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ALK3 is not required for the embryonic development, homeostasis and repopulation of epidermal Langerhans cells in steady and inflammatory states

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To the editor:

Epidermal Langerhans cells (LCs) are skin-resident dendritic cells (DCs), although recently LCs are also classified as specialized macrophages based on their embryonic development shared with tissue resident macrophages (Doebel et al., 2017, Hoeffel et al., 2012). LCs maintain skin immune surveillance and involve in skin disorder, including psoriasis (Eidsmo and Martini, 2018; Kaplan, 2017). Adult LCs homeostasis is maintained throughout lifetime self-renewal at steady-state but the impaired LCs could arise by bone marrow (BM)-derived precursors at inflammatory-state (Ginhoux et al., 2006, Sere et al., 2012). Quite a few well-

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Author Contributions

Conceptualization: QSM, LZ; Data Curation: QY, NP, LZ, QSM; Formal Analysis: QY, NP, LZ, QSM; Funding Acquisition: QSM, LZ, JTE; Investigation: QY, NP, LZ, QSM; Methodology: QY, NP, JTE, LZ, QSM; Project Administration: LZ, QSM; Resources: YM; Validation: NP, LZ, QSM; Visualization: QY, NP, LZ, QSM; Writing - Original Draft Preparation: QY, NP, LZ, QSM; Writing-review and editing: QY, NP, JTE, YM, LZ, QSM.

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identified genes, including transforming growth factor-β1 (TGF-β1), are essential for epidermal LCs (Zhang *et al.*, 2016). TGFβ1 regylates LC development, maturation, migration and repopulation (Kaplan et al., 2007, Sere et al., 2012). In the canonical TGF-β signaling, TGF-β1 binds TGFβ-R1 and TGFβ-R2 complex, mediating the phosphorylation of Smad2/3 complex, which forms a heterotrimeric complex with Smad4 and translocates into the nucleus and regulates TGFβ1 targeting genes (Derynck and Zhang, 2003). However, our genetic mouse models with deletion of Smad3 (Xu et al., 2012), Smad2 and Smad4 (Huang et al., 2020) showed no effect of Smad pathway loss on LCs development at steady-state, but did show an impact on LC repopulation under inflammatory-state. Nevertheless, recent studies revealed that both TGF-β family members, TGF-β1 and bone morphogenetic protein 7 (BMP7), can potentially signal via BMP receptor 1A (BMPR1A, or activin receptor-like kinase 3, ALK3), to induce LC differentiation *in vitro* (Borek et al., 2020, Yasmin et al., 2013), and BMP7-ALK3 signaling leads to the differentiation and proliferation of inflammation-associated LCs from BM–derived precursors in psoriatic lesions, which promotes the psoriatic epidermal changes in patients with psoriasis as well as in a psoriatic mouse model (Borek et al., 2020). However, it remains unclear whether ALK3 is required for LC development *in vivo*. Here, we reported that ALK3 is not required for LC development and repopulation *in vivo*, and ALK3 deletion in LCs did not significantly affect imiquimod (IMQ)-induced psoriatic dermatitis in mice.

To test the role of ALK3 in LC homeostasis *in vivo*, we first crossed $ALK3^{fl/fl}$ mice (Mishina et al., 2002) with $CD11c^{Cre}$ mice to generate DCs-specific ALK3 deletion mice. The expression of ALK3 in LCs from $CD11c^{Cre}ALK3^{fl/fl}$ knockout (KO) mice was dramatically reduced compared to wild-type (WT) littermates (Figure 1a). As shown in Figure 1b, there was no significant alteration in the frequencies of LCs ($CD45^{+}MHCII^{+}$) between $CD11c^{Cre}ALK3^{fl/fl}$ and WT mice, suggesting that ALK3 is inessential for LC homeostasis after birth. Recent studies confirmed that LCs are derived from embryonic yolk sac and fetal liver monocyte (Kaplan, 2017) and BMP7 highly expresses in embryonic keratinocytes (Borek et al., 2020, Yasmin et al., 2013). Colony stimulating factor 1 receptor (CSF-1R) Cre fate-mapper ($CSF\text{-}1R^{iCre}$) mice were used to study embryonic development of tissue resident macrophages and LC precursors (Hoeffel et al., 2015, Schulz et al., 2012, Yao et al., 2018). Hence, we crossed $CSF\text{-}1R^{iCre}$ mice with $ALK3^{fl/fl}$ mice to generate myeloid-exclusive ALK3 deletion mice. The deletion of ALK3 on LCs was confirmed by qRT-PCR and Western blot (Figure 1c). As shown in Figure 1d, the frequencies of LCs ($CD45^{+}F4/80^{+}$) at embryonic day 17.5 and postnatal day 0 were comparable between $CSF\text{-}1R^{iCre}ALK3^{fl/fl}$ and WT littermates, suggesting that the embryonic generation and skin-homing programs were unaffected by ALK3 loss. Furthermore, there was no significant difference in the frequency of LCs ($CD45^{+}MHCII^{+}$) from epidermal suspension (Figure 1e) and in the number of $MHCII^{+}$LC cells on the epidermal sheets (Figure 1f) between $CSF\text{-}1R^{iCre}ALK3^{fl/fl}$ and WT mice at 8-weeks old. Thus, overall, ALK3 is not required for LC ontogeny during embryonic development and homeostasis of LCs after birth.

LCs could be derived from BM under inflammatory conditions and we found that Smad2/4 is required for LC repopulation from BM in UVC treated skin (Huang et al., 2020). To explore the role of ALK3 in LC repopulation, the epidermal LCs ($CD45^{+}MHCII^{hi}CD207^{hi}$) in $CSF\text{-}1R^{Cre}ALK3^{fl/fl}$ and WT littermates were analyzed at 2 weeks post UVC treatment

and were found comparable (Figure 1g), indicating that ALK3 is not required for LCs repopulation. This observation is in line with the recent study demonstrating that BMP7 fails to induce the differentiation of LC-like cells in mouse BM cultures and fetal liver cells (Capucha et al., 2018).

The main functions of LCs include their phagocytosis and upregulating their CD80/86 expression upon stimulation. We next tested the role of ALK3 in LC function. Freshly-isolated epidermal LCs from *CSF-1R*^{iCre}*ALK3*$^{fl/fl}$ and WT mice were incubated with Dextran-Fluorescein isothiocyanate isomer (FITC) for 45 minutes at 37°C or 4°C (control), respectively. The LCs labeled with Dextran-FITC (CD45^{+}MHCII^{+} FITC^{+}) were defined to have effective phagocytosis. No significant differences were identified in LCs phagocytosis based on dextran-uptake (Figure 2a). Additionally, spontaneous maturation was evaluated after 72 hours *in vitro* culture and mature LCs based on the percentages of CD80^{hi}LCs and CD86^{hi}LCs after *in vitro* incubation were comparable between *CSF1R*^{Cre}*ALK3*$^{fl/fl}$ and WT mice (Figure 2b). Thus, these data suggest that ALK3 is dispensable for LC phagocytosis and maturation.

Lastly, we investigated the role of ALK3 in LCs in the imiquimod (IMQ)-induced psoriasis-like dermatitis mouse model. By monitoring the scores of erythema, scales and thickness of IMQ-treated mouse skin, we found no significantly altered inflammation based on psoriasis area and severity index (PASI) score between *CSF-1R*^{Cre}*ALK3*$^{fl/fl}$ and WT mice (Figure 2c). Furthermore, histopathological analysis of ear sections showed no significant changes in IMQ-induced epidermal thickening (acanthosis) and infiltrating cells (Figure 2d).

Overall, our results highly suggest that BMP7 or TGF-β1 induced ALK3 signaling is not required for the LC embryonic development, homeostasis after birth in steady-state and repopulation in inflamed-state in mice. ALK3 is dispensable for phagocytosis and maturation of LCs and lack of ALK3 in LCs does not significantly affect imiquimod-induced psoriatic dermatitis in mice. These results oppose recent studies demonstrating that BMP7 or TGF-β1 induced ALK3 signaling is required for LC differentiation and function. This discrepancy may be related to the differences between in vitro and in vivo studies or between human and mice, as well as due to the nonspecific ALK2–3-6 inhibitor dorsomorphin used. Therefore, further investigation is warranted to clarify potential involvement of BMP7-ALK2 or ALK6 in mouse LCs and BMP7/ALK3 signaling pathway in human LCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

Data related to this article are available on request

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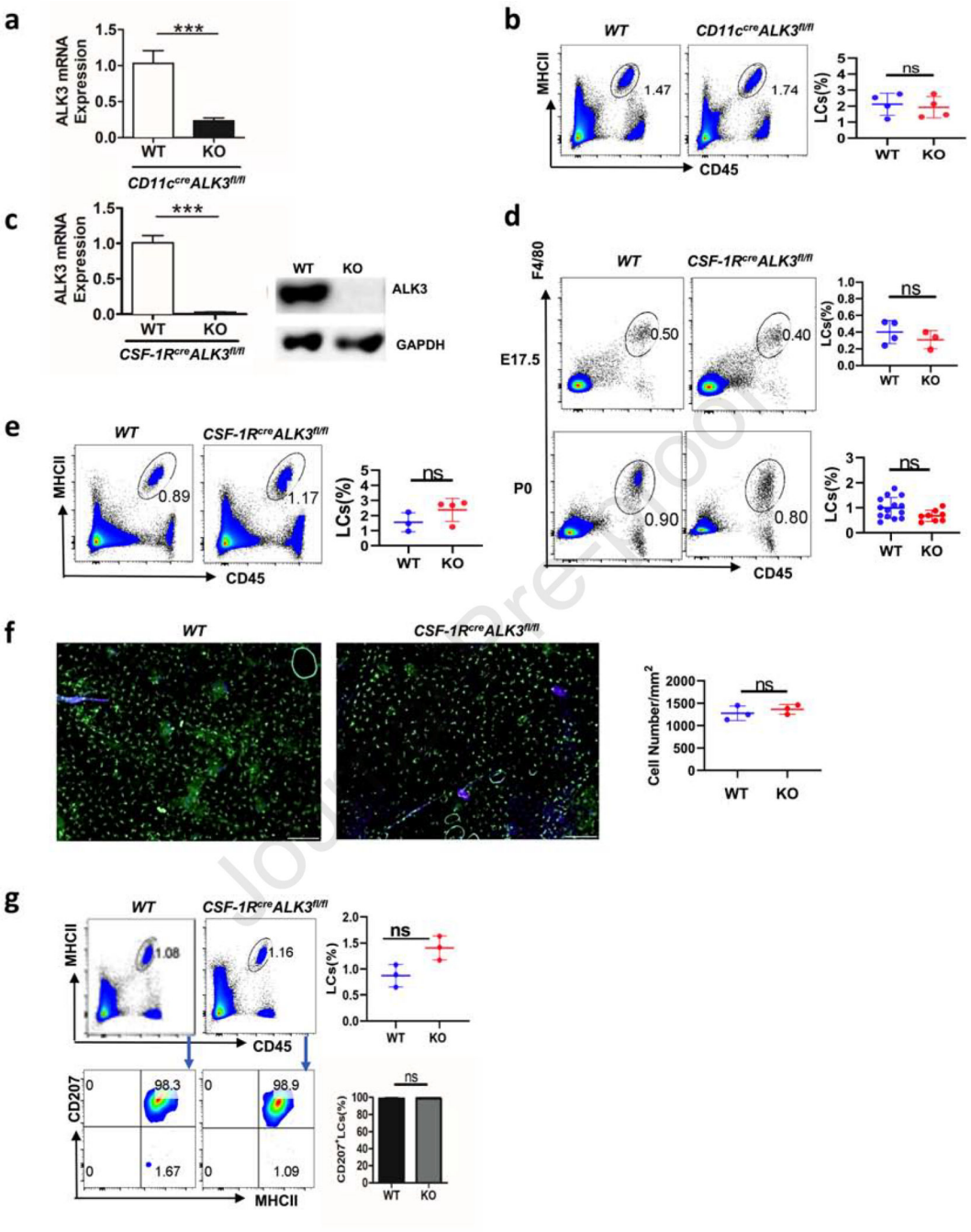

Figure 1. ALK3 is not required for LC development, homeostasis, and repopulation.

(a) Relative mRNA expression of ALK3 in sorted LCs from *CD11c*^{Cre}*ALK3*$^{fl/fl}$ (KO) and WT mice by qRT-PCR (n=3, ***P<0.001). (**b**) Flow cytometry analysis showing the frequencies of epidermal LCs (CD45^{+}MHCII^{+}) in eight weeks old *CD11c*^{Cre}*ALK3*$^{fl/fl}$ (KO) and WT mice (n=3–4, ns, no significant difference). (**c**) Relative mRNA and protein expression of ALK3 in sorted LCs from *CSF-1R*^{iCre}*ALK3*$^{fl/fl}$ (KO) and WT mice by qRT-PCR and Western blot, respectively (n=3, ***P<0.001). (**d**) Flow cytometry analysis showing the frequencies of epidermal LCs (CD45^{+}F4/80^{+}) at embryonic day 17.5 (E17.5)

and at birth (P0) in *CSF-1R*^{iCre}*ALK3*$^{fl/fl}$ (KO) and WT littermate mice (n=3–14). (**e**) Flow cytometry analysis showing the frequencies of epidermal LCs (CD45^{+}MHCII^{+}) in *CSF-1R*^{iCre}*ALK3*$^{fl/fl}$ (KO) and WT mice at eight weeks old (n=3–4). (**f**) Immuno-staining of MHCII (green) on epidermal sheets. Scale bar = 100μm, original magnification ×10. (**g**) A LCs repopulation study showing the frequencies of epidermal LCs (CD45^{+}MHCII^{+}) in the upper panel and the frequencies of BM-derived LCs (MHCII^{hi}CD207^{hi}) gated on CD45^{+}MHCII^{+} LCs in the lower panel between *CSF-1R*^{iCre}*ALK3*$^{fl/fl}$ (KO) and WT mice after UVC treatment (n=3).

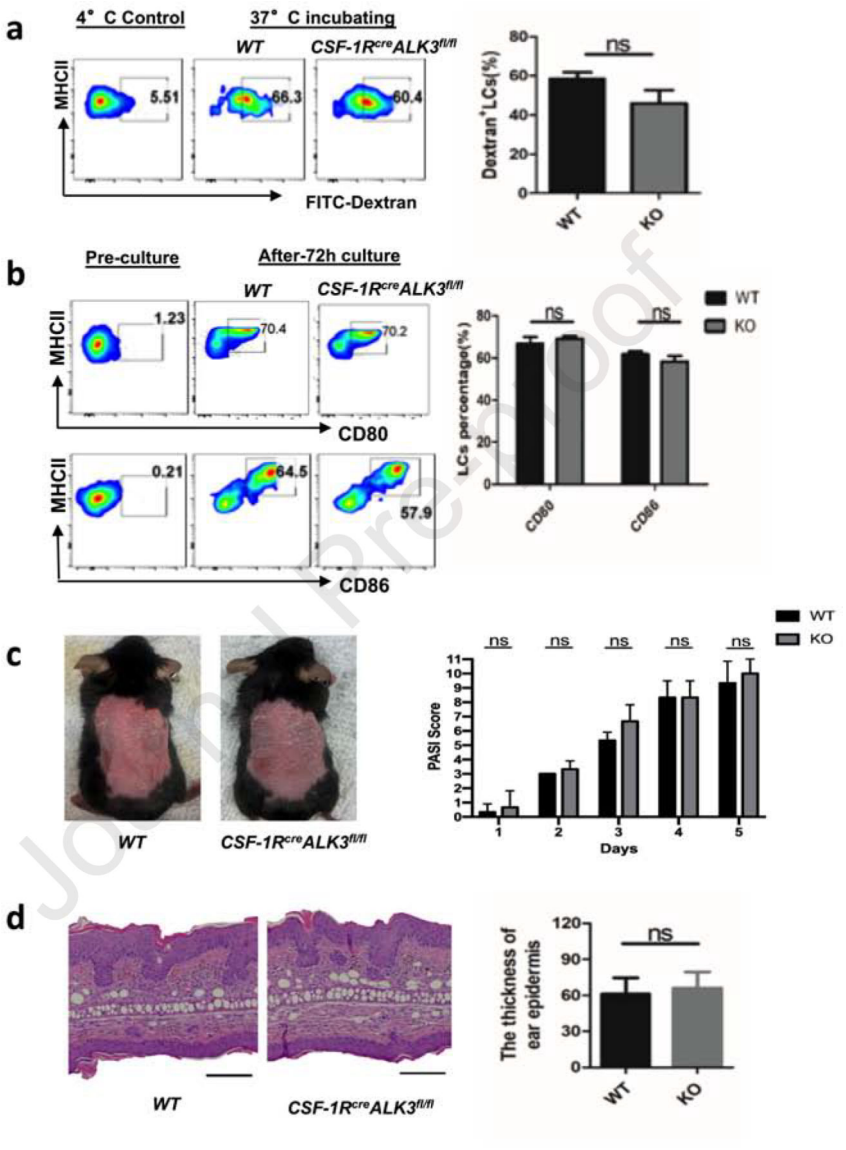

Figure 2. ALK3 is dispensable for LC phagocytosis and maturation and lack of ALK3 does not affect IMQ-induced psoriatic dermatitis in mice.

(**a**) Flow cytometry analysis showing the frequencies of epidermal LCs ($CD45^+MHCII^+FITC\text{-}Dextran^{hi}$) after 37°C or 4°C (as control) incubation for 45 minutes between *CSF-1R^{iCre}ALK3$^{fl/fl}$* (KO) and WT mice (n=3). (**b**) Flow cytometry analysis showing the ratio of mature LCs ($CD45^+MHCII^+CD80^{hi}$ and $CD45^+MHCII^+CD86^{hi}$) from *CSF-1R^{iCre}ALK3$^{fl/fl}$* (KO) and WT mice after 72 hours of *in vitro* culture (n=3). **(c)** The back skin and ears of mice were treated with 5% imiquimod (IMQ) for 5 consecutive days. PASI (cumulative scores of erythema, scaling and thickness) of their back skin was scored daily for 5 days, showing no significant difference between *CSF-1R^{iCre}ALK3$^{fl/fl}$* (KO) and WT mice. **(d)** Hematoxylin and eosin staining of ear skin sections on day 6 (scale bar =100μm), the epidermal thickness was measured with the assistance of Image-J software (ns, no significant difference).