TABLE 1. PRIMER PAIRS FOR REAL-TIME PCR.

Gene	Forward primer	Reverse primer	Product size (bp:Accession number)
IL-6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT	175 : M29150
IL-8	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCCTT	196 : BC013615
MIP-1α	TGCAACCAGTTCTCTGCATC	TTTCTGGACCCACTCCTCAC	198 : BC071834
MIP-1β	AAGCTCTGCGTGACTGTCCT	GCTTGCTTCTTTTGGTTTGG	211 : NM_002984
IFN-β	CATTACCTGAAGGCCAAGGA	CAGCATCTGCTGGTTGAAGA	178 : V00534
RANTES	GAGGCTTCCCCTCACTATCC	CTCAAGTGATCCACCCACCT	155 : BC008600
TLR3	AGCCTTCAACGACTGATGCT	TTTCCAGAGCCGTGCTAAGT	201 : NM_003265
G3PDH	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG	203 : BT006893

containing adaptor molecule (TICAM-1). TRIF/TICAM-1 activates the transcription factor NFκB and the interferon regulatory factor 3 (IRF3) [15,16]. The activation of NFκB leads to the production of inflammatory cytokines/ chemokines, and the activation of IRF3 elicits anti-viral responses, especially through the production of type I IFN [15,17,18]. The production of type I IFN is the first line of defense against viral infections, and it acts by limiting the early replication of viruses [19,20]. Deonarain et al. [21] demonstrated that IFN-β is crucial for this process, because IFN-β-deficient mice are highly susceptible to viral infections.

TLR3 recognizes dsRNA and would not be expected to detect DNA from a DNA virus, such as HSV. However, it is known that most viruses synthesize dsRNA during their replication [22], and therefore TLR3 should be able to recognize HSV. Recently, Kariko et al. [23] reported that TLR3 is stimulated by cellular mRNA, and Ashkar et al. [24] reported that the delivery of ligands for TLR3, but not TLR4, protected against HSV-2 infections. Hayashi et al. [25] reported that herpes simplex virus 1 (HSV-1) elicited inflammatory cytokines via TLR3 and TLR9 in the corneal epithelial cells. Thus, corneal epithelial cells may play a role as the first line of defense against viral infection, including HSV infection, through the TLRs.

The purpose of this study was to determine the role played by innate responses in controlling HSV-1 infection of the corneal epithelial cells. In addition, we examined whether immunosuppressive drugs altered the HSV-1 infection of the cornea. We shall show that polyriboinosinic-polyribocytidylic acid (poly(I:C)), a TLR3 agonist, can induce anti-viral responses in corneal epithelial cells. However, these anti-viral responses can be altered by dexamethasone (DEX) and cyclosporine A (CsA).

METHODS

Human subjects: All procedures on human subjects conformed to the tenets of the Declaration of Helsinki [26]. The experimental protocol for these experiments was approved by the Institutional Review Board of Ehime University.

Chemicals and cell cultures: All reagents used for the cell cultures were purchased from Invirogen (Carlsbad, CA). Primary human corneal epithelial cells (HCECs) were isolated from human corneoscleral buttons dissected from eyes acquired from an American Eye Bank(Sight Life Seattle WA) as reported [27]. Briefly, the buttons were carefully denuded of the endothelial cells and adherent iris. After digestion with 1.2 U/ml dispase at 4 °C for 24 h, the loosened epithelial sheets were removed and dispersed into single cells by enzyme digestion with 0.1% trypsin and 0.02% EDTA. Then, the HCECs were cultured in serum-free modified MCDB 153 type II medium, supplemented with insulin (5 μg/ml), hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), Insulin-like growth factor-1 (IFG-1; 10 ng/ml), Epidermal growth factor (EGF; 0.1 ng/ml), and Ca^{2+} (0.06 mM). The medium was changed every 2 days.

To determine the effects of DEX and CsA on the poly(I:C)-induced expression of cytokines/chemokines, HCECs were cultured with hydrocortisone-free, modified MCDB 153 type II medium for 24 h, then incubated with 100 ng/ml of poly(I:C) in the presence or absence of DEX (10^{-6} or 10^{-5} M) or CsA (10^{-6} or 10^{-5} M). In the CsA control, CsA was substituted with 0.01% dimethyl sulfoxide (DMSO), which was also used to reconstitute the CsA. After 24 h of stimulation the cells and supernatants were collected.

Real-time PCR analysis: Total RNA was extracted from the cultured HCECs using RNeasy kit (Qiagen, Valencia, CA), and then reverse-transcribed using Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer's protocols. Real-time PCR was performed with the DyNAmo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) as follows: 95 °C for 15 min; 40 cycles of denaturation at 95 °C for 10 s; annealing at 60 °C for 20 s; and extension at 72 °C for 30 s using the OPticon 2 DNA Engine (BioRad, Hercules, CA). The primer pairs used for real-time PCR are listed in Table 1. The C_t values were determined by the Opticon 2 software, and the amount of each mRNA was calculated relative to the amount of Glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) mRNA in the same samples [28]. Each run was completed with a melting curve analysis to confirm the specificity of the amplification and the absence of primer dimers.

Measurement of proinflammatory cytokines/chemokines production: The concentrations of MIP1-α, MIP1-β, IL-6,

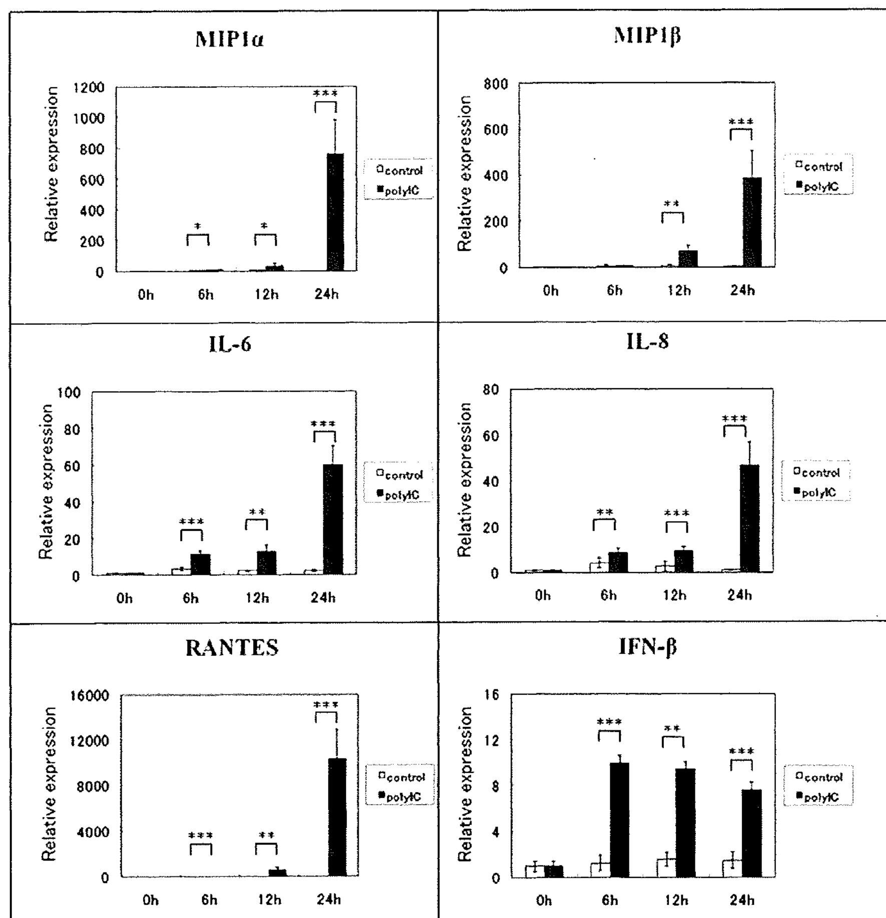

Figure 1. Expression of the mRNAs of cytokines and chemokines by HCEs exposed to poly(I:C), a TLR3 ligand. Total RNA was isolated from HCECs at 6, 12, and 24 h after poly(I:C) exposure, and the expressions of the mRNAs of *MIP1-α*, *MIP1-β*, *IL-6*, *IL-8*, *RANTES*, and *IFN-β* were determined by real-time PCR. The relative level of expression of each cytokine and chemokine mRNA is normalized to the level of *G3PDH* mRNA expression. The p values were calculated using two-tailed paired t-tests, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

IL-8, RANTES, and IFN-β in the supernatants of the cultured HCECs were determined using an ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's protocols.

Immunostaining for NFκB and IRF3: HCECs were cultured on CultureSlides (BD Falcon, Bedford, MA) with 100 ng/ml of poly(I:C) in the presence or absence of DEX (10^{-5} M) or CsA (10^{-5} M) for 3 h. Cells were washed three times with phosphate-buffered saline (PBS), then fixed for 15 min in 3.2% paraformaldehyde (PFA)/PB. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 for 5 min, followed by incubation with primary antibodies to NFκB p65 (0.2 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or to IRF3 (0.2 µg/ml; Santa Cruz Biotechnology) in 1% bovine serum albumin (BSA)/PBS at 4 °C for 16 h. After washing with PBS, the slides were incubated with specific secondary antibodies, then incubated with appropriate fluorescein (FITC) conjugated antibodies (Pierce, Rockford, IL). Finally, the slides were coverslipped using an anti-fading mounting medium (Vector, Burlingame, CA). For the controls, sections were treated with normal rabbit immunoglobulin G (IgG), and no positive staining was detected with any of the antibodies.

Herpes simplex virus 1 (HSV-1) infection: Stocks of the McKrae strain of HSV-1 were propagated on African green monkey kidney (Vero) cells grown in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin, and streptomycin. The titer of virus stocks was determined by the standard plaque assay on Vero cells, and titers were expressed as plaque-forming units (PFU)/ml. Stocks were stored at −70 °C in 1 ml aliquots, and a fresh aliquot of stock virus was used for each experiment.

HCECs were cultured in a hydrocortisone-free, modified MCDB 153 type II medium for 24 h, and cultured in the presence or absence of DEX (10^{-5} M) or CsA (10^{-5} M) prior to exposure to HSV-1. For the plaque assay, HCECs were inoculated with HSV-1 at a multiplicity of infection (MOI) of 50 for 48 h, and the cells were then fixed with 10% formalin and stained with crystal violet. The area of the plaques was measured by Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA) to evaluate the efficiency of infection. The supernatants were also collected to evaluate the concentration of HSV-1 DNA by real-time PCR. To examine

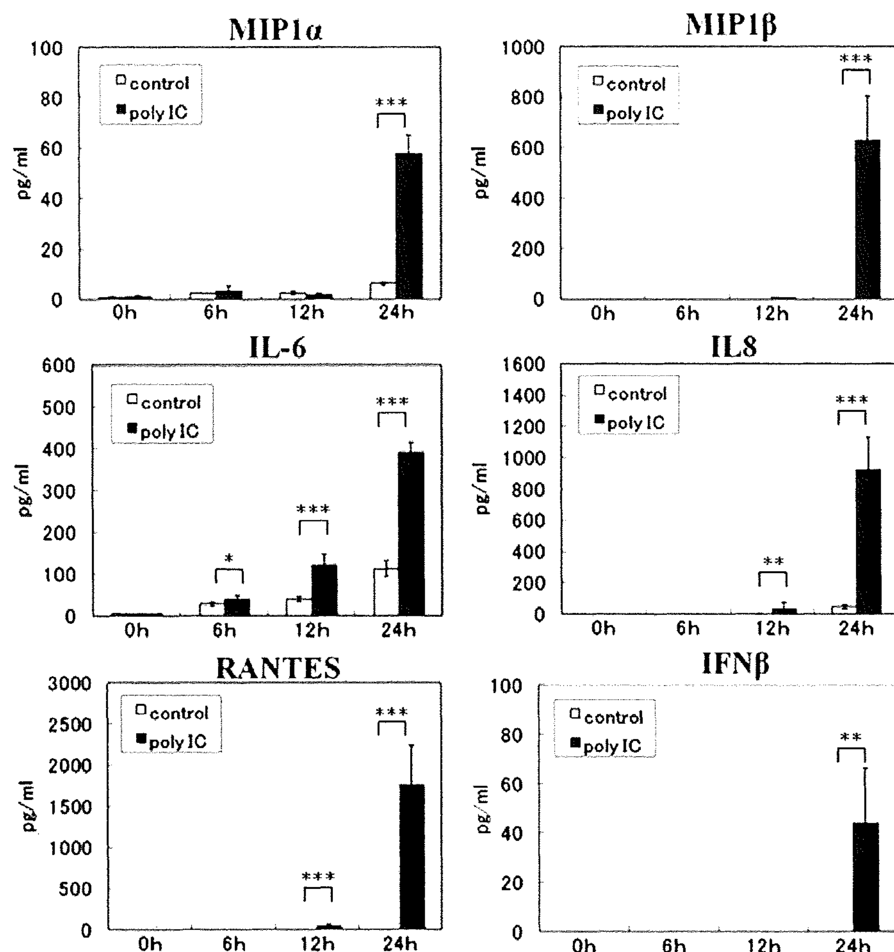

Figure 2. Cytokines and chemokines secreted by HCECs treated with poly(I:C). Culture medium was collected at 6, 12, and 24 h after poly(I:C) stimulation and analyzed for MIP1-α, MIP1-β, IL-6, IL-8, RANTES, and IFN-β protein by ELISA. The p values were calculated using two-tailed paired t-tests (*p<0.05, **p<0.01, ***p<0.001).

the participation of the TLR3 systems in signaling the HSV-1 infection on HCECs, the HCECs were pre-incubated with or without DEX, and then inoculated with HSV-1. To collect the cells before plaque formation, the time period from inoculation to testing was reduced to 24 h, and the inoculated dose increased to a MOI of 1,000, to allow detection of changes in inflammatory cytokines/chemokines. Therefore, HCECs were pre-incubated with or without DEX (10^{-5} M), followed by HSV-1 inoculation with a MOI of 1,000, and the cells collected for real-time PCR after 24 h.

Statistical analyses: Each experiment was repeated 3 times, and representative results are shown in the figures. Values are presented as means±standard deviations (SDs). Differences between the groups were determined by two-tailed paired t-tests. A p-value of <0.05 was considered to be statistically significant.

RESULTS

Poly(I:C)-induced TLR3 signaling pathway: To determine whether the TLR3/TRIF pathway is active in cultured HCECs, the HCECs were incubated with 100 ng/ml of poly(I:C) for 6, 12, and 24 h. Real time RT-PCR was then performed on the cells with primer pairs for *MIP1-α*, *MIP1-β*, *IL-6*, *IL-8*, *RANTES*, *IFN-β*, and *TLR3*. After stimulation by poly(I:C), the expression of the mRNA of *MIP1-α*, *IL-6*, *IL-8*, and *RANTES* were up-regulated as early as 6 h, and the level had increased 750 fold, 60 fold, 50 fold, and 10,000 fold, respectively, at 24 h. *MIP1-β* was also up-regulated at 12 h and reached about 400 fold at 24 h. *IFN-β* was up-regulated 9.9 fold within 6 h, which was maintained for 24 h (Figure 1). *TLR3* was also up-regulated at 12 h, and the level had increased about 40 fold after 24 h (Figure 2A). The expressions of inflammatory cytokines/chemokines and *TLR3* were not significantly altered without poly(I:C) stimulation (Figure 1 and Figure 2A).

The supernatants of the culture media were collected at 0, 6, 12, and 24 h, and the levels of MIP1-α, MIP1-β, IL-6, IL-8, RANTES, and IFN-β was evaluated using ELISA. The levels of MIP1-α, MIP1-β, and RANTES in the supernatant were elevated from undetectable levels at 0 h to 57.6 pg/ml,

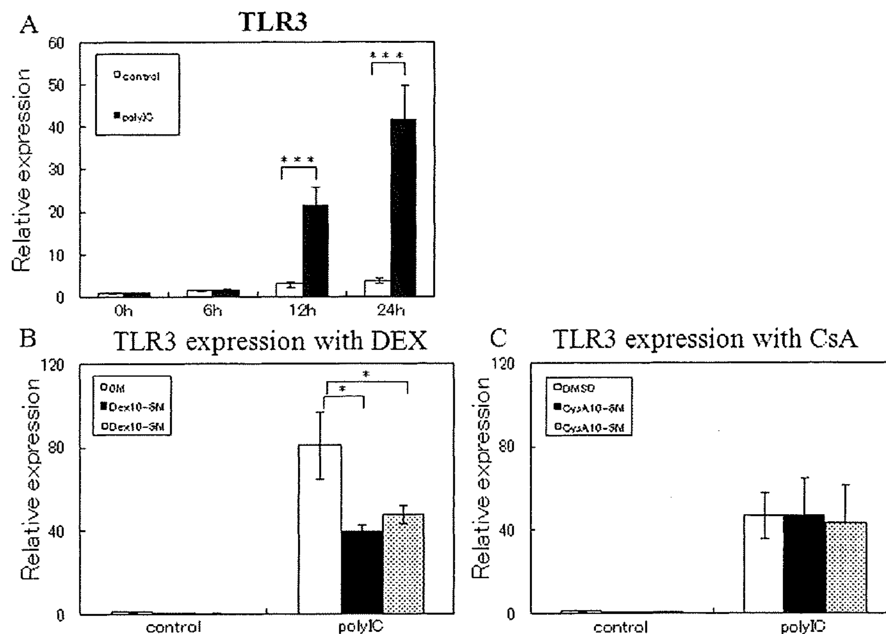

Figure 3. Effect of DEX and CsA on the expression of TLR3 by HCECs exposed to poly(I:C). Total RNA was isolated from HCECs at 6, 12, and 24 h after poly(I:C) stimulation (**A**), or from HCECs cultured with or without of DEX (**B**) or CsA (**C**) for 24 h and stimulated with poly(I:C) for 24 h. The expression of the mRNA of *TLR3* was determined by real-time PCR. The relative level of expression of each cytokine and chemokine mRNA is normalized against *G3PDH* mRNA expression. The p values were calculated using two-tailed paired t-tests (*p<0.05, **p<0.01, ***p<0.001).

630 pg/ml, and 1748.7 pg/ml, respectively, at 24 h after poly(I:C) stimulation. There was a slight but not significant elevation without poly(I:C) stimulation. The levels of IL-6 and IL-8 were slightly elevated without poly(I:C) stimulation, but were significantly elevated to 390 pg/ml and 920 pg/ml, respectively, at 24 h after poly(I:C) stimulation. The level of IFN-β was elevated to 43.8 pg/ml by poly(I:C) after 24 h, and no production of IFN-β was found without poly(I:C) stimulation (Figure 3).

Effect of DEX and CsA on TLR 3 signaling pathway: To determine whether DEX and CsA altered the expressions of the poly(I:C)-induced TLR3 and inflammatory cytokines/chemokines, HCECs were cultured with 100 ng/ml of poly(I:C) with or without DEX (10^{-6} or 10^{-5} M) or CsA (10^{-6} or 10^{-5} M). After 24 h, the cells and supernatants were collected, and the expression of the mRNAs and proteins of IL-6, IL-8, IFN-β, and TLR3 were evaluated by real-time PCR and ELISA.

Incubation with DEX down-regulated the poly(I:C)-induced expression of *TLR3* mRNA about 0.5 fold with 10^{-6} M and 0.6 fold with 10^{-5} M of DEX, whereas no effect was found when incubated with CsA (Figure 2B,C).

Incubation with DEX down-regulated the poly(I:C)-induced expression of the mRNA of *IL-6* about 0.4 fold with 10^{-6} M and 0.5 fold with 10^{-5} M of DEX (Figure 4). ELISA also showed that the poly(I:C) induced IL-6 production was decreased about 0.6 fold with 10^{-6} M and 0.5 fold with 10^{-5} M of DEX (Figure 5). The poly(I:C)-induced expressions of the mRNA and proteins of IL-8 were more significantly down-regulated by DEX, and the decrease was dose-dependent. Real-time PCR showed that the expression of the mRNA of *IL-8* was down-regulated about 0.4 fold with 10^{-6} M and 0.3 fold with 10^{-5} M of DEX. ELISA also showed a reduced production of IL-8 protein of about 0.5 fold with 10^{-6} M and 0.4 fold with 10^{-5} M of DEX (Figure 4 and Figure 5). DEX also down-regulated the poly(I:C)-induced mRNA expression of *IFN-β* by about 0.5 fold with 10^{-6} M and 10^{-5} M of DEX and decreased IFN-β production by about 0.6 fold with 10^{-6} M and 0.5 fold with 10^{-5} M of DEX (Figure 4 and Figure 5).

The effect of CsA on the poly(I:C)-induced inflammatory cytokine/chemokine expression was not as extensive as with DEX. However, the poly(I:C)-induced *IL-6* mRNA expression was down-regulated about 0.8 fold with 10^{-5} M of CsA, and ELISA showed that the poly(I:C) induced IL-6 production was reduced about 0.7 fold with 10^{-5} M of CsA (Figure 4 and Figure 5). The poly(I:C)-induced *IL-8* mRNA expression was also down-regulated about 0.65 fold with 10^{-5} M of CsA (Figure 4), and ELISA showed a decrease in production of about 0.65 fold with 10^{-5} M of CsA (Figure 5). Interestingly, CsA had no effect on poly(I:C)-induced *IFN-β* mRNA expression or production (Figure 4 and Figure 5).

Immunohistochemical staining for NFκB and IRF3: The effect of DEX (10^{-5} M) or CsA (10^{-5} M) on the activation of NFκB and IRF-3 was determined immunohistochemically after 3 h of stimulation by poly(I:C). NFκB p65 and IRF-3 staining were weakly detected in the cytosol of cultured HCECs without poly(I:C) stimulation (Figure 6A,E), but activated NFκB p65 and IRF-3 were clearly detected in the nuclei of most of cultured HCECs 3 h after stimulation by poly(I:C; Figure 6B,F). After stimulation by poly(I:C) in the presence of DEX, NFκB p65 and IRF-3 were detected in the nuclei of some HCECs but only in the cytosol of other HCECs

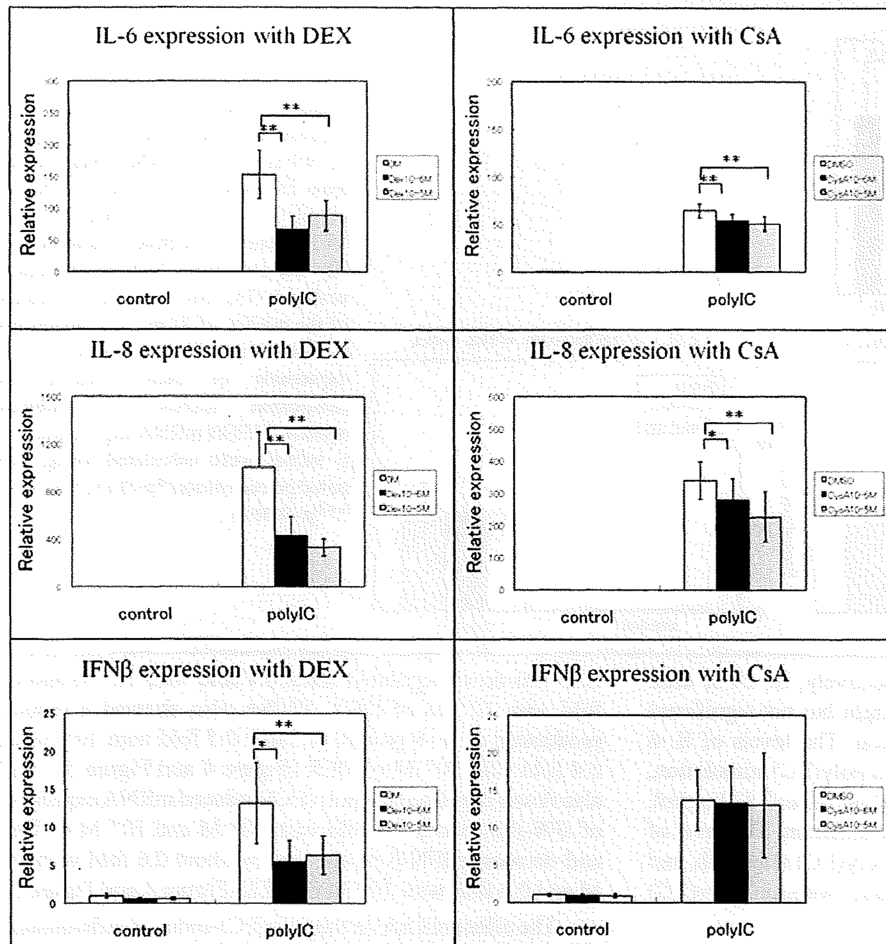

Figure 4. Effect of DEX and CsA on the expression of cytokines and chemokines by HCEs treated with poly(I:C). Total RNA was isolated from HCECs cultured with or without of DEX or CsA for 24 h and stimulated with poly(I:C) for 24 h. The expressions of the mRNAs of *IL-6*, *IL-8*, and *IFN-β* were determined by real-time PCR. The relative level of expression of each cytokine and chemokine mRNA is normalized to the level of *G3PDH* mRNA expression. The p values were calculated using two-tailed paired-tests (*p<0.05, **p<0.01, ***p<0.001).

(Figure 6C,G). After stimulation by poly(I:C), NFκB p65 staining was detected in more HCEC nuclei after exposure to CsA than to DEX, but some HCECs were stained only in the cytosol when exposed to CsA (Figure 6D). IRF3 was detected only in the nuclei of cultured HCECs after 3 h of stimulation by poly(I:C) in the presence of CsA (Figure 6H).

Effect of DEX and CsA on Herpes simplex virus 1 (HSV-1) infection: To determine whether DEX and CsA affected the HSV-1 infection of HCECs, HCECs were cultured in the presence or absence of DEX (10^{-5} M) or CsA (10^{-5} M), and inoculated with HSV-1 at a MOI of 50. The plaque area was increased when HCECs were pre-incubated with DEX, but CsA had no effect on HSV-1 infection (Figure 7A). Real time PCR showed more *HSV-1* DNA in the supernatant of DEX-exposed HCECs (Figure 7B).

In addition, we investigated the involvement of TLR3 signaling systems in HSV-1 infection of HCECs. Real-time PCR showed that the expressions of *IL6*, *IFN-β*, and *TLR3* were down-regulated by DEX when HCECs were inoculated with HSV-1 (Figure 8). *IL-6* and *IL-8* were also down-regulated, although the decrease was not statistically significant for *IL-8* (Figure 8).

DISCUSSION

Our results showed that poly(I:C), a TLR3 agonist, up-regulated the production of inflammatory cytokines/chemokines such as MIP1-α, MIP1-β, RANTES, IL-6, and IL-8, by activating NFκB. Incubation of HCECs with poly(I:C) also activated IRF3 followed by IFN-β production. The up-regulated expression of TLR 3 by poly(I:C) indicates that the TLR3/TRIF signaling pathways were most likely activated by poly(I:C) in HCECs. This is consistent with previous reports [1,15-17]. The cytokines and chemokines investigated are known to have powerful effects in recruiting immune cells and stimulating the maturation of dendritic cells [29-31]. Therefore, we suggest that corneal epithelial cells, when the TLR3s are activated de novo, are able to recruit and activate immune cells against viral infections.

Our results showed that DEX and CsA inhibit the poly(I:C)-induced NFκB activation and the subsequent

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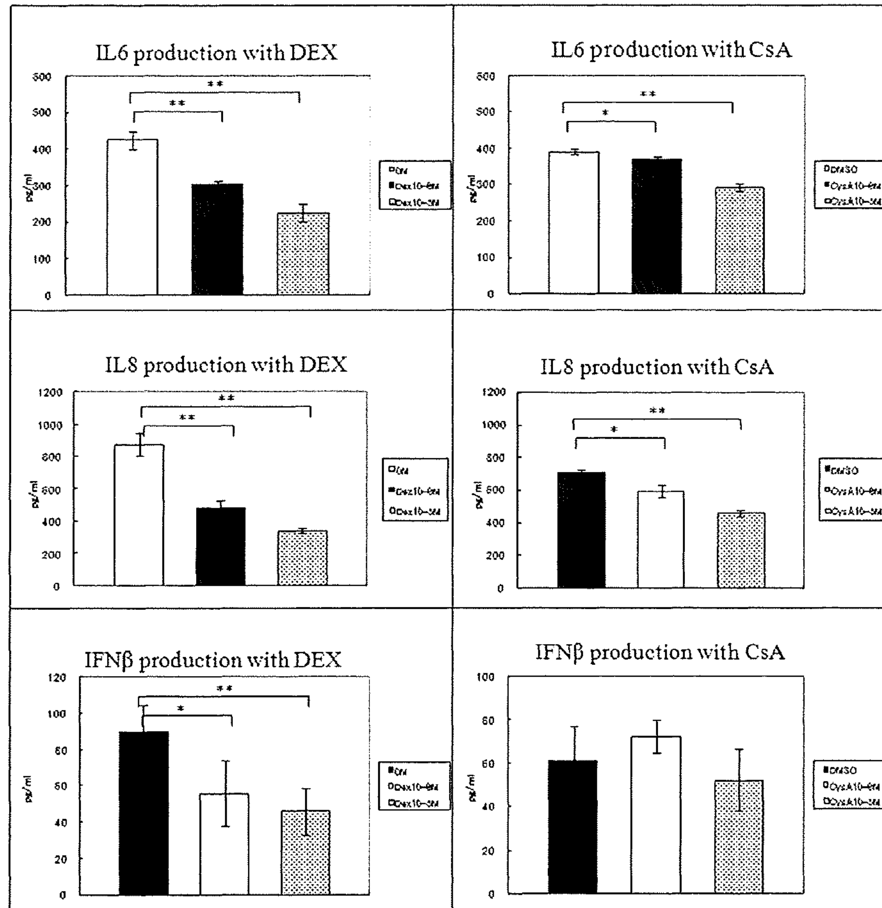

Figure 5. Cytokines and chemokines secreted by HCECs stimulated with poly(I:C) and cultured with or without DEX or CsA for 24 h. Culture medium was collected 24 hours after poly(I:C) stimulation and analyzed for the presence of IL-6, IL-8, and IFN-β protein by ELISA. The p values were calculated using two-tailed paired-tests, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

production of inflammatory cytokines/chemokines. Earlier studies have shown that the concentration of topically applied reagents in tears sharply decreases to less than 1/100 of the original concentration by one hour after administration, and keeps decreasing until only trace levels remain [32,33]. The concentrations of DEX and CsA used in this study were 1/500 and 1/5,000 of the concentration used in eye drops in a clinical setting (0.05%), and so the results should be clinically applicable.

Glucocorticoids, potent inhibitors of immune responses, act through glucocorticoid receptors (GRs) to depress the activities of other DNA-bound transcription factors, such as activator protein 1(AP-1) and NFκB [34-37]. CsA is known to inhibit T cell activation and proliferation [38]. Recent studies have shown that the inhibitory effects of CsA result from interference in the degradation of inhibitory kappaB (IκB) and a reduction in the transcriptional activity of the classic NFκB signaling pathway [39,40]. Our immunohistochemical results showed that DEX and CsA inhibit the poly(I:C)-induced nuclear translocation of NFκB, and these findings are in accord with earlier reports. Thus, the inhibition of inflammatory cytokines/chemokines by DEX and CsA in HCECs may result from the inhibition of NFκB, and this may be one of the mechanisms responsible for the immunosuppressive property of DEX and CsA.

DEX and CsA have different effects on the activation of IRF3 and IFN-β production, and both are part of the TRIF/TICAM-1 TLR3 signaling pathways [15,17,18]. DEX inhibited the poly(I:C)-induced IRF3 activation and the subsequent IFN-β production, while CsA inhibited neither IRF3 activation nor IFN-β production. The exact mechanism of action of DEX and CsA on IRF3 has still not been determined, however Reily et al. [41] have identified the glucocorticoid receptor-interacting protein 1 (GRIP1) to be an IRF3-interacting protein that facilitates IRF3-mediated transcription. They showed that the GRIP1:IRF3 interaction is blocked by the activation of GRs [41]. Our finding that DEX inhibited the poly(I:C)-induced IRF3 activation in HCECs is in accord with their findings.

The different effects of DEX and CsA on the activation of IRF3 and IFN-β production might also be explained by their differing effects on the expression of TLR3. Because the IFN-

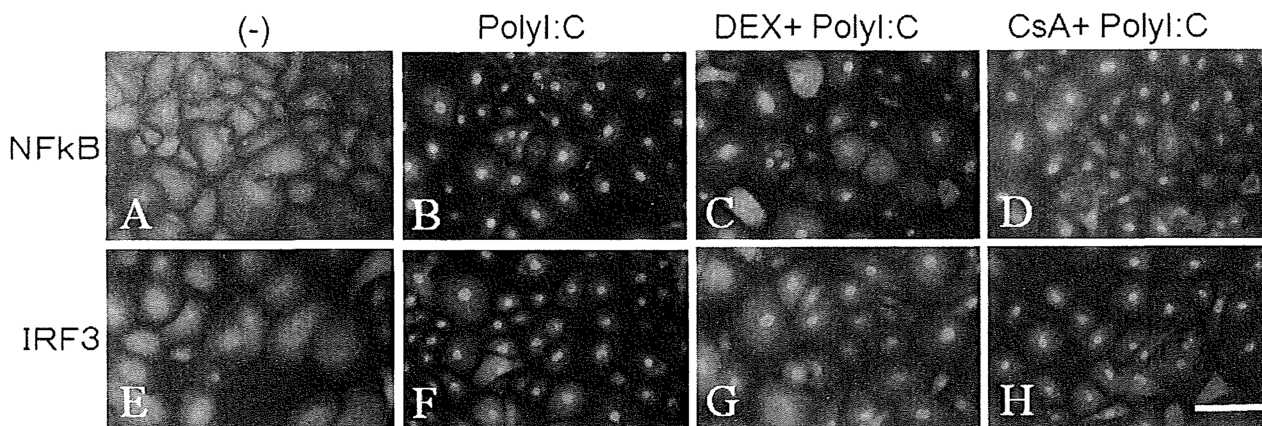

Figure 6. Immunohistochemical staining for NFκB and IRF3 in HCECs stimulated with poly(I:C) and cultured with or without of DEX or CsA for 24 h. NFκB p65 staining without poly(I:C, **A**), with poly(I:C, **B**), with DEX 10^{-5}M and poly(I:C, **C**), and with CsA 10^{-5}M and poly(I:C, **D**). IRF3 staining without poly(I:C, **E**), with poly(I:C, **F**), with DEX 10-5M and poly(I:C, **G**), and with CsA 10-5M and poly(I:C, **H**). Scale bar, 100 μm. Activated NFκB p65 and IRF-3 were clearly detected in the nuclei of most of cultured HCECs 3 h after stimulation by poly(I:C, **B** and **F**). In the presence of DEX, NFκB p65 and IRF-3 were detected in the nuclei of some HCECs but only in the cytosol of other HCECs (**C**, **G**). In the presence of CsA, NFκB p65 staining was detected in more HCEC nuclei after exposure to CsA than to DEX (D), while IRF3 was detected only in the nuclei of cultured HCECs (**H**).

responsive element (ISRE) is located on the human TLR3 promoter region, it has been suggested that IFNα/β induces the expression of TLR3 [42,43]. It has not been determined whether CsA regulates the IRFs or IFN, but our results showed no effect of CsA on IRF3 activation or on IFN-β production in HCECs.

The production of type I IFN is the first line of defense against viral infections, and it acts by limiting the early replication of viruses [19,20]. Deonarain et al. [21] demonstrated that IFN-β is crucial to this process because IFN-β-deficient mice were highly susceptible to viral infections. Our preliminary experiments showed that HSV infection was clearly depressed by poly(I:C) treatment prior to the HSV inoculation of the HCECs (data not shown). DEX treatment prior to HSV inoculation of HCECs led to the down-regulation of *TLR3* and *IFN-β* followed by increased *HSV-1* DNA and plaque formation. However, CsA did not interfere with the HSV-1 infection (data not shown). It is of interest to note that the anti-viral capabilities of corneal epithelial cells arise from their ability to produce IFN-β. Topical or systemic application of glucocorticoids results in the appearance of clinically active herpes keratitis, in which viral particles infect the corneal epithelial cells, leading to viral replication [9,10]. DEX has also been shown to increase the susceptibility of corneal epithelial cells to HSV-1 infection [44].

It has been known that TLR9 recognizes deoycytidylate-phosphate-deoxyguanosine (CpG) motifs in bacterial DNA, however, recent reports have demonstrated that TLR9 also recognizes CpG motifs in viral DNA, including HSV [24,45, 46]. In addition, retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), including RIG-I, melanoma differentiation-associated gene 5 (Mda5), and *Leishmania* G-protein 2 (LGP2), have recently been identified as cytoplasmic proteins that recognize viral RNA [47,48]. The RLRs also activate NFκB and IRF3 following viral infection and poly(I:C) stimulation. RLRs-mediated signaling induced by dsRNA has been demonstrated in epidermal keratinocytes [49]. Our results showed an elevated production of inflammatory cytokines/chemokines that was associated with an up-regulated expression of TLR3, indicating that TLR3/TRIF signaling pathways are involved in the anti-viral response of HCECs. However, the presence of signaling cannot be fully accounted for by the TLR3/TRIF signaling pathway alone. It is possible that the TLR9 and RLRs pathways may also play a role in the production of inflammatory cytokines/ chemokines, but we did not study the RLRs pathway. Further investigation will be needed to determine the exact mechanisms.

In summary, we have demonstrated that HCECs have ability to produce inflammatory cytokines/chemokines via the innate immune system, and these responses can be modified by DEX and CsA. DEX down-regulated both NFκB and IRF3, whereas CsA down-regulated only NFκB. This inhibition by DEX of IRF3 followed by IFN-β production may be another mechanism in the immunosusceptibility of HCECs to HSV infection. Thus, the innate corneal immune system may be involved in HSV infection of HCECs, and further studies to determine the function of the innate immune system might lead to new therapeutic agents, or the development of effective ways of preventing corneal infections.

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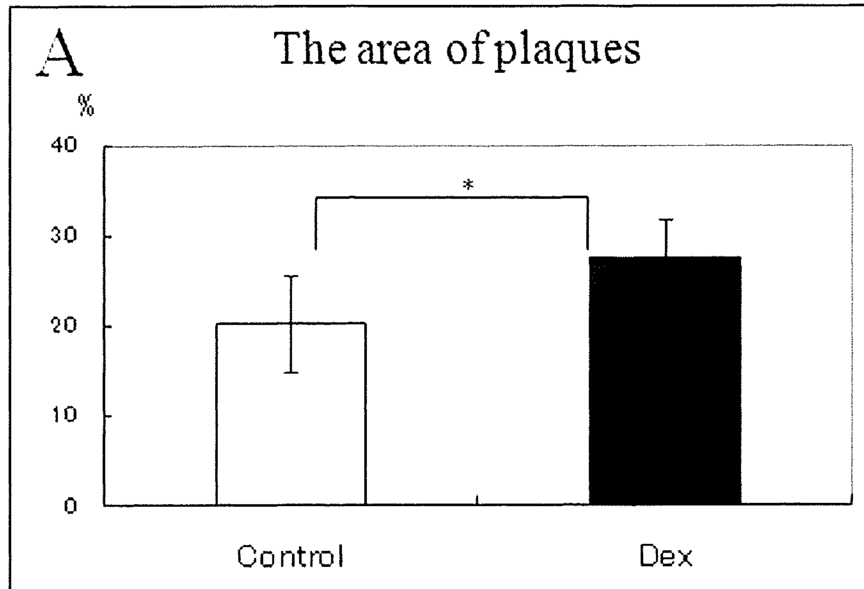

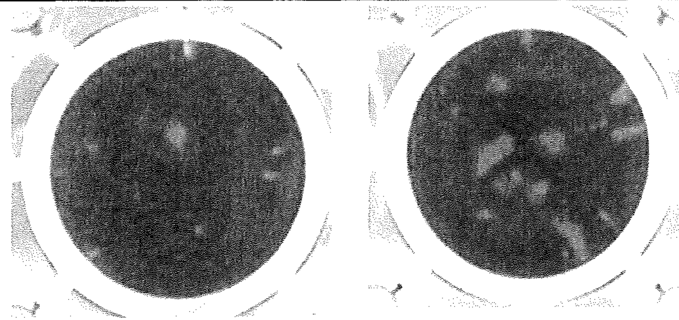

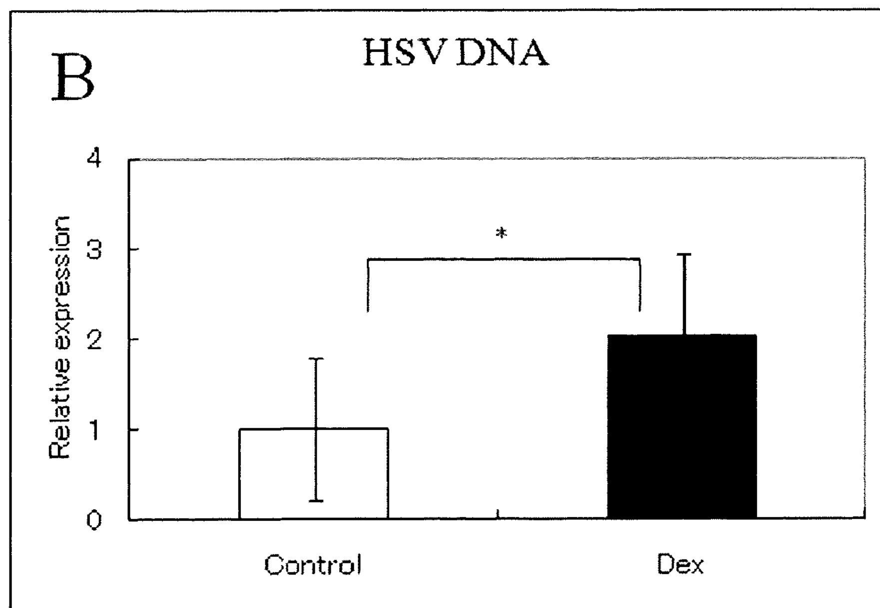

Figure 7. Effect of DEX and CsA on Herpes simplex virus 1 (HSV-1) infection. HCECs were cultured in the presence or absence of DEX (10^{-5} M), and inoculated with 50 MOI of HSV-1 for 48 h. The plaque area was increased when HCECs were pre-incubated with DEX (**A**). Real-time PCR results show a significantly higher level of *HSV-1* DNA in the supernatant with DEX (**B**). (*$p<0.05$)

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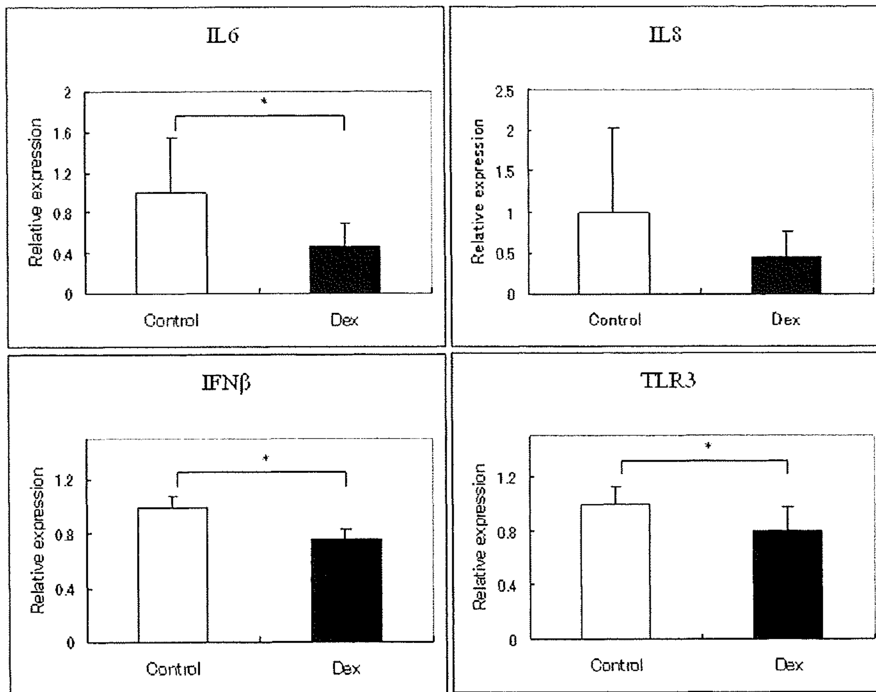

Figure 8. Effect of DEX and CsA on involvement of TLR3 signaling systems in HSV-1 infection of HCECs. HCECs were cultured in the presence or absence of DEX (10^{-5} M) and inoculated with 1,000 MOI of HSV-1 for 24 h. Real-time PCR shows that *IFN-β* and *TLR3* expression is down-regulated by DEX. *IL-6* and *IL-8* are also down-regulated, although the decrease of *IL-8* was not statistically significant. (*$p<0.05$)

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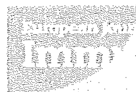

IL-17 and IL-22 mediate IL-20 subfamily cytokine production in cultured keratinocytes *via* increased IL-22 receptor expression

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IL-20 cytokine subfamily members, including IL-19, IL-20, and IL-24, are highly expressed in psoriatic skin lesions. Here, we demonstrate that psoriasis mediators IL-17 and IL-22 synergistically induce the production of IL-20 subfamily proteins in cultured human keratinocytes. Interestingly, expression of the IL-22 receptor (IL-22R) also increased in epidermal lesions *versus* normal skin. IL-22R over-expression using an adenoviral vector to mimic psoriatic conditions in cultured keratinocytes significantly enhanced IL-17- and IL-22-induced production of IL-20 subfamily cytokines. Furthermore, IL-17 and IL-22 coordinately enhanced MIP-3α, IL-8, and heparin-binding EGF-like growth factor (HB-EGF) production, depending on the amount of IL-22R expression. Additionally, because IL-20 and IL-24 share the IL-22R with IL-22, the function of IL-20 and IL-24 was also increased. IL-20 and IL-24 have effects similar to that of IL-22; IL-24 showed more potent expression than IL-20. A combination of IL-24 and IL-17 increased the production of MIP-3α, IL-8, and HB-EGF, as did a combination of IL-22 and IL-17. These data indicate that increased IL-22R expression in epidermal keratinocytes contributes to the pathogenesis of psoriasis through enhancing the coordinated effects of IL-22 and IL-17, inducing the production of the IL-20 subfamily, chemokines, and growth factors.

Key words: Chemokine · Cytokine · Dermatitis · Epithelial cells · Inflammation

Introduction

Psoriasis is a common chronic inflammatory skin disease, and typical lesions are well-circumscribed red plaques covered by a silvery white scale. Histologically, hyperkeratosis and epidermal hyperplasia with suprapapillary epidermal thinning are observed, with neutrophilic microabscesses and the disappearance of the granular layer. Vasodilation and infiltration of leukocytes are also seen in papillary dermis.

Epidermal keratinocytes from psoriatic lesions produce abundant chemokines, growth factors, and antimicropeptides, such as human beta-defensin 2 (HBD2) and LL-37 [1–5]. Chemokines produced by epidermal keratinocytes are important in recruiting inflammatory cells to the skin, and the chemokine expression pattern in psoriasis is different from that in atopic dermatitis, another common chronic inflammatory skin disease [3, 4]. In particular, expression of MIP-3α and IL-8 is strongly upregulated in psoriatic epidermis compared with atopic dermatitis; these chemokines recruit T lymphocytes and neutrophils, respectively. Over-expression of several growth factors, such as heparin-binding EGF-like growth factor (HB-EGF), TGF-α, epiregulin, and amphiregulin, has been reported, and may contribute to the epidermal hyperproliferation in psoriasis [6–9].

Cytokines produced by Th17 cells, including IL-17 and IL-22, are generally accepted to be involved in the development of psoriasis [10]. IL-17 acts directly on keratinocytes and regulates the

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production of MIP-3α, IL-8, and HBD2 [5, 11, 12], whereas IL-22 regulates keratinocyte differentiation [5, 13–15]. In addition, treatment of reconstructed skin with IL-22 induces epidermal hyperproliferation and the disappearance of the granular layer, characteristic features of psoriasis [5, 13, 15]. While the IL-17 receptor is highly and widely expressed in normal human tissue, expression of the receptor for IL-22 is relatively limited. Receptors for IL-22 are the IL-22 receptor (IL-22R) and the IL-10 receptor 2 (IL-10R2) [16]. Although IL-10R2 is expressed in a variety of normal human tissues, including immune cells, IL-22R mRNA is detected only in the pancreas, small intestine, colon, liver, lung, and skin, but not in immune cells [15, 17–20]. These findings indicate that skin is among the limited target tissues of IL-22.

IL-22 belongs to the IL-10 family, which also includes IL-19, IL-20, IL-24, IL-26, IL-28, and IL-29 [21]. Although the amino acid sequences of these cytokines are homologous to that of IL-10, their functions differ. IL-19, IL-20, and IL-24, the so-called IL-20 subfamily, have similar functions to IL-22 [13]. The IL-20 subfamily signals through IL-22R/IL-20R2 and/or the IL-20R1/IL-20R2 complex [22, 23], and the IL-20 subfamily and IL-22 induce STAT3 phosphorylation. Recently, STAT3 phosphorylation in epidermal keratinocytes has been implicated in the development of psoriasis [24]. Thus, the over-expression of IL-19, IL-20, and IL-24 in psoriatic skin lesions [17, 25, 26] may play an important role in the pathogenesis of this disease. The production of IL-20 subfamily in monocytes has been characterized by many groups [19, 27–29]. Furthermore, the regulation of IL-19, IL-20, and IL-24 expression in keratinocytes was also investigated [13, 17, 25]. Recently, the effect of Th17 cytokine in producing these mediators was suggested. In bronchial epithelial cells, IL-17 induces the production of IL-19 [20]. Sa *et al.* demonstrated that IL-22 increased IL-20 mRNA expression in epidermal keratinocytes [13]. Here, we demonstrate that Th17 cytokines induce IL-19, IL-20, and IL-24 production in keratinocytes. This production was especially enhanced by stimulation with IL-17 and IL-22 in combination. Additionally, we found that IL-22R expression was elevated in psoriatic epidermis. When IL-22R expression increased, the response to a combination of IL-17 and IL-22 was strengthened, producing the IL-20 subfamily, and the responses for IL-20 and IL-24 were also augmented *via* IL-22R.

Results

IL-19, IL-20, and IL-24 expression in psoriatic epidermis

Previous reports demonstrated that IL-19, IL-20, and IL-24 mRNA expression increased in psoriatic skin lesions compared with normal

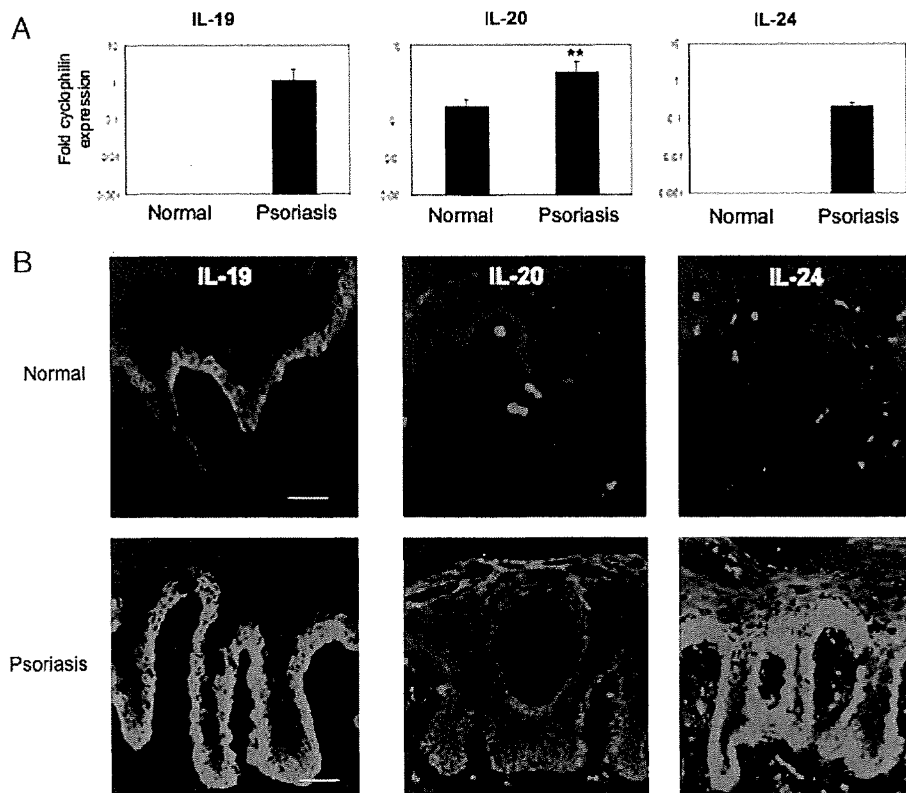

Figure 1. IL-19, IL-20, and IL-24 expression in normal and psoriatic epidermis. IL-19, IL-20, and IL-24 expression levels were determined by real-time RT-PCR and immunostaining. (A) Total RNA was extracted from the epidermis of nine normal and 11 psoriatic skin samples. The expression of IL-19, IL-20, and IL-24 was analyzed by real-time RT-PCR, with cyclophilin as house keeping control. Data show mean ± SD. $^{**}p<0.01$. (B) Frozen sections of four normal and six psoriatic skin samples were stained with antibodies against IL-19, IL-20, and IL-24. Scale bar, 50 μm.

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skin [17, 25, 26]. In these studies, total RNA was extracted from whole skin. However, this method results in the inclusion of RNA from dermal cells (*e.g.* fibroblasts, endothelial cells) and inflammatory cells. Therefore, we extracted total RNA from normal and psoriatic epidermis after first heat-separating the epidermis from the dermis. Heat separation does not influence the stability of RNA [9]. IL-19, IL-20, and IL-24 mRNA expression levels were then analyzed *via* real-time RT-PCR. After 40 PCR cycles, IL-20 mRNA was detected in normal epidermis, whereas IL-19 and IL-24 were not (Fig. 1A). In contrast, psoriatic epidermis showed up-regulated IL-19, IL-20, and IL-24 mRNA expression.

IL-19, IL-20, and IL-24 protein expression was analyzed *via* immunostaining. Normal epidermis expressed IL-19, IL-20, and IL-24 (Fig. 1B). Pretreatment of antibodies with their corresponding recombinant cytokines blocked this reactivity (data not shown). IL-20$^+$ cells were also observed in the upper dermis; they were also stained using macrophage-specific antibodies (data not shown), as described previously [30]. IL-24$^+$ cells in the epidermis were identified as Langerhans cells based on CD1a co-expression (data not shown). In psoriatic skin, IL-19, IL-20, and IL-24 expression increased significantly throughout the entire epidermis. These results suggest that psoriatic keratinocytes show increased IL-20 subfamily protein production.

IL-17 enhanced IL-19, IL-20, and IL-24 mRNA expression in cultured keratinocytes

We examined the effects of IL-17 on cultured keratinocytes, which express IL-17 receptor mRNA at a level almost equal to that of normal epidermis (data not shown). In cultured keratinocytes, IL-20 and IL-24 mRNA, but not IL-19 mRNA were detected in the steady state. IL-17 treatment induced time-dependent increases in IL-19, IL-20, and IL-24 mRNA expression (Fig. 2).

IL-22 enhanced IL-19, IL-20, and IL-24 mRNA expression in cultured keratinocytes

To evaluate the effect of IL-22 on IL-20 subfamily production in cultured epidermal keratinocytes, we first examined the expression of receptors for this cytokine. IL-22 receptors include both IL-22R and IL-10R2. The expression of IL-22R, but not of IL-10R2, was suppressed in cultured keratinocytes, as previously reported (Fig. 3A) [13]. Next, we examined whether IL-22R expression level was modulated by the differentiated state of keratinocytes. Keratinocytes were cultured with a high concentration of calcium, a differentiation-inducing factor, and IL-22R mRNA expression was analyzed. However, the IL-22R mRNA expression level was not increased, compared with undifferentiated keratinocytes (data not shown). Thus, we used an adenoviral vector (Ax) carrying IL-22R (AxIL22R) to increase expression in cultured keratinocytes. When cultured keratinocytes were infected with AxIL22R for 48 h, increased IL-22R expression was confirmed by Western blotting (Fig. 3B) and flow cytometry (Fig. 3C). Moreover, we detected IL-22R expression beginning 24 h after adenovirus vector infection, and the infection resulted in the constant expression of IL-22R, depending on the MOI. In keratinocytes infected with AxIL22R, IL-20, IL-24, and IL-22 induced STAT3 phosphorylation at 15 min and 24 h after stimulation (Fig. 3D).

In control keratinocytes, IL-22 enhanced the mRNA expression of IL-20 and IL-24 after 1 h, but did not increase IL-19 expression (Fig. 4). In keratinocytes infected with AxIL22R, IL-22 stimulation also induced a marked increase in IL-20 and IL-24 mRNA expression, which peaked at 1 h for IL-20 and at 3 h for IL-24, and was sustained for 24 h. IL-19 mRNA expression was also detectable between 3 and 12 h after stimulation. The vector control, AxLacZ, was not able to increase IL-20 subfamily expression (data not shown).

IL-17 and IL-22 coordinately enhanced IL-19, IL-20, and IL-24 production in cultured keratinocytes

The effect of various cytokines on the production of the IL-20 subfamily was examined. Keratinocytes infected with AxIL22R or AxLacZ were treated with IFN-γ, IL-1α, TNF-α, IL-17, or IL-22, which are involved in the pathogenesis of psoriasis. IL-19, IL-20, and IL-24 proteins in culture medium were analyzed by ELISA (Fig. 5A). IL-19 and IL-24 were

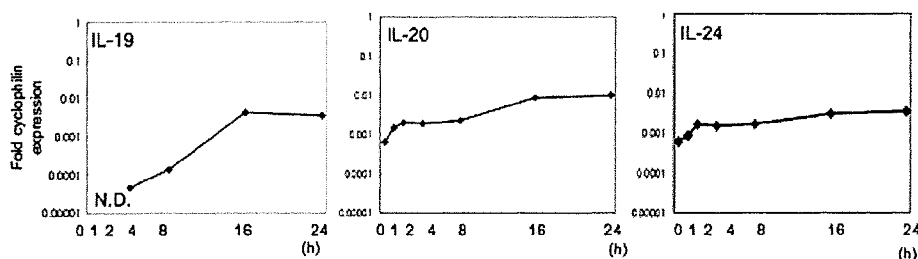

Figure 2. IL-19, IL-20, and IL-24 mRNA expression in cultured keratinocytes stimulated with IL-17. IL-17 (10 ng/mL) was added to culture media containing keratinocytes. Total RNA was extracted at various time points up to 24 h after stimulation. IL-19, IL-20, and IL-24 expression levels were determined by real-time RT-PCR, with cyclophilin as house keeping control. Representative data are shown from two independent experiments. N.D. indicates not detectable.

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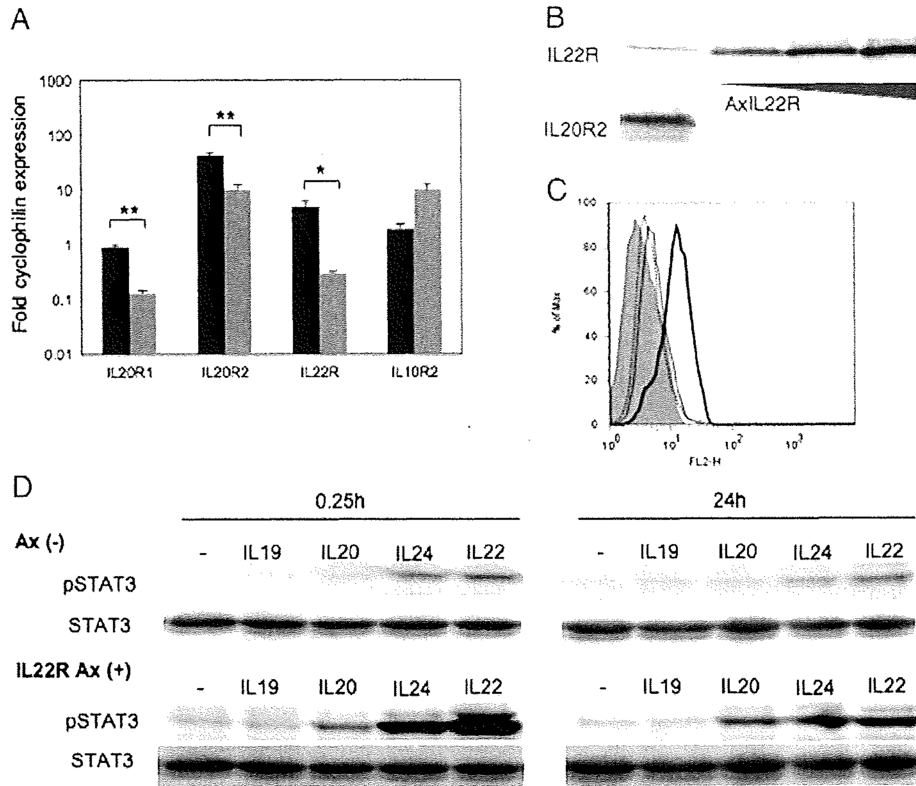

Figure 3. IL-20R1, IL-20R2, IL-22R, and IL-10R2 expression in cultured epidermal keratinocytes. (A) Normal epidermis was obtained from nine subjects and cultured keratinocytes were from eight other normal subjects. IL-20R1, IL-20R2, IL-22R, and IL-10R2 expression levels were determined by real-time RT-PCR, with cyclophilin as house keeping control, and compared between normal epidermis (black bar) and cultured keratinocytes (gray bar). Data show mean $\pm$ SD. *$p<0.05$; **$p<0.001$. (B) Western blot analysis of IL-20R1, IL-20R2, and IL-22R protein expression in cultured keratinocytes. (C) IL-22R expression levels were analyzed by flow cytometry (isotype control Ab: shaded area; anti-IL-22R Ab: thin line). Infection of AxIL22R at an MOI of 5 for 48 h increased IL-22R expression in keratinocytes (thick line), but not AxLacZ (dotted line). Data are representative of two independent experiments. (D) Uninfected control cultures or keratinocytes infected with AxIL22R were treated with 10 ng/mL IL-19, IL-20, IL-24, and IL-22. STAT-3 phosphorylation at 15 min or 24 h after stimulation was determined by Western blot. Data are representative of three independent experiments.

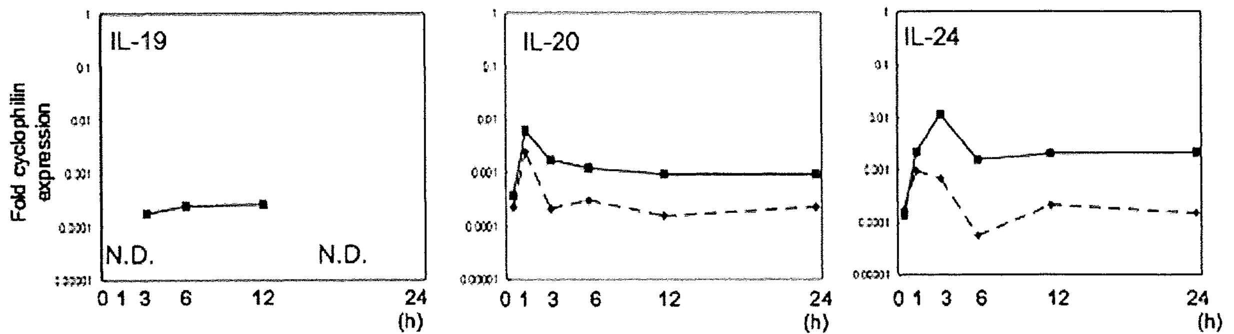

Figure 4. IL-19, IL-20, and IL-24 mRNA expression in cultured keratinocytes stimulated with IL-22. IL-22 (10 ng/mL) was added to culture media containing keratinocytes transduced with AxIL22R (black line) or uninfected control keratinocytes (dotted line). Total RNA was extracted at various time points up to 24 h after stimulation. IL-19, IL-20, and IL-24 expression levels were determined by real-time RT-PCR, with cyclophilin as house keeping control. Data are representative of two independent experiments. N.D. indicates not detectable.

not detected in the culture medium of keratinocytes treated with IFN-γ, TNF-α, or IL-1α. On the other hand, IL-20 was detected at low levels in the culture medium without stimulation, and the production was slightly increased by TNF-α and IL-1α treatment. Notably, IL-19, IL-20, and IL-24 were all produced by stimulation with IL-17. In addition,

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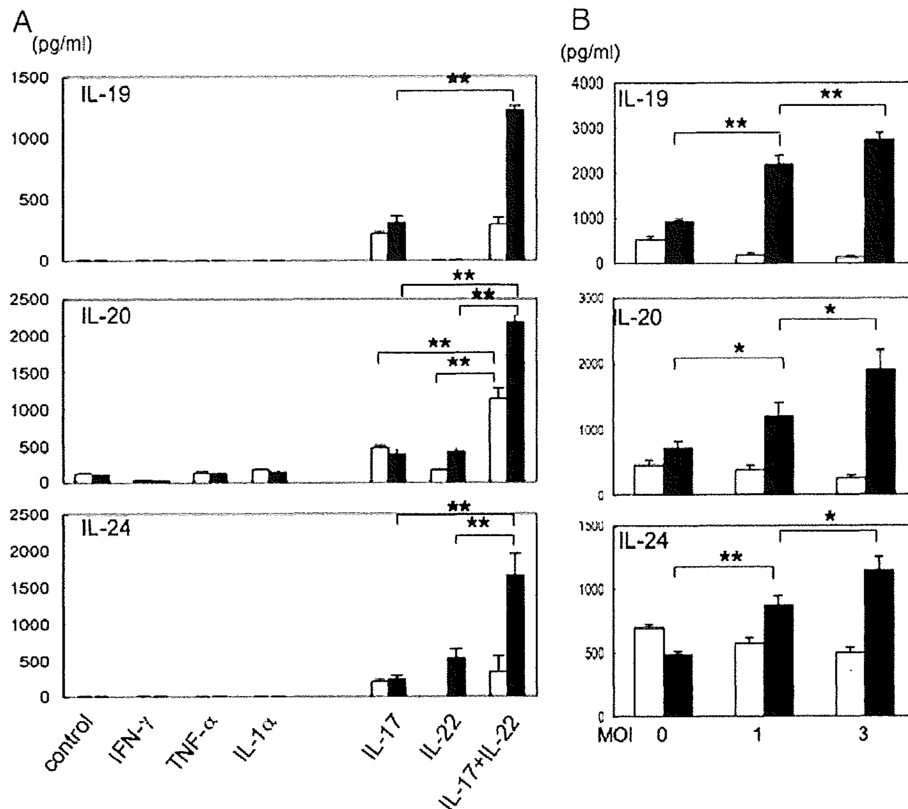

Figure 5. IL-19, IL-20, and IL-24 production in cultured keratinocytes. (A) Cultured keratinocytes infected with AxIL22R (black bar) or AxLacZ (white bar) at an MOI of 1 were treated with 50 IU/mL of IFN-γ, 10 ng/mL of IL-1α, TNF-α, IL-17, or IL-22 for 48 h. (B) Cultured keratinocytes were infected with AxIL22R (black bar) or AxLacZ (white bar) at an MOI of 1 or 3 for 24 h. After washing, cells were co-treated with 10 ng/mL IL-17 and 10 ng/mL IL-22 for 48 h. (A, B) IL-19, IL-20, and IL-24 in culture medium were measured by ELISA. Data show mean±SD ($n = 3$). Data are representative of three independent experiments. *$p<0.05$; **$p<0.01$.

increased IL-22R expression markedly augmented IL-22-stimulated IL-20 and IL-24 production. Moreover, a combination of IL-17 and IL-22 dramatically enhanced production of all of these cytokines (Fig. 5A), which was dependent on IL-22R expression (Fig. 5B).

Epidermal keratinocytes of psoriasis lesion skin express higher than normal levels of IL-22R

Because IL-20 subfamily production increased in response to IL-22R over-expression, we examined IL-22R expression in psoriatic epidermis. IL-22R mRNA levels increased significantly in psoriatic epidermis compared with normal epidermis, whereas mRNA levels for IL-20R1, IL-20R2, and IL-10R2 were similar to those of normal epidermis (Fig. 6A). Immunostaining revealed only weak IL-22R signals throughout the entire epidermis of normal skin. However, fluorescence staining intensity increased in psoriatic epidermis (Fig. 6B). Significant staining was primarily observed in the upper layer.

A combination of IL-17 and IL-22 mediated chemokine and growth factor production *via* increased IL-22R

We postulated that increased IL-22R expression might augment signal transduction by IL-22, and strengthen the coordinated effect of IL-22 and IL-17, other than by production of the IL-20 subfamily. Previous reports demonstrated that IL-17 induced HBD2 mRNA expression [5, 12] and that a combination of IL-17 and IL-22 markedly enhanced mRNA expression [31]. In our study, increased IL-22R augmented HBD2 mRNA expression stimulated by IL-17 and IL-22 in combination (Fig. 7A). Similarly, IL-22 treatment induced enhanced MIP-3α and IL-8 mRNA expression in cultured keratinocytes infected with AxIL22R, and the combination of IL-17 and IL-22 strongly enhanced their mRNA expression.

We further analyzed the production of growth factors. HB-EGF mRNA expression was enhanced by IL-22 stimulation in cultured keratinocytes expressing IL-22R, and co-stimulation with IL-17 and IL-22 markedly augmented the mRNA expression (Fig. 7A). In contrast, although TGF-α mRNA expression was also enhanced by IL-22 treatment, no combination effect was observed.

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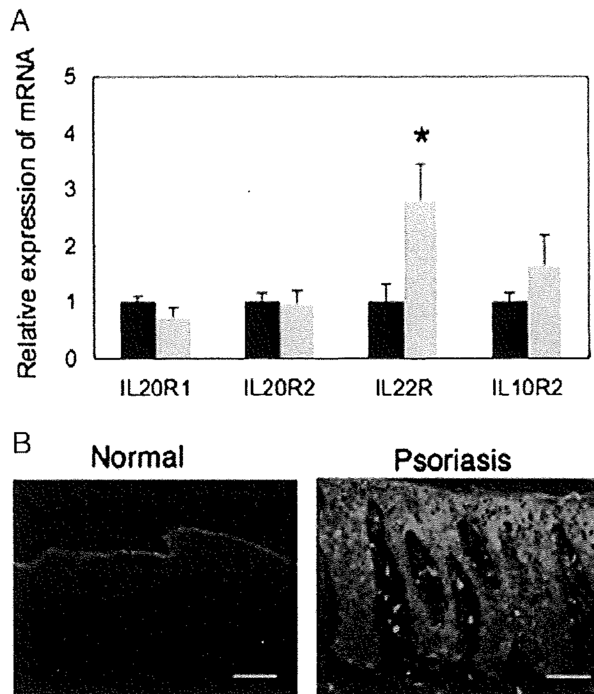

Figure 6. IL-22R expression in the epidermis of normal and psoriatic skin. (A) Total RNA was extracted from the epidermis of nine normal and 11 psoriatic skin samples. IL-20R1, IL-20R2, IL-22R, and IL-10R2 mRNA expression levels were determined by real-time RT-PCR, with cyclophilin as house keeping control. Results were expressed as relative expression levels of mRNA (gray bar) compared with that in normal epidermis (black bar). Data show mean±SD.*$p<0.05$. (B) IL-22R immunostaining of four normal and six psoriatic skin samples. In four psoriatic skin samples, fluorescence intensity increased markedly as a result of IL-22R over-expression. Scale bar, 50 μm.

A combination of IL-17 and IL-24 mediated chemokine and growth factor production *via* increased IL-22R

IL-20 and IL-24, but not IL-19, signal through IL-22R and IL-20R2 [22, 23]. Thus, the function of IL-20 and IL-24 is also considered to be enhanced by increased IL-22R. Because cultured keratinocytes express IL-20R2 protein (Fig. 3B), IL-20 and IL-24 treatment induced significant STAT3 phosphorylation in cultured keratinocytes infected with AxIL22R (Fig. 3D).

IL-24 stimulation augmented HBD2, MIP-3α, IL-8, and HB-EGF mRNA expression in cultured keratinocytes (Fig. 7A). A combination of IL-24 and IL-17 acted synergistically to enhance HBD2, MIP-3α, IL-8, and HB-EGF mRNA expression, as did a combination of IL-22 and IL-17. These effects were confirmed at the protein level for MIP-3α, IL-8, and HB-EGF (Fig. 7B). The effect of IL-20 was weaker than that of IL-24.

Discussion

We demonstrated that epidermal keratinocytes produced IL-19, IL-20, and IL-24, the so-called IL-20 subfamily, when stimulated with IL-17 and IL-22. Monocytes are generally accepted to be the source of IL-19, IL-20, and IL-24 [19, 27–29], and melanocytes are also candidates for producing IL-24 [32]. Although epidermal keratinocytes have been also suspected of producing the IL-20 subfamily [13, 17, 25], Wang *et al.* recently reported that keratinocytes may have no ability to produce IL-20 because IL-20 mRNA could not be detected in cultured keratinocytes [30]. This discrepancy may be due to a difference in the kind of keratinocytes cultured. They examined HaCaT cells, a malignant, transformed keratinocyte cell line. We demonstrated here that IL-17 was a potent inducer of IL-19, IL-20, and IL-24 productions in cultured normal epidermal keratinocytes. Moreover, IL-22 also induced the production of IL-20 and IL-24, and a combination of IL-17 and IL-22 strongly enhanced the production of all of these cytokines. Thus, we believe that IL-17 and IL-22 play important roles in the expression of the IL-20 subfamily by epidermal keratinocytes.

Co-stimulation with IL-17 and IL-22 also enhanced the production of MIP-3α, IL-8, and HB-EGF in keratinocytes. These chemokines and growth factor are known to be abundantly expressed in psoriatic epidermis and to play key roles in the pathogenesis of psoriasis [3, 4]. MIP-3α has been noted to have attractant activity for Th17 lymphocytes, and Th17 lymphocytes have been shown to express CCR6, a specific receptor for MIP-3α [33–35]. IL-8 recruits neutrophils and may contribute to the formation of subcorneal microabscesses, a characteristic feature of psoriasis. Moreover, hyperplasia of the epidermis is believed to arise from over-expression of several growth factors [6–9]. Because HB-EGF acts as a paracrine enhancer of other growth factors [36], production of HB-EGF may be an initial step in epidermal hyperproliferation in psoriasis.

Taken together, co-stimulation with IL-17 and IL-22 likely plays an important role in the pathogenesis of psoriasis. Thus, the fact that the production of the IL-20 subfamily, MIP-3α, IL-8, and HB-EGF, by co-stimulation with IL-17 and IL-22 was enhanced by increased IL-22R expression in cultured keratinocytes is of great interest. Furthermore, IL-22R expression increases in psoriatic epidermis.

IL-22R mRNA expression in psoriasis has previously been investigated using real-time RT-PCR [26, 30, 37]; in these studies, IL-20R1 and IL-20R2 mRNA decreased in psoriatic skin, whereas IL-22R mRNA expression remained unchanged. We suggest that this difference between our findings and these previous reports may be due to differences in the preparation of total RNA. In the previous studies, total RNA was extracted from whole skin, whereas we extracted total RNA from the epidermis alone. Because keratinocytes are the major component of the epidermis, mRNA expression in the epidermis reflects that of keratinocytes. Whole lesions from psoriatic skin contain dermal structures, with endothelial cells, fibroblasts, and numerous infiltrative cells that express a small amount of IL-20R1, IL-20R2, and IL-22R [19], thus affecting the results. Therefore, previous studies on skin mRNA expression may not reflect the mRNA expression of epidermal keratinocytes.

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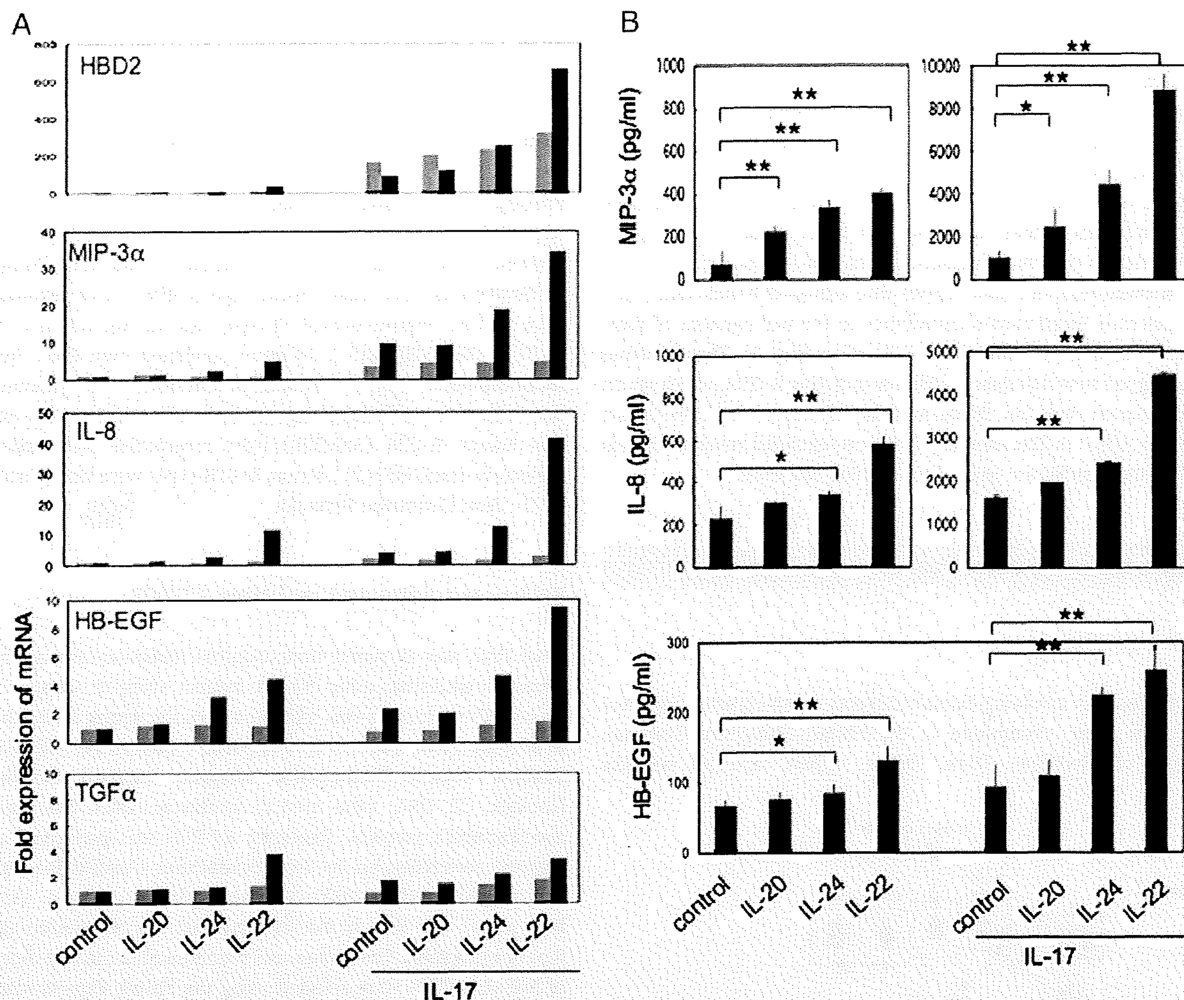

Figure 7. Effect of IL-20, IL-24, and IL-22 on cytokine production. (A) Cultured keratinocytes transduced with (black bar) or without (gray bar) IL-22R by an adenovirus vector were treated for 36 h with IL-20 (10 ng/mL), IL-24 (10 ng/mL), or IL-22 (10 ng/mL) in combination with or without IL-17 (10 ng/mL). The levels of mRNA expression were corrected using cyclophilin expression relative to the untreated cells (control) level. (B) Cultured keratinocytes transduced with IL-22R by an adenovirus vector were treated with IL-20 (10 ng/mL), IL-24 (10 ng/mL), or IL-22 (10 ng/mL) for 48 h in combination with (10 ng/mL) or without IL-17. Culture media were collected, and MIP-3α and HB-EGF were measured by ELISA. The analysis was performed in triplicate. Data show mean ± SD. Data are representative of three independent experiments. *$p<0.05$; **$p<0.01$.

We tried to determine whether IL-22R expression was increased in psoriasis epidermis, as compared with normal epidermis, by immunostaining. However, as Nograles *et al.* showed positive staining of IL-22R in normal epidermis [5], we stained all of the samples simultaneously and compared the fluorescence intensity. This showed that the IL-22R expression was clearly increased in the psoriasis lesion epidermis in four of six psoriasis skin samples, in comparison with normal epidermis. Significant staining for IL-22R was primarily observed in the upper layer, consistent with a previous report by Romer *et al.* [25]. Their study investigated IL-22R mRNA expression in normal and psoriatic skin by *in situ* hybridization and detected expression in the superficial part of the psoriatic epidermis.

Increased IL-22R expression augmented signal transduction by IL-22 and strengthened the coordinate effects of IL-22 and IL-17. Alternatively, IL-22R up-regulation may increase the production of the IL-20 subfamily, HBD2, MIP-3α, IL-8, and HB-EGF, in keratinocytes after IL-17 and IL-22 stimulation. Moreover, IL-20 and IL-24 also share the IL-22R [22, 23]. We confirmed here that IL-24 was more potent than IL-20 and acted synergistically with IL-17 to produce MIP-3α, IL-8, and HB-EGF. These findings suggest that IL-24 may contribute to maintaining the psoriatic lesion.

The role of IL-19 in psoriasis, however, remains unclear. IL-19 was also markedly elevated in epidermal psoriatic lesions and was produced by keratinocytes stimulated with IL-17 and IL-22 in combination *in vitro*. However, IL-19 signals through IL-20R1/IL-20R2, not through IL-22R. Several earlier reports suggested that IL-19 augmented Th2 cytokine secretion and played a role in the pathogenesis of allergic disease [38–40]. A more recent study reported a significant increase of IL-19 expression in the airway epithelia of patients with asthma *versus* that in patients with

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other diseases and stated that airway epithelial cells could produce IL-19 when stimulated with IL-17 and IL-4/IL-13 in combination [20]. IL-20R1 expression in cultured keratinocytes was up-regulated by IL-4 and down-regulated by IFN-γ ([17] and our observations). These findings suggest that IL-19 may act in Th2 diseases, rather than in Th1 or Th17 diseases. Furthermore, one may speculate that not just the amount of IL-20 subfamily cytokines, but rather the type and amount of the receptors expressed on cells may mediate the immune response.

In conclusion, our data suggest that increased IL-22R expression in epidermal keratinocytes contributes to the pathogenesis of psoriasis *via* an enhanced IL-22 and IL-24 effect (Fig. 8). These findings may suggest new therapies specifically targeting IL-22R, which act on keratinocytes, but not immune cells. However, the mechanism through which IL-22R expression was up-regulated remains unclear and further studies are needed to examine this issue.

Materials and methods

Skin samples

All procedures involving human subjects received prior approval from the ethics committee of the Ehime University Graduate School of Medicine, Toon, Ehime, Japan, and all subjects provided written informed consent. Normal human skin was obtained from plastic surgeries.

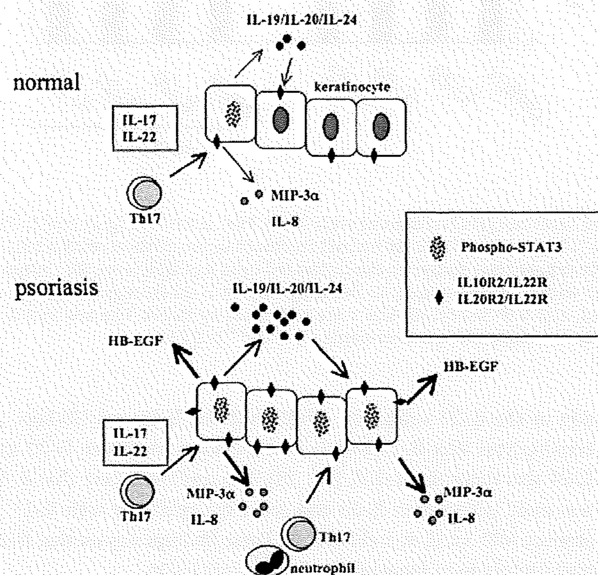

Figure 8. Role of IL-22R and the IL-20 subfamily in psoriasis. In psoriatic skin, IL-22R expression in the epidermis is elevated compared with normal skin. IL-22 and IL-17 produced by Th17 lymphocytes induce abundant expression of the IL-20 subfamily and chemokines such as MIP-3α and IL-8, and HB-EGF compared with normal skin. MIP-3α and IL-8 recruit further Th17 lymphocytes and neutrophils, respectively. HB-EGF may induce epidermal hyperproliferation. Surrounding keratinocytes treated with IL-20 and IL-24 strongly responded to IL-17 and produced more MIP-3α, IL-8, and HB-EGF. Phosphorylation of STAT3 was observed in the nuclei of keratinocytes stimulated with IL-22 or IL-20 subfamily members.

Cell culture and adenoviral vectors

Primary normal human keratinocytes were cultured under serum-free conditions as previously described [41], and treated with various cytokines. TNF-α and IL-1α were generous gifts from Dainippon Sumitomo Pharmaceutical (Osaka, Japan). IFN-γ was a generous gift from Otsuka Pharmaceutical (Tokyo, Japan). Recombinant IL-17, IL-19, IL-20, IL-22, and IL-24 were purchased from R&D Systems (Minneapolis, MN, USA). The optimal concentration of cytokines was determined in preliminary experiments. Adenoviral vectors expressing human IL-22R (AxIL22R) were constructed and purified as previously described [42]. Human IL-22R cDNA were kindly provided by Dr. Jean-Christophe Renauld.

RNA preparation and real-time RT-PCR

Total RNA was prepared from cultured keratinocytes and freshly prepared epidermis using Isogen reagent (Nippon Gene, Tokyo, Japan). The epidermis was separated from the dermis by incubation at 60°C for 1 min, followed by immediate cooling in ice-cold PBS [9]. TaqMan Reverse Transcription Reagents (Applied BioSystems, Norwalk, CT, USA) were used to synthesize cDNA, following the manufacturer's protocol. Real-time RT-PCR was performed using reagents recommended by the manufacturer (Applied Biosystems) and the ABI PRISM 7900 Sequence Detection system (Applied Biosystems). The level of mRNA expression was normalized against that of cyclophilin. The primer and probes used in the real-time PCR of MIP-3α mRNA were selected using the Primer Express software (Applied Biosystems); they were forward, 5′-AAGCCATTGAGACACCAGCTG-3′; reverse, 5′-ACCTCGCTCTGACTCCCAGA-3′; and 6FAM-ACGTCACGCGCCGGAGCAT-TAMRA. The primer and probes used for IL-20R2 were described previously [18]. TaqMan gene expression assays for the remaining genes were purchased from Applied Biosystems.

Western blot analysis

Western blot analysis was performed using a Vistra ECF kit (Amersham, Buckinghamshire, UK) and a FluoroImager (Molecular Dynamics, Sunnyvale, CA, USA). Anti-IL-20R2 and anti-IL-22R goat antibodies were purchased from R&D systems. Anti-STAT3 and anti-phospho-STAT3 mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Flow cytometry analysis

Cultured keratinocytes were treated with Cell Dissociation Buffer (Invitrogen, Tokyo, Japan) to prepare single-cell suspensions. Cells

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were incubated with PE-conjugated anti-IL-22R antibody (R&D systems) or FITC-conjugated control isotype antibody (BioLegend, San Diego, CA, USA). To exclude dead cells, 7-AAD (Invitrogen) was added just before analysis. Analysis was done with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Immunofluorescence microscopy

For immunostaining, four normal and six psoriasis lesion skin samples were used. Frozen skin sections (5 μm) embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) were fixed in cold acetone for 5 min and then reacted with the primary antibodies for 1 h at room temperature. After washing with PBS, the sections were incubated with donkey anti-mouse or anti-goat antibodies labeled with Alexa Fluor 488 (Molecular Probes) for 30 min at room temperature. Fluorescence was observed under a fluorescence microscope (Nikon, Tokyo, Japan). The following primary antibodies were used: goat anti-IL-19 antibody (AF1035, R&D Systems), mouse anti-IL-20 antibody (Abcam, Tokyo, Japan), mouse anti-IL-24 antibody (MAB 19651, R&D Systems), and goat anti-IL-22R antibody (AF2770, R&D Systems). Immunoreactivity of the anti-IL-22R antibody was confirmed in cultured keratinocytes infected with an adenoviral vector carrying the IL-22R gene (data not shown).

ELISA

The ELISA development kits for IL-24, MIP-3α, IL-8, and HB-EGF were purchased from R&D Systems and the kit for IL-20 was obtained from PeproTech (London, UK). They were used according to the manufacturers' protocol. ELISA for IL-19 was developed by using rabbit anti-IL-19 and biotinylated rabbit anti-IL-19 antibodies (PeproTech). Optical density was measured using an Immuno Mini NJ-2300 microplate reader (Nalge Nunc International K.K., Tokyo, Japan). The detection limits were 16 pg/mL, except for the IL-24 ELISA, which was 250 pg/mL.

Statistical analyses

Statistical significance was determined using Student's paired *t*-tests. Differences were deemed to be statistically significant at $p<0.05$.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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