

***Staphylococcus aureus* membrane and diacylated lipopeptide induce thymic stromal lymphopoietin in keratinocytes through the Toll-like receptor 2–Toll-like receptor 6 pathway**

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Background: *Staphylococcus aureus* heavily colonizes the lesions of patients with atopic dermatitis (AD) and is known to trigger a worsening of AD. However, the exact mechanism by which *S aureus* promotes AD is unknown. Thymic stromal lymphopoietin (TSLP), which is highly expressed by keratinocytes in skin lesions of patients with AD and bronchial epithelial cells in asthmatic patients, represents a critical factor linking responses at interfaces between the body and the environment to allergic type 2 immune responses.

Objectives: We sought to examine the ability of synthetic lipopeptides and *S aureus* to induce TSLP expression in human keratinocytes and identify the pathway of induction.

Methods: We stimulated primary human keratinocytes with lipopeptides and *S aureus*-derived materials. The release and gene expression of TSLP were measured by means of ELISA and quantitative PCR, respectively.

Results: Diacylated lipopeptide upregulated the expression of TSLP and other proinflammatory molecules. Heat-killed *S aureus* and the subcellular fractions of *S aureus* induced TSLP's release, with the membranous fraction having the greatest activity. Small interfering RNA-mediated knockdown of either Toll-like receptor (TLR) 2 or TLR6 inhibited the diacylated lipopeptide- and *S aureus* membrane-induced TSLP gene expression. *S aureus* membrane- and diacylated lipopeptide-induced release of TSLP was enhanced by T_H2/TNF- α cytokines and partially suppressed by IFN- γ and TGF- β .

Conclusions: The results suggest that ligands for the TLR2–TLR6 heterodimer in *S aureus* membranes, including diacylated lipoproteins, could promote T_H2-type inflammation through TSLP production in keratinocytes, providing an

overall picture of the vicious cycles between colonization by *S aureus* and AD in the T_H2-skewed sensitization process, exacerbation of the disease, or both. (J Allergy Clin Immunol 2010;126:985–93.)

Key words: Thymic stromal lymphopoietin, keratinocyte, diacylated lipopeptide, *Staphylococcus aureus*, Toll-like receptor 2, Toll-like receptor 6, cytokine milieu, atopic dermatitis, vicious cycle

Staphylococcus aureus heavily colonizes the lesions of patients with atopic dermatitis (AD).^{1–5} Deficiency in the function or expression of antimicrobial peptides (AMPs) in skin and in the T_H2 cytokine milieu accounts for the susceptibility of patients with AD to *S aureus*.^{2,3} The severity of AD is reduced by treatment with a combination of antistaphylococcal antibiotics and topical glucocorticoids, suggesting an important contribution of *S aureus* to the pathogenesis of AD.^{4,5} Very recently, we demonstrated that *S aureus* extracellular protease disrupts the epidermal barrier in mice,⁶ suggesting that it helps environmental allergens and *S aureus*-derived molecules penetrate the epidermis. However, the exact mechanism by which *S aureus*-derived factors condition the epidermis to promote a T_H2-skewed sensitization or the exacerbation of AD is unknown.

Thymic stromal lymphopoietin (TSLP), which is highly expressed by keratinocytes in the lesions of patients with AD and by bronchial epithelial cells of asthmatic patients, plays a key role in allergic diseases.^{7,8} TSLP-activated dendritic cells (DCs) secrete T_H2-recruiting chemokines, but not IL-12, and induce naive T cells to differentiate into inflammatory T_H2 cells producing IL-4, IL-5, IL-13, and TNF- α through the OX40 ligand. TSLP similarly activates epidermal Langerhans cells, a subset of DCs.⁹ The TSLP-activated DCs can cause allergen-specific T_H2 memory cells to undergo homeostatic expansion and further T_H2 polarization and to mediate recall responses. TSLP can also directly act on human mast cells synergistically with IL-1 and TNF- α to produce IL-5 and IL-13,¹⁰ on human CD4⁺ T cells activated with T-cell receptor stimulation to markedly proliferate,¹¹ on human eosinophils to survive longer and produce proinflammatory molecules,¹² and on human invariant natural killer T (iNKT) cells to express IL-4 and IL-13 and further IFN- γ by being cocultured with DCs.¹³ Thus TSLP represents a critical factor linking responses at interfaces between the body and environment to allergic type 2 immune responses.

Information on environmental and endogenous triggers for the release of TSLP in keratinocytes is still limited.¹⁴ Proinflammatory and T_H2 cytokines act synergistically to induce the release of TSLP from human skin explants obtained from healthy

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Abbreviations used

AD:	Atopic dermatitis
AMP:	Antimicrobial peptide
DC:	Dendritic cell
FSL-1:	(S,R)-(2,3-bisphalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe
iNKT:	Invariant natural killer T
LTA:	Lipoteichoic acid
Pam ₃ CSK ₄ :	(S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys ₄ -OH, 3HCl
PGN:	Peptidoglycan
PolyI:C:	Polyinosinic-polycytidylic acid
siRNA:	Small interfering RNA
SNP:	Single nucleotide polymorphism
TLR:	Toll-like receptor
Treg:	Regulatory T
TSLP:	Thymic stromal lymphopoietin

donors.¹⁵ Recently, we demonstrated that polyinosinic-polycytidylic acid (polyI:C), a synthetic double-stranded RNA recognized by sensors, including Toll-like receptor (TLR) 3, is a trigger for TSLP production in primary human keratinocytes.¹⁶⁻¹⁸ An endogenous protease, kallikrein 5, also has been reported to induce TSLP production in keratinocytes through protease-activated receptor 2.¹⁹

S aureus produces various molecules that can stimulate cells independently of IgE, such as peptidoglycan (PGN), lipoproteins, and so on.²⁰ To address the mechanisms by which *S aureus* conditions the epidermis, which is composed almost entirely of keratinocytes, to promote the T_H2-skewed sensitization and exacerbation of AD, we examined the ability of synthetic lipopeptides and *S aureus*-derived materials to induce production of TSLP in human keratinocytes and identified the pathway involved.

METHODS**Reagents**

The recombinant human cytokines used to stimulate keratinocytes were TNF- α (20 ng/mL), IL-4 (100 ng/mL), IL-13 (100 ng/mL), IFN- γ (100 ng/mL), TGF- β (10 ng/mL), and IL-17A (IL-17; 100 ng/mL; R&D Systems, Minneapolis, Minn). The TLR ligands used to stimulate keratinocytes were (S,R)-(2,3-bisphalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (10-1000 ng/mL; (FSL-1; InvivoGen, San Diego, Calif); (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys₄-OH, 3HCl (5 μ g/mL; Pam₃CSK₄; Calbiochem, San Diego, Calif); PGN from *S aureus* (20 μ g/mL; Sigma, St Louis, Mo); and polyI:C (10-1,000 ng/mL; GE Healthcare, Buckinghamshire, United Kingdom).

Subcellular fractionation of *S aureus*

S aureus strain MW2, which was isolated from a patient with fatal septicemia and septic arthritis,²¹ was subjected to subcellular fractionation (subcellular secreted, cell-wall, membranous, and cytoplasmic fractions), basically as described by Baba and Schneewind.²² Concentrations of the subcellular fractions were expressed in units per milliliter (1 U corresponds to the sample prepared from 1 mL of overnight bacterial culture of approximately 3×10^9 bacterial cells). For further information, see the Methods section in this article's Online Repository at www.jacionline.org.

Preparation of heat-killed *S aureus*

D-YSD1 and D-ISK1 were *S aureus* strains isolated from patients with AD. *S aureus* strains MW2, D-YSD1, and D-ISK1 were grown to the stationary

phase. The *S aureus* cells were harvested, followed by heat inactivation. For further information, see the Methods section in this article's Online Repository.

Cell culture and stimulation of keratinocytes

Primary human keratinocytes (Cascade Biologics, Portland, Ore) were cultured and stimulated with TLR ligands or materials from *S aureus* with or without cytokines.¹⁸ Culture supernatants for ELISA and total RNAs for quantitative real-time PCR were recovered. In some experiments, as described in the figure legends, keratinocytes were induced to differentiate by FBS and were stimulated, and culture supernatants for ELISA were recovered. For further information, see the Methods section in this article's Online Repository.

ELISA and real-time quantitative PCR

Concentrations of cytokines and chemokines were measured with ELISA kits (DuoSet, R&D Systems). Total RNA was extracted from the cells, and cDNA was synthesized as described previously.¹⁶ Real-time quantitative PCR was performed by using the Taqman method with an ABI7500 device (Applied Biosystems, Piscataway, NJ). The mRNA level was normalized to the gene expression of β -actin and is shown relative to the control group.

Transfection of keratinocytes with small interfering RNA

Keratinocytes were transfected with small interfering RNAs (siRNAs) for TLR1, TLR2, and TLR6 and control siRNAs with a similar guanine-cytosine content to the TLR siRNAs and were stimulated with FSL-1 or the *S aureus* membranous fraction. The expression of mRNA at 4 hours after stimulation relative to that in medium was analyzed by means of quantitative real-time PCR. For further information, see the Methods section in this article's Online Repository.

Statistical analysis

A 1-way ANOVA or the Mann-Whitney *U* test (2-tailed) was used. *P* values of less than .05 were regarded as statistically significant.

RESULTS**Diacylated lipopeptide induced release of TSLP in keratinocytes**

We examined whether PGN from *S aureus*, the synthetic triacylated lipopeptide Pam₃CSK₄, and the synthetic diacylated lipopeptide FSL-1 induce the release of TSLP. Keratinocytes treated with FSL-1 released detectable amounts of TSLP and IL-8 (Fig 1). PGN and Pam₃CSK₄ did not induce the release of TSLP at the concentrations tested. Pam₃CSK₄ induced the release of IL-8 but not TSLP.

The stimulation of keratinocytes with FSL-1 induced the gene (Fig 2, A) or protein (Figs 1 and 2, B) expression of not only TSLP but also other proinflammatory molecules, whereas Pam₃CSK₄ induced lower levels or no significant upregulation of their expression.

Diacylated lipopeptide-induced TSLP gene expression was dependent on expression of TLR2 and TLR6 in keratinocytes

TLR2 forms a heterodimer with either TLR6 or TLR1 for the specific recognition of diacylated or triacylated lipoproteins/

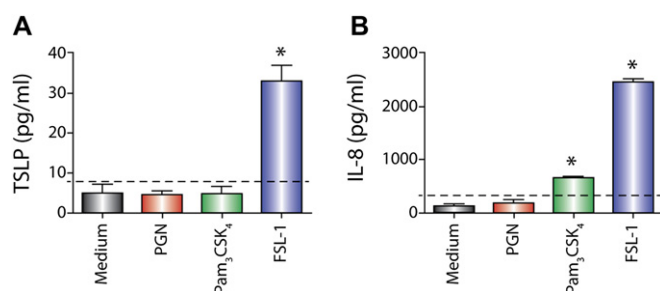


FIG 1. FSL-1 induced the release of TSLP in keratinocytes. Keratinocytes were stimulated with PGN (20 μ g/mL), triacylated lipopeptide (Pam₃CSK₄, 5 μ g/mL), or diacylated lipopeptide (FSL-1, 1 μ g/mL). **A**, TSLP. **B**, IL-8. *Broken line*, Minimum detection limit. Data shown are the means $\pm$ SDs for 3 wells and are representative of 3 independent experiments with similar results. * $P < .001$ versus the medium by means of ANOVA with the Tukey multiple comparison test.

lipopeptides, respectively.²³⁻²⁵ The gene expression of TLR1, TLR2, or TLR6 was knocked down by siRNAs to identify the receptors that recognize FSL-1 and contribute to the expression of TSLP in keratinocytes (Fig 3, A). Knockdown of TLR2 and TLR6, but not TLR1, significantly suppressed the FSL-1–induced gene expression of TSLP, resulting in a 67%, 82%, and 8% reduction by siRNA for TLR2, TLR6, and TLR1, respectively (Fig 3, B).

Th2 cytokines and TNF- α upregulated diacylated lipopeptide–induced release of TSLP in keratinocytes

To test whether a cytokine milieu that mimics the atopic environment modulates the diacylated lipopeptide–induced release of TSLP, we examined whether TNF- α together with the Th2 cytokines IL-4 and IL-13 (Th2/TNF cytokines) enhances the release of TSLP. FSL-1 alone at 0.1 and 1 μ g/mL or Th2/TNF cytokines without FSL-1 significantly induced the release of TSLP, whereas the combination of FSL-1 and Th2/TNF cytokines enhanced the release of TSLP and IL-8 (Fig 4).

S aureus membrane induced TSLP gene expression in a manner dependent on expression of TLR2 and TLR6 in keratinocytes

In patients with AD, colonization of lesional skin by *S aureus* is greater than colonization of nonlesional skin,¹ and TSLP is overexpressed in keratinocytes in the lesions.⁷ Very recently, diacylated²⁶ and triacylated²⁷ lipoproteins have been identified in *S aureus*. To test whether *S aureus*–derived molecules induce TSLP expression similarly to the synthetic diacylated lipopeptide, we examined the response of keratinocytes to *S aureus*–derived materials (Figs 5-8).

Stimulation of keratinocytes with the membranous fraction of *S aureus* induced the gene expression of TSLP and IL-8 similarly to FSL-1 (Fig 5, A). Knockdown of the expression of TLR2 and TLR6, but not TLR1, suppressed the membrane-induced TSLP gene expression, resulting in an 85% and 90% reduction by siRNAs for TLR2 and TLR6, respectively (Fig 5, B and C; see also Fig E1 in this article's Online Repository at www.jacionline.org).

Levels of mRNA for TLR1, TLR2, and TLR6 in unstimulated keratinocytes were determined. The average ratio of their

expression levels in 5 independent experiments was approximately 10:40:1, respectively (Fig 5, D).

S aureus membrane induced release of TSLP in keratinocytes

Next we examined the release of the TSLP protein in the response of keratinocytes to subcellular fractions of *S aureus* (Fig 6). In Figs 6 to 8 keratinocytes were induced to differentiate by culturing with 5% FBS before stimulation. The *S aureus* membrane–induced release of TSLP protein was efficient and highly reproducible in the differentiated keratinocytes, although undifferentiated keratinocytes released detectable amounts of TSLP in response to the *S aureus* membrane (our unpublished observations).

Supernatants of the overnight bacterial culture and fractions of the bacterial cell wall, membrane, and cytoplasm induced the release of TSLP and IL-8 (Fig 6, A and B). The membranous fraction achieved the most efficient release of TSLP (Fig 6, A). The membranous fraction, synthetic diacylated lipopeptide (FSL-1), and synthetic double-stranded RNA (polyI:C) induced TSLP's release in a dose-dependent manner, but the synthetic triacylated lipopeptide (Pam₃CSK₄) did not (Fig 6, C).

Effect of cytokines on *S aureus* membrane– and diacylated lipopeptide–induced release of TSLP in keratinocytes

Next we tested whether the cytokine milieu modulates the *S aureus* membrane– and FSL-1–induced release of TSLP in the differentiated keratinocytes (Fig 7). IFN- γ , TGF- β , IL-17, and Th2/TNF cytokines could be provided by cells such as Th1 cells, regulatory T (Treg) cells, Th17 cells, and inflammatory Th2 cells, respectively.^{8,28}

IFN- γ and TGF- β showed partial inhibition of the release of TSLP induced by FSL-1, the *S aureus* membranous fraction, Th2/TNF cytokines, and the *S aureus* membranous fraction plus Th2/TNF cytokines in the differentiated keratinocytes (Fig 7, A, IFN- γ and TGF- β). IL-17 showed smaller inhibition of the release of TSLP induced by FSL-1 and Th2/TNF cytokines (Fig 7, A, IL-17). IFN- γ and TGF- β showed partial inhibition of the induction of IL-8, whereas IL-17 showed enhancement (Fig 7, B).

Heat-killed *S aureus* induced release of TSLP in keratinocytes

Heat-killed whole bacterial cells of *S aureus* strains isolated from patients with AD, the D-YSD1 and D-ISK1 strains, showed the capacity to induce the release of TSLP and IL-8 similarly to the MW2 strain in the differentiated keratinocytes, and the release was enhanced in the presence of TNF- α (Fig 8).

DISCUSSION

The human innate immune system recognizes bacterial lipoproteins through TLR2. Some recent reports revealed that, among bacterial products reported as TLR2 agonists, only lipoproteins/lipopeptides are sensed at physiological concentrations by TLR2 and suggested that the TLR2 agonistic activity of the fractions prepared from bacteria, such as PGN and lipoteichoic acid (LTA), is likely due to contaminating, highly active natural lipoproteins/

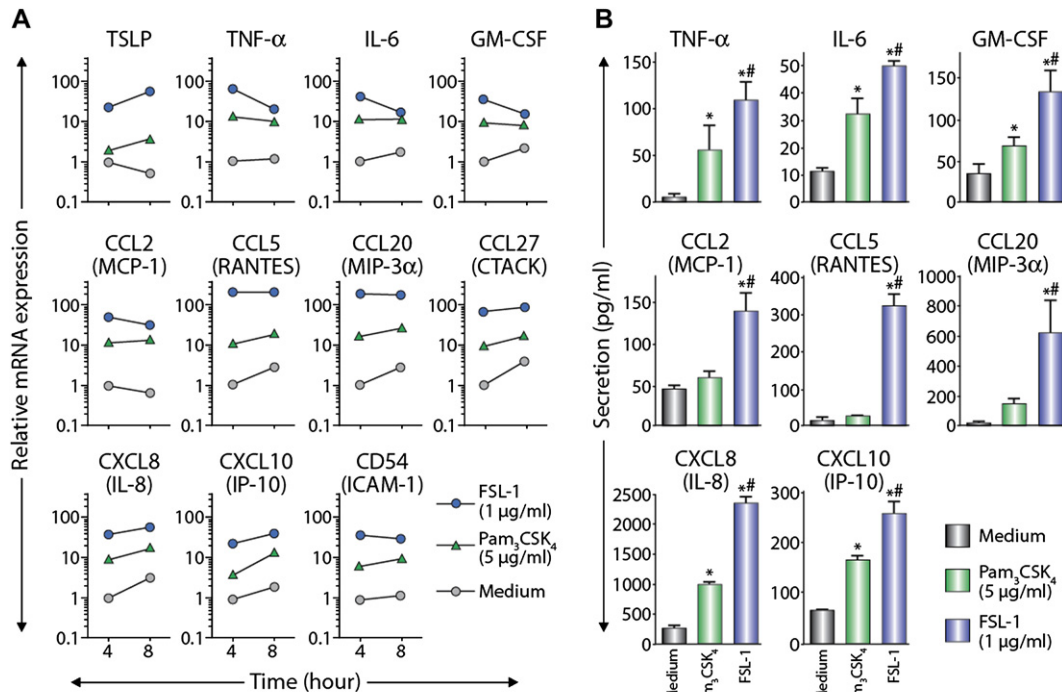


FIG 2. FSL-1 induced upregulation of the expression of TSLP and other proinflammatory molecules in keratinocytes. **A**, Levels of mRNA are presented relative to the medium at 4 hours. **B**, Protein concentrations in the culture supernatant. Data shown are the values for single wells (Fig 2, A) and means $\pm$ SDs for 3 wells (Fig 2, B) and are representative of 3 independent experiments with similar results. * $P < .05$ versus the medium and # $P < .05$ versus Pam₃CSK₄ by means of ANOVA with the Tukey multiple comparison test (Fig 2, B). Levels of cytokines (TSLP, TNF- α , IL-6, and GM-CSF), chemokines (CCL2/monocyte chemoattractant protein 1 [MCP-1], CCL5/RANTES, CCL20/macrophage inflammatory protein 3 α [MIP-3 α], CCL27/cutaneous T cell-attracting chemokine [CTACK], CXCL8/IL-8, and CXCL10/IFN-inducible protein 10 [IP-10]), and an adhesion molecule (CD54/intercellular adhesion molecule 1 [ICAM-1]) were measured.

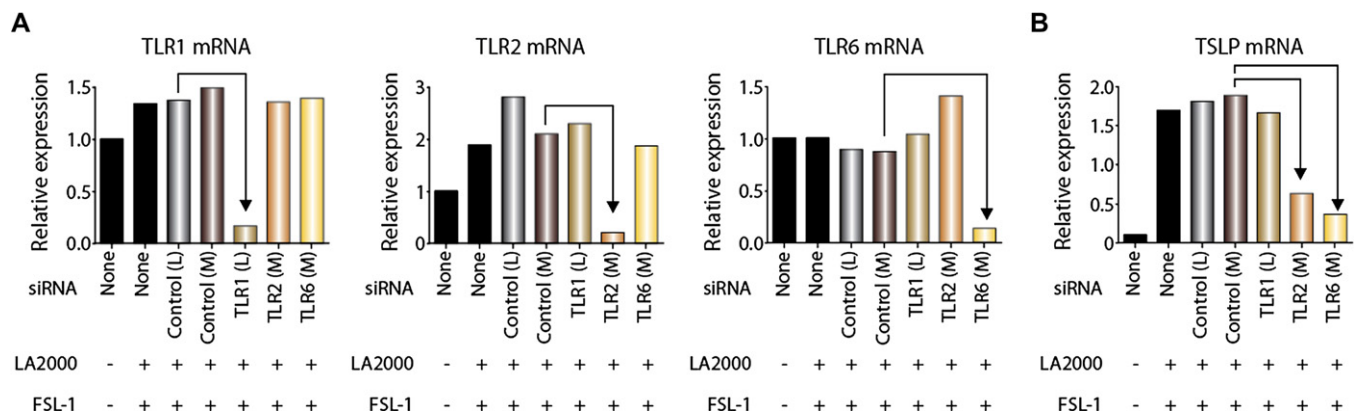


FIG 3. Knockdown of TLR2 and TLR6 inhibited the FSL-1-induced TSLP mRNA expression in keratinocytes. **A** and **B**, Keratinocytes transfected with siRNAs by using lipofectamine2000 (LA2000) were stimulated with FSL-1 (1 μ g/mL). L and M, Thirty-five percent to 45% and 45% to 55% guanine-cytosine content, respectively. TLR1-siRNA1, TLR2-siRNA3, and TLR6-siRNA3 were used. Data shown are the values for single wells and representative of 3 independent experiments with similar results.

lipopeptides.^{26,27,29,30} TLR2 forms a heterodimer with either TLR6 or TLR1 for the specific recognition of diacylated or triacylated lipoproteins/lipopeptides, respectively.²³⁻²⁵ In the present study we demonstrated that FSL-1 (a chemically synthesized lipopeptide composed of the diacylated N-terminal moiety of a *Mycoplasma* species-derived lipoprotein),³¹ subcellular fractions of

S aureus, and heat-killed whole bacterial cells of *S aureus* can stimulate human keratinocytes to release TSLP protein (Figs 1, 6, and 8). The *S aureus* membrane upregulated TSLP mRNA expression in a manner dependent on the expression of TLR2 and TLR6 (Fig 5 and Fig E1), as well as FSL-1 (Figs 2 and 3), although a disproportion between the expression levels of TLR2 and TLR6

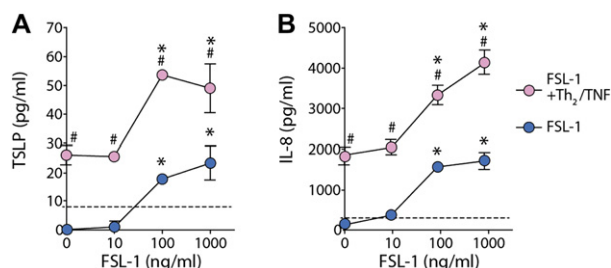


FIG 4. T_H2 cytokines and $TNF-\alpha$ upregulated the FSL-1-induced release of TSLP in keratinocytes. **A**, TSLP. **B**, IL-8. Broken line, Minimum detection limit. Data shown are the means $\pm$ SD for 3 wells and representative of 3 independent experiments with similar results. * $P < .001$ versus without FSL-1 and # $P < .001$ versus without $T_H2/TNF-\alpha$ by means of ANOVA with the Tukey multiple comparison test.

was observed (Fig 5, D, and our unpublished data obtained by means of flow cytometry). The *S aureus* membrane- and diacylated lipopeptide-induced release of TSLP were enhanced in the atopic cytokine milieu (Figs 4, A, and 7, A, + T_H2/TNF) and was partially inhibited in the presence of $IFN-\gamma$ and $TGF-\beta$ (Fig 7, A). The results suggest that the colonization by *S aureus* contributes to the pathogenesis or exacerbation of AD, being linked with TSLP expression through activation of the TLR2-TLR6 heterodimer by diacylated lipoproteins of *S aureus* (see Fig E2, A, in this article's Online Repository at www.jacionline.org), and that the cytokine milieu positively or negatively modulates the vicious cycles (see Fig E2, B). Cell numbers of heat-killed *S aureus* by area unit in the culture plates ($7.67 \log \text{ cells/cm}^2$ in Fig 8 and $6.67 \log \text{ cells/cm}^2$ in our unpublished data), which can induce the detectable amount of TSLP, are considered relevant in *S aureus* colonization in the skin lesions of patients with AD according to our calculation on the basis of the values reported previously (such as by Travers et al³²: the mean $\pm$ SD of $\log \text{ cells/cm}^2 = 5.1 \pm 2.0 = 3.1\text{--}7.1$ and >7.6 at the maximum levels).

Induction of TSLP by *S aureus* and lipopeptide through the TLR2-TLR6 pathway

The subcellular fractions of *S aureus* induced the release of TSLP in keratinocytes (Fig 6, A), although in the preparation of the cell-wall fraction, the rigid cell-wall structure was disrupted by enzymatic degradation of PGN with lysostaphin.²² The membranous fraction was most efficient at releasing TSLP (Fig 6, A). The prolipoprotein diacylglycerol transferase Lgt transfers a diacylglyceride moiety to bacterial proteins, and subsequent cleavage of the signal peptide results in diacylated lipoproteins. Subsequently, the apolipoprotein *N*-acetyltransferase Lnt adds a third fatty acid to the diacylated lipoproteins, resulting in triacylated lipoproteins.²³ The lack of conventional Lnt in *S aureus* suggests the efficient accumulation of diacylated lipoproteins in membranes, and very recently, Tawaratsumida et al²⁶ identified diacylated lipoproteins in the *S aureus* membrane. These results support our finding that the induction of TSLP expression by the *S aureus* membrane in keratinocytes is mostly dependent on TLR2 and TLR6 (Figs 5 and 6 and see Fig E1), those components of the heterodimeric receptor essential to the recognition of diacylated lipoproteins/lipopeptides.^{23,24}

The synthetic triacylated lipopeptide Pam₃CSK₄, a ligand for the TLR2-TLR1 heterodimer, and PGN did not induce the release

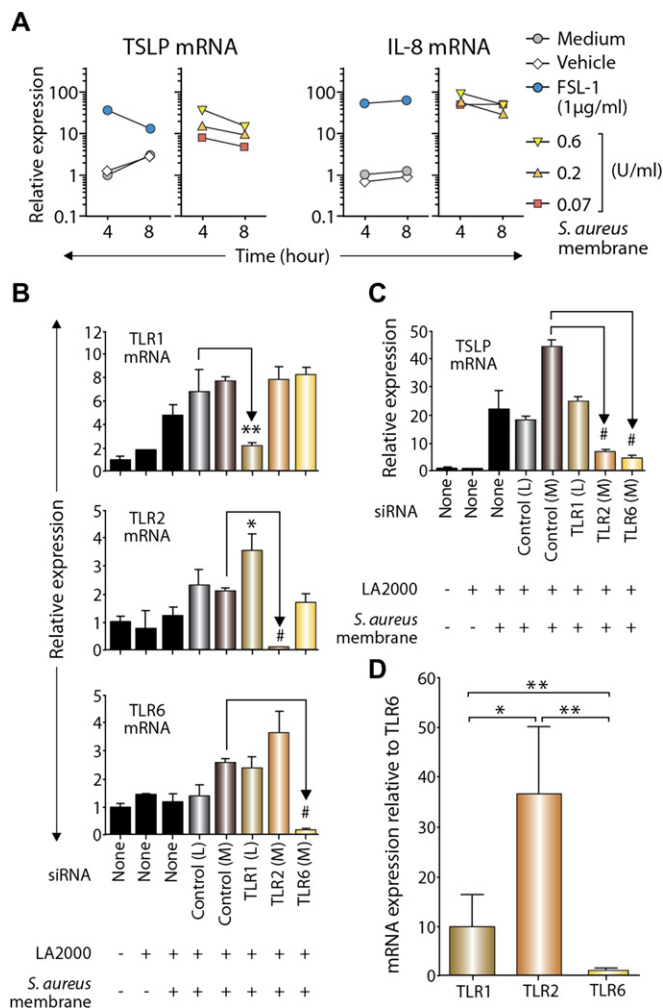


FIG 5. *S aureus* membranous fraction induced an upregulation of the gene expression of TSLP in a manner dependent on the expression of TLR2 and TLR6 in keratinocytes. **A**, Dose dependency. **B** and **C**, Keratinocytes transfected with siRNAs were stimulated with the membranous fraction of the *S aureus* MW2 strain (1.7 U/mL). L and M, Thirty-five percent to 45% and 45% to 55% guanine-cytosine content, respectively. TLR1-siRNA1, TLR2-siRNA3, and TLR6-siRNA2 were used. Data shown are the values for single wells (Fig 5, A) or the means $\pm$ SDs for 3 wells (Fig 5, B and C) and are representative of 3 independent experiments with similar results. * $P < .05$, ** $P < .01$, and # $P < .001$ versus the control siRNA by means of ANOVA with the Tukey multiple comparison test (Fig 5, B and C). **D**, mRNA expression of TLRs relative to the average of TLR6 expression. Data shown are the means $\pm$ SDs for 5 independent experiments. * $P < .05$ and ** $P < .01$ by using the Mann-Whitney *U* test (2-tailed).

of a detectable amount of TSLP, but the TLR2-TLR6 ligand FSL-1 did, even at a lower concentration (Figs 1 and 6, C). Pam₃CSK₄ induced an upregulation of TSLP gene expression in keratinocytes, although inefficiently compared with FSL-1 (Fig 2, A). Kurokawa et al²⁷ identified an *S aureus* triacylated lipoprotein, suggesting the presence of another type of *N*-acetyltransferase not homologous to the conventional Lnt. Therefore we do not exclude the possibility that Pam₃CSK₄, the PGN preparation, or the *S aureus*-derived triacylated lipoprotein could induce release of TSLP in keratinocytes in other experimental conditions, such as at higher concentrations. However, TLR1 was expressed at a higher level than TLR6 at the mRNA level (Fig 5, C), which is

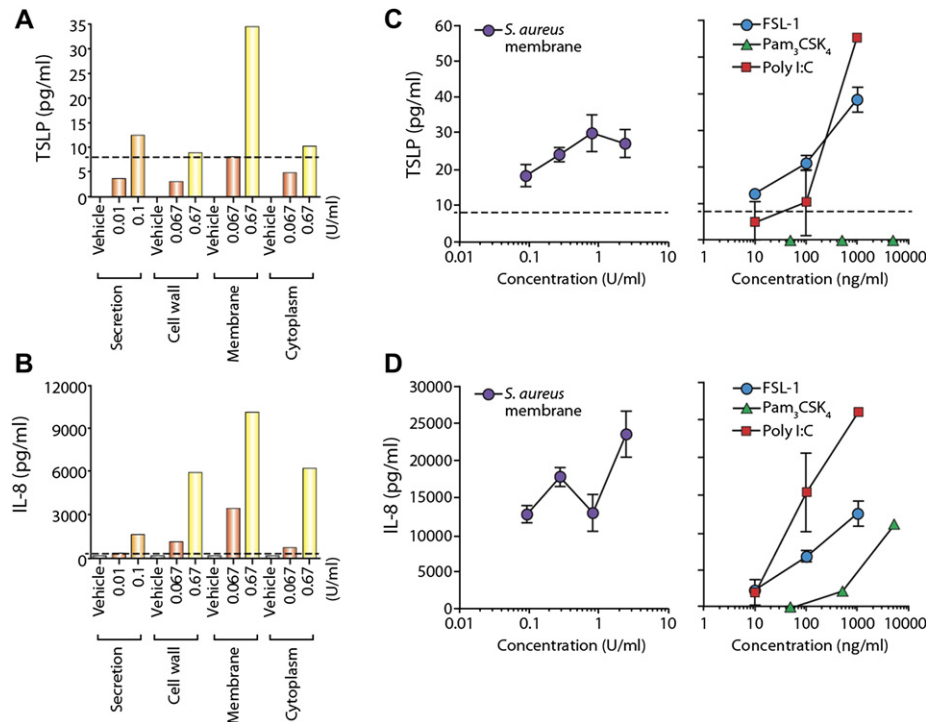


FIG 6. *S aureus* membranous fraction induced release of TSLP in keratinocytes. Keratinocytes were induced to differentiate by culturing with FBS before stimulation. **A** and **B**, Release of TSLP and IL-8 on stimulation with the subcellular fractions of the *S aureus* MW2 strain. **C** and **D**, Dose dependency of the release of TSLP (Fig 6, C) and IL-8 (Fig 6, D) by the *S aureus* membranous fraction, FSL-1, Pam₃CSK₄, and polyI:C. Broken line, Minimum detection limit. Data shown are the values for single wells (Fig 6, A and B) or the means $\pm$ SDs for 3 wells (Fig 6, C and D) and are representative of 3 independent experiments with similar results.

consistent with the findings of Kollisch et al.³³ Although the cause of the discrepancy is unknown, the ligand content in *S aureus*, the amino acid sequence, the receptor-ligand binding affinity, or expression levels of other coreceptors in keratinocytes might affect the keratinocyte response to *S aureus* lipoproteins and the synthetic lipopeptides.

Vicious cycles between *S aureus* and AD and modulation by the cytokine milieu

The important contribution of *S aureus* to the worsening of AD has been suggested.¹⁻⁵ Our demonstration that *S aureus*-derived factors induce TSLP expression in human keratinocytes (Figs 5 and 6), along with our very recent finding that extracellular protease of *S aureus* induced epidermal barrier dysfunction *in vivo* in mice,⁶ can well explain how colonization by *S aureus* contributes to the pathogenesis or exacerbation of AD (see Fig E2, A). First, *S aureus* could disrupt the skin barrier or enhance barrier dysfunction, a genetically disposed condition³⁴ or else one usually observed in infancy, by the *S aureus* protease,⁶ obtaining access to keratinocytes. In the second step the *S aureus*-derived ligands for the TLR2-TLR6 heterodimer could induce expression of TSLP, the master switch for T_H2 responses, in keratinocytes (Figs 5 and 6), leading to T_H2 skewing in the sensitization to environmental allergens and *S aureus*-derived allergens^{35,36} through the skin, the exacerbation of AD, or both.

A combination of TNF- α and the T_H2 cytokines IL-4 and IL-13 that mimics the atopic environment (Figs 4, A and 7, A, + T_H2/

TNF) or TNF- α alone (Fig 8, A, + TNF- α) enhanced the *S aureus*-induced release, diacylated lipopeptide-induced release, or both of TSLP in keratinocytes. Although contribution of the T_H2 cytokines to the *S aureus*- and diacylated lipopeptide-induced TSLP production is yet to be investigated, proinflammatory and T_H2 cytokines have been reported that act synergistically to induce the release of TSLP from human skin explants obtained from healthy donors.¹⁵ The release of TSLP induced by *S aureus* could be more effective in AD skin than in healthy skin because AD is associated with T_H2 inflammation and allergen-specific IgE, which stimulates mast cells and basophils to produce such cytokines through the high-affinity IgE receptor on exposure to allergens. The T_H2 cytokine milieu in the skin could promote colonization by *S aureus* through a reduction in the expression of inducible AMPs² and inhibition of the mobilization of an AMP, β -defensin 3,³ and could reduce the skin barrier function through a reduction in the expression of filaggrin,³⁷ which is critical for an effective skin barrier and whose genetic mutations are a major predisposing factor for AD,³⁴ in cooperation with *S aureus* protease,⁶ enabling the exposure of keratinocytes to *S aureus*-derived factors. In turn, the colonization could exacerbate the T_H2-type inflammation by inducing expression of TSLP, which contributes to differentiation of T_H2/TNF-producing inflammatory T_H2 cells,⁸ in keratinocytes (Figs 5 and 6) synergistically with the T_H2 and inflammatory microenvironment (T_H2/TNF cytokine milieu; Figs 4, A, and 7, A, + T_H2/TNF). This clearly explains the mechanism behind the vicious cycle between AD and *S aureus* colonization (see Fig E2, A).

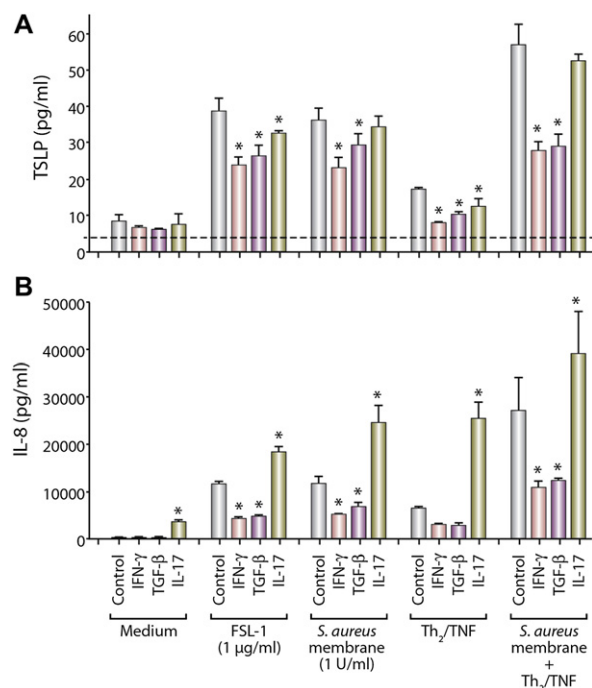


FIG 7. Effect of cytokines on *S aureus* membrane- and FSL-1-induced release of TSLP in keratinocytes. Keratinocytes were induced to differentiate by culturing with FBS before stimulation. Release of TSLP (**A**) and IL-8 (**B**) on stimulation with the membranous fraction of the *S aureus* MW2 strain was measured. Broken line, Minimum detection limit. Data shown are the means $\pm$ SDs for 3 wells and representative of 3 independent experiments with similar results. * $P < .05$ versus the medium by means of ANOVA with the Newman-Keuls multiple comparison test.

Interestingly, direct action of TSLP on iNKT cells to secrete IL-4 and IL-13 and further IFN- γ by being cocultured with DCs was reported.¹³ iNKT cells migrate into the AD skin lesions,³⁸ and their numbers in the skin lesions were greater in patients with severe AD than in those with mild and modest AD.¹³ Taken together with our results in the present study, TSLP being constitutively induced by *S aureus* in keratinocytes might stimulate the iNKT cells in the AD skin lesions to secrete the Th₂ cytokines, forming another vicious cycle between colonization by *S aureus* and AD. Direct action of TSLP on mast cells,¹⁰ Th₁ cells,¹¹ and eosinophils¹² can also contribute to the worsening of AD.

Previously, we demonstrated that IFN- γ , TGF- β , and IL-17 significantly suppressed polyI:C-induced release of TSLP in keratinocytes *in vitro*.¹⁶ In the present study we examined their capacity to modulate the *S aureus* membrane- and diacylated lipopeptide-induced release of TSLP in differentiated keratinocytes. IFN- γ and TGF- β partially inhibited the TSLP induction (Fig 7, A), suggesting that the interaction of keratinocytes with Th₁ or Treg cells could limit TSLP's release in *S aureus*-stimulated keratinocytes, even in the presence of Th₂/TNF cytokines. However, cytokine switch from Th₂ in the acute phase to Th₁ in the chronic phase is accepted for AD; apoptosis induction in keratinocytes indirectly by IFN- γ has been interpreted as an important event in eczema, mainly in patients with AD³⁹; and staphylococcal enterotoxin B inhibits natural Treg cells *in vitro*, although patients with AD have increased numbers of peripheral blood Treg cells.⁴⁰ Recently, Koga et al⁴¹ reported that the percentage of Th₁₇ cells in peripheral blood was higher in patients with AD

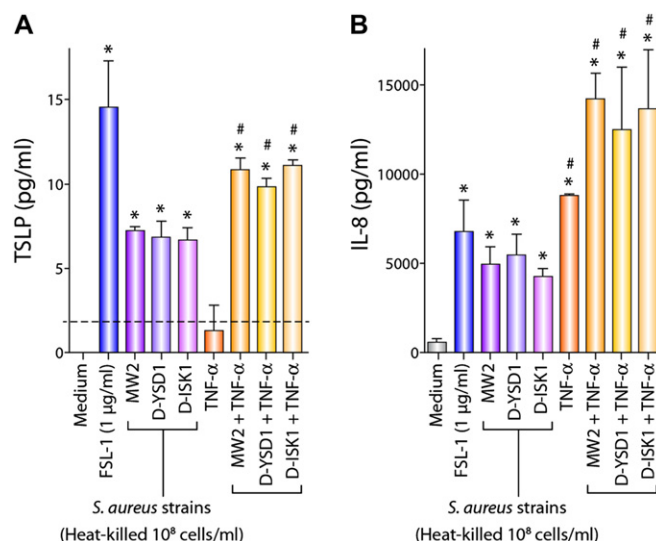


FIG 8. Heat-killed bacterial whole cells of *S aureus* strains isolated from patients with AD induced release of TSLP in keratinocytes. Keratinocytes were induced to differentiate by culturing with FBS before stimulation. Release of TSLP (**A**) and IL-8 (**B**) on stimulation with the heat-killed *S aureus* cells of the MW2 strain and 2 strains isolated from patients with AD (D-YSD1 and D-ISK1). Broken line, Minimum detection limit. Data shown are the means $\pm$ SDs for 3 wells and representative of 3 independent experiments with similar results. * $P < .05$ versus the medium and # $P < .05$ versus without TNF- α by means of ANOVA with the Newman-Keuls multiple comparison test.

and that IL-17⁺ cells infiltrated in the papillary dermis of patients with atopic eczema more markedly in acute lesions than in chronic lesions. IL-17 has been suggested to have important roles in skin innate immunity.⁴² Interestingly, very recently, IL-17 produced by epidermal $\gamma\delta$ T cells was reported to be essential for host defense against cutaneous *S aureus* infection in mice.⁴³ Overall relevance of IFN- γ , TGF- β , and IL-17 to suppression or worsening of AD in the acute and chronic phases remains to be elucidated. Possible contribution of IFN- γ , TGF- β , IL-17, IL-22 (another Th₁₇-derived cytokine), and so on to the modulation of the vicious cycles between *S aureus* and AD is schematically represented in Fig E2, B.

Gene-environment interaction

Two single nucleotide polymorphisms (SNPs) in the human *TLR2* gene, Arg753Gln within the intracellular portion^{36,44,45} and A-16934T in the promoter region,⁴⁶ have been reported to be associated with the severity of AD in Germany. Monocytes from patients with AD with the *TLR2* Arg753Gln SNP produced more IL-6 after stimulation with LTA or PGN⁴⁴ but less IL-8 after stimulation with PGN⁴⁵ than did those from patients with AD without the mutation. Monocytes from patients with AD homozygous for the *TLR2* -16934-A allele produced more IL-6 after stimulation with Pam₃CSK₄ than did those of patients homozygous for the *TLR2* -16934-T allele.⁴⁶ Whether keratinocytes from patients with AD show higher TSLP responses to *S aureus* and diacylated lipopeptide than those from healthy donors and how these SNPs function in the *S aureus*- or diacylated lipopeptide-dependent TSLP production seen in keratinocytes should be examined in future studies.

Interestingly, Travers et al³² very recently found that lesional skin of patients with AD with *S aureus* contained a considerable

amount of LTA by detecting a protein reported to be associated with the LTA fraction. Severity of AD is also associated with the amount of *S aureus* DNA in dust from the bed and floor,⁴⁷ suggesting that exposure to *S aureus*-derived factors in the home might also contribute to the severity and persistence of AD. We assume the amount of LTA³² or *S aureus* DNA⁴⁷ to correlate with that of *S aureus* lipoproteins/lipopeptides on the skin or in the indoor environment, respectively.

In summary, the effect of synthetic lipopeptides (Figs 1-4 and 6), subcellular fractions of *S aureus* (Figs 5 and 6 and see Fig E1), and heat-killed whole bacterial cells of *S aureus* strains, including those isolated from patients with AD (Fig 8), on human keratinocytes suggests a contribution of *S aureus*-derived factors, including diacylated lipoproteins/lipopeptides, to T_H2-type inflammation in the skin through expression of TSLP induced through the TLR2-TLR6 pathway. The dependency on TLR2 of the TSLP expression induced by the membranous fraction of *S aureus* (Fig 5, B and C) supports the recent findings that SNPs in the human *TLR2* gene are associated with the severity of AD.^{36,44-46} Given that the T_H2 cytokine milieu suppresses the function³ or induction² of AMPs to kill *S aureus* and downregulates filaggrin expression³⁷ and that *S aureus* protease disrupts epidermal barrier function,⁶ the present article helps provide an overall picture of the vicious cycles between *S aureus* colonization and AD (see Fig E2, A) and that of possible regulation of the cycles by other cytokine milieus (Fig 7 and see Fig E2, B). A reduction in levels of *S aureus*, a trigger of TSLP expression, is considered beneficial to the treatment of AD, and the recent elucidation of the structural basis of the ligand's recognition by the TLR2-TLR6 heterodimer²⁴ could lead to new therapeutic approaches (ie, the development of safe and effective topical drugs that specifically antagonize the TLR2-TLR6 pathway).

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Clinical implications: *S aureus* induces TSLP in keratinocytes through TLR2-TLR6, providing evidence of the vicious cycle between colonization by *S aureus* and AD and suggesting new drug targets in the treatment of AD.

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METHODS

Subcellular fractionation of *S aureus*

S aureus strain MW2^{E1} was grown overnight in tryptic soy broth (Becton, Dickinson, and Company, Sparks, Md) at 37°C and subjected to subcellular fractionation, basically as described by Baba and Schneewind.^{E2} Briefly, the cell wall was enzymatically solubilized by lysostaphin in a buffer osmotically equivalent to the cytoplasm after removal of the culture medium as an extracellular fraction. Isolation of the cell-wall fraction by means of centrifugation was then followed by the resuspension of protoplasts in the membrane buffer.^{E2} Freeze-thaw cycles were repeated 5 times to disturb the integrity of the cytoplasmic membrane that was recovered by means of subsequent ultracentrifugation. The supernatant after ultracentrifugation was then isolated as the cytoplasm. The membrane was resuspended in the membrane buffer by means of serial passage through a 27-gauge syringe needle, and the suspension was used for experiments.

Preparation of heat-killed bacterial cells of *S aureus* strains

S aureus strains were grown in tryptic soy broth at 37°C to the stationary phase. Cell numbers were determined by means of measurement of optical density at 562 nm with NovaspecII (Amersham, Amersham, United Kingdom). The *S aureus* cells were harvested by means of centrifugation, followed by heat inactivation by means of incubation at 70°C for 60 minutes.

Cell culture and stimulation of keratinocytes

Primary human keratinocytes (Cascade Biologics, Portland, Ore) were cultured in EpiLife KG2 (Kurabo, Osaka, Japan) supplemented with 0.1 ng/mL epidermal growth factor, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 50 µg/mL gentamicin, 50 ng/mL amphotericin B, and 0.4% vol/vol bovine brain pituitary extract. Cells were seeded at 8×10^3 cells/well in flat-bottomed 96-well microculture plates (for ELISA) or 7×10^4 in 12-well plates (for PCR) and 3×10^4 in 24-well plates (for PCR and multiple-cytokine ELISA) and cultured until 100% confluent. The medium was then changed to one without hydrocortisone.^{E3} After a further 24 hours in culture, cells were stimulated with TLR ligands or materials from *S aureus* with or without cytokines in fresh medium without hydrocortisone. Culture supernatants for ELISA were recovered at 24 hours after stimulation. Total RNAs for quantitative real-time PCR were recovered at 4 hours, 8 hours, or both after stimulation.

In some experiments, as described in the figure legends, keratinocytes were induced to differentiate by FBS as follows. Cells were cultured until they

reached 70% to 80% confluence, and the medium was changed to one with the supplements and 5% FBS and renewed every 3 days. At day 9, the medium was replaced with that not containing FBS. At day 10, the keratinocytes were stimulated in fresh medium without hydrocortisone and FBS. Culture supernatants for ELISA were recovered at day 12 (48 hours after stimulation).

Transfection of keratinocytes with siRNA

Cells were cultured until 70% to 80% confluent; then the medium was changed to basal medium without the supplements, and the cells were incubated for 8 hours. Keratinocytes were transfected with the following Stealth siRNAs using Lipofectamine2000 (Invitrogen, Carlsbad, Calif): TLR1-siRNA1, 5'-AGACCUUGCUGAUUAUCAAUGAGC-3'; TLR2-siRNA3, 5'-UUCAGAGUGAGCAAAGUCUCUCCGG-3'; TLR6-siRNA2, 5'-UGGGAAUGCUGUUCUGUGGAAUGGG-3'; TLR6-siRNA3, 5'-AAUAAGUCCGCGUGCAUGAGAGC-3'. We also used control siRNAs with a similar guanine-cytosine content to the TLR siRNAs (Stealth RNAi negative universal controls LO#2 and Med#2, Invitrogen). Lipofectamine2000 (2 µL) was mixed with 2 µL of a 20 µmol/L siRNA solution and 100 µL of OPTI-MEM (Gibco BRL, Gaithersburg, Md). After incubation for 30 minutes at room temperature, basal medium without the supplements was added to a total volume of 600 µL. Then 600 or 400 µL of solution was added per well to the 12-well or 24-well plates, respectively. After cultivation with siRNAs overnight, the medium was changed to hydrocortisone-free medium. After further cultivation for 24 hours, keratinocytes were stimulated with FSL-1 or the *S aureus* membranous fraction in hydrocortisone-free medium. The expression of mRNA at 4 hours after stimulation relative to that in the medium was analyzed by means of quantitative real-time PCR.

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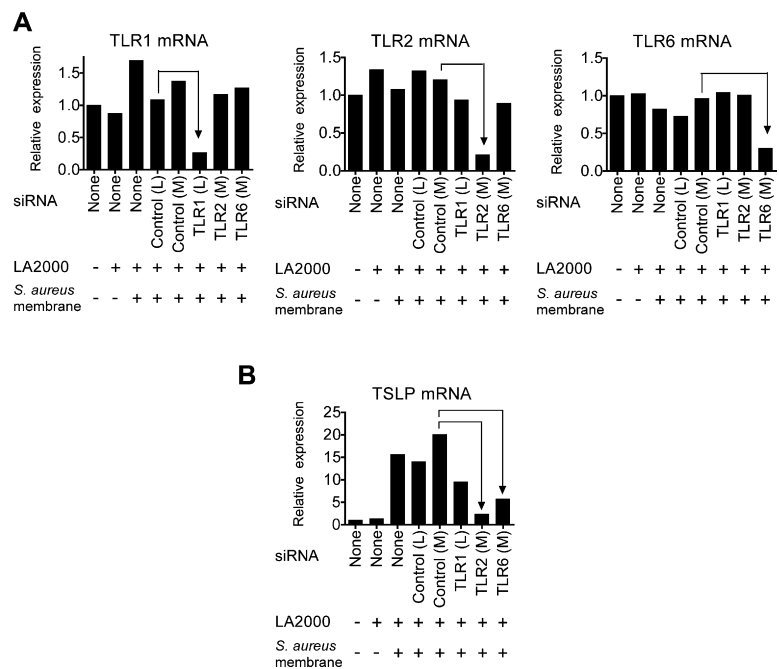


FIG E1. Knockdown of TLR2 and TLR6 inhibited *S aureus* membrane–induced upregulation of TSLP mRNA expression in keratinocytes. In Fig 5, B and C, the level of TLR1 mRNA after stimulation with the membranous fraction was suppressed by means of TLR1 siRNA compared with the control siRNA with the low guanine-cytosine content but was higher than that seen in unstimulated keratinocytes (Fig 5, B, TLR1 mRNA). Therefore results obtained in another experiment, in which the level of TLR1 mRNA in keratinocytes transfected with TLR1 siRNA was lower after stimulation than in the unstimulated keratinocytes, are shown here. Relatively little inhibition of the upregulation of TSLP gene expression by TLR1 siRNA (32% reduction) compared with that by TLR2 and TLR6 siRNAs (88% and 72% reduction respectively) was observed (Fig E1, B), suggesting that the *S aureus* membranous fraction induces TSLP in keratinocytes through the TLR2-TLR6 pathway similarly to that seen in Fig 5, B and C. Keratinocytes transfected with siRNAs using Lipofectamine2000 (LA2000) were stimulated with *S aureus* membrane (1.7 U/mL). L and M, Thirty-five percent to 45% and 45% to 55% guanine-cytosine content, respectively. TLR1-siRNA1, TLR2-siRNA3, and TLR6-siRNA2 were used.

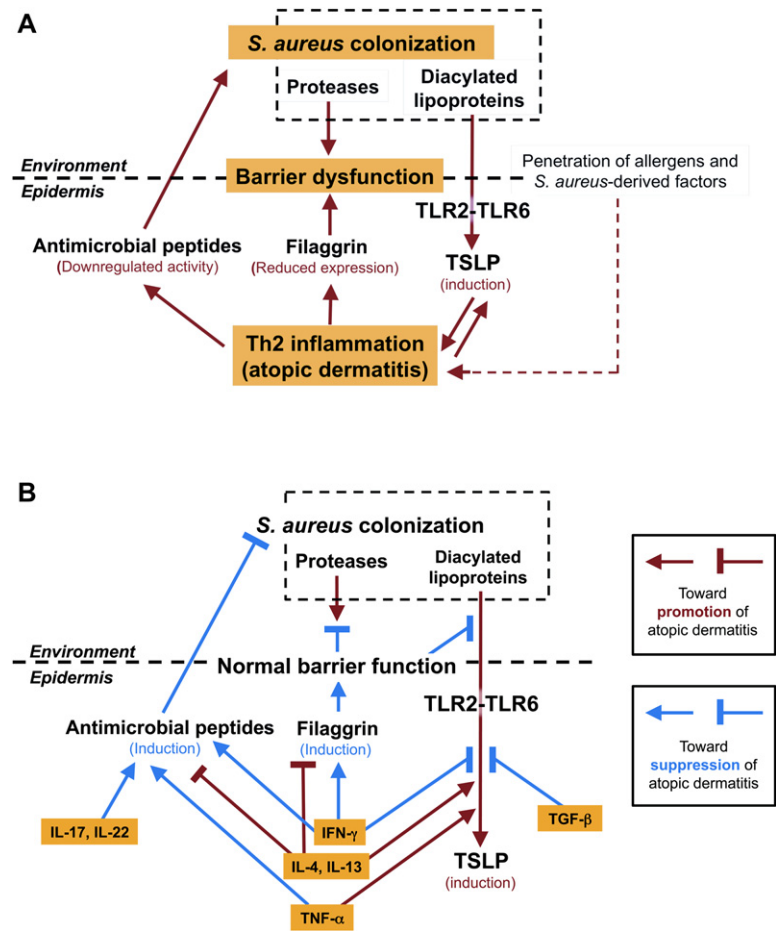


FIG E2. Vicious cycles between colonization with *S. aureus* and AD and modulation by cytokine milieu. **A**, Vicious cycles between colonization by *S. aureus* and AD. *S. aureus*-derived TLR2-TLR6 ligands and extra-cellular proteases promote AD. In turn, the inflammatory T_H2 (T_H2 /TNF) cytokine milieu in patients with AD further promotes the *S. aureus* colonization. **B**, The cytokine milieu modulates the vicious cycles. For further information, see the Discussion section.