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IL-4 and IL-13 Negatively Regulate TNF-α- and IFN-γ-Induced β-Defensin Expression through STAT-6, Suppressor of Cytokine Signaling (SOCS)-1, and SOCS-3[1]

Cristina Albanesi,* Heather R. Fairchild,† Stefania Madonna,* Claudia Scarponi,* Ornella De Pità,* Donald Y. M. Leung,† and Michael D. Howell†

Human β-defensins (HBDs) are a major class of antimicrobial peptides that play an important role in the innate immune response, however, the induction and regulation of these antimicrobial peptides is not well understood. We demonstrate here that stimulation of keratinocytes with TNF-α/IFN-γ induces HBD-2 and HBD-3 by activating STAT-1 and NF-κB signaling. We further demonstrate that IL-4 and IL-13 activate STAT-6 and induce the suppressors of cytokine signaling (SOCS)-1 and -3. This interferes with STAT-1 and NF-κB signaling, thereby inhibiting TNF-α/IFN-γ-mediated induction of HBD-2 and HBD-3. These data suggest that targeting the STAT-1-signaling pathway or suppressor of cytokine signaling expression enhances β-defensin expression and represents a new therapeutic strategy for reduction of infection in human diseases associated with β-defensin deficiency. ***The Journal of Immunology,*** **2007, 179: 984–992.**

Antimicrobial peptides (AMPs)[3] are integral components of the innate immune system found in mammalian and plant systems (1). Human β-defensins (HBDs) are one major class of AMPs that are primarily expressed in the epithelial cells (2–5). HBD-2 and HBD-3 are two members of the β-defensin family that are known to be important components of the innate immune response due to their antimicrobial activity (3–5). Additionally, defensins recruit dendritic and T cells to inflammatory sites through their interaction with CCR6 (6, 7), thereby linking the innate and adaptive immune responses. Defensins can be constitutively expressed or induced in response to exogenous stimuli or tissue injury (3, 4, 8).

Atopic dermatitis (AD) and cystic fibrosis (CF), which are complicated by recurrent infections, have been associated with decreased or impaired production of HBDs (5, 9–11). These diseases affect millions of people and are associated with significant reduction in quality of life. Recent studies have shown that HBD-2 and HBD-3 are effective at killing *Staphylococcus aureus* and *Pseudomonas aeruginosa* (3, 5), two bacteria associated with chronic infection in AD and CF patients, respectively (11, 12). Additionally, it has been shown that *S. aureus* and *P. aeruginosa* potentiate a Th2 environment (13, 14). Previously, we have shown that the Th2 cytokines, IL-4 and IL-13, down-regulate HBD expression in AD keratinocytes (5, 10, 15).

It has been postulated that IL-4 and IL-13 activate STAT-6 which inhibits subsequent signaling events (10, 16); however, the molecular events underlying the Th2 cytokine down-regulation of HBD-2 and HBD-3 in keratinocytes have not been delineated. In the current study, we show that TNF-α and IFN-γ signal through STAT-1 and NF-κB to induce the expression of HBD-2 and HBD-3 in human keratinocytes. We further demonstrate here that IL-4 and IL-13 activate STAT-6 and induce the suppressors of cytokine signaling (SOCS)-1 and -3 which in turn inhibit TNF-α- and IFN-γ-mediated induction of HBD-2 and -3.

Materials and Methods

Keratinocyte cultures

The HaCaT human keratinocyte cell line was grown in DMEM (Cellgro) as previously described (10). Primary keratinocyte cultures were obtained from Cascade Biologics or prepared from normal surgical tissue. Cells were maintained in serum-free EpiLife medium (Cascade Biologics) or serum-free keratinocyte growth medium (Clonetics) (10, 17). Keratinocytes were seeded at 2×10^5 cells/ml and allowed to adhere for 18–24 h before stimulation. Keratinocytes were then stimulated with 200 U/ml IFN-γ (R&D Systems), 20–50 ng/ml TNF-α (R&D Systems), 50 ng/ml IL-4 (R&D Systems), 50 ng/ml IL-13 (R&D Systems), or a combination of these cytokines.

In some experiments, keratinocytes were first treated for 24 h with a cell-permeable quinazoline compound (6-amino-4-(4-phenoxyphenylethylamino) quinazoline) (10 nM; Calbiochem) which inhibits NF-κB transcriptional activation (18). Cells were then stimulated for an additional 24 h with combinations of IFN-γ (R&D Systems), TNF-α (R&D Systems), IL-4 (R&D Systems), and IL-13 (R&D Systems).

RNA isolation and analysis

Total RNA was isolated using TRIzol (Invitrogen Life Technologies) or RNeasy Mini kits (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA and analyzed by real-time RT-PCR as previously described (10) or by RT2 Profiler PCR array (Superarray). HBD-2 primers were used with traditional real-time PCR. Primers and

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[3] Abbreviations used in this paper: AMP, antimicrobial peptide; HBD, human β-defensin; AD, atopic dermatitis; CF, cystic fibrosis; SOCS, suppressor of cytokine signaling; ChIP, chromatin immunoprecipitation; ODN, oligodeoxynucleotide; siRNA, short-interfering RNA; SH2, Src homology 2.

Table I. *Oligonucleotide sequences used for real-time RT-PCR*

Gene	Forward	Reverse
HBD-2	5′-TCCTCTTCTCGTTCCTCTTCATATTC-3′	5′-TTAAGGCAGGTAACAGGATCGC-3′
HBD-3	5′-GTCGCCTTCCAAAGGAGGA-3′	5′-TTCTTCGGCAGCATTTTCG-3′
β-actin	5′-CATCGAGCACGGCATCGTCA-3′	5′-TAGCACAGCCTGGATAGCAAC-3′
CXCL10	5′-TCTAAGTGGCATTCAAGGAGTACC-3′	5′-CAGTAAATTCTTGATGGCCTTCGA-3′
ICAM-1	5′-GTGACATGCACGACCTCCTG-3′	5′-TCCATGGTGATCTCTCCTCA-3′
DRα	5′-CGAGTTCTATCTGAATCCTG-3′	5′-GTTCTGCTGCATTGCTTTTGC-3′
GAPDH	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	5′-CATGTGGGCCATGAGGTCCACCAC-3′

probes for GAPDH, SOCS-1, and SOCS-3 were purchased from Applied Biosystems and Superarray. HBD-2 and HBD-3 primers and probes were prepared as previously described (10). β-actin was evaluated using sequences described in Table I. Quantitative analysis of CXCL10, ICAM-1, and HLA-DRα gene expression was performed using standard RT-PCR with titrated RNA concentrations, primers, and PCR cycles. Sequences for HBD-2, HBD-3, CXCL10, ICAM-1, DRα, and GAPDH are described in Table I. Relative expression levels (nanograms) were calculated by the relative standard curve method as outlined in the manufacturer's technical bulletin (Applied Biosystems). A standard curve was generated using the fluorescent data from 10-fold serial dilutions of total RNA of the highest expression sample. To allow for comparisons between samples and groups, quantities of all targets in test samples were normalized to the corresponding GAPDH levels and expressed as nanograms of target gene normalized to GAPDH.

Chromatin immunoprecipitation (ChIP) analysis

The ChIP assays were conducted as described by Hung et al. (19) with minor modifications. Briefly, 20×10^6 keratinocytes were stimulated with medium or IFN-γ and TNF-α for 45 min. Cells were then fixed with 1% formaldehyde and washed twice with PBS. The cell pellet was lysed and sonicated on ice to break the chromosomal DNA into fragments with an average length of 100–500 bp. After centrifugation, 1% of the extract was aliquotted and used for the total input control. The remaining extract was precleared with a protein A/G-agarose slurry (Santa Cruz Biotechnology) saturated by salmon sperm DNA. Immunoprecipitations were performed by adding normal rabbit Ig, anti-STAT-1 (sc-345), and anti-NF-κB p65 (sc-109) Abs (Santa Cruz Biotechnology). The subsequent steps of washing, eluting, and the purification of DNA were conducted with the ChIP assay kit (Upstate Biotechnology). The purified DNA was resuspended in 20 μl of Tris-EDTA buffer and analyzed by quantitative PCR. NF-κB-binding sites in the HBD-2 promoter region were revealed using the primer pairs: 5′-GGGATTTTCTGGGGTTTCCTG-3′ and 5′-CTGGGGAGGACA TCAAGCCTTC-3′ and (position from −161 to +26), and 5′-CAGAGA AAGCCCTGGCTCCC-3′ and 5′-AGATCTGAGCTGGTGAAATTTATG TG-3′ (position from −798 to −552). STAT-1 sites in the HBD-2 promoter region were detected using the oligonucleotides 5′-TGGGGAGT TTCAGGGGAAC-3′ and 5′-CAGGAAACCCCAGAAAATCCC-3′ (position from −574 to −161), 5′-ATCTTGGCTGCACAATATCGTTAC-3′ and 5′-CATCCCGGGCACTCAGG-3′ (position from −1303 to −1009). The putative NF-κB-binding site in the HBD-3 promoter region was amplified using the primer pair: 5′-CAGGCTGAGAGCTGGTGCTAA-3′ and 5′-CAGGAATCCAATGGATGGGTT-3′ (position from −1942 to −1266). Finally, STAT-1 sites were studied in the HBD-3 promoter region using the following oligonucleotides: 5′-ACCCCATCATAGGCCAA-3′ and 5′-AATATCTGGCAATAGGACTGGCTTT-3′ (position from −502 to −410), and 5′-TTGTCCCTTCCTATAGTATGTG-3′ and 5′-CAGGAA TCCAATGGATGGGTT-3′ (position from −1484 to −1266).

Oligodeoxynucleotide (ODN) technique

Double-stranded ODNs were prepared from complementary single-stranded primers (MWG Biotech) by melting at 95°C for 5 min, followed by a cool-down phase of 1 h at room temperature. The efficiency of the hybridization reaction was verified on 3% agarose gels and usually found to exceed 95%. The sequences of the single-stranded ODNs were as follows: STAT-1 decoy, 5′-CATGTTATGCATATTCCTGTAAGTG-3′; mutated STAT-1 decoy, 5′-CATGTTATGCATATTGGAGTAAGTG-3′; NF-κB decoy, 5′-CCTTGAAGGGATTTCCCTCC-3′; mutated NF-κB decoy, 5′-CATGTCGTCACTGCGCTCAT-3′. Human keratinocyte cultures were transfected in duplicate with 0.5 or 3 μg of decoy ODNs using Lipofectin reagent (Invitrogen Life Technologies). Typically, $1.5–2 \times 10^5$ cells were grown in 6-well plates for 24–48 h (60–80% confluency), transfected with ODNs for 8 h, and then stimulated with 200 U/ml IFN-γ and 50 ng/ml TNF-α for 18 h.

Transient and stable transfectants

HaCaT cells were permanently transfected with pcDNA-myc/SOCS1–3 or empty pcDNA3 plasmids, as previously described (17). HaCaT clones expressing SOCS-1 or SOCS-3 proteins were selected by Western blot analysis using anti-c-*myc* 9E10 mAb (Santa Cruz Biotechnology). Human keratinocytes grown in 6-well plates were transiently cotransfected with increasing concentrations (0.1–2 μg) of pcDNA3-myc/SOCS1–3 plasmids and 0.5 μg of pGAS-Luc (Stratagene). After overnight culture, the cells were incubated for 24 h with IFN-γ plus TNF-α in serum-free medium, and then lysed. Luciferase activities were measured using a luciferase assay system (Promega) and normalized to the β-galactosidase activities.

HaCat cells were transiently transfected at 60% confluency with 100 nM SOCS-3 short-interfering RNA (siRNA) (16811; Ambion), SOCS-1 siRNA, STAT-1 siRNA (51326; Ambion), STAT-6 siRNA (16811; Ambion), or control siRNA (4611; Ambion) using Lipofectamine 2000 (Invitrogen Life Technologies).

Western blot analysis

Protein extracts were prepared by solubilizing cells in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing a mixture of protease and phosphatase inhibitors. Alternatively, the protein fractions of keratinocyte membranes and cytosols were prepared following a procedure previously described (20). Proteins were subjected to SDS-PAGE or NuPage and transferred to a polyvinylidene difluoride membrane. Membranes were then blocked with nonfat dried milk or 3–5% BSA and probed with primary Abs against anti-STAT-1 (E-23; Santa Cruz Biotechnology), anti-phosphotyrosine STAT-1 (Tyr^{701}; New England Biolabs), anti-phosphoserine STAT-1 (Ser^{727}; Upstate Biotechnology), anti-STAT-1α (p91) (33-1400; Zymed Laboratories), anti-phosphotyrosine (pY701) STAT-1 (33-3400; Zymed Laboratories), anti-phosphoserine (pS727) STAT-1 (44-382G; BioSource International), anti-IκBα (sc-371; Santa Cruz Biotechnology), anti-NF-κB p65 (SA-171; Biomol Research Laboratories), and p50 subunits (SA-170; Biomol Research Laboratories). Filters were properly developed with secondary Ig Abs conjugated to HRP using the ECL-Plus Detection System (GE Healthcare Bio-Sciences) followed by autoradiography.

Testing of NF-κB-binding activity

NF-κB transcription factor activation was detected and quantified in untreated and IFN-γ/TNF-α-stimulated SOCS-expressing clones using the TransAM NF-κB family kit (Active Motif) following the manufacturer's instructions.

Statistical analysis

A minimum of three independent experiments were conducted for each keratinocyte experiment. Data from one representative experiment are shown. Experiments were conducted a minimum of three times. Data were analyzed by a one-way ANOVA and significant differences were determined by a Tukey-Kramer test (21). Differences were considered significant at $p \leq 0.05$. Independent experiments were conducted with a minimum of three replicates per condition to allow for statistical comparison.

Results

TNF-α and IFN-γ induce HBD-2 and HBD-3 through STAT-1 and NF-κB

TNF-α and IFN-γ are known to activate STAT-1 and NF-κB in fibroblasts (22). Therefore, we evaluated the promoter regions of

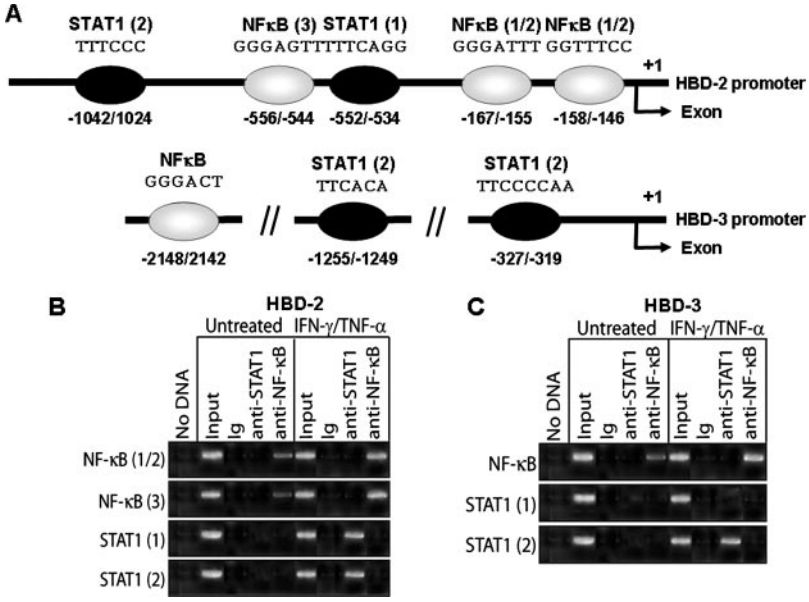

FIGURE 1. STAT-1 and NF-κB bind to the endogenous HBD-2 and HBD-3 promoter regions in keratinocytes treated with TNF-α and IFN-γ. *A*, Schematic representation of HBD-2 and HBD-3 promoter regions showing putative binding sites for STAT-1 and NF-κB transcription factors. *B*, ChIP analysis of STAT-1- and NF-κB-binding sites in the HBD-2 promoter following stimulation with TNF-α and IFN-γ. *C*, ChIP analysis of STAT-1- and NF-κB-binding sites in the HBD-3 promoter following stimulation with TNF-α and IFN-γ.

HBD-2 and HBD-3 for STAT-1- and NF-κB-binding sites using ChIP analysis. As shown in Fig. 1*A*, the HBD-2 promoter region contains three putative binding sites for NF-κB located at positions −158/−146 (NF-κB (1/2)), −167/−155 (NF-κB (1/2)) and −556/−544 (NF-κB (3)), with the upstream site partially overlapping with a STAT-1-binding sequence (−552/534) (STAT-1 (1)). An additional highly conserved STAT-1 site was found in the promoter region between −1042 and −1024 (STAT-1 (2)). The

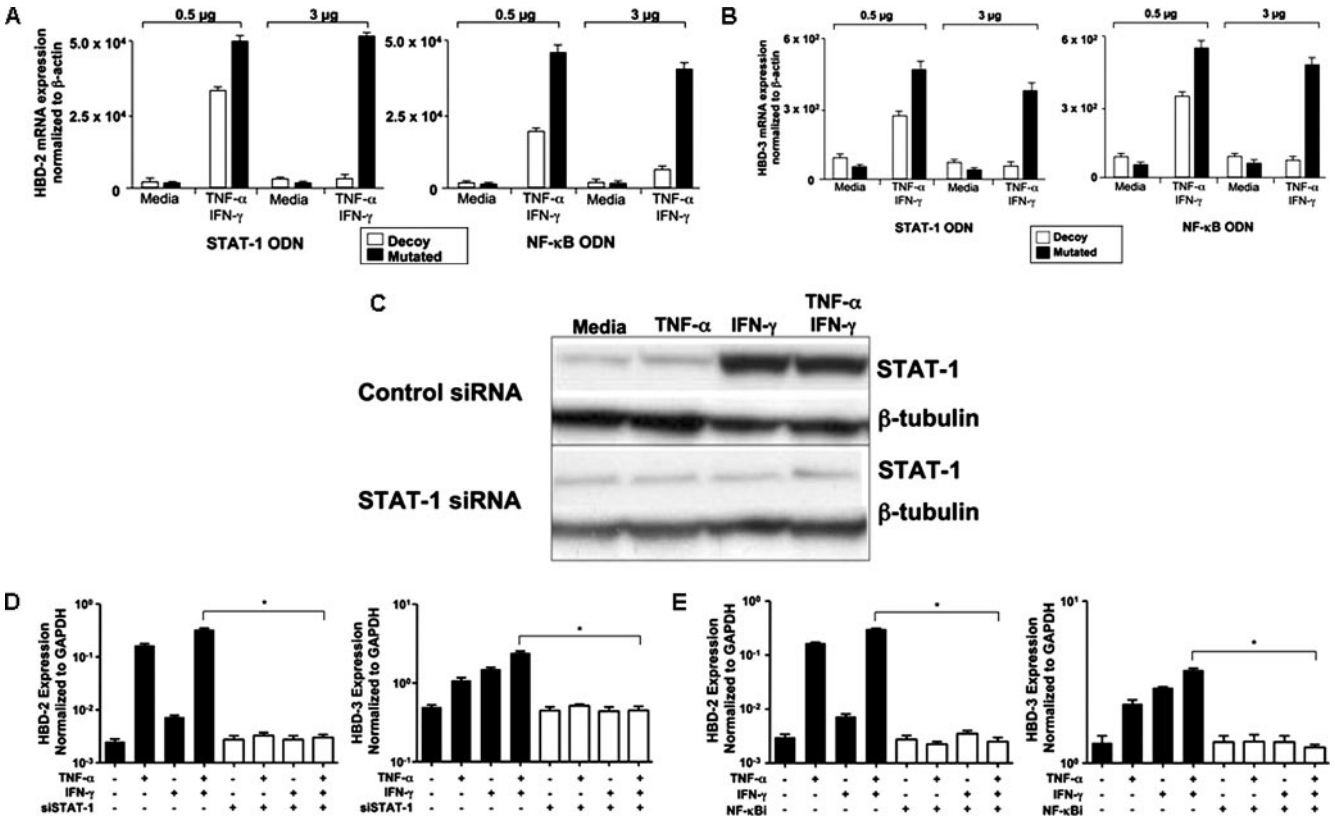

FIGURE 2. TNF-α and IFN-γ induce the expression of HBD-2 and HBD-3 through STAT-1 and NF-κB. HBD-2 (*A*) and HBD-3 (*B*) expression in keratinocytes transduced with 0.5 or 3 μg of decoy (□) or mutated (■) STAT-1 and NF-κB ODN for 8 h, and then left untreated or stimulated with IFN-γ and TNF-α for 18 h. *HBD-2* and *HBD-3* gene expression values were normalized using the housekeeping gene β-actin. ∗, A significant difference of $p < 0.001$; ∗∗, a significant difference of $p < 0.01$. *C*, TNF-α- and IFN-γ-mediated activation of STAT-1 activation is inhibited by STAT-1 siRNA. *D*, Keratinocytes were transfected with STAT-1 siRNA and then stimulated with TNF-α and IFN-γ for 24 h. HBD-2 and HBD-3 expression was evaluated using real-time RT-PCR. Data are expressed as the mean ± SE. ∗, A significant difference of $p < 0.001$. *E*, Keratinocytes were pretreated with an inhibitor to block the translocation of NF-κB into the nucleus and then stimulated with TNF-α and IFN-γ for 24 h. HBD-2 and HBD-3 expression was evaluated using real-time RT-PCR. Data are expressed as the mean ± SE. ∗, A significant difference of $p < 0.001$.

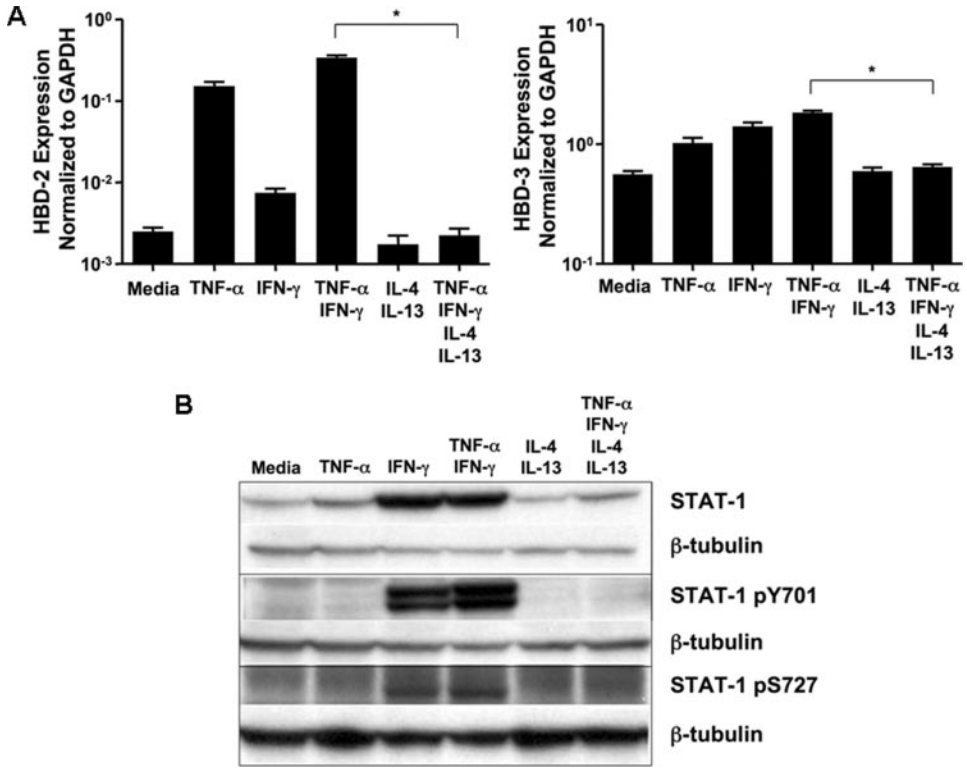

FIGURE 3. IL-4 and IL-13 downregulate HBD expression and inhibit STAT-1 activation. Keratinocytes were stimulated with TNF-α and IFN-γ in the presence/absence of IL-4 and IL-13. *A*, HBD-2 and HBD-3 expression were evaluated by real-time RT-PCR. Data are expressed as the mean $\pm$ SE. $*$, A significant difference of $p < 0.001$. *B*, Total STAT-1, pY701 STAT-1, and pS727 STAT-1 were analyzed by Western blotting.

analysis of the HBD-3 promoter region revealed that it contains only one putative NF-κB-binding site at position $-2148/2142$ and two conserved STAT-1 sites at positions $-327/-319$ (STAT-1 (1)) and $-1255/1249$ (STAT-1 (2)) (Fig. 1*A*). All the putative NF-κB sites in the HBD-2 promoter were detected in NF-κB immunoprecipitates from keratinocytes cultured in medium alone (Fig. 1*B*). As NF-κB sites at positions $-158/146$ and $-167/-155$ are partially overlapping, they could not be resolved by PCR. When nuclear extracts from TNF-α and IFN-γ-stimulated keratinocytes were analyzed, NF-κB immunoprecipitates contained more NF-κB (1/2) and NF-κB (3) site DNA, relative to untreated cells. In contrast to NF-κB, STAT-1 sites could not be detected in immunoprecipitates from untreated keratinocytes. STAT-1 binding to STAT-1 (1) and STAT (2) binding sites in the HBD-2 promoter occurred only in a TNF-α/IFN-γ-inducible manner (Fig. 1*B*). Although the putative NF-κB site predicted in the HBD-3 promoter was not totally adherent to its consensus motif, it showed specific binding of NF-κB in nuclei of keratinocytes. Additionally, NF-κB binding to the HBD-3 promoter was more abundant following IFN-γ/TNF-α treatment (Fig. 1*C*). Finally, STAT-1 associated with the putative STAT-1 (2), but not STAT (1) site, in a TNF-α/IFN-γ-dependent manner.

To determine whether STAT-1 and NF-κB signaling influenced the TNF-α/IFN-γ-induced expression of HBD-2 and HBD-3, a double-stranded decoy ODN, designed to mimic a STAT-1 or NF-κB target promoter sequence, was used to inhibit STAT-1 or NF-κB-dependent gene induction, respectively. Following introduction of the STAT-1 or NF-κB decoy ODN (wild type) or mutated control ODN by transfection, keratinocytes were stimulated with medium alone or IFN-γ and TNF-α, and levels of HBD-2 and HBD-3 were evaluated by real-time RT-PCR. As shown in Fig. 2, *A* and *B*, the STAT-1 decoy dose-dependently inhibited the TNF-α/IFN-γ-induced HBD-2 and HBD-3 mRNA expression in keratinocytes. *HBD-2* and *HBD-3* gene expression was reduced to background levels in keratinocytes transfected with 3 μg of the STAT-1 decoy (HBD-2: 0.31 ± 0.02, $p < 0.001$; HBD-3: 0.4 ± 0.05, $p < 0.001$) but not with the corresponding mutant ODN (HBD-2: 4.72 ± 0.44, $p < 0.01$; HBD-3: 3.75 ± 0.41, $p < 0.01$). Similarly, the highest dose of NF-κB-specific decoy markedly reduced the TNF-α/IFN-γ-induced *HBD-2* and *HBD-3* gene expression by 5.7- and 6.5-fold, respectively (HBD-2: 0.67 ± 0.05, $p < 0.001$; HBD-3: 0.74 ± 0.09, $p < 0.001$), when compared with mutated ODN (HBD-2: 3.78 ± 0.34, $p < 0.01$; HBD-3: 4.87 ± 0.41, $p < 0.01$). The efficacy of the STAT-1 and NF-κB decoy effect was confirmed by analyzing other genes (i.e., CXCL10, ICAM-1 and HLA-DRα) known to be regulated by IFN-γ and TNF-α (23). We further demonstrate the requirement for STAT-1 and NF-κB using siRNA and a NF-κB inhibitor, respectively. Stimulation of keratinocytes with TNF-α/IFN-γ strongly induced STAT-1; however, this was eliminated in keratinocytes treated with STAT-1 siRNA (Fig. 2*C*). Additionally, the combination of TNF-α/IFN-γ strongly induced HBD-2 (0.33 ± 0.02 ng HBD-2/ng GAPDH) and HBD-3 (2.41 ± 0.13 ng HBD-3/ng GAPDH) gene expression (Fig. 2*D*). However, this induction was significantly inhibited in keratinocytes first treated with STAT-1 siRNA (HBD-2: 0.00 ± 0.00, $p < 0.001$; HBD-3: 0.45 ± 0.05, $p < 0.001$). Similarly, treatment with a NF-κB inhibitor significantly inhibited the TNF-α/IFN-γ mediated induction of HBD-2 (0.00 ± 0.00) and HBD-3 (1.25 ± 0.05) as compared with treatment with TNF-α/IFN-γ alone (HBD-2: 0.30 ± 0.01, $p < 0.001$; HBD-3: 3.75 ± 0.10, $p < 0.001$) (Fig. 2*E*).

STAT-1 activation is inhibited by Th2 cytokines

Previously, we have shown that the expression of HBD-2 and HBD-3 is inhibited in the presence of the Th2 cytokines (5, 10). However, the exact mechanism by which Th2 cytokines modulate HBD expression is unknown. In the current study, we initially verified that IL-4 and IL-13 inhibit the TNF-α/IFN-γ-mediated induction of HBD-2 and HBD-3 (Fig. 3*A*). Because both HBD-2 and HBD-3 have STAT-1-binding sites in their promoter regions, we further investigated the effect of IL-4 and IL-13 on STAT-1 activation (Fig. 3*B*). Stimulation with TNF-α and IFN-γ induced

Table II. *Signaling genes induced by IL-4 and IL-13*

Gene	AVG C_t without Normalization[a]		Fold Up- or Down-Regulation	
	Media	IL-4 and IL-13	IL-4 and IL-13/media	*t* Test
STAT1	23.03	23.85	(1.27)	0.3650
STAT2	22.21	23.01	(1.25)	0.3743
STAT3	21.80	22.82	(1.46)	0.4276
STAT4	35.00	35.00	1.39	N/A
STAT5A	25.64	25.11	2.00	0.1654
STAT5B	25.26	26.71	(1.96)	0.3127
STAT6	23.32	20.41	10.47	0.0004
SOCS1	30.46	24.84	68.37	0.0037
SOCS2	25.25	24.68	2.06	0.0452
SOCS3	31.33	26.15	50.63	0.0002
SOCS4	22.29	22.68	1.06	0.5932
SOCS5	23.65	23.55	1.49	0.7884
GAPDH	15.91	16.51	(1.09)	0.4370
18S rRNA	13.28	13.65	1.08	0.2452
β-actin	14.67	14.85	1.23	0.6398

[a] AVG C_t, Average cycle threshold.

STAT-1 expression and activated STAT-1, as evidenced by phosphorylation at Y701 and S727; however, addition of IL-4 and IL-13 down-regulated STAT-1 expression and inhibited phosphorylation of Y701 and S727.

Th2 cytokines preferentially induce SOCS-1, SOCS-3, and STAT-6 to inhibit STAT-1 activation

Based on the results that IL-4 and IL-13 inhibit TNF-α- and IFN-γ-mediated STAT-1 activation, we investigated other genes involved in this signal transduction pathway using superarray technology. Stimulation of keratinocytes with IL-4 and IL-13 significantly induced STAT-6 (10.47-fold; $p = 0.0004$), SOCS-1 (68.37-fold; $p = 0.0037$), and SOCS-3 (50.63-fold; $p = 0.0002$) as compared with medium alone (Table II). SOCS-2 was also elevated in keratinocytes following IL-4 and IL-13 stimulation (2.06-fold; $p = 0.0452$), however, this increase was not considered to be biologically significant in our system based on previous data suggesting a primary role for SOCS-2 in growth hormone signaling (24). Additional *STAT* and *SOCS* genes were investigated but were not significantly modulated by stimulation with IL-4 and IL-13.

Using siRNA technology, we further investigated the roles of SOCS-1, SOCS-3, and STAT-6 in the IL-4/IL-13-mediated down-regulation of IFN-γ/TNF-α activation of STAT-1. siRNA sequences were designed for SOCS-1, SOCS-3, and STAT-6 and transfected into the HaCaT keratinocyte cell line. Cells were then stimulated with combinations of TNF, TNF-α/IFN-γ, IFN, IL-4, and IL-13. Real-time RT-PCR was used to confirm that SOCS-1, SOCS-3, and STAT-6 were inhibited by the siRNA sequences in keratinocytes stimulated with IL-4 and IL-13 (data not shown). Stimulation with TNF-α and IFN-γ strongly induced STAT-1 in keratinocytes transfected with control siRNA, however, this was

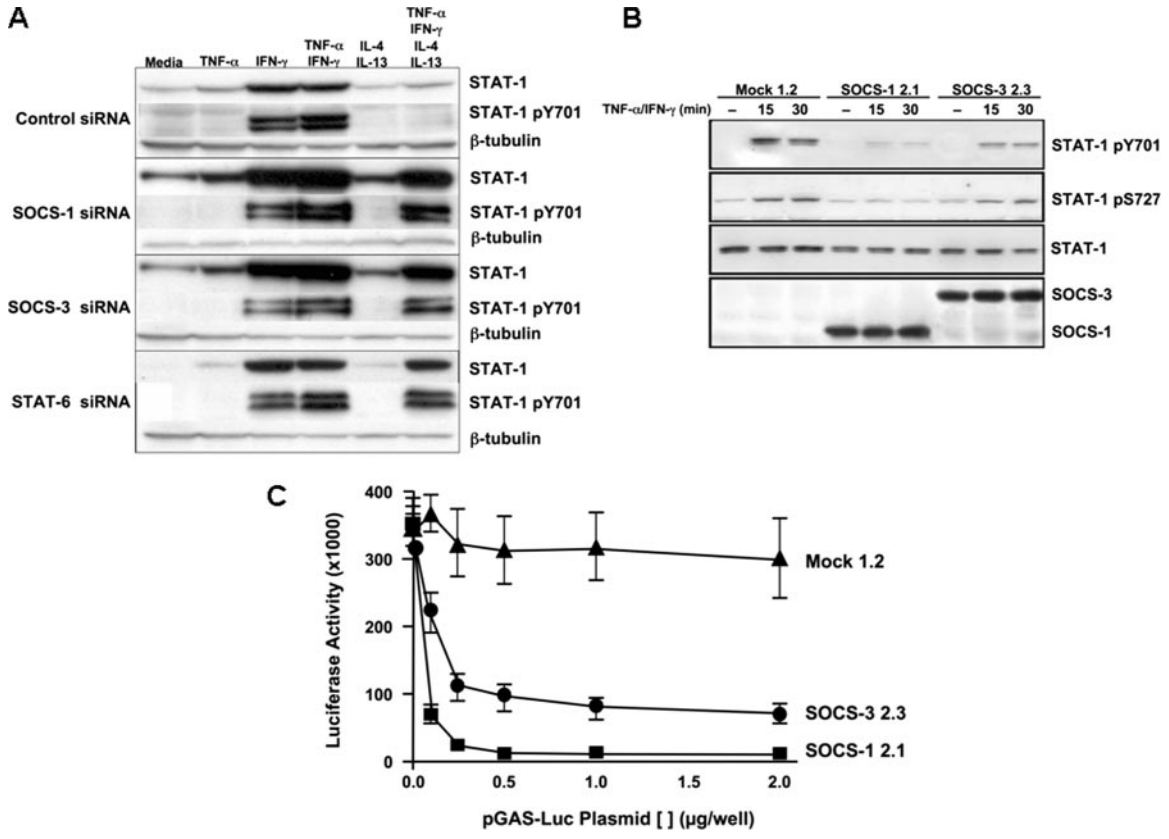

FIGURE 4. IL-4 and IL-13 inhibition of STAT-1 activation is mediated by STAT-6, SOCS-1, and SOCS3. *A*, Keratinocytes were transfected with STAT-6 siRNA, SOCS-1 siRNA, or SOCS-3 siRNA and then stimulated with a combination of TNF-α, IFN-γ, IL-4, and/or IL-13. Total STAT-1 and pY701 STAT-1 were analyzed by Western blotting. *B*, SOCS-1, SOCS-3, or mock-transfected keratinocyte clones were treated with medium or TNF-α and IFN-γ. Total STAT-1, pY701 STAT-1, pS727 STAT-1, and SOCS were analyzed by Western blotting. *C*, Cultured human keratinocytes were cotransfected with increasing amounts of pcDNA3 (mock), pcDNA3-myc-SOCS1, pcDNA3-myc-SOCS3 vectors, and 0.5 μg of pGAS-Luc reporter plasmid. After transfection, cells were incubated for 24 h with TNF-α and IFN-γ and then lysed to determine β-galactosidase and luciferase activities. Luciferase activity of each sample was normalized to the β-galactosidase activity. Data are expressed as the mean ± SE.

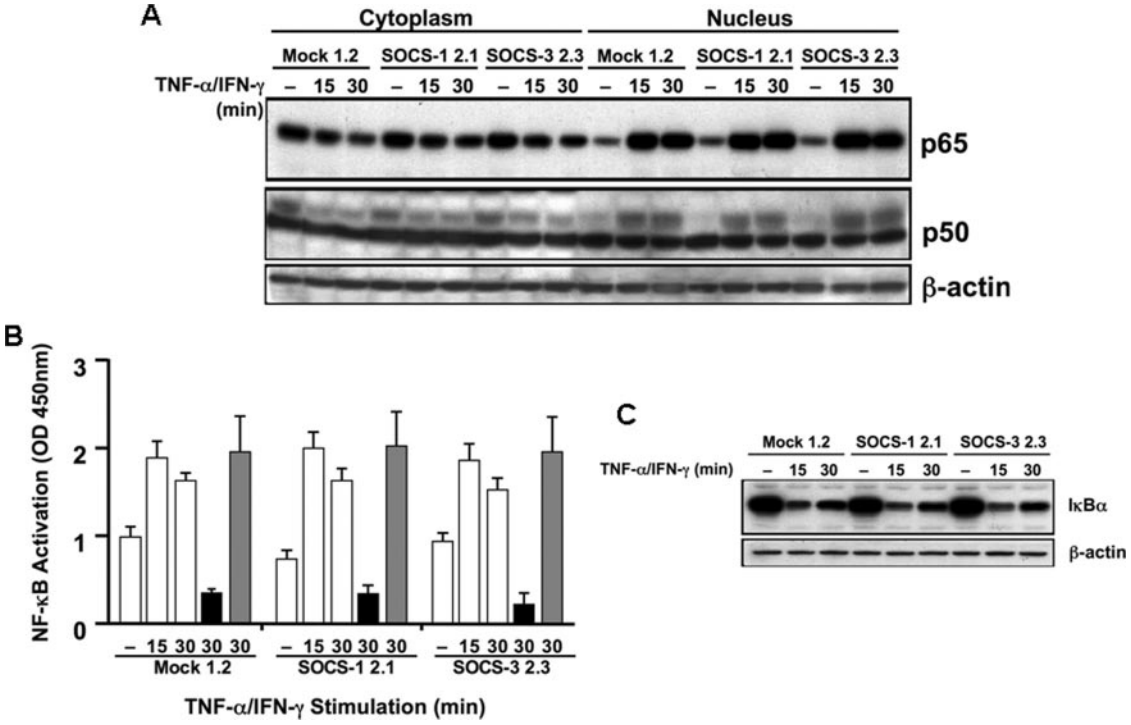

FIGURE 5. NF-κB signaling is not altered by SOCS-1 or SOCS-3. *A*, Nuclei and cytosol fractions were collected from TNF-α- and IFN-γ-stimulated SOCS-1, SOCS-3, or mock-transfected clones. Expression of the p65 and p50 NF-κB subunits was evaluated by Western blotting. *B*, Nuclei extracts from TNF-α- and IFN-γ-stimulated clones were assayed using the TransAM NF-κB family kit in the absence (□) or in presence of a molar excess of wild-type (■) or mutated (▨) consensus oligonucleotides. NF-κB-binding activity is expressed as mean OD at 450 nm of triplicate cultures. *C*, Western blot analyses of IκBα was performed on cytoplasmic lysates of medium or TNF-α- and IFN-γ-stimulated clones.

inhibited by IL-4 and IL-13 (Fig. 4*A*). In contrast, IL-4 and IL-13 no longer inhibited TNF-α- and IFN-γ-mediated-STAT-1 activation in keratinocytes transfected with SOCS-1, SOCS-3, or STAT-6 siRNA.

We additionally generated HaCaT clones which overexpressed SOCS-1 and SOCS-3. Analysis of STAT-1 signaling in SOCS-1 and SOCS-3 HaCaT clones revealed reduced STAT-1 phosphorylation at both Y701 and S727 residues in response to TNF-α and IFN-γ stimulation (Fig. 4*B*). Additionally, human keratinocytes transiently transfected with SOCS-1 and SOCS-3 showed inhibition of the TNF-α/IFN-γ-induced transactivation of a STAT-1-responsive reporter plasmid (pGAS-Luc) (Fig. 4*C*). In contrast to STAT-1, NF-κB signaling was not altered in SOCS-1 and SOCS-3 clones stimulated with TNF-α and IFN-γ, as assessed by analyzing NF-κB expression (p65 and p50 forms; Fig. 5*A*) and binding capacity to canonical sequences (Fig. 5*B*) as well as IκBα degradation (Fig. 5*C*).

SOCS-1, SOCS-3, and STAT-6 required for Th2-mediated down-regulation of HBDs

We further evaluated the role of SOCS-1, SOCS-3, and STAT-6 in the Th2-mediated down-regulation of HBD-2 and HBD-3. HaCaT cells transfected with SOCS-1, SOCS-3, and STAT-6 siRNA were stimulated with TNF-α and IFN-γ in the presence and absence of IL-4 and IL-13. Stimulation with TNF-α and IFN-γ in keratinocytes transfected with control siRNA significantly induced HBD-2 (0.339 ± 0.021; $p < 0.001$) and HBD-3 (2.47 ± 0.10; $p < 0.001$) expression as compared with medium alone (HBD-2: 0.003 ± 0.000, Fig. 6*A*; HBD-3: 0.34 ± 0.03, Fig. 6*B*). This induction was significantly inhibited by IL-4 and IL-13 (HBD-2: 0.003 ± 0.000, $p < 0.001$; HBD-3: 0.37 ± 0.02, $p < 0.001$); however, the inhibitory effect of IL-4 and IL-13 was eliminated by knocking out SOCS-1, SOCS-3, or STAT-6 using siRNA. Despite the addition of IL-4 and IL-13, TNF-α/IFN-γ-induced HBD-2 and HBD-3 expression in keratinocytes transfected with SOCS-1 (HBD-2: 0.354 ± 0.030, HBD-3: 2.76 ± 0.15), SOCS-3 (HBD-2: 0.333 ± 0.023; HBD-3: 2.34 ± 0.09), and STAT-6 (HBD-2: 0.351 ± 0.037, HBD-3: 2.40 ± 0.24) siRNA was similar to those seen in TNF-α/IFN-γ-stimulated keratinocytes transfected with control siRNA. Similarly, HBD-2 and HBD-3 mRNA was highly expressed by mock-transfected HaCaT clones following stimulation with TNF-α and IFN (Fig. 6*C*). However, TNF-α/IFN-γ-induced expression of HBD-2 and HBD-3 expression was significantly reduced in all SOCS-1 clones with 94–98% reduction for HBD-2 and 94–99% reduction for HBD-3 (Fig. 6*C*; $p < 0.001$). A 92–97% and 92–98% reduction of HBD-2 and HBD-3, respectively, was observed in TNF-α/IFN-γ-stimulated SOCS-3 clones ($p < 0.001$).

Effect of SOCS-1 and SOCS-3 is regulated by STAT-6

Although our data has shown that SOCS-1, SOCS-3, and STAT-6 are necessary for IL-4- and IL-13-mediated down-regulation of TNF-α/IFN-γ-induced HBD expression, the order in which these molecules regulate each other is unknown. Therefore, we investigated whether STAT-6 was necessary for the induction of SOCS-1 and SOCS-3. As shown in Fig. 7, stimulation of control siRNA-transfected keratinocytes with IL-4 and IL-13 significantly induced the expression of SOCS-1 (6.20 ± 0.35 ng SOCS-1/ng GAPDH; $p < 0.001$) and SOCS-3 (5.59 ± 0.23 ng SOCS-3/ng GAPDH; $p < 0.001$) as compared with medium alone (SOCS-1: 0.41 ± 0.03; SOCS-3: 0.32 ± 0.04). However, levels of SOCS-1 and SOCS-3 returned to baseline in keratinocytes initially transfected with STAT-6 siRNA (SOCS-1: 0.39 ± 0.06; SOCS-3: 0.36 ± 0.03), indicating that STAT-6 is necessary for the IL-4- and IL-13-induced expression of

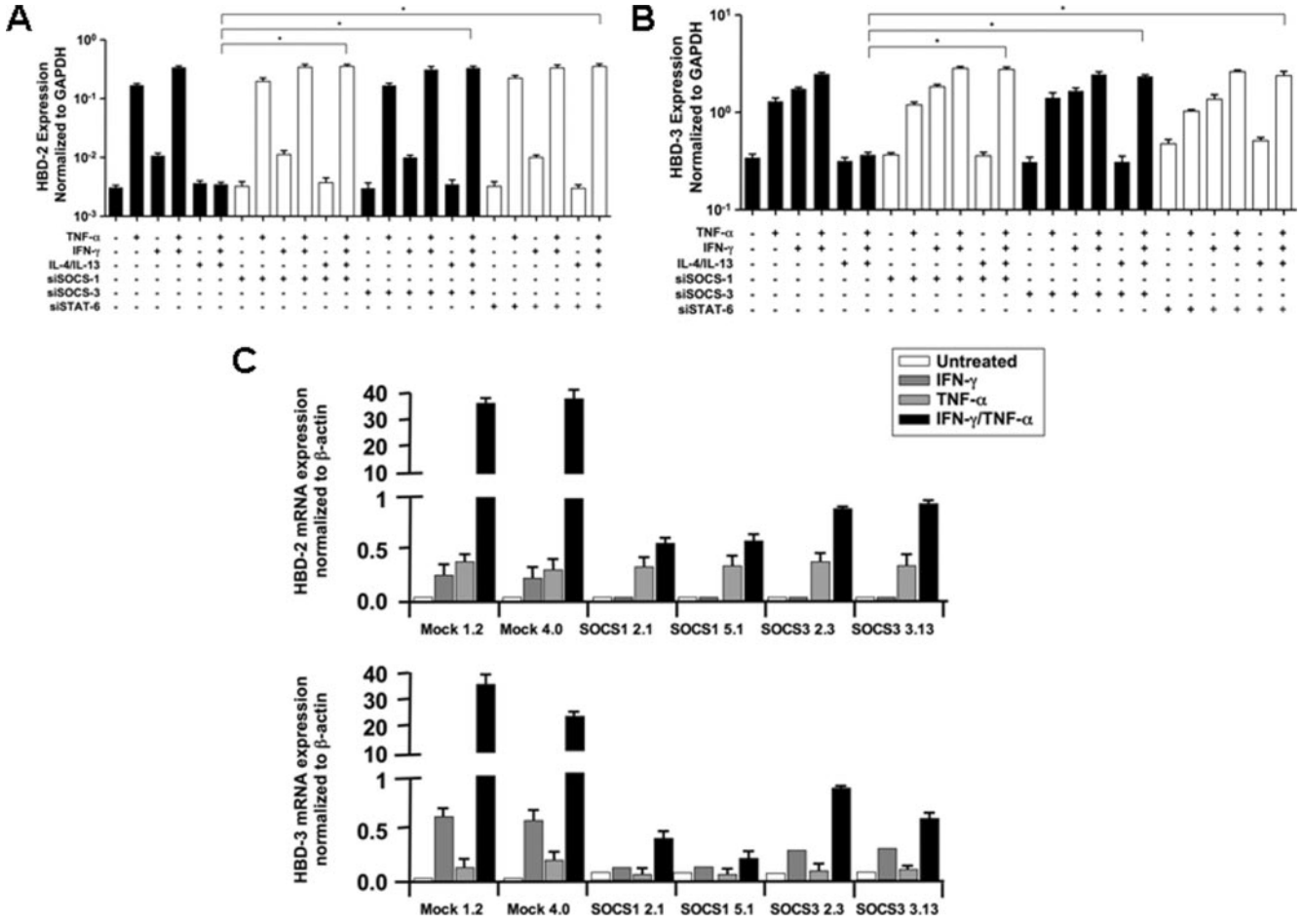

FIGURE 6. IL-4- and IL-13-mediated down-regulation of HBD-2 and HBD-3 is dependent on STAT-6, SOCS-1, and SOCS-3. Keratinocytes were transfected with STAT-6 siRNA, SOCS-1 siRNA, or SOCS-3 siRNA and then stimulated with a combination of TNF-α, IFN-γ, IL-4, and/or IL-13. HBD-2 (*A*) and HBD-3 (*B*) expression were evaluated using real-time RT-PCR. Data are expressed as the mean ± SE. ∗, A significant difference of $p < 0.001$. *C*, SOCS-1, SOCS-3, or mock-transfected keratinocyte clones were treated with medium or TNF-α and IFN-γ. HBD-2 and HBD-3 expression were evaluated using real-time RT-PCR. Data are expressed as the mean ± SE. Two representative SOCS1, SOCS3, and mock-transfected clones are shown.

SOCS-1. To see whether SOCS-1 or SOCS-3 in turn influence STAT-6 expression promoted by IL-4 and IL-13 in keratinocytes, we evaluated IL-4/IL-13-induced expression of STAT-6 in keratinocytes transfected with SOCS-1 or SOCS-3 siRNA. By real-time RT-PCR, we found that STAT-6 expression was not modulated by the presence or absence of SOCS-1 or SOCS-3 (Fig. 7*B*).

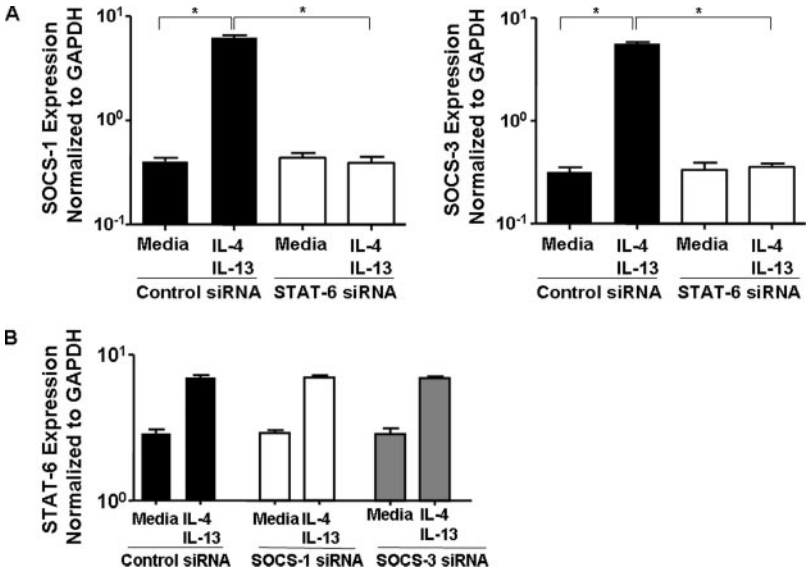

FIGURE 7. SOCS-1 and SOCS-3 expression is regulated by STAT-6. *A*, Keratinocytes were transfected with STAT-6 siRNA and then stimulated with IL-4 and IL-13 for 24 h. SOCS-1 and SOCS-3 expression was measured using real-time RT-PCR. Data are expressed as the mean ± SE. ∗, A significant difference of $p < 0.001$. *B*, Keratinocytes were transfected with SOCS-1 siRNA or SOCS-3 siRNA and then stimulated with IL-4 and IL-13 for 24 h. STAT-6 expression was measured using real-time RT-PCR. Data are expressed as the mean ± SE.

Discussion

Our current study delineates the mechanisms underlying the induction of both HBD-2 and HBD-3 by TNF-α and IFN-γ and their down-regulation by IL-4 and IL-13. Although IFN-γ and TNF-α have been found to be the most potent inducers of HBD-2 and HBD-3 expression in keratinocytes (3, 5, 10), the molecular mechanisms responsible for transcriptional activation of these genes have not yet been defined. We demonstrate here that TNF-α and IFN-γ induce *HBD-2* and *HBD-3* gene expression by signaling through STAT-1 and NF-κB. By ChIP analysis, we identified active STAT-1- and NF-κB-binding sites in the promoter regions of *HBD-2* and *HBD-3* genes. The importance of STAT-1 and NF-κB in the induction of HBD-2 and HBD-3 was further demonstrated in three ways: first, ODN sequences designed to mimic the target sequences for either STAT-1 or NF-κB inhibited TNF-α- and IFN-γ-induced expression of HBD-2 and HBD-3 expression in a dose-dependent manner. However, mutated ODN sequences failed to act in the same manner. Second, keratinocytes treated with STAT-1 siRNA showed significantly reduced levels of HBD-2 or HBD-3 following stimulation with TNF-α and IFN-γ. Third, keratinocytes treated with an inhibitor to block the translocation of NF-κB into the nucleus failed to induce HBD-2 and HBD-3 following stimulation with TNF-α and IFN-γ. Our observations are further supported by a previous study which demonstrated that inhibition of NF-κB prevents bacterially induced HBD-2 (25). As a whole, these data indicate that both STAT-1 and NF-κB transcription factors are required for the IFN-γ and TNF-α-induced expression of HBD-2 and HBD-3 in human keratinocytes.

Although TNF-α and IFN-γ induce the expression of HBD-2 and HBD-3, this induction is significantly inhibited by the addition of the Th2 cytokines IL-4 and IL-13. Understanding the mechanism by which IL-4 and IL-13 inhibit HBD expression is important because these cytokines are increased in AD skin and lavage fluid of CF patients (13, 26). Additionally, the increased expression of IL-4 and IL-13 in AD skin is thought to increase the propensity of these patients to recurrent bacterial and viral skin infections by reducing AMP expression (5, 16, 27). In this study, we found that STAT-1 activation was inhibited by IL-4 and IL-13. This suggests that IL-4 and IL-13 signal through a different pathway to inhibit TNF-α and IFN-γ-induced HBD-2 and HBD-3 expression. Using superarray technology, we investigated the effect of IL-4 and IL-13 on the expression of genes associated with signal transduction. In these experiments, we established that IL-4 and IL-13 up-regulated the expression of *STAT-6*, *SOCS-1*, *SOCS-2*, and *SOCS-3* genes.

STAT-6 is essential to IL-4- and IL-13-mediated functions (28, 29) and has been shown to be involved in the IL-4- and IL-13-mediated down-regulation of vaccinia virus-induced LL-37 (16) by human keratinocytes. STAT-6 is activated by the binding of IL-4 and/or IL-13 to the heterodimerized IL-4Rα:IL-13Rα1 (30). It then translocates to the nucleus where it inhibits the binding of NF-κB to promoter sequences and subsequent gene expression (31). IL-4- and IL-13-mediated repression of STAT-1 is not completely understood; therefore, we investigated the role of STAT-6 in the IL-4- and IL-13-mediated inhibition of STAT-1 activation. In the current study, we found that when keratinocytes were pretreated with STAT-6 siRNA, IL-4 and IL-13 no longer inhibited the TNF-α- and IFN-γ-mediated activation of STAT-1 or the subsequent induction of HBD-2 and HBD-3. Previous studies have shown, however, that STAT-6 does not directly inhibit the activation of STAT-1 (31), suggesting that STAT-6 regulates other proteins involved in signal transduction as well as the repression of STAT-1-mediated induction of HBD-2 and HBD-3.

The SOCS family of proteins is comprised of eight members: SOCS-1 through SOCS-7 and the cytokine-inducible Src homology 2 (SH2) domain-containing protein. Each member contains a SH2 domain and a conserved C-terminal 40-aa sequence termed the SOCS box (32, 33). In our current study, we found that *SOCS-1*, *SOCS-2*, and *SOCS-3* gene expression was increased in keratinocytes treated with IL-4 and IL-13. Because, SOCS-2 is primarily involved in the regulation of growth hormone (24), we focused our attention on SOCS-1 and SOCS-3. SOCS-1 and SOCS-3 function as negative regulators of the JAK/STAT-signaling pathway (34, 35). In our experiments, SOCS-1 and SOCS-3 inhibited TNF-α- and IFN-γ-mediated activation of STAT-1 by preventing phosphorylation of Y701 and S727. This additionally prevented the induction of HBD-2 and HBD-3. Similar results were observed in keratinocytes treated with IL-4 and IL-13 before stimulation with TNF-α and IFN-γ. Therefore, we transfected keratinocytes with SOCS-1 or SOCS-3 siRNA to demonstrate the necessity for SOCS-1 and SOCS-3 in the IL-4- and IL-13-mediated inhibition of STAT-1 activation and down-regulation of the defensins. When SOCS-1 and SOCS-3 were inhibited, IL-4 and IL-13 no longer prevented the TNF-α- and IFN-γ-mediated activation of STAT-1 or the induction of HBD-2 and HBD-3. SOCS-1 and SOCS-3 share similar N-terminal regions and SH2 domains, however, they inhibit the JAK/STAT signaling pathway differently. SOCS-1 has been shown to inhibit signaling by binding directly to JAK proteins (34) while SOCS-3 binds to phosphorylated tyrosine sites in the cytoplasmic domain of the respective receptor (36). This suggests that both SOCS-1 and SOCS-3 are necessary for the IL-4- and IL-13-mediated inhibition of STAT-1 activation and play a significant role in the down-regulation of HBD-2 and HBD-3 because HBD expression strongly depends on STAT-1 activation.

Because both STAT-1 and NF-κB are necessary for the induction of HBD-2 and HBD-3, we investigated the effect of SOCS-1 and SOCS-3 on NF-κB. Interestingly, NF-κB signaling was not altered in IFN-γ- and TNF-α-stimulated keratinocytes that overexpressed SOCS-1 and SOCS-3. We demonstrated this by first showing SOCS-1 and SOCS-3 failed to down-regulate the expression of the p65 and p50 subunits of NF-κB (Fig. 5*A*). We also show that the binding of NF-κB to canonical sequences and IκB degradation are unaffected by the overexpression of SOCS-1 and SOCS-3 (Fig. 5, *B* and *C*). This suggests that SOCS-1 and SOCS-3 work synergistically with STAT-6 to inhibit STAT-1 and NF-κB signaling.

The relationship between STAT-6 and SOCS proteins has previously not been defined. To our knowledge, the current study is the first demonstration that STAT-6 is required for the IL-4- and IL-13-mediated induction of SOCS-1 and SOCS-3. Keratinocytes treated with STAT-6 siRNA failed to express SOCS-1 and SOCS-3 following stimulation with IL-4 and IL-13. These data are in line with previous findings demonstrating that IL-4 and IL-13 induce SOCS-1 expression in epithelial cells through STAT-6 binding to three IFN-γ-activated sequence motifs situated 600 bp upstream of the transcriptional initiation site in the SOCS1 promoter (37, 38). Further investigation demonstrated that this relationship is unidirectional as the inhibition of SOCS-1 or SOCS-3 using siRNA did not alter the induction of STAT-6.

It has been postulated that the deficient production of human β-defensins may explain, in part, the increased susceptibility of AD and CF patients to recurrent skin and respiratory infections, respectively (5, 9, 10). In fact, the increased susceptibility toward bacterial and viral skin infections has prompted the Centers for Disease Control and Prevention to recommend that individuals

with AD refrain from smallpox vaccination due to potential adverse events (39). We recently demonstrated that HBD-3 exhibits potent activity against vaccinia virus, the vaccine for smallpox (40). Additional studies have demonstrated that β-defensins exhibit activity against HIV (41) and herpes simplex virus (40). These viral infections affect millions of individuals throughout the world. Our current study suggests that targeting the STAT-1-signaling pathway or SOCS expression would enhance β-defensin expression and represent a new therapeutic strategy for reduction of infection in human diseases associated with β-defensin deficiency.

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Disclosures

The authors have no financial conflict of interest.

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