Supplementary Information for

A rare subset of skin-tropic regulatory T cells expressing *Il10*/*Gzmb* inhibits the cutaneous immune response

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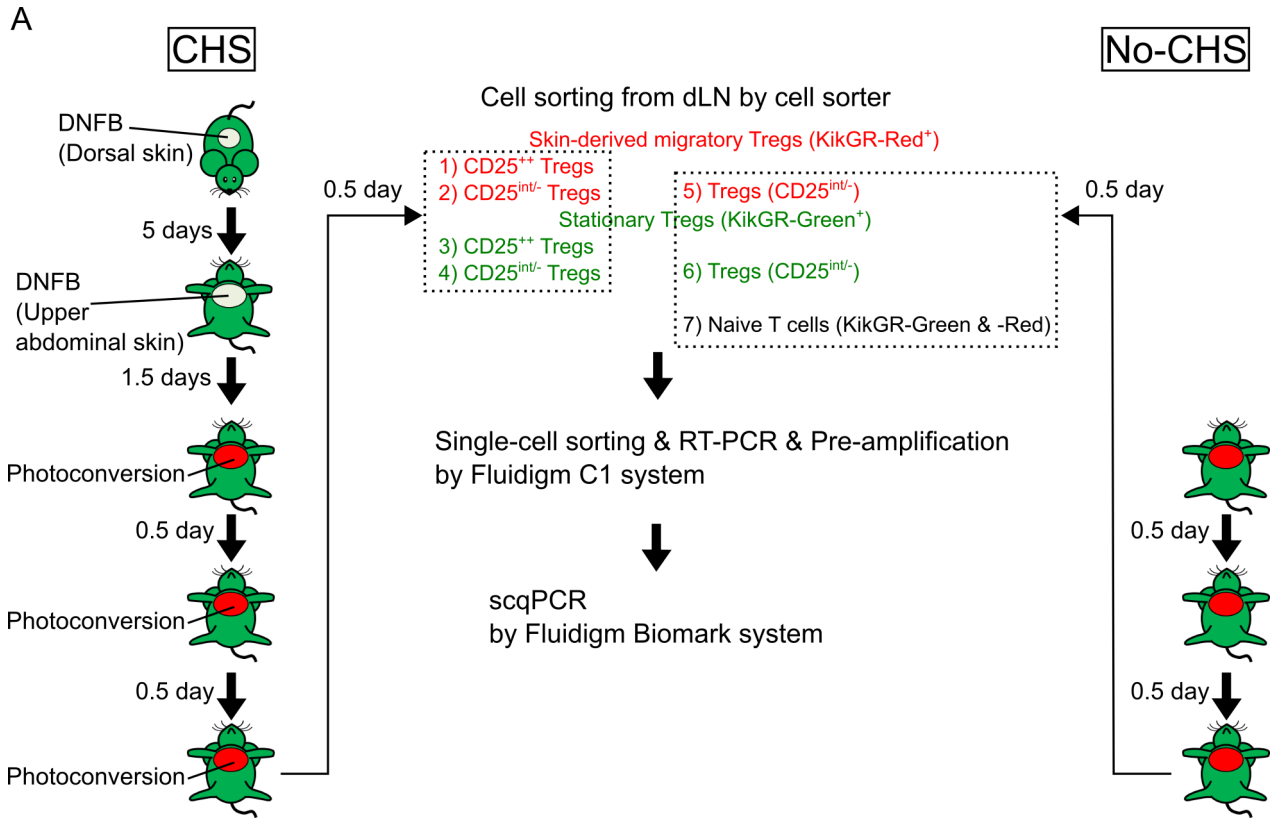
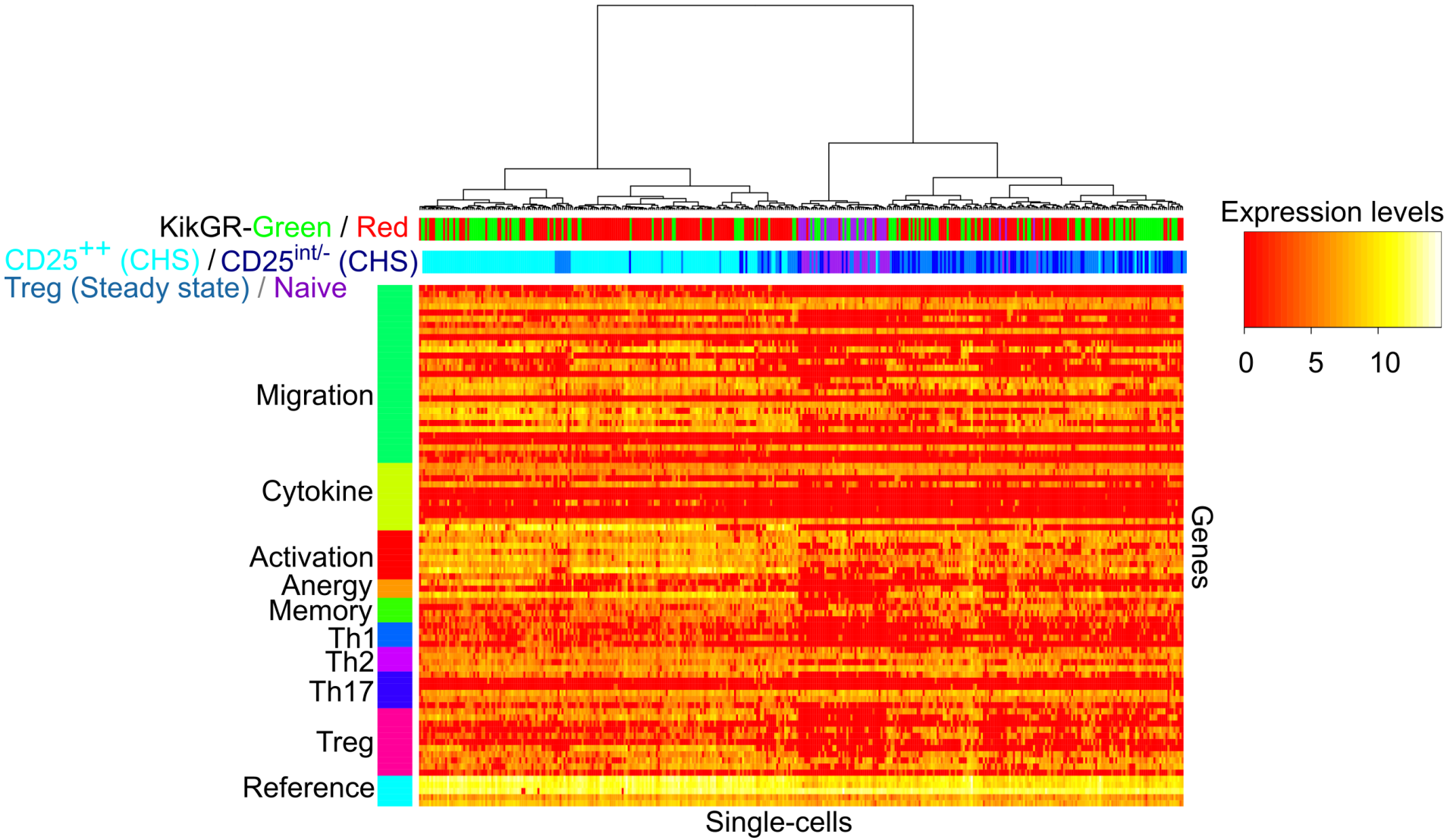
Supplementary Figures



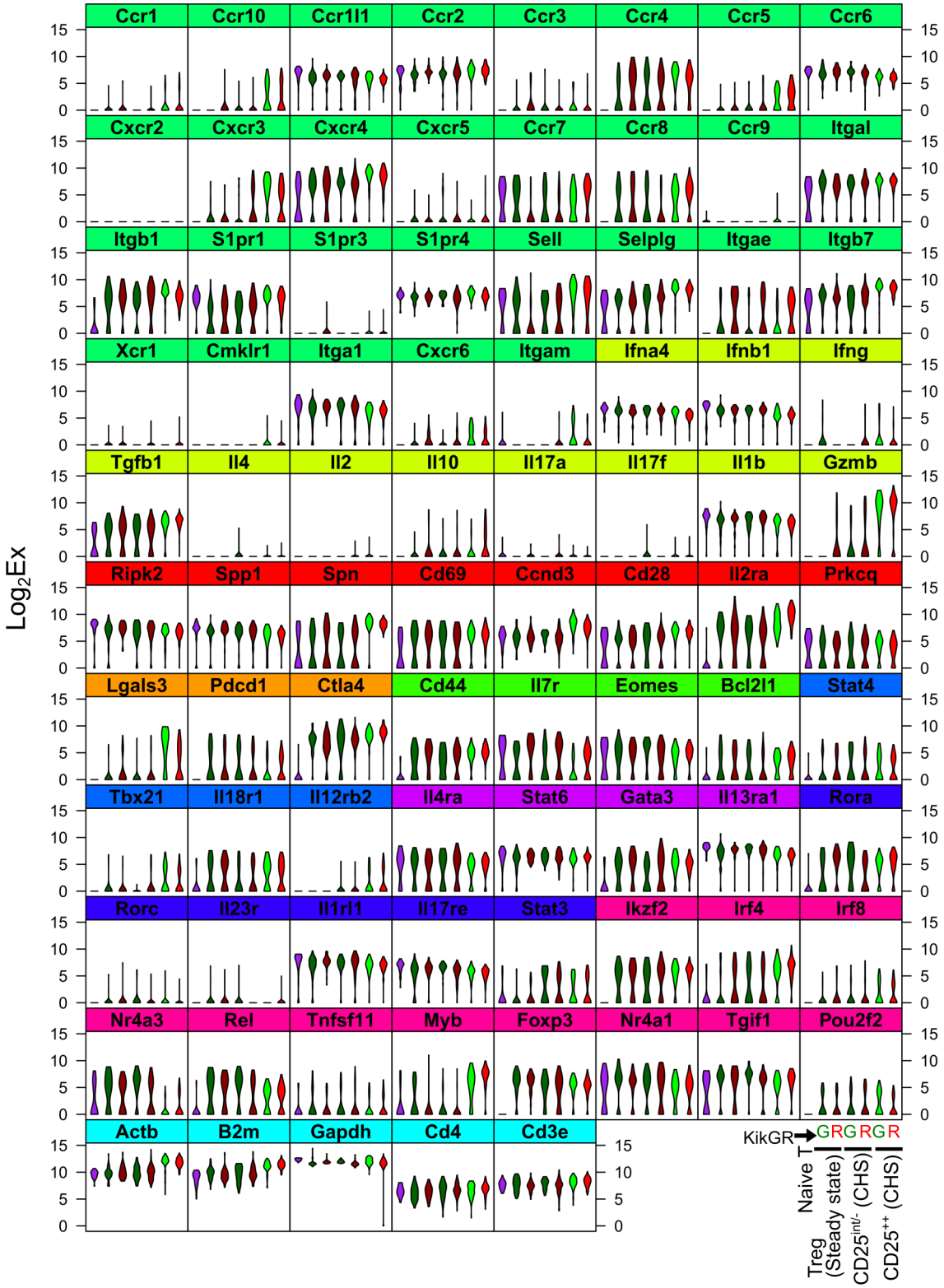
Supplementary Figure 1. Related to Figure 1. Experimental timeline for induction of CHS and scqPCR analysis.

(A) To induce CHS, KikGR/Foxp3$^{hCD2/hCD52}$ mice were immunized with 0.75% DNFB via the dorsal skin, then challenged with 0.45% DNFB via the abdominal skin. Cells in the skin were photoconverted following DNFB challenge. Half a day following the final photoconversion, Treg subsets isolated by cell sorter, and mRNA extracted from single-cells was synthesized into cDNA using a C1 system. scqPCR was performed using a Biomark system. Tregs in the steady state and naive T cells were analyzed as described above but without the CHS induction step. (B) Gene expression heatmap showing all single-cell expression data. The migratory status of each individual cell is indicated (KikGR-Green^{+} Tregs: green, KikGR-Red^{+} Tregs: red). Subpopulations of cells are depicted as follows: CD25^{++} Tregs from CHS mice (cyan), CD25$^{int/-}$ Tregs from CHS mice (navy), Tregs from steady-state mice (steel blue), and naive T cells (purple). Genes are grouped according to function as described in supplementary Table 1. Gene expression levels are represented in a gradient from red (low) to yellow (high). Illustration was created by R.I. using Power Point software.

B

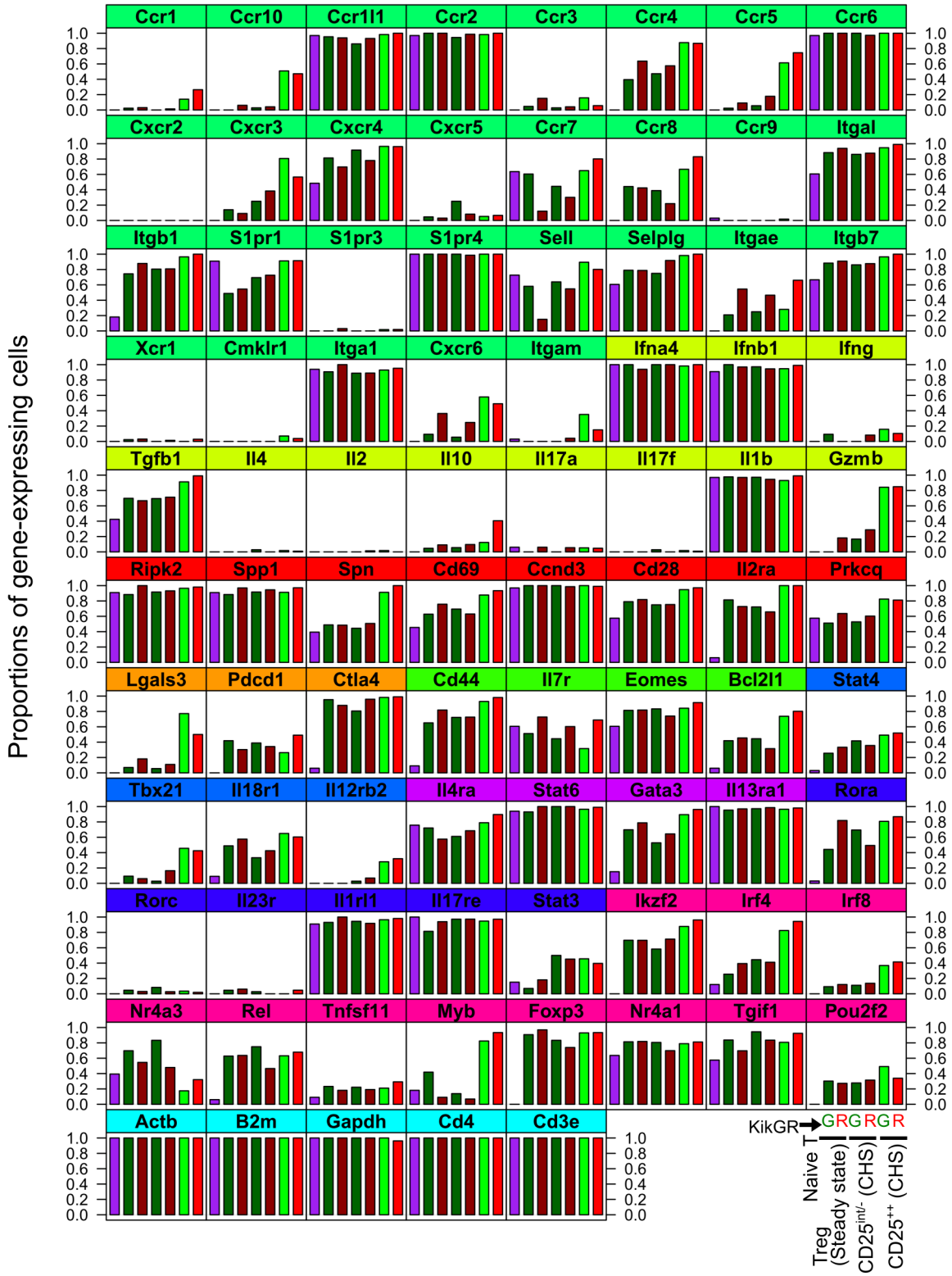

KikGR-Green / Red
CD25++ (CHS) / CD25int/- (CHS)
Treg (Steady state) / Naive
Migration
Cytokine
Activation
Anergy
Memory
Th1
Th2
Th17
Treg
Reference
Genes
Single-cells
Expression levels
0
5
10

Supplementary Figure 1. Continued



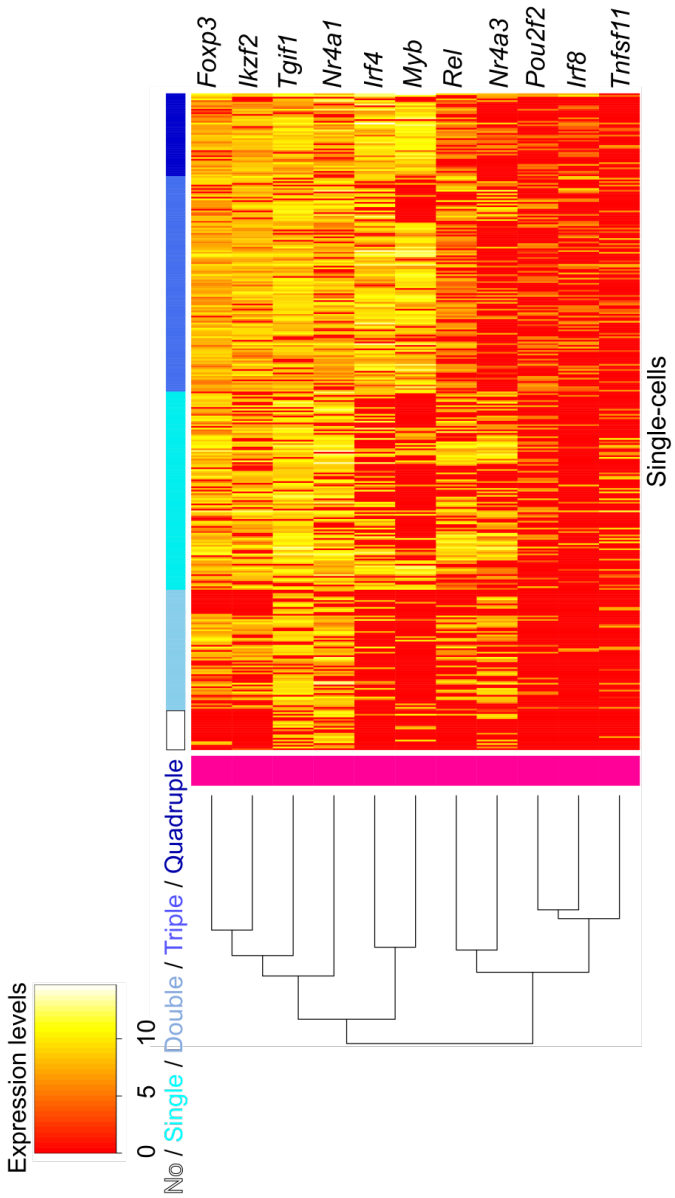
Supplementary Figure 2. Related to Figure 1. Gene expression profiles for all genes examined.

Gene expression levels for individual cells from each of the Treg subsets examined (from left: Naive T cells; KikGR-Green^{+} and KikGR-Red^{+} Tregs from steady-state mice; KikGR-Green^{+} and KikGR-Red^{+} CD25$^{int/-}$ Tregs from CHS mice; KikGR-Green^{+} and KikGR-Red^{+} CD25^{++} Tregs from CHS mice). Genes are grouped by function as described in supplementary Table 1.



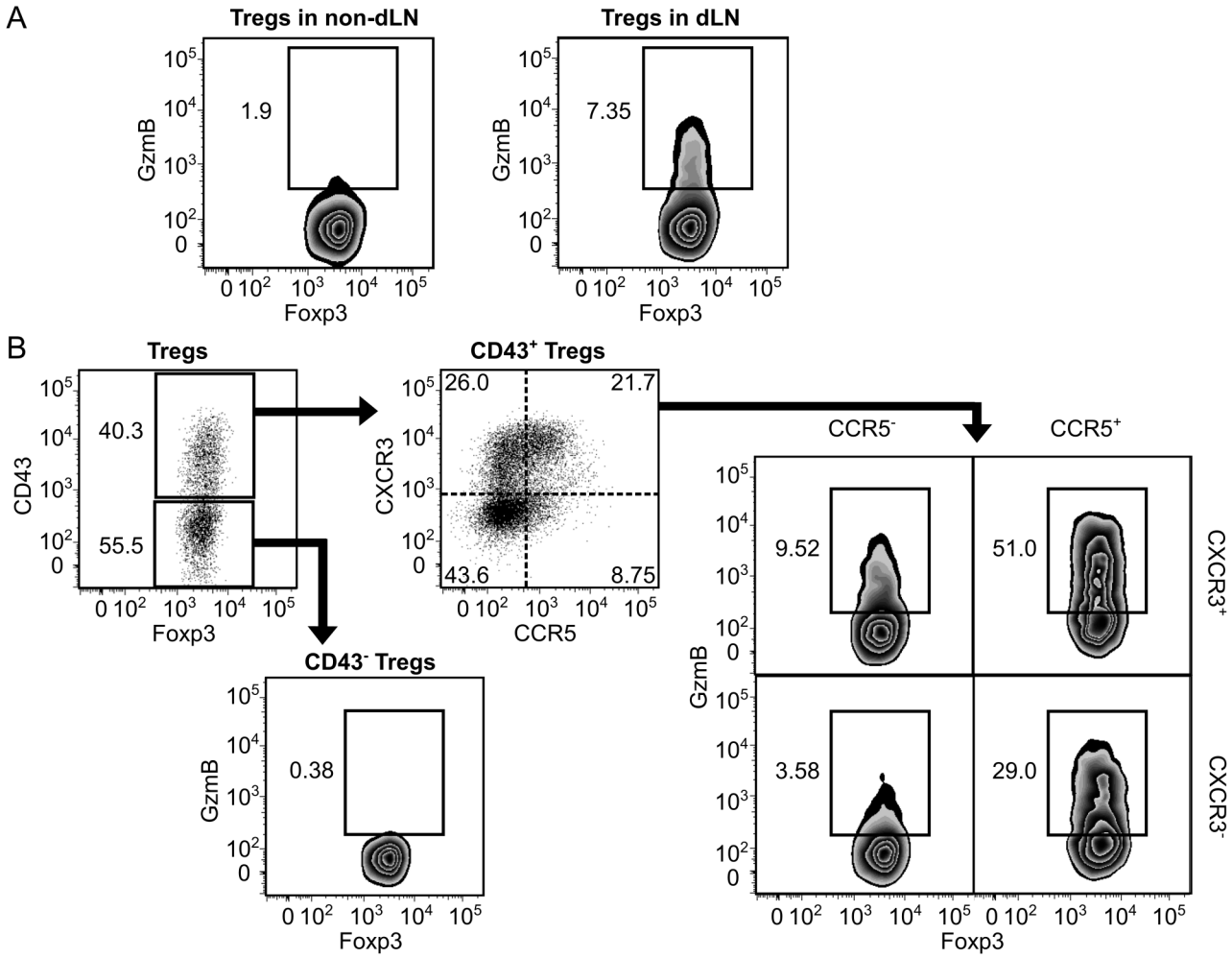
Supplementary Figure 3. Related to Figure 1. Proportions of individual cells expressing each gene.

Bar plots depict the proportion of cells expressing each gene for each of the Treg subsets examined (from left: Naive T cells; KikGR-Green^{+} and KikGR-Red^{+} Tregs from steady-state mice; KikGR-Green^{+} and KikGR-Red^{+} CD25$^{int/-}$ Tregs from CHS mice; KikGR-Green^{+} and KikGR-Red^{+} CD25^{++} Tregs from CHS mice). Genes are grouped by function as described in supplementary Table 1.



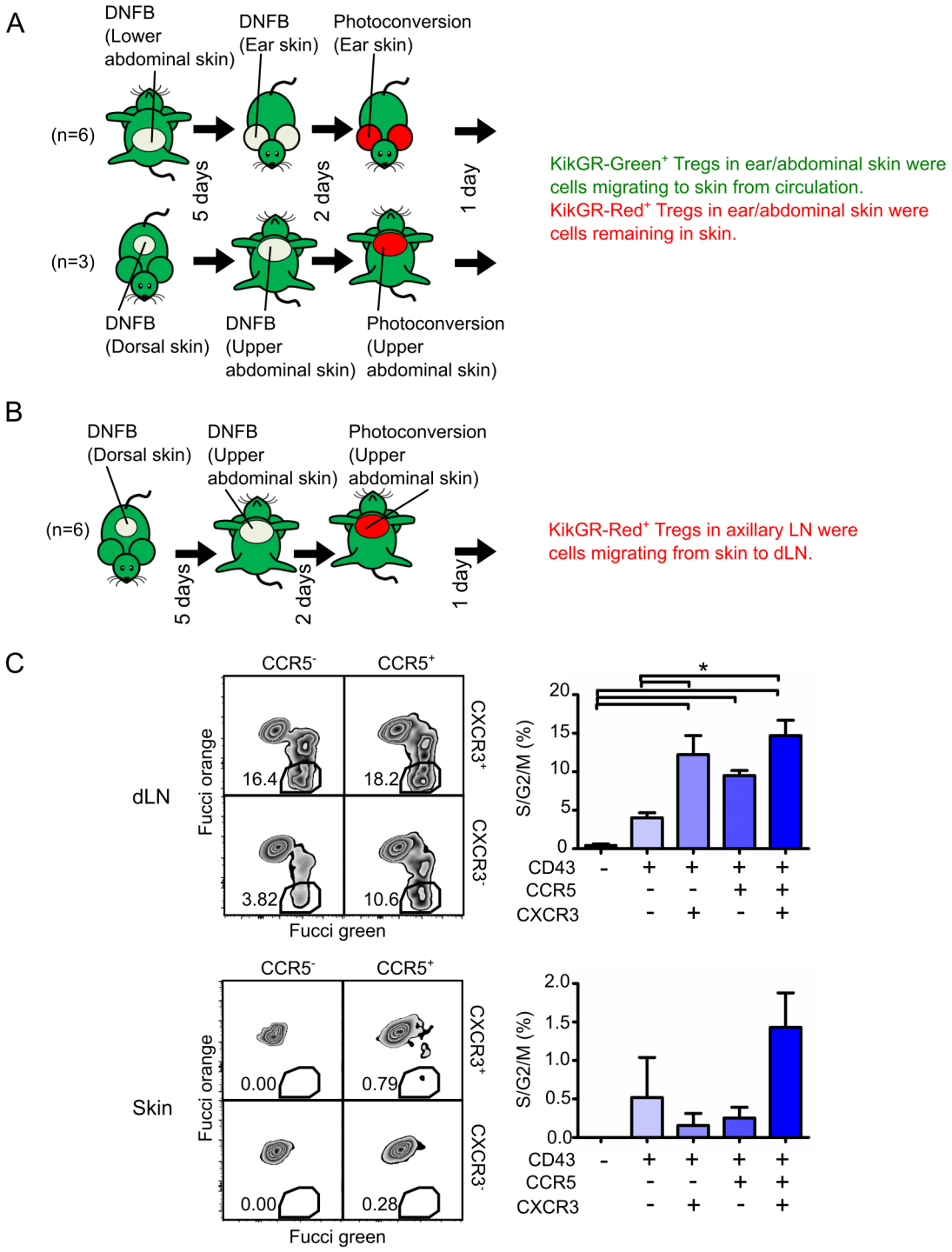
Supplementary Figure 4. Related to Figure 2. Gene expression heatmap for genes associated with Treg differentiation

Subpopulations of individual cells are grouped based on their co-expression of the genes *Il10*, *Gzmb*, *Tgfb1* and *Ctla4*: no expression (white), single-expressing (skyblue), double-expressing (cyan), triple-expressing (steel blue), and quadruple-expressing (navy) cells. Gene expression levels are represented in a gradient from red (low) to yellow (high).



Supplementary Figure 5. Related to Figure 3. GzmB, CD43, CCR5, and CXCR3 expression in Tregs

(A) Flow cytometric analysis of GzmB expression in Tregs isolated from the dLN and non-dLN of CHS mice. (B) Flow cytometric analysis of GzmB expression in $CD43^-$ Tregs and $CD43^+$ $CXCR3^{+/-}$ $CCR5^{+/-}$ Tregs from the dLN of CHS mice. Flow cytometry data are representative of three independent experiments; values on the plots indicate the percentage of the parent population.



Supplementary Figure 6. Related to Figure 5. Experimental design for the detection of Treg migration between the skin and the dLN.

(A, B) KikGR photoconversion was used to detect Tregs migrating from circulation to skin and remaining in skin (A) and Tregs migrating from skin to dLN (B). (C) Proportions of cells in the S/G_2/M phase among $CD43^-$ and $CD43^+$ $CXCR3^{+/-}$ $CCR5^{+/-}$ Treg subsets isolated from the dLN and skin of CHS mice (n = 3). Flow cytometric analyses were performed on day 2 following DNFB challenge. Flow cytometry data are representative of three independent experiments; values on the plots indicate the percentage of the parent population. Data in bar graphs represent means ± SEM. Statistical comparisons were performed by one-way ANOVA with Tukey's post-hoc test (* $p < 0.05$). Illustration was created by R.I. using Power Point software.

Supplementary Tables

Supplementary Table 1. Related to Figure 1. Examples of the function of the genes examined in this study.

Function	Gene Name
Migration	*Ccr1, Ccr10, Ccr1l1, Ccr2, Ccr3, Ccr4, Ccr5, Ccr6, Ccr7, Ccr8,*
Adhesion	*Ccr9, Cmklr1, Cxcr2, Cxcr3, Cxcr4, Cxcr5, Cxcr6, Itga1, Itgae, Itgal,*
	Itgam, Itgb1, Itgb7, S1pr1, S1pr3, S1pr4, Sell, Selplg, Xcr1
Cytokine	*Gzmb, Ifna4, Ifnb1, Ifng, Il10, Il17a, Il17f, Il1b, Il2, Il4, Il5, Tgfb1*
Activation	*Ccnd3, Cd28, Cd69, Il2ra, Prkcq, Ripk2, Spn, Spp1*
Proliferation	
Anergy	*Ctla4, Lgals3, Pdcd1*
Memory	*Bcl2l1, Cd44, Eomes, Il7r*
Th1	*Il12rb2, Il18r1, Stat4, Tbx21*, (*Ccr5*), (*Cxcr3*), (*Ifng*)
Th2	*Gata3, Il13ra1, Il4ra, Stat6, (Ccr4)*, (*Ccr8*), (*Il4*), (*Il5*)
Th17	*Il17re, Il1rl1, Il23r, Rora, Rorc, Stat3*, (*Il17a*), (*Il17f*)
Treg	*Foxp3, Ikzf2, Irf4, Irf8, Myb, Nr4a1, Nr4a3, Pou2f2, Rel, Tgif1, Tnfsf11*, (*Ccr6*), (*Ctla4*), (*Il10*), (*Tgfb1*), (*Gzmb*)
Reference gene	*Actb, B2m, Cd3e, Cd4, Gapdh*

Genes shown in parentheses appear in multiple categories.

Supplementary Methods

CHS protocol

Mice were anesthetized with isoflurane (DS Pharma Animal Health) and immunized by application of 25 μL of 0.75% DNFB (Nacalai Tesque) in 4:1 (wt/vol) acetone/olive oil to shaved dorsal or lower abdominal skin [1]. Five days later, the mice were challenged with 50 μL, 50 μL, or 20 μL of 0.45% DNFB to the shaved upper abdominal skin, to the shaved lower abdominal skin, or to the skin of each ear, respectively, which drain to the axillary, inguinal, and auricular LNs, respectively. The experimental timelines for the data shown in Fig. 1-4, 5, and 6 are depicted in supplementary Fig. 1A, 6, and Fig. 6.

Antibodies and staining for flow cytometry

The following antibodies were used for flow cytometry and cell sorting: anti-GzmB-PE (Life Technologies), anti-hCD2-APC-H7 (BD Biosciences), anti-IL-12RB2-APC (R&D systems), anti-IL-10-PE (BD Biosciences), anti-CCR5-PE or -APC or –biotin with streptavidin-PE-Cy5, anti-CXCR3-Brilliant Violet (BV) 421 or -APC, anti-CCR4-BV421, anti-CCR8-AlexaFluor647, anti-CD43-PE-Cy7, anti-CD4-BV785 or -Pacific blue or -APC-Cy7 or -AlexaFluor700, anti-CD25-BV650 or -BV421, anti-hCD2-APC or -PE-Cy7 or -FITC, anti-CD62L-APC, and anti-CD44-APC-Cy7. Antibodies other than anti-GzmB, anti-human CD2-APC-H7, anti-IL-10, and anti-IL12RB2 were obtained from BioLegend.

Single-cell suspensions of lymph node cells were incubated with anti-CD16/CD32 (TONBO biosciences) for 10 min, then stained with fluorochrome-conjugated antibodies. Dead cells were stained with 7-AAD (eBioscience) and excluded from our analyses. Stained samples were analyzed using an LSRFortessa (BD Biosciences) or SP6800 (SONY) flow cytometer. All data were analyzed using FlowJo software (Tree Star). Tregs were identified as $CD4^+$ $hCD2^+$ cells falling within a lymphocyte forward scatter and side scatter gate.

Measurement of gene expression at single-cell level

Experimental timeline is depicted in supplementary Fig. 1. Four types of $hCD2^+$ Treg subsets ($CD25^{++}$ KikGR-Red, $CD25^{++}$ KikGR-Green, $CD25^{int/-}$ KikGR-Red, and $CD25^{int/-}$ KikGR-Green) were sorted from the axillary LN of CHS-induced KikGR/$Foxp3^{hCD2/hCD52}$ mice using a Moflo Astrios flow cytometer (Beckman Coulter). Mice were immunized on the dorsal skin and challenged on the upper abdominal skin as described above. To detect KikGR-Red^+ and KikGR-$Green^+$ cells by flow cytometry, the skin was photoconverted as described above 36, 48 or 60 hours following DNFB immunization. Three days following the challenge, Treg cell subsets were isolated by cell sorting. Naive $CD4^+$ T cells ($hCD2^-$ $CD62L^{high}$ $CD44^{low}$), $CD25^{int/–}$ KikGR-Red, and $CD25^{int/–}$ KikGR-Green Tregs were also sorted from the axillary LN of steady-state mice. Samples for $CD25^{++}$ KikGR-Red^+ and $CD25^{int/–}$ KikGR-Red^+ Treg subsets in CHS were collected from duplicate mice.

The capture of individual Tregs, cell lysis, RTPCR, and pre-amplification specific for 85 genes were performed using C1 Single-Cell Auto Prep system (Fluidigm) with Single Cell-to-CT kit (Life Technologies) and C1 Single-Cell AutoPrep Reagent Kit (Fluidigm), as described in the manufacturer's protocol. Amplicons were collected and stored at –30°C prior to scqPCR assay. scqPCR was performed using 48.48 or 96.96 Dynamic Arrays (Fluidigm) with SsoFast EvaGreen Supermix with Low ROX (Bio-Rad Laboratories) and a BioMark system (Fluidigm). The primers used to amplify the 85 genes in the C1 system were designed and synthesized by Fluidigm and Life Technologies (see table below). To reduce nonspecific amplification, nested primers for 9 genes were purchased from Life Technologies (see table below). 76 primer pairs and 9 nested primer pairs were used for scqPCR with the Biomark system.

$CD43^+$ $CCR5^+$ $CXCR3^+$ and $CD43^+$ $CCR5^+$ $CXCR3^-$ Tregs were sorted from the axillary LN of $Foxp3^{hCD2/hCD52}$ CHS mice 3 days after DNFB challenge. RT-PCR and pre-amplification were performed using the C1 system and scqPCR was performed on 48. 48 Dynamic Arrays as described above.

Table. Sequences of primers using for pre-amplification and scqPCR

Gene Symbol	Forward primer sequence	Reverse primer sequence	Nested forward primer sequence	Nested reverse primer sequence
Actb	CCCTAAGGCCAACCGTGAAA	AGCCTGGATGGCTACGTACA		
B2m	ACTGACCGGCCTGTATGCTA	ATGTTCGGCTTCCCATTCTCC		
Bcl2l1	AGCCTTGGATCCAGGAGAAC	GGCTGCTGCATTGTTCCC		
Ccnd3	ATGCGGAAGATGCTGGCATA	GTTCATAGCCAGAGGGAAGACA		
Ccr1	TCCTCAAAGGCCCAGAAACA	GCTGAGGAACTGGTCAGGAA		
Ccr10	TAGCCAGAGATGGGGACCAA	CGAATAGGCCTCCTCATCGTAC		
Ccr1l1	TACACCCACTGCTGTGTCAA	CGAAACAACTGCCAGAGGTAC		
Ccr2	TGAGGCTCATCTTTGCCATCA	GGATTCCTGGAAGGTGGTCAA		
Ccr3	CTGGACTCATAAAGGACTTAGCA	GTGGTGCCCACTCATATTCA		
Ccr4	GACTGTCCTCAGGATCACTTTCA	AGCAGGAGAAGCCAATGAGAA		
Ccr5	TAGCCAGAGGAGGTGAGACA	CGGAACTGACCCTTGAAAATCC		
Ccr6	AAGGCACATATGCGGTCAAC	CCTGGACGATGGCAATGTAC		
Ccr7	GTGGTGGCTCTCCTTGTCA	GGTATTCTCGCCGATGTAGTCA		
Ccr8	AGTGGGCAGCTCTGAAACC	TTGGGCTCCATCGTGTAATCC		
Ccr9	CACCATGATGCCCACAGAAC	CTGTGGAAGCAGTGGAGTCATA		
Cd28	CTGCTGTTCTTGGCTCTCAAC	GGGCGACTGCTTTACCAAAA		
Cd3e	TGCTACACACCAGCCTCAAA	AGGTCCACCTCCACACAGTA		
Cd4	AAGGGACACTGCATCAGGAA	CCCATCACCTCACAGGTCAA		
Cd44	TTCCTTCGATGGACCGGTTA	TACTCGCCCTTCTTGCTGTA		
Cd69	GTGGTCCTCATCACGTCCTTA	ACAAGCCTGGGCAATTGTAC		
Cmklr1	GTAACAGACCAGCCAAGGAC	AGTCGTTGTAAGCGTCGTAC		
Ctla4	GGACTTGGCCTTTTGTAGCC	CTGAAGGTTGGGTCACCTGTA		
Cxcr2	CACAAACAGCGTCGTAGAACTA	AGGGCATGCCAGAGCTATAA		
Cxcr3	ACCAGCCAAGCCATGTACC	GGGAGAGGTGCTGTTTTCCA		
Cxcr4	GGTAACCACCACGGCTGTA	CAGGGTTCCTTGTTGGAGTCA		
Cxcr5	GGACATGGGCTCCATCACATA	GGGGAATCTCCGTGCTGTTA		
Cxcr6	TGAGCACACTTCACTCTGGAA	ACTCTTGATGCCCATCATCCA	CACACTTCACTCTGGAACAAA	TGATGCCCATCATCCATGGCA
Eomes	GCGGCAAAGCGGACAATAAC	ATCCAGTGGGAGCCAGTGTTA		
Foxp3	CCCACACCTCTTCTTCCTTGAA	GACGGTGCCACCATGACTA		
Gapdh	AGACGGCCGCATCTTCTT	TTCACACCGACCTTCACCAT		
Gata3	CCTACCGGGTTCGGATGTAA	CCGCAGTTCACACACTCC	CGGGTTCGGATGTAAGTCGA	GTTCACACACTCCCTGCC
Gzmb	GCATTCCCCACCCAGACTAT	AGTCCTCTTGGCCTTACTCTTC	TCCCCACCCAGACTATAATC	TGGCCTTACTCTTCAGCTTTA
Ifna4	AAGCCTGTGTGATGCAGGAA	CCTGTGGAAGTATGTCCTCACA		
Ifnb1	TACACTGCCTTTGCCATCCA	CCACCCAGTGCTGGAGAAA		
Ifng	CCACGGCACAGTCATTGAAA	GCCAGTTCCTCCAGATATCCAA		
Ikzf2	GGCCCAATGTGCTTATGGTAC	AAGAAGCTCCGCACTGGTTA		
Il10	AAAGGACCAGCTGGACAACA	TAAGGCTTGGCAACCCAAGTA		
Il12rb2	CAGCAAACAGCACTTGGGTAAA	GGCCATGCCATCAGGAGATTA		
Il13ra1	AGAGGACAAATGCCAGAATTCC	AGAACACCAGGGAGTTGGAA		
Il17a	TGAGTCCAGGGAGAGCTTCA	CGCTGCTGCCTTCACTGTA		
Il17f	AAGCAGCCATTGGAGAAACC	GGCAAGTCCCAACATCAACA		
Il17re	CCACACTGTAGACCTGCCTTA	CACAGTGTCCTCTTGCAGGTA	ACTGTAGACCTGCCTTATGAA	GTCCTCTTGCAGGTAGGAGGC
Il18r1	AAGAGGACAGCTCAGACCCTAA	GAAGCATGCAGTTTGCCTTCA		
Il1b	TGGCAACTGTTCCTGAACTCA	GGGTCCGTCAACTTCAAAGAAC	TGTTCCTGAACTCAACTGTGA	CGTCAACTTCAAAGAACAGGTC

Il1rl1	TTGGGCTTTGGCAATTCTGAC	TCATTTTCCAGACCCCAGGAC		
Il2	CCCAGGATGCTCACCTTCAAA	CCGCAGAGGTCCAAGTTCA		
Il23r	TGGAGATGCTCAGTGCTACA	CCATTCCCGACAAAAGTCCAA		
Il2ra	TGCGTTGCTTAGGAAACTCC	CTGGTGTTCAAGTTGAGCTGTA		
Il4	ACGGAGATGGATGTGCCAAA	GAAGCACCTTGGAAGCCCTA		
Il4ra	AACATCTCCAGAGAGGACAACC	CTCAGCCTGGGTTCCTTGTA		
Il7r	AAAGCATGATGTGGCCTACC	GGGATTGTTGTTCTTGTGTGGAA		
Irf4	TCCCCATTGAGCCAAGCATA	CGAGGATGTCCCGGTAATACA		
Irf8	GATATGCCGCCTATGACACA	CCCGTAGTAGAAGCTGATGAC		
Itga1	ACCGGCTTCAGTGCTCATTA	ATGACCACAGTTCCGTTCCA		
Itgae	CGCAGCCTTCAATTCAGACA	GGGCTTTGGCTTGTGTTCA		
Itgal	TGTCCTGTACCCTGTCAACA	TGGGACCTTTAGGGGTGAAA		
Itgam	AGCAGCTGAATGGGAGGAC	GGCCCCATTGGTTTTGTGAA		
Itgb1	AAGGGCCAACTTGTGAGACA	TGAAGGCTCTGCACTGAACA		
Itgb7	CTGAGTGAGGACTCCAGCAA	GGAGAGTGCTCAAGAGTCACA		
Lgals3	ATCATGGGCACAGTGAAACC	AGTGGAAGGCAACATCATTCC		
Myb	TCCTCCGTCAACAGCGAATA	CAATGCGACAGGATAGGGAAC		
Nr4a1	CAATGCTTCGTGTCAGCACTA	TGTTTGCCAGGCAGATGTAC		
Nr4a3	CTGCCTGTCAGCACTGAGTA	CTTGGTGCATAGCTCCTCCA		
Pdcd1	CTGGAAGCAAGGACGACAC	CTGGAAGTCCAGCTCCTCATA		
Pou2f2	CATTGTCCCAAGCTTCTAGCA	CCCACAGCTGAGGATAAGGTA		
Prkcq	GGCGACTTAATGTACCACATCC	AGGATGACCTCAGCAGCATAA		
Rel	CTGCCTCCCATTGTTTCTAACC	CTTGTTCACACGGCAGATCC		
Ripk2	CTCGTGTTCCTTGGCTGTAA	TCCACTGTTGGGCTATACCA		
Rora	AGAACCACCGAGAAGATGGAA	GTCGTCCACATAGGGCTCTTA		
Rorc	TGGAGCTCTGCCAGAATGAC	GGCCCTGCACATTCTGACTA		
S1pr1	CGGTGTAGACCCAGAGTCC	GAGAGGCCTCCGAGAAACA	AGACCCAGAGTCCTGCGGA	GCCTCCGAGAAACAGCAGC
S1pr3	GCCCCTAGACGGGAGTCTTA	ACTGCGGGAAGAGTGTTGAAA		
S1pr4	GGTGTACTACTGCCTGCTGAA	CTGACAGCAGCACGTTGAC		
Sell	CAGAGAGACTTGCAGAGAGACC	TCCTCGAGCCCCAGTAAGTA		
Selplg	AGGCAGAGTCGTTTGCTTCT	ACAAGGAAGCTTGGGGACAT		
Spn	CTGACCAAGCCTCAGGAAGAA	AAGGTGCAAGGCCATCTCC		
Spp1	TGCCTGACCCATCTCAGAA	AAGTCATCCTTTTCTTCAGAGGAC		
Stat3	TGGGCATCAATCCTGTGGTA	CCAATTGGCGGCTTAGTGAA	ATCAATCCTGTGGTATAACA	ATTGGCGGCTTAGTGAAGAA
Stat4	CCCAAGGAGATGAAGTGCAGTA	ATGGAATGCAACTCCTCTGTCA		
Stat6	TGACTTTCCACAACGCCTAC	CATCTGAACCGACCAGGAAC		
Tbx21	CAAGTTCAACCAGCACCAGAC	CCACGGTGAAGGACAGGAA		
Tgfb1	GCTGCGCTTGCAGAGATTAA	GTAACGCCAGGAATTGTTGCTA		
Tgif1	CCTCAGAGCAAGAGAAAGCA	GGGCGTTGATGAACCAGTTA		
Tnfsf11	CAGCCATTTGCACACCTCAC	GGTACCAAGAGGACAGAGTGAC		
Xcr1	GAAACCCTGACATGGACTCA	AAAGGCTGTAGAGGACTCCA		

Analysis of single-cell gene expression profiles

scqPCR data was initially analyzed using Real-Time PCR Analysis (Fluidigm) and Ct value data was exported, excluding data with failed PCR reactions. Preliminary analysis of the scqPCR data indicated a possible bias in mean expression levels depending on experimental batches (each array can be regarded as one batch containing a maximum of 96 cells). To reduce the effect of the bias, we performed a batch-wise normalization by adjusting the limit of detection (LOD) values for each batch so that the average expression levels (Log_2Ex) over each batch were the same. The data with no expression (Ct > LOD) were

converted to zero. To ensure that samples represented healthy $CD4^+$ T cells, we used only single cells expressing all of *Actb*, *B2m*, C*d3e*, and *Cd4* in the rest of the analyses.

For generating volcano plots, statistical comparisons were made using Student's t-test. For clustering analysis, we used the Euclidean distance and the Ward agglomeration methods. For the correlation network analysis, Spearman's rank correlation coefficients were used because the data values did not follow a Gaussian distribution. All analyses and data visualization were performed using the statistical software R 3.1.2 (the R Foundation for Statistical Computing).

Real-time PCR at the population level

To measure the expression levels of Treg effector molecules at the population level, Treg subsets were sorted from the axillary LN of CHS-induced $Foxp3^{hCD2/hCD52}$ mice 3 days after DNFB challenge. Cell lysis, cDNA synthetization, and pre-amplification were performed using a Single Cell-to-CT kit and a C1 Single-Cell AutoPrep Reagent Kit, according to the manufacturer's protocol. Real-time PCR was performed using a LightCycler 480 (Roche Diagnostics) with SsoFast EvaGreen Supermix with Low ROX. Each amplification was performed in duplicate, and the amounts of *Tgfb1*, *Ctla4*, *Gzmb*, *Il10* expression were divided by the mean expression of *Actb* and *Gapdh* as internal control genes. Gene expression data were presented as a ratio relative to expression levels in naive T cells.

Cell isolation from skin tissue

Fat tissue was removed from the skin and the skin was floated for 1 hr in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (HyClone), collagenase II (Worthington Biochemical, 500 U/mL), and DNaseI (Calbiochem) at room temperature. After mincing, the skin was incubated with shaking for 30 min at 37°C. After adding 500 mM EDTA, cell suspensions were filtered through a 70 μm of cell strainer (BD Biosciences).

Supplementary References

1. Otsuka, A. *et al.* Requirement of interaction between mast cells and skin dendritic cells to establish contact hypersensitivity. *PLoS One* **6,** e25538 (2011).