Supporting Information

Fujita et al. 10.1073/pnas.0911472106

SI Methods

Skin Sample Preparation for Flow Cytometry. Normal human skin samples were obtained as the discarded products of dermatologic surgery. Subcutaneous fat was excised, and remaining tissue was washed twice with PBS. The dermal layer was heavily scored with a scalpel and incubated in 2.4 U/mL Dispase II (Roche Diagnostics) overnight at 4 °C. Epidermis and dermis were separated with forceps. The epidermal sheet was cut in small pieces and incubated with 0.25% trypsin (Invitrogen) for 10 min at 37 °C. After washing, epidermal fragments were incubated in complete media consisting of RPMI 1640 (Invitrogen) supplemented with 5% pooled human serum (Mediatech), 0.1% gentamicin (Invitrogen), and 1% 1 M Hepes buffer (Sigma Aldrich) overnight at 37 °C. Afterward, a single cell suspension was obtained by filtration through 40 μm pore nylon cell strainers (BD Biosciences). Dermal sheets were transferred to complete media and incubated overnight. To obtain a dermal single cell suspension, the supernatants were collected and filtered with 40 μm pore nylon cell strainers.

Flow Cytometry. Cellular suspensions from epidermis were stained with the following anti-human, mouse monoclonal antibodies: HLA-DR-Alexa Fluor 700 (BioLegend) and CD207-Phycoerythrin (Immunotech) together with CD40-FITC/CD83-Allophycocyanin or CD80-FITC/CD86-Allophycocyanin (all from BD PharMingen). Cellular suspensions from dermis were also stained in the same way with the following antibodies: HLA-DR-Alexa Fluor 700, CD11c-Phycoerythrin-Cy7 (BioLegend), and BDCA-1-Phycoerythrin (Miltenyi Biotec) together with CD40-FITC/CD83-Allophycocyanin or CD80-FITC/CD86-Allophycocyanin. Detailed information of antibodies used is described in Table S1. Cells were stained in a total volume of 100 μL for 30 min on ice and then stained with 10 ng/mL DAPI (Sigma Aldrich) to label dead cells. Samples were acquired using a flow cytometer (LSR II; BD Biosciences). The expressions of CD40, CD80, CD83, and CD86 on HLA-DR$^+$CD207$^+$ cells (LCs) and HLA-DR^{hi}CD11c$^+$BDCA-1$^+$ cells (dermal DCs) were analyzed with FlowJo software (TreeStar). Appropriate isotype controls were used.

Generation of LC-Type DCs in Vitro. HPCs were isolated from buffy coats purchased from the New York Blood Center using CD34 MicroBeads kit (Miltenyi Biotec). Isolated HPCs were cultured at 2×10^4 cells/mL in RPMI medium 1640 supplemented with 10% FBS, 10 mM Hepes, 50 μM β-mercaptoethanol, 1% penicillin/streptomycin, GM-CSF (100 ng/mL; R&D Systems), FMS-like tyrosine kinase 3 ligand (Flt3L) (100 ng/mL; R&D Systems), and TNF-α (10 ng/mL; R&D Systems) for 8 days. Media and cytokines were refreshed at day 4 of culture. Cells were stained with CD1a-Phycoerythrin Ab and CD14-Allophycocyanin Ab (both from BD PharMingen). DAPI (10 ng/mL) was added to label dead cells. CD1a$^+$CD14$^-$ LCs and CD1a$^-$CD14$^+$ DCs were then sorted on a FACSAria (BD Biosciences) for subsequent MLR assay.

Generation of Monocyte-Derived Conventional DCs. Peripheral blood mononuclear cells were obtained by density centrifugation over Ficoll-Paque (Amersham Biosciences) and resuspended in complete media and plated in 6-well culture plates at 8×10^6 cells per well and allowed to adhere to plastic for 2 h at 37 °C. Plates were washed with RPMI 1640 to remove nonadherent cells. Plastic adherent cells were cultured in complete media supplemented with 25 ng/mL IL-4 and 100 ng/mL GM-CSF (both from R&D Systems) for 8 days. Cultures were supplemented with cytokines on days 2 and 4. On day 8, cells were harvested for subsequent MLR assay.

MLR. Single cell suspensions of epidermal cells were stained with mouse anti-human, HLA-DR-Alexa Fluor 700, and CD207-Phycoerythrin monoclonal antibodies, and sorted on a FACSAria. A population of LCs was collected by sorting HLA-DR$^+$CD207$^+$ cells. For dermal DCs, single cell suspensions of dermal cell crawl-outs were stained with mouse anti-human, HLA-DR-Alexa Fluor 700, CD11c-FITC (AbD Serotec), and BDCA-1-Phycoerythrin monoclonal antibodies, and sorted on a FACSAria. A population of dermal DCs was collected by sorting HLA-DR^{hi}CD11c$^+$BDCA-1$^+$ cells. T cells were obtained from a normal volunteer by treatment of whole peripheral blood with RosetteSep T Cell Enrichment Mixture (Stem Cell Technologies). Naive CD4$^+$ T cells were purified from peripheral blood mononuclear cells using human naive CD4$^+$ T Cell Isolation kit (Miltenyi Biotec). To evaluate proliferation, some of the purified peripheral T cells and naive CD4$^+$ T cells were subsequently labeled with 10 μM CFSE using the Vybrant CFDA SE Cell Tracer kit (Invitrogen/Molecular Probes). Thereafter, purified T cells (1×10^5 cells/well) were cultured with sorted LCs or DCs (2×10^3 cells/well) in 96-well round-bottom plates for 7 days. T cells alone were used as a negative control. T cell proliferation was analyzed on day 7 of culture. The cells were harvested, stained with CD3-Allophycocyanin (BD PharMingen), CD4-Phycoerythrin-Cy7 (eBioscience), and CD8-PerCp-Cy5.5 (BD PharMingen) for 30 min on ice. DAPI (10 ng/mL) was added to label dead cells. DAPI-negative and CD4$^+$ or CD8$^+$ cells were first gated and then plotted as CFSE vs. CD3. Samples were acquired using an LSR-II flow cytometer and analyzed with FlowJo software. The CFSE-low cells were quantified as a percentage of proliferating cells in the culture. Detailed information of antibodies used is described in Table S1.

Intracellular Cytokine Staining. Fresh T cells and T cells stimulated with allogeneic DCs for 7 days were restimulated for 4 h with 25 ng/mL PMA and 2 mg/mL ionomycin in the presence of 10 mg/mL BFA (all from Sigma Aldrich) at 37 °C. EDTA (2 mM; Fisher Scientific) was added for 10 min on ice to stop activation. Cells were then incubated in aqua marina live/dead dye (Invitrogen) on ice for 30 min for dead cell discrimination and subsequently fixed with 4% paraformaldehyde (BD Biosciences) on ice for 20 min. The cells were permeabilized in FACSPerm (BD Biosciences), blocked in 1:50 mouse serum (BD Biosciences), and incubated for 30 min on ice with the following anti-human, mouse monoclonal antibodies: CD3-Pacific Blue (eBioscience), CD4-Phycoerythrin-Cy7, CD8-PerCp-Cy5.5, IFN-γ-Alexa Fluor 700 (BD PharMingen), IL-4-Phycoerythrin (BD PharMingen), IL-17-Alexa Fluor 488 (eBioscience), and IL-22-Allophycocyanin (R&D Systems). Detailed information of antibodies used is described in Table S1. After incubation, cells were washed twice and collected. Samples were acquired using an LSR-II flow cytometer and analyzed with FlowJo software.

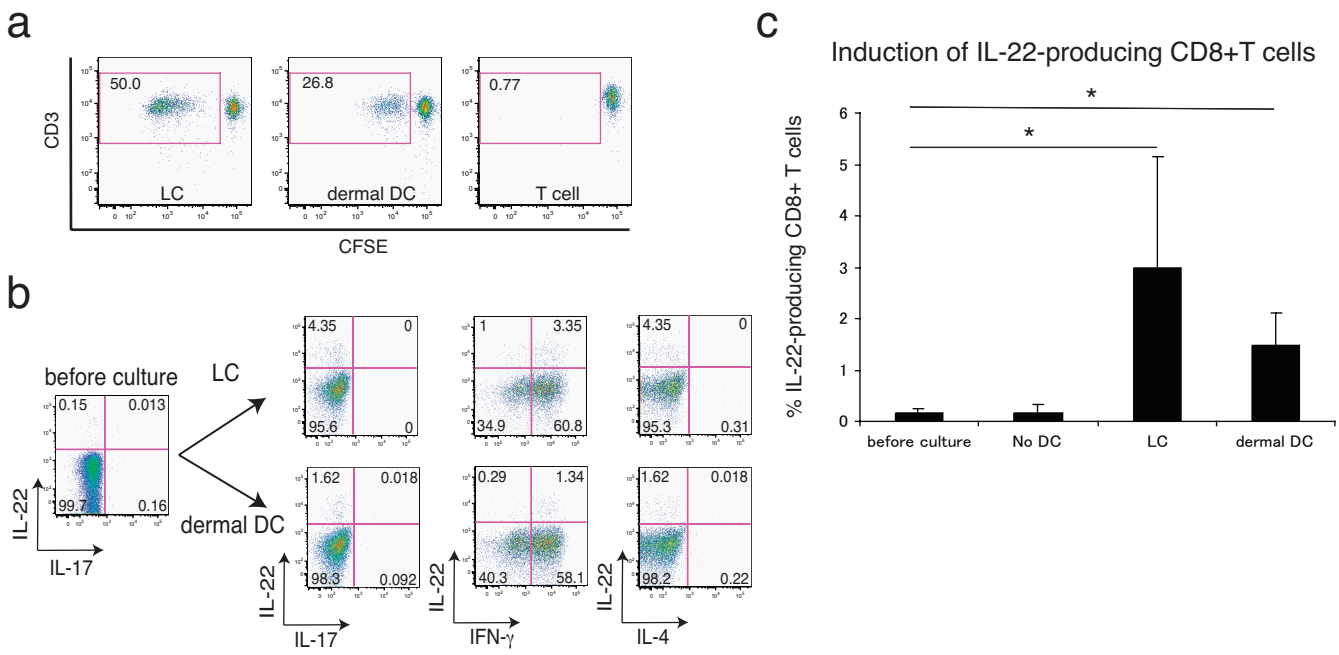

Fig. S1. LCs and dermal DCs activate CD8$^+$ T cells, leading to cell proliferation and IL-22 production. (*a*) Allogeneic whole peripheral T cells were labeled with CFSE and cultured for 7 days with sorted LCs or dermal DCs. Proliferation was determined by the dilution of CFSE using flow cytometry. Live, CD8$^+$ cells were first gated and then plotted as CFSE vs. CD3. Percentage of live, proliferating CD8$^+$ T cells is indicated in gate. Data are representative of four independent experiments. (*b*) Intracellular IL-22 vs. IL-17, IFN-γ, and IL-4 detected in proliferating CD8$^+$ T cell population following 7-day coculture of whole peripheral T cells and sorted allogeneic LCs or dermal DCs. T cells stimulated by LCs or dermal DCs were restimulated with PMA and ionomycin in the presence of BFA for 4 h and subsequently analyzed for the production of indicated cytokines by flow cytometry. Live, CD8$^+$ proliferating T cells were first gated and then cytokine production profile was analyzed. Numbers in quadrants indicate percentage of gated cells in each. Data are representative of five independent experiments. (*c*) Frequency of the cells producing IL-22 among CD8$^+$ T cells before culture and that among proliferating CD8$^+$ T cells after 7-day MLR assay. Horizontal axis indicates stimulators of T cells, and ''No DC'' group represents T cells cultured for 7 days without DCs. Asterisks (*) indicate statistical significance ($P < 0.05$). Data represent the mean (±SD) of five independent experiments.

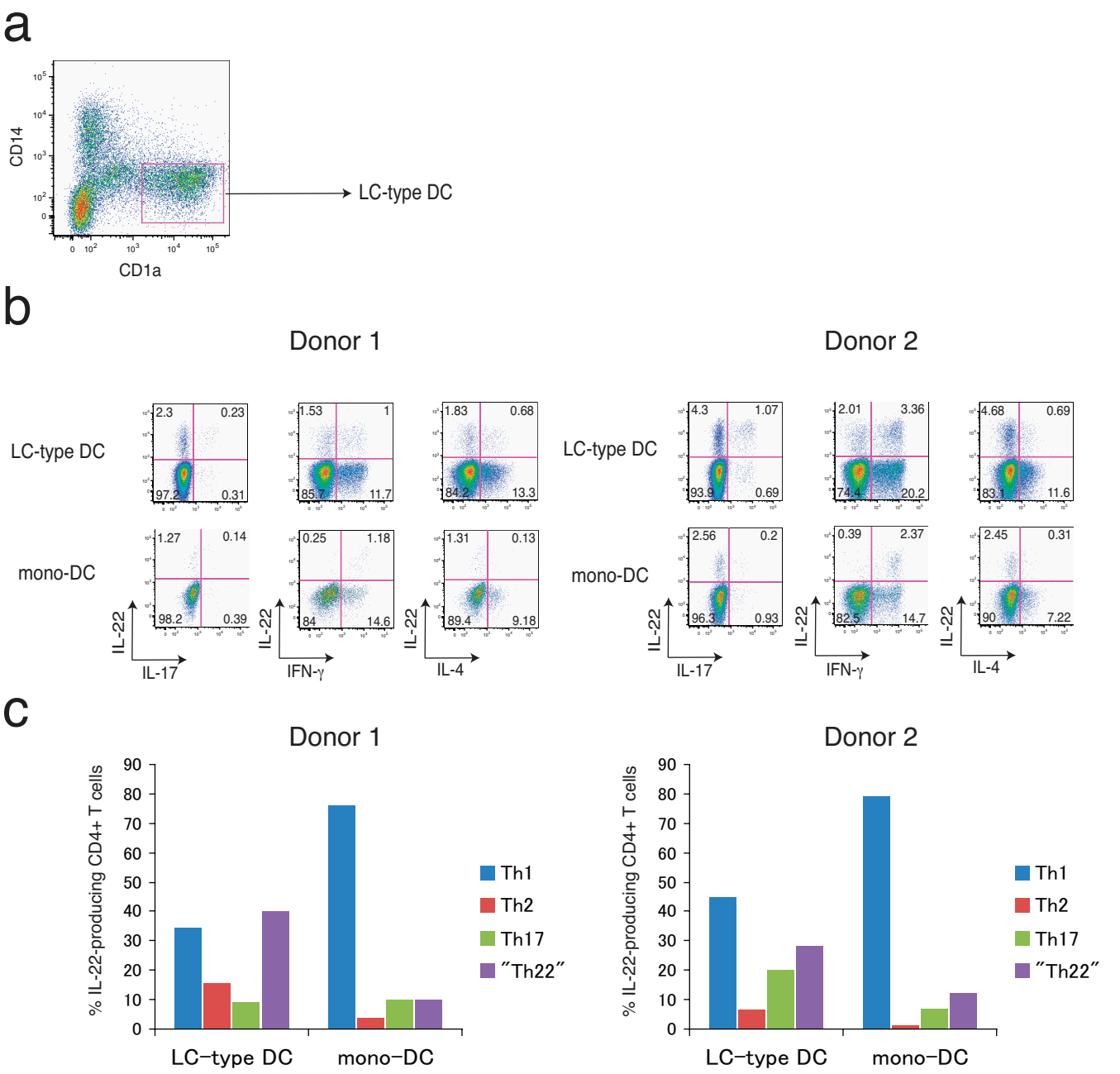

Fig. S2. In vitro-generated LC-type DCs are more efficient than monocyte-derived conventional DCs at inducing IL-22-producing $CD4^+$ T cells. (*a*) LC-type DCs were sorted as $CD1a^+CD14^-$ cells from $CD34^+$ HPCs cultured for 8 days with GM-CSF, TNF-α, and Flt3L. (*b*) Intracellular IL-22 vs. IL-17, IFN-γ, and IL-4 detected in proliferating $CD4^+$ T cells following 7-day coculture of allogeneic whole peripheral T cells and LC-type DCs or monocyte-derived DCs (mono-DCs). T cells stimulated by LC-type DCs or mono-DCs were restimulated with PMA and ionomycin in the presence of BFA for 4 h and subsequently analyzed for the production of indicated cytokines by flow cytometry. Live, $CD4^+$ proliferating T cells were first gated and then cytokine production profile was analyzed. Numbers in quadrants indicate the percentage of gated cells in each. (*c*) Frequency of Th1, Th2, and Th17 cells among IL-22-producing $CD4^+$ T cells. Analysis was performed as described in Fig. 3 *A* and *B*. Horizontal axis indicates stimulators of T cells.

Table S1. Antibodies used for flow cytometry

Antigen-fluorophor	Manufacturer	Clone*	Isotype	Dilution
BDCA-1-Phycoerythrin-Cy7	Miltenyi Biotec	AD5–8E7	IgG2a	1:50
CD11c-FITC	AbD Serotec	BU15	IgG1	1:50
CD11c-Phycoerythrin-Cy7	BioLegend	3.9	IgG1	1:50
HLA-DR-Alexa Fluor 700	BioLegend	L243	IgG2a	1:1,000
CD207- Phycoerythrin	Immunotech	DCGM4	IgG1	1:100
CD40-FITC	BD Pharmingen	5C3	IgG1	1:20
CD80-FITC	BD Pharmingen	L307.4	IgG1	1:20
CD83-Allophycocyanin	BD Pharmingen	HB15e	IgG1	1:33
CD86-Allophycocyanin	BD Pharmingen	2331(FUN-1)	IgG1	1:100
CD3-Allophycocyanin	BD Pharmingen	SK7	IgG1	1:500
CD3-Pacific Blue	eBioscience	500A2	IgG2a	1:40
CD4-Phycoerythrin-Cy7	eBioscience	RPA-T4	IgG1	1:200
CD8-PerCp-Cy5.5	BD Pharmingen	RPA-T7	IgG1	1:50
IFN-γ-Alexa Fluor 700	BD Pharmingen	L243	IgG1	1:200
IL-4-Phycoerythrin	BD Pharmingen	8D4–8	IgG1	1:20
IL-17-Alexa Fluor 488	eBioscience	eBio17B7	IgG1	1:20
IL-22-Allophycocyanin	R&D systems	142928	IgG1	1:20
CD1a- Phycoerythrin	BD Pharmingen	HI149	IgG1	1:33
CD14- Allophycocyanin	BD Pharmingen	MφP9	IgG2b	1:33

*All are murine monoclonals.