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# Regulation of Airway *MUC5AC* Expression by IL-1 $\beta$ and IL-17A; the NF- $\kappa$ B Paradigm<sup>1</sup>

Tomoyuki Fujisawa, Sharlene Velichko, Philip Thai, Li-Yin Hung, Fei Huang, and Reen Wu<sup>2</sup>

Mucin over-production is one of the hallmarks of chronic airway diseases such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis. NF- $\kappa$ B activation in airway epithelial cells has been shown to play a positive inflammatory role in chronic airway diseases; however, the role of NF- $\kappa$ B in mucin gene expression is unresolved. In this study, we have shown that the proinflammatory cytokines, IL-1 $\beta$  and IL-17A, both of which utilize the NF- $\kappa$ B pathway, are potent inducers of mucin (*MUC*)5AC mRNA and protein synthesis by both well-differentiated primary normal human bronchial epithelial cells and the human bronchial epithelial cell line, HBE1. *MUC5AC* induction by these cytokines was both time- and dose-dependent and occurred at the level of promoter activation, as measured by a reporter gene assay. These effects were attenuated by the small molecule inhibitor NF- $\kappa$ B inhibitor III, as well as p65 small-interfering RNA, suggesting that the regulation of *MUC5AC* expression by these cytokines is via an NF- $\kappa$ B-based transcriptional mechanism. Further investigation of the promoter region identified a putative NF- $\kappa$ B binding site at position-3594/-3582 in the promoter of *MUC5AC* as critical for the regulation of *MUC5AC* expression by both IL-1 $\beta$  and IL-17A. Chromatin immunoprecipitation analysis confirmed enhanced binding of the NF- $\kappa$ B subunit p50 to this region following cytokine stimulation. We conclude that an NF- $\kappa$ B-based transcriptional mechanism is involved in *MUC5AC* regulation by IL-1 $\beta$  and IL-17A in the airway epithelium. This is the first demonstration of the participation of NF- $\kappa$ B and its specific binding site in cytokine-mediated airway *MUC5AC* expression. *The Journal of Immunology*, 2009, 183: 6236–6243.

Chronic airway diseases such as asthma, chronic obstructive pulmonary disease (COPD),<sup>3</sup> and cystic fibrosis are all characterized by airway inflammation and mucus overproduction. Respiratory homeostasis in mucus production is essential for proper mucociliary and innate immune function in the airway (1). Mucins are the major protein components responsible for the viscoelasticity of mucus (2). Under diseased conditions, an excessive production of mucin can increase morbidity and mortality by obstructing mucociliary clearance and air flow (3, 4). To date, eleven mucin (*MUC*) genes (*MUC1*, 2, 3, 4, 5AC, 5B, 6, 7, 8, 13, and 19) have been described as being expressed in the lung (2). Among these, *MUC5AC* and *MUC5B* are the most prominent mucins in the airway. In particular, *MUC5AC* is considered to be a marker of mucus cell hyperplasia or metaplasia because of its high expression in mucus-secreting goblet cells (5). Thus, understanding the mechanism of *MUC5AC* gene expression regulation

may lead to a new therapeutic opportunity for the treatment of aberrant mucus production in various lung diseases.

NF- $\kappa$ B is a pleiotrophic transcription factor that has multiple critical roles in the regulation of immune responses (6–8). NF- $\kappa$ B becomes activated in response to inflammatory cytokines, mitogens, physical and oxidative stress, infection, and microbial products (9). Before stimulation, NF- $\kappa$ B subunits are sequestered in the cytoplasm by I $\kappa$ B. Following cell stimulation, I $\kappa$ B- $\alpha$  is phosphorylated by I $\kappa$ B kinase (IKK) 2. Phosphorylation of I $\kappa$ B- $\alpha$  results in the ubiquitination and degradation of I $\kappa$ B- $\alpha$ , leading to nuclear localization of NF- $\kappa$ B, and transcriptional activation of target genes (7). The importance of NF- $\kappa$ B in airway inflammation of chronic airway diseases has been well documented. Enhanced activation of NF- $\kappa$ B has been implicated in asthma and COPD (10, 11). Through the use of transgenic mice and conditional ablation strategies, activation of NF- $\kappa$ B within the airway epithelium has been shown to be necessary to induce airway inflammation and mucus overproduction (12, 13).

Although many studies have shown a critical role for NF- $\kappa$ B in airway inflammation, not much is known regarding the involvement of NF- $\kappa$ B in mucin gene regulation in the airway. Only a few studies have demonstrated the involvement of NF- $\kappa$ B in *MUC5AC* up-regulation by lipoproteins of *Haemophilus influenzae* or *Mycoplasma pneumoniae* (14, 15). Studies involving mice, as well as NCI-H292 cells infected with an adenoviral vector encoding a dominant-negative form of IKK $\beta$ , have suggested that IKK $\beta$ -mediated NF- $\kappa$ B activation is involved in TNF- $\alpha$ -induced mucus production (16). However, this information has not been extended to a primary human cell system, nor to other inflammatory cytokines that can up-regulate *MUC5AC*.

Numerous reports have shown that inflammatory cytokines (e.g., IL-1 $\beta$ , IL-4, IL-6, IL-9, IL-13, IL-17A, TNF- $\alpha$ ) can prominently stimulate *MUC5AC* gene expression in airway epithelial cells either in vitro or in vivo (2). However, most of these experiments were conducted in cell lines in vitro, or in the presence of inflammatory cells and other cell types in vivo. These studies have

Center of Comparative Respiratory Biology and Medicine, University of California, Davis, CA 95616

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<sup>2</sup> Address correspondence and reprint requests to Dr. Reen Wu, Center for Comparative Respiratory Biology and Medicine, Genome and Biomedical Science Facility, Suite 6510, University of California at Davis, 451 Health Sciences Drive, Davis, CA 95616. E-mail address: rwu@ucdavis.edu

<sup>3</sup> Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; MUC, mucin; IKK, I $\kappa$ B kinase; hBD-2, human  $\beta$ -defensin-2; NHBE, normal human bronchial epithelial; ALI, air-liquid interface; siRNA, small-interfering RNA; RO, random oligomer; ChIP, chromatin immunoprecipitation; CTL, control nuclear protein extract.

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been difficult to repeat *in vitro* in primary airway epithelial cell cultures. Our laboratory has previously utilized such an approach to show the importance of IL-17A in directly mediating MUC gene expression (17). Using this approach, we were able to repeat some of the previous studies demonstrating IL-1 $\beta$ - and IL-17A-induced mucin gene expression, but not induction by Th2 cytokines such as IL-4, IL-9, and IL-13, which stimulate MUC gene expression through the secretion of the Gob5 gene product (18). The purpose of this current study is to further elucidate the molecular events associated with these direct cytokine-induced *MUC5AC* expressions. We purposely selected IL-1 $\beta$  and IL-17A for the study, as both are especially potent mediators in the regulation of mucin gene expression.

IL-1 $\beta$  is a proinflammatory cytokine that is mainly secreted by immune cells in response to bacterial or viral challenge (19) and has been shown to play a role in airway diseases characterized by increased mucus production (20–22). The classical NF- $\kappa$ B signaling pathway could be stimulated by IL-1 $\beta$  and activated via the activation of the IKK complex, which phosphorylate I $\kappa$ B protein and release NF- $\kappa$ B to translocate into the nucleus.

IL-17A is a member of a novel family of proinflammatory cytokines, composed by six major isoforms: IL-17A, B, C, D, E, and F (23). IL-17A has been found to be associated with a variety of inflammatory conditions in the lung, such as asthma, COPD and Gram-negative bacterial pneumonia infection (24–26). IL-17A stimulates the production of inflammatory cytokines and chemokines and mediates pulmonary neutrophil migration (27, 28). Our recent studies have demonstrated that IL-17A induces *MUC5AC* and *MUC5B* (17), human  $\beta$ -defensin-2 (hBD-2) (29), CCL-20 (30), CXCL-1, -2, -3, -5, -6, and IL-19 (31) production by primary normal human bronchial epithelial (NHBE) cells. In addition, IL-17A stimulates the degradation of I $\kappa$ B- $\alpha$ , followed by the nuclear translocation of p50 and p65 NF- $\kappa$ B (31, 32).

In terms of airway *MUC5AC* expression, extensive work has been done to elucidate the signaling and the molecular mechanism in its regulation; however, the involvement of NF- $\kappa$ B remains to be determined. Therefore, we have investigated the role of NF- $\kappa$ B in IL-1 $\beta$  and IL-17A induced *MUC5AC* expression. In this study, we demonstrate that NF- $\kappa$ B is required for both IL-1 $\beta$ - and IL-17A-induced *MUC5AC* expression. Using site-directed mutagenesis and deletion analysis, we have identified a functional  $\kappa$ B response element in the promoter of the *MUC5AC* gene that is critical for regulation by these cytokines.

## Materials and Methods

### Culture conditions

Human bronchial tissues were obtained with patients' 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 procedure in the tissue procurement. Tissues were not collected from patients diagnosed with lung-related diseases. Protease-dissociated bronchial epithelial cells were plated on Transwell chambers (Corning; 24 mm) at  $5 \times 10^4$  cells/cm<sup>2</sup> in bronchial epithelial growth medium (Lonza). After 4–7 days in an immersed culture condition or when cultures reached confluency, cells were transferred to an air-liquid interface (ALI) culture condition in a Ham's F12/DMEM (1:1) with the addition of the following eight factors: transferrin (5  $\mu$ g/ml), insulin (5  $\mu$ g/ml), cholera toxin (10 ng/ml), epidermal growth factor (10 ng/ml), dexamethasone (0.1  $\mu$ M), bovine hypothalamus extract (15  $\mu$ g/ml), BSA (0.5 mg/ml), and all-*trans*-retinoic acid (100 nM), which facilitated polarization and mucociliary differentiation. Primary cells cultured for 7 days after transferring to ALI were used in this study. Immortalized normal human bronchial epithelial cell line, HBE1 (33), was used for most of the transfection experiments. The culture condition for HBE1 was described in previous studies (34).

### Cytokine and inhibitor treatments

Recombinant human IL-1 $\beta$  was purchased from Invitrogen and recombinant IL-17A was from R&D Systems. Both were dissolved in PBS with 0.1% BSA and used at 10 ng/ml, unless otherwise specified. NF- $\kappa$ B inhibitor III (20  $\mu$ M, Calbiochem), an NF- $\kappa$ B inhibitor, was dissolved in DMSO before use. The inhibitor was added to cultures 1 h before IL-1 $\beta$  or IL-17A treatment. We observed no cell cytotoxicity of the inhibitor at the doses used in this study. This was based on the nuclear dye (trypan blue) exclusion assay (data not included).

### RNA isolation and real-time RT-PCR

Total RNA was extracted with RNA TRIzol reagent (Invitrogen) and cDNA was generated from an equal amount of RNA (5  $\mu$ g per reaction) by Moloney's murine leukemia virus-reverse transcriptase (Promega) using oligo(dT) as the primer. SYBR Green Master Mix (Roche Applied Science) 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 the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The results were presented as  $2^{-(Ct \text{ of gene of interest} - Ct \text{ of GAPDH})}$  in 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 from different treated cells (data not included). We also used  $\beta$ -actin message for the normalization (data not included) and the results are compatible with those normalized with GAPDH. The primer sequences were as follows: GAPDH forward, TGGGCTACACTGAGCACCAG; GAPDH reverse, GGGTGTCTGCTGTGAAGTCA; and *MUC5AC* forward, GGAAGTGTGGGACAGCTCTT; *MUC5AC* reverse, GTCACA TTCCTCAGCGAGGTC; p65 forward, AGCTCAAGATCTGCCGAGTG; reverse, ACATCAGCTTGCGAAAAGGA.

### DNA plasmid constructs and small-interfering RNA (siRNA)

Four *MUC5AC* promoter-firefly luciferase reporter constructs were provided by Dr. J. D. Li in the Department of Microbiology and Immunology, Rochester University Medical Center (Rochester, New York) (35, 36). Two constructs contain different sized partial pieces of *MUC5AC* promoter region: *MUC5AC*3.7kb and *MUC5AC*3.4kb constructs contain 3752 and 3452 base pairs, respectively, upstream of the transcription start site. The *MUC5AC*-TK and *MUC5AC*-TK-NF $\kappa$ B-mt constructs contain a fragment of the *MUC5AC* promoter (–3752/–3452) upstream of a minimal TK promoter, encoding unmutated and mutated  $\kappa$ B site (–3594/–3582), respectively (14, 36). All constructs were further confirmed by DNA sequencing. pRL-TK (Promega) was used as the internal control for normalizing transfection efficiency. siRNA for p65 and random oligomer (RO) were purchased from Ambion Biotech.

### Transient transfection of HBE1 cells

Transfection of HBE-1 cells with *MUC5AC* promoter-luciferase constructs, pRL-TK (as an internal control), and other expression constructs were conducted using a Lipofectamine 2000-based gene transfer method (Invitrogen) according to the manufacturer's specifications. In brief, cells were plated onto 12-well plates at 80–90% confluency. One day after the plating, cultured cells were washed twice in Opti-MEM (Invitrogen) before transfection. The cells were incubated at 37°C with the mixture of DNA constructs and Lipofectamine 2000 in Opti-MEM for 5–6 h. Six hours before cytokine addition, transfected cultures were incubated in the growth factor-depleted F12:DMEM (1:1) medium. Sixteen hours after cytokines treatment, cells were lysed in luciferase lysis buffer (25 mM Tris-phosphate (pH 7.8); 8 mM MgCl<sub>2</sub>; 1 mM DTT; 1% Triton X-100; 15% glycerol). A dual luciferase reporter assay kit (Promega) was used for analyzing the firefly luciferase activity of the *MUC5AC* promoter and *Renilla* luciferase activity of the internal control. For each transfection, relative firefly luciferase activity was normalized to *Renilla* luciferase activity. For siRNA transfection, cells were plated at 40–60% density a day before transfection. A DharmaFECT-based transfection kit (Thermo Scientific) was used according to the manufacturer's instruction. Sixteen hours after transfection, the siRNA transfection mix was replaced with fresh culture medium. Two days later, cultures were depleted of hormonal supplements 6 h before IL-1 $\beta$  or IL-17A treatment. Sixteen hours after the treatment, cells were harvested for gene expression analyses.

### Western blot analysis

Total protein lysates from different treatments were harvested by modified RIPA buffer (50 mM Tris-HCl (pH 7.4); 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; and 1 mg/ml

each of aprotinin, leupeptin, pepstatin; 1 mM  $\text{Na}_3\text{VO}_4$ ; and 1 mM NaF). Cell pellets were removed after centrifugation. The concentration of the total protein from each sample was measured by a protein assay kit (Bio-Rad Laboratory). Equal amount of total protein from each sample was subjected to SDS-PAGE (3% bis-acrylamide upper gel and 10% lower bis-acrylamide gel) and then transferred to polyvinylidene difluoride membrane (Millipore) for Western blotting according to the standard protocol. Anti-p65 mAb from Santa Cruz Biotechnology and anti- $\beta$ -actin mAb from Sigma Aldrich were used for Western blotting.

For Western blot analysis of MUC5AC, NHBE cells were lysed with ice cold "keratin-extraction" buffer (20 mM Tris-Cl, (pH 7.0), 0.6M KCl, 1% Triton X-100, and 1 mM PMSF) and centrifuged at 5000 rpm for 10 min as described previously (34). The supernatant was recovered, and the protein concentration was quantified by DC-protein assay kit (Bio-Rad). Equal amounts of cell extracts (30  $\mu\text{g}/\text{lane}$ ) were separated via SDS-PAGE (4–12% BIS-Tris, Invitrogen) and Western blot analyzed using a mAb against mucin 5AC, 45M1 (Santa Cruz Biotechnology) (37), according to the manufacturer's specification.

#### Immunofluorescent staining

NHBE cells grown under air-liquid interface condition on Transwell membrane were fixed and permeabilized with cold methanol and stained with or without primary Ab, H160 (Santa Cruz Biotechnology), as described previously (17). H160 is a polyclonal Ab against a C-terminal mucin 5AC peptide (1214–1373). After an overnight incubation and washing, the membrane was stained with Alexa-Fluor 568 conjugated goat anti-rabbit Ab (red fluorescence). Nuclei were stained with DAPI (blue fluorescence).

#### Preparation of nuclear extracts

Nuclear lysates from cultured HBE1 cells were harvested according to Panomics nuclear extraction protocol. In brief, HBE1 cells were lysed with a lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.5% IGEPAL and Protease Inhibitor Cocktail) on ice for 10 min and harvested using a sterile scraper. Cytosolic fraction was collected from the supernatant by centrifugation at  $15,000 \times g$  for 3 min at  $4^\circ\text{C}$ . Pelleted nuclei were resuspended in extraction buffer (10 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT and Protease Inhibitor Cocktail). After incubation at  $4^\circ\text{C}$  for 2 h with gentle rocking, the nuclei were collected by centrifugation at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The resultant supernatants were collected and stored at  $-80^\circ\text{C}$ .

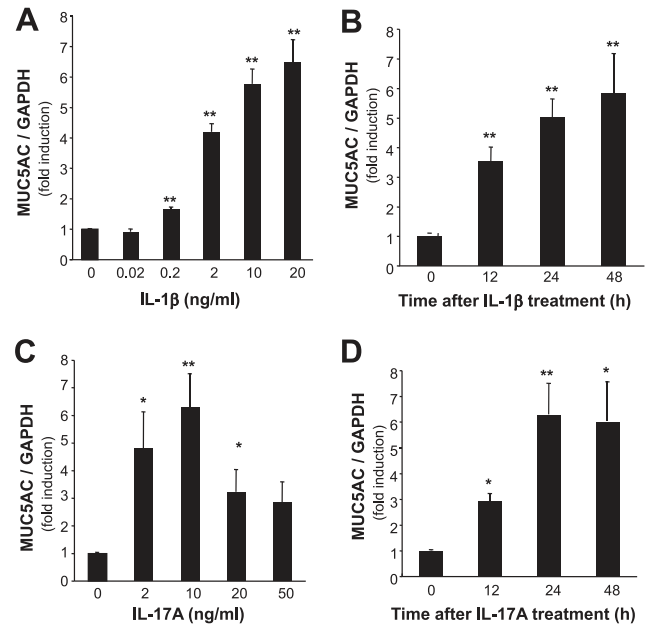
#### NoShift p50- and p65-binding assay

The NoShift transcriptional factor assay kit (Novagen) was used to measure binding of NF- $\kappa\text{B}$  p50 and p65 proteins to the NF- $\kappa\text{B}$  binding sequences on MUC5AC promoter (from  $-3602$  to  $-3577$ ) as described in the manual. In brief, 10  $\mu\text{g}$  each of sense and antisense 5'-biotinylated oligonucleotides flanking the  $\kappa\text{B}$  site ( $-3594/-3582$ , underlined) of MUC5AC distal promoter region (sense: 5'-ACACGACGGGGCACTCCCCTCTGAG-3', antisense: 5'-CTCAGAGGGGAGTGCCCCCTGCGTGT-3') were dissolved to 100  $\mu\text{l}$  of a mixture of  $0.5 \times \text{SSC}$  ( $1 \times \text{SSC}$  is 0.15 M NaCl plus 0.015 M sodium citrate), 75 mM NaCl, and 7.5 mM sodium citrate (pH 7.0); and heated to  $100^\circ\text{C}$  (in boiling water bath) for 10 min, slowly cooled to room temperature and then diluted to 10 pmol/ $\mu\text{l}$ . Nonbiotinylated oligonucleotides with same sequence duplex, which were used as competitor DNA, were prepared in the same way except with a final concentration of 50 pmol/ $\mu\text{l}$ .

For measurement of binding affinity, the reaction mixtures mainly contained 10 pmol of biotinylated target DNA duplex, and 30  $\mu\text{g}$  of nuclear extract were incubated on ice for 30 min. The reaction mixtures were then dispensed into freshly prepared streptavidin plates and incubated for 1 h at  $37^\circ\text{C}$ . The binding of p50 and p65 was detected by incubation for 1 h at  $37^\circ\text{C}$  with 100  $\mu\text{l}$  of anti-NF- $\kappa\text{B}$  p50 (Biolegend) and -p65 mouse mAbs diluted 1/1,000 in NoShift Ab dilution buffer. After repeated washing of the plate, HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was added (1/1,000 dilution in NoShift Ab dilution buffer). After 30 min of incubation at  $37^\circ\text{C}$ , wells were washed thoroughly and tetramethylbenzidine substrate was added. The reaction was quenched by 100  $\mu\text{l}$  of 1 N HCl/well, and binding intensity was measured as absorbance at 450 nm.

#### Chromatin immunoprecipitation (ChIP) assay

ChIP assays were conducted according to the ChIP protocol from Millipore, with minor modifications. In brief, cells were harvested and cross-linked with 1% formaldehyde at room temperature for 10 min. Isolated nuclei were sonicated with a Bioruptor (Diagenode) to fragment chromatin to  $\sim 500$  bp. The sonicated nuclear fractions were divided for input control and for overnight incubation with either anti-p50 Ab or the negative control



**FIGURE 1.** Stimulation of MUC5AC mRNA expression by IL-1 $\beta$  and IL-17A. **A** and **C**, Dose-response effects of IL-1 $\beta$  and IL-17A, respectively on MUC5AC mRNA expression in well-differentiated primary NHBE cells. NHBE cells grown in air-liquid interface for 1 wk supplemented with retinoic acid, as described in text, were starved for 16 h (without growth factors) before being treated with various concentrations of IL-1 $\beta$  (0 to 20 ng/ml) and IL-17A (0–50 ng/ml). Twenty-four hours after cytokine treatment, total RNA was harvested, and MUC5AC mRNA was analyzed using SYBR green quantitative real-time PCR and normalized to a housekeeping gene, GAPDH, as described in *Materials and Methods*. **B** and **D**, Time course effects of IL-1 $\beta$  (10 ng/ml) and IL-17A (20 ng/ml) treatments on MUC5AC mRNA expression, respectively in NHBE cells. RNA samples were harvested from these cultures at different time points (0, 12, 24, 48 h) following cytokine treatments. MUC5AC and GAPDH mRNA levels were quantified using real-time PCR. Triplicate dishes were used for each experiment, and experiments were repeated at least three times using different cultures derived from different human donors. Statistically significant: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , compared with saline control treatments.

IgG. The Ab protein-DNA complex was then pulled down with salmon sperm DNA-conjugated protein A agarose beads. Finally, genomic DNA recovered from the ChIP assay was PCR amplified with primers specific to the NF- $\kappa\text{B}$ -binding elements of MUC5AC promoter region (from  $-3685$  to  $-3540$ ); forward primer: 5'-TGAGGGAGGAGAAAGCTGTG-3', reverse primer: 5'-CGTGACCTCTGAACAGTCTGA-3'. The specificity of each primer set was verified by analyzing the dissociation curve of each gene-specific PCR product.

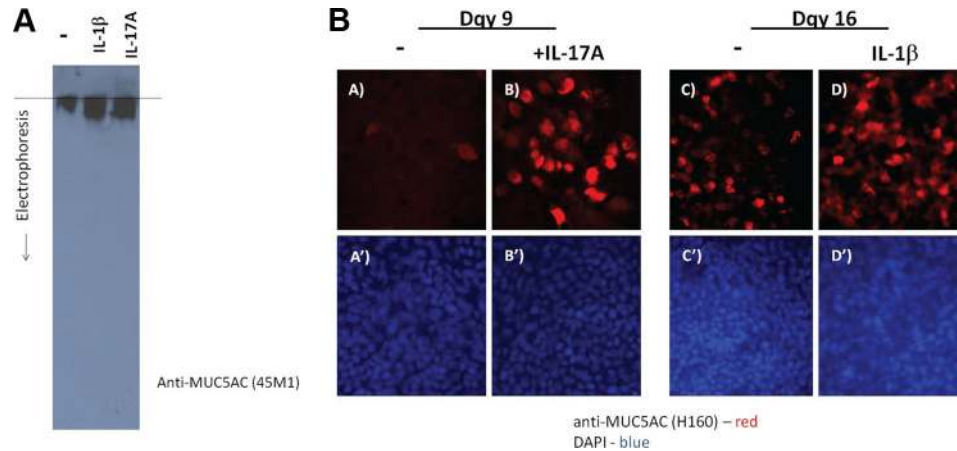
#### Statistical analysis

Data are expressed as mean  $\pm$  SE. Experiments were conducted in triplicate and at least in two independent cultures. Group differences were calculated using the Student's  $t$  test. Differences were considered significant for  $p$  values less than or equal to 0.05.

## Results

### Stimulation of MUC5AC gene expression by IL-1 $\beta$ and IL-17A

Initially, we examined the potency of MUC5AC stimulation by IL-1 $\beta$  and IL-17A in well-differentiated NHBE cells cultured under ALI condition. As shown in Fig. 1, both IL-1 $\beta$  and IL-17A induced MUC5AC mRNA in primary NHBE cells in a dose- and time-dependent manner. For IL-1 $\beta$ , significant stimulation of MUC5AC was observed at concentrations as low as 0.2 ng/ml and maximized at 10 ng/ml (Fig. 1A). A time course study indicated maximum stimulation of MUC5AC expression at 24 h (Fig. 1B) after the addition of 10 ng/ml IL-1 $\beta$ . A similar dose-dependent



**FIGURE 2.** Effects of cytokines on mucin 5AC production in NHBE cells. *A*, Western blot analysis of mucin 5AC protein expression in NHBE cells following IL-1 $\beta$  (10 ng/ml) or IL-17A (10 ng/ml) treatment, as described in Fig. 1. Cells were harvested 24 h poststimulation and extracted with “keratin-extraction” buffer (KEB) as described in the text. After a high-speed centrifugation to remove keratin-rich fraction, the total protein concentration was determined. Equal amounts of cell extracts (30  $\mu$ g/lane) were separated via SDS PAGEgel (4–12% BIS-Tris) and Western blot analyzed using a mAb against MUC 5AC, 45M1. Most mucin 5AC bands stayed at the gel entrance, as indicated by a dotted line. *B*, Immunofluorescent staining of cytokine-treated NHBE cultures with a rabbit polyclonal Ab generated against a C-terminal mucin 5AC peptide (1214–1373), H160. NHBE cells grown under air-liquid interface condition at day 8–9 (*A*, *B*, *A'*, and *B'*) or day 15–16 (*C*, *D*, *C'*, and *D'*) were treated with IL-17A (10 ng/ml) or IL-1 $\beta$  (10 ng/ml), respectively. Transwell membranes were permeabilized with cold (–20°C) methanol, and stained with or without primary Ab. Alexa-Fluor 568 conjugated goat anti-rabbit Ab was used as a secondary Ab (red fluorescence, *A*–*D*) and nuclei were stained with DAPI (blue, *A'*–*D'*). Controls, Transwell membranes stained with no primary Ab yielded no red fluorescent stain (data not included).

curve was seen for IL-17A, except with a slight decrease of stimulation when doses higher than 20 ng/ml were used (Fig. 1C). Maximum stimulation was seen 24 h after treatment with 10 ng/ml of IL-17A in NHBE cells (Fig. 1D). These studies demonstrated up to 6- to 7-fold stimulation of MUC5AC gene expression by IL-1 $\beta$  and IL-17A over untreated, confirming that both cytokines are potent stimulators for *MUC5AC* gene expression in well-differentiated NHBE cells. Similar time- and dose-dependent results with these cytokines were seen in the HBE1 cell line (data not included).

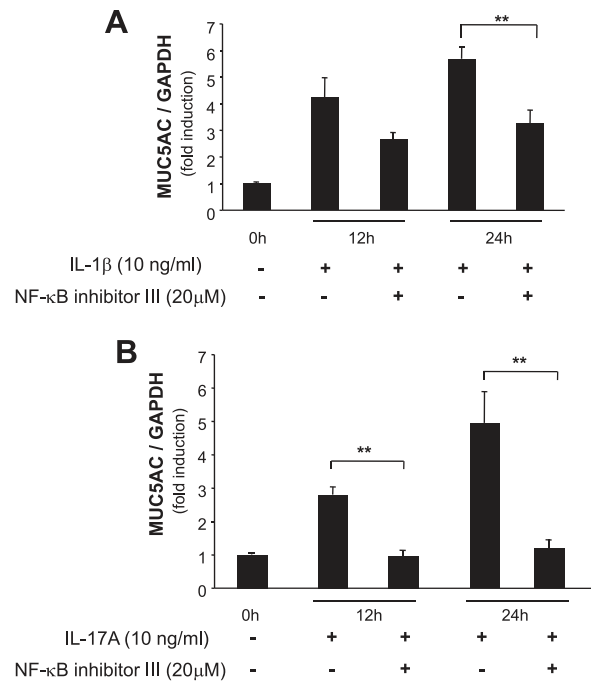
*Stimulation of MUC 5AC in NHBE cells by cytokines*

To further characterize stimulation at the protein level, two Abs commonly used for mucin 5AC identification were used. The 45M1 mAb was generated against purified gastric mucin (37), and used directly for Western blot analysis. As shown in Fig. 2A, most 45M1-stained bands were at the entrance of the SDS-PAGE gel, consistent with the high m.w. nature of MUC5AC. Based on the intensity, 45M1-specific bands were elevated in NHBE cultures after IL-17A and IL-1 $\beta$  treatment, compared with the untreated control. The polyclonal Ab, H160, generated against a C-terminal MUC5AC peptide, corresponding to amino acid sequence from 1214 to 1373 (38), was used for immunofluorescent staining. As shown in Fig. 2B, there were increased immunofluorescence-positive stained cells in cultures after IL-17A or IL-1 $\beta$  treatment, as compared with unstimulated controls. Interestingly, unstimulated NHBE cultures had more positively stained cells at day 16 than day 9, suggesting continuing mucous cell differentiation in primary cultures under air liquid interface.

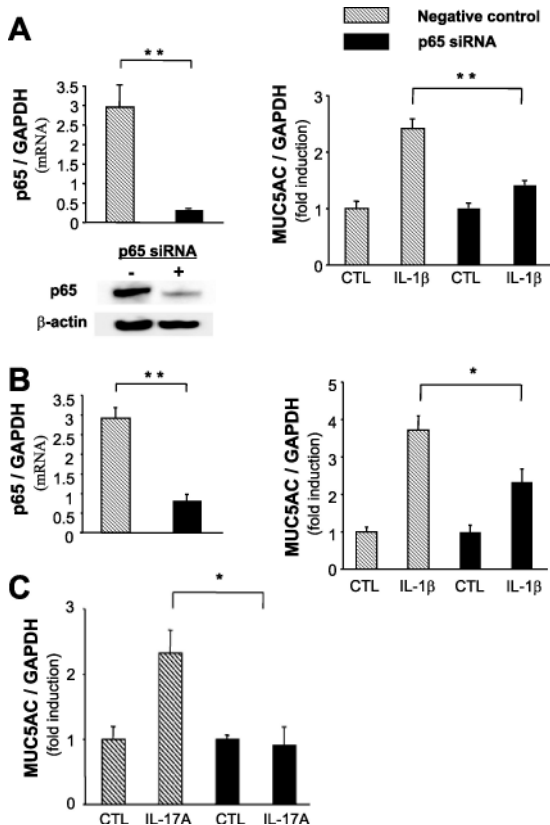
*NF- $\kappa$ B was required for both IL-1 $\beta$ - and IL-17A-induced MUC5AC expression*

We have previously shown that IL-17A-induced hBD-2 expression is regulated through an NF- $\kappa$ B based transcriptional mechanism (31, 32). To further evaluate the involvement of NF- $\kappa$ B in cytokine-induced MUC5AC expression, an NF- $\kappa$ B activation inhibitor and NF- $\kappa$ B siRNA were used. As shown in Fig. 3, *A* and *B*, both

IL-1 $\beta$  and IL-17A were able to enhance *MUC5AC* promoter-mediated reporter gene activity by 3- to 5-fold in transiently transfected cells. Though this stimulation was less than that

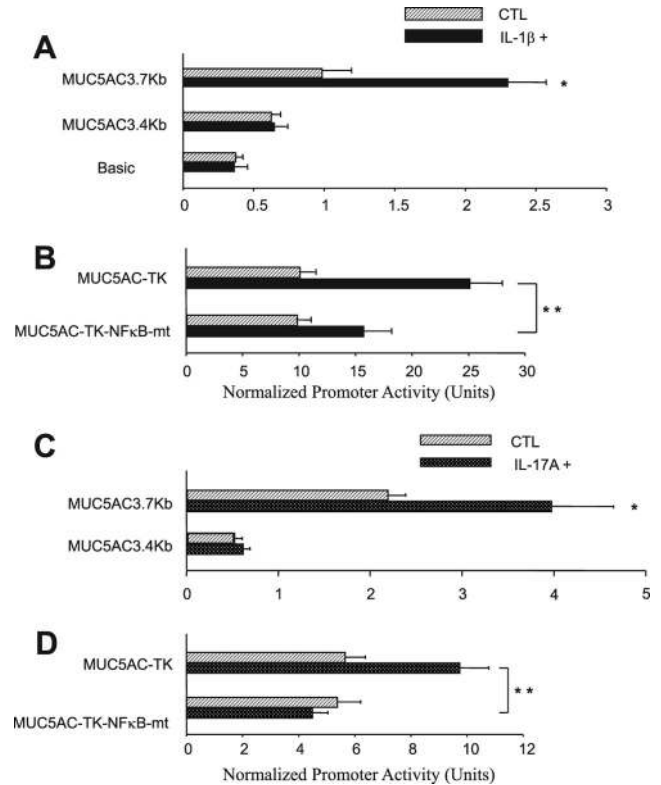


**FIGURE 3.** Effects of NF- $\kappa$ B inhibitor III on cytokine-induced *MUC5AC* expression. NF- $\kappa$ B inhibitor III (20  $\mu$ M) or equal amount of vehicle, dimethyl-sulfoxide (DMSO), was added 1 h before IL-1 $\beta$  (*A*, 10 ng/ml) or IL17A (*B*, 20 ng/ml) treatment on primary NHBE cells. At 12 and 24 h posttreatment, RNA samples were collected from these cultures. SYBR green quantitative RT-PCR was used to quantify the message levels of MUC5AC and GAPDH in these RNA samples. Triplicate dishes were used for each time point, and experiments were repeated three times for different cultures derived from different donors. Statistically significant: \*\*,  $p < 0.01$ .



**FIGURE 4.** Effects of p65 siRNA on cytokine-induced *MUC5AC* expression. Both NHBE (A) and HBE1 (B and C) cells were treated with p65 siRNA or RO, the negative control, as described in the text. Two days after siRNA treatment, cells were treated with IL-1 $\beta$  (A and B, 10 ng/ml) or IL-17A (C, 10 ng/ml) for 24 h as described in Fig. 1. The efficiency of siRNA-p65 in reducing endogenous p65 mRNA or protein in NHBE cells was confirmed by performing real-time PCR (top left) or Western blot analysis (bottom left) (A).  $\beta$ -actin served as the loading control for Western blot analysis. For HBE1 cells (B), the efficiency of siRNA-p65 in reducing endogenous p65 mRNA was confirmed by performing real-time RT-PCR (left panel). Relative *MUC5AC* message levels were averaged from triplicate dishes and the experiment was repeated three times with HBE1 cells from different passage numbers as well as primary NHBE cultures. Statistically significant: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; CTL; unstimulated control.

observed for mRNA (Fig. 1), any difference is probably due to differences in cell culture conditions, stimulation time, and the nature of the readout (quantitative RT-PCR vs luciferase activity). Despite lower stimulation, both IL-1 $\beta$ - and IL-17A-induced *MUC5AC* promoter reporter gene activities were sensitive to the NF- $\kappa$ B activation inhibitor in NHBE cells (Fig. 3B). To further confirm the results from inhibitor experiment, cells were transiently transfected with p65 NF- $\kappa$ B siRNA. As shown in Fig. 4A, p65 NF- $\kappa$ B siRNA was very effective in attenuating its own message, as well as its own protein in NHBE cells. The reduced expression of p65 NF- $\kappa$ B correlated with reduced IL-1 $\beta$ -induced *MUC5AC* expression. The control experiments with RO had no effect on either p65 NF- $\kappa$ B expression, nor IL-1 $\beta$ -mediated *MUC5AC* stimulation. A similar result was seen with the HBE1 cell line, in which IL-1 $\beta$  (Fig. 4B) or IL-17A (Fig. 4C) also induced *MUC5AC* expression, and this expression was attenuated by p65 NF- $\kappa$ B siRNA treatment. These results from Figs. 3 and 4 demonstrate that NF- $\kappa$ B is involved in cytokine-induced *MUC5AC* expression in both NHBE and HBE1 cells.

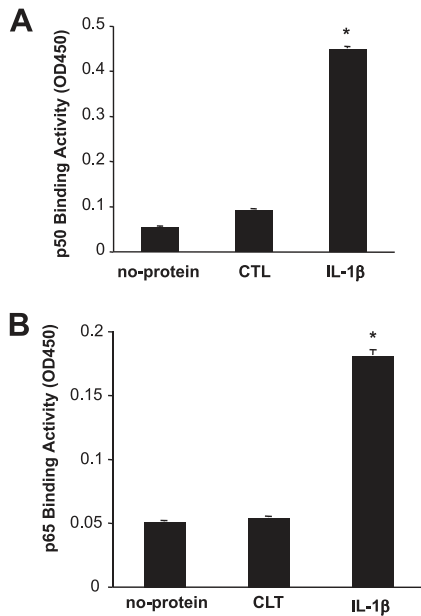


**FIGURE 5.** Deletion and site-directed mutagenesis analysis of *MUC5AC* promoter-reporter gene activities in response to cytokine treatments. HBE1 cells were cotransfected with the indicated luciferase reporter constructs and the control pRL-TK plasmid DNAs as described in *Materials and Methods*. Two days after transfection, cells were left unstimulated (CTL) or stimulated with IL-1 $\beta$  (A and B) or IL-17A (C and D) at 10 ng/ml level for 16 h. Luciferase activities in these cells were measured and normalized as described in *Materials and Methods*. A and C, Cells were transfected with *MUC5AC3.7kb* and *MUC5AC3.4kb* constructs. B and D, Cells were transfected with *MUC5AC-TK* and *MUC5AC-TK-NF $\kappa$ B-mt* constructs for enhancer analysis. Data from triplicate dishes were averaged and experiments performed in four independent cultures. Statistical significance: \*,  $p < 0.05$  compared with the CTL case without cytokine treatment; \*\*,  $p < 0.01$ .

#### Deletion and site-directed mutagenesis analysis of cytokine-induced *MUC5AC* promoter activity

To further determine whether the observed effects of IL-1 $\beta$  and IL-17A on *MUC5AC* expression was due to an NF- $\kappa$ B-mediated transcriptional activation, study of the effects of these cytokines on *MUC5AC* promoter activity was conducted in HBE1 cells using a transient transfection approach with *MUC5AC* promoter/luciferase chimeric construct DNA. As shown in Fig. 5A, IL-1 $\beta$  significantly increased the luciferase reporter gene activity when the *MUC5AC* 3.7 kb promoter construct was used to transfect cells; however, this stimulated phenomenon was diminished in cells transfected with the 3.4 kb *MUC5AC* promoter. A similar observation was seen with IL-17A-stimulated *MUC5AC* promoter luciferase activity (Fig. 5C). These results indicated that the region of the *MUC5AC* promoter spanning  $-3752$  to  $-3452$  bp contained the *cis*-acting element(s) required for both IL-1 $\beta$ - and IL-17A-stimulated gene expressions.

To identify the putative enhancer element(s) in this 300-bp region, sequence analysis using Genomatix-MatInspector software (Genomatix Software) revealed a putative NF- $\kappa$ B binding site ( $-3594/-3582$ , GGGGCACTCCCCT) on the 5'-flanking region of the *MUC5AC* promoter. To characterize the function of this



**FIGURE 6.** Analysis of the in vitro occupancy of IL-1 $\beta$  stimulated p50 and p65 binding activity to the NF- $\kappa$ B site on MUC5AC promoter. HBE1 cells were treated with IL-1 $\beta$  (10 ng/ml) for 20 min, and cells were lysed and the nuclear fraction was harvested. NoShift Transcription Factor Assay Kit from Novagen, was used to assay for p50 (A) and p65 (B) binding activity to the DNA oligomers flanking the distal NF- $\kappa$ B site (–3594/–3582) of MUC5AC promoter region. The binding activities were averaged from triplicate dishes and the experiment repeated twice on different passages of cells with similar results. \*,  $p < 0.01$ , compared with unstimulated control (CTL).

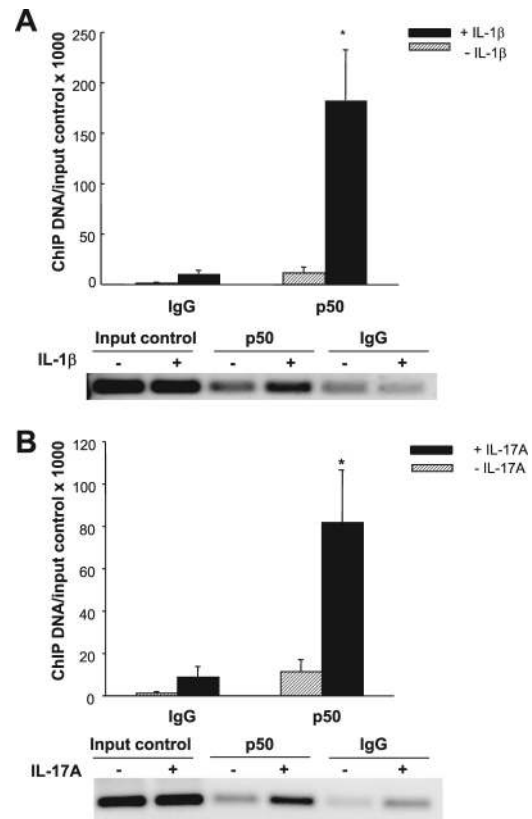
putative NF- $\kappa$ B binding site, 300-bp DNA fragments containing either the wild-type or the mutated NF- $\kappa$ B binding site (tcccgA CTCCCCT) were subcloned upstream of a thymidine kinase basal promoter-reporter construct. Using these constructs, MUC5AC-TK and MUC5AC-TK-NF $\kappa$ B-mt, for transfection studies, we observed enhanced luciferase activity in MUC5AC-TK-transfected HBE1 cells treated by either IL-1 $\beta$  (Fig. 5B) or IL-17A (Fig. 5D), but not in MUC5AC-NF $\kappa$ B-mt transfected cells. These results demonstrate the *cis*-enhancer nature of the NF- $\kappa$ B binding site at –3594/–3582 of MUC5AC 5'-flanking region in response to IL-1 $\beta$  and IL-17A stimulation.

#### Enhanced NF- $\kappa$ B translocation and binding to the NF- $\kappa$ B binding sequences on MUC5AC promoter by cytokines

Two of the critical events involved in NF- $\kappa$ B activation by cytokines is p50/p65 NF- $\kappa$ B nuclear translocation and binding to the putative  $\kappa$ B binding sequences on chromatin DNA. For IL-17A, we have previously demonstrated such a mechanism for hBD-2 expression (31, 32). As shown in Fig. 6, IL-1 $\beta$  stimulated not only the nuclear translocation of p50 and p65 subunits of NF- $\kappa$ B (31, 32), but also the enhanced binding of either subunit to the flanking region around the distal NF- $\kappa$ B binding site of the MUC5AC promoter. For the unstimulated control nuclear protein extracts (CTL), there was no increase of binding observed over the background.

#### Demonstration of NF- $\kappa$ B binding to MUC5AC promoter in situ by ChIP assay

To determine whether cytokine-induced MUC5AC promoter activity is due to a direct interaction between NF- $\kappa$ B and MUC5AC promoter on chromatin DNA, especially in the region flanking the putative NF- $\kappa$ B binding site (–3594/–3582), ChIP assays were



**FIGURE 7.** ChIP assays on p50 protein binding on MUC5AC promoter region in cells after IL-1 $\beta$  or IL-17A treatment. HBE1 cells were treated for 10 ng/ml of IL-1 $\beta$  (A) or IL-17A (B) for 1 h, then fixed with 1% formaldehyde as described in *Materials and Methods*. Cross-linked chromatin was isolated and fragmented to ~500 bp in size. Anti-p50 Ab or control IgG was used to precipitate DNA fragments bound with p50 protein or nonspecific proteins. Coprecipitated DNA and the corresponding input DNA before precipitation were quantified by real-time PCR analysis (*upper*) and PCR-gel electrophoresis analysis (*lower*) with primers flanking different MUC5AC promoter regions. Data shown were using primers covering the –3685/–3540 region of the MUC5AC promoter. Results from the quantitative real-time PCR, after normalization with input control DNA template, were averaged from three independent experiments. \*,  $p < 0.05$  compared with non-cytokine-treated control.

conducted in HBE1 cells before and after IL-1 $\beta$  and IL-17A treatments. As shown in Fig. 7, both cytokines stimulated the binding of the p50 subunit to the (–3685/–3540) DNA fragment in treated cells, as evidenced by the SYBR green quantitative real-time PCR quantification and the PCR DNA band in gel. Precipitations from control IgG Abs yielded very low real-time PCR activity and PCR DNA bands as compared with the equal input DNA in the precipitates. Binding to other “putative” NF- $\kappa$ B sites other than this distal site, such as two proximal NF- $\kappa$ B binding sites within the first 1 kb 5'-flanking region, were not changed by cytokine treatment (data not included). These results confirm the participation of this distal NF- $\kappa$ B binding site at –3594/–3582 in cytokine-enhanced MUC5AC expression.

## Discussion

NF- $\kappa$ B activation in airway epithelial cells plays a central role in airway inflammation; however, it remains to be determined whether an NF- $\kappa$ B-based mechanism is involved in cytokine-stimulated mucin up-regulation. In the present study, we have shown that NF- $\kappa$ B plays an indispensable role in both IL-1 $\beta$ - and IL-17A-induced MUC5AC expression in both well-differentiated primary NHBE and HBE1 cells. Both IL-1 $\beta$ - and IL-17A-stimulated

*MUC5AC* gene expression in a time- and dose-dependent manner. Attenuation of NF- $\kappa$ B using a specific inhibitor or siRNA suppressed *MUC5AC* induction by either cytokine. Using a reporter-based promoter study, we demonstrated the existence of a functional  $\kappa$ B site in the 5'-flanking region of the *MUC5AC* promoter and that it plays a critical role in IL-1 $\beta$ - and IL-17A-induced gene expression. Importantly, we also provide evidence of enhanced physical interaction between NF- $\kappa$ B proteins, especially p50, and the putative  $\kappa$ B binding element located at -3594/-3582 of the *MUC5AC* promoter by using ChIP analysis of cytokine-treated cells. This is the first report describing a crucial role of NF- $\kappa$ B in the transcriptional regulation of airway *MUC5AC* expression and the identification of a functional  $\kappa$ B response element in the promoter of *MUC5AC* gene in response to IL-1 $\beta$  and IL-17A treatments.

Before this report, there were several reports describing the participation of two proximal NF- $\kappa$ B binding sites within the first 1 kb region of the *MUC5AC* promoter in gene expression regulation (35, 38, 39). Rather than in well-differentiated primary cells, most of these studies were performed using immortalized cell lines. Furthermore, the direct binding of p50/p65 NF- $\kappa$ B to these putative  $\kappa$ B sites *in vivo* has not been demonstrated. In contrast to these results, our promoter deletion analysis indicated that a 300 bp region between -3.4 kb and -3.7 kb of the *MUC5AC* promoter is required for both IL-1 $\beta$  and IL-17A upregulations of *MUC5AC*. Both site-directed mutagenic analysis and ChIP assays have shown that the sole NF- $\kappa$ B binding site within this 300 bp region is involved in mediating cytokine-enhanced gene expression. We did not observe any participation of the proximal NF- $\kappa$ B binding sites in IL-1 $\beta$ - or IL-17A-mediated *MUC5AC* expression (data not included).

To date, the ability of IL-1 $\beta$  to stimulate *MUC5AC* gene expression in the airway epithelium remains controversial. Gray et al. (40, 41) reported that IL-1 $\beta$  increased the secretion of *MUC5AC* protein without affecting mRNA levels in NHBE cells. The mechanism responsible for such an up-regulation has not been further elucidated. We have found that IL-1 $\beta$  increases *MUC5AC* mRNA levels in NHBE cells in a time- and dose-dependent manner. Due to differences in cell culture conditions, examination of the discrepancy between our findings and theirs is difficult to assess. However, the finding that IL-1 $\beta$ -induced *MUC5AC* expression is dependent on NF- $\kappa$ B activation, is consistent with various studies that reported the involvement of NF- $\kappa$ B as the downstream mechanism of IL-1 $\beta$ -induced gene expression in various cell systems (42, 43).

Given the broad nature of NF- $\kappa$ B activation, it seems likely that other factors may be involved in the regulation of IL-1 $\beta$ -induced *MUC5AC* expression. Song et al. (44) have shown that IL-1 $\beta$  induced *MUC5AC* expression is through ERK, p38 MAPK, and CREB activation in NCI-H292 cells. In addition, they found that the CRE site at -878 bp of the *MUC5AC* promoter was critical for IL-1 $\beta$ -induced *MUC5AC* promoter activity. Various transcription factors, such as NF- $\kappa$ B, CREB, SP1, and AP1, are reported to be involved in *MUC5AC* expression in airway epithelial cells by various stimulants (14, 45-47). It is possible that an intricate network of transcriptional factors may be involved in the regulation of *MUC5AC* expression under various conditions. Recently, Chen et al. (14) demonstrated the involvement of both NF- $\kappa$ B and AP1 pathways in *MUC5AC* expression induced by *Haemophilus influenzae* lipoprotein P6 in a human colon-originated HM3 cell line. It is likely that several transcription factors or transcriptional coactivators may be involved in full expression of IL-1 $\beta$ -induced *MUC5AC* expression, which will be a topic of exploration in future studies.

We previously reported that IL-17A could stimulate *MUC5AC* expression in primary NHBE cultures (17) and that this stimulation could be partially blocked by an anti-IL-6 receptor neutralizing Ab. This result suggested an IL-17A-mediated IL-6 autocrine/paracrine loop in mucin gene expression regulation. Further studies have shown that this mechanism reached maximum stimulation at 48-72 h (Chen Y., unpublished observation). In contrast, the IL-1b and IL-17A induction of *MUC5AC* is an early event that occurs within 24 h after the treatment. In this study, we have found that the NF- $\kappa$ B signaling pathway is also required for IL-17A-induced *MUC5AC* expression. This finding is consistent with our previous studies, showing that IL-17A inductions of hBD-2, CCL-20, and IL-19 in NHBE and HBE1 cells partly involve NF- $\kappa$ B translocation and binding to  $\kappa$ B binding sites of these inducible genes (31, 32). We previously also showed that dual signaling pathways control IL-17A induced hBD-2 expression (31). One pathway involves the activation of the PI3K signaling pathway, while the other involves NF- $\kappa$ B activation through an Act1/TNF receptor-associated factor 6 (TRAF6)/TGF $\beta$ -activated kinase 1-mediated signaling pathway. Although PI3K is involved in IL-17A induced hBD-2 expression, its role in *MUC5AC* induction is undetermined and a further study is warranted.

In summary, we have found that both IL-1 $\beta$  and IL-17A, two prominent cytokines associated with chronic airway inflammation, are potent mediators in upregulating *MUC5AC* expression in airway epithelial cells. We further showed that NF- $\kappa$ B activation is an essential mechanism for both IL-1 $\beta$ - and IL-17A-induced *MUC5AC* expression in human airway epithelial cells, and we have further identified a critical NF- $\kappa$ B binding site in the distal portion of *MUC5AC* promoter essential for gene induction by these cytokines. As IL-1 $\beta$  and IL-17A have both been demonstrated as positively elevating airway inflammation in various disease states, our results are consistent with these findings and further suggest one manner in which these cytokines contribute to the pathogenesis of airway inflammatory diseases. This study highlights an important function of NF- $\kappa$ B as a regulator of mucin gene expression in airway epithelium and may provide new strategies for controlling mucus over production in chronic airway diseases.

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## Disclosures

The authors have no financial conflict of interest.

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