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Increased serum human β -defensin-2 levels in atopic dermatitis: Relationship to IL-22 and oncostatin M

Naoko Kanda*, Shinichi Watanabe

Department of Dermatology, Teikyo University, School of Medicine, 11-1, Kaga-2, Itabashi-Ku, Tokyo 173-8605, Japan

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ABSTRACT

Atopic dermatitis (AD) is associated with dysregulated expression of human β -defensins (hBDs) and infiltration of T cells producing cytokines which regulate hBD expression. We examined serum levels of hBDs and cytokines in AD patients, the effects of cytokines on hBD production in human keratinocytes, and those of hBDs on cytokine production in human peripheral blood-derived T cells. Levels of serum hBD-2, IL-22, and oncostatin M, but not hBD-3, were higher in AD patients than in normal donors. Serum hBD-2 levels of AD patients correlated with AD scoring and IL-22 levels. IL-22 and oncostatin M enhanced hBD-2 production and signal transducer and activator of transcription 3 (STAT3) activities strongly and hBD-3 production moderately in human keratinocytes. STAT3 inhibitor suppressed IL-22 and oncostatin M-induced production of hBD-2 and hBD-3. hBD-2 strongly and hBD-3 moderately enhanced IL-22 and oncostatin M production, whereas hBD-3 strongly and hBD-2 moderately enhanced IL-31, IL-13, and IL-4 production in CD3/28-stimulated T cells. hBD-2 induced phosphorylation of c-Jun N-terminal kinase, extracellular signal-regulated kinase, and Akt, while hBD-3 induced phosphorylation of inhibitory κ B kinase, p38 mitogen-activated protein kinase, and Akt in CD3/28-stimulated T cells. Inhibitors of these signals attenuated hBD-2- or hBD-3-induced production of cytokines. These results suggest that serum hBD-2 may be a biomarker of skin inflammation. IL-22 and oncostatin M may enhance hBD-2 production via STAT3 in keratinocytes, while hBD-2 may enhance IL-22 and oncostatin M production in T cells. hBD-3 may enhance T_H2 responses.

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Introduction

The antimicrobial peptides human β -defensin-2 (hBD-2) and hBD-3 are mainly produced by keratinocytes in the skin (de Jongh et al. 2005; Schlapbach et al. 2009) and potentiate innate and adaptive immunity (Yang et al. 1999; Röhl et al. 2010; Niyonsaba et al. 2007; Harder et al. 2010). hBD-2 and hBD-3 induce proliferation and production of cytokine/chemokines like IL-6 or CCL20 in keratinocytes (Niyonsaba et al. 2007). hBD-2 and hBD-3 bind CC

chemokine receptor 6 (CCR6) and chemoattract memory T cells and immature dendritic cells (Niyonsaba et al. 2007).

Atopic dermatitis (AD) is a chronic inflammatory dermatosis with skin barrier dysfunction and infiltration of activated T cells (Guttman-Yassky et al. 2011; Souwer et al. 2010). IL-4/IL-13-producing T_H2 cells act in the acute phase, whereas IFN- γ -producing T_H1 and T_H2 cells predominate the chronic phase of AD (Guttman-Yassky et al. 2011). IL-17A-producing T_H17 cells are increased in acute AD lesions (Souwer et al. 2010). IL-22, oncostatin M, IL-31, IL-25, or thymic stromal lymphopoietin (TSLP) are recently noted in the development of AD. IL-22 is produced by T_H17 , T_H1 , natural killer, or natural killer T cells. CD4⁺ or CD8⁺ T cells producing IL-22 alone (T22) infiltrate into chronic AD lesions (Nogales et al. 2009). IL-22 induces proliferation and suppresses terminal differentiation of keratinocytes (Guttman-Yassky et al. 2011; Souwer et al. 2010; Nogales et al. 2009). IL-31, preferentially produced by T_H2 cells but also by T_H1 cells (Bilsborough et al. 2006; Dillon et al. 2004), acts as a pruritogen and is expressed by skin homing T cells in AD lesions (Bilsborough et al. 2006). IL-25 is produced by T_H2 cells, mast cells, or dendritic cells; potentiates IL-4/5/13 production in a murine model of asthma (Angkasekwina et al. 2007); and suppresses filaggrin synthesis in keratinocytes (Hvid et al. 2011). T cells in AD lesions highly express oncostatin M (Boniface et al. 2007).

Abbreviations: AD, atopic dermatitis; ANOVA, analysis of variance; AP-1, activator protein-1; CCR6, CC chemokine receptor 6; C/EBP, CCAAT/enhancer binding protein; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATA3, GATA-binding protein 3; hBD-2, human β -defensin-2; IKK, inhibitory κ B kinase; JNK, c-Jun N-terminal kinase; KBM, keratinocyte basal medium; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T cells; NF- κ B, nuclear factor- κ B; PI-3K, phosphoinositide 3-kinase; pRLtk, herpes simplex virus thymidine kinase promoter-linked Renilla luciferase vector; SCORAD, scoring of atopic dermatitis; Sp1, specificity protein 1; STAT3, signal transducer and activator of transcription 3; TSLP, thymic stromal lymphopoietin.

* Corresponding author. Fax: +81 3 5375 5314.

E-mail address: nmk@med.teikyo-u.ac.jp (N. Kanda).

Oncostatin M, initially identified as a T_H1 cytokine, induces eotaxin-1 expression and eosinophilia in murine lung and may potentiate T_H2 responses (Fritz et al. 2011). TSLP, highly expressed in keratinocytes of AD lesions, stimulates dendritic cells to polarize naïve T cells to differentiate into T_H2 cells (He and Geha 2010).

AD patients are more susceptible to cutaneous infection with *Staphylococcus aureus* or *Herpes simplex* than healthy individuals (Boguniewicz et al. 2006). Recurrent infection in AD is attributed to reduced hBD expression (Ong et al. 2002) due to enhanced T_H2 activity because IL-4/13 suppress hBD-2/3 production (Albanesi et al. 2007). However, enhanced production of hBD-2/3 in AD lesions was also reported (Harder et al. 2010). Probable diffusion of lesional hBDs into the circulation may alter their systemic levels. AD lesions contain high levels of IL-22 (Nogales et al. 2009; Boguniewicz et al. 2006), oncostatin M (Boniface et al. 2007), IL-31 (Bilsborough et al. 2006), IL-25 (Hvid et al. 2011), or TSLP (He and Geha 2010), which could modulate hBD secretion from keratinocytes. Conversely, keratinocyte-derived hBDs may alter the expression of these cytokines in T cells or keratinocytes. However, few studies investigated the effects of hBDs on the production of these cytokines (Boniotto et al. 2006; Kanda et al. 2011).

In this study, we examined serum hBD-2/3 levels in AD patients and analyzed their correlation with serum IL-22, oncostatin M, IL-31, IL-25, or TSLP levels. We investigated the effects of these cytokines on hBD production in keratinocytes, the effects of hBDs on the production of these cytokines in T cells or keratinocytes, and the underlying mechanisms.

Materials and methods

Patients and normal donors

This study was conducted according to the principles of Declaration of Helsinki and was approved by the Institutional Review Board of Teikyo University School of Medicine (TUSM-RB 09-005). The study population comprised 26 patients with AD without infection (16 men and 10 women, aged 38.3 ± 13.0 years [mean $\pm$ SD]) and 27 normal volunteers without inflammation or infection (19 men and 8 women, aged 37.3 ± 6.3 years). Scoring of AD (SCORAD) (Kunz et al. 1997) was calculated for AD patients (mean $\pm$ SD, 27.43 ± 17.88). The patients were topically but not systemically treated with steroid or *tacrolimus*.

Reagents

RPMI 1640 supplemented with 10% fetal calf serum (complete RPMI) was purchased from PromoCell GmbH (Heidelberg, Germany). Synthetic hBD-2 and hBD-3 were from Peptide Institute Inc. (Osaka, Japan). Recombinant human IL-22, oncostatin M, IL-31, IL-4, IL-13, IL-17A, IFN- γ , TNF- α , IL-10, IL-25, and TSLP were from R&D Systems (Minneapolis, MN, USA). Stattic, Bay 11-7082, c-Jun N-terminal kinase (JNK) inhibitor I, SB202190, PD98059, wortmannin, and pertussis toxin were from Merck KGaA (Darmstadt, Germany) and were dissolved in dimethyl sulfoxide (DMSO).

ELISA

The following ELISA kits were used according to the manufacturers' instructions: hBD-2, hBD-3 (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), IL-13, IL-22, IL-31, IL-4, TSLP, TNF- α , IL-10, IFN- γ (R&D Systems), IL-17A (Gen-Probe Life Sciences Ltd., Livingston, UK), oncostatin M (RayBiotech, Inc., Norcross, GA, USA), and IL-25 (KOMA BIOTECH INC., Seoul, Korea).

Keratinocyte culture

Human neonatal foreskin keratinocytes (Clonetics, Walkersville, MD, USA) were cultured in keratinocyte growth medium (Clonetics) containing keratinocyte basal medium (KBM) supplemented with 0.5 μ g/ml hydrocortisone, 5 ng/ml epidermal growth factor, 5 μ g/ml insulin, and 0.5% bovine pituitary extract. Keratinocytes (5.0×10^4 cells/well) in 24-well plates were treated with the indicated cytokines in 0.4 ml KBM. After 48 h, supernatants were assayed for hBDs by ELISA.

T-cell culture

T cells ($>98\%$ CD3 $^+$) were isolated from heparinized blood of AD patients and normal donors, as described (Kanda et al. 2011). T cells (2.0×10^6 cells/well) in 24-well plates were stimulated with plate-bound anti-CD3 (0.1 μ g/ml) and soluble anti-CD28 (2 μ g/ml) (BD Pharmingen, San Diego, CA, USA) in the presence or absence of 0.2, 2, or 20 μ g/ml hBD-2 or hBD-3 in 2 ml complete RPMI. After 48 h, supernatants were assayed for cytokines using ELISA. hBD concentrations were determined according to a previous study (Boniotto et al. 2006) and did not reduce T-cell viability as determined by the trypan blue exclusion test ($>95\%$ of controls).

Real-time PCR

Keratinocytes or T cells were incubated for 4, 8, or 12 h as above. Total cellular RNA was extracted and reverse-transcribed. Quantitative real-time PCR was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), as described (Albanesi et al. 2007; Kanda et al. 2011). mRNA levels of hBDs or cytokines were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are shown as fold inductions relative to controls.

Transfection

Keratinocytes in 24-well plates were grown to approximately 60% confluence. p-Nuclear factor- κ B (NF- κ B)-luc, p-activator protein-1 (AP-1)-luc, or p-signal transducer and activator of transcription 3 (STAT3)-luc and herpes simplex virus thymidine kinase promoter-linked *Renilla* luciferase vector (pRL-tk) (Clontech, San Jose, CA, USA) were mixed with Eugene HD (Roche, Indianapolis, IN, USA), and added to keratinocytes. After 24 h, keratinocytes were incubated in KBM for 24 h and treated with the indicated cytokines. After 18 h, firefly and *Renilla* luciferase activities of cell extracts were quantified using Dual-Luciferase Assay System (Promega, Madison, WI, USA). NF- κ B, AP-1, or STAT3 activities, expressed as ratios of firefly/*Renilla* luciferase activity, are shown as fold inductions relative to controls.

Western blotting

Keratinocytes were incubated with 100 ng/ml IL-22 or oncostatin M, and T cells were stimulated with 20 μ g/ml hBD-2 or hBD-3 in the presence of CD3/28 for the indicated periods and lysed. Western blotting was performed as described (Kanda et al. 2011).

Statistical analysis

Spearman's rank test and linear regression were used to calculate linear relationships. The Mann-Whitney *U* test was used to determine differences between 2 groups. One-way analysis of variance (ANOVA) with Dunnett's test was used for Figs. 2, 3 and 6. One-way ANOVA with Scheffe's test were used for Figs. 4 and 7. Data in Figs. 6–8 represent 4 separate experiments using T cells from 2

Table 1

Comparisons of serum hBD and cytokine levels between AD patients and normal donors.

	Normal (n = 27)	AD (n = 26)	p
hBD-2 (ng/ml)	2.002 ± 1.652	5.232 ± 3.091	<0.0001**
hBD-3 (ng/ml)	0.166 ± 0.640	0.105 ± 0.417	0.843
IL-22 (pg/ml)	17.80 ± 13.40	37.97 ± 31.65	0.0056**
Oncostatin M (pg/ml)	3.6 ± 6.0	10.1 ± 7.0	0.0008**
IL-31 (pg/ml)	10.8 ± 19.4	3.8 ± 16.3	0.0055**
IL-25 (pg/ml)	66.8 ± 118.0	38.8 ± 57.0	0.0403 [†]
TSLP (pg/ml)	22.2 ± 27.4	16.8 ± 3.7	0.8882
IL-13 (pg/ml)	2.09 ± 2.93	2.17 ± 5.89	0.1327
IL-4 (pg/ml)	0.612 ± 0.938	0.587 ± 0.632	0.1261
TNF-α (pg/ml)	48.6 ± 38.5	18.1 ± 9.2	0.00001**
IL-17A (pg/ml)	8.95 ± 32.4	3.94 ± 4.78	0.6556
IL-10 (pg/ml)	6.16 ± 3.28	1.75 ± 0.84	0.00001**
IFN-γ (pg/ml)	15.01 ± 6.59	13.37 ± 15.84	0.3853

AD, atopic dermatitis; hBD, human β-defensin; TSLP, thymic stromal lymphopoietin. by Mann–Whitney U test.

[†] p < 0.05.

** p < 0.01.

normal donors and 2 AD patients. A p-value <0.05 was considered significant.

Results

Increased serum hBD-2, IL-22, and oncostatin M levels in AD patients

Serum hBD-2 levels in AD patients were higher than in normal donors (Fig. 1A) and correlated with SCORAD (Fig. 1B). Serum hBD-3 levels were not different in AD patients and in normal donors and were much lower overall than hBD-2 levels (Fig. 1C). Serum hBD-3 levels in AD patients did not correlate with SCORAD (Fig. 1D) and did not correlate with hBD-2 levels in patients ($r = 0.1579$, $p = 0.4411$) or normal donors ($r = 0.0055$, $p = 0.9782$, by Spearman's rank test and linear regression).

Serum IL-22 levels were higher in AD patients than in normal donors (Fig. 1E) and correlated with hBD-2 levels only in the former (Fig. 1G and H). Serum oncostatin M levels were higher in AD patients than in normal donors (Fig. 1F) but did not correlate with hBD-2 levels in either group (Supplementary Table 1). Serum IL-31, IL-25, TNF-α, and IL-10 levels were lower in AD patients than in normal donors (Table 1). None of these cytokine levels in AD patients correlated with SCORAD (Supplementary Table 2).

IL-22 and oncostatin M increase hBD-2 production strongly and hBD-3 production moderately.

We analyzed the effects of cytokines on hBD-2 or hBD-3 secretion from keratinocytes. hBD-2 secretion was increased by IL-22 and oncostatin M, was not altered by IL-31 and IL-25, and was reduced by TSLP, IL-13, and IL-4 (Fig. 2A). The magnitudes of hBD-2 induction by IL-22 (maximally 5.60-fold of controls) and oncostatin M (6.02-fold) were similar to that of TNF-α (5.32-fold) but lower than IL-17A (22.03-fold) or IFN-γ (24.98-fold) (Fig. 2A, inset). hBD-3 secretion was moderately increased by IL-22 and oncostatin M but was not altered by IL-31, IL-25, TSLP, IL-4, or IL-13 (Fig. 2B). The magnitudes of hBD-3 induction by IL-22 (maximally 1.69-fold of controls) and oncostatin M (1.92-fold) were similar to those of TNF-α (1.84-fold) and IL-17A (1.75-fold) but lower than IFN-γ (6.82-fold) (Fig. 2B, inset). These cytokines showed similar effects on hBD-2 and hBD-3 mRNAs (Fig. 2C and D).

hBD-2 or hBD-3 production is promoted by NF-κB, AP-1, or STAT3 (Kanda and Watanabe 2007; Ishikawa et al. 2009; Kanda and Watanabe 2008). We thus examined if IL-22 or oncostatin M may alter the activities of these transcription factors. STAT3 activities were enhanced by IL-22 and oncostatin M, and less potently by IL-31 (Fig. 3A). AP-1 activities were enhanced by IL-22, and less

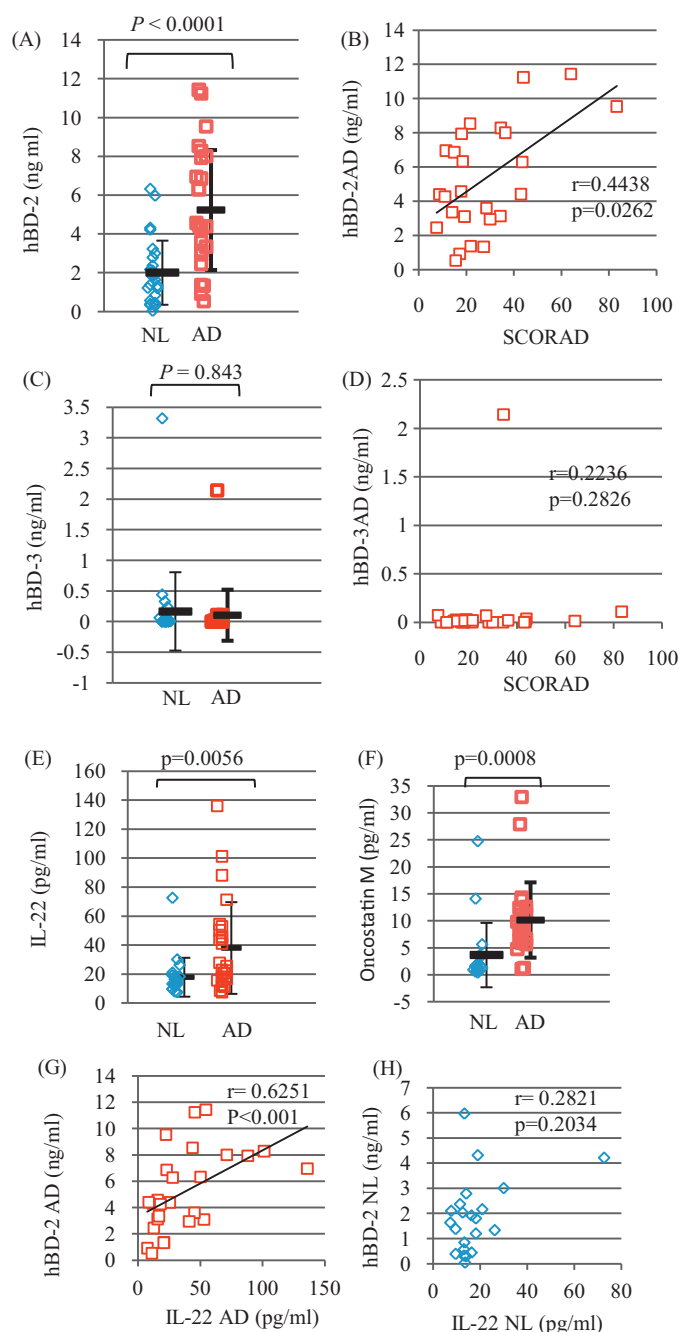


Fig. 1. Serum hBD-2 (A) but not hBD-3 levels (C) are higher in AD patients than in normal donors (NL) and correlate with SCORAD (B) (D). Serum IL-22 (E) and oncostatin M levels (F) are higher in AD patients than in normal donors, and IL-22 levels correlate with hBD-2 levels in AD patients (G) but not in normal donors (H). (A), (C), (E), and (F) Mann–Whitney U test was used to determine differences between 2 groups, and means ± SDs are shown. (B), (D), (G), and (H) Correlations were analyzed by Spearman's rank test and linear regression analysis, and regression lines are shown in (B) and (G).

potently by oncostatin M (Fig. 3B). NF-κB activities were moderately enhanced by oncostatin M (Fig. 3C). The activities of these transcription factors were not altered by TSLP or IL-25. These results indicate that STAT3 and/or AP-1 mediate IL-22-induced hBD-2/3 production and STAT3, AP-1, and/or NF-κB mediate oncostatin M-induced hBD-2/3 production.

We then analyzed the signals involved in IL-22 and oncostatin M-induced hBD-2/3 production, using specific inhibitors of the individual signals. STAT3 inhibitor stattic and p38

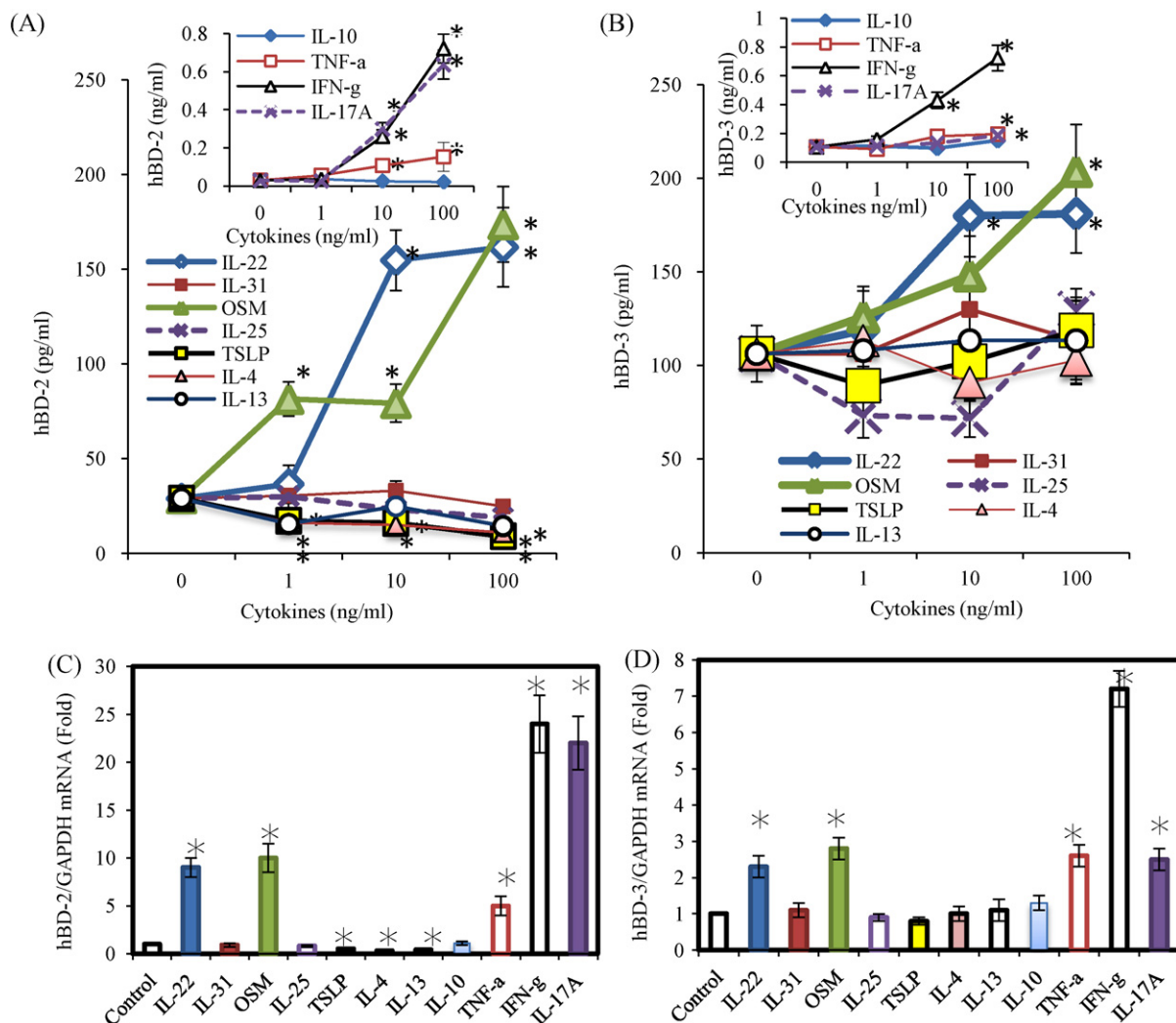


Fig. 2. IL-22 and oncostatin M (OSM) increase the secretion and mRNA levels of hBD-2 (A) and (C) or hBD-3 (B) and (D) in keratinocytes. (A and B) Keratinocytes were treated with the indicated concentrations of cytokines for 48 h, and supernatants were analyzed for hBD secretion. (C and D) Keratinocytes were treated with 100 ng/ml of specified cytokines for 8 h. mRNA levels of hBDs are shown as fold inductions relative to control keratinocytes with medium alone. * $p < 0.05$ versus controls, by one-way ANOVA with Dunnett's test. Data are means $\pm$ SDs of triplicate cultures and represent 4 separate experiments.

mitogen-activated protein kinase (MAPK) inhibitor SB202190 suppressed IL-22-induced hBD-2/3 secretion (Fig. 4A and B). Stat3 and MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor PD98059 suppressed oncostatin M-induced hBD-2/3 secretion (Fig. 4C and D) whereas inhibitory κ B kinase (IKK) inhibitor Bay 11-7082 suppressed oncostatin M-induced hBD-2 secretion but not hBD-3 (Fig. 4C and D). These inhibitors showed similar effects on hBD-2/3 mRNAs (data not shown). These results suggest that STAT3 and p38 MAPK mediate IL-22-induced hBD-2/3 production, while STAT3 and ERK mediate oncostatin M-induced hBD-2/3 production. IKK may mediate oncostatin M-induced hBD-2 production. We next determined whether IL-22 or oncostatin M activates STAT3, IKK, p38 MAPK, or ERK in keratinocytes. IL-22 induced phosphorylation of STAT3 and p38 MAPK (Fig. 5A and C, lane 2) but not of IKK or ERK (Fig. 5B and D, lane 2). Oncostatin M induced phosphorylation of STAT3, IKK, and ERK (Fig. 5A, B and D, lane 3) but not of p38 MAPK (Fig. 5C, lane 3).

hBD-2 strongly and hBD-3 moderately enhance IL-22 and oncostatin M production while hBD-3 strongly and hBD-2 moderately enhance IL-31, IL-13, and IL-4 production in CD3/28-stimulated T cells.

We analyzed whether hBD-2/3 alter the secretion of cytokines regulating their production in CD3/28-stimulated T cells. hBD-2

strongly and hBD-3 moderately enhanced IL-22 and oncostatin M secretion (Fig. 6A and B). hBD-3 strongly and hBD-2 moderately increased IL-31, IL-13, and IL-4 secretion (Fig. 6C–E). hBD-2 and hBD-3 similarly reduced IL-25 secretion (Fig. 6F). hBD-2 reduced IL-17A secretion (Fig. 6G) but increased IL-10, IFN- γ , and TNF- α secretion (Fig. 6H–J), which was consistent with our previous results (Kanda et al. 2011). hBD-3 did not alter IL-17A, IL-10, IFN- γ , or TNF- α secretion (Fig. 6G–J). hBD-2/3 did not alter the secretion of these cytokines without CD3/28 (data not shown). Because TSLP is preferentially produced by epithelial cells (He and Geha 2010), we examined TSLP secretion from TNF- α -stimulated keratinocytes. The TSLP secretion was similarly reduced by hBD-2 and hBD-3 (Fig. 6K). The patterns of hBD-2/3 alteration of individual cytokine mRNAs at 8 h in T cells or keratinocytes were similar to the effects on protein secretion (Fig. 6L), indicating transcriptional regulation. Similar results were obtained at 4 or 12 h (data not shown). T cells from normal donors and AD patients showed similar responses to hBD-2/3 (data not shown).

To analyze the signals involved in hBD-2/3-induced IL-22, oncostatin M, IL-31, IL-13, or IL-4 production, we inhibited IKK, JNK, p38 MAPK, MEK, phosphoinositide 3-kinase (PI-3K), and $G_{i/o}$ protein with Bay 11-7082, JNK inhibitor I, SB202190, PD98059, wortmannin, and pertussis toxin, respectively. In T cells stimulated with

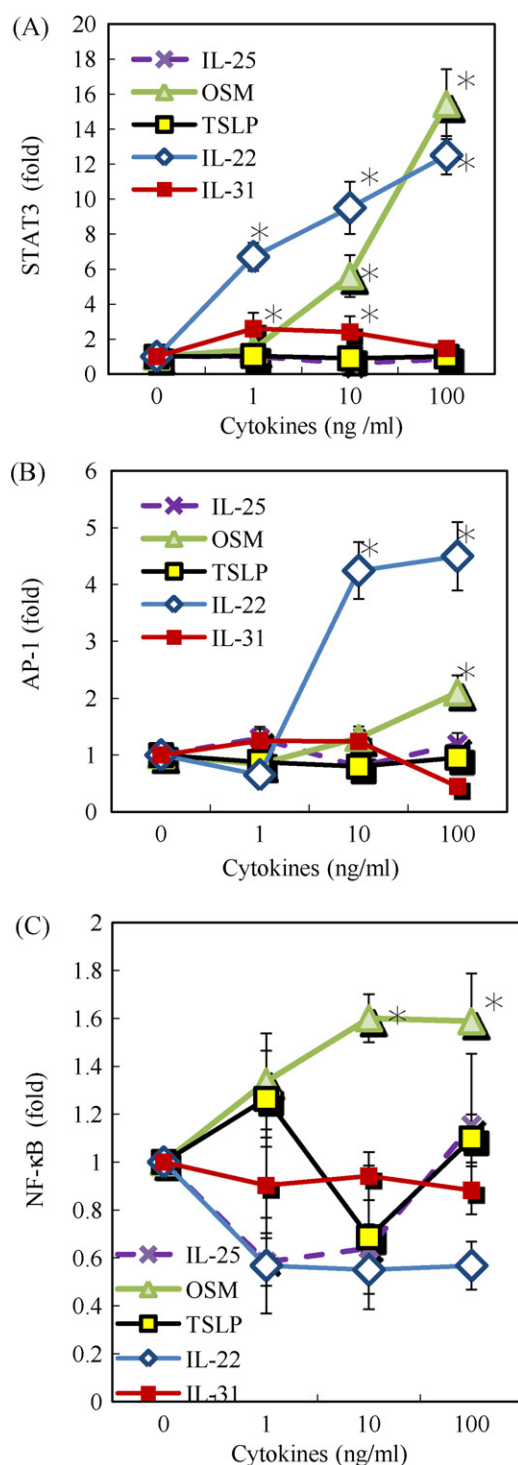


Fig. 3. Effects of IL-22, oncostatin M (OSM), IL-25, IL-31, and TSLP on STAT3 (A), AP-1 (B), and NF-κB activities (C). Keratinocytes transfected with firefly/*Renilla* luciferase vectors were incubated with the indicated doses of cytokines. Transcriptional activities are shown as fold inductions relative to controls. * $p < 0.05$ versus controls, by one-way ANOVA with Dunnett's test. Data are means $\pm$ SDs of triplicate cultures and represent 4 separate experiments.

CD3/28 alone (left one-third of each panel in Fig. 7), IL-22, oncostatin M, IL-13, and IL-4 secretion required IKK (Fig. 7A, B, D and E) but IL-31 did not (Fig. 7C). In T cells stimulated with hBD-2 plus CD3/28 (middle one-third of each panel in Fig. 7), IL-22 secretion required MEK (Fig. 7A), oncostatin M secretion required JNK (Fig. 7B), IL-13 secretion required MEK (Fig. 7D), IL-4 secretion

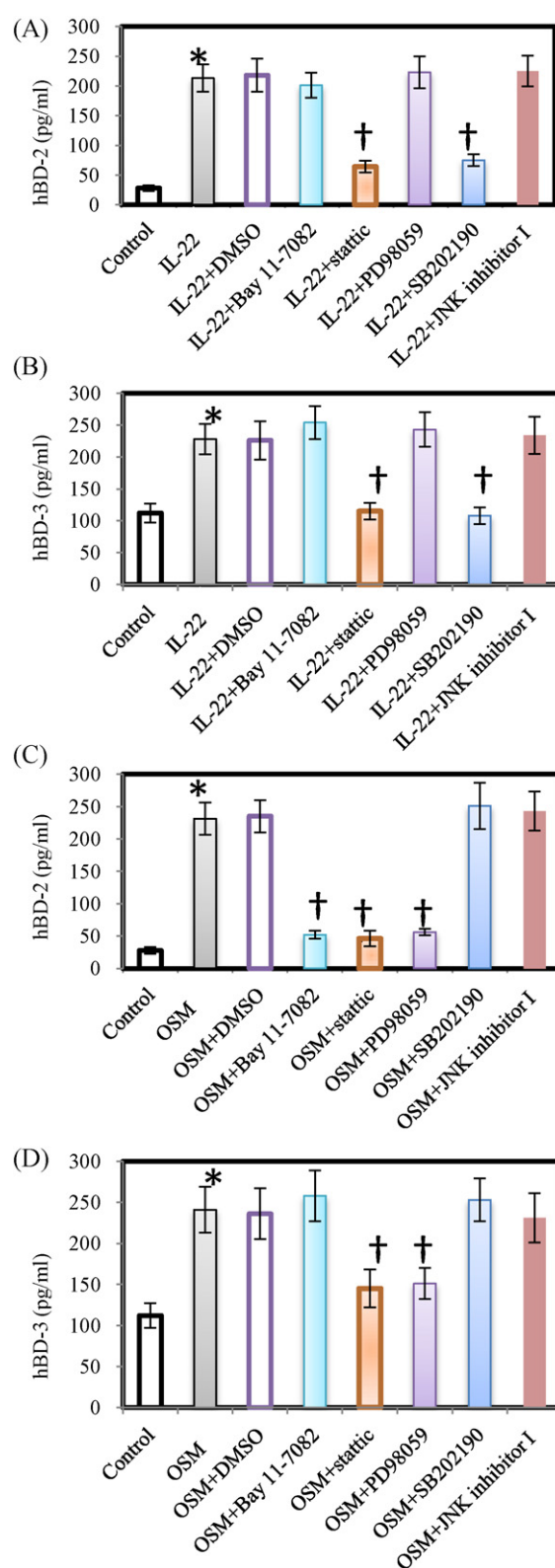


Fig. 4. Effects of signal inhibitors on hBD-2 and hBD-3 secretion induced by IL-22 (A and B) and oncostatin M (OSM) (C and D). Keratinocytes were preincubated with static (1 μ M), Bay 11-7082 (1 μ M), JNK inhibitor 1 (0.5 μ M), SB202190 (1 μ M), PD98059 (10 μ M), or 0.1% DMSO for 2 h, and stimulated with IL-22 or OSM at 100 ng/ml for 48 h. * $p < 0.05$ versus controls; † $p < 0.05$ versus IL-22 or OSM, by one-way ANOVA with Scheffe's test. Data are means $\pm$ SDs of triplicate cultures and represent 4 separate experiments.

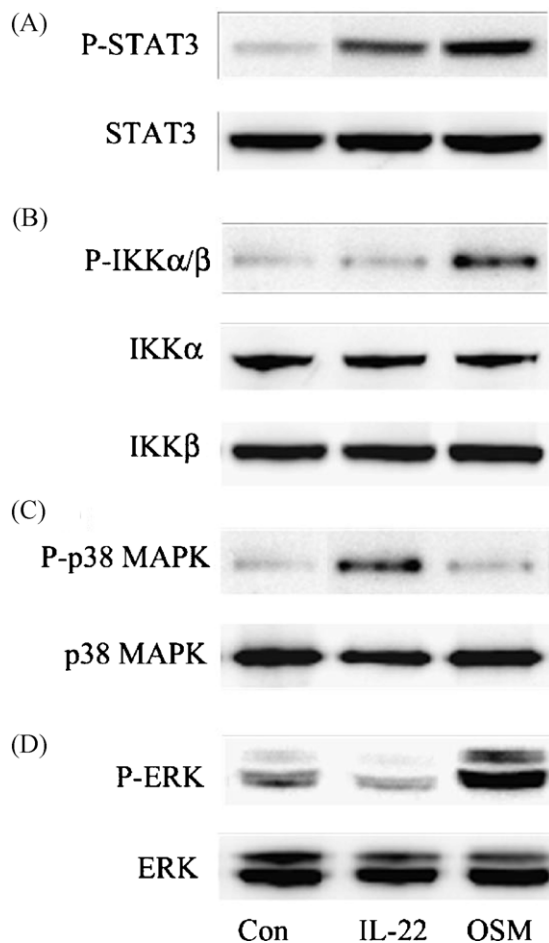


Fig. 5. Effects of IL-22 and oncostatin M (OSM) on the phosphorylation of STAT3 (A), IKK (B), p38 MAPK (C), and ERK (D). Keratinocytes were stimulated with medium alone (control [Con]) or IL-22 or OSM at 100 ng/ml for 10 min. The cell lysates were analyzed by western blotting for phosphorylated STAT3 (Tyr705), IKKα/β (Ser176/180), p38 MAPK (Thr180/Tyr182), or ERK (Thr202/Tyr204) versus total proteins. Data are representative of 4 separate experiments.

required PI-3K (Fig. 7E) in addition to IKK, and IL-31 secretion required JNK (Fig. 7C). The secretion of all these cytokines required $G_{i/o}$ proteins. These findings indicate that hBD-2-induced cytokine increases require $G_{i/o}$ proteins and involve MEK, JNK, or PI-3K in cooperation with IKK, depending on the cytokine. In T cells stimulated with hBD-3 plus CD3/28 (right one-third of each panel in Fig. 7), oncostatin M (Fig. 7B) and IL-13 secretion (Fig. 7D) required p38 MAPK, and IL-4 secretion required p38 MAPK and PI-3K (Fig. 7E) in addition to IKK. IL-22 or IL-31 secretion required IKK or p38 MAPK, respectively (Fig. 7A and C). The secretion of all these cytokines required $G_{i/o}$ proteins. These findings indicate that hBD-3-induced cytokine increases require $G_{i/o}$ proteins and involve PI-3K and p38 MAPK in cooperation with IKK, depending on the cytokine. These signal inhibitors showed similar effects on mRNAs of these cytokines (data not shown).

hBD-2 activates JNK, MEK/ERK, and PI-3K/Akt while hBD-3 activates IKK/NF-κB, p38 MAPK, and PI-3K/Akt.

We examined whether hBD-2 or hBD-3 activates IKK, JNK, p38 MAPK, MEK/ERK, or PI-3K/Akt in T cells alone or together with CD3/28. CD3/28 alone induced IKKα/β phosphorylation potently (Fig. 8A, first panel, lane 2) and JNK, ERK, p38 MAPK, and Akt phosphorylation moderately (Fig. 8B–E, first panel, lane 2). hBD-2 alone enhanced JNK, ERK, and Akt phosphorylation (Fig. 8B, C and E, first panel, lane 3) but not IKKα/β or p38 MAPK phosphorylation (Fig. 8A and D, first panel, lane 3). hBD-3 alone enhanced IKKα/β

phosphorylation moderately (Fig. 8A, first panel, lane 5) and p38 MAPK and Akt phosphorylation potently (Fig. 8D and E, first panel, lane 5) but did not enhance JNK or ERK phosphorylation (Fig. 8B and C, first panel, lane 5). hBD-2 plus CD3/28 or hBD-3 plus CD3/28 (Fig. 8A–E, first panel, lane 4 or 6, respectively) additively phosphorylated individual kinases. These findings suggest that hBD-2 activates JNK, MEK/ERK, and PI-3K/Akt, whereas hBD-3 activates IKK/NF-κB, p38 MAPK, and PI-3K/Akt.

Discussion

Serum hBD-2 levels in AD patients were higher than in normal donors and correlated with SCORAD. We recently found that serum hBD-2 levels in psoriasis patients are higher than in normal donors and correlate with psoriasis area and severity index (Kanda et al. 2011). These results totally indicate that serum hBD-2 levels in AD or psoriasis patients reflect levels of lesional skin and may be a biomarker of skin inflammation. Serum hBD-3 levels in AD patients and in normal donors were not different and much lower than hBD-2 levels, which is similar to the results in psoriasis patients (Kanda et al. 2011; Jansen et al. 2009). Being highly cationic, hBD-3 produced by keratinocytes may be trapped by negatively charged structures in the epidermis (Harder et al. 2010) and less likely to enter the circulation.

IL-22 and oncostatin M increased hBD-2 production strongly and hBD-3 production moderately in keratinocytes. STAT3 appeared essential for the production of both hBDs. NF-κB might mediate oncostatin M-induced hBD-2 production, and p38 MAPK or ERK might mediate IL-22- or oncostatin M-induced hBD-2/3 production, respectively. IL-22-induced p38 MAPK or oncostatin M-induced ERK may activate AP-1 through the synthesis and/or phosphorylation of AP-1 components like c-Jun or c-Fos, which may lead to the enhancement of hBD-2/3 transcription (Kanda and Watanabe 2007; Ishikawa et al. 2009; Schlee et al. 2008). In contrast, TSLP, IL-4, and IL-13 suppressed hBD-2 production in keratinocytes. STAT6 is reported to mediate IL-4/13-induced suppression of hBD-2 production via the expression of suppressor of cytokine signaling-1/3 (Albanesi et al. 2007). Thus TSLP-induced suppression of hBD-2 production may also involve STAT6 since TSLP is reported to induce tyrosine-phosphorylation of STAT6 in human myeloid dendritic cells (Arima et al. 2010).

hBD-2 increased IL-22 and oncostatin M production in CD3/28-stimulated T cells. The optimal hBD-2 concentration, 0.2–20 μg/ml, was more than 100-fold higher than serum concentrations but may be a possible level in inflammatory skin lesions, and thus the *in vitro* results above may reflect the events occurring in lesional skin. The *in vivo* hBD-2 concentration in the epidermis of psoriasis lesions is estimated 300 μM (1298 μg/ml) (Jansen et al. 2009) or 2.3–157 μM (10–683 μg/ml) (Ong et al. 2002) though the concentration in AD lesions is unknown. It is reported that IL-22 expression is induced by NF-κB, specificity protein 1 (Sp1), or CCAAT/enhancer-binding protein (C/EBP) (Kagami et al. 2010; Dumoutier et al. 2000; Ma et al. 2010), while AP-1, NF-κB, Sp1, or C/EBP induce oncostatin M expression (Ma et al. 1999; Amos et al. 2006; Kastl et al. 2008). CD3/28 activated NF-κB, which may possibly drive IL-22 and oncostatin M production. hBD-2-induced ERK or JNK might promote IL-22 or oncostatin M production, respectively. It is reported that ERK activates Sp1 and C/EBP by inducing their phosphorylation and/or expression (Chanteux et al. 2007; Cui et al. 2011; Lue et al. 2011), while JNK activates AP-1 via phosphorylation and/or expression of AP-1 components like c-Jun (Lue et al. 2011). Thus, hBD-2-induced ERK or JNK could activate Sp1/C/EBP or AP-1 to promote IL-22 or oncostatin M expression, respectively.

hBD-2 increased IL-31, IL-13, and IL-4 production in CD3/28-stimulated T cells. hBD-2-induced JNK may promote IL-31

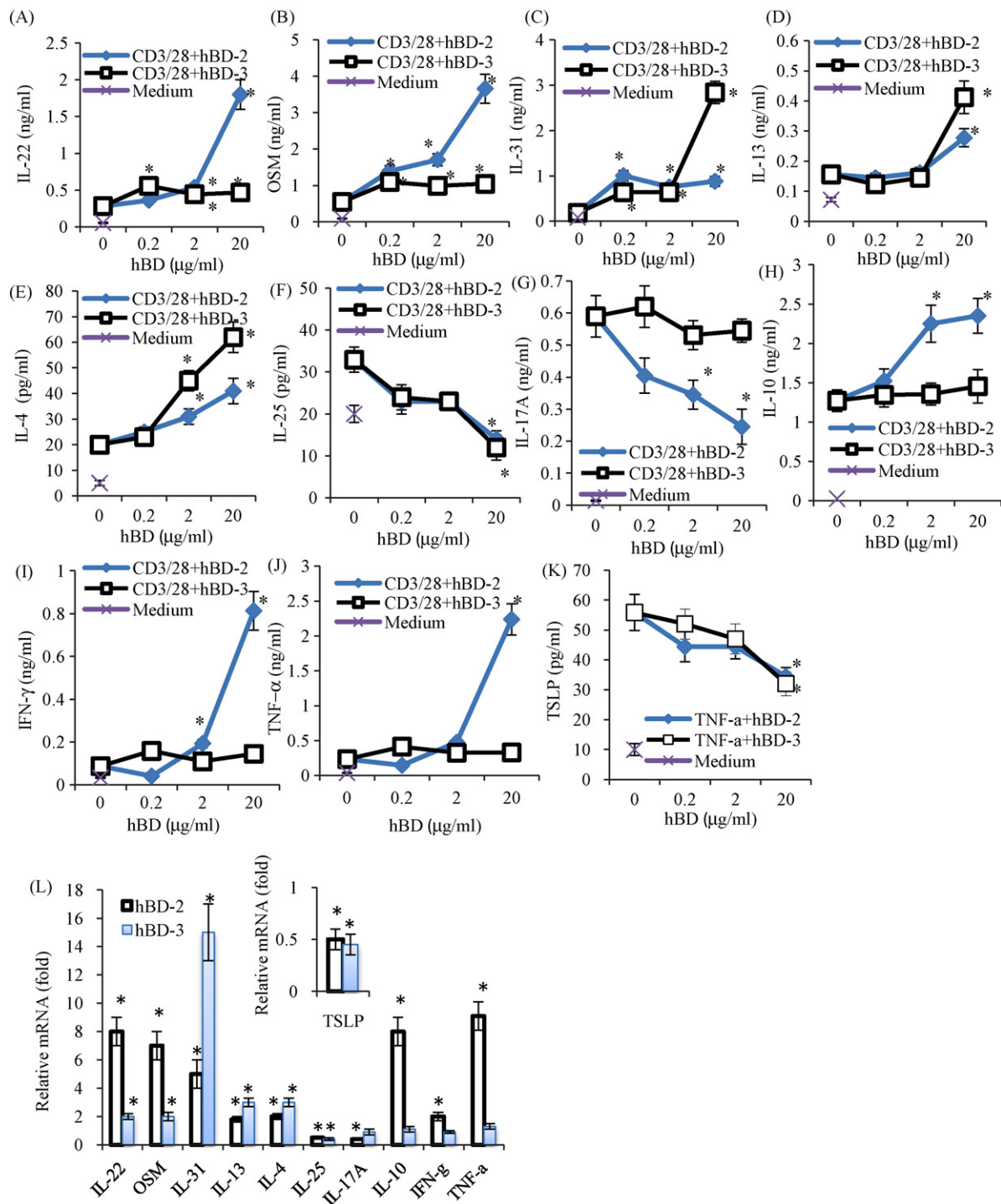


Fig. 6. Effects of hBD-2 and hBD-3 on secretion (A–K) and mRNA levels (L) in T cells and keratinocytes. (A–K) Normal donor T cells (A–J) or keratinocytes (K) were stimulated with CD3/28 (A–J) or TNF- α (100 ng/ml) (K), respectively, in the presence of the indicated hBD-2 or hBD-3 concentrations for 48 h. (L) mRNA levels of cytokines in CD3/28-stimulated T cells or TNF- α -stimulated keratinocytes (inset) after 8-h incubation with hBD-2 or hBD-3 (each 20 μ g/ml) are shown as fold inductions relative to control T cells with CD3/28 alone or keratinocytes with TNF- α alone, respectively. * p < 0.05 versus CD3/28 or TNF- α alone, by one-way ANOVA with Dunnett's test. Data are means $\pm$ SDs of triplicate cultures and represent 4 separate experiments.

production possibly through AP-1 (Gutzmer et al. 2009). It is reported that IL-13 and IL-4 expression is induced by NF- κ B, nuclear factor of activated T cells (NFAT), GATA-binding protein 3 (GATA3), and AP-1 (Hehner et al. 2000; Masuda et al. 2004; Manechotesuwan et al. 2007; Xue et al. 2007). CD3/28-activated NF- κ B might induce IL-13 and IL-4 production, and hBD-2-induced ERK or Akt might further augment IL-13 or IL-4 production, respectively. It is reported that Akt activates NFAT by phosphorylating and

inactivating glycogen synthase kinase-3 β , which phosphorylates NFAT and inhibits its nuclear import and DNA binding (Xue et al. 2007). Thus, hBD-2-induced ERK or Akt might stimulate AP-1 or NFAT to augment IL-13 or IL-4 expression, respectively.

hBD-3 moderately enhanced IL-22 and oncostatin M production. hBD-3 stimulated NF- κ B in cooperation with CD3/28, which may lead to the enhancement of IL-22 and oncostatin M expression. It is also known that NF- κ B mediates hBD-3-induced CCR7 expression

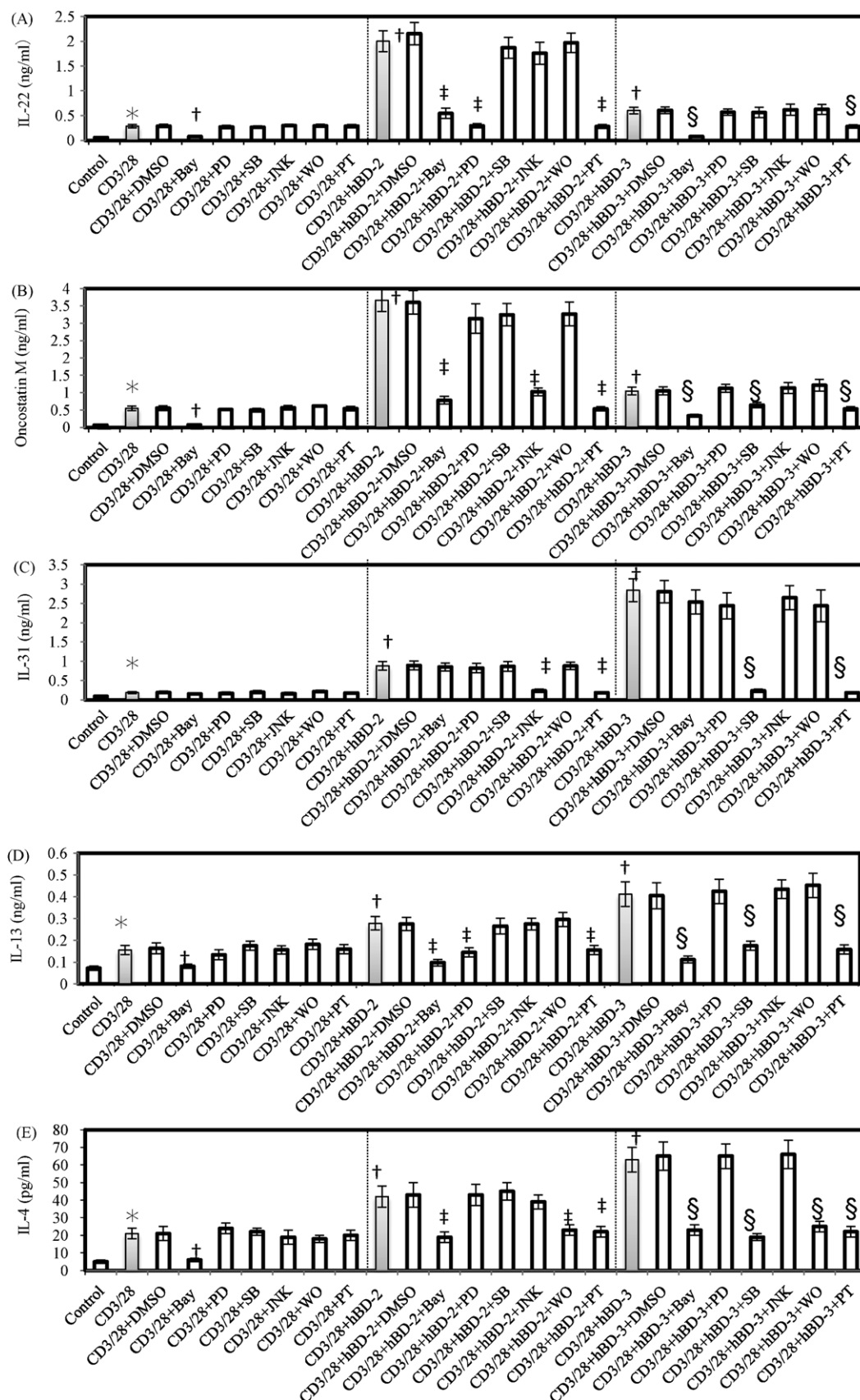


Fig. 7. Effects of signal inhibitors on cytokine secretion in CD3/CD28-stimulated T cells with or without hBD-2 or hBD-3. Normal donor T cells were preincubated with statin (1 μ M), Bay11-7082 (Bay) (1 μ M), JNK inhibitor I (JNK) (0.5 μ M), SB202190 (SB) (1 μ M), PD98059 (PD) (10 μ M), wortmannin (WO) (0.1 μ M), 100 ng/ml pertussis toxin (PT), or 0.1% DMSO for 2 h, and stimulated with CD3/28 in the presence or absence of hBD-2 or hBD-3 (20 μ g/ml) for 48 h. * $p < 0.05$ versus controls; † $p < 0.05$ versus CD3/28; ‡ $p < 0.05$ versus CD3/28 plus hBD-2; § $p < 0.05$ versus CD3/28 plus hBD-3, by one-way ANOVA with Scheffe's test. Data are means $\pm$ SDs of triplicate cultures and represent 4 separate experiments.

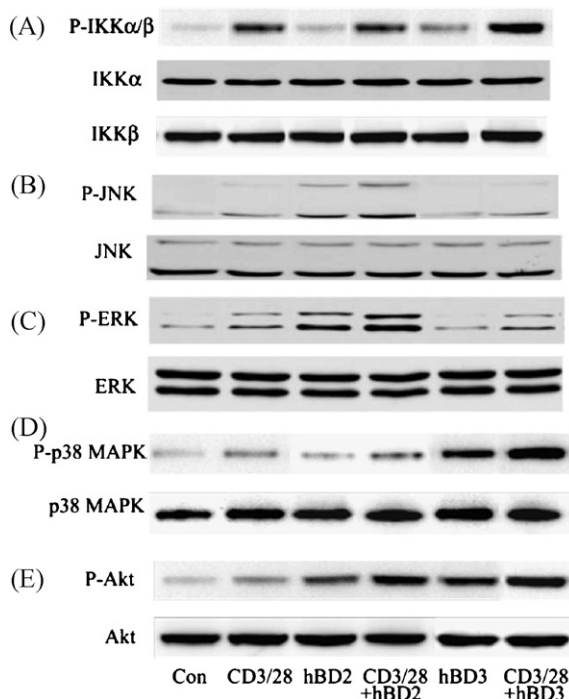


Fig. 8. Effects of hBD-2 or hBD-3 on IKK (A), JNK (B), ERK (C), p38 MAPK (D), and Akt phosphorylation (E). Normal donor T cells were stimulated with medium (Control [Con]), or CD3/28 with or without hBD-2 or hBD-3 (20 μ g/ml) for 10 min. The cell lysates were analyzed by western blotting for phosphorylated IKK α / β (Ser176/180), JNK (Thr183/Tyr185), ERK (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182), or Akt (Thr308) versus total proteins. Data are representative of 4 separate experiments.

in squamous cell carcinoma cell lines (Mburu et al. 2011). Besides hBD-3-induced p38 MAPK may augment oncostatin M expression via AP-1.

hBD-3 induced IL-31, IL-13, and IL-4 production more potently than hBD-2. This is the first study showing hBD-3-stimulated T_H2 responses. hBD-3-activated NF- κ B may promote IL-13, and IL-4 expression. Besides hBD-3-induced p38 MAPK may augment IL-13, IL-4, and IL-31 expression via AP-1 and/or GATA3 (Schulz et al. 2007) since p38 MAPK-mediated phosphorylation of GATA3 is known to induce its nuclear import and DNA binding (Maneechotesuwan et al. 2007). Furthermore, hBD-3-induced Akt might promote IL-4 expression via NFAT. In contrast, hBD-2/3 suppressed the production of other T_H2 -potentiating cytokines, IL-25 and TSLP. We should further elucidate the mechanisms for this suppression.

hBD-2/3 might promote T-cell cytokine production via receptor(s) coupled to pertussis toxin-sensitive G-proteins like $G_{i/o}$. Though it is reported that hBD-2/3 bind CCR2 (Röhrle et al. 2010) and CCR6 (Schlapbach et al. 2009), antibodies to these receptors did not block hBD-2/3-induced cytokine production in T cells (data not shown), indicating the effects through the other receptor(s), which should be identified.

Serum hBD-2 levels might represent the balance of hBD-2-inducing versus hBD-2-reducing effects such as IL-22/oncostatin M versus IL-13/IL-4/TSLP, respectively. The hBD-2-induced increase of IL-22/oncostatin M or of IL-13/IL-4 indicates positive or negative feedback to sustain or halt its production, respectively. Increased serum hBD-2 and IL-22 levels and their correlation in AD patients indicate the dominance of the former. We should extensively examine serum hBD-2 levels and their correlations with individual cytokine levels in patients with other inflammatory dermatoses like allergic contact dermatitis.

Conflict of interest

The authors have no conflicts of interest to be disclosed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imbio.2011.10.010.

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