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Langerhans cells arise from monocytes *in vivo*

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Abstract

Langerhans cells (LCs) are the only dendritic cells of the epidermis and constitute the first immunological barrier against pathogens and environmental insults. The factors regulating LC homeostasis remain elusive and the direct circulating LC precursor has not yet been identified *in vivo*. Here we report an absence of LCs in mice deficient in the receptor for colony-stimulating factor 1 (CSF-1) in steady-state conditions. Using bone marrow chimeric mice, we have established that CSF-1 receptor–deficient hematopoietic precursors failed to reconstitute the LC pool in inflamed skin. Furthermore, monocytes with high expression of the monocyte marker Gr-1 (also called Ly-6c/G) were specifically recruited to the inflamed skin, proliferated locally and differentiated into LCs. These results identify Gr-1^{hi} monocytes as the direct precursors for LCs *in vivo* and establish the importance of the CSF-1 receptor in this process.

Langerhans cells (LCs) are members of a family of highly specialized antigen-presenting cells called dendritic cells (DCs). Uniquely present in the epidermis, LCs form a tight cellular network that covers the entire body surface, constituting the first immunological barrier against the external environment. LCs are well equipped to ingest antigens present in the skin and to migrate to the regional lymph node in both steady-state and inflammatory conditions[1,2]. After being activated, LCs increase their expression of major histocompatibility complex (MHC) class II and costimulatory molecules[3] and migrate to T cell areas[2] of draining lymph nodes, where they secrete T cell–attractant chemokines[4] and interact with antigen-specific T cells. Whether migrating LCs serve mainly to carry antigens to blood-derived DCs in the draining lymph nodes or whether they directly prime or tolerize antigen-specific T cells is still debated[5–8].

Given their importance in skin immunity, the mobilization of LCs to draining lymph nodes and the subsequent replacement of migrating cells by newly differentiated LCs must be tightly regulated events. Indeed, LC homeostasis is differentially regulated in quiescent and

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inflamed skin, and LCs are maintained by local radioresistant hematopoietic precursors that self-renew *in situ* in quiescent skin[9]. In contrast, after substantial injury skin-resident LCs are lost and are subsequently replaced by circulating precursors[9]. The identification of circulating LC precursors is critical for understanding the mechanisms that regulate skin immunity against pathogens and environmental insults. However, despite considerable work in this area, the identity of the immediate circulating LC precursor that repopulates LC populations *in vivo* remains elusive.

The presence of fully differentiated and functional DCs and LCs in adult osteopetrotic mice lacking expression of colony-stimulating factor (*Csf1*$^{op/op}$ mice)[10,11], in which tissue macrophages and osteoclasts are much reduced[12], has provided the basis for the hypothesis that LC development is independent of mononuclear phagocytes[13]. It has been argued that in the mouse, circulating CD11c$^+$ cells distinct from monocytes give rise to all DC populations *in vivo*[14], although whether this is true for LCs has not been specifically examined. In addition, human circulating CD11c$^+$CD1a$^+$ cells can be made to differentiate into LCs *in vitro* without additional cytokines, and human CD34$^+$-derived CD1a$^+$ cells give rise to LCs *in vitro*, although circulating CD14$^+$ monocytes and CD34$^+$-derived CD14$^+$ are unable to do so[15,16], supporting the hypothesis that LCs have a common origin with all DCs *in vivo*. On the other hand, human circulating monocytes[17] and dermal CD14$^+$ cells[18] have been shown to differentiate into LC-like cells *in vitro* in the presence of a cytokine ‘cocktail’ containing transforming growth factor-β, a factor critical for LC development[19]. Thus, it is apparent that different precursors can be directed to differentiate into LCs *in vitro*, suggesting that some of the conditions used in those models are not physiologically relevant and that *in vivo* systems are much needed to delineate the mechanisms that regulate natural LC development. The fact that LC populations are repopulated by circulating LC precursors during skin injury provides a model with which to explore LC development directly *in vivo*[9]. Using that model here, we have characterized the direct circulating precursor that gives rise to LCs *in vivo*.

Results

LC development in *Csf1*$^{op/op}$ and CSF-1 receptor–deficient mice

Although LCs are present in adult *Csf1*$^{op/op}$ mice[13], LCs are greatly reduced in newborn *Csf1*$^{op/op}$ mice[20]. The fact that monocytes are increased by 72% in 2-week-old *Csf1*$^{op/op}$ mice[21] compared with their numbers in newborn mice suggests that monocyte recovery with age[21] may affect LC repopulation in adult *Csf1*$^{op/op}$ mice. As in *Csf1*$^{op/op}$ mice, LCs are absent from newborn mice that lack the receptor for CSF-1 (*Csf1r*$^{-/-}$)[20]. To explore if, like *Csf1*$^{op/op}$ mice, *Csf1r*$^{-/-}$ mice recover LCs with age, we first quantified the pool of LCs in 3-week-old *Csf1r*$^{-/-}$ mice. Unexpectedly, we found that in contrast to the LC status of *Csf1*$^{op/op}$ mice[13], LCs were totally absent from *Csf1r*$^{-/-}$ mice in steady-state conditions (Fig. 1a,b), suggesting a critical function for this cytokine receptor in LC homeostasis in the skin.

CSF-1 (also called macrophage CSF) is a hematopoietic factor that regulates the survival, proliferation and differentiation of monocytes[22]. Because the CSF-1 receptor (CSF-1R (CD115)) is a chief marker for monocytes[23–25] and macrophages[23], we hypothesized that monocytes participate in LC development *in vivo*. As LC replacement by circulating precursors occurs only during severe skin injury[9], we revisited how LC homeostasis is

restored in inflamed skin in both *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ mice. We began by re-examining the function of CSF-1 in *Csf1*$^{op/op}$ mice, as results obtained with these mice were initially used to claim ontogenic independence of LCs and monocytes[10,11]. We exposed 3-week-old *Csf1*$^{op/op}$ or *Csf1*$^{op/+}$ mice to ultraviolet light and monitored LC repopulation over time. We found that 2 weeks after exposure to ultraviolet irradiation, the percent of LCs among the total number of epidermal hematopoietic cells was substantially lower in *Csf1*$^{op/op}$ mice than in *Csf1*$^{op/+}$ mice (7% versus 40%), but those recovered to wild-type numbers at 4 weeks after injury (Fig. 1c). The delay in LC repopulation in *Csf1*$^{op/op}$ mice might have been due to a slight reduction of monocytes compared with that of *Csf1*$^{op/+}$ mice, although a more definite explanation was not possible because the number of monocytes and macrophages recovers with age in *Csf1*$^{op/op}$ mice and ultraviolet irradiation–mediated skin damage can be done only in adult mice. Therefore, we turned to more definitive approaches that would permit examination of the function of CSF-1 and monocytes in LC development.

Function of CSF-1R in LC differentiation *in vivo*

Csf1r$^{-/-}$ mice have multiple developmental defects[20], including severe osteopetrosis, that greatly reduce the bone marrow cavity size[20] and that may therefore indirectly affect the pool of hematopoietic progenitors, including LC precursors. In addition, CSF-1R deficiency in FVB/NJ mice (the genetic background on which the gene deletion is most viable) causes death at approximately 3 weeks of age[20,26], making any manipulation of these mice difficult. Therefore, to determine the function of CSF-1R in LC development in inflamed settings in adult mice, we reconstituted lethally irradiated CD45.1$^+$ C57BL/6 mice with CD45.2$^+$ *Csf1r*$^{-/-}$ hematopoietic precursors so that the absence of CSF-1 signaling was restricted to hematopoietic cells and was independent of the secondary effects resulting from osteopetrosis. Moreover, because recruitment of monocytes to inflammatory sites modulates the recruitment of LC precursors through the secretion of proinflammatory molecules[9], the failure of *Csf1r*$^{-/-}$ hematopoietic precursors to give rise to circulating monocytes could also affect LC recruitment without intrinsically affecting LC development. Thus, to test if CSF-1R is intrinsically required on the LC precursor, we reconstituted lethally irradiated CD45.1$^+$ recipient mice with mixed hematopoietic precursors (30% CD45.2$^+$ *Csf1r*$^{-/-}$ and 70% CD45.1$^+$ wild-type, or 30% CD45.2$^+$ *Csf1r*$^{+/-}$ (control littermate) and 70% CD45.1$^+$ wild-type) and monitored the reconstitution of CD45.2$^+$ *Csf1r*$^{-/-}$ or CD45.2$^+$ *Csf1r*$^{+/+}$ cells in the chimeric mice. The degree of blood B cell and granulocyte chimerism (30% CD45.2$^+$ and 70% CD45.1$^+$) reflected the percentage of mixed hematopoietic cells injected (Fig. 2a). *Csf1r*$^{-/-}$ progenitors were as efficient as *Csf1r*$^{+/+}$ cells in giving rise to circulating B cells and granulocytes (Fig. 2a). Circulating monocytes are defined as low forward- and side-scatter cells expressing F4/80, CD11b and CD115 (CSF-1R) molecules[25]. As expected, we did not detect any CD115$^+$ circulating cells in blood from *Csf1r*$^{-/-}$ mice and F4/80$^+$ CD11b$^+$ monocytes were variably reduced over time and were present at 50–60% of wild-type numbers (Fig. 2b). Consistent with the phenotype of *Csf1*$^{op/op}$ mice[13,21] and *Csf1r*$^{-/-}$ mice[20], *Csf1r*$^{-/-}$ peritoneal, kidney and dermal macrophages were much reduced in number in chimeric mice, confirming the idea that CSF-1R is intrinsically required for the differentiation of some tissue macrophages (Fig. 2c,d).

To explore the function of CSF-1 in LC development, we exposed the chimeric mice to ultraviolet light 3 weeks after hematopoietic reconstitution[9] and monitored the repopulation of mutant and wild-type LC populations in skin with ultraviolet irradiation injury. Analysis of chimeric mice 3 weeks after ultraviolet irradiation showed that CD45.2$^+$ *Csf1r*$^{-/-}$ LCs were absent (0.16% of total LCs) from all mice analyzed, whereas there was efficient reconstitution by CD45.2$^+$ LCs derived from *Csf1*$^{+/+}$ control littermates (33% of total LCs, which corresponds to the blood chimerism; Fig. 2e). The deficiency in *Csf1r*$^{-/-}$ LCs in the mixed chimeras persisted for at least 6 weeks after ultraviolet irradiation (Fig. 2f), establishing the idea that *Csf1r*$^{-/-}$ hematopoietic cells are unable to differentiate into LCs in inflamed skin. In contrast, there was similar reconstitution of mutant and wild-type splenic DCs (Fig. 2g), suggesting that the requirement for CSF-1R is restricted to LCs.

Labeling monocyte subsets *in vivo*

Because CSF-1R is important for monocyte and macrophage differentiation *in vivo*, we explored the capacity of monocytes to repopulate LC populations in inflamed skin. Two subsets of blood monocytes with different migrating properties have been described in mouse blood. These include the classical Gr-1^{hi}CCR2$^+$ monocytes and the more mature Gr-1^{lo}CCR2$^-$ monocyte subset[24,25,27]. In humans, these subsets correspond respectively to the classical CD14$^+$CD16$^-$ and the more infrequent CD14^{lo}CD16$^+$ monocyte subsets[27–29]. To avoid manipulating blood monocytes *ex vivo*, we monitored the fate of monocytes directly *in vivo* by labeling them with fluorescent latex beads[30]. We used congenic 'CD45.1$^+$ bone marrow→CD45.2$^+$' chimeric mice to distinguish circulating precursors (CD45.1$^+$) from skin-resident (CD45.2$^+$) LCs[9] (Supplementary Fig. 1 online). Intravenous injection of fluorescent latex beads labeled mainly Gr-1^{lo}CD115$^+$ monocytes (Fig. 3a), whereas bead injection 1 d after the elimination of circulating monocytes using clodronate-loaded liposomes[25] labeled mainly Gr-1^{hi}CD115$^+$ monocytes (Fig. 3b). Thus, all 'bead-positive' monocytes were either CD45.1$^+$Gr-1^{hi} monocytes (Gr-1^{hi} group) or CD45.1$^+$Gr-1^{lo} monocytes (Gr-1^{lo} group). At 4 h after injection of beads, we exposed chimeric mice to ultraviolet light (Supplementary Fig. 1 online).

Gr-1^{hi} monocytes infiltrate and proliferate in inflamed skin

We detected CD45.1$^+$ bead-positive cells only in inflamed skin from mice of the Gr-1^{hi} group (Fig. 3c,d). CD45.1$^+$Gr-1^{hi} circulating monocytes began to infiltrate the skin 18 h after injury (data not shown) but failed to do so after 4 d (Fig. 3e). At day 4 after ultraviolet treatment, bead-positive cells in the skin represented 6–10% of total infiltrating cells (Fig. 3c,d), which is similar to the labeling efficiency of blood monocytes (Fig. 3b). Gr-1^{hi} monocytes formed many clusters in the skin between day 4 and day 7 after ultraviolet injury (Fig. 4a, top) and began to detach between day 7 and day 10 (Fig. 4a, bottom). To explore whether these clusters corresponded to proliferating cells or only to aggregated cells, we administered 5-bromodeoxyuridine (BrdU) to mice 4 d after exposure to ultraviolet light. Because at that time point circulating monocytes failed to migrate to the skin (Fig. 3e), BrdU uptake by skin monocytes should have reflected only local proliferation. BrdU labeling showed that monocytes proliferated actively between day 4 and day 7 (Fig. 4b,c). We confirmed local proliferation of recruited bead-positive monocytes by *in situ*

immunostaining with the cell cycle protein Ki67 (Fig. 4d), establishing that the bead-positive clusters were formed by proliferating monocytes.

Differentiation of Gr-1^{hi} monocytes *in situ*

Beginning at day 7 after ultraviolet irradiation, skin-infiltrating monocytes decreased their proliferation rate (data not shown) and began to downregulate Gr-1 expression and upregulate expression of MHC class II molecules (Fig. 5a–c). Shortly thereafter, round monocytes detached from the clusters (Fig. 5d, left, and Fig. 4a) and began to express the LC-specific marker langerin (Fig. 5d). Along with those phenotypic changes, round monocytes developed dendrites at approximately day 14 (Fig. 5d), and by 3 weeks after ultraviolet treatment, all CD45.1$^+$ cells in the epidermis acquired an LC morphology and expressed langerin (data not shown). In addition to the development of some Gr-1^{hi} monocytes into LCs, a large fraction of these monocytes differentiated into F4/80^{hi}CD68^{hi} dermal macrophages. The differentiation of bead-positive monocytes into dermal macrophages in the dermis was faster and occurred within 6–7 d (Fig. 5e,f).

Adoptive transfer of Gr-1^{hi} monocytes

To confirm the LC differentiation potential of Gr-1^{hi} monocytes *in vivo*, we adoptively transferred purified CD45.1$^+$Gr-1^{hi} monocytes into CD45.2$^+$ recipient mice deficient in CCR2 and CCR6. Because CCR2 and CCR6 are required for LC repopulation *in vivo* in inflamed skin[9,31], the absence of CCR2 and CCR6 expression in recipient mice should decrease the recruitment of endogenous LC precursors to inflamed skin. This would give a competitive advantage to the adoptively transferred LC precursors, thereby facilitating their detection.

We injected Gr-1^{hi} monocytes intravenously 1 d after exposing recipient mice to ultraviolet irradiation. We detected a substantial population of CD45.1$^+$ Gr-1$^+$ cells in the recipient epidermis 4 d later, and a fraction of these cells had begun to downregulate Gr-1 and upregulate MHC class II molecules (Fig. 6a). Consistent with the results reported above, CD45.1$^+$Gr-1^{hi} monocytes recruited to inflamed skin differentiated into epidermal Gr-1$^-$I-A^{b+}langerin$^+$ LCs within 3 weeks (Fig. 6b–e). The number of CD45.1$^+$-derived LCs was proportional to the number of transferred CD45.1$^+$ monocytes (Fig. 6c), suggesting that Gr-1^{hi} monocytes are the precursors of LCs *in vivo*. Analysis of epidermal sheets 7 weeks after monocyte transfer and exposure to ultraviolet irradiation (Fig. 6e) showed accumulation of ‘islets’ of monocyte-derived LCs in defined area of the epidermis, consistent with the monocyte clustering noted above (Fig. 4a). In contrast, we detected no LCs in the control group injected with MHC class II–positive CD11c$^+$ bone marrow cells, further indicating that the common CD11c$^+$ precursor described in the blood[14] and the bone marrow[32], although able to give rise to all DC populations, probably does not participate in LC development *in vivo*.

Discussion

Many possible LC precursors have been described in the literature. One strong candidate has been the circulating CD11c$^+$ blood DC. Indeed, human CD11c$^+$ circulating blood DCs can

give rise to LCs without additional cytokines *in vitro*[16], whereas CD11c$^+$ circulating cells and bone marrow CD11c$^+$ cells in mice can give rise to all lymphoid organ DCs and plasmacytoid DCs *in vivo* and *in vitro*[14,32]. Although monocytes can be 'directed' to become LCs in certain conditions[17,33], the weight of the evidence *in vivo* and *in vitro* suggests that LCs do not derive from monocytes. Indeed, nonmonocytic precursors can give rise to LCs *in vitro*[15,16] and mice with fewer monocytes still contain LCs in the steady state[13].

In contrast to those results, here we have demonstrated that Gr-1^{hi} monocytes are the direct LC precursors *in vivo* and that CSF-1R is critical for this process. First, bone marrow progenitors that lacked CSF-1R failed to give rise to tissue macrophages and LCs in inflamed skin. Second, when we tagged circulating monocytes *in vivo* with fluorescent latex beads and monitored their fate in inflamed skin (in which LCs had been ablated) over time, Gr-1^{hi} monocytes homed to inflamed skin and proliferated *in situ* for 3–4 d before differentiating into LCs within 2–3 weeks. Third, adoptively transferred Gr-1^{hi} monocytes migrated to inflamed skin within a few days after LCs were ablated by ultraviolet irradiation and gave rise to new LCs within 3 weeks. Fourth, 3-week-old mice deficient for CSF-1R lacked LCs in quiescent skin, suggesting that expression of CSF-1R is required for LC development *in vivo* in both steady-state and inflammatory conditions.

We have also shown that the requirement for CSF-1R is specific to the LC precursor, as mice transplanted with a mixture of *Csf1r*$^{-/-}$ and *Csf1r*$^{+/+}$ hematopoietic precursors lacked CSF-1R-deficient LCs but not CSF-1R-deficient splenic DCs. Whether other DC populations in lymphoid and nonlymphoid organs, as well as plasmacytoid DCs, require CSF-1R for their development *in vivo* will require further study. In addition to the lack of CSF-1R-deficient LCs in the skin, chimeric mice also lacked CSF-1R-deficient dermal, peritoneal and kidney macrophages but not liver macrophages, suggesting that LCs share similar developmental pathways with some but not all tissue macrophages. These results established that CSF-1R is required for LC differentiation in inflamed skin.

Our finding that *Csf1r*$^{-/-}$ mice lacked LCs suggests that CSF-1R may also be required for LC development in the steady state; the fact that constitutive expression of CSF-1 occurs in quiescent skin further supports that hypothesis[34]. However, as *Csf1r*$^{-/-}$ mice have developmental defects that may indirectly affect LC differentiation *in vivo*[20], an experiment to establish the function of CSF-1R in LC development in the steady state will be the demonstration that *Csf1r*$^{-/-}$ LCs fail to develop normally in quiescent skin in wild-type mice reconstituted with *Csf1r*$^{-/-}$ bone marrow. Unfortunately, that is difficult to test experimentally because replacement of skin-resident LCs by circulating precursors occurs only in injured skin, a treatment that radically disrupts the steady state[9].

The absence of LC development in *Csf1r*$^{-/-}$ mice reported here contrasts with published reports of LC development in *Csf1*$^{op/op}$ mice[10,11]. The difference between *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ mice may be related to the difference in strain background (*Csf1*$^{op/op}$ is outbred; *Csf1r*$^{-/-}$ is inbred FVB/NJ), possible 'leakiness' of the *Csf1*^{op} mutation or stimulation of CSF-1R signaling directly or indirectly via a second ligand[20,22,35]. Indeed, CSF-1 is widely expressed in the mouse and although the two main forms of CSF-1 are absent from *Csf1*$^{op/op}$ mice[35], it is possible that expression of alternative CSF-1 splice variant depends on the cell

type and/or tissue[36]. CSF-1 is constitutively expressed in the skin[34] and it is likely that skin CSF-1 (ref. 34) is critical for LC development *in vivo*. Thus, it might be useful to analyze what isoform of CSF-1 is expressed in the skin and whether skin CSF-1 persists in *Csf1*$^{op/op}$ mice. Finally, another possible explanation for the strain difference is that other cytokines signal through CSF-1R and drive LC differentiation. In support of that possibility, the ligand for the tyrosine kinase receptor Flt3 is critical for LC differentiation *in vivo*[37]. Because Flt3 has some homology with CSF-1R[38], it might be useful to determine whether the Flt3 ligand can drive LC differentiation in *Csf1*$^{op/op}$ mice.

CSF-1 is critical to the survival, proliferation, differentiation and/or maturation of the mononuclear phagocyte lineage[22]. Injection of CSF-1 into animals increases the number of monocytes in peripheral blood, whereas no changes are noted in other myeloid cells[39]. The ability to respond to CSF-1 alone is an early event in monocyte commitment, and the specificity of CSF-1 for monocytic cells seems to be determined in part by increased expression of its receptor during or after commitment to the monocytic lineage[40]. However, the precise function of CSF-1 in monocyte commitment remains controversial. One hypothesis is that CSF-1 drives the differentiation of monocytes into tissue macrophages[41], although other studies have led to the hypothesis that CSF-1 provides a survival signal to the differentiating monocytes and that surviving cells use an intrinsic developmental program to become mature macrophages[42–44]. Our data showing that *Csf1r*$^{-/-}$ monocytes were only slightly fewer in number than wild-type monocytes, along with the capacity of *Csf1r*$^{-/-}$ monocytes to migrate to inflamed skin (data not shown), suggest that CSF-1 is not critical to monocyte production or recruitment to the skin but instead controls monocyte proliferation, survival or differentiation into LCs in the skin.

Intravenous injection of fluorescent beads into ‘CD45.1 bone marrow→CD45.2’ chimeric mice is a useful tool for monitoring the sequence of cellular events that lead a monocyte to become an LC in the skin. We found that once in the epidermis, Gr-1^{hi} monocytes formed many proliferating clusters, downregulated Gr-1 and upregulated MHC class II molecules. After a few days, the ‘maturing’ monocytes stopped proliferating, detached from the clusters and progressively acquired a dendritic morphology, including expression of the LC-specific marker langerin. Full differentiation into MHC class II–positive langerin-positive LCs occurred within 2 to 3 weeks. Notably, LC differentiation in newborn mice follows a similar pattern. Several studies have identified round CD45^{+} MHC class II–low F4/80^{+} myeloid cells in the epidermis of newborn mice[45,46]. Like the changes in monocytes that develop into LCs in inflamed adult skin, round epidermal F4/80^{+} hematopoietic cells progressively acquire a dendritic morphology and upregulate MHC class II expression, followed a few days later by expression of langerin[47]. Complete LC differentiation in the epidermis in newborn mice, as in adult mice treated with ultraviolet irradiation, occurs within approximately 3 weeks[45,47]. Those data, together with the fact that 3-week-old *Csf1r*$^{-/-}$ mice lack skin LCs, suggest that the round F4/80^{+} hematopoietic cells seen repopulating the ultraviolet-treated skin may correspond to an embryonic monocyte that progressively differentiates in a CSF-1-dependent way into an LC precursor able to self-renew in quiescent skin. We have also demonstrated that monocyte-derived LCs persisted more than 7 weeks after transfer, suggesting that LC differentiation was not transient. Whether

monocyte-derived LCs, like LCs in quiescent skin, are able to self-renew locally will require further study.

Gr-1^{hi} monocytes express the inflammatory chemokine receptor CCR2 and lose both Gr-1 and CCR2 after maturation[25,27]. The unique capacity of Gr-1^{hi} monocytes to home to inflamed skin and to differentiate into LCs is consistent with our finding that LC repopulation in inflamed skin was dependent on CCR2 (refs. 9,31). However, our finding that the classical Gr-1^{hi}CCR2^{+} monocyte subset served as the precursor for LCs was unexpected, as it represents evidence that these monocytes give rise to a distinct population of DCs *in vivo*. Past studies examining the development of DCs from monocytes *in vitro* and *in vivo* without the addition of exogenous cytokines (which may not represent physiological conditions) have indicated only the function of nonclassical monocyte subsets as DC precursors[24,29]. In addition, skin-draining lymph node DCs that do not take up residence in skin but instead transiently emigrate through the dermis do not derive from Gr-1^{hi} monocytes and are independent of CCR2 (ref. 24). Thus, it is evident that different skin-derived DC populations can, in common, arise from monocytes, although from different types of monocyte subsets. Although it is likely that most Gr-1^{hi} monocytes or their human equivalents differentiate into macrophages, a fraction of them, perhaps only in response to factors present in the epidermal environment, begin to proliferate and develop into LCs. Those results are in agreement with a report showing differentiation of human CCR2^{+}CD14^{+} monocytes into LCs in an *in vitro* model of reconstructed human skin[33], consistent with the importance of the epidermal environment in inducing monocyte differentiation into LCs.

Our results demonstrating the critical involvement of CSF-1 in LC development *in vivo* may have potential important therapeutic applications. Indeed, LC histiocytosis is characterized by the infiltration of large number of LCs organs and tissues throughout the body[48]. LC histiocytosis remains an ‘enigmatic’ disease with features of cancer and inflammatory processes, and treatment of patients with this disease has included both chemotherapy and immunosuppressors[48]. Our results demonstrating involvement of CSF-1 in LC development, together with the published finding that CSF-1 is increased in patients with LC histiocytosis[49], identify CSF-1 as a new potential therapeutic target for the treatment of these patients.

In summary, our results have identified Gr-1^{hi} monocytes as the direct circulating LC precursors *in vivo* and have established the critical involvement of CSF-1R in the migration and development of skin LCs. We also monitored the sequence of cellular events that lead a Gr-1^{hi} monocyte to become an LC in the skin. Gr-1^{hi} monocytes homed to inflamed epidermis and dermis, where they actively proliferated before beginning to differentiate into either dermal macrophages or epidermal LCs and repopulated the entire LC pool within 2–3 weeks. The identification of these mechanistic features of LC development and biology should contribute to ongoing efforts to engineer immune responses in vaccine design and tumor immunotherapy[50] and to a better understanding of the immune response to skin pathogens.

Methods

Animals

Female C57BL/6 (CD45.2$^+$) and congenic C57BL/6 CD45.1$^+$ mice 5–8 weeks of age were purchased from the Jackson Laboratory. CCR2- and CCR6-deficient C57BL/6 mice (*Ccr2*$^{-/-}$*Ccr6*$^{-/-}$ mice) were generated by crossing of *Ccr2*$^{-/-}$ mice[51] with *Ccr6*$^{-/-}$ mice[52] and were maintained in our animal facility (Mount Sinai School of Medicine, New York, New York). *Csf1r*$^{-/-}$ mice on the FVB/NJ background were generated as described[26]. *Csf1*$^{op/+}$ mice were purchased from the Jackson Laboratory. *Csf1*$^{op/op}$ mice were generated in our animal facility (Mount Sinai School of Medicine, New York, New York) by breeding of *Csf1*$^{op/+}$ mice. All mouse protocols were approved by the Institutional Committee on Animal Welfare of the Mount Sinai Medical School (New York, New York).

Flow cytometry

Multiparameter analyses were made on an LSR II (Becton Dickinson) and were analyzed with FlowJo software (Tree Star). Monoclonal antibodies to mouse I-A^b, CD11b, CD11c, CD45, CD45.1, CD45.2, CD115, F480 and Gr-1 (Ly6C/G), the corresponding isotype controls and the secondary reagents (allophycocyanin, peridinine chlorophyll protein and phycoerythrin–indotricarbocyanine–conjugated streptavidin) were purchased from PharMingen. Monoclonal antibody to langerin was purchased from Santa Cruz Biotechnology.

Transplantation of bone marrow and fetal liver cells and ultraviolet irradiation

Eight-week-old recipient CD45.1$^+$ C57BL/6 mice were lethally irradiated with 1,200 rads delivered in two doses of 600 rads each, 3 h apart, and were injected intravenously with a mixture of 30% *Csf1r*$^{-/-}$ CD45.2$^+$ fetal liver cells and 70% syngeneic wild type CD45.1$^+$ bone marrow or a mixture of 30% *Csf1r*$^{+/+}$ CD45.2$^+$ control littermate fetal liver cells and 70% syngeneic wild-type CD45.1$^+$ bone marrow. We used fetal liver as a source of *Csf1r*$^{-/-}$ hematopoietic precursors cells because C57BL/6 *Csf1r*$^{-/-}$ mice die *in utero*[26]. For bead labeling experiments, CD45.2$^+$ recipient C57BL/6 mice were lethally irradiated and were injected intravenously with 2×10^6 bone marrow cells isolated from congenic CD45.1$^+$ C57BL/6 mice. Hematopoietic engraftment was analyzed by measurement of blood chimerism 2 weeks after transplantation. Exposure to ultraviolet light was done between 3 and 4 weeks after transplant as described[9].

Labeling of blood monocytes

Fluorescein isothiocyanate–or phycoerythrin-conjugated plain microspheres 0.5 μm in diameter (Polysciences) were diluted 1:25 in PBS, and 250 μl of the solution was injected into the lateral tail vein for labeling of Gr-1^{lo} monocytes. For labeling of Gr-1^{hi} monocytes, 250 μl of liposomes containing clodronate were injected intravenously, followed by intravenous injection of 250 μl fluorescent microspheres 16–18 h later. Clodronate was a gift from Roche and was incorporated into liposomes as described[53].

Isolation of spleen DCs and peritoneal, kidney and dermis macrophages

Spleen, dermis and kidney cell suspensions were isolated as described[9] and were analyzed by flow cytometry for the presence of $CD45.2^+$ and $CD45.1^+$ $CD11c^+CD11b^+$ DCs or $CD11b^+F4/80^+$ macrophages. Peritoneal lavage cells were isolated by flushing of the peritoneal cavity with 4–6 ml of PBS supplemented with 0.5% FCS and were analyzed for the presence of $CD45.2^+$ and $CD45.1^+$ $CD11b^+F4/80^+$ macrophages.

LC isolation and analyses

The presence of LCs was assessed by flow cytometry of epidermal cell suspensions by gating on propidium iodide–negative $CD45^+$ $I\text{-}A^{b+}$ or by immunofluorescence analysis of epidermal sheets as described[9].

BrdU labeling and analysis

'CD45.1→CD45.2' bone marrow chimeric mice were injected intraperitoneally with 1 mg BrdU (Sigma) resuspended in PBS to ensure immediate availability and were given BrdU (0.4 mg/ml) in sterile drinking water changed daily for variable BrdU pulses. Epidermal cell suspensions were prepared at different times after BrdU administration and were analyzed for BrdU incorporation with the BrdU Flow Kit (Pharmingen) according to the manufacturer's instructions.

Monocyte transfer experiments

Bone marrow cell suspensions isolated from $CD45.1^+$ C57BL/6 mice were depleted of $I\text{-}A^{b+}$ and $CD11c^+$ cells by immunomagnetic cell depletion using MACS technology (Miltenyi Biotech) and were then positively selected for bone marrow monocytes by MACS immunomagnetic cell selection with monoclonal antibodies to Gr-1 (Ly6C/G) and CD115. The positive fraction contained less than 0.2% $CD11c^+$ MHC class II–positive contaminating cells. C57BL/6 $CD45.2^+$ $Ccr2^{-/-}Ccr6^{-/-}$ double-knockout recipient mice were exposed to ultraviolet light 24 h before intravenous injection with 9×10^6 $CD45.1^+Gr\text{-}1^+CD115^+$ bone marrow monocytes or 9×10^6 $CD45.1^+$I-A $CD11c^+$ bone marrow cells. The recruitment of $CD45.1^+$ monocytes to the skin of recipient $CD45.2^+$ mice was analyzed 4 d after transfer; the presence of $CD45.1^+$ LCs in the epidermis was analyzed 3 and 6 weeks later.

Histology and immunohistochemistry

Epidermal sheets were stained with mouse antibody to mouse CD45.1 (anti–mouse CD45.1; eBioscience) and goat anti-langerin (Santa Cruz Biotechnology) and detection was done with indocarbocyanine-conjugated monoclonal donkey anti-mouse and indodicarbocyanine-conjugated monoclonal donkey anti-goat (Jackson Immunoresearch). For staining of frozen sections, skin was 'snap frozen' in optimum cutting temperature compound and sections 8 mm in thickness were prepared, were fixed in 100% acetone for 5 min and were rinsed in PBS. Primary antibodies included biotinylated mouse anti-CD45.1 (eBioscience), rat anti-CD68 (Serotech), rat anti-$I\text{-}A^b$ (Pharmingen) and rat anti-Ki-67 (Becton Dickinson Biosciences). Carbocyanine-conjugated streptavidin and indocarbocyanine-conjugated monoclonal donkey anti-rat (Jackson Immunoresearch) were used for detection. Slides were

mounted with Vectashield containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories). Images were acquired with a Leica DMRA2 fluorescence microscope (Wetzlar) and a digital Hamamatsu CCD camera and were analyzed with Openlab software (Improvision).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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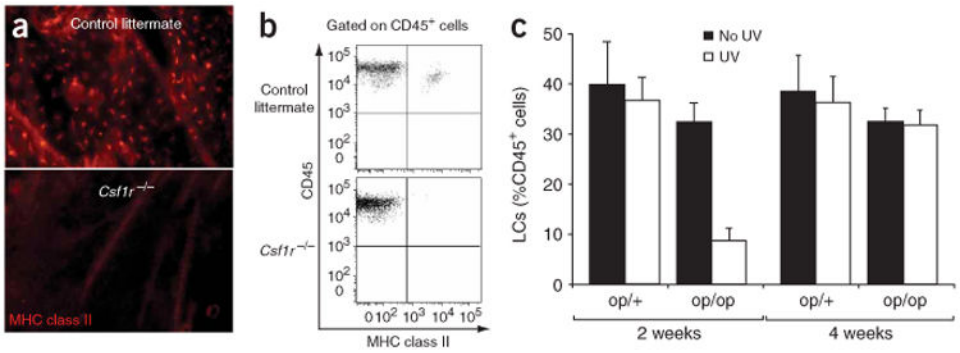

Figure 1.

LC development in CSF-1- and CSF-1R-deficient mice. (**a,b**) Immunofluorescence of epidermal sheets (**a**) and flow cytometry of cell suspensions (**b**) from 3-week-old *Csf1r*$^{-/-}$ or *Csf1r*$^{+/+}$ control littermate FVB/NJ mice and stained with monoclonal 'pan-anti–MHC class II'. Original magnification, ×10 (**a**). Dot plots (**b**) show propidium iodide–negative CD45^{+} epidermal cells that are positive for MHC class II. Results are representative of two separate experiments. (**c**) Flow cytometry of LCs repopulating the skin of 3-week-old *Csf1*$^{op/op}$ mice (op/op) or *Csf1*$^{op/+}$ control littermates (op/+) with (UV) or without (No UV) exposure to ultraviolet light for 30 min, assessed 2 or 4 weeks after exposure. Data represent the mean percent of LCs (CD45^{+}I-A^{b+}) among the total propidium iodide–negative CD45^{+} population in three individual mice.

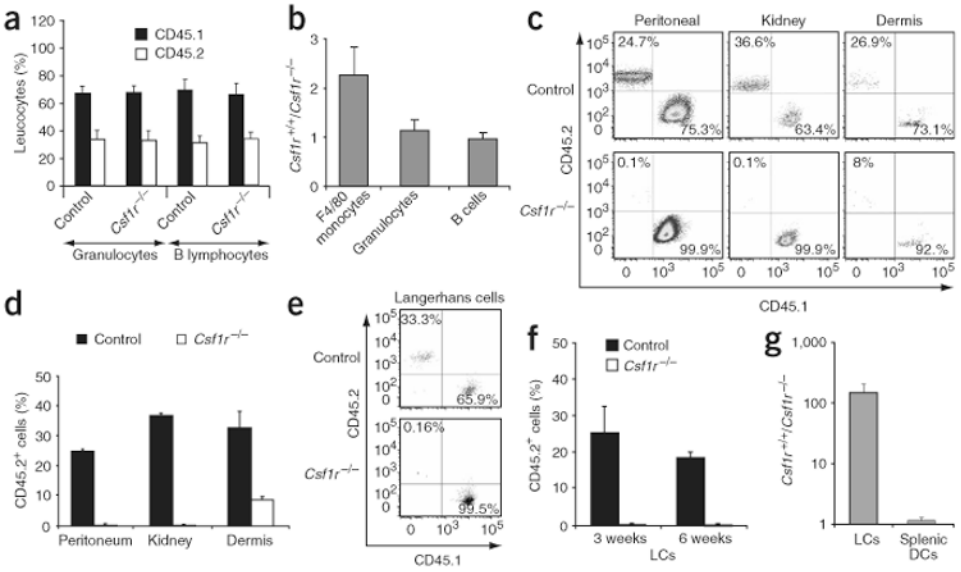

Figure 2.

Function of CSF-1R in LC differentiation *in vivo*. Lethally irradiated CD45.1$^+$ recipient mice were reconstituted with mixed chimera bone marrow and were exposed 3 weeks later to ultraviolet light. (**a**) Mean percent peripheral blood Gr-1 (Ly-6G)$^+$ granulocytes and B220$^+$ B lymphocytes in control and *Csf1r*$^{-/-}$ mice 3 weeks after transplantation in six individual mice. (**b**) Wild-type versus *Csf1r*$^{-/-}$ leukocyte ratio, calculated as percent CD45.2$^+$ *Csf1r*$^{+/+}$ leukocytes divided by percent CD45.2$^+$ *Csf1r*$^{-/-}$ leukocytes in six individual mice. (**c**) Propidium iodide–negative peritoneal, kidney and dermal F4/80$^+$ CD11b$^+$ macrophages that are CD45.1$^+$ or CD45.2$^+$ in control and *Csf1r*$^{-/-}$ chimeric mice, 3 weeks after exposure to ultraviolet irradiation. (**d**) Mean percent CD45.2$^+$ cells among total CD45$^+$ (CD45.1$^+$ and CD45.2$^+$) cells in the corresponding macrophage populations in control and *Csf1r*$^{-/-}$ mice. (**e**) Propidium iodide–negative–gated LCs that are CD45.1$^+$ or CD45.2$^+$ in control and *Csf1r*$^{-/-}$ mice, 3 weeks after exposure to ultraviolet irradiation. (**f**) Mean percent CD45.2$^+$ LCs in control and *Csf1r*$^{-/-}$ mice 3 and 6 weeks after exposure to ultraviolet irradiation. (**g**) Reduction in cells, calculated as percent CD45.2$^+$ *Csf1r*$^{+/+}$ LCs or spleen DCs divided by percent *Csf1r*$^{-/-}$ LCs or spleen DCs at 3 weeks after exposure to ultraviolet irradiation. Results in **c**–**g** are representative of three or more separate experiments. Numbers in quadrants (**c**,**e**) indicate percentage of propidium iodide–negative total CD45$^+$ gated cells that are CD45.1$^+$ (bottom right) or CD45.2$^+$ (top left).

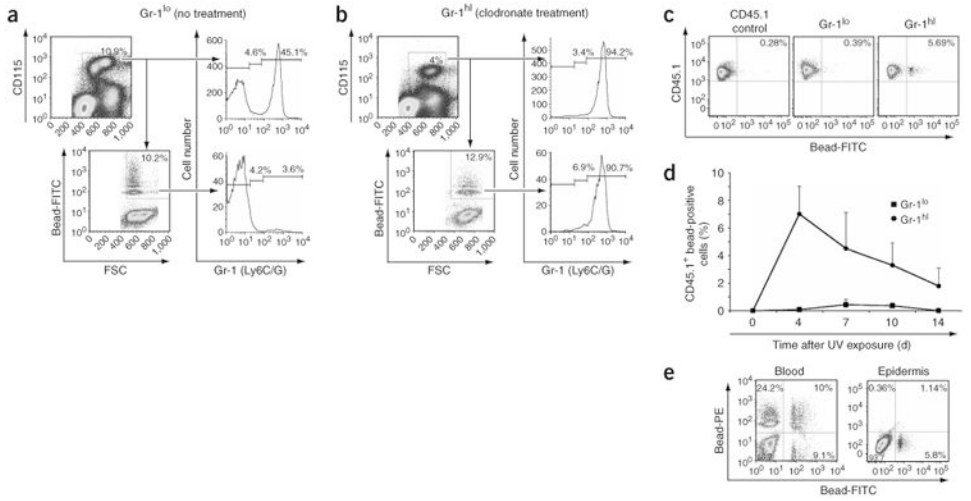

Figure 3.

Gr-1^{hi} monocytes infiltrate and proliferate *in situ* in ultraviolet irradiation–inflamed skin. Mice were treated as described in Supplementary Figure 1 online. (**a**,**b**) At 1 d after bead injection, all monocytes positive for fluorescein isothiocyanate–labeled beads are mainly Gr-1^{lo} in the absence of clodronate treatment (**a**) but are Gr-1^{hi} when beads are injected after mice are treated with clodronate-loaded liposomes (**b**). Data are representative of at least five separate experiments. (**c**) At 4 d after exposure to ultraviolet irradiation, bead-positive Gr-1^{hi} but not bead-positive Gr-1^{lo} monocytes are present in the skin. (**d**) Percent propidium iodide–negative CD45.1^{+} bead-positive cells that infiltrate the epidermis in Gr-1^{lo} and Gr-1^{hi} groups after exposure to ultraviolet irradiation. (**e**) Gr-1^{hi} monocytes labeled with fluorescein isothiocyanate–positive beads before exposure to ultraviolet light are recruited to the skin, whereas Gr-1^{hi} monocytes labeled with phycoerythrin-positive beads 4 d after exposure to ultraviolet light fail to enter the skin, suggesting that Gr-1^{hi} monocytes do not enter the skin 4 d after skin injury. Numbers in quadrants, beside outlined areas and above bracketed lines indicate percentage of cells in each area. FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; UV, ultraviolet. Data in **c**–**e** are representative of three separate experiments.

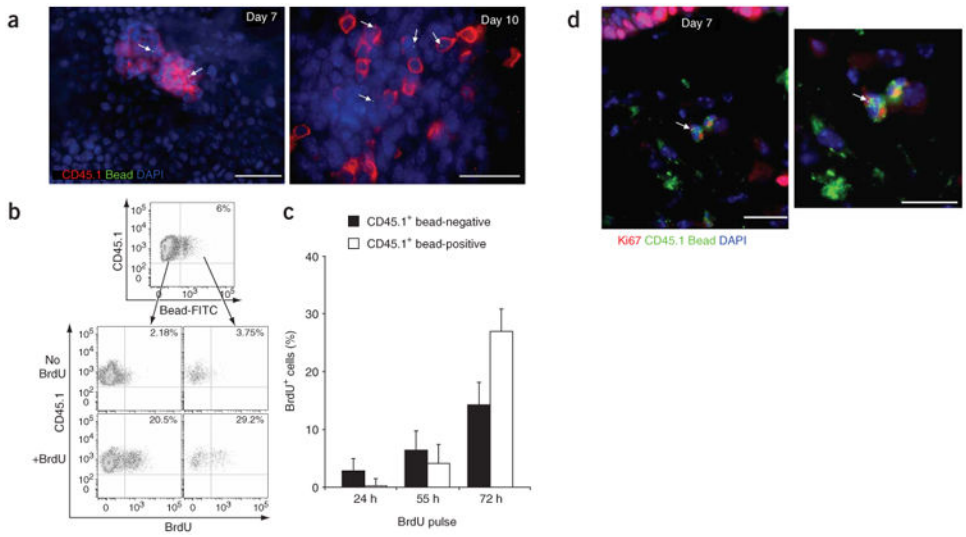

Figure 4.

Bead-positive Gr-1^{hi} monocytes differentiate into dermal macrophages and epidermal LCs in inflamed skin. (**a**) Epidermal sheets stained for CD45.1 (red) and DAPI (blue) at 7 d and 10 d after ultraviolet irradiation. Fluorescein isothiocyanate–positive beads are bright green dots (arrows). Scale bars, 10 μm. Images are representative of at least four separate experiments. (**b**) Percent BrdU$^+$ cells in recruited CD45.1$^+$ bead-negative or CD45.1$^+$ bead-positive populations in the epidermis after a 72-hour pulse of BrdU. Data are representative of three separate experiments. (**c**) Percent CD45.1$^+$ bead-negative BrdU$^+$ cells and CD45.1$^+$ bead-positive BrdU$^+$ cells after BrdU pulses of varying times. Data are one representative experiment (n = 3). (**d**) Frozen sections of skin stained for Ki67 (red), CD45.1 (green) and DAPI (blue), 7 d after ultraviolet treatment. Right, higher magnification. Fluorescein isothiocyanate–positive beads are bright green dots (arrows). Scale bars, 10 μm. Images are representative of two separate experiments.

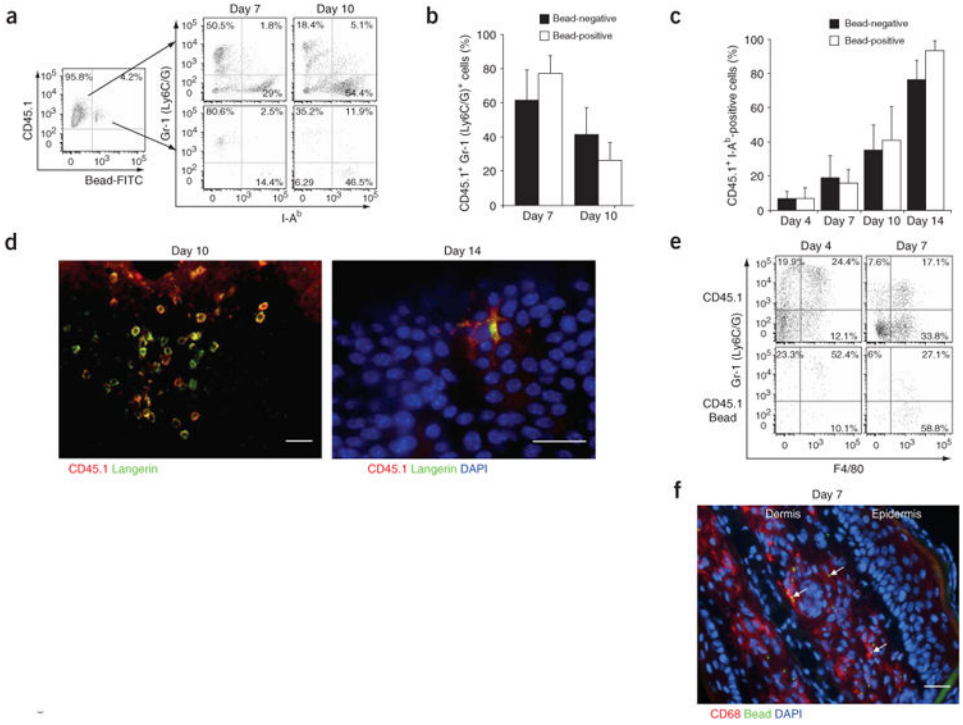

Figure 5.

Skin-infiltrating Gr-1^{hi} monocytes differentiate into LCs. (**a**) Gr-1 and I-A^{b} expression patterns in bead-negative CD45.1^{+} and bead-positive CD45.1^{+} populations recruited into inflamed epidermis at days 7 and 10 after exposure to ultraviolet irradiation. Data are representative of three separate experiments. (**b**,**c**) Mean percent bead-negative or bead-positive CD45.1^{+} cells that are Gr-1^{+} (**b**) or I-A^{b+} (**c**) at various times after exposure to ultraviolet irradiation (*n* = 3). (**d**) Epidermal sheets stained for CD45.1 (red), langerin (green) and DAPI (blue) at various times after exposure to ultraviolet irradiation. Scale bars, 10 μm. Images are representative of two individual mice. (**e**) Gr-1 and F4/80 expression patterns in bead-negative and bead-positive propidium iodide–negative CD45.1^{+} populations recruited into inflamed dermis at days 4 and 7 after exposure to ultraviolet irradiation (*n* = 2). (**f**) Frozen sections of skin stained for CD68 (red) and DAPI (blue), 7 d after ultraviolet treatment. Beads are bright green dots (arrows). Scale bar, 10 μm. Images are representative of two individual mice. Numbers in quadrants (**a**,**e**) indicate percentage of cells positive or negative for the marker.

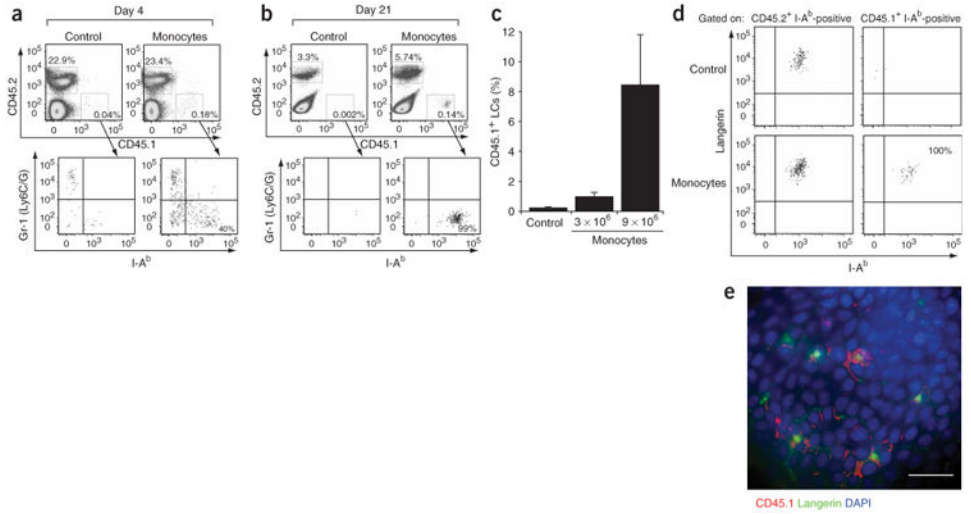

Figure 6.

Adoptive transfer of Gr-1^{hi} monocytes *in vivo*. (**a**,**b**) Top, percent propidium iodide–negative–gated epidermal cells that are CD45.1$^+$ or CD45.2$^+$ in mice injected with monocytes or control (I-A^{b+} CD11c$^+$) populations. Bottom, Gr-1 and I-A^b expression patterns of gated epidermal CD45.1$^+$ cells in mice injected with monocytes or control (I-A^{b+} CD11c$^+$) populations, 5 d (**a**) or 21 d (**b**) after exposure to ultraviolet irradiation. Results are representative of three separate experiments. (**c**) Percent CD45.1$^+$ LCs derived from adoptively transferred CD45.1$^+$ cells among total LCs. Data represent percent LCs derived from 3 × 10^6 or 9 × 10^6 monocytes or 9 × 10^6 enriched bone marrow I-A^{b+}CD11c$^+$ cells (Control) and are from one representative experiment (n = 3). (**d**) Langerin expression pattern of gated CD45.2$^+$ I-A^{b+} and CD45.1$^+$ I-A^{b+} epidermal cells in mice 6 weeks after adoptive transfer of CD45.1$^+$ monocytes. Results are representative of two individual mice. (**e**) Epidermal sheet stained with CD45.1 (red), langerin (green) and DAPI (blue). Epidermis is from a CD45.2$^+$ mouse, 6 weeks after adoptive transfer of 9 × 10^6 CD45.1$^+$ monocytes. Scale bar, 10 μm. Results are representative of two individual mice.