



SARS-CoV-2 Serological Assay

Antigens & Monoclonal Antibody Pairs

More >



Mapping the Transcriptional Machinery of the IL-8 Gene in Human Bronchial Epithelial Cells

This information is current as of May 1, 2020.

Valentino Bezzerri, Monica Borgatti, Alessia Finotti, Anna Tamanini, Roberto Gambari and Giulio Cabrini

J Immunol 2011; 187:6069-6081; Prepublished online 26 October 2011;

doi: 10.4049/jimmunol.1100821

<http://www.jimmunol.org/content/187/11/6069>

References This article **cites 53 articles**, 17 of which you can access for free at: <http://www.jimmunol.org/content/187/11/6069.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at: <http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at: <http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: <http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2011 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Mapping the Transcriptional Machinery of the IL-8 Gene in Human Bronchial Epithelial Cells

Valentino Bezzerri,* Monica Borgatti,[†] Alessia Finotti,[†] Anna Tamanini,*
Roberto Gambari,^{‡,§,1} and Giulio Cabrini*¹

IL-8 released from bronchial epithelial cells infected with *Pseudomonas aeruginosa* plays a crucial role in the chronic lung pathology of patients affected by cystic fibrosis. Novel anti-inflammatory approaches will benefit from a thorough understanding of the regulatory mechanisms involved in the transcription of this chemokine to identify potential pharmacological targets. We addressed this issue by investigating the role of phosphoproteins and transcription factors (TFs) on transcription of IL-8 gene in the human bronchial epithelial IB3-1, CuFi-1, and Calu-3 cells. *P. aeruginosa* increased the basal phosphorylation of the ERK1/2 pathway components 90-kDa ribosomal S6 kinase (RSK)1/2 and mitogen- and stress-activated kinase-2 and of the p38 MAPK pathway components p38 α / δ / γ and heat shock protein 27 (HSP27). The involvement of these kinases in the expression of IL-8 gene was confirmed with pharmacological inhibitors of ERK1/2, RSK, p38, and HSP27 both at transcription and secretion levels. Transfection of TF decoy oligodeoxynucleotides, designed to interfere with the interaction of the TFs NF- κ B, NF-IL6, AP-1, CREB, and CHOP with the corresponding consensus sequences identified in the IL-8 promoter, reduced the *P. aeruginosa*-dependent transcription of IL-8, suggesting their participation in the transcriptional machinery. Stimulation of IB3-1 cells with IL-1 β led to a similar pattern of activation, whereas the pattern of phosphoproteins and of TFs modulated by TNF- α differentiated sharply. In conclusion, the results highlight a novel role for RSK1/2 and HSP27 phosphoproteins and of the cooperative role of the TFs NF- κ B, NF-IL6, AP-1, CHOP, and CREB in *P. aeruginosa*-dependent induction of transcription of the IL-8 gene in human bronchial epithelial cells. *The Journal of Immunology*, 2011, 187: 6069–6081.

Cystic fibrosis (CF) is a severe inherited disease caused by mutations of a gene encoding a chloride channel termed CF transmembrane conductance regulator (CFTR) (1). Although most of the CF patients are affected by multiple organ pathologies, lung disease is the major cause of morbidity and mortality in CF. In the airway tract, CFTR mutations lead to defective chloride secretion, excessive sodium reabsorption, and

reduction of airway surface liquid volume and of mucociliary clearance, which favor, by still not completely defined mechanisms, the chronic bacterial colonization with *Pseudomonas aeruginosa* (2, 3). A hallmark of CF lung pathology is a hyper-inflammatory condition, characterized by predominant infiltrates of polymorphonuclear neutrophils in bronchial lumina and increased expression of proinflammatory cytokines and chemokines, in particular the CXC chemokine IL-8 (4–8). The excessive inflammation has been related with the molecular defect of CF (for review, see Ref. 9). Moreover, it has been proposed that excessive proteolytic activity of massive polymorphonuclear neutrophil infiltrates within the bronchial lumina of CF patients cleaves the chemokine receptor CXCR1 expressed on the membrane of neutrophils and inactivates their bacterial killing capacity (10). Recently, it has been established that excessive IL-8 and IL-6 release from lung epithelial cells may also play a role in bacterial proliferation and adhesion, enhancing the amount of sialyl Lewis X carbohydrate and its derivatives localized in respiratory mucins of CF patients (11). Thus, hyperexpression of the chemokine IL-8, which mainly originates from the surface of epithelial cells lining the conductive airways, is presently considered a critical pharmacological target to reduce the excessive inflammation in CF lungs. However, a thorough understanding of the transcription machinery of the IL-8 gene in human bronchial epithelial cells is still incomplete.

The interaction of *P. aeruginosa* with pattern recognition receptors (PRRs) expressed on the airway epithelial cells is a potent inducer of expression of IL-8. Among the PRRs involved in *P. aeruginosa* recognition, it has been reported that TLR5, in cooperation with TLR2 and/or asialo-GM1 receptor (ASGM1R), binds bacterial flagellum to transduce a MyD88-dependent signaling cascade activating protein kinases and a calcium signaling pathway, with the latter being mediated by the release of ATP into

*Laboratory of Molecular Pathology, Department of Pathology and Diagnostics, University Hospital of Verona, 37126 Verona, Italy; [†]Department of Biochemistry and Molecular Biology, University of Ferrara, 44100 Ferrara, Italy; and [‡]BioPharmaNet, Emilia Romagna-GenTech, Department of Biochemistry and Molecular Biology, University of Ferrara, 44100 Ferrara, Italy

¹R.G. and G.C. share senior authorship.

Received for publication March 22, 2011. Accepted for publication September 16, 2011.

This work was supported by Italian Cystic Fibrosis Research Foundation Grant FFC 8/2009 with the contribution of Delegazione Fondazione Ricerca Fibrosi Cistica di Roma 2 (evento "Centri Infiniti"), the White Gallery, Galà "Eleonora Abbagnato et ses amis", Grant FFC 18/2009 "con l'adozione della Delegazione Fondazione Ricerca Fibrosi Cistica di Belluno in ricordo di Silvia Sommarivilla" (to G.C. and R.G.), the Fondazione Cariparo (to R.G.), Ministero della Ricerca e dell'Università Grant COFIN-2007 (to R.G.), and Regione Veneto Grant 300/09 (to A.T.). V.B. is a fellow of the Italian Cystic Fibrosis Research Foundation (Project Quantigene).

Address correspondence and reprints requests to Dr. Giulio Cabrini or Dr. Roberto Gambari, Laboratory of Molecular Pathology, Department of Pathology and Diagnostics, University Hospital of Verona, Piazzale Stefani 1, 37126 Verona, Italy (G.C.) or Department of Biochemistry and Molecular Biology, University of Ferrara, Via Fossato di Mortara 74, 44100 Ferrara, Italy (R.G.). E-mail addresses: giulio.cabrini@univr.it (G.C.) and gam@unife.it (R.G.)

Abbreviations used in this article: ASGM1R, asialo-GM1 receptor; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ChIP, chromatin immunoprecipitation; Ct, threshold cycle; HSP27, heat shock protein 27; ISA, intermediate sequence A; ISB, intermediate sequence B; MSK, mitogen- and stress-activated kinase; ODN, oligodeoxynucleotide; PRR, pattern recognition receptor; RSK, 90-kDa ribosomal S6 kinase; TF, transcription factor; TIR, Toll/IL-1R.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/\$16.00

the extracellular milieu as a so-called “danger” signal (12–15). Even if it is known that TLR4 can bind the LPS of bacterial walls, generating a MyD88-dependent proinflammatory signaling, this pathway seems to be reduced in CF, although this remains controversial (16, 17). Activation of TLR2/4/5 and ASGM1R induces IL-8 expression mainly through the phosphorylation of three MAPKs, namely MAPK p38, ERK1/2, and JNK1/2/3. ERK1/2 and MAPK p38 can transduce signals to other substrates, such as the 90-kDa ribosomal S6 kinase (RSK) family. Among RSK components, RSK1 is the most represented form and its phosphorylation may occur downstream of both ERK and 3-phosphoinositide-dependent protein kinase-1 pathways, leading to activation of C/EBP β (also known as NF-IL6), as documented in human hepatocytes, and/or to activation of CREB, as reported in human airway epithelial cells (18). Another member of the RSK family, the mitogen- and stress-activated kinase-2 (MSK2), has been reported to be a substrate of p38 α , which is able to activate both CREB and AP-1 (19). The p38 effectors include also the small heat shock protein 27 (HSP27), which is involved in inflammatory processes, together with MK2 kinase, with both activating NF- κ B (20). Additionally, HSP27 has been reported to be recruited in IL-1 β - and TNF- α -dependent IL-8 gene transcription in HeLa cells (21).

These phosphorylations eventually activate transcription factors (TFs), first of all NF- κ B (22–28). Lung inflammation in CF has been related to an increased NF- κ B signaling causing the induction of IL-8 gene expression (22, 23, 29). CF airway epithelial cells exposed to proinflammatory stimuli, such as TNF- α and IL-1 β , regulate IL-8 gene transcription through the activation of NF- κ B, NF-IL6, AP-1, and CHOP (22, 23, 29, 30). Moreover, CREB has been proposed to be a TF involved in IL-8 gene transcription machinery in the U937 monocytic cell lines exposed to *Helicobacter pilori* VacA toxin (28), and it has been shown that CREB collaborates with NF- κ B in CXC chemokine expression in human respiratory carcinoma cells (31). Additionally, it has been recently observed that CREB and NF- κ B synergistically induce IL-6 but not IL-8 gene expression in astrocytes stimulated with TNF- α (32). Therefore, the complete understanding of all the different TFs specifically involved in *P. aeruginosa*-dependent IL-8 gene transcription in CF respiratory epithelial cells has not yet been reached.

To address this issue we applied in this study the TF “decoy” strategy (33, 34), based on oligodeoxynucleotides (ODNs) mimicking the consensus sequences for the major TFs identified within the proximal promoter region of the IL-8 gene in order to obtain a complete mapping of the TFs involved. In this paper, we report that the induction of IL-8 gene transcription by *P. aeruginosa* in human bronchial epithelial IB3-1 cells involves a cooperative activation of the MAPKs ERK and p38, as well as their substrate kinases RSK, MSK, and HSP27, and of the TFs NF- κ B, NF-IL6, AP-1, CHOP, and CREB.

Materials and Methods

Cell cultures and bacteria

IB3-1 cells (LGC Promochem) are human bronchial epithelial cells immortalized with adeno12/SV40, derived from a CF patient with a mutant F508del/W1282X genotype (35). Cells were grown in LHC-8 basal medium (Biofluids, Rockville, MO) supplemented with 5% FBS. CuFi-1 cells, a gift from A. Klingelutz, P. Karp, and J. Zabner (University of Iowa, Iowa City, IA), were derived from human bronchial epithelium from a patient with CF (CFTR DF-508/DF-508 mutant genotype) and had been transformed by a reverse transcriptase component of telomerase, hTERT, and human papillomavirus type 16, E6, and E7 genes. This cell line was grown on human placental collagen type IV (Sigma-Aldrich, St. Louis, MO)-coated flasks or plates in bronchial epithelial growth medium

(Cambrex Bio Science, Walkersville, MD). Calu-3 cells were obtained from a human lung adenocarcinoma and derived from serous cells of proximal bronchial airways. Calu-3 cells were cultured in culture flasks or plates in DMEM containing 4.5 g/l glucose, supplemented with 10% FBS in a humidified atmosphere of 5% CO₂. All culture flasks and plates were coated with a solution containing 35 μ g/ml bovine collagen (BD Biosciences, Franklin Lakes, NJ), 1 μ g/ml BSA (Sigma-Aldrich), and 1 μ g/ml human fibronectin (BD Biosciences). *P. aeruginosa*, PAO1 laboratory strain, was provided by A. Prince (Columbia University, New York, NY). Bacteria were grown in trypticase soy broth or agar (Difco, Detroit, MI).

Reagents and pharmacological inhibitors

Human rIL-1 β and rTNF- α were purchased from Sigma-Aldrich. Pharmaceutical inhibitors AG1288 and GSK3 inhibitor II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), KRIBB3, parthenolide, and SB203580 were from Sigma-Aldrich, and SL0101 and U1026 were from Tocris Bioscience (Ellisville, MO).

Human phospho-MAPK array

Cells were seeded in 6-cm Petri dishes at a density of 2.5×10^6 cells to obtain 1×10^7 cells for each array. Cells were starved in LHC-8 basal medium serum-free before stimulation with PAO1 (100 CFU/cell), human rIL-1 β (10 ng/ml), or human rTNF- α (50 ng/ml) for 30 min. According to the manufacturer’s protocol, 200 μ g cell lysate was incubated with each human phospho-MAPK array (R&D Systems, Minneapolis, MN). Arrays were exposed to chemoluminescent reagent, and nitrocellulose membranes were then exposed to x-ray films. Phospho-MAPK array spot signals developed on x-ray films were quantitated by scanning the film on a high-resolution transmission-mode scanner and analyzing the array image file using the image analysis software Digimizer (MedCalc Software, Maria-kerke, Belgium).

Preparation of cell nuclear extracts

Nuclear extracts were obtained from IB3-1 cells as previously described (36). IB3-1 cells were exposed to *P. aeruginosa* PAO1 strain (100 CFU/cell), IL-1 β (10 ng/ml), or TNF- α (50 ng/ml) for 4 h, washed twice with iced PBS, and detached by trypsinization. Nuclear proteins were separated by hypotonic lysis followed by high-salt extraction treatment of nuclei. Protein concentration was determined using the Bradford method. Nuclear extracts were brought to a concentration of 0.5 μ g/ml to perform EMSA.

EMSA experiments

EMSA experiments were performed as previously described (34). Double-stranded synthetic ODNs, designed on the putative consensus sequences of TFs NF- κ B, NF-IL6, AP-1, CHOP, and CREB localized within proximal promoter region of the IL-8 gene (see Table I), were used. ODNs were labeled with [γ -³²P]ATP using 10 U T4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany) in 500 mM Tris-HCl (pH 7.6), 100 mM MgCl₂, 50 mM DTT, and 1 mM EDTA in the presence of 50 mCi [γ -³²P]ATP in a volume of 20 μ l for 45 min at 37°C. Complementary ODNs (150 ng) were added in 150 mM NaCl buffer for annealing reaction, performed at 100°C for 5 min before being left diminish to room temperature overnight. Nuclear extracts (2.5 ng) from IB3-1 cells were used and poly(dI: dC) (1 mg/reaction) was also added to abolish nonspecific binding. After 30 min binding at room temperature, the samples were run at constant voltage (200 V) under low ionic strength conditions (0.25 \times TBE buffer: 22 mM Tris-borate, 0.4 mM EDTA) on 6% polyacrylamide gels. Gels were dried and finally subjected to standard autoradiographic procedures.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed by using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s instructions. Briefly, a total of 5×10^6 IB3 cells (from two 6-cm-diameter Petri dishes) were treated for 10 min at room temperature with 1% formaldehyde culture medium. Cells were washed in PBS, and then glycine was added to a final concentration of 0.125 M. The cells were then suspended in 0.5 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl [pH 8.1]) plus protease inhibitors (1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 mM PMSF) and the chromatin was subjected to sonication (using a Sonics Vibra-Cell VC130 sonicator with a 2-mm probe). Fifteen 15-s sonication pulses at 30% amplitude were required to shear chromatin to 200- to 1000-bp fragments. Aliquots (0.2 ml) of chromatin were diluted to 2 ml in ChIP dilution buffer containing protease inhibitors and then cleared with 75 μ l salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology) for 1 h at 4°C before

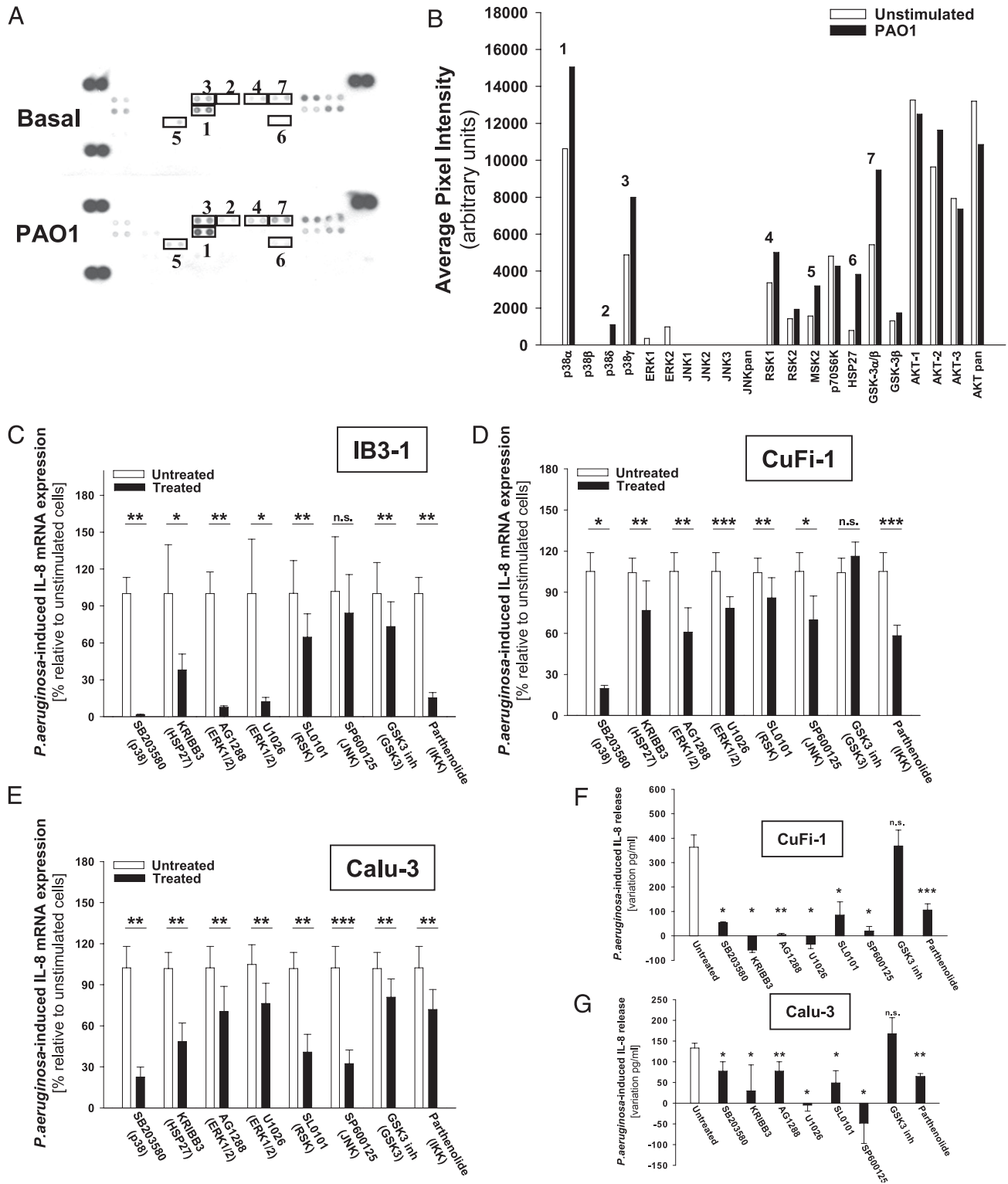


FIGURE 1. Phosphorylation pattern of kinases in cells infected with *P. aeruginosa* and the role of protein kinases in IL-8 gene expression. IB3-1 cells were exposed to *P. aeruginosa* PAO1 strain (100 CFU/cell) for 30 min or medium alone (unstimulated). Cell lysates were collected and analyzed using a human phospho-MAPK array as described in the *Materials and Methods*. *A*, Spots of the 21 phospho-kinases quantified with the phospho-MAPK array. *B*, The intensity of each spot presented in *A* was quantified with Digimizer image analysis software and is represented as a bar graph. The numbers indicate the correspondent kinases between *A* and *B*. *C*, IB3-1 cells were preincubated with inhibitors of different kinases before infection with *P. aeruginosa* PAO1 strain (100 CFU/ml) for 4 h. SB203580 (10 μ M) was added 1 h before infection; KRIBB3 (1 μ M) 1 h before infection; AG1288 (200 μ M) 2 h before infection; U1026 (10 μ M) 1 h before infection; SL0101 (2 μ M) 1 h before infection; SP600125 (3 μ M) 1 h before infection; GSK3 inhibitor II (100 nM) 1 h before infection; and parthenolide (10 μ M) 1 h before infection; total mRNA was extracted from cell lysates, and quantitative RT-PCR was performed to quantify IL-8 mRNA expression. *D* and *E*, CuFi-1 and Calu-3 cells, respectively, treated as in *C*. *F* and *G*, IL-8 protein released by CuFi-1 and Calu-3 cells, respectively, was measured by ELISA and represented as variation relative to basal release of IL-8 in starved cellular condition. Data are means \pm SEM of three independent experiments performed in duplicate and are expressed as percentages of unstimulated cells. * p < 0.05, ** p < 0.01 by Student *t* test.

incubation on a rocking platform with either 10 μ g NF- κ B p65-specific antiserum (sc-372X; Santa Cruz Biotechnology) or normal rabbit serum (Upstate Biotechnology). Diluted chromatin (20 μ l) was saved and stored for later PCR analysis as 1% of the input extract. Incubations occurred overnight at 4°C and continued an additional 1 h after the addition of 60 μ l protein A-agarose slurry. Thereafter the agarose pellets were washed consecutively with low-salt, high-salt, and LiCl buffers. DNA/protein complexes were recovered from the pellets with elution buffer (0.1 M NaHCO₃ with 1% SDS), and cross-links were reversed by incubating overnight at 65°C with 0.2 M NaCl. The samples were treated with RNase A and proteinase K, extracted with phenol/chloroform, and ethanol-precipitated. The pelleted DNAs were washed with 70% ethanol and dissolved in 40 μ l Tris-EDTA. Aliquots (2 μ l) were used for each real-time PCR reaction to quantitate immunoprecipitated promoter fragments.

Real-time PCR quantitation of immunoprecipitated promoter fragments

For quantitative real-time PCR reactions, a pair of primers that amplify a 301-bp region on the IL-8 promoter, containing the NF- κ B binding site, was designed (forward, 5'-TCA CCA AAT TGT GGA GCT TCA GTA T-3'; reverse, 5'-GGC TCT TGT CCT AGA AGC TTG TGT-3'). PCR reactions were also performed using negative control primers that amplify a 255-bp genomic region ~5 kb upstream of the IL-8 promoter, lacking

NF- κ B binding sites (forward, 5'-TCC CTA AGT CAC TTT CTT CAA GTT GC-3'; reverse, 5'-CGT GCA TTT AAT TGT GTC TTG TGG-3'). Each real-time PCR reaction was performed in 25 μ l final volume, using 2 μ l template DNA (from ChIPs), 10 pmol primers, and 1 \times iQ SYBR Green Supermix (Bio-Rad) for a total of 45 cycles (96°C for 15 s, 66°C for 30 s, 72°C for 20 s) using an iCycler IQ (Bio-Rad). The relative proportions of immunoprecipitated promoter fragments were determined based on the threshold cycle (*C_t*) value for each PCR reaction. Real-time PCR data analyses were obtained using the comparative *C_t* method: a ΔC_t value was calculated for each sample by subtracting the *C_t* value for the sample amplified with IL-8 promoter primers from the *C_t* value obtained for the same sample amplified with negative control primers. For each kind of immunoprecipitation (IgG or NF- κ B p65 antiserum), a $\Delta\Delta C_t$ value was then calculated by subtracting the ΔC_t value for the untreated cells sample from the ΔC_t value for the treated cell samples. Fold differences were then determined by the $2^{-\Delta\Delta C_t}$ method. Each sample was quantitated in duplicate for at least three separate experiments. Mean \pm SD values were determined.

Cell transfection with decoy ODNs

Human bronchial epithelial cells (IB3-1, CuFi-1, or Calu-3) were seeded in 24-well plates at a density of 30,000 cells/cm² and transfected with ODNs using cationic liposome vector Lipofectamine 2000 (Invitrogen, Carlsbad,

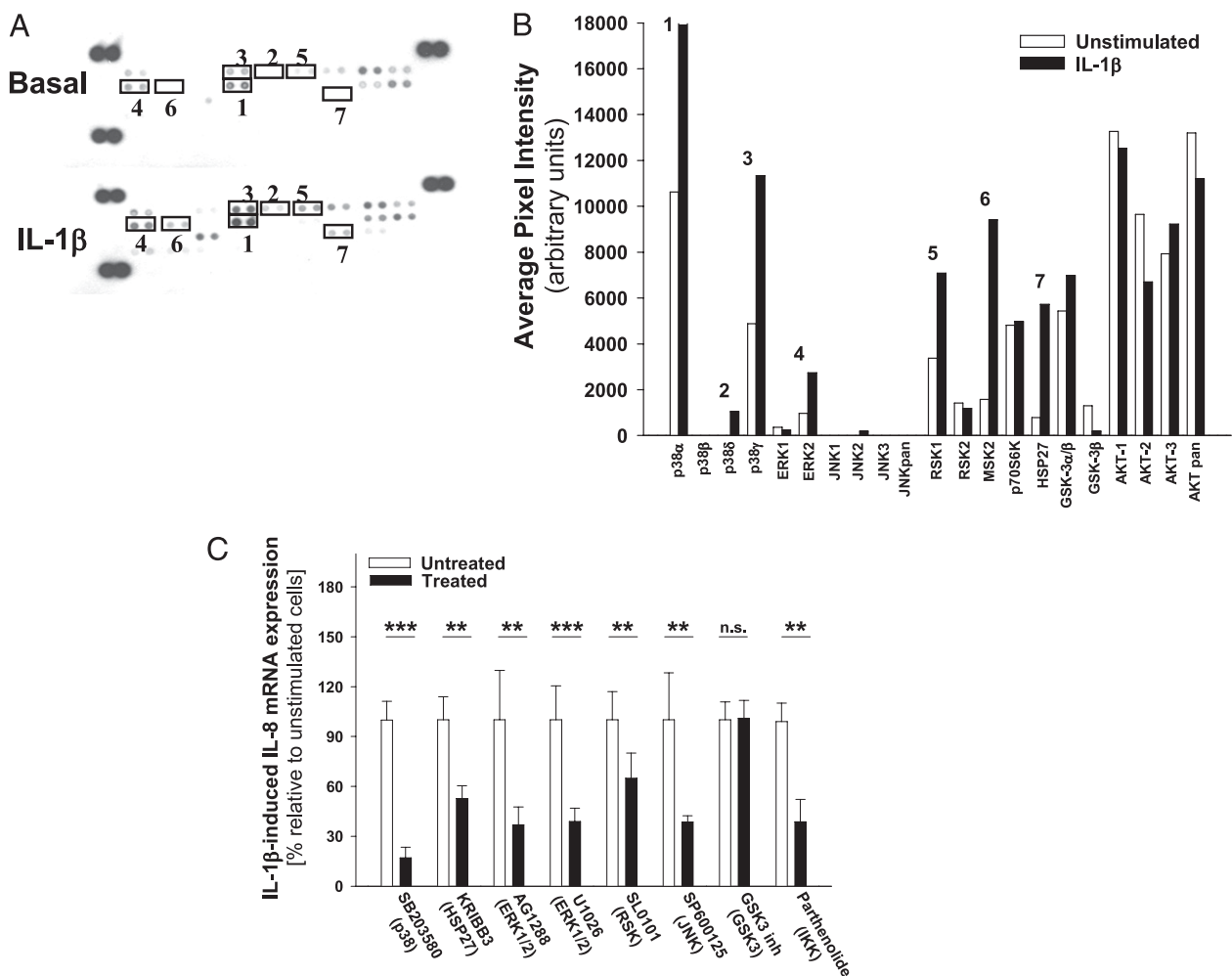


FIGURE 2. Phosphorylation pattern of kinases in cells stimulated with IL-1 β . IB3-1 cells were exposed to IL-1 β (10 ng/ml) for 30 min or medium alone (unstimulated). Cell lysates were collected and analyzed using the human phospho-MAPK array as described in *Materials and Methods*. *A*, Spots of the 21 phospho-kinases quantified with the phospho-MAPK array. *B*, The intensity of each spot presented in *A* was quantified with Digimager image analysis software and is represented as a bar graph. The numbers indicate the correspondent kinases between *A* and *B*. *C*, IB3-1 cells were preincubated with inhibitors of different kinases before stimulation with IL-1 β for 4 h. SB203580 (10 μ M) was added 1 h before infection; KRIBB3 (1 μ M) 1 h before infection; AG1288 (200 μ M) 2 h before infection; U1026 (10 μ M) 1 h before infection; SL0101 (2 μ M) 1 h before infection; SP600125 (3 μ M) 1 h before infection; GSK3 inhibitor II (100 nM) 1 h before infection; and parthenolide (10 μ M) 1 h before infection; total mRNA was extracted from cell lysates, and quantitative RT-PCR was performed to quantify IL-8 mRNA expression. Data are means \pm SEM of three independent experiments performed in duplicate and are expressed as percentages of unstimulated cells. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by Student *t* test.

CA) according to the manufacturer's protocol. Lipofectamine 2000 (4 μ l) was diluted in 1 ml serum-free LHC-8 basal medium (Biofluids) and double-stranded decoy or scrambled ODNs (2 μ g) were added and incubated for 10 min to generate liposome/DNA complexes as previously described (34). Liposome/DNA complexes were then added to IB3-1 cells and incubated for 6 h. After this time of incubation cells were washed twice with serum-free culture medium and left at 37°C and 5% CO₂ for 20–24 h before proinflammatory challenge with *P. aeruginosa* (100 CFU/ml), IL-1 β (10 ng/ml), or TNF- α (50 ng/ml) for a further 4 h.

RNA extraction and quantitative RT-PCR

Total RNA from IB3-1 cells was purified using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany), and 2.5 μ g RNA was reverse-transcribed to cDNA using the High Capacity cDNA Archive Kit and random primers (Applied Biosystems, Foster City, CA) in a final reaction volume of 100 μ l. cDNA (5 μ l) was mixed with Fast SYBR Green Master Mix (Applied Biosystems) and IL-8 primers (15 nM forward sequence, 5'-GACCACACTGCGCCAACA-3'; 15 nM reverse sequence, 5'-GCTCTC-TTCCATCAGAAAGTTACATAATTT-3'). GAPDH gene expression was determined as the normalizer gene (2.5 nM primer forward sequence, 5'-GTGGAGTCCACTGGCGTCTT-3'; 15 nM reverse sequence, 5'-GCA-AATGAGCCAGCCTTC-3'). Primer sets were purchased from Sigma-Genosys (The Woodlands, TX). Real-Time PCR was performed in duplicate for both target and normalizer genes using the ABI Prism 7900 HT

Fast real-time PCR system (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 20 s, and 40 two-step cycles of 95°C for 1 s and 60°C for 20 s. Results were collected with SDS 2.3 software (Applied Biosystems), and relative quantification was performed using the Ct method. Data were analyzed with RQ Manager software 1.2 (Applied Biosystems). Changes in mRNA expression levels were calculated after normalization to calibrator gene. The ratios obtained after normalization are expressed as fold change over untreated samples.

Results

P. aeruginosa-dependent phosphoprotein activation and IL-8 gene transcription in bronchial epithelial cells

It has been already defined that surface structures and soluble products from *P. aeruginosa* interact with TLR2/4/5, activating directly a MyD88-dependent cascade and leading to the expression of IL-8 and other proinflammatory genes in human bronchial epithelial cells (14, 15). *P. aeruginosa* is known to activate phosphorylation of MAPKs p38 and ERK1/2, which has been related to IL-8 gene expression (37). MAPK p38 was shown to phosphorylate HSP27, the latter leading to activation of IKK and NF- κ B, in human colonic epithelial cells upon induction by

Downloaded from <http://www.jimmunol.org/> by guest on May 1, 2020

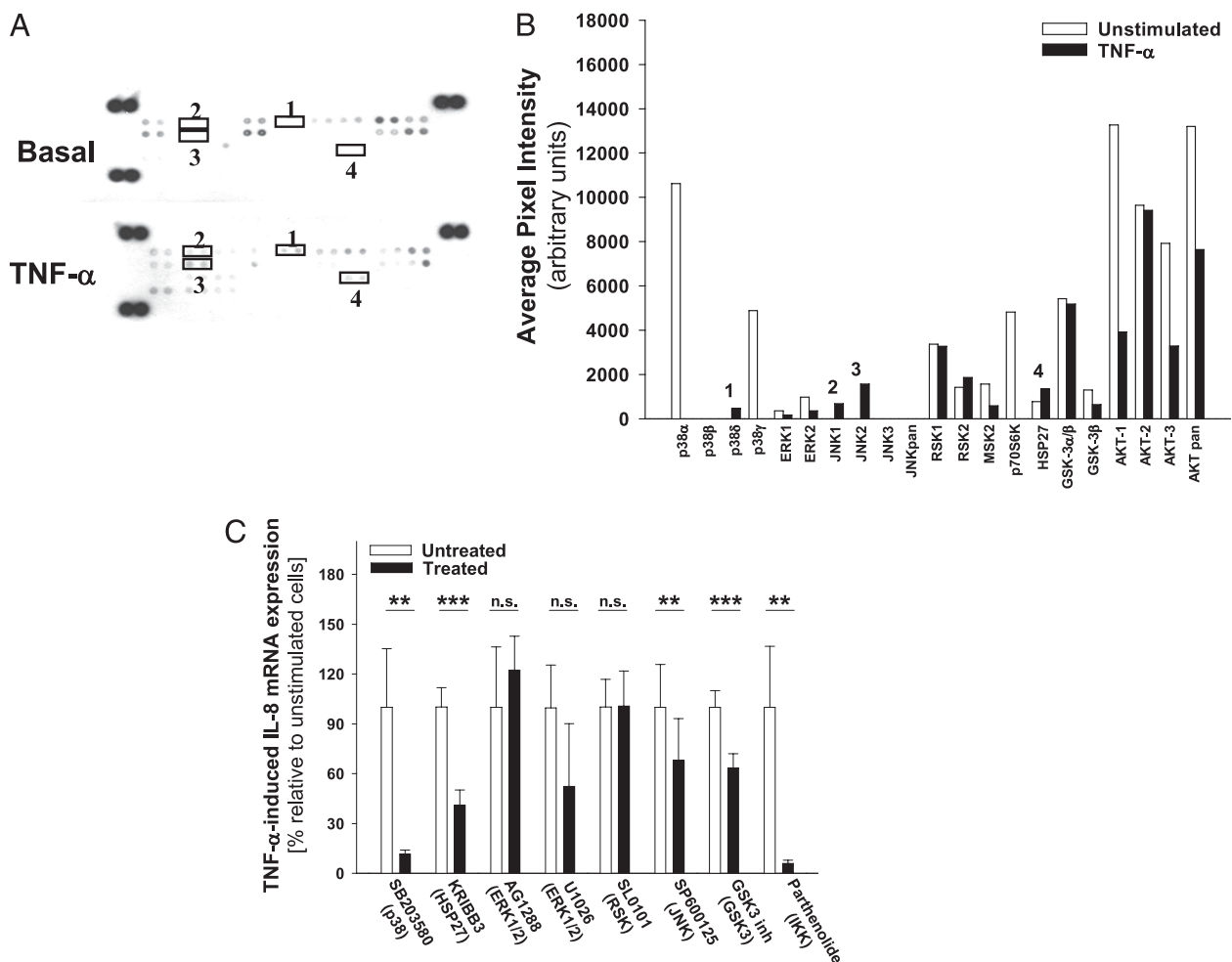


FIGURE 3. Phosphorylation pattern of kinases in cells stimulated with TNF- α . IB3-1 cells were exposed to TNF- α (50 ng/ml) for 30 min or medium alone (unstimulated). Cell lysates were collected and analyzed using a human phospho-MAPK array as described in *Materials and Methods*. **A**, Spots of the 21 phospho-kinases quantified with the phospho-MAPK array. **B**, The intensity of each spot presented in **A** was quantified with Digimizer image analysis software and is represented as a bar graph. The numbers indicate the correspondent kinases between **A** and **B**. **C**, IB3-1 cells were preincubated with inhibitors of different kinases before stimulation with TNF- α for 4 h. SB203580 (10 μ M) was added 1 h before infection; KRIBB3 (1 μ M) 1 h before infection; AG1288 (200 μ M) 2 h before infection; U1026 (10 μ M) 1 h before infection; SL0101 (2 μ M) 1 h before infection; SP600125 (3 μ M) 1 h before infection; GSK3 inhibitor II (100 nM) 1 h before infection; and parthenolide (10 μ M) 1 h before infection; total mRNA was extracted from cell lysates, and quantitative RT-PCR was performed to quantify IL-8 mRNA expression. Data are means \pm SEM of three independent experiments performed in duplicate and are expressed as percentages of unstimulated cells. * p < 0.05, ** p < 0.01, *** p < 0.001 by Student *t* test.

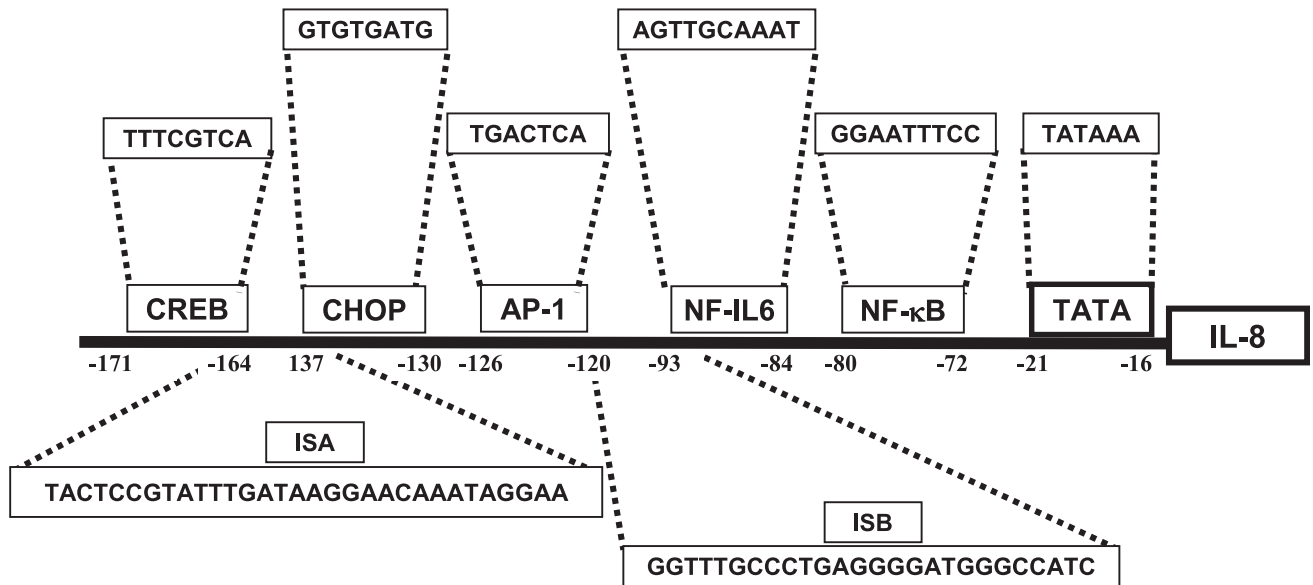


FIGURE 4. Proximal region of the promoter of IL-8 gene and putative consensus sequences for TFs. In silico study of the proximal region of the IL-8 gene promoter (up to -180 bp from start site) was performed using TESS search software to identify the putative consensus sequences for TFs. TESS found consensus sequences for TFs CREB, AP-1, NF-IL6, and NF- κ B. A previously reported CHOP consensus sequence was included (30). ISA, ISB, and TATA box are reported.

dextran sodium sulfate (38). Moreover, ERK1/2 phosphorylates RSK and MSK (39). RSK has been shown to be activated also by phosphorylation of AKT and it has been implicated in the activation of other transcription factors relevant to IL-8 gene expression, such as CREB, AP-1, and NF-IL6 (39). Another relevant kinase is GSK3, which has been shown to induce IL-8 expression in neuroblastoma cells (40). As a possible feedback mechanism, GSK3 activation can be inhibited through phosphorylation elicited by activated AKT (41). Thus, we started investigating the potential implication of MAPK JNK, RSK, MSK, HSP27, GSK3, and AKT, besides that known of MAPK ERK1/2 and p38, in the *P. aeruginosa*-dependent induction of IL-8 in the bronchial epithelial IB3-1 cell line. Cells were exposed to the *P. aeruginosa* laboratory strain PAO1 for 30 min, and cell lysates were extracted to detect the phosphorylation of these different kinase substrates with a human phospho-MAPK array.

As far as the MAPK p38 pathway is concerned, a clear phosphorylation of p38 isoforms α and γ and of the downstream substrate HSP27 can be observed (Fig. 1A, 1B). No striking phosphorylation of the MAPK ERK1/2 was found. However, a clear phosphorylation of RSK1 and MSK2, which are substrates of activated ERK1/2, was evident (Fig. 1A, 1B). With regard to the ERK1/2-independent kinases known to phosphorylate RSK1/2, we did not observe further phosphorylation of AKT-1/2/3 by *P. aeruginosa*, whereas phosphorylation of GSK3 was increased

(Fig. 1A, 1B). No significant basal or *P. aeruginosa*-stimulated phosphorylation of the MAPK JNK family was observed (Fig. 1A, 1B). To test whether the kinases activated by *P. aeruginosa* are implicated in the activation on IL-8 gene transcription, we preincubated the cells with the pharmacological inhibitors of MAPK p38 (SB203580), HSP27 (KRIBB3), ERK1/2 (AG1288 and U1026), RSK (SL0101), GSK3 (GSK3-inh), IKK (parthenolide),

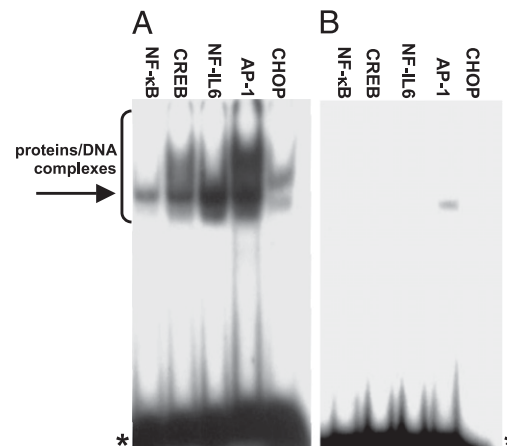


FIGURE 5. Activation of TFs by proinflammatory challenges and validation of TF decoy ODNs. *A*, Direct binding of [32 P]-labeled dsDNA, carrying the target sites for the transcription factors NF- κ B, CREB, NF-IL6, AP-1, and CHOP identified in the IL-8 promoter, to NFs isolated from IB3-1 cells induced with IL-1 β . [32 P]-labeled dsDNA molecules were incubated for 40 min in the presence of 2 μ g crude nuclear extracts. Protein/DNA complexes were separated by PAGE, and autoradiography was performed. *B*, Effects of dsDNA TF decoy hybrids, carrying the target sites for the transcription factors NF- κ B, CREB, NF-IL6, AP-1, and CHOP identified in the IL-8 promoter, on the interaction between NFs and the corresponding [32 P]-labeled dsDNA probe. Crude nuclear extracts (2 μ g) were incubated for 20 min in the presence of 200 ng TF dsDNA molecules, as indicated, and then incubated with radiolabeled dsDNA probes for 20 min. In *A* and *B*, arrows indicate complexes between proteins and target molecules, and asterisks indicate the free [32 P]-labeled probe.

Table I. Sequences of decoy ODNs based on IL-8 promoter regulatory elements

ODN Decoy	Sequence (5'-3')
IL-8 NF- κ B	AGAGGAATTTCCACGATT
IL-8 NF-IL6	CATCAGTTGCAAATCGTGG
IL-8 AP-1	TGTGATGACTCAGTTTG
IL-8 CHOP	CGCTGGTGTGATGCACGG
IL-8 CREB	AAAACCTTCGTCATACTC
IL-8 ISA	GGTTGCCCTGAGGGGATGGGCCATC
IL-8 ISB	TACTCCGTTTGGATAAGGAACAAATAGGAA
Scrambled	CACAAAGTGTAACAGTCT

and, as a negative control, of JNK (SP600125) before the 4-h infection with *P. aeruginosa*. As shown in Fig. 1C, the inhibitors of MAPK p38 and that of its substrate HSP27 sharply reduced IL-8 mRNA content, confirming and extending the role of this signaling pathway. Similarly, inhibition of ERK1/2 resulted in a significant inhibition of *P. aeruginosa*-dependent IL-8 gene transcription by testing both AG1288 and U1026 inhibitors, possibly partly dependent on its downstream kinase RSK (Fig. 1C). A partial reduction was also evident upon GSK3 inhibition, which is in agreement with the data reported in other cell models indicating GSK3 as an IL-8 inducer (40). Finally, IKK inhibition by parthenolide, which in turn inhibits NF- κ B activation, showed a strong reduction of IL-8 mRNA content (Fig. 1C). In summary, the results obtained in IB3-1 cells suggest that *P. aeruginosa*-dependent transmembrane signaling involved in IL-8 gene transcription appears associated with MAPK p38, ERK, and GSK3 pathways. Interestingly, MAPK p38 downstream IKK effector HSP27 and the ERK substrate RSK1 seem implicated in this model of inflammation. As previously reported (29), IKK was closely related to IL-8 gene expression in CF respiratory epithelial cells. The pilot experiments in IB3-1 cells were extended to the CuFi-1 and Calu-3 human epithelial bronchial cell lines, expressing either F508del-mutated CFTR or wild-type CFTR protein, by analyzing the effect of the same inhibitors on the expression of IL-8 gene both at the transcript and protein levels. As shown in Fig 1D–G, a consensus is evident for the involvement of p38, HSP27, ERK, RSK, and IKK in all the three cell lines. The modest, albeit statistically significant, involvement of GSK3 in IB3-1 cells is not consistently confirmed in the other cell lines, whereas SP600125, which inhibits JNK, shows an unexpected reduction of IL-8 mRNA expression and protein release in CuFi-1 and Calu-3 cells.

Comparison of MyD88-dependent and -independent signaling pathways in IL-8 gene transcription

Because *P. aeruginosa* activates in parallel both TLR/MyD88-dependent and cytosolic calcium-dependent pathways by promoting the release of nucleotides in the extracellular milieu, which bind to purinergic P2Y receptors (12, 13, 37), to further elucidate the role of the MyD88-dependent cascade in IL-8 gene expression, we stimulated bronchial IB3-1 cells with the proinflammatory cytokine IL-1 β , which activates MyD88 through its IL-1 receptor. Through MyD88 adaptor molecule and Toll/IL-1R (TIR) domain, IL-1R was shown to activate downstream kinases such as p38 and ERK in HeLa cells, finally inducing IL-8 gene expression (42). Additionally, IL-1 β has been proposed to activate a sequential ERK/RSK1 cascade both in human nasal epithelial cells and in rat vascular smooth muscle cells (43, 44). Interestingly, HSP27 has been reported to regulate IL-1-dependent IKK activation in HeLa cells (45). To verify and extend these findings in our experimental model, we tested the effect of IL-1 β -dependent phosphorylation with a human phospho-MAPK assay. As shown in Fig. 2A and 2B, IL-1 β strongly induced phosphorylation of MAPK p38 isoforms α and γ . A modest induction of the isoform δ and no phosphorylation of the isoform β were observed (Fig. 2A, 2B). Additionally, an increased phosphorylation of HSP27 was detected upon exposure of bronchial cells to IL-1 β stimulation (Fig. 2A, 2B). Furthermore, enhancement of phosphorylation of both ERK2 and its substrates RSK1 and MSK2 was observed. No changes of phosphorylation levels of JNK and AKT were detected, whereas a reduction of phosphorylation of GSK3 β was shown in respect to basal levels (Fig. 2A, 2B). Similar to observations obtained with *P. aeruginosa*-dependent stimulation, inhibition of MAPK p38 and HSP27 by SB203580 and KRIBB3,

respectively, caused a significant reduction of IL-1 β -dependent IL-8 gene transcription (Fig. 2C). Moreover, pharmacological inhibition of ERK1/2, with both AG1288 and U1026 and RSK and JNK, showed a sharp inhibition of IL-8 mRNA content (Fig. 2C). On the contrary, GSK3 inhibitor did not reduce IL-8 gene transcription elicited by IL-1 β (Fig. 2C). Finally, parthenolide strongly inhibited IL-8 gene transcription (Fig. 2C). To study a completely MyD88-independent signaling pathway of regulation of IL-8 gene transcription, we exposed IB3-1 cells to TNF- α , which has been reported to transduce p38 and JNK signaling through TNFR-associated factor protein activation (27, 46). Additionally, HSP27 has been reported to be activated after TNF- α stimulation through TAK1 kinase (21). As shown in Fig. 3A and 3B, TNF- α induced phosphorylation of MAPK p38 isoform δ but not α , β , and γ . Additionally, a 2-fold induction of HSP27 was found (Fig. 3A, 3B). In agreement with previously reported results (27, 46), we observed phosphorylation of JNK1 and JNK2 phosphokinases (Fig. 3A, 3B). Interestingly, TNF- α inhibited to different extents the basal phosphorylation of several substrates, such as AKT-1 and AKT-3, ERK 1/2, MSK2, and GSK3 β . As shown in Fig. 3C, pharmacological inhibition of MAPK p38 and HSP27 led to strong reduction of TNF-induced IL-8 gene transcription, confirming the role of the p38 pathway and extending the

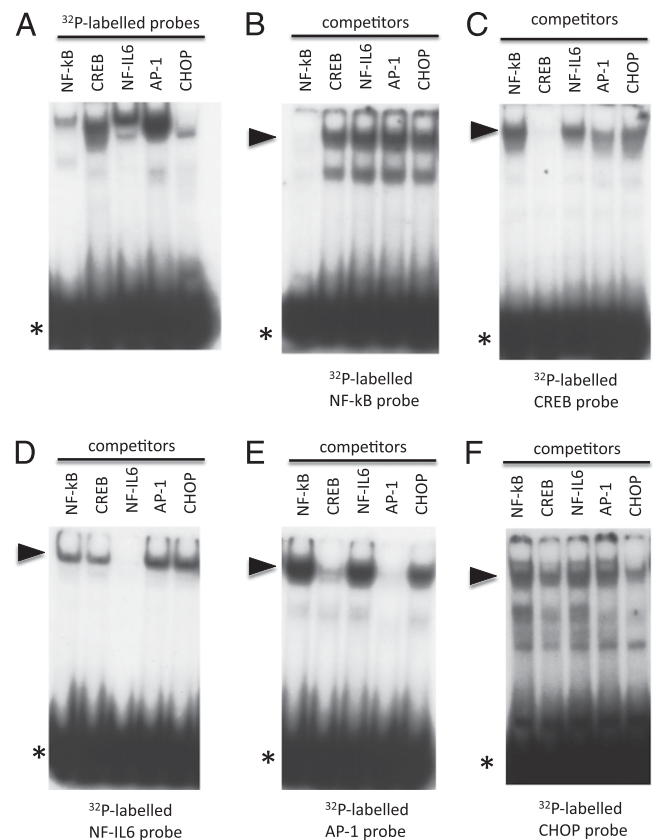


FIGURE 6. Competitive EMSA assay. EMSA assays were performed as described in Fig. 5 using [32 P]-labeled NF- κ B, CREB, NF-IL6, AP-1, and CHOP probes (A–F). Crude nuclear extracts (2 μ g) were incubated for 20 min in the presence of 200 ng cold NF- κ B, CREB, NF-IL6, AP-1, and CHOP competing dsDNA molecules, as indicated, and then incubated with [32 P]-labeled NF- κ B (B), CREB (C), NF-IL6 (D), AP-1 (E), and CHOP (F) dsDNA probes for a further 20 min. In B–F, arrows indicate complexes between proteins and target molecules, and asterisks indicate the free [32 P]-labeled probe. In A, the binding of nuclear extracts to [32 P]-labeled NF- κ B, CREB, NF-IL6, AP-1, and CHOP in the absence of competitors is shown.

comprehension of HSP27 activation. Also, inhibition of JNK kinases led to a significant decrease of IL-8 mRNA expression, whereas no effect was observed using both ERK1/2 inhibitors AG1288 and U1026 and RSK inhibitor SL0101 (Fig. 3C). Furthermore, the GSK3 inhibitor partially reduced the transcription of IL-8 gene (Fig. 3C). Finally, IKK inhibition by parthenolide showed a potent decrease of IL-8 mRNA expression mediated by TNF- α (Fig. 3C). Taken together, the consensus obtained with the results from the three different cell lines, expressing either the wild-type or mutated CFTR protein, so far suggests that the MyD88/TIR-dependent pathways elicited by *P. aeruginosa* and IL-1 β occur through the MAPK p38 isoforms α , δ , and γ , the p38 substrate HSP27, and the MAPK ERK2 and its substrate RSK. Additionally, the MyD88-independent TNF- α -induced pathway does not activate ERK1/2, but it does activate MAPK JNK and only the isoform δ of MAPK p38.

Development of TF decoy ODNs to interfere with IL-8 gene transcription

Despite the fact that NF- κ B and AP-1 are widely established as TFs implicated in the expression of IL-8 gene in different cell models (27, 34), other TFs have been recently reported as possible regulators of IL-8 gene transcription, such as the TF CHOP in T-lymphocytes and in human bronchial IB3-1 cells (30, 47), the TF CREB in U937 monocytic cells and in A549 cells (28, 48), and the TF NF-IL6 in human conjunctival and bronchial cells induced by *P. aeruginosa* (48). To build a comprehensive picture of the different TFs intervening in human bronchial epithelial cells challenged with *P. aeruginosa*, we first made an in silico analysis of the proximal region of the IL-8 gene promoter (from start site up to -180 bp) with the TF search software TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) to obtain a prediction of consensus sequences for TFs. Fig. 4 summarizes the major TF regulatory

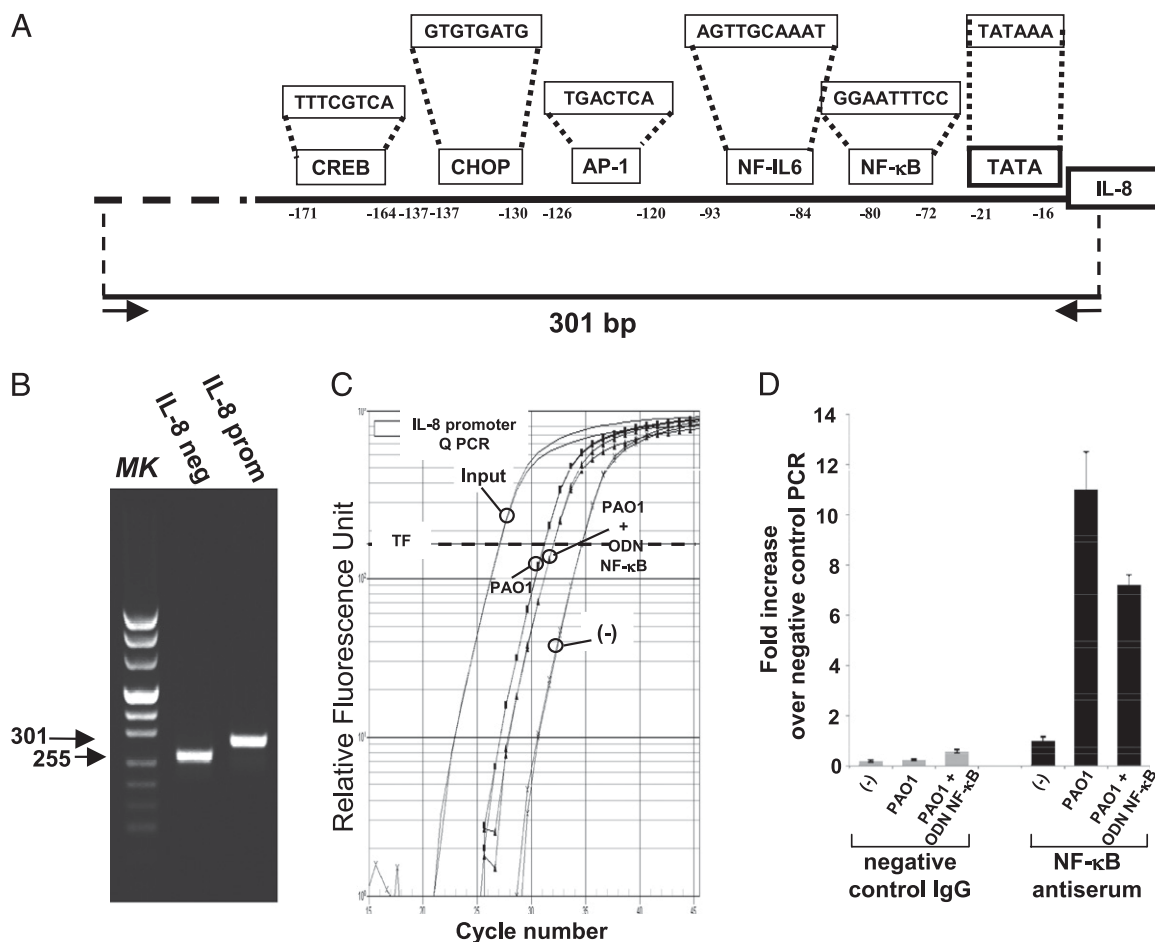


FIGURE 7. Interaction of NF- κ B transcription factor with the IL-8 gene promoter in IB3-1 cells treated with the NF- κ B decoy ODN. **A**, Schematic representation of IL-8 gene promoter region. The sequences homologous to transcription factor binding sites are boxed. The location of primers used for IL-8 promoter amplification in the ChIP assay and the product length are indicated by arrows. **B**, PCR product containing NF- κ B binding site, which was obtained from IL-8 promoter amplification (IL-8 prom) and PCR product obtained using control primers flanking a genomic region \sim 5 kb upstream of IL-8 promoter (IL-8 neg). MK, molecular marker. **C**, Quantitative real-time PCR profiles for the amplification of the IL-8 promoter are shown for a representative ChIP assay in which chromatin from IB-3 cells was immunoprecipitated using NF- κ B p65 antiserum. The data (from duplicate determinations) demonstrate the early exponential increase of fluorescence as a result of SYBR Green I incorporation into the amplifying IL-8 promoter fragment. PAO1 and PAO1 plus NF- κ B decoy ODN indicate duplicate curves from chromatin that have been immunoprecipitated with NF- κ B antiserum. (-), Curves from immunoprecipitations of untreated cells with NF- κ B antiserum (cross-marked lines). Input represents curves obtained from untreated IB3 cells chromatin (1%) before immunoprecipitation. The cycle at which the amplification curve reaches threshold fluorescence (TF), the threshold cycle, was used to determine the relative amounts of promoter in each sample. **D**, In vivo association of NF- κ B transcription factor with the IL-8 promoter. The results, obtained from ChIP assay quantitative real-time PCR using negative control IgG and NF- κ B antiserum, were analyzed following the methodology described in *Materials and Methods*. The fold increase compares the values obtained by IL-8 gene promoter amplification of untreated IB3 cells (-) and with IB3 treated with *P. aeruginosa* or *P. aeruginosa* plus NF- κ B decoy ODN.

elements within the proximal region of IL-8 gene promoter indicated by in silico analysis, namely the consensus sequences for NF- κ B (-80/-72 bp), NF-IL6 (-93/-84 bp), AP-1 (-126/-120 bp), and CREB (-171/-164 bp). Because a CHOP consensus sequence has been described at -137/-130 bp from the start site of the IL-8 promoter region (47), this has been included in our scheme, although it is not evidenced by TESS. We previously observed that TF decoy ODNs directed against the TF NF- κ B

strongly and selectively interfere with the transcription of the IL-8 gene in human bronchial IB3-1, CuFi-1, Calu-3, and BEAS-2B cell lines (34). Starting from our previous experience and this in silico analysis, decoy ODNs homologous to these sequences have been synthesized with the addition of short 5' and 3' flanking regions, as reported in Table I. In addition to the sequences identified for known TFs, the sequences localized between -119/-94 and -163/-138 bp were termed intermediate sequences A

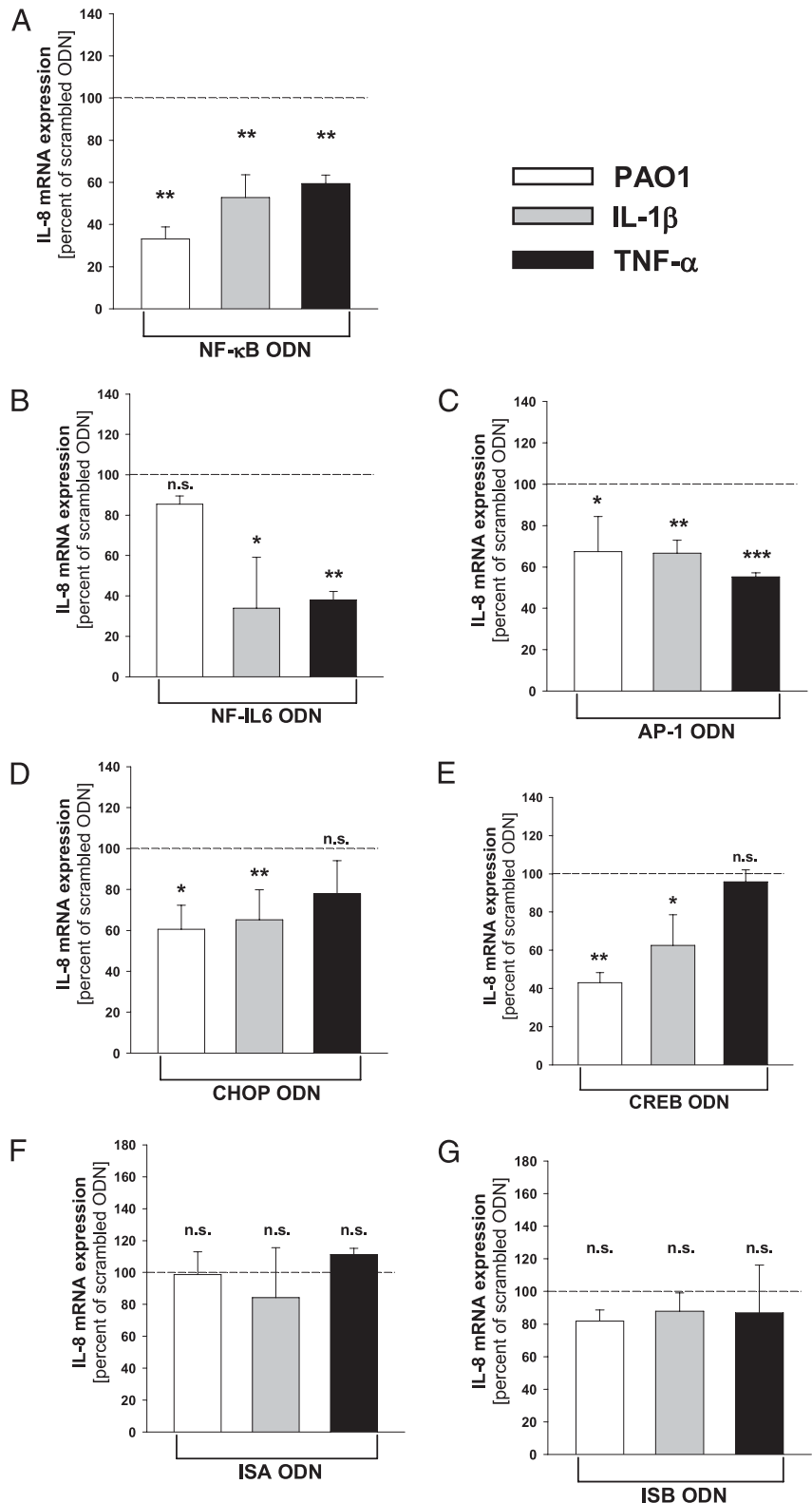


FIGURE 8. Effect of TF decoy ODNs on IL-8 gene transcription induced by *P. aeruginosa*, IL-1 β , and TNF- α in IB3-1 bronchial epithelial cells. IB3-1 cells were transfected from 20 to 24 h before infection with TF decoy ODNs against NF- κ B (A), NF-IL6 (B), AP-1 (C), CHOP (D), CREB (E), or with sequences ISA (F) and ISB (G), all complexed with Lipofectamine 2000. After this preincubation period, cells were challenged with *P. aeruginosa* (PAO1, 100 CFU/cell), IL-1 β (10 ng/ml), and TNF- α (50 ng/ml) for a further 4 h and IL-8 gene expression was measured by quantitative RT-PCR. Results are indicated as percentage of transcription obtained with transfection with scrambled ODN. Data shown are means (\pm SEM) of three independent experiments performed in duplicate. * p < 0.05, ** p < 0.01, *** p < 0.001 by Student t test.

and B (ISA and ISB) and tested to evaluate their effects on IL-8 gene transcription. To check whether the decoy ODN molecules synthesized are able to interfere with the biological activity of TFs NF- κ B, NF-IL6, AP-1, CHOP, and CREB, nuclear extracts of stimulated cells were produced and preincubated with each TF decoy ODN and run by EMSAs. As a source of TFs, IB3-1 cells were stimulated with IL-1 β , which induces abundant amounts of TFs in the unfractionated nuclear preparation. The results shown in Fig. 5 demonstrate that the decoy ODNs completely suppress the molecular interactions of these NFs with their specific target sequences. Additionally, similar inhibitory activity of the ODN decoys have been obtained, as expected, using nuclear extracts prepared from untreated IB3-1 cells, as well as cells stimulated with *P. aeruginosa* or TNF- α , in which >95% inhibition of DNA/protein interactions was obtained. Finally, the inhibitory effects were considered specific, since inhibition of DNA/protein interactions were obtained only with each specific ODN decoy molecule, with the others being nonactive or exhibiting a significantly lower activity. This is shown in Fig. 6, which reports the results of a competitive EMSA experiment in which 100 ng cold competitor decoy NF- κ B, NF-IL6, AP-1, CHOP, and CREB oligonucleotides

were incubated for 20 min with nuclear extracts before addition of the [³²P]-labeled EMSA probes. Fig. 6B clearly shows that NF-IL6, AP-1, CHOP, and CREB double-stranded oligonucleotides do not compete with the NF- κ B probe for binding to nuclear extracts. As expected, full suppression of the interactions between the [³²P]-labeled NF- κ B probe and nuclear extracts was observed when a cold NF- κ B double-stranded ODN competitor was employed. Similarly, no cross-competition was observed using [³²P]-labeled CREB (Fig. 6C), NF-IL6 (Fig. 6D), AP-1 (Fig. 6E), and CHOP (Fig. 6F), with the exception of the CREB oligonucleotide, which to some extent competes with AP-1 (but with lower efficiency, as expected; see Fig. 6E). The competitive EMSA analysis reported in Fig. 6 does support the concept that the decoy approach leads to specific inhibition of TF/DNA interaction; however, these results do not formally demonstrate that the decoy molecules interfere with the transcription factor activity on the IL-8 promoter in intact cells. This possibility was assessed using the NF- κ B model system with the objective of determining whether the decoy approach leads to reduction of the recruitment of NF- κ B to the IL-8 gene promoter; toward this end, CHIP experiments were performed. IB3-1 cells were treated with PAO1 or PAO1 plus NF- κ B decoy

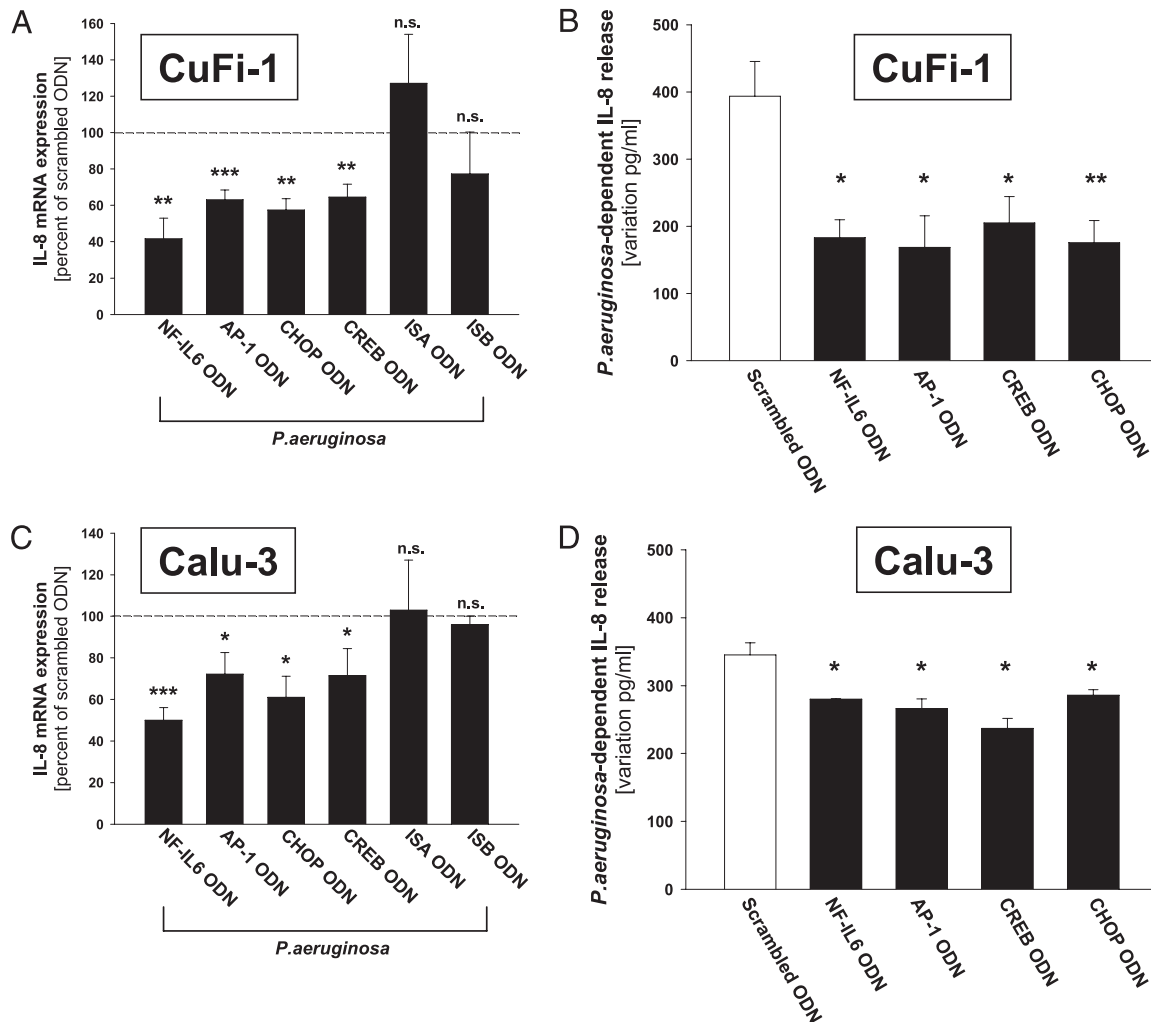


FIGURE 9. Effect of TF decoy ODNs on IL-8 gene expression induced by *P. aeruginosa* in CuFi-1 and Calu-3 bronchial epithelial cells. CuFi-1 and Calu-3 cells were transfected from 20 to 24 h before infection with TF decoy ODNs against NF- κ B, NF-IL6, AP-1, CHOP, CREB, or with sequences ISA and ISB complexed with Lipofectamine 2000. After this preincubation period, cells were challenged with *P. aeruginosa* (PAO1, 100 CFU/cell) for a further 4 h and IL-8 gene expression was measured with quantitative RT-PCR and ELISA assays. IL-8 mRNA expression in CuFi-1 (A) and Calu-3 (C) cells is shown. Results are indicated as percentage of transcription obtained by transfection with scrambled ODN. IL-8 protein release from CuFi-1 (B) and Calu-3 (D) cells, treated as for A and C, is shown. Results are indicated as variation relative to basal release of IL-8. Data are means (\pm SEM) of three independent experiments performed in duplicate. * p < 0.05, ** p < 0.01, *** p < 0.001 by Student t test.

ODN, nuclear/DNA interactions were stabilized with formaldehyde, and the shared chromatin was immunoprecipitated with Abs against NF- κ B. In parallel, the same procedure was performed with untreated IB3-1 cells as control. The DNA from immunoprecipitates was isolated and amplified by PCR with primers specific for the IL-8 promoter, as shown in Fig. 7A and 7B. Quantitative real-time PCR profiles for the amplification of the IL-8 promoter demonstrate that treatment of IB3-1 cells with PAO1 induces a fast and sharp recruitment of NF- κ B to the IL-8 gene promoter, as shown in Fig. 7C and 7D. This effect is inhibited in the presence of NF- κ B decoy ODN, indicating that the TF decoy approach is able to interfere with the recruitment and binding of TFs to the IL-8 gene promoter.

Differential effects of TF decoy molecules on IL-8 gene transcription

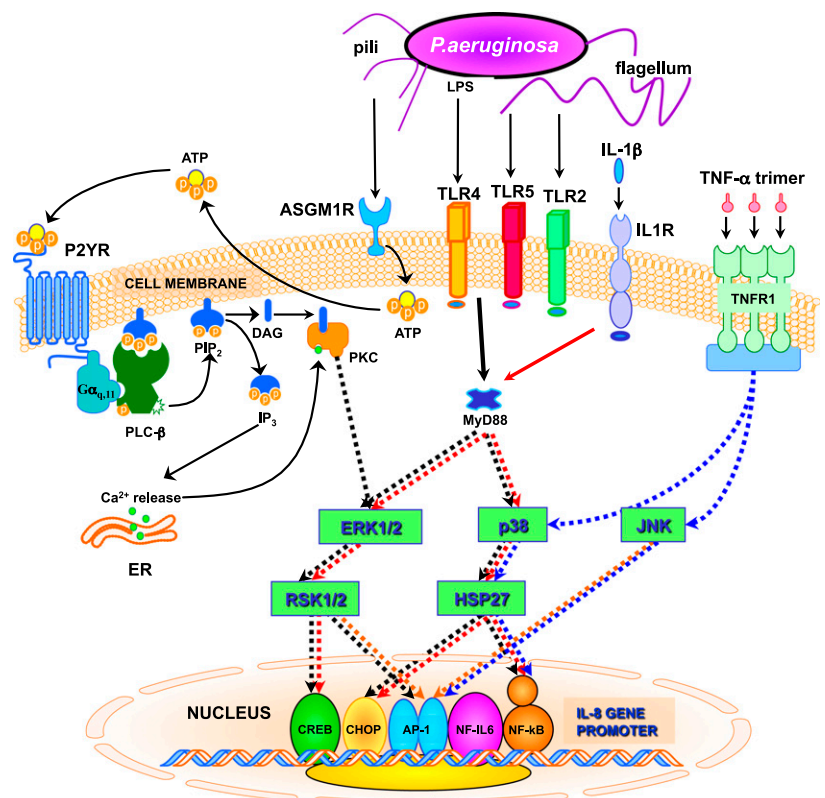
IB3-1 cells were transfected with IL-8 NF- κ B, IL-8 NF-IL6, IL-8 AP-1, IL-8 CHOP, IL-8 CREB decoy ODNs, ISA and ISB ODNs, or scrambled ODN, each complexed with cationic liposomes for 20–24 h before exposure to *P. aeruginosa*, IL-1 β , or TNF- α for further 4 h. As shown in Fig. 8, *P. aeruginosa*-dependent IL-8 transcription in bronchial epithelial cells was significantly inhibited using decoy ODNs against the TFs NF- κ B, AP-1, CHOP, and CREB, whereas NF-IL6 decoy ODN showed no inhibitory activity in IB3-1 cells. Instead, IL-8 gene transcription upon exposure to IL-1 β was diminished after preincubation of decoy ODNs interfering with TFs NF- κ B, NF-IL6, AP-1, CHOP, and CREB. Third, TNF- α -mediated IL-8 gene transcription was reduced only by preincubation of decoy ODNs against NF- κ B, NF-IL6, and AP-1. These results overall suggest a differential interference of TF decoy ODNs depending on the proinflammatory stimulus in that the TNF- α pathways seem independent of the participation of the TFs CHOP and CREB. Focusing again on *P. aeruginosa*, we previously demonstrated the strong inhibitory ef-

fect of TF NF- κ B decoy ODNs on the expression of IL-8 gene in CuFi-1 and Calu-3 cells. In this study, we extended the analysis on the effect of the TF decoy ODNs against NF-IL6, AP-1, CHOP, and CREB. As shown in Fig. 9, all of these TF decoy ODNs significantly inhibited IL-8 gene transcription and protein secretion in CuFi-1 and Calu-3 cells. Finally, no effect on IL-8 gene transcription was observed by preincubating ISA and ISB ODNs before stimulation with *P. aeruginosa*, IL-1 β , or TNF- α . The lack of inhibition of IL-8 transcription always observed with ISA and ISB ODNs, based on the sequence localized from -119 to -94 and from -163 to -138 bases from the transcription start site of the IL-8 gene promoter, suggests the absence of putative regulatory elements, as anticipated with in silico TESS analyses, in these intervening sequences. Collectively, the results presented in this study suggest that the induction of IL-8 gene transcription by *P. aeruginosa* in our human bronchial epithelial cell models is associated with the activation of MAPK ERK and its substrate kinase RSK, of the MAPK p38 and its substrate HSP27, and of the TFs NF- κ B, NF-IL6, AP-1, CHOP, and CREB.

Discussion

A thorough understanding of the molecular pathways regulating the chronic lung inflammation in CF is relevant to design novel approaches to reduce the progressive damage of the pulmonary tissue. Lung damage is thought to be mediated by an excessive recruitment of neutrophils, lead by overexpression of the chemokine IL-8, which is now considered a key therapeutic target. It is already known that *P. aeruginosa*, upon interaction with PRRs expressed in respiratory epithelial cells, induces a proinflammatory response involving the participation of the MAPKs p38 and ERK, as well as of the nuclear transcription factors NF- κ B, NF-IL6, and AP-1, via MyD88-dependent cascades and intracellular calcium (13, 24). In this paper, we basically confirm this scenario also in our experimental model, and we further propose that RSK,

FIGURE 10. Model of cooperative activation of IL-8 gene transcription after *P. aeruginosa* induction in bronchial epithelial cells. Stimulation of *P. aeruginosa*, IL-1 β , and TNF- α activates transmembrane signaling leading to phosphorylation of different pattern of kinases and transcription factors. Black solid arrows indicate putative activatory interactions induced by *P. aeruginosa* at transmembrane receptor levels, including the interactions with TLR2/4/5 and ASGM1R, as well as the release on nucleotides leading to activation of purinergic receptors, phospholipase C isoform β , protein kinase C, and cytosolic calcium, as reported previously (12–17). The major phosphoproteins and nuclear transcription factors evidenced in this investigation are reported. Arrows with dashed lines indicate the putative interactions between the different phosphoproteins and transcription factors as obtained from the literature and described in the text (18–32, 37–51). Dashed arrows are related to the putative transmembrane signaling of *P. aeruginosa* (black lines), IL-1 β (red lines), or TNF- α (blue lines). DAG, 1,2-diacylglycerol; G $\alpha_{q,11}$, α subunit of heterotrimeric G protein; IP₃, inositol 1,4,5-trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC- β , phospholipase C isoform β ; P2YR, purinergic receptor P_{2Y}.



GSK3, HSP27, and the transcription factors CREB and CHOP are implicated in the process.

To investigate the cascade of kinases activated by *P. aeruginosa*, we performed a phosphokinase assay. We confirm that *P. aeruginosa* induces phosphorylation of the MAPK p38 pathway and, specifically, of the p38 isoforms α , δ , and γ and of their downstream effector HSP27. Its role in IL-8 gene expression is confirmed by the pharmacological inhibition of both p38 and HSP27 with SB203580 and KRIBB3, respectively. As HSP27 was reported to participate always in IL-8 mRNA expression, our data may suggest a p38 δ -dependent activation of HSP27, also because p38 δ is the only isoform activated by all three proinflammatory challenges. We did not observe a direct *P. aeruginosa*-dependent phosphorylation of ERK1/2, as reported by other investigators (13). However, we found a clear increase of phosphorylation of RSK1 and MSK2, which are substrates of ERK1/2, and, in parallel, we found an inhibitory effect of *P. aeruginosa*-dependent IL-8 gene transcription with the inhibitors AG1288 and U1026 (ERK1/2) and SL0101 (RSK). A possible explanation of the lack of direct phosphorylation of ERK1/2 could be due to a very quick dephosphorylation of ERK within the 30 min incubation performed in the assay. With everything considered, we are in favor of a role of the ERK pathway in *P. aeruginosa*-dependent expression of the IL-8 gene. The phosphokinase assay showed also the increased phosphorylation of GSK3, and that its pharmacological inhibition significantly but modestly decreased *P. aeruginosa*-dependent transcription of IL-8 in IB3-1 cells. GSK3 has been described to phosphorylate >40 different substrates and to modulate the activation of 18 transcription factors, including NF- κ B, AP-1, CREB, and C/EBP (49), which could be relevant in IL-8 gene expression. However, the role of GSK3 in our experimental model is very controversial, as its pharmacological inhibition reduces only slightly IL-8 gene transcription in IB3-1 cells and does not change it significantly in CuFi-1 and Calu-3 cells. Thus, the complete mechanisms of activation and the pattern of transcription factors modulated by GSK3 in our model deserve further specific investigations.

The results obtained in this study with three proinflammatory challenges could provide suggestions related to MyD88-dependent cascades involved in the regulation of transcription of the IL-8 gene. Both *P. aeruginosa* and the proinflammatory cytokine IL-1 β are known to transduce intracellular signals through a MyD88/TIR-dependent cascade, whereas TNF- α utilizes alternative pathways (46). As expected from MyD88 activation, both *P. aeruginosa* and IL-1 β activate MAPK p38 and ERK, whereas TNF- α involves p38 and JNK, but not the ERK pathway. Additionally, in this study we observed the involvement of RSK1 in the MyD88 pathway and of HSP27 in the common p38-related pathway.

A role for CHOP in the activation of transcription of the IL-8 gene induced by IL-1 β and PGE₂ in the same IB3-1 bronchial epithelial cells has recently been proposed (30). In this study, we confirm the involvement of CHOP after exposure of IB3-1 cells to both IL-1 β and *P. aeruginosa* by a TF decoy approach. The MAPK p38/HSP27 pathway has been related to CHOP activation in melanoma cells (50). Consistent with this hypothesis, we also observed phosphorylation of MAPK p38 α and p38 γ and a very relevant phosphorylation of HSP27 after testing proinflammatory stimuli in which CHOP seems involved, namely *P. aeruginosa* and IL-1 β .

CREB has been previously related to IL-8 gene transcription in U937 monocytic cells exposed to VacA toxin (28), but never in bronchial epithelial cells challenged with *P. aeruginosa* and IL-1 β , as shown in this study. As RSK has been reported as a po-

tential activator of CREB (37), and we showed that RSK1 was phosphorylated after exposure to *P. aeruginosa* and IL-1 β , our transcription factor decoy approach seems to confirm the relationship between the activations of RSK and CREB, at least in our experimental model. In parallel, the lack of inhibition of CREB by TF decoy ODNs in IB3-1 cells stimulated with TNF- α is consistent with the suppression of CREB activity by TNF- α , previously reported in murine Leydig tumor cells (51). In any case, we were not able to reach full suppression of IL-8 gene transcription using single TF decoys, suggesting that the single transcription factors in this study can be judged as required, but not sufficient, to sustain full activated transcription of the IL-8 gene.

In conclusion, the present study is intended to increase knowledge on different activatory pathways leading to transcription of the IL-8 gene in the specific context of bronchial epithelial cells. The working hypothesis, derived from the studies of others (13–32, 37–51) and from our results (12, 52, 53), is summarized in the model depicted in Fig. 10. From a practical point of view, our model confirms the redundancy of the phosphoproteins and TFs involved, a model in which no single step can completely inhibit IL-8 gene transcription. Additionally, this suggests several possible regulatory targets to modulate the excessive expression of this chemokine, besides the well-known TF NF- κ B, for the treatment of the lung pathology of patients affected by CF.

Acknowledgments

We are grateful to Alice Prince for donating the *P. aeruginosa* strain PAO1, to Stephan J. Reshkin for critical revision of the manuscript and helpful discussions, to Maria Cristina Dececchi and Elena Nicolis for helpful discussions, and to Federica Quiri for excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

- Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou, et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245: 1066–1073.
- Boucher, R. C. 2007. Evidence for airway surface dehydration as the initiating event in CF airway disease. *J. Intern. Med.* 261: 5–16.
- Zhang, Y., X. Li, A. Carpinteiro, J. A. Goettel, M. Soddemann, and E. Gulbins. 2011. Kinase suppressor of Ras-1 protects against pulmonary *Pseudomonas aeruginosa* infections. *Nat. Med.* 17: 341–346.
- Boucher, R. C. 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur. Respir. J.* 23: 146–158 (Review).
- Muhlebach, M. S., P. W. Stewart, M. W. Leigh, and T. L. Noah. 1999. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. *Am. J. Respir. Crit. Care Med.* 160: 186–191.
- Eidelman, O., M. Srivastava, J. Zhang, X. Leighton, J. Murtie, C. Jozwik, K. Jacobson, D. L. Weinstein, E. L. Metcalf, and H. B. Pollard. 2001. Control of the proinflammatory state in cystic fibrosis lung epithelial cells by genes from the TNF- α R/NF κ B pathway. *Mol. Med.* 7: 523–534.
- Sagel, S. D., J. F. Chmiel, and M. W. Konstan. 2007. Sputum biomarkers of inflammation in cystic fibrosis lung disease. *Proc. Am. Thorac. Soc.* 4: 406–417.
- Muhlebach, M. S., and T. L. Noah. 2002. Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 165: 911–915.
- Jacquot, J., O. Tabary, P. Le Rouzic, and A. Clement. 2008. Airway epithelial cell inflammatory signalling in cystic fibrosis. *Int. J. Biochem. Cell Biol.* 40: 1703–1715.
- Hartl, D., P. Latzin, P. Hordijk, V. Marcos, C. Rudolph, M. Woischnik, S. Krauss-Etschmann, B. Koller, D. Reinhardt, A. A. Roscher, et al. 2007. Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. *Nat. Med.* 13: 1423–1430.
- Groux-Degroote, S., M. A. Krzewinski-Recchi, A. Cazet, A. Vincent, S. Lehoux, J. J. Lafitte, I. Van Seuningen, and P. Delannoy. 2008. IL-6 and IL-8 increase the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialylated and/or sulfated Lewis^x epitopes in the human bronchial mucosa. *Biochem. J.* 410: 213–223.
- Bezzeri, V., P. d'Adamo, A. Rimessi, C. Lanzara, S. Crovella, E. Nicolis, A. Tamanini, E. Athanasakis, M. Tebon, G. Bisoffi, et al. 2011. Phospholipase C- β 3 is a key modulator of IL-8 expression in cystic fibrosis bronchial epithelial cells. *J. Immunol.* 186: 4946–4958.

13. McNamara, N., M. Gallup, A. Sucher, I. Maltseva, D. McKemy, and C. Basbaum. 2006. AsialoGM1 and TLR5 cooperate in flagellin-induced nucleotide signaling to activate Erk1/2. *Am. J. Respir. Cell Mol. Biol.* 34: 653–660.
14. Chun, J., and A. Prince. 2006. Activation of Ca²⁺-dependent signaling by TLR2. *J. Immunol.* 177: 1330–1337.
15. Adamo, R., S. Sokol, G. Soong, M. I. Gomez, and A. Prince. 2004. *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and Toll-like receptor 2 as well as Toll-like receptor 5. *Am. J. Respir. Cell Mol. Biol.* 30: 627–634.
16. Raoust, E., V. Balloy, I. Garcia-Verdugo, L. Touqui, R. Ramphal, and M. Chignard. 2009. *Pseudomonas aeruginosa* LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. *PLoS ONE* 4: e7259.
17. John, G., A. O. Yildirim, B. K. Rubin, D. C. Gruenert, and M. O. Henke. 2010. TLR4-mediated innate immunity is reduced in cystic fibrosis airway cells. *Am. J. Respir. Cell Mol. Biol.* 42: 424–431.
18. Lee, S. J., and S. G. Kim. 2006. Role of p90 ribosomal S6-kinase-1 in oltipraz-induced specific phosphorylation of CCAAT/enhancer binding protein-beta for GSTA2 gene transactivation. *Mol. Pharmacol.* 69: 385–396.
19. Pierrat, B., J. S. Correia, J. L. Mary, M. Tomás-Zuber, and W. Lesslauer. 1998. RSK-B, a novel ribosomal S6 kinase family member, is a CREB kinase under dominant control of p38 α mitogen-activated protein kinase (p38 α MAPK). *J. Biol. Chem.* 273: 29661–29671.
20. Gorska, M. M., Q. Liang, S. J. Stafford, N. Goplen, N. Dharajiya, L. Guo, S. Sur, M. Gaestel, and R. Alam. 2007. MK2 controls the level of negative feedback in the NF- κ B pathway and is essential for vascular permeability and airway inflammation. *J. Exp. Med.* 204: 1637–1652.
21. Alford, K. A., S. Glennie, B. R. Turrell, L. Rawlinson, J. Saklatvala, and J. L. Dean. 2007. Heat shock protein 27 functions in inflammatory gene expression and transforming growth factor- β -activated kinase-1 (TAK1)-mediated. *J. Biol. Chem.* 282: 6232–6241.
22. Verhaeghe, C., C. Remouchamps, B. Hennuy, A. Vanderplasschen, A. Chariot, S. P. Tabruyn, C. Oury, and V. Bours. 2007. Role of IKK and ERK pathways in intrinsic inflammation of cystic fibrosis airways. *Biochem. Pharmacol.* 73: 1982–1994.
23. Richmond, A. 2002. NF- κ B, chemokine gene transcription and tumour growth. *Nat. Rev. Immunol.* 2: 664–674.
24. Fu, Z., K. Bettega, S. Carroll, K. R. Buchholz, and T. E. Machen. 2007. Role of Ca²⁺ in responses of airway epithelia to *Pseudomonas aeruginosa*, flagellin, ATP, and thapsigargin. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 292: L353–L364.
25. Boncoeur, E., V. S. Criq, E. Bonvin, T. Roque, A. Henrion-Caude, D. C. Gruenert, A. Clement, J. Jacquot, and O. Tabary. 2008. Oxidative stress induces extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase in cystic fibrosis lung epithelial cells: potential mechanism for excessive IL-8 expression. *Int. J. Biochem. Cell Biol.* 40: 432–446.
26. Muslet-Charlier, C., T. Roque, E. Boncoeur, K. Chadelat, A. Clement, J. Jacquot, and O. Tabary. 2007. Enhanced IL-1 β -induced IL-8 production in cystic fibrosis lung epithelial cells is dependent of both mitogen-activated protein kinases and NF- κ B signaling. *Biochem. Biophys. Res. Commun.* 357: 402–407.
27. Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* 72: 847–855.
28. Hisatsune, J., M. Nakayama, H. Isomoto, H. Kurazono, N. Mukaida, A. K. Mukhopadhyay, T. Azuma, Y. Yamaoka, J. Sap, E. Yamasaki, et al. 2008. Molecular characterization of *Helicobacter pylori* VacA induction of IL-8 in U937 cells reveals a prominent role for p38MAPK in activating transcription factor-2, cAMP response element binding protein, and NF- κ B activation. *J. Immunol.* 180: 5017–5027.
29. Saadane, A., S. Masters, J. DiDonato, J. Li, and M. Berger. 2007. Parthenolide inhibits I κ B kinase, NF- κ B activation, and inflammatory response in cystic fibrosis cells and mice. *Am. J. Respir. Cell Mol. Biol.* 36: 728–736.
30. Vij, N., M. O. Amoako, S. Mazur, and P. L. Zeitlin. 2008. CHOP transcription factor mediates IL-8 signaling in cystic fibrosis bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 38: 176–184.
31. Sun, H., W. C. Chung, S. H. Ryu, Z. Ju, H. T. Tran, E. Kim, J. M. Kurie, and J. S. Koo. 2008. Cyclic AMP-responsive element binding protein- and nuclear factor- κ B-regulated CXC chemokine gene expression in lung carcinogenesis. *Cancer Prev Res (Phila)* 1: 316–328.
32. Sporeen, A., R. Kooijman, B. Lintermans, K. Van Craenenbroeck, L. Vermeulen, G. Haegeman, and S. Gerlo. 2010. Cooperation of NF κ B and CREB to induce synergistic IL-6 expression in astrocytes. *Cell. Signal.* 22: 871–881.
33. Gambari, R. 2004. New trends in the development of transcription factor decoy (TFD) pharmacotherapy. *Curr. Drug Targets* 5: 419–430.
34. Bezzerri, V., M. Borgatti, E. Nicolis, I. Lampronti, M. C. Dececchi, I. Mancini, P. Rizzotti, R. Gambari, and G. Cabrini. 2008. Transcription factor oligodeoxynucleotides to NF- κ B inhibit transcription of IL-8 in bronchial cells. *Am. J. Respir. Cell Mol. Biol.* 39: 86–96.
35. Zeitlin, P. L., L. Lu, J. Rhim, G. Cutting, G. Stetten, K. A. Kieffer, R. Craig, and W. B. Guggino. 1991. A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. *Am. J. Respir. Cell Mol. Biol.* 4: 313–319.
36. Andrews, N. C., and D. V. Faller. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* 19: 2499.
37. Ratner, A. J., R. Bryan, A. Weber, S. Nguyen, D. Barnes, A. Pitt, S. Gelber, A. Cheung, and A. Prince. 2001. Cystic fibrosis pathogens activate Ca²⁺-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J. Biol. Chem.* 276: 19267–19275.
38. Bhattacharyya, S., P. K. Dudeja, and J. K. Tobacman. 2009. ROS, Hsp27, and IKK β mediate dextran sodium sulfate (DSS) activation of I κ B, NF κ B, and IL-8. *Inflamm. Bowel Dis.* 15: 673–683.
39. Roux, P. P., and J. Blenis. 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68: 320–344.
40. De Ketelaere, A., L. Vermeulen, J. Vialard, I. Van De Weyer, J. Van Wauwe, G. Haegeman, and I. Moelans. 2004. Involvement of GSK-3 β in TWEAK-mediated NF- κ B activation. *FEBS Lett.* 566: 60–64.
41. Sugden, P. H., S. J. Fuller, S. C. Weiss, and A. Clerk. 2008. Glycogen synthase kinase 3 (GSK3) in the heart: a point of integration in hypertrophic signalling and a therapeutic target? A critical analysis. *Br. J. Pharmacol.* 153(Suppl. 1): S137–S153.
42. Yang, H. T., P. Cohen, and S. Rousseau. 2008. IL-1 β -stimulated activation of ERK1/2 and p38 α MAPK mediates the transcriptional up-regulation of IL-6, IL-8 and GRO- α in HeLa cells. *Cell. Signal.* 20: 375–380.
43. Song, K. S., J. K. Seong, K. C. Chung, W. J. Lee, C. H. Kim, K. N. Cho, C. D. Kang, J. S. Koo, and J. H. Yoon. 2003. Induction of MUC8 gene expression by interleukin-1 β is mediated by a sequential ERK MAPK/RSK1/CREB cascade pathway in human airway epithelial cells. *J. Biol. Chem.* 278: 34890–34896.
44. Xu, S., H. Bayat, X. Hou, and B. Jiang. 2006. Ribosomal S6 kinase-1 modulates interleukin-1 β -induced persistent activation of NF- κ B through phosphorylation of I κ B β . *Am. J. Physiol. Cell Physiol.* 291: C1336–C1345.
45. Wu, Y., J. Liu, Z. Zhang, H. Huang, J. Shen, S. Zhang, Y. Jiang, L. Luo, and Z. Yin. 2009. HSP27 regulates IL-1 stimulated IKK activation through interacting with TRAF6 and affecting its ubiquitination. *Cell. Signal.* 21: 143–150.
46. Bouwmeester, T., A. Bauch, H. Ruffner, P. O. Angrand, G. Bergamini, K. Croughton, C. Cruciat, D. Eberhard, J. Gagneur, S. Ghidelli, et al. 2004. A physical and functional map of the human TNF- α /NF- κ B signal transduction pathway. *Nat. Cell Biol.* 6: 97–105.
47. Cucinotta, M., M. Visalli, M. Aguenouz, A. Valenti, S. Loddo, L. Altucci, and D. Teti. 2008. Regulation of interleukin-8 gene at a distinct site of its promoter by CCAAT enhancer-binding protein homologous protein in prostaglandin E₂-treated human T cells. *J. Biol. Chem.* 283: 29760–29769.
48. Venza, I., M. Cucinotta, M. Visalli, G. De Grazia, S. Oliva, and D. Teti. 2009. *Pseudomonas aeruginosa* induces interleukin-8 (IL-8) gene expression in human conjunctiva through the recruitment of both RelA and CCAAT/enhancer-binding protein beta to the IL-8 promoter. *J. Biol. Chem.* 284: 4191–4199.
49. Jope, R. S., and G. V. Johnson. 2004. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem. Sci.* 29: 95–102.
50. Sarkar, D., Z. Z. Su, I. V. Lebedeva, M. Sauean, R. V. Gopalkrishnan, K. Valerie, P. Dent, and P. B. Fisher. 2002. Mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc. Natl. Acad. Sci. USA* 99: 10054–10059.
51. Arai, K. Y., K. F. Roby, and P. F. Terranova. 2005. Tumor necrosis factor alpha (TNF) suppresses cAMP response element (CRE) activity and nuclear CRE binding protein in MA-10 mouse Leydig tumor cells. *Endocrine* 27: 17–24.
52. Gambari, R., M. Borgatti, V. Bezzerri, E. Nicolis, I. Lampronti, M. C. Dececchi, I. Mancini, A. Tamanini, and G. Cabrini. 2010. Decoy oligodeoxynucleotides and peptide nucleic acids-DNA chimeras targeting nuclear factor κ -B: inhibition of IL-8 gene expression in cystic fibrosis cells infected with *Pseudomonas aeruginosa*. *Biochem. Pharmacol.* 80: 1887–1894.
53. Cabrini, G., V. Bezzerri, I. Mancini, E. Nicolis, M. C. Dececchi, A. Tamanini, I. Lampronti, L. Piccagli, N. Bianchi, M. Borgatti, and R. Gambari. 2010. Targeting transcription factor activity as a strategy to inhibit pro-inflammatory genes involved in cystic fibrosis: decoy oligonucleotides and low-molecular weight compounds. *Curr. Med. Chem.* 17: 4392–4404.