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Mechanism of interleukin-1α transcriptional regulation of *S100A9* in a human epidermal keratinocyte cell line

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Abstract

S100A9 is a calcium-binding protein and subunit of antimicrobial calprotectin complex (S100A8/A9). Produced by neutrophils, monocytes/ macrophages and keratinocytes, *S100A9* expression increases in response to inflammation. For example, IL-1α produced by epithelial cells acts autonomously on the same cells to induce expression of *S100A8/A9* and cellular differentiation. Whereas it is well known that IL-1α and members of the IL-10 family of cytokines upregulate *S100A8* and *S100A9* in several cell lineages, the pathway and mechanism of IL-1α-dependent transcriptional control of *S100A9* in epithelial cells is not established. Modeled using human epidermal keratinocytes (HaCaT cells), IL-1α stimulated phosphorylation of p38 MAPK and induced *S100A9* expression, which was blocked by IL-1 receptor antagonist, RNAi suppression of p38, or a p38 MAPK inhibitor. Transcription of *S100A9* in HaCaT cells depended on nucleotides -94 to -53 in the upstream promoter region, based upon use of deletion constructs and luciferase reporter activity. Within the responsive promoter region, IL-1α increased the binding activity of CCAAT/enhancer binding protein β (C/EBPβ). Mutated C/EBPβ binding sequences or C/EBPβ-specific siRNA inhibited the *S100A9* transcriptional response. Hence, IL-1α is strongly suggested to increase *S100A9* expression in a human epidermal keratinocyte cell line by signaling through the IL-1 receptor and p38 MAPK, increasing C/EBPβ-dependent transcriptional activity.

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Keywords

S100A9; IL-1α; keratinocytes; p38; C/EBPβ

1. Introduction

S100A9 belongs to the S100 family of calcium-binding proteins; the gene is encoded in the epidermal differentiation complex located on chromosome 1q21 [1]. Although homodimers can form, S100A9 typically forms intracellular heterodimers with S100A8. The complex of S100A8 and S100A9, known as calprotectin (S100A8/A9), localizes in the cytosol of neutrophils, monocytes/ macrophages and epithelial cells in the suprabasal spinous cell layer of epithelium [2-7]. S100A8/A9 shows broad-spectrum antimicrobial activity, chemotactic activity for neutrophils, and apoptosis-inducing activity in tumor cells and fibroblasts [8-12]. Furthermore, we have recently reported that S100A8/A9 maintains the cell cycle checkpoint at G2/M in carcinoma cells (Khammanivong et al, 2013, accepted with minor revisions 01.11.13). Whereas expression decreases in head and neck carcinoma, S100A8/A9 increases in inflamed tissues, healing wounds and hyperproliferative epidermis [6, 13-15] and the transcriptional mechanisms are not well understood.

S100A8/A9 is regulated as part of an epithelial cell autonomous response to infection and inflammation as we have reported [5, 16-20]. Hence, IL-1α produced autonomously by epithelial cells in response to bacterial pathogens such as *Listeria monocytogenes* interacts with IL-1R to upregulate endogenous S100A8/A9 [20]. IL-1R antagonists can block the S100A8/A9 response, indicating that an IL-1α autocrine loop could contribute to innate epithelial immunity during infection. Exogenous proinflammatory factors such as LPS, IL-1β, TNF-α also regulate expression of S100A8/A9 in epithelial cells suggesting that activation of proximal immune cells also exert control of the antimicrobial peptide response [21, 22]. S100A8/A9 production is increased by IL-1α and added calcium, but decreased by TGF-β [22]. In epithelial cells, *S100A8/A9* expression is also modulated by keratinocyte proliferation- or differentiation- related factors [22, 23]. For example, retinoic acid inhibited the IL-1α-mediated induction of S100A8/A9 and keratinocyte differentiation [22]. Appearing to exert another level of control of expression, S100A8/A9 can also be regulated by epithelial-mesenchymal interactions as we have also reported [23]. IL-1α produced by epithelial cells upregulates keratinocyte growth factor (KGF) produced by co-cultured fibroblasts. KGF in turn down-regulates S100A8/A9 in the epithelial cells [23]. Epithelial-mesenchymal interactions mediated, therefore, by epithelial IL-1α up-regulation of fibroblast KGF appears to function to maintain epithelial differentiation by providing tight and complex control of *S100A8* and *S100A9* expression.

Transcriptional control of the human *S100A9* promoter region has been partially characterized for both common and either myeloid or epithelial (TR146 cells) cell-specific regulatory elements [24, 25]. In different cell lines, transcription factors (TFs) that can regulate *S100A9* expression include c-myb [24], C/EBPα [24], C/EBPβ [24, 26], and Ku70/ Ku80 [27]. Myeloid-specific regulatory elements (MRE) were found in both the *S100A9* -114/-419 [24] and -400/-374 [28] promoter regions. TF binding sites can be promiscuous. For example, involucrin is regulated by C/EBPα and like *S100A9* presents in the epithelial differentiation complex in chromosome 1q21 [29]. In response to phorbol ester in human keratinocytes, C/EBPα-mediated involucrin promoter activity is inhibited by C/EBPβ and C/EBPδ [29]. Since *S100A9* and involucrin genes are both upregulated by IL-1α, C/EBPα was thought to regulate expression of both genes [22]. In the present report, we characterize the mechanism of transcriptional regulation of *S100A9* in response to IL-1α to better understand the role of epithelial cells in innate immunity.

To understand the epithelial cell-specific mechanism of transcriptional regulation of *S100A9*, we linked the (i) MAPK pathway activated in response to IL-lα with the (ii) responsible promoter elements in the upstream region of the *S100A9* and the (iii) required transcription factor for regulation of *S100A9* expression in IL-1α-stimulated epidermal keratinocytes (HaCaT cells).

2. Materials and Methods

2.1. Cell culture

An epidermal human keratinocyte cell line, HaCaT [30], was supplied by Dr. Norbert Fusenig (German Cancer Research Center), seeded at 4,800 cells/cm^2 and cultured in DMEM (NISSUI Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, HyClone, Utah, USA), 100 U/ml penicillin and 100 μg/ml streptomycin for 5 days. At 90% confluence, HaCaT cells were further cultured with or without IL-1α (20 ng/ml; Wako Pure Chemical Industries, Osaka, Japan) for 24 h. In the experiments using inhibitors, cells were cultured in DMEM-2% FBS for 12 *h* and then pre-cultured for 1 h with anti-human IL-1α antibody (1/100 dilution; IgG fraction of anti-human IL-1α, Rockland, Gilbertsville, PA, USA), IL-1α receptor antagonist (100 ng/ml; recombinant human IL-1ra/IL-1F3; IL-1ra, R&D SYSTEMS, Minneapolis, USA) or MAPK inhibitors dissolved in DMSO (included in buffer controls as appropriate) including SB203580 (30 μM; Wako), U0126 (10 μM; Wako) or SP600125 (10 μM; CALBIOCHEM, Darmstadt, Germany), and cultured with IL-1α (20 ng/ml) for 30 min (Western blotting for MAP kinase phosphorylation) or 24 h (gene expression studies using Northern blotting and quantitative PCR).

2.2 Generation of reporter vectors

Promoter fragments of the human *S100A9* were prepared either by enzyme digestion or PCR. A 1.43 kb DNA upstream fragment of *S100A9* promoter [31] was amplified by PCR according to a modified method of Kerkhoff *et al.* [28]. Briefly, the PCR mixture contained human genome DNA (LA PCR™ Genome DNA Set; TaKaRa Bio) as a Template DNA, primers (Forward: 5′-TCCCCCGGGATCACTGTGGAGTAGGGGAAGGG-3′ Reverse: 5′-GTAGATCTCGTC TTGCACTCTGTCTGTGTAA-3′) and Taq polymerase (TaKaRa Ex Taq™ HS, TaKaRa Bio). The PCR product was digested by *Sma I* and *Bgl II*, and inserted into the cloning site of pGL3 Luciferase Reporter Vector (Promega, Madison, WI, USA). The plasmid containing the cloned fragment was digested with *Kpn I* and *Nhe I*, followed by treatment with exonuclease III (TaKaRa Bio) at 25°C for 5 to 8.5 min as described by Kerkhoff *et al.* [28], and S1-nuclease (TaKaRa Bio) at 23°C for 15 min. The digested plasmid was treated with KOD DNA polymerase (TOYOBO, Osaka, Japan) and ligated with Ligation high (TOYOBO). Promoter construct generation for mapping analysis is detailed in the supplemental data.

2.3 Transient transfection and luciferase reporter assay

HaCaT cells (16.7×10^4 cells/cm^2) were cultured in DMEM-10% FBS for 24 h and were transiently transfected with deletion constructs (2 μg), internal control reporter phRL-TK (0.04 μg; Promega) and Lipofectamine™ 2000 (6 μl; Invitrogen, Life Technologies, Carlsbad, CA, USA) in Opti MEM® (Invitrogen). After 12 h of culture, the medium was changed to DMEM-1% FBS and cells were cultured in the presence or absence of IL-1α (100 ng/ml) for 12 h, washed twice with PBS and frozen at -80°C. Luciferase activity in thawed cells was determined using the Dual-Glo™ Luciferase Assay System (Promega). Relative luciferase activity was normalized to Renilla luciferase expression and expressed as the percentage activity of the pGL3(-500/+430) deletion construct in the absence of IL-1α.

Transcriptional activity attributable to IL-1α was represented as the difference in activity in the presence and absence of IL-1α.

2.4 Northern blot analysis

Total RNA was isolated from the cultured cells using RNAiso® Reagent (TaKaRa Bio Inc., Otsu, Japan). RNA (10-15 μg) was electrophoretically separated on 6% formaldehyde - 1% agarose gels and transferred to Hybond N+ membranes (GE Healthcare Bio-Science Co., Piscataway, USA). The cDNA probes for *S100A9* and *GAPDH* were prepared by PCR amplification. The primers used for *S100A9:* forward, 5′-TCGCAGCTGGAACGCAACATA-3′; reverse, 5′-AGCTCAGCTGCTTGTCTGCAT-3′ and *GAPDH:* forward, 5′-TCCACCACCCTG TTGCTGTA-3′; reverse; 5′-ACCACAGTCCATGCCATCAC-3′. The probes were labeled with [α-^{32}P]dCTP using BcaBest Labeling Kit (TaKaRa Bio). Prehybridization was performed for 2 h at 42°C in 50% formamide, 5× saline sodium phosphate- EDTA (SSPE), 5× Denhardt's solution, 0.5% SDS and 200 μg/ml salmon sperm DNA. Hybridization was performed for 12 h at 42°C in the same solution containing ^{32}P-labeled cDNA probes. After washing the membrane with 2× SSPE-0.1% SDS at 65°C, the autoradiography signals were analyzed using the BAS 2000 Bio-Imaging Analyzer (Fuji Photofilm Co., Tokyo, Japan).

2.5 RT-PCR and quantitative PCR

The expression of IL-1 receptor (IL-1R1) in HaCaT cells was investigated by RT-PCR. The PCR mixture consisted of the synthesized cDNA, primers for IL-1R1 (forward: 5′-TGCCGC TCTTCTGTCATCCCGCTC-3′, reverse: 5′-GGGGGGACCGTTATTGACCTGAAA-3′) and Taq HS (TaKaRa Bio). The *IL-1R1* was amplified 30 cycles under these conditions: denatured at 94°C for 1 min, annealed at 55°C for 1 min and extended at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

The effect of IL-1α antibody and IL-1α antagonist on IL-1α-induced *S100A9*-specific mRNA expression was determined using quantitative PCR One Step SYBR® PrimeScript® RT-PCR Kit (TaKaRa Bio). Briefly, 10 μg of total RNA (*S100A9*) or 1 μg of RNA (*GAPDH*) was mixed with One Step SYBR® RT-PCR Buffer III, TaKaRa Ex Taq HS, PrimeScript® RT enzyme Mix II, and *S100A9* primers (forward: 5′-CAGCTGGAACGCAACATAGA-3′, reverse: 5′-TCAGCTGCTTGTCTGCATTT-3′) or *GAPDH* primers (forward: 5′-GACCCCTTCATT GACCTCAACTAC-3′, reverse: 5′-AGCCTT CTCCATGGTGGTGAAGAC-3′). The mixture was incubated for reverse transcription at 42°C for 5 min and at 95°C for 10 sec, and then for PCR at 95°C for 5 sec and 60°C for 30 sec (40 cycles) using the Thermal Cycler Dice® Real Time System (TaKaRa Bio). The expression level of *S100A9*-specific mRNA was normalized to *GAPDH mRN*A.

2.6 RNA Interference

C/EBPβ and p38 MAP kinase (p38) were silenced independently by transfecting HaCaT cells with C/EBPβ or p38 small interfering RNA (siRNA, Cell Signaling, Danvers MA; 25nM) using *Trans*IT-siQUEST™ (Mirus, Madison WI) and analyzing for protein expression. Nonspecific siRNA (Santa Cruz Biotech) served as control.

2.7 Western blot analysis

Cells treated for 30 min with IL-1α (20 ng/ml) or MAPK inhibitors were collected in 125 mM Tris-HCl (pH = 6.8) with 10% glycerol, 4% SDS and protease inhibitors (leupeptin, pepstatin, *N-p*-tosyl-L-phenylalanine chloromethyl ketone and *N-a-p*-tosyl-L-lysine

chloromethyl ketone hydrochloride). The optimal time of incubation with IL-1α was determined in preliminary experiments. The phosphorylation of MAPK was investigated using western blot analysis as we described previously [23]. Briefly, the protein extract (30 μg of protein) was electrophoretically separated on SDS-polyacrylamide gels (10 or 12.5% acrylamide) under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P: GE Healthcare, Buckinghamshire, UK). After blocking with 5% skim milk and 0.1% Tween-20, the membrane was reacted with 1/2000 diluted rabbit antibodies against phospho- or total phospho-extra cellular regulated kinase (p44/42 MAPK antibody, #9102; phospho-p44/42 MAPK antibody, #4376, Cell Signaling Technology, Danvers, MA, USA), p38 (p38 MAPK antibody, #9212; phospho-p38 MAPK antibody, #4631;, Cell Signaling Technology), c-Jun N-terminal kinase (SAPK/JNK antibody, #9252; phospho-SAPK/JNK antibody, #9251), S100A9 (sc-20173, Santa Cruz Biotech), C/EBPβ (sc-7962X, Santa Cruz Biotech) or mouse anti-β-actin (1/500 dilution, clone AC-15 A5441, Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight, and then reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (#7074, Cell Signaling Technology) for 1.5 h at room temperature. The membranes were developed using the ECL Western Blocking Detection System (GE Healthcare) and exposed to Hyperfilm-ECL (GE Healthcare).

2.8 Electrophoretic mobility shift assay (EMSA)

For EMSA, HaCaT cells (4,800 cells/cm^2) were cultured for 5 days, further cultured with IL-1α (10 ng/ml) for 6 h and nuclear proteins were extracted using the NucBuster™ Protein Extraction Kit (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. EMSA was performed using the DIG Gel Shift Kit, 2nd Generation (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. Briefly, oligonucleotides containing a putative C/EBPβ binding site (sense:5′-GCTCACCTGTGAAGCAATCTT-3′, antisense: 5′-AAGATTGCTTCACAGGTGAGC-3′) were synthesized by Hokkaido System Science Co. Ltd. (Sapporo, Japan) and annealed for 10 min at 95°C. Cold mutated (sites underlined) oligonucleotides for C/EBPβ binding (sense: 5′-GCTCACCTACTGAGTGCTCTT-3′, antisense: 5′-AAGAGCACTCAGTAGGTGAGC-3′) were used for competitive assay. Oligonucleotides containing putative GATA2 or HNF4 binding sites are shown in Supplementary Information. Double-stranded oligonucleotides were labeled by incubating with digoxigenin (DIG)-ddUTP, terminal transferase and $CoCl_2$ solution for 1 h at 37°C. Nuclear proteins (10 μg) were incubated with DIG-labeled probes, poly [d(I-C)] and poly L-lysine for 30 min at room temperature. For competitive assays, reaction mixtures were preincubated with 100-fold cold probe for 20 min at room temperature before addition of the labeled probe. For supershift assays, nuclear extracts were incubated on ice for 30 min with 1 μl anti-C/EBPα (D-5, sc-365318 X) or C/EBPβ (H-7, sc-7962 X) antibody (Santa Cruz Biotech, CA). Labeled oligonucleotides were then added and incubated at room temperature for another 30 min. The oligonucleotide-nuclear protein complex was electrophoretically separated on 6% native polyacylamide gels in 0.5× Tris-borate EDTA (TBE) buffer (pH = 8.0) at 50V, transferred to a positively charged Nylon membrane (Roche) at 40 mA and fixed using a UV-crosslinker. The membrane was incubated in blocking solution (Roche) for 1.5 h at room temperature and then reacted with anti-DIG antibody conjugated with alkaline phosphatase for 1 h at room temperature. The membrane was then incubated with a chemiluminescent substrate (CSPD®, Roche) for 15 min in the dark and exposed to Amersham Hyperfilm ECL (GE Healthcare).

2.9 Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using Magna ChIP™ A/G kit (Milipore, Billerica, MA) according to manufacturer's instructions. For amplification of the *S100A9* promoter

(-203/+50), primer pairs 5′-CCCAAACCAGTTTCAGGCCAG-3′/5′-GGGAAGCTGGCAGCTCACTTACC-3′ were used. Serving as the control for absence of C/EBPβ binding sites, the primer pairs 5′-CCACAAAAGCTCTTTGGGATACTCAATAACTTCTATG-3′/5′-GTAAGGAAGGAAGT GGAAGGGAGTGATGG-3′ were designed to amplify -5360/-5130 of the human *S100A9* upstream fragment. C/EBPβ (H-7, sc-7962 X) antibody (Santa Cruz Biotech) was used for immunoprecipitation. β-actin antibody and mouse IgG (Santa Cruz Biotech) were used as negative controls. Cycling conditions were 2 min at 95°C, 32 cycles at 95°C for 20s, 61°C for 20s, and 72°C for 30s, followed by final extension at 72 °C for 3 min.

3. Results

3.1 IL-1α signals S100A9-specific mRNA expression via IL-1R1 in HaCaT cells

IL-1R1-specific mRNA was expressed in HaCaT cells; expression appeared unaffected by IL-1α (Fig. 1A). In response to IL-1α, *S100A9*- specific mRNA expression increased (Fig. 1B, C, S1) as shown previously [25] and was significantly inhibited by either anti-IL-1α antibody or IL-1R1 antagonist (Fig. 1B, C).

3.2 IL-1α signals S100A9-specific mRNA expression via p38 pathway

IL-1α stimulated phosphorylation of p38, ERK and JNK (Fig. 2A). In the presence of the p38 inhibitor, SB203580, IL-1α-stimulated phosphorylation of p38 decreased, whereas phosphorylation of ERK was unaffected and p-JNK appeared to increase (Fig. 2A). In the presence of IL-1α, U0126 selectively inhibited phosphorylation of ERK whereas SP600125 selectively inhibited p-JNK (Fig. 2A). Consistent with signaling through p38, SB203580 also inhibited IL-1α-upregulated S100A9 expression (Fig. 2B). Although IL-1α appeared to increase p-JNK phosphorylation in the presence of SB203580, S100A9 expression was not affected by SP600125, a JNK inhibitor, or U0126, an ERK inhibitor (Fig. 2B). In the absence of IL-1α, S100A9-specific mRNA expression appeared unaffected by any of the inhibitors (data not shown). Moreover, RNAi suppression of p38 reduced IL-1α-stimulated S100A9 expression (Fig. 2C, D). Taken together, these results suggest that IL-1α signals increased expression of *S100A9*-specific mRNA via the p38 MAPK pathway.

3.3 IL-1α increases transcriptional activity of the S100A9 gene

To characterize the active *S100A9* promoter region, transcriptional activity was monitored using luciferase reporter-deletion constructs in HaCaT cells. When deletion constructs of -2574 to -500 nucleotides were transfected into HaCaT cells, IL-1α significantly increased luciferase activity (Fig. 3A). When the -500 to +1 sequence was deleted, *S100A9* transcriptional activity was not stimulated in response to IL-1α (Fig. 3A). The promoter region between -500 and +1 of the *S100A9* was further analyzed using the promoter deletion constructs *S100A9/-500,* -398, -324, -185, -94, -53 and +1. IL-1α promoted luciferase activity of deletion constructs containing bases -500 to -94, but activity was significantly reduced for constructs *S100A9/*-53 and +1 (Fig. 3B).

3.4 IL-1α stimulates S100A9 expression by activating C/EBPβ

In the -94 to +1 region of *S100A9,* four potential transcription factor binding sites were identified using bioinformatic analysis (TFSEARCH, Computational Biology Research Center, AIST, Japan) [33], including C/EBPβ(-89 to -76), hepatocyte nuclear factor 4 (HNF4: -68 to -50), GATA binding protein 2 (GATA2: -83 to -74) and E2F (-65 to -58 nucleotides) (Fig. S2). To determine which of these transcription factors contribute to IL-1α-stimulated *S100A9* mRNA expression, the DNA binding activity C/EBPβ, HNF4, GATA2, and E2F were investigated by EMSA using oligonucleotides synthesized from the

sequence shown in Fig. 4A. IL-1α increased binding of labeled oligonucleotide probe to the C/EBPβ binding site in the nuclear protein extract (Fig. 4B); putative GATA2 and HNF4 binding activity appeared unaffected by IL-1α (Fig. S2). More DIG-labeled C/EBPβ motif oligonucleotide probe bound to nuclear proteins from cells cultured in the presence of IL-1α than in the absence (Fig. 4B). Adding 100-fold unlabeled (cold) oligonucleotide eliminated the binding of labeled probe (Fig. 4B). In separate experiments, treatment with 100-fold mutated unlabeled (cold) oligonucleotide was without effect on the binding of the labeled oligonucleotide to C/EBPβ (Fig. 4B). When anti-C/EBPβ antibody was added to labeled oligonucleotide and the nuclear extract, the complex showed a supershift (see asterisk, Fig. 4B). In contrast, no supershift was apparent when anti-C/EBPα antibody was added, consistent with the specificity indicated for C/EBPβ. The oligonucleotide with a putative C/EBPβ binding site appeared to bind specifically to C/EBPβ in the nuclear protein extract.

The *in vivo* binding status of C/EBPβ was assessed by chromatin immunoprecipitation (ChIP). The *S100A9* promoter (-203/+50) was immunoprecipitated by C/EBPβ antibody (Fig.4A, Fig. 4C), but not by β-actin antibody or mouse IgG (Fig. 4C). No signal was observed using a control primer pair specific for an *S100A9* upstream fragment (-5360/-5130) (Fig. 4C). The results indicate that C/EBPβ binds *in vivo* to the *S100A9* promoter. In addition, more *S100A9* promoter fragments were recovered from C/EBPβ immunoprecipitates after treatment with 2.5 or 10 ng/ml IL-1α than vehicle treatment (Fig. 4D).

3.5 C/EBPβ binding at -89/-76 is crucial for IL-1α-induced S100A9 expression

To evaluate the effect of C/EBPβ on *S100A9* promoter activity, siRNA was used to knock down C/EBPβ expression (Fig. 5A), reducing *S100A9* promoter activity by about 42% (Fig. 5B). The C/EBPβ binding site in the *S100A9* reporter construct was then mutated to confirm a role in activating IL-1α-stimulated transcription of S100A9. IL-1α significantly increased *S100A9/-500* (wild type) luciferase reporter activity by about 2-fold of control, whereas a substitution or deletion in the C/EBPβ binding site abrogated IL-1α-stimulated transcriptional activity (Fig. 5C). Taken together, these data indicate that C/EBPβ binding at -89/-76 is crucial for IL-1α-induced *S100A9* expression.

4. Discussion

We now present data that strongly suggests that IL-1α-induced *S100A9* expression in a human epidermal keratinocyte cell line (HaCaT) is signaled through the IL-1 receptor and p38 MAPK pathways, resulting in binding of C/EBPβ to the upstream *S100A9* promoter. Although we previously showed that IL-1α upregulated S100A8/A9 expression in HaCaT cells [33] and primary keratinocytes from human foreskin [34], the mechanism of response to IL-1α was not known. We observed the expression of IL-1R1-specific mRNA in HaCaT cells and human gingival keratinocytes [19] and confirmed that IL-1α functions as a signaling ligand by binding to IL-1R1; whereas it is possible that IL-1α increases IL-1R1 expression, this was not seen in HaCaT cells. Hence, the increase in S100A9 transcriptional activity in response to IL-1α appeared attributable to signaling through the p38 pathway and was not likely to reflect an increase in the number of surface receptors on the cells.

IL-1α is constitutively produced by keratinocytes from skin, oral mucosa and gingiva, and induces keratinocyte differentiation in an autocrine manner [35-38]. In keratinocytes, IL-1α also upregulates the expression of other cytokines such as IL-1β and IL-8, chemokine ligands and differentiation-related factors [33, 39]. We reported that IL-1α increased the expression of AMPs including calprotectin (S100A8/A9), lipocalin 2 and secretory leukocyte protease inhibitor in human epidermal keratinocytes, as modeled using HaCaT [33] and TR146 buccal carcinoma cells [20], and suggested that IL-1α modulates epithelial

innate immunity. IL-1α modulation of innate epithelial immunity is signaled through MAP kinases. IL-1α binds to the IL-1 receptor (IL-1R1) and signals through ERK, JNK and p38, whereas the responses are blocked in the presence of IL1R antagonist. Activation of an IL-1α-stimulated MAPK pathway depends on the target cell type and is uncharacterized in human keratinocytes [40-43].

The response to IL-1α can reflect pathway furcations. As we show here, *S100A9* expression is signaled through p38 MAP kinase. In contrast, IL-1α increases human β-defensin 2 expression in middle ear epithelial cells by activating ERK MAPK activity [44], induces IL-8 secretion from human pancreatic cancer cells via p38 and ERK pathways [42], and upregulates cyclooxygenase-2 expression via p38, ERK and JNK pathways in fibroblasts [43]. In the present study, IL-1α activated phosphorylation of p38, ERK and JNK in epidermal keratinocytes, but only a p38 inhibitor inhibited IL-1α-up-regulation of *S100A9* expression. Hence, the transcription of *S100A9* in keratinocytes involved only the p38 MAPK pathway, although IL-1α activated all three pathways. Although the downstream outcomes of IL-1α-induced activation of ERK and JNK in keratinocytes are not known, IL-1α activated ERK, p38 and JNK kinases in human cardiac myofibroblasts to increase expression of IL-1β, TNF-α and IL-6 [45]. We previously showed that IL-1α increased the expression of 37 genes in HaCaT cells including *S100A8, S100A9, IL-1α, IL-1β, IL-8* and several chemokine ligands using DNA microarray [33]. Whereas p38 MAPK signals for selective activation of C/EBPβ in response to IL-1α, we speculate that concurrent furcated signaling for ERK and JNK phosphorylation will signal for expression of other cytokines and chemokines and S100A8.

In myeloid or epithelial cells, the S100A9 promoter contains both negative and positive regulatory regions, which show cell-type and species differences [24, 26, 28]. *S100A9* expression in myeloid cells, oral keratinocytes and fibroblasts is regulated via different MAPK and transcription factor pathways, which consistent with previous reports in other epithelial cell lines [33, 46, 47]. In basal conditions (when no IL-1α was included), promoter activity attributable to upstream promoter regions differed in the oral buccal carcinoma cell line, TR146 [25] and HaCaT cells. Basal TR146 cells showed *S100A9* promoter activity in a construct 40 base pairs upstream of the start codon and containing exon/intron I. *S100A9* promoter activity in TR146 cells decreased when sequences between -40 and -600 were deleted or when sequences extended to -6500 bp. Between -600 and 1000 bp there was a 6.8-fold increase in activity. In the present study, basal HaCaT promoter activity increased as the length of constructs increased, and appeared to maximize in the -398 to -999 bp region. Our study using HaCaT cells shows for the first time the effect of IL-1α on *S100A9* promoter activity.

IL-1α and high calcium, both keratinocyte differentiation factors, function independently to increase C/EBPα binding to DNA [22]. Yet, it is not clear that C/EBPα binding to the upstream promoter of *S100A9* accounts for the increase in transcription after stimulation of cells with IL-1α. While co-regulated, the *S100A9* promoter does not contain the C/EBPα-consensus binding site reported for involucrin [22]. The *S100A9* promoter is predicted to contain three C/EBPα binding sites using bioinformatics tools. As analyzed in our gel shift/supershift studies, these C/EBPα sites are not localized in the C/EBPβ -53 to -94 bp region and cannot explain the IL-1α-dependent *S100A9* transcriptional activity. The S100A9 promoter region in HaCaT cells contains putative binding sites for the four transcription factors: C/EBPβ; NHF-4; inverse GATA2; and E2F. The IL-1α-induced increase in transcriptional activity of *S100A9* was abrogated by mutation of the C/EBPβ binding sequence (the region from -89 to -76). *S100A9* transcriptional activity was also inhibited by 100-fold excess of specific oligonucleotide, which competed for promoter binding, but not by a mutated sequence. Furthermore, culture medium with high calcium concentrations also

induces C/EBPβ expression in human and murine skin keratinocytes [48]. In other cell lineages, the expression of *S100A9* is regulated by transcription factors including C/EBP, NF-κB and STAT3 [26, 43, 49-51], which may reflect tissue-specific promoter sequences. Using TFSEARCH, we determined that binding sites for NF-κB and STAT3 do not exist in the IL-1α-responsive *S100A9* promoter region (-94 to -53) in HaCaT cells, but are found upstream (NF-κB: -178 ~ -169; STAT isoforms -748 ~ -740 and -2254 ~ -2246). IL-1α increased C/EBPβ DNA-binding activity in HaCaT cells, without affecting DNA-binding of NF-κB and STAT3 (EMSA data not shown). Although C/EBPβ appears to be the responsive transcription factor for *S100A9* in HaCaT cells in response to IL-1α, the IL-1α-responsive *S100A9* regulatory pathway in other tissues may be under control of tissue-specific response elements. Furthermore, other bacterial components such as flagellin also upregulate S100A8/A9 [34] and S100A7 [52] through TLR5 signaling, but the transcriptional mechanism has not been reported.

IL-1α plays an important role in maintaining homeostasis of epithelial and mesenchymal tissues. Acting directly on keratinocytes to stimulate cell differentiation and expression of cytokines, antimicrobial peptides and chemokines [22, 33, 35, 40, 53-55], IL-1α also stimulates fibroblasts to upregulate keratinocyte growth factor, which signals keratinocyte expression of S100A8/A9, cytokines and keratinocyte proliferation [23, 56-58]. Upregulation of calprotectin augments intracellular resistance to invading bacteria in keratinocytes as we have reported [17, 18], and, likewise, IL-1α signaling in keratinocytes regulates S100A8/A9-dependent resistance to bacterial invasion [20]. Whereas expression of S100A8/A9 is at the limits of detection in normal, uninflamed skin, it would be interesting to study the role of IL-1R signaling on the developmental program for epidermal differentiation. Building on previous evidence, IL-1α is strongly suggested to regulate *S100A9* expression via a pathway including IL-1R1, p38 MAPK and C/EBPβ promoter activity in a human epidermal keratinocyte cell line. Hence, we now link the IL-1 intracellular signaling pathway to cell autonomous innate immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ERK	extracellular-regulated kinase
JNK	c-JUN N-terminal kinase
p38	p38 kinase
IL-1R1	IL-1 receptor
siRNA	small interfering RNA
TLR5	Toll-like receptor 5

Highlights

S100A9 expression in epidermal keratinocytes is upregulated by IL-1α.

IL-1α signals *S100A9* expression via IL-1R1, p38 and C/EBPβ.

Blocking IL-1R or suppression of p38 MAPK reduced IL-1α-induced S100A9 expression.

IL-1α induced C/EBPβ binding activity, which was required for S100A9 expression.

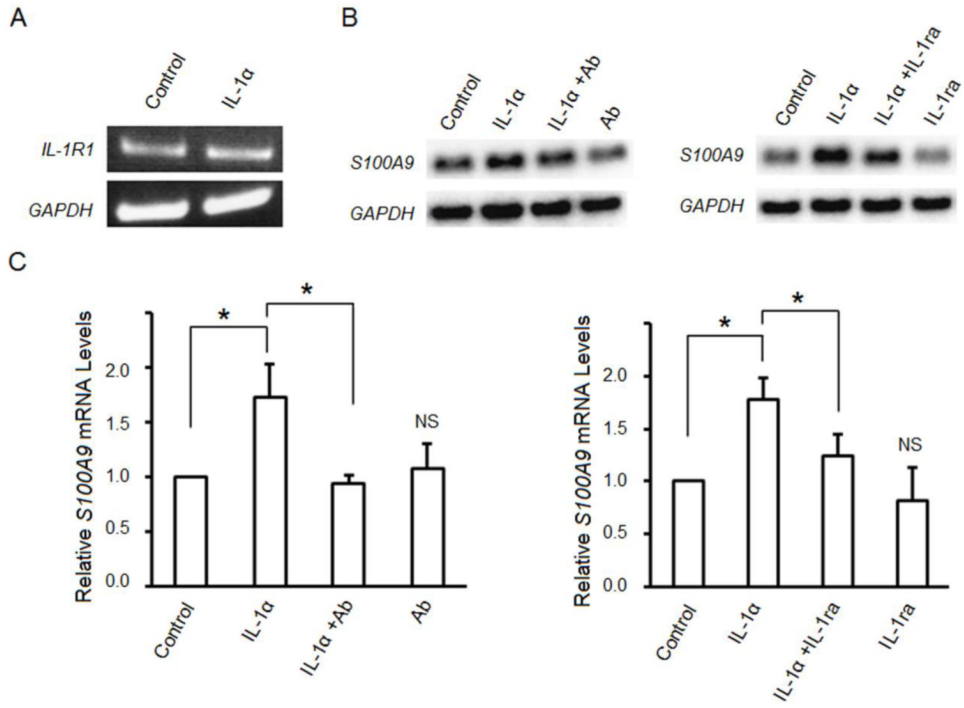

Figure 1. Effect of IL-1α, IL-1α antibody and IL-1R1 antagonist on the expression of *IL-1R1* and *S100A9* mRNA in HaCaT cells

(**A**) HaCaT cells were cultured for 5 days. To some cells, IL-1α (20 ng/ml) was added and all cultures continued for another 24 h. After RNA isolation from the cultured cells, IL-1R1-specific mRNA expression was analyzed by RT-PCR. After culture for 5 days, HaCaT cells were pre-cultured with anti-IL-1α antibody (1/100 dilution) or IL-1α receptor antagonist (100 ng/ml) for 1 h and further cultured with IL-1α (20 ng/ml) for 24 h. The expression of *S100A9* mRNA was analyzed by northern blot analysis (B) and quantitative PCR (C). The RT-PCR and northern blot analyses were repeated three times and a typical result is shown. *S100A9* mRNA expression was normalized to *GAPDH* mRNA. Data are expressed as fold change relative to the vehicle treatment. NS = not significant. The vehicle only control reflected addition of DMEM into the culture medium. Bars represent the mean ± S.D. of three independent RNA samples. *P < 0.05.

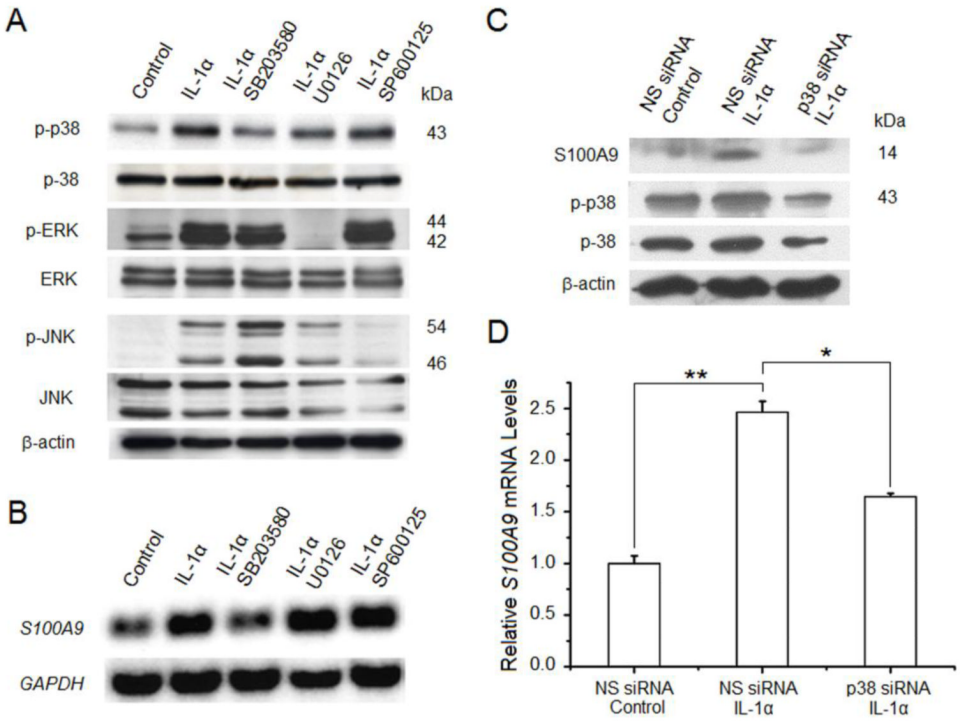

Figure 2. Effect of MAPK inhibitors on *S100A9* expression and phosphorylation of MAPK in HaCaT cells

HaCaT cells were cultured for 5 days and pre-cultured with SB203580 (30 μM), U0126 (10 μM) or SP600125 (10 μM) for 1 h, and further cultured with IL-1α (20 ng/ml) for 30 min (Western blotting) or 24 h (Northern blotting). (**A**) MAPK phosphorylation was analyzed by a western blot analysis of the cell protein fraction using anti-p38 and phospho-p38, anti-ERK and phospho-ERK, anti-JNK and phospho-JNK antibodies. (**B**) RNA was isolated from the cultured cells and *S100A9* mRNA expression was quantified by a northern blot analysis. The vehicle only control reflected addition of DMSO (0.05% concentration) into the culture medium. (**C**) Cells were grown to approximately 60-80% confluency and transfected with control siRNA or p38 siRNA (25 nM) for 24 h. The medium was then removed, and cells were treated with fresh medium containing IL-1α (20 ng/ml) or vehicle (DMEM medium) for another 24 h before harvesting for (**C**) Western blot analysis or (D) quantitative PCR analysis. Data are expressed as fold change relative to the control siRNA transfection and vehicle treatment. These results were confirmed by three independent experiments. *P < 0.05. **P<0.01.

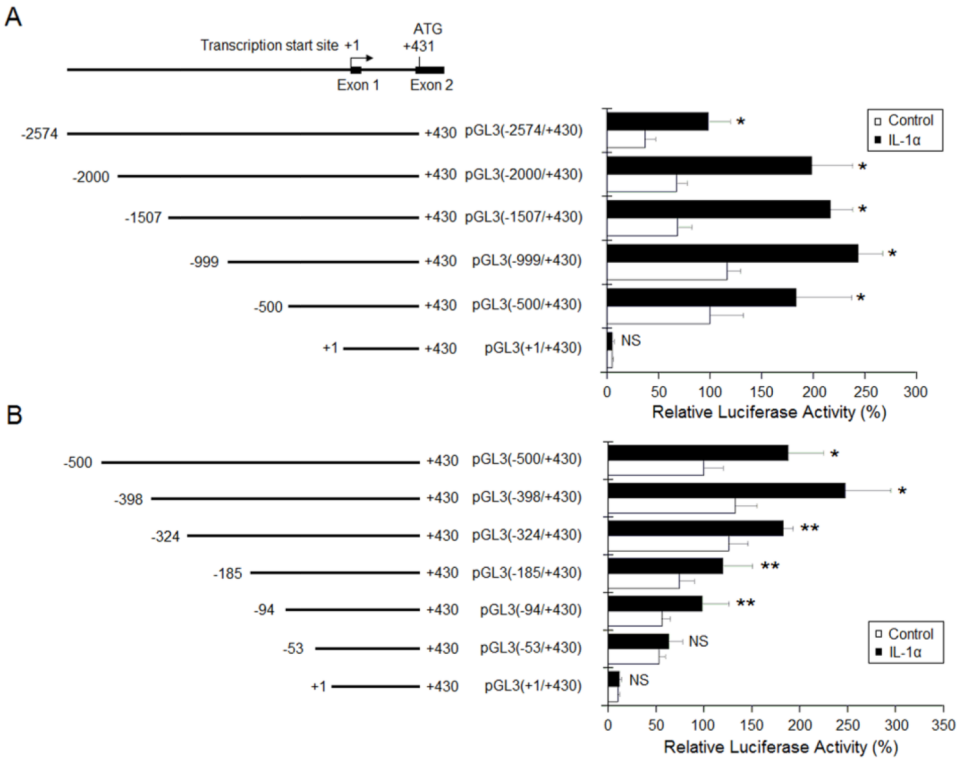

Figure 3. Effect of IL-1α on *S100A9* transcription in HaCaT cells

HaCaT cells were transiently co-transfected with deletion constructs in the *S100A9* promoter region (**A**: -2574 to +1; **B**: -500 to +1) and an internal control Renilla vector (phRL-TK), and cultured with or without IL-1α (100 ng/ml) for 12 h. The transcriptional start site was set as +1 (57). Luciferase activity was measured using Dual-Luciferase Reporter Assay System. Relative luciferase activity was normalized to Renilla luciferase expression using the phRL-TK Vector control and expressed as the percentage activity of the pGL3(-500/+430) deletion construct in the absence of IL-1α as described in the Experimental Procedures. Cell samples from IL-1α-stimulated (black bar) and non-stimulated cells (white bar; vehicle) were analyzed and the data expressed as the mean ± S.D. of cell samples from six separate experiments. The vehicle only control reflected an addition of Opti-MEM into the culture medium. *P < 0.01 **P < 0.05.

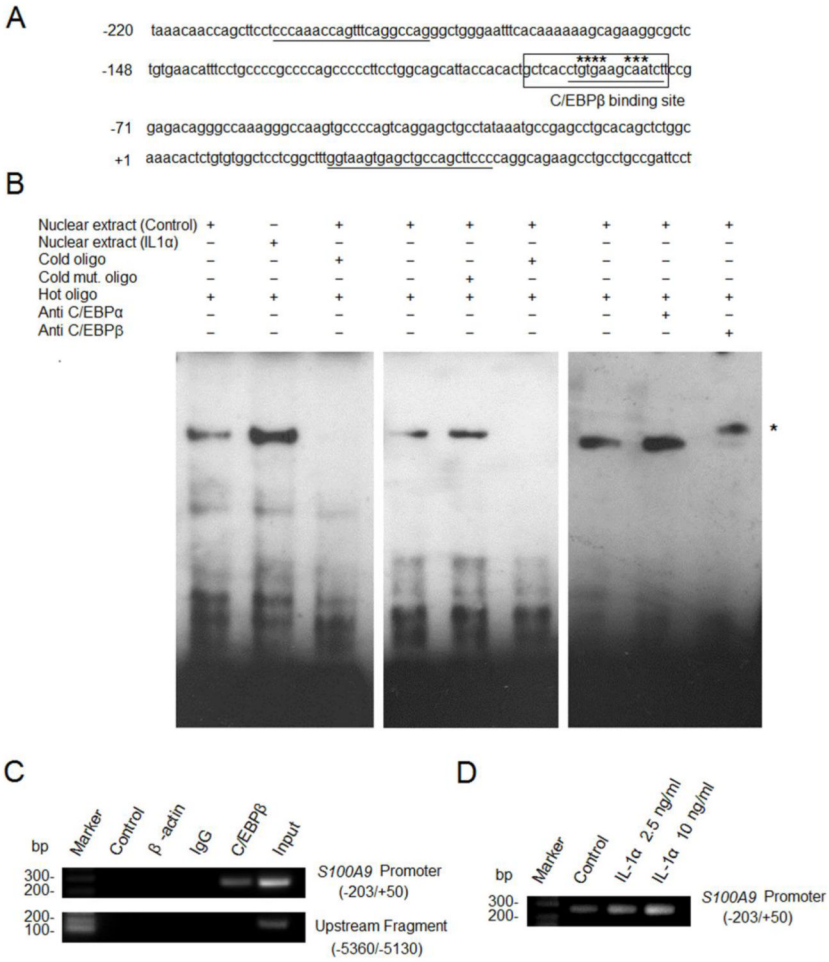

Figure 4. C/EBPβ associated with IL-αa-stimulated *S100A9* expression

(**A**) Location of oligonucleotide sequence for EMSA is boxed and asterisks (*) show mutated nucleotide positions. *S100A9* promoter (-203/+50) sequences amplified for ChIP assay primers are underlined. (**B**) C/EBPβ binding to the *S100A9* promoter region as analyzed by EMSA. HaCaT cells were cultured for 5 days followed by culture for 6 h with or without IL-1α (10 ng/ml). The nuclear protein (10 μg) extracted from the cultured cells was incubated with DIG-labeled double-stranded oligonucleotide and used for EMSA as described in the Experimental Procedures. Oligonucleotide competition was performed using 100-fold excess of each unlabeled oligonucleotide or mutated oligonucleotide. A supershift assay was performed by preincubation with anti-C/EBPα or anti-C/EBPβ antibody. An asterisk identifies the supershift band. (**C**) C/EBPβ binding to the S100A9 promoter in vivo. The S100A9 promoter region (−203/+50) containing the putative C/EBPβ binding site was amplified as described in Experimental Procedures. The S100A9 upstream fragment (-5360/-5130) was amplified and served as control for the absence of C/EBPβ binding sites. The vehicle only control consisted of addition of DMEM into the culture medium. β-actin antibody and mouse IgG were used as negative controls, whereas “Input” was sheared DNA prior to immunoprecipitation. Detailed methods can be seen in Experimental Procedures. (**D**) Dose-response increase in C/EBPβ-binding after treatment with IL-1α. Amplification of C/EBPβ immunoprecipitates after treatment with IL-1α (2.5 and 10 ng/ml) shows increasing recovery of *S100A9* promoter fragments when compared

with vehicle control. The transcriptional start site is set as +1. The analyses reported in panels B, C, and D were repeated three separate times and a typical result is shown for each.

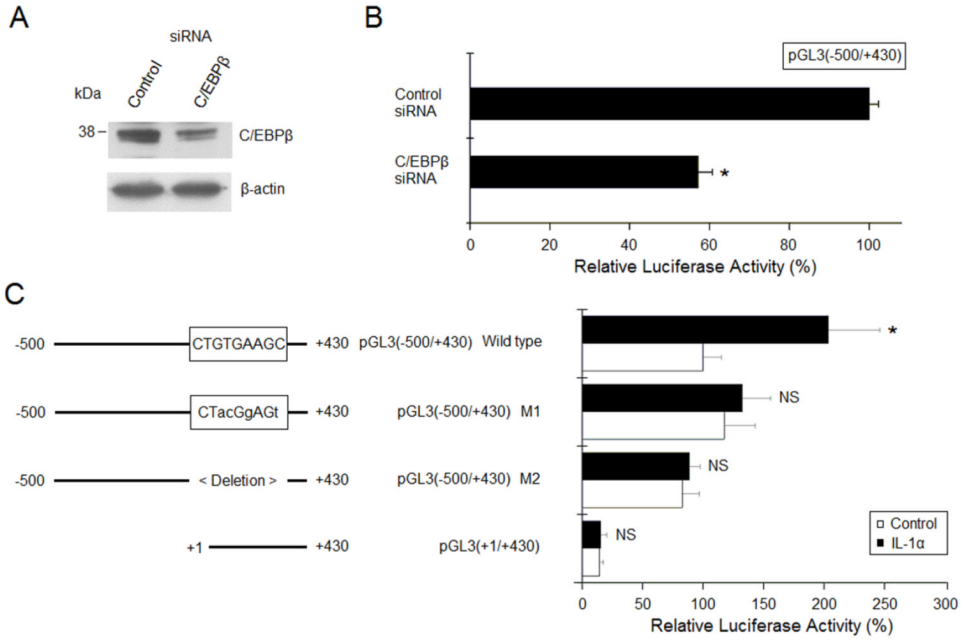

Figure 5. C/EBPβ binding site at -89/-76 critical for IL-1α-stimulated *S100A9* expression

(A, B) Downregulation of C/EBPβ by siRNA reduces *S100A9* promoter activity. Cells were grown to approximately 60-80% confluency and transfected with pGL3(−500/+430) and pRL-TK for 24 h. Cells were then were transfected with 25 nM C/EBPβ siRNA or non-specific control siRNA, medium removed, and growth continued for an additional 24 h before (**A**) Western blot analysis and (**B**) luciferase activity assay. (**C**) Mutational analysis of the C/EBPβ binding site in the *S100A9* promoter region using a luciferase reporter as described in the Experimental Procedures. HaCaT cells were transiently transfected with the luciferase reporter construct with a mutation (M1) or deletion (M2) of the C/EBPβ binding site and cultured with or without IL-1α (100 ng/ml) for 12 h. Luciferase activity from IL-1α-stimulated (black bar) and non-stimulated cells (white bar; vehicle) is expressed as the mean ± S.D. of cell samples from six separate experiments. The vehicle only control reflected an addition of Opti-MEM into the culture medium. *P < 0.01.