IL-4 Regulates Chemokine CCL26 in Keratinocytes through the Jak1, 2/Stat6 Signal Transduction Pathway: Implication for Atopic Dermatitis

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Abbreviations: AD, atopic dermatitis; DN, Dominant negative; FBS, fetal bovine serum; Jak, Janus kinase; Stat, signal transducer and activator of transcription; TBS, Tris-buffered saline; Tg, transgenic.

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Abstract

Atopic dermatitis (AD), a chronic, pruritic, inflammatory skin disease, is histopathologically characterized by epidermal hyperplasia and infiltration of T cells, mast cells, and eosinophils. Clinical study and basic research have established that IL-4 plays an important role in the pathogenesis of AD. In this report, using HaCat cells, we show that CCL26, a chemokine for eosinophils, is up-regulated by IL-4 at both the mRNA and protein levels. IL-4 also enhances CCL26 promoter activity. Serial 5' deletion of the promoter and mutagenesis study reveal that the proximal Stat site is the key response element for IL-4 regulation of CCL26. Although IL-4 increases phosphorylation of both Stat3 and Stat6, it only activates Stat6 as shown by dominant negative studies. In addition, we found that IL-4 induces Stat6 nuclear translocation and stimulates phosphorylation of Jak1 and Jak2 but not Tyk2. IL-4 up-regulation of CCL26 can be suppressed by Jak inhibitors in a dose-dependent manner. Taken together, results of this investigation reveal that IL-4 signals through the Jak1, 2/Stat6 pathway in keratinoctyes to stimulate CCL26 expression and this may provide an explanation for the pathogenesis of AD.

Key Words: IL-4, CCL26, Gene regulation, Atopic dermatitis

1. Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by T cells, eosinophils and mast cells infiltrating the skin. Its prevalence has steadily increased in developed countries, affecting 10-20% of children and 1-3% of adults (Saito, 2005). More than 50% of AD patients eventually develop other atopic disorders, such as asthma and allergic rhinitis (Kapoor et al., 2008). In fact, AD is regarded to be the skin manifestation of a systemic disorder by many researchers (Leung and Bieber, 2003; Spergel and Paller, 2003). Intense itch, skin inflammation, and other atopic symptoms create an enormous burden on the individual and society.

The etiology and pathogenesis of AD are not fully delineated. Current evidences indicate that it involves a complex interaction of skin barrier dysfunction, hypersensitivity, and exposure to offensive allergens or microbes. We and others have shown that IL-4 plays an important role in the pathogenesis of AD (Chen et al., 2006a; Chen et al., 2008a; Chen et al., 2004; Morioka et al., 2009; Sehra et al.). Acute and chronic AD skin lesions have more IL-4 expressing cells than the unaffected skin of AD patients or skin samples from healthy people (Ou and Leung, 2005). IL-4 has been reported to suppress the production of filaggrin (Howell et al., 2008) and ceramide (Hatano et al., 2005) in the skin, disrupting skin barrier functions. IL-4 was shown to decrease the expression of human beta defensin, a potent antimicrobial peptide (AMP), leading to recurrent skin bacterial infection (Howell et al., 2006). We have generated a mouse AD animal model over-expressing IL-4 in the basal epidermis using a basal keratinocyte-specific keratin-14

promoter/enhancer (Chan et al., 2001). The transgenic (Tg) mice spontaneously develop skin lesions satisfying the clinical and histological diagnostic criteria for human AD (Chen et al., 2006a).

Although all cell types show some degree of dysfunction in AD, the major effector cells are thought to be the Th2-polarized T cells, IgE-bearing antigen presenting cells, and keratinocytes (Esche et al., 2004). We focus our attention on keratinocytes because they produce cytokines and inflammatory mediators that may be involved in initiation and exacerbation of inflammation, immunomodulation, inflammatory cell trafficking, and keratinocyte apoptosis (Esche et al., 2004; Pastore et al., 2006). We are interested in identifying genes regulated by IL-4 in keratinocytes that may play key roles in the pathophysiology of AD. Using the Inflammatory Response and Autoimmunity 384HT PCR Array (SABiosciencs, Frederick, MD), we found that among all the genes analyzed, the chemokine (C-C motif) ligand 26 (CCL26)/eotaxin-3 is stimulated most significantly (Bao and Chan, 2011) .

CCL26 is a member of the eotaxin subfamily that consists of CCL11/eotaxin-1, CCL24/eotaxin-2 and CCL26/eotaxin-3, all of which are well known for their major chemotactic roles for eosinophils (Amerio et al., 2003; Teran et al., 2006). Eotaxins are up-regulated in skin lesions of AD patients, and they may play important roles in the pathogenesis of AD (Kagami et al., 2003; Owczarek et al.; Yawalkar et al., 1999). They bind to the chemokine cysteine–cysteine receptor 3 (CCR3), which is expressed predominantly on eosinophils as well as on other cells, such as T lymphocytes, basophils, mast cells, and macrophages (Daugherty et al., 1996), recruiting these cells to the inflammatory site.

Eosinophil skin infiltration is an important characteristic of atopic dermatitis(Kapp, 1993; Leiferman et al., 1985). Activated eosinophils may release a variety of cytotoxic granule proteins to cause tissue damage (Kapp, 1993). These granular proteins include major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin (Gleich and Adolphson, 1986), which were found to be elevated in AD patients (Bruijnzeel-Koomen et al., 1992; Leiferman et al., 1985; Simon et al.). Clinically, Halmerbauer *et al* recommended measuring serum eosinophil cationic protein levels to monitor AD progression and treatment response (Halmerbauer et al., 1997).

Previously it was reported that IL-4 stimulates CCL26 expression in both primary keratinocytes and HaCat cells (Kagami et al., 2005; Nishi et al., 2008), but the detailed signal transduction pathway has not been characterized. In this study, using molecular biology techniques, we show that the regulation is at the transcriptional level and involves the Janus kinase (Jak)1 and Jak2/signal transducer and activator of transcription (Stat)6 pathway. Only the proximal Stat site in CCL26 promoter area is involved in the up-regulation of this gene by IL-4.

2. Materials and methods

2.1. Materials

IL-4, cell culture medium DMEM with or without phenol red, fetal bovine serum (FBS), charcoal stripped FBS, Penicillin/Streptomycin, nonessential amino acids, sodium pyruvate, the reverse transcriptase (RT) kit, SYBR Green PCR Master Mix, and Trizol reagent were obtained from Invitrogen (Carlsbad, CA); IRDye Secondary Antibodies, Blocking Buffers, Stripping Buffers, and PVDF membranes were from Li-Cor (Lincoln, NE); trypsin-EDTA was purchased from Mediatech (Herndon, VA); RIPA buffer, protease inhibitor cocktail, and sodium orthovanadate were purchased from Sigma (St. Louis, MO); Passive lysis 5X buffer, Dual-Luciferase Reporter Assay were purchased from Promega (Madison WI); Effectene Transfection Reagent was obtained from QIAGEN Inc. (Valencia, CA); AG490 and Jak inhibitor I were purchased from Calbiochem (La Jolla, CA). The DN-Stat6 lacking the C-terminal 186 amino acids was kindly provided to us by Dr. Steven M. Dubinett (University of California at Los Angeles, Los Angeles, CA). The DN-Stat3 is a gift from Dr. Toshio Hirano (Osaka University, Osaka, Japan). Tyr705 is replaced by phenylalanine so that it can not be phosphorylated. The DN-Stat5a was kindly given to us by Dr. Alice Mui (University of British Columbia, British Columbia, Canada). It is transcriptionally inactive because of its transactivation domain deletion at the C-terminal. The DN-Stat5b, a gift from Dr. Li-yuan Yu-Lee (Baylor College of Medicine, Houston, TX), contains a C-terminal deletion in the transactivation domain.

2.2. Cell culture

Immortalized human keratinocytes, HaCat cells, were grown in DMEM medium supplemented with FBS (10%), nonessential amino acids, and antibiotic-antimycotic solution. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 C. Culture media were replaced every 48 hours. Cells were treated with various concentrations of IL-4 in DMEM medium supplemented with 1% charcoal-dextran-treated FBS. 24 hours later, cells were washed twice with ice cold PBS and were then frozen at –80 C until RNA extraction.

2.3. RNA isolation and real-time RT-PCR analysis

Total RNA from cell culture was isolated using Trizol reagent according to the manufacturer's instructions. The RT and real-time PCRs were performed as previously described (Bao et al., 2007). Briefly 1 μg of total RNA was reverse-transcribed and the final volume was 100 μl. 5 μl and 10 μl of cDNA was used for GAPDH and CCL26 respectively. For each real-time PCR (25 μl total volume), cDNA was mixed with 2x SYBR Green PCR Master Mix containing the following primers. The human GAPDH primers used were 5'- ACA CCC ACT CCT CCA CCT TT-3' and 5'-TGC TGT AGC CAA ATT CGT TG-3'. The human CCL26 primers used were 5'-AAC TCC GAA ACA ATT GTG ACT CAG CTG-3' and 5'-GTA ACT CTG GGA GGA AAC ACC CTC TCC-3' (Kagami et al., 2005). The PCR were carried out in duplicate, and the samples were analyzed on a Stratagene Mx3000 real-time PCR machine (Santa Clara, CA). GAPDH was used as the internal reference. Melting curve analyses were done for both CCL26 and GAPDH to confirm the product specificity.

2.4. Immunocytochemistry (ICC)

ICC was performed as described previously (Bao et al., 2006). Briefly, HaCat cells were grown for 24 hours in 1% charcoal-dextran-treated FBS medium on DB chamber slides (BD Biosciences, Bedford, MA). Cells were treated with either IL-4 (20 ng/ml) or vehicle for 0.5 or 24 hours. At the end of the treatment, medium was discarded and cells were fixed for 20 minutes with 4% paraformaldehyde solution at room temperature. The cells were permeabilized for 30 minutes at room temperature in PBS containing 10% BSA, 0.1% Triton X-100, and 0.2% Tween 20. The cells were then incubated overnight at 4 C with either anti-CCL26 (Abcam, Cambridge, MA) or anti-Stat6 (Millipore, Billerica, MA). Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were then used to visualize the binding of the primary antibodies. The slides were mounted in Vectashield medium (Vector Laboratories, Inc., Burlingame, CA) containing DAPI and were observed with a Zeiss LSM 510 META Confocal Microscope (Oberkochen, Germany) equipped with a X63 water immersion objective lens.

2.5. Western blot analysis

Western blots were performed as described previously (Chen et al., 2006b). Anti-Stat6, anti-phospho-Stat6, anti-Stat5a/b, anti-phospho-Stat5a/b, anti-Stat3, anti-phospho-Stat3 (Millipore, Billerica, MA), anti-Jak1, anti-Jak2, anti-phospho-Jak1, (invitrogen, Carlsbad, CA), anti-phospho-Jak2, anti-phospho-Tyk2 (Cell Signaling, Danvers, MA) and anti-Tyk2 (Sigma, St. Louis, MO) were used. Briefly, 80 μg of cellular proteins were separated on a 10% SDS-PAGE gel under reducing condition and transferred to a PVDF membrane. The membrane was blocked by Li-Cor blocking buffer for an hour. Membranes were then incubated with the primary antibody overnight at 4 C on a rocking platform. After a series of washes, membranes were incubated with secondary antibodies linked to infrared dyes (IRDye 680 or IRDye 800) for 1–2

hours at room temperature. Finally blots were scanned by a Li-Cor Odyssey machine to obtain immunoflourescence images and quantification was done by ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA)

2.6. Transfection of HaCat cells

Transfection was done by using Effectene transfection reagent according to the manufacturer's instructions. Briefly, HaCat cells were seeded in 12-well plates and cultured until 60–80% confluency. A total of 0.4–0.6 μg DNA was transfected per well. 18-19 hours after transfection, the cells were treated with IL-4 (20 ng/ml) for another 24 hours. Transient expression of the reporter gene was quantified by a standard luciferase assay and was normalized against renilla luciferase according to the manufacturer's instructions. Luciferase activities were measured using a luminometer (Lumat LB 9507 luminometer; EG&G Berthold, Oak Ridge, TN).

2.7. Statistical Analysis

Data were examined by one-way ANOVA followed by the Tukey test (Figs. 1, 3, 5 and 6), or two-way ANOVA followed by Bonferroni post-tests (Figs. 2 and 4) using Prism software (GraphPad Software, Inc., San Diego, CA). Values were considered statistically significant at $P < 0.05$.

3. Results

3.1. IL-4 stimulates CCL26 expression in HaCat cells

To examine whether IL-4 indeed regulates CCL26 expression, we used a human epidermal cell line, HaCat cells. Cells were treated with different doses of IL-4 for 24 hours, and CCL26 mRNA expression was examined by real-time RT-PCR. The results indicate that IL-4 greatly stimulates the mRNA expression of CCL26 (Fig. 1A). The highest level of stimulation was achieved at 20 ng/ml. Interestingly, the stimulatory effect of IL-4 at doses higher than 20 ng/ml is substantially decreased. In comparison, we also examined whether IL-5 and IL-13, another two Th2 cytokines, stimulate CCL26 expression in keratinocytes. While IL-13 induced a similar pattern of CCL26 up-regulation, IL-5 had no effect on CCL26 expression. To correlate the protein change with the mRNA change, HaCat cells were either treated with or without IL-4, followed by ICC analysis of CCL26 using confocal microscopy. Consistent with the mRNA change, cells treated with IL-4 (20 ng/ml) demonstrated markedly increased CCL26 expression at the protein level as compared to the vehicle-treated cells (Fig. 1B).

3.2. Localization of the IL-4 responsive element in the CCL26 promoter

To further delineate the molecular effect of IL-4 on CCL26 expression, we attempted to locate the proximity of the CCL26 promoter where IL-4 confers its regulatory function. Towards that end, we transfected HaCat cells with CCL26 promoters truncated with serial 5' deletion mutations. The cells were cotransfected with a renilla luciferase plasmid, allowing for

normalization of transfection efficiency. Then IL-4 was introduced to assess the effects. As shown in Fig. 2, IL-4 treatment induced a 3.5-fold increase in the activity of the 800 bp CCL26 promoter. Serial 5' deletion of the promoter revealed that the region between -500 bp and -93 bp is essential for the regulation of the promoter by IL-4. Analysis of the CCL26 promoter revealed one proximal Stat site (pStat: TTCTCTGGAA -98 to -89 bp) and one distal Stat homology site (dStat: TTCCACTGGAA, -703 to -693 bp). To examine whether these two Stat sites are necessary for IL-4 stimulation of CCL26, mutagenesis studies were performed. We found that proximal Stat site mutation or double mutation eliminated IL-4 action, whereas distal site mutation had no effect on IL-4 regulation of the promoter. This data suggests that the proximal Stat site is the key transcription regulation site for the activation of this gene by IL-4. As expected, IL-4 did not induce any luciferase activity with control empty vector.

3.3 Stat6 is functionally involved in the IL-4 regulation of CCL26 in HaCat cells

Having determined the proximal Stat site as the important promoter location for IL-4 up-regulation of CCL26 activity in HaCat cells, we next asked the question of which Stat(s) is/are involved in IL-4 action. HaCat cells were treated with IL-4 (20 ng/ml) for different time points and then the cellular proteins were subjected to Western blot analysis to examine both total and phosphorylated Stats. We showed that IL-4 induced phosphorylation of both Stat3 and Stat 6 within 10–20 minutes of stimulation. However, we observed no apparent effect of IL-4 on Stat5a/b phosphorylation (Fig. 3A). Our next experiment, ICC analysis, showed nicely that IL-4 induces Stat6 nuclear translocation whereas Stat6 remains in the cytosol with vehicle treatment, thus providing additional support for Stat6 being the IL-4-regulated transcription factor (Fig. 3B).

To definitively determine which Stat is functionally involved in the IL-4 regulation of CCL26, we employed dominant negative methodology. We first transfected HaCat cells with CCL26 promoter, followed by cotransfecting these cells with dominant negative (DN)-Stat5a, DN-Stat5b, DN-Stat3, DN-stat6 or empty vector. 18-19 hours after the transfection, the cells were treated with IL-4 for another 24 hours and CCL26 promoter activity was examined by luminometry. As shown in Fig. 4, DN-Stat6, but not DN-Stat3 or DN-Stat5a/b, completely eliminated IL-4 stimulation of CCL26 promoter activity. As expected, control empty vector did not prevent IL-4 effect. Interestingly, although IL-4 stimulates phosphorylation of Stat3 protein, Stat3 has no role in the regulation of CCL26 by IL-4.

3.4. Jak1 and Jak2 are important for IL-4 regulation of CCL26 in HaCat cells

Having concluded the role of Stat6 in the IL-4 regulation of keratinocyte chemokine CCL26, we next investigated which Jak(s) is/are involved in IL-4 signal pathway. We treated HaCat cells with IL-4 for different lengths of time and the cellular protein was subjected to Western blot analysis to examine the total and phosphorylated Jak1, Jak2, and Tyk2. As shown in Fig. 5, we found that IL-4 enhanced phosphorylation of Jak1 and Jak2 but had no apparent effect on Tyk2 phosphorylation. Jak3 was not examined since it is not expressed in HaCat cells (David et al., 2001). To further confirm the role of Jak1 and Jak2 in the regulation of CCL26, we utilized both a Jak2-specific inhibitor (AG490) and a nonspecific Jak inhibitor (Jak inhibitor I), which inhibits all the Jak family members. HaCat cells were either untreated or treated with combination of IL-4 (20 ng/ml) plus various concentrations of AG490 or Jak inhibitor I, and real time RT-PCR was performed to examine CCL26 expression normalized to GAPDH. As shown in Fig. 6, AG490

suppressed significantly CCL26 expression in a dose-dependent manner. Jak Inhibitor I totally suppressed the expression of CCL26 event at 5 μM. Thus, our data suggest that Jak1 and Jak 2 may participate in the regulation of CCL26 by IL-4 in a synergistic manner. Since a Jak1-specific inhibitor is currently unavailable, we could not definitively determine the relative importance of these two Jak family members. Interestingly, in human B lymphocytes, IL-4 induces phosphorylation of Jak1 and Jak3 (Wery-Zennaro et al., 1999), indicating that IL-4 may phosphorylate different Jaks in different cells.

4. Discussion

IL-4, a T lymphocyte derived glycoprotein, is a multifunctional cytokine with a wide spectrum of biological activities. It induces the differentiation of Th0 cells to Th2 cells, IgE switching, regulation of MHC class II production (Del Prete, 1992; Lebman and Coffman, 1988; O'Keefe et al., 1999). Once activated by IL-4, Th2 cells will produce more IL-4, thus strengthening IL-4 bioactivity.

There are two types of IL-4 receptors. The type I receptor consists of IL-4Rα and IL-2Rγc chains, and the type II receptor consists of IL-4Rα and IL-13Rα1, which binds to both IL-4 and IL-13 (Aman et al., 1996; Wery-Zennaro et al., 1999). There is no γc chain expression in keratinocytes; therefore IL-4 signal transduction is through either homodimerization of IL-4Rα or heterodimerization of IL-4Rα and IL-13Rα1(Wery-Zennaro et al., 1999).

We have shown that in HaCat cells, IL-4 stimulates phosphorylation of Stat3, but phosphorylated Stat3 is not involved in the regulation of CCL26 by IL-4. It is likely that activated Jak1 and Jak2 phosphorylate adjacent Stat3, but Stat3 full activation may need other protein modifications, such as serine phosphorylation (Decker and Kovarik, 2000). Furthermore, Yuan *et al* recently reported that a mutant Stat3 lacking lysine 685 for acetylation actually loses its ability to transactivate its target genes although it can still translocate to the nucleus after tyrosine and serine phosphorylation (Yuan et al., 2005). Interestingly, it has been reported that Stat3 nuclear translocation is independent of tyrosine phosphorylation (Liu et al., 2005), and unphosphorylated

Stat3 can even transactivate genes (Yang et al., 2005). Taken together, tyrosine phosphorylation is neither necessary nor sufficient for activation of Stat3. The molecular mechanisms involved may need further investigation. Stat6 knockout mice show defects in IgE switching and Th2 cell development (Akira, 1999), and these mice lack eosinophil tissue infiltration even when challenged with ovalbumin that is often used to induce AD in animal studies (Miyata et al., 1999). On the other hand, although Stat3 Cre-loxP knockout mice show defects in T cells, macrophages and neutrophils, no eosinophil malfunction has been reported (Akira, 1999), lending further support to a major role of Stat6 in IL-4 regulated CCL26 expression.

Phosphorylated Stat6 dimerizes and tranlocates to the nucleus to transactivate its target genes. The Stat response elements consist of two palindromic half sites TTC and GAA, which are separated by several random nucleotides. Different from most other Stat proteins, which usually sit on the palindromic core motif with three spacing nucleotides, Stat6 prefers four spacing nucleotides in its response elements (Ehret et al., 2001). This can partly explain why it is the proximal Stat site rather than the distal site that is involved in the IL-4 regulation of CCL26 in keratinocytes.

IL-4 has been shown to activate different signal pathways in different tissues including Jak/Stat, NF-κB, MAPK and PI3 pathways (Benczik and Gaffen, 2004; Jiang et al.). It is clear from this study that IL-4 up-regulation of CCL26 in the skin involves Jak1, 2/Stat6 pathway. Previously we have shown that CCL27, a T cell skin-homing factor that is elevated in sera of AD patients, is over-expressed in the skin of IL-4 transgenic mice (Chen et al., 2006a). Different from the regulation of CCL26, the high level of CCL27 is not due to its direct regulation by IL-4; instead,

it is up-regulated by high levels of IL-1β and TNF-α in the skin lesion of the IL-4 Tg mice as compared to wild type mice (Chen et al., 2006a; Chen et al., 2004).

Skin eosinophil infiltration is one of the most important characteristics of AD. Among different eosinophil chemoattractants, the eotaxin subfamily is considered to play important roles. In our gene array study, we found that in addition to CCL26, CCL24 was also up-regulated (Bao and Chan, 2011). IL-4 induced a 500-fold up-regulation of CCL26, whereas it only increased CCL24 expression by 5-fold. CCL11 expression in keratinocytes was too low for us to examine whether it was regulated by IL-4 in our gene array study. It has been reported that CCL11 is expressed in the skin fibroblast (Mochizuki et al., 1999), and it is up-regulated by IL-4 (Gahr et al.). Furthermore, fibroblasts from AD lesions show increased CCL11 responsiveness to IL-4 stimulation (Gahr et al.). This suggests that in the skin under the action of IL-4, CCL11, CCL24 and CCL26 may work synergistically to attract eosinophils to cause tissue damage. It was reported that serum levels of CCL26 alone but not CCL24 or CCL11 are correlated with AD severity (Kagami et al., 2003), suggesting that CCL26 may play a major role in the pathogenesis of AD among eotaxin subfamily members. Interestingly in mice, while CCL11 and CCL24 have been reported to be involved in eosinophil recruitment and activation (Oshio et al., 2009), mouse CCL26 is a pseudogene (Pope et al., 2005). IL-4 Tg mice displayed CCL24 up-regulation and increased eosinophil skin infiltration as compared to wild type mice, but we did not see any difference in CCL11 expression (Chen et al., 2008b). Additionally, when sensitized with ovalbumin, the skin from IL-4 knockout mice failed to manifest AD skin changes or skin eosinophil infiltration. All these suggest that in mice CCL24 may play similar roles in AD pathogenesis as CCL26 does in humans, both of which are up-regulated by IL-4 in the skin. In

addition to binding to CCR3 receptors, CCL26 also binds to CX3CR1 receptors (Nakayama et al.). The up-regulation of CCR3 and CX3CR1 was observed in AD patients (Echigo et al., 2004; Kato et al., 2006). Besides the eotaxin subfamily, eosinophil attractractants also include CCL5, monocyte chemotactic protein-3, IL-5, granulocyte-macrophage colony-stimulating factor, and platelet-activating factor (Schroder et al., 1996).

Taken together, results from this investigation provide an explanation for the pathogenesis of AD and describe the detailed signal transduction pathway for IL-4 regulation of CCL26 in the skin. Targeting these pathway components with chemical inhibitors or molecular biology techniques may ultimately lead to effective treatment of AD.

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Figure 1. IL-4 and IL-13 stimulate CCL26 expression in HaCat cells.

(A) HaCat cells, a human keratinocyte cell line, were treated with different doses of IL-4, IL-5 and IL-13 for 24 hours. CCL26 mRNA levels were analyzed by real-time RT-PCR. Values are expressed as the mean+- SEM (n=4). *, $P < 0.05$ *vs.* 0 ng/ml. (B) HaCat cells were treated with IL-4 (20 ng/ml) or vehicle for 24 h and were subjected to immunocytochemistry as described in *Methods. CCL26, Red; nucleus, blue.* Experiments were repeated four times.

Figure 2. IL-4 activates CCL26 promoter in HaCat cells.

Using Effectene reagent, HaCat cells were transfected with equal amounts of different CCL26 promoter reporter constructs obtained by serial 5 deletion, internal deletion or mutation. 18-19 hours after transfection, cells were treated with IL-4 (20 ng/ml) for another 24 h. Transient expression of the reporter gene was quantified by a standard luciferase assay and normalized against renilla luciferase. The experiment was repeated three times with triplicate wells for each group. Values are expressed as the mean+- SEM. *, $P < 0.05$ *vs.* vehicle.

Figure 3. Stat6 phosphorylation and nuclear translocation are stimulated by IL-4 treatment in HaCat cells.

(A) HaCat cells were cultured in 1% charcoal-dextran-treated FBS for 24 hours. The cells were serum starved for 4 hours and then treated with IL-4 (20 ng/ml) for different lengths of time. Phosphorylation of Stat3, Stat5 and Stat6 was measured by Western analysis and quantified by ImageQuant. Values are expressed as the mean+- SEM (n=3). *, $P < 0.05$ *vs.* 0 min. (B) The cells were treated with either vehicle (a-c) or IL-4 (20 ng/ml) (d-f) for 30 min. The Cells were

subjected to immunocytochemistry as described in *Materials and Methods. Stat6, Red; nucleus, blue.* Experiments were repeated four times.

Figure 4. DN-Stat6 suppresses the stimulation of CCL26 promoter by IL-4 in HaCat cells. HaCat cells were cotransfected with the CCL26 promoter reporter construct and with either empty vector, DN-Stat3, DN-Stat5a, DN-Stat5b or DN-stat6 for 18-19 hours. Cells were then treated with either IL-4 or vehicle for another 24 h. Transient expression of the reporter gene was quantified by the standard luciferase assay and normalized against renilla luciferase. The experiment was repeated three times with triplicate wells for each group. Values are expressed as the mean+- SEM. *, $P < 0.05$ *vs.* vehicle.

Figure 5. Jak1 and Jak2 but not Tyk2 are phosphorylated by IL-4 in HaCat cells. HaCat cells were cultured in 1% charcoal-dextran-treated FBS for 24 hours. The cells were serum starved for 4 h and then treated with IL-4 (20 ng/ml) for different lengths of time. Phosphorylation of Jak1, Jak2 and Tyk2 was measured by Western analysis and quantified by ImageQuant. Values are expressed as the mean+- SEM (n=3). *, $P < 0.05$ *vs.* 0 min.

Figure 6. Jak inhibitors suppress the up-regulation of CCL26 by IL-4 in HaCat cells. Total RNA obtained from HaCat cells treated with different doses of AG490 (a Jak2 specific inhibitor) or Jak inhibitor I (a nonspecific Jak inhbitor) was subjected to real-time RT-PCR analysis. Values are expressed as the mean+- SEM (n=4). *, $P < 0.05$ *vs.* control.