

CCCTC-binding factor controls the homeostatic maintenance and migration of Langerhans cells

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Background: Langerhans cells (LCs) are skin-resident dendritic cells (DCs) that orchestrate skin immunity. CCCTC-binding factor (CTCF) is a highly conserved DNA-binding protein that regulates higher-order chromatin organization and is involved in various gene regulation processes.

Objective: We sought to clarify a possible role for CTCF in LC homeostasis and function *in vivo*.

Methods: We used a conditional gene deletion mouse system to generate DC- and LC-specific CTCF-ablated mice. Short hairpin RNA-mediated RNA interference was used to silence CTCF expression in human monocyte-derived Langerhans cells. DC populations were assessed by using flow cytometry and immunofluorescence. Gene expression arrays were performed to identify genes regulated by CTCF in LCs. Contact hypersensitivity and epicutaneous sensitization responses were measured to examine the functional significance of CTCF ablation.

Results: DC-specific CTCF deletion led to a reduced pool of systemic DCs, with LCs most severely affected. Decreases in epidermal LC numbers were specifically associated with self-turnover defects. Interestingly, CTCF-deficient LCs demonstrated impaired migration out of the epidermis. Whole-transcriptome analyses revealed that genes that promoted cell adhesion were highly expressed, but CCR7 was downregulated in CTCF-depleted LCs. Hapten-induced contact hypersensitivity responses were more sustained in LC-specific CTCF-deficient mice, whereas epicutaneous sensitization to protein antigen was attenuated, indicating that CTCF-dependent LC homeostasis is required for

optimal immune function of LCs in a context-dependent manner.

Conclusion: Our results show that CTCF positively regulates the homeostatic pool and the efficient emigration of LCs, which are required for modulating the functional immune network of the skin. (J Allergy Clin Immunol 2015;■■■■:■■■-■■■.)

Key words: CCCTC-binding factor, dendritic cells, homeostasis, immune modulation, Langerhans cells, migration

Dendritic cells (DCs) are specialized antigen-presenting cells comprising a heterogeneous population of cells arising from hematopoietic precursors.¹ Langerhans cells (LCs) are nonlymphoid migratory DCs that reside in the skin.² Although LCs are known to promote T-cell immunity, recent evidence has shown that LCs can suppress immune responses in a context-dependent manner.³⁻⁷ LCs develop from myeloid precursors that infiltrate the fetal epidermis in the late stage of embryogenesis.⁸ Immediately after birth, epidermal LC precursors rapidly proliferate and differentiate into mature LCs to complete adult-like LC networks.^{9,10} Unlike other DCs, which are replaced by blood-borne committed precursors,¹¹ LCs are maintained by slow self-turnover in the quiescent skin throughout life.^{12,13} LC homeostasis depends on the signaling of specific cytokines, such as TGF- β /TGF- β receptor II and IL-34/colony-stimulating factor 1 receptor (CSF-1R).¹⁴

CCCTC-binding factor (CTCF) is a highly conserved, 11-zinc finger protein that contains a DNA-binding domain.¹⁵ Its DNA occupancy is widely distributed throughout the mammalian genome, and 30% to 60% of binding is cell type specific, partly because of differences in DNA methylation.¹⁶ CTCF function is largely attributed to its specific ability to mediate long-range DNA interactions that affect the 3-dimensional chromatin structure.^{17,18} Topologic genetic remodeling can potentially regulate the expression of cell differentiation- and cell function-associated genes.^{15,19} CTCF plays multiple roles in hematopoietic cell lineages; it regulates early thymocyte development,²⁰ cell differentiation, and cytokine production in helper T cells,^{21,22} as well as T-cell receptor α gene recombination.²³ CTCF-dependent chromatin organization essentially modulates immunoglobulin gene rearrangement in B lymphocytes.²⁴⁻²⁶ In the myeloid lineage CTCF is involved in macrophage development and function,²⁷ and CTCF overexpression alters DC survival, proliferation, and differentiation.²⁸ However, the precise role of CTCF in controlling DC and LC homeostasis *in vivo* is largely unclear.

In this study we used a gene conditional knockout (cKO) mouse system to demonstrate the role of CTCF in epidermal LC homeostasis and function. Our results highlight an essential association between CTCF and LCs and position CTCF as a crucial homeostatic regulator of LCs.

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Abbreviations used

BM:	Bone marrow
cDC:	Conventional dendritic cell
CHS:	Contact hypersensitivity
cKO:	Conditional knockout
CSF-1R:	Colony-stimulating factor 1 receptor
CTCF:	CCCTC-binding factor
DC:	Dendritic cell
dDC:	Dermal dendritic cell
DNFB:	2,4-Dinitrofluorobenzene
FACS:	Fluorescence-activated cell sorting
FITC:	Fluorescein isothiocyanate
LC:	Langerhans cell
LN:	Lymph node
MDLC:	Monocyte-derived Langerhans cell
MHCII:	MHC class II
OVA:	Ovalbumin
SDLN:	Skin-draining lymph node
WT:	Wild-type

METHODS

Full details of the methods used in this study are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**Decreased pool of multiple DC lineages in DC-specific CTCF-deficient mice**

To examine the role of CTCF in the DC lineage *in vivo*, we generated DC-specific CTCF-deficient mice (CD11c-cKO; see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). Depletion of endogenous CTCF in the DC lineage was confirmed in purified splenic CD11c⁺ DCs (see [Fig E1, B](#)). To assess whether CTCF deletion influenced overall DC homeostasis, we compared the proportions of individual DC subsets in the lymphoid and nonlymphoid organs. Lymphoid conventional dendritic cells (cDCs) in the spleen and skin-draining lymph nodes (SDLNs) were decreased by approximately 50% in CD11c-cKO mice compared with those seen in wild-type (WT) mice, and the numbers of plasmacytoid DCs were slightly reduced ([Fig 1, A](#), and [C](#), and see [Fig E2](#) in this article's Online Repository at www.jacionline.org). Despite a decrease in lymphoid cDC numbers, their subset division into CD8 α ⁺ and CD11b⁺ cells was comparable ([Fig 1, A](#)).

We then analyzed heterogeneous DC populations in the skin and their corresponding migratory counterparts as a representative of nonlymphoid tissue DCs, which are comprised of epidermal LCs and dermal dendritic cells (dDCs). LCs were diminished by up to 40% in the epidermis of CD11c-cKO mice ([Fig 1, B](#) and [C](#), and see [Fig E2](#)). Interestingly, they exhibited far greater decreases in the dermis and SDLNs, indicating impaired distribution of CTCF-deficient LCs in their migrating compartments. CD103⁺ and CD103[−] dDC numbers were also reduced in the dermis of cKO mice and were further decreased in SDLNs ([Fig 1, B](#) and [C](#), and see [Fig E2](#)). Lymphoid cDCs commonly develop from pre-cDCs, which are late-stage committed progenitors.²⁹ However, the percentage of pre-cDCs was unchanged in WT and CD11c-cKO bone marrow (BM; [Fig 1, D](#)), suggesting that CTCF-deficient cDC quantities are not affected by pre-cDC level. These findings demonstrate a critical role for CTCF in

maintaining the overall population size of terminally differentiated DCs *in vivo*.

Reduced cell numbers with distinct morphologic and surface phenotypes of CTCF-deficient LCs

Because the homeostasis of LCs is distinct from that of other DCs,³⁰ we next established LC-specific CTCF-deficient mice (huLang-cKO; see [Fig E1, A](#)). The efficient excision of the *Ctcf* locus was confirmed by using genomic DNA PCR with LCs sorted by means of flow cytometry (see [Fig E3, A](#) and [B](#), in this article's Online Repository at www.jacionline.org). Whole epidermal sheet staining revealed fewer epidermal MHC class II (MHCII)⁺ LCs in huLang-cKO mice, which was similar to results obtained in the CD11c-cKO epidermis ([Fig 2, A](#)). The reduced number of LCs did not affect the frequency of dendritic epidermal T cells in accordance with a previous report (see [Fig E3, C](#)).³¹ CTCF-deficient LCs increased in cell size and projected more elongated dendrites, and the MHCII staining intensity was higher in cKO LCs than in WT cells ([Fig 2, A](#) and [B](#)). In the steady state CTCF-deficient LCs displayed slightly lower basal levels of CD86 but profoundly greater CD80 and CD40 expression ([Fig 2, B](#)). Moreover, *in vivo* oxazolone treatment also led to an altered pattern of CD86 and CD80 expression in cKO LCs, indicating that CTCF differentially regulates each costimulatory molecule in LCs ([Fig 2, C](#)). Levels of the LC marker langerin were decreased in CTCF-deficient LCs ([Fig 2, B](#)), and a sparse number of Birbeck granules in CTCF-ablated LCs was observed (see [Fig E3, D](#)), thus confirming a positive relationship between langerin and Birbeck granule formation.³²

CTCF-deficient LCs exhibit reduced homeostatic self-turnover

Because CTCF deletion led to fewer epidermal LCs, we determined whether CTCF was responsible for maintaining the epidermal LC pool. CTCF variably contributes to apoptosis in a cell type-specific manner.^{33,34} Enhanced CTCF expression leads to an increase in the apoptosis of BM-derived DCs *in vitro*.²⁸ However, no increases in apoptosis were observed in CTCF-ablated LCs *in situ* (see [Fig E4, A](#), in this article's Online Repository at www.jacionline.org). Moreover, we did not observe differences between the annexin V⁺ population of WT and cKO LCs regardless of LC activation, indicating that apoptosis does not contribute to the decrease in CTCF-deficient LC numbers ([Fig 3, A](#)). LCs sustain their pool through slow self-renewal in the steady-state epidermis.¹² Notably, significantly lower numbers of bromodeoxyuridine-incorporated LCs were quantified in huLang-cKO mice ([Fig 3, B](#)). Intracellular Ki-67 staining revealed a lower proportion of cells within the active phases of the cell cycle in CTCF-deficient LCs (see [Fig E4, B](#)). However, surface TGF- β receptor II and CSF-1R were similarly expressed in WT and cKO LCs ([Fig 3, C](#)). This suggests that the CTCF deficiency-induced reduction in LC numbers is due to an intrinsic defect of self-perpetuation rather than insensitivity to external cytokine signals.

We next examined the initial phase of LC development. Similar infiltrations of CD11b⁺F4/80⁺ myeloid precursors were observed in the WT and huLang-cKO postnatal day 1 epidermis (see [Fig E5, A](#) and [B](#), in this article's Online Repository at www.jacionline.org). Equivalent expansion of MHCII⁺ cells

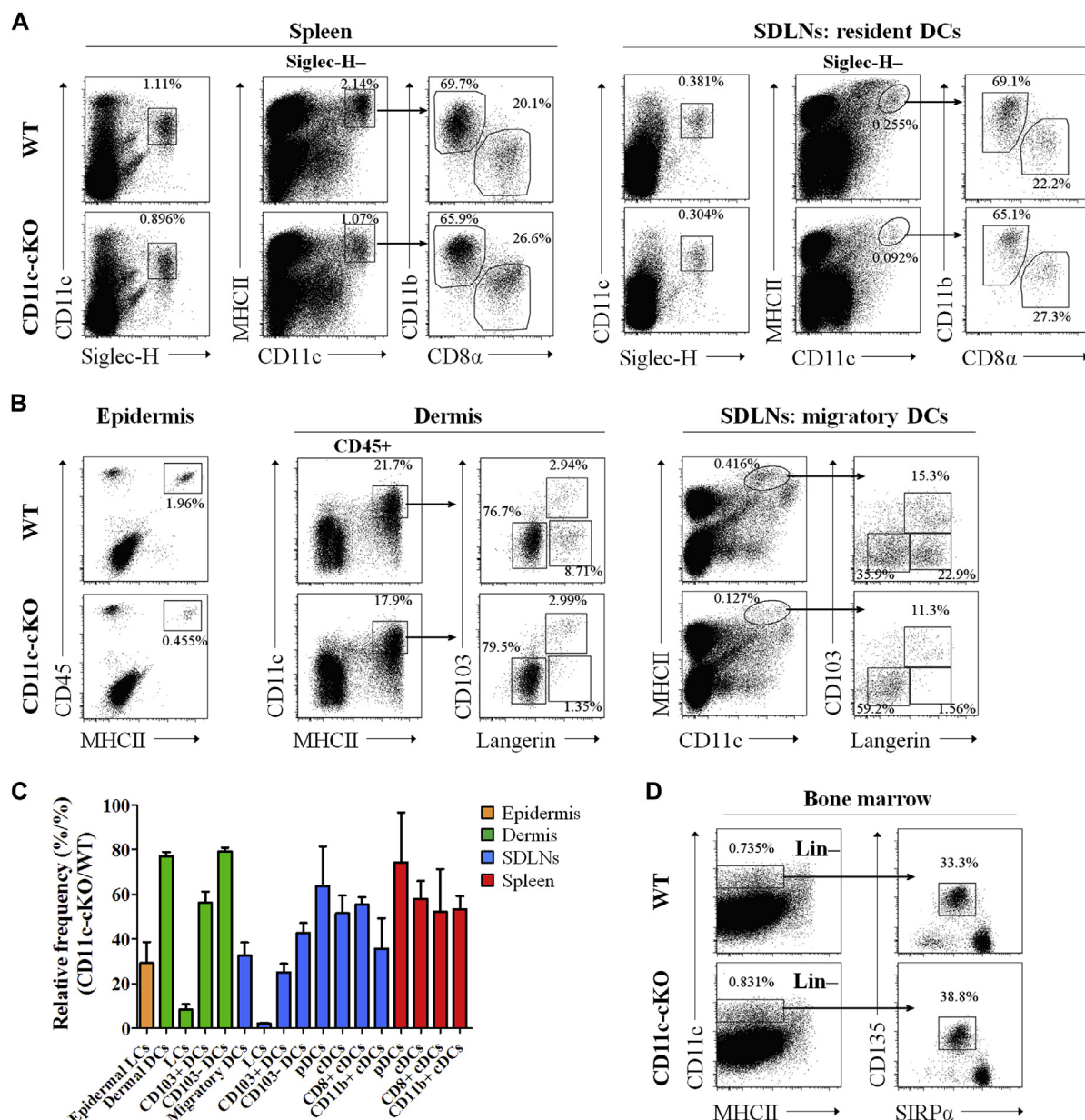


FIG 1. Decrease in the general pool of DCs in mice with DC-specific CTCF depletion *in vivo*. **A**, Lymphoid organ DCs, including plasmacytoid DCs (Siglec-H⁺CD11c^{int}), cDCs (Siglec-H⁺CD11c⁺MHCII⁺), CD8 α ⁺ cDCs (Siglec-H⁺CD11c⁺MHCII⁺CD8 α ⁺CD11b⁺), and CD11b⁺ cDCs (Siglec-H⁺CD11c⁺MHCII⁺CD8 α ⁺CD11b⁺) from the spleen and SDLNs were analyzed by using flow cytometry. **B**, Population frequencies of cutaneous DCs in single-cell suspensions derived from the epidermis, dermis, and SDLNs were analyzed by using flow cytometry. Epidermal LCs (CD45⁺MHCII⁺), DC populations among CD45⁺ leukocytes in the dermis (including LCs [CD11c⁺MHCII⁺Langerin⁺CD103⁺], CD103⁺ dDCs [CD11c⁺MHCII⁺Langerin⁺CD103⁺], and CD103⁺ dDCs [CD11c⁺MHCII⁺Langerin⁺CD103⁺]), and migratory skin DCs (CD11c⁺MHCII^{hi}) in SDLNs were analyzed. **C**, Normalized ratios (relative frequencies) of the percentages of DCs in CD11c-cKO mice against the percentages of the corresponding WT DC subsets. **D**, The proportion of pre-cDCs (Lin⁺CD11c⁺MHCII⁺CD135⁺Sirp α ^{lo}) in the BM. Lin, Lineage (CD3, CD19, NK1.1, Ter119, and B220). Data were pooled from 2 independent experiments with 4 to 5 mice per group. Error bars indicate SEMs.

was seen in day 7 epidermis, and almost all epidermal myeloid leukocytes strongly expressed MHCII and Langerin (see Fig E5, B and C). Thus the decreased number of CTCF-deficient LCs was largely attributed to reduced LC proliferation capacity after completion of the LC network.

CTCF deficiency impairs emigration of LCs from the epidermis

Because the relative LC frequencies in the dermis and SDLNs were lower than those in the epidermis of CD11c-cKO mice (Fig 1, C), we next examined the efficacy of LC migration. In

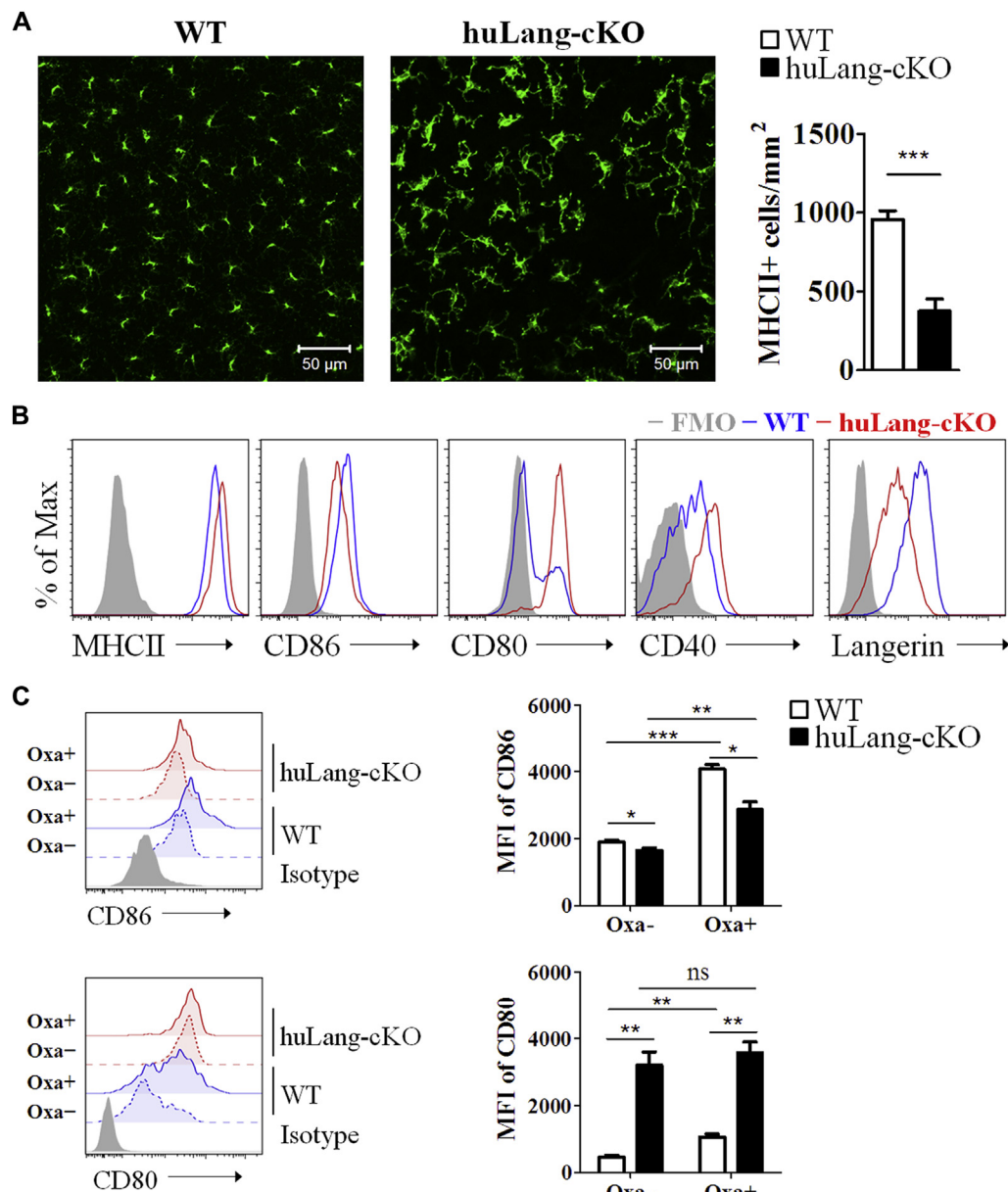


FIG 2. LC-selective loss of CTCF leads to decreases in epidermal LC numbers and distinct phenotypic characteristics of LCs. **A**, Epidermal whole mounts from WT and huLang-cKO mice were stained for MHCII (green), and the cellular densities for MHCII⁺ LCs were calculated (bar graph). **B**, Expression of MHCII, CD86, CD80, CD40, and langerin in WT and CTCF-deficient LCs (CD45⁺CD11c⁺). The shaded line represents the fluorescence minus one (FMO) control. **C**, Surface CD86 and CD80 expressions of LCs were assessed by using flow cytometry with or without 1% oxazolone (Oxa) treatment for 24 hours. Data represent at least 2 independent experiments with 3 to 6 mice per group. Error bars indicate SEMs. **P* < .05, ***P* < .01, and ****P* < .001. ns, Not significant.

huLang-cKO mice discordant relative ratios of LCs in the epidermis (approximately 30%), dermis (<10%), and SDLNs (<10%) were found, suggesting hampered migration of CTCF-deficient LCs out of the epidermis (Fig E6 in this article's Online Repository at www.jacionline.org). To assess the full migratory capacity of CTCF-deficient LCs *in vivo*, we evaluated antigen-bearing LCs in regional lymph nodes (LNs) by painting the skin with fluorescein isothiocyanate (FITC). Consistent with a previous report, the number of FITC⁺ migrating LCs gradually increased in WT mice (peak at 72-96 hours; Fig 4, A, and see

Fig E7 in this article's Online Repository at www.jacionline.org).³⁵ Importantly, the draining LNs of huLang-cKO mice had a remarkably smaller number of FITC⁺ LCs, whereas similar proportions of FITC⁺ dDC migrants were observed (Fig 4, A, and see Fig E7). CCR7 is critical for peripheral DC migration to the draining LNs.³⁶ Notably, CTCF-deficient LCs expressed a significantly lower level of surface CCR7 (Fig 4, B).

Next, we sought to examine whether CTCF-deficient LCs could leave the epidermis. We cultured mouse ear skin explants for 72 hours to induce LC migration and performed flow

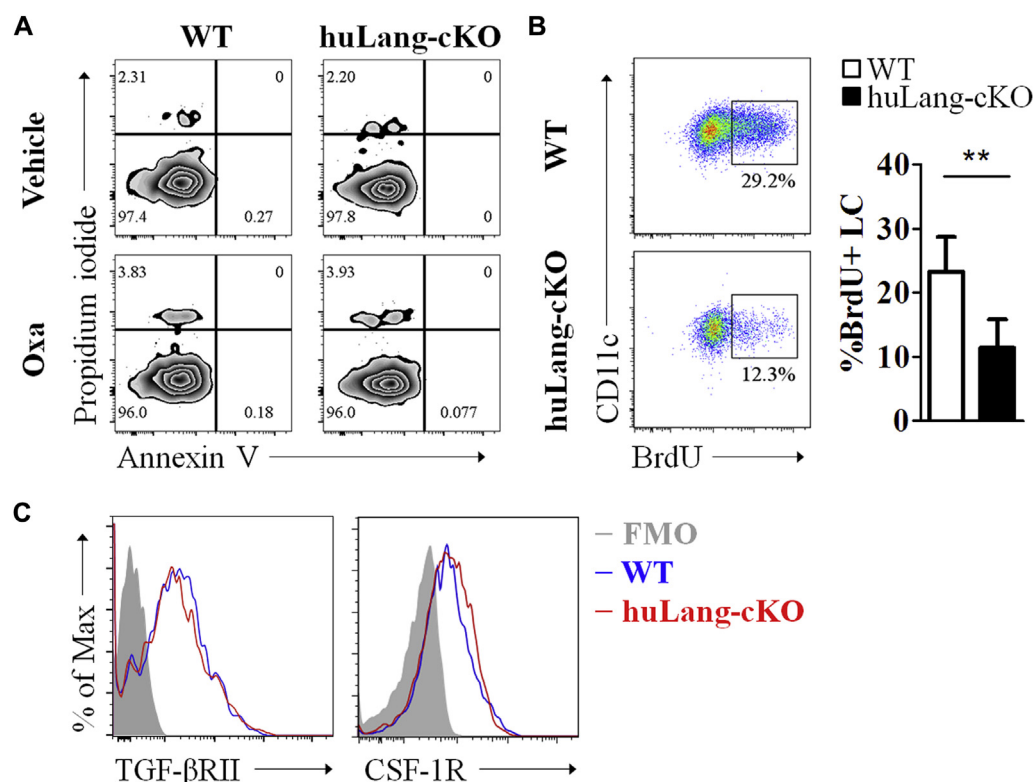


FIG 3. CTCF-deficient LCs exhibit self-proliferation impairment. **A**, LC activation was induced by 1% oxazolone (*Oxa*) treatment, and apoptosis of LCs ($CD45^+MHCII^+$) in epidermal single-cell suspensions from mouse ears was analyzed by using flow cytometry. **B**, Intracellular bromodeoxyuridine (*BrdU*) levels in LCs ($CD45^+MHCII^+Langerin^+$) were assessed by using flow cytometry. **C**, TGF- β receptor II (*TGF- β RII*) and CSF-1R expression levels in LCs were analyzed. Data represent at least 2 independent experiments with 3 to 7 mice per group. Error bars indicate SEMs. ** $P < .01$.

cytometry for crawl-out cells and epidermal sheet staining for remnant ear tissues.^{37,38} The number of crawl-out LCs from cultured huLang-cKO ear skin was strikingly reduced compared with that from WT skin cultures (Fig 4, C). Moreover, CTCF-deficient LCs were retained in the cultured epidermis, whereas WT epidermal LCs disappeared to a large extent after 72 hours of skin culture (Fig 4, D). Therefore our *in vivo* and *in vitro* findings demonstrate a critical role for CTCF in efficient LC egress from the epidermis.

Human LCs can be derived from peripheral monocytes *in vitro* (see Fig E8, A, in this article's Online Repository at www.jacionline.org).³⁹ We further assessed the role of CTCF in human LCs using lentiviral vector-mediated RNA interference of CTCF. The use of Vpx-containing virus-like particles significantly enhanced lentiviral transduction efficiency (see Fig E8, B),⁴⁰ and human monocyte-derived Langerhans cells (MDLCs) that were green fluorescent protein positive (indicative of successful viral transduction) were subjected to fluorescence-activated cell sorting (FACS). Expressions of CTCF mRNA and protein were significantly decreased in MDLCs infected with lentiviruses containing short hairpin RNA against CTCF (see Fig E8, C and D). Although the transduction efficiencies between control and shCTCF-expressing lentiviruses were comparable, the number of recovered MDLCs was reduced in the CTCF knockdown group (see Fig E9, A, in this article's Online Repository at www.jacionline.org). CTCF-diminished human MDLCs showed greater increases in CD80 expression after treatment with LPS,

but CD86 levels were modestly affected by CTCF silencing (see Fig E9, B). Notably, CTCF knockdown in LPS-treated MDLCs resulted in a lower levels of surface CCR7 compared with those seen in control cells, which is similar to results obtained with murine CTCF-deficient LCs (Fig 4, E). Moreover, an *in vitro* chemotaxis assay revealed that MDLC migration toward CCL19 was significantly abrogated by CTCF knockdown (Fig 4, F). These data suggest that CTCF plays an important role in the CCR7-dependent migration of LCs in both human subjects and mice.

CTCF inactivation in LCs augments cell adhesion molecule gene expression

To identify CTCF-regulated genes in LCs, we purified WT and CTCF-deficient LCs using FACS and performed microarray-based gene expression profiling (Fig 5, A, and see Fig E10 and Table E1 in this article's Online Repository at www.jacionline.org). We found lower *Ccr7* expression in cKO LCs, which was confirmed by means of flow cytometry (Figs 4, B, and 5, A, and see Fig E10). In particular, systematic Gene Ontology analysis showed that cell adhesion-associated genes were significantly different (Fig 5, B, and see Table E2 in this article's Online Repository at www.jacionline.org). Among them, genes that promote cellular adhesion, such as cell adhesion molecule 3 (*Cadm3*), cadherin 17 (*Cdh17*), and nidogen-1 (*Nid1*) were highly expressed in CTCF-deficient LCs (Fig 5, B, and see Fig E10).

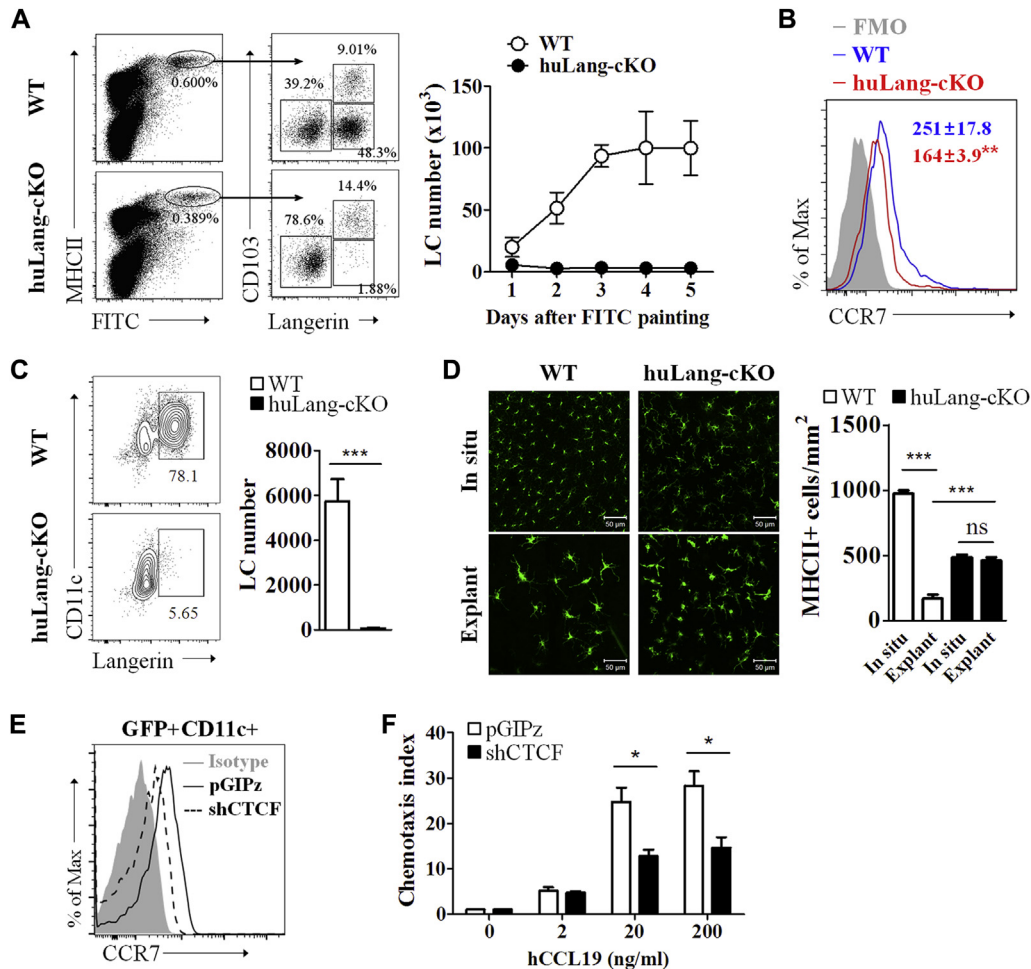


FIG 4. CTCF-deficient LCs fail to egress from the epidermis. **A**, Mice were painted with 0.5% FITC, and migrating LCs in SDLNs (FITC⁺MHCII^{hi}Langerin⁺CD103⁺) were quantified by using flow cytometry at the indicated time points. A representative FACS plot at the 72-hour time point is presented. **B**, Surface CCR7 expression of CD45⁺CD11c⁺MHCII⁺ LCs. The number indicates the mean fluorescence intensity ± SEM for CCR7 staining. FMO, Fluorescence minus one. **C**, Mouse ear skin explants were cultured for 72 hours, and crawl-out cells in the supernatants were analyzed by using flow cytometry to quantify migrating LCs (MHCII⁺CD103⁺CD11c⁺Langerin⁺). **D**, Epidermal sheets were prepared from resting and cultured skin, and the number of LCs in the epidermis was assessed by means of immunofluorescence after staining for MHCII (green). Data are representative of 2 to 3 independent experiments with 3 to 6 mice per group. **E**, CCR7 levels of transduced human MDLCs stimulated with LPS (representative data of 3 subjects). **F**, Chemotaxis indices of green fluorescent protein-positive (GFP⁺) MDLCs migrating toward the indicated concentrations of human CCL19 (pooled data from 4 subjects). Error bars indicate SEMs. **P* < .05, ***P* < .01, and ****P* < .001. ns, Not significant.

To explore whether these adhesion molecules were physiologically relevant to LC migration, we treated mouse ears with oxazolone to initiate LC migration and analyzed gene expression changes from the sorted LCs.⁴¹ Notably, *Cadm3*, *Cdh17*, and *Nid1* levels were significantly decreased in WT LCs on oxazolone treatment (Fig 5, C). However, both resting and stimulated CTCF-deficient LCs expressed strikingly higher levels of those adhesion molecules compared with WT LCs (Fig 5, C). Interestingly, the *Ctcf* level in LCs was not significantly influenced by LC activation *in vivo* (Fig 5, D). This suggests that those adhesion molecules are not regulated by direct changes in CTCF expression level but possibly through other mechanisms, such as a differential protein interaction of CTCF, which modulates its target gene's chromatin structures.

It has been known that LC motility in the epidermis is partly mediated by some adhesion molecules, including E-cadherin and epithelial cell adhesion molecule.^{38,42} Although no differences in E-cadherin levels were observed, CTCF-deficient LCs expressed significantly lower levels of epithelial cell adhesion molecule, which led to enhanced binding of LCs to keratinocytes (Fig 5, E).³⁸ Collectively, these results indicate that CTCF is indispensable for efficient LC emigration by regulating the balanced expression of CCR7 and cell adhesion molecules.

CTCF-dependent LC homeostasis is required for optimal immunity of the skin

Recent studies have proposed an immunosuppressive role for murine LCs by showing enhanced delayed-type hypersensitivity

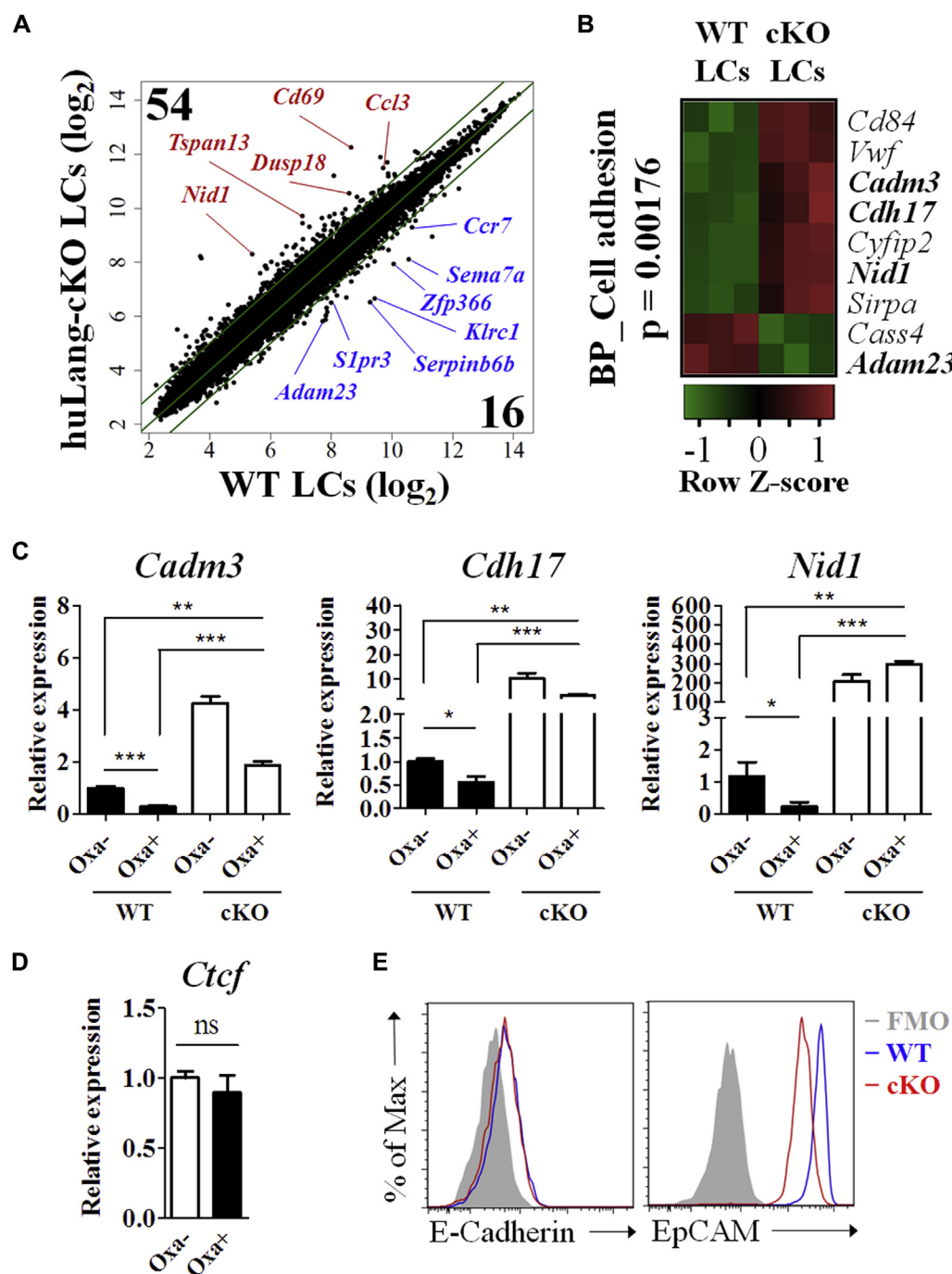


FIG 5. CTcf-deficient LCs express higher levels of cell adhesion molecules. **A**, LCs (CD45⁺CD11c⁺MHCII⁺) were sorted from epidermal single-cell suspensions. Scatter plots show raw expression levels ($\log_2$), and selected genes are indicated in red (upregulated in cKO LCs) or blue (upregulated in WT LCs). Green lines indicate the 2-fold boundary. Black indicates the number of genes that exhibited greater than 2-fold increases in WT (lower) or cKO (upper) LCs with a false discovery rate of less than 0.05. **B**, The heat map shows genes that belong to the cell adhesion biological process, as revealed by Gene Ontology analysis. Genes confirmed by using real-time quantitative polymerase chain reactions are indicated in boldface. **C** and **D**, Epidermal LCs were sorted with or without treatment with 1% oxazolone (Oxa), and expression of *Cadm3*, *Cdh17*, *Nid1* (Fig 5, C), and *Cctf* (Fig 5, D) genes were examined by using real-time quantitative PCR. **E**, Surface E-cadherin and epithelial cell adhesion molecule (*EpCAM*) levels in LCs. FMO, Fluorescence minus one. Data were pooled from 2 experiments with 3 to 6 mice. Error bars indicate SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

to contact allergens in mice with selective LC deficiency⁴³ and impaired LC migration,³⁸ although the exact role for LCs remains controversial.^{44,45} Because our huLang-cKO mice showed

decreases in LC numbers and defective LC migration, we first assessed 2,4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity (CHS) responses. DNFB treatment induced similar

extents of ear swelling in WT and huLang-cKO mice at 24 hours after challenge. However, cutaneous inflammation was significantly sustained in huLang-cKO mice from 48 hours after treatment (Fig 6, A). Ear swelling without DNFB sensitization (Fig 6, A) and croton oil-induced irritant dermatitis was equivalent between WT and cKO mice (see Fig E11 in this article's Online Repository at www.jacionline.org), indicating that the residual CTCF-deficient LCs were sufficient to reduce irritant reactions as opposed to the results from mice with complete LC depletion.^{46,47} Because IFN- γ -producing CD4⁺ and CD8⁺ T cells mediate CHS in response to DNFB, we examined the effect of LC-specific CTCF deficiency on the proliferation and priming of IFN- γ -producing T cells in draining LNs.⁴⁸ Antigen-specific T-cell proliferation was not significantly different between sensitized LN cells from WT and cKO mice (see Fig E12 in this article's Online Repository at www.jacionline.org). However, total sensitized LN cells from huLang-cKO mice produced greater amounts of IFN- γ than those from WT mice (Fig 6, B). This was associated with significantly enhanced induction of IFN- γ -producing CD8⁺ T cells in huLang-cKO LNs (Fig 6, C). In contrast, the proportion of the IFN- γ ⁺ population within CD4⁺ T cells was marginally affected by LC-selective CTCF deficiency (Fig 6, D). In addition, the frequency of forkhead box protein 3-positive regulatory T cells in SDLNs was comparable between WT and cKO mice, irrespective of sensitization (see Fig E13 in this article's Online Repository at www.jacionline.org). These findings suggest that the migratory failure of LCs caused by CTCF deficiency facilitates the cross-priming of IFN- γ -producing effector CD8⁺ T cells and in turn enhances CHS reactions, which indicate a suppressive role for LCs against the haptenized antigen.

Aside from a CHS response, LCs are known to elicit T_H2 and humoral immunity to protein antigens that encounter the epidermal barrier.^{49,50} To further understand the consequence of CTCF deficiency in LCs, we epicutaneously sensitized mice with ovalbumin (OVA) patches.⁵⁰ Repeated OVA application led to local dermatitis in WT mice; however, huLang-cKO mice showed less severe inflammation (Fig 7, A and B, and see Table E3 in this article's Online Repository at www.jacionline.org), which correlated with reduced T_H2-associated OVA-specific serum IgE and IgG₁ levels in cKO mice (Fig 7, C). Furthermore, huLang-cKO mice exhibited decreased OVA-specific T-cell proliferation after a single epicutaneous exposure to OVA (Fig 7, D, and see Fig E14 in this article's Online Repository at www.jacionline.org). Altogether, our data demonstrate that CTCF-dependent LC homeostasis is functionally required for full induction of cutaneous adaptive immunity to a protein antigen.

DISCUSSION

In the steady state most DCs, except for epidermal LCs and brain microglia, are continuously replenished by precursors from the blood.^{51,52} Moreover, DCs undergo a small number of divisions in the periphery that are important for maintaining homeostasis.⁵³⁻⁵⁵ In this study we used DC-specific CTCF-deficient mice to demonstrate a common but differential extent of reduction in each DC subset. Because gene recombination in CD11c-Cre mice fully occurs in differentiated DCs,⁵⁶ an overall decrease in DC quantity might result from CTCF deficiency in DCs. Similar proportions of pre-cDCs in the WT and CD11c-cKO BM and decreases in numbers of plasmacytoid DCs and

epidermal LCs, which develop independent from pre-cDCs,⁵² highlight a critical role for CTCF in sustaining terminally differentiated DC pools *in vivo*.

We then used a LC-specific CTCF-deficient mouse line to show that CTCF is essential for epidermal LC homeostasis. The reduction in epidermal LC numbers in huLang-cKO mice was not attributed to a failure of the initial LC network formation or a subsequent increase in cell apoptosis. Instead, CTCF-deficient LCs demonstrated a significant deterioration in self-renewal capacity. Previous studies have shown that conditional CTCF deletion in T cells leads to cell-cycle blockage²⁰ and impaired proliferation on T-cell receptor stimulation.²² In line with those results from lymphoid lineage cells, our findings suggest that CTCF played an important supporting role in myeloid LC proliferation. However, CTCF overexpression during GM-CSF-supplemented BMDC culture was reported to lead to the diminished proliferation.²⁸ These discrepant observations might be due to the fact that LC development strictly depends on TGF- β and IL-34 but not GM-CSF.⁵⁷

CTCF-deficient LCs demonstrated unexpected impairments in their egress from the epidermis. LC migration is initiated by downregulation of cell adhesion molecules in response to proinflammatory cues, which facilitates the release of LCs from the epidermis.⁴² A previous report suggested a role for CTCF in the migration of transformed corneal cell lines *in vitro*, but the exact regulatory mechanisms were unclear.⁵⁸ In our microarray experiments the expression levels of cell adhesion molecules, including *Cadm3*, *Cdh17*, and *Nid1*, were significantly greater in CTCF-deficient LCs, which might augment LC attachment within the epidermis. *Cadm3* is associated with cell-cell adhesion of neuronal cells.⁵⁹ *Cdh17* is a noncanonical cadherin that is frequently upregulated in cancer cells and mediates their adhesion.^{60,61} Notably, CTCF-deficient LCs expressed high levels of *Nid1*, which can bind to laminin and collagen IV in the basement membrane.⁶² Because CTCF-deficient LCs were unable to efficiently transmigrate the dermoepidermal junction, we speculate that cKO LCs might be entrapped within the epidermis by an increased affinity to the juxtaposed keratinocytes and basement membrane structure. Importantly, levels of those adhesion molecules were consistently diminished in WT LCs after *in vivo* activation. Collectively, these results strongly suggest that CTCF intrinsically supports the efficient egress of LCs from the epidermis by negatively regulating LC adhesion intensity.

In addition to the adhesion molecules, both mouse and human CTCF-depleted LCs showed lower levels of CCR7 expression and impaired migration toward CCR7 ligand-rich areas. Thus our findings provide relevant evidence supporting the hypothesis that CTCF is involved in LC migration. At the molecular level, the constitutive expression of CTCF, even after LC activation, proposed that CTCF in LCs might work through differential DNA occupancy patterns¹⁶; collaboration with other interacting proteins, such as transcription factors, cofactors, and cohesin complexes; or both.^{25,26,63,64} Further studies will be necessary to dissect global CTCF binding profiles and its protein interactomes in LCs to illuminate CTCF-dependent molecular mechanisms for regulating its target genes.

Although there are conflicting results regarding the role of LCs, several recent studies have shown that LCs can suppress CHS and other types of inflammation.^{5,7,43,65,66} In huLang-cKO mice few antigen-laden LCs could reach the draining LNs, whereas dDC migration was comparable with that seen in WT mice. A recent

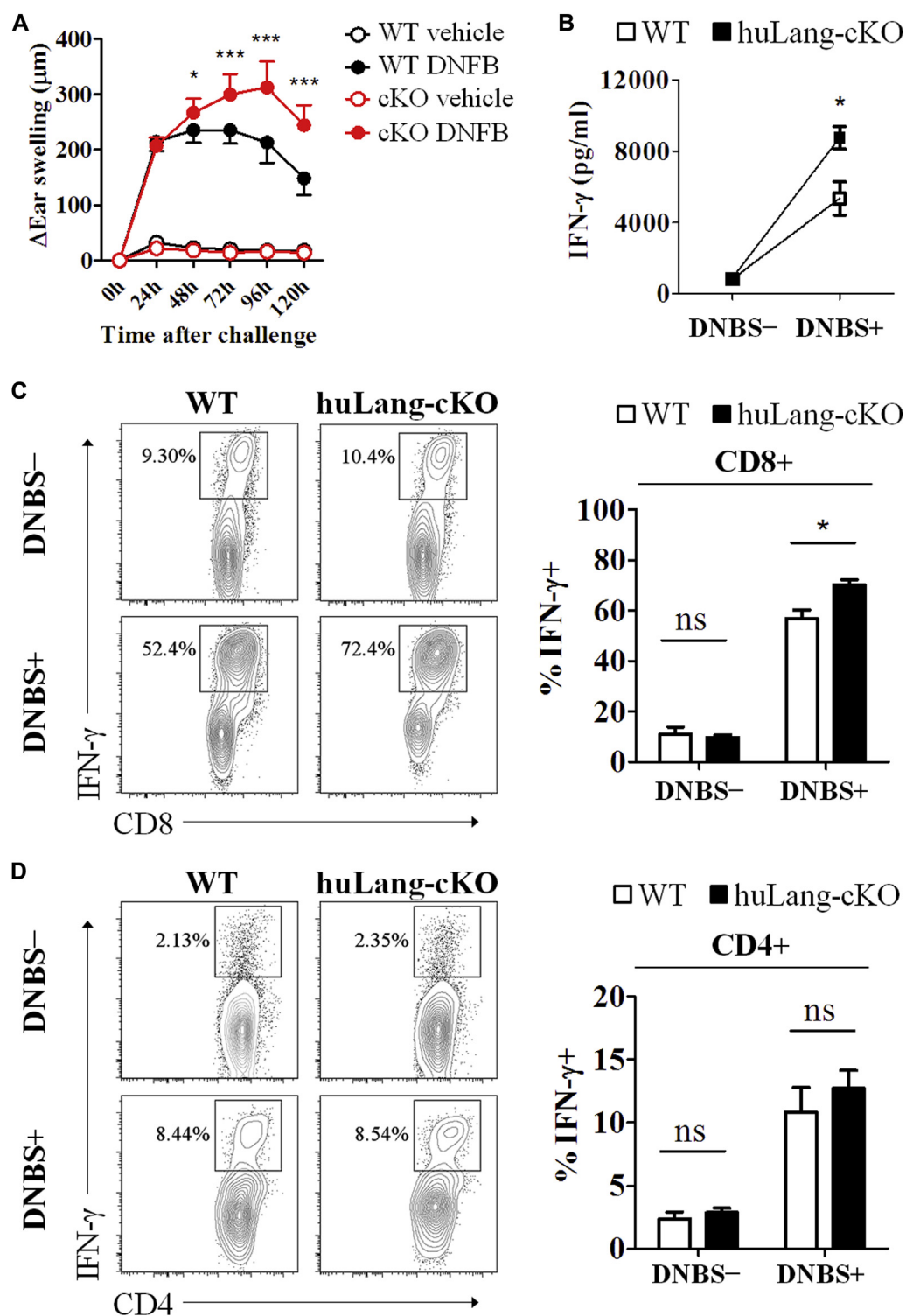


FIG 6. Prolonged and exaggerated CHS responses with higher priming of IFN- γ -producing CD8 $^{+}$ T cells in huLang-cKO mice. **A**, Changes in ear thickness after DNFB challenge were measured at the indicated time points. **B**, DNFB-sensitized total LN cells were cultured for 72 hours with or without 2,4-dinitrobenzene sulfonic acid (DNBS). IFN- γ levels in the supernatants were evaluated. **C** and **D**, Cultured total LN cells were restimulated with phorbol 12-myristate 13-acetate/ionomycin, and intracellular IFN- γ production among CD8 $^{+}$ (Fig 6, C) and CD4 $^{+}$ (Fig 6, D) T cells was assessed by using flow cytometry. Data represent 3 independent experiments with 5 to 7 mice per group. Error bars indicate SEMs. * P < .05 and *** P < .001. ns, Not significant.

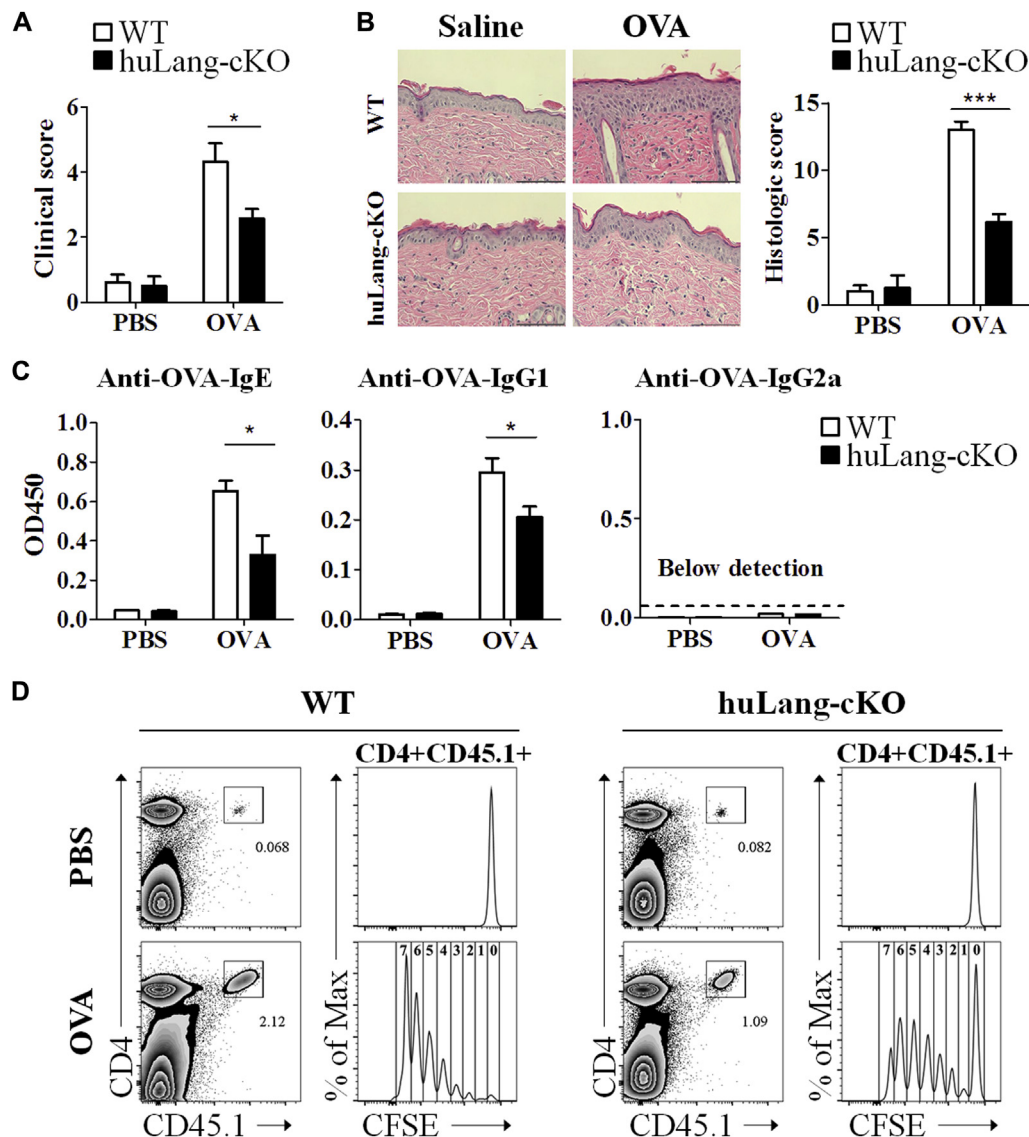


FIG 7. Attenuated epicutaneous sensitization to OVA in huLang-cKO mice. **A**, Total clinical severity scores. **B**, Hematoxylin and eosin staining of patch-applied back skin. The sum of each histologic score is presented. Scale bar = 100 μ m. **C**, Serum OVA-specific antibodies were assessed by means of ELISA. OD values for IgE, IgG₁, and IgG_{2a} were measured at a wavelength of 450 nm. **D**, Carboxyfluorescein succinimidyl ester (CFSE)-labeled CD45.1⁺ OT-II T cells were adoptively transferred to WT and huLang-cKO mice, and mice were treated with an OVA patch. SDNs were analyzed to measure OVA-specific T-cell proliferation. Numbers in the histogram indicate the number of CD45.1⁺ OT-II T-cell divisions. Data represent at least 2 independent experiments with 5 to 7 mice per group. Error bars indicate SEMs. * $P < .05$ and *** $P < .001$.

study with a langerin-targeting antigen delivery strategy demonstrated that priming of CD8⁺ T cells was mediated by langerin-positive dDCs, but surprisingly, LCs actively suppressed immunity.⁶⁷ Because our data revealed greater priming of DNFB-specific IFN- γ ⁺CD8⁺ T cells in huLang-cKO mice, LCs are likely to restrict the cross-priming of cytotoxic T cells when antigens are captured and presented by both LCs and dDCs.

Another murine model that used epicutaneous sensitization allowed us to demonstrate a distinct role for migratory LCs in promoting allergic immunity to protein antigens. In the skin LCs are major players that uptake external protein antigens and activate antigen-specific immune responses,^{50,68} but their function is somewhat redundant, suggesting the potential contribution

of other types of antigen-presenting cells.^{50,69} Nevertheless, our huLang-cKO mice showed a lower degree of epicutaneous sensitization to OVA, indicating that CTCF-dependent LC homeostasis is pivotal for cutaneous allergic responses to proteins. Interestingly, an association between an increased risk of asthma and a disease-related allele linked to changes in CTCF DNA-binding was reported, opening a possible causality between allergic diseases and CTCF.⁷⁰ Moreover, DCs in different barrier tissues possess an important role in allergy pathogenesis.⁷¹ Therefore future studies are needed to explore the link between genome-wide binding patterns of CTCF and immunologic functions of DCs from allergic patients to clarify the functional implications of the CTCF-DC axis in allergic inflammation.⁷²

In summary, we elucidated a role for CTCF in the DC lineage using genetic approaches. CTCF supported quantitative maintenance of the systemic DC pool and promoted LC self-turnover. We also demonstrated that CTCF facilitated LC egress from the epidermis and conferred them to differentially regulate cutaneous immunity. This study expands our knowledge of CTCF, which is actively involved in multiple facets of LC homeostasis. Further study is required to fully understand the underlying mechanisms of CTCF in the control of the development and function of each DC subset and their associations with inflammatory disorders.

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Clinical implications: Molecular targeting of CTCF to modulate its activity can be a future therapeutic strategy to regulate LC function and dampen cutaneous inflammations *in vivo*.

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Online Repository Materials

Mice

Mice carrying the targeted *CCCTC-binding factor* (*Ctcf*) allele, *Ctcf^{tm1a(EUCOMM)Wtsi}*, were purchased from European Conditional Mouse Mutagenesis Consortium. For deletion of the lacZ-neomycin-resistance cassette and the generation of mice with a loxP-flanked *Ctcf* allele (*Ctcf^{fl/+}*), *Ctcf^{tm1a(EUCOMM)Wtsi}* mice were bred to ACT-FLPe C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA),¹ which express a transgene of an enhanced form of the recombinase FLP that is driven by a chicken β -actin-rabbit β -globin hybrid promoter and the human cytomegalovirus-IE enhancer (Fig E1, A). *Ctcf^{fl/+}* mice were intercrossed to generate *Ctcf^{fl/fl}* mice. Mice were genotyped by polymerase chain reaction (PCR) with the following oligonucleotides: forward primer 5'- AACTCCCTGGTTCCACTCCT -3' and reverse primer 5'- GACCCTAGTGCTGTTTCGGC -3'; wild-type product, 420 bp; *Ctcf^{fl}* product, 539 bp. To generate mice with DC-specific *Ctcf* deficiency (CD11c-cKO), *Ctcf^{fl/fl}* mice were crossed with CD11c-Cre BAC transgenic mice.² To generate mice with LC-specific *Ctcf* deficiency (huLang-cKO), we crossed *Ctcf^{fl/fl}* mice with human Langerin-Cre BAC transgenic mice (obtained from NCI Mouse Repository).³ After crossing *Ctcf^{fl/fl}* mice with Cre mice, the deletion efficiency of the conditional *Ctcf* allele was confirmed by genomic DNA PCR with the following oligonucleotides: forward primer 5'- AACTCCCTGGTTCCACTCCT-3' and reverse primer 5'- TGGAGACCCAGATTCAAATG-3'; *Ctcf^{fl}* product, 1226 bp; *Ctcf^Δ* product, 435 bp. CD45.1 congenic mice and OT-II transgenic mice expressing T-cell receptor specific for OVA₃₂₃₋₃₃₉ were purchased from the Jackson Laboratory. CD45.1 and OT-II mice were interbred to obtain CD45.1⁺OT-II mice. Six- to 12-week-old mice that were bred in specific pathogen-free facilities at Yonsei University College of Medicine were used for all experiments. Age- and sex-matched *Ctcf^{fl/fl}* littermate mice were used as wild-type (WT) controls throughout the study. All animal studies were approved by the Department of Laboratory Animal Resources Committee of Yonsei University College of Medicine.

Generation of human monocyte-derived Langerhans cells (MDLCs)

Peripheral blood mononuclear cells (PBMCs) were obtained from normal volunteers (n=15; age range 25-40) under the Yonsei University IRB-approved protocol. Written informed consent

was obtained and study was performed following the Declaration of Helsinki Principles. PBMCs were isolated by gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) and CD14⁺ monocytes were purified by anti-human CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was confirmed by flow cytometry (>92%). Human MDLCs were cultured as described previously.⁴ Briefly, 1 x 10⁶ monocytes were placed in 24-well tissue culture plates (SPL, Seoul, Korea) and cultured in complete RPMI medium, which consisted of RPMI 1640 (HyClone, Logan, UT, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone), 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μ g/mL streptomycin. For MDLC differentiation, 100 ng/ml rhGM-CSF, 20 ng/ml rhIL-4 (Creagene, Seoul, Korea), and 10 ng/ml TGF- β 1 (Peprotech, Rocky Hill, NJ, USA) were used. MDLCs were harvested at day 6 and stimulated with 1 μ g/ml of lipopolysaccharide (LPS) (Invivogen, San Diego, CA, USA) for additional 2 days.

Viruses and Vpx-containing virus-like particle (Vpx-VLP) production

Due to the strikingly low transduction efficiency of monocytes and DCs with HIV-1-derived lentiviruses, we took advantage of co-transduction of Vpx-VLP which drastically assisted MDLCs with lentiviral infection.^{5,6} The pGIPz and pGIPz-shCTCF lentiviral vectors were obtained from Dr. Jianrong Lu (University of Florida College of Medicine).⁷ pSIV3+ plasmid was kindly provided by Dr. Kwangseog Ahn (Seoul National University).⁸ Lentiviral infection and gene transduction to MDLCs were performed as previously described.⁶ Briefly, lentiviruses were generated from HEK-293 cells co-transfected with psPAX2 and pMD2.G (Addgene, Cambridge, MA, USA) using polyethylenimine (Polysciences, Warrington, PA, USA). Vpx-VLP was generated by co-transfection of pSIV3+ with pMD2.G.^{5,6} Lentiviral infections were carried out during the first 2 days of monocyte culture in the presence of 8 μ g/mL polybrene (Sigma, St. Louis, MO, USA). Green fluorescent protein (GFP)-positive transduced MDLCs were underwent FACS sorting and used for further analyses.

Tissue preparation and flow cytometry

For epidermal and dermal single-cell suspensions, excised ears were placed in 2.5 mg/mL dispase II (Roche Diagnostics, GmbH, Mannheim, Germany) for 30 min at 37°C. Isolated

epidermal and dermal sheets were incubated in 1 mg/mL collagenase IV (Worthington, USA) for 1 h at 37°C. In some experiments, mouse ears were treated with 20 µL of 1% oxazolone (Sigma) dissolved in acetone/olive oil (4:1), and after 24 h, the skins were prepared and subsequently examined. Total cells from the skin-draining lymph nodes (SDLNs) and spleen were incubated in 1 mg/mL collagenase IV for 1 h at 37°C. Suspension cells were stained with antibodies and acquired by the FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

Antibodies

Fluorochrome-conjugated anti-mouse CD11b, CD11c, CD40, CD44, CD45, CD45.1, CD69, CD80, CD86, CD103, CD115 (colony-stimulating factor-1 receptor [CSF-1R]), CD135, CD207 (Langerin), Annexin V, C-C chemokine receptor 7 (CCR7), E-cadherin, epithelial cell adhesion molecule (EpCAM), F4/80, Foxp3, I-A^b (MHC II), interferon (IFN)-γ, Ki-67, Siglec-H, and signal-regulatory protein alpha (Sirpα) monoclonal antibodies (mAbs) were obtained from eBioscience (San Diego, CA, USA), and anti-mouse CD4 and CD8α antibodies were from BD Biosciences. Biotin-conjugated anti-mouse CD3, CD19, NK1.1, Ter119, and B220 mAbs were used for discriminating lineage-positive populations in the bone marrow to analyze pre-cDCs (eBioscience). Anti-mouse CTCF (Abcam, Cambridge, MA, USA) and transforming growth factor-beta receptor II (TGF-βRII; R&D Systems, Minneapolis, MN, USA) antibodies were also used in this study. Tissue suspensions containing cells were incubated in violet fluorescent live/dead dye (Molecular Probes, Eugene, OR, USA) for 30 min on ice and stained with the antibodies described above at the appropriate dilutions using FACS buffer (phosphate-buffered saline supplemented with 1% bovine serum albumin). For the intracellular detection of IFN-γ and Langerin, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) and incubated for 30 min on ice with antibodies diluted in Perm/Wash buffer (BD Biosciences). Intracellular detection of Foxp3 and Ki-67 was performed using Foxp3/Transcription Factor Staining Kit (eBioscience). For human MDLC staining, we used fluorochrome-conjugated anti-human CCR7, CD11c, CD14, CD80 (BD Biosciences), CD86, and E-cadherin (eBioscience) mAbs. All flow cytometry data were analyzed with the FlowJo software (Treestar, Ashland, OR, USA).

Whole-mount epidermal sheet staining

Separated epidermal sheets were fixed with cold acetone for 15 min at room temperature. Epidermal sheets were incubated with a purified anti-mouse I-A^b mAb (clone M5/114, Biolegend, San Diego, CA, USA), and signals were amplified by Alexa 488-conjugated goat anti-rat IgG (Invitrogen, Carlsbad, CA, USA). After staining, sheets were mounted on glass slides with mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired with the LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) and analyzed with the ZEN software (Zeiss).

Immunofluorescence

Tissue sections (6 µm thick) from frozen ear samples that were preserved in optimal cutting temperature (Sakura, Tokyo, Japan) were prepared by cryostat. Skin sections were fixed in ice-cold acetone and blocked in 10% goat serum. After antibody staining, images were generated with the LSM 700 confocal microscope (Zeiss) and analyzed with the ZEN software (Zeiss). The TUNEL assay was performed using the In Situ Cell Death Detection Kit, according to the manufacturer's instructions (Roche Diagnostics).

Electron microscopy

For immersion-fixation, mouse ear tissues were fixed with a mixture of 3% formaldehyde (freshly prepared from paraformaldehyde) and 1% glutaraldehyde (vacuum distilled) in 0.1 M cacodylate buffer (pH 7.2) for 15 min at 37°C. Afterwards, the tissues were sliced into thin pieces, followed by immersion fixation in the same fixative at ambient temperature for 4 h. To stop fixation, free aldehyde groups were blocked by soaking the tissue slices in 50 mM NH₄Cl in buffer for 1 h. The tissue slices were then post-fixed with 1% osmium tetroxide in distilled water for 1 h, followed by en bloc staining with 1% aqueous uranyl acetate for 1 h. After dehydrations in a series of graded ethanol, embedding in Epon-Araldite (Fluka) was performed, according to the standard protocol. Ultrathin 80-nm sections were prepared on copper slot grids, stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7650 electron microscope (Hitachi, Tokyo, Japan) at 80 kV. Electron micrographs were taken with an 11-megapixel CCD XR611-M digital camera (Advanced Microscopy Techniques, Woburn, MA, USA).

Whole-skin explant culture

Whole-skin explant culture was performed as described previously.⁹ Ears of mice were cut off the base and split into dorsal and ventral halves. Both skin halves were cultured in 12-well plates containing complete RPMI culture medium. After 3 days, supernatants were pooled from the four skin explants and analyzed by flow cytometry. Epidermal sheets were prepared from the cultured explants and subsequently assessed by whole-mount epidermal sheet staining.

***In vivo* bromodeoxyuridine (BrdU) labeling and analysis**

Each mouse received daily intraperitoneal injections of 1 mg BrdU (Sigma) for 7 days. One day after the final injection, isolated LCs were stained using the BrdU Flow Kit, according to the manufacturer's instructions (BD Pharmingen).

Fluorescein isothiocyanate (FITC)-induced cutaneous DC migration assay

Mice were shaved on the abdomen, and 200 μ L of the 0.5% FITC (Sigma) solution in acetone/dibutylphthalate (1:1) was applied to the shaved area. The draining axillary and inguinal LNs were harvested, and cells were analyzed by flow cytometry at the indicated time points.

Contact hypersensitivity and hapten-specific T-cell responses

2,4-dinitrofluorobenzene (DNFB; Sigma) was used to induce CHS, as described previously.^{10,11} Mice were sensitized by the application of 25 μ L of 0.5% (wt/vol) DNFB in acetone/olive oil (4:1) to their shaved abdomens on day 0 and challenged on the right ear on day 5 with 20 μ L of 0.3% (wt/vol) DNFB. Ear thickness was measured before and after challenge using a thickness gauge. Hapten-specific T-cell responses were examined as described elsewhere with slight modifications.¹¹ Mice were sensitized with 25 μ L of 0.5% DNFB, and inguinal and axillary LNs were harvested 5 days later. Total LN cells were stained with 2.5 μ M carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) for 15 min at 37°C and washed, and 1×10^6 LN cells/well in a 96-well plate were cultured in the complete RPMI medium with or without 50 μ g/mL 2,4-dinitrobenzene sulfonic acid (DNBS; Alfa Aesar, Ward Hill, MA, USA), which is a water-soluble compound with the same antigenicity as DNFB, for 3 days. The cells were then harvested and re-stimulated with 50 ng/mL phorbol myristate acetate (PMA; Sigma) and 500 ng/mL ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences) for 5 h at 37°C, and subsequent intracellular staining for IFN- γ was performed. The secretion of IFN- γ was

examined by using the Cytometric Bead Array (BD Biosciences) following the manufacturer's instructions.

Croton oil-induced irritant dermatitis

Twenty μL of 1% (vol/vol) croton oil (Sigma) in acetone was applied to ear skin and ear thickness was measured before and after treatment at the indicated time points.¹²

Epicutaneous sensitization and T cell adoptive-transfer

Mice were shaved on the back with an electric razor and a 1 x 1 cm size of skin site was tape-stripped at least 4 times using cellophane tape (Nichiban, Tokyo, Japan). One hundred μg of ovalbumin (OVA) (Sigma) in 50 μL of normal saline was applied using Finn Chamber (SmartPractice). A total of three 2 days exposures separated by 1 day intervals were performed as described previously.¹³ For T cell adoptive-transfer, CD4^+ T cells were isolated from $\text{CD45.1}^+\text{OT-II}$ mice using beads-conjugated anti- CD4 mAb and magnetic bead separation (Miltenyi Biotec), and labeled with 10 μM of CFSE. Totally, 4×10^6 labeled cells were adoptively transferred into recipient mice via retro-orbital routes and mice were epicutaneously sensitized with OVA 24 h later. Brachial SDLNs were harvested 72 h after OVA sensitization, and cell suspensions were analyzed for diminution of CFSE by flow cytometry.

Clinical and histological evaluation of epicutaneously sensitized skin

The clinical score of the inflamed skins was evaluated as previously described.¹³ Briefly, the sum of severity of each clinical category for skin lesions was calculated, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for the symptoms of erythema, edema, erosion, scaling, and pruritus. Skin tissues were fixed in 10% formalin and embedded in paraffin for the histological assessment. Skin sections (6 μm thick) were prepared and stained with hematoxylin and eosin. The histological examination was performed as reported previously with slight modification.^{13,14} As epicutaneously sensitized skins revealed the features of eczematous dermatitis, we added a score for epidermal spongiosis. A grading scale we used is as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; and 4, marked response.

Enzyme-linked immunosorbent assay (ELISA) for OVA-specific antibodies

OVA-specific IgE/IgG1/IgG2a levels were measured by an indirect ELISA method using mouse IgE/IgG1/IgG2a ELISA kit (eBioscience) with some modifications. ELISA plates were coated with OVA (500 ng/well) at 4°C overnight. After blocking, 50 µL of 20x diluted serum was added to each well and incubated for 2 h. IgE levels were determined using biotin-conjugated anti-mouse IgE Abs and streptavidin-conjugated horseradish peroxidase (eBioscience). Alkaline phosphatase-conjugated anti-mouse IgG1/IgG2a Abs (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also used to detect OVA-antibody complex. Optical density was measured at 450nm.

Chemotaxis migration assay

Cell migration was evaluated using the 24-well, 5 µm pore size polycarbonate Transwell system (Costar, Cambridge, MA, USA) as described in our previous study.¹⁵ Total of 1×10^5 LPS-stimulated GFP⁺ MDLCs were placed on the top of the Transwell in complete RPMI medium and incubated with or without the indicated concentrations of recombinant human C-C chemokine ligand 19 (CCL19) (Peprotech) in the bottom chamber for 3 h at 37°C. The number of migrating cells was counted for a fixed time period of 1 min using flow cytometry. Chemotaxis index was calculated as the ratio of the number of cells migrating toward CCL19 divided by the number of migrating cells in the negative control medium.

Quantitative real-time polymerase chain reaction

Total RNA from purified cells was isolated with the Hybrid-R Total RNA kit (GeneAll Biotechnology, Seoul, Korea). cDNA was synthesized using PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan). Quantitative real-time PCR reaction was performed with the ABI StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) by monitoring the synthesis of double-stranded DNA during the various PCR cycles using SYBR Green (Takara Bio). For each sample, duplicate test reactions were analyzed for the expression of the gene of interest, and results were normalized to *Gapdh* mRNA. Primer sequences are listed in Table E4.

Western blot

Total splenocytes were labeled with magnetic bead-conjugated anti-CD11c mAb (Miltenyi Biotec), and DCs were purified by two rounds of positive selection on paramagnetic columns (Miltenyi Biotec). Cells were lysed in 100 μ L cell lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol, 1 mM ethylenediaminetetraacetic acid, 5% glycerol, and protease inhibitor cocktail (Sigma). Whole cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% skim milk, the membrane was incubated with the antibodies, followed by incubation with the horseradish peroxidase-conjugated secondary antibody. Target proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Microarray

Epidermal Langerhans cells (LCs) were sorted by the FACS Aria II cell sorter (BD Biosciences) at the flow cytometry core in the Avison Biomedical Research Center (Yonsei University College of Medicine). Sorted LCs were immediately collected in 500 μ L TRIzol (Invitrogen), and total RNA was extracted using the isopropanol precipitation method. Isolated RNA was linearly amplified by the WT Expression Kit (Ambion, Austin, TX, USA) and labeled using WT Terminal Labeling Kit (Ambion). Labeled cRNA was hybridized on the Affymetrix Mouse GeneChip 2.0 ST Arrays (Affymetrix, Santa Clara, CA, USA). Acquired expression data were analyzed by the R software (www.R-project.org). Quality control was done using a different R package from the Bioconductor project (www.bioconductor.org). The Significance Analysis of Microarray (SAM) was used to determine genes that were significantly and differentially expressed between WT and CTCF-deficient LCs (FDR; $q < 5\%$).¹⁶ We performed functional annotation with the Gene Ontology biological process using the DAVID Functional Annotation tool (<http://david.abcc.ncifcrf.gov>) from an expanded subset of gene whose expression differed by at least 1.5-fold.

Statistics

Data were analyzed with the unpaired Student's two-tailed t-test, unless otherwise stated, using the Prism software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered to be significant.

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Supplementary Figure Legends

Figure E1. Schematic diagram showing *Ctcf*^{fl/fl} mice generation and CTCF deletion efficiency.

(A) Mice carrying the targeted *Ctcf*^{tm1a(EUCOMM)Wtsi} allele were bred to ACT-FLPe transgenic mice to remove the LacZ/NeoR cassette and generate mice carrying a conditional *Ctcf*^{fl} allele in which exon 8 was flanked by loxP sites. Mice carrying a conditional *Ctcf*^{fl} allele were bred to Cre-expressing transgenic mice to generate conditional *Ctcf*-deleted (*Ctcf*^Δ) knockout mice. Exons are represented by black boxes. (B) The efficiency of CTCF deletion was determined by western blotting of sorted CD11c⁺ cells of the spleen from WT and CD11c-cKO mice. Beta-actin served as the protein loading control. Data represent two independent experiments with three mice per group.

Figure E2. DC-selective CTCF deletion results in a decreased general DC lineage.

The proportion of lymphoid DCs in the spleen and SDLNs and non-lymphoid skin DCs were analyzed by flow cytometry. Error bars represent the mean $\pm$ SEM of pooled data from two experiments with four to five mice per group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant.

Figure E3. Efficient LC-specific deletion of the *Ctcf* conditional allele in huLang-cKO mice.

(A) FACS gating strategies for isolating LCs and dendritic epidermal T cells (DETCs). (B) Genomic DNA PCR for isolated DNA from LCs and DETCs from WT and huLang-cKO mice as separated by agarose gel electrophoresis. (C) The epidermal CD103⁺ DETC population was assessed by flow cytometry. (D) Tennis-racket-like Birbeck granules (black arrows) from WT and CTCF-deficient LCs in the epidermis were examined by electron microscopy. Bar=100 nm. Data represent at least two independent experiments with three to six mice per group. ns, not significant.

Figure E4. No increase in cellular apoptosis but impaired self-proliferation in CTCF-deficient LCs.

(A) *In situ* TUNEL assays were performed using MHC II and DAPI co-staining in frozen ear skin sections from WT and huLang-cKO mice. Original magnification, $\times 200$. (B) The actively cycling LC subpopulation was analyzed by detecting Ki-67 positivity (same LC gating as Fig 3, B). Data represent at least two independent experiments with three to seven mice per group. Error bars indicate SEM. **, $P < 0.01$.

Figure E5. Early epidermal LC network formation is comparable between WT and huLang-cKO mice.

(A) The frequency of myeloid LC precursors ($CD45^+CD11b^+F4/80^+$) in the epidermis on postnatal day 1 was assessed by flow cytometry. (B and C) The numbers of MHC II⁺ cells (green) in the epidermis from postnatal days 1 and 7 were quantified by immunofluorescence (B), and the expressions of MHC II and intracellular langerin were examined by flow cytometry (C). Data were pooled from three experiments with four to six mice per group. Error bars indicate SEM. ns, not significant.

Figure E6. CTCF-deficient LCs are significantly less distributed in their sequential migratory compartments.

(A-C) DC populations in the epidermis (A), dermis (B), and SDLNs (C) from WT and huLang-cKO mice were analyzed by flow cytometry, using the same gating strategies described in Fig 1. (D) Normalized ratios (relative frequencies) of the percentages of LCs in huLang-cKO mice against the percentages of corresponding WT LCs in the epidermis, dermis, and SDLNs. Data were pooled from five experiments (n=11). Error bars indicate SEM. ***, $P < 0.001$; ns, not significant.

Figure E7. Time kinetics of cutaneous DC migration after FITC painting.

SDLNs were analyzed by flow cytometry to quantify migrating DCs after FITC application. Representative FACS plots for the migrating cutaneous DCs at the indicated time points (upper) are presented (three to four mice per group). Graphs indicate the numbers of migrating CD103⁺ (lower left) and CD103⁻ (lower right) dDCs. Error bars indicate SEM.

Figure E8. Vpx-VLP-assisted lentiviral transduction of human MDLCs and shRNA-mediated CTCF knockdown.

(A) Differentiation of MDDCs and MDLCs assessed by flow cytometry. (B) MDLCs were infected with pGIPz control lentivirus in the absence or presence of Vpx-VLP, and transduction efficiency was determined by frequency of GFP⁺ populations at day 8 (multiplicity of infection=2.0). (C and D) Transduced MDLCs were sorted, and the knockdown efficiency of CTCF was determined by real-time qPCR (C) and immunoblotting (D). Data are representative of five individuals.

Figure E9. Phenotypes of CTCF knockdown MDLCs.

(A) Representative FACS plot for the transduction efficiency of MDLCs with control and shCTCF-containing lentiviruses (left), and the number of recovered MDLCs after gene transduction (right). (B) Surface CD80 and CD86 levels of FACS-sorted GFP⁺ MDLCs were analyzed after 2 days of LPS treatment. Data are representative of four individuals. Error bars indicate SEM. *, P<0.05.

Figure E10. Semi-quantitative real-time qPCR and flow cytometric confirmation of microarray-based differential gene expression between WT and CTCF-deficient LCs.

Expressions of the indicated genes in FACS-sorted WT and cKO LCs (same gating strategies from Fig E3, A) were analyzed by real-time qPCR (top). CD69 protein expression in WT and cKO LCs was assessed by flow cytometry (bottom). Data were pooled from two experiments with four to six mice. Error bars indicate SEM. **, $P < 0.01$; ***, $P < 0.001$.

Figure E11. No differences in severity of cutaneous irritant reaction between WT and CTCF-deficient LCs.

Changes in ear thickness after application of croton oil or vehicle were measured at the indicated time points.

Figure E12. Antigen-specific T-cell proliferation against DNBS in WT and huLang-cKO mice.

(A and B) DNFB-sensitized total LN cells were labeled with CFSE and cultured with or without DNBS for 72 h. CD44⁺CFSE^{low} antigen-specific T-cell proliferation was examined by flow cytometry. Data represent three independent experiments with five to seven mice per group. Error bars indicate SEM. ns, not significant.

Figure E13. The frequencies of regulatory T cells in SDLNs are comparable between WT and huLang-cKO mice irrespective of DNFB sensitization.

Foxp3⁺ regulatory T cells among the CD4⁺ T cell population in SDLNs were analyzed by flow cytometry 5 days after treatment with vehicle or DNFB. Data represent two independent experiments with five mice per group. Error bars indicate SEM. ns, not significant.

Figure E14. CTCF in LCs is required for optimal antigen-specific T cell proliferation in response to OVA.

(A and B) Mice were transplanted with CFSE-labeled CD45.1⁺OT-II T cells and treated with OVA patches. Proliferation of CD45.1⁺OT-II T cells in SDLNs was determined by the decrease of CFSE and the percentage of T cells in each generation of cell divisions was shown. Five to six mice per group were assessed. Error bars indicate SEM. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

Table E1. Genes exhibiting two-fold changes in expression between WT and CTCF-deficient LCs with a false discovery rate (FDR) less than 0.05.

Fold Change*	GeneID	Description
12.083	CD69	CD69 antigen
7.437	NID1	similar to Nidogen precursor (Entactin); nidogen 1; similar to Nid1 protein
6.301	TSPAN13	tetraspanin 13
4.143	MILR1	Mus musculus mast cell immunoglobulin like receptor 1 (Milr1), transcript variant 6
4.075	MEX3A	Mus musculus mex3 homolog A (C. elegans) (Mex3a)
3.954	KLRA17	killer cell lectin-like receptor, subfamily A, member 17; killer cell lectin-like receptor, subfamily A, member 17B
3.913	DUSP18	dual specificity phosphatase 18
3.839	LILRB4	glycoprotein 49 A; leukocyte immunoglobulin-like receptor, subfamily B, member 4
3.709	CYBB	cytochrome b-245, beta polypeptide
3.508	PTPN5	protein tyrosine phosphatase, non-receptor type 5
3.434	CLEC4A3	C-type lectin domain family 4, member a3
3.432	CCL3	chemokine (C-C motif) ligand 3
3.431	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3
3.204	NID1	similar to Nidogen precursor (Entactin); nidogen 1; similar to Nid1 protein
3.009	CD180	CD180 antigen
2.977	EMB	embigin
2.829	LACC1	Mus musculus laccase (multicopper oxidoreductase) domain containing 1 (Lacc1)
2.728	DAB2	disabled homolog 2 (Drosophila)
2.726	CYFIP2	cytoplasmic FMR1 interacting protein 2
2.721	RGS18	regulator of G-protein signaling 18
2.716	IGSF6	immunoglobulin superfamily, member 6
2.709	MCOLN2	mucolipin 2
2.689	STXBP1	syntaxin binding protein 1
2.651	SCN1B	sodium channel, voltage-gated, type I, beta
2.636	EFNA3	ephrin A3; similar to Ephrin A3
2.606	NDNF	Mus musculus neuron-derived neurotrophic factor (Ndnf)
2.538	AQP11	aquaporin 11
2.501	GPRC5A	G protein-coupled receptor, family C, group 5, member A
2.482	EAF2	ELL-associated factor 2
2.480	VAX2	ventral anterior homeobox containing gene 2
2.471	DMKN	dermokine
2.416	CADM3	cell adhesion molecule 3
2.407	FSCN1	fascin homolog 1, actin bundling protein (Strongylocentrotus purpuratus)
2.296	ZDBF2	zinc finger, DBF-type containing 2
2.269	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
2.253	BANK1	B-cell scaffold protein with ankyrin repeats 1
2.229	DCSTAMP	Mus musculus dendrocyte expressed seven transmembrane protein (Dcstamp)

2.226	IDO2	indoleamine 2,3-dioxygenase 2
2.206	SIRPA	signal-regulatory protein alpha
2.181	CDH17	cadherin 17
2.175	EML5	echinoderm microtubule associated protein like 5
2.127	PDE4A	phosphodiesterase 4A, cAMP specific
2.109	VHL	von Hippel-Lindau syndrome
2.091	TMEM206	transmembrane protein 206
2.088	FGD2	FYVE, RhoGEF and PH domain containing 2
2.082	H2-OB	histocompatibility 2, O region beta locus
2.052	GPR18	G protein-coupled receptor 18
2.052	AA667203	Mus musculus 3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630075J08 product:hypothetical protein, full insert sequence.
2.047	GM19760	Mus musculus activated spleen cDNA, RIKEN full-length enriched library, clone:F830221E22 product:hypothetical protein, full insert sequence.
2.044	H2-OA	histocompatibility 2, O region alpha locus
2.038	ATP8B4	ATPase, class I, type 8B, member 4
2.031	TNNI3	troponin I, cardiac 3
2.005	GPR82	G protein-coupled receptor 82
2.000	SLC7A7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
0.049	MMD	monocyte to macrophage differentiation-associated; similar to monocyte to macrophage differentiation-associated
0.046	MIR3095	Mus musculus microRNA 3095 (Mir3095)
0.045	CASS4	Cas scaffolding protein family member 4
0.044	STXBP5L	syntaxin binding protein 5-like
0.043	KLRC2	killer cell lectin-like receptor subfamily C, member 2
0.042	CCR7	chemokine (C-C motif) receptor 7
0.034	CRISP1	cysteine-rich secretory protein 1
0.031	SCN3A	sodium channel, voltage-gated, type III, alpha
0.030	TMEM45B	transmembrane protein 45b
0.023	ADAM23	a disintegrin and metallopeptidase domain 23; similar to ADAM23
0.015	S1PR3	sphingosine-1-phosphate receptor 3
0.015	EGFL8	EGF-like domain 8
0.007	KLRC1	killer cell lectin-like receptor subfamily C, member 1
0.005	SEMA7A	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A
0.003	ZFP366	zinc finger protein 366
0.001	SERPINB6B	serine (or cysteine) peptidase inhibitor, clade B, member 6b

* Fold change: Ctcf-deficient LCs / WT LCs

Table E2. Gene Ontology analysis for the differentially expressed genes exhibiting 1.5-fold changes in expression between WT and CTCF-deficient LCs with a FDR less than 0.05.

GO ID	GO term - BP FAT	P-value
GO:0042330	Taxis	9.76E-04
GO:0006935	Chemotaxis	9.76E-04
GO:0007155	Cell adhesion	1.76E-03
GO:0022610	Biological adhesion	1.78E-03
GO:0006955	Immune response	2.77E-03
GO:0009611	Response to wounding	1.29E-02
GO:0006814	Sodium ion transport	1.41E-02
GO:0007626	Locomotory behavior	1.58E-02
GO:0031589	Cell-substrate adhesion	2.23E-02
GO:0055080	Cation homeostasis	3.84E-02
GO:0010324	Membrane invagination	4.05E-02
GO:0006897	Endocytosis	4.05E-02
GO:0019882	Antigen processing and presentation	4.84E-02

GO ID	GO term - CC FAT	P-value
GO:0005886	Plasma membrane	5.00E-05
GO:0031224	Intrinsic to membrane	1.89E-02
GO:0044459	Plasma membrane part	2.86E-02
GO:0042613	MHC class II protein complex	4.01E-02

GO ID	GO term - MF FAT	P-value
GO:0019865	Immunoglobulin binding	4.06E-02

Table E3. Histological scoring of the epicutaneously sensitized skins (*, P<0.05 between WT-OVA and huLang-cKO-OVA).

	WT		huLang-cKO		
	Saline	OVA	Saline	OVA	
Inflammation	0.20±0.45	2.67±0.52	0.25±0.50	1.43±0.53	*
Neutrophils	0.00±0.00	1.33±0.52	0.00±0.00	0.86±0.69	
Mononuclear cells	0.60±0.55	2.67±0.52	0.75±0.96	1.43±0.53	*
Edema	0.00±0.00	1.67±0.52	0.00±0.00	1.00±0.58	
Epidermal spongiosis	0.00±0.00	2.17±0.41	0.00±0.00	0.43±0.53	*
Epithelial hyperplasia	0.20±0.45	2.5±0.55	0.25±0.50	1.00±0.58	*

Table E4. Primer sequences used for real-time qPCR.

Genes	Forward	Reverse
<i>Cadm3</i>	AGGGATTGTGGCTTTCATTG	TCGCTTCGTGTGTCAGGTAG
<i>Ccl3</i>	GTACCATGACACTCTGCAACC	GTCAGGAAAATGACACCTGGCTG
<i>Ccr7</i>	CCAGCAAGCAGCTCAACATT	GCCGATGAAGGCATACAAGA
<i>Cd69</i>	GGATTGGGCTGAAAAATGAA	CTCACAGTCCACAGCGGTAA
<i>Cdh17</i>	CACGACCAATGCAGGATATG	CACCAGTGGCTCAGGATTTT
<i>Ctcf (mouse)</i>	AGCGCTATCATGATCCCAAC	CGGCTCAGCATTTTCTTCAC
<i>CTCF (human)</i>	TGACACAGTCATAGCCCGAAAA	TGCCTTGCTCAATATAGGAATGC
<i>Emb</i>	CCCCGGTACAGAAAAACTGA	ACGTTGAGGGCATCTTTGTC
<i>Gapdh</i>	TGGCCTTCCGTGTTCTTAC	GAGTTGCTGTTGAAGTCGCA
<i>Klrc1</i>	TGCAAAGGTTTTCCATCTCC	TGCTTCGGTATATGGTGTGG
<i>Nid1</i>	TGGTTCCTCCTACACCTGCT	TGTTGTAGCAGAAGGCATCG
<i>Sema7a</i>	GGCGGAAGCTCTATGTGACC	GACTGCAGCACTGATCGTTGG

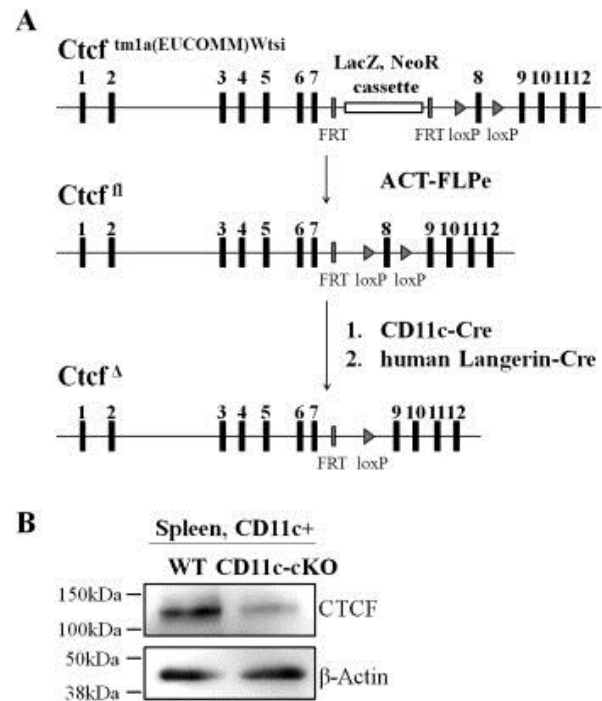


Fig E1

Tae-Gyun Kim , Mikyoung Kim , Jong-Joo Lee , Sung Hee Kim , Jeong Hwan Je , Yangsin Lee , Min-Ji Song , Yeeun Ch...

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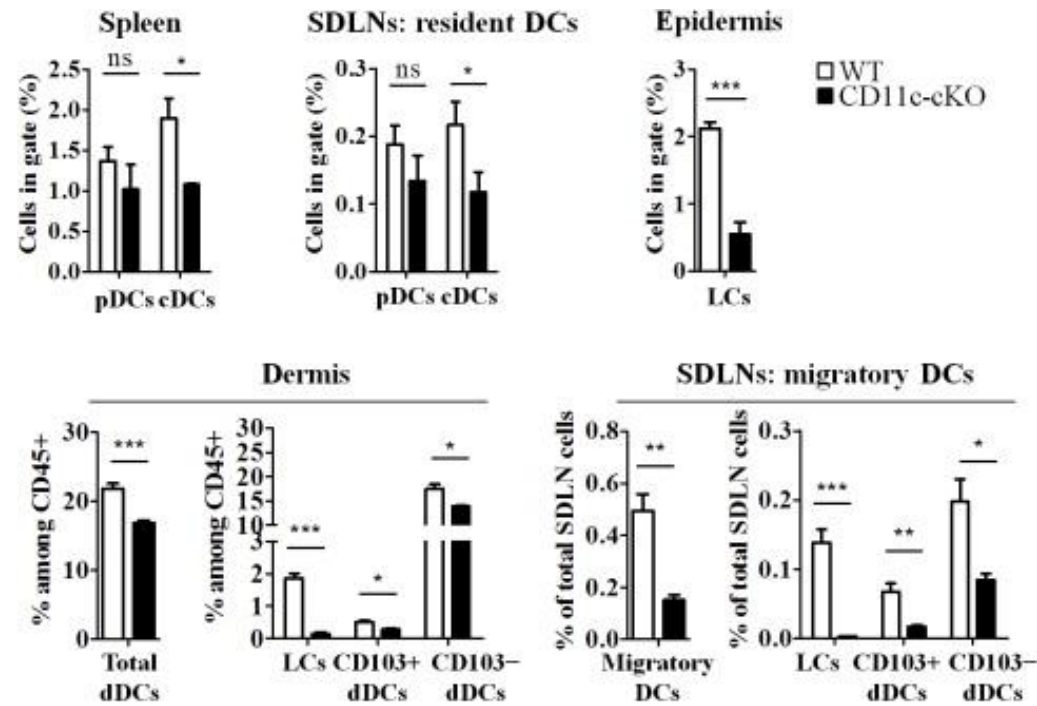


Fig E2

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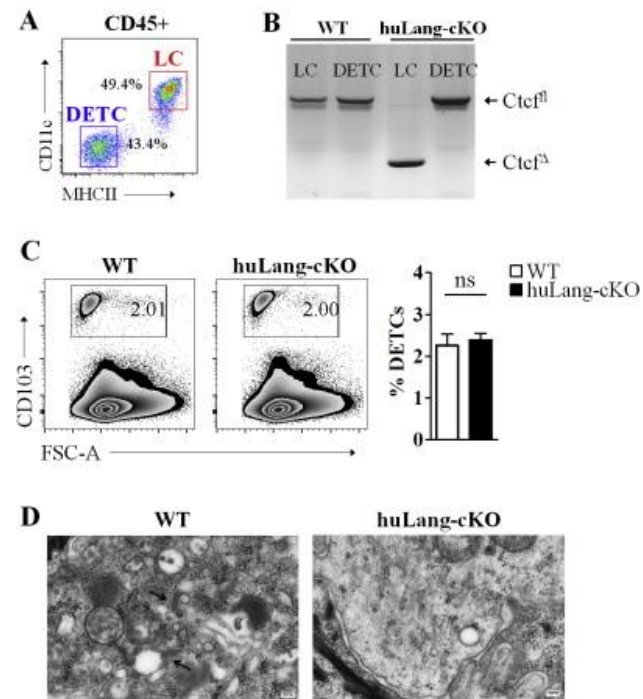


Fig E3

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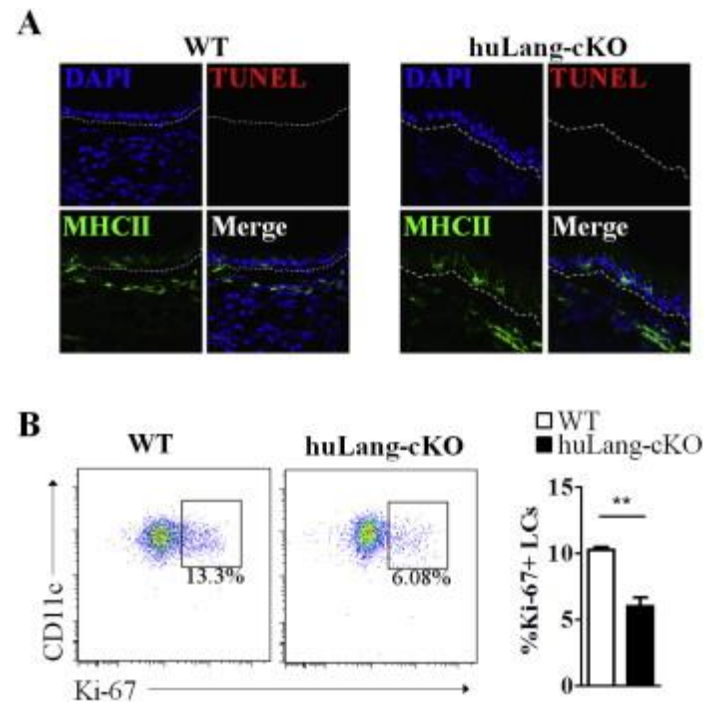


Fig E4

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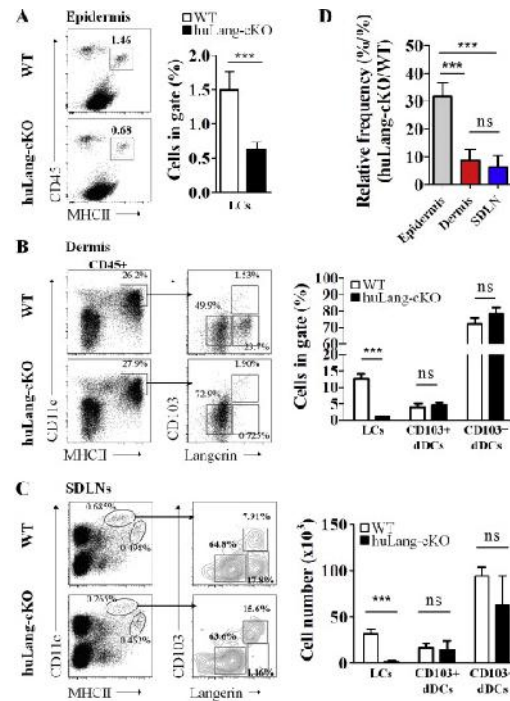


Fig E6

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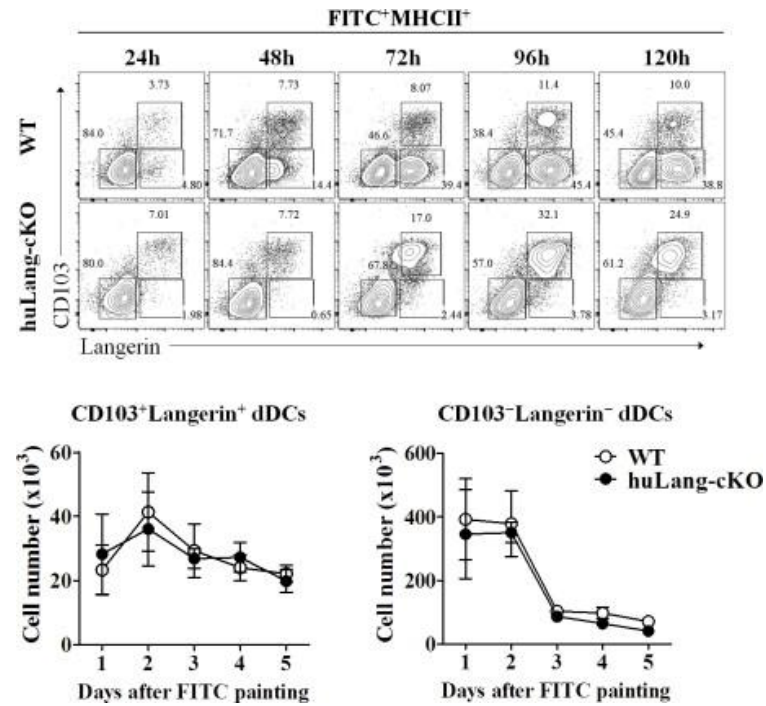


Fig E7

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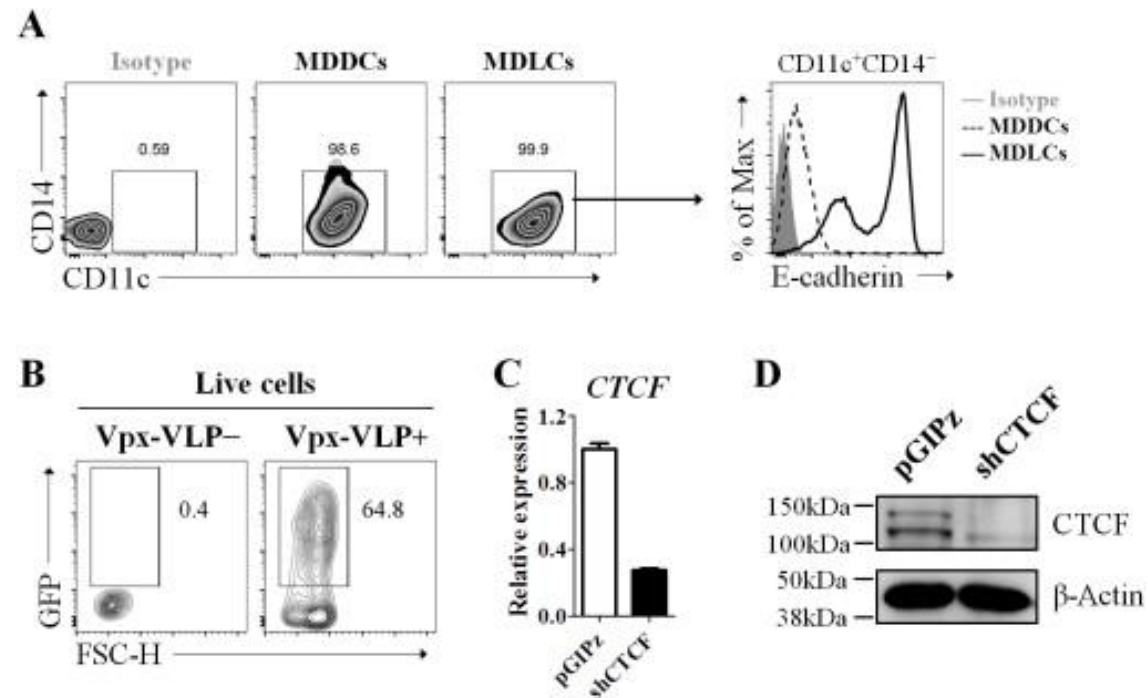


Fig E8

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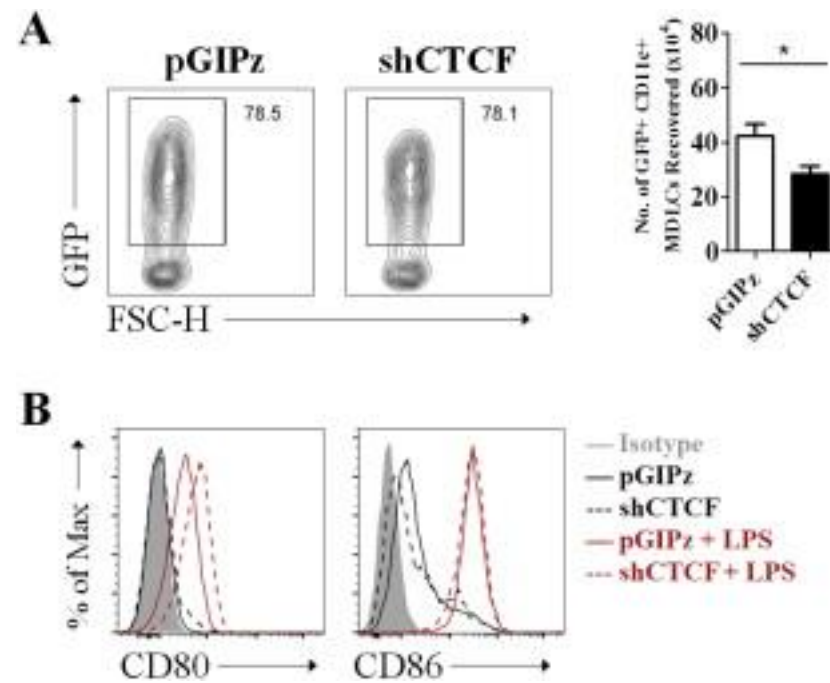


Fig E9

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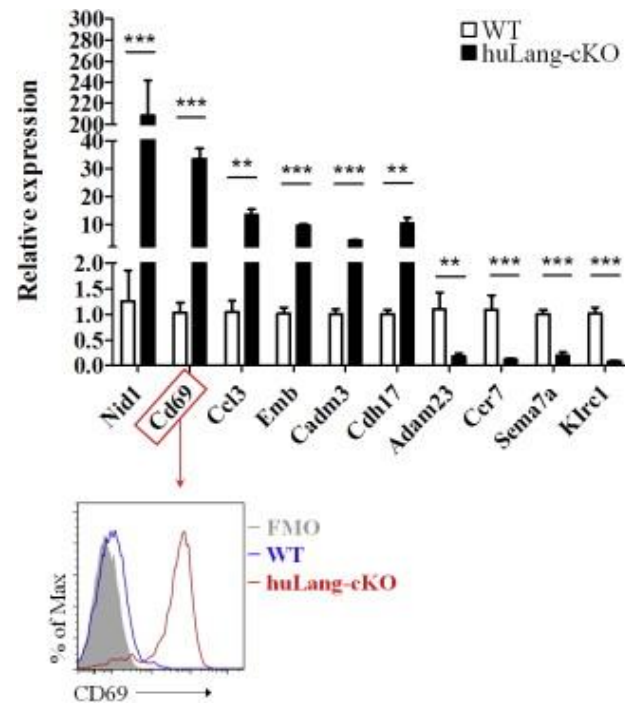


Fig E10

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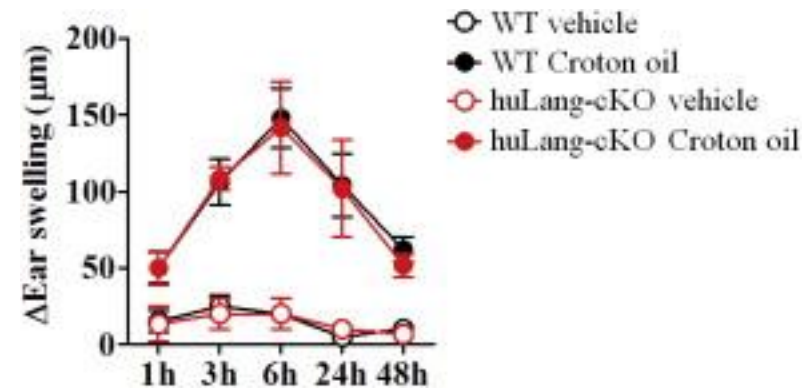


Fig E11

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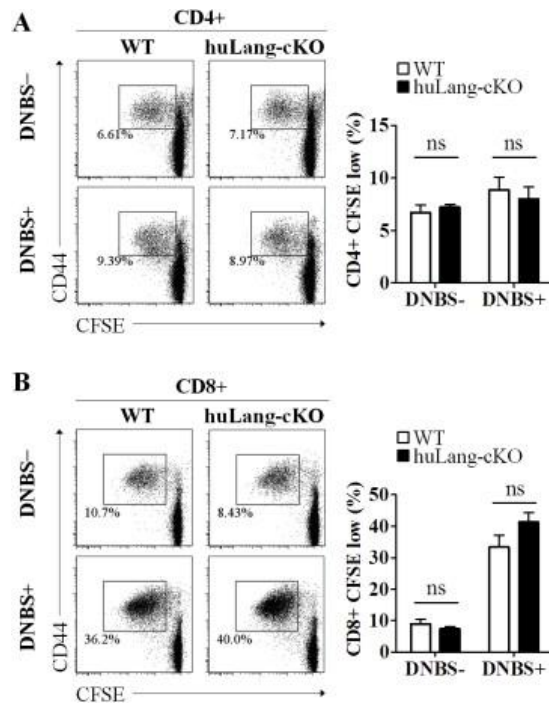


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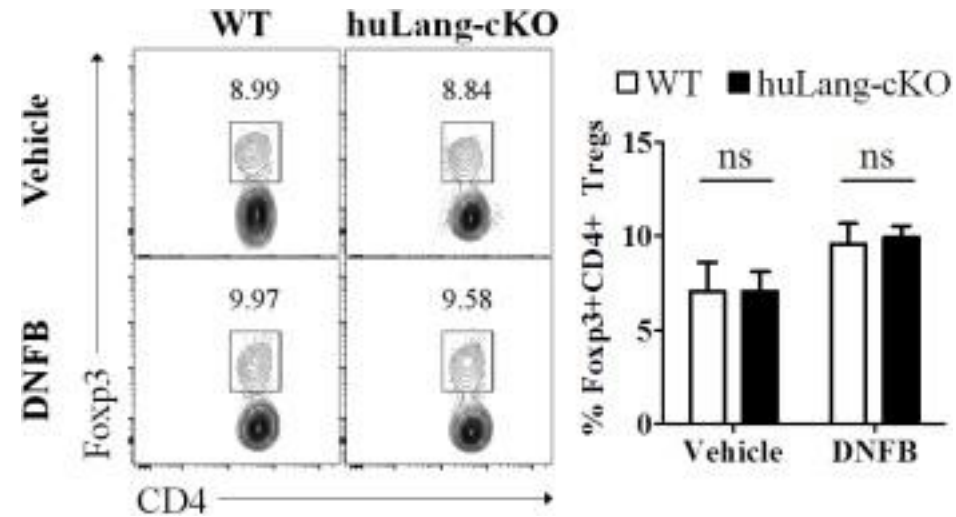


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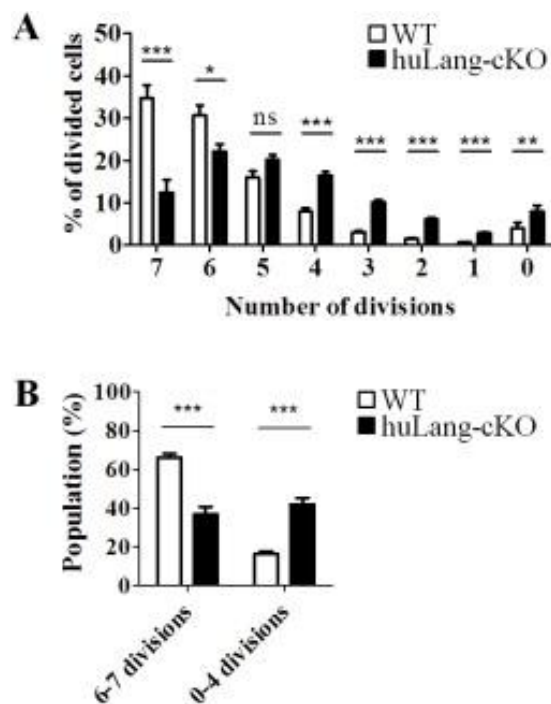


Fig E14

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