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Flt3L Dependence Helps Define an Uncharacterized Subset of Murine Cutaneous Dendritic Cells

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Skin-derived dendritic cells (DCs) are potent antigen-presenting cells with critical roles in both adaptive immunity and tolerance to self. Skin DCs carry antigens and constitutively migrate to the skin-draining lymph nodes (LNs). In mice, Langerin–CD11b− dermal DCs are a low-frequency, heterogeneous, migratory DC subset that traffics to LNs (Langerin–CD11b− migDCs). Here, we build on the observation that Langerin–CD11b− migDCs are Fms-like tyrosine kinase 3 ligand (Flt3L) dependent and strongly Flt3L responsive, which may relate them to classical DCs. Examination of DC capture of FITC from painted skin, DC isolation from skin explant culture, and from the skin of CCR7 knockout mice, which accumulate migDCs, demonstrate these cells are cutaneous residents. Langerin–CD11b− Flt3L-responsive DCs are largely CD24(+) and CX_3CR1^{low} and can be depleted from Zbtb46-DTR mice, suggesting classical DC lineage. Langerin–CD11b− migDCs present antigen with equal efficiency to other DC subsets *ex vivo*, including classical CD8α cDCs and Langerin+CD103+ dermal DCs. Finally, transcriptome analysis suggests a close relationship with other skin DCs, and a lineage relationship with other classical DCs. This work demonstrates that Langerin– CD11b− dermal DCs, a previously overlooked cell subset, may be an important contributor to the cutaneous immune environment.

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INTRODUCTION

As the primary barrier between the body and the outside world, the skin is a unique immune organ. The importance of cutaneous immunity is demonstrated in several ways. First, successful vaccination strategies rely on delivery of vaccine antigens to the skin, including vaccinia (Liu *et al.*, 2010) and dermally delivered influenza (Kenney *et al.*, 2004). Second, innate immune adjuvants that act on the cutaneous immune network (imiquimod, resiquimod) can lead to the regression of lentigo maligna and superficial spreading of basal cell carcinomas (Schon and Schon, 2008). Third, steady-state immune surveillance is critical to prevent skin cancers, as evidenced by the markedly increased incidence of neoplasia observed in renal transplant recipients maintained on immunosuppressive agents (Clark, 2010). Despite the immune-responsive nature of skin cancers and potential for skin-resident immune cells to mediate adaptive responses to cancer and infection, therapies have not been generated to prevent skin cancer formation. Improved understanding of adaptive immunity in the cutaneous environment is needed.

Adaptive cutaneous immunity depends on dendritic cells (DCs) that abundantly populate the skin and skin-draining lymph nodes (LNs). DCs are low-frequency hematopoietic cells, specialized at antigen presentation. DCs educate T cells to respond to tumor and microbial antigens, control the developing immune response, and maintain long-term immune memory to tumors and infections. In the noninflamed state, human skin contains four known phenotypically distinct subsets of DCs (Haniffa *et al.*, 2012; Teunissen *et al.*, 2012), whereas murine skin contains five (Henri *et al.*, 2010). These DCs continuously migrate from skin to the draining LNs and are collectively termed migratory dendritic cells (migDCs). Given such phenotypic heterogeneity amongst skin DCs, increasing efforts are aimed at identifying their functional and developmental attributes and correlating these with well-described DC subsets in other tissues.

Classical DCs are lymphoid and non-lymphoid DCs that derive from pre-DCs originating from a common bone marrow precursor (common DC precursor). The term classical is used

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Abbreviations: cDCs, classical DCs; DCs, dendritic cells; Flt3L, Fms-like tyrosine kinase 3-ligand; LN, lymph node; migDCs, migratory dendritic cells; PDCs, plasmacytoid DCs; PCA, principal component analysis

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to distinguish these populations from plasmacytoid DCs (PDCs) (Merad *et al.*, 2013) and monocyte-derived DCs, which develop in the setting of inflammation. Classical DCs broadly may be divided into CD11b+ and CD11b− populations. Classical DCs that are LN- and spleen-resident and are CD11b− also express CD8α and CD205 (termed CD8α+ cDC), whereas CD11b+ LN- and spleen-resident DCs lack expression of CD8α- (termed CD11b+ cDC). In peripheral tissue such as skin, CD11b− and CD11b+ may be additionally divided on the basis of other markers such as CD103 and Langerin.

In draining LNs, cell-surface expression of CD11c and MHCII is used to separate the three major DC groups: plasmacytoid DCs, LN-resident classical DCs (cDCs), and migDCs. MigDCs include some DCs of classical lineage that are CD11b− and CD11b+, such as Langerin+CD103+CD11b− DCs and Langerin–CD11b+ DCs, respectively (Ginhoux *et al.*, 2009). MigDCs also include Langerhans cells (LCs) of the epidermis, whose origin is distinct and which are not seeded from pre-DCs, but derive instead from radio-resistant precursors (Helft *et al.*, 2010). Although an additional migratory population of CD11b− cells was previously identified in skin (Langerin–CD11b−) (Henri *et al.*, 2010), these cells have been largely overlooked and incompletely characterized because they exist in low abundance in other peripheral tissue such as the lung and mucosa, although they comprise a significant fraction of DCs from the skin.

Some DCs are functionally specialized at cross-presentation, the major pathway to present viral and tumor antigens. These DCs have unique properties that make them attractive cellular targets for vaccination (Poulin *et al.*, 2010). When mature, they are superior to cross-present exogenous antigens on MHC I, ingest dead or dying cells, and produce IL-12 in response to innate and T cell–derived cues (Yamazaki *et al.*, 2008; Shortman and Heath, 2010). In skin, Langerin+ CD103+ DCs cross-present antigens, and CD8α+CD11b− DCs, which also express the C-type lectin CD205 (CD8α DC), cross-present antigens in lymphoid organs. In skin and other epithelial tissue, CD103+ DCs cross-present antigens from cytolytic viruses and epithelial pathogens (Bedoui *et al.*, 2009) including influenza (Helft *et al.*, 2012), herpes virus (Bedoui *et al.*, 2009), and erosive candida (Igyarto *et al.*, 2011). Several lines of evidence suggest that other skin DCs beyond Langerin+ CD103+ DCs may have an important role in priming to vaccines and pathogens. Langerin+ CD103+ DCs are not required in dermal vaccine administration (Flacher *et al.*, 2012). Furthermore, residual antigen presentation capacity to cutaneous pathogens including erosive candida (Igyarto *et al.*, 2011) and vaccinia (Seneschal *et al.*, 2013) is observed in the absence of Langerin+CD103+ DCs. These data suggest that another DC subset may cross-present antigens locally in the skin and skin-draining LNs.

Flt3L, a DC hematopoietin, and its receptor (Flt3, FLK2) regulate classical DC homeostasis of both DCs in lymphoid organs and DCs at, or arriving from, peripheral tissue (CD103+ DC in skin and lungs) (Waskow *et al.*, 2008; Liu *et al.*, 2009). Migratory Langerin+ CD103+ DCs, CD8α lymphoid resident cDCs, and human BDCA3+ cross-presenting DCs share developmental dependence on Flt3L (Ginhoux *et al.*, 2009; Haniffa *et al.*, 2012). Flt3L treatment biases cDC development to cross-presenting DCs (Bozzacco *et al.*, 2010). Flt3L expansion of DCs has helped identify very infrequent but functionally important DCs at epithelial sites such as blood–brain barrier (Anandasabapathy *et al.*, 2011) and aortic intima (Choi *et al.*, 2011). However, Flt3L dependence has not been used to characterize infrequent DCs in the skin or migDCs in the skin-draining LNs.

Here we analyze the cutaneous residence, migratory properties, developmental dependence, transcriptome, and functional properties of a cutaneous Langerin–CD11b− migratory DC subset (abbreviated Langerin– CD11b− migDC). Our data suggest that this subset is heterogeneous but contains highly Flt3L-responsive DCs with potent antigen-presentation capacity. We demonstrate that Langerin–CD11b− migDCs share many features with other classical Flt3L-dependent DCs.

RESULTS

Langerin–CD11b− migDCs are Flt3L dependent

We examined the expansion of migDC in the skin-draining LNs of mice in the presence and absence of *in vivo* Flt3L treatment. MigDCs can be gated as $CD11c^{int}$ and IAIE $(MHCII)^{hi}$ and further divide into Langerin+ known DC subsets including Langerin+ CD103+ DCs and Langerin+ CD103− DCs (which include Langerhans cells). Langerin– migDC subsets include Langerin–CD11b+ DCs and Langerin–CD11b− DCs (Henri *et al.*, 2010). (Figure 1a, and gating in Supplementary Figure S1A online). In C57BL/6 mice (Figure 1a) and Langerin GFP mice (Figure 1b) treated with Flt3L, we observed a major expansion of LN migDCs within the Langerin– compartment, comprising both CD11b+ and CD11b− cells. This was intriguing because our lab and others have shown a parallel expansion of CD11b− (CD8α+) and CD11b+ (CD8α-) cDCs in lymphoid tissue after Flt3L treatment (Bozzacco *et al.*, 2010). LN DC subset numbers were quantified from Flt3L-treated, untreated, and knockout mice (Figure 1c). Langerin–CD11b+ and Langerin–CD11b− subsets were expanded most significantly by Flt3L treatment. Flt3L loss affected Langerin+CD103+ DCs, and Langerin–CD11b+ and Langerin–CD11b− subsets.

CD11b− migDCs are skin-resident and traffic to the skin-draining LNs

To ascertain whether Langerin–CD11b− cells migrated to the LNs from skin, or were a contaminant from LN-resident populations such as CD8α cDCs, we examined skin explants in which DCs are directly isolated from the skin. CD11b− cells were present and expanded with Flt3L in ''crawlout'' DC isolation from skin (gating, Supplementary Figure S1B online). We established this in several models: Flt3L doxycycline-inducible mice (Manfra *et al.*, 2003) (Figure 1d), recombinant Flt3L-treated Langerin GFP mice (Figure 1e, Supplementary Figure S1B online), recombinant Flt3L-treated C57Bl/6 mice (Supplementary Figure S1C online), and Flt3L-secreting B16 tumor–treated mice (Supplementary Figure S1D online). In cDCs, Flt3L creates bias to CD8α CD205+ cross-presenting cDCs. Analysis of CD205+ expression on migDC explants

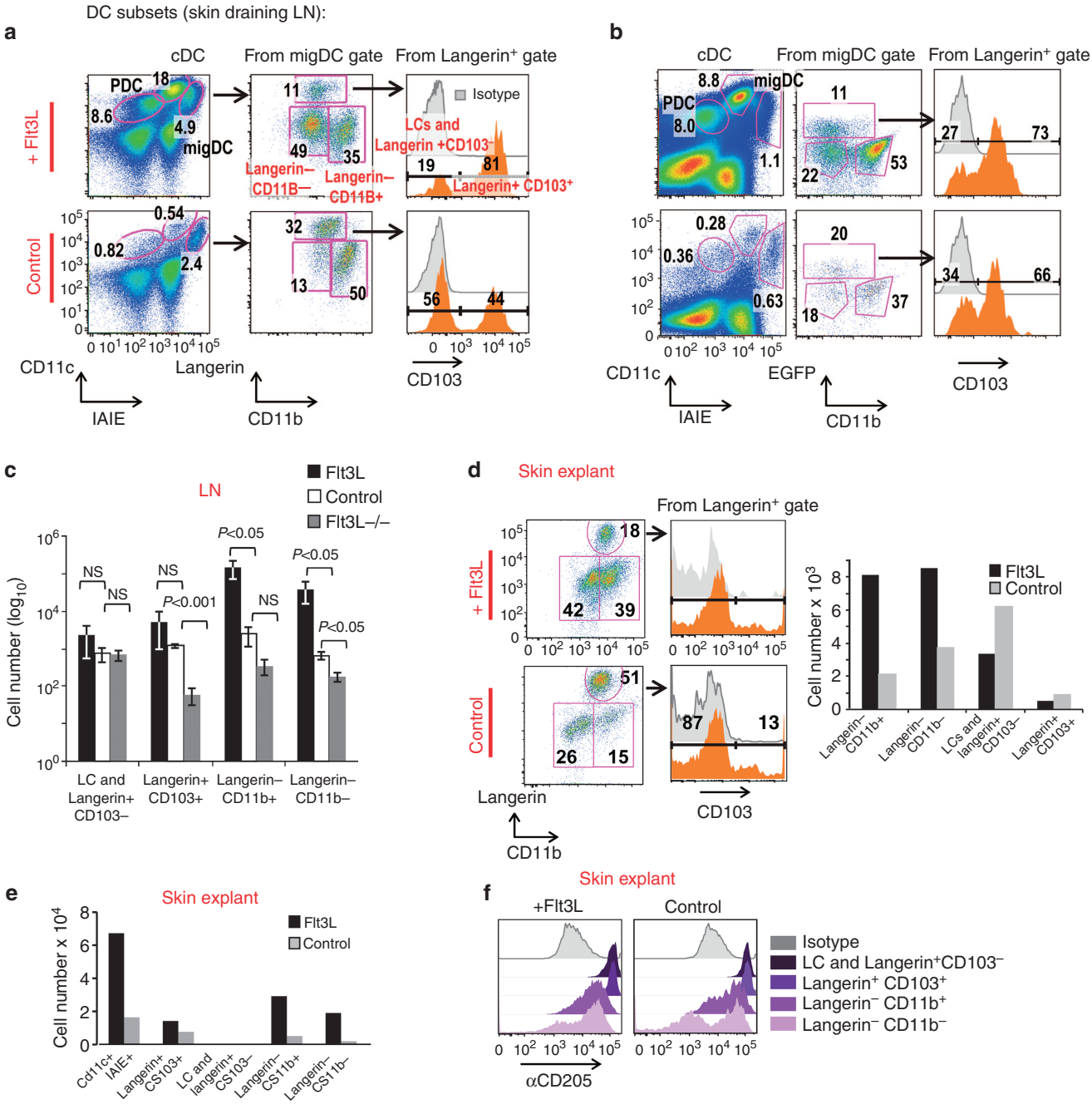

Figure 1. Flt3L dependence of migratory dendritic cell (DC) subsets, including Langerin–CD11b – DCs. (**a**) Schema of lymph node (LN) DCs from Flt3L-tumor-treated versus C57Bl/6 (B6) control mice at day 13. PDCs (plasmacytoid DCs), cDCs (classical LN resident DCs), and migDCs (migratory DCs) are labeled. (**b**) LN DC subsets Flt3L-treated (upper) versus untreated Langerin–GFP mice. (**c**) Quantification of migratory DCs from Flt3L-treated, control, Flt3L – / – mice (quantified per skin-draining LN; error bars show mean ± SD of three individual mice per group, analyzed by an unpaired *t*-test). (**d**) Flt3L-induced expansion of skin-resident DC subsets from ''crawlout'' skin explants of doxycycline-inducible Flt3L mice given 8 days of doxycycline in their drinking water (top) versus untreated controls (bottom) (one representative experiment of two). (**e**) Quantification of DCs isolated from skin explant ''crawlouts'' of Flt3L-treated versus control Langerin GFP reporter mice (pooled from the ears of three mice per experiment, one representative experiment of three). (**f**) αCD205 + staining of skin DC subsets from explant cultures of doxycycline-treated (Flt3L +) versus untreated (control) mice (pooled from $n = 2$ mice per condition).

revealed that Langerin–CD11b – DCs contained both CD205^{high} and CD205^{low} cells in the steady state. However, a higher percentage of CD11b – cells are CD205^{high} after treatment with Flt3L (Figure 1f).

Skin migDCs depend on CCR7 for migration to LNs (Forster *et al.*, 1999). To further validate that Langerin–CD11b – cells in LNs have migrated from the skin, we examined CCR7 – / – mice treated with Flt3L versus untreated controls. Irrespective

of Flt3L treatment, LNs from CCR7 knockout mice lack cells within the CD11c^{int}IAIE^{hi} DC migratory gate (Figure 2a, upper panel). MigDC subsets including CD11b – cells preferentially accumulated in skin explants and expanded with Flt3L locally in the skin (Figure 2a, lower panel). When FITC is applied with a contact-sensitizing agent to the epidermis of mice, migDCs rapidly transport FITC from skin to skin-draining LNs. FITC label was present only in the IAIE^{hi} migratory cells (Figure 2b).

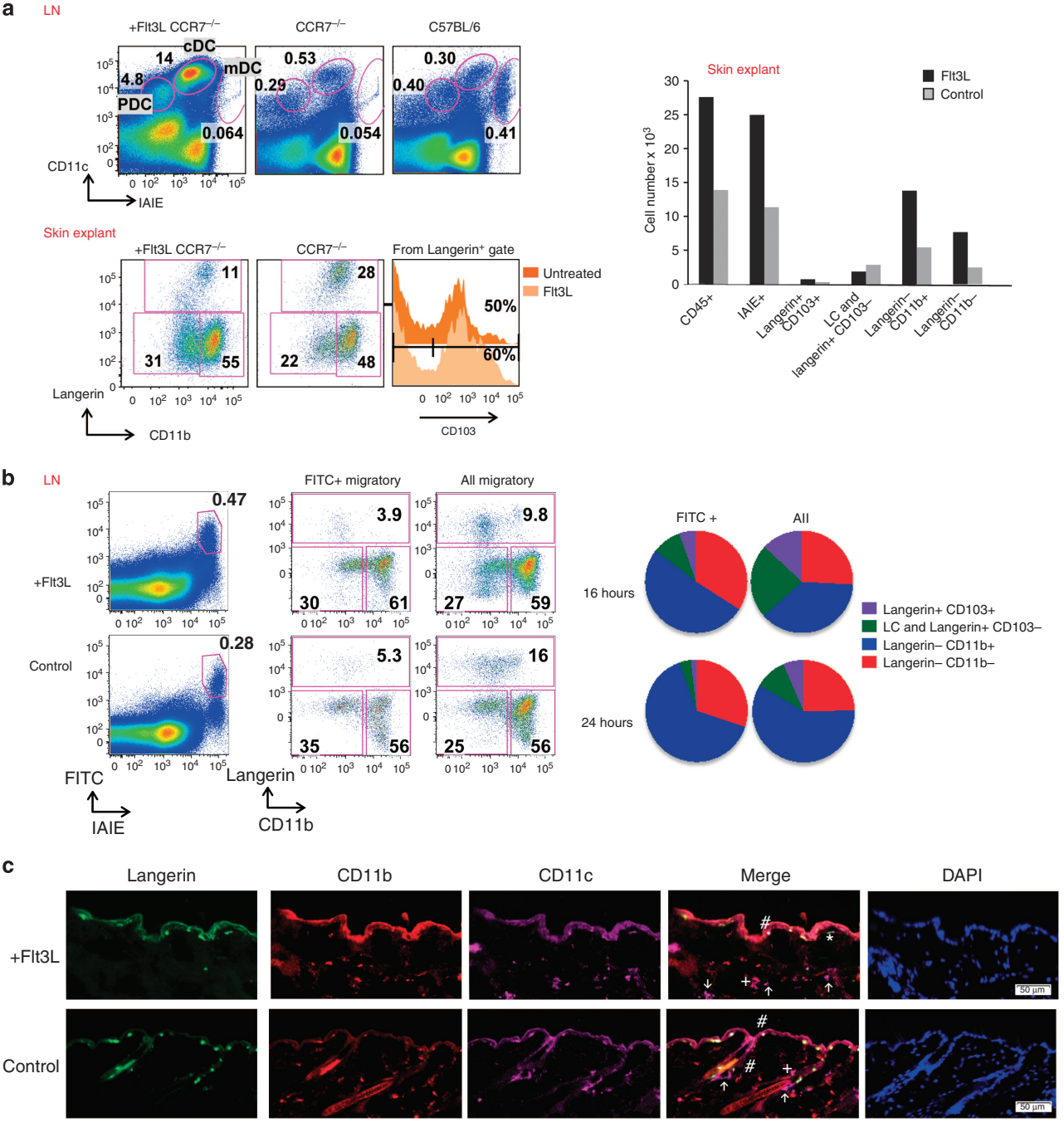

Figure 2. CD11b – dendritic cells (DCs) are skin-resident and migrate to the skin-draining lymph nodes (LNs). (**a**) Upper: representative skin-draining LN DCs gating from CCR7 –/– mice treated with Flt3L (left), CCR7 –/– untreated controls (middle), and B6 controls (right). Lower: CCR7 –/– skin-resident DC subsets from ''crawlout'' explants (Flt3L treated versus untreated controls). One of three representative experiments is shown. (**b**) FITC painting assay with gating on FITC + migratory DC versus total migratory DCs in Flt3L-treated (upper) and control mice (lower). CD11b – migDCs (red) directly capture FITC in the skin and traffic to the skin-draining LNs at 16 hours and 24 hours in C57BL6/WT mice ($n = 3$ mice per time point). (**c**) Immunofluorescence of flank sections of Flt3L-treated (upper) and control (lower) mice co-stained with langerin (green), CD11b (red), and CD11c (magenta) and counterstained with DAPI to visualize cell nuclei. Positions of Langerhans cells (LCs: Langerin +, CD11b +, CD11c +, #). Dermal DC (CD11c +) were further distinguished as Langerin–CD11b – (↑), Langerin–CD11b + (+), and Langerin + CD11b – (*) were marked in the merged images. Bar = 50 µm for all images.

Examination of FITC + migDCs versus all migDCs demonstrated an enhanced representation of FITC + Langerin– CD11b – cells during inflammation and FITC painting. Several populations of DCs transported FITC to the skin-draining LNs, yet a greater fraction of Langerin–CD11b – and Langerin– CD11b + DC subsets were present that labeled with FITC when compared with all migDCs after FITC painting (Figure 2b). These data suggest that Langerin–CD11b – DCs, similar to other migDCs, can transport FITC from the skin to the skin-draining LNs. To visualize Langerin–CD11b – DCs directly in skin, immunofluorescence staining was performed in Flt3L-treated and untreated mice. LCs were marked in the epidermis (Langerin + CD11b + CD11c +). Contained within dermal CD11c + cells, we observed Langerin– CD11b + , Langerin–CD11b – , and Langerin + CD11b – DCs (Figure 2c).

Langerin–CD11b – migDCs are heterogenous but contain a Flt3L-responsive CD24^{hi} CX3CR1^{low} DC population

Given that CD205^{high}- and CD205^{low}-expressing cells were present in the steady state within Langerin– CD11b – migDCs, yet enhanced numbers of CD205^{high} expressing cells were noted after Flt3L treatment, we speculated that the Langerin–CD11b – subset might be heterogeneous in the steady state. To address this, we examined CD24 expression, an additional marker for CD8α cDCs, on migDCs before and after Flt3L treatment. We observed that Flt3L treatment expands the percentage of Langerin–CD11b – cells expressing CD24 in the LNs from ~40% to ~80%, with a more modest and variable percentage expansion in skin (Figure 3a, Supplementary Figure S1C-D online). We also noted that in skin and LNs, an increase in the absolute number of Langerin– CD11b – CD24 + cells occurred after Flt3L and a correspondingly higher ratio of Langerin–CD11b – CD24 + cells in Flt3L treated versus control mice (quantification, Supplementary Figure S2 online). An increased percentage of CD24 + cells after Flt3L was also observed on CD8α + cross-presenting cDCs in LNs. Langerin + DC subsets have high CD24 expression, and CD11b + DCs have lower CD24 + levels at baseline. In CD11b + migDCs, CD24 + percentage was not markedly altered after Flt3L treatment in LNs and skin (Figure 3a and Supplementary Figure S1C-D online). These data suggest that Flt3L expands CD24 + Langerin–CD11b – cells. In contrast, during FITC painting in the presence of a

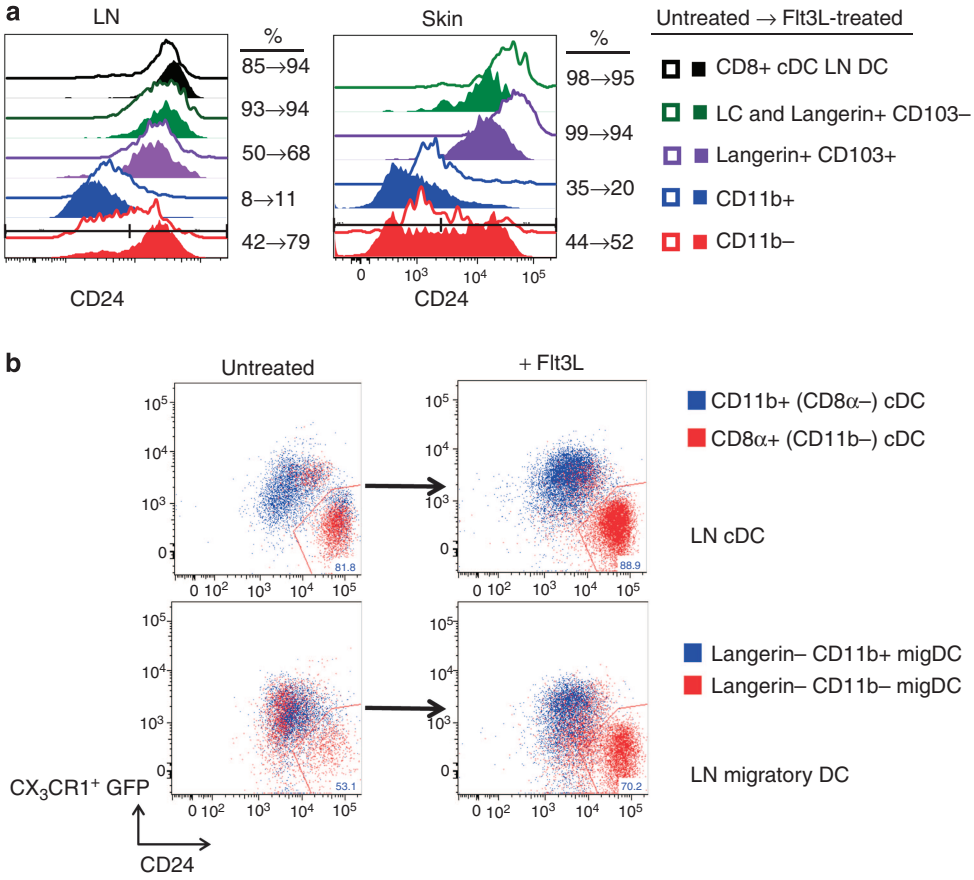

Figure 3. Flt3L-responsive CD11b – migratory dendritic cells (migDCs) include CD24^{high} CX$_3$CR1^{low} subsets. (**a**) In skin and skin-draining lymph nodes (LNs), a subset of CD11b – migDCs are CD24 + and expand with Flt3L. Gated pre (open) and post (closed) Flt3L in the LN and skin explant (lower) as compared with classical CD8α + DCs and other migDCs ($n = 3$ mice per group, one representative experiment of three). (**b**) Lower: Flt3L-expanded CD24 + cells are CX$_3$CR1^{low} within the CD11b – migDC subset (red) as compared with migratory CD11b + cells (blue). Upper: Comparison of LN-resident CD11b + CD8α- cDCs (blue) and CD8α + cDCs, which are CD24 + CX$_3$CR1^{low} (red) ($n = 2$ Flt3L-treated or control CX$_3$CR1 mice per group, one representative experiment of two).

proinflammatory contact-sensitizing agent, we observe that the expanded CD11b − population is predominantly $CD24^{low}$ (Supplementary Figure S1E online).

To further investigate Langerin–CD11b − heterogeneity, we examined the expression of CX_3CR1 using CX_3CR1-GFP reporter mice (Jung *et al.*, 2000). The chemokine receptor CX_3CR1 (fractaline) is abundantly expressed on CD11b + LN-resident cDCs and largely absent from the majority of CD8α LN-resident cDCs, which helps distinguish these two subsets (Figure 3b). The small fraction of CD8α + DCs that express CX_3CR1 lack cDC capacity for IL-12 secretion, cross-presentation of antigen, and resemble CD8α cDCs (Bar-On *et al.*, 2010). After Flt3L treatment, CD8α + $CD24^{hi}$ CX_3CR1^{low} cDCs (red, upper) remain well distinguished from CD11b + $CD24^{low}$ $CX3CR1^{hi}$ cDCs (blue, upper) (Figure 3b). In the steady state, Langerin–CD11b − cells are ∼50% $CD24^{high}$ CX_3CR1^{low} as compared with Langerin–CD11b + migDCs, which were predominantly $CD24^{low}CX_3CR1^{hi}$ in the steady state (lower, left). After Flt3L, 70% of Langerin–CD11b − migDCs became $CD24^{hi}$ CX_3CR1^{low}, suggesting that Flt3L expands this population selectively (lower, right). Collectively, these data suggest that in the steady state Langerin–CD11b − cells are heterogeneous but Flt3L selectively expands a $CD24^{hi}$ CX_3CR1^{low} subset, distinct from CD11b + $CD24^{low}$ CX_3CR1^{hi} migDCs.

Langerin–CD11b − migDCs, similar to other cDCs, depend on the transcription factor Zbtb46

To further characterize Langerin–CD11b − cells, we took advantage of recently generated Zbtb46-diptheria toxin receptor (DTR) mice, which allows specific depletion of cDCs and their precursors, but does not affect PDCs, monocytes, or macrophages (Meredith *et al.*, 2012a,b; Satpathy *et al.*, 2012). At 24 and 48 hours post DT treatment, an overall gross reduction in total number of LN-resident cDCs was observed, as expected (Figure 4a, gating Supplementary Figure S3A online). MigDCs were also rapidly depleted (Figure 4b). Langerin–CD11b − cells were efficiently depleted along with other cDCs in the LNs (Figure 4b) and in skin (Figure 4c and Supplementary Figure S3B online) with few residual cells left. These data suggest that Langerin–CD11b − cells are a Zbtb46-expressing classical DC subset that shares the same origin as cDCs.

Langerin–CD11b − migDCs perform key DC functions

To further examine whether the Langerin–CD11b − subset could perform key DC functions, we sorted CD8α LN-resident cDCs and several migDC subsets from Langerin GFP mice treated with Flt3L. We tested individual DC subsets (C57BL/6) in the mixed leukocyte reaction (MLR) using T cells from BALB/c mice. All DC types could effectively stimulate allogeneic T cells, including Langerin–CD11b − migDCs (Figure 5a and b). In addition, we sorted and cultured DC subsets with titrated doses of OVA protein and CD8 + OT-1 transgenic T cells. Again, Langerin–CD11b − DCs were comparable to other LN-resident and migDC subsets at OVA antigen presentation to CD8 T cells *ex vivo* (Figure 5c and d, gating, and representative flow data Supplementary Figure S4 online). These data suggest that Langerin–CD11b − migDCs

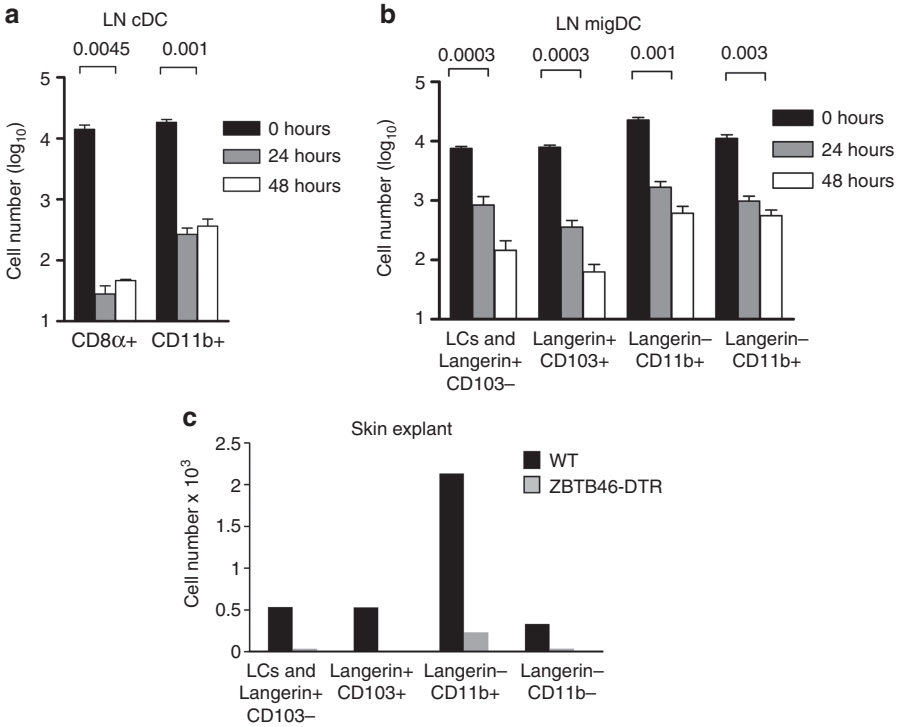

Figure 4. CD11b − migratory dendritic cells (DCs) are Zbtb46-dependent. (**a**) Lymph node (LN) classical (cDC) versus total migratory DCs (migDC) and (**b**) migratory DC subset counts from skin-draining LNs after single-dose diptheria toxin (DT) versus PBS administration 24 or 48 hours before harvest ($n = 3$ mice per time point, one representative experiment of three). (**c**) Skin explants pooled from three Zbtb46DTR versus C57BL/6 controls 24 hours after DT treatment ($n = 3$ mice pooled per group, one representative experiment of two).

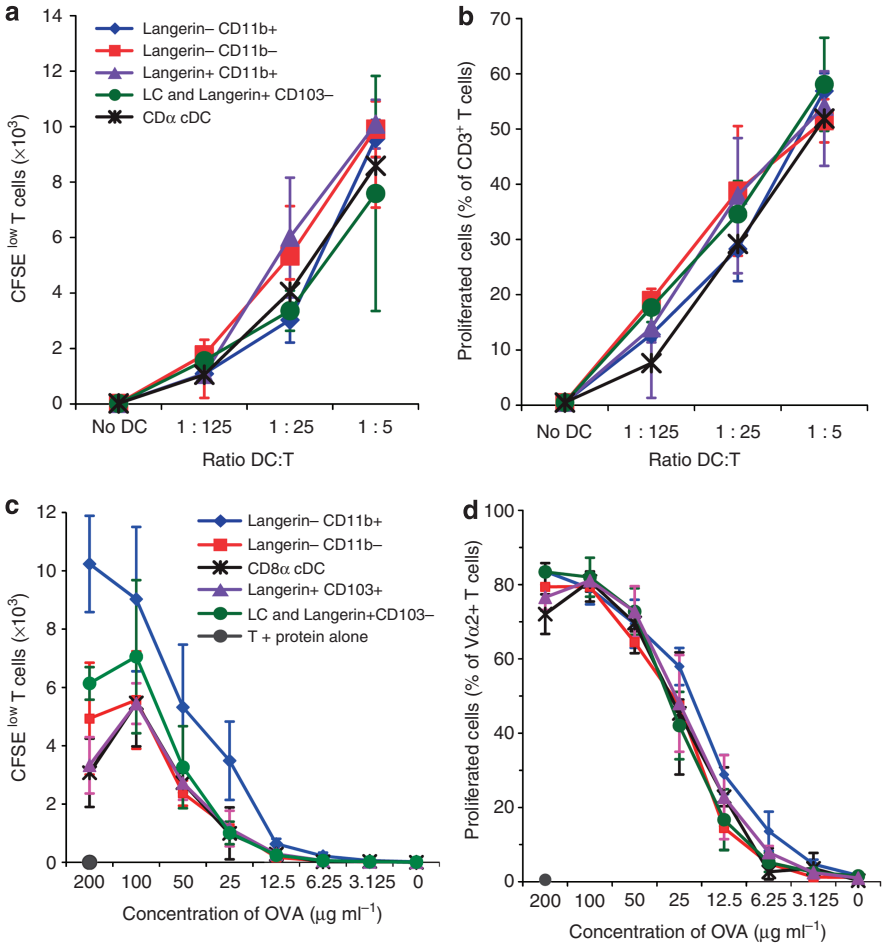

Figure 5. CD11b − migratory dendritic cells (migDCs) present antigen. CD11b − migDCs present allo-antigens in the mixed leukocyte reaction (**a**-**b**) and present OVA protein (**c**-**d**) to CD8 + Vα2 OT-1 T cells with equal efficiency to other DC subsets *ex vivo*. One of two duplicate experiments is shown with triplicate wells per sample.

perform equivalent antigen presentation of OVA to CD8 + T cells when compared with other DCs, including CD8α and CD103 + Langerin + DCs.

Langerin–CD11b − migDCs relate closely to other skin DCs and other cDCs, and express core DC signature genes

To further address the relationship of individual migDC populations to each other and to cDCs in LNs, we performed transcriptome analysis. We also included two additional subsets of LN DCs in our analysis obtained from LPS-treated mice: DC-SIGN + monocyte-derived DCs and CD205 + DCs (Cheong *et al.*, 2010) and peritoneal macrophages. Using principal component analysis (PCA), we identified four distinct clusters (data not shown): one cluster was formed by migDC subsets including Langerin CD103 + DCs, Langerin–CD11b + DCs, and Langerin–CD11b − DCs, consistent with prior work suggesting that tissue DCs cluster together (Miller *et al.*, 2012). Hierarchy analysis (dendrogram) (Figure 6a) revealed close relatedness of cutaneous Langerin–CD11b + and Langerin–CD11b − subsets to each other, and to Langerin + CD103 + and CD8α cDCs when compared against macrophages and LPS-treated DC subsets.

Recent studies have defined a ''core-DC gene signature'' when comparing multiple cDCs with macrophages (Miller *et al.*, 2012). By heat-map analysis, we analyzed the expression of many of these previously defined core DC genes in our subsets, shown as a relative fold expression against macrophages (Figure 6b). Many core DC genes including Flt3, Ccr7, and Zbtb46 were upregulated in all DC subsets examined, including Langerin–CD11b − migDCs. Heat-map comparison of additional selected DC genes was also performed across all populations (Figure 6c). These include Irf8, Clec9A, Ly75 (CD205, Dec205), Stat3, Zbtb46, Flt3, and Ccr7. Irf8, a transcription factor necessary for CD8α (and its equivalents) lineage development (Seillet *et al.*, 2013), was observed at the highest expression intensity in CD8α + and Langerin + CD103 + and in CD205 + DCs despite LPS treatment. CD11b − migDC levels of Irf8 were intermediate but higher than in CD11b + DCs, DC-Sign + LPS-treated monocyte-derived DCs, and macrophages.

DISCUSSION

This study characterizes a previously identified skin-resident subset of immune cells (Henri *et al.*, 2010) that we now

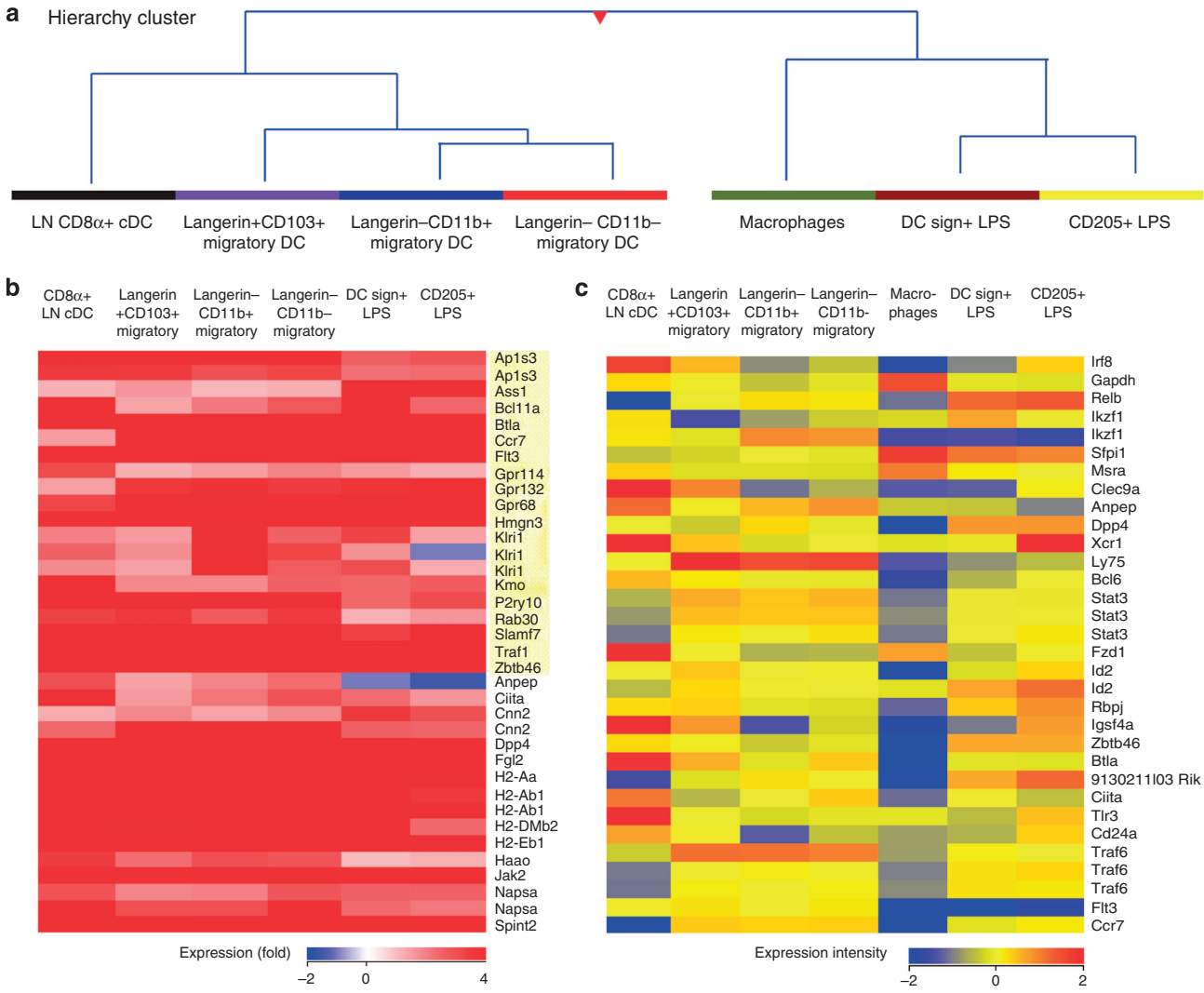

Figure 6. Langerin– CD11b – dendritic cells (DCs) relate closely to classical DCs. (**a**) Hierarchical clustering of subsets based on a twofold change or greater of all genes ($n = 8,601$). (**b**) Heat-map comparison of DC signature gene expression across DC groups as compared with macrophages (yellow indicates the core DC signature). (**c**) Heat-map comparison of normalized intensity values from selected DC genes across all subsets.

demonstrate as migDCs sharing many properties to cDCs, including Flt3L and Zbtb46 dependence. The current study builds on prior work in which Langerin–CD11b – DCs along with several other subsets were isolated from skin and skin-draining LNs of mice. Previously, Langerin–CD11b – DCs were noted to share division and repopulation kinetics along with other dermal DCs such as Langerin + CD103 + DCs, suggesting a common precursor (Henri *et al.*, 2010), yet they have been largely overlooked given the cross-presenting properties of Langerin + CD103 + DCs. Here we provide a dedicated characterization of Langerin–CD11b – migDCs based on developmental, phenotypic, and functional DC criteria, establishing these cells as a distinct DC subset by transcriptome analysis and relating them to other Flt3L-dependent classical DC subsets, distinct from monocytes and macrophages.

We establish that Langerin–CD11b – DCs are heterogeneous in the steady state but contain a highly Flt3L-dependent and -responsive population. Markedly reduced dermal Langerin– CD11b + and Langerin–CD11b – DC subsets were gated from LNs in Flt3L – / – mice. Flt3L treatment preferentially expanded Langerin–CD11b – and Langerin–CD11b + dermal DC subsets in the LNs and in the skin by crawlout explant. This may parallel spleen and BM where Flt3L-expanded DCs consistently divide into $CD11b^{hi}$ DC subsets (CD8α – cDC equivalents) and $CD11b^{low}$ $CD24^{hi}$ $SIRP\alpha^{low\text{-}intermediate}$ (CD8α + cDC equivalents) (Naik *et al.*, 2005). Langerin–CD11b – Flt3L-responsive DCs, similar to classical CD8α + CD11b – cDCs, are CD24 + and CX_3CR1^{low} and are efficiently depleted in DT-treated Zbtb46 DTR mice. Similar to other migDCs, we determine that Langerin–CD11b – are CCR7 dependent, are skin residents by explant and FITC painting, expand locally in the skin in response to Flt3L, and may be visualized in the dermis. Langerhans cells are radio-resistant, do not express Flt3 (Ginhoux *et al.*, 2012), and were not anticipated to expand. These data suggest that Langerin– DCs comprise the majority of the 14-fold Flt3L-based CD11c + expansion previously noted in the dermis (Esche *et al.*, 1999).

Although our study was conducted in mice, Flt3L dependence and Flt3L expansion of local cutaneous DC subsets is of particular importance to the cancer vaccine arena, as current and prior translational efforts aim to deliver cancer antigens to DC after subcutaneous administration of Flt3L.

We observed that Langerin–CD11b − DCs from mice treated with Flt3L performed allo-MLR and cross-presentation to OVA at equal efficiency to LN-resident cDCs. This suggests an efficient role in antigen presentation and *bona fide* DC function. This may be of significance to priming against erosive candida and cytolytic viruses where residual T-cell priming by DCs occurs even in the absence of Langerin + CD103 + dermal DCs, yet no other DC subset in skin has been identified as responsible for residual priming. However, strictly *ex vivo* analysis of DC function is somewhat limited by the fact that migDCs mature in culture after leaving the skin, and distinct individual *in vivo* roles of cutaneous DC subsets are still incompletely characterized. Current models of acute DC depletion (Langerin–DTR, CD11B − DTR, Zbtb46-DTR, and CD11c − DTR) cannot selectively isolate Langerin–CD11b − migDCs at this time; therefore, it will be difficult to immediately resolve their *in vivo* role in mediating cutaneous immunity. It is possible that, when licensed to prime, DC subset activity in the skin could relate to specialization or nonredundant coverage of various pathogens. Differing susceptibility of DC subsets to infection may permit discrete roles in antigen presentation, as observed for tissue-resident CD103 + DCs versus alveolar macrophages during influenza infection (Helft *et al.*, 2012).

We have determined that migDC group together by hierarchy clustering. These data suggest that three dermal DC subsets are closely related at the transcriptome level to each other and may suggest that tissue microenvironment affects terminal DC differentiation. As such, Langerin + CD103 + DCs group more closely with other migDCs than with their lymphoid developmental and functional equivalents: CD8α cDCs. Perhaps in the steady state, tissue-resident migDCs may act in concert. Intriguingly, LN CD205 + cDCs from LPS-treated mice cluster with LPS-treated DCs such as DC-SIGN + monocyte-derived DCs than to cDCs. The dominant gene signatures relating these subsets in this context are likely related to LPS treatment.

A lack of clarity surrounding the function and development of diverse DC subsets present in the skin and skin draining LN has long hindered the development of vaccines and clinical therapeutics to skin cancer. This work suggests that the previously overlooked Langerin–CD11b − DC subset contains a distinct, previously uncharacterized Flt3L-responsive migratory population with many properties of cDCs. As such, Langerin–CD11b − DCs may serve as a potential target of therapeutics aimed to enhance cutaneous immunity and as a mediator of the cutaneous immune response.

MATERIALS AND METHODS

Tissue harvest and DC cell preparation

''Flt3L-treated'' mice were injected by the intraperitoneal (IP) route with endotoxin-free ($<0.0064\,EU\,mg^{-1}$), GMP-grade, recombinant human Flt3L (Celldex) at 10 μg per mouse per day, diluted in sterile PBS versus sterile PBS-treated or untreated C57Bl/6 controls by IP injection. ''Tumor-treated'' 8–12-week C57BL/6F or Langerin–GFP mice were administered 5×10^6 murine Flt3L-secreting B16 melanoma tumor cells by subcutaneous injection. Doxycycline-inducible models of Flt3L induction have been previously characterized (Manfra *et al.*, 2003). At 10–14 days of IP injection of Flt3L, ~10 mm tumor size, or after 8–12 days of doxycycline addition to drinking water, mice were killed. Skin-draining LNs (inguinal, brachial, axillary, popliteal, and superficial cervical pooled) and spleen were isolated from individual mice. LNs and spleen were added to Collagenase D ($400\,U\,ml^{-1}$, Roche) in Hanks' Balanced Salt Buffer (Gibco) solution. LNs were teased apart and spleens were injected with this solution using a 22- to 23-gauge needle attached to a 3-ml syringe, and incubated for 25 minutes at 37 °C (Steinman *et al.*, 1979). After incubation, 0.5 M EDTA was added to a final concentration of 10 mM EDTA for disruption of DC:T-cell complexes, and the sample was further incubated for 5 minutes at 37 °C. For spleen cell preparation, ACK lysis of red cells was performed. Undigested fibrous material was filtered through a 70-μm cell strainer. Subsequent washed were performed with ice-cold PBS with 2% fetal calf serum (FCS). For crawlout assay, individual ears were harvested, washed in 70% EtOH, and both sides of ear halves were split dermal side down into R5-RPMI medium with 10% FCS. Crawlouts were harvested in 6-well plates (one ear per well), leading to some variability in total cell counts across explants. At 72 hours, cell suspensions were isolated and filtered. The pellet was washed twice and incubated in Fc block with 2% rat serum before cell surface marker antibody staining.

Mice

C57BL/6 mice (B6) were purchased from Taconic Labs or bred at Rockefeller University. CCR7 − / − mice were bred at Rockefeller University after being purchased from Jackson Laboratory (Bar Harbor, ME) and are described with respect to defects in the skin-derived DC migration (Martin-Fontecha *et al.*, 2003; Martin-Fontecha *et al.*, 2008). Langerin–GFP mice were generously provided by Bernard Malissen, bred as homozygotes at Rockefeller University, and have been previously described (Kissenpfennig *et al.*, 2005). CX_3CR1-GFP mice were purchased from Jackson Laboratory and previously described (Jung *et al.*, 2000). Zbtb46-DTR mice were generously provided by Matthew Meredith and Michel Nussenzweig. Doxycycline-inducible Flt3L were kindly provided by Sergio Lira. All mice were housed in specific pathogen-free conditions. Protocols were approved by the Rockefeller University and Harvard University Animal Care and Use Committees.

DC isolation

For allo-MLR, OVA presentation, and microarray RNA isolation, DC subsets were sorted from 5–10 pooled Flt3L-treated Langerin–GFP mice per experiment. Following size, live/dead, and exclusion criteria (CD3, CD19, NK1.1 exclusion) DC subsets were sorted following the general gating schema depicted in S1a. DC-SIGN + and CD205 + DCs were sorted from the skin-draining LNs of C57BL/6 mice 24 hours after intravenous injection of 5 μg of LPS (Cheong *et al.*, 2010), and macrophages were isolated from the peritoneum.

Immunofluorescence

Back skin from Flt3L-treated (12 days) or -untreated C57BL/6 controls was isolated and frozen in tissue-tek OCT (Sakura Finetek, Torrance,

CA). Sections (6 μm) were cut by cryomicrotome (Leica, Norcross, GA) sectioning, air-dried, and fixed in ice-cold acetone. Sections were pretreated with endogenous avidin/biotin block (Invitrogen, Carlsbad, CA) and further blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories). Sequential immunofluorescence staining was performed using anti-langerin (clone 929F3, Dendritics, Lyon, France), anti-CD11b (clone M1/70; Biolegend, San Diego, CA), and anti-CD11c (clone HL3, BD Biosciences, San Jose, CA) with the appropriate secondary antibodies (anti-rat IgG2a FITC (eBioscience, San Diego, CA), anti-rat IgG2b eFluor570 (eBioscience), and streptavidin-Alexa Fluor 647 (Invitrogen). Sections were mounted with Vectashield with DAPI (Vector Labs, Burlingame, CA). Images were acquired on a VS120 Whole Slide Scanner (Olympus, Center Valley, PA) at ×10 magnification using the ASW software and analyzed using the Olyvia software (Olympus).

Microarray analysis, normalization, and data analysis

RNA samples were prepared by standard methods using Trizol (Invitrogen) and further purified using RNeasy MinElute cleanup (QIAGEN, Valencia, CA). Purity analysis was performed by nanodrop and Eukaryote Total RNA Pico Series II (Agilent). RNA was amplified and hybridized on the Illumina MouseRef-8 v2.0 Expression BeadChip. For subset analysis, at least three replicates were analyzed from individual sorts to achieve statistical significance. Raw data from Illumina single-color chips were analyzed with GeneSpring 12.5 (Agilent Technologies, Santa Clara, CA). Intensities below background were replaced with the average background over all samples. Quantile normalization was applied to have a common distribution of intensities, followed by $\log_2$ transformation. Normalized values were baseline-transformed to the median of all samples. Normalized data were filtered to eliminate probes with low expression with a coefficient of variation of >0.5 in population samples. One-way ANOVA was used to find statistically significant differences among probes (*P*-values corrected with Benjamini–Hochberg false discovery method set at $P<0.05$). All DC subsets were arranged by a hierarchical clustering algorithm using normalized intensity values and based on twofold change or greater of all genes ($n=8601$). Heat maps for the differential expressions of core DCs relative to macrophages (two-fold) were generated and analyzed (Figure 6c). For selected DC genes of interest, heat maps of normalized intensity values were generated (Figure 6d). All data sets have been deposited at the National Center for Biotechnology Information/GSE53588.

Flow cytometry and gating

Cells were stained on ice in PBS with 2.0% (vol/vol) FCS. LSR II (Becton Dickinson, Franklin Lakes, NJ) was used for multiparametric flow cytometry of stained cell suspensions, followed by analysis with the FlowJo software (TreeStar).

Antibodies, live/dead dye, CFSE, FITC painting, and staining reagents

The following reagents were from BD Biosciences, eBioscience, or Biolegend: anti-Langerin (eBioL31), anti-CD11c (N418), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD11b (M1/70), anti-CD103 (2E7), anti-Armenian Hamster IgG Isotype Control (eBio299Arm), anti-rat IgG2a Isotype Control (eBR2a), anti-CD3 (500A2), anti-CD45 (30-F11), anti-CD205 (NLDC-145), anti-EPCAM (G8.8), anti-CD24 (M1/69), anti-F4/80 (BM8), anti-CD115 (AFS98), anti-PDCA1 (ebio927), anti-Ly6c (HK1.4), anti-B220 (RA3-6B2), anti-I-A/I-E (M5/114.15.2), anti-CD3 (17A2), anti-CD19 (eBio1D3), and anti-NK1.1 (PK136). AQUA (L34957) was from Invitrogen. Cytofix/cytoperm kit was from BD Biosciences. CFSE was from Sigma. Anti-CD205 (NLDC-145) was produced in the Steinman lab and conjugated to Alexafluor 488 or 647. Other reagents included PBS and FBS (Gibco-BRL), and ACK lysing buffer (BioSource, Invitrogen, Carlsbad, CA). Staining with anti-Langerin and anti-CD205 was performed with cell surface and intracellular label as described (Cheong *et al.*, 2007). For intracellular blocking, 2% rat serum was diluted into perm/wash buffer. All extracellular staining was performed in ice-cold PBS with 2% FCS. FITC painting was performed on the flank with 1:1 1% FITC in acetone:Dibutyl phthalate (.5% FITC final) for 16–30 hours before harvest.

OVA presentation

Endotoxin-free (<0.001 EU μg^{-1}) OVA was purchased from Hyglos (Bernried, Germany). For all groups, equal total numbers of DC subsets and CFSE-labeled T cells were cultured at a 1:10 ratio with increasing concentrations of soluble OVA protein.

MLR

Allo-MLR was performed as previously described (Anandasabapathy *et al.*, 2011) from FACS-sorted LN-resident and migDC subsets isolated from the skin-draining LNs.

CONFLICT OF INTEREST

Ralph Steinman was on the scientific advisory board of Celldex Pharmaceuticals and held a significant financial interest.

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SUPPLEMENTARY MATERIAL

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

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