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Role of IKK and ERK pathways in intrinsic inflammation of cystic fibrosis airways

Catherine Verhaeghe [a], Caroline Remouchamps [a], Benoît Hennuy [b], Alain Vanderplasschen [c], Alain Chariot [a], Sebastien P. Tabruyn [d], Cecile Oury [a], Vincent Bours [a,*]

[a] Units of Medical Chemistry and Human Genetics (GIGA-R), University of Liège, CHU Sart-Tilman B35, B-4000 Liège, Belgium
[b] GIGA Transcriptomic Platform (GIGA-R), University of Liège, Belgium
[c] Department of Infectious and Parasitic Diseases, Immunology-Vaccinology, University of Liège, Belgium
[d] Department of Pathology, Angiogenesis Laboratory, GROW, University of Maastricht, The Netherlands

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ABSTRACT

In cystic fibrosis (CF) patients, pulmonary inflammation is a major cause of morbidity and mortality and may precede bacterial colonization. The aim of the present study was to investigate the molecular mechanisms underlying intrinsic inflammation in cystic fibrosis airways. Using different cystic fibrosis cell models, we first demonstrated that, beside a high constitutive nuclear factor of kappaB (NF-κB) activity, CF cells showed a higher activator protein-1 (AP-1) activity as compared to their respective control cells. Gene expression profiles, confirmed by RT-PCR and ELISA, showed over-expression of numerous NF-κB and AP-1-dependent pro-inflammatory genes in CF cells in comparison with control cells. Activation of NF-κB was correlated with higher inhibitor of κB kinase (IKK) activity. In addition, Bio-plex phosphoprotein assays revealed higher extracellular signal-regulated kinase (ERK) phosphorylation in CFT-2 cells. Inhibition of this kinase strongly decreased expression of pro-inflammatory genes coding for growth-regulated proteins (Gro-α, Gro-β and Gro-γ) and interleukins (IL-1β, IL-6 and IL-8). Moreover, inhibition of secreted interleukin-1β (IL-1β) and basic fibroblast growth factor (bFGF) with neutralizing antibodies reduced pro-inflammatory gene expression. Our data thus demonstrated for the first time that the absence of functional cystic fibrosis transmembrane conductance regulator (CFTR) at the plasma membrane leads to an intrinsic AP-1, in addition to NF-κB, activity and consequently to a pro-inflammatory state sustained through autocrine factors such as IL-1β and bFGF.

1. Introduction

While multiple organs are affected in cystic fibrosis (CF) patients, chronic lung disease is the most severe clinical manifestation and 80–90% of CF patients succumb to respiratory failure caused by chronic bacterial infection and concomitant airway inflammation [1]. The sequence of events at the onset of pulmonary infection and inflammation is

* *Corresponding author*. Tel.: +32 4 366 81 44; fax: +32 4 366 81 46.
E-mail address: vbours@ulg.ac.be (V. Bours).

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ICAM-1, intracellular adhesion molecule-1; COX-2, cycloxygenase-2; NF-κB, nuclear factor-kappaB; AP-1, activator protein-1; IκB, inhibitor of kappaB; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase; MEK 1, mitogen-activated protein kinase kinase 1; EMSA, electrophoresis mobility shift assay; PCR, polymerase chain reaction; IL, interleukin; bFGF, basic fibroblast growth factor; ALLN, *N*-acetyl-*L*-leucyl-*L*-leucyl-*L*-norleucinal; Gro, growth-regulated protein; MMP, matrix metalloproteinase

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controversial and not fully characterized. At birth, the lungs of CF patients appear normal; however, autopsy examination of infants revealed an abnormal mucus secretion [2,3] demonstrating pre-clinical signs in the very first months of life. Moreover, our recent study showed the over-expression of pro-inflammatory proteins in the airways of a 24-week-old CF fetus [4]. Several studies also described neutrophil infiltration, elevated levels of elastase and interleukin-8 (IL-8) in broncho-alveolar lavage fluids of CF newborns as compared to healthy individuals in the absence of any pathogen, thus, opening the debate on the origin of this inflammation [1,5–7]. Moreover, although the abnormal composition of airway secretions and/or their depletion are frequently cited as host factors that predispose CF patients to chronic colonization by *P. aeruginosa* and resultant inflammation, a recent study [8] showed that the over-expression of the epithelial Na^+ channel ENaC in mice bronchiolar epithelium reduced the volume of preciliary liquid leading to neutrophil influx and increased levels of IL-8 in airways. Finally, several reports showed, in various CF cell lines, an increased activation of the transcription nuclear factor-κB (NF-κB) [9–12], a central player in inflammation supporting the idea that cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction can intrinsically cause the expression of pro-inflammatory mediators [1,5–7,13–15]. These results thus indicate that inflammation can arise from dysregulated ion transport and/or signaling pathways in airway epithelium in the absence of any infection. Nevertheless, very little evidence for an inflammatory state existing before any infection has been reported so far, leaving the debate open.

In order to investigate the molecular mechanisms underlying intrinsic airway pro-inflammatory state in CF, we studied the NF-κB and activator protein-1 (AP-1) activities and their signaling pathways in different cell models. A gene expression profile analysis was also performed on CFT-2 (CF cells) and NT-1 cells (control cells) that revealed the over-expression of several pro-inflammatory molecules in CF cells. In addition to the dependence on NF-κB classical pathway, the expression of most of these genes was found to be extracellular signal-regulated kinase (ERK)-dependent. We also demonstrated that IL-1β and basic fibroblast growth factor (bFGF) contributed to up-regulate the expression of selected NF-κB and AP-1 target genes through an amplification loop.

2. Materials and methods

2.1. Cell culture

Immortalized human fetal tracheal cells (CFT-2, cell line carrying the homozygous mutation ΔF508 and NT-1, control cell line) were previously characterized [16,17]. WT-CFTR and ΔF508-CFTR expressing HeLa cells were maintained as described [18]. The Human tracheal cell lines 16HBE14o$^-$ CFTR sense and anti-sense were plated onto dishes coated with fibronectin (VWR International, Leuven, Belgium), collagen I (Nutacon, Leimuiden, The Netherlands) and bovine serum albumin, as described [19,20].

2.2. Reagents

BAY 11-7085, U0126 were purchased from Alexis (San Diego, CA), *N*-acetyl-*L*-leucyl-*L*-leucyl-*L*-norleucinal (ALLN) and cycloheximide were from Sigma (Schnelldorf, Germany), the anti-bFGF and -IL-1RI antibodies were from R&D systems (Lille, France).

2.3. Western blot analyses

Western blot analyses were performed using conventional procedures. The polyclonal anti-HA, -ICAM, -IκBα, -IKKγ, -α-tubulin, -Erk1/2 and -COX-2 antibodies and the monoclonal anti-P-Erk1/2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-IKKα and -IκBα-P Ser 32/36 antibodies were from Pharmingen (San Diego, CA) and Cell Signaling (Beverly, MA), respectively.

2.4. Nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts and EMSA were performed as previously described [21]. The sequence of the consensus and mutated AP-1 probes were as follows: 5′-CGCTTGATGACTCAGCC GGAA-3′ (Santa Cruz Biotechnology); 5′-TTGGGCCCCGCGGA-GACAGCAGCCG-3′, respectively.

2.5. Immunoprecipitation and kinase assays

Immunoprecipitations were performed as described [22,23]. Briefly, whole cell lysates were immunoprecipitated with 1 μg of an anti-IKKγ or -HA antibody before incubation with protein A-agarose beads. The beads were washed before being incubated with a glutathion-S-transferase (GST)-IκBα (1–55) and ATP.

2.6. Transfection and reporter gene analyses

Luciferase activity was determined as described [24]. Briefly, cells were transfected using FuGENE transfection reagent (Roche, Vilvorde, Belgium) with the Ig-5κB-Luc reporter gene plasmid or a pRAP-1-Luc plasmid. Data were normalized with the pRSV-β-galactosidase vector.

2.7. Microarray analyses

Three independent RNA extractions were performed at different NT-1 and CFT-2 cell passages using the RNeasy extraction kit (Qiagen, Valencia, CA). The expression profiles were analyzed with the Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) in the GIGA Microarray Facility (University of Liège). The microarray expression data were generated by the MAS 5.0 Affymetrix software suite. We averaged the expression values within both groups and we generated fold changes between our two experimental conditions. Only genes for which differences in expression levels were higher than two-fold were considered.

2.8. Real-time RT-PCR

Real-time polymerase chain reaction (RT-PCR) was carried out on a TaqMan platform using SYBR Green reagent (Applied

Biosystems, Foster city, CA) as described previously [25]. Primers were designed using the Primer Express software (Applied Biosystems). Transcript levels were normalized using the housekeeping gene β_2-microglobulin that does not show expression changes among the different cell pairs tested.

2.9. ELISA

Concentrations of IL-6 and IL-8 in NT-1 and CFT-2 supernatants were determined by ELISA according to the manufacturer's recommendations (Biosource Europe, Fleurus, Belgium).

2.10. Bio-plex phosphoprotein assay

NT-1 and CFT-2 protein lysates were prepared by using the Cell lysis kit (Bio-Rad, Nazareth, Belgium). Phosphorylated p38, ERK1/2 and JNK were detected with the Bio-Plex 3-plex phosphoprotein Assay kit (Bio-Rad) according to the manufacturer's protocol. Briefly, 25 μg of proteins were incubated overnight at RT with anti-phospho-p38, -ERK1/2 and -JNK antibody in 96 well filter plates. After addition of detection antibodies, beads were washed with resuspension buffer. The plate was then read with the Luminex 100 instrument (Bio-Rad).

2.11. Statistical analysis

Data are expressed as mean values ± S.E.M. and were analyzed by a Student's *t*-test.

3. Results

3.1. Constitutive activation of the classical NF-κB activating pathway in CFT-2 cells

The NF-κB transcription factor plays a central role in inflammatory processes [26,27]. In order to assess NF-κB activation in NT-1 and CFT-2 cells, we first performed electrophoretic mobility shift assays (EMSA) on nuclear extracts from these cells. EMSA analyses showed a higher nuclear DNA binding activity of NF-κB in CFT-2 cells as compared to NT-1 cells (Fig. 1A). The specificity of these complexes was demonstrated as the binding was reduced after competition with a 100-fold molar excess of unlabeled wild type probes (wt.p) but not with an excess of unlabeled mutated probes (mt.p). Supershift studies identified the upper and the lower band of NF-κB complexes as p50/p65 heterodimers and p50/p50 homodimers, respectively.

Translocation of NF-κB subunits to the nucleus is required but not sufficient to activate the transcription of their target genes [27,28]. Therefore, to further demonstrate enhanced NF-κB activity in CFT-2 versus NT-1 cells, luciferase assays were performed using a reporter gene driven by five κB sites and showed a luciferase activity 40-fold higher in CFT-2 cells than in NT-1 cells (Fig. 1B).

In most cells, NF-κB resides in the cytoplasm as an inactive form bound to the inhibitory IκB-α protein. In the classical pathway for NF-κB activation, IκB-α is phosphorylated by

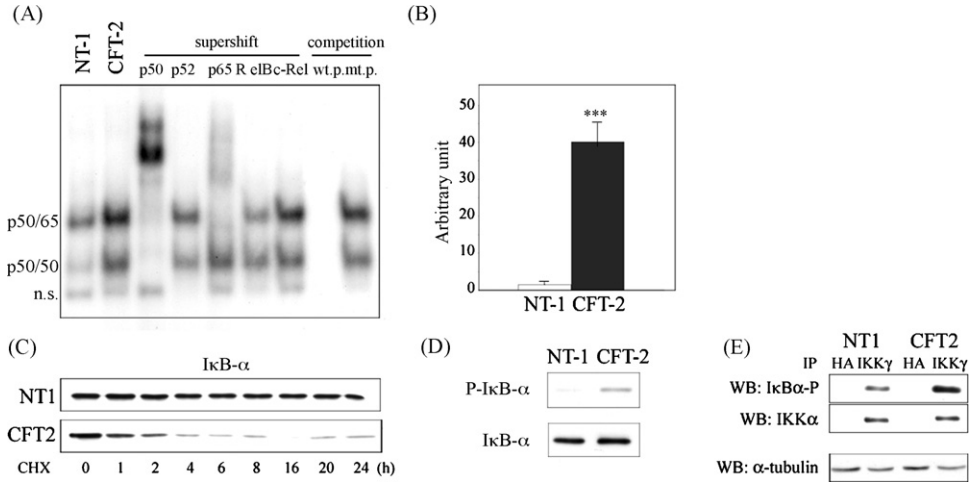

Fig. 1 – Constitutive NF-κB activation in CFT-2 cells. (A) Higher NF-κB DNA binding activity in CFT-2 than in NT-1 cells. Nuclear extracts from NT-1 and CFT-2 cells were assessed for NF-κB DNA-binding activity by EMSA. Supershift analyses were conducted on CFT-2 cells with antibodies directed against p50, p52, p65, RelB and c-Rel. In competition assays, CFT-2 nuclear extracts were pre-incubated with a 100-fold molar excess of wild-type (wt.p.) or mutated (mt.p.) unlabeled NF-κB probe. (B) Reporter gene assays on NT-1 and CFT-2 cell lines. Both cell lines were transfected with a κB-luc plasmid. Values (in arbitrary units) represent the means ± S.D. ($n = 6$) of luciferase activity normalized for β-galactosidase activity. $^{***}p < 0.001$. (C) Increased IκB-α half life in CFT-2 cells. NT-1 and CFT-2 cells were treated with cycloheximide (50 μg/ml) during the indicated times and IκB-α expression was detected by Western blot. (D) Enhanced IκB-α phosphorylation in CFT-2 cells. NT-1 and CFT-2 cells were treated for 45 min with 100 μM ALLN (proteasome inhibitor). Cells were lyzed and IκB-α was immunoprecipitated. P-IκB-α detection was performed by Western blot. (E) Enhanced IKK activity in CFT-2 cells. NT-1 and CFT-2 cells were lyzed and the IKK complex was immunoprecipitated with an IKKγ antibody and incubated with a GST-IκB substrate. Detection of P-IκB-α was performed by Western blot. Detection of IKKα indicated proper immunoprecipitation of the IKK complex. An anti-HA antibody was used as negative control of immunoprecipitation. Equal protein amounts before immunoprecipitation were verified by α-tubulin Western blotting.

IKKβ, an IκB kinase (IKK) subunit, ubiquitinated and degraded upon cell stimulation by cytokines, lipopolysaccharides (LPS) or stress inducers. NF-κB then translocates into the nucleus where it binds to specific gene promoter sequences [29]. To determine whether NF-κB activity in CFT-2 cells relied on the activation of the classical pathway, we compared IκB-α half life in NT-1 and CFT-2 cells after a treatment with the protein synthesis inhibitor, cycloheximide (50 μg/ml). As shown in Fig. 1C, IκB-α half life in CFT-2 cells is below 1 h while IκB-α is stable for up to 24 h in NT-1 cells. The phosphorylation status of IκB-α was then examined in NT-1 and CFT-2 cells treated with the proteasome inhibitor ALLN. We observed a much stronger IκB-α phosphorylation in CFT-2 cells as compared to NT-1 cells (Fig. 1D). To confirm that IκB-α phosphorylation on Ser-32 and -36 occurs through an IKK-dependent mechanism in CFT-2 cells, kinase assays were performed. Enhanced IKK activation was detected in CFT-2 versus NT-1 cells (Fig. 1E), thus confirming an IKK-dependent constitutive NF-κB activation in these cells. These results demonstrated a constitutive activity of NF-κB through enhanced IKK activity in CFT-2 cells.

3.2. Constitutive AP-1 activation in CFT-2 cells

The transcription factor AP-1 is also known to regulate pro-inflammatory gene expression [30,31]. Therefore, to determine whether AP-1 activity is also deregulated, we performed EMSAs on nuclear extracts from NT-1 and CFT-2 cells and detected a higher nuclear DNA binding activity of AP-1 in CFT-2 cells as compared to NT-1 cells (Fig. 2A). The specificity of this complex was demonstrated as the binding was reduced after competition with a 100-fold molar excess of unlabeled wild type probes (wt.p) but not with an excess of unlabeled mutated probes (mt.p). Antibodies against JunB, c-Jun, cFos, ATF1 and ATF2 were used for supershift experiments, and revealed, by the disappearance of the complex, the presence of the c-Jun subunit in the AP-1 complex. We also investigated the transactivation activity of the AP-1 factor by luciferase reporter gene assay. NT-1 and CFT-2 cells were transiently transfected with a plasmid coding for a luciferase reporter gene under the control of seven AP-1 binding elements [32]. In these conditions, the luciferase activity was increased by about nine-fold in CFT-2 cells as compared to NT-1 cells (Fig. 2B).

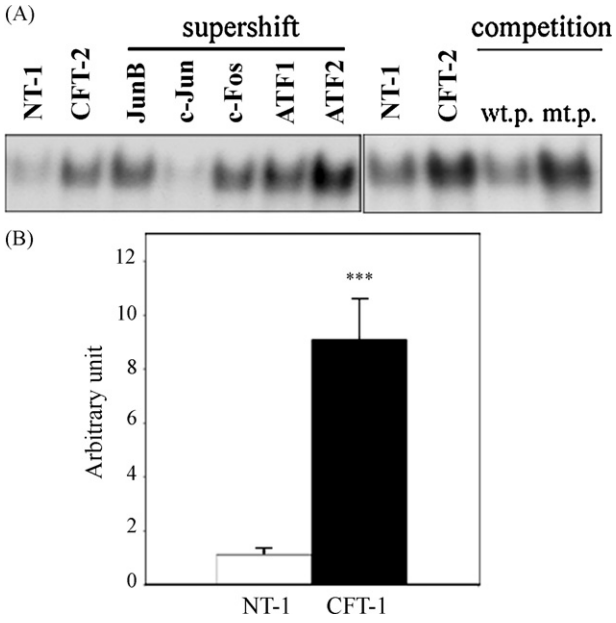

Fig. 2 – Constitutive AP-1 activation in CFT-2 cells. (A) Higher AP-1 DNA binding activity in CFT-2 than in NT-1 cells. Nuclear extracts from NT-1 and CFT-2 cells were assessed for AP-1 DNA-binding activity by EMSA. Supershift analyses were conducted with antibodies directed against JunB, c-Jun, c-Fos, ATF1 and ATF2. In competition assays, CFT-2 nuclear extracts were pre-incubated with a 100-fold molar excess of wild-type (wt.p.) or mutated (mt.p.) unlabeled AP-1 probes. (B) Reporter gene assays on NT-1 and CFT-2 cell lines. Both cell lines were transfected with a AP-1-Luc plasmid. Values (in arbitrary units) represent the means ± S.D. ($n = 6$) of luciferase activity normalized for β-galactosidase activity. $^{***}p < 0.001$.

3.3. ΔF508-CFTR-dependent constitutive NF-κB and AP-1 activation

In order to determine whether the expression of the mutated ΔF508-CFTR leads to NF-κB or AP-1 activation, we studied HeLa cells transiently or stably transfected with expression vectors for wild type (WT) or ΔF508-CFTR. WT-CFTR is initially synthesized as a ~140-kDa core-glycosylated precursor that matures into a ~160-kDa glycosylated protein. In contrast, ΔF508-CFTR fails to mature and does not reach the plasma membrane [33,34]. In stably expressing WT-CFTR or ΔF508-CFTR HeLa cells, both the 140-kDa core-glycosylated precursor (Band B) and the 160-kDa mature protein (Band C) were detected (Fig. 3A). However, while WT-CFTR was largely expressed as a mature protein, the ΔF508-CFTR protein was expressed at lower levels and mainly as a precursor protein.

We thus studied NF-κB and AP-1 activities in HeLa cells stably (Fig. 3B and C) or transiently (Fig. 3D and E) expressing WT-CFTR or ΔF508-CFTR. In a reporter gene assay, the luciferase activity measured in cells transfected with the κB-luc reporter gene was between 2- and 3.5-fold higher in HeLa cells expressing ΔF508-CFTR than in HeLa cells expressing CFTR (Fig. 3B and D) whereas luciferase activity measured after transfection with the AP-1-luc construct was increased by about 4- and 2.7-fold in ΔF508-CFTR HeLa cells (Fig. 3C and D). These data indicated that the ΔF508 mutation of CFTR is sufficient to cause constitutive NF-κB and AP-1 activities.

3.4. Up-regulation of pro-inflammatory genes in CF cells

In order to investigate the molecular mechanisms contributing to the inflammatory state in CF airways, we compared the gene expression profiles of NT-1 and CFT-2 cells. Among 54.675 transcripts, 4.5% (2424) were differentially expressed between NT-1 and CFT-2 cells. 1150 transcripts showed a reduced expression and 1274 an increased expression in CFT-2 cells as compared to NT-1 control cells. If redundant transcripts and expressed sequence tag (EST) were excluded, the expression of 1192 genes were found to be modified. The

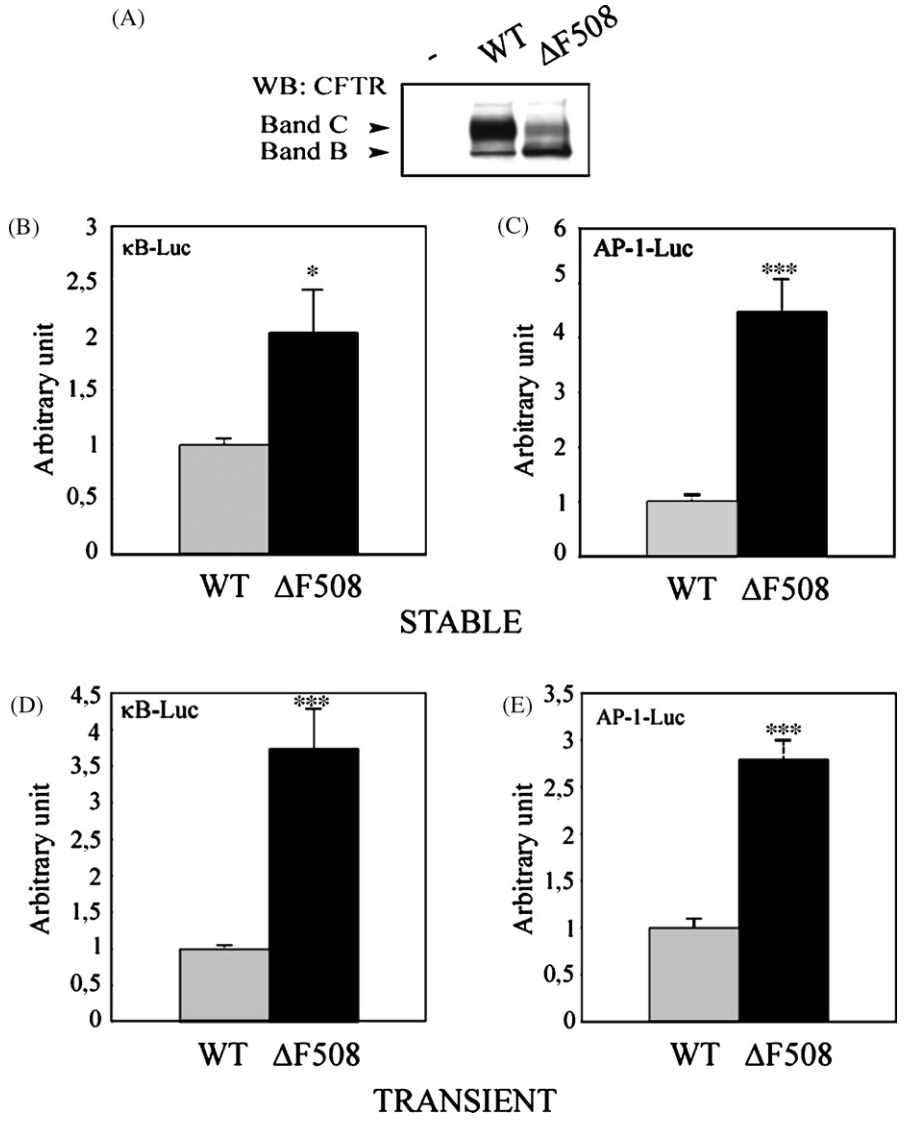

Fig. 3 – ΔF508-CFTR-dependent constitutive NF-κB and AP-1 activation. (A) CFTR expression. CFTR expression was analyzed by Western blot in HeLa and in stably expressing WT or ΔF508-CFTR HeLa cells. Band B represents the 140-kDa core-glycosylated precursor and Band C indicates the 160-kDa mature protein. (B–E) Reporter gene assays in stably (B and C) or transiently (D and E) transfected HeLa cells. HeLa cells were transfected with a κB-luc (B and D) or an AP-1-luc (C and E) plasmid together with the WT or ΔF508-CFTR vectors. Values (in arbitrary units) represent the means ± S.D. ($n = 4$) of luciferase activity normalized for β-galactosidase activity. $^{*}p < 0.05$, $^{***}p < 0.001$.

Table 1 – Pro-inflammatory gene expression in CFT-2 cells compared to NT-1 cells

Category	Gene name	Fold change
Inflammation and fibrosis (up-regulation in CFT-2 cells)		
Adhesion molecules	Intercellular adhesion molecule 1 (ICAM-1)	>2-fold
	Integrin, alpha 2 (ITGA2)	>7-fold
	Integrin, alpha 3 (ITGA3)	>2-fold
	Integrin, alpha 4 (ITGA4)	>14-fold
	Integrin, alpha 6 (ITGA6)	>23-fold
	CD44 antigen (CD44)	>2-fold
	KIT ligand (KITLG)	>3-fold
	Collagen, type XIII, alpha 1 (COL13A1)	>2-fold
Chemokines	Interleukin-8	>41-fold
	Chemokine ligand 1 (CXCL1)	>30-fold
	Chemokine ligand 2 (CXCL2)	>20-fold

Table 1 (*Continued*)

Category	Gene name	Fold change
	Chemokine ligand 3 (CXCL3)	>11-fold
	Chemokine ligand 5 (CXCL5)	>12-fold
	Chemokine (C-C motif) ligand 2 (CCL2)	>7-fold
	Chemokine ligand 12 (CXCL12)	>2-fold
Cytokines/growth factors	Interleukin-1B (IL-1β)	>10-fold
	Interleukin-6 (IL-6)	>8-fold
	Platelet-growth factor derived alpha polypeptide (PDGFA)	>2-fold
	Platelet-growth factor C (PDGFC)	>2-fold
	Vascular endothelial growth factor (VEGF)	>2-fold
	Vascular endothelial growth factor C (VEGFC)	>2-fold
	Basic fibroblast growth factor (bFGF)	>5-fold
Cytokines receptors	Interleukin-1 receptor, type I (IL-1R)	>3-fold
	Interleukin-7 receptor (IL-7R)	>2-fold
	Transforming growth factor beta, receptor 2 (TGFBR2)	>7-fold
NF-κB and/or AP-1 pathway	Prostaglandin-endoperoxide synthase-2 (PTGS2)	>15-fold
	Tumor necrosis factor receptor superfamily, member 21 (TNFRSF21)	>9-fold
	Tumor necrosis factor, alpha-induced protein 6 (TNFAIP6)	>3-fold
	Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3/A20)	>3-fold
	Tumor necrosis factor receptor superfamily, member 11a (TNFRSF11A)	>2-fold
	Tumor necrosis factor ligand superfamily, member 10a (TNFSF10A)	>6-fold
	Tumor necrosis factor receptor superfamily, member 10a (TNFRSF10A)	>2-fold
	Tumor necrosis factor receptor superfamily, member 19 (TNFRSF19)	>12-fold
	IκB-α (NFKBIA)	>3-fold
	Interleukin-1 receptor-associated kinase 2 (IRAK2)	>3-fold
Matrix remodeling	Plasminogen activator, urokinase (PLAU)	>5-fold
	Plasminogen activator, urokinase receptor (PLAUR)	>3-fold
	Matrix metalloproteinase-14 (MMP-14)	>2-fold
	Matrix metalloproteinase-2 (MMP-2)	>30-fold
	Matrix metalloproteinase-3 (MMP-3)	>40-fold
	Matrix metalloproteinase-1 (MMP-1)	>100-fold
	ADAM with thrombospondin motif 3 (ADAMTS3)	>6-fold
Inflammation and fibrosis (down-regulation in CFT-2 cells)		
Adhesion molecules	Integrin, alpha 11 (ITGA11)	>4-fold
	Integrin, alpha 7 (ITGA7)	>3-fold
	Collagen, type V, alpha 3 (COL5A3)	>7-fold
	Collagen, type XI, alpha 1 (COL11A1)	>9-fold
	Collagen, type XV, alpha 1 (COL15A1)	>5-fold
	Collagen, type IV, alpha 1 (COL4A1)	>5-fold
	Collagen, type VIII, alpha 1 (COL8A1)	>4-fold
	Collagen, type IV, alpha 2 (COL4A2)	>3-fold
Cytokines/growth factors	Transforming growth factor beta 2 (TGFB2)	>5-fold
Cytokines receptors	Interleukin-6 receptor (IL-6R)	>2-fold
	Platelet-growth factor derived receptor, alpha polypeptide (PDGFRA)	>7-fold
	Platelet-growth factor derived receptor, beta polypeptide (PDGFRB)	>5-fold
	KIT	>9-fold
	IL1 receptor accessory protein (IL1RAP)	>2-fold
	Iinhibin, beta E (INHBE)	>4-fold
	CCR5	>3-fold
	Vascular permeability factor receptor (FLT1)	>17-fold
NF-κB and/or AP-1 pathway	TNF receptor-associated factor 5 (TRAF5)	>2-fold
	Toll-like receptor 4 (TLR4)	>4-fold
Matrix remodeling	Matrix metalloproteinase-16 (MMP-16)	>3-fold
	Disintegrin and metalloproteinase 33 (ADAM33)	>16-fold
	ADAM with thrombospondin motif 5 (ADAMTS5)	>3-fold
	ADAM with thrombospondin motif 2 (ADAMTS2)	>3-fold

Microarray analyses were carried out on RNAs extracted from three different passages of NT-1 and CFT-2 cells. The table shows a list of inflammatory genes identified to be reproducibly up- or down-regulated in CFT-2 cells. The indicated values correspond to the ratios of gene expression levels in CFT-2 cells vs. NT-1 cells.

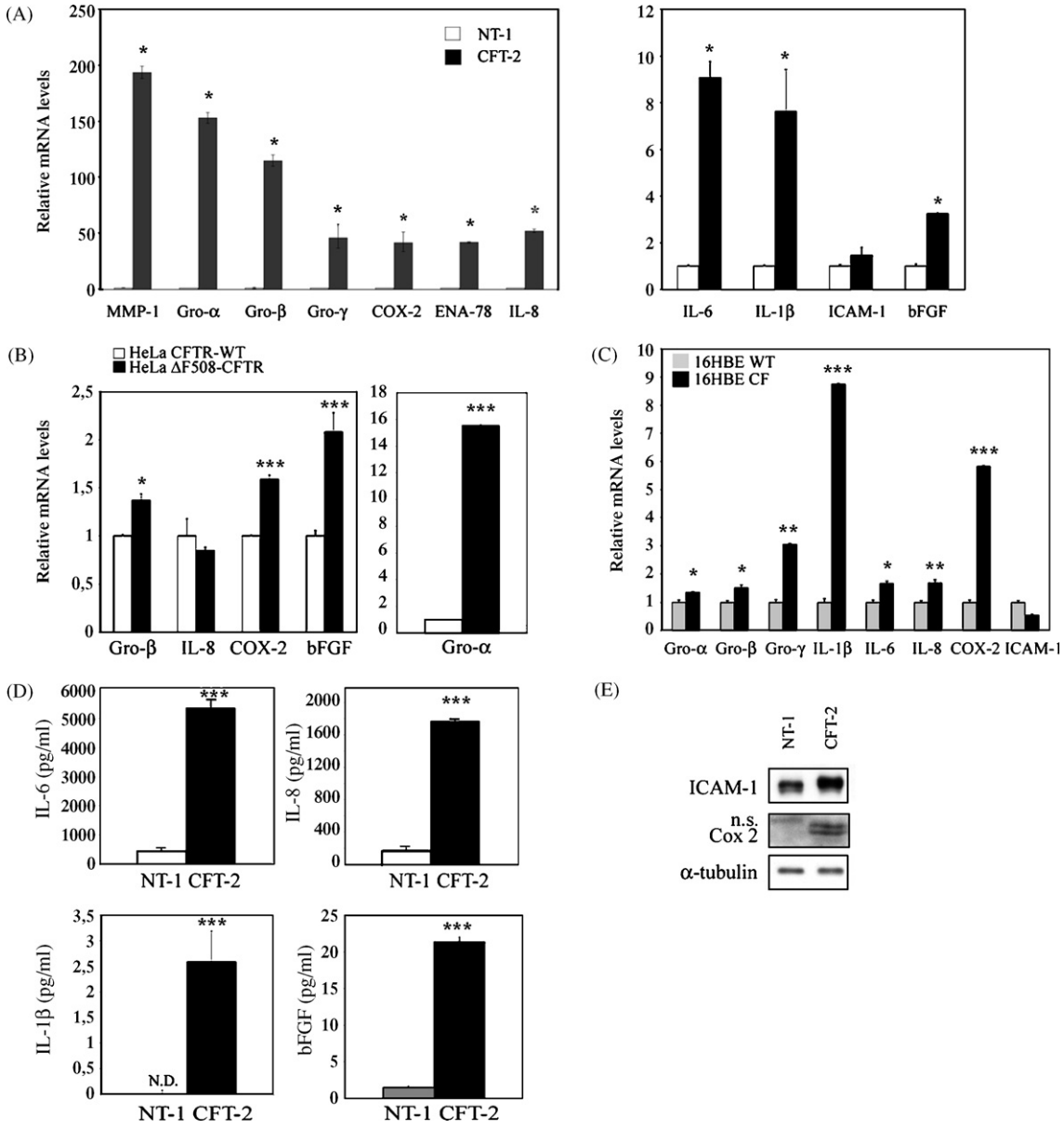

Fig. 4 – Validation of up-regulated pro-inflammatory genes and proteins in three CF cell lines. (A) Up-regulated pro-inflammatory genes in CFT-2 cells. RNAs from NT-1 and CFT-2 cells were extracted and levels of transcript expression of MMP-1, Gro-α, Gro-β, Gro-γ, ENA-78, IL-1β, IL-6, IL-8, COX-2, ICAM-1 and bFGF were analyzed by real-time RT-PCR. Values are expressed as fold increased expression in CFT-2 cells relative to NT-1 cells. Data were normalized by quantification of the β_2-microglobulin transcripts and are representative of at least three distinct experiments. Each column represents the mean ± S.D. ($n = 3$). $^{*}p < 0.01$ vs. NT-1. (B and C) Up-regulated pro-inflammatory genes in HeLa ΔF508-CFTR and in 16HBE CF cells. RNAs from HeLa WT-CFTR and ΔF508-CFTR were extracted and expression levels of Gro-α, Gro-β, IL-8, COX-2 and bFGF transcripts were analyzed by real-time RT-PCR (B) as well as expression levels of Gro-α, Gro-β, Gro-γ, IL-1β, IL-6, IL-8, COX-2 and ICAM-1 transcript in 16HBE control and CF cells (C). Values are expressed as fold increased expression in CF cells relative to control cells. Data were normalized by quantification of the β_2-microglobulin transcripts. (D) Quantification by ELISA of IL-6, IL-8, IL-1β and bFGF production in supernatants of NT-1 and CFT-2 cells. Each column represents the mean ± S.D. ($n = 3$). $^{***}p < 0.001$ vs. NT-1, N.D. = non-determined. (E) Western blot detection of ICAM-1 and COX-2 in whole cell protein extracts from NT-1 and CFT-2 cells. These results are representative of three independent experiments. n.s. = non-specific.

data were further analyzed with the DAVID 2.0 software [35] that allows the determination of biological and (patho)physiological functions significantly modified between NT-1 and CFT-2 cells. This software notably identified genes involved in "inflammation, fibrosis and matrix remodeling" (Table 1) and thus possibly relevant for cystic fibrosis. Moreover, it identified the pathway "cytokine–cytokine receptor interaction" to include the most significant group of differentially expressed

genes (p value = 0.0027). This gene expression profile analysis highlighted a large number of pro-inflammatory genes over-expressed in CFT-2 cells.

Within the panel of pro-inflammatory genes identified in our microarray experiments, 11 genes were selected for further analyses. These genes code for chemokines from the CXCL family [CXCL1 (Gro-α), CXCL2 (Gro-β), CXCL3 (Gro-γ), CXCL5 (ENA-78) and CXCL8 (IL-8)], cytokines (IL-1β and IL-6), the basic fibroblast growth factor (bFGF), the pro-inflammatory enzyme COX-2, the matrix metalloproteinase MMP-1 and the adhesion molecule ICAM-1 and thus include the most relevant groups of pro-inflammatory molecules. Up-regulation of these genes was confirmed by real-time RT-PCR in CFT-2 cells (Fig. 4A) and was analyzed in two other CF cell lines in comparison with their respective control cells. In ΔF508-CFTR HeLa cells, the expression of the genes coding for Gro-β (1.4-fold), COX-2 (1.6-fold), bFGF (2.5-fold) and especially Gro-α (16-fold) was up-regulated as compared to WT-CFTR HeLa cells (Fig. 4B). More strikingly, we found an up-regulation of Gro-α (1.35-fold), Gro-β (1.52-fold), Gro-γ (3.05-fold), IL-1β (8.75-fold), IL-6 (1.67-fold), IL-8 (1.69-fold) and COX-2 (5.58-fold) coding genes in 16HBE CF cells as compared to 16HBE control cells (Fig. 4C). Moreover, ELISA and Western blot analyses confirmed higher production of IL-6, IL-8, IL-1β, bFGF, ICAM-1 and COX-2 by CFT-2 cells as compared to NT-1 control cells (Fig. 4D and E). These results demonstrate that the absence of functional CFTR or the exogenous expression of mutated ΔF508-CFTR in unrelated cell lines activate pro-inflammatory gene transcription independently of any infection.

3.5. *Involvement of ERK and IKK in pro-inflammatory gene up-regulation in CFT-2 and HeLa ΔF508-CFTR cells*

It is well established that AP-1 is stimulated through the activation of different members of the mitogen-activated protein kinase family (MAPK), such as extracellular signal-regulated kinases (ERK1/2), c-JUN N-terminal kinase (JNK) and p38 [36]. In order to identify the MAPK responsible for the constitutive AP-1 activation, a Bio-Plex phosphoprotein assay was performed on NT-1 and CFT-2 cell lysates. The level of P-p38 MAPK (Thr^{180}/Tyr^{182}) and P-JNK (Thr^{183}/Tyr^{185}) were similar in both cell lines (Table 2) while the level of P-ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}) was significantly higher in CFT-2 cells in comparison with the NT-1 cells. In order to confirm these results, we analyzed the level of P-JNK, P-p38 and P-ERK1/2 by Western blot. We observed a higher level of P-ERK1/2 in CFT-2 than in NT-1 cells (Fig. 5A) while P-p38 and P-JNK were not detected in any cell line (data not shown).

Table 2 – Higher ERK phosphorylation in CFT-2 cells compared to NT-1 cells

	NT-1	CFT-2
P-p38 MAPK	181.7 ± 21.99	126.7 ± 62.18
P-JNK	73.7 ± 28.22	95.7 ± 92.15
P-ERK1/2	12 632.5 ± 860.68	16 406.8 ± 799*

Proteins were extracted from NT-1 and CFT-2 cells and were tested for the presence of phosphorylated p38 MAPK, JNK and ERK by a Bio-plex phosphoprotein assay kit. Values indicate the relative fluorescence of each phosphoprotein. Data are expressed as mean ± S.D. of triplicate values. *$p < 0.01$.

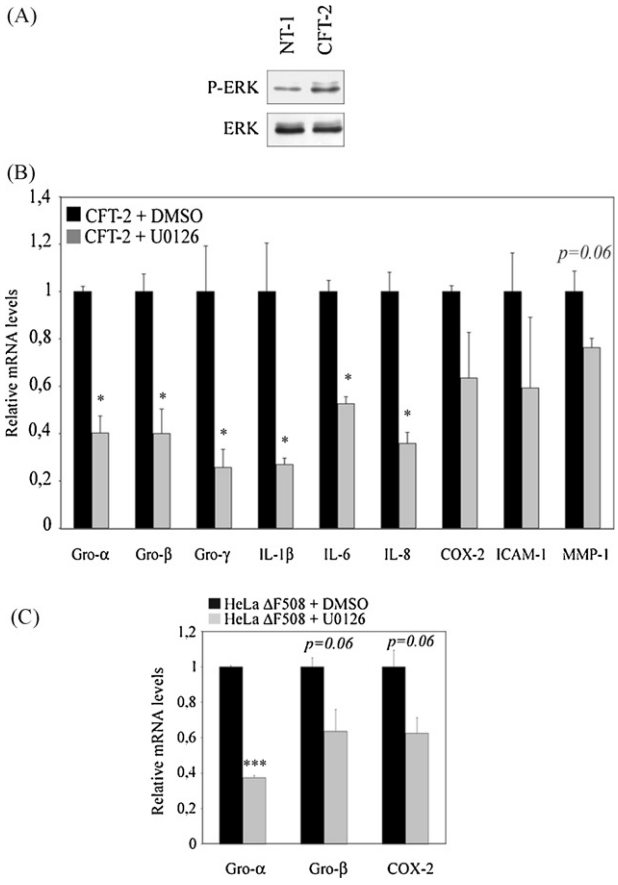

Fig. 5 – ERK regulates pro-inflammatory gene expression in CF cells. (A) Higher P-ERK1/2 in CFT-2 cells. Western blot detection of P-ERK1/2 in whole cell protein extracts from NT-1 and CFT-2 cells. Equal protein amounts were verified by ERK1/2 Western blotting. (B and C) Identification of ERK dependent gene expression. RNAs were extracted from CFT-2 or ΔF508-CFTR HeLa cells, treated or not for 6 h with U0126, 10 μM or vehicle. Levels of Gro-α, Gro-β, Gro-γ, IL-1β, IL-6, IL-8, COX-2, ICAM-1 and MMP-1 transcripts were analyzed by real-time RT-PCR. Values are expressed as fold change relative to untreated CFT-2 or ΔF508-CFTR HeLa cells. Data were normalized by quantification of the β_2-microglobulin transcripts and are representative of at least three distinct experiments. Each column represents the mean ± S.D. ($n = 3$). *$p < 0.05$ vs. DMSO.

To investigate the role of ERK in the expression of CF-related pro-inflammatory genes, CFT-2 cells and ΔF508-CFTR HeLa expressing cells were treated with the MEK1/2 (ERK kinase) inhibitor U0126. After this treatment, the expression levels of Gro-α, Gro-β, Gro-γ, IL-1β, IL-6 and IL-8 were significantly reduced in CFT-2 cells (Fig. 5B). Interestingly, ERK inhibition reduced IL-1β and IL-6 mRNA levels near to those measured in NT-1 cells, indicating a major role of this pathway. In NT-1 cells, the expression levels of Gro-α, Gro-β, Gro-γ, IL-1β and IL-8 were not significantly affected by the U0126 treatment while the expression level of IL-6 decreased

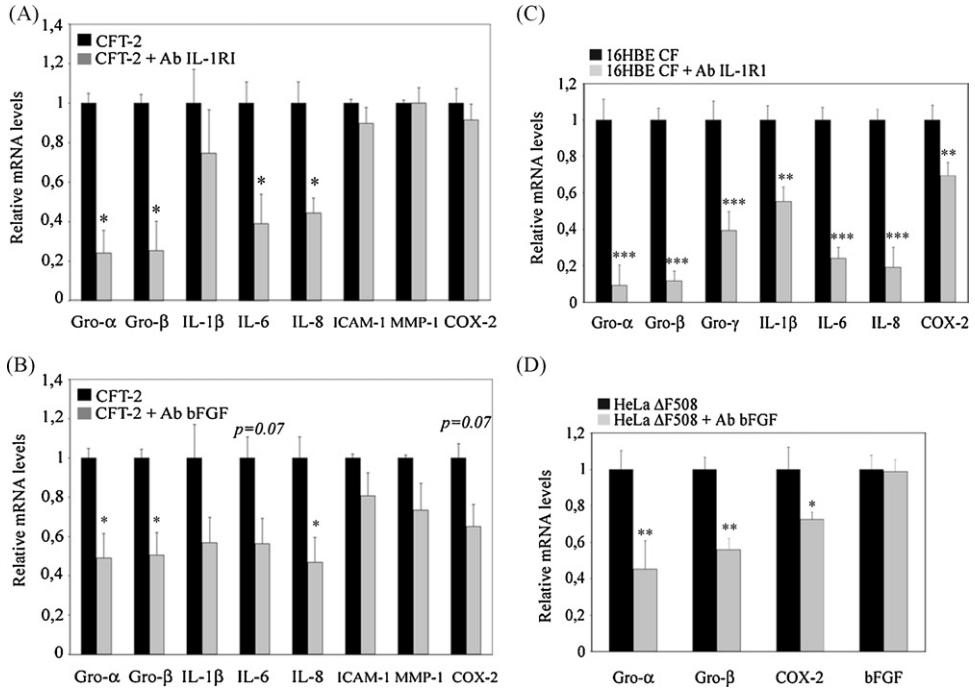

Fig. 6 – An amplification loop through IL-1β and bFGF. (A–D) Down-regulation of inflammatory genes after inhibition of IL-1β and bFGF pathways. RNAs were extracted from CFT-2 cells, treated for 8 h with anti-IL-1RI (10 μg/ml) (A) or -bFGF (2 μg/ml) antibodies (B). Expression levels of Gro-α, Gro-β, IL-1β, IL-6, IL-8, ICAM-1, MMP-1 and COX-2 transcripts were analyzed by real-time RT-PCR. RNAs were extracted from 16HBE CF cells, treated for 8 h with an anti-IL-1RI (10 μg/ml) antibody (C). Expression levels of Gro-α, Gro-β, Gro-γ, IL-1β, IL-6, IL-8 and COX-2 transcripts were analyzed by real-time RT-PCR. RNAs were extracted from HeLa ΔF508-CFTR, treated for 8 h with an anti-bFGF (2 μg/ml) antibodies (D). Expression levels of Gro-α, Gro-β, COX-2 and bFGF transcripts were analyzed by real-time RT-PCR. Values are expressed as fold change relative to untreated CF cells. Data were normalized by quantification of the β_2-microglobulin transcripts. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. untreated CF cells.

by about 30% (data not shown). The expression of the three genes studied in HeLa ΔF508-CFTR was also reduced after the treatment with U0126, although only the reduction of Gro-α expression was statistically significant (Fig. 5C). Treatment with the NF-κB inhibitor BAY 11-7085 significantly reduced the expression of the genes coding for Gro-α, Gro-β, Gro-γ, IL-1β, IL-6, IL-8, COX-2, ICAM-1 and ENA-78, indicating that the transcription of these genes is controlled by NF-κB in CFT-2 cells, as expected (data not shown). In addition, the combination of both inhibitors, BAY 11-7085 and U0126, decreased the expression levels of these pro-inflammatory genes in an additive manner (data not shown). These results indicated that, in addition to NF-κB, ERK signaling also controls the expression of several pro-inflammatory genes in CF cells.

3.6. *Secreted IL-1β and bFGF participate in the control of pro-inflammatory gene expression*

In order to determine whether the secretion of IL-1β and bFGF (see Fig. 4D) is implicated in pro-inflammatory gene expression in CF cells, CFT-2 cells were treated for 8 h with neutralizing antibodies against IL-1RI or bFGF prior to RNA extraction. While IL-1β, ICAM-1 and MMP-1 expression levels were not affected by any antibody, the expression of Gro-α, Gro-β, IL-6 and IL-8 was statistically reduced by neutralizing IL-1RI (Fig. 6A). The anti-bFGF antibody largely reduced the expression levels of Gro-α, Gro-β and IL-8, three genes with a significantly reduced expression in the presence of the ERK inhibitor, while it mildly affected the expression of IL-6 and COX-2 (Fig. 6B). Since 16HBE CF over-expressed IL-1β (Fig. 4C), the neutralizing antibody against IL-1R was tested on the expression levels of pro-inflammatory genes. In these cells, the expression levels of all the tested pro-inflammatory genes were significantly reduced when the IL-1 pathway was blocked (Fig. 6C). A similar experiment was performed with the anti-bFGF antibody on HeLa ΔF508-CFTR, which showed an up-regulation of the bFGF expression level as compared to HeLa WT-CFTR (Fig. 4B). The expression levels of up-regulated genes in HeLa ΔF508-CFTR were significantly reduced after treatment with this anti-bFGF antibody, with the exception of the bFGF gene itself (Fig. 6D). These results demonstrated that IL-1β and bFGF participate in an amplification loop to induce pro-inflammatory gene expression in CF cells, possibly through NF-κB and AP-1 activation.

4. Discussion

The origin of the chronic inflammatory reaction in the lung of cystic fibrosis patients remains controversial. It was

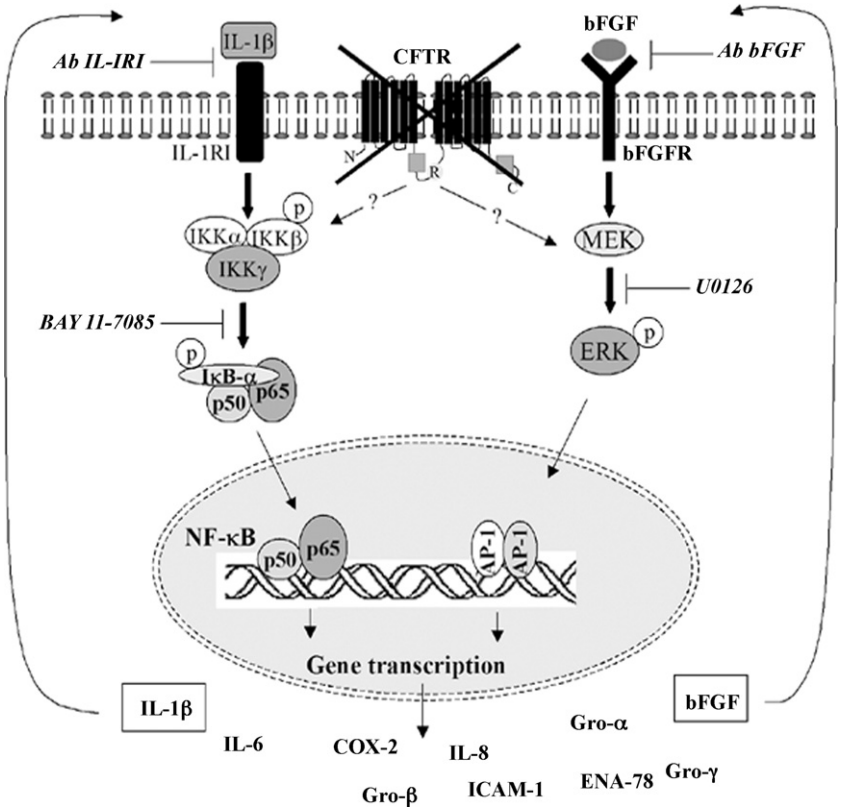

Fig. 7 – Molecular mechanisms of early inflammation in CF airway cells. Signaling pathways linking CFTR dysfunction to induction of pro-inflammatory gene expression through IKK and ERK phosphorylation. In cells lacking functional CFTR, the IKK and the ERK kinases are constitutively activated. The activation of these kinases leads to up-regulation of NF-κB- and AP-1-dependent pro-inflammatory genes. Among them, secreted IL-1β and bFGF can bind to their own receptors and participate in NF-κB and/or AP-1 activation. Cross-talks may exist between the two pathways.

generally considered that the abnormal mucus favors infections and lung colonization by bacteria, which in turn lead to a chronic inflammation and fibrosis [1]. However, several authors reported evidence from *in vitro* or *in vivo* studies that some degree of inflammation is observed in CF cells or lungs even before any infection [5–7,9–12,37–39] and we recently reported [4] that the airways of a 24-week-old human CF fetus expressed higher pro-inflammatory molecules compared to the lungs of a non-cystic fibrosis fetus indicating the presence of an intrinsic inflammatory state. However, this matter remains controversial as there is not any animal or cell model that could recapitulate the biochemical events induced by the *cftr* gene mutation in patients' cells. Indeed, mouse models do not show any lung inflammation and cell lines differ genetically and have modified gene expression in the process of transformation. Patients' samples are difficult to obtain and often infected while primary cell cultures showed variable results according to the number of passages, possibly as a consequence of environment modifications. Therefore, to study the molecular mechanisms underlying this early inflammatory process, we chose to investigate in parallel different cellular models in order to identify common mechanisms that are more likely to be relevant for the pathogenesis of CF-related inflammation.

We thus compared the fetal CFT-2 cells, originating from a 4-month-old fetus homozygous for the ΔF508 mutation and the NT-1 cells as control fetal cells. Nevertheless, as the genetic background of these two cell lines is different, we also compared HeLa cells stably transfected with the ΔF508-CFTR to isogenic HeLa cells expressing wild-type CFTR and the human tracheal 16HBE14o- cells, transfected with CFTR sense or anti-sense constructs.

In the present study, we observed a constitutive activation of two important transcription factors that play central roles in the onset and persistence of an inflammatory process: NF-κB and AP-1. Their activation is associated with the expression of several pro-inflammatory molecules such as cytokines, chemokines, enzymes, growth factors or adhesion molecules. Although this constitutive activity is much weaker than the one induced in response to exogenous cytokines, it is nonetheless observed in the different CF *in vitro* models we explored and is sufficient to drive gene expression. Interestingly, transient or stable expression of a mutant CFTR protein in HeLa cells was sufficient to induce some AP-1 and NF-κB activity as well as pro-inflammatory gene expression, thus indicating that the expression of the mutated CFTR, in contrast to the wild type CFTR, was by itself responsible for the induction of a pro-inflammatory response. Moreover, inhibition of CFTR expression in 16HBE cells by an anti-sense

plasmid also leads to pro-inflammatory gene expression, thus, suggesting that not only mutations of CFTR but also the loss of CFTR membrane expression could favor the onset of a pro-inflammatory state in epithelial cells.

NF-κB activity in CF cells was previously reported [5–7,9–12,37–39] while a single study indicated an AP-1 activity in 16HBE CF cells [39]. Several reports indirectly suggested a role of AP-1 in cystic fibrosis because of the presence of AP-1 sites in the IL-8 promoter [40–43]; however, the responsible signaling pathways remained unknown. Our data indicate that NF-κB is activated through the canonical pathway that involves IKK-dependent IκB-α phosphorylation while ERK activation plays a major role in some inflammatory gene expression such as IL-1β and IL-6. Interestingly, these two pathways are not completely independent as cross talks were reported between ERK and NF-κB [44,45]. For instance, it has been demonstrated that the ERK/Msk-1 pathway participates in the regulation of RelA/p65 activity through its phosphorylation on Ser276 [46,47]. In our study, a significant inhibition of the NF-κB transcriptional activity was observed after a treatment with the inhibitor U0126, indicating a possible cross-talk between ERK pathway and NF-κB (data not shown). Moreover, we identified an amplification loops leading, through IL-1β and bFGF expression, to constitutive pro-inflammatory gene expression, most likely through AP-1 and NF-κB pathways as genes down-regulated in response to the inhibition of kinases or secreted agents are partially common (Fig. 7). Although IL-1β is known to activate the NF-κB pathway, several studies have reported that IL-1β induces secretion of cytokines such as IL-6 through ERK pathways [48–50]. Such loops, if confirmed *in vivo*, could provide interesting targets for novel therapies in CF. Therefore, targeting both the AP-1 and NF-κB pathways with novel therapeutic agents is certainly a promising strategy and it was recently reported that, indeed, moxifloxacin selectively inhibited ERK, JNK and NF-κB [51].

Relationship between mutated CFTR and activation of NF-κB and AP-1 has not been elucidated yet. However, several hypotheses were reported. Since the mutated ΔF508-CFTR protein is misfolded, an endoplasmic reticulum (ER) stress was proposed to be involved. However, Nanua et al. recently demonstrated the absence of ER stress in CF cells [52]. A second hypothesis, suggested by Ribeiro et al., involved higher intracellular Ca^{2+} responses in CF than in normal epithelial cells, which reflects an expansion of the ER Ca^{2+} stores [53] and could contribute to NF-κB activation and subsequent cytokine secretions [54–57]. Finally, another hypothesis considered CFTR as a complex molecule implicated in signal transduction through specific interactions between its intracytoplasmic domain and various proteins such as CAL70, EBP50, NHERF2 [58–60]. A recent publication identified a hundred CFTR interacting proteins required for folding, trafficking and function of CFTR [61]. These proteins include Hsp90 which also interacts with the IKK complex. Such interactions, or their absence, could possibly lead to NF-κB and AP-1 activation.

The present paper thus investigated the pathways and the genes responsible for a torpid pro-inflammatory state pre-existing in the absence of bacterial colonization. These factors are also likely linked to the exaggerated response to pro-inflammatory stimuli observed in CF cells [10,11,51,62]. Therefore, a preventive treatment could be imagined, based on the inhibition of the NF-κB and/or AP-1 pathways but it is today not clear whether the NF-κB inhibitors that are being tested for the treatment of some cancers [63,64] could be safely administered on a regular basis to CF patients. Possibly, other inhibitors will have to be considered and several of them, such as curcumin or moxifloxacin, have already been investigated, at least *in vitro* [51,65,66].

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