A wave of monocytes is recruited to replenish the long-term Langerhans cell network after immune injury

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A dense population of embryo-derived Langerhans cells (eLCs) is maintained within the sealed epidermis without contribution from circulating cells. When this network is perturbed by transient exposure to ultraviolet light, short-term LCs are temporarily reconstituted from an initial wave of monocytes but thought to be superseded by more permanent repopulation with undefined LC precursors. However, the extent to which this process is relevant to immunopathological processes that damage LC population integrity is not known. Using a model of allogeneic hematopoietic stem cell transplantation, where alloreactive T cells directly target eLCs, we have asked whether and how the original LC network is ultimately restored. We find that donor monocytes, but not dendritic cells, are the precursors of long-term LCs in this context. Destruction of eLCs leads to recruitment of a wave of monocytes that engraft in the epidermis and undergo a sequential pathway of differentiation via transcriptionally distinct EpCAM+ precursors. Monocyte-derived LCs acquire the capacity of self-renewal, and proliferation in the epidermis matched that of steady-state eLCs. However, we identified a bottleneck in the differentiation and survival of epidermal monocytes, which, together with the slow rate of renewal of mature LCs, limits repair of the network.

Furthermore, replenishment of the LC network leads to constitutive entry of cells into the epidermal compartment. Thus, immune injury triggers functional adaptation of mechanisms used to maintain tissue-resident macrophages at other sites, but this process is highly inefficient in the skin.

In this non-inflammatory context, the replenishment of the empty niche is characterized by the slow kinetics by which emerging LCs expand to fill the epidermis, reflecting the quiescent nature of mature LCs within the skin environment (3). By contrast, severe perturbation of eLCs in the context of inflammation leads to recruitment of BM-derived cells into the epidermis and repopulation of the empty niche (10, 16). The cellular mechanisms by which this occurs have largely been defined using models in which transient acute exposure of murine skin to ultraviolet (UV) irradiation leads to cell death within the epidermis and eLC replacement. Under these conditions, Gr-1+ monocytes are recruited to the epidermis (17, 18). Whereas some studies suggest that these monocytes can differentiate into long-term LCs (17, 19), others have proposed a two-wave model of eLC replacement, in which monocytes can persist for up to 3 weeks as “short-term” LC-like cells but are superseded by undefined precursors, which become long-lived replacement LCs (18). Central to this model is the observation that LCs require the transcription factor Id2 for their development and persistence as long-lived quiescent cells (18). However, it remains controversial whether Id2 is required for repopulation of the LC niche after UV irradiation (20). The nature of the long-term LC precursors remains a key question in the field, with a number of studies suggesting the potential for DCs or their precursors to seed epidermal LCs in the adult (21–25).

The resident macrophage population in most tissues is maintained by recruitment of Ly6C+ classical monocytes from the blood (26). Ly6C+ monocytes are short-lived, nondividing cells once they have left the BM; however, they show remarkable plasticity upon differentiation, and recent studies have defined a dominant role of the local tissue niche in shaping differentiation of recruited cells (27, 28). One active area of investigation is whether monocyte-derived macrophages can transcriptionally and functionally replace the resident...
We have previously shown that alloreactive T cells infiltrate the epidermis in this model, wherein interaction with eLCs leads to enhanced cytotoxic function and survival (35). Activated T cells target recipient keratinocytes and eLCs (16), leading to graft-versus-host disease (GVHD) (35). We demonstrate that T cell–mediated destruction of eLCs leads to the recruitment of monocytes that seed long-term monocyte-derived LCs (mLCs), which are indistinguishable from the embryo-derived cells that have been replaced. We provide evidence for a surge of monocyte recruitment to the epidermis, but differentiation of these cells into mLCs is a rate-limiting step, resulting in inefficient rebuilding of the mature LC network. In addition, the epidermal compartment is not reseeded after entry of T cells and remains open to circulating cells. Thus, immune injury triggers an adaptive process that converges with mechanisms to regenerate other tissue-resident macrophages, but this is highly inefficient in the skin.

**RESULTS**

Immune injury leads the gradual replenishment of the epidermis with LC-like cells

We and others have previously shown that transfer of CD8+ male minor histocompatibility antigen–reactive Matahari (Mh) T cells with BM transplantation (BMT) (35, 36) leads to infiltration of pathogenic T cells into target organs and the development of sublethal GVHD (35). Intravital imaging by our laboratory demonstrated direct interactions between Mh T cells and host LCs within the epidermis (35) and the onset of GVHD pathology 2 weeks after transplant (35). Once in the epidermis, CD8+ T cells kill host LCs via Fas ligand–dependent cytotoxicity (16). We determined the kinetics of LC turnover after immune injury and eLC destruction in our transplant model (fig. S1). eLCs are radiosensitive (16) and persisted in control mice that received BMT alone. A small population of donor BM-derived cells was evident in some mice 10 weeks after transplant, but we observed substantial variability between mice [Fig. 1, A (top panel) and B]. Loss of eLCs was complemented by the emergence of donor BM-derived CD11b+Langerin+ LC-like cells in the epidermis 2 weeks after transplant, which increased sharply in frequency and number between 2 and 3 weeks [Fig. 1, A (bottom panel) and B], concomitant with the peak of Mh T cell numbers in the epidermis (Fig. 1C). The development of full donor LC chimerism was gradual and evident by week 10 after transplant. At this time point, repopulating donor CD11b+Langerin+ LC-like cells were phenotypically distinct from resident macrophages that were originally seeded from intrinsically distinct precursors at birth. In the steady state, genetic ablation of tissue-resident cells results in the differentiation of Ly6C+ monocytes into Kupffer cells (29) and alveolar macrophages (30) in the liver and lung, respectively, that show few differences from the cells they replace. By contrast, genetic ablation of microglia leads to repopulation by monocyte-derived cells that appear to fulfill the functional roles of their resident counterparts, but remain morphologically distinct, and continue to express monocyte-related genes (31, 32). Whether reemerging skin LCs, driven by immune injury and inflammation in the skin, also retain evidence of their cellular origin and to what extent repopulating cells can become a long-term quiescent LC network remain pertinent questions, not least because monocyte-derived cells in other inflamed tissues remain transcriptionally and functionally distinct from resident cells (33, 34).

We have exploited a murine model of hematopoietic stem cell transplant to define the cellular mechanisms that control rebuilding of the LC network after immune-mediated pathology. We have previously shown that alloreactive T cells infiltrate the epidermis in this model, wherein interaction with eLCs leads to enhanced cytotoxic function and survival (35). Activated T cells target recipient keratinocytes and eLCs (16), leading to graft-versus-host disease (GVHD) (35). We demonstrate that T cell–mediated destruction of eLCs leads to the recruitment of monocytes that seed long-term monocyte-derived LCs (mLCs), which are indistinguishable from the embryo-derived cells that have been replaced. We provide evidence for a surge of monocyte recruitment to the epidermis, but differentiation of these cells into mLCs is a rate-limiting step, resulting in inefficient rebuilding of the mature LC network. In addition, the epidermal compartment is not reseeded after entry of T cells and remains open to circulating cells. Thus, immune injury triggers an adaptive process that converges with mechanisms to regenerate other tissue-resident macrophages, but this is highly inefficient in the skin.

**Fig. 1.** Immune injury leads the gradual replenishment of the epidermis with LC-like cells. (A) Male recipients received female BM alone (BMT) or with CD4 and CD8 (Mh) T cells. Chimerism was measured within the mature CD11b+Langerin+ LC population at different time points. Representative flow plots show the relative frequency of host (CD45.2)–derived and donor (CD45.1)–derived cells. (B) Graph showing the frequency ± SD of donor LCs in mice receiving BMT with (circles) or without (triangles) T cells. Significance was determined with a two-way ANOVA, ***P < 0.001. Data are pooled from two independent experiments for each time point (n = 5 to 10). (C) Graph shows the number ± SD of Vj8.3+ Mh T cells in the epidermis over time per 0.1 g of total ear tissue (n = 7 to 8). (D) Top: Representative histogram overlays show the expression of LC-associated proteins on donor-derived LCs (from mice that received BMT + T cells) or host eLCs (BMT alone) 10 weeks after transplant. Bottom: Summary data showing the median fluorescent intensity (MFI) for each sample. Each symbol is one mouse. Data are pooled from two independent experiments (n = 8) and are representative of more than three different experiments.
indistinguishable from host eLCs in BMT controls according to markers defined in previous studies (Fig. 1D) (18, 38).

**DC lineage cells do not become long-term replacement LCs**

The DC-like nature of LCs has led to the suggestion that DC lineage cells may contribute to adult LCs and/or seed repopulating cells after damage (21, 23). This hypothesis has recently been supported by work demonstrating that circulating human CD1c+ DCs have the potential to become monocyte-derived DCs (moDCs) (18, 19, 24, 41). Therefore, we investigated the possibility that DC lineage cells may contribute to LC repopulation after immune injury by transplanting irradiated male recipients with a 1:1 mixture of BM from Vav-Cre,Rosa26LSLTomato (VavTom) and Clec9a-Cre,Rosa26LSLEYFP (Clec9aYFP) reporter lines. The Vav-Cre transgene is expressed by all hematopoietic cells (39) and provides an internal control for the development of BM-derived LCs, whereas Clec9a-dependent yellow fluorescent protein (YFP) marked cells are restricted to the DC lineage (Fig. 2A) (40). Ten weeks later, an average of 37 ± 14.1% (SEM) of splenic CD11c+ MHCII+ cells were derived from VavTom BM in the BMT + T group. Of the Clec9aYFP-derived cells, 36 ± 2.6% (SEM) expressed YFP. By contrast, although there was a clear contribution of donor VavTom cells to repopulating LCs after BMT with T cells, we did not detect any YFP+ LCs (Fig. 2, B and C).

**LC repopulation is preceded by influx of donor CD11b+ cells**

Mature LCs are identified by the unique concomitant expression of high levels of the cell adhesion molecule EpCAM (CD326) and the C-type lectin receptor Langerin (CD207), which are simultaneously up-regulated upon differentiation of eLCs (9, 19, 41). By contrast, short-term LCs do not up-regulate EpCAM after UV irradiation (18). We observed the T cell–dependent accumulation of CD11bint to high cells within the epidermis (Fig. 3, A and B) that peaked 3 weeks after transplant and before the shift to donor LC chimerism, as shown in Fig. 1A. Phenotypic analysis of these cells demonstrated the presence of three populations subdivided by expression of EpCAM and Langerin (Fig. 3C): Donor CD11bhigh cells contained two subpopulations that either were negative/low for both markers, suggesting recent arrival in the epidermis, or solely expressed EpCAM; by comparison, CD11bint cells were EpCAMhighLangerinhight and therefore resembled mature LCs. For this paper, we will refer to these populations as “CD11bhigh,” “EpCAM+,” and “donor LCs,” respectively. We observed a peak in the frequency and number of EpCAM+ cells between 2 and 3 weeks after transplant, preceding the gradual accumulation of donor LCs over time (Fig. 3D). These data strongly suggested a developmental trajectory, whereby a wave of epidermal CD11bhigh cells repopulated the mature LC network. We reasoned that acquisition of LC-defining proteins would be consistent with the developmental transition of these subpopulations. We observed the gradual loss of CD11b and up-regulation of CD24 and DEC205 with differentiation of donor LCs (Fig. 3E).

**EpCAM+ monocyte-derived cells are distinct from donor LCs**

Our phenotyping data suggested that incoming CD11b+ cells differentiated into a unique EpCAM+ intermediate before becoming donor LCs, but it was possible that EpCAM+ cells were already immature LCs. To distinguish between these possibilities, we compared the transcriptional profile of EpCAM+ cells with donor LCs or other CD11b+ populations in the skin and blood (Fig. 4A and see fig. S2 for sorting strategy). Hierarchical clustering demonstrated that eLCs and donor LCs were interchangeable, but that EpCAM+ cells clustered as a distinct population, and were more closely aligned to blood and dermal monocytes than mature LCs (Fig. 4B). Analysis of the genes that contributed to differences along the PC1 axis demonstrated that EpCAM+ cells were distinguished by the down-regulation, but not loss, of expression of genes associated with monocyte development and function (e.g., Prr5, Ccl9, Fcgr3 and Fcgr4, Trem3, Trl7) (fig. S3B), including Trem4, which is expressed by Ly6C+ monocytes with the potential to become monocyte-derived DCs (moDCs) (42). However, EpCAM+ cells had not up-regulated genes associated with changes to cell structure, adhesion, and signaling that defined donor LCs (e.g., Emp2, Kremen2, Nedd4, and Ptk7). Given the distinct clustering of monocytes/EpCAM+ cells and eLCs/donor LCs,
we directly tested the contribution of monocytes to emerging LCs by transferring mixed congenic Ccr2+/+ and syngeneic Ccr2−/− BM with T cells (Fig. 4C). CCR2 is required for both egress of monocytes from the BM and entry into tissues (43). We found that only Ccr2−/− cells contributed to CD11bhigh, EpCAM*, and donor LC subpopulations (Fig. 4, D and E), suggesting a monocytic origin for these cells.

Ly6C+ monocytes mature into MHCII+ activated monocytes (or mDCs) in the dermis (44). Therefore, we directly compared the outcomes of monocyte differentiation within different skin compartments using panels of genes associated with monocyte maturation and function described by Schridde and colleagues (45) (Fig. 4F). This analysis demonstrated the clear divergence between Ly6C+ monocytes differentiating within the dermis or epidermis. EpCAM* cells displayed a unique gene signature associated with tissue homeostasis and modulation of the epidermal niche by matrix metalloproteinases [mmp12, mmp13, mmp14, and mmp25, but not mmp2 and mmp9, which are associated with egress of mature LCs out of the epidermis (46)], phagocytosis and uptake of apoptotic cells (cda9, axd, cd36, itgb5, and itgav), and activation of complement (c1qa, c1qb, and c1qc). However, EpCAM* also retained shared patterns of gene expression with mDCs that suggested recent extravasation from the blood (gpr35, itgai, and ccr2) and the potential to activate T cells (b2m, tapbp, cd80, cd40, and fcgrt), which was lacking from LCs.

Proliferation of monocytes and LCs in situ combine to replenish the LC network

Our data implied that monocytes were sufficient to replenish the LC network, but Ly6C+ monocytes are short-lived non-cycling cells, whereas proliferation of eLCs at birth and in adults determines the LC density within the epidermis (9, 12). Therefore, we considered the relative importance of recruitment versus proliferation of epidermal CD11b+ cells for the rebuilding of the LC network. We constructed mathematical models to quantify the flows between CD11bhigh to EpCAM* populations and the donor mLC pool using time courses of Ki67 expression (Fig. 5, A and B). All epidermal populations showed evidence of active or recent cell division after transplant, which decreased to homeostatic rates equivalent to eLCs by 10 weeks (9.8 ± 1.49% (SEM)) (9). Models of the flow from CD11bhigh cells to EpCAM* were fitted simultaneously to the time courses of total cell numbers and Ki67 expression in the two populations. The kinetics of EpCAM* cell numbers closely tracked that of the CD11bhigh population, suggesting that EpCAM* cells were short lived and/or rapidly underwent forward differentiation (Table 1). We first explored whether the flow of CD11bhigh and EpCAM* cells into donor LCs was consistent with a linear developmental pathway, as predicted by our experimental data. This was compared with the alternative scenario in which EpCAM* cells...
were a “dead-end” population and wherein monocytes differentiated directly into LCs, which we named the branched pathway (fig. S4A). The fitted predictions of the two models were visually similar (Fig. 5C). Nevertheless, we found about 10-fold greater statistical support for the linear pathway, as measured by weights calculated using the Akaike information criterion (47). However, maturation of EpCAM+ cells was highly inefficient, with only 4% becoming donor mLCs (Fig. 5D and Table 1). Gene set enrichment analysis of EpCAM+ cells compared with donor LCs suggests that most EpCAM+ cells underwent apoptosis within the epidermis (fig. S4B).

Production of donor LCs was initially dominated by recruitment from CD11bhigh and EpCAM+ cells, but proliferative self-renewal replaced recruitment by week 10 when the mature LC pool had reached steady state (Fig. 5E and Table 1). At this point, donor LCs resided in the skin for about 10 weeks on average and divided once every 78 days (Table 1). These estimates were consistent with other observations of the rates of turnover and division of eLCs in the steady state (10) and matched eLC doubling time within the unperturbed eLC network (12, 48). Fits were based on the assumption that the CD11b+ and EpCAM+ cells died or differentiated at a constant per cell rate. However, we had to include density-dependent proliferation of donor LCs to explain the waning of Ki67+ cells over time. Thus, division occurred more frequently at low cell densities (Fig. 5F and Table 1).

Expression of Ki67 by CD11b+ cells suggested that accumulation of LC precursors required local proliferation of undifferentiated cells. This hypothesis was supported by the overrepresentation of cell cycle pathways in EpCAM+ cells (fig. S4C). This gene signature was in contrast to dermal monocytes, which up-regulated pathways associated with innate receptors and T cell activation (fig. S4D). To pinpoint active cell division within epidermal cells in vivo, we injected 5-ethynyl-2′-deoxyuridine (EdU) into mice 3 weeks after BMT with T cells and analyzed the frequency of EdU+ cells 4 hours later. Within this window, we detected incorporation of EdU by cycling CD11b+ EpCAM+ cells in the epidermis and less so in donor LCs (Fig. 5, G and H).
Fig. 5. Proliferation of monocytes and LCs in situ combine to replenish the LC network. Mice received BMT with T cells. Total numbers and Ki67 expression of epidermal cells were analyzed at different time points and described with mathematical models. (A) Representative histograms show gating of Ki67+ cells in the EpCAM+ and donor LC populations 3 weeks after BMT with T cells. (B) Graphs show the frequency ± SD of EdU+ cells in the different groups. Circles are Ki67−, squares, EpCAM+, triangles, donor LCs. Data are pooled from two independent experiments. Mo., blood monocytes; dLC, donor LCs. (C) The model of a linear development pathway had the strongest statistical support. Numbers indicate parameter estimates from the model. (D) Graph showing the relative contribution of proliferation in donor LCs to influx (with 95% confidence interval) over time. (E) Graph showing the estimated mean interdivision time (with 95% confidence interval) of donor LCs at different times after BMT with T cells. Parameter estimates are displayed in full in Table 1. (G) Mice received EdU 3 weeks after BMT with T cells. Four hours later, the skin and blood were harvested, and cells were analyzed for incorporation of EdU. Representative contour plots show overlaid gated CD11b−Langerinlo (yellow) or CD11b−Langerinhi (magenta) populations in the epidermis, or Ly6C+CD115+ monocytes in the blood. FMO is the fluorescent minus one stain without the EdU detection reagent. (H) Summary graph showing the mean ± SD frequency of EdU+ cells in the different groups. Circles are individual mice (n = 6). Data are pooled from two independent experiments. Mo., blood monocytes; dLC, donor LCs.
in EpCAM+ cells showed evidence of early responsiveness to the dominant epidermal cytokine tumor growth factor–β (TGFβ) (fig. S5E) and matured into LC-like cells upon culture with TGFβ ex vivo (fig. S5F). Therefore, these data suggested that LDTFs and responsiveness to TGFβ are switched on in EpCAM+ cells within the epidermal environment before differentiation into mature LCs. We next used an in vitro screen to identify the growth factors, in addition to TGFβ, that controlled Id2 expression and LC identity after differentiation from BM cells. We selected bone morphogenetic protein 7 (BMP7), colony-stimulating factor–1 (CSF-1), and interleukin-34 (IL-34) based on expression of their cognate receptors (BMPR1a and CSF1R, respectively) by EpCAM+ cells in vivo (fig. S5G) and their requirement for LC repopulation after UV irradiation (41, 49, 50) and tested the impact of each factor on LC development in BM cultures (fig. S6) (20, 41, 51). IL-34, but not CSF-1 or BMP7, enhanced LC numbers (Fig. 6C), and this was associated with the specific up-regulation of Id2 by LCs in IL-34 cultures (Fig. 6D). BM-LCs were derived from cells that proliferated before maturation in these cultures (Fig. 6E), mirroring cycling of CD11b+ cells in the epidermis. Cell division was not affected by addition of IL-34, which instead increased the survival of LCs (Fig. 6F).

**Immune damage and loss of eLCs open the epidermal compartment**

Having considered LC repopulation at the cellular level and demonstrated that monocytes differentiate into bona fide LCs within the epidermis, we now considered the impact of immune pathology on the LC network and integrity of the epidermal compartment.

The kinetics of LC repopulation demonstrated that monocytes failed to completely replenish the LC network in most mice 10 weeks after transplant, suggesting a prolonged reduction in LC density. However, this decrease in LC numbers was also evident in mice that had received BMT alone, demonstrating that the slow rate of division by mature LCs ultimately dictated the speed at which the network was repaired, rather than LC origin (Fig. 7A). Confocal analysis of epidermal sheets revealed notable heterogeneity in the density of mLCs in different fields of view (Fig. 7B), but mLCs tended to be smaller than eLCs from BMT controls (Fig. 7C). Although mLCs and dendritic epidermal T cells (DETCs) were closely colocated within the epidermis, we found no difference in the frequency of mLCs 3 weeks after BMT into Tcrbd−/− recipients that lacked all endogenous T cells (fig. S7A).
Given the smaller volume of mLCs, we considered whether they were less integrated within the epidermis than eLCs and therefore migrated more readily to draining lymph nodes (LNs). To test this, we topically applied fluorescein isothiocyanate (FITC) to the ear of mice that had received BMT 10 weeks earlier, with or without T cells. LN cells were divided into migratory and resident populations on the basis of expression of CD11c and major histocompatibility complex II (MHC II) as published by others (40, 52, 53), and we determined the number of EpCAM⁺Langerin⁺LCs in the migratory gate (fig. S7B). At this time point, all migrating LCs were derived from donor BM (fig. S7B), and therefore, we compared their frequency with that of host eLCs in mice that had received BMT without T cells. The frequency of FITC⁺ cells within LN LCs was equivalent irrespective of LC origin (Fig. 7D), demonstrating that, although donor mLCs acquired the capacity to migrate to LNs, this was not to a greater extent than eLCs. It was evident from flow cytometry plots that mLCs picked up less FITC than eLCs from untransplanted controls. Comparison of the median intensity of FITC⁺ cells demonstrated that this was indeed the case, but a similar effect was also observed by eLCs in BMT controls (fig. 7E and F). Thus, mLCs migrate to LNs, but irradiation and BMT may also affect the acquisition of topical antigen by LCs.

We next considered how changes to the density of the LC network would affect the entry of cells into the epidermis in the absence of T cell–mediated injury. Thus, transplanted mice received a second round of total body irradiation with BMT (without T cells), and we tracked the origin of epidermal LCs 8 weeks later (Fig. 7G). Host eLCs were replaced by donor mLCs after BMT with T cells (Tx1 only), but not BMT alone (Tx2 only), as expected. However, the epidermis of mice that had received both transplants (Tx1 and Tx2) contained three populations of coexisting LCs. These were identified as radiosensitive host eLCs and donor mLCs (Fig. 7H, Langerin.GFP⁺CD45.1⁺ and Langerin. GFP⁺CD45.1⁻, respectively) and new BM-derived LCs (Langerin.GFP⁺CD45.1⁻).
consensus on the role of adult monocytes in maintaining and replenishing tissue macrophages in other organs, the nature of the precursor that repopulates LCs in the skin has remained elusive and controversial. Previously, studies that addressed the nature of LC replacement in the epidermis have depended on the destruction of resident eLCs by acute (15 to 30 min) exposure of ear skin to UV irradiation. However, these studies have produced conflicting data on whether Gr-1+ monocytes persist in the epidermis of UV-treated mice to become LCs (17, 18). This work has led to the concept that an alternative “long-term” LC precursor was required to replenish the LC network.
after UV-induced damage, and studies using human cells have since invoked a role for blood DCs as LC precursors (24, 25). By contrast, in our stem cell transplant model, allogeneic T cells are recruited to the epidermis over a period of weeks, resulting in prolonged immune pathology and inflammation (35). Under these conditions, monocytes can become long-term LCs, and DC precursors do not contribute to the emerging LC network. It is conceivable that transient exposure to UV irradiation compared with the prolonged inflammation caused by alloreactive T cells may trigger different mechanisms of LC repopulation in the skin. However, the adoptive transfer experiments previously used to define monocytes as LC precursors after UV irradiation are challenging and require injection of large numbers of cells into Ccr2 \textsuperscript{-/-} Ccr6 \textsuperscript{-/-} mice to reduce competition from endogenous cells (17, 18). We suggest that the physiological recruitment of monocytes from the BM in our model has revealed their role in the repair of the damaged eLC network after immune pathology.

Statistical analysis of our mathematical models favors the linear differentiation of CD11b \textsuperscript{high} monocytes into EpCAM\textsuperscript{+} precursors of mLCs. However, this conclusion is specific to the models that we considered; the transient nature of the EpCAM\textsuperscript{+} population and similar kinetics of CD11b \textsuperscript{high} and EpCAM\textsuperscript{+} cells mean that it remains possible that the branched pathway may also occur with EpCAM\textsuperscript{+} cells as a developmental endpoint. We think that this is unlikely based on our evidence that EpCAM\textsuperscript{+} cells express an intermediate phenotype and LDFTs compared with CD11b \textsuperscript{high} monocytes and mLCs, and culture of EpCAM\textsuperscript{+} cells induces up-regulation of Langerin. Despite the surge of monocytes entering the epidermis, and proliferation of CD11b \textsuperscript{high} cells in situ, we have identified a bottleneck, with only 4% of CD11b \textsuperscript{high}/EpCAM\textsuperscript{+} cells becoming mLCs. The reasons for this inefficiency remain to be determined. One possibility is that monocyte differentiation is an intrinsically inefficient process and may also occur for the generation of tissue-resident macrophages at other sites. Alternatively, monocyte-derived EpCAM\textsuperscript{+} Langerin\textsuperscript{-neg} cells can be identified within the oral mucosa, wherein inflammation blocks the transition to mucosal LCs (13, 54). Therefore, it is possible that continued inflammation in our model blocks differentiation of EpCAM\textsuperscript{+} cells.

Entry of CD11b \textsuperscript{high} cells into the epidermis triggers a burst of proliferation that has also been reported when phagocytic monocytes enter the skin after UV irradiation (17). Subsequent to this, mLCs divide at a rate that matches that of steady-state eLCs (12, 48). This concordance both validates our modeling approach and reveals a developmental convergence of quiescent mLCs with their embryonic counterparts. It has been suggested that the density of tissue macrophage populations is controlled by mechanisms of quorum sensing in response to CSF-1 (55), but this has not been directly demonstrated experimentally. We found that density-dependent proliferation was required to fit mathematical models to our data, supporting the concept of quorum sensing within the epidermal niche. CSF-1 and IL-34 compete for the CSF-1 receptor (56). Given the dominance of IL-34, rather than CSF-1 in the epidermal environment (57) and our in vitro data showing that IL-34 increases expression of Id2 and promotes BM-LC survival, it is possible that IL-34 fulfills this function in the skin.

We have shown that the epidermal compartment is not resealed after immune-mediated destruction of eLCs, and BM-derived cells continue to be recruited into the epidermis. Small numbers of donor CD11b \textsuperscript{+} cells constitutively enter the epidermis of transplanted mice in the steady state, as observed 10 weeks after transplant (see Fig. 3D). One possibility is that the inefficient repair of the mLC network reduces competition between established and incoming cells (55).

In this sense, the LC network in the epidermis more closely resembles that of the oral mucosa (1, 13) after exposure to immune injury.

The activation of auto- or alloreactive T cells and destruction of tissue-resident cells can have profound impacts on the balance of immune cells within tissue compartments with long-term consequences for the control of infection and cancer at these sites. The skin is highly sensitive to such changes and is a major target organ for T cells in patients suffering from GVHD after hematopoietic stem cell transplantation and those receiving immune checkpoint blockade (58). However, we know little about the impact of immune injury in these patients on the regulation of immunity in the skin.

Here, we provide insights into the cellular mechanisms by which the LC compartment is replenished and maintained after damage, and demonstrate that immune injury triggers an adaptive process that converges closely with mechanisms to regenerate other tissue-resident myeloid cells.

**MATERIALS AND METHODS**

**Study design**

The aims of this study were to define the nature of long-term repopulating LCs and to identify the cellular processes by which the network was repaired after immune injury. We used an in vivo model of LC replacement and measured changes to cell populations using flow cytometry and confocal microscopy. This was combined with mathematical modeling of the flow between epidermal populations and RNA sequencing (RNA-seq) to determine changes in gene expression. 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. There was no randomization or blinding. Recipients were co-housed, where possible.

**Mice**

C57BL/6 (B6) mice were purchased from Charles River and bred in-house by the University College London (UCL) Comparative Biology Unit. Langerin.DTR.EGFP mice (59) were originally provided by A. Kissenpfennig and B. Malissen. C57BL/6 T cell receptor (TCR)–transgenic anti-HY MataHari mice (60) were provided by J. Chai (Imperial College London, London, UK). Ccr2\textsuperscript{-/-} mice were a gift from F. Geissmann (61). All strains were bred in-house at UCL. TCRBd\textsuperscript{-/-} mice were generated by crossing Tcrd\textsuperscript{d-/-} (62) and Tcrb\textsuperscript{b-/-} (63) lines and bred in-house at the Imperial College London, Hammersmith Campus. CD45.1\textsuperscript{+} OT-I TCR transgenic mice were bred in-house. 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).

**BM transplants**

Recipient male CD45.2 B6 mice were lethally irradiated (11 Gy of total body irradiation, split into two fractions over a period of 48 hours) and reconstituted 4 hours after the second dose with 5 \times 10\textsuperscript{8} female CD45.1 C57BL/6 T cell–depleted BM cells and 2 \times 10\textsuperscript{6} CD4 T cells, with 1 \times 10\textsuperscript{6} CD8 Thy1.1\textsuperscript{+} Mh T cells administered by intravenous injection through the tail vein. CD4 and CD8 T cells were isolated by magnetic selection of CD4 or CD8 BM cells or splenocytes using
the Miltenyi MACS system [QuadroMACS separator, LS columns, CD4 (L3T4) MicroBeads, CD8a (Ly-2) MicroBeads; Miltenyi Biotec] according to the manufacturer’s instructions, either to be discarded from T cell–depleted BM or selected for injection of splenic T cells. In some experiments, Langerin.DTR.EGFP recipients or BM donors were used to track Langerin* host or donor LCs, respectively.

For secondary irradiation experiments, BMT recipients initially received BM from Langerin-DTR.GFP donors with CD4 and CD8 Mh T cells. Eight weeks later, mice received 11 Gy of split-dose total-body irradiation and CD45.1+ C57BL/6 female T cell–depleted BM alone.

Mixed chimera experiments
BM from Vav-Cre.Rosa26SLTomato and Clec9a-Cre.Rosa26SL EYFP donors was a gift from C. Reis e Sousa (Francis Crick Institute). Irradiated B6 male mice received a 50:50 mix of BM from the reporter mice with T cells. For CCR2 competitive chimeras, irradiated CD45.2+ Langerin-DTR.EGFP recipients received a 50:50 mix of CD45.2+ Ccr2+/+ and CD45.1+ Ccr2−/− BM with T cells.

FITC painting
The dorsal side of ear pinna was coated with 25 μl of a 1:1 mixture of 0.5% FITC (Sigma-Aldrich, UK) in acetone and dibutyl phthalate (Sigma-Aldrich, UK). Seventy-two hours later, the draining auricular and cervical LNs were harvested and analyzed.

Generation of tissue single-cell suspensions
Epidermal single-cell suspensions were generated as described (35). Split dorsal and ventral sides of the ear pinna were floated on Dis-pase II (2.5 mg/ml; Roche) in Hank’s balanced salt solution (HBSS) and 2% fetal bovine serum (FBS) for 15 hours at 4°C, followed by mechanical dissociation of the epidermal layer in phosphate-buffered saline (PBS)/1 mM EDTA/1% FBS by mincing with scissors or using the GentleMACS tissue dissociator (Miltenyi Biotec). Epidermal cells were passed sequentially through 70- and 40-μm cell strainers to remove clumps of cells. Numbers of cells were calculated by addition of counting beads (Invitrogen) before staining of samples for flow cytometry and normalized to 0.1 g weight for both complete ears before processing. After separation from the epidermis, the dermis was minced into small pieces and digested with collagenase IV (250 U/ml; Worthington) and deoxyribonuclease (DNase) I (800 U/ml; AppliChem, USA) at 37°C for 1 hour. Dermal single-cell suspensions were then generated using a GentleMACS tissue dissociator (Miltenyi Biotec).

LNs were teased apart with needles and digested in HBSS/collagenase IV (4000 U/ml; Worthington) for 40 min at 37°C. Digestion was quenched with 10 mM EDTA, and the cells were passed through a 40-μm cell strainer, washed, and resuspended in PBS/1 mM EDTA/1% FBS. For blood cells, erythrocytes were removed by hypotonic lysis with distilled water, and cells were then resuspended in PBS/1 mM EDTA/1% FBS.

BM cultures
BM was flushed from femurs and tibias of donors, and red blood cells were lysed in 1 ml of ammonium chloride (ACK buffer; Lonza, UK) for 1 min at room temperature. Cells were washed and resuspended at 2.5 × 10⁶/ml in R5 medium (RPMI 1640; Lonza, Switzerland), 5% heat-inactivated FBS (Life Technologies, USA), 1% l-glutamine (2 mM; Life Technologies, USA), 1% penicillin-streptomycin (100 U/ml; Life Technologies, USA), and 50 μM β-mercaptopethanol (Sigma-Aldrich, UK). Cell suspension (1 ml) was plated per well in tissue culture–treated 24-well plates and supplemented with recombinant granulocyte-macrophage CSF (GM-CSF) (20 ng/ml) and TGFβ (5 ng/ml; PeproTech, USA). Cells were cultured at 37°C. The medium was partially replaced on day 2 of culture and completely replaced on day 3, and the cells were harvested on day 6. Some cultures were supplemented with combinations of IL-34 (8 μg/ml; Generon, UK), BMP7 (100 μg/ml; R&D Systems, USA), and CSF-1 (10 μg/ml; BioLegend, USA) for the duration of the culture.

Flow cytometry
Cells were distributed in 96-well conical bottom plates and incubated in 2.4G2 hybridoma supernatant (containing cCD16/32) for at least 10 min at 4°C to block Fc receptors. For cell surface labeling, cells were incubated with fluorochrome-conjugated antibodies diluted in 100 μl of fluorescence-activated cell sorting (FACS) buffer (PBS/1 mM EDTA/1% FBS) at 4°C for at least 20 min in the dark: EpCAM (G8.8, ebioscience, USA), CD11b (M1/70, ebioscience, USA), CD45.1 (A20, BD Biosciences, Germany), CD45.2 (104, ebioscience, USA), MHC II I-A-I-E (M5/114.15.2, ebioscience, USA), CD11c (HL3, BD Pharingen, USA), CD24 (M1/69, BD Biosciences or BioLegend), CD205 (475301, R&D systems), CD64 (x54-5.7.1, Biolegend), B220 (RA3-6B2, BD Biosciences), and Vβ8.3 TCR (1B3.3, BD Biosciences, Germany). To exclude lineage cells, we used a cocktail of CD3 (145-2C11, BD Biosciences, Germany), CD19 (1D3BD, Biosciences, Germany), NK1.1 (PK136, BioLegend), and Ly6G (1A8, BD Biosciences, Germany) all conjugated to allophycocyanin (APC)–Cy7.

Intracellular staining with Langerin (CD207) antibodies (eBioL31, ebioscience, USA) was performed after cell surface immunolabeling. Samples were washed with FACS buffer, fixed in 100 μl of fixation solution (BD Cytofix/Cytoperm solution, BD Biosciences, UK) for 15 min at 4°C, washed twice with permeabilization buffer (BD Perm/ Wash, BD Biosciences, UK), and incubated with 100 μl of Langerin diluted in permeabilization solution at 4°C for 30 min in the dark.

Live cells were identified by exclusion of propidium iodide (unfixed cells) (Life Technologies, USA) or a fixable viability dye (ebioscience, USA or Life Technologies, USA). Multicolor flow cytometry data were acquired with BD LSRRFortessa and BD LSR II cell analyzers equipped with BD FACSVerse v6.2 software (BD Biosciences, Germany). FACS was performed on a BD FACSaria equipped with BD FACSVerse v5.0.3 software (BD Biosciences, Germany). All samples were maintained at 4°C for the duration of the sort. The cells were sorted into PBS/2% FBS before resuspension in Buffer RL7 (Qiagen, USA) or directly into Buffer RL7 with 1% β-mercaptoethanol (Sigma, UK), disrupted through vortexing at 3200 rpm for 1 min, and immediately stored at −80°C until further processing. Two to three biological replicates were obtained for each sample from at least two independent experiments, each containing a minimum of 4000 cells (pooling where necessary from multiple mice from individual experiments). Flow cytometry data were analyzed with FlowJo X v9 and 10 (LLC, USA), and cells were pregated on singlets (FSC-A versus FSC-H) and a morphological forward scatter (FSC)/side scatter (SSC) gate.

Measurement of cell proliferation
In vivo
Epidermal single-cell suspensions were immunolabeled with surface antibodies, then fixed and permeabilized using the ebioscience
intranuclear staining kit, before incubation with αKi67–v450 antibodies (SolA15, eBioscience, USA). Gates were set on non-proliferating cells and unstained cells. Alternatively, mice were injected intraperitoneally with 100 μg of EdU (Invitrogen, USA) and euthanized 4 hours later.

**In vitro**
Cells were pulsed with 10 μM EdU on day 2 or 5 of culture, and the medium was replaced 24 hours later. For both in vivo and in vitro studies, cells were labeled for flow cytometry using the Click-IT Plus EdU Flow Cytometry Assay Kit (Invitrogen, USA), according to the manufacturer’s instructions.

**Immunohistochemistry**
Biopsy punches (4 mm) were excised from the dorsal and ventral sides of split ears and incubated in 0.5 M ammonium thiocyanate for 30 min at 37°C to remove the epidermis. Epidermal sheets were collected in Eppendorf tubes, washed twice with PBS, and fixed with cold (stored at −20°C) acetone for 10 min. Sheets were washed twice with PBS and blocked using 0.25% fish gelatin and 10% normal goat serum in PBS for 1 hour at room temperature. Sheets were collected in Eppendorf tubes, washed twice with PBS, and fixed with cold (stored at −20°C) acetone for 10 min. Sheets were washed twice with PBS and blocked using 0.25% fish gelatin and 10% normal goat serum in PBS for 1 hour at room temperature. Sheets were then incubated with primary rat αLangerin (eBioL31, eBioscience, USA), αCD45.2–biotin (A20, eBioscience, USA), and anti-β2-FITC (536, BD Pharmingen, USA) antibodies (diluted 1:100 in blocking normal goat serum in PBS), and then incubated for 1 hour at room temperature. Stained sheets were washed four times with PBS and mounted on slides with ProLong Diamond anti-fade mountant (Invitrogen). Samples were imaged on a Nikon Ti inverted microscope, through a 20× objective for epidermal sheets (Plan Apochromat N.A. 0.75) and a 64× objective for epidermal sheets (Plan Apochromat N.A. 0.75 W.D. 1 mm) or 40× for sorted EpCAM+ cells (Plan Apochromat N.A. 0.95 W.D. 0.21 mm), using a C2 confocal scan head with 488-nm, 561-nm, and 635-nm laser illumination (Nikon Instruments, Tokyo, Japan). Multiple Z-stacks were acquired for each sample. Data were saved as nd2 files using FIJI/ImageJ for quantification.

**Confocal analysis**
Quantification of confocal records was performed using Definiens Developer software. Each channel in a record was processed with Gaussian filter followed by application of multiresolution segmentation. Individual cells were detected on the basis of their relative intensity in Langerin and CD45.2 channels. The cell volume (in cubic micrometers, based on the total number of voxels occupied by a cell) was measured for each cell type.

**Statistics**
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 expressed as means ± SD. Significance was 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 as specified in individual figures. A paired t test was used in Fig. 6 to compare cells cultured under different conditions. Significance was defined as *P < 0.05, **P < 0.01, ***P < 0.001. The false discovery rate was used to compare ontogeny pathways enriched in dermal or epidermal cells.
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N. C., Acton, N.

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M. Teno, K.

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Acknowledgments: We thank the UCL Comparative Biology Unity for their support with animal work and UCL Genomics for performing the RNA-seq. We are grateful to C. Reis e Sousa for providing BM from Clec9a and Vav reporter mice, J. Grove and B. Seddon for constructive discussions during this work, and H. Strauss for support. Funding: This work was supported by a BBSRC project grant (BB/L001608/1) and Royal Free charity funding (award 174418) to C.L.B., a Bloodwise programme continuity grant (17007) to R.C. and C.L.B., an MRC PhD studentship (1450227) to H.C.W., and the NIH (R01 AI093870) to A.J.Y. D.S.U. acknowledges the support of the Wellcome Trust (grant 100156) and the Infection and Immunity Immunophenotyping (3i) Consortium. Author contributions: Conceptualization: C.L.B.; methodology: I.R.F., H.C.W., and P.S.E.S.; formal analysis: S.H., D.S.U., and A.J.Y.; investigation: I.R.F., H.C.W., and P.S.E.S.; data curation: S.H., D.S.U., and A.J.Y.; writing—original draft: C.L.B. and A.J.Y.; writing—reviewing and editing: I.R.F., H.C.W., R.C., A.J.Y., and C.L.B.; funding acquisition and supervision: C.L.B.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: The RNA-seq data for this study have been deposited in the NCBI Gene Expression Omnibus database (GSE130257).

Submitted 30 April 2019
Accepted 15 July 2019
Published 23 August 2019
10.1126/sciimmunol.aax8704

Resurrecting sentinels in the skin

Langerhans cells are resident innate immune cells in the skin that play essential roles in promoting local immune responses and in maintaining skin homeostasis. Langerhans cells arise from fetal progenitors that seed the skin early in development. Here, Ferrer et al. have used a hematopoietic stem cell transplant model to examine the consequences of immune damage and loss of Langerhans cells in adult mouse skin. They report that monocytes from the blood infiltrate the skin and eventually replenish the Langerhans cell network, albeit the process by which monocytes give rise to Langerhans cells is not particularly efficient. Their findings are in agreement with studies reporting the ability of monocytes to replenish tissue-resident macrophages at other sites.