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Non-redundant roles of keratinocyte-derived IL-34 and neutrophil-derived CSF1 in Langerhans cells renewal in the steady-state and skin inflammation

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Abstract

IL-34 and colony stimulating factor 1 (CSF1) are two alternative ligands for the CSF1 receptor that play non-redundant roles in the development, survival and function of tissue macrophages and Langerhans cells (LCs). In this study, we investigated the spatio-temporal production of IL-34 and its impact on skin LCs in the developing embryo and adult mice in the steady state and during inflammation using *Il34*^{LacZ} reporter mice and newly generated inducible *Il34*-knockout mice. We found that IL-34 is produced in the developing skin epidermis of the embryo, where it promotes the final differentiation of LC precursors. In adult life, LCs required IL-34 to continually self-renew in the steady-state. However, during UV-induced skin damage, LCs regeneration depended on neutrophils infiltrating the skin, which produced large amounts of CSF1. We conclude that LCs require IL-34 when residing in fully differentiated and anatomically intact skin epidermis, but rely on neutrophil-derived CSF1 during inflammation. Our demonstration that neutrophils are an important source of CSF1 during skin inflammation may exemplify the mechanism through which neutrophils promote their subsequent replacement with mononuclear phagocytes.

Keywords

Langerhans cells; cell development; cytokines; IL-34; CSF1; skin inflammation

Introduction

Langerhans cells (LCs) are a unique subset of dendritic cells (DCs) that reside in the epidermis of the skin [1-3]. Because of their location, LCs are considered to be critical inducers of skin immunity, although their precise role in the initiation and control of

Authorship Contributions

Y.W. designed, performed, analyzed experiments and wrote the manuscript; M.B. and W.V. carried out immunohistochemical analysis; T.K.U. performed bone marrow transplant. S.G. generated the UBC-Cre/ERT2.*Il34*$^{Flox/LacZ}$ mice and setup timed-mating experiments; M.Co. supervised research and wrote the manuscript.

Conflict of Interest

Yaming Wang is an employee of Lilly & Company.

cutaneous immune responses remains a matter of active debate [4, 5]. Additionally, LCs have been implicated in the pathogenesis of skin tumors independent of immune mechanisms, because of their capacity to transform polyaromatic hydrocarbons into potent carcinogens [6]. The development of LCs encompasses several steps [7-11]. Initially LC progenitors develop in the yolk sac and fetal liver and migrate into the primitive skin that lacks a mature epidermal layer. Towards the end of the embryonic life, the skin develops an epidermal layer and acquires barrier function conferred by the stratus corneum. LC precursors move into the epidermis and differentiate into mature LCs shortly after birth. During the first week of life, LCs undergo a burst of proliferation, after which LCs self-renew by slowly dividing throughout life.

Colony-stimulating factor-1 receptor (CSF1R) is crucial for both development and maintenance of LCs, as demonstrated by the severe defect in LCs in *Csf1r*$^{-/-}$ mice [12, 13] and wild-type (WT) mice treated with CSF1R-blocking antibodies. CSF1R has two distinct ligands: colony-stimulating factor-1 (CSF1), which is produced by various cell types [14, 15]; and interleukin-34 (IL-34), which is produced by keratinocytes and neurons [15-18]. CSF1 and IL-34 play distinct roles in LCs development. LCs numbers are considerably reduced in newborn but are normal in adult CSF1-deficient mice (*Csf1*$^{op/op}$) [12, 13, 19], suggesting that CSF1 plays an early role in LCs development but is not required for LCs maintenance. IL-34-deficient mice (*Il34*$^{LacZ/LacZ}$) have a marked defect in LCs at birth that persists in adult mice [17, 18], suggesting that IL-34 promotes LCs development and possibly LCs persistence throughout life.

Following exposure to ultraviolet (UV) light irradiation or other inflammatory stimuli, LCs migrate into skin-draining lymph nodes or die, depleting the LCs niche [20-22]. Regeneration of skin LCs relies on bone marrow-derived monocytes that migrate into the skin and differentiate into LCs [13, 23-25]. While genetic ablation of *Csf1r* in LCs impairs LCs regeneration following inflammation [18], IL-34 and CSF1 play non-redundant roles. In *Il34*$^{LacZ/LacZ}$ mice, initial LCs regeneration is intact but the persistence of newly generated LCs is impaired. In contrast, LCs repopulation is delayed in *Csf1*$^{op/op}$ mice, indicating that CSF1 is essential for the early phase of LCs regeneration [13], although the source of CSF1 during this process is unknown.

In this study, we investigated the spatio-temporal production of IL-34 and its impact on skin LCs in the developing embryo and adult mice in the steady state and during inflammation. We found that IL-34 promotes LCs differentiation and survival in synchrony with maturation of the skin epidermis. Moreover, we generated a novel inducible *Il34*-knockout mouse line and demonstrated that IL-34 is required for the maintenance of LCs in the intact epidermis of adult mice. Remarkably, after UV-mediated skin damage, LCs repopulation in the skin depended on infiltrating neutrophils that produced CSF1, whereas IL-34 was dispensable. Collectively, our results demonstrate distinct spatio-temporal roles of IL-34 and CSF1 in LCs development. Renewal of LCs residing in a fully differentiated and anatomically intact skin epidermis requires IL-34, whereas renewal of LCs in the inflamed skin requires neutrophil-derived CSF1.

Results

IL-34 controls LCs differentiation and survival in synchrony with maturation of the skin epidermis

To determine the spatial-temporal expression of IL-34 at successive developmental stages of the skin, we stained *Il34*$^{+/LacZ}$ embryos with Xgal, which detects expression of the β-galactosidase reporter driven by the IL-34 promoter. Early on (embryonic day 12.5, E12.5), β-galactosidase was expressed neither in the yolk sac nor in the limb buds that comprise the primitive skin (**Figure 1A, B**). β-galactosidase expression became detectable in the skin at E17.5, concomitant with the appearance of mature epidermis and was sustained after birth (**Figure 1C**). Real time PCR confirmed that *Il34* mRNA is highly expressed in the epidermis of E18.5 embryos, whereas *Csf1* mRNA is predominantly expressed in the dermis (**Figure 1D**). Thus, IL-34 production substantially overlaps with maturation of the skin epidermis.

We next analyzed the impact of IL-34 deficiency on LC precursors and mature LCs before and after development of the epidermis. At E12.5, we examined primitive macrophages (defined as CD11b^{+}F4/80^{+} cells) and found that they were equally represented in the yolk sac and in the total skin of *Il34*$^{LacZ/LacZ}$ and *Il34*$^{+/LacZ}$ embryos (**Figure 1E**). At E18.5, we examined fetal liver-derived monocytes (CD11b^{HI}F4/80^{LO} cells) and LC precursors (CD11b^{LO}F4/80^{HI} cells) as defined by Hoeffel *et al.* [7] (**Supporting information Fig. 1**). Equivalent numbers of both populations were present in the skin of *Il34*$^{LacZ/LacZ}$ and *Il34*$^{+/LacZ}$ embryos (**Figure 1F)**. Thus, IL-34 deficiency has no detectable effect on LC precursors before the complete maturation of the epidermis. In contrast, LC precursors (CD11b^{LO}F4/80^{HI} cells) and mature LCs (CD11b^{+}MHCII^{+} cells) were markedly reduced in the skin of *Il34*$^{LacZ/LacZ}$ neonates in comparison to *Il34*$^{+/LacZ}$ neonates (**Figure 1G, H**). Histological analysis confirmed a selective reduction of CD11b^{+} LCs in the epidermis of *Il34*$^{LacZ/LacZ}$ 2-day-old (P2) neonates and E18.5 embryos compared to *Il34*$^{+/LacZ}$ controls (**Table 1, Supporting information Fig. 2A, B**), suggesting that LCs require IL-34 once they migrate into the epidermis. Consistent with these results, cell cycle analysis revealed less proliferation and more apoptosis among LC precursors in the skin of *Il34*$^{LacZ/LacZ}$ neonates than in the skin of *Il34*$^{+/LacZ}$ neonates (**Figure 1I**). We conclude that IL-34 is produced as soon as the skin epidermis matures and that it becomes essential for LCs when they move into the epidermis to take up their final residence. In contrast, IL-34 deficiency has no detectable impact on LC precursors before maturation of the epidermis, most likely because LC precursors are confined to the prospective dermis of the immature skin, where CSF1 can support their survival (**Figure 1J**).

IL-34 maintains LCs homeostasis

Although *Il34*$^{LacZ/LacZ}$ mice lack LCs at birth and throughout life [17, 18], it is unclear whether IL-34 is essential only for LCs development or also for LCs maintenance in the epidermis. To answer this question, we generated an inducible *Il34*-knockout mouse that harbors one *Il34*^{LacZ} allele and one *Il34* floxed allele (*Il34*^{Flox}) as well as a transgene encoding Cre recombinase fused to a triple mutant form of the human estrogen receptor under control of the human ubiquitin C (UBC) promoter (UBC-Cre/ERT2). Administration of Tamoxifen to these mice effectively deleted *Il34* in the skin, resulting in marked reduction

of *Il34* mRNA (**Figure 2A**) as well as an almost complete depletion of LCs within 10 days (**Figure 2B**). We conclude that IL-34 is essential for maintenance of LCs in steady state and that induction of IL-34 deficiency in the skin leads to rapid depletion of LCs.

LCs regeneration after UV-induced skin injury requires neutrophil-derived CSF1

It has been shown that regeneration of LCs from circulating monocytes after UV-induced damage is IL-34 independent, whereas maintenance of newly generated LCs requires IL-34 [17, 18]. Accordingly, LCs repopulated the epidermis of $Il34^{LacZ/LacZ}$ mice 3 weeks after UV-induced injury of ear skin but disappeared after 9 weeks (**Figure 3A, B**). A transient increase in *Csf1* mRNA in UV-damaged skin has been reported [18], which suggests that CSF1 may be sufficient for the generation of monocyte-derived LCs in the absence of IL-34. However, it is possible that LCs generated in the presence or absence of IL-34 are functionally distinct. To address this question, we compared various features of LCs repopulating WT and $Il34^{LacZ/LacZ}$ mice 21 days after UV treatment and found them virtually indistinguishable in terms of phenotypic markers (**Fig. 3C**), morphology (**Fig. 3D**) and gene expression profiles (**Fig. 3E, F**). Thus, these data indicate that IL-34 does not play a relevant role in the regeneration of LCs after UV-induced skin injury.

We next asked: what is the cellular source of CSF1 for LCs regeneration? We found that CSF1 expression peaked one week after skin injury, coincident with the appearance of inflammation and swelling, whereas IL-34 expression decreased, likely due to keratinocytes injury (**Fig. 4A**). To determine the origin of CSF1 during LCs repopulation, we reconstituted $Il34^{LacZ/LacZ}$ mice with bone marrow from WT or CSF1-deficient ($Csf1^{op/op}$) mice after lethal irradiation. We then exposed mice to UV irradiation and determined the frequencies of LCs in the epidermis after 21 days. Repopulation of LCs in $Il34^{LacZ/LacZ}$ mice reconstituted with $Csf1^{op/op}$ bone marrow was significantly attenuated (**Figure 4B**), indicating that the CSF1 required for inducing the generation of LCs after inflammation derives at least in part from cells of hematopoietic origin. Major $CD45^+$ leukocyte populations present in the skin after UV radiation included $Ly6G^+Ly6C^+$ neutrophils and $Ly6C^+$ monocytes, both of which were recruited in an IL-34-independent fashion (**Fig. 4C, D**). We noticed that neutrophils represented the major population of $CD45^+$ leukocytes in the skin and expressed more *Csf1* mRNA than all other cell types in the skin (**Fig. 4E**), suggesting that neutrophils may be the major source of CSF1 for LCs regeneration. Indeed, systemic depletion of neutrophils in $Il34^{LacZ/LacZ}$ mice during UV-mediated skin injury using an anti-Ly6G antibody significantly attenuated generation of LCs (**Fig. 4F**), even though the efficiency of depletion in the skin was approximately 50% (data not shown). We conclude that that the generation of monocyte-derived LCs after UV-induced damage is dependent on neutrophil-derived CSF1.

Discussion

In this study, we defined the spatio-temporal expression of IL-34 and its impact on the generation of LCs in the embryo and adult in the steady-state and during inflammation. It was previously shown that IL-34 deficiency affects the skin content of LC precursors [18]; however, the specific stage of LCs differentiation at which IL-34 is required was not

delineated. We showed that IL-34 is produced in the developing epidermis in the embryo and is selectively required during the final stage of LCs development, when LC precursors migrate into the epidermis and differentiate into mature LCs. Using a newly generated inducible *Il34*-knockout mouse, we demonstrated that LCs are immediately affected by the induced depletion of IL-34. Thus, in adult life, LCs continually require IL-34 for self-renewal in the steady-state.

After UV-induced skin damage, however, LCs regeneration was independent of IL-34 and relied on CSF1. Moreover, we noted that the morphological, phenotypic and transcriptional profiles of monocyte-derived LCs repopulating the skin in the presence or absence of IL-34 were almost superimposable. We postulate that once LC progenitors have differentiated in the epidermis, their features are largely driven by the tissue microenvironment, which dictates the final functional profile regardless of whether their initial generation was IL-34- or CSF1-dependent. Importantly, we found that neutrophils infiltrating the skin were a major source of CSF1 required for LCs reconstitution from circulating monocytes. Our study demonstrates for the first time that neutrophils are substantial producers of CSF1 during skin inflammation. Since $Csf1^{op/op}$ mice have been shown to have reduced cutaneous expression of *Il34* mRNA [26], it is possible that neutrophil-derived CSF1 also contributes to subsequent production of IL-34 during the repair phase. The capacity of neutrophils to produce large amounts of CSF1 is consistent with the neutrophil RNA profile reported by the Immgen consortium (http://www.immgen.org). Moreover, neutrophil production of CSF1 has been noted in another context, specifically during activation of neutrophils by sodium caseinate, which is used in a mouse experimental model of peritoneal inflammation [27, 28]. The demonstration that neutrophils are an important source of CSF1 illustrates a mechanism through which neutrophils promote their subsequent replacement with mononuclear phagocytes during skin inflammation. In conclusion, our study shows that LCs depend on IL-34 when residing in a fully differentiated and anatomically intact skin epidermis, but rely on neutrophil-derived CSF1 in inflamed skin.

Materials and Methods

Mice and Treatment

$Il34^{LacZ/LacZ}$ and $Il34^{Flox/LacZ}$ mice were generated as previously described [17]. UBC-Cre/ERT2 mice were purchased from the Jackson Laboratory. C57BL/6 WT control mice were bred in house. All animal studies were approved by the Washington University Animal Studies Committee. Tamoxifen working solution was prepared by first dissolving 100mg of Tamoxifen citrate (Sigma) in 1mL 100% ethanol before diluting into sunflower oil to a final concentration of 10mg/mL. Mice were injected with 200uL of Tamoxifen working solution daily i.p. for 7 days. To induce skin inflammation, mice were treated with UVB for 30min as previously described [13].

Bone marrow chimeras

Bone marrow cells were isolated from WT and $Csf1^{op/op}$ mice and retro-orbitally injected into lethally irradiated $Il34^{LacZ/LacZ}$ mice. Chimeric mice were housed for 6 weeks before UV treatment.

Immunohistochemistry

Sections for immunohistochemistry were obtained from formalin-fixed paraffin-embedded or frozen tissues. For morphology, H&E stained slides were evaluated. Five micron cryostat sections were air dried overnight, fixed in acetone for 10 min and stained by using the β-Galactosidase Reporter gene staining kit (Sigma) as described previously. Nuclei were counterstained with fast red or giemsa. A similar method was also used to detect β-Galactosidase reporter activity in embryos. Four-micron sections from fixed tissues were stained using anti-CD11b mAb (M1/70) and reactivity revealed using Rat-on-mouse HRP-Polimer (Biocare Medical) followed by diaminobenzidine (DAB). Nuclei were counterstained with haematoxylin. To examine LCs morphology, sorted LCs were centrifuged onto glass slides, air dried overnight and counterstained with Hema3 Stat Pack (Fisher Diagnostics). All images were acquired using an Olympus DP70 camera mounted on an Olympus Bx60 microscope.

Cell isolation

Yolk sacs and limb buds were digested with collagenase D (100ug/mL, Sigma) for 30 min and vortexed to achieve single cell suspension. Epidermis was isolated as previously described [17]. To examine skin infiltrating monocytes and neutrophils, ear skin was cut into fine pieces and digested with collagenase VIII (4830U/mL, Sigma) and DNase I (0.1mg/mL, Sigma) for 60min.

Flow cytometry analysis

LCs and other cell types in single cell suspension were incubated with HB-197 supernatant (ATCC) for Fc receptor blockade and stained for CD11b, CD11c, CD326 (eBioscience), CD45, F4/80, Ly6C, MHC-II (BD Pharmingen), Ly6G (BioLegend). For intracellular staining with CD207 (L31, eBioscience), cells after surface staining were fixed and permeabilized with Cytofix/Cytoperm buffer (BD Bioscience). Cells were processed on a FACSCalibur or FACSCanto II (BD Bioscience) and analyzed with FlowJo (Treestar).

Quantitative PCR and microarray analysis

RNA was extracted from skin using the RNeasy Fibrous Tissue Mini kit (QIAGEN) as recommended by the manufacturer. cDNA was synthesized from RNA with Superscript III first-strand synthesis system for RT-PCR (Invitrogen), and relative levels of RNA expression were determined by qPCR. SYBR Green PCR master mix (Bio-Rad Laboratories) and an ABI7000 machine (Applied Biosystems) were used according to the manufacturer's instructions. Follo wing oligos were used: *Il34* Forward: 5'-ACTCAGAGTGGCCAACATCACAAG-3', *Il34* reverse: 5'-ATTGAGACTCACCAAGACCCACAG-3', *Csf1* forward: 5'-TACAAGTGGAAGTGGAGGAGCCAT-3', *Csf1* reverse: 5'-AGTCCTGTGTGCCCAGCATAGAAT-3', *Hprt* Forward: 5'-GCAGTACAGCCCCAAAAT-3', *Hprt* Reverse: 5'-AACAAAGTCTGGCCTGTATCCAA-3'. For Mouse Gene 1.0 ST Arrays, RNA was extracted from sorted LCs using the Arturus PicoPure RNA Isolation Kit (Applied BioSystems), amplified with a WT Expression kit (Ambion) and labeled, fragmented and

hybridized with a WT Terminal Labeling and Hybridization kit (Affymetrix). Data were processed by robust multiarray average (RMA) normalization, and expression values were modeled with ArrayStar software (DNASTAR).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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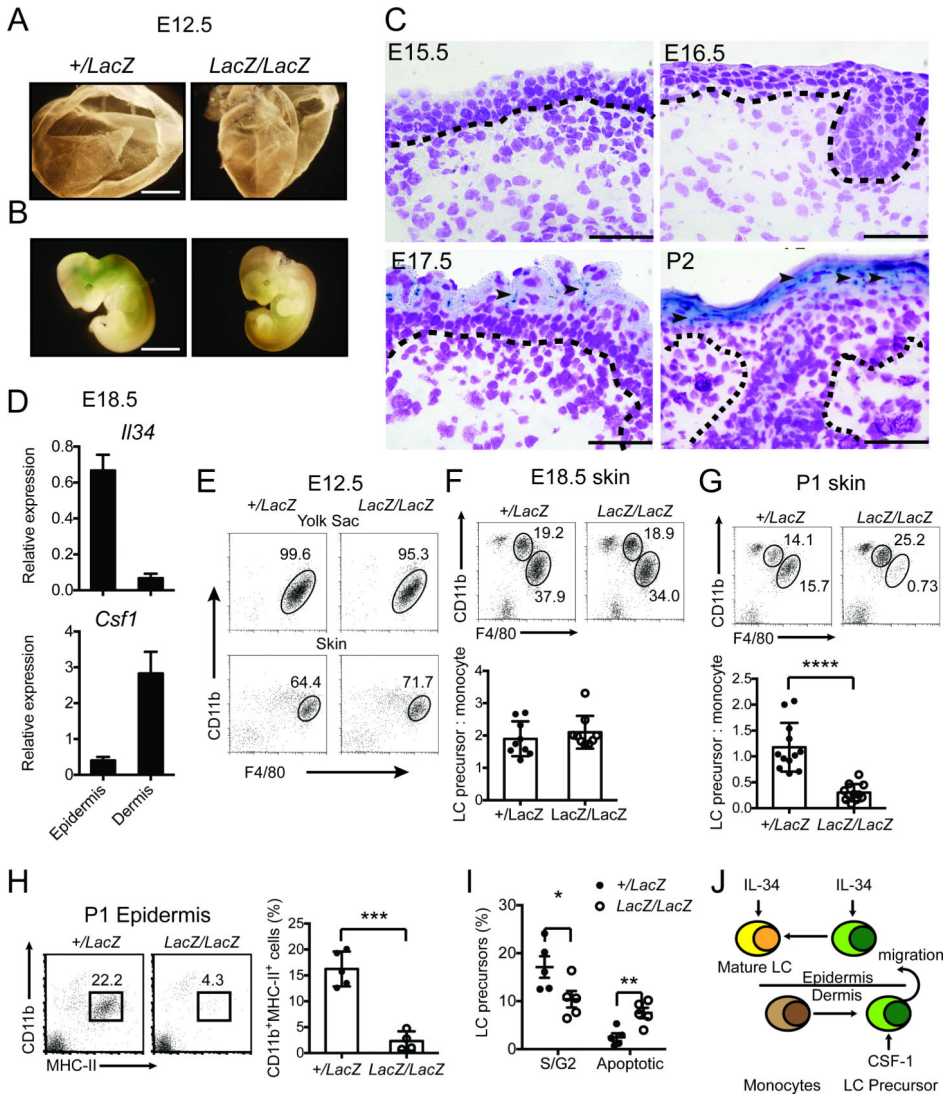

Figure 1. IL-34 promotes survival and differentiation of LC precursors in synchrony with maturation of the skin epidermis in the embryo

(**A-J**) *Il34*$^{+/LacZ}$ and *Il34*$^{LacZ/LacZ}$ embryos (n=3-12) were examined at different stages of embryogenesis. LacZ expression and frequencies of tissue monocytes, macrophages, LC precursors and mature LCs were examined. (**A, B**) X-gal staining of E12.5 yolk sac (A) and embryos (B). Original magnification 4X. Scale bar=2mm. (**C**) X-gal staining of the skin of E15.5, E16.5, E17.5 embryos and P2 neonates. Original magnification 400X. Scale bar= 50μm. (A-C) Images shown are representative of 2 independent experiments with 3-4 embryos per group. (**D**) *Il34* and *Csf1* mRNA expression in E18.5 epidermis and dermis. (**E**) FACS analysis of frequencies of primitive macrophages among CD45$^+$ cells in the yolk sac and skin of E12.5 embryos. (**F, G**) FACS analysis of frequencies of CD11b^{hi}F4/80^{lo} fetal liver-derived monocytes and CD11b^{lo}F4/80^{hi} LC precursors among CD45$^+$ cells in the skin of E18.5 embryos (F) and P1 neonates (G). (**H**) FACS analysis of LC precursors in the epidermis of P1 neonates. (**I**) Cell cycle analysis of LC precursors in the skin of P1 neonates. (D-I) Data are shown as mean ±SD (n= 3-5) and are representative of two

independent experiments (D, E) or pooled from three independent experiments (F-I). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Student's *t*-test. (**J**) Proposed model for the role of IL-34 and CSF1 in prenatal and perinatal development of LCs..

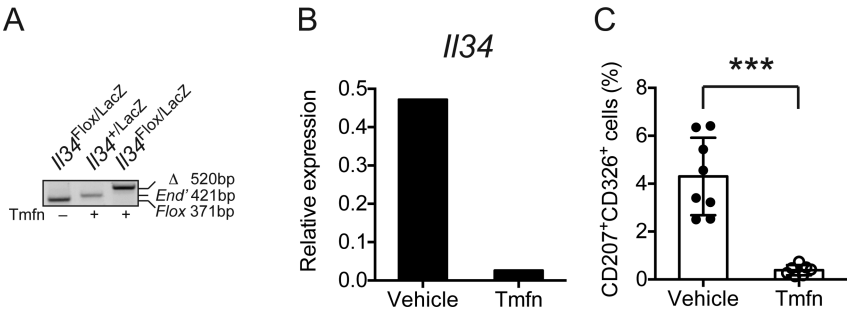

Figure 2. LCs survival in the adult requires constant IL-34 signaling

(**A-C**) UBC-Cre/ERT2.*Il34*$^{Flox/LacZ}$ mice (n=4) were treated with Tamoxifen (Tmfn) i.p. for 7 days. Ear skin was analyzed 9 days after the last treatment. UBC-Cre/ERT2.*Il34*$^{+/LacZ}$ mice were used as a negative control. (**A**) Efficacy of Cre-mediated recombination after Tamoxifen treatment was assessed by a PCR assay that detected endogenous wild-type *Il34* allele (End), floxed *Il34* allele (Flox) and Cre excised allele (Δ). Blot is representative of three independent experiments. (**B**) Ablation of *Il34* mRNA after Tamoxifen treatment was confirmed by real time PCR. (**C**) Frequencies of CD207$^+$ (Langerin$^+$) CD326$^+$ (Epcam$^+$) LCs after Tmfn treatment were measured by flow cytometry. Data are shown as mean±SD (n=6-8 mice/group) and are pooled from two independent experiments. ***p<0.001, Student's *t*-test.

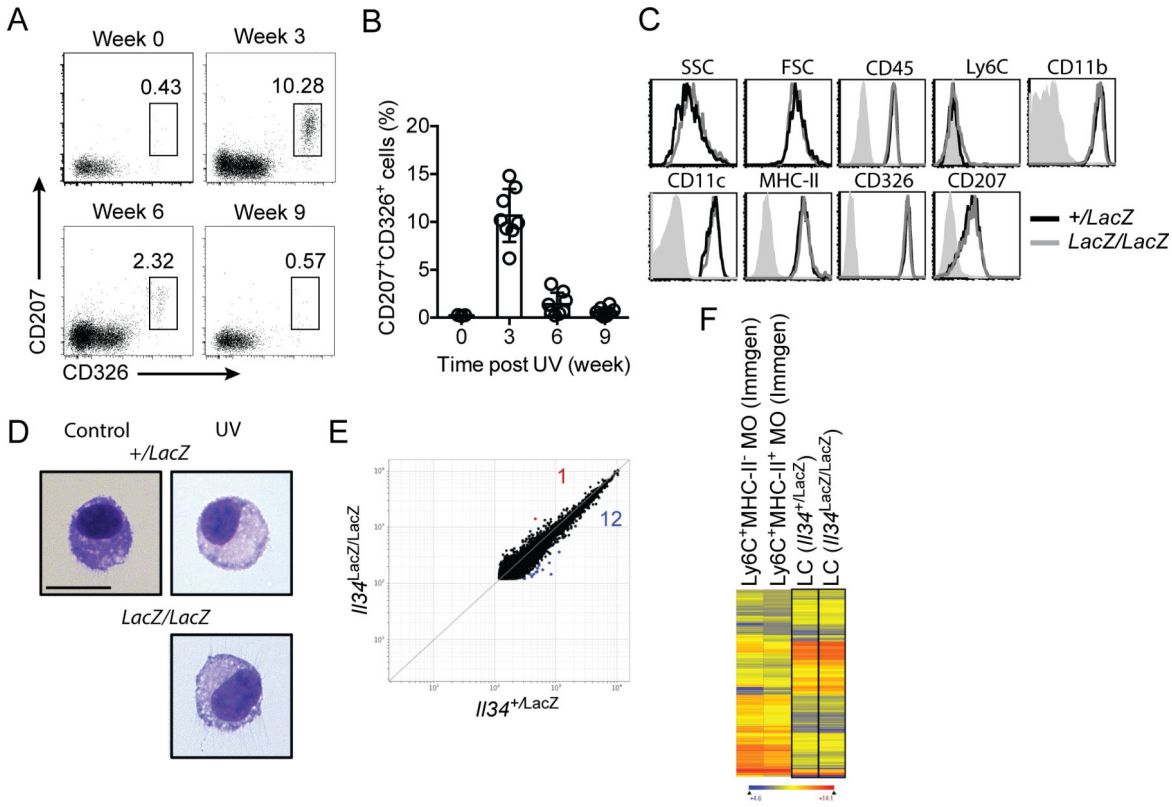

Figure 3. IL-34 is essential for long-term maintenance of LCs after UV injury

(**A, B**) Frequencies of LCs in the epidermis of *Il34*$^{LacZ/LacZ}$ mice at various time points post UV treatment. Data are shown as mean±SD (n=8-10) and are pooled from two independent experiments. (**C, D**) Comparison of phenotypic markers by flow cytometry (C) and cellular morphology by cytospin (D) of LCs in *Il34*$^{+/LacZ}$ and *Il34*$^{LacZ/LacZ}$ mice 21 days post UV treatment. Data shown are representative of two independent experiments. Original magnification 400X. Scale bar= 10μm. (**E, F**) Gene expression analysis of FACS-sorted LCs from *Il34*$^{LacZ/LacZ}$ or *Il34*$^{+/LacZ}$ mice 21 days post UV treatment. (E) Scatter plot shows direct comparison of genes with expression value greater than 120 between LCs from *Il34*$^{LacZ/LacZ}$ and *Il34*$^{+/LacZ}$ mice. (F) Heat map shows very similar expression of a set of genes in LCs purified from *Il34*$^{LacZ/LacZ}$ and *Il34*$^{+/LacZ}$ mice after UV irradiation, as compared to Ly6C^{+} MHC class II^{-} monocytes and Ly6C^{+} MHC class II^{+} monocytes, which were used as reference. This set of genes includes genes that are at least 4-fold enriched in either LCs (GSE) or Ly6C^{+} blood monocytes (www.immgen.org, GSE15907). Gene expression data shown are representative of the average of two independent experiments.

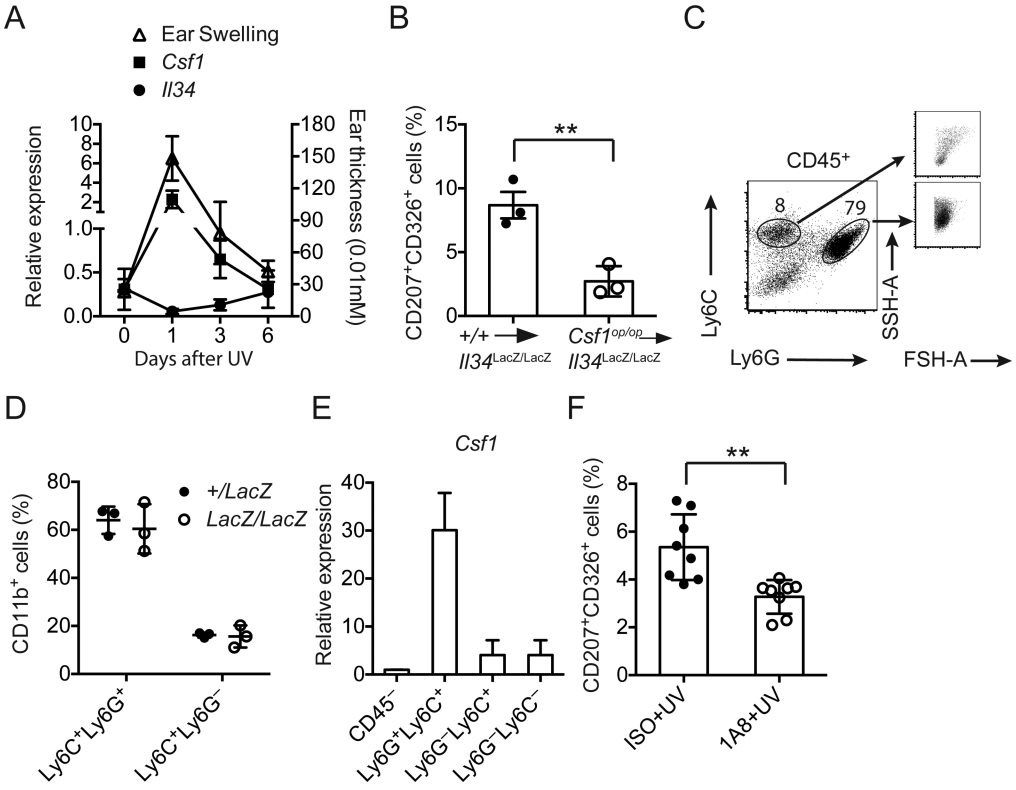

Figure 4. LCs regeneration following UV-induced skin damage requires neutrophil-derived CSF1

(**A**) Induction of inflammation (as measured by skin thickness) and the expression of *Csf1* and *Il34* mRNA were determined in the ears WT mice after UV treatment. (**B**) $Il34^{LacZ/LacZ}$ mice were reconstituted with bone marrow cells from WT or $Csf1^{op/op}$ mice after lethal irradiation. LCs regeneration was determined 21 days after UV treatment by flow cytometry. (**C, D**) $Ly6G^+Ly6C^+$ neutrophils and $Ly6C^+$ monocytes represent ~90% of the $CD45^+$ cells that are recruited to the UV damaged skin. (C) Right panels show differences in size (FSH-A) and granularity (SSH-A) between $Ly6G^+Ly6C^+$ neutrophils and $Ly6C^+$ monocytes. (D) IL-34 deficiency does not affect the percentages of infiltrating $Ly6G^+Ly6C^+$ neutrophils and $Ly6C^+$ monocytes after UV irradiation. (**E**) qPCR analysis of *Csf1* mRNA expression in several population of cells in UV damaged skin. Infiltrating neutrophils express highest level of *Csf1*. (**F**) Anti-Ly6G 1A8 mAb injection attenuates the regeneration of LCs in $Il34^{LacZ/LacZ}$ mice in response to UV. $Il34^{LacZ/LacZ}$ mice were treated with 200 μg 1A8 mAb i.p. on day 5 post UV treatment and every other day for 15 days. Frequencies of LCs were determined 21 days post UV. (A-F) Data are shown as mean±SD (n=3-8) and are representative of two independent experiments. **p<0.01, Student's *t*-test.

Table 1

Impact of IL-34 deficiency on $CD11b^+$ cells in the epidermis of E18.5 embryos and 2-day-old (P2) neonates

E18.5			P2		
+/LacZ	*LacZ/LacZ*	*P* value	*+/LacZ*	*LacZ/LacZ*	*P* value
2.93±1.79	0.75±1.29	0.01	4.08±1.87	0.67±1.30	0.04