RARα supports the development of Langerhans cells and langerin-expressing conventional dendritic cells

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Langerhans cells (LC) are the prototype langerin-expressing dendritic cells (DC) that reside specifically in the epidermis, but langerin-expressing conventional DCs also reside in the dermis and other tissues, yet the factors that regulate their development are unclear. Because retinoic acid receptor alpha (RARα) is highly expressed by LCs, we investigate the functions of RARα and retinoic acid (RA) in regulating the langerin-expressing DCs. Here we show that the development of LCs from embryonic and bone marrow-derived progenitors and langerin+ conventional DCs is profoundly regulated by the RARα-RA axis. During LC differentiation, RARα is required for the expression of a LC-promoting transcription factor Runx3, but suppresses that of LC-inhibiting C/EBPβ. RARα promotes the development of LCs and langerin+ conventional DCs only in hypo-RA conditions, a function effectively suppressed at systemic RA levels. Our findings identify positive and negative regulatory mechanisms to tightly regulate the development of the specialized DC populations.
angerhans cells (LCs) are the prototype dendritic cells that reside specifically in the epidermis. At steady state, LCs are the only MHC-II-expressing antigen-presenting cells in the epidermis. Langerin\(^+\) conventional dendritic cells (cDCs), similar to LCs, are also found in other tissues, including dermis, lymph nodes, spleen and lungs, albeit at significantly lower frequencies. A long-standing question is how LC development occurs selectively in the epidermis. 

The developmental origin of LCs is different from that of cDCs. LCs are developed from embryonic myeloid precursors from the yolk sac and fetal liver, and fully differentiated langerin\(^+\) LCs appear within a few days following birth in mice\(^{4-6}\). These cells can self-renew and persist in the skin throughout the life\(^6\). However, the LCs of embryonic origin can be replaced by bone marrow (BM)-derived LCs in inflammatory conditions\(^6\). Other langerin\(^+\) cDCs are thought to be generated from BM-derived precursors\(^7,8\). LC development is positively regulated by two cytokines, TGF-\(\beta\) and IL-34\(^9\)\(^-\)\(^15\). LC development is promoted by certain transcription factors, such as PU.1, inhibitor of DNA binding (Id2), and runt-related transcription factors 3 (Runx3), and suppressed by C/EBP\(\beta\) (CCAAT/enhancer-binding protein \(\beta\))\(^{16-18}\). Tissue factors that tightly control the development of LC and langerin\(^+\) cDCs in the body remain unclear. 

Retinoic acids (RAs) and their receptors play pivotal roles in embryo morphogenesis and immune regulation\(^9,20\). RA influences myeloid cell differentiation\(^{21,22}\) and generates mucosal DCs that express retinal aldehyde dehydrogenase 2 (RALDH2), Arg1, and TGF-\(\beta\) receptor 1 (TβR1), which are involved in myeloid cell differentiation\(^{21,22}\) and generates mucosal DCs in the skin\(^6,23\). These cells express RAR\(\alpha\) and RAR\(\beta\) and are found in other tissues, including dermis, lymph nodes, lung and spleen of RA-deficient mice compared to WT mice. In vitro, these cells were most clearly affected by RAR\(\alpha\) deficiency. 

We here report that the development of LCs and langerin\(^+\) cDCs is regulated by RAR\(\alpha\) in a RA-concentration-dependent manner. RAR\(\alpha\) promotes the development of these DC populations in hypo-RA conditions. However, systemic concentrations of RA effectively inhibit the generation of these DC populations. Our results provide new insights into the development of LCs and langerin\(^+\) cDCs.

**Results**

**LC development is defective in Rara-deficient mice.** There have been efforts to develop LC-like cells in vitro from mouse BM cells\(^{10,33}\), but none were able to effectively induce langerin expression. In line with this, the generation of langerin\(^+\) cells was ineffective in a medium containing regular fetal bovine serum (FBS) (Supplementary Fig. 1a). We used a medium with charcoal-treated FBS, which contains reduced levels of RAs\(^{34}\), to induce LC differentiation with the two LC-promoting cytokines GM-CSF and TGF-\(\beta\). CD11c\(^+\) cells expressing the well-established LC markers, langerin and EpCAM, were readily generated in the charcoal-treated FBS medium (Supplementary Fig. 1b). The frequencies of langerin\(^+\)/EpCAM\(^+\) cells in the culture were highest on day 3. Rara mRNA is expressed by the BM-derived LC-like cells, and this expression was decreased by RA (Supplementary Fig. 2a). Rara expression was higher in CD11c\(^+\) cells cultured in the BM-LC than in a BM-DC condition. Moreover, it was highly expressed by primary LC cells from 3-day old mice (Supplementary Fig. 2a). This expression level was higher than those of epidermal CD11c\(^+\) MHC-II\(^+\) cells that had not yet expressed langerin (pre-LCs) from newborn mice and of dermal CD11c\(^+\) MHC-II\(^+\) and CD45-negative epidermal tissue cells from 3-day old mice (Supplementary Fig. 2b). Publicly available microarray data also indicate that LCs expressed Rara at a level higher than many DC populations in lymphoid tissues (Supplementary Fig. 2c, ImmGen). To determine the function of RAR\(\alpha\) in LC development, we created ΔRara\(^{CD11c}\) (CD11c-Cre \(\times\) floxed Rara) mice with the exon 3 of the Rara gene deleted specifically in CD11c\(^+\) cells (Supplementary Fig. 3). The frequency and numbers of CD11c\(^+\) MHC-II\(^+\) cells were drastically decreased in the epidermis of ΔRara\(^{CD11c}\) mice (Fig. 1a). Interestingly, langerin\(^+\) CD11c\(^+\)MHC-II\(^+\) cells were almost absent in the epidermis of ΔRara\(^{CD11c}\) mice (Fig. 1a, Supplementary Fig. 4). The few langerin\(^+\) CD11c\(^+\) cells, present in the epidermis of ΔRara\(^{CD11c}\) mice, were abnormal in that they also lack CD24 expression (Fig. 1a, Supplementary Fig. 4). cDCs are largely divided into DC1 and DC2 in many tissues with high heterogeneity within each subset. Numbers of both XCR1\(^+\) CD172\(^+\) DC1 and XCR1\(^+\) CD172\(^-\) DC2 subsets were decreased in the dermis of ΔRara\(^{CD11c}\) mice (Supplementary Fig. 5). Immunofluorescence microscopy confirmed that epidermal MHC-II\(^+\) cells (i.e., LCs) were largely absent in ΔRara\(^{CD11c}\) mice with decreased numbers of dermal DCs (Fig. 1b). A few epidermal langerin\(^+\) cells, not readily detected by flow cytometry, were found in the epidermis of ΔRara\(^{CD11c}\) mice. These cells had an abnormally elongated cell shape different from that of typical LCs (Fig. 1c). Significantly fewer langerin\(^+\) DC1 cells were found in the dermis, skin-draining lymph nodes, lung and spleen of ΔRara\(^{CD11c}\) mice compared to WT mice (Fig. 1d and Supplementary Fig. 6). Additionally, a moderate decrease in the number or frequency of the DC subsets was observed in the lung (Supplementary Fig. 7), indicating the possibility that RAR\(\alpha\) may also affect some non-LC conventional DC subsets. We focused this study on LCs and langerin\(^+\) cells, which were most clearly affected by RAR\(\alpha\) deficiency. 

**Defective LC development in Rara-deficient progenitors.** Langerin up-regulation on CD11c\(^+\) MHC-II\(^+\) CD11b\(^+\) cells occurs right after birth in mice with massive proliferation of this cell population\(^35\). However, the early langerin up-regulation was aborted in ΔRara\(^{CD11c}\) mice (Fig. 2a). In the epidermis of newborn ΔRara\(^{CD11c}\) mice, the frequency and number of CD11c\(^+\) MHC-II\(^{low}\) cells were abnormally increased at the expense of CD11c\(^+\) MHC-II\(^{high}\) cells (Fig. 2b, Supplementary Fig. 8a). The CD11c\(^+\) MHC-II\(^{low}\) cells did not persist and soon disappeared on day 3 in the skin of ΔRara\(^{CD11c}\) mice (Supplementary Fig. 8a). The langerin\(^+\) CD11c\(^+\) MHC-II\(^{high}\) cells slightly increased in number in the skin of 9-day-old ΔRara\(^{CD11c}\) mice but failed to expand to the level of langerin\(^+\) CD11c\(^+\) MHC-II\(^{high}\) cells (Supplementary Fig. 8a). Ki-67 expression, which is strictly expressed by proliferating cells, was decreased on both the CD11c\(^+\) subsets from the ΔRara\(^{CD11c}\) compared to WT newborn mice (Supplementary Fig. 8b), which is in line with defective expansion and differentiation. Annexin V staining indicates that there were few dead cells in the CD11c\(^+\) MHC-II\(^{low}\) and MHC-II\(^{high}\) populations. While the importance is unclear, we observed somewhat lower cell death in the ΔRara\(^{CD11c}\) CD11c\(^+\) MHC-II\(^{low}\) population, compared to their WT counterpart (Supplementary Fig. 8b).

In postnatal life, LCs can be generated from BM-derived precursors. In order to determine if RAR\(\alpha\) is required for LC generation from BM-derived cells, we performed a BM reconstitution study from WT or ΔRara\(^{CD11c}\) mice to ΔRara\(^{CD11c}\) mice (Supplementary Fig. 9a). While WT BM transfer into ΔRara\(^{CD11c}\) mice generated langerin\(^+\) cells, BM transfer from ΔRara\(^{CD11c}\) to ΔRara\(^{CD11c}\) mice failed to generate them (Fig. 2c, Supplementary Fig. 9b). BM transfer into WT mice did not lead to successful reconstitution with donor cells in the epidermis (Supplementary Fig. 9a) as expected due to the known
radio-resistance of recipient LCs in the skin. Overall, these results show that ΔRara-CD11c progenitors are defective in generating langerin+CD11c+MHC-II+CD11b+ cells in the skin after BM transplantation.

RA negatively regulates BM-LC generation in vitro. We, next, investigated the effects of RAR agonists and antagonists on generation of langerin+ cells in vitro. Interestingly, RA, when added to cultures of BM cells in the LC-inducing condition, effectively suppressed the up-regulation of EpCAM and langerin, whereas BMS493, which blocks the effect of RA by enhancing nuclear corepressor (NCoR) interaction with RARα36, enhanced expression of the LC markers (Fig. 3a). Suppression was clear even at 0.1 nM of RA, and complete suppression was achieved even at 1 nM of RA. Unlike WT BM cells, only a few percent of ΔRara-CD11c BM cells
expressed langerin in charcoal-treated FBS (Fig. 3b). While the effects were not statistically significant (Supplementary Fig.10a–c), EpCAM up-regulation was detected on some ΔRaraCD11c CD11c+ cells, and this expression was somewhat regulated by RA and BMS493 (Supplementary Fig.10a–c).

We further verified the regulation of langerin expression by a surface and intracellular co-staining approach. Both ΔRara epidermal CD11c+ MHC-II+ cells and ΔRara BM cells, cultured in the LC-induction condition, have defective surface and intracellular langerin expression (Supplementary Fig. 11a, b). This indicates that the defective langerin expression is not the result of simple internalization of langerin. Also, confocal imaging revealed that langerin protein expression was defective in both surface and intracellular compartments of ΔRaraCD11c cells and was suppressed by RA (Fig. 3c). We observed that langerin expression in BM-LCs was suppressed at the mRNA level by RA, as well as by Rara deficiency (Fig. 3d). RA did not decrease existing langerin expression on and in primary LCs (Supplementary Fig. 11c).

Upon culture, LCs up-regulate the expression of CD40, CD86 and CCR7, but down-regulate E-cadherin (Supplementary Fig. 12a). During the culture, RA did not affect the change of these surface markers on primary LCs (Supplementary Fig. 12a), RA, when added during BM-LC differentiation, even suppressed the expression of CD40 and CD86 (Supplementary Fig. 12b). We also examined the possibility that RA at a physiological concentration influences the emigration of langerin+ CD11c+ MHC-II+ cells from WT and ΔRaraCD11c cells to WT and ΔRara ear explants in response to a CCR7 ligand (CCL19). Both WT langerin+ and langerin− CD11c+ MHC-II+ cells emigrated in response to CCL19, but RA had no effect on this migration (Supplementary Fig. 13). No migration of ΔRara langerin+ CD11c+ MHC-II+ cells was detected which is probably due to their paucity in the skin. On the other hand, CCL19-dependent migration of ΔRara langerin− CD11c+ MHC-II+ cells was detected which was unlikely to affect maturation or emigration of skin langerin-expressing cells.

Next, the impact of decreased RA levels on LCs was examined utilizing vitamin A deficient (VAD) mice. While the already high LC frequency in the epidermis was unchanged, the frequency of dermal langerin+ cells and their expression of langerin were significantly increased in adult VAD mice (Supplementary Fig. 14a–c). Thus, vitamin A metabolites negatively regulate the langerin+ dermal DCs.
Fig. 3 RA negatively regulates LC development. a RA suppresses langerin+ cell (BM-LC) generation in vitro, while the RAR antagonist BMS493 enhanced BM-LC generation. For BM-LC culture, BM cells were cultured in GM-CSF and TGFβ1 for 3 days in the presence of At-RA (0.01, 0.1, 1 and 10 nM) or BMS493 (100 nM) in media containing charcoal-treated or regular FBS. Frequencies of indicated CD11c+ cells are shown in graphs.

b Defective BM-LC generation from BM cells of ΔRaraCD11c mice. BM cells were cultured in a medium containing charcoal-treated FBS.

c Confocal fluorescent microscopy of langerin expression by CD11c+ BM cells cultured in the LC-inducing condition without and with RA (1 nM).

d Expression of Rara mRNA by CD11c+ BM cells cultured in the LC-induction condition without or with RA (1 nM). Normalized values for a housekeeping gene (GAPDH) are shown. Representative and combined data (n=3–8) from at least 3 experiments are shown. *Significant differences from control or between indicated groups by one-way ANOVA with Bonferroni multiple comparisons (a) or Mann–Whitney U test (p < 0.05, unpaired, 2-sided, b, d). Error bars are defined as s.e.m.
Impact of RARα deficiency and RA on gene expression. To gain insights into the functions of RARα and RA in regulating gene expression during LC differentiation, we performed an RNA-seq study on cultured BM cells from WT and ΔRara<sup>CD11c</sup> mice in the LC-inducing condition (Fig. 4). Multiplot, hierarchical clustering and principal component analyses (PCA) identified a high level of similarity between the effects of RA and RARα deficiency, increasing or decreasing largely overlapping groups of genes (Fig. 4a–c). Genes associated with LC or DC development, such as Runx3 and Cebp, were co-regulated by RARα deficiency and RA (Fig. 4d). Also, the expression of EpCAM1, Cer7, and Cd207 were co-suppressed by RA and RARα deficiency. The expression of Ilg-aM, Sirpa and Cx3cr1 was increased by RA, whereas the expression of Lamp2a1 and Xcr1 was increased by RARα deficiency (Fig. 4d). Additionally, top 50 up- and down-regulated by RA and RARα deficiency, including Snord88a (Small Nucleolar RNA SNORD88A), Cd276 (B7-H3), and Mfge8 (Milk fat globule-EGF factor 8 protein), are listed in Supplementary Fig. 15a and b. These results indicate that RARα deficiency exerts a powerful influence on gene expression during LC-like cell differentiation. The RNA-seq analysis revealed that the effects of RARα deficiency and RA treatments have largely similar negative effects on BM-LC development.

Runx3 and C/EBPβ are reciprocally regulated by RARα and RA. Runx3, a transcription factor that promotes LC generation<sup>14</sup>, was one of the genes down-regulated by both RA and RARα deficiency. We confirmed this down-regulation at mRNA and protein levels (Fig. 5a, b). Down-regulation of Runx3 provides a potential mechanism for the decreased LC generation by RA. We over-expressed Runx3 with a retroviral gene transfer method and examined if this abolished the inhibitory effect of RA and RARα deficiency (Fig. 5c). The enforced Runx3 expression abolished the suppressive effect of RA. However, the enforced Runx3 expression did not normalize the LC generation defect of ΔRara<sup>CD11c</sup> cells, indicating that the effects of RA and RARα deficiency on LC development are not identical.

Cebp, a gene that encodes a transcription factor that suppresses LC but promotes macrophage differentiation<sup>18,37</sup>, was up-regulated by RA and in RARα deficiency. C/EBPβ up-regulation was confirmed at mRNA and protein levels (Fig. 5d, e). To assess the role of up-regulated C/EBPβ by RA and RARα deficiency in LC-like cell development, we suppressed C/EBPβ activity by enforced expression of dominant negative (dn) C/EBPβ with a retroviral approach. Hyper-induction of WT LCs by dnc/C/EBPβ indicates C/EBPβ’s powerful suppressive effect on LC generation (Fig. 5f). Importantly, enforced expression of dnC/EBPβ abolished not only the suppressive effect of RA but also that of RARα deficiency (Fig. 5f). Thus, C/EBPβ regulation by the RARα axis is functionally important for LC development.

DNA-binding is important for the function of RARα. To gain further mechanistic insights into the RARα function in regulating LC differentiation, we expressed genes for WT and mutant forms of RARα in ΔRara<sup>CD11c</sup> cells. Retrosuduction of wild type Rara restored the LC differentiation defect in ΔRara<sup>CD11c</sup> cells. Interestingly, the Rara G303E mutant gene with a defective ligand binding domain (Rara-DLβ) was able to rescue the differentiation defect (Fig. 6). However, the Rara C105G mutant gene with a defective DNA binding domain (Rara-ΔDB) could not rescue the defect, underlining the importance of DNA binding capacity of RARα in regulating LC development. Thus, the positive function of RARα is mediated through DNA binding.

The RARα-RA axis also regulates human LC differentiation. Finally, we studied whether RA also regulates human LC differentiation. Langerin<sup>+</sup> LC-like cells can be differentiated from peripheral blood CD14<sup>+</sup> monocytes or CD1c<sup>+</sup> DCs<sup>17,38</sup>. As reported by others, normal fetal bovine serum (FBS) does not support langerin<sup>+</sup> LC-like cell generation (Fig. 7a). However, we found that charcoal-treated FBS was conducive for LC-like cell generation from CD14<sup>+</sup> monocytes. This indicates that there is a factor (or factors) in blood that inhibits human LC differentiation. The results with mouse LC cells strongly suggest that RA is such a factor that restrains LC differentiation. Indeed, RA, when added to culture even at a low concentration (1 nM), completely suppressed the generation of langerin<sup>+</sup> CD1a<sup>+</sup> LC cells (Fig. 7b). Considering the significant concentrations (~5 nM) of RA in human blood<sup>39</sup>, our results indicate that physiological levels of RA have the potential to effectively suppress LC differentiation in systemic tissues. Two different RARα antagonists, BMS493 and Ro41-5253, enhanced human LC-like cell generation (Fig. 7c), which suggests that RA in regular FBS is indeed a factor that suppresses LC generation. Langerin<sup>+</sup> cells were mostly C/EBPβ<sup>−</sup>- and the generation of these cells was effectively suppressed by RA (Fig. 7b). At mRNA level, CEBPβ expression was decreased by BMS493 in the regular-FBS medium and increased by RA in the charcoal-treated FBS medium (Fig. 7d). The pattern of RUNX3 expression was exactly the opposite, increased by BMS493 and decreased by At-RA in the human LC-like cells. Overall, RARα ligands and antagonists reciprocally regulate LC-like cell differentiation from human blood monocytes.

Discussion
In this study, we demonstrated that a regulatory pathway shaped by RARα plays a crucial role in the development of LCs and langerin-expressing DCs. We demonstrated that RARα is required for LC differentiation in vitro and in vivo. Interestingly, RARα drives LC differentiation and generation of langerin-expressing DCs in hypo-RA conditions. In systemic concentrations, RA generates a negative signal to effectively suppress LC differentiation. Thus, RARα plays a highly sophisticated role in the development of LCs and langerin<sup>+</sup> cDCs in a RA-concentration-dependent manner.

LCs are developed from both embryonic progenitors and bone marrow-derived cells. We demonstrated that RARα-deficient progenitors fail to give rise to LCs and langerin<sup>+</sup> cDCs in the skin. We speculate that the CD11c<sup>+</sup> MDSC<sup>II</sup> cells in the epidermis of newborn mice could be the precursors unable to develop into LCs in Rara deficiency. Langerin expression on DCs in non-skin tissues was also decreased in RARα-deficient mice. We also demonstrated that RARα-deficient CD11c<sup>+</sup> BM progenitors fail to differentiate to LCs in the skin during adult life. Therefore, RARα is required for LC development from both fetal and BM origins in newborn and adult mice respectively. A question is how RARα regulates LC development. RARα is a transcription factor that functions through both DNA binding and non-binding mechanisms<sup>40</sup>. A major way to trigger RARα activation is through ligand binding, which induces conformational changes to recruit co-activators and dissociate co-repressors. This can cause epigenetic changes and regulates gene expression. However, our results regarding the regulation of LC development indicate that RARα has a clear positive function in hypo-ligand conditions. This phenomenon is supported by the fact that RARα can epigenetically regulate gene expression in a manner independent of retinoid ligands as documented previously<sup>41</sup>. It seems that RARα shapes the gene expression program conducive for LC generation. As we demonstrated,
**Fig. 4** The effects of RARα deficiency vs. RA on gene expression in BM-derived DCs cultured in a LC-inducing condition. For all panels in this figure, RNA-seq was performed on WT and ΔRara<sup>CD11c</sup> BM cells cultured in charcoal-treated FBS medium containing GM-CSF and TGFβ1 for 3 days. WT cells were cultured with or without At-RA (10 nM). 2–4 independent samples were examined for each group. a) Scatter plot analysis of RPKM (Reads Per Kilobase of transcript per Million mapped reads) values for ΔRara<sup>CD11c</sup> vs. WT for X-axis and RA vs. WT for Y-axis. 2619 differentially expressed genes were selected based on T-test (P < 0.2) adjusted with Benjamini–Hochberg procedure of the Multiplot Studio (Version 1.5.29, GenePattern). Total 8 samples were examined by RNA-seq (4 WT, 2 ΔRara<sup>CD11c</sup>, and 2 At-RA). Genes highlighted in orange are the top 10 up- or down-regulated genes. b) Principal component analysis (PCA) of the 2619 selected genes based on their RPKM values. c) A Treeview showing hierarchical clustering of 2619 genes commonly or differentially regulated in BM-derived DCs cultured in a LC-inducing condition. Pearson correlation indices are shown. d) A Treeview showing hierarchical clustering of manually selected genes from the differentially regulated 2619 genes. The green and red triangles respectively highlight Runx3 and Cebpb genes. Error bars are defined as s.e.m.
**Fig. 5** RA and RARα reciprocally regulate the expression of the positive and negative LC-regulating transcription factors, Runx3 and C/EBPβ. 

a. Impact of RA and RARα deficiency on Runx3 expression at mRNA level. 

b. Impact of RA and RARα deficiency on Runx3 expression at protein level. 

c. Effect of enforced Runx3 expression on BM-derived LC differentiation in the presence and absence of RA. The data shown are gated for transduced Thy1.1+ CD11c+ cells. 

d. Impact of RA and RARα deficiency on expression of Cebpb mRNA. 

e. Impact of RA and RARα deficiency on expression of C/EBPβ protein. 

f. Effect of dncC/EBPβ on BM-derived LC differentiation in the presence and absence of RA. BM cells from WT or ΔRara CD11c+ mice were cultured with GM-CSF and TGFβ1 for 5 days (3 days following retroviral transduction) in the presence of At-RA (10 nM except in panels c and f where 0.1 nM was used) in a medium containing charcoal-treated FBS. Representative and combined data (n = 3–7) from at least 3 experiments are shown. Significant differences from controls by one-way ANOVA with Bonferroni adjustments (p < 0.05)* or between indicated groups by two-way ANOVA with Tukey adjustments (p < 0.05)**. Error bars are defined as s.e.m.
However, the function of RARα in regulating LC differentiation changes depending on the presence of its ligands (i.e., RAs).

While RARα is required for LC development, this appears to occur in hypo-RA conditions. The in vitro culture systems that were used in this study with charcoal-treated vs. regular FBS were useful to determine the negative role of RA in suppressing mouse BM-LCs. Charcoal depletes hydrophobic components, such as RAs, from FBS. Thus, the medium with charcoal-treated FBS represents a hypo-RA condition. The medium with regular FBS represents sub-systemic RA levels because it contains only 10% of RA. For example, it has been reported that RA rapidly activates several kinases including p38 mitogen-activated protein kinase (MAPK) and p42/p44 extracellular signal-regulated kinases (Erk) 44,45.

We demonstrated that the DNA-binding ability of RARα is important for its regulation of BM-LCs. The DNA binding function of RARα can be mediated by several different ways, including direct binding to canonical retinoid acid response element (RARE) sequences, half RARE sites, and other incompletely identified DNA sequences. RARα can also bind DNA via tethering through protein-protein interaction with other proteins such as BZLF1 to exert their regulatory effects42,43. Indeed, many of the genes regulated by RA lack canonical RAREs43. Therefore, it is likely that the function of RARα in regulating LCs is the result of both direct and indirect or primary and secondary regulation of gene expression by the RARα-RA axis. The RA effect on LC differentiation could be mediated also by non-genomic functions of RA. For example, it has been reported that RA rapidly activates several kinases including p38 mitogen-activated protein kinase (MAPK) and p42/p44 extracellular signal-regulated kinases (Erk) 44,45.

RARα-deficient BM progenitors, while they can’t effectively express langerin, can still express EpCAM albeit at reduced levels compared to WT BM progenitors. This indicates differential dependence of the expression of LC-associated molecules on RARα. Alternatively, some cells, which may have escaped the CD11c-Cre-induced Rara gene deletion or expressed the molecules before Rara gene deletion, have the potential to express the LC-associated molecules. Alternatively, major RA, such as At-RA
and 9-cis-RA, work through additional retinoid receptors such as RARβ and RARγ isotypes. Moreover, RAs activate other receptors such as PPARγ, RORγ, COUP-TFI and TR2/46, which may also explain the difference between the effects of RARα and RA. This possibility, however, is not high because the affinity of RA to these receptors is significantly lower than that to RARα46. Moreover, functional expression of these receptors should be established in LCs and langerin-expressing DCs.

Our results indicate that RARα and RA can regulate a number of genes in LC precursor cells. Indeed, we found that the Cebpβ gene expression is up-regulated in RARα deficiency, and it is also increased by RA, effectively suppressing LC differentiation. The Cebpβ gene encodes C/EBPβ, a transcription factor required for the development of certain macrophage subsets but suppressive of LC differentiation18,37,47. On the other hand, the expression of the pro-LC transcription factor Runx3 is suppressed in RARα deficiency. Therefore, these two genes are potential mediators of the RARα function to regulate LC differentiation. While some genes are direct targets of RARα, many genes are regulated indirectly. Further studies in this regard are necessary to delineate the regulatory mechanism in the reciprocal expression of the two transcription factors. Our RNA-seq study revealed that many more genes in addition to the two genes are regulated by RARα.

The genes for EpCAM, CD207 (langerin), and MHC II molecules expressed by LCs were suppressed in RARα deficiency. Genes upregulated in RARα deficiency include macrophage-associated genes such as Arg1, CD38, IL-6, CXCL14, and Snai3. Interestingly, RA regulates the expression of these genes in a manner highly similar to RARα deficiency. We reason that the similar effects of RARα deficiency and RA on the global gene expression in developing LCs is probably due to the shared suppressive effect by these two conditions.

The RA-RARα axis also regulates human LC development, which was demonstrated in this study in an in vitro setting. It has been reported that serum (i.e., blood) contains fat-soluble factors that potently suppress human LC differentiation48 but such a suppressive factor has not been identified to date. Our results identify that RA is such a suppressive factor. Serum concentration of RA is ~5 nM. In general, 0.1–10 nM is a physiologically relevant concentration range of RA, which was effective in suppressing LC-like cell generation in vitro. As exemplified well in developing embryos49, RA levels in tissues are likely to be highly regulated by RA-producing and degrading cells. The epidermis-specific tissue tropism of LCs in the body together with the fairly high RA concentrations in the blood suggest that the RARα has the potential to effectively promote LC differentiation from
precursors in the skin epidermis but probably suppress ectopic LC differentiation in relatively high-RA environments, such as blood circulation, lymphoid tissues, and gut tissues. In this regard, the topological correlation between RA activity and LC development in the body should be established in the future. In regard, the topological correlation between RA activity and LC

**Methods**

**Animals, vitamin A deficiency, and topical retinoid treatment.** All animal experiments were approved by the Animal Care and Use Committees at the University of Michigan and Purdue University. CD11c-Cre GFP mice (stock # 007567) were purchased from the Jackson Laboratory (Bar Harbor, ME) and mated with RARA<sup>−/−</sup> mice to generate ΔRARA<sup>CD11c</sup> mice. As WT control mice, we used either C57BL/6, RA<sup>−/−</sup> (if), and CD11c-Cre-GFP (including littermate and non-litter-mate) mice, all of which were not different from each other in LC development.

The data from male and female mice were indistinguishable and combined. Vitamin A-deficient (VAD) and vitamin A-normal (VAN) mice were generated by feeding late-term (15–16 post coitus-day) pregnant females with AIN-93G-based custom diets containing retinyl acetate at 2500 IU/kg (VAD, TN. 07267, Harlan Teklad, Indianapolis, IN) or 0 IU/kg of diet (VAD. 00158). Weaned mice were kept on the same diet for at least 10 weeks before examination of DC subsets. No randomization or blinding was performed for animal experiments.

**BM reconstitution study.** BM cells (5 x 10<sup>5</sup> cells per mouse) from WT or ΔRARA<sup>CD11c</sup> mice were transferred i.v. into lethally irradiated (1,100 rads) ΔRARA<sup>CD11c</sup> mice. Mice were examined 12–15 weeks after bone marrow transplantation.

**Cell isolation.** Mouse ears were separated into two skin halves and treated with 0.5% trypsin to remove dermis and epidermis. Dermal layers were separated and dermis was digested with collagenase IV (1–2 mg ml<sup>−1</sup>, Worthington, Lakewood, NJ). Epidermal cells were released after trypsin digestion without using collagenase treatment. The trunk skin of neonatal mice was similarly processed to obtain single cell suspensions. For qR-PCR analysis of Rara<sup>−/−</sup> and Ccl20 genes, CD11c<sup>+</sup> cells from BM culture or CD11c<sup>+</sup> cells and CD45-negative tissue cells from single suspensions of epidermis or dermis were isolated by magnetic selection with biotin-labeled CD11c (clone 4184, BioLegend) and Mojosort<sup>TM</sup> streptavidin nanobeads (BioLegend; purity > 90%).

Gradient centrifugation of tissue cells from single suspensions of epidermis or dermis were isolated by magnetic selection with biotin-labeled CD11c (clone 4184, BioLegend) and Mojosort<sup>TM</sup> streptavidin nanobeads (BioLegend; purity > 90%). Gradient centrifugation of skin epidermal and dermal cells released by collagenase digestion was performed on a 40/70% Percoll gradient to enrich Lc Dedicated LC cells.

**Induction of mouse BM-derived LC-like cells.** For most experiments, BM cells were cultured for 3 days in complete RPMI-1640 medium supplemented with regular or charcoal-treated FBS (10%, Thermo-Fisher). GM-CSF (20 ng ml<sup>−1</sup>) and BMS493 (10 nM) were added to generate paired-end 100 base reads. Sequencing data were analyzed with KAPA Library Quanti-fication software, the libraries were sequenced with a HiSeq 2500 using v3 chemistry to generate paired-end 100 base reads. Sequencing data were analyzed using FastQC (Babraham Bioinformatics, Cambridge, UK) for quality control and mapped to the mouse genome (UCSC mm10) using STAR RNA-seq aligner. Results distribution across the genome was assessed using bamtools. Uniquely mapped sequencing reads were assigned to mm10 refGene genes using featureCounts with the following parameters: -s 2 -p Q10. Quality control of sequencing and mapping results was summarized using MultiQC. Genes with read count per million (CPM) > 0.5 in more than 2 of the samples were removed. The data were normalized using the trimmed mean of M (TMM) values method. Differential expression analysis was performed using edgeR. T-test P-value adjusted by Benjamini-Hochberg procedure was computed using Multiplot Studio (GenePattern, Broad Institute) to select a set of genes for further analysis. Multiplot Studio, hierarchical clustering, and TreeView from GenePattern were used to visualize differentially expressed genes. Total RNA was isolated from BM cells using TRIzol (Thermo Fisher Scientific) using the primers described in Supplementary Table 1. All data are given as relative expression levels following normalization with GAPDH expression.
12. Greter, M. et al. Stroma-derived interleukin-34 controls the development and function of dendritic cells in the skin. Nat. Immunol. 14, 417–428 (2013).

13. Duester, G. Mechanisms of retinoic acid signalling and function independently of epidermal Langerhans cells. J. Exp. Med. 204, 3119–3131 (2007).

14. Capucha, T. et al. Distinct murine mucosal langerhans cell subsets develop under the accession code (GSE101991). The BM-LC RNA sequencing data have been deposited in Gene Expression Omnibus (GSE101991).

15. Harris, M. et al. Retinoic acid receptor inhibition of IFN-gamma-stimulated expression of MHC class II and costimulatory molecules. J. Exp. Med. 187, 961–966 (1998).

16. Zeng, R., Bscheider, M., Lahl, K., Lee, M. & Butcher, E. C. Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. J. Immunol. 165, 5915–5925 (2000).

17. Jaensson, E. et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J. Exp. Med. 205, 2139–2149 (2008).

18. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

19. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

20. Klebanoff, C. A. et al. Retinoic acid controls the homeostasis of pre-DC-derived splenic and intestinal dendritic cells. J. Exp. Med. 210, 3514–3518 (2013).

21. Fainaru, O. et al. Impaired granulocytic differentiation in vitro in hematopoietic cells lacking retinoic acid receptors alpha1 and gamma. Blood 103, 607–615 (1999).

22. Purton, L. E. et al. RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. J. Exp. Med. 203, 1283–1293 (2006).

23. Zeng, R. et al. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. Mucosal Immunol. 6, 847–856 (2013).

24. Saurer, L., McCullough, K. C. & Summerfield, A. In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. J. Immunol. 179, 3504–3514 (2007).

25. Chang, J. et al. Retinoic acid promotes the development of Arg1-expressing dendritic cells for the regulation of T-cell differentiation. Eur. J. Immunol. 43, 967–978 (2013).

26. Hill, J. A. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

27. Hill, J. A. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

28. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

29. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

30. Klebanoff, C. A. et al. Retinoic acid controls the homeostasis of pre-DC-derived splenic and intestinal dendritic cells. J. Exp. Med. 210, 3514–3518 (2013).

31. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

32. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

33. Poulin, L. F. et al. The dermis contains langerin+ cells. Blood 115, 1135–1141 (2002).

34. Ginhoux, F. et al. Langerhans cells are generated by two distinct PU.1-expressing hematopoietic progenitor cells to generate Langerhans cell-like dendritic cells in the skin. Nat. Immunol. 14, 407–416 (2013).

35. Poulin, L. F. et al. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. J. Exp. Med. 207, 1383–1395 (2010).

36. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

37. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

38. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

39. Hill, J. A. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

40. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

41. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

42. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

43. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

44. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

45. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

46. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

47. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

48. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).

49. Zeng, R. et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. Immunity 29, 758–770 (2008).

50. Fainaru, O. et al. Runx3 regulates mouse TGF-beta-mediated dendritic cell differentiation and its absence results in airway inflammation. J. Exp. Med. 210, 1961–1976 (2013).
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Author contributions

C.K. conceived the study and obtained funding. S.H., L.F. and S.P. performed the experiments and plotted graphs. M.K. provided retroviral vectors. S.I. performed GenePattern and Ingenuity analysis of RNA-seq data. C.K. drafted the manuscript, and all participated in completing the final version of the manuscript.

Additional information

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