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Viral Inhibition of IL-1- and Neutrophil Elastase-Induced Inflammatory Responses in Bronchial Epithelial Cells[1]

Tomás P. Carroll,* Catherine M. Greene,[2]* Clifford C. Taggart,* Andrew G. Bowie,† Shane J. O'Neill,* and Noel G. McElvaney*

Previously, we elucidated the intracellular mechanisms by which neutrophil elastase (NE) up-regulates inflammatory gene expression in bronchial epithelial cells. In this study, we examine the effects of both IL-1 and NE on inflammatory gene expression in 16HBE14o$^-$ bronchial epithelial cells and investigate approaches to abrogate these inflammatory responses. IL-1 induced IL-8 protein production in time- and dose-dependent fashions, an important observation given that IL-8 is a potent neutrophil chemoattractant and a key inflammatory mediator. IL-1 and NE were shown to activate the p38 MAPK pathway in 16HBE14o$^-$ cells. Western blot analysis demonstrated IL-1R-associated kinase 1 (IRAK-1) degradation in response to stimulation with both IL-1 and NE. In addition, the expression of dominant negative IRAK-1 (IRAK-1Δ), IRAK-2Δ, or IRAK-4Δ inhibited IL-1- and NE-induced NF-κB-linked reporter gene expression. Dominant negative versions of the intracellular adaptor proteins MyD88 (MyD88Δ) and MyD88 adaptor-like (Mal P/H) abrogated NE-induced NF-κB reporter gene expression. In contrast, only MyD88Δ was found to inhibit IL-1-induced NF-κB reporter activity. We also investigated the vaccinia virus proteins, A46R and A52R, which have been shown to antagonize IL-1 signaling. Transfection with A46R or A52R cDNA inhibited IL-1- and NE-induced NF-κB and IL-8R gene expression and IL-8 protein production in primary and transformed bronchial epithelial cells. Furthermore, cytokine array studies demonstrated that IL-1 and NE can up-regulate the expression of IL-6, oncostatin M, epithelial cell-derived neutrophil activating peptide-78, growth-related oncogene family members, vascular endothelial growth factor, and GM-CSF, with induction of these proteins inhibited by the viral proteins. These findings identify vaccinia virus proteins as possible therapeutic agents for the manifestations of several inflammatory lung diseases. ***The Journal of Immunology,*** **2005, 175: 7594–7601.**

Interleukin-1 is the prototypical proinflammatory cytokine implicated in a range of inflammatory conditions, including chronic pulmonary inflammation. It induces the expression of multiple genes involved in inflammatory cascades and affects nearly every cell type (1). The IL-1 signaling pathway leading to activation of the transcription factor NF-κB, and MAPK activation has been extensively elucidated (2–4). Binding of IL-1 to type I IL-1R (IL-1RI)[3] and the IL-1R accessory protein (5) triggers recruitment of the adaptor protein MyD88, which associates with IL-1RI through its C-terminal Toll/IL-1 receptor (TIR) domain (6). MyD88 then interacts with two IL-1R-associated kinases, IRAK-1 and IRAK-2 (7), whereas IRAK-4 signals upstream of the other IRAKs (8) and is thought to function as an IRAK-1 kinase (9). After its phosphorylation, IRAK-1 dissociates from MyD88 and associates with TNFR-associated factor 6 (10). This complex then associates with TGF-β-activated kinase-1, which phosphorylates and activates the IκB kinase complex, leading to activation of NF-κB (11, 12).

Neutrophil accumulation on the airway epithelial surface is an essential component of normal host defense against infection. However, when exaggerated, it can cause progressive damage to the bronchial epithelium. In chronic lung disease, this damage is mediated significantly by neutrophil elastase (NE), a powerful proteolytic enzyme released by activated neutrophils (13). NE can impair local host defense mechanisms by degrading many extracellular matrix molecules and adversely affecting mucociliary clearance (14). NE can also degrade surfactant proteins, vital components of lung innate immune defenses (15, 16). NE has been identified as a major signal capable of inducing IL-8 expression in bronchial epithelial cells. IL-8, a member of the CXC chemokine family, is a potent activator and chemoattractant of neutrophils (17) and is expressed in bronchial epithelial cells in response to a variety of stimuli (18–20). IL-8-induced recruitment of neutrophils into the airways results in additional release of NE and induction of IL-8 gene expression by bronchial epithelial cells, thereby perpetuating a chronic cycle of inflammation in the lung (13, 21). Work from our group has elucidated that NE signals through several intracellular transducers common to both IL-1R and TLR pathways, including MyD88, IRAK-1, and TNFR-associated factor 6 (TRAF-6) (22).

The TIR family comprises two groups of transmembrane proteins that share functional and structural properties (23). A hallmark of the TIR family is the cytoplasmic TIR domain, which is

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[3] Abbreviations used in this paper: IL-1RI, IL-1R type I; ENA-78, epithelial cell-derived neutrophil-activating peptide; GRO, growth-related oncogene; IRAK, IL-1R-associated kinase; Mal P/H, MyD88 adaptor-like; NE, neutrophil elastase; NHBE, normal human bronchial epithelial cell; TIR, Toll/IL-1R; VEGF, vascular endothelial growth factor; TRAF-6, TNFR-associated factor 6.

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indispensable for signal transduction and serves as a scaffold for a series of unique protein-protein interactions (24). Recently, two virus proteins have been shown to antagonize signaling by TIR family members. During a search for novel TIR domain-containing proteins, two peptides from vaccinia virus, A46R and A52R, were identified. These are now known to be inhibitors of TIR-dependent signaling (25). Functional analyses of the vaccinia proteins revealed that both A46R and A52R blocked IL-1 signaling (25). In addition, A52R inhibited signaling through TLR2/1, TLR2/6, TLR3, TLR4, TLR5, and IL-18, indicating that it acted at some common step in the pathway (26). In support of this hypothesis, it was found that A52R coimmunoprecipitated with both IRAK-2 and TRAF-6, downstream components of TIR signaling. A46R and A52R are the first viral proteins identified that are capable of blocking IL-1R/TLR signaling. Furthermore, A46R and A52R block separate cell-signaling cascades by targeting conserved protein domains that have overlapping functions. Although many viral proteins function as decoy receptors for specific cytokines or chemokines, these poxvirus proteins have the potential to affect multiple host immune responses in infected cells.

In this study we examine the effects of mutant versions of the endogenous signaling mediators MyD88, MyD88 adaptor-like (Mal), IRAK-1, IRAK-2, and IRAK-4 on IL-1- and NE-induced inflammatory gene expression in bronchial epithelial cells. We also investigate the potential inhibitory effects of the vaccinia virus proteins A46R and A52R, known antagonists of TIR domain signaling, on inflammatory responses in the bronchial epithelium (25) and demonstrate that the viral proteins interfere not only with IL-8 production, but also with the expression of several other cytokines, chemokines, and growth factors. The findings implicate A46R and A52R as potential therapeutics for chronic inflammatory lung disorders.

Materials and Methods

Cell culture and treatments

16HBE14o$^-$ cells, an SV-40-transformed human bronchial epithelial cell line, were obtained as a gift from D. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA). The cells were cultured at 37°C in Eagle's MEM (BioWhittaker) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin (Invitrogen Life Technologies). Normal human bronchial epithelial (NHBE) primary cells were obtained from Cambrex. The NHBE cells were cultured at 37°C in bronchial epithelial basal cell medium supplemented with 0.4% bovine pituitary extract, 0.1% hydrocortisone, 0.1% human epithelial growth factor, 0.1% epinephrine, 0.1% transferrin, 0.1% insulin, 0.1% retinoic acid, 0.1% triiodothyronine, and 0.1% gentamicin sulfate/amphotericin B solution (Cambrex). IL-1β was purchased from R&D Systems, NE was purchased from Elastin Products, and PMA was purchased from Sigma-Aldrich.

Measurement of IL-1RI and TLR4 expression

16HBE14o$^-$ cells (1×10^5/ml) were washed in PBS (Invitrogen Life Technologies) and fixed in methanol (BDH). Cells were Fc blocked with 1 μg/ml goat IgG1 for 15 min at room temperature and labeled with 10 μg/ml of a mAb directed against human IL-1RI (QED Bioscience), human TLR4 (Santa Cruz Biotechnology), or mouse IgG1 or IgG2a isotype control Abs (R&D Systems). After washing, the cells were stained with anti-mouse $F(ab)_2$-FITC (DakoCytomation), washed, permeabilized, and labeled with propidium iodide (Molecular Probes). Receptor expression was quantified on a CompuCyte laser scanning cytometer. Individual cells expressing IL-1RI and TLR4 were identified and quantified on the basis of integrated green fluorescence reflecting binding of anti-IL-1RI-FITC and anti-TLR4-FITC Ab.

IL-8 protein production

16HBE14o$^-$ cells were seeded at 1×10^5 on 24-well plates 24 h before stimulation. Cells were left untreated or were stimulated with different doses of IL-1β for different time periods, NE (10 nM, 4 h), or PMA (50 ng/ml, 24 h). IL-8 protein concentrations in the cell supernatants were determined by ELISA (R&D Systems). After removal of supernatants, the cells were lysed using lysis 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-Aldrich), and protein concentrations were determined by the method of Bradford (27).

Transfection and reporter gene studies

16HBE14o$^-$ or NHBE cells were seeded at 1×10^5 on 24-well plates 24 h before transfection. Transfections were performed with TransFast transfection reagent (Promega) using 200 ng of an NF-κB_5-luciferase reporter gene, an IL-8 promoter-linked reporter gene or a MAPK reporter gene, pFR-luciferase. In combination with the luciferase reporter genes, dominant negative expression vectors IRAK-1Δ, IRAK-2Δ, IRAK-4Δ (gifts from Tularik), MyD88Δ (a gift from M. Muzio, Mario Negri Institute, Milan, Italy:), Mal P/H, or cDNAs encoding the viral proteins A46R and A52R were cotransfected into the cells. IRAK-1Δ and IRAK-2Δ are truncated death domain-containing N terminus versions of both IRAK proteins that lack their kinase-binding domains. IRAK-1Δ, comprising aa 1–215, and IRAK-2Δ, comprising aa 1–96. IRAK-4Δ (KK213AA), is a site-directed kinase-inactive mutant of IRAK-4. A46R and A52R are both inhibitors of TIR domain signaling, MyD88Δ contains only a functional TIR domain and lacks the death domain required for downstream signaling, whereas Mal P/H is a dominant negative version of Mal with a proline to histidine point mutation in box 2 of the TIR domain. For the MAPK reporter system (PathDetect In Vivo Signal Transduction Pathway Trans-Reporting System; Stratagene), 20 ng of pFC2-DNA-binding domain (negative control) and pFA-CHOP (p38 fusion *trans*-activator) were transfected into the cells in combination with pFR-luciferase. The total amount of DNA introduced into the cells was kept constant by supplementation with the relevant empty vectors. Transfection efficiencies were quantified using a *Renilla* luciferase vector (Promega). Transfections were left untreated or were stimulated with IL-1β or NE. After 48 h, supernatants were recovered for IL-8 ELISA. Cells were lysed with Reporter Lysis Buffer (Promega), protein concentrations were determined, and reporter gene activity was quantified by luminometry on a Wallac Victor^2 1420 multilabel counter (PerkinElmer) using the Promega luciferase assay system. Data are expressed as the relative luciferase activity ± SE.

Preparation of subcellular fractions

16HBE14o$^-$ cells (1×10^6/ml) were seeded on 6-well plates 24 h before stimulation. Cells were stimulated with IL-1β (10 ng/ml) or NE (10 nM) for different time periods, and cytoplasmic extracts were isolated as previously described (22). Protein concentrations of cytoplasmic extracts were determined by the method of Bradford (27) and stored at −80°C until required for use.

Western blot analysis

Cytoplasmic extracts (10 μg of protein) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Sigma-Aldrich). Nonspecific binding was blocked with 0.2% I-Block (Tropix) and PBS containing 0.1% Tween 20 (Sigma-Aldrich). Immunoreactive proteins were detected with Abs to IRAK-1 (BD Transduction Laboratories) or phospho-p38 (Cell Signaling Technology) and detected using alkaline phosphatase-conjugated anti-mouse IgG (Promega) and CDP-Star chemiluminescent substrate solution (Sigma-Aldrich).

Cytokine array

16HBE14o$^-$ cells were seeded at 1×10^5 on 24-well plates 24 h before transfection. Transfections were performed with TransFast transfection reagent (Promega) as described, using 200 ng of A46R, A52R, or empty vector pRK5 and 200 ng of pGL3 control constitutive luciferase plasmid to assess transfection efficiency. Cells were then left untreated or were stimulated with IL-1β (10 ng/ml) or NE (100 nM) for 4 h at 37°C. After 4 h, supernatants of transfected cells were incubated with cytokine array membranes (RayBiotec) and developed according to the manufacturer's instructions. Relative protein levels were quantified by densitometry using the SynGene GeneSnap and GeneTools software (Cambridge), and individual values were expressed per light unit of control luciferase reporter activity.

Statistical analysis

Data were analyzed with the GraphPad PRISM 3.0 software package. Results are expressed as the mean ± SE and were compared by ANOVA with post-hoc analysis. Differences were considered significant at $p \leq 0.05$.

Results

IL-1RI and TLR4 are expressed on bronchial epithelial cells

The presence of the cognate receptor for IL-1 and the putative receptor for NE on 16HBE14o$^-$ cells was investigated. Functional IL-1R is a heterodimer of two components, IL-1RI and IL-1RAcP, whereas the functional TLR4 complex requires the glycoprotein CD14 and the helper molecule MD-2 for optimal signaling. IL-1RI, IL-1RAcP, TLR4, CD14, and MD-2 transcripts were detected in 16HBE14o$^-$ RNA (data not shown), confirming the presence of RNA for the IL-1R and TLR4 in 16HBE14o$^-$ cells. IL-1RI and TLR4 protein expressions were detected on the surface of 16HBE14o$^-$ cells by laser scanning microscopy (Fig. 1). Cytometric analysis revealed that 16HBE14o$^-$ cells constitutively express IL-1RI and TLR4. Median channel fluorescence, detected at >610 nm, was significantly higher for anti-IL-1RI (anti-IL-1RI, 336,449 ± 3,351; isotype, 197,757 ± 16,054) and for anti-TLR4 (anti-TLR4, 229,955 ± 40,698; isotype, 144,854 ± 14,186) compared with isotype control Ab-labeled 16HBE14o$^-$ cells.

IL-1β and NE stimulate IL-8 protein production and activate p38 MAPK in bronchial epithelial cells

Basal, IL-1-induced, and NE-induced IL-8 protein productions in cell supernatants from 16HBE14o$^-$ cells were measured by ELISA. We previously optimized conditions for NE-induced IL-8 expression in 16HBE14o$^-$ cells (10 nM, 4 h) (22). Dose-response experiments conducted across a range of IL-1 concentrations for 24 h demonstrated that 10 ng/ml IL-1 was the most potent treatment (Fig. 2*A*). 16HBE14o$^-$ cells treated with 10 ng/ml IL-1 for 24 h produced a 6-fold induction above basal IL-8 levels. Time-course experiments, shown in Fig. 2*B*, demonstrate that 10 ng/ml IL-1 for 6 h elicited the maximal IL-8 protein production from 16HBE14o$^-$ cells, increasing cytokine levels from 1437 ± 134 to 6317 ± 665 pg IL-8/mg protein. Control PMA stimulations (50 ng/ml for 24 h) also significantly increased IL-8 protein production. It has been shown that p38 MAPK pathways are activated upon stimulation with a variety of cytokines and extracellular stimuli. Western analysis demonstrated that phospho-p38, but not phospho-ERK or phospho-JNK (data not shown), was activated after stimulation with both IL-1 and NE in bronchial epithelial cells (Fig. 2*C*). This was confirmed by MAPK reporter gene experiments using the pFR luciferase reporter gene and a CHOP expression plasmid (p38 MAPK dependent), which demonstrated that CHOP expression was increased upon stimulation with IL-1 and NE (Fig. 2*D*).

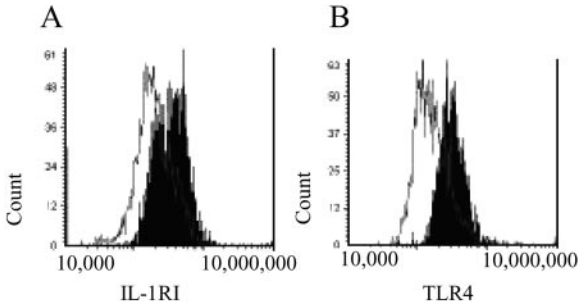

FIGURE 1. IL-1RI and TLR4 expression in bronchial epithelial cells. *A*, 16HBE14o$^-$ cells (1×10^4/ml) were stained for IL-1RI with 10 μg/ml mouse monoclonal anti-human IL-1RI or a mouse-conjugated IgG1 isotype control Ab and analyzed by laser scanning cytometry. *B*, 16HBE14o$^-$ cells (1×10^4/ml) were stained for TLR4 with 10 μg/ml mouse monoclonal anti-human TLR4 or a mouse conjugated IgG2a isotype control Ab and analyzed by laser scanning cytometry.

IRAK involvement in bronchial epithelial cells stimulated with IL-1β and NE

We investigated the roles of IRAK-1, IRAK-2, and IRAK-4 in IL-1 and NE signaling pathways in 16HBE14o$^-$ cells. Previous work has shown that upon IL-1 stimulation of cells, IRAK-1 is degraded (28). In addition, we have demonstrated that NE signaling occurs through an IRAK pathway (22). Western analysis confirmed the involvement of IRAK-1 in 16HBE14o$^-$ cells, with degradation occurring between 1 and 2 h after IL-1β stimulation (Fig. 3*A*). NE treatment (10 nM, 5 min) also induced IRAK-1 degradation, reflecting our previous findings. To further characterize IRAK involvement, we cotransfected 16HBE14o$^-$ cells with an NF-κB luciferase reporter gene and cDNAs encoding IRAK-1Δ, IRAK-2Δ, or IRAK-4Δ. Treatment with IL-1β (10 ng/ml, 4 h) or NE (10 nM, 4 h) caused a significant induction of NF-κB reporter gene expression (Fig. 3*B*). Cotransfection with IRAK-1Δ, IRAK-2Δ, or IRAK-4Δ abrogated these responses. IRAK-1Δ, IRAK-2Δ, or IRAK-4Δ had no effect on PMA-induced IL-8 protein production (data not shown).

Roles of MyD88 and Mal in IL-1- and NE-induced NF-κB reporter gene expression

Basal, IL-1-induced, and NE-induced NF-κB-linked promoter activities in cell lysates from transfected 16HBE14o$^-$ cells were measured by luminometry. We investigated the roles of the adaptors, MyD88 and Mal, which have no role in IL-1 signaling (29, 30). As before, treatment with IL-1β (10 ng/ml, 4 h) caused a significant induction of NF-κB promoter activity. Cotransfection with MyD88Δ significantly inhibited this response, whereas transfection with Mal P/H had no effect on IL-1-induced NF-κB activity (Fig. 4*A*). NE stimulation (10 nM, 4 h) caused a significant induction of NF-κB activity, which was abrogated by MyD88Δ (Fig. 4*B*). Cotransfection with Mal P/H, which acts in a dominant negative fashion in TLR4 signaling (30), inhibited NE-induced NF-κB-linked reporter gene expression.

A46R and A52R inhibit IL-1- and NE-induced NF-κB and IL-8R gene activities

We next investigated the effect of the vaccinia virus proteins, A46R and A52R, on IL-1β and NE signaling in 16HBE14o$^-$ cells. Cotransfection with 50, 100, or 200 ng of A52R expression plasmid abrogated IL-1-induced NF-κB reporter gene expression dose dependently. We observed a similar, less potent effect with A46R (Fig. 5*A*). In addition, cotransfection with 50, 100, or 200 ng of A46R or A52R expression plasmids abrogated NE-induced NF-κB reporter gene expression dose dependently (Fig. 5*B*). We also investigated the effects of A46R and A52R on IL-1β and NE signaling in primary bronchial epithelial cells (NHBE). Cotransfection with 200 ng of A46R or A52R inhibited IL-1- and NE-induced IL-8 gene expression in NHBE cells (Fig. 5*C*).

A46R and A52R inhibit IL-1- and NE-induced IL-8 protein production

The effects of the vaccinia proteins, A46R and A52R, on IL-1β- and NE-induced IL-8 protein production in 16HBE14o$^-$ cells were next investigated. Cotransfection with A46R and A52R abrogated IL-1-induced (Fig. 6*A*) and NE-induced (Fig. 6*B*) IL-8 production significantly. PMA-induced IL-8 protein production was not inhibited by A46R or A52R (Fig. 6*C*).

A46R and A52R inhibit a plethora of IL-1- and NE-induced genes with inflammatory roles

The effects of IL-1 and NE on the expression of a range of cytokines and chemokines in bronchial epithelial cells were studied

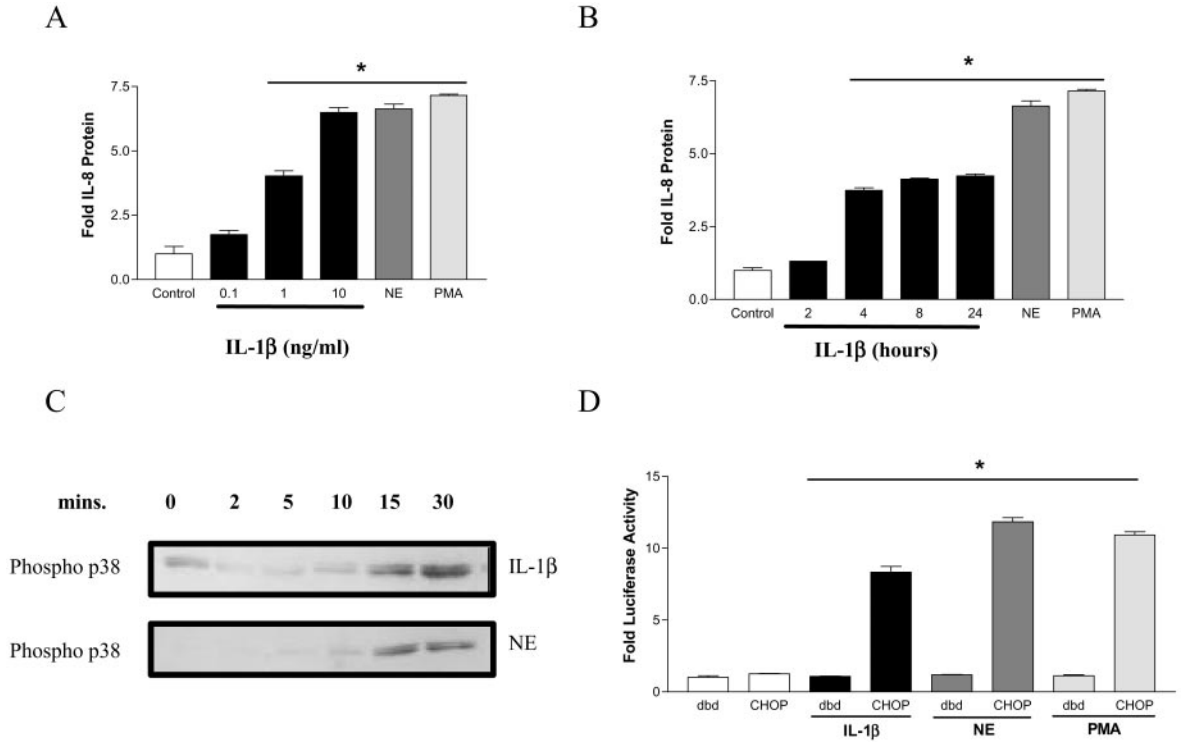

FIGURE 2. NE and IL-1β induce IL-8 protein production and p38 MAPK activation in bronchial epithelial cells. *A*, 16HBE14o$^-$ cells (1×10^5/ml) seeded on 24-well plates 24 h before stimulation. Cells were left untreated or were stimulated with IL-1β at different concentrations, NE (10 nM, 4 h), or PMA (50 ng/ml) for 24 h (*, $p \leq 0.05$ compared with unstimulated cells). *B*, 16HBE14o$^-$ cells (1×10^5/ml) seeded on 24-well plates 24 h before stimulation. Cells were left untreated or were stimulated with an optimal IL-1β concentration (10 ng/ml) for different time periods, NE (10 nM, 4 h), or PMA (50 ng/ml, 24 h; *, $p \leq 0.05$ compared with unstimulated cells). Levels of IL-8 in supernatants were measured by ELISA, and values were corrected to picograms per milligram of total protein. Data are expressed as relative IL-8 protein levels. Assays were performed in triplicate and are representative of at least three separate experiments. *C*, Phospho-p38 activation was analyzed by Western blot using anti-phospho-p38 Abs on cytoplasmic extracts (10 μg) from control, IL-1β-treated (10 ng/ml), and NE-treated (10 nM) cells for different time periods. *D*, 16HBE14o$^-$ cells (1×10^5/ml) cotransfected with 200 ng of pFR luciferase reporter and 20 ng of DNA-binding domain (negative control) or CHOP (p38 *trans*-activator plasmid). Twenty-four hours after transfection, cells were left untreated or were stimulated with IL-1β (10 ng/ml, 4 h), NE (10 nM, 4 h), or PMA (50 ng/ml, 4 h) and lysed. Reporter gene activity was quantified by luminometry, and values were corrected to light units per microgram of total protein. Data are expressed as the fold luciferase stimulation over the control value (*, $p < 0.05$ compared with unstimulated cells). Assays were performed in triplicate and are representative of at least three separate experiments.

using a cytokine protein array. Both IL-1 and NE induced the expression of the CXC chemokines IL-8, epithelial cell-derived neutrophil-activating peptide (ENA-78), and growth-related oncogene (GRO) family members (GRO family members GRO-α/CXCL1, GRO-β/CXCL2, and GRO-γ/CXCL3 are represented as a single GRO identity on the cytokine array), as well as the CC cytokine IL-6 and related oncostatin M in 16HBE14o$^-$ cells. GM-CSF and vascular endothelial growth factor (VEGF) were also up-regulated by IL-1 and NE (Fig. 7). Transfection with A46R or A52R cDNAs either partially or completely abrogated the IL-1- and NE-induced up-regulation of the CXC chemokines (IL-8, ENA-78, and GRO family members), IL-6 cytokine family members (IL-6 and oncostatin M), GM-CSF, and VEGF (Fig. 7).

Discussion

Airway epithelial cells represent a significant portion of the cellular content of the airways, and together constitute a vast surface area. The contribution of epithelial cells to the inflammatory response in the lung is an increasingly important area of research. IL-1 and NE are key proinflammatory factors present at high concentrations on the epithelial surface of the lung during acute and chronic inflammatory lung diseases, and excessive chronic inflammation, such as is seen in cystic fibrosis, could benefit from targeted therapeutic approaches to inhibit proinflammatory gene expression. In this study, we investigated potential ways to inhibit IL-1- and/or NE-induced inflammatory gene expression in human bronchial epithelial cells.

Intracellular signaling pathways activated by IL-1 and NE in human bronchial epithelial cells were investigated, and the inhibitory capacities of endogenous and viral proteins were evaluated with respect to NF-κB activation, IL-8 production, and the expression of multiple cytokines. The data show that both IL-1 and NE up-regulate IL-8 gene expression and activate NF-κB and p38 MAPK. The p38 MAPK pathway shares many similarities with ERK1/2 and JNK cascades and is associated with inflammation, cell growth, cell differentiation, and cell death. Extracellular stimuli of the p38 MAPK pathway include a variety of cytokines and a number of pathogens that activate p38 through different TLRs, including LPS, peptidoglycan, and staphylococcal enterotoxin (31). The downstream targets of p38 MAPK are kinases and transcription factors such as AP-1. Indeed, the promoter for IL-8 contains AP-1 binding sites, and in this way, p38 can also regulate inflammatory gene expression (32). A recent study has supported our findings with regard to NE-induced p38 MAPK activation, demonstrating that NE in similar doses can activate p38 MAPK in A549 epithelial cells, up-regulate NF-κB and AP-1, and induce IL-8 mRNA expression and protein synthesis (33).

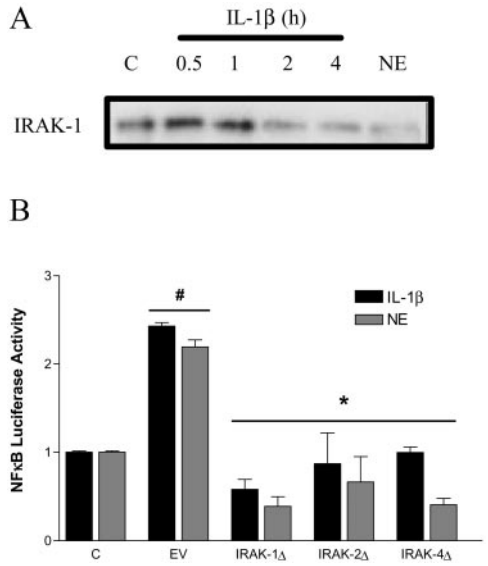

FIGURE 3. IRAK involvement in bronchial epithelial cells stimulated with IL-1β and NE. *A*, IRAK degradation was analyzed by Western blot using anti-IRAK-1 Abs on cytoplasmic extracts (10 μg) from control (C) and IL-1β-treated cells (10 ng/ml) for different time periods. NE (10 nM, 5 min) was used as a positive control. *B*, 16HBE14o$^-$ cells (1×10^5/ml) seeded on 24-well plates 24 h before transfection. Cells were transfected with 200 ng of NF-κB-linked luciferase reporter vector and cotransfected with 200 ng of empty vector (EV), IRAK-1Δ, IRAK-2Δ, or IRAK-4Δ. 48 h after transfection, then were left untreated or were stimulated with IL-1β (10 ng/ml, 4 h), NE (10 nM, 4 h), or PMA (50 ng/ml, 4 h), and cells were lysed. Reporter gene activity was quantified by luminometry, and values were corrected to light units per microgram of total protein. Data are expressed as relative luciferase stimulation over the control value (#, $p \leq 0.05$ compared with unstimulated cells; *, $p \leq 0.05$ compared with empty vector control stimulated with IL-1β or NE). Assays were performed in triplicate and are representative of at least three separate experiments.

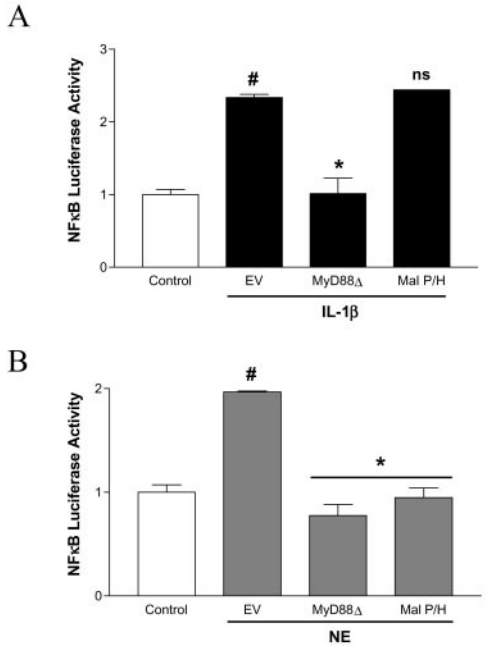

FIGURE 4. Roles of MyD88 and Mal in IL-1β- and NE-induced NF-κB reporter gene expression in bronchial epithelial cells. *A* and *B*, 16HBE14o$^-$ cells (1×10^5/ml) were transfected with 200 ng of NF-κB luciferase reporter and cotransfected with 200 ng of empty vector, MyD88Δ, or Mal P/H. Forty-eight hours after transfection, cells were left untreated or were stimulated with IL-1β (*A*; 10 ng/ml, 4 h) or NE (*B*; 10 nM, 4 h), and cells were lysed. Reporter gene activity was quantified by luminometry, and values were corrected to light units per microgram of total protein. Data are expressed as relative luciferase stimulation over the control value (#, $p \leq 0.05$ compared with unstimulated cells; *, $p \leq 0.05$ compared with empty vector control stimulated with IL-1β or NE). Assays were performed in triplicate and are representative of at least three separate experiments.

Both IL-1 and NE can induce IL-8 protein via an MyD88/IRAK mechanism, involving IRAK-1, IRAK-2, and IRAK-4 (9). IRAK proteins are a family of receptor-associated serine/threonine kinases, identified as important mediators in the signal transduction TIR family members (34). Our data implicate IRAK-1 in IL-1 signaling in bronchial epithelial cells, as indicated by its degradation in response to treatment with IL-1. NF-κB-linked reporter gene experiments clarified the involvement of IRAK-1, IRAK-2, and IRAK-4 in IL-1 signaling. This supports a recent study that showed that IL-1β from tracheal aspirates of premature infants can induce IL-8 expression via NF-κB in 16HBE14o$^-$ cells (35). We also demonstrated that NE signaling involves IRAK-2 and IRAK-4 as well as IRAK-1 (22). IRAK-4 acts upstream of IRAK-1, functioning as an IRAK-1 kinase and regulating IRAK-1 modification and turnover (36).

Previous studies of IL-1 signaling have demonstrated the involvement of the various signaling components in vitro in HEK293 cells and by the use of knockout mice (7, 8, 37, 38). In this study we report that the intracellular IL-1 signaling pathway involving MyD88 and IRAK1, -2 and -4 is conserved in human bronchial epithelial cells. Transfection of 16HBE14o$^-$ cells with MyD88Δ abrogated IL-1-induced NF-κB reporter gene activity, with Mal P/H having no effect. However, both MyD88Δ and Mal P/H were shown to abrogate NE-induced NF-κB reporter gene activity in bronchial epithelial cells. This apparent dichotomy in adaptor engagement between TLR4 and IL-1R signaling is supported by knockout mouse studies (30). Work from our group has demonstrated that NE induces IL-8 expression through the TLR signal transducers, MyD88, IRAK-1, and TRAF-6 (22), and that NE can induce LPS tolerance, indicating that NE exerts its effects via TLR4 (39). The fact that Mal P/H abrogates NE-induced NF-κB activity suggests that Mal is involved in downstream NE signaling pathways and provides additional evidence supporting NE signaling through TLR4. However, this raises the possibility that NE could also signal through TLR2, because TLR2 signaling uses both MyD88 and Mal (40), and TLR2 agonists have been shown to induce tolerance (41).

In addition to using dominant negative versions of intracellular signal transducers, we exploited naturally occurring viral proteins, shown to interfere with inflammatory signaling pathways. We evaluated whether the vaccinia virus proteins A46R and A52R could also abrogate IL-1- and NE-induced inflammatory gene expression in bronchial epithelial cells. A46R was first identified as sharing amino acid sequence similarity with the TIR domain, whereas A52R has sequence similarity to A46R, but not to TIR, domains (25). Both A46R and A52R can antagonize IL-1 signal transduction in mammalian cells, inhibiting proinflammatory gene expression by mimicking the effect of a dominant negative MyD88. Thus, vaccinia virus uses a mechanism of suppressing TIR domain-dependent intracellular signaling, representing a route of infection by stealth, with the virus switching off the host immune defenses. Furthermore, although both A52R and A46R can block IL-1 signaling, A52R was also shown to interfere with NF-κB activation by IL-18 and multiple TLRs, including TLR3 and TLR4, essential in the respective recognition of viral RNA and LPS (26).

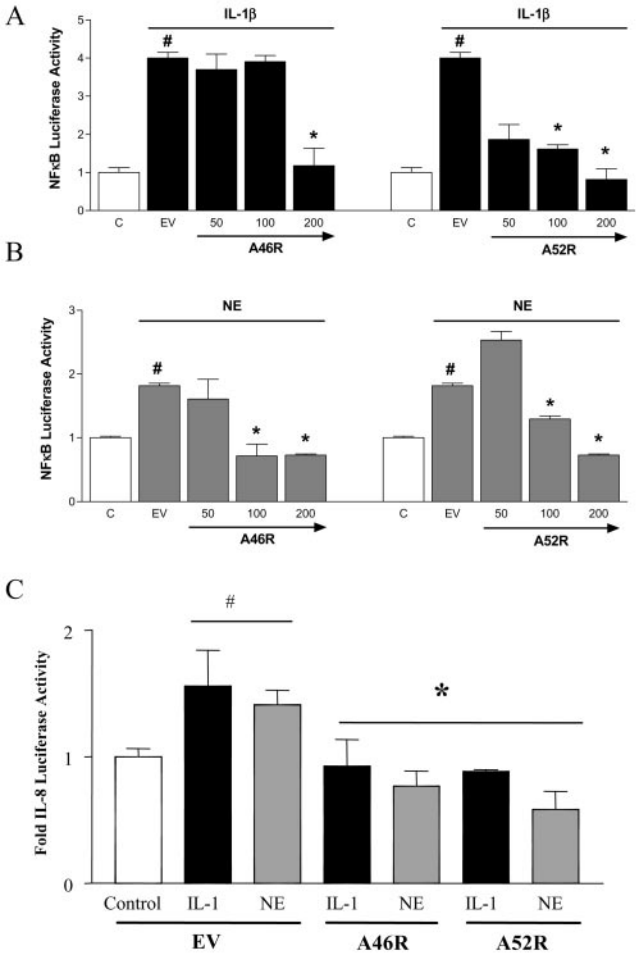

FIGURE 5. Vaccinia virus proteins A46R and A52R inhibit IL-1β- and NE-induced NF-κB reporter and IL-8R gene expression. *A* and *B*, 16HBE14o$^-$ cells (1×10^5/ml) transfected with 200 ng of NF-κB luciferase reporter gene and cotransfected with empty vector (EV) or 50, 100, or 200 ng of either A46R or A52R. Forty-eight hours after transfection, cells were left untreated or were stimulated with IL-1β (*A*; 10 ng/ml, 4 h) or NE (*B*; 10 nM, 4 h), supernatants were retained, and cells were lysed. *C*, NHBE cells (1×10^5/ml) transfected with 200 ng of IL-8 luciferase reporter gene and cotransfected with empty vector or 200 ng of either A46R or A52R. Forty-eight hours after transfection, cells were left untreated or were stimulated with IL-1β (10 ng/ml, 4 h) or NE (10 nM, 4 h), and cells were lysed. Reporter gene activity was quantified by luminometry, and values were corrected to light units per microgram of total protein. Data are expressed as relative luciferase stimulation over the control value (#, $p \leq 0.05$, agonist vs control; $*$ or $**$, $p \leq 0.05$, inhibitor vs agonist). Assays were performed in triplicate and are representative of at least three separate experiments.

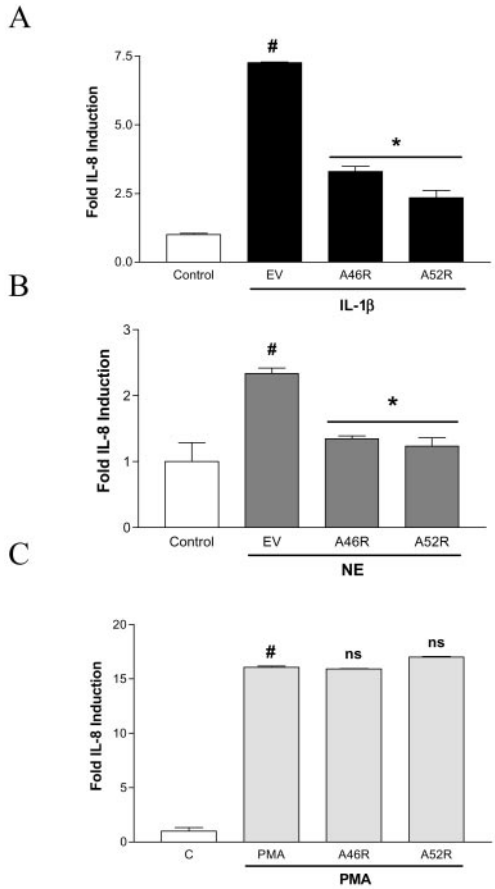

FIGURE 6. Vaccinia virus proteins A46R and A52R inhibit IL-1β- and NE-induced IL-8 protein production. *A–C*, 16HBE14o$^-$ cells (1×10^5/ml) transfected with 100 ng of empty vector, A46R, or A52R. Forty-eight hours after transfection, cells were left untreated or were stimulated with IL-1β (*A*; 10 ng/ml, 4 h), NE (*B*; 10 nM, 4 h), or PMA (*C*; 50 ng/ml, 4 h), and supernatants were retained. IL-8 levels in supernatants were measured by ELISA, and values were corrected to picograms per milligram of total protein. Data are expressed as relative IL-8 protein levels over the control value (#, $p \leq 0.05$, agonist vs control; $*$ or $**$, $p \leq 0.05$, inhibitor vs agonist). Assays were performed in triplicate and are representative of at least three separate experiments.

In this study we demonstrated that both A46R and A52R significantly inhibit IL-1- and NE-induced NF-κB reporter and IL-8 gene activities and IL-8 protein production in primary and transformed bronchial epithelial cells, with the poxvirus proteins mimicking the effects of truncated versions of endogenous signal transducers. This would suggest that A46R and A52R represent possible therapeutics in combating inflammation in the pulmonary environment. IL-8 is a member of the CXC chemokine superfamily and is a potent neutrophil chemotactic factor (20). In addition, cytokine array data implicate several other CXC chemokines in IL-1- and NE-driven inflammation, including ENA-78 and GRO family members, with induction of these chemokines also inhibited by A46R and A52R. Bronchial epithelial cells are known to produce ENA-78 in vitro in response to viral infection (42), and ENA-78 plays a role in lung inflammation, for example, in asthma, activating neutrophils, and helping to promote connective tissue remodeling (43). GRO family members, GRO-α, GRO-β, and GRO-γ, are neutrophil-specific chemokines and, as such, play a major role in the development of inflammatory responses. All three can be induced by respiratory syncytial virus in airway epithelial cells (44), and GRO-α was found to be present at higher concentrations than IL-8 in the bronchoalveolar fluid of adult respiratory distress syndrome patients (45). Along with these chemokines, both IL-1 and NE induce the cytokines IL-6 and oncostatin M, and this up-regulation is abrogated by A46R and A52R. Although IL-6 is a well-known proinflammatory cytokine, oncostatin M belongs to the same superfamily and has recently been implicated in the inflammatory responses in rheumatoid arthritis and multiple sclerosis (46, 47).

Several lines of evidence suggest that IL-8 has a wide range of actions not only on neutrophils, but also on various other cell types, including lymphocytes, monocytes, endothelial cells, and fibroblasts (48). Thus, IL-8, in concert with ENA-78 and the GRO family, plays a vital role in the pathophysiology of inflammatory

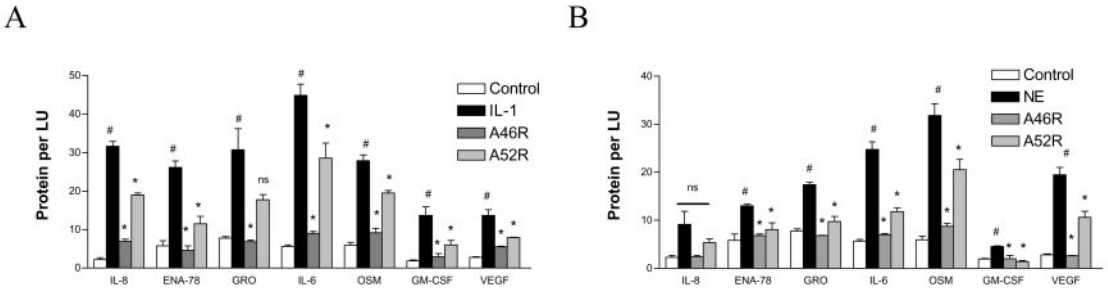

FIGURE 7. IL-1 and NE up-regulate multiple inflammatory genes that are inhibited by vaccinia virus proteins A46R and A52R. *A* and *B*, 16HBE14o$^-$ cells (1×10^5/ml) transfected with 200 ng of empty vector, A46R, or A52R. Twenty-four hours after transfection, cells were left untreated or were stimulated with IL-1β (*A*; 10 ng/ml, 4 h) or NE (*B*; 100 nM, 4 h), and supernatants were retained. Cytokine array membranes were incubated with cell supernatants, relative cytokine levels were measured using densitometry, and values were normalized according to transfection efficiency. Data are expressed as relative protein levels over the control value (#, $p \leq 0.05$ compared with unstimulated cells; ∗, $p \leq 0.05$ compared with empty vector control stimulated with IL-1β or NE). Assays were performed in triplicate and are representative of at least three separate experiments.

lung disease. These potent chemokines attract neutrophils to inflammatory sites, where their transepithelial passage is facilitated by ICAM-1 and their survival is prolonged by the hemopoietic cytokine GM-CSF (49, 50). In this study both A46R and A52R were found to inhibit IL-1- and NE-induced GM-CSF up-regulation in bronchial epithelial cells. Along with its proinflammatory and antiapoptotic effects, GM-CSF can increase TLR2 and TLR9 expression levels in neutrophils (51) and has a long-established role in lung inflammation (52). The viral proteins were also shown to interfere with IL-1- and NE-induced VEGF up-regulation. VEGF is induced by hypoxemia and inflammation and is expressed by many cells, including neutrophils and airway epithelial cells, with VEGF expression stimulated by IL-1, IL-6, and NE (53, 54). Although VEGF is known to play multifunctional roles in both the development of vasculature and the maintenance of vascular structure and function, it may have as yet to be described roles in the pulmonary inflammatory response.

In addition to inhibiting IL-1- and NE-induced inflammatory responses, the viral approach used in this study could block inflammation by many other proinflammatory stimuli, because multiple inflammatory mediators share the common pathway described. For example, lipopeptide (41), LPS (55), flagellin (56), and microbial DNA (57) use this conserved intracellular pathway via engagement of their cognate receptors, TLR2, TLR4, TLR5, and TLR9, respectively, and, as such, could be targeted by the viral proteins.

In the past, various attempts have been made to inhibit the separate and diverse inflammatory stimuli found in chronic inflammatory lung disease. These have focused primarily on antiproteases (21, 58), antioxidants (59), antimicrobials (60), recombinant human DNases (61), and IL-1R antagonist (62) with limited success due to the marked and disparate inflammatory burden. Because inflammation is a key contributor to the pathogenesis of chronic lung disease, directed anti-inflammatory therapy must assume a larger role in treatment. The discovery that vaccinia virus proteins can interfere with the expression of multiple inflammatory genes induced by IL-1 and the serine protease NE in bronchial epithelial cells is promising and raises the prospect of developing them as therapeutic anti-inflammatory agents for the treatment of chronic inflammatory lung diseases.

Disclosures

The authors have no financial conflict of interest.

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