JEM

SUPPLEMENTAL MATERIAL

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Mouse genotyping. Genomic DNA of the offspring was isolated from tail biopsies and the genotype was determined by PCR. For the identification of the Tie2-Cre gene, the F-Tie2-Cre primer (5′-CCCTGTGCTCAGACAGAAATGAGA-3′) and the antisense primer Rev-Cre (5′-CCAGCAGGCGCAC-CATTGCCCCTG-3′) yielded a 1,263-bp PCR product. For the L1 gene, the L1-4F primer (5′-GAGCCACCTGTCATCACGGAAC-3′) and the antisense primer L1-4R (5′-CATGGATAAGAGGTTCTAGCACTC-3′) were used. These two primers give two PCR products of 490 and 320 bp for the $L1^{floxed}$ and the $L1^{wild\text{-}type}$ alleles, respectively.

Lymph node analysis. Lymph node and spleen cells were derived by tissue disaggregation with 500 µl of 3.2 mg/ml of collagenase (Sigma-Aldrich) and 0.4 mg/ml DNase (Roche) in RPMI medium for 40 min at 37°C. To characterize lymph node and spleen cells, FACS analysis was performed using the following antibodies: Alexa Fluor 647–conjugated rat anti-L1 (clone S10.33); allophycocyanin (APC)-conjugated anti–mouse CD3 (145-2C11; BD); APC-conjugated anti-CD19 (1D3; BD); APC- and FITC-conjugated anti-CD11b (M1/70; BD); PE-conjugated anti-CD11c (HL3; BD); PerCP-Cy5.5–conjugated anti–Gr-1 (RB6-8C5; BD); FITC-conjugated anti-B220 (RA3-6B2; BD); FITC- or APC-conjugated anti–CD8-α and anti-CD4 (BD); FITC-conjugated anti-F4/80 (Ci:A3-1; Invitrogen); Alexa Fluor 488–conjugated rat anti–mouse langerin (clone 929F3; Dendritics).

Chemotaxis assays. Chemotaxis assays were performed using polycarbonate Transwell inserts (5-µm pore; Corning) as described previously (1). CFSE-labeled DCs were seeded in the upper compartment and chemoattractants (100 ng/ml CCL3 or CCL9; PeproTech) were added to the lower compartment. After different incubation times at 37°C, Transwell inserts were thoroughly washed with PBS, fixed in paraformaldehyde (PFA), and mounted onto a microscope slide. Images of CFSE-labeled DCs were obtained with a microscope (Biosystems BX-71; Olympus). DC transmigration was quantified by measuring the green fluorescent area on the bottom side of the filters with ImageJ software (National Institutes of Health).

TAT-Cre transduction of $L1^{floxed}$ bone marrow precursor cells. Purified TAT-Cre protein (2) was used to transduce $L1^{floxed}$ bone marrow precursors. The transduction was performed at days 2 and 7 of the DC differentiation period as described below. Nonadherent and weakly adherent bone marrow cells were centrifuged and washed three times in 10 ml HyQ ADCF mAb medium (Thermo Fisher Scientific). Afterward, cells were counted and resuspended at the density of 10^7/ml in HyQ ADCF mAb medium. A 100-µg/ml solution of TAT-Cre in HyQ ADCF mAb medium was prepared and both cells and TAT-Cre solution were prewarmed for 10 min at 37°C. Then cells and TAT-Cre solution were combined at a 1:1 ratio (vol/vol) and incubated for 45 min at 37°C. After the incubation period, 10 ml of fresh GM-CSF–containing medium were added and cells were centrifuged, counted, and seeded at the density of 2×10^6 ml.

Western blotting. For Western blotting analysis, DCs and ECs were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-100). Protein extracts (50 mg/lane) were separated on an SDS polyacrylamide gel (SDS-PAGE) and then transferred onto nitrocellulose membrane (GE Healthcare). The proteins of interest were detected using specific antibodies. Rat anti–mouse L1 (clone I4.2) was used to analyze L1 expression in mouse DCs and ECs. Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Thermo Fisher Scientific). The signal was revealed by an enhanced chemiluminescence kit (ECL kit; GE Healthcare).

Cell immunofluorescence staining. The immunofluorescence analysis of endothelial cells was performed as described previously (3). In brief, cells were seeded on fibronectin-coated glass coverslips and allowed to reach confluence. Cells were then fixed with 3.7% PFA and permeabilized with 0.5% Triton X-100 before staining. After incubation with the primary antibody (anti-L1 [clone S10.33] for mouse cells and rabbit anti–human L1 for human cells), cells were washed and incubated with the fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Cell nuclei were counterstained with DAPI (Jackson ImmunoResearch Laboratories).

Injection of FITC-latex particles. Mice were anesthetized by intraperitoneal injection of 2.5% avertin and shaved at four sites of the dorsal skin, which were drained either by the inguinal or brachial lymph nodes. Ten million FITC-conjugated latex particles (Invitrogen) were injected intradermally, as tracers, using a Hamilton syringe (Thermo Fisher Scientific). $CD11c^+$ cells carrying latex particles to draining lymph nodes were analyzed 3 d after treatment. For quantification, the entire population of lymph node cells was analyzed by cytofluorimetry and the numbers of $CD11c^+/FITC^+$ cells per lymph node were determined.

Staining of mouse epidermal sheets. Epidermal sheets were prepared from the ears of C57BL/6 mice as described previously (4). After fixation in methanol/acetone (1:1), epidermal sheets were subjected to immunofluorescence costaining with anti-langerin (clone 929F3) and anti-L1 (clone S10.33), according to the same procedure described in Materials and methods. Stained tissues were then analyzed by confocal microscopy.

Ex vivo staining of mouse vessels. 6-wk-old C57BL/6 mice were sacrificed and the ears were removed. The skin was separated from the ear cartilage and placed in a 2-cm well in RPMI medium supplemented with 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The skin tissue was incubated, where indicated, with 20 ng/ml TNF-α for 16 h at 37°C. After fixation with PFA, staining was performed using rat anti–PECAM-1 and rabbit anti–LYVE-1 (Millipore) followed by Cy3- and Cy5-conjugated secondary antibodies. Tissue was fixed again, blocked with excess rat IgG, and then incubated with Alexa Fluor 488–conjugated rat anti–mouse L1 (clone S10.33). The tissue was then mounted onto microscope slides and images were obtained as described for cell immunofluorescence.

Retroviral transduction of 1G11 cells. Mouse cDNA encoding full-length L1 was excised with EcoRI from the pcDNA3.1-L1 vector (a gift from M. Schachner) and cloned into the retroviral vector PINCO (5) according to standard DNA cloning procedures. The resulting PINCO-L1 vector was amplified in competent bacterial cells (DH5-α) and purified using the Mini- or Megaprep kits (QIAGEN) according to the manufacturer's instructions.

The packaging cell line Phoenix was transfected with the PINCO vector or with PINCO-L1 vector by the calcium phosphate chloroquine method (6, 7). Culture supernatants containing viral particles were collected 48 h after transfection. 1G11 cells were cultured in the presence of viral supernatant (filtered with a 0.45-µm filter) supplemented with 4 µg/ml polybrene (Sigma-Aldrich) for 3 h. Four infection cycles were performed. After infection, 1G11 cells were plated in their complete medium for 24 h.

Because PINCO vectors contain GFP (5), transmigration assays with transduced 1G11 cells were performed using DCs prelabeled with PKH26 red fluorescent dye (Sigma-Aldrich) according to the manufacturer's instructions. Transmigrated PKH26-labeled DCs were counted as described for chemotaxis assays.

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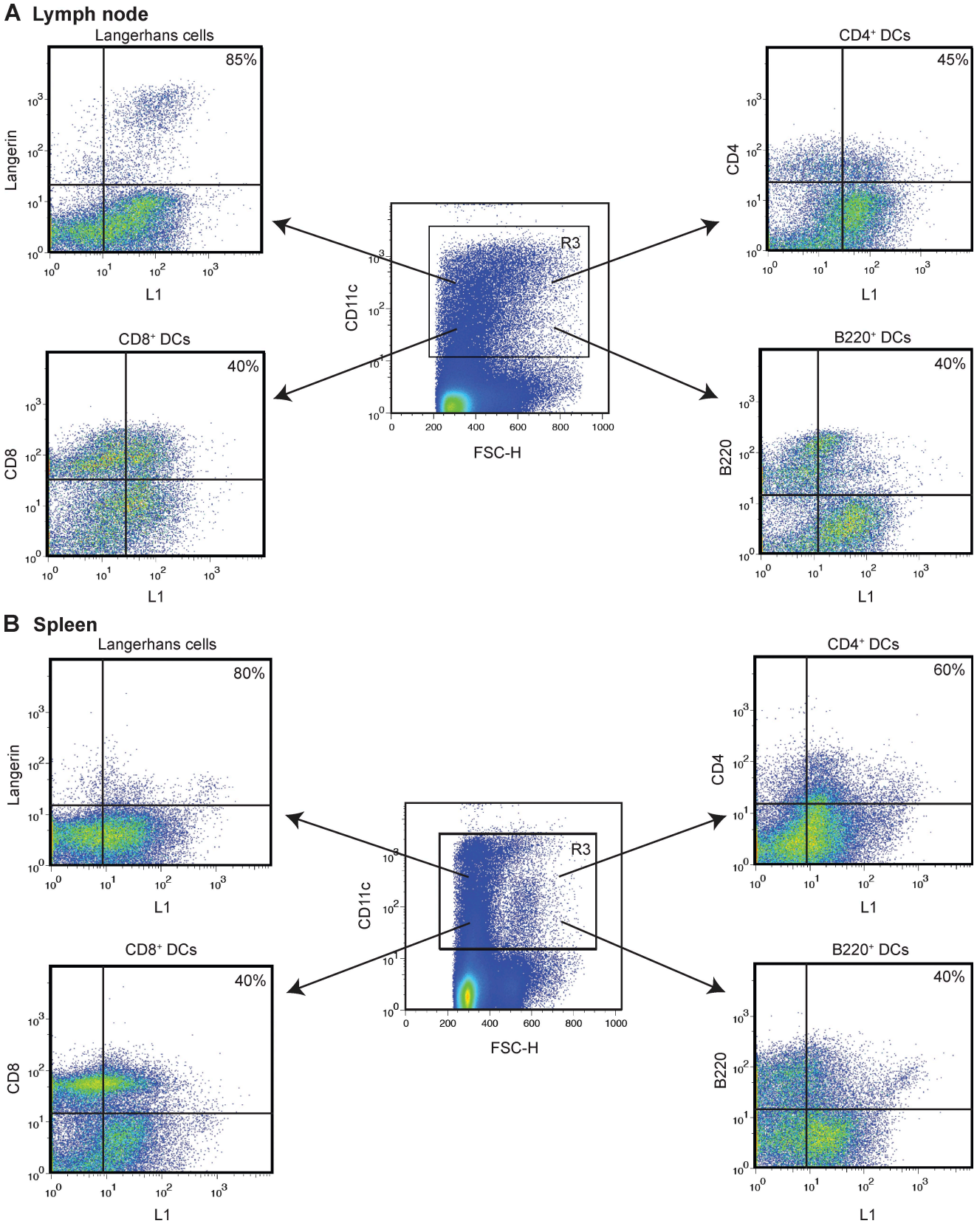
Figure S1. Characterization of L1-expressing DCs in lymph nodes and spleen. Single-cell suspensions from mouse lymph nodes (A) or spleen (B) were first stained for surface antigens (CD11c, L1, CD4, CD8, and B220) and then fixed and permeabilized before costaining with clone 9292F3 which recognizes intracellular langerin (see Material and methods). Cells were gated for CD11c expression and analyzed for the coexpression of L1 with langerin, CD4, CD8, and B220. The percentages of $CD11c^+/L1^+$ DCs coexpressing langerin, CD4, CD8, or B220 are indicated. The figure refers to a representative analysis performed on pooled cells from the lymph nodes and spleens of five mice. The analysis was independently repeated three times.


A Lymph node
Langerhans cells
85%
Langerin
L1
CD4⁺ DCs
45%
CD4
L1
CD8⁺ DCs
40%
CD8
L1
B220⁺ DCs
40%
B220
L1
R3
CD11c
FSC-H
B Spleen
Langerhans cells
80%
Langerin
L1
CD4⁺ DCs
60%
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L1
R3
CD11c
FSC-H

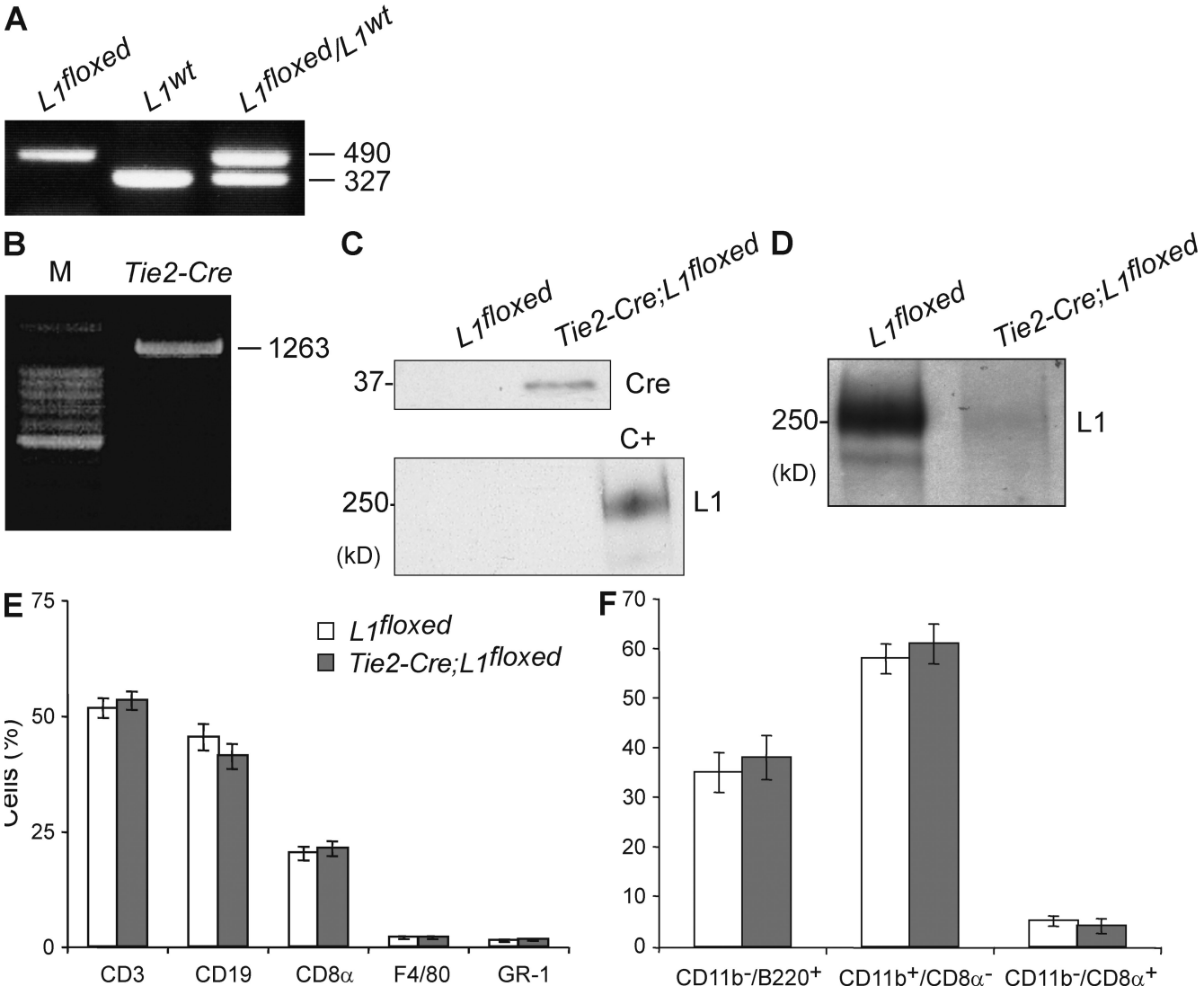


Figure S2. **Characterization of *L1*^{floxed} and *Tie2-Cre;L1*^{floxed} mice.** (A and B) Mouse genotyping. Representative results of PCR performed on tail biopsies to detect the *L1*^{floxed} allele (A) or the Tie2-Cre transgene (B). L1 primers amplified a 327-bp fragment for the wild-type allele and a 490-bp fragment for the *L1*^{floxed} allele. Tie2-Cre primers amplified a 1,263-bp fragment. (C) Representative results of the immunoblotting analysis for the expression of Cre (top) or L1 (bottom) in bone marrow precursors from *L1*^{floxed} and *Tie2-Cre;L1*^{floxed} mice. Similar results were obtained with bone marrow precursors from at least five mice per genotype. C+, positive control. (D) Expression of L1 in bone marrow–derived DCs from *L1*^{floxed} and *Tie2-Cre;L1*^{floxed} mice was analyzed by immunoblotting. Similar results were obtained with bone marrow–derived DCs from at least five mice per genotype. (E) Inguinal lymph nodes from *L1*^{floxed} or *Tie2-Cre;L1*^{floxed} mice were subjected to collagenase/DNase treatment, followed by FACS analysis for the expression of the following markers: CD3 (T lymphocytes), CD19 (B lymphocytes), CD8-α (cytotoxic T lymphocytes), F4/80 (macrophages), and GR-1 (granulocytes). (F) CD11c-positive cells were gated and analyzed for the following markers of DC subpopulations: CD11b^{-}/B220^{+} (plasmacytoid DCs), CD11b^{+}/CD8-α^{-} (myeloid DCs), and CD11b^{-}/CD8-α^{+} (lymphoid DCs). Data refer to the means ± SD from four mice for each genotype. No difference could be observed in the relative amount of either hematopoietic cell types or DC subpopulations.

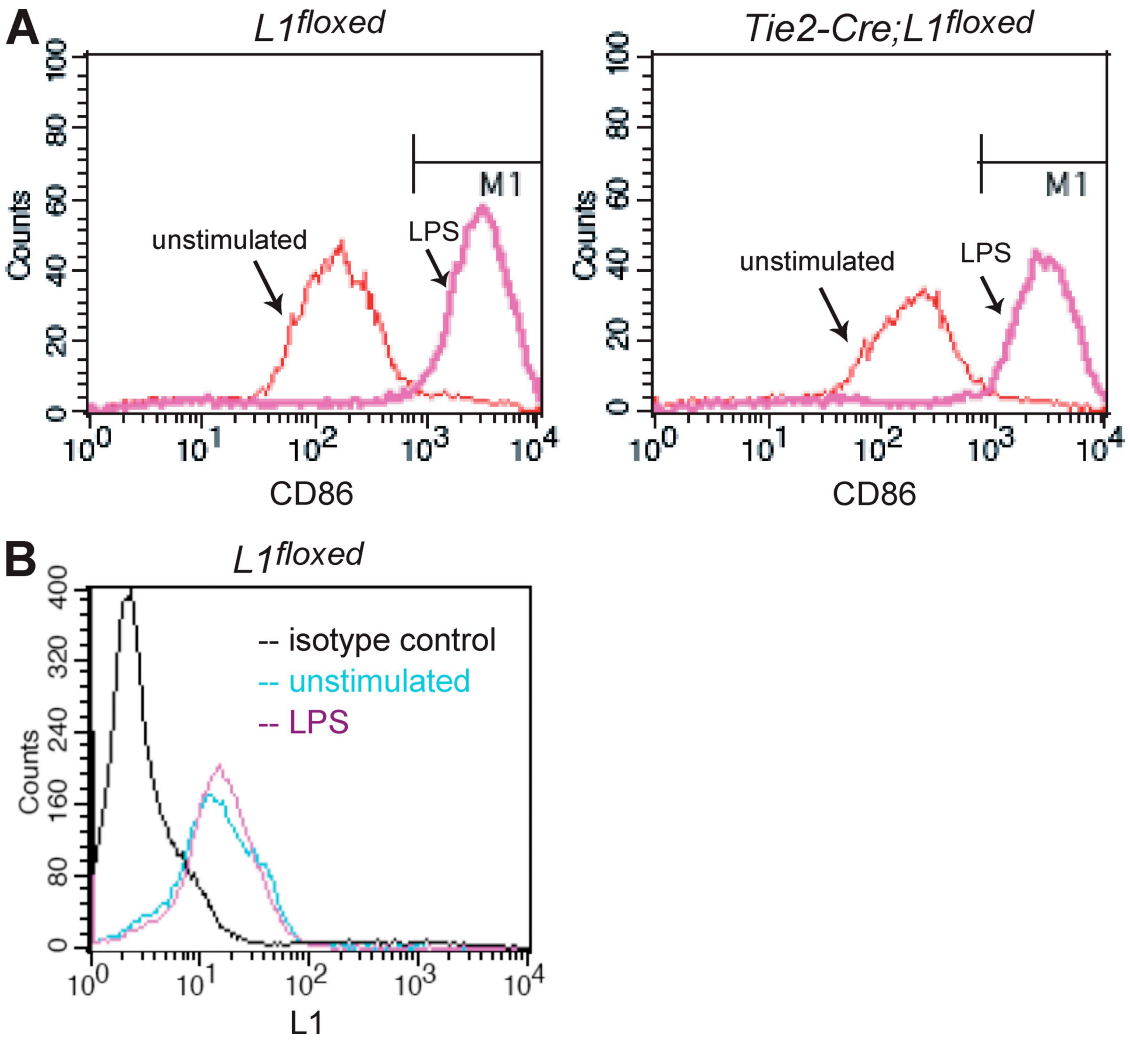


Figure S3. LPS-induced activation of DCs. (A) Bone marrow–derived DCs from *L1*^{floxed} (left) or *Tie2-Cre;L1*^{floxed} (right) mice were left untreated or were stimulated with 100 ng/ml LPS for 16 h, followed by FACS analysis for CD86 expression. The latter was induced by LPS in both *L1*^{floxed} and *Tie2-Cre;L1*^{floxed} DCs. (B) *L1*^{floxed} DCs were stimulated with LPS or left untreated. Cells were then stained with anti-L1 or with an isotype-matched antibody. Data refer to a representative experiment which was independently repeated three times using different DC batches.

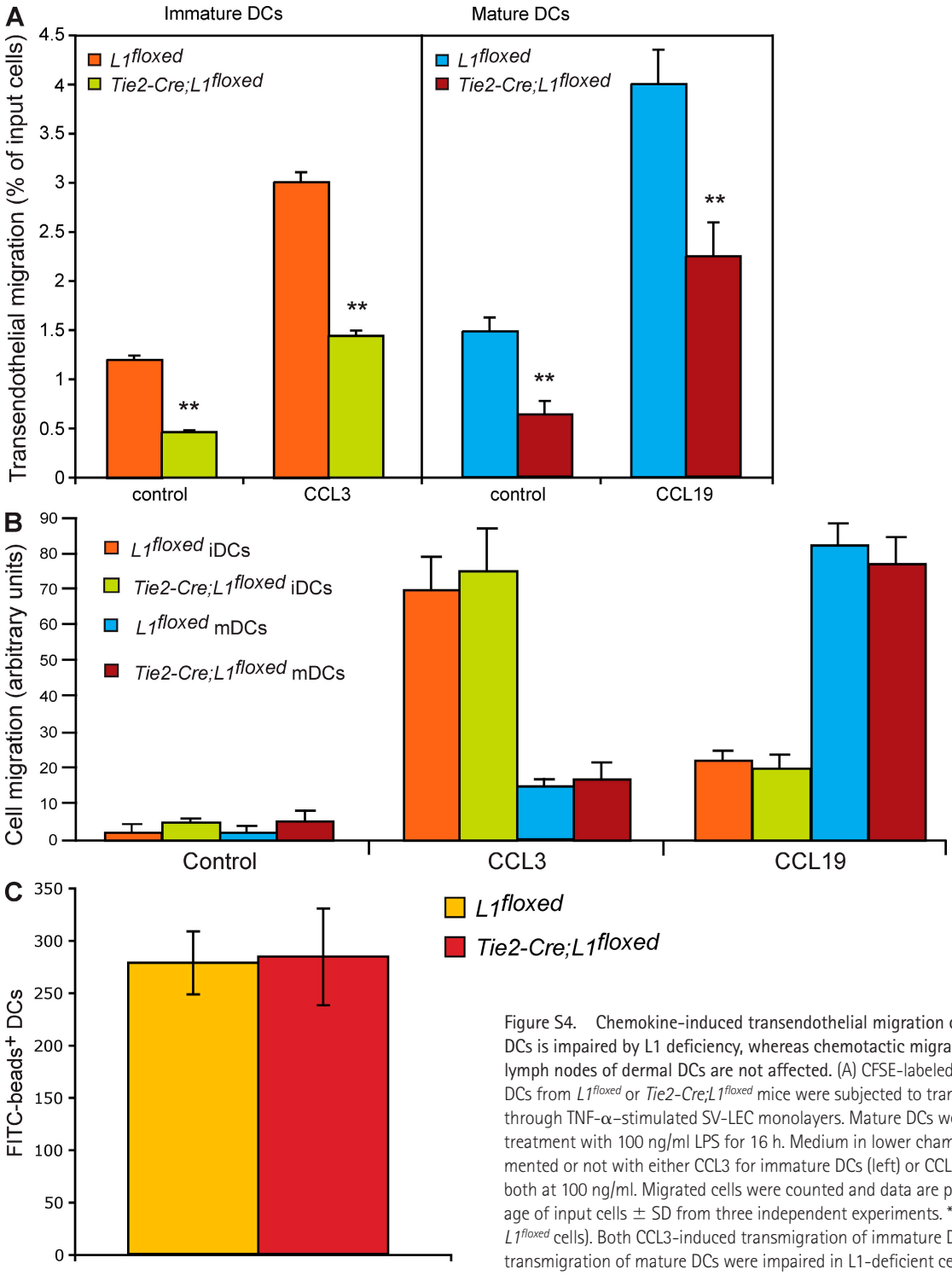


Figure S4. Chemokine-induced transendothelial migration of bone marrow–derived DCs is impaired by L1 deficiency, whereas chemotactic migration and migration to lymph nodes of dermal DCs are not affected. (A) CFSE-labeled bone marrow–derived DCs from *L1*^{floxed} or *Tie2-Cre;L1*^{floxed} mice were subjected to transmigration assays through TNF-α–stimulated SV-LEC monolayers. Mature DCs were obtained by a pretreatment with 100 ng/ml LPS for 16 h. Medium in lower chambers was either supplemented or not with either CCL3 for immature DCs (left) or CCL19 for mature DCs (right), both at 100 ng/ml. Migrated cells were counted and data are presented as the percentage of input cells ± SD from three independent experiments. **, $P < 0.005$ (relative to *L1*^{floxed} cells). Both CCL3-induced transmigration of immature DCs and CCL19-induced transmigration of mature DCs were impaired in L1-deficient cells. (B) Bone marrow–derived DCs from *L1*^{floxed} or *Tie2-Cre;L1*^{floxed} mice were subjected to chemotaxis assays using either CCL3 or CCL19 (both at 100 ng/ml) as chemoattractants in the lower compartment of modified Boyden chambers. Mature DCs (mDCs) were obtained by a pretreatment with 100 ng/ml LPS for 16 h. As expected, CCL3 induced the migration of immature DCs (iDCs), whereas CCL19 induced the migration of mDCs. No difference in chemotactic migration was observed between *L1*^{floxed} and *Tie2-Cre;L1*^{floxed} DCs. Migrated cells were counted and data are presented as the means ± SD from a representative experiment performed in triplicate. The experiment was independently repeated three times. (C) FITC-labeled latex beads (10^7 beads in 20 µl) were injected intradermally into *L1*^{floxed} or *Tie2-Cre;L1*^{floxed} mice, as described in the supplemental Materials and methods. After 72 h, inguinal draining lymph nodes were excised and subjected to FACS analysis to count CD11c$^+$ cells that internalized FITC-labeled beads. Data are expressed as CD11c$^+$/FITC$^+$ cells and represent the means ± SD of a representative experiment with six mice per group. The experiment was repeated twice. No difference between the two mouse genotypes in the uptake of latex beads by dermal DCs and migration to lymph nodes was observed.

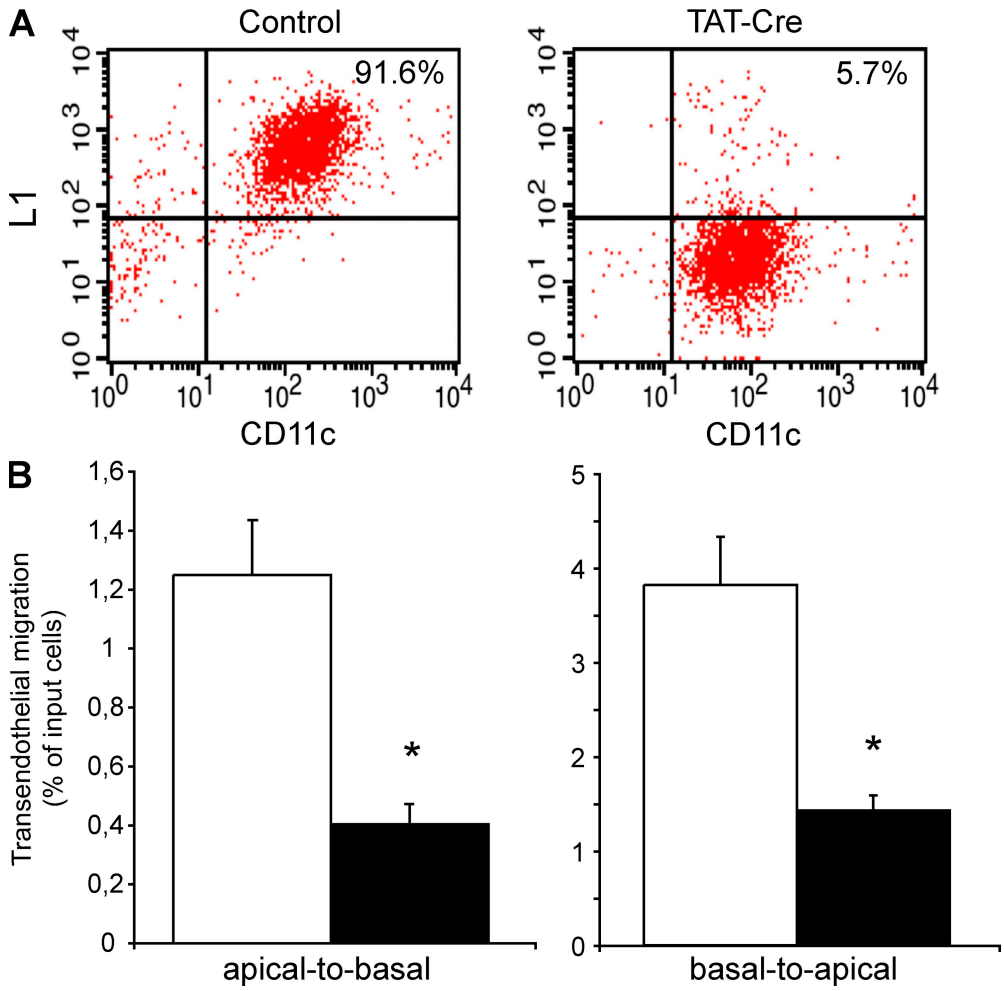
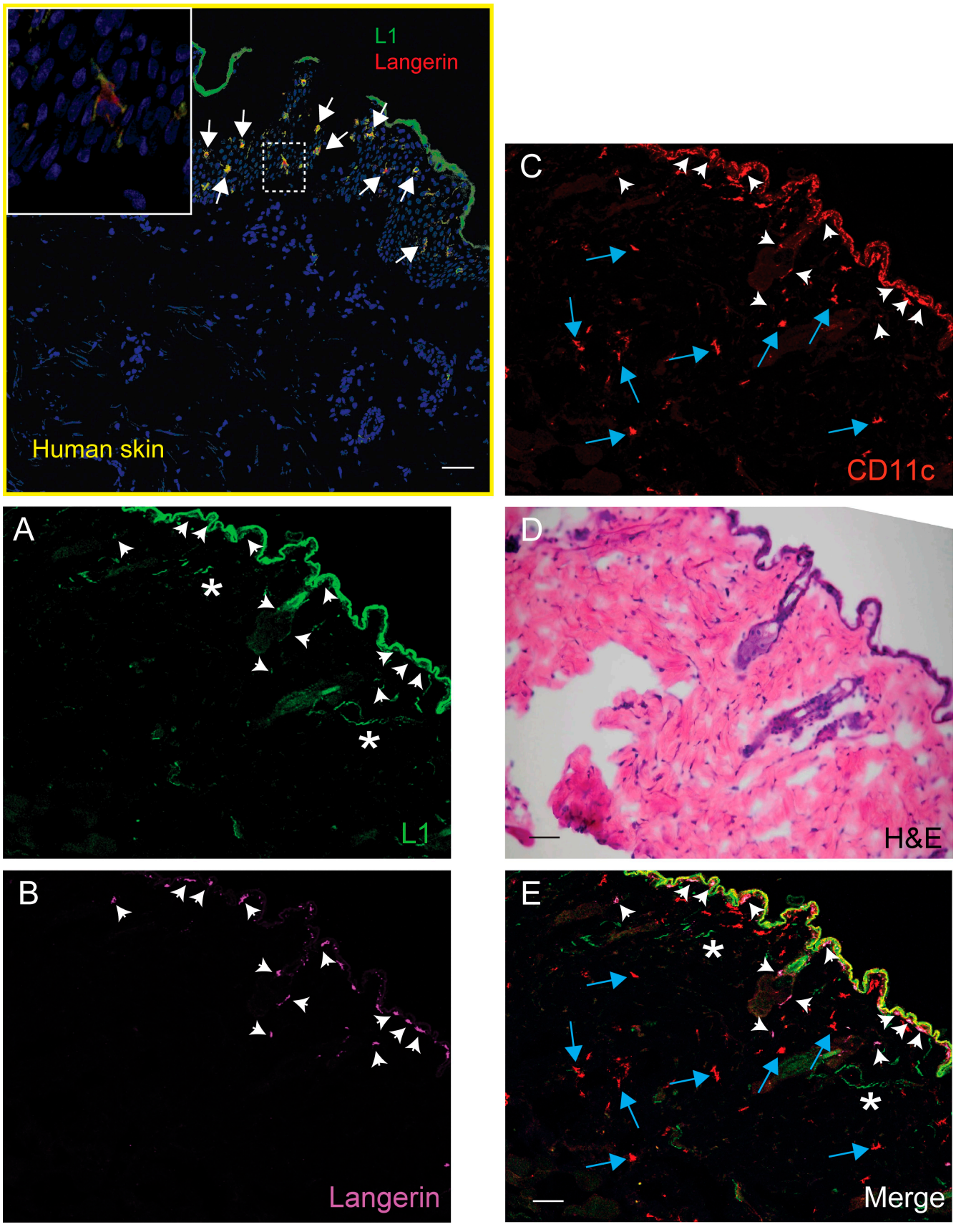


Figure S5. Tat-Cre–mediated ablation of L1 leads to impaired transendothelial migration of *L1*^{floxed} DCs. (A) Bone marrow–derived progenitors from *L1*^{floxed} mice were transduced with purified Tat-Cre during their differentiation to DCs (see Materials and methods). L1 expression in CD11c^{+} Tat-Cre–treated (right) or untreated cells (left) was then analyzed by FACS analysis using Alexa Fluor 488–conjugated anti-L1 antibody. Data refer to a representative experiment. The analysis was independently repeated three times using different DC batches. (B) CFSE-labeled DCs derived from control (white) or Tat-Cre–treated progenitors (black) were subjected to apical-to-basal or basal-to-apical transmigration assays through SV-LEC monolayers (see Fig. 3 legend). Data represent the means ± SD of a representative experiment performed in triplicate. The experiment was independently repeated three times (with L1^{floxed} progenitors from three different mice) with similar results. *, $P < 0.005$ (relative to control cells).


L1
Langerin
Human skin
C
CD11c
A
L1
D
H&E
B
Langerin
E
Merge

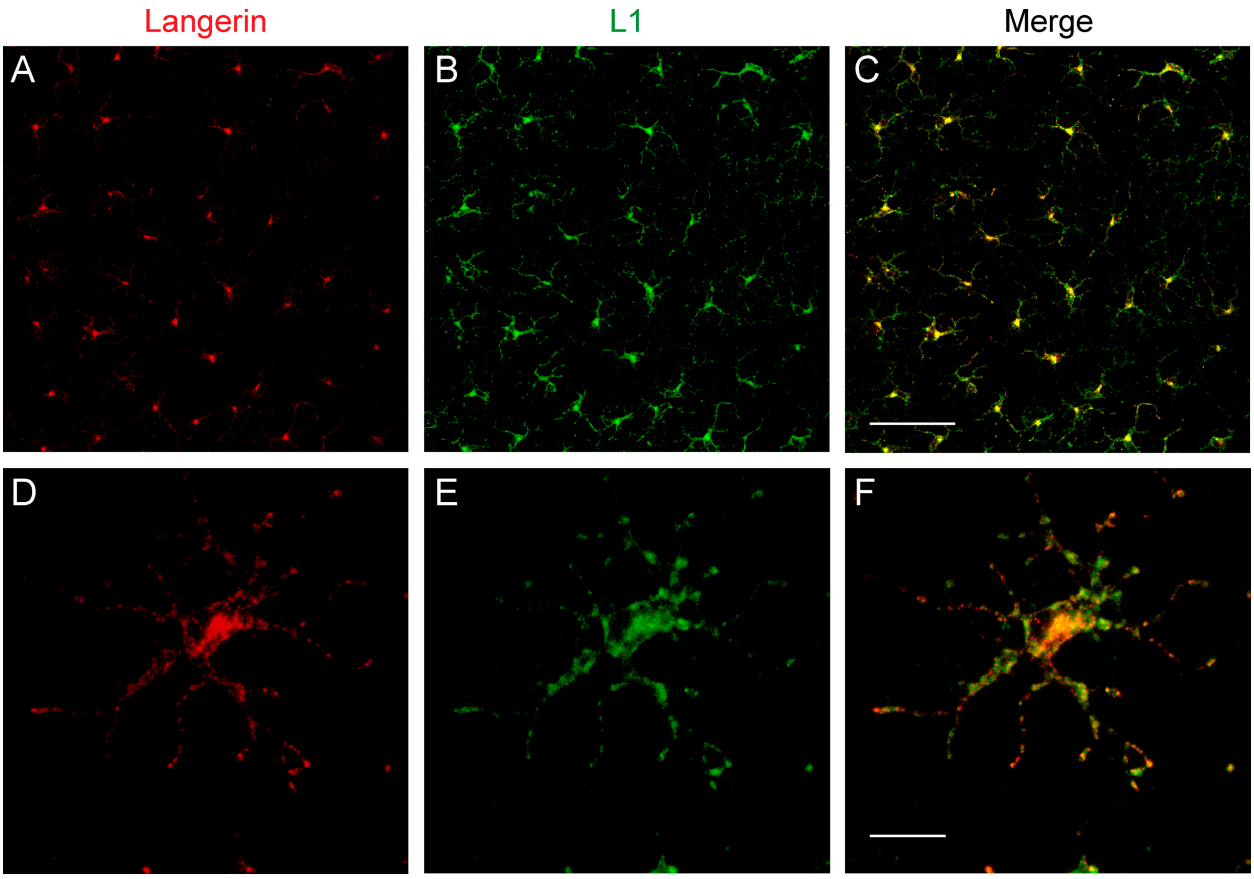


Figure S7. Widespread expression of L1 in mouse epidermal Langerhans cells. Epidermal sheets were prepared from the ears of C57BL/6 mice and then subjected to immunofluorescence costaining for langerin (clone 929F3; red) and L1 (clone S10.33; green). L1 is expressed in all langerin-positive cells. Similar results were obtained with epidermal sheets from three mice. Bars: (C) 50 µm; (F) 10 µm.

Figure S6. L1 is specifically expressed in cutaneous Langerhans cells but not in dermal DCs. Skin tissue sections from C57BL/6 mice were subjected to immunofluorescence triple staining for CD11c (red), Langerin (purple), and L1 (green), as described in Material and methods and in the Fig. 4 B legend, before confocal analysis. Arrowheads indicate $CD11c^{+}$/$langerin^{+}$ Langerhans cells that express L1, and arrows indicate $CD11c^{+}$/$Langerin^{-}$ dermal DCs that do not express L1. The asterisks indicate L1-positive nerves that served as internal control. E shows the merge of the different colors, whereas a serial tissue section stained with hematoxylin and eosin is shown in D. The staining was independently repeated three times with sections from three different mice. The costaining for L1 and Langerin in a human skin section is shown on the bottom left. A higher magnification of the boxed area is shown in the inset. L1 is specifically expressed in Langerhans cells in both mouse and human skin, whereas no L1 is detectable in dermal DCs. Bars, 50 µm.

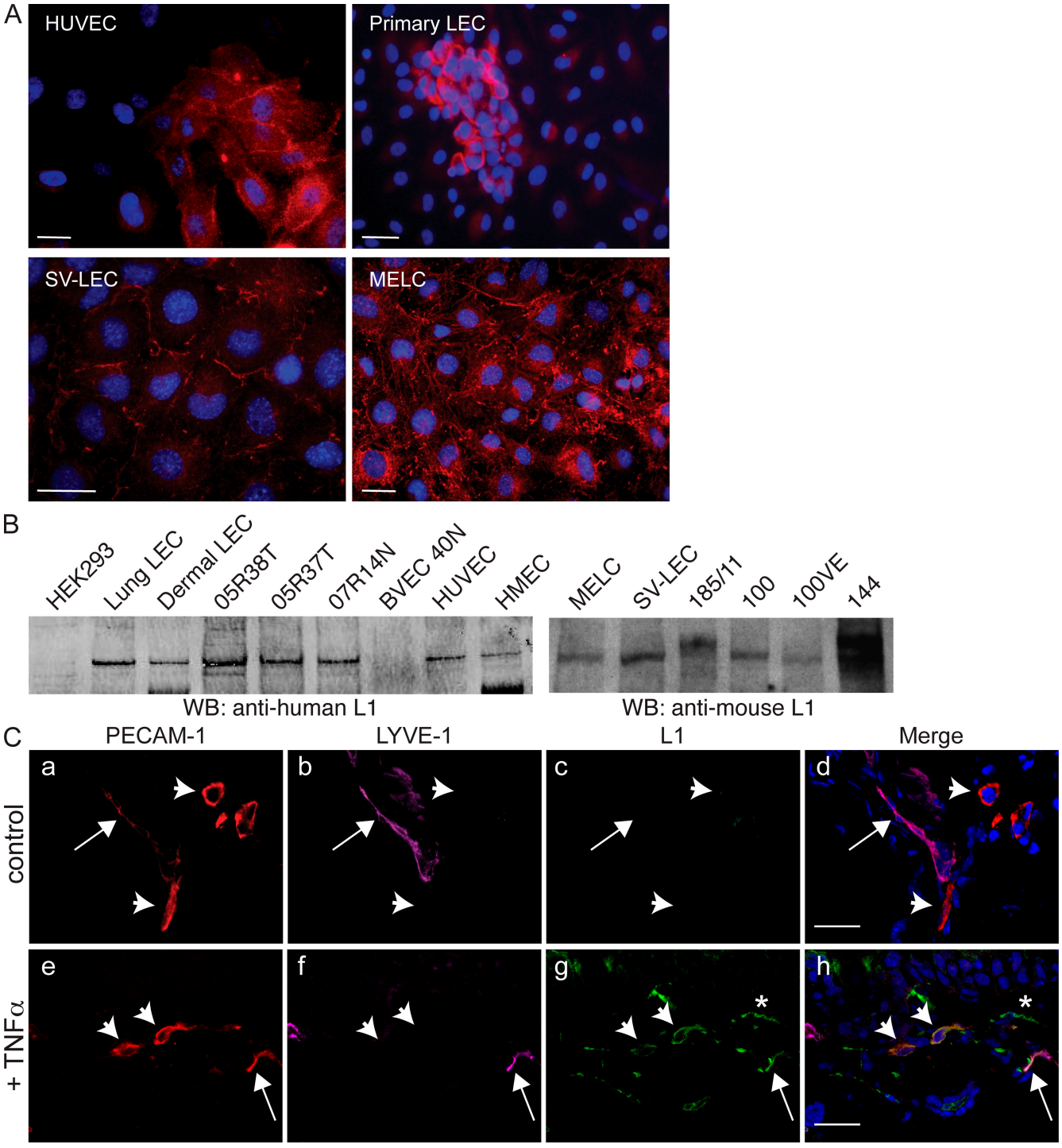


Figure S8. L1 expression in blood and lymphatic vascular endothelial cells. (A) HUVEC, primary prostate LEC, SV-LEC, and MELC, cultured in complete growth medium, were fixed and subjected to immunofluorescence staining for L1. All four cell types showed L1 expression, with an enrichment at the cell–cell borders. Bars, 10 µm. (B) A panel of protein lysates from primary or established endothelial cell populations was immunoblotted for L1, revealing its widespread expression in blood and lymphatic vascular endothelial cells. HEK293, human embryonic kidney; 05R38T, 05R37T, and 07R14N, primary LEC from human prostate; BVEC 40N, primary blood vessel ECs from human prostate; HMEC, human dermal ECs; 185/11, 100, 100VE, and 144, immortalized mouse blood vessel ECs. (C) Ex vivo assay for TNF-α–induced expression of L1 in endothelium. Skin tissue fragments from mouse ears were treated either with vehicle (a–d) or with 20 ng/ml TNF-α for 16 h (e–h) before immunofluorescence costaining for PECAM-1, LYVE-1, and L1 as described in the supplemental Materials and methods. Arrowheads indicate blood vessels (strongly positive for PECAM-1 and negative for LYVE-1), and arrows indicate lymphatic vessels (weakly positive for PECAM-1 and strongly positive for LYVE-1). The asterisks indicate an L1-positive nerve that served as an internal control. No L1 immunoreactivity was observed in the vessels untreated ears, whereas TNF-α induced L1 expression in both blood and lymphatic vessels. The experiment was independently repeated three times with three different mice. Bars, 25 µm.

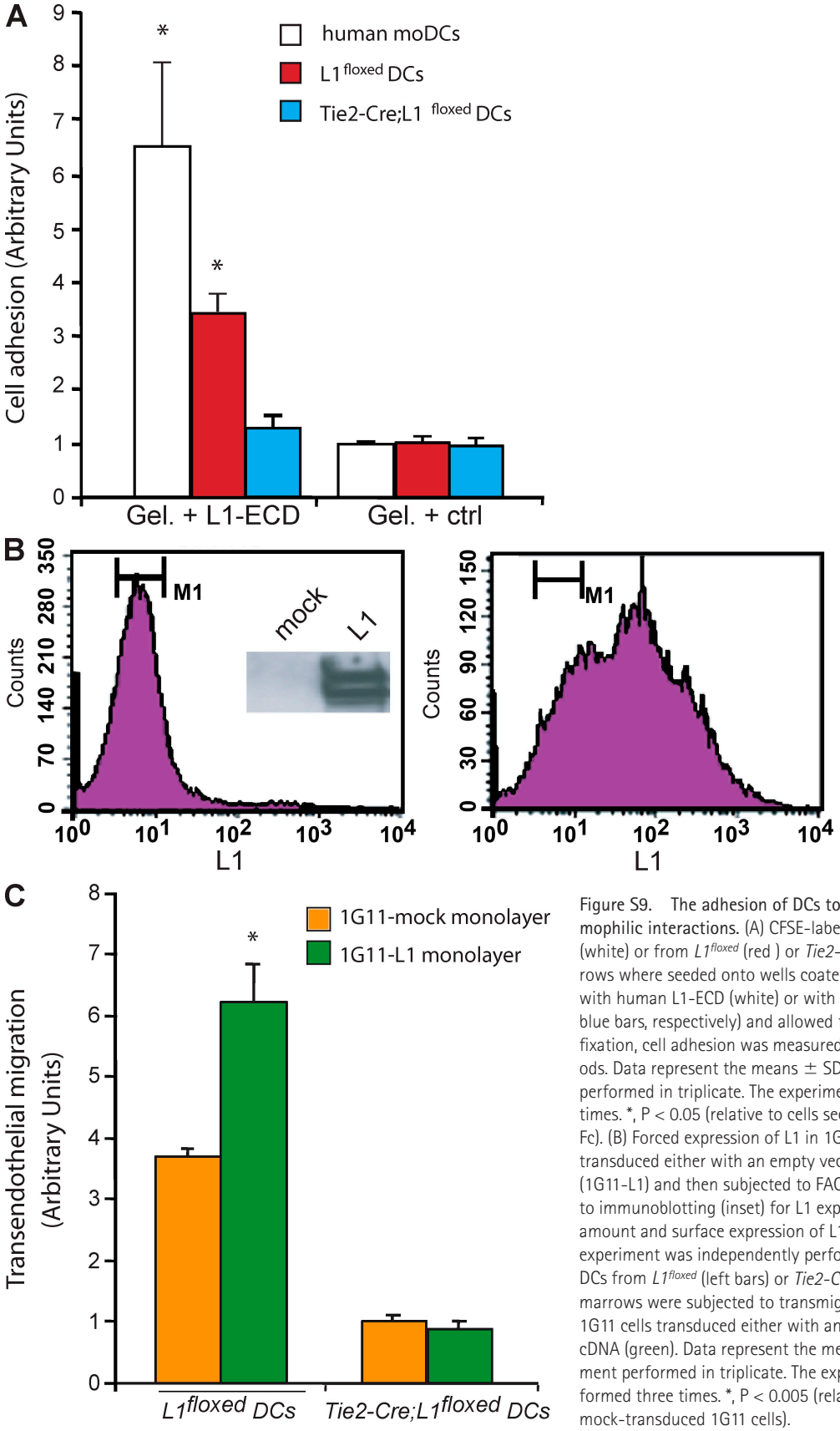


Figure S9. The adhesion of DCs to endothelium is mediated by L1 homophilic interactions. (A) CFSE-labeled DCs from human monocytes (white) or from *L1floxed* (red) or *Tie2-Cre;L1floxed* (blue) mouse bone marrows where seeded onto wells coated with gelatin supplemented either with human L1-ECD (white) or with mouse L1-Fc or Fc alone (red and blue bars, respectively) and allowed to adhere for 1 h. After washing and fixation, cell adhesion was measured as described in Materials and methods. Data represent the means ± SD of one representative experiment performed in triplicate. The experiment was independently repeated three times. *, $P < 0.05$ (relative to cells seeded onto gelatin supplemented with Fc). (B) Forced expression of L1 in 1G11 endothelial cells. 1G11 cells were transduced either with an empty vector (1G11-mock) or with L1 cDNA (1G11-L1) and then subjected to FACS analysis (nonpermeabilized cells) or to immunoblotting (inset) for L1 expression. A dramatic increase in the amount and surface expression of L1 was observed in 1G11-L1 cells. The experiment was independently performed three times. (C) CFSE-labeled DCs from *L1floxed* (left bars) or *Tie2-Cre;L1floxed* (right bars) mouse bone marrows were subjected to transmigration assays through monolayers of 1G11 cells transduced either with an empty vector (orange) or with L1 cDNA (green). Data represent the means ± SD of a representative experiment performed in triplicate. The experiment was independently performed three times. *, $P < 0.005$ (relative to cells seeded onto mock-transduced 1G11 cells).