Supplementary Information for

Altered ratio of DC subsets in skin draining lymph nodes promotes Th2 driven contact hypersensitivity

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SI Appendix, Materials and Methods

Mice. All animal studies were approved by the Washington University Institutional Animal Care and Uses Committee. CLEC4C-DTR^{+} male and female C57BL/6 mice (CD45.2 and CD45.1 x CD45.2) were bred in house in a specific pathogen free facility. Littermate transgene negative mice were used as controls for all experiments. CD45.1 male and female mice used as recipients in bone marrow chimera studies were bred in house in a specific pathogen free facility. Female *Ifnar*$^{-/-}$ mice were kindly provided by A. French (Washington University School of Medicine). Female *Il28ra*$^{-/-}$ mice were kindly provided by M. Diamond (Washington University School of Medicine). All mice were used between 10-12 weeks of age. *Zbtb46*^{GFP} homozygous mice were purchased by Jackson in a C57BL6 background and crossed to CLEC4C-DTR^{+} mice to generate *Zbtb46*^{GFP} x CLEC4C-DTR^{+} and DTR^{-} littermates.

CHS. In the FITC contact hypersensitivity protocol, mice were anesthetized with ketamine/xylazine mixture and a 3 cm x 3 cm patch was shaved on the abdomen. 100μL of 0.5% FITC solution dissolved in 1:1 mixture of acetone and dibutyl phthalate was applied epicutaneously to the shaved skin on day 0 and day 2. On day 6, 20μL of 0.5% FITC solution was applied to the right ear and 20μL of acetone/dibutyl phthalate solvent was applied to the left ear. Ear swelling was measured 24 h later and mice were sacrificed for analysis. In the DNFB contact hypersensitivity protocol, mice were anesthetized with ketamine/xylazine mixture and 1 cm x 1 cm patch was shaved on the abdomen. 20μL of 0.5% DNFB in a 4:1 mixture of acetone and olive oil was applied epicutaneously to the shaved skin on day 0 and 1. On day 5, 20μL of 0.2% DNFB was applied to the right ear

and 20μL of acetone/olive oil was applied to the left ear. Ear swelling and analysis was conducted 24 h later. CLEC4C-DTR$^+$ mice and littermate transgene negative WT controls were injected with 200-400ng of DT on day -1, 1, 3, and 5 in both the FITC and DNFB CHS protocols.

Calcipotriol Skin Model. Calicipotriol (MC903) was applied topically to the right ear at a dose of 2nmol every 2 days for 21 days. Ethanol was applied to the left ear as a solvent control. For these experiments, CLEC4C-DTR$^+$ mice were injected with 200ng DT i.p. on days -1, 1, 3, 5, and 7.

Cell Preparations. Cell suspensions of spleen and lymph nodes were prepared by collagenase D digestion and filtered through nylon mesh cell strainers (BD Biosciences). BM was collected from tibias and femurs. Red blood cells were lysed with RBC lysis buffer (Sigma-Aldrich). Cell suspensions of skin were prepared by Liberase TL digestion (Roche) and filtered through 70 μm nylon mesh cell strainers. For analysis of skin DCs, epidermis was removed with dispase digestion and remain dermal layer was incubated in collagenase D and passed through a 70 μm nylon mesh cell strainer after mincing. An additional passage through a 40 μm cell strainer was performed immediately prior to staining for flow cytometry.

Antibodies, Flow Cytometry and Sorting. The following reagents were from BD Biosciences, eBioscience or Biolegend: fluorochrome labeled anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD45 (30-F11) anti-CD11c (HL3), anti-SIGLECH (551 or 440c), anti-

B220 (RA3-6B2), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD3 (145-2C11), anti-CD11b (M1/70), anti-CD103 (2E7), anti-CD172a (P84), anti-SIGLECF (E50-2440), anti-Ly6G (1A8), anti-GATA3 (TWAJ), anti-FOXP3 (150D/E4), anti-IFNγ (XMG1.2), anti-IL17A (TC11-18H10), anti-CD127 (A7R34), anti-ST2 (RMST2-2), anti-CD19 (1D3/CD19), anti-CD25 (PC61.5), anti-CD86 (GL1), anti-I-A/I-E (M5/114.15), anti-CD326 (G8.8), anti-CD24 (M1/69), anti-NK1.1 (PK136) and anti-IRF4 (3E4). Fc receptors were blocked before surface staining and dead cells were excluded by LiveDead Aqua stain (Life Technologies). For transcription factor detection, cells were stained for surface markers followed by fixation and permeabilization with the Foxp3 staining buffer set (eBioscience). Cells stained with intracellular antibodies diluted in 1X Perm Wash buffer (eBioscience). For detection of intracellular cytokines, PMA/ionomycin stimulated cells and controls were washed, surface stained, fixed and permeabilized using Cytofix/Cytoperm solution (BD Bioscience). Fixed cells were stained for intracellular cytokines with antibodies diluted in 1 x Perm Wash solution (BD Bioscience). Stained cells were analyzed on a FACS Canto or on a BD FACS Symphony A3 Cell analyzer with Diva software. Flow cytometry data were analyzed with FlowJo software (Tree Star, Inc.). Splenic pDCs were sorted on a FACSAria II (BD Biosciences) after enrichment using a negative selection microbeads kit, Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi). For single cell RNAseq, cells were sorted without any enrichment on an FACSAria II (BD Biosciences).

Cell Culture and Stimulations. Primary cells were cultured in complete RPMI 1640 with 10% bovine calf serum (BCS), 1% glutamax, 1% nonessential amino acids, 1% sodium pyruvate, and 1% kanamycin sulfate (Gibco-Invitrogen). For secreted cytokine analysis,

cells were cultured for 12h at 1 x 10^6 cells/mL with 10^{-7} M Phorbol 12-myristate 13-acetate (PMA) and 500ng/mL ionomycin (Sigma). For intracellular cytokine staining, primary cells were cultured in complete RPMI at 1 x 10^6 cells/mL with PMA/ionomycin for 6 hours with Brefeldin A (BD Biosciences) added 2 hours into the culture. Splenic pDCs were stimulated for 18 hours with CpGA 2216 (6 μg/ml, Operon); CpGB 1826 (6 μg/ml, Operon) or Imiquimod (6 μM, 3M).

ELISA and Cytometric Bead Array. Supernatants from lymph node cultures were collected and stored at -20°C until analysis. IFN-γ and IL-4 were measured by cytometric bead array (BD Biosciences). IL-13 levels were measured by ELISA (eBiosciences).

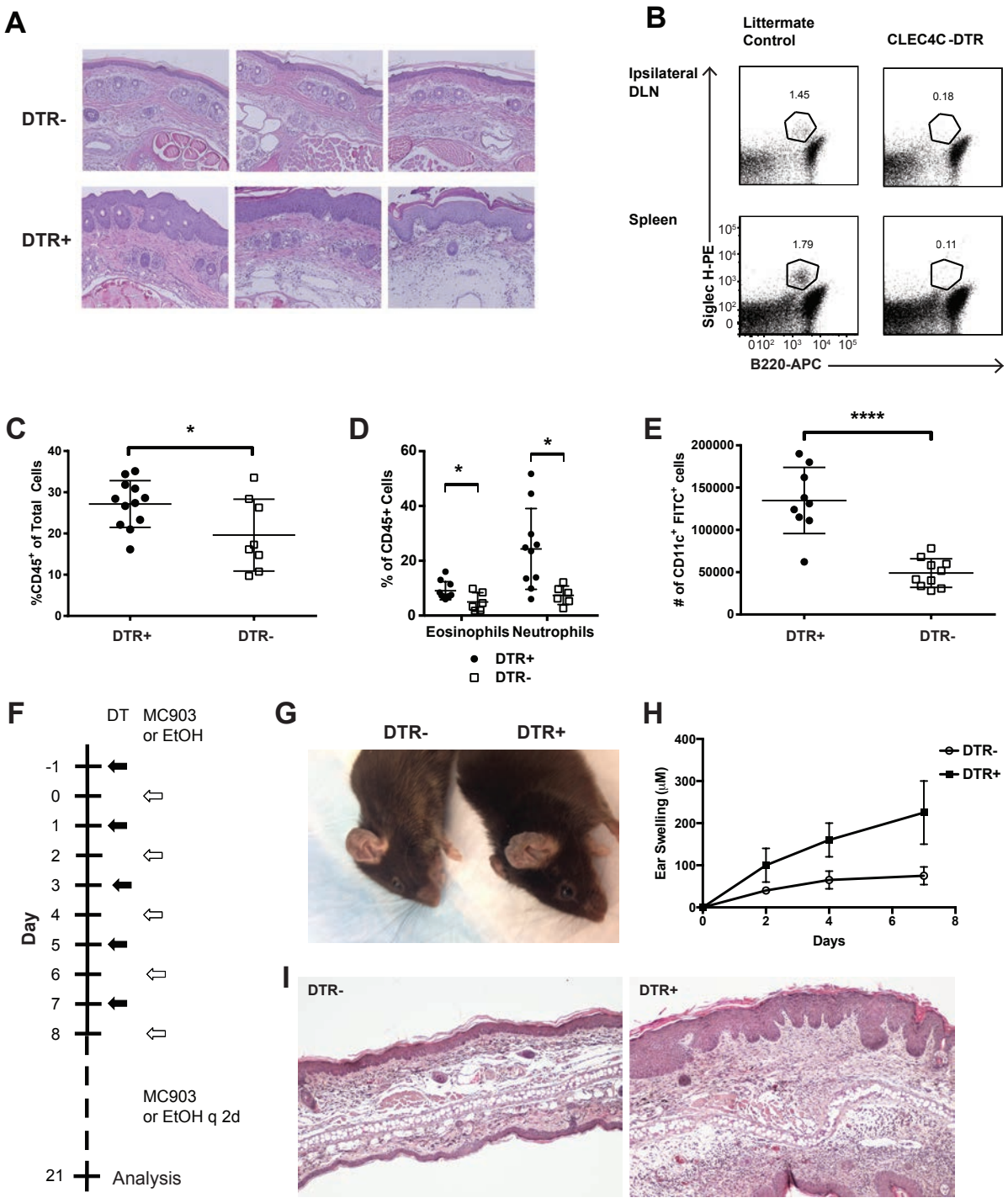
Microarray Analysis. RNA was prepared from whole skin using RNeasy Mini (Qiagen). For skin preparation, one mouse was used per replicate and each replicate contained 1 FITC treated ear. Three replicates from each group that passed quality control were amplified and hybridized to the Affymetrix Mouse Gene 1.0 ST array by the Genome Technology Access Center at Washington University, St. Louis, MO. For normalization, raw data were processed by Robust Multi-Array (RMA) method. Pathway analysis was performed with GSEA software from the Broad Institute using MSigDB Hallmark and Canonical Pathways gene sets (http://software.broadinstitute.org/gsea/index.jsp). Volcano plots and differential expression analysis was performed using Multiplot studio and Morpheus with a threshold of 2-fold change and a minimum P value significance threshold of 0.05 (after False Discovery Rate [FDR] correction for the number of tests). Microarray data has been deposited at GEO (accession number: GSE160261).

scRNA-seq. Cells were sorted on an Aria II and preserved in 10% FCS RPMI until input into 10X Genomics Single Cell Protocol. Isolated mouse cells were subjected to droplet-based 3' end massively parallel single-cell RNA sequencing using Chromium Single Cell 3' Reagent Kits as per manufacturer's instructions (10x Genomics). The libraries were sequenced using Illumina HiSeq4000 sequencers at the McDonnell Genome Institute. Sample demultiplexing, barcode processing, and single-cell counting was performed using the Cell Ranger Single-Cell Software Suite (10x Genomics). Cellranger count was used to align samples to the reference genome (mm10), quantify reads, and filter reads with a quality score below 30. The Seurat package in R was used for subsequent analysis (1). Genes expressed in fewer than 3 cells and cells that expressed less than 400 or greater than 3500 genes were removed for downstream analysis. Data was normalized using a scaling factor of 10,000, and nUMI was regressed with a negative binomial model. Principal component analysis was performed using the top 3000 most variable genes and UMAP analysis was performed with the top 20 PCAs. Clustering was performed using a resolution of 0.6. For identifying the markers for each cluster, we performed differential expression of each cluster against all other clusters using MAST package in R (2), identifying negative and positive markers for that cluster. scRNA-seq data has been deposited at GEO (accession number: GSE161604).

Statistical Analysis. The statistical significance of differences in mean values was analyzed with unpaired, two-tailed Student's t test. P values less than 0.05 were considered statistically significant.

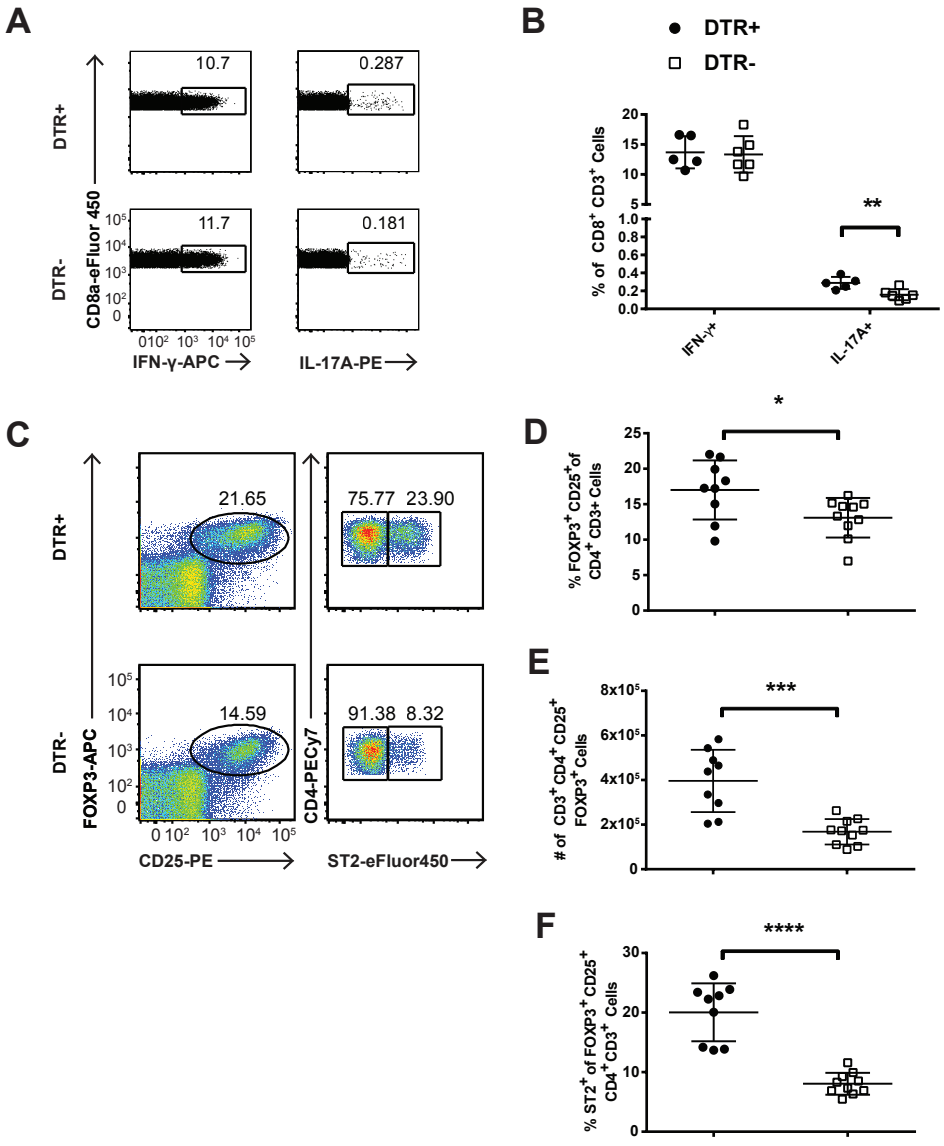
References for SI Appendix Materials and Methods

1. A. Butler, P. Hoffman, P. Smibert, E. Papalexi, & R. Satija, Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* **36**, 411-420 (2018).
2. G. Finak*, et al.*, MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. *Gen. Biol.* **16**, 278-278 (2015).



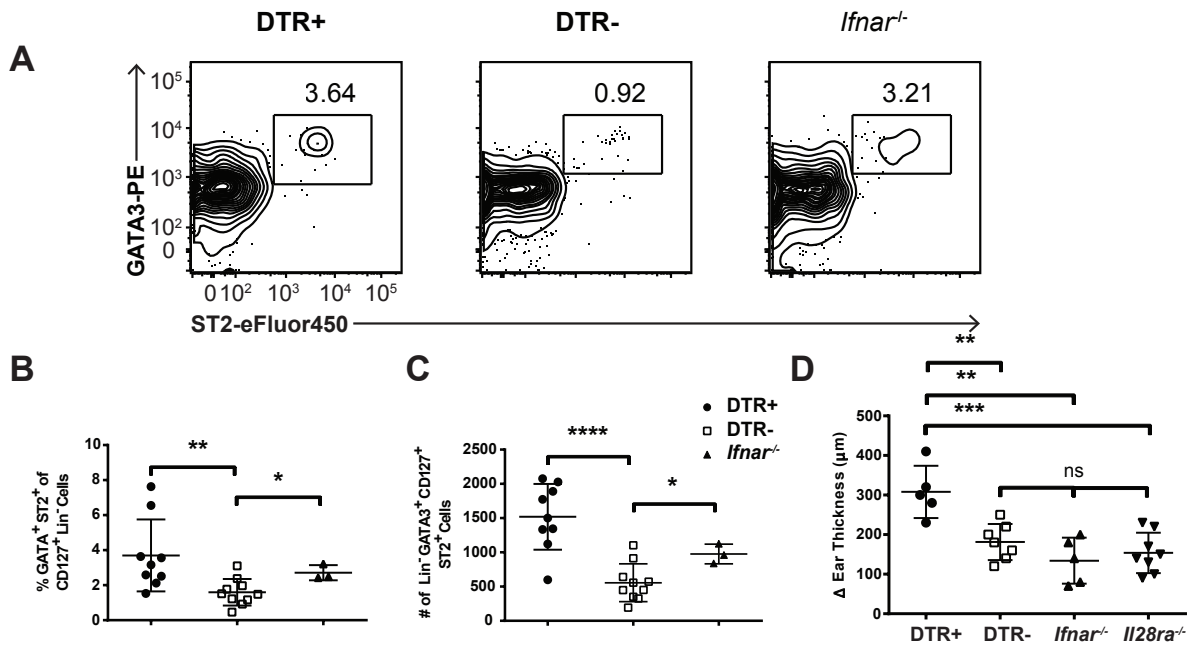
SI Appendix, Fig. S1. CLEC4C-DTR^{+} mice exhibit increased type 2 immune responses in the skin after DT treatment. Related to Fig. 1. (*A*) Representative tail H&E histology

from CLEC4C-DTR^{+} and CLEC4C-DTR^{-} controls treated with DT and sensitized with permanent marker (Sharpie) from 3 individual mice per group. (*B*) pDC depletion. Representative staining of SIGLECH^{+}B220^{+} cells in the ipsilateral DLN and spleen 24h post FITC challenge. Cells are gated on CD11c^{int}. Representative of 8 separate experiments (n=28 CLEC4C-DTR^{+}, n=29 DTR^{-} littermates). (*C*) Percent CD45^{+} cells isolated after 90 minute digestion of the ear skin with Liberase TL and analysis by flow cytometry. Data pooled from 3 experiments (n=12 CLEC4C-DTR^{+}, n=8 DTR^{-} littermates). (*D*) Percent eosinophils (SIGLECF^{+}CD11b^{+}CD11c$^{low/-}$) and neutrophils (Ly6G^{+}CD11b^{+}CD11c^{-}). Data pooled from 2 experiments (n=10 CLEC4C-DTR^{+}, n=6 DTR^{-} littermates). (*E*) Percent CD11c^{+} FITC^{+} cells in the ipsilateral DLN 24h post challenge. Data pooled from 3 experiments (n=9 CLEC4C-DTR^{+}, n=10 DTR^{-} littermates). (*F*) MC903 experimental design. (*G*) Gross appearance of ears from mice treated with protocol in (*F*). Representative of 2 experiments (n=6 CLEC4C-DTR^{+}, n=6 DTR^{-} littermates). (*H*) Mean ear swelling for first 7 days of protocol. Data representative of 1 of 2 experiments (depicted: n=3 CLEC4C-DTR^{+}, n=3 DTR^{-} littermates, total; n=6 CLEC4C-DTR^{+}, n=6 DTR^{-} littermates). (*I*) H&E staining of depleted and non-depleted mice treated with MC903. Representative of 2 experiments (n=6 CLEC4C-DTR^{+}, n=6 DTR^{-} littermates). *p<0.05, ****p<0.0001; statistics by unpaired two tailed students T test. Data in *C*-*E*, *H* are mean ± st. dev. and each symbol representative of an individual mouse.

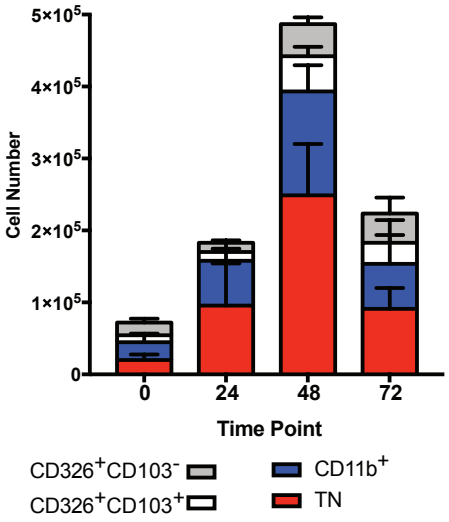


SI Appendix, Fig. S2. CLEC4C-DTR^{+} mice show increased IL-17-producing CD8^{+} T cells and enhanced Treg in the FITC CHS model upon DT treatment. Related to Fig. 2. Cells from the ipsilateral superior cervical lymph nodes were isolated from CLEC4C-DTR^{+} and CLEC4C-DTR^{-} littermate controls 24h post FITC challenge. (*A*) Intracellular cytokine staining of CD8^{+} T cells stimulated with PMA/ionomycin. (*B*) Quantification of IFN-γ^{+} and IL-17A^{+} cells within the CD8^{+}CD3^{+} population. Data is representative of 1 of 2

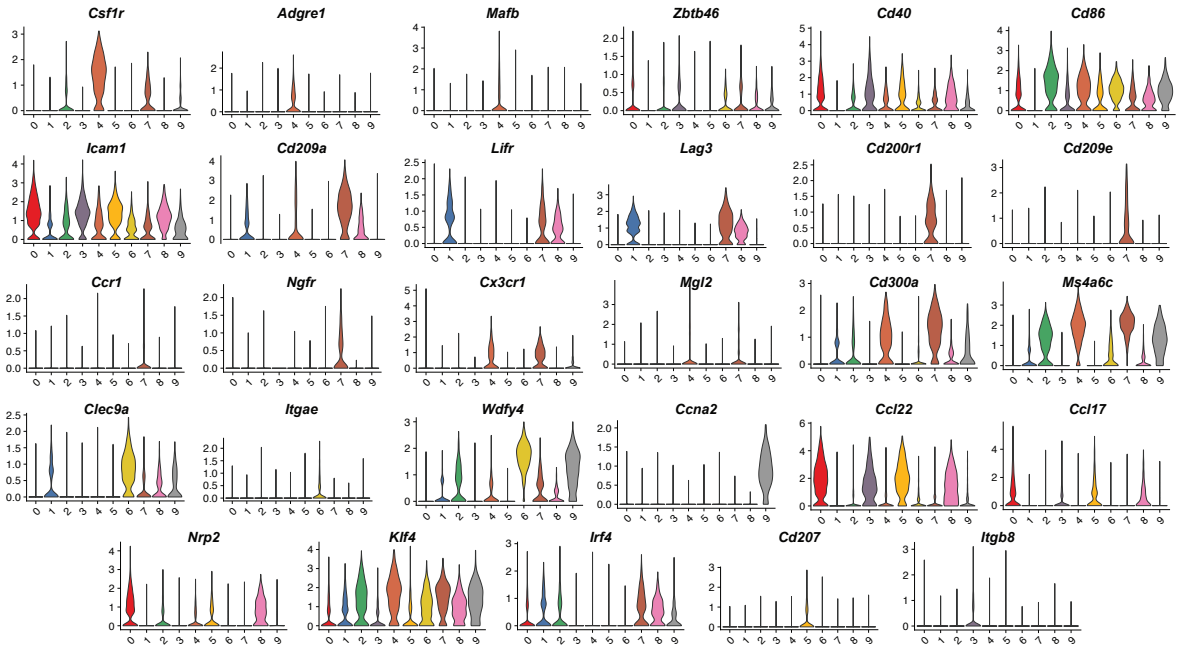
experiments (depicted: n=5 CLEC4C-DTR^{+}, n=6 DTR^{-} littermates, total: n=10 CLEC4C-DTR^{+}, n=10 DTR^{-} littermates). (*C*) Representative Treg staining, gated on CD4^{+}CD3^{+} cells, and representative ST2 staining, gated on FoxP3^{+}CD25^{+}CD4^{+}CD3^{+} cells. (*D*) Percent of FoxP3^{+}CD25^{+} within CD4^{+}CD3^{+} cells and (*E*), number of FoxP3^{+}CD25^{+}CD4^{+}CD3^{+} cells per lymph node. (*F*) Percent of ST2^{+} cells within FoxP3^{+}CD25^{+}CD4^{+}CD3^{+} cells. Data in *D-F* is pooled from 3 experiments (n=10 CLEC4C-DTR^{+}, n=11 DTR^{-} littermates). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; statistics by unpaired two tailed students t test. Data in *B*, *D*, *E*, and *F* are mean ± st. dev.; each symbol represents an individual mouse.



SI Appendix, Fig. S3. Enhanced CHS in CLEC4C-DTR^{+} mice is independent of IFN-I or IFN-λ. (*A*) Representative ILC2 staining from ipsilateral DLN 24h post FITC challenge, gated on CD127^{+}CD3^{-}CD19^{-} cells. (*B*) Percent of GATA3^{+}ST2^{+} within CD127^{+}CD3^{-}CD19^{-} cells in the ipsilateral DLN 24h post FITC challenge and (*C*) number of CD3^{-}CD19^{-}CD127^{+}GATA3^{+}ST2^{+} cells. Data in *B* and *C* represent 2 pooled experiments (n=10 CLEC4C-DTR^{+}, n=10 DTR^{-} littermates, n=3 *Ifnar*$^{-/-}$). (*D*), *Ifnar*$^{-/-}$ and *Il28ra*$^{-/-}$ mice were treated with the FITC CHS protocol along with CLEC4C-DTR^{+} and WT mice as controls. All mice were administered DT. Ear swelling was measured 24h post challenge in the FITC treated ear. Data represents 2 pooled experiments (n=5 CLEC4C-DTR^{+}, n=7 DTR^{-} littermates, n=5 *Ifnar*$^{-/-}$, n=8 *Il28ra*$^{-/-}$). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; statistics by unpaired two tailed students t test. Data in *B-D* are mean ± st. dev. and each symbol represents an individual mouse.



SI Appendix, Fig. S4. DC migration peaks 48 hours following FITC sensitization. Related to Fig. 4. Number of migratory DC ($CD11c^{int}MHCII^{hi}$) subsets in inguinal and axillary lymph nodes at baseline (0) and 24, 48, and 72h after epicutaneous FITC application. Data is representative of 1 of 2 experiments (depicted: n= 3 mice per time point, total: n=7 WT mice per time point). Data are mean ± st. dev.



SI Appendix, Fig. S5. Heterogeneity of cDC and pDC populations in skin DLN revealed by scRNAseq. Related to Fig. 5. Violin plots of the indicated markers show unique or shared expression of selected genes in each DC cluster.