Skin infection generates non-migratory memory CD8\(^+\) T\(_{RM}\) cells providing global skin immunity

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Protective T-cell memory has long been thought to reside in blood and lymph nodes, but recently the concept of immune memory in peripheral tissues mediated by resident memory T (T\(_{RM}\)) cells has been proposed\(^{1–6}\). Here we show in mice that localized vaccinia virus (VACV) skin infection generates long-lived non-recirculating CD8\(^+\) skin T\(_{RM}\) cells that reside within the entire skin. These skin T\(_{RM}\) cells are potent effector cells, and are superior to circulating CD4\(^+\), CD8\(^+\) T cells in non-involved skin. These findings have important implications for our understanding of protective immune memory at epithelial interfaces with the environment, and suggest novel strategies for vaccines that protect against tissue tropic organisms.

CD8\(^+\) T cells have a pivotal role in antiviral immunity in target tissues\(^{6–9}\). We infected the skin of control, CD4\(^{-/–}\), or CD4\(^+\) T-cell-depleted mice with VACV and assessed VACV-specific pentamer\(^\dagger\) CD8\(^+\) T cells\(^{10}\). Absence of CD4\(^+\) T cells did not impair either antigen-specific CD8\(^+\) T-cell proliferation in draining lymph nodes or subsequent accumulation in skin; in fact, the latter was enhanced (Fig. 1a, b). We then infected mice infused with OT-I (CD8\(^+\)) and OT-II (CD4\(^+\)) T cells with an ovalbumin-expressing VACV (VACV-Ova\(^\dagger\))\(^{11}\). OT-I T cells are a transgenic CD8\(^+\) T-cell population that recognize ovalbumin residues 257–264 in the context of H-2K\(^b\), whereas OT-II T cells are a transgenic CD4\(^+\) T-cell population recognizing ovalbumin residues 323–339 in the context of I-\(^A\)\(^b\) (ref. 1). After skin infection, both OT-I and OT-II cells proliferated similarly in draining lymph nodes, and OT-I cells but not OT-II cells accumulated infiltration of Thy1.1\(^+\) OT-I and CD45.1\(^+\) OT-II cells to infected skin after their co-transfer to naive mice. e–g. The numbers of OT-I cells in infected skin 7 days after infection in the absence of CD4, IFN-\(\gamma\) or FucT IV/VII, respectively. All data are representative of at least three independent experiments (\(n = 5\) mice per time point per group). b, d–g. Error bars show standard error of the mean (s.e.m.); **\(P < 0.01\); N.S., not significant.

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significantly in infected skin (although other CD4+ T cells showed some accumulation) (Supplementary Fig. 1g and 1h). Interestingly, OT-I cells accumulated in infected skin efficiently in the absence of either CD4+ T cells or interferon (IFN)-γ (Fig. 1e, f), in contrast to a recently reported herpes simplex virus (HSV) vaginal infection model 12. However, skin accumulation (but not lymph-node proliferation) of OT-I cells from FucT IV/VII−/− mice, which cannot make E- and P-selectin ligands, was significantly impaired (Fig. 1g and Supplementary Fig. 2a). Both E- and P-selectin were significantly upregulated in VACV-infected skin (Supplementary Fig. 2b). Thus, CD8+ T-cell accumulation in skin after VACV infection does not require CD4+ T cells or IFN-γ, but does require expression of E- and P-selectin ligands.

Murine models of viral infections of skin and other tissues have been useful in the study of T-cell memory 13–16. We explored the ability of CD8+ memory T cells, generated by VACV infection to recirculate after resolution of the cutaneous infection. We infected the skin of mice infused with OT-I cells with VACV, and waited until complete resolution of the infection (30 days). At 30 days we could identify TCM cells in lymph nodes and effector memory T (TEM) cells in skin (Supplementary Fig. 3a, b). We then surgically created parabiotic pairs between the infected mice and never-infected naive mice that had not been given OT-I cells. Parabiotic pairs were maintained for 2, 4, 8, 12 and 24 weeks, at which point they were surgically separated for the analysis of VACV-specific OT-I T cells (Fig. 2a). Mice joined for 8 weeks had similar numbers of OT-I TCM cells in the spleen and lymph nodes of both parabionts, indicating rapid recirculation and equilibration of TCM cells (Fig. 2b, d). However, at 2–24 weeks there were no OT-I TRM cells in the skin of the unimmunized parabiont (Fig. 2c, e). These early kinetics of TCM recirculation and TRM non-recirculation were confirmed by parabiotic mice that received no OT-I cells, using pentamer expression to identify VACV-specific memory cells (Supplementary Fig. 4). OT-I TRM cells were readily identified in the skin of previously infected parabionts and mice that had been infected in parallel but never joined. These OT-I TRM cells represented a significant fraction of total skin cells (Fig. 2c), and persisted for long periods of time: 30% of skin CD8+ T cells at 12 weeks, and 15–20% of skin CD8+ T cells at 24 weeks. In contrast, naive mice joined to previously infected mice had no OT-I TRM cells in the skin, even after 24 weeks of parabiosis (Fig. 2c, e). Thus, skin TCM cells persisted in skin for at least 6 months after infection and did not recirculate appreciably. TRM and TCM cells were further analysed by fluorescence-activated cells sorting (FACS) for expression of CD69, CD103, E- and P-selectin ligands, and production of IFN-γ and TNF-α upon activation. A subset of TCM cells expressed BCL2, and TCM cells lacked CD122 and CD127 (Supplementary Fig. 5). Immunofluorescence staining showed that many TCM cells localize in epidermal and follicular epithelium, as reported in skin HSV infection 17, but also localize in the dermis (Fig. 2f).

To compare directly the ability of TCM and TRM cells to eliminate a subsequent VACV infectious challenge, we infused μMT mice with OT-I cells and infected them through the skin with VACV-OVA. After 30 days, mice were joined parabiotically to naive μMT mice for 8 weeks, and then surgically separated. The infected parabiont contained

Figure 2 | CD8+ TCM cells recirculate quickly between parabiotic mice, but skin CD8+ TRM cells remain in place long term. a, 2 × 106 Thy1.1+ OT-I cells were intravenously transferred into Thy1.1+ OT-I cells and infected in parallel but never joined. These OT-I TRM cells represented a significant fraction of total skin cells (Fig. 2c), and persisted for long periods of time: 30% of skin CD8+ T cells at 12 weeks, and 15–20% of skin CD8+ T cells at 24 weeks. In contrast, naive mice joined to previously infected mice had no OT-I TRM cells in the skin, even after 24 weeks of parabiosis (Fig. 2c, e). Thus, skin TCM cells persisted in skin for at least 6 months after infection and did not recirculate appreciably. TRM and TCM cells were further analysed by fluorescence-activated cells sorting (FACS) for expression of CD69, CD103, E- and P-selectin ligands, and production of IFN-γ and TNF-α upon activation. A subset of TCM cells expressed BCL2, and TCM cells lacked CD122 and CD127 (Supplementary Fig. 5). Immunofluorescence staining showed that many TCM cells localize in epidermal and follicular epithelium, as reported in skin HSV infection 17, but also localize in the dermis (Fig. 2f).
T<sub>CM</sub> cells in both the spleen and lymph nodes and T<sub>RM</sub> cells within the skin, whereas the uninfected parabiont contained T<sub>CM</sub> cells only in the spleen and lymph nodes (Fig. 2). At 2 weeks after separation, the skin of these mice was challenged with VACV-Ova, and assessed 6 days later for viral load (Fig. 3a). Despite the presence of abundant circulating OT-I T<sub>CM</sub> cells, uninfected parabionts cleared the virus only 30-fold more effectively than naive mice (Fig. 3b). In contrast, the infected parabiont cleared the virus completely, 10<sup>4</sup>-fold more effectively than the uninfected parabiont (Fig. 3b). Viral clearance was efficient even in mice treated with FTY720, a S1P inhibitor that blocks egress of T<sub>CM</sub> cells from lymph nodes into blood (Fig. 3b). To show that this was not an artefact of OT-I cell transfer, we reproduced and extended the experiment in a parabiotic model not involving transfer of OT-I cells (Fig. 3c). Parabiotic pairs were separated at 4 weeks, challenged with VACV, and assessed for viral load at 6, 14 and 26 days after challenge. There were again striking differences in the ability of endogenous T<sub>RM</sub> mice without OT-I transfer but with VACV skin infection were used to create immunized:unimmunized parabiotic mice. Four weeks after surgery, the same VACV skin challenge protocol was applied. a, μMT mice with OT-I transfer and VACV-Ova skin infection were used to create OT-I:Normal parabiotic mice as described in Fig. 2. Eight weeks after surgery, parabiotic mice were separated. Two weeks later, separated mice were challenged with VACV-Ova on the skin. Half mice were injected daily with FTY720. b, Six days after challenge, skin viral load was assayed by quantitative polymerase chain reaction (qPCR). ctl, control; immu., immunized; nor., normal; para., parabiont. c, In separate experiments, μMT mice without OT-I transfer but with VACV skin infection were used to create immunized:unimmunized parabiotic mice. Four weeks after surgery, the same VACV skin challenge protocol was applied. d-f, Six, fourteen or twenty-six days after challenge, viral load was assayed. The results from duplicate qPCR runs are plotted. Horizontal bars indicate the mean. Data are representative of two independent experiments (n = 5 mice per group). **P < 0.01; N.S., not significant; N.D., not detectable.

Figure 3 | Skin CD8<sup>+</sup> T<sub>RM</sub> cells are superior to T<sub>CM</sub> cells at protecting against re-infection. a, μMT mice with OT-I transfer and VACV-Ova skin infection were used to create OT-I:Normal parabiotic mice as described in Fig. 2. Eight weeks after surgery, parabiotic mice were separated. Two weeks later, separated mice were challenged with VACV-Ova on the skin. Half mice were injected daily with FTY720. b, Six days after challenge, skin viral load was assayed by quantitative polymerase chain reaction (qPCR). ctl, control; immu., immunized; nor., normal; para., parabiont. c, In separate experiments, μMT mice without OT-I transfer but with VACV skin infection were used to create immunized:unimmunized parabiotic mice. Four weeks after surgery, the same VACV skin challenge protocol was applied. d-f, Six, fourteen or twenty-six days after challenge, viral load was assayed. The results from duplicate qPCR runs are plotted. Horizontal bars indicate the mean. Data are representative of two independent experiments (n = 5 mice per group). **P < 0.01; N.S., not significant; N.D., not detectable.
and T_{CM} cells to mediate viral clearance at days 6 (Fig. 3d) and 14 (Fig. 3e), which began to normalize by day 26 (Fig. 3f). However, FTY720-treated T_{CM} mice (the parabiont containing T_{CM} cells but no T_{RM} cells) are unable to clear virus even 26 days after infection, a time point by which naive mice have cleared the skin infection (Fig. 3f), presumably by generating protective T_{RM} cells. Thus, although T_{CM} cells are superior to naive T cells, they are inferior to T_{RM} cells at mediating rapid viral clearance from skin.

E- and P-selectin, CCL17 and ICAM1 are expressed constitutively on the blood vessels of normal skin and can support entry into uninflamed skin of T_{EM} cells via VCAM1-mediated VACV infection. To study this phenomenon, we infected the left ears of OT-I-loaded mice with VACV-Ova and then measured the accumulation of OT-I cells in both infected (left) and uninfected (right) ears. There was measurable accumulation of OT-I cells in both ears, with similar kinetics. The absolute number of OT-I cells was always higher in the infected ear, but even 30 days after infection, OT-I cells represented a measurable fraction of all cells present in the uninfected ear (Fig. 4a). Thus, VACV skin infection generates CD8^{+} T_{RM} cells that distribute to distant skin sites as well as the site of infection. The accumulation of OT-I T_{RM} cells in distant skin sites is increased further after multiple sequential infections to other sites of skin (Fig. 4b–d), suggesting that skin T_{RM} cells continue to accumulate throughout skin in response to repeated cutaneous infections at distant sites.

To determine if CD8^{+} T_{RM} cells in distant skin sites were as protective as those at previously infected sites, we challenged μMT mice previously infected on one ear with a second VACV skin infection on both ears either 7 or 30 days after the initial infection. FTY720 was administered to limit the contribution of T_{CM} cells (Fig. 4e). Notably, at both day 7 and day 30, T_{RM} cells in distant skin sites markedly reduced viral loads to levels comparable to those observed at the actual site of previous infection, indicating that these distant T_{RM} cells were highly effective at rapidly eliminating virus. In contrast, viral loads in the skin of intraperitoneally (i.p.) immunized mice were between 10^5 and 10^6 higher at these time points (Fig. 4f–g). Thus, skin infection with VACV generates populations of T_{EM} cells that distribute to the entire skin surface, become T_{RM} cells, and mediate protection of the skin against re-infection with VACV in the absence of antibodies or T_{CM} cells.

T_{RM} cells have now been identified in the skin, gut, lung and brain in murine models and human subjects in both health and in the setting of skin disease. We demonstrate that after VACV viral infection through the skin, CD8^{+} T_{RM} cells are generated and distribute not only to the site of infection but also throughout the entire skin surface. These CD8^{+} T_{RM} cells produce effector cytokines, persist for many months, and are highly effective at rapidly controlling subsequent VACV skin infection. T_{RM} cells were orders of magnitude more effective than T_{CM} cells at controlling viral re-infection of the skin, at all time points examined in this study. The use of parabiotic mice allowed us to examine rigorously the tissue distribution and relative roles of T_{CM} and T_{RM} cells in VACV immune responses. The relatively minor role of CD4^{+} T cells in VACV skin infection may reflect differences in immune responses to different viruses and/or infection of different tissues, as CD4^{+} T cells are clearly more important in HSV infection. Moreover, CD4^{+} T_{RM} cells predominately in human skin and lung, and are enriched for memory cells specific for pathogens encountered through those tissues.

Pathogens typically invade the host through epithelial interfaces with the environment. Our studies suggest that T_{EM} cells generated as a result of epithelial tissue infections accumulate as T_{RM} cells at both sites of infection as well as at distant sites within the same epithelial tissue, providing broad and long-lived protective T-cell immunity against
re-infection. A more complete understanding of TRM-cell-mediated immune memory should enhance our understanding of adaptive immunological memory, influence rational vaccine design, and illuminate the pathophysiology of human T-cell-mediated diseases.

METHODS SUMMARY

VACV skin infection. 2 × 10^6 p.f.u. of recombinant VACV Western Reserve strain or VACV expressing full-length ovalbumin (VACV-Ova) was used for epistemic interference with skin scarification or i.p. injection, as described previously. Parabiotic mice. Well-matched (sex and age) mouse partners were anaesthetized to full muscle relaxation with ketamine and xylazine (10 mg kg^-1) or 2.5% avertin (15 μg g^-1) by i.p. injection. The corresponding lateral aspects of mice were shaved and the excess hair was wiped off with alcohol prep pad. The disinfection was performed by wiping with betadine solution and 70% ethanol three times. Matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about 0.5 cm of free skin. The olecranon and knee joints were attached by a single 5-0 silk suture and tie, and the dorsal and venal skins were approximated by staples or continuous suture. Betadine solution was used to cover the full length of the dorsal and venal incision. The mice were then kept on heating pads and continuously monitored until recovery. For analgesic treatment, mice were injected subcutaneously with 2.5 μg kg^-1 flunixin every 8–12 h for 48 h after the operation.

Cell isolation from skin. Before harvest, skin hair was removed using Nair Hair Remover. Skin samples were then chopped into small fragments and incubated in Hanks balanced salt solution (HBSS) supplemented with 1 mg ml^-1 collagenase A (Roche) and 40 μg ml^-1 DNase I (Roche) at 37 °C for 30 min. After filtrating through a 70-μm nylon cell strainer, cells were collected and washed thoroughly with cold PBS before staining.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions X.J. and T.S.K. designed research; X.J. performed research; LL helped to establish the VACV skin scarification model; A.J.W. helped to create parabiotic mice; X.J., R.A.C., R.C.F., L.L. and T.S.K. analysed data; and X.J., R.A.C. and T.S.K. wrote the paper.

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METHODS

Mice. C57BL/6, CD4−/−, IFN-γ−/− and μMT mice were purchased from The Jackson Laboratory. Thy.1.1 Rag−/− OT-I, CD45.1+ OT-II, FucT IV/VII−/− mice were housed at the animal facility of Harvard Institute of Medicine, Harvard Medical School. Thy.1.1 Rag−/− OT-I mice were crossed with FucT IV/VII−/− mice to yield Thy.1.1+ FucT IV/VII−/− OT-I mice. OT-I and OT-II are T-cell-receptor-transgenic mice recognizing chicken ovalbumin residues 257–264 in the context of H-2Kd and 323–339 in the context of I-Ak, respectively. Animal experiments were performed in accordance with the guidelines set out by the Center for Animal Resources and Comparative Medicine at Harvard Medical School.

Viruses and infections. Recombinant VACV (Western Reserve strain) and OT-I, CD45.1+ and IL-2 staining was performed using Intracellular Cytokine Detection Kits (BD (MP6-XT22), IL-2 (JES6-5H4).

Parabiosis surgery was performed as described elsewhere

Total RNA was extracted from OT-II, FucT IV/VII−/− and u(XMG1.2), TNF-α pentamers were, The following anti-mouse antibodies were

OT-I mice were crossed with FucT IV/VII−/− and 323–339 in the context of I-Ak, respectively. Animal experiments were performed in accordance with the guidelines set out by the Center for Animal Resources and Comparative Medicine at Harvard Medical School.

Parabiotic mice. Parabiosis surgery was performed as described elsewhere with some modifications. Briefly, sex- and age-matched mouse partners were anesthetized to full muscle relaxation with ketamine and xylazine (10 µg/g) or with 2.5% avertin (15 µg/g) by i.p. injection. The corresponding lateral aspects of mice were shaved and the excess hair was wiped off with alcohol prep pad. After skin disinfection by wiping with betadine solution and 70% ethanol three times, two matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about 0.5 cm of free skin. The olecranon and knee joints were attached by a single 5-0 silk suture and tie, and the dorsal and ventral skins were approximated by staples or continuous suture. Betadine solution was used to cover the full length of the dorsal and ventral incision. The mice were then kept on heating pads and continuously monitored until recovery. 2.5 µg g−1 flunixin was used for analgesic treatment by subcutaneous injection every 12–24 h for 48 h after the operation. After an interval of the indicated weeks, parabiotic mice were surgically separated by a reversal of the above procedure for the next experiments.

Adoptive transfer and T-cell depletion. Lymph nodes were collected from the naive female Thy.1.1+ Rag−/− OT-I, CD45.1+ OT-II, or Thy.1.1+ FucT IV/VII−/− OT-I mice at the age of 6–8 weeks. OT-I or OT-II cells were purified by magnetic cell sorting using mouse CD8z or CD4z T-cell isolation kit (Miltenyi Biotec), respectively. 2 × 106 isolated OT-I and/or OT-II cells were then intravenously transferred to female recipient mice. In some experiments, OT-I and OT-II cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) before co-transfer. To deplete CD4z T cells in vivo, the recipient mice were injected i.p. with 500 µg anti-CD4 (GK1.5) in 100 µl PBS 4 days and 1 day before and on day 2 and 5 after infection.

Preparation of cell suspensions. Lymph nodes and spleen were harvested and mashed through a 70-µm nylon cell strainer to prepare cell suspensions. Red blood cells were lysed using lysing buffer. Skin tissue was removed after hair removal, chopped into small fragments and incubated in Hanks balanced salt solution (HBSS) supplemented with 1 mg ml−1 collagenase A and 40 µg ml−1 DNase I at 37 °C for 30 min. After filtering through a 70-µm nylon cell strainer, cells were collected and washed thoroughly with cold PBS before staining.

Intracellular cytokine detection. The infected skin from memory OT-I-bearing mice was harvested at 35 days after infection, and single-cell suspensions were prepared as described above. In some cases, 2 months after skin scarification with VACV-Ova, mice transferred with 2 × 106 OT-I cells were i.p. challenged with VACV-Ova. Five days after challenge, splenocytes were prepared. Red blood cells were lysed using lysing buffer. Cells were then incubated with 2 µg ml−1 SINEKFL peptide of ovabulmin at the presence of Brefeldin A for 7 h. Fc receptors were blocked with CD16/CD32 monoclonal antibodies and intracellular IFN-γ, TNF-α and IL-2 staining was performed using Intracellular Cytokine Detection Kits (BD Bioscience) before flow cytometry.

Determination of viral load. Mice were challenged with 2 × 105 p.f.u. VACV or VACV-Ova on the skin. In some cases, mice were simultaneously injected (i.p.) with 1 µg g−1 FT7200 each day. At indicated time points, viral load of skin was examined by quantitative real-time PCR, as described previously.

Isolation of mRNA and real-time PCR. Total RNA was extracted from homogenized skin tissue and cDNA was generated with iScript cDNA synthesis kit (Bio-Rad). Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad) was used with the following settings: 45 cycles of 15 s of denaturation at 95 °C, and 1 min of primer annealing and elongation at 60 °C. Real-time PCR was done with 1 µl cDNA plus 12.5 µl of 2× QiQ SYBR Green Mix (Bio-Rad) and 0.5 µl (10 µM) specific primers: mouse E-selectin 1 (5′-GGACACCAACATTCCTGGCCTGCT-3′) and mouse E-selectin 2 (5′-TGGAGAGAGATGCAGGCTCAGAGCCACAGCTG-3′); mouse P-selectin 1 (5′-AAGATGCTGCTGCACTGACCGAG-3′) and mouse P-selectin 2 (5′-CAAGAGGGCTGACCCGAGTCT-3′); mouse β-actin 1 (5′-CA TTGCTGACAGATGCAAGG-3′) and mouse β-actin 2 (5′-CGTCTGGAAGTTGACAGTGACG-3′). All samples were run in duplicate and fold change of gene expression was calculated using the reference sample (naive skin).

Antibodies and flow cytometry. The following anti-mouse antibodies were obtained from BD PharMingen: CD8α (53-6.7), Thy.1.1 (OX-7), CD4 (L3T4), CD45.1 (A20), CD19 (1D3), CD16/CD32 (2.4G2), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD103 (M290), CD122 (TM-Beta 1), BCL2 (A19-3), Aβ (D1243), IFN-γ (XMG1.2), TNF-α (MP6-XT22), IL-2 (JES6-5H4). Fluorescence-conjugated anti-mouse CD127 (A7R34) and PD-1 (RMP1-30) were purchased from eBioscience. PE-conjugated BBR10.2/II-2H2k pentamers were obtained from Prolimmune Ltd and pentamer+ CD8z T-cell staining was performed according to the protocol provided by the company. E- or P-selectin ligand expression was examined by incubating cells with rmE-Selectin/Fc Chimera or rmP-Selectin/Fc Chimera (R&D System) in conjunction with APC-conjugated F(ab’)2 fragments of goat anti-human IgG (c) antibody (Jackson Immuno-research). Dead cells were excluded using 7-AAD staining. Data were analysed on FACSCanTo Flow Cytometer using FACSData software.

Immunoﬂuorescence microscopy. One centimetre of tail containing the infected skin was cut into small pieces, homogenized skin tissue and cDNA was generated with iScript cDNA synthesis kit (Bio-Rad). Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad) was used with the following settings: 45 cycles of 15 s of denaturation at 95 °C, and 1 min of primer annealing and elongation at 60 °C. Real-time PCR was done with 1 µl cDNA plus 12.5 µl of 2× QiQ SYBR Green Mix (Bio-Rad) and 0.5 µl (10 µM) specific primers: mouse E-selectin 1 (5′-GGACACCAACATTCCTGGCCTGCT-3′) and mouse E-selectin 2 (5′-TGGAGAGAGATGCAGGCTCAGAGCCACAGCTG-3′); mouse P-selectin 1 (5′-AAGATGCTGCTGCACTGACCGAG-3′) and mouse P-selectin 2 (5′-CAAGAGGGCTGACCCGAGTCT-3′); mouse β-actin 1 (5′-CA TTGCTGACAGATGCAAGG-3′) and mouse β-actin 2 (5′-CGTCTGGAAGTTGACAGTGACG-3′). All samples were run in duplicate and fold change of gene expression was calculated using the reference sample (naive skin).

Statistical analysis. Statistical significance in values between experimental groups was determined by one-way analysis of variance (ANOVA) followed by Tukey post-test. P < 0.05 was considered statistically significant.