Interleukin-10 Inhibits Elevated Chemokine Interleukin-8 and Regulated on Activation Normal T Cell Expressed and Secreted Production in Cystic Fibrosis Bronchial Epithelial Cells by Targeting the I_kB Kinase α/β Complex

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Accumulating evidence suggests that in cystic fibrosis (CF) patients, airway fluids are characterized by decreased antibacterial activity, elevated NaCl concentration, and high levels of chemokines, resulting in exaggerated activation of the transcriptional nuclear factor (NF)- κ B in airway epithelial cells. The present study was undertaken to evaluate the effects of anti-inflammatory cytokine interleukin-10 (IL-10) on NaCl-induced chemokine IL-8 and regulated on activation normal T cell expressed and secreted (RANTES) expression through the NF- κ B signaling in primary Δ F508 CF and non-CF (control) human bronchial epithelial cells. Exposure of CF and non-CF bronchial epithelial cells to hypertonic (170 mmol/L NaCl) milieu compared to isotonic (115 mmol/L NaCl) and hypotonic (85 mmol/L NaCl) milieu caused a significant, NaCl-dependent increase in IL-8 and RANTES gene expression and protein production. Compared to non-CF cells, CF bronchial epithelial cells were characterized by a higher susceptibility to produce elevated IL-8 and RANTES production in an hypertonic NaCl milieu in response to IL-1 β activation. Treatment with IL-10 suppressed IL-8 and RANTES gene expression in both non-CF and CF bronchial epithelial cells was associated with a reduced expression of I_kB (IKK) α/β kinases, particularly for IKK α which is greater expressed in CF bronchial epithelial cells, and resulting in reduced NF- κ B activation. These findings suggest that IL-10 might have anti-inflammatory benefits in airways of CF patients. (*Am J Pathol* 2003, 162:293–302)

In human airways, inflammatory mediator production by epithelial cells is a critical determinant of chronic airway inflammation. In CF patients, the chloride transport defect¹ caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein in surface airway epithelium and submucosal gland cells^{2,3} is translated into early excessive inflammation and bacterial infection, which are the main causes of progressive lung destruction and mortality. Infants with CF, even without detectable pulmonary bacterial infection, have elevated numbers of neutrophils and high levels of the neutrophil chemoattractant interleukin (IL)-8 in their airways.^{4,5} Recent clinical data from CF infants have confirmed that the lung inflammatory response is excessive relative to the endotoxin activity and bacterial count evaluated in bronchoalveolar lavage fluids compared to non-CF infants with a similar burden of endotoxin and bacteria.⁶ Elevated IL-8 levels have been shown in airway lumen of human fetal tracheal grafts from uninfected CF bronchial tissues compared to non-CF bronchial tissues.⁷ Absence of functional CFTR is associated with defective regulation of gap junctional communication in CF airway epithelial cells, contributing to an excessive inflammatory response to tumor necrosis factor (TNF)- α .⁸ Moreover, the expression of Δ F508 CFTR in Chinese hamster ovary cells, which do not express CFTR, revealed a sevenfold activation of nuclear factor (NF)- κ B compared to wild-type CFTR or the G551D mutant, which was paralleled with high IL-8 expression.⁹ Thus, accumulating evidence suggests that in CF, excessive NF- κ B activation causes a primary inflammatory disorder in airways.

Changes in airway osmolarity have been involved in the pathogenesis of CF. Defective CFTR function in airways leads to reduced absorption of NaCl in surface epithelium, generates a hypertonic microenvironment (170 mmol/L)¹⁰ so that salt-sensitive antibacterial pep-

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tides appear inefficient against *Pseudomonas aeruginosa* infection in CF airway epithelium.^{11,12} Not all investigators have found CF airway surface fluid to have elevated NaCl concentration (called the "high salt hypothesis"), but rather found evidence for high rates of absorption of the liquid layer (called the "isotonic/low volume hypothesis") coating CF airway epithelial cells.^{13,14} The resolution of this controversy (abnormal ion composition or abnormal water volume) is key to understanding the lung pathophysiology and to treat the initial events of CF disease.^{15,16}

How defective CFTR function affects the regulation of NF- κ B-dependent genes remains to be determined. New evidence supports the idea that defective CFTR causes a dysregulation of cytokine expression both in airway epithelial cells and blood peripheral T cells of CF patients. Dysregulation includes absence or low levels of the anti-inflammatory cytokine IL-10^{17,18} and elevated production of NF- κ B-dependent chemokines.^{9,19} Exaggerated activation of NF- κ B associated with altered I κ B- β processing and high IL-8 production has been described in a CF bronchial epithelial cell line (IB3 cells).^{20,21} In CF patients, an absence or decrease in IL-10 may be a pre-existing immunoregulatory abnormality that allows inflammation to persist in lung tissues.²² Using animal models, it has been reported that IL-10 blocks lipopolysaccharide-induced biosynthesis of IL-1 β and TNF- α and reduced NF- κ B activation in rat alveolar epithelium.²³ Recent evidence suggests that local IL-10 deficiency contributes to prolonged inflammatory responses that is coupled with activation of NF- κ B and impaired regeneration of I κ B α inhibitor in lungs of IL-10 knockout mice to acute *P. aeruginosa* challenge.²⁴

The action mechanisms of IL-10 in CF human bronchial epithelium are as yet undefined. Understanding of molecular targets of IL-10 is of great interest because it may lead to the development of novel therapeutic strategies in airways of CF patients. To examine the effects of IL-10 in NF- κ B-dependent genes in bronchial epithelial cells, we investigated whether exogenous IL-10 diluted in hypotonic, isotonic, and hypertonic NaCl milieu affected the production of chemokines IL-8 and regulated on activation normal T cell expressed and secreted (RANTES) by targeting the NF- κ B/I κ B α signaling in primary CF and non-CF human bronchial gland epithelial cells. We demonstrate, for the first time, that IL-10 blocks the IL-8 and RANTES expression in both CF and non-CF bronchial epithelial cells through a marked reduction of NF- κ B activity and a decreased I κ B kinase α/β (IKK α/β) expression, particularly for IKK α which is greater expressed in CF bronchial epithelial cells. Therefore, blocking NF- κ B activity in epithelial cells with IL-10 treatment seems to be a very attractive strategy for reducing inflammation in airways of CF patients.

Materials and Methods

Cell Culture

Cell isolation and subculture procedures of human bronchial gland epithelial cells were performed on human

bronchial tissues collected from eight Δ F508 homozygous CF patients and four non-CF patients, as described previously.²⁵ Briefly, cells were isolated by enzymatic digestion from bronchial submucosa and grown on type I collagen-coated 25-cm² tissue culture flasks in a Dulbecco's modified Eagle's medium/Ham's F12-mixture (50/50%, v/v) supplemented with 1% Ultrosor G (a serum substitute from Sepracor, Villeneuve-la-Garenne, France), glucose (10 g/L) and sodium pyruvate (0.33 g/L). Penicillin G (100 U/ml) and streptomycin (100 μ g/ml) were also added. Using the halide-sensitive fluorescent dye 6-methoxy-N-(3-sulfopropyl)-quinolinium, we have previously shown a significant increase in Cl⁻ channel activity via the CFTR protein in non-CF bronchial epithelial cells in response to forskolin treatment (demonstrating a cAMP-dependent activation of a Cl⁻ efflux), which is not preserved in cultured CF bronchial gland cells.²⁶

Exposure of Bronchial Epithelial Cells to Different Extracellular NaCl Solutions

Subconfluent cultures of non-CF and CF bronchial gland cells were placed in an Ultrosor G-free RPMI 1640 medium for 16 hours and exposed for a further 4-hour period to Cl⁻ solutions containing either 85 mmol/L, 115 mmol/L, or 170 mmol/L Cl⁻, respectively, with or without IL-1 β (20 ng/ml), TNF- α (20 ng/ml), or recombinant human IL-10 (rhIL-10, 20 ng/ml) (Calbiochem, San Diego, CA). The three chloride-containing solutions used in this study (85 mmol/L Cl⁻, 115 mmol/L Cl⁻, and 170 mmol/L Cl⁻) contained 1 mmol/L CaCl₂, 20 mmol/L KCl, and either 60 mmol/L, 85 mmol/L, or 148 mmol/L NaCl, respectively, at pH 7.4, as previously reported.²⁵ All reagents were molecular biology grade, and all buffers and solutions were prepared using pyrogen-free grade water for which no endotoxins were detected by chromogenic limulus amoebocyte lysate (LAL) assay (BioWhittaker, Emerainville, France). Immediately after each period of CF and non-CF bronchial gland cell exposure, supernatants were collected and stored at -80°C until tested by enzyme-linked immunosorbent assay (ELISA) for the presence of cytokines IL-8 and RANTES. Cell viability of cultured CF and non-CF bronchial gland cells always exceeded 97%, as determined by the trypan blue exclusion test after all experimental interventions.

ELISA for IL-8 and RANTES

The ELISAs for IL-8 and RANTES detection, which were sensitive down to a level of 5 pg/ml and 2 pg/ml, respectively, were performed in the culture supernatants by following the manufacturer's instructions in commercially available ELISA kits (Biosource International, Camarillo, CA). Data were expressed either as pg/ml/viable 10⁶ cells or as released cytokine percentage compared to 100% control value.

RNase Protection Assay

Quantification of steady-state cytokine mRNA levels of non-CF and CF bronchial gland cell cultures placed in an Ultrosor G-free DMEM/F12 medium for 16 hours and exposed for a further 1-hour period to Cl^- solutions containing either 85 mmol/L, 115 mmol/L, or 170 mmol/L Cl^- , with or without rhIL-10 (20 ng/ml) were performed by the RiboQuant multi-Probe RNase protection assays (RPA) using the hCK-5 RPA template set (Pharmingen, San Diego, CA). In brief, total RNA was isolated with TRIzol reagent (Life Technologies, Paris, France) according to the manufacturer's instructions. Lysate was run through a 22-gauge needle and extracted with phenol/chloroform. Samples were mixed vigorously and incubated at room temperature for 2 minutes then spun at $12,000 \times g$ for 15 minutes, the aqueous phase collected and the RNA precipitated with equal volumes of isopropyl alcohol. Each final RNA pellet was resuspended in 50 μl of diethylpyrrocarbonate-treated water. Multiprobe, hCK5, which contains templates for Ltn, RANTES, MCP-1, IP-10, MIP-1 β , MIP-1 α , MCP-1, IL-8, I309, and the housekeeping genes L-32 and GAPDH, was labeled with [α - ^{32}P]-UTP using T7 RNA polymerase. Labeled probe (3×10^6 cpm) was hybridized to 5 μg of total RNA for 16 hours at 56°C . The protected RNA duplexes were purified by phenol-chloroform extraction and ethanol precipitation, and the pellets were resuspended in 5- μl portions of RPA loading buffer (80% formamide, $0.5\times$ Tris-borate-acetate, 0.05% bromophenol blue). Protected hybrids were resolved on a 6% polyacrylamide-8 mol/L urea-denaturing sequencing gel and exposed to radiographical film (Kodak X-Omat AR film; Kodak, Paris, France). To correct for RNA loading, each intensity score was normalized to the intensity of hybridization for the GAPDH gene.

Nuclear Protein Extracts and Electrophoretic Mobility Shift Assay

NF- κB DNA-binding activity was assessed by electrophoretic mobility shift assay, as previously described.²⁵ Briefly, nuclear extracts of CF and non-CF bronchial epithelial cells placed in Ultrosor G-free DMEM/F12 medium for 16 hours and exposed for a further 1-hour period to 170 mmol/L Cl^- solution, with or without rhIL-10 (20 ng/ml), were incubated with ^{32}P -labeled NF- κB oligonucleotide. The consensus κB DNA sequence used for the electrophoretic mobility shift assay was 5'AGT TGA GGG GAC TTT CCC AGG C3' (Promega Corp., Madison, WI). The oligonucleotide was radiolabeled by the T4 polynucleotide kinase (Pharmacia Biotech, Paris, France) enzyme with [α - ^{32}P]-ATP. The protein-DNA complexes were electrophoresed on a nondenaturing 5% polyacrylamide gel, then dried under vacuum and exposed at -80°C with autoradiographic film. In competition studies and supershift assays, a 100-fold molar excess of unlabelled oligonucleotide or 1 μg of antibodies was added to the binding reaction mixture, before the addition of the labeled κB probe. Identification of the different NF- κB heterodimeric proteins was performed by incubating the

nuclear extracts with polyclonal antibodies against the NF- κB proteins NF- κB 1 (p50) and Rel (p65) RelA (Santa Cruz Biotechnology, Santa Cruz, CA), before the addition of the labeled κB probe.

Cell Extracts and Western Blot Analysis

Subconfluent cultures of non-CF and CF bronchial epithelial cells were placed in Ultrosor G-free DMEM/F12 medium for 16 hours and exposed for a further 4-hour period to Cl^- solutions containing either 85 mmol/L, 115 mmol/L, or 170 mmol/L Cl^- , with or without rhIL-10 (20 ng/ml). After each saline treatment, cells were washed in phosphate-buffered saline (pH 7.2) and total protein was extracted in RIPA buffer, as previously described.²⁵ Protein extracts were centrifuged ($12,000 \times g$, 5 minutes, 4°C) and protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein were boiled for 4 minutes in Laemmli buffer, electrophoresed under denaturing conditions using 4 to 15% polyacrylamide gels (Pharmacia Biotech, Orsay, France) that were then transferred onto a nitrocellulose membrane (Millipore, Bedford, MA). Membranes were blocked with Tris-buffered saline containing Tween 20 (40 mmol/L Tris, pH 7.6, 300 mmol/L NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 1 hour at room temperature before exposure to rabbit polyclonal anti-human $\text{I}\kappa\text{B}\alpha$ and anti- $\text{I}\kappa\text{B}\beta$ (Santa Cruz Biotechnology, Santa Cruz, CA). For analysis of $\text{I}\kappa\text{B}$ α/β kinases (IKK α and IKK β), membranes were exposed to rabbit polyclonal anti-IKK α and IKK β antibodies, respectively (Santa Cruz Biotechnology Inc.). The level of phosphorylated $\text{I}\kappa\text{B}\alpha$ was analyzed by Western blot using a polyclonal phospho-specific anti- $\text{I}\kappa\text{B}\alpha$ (New England Biolabs, Beverly, MA) antibody that detects $\text{I}\kappa\text{B}\alpha$ only when activated by phosphorylation at Ser-32. Proteins were visualized using horseradish peroxidase-conjugated donkey anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) and the enhanced chemiluminescence detection kit (Amersham Life Science, Arlington Heights, IL). Densitometric analyses of Western blots were performed on a Fujifilm model LAS 1000 imaging densitometer using AIDA 2D densitometry software (Aida Ray test, Paris, France). The intensities of bands were compared on the basis of adjusted volume (mean optical density $\times$ area in square millimeters).

Results

IL-8 and RANTES Gene Expression in Non-CF and CF Bronchial Epithelial Cells Are Increased with Increasing Extracellular NaCl Content

To mimic the *in vivo* situation, in which a change in NaCl concentration is observed in human CF bronchial liquids,¹¹ we first analyzed the effects of increasing extracellular NaCl solution (85 mmol/L, 115 mmol/L, and 170 mmol/L of NaCl) on the gene expression of several chemokines. As shown in Figure 1, IL-8 and RANTES mRNA

Figure 1. Steady-state levels of IL-8 and RANTES mRNA from non-CF *versus* $\Delta F508$ homozygous CF bronchial epithelial cells after their exposure for 1 hour to increasing extracellular NaCl concentrations (85 mmol/L, 115 mmol/L, and 170 mmol/L NaCl). RPAs were performed by the RiboQuant multi-Probe RPA using the hCK-5 RPA template set (Pharmingen). Note that the IL-8 and RANTES mRNA levels increased with increasing extracellular NaCl content. Compared to the positive control (lane 1), no mRNA of lymphotactin (Ltn), IP-10, MIP-1 β , MIP-1 α , MCP-1, and, I-309 were detected under any conditions. Band intensities for the housekeeping genes, L32 and GAPDH, indicate that similar amounts of sample were loaded in each lane. Data are representative of three separate experiments.

expression could be detected in non-CF and in CF human bronchial epithelial cells using the RPA method. There was no detectable expression of mRNA for other chemokines, ie, lymphotactin, IP-10, MIP-1 β , MIP-1 α , MCP-1, and I-309 in NaCl-treated CF and non-CF bronchial epithelial cells. Interestingly, increasing the extracellular NaCl milieu, from 85 mmol/L to 170 mmol/L, increased the steady-state level of RANTES and IL-8 mRNA. When normalized to the housekeeping genes, L32 and GAPDH, levels of RANTES and IL-8 mRNA were higher in CF bronchial epithelial cells as compared to non-CF bronchial epithelial cells, regardless of the NaCl concentration. In CF bronchial epithelial cells, we demonstrated a significant threefold and 2.5-fold increase in RANTES and IL-8 mRNA expression compared to that obtained in non-CF bronchial epithelial cells in 85- and 170-mmol/L NaCl milieu, respectively, as evaluated by densitometric analyses (data not shown).

IL-1 β but Not TNF- α Stimulates NaCl-Mediated Production of IL-8 and RANTES in Non-CF and CF Bronchial Epithelial Cells

Given that IL-1 β and TNF- α are considered to be pivotal cytokines in the initiation of the early inflammatory process in the response to infection, and to determine the pattern of later cytokine production in airways, it was important to investigate the effect of these two proinflam-

Figure 2. Levels of IL-8 (A and B) and RANTES (C and D) protein secreted in cultured human non-CF *versus* CF bronchial epithelial cells after their exposure for 4 hours to increasing extracellular NaCl concentrations (85, 115, and 170 mmol/L) in the presence or absence of TNF- α (20 ng/ml) or IL-1 β (20 ng/ml). Note different y axis scale for each cytokine panel. Values in the ELISAs (means $\pm$ SD) are representative of three experiments and are reported as pg of IL-8 or RANTES/ml/ 10^6 cells. Each assay was performed at least in duplicate. *, $P < 0.05$ compared with the control (in the absence of IL-1 β or TNF- α); ***, $P < 0.001$.

matory cytokines on NaCl-induced IL-8 and RANTES production in CF and non-CF bronchial epithelial cells. Cultures of CF and non-CF bronchial epithelial cells were stimulated for 4 hours with 20 ng/ml of IL-1 β or TNF- α in increasing extracellular NaCl milieu from 85 mmol/L to 170 mmol/L NaCl. Exposure of resting CF bronchial epithelial cells to isotonic (115 mmol/L) and hypertonic (170 mmol/L) NaCl milieu, resulted in statistically significant threefold and fivefold increases ($P < 0.001$) in IL-8 and RANTES release (Figure 2, B and D), compared to similarly treated non-CF bronchial epithelial cells (Figure 2, A and C). Interestingly, IL-1 β stimulation, but not TNF- α , significantly enhanced ($P < 0.001$) the NaCl-induced IL-8 and RANTES production in CF and non-CF bronchial epithelial cells. In CF bronchial epithelial cells, the IL-8 and RANTES production induced by IL-1 β (Figure 2, B and D) was markedly increased with increasing extracellular NaCl milieu (from 1.5-fold in an isotonic milieu to 6.5-fold in an hypertonic milieu). Thus, these data clearly show that CF bronchial epithelial cells are characterized by a higher susceptibility to produce high IL-8 and RANTES protein level in response to IL-1 β activation associated with an hypertonic NaCl milieu. The stimulation of both non-CF and CF bronchial epithelial cells by IL-1 β was therefore chosen for the following experiments.

IL-10 Is a Potent Inhibitor of IL-8 and RANTES Expression in Non-CF and CF Bronchial Epithelial Cells

It was subsequently of interest to determine whether increasing extracellular NaCl milieu may modulate the inhibitory effect of IL-10 on IL-8 and RANTES expression in unstimulated and IL-1 β -stimulated non-CF and CF bronchial epithelial cells. Under similar culture conditions (ie, throughout a 4-hour period in an unstimulated resting

Figure 3. Effects of IL-10 treatment on the levels of IL-8 (A and B) and RANTES (C and D) protein released by unstimulated and IL-1 β -stimulated non-CF and CF human bronchial epithelial cells in increasing extracellular NaCl concentrations (85 mmol/L, 115 mmol/L, and 170 mmol/L). Note different y axis scale for each cytokine panel. IL-8 and RANTES levels were assayed in 4-hour supernatants collected from cell cultures exposed to IL-1 β (20 ng/ml), IL-10 (20 ng/ml), or both. Values in ELISAs (means $\pm$ SD) are representative of three experiments and are reported as pg of IL-8 or RANTES/ml/10⁶ cells. Each assay was performed at least in duplicate. *, $P < 0.05$ compared with the control (in the absence of IL-1 β or TNF- α); ***, $P < 0.001$.

state), exposure of non-CF and CF bronchial epithelial cells to IL-10 (20 ng/ml) caused a significant two-fold reduction ($P < 0.001$) of IL-8 and RANTES secretion in the two cell types, regardless of the hypoisotonic and hypertonic NaCl milieu used (Figure 3). Interestingly, the highest IL-8 and RANTES protein production that was observed in IL-1 β -stimulated CF bronchial epithelial cells placed in hypertonic milieu (Figure 3, B and D) was significantly reduced ($P < 0.001$) with IL-10 treatment to a level similar to that observed in unstimulated CF bronchial epithelial cells.

To evaluate whether or not the reduction in NaCl-induced IL-8 and RANTES production was because of decreased mRNA expression in IL-10-treated non-CF and CF bronchial epithelial cells, the cells were exposed to IL-10 (20 ng/ml) for 1 hour and analyzed using the RPA method. As shown in Figure 4, we observed that NaCl-induced IL-8 and RANTES mRNA expression was blocked by IL-10 treatment in non-CF and CF bronchial epithelial cells.

IL-10 Reduces the NF- κ B Activation in Non-CF and CF Bronchial Epithelial Cells

Nuclear extracts of non-CF and CF bronchial epithelial cells maintained for 1 hour in an hypertonic NaCl milieu were prepared and incubated with an end ³²P-labeled DNA oligonucleotide containing the NF- κ B recognition site. As demonstrated by electrophoretic mobility shift assay (Figure 5), a higher NF- κ B-DNA-binding activity was demonstrated in the nuclear protein extracts of CF bronchial epithelial cells compared with that observed in non-CF bronchial epithelial cells (Figure 5A, compare lane 3 to lane 1). Exposure of 170 mmol/L NaCl-treated non-CF bronchial epithelial to IL-10 cells suppressed the activation of NF- κ B (Figure 5A, lane 2) and also led to a

Figure 4. Steady-state levels of IL-8 and RANTES mRNA of non-CF versus Δ F508 homozygous CF bronchial epithelial cells after their exposure for 1 hour to IL-10 (20 ng/ml) in increasing extracellular NaCl concentrations (85 mmol/L, 115 mmol/L, and 170 mmol/L). IL-10 treatment of non-CF and CF cell cultures markedly reduced the NaCl-induced IL-8 and RANTES mRNA expression, regardless of the extracellular NaCl concentration. A representative experiment is shown and similar results were seen in two other independent experiments.

significant reduction of NF- κ B binding in CF bronchial epithelial cells (Figure 5A, lane 4). The specificity of NF- κ B-DNA binding was confirmed in competition experiments with a 100-fold excess of unlabeled cold NF- κ B oligonucleotide, which led to a complete inhibition of the binding activity (Figure 5B, lane 5). Supershift assay confirmed the presence of p65 subunits of NF- κ B (Figure 5B, lane 6), and a similar result was observed with antibodies to p50 (data not shown).

IL-10 Reduces Phosphorylated I κ B α and Increases I κ B β Inhibitor in Non-CF and CF Bronchial Epithelial Cells

Because NF- κ B exists as an inactive form bound to two major inhibitory proteins I κ B α and I κ B β in the cytoplasm, activation of NF- κ B occurs via phosphorylation of two serine residues of I κ B α at positions 32 and 36 followed by the degradation of I κ B α .^{27,28} To measure I κ B α phosphorylation, we used a phospho-specific anti-I κ B α -antibody that detects I κ B α only when activated by phosphorylation at Ser-32. We examined the phosphorylation of I κ B α over the hypotonic, isotonic, and hypertonic salt conditions in non-CF and CF bronchial epithelial cells with or without IL-10 treatment. In striking contrast to non-CF bronchial epithelial cells in which levels of phosphorylated I κ B α were salt-dependent, high levels of phosphorylated I κ B α were found in CF bronchial epithelial cells (up to a 2.2-fold increase), even in hypotonic (85 mmol/L) NaCl solution and did not change in accordance to the hypotonic, isotonic, and hypertonic salt conditions (Figure 6, A and B). Interestingly, exposure of CF bronchial epithelial cells to IL-10 resulted in a marked reduction of phosphorylated I κ B α to levels similar of that observed in untreated non-CF bronchial epithelial cells, regardless of the NaCl milieu used (Figure 6). We also demonstrated that CF bronchial epithelial cells expressed an elevated basal level of I κ B β

Figure 5. NF- κ B binding activity in non-CF *versus* Δ F508 homozygous CF bronchial epithelial cells after their exposure in 170 mmol/L of NaCl in the presence and absence of IL-10 (20 ng/ml, 1 hour) (A, lanes 1 to 4). To demonstrate the specificity of binding of the NF- κ B oligonucleotide, a 100-fold molar excess of unlabelled NF- κ B (B, lane 5, cold κ B) was used to compete with the labeled NF- κ B probe. The addition of an antibody to RelA (p65 subunit) (B, lane 6, p65) caused a supershift, as indicated.

compared with similarly NaCl-treated non-CF bronchial epithelial cells, and that IL-10 treatment enhanced its expression in non-CF (up to 250%) and CF (up to 20%) bronchial epithelial cells (Figure 7).

IL-10 Reduces $I_{\kappa}B$ Kinase α/β (IKK α and IKK β) Expression in Non-CF and CF Bronchial Epithelial Cells

The IKK complex is made up of two kinases IKK α and IKK β , which phosphorylate $I_{\kappa}B\alpha$ leading to its degradation and the translocation of NF- κ B to the nucleus.²⁷ To determine whether or not the IL-10-induced reduction of

Figure 6. Expression levels of phosphorylated $I_{\kappa}B\alpha$ ($I_{\kappa}B\alpha$ -P) in non-CF (lanes 1 to 6) and CF (lanes 7 to 12) bronchial epithelial cells after their exposure to increasing extracellular NaCl concentrations (from 85 mmol/L to 170 mmol/L) in the presence or absence of rhIL-10 (20 ng/ml, 4 hours). Levels of $I_{\kappa}B\alpha$ -P were detected with a phospho-specific anti- $I_{\kappa}B\alpha$ antibody that only recognizes $I_{\kappa}B\alpha$ -phosphorylated on Ser-32. Equal amounts of total protein (10 μ g/lane) were loaded in each lane, and proteins were resolved by electrophoresis in a 10% SDS-polyacrylamide gel. A representative experiment is shown in A, and results of three experiments are represented in B expressed in arbitrary units (a.u.) compared to the $I_{\kappa}B\alpha$ -P level found in non-CF bronchial gland cells exposed to 85 mmol/L NaCl (lane 1).

phosphorylated $I_{\kappa}B\alpha$ (Figure 6) is related to reduced IKK α and IKK β levels, Western blot analyses of two kinases were performed from cytoplasmic extracts of CF and non-CF bronchial epithelial cells placed in hypotonic, isotonic, and hypertonic NaCl milieu with or without the addition of IL-10. Interestingly, image analysis of digitized Western blots demonstrated that IKK α levels in CF bronchial epithelial cells were increased by more than 300% whatever the NaCl milieu used, as compared to the IKK α level obtained in non-CF bronchial epithelial cells in hypotonic NaCl solution (Figure 8, A and B, compare lanes 7 to 9 to lane 1). Exposure of non-CF bronchial epithelial cells to IL-10 markedly reduced the IKK α level (by 250%) and to a lesser extent (by 30%) in CF bronchial epithelial cells (Figure 8; compare lanes 4 to 6 to lane 1 and lanes 10 to 12 to lane 7, respectively). In contrast to elevated IKK α levels that were found to be NaCl-dependent in non-CF bronchial epithelial cells, we detected low IKK β levels in both non-CF and CF bronchial epithelial cells (Figure 9). After IL-10 treatment, we noted a similar reduction of IKK β expression (45% to 50%) in both non-CF

Figure 7. Expression of $I_{\kappa}B\beta$ inhibitor in non-CF and Δ F508 homozygous CF bronchial epithelial cells after their exposure to increasing extracellular NaCl concentrations (from 85 mmol/L to 170 mmol/L) in the presence or absence of IL-10 (20 ng/ml). Equal amounts of total protein from non-CF and Δ F508 homozygous CF cells treated with or without IL-10 were analyzed for levels of cytosolic $I_{\kappa}B\beta$ by Western blotting using specific antibodies to $I_{\kappa}B\beta$ (A). Densitometric analyses of the data combined with three similar studies are expressed in arbitrary units (a.u.) and are reported as the percentage of $I_{\kappa}B\beta$ compared with untreated non-CF bronchial epithelial cells (B, lane 1). One representative experiment of three independent experiments is shown.

Figure 8. Expression of IKK α levels in non-CF and Δ F508 homozygous CF bronchial epithelial cells after their exposure to increasing extracellular NaCl concentrations (from 85 to 170 mmol/L) in the presence or absence of rIL-10 (20 ng/ml) for 4 hours, as demonstrated by Western blotting (**A**). Equal amounts of total protein from non-CF and Δ F508 homozygous CF cells treated with or without IL-10 were analyzed for levels of IKK α . Densitometric analyses of the data combined with three similar studies are expressed in arbitrary units (a.u.) and are reported as the percentage of IKK α compared with untreated non-CF bronchial epithelial cells (**A** and **B**, lane 1). One representative experiment of three independent experiments is shown.

and CF bronchial epithelial cells (Figure 9, compare lanes 4 to 6 to lanes 1 to 3 and lanes 10 to 12 to lanes 7 to 9, respectively).

Discussion

In CF disease, there is increasing evidence showing that dysregulation of inflammatory and immune response involves surface airway epithelial cells,^{21,22} submucosal airway gland epithelial cells,^{26,29} and blood T lymphocytes,³⁰ by demonstrating a severe deficiency in the production of anti-inflammatory cytokine IL-10. A recent study confirms that functional IL-10 deficiency in the lung of CF (cfr^{-/-}) and IL-10 knockout mice causes excessive lung immune responses coupled with increased production of B7 co-stimulatory molecules of alveolar macrophages.³¹

In the present study, we demonstrate for the first time that exogenous rIL-10 inhibits constitutive NF- κ B activation in Δ F508 homozygous CF human bronchial epithelial cells that is paralleled with a strong reduction of chemo-

Figure 9. Expression of IKK β levels in non-CF and Δ F508 homozygous CF bronchial epithelial cells after their exposure to increasing extracellular NaCl concentrations (from 85 mmol/L to 170 mmol/L) in the presence or absence of IL-10 (20 ng/ml) for 4 hours, as demonstrated by Western blotting (**A**). Equal amounts of total protein from non-CF and Δ F508 homozygous CF cells treated with or without IL-10 were analyzed for IKK β levels. Densitometric analyses of the data combined with three similar studies are expressed in arbitrary units (a.u.) and are reported as the percentage of IKK β compared with untreated non-CF bronchial epithelial cells (**A** and **B**, lane 1). One representative experiment of three independent experiments is shown.

kines IL-8 and RANTES gene and protein expression. Endogenous NF- κ B activation associated with elevated IL-8 expression had been recently shown to be dependent on Δ F508 CFTR mistrafficking in transfected Chinese hamster ovary cells by eliciting an estrogen receptor stress reaction and impaired CFTR Cl⁻ channel activity.^{9,32}

Airway epithelium play a pivotal role in the regulation of ionic content of the connective tissue in the airway wall, and modulation of the composition of the airway liquid or influencing signal transduction pathways will provide a promising target for new therapies in CF. Our interest in studying the molecular mechanisms by which IL-10 down-regulates IL-8 and RANTES expression in different extracellular NaCl milieu stems from the salt controversy of CF airway liquids over whether abnormalities in NaCl level (the salt hypothesis)¹⁰ or water volume (the hydration hypothesis)^{13,14} of airway epithelium initiate CF airway disease. In the increased salt model,¹⁰ NaCl transport depends on CFTR, and the high CFTR expression demonstrated in submucosal gland cells^{2,3} compared to low expression in surface airway epithelial cells, suggests that submucosal gland cells have higher absorptive fluid rates. In CF disease, the lack of functional CFTR is predicted to abolish transcellular absorption and increase NaCl content of airway liquid. Consequently, the salt model predicts to add a hypotonic solution to decrease NaCl concentration in CF airways. At the opposite, the hydration model^{14,33} shows lower water volume with Cl⁻ level similar to normal airway fluid and isotonic with plasma. Thus, therapeutic goal predicted by the isotonic volume model is to add salt, and consequently water, to volume-depleted CF airways.¹⁴ In agreement with other reports,^{34,35} we found that increasing extracellular NaCl concentration led to elevated IL-8 and RANTES expression by human bronchial epithelial cells. Both elevated IL-8 and RANTES production contribute to the massive recruitment of eosinophils and neutrophils into CF airways. A high level of released cytotoxic proteins from eosinophils from CF patients has been reported, suggesting that these inflammatory cells and their mediators may contribute to progressive tissue damage in CF.³⁶ Chemokine IL-8-induced recruitment of neutrophils to the airways results in release of elastase and additional induction of IL-8 gene expression by bronchial epithelial cells, thereby perpetuating a chronic cycle of CF airway inflammation.³⁷ A recent study suggests that neutrophil elastase induces IL-8 gene up-regulation in bronchial epithelial cells through a MyD88/IRAK/TRAF-6-dependent pathway, resulting in degradation of I κ B β and enabling NF- κ B translocation to the nucleus.³⁸

Our findings that elevated RANTES mRNA and protein production were increased in CF bronchial epithelial cells compared to those observed in non-CF bronchial gland cells contrasts with results reported by Schwiebert and colleagues,³⁹ showing no detectable RANTES expression from cultured CF surface epithelial cell lines. One possible explanation for this difference is that signaling transduction pathways may be differently implicated in primary CF bronchial epithelial cells and bronchial epithelial cell lines. Recent reports demonstrate that the

induction of RANTES expression requires activation of NF- κ B in primary airway epithelial cells,⁴⁰ and the RANTES and IL-8 gene expression are differently regulated by NF- κ B.⁴¹ In agreement with our results, the differential regulation of RANTES and IL-8 production might be cell-type-specific.

Our data also show that treatment with IL-10 of CF and non-CF bronchial epithelial cells resulted in the reduction of phosphorylated I κ B α , an enhanced production of I κ B β inhibitor and a marked reduction of IKK α / β kinase expression associated with an inhibition of activated NF- κ B in both cell types. It has been reported that IL-10 knockout mice have prolonged and excessive proinflammatory cytokine production and neutrophil infiltration in the airways compared to wild-type mice.²⁴ Both wild-type and IL-10 knockout mice displayed reduced lung I κ B α levels after acute lung infection; but the IL-10 knockout mice fail to recover I κ B α at a normal rate once degraded and subsequently prolonged the activation of NF- κ B.

In our study, we show that IL-10 inhibits IL-8 and RANTES gene and protein expression through preventing NaCl-induced phosphorylation of I κ B α and reduced NF- κ B activity in CF bronchial epithelial cells. Whether or not destabilization or impaired regeneration of I κ B α is specific for Δ F508 CF human airway epithelium is currently unknown. We previously demonstrated that a lack of I κ B α and increased I κ B β inhibitor in Δ F508 homozygous CF human bronchial epithelial cells were associated with activated NF- κ B and increased IL-8 production *in vivo* and *in vitro*.²⁶ The I κ B β inhibitor has been described to be involved in the regulation of the persistent response in a biphasic activation of NF- κ B activity in tissues lacking I κ B α , because no feedback inhibition through increased synthesis of I κ B α would occur in these tissues.⁴² As noted in our study, elevated levels of I κ B β has been also reported in a CF (IB3) pulmonary epithelial cell line and is hypophosphorylated under basal conditions enabling NF- κ B with bound I κ B β complexes to direct transcription.²¹

The I κ B α / β kinase (IKK α / β) complex represents a potential convergence point for multiple signaling stimuli that activate NF- κ B via the phosphorylation of I κ B α .²⁷ Several reports have yielded promising results that suggest that NF- κ B is a key target for development of anti-inflammatory therapeutics for CF. We have previously demonstrated that genistein inhibited constitutive NF- κ B activity in CF bronchial epithelial cells, and down-regulated IL-8 production.⁴³ Overexpression of adenovirus-mediated I κ B α and liposome-mediated transfection with decoy oligonucleotides in normal and CF respiratory epithelial cell lines has been shown to reduce TNF- α -induced IL-8 secretion and NF- κ B activation.^{44,45}

In the present study, we show that, in non-CF and CF human bronchial epithelial cells, the intracellular targets of IL-10 action involve the I κ B kinase (IKK α / β) complex that play a pivotal role of cytokine-mediated NF- κ B activation. By IL-10 treatment, we observed a reduced NF- κ B activity and a decreased IKK α / β kinase expression, particularly for IKK α which is greater expressed in CF bronchial epithelial cells compared to non-CF bronchial epithelial cells. Surprisingly, we observed that

NaCl-dependent IL-8 and RANTES gene and protein expression was accompanied with no significant modification of phosphorylated I κ B α and IKK α / β expression in CF epithelial cells. These findings suggest the involvement of other factors potentially regulating the NaCl-dependent IL-8 and RANTES expression. Recent studies,^{34,35} have demonstrated that the MAP kinase pathway plays a prominent role in the IL-8 expression by normal bronchial epithelial cells in response to hypertonic milieu. It has also been reported that CFTR gene expression itself is modulated by hyperosmolarity in a p38 MAPK-dependent manner.⁴⁶ A recent study has shown that modest elevations of extracellular NaCl differentially regulated the activation of MAP kinases and Akt in mouse macrophages and potentiate macrophage apoptosis.⁴⁷ The phosphorylation and the activation of p38 MAPK could be the result of the activation of Rap-1, a small G-protein upstream of MAPKK kinase and MEK1/6.⁴⁸ Further investigations are required to better characterize the role of the MAP kinase pathway [ie, the p38 MAP kinase, extracellular signal-regulated kinase-1 and -2 (ERK1/2) and c-Jun-NH2-terminal kinase (JNK) activation] in the regulation of chemokine expression in CF airway epithelial cells.

It is well recognized that IKK α / β kinases are required for activation of NF- κ B.^{49,50} The blockage of IKK α / β complex has been identified as a molecular target of aspirin- and cyclopentenone prostaglandin-mediated inhibition of NF- κ B activity in HeLa cells,⁵¹ and further emphasizes IKK complex as a crucial target of anti-inflammatory drugs.⁵² New treatment modality with IL-10 has recently been proposed for several inflammatory diseases, including Crohn's disease⁵³ and rheumatoid arthritis.⁵⁴

Therefore, it may be possible to devise a therapy through the IKK α / β complex to modulate NF- κ B-dependent inflammatory gene transcription in CF airway epithelial cells. The results of our study suggest that IL-10 may hold therapeutic potential for the reduction of excessive inflammation in CF airways. The clinical implications of such therapy with IL-10 treatment in airways of CF patients require clinical trials. The ultimate benefits of such therapy will depend on the balance between suppressing inflammation and interfering with normal cellular functions of airway epithelium in CF patients.

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