $CD45^{neg}$ cells were sorted into RPMI medium supplemented 2% FCS and counted manually. Cell concentrations were adjusted to 500-1200 cells/µl and loaded at 7,000-15,000 cells per chip position using the 10x Chromium Single cell 5' Library, Gel Bead and Multiplex Kit and Chip Kit (10X Genomics, V3 barcoding chemistry) according to manufacturer's instructions. All subsequent steps were performed following standard manufacturer's instructions. Purified libraries were analyzed by an Illumina Hiseq X Ten sequencer with 150-bp paired-end reads.

Single-cell RNA sequencing data processing and analyses

Generated scRNA-seq data preprocessed using the kallisto and bustools workflow (97). Downstream analysis was performed using the Seurat package in R (98). Cells with <500 detected genes and >20% mitochondrial gene expression were removed from the dataset. DoubletFinder was used to identify and remove any likely doublets. These were typically less than 1% of each batch.

PCA was performed on the 2000 most variable genes and clusters were identified using the Leiden algorithm. Clusters were annotated based on the expression of key cell-type defining genes. Differentially expressed genes (DEGs) were identified using the FindMarkers function with significance cut offs of log2-fold change > 2 and adjusted p-values < 0,05.

Enrichment scores

To calculate enrichment scores for specific gene signatures the Seurat function AddModuleScore was used. The human migLC gene signature included 101 genes (38). The MDP-Mo and GMP-Mo signatures included 140 and 108 genes respectively (35).

Trajectory analyses

Pseudotime trajectory inference of differentiation and RNA velocity analysis based upon spliced and unspliced transcript ratios were performed respectively using the Slingshot (45) and velociraptor packages for R (44). Expression of genes changing along the trajectories were identified using general additive models fitted by tradeseq.

Receptor-ligand interaction analysis

Potential receptor-ligand (R-L) interactions between the follicular keratinocyte subset and monocyte-derived cell clusters were investigated using the LIANA package (60). LIANA is an umbrella framework which creates a consensus R-L score from the methods and pathway tools of several other software packages. It encompasses CellPhoneDB (v2), CellChat, NATMI, iTALK and CytoTalk (60).

SCENIC analysis

SCENIC was used to identify regulons, sets of transcription factors and their cofactors co-expressed with their downstream targets in single cell data (66). This analysis was applied to the 10X scRNA-seq dataset and each of the regulon AUC per cell scores was used to identify regulons with the greatest mean difference between Res-mLC and all other clusters.

COMPASS analysis

In silico flux balance analysis was conducted via COMPASS (65). Normalised scRNA-seq counts per million (CPM) gene expression profiles were exported and COMPASS analysis was conducted using standard settings on a high-performance computing cluster (99). Metabolic reactions were mapped to RECON2 reaction metadata (100), and reaction activity scores were calculated from reaction penalties. Reactions which do not have an enzyme commission number or for which there is no biochemical support (RECON2 confidence score = 1-3) were excluded from the analysis. Differential reaction activities were analysed via Wilcoxon rank-sum testing and resulting p-values were adjusted via the Benjamini-Hochberg (BH) method. Reactions with an adjusted p-value of less than 0.1 were considered differentially active. Effect sizes were assessed with Cohen's d statistic.

Bulk RNA-sequencing and analyses

Mouse. Up to 100,000 cells were FACS sorted into RLT lysis buffer (Qiagen) supplemented with 14mM ß-mercaptoethanol. Cells were vortexed immediately after being sorted to ensure cell lysis. RNA was extracted using the RNeasy Micro kit (Qiagen) as per manufacturer's instructions, with an additional DNA clean-up step using RNase-free DNase I (Qiagen). RNA quantification and quality check was carried out by Novogene (UK), as well as subsequent library preparation and sequencing. Sequencing was performed on a NovaSeq 600 System (Illumina) to yield an average of 30 million reads per sample. RNA-seq transcript abundance was quantified using the salmon read mapper and an Ensembl GRCm39 transcript model. The data were imported to the R statistical environment and summarized at the gene level (that is, transcript counts summed) using tximport. Statistical transformations for visualisation (vst and log10) and analyses of differential expression were performed using the DESeq2 package (101). Multiple testing adjustments of differential expression utilised the Benjamini–Hochberg false discovery rate (fdr).

Human. RNA was isolated using Direct-zol RNA micro prep (Zymo, UK) as per the manufacturer's protocol. RNA concentration and integrity was determined with an Agilent Bioanalyser (Agilent Technologies, Santa Clara, CA). Preparation of RNA-seq libraries and sequencing were carried out by Oxford Genomics Centre, UK. cDNA libraries were generated using SMART-Seq Stranded

Library Preparation for Ultra Low Input according to the SMART-Seq Stranded Kit User Manual following the Ultralow input workflow (Takara Bio). Samples were pooled (12/batch) for library preparation. Amplified libraries were validated on the Agilent BioAnalyzer 2100 to check the size distribution and on the Qubit High Sensitivity to check the concentration of the libraries. All the libraries passed the QC step. Sequencing was done on Illumina HiSeq 4000 instrument, 150bp PE runs, 20 $x10^6$ reads per sample.

RT-PCR

RNA was extracted from samples as described above. RNA quantification was carried out using a Nanodrop and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer's instructions. RT-PCR was run on a QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific) using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) according to manufacturer's instructions. Primers used in this study: Ahr forward, AGC CGG TGC AGA AAA CAG TAA; Ahr reverse, AGG CGG TCT AAC TCT GTG TTC; Cyp1b1 forward, ACG ACG ATG CGG AGT TCC TA; Cyp1b1 reverse, CGG GTT GGG AAA TAG CTG C; GAPDH forward, CGGGTTCCTATAAATACGGACTGC; GAPDH reverse, GTTCACACCGACCTTCACCA

Statistical Analyses

All data, apart from RNA-seq data, were analyzed using GraphPad Prism Version 6.00 for Mac OsX (GraphPad Software, USA). All line graphs and bar charts are shown as mean±SD. Protein expression data for flow cytometry is shown as geometric mean fluorescent intensity (as specified in figure legends) with the range. Significant differences were determined using one-way analysis of variance (ANOVA) to measure a single variable in three groups or two-way ANOVA for experiments with more than one variable, with post-tests specified in individual figure captions. For comparisons between 2 paired groups, a paired t test was used according to a normality test. Significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$. Statistical details of the data can be found in each figure caption. Analysis of bulk and scRNA-seq data was performed in the R and Python environments using tests described in the method details.

List of supplementary materials

Figure S1. scRNA-seq of monocyte-derived cells isolated from the epidermis 3 weeks post-BMT with T cells, related to Figure 1.

Figure S2. Analysis of scRNAseq data from epidermal myeloid cells, related to Figure 1.

Figure S3. Analysis of monocyte heterogeneity and differentiation to mLCs, related to Figure 2.

Figure S4. Transcription factors associated with mLC differentiation, related to Figure 3.

Figure S5. scRNA-seq of keratinocytes from the GVHD epidermis, related to Figure 4.

Figure S6. Metabolic adaptation of resident mLC, related to Figure 5.

Figure S7. Bulk RNA-seq of in vitro-generated mLCs, related to Figure 5.

Figure S8. Bulk RNA-seq of human LCs from newborns, infants and children, related to Figure 6.

Data file S1. Murine_BulkRNAseq_DEGs.

Data file S2. Human_BulkRNAseq_DEGs.

Data file S3. Raw data file.

Table S1. List of flow cytometry antibodies used in this study.

Table S2. List of primary antibodies used for confocal microscopy in this study.

Table S3. List of pediatric donor information

Reproducibility checklist.

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Competing interests: MEP is currently employed at Johnson and Johnson Innovative Medicine. Johnson and Johnson Innovative Medicine or any of employees/stakeholders have not been involved in any part or aspect of the project or manuscript. All other authors declare no competing interests.

Materials and data availability: The sequencing data for this study have been deposited in the Geo genomics data repository database and can be found as NCBI GEO GSE247878 (murine scRNAseq epidermal $CD11b^{+}MHCII^{+}$ cells), GSE247874 (murine bulk RNAseq monocyte-derived $EpCAM^{+}$ cells), and GSE251705 (human bulk RNAseq $CD207^{+}CD1a^{+}$ LCs). Tabulated data underlying the figures is provided in Data file S3. All other data needed to support the conclusions of the paper are present in the paper or the Supplementary Materials.

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Figure captions

Figure 1. scRNA-seq reveals monocyte-derived cell heterogeneity in the inflamed epidermis. **A.** Experimental design showing murine bone marrow transplant model and cells sorted for scRNA-seq from murine epidermis. M, male; F, female; BM, bone marrow. For full gating strategy see figure S1A. **B.** UMAP and clustering of murine donor $CD11b^{+}MHCII^{+}$ cells from murine GVHD epidermis analyzed by scRNA-seq. Data are from 2 combined independent sorting and sequencing experiments using epidermis from 4 and 10 pooled mice. BMT, bone marrow transplant; Mac, macrophage; MC, monocyte-derived cell; res. mLC, resident monocyte-derived Langerhans cell; mig. mLC, migratory mLC; mono, monocyte; cyc. mLC, cycling mLC. **C.** Heatmap overlays showing expression of indicated genes across dataset. Expression scales: *Cd207* 0-4, *Mki67* 0-4, *Mrc1* 0-5, *Ccr7* 0-4, *Plac8* 0-5, *Isg15* 0-4, *S100a9* 0-6, *Hmox1* 0-5. **D.** Violin plot showing enrichment scores for a human mig. LC gene signature across clusters. **E.** Representative flow plots showing donor $CD11b^{+}MHCII^{+}$ cells from murine GVHD epidermis at the indicated time-points following BMT+T cells. Gated on live, singlets, $CD45.1^{+}$ (donor) cells. **F.** Quantification of populations indicated in (E). Data are represented as mean±SD, (n=2 for 2 weeks, 8 for 3 weeks, 2 for 4 weeks). Data are pooled from three independent experiments. **G.** Differentiation trajectories calculated using Slingshot overlaid onto UMAP from (B) (above), normalized expression of indicated genes (y-axis) across pseudotime (x-axis) for the indicated trajectories (middle) and feature plots showing normalized expression of indicated genes overlaid onto UMAP from (B) (below). Expression scales: Clec4d 0-4, Ccl22 0-6, *Epcam* 0-4 **H.** RNA velocity analysis applied to data from (B). Arrow directions indicate inferred cell trajectory.

Figure 2. Monocyte ontogeny determines mLC repopulation. A. UMAP and sub-clustering of monocytes from GVHD epidermis. **B.** Clusters from (A) overlaid onto UMAP from figure 1B. **C.** Violin plots showing enrichment scores for MDP-Mo (above) and GMP-Mo (below) gene signatures across clusters from (A). **D.** Heatmap overlays showing normalized expression of indicated genes. Expression scales: *Slamf7* 0-2, *Cd177* 0-1.5. **E.** Volcano plot showing differentially expressed genes (DEGs) between cluster 2 and cluster 3 from (A). Top 10 significant DEGs are highlighted. **F.** Scatter plots of selected genes across monocyte clusters. **G.** Schematic and representative flow plot

showing Ms4a3-tdTomato and Cx3cr1-GFP expression on live cells isolated from Ms4a3$^{Cre/+R26LSL-TdTomato}$:Cx3cr1$^{GFP/+}$ bone marrow (BM). **H.** Representative contour plots showing Ms4a3-tdTom and Cx3cr1-GFP expression on epidermal CD11b^{high} monocytes and LCs 3 weeks post-BMT+T cells. **I.** Bar graph showing the frequency of GMP-derived (tdTom+) and MDP-derived (tdTom-) cells within epidermal CD11b^{high} cells. Data are represented as mean±SD (n=6; p=0.03, Wilcoxon matched-pair test) **J.** Left – Bar graph showing the frequency of tdTom+ and tdTom- epidermal LCs. Right - Bar graph showing the frequency of host (CD45^{+}) and donor (CD45^{++}) cells within the tdTom-EpCAM^{+}CD24^{+} LC gate. Data are represented as mean±SD (n=6). Data are pooled from 2 independent experiments.

F**igure 3. mLC differentiation is associated with loss of Zeb2 and upregulation of Ahr. A.** Heatmap showing scaled gene expression of transcription factors that are differentially expressed along the differentiation trajectory (Pseudotime) from monocyte to res. mLC. **B.** Heatmap overlays showing normalized expression of indicated genes across UMAP from Fig. 1B. Expression scales: *Epcam* 0-4, *Zeb2* 0-3. **C.** Correlation of selected LC-defining genes (y-axis) across all clusters of the scRNA-seq dataset. **D.** Density plot showing expression *Ahr* across cells from scRNA-seq dataset, expression scale 0-0.06. **E.** Violin plot showing normalized expression of *Ahr* across clusters from scRNA-seq dataset. **F.** Bar graphs showing the relative expression (mean ± SD) of *Ahr* and *Cyp1b1* in sorted CD11b^{+}EpCAM^{neg} and CD11b^{+}EpCAM^{+} cells generated *in vitro* in the presence of FICZ. Expression is normalized to cells treated with GM-CSF+TGFβ+IL34 alone (n=2 independent experiments); GM, GM-CSF. **G.** Representative histogram overlay (left) of EpCAM expression by monocytes cultured for 6 days under the indicated conditions and summary bar graph (right) of mLC-like cells generated from these conditions (for gating see Figure S4D). Data are represented as mean±SD (n=5 independent experiments). Statistical differences were assessed using Kruskal-Wallis with Dunn's multiple comparison test, * p<0.05. **H.** Left - Schematic showing the experimental set up to generate competitive chimeras. Male Langerin$^{DTR.GFP}$.B6 mice received a 1:1 mix of BM from female Ahr-replete (Ahr$^{+/+}$.Id2^{BFP}.B6 reporter mice, WT) or Ahr-deficient (Ahr$^{-/-}$.B6) donors with Matahari T cells. and donor chimerism was assessed in the epidermis and spleen 3 weeks post-transplant. Right – representative contour plot showing gating of the different populations in the

epidermis; M, male; F, female. **I.** Bar graph showing ratio of Ahr-/- to WT frequencies of indicated cell types in spleen and epidermis of transplanted mice. Data are represented as mean±SD (n=6 for epidermis and 9 for spleen, from 2 or 3 independent experiments). Significant differences were assessed using ruskal-Wallis with Dunn's multiple comparison test, ** p<0.01.

Figure 4. A specialized follicular keratinocyte niche imprints mLC fate. A. Immunofluorescence (IF) image of murine epidermis 4 weeks post-BMT+T cells: $MHCII^+$ cells (green), $KRT14^+$ keratinocytess (magenta), nuclei (blue). Scale bar = 20µm. **B.** IF merged and single images of murine epidermis highlighting $CD11b^+MHCII^+$ cells at $KRT14^+$ upper hair follicle,. Scale bar = 50µm. **C.** Schematic of murine hair follicle; IM, isthmus. **D.** UMAP visualization of keratinocytes 3 weeks post-BMT+T cells analyzed by scRNA-seq. Data are from epidermal cells of 5 pooled mice 3 weeks post-BMT+ T cells KC, keratinocytes. **E.** Heatmap overlays showing normalized expression of indicated genes overlaid onto UMAP from (D). Expression scales: *Csf1* 0-1.5, *Il34* 0-2, *Bmp7* 0-2.5, *Tgfb1* 0-2, *Tgfb2* 0-2, *Epcam* 0-3 **F.** Merged and single IF images of murine epidermis 4 weeks post-BMT+T cells: EpCAM (white), CD11b (green) and KRT14 (magenta). Scale bar = 20µm. **G.** Bar graphs showing frequency and gMFI of $EpCAM^+$ expressing hair follicle cells from untransplanted (Un-tx) mice or post-BMT+T cells. Data are mean±SD (n=3 control, 2 weeks n=3, 3 weeks n=9, 4 weeks n=7, 7 weeks n=3), pooled from three independent experiments. Significance was calculated using Kruskal-Wallis with Dunn's multiple comparison test, p*<0.05. **H.** Chord plot showing receptor-ligand interactions between follicular KC (grey) and monocytes (blue), ISG monocytes (orange), MC (purple) and res.mLC (red) assessed by LIANA. The width/weight of each arrow indicates the number of potential interactions identified. **I.** Dot plot showing the specificity (NATMI edge specificity) and magnitude (sca LR score) of interactions between follicular KC (grey) and indicated populations (blue). **J.** Representative histograms of Jag1 and Jag2 expression by $EpCAM^+$ KC in the epidermis. FMO, fluorescence minus one. **K.** Bar graphs showing frequency of $CD11b^{high}$, $EpCAM^+$ precursors and mLCs in mice treated with anti-Jag2 antibodies or anti-IgG isotype control (Ctrl). Data are shown as mean±SD (control n=4, anti-Jag2 n=7), pooled from 2 independent experiments.

Figure 5. Notch signaling is sufficient to program mLC differentiation. A. Bar graph showing the proportion of mLC-like cells generated from monocytes cultured with GM-CSF alone or GM-CSF, TGFβ and IL34 in the presence or absence of indicated Notch ligands (see figure S7A for gating strategy). Data is shown as mean±SD (n=5). Significance was calculated by 2-way ANOVA with uncorrected Fisher's LSD for multiple comparisons, * $p<0.05$; ***$p<0.001$. **B.** Heatmap showing average gMFI of indicated markers from bone-marrow derived monocytes cultured and analyzed by flow cytometry as indicated in (A) (n=5). **C.** Experimental set up for bulk RNA-seq of mLC-like cells generated under indicated conditions. **D.** Principal component analysis (PCA) plot of bulk RNA-seq samples colored by culture condition. **E.** Venn diagram showing numbers of common and unique DEGs between indicated conditions. **F.** Heatmap showing scaled expression of LC signature genes across samples. **G.** Heatmap showing expression of gene signatures from epidermal myeloid cell clusters (defined as top 20 DEGs) (y-axis) across bulk RNA-seq samples (x-axis).

Figure 6. Post-natal maturation of eLCs in human skin induces expression of DC-like immune gene programs that mirror mLC development. A. Schematic showing human LCs isolation workflow. Skin samples were collected from healthy donors aged 0-15 years old and epidermal cell suspensions were obtained. $CD207^+CD1a^+$ cells were FACS purified directly into Trizol; d, days; mo, months; yo, years old. **B.** Percentage of $CD207^+CD1a^+$ cells across newborns, infants and children. Significance was calculated by one way ANOVA with Tukey's multiple comparisons test, **$p<0.01$. **C.** Transcript to transcript clustering with visualization using Graphia, 2447 genes, r=0.75, MCL=1.7 identified 21 clusters with n>10 genes, encoding distinct transcriptional programs in human LCs. Arrows indicate enrichment. **D.** Average TMM normalized gene expression levels in cluster 5 across newborns, infants and children. Significance was calculating using one way ANOVA. **E.** Gene ontology ranked with FDR corrected p-values given for cluster 5. **F.** Heatmap showing normalized expression of indicated genes.