FIGURE 2. hBDs and LL-37 induce IL-31 production and release from LAD2 mast cells. *A*, LAD2 cells (2×10^6) were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4 or 10 μg/ml LL-37 for 3–24 h, and the amounts of IL-31 in cell-free supernatants were determined by ELISA. *B*, hBD– and LL-37–stimulated cell lysates were obtained by lysing mast cells in lysis buffer containing protease inhibitor mixture. The IL-31 content of cell lysates was analyzed by ELISA. *C*, Cells (2×10^6) were sensitized with 1 μg/ml IgE overnight and stimulated with 1 μg/ml anti-IgE, 5 μg/ml hBD-1–4 or 5 μg/ml LL-37 alone or in combination for 12 h. The amounts of IL-31 in the cell-free supernatants were determined by ELISA. *p < 0.05 and **p < 0.01, when values were compared with the untreated group (Med, medium); #p < 0.05 when values were compared between each combination and the corresponding peptide used alone. Each bar represents the mean ± SD of four separate experiments.

whether the increased release was due to increased membrane permeability or to increased IL-31 synthesis. We therefore examined IL-31 amounts in cell lysates. As seen in Fig. 2*B*, large amounts of IL-31, almost 2-fold higher than the amounts extracellularly released into the respective cell culture supernatants, were detected in hBD- and LL-37-stimulated cell lysates. This finding suggests that hBDs and LL-37 induce both the production and release of IL-31.

Because IgE/anti-IgE also stimulated IL-31 gene expression and protein production in LAD2 cells (unpublished observation), we investigated whether IgE/anti-IgE and hBDs or LL-37 could cooperate to further increase IL-31 release. Mast cells were sensitized with 1 μg/ml myeloma IgE overnight and stimulated with 1 μg/ml anti-IgE alone or in combination with 5 μg/ml hBDs or LL-37. Combined treatment with anti-IgE and hBDs or LL-37 was found to significantly augment IL-31 release into the culture supernatant compared with the amounts released after treatment with each peptide alone (Fig. 2*C*). However, the simultaneous stimulation with anti-IgE and peptides showed additive but not synergistic

effect. In preliminary experiments, we titrated anti-IgE, hBDs and LL-37, determined the lowest concentration required to maximally increase IL-31 release, and used this concentration for combination experiments. An additive effect was defined as a combined activity that equaled the sum of individual activities, whereas a synergistic effect was defined as a combined activity greater than the sum of individual activities.

The ability of hBDs and LL-37 to stimulate IL-31 secretion by human mast cells was not unique to the LAD2 cell line. In fact, these peptides also significantly increased both IL-31 gene expression (Fig. 3*A*) and protein production/release (Fig. 3*B*) in human peripheral blood-derived cultured mast cells. However, peripheral blood-derived mast cells had a weaker response to hBDs and LL-37 than LAD2 cells, suggesting different characteristics of these two cell types.

hBDs and LL-37 enhance the expression of IL-31 in mast cells in vivo

To verify that mast cells express IL-31 and that hBDs and LL-37 enhance this expression in vivo, we performed double-immuno-labeling studies in rat skin using Abs to mast cells and to IL-31. We detected a feeble constitutive expression of IL-31 in rat skin mast cells; this constitutive expression was not affected by the presence of hBD-1. However, in the presence of hBD-2–4 or LL-37, the expression of IL-31 in skin mast cells was prominent (Fig. 4). Most of the mast cells showed positive immunostaining for IL-31, implying that this cell population forms a key source of IL-31 in the skin. The omission of primary Abs resulted in loss of specific staining (data not shown).

The expression of IL-31 in mast cells was also confirmed in human skin by double-staining with IL-31 and mast cell tryptase
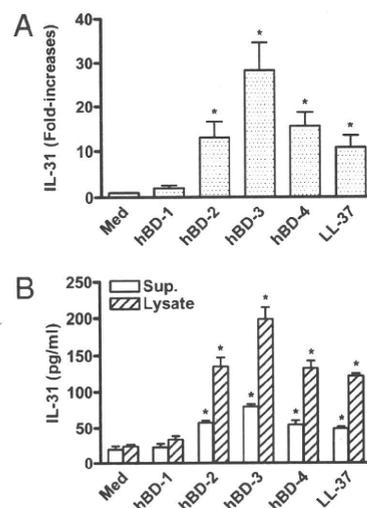

FIGURE 3. hBDs and LL-37 enhance IL-31 gene expression and protein production and release from human peripheral blood-derived cultured mast cells. *A*, Cells (1×10^6) were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 (6 h treatment with hBD-1 and 3 h treatment with hBD-2–4 or LL-37). Real-time PCR was performed to analyze changes in IL-31 gene expression. Each bar shows the mean ± SD from four separate experiments, each of which was run in triplicate. Values represent fold increases in gene expression compared with cells incubated with medium alone. Med, medium. *p < 0.01. *B*, Cells were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 for 12 h, and the concentrations of IL-31 in cell lysates (Lysate) and cell-free supernatants (Sup.) were determined by ELISA. Values are the mean ± SD of four separate experiments compared with the untreated group. *p < 0.05. Med, medium.
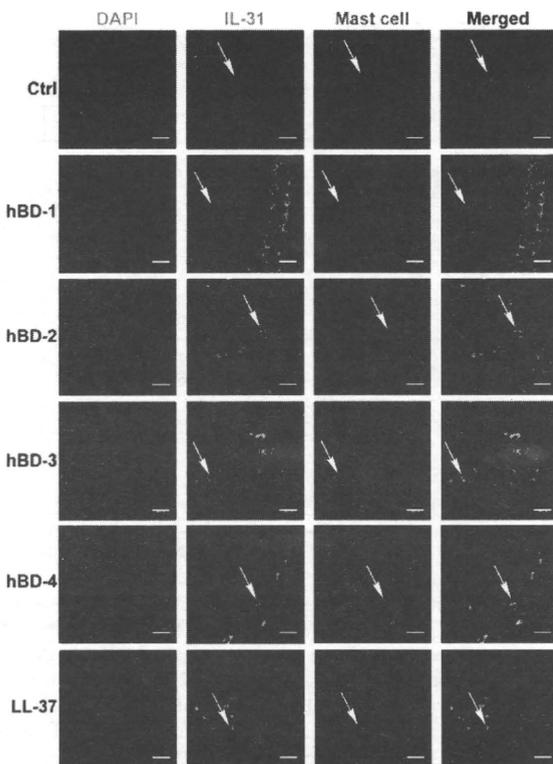
FIGURE 4. hBDs and LL-37 increase expression of IL-31 in mast cells in vivo. Rat ears were injected intradermally with 50 μl hBDs or LL-37 (500 ng) or with vehicle (0.9% normal saline) for 12 h. Frozen sections (5 μm) were incubated with either IL-31 (green) or mast cell (red) specific Abs, and immunofluorescence staining was performed using goat anti-rabbit Alexa Fluor 488 or goat anti-mouse Alexa Fluor 594. Arrows indicate IL-31 positive cells (green), mast cells (red), or colocalization of IL-31 and mast cells (merged). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). Results are representative of five independent experiments. Original magnification ×40. Scale bar, 50 μm. Ctrl, control.

FIGURE 5. Expression of IL-31 protein in mast cells from human skin. Frozen sections (7 μm) of skins from healthy volunteers (normal skin) and inflamed lesional psoriasis (psoriatic skin) were stained with either IL-31 (red) or mast cell tryptase (green) specific Abs, and immunofluorescence staining was performed using Alexa Fluor 488 or Alexa Fluor 594. Arrows indicate IL-31 positive cells (red), mast cells (green), or colocalization of IL-31 and mast cells (Merged). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). Results are representative of three independent experiments from three healthy volunteers and three psoriatic patients. The white dotted line in each panel indicates the border between the epidermis and the dermis. Scale bar, 50 μm.

Abs. In comparison with healthy volunteers, the expression level of IL-31 was greater in patients with psoriasis, and mast cells from psoriatic skin showed colocalization with IL-31 (Fig. 5). Similarly, skin mast cells from chronic atopic dermatitis patients also highly expressed IL-31 (data not shown).

Mast cells induce the release of various pruritus-promoting mediators upon stimulation with hBDs and LL-37

We next tested whether hBDs and LL-37 stimulate the release of other pruritogenic mediators in addition to IL-31. The results shown in Fig. 6A reveal that hBD-2–4 and LL-37, but not hBD-1, significantly induced the release of various cytokines, including IL-2, IL-4, IL-6, and GM-CSF, which have been reported to contribute to itching (42, 52–55). In addition, we verified that hBD-2–4 and LL-37 augmented the production of other cytokines, such as IL-8 and TNF-α, that also likely play a role in itching (56, 57) (data not shown). We also examined the effects of hBDs and LL-37 on the expression or release of the important pruritus-inducing factors substance P, NGF, PGE_2 and LTC_4. The results revealed that although hBD-2–4 and LL-37 drastically induced substance P mRNA expression (Fig. 6B), they failed to stimulate its protein production (data not shown). Increased release of both PGE_2 and LTC_4 was observed within 30 min, after which it decreased gradually, whereas NGF release remained high even after 6 h of stimulation by hBDs and LL-37 (Fig. 6B).
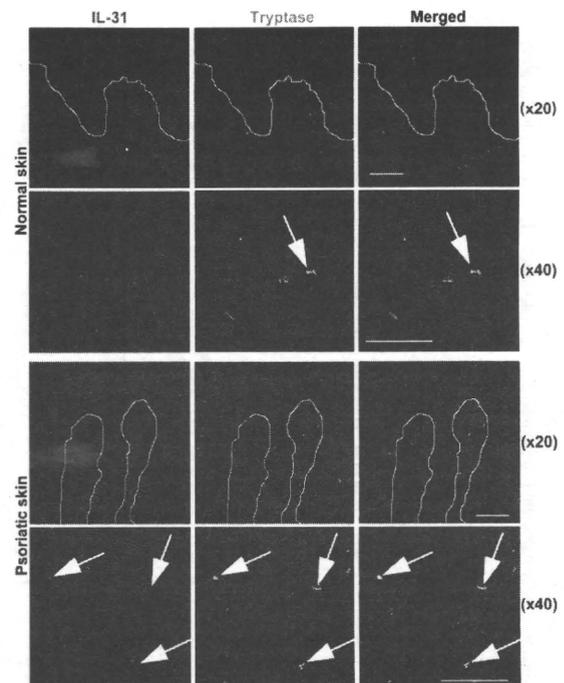
Effects of pertussis toxin and wortmannin on hBD- and LL-37–mediated IL-31 production/release

hBDs and LL-37 are known to activate rat mast cells via G-protein coupled receptors (32, 35). We therefore investigated the effects of pertussis toxin, an inhibitor of G-protein, on hBD- and LL-37-induced IL-31 secretion. Pretreatment of mast cells with pertussis toxin significantly decreased the production/release of IL-31 (Fig. 7). The role of PI3K in hBDs and LL-37 activities was examined by preincubating mast cells with a PI3K inhibitor, wortmannin. Like pertussis toxin, wortmannin noticeably reduced the production/release of IL-31 induced by hBDs and LL-37 (Fig. 7). These observations indicate that hBDs and LL-37 stimulate human mast cells via G-protein-coupled receptors and PI3K pathways.

Activation of MAPKs by hBDs and LL-37 is necessary for the production/release of IL-31

Because hBDs and LL-37 have been reported to induce phosphorylation of MAPKs in various cell types (35, 36, 51), we reasoned that they might activate MAPKs in human mast cells. As seen in Fig. 8, hBD-2–4 and LL-37 markedly induced phosphorylation of p38, ERK, and JNK. In preliminary experiments, the maximal activation of p38, ERK, or JNK induced by hBDs and LL-37 was observed after 30 min.

The activation of MAPKs was required for the secretion of IL-31 by hBDs and LL-37. This is shown by the noteworthy suppression of IL-31 production and release by specific inhibitors of p38, ERK, and JNK (Fig. 9). The presence of SB203580, PD98059, or
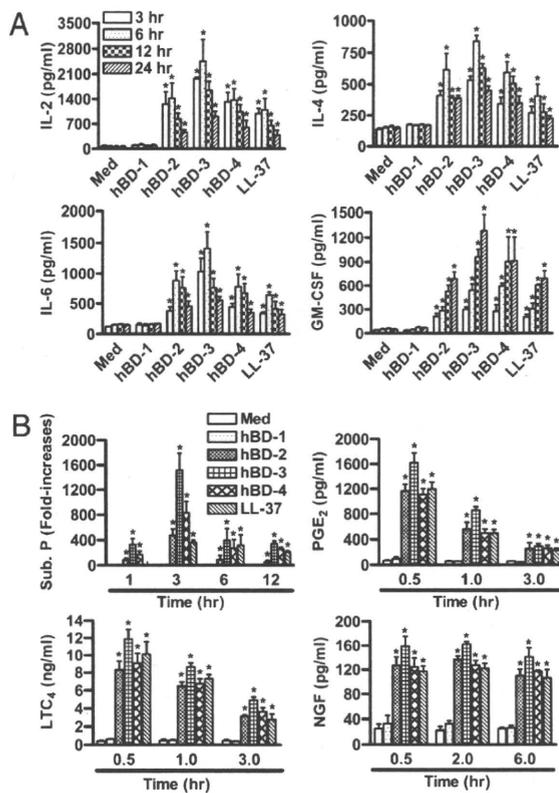
FIGURE 6. hBDs and LL-37 induce the expression or release of various pruritogenic mediators by LAD2 mast cells. A, Cells (2×10^6) were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 for 3–24 h, and the concentrations of IL-2, IL-4, IL-6, and GM-CSF released into cell-free supernatants were determined by ELISA. Values were compared between stimulated and nonstimulated groups. Each bar represents the mean $\pm$ SD of four to six separate experiments. $*p < 0.01$. B, Cells were stimulated with hBDs or LL-37 for the indicated times. Real-time PCR was performed to analyze changes in substance P gene expression (Sub. P). Values represent fold increases in gene expression above cells incubated with medium alone. $*p < 0.01$. After cell stimulation, enzyme immunoassays were used to analyze the amounts of PGE$_2$ and leukotriene C$_4$ (LTC$_4$) in cell-free supernatants, and NGF levels were detected using an ELISA. Values were compared between stimulated and non-stimulated cells. Med, medium. $*p < 0.01$. Each bar represents the mean $\pm$ SD of three to five separate experiments.

SP600125 partially inhibited the production/release of IL-31 caused by hBD-2–4 and LL-37. Interestingly, the combination of all MAPK inhibitors further strikingly decreased the production and release of IL-31. The doses of MAPK inhibitors used in this study were not toxic to mast cells, as analyzed by trypan blue exclusion and lactate dehydrogenase activity (data not shown).

We further showed that IgE/anti-IgE treatment, which elevated IL-31 expression at both the gene and protein levels in mast cells, significantly stimulated p38, ERK, and JNK phosphorylation; this activation reached a peak at 5 min (Fig. 10).

Discussion

In the current study, we investigated the ability of antimicrobial hBDs and LL-37 to stimulate human mast cells to secrete the novel pruritogenic cytokine IL-31. We demonstrated that hBDs and LL-37 induced both IL-31 mRNA expression and IL-31 protein production and release, and that they also stimulated the release of other pruritogens, including cytokines, NGF, PGE$_2$, and LTC$_4$. The production and release of IL-31 was under the control of G-protein, PI3K, and MAPK pathways. Thus, our data suggest a novel
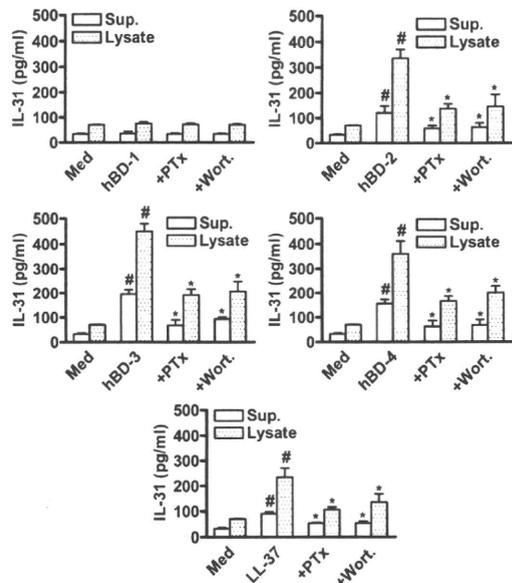

FIGURE 7. Effects of pertussis toxin and wortmannin on hBD– and LL-37–induced IL-31 production and release by LAD2 mast cells. Cells (2×10^6) were pretreated with 200 ng/ml pertussis toxin (PTx), 20 μM wortmannin (Wort.), or solvent (Med, medium) for 2 h, and were then challenged for 12 h with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37. The concentrations of IL-31 in cell lysates (Lysate) and cell-free supernatants (Sup.) were then determined by ELISA. Values are the mean $\pm$ SD of five separate experiments. Med, medium with solvent. $^\#p < 0.05$ as compared between stimulated group without inhibitor and untreated group; $*p < 0.05$ as compared between the presence and absence of each MAPK inhibitor.

mechanism for hBDs and LL-37 in inflammatory and/or allergic reactions.

The human skin is permanently exposed to a variety of potentially harmful microorganisms, but usually remains free of infection. This is not only due to the physical barrier of the stratum corneum, but also to the production of AMPs that form an innate epithelial chemical shield and inhibit microbial invasion (1). Among these AMPs, hBDs, and LL-37 have been reported to exhibit a broad spectrum of microbiocidal properties; they also display various stimulatory activities in mammalian cells (1). We and other investigators have demonstrated that hBDs and LL-37 recruit and stimulate mast cells to release inflammatory mediators such as histamine and PGD$_2$ (32, 35), which promote pruritus (42, 43). In this study, we show that, in addition to T cells, mast cells constitute another source of a pruritogenic factor, IL-31 (44). Upon stimulation with hBD-2–4 or LL-37, IL-31 was highly expressed in the LAD2 mast cell line and in rat skin mast cells. In addition, compared with cells from healthy human skin, the expression of IL-31 was notably elevated in skin mast cells from patients with psoriasis, a skin disease in which pruritus is observed in 70–90% of patients (42), and that has been associated with markedly enhanced levels of hBDs and LL-37 (1, 30). However, the fact that hBDs and LL-37 are downregulated in atopic dermatitis (30), in which the expression of IL-31 in mast cells was also augmented (data not shown), implies that these peptides are not exclusive IL-31 inducers in mast cells. We observed that IgE/anti-IgE also induced IL-31 production in mast cells and that it, like hBDs and LL-37, could also activate MAPKs. However, given that the combination of IgE/anti-IgE with hBDs or LL-37 resulted in additive but not synergistic stimulation of IL-31 secretion, our results suggest that the receptor signaling pathways involved in the stimulation of IL-31 release by IgE/anti-IgE and by hBDs or
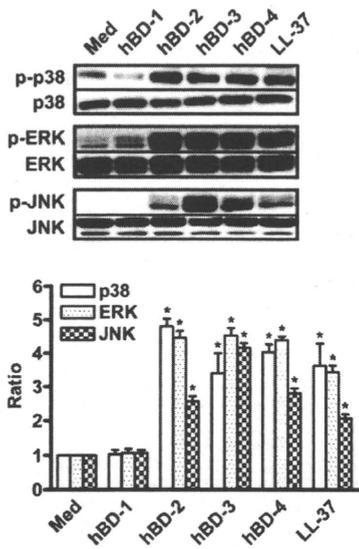
FIGURE 8. hBDs and LL-37 induce the phosphorylation of MAPKs in LAD2 mast cells. Cells (2×10^6) were stimulated for 30 min with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37. The levels of phosphorylated and unphosphorylated p38 (p-p38, p38), ERK (p-ERK, ERK) and JNK (p-JNK, JNK) in cellular lysates were then determined by Western blot analysis. *Upper panel,* Representative of four separate experiments with similar results. *Lower panel,* Bands were quantified by densitometry using the software program Image Gauge (LAS-4000plus) to allow correction for protein loading. Data represent the ratio of the intensity of each phosphorylated protein (p-p38, p-ERK or p-JNK) divided by the amount of the respective unphosphorylated protein (p38, ERK or JNK). Values are the mean ± SD of four independent experiments. *$p < 0.05$ as compared between stimulated and non-stimulated cells. Med, medium.

FIGURE 9. Effects of MAPK inhibitors on hBD- and LL-37-induced IL-31 production and release by LAD2 mast cells. Cells (2×10^6) were preincubated with 10 μM SB203580 (SB), 10 μM PD98059 (PD), 20 μM SP600125 (SP), or with a combination of 10 μM SB203580, 10 μM PD98059, and 20 μM SP600125 (All), or solvent (Med, medium) for 2 h. Cells were then stimulated for 12 h with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37. The concentrations of IL-31 in cell lysates (Lysate) and cell-free supernatants (Sup.) were determined using an ELISA. Values are the mean ± SD of five separate experiments. [#]$p < 0.05$ as compared between stimulated group without inhibitor and the untreated group (Med, medium with solvent); *$p < 0.05$ as compared between the presence and absence of each MAPK inhibitor.

LL-37 may not interact. The observation that all hBDs except hBD-1 stimulated the production and release of IL-31 is probably not that the hBD-1 used was biologically inactive, because hBD-1 significantly enhanced IL-31 gene expression (Fig. 1); also, the same reagent markedly activated differentiated keratinocytes, as previously reported (51). Further investigations to determine whether the secondary structure of hBD-1 may affect its stimulatory ability are necessary.

Apart from the secretion of IL-31, hBDs and LL-37 also enhanced the release of numerous cytokines, including IL-2, IL-4, IL-6, IL-8, GM-CSF, and TNF-α, which are known to play a role in the elicitation of itching (52–57). However, several experimental studies have suggested an indirect pruritogenic effect of above cytokines via other mediators (55, 58). Furthermore, hBDs and LL-37 elicited the expression or release of the most potent pruritogens, including substance P, PGE_2, LTC_4, and NGF (42, 59–61). These observations provide novel evidence that hBDs and LL-37 may participate in inflammatory and/or allergic reactions via the activation of mast cells.

To gain insight into the cellular signaling mechanism by which hBDs and LL-37 stimulate IL-31 secretion in mast cells, the receptor-mediated process was examined. The results revealed that hBD- and LL-37-induced IL-31 production/release is controlled by a G-protein pathway. Until now, the receptors through which hBDs activate mast cells have not been well characterized. It has been reported that hBD-1 and hBD-2 chemoattract dendritic cells and T cells via a G-protein coupled chemokine receptor, CCR6 (37). Furthermore, hBD-3 reportedly chemoattracts monocytes and CCR6-transfected human embryonic kidney 293 cells (38), and suppresses neutrophil apoptosis through the activation of CCR6 (62). However, Soruri et al. (31) recently
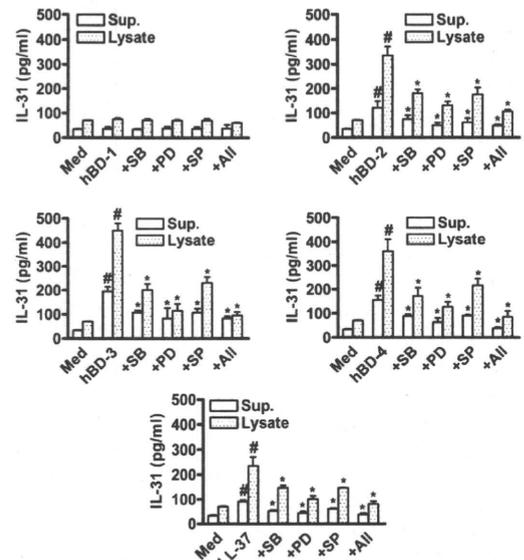
demonstrated that hBD-1–4 and mBD-8 were unable to induce the migration of dendritic cells, T cells or RBL-2H3 and 300.19 cells stably expressing CCR6. Likewise, a specific CCR6 ligand, MIP-3α/CCL20, could not compete with hBD-2 binding to rat mast cells (33). Similarly, a functional receptor for LL-37 on mast cells has not yet been identified. LL-37 likely activates FPRL1 (39) and purinergic receptor $P2X_7$ (40), and transactivates epidermal growth factor receptor in various cells (41). However, it is not clear whether these receptors are involved in LL-37-mediated mast cell activation. The failure of MMK1, an FPRL1-specific agonist, to compete with LL-37 binding to mast cells suggests that FPRL1 may not be a functional LL-37 receptor in mast cells (34). Other studies have shown that LL-37 activation of airway epithelial cells and human keratinocytes does not occur via FPRL1 (41, 63). Further studies are required to identify the functional receptors of hBDs and LL-37 on mast cells. We found that, in addition to G-protein coupled pathway,
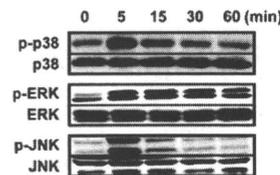
FIGURE 10. IgE/anti-IgE induces the phosphorylation of MAPKs in LAD2 mast cells. Cells (2×10^6) were sensitized with 1 μg/ml IgE overnight and stimulated with 2 μg/ml anti-IgE for 5–60 min; the levels of phosphorylated and unphosphorylated p38 (p-p38, p38), ERK (p-ERK, ERK), and JNK (p-JNK, JNK) in cell lysates were then determined by Western blot analysis. One representative experiment of three separate experiments with similar results is shown.

hBDs and LL-37 also activated the PI3K pathway. However, given that neither G-protein nor PI3K pathways were completely suppressed by their specific inhibitors, the presence of additional pathways for hBDs and LL-37 (e.g., another activating receptor or transactivation) cannot be excluded.

This study also showed that MAPK activation is involved in hBD- and LL-37-mediated mast cell activation. The mammalian MAPK family mainly consists of p38, ERK, and JNK, which are activated by different stimuli, and target different downstream molecules, thereby performing different functions, including regulation of growth, differentiation, inflammation, and production of cytokines and chemokines (51). We demonstrated that hBDs and LL-37 induced the activation of p38, ERK, and JNK, and that the activation of these molecules was further required for mast cell stimulation, as their specific inhibitors significantly suppressed IL-31 secretion caused by hBDs and LL-37.

IL-31 is a recently identified pruritogenic factor mainly produced by activated T cells (44); its mRNA expression is upregulated in pruritic skin from humans with atopic dermatitis and other pruritic skin lesions (46, 47). In NC/Nga mice, increased IL-31 mRNA significantly coincides with increased scratching counts (64). In human keratinocytes, IL-31 induces the expression of chemokine genes associated with atopic skin inflammation (44). Hence, through the induction of chemokines, IL-31 may recruit inflammatory cells, which become activated and in turn produce more IL-31, thereby aggravating skin inflammation and pruritus. Besides its pruritogenic properties, IL-31 has been shown to be involved in the regulation of cell proliferation in lung epithelial cells and colorectal cancer cells, and it plays an important role in regulating hematopoiesis via the activation of STAT-3 and STAT-5 (44, 65). Moreover, IL-31 stimulates the secretion of proinflammatory cytokines, chemokines, and matrix metalloproteinases, and it likely plays a crucial role in the regulation of inflammation and the immune response (65–67). Although the concentrations of IL-31 produced by hBDs and LL-37 are low (pg/ml level) compared with the doses of IL-31 (ng/ml level) needed to exert its stimulatory functions (66, 67), IL-31 may cooperate with other molecules in stimulating its biologic functions. For instance, the combination of IL-31 with IL-17 and of IL-31 with IL-4 or IL-13 has resulted in increased production of numerous cytokines and chemokines in epithelial cells (66, 67). Thus, the ability of hBDs and LL-37 to stimulate IL-31 secretion by mast cells suggests a key role of these AMPs in the immune and inflammatory responses mediated by IL-31.

In the skin, mast cells are distributed within the dermis and are involved in the pathogenesis of several skin disorders, including wounding and lichen planus, in which concentrations of AMPs are abundantly enhanced (1, 68). Because the basal membrane between the dermis and epidermis is impaired in wounding and lichen planus, this may lead to direct contact between skin-derived AMPs and mast cells, resulting in subsequent activation of mast cells. Human epithelium contains elevated amounts of hBDs and LL-37 at sites of infection or inflammation. For example, the concentrations of hBD-2 and LL-37 have been estimated at ~157 μM and $\geq$1605 μM, respectively, in a pruritic skin disease—psoriasis (30). This observation supports the pathophysiologic relevance of the doses of hBDs and LL-37 (ranging 1–20 μg/ml, equivalent to 0.2–4.0 μM) used in this study.

In conclusion, our study demonstrated novel functions of hBDs and LL-37 in human mast cells and showed that mast cells constitute another source of IL-31. The hBD- and LL-37–mediated stimulation of the production of pruritogenic factors by mast cells provides a novel mechanism by which human AMPs may contribute to inflammatory reactions and suggests a role for these AMPs in the pathogenesis of skin disorders.

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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-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe
iNKT:	Invariant natural killer T
LTA:	Lipoteichoic acid
Pam_3CSK_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

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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-bispalmitoyloxypropyl)-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)-Lys$_4$-OH, 3HCl (5 μg/mL; Pam_3CSK_4; Calbiochem, San Diego, Calif); PGN from *S aureus* (20 μg/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 Pam_3CSK_4, 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 Pam_3CSK_4 did not induce the release of TSLP at the concentrations tested. Pam_3CSK_4 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 Pam_3CSK_4 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/
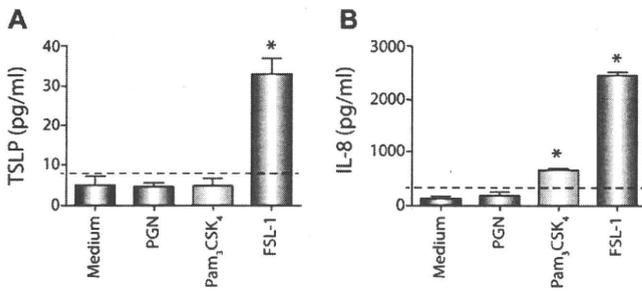
A

B

FIG 1. FSL-1 induced the release of TSLP in keratinocytes. Keratinocytes were stimulated with PGN (20 μg/mL), triacylated lipopeptide (Pam₃CSK₄, 5 μg/mL), or diacylated lipopeptide (FSL-1, 1 μg/mL). **A**, TSLP. **B**, IL-8. *Broken line*, Minimum detection limit. Data shown are the means ± 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*).

T$_H$2 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 T$_H$2 cytokines IL-4 and IL-13 (T$_H$2/TNF cytokines) enhances the release of TSLP. FSL-1 alone at 0.1 and 1 μg/mL or T$_H$2/TNF cytokines without FSL-1 significantly induced the release of TSLP, whereas the combination of FSL-1 and T$_H$2/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 over-expressed in keratinocytes in the lesions.[7] Very recently, diacylated[26] and triacylated[27] 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 siR-NAs 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 T$_H$2/TNF cytokines could be provided by cells such as T$_H$1 cells, regulatory T (Treg) cells, T$_H$17 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, T$_H$2/TNF cytokines, and the *S aureus* membranous fraction plus T$_H$2/TNF cytokines in the differentiated keratinocytes (Fig 7, *A*, IFN-γ and TGF-β). IL-17 showed smaller inhibition of the release of TSLP induced by FSL-1 and T$_H$2/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/
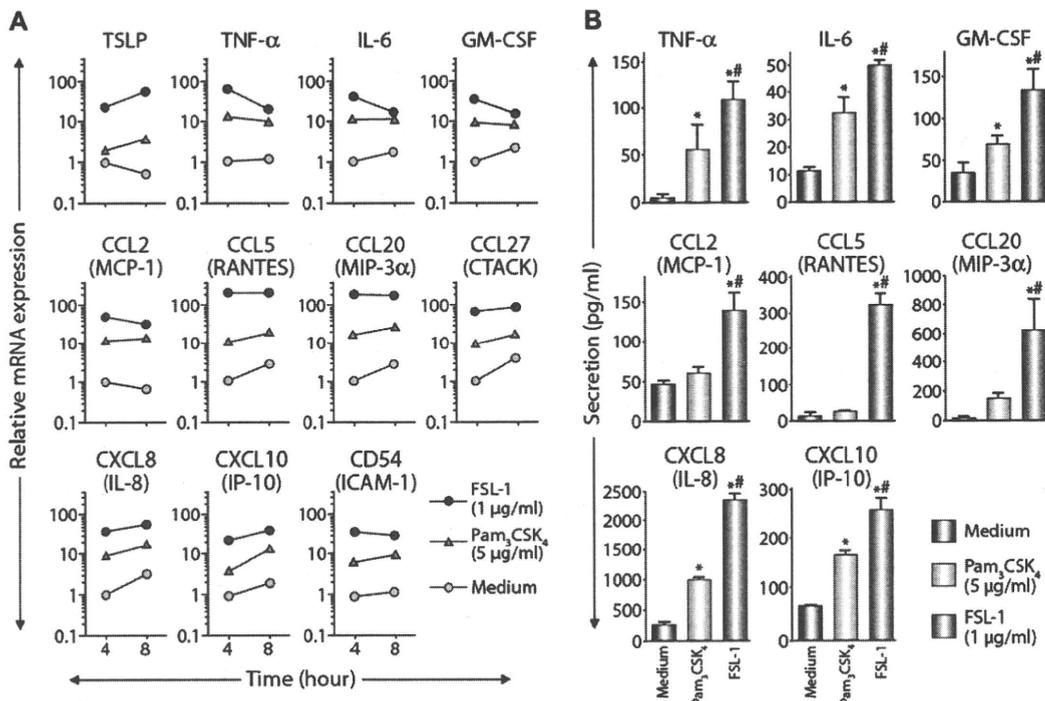
FIG 2. FSL-1 induced upregulation of the expression of TSLP and other proinflammatory molecules in keratinocytes. **A,** Levels of mRNA are presented relative to the medium at 4 hours. **B,** Protein concentrations in the culture supernatant. Data shown are the values for single wells (Fig 2, A) and means ± 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 Pam$_3$CSK$_4$ 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 3α [MIP-3α], 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.
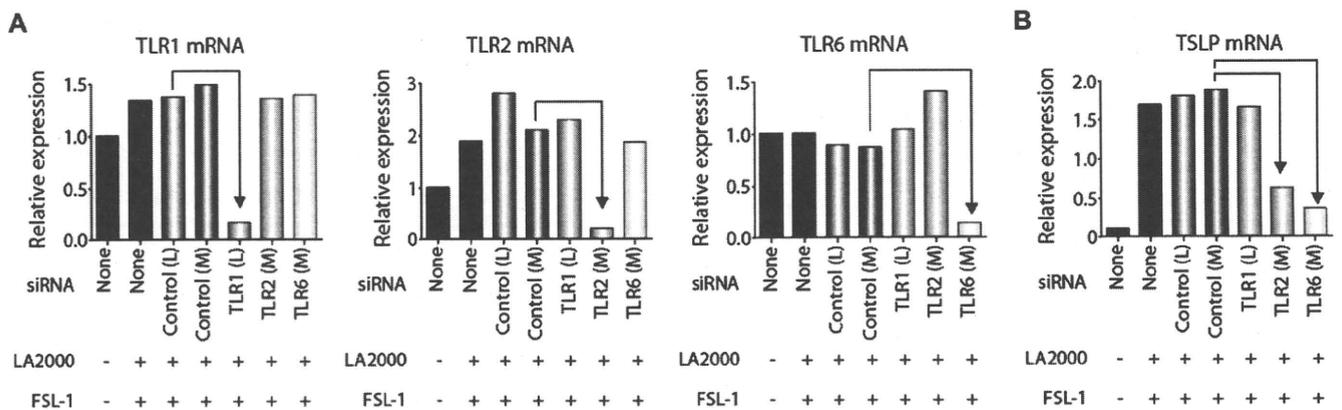

FIG 3. Knockdown of TLR2 and TLR6 inhibited the FSL-1–induced TSLP mRNA expression in keratinocytes. **A** and **B,** Keratinocytes transfected with siRNAs by using lipofectamine2000 (LA2000) were stimulated with FSL-1 (1 μg/mL). L and M, Thirty-five percent to 45% and 45% to 55% guanine-cytosine content, 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
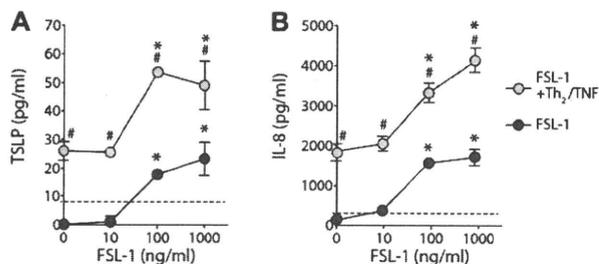
FIG 4. T_H2 cytokines and TNF-α upregulated the FSL-1–induced release of TSLP in keratinocytes. **A**, TSLP. **B**, IL-8. *Broken line*, Minimum detection limit. Data shown are the means ± SD for 3 wells and representative of 3 independent experiments with similar results. *$P < .001$ versus without FSL-1 and #$P < .001$ versus without T_H2/TNF-α 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-γ and TGF-β (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 cells/cm^2 in Fig 8 and 6.67 log 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 al[32]: the mean ± SD of log cells/cm^2 = 5.1 ± 2.0 = 3.1-7.1 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 diacylglyceryl 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 al[26] 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 Pam_3CSK_4, a ligand for the TLR2-TLR1 heterodimer, and PGN did not induce the release
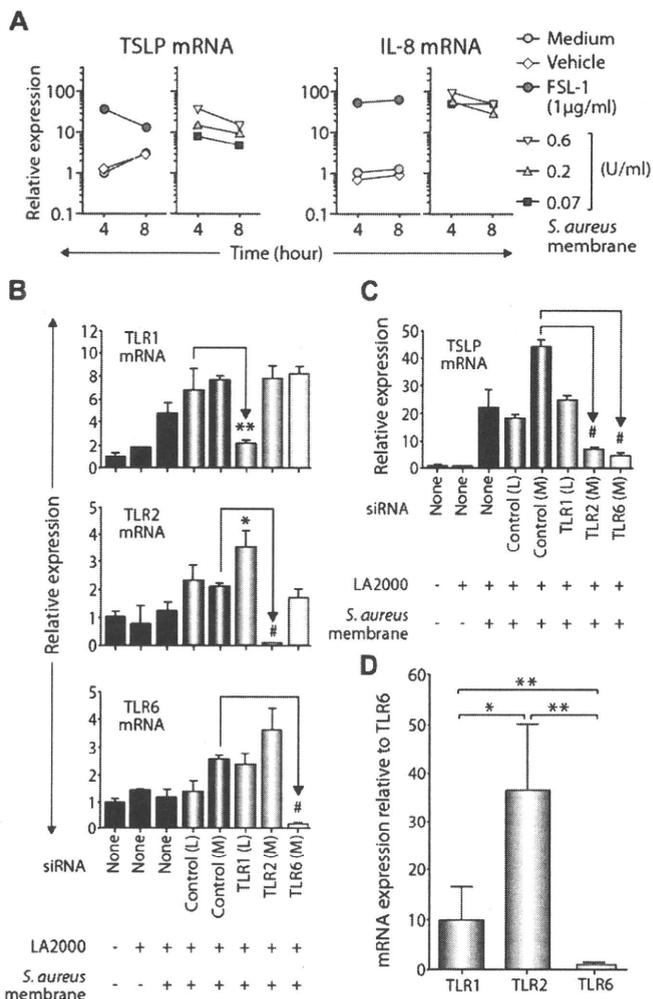

FIG 5. *S aureus* membranous fraction induced an upregulation of the gene expression of TSLP in a manner dependent on the expression of TLR2 and TLR6 in keratinocytes. **A**, Dose dependency. **B** and **C**, Keratinocytes transfected with siRNAs were stimulated with the membranous fraction of the *S 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 ± 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 ± 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*). Pam_3CSK_4 induced an upregulation of TSLP gene expression in keratinocytes, although inefficiently compared with FSL-1 (Fig 2, *A*). Kurokawa et al[27] 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 Pam_3CSK_4, 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
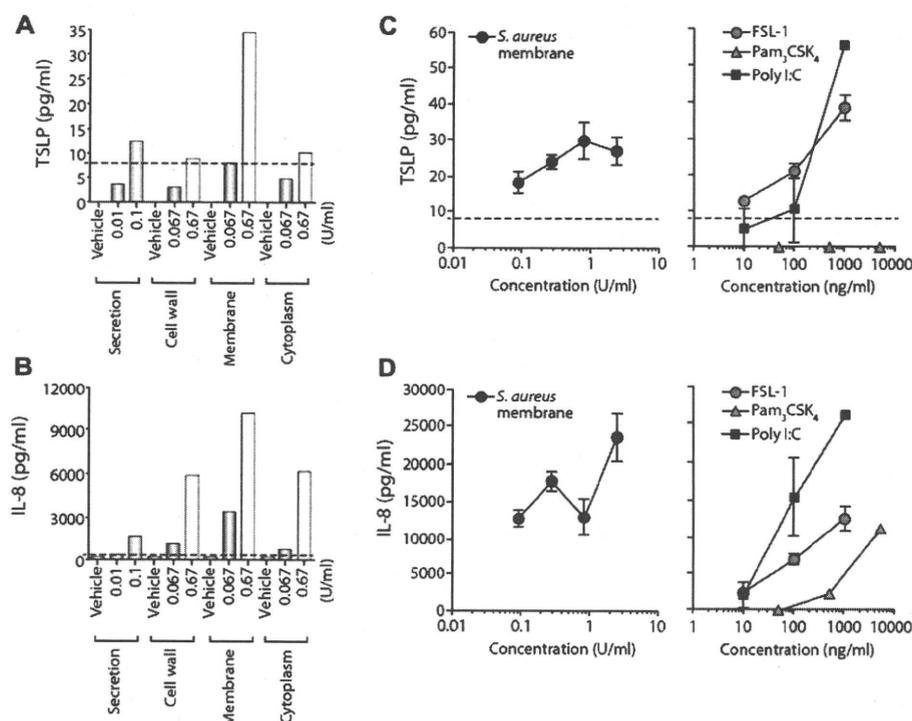
FIG 6. *S aureus* membranous fraction induced release of TSLP in keratinocytes. Keratinocytes were induced to differentiate by culturing with FBS before stimulation. **A** and **B**, Release of TSLP and IL-8 on stimulation with the subcellular fractions of the *S aureus* MW2 strain. **C** and **D**, Dose dependency of the release of TSLP (Fig 6, *C*) and IL-8 (Fig 6, *D*) by the *S aureus* membranous fraction, FSL-1, Pam$_3$CSK$_4$, and polyI:C. *Broken line*, Minimum detection limit. Data shown are the values for single wells (Fig 6, *A* and *B*) or the means ± 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 condition[34] 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$_H$2 responses, in keratinocytes (Figs 5 and 6), leading to T$_H$2 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$_H$2 cytokines IL-4 and IL-13 that mimics the atopic environment (Figs 4, *A* and 7, *A*, + T$_H$2/

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$_H$2 cytokines to the *S aureus*– and diacylated lipopeptide–induced TSLP production is yet to be investigated, proinflammatory and T$_H$2 cytokines have been reported that act synergistically to induce the release of TSLP from human skin explants obtained from healthy donors.[15] The release of TSLP induced by *S aureus* could be more effective in AD skin than in healthy skin because AD is associated with T$_H$2 inflammation and allergen-specific IgE, which stimulates mast cells and basophils to produce such cytokines through the high-affinity IgE receptor on exposure to allergens. The T$_H$2 cytokine milieu in the skin could promote colonization by *S aureus* through a reduction in the expression of inducible AMPs[2] and inhibition of the mobilization of an AMP, β-defensin 3,[3] and could reduce the skin barrier function through a reduction in the expression of filaggrin,[37] which is critical for an effective skin barrier and whose genetic mutations are a major predisposing factor for AD,[34] in cooperation with *S aureus* protease,[6] enabling the exposure of keratinocytes to *S aureus*–derived factors. In turn, the colonization could exacerbate the T$_H$2-type inflammation by inducing expression of TSLP, which contributes to differentiation of T$_H$2/TNF-producing inflammatory T$_H$2 cells,[8] in keratinocytes (Figs 5 and 6) synergistically with the T$_H$2 and inflammatory microenvironment (T$_H$2/TNF cytokine milieu; Figs 4, *A*, and 7, *A*, + T$_H$2/TNF). This clearly explains the mechanism behind the vicious cycle between AD and *S aureus* colonization (see Fig E2, *A*).
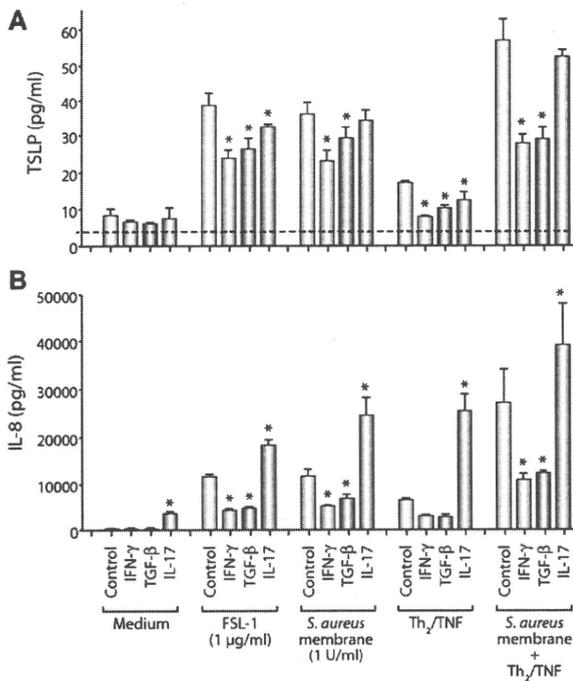
FIG 7. Effect of cytokines on *S aureus* membrane– and FSL-1–induced release of TSLP in keratinocytes. Keratinocytes were induced to differentiate by culturing with FBS before stimulation. Release of TSLP **(A)** and IL-8 **(B)** on stimulation with the membranous fraction of the *S aureus* MW2 strain was measured. *Broken line,* Minimum detection limit. Data shown are the means ± SDs for 3 wells and representative of 3 independent experiments with similar results. *$P < .05$ versus the medium by means of ANOVA with the Newman-Keuls multiple comparison test.
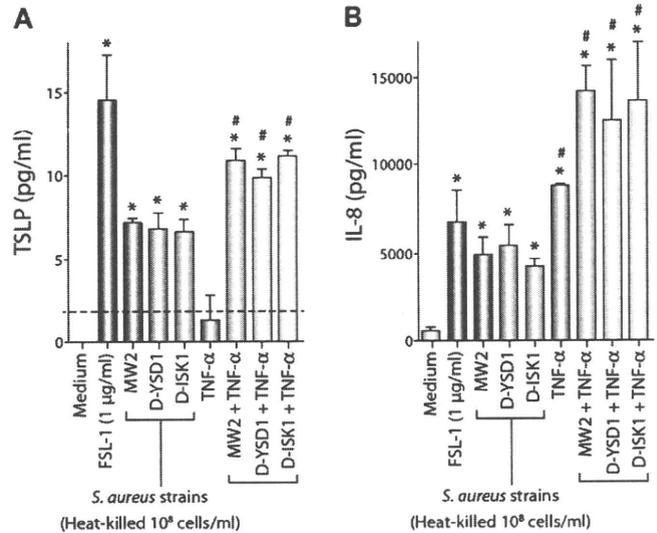

FIG 8. Heat-killed bacterial whole cells of *S aureus* strains isolated from patients with AD induced release of TSLP in keratinocytes. Keratinocytes were induced to differentiate by culturing with FBS before stimulation. Release of TSLP **(A)** and IL-8 **(B)** on stimulation with the heat-killed *S 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 ± 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.

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 T_H2 cytokines, forming another vicious cycle between colonization by *S aureus* and AD. Direct action of TSLP on mast cells,[10] T_H cells,[11] and eosinophils[12] 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 T_H1 or Treg cells could limit TSLP's release in *S aureus*-stimulated keratinocytes, even in the presence of T_H2/TNF cytokines. However, cytokine switch from T_H2 in the acute phase to T_H1 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 AD[39]; 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 al[41] reported that the percentage of T_H17 cells in peripheral blood was higher in patients with AD

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 γδT 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 T_H17-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 PGN[44] but less IL-8 after stimulation with PGN[45] 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 al[32] 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 LTA[32] or *S aureus* DNA[47] 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 function[3] or induction[2] of AMPs to kill *S aureus* and downregulates filaggrin expression[37] 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 heterodimer[24] could lead to new therapeutic approaches (ie, the development of safe and effective topical drugs that specifically antagonize the TLR2-TLR6 pathway).

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′-AGACCUUGCUGAUAUUCAAAUGAGC-3′; TLR2-siRNA3, 5′-UUCAGAGUGAGCAAAGUCUCUCCGG-3′; TLR6-siRNA2, 5′-UGGGAAUGCUGUUCUGUGGAAUGGG-3′; TLR6-siRNA3, 5′-AAUAAGUCCGCUGCGUCAUGAGAGC-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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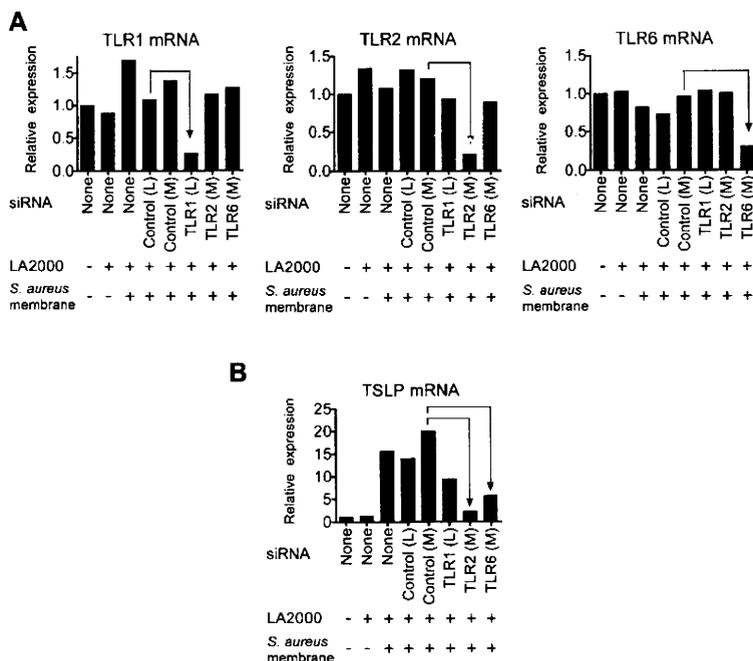
FIG E1. Knockdown of TLR2 and TLR6 inhibited *S aureus* membrane–induced upregulation of TSLP mRNA expression in keratinocytes. In Fig 5, *B* and *C*, the level of TLR1 mRNA after stimulation with the membranous fraction was suppressed by means of TLR1 siRNA compared with the control siRNA with the low 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.
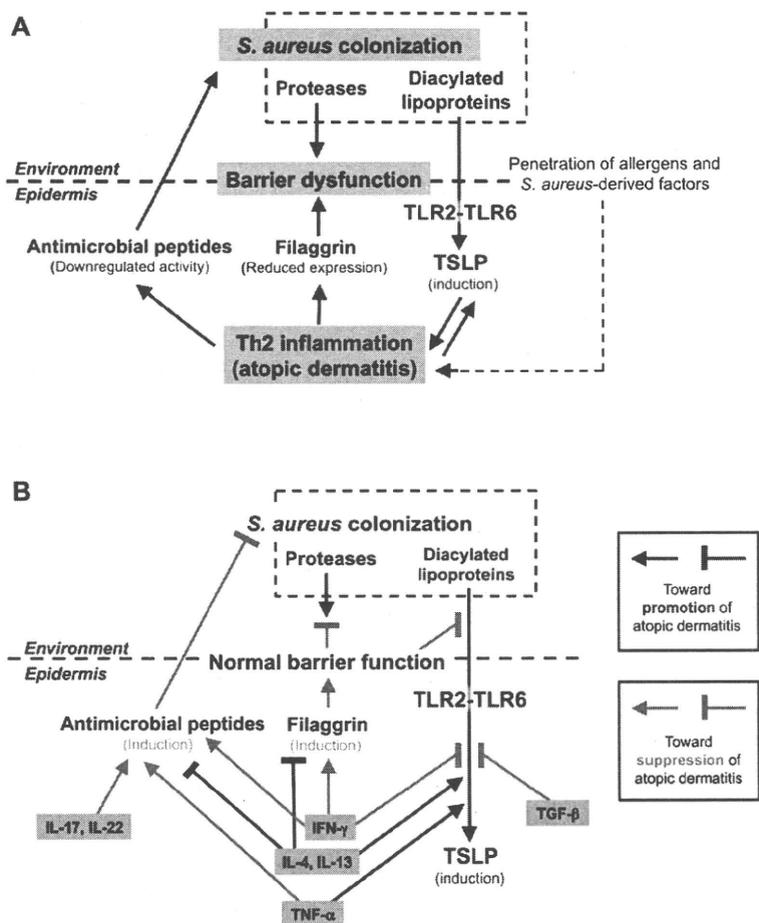
FIG E2. Vicious cycles between colonization with *S aureus* and AD and modulation by cytokine milieu. **A**, Vicious cycles between colonization by *S aureus* and AD. *S aureus*–derived TLR2-TLR6 ligands and extracellular proteases promote AD. In turn, the inflammatory T$_H$2 (T$_H$2/TNF) cytokine milieu 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.

In conclusion, we have demonstrated the direct correlation that exists between the lateral molecular organization of the SC lipid matrix and the efficiency of the skin barrier: as previously suggested, but to our knowledge never shown *in vivo*, the higher the extent of purely OR phases, the lower is the inside-out flux of water. Our results lend further support to the domain mosaic and sandwich models of the lipid organization in SC. This work raises several interesting questions relevant to the areas of skin biophysics, dermatology, and transdermal drug delivery, such as the changes in the lipid molecular organization that might be induced by environmental conditions (for example, temperature and humidity) and by topical application of products, the relationship between the inside-out and the outside-in fluxes of water and chemicals through human skin, and the reversibility and time scale of recovery of the molecular organization in SC *in vivo* after a chemical or environmental insult.

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Staphylococcus aureus Extracellular Protease Causes Epidermal Barrier Dysfunction

TO THE EDITOR

Dry skin and impaired barrier function are hallmarks of the pathogenesis of atopic dermatitis (AD). The combination of genetically determined barrier deficiency (Walley *et al.*, 2001; Kato *et al.*, 2005; Palmer *et al.*, 2006; Vasilopoulos *et al.*, 2007; Ogawa *et al.*, 2008) and barrier disruption by exogenous proteases (Nakamura *et al.*, 2006; Jeong *et al.*, 2008), endogenous proteases (Rogalski *et al.*, 2002; Hachem *et al.*, 2006), and detergents (Okuda *et al.*, 2002) might increase the risk of sensitization to allergens toward IgE production and contribute to AD exacerbations (Ogawa and Yoshiike, 1993; Cork *et al.*, 2006). Recently, we

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Abbreviations: AD, atopic dermatitis; SC, stratum corneum; TEWL, transepidermal water loss

reported that cysteine protease activity of recombinant house dust mite major group 1 allergens (Kato et al., 2005; Kikuchi et al., 2006) impairs the epidermal permeability barrier (Nakamura et al., 2006). Colonization by Staphylococcus aureus, another common feature of AD, has been implicated as

an environmental factor in its pathogenesis (Aly et al., 1977; Cork et al., 2006). S. aureus produces extracellular proteases (Dubin, 2002); however, the possible involvement of S. aureus proteases in the pathogenesis of AD has not been investigated. In this study, we examine whether an S. aureus extra-

cellular protease, commonly referred to as V8 protease, impairs the epidermal permeability barrier in mice.

S. aureus V8 protease exhibited proteolytic activity in zymography (Figure 1a) and cleaved the peptide bonds at the carboxy-terminal side, preferentially after glutamate and to a
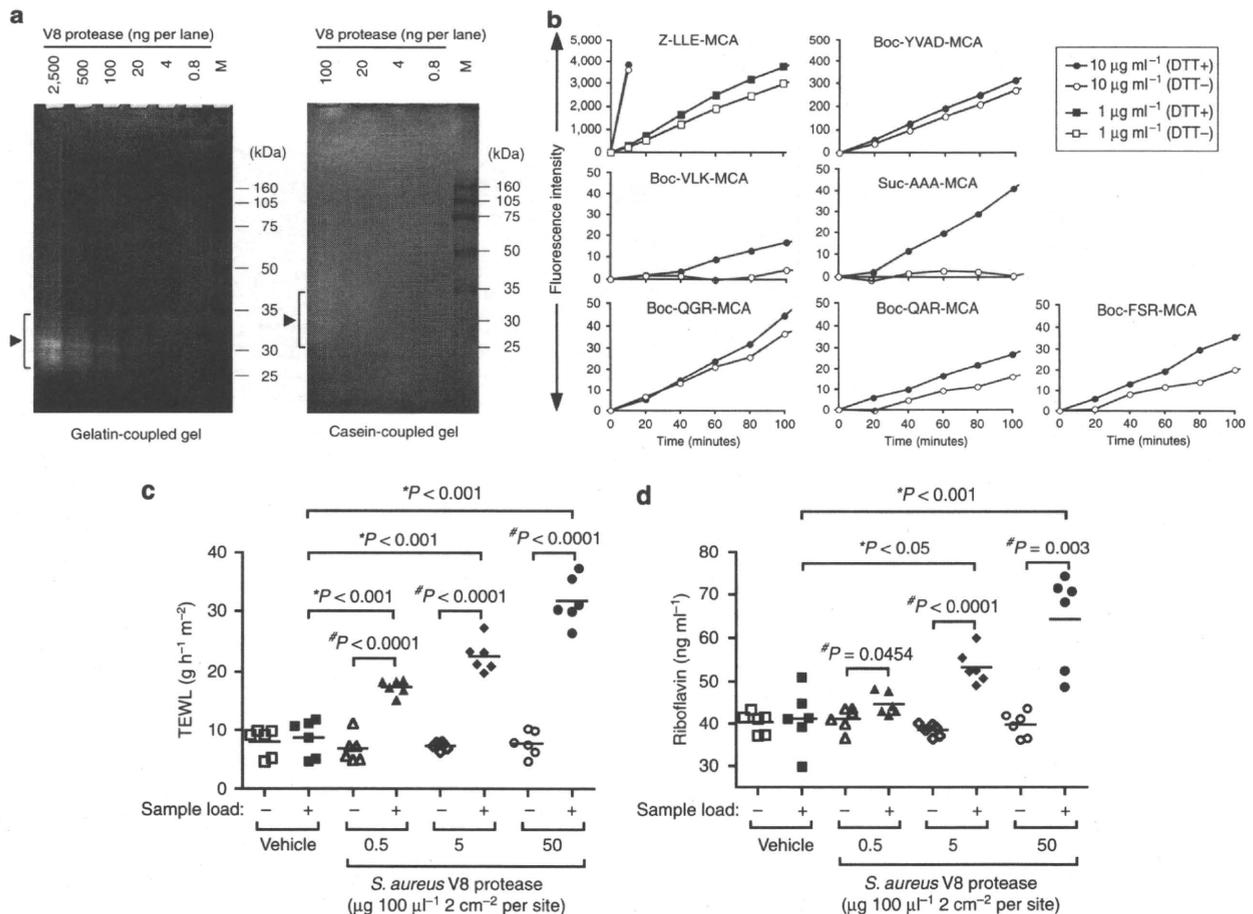

Figure 1. **Proteolytic activity of _Staphylococcus aureus_ V8 protease and epidermal permeability barrier dysfunction in the skin of nude mice, caused by S. aureus V8 protease.** In **a** and **b**, the proteolytic activity of V8 protease (Pierce, Rockford, IL) was analyzed as previously described (Gunawan et al., 2008a, b). (**a**) Gelatin and casein zymography. Arrows indicate transparent bands, where the protease digested the substrate. V8 protease exhibited proteolytic activity in zymography at low doses (100 and 20 ng per lane in gelatin and casein zymography, respectively). The mobility of the bands does not always correspond to the molecular weight of the protease because, to minimize inactivation of protease activity, the samples were not boiled. (**b**) Cleavage of fluorogenic substrates. Benzyloxycarbonyl-Leu-Leu-Glu-MCA (Z-LLE-MCA), Boc-Tyr-Val-Ala-Asp-MCA (Boc-YVAD-MCA), Boc-Gln-Gly-Arg-MCA (Boc-QGR-MCA), Boc-Gln-Ala-Arg-MCA (Boc-QAR-MCA), Boc-Phe-Ser-Arg-MCA (Boc-FSR-MCA), succinyl-Ala-Ala-Ala-MCA (Suc-AAA-MCA), and Boc-Val-Leu-Lys-MCA (Boc-VLK-MCA) were used as substrates. The vertical scale is the same among substrates, except for Z-LLE-MCA and Boc-YVAD-MCA. V8 protease cleaved the peptide bonds of the short peptide substrates at the carboxy-terminal side, preferentially after glutamate (Z-LLE-MCA) and to a lesser extent after aspartate (Boc-YVAD-MCA). Contamination by cysteine protease, which can be activated in the presence of DTT (particularly in Suc-AAA-MCA and Boc-VLK-MCA), was very minor. (**c** and **d**) Epidermal permeability barrier dysfunction. A patch containing V8 protease with occlusion using a polyurethane film was applied to the backs of 6-week-old female nude mice with the BALB/c background (Charles River Japan, Yokohama, Japan) every other day for 1 week. The final concentrations of the protease were 5, 50, or 500 μg ml^{-1} (0.5, 5, or 50 μg 100 μl^{-1} 2 cm^{-2} per site), the first two of which could be considered relevant in staphylococcal colonization according to our calculation on the basis of the V8 protease concentration released into the culture supernatant (Miedzobrodzki et al., 2002). The day after the last application, after removal of the patch, transepidermal water loss (TEWL) measurement (**c**) and riboflavin test (**d**) were carried out. +, treated sites; −, untreated sites. Number of mice per group was six. Bars indicate means. *$P < 0.05$ by one-way analysis of variance and Tukey _post hoc_ test among the treated sites, and #$P < 0.05$ by t-test (two-tailed) between treated and untreated sites were considered statistically significant. Animals were maintained in a specific pathogen-free animal facility at Juntendo University, and all animal studies were approved by the review board of Juntendo University.