The number of lymphocytes in the BALF of *Hdc-/-* mice was twenty times that of wild-type mice, although no significant difference in the concentration of IL-2 in the BALF was found between knock-out and wild-type mice. The number of cells and cytokine levels in BALF are not always concordant in the time courses of *in vivo* experiments. The level of IL-2 in BALF peaked within 1 week during exposure to OVA (unpublished data). However, in this study we examined the cytokine levels and the cell differentials in BALF only on the 14th day. To explain the discrepancy between the IL-2 level and the number of lymphocytes in BALF in the study, it would have been helpful to evaluate the time course of IL-2 concentration and the number of lymphocytes in BALF.

Although we designed a 2- week exposure program to evaluate the extent of goblet cell hyperplasia in the *Hdc-/-* mice, we did not examine airway hyperresponsiveness (AHR). However, we found no difference in AHR between the *Hdc-/-* and the wild mice in our preliminary study as was reported in the previous acute model (unpublished data).[29]

In the present study, we did not measure the anti-OVA specific IgE in serum. Although the levels of serum OVA-specific IgE and total IgE are not always consistent, the procedures for sensitization with OVA and exposure to OVA were directly associated with the increase of IgE in serum. Despite the fact that the levels of serum OVA-specific IgE were different from the level of total IgE in serum, the level of total IgE in serum reflected strongly the levels of serum OVA-specific IgE in our study.

Anti-histamine drugs available for therapy consist of H1 and H2 blockers. H1 blockers have not been recommended for therapy for asthma, but their anti-allergic actions have been recognized. According to the results of this study, the H2 blocker may have the possibility of stimulating goblet cell hyperplasia in patients with allergic asthma. It will be necessary to evaluate the effects of H2 blockers in a clinical study in the future.

In conclusion, we demonstrated enhanced goblet cell hyperplasia in the airway of OVA sensitized histamine-deficient mice. The results of our study suggested that histamine may play a significant role in goblet cell hyperplasia in the airway with allergic inflammation.

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Contact immunotherapy-induced Renbök phenomenon in a patient with alopecia areata and psoriasis vulgaris

Happle *et al.* first introduced the term "Renbök phenomenon", derived from the reversal of "Köbner", to describe the observation of normal hair growth in psoriatic lesions in patients with co-existing psoriasis and alopecia areata (AA) [1]. Although the exact mechanism of this unique phenomenon is unknown, it has been proposed that biological events inherent in psoriasis may act on hair follicles to restore hair growth in AA. We herein describe the Renbök phenomenon related to contact immunotherapy in a patient suffering from AA and psoriasis vulgaris.

A 15-year-old girl presented with a 3-year history of patchy hair loss on the vertex of the scalp with a gradual increase in the number of lesions. In addition, scaly and erythematous eruptions appeared on her scalp and upper extremities one year after the onset of scalp hair loss. Initial examination at our outpatient clinic revealed multiple hair loss patches on her scalp and scaly lesions on her scalp and upper extremities. Interestingly, the presence of terminal hair coincided with the scaly lesions *(figure 1A)*. There was no associated systemic disease nor was there any family history of AA or psoriasis vulgaris.

Hematoxylin and eosin (H-E)-stained sections of a biopsy specimen from a hair loss patch revealed lymphocyte infiltration around atrophic hair follicles, compatible with the diagnosis of AA. The biopsy specimen from a scaly lesion of the right antebrachium showed parakeratosis, psoriasiform acanthosis, thin but club-shaped rete ridges, Munro's microabscesses and perivascular infiltration of lymphocytes. Results of laboratory studies including a hemogram and blood chemistry tests and thyroid autoantibodies were normal or negative. She had HLA-DQB1* 0301 (a gene susceptibility to AA) but was negative for HLA-Cw6 and HLA-DR7, which are genetic loci relating to psoriasis [2].

Contact immunotherapy with squaric acid dibutylester (SADBE), and topical maxacalcitol and betamethasone butyrate propionate were instituted for AA and psoriasis, respectively. Three months following contact immunotherapy, terminal hairs re-grew but psoriatic lesions developed at the sites of SADBE application *(figure 1B)*, which were then successfully treated with maxacalcitol lotion. Other pre-existing psoriatic lesions responded well with the 4 weeks of the topical treatment. One year later, the hair loss and psoriasis were markedly improved, although a few hair loss patches occasionally recurred.

In our case, contact immunotherapy was effective for AA but also induced psoriatic lesions at the site of treatment. Orecchia *et al.* report a case of alopecia universalis in which there was a concomitant appearance of hair regrowth and psoriatic plaques in the same area following contact immunotherapy with SADBE [3]. Contact immunotherapy appears to modulate cytokine production in the skin with a decrease in the mRNA expression of interferon (IFN)-γ while mRNA for IL-2, IL-8, IL-10 and tumor necrosis factor (TNF)-α is increased [4]. AA is regarded as a tissue-specific autoimmune disease against melanin-associated proteins in the hair follicle [5]. IFN-γ may contribute to initiating the disease process by collapsing the hair follicle immune privilege and resulting in the exposure of autoantigens [5]. On the other hand, TNF-α is a crucial cytokine in psoriatic lesions [6]. Therefore, we propose that a change in the cytokine milieu due to contact immunotherapy may play an important role in both the improvement of alopecia and the induction of psoriasis. ■

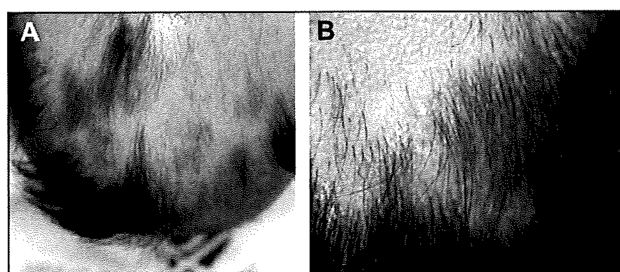

Figure 1. A) Extensive hair loss was observed on the vertex and occipital region of the head. **B)** Concomitant hair regrowth and psoriasis on the scalp 1 month after contact immunotherapy.

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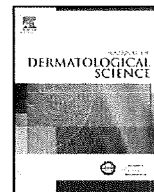

Roxithromycin inhibits chemokine-induced chemotaxis of Th1 and Th2 cells but regulatory T cells

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ABSTRACT

Background: Roxithromycin (RXM), a 14-member macrolide antibiotic, has a variety of bioregulatory functions such as anti-inflammatory effects, anti-oxidant effects, and modulation of immune responses.
Objectives: In this study, we analyzed the effect of RXM on chemokine-induced chemotaxis of Th1, Th2, and regulatory T (Treg) cells established from three normal human peripheral blood lymphocytes by the reported methods.
Methods and results: Incubation with 10 μM RXM for 18 h did not alter the expression profile of CXCR3 on Th1 cells and CCR4 on Th2 and Treg cells. However, upon RXM preincubation, the migration of Th1 cells to IP-10 and Th2 cells to TARC was partially suppressed, although RXM did not influence Treg cell migration. Erythromycin and clarithromycin at the same concentration did not exert such effects. F-actin polymerization and Ca^{++} influx induced by IP-10 and TARC in Th1 and Th2 cells, respectively, was down-regulated by RXM pretreatment.
Conclusion: These results imply that RXM exhibits bioregulatory function by influencing chemotaxis of Th1 and Th2 cells while leaving Treg cell migration unaffected.
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1. Introduction

Macrolides were originally exploited as antibiotics against *Staphylococcus*, *Streptococcus pneumoniae*, *Moraxella*, and *Propionibacterium acnes* [1]. Some macrolides have a variety of bioregulatory functions such as anti-inflammatory and anti-oxidant effects, and modulation of immune responses including immunosuppression [2,3]. The 14-member macrolide roxithromycin (RXM) inhibits T cell proliferation responses, cytokine production by T cells and macrophages [4,5,6], and accessory functions of professional and non-professional antigen presenting cells [7,8]. In fact, RXM exerts beneficial effects on various skin diseases such as psoriasis [9,10], atopic dermatitis [7], prurigo, and eosinophilic pustule folliculitis [10] partly through these immunomodulatory activities.

The interaction between chemokines and chemokine receptors is crucial in cell trafficking such as steady-state circulation, inflammation, and tumor metastasis [11]. Among chemokines, IFN-γ-inducible protein 10 (IP-10/CXCL10) is a T helper (Th)1 chemokine with affinity to CXC chemokine receptor 3 (CXCR3) on Th1 cells [12]. On the other hand, thymus and activation-regulated chemokine (TARC/CCL17) is known as a Th 2 chemokine that binds to CC chemokine receptor 4 (CCR4) on Th2 cells. It has been reported that RXM influences production of chemokines and expression of chemokine receptors in relation to skin immunity [13,14]. In order to further clarify these issues, we assessed the effect of RXM on TARC and IP-10-induced chemotaxis of established Th1, Th2, and regulatory T (Treg) cells.

2. Materials and methods

2.1. Study participants

Two healthy males (age, 46 and 38 years) and a female (33 years) were enrolled in this study after written informed consent was obtained. The study was approved by the ethical committee of the Hamamatsu University School of Medicine, and conducted according to the Declaration of Helsinki principles.

2.2. Reagents and kits

RXM, erythromycin (EM), and clarithromycin (CAM) were obtained from Wako Pure Chemical Industries (Osaka, Japan); $CD4^+$ T Cell Isolation Kit II and CD45RO microbeads from Miltenyi Biotec (Auburn, CA); staphylococcal enterotoxin B (SEB), anti-human interleukin (IL)-4 monoclonal antibody (mAb), anti-human IL-12 mAb, recombinant (r)IL-2, phycoerythrin (PE)-labeled mouse IgG1 anti-human CD183 (CXCR3) mAb, PE-labeled mouse IgG1 anti-human CCR4 mAb, fluorescein isothiocyanate (FITC)-labeled mouse IgG1 anti-human CD4 mAb, FITC-labeled mouse IgG1 anti-human CD25 mAb, peridinin chlorophyll protein-labeled mouse

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IgG1 anti-human CD4 mAb, Cytoperm solution, and Perm/Wash buffer from BD Bioscience (San Jose, CA); FITC-conjugated anti-human interferon (IFN)-γ mAb, PE-conjugated anti-human IL-4 mAb, Goldiplug, and Cytofix/Cytoperm Plus Kit from BD Pharmingen (San Diego, CA); human rIL-12, and human rIL-4 from PeproTech (Rocky Hill, NJ); phytohemagglutinin (PHA), human rTGF-β thalidomide, dimethylsulfoxide (DMSO), FITC-conjugated phalloidin, and Fluo-3AM from Sigma–Aldrich (St. Louis, MO); 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) from Molecular Probes (Eugene, OR); and CD3/CD28 molecules from Dynal Biotec (Oslo, Norway).

2.3. Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation with Ficoll-Paque PLUS in LeucoSep (Greiner Bio-One, Frickenhausen, Germany) from three volunteers. $CD4^+$ T cells were prepared from PBMC with a $CD4^+$ T Cell Isolation Kit II by negative selection. The remaining $CD4^-$ cells were used as antigen-presenting cells (APC) to establish Th1, Th2, and Treg cells. $CD45RA^+CD4^+$ cells were purified from $CD4^+$ T cells with the CD45RO microbeads.

2.4. Cell culture

Culture medium was RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin from Gibco-BRL (Carlsbad, CA); human TARC and IP-10 from R&D systems (Minneapolis, MN). PBMC (5×10^6 cells/well) in culture medium were incubated in 12-well tissue culture plates at 37 °C in 5% CO_2 in air.

2.5. Generation of Th1, Th2 and Treg cells

All type of cells was established in serum-free medium in the presence of APC and various stimulants according to the reported methods [15–17]. Each cell type was come from PBMCs of three volunteers. In brief, SEB, an anti-human IL-4 antibody, rIL-12, and rIL-2 were employed to establish Th1 cells [15]. For establishment of Th2 cells, PHA, thalidomide, anti-IL-12 antibody, rIL-4, and rIL-2 were used [16]. Treg cell were established with SEB and human rTGF-β [17].

2.6. Immunofluorescence staining and flow cytometric analysis

For cell surface staining, cells were doubly stained with PE-labeled and FITC-labeled mAb in PBS for 30 min at room temperature. For intracytoplasmic cytokine staining, 1 μl of Goldiplug was added to each well during the last 6 h of culture according to the manufacturer's protocol. The cells were then reacted with 100 μl of CytoFix for 15 min at 4 °C, washed with phosphate-buffered saline containing 0.1% saponin, and stained with FITC-conjugated anti-human IFN-γ mAb and PE-conjugated anti-human IL-4 mAb for 30 min, followed by reaction with peridinin chlorophyll protein-labeled mouse IgG1 anti-human CD4 mAb. After washing in PBS, 10,000 cells were analyzed on a FACSCalibur (Becton Dickinson). Mean fluorescence intensity (MFI) was calculated on a log scale. Cells stained with isotype-matched mAbs served as the control.

2.7. Cell proliferation assay

$CD4^+$ T cells were labeled with 5 μM CFSE in DMSO for 15 min at 37 °C as previously described [18], stimulated with CD3/CD28 for 4 days, and analyzed by flow cytometry [18]. Cells once divided showed half the CSFE intensity of parental cells shown as the multipeak histogram of several divisions. In order to examine the suppressive effect of Treg cells on lymphocytic proliferation, autologous Treg cells were added to $CD4^+$ T cells ranging from 0.05 to 1.0 for 24 h, and the cell mixtures were subjected to CFSE analysis. Control culture continued with Treg cells.

2.8. Incubation of cells with macrolide

Cells were cultured for 8 h in the presence or absence of either 10 μM RXM, EM, or CAM. Cell viability after incubation with macrolides was >90% as judged by dye exclusion.

2.9. Real-time horizontal chemotaxis assay

Time-lapse images of cell migration during chemotaxis were observed directly with an optically accessible horizontal chemotaxis apparatus TAXIScan *via* a CCD camera (EZ-TAXIScan; GE Healthcare, Tokyo, Japan) as described [19]. The apparatus consisted of front and back chambers containing cells and a chemoattractant, respectively, which were connected by a microchannel. A 1-μl suspension of cells (5×10^6 cells/ml) was placed in one compartment, and 1 μl of either TARC at 25 mg/ml or IP-10 at 10 mg/ml was injected into the other compartment to initiate chemotaxis under the concentration gradient in the channel. Data were analyzed using the Image J software (NIH, Bethesda, MD) and the Manual Tracking plug-in produced by FP Cordelieres (Institut Curie, Orsay, France; http://rsb.info.nih.gov/ij/plugins/manual-tracking.html).

2.10. F-actin polymerization

Phalloidin has been found to bind only to polymeric and oligomeric forms of actin and not to monomeric actin. Therefore, the level of polymerized actin was determined by staining cells with phalloidin as described before. Cells were permeabilized in a Cytofix/Cytoperm solution for 20 min, washed in Perm/Wash buffer for 10 min, and incubated with 5 mg/ml FITC-conjugated phalloidin for 30 min. All procedures were done at 4 °C. Cells were analyzed on a FACSCalibur, and the level of actin-polymerization was expressed by MFI. Because MFI varied among each cell type, the percentage of fluctuation was calculated by the ratio before and after treatment with chemokines as follows: %MFI = MFI of treated cells/MFI of control, non-treated cells.

2.11. Calcium influx

Cells were incubated with 0.8 mM Fluo-3AM/DMSO in RPMI supplemented with 5% FCS for 30 min at 37 °C as described [20]. Fluorescence intensity was continuously measured on a FACSCalibur for 50 s after incubation with Fluo-3AM/DMSO. Chemokine was added into cell suspensions at 10 s after starting the measurement of Ca^{++} concentrations. For quantitative evaluation, the variation of intracellular Ca^{++} concentrations was calculated according to the following equation: $(F - F_{min})/(F_{max} - F) \times 100$, in which F was MFI of chemokine-treated cells, F_{min} was MFI of cells incubated with 6 mM EGTA to chelate Ca^{++}, and F_{max} was MFI of cells incubated with 700 mM ionophore in DMSO [21].

2.12. Statistical analysis

All values were expressed as means ± standard deviation (SD). Data were analyzed with Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Immunological characteristics of established cells

More than 80% of Th1 cells expressed CD4 and CXCR3 and produced intracellular IFN-γ (Fig. 1A and B), while 75% of Th2 cells were positive for CD4 and CCR4, and 12% synthesized IL-4 (Fig. 1C and D). Since Th2 cells established according to the reported method [16] express relatively low levels of IL -4 and no IFN-γ, we used this cell line. Around 90% of Treg cells expressed CD4, CCR4, and CD25 (Fig. 1E and F), and none produced intracellular IFN-γ or IL-4 (Fig. 1G). The CSFE assay revealed that Treg cells suppressed proliferation of autologous $CD4^+$ T cells in response to immobilized CD3/CD28 in a dose-dependent manner (Fig. 2).

3.2. RXM down-regulates the migration of Th1 and Th2 cells, but not of Treg cells, toward IP-10 and TARC without influencing chemokine receptor expression

The migration of Th1, Th2, and Treg cells toward IP-10 or TARC was observed on a TAXIScan for 60 min. Non-treated control Th1 and Th2 cells ran toward IP-10 and TARC, respectively (Fig. 3). On the other hand, RXM-treated cells did not migrate toward the corresponding chemokine. Both non-treated control and RXM-treated Treg cells exhibited comparable chemotaxis to TARC. RXM did not affect chemokine receptor expression such as CXCR3 on Th1 cells, CCR4 on Th2 cells (Fig. 4A–D), or CCR4 on Treg cells (data not shown) as revealed by flow cytometry. Preincubation with CAM and EM did not influence the migration pattern of Th1, Th2, or Treg cells toward corresponding chemokines (data not shown).

3.3. F-actin polymerization to chemokines in Th1, Th2, and Treg cells

Actin polymerization is an early event that controls cell migration and reorganization of the actin cytoskeleton [22]. In Th1 cells, the baseline MFI of phalloidin was 503.7 ± 13.4. MFI increased by 106.2 ± 0.46% with IP-10 compared to control ($p < 0.05$). MFI significantly decreased by 92.23 ± 2.6% with RXM

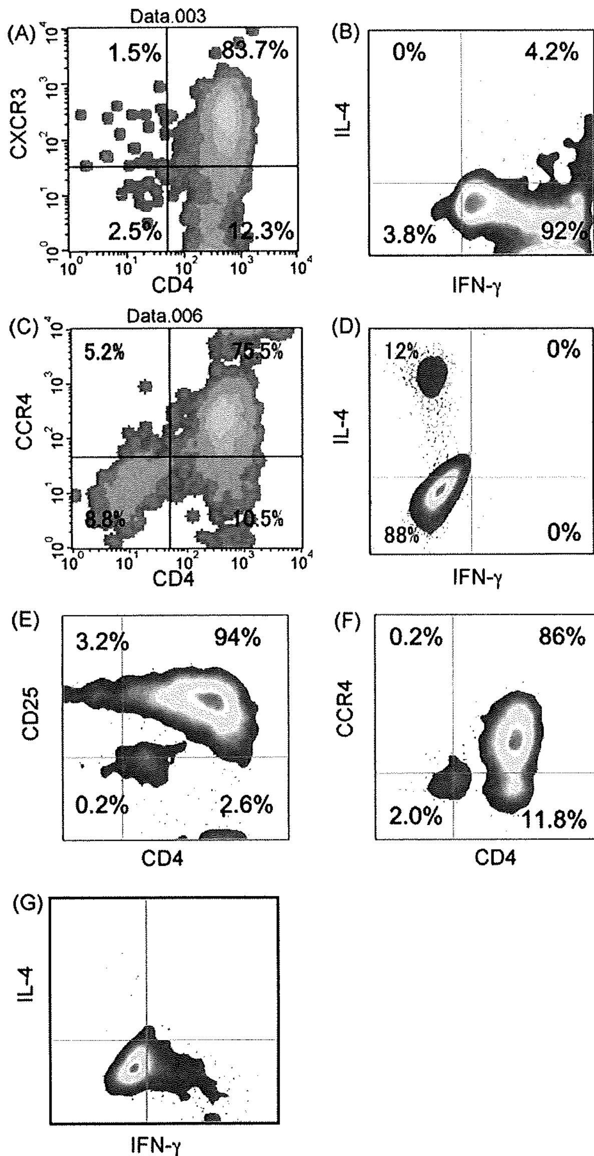

Fig. 1. Surface phenotypes and intracellular cytokine expression on established Th1, Th2, and Treg cells. (A) Expression of CD4 and CXCR3 on Th1 cells; (B) intracellular IL-4 and IFN-γ expression in Th1 cells; (C) expression of CD4 and CCR4 on Th2-t cells; (D) intracellular IL-4 and IFN-γ expression on Th2-t cells; (E) expression of CD4 and CD25 on Treg-h cells; (F) expression of CD4 and CCR4 on Treg-h cells; (G) intracellular IL-4 and IFN-γ expression in Treg-h cells.

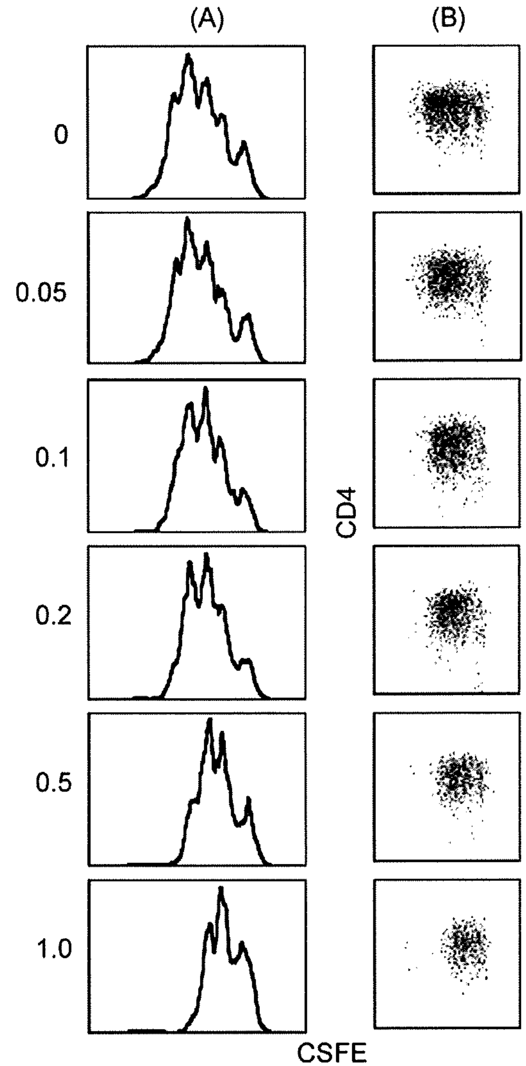

Fig. 2. CFSE analysis of Treg cell division. Tregs were coincubated with CD4$^+$ T cells for 24 h, and then labeled with 5 μM CFSE for 15 min at 37 °C. To prepare CFSE-labeled CD4$^+$ T cells, an equal volume of 10 mM CFSE was added such that the final concentration was 5 mM and the mixture was incubated for 10 min at 37 °C. After labeling, CD4$^+$ T cells were stimulated by CD3 (2C11) molecules. 3 and 4 days after stimulating with CD3 molecules, CD4$^+$ T cells were analyzed on a FACSCaliber.

pretreatment compared to non-treatment ($p < 0.05$). The MFI also significantly decreased by 92.0 ± 5.5% in cells pretreated with RXM and subsequently incubated with IP-10 compared to non-treated cells ($p < 0.05$) (Fig. 5A). In Th2 cells, the baseline of phalloidin MFI was 352.6 ± 23.0, and MFI increased by 107.5 ± 3.3% with TARC compared to control ($p < 0.05$). MFI significantly decreased by 94.5 ± 2.23% with RXM pretreatment compared to non-treatment ($p < 0.05$). The MFI significantly increased by 104.8 ± 2.9% in cells pretreated with RXM and subsequently incubated with TARC compared to non-treated cells ($p < 0.05$). This MFI was significantly lower than that of TARC-treated cells ($p < 0.05$) (Fig. 5A). In Treg cells, the baseline of MFI was 72.2 ± 8.9, and MIF did not change with TARC (MFI = 101.2 ± 0.2%), RXM (MFI = 100.8 ± 0.02%), or RXM + TARC (99.9 ± 2.2%) compared to non-treatment (Fig. 5A). Therefore, RXM hampered chemokine-induced F-actin polymerization in Th1 and Th2 cells but not in Treg cells. The representative data is shown in Fig. 5B and C.

3.4. *Calcium influx*

In the control Th1 cells, the baseline value of Ca^{++} influx was 164.3 ± 2.2, and the Ca^{++} influx (as expressed by %MFI ± SD) increased by 12 ± 2.5% ($p < 0.01$) by the addition of IP-10 compared to the influx before IP-10 addition (baseline Ca^{++} influx) (Fig. 6A). In RXM-treated Th1 cells, the baseline Ca^{++} influx was 158.9 ± 3.03, and it increased by 0.53 ± 0.04% upon IP-10 addition compared to baseline; this was lower than in non-treated cells. In the control Th2 cells, the baseline value of Ca^{++} influx was 238.0 ± 18.8, and TARC increased it by 81.3 ± 7.28%. In RXM-treated Th2 cells, the baseline Ca^{++} influx was 230.5, and TARC increased it by 55.9 ± 7.71%; this was significantly lower than in non-treated Th2 cells ($p < 0.01$) (Fig. 6B). In non-treated and RXM-treated Treg cells, the baseline value of Ca^{++} influx was 315.5 ± 53.2 and 333.1 ± 73.5, respectively. RXM did not influence Ca^{++} influx following the addition of TARC in non-treated and RXM-treated cells (Fig. 6C). These results indicate that RXM significantly suppresses chemokine-induced Ca^{++} influx in Th1 and Th2 cells, but not in Treg cells. The representative data is shown in Fig. 6D.

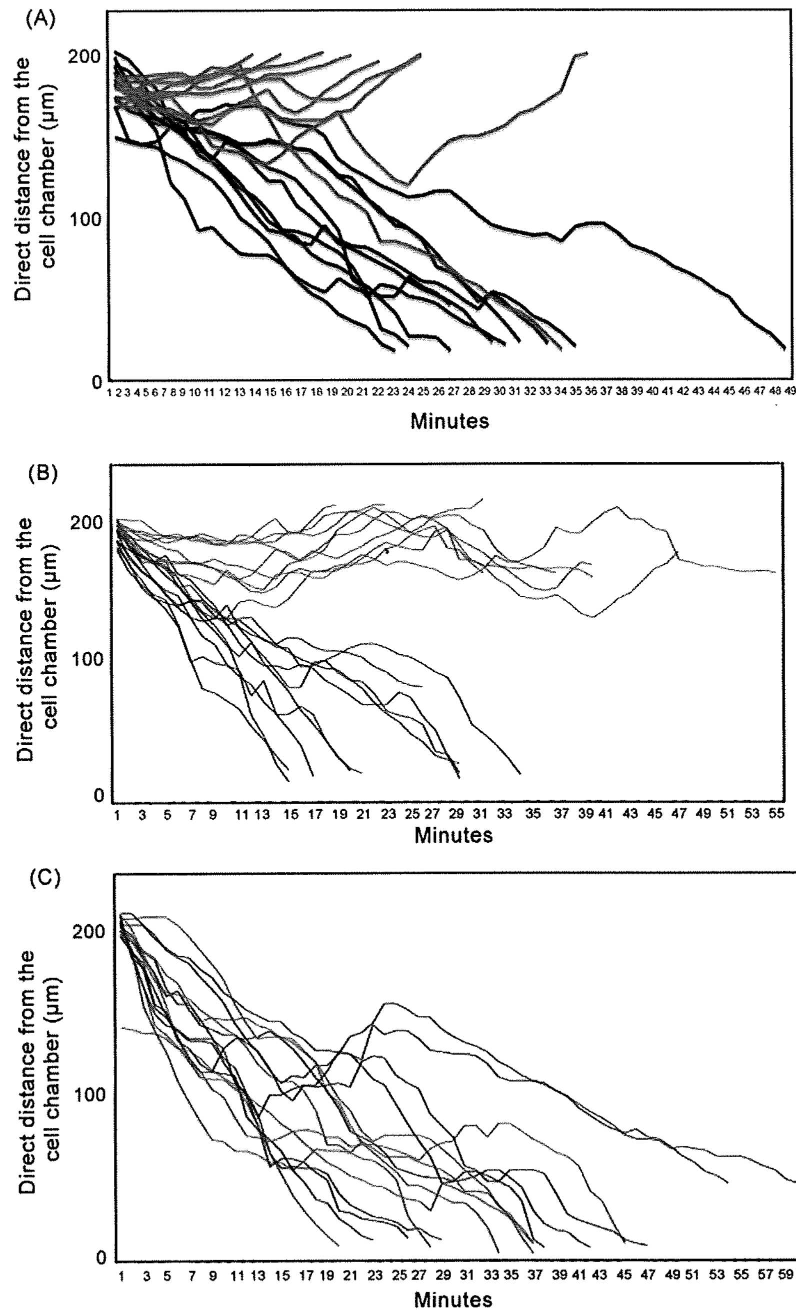

Fig. 3. Chemotaxis assay of RXM-treated T cells by TAXIScan®. Ten randomly selected cells were traced and the direct distances from the cell chamber measured at each time-point were plotted. Black and red lines indicate non-treated and RXM-treated Th1 (A), Th2 (B), and Treg (C) cells, respectively. The *x*-axis indicates minutes after starting chemotaxis. The *y*-axis indicates the direct distance from the cell chamber.

4. Discussion

Our study clearly showed that RXM down-regulated migration of Th1 and Th2 cells, but not Treg cells, toward corresponding chemokines without influencing chemokine receptor expression. These inhibiting effect are only found in RXM but CAM and EM. CAM and EM also have some immunomodulatory effects, the reason of the differences has been uncertain in this study. So far, there has been no clear data of CAM and EM on T cell chemotaxis. Of coarse this study used only one concentration of RXM, Kobayashi et al. reported that RXM at 1 or 10 μM significantly suppressed the production/expression of Th2 chemokines MDC and TARC in these keratinocytes [14]. In addition, the IL-2-enhanced expression level of Th2 chemokine receptor CCR4 was decreased by RXM at 10 μM [14]. Therefore, 10 μM RXM can be compared to the study. In the absence of RXM-pretreatment, Th1 cells migrated to IP-10, and Th2 cells to TARC. These events were associated with an increase in Ca^{++} influx and F-actin polymerization. Interestingly, our results indicate that RXM itself down-regulate F-actin polymerization in Th1 and Th2 but in Treg cells. Furthermore, chemokine-induced migration was down-regulated in RXM-pretreated Th1 and Th2 cells. In these cells, Ca^{++} influx was not as intense and F-actin polymerization was not as remarkable compared to cells without RXM pre-treatment upon incubation with corresponding chemokines. So far, there has been no report showing the effect of RXM on Ca^{++} concentration and actin polymerization in T cells. Thus, we can only speculate that RXM mainly affects the intracellular signaling pathways that lead to chemokine-induced

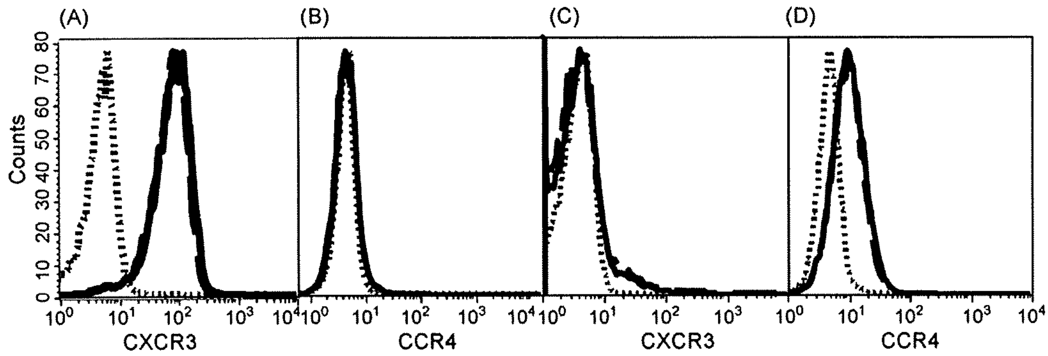

Fig. 4. Expression of CXCR3 and CCR4 on Th1, Th2, and Treg cells. (A and B) CXCR3 and CCR4 expression on Th1 cells, respectively. (C and D) CXCR3 and CCR4 expression on Th2 cells, respectively. The x-axis and y-axis indicate cell number and MFI, respectively. (—) Isotype control; (- - -) intensity of non-treated cells; (▬▬) intensity of RXM treated cells.

chemotaxis. Kobayashi et al. reported that RXM may down-regulate chemokine receptor expression on Th2 cells derived from patients with mycosis fungoides [14]. The different effect of RXM on chemokine receptor expression may come from the different cell origin, i.e., our T cells were established from normal human PBMC, and Kobayashi's from cutaneous lymphoma cells.

We do not know why RXM did not change the migration pattern in Treg cells. Chemotaxis in Treg cells has been studied in various

Fig. 5. F-actin polymerization assay in response to chemokines in Th1, Th2, and Treg cells. F-actin polymerization is expressed as % change compared to the baseline Ca^{++} influx in Th1, Th2, and Treg cells. (■) Ca^{++} influx induced with chemokines in untreated cells. (□) Ca^{++} influx induced with chemokines in RXM-pretreated cells. (▨) Ca^{++} influx induced with chemokines in RXM-pretreated cells. (B and C) The representative data of F-actin polymerization assay.

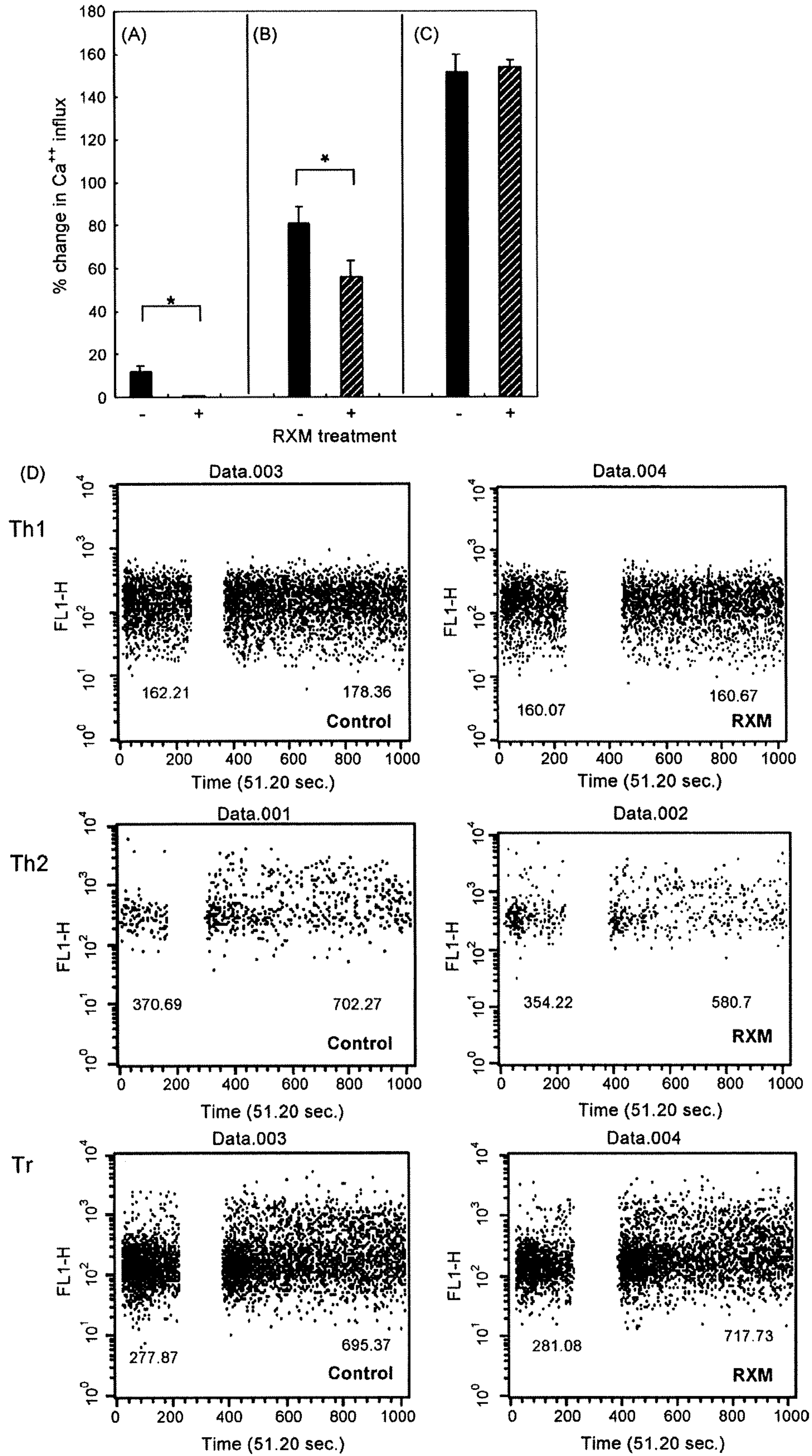

Fig. 6. (A–C) Ca^{++} influx in response to chemokines in Th1, Th2, and Treg cells. The *y*-axis indicates the % increase of MFI upon culture with chemokines (A: IP-10, B and C: TARC) compared to MFI without chemokine stimulation in Th1 (A), Th2 (B), and Treg (C) cells. (D) The representative data of Ca^{++} influx in Th1, Th2, and Treg cells.

diseases. The migratory capacity of Treg cells decreases in patients with SLE [23]. On the other hand, an increased migratory capacity of Treg cells is one of the reasons for impaired anti-tumor immunity in patients with cancer [24]. Thus, the altered migratory profiles may contribute to defective protective immunity, which underlies the pathogenesis of these disorders.

Cell migration is controlled by the formation of lamellipodia and filopodia at the leading edge [25–27]. To form these structures, actin polymerization has to occur in the leading edge in association with the redistribution of integrins, chemokine receptors, and signaling molecules [28]. Not only the activating processes, but also inactivating signals of deploymerization are important for chemotaxis [29–31]. RXM may affect some molecules in the chemotaxis cascade or Ca^{++} channels on Th1 and Th2 cells without changing chemokine receptor expression. Our study suggests that RXM exerts an immunomodulatory effect on both of Th1 and Th2 diseases through influencing chemotaxis of Th1 and Th2 cells. Lack of the RXM effect in Treg cells may also be critical in immunomodulation.

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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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Summary

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Conflicts of interest
None declared.

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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

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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.).

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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]

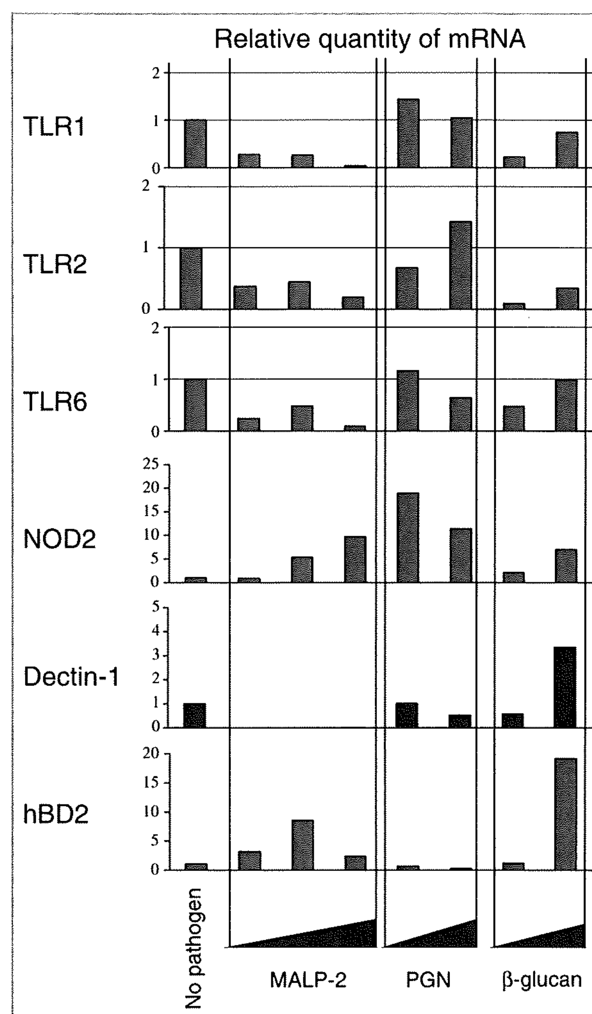

Fig 1. Expression of toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) in normal human epidermal keratinocytes (NHEK). NHEK were cultured for 2 h with MALP-2 (1, 10, 100 ng mL^{-1}), peptidoglycan (PGN) (0·2, 2 μg mL^{-1}) or β-glucan (1,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

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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.

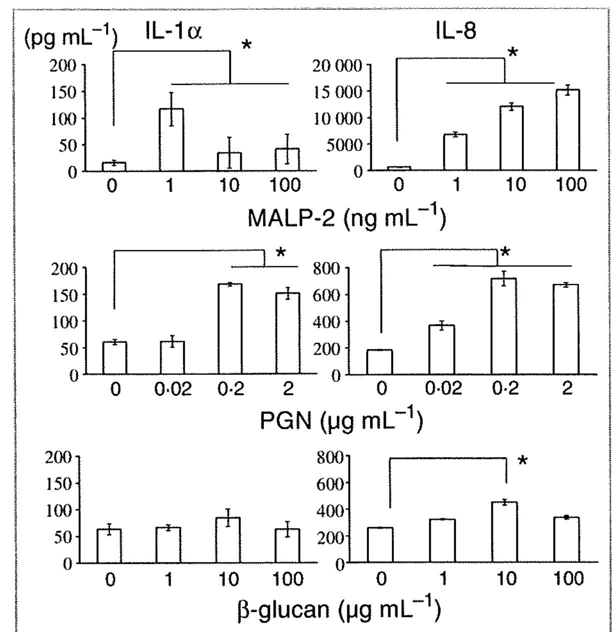

Fig 3. Augmentative effect of MALP-2, peptidoglycan (PGN) and β-glucan on cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h in the presence or absence of pathogen-associated molecules at the indicated concentration. The amounts of interleukin (IL)-1α 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.

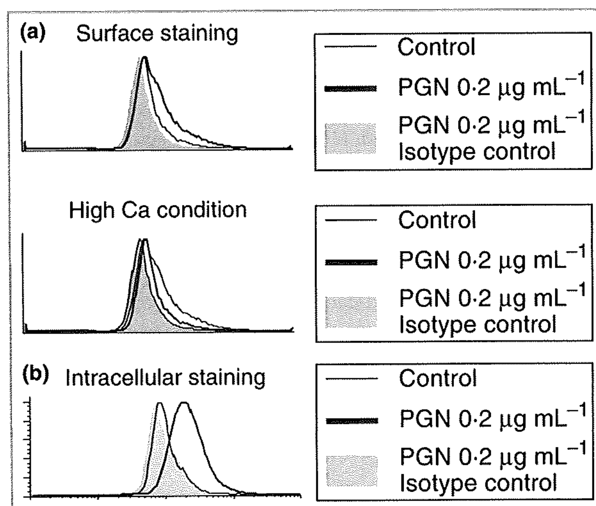

Fig 2. Flow cytometric detection of toll-like receptor (TLR) 2 expression in the cytoplasm but not on the surface of normal human epidermal keratinocytes (NHEK). NHEK were cultured in the presence or absence of peptidoglycan (PGN) for 48 h. Cells were stained with anti-TLR2 monoclonal antibody (mAb) 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).

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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.

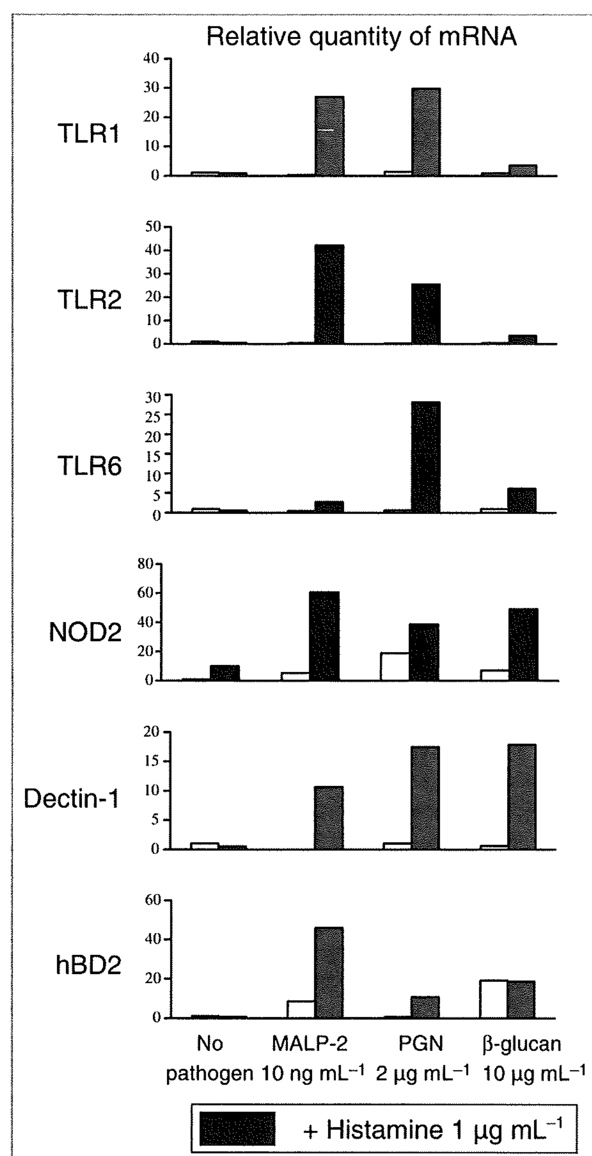

Fig 4. Synergism of MALP-2, peptidoglycan (PGN) or β-glucan, with or without histamine, in mRNA expression for toll-like receptor (TLR) 1, TLR2, TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) by real-time polymerase chain reaction (PCR) analysis. Normal human epidermal keratinocytes were cultured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

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, 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

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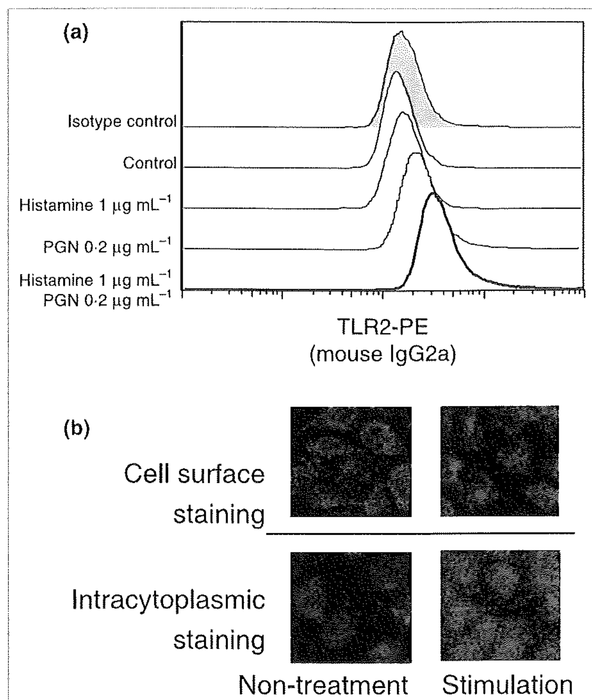

Fig 5. Synergism between peptidoglycan (PGN) and histamine in intracellular toll-like receptor (TLR) 2 expression and confocal microscopic analysis for detection of TLR2 in normal human epidermal keratinocytes (NHEK). (a) NHEK were cultured in the presence of histamine (1 μg mL^{-1}) and/or PGN (0·2 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.

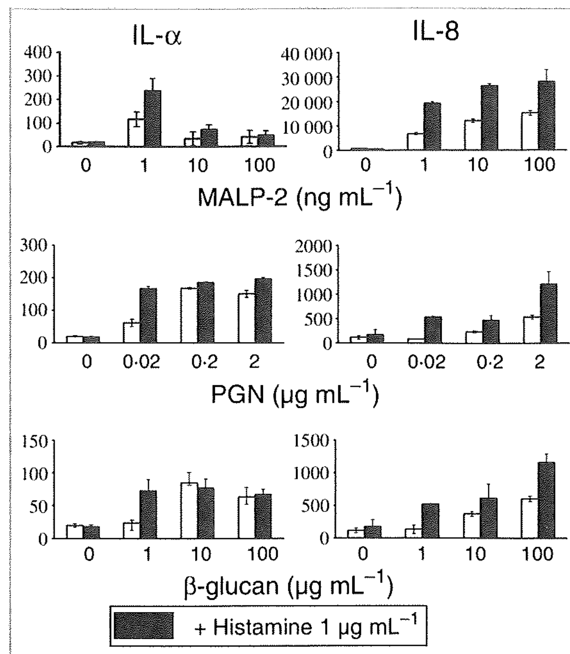

Fig 6. Synergism between pathogen-associated molecules and histamine in cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h with each of the pathogen-associated molecules and/or histamine. The concentrations of interleukin (IL)-α and IL-8 in the supernatants 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

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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]

Acknowledgments

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In the light of our patient's history, symptoms and clinical presentation we diagnosed AGEP following the intake of moxifloxacine. After discontinuation of the antibiotic and intravenous administration of corticosteroids (100 mg prednisolone daily) and antihistaminics (30 mg diphenhydramine hydrochloride twice a day), the rash cleared with typical generalized desquamation within one week. A consecutive patch test with moxifloxacine remained negative. A provocation test with the drug was not performed.

Recent reports suggest an association between the administration of moxifloxacine and elevated liver enzymes, potentially ending in hepatitis fulminans and Stevens-Johnson-Syndrome or TEN (toxic epidermal necrolysis). In this regard our patient remained without relevant pathological findings. AGEP is usually caused by a large variety of drugs, including antibiotics (ß-lactams, pristinamycine, co-trimoxazole, metronidazole), antifungal agents (nystatin, terbinafine, fluconazole, amphotericin B), carbamazepine, hydroxy-chloroquine, azathioprine, diltiazem, nimesulide, non-ionic contrast media and others [1, 3]. Occasional cases of acute pustular drug reactions after the intake of other quinolone antibacterial agents have already been reported [4-6]. Our case is the first description in the literature of AGEP after treatment with moxifloxacine. ■

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Cisplatin-induced acral erythema

Chemotherapy-induced acral erythema (CAE) is a cutaneous reaction associated with the use of various systemic chemotherapeutic agents, usually administered at high doses. Since 1982, when CAE was first described, it has been reported under a variety of names, such as palmoplantar erythema, palmoplantar erythrodysaesthesia syndrome and hand-foot syndrome. CAE tends to occur in patients with malignancies, especially those receiving cumulative high-dose chemotherapy with concomitant irradiation and bone-marrow transplantation. The incidence of acral erythema during chemotherapy is about 2% [1] and the main agents responsible are fluorouracil and doxorubicine, followed by docetaxel, pacritaxel, methotrexate, vinorelvine, gemcitabine, cytarabine and cyclophosphamide [1, 2]. We report a patient who developed an erythematous eruption on the palms after systemic administrations of cisplatin, and review the literature of cisplatin as the causative agent of CAE.

A 35-year-old woman was diagnosed as having cervical cancer. After hysterectomy, a combination therapy with irradiation (30 Gy/total) and cisplatin (30 mg/m^2) was started. Cisplatin was administered intravenously through her left cephalic vein. At night on the initial day of the treatment, she felt a burning pain in her reddish swollen forearm. The swelling subsided within a day by topical application of betamethasone 1 g per day. However, on the 6th day of chemotherapy, she again developed swelling on her left upper limb and was referred to us.

On physical examination, her left forearm was swollen and slightly reddish. There was symmetrical erythema on the bilateral palms extending to the palmar surface of the fingers *(figures 1A, B)*. The eruption consisted of multiple, salmon-pink, faintly demarcated, non-tender lesions. No eruption was noted on the dorsum of hands and fingers. Laboratory data revealed no leucocytosis or peripheral eosinophilia. We did not perform any skin tests for ethical reasons, because of the cytotoxic properties. Drug-

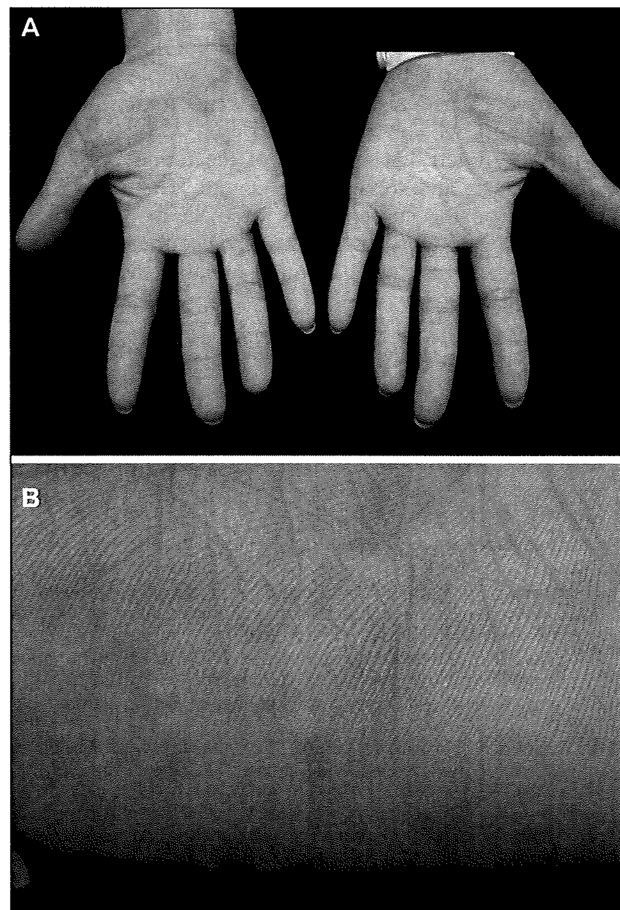

Figure 1. Acral erythematous eruption on both palms **(A)**, showing multiple, demarcated, salmon-pink lesions **(B)**.