Human β-defensin-3 increases the expression of interleukin-37 through CCR6 in human keratinocytes

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Abbreviations: CCR: CC chemokine receptor; **EGF:** epidermal growth factor; **hBD**: human β-defensin; **HDP**: host defense peptide; **IFN:** interferon; **IL:** interleukin; **LPS:** lipopolysaccharide; **MAPK:** mitogen-activated protein kinase; **NF-κB:** nuclear factor-κB; **PBMC:** peripheral blood mononuclear cells; **TLR:** Toll-like receptor; **TNF:** tumor necrosis factor.

Abstract

Background: Interleukin (IL)-37, a new member of the IL-1 family, is characterized as a fundamental inhibitor of innate immunity: it dampens the production of proinflammatory cytokines, protects against inflammatory and autoimmune diseases, and plays a potent immunosuppressive role in the pathogenesis of psoriasis. IL-37 is highly expressed in psoriatic skin, in which human β-defensins (hBDs) have been detected. Although hBDs enhance the production of cytokines, including IL-1 cytokines, whether they stimulate the production of IL-37 remains unclear.

Objectives: To assess the ability of hBDs to stimulate IL-37 expression/production by human keratinocytes and to determine the mechanism involved.

Methods: Real-time PCR and Western blotting were used to evaluate IL-37 expression. Caspase activities were assessed using colorimetric assay kits. A CCR6 antibody, siRNA, and caspase, Smad3, MAPK and NF-κB inhibitors were used to investigate the signaling mechanism of hBDs.

Results: Among the four hBDs used, only hBD-3 up-regulated the mRNA and protein expression of IL-37. The combination of TNF-α, EGF and poly (I:C) with hBD-3 synergistically enhanced the mRNA but not the protein expression of IL-37. Furthermore, hBD-3 increased the release of IL-37 into the culture supernatants. Evaluation of the signaling mechanism of hBD-3 suggested that caspases 1 and 4, Smad3, CCR6, MAPKs and NF-κB were required for hBD-3-mediated IL-37 expression.

Conclusions: The finding that hBD-3 stimulates IL-37 expression, a novel target for the pathogenesis and therapy of cutaneous inflammatory diseases, provides evidence that hBDs contribute to the suppression of inflammatory and innate immune responses through the regulation of IL-37 expression.

Introduction

Skin-derived host defense peptides (HDPs), also known as antimicrobial peptides, have been proposed to provide the first line of defense against infection by acting not only as 'natural antibiotics' but also as immunoregulators [1, 2]. The major HDPs found in human skin include the defensins and cathelicidin LL-37. Based on gene organization, cellular location, expression pattern and disulfide bond connectivity, human defensins are divided into α- and β-defensins [2, 3]. In contrast to α-defensins, which are distributed in neutrophils and intestinal Paneth cells [4, 5], human β-defensins (hBDs) are mainly generated by the epithelia of several organs, including the skin [1, 2, 6]. To date, four hBDs, hBD-1 through hBD-4, have been identified in human skin. hBD-1 is constitutively expressed by various epithelial tissues, particularly those of the skin and urogenital and respiratory tissues [7, 8]. hBD-2 was initially identified in psoriatic lesions and was later shown to be inducible in activated normal keratinocytes [9, 10]. Similarly to hBD-2, hBD-3 was first isolated from lesional psoriatic scales, but it is also abundant in non-epithelial tissues [11]. hBD-4, the fourth member of the hBDs, has only been identified in the skin at the mRNA level [12] and is inducibly expressed in differentiated keratinocytes [13]. The expression of hBDs is up-regulated in cases of wounding, acne vulgaris, and psoriasis [14-16]; on the contrary, their expression is down-regulated in atopic dermatitis [17, 18]. In addition to their antimicrobial activities, hBDs stimulate keratinocyte cytokine/chemokine production, as well as cell migration and proliferation, regulate skin barrier function and accelerate wound healing [1, 2, 19].

Interleukin (IL)-37 (formerly known as IL-1F7) is a new member of the IL-1 family and comprises 5 isoforms (IL-37a-e), among which IL-37b is the largest and comprises 5 of the 6 exons [20-22]. It has been reported that mature IL-1F7b translocates into the nucleus via a caspase-1-dependent process [23]. Therefore, it was proposed that IL-1F7b may act as a cytokine with intracellular as well as extracellular functionality [20]. IL-37 can be induced by

several toll-like receptor (TLR) agonists and proinflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in peripheral blood mononuclear cells (PBMCs) and dendritic cells [20, 24, 25]. IL-37 is also detectable in keratinocytes and fibroblasts [26, 27], and its expression has also been identified in diverse human tissues, including the skin, tonsils, esophagus, and placenta, as well as in melanoma and in breast, prostate, colon and lung carcinomas [24].

IL-37 inhibits TLR-induced proinflammatory cytokines in macrophages, epithelial cells and keratinocytes [28, 29]. Consequently, it has been characterized as a fundamental inhibitor of innate immunity and inflammatory responses [20] and is therefore distinct from most IL-1 members such as IL-1 and IL-18, which initiate inflammatory reactions [20]. IL-37 protects mice from dextran sulfate sodium-induced colitis, ischemia and reperfusion injury, obesity-induced inflammation, and lipopolysaccharide (LPS)-induced shock and ameliorates inflammatory cytokine production [30, 31]. Furthermore, IL-37 has been shown to mediate antitumor activity via the regulation of innate immunity [30, 31] and to play a potent immunosuppressive role in the pathogenesis of psoriasis [32].

Recently, IL-37 was detected to be abundant in psoriatic skin [32], in which several HDPs, including hBDs, have been shown to be overproduced [1, 2]. Because hBDs have been shown to enhance the production of IL-1 members [1, 14], we hypothesized that these peptides may also stimulate keratinocytes to produce IL-37, thereby participating in the regulation of inflammation in cutaneous diseases. Here, we demonstrated that hBD-3 enhanced the expression and extracellular release of IL-37 in human keratinocytes. hBD-3-mediated IL-37 expression was controlled by caspase-1 and caspase-4, Smad3, CC chemokine receptor 6 (CCR6), and the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways. These observations provide novel evidence that, in addition to their antimicrobial

and immunomodulatory functions, skin-derived HDPs may also contribute to the suppression of inflammatory and innate immune responses through the regulation of IL-37 expression.

Materials and Methods

1. Reagents

Synthetic hBD-1, hBD-2, hBD-3 and hBD-4 were obtained from the Peptide Institute (Osaka, Japan). Anti-Smad3 and anti-phosphorylated Smad3 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-IL-37 antibody was purchased from Abcam (Tokyo, Japan) and anti-CCR6 antibody was from R&D Systems (Minneapolis, MN). ATP, IL-1β, IFN-γ, TNF-α, epidermal growth factor (EGF), flagellin, LPS, peptidoglycan and polyinosinic: polycytidylic acid (poly I:C) were purchased from Sigma-Aldrich (St Louis, MO). Caspase-1 inhibitor I, caspase-4 inhibitor I, U0126 (ERK inhibitor), SB203580 (p38 inhibitor), JNK inhibitor II, NF-κB activation inhibitor II and SIS3 (Smad3 inhibitor) were purchased from Calbiochem (La Jolla, CA).

2. Keratinocyte culture and stimulation

Primary human epidermal keratinocytes isolated from neonatal foreskins were purchased from Kurabo Industries (Osaka, Japan) and were cultured in serum-free HuMedia-KG2 keratinocyte growth medium (Kurabo Industries) as previously described [15]. The cells were serially passaged at 60-70% confluence, and the experiments were conducted at passage three using subconfluent cells (60-80% confluence) in the proliferative phase, unless otherwise specified. For total RNA extraction and Western blot analysis, keratinocytes were cultured in 12-well tissue culture plates. After the 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 peptides or stimulants.

3. Total RNA extraction and real-time PCR

Total RNA was extracted from keratinocytes using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized from 1 μg total RNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's

instructions. Real-time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ). Amplification and detection of mRNA were performed using the StepOne Plus Real-time PCR System (Applied Biosystems) following the manufacturer's specifications. All of the primer/probe sets used in this study were obtained from Assays-on-Demand (Applied Biosystems). All of the real-time PCR reactions were performed in triplicate, and the changes in gene expression are reported as fold increases relative to untreated controls.

4. Western blot analysis

Sub-confluent keratinocytes were incubated with various stimulants, and lysates were obtained by lysing the cells in RIPA buffer (Cell Signaling Technology). The total protein concentrations were determined using the Precision Red Advanced Protein Assay kit (Cytoskeleton, Denver, CO), and equal amounts of total protein (20 - 50 μg in each well) were subjected to 12.5% SDS-PAGE. Non-specific binding sites were blocked with Immunoblock (Dainippon-Pharm, Osaka, Japan) for 1 h at room temperature. The blots were incubated overnight with the appropriate antibodies diluted in a mixture of TBS containing 0.1% Tween 20 and Immunoblock (1:1), according to the manufacturer's instructions. The membranes were developed with the Luminata Forte Western HRP substrate (Millipore, Billerica, MA) and were imaged using Fujifilm LAS-4000 Plus (Tokyo, Japan). In some of the experiments, the cells were pretreated with various inhibitors, and Western blotting was performed as described above. The doses of inhibitors used in this study were not toxic, as tested by lactate dehydrogenase activity (data not shown). All of the experiments included DMSO vehicle controls, and the levels of DMSO in the cell cultures never exceeded 0.1%.

5. Release of IL-37 from keratinocytes

Following stimulation with hBD-3, the cells were treated with ATP for 20 min. The supernatants were harvested via centrifugation and subjected to a membrane-based

concentration step (Amicon Ultra-0.5 Centrifugal Filter, Millipore, Billerica, MA) as a means of concentrating the low molecular weight analytes present in the supernatants, according to the manufacturer's instructions. The 30-fold concentrated solutes were then subjected to 12.5% SDS-PAGE analysis.

6. Caspase enzymatic activity assay

Caspase-1 and caspase-4 enzymatic activities were assayed using caspase-1 and caspase-4 colorimetric assay kits (Biovision, Milpitas, CA) according to the manufacturer's instructions. Briefly, 2 - 5 $\times$ 10^6 cells were lysed in 50 μl cold cell lysis buffer for 10 min, and the cell lysates were centrifuged at 10,000 x *g* for 1 min. A volume of 50 μl cell lysate was added to 50 μl caspase reaction buffer containing 10 mM DTT. Each sample was added to 200 μM caspase-1 substrate (YVAD-*p*NA) or caspase-4 substrate (LEVD-*p*NA), followed by incubation for 2 h at 37ºC. The enzymatic activities were monitored on a microplate reader at a wavelength of 405 nm.

7. Statistical analysis

The statistical analysis consisted of ANOVA followed by the appropriate post hoc test. The statistical analyses were performed with GraphPad Prism for Windows (Prism 5, GraphPad Software, San Diego, CA). A value of $P < 0.05$ was considered significant. The results are presented as the means ± SD.

Results

1. hBD-3 increases IL-37 mRNA and protein levels in keratinocytes

Human defensins have been reported to enhance the expression and secretion of IL-1 members such as IL-1 and IL-18 [14, 33]; therefore, we investigated whether these peptides may also stimulate keratinocytes to express IL-37. As shown in Fig. 1a (left panel), among the four hBDs tested, only hBD-3 significantly and dose-dependently up-regulated the mRNA expression of IL-37b, the largest isoform of the IL-37 splice variants [22]. Surprisingly, other hBDs, even at doses as high as 40 μg/ml, did not increase the expression of IL-37 (data not shown). In preliminary experiments, the mRNA expression of IL-37 peaked within 24 h after exposure to hBD-3. The expression of IL-37 at the protein level was detected via Western blotting; the blots were probed with anti-IL-37 antibody, which corresponds to 24-kDa, mature IL-37. As observed in Fig. 1a (right panel), strong bands representing IL-37 protein in hBD-3-stimulated keratinocytes were identified, demonstrating that hBD-3 induces IL-37 expression at both the mRNA and protein levels.

Because the expression of IL-37 in PBMCs and dendritic cells was shown to be up-regulated by several proinflammatory cytokines, as well as by TLR ligands [20], the effects of these stimulants on IL-37 expression in the presence or absence of hBD-3 were evaluated. The results showed that among the stimulants tested, only TNF-α and EGF alone increased the mRNA expression of IL-37 (6-fold increases, each), and the combination of these stimulants with hBD-3 synergistically enhanced IL-37 expression (17-fold and 23-fold, respectively) (Fig. 1b, left panel). However, at the protein level, neither TNF-α nor EGF up-regulated IL-37 expression, and their combination with hBD-3 failed to further increase IL-37 protein expression (Fig. 1b, right panel). Furthermore, flagellin, LPS, peptidoglycan and poly (I:C) independently elevated IL-37 mRNA expression (between 3- and 5-fold increases), and only the combination of poly (I:C) with hBD-3 resulted in a 24-fold

synergistic increase in IL-37 expression (Fig. 1c, left panel). However, poly (I:C) alone failed to stimulate IL-37 protein expression, and an hBD-3/poly (I:C) combination did not further increase IL-37 protein levels (Fig. 1c, right panel) compared with hBD-3 alone. These observations suggest that different cell types possess different IL-37 expression profiles.

2. hBD-3 enhances the release of IL-37

To determine whether hBD-3 induces the externalization of IL-37, the presence of IL-37 in the cell culture supernatants was assessed. Because IL-37 protein was not detectable in keratinocyte supernatants by specific ELISA (data not shown), we therefore used Western blot analysis. As depicted in Fig. 2, a band representing IL-37 released into the culture supernatants of hBD-3-stimulated keratinocytes was detected, suggesting that hBD-3 induces not only intracellular but also extracellular IL-37 release. Because macrophages have been reported to release IL-37 upon stimulation with LPS and ATP [34], we investigated whether ATP also increases hBD-3-mediated IL-37 release. The addition of extracellular ATP to hBD-3-stimulated keratinocytes for 20 min caused a burst of IL-37 release into the culture supernatants. ATP stimulation alone was insufficient to trigger IL-37 release.

3. hBD-3 increases the expression of IL-37 through the activation of caspase-1 and caspase-4

IL-37 has been reported to be a substrate for both caspase-1 and caspase-4, although caspase-1 appeared to be more efficient than caspase-4 in generating mature IL-37 [24]. To test whether hBD-3-induced IL-37 expression was dependent on caspase activity, the caspase-1 and caspase-4 enzymatic activities in hBD-3-stimulated keratinocytes were assayed using colorimetric assays. We found that hBD-3 markedly increased caspase-1 and caspase-4 activities by up to 6- and 5-fold, respectively (Fig. 3a). We further confirmed that the activation of both caspase-1 and capsase-4 was required for hBD-3-mediated IL-37 expression using various amounts of caspase-1 and caspase-4 inhibitors. As shown in Fig. 3b,

the addition of increasing amounts of caspase-1 and caspase-4 specific inhibitors resulted in the dose-dependent inhibition of IL-37 expression; the highest doses completely abolished IL-37 expression.

4. Activation of Smad3 is necessary for the hBD-3-induced increase of IL-37 expression

Transforming growth factor (TGF)-β is effective in inducing endogenous IL-37, which is relevant for the intracellular interaction with Smad3, and IL-37 has been shown to colocalize with phospho-Smad3 [20]. Therefore, to gain a better understanding of the mechanism by which hBD-3 induces IL-37 expression, we tested whether hBD-3 might induce phosphorylation of Smad3, leading to increased IL-37 expression. As shown in Fig. 4a, Smad3 activation was strongly elevated at 30-120 min during stimulation with hBD-3. We tested the hypothesis that this activation of Smad3 is required for hBD-3-induced IL-37 expression in keratinocytes. We used the specific Smad3 inhibitor, SIS3, to block the activation of Smad3 [35] and found that this inhibitor almost completely suppressed hBD-3-mediated IL-37 expression (Fig. 4b). This result suggests that hBD-3 increases IL-37 expression via the activation of Smad3.

5. hBD-3 enhances the expression of IL-37 through CCR6

Only hBD-3, not other hBDs, induced IL-37 expression, implying that hBD-3 may trigger certain molecules that other hBDs cannot activate. CCR6 appears to be the most interesting of these molecules because only hBD-3 (not other hBDs) has been reported to regulate neutrophil apoptosis and skin barrier function via CCR6 [19, 36]. Therefore, the possible role of CCR6 in hBD-3-mediated IL-37 expression was of particular interest. In the current study, we observed that a CCR6 neutralizing antibody markedly reduced hBD-3-mediated IL-37 expression in keratinocytes (Fig. 5a). To further confirm the requirement for CCR6 in hBD-3-mediated IL-37 expression, keratinocytes were transfected with CCR6 siRNA to knock down CCR6 (Fig. 5b, left panel). CCR6 siRNA markedly reduced hBD-3-increased

IL-37 expression (Fig. 5b, right), suggesting the necessity of CCR6 in hBD-3-induced IL-37 expression.

6. MAPKs and NF-κB are responsible for the hBD-3-induced increase of IL-37 expression

The activation of MAPK and NF-κB targets various downstream molecules, leading to diverse cellular functions, including the production of cytokines and chemokines [37, 38]. Because HDP-induced MAPK and NF-κB activation results in the production of cytokines/chemokines, including IL-1 family members [39], we explored the molecular mechanism by which hBD-3 induces IL-37 expression by investigating the involvement of MAPKs and NF-κB in hBD-3-mediated IL-37 expression. The treatment of keratinocytes with specific inhibitor of ERK (U0126), p38 (SB203580), JNK (JNK inhibitor II), and NF-κB (NF-κB activation inhibitor II) revealed that hBD-3-mediated IL-37 expression was mainly dependent on ERK and also partially required JNK and NF-κB but not p38 activation (Fig. 6).

Discussion

IL-37, a new member of the IL-1 cytokine family, is a potent inhibitor of inflammation and innate immunity in various diseases such as psoriasis [28, 32] where hBDs are overproduced [1, 2]. To further understand the regulation of IL-37, we investigated the effects of hBDs on IL-37 production. This study demonstrated that hBD-3 up-regulated the expression and release of IL-37 in human keratinocytes. hBD-3-induced IL-37 expression was regulated by caspase-1, caspase-4, Smad3, CCR6, and the MAPK and NF-κB pathways. These observations provide novel evidence of the contribution of hBDs to the suppression of inflammatory and innate immune responses through the regulation of IL-37 expression.

In vitro, several proinflammatory cytokines (IL-1β, IL-18, IFN-γ and TNF-α) and TLR ligands (LPS, Pam_3CSK_4 and CpG) have been shown to induce IL-37 protein expression in PBMCs and dendritic cells [20]. However, among the stimulants tested, we found that TNF-α, EGF, flagellin, LPS, peptidoglycan and poly (I:C) independently increased IL-37 mRNA but not protein expression, suggesting that different cell types possess different IL-37 expression profiles. Moreover, we found that the combination of TNF-α, EGF and a TLR3 ligand, poly (I:C), with hBD-3 synergistically enhanced IL-37 mRNA levels but failed to further increase the protein levels. Although the mechanism of synergism between cytokines, TLR ligands and HDPs is unclear, interactions between these stimulants have been reported. For instance, TNF-α has been shown to enhance hBD expression [13], and the induction of hBDs in keratinocytes was found to be mediated by EGF receptor (EGFR) transactivation [40]. Furthermore, poly (I:C) has been reported to induce hBD expression in epithelial cells [41]. Interestingly, HDPs, including hBDs themselves, have been reported to induce TNF-α production [42], to indirectly stimulate EGFR and downstream cascades [15], and to bind to poly (I:C), leading to enhanced TLR3 signaling [43]. Taken together, we assume that these interactions may lead to the synergistic enhancement of IL-37 expression mediated by

cytokines, TLR ligands and hBD-3. However, it is important to note that although these stimulants cooperatively increased the synthesis of IL-37 mRNA, this synergistic effect did not necessarily lead to further increased protein synthesis. We suspect that this is may be due to mechanisms of IL-37 protein posttranslational control, including degradation, that have yet to be identified.

Similarly to IL-1 and IL-18, IL-37 is also cleaved by caspases [24]. It has been proposed that caspase-processed IL-37 either is secreted extracellularly to inhibit the actions of proinflammatory cytokines or their receptors [34] or translocates to the nucleus, where it interacts with Smad3 to suppress the transcription of proinflammatory cytokines [20, 23]. Using Western blot analysis, we found that hBD-3 induced the extracellular secretion of IL-37 into the culture supernatants and that this secretion was further enhanced by exposure of hBD-3-stimulated keratinocytes to ATP, which is known to be responsible for the maturation and secretion of IL-1β, IL-18 and IL-37 in PBMCs and macrophages [34, 44]. An attempt to assess IL-37 concentrations in keratinocyte supernatants failed because IL-37 protein was not detectable by ELISA, suggesting relatively low extracellular concentrations of IL-37 in human keratinocytes.

Although both caspase-1 and caspase-4 have been reported to cleave precursor IL-37, caspase-1 appeared to be more efficient than caspase-4 in generating mature IL-37 [24]. Here, we showed that hBD-3-induced IL-37 expression was dependent on both caspases because treatment with caspase-1 and caspase-4 specific inhibitors abolished IL-37 expression and hBD-3 induced the activation of both caspase-1 and caspase-4 to almost comparable levels. In addition, we found that hBD-3 induced the activation of Smad3 and that hBD-3-mediated IL-37 expression was inhibited by the specific Smad3 inhibitor SIS3. This finding is in accordance with previous reports showing the association of IL-37 with phosphorylated Smad3 and the observation of a IL-37-Smad3 complex in IL-37b-overexpressing cells [20].

Moreover, inhibition of Smad3 with SIS3 or Smad3-specific siRNA increased the production of various proinflammatory cytokines [20]. These *in vitro* findings were confirmed *in vivo*, where the reduction of LPS-induced lung inflammation in IL-37 transgenic mice was reversed in transgenic mice with Smad3 knocked down in the lungs [20]. These observations imply that Smad3 is a key molecule in the IL-37-mediated inhibition of inflammatory responses.

hBD-1 through hBD-4 are known to activate keratinocytes [1, 2]. Here, we found that only hBD-3, not other hBDs, induced IL-37 expression, raising the possibility that hBD-3 may interact with certain specific molecules in keratinocytes, to which other hBDs cannot bind. It has been noted that although hBDs activate various cell types through receptors such as CCR2, CCR6, EGFR, and TLRs [1, 2], only hBD-3 regulates neutrophil apoptosis and skin barrier function via CCR6 [19, 36]. In this study, CCR6 was in fact necessary for the hBD-3-induced IL-37 expression because both the CCR6 neutralizing antibody and CCR6 siRNA suppressed IL-37 induction by hBD-3. However, the inhibitory effects of the CCR6 antibody and CCR6 siRNA were not complete, indicating the involvement of other signaling pathway(s). For instance, due to the very high cationicity of hBD-3 (compared with other hBDs), we assume that hBD-3 might also utilize non-selective membrane receptors to activate keratinocytes, as has been reported for many cationic peptides [45].

To further identify the downstream target of hBD-3, we investigated the involvement of MAPKs and NF-κB in hBD-3-induced IL-37 expression. The activation of MAPKs and NF-κB by different stimuli targets various downstream molecules, resulting in the induction of cytokines/chemokines, adhesion molecules, cell growth and differentiation, and antimicrobial peptides, as well as the initiation of innate epithelial immune responses against infection [37, 38]. MAPKs and NF-κB have been implicated in cytokine/chemokine production by keratinocytes stimulated by HDPs, including hBDs, LL-37 and Dermcidin [14, 46, 47]. Here, we showed that hBD-3-mediated IL-37 expression was mainly dependent on

ERK activation and moderately required JNK but not p38 activation. Interestingly, a very recent report demonstrated that triptolide- and triptonide-induced IL-37 expression in THP-1 cells occurred via ERK and p38 activation [48], suggesting that the involvement of MAPK in IL-37 expression may differ depending on the stimulants as well as the cell types used. In addition to the MAPK pathway, we observed that IL-37 expression was partially controlled by NF-κB, a downstream target of the MAPK pathway that regulates the transcription of various genes, including those that lead to the expression of IL-1 cytokine family members [49, 50].

IL-37 expression is likely linked to inflammation. IL-37 is constitutively expressed in various cells, and its expression is increased by inflammatory cytokines and TLR ligands [20]. IL-37 expression has been detected in human inflammatory and autoimmune diseases such as rheumatoid arthritis, *Mycobacterium avium* infection, atherosclerotic coronary and carotid artery plaques, Crohn's disease, obesity, lupus, carcinoma, and psoriasis [27, 30, 32]. Teng *et al*. recently reported that IL-37 was highly expressed in psoriatic lesions compared with healthy skin and that its source seemed to be T cells and macrophages rather than keratinocytes and fibroblasts, as analyzed by immunohistochemistry [32]. In contrast, in another study, IL-37 was only detected at the mRNA level in psoriatic keratinocytes; however, this expression did not differ from that in normal keratinocytes and was not increased upon IL-17 stimulation [27]. Fujita *et al*. also detected IL-37 mRNA but not protein in normal keratinocytes and fibroblasts [26]. In the above reports, we suspect that the failure to detect IL-37 protein in keratinocytes/fibroblasts was most likely due to the experimental assays, which consisted of either immunohistochemical or ELISA analysis. In our study, we detected IL-37 protein both in the cell lysates and culture supernatants of hBD-3-stimulated keratinocytes using Western blot analysis, but could not detect it using ELISA (data not shown).

IL-37 represents a potent immunosuppressor in the pathogenesis of psoriasis, in which HDPs such as hBDs and LL-37 have been shown to be overproduced [1, 2], and has emerged as a novel research target for the therapy of autoimmune and cutaneous inflammatory diseases [32]. We observed that cathelicidin LL-37 also induced IL-37 expression by a different mechanism than hBD-3 (manuscript submitted). Therefore, our finding that HDPs induce IL-37 expression in keratinocytes provides novel evidence that, in addition to their antimicrobial and immunomodulatory functions, these peptides may also contribute to the suppression of inflammatory and innate immune responses in skin diseases such as psoriasis through the regulation of IL-37 expression.

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Figure legends

Figure 1. hBD-3 enhances the expression of IL-37 by human keratinocytes

(a) Effects of hBDs on IL-37 expression. **Left panel:** Keratinocytes were stimulated with 5-20 μg/ml of hBD-1 through hBD-4 or the diluent (Ctrl, control) for 24 h. Following incubation, real-time PCR was performed to analyze the changes in gene expression of IL-37b. **Right panel:** Keratinocytes were stimulated with hBD-3 or the diluent (control) for 48 h. After stimulation, cell lysates were obtained, and equal amounts of total protein were subjected to 12.5% SDS-PAGE analysis. The results are representative of three separate experiments with similar results. **(b)** Keratinocytes were stimulated for 24 h with 20 ng/ml IL-1β, 200 ng/ml IFN-γ, 40 ng/ml TNF-α, 80 ng/ml EGF, and 20 μg/ml hBD-3 alone or in combination. Real-time PCR was performed as described above. **Right panel:** Keratinocytes were stimulated for 48 h with 40 ng/ml TNF-α, 80 ng/ml EGF, and 20 μg/ml hBD-3 alone or in combination. IL-37 immunoblotting was performed as described above. **(c) Left panel:** Keratinocytes were stimulated for 24 h with 100 ng/ml flagellin, 100 ng/ml LPS, 100 μg/ml peptidoglycan (PGN), 25 μg/ml poly (I:C), and 20 μg/ml hBD-3 alone or in combination. Real-time PCR was performed as described above. **Right panel:** Keratinocytes were stimulated for 48 h with 25 μg/ml poly (I:C) and 20 μg/ml hBD-3 alone or in combination (or with the diluent), followed by IL-37 immunoblotting. The values were compared between stimulated and non-stimulated cells. Each bar represents the means ± SD of four to six separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ when values were compared between stimulated and non-stimulated cells (Ctrl, control). # $P < 0.05$, ### $P < 0.001$, #### $P < 0.0001$ when values were compared between combined stimulation and hBD-3-stimulated cells.

Figure 2. hBD-3 enhances the release of IL-37 by human keratinocytes

Keratinocytes were cultured in the presence or absence of 20 μg/ml hBD-3 for 48 h; the cells were then exposed to 5 mM ATP for 20 min. The supernatants were harvested via centrifugation and subjected to a membrane-based concentration step, according to the manufacturer's instructions. The concentrated solutes were then subjected to 12.5% SDS-PAGE analysis. The results are shown for one experiment; two additional experiments yielded similar results.

Figure 3. hBD-3 induces IL-37 expression through the activation of caspase-1 and caspase-4

(a) Effect of hBD-3 on caspase-1 and caspase-4 enzymatic activities. Keratinocytes were incubated with 20 μg/ml hBD-3 for 48 h, and enzymatic activities were assayed using caspase-1 and caspase-4 colorimetric assay kits. The data normalized to the negative control (Ctrl: control) are shown as fold increases in caspase activity and are indicated as the means ± SD of three to five separate experiments. Values are compared between stimulated and non-stimulated cells. *** $P < 0.001$. **(b)** The effects of caspase-1 and caspase-4 inhibitors on hBD-3-mediated IL-37 expression. Keratinocytes were pre-treated with 25-100 μM caspase-1 inhibitor I (caspase-1 inh), 2.5-10 μM caspase-4 inhibitor I (caspase-4 inh) or 0.1% DMSO for 2 h, and the cells were then exposed to 20 μg/ml hBD-3 for 48 h, followed by IL-37 immunoblotting. The results are shown for one experiment; two additional experiments yielded similar results.

Figure 4. hBD-3 activates Smad3, which is necessary for IL-37 expression

(a) Keratinocytes were incubated with 20 μg/ml hBD-3 for 10-120 min and lysed. Equal amounts of protein were immunoblotted using antibodies directed against Smad3 and phosphorylated (p-) Smad3 proteins. The results are shown for one experiment; two

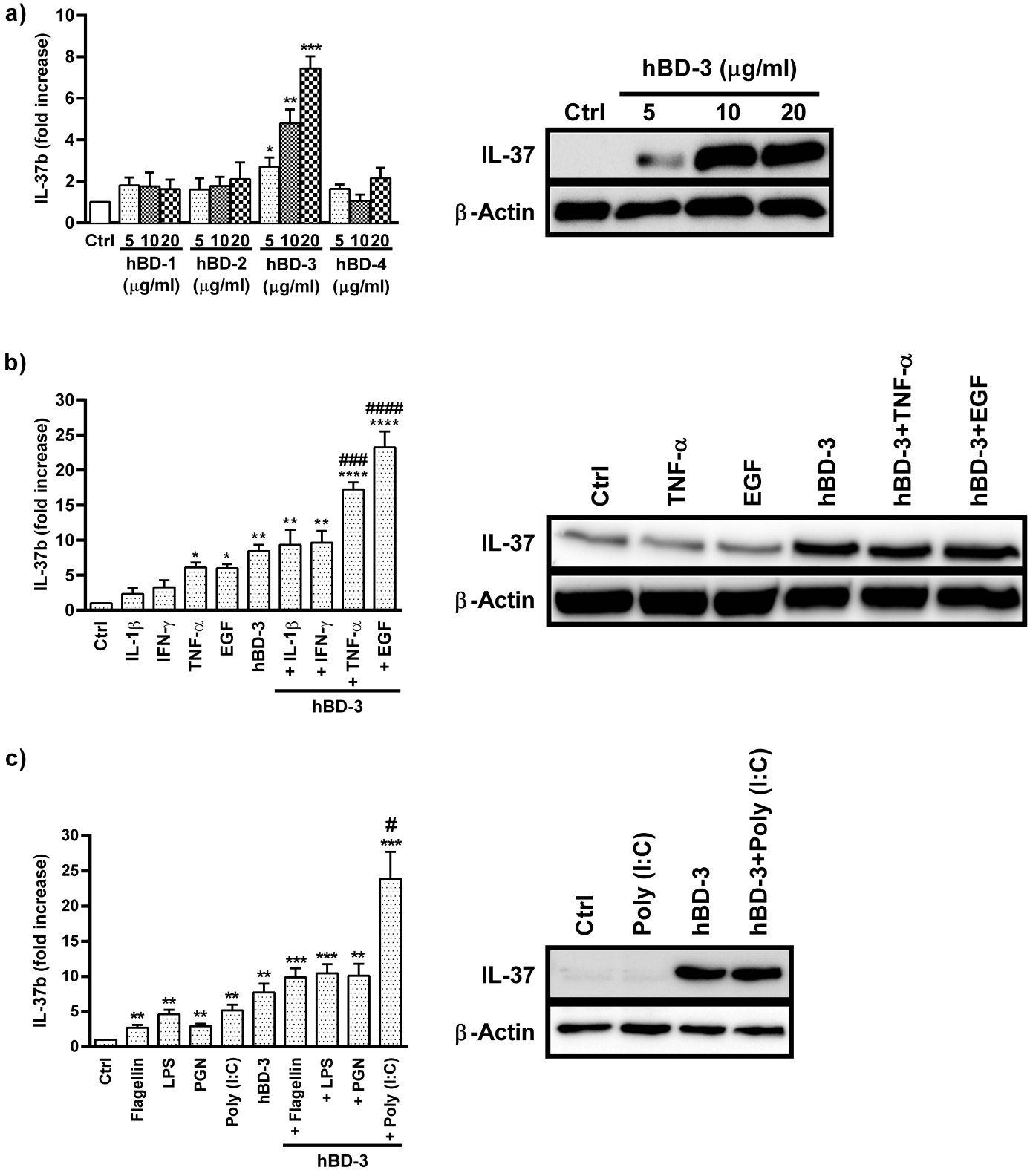
additional experiments yielded similar results. **(b)** hBD-3 increases IL-37 expression through Smad3 activation. Keratinocytes were pre-treated with 10 μM SIS3 or 0.1% DMSO for 2 h; the cells were then exposed to 20 μg/ml hBD-3 for 48 h, followed by IL-37 immunoblotting. The results are shown for one experiment; three additional experiments yielded similar results.

Figure 5. Effects of anti-CCR6 antibody and CCR6 siRNA on hBD-3-induced IL-37 expression

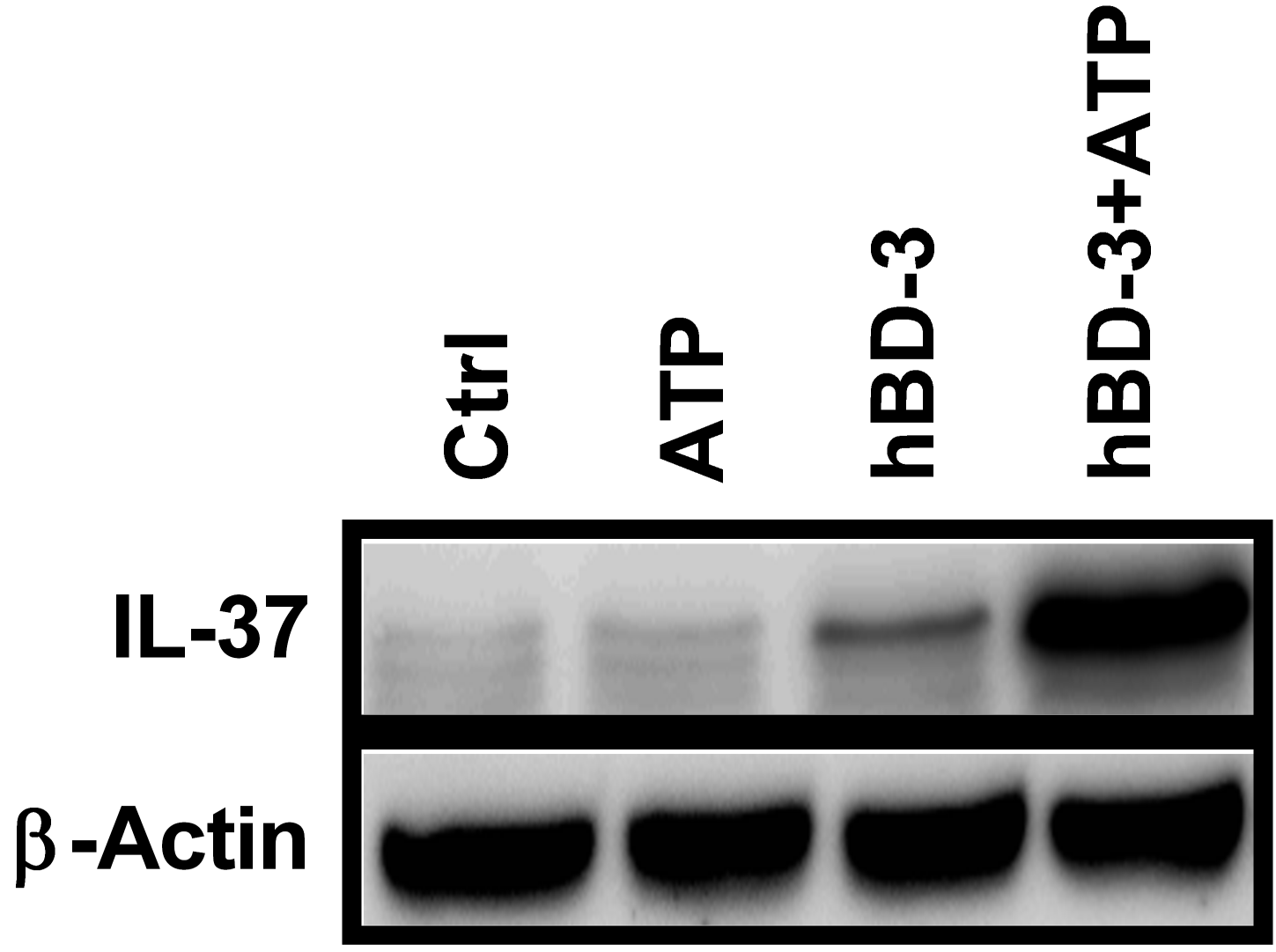
(a) Keratinocytes were pre-treated with 50 μg/ml anti-CCR6 (+CCR6 Ab) or isotype control antibodies for 24 h and stimulated with 20 μg/ml hBD-3 for 48 h, followed by IL-37 immunoblotting. **(b) Left panel:** Keratinocytes were transfected with 400 nM CCR6 siRNA or control siRNA for 48 h for gene silencing, and CCR6 mRNA expression was evaluated by qRT-PCR using CCR6-specific primers/probes. Values are the mean ± SD of three separate experiments and were compared between CCR6 siRNA-transfected (CCR6 siRNA) and control siRNA-transfected cells (Ctrl siRNA). **** $P < 0.0001$. **Right panel:** Transfected keratinocytes were stimulated with 20 μg/ml hBD-3 for 48 h; IL-37 immunoblotting was then performed. The results are shown for one experiment; two additional experiments yielded similar results.

Figure 6. hBD-3 enhances IL-37 expression through MAPKs and NF-κB

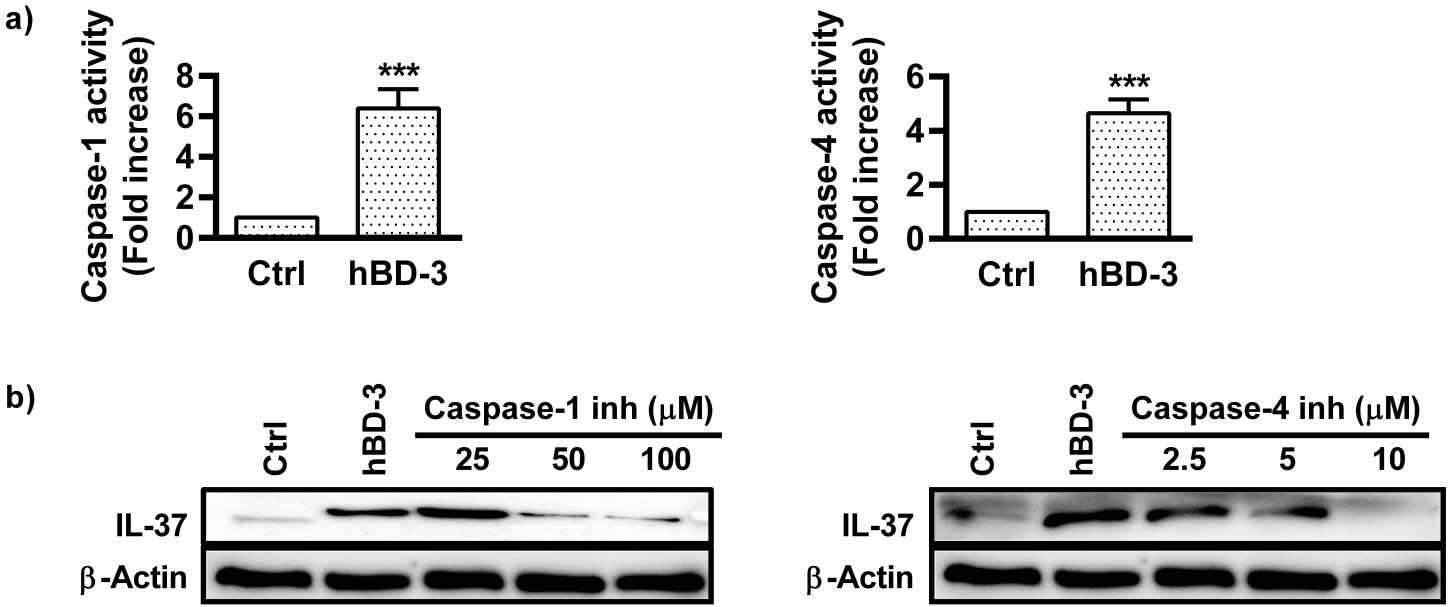
Keratinocytes were pre-treated with 10 μM U0126, SB203580 (SB), JNK inhibitor II (JNK inh), NF-κB activation inhibitor II (NFκBAI II) or 0.1% DMSO for 2 h; the cells were then exposed to 20 μg/ml hBD-3 for 48 h. IL-37 immunoblotting was performed as described above. The results are shown for one experiment; two additional experiments yielded similar results.



Smithrithee R. *et al.* Figure 1



Smithrithee R. *et al.* Figure 2



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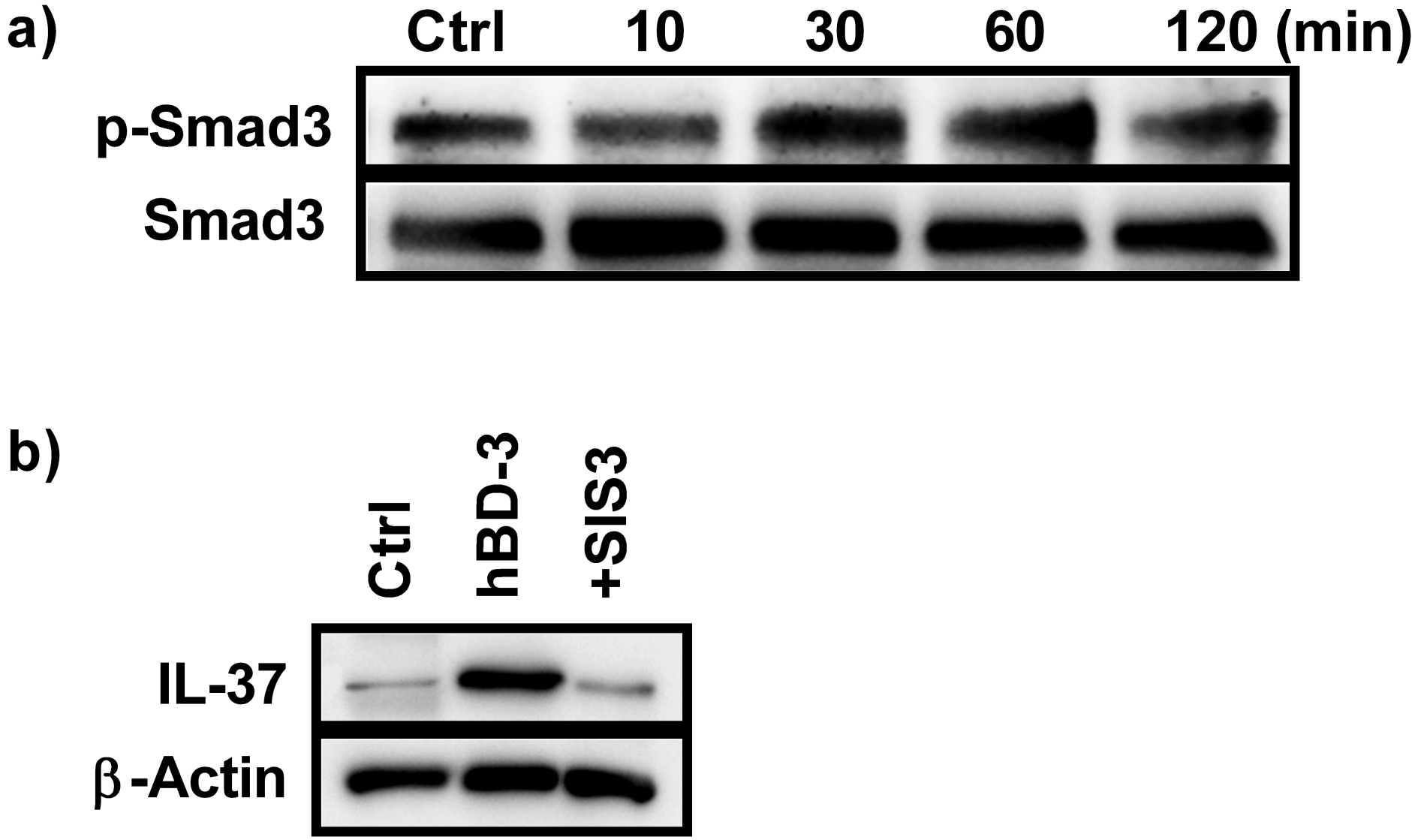

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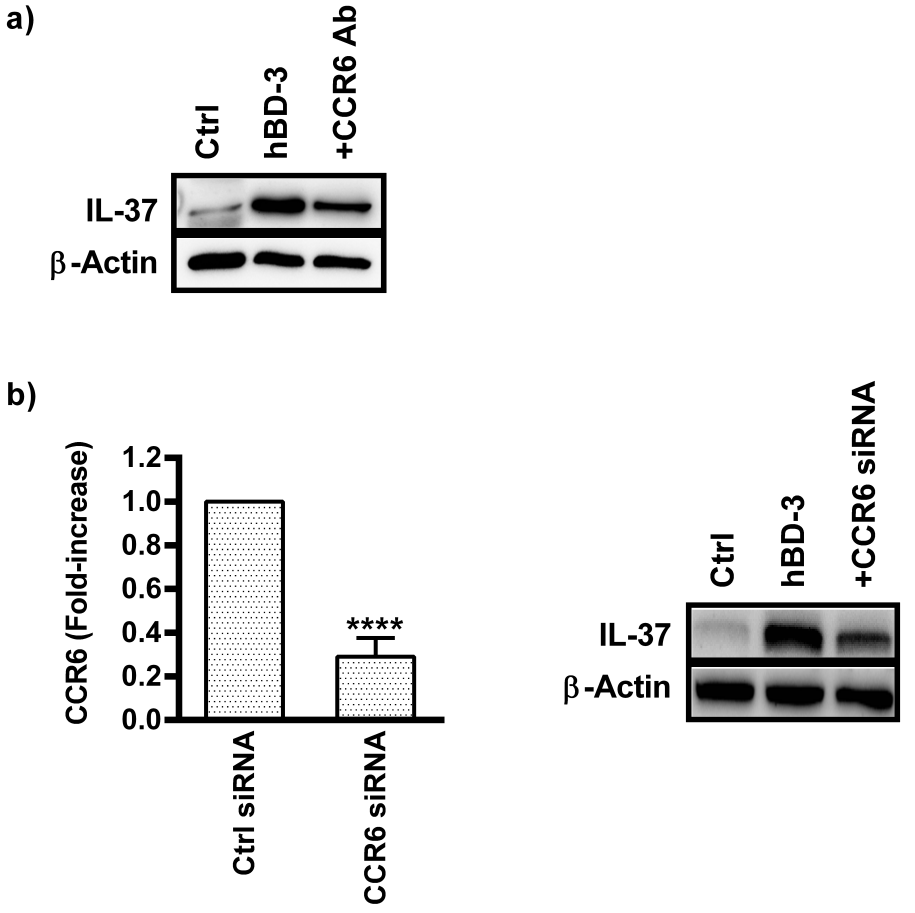
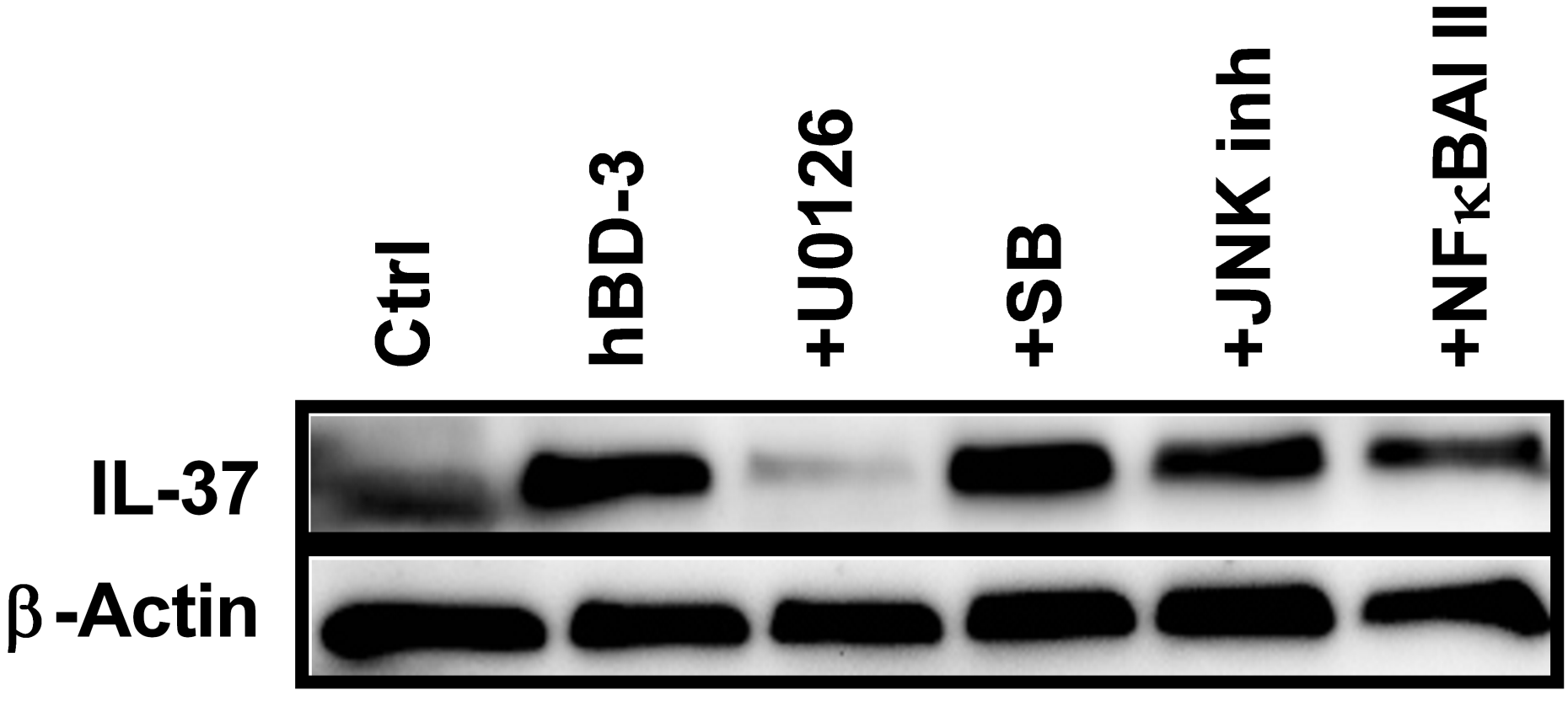


Smithrithee R. *et al.* Figure 5



Smithrithee R. *et al.* Figure 6