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*J Immunol* 2007; 179:6504-6513; doi: 10.4049/jimmunol.179.10.6504

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Requirement for Both JAK-Mediated PI3K Signaling and ACT1/TRAFl6/TAK1-Dependent NF-κB Activation by IL-17A in Enhancing Cytokine Expression in Human Airway Epithelial Cells

Fei Huang, Cheng-Yuan Kao, Shinichiro Wachi, Philip Thai, Jisu Ryu, and Reen Wu

Through DNA microarray analysis and quantitative PCR verification, we have identified additional IL-17A-inducible genes—IL-19, CXCL-1, -2, -3, -5, and -6—in well-differentiated normal human bronchial epithelial cells. These genes, similar to previously described human β-defensin-2 (HBD-2) and CCL-20, were induced by a basolateral treatment of IL-17A, and regulated by PI3K signaling and NF-κB activation. For PI3K signaling, increases of cellular PIP3 and phosphorylation of downstream molecules, such as Akt and glycogen synthase kinase-β (GSKβ) (S9), were detected. Induced gene expression and HBD-2 promoter activity were attenuated by LY294002, p110α small-interfering RNA (siRNA), as well as by an overexpression of constitutively active GSK3β (S9A) or wild-type phosphatase and tensin homolog. Increased phosphorylation of JAK1/2 after IL-17A treatment was detected in primary normal human bronchial epithelial cells. Transfected siRNAs of JAK molecules and JAK inhibitor I decreased IL-17A-induced gene expression and GSK3β phosphorylation. However, both JAK inhibitor I and PI3K inhibitor had no effect on the DNA-binding activities of p65 and p50 to NF-κB consensus sequences. This result suggested a JAK-associated PI3K signaling axis is independent from NF-κB activation. With siRNA to knockdown STIR (similar expression to fibroblast growth factor and IL-17R; Toll-IL-1R)-related signaling molecules, such as Act1, TNFR-associated factor 6 (TRAF6), and TGF-β-activated kinase 1 (TAK1), and transfection of A52R, an inhibitor of the MyD88/TRAFl6 complex, or dominant-negative TAK1, IL-17A-inducible gene expression and HBD-2 promoter activity were reduced. Additionally, IL-17A-induced p65 and p50 NF-κB activations were confirmed and their nuclear translocations were down-regulated by siRNAs of TRAF6 and TAK1. These results suggest that two independent and indispensable signaling pathways—1) JAK1-associated PI3K signaling and 2) Act1/TRAFl6/TAK1-mediated NF-κB activation—are stimulated by IL-17A to regulate gene induction in human airway epithelial cells. The Journal of Immunology, 2007, 179: 6504–6513.

Increasing evidence suggests that the airway epithelium plays an essential role in the regulation of airway innate and adaptive immune responses in addition to serving merely as a physical barrier against microbial infection and environmental air pollutant insults. The nature of this modulator and the potential mediators involved are currently unresolved. IL-17A has recently emerged as a potential candidate of bridging innate and adaptive immune responses in the lung, especially in response to bacterial infection (1, 2).

IL-17A has been detected in various cell types such as neutrophils and macrophages, but the major source of IL-17A is CD4+ T cells (3, 4). A subtype of CD4+ T cells termed Th-17 or Th-IL-17 has recently been identified to have a distinct function of producing IL-17A (5–8). Despite the obvious presence of IL-17A in inflamed airways, little information is known regarding its biological function and related cell signaling in the lung. Studies from our laboratory have recently shown that IL-17A is one of the most potent cytokines among a panel of 21 cytokines (IL-1α, IFN-γ, GM-CSF, and TNF-α) to stimulate the expression of mucins, human β-defensin 2 (HBD-2), and CCL-20 (MIP-3α) in disease and rheumatoid arthritis in humans and experimental autoimmune encephalomyelitis in mice (11–16).

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Received for publication September 13, 2006. Accepted for publication September 5, 2007.

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1 This work was supported in part by grants from National Institutes of Health (HL077902, HL077315, and ES00628) and T32 HL07103 (to P.T.).

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primary human airway epithelial cells (22–24). Additionally, Linden and colleagues (25, 26) have shown that IL-17A induced CXCL-6 (granulocyte chemotactic protein (GCP)-2), CXCL-1 (growth-related oncogene (GRO)-α), and IL-8 (CXCL-8) production by human bronchial epithelial cells. However, the common signaling pathway involved in IL-17A-induced gene expression is still unclear. Recent studies have identified a SEFIR (similar expression to fibroblast growth factor and IL-17R Toll-IL-1R domain) factor 6 (TRAF6) and TGF-β-activated kinase 1 (TAK1) and IL-17AR signal pathways. Recently, several studies have shown the potential involvement of Act1, another SEFIR-domain containing protein, and TRAF6, a TIR-associated protein, in IL-17AR-mediated signaling transduction in various cell lines and embryonic fibroblasts in vitro (28, 29, 30).

In this study, we sought to characterize the proinflammatory nature of IL-17A on the targeted airway epithelial cells and to examine the signaling pathways that are involved in IL-17A-induced gene expression. Through DNA microarray and real-time RT-PCR studies, we have identified several inducible cytokine genes, including IL-19, CXCL-2 (GRO-β), CXCL-3 (GRO-γ), and CXCL-5, in addition to previously described HBD-2, CCL-20, CXCL-1, CXCL-6, and IL-8 (22–26) in well-differentiated primary normal human bronchial epithelial (NHBE) cells. Signal transduction analyses demonstrated the involvement of both the activation of the PI3K-signaling pathway and the nuclear translocation of NF-κB in IL-17A-induced gene expression. In this study, we demonstrated two independent signaling pathways for IL-17A-induced gene expression: one is related to the JAK-associated PI3K signaling pathway, and the other involves Act1/TRAF6/TAK1/NF-κB activation. Both of them are required for IL-17A-induced gene expression and HBD-2 promoter activity.

Materials and Methods

Culture conditions

Human bronchial tissues were obtained with informed consent from the University of California–Davis (Medical Center, Sacramento, CA) and the National Disease Research Interchange (Philadelphia, PA). The University Human Subjects Review Committee approved and periodically reviewed the procedures in the tissue procurement. Tissue was not collected from patients diagnosed with lung-related diseases (30). Protease-dissociated bronchial epithelial cells were plated on transwell chambers (Corning; 25-mm) at 1–3 × 10^6 cells/cm² in a Ham’s F12/DMEM (1:1) with the addition of the following eight factors: transferrin (5 μg/ml), insulin (5 μg/ml), cholera toxin (10 ng/ml), epidermal growth factor (10 ng/ml), dexamethasone (0.1 μM), bovine hypothalamus extract (15 μg/ml), BSA (0.5 mg/ml), and all-trans-retinoic acid (30 nM). After a week in an immersed culture condition, these primary NHBE were transferred to an air-liquid interface culture condition, which facilitates mucociliary differentiation. Primary cells cultures reaching full differentiation at day 14–21 after plating were used in this study. At full confluence, the cells in the chamber achieved an elevated transepithelial resistance as measured by voltmeter (Millicell-ERS; Millipore), as previously described (14). Immortalized NHBE cell line, HBE-1, was used for most of the transfection experiments. The culture condition for HBE-1 was described in previous studies (31).

DNA plasmid constructs and small-interfering RNA (siRNA)

The construction of the HBD-2 promoter (2 kb) in the pGL-3 basic vector was described in the previous study (23). The wild-type phosphatase and tensin homolog (wt-PTEN), which was well-studied as a negative regulator of the PI3K pathway, and its phosphatase mutant PTEN (mut-PTEN) were provided by Dr. G. B. Mills (M. D. Anderson Cancer Center, Houston, TX). pHR-TK (Promega) was used as the internal control for normalizing transfection efficiency. The clone of A52R, a vaccinia virus protein with inhibitory effects on the MYD88/TRAF6 complex, was gifted by Dr. L. A. O’Neill (Trinity College, Dublin, Ireland). The dominant-negative TAK1 (dnTAK1) clone was provided by Dr. X. Li (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH). The constitutively active (ca)-GSK3β/8 (S9) clone was provided by Dr. M. C. Hung (M. D. Anderson Cancer Center, Houston, TX). siRNAs of JAK1, 2, 3, TAK1, p110α, TRAF6, Act1, and random oligomer (RO) were purchased from Ambion Biotech.

Transient transfection of HBE-1 cells

Transfection of HBE-1 cells with HBD-2 promoter-luciferase construct, pHR-TK, and other expression constructs or siRNA was conducted by FuGENE 6-based (Roche Diagnostics) or Lipofectamine 2000-based gene transfer methods (Invitrogen Life Technologies) according to the manufacturer’s specifications. Briefly, cells were plated onto 12-well plates at 40–60% (FuGene 6) or 95% (Lipofectamine 2000) confluency. One day after the plating, cultured cells were washed twice with Opti-MEM (Invitrogen Life Technologies) before transfection. The cells were incubated at 37°C with the mixture of DNA constructs/siRNA and FuGene 6/Lipofectamine 2000 in Opti-MEM for 16 h. One day before IL-17A addition, transfected cultures were incubated in hormone-depleted and serum-free medium. One more day after IL-17A treatment, cells were harvested by luciferase lysis buffer (25 mM Tris-phosphate (pH 7.8); 8 mM MgCl₂; 1 mM DTT; 1% Triton X-100; 150 mM glycerol). A dual luciferase reporter assay kit (Promega) was used for analyzing the firefly luciferase activity of the HBD-2 promoter and Renilla luciferase activity of the internal control. The firefly luciferase activity of HBD-2 promoter-pGL3-basic construct-transfected cells was calculated by subtracting the firefly luciferase activity of the promoterless pGL3-basic plasmid transfected cells, and then normalized to the control (Renilla luciferase) activity.

For siRNA transfection, cells were plated at 40–60% density a day before transfection. An Oligofectamine-based transfection kit (Invitrogen Life Technologies) was used according to the manufacturer’s instruction. Six hours after transfection, transfection mix was replaced with fresh culture medium. Two days later, cultures were depleted of hormonal supplements 24 h before IL-17A treatment. At various times after the treatment, cells were harvested for protein and gene expression analyses.

Cytokine inhibitor treatment

Recombinant human IL-17 (or IL-17A) was purchased from R&D Systems, and was dissolved in PBS with 0.1% BSA and used at 20 ng/ml, unless specified in the text. LY294002, a PI3K-specific inhibitor, and hel- notaxol (3–2 μg/ml; an Akt inhibitor), were used and added 3 h before IL-17A treatment. These inhibitors and their corresponding vehicles (<0.1%) were added to the cultures 3 h before IL-17A treatment. We observed no cell cytotoxicity of these inhibitors at the doses used in this study. This was based on the nuclear dye exclusion assay (data not included).

RNA isolation and real-time RT-PCR

Total RNA was extracted with RNA TRIzol reagent (Invitrogen Life Technologies) and cDNA was generated from an equal amount of RNA by M-MuLV’s murine leukemia virus reverse transcriptase (Promega) using oligo(dT) as the primer. SYBR Green Master Mix (Applied Biosystems) and the ABI7900HT Detection System (Applied Biosystems) were used following the manufacturer’s protocol for real-time PCR analysis. The relative mRNA amount of each sample was calculated based on its threshold cycle, Ct, in comparison to the Ct of housekeeping genes, such as GAPDH and β-actin, as described before (23, 24). The results were presented as 2^ΔCt of gene of interest – Ct of GAPDH or β-actin in an arbitrary units. The purity of amplified product was determined as a single peak of dissociation curve. Throughout the study, there was no observable fluctuation in the Ct values of GAPDH and β-actin from different treated cells (data not included). For data presentation, the relative mRNA level of each gene of interest was normalized with GAPDH in this study.

In the present study, the expression of HBD-2 and HBD-2 for real-time PCR were described in our previous study (23). For CXCL-1, -2, -3, -5, -6, HBD-2, IL-19, TAK1, TRAF6, p110α, JAK1, 2, and Act1, the primer sequences are listed below. The specificity of the primers for each gene was determined by dissociation curve for each gene-specific PCR product. CTCL-1 forward. AGATCTCATGGATATTTTAATGTTGTTAAAATATGCAAT; CTCL-1 reverse. AATACCTATGGGTTACCTTCTG; CXCL-2 forward, CTCTCA TTTGGTATTATATATATGCTCTGTT; CXCL-2 reverse, GAGAC TAACTGGGTTAAGCCATTTAAA; CXCL-3 forward, TGAAGAAGGACAGCAGCTTCT; CXCL-3 reverse, AGGAACTGACGATGGTTTGATGAAACA; CXCL-5 forward, CGAGCCTTCTTCAGAGCAGTT; CXCL-5 reverse, CTTTAA CACCGCAGAGCAGCTT; CXCL-6 forward, AGTTTACAG CTCGACTAATGGAGCGATACT; CXCL-6 reverse, CGGTTAGCCTTTGAACGTG; CXCL-8 forward, CAGGAGTATGTGCACTGACA; CXCL-8 reverse, TGGGCCCCCTTCAGAGGAGTA; CXCL-8 reverse, CTGCTGCTACAACGACCCGAGCTT; CXCL-8 reverse, GGGCTGCTACAACGACCCGAGCTT; CXCL-9 reverse, TGGGCCCCCTTCAGAGGAGTA; CXCL-9 reverse, CTGCTGCTACAACGACCCGAGCTT; CXCL-9 reverse, GGGCTGCTACAACGACCCGAGCTT; CXCL-10 reverse, TGGGCCCCCTTCAGAGGAGTA; CXCL-10 reverse, CTGCTGCTACAACGACCCGAGCTT; CXCL-10 reverse, GGGCTGCTACAACGACCCGAGCTT; CXCL-11 reverse, TGGGCCCCCTTCAGAGGAGTA; CXCL-11 reverse, CTGCTGCTACAACGACCCGAGCTT; CXCL-11 reverse, GGGCTGCTACAACGACCCGAGCTT.
anti-phospho-Ser429 Akt rabbit mAb, anti-phospho-Thr 308 Akt rabbit polyclonal Ab, anti-phospho-Ser9 GSK3β rabbit polyclonal Ab, anti-phospho-Thr 202/204 TAK1 rabbit polyclonal Ab, anti-phospho-Tyr 1022/1023 JAK1 (Cell Signaling Technology); anti-4E-BP-1 mAb (BD Biosciences); anti-p65 mAb, anti-JAK1, anti-JAK2, and anti-TRAFL6 rabbit polyclonal Ab (Santa Cruz Biotechnology); anti-p50 mAb (Biolegend); anti-TAK1 rabbit polyclonal Ab (Upstate Biotechnology); and anti-nucleolin mAb and anti-β-tubulin mAb (Sigma Aldrich).

ELISA-based transcription factor and DNA-binding assay

Binding of p65 and p50 NF-κB subunits to their consensus DNA sequences were determined quantitatively by the ELISA-based Mercury Transfactor kit (BD Biosciences) following the manufacturer’s protocol. Briefly, equal amounts of nuclear extract from each cell sample were loaded on a 96-well plate, which was coated with DNA of the consensus sequences for p65 and p50. After a 1-h incubation, the unbound protein was washed away, and the primary Ab to the target transcription factor was applied. The amount of the Ab bound to transcription factor was detected by HRP-conjugated secondary Ab and its substrate (Pierce Biotechnology). Absorbance was measured by a standard microtiter plate reader at 655 nm wavelength.

\[ P(3,4,5)IP_3 \text{ quantitation} \]

A PIP3 Mass ELISA kit was purchased from Echelon Biosciences. HBE-1 of 10, 30 min after IL-17A and insulin (as a positive control) treatments were harvested and precipitated by trichloroacetic acid. PIP3 lipid was extracted twice from the trichloroacetic acid precipitated fraction by methanol:chloroform (2:1), according to the manufacturer’s specification. After acidification, organic-phase lipids were used for PIP3 quantitation, based on the standard protocol came with the ELISA kit. Briefly, the lipid extract from cultured cells were first mixed with the PIP3-specific detector protein, which was then incubated in a PIP3-coated microplate for competitive binding. After several washes, the microplate was then incubated with a HRP-linked secondary detector and tetramethylbenzidine substrate for color development. To stop further color development, 2 M H2SO4 solution was then added. Microplates were read at 450 nm wavelength for absorbance. A series of different dilutions of PIP3 standards were used for establishing a standard curve for each reaction. Cellular PIP3 amounts could be estimated by comparing the absorbance in the wells with the values in the standard curve. Experiments were conducted in triplicate dishes and repeated in two independent cultures with cell density >5 million cells per 100-mm dish.

Statistical analyses

Data from at least triplicate dishes and transwell chambers were used to determine mean ± SE of mRNA expression of cytokines and chemokines. The results were repeated for at least three representative cultures derived from different donors or passages. Group differences were calculated by Student’s t test as described in figures. Value of \( p < 0.05 \) was considered significant.

Results

Induction of cytokine/chemokine expression by IL-17A

The proinflammatory nature of IL-17A has been well-recognized, but the extent of this effect on airway epithelial cells has not been demonstrated. DNA microarray analysis that compares the expression values between the control and IL-17A-treated NHBE cells showed >2-fold induction of several chemokine/cytokine genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>No Treatment (Expression of Gene/GAPDH)</th>
<th>+IL-17A (Expression of Gene/GAPDH)</th>
<th>Fold Induction by IL-17A</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD-2</td>
<td>0.0072 ± 0.00608</td>
<td>*0.5542 ± 0.20757</td>
<td>77.9</td>
</tr>
<tr>
<td>IL-19</td>
<td>0.0006 ± 0.00060</td>
<td>*0.0999 ± 0.006534</td>
<td>160</td>
</tr>
<tr>
<td>CXCL-1(GRO-α)</td>
<td>0.00416 ± 0.00228</td>
<td>*0.2220 ± 0.11480</td>
<td>5.3</td>
</tr>
<tr>
<td>CXCL-2(GRO-β)</td>
<td>0.0037 ± 0.000227</td>
<td>*0.0111 ± 0.009479</td>
<td>3.6</td>
</tr>
<tr>
<td>CXCL-3(GRO-γ)</td>
<td>0.0062 ± 0.004222</td>
<td>*0.0281 ± 0.01974</td>
<td>4.5</td>
</tr>
<tr>
<td>CXCL-5</td>
<td>0.0021 ± 0.00138</td>
<td>*0.0029 ± 0.01142</td>
<td>11.4</td>
</tr>
<tr>
<td>CXCL-6(GCP-2)</td>
<td>0.0044 ± 0.00196</td>
<td>0.0250 ± 0.03138</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Primary NHBE cells were cultured as described in the text. The inducible genes were measured by real-time PCR and the relative gene expression in each sample was normalized to GAPDH. Data were presented as mean ± SE from five independent primary NHBE cultures derived from different donors. The significance in each gene induction was determined by Student’s t test; *, \( p < 0.05 \).
Effects of IL-17A on polarized NHBE cultures and the inhibitor effects. Primary NHBE cells under air-liquid interface culture condition were treated with IL-17A (20 ng/ml) and/or inhibitors, as indicated. Cultures were harvested 24 h later for RNA and real-time RT-PCR analysis. Data were averages from triplicates, and similar experiments were independently performed in three primary NHBE cultures derived from different donors. A, Effects of IL-17A treatments on polarized NHBE cultures. NHBE cultures were treated with IL-17A from the apical, basolateral, and both sides of transwell chambers, as indicated. **, p < 0.01 for samples treated with IL-17A compared with the control. B, Effects of JAK inhibitor I on IL-17A-induced gene expression. JAK inhibitor I (1 μM) was added 3 h before IL-17A (20 ng/ml) treatment (lane 3). C, Effects of PI3K inhibitor, LY294002, on IL-17A-induced gene expression. LY294002 was added to NHBE cultures at concentration of 0, 2, 10, and 50 μM (lanes 2–5, respectively) 3 h before IL-17A (20 ng/ml, lanes 2–5) treatment. D, Effects of helenalin on IL-17A-induced gene expression. Helenalin was used at 0, 5, 20, and 50 μM levels (lanes 2–5, respectively). For inhibitor studies (B–D), cultures were harvested 24 h later after IL-17A treatment for RNA and real-time RT-PCR. There was no change in the Ct values of the housekeeping genes, GAPDH and β-actin, with these inhibitor treatments. The relative gene expression to GAPDH was determined, and fold induction of each was presented in the graph. Data were analyzed from three independent experiments and are presented as mean ± SE. Group differences were analyzed by Student’s t test. *, p < 0.05 for IL-17A-inducible gene expression as compared with control (lane 1), untreated samples. #, p < 0.05 for the reduction of IL-17A-induced gene expression by these inhibitors (lanes 3–5) as compared with the IL-17A-treated cultures with vehicle (lane 2).

FIGURE 2. Effects of IL-17A on polarized NHBE cultures and the inhibitor effects. Primary NHBE cells under air-liquid interface culture condition were treated with IL-17A (20 ng/ml) and/or inhibitors, as indicated. Cultures were harvested 24 h later for RNA and real-time RT-PCR analysis. Data were averages from triplicates, and similar experiments were independently performed in three primary NHBE cultures derived from different donors. A, Effects of IL-17A treatments on polarized NHBE cultures. NHBE cultures were treated with IL-17A from the apical, basolateral, and both sides of transwell chambers, as indicated. **, p < 0.01 for samples treated with IL-17A compared with the control. B, Effects of JAK inhibitor I on IL-17A-induced gene expression. JAK inhibitor I (1 μM) was added 3 h before IL-17A (20 ng/ml) treatment (lane 3). C, Effects of PI3K inhibitor, LY294002, on IL-17A-induced gene expression. LY294002 was added to NHBE cultures at concentration of 0, 2, 10, and 50 μM (lanes 2–5, respectively) 3 h before IL-17A (20 ng/ml, lanes 2–5) treatment. D, Effects of helenalin on IL-17A-induced gene expression. Helenalin was used at 0, 5, 20, and 50 μM levels (lanes 2–5, respectively). For inhibitor studies (B–D), cultures were harvested 24 h later after IL-17A treatment for RNA and real-time RT-PCR. There was no change in the Ct values of the housekeeping genes, GAPDH and β-actin, with these inhibitor treatments. The relative gene expression to GAPDH was determined, and fold induction of each was presented in the graph. Data were analyzed from three independent experiments and are presented as mean ± SE. Group differences were analyzed by Student’s t test. *, p < 0.05 for IL-17A-inducible gene expression as compared with control (lane 1), untreated samples. #, p < 0.05 for the reduction of IL-17A-induced gene expression by these inhibitors (lanes 3–5) as compared with the IL-17A-treated cultures with vehicle (lane 2).

FIGURE 3. Activation of PI3K signaling by IL-17A in HBE-1 and NHBE cells. Before the treatment, cultures were deprived in serum-free medium (DMEM/F12) for 24 h. A, Quantitation of PIP3 levels in HBE1 cells after cytokine treatments. IL-17A (20 ng/ml) or insulin (500 μg/ml) was added to HBE-1 cultures for 10 and 30 min. Cells were harvested for lipid extraction as described in the text. The level of PIP3, was determined by the PIP, ELISA Mass kit. The data are presented as the mean ± SE from three independent experiments, and the Student t test was used for determining group differences. *, p < 0.05 for IL-17A-treated or insulin-treated samples compared with control. B, Effects of IL-17A on the phosphorylation of PI3K-signaling molecule Akt. NHBE cells were treated with IL-17A (20 ng/ml) in a time course. NHBE cells at various times, as indicated, after IL-17A (20 ng/ml) treatment were harvested for protein extracts and subjected to Western blot analysis with Abs specific to phosphorylated Akt at serine (473) and Akt at threonine (308). Total Akt proteins were used as input controls. C, Effects of IL-17A on the phosphorylation of PI3K-signaling molecule GSK3β. NHBE cells were treated with IL-17A (20 ng/ml) in the presence or absence of JAK inhibitor I (1 μM) and harvested at various times after the treatment. Protein extract were analyzed with anti-phosphorylated GSK3β(S9) Ab in a Western blot. Protein inputs were shown by β-tubulin expression in the same blot.

In addition to the previously known induction of HBD-2, CCL-20, IL-8, CXCL-1, CXCL-6, CSF-3, and CSF-2 (22, 23, 34), we have found that IL-19, CXCL-2, -3, and -5 were highly stimulated by IL-17A in primary NHBE cells. Real-time RT-PCR demonstrated a 78-, 160-, and 3- to 12-fold stimulation of IL-19, CXCL-2, -3, and -5 by IL-17A (Fig. 1). To reveal whether IL-17A-induced genes were regulated through a common mechanism, we first examined whether the induction depended on basolateral localization of IL-17AR, JAK, and PI3K signaling, and NF-kB activation

To reveal whether IL-17A-induced genes were regulated through a common mechanism, we first examined whether the induction depends on the interactions between IL-17AR and its ligand, IL-17A. As shown in Fig. 2A, the induction of all genes depended on the basolateral treatment of IL-17A. No stimulation was seen when
anti-p110 of IL-17A-induced gene expression by siRNA. Western blot analysis compared with control, no IL-17A treatment. #, GAPDH.

Gene promoter activity. Experiments were conducted as described in text. RNA and protein were harvested 24 h after IL-17A treatment. Relative message levels of HBD-2 and IL-19 that were normalized to GAPDH are displayed. *, p < 0.05 for IL-17A- and RO-treated cultures compared with control, no IL-17A treatment. #, p < 0.05 for the suppression of IL-17A-induced gene expression by siRNA. Western blot analysis with anti-p110 Ab was conducted to determine the effect of siRNA at the protein level. Anti-β-tubulin Ab bands were used for the normalization of protein input in the blot. B, Effects of siRNA p110x on IL-17A-induced HBD-2 promoter activity. Experiments were conducted as described in A and text. HBD-2 promoter activity normalized with phRL-TK promoter activity is displayed. *, p < 0.05 for IL-17A and RO treatments on induced HBD-2 promoter activity compared with control with no IL-17A treatment. #, p < 0.05 for the suppression of IL-17A-induced HBD-2 promoter activity by siRNA.

C, Dose-dependent inhibition of IL-17A-induced HBD-2 promoter activity by PI3K inhibitor, LY294002. #, p < 0.05 for IL-17A induced HBD-2 promoter activity compared with the control with no IL-17A treatment. #, p < 0.05 for the suppression of IL-17A-induced HBD-2 promoter activity by LY294002. D, Effects of wt-PTEN and mut-PTEN cotransfections on IL-17A-induced HBD-2 promoter activity. *, p < 0.05 for IL-17A-induced HBD-2 promoter activity compared with the control with no IL-17A treatment. #, p < 0.05 for the suppression of the stimulation of IL-17A-induced HBD-2 promoter activity by cotransfection with wt- and mut-PTEN, respectively. E, Suppression of IL-17A-induced HBD-2 promoter activity by ca-GSK3β(S9A) cotransfection. *, p < 0.05 for IL-17A-induced HBD-2 promoter activity compared with the control with no IL-17A treatment. #, p < 0.05 for the suppression of IL-17A-induced HBD-2 promoter activity by ca-GSK3β(S9A). The data are presented as the mean ± SE from three independent experiments.

FIGURE 5. Activation of JAK1, 2 by IL-17A in primary NHBE and HBE-1 cells. A, Western blot analysis of JAK1, 2 phosphorylation. Primary NHBE cells were treated with/without IL-17A (20 ng/ml) and harvested at various times as indicated (left panel). The IL-17A-treated culture was used as a positive control. For JAK inhibitor effects (right panel), NHBE cells were pretreated with the inhibitor and then treated with IL-17A and IL-13. Cells were harvested 10 min after the cytokine treatment. Increases of phospho-JAK1 (Tyr1022/1023) (p-JAK1) and phospho-JAK2 (Tyr1007/1008) (p-JAK2) upon IL-17A treatment were detected by Western blot. Total JAK1 and JAK2 proteins in the membrane were used as the input controls. In B, C, and E, siRNA transfections were conducted at 2 days before IL-17A treatment as described in Fig. 4 and text. RNAs were harvested 24 h after IL-17A treatment for real-time RT-PCR quantifications of HBD-2 (B), IL-19 (C), and JAK1/2 protein (E). D, HBE1 cells were co-transfected with siRNAs, RO, HBD-2 promoter-firefly luciferase reporter constructs and phRL-TK control promoter 2 days before IL-17A treatment. HBD-2 promoter luciferase activity was normalized with phRL-TK promoter activity. The values are presented as the mean ± SE from triplicates of three independent experiments. Group differences were analyzed by Student t test. In B−D, * indicates p < 0.05 for siRNA effects on IL-17A-induced gene expression and HBD-2 promoter activity, as compared with IL-17A-treated RO transfections.

IL-17A was added to the polarized cultures from the apical surface only. These results suggest a preferential presence of IL-17AR at the basolateral side of the polarized airway epithelia in vitro. This is consistent with our previous finding of anti-IL-17AR Ab staining that showed the basolateral presence of the receptor in well-differentiated NHBE cultures (24) and the in vivo lung tissue staining by the other group (33).

Previously, we demonstrated that IL-17A-induced HBD-2 is sensitive to inhibitors of the JAK- and NF-kB-signaling pathways. However, to our current knowledge, CCL-20, another IL-17A-inducible gene, is only sensitive to NF-kB inhibitor (24). Here, we sought to determine whether the induction of IL-19, CXCL-2, -3, and -5 are through a common mechanism as well. Through inhibitor screening, we found that JAK inhibitor I, LY294002, and helinalin, significantly attenuated IL-17A-induced cytokine expression (Fig. 2, B–D, respectively). The inhibitory effects of JAK
This PIP3 elevation is about one-third of insulin.

**FIGURE 3.** The requirement of the PI3K-signaling pathway by IL-17A-induced gene expression.

To further elucidate the role of PI3K signaling, we first measured the increase of PIP3 lipid in HBE-1 after IL-17A treatment. Using a commercial PIP3 ELISA Mass kit, we found that IL-17A stimulated the PIP3 production within 10 min, and then declined after 60–120 min. Interestingly, JAK inhibitor I suppressed the phosphorylation of GSK3β(S9) (Fig. 3C), suggesting a potential role of JAK in PI3K-signaling pathways.

The phosphorylation of GSK3β(S9), which leads to inactivation of GSK3β, is of special interest for its involvement in numerous cellular signaling pathways (35–37). Therefore, we further tested the impact of PI3K signaling and PI3K-dependent inactivation of GSK3β on IL-17A-induced HBD-2 and IL-19 expression. As shown in Fig. 4A, p110α siRNA transfection significantly decreased p110α protein (lower panel) as well as the IL-17A-induced HBD-2 and IL-19 mRNA. The same inhibitory effect was also observed in HBD-2 promoter activity (Fig. 4B).

To further investigate the significance of PI3K signaling, effects of LY294002 and forced expression of wt-/mut-PTENs and cag-GSK3β on IL-17A-induced HBD-2 promoter activity were tested. As shown in Fig. 4C, IL-17A-stimulated HBD-2 promoter activity was inhibited by LY294002 in a dose-dependent manner. The inducible HBD-2 promoter activity was significantly changed by cotransfections with wt- and mut-PTEN constructs (Fig. 4D). Because PTEN is known as an intrinsic negative regulator of PI3K activation, overexpression of wt-PTEN decreased IL-17A-induced HBD-2 promoter activity while overexpression of mut-PTEN increased HBD-2 promoter activity. As GSK3β inactivation is regulated by PI3K signaling, we thereby examined whether GSK3β inactivation is also required for HBD-2 promoter activity. As shown in Fig. 4E, cotransfection with ca-GSK3β(S9A) significantly suppressed IL-17A-induced HBD-2 promoter activity.

**FIGURE 6.** Involvement of STIR family members in IL-17A-induced gene expression. Two days before IL-17A treatment, HBE-1 cells were transfected with TRAF6, TAK1, and Act1 siRNAs (A–D). RNAs were harvested 1 day after the cytokine treatment for real-time RT-PCR for the measurement of relative HBD-2 (A), IL-19 (B), and various STIR family genes TRAF6, TAK1, and Act1 (D) messages. C. The effects of the siRNAs and their control (random oligomer) on the inhibition of IL-17A-induced HBD-2 promoter activity were measured by luciferase assay described in Fig. 5. E. The effects of A52R and dnTAK1 cotransfections were shown on the attenuation of IL-17A-induced HBD-2 promoter activity. The values are presented as the mean ± SE from triplicates. Group differences were analyzed by Student t test. In A–C and E. (+) indicates p < 0.05 for siRNA, A52R, or dnTAK1 suppression on IL-17A-induced gene expression activity, as compared with their corresponding controls. In D, (±) indicates p < 0.05 for the reduction of relative mRNA amounts due to their siRNA treatment as compared with their RO-transfected cultures.
These results suggest the involvement of the PI3K-signaling pathway in regulating IL-17A-inducible gene expression.

**JAK1 and JAK2 are required for IL-17A-induced gene expression**

As described above, JAK inhibitor I effectively suppressed IL-17A-induced gene expression (Fig. 2) and down-regulated GSK3β/H9252 phosphorylation (Fig. 3C). These data suggest the importance of the JAK family in the regulation of IL-17A signaling. We therefore examined whether JAK1 and JAK2 protein are phosphorylated upon IL-17A stimulation in primary NHBE cells. In Fig. 5A, primary NHBE cells were treated with IL-17A in a time course (left panel) with IL-13 as a positive control (right panel) for JAK phosphorylation. Western blot was used to analyze the phosphorylation of JAK1 at Tyr 1022/1023 and JAK2 at Tyr1007/1008. Upon IL-17A stimulation, phosphorylations of both JAK1 and JAK2 were detected from 5 min to 2 h with the peak time of around 10–20 min after treatment. The induction of phospho-JAK1/2 by IL-17A and IL-13 can be abolished by JAK inhibitor I (right panel). The necessity of JAK1 and JAK2 for signaling was further confirmed by siRNA approach in HBE-1 cells. As shown in Fig. 5, B, C, and E, siRNAs of JAK1 and JAK2 decreased IL-17A-dependent HBD-2 (Fig. 5B) and IL-19 (Fig. 5C) induction, as well as their own protein expression (Fig. 5E). The specificity of siRNA was confirmed in Fig. 5E as siRNA of both JAK1 and JAK2 specifically knockdown its target protein but not the other while RO has no effect on neither of the protein. Moreover, the inhibition of induced-HBD-2 by JAK1/2 siRNA is specific to IL-17A stimulation while HBD-2 gene induction by other stimulants, such as IL-1β and TNF-α, is not affected by JAK siRNAs (data not shown). Because the expression of JAK3 in airway epithelial cells is low, and minimal effect of JAK3 siRNA on IL-17A-induced IL-19 expression (data not shown), we mainly focus our study on JAK1 and JAK2 in the regulation of IL-17A signaling. Fig. 5D show that JAK1 siRNA effectively hampered IL-17A-induced HBD-2 promoter activity while JAK2 siRNA did not have much impact (Fig. 5D). These results imply that the contributions of JAK1 and JAK2 are not equal.

**Involvement of SEFIR family signaling molecules in IL-17A-induced gene expression**

It has been reported that IL-17AR has a SEFIR domain, which belongs to the STIR superfamily (27, 28, 29). Therefore, we sought to tested the roles of other STIR-related signal molecules,
such as TRAF6, TAK1, and Act-1, in IL-17A-induced gene expression regulation in our system (28, 38–41). We used an siRNA knockdown approach to determine which signaling molecules are involved in IL-17A-mediated gene expression in human airway epithelial cells. As shown in Fig. 6D, the siRNAs of Act1, TRAF6, and TAK1 effectively knockdown their own mRNA expressions as compared with the RO controls. Furthermore, the siRNAs of Act1, TRAF6, and TAK1 effectively attenuated IL-17A-induced HBD-2 and IL-19 mRNA, and the induced HBD-2 promoter activity (Fig. 6, A–C, respectively). These results implicate the involvement of Act1, TRAF6, and TAK1 in IL-17A-induced signaling and gene expression.

In addition to the siRNA approach, we transfected some modified genes that are involved in TRAF6 signaling. As shown in Fig. 6E, a forced expression of virus-derived A52R (42) which interferes the docking of TRAF6 to MyD88 and dominant-negative TAK1 which attenuates downstream signaling of TRAF6 significantly suppressed IL-17A-induced HBD-2 promoter activity. The data indicate that HBD-2 expression is dependent on TRAF6 and TAK1. Whether MyD88 is responsible for gene induction by IL-17A is still unclear. Nonetheless, these results further strengthened the potential involvement of the STIR family molecules and signaling pathways of IL-17A-induced gene expression.

**TRAF6/TAK1 is responsible for IL-17A-dependent NF-κB transactivation**

The inhibitory effect of helenalin on IL-17A-induced genes (Fig. 2D) (23, 24) indicated the importance of NF-κB-dependent transcriptional activities in these gene induction. However, it is less clear how NF-κB is activated by IL-17A in airway epithelial cells. In Western blot analyses, we observed decreases on IkBα level in both NHBE and HBE1 total lysates within 3–10 min after IL-17A treatment (Fig. 7, A and B, respectively). This reduction was followed by an increase of nuclear translocation of p65 and p50 NF-κB subunits (Fig. 7C). Moreover, p65 and p50 nuclear translocation coincided with increases of their DNA-binding activities in nuclear fractions, which were assayed by ELISA-based Mercury Transfactor kit (Fig. 7D). Interestingly, both JAK inhibitor I and PI3K inhibitor (LY294002) had no effect on p65/p50 element-binding activity (Fig. 7D). These results suggest that neither JAK nor PI3K pathways are involved in NF-κB activation by IL-17A.

Because TRAF6 has been reported as an important regulator of IL-17A signaling, we further examined whether TRAF6 and its related downstream signaling molecule, TAK1, regulate the nuclear translocation of NF-κB induced by IL-17A. HBE-1 cells transfected with TRAF6 or TAK1 siRNA showed significant decreases of their protein production compared with the negative controls which were transfected by RO (Fig. 7C). A down-regulation of p50 and p65 nuclear translocation was also observed in HBE-1 cells transfected with TRAF6 siRNA and, to a lesser extent, TAK1 siRNA as compared with the results of RO controls. These results indicate that IL-17A-induced NF-κB translocation is through a TRAF6/TAK1-dependent pathway. In contrast, siRNA of TRAF6 had no effect on IL-17A-mediated PI3K signaling, such as the phosphorylation on GSK3β (S9) (data not shown), suggesting that PI3K pathway is independent from TRA6 activation in IL-17A-treated cells.

**Discussion**

IL-17A is a novel proinflammatory cytokine that plays a significant role in host defense (1, 10). Airway epithelial cells are the major target of this cytokine. Previously, we have shown that IL-17A is one of the most potent cytokines among a panel of 21 cytokines to stimulate gel-forming mucins (22), HBD-2 (23), and CCL-20 (24) expression in well-differentiated NHBE. This study, along with the others, found that IL-17A can also up-regulate IL-19, CXCL-2, -3, and -5 in airway epithelial cells (25). Furthermore, we demonstrated that IL-17A elicits two independent and indispensable signaling pathways for gene induction in airway epithelial cells: 1) JAK-associate PI3K/GSK3β signaling and 2) Act1/TRA6/TAK1-dependent NF-κB activation (Fig. 8).

IL-17A is a potent mediator of neutrophil recruitment. Our studies support that the neutrophil recruitment is through the up-regulation of chemokine expression by IL-17A on the target epithelial cells. IL-17A also activates expression of HBD-2 and CCL-20 (23, 24), which are known to interact with CCR6 (43, 44). CCR6 is an important receptor for dendritic cell localization and lymphocyte homeostasis in mucosal tissues (24). Therefore, the IL-17A might not only enhance neutrophil recruitment, but also act indirectly on other inflammatory cells. We as well as others have shown that IL-17A induces CXC cytokine family members in a number of cell types (45, 46). IL-17A stimulates CXCL-1 in bronchial epithelial cells and osteoclasts, CXCL-2 in fibroblast-like synoviocytes, CXCL-5 in osteoclasts, and CXCL-6 in bronchial epithelial cells (25, 47, 48). Using primary NHBE cells, we further demonstrate that CXCL-2, -3, and -5 genes are also induced by IL-17A in airway epithelial cells. More importantly, IL-19, an emerging IL-10 family member, is strongly induced by IL-17A in airway epithelial cells. IL-19 is mainly produced by monocytes and is up-regulated in skin of psoriatic patients (49, 50). The function of IL-19 has been extensively studied in chronic skin inflammatory diseases and its significance in asthmatic airways and the potential role in the regulation of Th2 cytokine expression have recently been reported (51). Our finding indicates that airway epithelium could be a major source of IL-19 through the stimulation by IL-17A.

We also confirmed that the polarity of IL-17AR expression in airway epithelium is crucial for downstream gene activation. Our data indicate that basolateral treatment of IL-17A is required for the gene induction. The immunostaining of IL-17AR also confirmed its distribution in the basal region of the polarized epithelia in both culture and tissue sections (data not shown). These are all
Pathways are needed for IL-17A-induced gene expression as well as the antimicrobial activity (24, 52). Our data reaffirm the significance of IL-17AR polarization in IL-17A signaling (23, 24, 33).

Following the receptor engagement, we have observed a variety of signaling pathways activated by IL-17A, including: 1) JAK1-associated PI3K/GSK3β signaling and 2) Act1/TRAf6/TAK1-dependent NF-κB activation. The sensitivity of NHBE cells to JAK, PI3K, and NF-κB inhibitors is the first evidence indicating that these pathways are crucial to IL-17A signaling. The importance of these pathways were further confirmed by various biochemical studies showing an increase of cellular PI3P level, increases of the phosphorylation of PI3K-signaling molecules Akt and GSK3β, and the elevation of p50 and p65 NF-κB DNA-binding activities in cells after IL-17A treatment. Additionally, using siRNA knockdowns and forced expression of modified genes of JAK-, PI3K-, and STIR-related signal molecules, we were able to see the attenuation of IL-17A-induced gene expression and HBD-2 promoter activity. These studies further support the involvement of these pathways in IL-17A-induced cell signaling and gene expression. It is worthwhile to emphasize that GSK3β, a downstream target of PI3K-Akt signaling (53–55), is found to be phosphorylated at serine 9, which inactivates its kinase activity (56). Our study with the ca-GSK3β(S9A) transfection (Fig. 4E) confirms the importance of the PI3K-GSK3β axis signaling. In fact, the inactivation of GSK3β is essential to IL-17A-mediated gene expression. We also found that the phosphorylation of GSK3β was influenced by the JAK pathway (Fig. 3C) rather than TRAf6 (data not shown). These results indicate the association between JAK and PI3K signaling, which leads to GSK3β inactivation and the subsequent gene activation (Fig. 8).

For IL-17A-mediated NF-κB activation, we have identified a rapid degradation (within 3–10 min) of IκB-α in cells after IL-17A treatment, followed by the p65 and p50 nuclear translocation, and significant increases in their DNA-binding activities to NF-κB consensus sequences. These activities were inhibited by the treatment of siRNAs of TRAf6 and TAK1, but not by JAK inhibitor I or PI3K inhibitor (LY294002), suggesting a role of TRAf6/TAK1-dependent NF-κB activation. In addition, siRNA and dominant-negative gene transfection of these STIR-signaling molecules have showed that Act1, TRAf6, and TAK1 are all required in mediating IL-17A-induced gene expression.

This is the first reporting of two signaling pathways mediated by IL-17A and their roles in coordinating the gene induction in airway epithelial cells. However, these two independent signaling pathways are needed for IL-17A-induced gene expression is unclear. The importance of NF-κB activation is obvious because most of these IL-17A-induced genes have putative NF-κB consensus sequences in their promoter regions. The role of PI3K is less clear, because it has no effect on NF-κB activation. A recent publication has shown that C/EBP promotes the transcription of IL-17A-targeting genes (57). It has also been shown that GSK3β inhibition through PI3K signaling regulates the function and phosphorylation of certain transcriptional coactivators, such as C/EBP and β-catenin, or leads to the dysfunction of some transcriptional repressors (58, 59). Therefore, it is possible that the signaling axis of JAK1-associated PI3K/Akt/GSK3β is involved in the modulation of the transcriptional coactivators (such as C/EBP) or repressors. Further studies are needed to resolve these complicated pathways.

In summary, we demonstrated a profound effect of IL-17A on the expression of several cytokines in human bronchial epithelial cells. The importance of IL-17A has been reported in a number of airway diseases including those related to microbial infection and Th2-dominated diseases (25, 60). Our study shows that the majority of these inducible gene expressions are commonly regulated through two independent and indispensable pathways: JAK1-associated PI3K/GSK3β and Act1/TRAf6/TAK1/NF-κB activation.

Acknowledgments
We express our thanks to the generous offers of various constructs by Drs. G. B. Mills and M. C. Hung (M. D. Anderson, Houston, TX), Dr. L. A. O‘Neill (Trinity College, Dublin, Ireland), Dr. X. Li (Lerner Research Institute, Cleveland, OH), and the HBE1 cell line by Dr. J. Yankaskas (University of North Carolina, Chapel Hill, NC) for this study. We thank Dr. S. Smiley-Jewell for her editing of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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