Online supplemental material

Dendritic epidermal T cells regulate skin antimicrobial barrier function

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Supplementary data contains Methods, one supplementary table and five supplementary figures with figure legends.

Methods

Flow cytometry. Antibodies and appropriate IgG controls were conjugated to FITC, PE, PerCP-Cy5.5, Pe-Cy7 or APC. Antibodies to Vγ3 (536), TCRβ (H57-597), NK1.1 (PK136), CD62L (MEL-14), pSTAT3 (pY705), and pan-γδTCR (GL-3) were purchased from BD Bioscience. Thy1.2 (53-2.2), CD3 (17A2 and 145-2C11), CD4 (GK1.5), CD27 (LG.3A10), CD45RB (C363-16A), IL-7R (A7R34), CD122 (5H4), IL-17A (TC11-18H10.1), IL-22 (Poly5164), T-bet (4B10), and CCR6 (29-2L) antibodies were purchased from Biolegend. Antibodies to IL-17A (TC11-18H10.1), IFNγ (XMG 1.2), IL-17F (ebio18F10) and RORγt (AKFJS-9) were purchased from eBioscience. For intracellular cytokine staining, cells were fixed and permeabilized with cytofix and cytoperm reagents from BD Bioscience. For staining of RORγt, the FoxP3 staining buffer set was used (eBioscience). For staining pSTAT3, a Phosflow kit (BD Bioscience) was used. Cells were acquired with DiVa 5.0 software on a Digital LSRII or FACS Canto and analyzed with FlowJo software (Tree Star, Inc.).

Epidermal ear sheet and skin immunofluorescence. Epidermal ear sheets were prepared as previously described (1) and subsequently stained with FITC-conjugated antibodies to Vγ3 and DAPI (Sigma-Aldrich). Quantification of DETC numbers was performed using Image J software. For histological analysis, back skin wounds and surrounding skin were excised, placed in Tissue-Tek O.C.T. compound (Sakura), and stored at -80°C until use. 8μm-thick sections were cut, methanol- or paraformaldehyde-fixed and incubated in a blocking solution of PBS containing 2.5% normal goat serum (Jackson Immuno Research), 2.5% normal donkey serum (Jackson Immuno Research), 1% bovine serum albumin (Calbiochem), 2% fish gelatin (Sigma), and 0.1% Triton-X100 for 1 hour at room temperature or overnight at 4°C before incubation with primary antibodies overnight at 4°C. Antibodies recognizing Ki67 (Millipore) were used at 1:250, β-defensin 3 (Alpha Diagnostics) or RegIIIγ (Biorbyt) were used at 5μg/ml; antibodies recognizing S100A8 or IgG_{2B} (R&D Systems) were used at 8μg/ml. Appropriate IgG or blocking peptides were used as controls. Secondary antibodies were PE- or Cy3-conjugated anti-rabbit or Cy3-conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories) used at 1:200. After subsequent washing, sections were mounted with Prolong Gold antifade containing DAPI (Invitrogen). Sections were visualized using a Nikon Eclipse E800 microscope and digital images were acquired with a Zeiss AxioCam HRc camera.

In vivo wounding. 3mm punch biopsy wounds were made on the back skin of anesthetized C57BL/6, *Il17a*$^{-/-}$, and *Tcrd*$^{-/-}$ mice as previously described (2). For IL-17A FACS analysis, epidermal cells were isolated from skin areas immediately surrounding the wounds. Belly skin from the same mouse was used as non-wounded control skin.

Prior to intracellular IL-17A staining, cell suspensions were incubated in DMEM containing brefeldin A (10μg/ml) and monensin (1:1000) for 4 hours. Alternatively, for RNA analyses, DETC were isolated and FACS-sorted based on Thy1.2 and Vγ3 expression for DETC and Thy1.2 and TCRβ expression for epidermal T cells from TCR$\delta^{-/-}$ mice. For detection of AMP gene expression mice were wounded and epidermal cells from back or ear skin were separated by trypsin digestion as previously described (Jameson et al., 2002). For some experiments, 200ng recombinant IL-17A, 10μg monoclonal mouse IL-17A antibody (R&D) or IgG_{2a} (R&D), 500ng recombinant β-defensin 3 (Alpha Diagnostics), 70ng recombinant S100A8 (Creative Biomart) or vehicle controls were topically applied into the wound bed of skin wounds on the day of wounding while mice were anesthetized, unless otherwise indicated. Topical administration of neutralizing IL-17A antibody was repeated on day 2 post-wounding. To determine wound closure kinetics, wounds were photographed daily and wound areas were measured using Image J software.

Skin organ culture. Sections of skin that included a 3mm punch biopsy and approximately 10mm of surrounding non-wounded tissue wound were excised and placed on Gelfoam (Pfizer) in 500μl DMEM or RPMI-1640 (Gibco) with 10% FCS as previously described (2). Media was replaced every other day. In some experiments, 100ng/ml recombinant IL-17A (eBiocscience) or vehicle control were added to SOC on day 0 and day 2 post-wounding. In some experiments, freshly isolated and FACS-sorted WT or IL-17A-deficient DETC were added to SOC. The number of DETC added to SOC was based on the calculation that approximately 550-600 DETC populate 1mm^2 epidermis. Each SOC had an approximate size of 2.55 cm^2 and therefore, approximately 150,000 cells were added per SOC condition. DETC were pre-activated for 3-6 hours with plate-bound anti-CD3ε (10μg/ml) before adding into the wound bed. For IL-17A-neutralization experiments, activated DETC and their supernatants were preincubated for 30 minutes with anti-IL-17A antibody, before addition into the wound bed. The final concentration of neutralizing anti-IL-17A antibody per wound and per well was 100ng/ml. Wound closure kinetics were measured daily as described above.

RNA isolation and RT-PCR. Total RNA was isolated from cells using RNeasy Micro Kit (Qiagen) or from tissue using the TRIZOL reagent (Invitrogen). RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad) and resulting cDNA was amplified using FastStart Universal SYBR Green Master Mix (Roche). Primers for amplification are listed in Supplementary Table 1. Fold induction of gene expression was normalized to β-actin and calculated using the $2^{(-\Delta\Delta Ct)}$ method.

Figure Legends

Supplementary Figure 1. *Il17a*$^{-/-}$ mice have impaired keratinocyte proliferation during wound healing, but normal DETC numbers and DETC proliferative capacities

(A) Ki67 staining in WT and *Il17a*$^{-/-}$ skin wounds 3 days post-wounding. DAPI was used to visualize cell nuclei. Dotted white line represents the epidermal-dermal border. Scale bar: 50μm; e, eschar; *, wound edge (B and C) Vγ3^{+} DETC were quantified in epidermal ear sheets from WT and IL-17A$^{-/-}$ mice. (B) Epidermal ear sheets were stained for Vγ3 (green). Scale bar: 50 μm (C) Approximately 200 cells were counted for each epidermal sheet and numbers are expressed as DETC per mm^{2}. Data are presented as mean $\pm$ SEM from 4-5 epidermal ear sheets per genotype. (D) Freshly isolated and sorted Vγ3^{+}Thy1.2^{+} DETC were stimulated with anti-CD3ε (10μg/ml) and ^{3}H thymidine incorporation was measured. Data are presented as mean $\pm$ SEM from duplicates. (E) Effect of different doses of IL-17A on the wound healing response. Skin wounds from *Il17a*$^{-/-}$ mice were supplemented with rIL-17A or vehicle control. 2, 20, or 200ng rIL-17 was added into the wound beds on day 0 and day 2 post-wounding. Data are presented as mean $\pm$ SEM from 6-8 wounds.

Supplementary Figure 2. Cytokine and transcription factor expression in TCR-activated DETC

(A) DETC produce IL-17A but not IL-17F or IL-22. DETC were stimulated with anti-CD3ε (10μg/ml) for 24 hours and intracellular cytokine expression was measured by FACS. Data are representative of 3 independent experiments.

(B) Signal strength alters cytokine kinetics in DETC. Sorted Vγ3$^+$ Thy1.2$^+$ DETC short term cell lines were stimulated with 1 or 10μg/ml doses of anti-CD3ε and supernatants were harvested at indicated time points. IL-17A, TNFα and IFNγ secretion was assayed by ELISA. Data are shown as mean $\pm$ SEM from triplicate measurements and are representative of 3 independent experiments. ***P < 0.001; **P < 0.01; *P < 0.05 by two-way ANOVA. (C) DETC upregulate *Rorc* and *IFNγ* upon TCR stimulation. Quantitative real-time PCR on the expression of *Rorc*, *STAT3*, *Ahr, Tbet and IFNγ* in freshly isolated and sorted Vγ3$^+$Thy1.2$^+$ DETC stimulated with anti-CD3ε (10μg/ml) for 3 hours. The same cDNA was used as for quantification of IL-17A in Figure 2A. Data is presented as relative fold change compared to resting DETC. (D) DETC short term cell line or (E) freshly isolated DETC were stimulated with anti-CD3 (10μg/ml) and (D) pSTAT3 expression or (E) T-bet and IFNγ expression was measured by FACS at 3 or 24 hours, respectively. Cells are gated on Vγ3$^+$Thy1.2$^+$.

Supplementary Figure 3. Surface markers that are not expressed on DETC are detectable on splenocytes

(A) Splenocytes were stained for CD3, CD4, CD27, CD62L, and IL-7R expression. Cells are gated on CD3$^+$CD4$^+$. (B) Splenocytes were stained for CD3 and NK1.1. Gate displays NK1.1$^+$CD3$^-$ cells resembling NK cells.

Supplementary Figure 4. Effect of different doses of β-defensin 3 on the wound healing response

Skin wounds from *Il17a*$^{-/-}$ mice were supplemented with β-defensin 3 or vehicle control. 5, 50, or 500ng β-defensin 3 was added into the wound beds on day 0 and day 2 post-wounding. Data are presented as mean $\pm$ SEM from 6-8 wounds.

Supplementary Figure 5. IL-17A-sufficient DETC restore β-defensin 3 expression in *Il17*a$^{-/-}$ skin

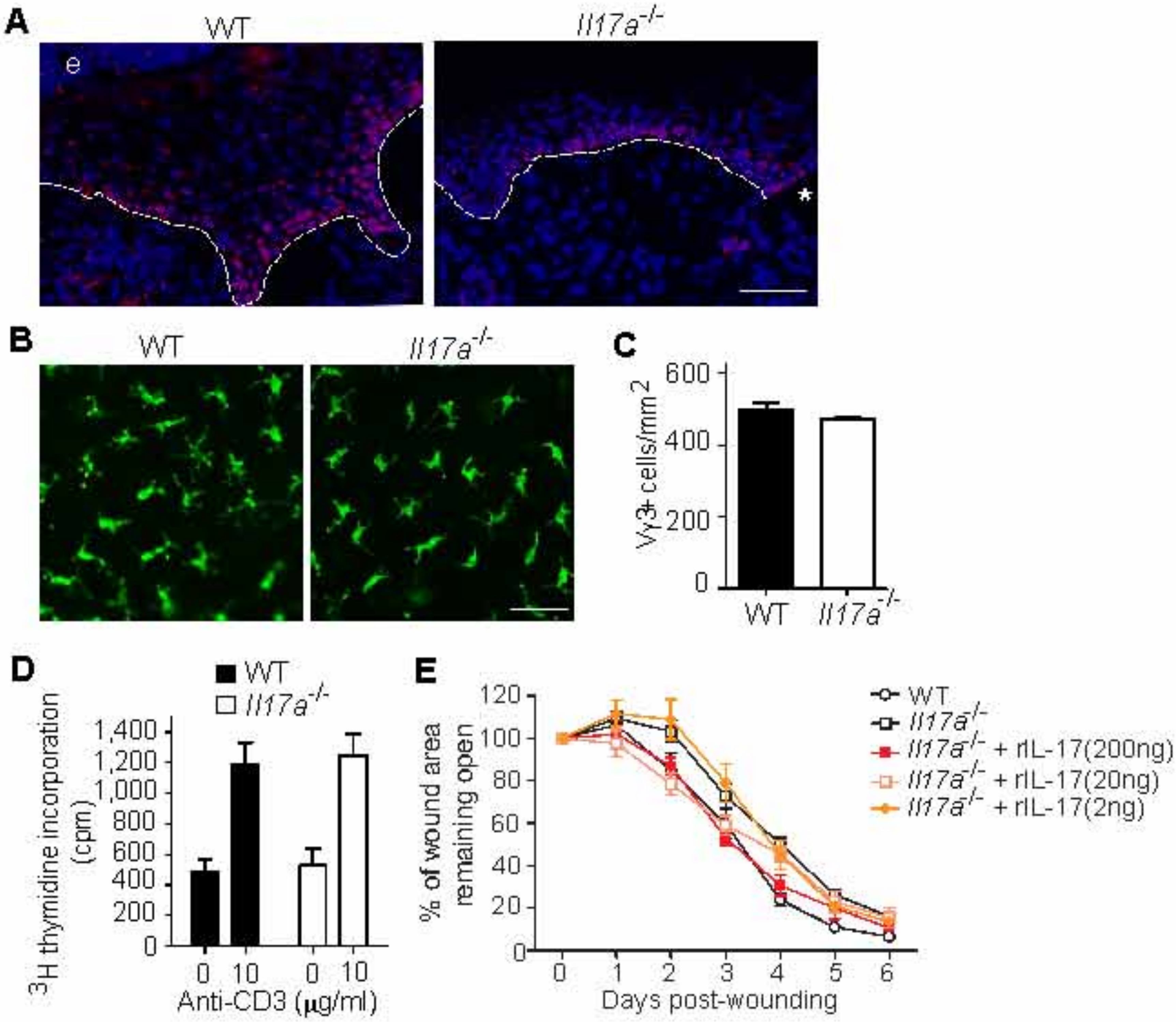
Expression of β-defensin 3 was analyzed 24 hour post-wounding by immunofluorescence staining (red). DAPI was used to visualize cell nuclei. Dotted white lines represent the epidermal-dermal border. Wounded skin organ culture from WT mice served as control (far left panel). Data are representative of 3 independent experiments with 1-2 wounds per condition in each experiment. Scale bar: 50μm.

Supplementary Table 1. PCR primers used for qPCR.

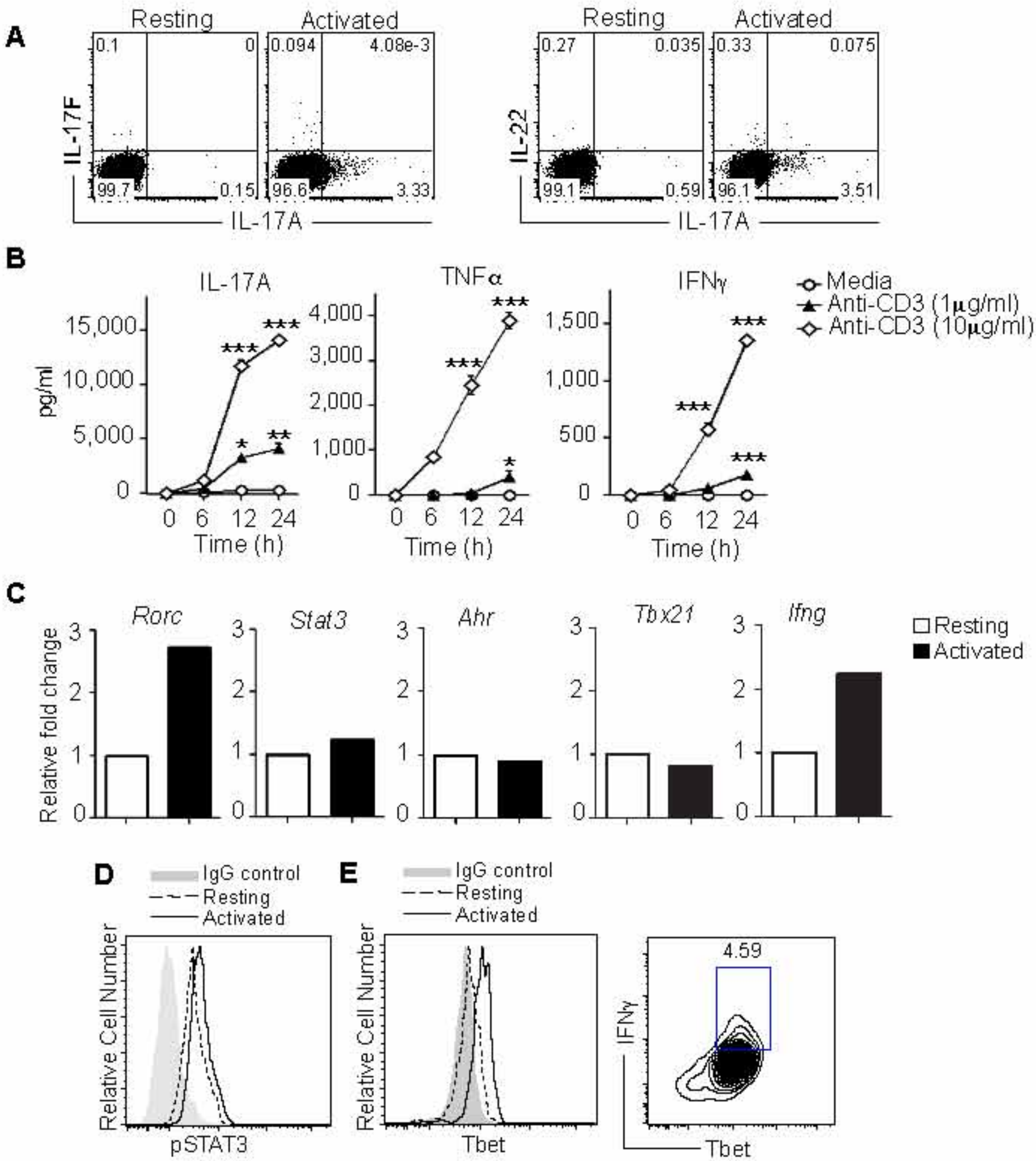
References

1. Tschachler E, Schuler G, Hutterer J, Leibl H, Wolff K, and Stingl G. Expression of Thy-1 antigen by murine epidermal cells. *J Invest Dermatol*. 1983;81(3):282-285.
2. Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, and Havran WL. A role for skin γδ T cells in wound repair. *Science*. 2002;296(5568):747-749.

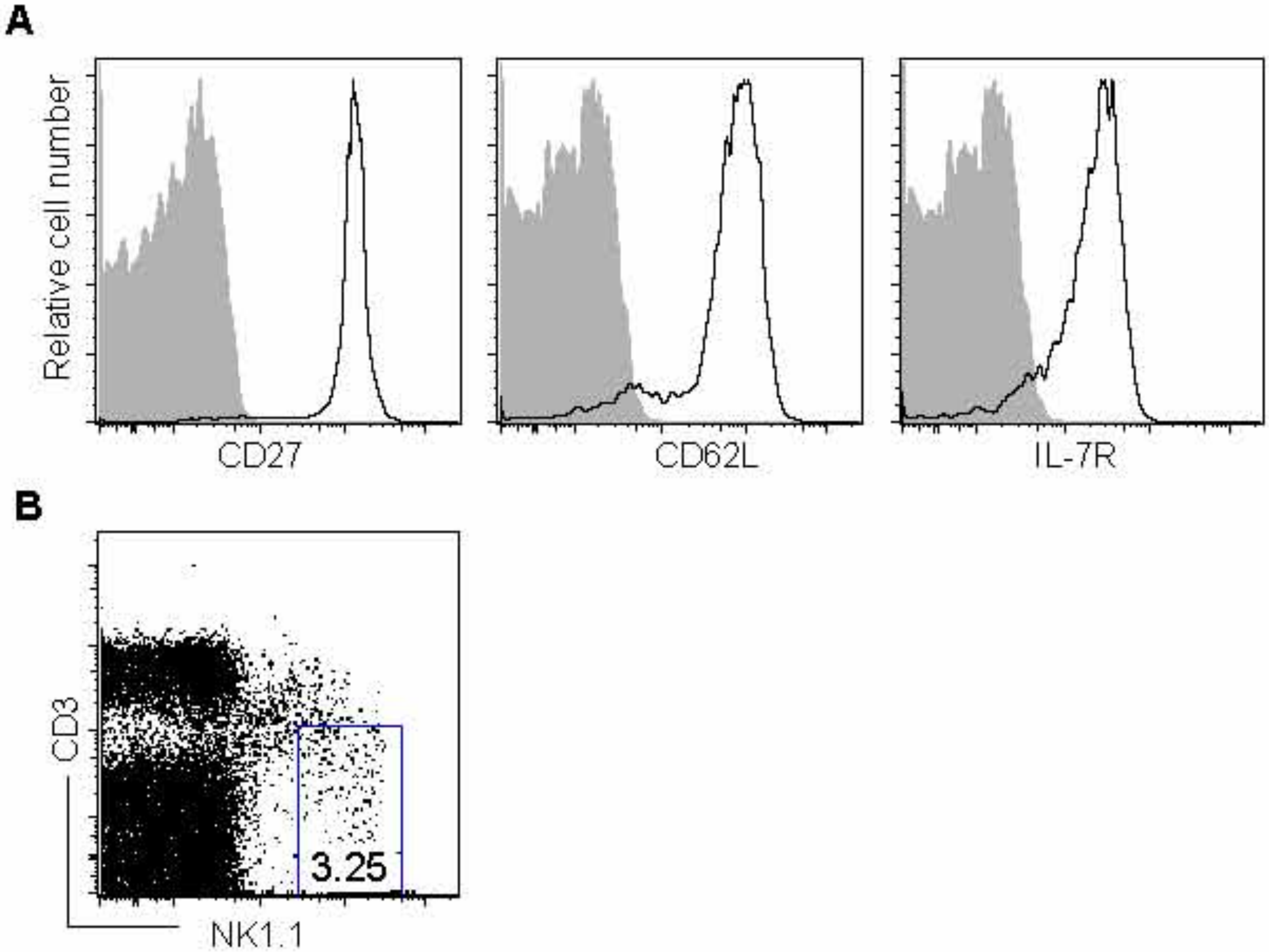
Supplementary Figure 1, MacLeod et al.



Supplementary Figure 2, MacLeod et al.



Supplementary Figure 3, MacLeod et al.



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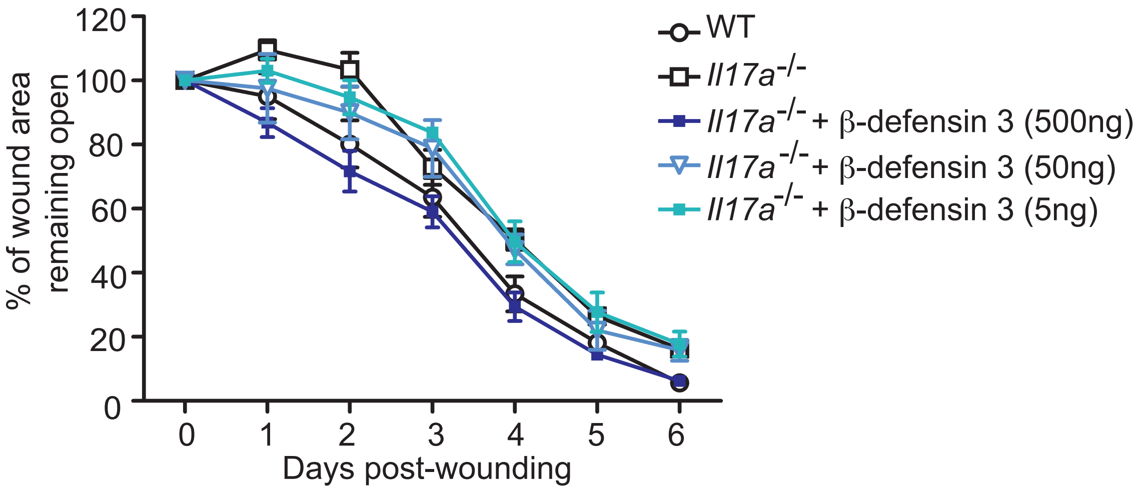
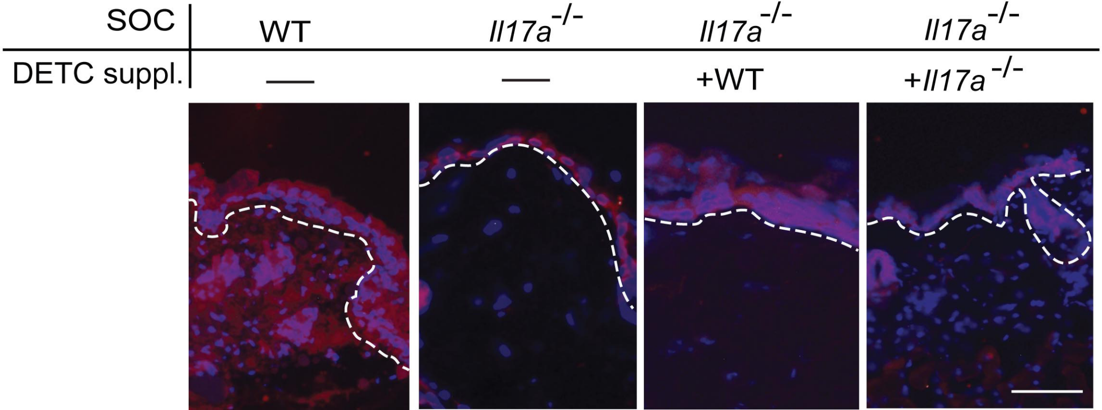


Supplementary Figure 5, MacLeod et al.



Supplementary Table 1, MacLeod et al.

Sequence name	Primers 5' to 3'
Il17a	F-tttaactcccttggcgcaaaa R-ctttccctccgcattgacac
Defb3	F-tctcctggtgctgctgtctc R- actccactgccaatctgac
Defb1	F-aggtgttggcattctcacaag R-gcttatctggtttacaggttccc
Defb2	F-tatgctgcctcctttctca R-gacttccatgtccttccttc
Reg3g	F-ttcctctccctccatgatcaaaa R-catccacctctgttgggttca
S100a8	F-ggaaatcaccatgccctctacaa R-atgccacacccactttatca
Rorc	F-acagccactgcattcccacttt R-tctcggaaggacttgcagacat
Ahr	F-tgcacaaggagtg R-aggaagctggtct
STAT3	F-acccaacagccgc R-cagactggttgtt
Ifng	F-cctttggaccctc R-agccaagatgcag
Tbx21	F-gccagggaaccgc R-gacgatcatctgg