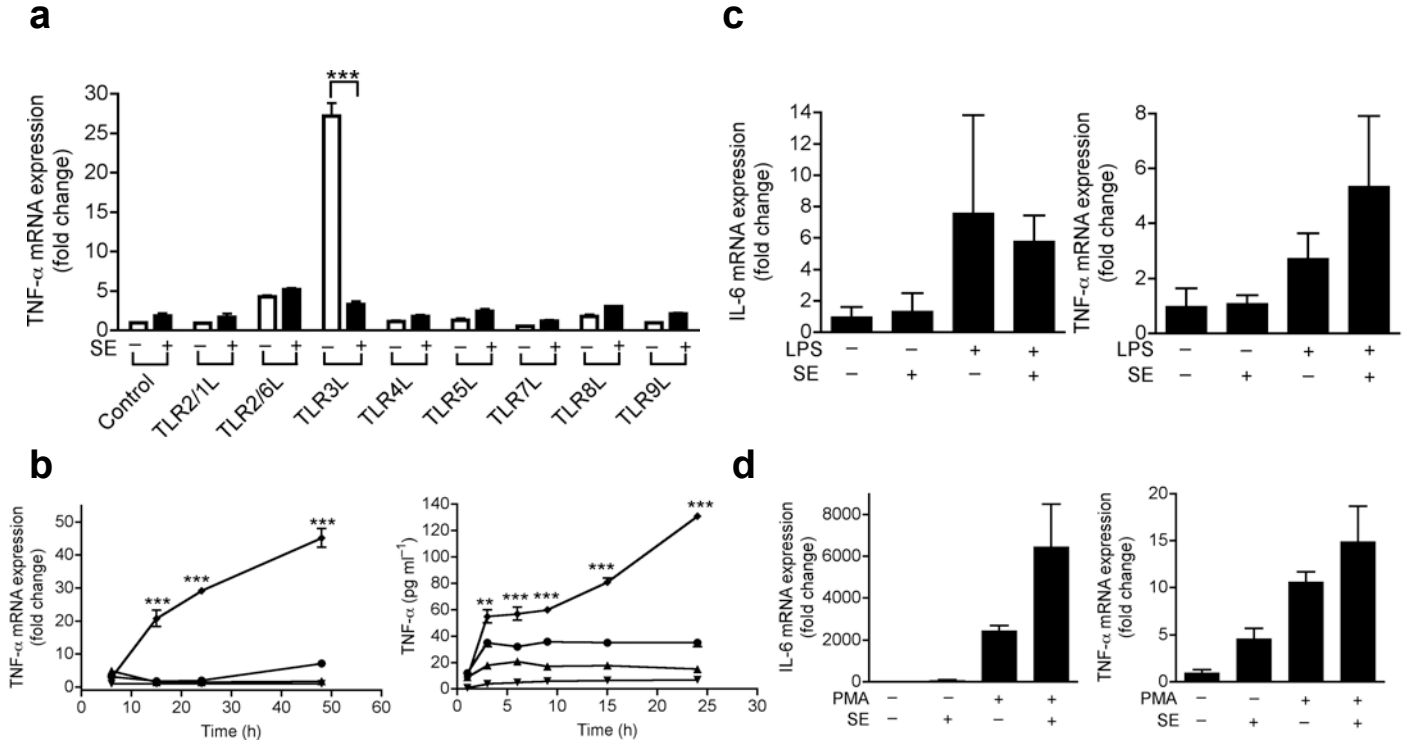# Supplementary Information

Commensal bacteria regulate TLR3-dependent inflammation following skin injury

Yuping Lai, Anna Di Nardo,Teruaki Nakatsuji, Anke Leichtle, Yan Yang, Anna L. Cogen, Zi-Rong Wu, Lora V. Hooper, Sonja von Aulock, Katherine A. Radek, Chun-Ming Huang, Allen F. Ryan, Richard L. Gallo
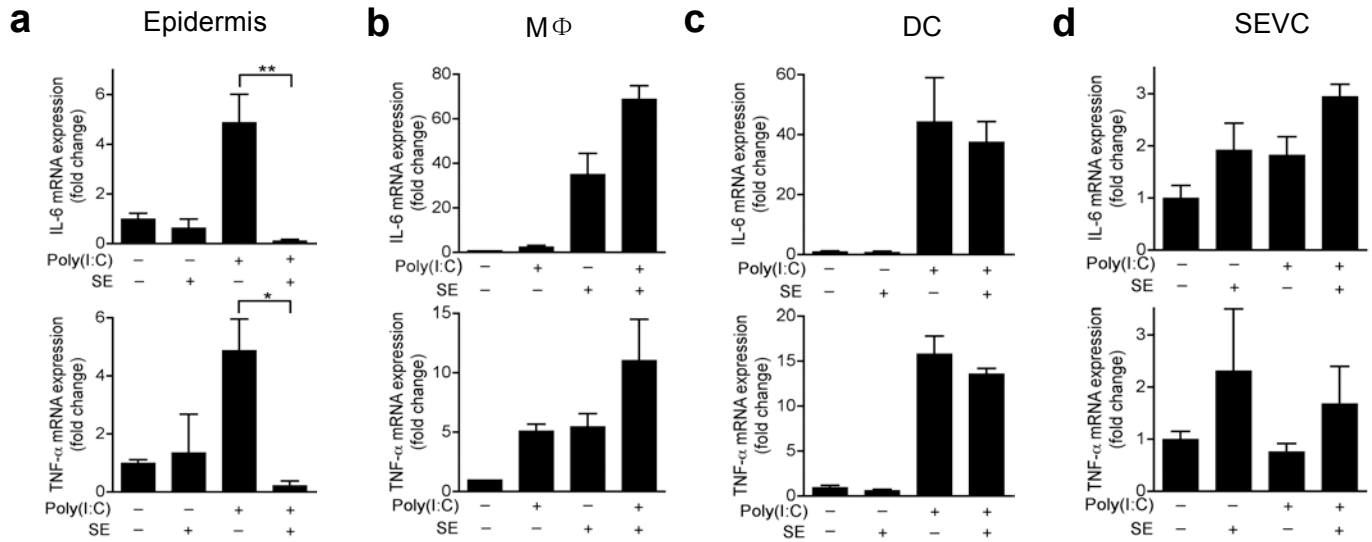
**Supplementary Figure 1. Staphylococci suppress inflammatory cytokines induced by TLR3 ligand but not by LPS or PMA. (a**) A sterile <10 kDa product of *S. epidermis* conditioned culture media (SE) suppressed TLR3 ligand-induced TNF-α, but not TLR2/6 ligand-induced TNF-α in cultured human keratinocytes. White bar: without SE; Black bar: with SE. The ligands used here and their concentrations are TLR2/1L: Pam3CSK4, 1 μg ml^{-1}; TLR2/6L: Malp-2**,** 100 ng ml^{-1}; TLR3L: poly(I:C), 10 μg ml^{-1}; TLR4L: LPS, 100 ng ml^{-1}; TLR5L: Flagellin, 50 ng ml^{-1}; TLR7L: imiquimod, 10 μg ml^{-1}; TLR8L: sspolyU/LyoVec, 25 μg ml^{-1}; TLR9L: CpG, 10 μg ml^{-1}. (**b**) In cultured human keratinocytes SE suppressed poly(I:C)-induced TNF-α mRNA and protein release in time-dependent manner. *Triangle*-control; *inverted triangle*- SE; *diamond*- poly(I:C); *circle*- poly(I:C) plus SE. (**c**) SE failed to suppress LPS-induced IL-6 and TNF-α in mice ears. Mice ears were preinjected by 20 μL of SE (12 μg) 2 h prior to injection of 20 μL of LPS (20 μg). After 24 h, ears were taken and RNA was isolated for real-time RT-PCR. (**d**) SE was unable to suppress PMA-induced IL-6 and TNF-α in mice ears. Mice ears were preinjected by 20 μL of SE (12 μg) 2 h prior to topically application of 20 μL of 1 mg ml^{-1} PMA. After 6 h, ears were taken and RNA was isolated for real-time RT-PCR. (**a**), *** P <0.001. *P*-value was determined by Two-tailed t tests. (**b**), ** P <0.01 and *** P <0.001. *P*-values were evaluated by Two-way ANOVA. Error bars represent SEM. Data shown are representative of three independent experiments with *n* = 3–6 per group.
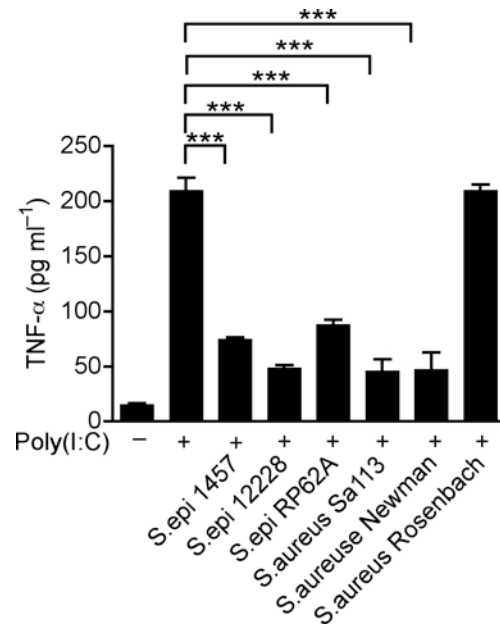
Supplementary Figure 2. Multiple staphylococcal strains suppress poly(I:C)-induced TNF-α in cultured human keratinocytes. Less than 10 kDa sterile products from multiple staphylococcal strains were added to cultured human keratinocytes that were treated with 10 μg ml^{-1} poly(I:C). After 24 h, media were collected for ELISA assay. *** $P < 0.001$. *P*-values were analyzed by One-way ANOVA. Data are the mean ± SEM of triplicate cultures and are representative of two independent experiments.

Supplementary Figure 3. *Staphylococcus epidermidis* **suppresses poly(I:C)-induced IL-6 and TNF-α in mouse epidermis, but does not suppress these cytokines in mouse dermal cells.** (**a**) SE suppressed poly(I:C)-induced IL-6 and TNF-α in intact mouse epidermis sheets. (**b**) The expression of IL-6 and TNF-α increased in response to poly(I:C) in mouse macrophages (MΦ), but SE was not able to suppress these poly(I:C)-induced proinflammatory cytokines. (**c**) SE failed to suppress poly(I:C)-induced IL-6 and TNF-α in mouse dendritic cells (DC). (**d**) Mouse endothelial cells (SEVC) failed to response to poly(I:C). * P <0.05 and ** P <0.01. P-values were determined by Two-tailed t tests. Data are the mean ± SEM of triplicate cultures and are representative of two independent experiments.
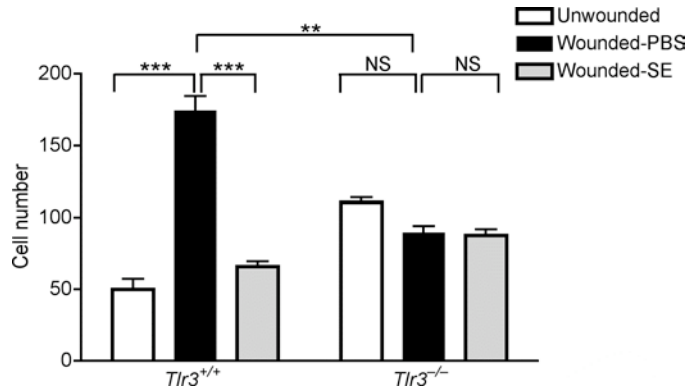
Supplementary Figure 4. Leukocyte infiltration in upper dermis at wound edge. Inflammatory cell infiltrate measured in *Tlr3*-deficient and wild-type mouse skin treated as in Figure 2a. Data shown are mean ± SD cell number per high power field in three adjacent sections. In wild-type mice the infiltrated leukocytes in upper dermis was significantly higher in wounded skin pretreated with PBS compared to leukocytes in wounded mouse skin pretreated with SE. In *Tlr3*-deficient mice leukocytes in upper dermis of wounded skin were less than wild-type mice, and when pretreated with SE this did not have a suppressive effect on *Tlr3*$^{-/-}$ mice. White bar: unwounded skin injected twice with 100 μL of PBS as control; Black bar: wounded skin injected with 100 μL of PBS 2 h and 24 h before wounding; Grey bar: wounded skin injected with 100 μL of SE (24 μg) 2 h and 24 h before wounding.** P <0.01, *** P <0.001. n.s. no significance. *P*-values were analyzed by using Two-way ANOVA. Data are the mean ± SEM of cell number from three adjacent sections.

a

b
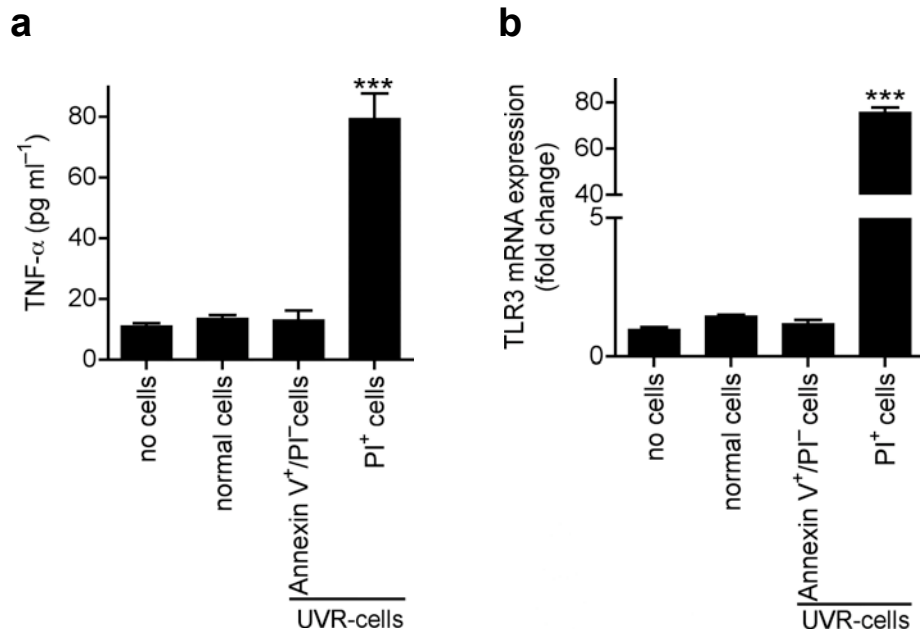
Supplementary Figure 5. PI⁺ UVR-cells activate TLR3 to produce TNF-α in cultured human keratinocytes. Cultured human keratinocytes were irradiated by UVB at 15 mJ/cm^2 for 1 min as Fig.2**c**. After 24 h, UVR-cells were collected and sorted into Annexin V⁺/PI⁻ cells and PI⁺ cells. 200,000 of Annexin V⁺/PI⁻ cells or PI⁺ cells were added to untreated normal human keratinocytes in culture, respectively. 24 h later, culture media were collected for TNF-α ELISA (**a**) and cells were collected for RNA isolation to evaluate TLR3 mRNA expression (**b**). Sonicated-non-irradiated cells (normal cells) were used as control.
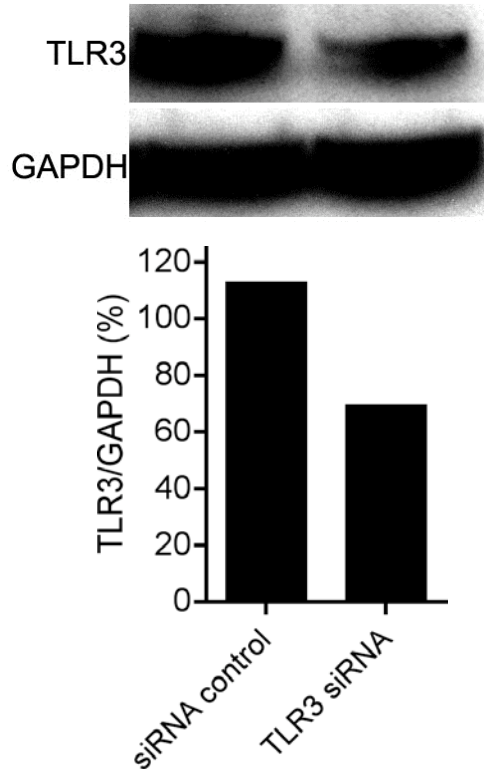
Supplementary Figure 6. Efficiency of *TLR3* siRNA knock-down in cultured human keratinocytes. Cells were transfected with four pairs of siRNA oligonucleotides targeted to *TLR3* or non-targeted control siRNA. After 24 h, cell lysates were collected for Western blot analysis of *TLR3* expression. The knock-down efficiency of *TLR3* siRNA was around 50% by Image J analysis.

a

Control · Poly(I:C) · SE · Poly(I:C) + SE

b

Control · Poly(I:C) · SE · Poly(I:C) + SE
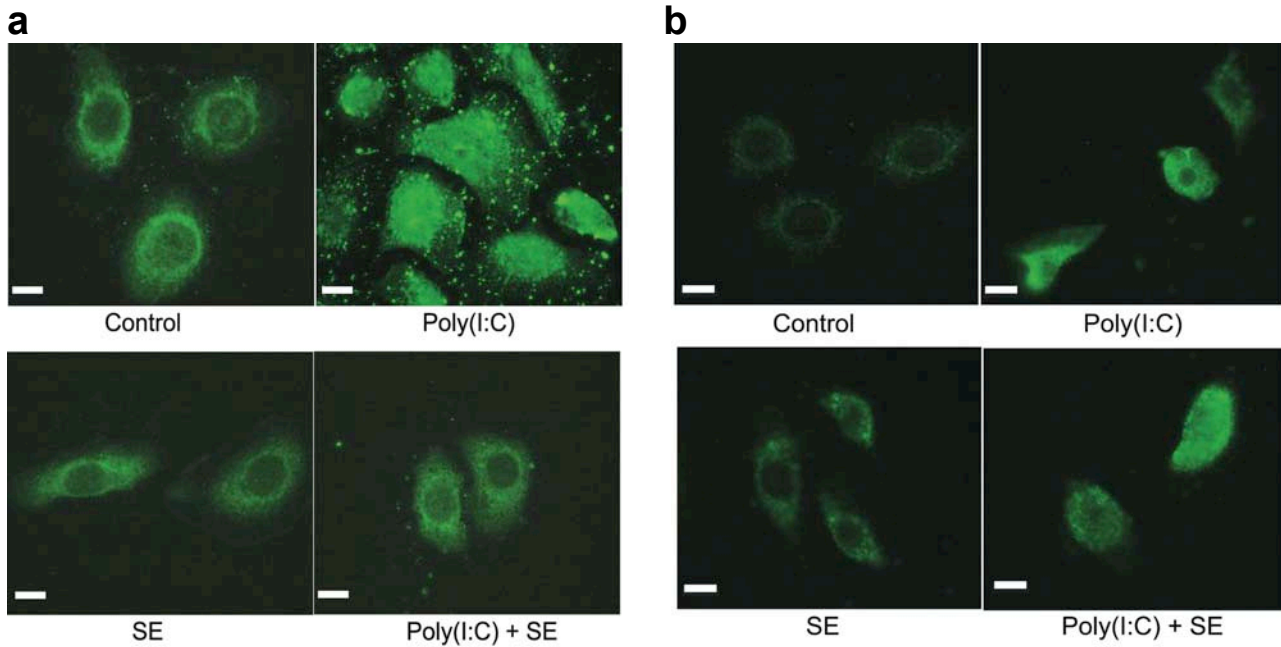
Supplementary Figure 7. *Staphylococcus epidermidis* **blocked the translocation of TLR3-induced NF-κB into the nucleus, but did not block IRF3 translocation.** (**a**) SE inhibited nuclear translocation of poly(I:C)-induced NF-κB1/p50 in cultured human keratinocytes. (**b**) SE failed to block the translocation of poly(I:C)-induced IRF3 in cultured human keratinocytes. Scale bars represent 50 μm.
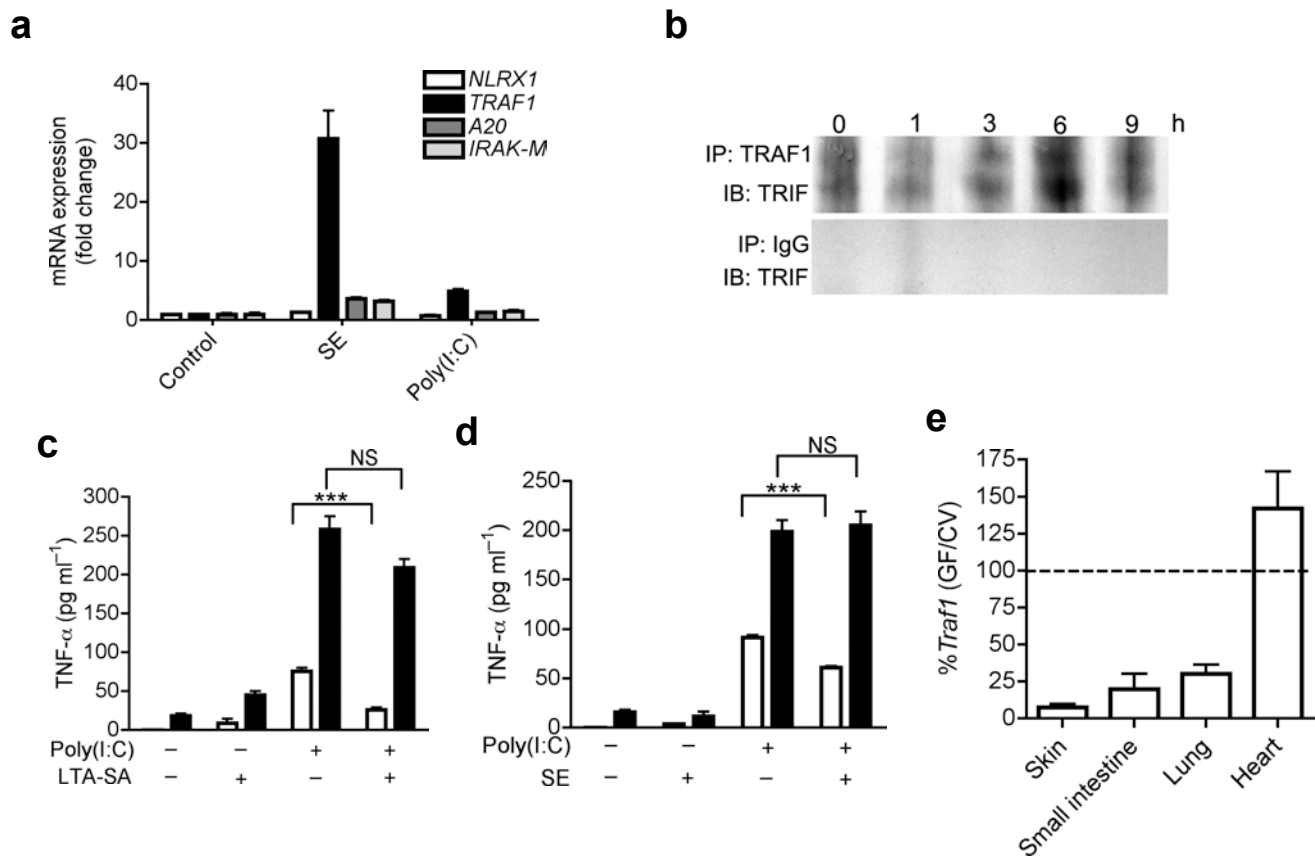
Supplementary Figure 8. *Staphylococcus epidermidis* **induces TRAF1 to suppress the production of poly(I:C)-induced proinflammatory cytokines.** (**a**) In cultured human keratinocytes SE significantly induced negative regulator *TRAF1* whereas poly(I:C) slightly induced *TRAF1* at 6 h. Both failed to induce other negative regulators, *NLRX1*(nucleotide-binding domain (NBD)- and leucine-rich-repeat (LRR) - containing family member), *A20* and *IRAK-M.* (**b**) TRAF1 induced by SE bound to TRIF in cultured human keratinocytes. TRAF1 from the cell lysate of cultured human keratinocytes treated with SE was immunoprecipitated by TRAF1-specific antibody and the association with TRIF was detected by antibody to TRIF. In the upper panel the cell lysate of cultured human keratinocytes treated with SE was immunoprecitpitated by TRAF1-specific antibody, whereas in the lower panel the same cell lysate was immunoprecitpitated by IgG control, and then both was immunoblotted by antibody to TRIF. Arrow indicates the association of TRAF1 and TRIF. (**c**) In cultured human keratinocytes caspase 8 inhibitor increased TNF-α production and completely restored the production of poly(I:C)-induced TNF-α that was suppressed by LTA-SA. White bar: without caspase 8 inhibitor; Black bar: with caspase 8 inhibitor. (**d**) Caspase 8 inhibitor restored the production of poly(I:C)-induced TNF-α that was suppressed by SE. White bar: without caspase 8 inhibitor; Black bar: with caspase 8 inhibitor. (**e**)The expression of *Traf1* mRNA was decreased in skin, small intestine and lung of germ-free mice compared to the same tissues from conventional mice, but a sterile tissue such as heart showed no significant difference in *Traf1* expression. Data shown here is the %*Traf1* expression of germ-free mice compared to the identical tissue harvested from conventionally housed mice. *Traf1* expression for each tissue from conventionally housed mice was normalized to 100% (dash line). (**c**) and (**d**), *** *P* <0.001. *P*-values were evaluated by Two-way ANOVA. n.s. no significance. Error bars represent SEM. Each data represents two independent experiments with *n* = 3 per group.

Supplementary methods

Bacterial strains

S. epidermidis strains 1457 and RP62A, *S. aureus* strains Rosenbach, Sa113 and Sa113 dltA mutant, were either stored in our laboratory or obtained from Dr Victor Nizet (University of California, San Diego). *S. aureus* Newman and *S. epidermidis* 12228 were purchased from ATCC.

Primary epidermis sheet / cell culture and stimulation

We cultured neonatal human epidermal keratinocytes (Cascade Biologics) in EpiLife medium (Cascade Biologics) and mouse macrophages (MHS) and endothelial cells (SEVC) in RPMI medium 1640 (GIBCO). We made dendritic cells from mouse bone marrow. We seeded cells in 12-well or 24-well plates to grow to about 70% confluence. After we pretreated cells with 10 μg ml^{-1} of poly(I:C) (InvivoGen) for 30 min, we added 36 μg ml^{-1} of bacterial extracts or different TLR ligands (InvivoGen) to stimulate cells for another 24 h. We harvested cells and analyzed the expression of genes by Real-time RT-PCR. We measured the concentration of TNF-α release by using an ELISA Kit (R&D Systems).

For primary isolates from mouse epidermis sheet culture, we removed the dermis layers from mouse skin biopsies after the incubation with Dispase II (Roche) at 37 °C for 1-2 h. We cultured the epidermis sheets in Medium 154CF (Cascade Biologics) and stimulated them with 36 μg ml^{-1} of SE with or without 10μg ml^{-1} of poly(I:C). After 24 h, we had epidermis sheets homogenization and isolated RNA for real-time RT-PCR analysis.

Real-time quantitative RT-PCR

We used Trizol Reagent (Invitrogen) to isolate total RNA. We used 1 µg of total RNA for cDNA synthesis by the iSCRIPT cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's

instructions. We performed Real-time RT-PCR in an ABI PRISM 7000 sequence detector (PE Applied Biosystems). We purchased the primers and probes used for real-time RT-PCR from Applied Biosystems. We did RNA analysis by using the TaqMan Master Mix reagents kit (Applied Biosystems). We used the comparative $\Delta\Delta C_T$ method to determine the quantification of gene expression. We normalized the target gene expression in the test samples to the endogenous reference GAPDH level and reported them as the fold difference relative to GAPDH gene expression. We performed all the assays in triplicate and repeated the experiments at least 2 times.

Immunofluorenscent staining

We grew second passage neonatal human epidermal keratinocytes in Lab-Tek Chamer Slide (Fisher). We added SE to stimulate cells in final concentration of 36 μg ml^{-1} for 24 h. After cold acetone fixation and subsequent washing by phosphate-buffered saline (PBS), we blocked cells with 3% BSA (Sigma) for 30 min at room temperature and stained cells with NF-κB1/p50-specific antibody (Cell signaling) or antibody to IRF3 (San Cruz Biotechnology Inc) or negative control Rabbit Immunoglobulin Fraction (Dako) at 4 °C for overnight. After washed by PBS, we reprobed the cells with anti-rabbit IgG FITC conjugate antibody (Sigma). After subsequent washing by PBS, we mounted the slides in ProLong Gold antifade reagent with DAPI (Invitrogen) and visualized them by Olympus BX41 microscope.