Supplemental Information for:

Dendritic cells maintain dermal adipose-derived stromal cells in skin fibrosis

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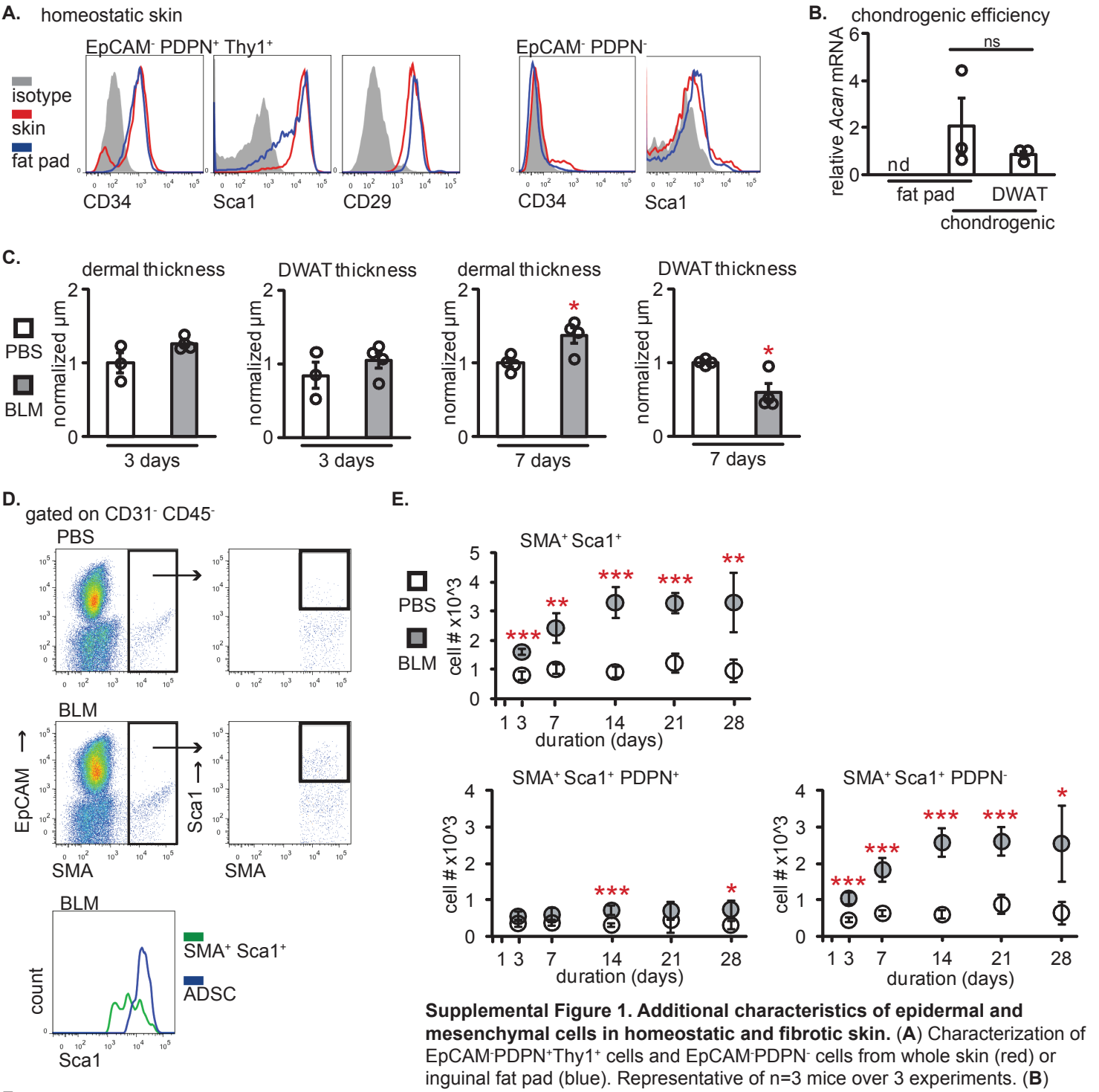
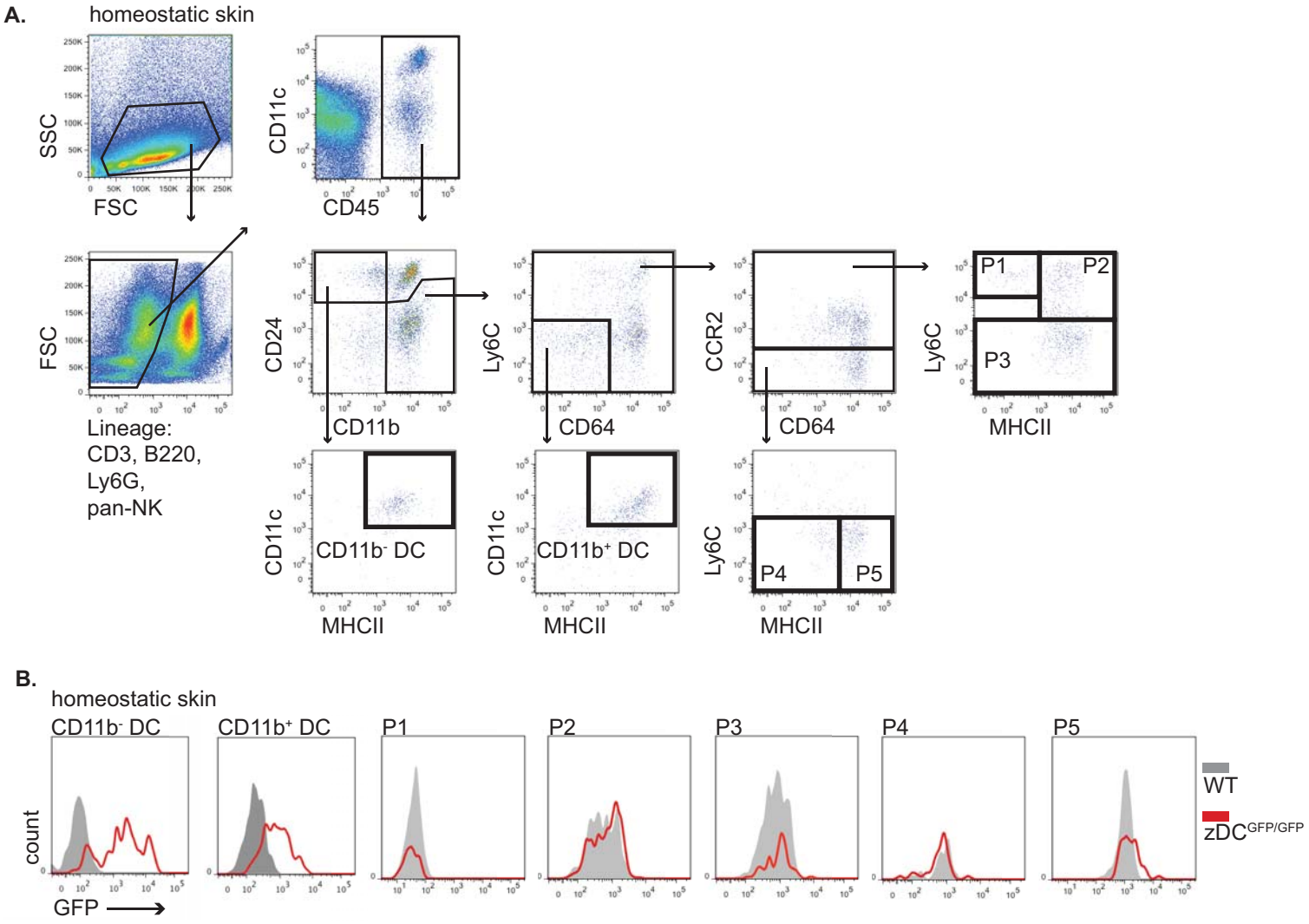
Supplemental Figure 1



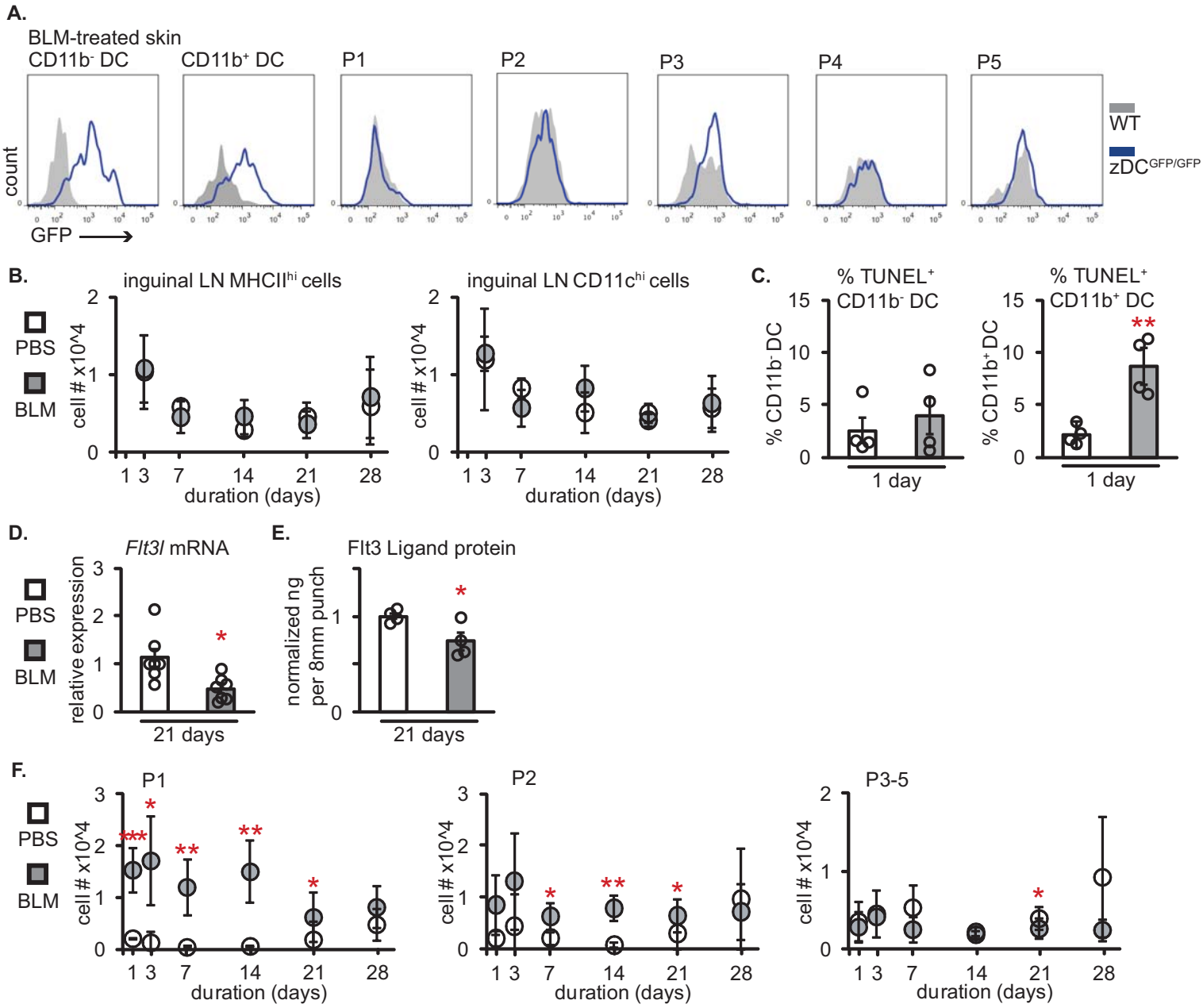
Supplemental Figure 1. Additional characteristics of epidermal and mesenchymal cells in homeostatic and fibrotic skin. (**A**) Characterization of EpCAM$^-$PDPN$^+$Thy1$^+$ cells and EpCAM$^-$PDPN$^-$ cells from whole skin (red) or inguinal fat pad (blue). Representative of n=3 mice over 3 experiments. (**B**) Chondrogenic differentiation of inguinal fat pad ADSCs or isolated DWAT ADSCs. Chondrogenic efficiency as assayed by Acan mRNA assessed by qPCR. n=3 over 3 experiments. (**C-F**) Mice were injected with PBS or BLM over duration indicated. (**C**) Dermal and DWAT thicknesses normalized to PBS condition. n=3-4 mice over 2 experiments. (**D**) Gating strategy for SMA$^+$ cells, and Sca1 expression profile of SMA$^+$Sca1$^+$ presumed myofibroblasts (green) versus ADSCs (blue) in 21 day PBS vs BLM treated skin. (**E**) Cell numbers of the indicated SMA$^+$ subpopulation per punch. n=4-6 mice over 2-3 experiments. (**F**) EpCAM$^+$ and EpCAM$^-$PDPN$^-$ cell numbers per punch. n=8 mice over 4 experiments. *p <0.05, **p <0.01, ***p <0.001 using two-tailed unpaired student's t test. Error bars depict S.D. in (E) and S.E.M in all other graphs.

Supplemental Figure 2



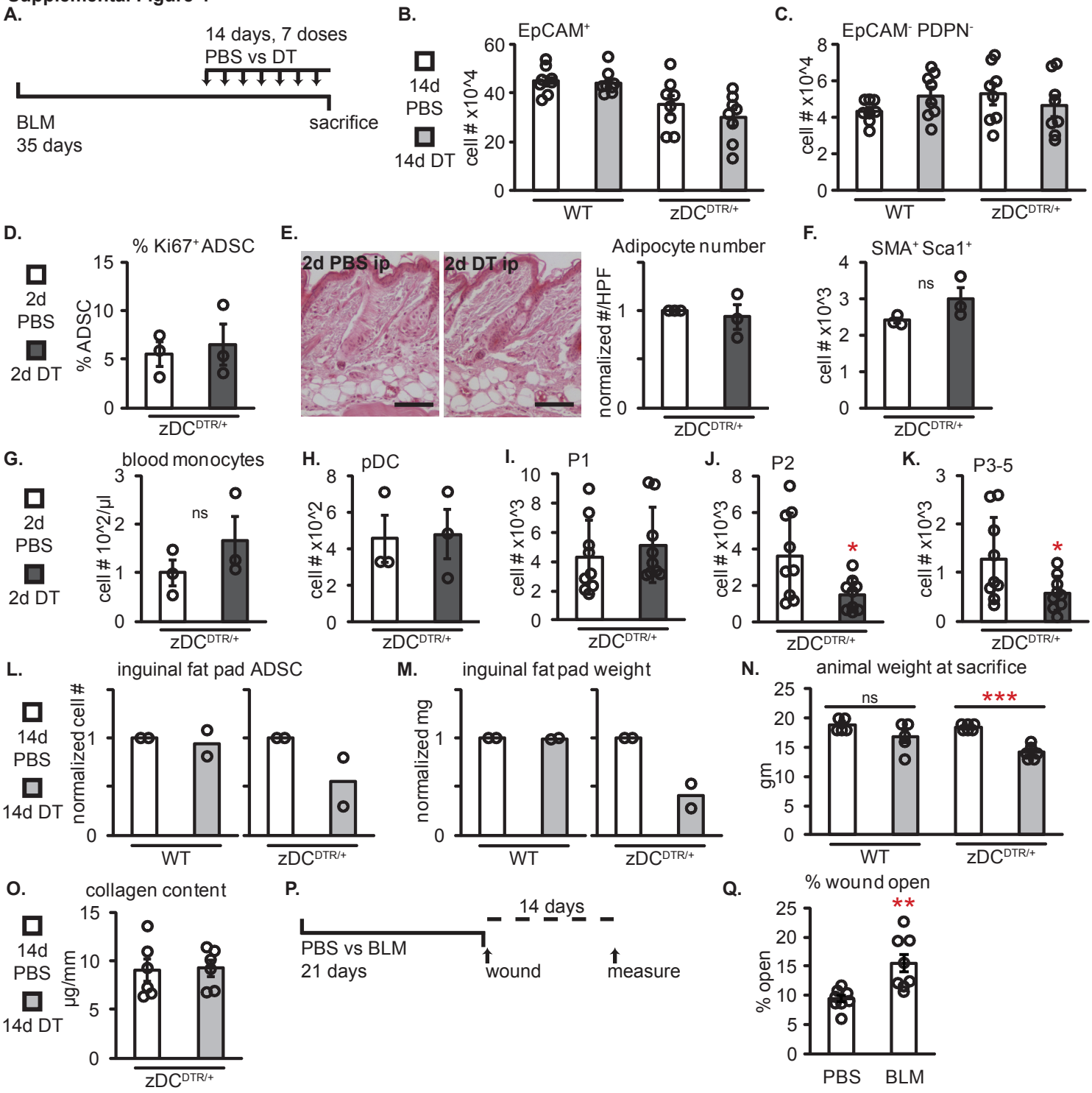
Supplemental Figure 2. Gated CD11b$^-$ and CD11b$^+$ DCs in homeostatic skin express zbtb46 and are Flt3 ligand-sensitive. (**A**) Gating strategy for CD11b$^-$ and CD11b$^+$ DC and other mononuclear phagocyte populations, based on the strategy of Tamoutounour et al (27). For non-DC populations, the Tamoutounour designations are used: P1 are monocytes, P2 and P3 are 2 different populations of monocyte-derived DCs, and P4 and P5 are MHCII$^-$ and MHCII$^+$ macrophages, respectively. As also stated in the text, cells in the CD11b$^-$ and CD11b$^+$ DC gates are "DCs," while P2 and P3 are "monocyte-derived DCs." (**B**) GFP expression by DCs and other myeloid cell populations in back skin of homeostatic WT (shaded) or zDC$^{GFP/GFP}$ (red) mice. Representative of 6 mice over 3 experiments. (**C**) DC numbers in homeostatic back skin of WT or *Flt3l*$^{-/-}$ mice. Numbers are reported per 8mm punch. n=4-5 mice per genotype over 3 experiments. **p <0.01, ***p <0.001 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Figure 3



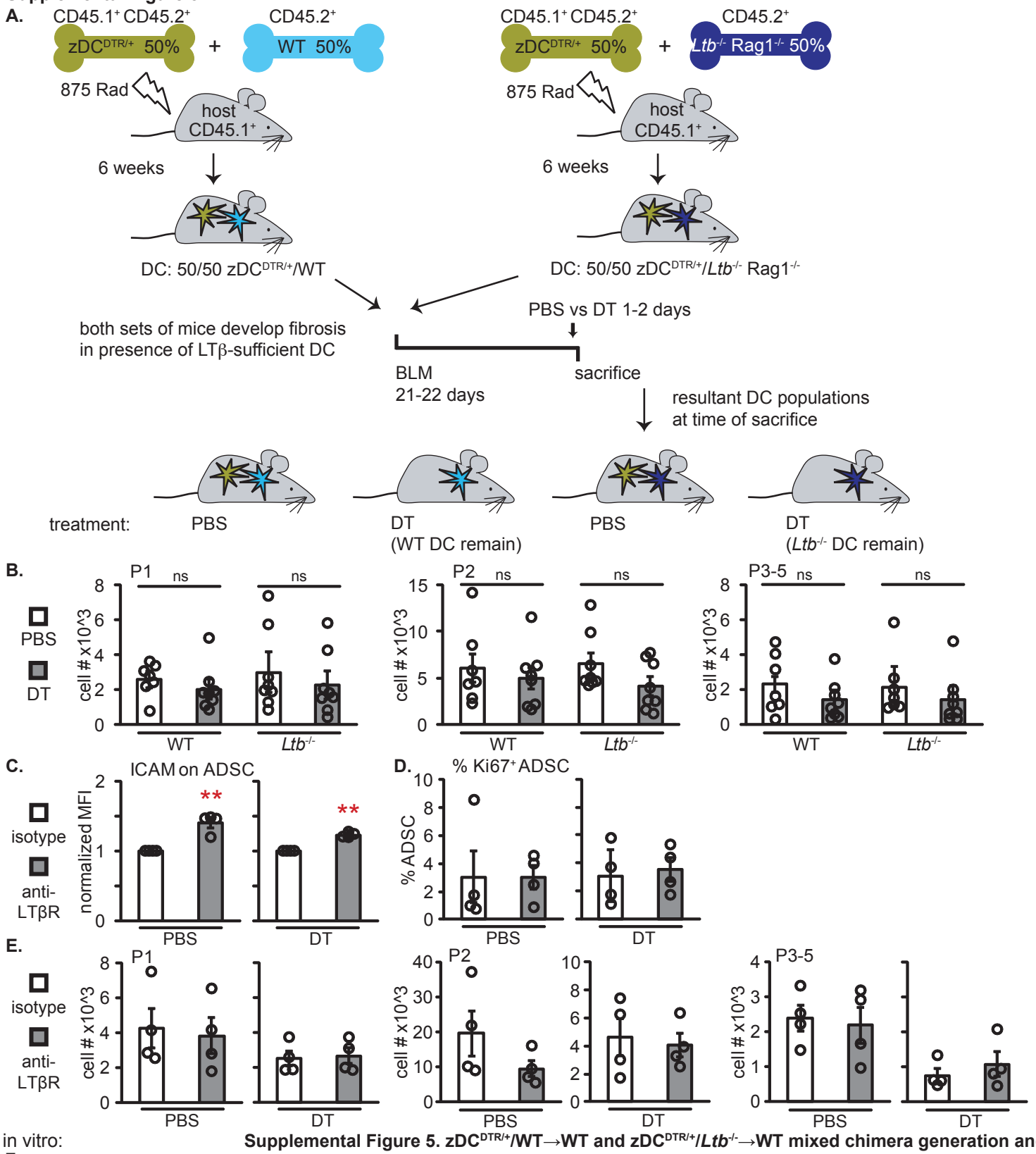
Supplemental Figure 3. DCs and other mononuclear phagocyte populations with BLM treatment. (**A**) GFP expression by DCs and other mononuclear phagocyte populations in back skin of WT (shaded) or zDC$^{GFP/GFP}$ (blue) mice treated for 21 days with BLM. Representative of 6 mice over 3 experiments. (**B-F**) Mice were treated with PBS or BLM for the duration indicated. (**B**) CD11c^{+}MHCII^{hi} and CD11c^{hi}MHCII^{+} DC numbers per draining inguinal lymph node (LN). DCs that migrate from skin to nodes are mainly in these gates (17, Supp.Ref. 1), n=4-8 over 2-4 experiments. (**C**) Percentage of DCs in the skin that are TUNEL^{+} after 1 day of BLM. n=4 over 2 experiments. (**D**) Relative *Flt3l* mRNA in skin assayed by qPCR. (**E**) Flt3 ligand protein in skin measured by ELISA and normalized to PBS group. (**D-E**) n=4-7 from 2-3 experiments (**F**) Enumeration of Tamoutounour populations (27) P1, P2, and P3-P5 expressed as cell numbers per punch. n=4-8 over 2-4 experiments. *p <0.05 **p <0.01, ***p <0.001 using two-tailed unpaired student's t test. Error bars depict S.E.M. in (B,F) and S.D. in all other graphs.

Supplemental Figure 4



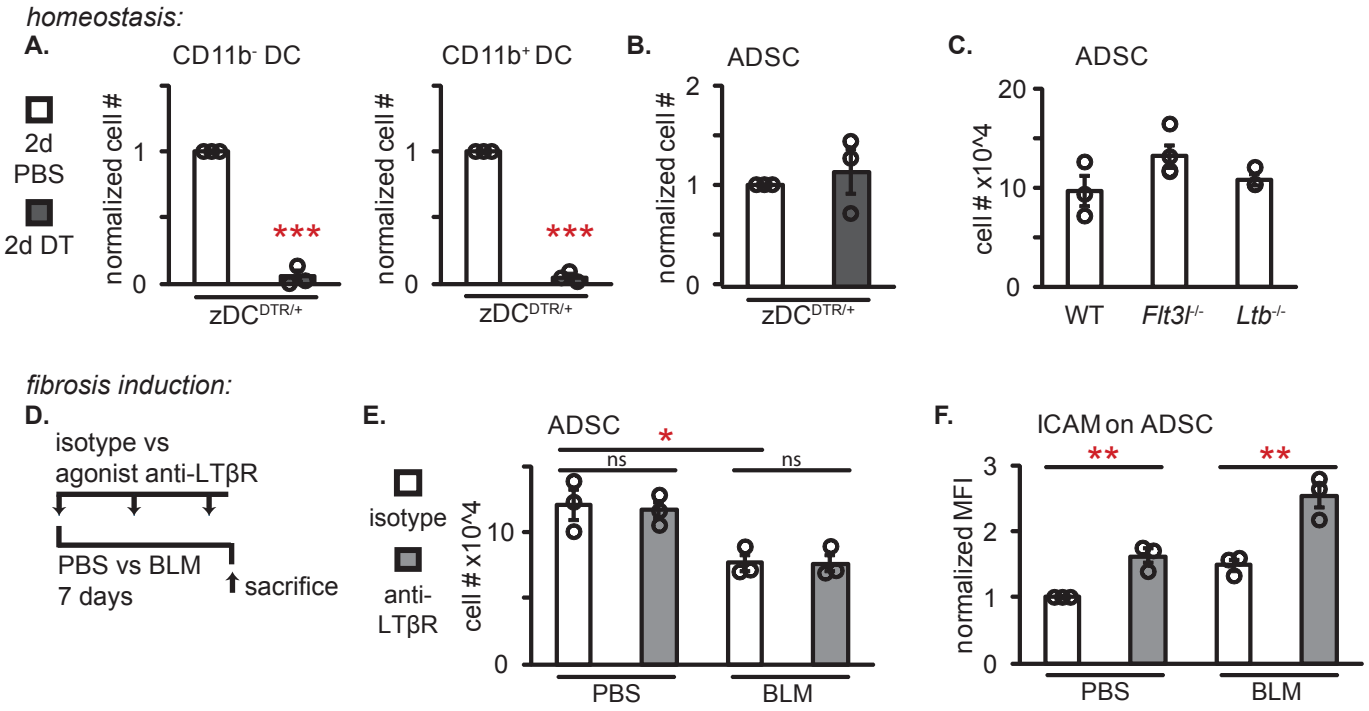
Supplemental Figure 4. Additional effects of DC depletion in fibrosis. (**A**) Experimental schematic for WT→WT chimeras (WT) or zDC$^{DTR/+}$→WT chimeras (zDC$^{DTR/+}$) in Figures 4A-D,H and Supplemental Figures 4B-C,L-O. (**B-C**) EpCAM$^+$ and EpCAM$^-$PDPN$^-$ cell numbers. Numbers are reported per 8mm punch. n=8 chimeras per condition over 5 experiments. (**D-K**) zDC$^{DTR/+}$ chimeras were treated with subcutaneous (sc) BLM over 22 days, with intraperitoneal (ip) PBS or DT for the final 2 days before analysis. (**D**) Percentage of skin ADSC that are Ki67$^+$. (**E**) (Left) Representative H&E stained sections and (right) number of adipocytes per high-powered field. Scale bars indicate 100µm. (**F**) SMA$^+$Sca1$^+$ cell numbers per punch. (**G**) Ly6C^{hi} monocyte numbers per µl blood. (**H**) pDC (identified as CD45$^+$B220$^+$CD11b$^-$CD11c^{med}Ly6C^{low}) per punch. (**D-H**) n=3 chimeras per condition over 3 experiments. (**I-K**) Enumeration of Tamoutounour populations (36) (**I**) P1, (**J**) P2, and (**K**) P3-5 per punch. n=9 chimeras per condition over 6 experiments. (**L**) ADSC numbers per fat pad normalized to PBS group. (**M**) Inguinal fat pad weight normalized to PBS group. (**L,M**) n=2 mice per condition over 2 experiments. (**N**) Animal weight at time of sacrifice. n=5-6 over at least 3 experiments. (**O**) Collagen content of skin. n=6 chimeras per condition over 4 experiments. (**P**) Experimental schematic for wound healing assay in Supplemental Figure 4Q. (**Q**) Percent open of wounds at day 14 relative to wound size at day 0 in experiments performed as in (**P**). n=8 wounds in 4 mice per condition in 2 experiments. (**R**) Experimental schematic for wound healing assay in Figures 4I-J. *p <0.05, **p <0.01, ***p <0.001 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Figure 5



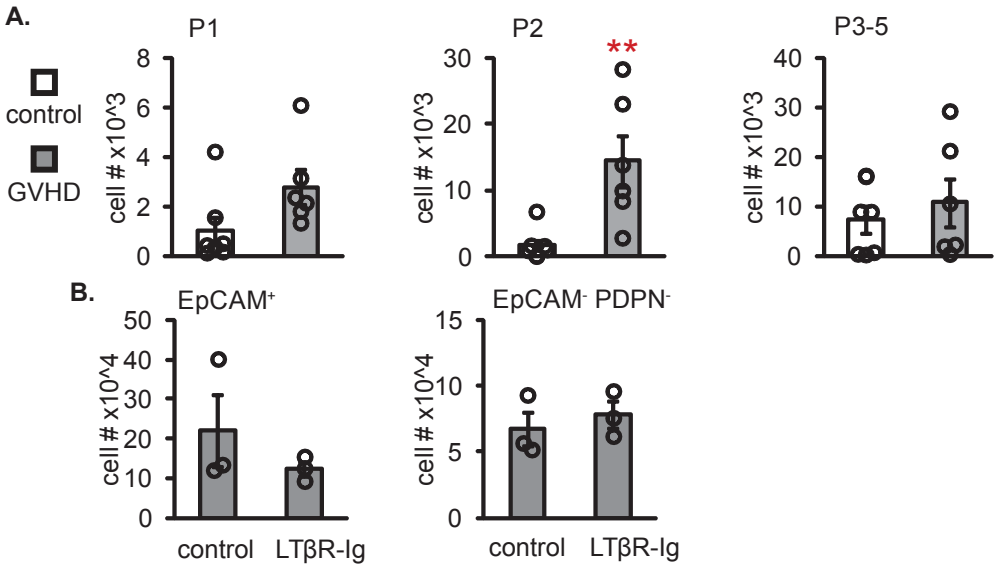
Supplemental Figure 5. zDC$^{DTR/+}$/WT→WT and zDC$^{DTR/+}$/*Ltb*$^{-/-}$→WT mixed chimera generation and mononuclear phagocyte populations, and ADSC features and mononuclear phagocyte populations upon DC depletion with anti-LTβR treatment. (**A**) Schematic for mixed chimeras used in Figure 5B-D, Supplemental Figure 5B. (**B**) Enumeration of Tamoutounour populations (36). P1, P2, and P3-5 per punch in the mixed chimeras. n=7-8 chimeras per condition over 6 experiments. (**C-E**) 100% zDC$^{DTR/+}$ chimeras were treated with BLM over 22 days, with PBS or DT for the final 2 days, and with 20μg anti-LTβR or isotype control 6 hours prior to the first dose of PBS or DT, as in Figure 5E-F. n=4 chimeras per condition over 4 experiments. (**C**) Geometric mean fluorescence intensity (MFI) of ICAM-1 on ADSC. (**D**) Percentage of ADSC that are Ki67^{+}. (**E**) Enumeration of Tamoutounour populations (36) P1, P2, and P3-5 per punch. (**F**) Geometric mean fluorescence intensity (MFI) of ICAM-1 on murine ASDC in vitro, treated as in Figure 5H. *p <0.05, **p <0.01 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Figure 6



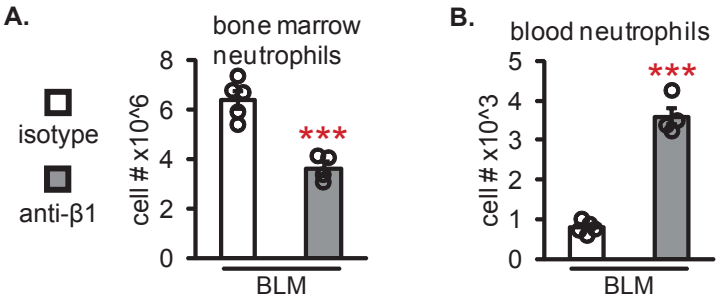
Supplemental Figure 6. DC signals are not necessary for ADSC maintenance in homeostatic skin, and LTβR stimulation does not prevent ADSC loss during fibrosis induction. (**A-B**) Homeostatic zDC$^{DTR/+}$ chimeras were treated with PBS or DT for 2 days before skin analysis. n=3 chimeras per condition over 3 experiments. (**A**) DC numbers per punch, normalized to PBS group. (**B**) Skin ADSC numbers per punch, normalized to PBS group. (**C**) ADSC numbers per punch in homeostatic skin of WT, *Flt3l*$^{-/-}$ or *Ltb*$^{-/-}$ mice. n=3-4 mice per genotype over 2 experiments. (**D-F**) Mice were treated PBS or BLM subcutaneously for 7 days before skin analysis, and with isotype or agonist anti-LTβR at the time of the 1st dose of BLM and then every 3 days. n=3 mice per condition over 3 experiments. (**D**) Experimental schematic. (**E**) ADSC numbers per punch. (**F**) Geometric mean fluorescence intensity (MFI) of ICAM-1 on ADSC. *p <0.05, **p <0.01, ***p <0.001 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Figure 7



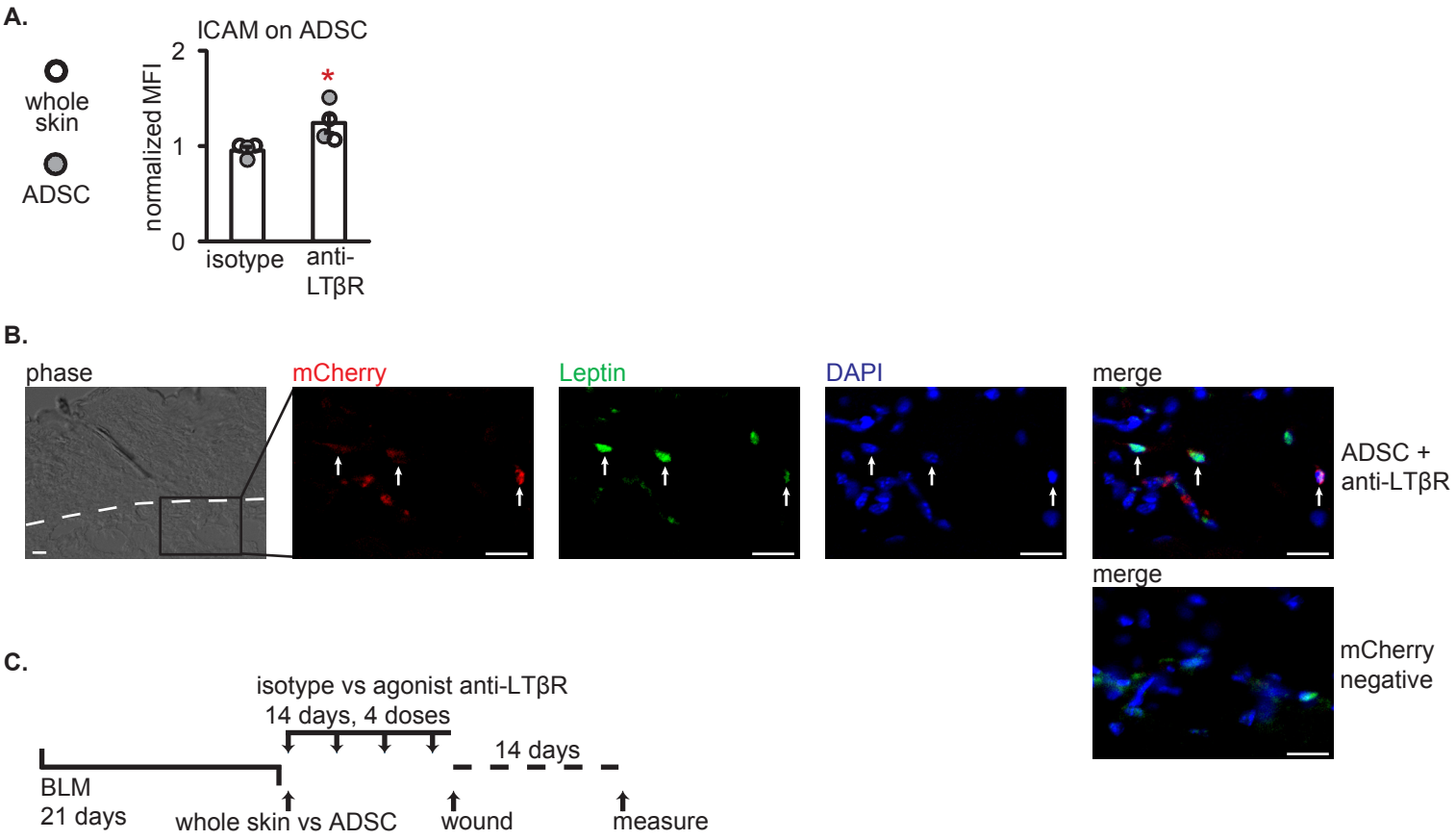
Supplemental Figure 7. Additional characterization of systemic sclerosis-GVHD fibrosis and effect of LTβR-Ig on EpCAM$^+$ and EpCAM$^-$PDPN$^-$ populations. Congenic (control) or B10.D2 (GVHD) splenocytes were adoptively transferred into Balb/C *Rag2*$^{-/-}$ hosts 22-23 days before animal sacrifice, as in Figure 6. Back skin was analyzed. (**A**) Enumeration of Tamoutounour mononuclear phagocyte populations (36) P1, P2, and P3-5 per punch. n=6-7 mice per group over 4 experiments. (**B**) Effect of LTβR-Ig on EpCAM$^+$ and EpCAM$^-$PDPN$^-$ population numbers per punch in systemic sclerosis-GVHD mice. Control or LTβR-Ig was given on day 20 after GVHD induction and animals were sacrificed on day 22, as in Figure 6F. n=3 mice per condition over 2 experiments. **p <0.01 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Figure 8



Supplemental Figure 8. β1 integrin neutralizing antibody is given at a dose that inhibits neutrophil retention in the bone marrow. Mice were treated with BLM over 22 days and with isotype control or neutralizing anti-β1 integrin antibody 6 hours before analysis, as in Figure 7A. (**A**) Bone marrow neutrophil number per femur. (**B**) Blood neutrophil number per µl blood. Neutrophils were identified as $CD3^{-}B220^{-}Ly6G^{+}Ly6C^{med}$. n=4-5 mice per condition over 2 experiments. **p <0.01, ***p <0.001 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Figure 9



Supplemental Figure 9. Features of ADSCs upon combined ADSC+anti-LTβR treatment, and schematic of associated wound healing assay. (**A-B**) Mice were treated as in Figure 8A-H. (**A**) Geometric mean fluorescence intensity (MFI) of ICAM-1 on total ADSCs. n=4 mice per antibody treatment condition over 2 experiments. Open circles indicate animals receiving whole skin cells, and shaded circles indicate animals receiving DWAT ADSCs. (**B**) Representative fixed frozen tissue sections stained for mCherry (red), leptin (green) and DAPI (blue). Arrows point to mCherry^{+} leptin^{+} nucleated cells. Scale bars indicate 20μm. n=3 mice over 3 experiments. (**C**) Experimental schematic for Figure 8I. *p <0.05 using two-tailed unpaired student's t test. Error bars depict S.E.M.

Supplemental Table 1. Statistics for gene expression changes between Cell+Antibody treatment groups. Value of two-tailed unpaired student's t test for gene expression changes depicted in Figure 8H, comparing the listed treatment condition to the whole skin+isotype condition. † identifies genes that changed with BLM treatment in Figure 4H. Underlined genes change in the opposite direction as with BLM treatment. Values p <0.05 are highlighted in red, p <0.01 in yellow and p <0.001 in green.

gene	whole skin + anti-LTβR	ADSC + isotype	ADSC + anti-LTβR
Mmp13	0.257	0.402	0.568
Lox	0.559	0.361	0.139
Ccl27a	0.549	0.856	0.980
Fmod	0.665	0.532	0.153
Ctgf	0.176	0.046	0.850
Spp1	0.965	0.416	0.473
Serpine1 †	0.677	0.373	0.023
Timp1 †	0.272	0.199	0.007
Vwf	0.999	0.056	0.514
Angpt2 †	0.294	0.060	0.019
Ccl5	0.085	0.411	0.373
Cxcl10 †	0.078	0.815	0.440
Mx2	0.909	0.830	0.411
Ccl2 †	0.548	0.453	0.00001
Oas1 †	0.821	0.030	0.615
Irf7 †	0.981	0.809	0.003
Wisp1 †	0.800	0.483	0.091
Sfrp2 †	0.451	0.203	0.075
Pparg †	0.735	0.714	0.706
Adipoq	0.485	0.323	0.549
Plin1 †	0.190	0.906	0.324

Supplemental methods

Mice

Flt3l$^{-/-}$ mice were from Taconic Farms and bred at our facility.

Mouse treatments

For anti-LTβR treatment during the first 7 days of BLM induction, three doses of 20μg were given by retro-orbital injection every 3rd day beginning at the time of first BLM or PBS injection.

PCR primer sequences

Flt3l

Forward GCAGGGTCTAAGATGCAAACG

Reverse ACGAATCGCAGACATTCTGGTA

Acan

Forward CCGCTTGCCAGGGGGAGTTG

Reverse CCTGCAGCCAGCCAGCATCA

Flt3 Ligand ELISA

One 8mm biopsy punch was incubated in 220μl DMEM (VWR) with 50 IU/ml Penicillin/Streptomycin and without FCS for 20 hours at 37°C. 100μl of supernatant was used for quantification in Flt3 Ligand ELISA (R&D Systems) according to the manufacturer's protocol.

Supplemental Reference

1. Jakubzick, C., Bogunovic, M., Bonito, A.J., Kuan, E.L., Merad, M., and Randolph, G.J. 2008. Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. *The Journal of Experimental Medicine* **205**:2839-2850.