

Supporting Information

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SI Materials and Methods

Mice and Contact Hypersensitivity Sensitization. IL-37tg mice on a C57BL/6 background have been described previously (1). C57BL/6 (WT) mice and FVB/N mice were from Jackson Laboratory. All mouse experiments were approved by the Colorado Multiple Institutional Review Board. All mice were maintained in a specific pathogen-free animal facility at the University of Colorado Denver and handled in accordance with the institutional guidelines. Six- to 8-wk-old age- and sex-matched WT and IL-37tg mice were sensitized by application of 25 μ L of 0.5% 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich) in 4:1 (vol/vol) acetone/olive oil onto their shaved abdomen on day 0. Mice were challenged on day 5 by applying 10 μ L of 0.2% DNFB in 4:1 (vol/vol) acetone/olive oil onto their right ear. As a control, the left ear was painted with 10 μ L vehicle. Ear thickness was measured with an engineer's micrometer (Mitutoyo). Results were expressed as net ear swelling, which was calculated by subtracting the thickness of the ear before treatment from the thickness of the ear after treatment.

Histology. Ears from vehicle or hapten-sensitized mice were excised 48 or 72 h after challenge. They were fixed in formalin and embedded in paraffin. Five- μ m sections of paraffin-embedded tissues were stained with H&E.

Skin Cell Preparation. Epidermal and dermal single-cell suspensions were prepared as described previously (2). Ears were split into dorsal and ventral halves and incubated in 20 mM EDTA (Sigma-Aldrich) for 30 min at 37 °C. Epidermis was peeled from the dermis, and epidermal sheets and dermal sheets were obtained. To prepare single-cell suspensions, epidermal sheets were incubated with 0.125% trypsin for 20 min at 37 °C whereas dermal sheets were incubated in 2 mg/mL collagenase I (Sigma-Aldrich) and 2 mg/mL hyaluronidase (Sigma-Aldrich) for 2 h at 37 °C. Cells were stained with CD11c, MHC II, and EpCAM for flow cytometric analysis. FITC-labeled anti-mouse MHC II (M5/114, 15.2), FITC-labeled anti-mouse CD11c (N418), and FITC-labeled anti-mouse EpCAM (G8.8) were all purchased from eBioscience.

Immunostaining of Epidermal Sheets. Epidermal sheets were prepared as described previously with slight modifications (2). They were fixed with cold acetone for 10 min and stained with FITC-labeled anti-mouse MHC II antibody. MHC II⁺ cells were counted using a micrometer grid in five random 10 \times fields per section and used to calculate Langerhans cell density per square millimeter.

Flow Cytometry. Flow cytometry was performed by CyAn (DakoCytomation), and the results were analyzed with Summit software (DakoCytomation). DAPI was used to remove dead cells at a final concentration of 2 μ g/mL. Cell sorting was conducted using a MoFlo machine (DakoCytomation). Intracellular Langerin and Foxp3 staining were performed according to the intracellular staining protocol. Allophycocyanin (APC)-labeled anti-mouse Foxp3 (FJK-16s) and phycoerythrin (PE)-labeled anti-mouse Langerin (eBioL31) antibody were purchased from eBioscience.

Generation of Bone Marrow-Derived Dendritic Cells. Dendritic cells (DCs) were generated as described by Inaba et al. (3). Bone marrow cells were obtained from the tibia and femur of mice. Cells were resuspended at 1×10^6 cells/3 mL in complete RPMI-1640

(Cellgro) with 20 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (R&D Systems) and 20 ng/mL recombinant murine IL-4 (R&D Systems). On day 2, nonadherent cells were aspirated off following gentle swirling of the plate. Loosely adherent cells, including DCs, were harvested by gentle pipetting on day 6 of culture. The harvested cells were purified for CD11c⁺ subset using anti-CD11c microbeads and auto MACS (Miltenyi Biotec). The purity of isolated cells was analyzed by flow cytometry using anti-mouse CD11c antibody and was shown to be greater than 90%. Bone marrow-derived dendritic cells (BMDCs) were incubated for 24 h with LPS (100 ng/mL) and immunolabeled with FITC-labeled anti-mouse MHC II, FITC-labeled anti-mouse CD40 (HM40-3), FITC-labeled anti-mouse CD80 (16-10A1), and FITC-labeled anti-mouse CD86 (GL1) (all from eBioscience).

RNA Extraction and Quantitative RT-PCR Analysis. RNA was extracted from BMDCs using the RNeasy mini kit (Qiagen) or mouse skin following the TRIZOL (Life Technologies) RNA isolation protocol and subsequently reverse-transcribed. Quantitative RT-PCR (qRT-PCR) was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) on the MX3000P PCR system (Stratagene). Primer sets used were the following: GAPDH forward—5'-TGC ACC ACC AAC TGC TTA GC-3'; reverse—5'-GGC ATG GAC TGT GGT CAT GAG-3'; IL-37 forward—5'-GCA TTC ATG ACC AGG ATC AC-3'; reverse—5'-CAA AGA AGA TCT CTG GGC GTA-3'; CCR7 forward—5'-GAC CAT GAC GGA TAC CTA CCT-3'; reverse—5'-AGA CGC CAA AGA TCC AGG ACT-3'; IL-1 β forward—5'-TCA GGC AGG CAG TAT CAC TC -3'; reverse—5'-AGG ATG GGC TCT TCA AA-3'; IL-10 forward—5'-CCC AGA AAT CAA GGA GCA TT-3'; reverse—5'-TCA CTC TTC ACC TGC TCC AC-3'; TGF- β 1 forward—5'-CTG AAC CAA GGA GAC GGA AT-3'; and reverse—5'-TTG CTG TCA CAA GAG CAG TG-3'.

Western Blot. Analysis was performed as previously described (4). Goat anti-human IL-37 (IL-1F7) polyclonal antibody (Lys27Asp192) for Western blot was obtained from R&D Systems. Mouse β -actin monoclonal antibody (AC-15), HRP-conjugated anti-goat, and anti-mouse IgG were purchased from Sigma-Aldrich. Goat anti-human IL-37 polyclonal antibody was diluted at 1:2,000. Mouse β -actin antibody was diluted at 1:30,000.

Phagocytotic Assay. BMDCs from WT and IL-37tg mice (5×10^4 cells per well in 96-well plates) were pretreated with 100 ng/mL LPS for 30 min or 2 h. They were incubated with 1 mg/mL FITC-dextran (Sigma-Aldrich) at 4 °C or 37 °C for 30 min or 2 h, washed, and analyzed by flow cytometry.

Chemotaxis Assay. QCM Chemotaxis Cell Migration Assay (Millipore) was used. CCL21 (500 ng/mL, R&D Systems) was added to serum-free RPMI-1640 in the feeder tray (lower well). BMDCs from WT and IL-37tg mice were pretreated with 100 ng/mL LPS for 24 h, placed in a migration chamber (1×10^5 cells per upper well in 100 μ L serum-free RPMI-1640), and cultured for 2 h. Cells that migrated to the underside or through the membrane were mixed with Lysis Buffer/CyQuant GR Dye Solution (Millipore), incubated at room temperature for 15 min, and analyzed with a fluorescence plate reader using 480/520 nm filter set.

DC Migration Assay in Vivo. The FITC-based in vivo cell migration assay was performed. Mouse abdominal skin was shaved and painted with 0.5% FITC (AbD serotec) in ADBP (acetone:

dibutyl phthalate, 1:1, vol/vol) for DC tracing. Twenty-four hours later, draining lymph nodes (dLNs) were removed from axillary and inguinal dissection, and single-cell suspensions were analyzed by flow cytometry.

DC Adoptive Transfer. Donor mice (WT and IL-37tg) were painted with 0.5% DNFB on their shaved abdomen. After 24 h, DCs were isolated from dLN of WT and IL-37tg mice and purified using CD11c MACS beads (Miltenyi Biotec). Donor DCs were resuspended in serum-free medium (Invitrogen) with 4 μ M 4,6-dinitrobenzothiadiazole (DNBS) and incubated at 37 °C for 30 min. A total of 0.2 million DCs from WT or IL-37tg mice were washed, resuspended in 1 \times PBS, and injected subcutaneously into the abdomen of age- and sex-matched WT recipient mice. After 5 d, recipient mice were challenged by the application of 10 μ L 0.2% DNFB on their right ear, whereas the left ear was treated with vehicle. Contact hypersensitivity (CHS) response was determined by measuring ear swelling as described in *Mice and Contact Hypersensitivity Sensitization*.

Cytokine Measurements. The levels of cytokines were determined by a multiplex ELISA cytokine array (Q-Plex Mouse Cytokine Array, Quansys Biosciences). Ninety-six-well plates were coated with capture antibodies. The cytokine standard or supernatant was added, followed by incubation and washes. Plates were then probed with streptavidin–HRP. After washing and adding substrate, the signals were detected by a Q-view imager (Quansys Biosciences) and the results were analyzed by Q-view software (Quansys Biosciences). The levels of IL-10 and TGF- β 1 were measured by ELISA kit obtained from R&D Systems and eBioscience, respectively, according to the manufacturer's instructions. The limits of sensitivity for IL-10 and TGF- β 1 were 4 and 8 pg/mL, respectively.

T-Cell Proliferation Assay. Splenocytes from C57BL/6 mice or FVB/N mice were purified using Mouse Pan T Cell Isolation Kit II (Miltenyi Biotec), stained with carboxyfluorescein diacetate, succinimidyl ester (CFSE) (0.25 μ M, Invitrogen), and used as responders for syngeneic or allogeneic responses, respectively. BMDCs were incubated in serum-free medium with or without LPS (100 ng/mL) at 37 °C for 2 h and used as stimulators. For antigen-specific responses, cells from dLNs were obtained 5 d after DNFB sensitization on abdomens, purified using Mouse Pan T Cell Isolation Kit II, stained with 0.25 μ M CFSE, and used as responders. BMDCs were resuspended in serum-free medium containing 4 μ M DNBS (Sigma-Aldrich) or 6 μ g/mL 4-AP (Sigma-

Aldrich), incubated at 37 °C for 30 min, and used as stimulators. BMDCs resuspended without compounds were used as control. The purity of isolated T cells was analyzed by flow cytometry using APC-labeled anti-mouse CD3 antibody (17A2) (eBioscience) and was shown to be greater than 95%. Responders (1×10^6) were cocultured with 1×10^5 (for syngeneic or allogeneic response), 2×10^4 (for antigen-specific response) stimulators for 3 d in 100 μ L RPMI-1640, supplemented with 10% (vol/vol) FBS and 50 μ M 2-mercaptoethanol and 100 U of penicillin and streptomycin. Cultured cells were collected and subjected to flow cytometric analysis using APC-labeled anti-mouse CD4 (RM4-5) or PE-cy7–labeled anti-mouse CD8 (53-6.7) (both from eBioscience).

Analysis of DC-Induced T Regulatory Cells. BMDCs from WT or IL-37tg mice were cocultured with T cells from WT mice, and T regulatory (Treg) cells were determined by flow cytometry. T cells were isolated from mouse spleen of WT mice and purified using Pan T Cell Isolation Kit II. BMDCs (1×10^5) from WT or IL-37tg mice were cocultured with T cells (1×10^6) for 3 d in RPMI-1640 medium supplied with 50 μ M 2-mercaptoethanol and 100 U/mL IL-2. Cultured cells were then collected and stained with FITC-labeled anti-mouse CD4 (GK1.5, eBioscience), PE-labeled anti-mouse CD25 (PC61, eBioscience), and APC-labeled anti-mouse Foxp3. Treg (CD4⁺CD25⁺Foxp3⁺) cells were determined by flow cytometry.

Immunofluorescent Staining. Five- μ m sections of paraffin-embedded tissues were stained with rat anti-mouse CD8a antibody (LS-B3638, LifeSpan BioSciences) (1:100), followed by Alexa Fluor 594-labeled anti-rat IgG secondary antibody (Invitrogen). Five to 10- μ m sections of frozen tissues embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek) were fixed in cold acetone for 10 min and stained with purified rat anti-mouse Foxp3 (FJK-16a, eBioscience) (1:100) followed by Alexa Fluor 488-labeled anti-rat IgG (Invitrogen) secondary antibody. Slides were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) and analyzed by a Nikon Eclipse 90i microscope with NIS-Elements AR 3.0 imaging software (Nikon). Positive cells were counted in five random 8- \times 8-mm fields per section and used to calculate cell density per square millimeter.

Statistical Analysis. A paired two-tailed Student *t* test was performed in all cases. Where appropriate, mean \pm SE is represented on graphs. A *P* value of <0.05 was considered statistically significant.

1. Nold MF, et al. (2010) IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol* 11(11):1014–1022.
2. Nagao K, et al. (2009) Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proc Natl Acad Sci USA* 106(9):3312–3317.
3. Inaba K, et al. (1986) Immunologic properties of purified epidermal Langerhans cells. Distinct requirements for stimulation of unprimed and sensitized T lymphocytes. *J Exp Med* 164(2):605–613.

4. Okamoto M, et al. (2010) Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1 β . *J Biol Chem* 285(9):6477–6488.

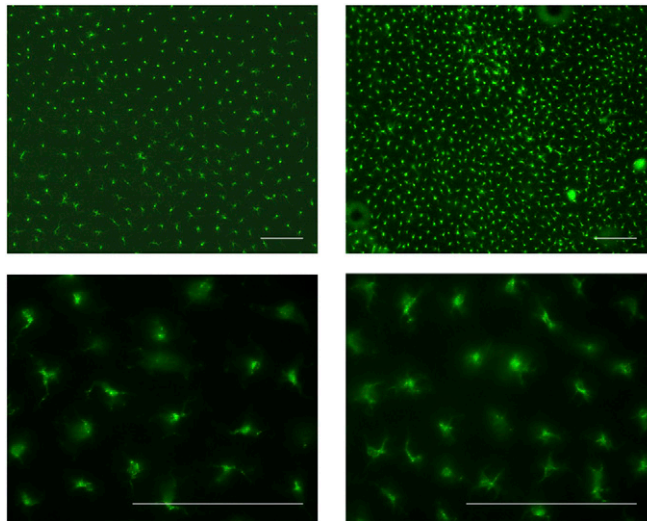


Fig. S1. Distribution and morphology of skin DCs. Untreated epidermal sheets from WT and IL-37tg mice were stained with FITC-labeled anti-mouse MHC II. (Scale bar, 100 μ m.)

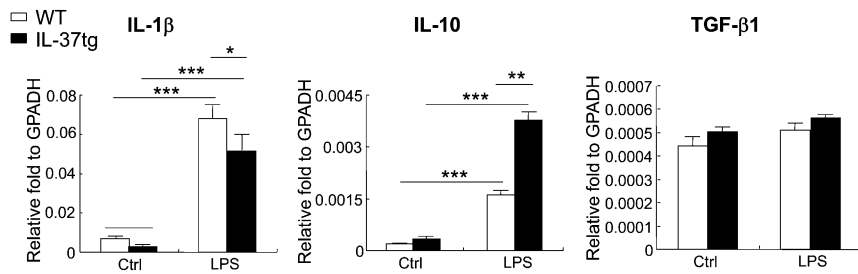


Fig. S2. Expression levels of cytokines in DCs. BMDCs from WT and IL-37tg mice were treated with LPS (100 ng/mL) for 24 h. RNAs were analyzed for qRT-PCR of IL-1 β , IL-10, and TGF- β 1. Expression levels were normalized to GAPDH. The data are shown as the mean \pm SE ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.