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Commensal bacteria regulate Toll-like receptor 3–dependent inflammation after skin injury

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The normal microflora of the skin includes staphylococcal species that will induce inflammation when present below the dermis but are tolerated on the epidermal surface without initiating inflammation. Here we reveal a previously unknown mechanism by which a product of staphylococci inhibits skin inflammation. This inhibition is mediated by staphylococcal lipoteichoic acid (LTA) and acts selectively on keratinocytes triggered through Toll-like receptor 3(TLR3). We show that TLR3 activation is required for normal inflammation after injury and that keratinocytes require TLR3 to respond to RNA from damaged cells with the release of inflammatory cytokines. Staphylococcal LTA inhibits both inflammatory cytokine release from keratinocytes and inflammation triggered by injury through a TLR2-dependent mechanism. To our knowledge, these findings show for the first time that the skin epithelium requires TLR3 for normal inflammation after wounding and that the microflora can modulate specific cutaneous inflammatory responses.

All complex metazoans are colonized with a myriad of microbial organisms, a group that has been referred to as the 'microbiome'. On the skin, bacterial colonization is abundant, diverse and constant, but inflammation is undesirable and is an indication of disease. The two principal normal stimuli of inflammation are injury and infection. During infection, the detection of microbes is accomplished in part by TLRs, which are best known as stimuli of inflammation. Although the role of TLRs in response to infection is well defined, the mechanisms involving TLRs that regulate inflammation in the skin injury are poorly understood.

Epithelial surfaces in the gut regulate the magnitude and duration of TLR signaling, thus shaping and maintaining normal mucosal immunity[1–3] via the induction of proinflammatory and anti-inflammatory cytokine synthesis[4,5]. Moreover, the commensal bacteria *Lactococcus lactis* and *Bacteroides vulgatus* can prime the host to produce interleukin-10 (IL-10), trefoil factors and transforming growth factor-β (TGF-β), which are suggested to modulate inflammation in the intestine[6–8]. However, TLRs that have been generally thought to act as proinflammatory signals in response to microbial products also may recognize self-epitopes that are released from damaged cells or are present at the surface of apoptotic cells in autoimmune diseases[9,10]. These observations have raised the possibility that TLRs may have a role in initiating inflammation in response to injury[11,12].

In skin injury, tissue damage results in necrosis and apoptosis, but it is not known if this event is involved in the initiation of the inflammatory response that is crucial to normal wound repair. In the absence of infection, uncontrolled inflammation during wound repair is undesirable and may cause dysfunction during healing. Uncontrolled inflammation after minor trauma is also well known to exacerbate several human skin diseases, such as psoriasis. Therefore, normal immune defense requires that a balance is maintained to minimize unnecessary inflammation yet rapidly respond to infection and injury. This balance is particularly difficult to maintain at epithelial surfaces that are in contact with the external environment and have frequent trauma and exposure to the products of the microbiome. The innate systems that enable the control of this inflammatory response are generally unknown.

Given the hypothesis that epithelial flora may serve to protect the host from unintended inflammatory diseases and the importance of TLRs in the recognition of microbial products and potential role after tissue damage, we set out to study how these systems may be involved in homeostatic control of skin inflammation. Staphylococcal species are the most frequently cultured normal inhabitants of the healthy human skin[13] and have been hypothesized to serve a role in human health[14]. It has been entirely unknown how these bacteria might affect cutaneous homeostasis. In this study, we describe a mechanism by which a product of staphylococci, LTA, suppresses skin inflammation during wound repair.

RESULTS

Staphylococcal products suppress inflammation

Recent observations in several systems have suggested that activation of TLRs on keratinocytes will trigger inappropriate production

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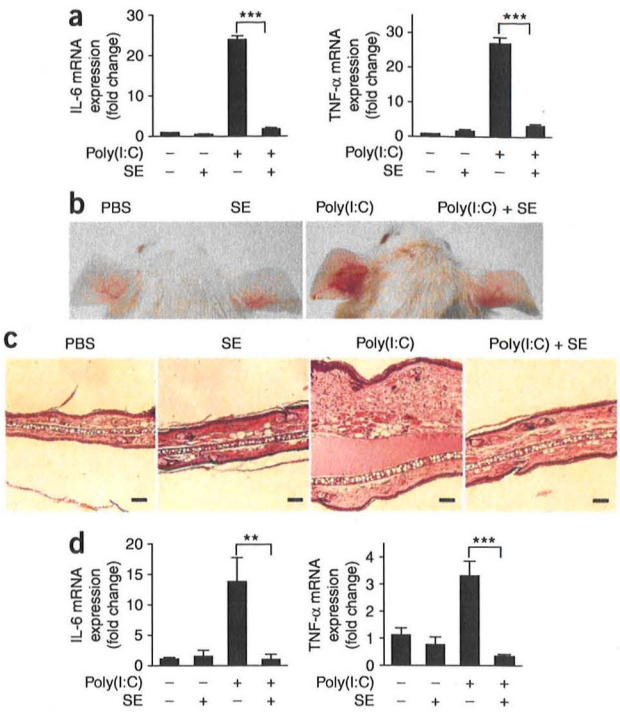

Figure 1 *S. epidermidis* inhibits poly(I:C)-induced inflammatory cytokines produced by keratinocytes, and inflammation in mouse skin. (**a**) Quantification of IL-6 and TNF-α mRNA expression of cultured normal human keratinocytes treated with 36 μg ml^{-1} of a sterile <10-kDa product of *S. epidermidis*–conditioned culture medium (SE) and 10 μg ml^{-1} of the TLR3 ligand poly(I:C) for 24 h. (**b**) Photographs of the ears of BALB/c mice 24 h after subcutaneous injection with PBS alone, the <10-kDa product of *S. epidermidis* alone (SE), poly(I:C) alone or the <10-kDa product of *S. epidermidis* and poly(I:C). (**c**) H&E staining of ears treated as in **b**. Scale bars, 100 μm. (**d**) Quantification of IL-6 and TNF-α expression in tissue from mouse ears treated as in **b**. $^{**}P < 0.01$ and $^{***}P < 0.001$. *P* values were determined by two-tailed *t* tests. All data are representative of three independent experiments with $n = 3$–6 per group and are means ± s.e.m.

of proinflammatory cytokines in the epidermis as an element in the pathogenesis of some skin diseases[15–17]. However, despite production of TLR ligands, commensal bacterial species, including *Staphylococcus epidermidis*, normally reside in contact with keratinocytes in the epidermis and do not initiate inflammation. We therefore hypothesized that *S. epidermidis* can influence the inflammatory response of keratinocytes through negative regulation of TLR signaling. Primary human keratinocytes treated with a panel of TLR ligands showed that polyriboinosinic polyribocytidylic acid (poly(I:C)), a stimulus for TLR3 (ref. 18,19), was the most potent stimulus for expression of tumor necrosis factor-α (TNF-α) by keratinocytes and that previous exposure to a <10-kDa product of *S. epidermidis* suppressed poly(I:C)-induced IL-6 and TNF-α expression (**Fig. 1a** and **Supplementary Fig. 1a**). This suppression persisted over time and affected both messenger RNA and protein levels of TNF-α (**Supplementary Fig. 1b**).

We next examined cutaneous inflammation induced by poly(I:C) in mice to determine the *in vivo* relevance of the response observed to *S. epidermidis*. Ears pretreated with the <10-kDa product of *S. epidermidis* before poly(I:C) showed less inflammation compared to those treated only with poly(I:C) (**Fig. 1b,c**). The decrease in inflammation corresponded with a decrease in the expression of IL-6 and TNF-α mRNA (**Fig. 1d**). However, we did not observe the inhibitory effects of *S. epidermidis* <10-kDa product in whole skin when inflammation was induced by lipopolysaccharide (LPS) (**Supplementary Fig. 1c**), or phorbol 12-myristate 13-acetate (PMA) (**Supplementary Fig. 1d**). Thus, these data show that a product of *S. epidermidis* functions as a selective suppressor of poly(I:C)-mediated inflammation in the skin.

Analysis of similar soluble <10-kDa products of other staphylococcal strains showed that the capacity to inhibit poly(I:C)-induced TNF-α expression was present in multiple staphylococcal strains, except one hospital-isolated strain of *Staphylococcus aureus* (**Supplementary Fig. 2**). The <10-kDa product of *S. epidermidis* also suppressed the expression of IL-8 but was not able to suppress interferon-β (IFN-β) or IL-1β (data not shown). Moreover, the <10-kDa product of *S. epidermidis* inhibited the capacity of mouse epidermal sheets to express TNF-α and IL-6 in response to poly(I:C) (**Supplementary Fig. 3a**). However, in contrast to keratinocytes and epidermal sheets, other cell types representative of those present in skin but below the epidermis were not inhibited by the <10-kDa product of *S. epidermidis* and had increased expression of IL-6 and TNF-α in response to poly(I:C) and *S. epidermidis* (**Supplementary Fig. 3b–d**). Taken together, these results show poly(I:C) is a potent stimulus to release proinflammatory cytokines in the skin, but a soluble, low-molecular-weight product from some bacteria will inhibit this response selectively in keratinocytes.

TLR3 mediates a response to injury

To understand the immunological relevance of suppression of TLR3-induced cytokine release by keratinocytes, we next sought to investigate conditions in which TLR3 might be activated in skin. In gastrointestinal epithelium, TLR3 has been implicated as a mechanism for detection of cell death[20]. Skin injury results in the generation of necrotic and apoptotic cells[21,22]. Therefore, we examined whether TLR3 activation might be a mechanism for detection of skin injury and initiation of inflammation. To test this hypothesis, we made aseptic, full-thickness incisions on the backs of *Tlr3*-deficent mice and matched wild-type controls. *Tlr3*-deficent mice showed significantly less production of IL-6 and TNF-α at the wound edge compared to wild-type controls (**Fig. 2a**). This correlated with a decrease in inflammation and leukocyte recruitment (**Fig. 2b** and **Supplementary Fig. 4**). Furthermore, consistent with the inhibitory effects of *S. epidermidis* on poly(I:C)-induced skin inflammation, application of the <10-kDa product of *S. epidermidis* also inhibited wound-induced IL-6, TNF-α and inflammation in wild-type mice but had no marked effect on the injury response in *Tlr3*$^{-/-}$ mice (**Fig. 2a,b** and **Supplementary Fig. 4**). Thus, these findings establish that *Tlr3* is required for part of the normal inflammatory response after injury and that *S. epidermidis* acts on this pathway to decrease the magnitude of inflammation.

Wounding results in the rapid generation of abundant amounts of damaged cells, including necrotic and apoptotic keratinocytes. To test whether a product of damaged keratinocytes can stimulate TLR3-dependent inflammation in adjacent normal cells, we treated cultured human keratinocytes with ultraviolet B (UVB) radiation to induce apoptosis and cell death. We then collected these cells (UVR cells) and added them to separate cultures of normal human keratinocytes. TNF-α release significantly increased when we exposed normal keratinocytes to UVR cells but not when we exposed them to equal amounts of sonicated, nonirradiated cells (**Fig. 2c** and **Supplementary Fig. 5a**). This response was dependent on *TLR3*, as targeted knockdown of

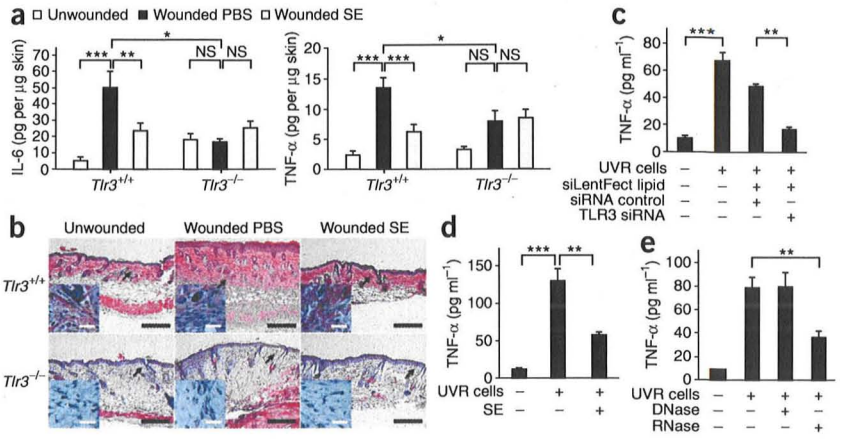

Figure 2 Inflammation in wounds is dependent on TLR3 and inhibited by *S. epidermidis*. (**a**) Quantification of IL-6 and TNF-α production by ELISA in extracts of skin taken from 2 mm surrounding the wound edge 3 d after aseptic injury. (**b**) H&E staining of skin 2 mm adjacent to mouse wounds of wild-type (*Tlr3*$^{+/+}$) and *Tlr3*-deficient (*Tlr3*$^{-/-}$) mice treated as in **a**. Black scale bars represent 200 μm, and white scale bars represent 25 μm. Arrows designate region of 400× magnification shown in insets. (**c**) The induction of TNF-α by UVB-irradiated keratinocytes (UVR cells) in untreated normal human keratinocytes and the blockage of UVR cell–induced TNF-α by *TLR3*-specific siRNAs. (**d**) Effect of the <10-kDa product of *S. epidermidis* on TNF-α production stimulated by UVR cells. (**e**) Effects of RNase treatment or DNase treatment on the capacity of UVR cells to induce TNF-α in normal human keratinocytes in culture. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. NS, no significance. *P* values were analyzed by two-way analysis of variance (ANOVA) in **a** or one-way ANOVA in **c**–**e**. Data are the means ± s.e.m. and are representative of two to four independent experiments with $n = 4$–7 per group.

TLR3 by siRNA abrogated the response to UVR cells (**Fig. 2c** and **Supplementary Fig. 6**). Consistent with the response to poly(I:C) or wounding, the <10-kDa product of *S. epidermidis* also inhibited TNF-α release induced by UVR cells (**Fig. 2d**). The product of UVR cells that activated TLR3 in normal keratinocytes was RNA, as RNase abrogated the capacity of UVR cells to stimulate the production of TNF-α, but treatment with DNase did not (**Fig. 2e**). We also sorted UVR cells into intact, annexin V^{+} cells that excluded propidium iodide and dead cells whose membrane was permeable after UVB irradiation and thus stained with propidium iodide. Nonirradiated cells (normal cells) disrupted by sonication, or early apoptotic cells that were annexin V–positive but excluded propidium iodide (annexin V–positive, propidium iodide–negative cells), could not induce TNF-α release or *TLR3* expression (**Supplementary Fig. 5a,b**). However, UVR cells staining with propidium iodide (propidium iodide–positive cells) substantially increased TNF-α release and *TLR3* expression (**Supplementary Fig. 5a,b**). Thus, these results show that RNA released from necrotic cells can induce TNF-α and *TLR3* expression in keratinocytes.

LTA suppresses TLR3 inflammation

TLR-TLR cross-talk can suppress inflammatory responses[23,24], and LTA is a known molecular signal for recognition of staphylococci; thus, we sought to determine whether this molecule might be responsible for the observed effects. LTA from *S. epidermidis* has two forms, cellular and exocellular LTA[25]. Exocellular LTA can be recovered from liquid growth medium and is the form present in the <10-kDa product of *S. epidermidis*, whereas commercial LTA is derived from the cell wall and membrane. Commercially available LTA from *S. aureus* (LTA-SA), but not LPS or flagellin, suppressed the production of TNF-α induced by poly(I:C) (**Fig. 3a**). LTA directly purified from *S. epidermidis* (LTA-SE) or commercial LTAs from some other staphylococcal species suppressed poly(I:C)-induced TNF-α, whereas other ligands of TLR2 either synergistically induced TNF-α or failed to suppress TNF-α when combined with poly(I:C) (**Fig. 3b**). TLR2 ligands tested included PGN-SA (peptidoglycan from *Staphylococcus aureus*), PGN-EK (peptidoglycan from *Escherichia coli K12*) and zymosan, as well as ligands that activate heterodimers of TLR2 and TLR1 such as the synthetic triacylated lipoprotein Pam3CSK4, or ligands of heterodimers of TLR2 and TLR6 such as diacylated lipoprotein FSL-1 ((*S,R*)-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp- RPro-Lys-His-Pro-Lys-Ser-Phe) and macrophage-activating lipopeptide (Malp-2). Notably, LTAs from *Streptococcus fecalis* and *Bacillus subtilis* (LTA-SF and LTA-BS) were not active (**Fig. 3b**).

We next confirmed the identity of LTA as the molecule in *S. epidermidis* responsible for suppression of keratinocyte TNF-α production by use of a specific LTA-neutralizing antibody[25] (**Fig. 3c**). Furthermore, synthetic LTAs containing two or three D-alanines suppressed poly(I:C)-induced TNF-α, whereas synthetic LTAs only containing an anchor or containing two or five *N*-acetylglucosamine failed to suppress TNF-α (**Fig. 3d**). We then confirmed the necessity of D-alanine modification by using LTA from a *S. aureus* dltA mutant that lacks D-alanine modifications[26]. Preparations from this mutant bacterial culture supernatant (SA dltA) partially lost the capacity to suppress poly(I:C)-induced TNF-α compared to its parental strain (**Fig. 3e**).

N-TRAF1 mediates LTA inhibition of TLR3

We observed that *S. epidermidis* blocked nuclear translocation of the p50 subunit of nuclear factor-κB1, but not the translocation of interferon regulatory factor-3, both stimulated by poly(I:C) in keratinocytes (**Supplementary Fig. 7a,b**). Thus, we next investigated negative regulators involved in TLR-mediated NF-κB signaling, including TNF receptor–associated factor-1 (TRAF1), TNF-α–induced protein-3 (TNFAIP3) and interleukin-1 receptor–associated kinase M (IRAK-M)[27]. LTA and the <10-kDa product of *S. epidermidis* significantly induced TRAF1 protein and mRNA in keratinocytes within 1 h and maximally between 6 and 9 h (**Fig. 4a** and **Supplementary Fig. 8a**). The expression of other negative regulatory genes such as nucleotide-binding domain (NBD)- and leucine-rich-repeat (LRR)-containing family member X1 (*NLRX1*), *TNFAIP3* or *IRAK3* (encoding IRAK-M) involved in both the NF-κB and retinoic acid–inducible gene (RIG)-like helicase pathways, were not induced (**Supplementary Fig. 8a**). We confirmed the role of TRAF1 as a mediator of the LTA inhibitory effect by experiments in *Traf1*$^{-/-}$ mice where LTA was unable to suppress the production of IL-6 and TNF-α at wound edge compared to wild-type controls (**Fig. 4b**).

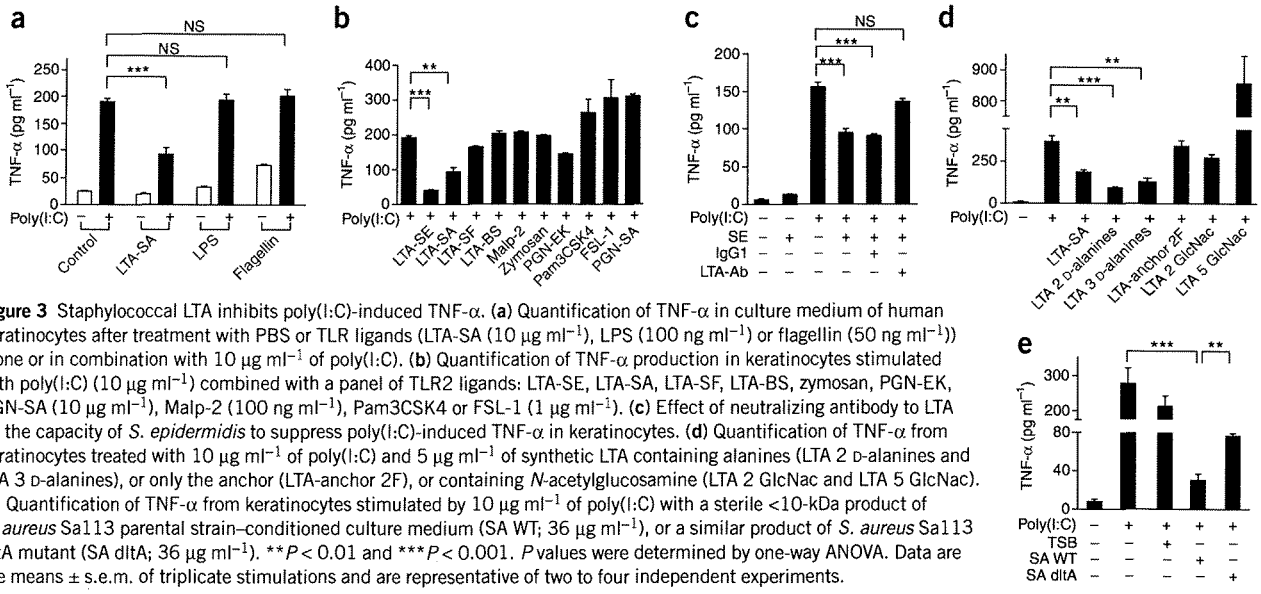

Figure 3 Staphylococcal LTA inhibits poly(I:C)-induced TNF-α. (**a**) Quantification of TNF-α in culture medium of human keratinocytes after treatment with PBS or TLR ligands (LTA-SA (10 μg ml^{-1}), LPS (100 ng ml^{-1}) or flagellin (50 ng ml^{-1})) alone or in combination with 10 μg ml^{-1} of poly(I:C). (**b**) Quantification of TNF-α production in keratinocytes stimulated with poly(I:C) (10 μg ml^{-1}) combined with a panel of TLR2 ligands: LTA-SE, LTA-SA, LTA-SF, LTA-BS, zymosan, PGN-EK, PGN-SA (10 μg ml^{-1}), Malp-2 (100 ng ml^{-1}), Pam3CSK4 or FSL-1 (1 μg ml^{-1}). (**c**) Effect of neutralizing antibody to LTA on the capacity of *S. epidermidis* to suppress poly(I:C)-induced TNF-α in keratinocytes. (**d**) Quantification of TNF-α from keratinocytes treated with 10 μg ml^{-1} of poly(I:C) and 5 μg ml^{-1} of synthetic LTA containing alanines (LTA 2 D-alanines and LTA 3 D-alanines), or only the anchor (LTA-anchor 2F), or containing *N*-acetylglucosamine (LTA 2 GlcNac and LTA 5 GlcNac). (**e**) Quantification of TNF-α from keratinocytes stimulated by 10 μg ml^{-1} of poly(I:C) with a sterile <10-kDa product of *S. aureus* Sa113 parental strain–conditioned culture medium (SA WT; 36 μg ml^{-1}), or a similar product of *S. aureus* Sa113 dltA mutant (SA dltA; 36 μg ml^{-1}). $^{**}P < 0.01$ and $^{***}P < 0.001$. *P* values were determined by one-way ANOVA. Data are the means ± s.e.m. of triplicate stimulations and are representative of two to four independent experiments.

It has been hypothesized that TRAF1 is processed by caspase-8 to an active N-terminal fragment (N-TRAF1) followed by binding to TRIF to serve as a negative regulator of TLR3. We observed the cleavage of TRAF1 to N-TRAF1 in the presence of the <10-kDa product of *S. epidermidis* and poly(I:C) (**Fig. 4c**), and it occurred in a caspase-8–dependent manner, as treatment with a caspase-8 inhibitor blocked the generation of N-TRAF1 (**Fig. 4d**). We found that TRAF1 associated with TRIF by immunoprecipitation of *S. epidermidis*–treated keratinocytes with TRAF1-specific antibody and detection with antibody to TRIF (**Supplementary Fig. 8b**). Furthermore, addition of a caspase-8 inhibitor increased TNF-α production and completely abrogated the inhibitory effects of *S. epidermidis* and LTA (**Supplementary Fig. 8c,d**).

TLR2 inhibits TLR3

Having established the involvement of TRAF1 in the suppression of keratinocyte cytokine release, and the role of the TLR2 ligand LTA in initiating this effect, we next sought to further confirm the mechanism and physiological relevance of these observations *in vivo*. Analysis of the skin from germ-free wild-type mice that were never exposed to bacterial products showed that the expression of *Traf1* in their skin was markedly decreased compared to skin of normal mice housed in pathogen-free but nonsterile conditions (**Fig. 4e**). In addition to that in skin, the expression of *Traf1* in small intestine and lung, but not in heart, of germ-free wild-type mice was decreased compared to those tissues from conventional wild-type mice (**Supplementary Fig. 8e**). Furthermore, because recognition of LTA requires TLR2 and the downstream adapter protein myeloid differentiation factor-88 (MYD88), we also observed diminished expression of *Traf1* in *Tlr2*$^{-/-}$ or *Myd88*$^{-/-}$mice exposed to LTA (**Fig. 4f**). Therefore, *Tlr2*$^{-/-}$ mice might be used as another model to test the effect of diminished Traf1 on suppression of Tlr3-dependent skin inflammation after injury. As expected, LTA failed to suppress inflammation and the production of IL-6 and TNF-α at wound

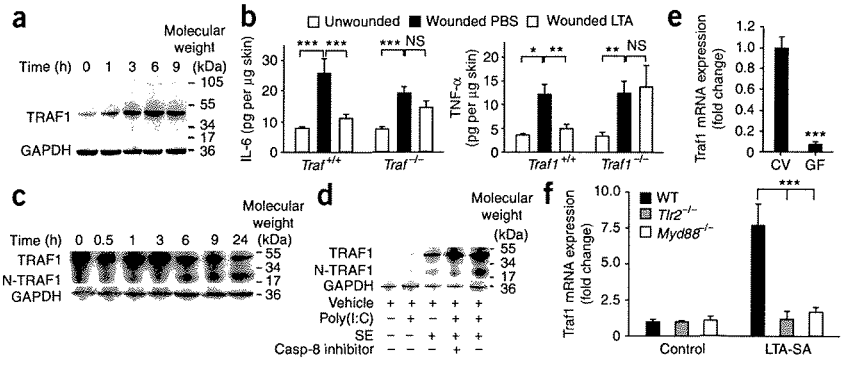

Figure 4 Staphylococcal LTA induces TRAF1 to inhibit TNF-α. (**a**) Western blot of TRAF1 in cultured human keratinocytes stimulated by LTA-SA for various times. The predicted molecular weight of TRAF1 is ~52 kDa. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. (**b**) Quantification of IL-6 and TNF-α of skin extracts from C57BL/6 *Traf1*-deficient mice and wild-type controls preinjected with PBS or LTA-SA 24 h and 2 h before wounding. Two millimeters of skin around the wound edges was collected for ELISA. (**c**) Western blot with antibody raised against the N-terminus of human TRAF1 in keratinocyte extract treated with the <10-kDa product of *S. epidermidis* and poly(I:C) for the indicated time periods. The predicted molecular weight of N-TRAF1 is ~22 kDa. (**d**) Western blot showing that caspase-8 inhibitor prevented the cleavage of TRAF1 in cultured human keratinocytes. (**e**) Quantification of Traf1 mRNA expression in skin of germ-free mice and conventionally housed mice. CV, conventionally housed wild-type mice; GF, wild-type mice housed in a germ-free environment. (**f**) Quantification of Traf1 mRNA expression induced by LTA-SA in skin of wild-type, *Tlr2*$^{-/-}$ and *Myd88*$^{-/-}$ mice. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. *P* values were determined by two-way ANOVA in **b** and **f** and two-tailed *t* tests in **e**. Data are means ± s.e.m. and are representative of two independent experiments with $n = 3$–7 per group.

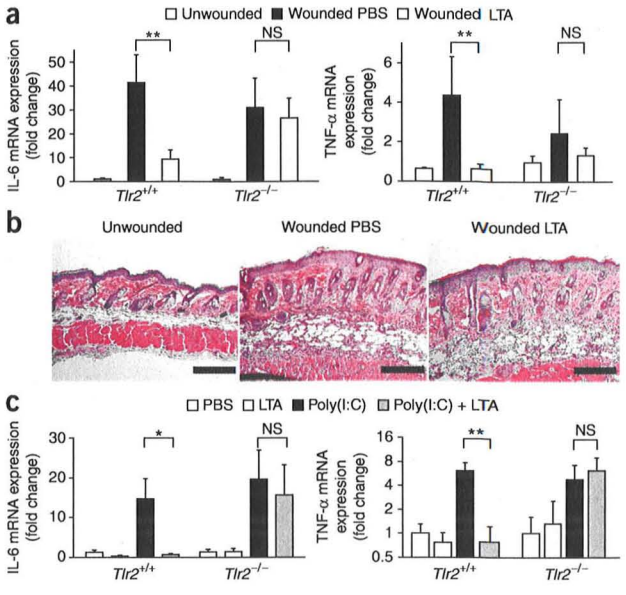

Figure 5 TLR2 is required for LTA to inhibit skin inflammation after injury or injection of poly(I:C). (**a**) Quantification of IL-6 and TNF-α mRNA expression in skin from C57BL/6 *Tlr2*-deficient mice and wild-type controls preinjected with PBS or LTA-SA 24 h and 2 h before wounding. Two millimeters of skin around the wound edges was collected for real-time RT-PCR. (**b**) H&E staining of skin in *Tlr2*-deficient mice treated as in **a**. Scale bars, 200 μm. (**c**) Quantification of IL-6 and TNF-α mRNA expression in skin from C57BL/6 *Tlr2*-deficient mice stimulated by subcutaneous injection of poly(I:C). Some ears were preinjected with LTA-SA or PBS 2 h before injection of poly(I:C). $*P < 0.05$ and $**P < 0.01$. *P* values were evaluated by two-way ANOVA. Data are the means ± s.e.m. of $n = 5–7$ and are representative of three independent experiments.

edge in $Tlr2^{-/-}$ mice (**Fig. 5a,b**), and we observed a similar loss of responsiveness to LTA in $Tlr2^{-/-}$ mice treated with poly(I:C) (**Fig. 5c**).

DISCUSSION

Commensal microorganisms have been proposed to influence host immune responses and host-pathogen interactions in the gut[28]. Here we hypothesized that specific elements of the resident microbiota of normal human skin can modulate cutaneous immune responses triggered by TLR ligands. Our results confirmed this hypothesis while also revealing that TLR3 is a key element in the induction of inflammation after skin injury. Inhibition of this inflammatory event is accomplished by specific staphylococcal LTAs and is mediated by TLR2 on keratinocytes. The mechanism for LTA-TLR– mediated suppression of TLR3 signaling is by induction of the negative regulatory factor TRAF1, an event we show has a major role in limiting the extent of cutaneous inflammation. Thus, we show that a sensitive balance exists between stimulating and inhibitory mediators during wound healing and that epithelial cells uniquely detect these signals to achieve tissue homeostasis after injury.

It was previously unclear why keratinocytes are highly sensitive to TLR3 ligands, as the classical ligand for TLR3, viral double-stranded RNA, is not a frequent initiator of major inflammatory responses in the skin. The epidermis can be exposed to double-stranded RNA from viral infections, but such infections are relatively uncommon compared to infections by other skin pathogens. We show here that the high sensitivity to TLR3 ligands is probably because the epidermis uses TLR3 for recognition of injury to self. Our data show that RNA from necrotic cells triggers TLR3 on undamaged keratinocytes, leading to a local release of proinflammatory cytokines. This is probably a frequent mechanism for detection of injury and maintenance of homeostasis, as necrotic cells are abundant at the wound edge. Therefore, it is reasonable to speculate that TLR3 in the normal epidermis is an important sensor of injury and that systems must exist to modulate this response to prevent excessive or unwanted inflammation. Furthermore, given that *S. epidermidis* or LTA not only inhibited proinflammatory cytokine production by isolated keratinocytes in culture but also inhibited inflammation *in vivo*, the production of proinflammatory cytokines by keratinocytes is probably a major contributor to some forms of skin inflammation.

Our findings show that LTA produced by staphylococcal species have a unique anti-inflammatory action on keratinocytes. In contrast, LTA initiates the opposite response when exposed to other immune cells. It is logical that LTA would have distinct effects on cytokine release depending on the cell type exposed. LTA acts as a proinflammatory factor for cells that normally exist in a sterile environment, such as macrophages (**Supplementary Fig. 3b**), monocytes and mast cells[29,30]. These cells are not normally exposed to the surface microbiome and appropriately recognize LTA as foreign. However, keratinocytes are unique in that they are frequently exposed to LTA. Furthermore, the structure of LTA seems to determine the nature of the keratinocyte response. Addition of D-alanine to the LTA core seemed to be a key factor in dictating activity, but further analysis is necessary to understand these LTA structure-function relationships. Moreover, other TLR2 ligands that depend on formation of a heterodimer with TLR1 or TLR6 do not inhibit TLR3 in keratinocytes but rather have a proinflammatory effect. Thus, the specificity of the response is dictated by cell type and the specific structure of the TLR2 ligand produced by the microbe. In particular, *S. epidermidis*, a normal inhabitant of the skin, may have a uniquely structured TLR2 ligand that maximizes anti-inflammatory action yet minimizes the capacity to initiate inflammation.

To limit TLR-induced inflammation, several negative regulatory systems exist, including sequestration of signaling molecules, blockade of their recruitment, degradation of target proteins and inhibition of transcription[27,31]. These negative regulators can be part of microbial virulence. Examples of TLR inhibition by microbes include decoy receptors in some bacterial infections that prevent a direct interaction between TLRs and their microbial ligands, and vaccinia virus production of several proteins that interfere with viral recognition through both TLR3 and helicases[32–34]. Therefore, there is potential for pathogenic staphylococci such as *S. aureus* to exploit suppression of keratinocyte activation as a mechanism of virulence, whereas *S. epidermidis* may benefit the host by dampening unwanted inflammation. This hypothesis requires further testing but is supported by the data we obtained from germ-free mice that shows such mice lack normal expression of *Traf1* in the skin. These observations support additional findings that the ability of TLR2 to recognize commensal bacteria is not irrelevant under normal conditions. Rather, activation of TLR2 in keratinocytes has its own beneficial effect in maintaining homeostasis.

Taken together, these findings are best appreciated when one recognizes that inflammation is an undesirable condition on skin but is fundamentally a necessary protective response after injury. Prolonged and dysregulated production of inflammatory cytokines leads to excessive neutrophil influx, resulting in sustained inflammatory responses and poor healing, subsequently causing extensive tissue damage[35,36]. In contrast, without an appropriate inflammatory response, wound

healing is also delayed, and the host is more susceptible to microbial invasion. Local modulation of the inflammatory response by products of bacterial commensals at the site of such an injury might be a beneficial therapeutic strategy for management of wound healing complicated by excessive inflammation or control of other inflammatory skin disorders. The trick will be to evoke a reduction in the detrimental aspects of inflammation without increasing the risk of wound infection. Our findings emphasize the potential benefit of the resident bacteria on skin and the potential negative consequences of complete depletion of microflora from skin by indiscriminate use of topical and systemic antibiotics.

METHODS

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

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AUTHOR CONTRIBUTIONS

Y.L. and R.L.G. designed the experiments; Y.L. performed most of the experiments and analyzed data; A.D.N. helped in culture of bone-marrow-derived dendritic cells and preparation of UVB-irradiated apoptotic and necrotic cells; T.N. and A.L. helped with mouse experiments; Y.Y. and Z.-R.W. designed the LPS-induced inflammation model; A.L.C. helped to perform initial experiments; L.V.H. provided germ-free mouse ear and intestine samples; R.R.S. and S.v.A. provided synthetic LTAs; K.A.R. showed how to make mouse wound models; C.-M.H. helped to modify the protocol for animal study; A.F.R. provided *Tlr2*-deficient mice; Y. L. and R.L.G. wrote and prepared the manuscript.

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ONLINE METHODS

Mice. We housed C57BL/6 wild-type, *Tlr3*-deficient, *Myd88*-deficient and *Tlr2*-deficient mice and BABL/c wild-type mice in the VA San Diego Healthcare System Veterinary Medical Unit, and we purchased *Traf1*-dificient mice from the Jackson Laboratory. We purchased Swiss-Webster conventional and germ-free mice from Taconic. All animal experiments were approved by the VA San Diego Healthcare System Institutional Animal Care and Use Committee.

Bacterial extracts preparation and lipoteichoic acid purification. We grew bacteria in tryptic soy broth at 37 °C for 15–16 h. For scale-up preparation, we diluted overnight cultures 1 in 100 into tryptic soy broth and grew them for another 15–16 h. We then collected bacterial cultures and filtered them by 0.22 μM Stericup (Millipore). We used the MacroSep 10K OMEGA column (VWR) to collect the <10-kDa fractions from bacterial supernatants and determined concentrations of bacterial extracts by BCA Protein Assay Kit (Pierce). We purified LTA from *S. epidermidis* conditioned culture medium by using a previously described protocol[37].

Ultraviolet B–induced apoptosis and necrosis. We irradiated cultured human keratinocytes by UVB at 15 mJ cm^{-2} for 1 min. After 24 h, we collected the UVB-irradiated cells and sorted them into annexin V–positive, propidium iodide–negative cells and propidium iodide–positive cells. To stimulate TNF-α, we added these sorted UVR cells to untreated normal human keratinocytes in culture. We used sonicated nonirradiated cells (normal cells) as controls. We measured TNF-α in culture medium 24 h after treatment with these cells.

Lipoteichoic acid neutralization. We used 300 μg ml^{-1} of *S. epidermidis* LTA monoclonal antibody (Abcam) or monoclonal mouse IgG1 isotype control (clone PPV-06) (EXBIO, 11-457-C100) to incubate with 30 μg of the <10-kDa product of *S. epidermidis* for 20 h at 4 °C before we added the <10-kDa product of *S. epidermidis* to stimulate cells. After 24 h stimulation, we collected cell supernatants for TNF-α ELISA assay.

RNA interference. We seeded neonatal human epidermal keratinocytes at first or second passage in 24-well plates and transfected cells with 10 nM of four pairs of siRNA oligonucleotides targeted to *TLR3* (Dharmacon; SMART Pool) and nontargeted control siRNA (Dharmacon) by using silentFect (BioRad), as we have described previously[38]. We tested the efficiency of TLR3 siRNA blockage by western blotting. After 24 h transfection, we added UVR cells to stimulate cells for 24 h. We evaluated the production of TNF-α by ELISA.

Caspase-8 inactivation. We applied 100 nM caspase-8 inhibitor (Z-IETD; R&D) 10 min before we added 10 μg ml^{-1} of poly(I:C) with or without 36 μg ml^{-1} of the <10-kDa product of *S. epidermidis* or 10 μg ml^{-1} of LTA in cultured human keratinocytes and waited for 6 h or 48 h. We analyzed the inhibition of TRAF1 processing by western blotting, and we measured the production of TNF-α by ELISA.

Immunoprecipitation and immunoblotting. We stimulated cultured human keratinocytes at the time points indicated in **Figure 4a,c** and **Supplementary Figure 8b**. We lysed cells by using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.1% SDS) containing protease inhibitor cocktail (Roche) and then sonicated them on ice-cold water. We measured protein concentrations of the extracts by BCA Protein Assay Kit (Pierce) and used 15 μg of total protein for immunoprecipitation and 3 μg of total protein for western blotting. We used SDS-PAGE to separate bands, and detected TRAF1 by immunoblotting with the antibody to TRAF1 (Santa Cruz, sc-983) or the association of TRAF1 and TRIF by immunoblotting with TRIF-specific antibody (Cell Signaling, 4596).

Cutaneous inflammation *in vivo*. We shaved the backs of age-matched adult littermates and removed hair by chemical depilation (Nair; Church & Dwight), as previously described[39]. We intradermally injected 100 μl of PBS or 100 μl of the <10-kDa product of *S. epidermidis* (24 μg) or 100 μl of LTA-SA (50 μg) 24 h and 2 h before wounding back skin of wild-type or *Tlr3*-deficient mice or *Traf1*-deficient mice by biopsy punches. Three days later, we collected 2 mm of skin around wound edges either for ELISA assay or stored it in formalin (Protocol) for H&E staining.

For cutaneous inflammation in ears, we injected 12 μg of the <10-kDa product of *S. epidermidis* or 10 μg of LTA-SA into the ear lobes of C57BL/6 wild-type (or BABL/c wild-type mice) and *Tlr2*-deficient mice 2 h before we injected 50 μg of poly(I:C) or 20 μg of LPS. After 24 h, we cut mouse ears to either store them in formalin for H&E staining or homogenize them for RNA isolation. We analyzed the expression of cytokines by real-time RT-PCR.

For PMA-induced skin inflammation, we injected 12 μg of the <10-kDa product of *S. epidermidis* into BALB/c wild-type mouse ear lobes 2 h before we topically treated mouse ears with 20 μl of 1 mg ml^{-1} PMA (Sigma). After 6 h, we took ears for cytokines analysis.

Statistical analysis. All data are presented as means ± s.e.m. We used two-tailed *t* tests to determine significances between two groups. We did analyses of multiple groups by one-way or two-way ANOVA with Bonferroni's *post* test of GraphPad Prism Version 4. For all statistical tests, we considered P values <0.05 to be statistically significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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