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Interleukin-8 Up-regulation by Neutrophil Elastase Is Mediated by MyD88/IRAK/TRAF-6 in Human Bronchial Epithelium*

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Cystic fibrosis is characterized in the lungs by neutrophil-dominated inflammation mediated significantly by neutrophil elastase (NE). Previous work has shown that NE induces interleukin-8 (IL-8) gene expression and protein secretion in bronchial epithelial cells. We sought to determine the intracellular mechanisms by which NE up-regulates IL-8 in bronchial epithelial cells. The data show that stimulation of 16HBE14o$^-$ cells with NE induced IL-8 protein production and gene expression. Both responses were abrogated by actinomycin D, indicating that regulation is at the transcriptional level. Electrophoretic mobility shift assays demonstrated that nuclear factor κB (NFκB) was activated in 16HBE14o$^-$ cells stimulated with NE. Western blot analysis demonstrated that activation of NFκB by NE was preceded by phosphorylation and degradation of IκB proteins, principally IκBβ. In addition, we observed that interleukin-1 receptor-associated kinase (IRAK) was degraded in 16HBE14o$^-$ cells stimulated with NE. Quantification of IL-8 reporter gene activity by luminometry demonstrated that dominant negative MyD88 (MyD88Δ) or TRAF-6 (TRAF-6Δ) inhibited IL-8 reporter gene expression in response to NE. Furthermore, MyD88Δ inhibited NE-induced IRAK degradation. These results show that NE induces IL-8 gene up-regulation in bronchial epithelial cells through an IRAK signaling pathway involving both MyD88 and TRAF-6, resulting in degradation of IκBβ and nuclear translocation of NFκB. These findings may have implications for therapeutic treatments in the cystic fibrosis condition.

Cystic fibrosis (CF)[1] is the most common life-threatening hereditary disorder affecting the Caucasian population. It is caused by mutations of the CF transmembrane conductance regulator gene, a 27-exon, 250-kilobase gene on chromosome 7 at q31, whose predicted primary translation product is a 1480-amino acid protein (1, 2). The major cause of mortality and morbidity in CF is lung disease. Although airway obstruction and infection are clearly major pathogenic factors in CF, there is increasing recognition that the pulmonary inflammatory response is of key importance (3–7). One of the essential components of this inflammation is the neutrophil (8). Even in young infants with CF, studies have demonstrated elevated numbers of neutrophils and increased neutrophil products on the respiratory epithelial surface (4, 6, 9).

While neutrophil accumulation on the airway epithelial surface is an essential component of normal host defense against infection, when exaggerated it can cause progressive damage to the bronchial epithelium. In CF, this damage is mediated most significantly by neutrophil elastase (NE), a powerful proteolytic enzyme released by activated neutrophils (10, 11). NE can impair local host defense mechanisms by degrading many extracellular matrix molecules and adversely affecting mucociliary clearance (5, 11). NE also cleaves immunoglobulin and complement proteins, thereby reducing phagocytosis and killing of *Pseudomonas aeruginosa* by neutrophils (12–15). In CF epithelial lining fluid (ELF), NE has been identified as a major signal capable of inducing the expression of IL-8 in bronchial epithelial cells (BECs) (10). IL-8, a member of the CXC chemokine family, is a potent activator and chemoattractant of neutrophils (16) and has been shown to be expressed in BECs in response to a variety of stimuli (17–19). In CF, IL-8-induced recruitment of additional neutrophils to the airways results in further release of NE and additional induction of IL-8 gene expression by bronchial epithelial cells, thereby perpetuating a chronic cycle of respiratory inflammation (10). *In vivo* studies have demonstrated that NE induction of IL-8 gene expression in the CF respiratory epithelial surface and subsequent neutrophil-dominated inflammation may be attenuated by NE inhibitors (20), demonstrating a new potential therapeutic target for the respiratory manifestations of CF.

While NE appears to be a major signal for CF bronchial epithelial IL-8 gene expression, the intracellular mechanisms of this response are unknown. NE increases the relative rate of transcription of the IL-8 gene in BECs but does not influence the IL-8 mRNA transcript stability, suggesting that the transcriptional process dominates in the response (10). With this as background, we have elucidated the intracellular signaling pathways involved in NE induction of IL-8 gene expression in BECs. The data demonstrate that as previously shown BECs are capable of producing IL-8 protein and expressing the IL-8 gene, and these responses are up-regulated by NE. In addition, we have demonstrated that NE-stimulated IL-8 gene expression results in increased activation of NFκB in BECs via a pathway involving IκBβ. We have also identified that interleu-

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[1] The abbreviations used are: CF, cystic fibrosis; NE, neutrophil elastase; IL, interleukin; TNF, tumor necrosis factor; BEC, bronchial epithelial cell; NFκB, nuclear factor κB; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; TRAF-6/2Δ, dominant negative TRAF-6/2; ELF, epithelial lining fluid; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; PMSF, phenylmethylsulfonyl fluoride; rSLPI, recombinant secretory leukoprotease inhibitor; TLR, Toll-like receptor; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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kin-1 receptor-associated kinase (IRAK) (21–25) is involved in NE-induced signaling events in BECs and provided evidence of the involvement of the signal transducing molecules MyD88 (24, 26–29) and TRAF-6 (24, 25, 30) in the NE-induced IL-8 signaling pathway.

In summary, these data show that NE activates IL-8 gene expression through a MyD88/IRAK/TRAF-6-dependent pathway, a breakthrough that may enable better targeting of novel anti-inflammatory therapy for the CF condition.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—16HBE14o$^-$ cells, an SV-40-transformed human bronchial epithelial cell line (31), were obtained as a gift from D. Gruenert (University of Vermont). The cells were cultured at 37 °C in Eagle's minimal essential medium (Biowhittaker, Berkshire, United Kingdom) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (Life Technologies, Inc.). The surface of the culture dishes was coated with a mixture of fibronectin (1 mg/ml; Sigma), collagen (Vitrogen 100, 2.9 mg/ml; Collagen Corp., Palo Alto, CA), and bovine serum albumin (1 mg/ml; Sigma). To eliminate the effect of different factors in the growth media and any effect contributed by trypsin, 24 h prior to agonist treatment cells were washed twice with 1× PBS and placed under serum-free conditions. Before agonist treatment, cells were again washed twice with 1× PBS and covered with serum-free media for 1 h.

Neutrophil elastase was purchased from Elastin Products Company, Inc. (Owensville, MO). Recombinant human TNFα and secretory leukoprotease inhibitor (rSLPI) were purchased from R&D Systems (Oxon, UK), and actinomycin D, MeO-Suc-Ala-Ala-Pro-Val-chloromethyl ketone, and PMSF were all purchased from Sigma.

IL-8 Protein Production—HBE cells were seeded at 1×10^6 on coated 24-well plates (16-mm diameter) 24 h before stimulation. Cells were left untreated or were stimulated with different doses of NE for different time periods or with TNFα (10 ng/ml, 3 h). In some experiments, cells were pretreated with actinomycin D (10 μg/ml) for 1 h before NE or TNFα treatment. The dose dependence studies with NE were carried out using 0–50 nM purified NE for 4 h. The time course studies used 10 nM NE and subsequent incubation of the HBE cells for 0–24 h. IL-8 protein concentrations in the cell supernatants were determined by enzyme-linked immunosorbent assays (R&D Systems). Following the removal of supernatants, the cells were lysed using RIPA buffer (1% Igepal CA-630, 0.5% deoxycholic acid, 0.1% SDS, 1% PMSF (10 mg/ml), 1% sodium orthovanadate (100 mM), and 3% aprotinin) (Sigma), and protein concentrations were determined by the method of Bradford (32). Cell viability assessed by trypan blue exclusion was >95% in all studies.

IL-8 mRNA Analysis—Total RNA was isolated from 1×10^6 HBE cells that had been left untreated or been stimulated with NE (10 nM, 4 h) or TNFα (10 ng/ml, 3 h) ± actinomycin D (10 μg/ml, 1 h) using TRI reagent (Sigma) according to the manufacturer's instructions. For both quantitative LightCycler™ (Roche Molecular Biochemicals) PCRs and semiquantitative RT-PCRs, 1 μg of total RNA was reverse-transcribed into cDNA with an oligo(dT)$_{15}$ primer using the first strand cDNA synthesis kit (Roche Molecular Biochemicals) as described in the manufacturer's protocol. cDNA amplifications were done by quantitative PCR using the light cycler and the double-stranded DNA binding dye SYBR Green 1 (Roche Molecular Biochemicals). The samples were continuously monitored during the PCR, and fluorescence was acquired every 0.1 °C. PCR mixtures contained 0.5 μM of either GAPDH- (MWG Biotech, Milton Keynes, UK) or IL-8-specific primers (BIOSOURCE). The samples were denatured at 95 °C for 10 min followed by 45 cycles of annealing and extension at 95 °C for 15 s, 55 °C for 5 s, and 72 °C for 10 s. The melting curves were obtained at the end of amplification by cooling the samples to 65 °C for 15 s followed by further cooling to 40 °C for 30 s. Serial 10-fold dilutions were prepared from known quantities of GAPDH and IL-8 PCR products, which were then used as standards to plot against the unknown samples. Quantification of data was analyzed using the LightCycler™ analysis software, and values were normalized to GAPDH expression.

In semiquantitative RT-PCR, the integrity of RNA extraction and cDNA synthesis was verified by PCR by measuring the amounts of GAPDH cDNA in each sample using GAPDH-specific primers. PCR mixtures contained 10× reaction buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 1.25 units of *Taq* polymerase, and 0.2 mM of each dNTP (Promega). Thermocycling conditions for IL-8 cDNA were 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Twenty cycles were used to amplify the more abundant GAPDH cDNA. A final extension step of 72 °C for 10 min was followed by resolution of the 227-base pair IL-8 products and the 211-base pair GAPDH products on a 1.5% Tris borate-EDTA agarose gel containing 0.5 μg/ml ethidium bromide (Sigma). IL-8 PCR products were quantified by densitometry using the GeneGenius Gel Documentation and Analysis System (Cambridge, UK) and Syngene GeneSnap and Gene-Tools software.

Preparation of Subcellular Fractions—HBE cells (1–4 × 10^6/ml) were seeded on coated six-well plates (34-mm diameter) 24 h before stimulation. Cells were activated with NE (10 nM) or TNFα (10 ng/ml) for different time periods ± actinomycin D (10 μg/ml, 1 h), and nuclear and cytoplasmic extracts were isolated. Briefly cells were washed and resuspended in 1 ml of ice-cold PBS and kept on ice for 5 min. Cells were lifted from plates with a cell scraper and pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 1 ml of hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, and 0.5 mM dithiothreitol) (Sigma). Cells were pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C and then lysed for 10 min on ice in 20 μl of hypotonic buffer containing 0.1% Igepal CA-630. Lysates were centrifuged as before, and the cytoplasmic extract was removed. The remaining nuclear pellet was lysed in 15 μl of lysis buffer (20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF) (Sigma) for 15 min on ice. After centrifugation at 14,000 rpm for 10 min at 4 °C, nuclear extracts were removed into 35 μl of storage buffer (10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM PMSF, and 0.5 mM dithiothreitol). Protein concentrations of cytoplasmic and nuclear extracts were determined (32), and extracts were stored at −80 °C until required for use.

Electrophoretic Mobility Shift Assay—Nuclear extracts (5 μg of protein) were incubated with 10,000 cpm of a 22-base pair oligonucleotide containing the NFκB consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA) that previously had been labeled with [γ-^{32}P]ATP (10 mCi/mmol) (Amersham Pharmacia Biotech) by T4 polynucleotide kinase (Promega). Incubations were performed for 30 min at room temperature in binding buffer (4% (v/v) glycerol, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM dithiothreitol, and 0.1 mg/ml nuclease-free bovine serum albumin) and 2 μg of poly(dI-dC)·poly(dI-dC) (Sigma). In some experiments, unlabeled mutant or wild-type oligonucleotides or antibodies to p50, p65, or c-Rel (Santa Cruz Biotechnology) were added to the extracts before incubation with the labeled oligonucleotide. All incubations were subjected to electrophoresis on native 4% (w/v) polyacrylamide gels that were subsequently dried, analyzed on a Molecular Dynamics Storm 820 Phosphorimagery Scanner (Amersham Pharmacia Biotech) for quantification, and autoradiographed.

Western Blot Analysis—Cytoplasmic extracts (10 μg of protein) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Sigma) in 20 mM Tris, 150 mM glycine, 0.01% SDS, and 20% (v/v) methanol at 75 mA for 2 h using a semidry electrophoretic blotting system. Nonspecific binding was blocked with 0.2% I-Block (Tropix, Bedford, MA) and PBS containing 0.1% Tween 20 (Sigma). Immunoreactive proteins were detected by incubating the membrane with specific antibodies (IκBα and IκBβ from Santa Cruz Biotechnology and IRAK from Transduction Laboratories, Lexington, KY). Following six 5-min washes with PBS containing 0.1% Tween 20, immunoreactive proteins were detected using alkaline phosphatase-conjugated anti-mouse IgG (Promega) (IRAK and IκBα) or goat anti-rabbit IgG (Tropix) (IκBβ) and CDP-Star chemiluminescent substrate solution (Sigma) according to the manufacturer's instructions.

Transfection and Reporter Gene Studies—To construct the IL-8 luciferase reporter plasmid, the human IL-8 promoter was PCR-amplified using *Pfu* polymerase (Stratagene, Cambridge, UK) from genomic DNA with 5′-GCACTCGAGTAACCCAGGCATTATT-3′ (forward) and 5′-GCTAAGCTTAGTGCTCCGGTGGCTTTT-3′ (reverse) and was cloned on a *Xho*I-*Hin*dIII fragment (underlined) into pGL3-PV (Promega). HBE cells were seeded at 5×10^5 on coated six-well plates 24 h before transfection. Transfections were performed with TransFast Transfection Reagent (Promega) in a 1:1 ratio according to the manufacturer's instructions using 500 ng of IL-8 luciferase and 500 ng of either pRK5, TRAF-6Δ, TRAF-2Δ (a gift from Tularik, San Francisco, CA), pCDNA3, or MyD88Δ (a gift from M. Muzio (26, 27)). Uniform transfection efficiencies were achieved by initially optimizing transfection conditions using a constitutive luciferase expression vector, pGL3-control (Promega). Transfections were incubated for 1 h at 37 °C. Cells were then supplemented with additional growth medium (4 ml/well) for 48 h at 37 °C before being left untreated or stimulated with NE or TNF as indicated. Cells were lysed with Reporter Lysis buffer (Promega) (250 μl/well), protein concentrations were determined (32), and IL-8

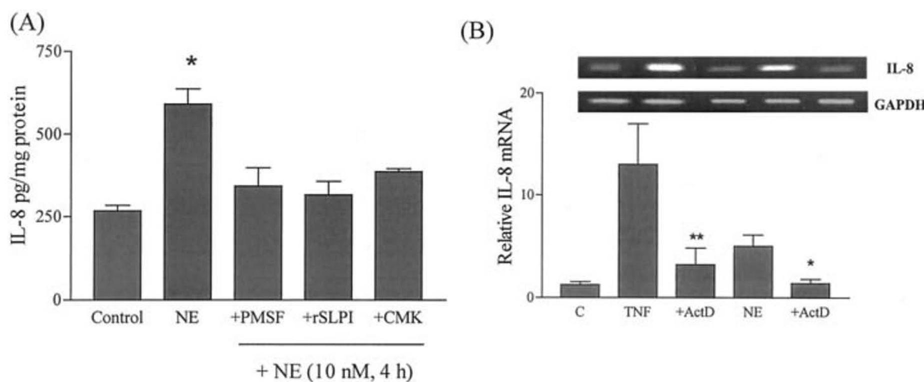

FIG. 1. **NE induction of IL-8 protein production and IL-8 gene expression in bronchial epithelial cells.** *A*, 16HBE14o$^-$ cells (1 × 10^6/ml) were left untreated or were stimulated with NE (10 nM, 4 h) ± a 20-min preincubation with PMSF (1 μM), rSLPI (500 nM), or MeO-Suc-Ala-Ala-Pro-Val-chloromethyl ketone (*CMK*) (1 μM). Levels of IL-8 in supernatants were measured by enzyme-linked immunosorbent assay, and values were corrected to pg/mg of total protein (*, $p \leq 0.005$, analysis of variance). *B*, 16HBE14o$^-$ (1 × 10^6/ml) cells ± actinomycin D (*ActD*; 10 μg/ml, 1 h) were left untreated or were stimulated with TNFα (10 ng/ml, 3 h) or NE (10 nM, 4 h). Total RNA was extracted and 1 μg was reverse-transcribed into cDNA and used as template in quantitative LightCycler™ PCRs. Values are expressed as relative IL-8 mRNA after normalization to GAPDH (*, $p \leq 0.05$; **, $p \leq 0.02$). Replica 16HBE14o$^-$ RNA samples were also used to measure IL-8 gene expression in semiquantitative RT-PCRs. Control (*C*) reactions measured levels of GAPDH mRNA. Products were electrophoresed in 1.5% Tris borate-EDTA agarose gels containing 0.5 μg/ml ethidium bromide and visualized under UV light. Assays were performed in triplicate and are representative of at least three separate experiments.

reporter gene activity was quantified by luminometry (Wallac Victor[2], 1420 multilabel counter) using the Promega luciferase assay system according to the manufacturer's instructions. Reporter gene expression was expressed as light units/μg of total protein.

Statistical Analysis—Data were analyzed with the GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA). Results are expressed as mean ± S.E. and were compared by Mann-Whitney test or analysis of variance with post hoc analysis. Differences were considered significant when the p value was ≤0.05.

RESULTS

NE Stimulates IL-8 Protein Production and IL-8 Gene Expression in Bronchial Epithelial Cells—Basal and NE-induced IL-8 protein levels in cell supernatants from HBE cells were quantified by enzyme-linked immunosorbent assay. HBE cells produced a mean basal level of IL-8 of 267 ± 17 pg/mg of protein. Time course and dose-response experiments (data not shown) demonstrated that 10 nM NE for 4 h induced maximal IL-8 protein production from HBE cells, increasing IL-8 levels to 538 ± 24 pg/mg of protein ($p \leq 0.001$) and confirming a previous report using a Bet1A cell line (10). It was not feasible to investigate whether doses higher than 10 nM NE could further potentiate IL-8 protein production because at higher concentrations NE degrades IL-8 protein (33) and renders the enzyme-linked immunosorbent assay inaccurate. NE did not induce apoptosis in HBE cells at doses up to 50 nM NE over 4 h, and the cells retained >90%viability (data not shown). Control TNFα stimulations (10 ng/ml for 3 h) increased IL-8 protein to 4134 ± 125 pg/mg of protein ($p \leq 0.007$).

Preincubation of NE with the synthetic serine protease inhibitor PMSF (1 μM), a recombinant form of the naturally occurring serine protease inhibitor, rSLPI (500 nM), or the specific NE inhibitor MeO-Suc-Ala-Ala-Pro-Val-chloromethyl ketone (1 μM) abolished its ability to induce IL-8 production (Fig. 1*A*) ($p < 0.005$, analysis of variance). This indicated that the elastase activity of NE is required for IL-8 up-regulation.

In Fig. 1*B* IL-8 gene expression in response to NE and TNFα was measured in HBE cells by quantitative LightCycler™ PCRs and also by semiquantitative RT-PCRs using IL-8-specific primers and comparison with expression of GAPDH mRNA. To evaluate whether NE increased IL-8 by a transcriptional mechanism, HBE cells were pretreated with actinomycin D (10 μg/ml, 1 h). Real time RT-PCR demonstrated that in HBE cells, NE induced a 4-fold increase in IL-8 mRNA that was inhibited by actinomycin D pretreatment ($p \leq 0.05$) (Fig. 1*B*). TNF strongly induced IL-8 mRNA, an effect that was also blocked by actinomycin D ($p \leq 0.02$). Resolution of the semiquantitative RT-PCR samples on a 1.5% Tris borate-EDTA agarose gel qualified the real time RT-PCR data. Actinomycin D also significantly decreased NE- and TNFα-induced IL-8 protein levels to lower than basal levels (171 ± 21 pg/mg of protein ($p \leq 0.0001$) and 191 ± 11 pg/mg of protein ($p \leq 0.0001$), respectively).

NE Stimulates NFκB Activation in Bronchial Epithelial Cells—The transcription factor NFκB regulates IL-8 gene expression (34). NFκB activation in nuclear extracts from HBE cells was measured by electrophoretic mobility shift assay. Cells were left untreated or were stimulated with either NE or TNFα. Time course studies (data not shown) demonstrated that 10 nM NE induced maximum NFκB activation at 5 min, while TNFα (10 ng/ml) induced a similar effect at 10 min. NFκB nuclear translocation was increased 3- and 4-fold compared with control for NE and TNFα, respectively, as measured by phosphorimagery (Fig. 2*A*). Concomitant Western blotting of cytoplasmic extracts was performed using anti-IκBα and anti-IκBβ antibodies. Stimulation with either NE or TNFα led to degradation of IκBβ. TNF also degraded IκBα, however, NE had only a modest effect on IκBα degradation.

In Fig. 2*B* competition studies with unlabeled mutant and wild-type NFκB probes demonstrated that NE activated NFκB specifically. Antisera to NFκB components identified the subunit composition of the NE-induced NFκB complexes as heterodimers of p50 and p65 but not c-Rel (Fig. 2*C*).

NE Stimulates IRAK Degradation in Bronchial Epithelial Cells—Given our interest in Toll/IL-1 receptor signaling (24–25), we examined a role for IRAK in the activation of NFκB in response to NE. Cytoplasmic extracts from control HBE cells or cells stimulated with either NE (10 nM, 5 min) or TNFα (10 ng/ml, 10 min) were Western blotted to measure IRAK degradation (Fig. 3). An immunoreactive band of 80 kDa was detected in extracts from control cells. This was degraded following stimulation with NE but not TNFα, implicating IRAK in the NE pathway.

TRAF-6, but Not TRAF-2, Is a Component of the NE/IL-8 Signaling Pathway—To further elucidate signaling events activated in response to stimulation with NE, IL-8 promoter activity using an IL-8 promoter luciferase reporter gene was measured in HBE cells. NE time course (data not shown) and dose-response studies demonstrated that NE stimulation (50 nM, 4 h) induced optimal IL-8 promoter activity (Fig. 4*A*) and indicated that in-

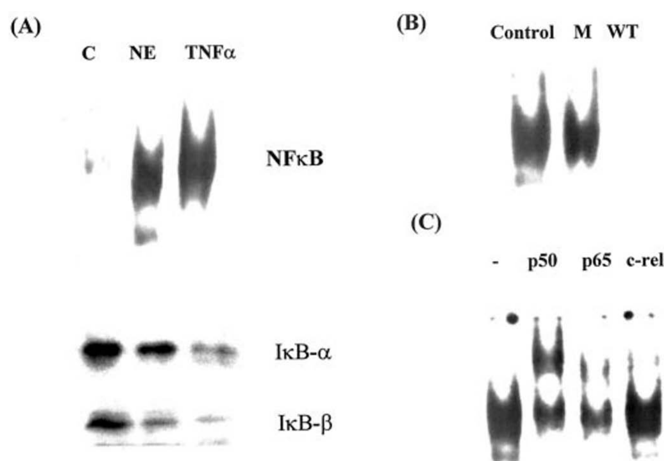

FIG. 2. **NFκB activation and IκB degradation in bronchial epithelial cells stimulated with NE.** *A*, NFκB activation was measured by electrophoretic mobility shift assay using a [γ^{32}P]ATP end-labeled NFκB consensus sequence (10,000 cpm) in nuclear extracts (5 μg) from control 16HBE14o$^-$ cells (*C*) and cells stimulated with NE (10 nM for 5 min) or TNFα (10 ng/ml for 10 min) ($n = 5$). IκBα and IκBβ degradation were analyzed concomitantly by Western blot using antibodies against IκBα and IκBβ. *B*, nuclear extracts (5 μg) from 16HBE14o$^-$ cells stimulated with NE (10 nM for 5 min) were incubated with γ^{32}P-labeled NFκB (*Control*) + preincubation for 30 min with mutant (*M*) NFκB or wild-type (*WT*) NFκB ($n = 2$). *C*, supershift assays were performed with 5 μg of nuclear extract from NE-treated 16HBE14o$^-$ cells ± anti-p50, anti-p65, or anti-c-Rel antibodies for 30 min ($n = 2$).

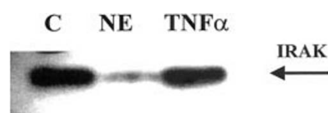

FIG. 3. **IRAK degradation in bronchial epithelial cells stimulated with NE.** IRAK degradation was analyzed by Western blot using an anti-IRAK antibody and cytosolic extracts (10 μg) from control (*C*), NE-treated (10 nM, 5 min), or TNFα-treated (10 ng/ml, 10 min) 16HBE14o$^-$ cells ($n = 5$).

creasing doses of NE can potentiate IL-8 expression. However, at higher doses (100 nM) NE caused cell detachment and necrosis and thereby prevented further IL-8 promoter activity.

The induction of IL-8 promoter activity was significantly down-regulated by transfection of HBE cells with dominant negative TRAF-6 (TRAF-6Δ) but not dominant negative TRAF-2 (TRAF-2Δ) (Fig. 4*B*), indicating that TRAF-6, but not TRAF-2, is a component of the NE/IL-8 signaling cascade. TNFα (10 ng/ml, 3 h) also increased IL-8 promoter activity. The TNFα effect was not affected by transfection with TRAF-6Δ but was significantly inhibited by TRAF-2Δ ($p \leq 0.0001$).

MyD88Δ Inhibits NE-induced IRAK Degradation and IL-8 Reporter Gene Expression in Bronchial Epithelial Cells—To elucidate additional upstream components in the NE/IL-8 signaling pathway in HBE cells, the effect of dominant negative MyD88 (MyD88Δ) on NE-induced IRAK degradation was determined (Fig. 5*A*). HBE cells were left untreated or were stimulated with NE (10 nM, 5 min) in the absence or presence of MyD88Δ. Western blotting using an anti-IRAK antibody showed, as before, that NE could induce IRAK degradation. However, this effect was blocked by MyD88Δ, indicating that MyD88 is positioned upstream from IRAK and both components are involved in intracellular events activated by NE. This result was further verified in Fig. 5*B* by measuring the effect of MyD88Δ on IL-8 promoter activity in HBE cells in response to NE. IL-8 reporter gene activity was significantly up-regulated in HBE cells following NE stimulation. However, this response was inhibited when the cells were co-transfected with MyD88Δ

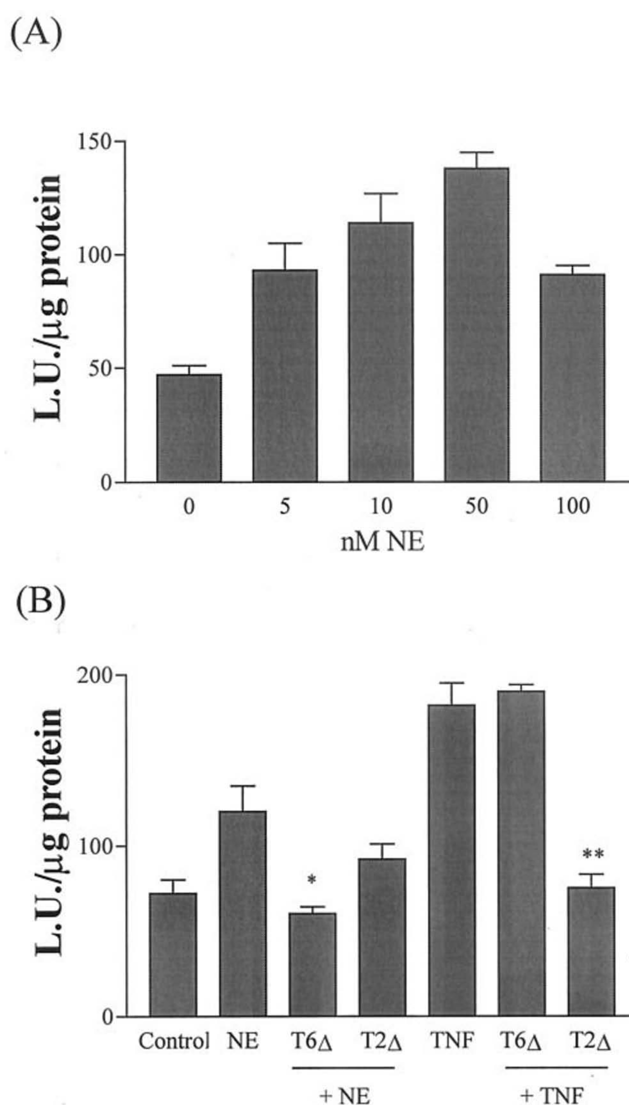

FIG. 4. **TRAF-6Δ, but not TRAF-2Δ, inhibits NE-induced IL-8 reporter gene expression in bronchial epithelial cells.** Triplicate 16HBE14o$^-$ cultures (5×10^5 cells) were transfected with an IL-8 luciferase reporter vector (IL-8-luc) (*A*) or co-transfected with IL-8-luc and either pRK5 (empty vector) (*Control*, *NE*, *TNF*), TRAF-6Δ (*T6Δ*), or TRAF-2Δ (*T2Δ*) expression plasmids (*B*). 48 h post-transfection, cells were left untreated or were stimulated with increasing doses of NE for 4 h (as indicated) (*A*) or NE (50 nM, 4 h) or TNFα (10 ng/ml, 3 h) (*B*) and lysed. IL-8 reporter gene activity was quantified by luminometry (*, $p \leq 0.001$; **, $p \leq 0.0001$). Data are expressed as light units (*L.U.*)/μg of total protein ($n = 3$).

($p < 0.0001$), providing further evidence for the involvement of MyD88 in the NE/IL-8 signaling pathway.

DISCUSSION

We have previously shown that IL-8, an 8.5-kDa potent chemoattractant and activator of neutrophils, is released by bronchial epithelial cells in response to NE (10). Thus begins a vicious cycle of inflammation whereby NE stimulates IL-8 production, leading to enhanced neutrophil accumulation and further NE release. In this study, we elucidate the mechanisms by which NE induces IL-8 gene expression and IL-8 release from the bronchial epithelium. We demonstrate that this response is inhibited by actinomycin D, indicating that the mechanism of IL-8 regulation by NE is transcriptional. We further show that following NE stimulation of HBE cells IκB kinases are activated, resulting in phosphorylation and degradation of IκBβ and enabling NFκB translocation to the nucleus where it in-

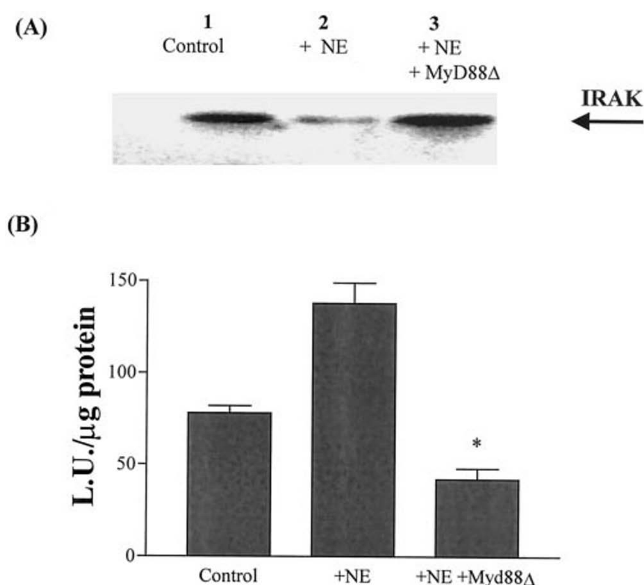

FIG. 5. **Inhibition of NE-induced IRAK degradation and IL-8 reporter gene expression by MyD88Δ.** *A*, IRAK degradation was analyzed by Western blot in cytosolic extracts (10 μg) from 16HBE14o$^-$ cells that were transfected with pCDNA3 (empty vector) (*lanes 1* and *2*) or an MyD88Δ expression vector (*lane 3*) and left untreated or stimulated with NE (10 nM for 5 min) 48 h post-transfection (n = 3). *B*, triplicate 16HBE14o$^-$ cultures (5×10^5 cells) were co-transfected with an IL-8 luciferase reporter construct and either pCDNA3 (empty vector) or an MyD88Δ expression plasmid. 48 h post-transfection, cells were left untreated or were stimulated with NE (50 nM, 4 h) and lysed, and IL-8 reporter gene activity was quantified by luminometry (*, $p \leq 0.0001$). Data are expressed as light units (*L.U.*)/μg of total protein (n = 3).

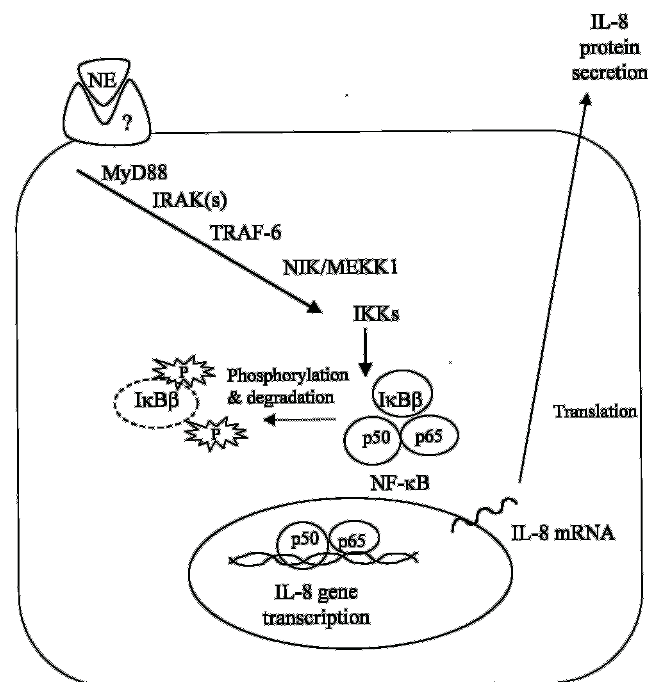

FIG. 6. **Schematic representation of NE-induced IL-8 signal transduction pathway in human bronchial epithelial cells.** In bronchial epithelial cells, NE activates an as yet unidentified receptor. The cytosolic protein MyD88 acts as an adaptor coupling the receptor complex to IRAK. The signal is relayed downstream via TRAF-6 to the IκB kinases (*IKKs*) (via NIK/MEKK1), resulting in phosphorylation and degradation of IκBβ, and subsequent release and translocation of NFκB to the nucleus where it transactivates IL-8 gene expression. *NIK*, NFκB-inducing kinase; *MEKK1*, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1.

duces IL-8 gene expression (Fig. 6). Upstream components of the NE-induced IL-8 signaling pathway in bronchial epithelial cells were also identified. Following stimulation with NE, IRAK is degraded. IRAK was originally identified as a signal transducer involved in IL-1 signaling (23, 25) and has subsequently been shown to have an important role in Toll-like receptor (TLR) signal transduction (27, 36–38). This prompted us to examine the potential involvement of other TLR signal transducers in the regulation of IL-8 expression by NE. In particular we focused on two novel signal transducing molecules: TRAF-6, a member of the TNF receptor-associated factor family (24–25, 30), and MyD88, a member of the IL-1 receptor family (24, 26–29). In this study we have demonstrated that both TRAF-6 and MyD88 are involved in the NE/IL-8 pathway. Transfection of HBE cells with either dominant negative TRAF-6 (TRAF-6Δ) or dominant negative MyD88 (MyD88Δ) inhibited NE-induced IL-8 reporter gene activity. In addition, MyD88Δ blocks NE-induced IRAK degradation, establishing its position upstream from IRAK where it most likely functions as an adaptor coupling the receptor complex to IRAK (28–29) (Fig. 6).

These data demonstrate that NE up-regulates IL-8 gene transcription via a MyD88/IRAK/TRAF-6 mechanism. This is of importance in conditions such as CF and pneumonia where free NE is present on the airway epithelial surface. In addition to NE, other constituents of the ELF may also be important in inducing inflammation. Like NE, TNFα stimulates IL-8 gene expression and protein production via a transcriptional mechanism. To eliminate the possibility that NE may be inducing TNFα expression and inducing an autocrine effect we investigated whether NE could increase TNFα expression in HBE cells in dose-response studies (data not shown). NE failed to induce TNFα expression even at a dose of 50 nM up to the 4-h time point used in this study. Furthermore, Nakamura *et al.* (10) provide additional evidence to support our hypothesis that NE rather than TNFα is responsible for IL-8 induction, having shown that anti-TNFα neutralizing antibodies have no inhibitory effect on CF ELF induction of IL-8 expression in human bronchial epithelial cells.

NFκB activation requires its release from inhibitory IκB proteins via their phosphorylation and degradation. We have shown in this study that while stimulation of HBE cells with TNFα degrades both IκBα and IκBβ, treatment with NE results predominantly in degradation of IκBβ. IRAK is also phosphorylated and degraded following NE stimulation unlike TNFα, which does transduce its signal via IRAK. The distinction between the NE and TNFα inflammatory pathways is further exemplified by demonstrating that TNFα-induced IL-8 promoter activity is unaffected by TRAF-6Δ but significantly reduced by TRAF-2Δ (39, 40). TRAF-2Δ, however, fails to alter NE-induced IL-8 gene expression.

To date, MyD88/IRAK/TRAF-6 have been reported to be the transducers for the IL-1 receptor/TLR superfamily, a recently defined and expanding group of receptors that participate in innate immune and inflammatory responses (24, 41–43). The superfamily is defined by the Toll/IL-1 receptor (TIR) domain and is further subdivided into two groups based on homology to the extracellular domains of either the type 1 IL-1 receptor or *Drosophila* Toll receptor (24, 41, 42). In *Drosophila*, the ligand for Toll is Spaetzle, the generation of which involves a protease cascade (43). It has been hypothesized that mammalian TLRs may be receptors for Spaetzle-like ligands generated by protease cascades involving potential human Snake or Easter homologues (42). Given that the elastase activity of NE is essential for IL-8 induction an intriguing parallel may be made to the NE-induced IL-8 signaling pathway illustrated in this study whereby NE fulfills a similar role. Signaling pathways activated via TLRs trigger the activation of downstream kinases and transcription factors including NFκB and involve the sig-

nal transducer IRAK and the adaptor protein MyD88 (24, 42). We hypothesize that in bronchial epithelial cells NE may engage or cleave a receptor belonging to the TLR family to activate this pathway. Alternatively NE may activate an as yet unidentified receptor(s) that transduces its signal via MyD88, IRAK, and TRAF-6. It remains to be elucidated whether other mammalian lung proteases such as collagenases or proteinase-3 can induce similar effects (44, 45). *P. aeruginosa* is the most important pathogen associated with CF and expresses an elastase that potently induces IL-8 (46). It is exciting to speculate that this enzyme exerts its effects via a similar mechanism to NE.

We have elucidated one of the intracellular mechanisms involved in the NE-induced inflammatory response in bronchial epithelial cells. Given the fact that pulmonary inflammation contributes significantly to morbidity in CF and in a variety of other lung conditions, our findings may help identify potential therapeutic targets. This may be achieved by inhibiting one or more of the signaling components identified by this study to be involved in NE-regulated IL-8 gene expression. A number of ways of countering NE-induced airway inflammatory damage have been proposed. These range from specific anti-protease therapy such as α_1-antitrypsin or rSLPI directed at NE and other proteolytic enzymes (20, 47) to antioxidant medication such as glutathione (48). Studies have demonstrated that administration of aerosolized rSLPI to CF individuals decreases active NE levels, neutrophil numbers, and IL-8 levels in CF ELF (20). Aerosol administration of glutathione, a potent antioxidant has also been attempted to restore the oxidant-antioxidant imbalance in CF ELF, thereby down-regulating the airway inflammatory manifestations associated with CF (48). Recent efforts to develop effective CF therapies have focused on other anti-inflammatory strategies directed at the airway epithelium using either liposome-mediated gene transfer or pharmacological intervention concentrating on NFκB as a target (49–50). Genistein has been shown to inhibit NFκB activity in CF bronchial cells and in this way down-regulate IL-8 production (49). Adenovirus-mediated overexpression of IκBα and liposome-mediated transfection with decoy oligonucleotides in a CF airway epithelial cell line has been shown to decrease TNFα-induced IL-8 secretion and NFκB activity (50). In addition, the nonsteroidal anti-inflammatory drug ibuprofen has been shown to inhibit NFκB activity, thereby preventing the expression of inflammatory cytokines (51–53). These studies have yielded promising results that suggest that NFκB may indeed be a key target for development of anti-inflammatory therapeutics for CF. Better understanding of the intracellular signaling cascades involved in this response, as afforded by this study, may open up new and interesting perspectives for treatment in this and other inflammatory conditions.

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