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Stimulation of Airway Mucin Gene Expression by Interleukin (IL)-17 through IL-6 Paracrine/Autocrine Loop*

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Yin Chen‡, Philip Thai‡, Yu-Hua Zhao‡, Ye-Shih Ho§, Mary M. DeSouza¶, and Reen Wu‡‖

From the ‡Center for Comparative Respiratory Biology and Medicine and Division of Pulmonary and Critical Care Medicine, University of California, Davis, California 95616, §Institute of Environmental Health Sciences, Wayne State University, Detroit, Michigan 48201, and ¶GlaxoSmithKline Pharmaceuticals, King of Prussia, Pennsylvania 19406

Mucus hypersecretion and persistent airway inflammation are common features of various airway diseases, such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis. One key question is: does the associated airway inflammation in these diseases affect mucus production? If so, what is the underlying mechanism? It appears that increased mucus secretion results from increased mucin gene expression and is also frequently accompanied by an increased number of mucous cells (goblet cell hyperplasia/metaplasia) in the airway epithelium. Many studies on mucin gene expression have been directed toward Th2 cytokines such as interleukin (IL)-4, IL-9, and IL-13 because of their known pathophysiological role in allergic airway diseases such as asthma. However, the effect of these cytokines has not been definitely linked to their direct interaction with airway epithelial cells. In our study, we treated highly differentiated cultures of primary human tracheobronchial epithelial (TBE) cells with a panel of cytokines (interleukin-1α, 1β, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, and tumor necrosis factor α). We found that IL-6 and IL-17 could stimulate the mucin genes, *MUC5B* and *MUC5AC*. The Th2 cytokines IL-4, IL-9, and IL-13 did not stimulate *MUC5AC* or *MUC5B* in our experiments. A similar stimulation of *MUC5B*/*Muc5b* expression by IL-6 and IL-17 was demonstrated in primary monkey and mouse TBE cells. Further investigation of *MUC5B* expression demonstrated that IL-17's effect is at least partly mediated through IL-6 by a JAK2-dependent autocrine/paracrine loop. Finally, evidence is presented to show that both IL-6 and IL-17 mediate *MUC5B* expression through the ERK signaling pathway.

Chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD),[1] and cystic fibrosis are all characterized by inflammation of the airways and mucus hypersecretion (1). The mucus hypersecretion by itself might increase morbidity and mortality in these conditions by obstructing the airways and impairing gas exchange (2). One of the main components of mucus secretion is the mucin protein. Mucins are a family of large glycoproteins that have a molecular mass of several thousand kilodaltons, and they are a major determinant of the viscoelasticity of mucus secretion (3). There are currently 19 identified mucin genes highly expressed in tissues, such as lung, nose, salivary glands, GI tract, and uterus (4, 5). In the lung, synthesis and secretion of mucins are restricted largely to the airway with little to no expression in alveolar airspaces (1). Although at least eight mucin genes (*MUC 1, 2, 4, 5AC, 5B, 7, 8, 13*) have been found to be expressed in adult human lung (4), *MUC5AC* and *MUC5B* appear to be the predominant genes expressed, and their glycoprotein products are the most abundant in mucus secretions (4, 6–9). *MUC5AC* appears to be produced mainly in the airway epithelium by goblet cells (8, 10), while *MUC5B* is mostly produced in the underlying submucosal glands (10). In contrast to this normal distribution pattern, we previously showed that *MUC5B* could be expressed by surface airway epithelial cells in addition to the expression by submucosal gland cells in airway tissue sections obtained from COPD and asthma (10), while *MUC5AC* expression was still restricted at the surface epithelial cells in these tissue sections. These results suggest that changes in *MUC* gene expression, especially *MUC5B*, are associated with airway diseases. The source of increased mucus production in diseases such as asthma and COPD is due at least partially to an increased number of goblet cells in the airway epithelium (11–13). Studies have linked goblet cell metaplasia to the increase of mucin gene expression in airway epithelial cells (10, 14).

In recent years, inflammatory cytokines have been linked to increased mucus production by their effects on mucin gene expression in the airway epithelium (15). For example, tumor necrosis factor (TNF) α has been shown to induce expression of *MUC2* (16) and *MUC5AC* (17) in NCI-H292 cells. More recently, IL-1β has also been shown to induce *MUC2* and *MUC5AC* expression in NCI-H292 cells (18, 19). From studies of asthma, evidence suggests that Th2 cytokines IL-4, 9, and 13 can also affect mucin gene expression. Transgenic overexpression of IL-4 in murine lungs causes mucous cell metaplasia and an induction of MUC*5AC* expression in the airway epithelium (20). However, direct treatment of IL-4 on airway epithelial cell culture has produced conflicting results. One group reported a decrease in *MUC5AC* expression (21), another reported no change (22), and a third reported an increase of *MUC2* expression by IL-4 (23).

It was later suggested that IL-13 was also important for the development of asthmatic phenotypes such as airway hyperre-

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‖ To whom correspondence should be addressed: Center for Comparative Respiratory Biology and Medicine, Surge 1 Annex, Room 1121, University of California at Davis, One Shields Ave., Davis, CA 95616. Tel.: 530-752-2648; Fax: 530-752-8632; E-mail: rwu@ucdavis.edu.

[1] The abbreviations used are: COPD, chronic obstructive pulmonary disease; TBE, tracheobronchial epithelial cells; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; MAP, mitogen-activated protein; PI, phosphatidylinositol; ELISA, enzyme-linked immunosorbent assay; nt, nucleotides; STAT, signal transducer and activator of transcription; FITC, fluorescein isothiocyanate; IL, interleukin; TNF, tumor necrosis factor.

activity, eosinophilic infiltration, and mucous cell metaplasia (24, 25). *In vivo* models using transgenic mice (26) and intranasal (14) or intratracheal (27) injections of IL-13 consistently showed increased goblet cells in the airways of mice. However, one limitation of these *in vivo* experiments was their inability to determine the exact mechanism of how the cytokine affects mucin gene expression. Does IL-13 interact directly with receptors on the airway epithelium to induce mucin gene expression, or are its effects mediated through inflammatory cell recruitment or the induction of local mediator release from surrounding cells such as fibroblasts or smooth muscle cells? For instance, significant infiltration of eosinophils and neutrophils within 4–8 h after the instillation of IL-13 was observed (28). Since products from both neutrophils and eosinophils can induce mucin gene expression (29–31), one cannot determine convincingly whether it is IL-13 or the inflammatory cells and their products that are responsible for the mucous cell metaplasia. In airway epithelial cell cultures, IL-13 has been shown to enhance mucous cell differentiation in human nasal (32) and pig tracheal (33) epithelial cells. However, in these studies, the requirement of IL-13 treatment for 10–14 days is difficult to understand. In another recent study, IL-13 was also shown to inhibit *MUC5AC* gene expression in nasal epithelial cells (34) and had no effect in NCI-H292 cells (22).

IL-9 has also been shown to have the ability to stimulate mucous cell hyperplasia *in vivo* (35) as well as mucin gene expression *in vitro* (36, 37). However, gene knockout mice of IL-9 showed that IL-9 was necessary for mucous cell hyperplasia in a granuloma model of disease (38) but not in an allergic asthma model (39). In cell cultures, stimulation of *MUC5AC* by IL-9 could be seen (36, 37) but the cells used were undifferentiated TBE cells or cancerous cell lines. To further define the roles of cytokines in mucin gene expression, we used well differentiated primary cultures of human tracheobronchial epithelial (TBE) cells (40, 41) to determine which cytokines can stimulate *MUC5AC* and *MUC5B* expression. Our study has demonstrated that only IL-6 and IL-17, not Th2 cytokines, can directly stimulate mucin gene expression in these primary human TBE cells. A similar observation was extended to primary TBE cells derived from monkey and mouse.

MATERIALS AND METHODS

Primary Cell Culture from Human, Monkey, and Mouse Airway Tissues—Human tracheobronchial tissues were obtained from the University of California at Davis Medical Center (Sacramento, CA) by patient consent. The University Human Subjects Review Committee approved all procedures involved in tissue procurement. In this study, tissues were collected only from patients without diagnosed lung-related disease. Monkey tissues were obtained from the California Regional Primate Research Center at the University of California, Davis, CA. Transgenic mice were generated by the in-house transgenic animal facility. Primary cultures derived from these airway tissues have been established before (42). Normally, tracheobronchial epithelial (TBE) cells were plated on a collagen gel substratum-coated Transwell™ (Corning Costar, Corning, NY) chamber (25 mm) at 1–2 $\times$ 10^4 cells/cm^2, in a Ham's F12/Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (10 ng/ml), dexamethasone (0.1 μM), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 μg/ml), bovine serum albumin (0.5 mg/ml), and all-*trans*-retinoic acid (30 nM). These primary TBE cultures, after a week in an immersed cultured condition, were transferred to an air-liquid interface (biphasic) culture condition. Under biphasic culture conditions, a mucociliary epithelium with the formation of cilia and mucus-secreting granules was observed (40).

Cytokine, Antibody, and Inhibitor Treatment—Cytokines, IL-1α, 1β, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, and TNFα were purchased from R&D systems Inc. (Minneapolis, MN). They were dissolved in phosphate-based saline with 1% bovine serum albumin and added directly to the primary TBE cultures at concentrations of 10 and 50 ng/ml. For additional dosage study (as indicated under "Results"), cytokine concentration was gradually increased up to 200 ng/ml. For the IL-6 neutralizing antibody study (R&D systems Inc. Minneapolis, MN), the antibody was added to culture at 0.05, 0.1, and 0.2 μg/ml at the time of IL-17 treatment and its continuous presence was maintained until the time of harvest. For the inhibitor study, AG490, PD98059, U0126, and wortmannin were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA), and they were dissolved in Me_2SO. The dose for each of these selected inhibitors was AG490 (5 μM), U0126 (1 μM), PD98059 (25 μM), and wortmannin (10 μM). Each dose was determined to be optimal in the initial literature search and the following experimental trials.

RNA Isolation and Northern Blot Hybridization—RNA was isolated from the cultures by a single step phenol/chloroform extraction (43). For Northern blot hybridization, an equal amount of total RNA (20 μg/lane) was subjected to electrophoresis on a 1.0% agarose gel in the presence of 2.2 mM formaldehyde and transblotted onto Nytran membranes as described above (44). For human and monkey cells, single-stranded antisense oligonucleotides corresponding to the tandem repeat unit of human *MUC5B* and *MUC5AC*, 5′-TGTGGTCAGCTTTGTGAGGATCC-AGGTCGTCCCCGGAGTGGAGGAGGG-3′ (423–376 nt, GenBank™: U63836) and 5′-AGGGGCAGAAGTTGTGCTGGTTGTGGGAGCAGAG-GTTGTGCTGGTTGT-3′ (582–535 nt, GenBank™: Z34277), respectively, were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. For mouse *Muc5b* gene detection, the clone corresponding to the 3′-end sequence of mouse *Muc5b* (42) was labeled with [α-^{32}P]dCTP by a ready-to-go™ random labeling kit (Amersham Biosciences). All blots were exposed overnight to a phosphor screen and read by the STORM™ system (Molecular Dynamics, Sunnyvale, CA). The relative abundance of *MUC5B*/*MUC5AC* message in Northern blots was normalized with the 18 S ribosomal RNA (rRNA) band.

RT-PCR Analysis of MUC5AC and MUC5B Gene Expression—PCR approach was carried out to examine *MUC5AC* and *MUC5B* gene expression. cDNAs were generated from the RNA mentioned above by oligo(dT) primer. *MUC5AC* and *MUC5B* gene-specific primers were designed according to sequences retrieved from GenBank™. Specifically, for *MUC5AC,* forward primer (5′-ACCCAGATCTGCAACACAC-ACT-3′) and reverse primer (5′- GAGCGAGTACATGGAAGAGCTG-3′) were designed based on *MUC5AC* sequence (AJ001403). For *MUC5B*, forward primer (5′-ACATGTGTACCTGCCTCTCTGG-3′) and reverse primer (5′-TCTGCTGAGTACTTGGACGCTC-3′) were designed based on *MUC5B* sequence (Y09788). Each PCR reaction contained 10 μM primers for a total volume of 50 μl of PCR reaction solution. The initial denaturing step was 94 °C for 2 min, and the last elongation step was 72 °C for 7 min. These PCR reactions were all carried out the same way: denaturing at 94 °C for 30 s, annealing at 55 °C for 45 s and extension (or polymerizing) at 72 °C for 1 min per cycle. Cycle number was determined by experiment with different dilutions of the cDNA samples to avoid saturation. Ultimately 25 cycles were chosen for the PCR. β-actin band was used as an internal control. The PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide post-staining.

Generation of the MUC5B-Luciferase Transgenic Mouse and the Luciferase Assay—A chimeric construct containing the proximal 4169 bp of the human *MUC5B* promoter region (10), and a luciferase reporter gene was prepared using pGL-3 vector. Transgenic mice were generated using B6 mice from Targeted Genomics Laboratory (University of California, Davis). The transgenic positive mice were determined by Southern blot and RT-PCR. The expression profiles of the human *MUC5B* promoter-driven luciferase were consistent with mouse *Muc5b* gene expression in the mouse tissues. Two different founder mice were used for this study. For the luciferase assay, cell extracts were prepared from mouse TBE cultures and incubated with Luclite plus™ (Packard Instrument, Meriden, CT) according to the manufacturer's protocol. The relative luciferase activity was expressed with the total protein concentration after normalization. The results were averaged from triplicate dishes of two separate cultures derived from two different founder mice.

ELISA Measurement—A human IL-6 Quantikine™ ELISA kit (R&D systems Inc. Minneapolis, MN) was used to measure the secreted IL-6 concentrations in both the apical and basal media of biphasic cultured cells following the manufacturer's instructions.

Immunohistochemistry—Anti-human IL-6R antibody (R&D systems Inc., Minneapolis, MN) was used to characterize the expression of IL-6 receptors in these primary TBE cultures and various human tracheal tissue sections. The staining was carried out by using FITC-conjugated anti-mouse secondary antibody (Vector Laboratories Inc. Burlingame, CA) and Vectashield mounting medium with propidium iodide (1.5 μg/ml), following the manufacturer's instructions. The staining pictures were captured by a digital camera attached to a Zeiss fluorescent microscope.

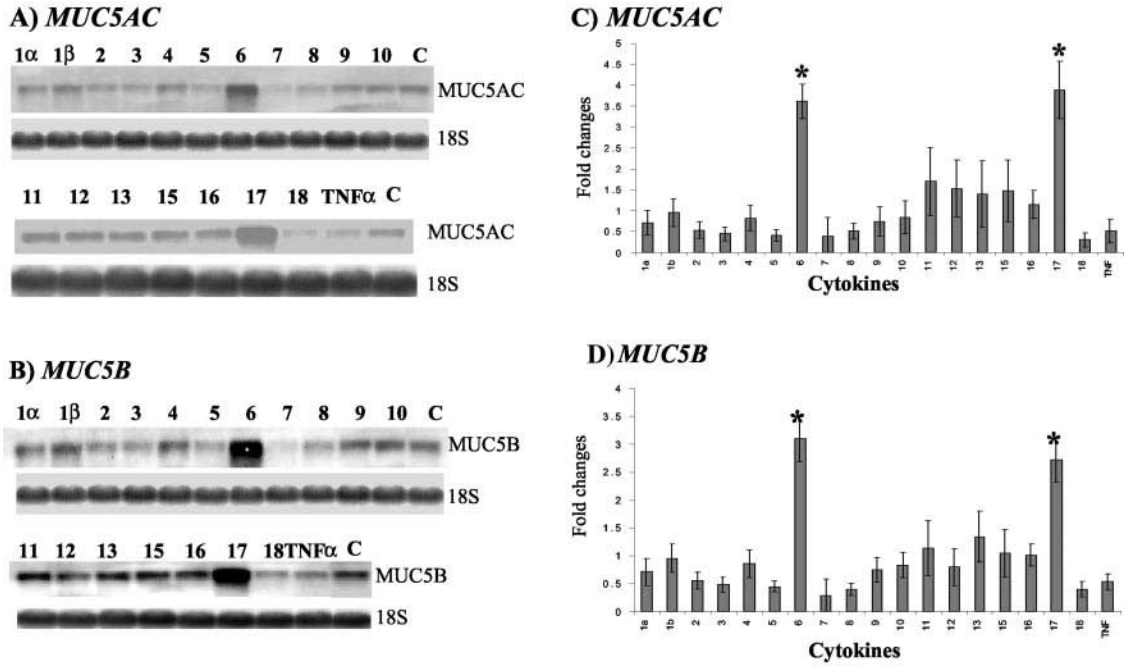

FIG. 1. **Northern blot analysis of MUC5AC and MUC5B mRNA levels in primary human TBE cells after cytokine treatment.** Primary cultures were carried out under air-liquid interface culture condition as described in the text. At day 21 after plating, cytokines (10 ng/ml) were added to both the apical and basal sides of the culture. Total RNA was collected after overnight incubation (16 h). Cytokines: IL-1α, IL-1β, IL-2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, TNFα. *C*, control. *A* and *B*, representative Northern blots for *MUC5AC* and *MUC5B* as well as 18 S ribosomal RNA, respectively, from one of these primary cultures. *C* and *D*, cumulative quantification of Northern blot analyses of *MUC5AC* and *MUC5B* mRNA levels, respectively, from three independent primary TBE cultures derived from different donors. The intensity of mucin mRNA was normalized to the intensity of the 18 S ribosomal RNA band, and the relative intensity was further normalized with the control. *C*, vehicle (phosphate-buffered saline/1% bovine serum albumin)-treated cultures. $n = 3$; *, $p < 0.01$.

Statistical Analysis—Data are expressed as mean ± S.D. The number of repeats are described under "Results" and in the figure legends. Group differences were calculated by analysis of variance. When $p < 0.01$, the difference was considered significant.

RESULTS

Effects of Various Cytokines—We treated primary tracheobronchial epithelial cells grown in an air liquid interface with a panel of cytokines (IL-1α, 1β, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, and TNFα) for 24 h. As shown in Fig. 1, our cytokine panel at 10 ng/ml showed that only IL-6 and IL-17 could induce a dramatic increase in expression of *MUC5AC* and *MUC5B*. The Th2 cytokines IL-4, IL-9, and IL-13 did not stimulate either *MUC5AC* or *MUC5B*. Treatment with a higher dose of 50 ng/ml yielded identical results (data not shown). *MUC5B*/*5AC* mRNA signals from three independent primary TBE cultures from different donors were normalized to 18 S rRNA signals and quantified. A 3–4-fold stimulation of both *MUC5B* and *MUC5AC* messages was consistently observed after an overnight (16 h) treatment by IL-6 and IL-17 (Fig. 1, *C* and *D*). The effects of IL-6 and IL-17 on mucin gene expression were dose-dependent (Fig. 2, *A* and *B*). Concentrations as low as 2 ng/ml of IL-6 or 10 ng/ml of IL-17 elicited a significant stimulation of *MUC5B* gene expression after 16 h of treatment on human primary TBE cultures. To ensure that our failure to find any induction by IL-4, IL-9, or IL-13 was not due to the generally lower sensitivity of northern blots (45), we repeated the experiments for them as well as IL-6 and IL-17 with reverse transcription polymerase chain reaction (RT-PCR). In addition, we used concentrations of up to 200 ng/ml to rule out that an inadequate cytokine concentration was a factor. As shown in Fig. 2*B*, even at 200 ng/ml, no effects on *MUC5AC* and *MUC5B* were detected in the IL-4, IL-9, or IL-13 treatments. In contrast, IL-6 showed a dose-dependent mucin-inducing activity at all concentrations up to 200 ng/ml, while IL-17 induced mucin genes at lower doses (10 and 50 ng/ml) but not at higher doses(100 and 200 ng/ml). The reason why lower levels of *MUC5AC* and *MUC5B* were seen at the higher concentrations of IL-17 is unclear. But it was apparently not due to toxicity because we routinely checked cell viability by the trypan blue dye exclusion test and found no evidence that higher levels of IL-17 were harmful to the cultures. For IL-4, IL-9, and IL-13, it was unlikely that a low level induction of *MUC5AC* or *MUC5B* occurred that could not be detected with the more sensitive RT-PCR (45). IL-2, 3, 5, 7, 8, and 18 appeared to have inhibited the expression of *MUC5AC* and *MUC5B*. These effects were not related to the cytotoxicity of the cytokines since the viability of the cells (greater than 95%) was routinely checked by the trypan blue dye exclusion test with consistently positive results.

A similar stimulation of *MUC* gene expression by these two cytokines was also seen in primary TBE cultures derived from monkey and mouse tissues (Figs. 2*C* and 4*A*, respectively). However, both animal cultures required a longer treatment time than human cells in order to see the stimulation by Northern blot analysis, which was probably related to the fact that human TBE cell culture has many mucous cells (goblet cell) while primary monkey and mouse TBE cell cultures have very few.[2]

The significance of the stimulation by these two cytokines was further supported by the dramatic increase of the Alcian blue-PAS stained cell population in primary human TBE cultures (*arrows* in Fig. 3, *A–C*), suggesting the elevation of mucous cell phenotypes in these cultures after IL-6 and IL-17 treatment. It is noteworthy to point out that throughout these studies, Th2 cytokine treatments had no stimulatory effect in these cultures (data not shown).

Stimulation of MUC5B Promoter Activity by IL-6 and IL-17—In order to further clairfy the nature of mucin gene regu-

[2] R. Wu, Y. Chen, and Y. Zhao, unpublished observations.

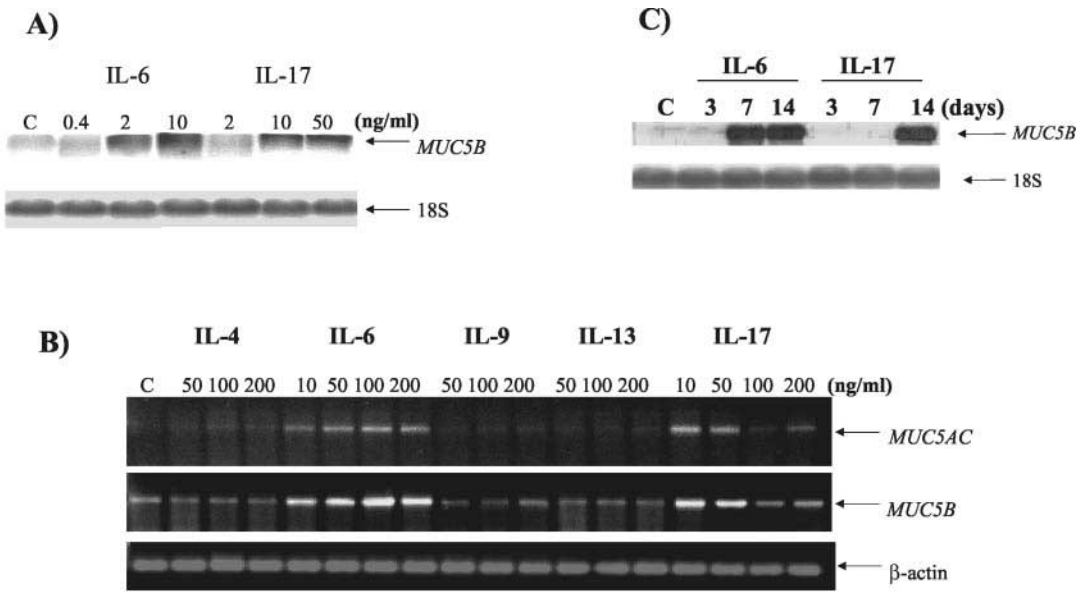

FIG. 2. **Dose- and time-dependent elevation of *MUC5B* gene expression by cytokines.** *A*, dose-dependent elevation of MUC5B message by IL-6 and IL-17, examined by Northern blot. IL-6 or IL-17 of various concentrations, as indicated, was used to treat primary human TBE cells overnight (16 h), as described in the legend to Fig. 1. *B*, IL-4, 6, 9, 13, 17 of various concentrations, as indicated, were used to treat primary human TBE cells overnight (16 h), as described in the legend to Fig. 1. Dose-dependent elevation of *MUC5AC* and *MUC5B* messages by IL-6 and IL-17 examined by RT-PCR. IL-17 had no induction at higher doses (100 ng/ml and 200 ng/ml). Notably, no inductions of *MUC5B* message were seen in cultures treated with higher levels (up to 200 ng/ml) of IL-4, 9, and 13. *C*, time-dependent elevation of *MUC5B* message in monkey TBE cells after IL-6 (10 ng/ml) and IL-17 (10 ng/ml) treatments.

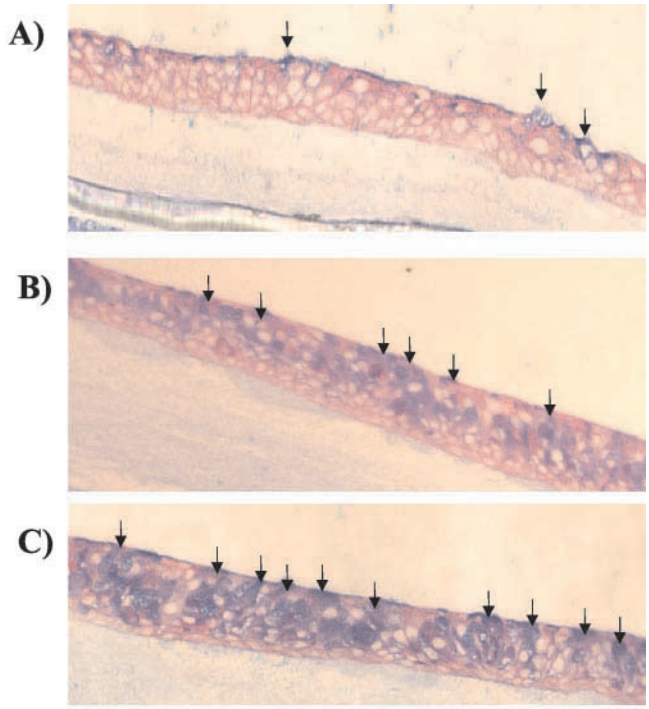

FIG. 3. **Increase of mucous cell population by IL-6 and IL-17.** *A–C*, Alcian blue/PAS staining of primary human TBE cultures after treatment with vehicle (phosphate-buffered saline/1% bovine serum albumin), IL-6, and IL-17, respectively. The *arrows* indicate "purple" cells stained positively with Alcian blue/PAS.

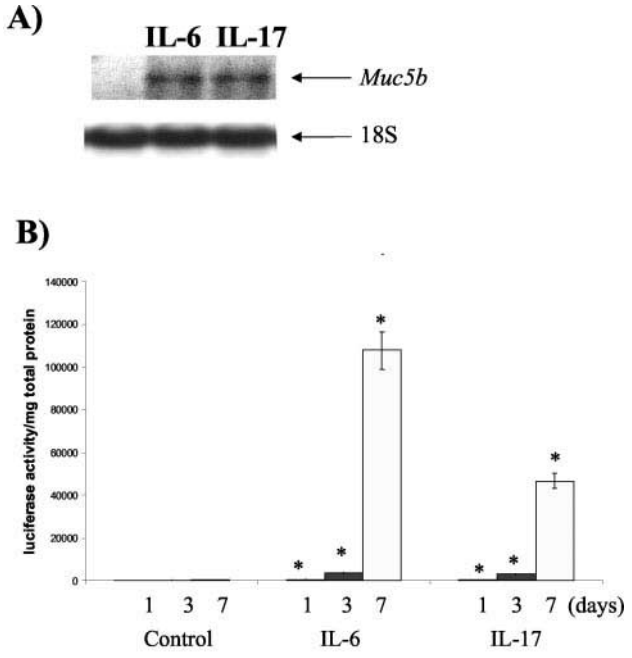

FIG. 4. **Effects of IL-6 and IL-17 on primary mouse tracheal epithelial cells in culture.** Primary tracheal epithelial (*TE*) cells were isolated from transgenic mice carrying multiple copies of *MUC5B* promoter-luciferase construct as described in the text. These TE cells were cultured under an air-liquid interface culture condition as described in the text. At day 14 after plating, cells were treated with vehicle (phosphate-buffered saline/1% bovine serum albumin) or 10 ng/ml each of IL-6 and IL-17, respectively. At day 1, 3, and 7 after cytokine treatments, cultures were harvested for luciferase activity assay, protein quantitation, and RNA isolation. *A*, representative RNA Northern blot analysis from TE cultures 7 days after cytokine treatments. *Lane 1*, control; *lane 2*, IL-6; *lane 3*, IL-17. *B*, promoter-reporter gene activity after IL-6 and IL-17 treatments. The luciferase activity was standardized to the total protein concentration. Triplicate culture chambers were used for each treatment. And the whole experiment was repeated using the TBE cells derived from another founder mouse. $n = 6$; *, $p < 0.01$.

lation, we looked into its transcriptional regulation. Because of the difficulty in transfecting well-differentiated primary human TBE cells, we examined the effects of IL-6 and IL-17 on primary cultures of mouse TBE cells derived from transgenic mice carrying multiple copies of a *MUC5B* promoter-luciferase reporter gene construct (10). These cells essentially acted as "stable-transfected" cells. We grew mouse TBE cells from two independent transgenic lines in culture and treated each of

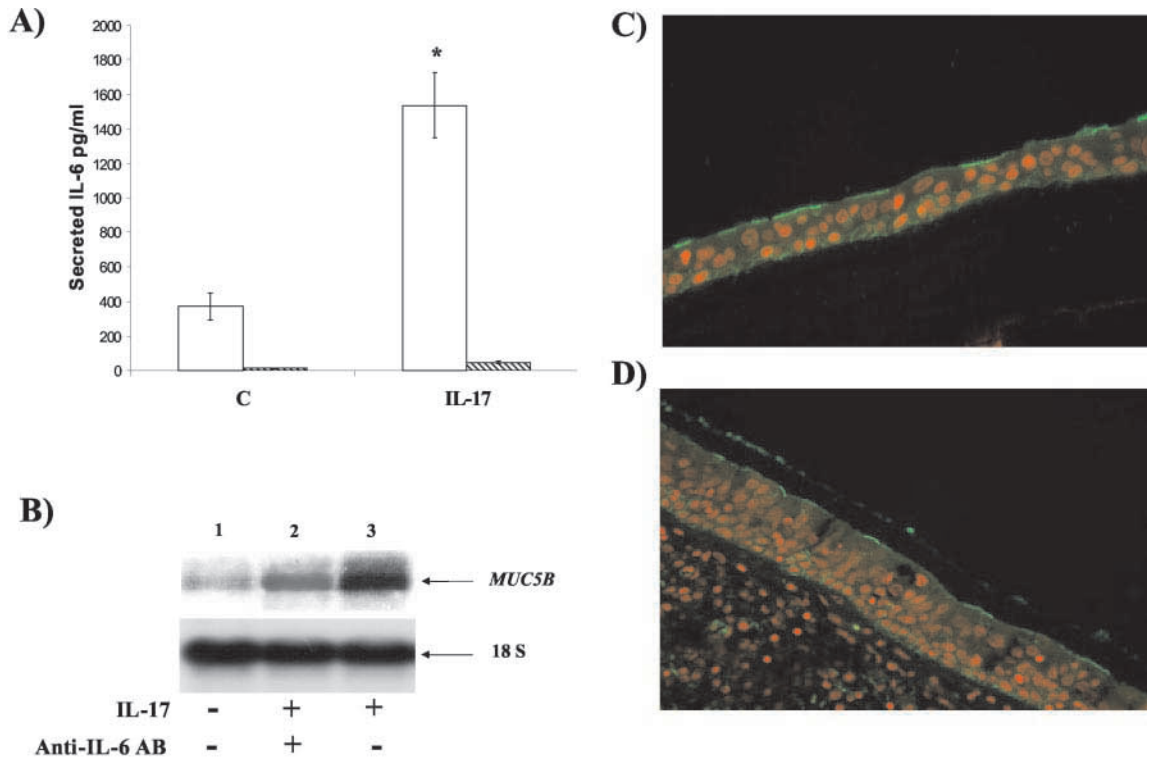

FIG. 5. **IL-17-mediated IL-6 secretion and MUC5B expression.** *A*, ELISA analysis of IL-6 secretion by human TBE cells in primary culture after IL-17 (10 ng/ml) treatment. Apical and basal media were collected after an overnight treatment (16 h). *Empty bars*, apical secretion of IL-6. *Streaked bars*, basal secretion of IL-6. Each point represents the average of triplicate dishes and a similar experiment has been repeated in three primary cultures from different donors. $n = 9$; *, $p < 0.01$. *B*, effects of IL-6 neutralizing antibody (200 ng/ml) on IL-17-mediated *MUC5B* expression. *Lane 1*, control cultures without IL-17 and antibody treatments; *lane 2*, cultures treated with both IL-6 neutralizing antibody and IL-17; *lane 3*, cultures treated with control, nonspecific serum and IL-17. *C* and *D*, immunofluorescent staining with IL-6 receptor antibody in primary human TBE culture and human tracheal tissue section, respectively. Anti-IL-6R antibody was used and stained with FITC-conjugated secondary antibody. Nuclei were stained with Vectashield mounting medium with propidium iodide (1.5 μg/ml).

these cultures with 10 ng/ml of IL-6 and IL-17. As shown in Fig. 4*B*, the background luciferase activity was very low in these TBE cultures, which was consistent with the observation that there are not many mucous cells in mouse TBE cell culture. One day after treatment with IL-6 and IL-17, there was a significant (1.6-fold) increase of the luciferase activity. This increase continued to 9- and 8-fold at day 3, and it reached 223- and 96-fold by day 7 after IL-6 and IL-17 treatments, respectively. These increases were also consistent with the Northern blot analysis of mouse *Muc5b* message in these cultures (Fig. 4*A*).

IL-17-mediated IL-6 Autocrine/Paracrine Loop—Since IL-17 is known to stimulate IL-6 secretion in bronchial epithelial cells (46), we suspected that part of the IL-17 stimulatory effect on *MUC* expression was through the IL-6 autocrine/paracrine loop. Consistent with this notion, we found that IL-17 also stimulated IL-6 production in primary human TBE cultures (Fig. 5*A*). This secretion occurred mostly at the apical side of the stimulation. By use of a neutralizing anti-IL-6 antibody, IL-17-mediated *MUC5B* expression was significantly attenuated in primary human TBE cells (Fig. 5*B*, *lane 2*). This effect was not observed in the control treatment (Fig. 5*B*, *lane 3*) when a nonspecific mouse antibody was used.

The predominantly apical secretion of IL-6 prompted us to examine if the IL-6 receptor was also present apically. By using anti-IL-6R antibody and FITC based fluorescent microscopy, we found a strong staining on the apical surface membranes of both cultured cells (Fig. 5*C*) and human tracheal tissue sections (Fig. 5*D*). These stains were apparently located in both secretory and non-secretory cell types, including a weak staining on the basal cell type. These results support the functional relevance of the apical IL-6 secretion and IL-6-mediated *MUC* expression in IL-17-treated cells.

Effects of Inhibitors on Signaling Pathways—Janus kinases (JAKs) are a family of protein tyrosine kinases known to have a major role in mediating cytokine receptor signaling (47). Because JAK/STAT and MAP kinase pathways have been shown to be activated by IL-17 and IL-6 (48–52), we want to determine if they were involved in regulating mucin expression as well. Treatment of cells with AG490, a specific JAK2 inhibitor, at 5 μM significantly diminished IL-17-mediated IL-6 secretion by 50% (Fig. 6*A*). A similar inhibition by AG490 was seen in IL-17-mediated *MUC5B* expression (Fig. 6*B*). In contrast, IL-6-mediated *MUC5B* expression was not attenuated by AG490 (Fig. 6*B*). These results suggest that IL-17-mediated IL-6 secretion is at least partly mediated through JAK2 but that IL-6-mediated *MUC5B* expression is not. The decrease in *MUC5B* induction by IL-17 in AG490-treated cells further supports the notion that part of the IL-17-mediated effects on *MUC5B* is through IL-6 action in an autocrine/paracrine loop. In order to examine the role of MAP kinase pathways, we used two inhibitors (U0126 and PD98059) of the ERK pathways. U0126 at 1 μM (Fig. 6*C*) significantly diminished *MUC5B* gene expression by both IL-6 and IL-17, while PD98059 at 25 μM had similar effects (data not shown). These inhibitors did not affect IL-17 induction of IL-6 secretion (Fig. 6*A*), but significantly attenuated *MUC5B* expression in both IL-6- and IL-17-treated cells. Because the PI-3 pathway can also be activated by JAK kinases, we also treated cells with wortmannin, an inhibitor of PI 3-kinase, and noted no inhibitory effect on *MUC5B* expression or IL-6 secretion (data not shown).

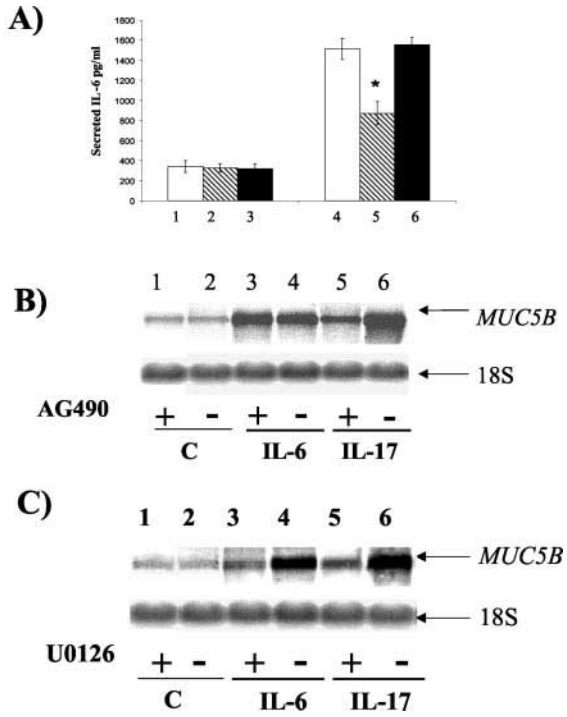

FIG. 6. **Effects of JAK2 and ERK inhibitors on IL-6 secretion and MUC5B expression.** *A*, ELISA analysis of IL-6 produced in primary human TBE cultures after IL-17 treatment. Cultured human TBE cells were treated with vehicle, AG490, and UO126, 2 h before IL-17 treatment (*lanes 4–6*, respectively). Controls (*lanes 1–3*, respectively), without IL-17 treatment, were treated with the same inhibitors. Media were collected, and IL-6 secretion was measured as described in the legend to Fig. 5. Only the apical secretions were quantified. Each point represents the average of triplicate dishes and a similar experiment has been repeated in three primary cultures from different donors. $n = 9$; *, $p < 0.01$. *B*, effects of AG490 (JAK2-specific inhibitor) on cytokine-induced *MUC5B* expression. Primary cultures were pretreated with AG490 (*lanes 1*, *3*, and *5*), then treated with IL-6 (*lanes 3* and *4*) and IL-17 (*lanes 5* and *6*). *Lane 2*, *C*, control without any treatment. *C*, effects of ERK-specific inhibitor, UO126, on cytokine-induced *MUC5B* expression. Primary cultures were pretreated with U0126 (*lanes 1*, *3*, and *5*) and then treated with IL-6 (*lanes 3* and *4*) and IL-17 (*lanes 5* and *6*). *Lane 2*, *C*, control without any treatment. Similar results treated with PD98059 were obtained (data not included).

DISCUSSION

In order to determine the direct effect of the cytokines on mucin expression and mucous cell differentiation, we chose *MUC5B*/*5AC* as our markers for the screening because of their predominant roles in airway secretion. *MUC2* was not included due to its very small contribution to airway mucus (53, 54) as well as the mucin secretion in cell culture (55). In various chronic airway diseases, *MUC5B* molecules have been shown to be present in the most tenacious portion of the mucus (7, 9, 54). Although *MUC5B* is believed to be expressed mainly in the submucosal gland, we have previously reported that it can also be expressed on the airway surface epithelial cells in patients with chronic airway diseases (10) as well as in a mouse asthma model (42). These observations suggest that the expression of *MUC5B* might be a marker of goblet cell hyperplasia and mucus hypersecretion associated with various airway diseases.

Using primary tracheobronchial epithelial cells grown in an air/liquid interface, we treated them with a panel of 19 common cytokines and found that IL-6 and IL-17 could directly stimulate *MUC5B*/*5AC* expression. This finding was reproduced in cultures of human, monkey, and mouse airway cells although the latter two required a longer treatment time. However, we could not find a stimulation of *MUC5AC* or *MUC5B* from TNFα, IL1β, and Th2 cytokines such as IL-4, IL-9, and IL-13, which have all been previously shown by other investigators to stimulate *MUC5AC* expression. We believe the discrepancy between our findings and those of other investigators may be due to the different cell culture systems used. Many other studies looking at cytokines and their effects on mucin gene expression in cell cultures made use of undifferentiated primary TBE cells and immortalized or cancerous cell lines such as NCI-H292 or A549 cells. We believe one limitation of using such cells is that morphologically, they bear very little resemblance to airway epithelial cells in the intact organism. For example, normal airway epithelial cells in animals typically have cilia with tight junctions separating the cells as well as apical and basolateral polarity (56, 57). These characteristics are present in airway epithelial cells grown under our conditions (40, 41) but are absent in the undifferentiated TBE cells grown in culture dishes and those immortalized or cancerous cell lines. For these reasons, we believe that our primary cell cultures, under an air-liquid interface culture condition, may better represent the native airway epithelium in animals for *in vitro* studies.

It is unclear why a longer treatment time was needed for IL-17 and IL-6 stimulation of *MUC5B*/*Muc5b* in monkey and mouse TBE cells. We suspect that it could be due to the fact that in the primary mouse and monkey TBE cultures, there was a paucity of mucous cells as compared with the human cell culture. Therefore, at the time of treatment, human cells may already have had a greater percentage of cells with a mucous cell phenotype ready to express mucin genes. In contrast, mouse and monkey cells may require a lag time so that the treatment will make them more capable of expressing mucin genes.

Some of the cytokines in our initial screening panel appeared to have inhibitory effects on *MUC5AC* and *MUC5B* expression. Because our main purpose was to identify cytokines that stimulated mucin gene expression, we did not further explore those that had inhibitory effects. However, it is logical to believe that physiologically, mucin gene expression may be regulated by both stimulatory and inhibitory influences. In inflammatory diseases such as asthma (58) and COPD (59), the local cytokine milieu in the airways and the balance between the stimulatory and inhibitory influences may determine the degree of mucous cell hyperplasia and mucus production.

IL-17 is a proinflammatory cytokine that is secreted primarily by T cells (60) while IL-6 is secreted by a wide variety of cells including inflammatory (*e.g.* T-cells, macrophages), stromal (*e.g.* fibroblast, smooth muscle), and epithelial cells (*e.g.* airway, renal tubular) (61). IL-17 is a 20–30-kDa polypeptide that exists as a homodimer (60). Its normal physiological role may involve recruitment of neutrophils to sites of infection (62, 63). Mice carrying knockout of the IL-17 receptor show impairment of neutrophil influx and clearance of bacteria in a Klebsiella pneumonia model (64). IL-17 may also play a role in allergic inflammatory responses. IL-17 is found in high level in a mouse model of asthma and its inhibition by an anti-IL-17 antibody reduces granulocyte influx (65). In asthmatic patients, more IL-17 positive cells have been found in sputum compared with control subjects (66). Targeted disruption of the *IL-17* gene in mice shows a reduction in both pulmonary inflammation and airway hyperreactivity in an asthma model (67). IL-17 has also been implicated in the development of rheumatoid arthritis and organ rejection (68–70). IL-6 is a 22–27-kDa polypeptide with a wide variety of physiological functions. IL-6 has pleiotrophic effects. In the lungs, IL-6 may play a role in causing subepithelial fibrosis and airway remodeling in asthma (71). To our knowledge, no studies have previously shown that IL-6 or IL-17 can directly induce mucin gene expression in cell cultures

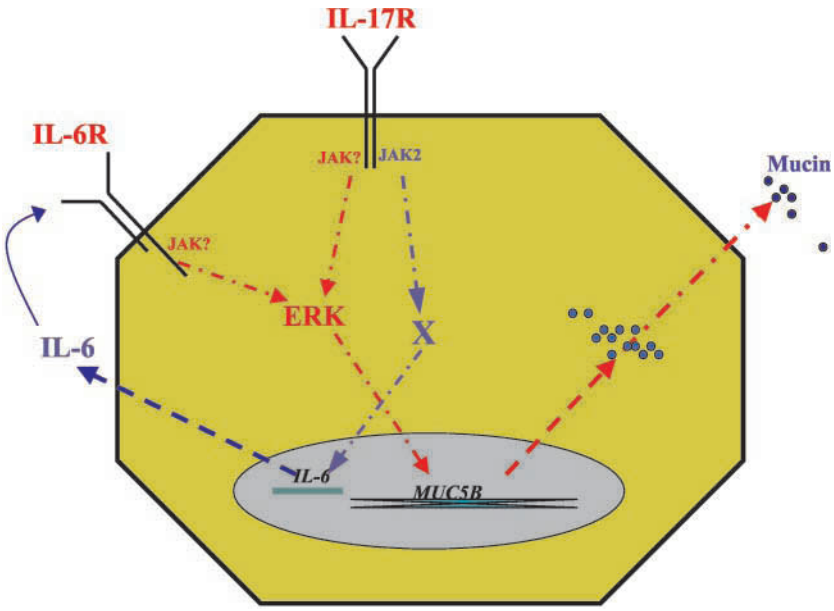

FIG. 7. **A summary of the IL-6 and IL-17 signalings on the regulation of MUC5B gene expression.** *JAK?* represents the unidentified AG490 insensitive JAK(s). *Blue lines* delineate the IL-6 autocrine/paracrine loop. *Red lines* delineate the mucin synthesis pathway. *X* represents the unidentified STAT that transduces the receptor-JAK signal into the nucleus and is involved in the transcription of *MUC5B* genes.

of airway epithelial cells. Our results add to the growing body of evidence of the important role cytokines play in regulating mucin gene expression. We believe that the ability of IL-6 and IL-17 to induce mucin gene expression could play a role in the mucous cell metaplasia as seen in various airway diseases characterized by profound inflammation. IL-17 in particular is most notable for its ability to recruit neutrophils to sites of inflammation. It would be tempting to speculate whether it may play a role in diseases characterized by neutrophilic infiltration such as COPD and cystic fibrosis. Furthermore, because IL-17 is secreted mainly by T-cells, it may also be involved in regulation of mucus secretion in diseases such as asthma where sensitivity and reaction to environmental antigens occur. Future studies focusing on measuring this cytokine in clinical samples or animal models might shed some light on these issues.

In this study, we further studied *MUC5B* to determine how IL-17 and IL-6 could effect mucin gene expression. We decided to focus on *MUC5B* for two reasons. First, its expression could be elevated dramatically in the epithelium of the human chronic airway disease (10) and mouse disease model (42), and second, its induction by cytokines was surprising and a novel finding to us. Mucin gene expression can be regulated either by the rate of its transcription or the stability of its RNA. The use of *MUC5B*-luciferase transgenic mouse tracheal epithelial cells clearly demonstrated that both IL-6 and IL-17 could directly regulate *MUC5B* expression at the promoter level. Since mucin RNA messages are relatively stable in cultured cells (72), our studies showing the early time of stimulation of the *MUC5B* gene in human cells as well as the stimulation of its promoter in transgenic mouse cells support a transcriptional mechanism for the role of IL-6 and IL-17 in mucin gene expression.

Since IL-17 is known to be capable of causing IL-6 secretion in many cell types (46), it was possible that IL-17's affect on *MUC5B* gene expression was mediated through locally secreted IL-6 acting in a autocrine/paracrine manner. Indeed, we showed that IL-17 could induce IL-6 secretion in human airway epithelial cells. Moreover, treatment of IL-17-treated cells with an anti-IL-6 antibody blocked *MUC5B* gene expression suggesting that IL-17's effect was partly mediated through IL-6 (Fig. 7).

To determine what signal transduction mechanisms were involved in IL-17 or IL-6 receptor binding, we did some studies looking specifically at the JAK/STAT and the MAP kinase pathways. Four JAK family kinases, JAK1, 2, 3, and Tyk2 have been reported (73). IL-17 has been reported to activate JAK/STAT proteins (48) as well as MAP kinase (49, 50) in U937 and renal epithelial cells. The IL-6 receptor has been shown to interact with JAK1, 2, and Tyk2 in various cell types (74). We demonstrated that IL-17 could stimulate IL-6 secretion and the stimulation was attenuated by AG490, a JAK2 inhibitor, suggesting a JAK2-dependent pathway is involved in the regulation of IL-6 production. In addition to the IL-6 secretion effect, AG490 also suppressed IL-17-stimulated *MUC5AC/MUC5B* expression. This effect is consistent with the treatment of IL-6 neutralizing antibody on IL-17-treated cells. Taking these data together, it is suggested that a part of IL-17-stimulated *MUC5AC/MUC5B* expression is through a JAK2-dependent IL-6 autocrine/paracrine loop (Fig. 7).

Stimulation of *MUC5B* by IL-6 was not affected by AG490, suggesting it uses a different signaling pathway. To determine if MAP kinase pathways may have been involved, we used U0126 and PD98059, specific inhibitors of ERK signaling. Both inhibitors reduced IL-17 and IL-6 stimulation of *MUC5B* but neither had an effect on IL-17-mediated IL-6 secretion. This suggests that ERK signaling is required distal to IL-6 signaling but is not required for IL-17's effect on IL-6. We also looked to see if the PI-3 pathway could be involved as it has been shown to act distally to JAK kinases. However, wortmannin, a PI-3 pathway inhibitor, had no effect on either IL-6 secretion by IL-17- or IL-6-mediated *MUC5B* expression. Thus IL-6 and IL-17's effect is likely independent of the PI-3 pathway. The signaling pathway is summarized in Fig. 7. Since other JAK and MAP kinase pathways exist that were not evaluated in this study, further studies will be needed to define their potential roles in IL-6- and IL-17-mediated mucin gene expression.

In summary, after screening 19 cytokines, we have identified two cytokines (IL-6 and IL-17) that have direct stimulatory effects on *MUC5AC* and *MUC5B* gene expression in primary airway epithelial cells. We further demonstrated that IL-17 mediates its effect on *MUC5B* partly through IL-6 by acting in an autocrine/paracrine manner and that JAK2 may be involved

in the signaling events. Furthermore, IL-6 and IL-17's effect on *MUC5B* may depend on ERK signaling pathways. Future studies should address whether other MAP kinase and JAK/STAT pathways are also involved in IL-6- or IL-17-mediated gene expression in airway epithelial cells. More experiments will also be needed to determine whether these two cytokines play a role in the mucous cell metaplasia seen in chronic airway diseases.

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