

IL-17A Upregulates Keratin 17 Expression in Keratinocytes through STAT1- and STAT3-Dependent Mechanisms

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Psoriasis, an immunological skin disease, is characterized by epidermal hyperproliferation, chronic inflammation, and an accumulation of infiltrating T cells. IL-17A is a key cytokine that has a critical role in the pathogenesis of psoriasis. Keratin 17 (K17) is strongly expressed in psoriatic lesions but not in normal skin. Thus, K17 expression is regarded as a hallmark of psoriasis. We previously reported that the K17/T cells/cytokine autoimmune loop was involved in psoriasis. However, the relationship between IL-17A and K17 has yet to be determined. In the present study, IL-17A-induced K17 expression was confirmed in cultured keratinocytes in both mRNA and protein levels. In addition, increased K17 expression was found in the epidermis of IL-17A-injected mouse skin. The regulatory mechanism of K17 expression was further investigated. We found that both the signal transducer and activator of transcription (STAT) 1 and STAT3 pathways were involved in the upregulation of K17 expression induced by IL-17A, and that such regulation could be partially suppressed by STAT1 or STAT3 small interfering RNA and inhibitor. Our data suggest that IL-17A can upregulate K17 expression in keratinocytes in a dose-dependent manner through STAT1- and STAT3-dependent mechanisms. The results indicate that IL-17A might be an important cytokine in the K17/T cells/cytokine autoimmune loop associated with psoriasis.

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INTRODUCTION

Keratin 17 (K17), a myoepithelial keratin, is overexpressed in psoriatic lesions, and is not found in healthy epidermis. Hence, K17 is considered to be a hallmark of psoriasis (de Jong *et al.*, 1991). It has been shown that IFN- γ can upregulate K17 expression by activating signal transducer and activator of transcription (STAT) 1, a transcription factor (Jiang *et al.*, 1994). K17 may function as an autoantigen in the immunopathogenesis of psoriasis, which may be a major target for autoreactive T cells (Fierlbeck *et al.*, 1990). Some restricted T-cell epitope regions, found on the K17 molecule, can promote the proliferation of psoriatic T cells and induce the production of IFN- γ effectively (Shen *et al.*, 2005). Thus, a positive-feedback mechanism, previously described as a K17/T cell/cytokine autoimmune loop, may exist to drive the pathogenesis of

psoriasis (Bockelmann *et al.*, 2005; Shen *et al.*, 2006). Recently, the relationship between K17 overexpression and psoriasis has captured the attention of dermatologists, but the regulation and biological roles of K17 in psoriasis remains unknown.

Psoriasis is now believed to be a mixed Th1/Th17 cell-mediated autoimmune disease, in which the likely induction of IFN- $\gamma^{(+)}$ IL-17 $^{(+)}$ cells is considered to be pathogenic (Ksatelan *et al.*, 2004; Arican *et al.*, 2005). IL-17A is a cytokine produced by Th17 cells that helps to recruit neutrophils and drive inflammatory responses (Albanesi *et al.*, 2000; Weaver *et al.*, 2007). IL-17A expression is detectable in psoriatic skin lesions and allergic contact dermatitis, but not in normal skin (Teunissen *et al.*, 1998; Albanesi *et al.*, 1999). Overexpression of IL-17A at both gene transcript and protein levels has been observed in serum and skin lesions of psoriatic patients, and is correlated with the severity of the disease (Arican *et al.*, 2005; Caproni *et al.*, 2009; Kagami *et al.*, 2010). This evidence strongly suggests that IL-17A is involved in the pathophysiology of psoriasis (Fitch *et al.*, 2007; van Beelen *et al.*, 2007).

On the basis of two previously reported findings, (1) that the K17/T cells/cytokine loop functions in the pathogenesis of psoriasis (Shen *et al.*, 2006), and (2) that IL-17A is a Th17-produced cytokine that activates three signaling pathways, which is detectable in psoriatic skin lesions, we hypothesized that IL-17A may be a key cytokine member of the K17/T cells/cytokine autoimmune loop and induce K17 expression by activating signaling pathways, and therefore participate in the

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Abbreviations: K17, keratin 17; NHEK, normal human epidermal keratinocyte; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription

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development of psoriasis. We tested this hypothesis in the present study by observing the effect of IL-17A on the expression of K17 in HaCaT human keratinocytes, normal human epidermal keratinocytes (NHEKs), and mice model.

RESULTS

The upregulation of K17 expression in IL-17A-induced keratinocytes

To determine whether IL-17A can upregulate K17 expression, we pretreated HaCaT cells with IL-17A in different concentrations (10, 50, 250, or 500 U ml⁻¹), and then analyzed K17 mRNA levels with real-time PCR. As expected, we found that K17 mRNA levels increased with IL-17A concentration in a

dose-dependent manner, especially at higher concentrations (250 and 500 U ml⁻¹), as compared with the levels in untreated cells (Figure 1a). Meanwhile, we constructed two reporter vectors containing different regions of K17 promoter spanning -244 to +18 bp (Δ K17p1) and -686 to +18 bp (Δ K17p2). IL-17A upregulated the activity of Δ K17p2 promoter, whereas Δ K17p1 did not respond to IL-17A, suggesting that the element in response to IL-17A is located between -686 and -244 bp of the K17 promoter (Figure 1b). To further confirm this finding, ELISA and western blot assays were performed to measure K17 protein expression. K17 protein expression was upregulated by IL-17A at concentrations of 250 U ml⁻¹ or higher. However, no significant difference in the expression levels of K17 protein was

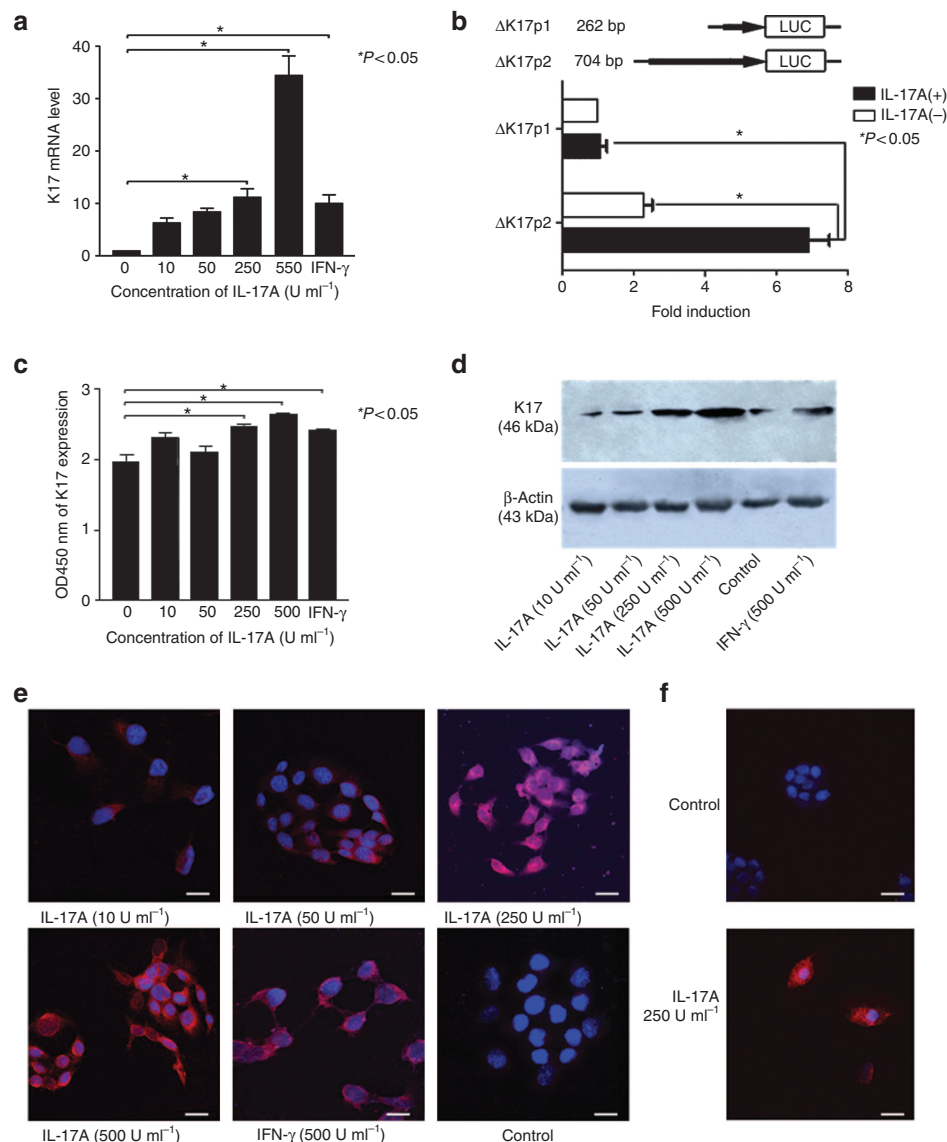


Figure 1. Upregulation of keratin 17 (K17) expression in IL-17A-induced keratinocytes. (a) Real-time PCR analysis of K17 mRNA level. Data were expressed as $2^{-\Delta\Delta CT}$ relative to untreated HaCaT cells. (b) K17 promoter activity was examined via reporter assay under the stimulation of IL-17A (250 U ml⁻¹). Filled arrows represent the K17 promoter region, the box the firefly luciferase gene. (c) ELISA analysis of K17 expression. (d) Protein expression of K17 was examined using western blot. (e) Immunofluorescence was performed on HaCaT cells to measure K17 expression. 4'-6-Diamidino-2-phenylindole staining for nuclei is blue. (f) Immunofluorescence was performed on normal human epidermal keratinocytes to measure K17 expression. Bar = 30 μ m. Results represent mean \pm SEM from three independent experiments. * $P < 0.05$ was considered significant.

observed when the concentration of IL-17A was lower than 50 U ml^{-1} (Figure 1c and d). To characterize K17 protein expression in IL-17A-treated HaCaT cells, we evaluated these cells with two-color immunofluorescence. Immunostaining revealed weak K17 signals in the cytoplasm of untreated cells. In contrast, there was a gradual increase in the staining intensity of intracellular K17, which was correlated with an increasing concentration of IL-17A from 10 to 500 U ml^{-1} . In particular, HaCaT cells treated with an IL-17A concentration of 250 U ml^{-1} were similar to control HaCaT cells treated with IFN- γ (Figure 1e). K17 expression (Figure 1f and Supplementary Figure S4 online), as well as K6 and K16 expression (Supplementary Figure S1 online), was also found in the cultured NHEKs treated with IL-17A (250 U ml^{-1}). Taken together, IL-17A upregulates K17 expression in keratinocytes in a dose-dependent manner.

The effects of STAT1 and STAT3 signaling pathways on K17 expression in IL-17A-induced HaCaT cells

As K17 expression in HaCaT cells is primarily regulated by STAT-dependent signaling pathways, phosphorylation of STAT1 and STAT3 was observed in IL-17A-treated HaCaT cells. The results of ELISA, western blot, and immunofluorescence showed that IL-17A could promote STAT1 and STAT3 phosphorylation at a concentration of 250 U ml^{-1} , indicating an enhanced STAT1 and STAT3 activation (Figure 2). To characterize that the signaling pathways of IL-17A increase K17 expression in HaCaT cells, fludarabine and piceatannol were used to selectively inhibit STAT1 and

STAT3 signaling, respectively. Preincubation of HaCaT cells with fludarabine or piceatannol partially suppressed the effect of IL-17A on K17 expression. K17 mRNA was then quantified by real-time PCR (Figure 3a). These data show that both fludarabine and piceatannol significantly reduce the expression of K17. Next, the inhibition of K17 expression was confirmed at the protein level by ELISA, western blot, and immunofluorescence (Figure 3b, c and d). Taken together with real-time PCR, fludarabine and piceatannol partially suppressed the effect of IL-17A on K17 expression at the mRNA and protein level. Thereafter, we knocked down the expression of STAT1/3 via small interfering RNA (siRNA) before IL-17 stimulation, which resulted in the same K17 expression variation as the specific inhibitors did (Figure 4). These results indicate that activation of the STAT1 and STAT3 pathways are required for IL-17A-induced K17 expression in HaCaT cells.

The role of p65 and p38 signaling pathways in K17 expression in IL-17A-induced HaCaT cells

We examined the role that the p65 and p38 signaling pathways have in IL-17A-induced K17 expression. HaCaT cells were incubated with IL-17A at a concentration of 250 U ml^{-1} for 15, 30, or 60 minutes, following which levels of p65/p38 expression were determined by ELISA, western blot, and Immunofluorescence analyses. As shown in Supplementary Figure S2 online, increased phosphorylated forms of both p65 and p38 were found in IL-17A pretreated HaCaT cells, which indicated that p65 and p38 pathways

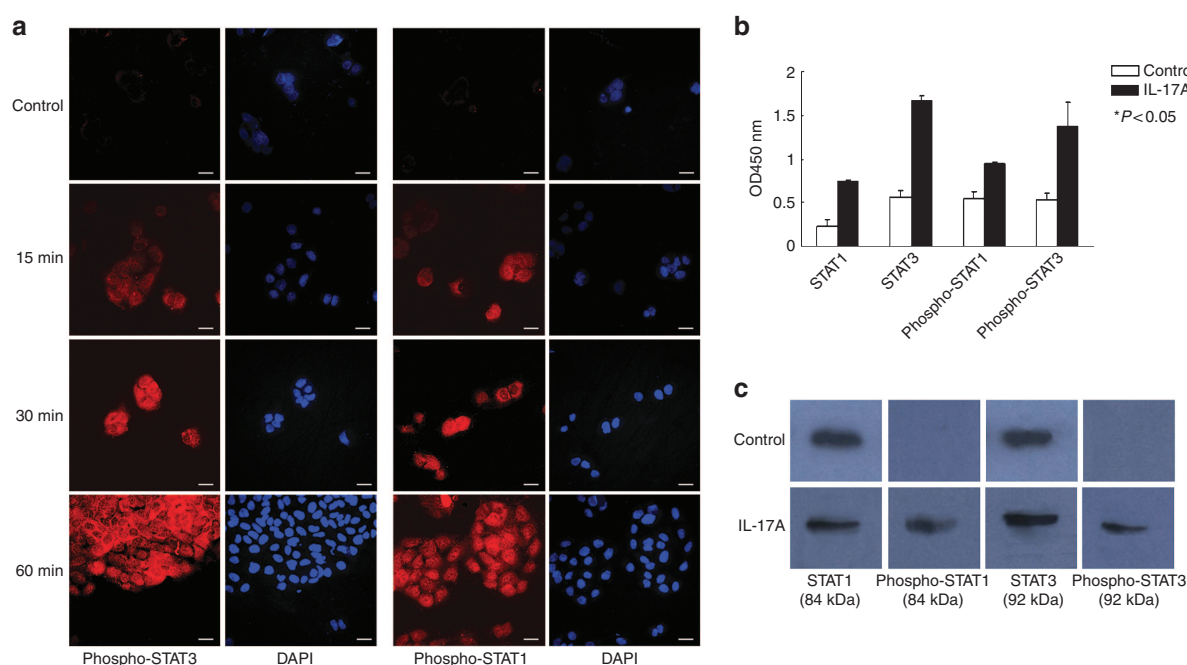


Figure 2. Activation of signal transducer and activator of transcription (STAT) 1 and STAT3 signaling pathways in IL-17A-treated HaCaT cells. HaCaT cells were stimulated with IL-17A (250 U ml^{-1}) and the expression of STAT1, STAT3, phospho-STAT1, or phospho-STAT3 was tested with corresponding antibodies. (a) Immunofluorescence staining of phospho-STAT1 and phospho-STAT3 in IL-17A-treated HaCaT cells at different time points. Note that the signals obtained in cultures at 15, 30, and 60 minutes showed a progressive increase in intensity. Bars = $30 \mu\text{m}$. (b) Cell-based ELISA analysis revealed that STAT1 and STAT3 pathways were markedly activated at 60 minutes following IL-17A treatment. The graph shows the means of three samples and error bars represent SEM values (* $P < 0.05$). (c) The activation of phospho-STAT1 and phospho-STAT3 in IL-17A-treated HaCaT cells was analyzed by western blot analysis at 60 minutes following IL-17A treatment. DAPI, 4',6-diamidino-2-phenylindole.

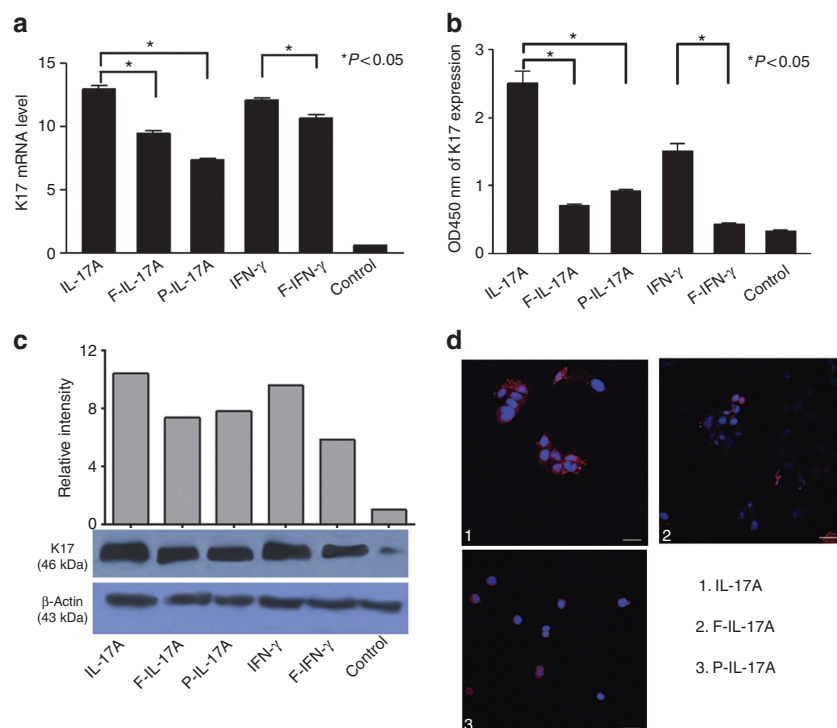


Figure 3. Inhibition of signal transducer and activator of transcription 1/3 (STAT1/3) pathways partially suppresses the effect of IL-17A on keratin 17 (K17) expression via a specific inhibitor. (a) Examination of the inhibitory effect of fludarabine and piceatannol on IL-17A (250 U ml⁻¹)-induced K17 mRNA expression level with real-time PCR analysis. (b) The effects of STAT1/3 inhibitor on K17 expression were analyzed with ELISA. (c) Western blot of the inhibitory effect of fludarabine and piceatannol on K17 expression. (d) Immunofluorescence analysis of K17 expression in fludarabine (2), piceatannol-treated (3), or untreated HaCaT cells (1). 4'-6-Diamidino-2-phenylindole staining for nuclei is blue. IL-17, HaCaT cells treated with IL-17; F-IL-17, treated with fludarabine and IL-17; P-IL-17, treated with piceatannol and IL-17; IFN- γ , treated with IFN- γ ; F-IFN- γ , treated with fludarabine and IFN- γ . * P <0.05. Bars = 30 μ m.

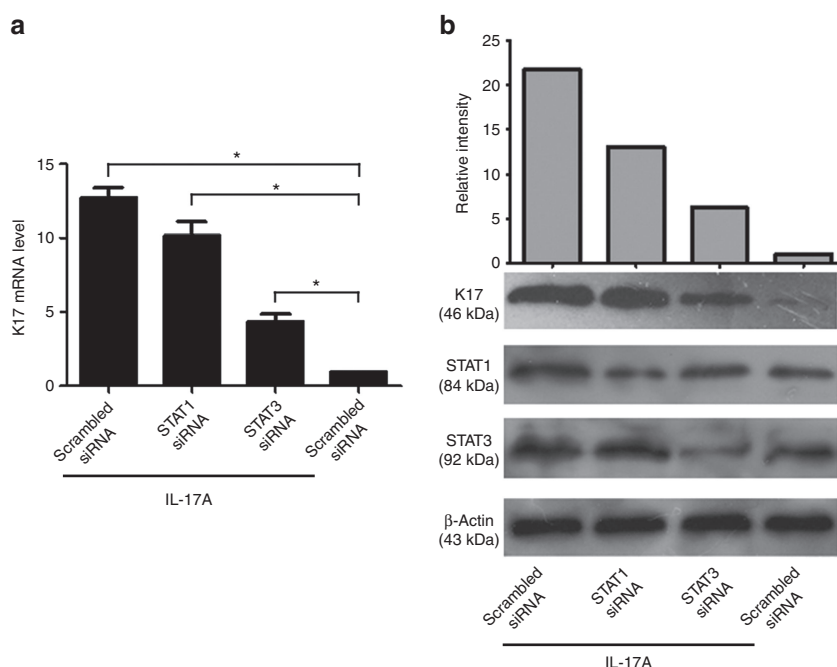


Figure 4. Inhibition of signal transducer and activator of transcription 1/3 (STAT1/3) pathways partially suppresses the effect of IL-17A on keratin 17 (K17) expression via STAT1/3 small interfering RNA (siRNA). (a) Examination of the inhibitory effect of STAT 1/3 siRNA on IL-17A (250 U ml⁻¹)-induced K17 mRNA expression level with real-time PCR analysis. HaCaT cells were transfected with scrambled siRNA or STAT1/3 siRNA using Lipofectamine 2000 24 hours before undergoing a 48-hour stimulation with IL-17A or not. RNA samples were then collected to perform real-time PCR analysis (* P <0.05). (b) Western blot analysis of the inhibitory effect of STAT1/3 siRNA on K17 expression. HaCaT cells were transfected with scrambled siRNA or STAT1/3 siRNA using Lipofectamine 2000 24 hours before undergoing a 48-hour stimulation with IL-17A or not. Protein samples were then collected to perform western blot analysis.

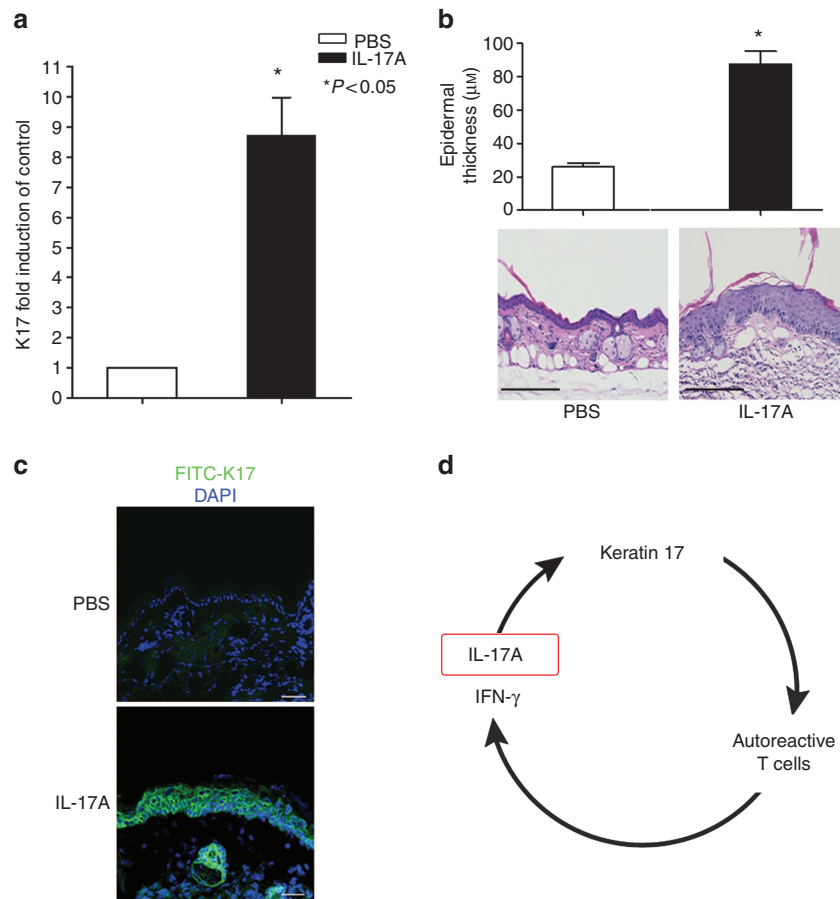


Figure 5. Epidermal proliferation and upregulation of keratin 17 (K17) in the mouse skin induced by IL-17A. (a) Real-time PCR analysis of K17 mRNA level after IL-17A treatment (500 U q.d.). Results represent mean \pm SEM from three independent experiments. (b) Hematoxylin and eosin-stained sections of phosphate-buffered saline (PBS)- or 500 U IL-17A-injected ears after daily injection for 7 days. Epidermal thickness was measured. Data are from three experiments in five mice per groups. * $P < 0.05$, IL-17A-injected versus PBS control. Bars = 200 μ m. (c) Frozen sections of ears from PBS-injected or IL-17A-injected mouse stained with rabbit anti-mouse K17 pAb, followed by FITC-conjugated goat anti-rabbit IgG (green). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Bars = 30 μ m. (d) A loop working in the pathogenic process of psoriasis.

were activated by IL-17A in HaCaT cells. To further confirm this finding, HaCaT cells were preincubated with PDTC and SB203580, which are p53 and p38 inhibitors, respectively, for 2 hours before IL-17A treatment. We found that pretreatment with p53 and p38 inhibitors did not block increased K17 expression induced by IL-17A at both mRNA and protein levels (Supplementary Figure S3 online). These results suggest that the p53 and p38 signaling pathways are not involved in the induction of K17 expression in HaCaT cells stimulated with IL-17A.

Epidermal proliferation and K17 expression induced by IL-17A *in vivo*

We performed additional experimentation *in vivo* in order to further confirm that IL-17A can upregulate K17 expression in keratinocytes. We examined whether IL-17A increased the expression of K17 after IL-17A injection in mouse skin using real-time PCR and immunohistological analysis. The expression of K17 mRNA after two injections was 8.9-fold higher than that in the control group (Figure 5a). Hematoxylin and eosin staining of sections from mouse ears after daily

injection for seven times showed the epidermal changes, namely acanthosis (thickening of the spinous layer). The mouse epidermal thickness was $87.55 \pm 15.06 \mu$ m (IL-17A group) and $26.08 \pm 4.57 \mu$ m (phosphate-buffered saline (PBS) group; Figure 5b). Immunofluorescence microscopy analysis with anti-K17 antibody revealed prominent upregulation of K17 in acanthotic mouse epidermis (Figure 5c). These results suggest that IL-17A can also upregulate K17 expression that may result in acanthosis *in vivo*.

DISCUSSION

In this study, we demonstrated that IL-17A induces K17 expression in keratinocytes in a dose-dependent manner, which, to our knowledge, is previously unreported. K17 is a putative major autoantigen recognized by autoreactive T cells in psoriasis. Previous studies demonstrated that K17 shares the ALEEAN amino-acid sequence with streptococci M protein (Sigmundsdottir *et al.*, 1997), which has been proven to be one of the exogenous antigens of psoriasis and is associated with acute guttate psoriasis (Valdimarsson *et al.*, 1997, 2009; McFadden *et al.*, 2009). In addition, it was

confirmed that the K17 molecule contains at least another five psoriasis-related T-cell epitopes without the ALEEN sequence, and that these epitopes can stimulate the proliferation of psoriatic T cells and promote the release of IFN- γ . Therefore, K17 has the ability to stimulate psoriatic T cells, which produce high levels of IFN- γ (Shen *et al.*, 2006). Moreover, it has been reported that K17 can promote hyperplasia and Th1- and Th17-dominated inflammatory responses in the epidermis (Depianto *et al.*, 2010). Meanwhile, it has been demonstrated that the expression of K17 can be induced *in vitro* by IFN- γ (Bonnekoh *et al.*, 1995). Moreover, K17 is the only keratin reported to be induced by IFN- γ (Arican *et al.*, 2005; Van Beelen *et al.*, 2007). Hence, we suggested that a K17/T cell/cytokine autoimmune loop exists in the psoriatic epidermis with IFN- γ as one of the cytokine members. Such an autoimmune loop may have a key role in the maintenance, aggravation, and relapse of psoriatic lesions.

IL-17A is secreted predominantly by a special subset of T-helper (Th) cells (CD4 cells) termed Th17 cells, which are phenotypically and functionally distinct from Th1 cells, Th2 cells, and T-regulatory cells (Harrington *et al.*, 2005; Park *et al.*, 2005; Wynn, 2005; Ivanov and Lindén, 2007). Atypically elevated IL-17A levels are found in skin lesions and serum of psoriatic patients, whereas IL-17A remains at a baseline level in normal skin and serum. It is widely accepted that IL-17A is involved in the pathogenesis of psoriasis (Lowe *et al.*, 2008; Lee *et al.*, 2010). As we have revealed that IL-17A induces K17 expression by increasing the K17 promoter activity, it is safe to say that IL-17A may be an additional important cytokine member in the K17/T cell/cytokine autoimmune loop. Moreover, IL-17A has at least one role in the pathogenesis of psoriasis as the inducer of K17 expression (Figure 5d).

Furthermore, we investigated the mechanism of IL-17A-induced expression of K17. Intracellular IL-17A signaling includes at least three pathways. IL-17A can potentially activate JAK/STAT, mitogen-activated protein kinase, and NF- κ B pathways (Gaffen, 2008; Shen and Gaffen, 2008; Ivanov and Lindén, 2009). In particular, IL-17A induces tyrosine phosphorylation of STAT1, 2, 3, and 4 in U937 monocytic leukemia cells (Subramaniam *et al.*, 1999; Miyoshi *et al.*, 2011). The STAT family can be activated by the specificity of a cytokine signal at the cell surface. For example, STAT1 is induced by IFN- γ , IL-6, and epidermal growth factor; STAT2 by IFN- α ; STAT3 by epidermal growth factor and IL-6; and STAT4 by IL-12. As we know, IFN- γ and IL-6 induce K17 expression through the STAT1 signaling pathway. IFN- α does not influence K17 gene expression (Jiang *et al.*, 1994; Komine *et al.*, 1996). IL-12 (p35/p40) is present during the activation of naive T cells, which are polarized into Th1 cells through the activation of transcription factors STAT4 and T-bet (Weaver *et al.*, 2006). It is also well established that members of the mitogen-activated protein kinase family, such as c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38, are clearly involved in IL-17A-induced cytokine production (Kolls and Lindén, 2004). Analysis of psoriatic skin has shown that the levels of p38 activity are increased, which is consistent with

the potential role that these kinases have in the development of psoriasis (Johansen *et al.*, 2005). NF- κ B is highly activated in inflammatory diseases such as in rheumatoid arthritis and psoriasis, and is often used as a potential therapeutic target in clinical applications. Antisense oligonucleotide therapy has been used to block p65 activation, a member of NF- κ B signaling pathways, and is an effective treatment for a murine model of colitis (Murano *et al.*, 2000).

We examined the aforementioned signaling pathways and found that HaCaT cells pretreated with IL-17A expressed phosphorylated proteins of STAT1 and STAT3, whereas untreated HaCaT cells did not. The induction of K17 expression by IL-17A was partially suppressed by STAT1- or STAT3-specific inhibitor and siRNA, but not by p65 or p38 inhibitor. Taken together, we concluded that IL-17A upregulates K17 expression by activating STAT1 and STAT3 signaling transduction pathways. However, we can see that blocking the STAT 1/3 pathway cannot completely suppress K17 expression induced by IL-17A. We therefore deduce that there may be some other pathways involved in the regulation of K17 expression induced by IL-17A, such as the c-Jun N-terminal kinase and extracellular signal-regulated kinase pathways, which our group is currently investigating.

Improved comprehension of the complex interplay between cytokines, their molecular signaling pathways in affected cells, and the resulting changes in these affected cells will make way for a better understanding of the pathomechanisms involved in psoriasis. Future studies will continue to investigate and profile the actions of particular cytokines in both healthy and psoriatic keratinocytes. Our study has demonstrated that K17 expression is induced by IL-17A stimulation in keratinocytes via the STAT1 and STAT3 signaling pathways, and that IL-17A might be an important cytokine in a K17/T cells/cytokine autoimmune loop in psoriasis. Our findings help to further elucidate the molecular and cellular mechanisms underlying the pathogenesis of psoriasis, which provides the field with new strategies for the treatment of this chronic disease.

MATERIALS AND METHODS

Cell cultures and IL-17A treatment

HaCaT cells were cultured in DMEM (Gibco-Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Gibco-Invitrogen) under a humidified atmosphere containing 5% CO₂ at 37 °C. NHEKs were obtained from skin biopsies of healthy volunteers (N=6), who provided written, informed consent. NHEKs were cultured in the serum-free medium, keratinocyte growth medium (Gibco-Invitrogen), for at least 3–5 days. Cells at 40–60% confluence were stimulated with IL-17A (10, 50, 250, or 500 U ml⁻¹) for 48 hours, and IFN- γ (Peprotech, Rocky Hill, NJ)-treated HaCaT cells (250 U ml⁻¹) were used as positive control. To inhibit STAT1, STAT3, p65, and p38 signaling pathways, fludarabine, piceatannol, PDTC, and SB203580 (100 μ M, respectively; Sigma, St Louis, MO) were added 2 hours before stimulation with IL-17A (Peprotech), respectively.

siRNA targeting STAT1 and STAT3 were synthesized from Gene Pharma (Shanghai, China) and dissolved in diethylpyrocarbonate-treated H₂O at a concentration of 20 μ mol l⁻¹ as a stock. According to the manufacturer's instructions, HaCaT cells were transfected with

plasmids or siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 24 hours before stimulation with IL-17A.

Real-time PCR

Total cellular RNA was isolated using a Total RNA Extraction Kit (Anmei Biologicals, Xi'an, China) following the manufacturer's instructions, and cDNA was synthesized with PrimeScript RT reagent Kit (Takara, Ohtsu, Japan) according to the manufacturer's protocol and used as the template for quantitative PCR. Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa) on a Chromo4 continuous fluorescence detector with a PTC-200 DNA Engine Cycler (Bio-Rad, Hercules, CA). The reaction components were 0.5 µl of forward and reverse primers for K17 and β-actin as an internal control (Supplementary Table S1 online). The cycling conditions were as follows: 95 °C for 2 minutes, followed by 45 cycles of denaturation at 95 °C for 5 seconds, annealing at 60 °C for 10 seconds, and extension at 72 °C for 15 seconds. All reactions were run in triplicates for at least three independent experiments. Relative quantification was performed according to the $\Delta\Delta C_T$ method, and results were expressed in the linear form using the formula $2^{-\Delta\Delta C_T}$. Results were considered significant when at least a 2-fold difference in expression levels was detected.

Plasmid construction and reporter assay

For the construction of the pGL3-ΔK17p1 and pGL3-ΔK17p2 reporter vectors, different regions of K17 promoter were amplified by reverse transcription-PCR from the chromosome of primary keratinocytes using the primers listed in Supplementary Table S1 online. The PCR products were digested with indicated restriction enzymes (Takara) before being ligated into pGL3 basic vector (Promega, Madison, WI).

HaCaT cells were seeded in 24-well plates and transfected with 1 µg of pGL3-ΔK17p1 or pGL3-ΔK17p2. Cells were co-transfected with 50 ng of pRL-TK (a plasmid constitutively expressing Renilla luciferase; Promega) to normalize for transfection efficiency. Cells were treated with IL-17A (250 U ml⁻¹) for 24 hours, and then lysed using passive lysis buffer and analyzed for firefly and Renilla luciferase activities using the dual-luciferase reagent assay kit (Promega) according to the manufacturer's instructions. Values were corrected for transfection efficiency and expressed as Mean ± SEM from at least three independent experiments.

ELISA

HaCaT cells were seeded into 96-well plates precoated with 10 µg ml⁻¹ poly-L-lysine with a cell density of 1.5×10^4 cells per well, and then were cultured at 37 °C for 24 hours. After induction of IL-17A as described above, the expressions of K17, STAT1, STAT3, p65, p38, phospho-STAT1, phospho-STAT3, phospho-p65, and phospho-p38 were determined by cell-based ELISA. In brief, after adherence of cells, the cells were stimulated by IL-17A at concentrations of 10–500 U ml⁻¹ at 37 °C for 48 hours. After washing, the cells were blocked with blocking buffer for 1 hour at room temperature and then were incubated with primary antibodies for K17, STAT1, STAT3, p65, p38, phospho-STAT1, phospho-STAT3, phospho-p65, and phospho-p38 (Cell Signaling Technology, Danvers, MA) at 37 °C for 1 hour. Next, the cells were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Dako Cytomation, Glostrup, Denmark) at 37 °C for 1 hour. Subsequently, the cells were incubated

with 100 µl of a developing solution for 10 minutes at room temperature. The reaction was stopped with 100 µl of a stop solution and the absorbance was measured at an optical reference wavelength of 450 nm with an ELISA reader.

Western blot

Total proteins of HaCaT cells were prepared for western blot analysis. Briefly, 5×10^6 IL-17A-treated cells were harvested and proteins from cell lysates were denatured in SDS, and 30 µg of total extracted proteins were applied per lane for 10% SDS-PAGE. After proteins were transferred to Invitrolon PVDF membrane (Invitrogen), nonspecific binding was blocked for 2 hours in the blocking buffer. The membranes were incubated with specific antibodies against β-actin (Abcam, Cambridge, UK), K6, K16 (Epitomics, Burlingame, CA), K17, STAT1, STAT3, p65, p38, phospho-STAT1, phospho-STAT3, phospho-p65, and phospho-p38 (Cell Signaling Technology) overnight. Blots were washed with Tris-buffered saline Tween-20, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Dako Cytomation) for 1 hour. Bound antibodies were revealed using chemiluminescence reaction. The expression amounts of K17 were quantitated using the Alphamager Gel (Alpha Innotech, San Leandro, CA) Imaging System.

Immunofluorescence staining and confocal microscopy

IL-17A-treated HaCaT cells and NHEKs were first fixed in acetone, blocked with 20% goat serum, and then incubated with rabbit anti-human K6 mAb, anti-human K16 mAb, anti-human K17 mAb, anti-human phospho-STAT1 mAb, anti-human phospho-STAT3 mAb, anti-human phospho-p65 mAb, or anti-human phospho-p38 mAb (Epitomics). To detect bound antibodies, CY3-labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD) was used. 4'-6-Diamidino-2-phenylindole (KPL) was used for nuclear counterstaining in all cells. Laser scanning confocal microscopy was done using a FV-1000/ES confocal microscope (Olympus, Tokyo, Japan).

Animal experiments of IL-17A-induced epidermal changes

Female BALB/c mice were obtained from the Department of Laboratory Animal Medicine of the University and used at 8–10 weeks of age. We performed intradermal injection of 20 µl PBS, either alone or containing 500 U recombinant mouse IL-17A (Peprotech), into the ears of anesthetized mice using a 30-gauge needle daily for 2 days or 7 days. Five mice were used in each experimental group, and each experiment was repeated at least three times. After injection, the mice ears were collected and frozen immediately in liquid nitrogen for RNA quantification, hematoxylin and eosin staining, and immunofluorescence microscopy analysis.

The 4 µm sections of mouse ear were fixed in 10% formalin in PBS and stained with hematoxylin and eosin. Frozen sections of mouse ear were prepared for immunofluorescence staining. Sections were fixed with precooled acetone for 10 minutes at room temperature, blocked for 30 minutes at room temperature with 5% goat serum in PBS, and incubated with rabbit anti-mouse K17 pAb (Abcam) for 1 hour at room temperature. After washing in PBS, the sections were incubated for 30 minutes at room temperature with an FITC-conjugated goat anti-rabbit IgG (KPL), then washed in PBS and counterstained with 4'-6-diamidino-2-phenylindole nuclear stain (KPL). Laser scanning confocal microscopy was done using an FV-1000/ES confocal microscope.

Statistics

All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Student's *t*-test was used to compare differences of K17 expression. *P*-values <0.05 were considered statistically significant.

This study was approved by the local medical research ethics committee at Xijing Hospital, the Fourth Military Medical University, Xi'an, China.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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