Tumor Necrosis Factor-α Triggers Mucus Production in Airway Epithelium through an IκB Kinase β-dependent Mechanism*

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Excessive mucus production by airway epithelium is a major characteristic of a number of respiratory diseases, including asthma, chronic bronchitis, and cystic fibrosis. However, the signal transduction pathways leading to mucus production are poorly understood. Here we examined the potential role of IκB kinase β (IκKB) in mucus synthesis in vitro and in vivo. Tumor necrosis factor-α (TNF-α) or transforming growth factor-α (TGF-α) stimulation of human epithelial cells resulted in mucus secretion as measured by MUC5AC mRNA and protein. TNF-α stimulation induced IκKB-dependent p65 nuclear translocation, mucus synthesis, and production of cytokines from epithelial cells. TNF-α, but not transforming growth factor-α, induced mucus production dependent on IκKB-mediated NF-κB activation. In vivo, TNF-α induced NF-κB as determined by whole mouse body bioluminescence. This activation was localized to the epithelium as revealed by LacZ staining in NF-κB-LacZ transgenic mice. TNF-α-induced mucus production in vivo could also be inhibited by administration into the epithelium of an IκKB dominant negative adenovirus. Taken together, our results demonstrated the important role of IκKB in TNF-α-mediated mucus production in airway epithelium in vitro and in vivo.

The airway epithelium is the first layer of cellular interaction with airborne antigens and plays a key role in initiating allergic responses. Research over the last decade has established a critical role for the epithelium not only by providing a physical boundary but also in transducing and integrating signals that help mount optimal responses to a wide variety of insults (1–3). Airway mucus is a highly hydrated glycoprotein exhibiting a gel state and, in combination with the ciliated cells in the airways, constitutes a mucociliary clearance system critical for protection of the respiratory tract (4). The apoprotein component of mucus is the product of a family of mucin genes (generally referred to as MUC genes), of which MUC1, -2, -3, -4, -5AC, -5B, and -7 have been shown to be expressed in airway epithelium (5). Both MUC2 and MUC5AC expression have been shown to be highly regulated (6). Excessive mucus production is a common feature of a wide variety of respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis, and constitutes a significant unmet clinical need given the lack of appropriate and effective therapies for decreasing mucus production.

Despite the clinical importance of excessive mucus production, the signal transduction pathways leading to its synthesis and release from the epithelium are poorly understood. Tumor necrosis factor-α (TNF-α) is produced by diverse cell types, including macrophages, lymphocytes, fibroblasts, and keratinocytes (7, 8). TNF-α is rapidly produced in response to inflammation, infection, and tissue injury and triggers a broad array of systemic and cellular responses. Through binding to its major receptor, TNFR1, TNF-α activates several intracellular signal transduction cascades, among which the nuclear factor-κB (NF-κB) pathway is of pivotal importance. In its canonical form, NF-κB is a heterodimer composed of the p50 and p65 proteins and is held in an inactive form in the cytoplasm of cells by the inhibitor of the NF-κB protein, IκBα. Upon TNFR1 engagement, a number of docking proteins are recruited to its intracellular domain, leading to the activation of the IκB kinase (IκK) complex (composed of IκKα, IκKβ, and NEMO). Subsequently, IκKβ phosphorylates IκBα at two critical serine residues, which leads to its proteasome-mediated degradation and release of the NF-κB complex. NF-κB then translocates to the nuclear compartment and acts as a transcription factor regulating the expression of numerous genes (9, 10).

There is limited information linking NF-κB and mucus production. Proximal regulatory regions from MUC2 and MUC5AC genes have been cloned and characterized in detail (11, 12). Sequence analyses of these regions have indicated the presence of NF-κB binding sequences, and electromobility shift assays and supershift experiments with specific antibodies have demonstrated binding of p50/p65 complexes to these sequences upon stimulation of cell cultures with Pseudomonas aeruginosa-derived LPS. NF-κB has been shown to play an important role in mucus production in mouse models of allergic lung inflammation (13, 14). Cell lines transfected with MUC2 reporter constructs have also suggested a role for NF-κB in LPS-induced mucus production in a pathway involving Ras-MAPK-pp90/S6K (15). Along similar lines, p38 MAPK has been shown to play a role in MUC5AC up-regulation upon stimulation of normal human nasal epithelial cells and human epithelial cell lines with IL-1, TNF-α, and cytoplasmic proteins of nontypeable Haemophilus influenzae (16–18). TGF-α-mediated EGFR tyrosine phosphorylation has also been found to play an important role in mucus elaboration in human airway epithelial cells (19–21). Moreover, studies where TNF-α and TGF-α were used in combination to stimulate NCI-buffered saline: X-gal, 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside; pfu, plaque-forming units; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; IL, interleukin.

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‡ The abbreviations used are: COPD, chronic obstructive pulmonary disease; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; IκK, IκB kinase; GFP, green fluorescent protein; WT, wild type; DN, dominant negative; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside; pfu, plaque-forming units; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; IL, interleukin.

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NF-κB-mediated Mucus Production in Lung Epithelium

H292 human lung epithelial cells showed an increased level of mucin protein synthesis compared with either cytokine alone, and a putative cross-talk between both signaling pathways has been hypothesized (19).

In the present work we have investigated the role of IKKβ in transducing the pro-inflammatory signal delivered by TNF-α and TGF-α to trigger mucus production by airway epithelial cells. Following a combination of cell culture and in vivo experiments, we show the following: first, TNF-α induces mucin and cytokine production and activates NF-κB in a human lung epithelial cell line; second, IKKβ is required for TNF-α-induced mucin and cytokine production but not for TGF-α-dependent mucus production by this human lung epithelial cell line; and third, TNF-α activates NF-κB in mouse airway epithelium in vivo and leads to goblet cell metaplasia and mucous production in an IKKβ-dependent manner.

MATERIALS AND METHODS

Cell Culture—NCI-H292 cells (a human pulmonary mucociliary carcinoma cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Hepes (25 mM) at 37 °C in a humidified 5% CO₂ water-jacketed incubator. When 60–80% confluent, cells were incubated with human TNF-α (PeproTech 300–01A) for 30 min to 24 h.

Adenoviral Infection Studies—Adenoviral vectors were prepared as described (22); gene expression was driven by the cytomegalovirus promoter. NCI-H292 cells were subcultured into 4-well chamber slides overnight to adhere. Each well was seeded with 2 × 10⁵ cells. When the cultures turned 60–80% confluent, they were washed with OPMI medium and incubated in OPMI medium for 1 h, and the following adenoviral vectors added: GFP vector alone (2 × 10⁵ pfu/ml), IKKβ DN (2 × 10⁶ pfu/ml), and IKKβ WT (2 × 10⁶ pfu/ml) for 1 h at 37 °C. After 1 h of infection, wells were washed with the culture medium and replaced with growth medium overnight. 16–20 h later, culture medium was replaced and replaced with fresh medium with or without TNF-α (50 ng/ml).

Immunofluorescence Staining—Cells were fixed in 2% paraformaldehyde for 15 min or with a 10% low odor formalin solution for 30 min, treated with 0.2% Triton X-100 in TBS (20 mM Tris base, 154 mM NaCl, pH 7.4) for 20 min, blocked with 10% HISS in TBS for 1 h at room temperature, and washed with TBST (0.1% Tween 20 in TBS) for 15 min, and then incubated with rabbit polyclonal anti-p65 antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or MUC5AC monoclonal antibody (1:1000 dilution, NeoMarkers, Inc., Fremont, CA) for 1 h at room temperature. Cells were then washed and incubated with a Cy3-conjugated anti-rabbit IgG (1:300 dilution, Jackson ImmunoResearch, West Grove, PA) or fluorescein isothiocyanate-conjugated anti-mouse IgG + IgM (1:200 dilution, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Cells were washed and stained with Hoescht nuclear dye for 3 min at room temperature, mounted with coverslips, and analyzed under the fluorescence microscope using the MetaMorph 6.0 software package (Universal Imaging Corp. Ltd., Marlow, Buckinghamshire, UK).

Immunohistochemistry—Cells were fixed with a 10% low odor formalin solution for 30 min, permeabilized with 0.1% Tween 20 in PBS for 10 min, and peroxidase-blocked in 0.3% H₂O₂ containing 1% horse serum for 5 min. For color development, an AEC substrate kit for peroxides from Vector Laboratories ( Burlingame, CA) (SK-4200) and ABC kit (Vector PK-6102) were used. Counter stain was Mayer’s hematoxylin (Polyscientific, Bay Shore, NY, S216/160z).

RNA Isolation/cDNA Preparation—Resting and activated NCI-H292 cells were lysed in 2 ml of lysis buffer containing guanidine isothiocyanate (RLT buffer) with β-mercaptoethanol (10 μl/ml). Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer’s protocol. DNA was degraded using Ambion DNase I at 37 °C for 30 min followed by 75 °C heat inactivation of the enzyme for 5 min. The RNA was purified by ethanol precipitation and then reverse-transcribed by using the Qiagen Omniscript reverse transcriptase kit using oligo(dt) primers and random hexamers in accordance with the manufacturer’s protocol. After reverse transcription, any remaining RNA was digested by adding 1 μl of RNase H, and the mixture was incubated at 37 °C for 20 min. The cDNA was diluted 1:10, and 5 μl was used for real time PCR analysis.

Real Time PCR—mRNA expression was assessed by quantitative PCR (TaqMan®, PE Applied Biosystems, Foster City, CA). Total RNA was isolated using the Qiagen RNeasy kit, and reverse transcription was performed as described above. An oligonucleotide probe was designed to anneal to the gene of interest between two PCR primers. The probe was then fluorescently labeled with 6-carboxyfluorescein (reporter dye) on the 5’ end and 6-carboxytetramethylrhodamine (quencher dye) on the 3’ end. A similar probe and PCR primers were designed for β₂-microglobulin (housekeeping internal control gene). The probe for this gene incorporated VIC as the reporter dye. PCRs were run that included the primers and probes for these two genes, as well as the input cDNA. As the polymerase moves across the gene during the reaction, it cleaves the reporter dye from one end of each probe, which causes a fluorescent emission that is measured by the ABI Prism 7700 sequence detector. The emissions recorded for each cDNA can then be converted into the level of expression for the gene normalized to the expression of β₂-microglobulin. Sequences for MUC5AC transcript were as follows: forward primer 5’-GGCAACAATGTCACTCCTTGAG-3’, reverse primer 5’-TCAGAGGTGGGTAACCATTGG-3’, and probe 5’-TGCCCAGAGCTGAATC57BL/6 mice and NF-κB-LacZ transgenic mice (23) were maintained in a temperature-controlled room with standard laboratory food and water freely available. Mice were lightly anesthetized with isofluorene, and mouse TNF-α (5 μg/50 μl/mouse) was intranasally delivered on days 1–3. 24 h after the third dose, animals were euthanized, and lungs were harvested.

Tissue Preparation—For frozen sections, tissues were removed, placed in dry ice, and stored at −80 °C. Tissues were embedded in optimal cutting temperature compounds, cryostat-sectioned to 20 μm, and stored at −80 °C. For Alcian Blue/periodic acid-Schiff and hematoxylin and eosin staining, tissues were fixed in 10% low odor formalin. Mice were lightly anesthetized with isofluorene, and mouse TNF-α (5 μg/50 μl/mouse) was intranasally delivered on days 1–3. 24 h after the third dose, animals were euthanized, and lungs were harvested.

Immunofluorescence Localization of p65 in Mouse Lung Epithelium—Frozen sections of mice lung were processed essentially as described for the in vitro studies.

β-Galactosidase Staining—After cryostat cutting of frozen lungs, sections were air-dried at room temperature for 15 min, and then the slides were washed with 1× PBS and fixed in 2% paraformaldehyde, 0.2% glutaraldehyde for 5 min. After fixation, slides were washed three times with PBS, and X-gal staining solutions (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, and 1 mg/ml X-gal in PBS) were added. The counterstain was eosin Y.
**Bioluminescence of NF-κB in Vivo**—Adenovirus (2 × 10^8 pfu) carrying an NF-κB-luciferase reporter construct (kind gift from Aventis Pharmaceuticals) along with either GFP or IKKαDN adenoviruses (1 × 10^9 pfu) in 50 μl of PBS were intranasally delivered to isoflurane-anesthetized BALB/c female mice at 6–8 weeks of age. Three days later, mice were challenged with 0.5 g/40 μl/mouse of TNF-α in PBS given intranasally. After 2 h of stimulation, luciferin at a dose of 150 mg/kg in 150 μl of PBS was administered intraperitoneally for 10 min. NF-κB-luciferase activity was captured as images and as emitted total photons/min using the IVIS200 imaging system (Xenogen, Alameda, CA).

**Bioluminescence of MUC5AC in Vivo**—The promoter region from −1100 to −1 of the human MUC5AC gene was PCR-amplified from genomic DNA and cloned into a vector derived from pMCS-luciferase (Stratagene, La Jolla, CA) in which the minimal TATA sequence had been removed. To generate the MUC5AC adenoviral reporter construct, the entire promoter/luciferase/poly(A) transcription cassette was excised from this vector as a BamHI restriction fragment, blunt-ended using T4 DNA polymerase, and cloned into XmnI/EcoRV-digested pENTR1A (Invitrogen). The transcription cassette was then introduced into pAd/PL-DEST (Invitrogen) via an LR clonase reaction. MUC5AC-luciferase adenoviral vector plasmid was linearized by SwaI digestion and sent to ViraQuest (North Liberty, IA) for large scale growth and CsCl purification of recombinant virus. Adenoviruses (1 × 10^9 pfu) carrying the MUC5AC-luciferase reporter in 50 μl of PBS were intranasally delivered to isoflurane-anesthetized BALB/c female mice at 6–8 weeks of age. Three days later, mice were challenged for 3 consecutive days with 50 μl of PBS or 5 μg/50 μl/mouse of TNF-α or IL-13 in PBS given intranasally. 24 h after the last challenge, luciferin at a dose of 150 mg/kg in 150 μl of PBS was administered intraperitoneally for 10 min. MUC5AC-luciferase activity was captured as images and as emitted total photons/min using the IVIS200 imaging system (Xenogen).

**RESULTS**

TNF-α Induces Mucin and Cytokine Production by NCI-H292 Human Lung Epithelial Cells—In order to investigate if mucus and/or cytokine production can be modulated by TNF-α or TGF-α in human lung epithelial cells, NCI-H292 cells were cultured in the presence of TNF-α (50 ng/ml) or TGF-α (50 ng/ml) alone or in combination for 12 hours.
or 24 h, and RNA and protein levels for MUC5AC and several cytokines were measured (Fig. 1). Real time PCR showed that both TNF-α and TGF-α induced steady-state MUC5AC transcript accumulation by 12 h (Fig. 1A), with no further increase at 24 h (data not shown). TGF-α and TNF-α stimulation exerted an additive effect on this process (Fig. 1A). Similarly, MUC5AC protein levels increased with TNF-α and TGF-α stimulation, and this response was enhanced when TGF-α was used in combination with TNF-α, as revealed by immunohistochemistry (Fig. 1B). TNF-α, alone or in combination with TGF-α, also induced IL-6 and IL-8 production (Fig. 1C). Most interestingly, TGF-α was much less efficient than TNF-α in inducing cytokine secretion. Indeed, TGF-α failed to induce IL-6 and modestly increased IL-8 secretion.

**TNF-α Activates NF-κB and MUC5AC Synthesis in NCI-H292 Cells**—Because TNF-α is a potent activator of the NF-κB pathway, we used an anti-p65 antibody to follow its expression and intracellular trafficking in human lung epithelial cells. Immunofluorescence experiments demonstrated high expression of p65 in resting NCI-H292 cells (Fig. 2), with the protein homogeneously distributed throughout the cytoplasm and absent from the nucleus. However, upon TNF-α treatment, this pattern shifted, and p65 was found primarily around or within the nucleus (Fig. 2, purple dots, resulting from merging red and blue channels). Nuclear translocation was clear after 1 h (data not shown) and was maximal after 3 h (Fig. 2). From these experiments we conclude that NCI-H292 cells have a functional NF-κB signaling pathway, and this pathway can be activated by TNF-α. Moreover, expression of MUC5AC was concomitant with NF-κB activation. As shown in Fig. 2, at 3 h of TNF-α stimulation (maximal NF-κB activation), the MUC5AC protein starts to be detected in the cytoplasm (Fig. 2, green channel).

**NF-κB Is Required for TNF-α-induced, but Not TGF-α-induced, Mucin Production in NCI-H292 Cells**—In order to determine whether NF-κB is functionally required for TNF-α- and/or TGF-dependent mucin production in the lung epithelium, we undertook adenoviral

![FIGURE 2. p65 and MUC5AC immunofluorescence analysis in NCI-H292 cells. Nuclei are shown in blue after staining with the Hoechst 33342 dye, which binds to DNA. The secondary antibody for p65 was Cy3-conjugated (revealed through the red channel) and for MUC5AC was fluorescein isothiocyanate-conjugated (revealed through the green channel). Purple dots represent co-localization of DNA and p65 and are the consequence of merging the red and blue colors.](http://www.jbc.org/)

![FIGURE 3. IKKβ dominant negative adenovirus inhibition of TNF-α-induced but not TGF-α-induced MUC5AC up-regulation in NCI-H292 cells.](http://www.jbc.org/)
mediated gene delivery experiments using mutant forms of IKKβ to block specifically the NF-κB-dependent gene transcription. Adenoviral vectors harboring dominant negative (DN, K44M) or wild type (WT) forms of IKKβ or a GFP control construct were used. Only cultures with 75–80% infected cells were used in subsequent analyses (as determined by GFP expression, not shown). The experiments were done in triplicate, and representative pictures are shown in Fig. 3A. IKKβ DN delivery almost completely abrogated MUC5AC expression in TNF-α-stimulated cells (Fig. 3A, compare panels 2 and 6). However, IKKβ DN had no effect on TGF-α-induced mucin elaboration (Fig. 3A, panels 3 and 7). Transduction of IKKβ DN to TNF-α+ TGF-treated cells resulted in a diminution of MUC5AC-positive cells to levels similar to those detected in TGF-only stimulated cells (Fig. 3A, panels 4 and 8). Fig. 3A, panel B, shows the quantification of the images shown in panel A (in triplicate). These data strongly suggest a role for NF-κB in TNF-α-induced but not in TGF-α-induced mucus production. Furthermore, unlike TNF-α, TGF-β fails to activate NF-κB in NCI-H292 cells, as determined by p65 nuclear translocation experiments (Fig. 4).

NF-κB Mediates Cytokine Production in TNF-α-stimulated NCI-H292 Cells—Supernatants from adenoviral transfer experiments were collected and protein levels for IL-6 and IL-8 measured (Fig. 5). Interference with NF-κB activation (IKKβ DN delivery) resulted in a decrease in IL-6 and IL-8 secretion. Conversely, enhancement of NF-κB activation by overexpression of IKKβ (IKKβ WT delivery) was followed by an increase in cytokine production.

TNF-α Activates NF-κB in Airway Epithelium in Vivo—In order to determine whether TNF-α was sufficient for NF-κB activation in lung epithelium, we next used bioluminescent imaging in vivo. TNF-α delivery to the lungs of animals that had been infected with an NF-κB-luciferase reporter adenovirus resulted in a 5-fold increase in bioluminescence as compared with PBS-treated animals (Fig. 7A). As expected, IL-13 administration led to an increase in MUC5AC-driven luciferase activity. Therefore, TNF-α is sufficient to engage MUC5AC gene expression in mouse lung epithelium. We next delivered TNF-α to mice intranasally for 3 consecutive days, and lung sections were prepared and stained for Acanth Blue/periodic acid-Schiff. Several independent experiments were carried out, with a total of 17 mice analyzed. Representative views are presented in Fig. 7, B and C. As shown in Fig. 7B, airways from saline-treated mice were Acanth Blue/periodic acid-Schiff-negative. However, epithelial cells from TNF-α-treated mice were characterized by mucin production and goblet cell metaplasia (Fig. 7C).

IKKβ-dependent Mucus Production in Airway Epithelium in Vivo—The observation of TNF-α-induced p65 nuclear translocation, NF-κB activation, and mucus production by epithelial cells prompted us to determine whether NF-κB was required for mucus production in vivo. To tackle that question, we administered TNF-α intranasally for 3 consecutive days to mice treated with a control adenovirus (GFP) or an IKKβ DN adenovirus. Lungs from animals after saline treatment were periodic acid-Schiff-negative (Fig. 7D, upper panel). Airway epithelium from TNF-α-treated GFP-infected mice developed a response that included mucus production by airway epithelial cells (Fig. 7D, middle panel). However, no mucus was detected upon TNF-α provocation in IKKβ DN adenovirus-treated ani-
NF-κB-mediated Mucus Production in Lung Epithelium

**DISCUSSION**

The epithelium lining the airways has evolved to respond to viral and bacterial infections by mounting an immune response characterized by the release of cytokines and chemokines and the production of mucus to provide additional protection. However, in cases of chronic inflammation, secondary tissue damage can be substantial, and excessive mucus production can compromise normal lung function. In fact, mucus overproduction is a common and pervasive clinical problem in COPD, and cystic fibrosis. Therapies have yet to be developed. The notion of NF-κB activation in lung epithelium after TNF-α intranasal delivery, using the imaging system from Xenogen, bioluminescent images of BALB/C mice were treated with either PBS or TNF-α. Mice were infected intranasally with NF-κB-luciferase adenoviruses along with either GFP or IKKβ DN adenoviruses for 3 days. NF-κB luciferase activities were measured as emitted total photons/min and were graphed as average value per group with standard deviation. 24 h after receiving the third of three daily intranasal doses of saline (C) or TNF-α (D) to NF-κB-LacZ transgenic mice, lungs were harvested and frozen. β-Galactosidase-positive cells (blue) were revealed by X-gal staining. E, kinetics of TNF-α-induced NF-κB nuclear translocation in airway epithelium in vivo. C57BL/6 naive mice received one single intranasal dose of saline or TNF-α and were sacrificed at the indicated times after challenge. Lungs were collected, frozen, and assessed for p65 localization. White arrows indicate representative areas of p65/DNA co-localization.

**FIGURE 6.** NF-κB activation in lung epithelium after TNF-α intranasal delivery. A, using the imaging system from Xenogen, bioluminescent images of BALB/C mice were treated with either PBS or TNF-α. Mice were infected intranasally with NF-κB-luciferase adenoviruses along with either GFP or IKKβ DN adenoviruses for 3 days. B, NF-κB luciferase activities were measured as emitted total photons/min and were graphed as average value per group with standard deviation. 24 h after receiving the third of three daily intranasal doses of saline (C) or TNF-α (D) to NF-κB-LacZ transgenic mice, lungs were harvested and frozen. β-Galactosidase-positive cells (blue) were revealed by X-gal staining. E, kinetics of TNF-α-induced NF-κB nuclear translocation in airway epithelium in vivo. C57BL/6 naive mice received one single intranasal dose of saline or TNF-α and were sacrificed at the indicated times after challenge. Lungs were collected, frozen, and assessed for p65 localization. White arrows indicate representative areas of p65/DNA co-localization.

mals (Fig. 7D, lower panel). These results provide compelling evidence of a pivotal role for NF-κB in controlling TNF-α-induced mucus production in airway epithelium in vivo.

Several epithelial cell lines, including NCI-H292, HT29-MTX, Calu-3, and HM3, have been used to study mucus elaboration in cell culture systems (15, 16, 19, 24, 25). These studies showed a potential for a number of stimuli to induce mucus production. TNF-α induces MUC5AC protein expression by HT29 cells in a dose-dependent manner, with a peak at about 20 ng/ml (24). In studies carried out with NCI-H292 cells, Takeyama et al. (19) provided data showing mucin up-regulation by EGFR ligands, such as TGF-α. Moreover, pharmacological inhibition with specific tyrosine kinase inhibitors suggested that tyrosine phosphorylation events downstream of EGFR engagement were necessary for TGF-α-induced mucin up-regulation. These studies also suggested that TNF-α triggered this response presumably by up-regulating the expression of EGFR (19, 26). However, in vivo evidence of this proposed pathway is not firmly established, and the actual signal transduction pathway presumably engaged by tyrosine phosphorylation and leading to mucus production remains unknown. Our results have confirmed the observation of enhanced mucus production by NCI-H292 cells when both TNF-α and TGF-α are used to stimulate the cells. In addition, we show that TNF-α alone is sufficient to induce mucin up-regulation in NCI-H292 cells and that this process is mediated by IKKβ. There have been recent reports providing evidence of cross-talk between the NF-κB and EGFR pathways. Hirota et al. (27) report enhanced TNF-α-dependent NF-κB activation by overexpression of EGFR in NIH3T3 cells. Moreover, Wu et al. (28) further support the notion of NF-κB/EGFR pathway interactions based on their experiments using BEAS-2B airway epithelial cells. In the context of mucus production, the TGF-α/Smad pathway has been shown to interact with the NF-κB pathway to mediate H. influenzae-induced MUC2 expression (29), and EGFR has been shown to induce MUC2 and MUC5AC expression through Ras and Sp1 (30). To our knowledge, there are no reports addressing the specific molecular mechanism that mediates the putative NF-κB/EGFR interaction to enhance mucus production in airway epithelial cells. Our results demonstrate a critical role for IKKβ to...
mediate TNF-α-induced mucus production both in vitro and in vivo, and provide an experimental framework to address the molecular mechanism of TNF-α-induced mucus production in lung epithelium. We also demonstrate that TGF-β-induced mucus production is completely independent of NF-κB activation, at least in NCI-H292 cells. We show evidence of NF-κB nuclear translocation and MUC5AC message and protein accumulation upon TNF-α stimulation of NCI-H292 cells. Moreover, we were able to block MUC5AC protein expression in these cells by delivering a dominant negative form of the IKK protein. Previous studies with reporter constructs and kinase inhibitors provide indirect evidence for an LPS-induced mucin expression and NF-κB activation in NCI-H292 and HM3 cell lines (15). In this system, LPS presumably acts through the toll-like receptor 4-CD14 complex, which is known to activate NF-κB (10). Our data further support a role for IKKβ in MUC5AC expression. Furthermore, we also show that NCI-H292 cells also produce cytokines and chemokines after TNF-α stimulation and that this response is also mediated by IKKβ.

Because NF-κB has a profound effect in immunity and inflammation (10, 31), its particular role in specific conditions has been tested in vivo in animal models. Experiments carried out with p50-deficient animals have established a pivotal role for NF-κB in lung inflammation, participating in Th2 differentiation, chemokine production, eosinophil migration to the airways, and airway hyperresponsiveness (32, 33). Moreover, Poynter et al. (34) showed expression of NF-κB in airway epithelium of ovalbumin-sensitized and challenged mice. In mouse models of allergic lung inflammation, inhibiting NF-κB can block inflammation and mucus production (13, 14). Our cell culture data led us to investigate the potential TNF-α-induced, NF-κB-mediated mucus production in mouse airways. We demonstrate that TNF-α administration causes mucus production by lung epithelial cells in vivo. Further-

FIGURE 7. TNF-α stimulation in vivo leads to MUC5AC expression and mucus production. A, bioluminescence imaging analysis of mice infected with the MUC5AC adenoviral construct and activated with saline, IL-13 (positive control), or TNF-α. B and C, histological analysis of TNF-α-challenged mice, 24 h after receiving the third of three daily intranasal doses of saline (B) or TNF-α (C), C57BL/6 mice were sacrificed, and lung sections were Alcian Blue/periodic acid-Schiff-stained to reveal mucin-producing cells (intense blue staining). D, NF-κB mediates TNF-α-induced mucus production in the airways. Periodic acid-Schiff staining of mouse lungs after saline (upper panel) or TNF-α (middle and lower panels) intranasal administration are shown. Adenoviral constructs (GFP control or IKKβ DN) were delivered 4 days prior to TNF-α challenge.
more, following the same protocol of cytokine intranasal delivery, we show that NF-κB becomes activated in the airways after TNF-α administration by bioluminescent imaging as measured using adenoviral delivery of an NF-κB-luciferase construct. Moreover, using NF-κB-LacZ mice, we localized the site of NF-κB activation to the lung epithelium. Based on these findings, we hypothesize an NF-κB-dependent, TNF-α-induced mucus production mechanism in the airway epithelium in vivo. This hypothesis was supported by the demonstration that in vivo, TNF-α-mediated mucus secretion was inhibited by an IKKβ dominant negative adenovirus.

Descriptive data showing the presence of NF-κB in lungs of asthma and COPD patients are rapidly accumulating (35–37). Our work suggests a possible new role for NF-κB in mediating some aspects of the pathogenesis associated with these conditions beyond cellular infiltration, namely TNF-α pathway associated with these conditions beyond cellular infiltration, namely TNF-α–mediated mucus production but also to an amelioration of emphysema. This notion is supported by cytokine intranasal delivery of an NF-κB dominant negative adenovirus.

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