and 1% penicillin, without any additional stimulant, for 48 h at 37°C in humidified 5% CO_2 in air. When dispersed ECs are cultured for different culture periods, the amount of cytokines in the culture supernatants are markedly increased between 24 and 48 h and reached maximum at 72 h (9). As the 72-h culture possibly masks the influence of barrier disruption, we used the 48-h culture for the assessment. The Quantikine (R & D Systems, Minneapolis, MN) protocol for sandwich ELISA was used to quantify total amount of CCL17, CCL22 and CCL5 in the culture supernatants.

Histological assessment

Skin specimens were obtained from earlobes and fixed in 20% buffered formalin and embedded in paraffin. Multiple 3 mm sections were stained with haematoxylin and eosin (H&E) for eosinophil and lymphocyte counting. The numbers of eosinophils and lymphocytes in the dermis were enumerated in three high power fields of microscopy and expressed per one section (0.25 mm^2) at 400×. Each section was assessed in random order by two observers of us without the knowledge of patient identification.

Preparation of skin homogenized samples and real-time quantitative PCR analysis

To examine the cytokines and chemokine receptors of T cells, homogenized samples of whole ears were used. BALB/c mice were sensitized on the shaved abdomen with 200 μl of 1% FITC in acetone/dibutyl phthalate (1:1 ratio) three times a week for 2 weeks and earlobes were provoked by painting of 40 μl of 1% FITC 24 h after tape-stripping. At 3, 6 and 12 h after challenge, the earlobes were prepared and homogenized using a T 10 basic Ultra-Turrax (Ika-Werke, Staufen, Germany) with the Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA). Total cellular RNA was extracted with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen Inc.) from Trizol samples. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (Applied Biosystems) interferon-γ (IFN-γ), interleukin-4 (IL-4), CXCR3, and CCR4. Quantitative PCR was performed as described above.

Contact hypersensitivity (CHS)

Mice were sensitized with FITC by painting of the shaved abdomen with 200 μl of 1% FITC. Five days after sensitization, the earlobes were barrier disrupted or untreated. After 24 h, mice were elicited by painting of both sides of earlobes with 40 μl of 0.5% FITC and the increase in ear thickness was measured immediately before and 1, 4, 8 and 24 h after painting using a dial thickness gauge (Ozaki Co, Tokyo, Japan). Ear swelling was calculated as (ear thickness after challenge) – (ear thickness before challenge).

Statistical analysis

Data were analysed using an unpaired two-tailed *t*-test. $P < 0.05$ was considered to be significant.

Results

Absence of differences in TEWL following treatment between tape-stripping and acetone-rubbing and between BALB/c and B6

In advance of testing the actions on the epidermal chemokine production and cell infiltration, we compared tape-stripping and acetone-rubbing in their effects on TEWL, a representative marker for the barrier function. We used two strains of mice, Th2-preponderant BALB/c and Th1-preponderant B6 mice. Earlobes of mice were stripped with cellophane tape or rubbed with acetone and TEWL was monitored after treatment. TEWL was elevated immediately after either of the treatments and declined thereafter at comparable levels (Fig. 1). Therefore, there was no difference between tape-stripped and acetone-rubbed skin sites in the increment and recovery of TEWL. Furthermore, BALB/c (Fig. 1a) and B6 mice (Fig. 1b) had virtually the same TEWL values following the treatments.

Higher expression of Th2 and eosinophil chemokine mRNAs by tape-stripping than acetone-rubbing in ECs of BALB/c mice

Earlobes of mice were stripped with cellophane tape or rubbed with acetone and EC suspensions were prepared from the ears at 6, 12 or 24 h after treatment and subjected to real-time PCR analysis. As shown in Fig. 2, the two barrier disruption procedures differentially induced the expression of mRNA for Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil-chemoattracting chemokine (CCL5) (10,11), depending on the mouse strains and the timing after treatment. BALB/c mice exhibited higher expression levels of all the chemokines than did B6 mice. In BALB/c mice, the increased expression was discernible at 12 h and remarkable at 24 h after treatment. CCL17, CCL22 and CCL5 were more strongly induced by tape-stripping than acetone-rubbing, but inversely, CXCL10, CXCL9 and CXCL11 were expressed more remarkably by acetone-rubbing than tape-stripping. Thus, tape-stripping is capable of inducing the production of Th2 chemokines by ECs in BALB/c mice.

To confirm the expression of Th2 chemokines and CCL5 promoted by tape-stripping, EC suspensions were prepared from BALB/c mice at 6, 12 or 24 h after the treatment and cultured for 48 h. The chemokine concentration in the culture supernatants was measured using ELISA. ECs from the treated mice produced higher levels of CCL17, CCL22 and

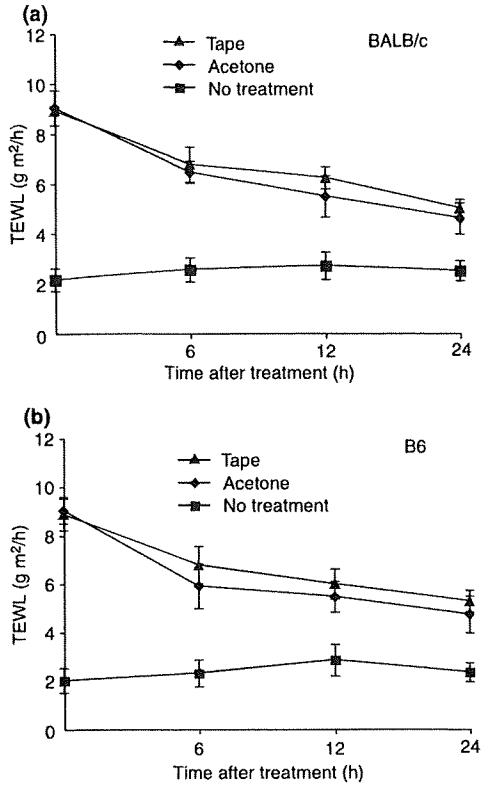

Figure 1. TEWL following tape-stripping or acetone-rubbing. Earlobes of BALB/c (a) and B6 mice (b) were stripped with tape or rubbed with acetone. Immediately after treatment (time 0), or 6, 12 or 24 h after treatment, TEWL was measured. Data are expressed as the mean ± SD of five mice.

CCL5 than those of untreated mice (Fig. 3). Again, tape-stripping induced the production of these chemokines, particularly CCL17, at significantly higher levels than acetone-rubbing.

Infiltration of eosinophils in tape-stripped ears of BALB/c mice

As tape-stripping stimulated ECs to produce chemokines, we monitored the infiltrate of inflammatory cells in the tape-stripped earlobes of BALB/c and B6 mice. Interestingly, we found that the tape-stripped ears of BALB/c mice showed infiltration of eosinophils in the dermis (Fig. 4a,b). Eosinophils appeared in the dermis at 8 h after treatment and increased in number at 24 h (Fig. 4c). Acetone-rubbing did not induce such an infiltrate of eosinophils. In B6 mice, the tape-stripping-provoked infiltrate of eosinophils was barely perceptible (Fig. 4d). Thus, the enhanced expression of CCL5 seems to be significant *in vivo*. In both strains, lymphocytes also infiltrated after either treatment with acetone or tape (Fig. 4e,f).

Augmented expression of IL-4 and CCR4 by challenge *via* tape-stripped skin in repeatedly sensitized mice

To examine the effect of tape-stripping on the induction of Th2 cells, we used the repeated sensitization method. As even the repeated sensitization with a hapten does not exclusively induce Th2 cells, Th1 cytokines are simultaneously increased by the challenge to some extent. BALB/c mice were sensitized with 1% FITC three times a week for 2 weeks on the abdomen and challenged with 1% FITC on the earlobes untreated or stripped with tape 24 h before. The ears were taken at 3, 6 or 12 h after challenge, homogenized and subjected to real-time PCR analysis for the expression of IFN-γ, IL-4, CXCR3 and CCR4. IFN-γ and IL-4 are representative Th1 and Th2 cytokines respectively, and CXCR3 and CCR4 are Th1 and Th2 chemokine receptors respectively (11). The expression of IFN-γ was increased at 3–12 h after challenge and the challenge *via* tape-stripped skin elevated its expression compared with the challenge *via* untreated skin (Fig. 5a). IL-4 expression was also enhanced by the challenge *via* tape-stripped skin at 3, 6 and 12 h (Fig. 5b). As to the chemokine receptors, while CXCR3 was not affected by challenge through the tape-stripped skin (Fig. 5c), CCR4 was augmented by the challenge (Fig. 5d). Thus, the expression of Th2 cytokine as well as IFN-γ and Th2 chemokine receptor was augmented in the tape-stripped and challenged skin, suggesting promoted accumulation of Th2 cells by tape-stripping.

Enhancement of both late-phase and delayed-type hypersensitivities in tape-stripped mice

It has been reported that the delayed-type contact hypersensitivity is enhanced through the barrier-disrupted skin (2). We further explored the *in vivo* significance of tape-stripping-augmented chemokine production in cutaneous hypersensitivities. BALB/c mice were sensitized with 1% FITC and challenged with 0.5% FITC on the tape-stripped or untreated earlobes. At 8 and 24 h after challenge, higher ear swelling responses were observed in mice challenged *via* tape-stripped ears than those challenged *via* untreated skin (Fig. 6). Acetone-rubbing, instead of tape-stripping, did not enhance the ear swelling responses at 8 h after challenge, while the treatment augmented the response 24 h after challenge (data not shown). Therefore, elicitation through the tape-stripped skin augmented not only the delayed-type but also the late-phase reactions of contact hypersensitivity where Th2 cells and eosinophils are involved (12).

Discussion

Our study showed that acute barrier disruption upregulates the production/expression of chemokines by ECs, depend-

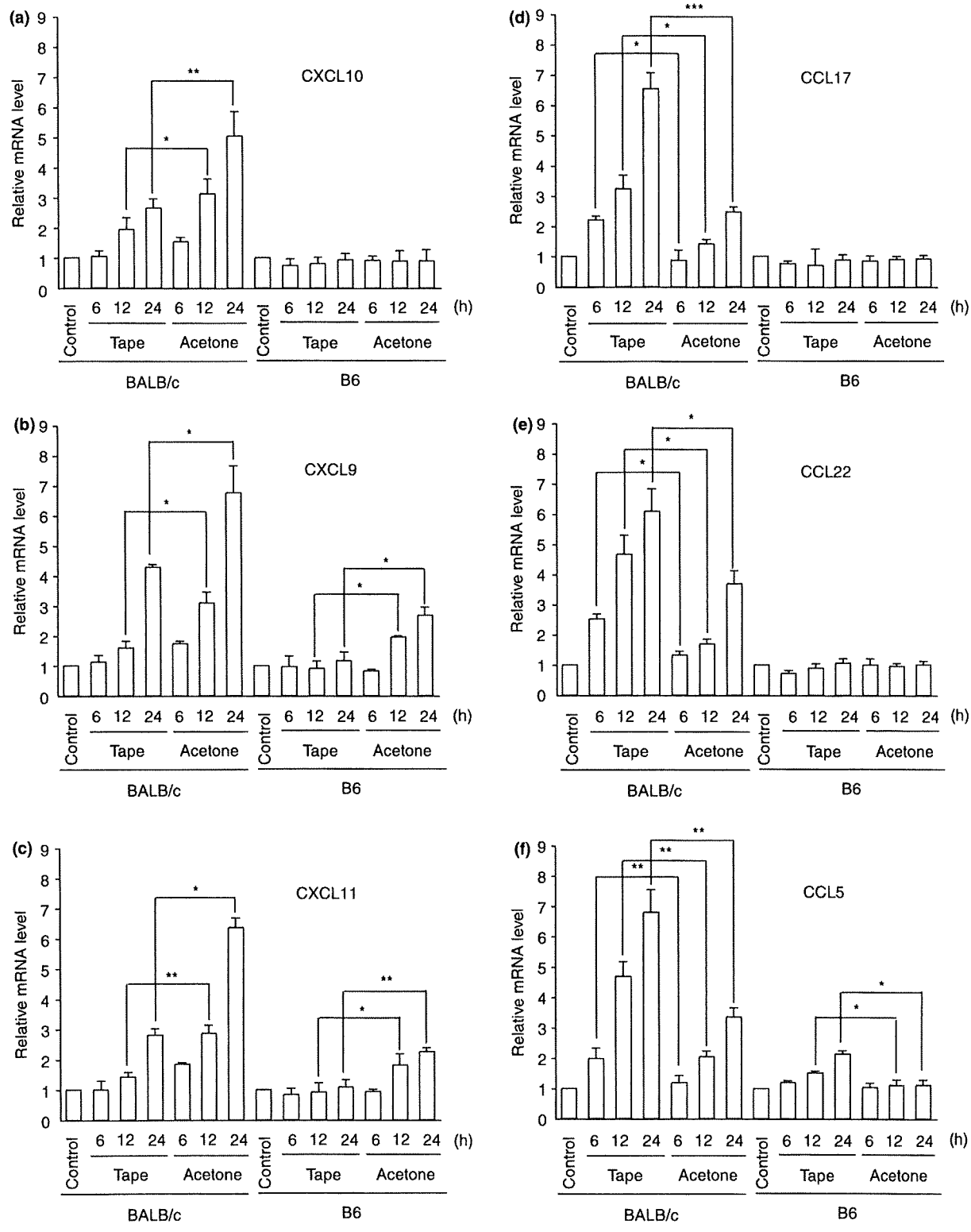

Figure 2. Real-time PCR analysis of mRNA expression for chemokines in ECs from barrier-disrupted earlobes. Earlobes of BALB/c and B6 mice were stripped with tape or rubbed with acetone. At 6, 12 and 24 h after treatment, EC suspensions were prepared and subjected to real-time PCR analysis for chemokines, including Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokine (CCL17 and CCL22) and CCL5. The expression of mRNA is represented as fold increase (2^{-DDC_t}), where $DDC_t = [DC_t \text{ (sample)}] - [DC_t \text{ (ECs without treatment)}]$ and $DC_t = [C_t \text{ (sample)}] - [C_t \text{ (b-actin)}]$. Data are expressed as the mean ± SD of five mice (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).

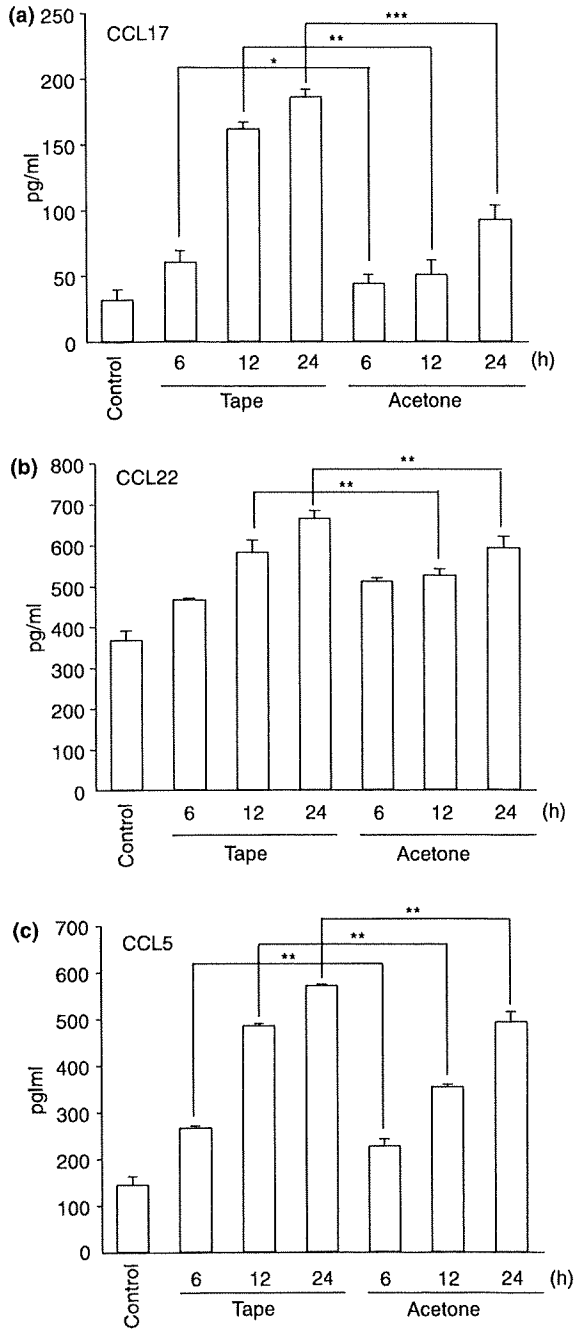

Figure 3. ELISA for chemokines in culture supernatants from ECs obtained from barrier-disrupted earlobes in BALB/c mice. Earlobes of BALB/c mice were stripped with tape or rubbed with acetone. At 6, 12 and 24 h after treatment, EC suspensions were prepared and cultured for 48 h. The concentration of CCL17, CCL22 and CCL5 in the culture supernatants was measured using ELISA. Data are expressed as the mean ± SD of five mice ($*P < 0.05$, $**P < 0.005$, $***P < 0.0001$).

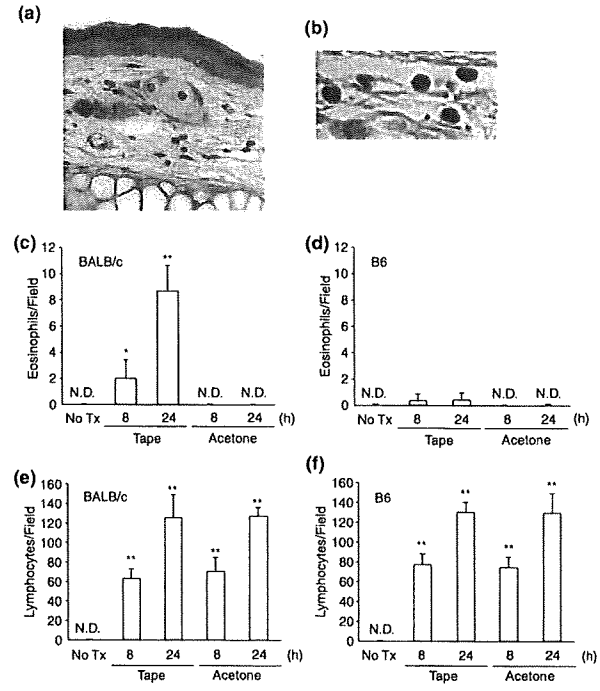

Figure 4. Histological pictures and numbers of infiltrating eosinophils and lymphocytes in tape-stripped earlobes of BALB/c mice. Earlobes of BALB/c and B6 mice were tape-stripped and acetone-rubbed and histological sections (H&E) were prepared 8 and 24 h later. Control sections were obtained from non-treated earlobes. (a, b) Histological picture of BALB/c mice at 24 h after tape-stripping (original magnification 200× and 400×). The numbers of eosinophils and lymphocytes were counted per one section (0.25 mm^2) at 400×. (c) Eosinophil counts in BALB/c mice treated with acetone-rubbing or tape-stripping. (d) Eosinophil counts in B6 mice treated with acetone-rubbing or tape-stripping. (e) Lymphocyte counts in BALB/c mice treated with acetone-rubbing or tape-stripping. (f) Lymphocyte counts in B6 mice treated with acetone-rubbing or tape-stripping. Data are expressed as the mean ± SD of five mice ($*P = 0.0061$, $**P < 1.0 \times 10^{-6}$). No Tx, no treatment; and N.D., not detected.

ing on the procedure of disruption, type of chemokines and strain of mice. Th2 chemokines CCL17 and CCL22 and eosinophil chemoatrractant CCL5 (10,11) were augmented by tape-stripping more markedly than acetone-rubbing, while Th1 chemokines CXCL10, CXCL9 and CXCL11 (10,11) were enhanced by acetone-rubbing. The increased production of CCL17, CCL22 and CCL5 was clearly observed in Th2-polarized BALB/c mice but not in Th1-dominant B6 mice. It should be stressed that, in accordance with this observation, tape-stripping allowed eosinophils to infiltrate in the dermis of BALB/c mice. In addition, FITC challenge *via* tape-stripped ears of sensitized BALB/c mice induced the expression of IL-4 and CCR4, indicating accumulation of Th2 cells in the tape-stripped and hapten-challenged skin. Accordingly, the tape-stripped mice showed increased responses at 8 h as well as 24 h when they were challenged *via* the treated ears. These findings suggest that tape-stripping stimulates ECs to express/produce Th2 chemokines and eosinophil chemoattractant and hapten application *via* the tape-stripped skin evokes the late phase reaction.

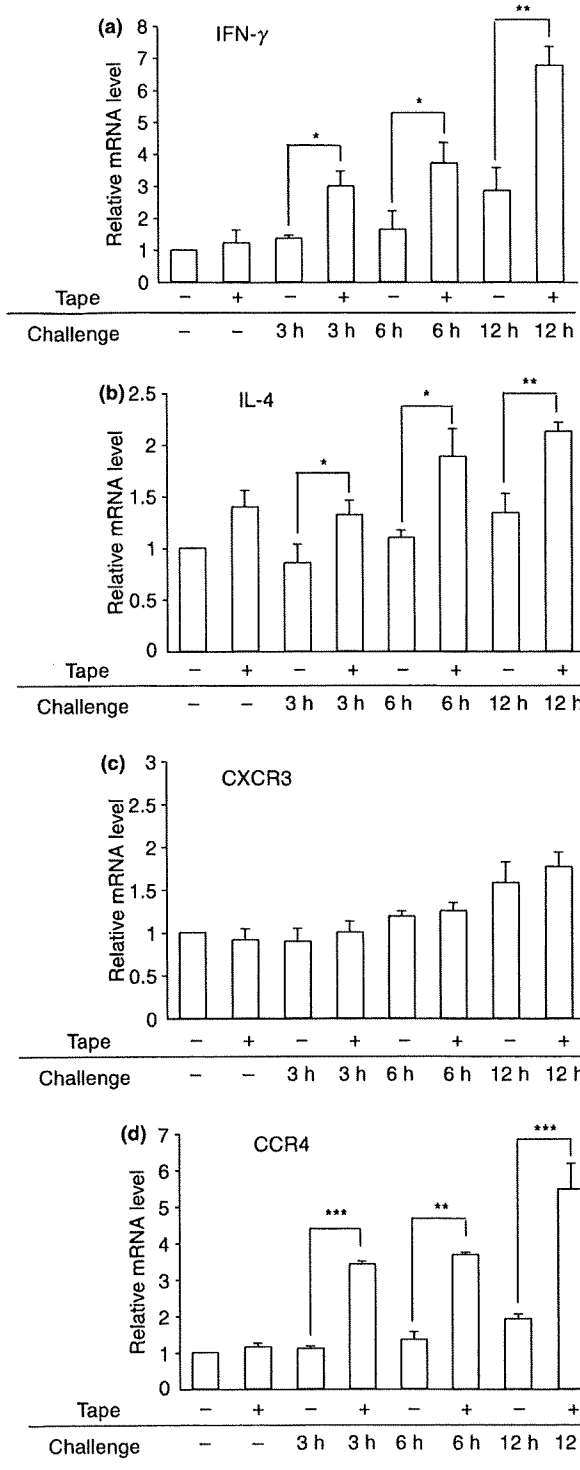

Figure 5. Real-time PCR analysis of mRNA expression for IFN-γ, IL-4 and CCR4 in the challenged skin. BALB/c mice were sensitized on the shaved abdomen with 1% FITC three times a week for 2 weeks and earlobes were provoked by painting of 1% FITC 24 h after tape stripping. At 3, 6 and 12 h after challenge, the earlobes were prepared and homogenized and subjected to real-time PCR analysis to assess the expression of IFN-γ, IL-4 and CCR4. Data are expressed as the mean ± SD of five mice (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$).

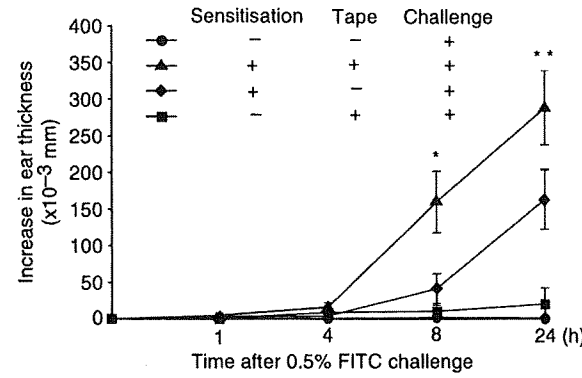

Figure 6. Augmentation of CHS to FITC n barrier-disrupted mice. BALB/c mice were repeatedly sensitized on the shaved abdomen with 1% FITC, and earlobes were provoked by painting of 0.5% FITC after tape stripping. Ear swelling responses were measured 1, 4, 8 and 24 h after challenge. Data represent Δ ear swelling from the basal ear thickness and are expressed as the mean ± SD of five mice (*$P = 0.00046$, **$P = 0.0026$).

Both tape-stripping and acetone-rubbing are known as a procedure for the acute barrier disruption, and in fact, the TEWL values of the treated skin were comparable in our study. However, the two treatments had different capacities to stimulate ECs to produce Th1 and Th2 chemokines. As keratinocytes can produce both Th1 and Th2 chemokines (11), this observation might be interpreted as an indication that tape-stripping and acetone-rubbing preferentially stimulate keratinocytes to produce Th2 and Th1 chemokines respectively. However, our recent study suggests that the main sources of Th1 (CXCL10, CXCL9 and CXCL11) and Th2 chemokines (CCL17 and CCL22) are keratinocytes and Langerhans cells respectively (13). We found that repeated application of hapten induces Th2 chemokine production by Langerhans cells. This raises an alternative possibility that tape-stripping stimulates Langerhans cells to produce Th2 chemokines and does keratinocytes to produce Th1 chemokines. In this scenario, acetone-rubbing possibly induces keratinocyte Th1 chemokine production without stimulating Langerhans cells to produce Th2 chemokines. Langerhans cells are also known to release CCL5 (14) and the observed increment of this eosinophil attractant might be derived from Langerhans cells. Tape-stripping has been shown to activate keratinocytes to produce Langerhans cell-maturating cytokines including interleukin-1α, tumor necrosis factor-α and granulocyte/macrophage colony stimulating factor (2). In parallel with this maturation, Langerhans cells might also release Th2 and eosinophil-chemoattracting chemokines.

The differences in the chemokine expression between the two stains were clearly seen with tape-stripping. BALB/c mice were more susceptible to tape-stripping than B6 mice in the

expression of all the chemokines examined. In particular, the expression of the Th2 chemokines and eosinophil chemoatrractant was markedly promoted in BALB/c mice. Although the increased expression of the Th2 and eosinophil chemokines in BALB/c mice is in accordance with the Th2-skewing property of this mouse strain, the exact mechanism underlying this preponderant expression remains unknown. To address this issue, we cultured keratinocytes from BALB/c and B6 mice, examined the production of Th2 chemokines after stimulation with interferon-γ and/or tumor necrosis factor-α and found no difference in the chemokine production between the two strains of mice. Therefore, the keratinocytes themselves are considered not to differ from each other. Given that interferon-γ suppresses Langerhans production of Th2 chemokines (13), the difference in the Th2 chemokine production might be attributable to the different Th1 or Th2 cytokine dominancy in each strain.

The tape-stripped skin exhibited enhanced degrees of the late-phase as well as delayed-type reactions upon challenge with hapten. This is thought to be a reflection of the infiltrates of eosinophils and Th2 cells (15). It is well known that patients with atopic dermatitis have skin barrier impaired by loss of filaggrin (16), decreased amounts of ceramide (8) and secondary damage following inflammation (17). Scratching caused by itch further exaggerates the barrier damage in atopic patients (18). Both the delayed-type and late-phase reactions have been put forward for the mechanisms underlying skin lesions of atopic dermatitis (19). The delayed-type reaction is clinically represented by eczematous dermatitis and mediated by Th1 and Tc1 cells (20). On the other hand, the late-phase reaction is clinically recognized by edematous erythema and mediated by Th2 and eosinophils (21). The late-phase reaction is prone to occur in the stratum corneum-removed skin and scratching exacerbates certain skin disorders such as atopic dermatitis by inducing Th2 and eosinophil-attracting chemokines. Moreover, it is tempting to speculate from our study that scratching could induce eosinophil infiltration in healthy skin and yield a late phase reaction without application of an allergen. Studies in human skin may clarify these important issues.

Acknowledgement

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Conflict of interest statement

The authors state no conflict of interest.

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LETTER TO THE EDITOR

Antihistaminic drug olopatadine downmodulates CCL17/TARC production by keratinocytes and Langerhans cells

Dear Editor,

Olopatadine hydrochloride (OLP; [Z]-11-[3-dimethylaminopropylidene]-6,11- dihydrodinenz [b,e] oxepin-2-acetic acid monohydrochloride) is a histamine H_1-receptor-blocking agent that possesses both acidic and basic residues.[1] This H_1 blocker also suppresses the production by epithelial cells or mast cells of various chemical mediators and cytokines, such as leukotrienes, arachidonic acid, interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α),[2,3] and inhibits intracellular adhesion molecule 1 (CD54) expression on conjunctival cells[4] and activity/migration of eosinophils.[5] Based on these findings, OLP is now widely used for the treatment of allergic rhinitis, urticaria and various itchy skin diseases including eczematous dermatitis.[6]

It has been reported that OLP has a unique anti-allergic property, which may provide implications for the mechanisms underlying its therapeutic actions. Thymus and activation-regulated chemokine (CCL17/TARC) is one of the T-helper (Th)2-associated chemokines, and an important regulator of Th2 cell recruitment into the skin.[7] Serum CCL17 level is proportional to the disease activity of atopic dermatitis (AD), and OLP inhibits CCL17 production by peripheral blood mononuclear cells from AD patients.[7] Serum CCL17 level is also related to the disease activity of bullous pemphigoid, mycosis fungoides, chronic actinic dermatitis and papuloerythroderma.[8,9] In the skin, CCL17 is secreted by keratinocytes (KC) and Langerhans cells (LC). LC are professional antigen-presenting cells in the epidermis, and we have recently shown that they are the main source of CCL17 among epidermal cells.[10] These findings urged us to investigate whether OLP induces inhibition of CCL17 production by KC or LC *in vitro*. To examine the effects of OLP on KC, we used human KC cell line HaCaT cells. LC-enriched epidermal cells (LC-EC) and bone marrow-derived dendritic cells (BMDC) were prepared from BALB/c mice. Our results suggest that OLP exerts its therapeutic effectiveness by inhibiting CCL17 production by both KC and LC.

First, to explore whether olopatadine suppresses CCL17 production by KC, we added olopatadine into the culture medium of KC cell line HaCaT cells. Three-day culture supernatants from HaCaT cells were collected, stored at −80°C and measured for CCL17, CCL22/MDC, monokine induced by γ-interferon (IFN-γ) (CXCL9/Mig) and IFN-γ-inducible protein 10 (CXCL10/IP-10) using enzyme-linked immunosorbent assay (ELISA) kits (Genzyme/Techne, Minneapolis, MN, USA) according to the manufacture's directions. It has been reported that the concentrations of OLP at 10^{-5} to 10^{-7} mol/L suppresses *in vitro* activities of both KC and LC.[11] Therefore, we followed the protocols to examine the inhibitory activity of olopatadine in our experiments. As shown in Figure 1, the IFN-γ/TNF-α-augmented production of CCL17 was suppressed significantly by the addition of olopatadine at a concentration of 10^{-6} or 10^{-5} mol/L. The concentrations of OLP in this *in vitro* study were chosen on the basis of the therapeutic dose of this drug.[12]

To see the effects of olopatadine on CCL17 production by LC-EC, epidermal cell (EC) suspensions freshly isolated from naïve BALB/c mice were subjected to Ficoll gradient separation of LC-EC as described previously.[13] The percentage of LC in LC-EC fraction was 15–20%, as assessed by flow cytometric analysis with anti-I-A^d phycoerythrin (PE)-labeled monoclonal antibody (BD PharMingen, San Diego, CA, USA). OLP

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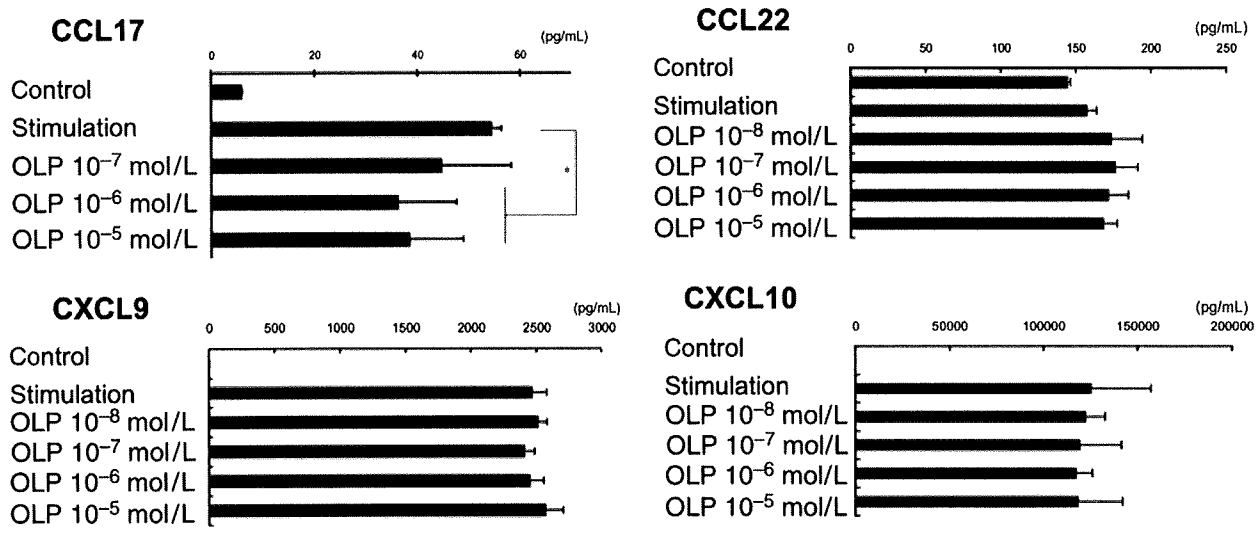

Figure 1. To examine chemokine production, semiconfluent HaCaT cells in 24-well plates were stimulated with 2000 units/mL of recombinant γ-interferon (IFN-γ) (Biogamma; Maruho Pharmaceutical, Osaka, Japan) and 4000 units/mL of tumor necrosis factor-α (TNF-α) (Invitrogen, Carlsbad, CA, USA) for the first 2 h, followed by 200 units/mL IFN-γ and 400 units/mL TNF-α thereafter. Olopatadine (OLP) was added at the starting of culture. Three-day culture supernatants were measured for CCL17, CCL22, CXCL9 and CXCL10 by enzyme-linked immunosorbent assay. Data represent the mean ± standard deviation. $^{*}P < 0.05$.

downregulated the expression of mRNA for CCL17 but not CCL22 (Fig. 2a). Because KC coexist with LC in LC-EC fraction, we also investigated the production of CCL17 by BMDC, a mimicry of pure LC. Murine immature DC were generated from bone marrow according to standard protocols.[14,15] Minor modification included feed culture medium on day 3 containing granulocyte-macrophage colony-stimulating factor (10 ng/mL). On day 6, BMDC (5×10^6/well) were cultured for 24 h with the two indicated concentrations of OLP. As shown in Figure 2(b), OLP decreased the mRNA expression of CCL17 and CCL22 in mature BMDC. Three independent series of experiments confirmed the result. CCL17 in culture supernatants was quantified by ELISA. OLP significantly suppressed the production of CCL17 by 37%, while the production of CCL22, CXCL9 or CXCL10 was not inhibited (data not shown). The above findings suggested that OLP directly downregulates Th2 chemokine production by DC and LC.

It has been reported that PAM 212 cells, a murine KC cell line, and normal human KC produce CCL17 after stimulation with TNF-α and IFN-γ.[16,17] Consistent with these *in vitro* data, CCL17 is expressed in the lesional KC of AD skin, suggesting that KC is one of the main sources of CCL17.[17] CCL17-transgenic mice showed enhanced Th2 type contact hypersensitivity and reduced Th1 type reactivity.[18] In this study, we demonstrated that OLP downmodulates the production of CCL17 by epidermal KC.

We have previously demonstrated that the ability of LC to present hapten to prime T cells was reduced by OLP with decreased expression of major histocompatibility complex class II and co-stimulatory molecules.[11] LC are capable of producing a high level of CCL17 constitutively during culture even without exogenous stimuli,[19] and we have recently shown that LC are responsible for the production of CCL17 by epidermal cells.[10] The present study showed that OLP inhibits the production of CCL17 by LC-EC. Thus, OLP is effective for the treatment of Th2-associated skin disorders not only by suppressing antigen-presenting ability but also by inhibiting CCL17 production. In our experiment system using LC-EC, KC coexisted with LC, raising the possibility that OLP alters CCL17 production by LC indirectly by modulating bystander KC. Therefore, another DC population without contamination of KC was tested for the modulatory effect of OLP on the chemokine production. Because Th2 chemokines including CCL17 was

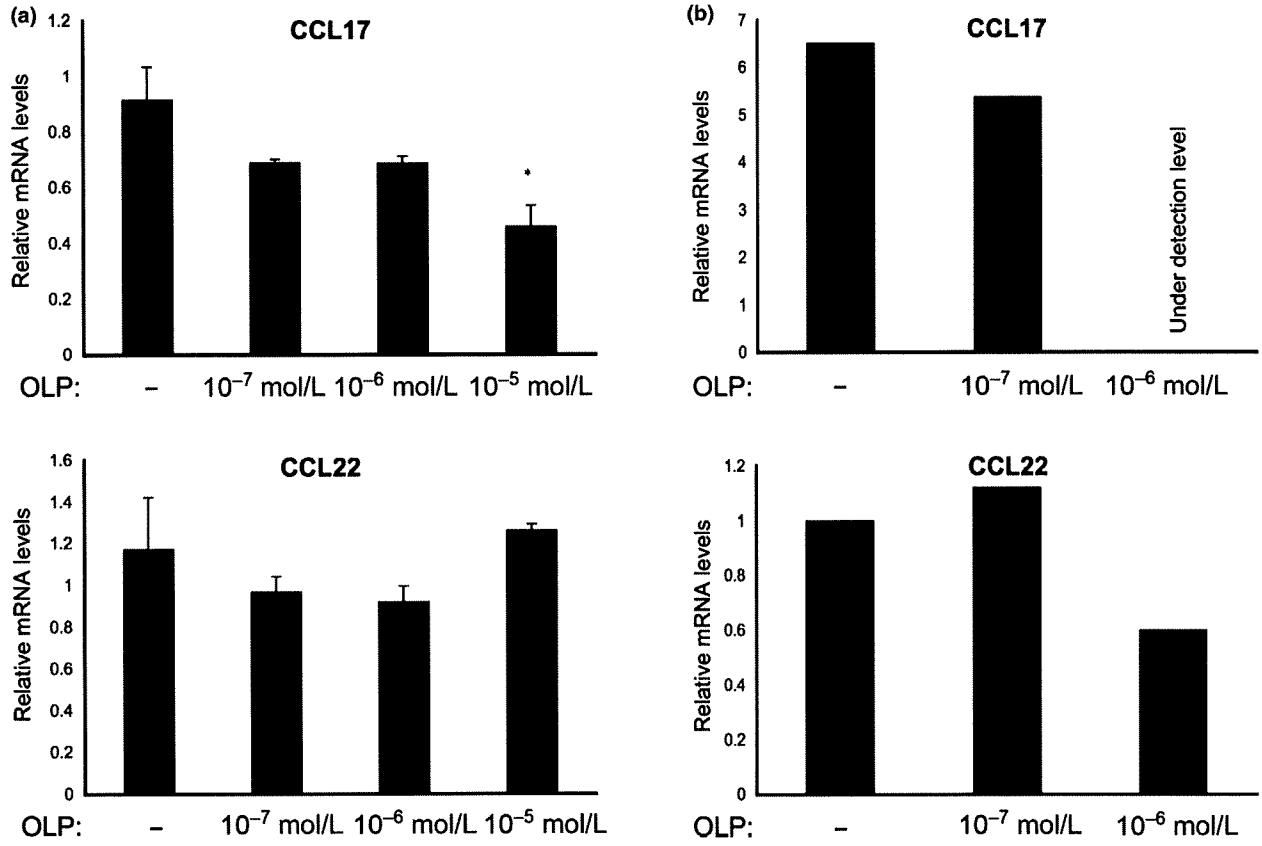

Figure 2. Total cellular RNA was extracted with an RNA extraction kit (Promega, Madison, WI, USA) from cultured Langerhans cell-enriched epidermal cells (LC-EC) and bone marrow-derived dendritic cells (BMDC). RNA was then reverse-transcribed and amplified by random hexamer in single-tube assay using the TaqMan Reverse Transcription Reagants (Applied Biosystems, Foster City, CA, USA) with gene-specific sense and antisense primers and a detection probe labeled on the 5′-end with the reporter dye 6-FAM. Primers and probes were obtained from TaqMan Gene Expression Assays Inventories (accession numbers: CCL17, Mm00516136-m1; CCL22, Mm00436439-m1; β-actin, 4352933E; all for Applied Biosystems). Using an ABI Prism 7000 Sequence Detection Systems (Applied Biosystems), samples were reverse-transcribed and amplified. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of standard curve generated from control mRNA levels. Amplification of the gene for mouse β-actin was performed on all samples to control interspecimen variations in RNA amounts. (a) mRNA expression for chemokines in LC-EC. LC-EC from naïve mice were cultured with or without olopatadine for 24 h. The cultured cells were subjected to real-time polymerase chain reaction analysis for CCL17 and CCL22. Data are expressed as the mean ± standard deviation of triplicate culture. $^*P < 0.05$, compared with the olopatadine non-added one. (b) mRNA expression for chemokines in BMDC. BMDC were cultured for 24 h with or without olopatadine. The cultured cells were subjected to real-time polymerase chain reaction analysis for CCL17 and CCL22. The data are from a representative experiment out of three.

expressed in a subset of BMDC, we investigated the effect of OLP on CCL17 production by BMDC. OLP downregulated both CCL17 and CCL22 production by BMDC. Besides the effects of OLP on KC and LC, another study has shown that antihistamines regulate immune responses by affecting the interaction between DC and $CD4^+$ T cells.[20]

In summary, OLP suppresses the production of CCL17 by KC and DC. This suggests that OLP may exerts its therapeutic effect at least partly by down-modulating Th2 chemokine production by epidermal cells.

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CONFLICT OF INTEREST

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Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes

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Conflicts of interest
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Summary

Background Epidermal keratinocytes are involved in the skin innate immunity and express toll-like receptors (TLRs) and other innate immune proteins. The epidermis is continuously exposed to pathogenic Gram-positive bacteria or fungi. However, few studies have examined the function and expression of innate immune proteins in keratinocytes. Histamine, which is well known for itch and allergy, is closely associated with innate immunity, but its influence on epidermal innate immunity is still unclear.

Objectives To clarify the expression of innate immune proteins in keratinocytes stimulated by ligand pathogen-associated molecules, and the function of histamine in this process.

Methods We investigated the effects of lipopeptide (MALP-2, 1–100 ng mL^{-1}; ligand for TLR2), peptidoglycan (PGN, 0·02–2 μg mL^{-1}; ligand for NOD2) and β-glucan (1–100 μg mL^{-1}; ligand for dectin-1) in the presence or absence of histamine on mRNA expression of TLR2, NOD2 and dectin-1 as well as human β-defensin 2 by quantitative real-time polymerase chain reaction in cultured normal human epidermal keratinocytes. TLR2 expression was also examined at the cell surface and intracellularly, as determined by flow cytometry and confocal microscopy. The quantities of interleukin (IL)-1α and IL-8 produced by keratinocytes were measured using enzyme-linked immunosorbent assay.

Results At the mRNA level, TLR2 was enhanced by PGN but not by its ligand MALP-2 or by β-glucan; NOD2 was easily induced by all three ligands; and dectin-1 was enhanced by its ligand β-glucan. These enhanced expressions were further augmented by histamine at 1 μg mL^{-1}. While the surface expression of TLR2 was barely detectable by flow cytometry even after stimulation, the intracellular expression of TLR2 was apparently elevated by PGN and further promoted by histamine. A confocal microscopic analysis also revealed the enhanced expression of TLR2 in the cytoplasm. The expression of TLR2, NOD2 and dectin-1 was functional, as these pathogen-associated molecules induced the production of IL-1α, IL-8 and defensin, and again, histamine greatly enhanced this production.

Conclusions Our study demonstrated that the expression of functional innate immune receptors is augmented by the pathogen-associated molecules in a ligand-feed forward or nonrelated manner in keratinocytes, and histamine promotes their expression and the resultant production of cytokines and defensins.

Toll-like receptors (TLRs) are involved in the innate immune system and recognize various pathogen-associated molecular patterns of microorganisms, such as lipopeptide, lipopolysaccharide, RNA and unmethylated CpG DNA.[1] It has been shown by many studies that epithelial cells from several defensive organs play a pivotal role in the primitive defence system against microorganisms such as bacteria, fungi and viruses, and accordingly, they express TLRs.[2–4] TLR2 is a key

receptor for epithelial cells, because the epithelium is continuously exposed to pathogenic Gram-positive bacteria whose products stimulate TLR2. The expression of TLRs is induced by cytokines such as tumour necrosis factor (TNF)-α and interferon-γ,[4,5] and by TLR agonists *per se*.[6] In addition, it has been strongly suggested that mast cells are deeply involved in innate immunity[7] and in the epithelial defence system.[8,9] A recent finding that histamine, a key product from mast cells, induces TLR expression on endothelial cells,[10] implicates the ability of histamine to increase TLR expression in epithelial cells as well.

In the skin, keratinocytes are the first responders to external invaders and serve as initiators in innate immunity by producing cytokines/chemokines and antimicrobial peptides.[11–13] Several studies have documented that keratinocytes express TLRs such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR10.[14] However, the induction and detection of these molecules remain unclear or even controversial among the previous reports. As assessed by flow cytometry, human keratinocytes were shown to express TLR4 as well as CD14,[15] whereas another group of investigators reported that only TLR2 was detected on the surface of cultured normal human keratinocytes.[16] The HaCaT keratinocyte cell line was reported to express both TLR2 and TLR4 by flow cytometry.[17] On the other hand, an immunohistochemical study of human skin demonstrated that TLR1, TLR2 and TLR5 are constitutively expressed in the cytoplasm of normal keratinocytes and that TLR2 expression is increased in psoriasis.[18]

Upon stimulation of keratinocytes via TLRs, they produce cytokines/chemokines and antimicrobial peptides as an outcome of operation of the innate immunity. These substances include interleukin (IL)-1α, TNF-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-8, macrophage inflammatory protein-1α,[13,16,19–23] and major antimicrobial peptides, the β-defensins.[24–26] The production of these molecules is one of the hallmarks of expression of functional TLRs by keratinocytes. As a consequence, IL-1α, TNF-α and GM-CSF produced by keratinocytes subsequently activate the cutaneous acquired immunity by enhancing the antigen-presenting ability of dendritic cells.[13]

In this study, we aimed firstly to clarify the agents that induce the expression of TLR2 and its cooperating receptors in keratinocytes, focusing on lipopeptide, peptidoglycan (PGN), the fungal element β-glucan, and histamine. Secondly, we explored whether TLR2 expression can be assessed by flow cytometry at the surface or intracellular level. We chose these agents because lipopeptide is a TLR2 ligand[27] and β-D-glucan binds to dectin-1.[28] PGN, which is recognized by NOD2,[29,30] also augments expression of TLR2.[27,31] In addition to these external stimulants for epidermal keratinocytes, we also examined histamine, which is a physiological stimulator for TLR2 expression,[10] and whose receptors, H_1 and H_2,[32] are expressed on keratinocytes. Results suggest that PGN and histamine strongly elevate the intracellularly detectable TLR2 and augment the production/expression of cytokines/chemokines and β-defensin in normal human keratinocytes. It is notable that there is a synergism between the pathogen-associated molecules and histamine in the expression of TLR2 and the production of inflammatory and antimicrobial molecules.

Materials and methods

Culture and stimulation of keratinocytes

Normal human epidermal keratinocytes (NHEK) isolated from neonatal foreskin were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, U.S.A.) and grown in the serum-free keratinocyte growth medium KGM-2 (Clonetics, San Diego, CA, U.S.A.) or Dulbecco's modified Eagle's medium (Gibco BRL Life Technology Inc., Gaithersburg, MD, U.S.A.) at 37 °C in a 5% CO_2 incubator. NHEK were subcultured using trypsin–ethylenediamine tetraacetic acid (Clonetics), and semiconfluent cells at third passage were used in all experiments. Unless otherwise mentioned, semiconfluent keratinocytes in six-well plates (Corning Glass Works, Corning, NY, U.S.A.) with 2 mL of medium were stimulated with the following substances: lipopeptide (MALP-2; Axora, San Diego, CA, U.S.A.), PGN (InvivoGen, San Diego, CA, U.S.A.), β-glucan (MP Biomedicals, Aurora, OH, U.S.A.) and histamine (MP Biomedicals).

Quantitative real-time polymerase chain reaction

Total RNA from NHEK was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions with inclusion of the DNase step. Purified RNA was reverse transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, U.S.A.) with oligo d(T)16 primers. TaqMan experiments were carried out in an ABI PRISM 7000 Sequence Detector System using TaqMan Gene Expression Assays for TLR1, TLR2, TLR6, NOD2, human β-defensin 2 (hBD2) and dectin-1 (Applied Biosystems). Endogenous β-actin was used to normalize the gene expression between different samples.

Flow cytometry of toll-like receptor 2 expression

NHEK were incubated for 48 h with the stimulants. For the surface expression of TLR2, cells were stained with phycoerythrin (PE)-conjugated anti-TLR2 (TL2.1) monoclonal antibody (mAb) or isotype control mouse IgG2a (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). For the intracellular expression of TLR2, cells were first stained with nonconjugated anti-TLR2 mAb, fixed with Cytofix/Cytoperm (BD Pharmingen, San Jose, CA, U.S.A.) for 40 min at 4 °C, and stained with the PE-conjugated anti-TLR2 mAb as above. Hanks' balanced salt solution containing 0·1% NaN_3 and 1% fetal calf serum was used as the staining buffer. After incubation for 60 min at room temperature, cells were washed twice and analysed: fluorescent profiles were generated using a FACSCanto (Becton Dickinson, San Jose, CA, U.S.A.).

Quantification of cytokines and chemokines in keratinocyte culture supernatants

Three-day culture supernatants from NHEK were collected, stored at −80 °C, and assayed for IL-1α and IL-8 using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's directions. Optical density was measured with a microplate reader (model 3550; Bio-Rad, Hercules, CA, U.S.A.).

Construct and transfection

The pCMV-SPORT6-TLR2 (IMAGE clone 5213439) including full-length TLR2 cDNA (GenBank BC033756) and pCMV-SPORT6 (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) were purchased from Open Biosystems (Huntsville, AL, U.S.A.) and used for the transfection study. NHEK (5×10^5 cells) were cultured without serum and antibiotics in a 60 × 15 mm TC dish (Nalge Nunc International, Naperville, IL, U.S.A.) and used at 40–50% confluency. Transfection was performed with 4 μg of plasmid DNA and 8 μL of Hily Max (Dojindo Laboratories, Kumamoto, Japan) in Opti-MEM I (Gibco). Four hours after transfection, the cells were washed and soaked in the fresh medium.

Toll-like receptor (TLR) 2 blocking with anti-TLR2 antibody in interleukin-8 production

Semiconfluent NHEK were incubated with 10 μg mL^{-1} of Function Grade (FG) antihuman TLR2, clone TL2.1, or FG mouse IgG2a isotype control (eBioscience, San Diego, CA, U.S.A.) at room temperature for 1 h. PGN (0·02 μg mL^{-1}) or MALP-2 (1 ng mL^{-1}) was then added to the culture and incubated for 72 h. The concentration of IL-8 in the supernatants was measured by ELISA.

Immunocytoplasmic staining

NHEK were cultured in chamber slides (Nalge Nunc International). Slides were fixed and stained as for flow cytometric analysis. After staining, cells were analysed by confocal microscopy (LSM5 Pascal; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical differences were determined by Student's t-test or Welch's t-test; $P < 0·05$ was considered to be significant.

Results

Expression of mRNA for toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, dectin-1, NOD2 and human β-defensin 2 in normal human epidermal keratinocytes

We first examined whether TLR2, its cooperating receptors (TLR1 and TLR6), dectin-1, NOD2 and hBD2 are expressed in unstimulated or stimulated NHEK. TLR2 is a receptor for lipopeptide (MALP-2), and dectin-1 in combination with TLR2 is a receptor for zymosan, and they collaborate with each other in recognition of microbes and induction of inflammation.[33–35] NOD2 is an intracytoplasmic molecule that recognizes PGN.[29,30] hBD2 is an antimicrobial peptide known to be produced following TLR ligation.[36,37]

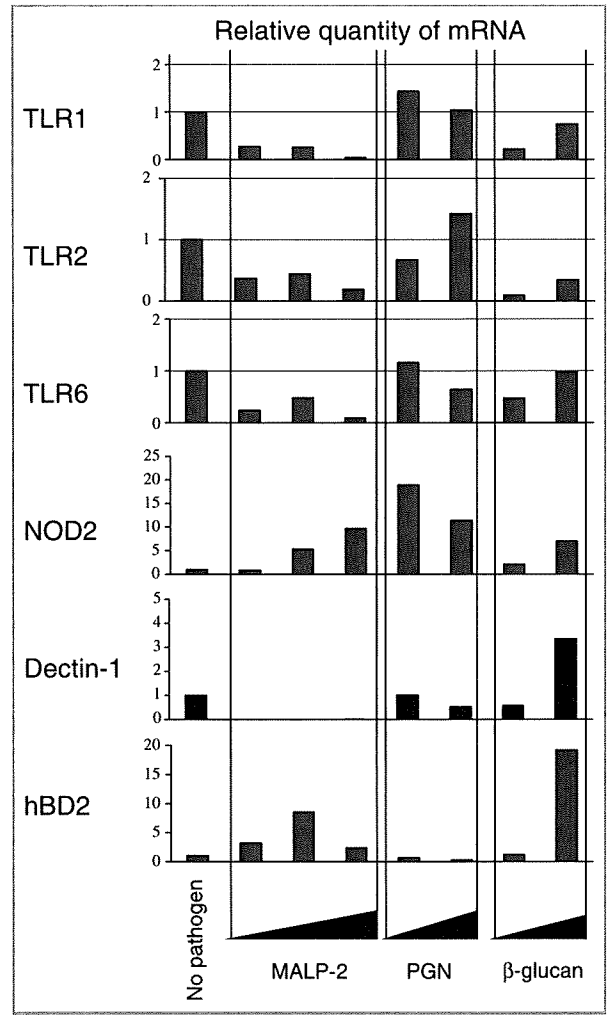

Fig 1. Expression of toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) in normal human epidermal keratinocytes (NHEK). NHEK were cultured for 2 h with MALP-2 (1, 10, 100 ng mL^{-1}), peptidoglycan (PGN) (0·2, 2 μg mL^{-1}) or β-glucan (1, 10 μg mL^{-1}) and subjected to real-time polymerase chain reaction analysis.

The levels of mRNA for TLR2, TLR1 and TLR6 were augmented by PGN to some extent at certain concentrations, but not by MALP-2 or β-glucan (Fig. 1). NOD2 expression was remarkably enhanced by all the stimulants, with variations. The expression of dectin-1 was elevated by β-glucan but not by MALP-2 or PGN. hBD2 expression was increased by MALP-2 and β-glucan. It is thus suggested that (i) NOD2 is easily inducible by various pathogenic stimulants, (ii) TLR2 is enhanced by PGN but not by its ligand lipoprotein or by

β-glucan; and (iii) dectin-1 is enhanced by β-glucan. These findings partly support the concept that TLR expression is often augmented by pathogen-associated molecules other than the corresponding specific ligand.[5,6]

Detection of augmented expression of intracellular toll-like receptor 2 by flow cytometry

We tested whether TLR2 is detectable at the protein level in NHEK, either unstimulated or following stimulation with PGN and β-glucan at relatively high doses. A flow cytometric analysis showed that the surface expression of TLR2 was very low, and that it was not enhanced by PGN (Fig. 2a) or β-glucan stimulation at either low or high Ca concentration, which induces basal and cornified keratinocytes, respectively. However, when these cells were subjected to intracellular staining, we found that PGN upregulated the expression of TLR2 (Fig. 2b). The addition of β-glucan also elevated TLR2 expression in the cytoplasm but not at the surface. Thus, an increased level of TLR2 was detected by flow cytometry in the cytoplasm but not at the surface of keratinocytes.

Functional expression of toll-like receptor 2, NOD2 and dectin-1 assessed by cytokine/chemokine production

To confirm the functional expression of TLR2, NOD2 and dectin-1, NHEK were cultured with MALP-2, PGN or β-glucan and the amounts of IL-1α and IL-8 secreted in the supernatants were measured. As shown in Figure 3, MALP-2 stimulated NHEK to produce these cytokines/chemokines. The NOD2 ligand PGN also augmented the production at an optimal concentration as low as 0·2 μg mL^{-1}. Similarly, β-glucan, a ligand for TLR2 and dectin-1, promoted the production of these cytokines/chemokines at an optimal concentration of 10 μg mL^{-1}. Thus, TLR2 and NOD2 seemed to be functionally expressed in NHEK.

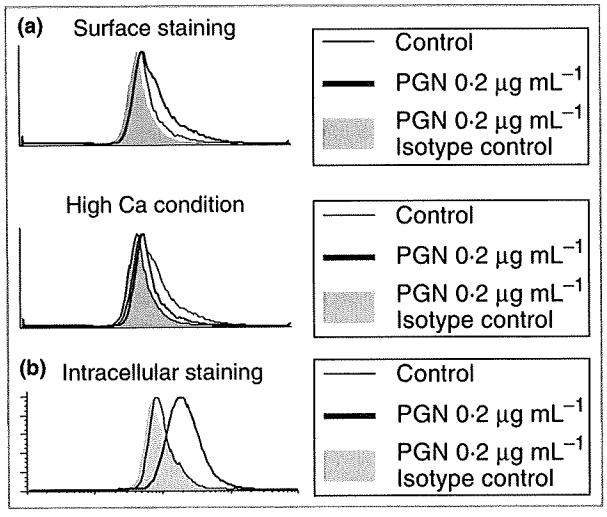

Fig 2. Flow cytometric detection of toll-like receptor (TLR) 2 expression in the cytoplasm but not on the surface of normal human epidermal keratinocytes (NHEK). NHEK were cultured in the presence or absence of peptidoglycan (PGN) for 48 h. Cells were stained with anti-TLR2 monoclonal antibody (mAb) or isotype-matched control antibody (a). The levels of isotype-matched control were evaluated in the stimulated NHEK. Simultaneously, NHEK cultured with the stimulant were intracellularly stained with anti-TLR2 mAb (b).

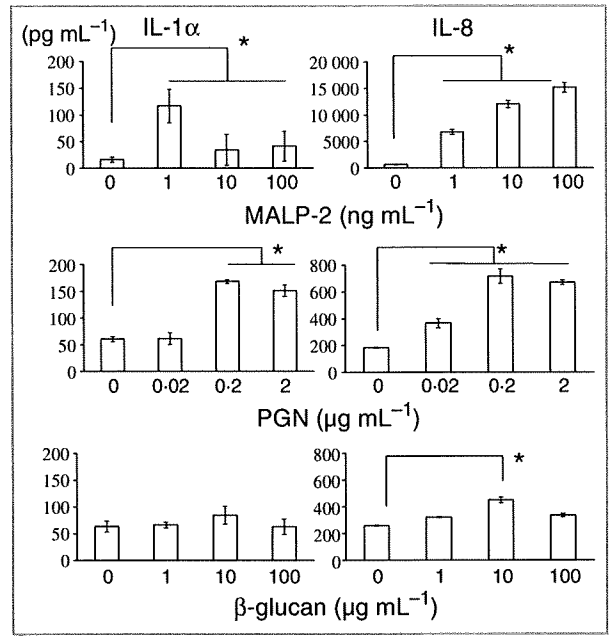

Fig 3. Augmentative effect of MALP-2, peptidoglycan (PGN) and β-glucan on cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h in the presence or absence of pathogen-associated molecules at the indicated concentration. The amounts of interleukin (IL)-1α and IL-8 in the supernatants were measured by enzyme-linked immunosorbent assay. The values are means of quadruplicate determinations with SD shown by vertical bars. *$P < 0·05$, compared with the nonaddition group.

It is known that PGN is not a ligand for TLR2 but is an activator.[31,38,39] To confirm this notion, we performed two studies. In one study, TLR2-transfected keratinocytes were stimulated with PGN or MALP-2, and the concentration of IL-8 was measured in the culture supernatants. Compared with the control (mean ± SD 1970·45 ± 15·14 pg mL^{-1}), addition of MALP-2 at 1 ng mL^{-1} (2518·01 ± 26·34 pg mL^{-1}) but not PGN at 0·02 μg mL^{-1} (1957·83 ± 28·44 pg mL^{-1}) gave a significantly higher level of IL-8 secretion in the supernatants. In the other study, NHEK were cultured with PGN (0·02 μg mL^{-1}) or MALP-2 (1 ng mL^{-1}) in the presence of blocking antibody to TLR2 or IgG2a as isotype-matched control. Following stimulation with PGN, neither anti-TLR2 antibody-treated nor isotype-matched control showed decreased production of IL-8. Following stimulation with MALP-2, however, treatment with anti-TLR2 antibody, but not control IgG2a, significantly decreased IL-8 production by 20%. Taken together, these findings suggest that PGN is not a specific ligand for TLR2.

Synergistic effects between pathogen-associated molecules and histamine on toll-like receptor 2 expression and cytokine production

NHEK were cultured with PGN or β-glucan in the presence or absence of histamine, and the levels of mRNA expression for TLR1, TLR2 and TLR6, NOD2, dectin-1 and hBD2 were measured by quantitative real-time polymerase chain reaction (PCR). Histamine at 1 μg mL^{-1} clearly amplified the expression of all the receptors and hBD2 that were induced by MALP-2 at 10 ng mL^{-1}, PGN at 2 μg mL^{-1} or β-glucan at 10 μg mL^{-1} (Fig. 4), demonstrating their synergistic effects.

Further to confirm the synergism by intracellular flow cytometry, we tested the combined effect of PGN at 0·2 μg mL^{-1} and histamine at 1 μg mL^{-1} on the expression of TLR2. The synergism between PGN and histamine was clearly observed, as the addition of both further elevated the TLR2 expression induced by individual stimulants (Fig. 5a), but again the surface expression was undetectable (data not shown). A confocal image analysis of keratinocytes showed that augmentation of the cytoplasmic level of TLR2 induced by synergism between PGN and histamine was greater than that of the cell surface level (Fig. 5b).

Finally, the synergism with histamine in cytokine/chemokine production was examined. NHEK were cultured with MALP-2, PGN or β-glucan in the presence or absence of histamine, and the amounts of IL-1α and IL-8 in the supernatants were measured. As shown in Figure 6, histamine at 1 μg mL^{-1} alone did not increase the production of IL-1α or IL-8 (solid bars of ligand 0). However, histamine upmodulated IL-1α and IL-8 production in the presence of MALP-2, PGN or β-glucan.

Discussion

Our study demonstrated that NHEK express both TLRs and nontoll-like innate immune proteins, including the intracellular Nod-like protein NOD2 and the surface C-type lectin dectin-1. As MALP-2, PGN and β-glucan stimulated keratinocytes to produce cytokines and antimicrobial peptides, it is considered that TLR2, NOD2 and dectin-1 are functionally expressed and efficiently mount the innate immunity in keratinocytes. More interestingly, the innate immune receptors were augmented by pathogen-associated molecules relevant or irrelevant to the corresponding receptors, as has been suggested in other types of cells.[5,6] The feed-forward stimulation was observed between PGN and NOD2 and between β-glucan and dectin-1, while TLR2 was induced by PGN but not by its ligand MALP-2. Therefore, the pathogen-driven acceleration of receptor expression appears to be complicated. However, NOD2 might be a key molecule, because all the pathogen-associated molecules tested enhanced NOD2 expression, and its ligand PGN was most stimulatory for the cytokine/hBD2 production among the pathogen-associated molecules.

TLR2 was induced by PGN and was detected at both protein and mRNA levels. We successfully evaluated the intracellular,

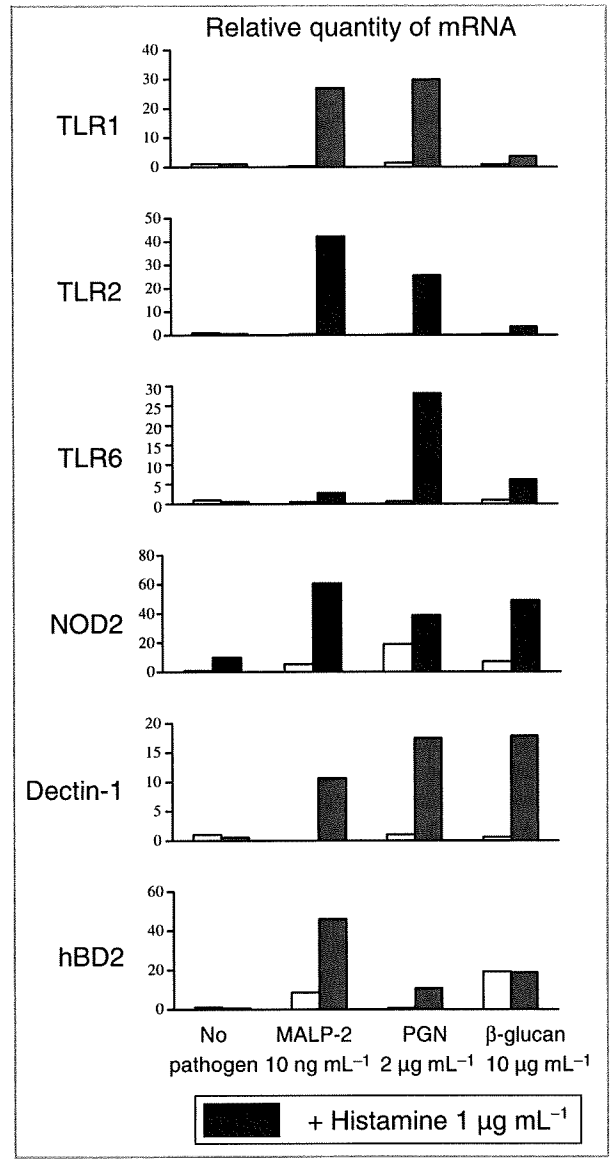

Fig 4. Synergism of MALP-2, peptidoglycan (PGN) or β-glucan, with or without histamine, in mRNA expression for toll-like receptor (TLR) 1, TLR2, TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) by real-time polymerase chain reaction (PCR) analysis. Normal human epidermal keratinocytes were cultured for 2 h with each of the pathogen-associated molecules and/or histamine and subjected to real-time PCR analysis.

but not surface, expression of TLR2 by flow cytometry. The expression of each TLR in keratinocytes has been a matter of debate.[40] In the present study, the surface expression of TLR2 was very low compared with the isotype-matched control, and was not enhanced by any of the stimuli at either low or high Ca concentration. Nevertheless, we found that the intracellular expression of TLR2 was significantly elevated upon stimulation. As the TLR2 ligand MALP-2 promoted the production/expression of cytokines and of hBD2, it is considered that TLR2 is expressed on keratinocytes as a functional surface molecule. Although the change of surface expression was

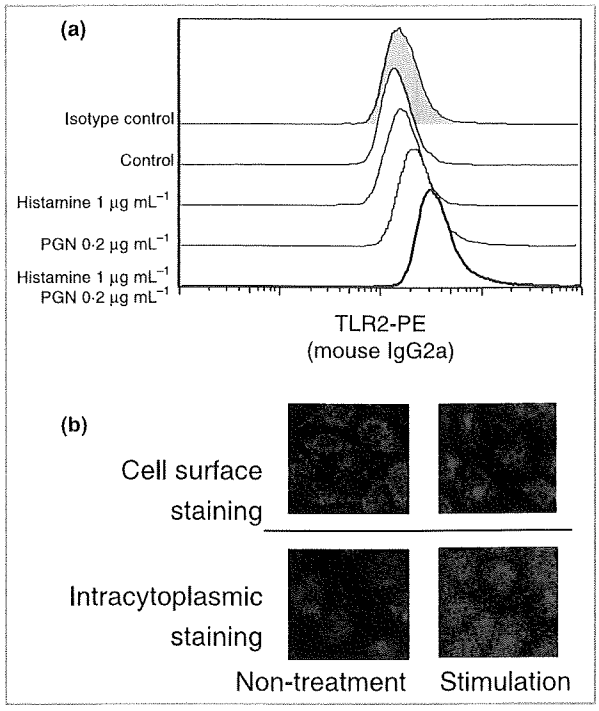

Fig 5. Synergism between peptidoglycan (PGN) and histamine in intracellular toll-like receptor (TLR) 2 expression and confocal microscopic analysis for detection of TLR2 in normal human epidermal keratinocytes (NHEK). (a) NHEK were cultured in the presence of histamine (1 μg mL^{-1}) and/or PGN (0·2 μg mL^{-1}), and were subjected to intracellular staining for TLR2. The level in the isotype-matched control was evaluated in NHEK stimulated with both histamine and PGN. (b) NHEK were cultured in a chamber slide in the presence or absence of PGN at 0·2 μg mL^{-1} and histamine at 1 μg mL^{-1} for 48 h. After incubation with nonconjugated isotype control mouse IgG2a, intracellular or cell surface staining of keratinocytes was performed with phycoerythrin (PE)-conjugated anti-TLR2 monoclonal antibody and visualized by confocal microscopy.

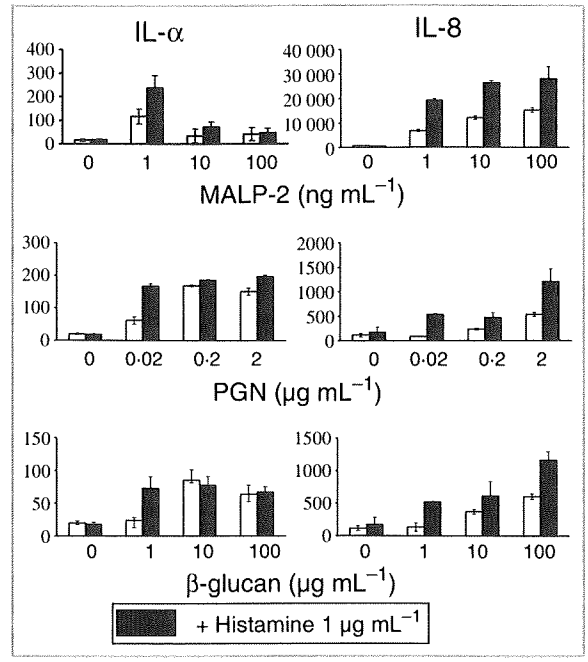

Fig 6. Synergism between pathogen-associated molecules and histamine in cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h with each of the pathogen-associated molecules and/or histamine. The concentrations of interleukin (IL)-α and IL-8 in the supernatants were measured by enzyme-linked immunosorbent assay. The values are means of quadruplicate determinations with SD shown by vertical bars. PGN, peptidoglycan.

under the detection level, the intensity of its intracellular expression may predict an alteration of the surface expression. A similar finding has been reported in dendritic cells[41] and has recently been reported in keratinocytes.[42] On the other hand, a recent finding has suggested that the intracellular TLR2 functions as a receptor for the infecting pathogen.[43] Considering that skin is constantly exposed to microorganisms, the low level of surface expression of TLR2 might be reasonable. If the quantity of TLR of the surface of keratinocytes were easily increased, this would always give rise to an irritable response to pathogens and to the occurrence of inflammation. In this respect, keratinocytes should be different from monocytes/macrophages, and intracellular TLR2 appears to recognize the pathogens when they invade the cell. There might exist a certain protein that regulates the surface expression of TLR2 like that of TLR4,[44] and it may act on the homeostasis of the epithelial tissue.

It is already known that histamine plays an important role for innate immunity.[45] We stimulated keratinocytes with the pathogen-associated molecules in combination with histamine. Histamine amplified the expression of TLRs and proinflammatory cytokines synergistically with the pathogen-associated molecules. The augmentation of TLR2 expression by histamine was also observed in endothelial cells.[10] We found that there was synergism between histamine and MALP-2, PGN or β-glucan in TLR2 expression, as assessed by both intracellular staining and real-time PCR. Such synergistic effects were also found in the expression of hBD2 and dectin-1 and the production of IL-1α and IL-8. In the skin, keratinocytes are potentially exposed to histamine that is released from dermal mast cells in certain pathological conditions. As mast cells also express TLRs and can produce histamine by TLR ligation,[46] pathogens may stimulate keratinocytes directly or indirectly via mast cells with their produced histamine. This scenario suggests the potential involvement of histamine in the natural defence system and may result in an exaggerated response to pathogens.

Our findings are of clinical significance. As the skin is constantly exposed to Gram-positive bacteria and fungi as exemplified by *Staphylococcus aureus* colonization[47] and superficial fungal infection,[48] the upmodulation of TLR2, NOD2 and dectin-1 in keratinocytes may be beneficial for the defence system. The expression of these molecules is enhanced by pathogens, resulting in the augmented production of

proinflammatory cytokines and chemokines for neutrophils and lymphocytes. These cytokines/chemokines eventually protect the host from bacteria and fungi by inducing inflammation and immune reactions. In addition, the upregulated production of the defensins effectively eliminates microorganisms. Histamine may support these events as an internal stimulus for innate immune protein expression. It is suggested that the activation of keratinocytes via innate immune proteins leads to pluripotential responses in the cutaneous innate immunity and subsequent acquired immunity.[13]

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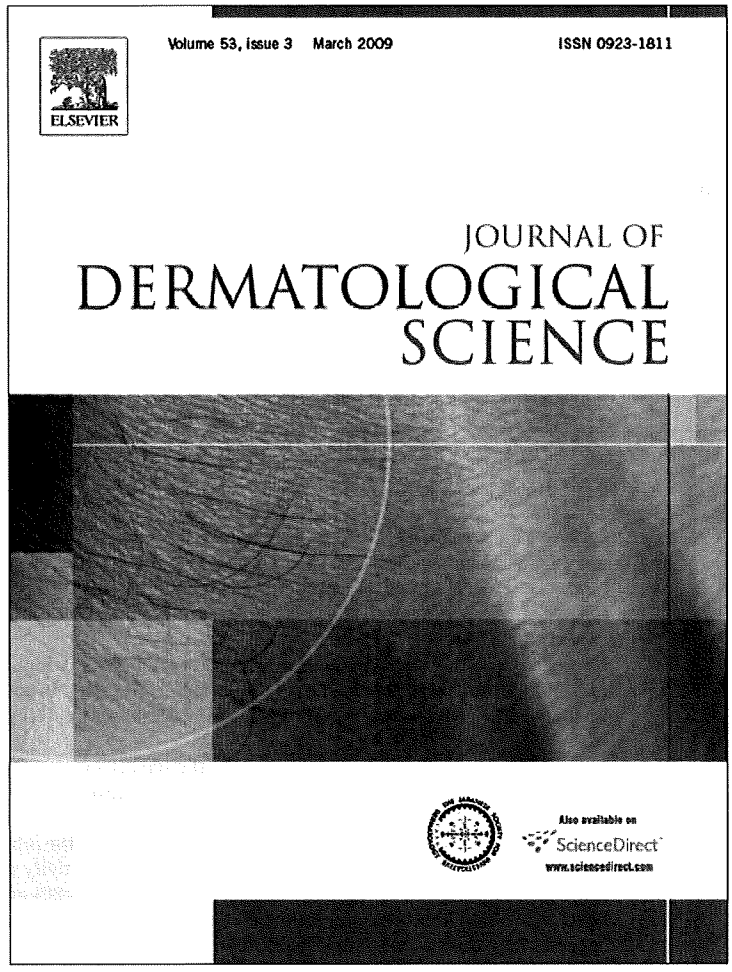

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Skin application of ketoprofen systemically suppresses contact hypersensitivity by inducing CD4$^+$ CD25$^+$ regulatory T cells

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ABSTRACT

Background: Ketoprofen (KP) is a widely used nonsteroidal anti-inflammatory drug that inhibits prostaglandin biosynthesis. We have previously shown that topical KP treatment at the sensitizing site inhibits the development of contact hypersensitivity (CHS) to picryl chloride (PCl).
Objective: We investigated the mechanism underlying the KP-induced immunosuppression of CHS by application of KP.
Methods: We analyzed the CHS responses to the non-sensitizing site and subsequent sensitization with PCl, and by transfer of the draining lymph node cells (LNCs) from KP-tolerated mice to recipient mice. Changes in the Foxp3 expression of LNCs from KP-phototreated skin were also examined by real-time PCR.
Results: Topical application of KP to not only the sensitizing but also non-sensitizing site suppressed CHS response. The immunosuppression was transferred with LNCs from mice treated with PCl plus KP, but not from mice treated oxazolone plus KP. In this transfer study, the CD4$^+$ CD25$^+$ subset of LNCs exerted the suppressive effect, while CD25$^+$ cell-depleted LNCs lost the inhibitory ability. CTLA-4 blocking with a specific antibody, but not IL-10 blocking, abrogated the activity of CD4$^+$ CD25$^+$ cells. Moreover, Foxp3 mRNA expression was remarkably increased in LNCs from PCl and KP-treated mice.
Conclusion: The immunosuppression of CHS by topical application of KP is systemic and hapten-specific. Treg cells play an important role in the suppressive effect by KP.

1. Introduction

Contact hypersensitivity (CHS) is a delayed-type cutaneous reaction in which various immunocompetent cells, including epidermal Langerhans cells (LCs), dermal dendritic cells (dDCs), keratinocytes and T cells, and their cytokines and chemokines are involved [1,2]. Ketoprofen (KP) is a nonsteroidal anti-inflammatory drug (NSAID) that inhibits prostaglandin (PG) biosynthesis and widely used topical NSAID to remove pain. We have previously shown that *in vivo* application or *in vitro* addition of KP inhibits the maturation of LCs [3]. As a result, topical application of KP to the sensitizing site inhibits CHS responses to hapten. Aspirin, a representative NSAID, has an inhibitory effect on the *in vitro* maturation of LCs, but aspirin did not suppress CHS response to hapten. Thus, KP is unique in this suppressive ability, and in addition to the topical modulation of LCs, another mechanism might exist in KP-induced immunosuppression. In this respect, it remains unclear whether KP induces systemic or local immunosuppression and how KP application induces the immunological tolerance in CHS.

Regulatory T cells (Treg) prevent from harmful immune responses to self and nonself antigens in a dominant manner [4–7]. In mouse models, the responses of CHS are reduced by Treg cells-inducing treatments such as irradiation of skin with UVB, skin graft, and oral application of antigen. Treg cells can be classified into two major categories: thymus-derived natural Treg cells and those induced in the periphery [8,9]. The principal subset of natural Treg cells is CD4$^+$ cells that constitutively express CD25 [10,11]. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is an important molecule that is expressed on the surface of Treg cells and exhibits the ability to ligate the B7 family molecules (CD80 and CD86) like CD28 [6,7]. Unlike CD28, however, cross-linking of CTLA-4 downregulates interleukin (IL)-2 production and cell cycle progression, and therefore, blockade of CTLA-4 reduces CD4$^+$

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