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# The Human β-Defensins (-1, -2, -3, -4) and Cathelicidin LL-37 Induce IL-18 Secretion through p38 and ERK MAPK Activation in Primary Human Keratinocytes[1]

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# The Human β-Defensins (-1, -2, -3, -4) and Cathelicidin LL-37 Induce IL-18 Secretion through p38 and ERK MAPK Activation in Primary Human Keratinocytes[1]

**François Niyonsaba,[2]* Hiroko Ushio,* Isao Nagaoka,† Ko Okumura,‡ and Hideoki Ogawa§**

**In addition to its physical barrier against invading microorganisms, the skin produces antimicrobial peptides, human β-defensins (hBDs) and cathelicidin LL-37, that participate in the innate host defense. Because IL-18 is produced by keratinocytes and involved in skin diseases in which hBDs and LL-37 are highly expressed, we hypothesized that these peptides would activate keratinocytes to secrete IL-18. We found that hBD-2, -3, and -4 and LL-37, but not hBD-1, activated normal human keratinocytes to secrete IL-18; this secretion reached peak strength at 3 h. In addition, the combination of peptides resulted in a synergistic effect on IL-18 secretion. We also revealed that hBD-2, -3, and -4 and LL-37 increased IL-18 mRNA expression, and that IL-18 secretion was more enhanced in keratinocytes differentiated in vitro with high $Ca^{2+}$-containing medium. Furthermore, because IL-18 secretion induced by hBDs and LL-37 could not be suppressed by caspase-1 or caspase family inhibitors, and because these peptides failed to increase caspase-1 activity, we suggest that hBD- and LL-37-induced IL-18 secretion is probably via a caspase-1-independent pathway. To determine the molecular mechanism involved, we demonstrated that IL-18 secretion was through p38 and ERK1/2 MAPK pathways, because the inhibitors of p38 and ERK1/2, but not JNK, almost completely nullified IL-18 secretion. Moreover, hBD-2, -3, and -4 and LL-37 could induce the phosphorylation of p38 and ERK1/2, but not JNK. Thus, the ability of hBDs and LL-37 to induce IL-18 secretion by keratinocytes provides a new mechanism for these peptides in innate immunity and an understanding of their role in the pathogenesis of skin disorders.** ***The Journal of Immunology,*** **2005, 175: 1776–1784.**

The skin's natural properties to kill invading microorganisms have been attributed to several antimicrobial agents, including endogenous antimicrobial peptides. In humans, more than a dozen of these peptides have been identified; they comprise histatins, granulysin, lactoferricin, α- and β-defensins, and cathelicidin LL-37 (reviewed in Ref. 1). Among these antibacterial peptides, two major classes of human defensins and cathelicidin kill a broad spectrum of pathogens, including Gram-positive and Gram-negative bacteria, fungi, and viruses (1).

The α- and β-defensins differ from one another in the spacing and connectivity of their characteristic six-cysteine residues. Additionally, α-defensins are found in neutrophils as well as Paneth cells of the small intestine, whereas human β-defensins (hBD)[3] are products of epithelial tissues (1). To date, six hBDs, hBD-1 through -6, have been identified in human tissues. hBD-1 is constitutively produced by various epithelial tissues, including urogenital and respiratory tracts and skin (2). hBD-2 was originally isolated from extracts of lesional scales from psoriatic skin and is mainly present in skin and respiratory as well as gastrointestinal tracts (3). It is inducibly expressed in inflamed skin lesions upon treatment with LPS and cytokines such as TNF-α and IL-1β (3–5). The third β-defensin, termed hBD-3, was also isolated from human lesional psoriatic scales and is expressed in epithelial and nonepithelial tissues, such as heart, liver, and skeletal muscle (6). The expression of hBD-3 is inducible in airway epithelium and gingival keratinocytes upon IFN-γ stimulation. hBD-4 was discovered by screening the human genome database and is up-regulated by infection with Gram-positive and Gram-negative bacteria in response to PMA, but not in response to other inflammatory factors that up-regulate the expression of hBD-2 or hBD-3 (7). In addition to their antibacterial activity, hBD-1, -2, -3, and -4 have been implicated in several cellular activities, such as production of proinflammatory mediators and chemotaxis of dendritic cells, T cells, mast cells, and neutrophils (8–11). The newly discovered hBD-5 and hBD-6 are found in epididymis; however, their antibacterial or other cellular activities are not yet well known (12).

Although ~30 cathelicidin members have been found in mammalians, only one cathelicidin, named human cationic antibacterial protein of 18 kDa (hCAP18), has been identified in humans to date. Its mature antibacterial peptide, LL-37, was first identified in neutrophils (13) and was later shown to be expressed in various squamous epithelia (14), surface epithelial cells of the conducting airways, and serous and mucous cells of the submucosal glands (15); by keratinocytes in inflamed skin (16); and by specific lymphocyte and monocyte populations (17). LL-37 displays antimicrobial activity against a broad spectrum of pathogenic microorganisms and neutralizes LPS bioactivity (18). The crucial role of cathelicidins in host defense against infection has been clarified in a recent report demonstrating that mice deficient in the murine cathelin-related antimicrobial peptide suffer from more severe bacterial skin infections than do wild-type mice (19). Besides the killing properties, other activities for LL-37 have also been described, including stimulation of mast cell degranulation and chemotactic

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[3] Abbreviations used in this paper: hBD, human β-defensin; DNCB, 1-chloro-2,4-dinitrobenzene; LDH, lactate dehydrogenase.



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activity toward neutrophils, monocytes, mast cells, and macrophages (9, 20–22).

IL-18, an IFN-$\gamma$ inducer, is a proinflammatory cytokine intracellularly produced from a biologically inactive precursor, pro-IL-18. Pro-IL-18 is proteolytically activated to mature IL-18 in the cytoplasm, and caspase-1 is the protease responsible for activation of IL-18, in a similar manner as the activation of IL-1$\beta$ (23, 24). Activated IL-18 acts together with IL-12 as a potent inducer of IFN-$\gamma$ and GM-CSF production by T cells (25, 26). It has been reported that IL-18 is expressed in a wide range of cells, including keratinocytes, Kupffer cells, macrophages, T cells, B cells, osteoblasts, dendritic cells, and astrocytes (reviewed in Ref. 27). IL-18 was originally considered a Th1 cytokine, acting through its ability to induce IFN-$\gamma$ production, and its expression is highly enhanced in psoriasis. However, recent studies have indicated a more complicated pleiotropic role for IL-18 than simply induction of IFN-$\gamma$ production, and IL-18 was reported to induce the production of IgE and Th2 cytokines, including IL-4, IL-5, IL-10, and IL-13 (reviewed in Ref. 27). Indeed, IL-18 was also recently found to be associated with the pathogenesis and severity of atopic dermatitis and asthma (28).

Although hBD-1, -2, -3, and -4 and LL-37 are expressed in various epithelial tissues, including skin, their activities in epithelial cells, such as keratinocytes, are not well known. Because hBD-1, -2, -3, and -4 and LL-37 are produced by keratinocytes and involved in skin diseases such as psoriasis and chronic atopic dermatitis (1, 16, 29), and because IL-18, a product of several cells, including keratinocytes, plays a crucial role in the above-mentioned skin diseases, we hypothesized that hBDs and LL-37 could activate human keratinocytes to secrete IL-18. To this end, individual and synergistic effects of hBD-1, -2, -3, and -4 and LL-37 on IL-18 secretion were evaluated. Given the key role of MAPK in the production of cytokines and/or chemokines, we investigated the effects of MAPK inhibitors on IL-18 secretion and explored the activation of p38, ERK1/2, and JNK by hBD-1, -2, -3, and -4 and LL-37. Our results indicate that in addition to their antimicrobial and chemotactic activities, epithelial cell-derived antibacterial peptides hBDs and LL-37 may also contribute to innate or adaptive immunity trough the activation of keratinocytes to secrete the proinflammatory cytokine IL-18.

## Materials and Methods

### *Reagents*

Antibacterial peptides hBD-1, hBD-2, hBD-3, hBD-4, a caspase-1 inhibitor Ac-YVKD-CHO, and a caspase family inhibitor z-VAD-FMK were obtained from Peptide Institute. LL-37 (L[1]LGDFFRKSKEKIG KEFKRIVQRIKDFLRNLVPRTES[37]) was synthesized by the solid phase method on a peptide synthesizer (model PSSM-8; Shimadzu) by F-moc chemistry, and the molecular mass was confirmed on a mass spectrometer (model TSQ 700; Thermo Quest Finnigan). Rabbit polyclonal anti-phosphorylated p38, ERK1/2, and JNK Abs and total p38, ERK1/2, and JNK Abs were purchased from Cell Signaling Technology. The inhibitors SB203580 (Sigma-Aldrich), PD98059 (Cell Signaling Technology), and SP600125 (Calbiochem) were used to study the MAPK pathways involved in the activation of keratinocytes. 1-Chloro-2,4-dinitrobenzene (DNCB) and nigericin were purchased from Sigma-Aldrich.

### *Cell culture and stimulation*

Second-passage neonatal foreskin normal human epidermal keratinocytes were purchased from Kurabo Industries and cultured in serum-free keratinocyte growth medium, HuMedia-KG2 (Kurabo Industries), containing human epidermal growth factor (0.1 ng/ml), insulin (10 $\mu$g/ml), hydrocortisone (0.5 $\mu$g/ml), gentamicin (50 $\mu$g/ml), amphotericin B (50 ng/ml), and bovine brain pituitary extract (0.4%, v/v) at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. The $Ca^{2+}$ concentration in HuMedia-KG2 was 0.15 mM. Cells were passaged at 60–70% confluence to avoid differentiation, and the experiments were conducted with subconfluent cells at passage 3 or 4 in the proliferative phase at 60–80% confluence. Induction of keratinocyte differentiation was achieved by culturing the keratinocytes for 48 h in the culture medium containing 1.35 mM $Ca^{2+}$ as previously described (30). Keratinocyte differentiation was confirmed by morphological change using microscopy. For stimulation, keratinocytes were cultured in 12-well tissue culture plates, and for subsequent RNA or protein isolation, cells were cultured in six-well plates. After removal of growth medium, the cells were washed twice with PBS before being cultured in HuMedia-KG2 supplemented with only antibiotics for 24 h. The keratinocytes were subsequently stimulated with various doses of antibacterial peptides (hBD-1, -2, -3, and -4 and LL-37) or with 0.001% DNCB for the indicated time periods. In some experiments, keratinocytes were pretreated with different inhibitors for 1 h before stimulation with hBD-1, -2, -3, and -4 and LL-37.

THP-1 monocytic cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich) and 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 10 $\mu$M MEM nonessential amino acids in a humidified atmosphere at 37°C in 5% $CO_2$. Cells were passaged every 3 days. For experiments, cells were centrifuged for 4 min at 500 $\times$ *g* at 4°C, resuspended in the above-described medium, plated into 24-well plates ($1 \times 10^6$ cells/well), and incubated with 20 $\mu$M nigericin for 2 h as reported previously (31).

### *ELISA*

The amounts of IL-18, IL-20, and IL-8 in the cell-free supernatants from stimulated or nonstimulated keratinocyte culture were collected at the indicated time points after treatment and stored at −20°C until used for ELISA according to the manufacturer's instructions. The ELISA kit specific for the mature and active form of IL-18 was purchased from Medical and Biological Laboratories. The IL-20 ELISA development kit was obtained from PeproTech EC, and the ELISA kit for IL-8 was purchased from R&D Systems.

### *Measurement of lactate dehydrogenase (LDH) activity*

For quantification of the cytotoxicity of antibacterial peptides to keratinocytes, LDH activity in the keratinocyte culture supernatants prepared for IL-18 ELISA was measured using a commercially available Cytotoxicity Detection Kit-LDH (Roche) according to the manufacturer's instructions. The percentage of LDH activity in the supernatants was calculated as ((experimental value − LDH activity released from untreated cells)/(maximum releasable LDH activity in the cells by 1% Triton X-100 − LDH activity released from untreated cells)) $\times$ 100.

### *Caspase-1 enzymatic activity assay*

Caspase-1 enzymatic activity was determined using a caspase-1 colorimetric assay kit (R&D Systems) according to the manufacturer's instructions. Briefly, $2 \times 10^6$ cells were lysed in 50 $\mu$l of cold lysis buffer. The cell lysates were incubated on ice for 10 min and centrifuged at 10,000 $\times$ *g* for 1 min, then the supernatants were collected. A volume of 50 $\mu$l of cell lysate was added to 2.5 mM DTT and 50 $\mu$l of caspase reaction buffer. Each sample was added with 40 $\mu$M caspase-1 substrate WEHD-pNA, followed by a 2-h incubation at 37°C. The enzymatic activity of caspase-1 was monitored on a microplate reader using a 405-nm wavelength.

### *Western blot analysis*

After stimulation of keratinocytes for the indicated time periods, the supernatants were removed, and cells were collected using a cell scraper. The lysates were obtained by lysing cells in 40 $\mu$l of lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.02% $NaN_3$, 0.1% SDS, 1% Nonidet P-40, containing 1 $\mu$M PMSF, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin A, and 50 $\mu$g/ml aprotinin). The amounts of total protein were quantitated using a bicinchoninic acid assay (Pierce), and equal amounts of total protein were subjected to 15% SDS-PAGE. Nonspecific binding sites were blocked, and the blots were incubated with polyclonal Abs against phosphorylated p38, ERK1/2 or JNK, and total p38, ERK1/2, or JNK overnight, according to the manufacturer's instructions. The membrane was developed with an ECL detection kit (Amersham Biosciences).

### *RNA extraction and real-time quantitative PCR*

Total RNA was extracted from keratinocytes using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 $\mu$g of total RNA with oligo$(dT)_{12-18}$ primers using SuperScript II RNase $H^-$ reverse transcriptase (Invitrogen Life Technologies). Furthermore, to remove RNA complementary to the cDNA, 2 U of RNase H (Invitrogen Life Technologies) was added to the reaction mixtures, which were then incubated at 37°C for 20

min. Real-time quantitative PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). Amplification and detection of IL-18 mRNA were analyzed by a real-time PCR system (model 7500; Applied Biosystems), according to the manufacturer's instructions. The IL-18 primer/probe set was obtained from Applied Biosystems (Assays-on-Demand, Hs0015557_m1). PCR was performed as follows: one initial step at 50°C for 2 min and 95°C for 10 min was followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. To standardize IL-18 mRNA concentrations, transcript levels of the housekeeping gene GAPDH were determined in parallel for each sample, and relative IL-18 transcript levels were corrected by normalization based on the GAPDH transcript levels. For GAPDH, we used a predeveloped assay (Applied Biosystems). All real-time PCRs were performed in triplicate. Changes in gene expression were reported as fold increases relative to untreated controls (medium alone).

### *Statistical analysis*

The statistical analysis was performed using Student's $t$ test, and $p < 0.05$ was considered significant. The results are shown as the mean ± SD.

## Results

### *Antibacterial peptides hBD-2, -3, and -4 and LL-37 induce IL-18 protein release*

To study the effects of hBDs and LL-37 on IL-18 protein release in skin, keratinocytes were stimulated with hBD-1, -2, -3, and -4 and LL-37 for different time periods, and IL-18 release in cell-free supernatants was determined by IL-18-specific ELISA. Low baseline production of IL-18 protein was almost consistently detected by ELISA. As shown in Fig. 1*A*, the incubation of keratinocytes with 10 μg/ml hBD-2, -3, and -4 and LL-37 for 1–48 h resulted in a significant induction of IL-18 secretion, reaching peak strength within 3 h of peptide exposure before decreasing gradually. Forty-eight hours after stimulation with peptides, secretion had returned to baseline. In contrast, hBD-1 did not notably increase IL-18 secretion. We confirmed that the hBD-1 used was biologically active, because it could effectively kill *Escherichia coli* and *Staphylococcus aureus* (32). The contact sensitizer DNCB was used as a positive control (33).

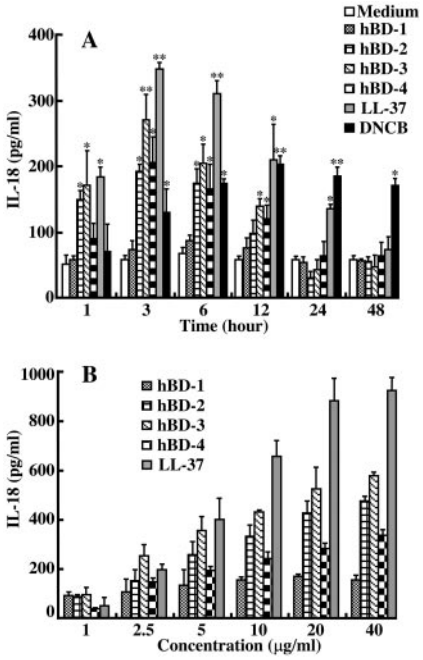


**FIGURE 1.** hBDs and LL-37 induce IL-18 release from human keratinocytes. *A*, Normal human keratinocytes were stimulated with 10 μg/ml hBD-1, -2, -3, and -4 and LL-37 or 0.001% DNCB for 1–48 h, and the concentration of IL-18 in the supernatants was determined by ELISA. Values are compared between stimulated and nonstimulated cells (Medium). *, $p < 0.05$; **, $p < 0.01$. Each bar represents the mean ± SD of five separate experiments. *B*, The dose-dependent experiments were performed by incubating keratinocytes with various doses of hBD-1, -2, -3, and -4 and LL-37 (1–40 μg/ml), and the amounts of IL-18 secretion in the supernatants were determined by ELISA. In contrast to hBD-2, -3, and -4 and LL-37, which induced significant amounts of IL-18 in a dose-dependent fashion, hBD-1 could not activate keratinocytes (compared with medium alone, 79.35 ± 11. 28 pg/ml). Each bar represents the mean ± SD of five to seven separate experiments.

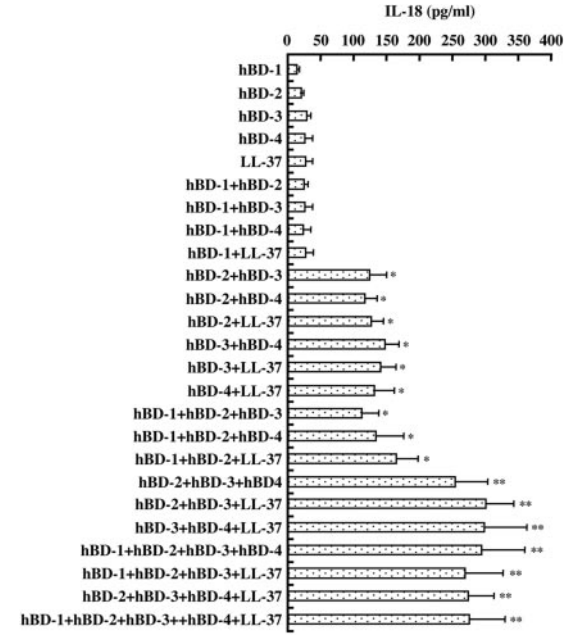


**FIGURE 2.** Synergistic effect of hBDs and LL-37 on IL-18 secretion by keratinocytes. Human keratinocytes were incubated alone or in combination with 5 μg/ml hBD-1, 1 μg/ml hBD-2, 0.5 μg/ml hBD-3, 1 μg/ml hBD-4, or 0.5 μg/ml LL-37. Then, the levels of IL-18 secretion in the supernatants were determined by ELISA. Data represent the mean ± SD of five separate experiments. The amounts of IL-18 induced by each combination and the lowest value of peptide used alone were compared. *, $p < 0.05$; **, $p < 0.01$.

By incubating keratinocytes for 3 h with 1–40 μg/ml of each peptide, we observed that hBD-2, -3, and -4 and LL-37 could induce IL-18 secretion in a dose-dependent fashion (Fig. 1*B*). No LDH activity was detected in the supernatants after stimulation with peptides (1–40 μg/ml) for 1–48 h, indicating that these peptides at the concentrations used were not toxic to keratinocytes (data not shown). However, 80–100 μg/ml hBD-2, -3, and -4 and LL-37 induced significant amounts of LDH release (≥30%; data not shown).

### *hBDs and LL-37 synergistically induce IL-18 secretion by human keratinocytes*

We and other investigators have reported that human antibacterial peptides, including hBDs and LL-37, act synergistically to enhance their killing effect against microorganisms (29, 32, 34, 35). Thus, we thought that hBDs and LL-37 could also synergize to increase their activity of inducing IL-18 secretion by keratinocytes.

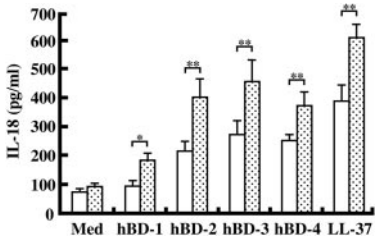


**FIGURE 3.** Differentiated keratinocytes secrete more IL-18 in response to hBDs and LL-37. Human keratinocytes were cultured in the presence of 0.15 mM $Ca^{2+}$ (□) or 1.35 mM $Ca^{2+}$ (▨) for 48 h, then stimulated with 30 $\mu$g/ml hBD-1, -2, -3, or -4 or LL-37 for 3 h. The IL-18 concentration in the culture supernatants was measured by ELISA. Data are the mean ± SD of four independent experiments. *, $p < 0.05$; **, $p < 0.01$ (significantly different from the mean value of the corresponding control response).

In preliminary experiments, hBD-1, -2, -3, and -4 and LL-37 were titrated to determine a concentration at which they did not have a significant effect on IL-18 secretion alone. We found that the combinations of two to five peptides comprising hBD-1, -2, -3, or -4 or LL-37 had a synergistic effect on IL-18 secretion by keratinocytes (Fig. 2). These results suggest that epithelial cell-derived hBDs and LL-37 work cooperatively to activate human keratinocytes.

### *Differentiated human keratinocytes produce more IL-18 upon stimulation with hBDs and LL-37*

It was reported that culturing human keratinocytes in the presence of high $Ca^{2+}$ (1.2–1.7 mM) resulted in keratinocyte differentiation (30, 36). We thus investigated the effects of hBD-1, -2, -3, and -4 and LL-37 on IL-18 secretion by differentiated keratinocytes cultured in 1.35 mM $Ca^{2+}$-containing medium. We found that differentiated keratinocytes produced significantly enhanced amounts of IL-18 upon stimulation with hBD-1, -2, -3, and -4 and LL-37 (30 $\mu$g/ml each peptide) compared with the undifferentiated keratinocytes that were maintained in the culture medium containing 0.15 mM $Ca^{2+}$. Interestingly, even hBD-1 that was unable to induce IL-18 secretion in undifferentiated keratinocytes could activate differentiated keratinocytes to secrete significant amounts of IL-18 (Fig. 3). Thus, the ability of hBDs and LL-37 to induce IL-18 secretion was further enhanced in differentiated human keratinocytes.



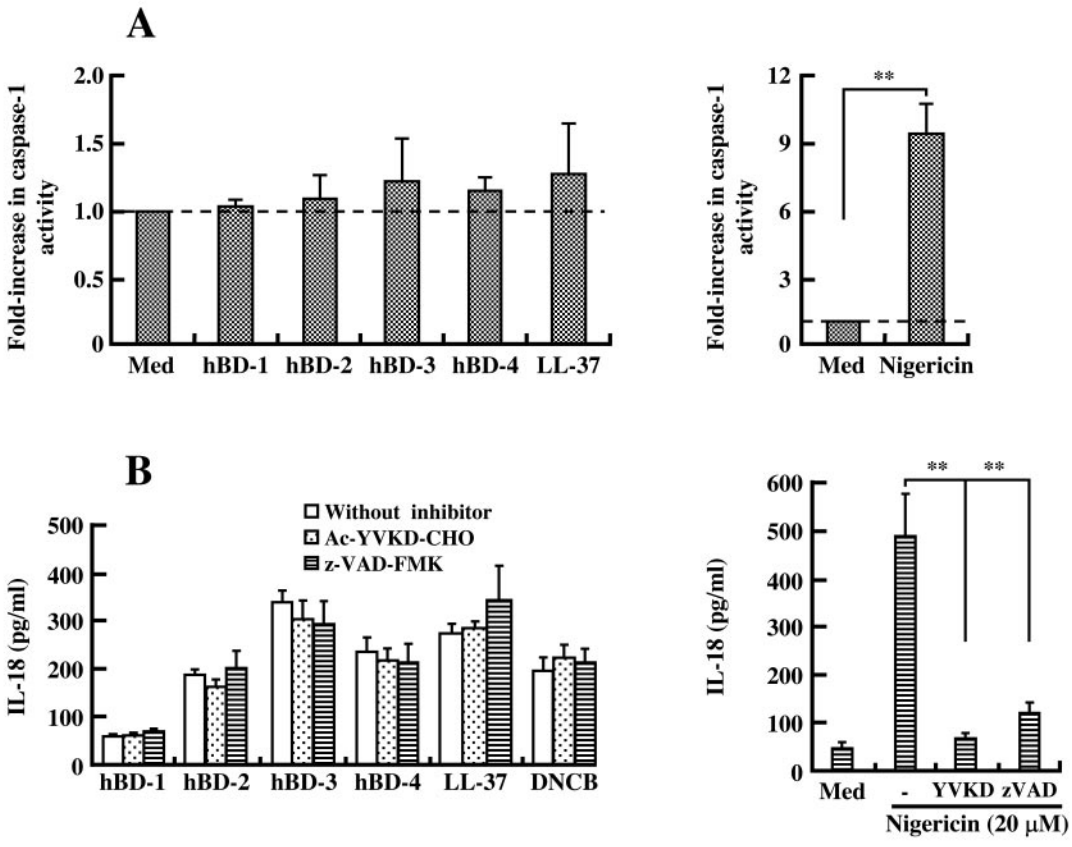


**FIGURE 4.** hBD- and LL-37-induced IL-18 secretion is caspase-1 independent. *A*, Human keratinocytes were stimulated with 40 $\mu$g/ml hBD-1, -2, -3, or -4 or LL-37 for 3 h (*left panel*), and THP-1 cells were stimulated with 20 $\mu$M nigericin for 2 h (*right panel*). Caspase-1 enzymatic activity was assayed with a caspase-1 colorimetric assay kit according to the manufacturer's instructions. The data normalized to the negative control (medium alone) are shown as fold increases in caspase activity and are the mean ± SD of three to five separate experiments. Values are compared between stimulated and non-stimulated (Med, medium) cells. **, $p < 0.01$. *B*, Keratinocytes (*left panel*) or THP-1 cells (*right panel*) were pretreated with 100 $\mu$M of the caspase-1 inhibitor Ac-YVKD-CHO or 100 $\mu$M of the caspase family inhibitor z-VAD-FMK for 1 h, then challenged with 40 $\mu$g/ml hBD-1, -2, -3, or -4; LL-37; or 0.001% DNCB for 3 h for keratinocytes or with 20 $\mu$M nigericin for 2 h for THP-1 cells. The IL-18 concentration in the culture supernatants was measured by ELISA. Values are the mean ± SD of three or four independent experiments, and comparisons are made between the presence and the absence of each inhibitor. **, $p < 0.01$.

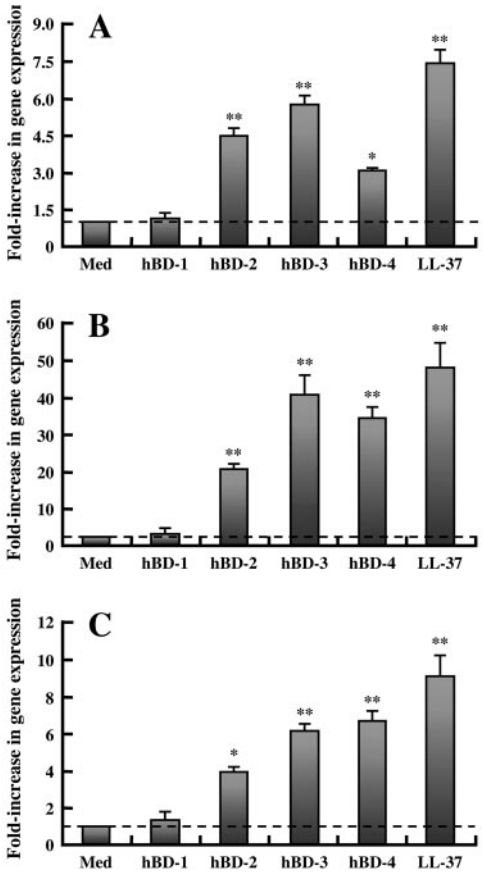


**FIGURE 5.** hBDs and LL-37 increase the gene expression of IL-18 in human keratinocytes. Human keratinocytes were stimulated with 40 $\mu$g/ml hBD-1, -2, -3, or -4 or LL-37 for 1 h (*A*), 3 h (*B*), or 6 h (*C*). After incubation, RNA was extracted and converted into cDNA, and real-time PCR was performed to analyze the changes in IL-18 gene expression. Each bar shows the mean ± SD from four independent experiments; each experiment was performed in triplicate. Values represent fold increases in gene expression above cells incubated with medium alone (Med). *, $p < 0.01$; **, $p < 0.001$.

### *Secretion of IL-18 induced by hBDs and LL-37 is caspase-1-independent*

Because caspase-1 is required for the cleavage of pro-IL-18 leading to the generation of mature IL-18, we next investigated the molecular mechanism underlying hBD- and LL-37-induced IL-18

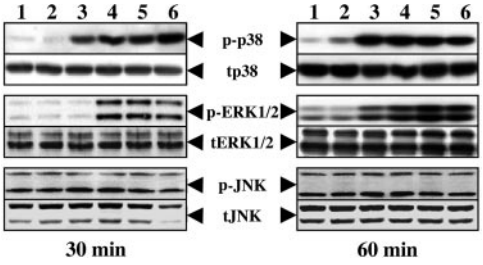


**FIGURE 6.** hBDs and LL-37 induce the activation of p38 and ERK, but not JNK MAPK. Keratinocytes were stimulated with 40 $\mu$g/ml hBD-1, -2, -3, or -4 or LL-37 for 30 or 60 min, and phosphorylated p38 (P-p38), ERK1/2 (P-ERK1/2), JNK (P-JNK), and total p38 (tp38), ERK1/2 (tERK1/2), or JNK (tJNK) levels in cellular lysates were determined by Western blot analysis. *Lane 1*, Medium alone; *lane 2*, hBD-1; *lane 3*, hBD-2; *lane 4*, hBD-3; *lane 5*, hBD-4; *lane 6*, LL-37. Shown is one representative of five repeated independent experiments with similar results.

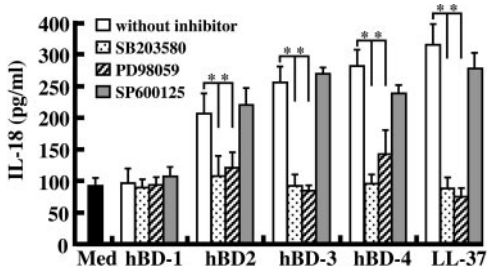


**FIGURE 7.** hBDs and LL-37 induce the secretion of IL-18 through p38 and ERK, but not JNK MAPK, pathways. Keratinocytes were preincubated with 20 $\mu$M of the p38 kinase-specific inhibitor SB203580, 20 $\mu$M of the ERK1/2-specific inhibitor PD98059, or 100 $\mu$M of the JNK-specific inhibitor SP600125 for 1 h, then the cells were exposed for 3 h to 40 $\mu$g/ml hBD-1, -2, -3, or -4 or LL-37. As a control, cells were incubated with medium alone (Med; ■). The IL-18 concentration in the culture supernatants was measured by ELISA. Values are the mean ± SD of four separate experiments. *, $p < 0.05$. Values are compared between the presence and the absence of each MAPK inhibitor.

secretion by performing the caspase-1 colorimetric assay. Cells were stimulated with 40 $\mu$g/ml hBD-1, -2, -3, or -4 or LL-37 for 3 h, and caspase-1 enzymatic activity was determined using a specific substrate, WEHD-pNA. The results revealed that neither hBDs nor LL-37 could increase caspase-1 activity (Fig. 4*A*, *left panel*). The stimulation of keratinocytes with peptides for 30 min, 1 h, 6 h, or 12 h did not further increase the activity of caspase-1 (data not shown). The caspase-1 colorimetric assay used could detect the increased (9-fold) caspase-1 activity in THP-1 monocytic cells stimulated by nigericin, a potassium ionophore known to induce IL-18 production through the activation of caspase-1 (31) (Fig. 4*A*, *right panel*).

Next, the involvement of caspase-1 in IL-18 secretion by keratinocytes was examined by pretreating keratinocytes for 1 h with the caspase-1-specific inhibitor Ac-YVKD-CHO or with the caspase family inhibitor z-VAD-FMK before stimulation with hBD-1, -2, -3, or -4 or LL-37. We observed that neither Ac-YVKD-CHO nor z-VAD-FMK (100 $\mu$M each) could suppress the secretion of IL-18 induced by hBDs and LL-37 (Fig. 4*B*, *left panel*). These inhibitors were actually active because they could almost completely suppress IL-18 production by nigericin-stimulated THP-1 cells (Fig. 4*B*, *right panel*). These findings suggest that hBDs and LL-37 are involved in IL-18 secretion by keratinocytes probably via a caspase-1-independent pathway.

### *hBD-2, -3, and -4 and LL-37 induce expression of IL-18 mRNA*

We next sought to determine whether hBDs and LL-37 have an effect on the mRNA expression of IL-18. Keratinocytes were incubated with 40 $\mu$g/ml hBD-1, -2, -3, and -4 and LL-37 for the indicated time periods, and the gene expression of IL-18 was analyzed using real-time PCR. As shown in Fig. 5, exposure of keratinocytes to hBD-2, -3, and -4 and LL-37 rapidly induced the expression of IL-18 mRNA. Time-course experiments demonstrated that IL-18 mRNA signals were significantly induced within 1 h of hBD-2, -3, and -4 and LL-37, but not hBD-1, stimulation (2.56- to 7.09-fold increases; Fig. 5*A*), reaching peak strength 3 h after stimulation (20.06- to 46.37-fold increases; Fig. 5*B*), and then decreasing after 6 h of incubation (3.74- to 8.79-fold increases; Fig. 5*C*).

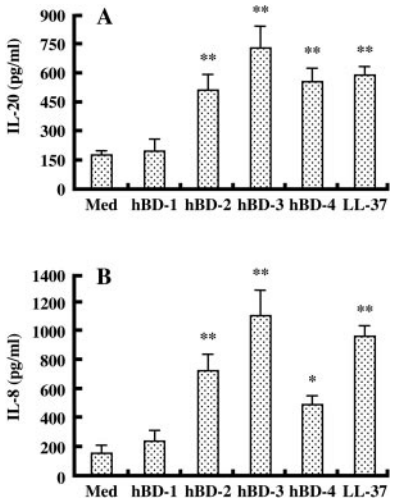


**FIGURE 8.** hBDs and LL-37 induce IL-20 and IL-8 production by human keratinocytes. Keratinocytes were stimulated with 40 $\mu$g/ml hBD-1, -2, -3, and -4 and LL-37 for 24 h for IL-20 (*A*) or for 48 h for IL-8 (*B*), and the concentrations of IL-20 and IL-8 in the supernatants were determined by ELISA. Values are compared between stimulated and nonstimulated cells (Med, medium). *, $p < 0.05$; **, $p < 0.01$. Each bar represents the mean ± SD of four separate experiments.

### *hBD-2, -3, and -4 and LL-37 induce phosphorylation of p38 and ERK1/2 in human keratinocytes*

Because LL-37 has been recently shown to activate MAPK pathways in monocytes and airway epithelial cells (37, 38), we explored the mechanisms by which hBDs and LL-37 induce IL-18 secretion from human keratinocytes by investigating the effects of these peptides on p38, ERK1/2, or JNK phosphorylation in keratinocytes. Cells were stimulated with hBD-1, -2, -3, and -4 and LL-37 for 30 or 60 min, and both phosphorylated and total p38, ERK1/2, or JNK levels were determined using Western blot analysis. As shown in Fig. 6, after stimulation for 30 and 60 min, hBD-2, -3, and -4 and LL-37, but not hBD-1, induced the phosphorylation of p38. Furthermore, the phosphorylation of ERK1/2 was induced by hBD-3, hBD-4, and LL-37 from 30 min after stimulation, whereas hBD-2 could activate ERK1/2 after 60 min of stimulation. In contrast, we could not detect the activation of JNK after stimulation with hBDs and LL-37 for 15, 30, 60, 90, or 120 min (Fig. 6 and data not shown). An effect of hBD-1 on p38, ERK1/2, or JNK was not observed.

### *Activation of p38 and ERK1/2, but not JNK MAPK, is necessary for the secretion of IL-18 induced by hBD-2, -3, and -4 and LL-37*

To determine whether activation of MAPKs was indeed required for hBD- and LL-37-induced IL-18 secretion, cells were incubated with a p38 kinase-specific inhibitor, SB203580 (20 $\mu$M); an ERK1/2-specific inhibitor, PD98059 (20 $\mu$M); and a JNK-specific inhibitor, SP600125 (100 $\mu$M), for 1 h before a 3-h incubation with hBD-1, -2, -3, and -4 and LL-37. IL-18 in the culture medium was assayed by ELISA. We found that IL-18 secretion induced by hBD-2, -3, and -4 and LL-37 was almost completely abolished in the presence of either p38 or ERK1/2 inhibitor, but not JNK inhibitor (Fig. 7). Various doses of JNK inhibitor could not reduce the level of IL-18 secretion (data not shown). These data demonstrate that hBDs and LL-37 induce IL-18 secretion through p38 and ERK1/2, but not JNK, pathways.

### *hBDs and LL-37 induce production of other proinflammatory mediators from keratinocytes*

To further investigate whether, in addition to IL-18, hBDs and LL-37 could induce the production of other proinflammatory mediators, we evaluated the abilities of hBD-1, -2, -3, and -4 and LL-37 to up-regulate the protein levels of IL-20 and IL-8, cytokines that have been demonstrated to be involved the pathogenesis of psoriasis (39, 40). Except hBD-1, all peptides used significantly induced the production of both IL-20 and IL-8 (Fig. 8). Supporting previous reports that the production of IL-20 and IL-8 is through MAPKs (41–43), we confirmed that hBD- and LL-37-induced IL-20 as well as IL-8 production could be significantly abolished by treatment of keratinocytes with either p38 or ERK1/2 inhibitor (data not shown).

## Discussion

In the current study we demonstrate that epithelial cell-derived hBDs and the cathelicidin LL-37 individually or synergistically induce cultured human keratinocytes to secrete IL-18. Differentiated keratinocytes produce more IL-18 upon stimulation of hBDs and LL-37, and these peptides significantly increase the expression of IL-18 mRNA. Furthermore, hBD-2, -3, and -4 and LL-37 activate the MAPK p38 and ERK1/2, but not JNK, pathways that are further required for IL-18 secretion. Thus, because IL-18 is involved in both Th1 and Th2 functions, our data suggest a new mechanism for the implication of human antibacterial peptides in innate and adaptive immunity and their roles in the pathogenesis of certain skin diseases through the secretion of IL-18.

To avoid opportunistic infections, human skin has developed in the epithelia antimicrobial peptides such as hBDs and LL-37 that play a crucial role in innate and adaptive immunity to protect the body against invading microorganisms. Besides their bactericidal activities, antimicrobial peptides have been implicated in the activation of dendritic cells, T cells, mast cells, neutrophils, and monocytes (8–11, 20–22). It is known that some antibacterial peptides are involved in the production of cytokines or chemokines. For example, human $\alpha$-defensins human neutrophil peptide-1 to -3 enhance the production of cytokines such as IL-1, IL-4, IL-6, TNF-$\alpha$, and IFN-$\gamma$ in monocytes (44) and chemokines such as MCP-1 and MIP-2 in lung epithelial cells (45). Furthermore, LL-37 stimulates monocytes and airway epithelial cells to generate IL-8, MCP-1, and MCP-3 (37, 38). The current study is the first report to demonstrate that the antibacterial peptides hBDs and LL-37 activate keratinocytes to secrete not only the proinflammatory IL-18, but also IL-20 and IL-8, which are involved in the pathogenesis of psoriasis (39, 40). Our results revealed that hBD-2, -3, and -4 and LL-37 individually and synergistically induce IL-18 secretion by keratinocytes. Although we and others have demonstrated that antibacterial peptides synergize to kill the pathogenic microorganisms, the synergistic effect of hBDs and LL-37 on normal human cell activities has not been reported previously.

There are four layers of keratinocytes in the epidermis, and each layer expresses a unique differentiation pattern. Only basal cells maintain proliferative capacity as well as the capacity to differentiate as they move upward to the skin surface (46). Previous studies have shown that basal keratinocytes constitutively express the pro-IL-18 protein, but whether pro-IL-18 is processed and released as the mature form is still controversial (33, 47, 48). Interestingly, it has been reported that the expression of IL-18 is markedly increased in suprabasal keratinocytes in psoriatic lesions (33, 49),

raising the possibility that IL-18 expression is up-regulated in differentiated keratinocytes. To confirm whether differentiated keratinocytes indeed produce more IL-18, we examined IL-18 secretion in cultured keratinocytes induced to differentiate by extracellular $Ca^{2+}$. $Ca^{2+}$ is a potent regulator of terminal differentiation for keratinocytes in vitro, because at a low extracellular $Ca^{2+}$ concentration (0.05 mM), keratinocytes maintain a proliferative, basal cell-like phenotype; increasing the $Ca^{2+}$ concentration in the medium triggers stepwise changes in gene expression similar to those seen in the epidermis (50). Parallel with this finding, the expression of hBD-1, -2, -3, and -4 has been reported to be enhanced during $Ca^{2+}$-induced differentiation of keratinocytes (36). Consistently, our results revealed that differentiated keratinocytes produce more IL-18 upon stimulation of hBDs and LL-37 compared with undifferentiated keratinocytes. Because keratinocytes are unique cells that produce hBDs in skin tissue, one can conclude that the hBD-induced IL-18 secretion by keratinocytes is via an autocrine effect. In contrast with that of hBDs, the production of LL-37 in skin can derive not only from keratinocytes, but also from neutrophils, monocytes, and lymphocytes (13–17). Because hBDs and LL-37 are abundantly expressed in psoriasis, and because the expression of IL-18 is markedly up-regulated in this disease (33, 49), it is suggested that hBDs and LL-37 are among the inducers of IL-18 secretion in psoriatic skin, where Th1 induction may exacerbate the disease state, and IL-18 is implicated in the disease progression (47). Therefore, hBD- and LL-37-induced IL-18 secretion by keratinocytes may provide new insights into the mechanism underlying the pathogenesis of skin disorders that may be primarily initiated by the activation of keratinocytes.

It is known that caspase-1 is responsible for cleavage of pro-IL-18 as well as pro-IL-1$\beta$ to generate mature IL-18 and IL-1$\beta$, respectively (23, 24). However, the current study showed that neither hBDs nor LL-37 could increase caspase-1 activity, and both caspase-1 inhibitor and caspase family inhibitor failed to suppress hBD- and LL-37-induced IL-18 secretion. Moreover, the finding that hBDs and LL-37 could not induce IL-1$\beta$ production (data not shown) led us to conclude that hBD- and LL-37-induced IL-18 secretion is unlikely to be mediated through the caspase-1 pathway. Our suggestion is consistent with that by Nakano et al. (51), who showed that caspase-1 inhibitor did not suppress IL-18 secretion from *S. aureus* protein A-stimulated mouse keratinocytes, and that caspase-1 was not involved in IL-18 secretion by keratinocytes, because caspase-1-deficient keratinocytes could secrete similar amounts of IL-18 as wild-type keratinocytes. To date, the mechanism of IL-18 secretion by keratinocytes remains unclear. Although it has been suggested that caspase-1 is responsible for proteolytic activation of IL-18 in human keratinocytes (33), another report has controversially proposed that caspase-1 in human keratinocytes exists in the unprocessed, biologically inactive form (49). These controversial conclusions complicate our understanding of the role of caspase-1 in IL-18 secretion by keratinocytes, and we thus assume the existence of another pathway of IL-18 secretion from keratinocytes. Indeed, a caspase-1-independent, Fas/Fas ligand-mediated IL-18 secretion from macrophages has been reported (52). However, it is to be elucidated whether this pathway is also involved in keratinocytes. Additional study is required for identification of the accurate molecular mechanism involved in hBD- and LL-37-induced IL-18 secretion from keratinocytes.

To gain insight into the cellular mechanisms by which hBDs and LL-37 activate keratinocytes, we focused on the pathways of three major downstream MAPK cascades: p38, ERK1/2, and JNK. In cells, these kinase cascades act as signal sorters for a variety of upstream signals before entering the nucleus. The p38, ERK, and JNK kinase cascades have been shown to be involved in a large variety of cellular activities, ranging from cell survival and proliferation to expression of proinflammatory cytokines (53, 54). Very recently, LL-37 has been shown to activate monocytes through p38 and ERK1/2 (38). Furthermore, p38 and ERK1/2, but not JNK, have been implicated in human keratinocyte motility (55). By studying these three MAPKs, we showed how they participate in IL-18 secretion upon stimulation of hBDs and LL-37. We demonstrated that both activated p38 and ERK1/2, but not JNK, are required for hBD- and LL-37-induced IL-18 secretion, as shown by the ability of the respective inhibitors of p38 and ERK1/2 to almost completely nullify IL-18 production. The involvement of MAPKs was also observed in the production of IL-20 and IL-8 by hBD- and LL-37-stimulated keratinocytes (data not shown). In addition, we found that hBD-2, -3, and -4 and LL-37 could induce the phosphorylation of p38 and ERK1/2, but not that of JNK.

Reportedly, stimulants such as *S. aureus* protein A, DNCB, PMA, and LPS induce IL-18 secretion by keratinocytes without increasing IL-18 mRNA expression (33, 51). It has been proposed that this was due to the fact that the IL-18 gene possesses two promoters, one of which is constitutive, and another, the inducible IL-18 promoter, which is inactive in human keratinocytes (56). The finding that hBDs and LL-37 strongly increase the levels of IL-18 mRNA from 20- to 46-fold (Fig. 5*B*) suggests that these peptides activate keratinocytes through pathways different from those for other IL-18 inducers. In aggregate, the ability of hBDs and LL-37 to induce IL-18 mRNA expression as well as IL-18 protein release suggests that human antibacterial peptides are involved in primary immune responses induced by skin cells.

Human antibacterial peptides exhibit their bactericidal activities from 0.1–50 $\mu$g/ml (equivalent to 0.02–12.5 $\mu$M) (34, 57) and are highly concentrated in human epithelial tissues. For instance, hBD-2 is estimated to be $\geq$2.3 $\mu$M in airway surface liquid (58), $\sim$16 $\mu$M in IL-1$\alpha$-stimulated epidermis (59), and $\sim$157 $\mu$M in psoriatic skin lesions (29). Furthermore, LL-37 is found at a concentration of $\sim$4.4 $\mu$M in bronchoalveolar lavage liquid (60) and is estimated to be $\geq$1605 $\mu$M in psoriatic skin (29). Thus, the concentrations of hBDs and LL-37 (ranging from 1 to 40 $\mu$g/ml, equivalent to 0.2 to 10 $\mu$M) that we used in this study, which did not cause cytotoxicity, are supposed to be adequate for evaluating the physiological roles of these antibacterial peptides.

In conclusion, we demonstrated a novel activity of the epithelial cell-derived antibacterial peptides hBDs and LL-37 on normal human keratinocytes. Because IL-18, IL-20, and IL-8 are involved in the pathogenesis of skin diseases such as psoriasis, the ability of hBDs and LL-37 to induce the secretion of these proinflammatory mediators by keratinocytes provides a new mechanism for the involvement of human antibacterial peptides in innate and adaptive immunity and their roles in the pathogenesis of skin disorders.

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## Disclosures

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