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Proinflammatory signal transduction in epithelial cells: the model of cystic fibrosis lung disease

S.S.D. BIO/11

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ABSTRACT

Cystic Fibrosis (CF) is a severe inherited disease caused by mutations of the gene encoding for a chloride channel termed CFTR. Albeit CF is a multiple-organ disease, lung inflammation is the most common cause of morbidity and mortality. CF lung pathology is characterized by huge infiltrates of neutrophils (PMNs) into the lung lumen and by excessive release of cytokines and chemokines, in particular IL-8. After bacterial infection, sustained mainly by *Pseudomonas aeruginosa*, this inflammatory process is amplified, leading to massive recruitment of PMNs which contributes to lung tissue damage. Our goal is gain further insights into the pro-inflammatory signal transduction that underlies the CF lung inflammation. Here we reported that *P. aeruginosa*-dependent transmembrane signalling pathway in bronchial epithelia occurs on the one hand via Toll Like Receptors (TLRs) activation, thus by activation of MAPK p38, ERK-1/2, JNK and their downstream effectors HSP27, RSK and IKK; on the other hand via ATP release and purinergic activation which in turn activates Phospholipase-C beta (PLCB). This enzyme is able to induce intracellular calcium signalling, triggering the PKC activation. Furthermore, here we reported that many of the MAP kinases involved are able to promote activation of several Transcription Factors (TFs), such as CREB, CHOP, AP-1 and NF-IL6, beside the well known nuclear factor NF-κB. These TFs bind to the proximal promoter region of IL-8 gene causing its expression. Moreover, results indicate that PLCB1, PLCB3 and PLCB4 isoforms seem to be redundantly activated by *P. aeruginosa*-dependent ATP release through purinergic receptor binding. Concluding, the final aim of this Ph.D program has been deepen the pro-inflammatory transmembrane signalling in order to provide a panel of molecular targets which may support the future development of novel therapies for CF lung inflammation.

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1. INTRODUCTION

1.1 State of the art of the lung pathology in cystic fibrosis

Cystic fibrosis (CF) is a lethal inherited disease caused by mutations of a gene encoding a chloride channel which belong to the superfamily of ABC transporter ATPase, termed Cystic Fibrosis Transmembrane conductance Regulator (CFTR). To date, more than 1800 different CFTR gene mutations, divided into five classes, are associated to CF illness. The most common CFTR mutation, F508del/F508del, results in a misfolded protein that is improperly glycosylated. The homozygous F508del CFTR protein is degraded into endosomal vesicles failing to reach the apical surface of the epithelium. Other CFTR mutations, such as G551D, form a reduced functional channel [Welsh, 2001]. Furthermore, there is a large number of modifier genes which should affects the clinical symptoms of the disease.

Although CF is a multiple-organ disease, affecting pancreas, liver, bowel, sweat glands and reproductive organs, the lung pathology is the major cause of morbidity and mortality in CF. Even if huge progresses were made in understanding the molecular biology and the physiology of the CFTR channel, it remains so far unknown why CFTR mutations cause the hyper responsiveness to pulmonary inflammation. One hypothesis is that CFTR dysfunction, which leads to lesser chloride efflux from epithelia and subsequently excessive sodium reabsorption, causes dehydration of the airway surface liquid (ASL) impairing the mucociliary clearance. In this condition, mucus accumulates into lung lumen promoting some bacterial and fungal colonization by opportunistic pathogens like *Pseudomonas aeruginosa*, *Staphilococcus aureus* and *Aspergillus fumigatus* [Cohen, 2012]. However, excessive infiltrates of neutrophils (PMNs) and release of pro-inflammatory cytokines and chemokines, were found also in broncoalveolar lavage fluids from young CF children which did not present any bacterial or fungal infection [Bonfield, 1999; Muhlebach, 2004]. These evidences suggest that CFTR dysfunction prejudices the innate immune system inducing inflammatory processes in infants with CF. In this regard, anti-inflammatory drugs have been proposed in order to ameliorate CF pulmonary pathology. Both non-corticosteroid anti-inflammatory drugs such as Ibuprofen and steroids such as Beclomethasone have been not successful treatments so far.

Another hypothesis regard the presence of constitutive activation of the nuclear factor κ light-chain enhancer of activated B cells (NF-κB) in airway epithelia [Cohen, 2012]. NF-κB plays a key role on Tumor Necrosis Factor alpha (TNFα) and IL-8 gene expression. IL-8 is the major chemoattractant for PMNs, and dysregulation of NF-κB may reflects the excessive IL-8 releasing observed in CF lung pathology, which, at least in part, may explain the excessive accumulation of PMNs and their products [Cohen, 2012]. Furthermore, constitutive activation of NF-κB signaling results in increased amounts of reactive oxygen species (ROS) which are also commonly generated by PMNs which are continuously accumulated in the airway. Normally, CFTR maintain the downregulation of NF-κB signaling during oxidative stress by controlling the degradation of its inhibitor, IκB-α. Unfortunately, this response lacks in the CF lung. Moreover, bacterial infection per se increases the pro-oxidant balance since airway epithelial cells produce detectable levels of H_2O_2 in response to *P. aeruginosa* and *S.aureus* infection (see fig. 1), as shown both in human epithelial cells *in vitro* and in murine lung models *in vivo* [Moskwa, 2006]. It has also been shown that H_2O_2 released from pulmonary epithelia in response to different *P. aeruginosa* strains is due to the activation of the NADPH oxidase Duox-1 [Rada, 2010]. ROS such as O_2, H_2O_2 and HOCl have been associated with increased secretion of IL-8 and IL-6 from lung epithelia upon *P. aeruginosa* infection, and with defective autophagy and reduced CFTR expression [Bérubé, 2010; Luciani, 2010]. Epithelial cells may control oxidative damage through the production of several antioxidant molecules, such as glutathione (GSH) and thiocyanate (SCN^-) that are released into the ASL. However, also GSH secretion is markedly reduced both in patients with cystic fibrosis and in the cystic fibrosis mouse model, due to a protein trafficking defect reported in the epithelial cells (for review see Galli, 2012). Thus, deficient epithelia CFTR function results in a decreased capability to counteract the oxidative stress which continuously takes place in CF lungs (see fig. 2) [Cantin, 1987; Xu, 2009; Galli, 2012].

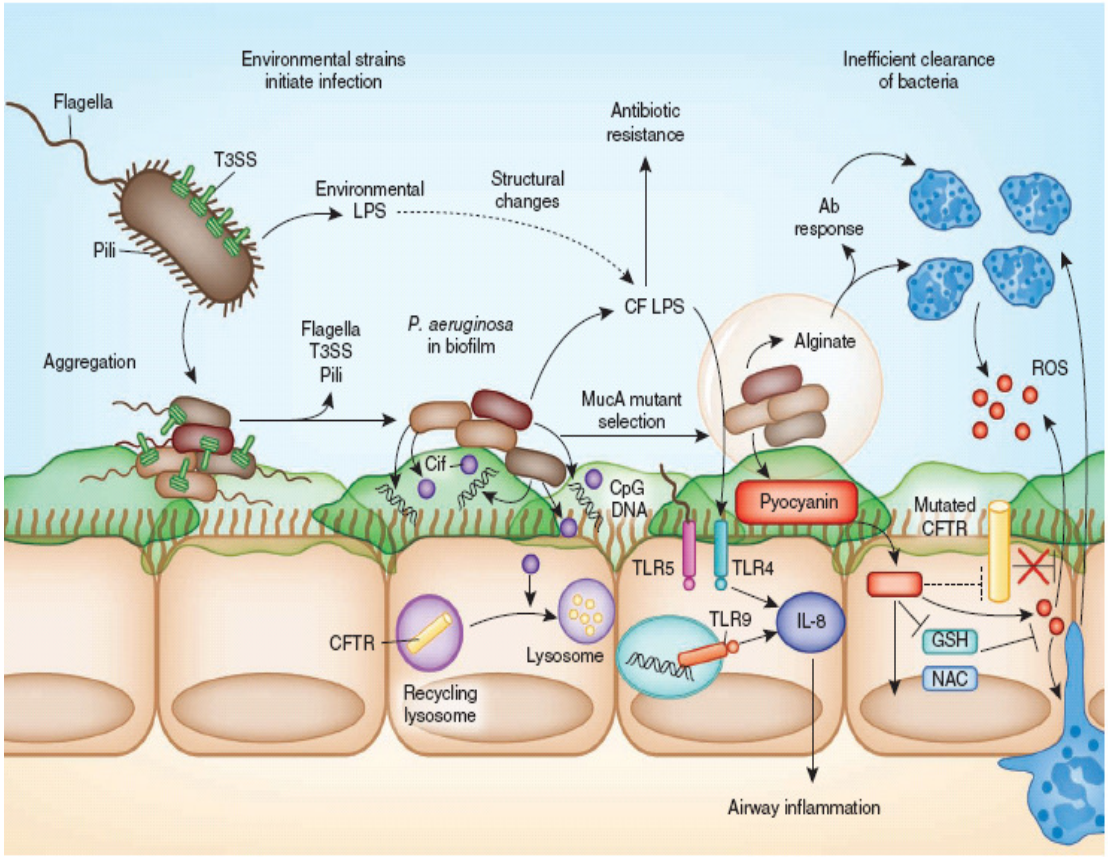

Figure 1

The adaptation process of inhaled bacteria to the cystic fibrosis lung environment. *Inhaled bacteria expressing flagella, pili and a type 3 secretion system (T3SS) aggregate within the cystic fibrosis lung, producing biofilm. Within the biofilm, bacteria lose flagella, pili and the T3SS, increasing the production of alginate, releasing CpG DNA and expressing a diverse range of virulence factors which promote the evasion of the host immune system. P. aeruginosa also releases the CFTR inhibitor factor (Cif), a protein that inhibits the recycling of CFTR in the host. Furthermore, the lipid A structure of the LPS is altered through the addition of palmitate and aminoarabinose (CF LPS), resulting in increased antibiotic (Ab) resistance and increased induction of IL-8 production by host cells (from: Cohen TS and Prince A. Nat Med 2012; 18:509-1-519).*

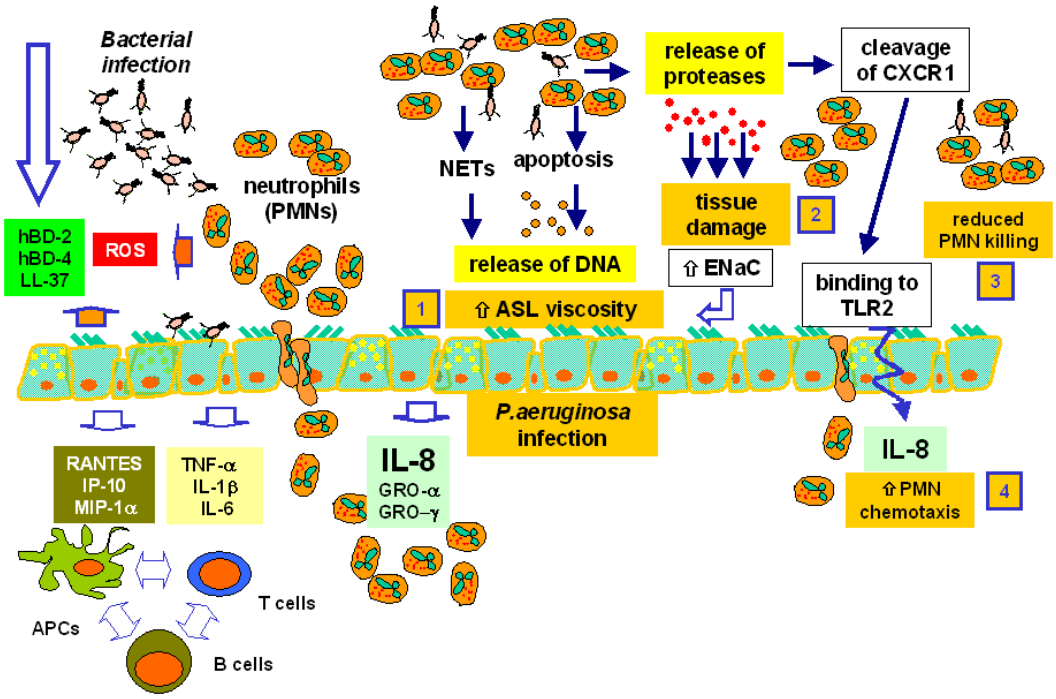

Figure 2

Inflammation: a double-edged sword in CF chronic lung disease. *Upon exposure to bacteria, respiratory epithelial cells release Reactive Oxygen Species (ROS) as an innate anti-infective mechanism, together with several anti-microbial peptides such as human beta defensins (hBD-1/2/4) and cathelicidins (LL-37). Recruitment of leukocytes also starts from epithelial cells releasing chemokines directed to recruit neutrophils (e.g. IL-8, GRO-α/γ), or recruiting lympho-monocytes (e.g. RANTES, IP-10, MIP-1a). Also the major pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNFa) are initially expressed and released by surface epithelial cells of the conductive airways. Different undesired effect of the activation of PMNs are schematically represented: [1] Neutrophil Extracellular Traps (NETs), a pulsed release of DNA from PMNs, originally directed to immobilise bacteria and favour their phagocytosis, produce a worsening of the already reduced muco-ciliary clearance in CF airways by increasing the viscosity of the Airway Surface Liquid (ASL), an undesired effect amplified by the extensive DNA release dependent on cell death of the short-living PMNs; [2] proteases released from the granules of activated PMNs produce tissue damage, increase activation of the Epithelial Sodium Channel (ENaC), further reducing ASL hydration and fluidity, [3] cleave the CXC Receptor 1 expressed on PMNs, reducing their bacterial killing capacity, and peptides cleaved by proteases from CXC Receptor 1 bind to TLR2 and further activate PMN chemotaxis [4] (from: Cabrini G et al. 2010; Chem Med Chem 17:4392-4404)*

1.2 The CFTR channel

CFTR is a glycosylated protein that functions as a plasma membrane chloride and bicarbonate channel [Anderson, 1991] and modulates salt and water transport across epithelial cell membranes of various tissues [Huang, 2004; Li, 2005]. CFTR is comprised of two membrane-spanning domains (MSD1 and MSD2), two nucleotide-binding domains (NBD1 and NBD2) and a regulatory domain (R) [Riordan, 2004] (see fig. 3) . CFTR activation requires both ATP binding to the interface between NBD1 and NBD2 and PKA mediated phosphorylation of the R domain [Cheng, 1991; Berger, 2005]. A large number of cellular chaperones and co-chaperones participate in regulating the correct folding of CFTR [Kim, 2012]. The deletion of the phenylalanine residue at position 508 (*F508del*) in the NBD1 domain is the most common class II mutation in cystic fibrosis (CF) and results in a folding defect of F508del CFTR, its retention in the endoplasmic reticulum (ER) and the premature degradation by the ubiquitin-proteasome system (UPS). The small amount of F508del CFTR that escapes from UPS and reaches the plasma membrane functions poorly and is unstable on the plasma membrane with a half-life on the cell surface significantly shorter than that of the wild type (wt) protein [Lukacs, 1993; Heda, 2001]. The consequent F508del CFTR-dependent ionic imbalance and the aberrant fluid homeostasis at epithelial surfaces in the lung results in the secretion of thick mucus which impairs the mucociliary clearance favoring the bacterial infections, inflammation and consequent impairment of lung function [Boucher, 2007]. It has been shown that culturing cells at low temperature (27^{o}C) can rescue the trafficking defect of F508del CFTR and its expression at the cell surface, although still with altered gating of the channel and reduced cell surface density and recycling efficiency [Denning, 1992; Cholon, 2007; Jurkuvenaite, 2010]. In addition to low temperature culture, chemical compounds such as DMSO [Bebok, 1998], glycerol [Sato, 1996] or organic solutes [Zhang, 2003] increase the processing of the core-glycosylated, endoplasmic reticulum-arrested F508del CFTR into the fully glycosylated mature form of CFTR. These findings, together with the evidence that restoration of small amounts of functional CFTR protein (20-30% of normal levels) [Zhang, 2009] can greatly ameliorate the disease severity, have stimulated a great effort to identify membrane permeable small molecule compounds which could

either rescue the biosynthetic defect of F508del CFTR thus restoring its folding, trafficking and insertion into the plasma membrane (correctors) and/or enhance its regulated function once rescued to the surface (potentiators).

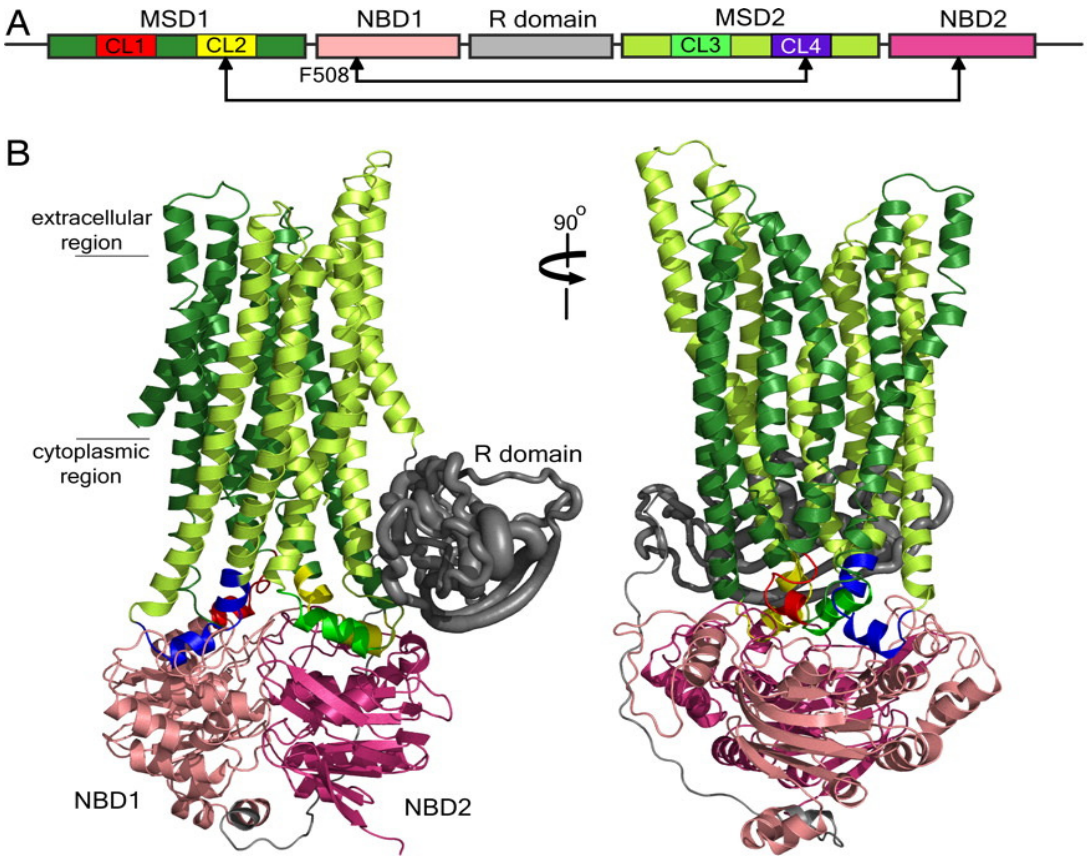

Figure 3

A theoretical model of CFTR structure. *(*A*) CFTR primary structure containing two nucleotide-binding domains (NBD1 and NBD2), two membrane-spanning domains (MSD1 and MSD2), and a regulatory region (R domain). Each MSD contains two cytoplasmic loops (CL) that form interfaces with the NBDs. (*B*) Model of CFTR where the domains are coloured as in panel A (adapted from: Serohijos A W R et al. PNAS 2008; 105:3256-3261)*

The potentiators, interacting directly with the NBD1 and NBD2 of CFTR [Cai, 2006], rapidly increase CFTR channel activation by either an ATP-dependent [Hwang, 2009] or -independent [Eckford, 2012] mechanism, while the correctors

require several hours to rescue the functional expression of CFTR. Most of the presently identified potential correctors have been selected by high-throughput screening which allows a rapid screening of thousands of small molecules before being validated in *in vitro* culture systems [Verkman, 2006; Verkman, 2009]. Some of them have displayed some efficacy in restoring F508del CFTR plasma membrane localization and improving chloride transport [Pedemonte, 2006; Wang, 2006; Carlile, 2007]. In particular, VX-809 [Van Goor, 2011], developed by Vertex Pharmaceuticals, has resulted to be efficacious in improving F508del CFTR surface expression and chloride transport in cultured human bronchial epithelial cells isolated from CF patients homozygous for the *F508del CFTR* mutation and is presently considered suitable for advancement into Phase III clinical studies in combination with the potentiator VX-770 "Kalydeco" [Van Goor, 2009]. This combined administration of a potentiator and a corrector together is required to rescue a sufficient functional expression of F508del CFTR at the cell surface as the *F508del CFTR* mutation displays both trafficking and gating defects. The mechanism of action of many correctors remains relatively poorly known and studies to better understand the structure of the CFTR protein and the conformational destabilization of F508del NBD1 are useful to develop new compounds that can bind directly to the mutant protein. In this respect, the identification of a number of folding correctors of F508del CFTR has been discovered *in silico* structurebased screening utilizing homology models of CFTR [Kalid, 2010]. Correctors may rescue the functional expression of F508del CFTR either acting as pharmacological chaperones which interact directly with the misfolded F508del CFTR protein favoring its folding and trafficking to the cell surface [Loo, 2008; Sampson, 2011; Wang, 2007; Yu, 2011] or as proteostasis regulators modulating the activity of a plethora of pathways involved in F508del CFTR folding and degradation [Balch, 2011; Hanrahan, 2013; Mu, 2008]. However, as F508del CFTR trafficking is inhibited by various quality control systems, it is becoming evident that a combination of two different correctors or a corrector and a potentiator together might be necessary to achieve a significant increase in F508del CFTR functional expression and restore the CF symptoms. In this respect, compounds having both corrector and potentiator functions would alleviate this need for multiple combined administrations but until now very few

compounds have been identified with both corrector and potentiator activities [Pedemonte, 2011; Phuan, 2011; Knapp, 2012]. It has been recently demonstrated that the psoralen-related compound, 4,6,4'-trimethylangelicin (TMA), strongly potentiates the cAMP/PKA-dependent activation of wt CFTR in airway cells. Moreover, in the same work it has been demonstrated that TMA significantly inhibits the expression of the IL-8 gene in airway cells in which the inflammatory response has been challenged with *P. aeruginosa*, suggest that TMA may represent a promising lead compound for the development of a single drug therapy useful for overcoming the CF defect [Tamanini, 2011].

1.3 Inside the lung inflammatory process in cystic fibrosis: the MAP kinases-mediated pro-inflammatory signal transduction

As previously discussed, CF lung pathology is also featured by exuberant neutrophils (PMNs) infiltration in bronchial lumen and increased levels of pro-inflammatory cytokines and chemokines expression, in particular Interleukin-8 (IL-8), and free proteases [Welsh, 2001]. After bacterial colonization, sustained mainly by *P. aeruginosa* in the early phases, and by *Burkolderia Cepacia* in the advanced phases, the pro-inflammatory condition tends to worsen dramatically. Indeed, on the bacterial surfaces are present several immunostimulatory pathogen–molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagella. PAMPs are able to bind pattern recognition receptors (PRRs) expressed on the cellular surface of the host, inducing multiple signal transduction pathways which finally unleash inflammatory responses [Welsh, 2001]. In this regard, Toll-like receptors (TLRs) play a significant role as PRRs involved in bacterial recognition. For instance, the recognition of flagellin subunits released by *P. aeruginosa* flagella is mediated by TLR5, whereas LPS is known to bind TLR4. TLR4 and TLR5 signal transduction is mainly mediated through the adaptor protein myeloid differentiation primary response gene-88 (MyD88) which in turn leads to phosphorylation of three Mithogen Activated Protein Kinase (MAPK): p38 MAPK, Extracellular signal Regulated Kinase 1/2 (ERK1/2) and c-Jun-N-terminal Kinase (JNK) [Cohen, 2012]. However, knockdown of MyD88 in cystic fibrosis epithelial cells prevented bacterial-induced signaling as expected, but did not inhibit NF-κB signaling to the control non-CF cells

level [Cohen, 2012]. This data strengthen the hypothesis of constitutive activation of the NF-κB pathway in cystic fibrosis cells. Anyway, other mediators of signal transduction are activated downstream ERK1/2 and p38 MAPK, such as mitogen and stress-activated kinases (MSK) and 90kDa ribosomal S6 kinases (RSK), which lead to activation of transcription factors (TFs) like NF-κB and cAMP response element-binding protein (CREB) [Pierrat, 1998; Roux, 2004]. 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/EBPβ (also known as NF-IL6), as documented in human hepatocytes, and/or to activation of CREB, as reported in human airway epithelial cells [Lee, 2006]. Another member of the RSK family, the mitogen- and stress-activated kinase-2 (MSK2), has been reported to be a substrate of p38α, which is able to activate both CREB and AP-1 [Pierrat, 1998]. 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-κB [Gorska, 2007]. Additionally, HSP27 has been reported to be recruited in IL-1β– and TNFα–dependent IL-8 gene transcription in HeLa cells [Alford, 2007]. These phosphorylations eventually activate transcription factors (TFs), first of all NF-κB [Muselet-Charlier, 2007; Boncoeur, 2008]. Moreover, several *in vitro* studies using human epithelial cystic fibrosis cell lines confirmed increased NF-κB signaling and the involvement of several proinflammatory cascades, including activation of Ca^{2+}-dependent signaling and MAPK-dependent activation of activator protein 1 (AP-1), in cystic fibrosis epithelia compared to control epithelia [Fu, 2007]. CF airway epithelial cells exposed to proinflammatory stimuli, such as TNFα and IL-1β, regulate IL-8 gene transcription through the activation of NF-κB, NF-IL6, AP-1, and CHOP [Verhaeghe, 2007; Saadane, 2007; Vij, 2008]. 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 [hisatsune, 2008], and it has been shown that CREB collaborates with NF-κB in CXC chemokine expression in human respiratory carcinoma cells [Sun, 2008]. Additionally, it has been recently observed that CREB and NF-κB synergistically induce IL-6 but not IL-8 gene expression in astrocytes stimulated with TNFα [Spooren, 2010].

1.4 Inside the lung inflammatory process in cystic fibrosis: the calcium-dependent signal transduction pathways

Also extracellular ATP activates cytosolic calcium signaling contributing to the expression of pro-inflammatory genes. In this regard, *P. aeruginosa* has been shown to interact with asialo-GM1 receptor (ASGM1R) which co-localizes with TLR5, promoting sustained release of nucleotides. The ATP which accumulates within the cellular milieu bind the G-coupled seven transmembrane spanning domain purinergic receptors (P2Y2) which in turn activate cytosolic calcium signalling, through the activation of the enzyme Phospholipase-C (PLC), contributing to IL-8 expression. In this regard, PLC-β isoforms are implicated in signal transduction by receptors for hormones, growth factors, neurotransmitters and other ligands involved in regulation of different cellular processes, including the immune response. It has been shown that PLCB3 isoform is selectively coupled to the P2Y2 receptor-activated calcium transients in recombinant CHO cells. PLCB3 catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to generate 1,2-diacylglicerol (DAG) and inositol 1,4,5 triphosphate (IP_3). DAG is an activator of protein kinase C (PKC) which translocate to the plasmamembrane upon its phosphorylation, whereas IP_3 activate intracellular calcium transients. Notably, it has been recently shown that UDP can activate cytosolic calcium rise in murine macrophages through the activation of purinergic receptors which in turn activate both PLCB3 and PLCB4, whereas other ligands like C5a activate only PLCB3 isoform (25).

2.AIMS OF THE DOCTORAL PROJECT

2.1 Aims overview

The general aim of this doctoral project is to explore the signal transduction pathways which regulate the pro-inflammatory gene expression, in particular the chemokine IL-8, in respiratory epithelial cells exposed to pro-inflammatory stimuli specific of CF lung disease, in order to highlight molecular targets for novel anti-inflammatory approaches.

Specific aims can be summarized as follows:

1. Comprehensive analysis of the molecular mechanisms that underlie the IL-8 transcription in bronchial epithelial cells derived from patients affected by cystic fibrosis.
2. Investigate the transmembrane calcium-mediated signal transduction during *P. aeruginosa*-dependent pro-inflammatory gene expression.

2.2 Rationale of specific aim 1

Activation of the transcription machinery of IL-8 could be regulated by the intervention of one or more TFs among those reported in different cell models (see chapter 1.3), but the precise map of those intervening in cells derived from the epithelium lining the conductive airways of bronchi, specifically exposed to *P. aeruginosa* or soluble pro-inflammatory stimuli, is not fully understood. First, we scanned the role of TFs with the so-termed TF "decoy" strategy. The aim of the TF "decoy" strategy is based on the competition for trans-acting factors between endogenous cis-elements present within the regulatory regions of the target gene and exogenously added DNA decoys (or functionally active analogues) mimicking the specific cis-elements. The objective of this molecular intervention is to cause an attenuation of the authentic interactions of trans-factors with their cis-elements, leading to a removal of the trans-factors from the endogenous cis-element inside the cell (see fig. 4). The most important feature of potential decoy molecules is the ability to tightly bind to target transcription factors. In this case, the expression of genes directly regulated by the targeted transcription factors will be deeply altered.

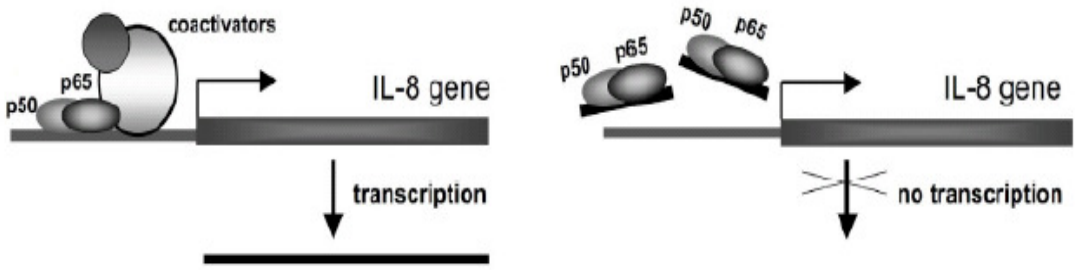

Figure 4

The decoy strategy targeting NF-κB. *The interactions between decoy oligonucleotides and p50/p65 heterodimers inhibit transcription (from: Cabrini G et al. 2010; Chem Med Chem 17:4392-4404).*

With this approach the host laboratory of this doctoral project previously observed the novel role of the TF Sp1 in the regulation of expression of IL-6 [Borgatti, 2007] and confirmed that of NF-κB for IL-8 in human bronchial epithelial cell lines derived from a CF patient [Bezzerri, 2008]. After *in silico* analysis of the putative consensus sequences for binding of TFs in IL-8 promoter, short 18-22 bp oligodeoxynucleotides (ODNs) mimicking in part or completely the consensus sequences identified, with proper flanking strands to confer resistance to nucleases, have been designed, synthesized and transfected in bronchial cells as TF "decoy" short molecules as previously described [Borgatti, 2007; Bezzerri, 2008]. In order to validate that interaction of TF "decoy" molecules in bronchial epithelial cells is actually related to each TF, the Electrophoretic Mobility Shift Assays (EMSA) have been performed, as previously described [Bezzerri, 2008]. Short sequence TF "decoy" ODNs should provide the possibility of dissecting the different parts of the IL-8 promoter and ultimately rebuild a complete scanning of the promoter sequences relevant to IL-8 transcription.

Moreover, it is already known that anti-inflammatory drugs can intervene by modulating the function of kinases and adapters upstream the TFs. For instance, corticosteroids exert pleiotropic effects by inhibiting cytosolic phospholipase A2α through induction of transcription of annexin I and induce MAPK phosphatase

1, thus inhibiting c-Jun-mediated transcription (for review see Rhen, 2005). Therefore the analysis of the effect of *P. aeruginosa* on the activation of the major kinases, either by direct analysis of phosphoproteins, integrated by study of chemical inhibitors, could provide an important framework of the intracellular signalling leading to IL-8 expression, on which novel potential anti-inflammatory molecules can be verified.

2.3 Rationale of specific aim 2

Chronic airway infection by *P. aeruginosa* is a common pathological manifestation in Cystic Fibrosis (CF) patients [Welsh MJ, 2001]. This manifestation is associated with an excessive inflammatory response characterized by the accumulation of large amounts of chemokines and cytokines, including IL-8 and IL-1β [Bonfield, 1995]. In our specific model, interaction of *P. aeruginosa* with ASGM1R, colocalized with TLR5, is known to promote the release of nucleotides from epithelial cells, activating an autocrine loop with P2Y2 receptors [McNamara, 2001; Adamo, 2004], and this is at least partly mediated by bacterial flagellin [McNamara, 2006]. Interestingly, some PLCB isoforms, such as PLCB3, have been shown to be selectively coupled to the P2Y2 receptor-dependent activation of intracellular Ca^{2+} transients in recombinant CHO cells [Strassheim, 2000]. Activation of PLC gamma and epsilon isoforms is known to be dependent on tyrosine kinase coupled receptors, whereas the PLC delta isoform activation is dependent on the elevation of cytosolic calcium, and only the PLC beta isoforms is known to be coupled to seven membrane spanning domain receptors, such as purinergic receptors, through GTP binding proteins [Suh, 2008]. Also, among the surface receptors expressed in bronchial epithelial cell that are engaged by *P. aeruginosa*, TLRs and ASGM1R have not been described as coupled to GTP binding proteins [Wettschureck, 2005]. Therefore, as human bronchial epithelial cells utilized in our experimental model express the transcripts of all the PLC beta isoforms, e.g. PLCB1, B2, B3 and B4, albeit at different levels, other PLCB isoforms, besides the PLCB3, are the most likely participants in this signaling pathway, but their precise role should be completely verified and characterized. However, the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to generate two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3),

which in turn activate intracellular calcium transients [Katan, 1998; Suh, 2008], as mediated by PLC beta isoforms, is a biochemical event which is very upstream the intracellular signaling cascade inducing the expression of pro-inflammatory genes. Therefore it would be likely that the calcium-associated signaling could result in the activation of a differentiated pattern of pro-inflammatory genes in bronchial epithelial cells, which could help understanding the relevance and selectivity of PLCB isoforms as therapeutic targets in CF lung disease.

3. MATERIAL AND METHODS

3.1 Reagents and pharmacological inhibitors

Human rIL-1β, rTNFα, ATP, Apyrase, pharmaceutical inhibitors KRIBB3, parthenolide, and SB203580, have been purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal Ab anti-PLCB3 (sc-13958), goat polyclonal Ab anti-β-actin (sc-1615), HRP-conjugated goat anti-rabbit IgG (sc-2004), HRP-conjugated donkey anti-goat IgG (sc-2020) and pharmaceutical inhibitors AG1288 and GSK3 inhibitor II have been purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SL0101 and U1026 were from Tocris Bioscience (Ellisville, MO). The calcium chelator BAPTA-AM was purchased from Molecular Probes (Eugene, OR), the broad protein kinaseC(PKC) inhibitor bisindolylmaleimide- I (BIM-I) was purchased from Merck KGaA (Darmstadt, Germany)

3.2 Airway epithelial cell culture

IB3-1 cells (LGC Promochem Europe) are human bronchial epithelial cells immortalized with adeno12/SV40, derived from a CF patient with a mutant F508del/W1282X genotype. Cells will be grown in Laboratory of Human Carcinogenesis (LHC)-8 basal medium (Biofluids, Rockville, MO) supplemented with 5% FBS. All culture flasks and plates will be coated with a solution containing 35 mg/ml bovine collagen (BD Biosciences, Franklin Lakes, NJ), 1 μg/ml BSA (Sigma-Aldrich), and 1 μg/ml human fibronectin (BD Biosciences). CuFi-1 and NuLi-1 cells, kindly donated by A. Klingelhutz, P. Karp, and J. Zabner (University of Iowa, Iowa City, IA), have been derived from human bronchial epithelia. CuFi-1 cells have been derived from a patient with CF (CFTR mutant genotype F508del/F508del), whereas NuLi-1 cells have been derived from a non-CF subject (wild type CFTR), and were transformed by reverse transcriptase component of telomerase, hTERT, and human papillomavirus type 16 E6 and E7 gene. These cells will be grown on human placental collagen type IV (Sigma-Aldrich)-coated flasks in bronchial epithelial growth medium (Cambrex Bioscience, Walkersville, MD). Human A549 alveolar type II-derived epithelial cells (American Type Culture Collection, Manassas, VA) will be maintained in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% low-endotoxin fetal bovine serum (FBS; Bio-Whittaker, Walkersville, MD). Calu-3 cells were obtained from a human lung adenocarcinoma and derived from serous cells of proximal bronchial airways. Calu-3 cells will be cultured in DMEM containing 4.5 g/l glucose and supplemented with 10% FBS. MucilAir® (Epithelix Sa` rl, Geneva, Switzerland) primary cultures of human bronchial epithelial cells have been purchased from are human airway epithelia reconstituted *in vitro* with cells isolated from CF patients or from healthy donors, cultivated on microporous filters at an air–liquid interface (ALI).

3.3 Infection with *P. aeruginosa*

The well-characterized motile nonmucoid laboratory strains of *P. aeruginosa* named PAO1, PAK, PAK/Δfl have been kindly donated by A. Prince (Columbia University). Bacteria colonies from overnight cultures on trypticase soy agar plates (Difco, Detroit, MI) will be grown in 20 ml trypticase soy broth (Difco) at 37°C until an OD (A660 nm wavelength), corresponding to 1×10^7 CFU/ml, will be reached. Bacteria will be washed twice with PBS and diluted in each specific serum-free medium before infection and will be added to cells at the concentration of about 100 CFU per cell.

3.4 Quantitative RT-PCR

Total RNA from airway epithelial cells has been purified using High Pure RNA Isolation Kit (Roche, Mannheim, Germany), and 2.0 μg RNA has been reversetranscribed to cDNA using the High Capacity cDNA Archive Kit and random primers (Applied Biosystems, Foster City, CA). For the Real-time qPCR, 50 ng of cDNA has been used for each Sybr Green real-time PCR to quantify the relative gene expression. The cDNA amplification has been performed for 40 PCR cycles using the SYBR Green PCR Master Mix (Applied Biosystems), using 7900HT Fast Real-Time PCR apparatus (Applied Biosystems, Foster City, CA). In order to perform the PCR reaction QuantiTect Primer assays (Qiagen, Hilden, Germany) for IL-8 (Hs_IL8_1_SG, NM_000584), GROγ (Hs_CXCL3_1_SG, NM_002090), ICAM-1 (Hs_ICAM1_1_SG, NM_000201), IL-6 (Hs_IL6_1_SG, NM_000600), TNFα (Hs_TNF_1_SG, NM_000594), PLCB1 (Hs_PLCB1_1_SG, NM_015192,

NM_182734), PLCB3 (Hs_PLCB3_1_SG, NM_000932, NM_001184883), PLCB4 (Hs_PLCB4_1_SG, NM_000933, NM_001172646, NM_182797), PLCG2 (Hs_PLCG2_1_SG, NM_002661), GAPDH (HS_GAPDH_1_SG, NM_002046) and Actin-beta (ACTB) (Hs_ACTB_1_SG, NM_001101) have been used. Changes in mRNA expression level have been calculated following normalization with the ACTB or GAPDH calibrator genes. Results have been collected with SDS 2.3 software (Applied Biosystems), and relative quantification has been performed using the Ct method. Data have been analyzed with RQ Manager software 1.2 (Applied Biosystems).

3.5 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, CA) according to the manufacturer's protocol. Lipofectamine 2000 (2 μl) was diluted in 1 ml serum-free LHC-8 basal medium (Biofluids) and double-stranded decoy or scrambled ODNs (10 nM) were added and incubated for 10 min to generate liposome/DNA complexes as previously described [Bezzerri, 2008]. 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% CO_2 for 20–24 h before proinflammatory challenge with *P. aeruginosa* (100 CFU/ ml), IL-1β (10 ng/ml), or TNFα (50 ng/ml) for a further 4 h.

3.6 Human phospho-MAPK array

Cells were seeded in 6-cm Petri dishes at a density of 2.5 x 10^6 cells to obtain 1 x 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-1β (10 ng/ml), or human rTNFα (50 ng/ml) for 30 min. According to the manufacturer's protocol, 200 μg 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 quantified 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, Mariakerke, Belgium).

3.7 Preparation of cell nuclear extracts

Nuclear extracts were obtained from IB3-1 cells as previously described [Andrews, 1991]. Briefly, IB3-1 cells were exposed to *P. aeruginosa* PAO1 strain (100 CFU/cell), IL-1β (10 ng/ml), or TNFα (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 final concentration of 0.5 μg/ml.

3.8 Electrophoretic Mobility Shift Assay

EMSA experiments were performed as previously described [Borgatti, 2007]. Double stranded synthetic ODNs, designed on the putative consensus sequences of TFs NF-κB, NF-IL6, AP-1, CHOP, and CREB localized within proximal promoter region of the IL-8 gene (see fig. 9), were used. ODNs were labeled with [γ-^{32}P]ATP using 10 U T4 polinucleotide 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 [g-32P]ATP in a volume of 20 ml 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 (0.253 TBE buffer:22 mM Tris-borate, 0.4 mM EDTA) on 6% polyacrilamide gels. Gels were dried and finally subjected to standard autoradiographic procedures.

3.9 PLCB gene silencing

To perform gene silencing experiments of PLCB isoforms, a TriFECTa RNAi Kit (Integrated DNA technologies, Coralville, Iowa, IA) was used accordingly to the

manufacturer's instructions. IB3-1 cells were transiently transfected with specific siRNA for PLCB1, (sequence 1, 5'-CGCUAAGAAAUAAUUGAU-3'; sequence 2, 5'-UGGCUCCAUCAAUUAUUU-3'), PLCB3 (sequence 1, 5'-AGAUGAGGGACAAGCAUAAGAAGGA- 3'; sequence 2, 5'-GCUCGAAAGAGGAACCGAAGCAUUUGUUCCU- 3') PLCB4 (sequence 1, 5'-AGUAAGGAUAGAAGACUU-3'; sequence 2, 5'-GGAGUAUUACUAGAACAU-3') PLCG2 (sequence 1, 5'-GCGCUACAAUAUGGAAAG-3'; sequence 2, 5'-GGAGAAACAACAUGAAGUA-3'; sequence 3, 5'-GGACUACCAAGAUCAAGU-3' or scrambled (sequence, 5'-CUUCCUCUCUUUCUCUCCCUUGUGA- 3') duplexes complexed with cationic liposomes Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Lipofectamine 2000 (4 μl) was diluted in 1 ml LHC-8 serum-free cell culture medium. PLCB3 siRNA or scrambled duplexes (10 nM) were added and incubated for 10 min. Liposome:duplexes complexes in LHC-8 serum-free medium (500 μl) were added to IB3-1 cells grown in 2 cm^2 wells and incubated at 37°C/5% CO_2 for 6 h. Cells were washed twice with culture medium and left at 37°C/5% CO_2 for an additional 18 h.

3.10 Immunofluorescence

PLCB3 protein was detected by immunofluorescence. IB3-1 cells were seeded on 8-well chamber slides (Nunc, Naperville, IL) and preincubated with PLCB3 small-interfering RNA (siRNA) or scrambled duplexes for 24 h in LHC-8 basal serum-free medium as indicated below in the silencing protocol. Cells were washed three times with PBS and fixed with 4% paraformaldehyde (w/v) in PBS for 20 min at room temperature. After three washes with PBS, cells were permeabilized with methanol at 220°C for 5 min and dried for 1 h. Slides were incubated with 5% BSA in PBS for 90 min at room temperature and then subjected to three incubations at room temperature with the following: 1) 1/200 dilution of rabbit polyclonal Ab anti-PLCB3 (sc-13958 from Santa Cruz Biotechnology, Santa Cruz, CA) in 5% BSA for 1 h or with an irrelevant rabbit IgG Ab; 2) 1/200 dilution of biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology) in 1% BSA/PBS-0.1% Tween 20; and 3) 1/60 dilution of FITC conjugated streptavidin (Sigma-Aldrich) in 1% BSA/PBS-0.1% Tween 20. Coverslips were mounted with Prolong Antifade (Molecular Probes) and

stored at room temperature. Fluorescence was examined with a digital imaging system based on a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated charge-coupled device camera (Roper Scientific), excitation, and emission filter wheels (Sutter Instruments, Novato, CA) and piezoelectric motoring of the z stage (Physik Instrumente, Karlsruhe, Germany) for rapid focusing in the Z plane. The data were acquired and processed using the MetaMorph analyzing program (Universal Imaging, Downingtown, PA). The Z-steps were then turned into projections, and the average intensity after background subtraction was determined. All intensity comparisons were determined from at least 10 different cells to minimize cell-to-cell staining variations. The levels of PLCB3-silencing were expressed as the percentage of arbitrary unit of fluorescence, in respect to scrambled condition.

3.11 ELISA

IL-8 protein release was measured with an ELISA kit. Airway epithelial cells were grown and infected as previously described, then supernatants were collected from each well, and an ELISA for the quantitative detection of human IL-8 was performed using the Human IL-8 Instant ELISA kit (Bender MedSystems, Vienna, Austria), according to the manufacturer's protocol. Briefly, IL-8 present in 100 µl of supernatants or standard have been incubated with the antibodies which are adsorbed to the microwells furnished by the kit; a biotin-conjugated anti-human IL-8 antibody has been incubated to the IL-8 previously captured by the first antibody. Subsequently, streptavidin-HRP has been added to the biotin-conjugated anti-human IL-8. Following incubation, unbound biotin-conjugated anti-human IL-8 and streptavidin-HRP was removed during a wash step, and substrate solution reactive with HRP was added to the wells. A coloured product was formed in proportion to the amount of soluble human IL-8 present in the sample. The reaction has been terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from 7 different human IL-8 standard dilutions and human IL-8 sample concentration was determined.

4. RESULTS

4.1 Pattern of activation of phosphoproteins in bronchial epithelial cells infected with *P. aeruginosa*

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 pro-inflammatory genes in human bronchial epithelial cells [Adamo 2004; Chun, 2006]. *P. aeruginosa* is known to activate phosphorylation of MAPKs p38 and ERK1/2, which has been related to IL-8 gene expression [Ratner, 2001]. MAPK p38 was shown to phosphorylate HSP27, the latter leading to activation of IKK and NF-κB, in human colonic epithelial cells upon induction by dextran sodium sulfate [Bhattacharyya, 2009]. Moreover, ERK1/2 phosphorylates RSK and MSK [Roux, 2004]. 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 [Roux, 2004]. Another relevant kinase is GSK3, which has been shown to induce IL-8 expression in neuroblastoma cells [De Ketelaere, 2004]. As a possible feedback mechanism, GSK3 activation can be inhibited through phosphorylation elicited by activated AKT [Sugden, 2008]. 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. 5). No striking phosphorylation of the MAPK ERK1/2 was found in *P. aeruginosa*-stimulated IB3-1 cells (Fig. 5 A-B), whereas a clear up-regulation of ERK1/2 phosphorylation levels was observed in stimulated CuFi-1 cells (Fig. 5 C-D). However, a clear phosphorylation of RSK1 and MSK2, which are substrates of activated ERK1/2, was evident on both bronchial epithelial cell lines (Fig. 5). 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 total phosphorylation of GSK3 was increased (Fig. 5). A significant *P. aeruginosa*-stimulated phosphorylation of the MAPK JNK family was observed in CuFi-1 cells (Fig. 5 C-D).

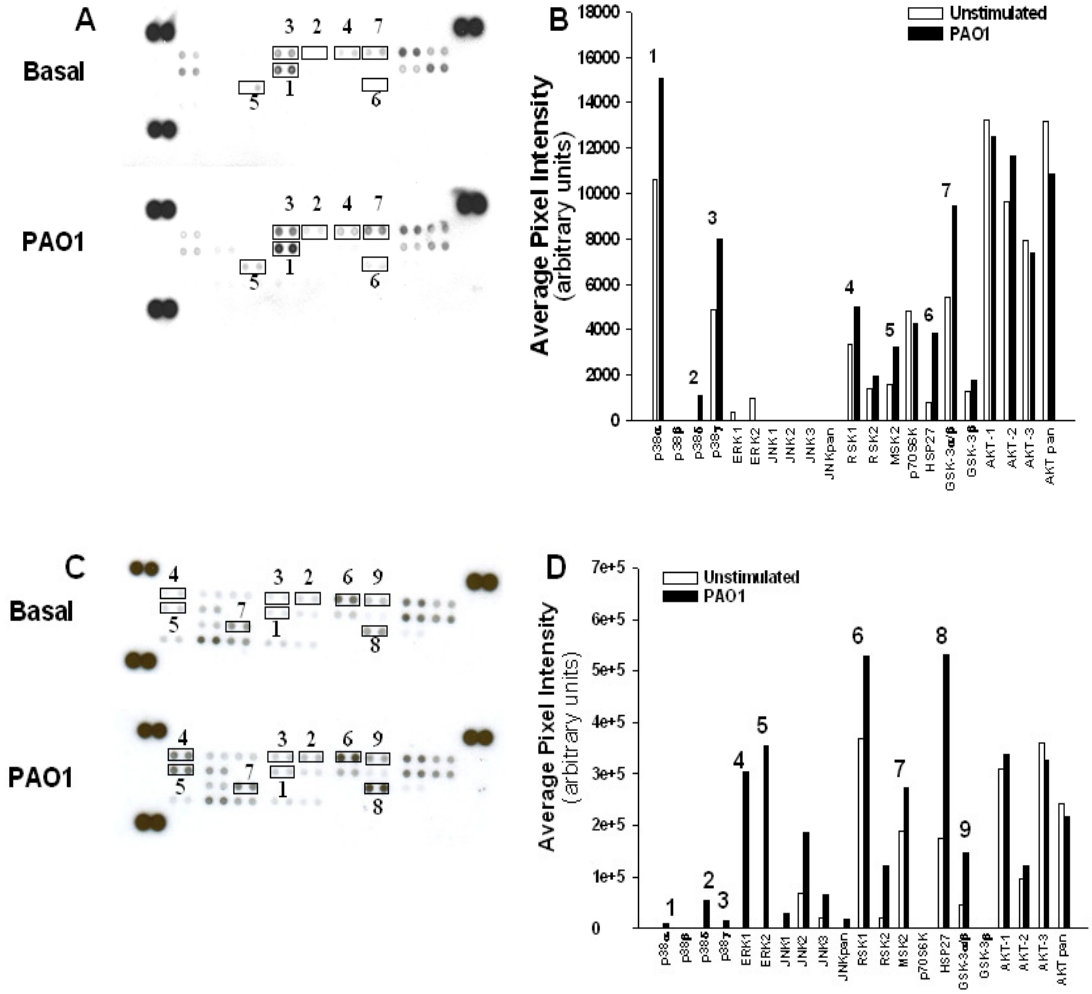

FIGURE 5

Phosphorylation pattern of kinases in bronchial epithelial cells infected with P. aeruginosa. *IB3-1 cells (panels A and B) and CuFi-1 cells (panels C and D) 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, or between C and D.*

4.2 Effect of phospho-kinases on IL-8 gene expression in bronchial epithelial cells

In order to test whether the kinases activated by *P. aeruginosa* are implicated in the activation on IL-8 gene transcription, we preincubated three different bronchial epithelial cell lines, derived by CF patients (IB3-1 and CuFi-1) or by non-CF individuals (Calu-3) with the pharmacological inhibitors of MAPK p38 (SB203580), HSP27 (KRIBB3), ERK1/2 (AG1288 and U1026), RSK (SL0101), GSK3 (GSK3-inh), IKK (parthenolide), and, of JNK (SP600125) before the 4-h infection with *P. aeruginosa.* As shown in Fig. 6, 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. IKK inhibition by parthenolide, which in turn inhibits NF-κB activation, showed a strong reduction of IL-8 mRNA content (Fig. 6). In summary, the results obtained suggest that *P. aeruginosa*-dependent transmembrane signaling involved in IL-8 gene transcription appears associated with MAPK p38 and ERK. However, also JNK inhibitors showed inhibitory effect in *P. aeruginosa*-dependent IL-8 expression in CuFi-1 and Calu-3 cells, whereas no effect was observed in IB3-1 cells. Interestingly, MAPK p38 downstream effector HSP27 and the ERK substrate RSK1 seem implicated in this model of inflammation. As previously reported [Saadane, 2007], IKK was closely related to IL-8 gene expression in CF respiratory epithelial cells. The results obtained by mRNA analysis were extended in 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 at the protein levels. As shown in Fig 6C and 6E, a consensus is evident for the involvement of p38, HSP27, ERK, RSK, and IKK. The modest, albeit statistically significant, involvement of GSK3 in IB3-1 cells is not consistently confirmed in the other cell lines.

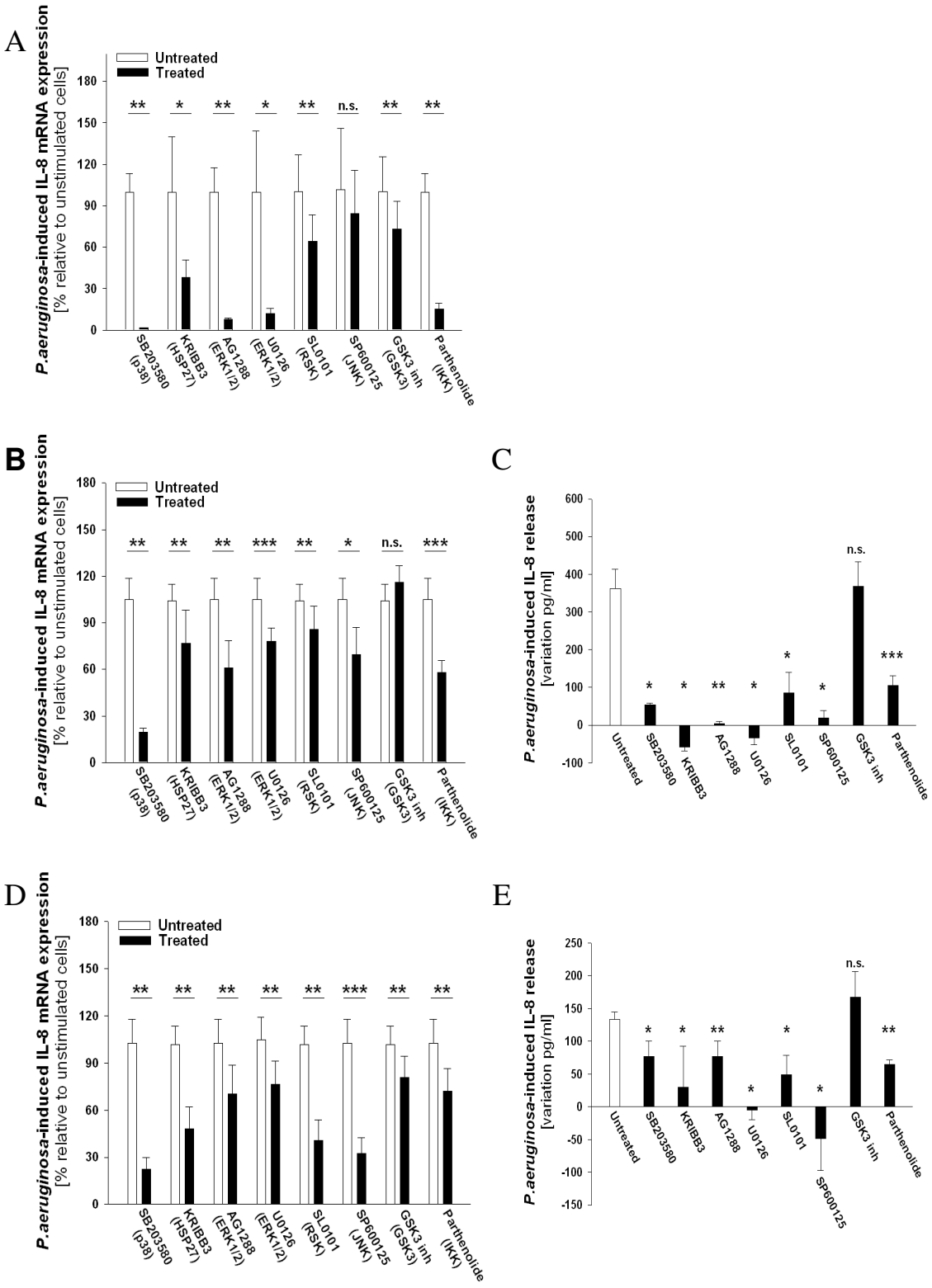

Figure 6

Role of protein kinases in P. aeruginosa-dependent IL-8 gene expression. A, 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 μM) was added 1 h before infection; KRIBB3 (1 μM) 1 h before

*infection; AG1288 (200 μM) 2 h before infection; U1026 (10 μM) 1 h before infection; SL0101 (2 μM) 1 h before infection; SP600125 (3 μM) 1 h before infection; GSK3 inhibitor II (100 nM) 1 h before infection; and parthenolide (10 μM) 1 h before infection; total mRNA was extracted from cell lysates and quantitative RT-PCR was performed to quantify IL-8 mRNA expression. B and D, CuFi-1 and Calu-3 cells, respectively, treated as in A. C and E, 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 ± SEM of three independent experiments performed in duplicate and are expressed as percentages of unstimulated cells. *p , 0.05, **p , 0.01 by Student's t test.*

4.3 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 [Chun, 2006; McNamara, 2006; Okada, 2013], 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-1β, 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 [Yang, 2008]. Additionally, IL-1β has been proposed to activate a sequential ERK/RSK1 cascade both in human nasal epithelial cells and in rat vascular smooth muscle cells [Song, 2003; Xu, 2006]. Interestingly, HSP27 has been reported to regulate IL-1–dependent IKK activation in HeLa cells [Wu, 2009]. To verify and extend these findings in our experimental model, we tested the effect of IL-1β–dependent phosphorylation with a human phospho-MAPK assay. As shown in Fig. 7A and 7B, IL-1β 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. 7A, 7B). Additionally, an increased phosphorylation of HSP27 was detected upon exposure of bronchial cells to IL-1β stimulation (Fig. 7A, 7B). 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 GSK3β was shown in respect to basal levels (Fig. 7A, 7B).

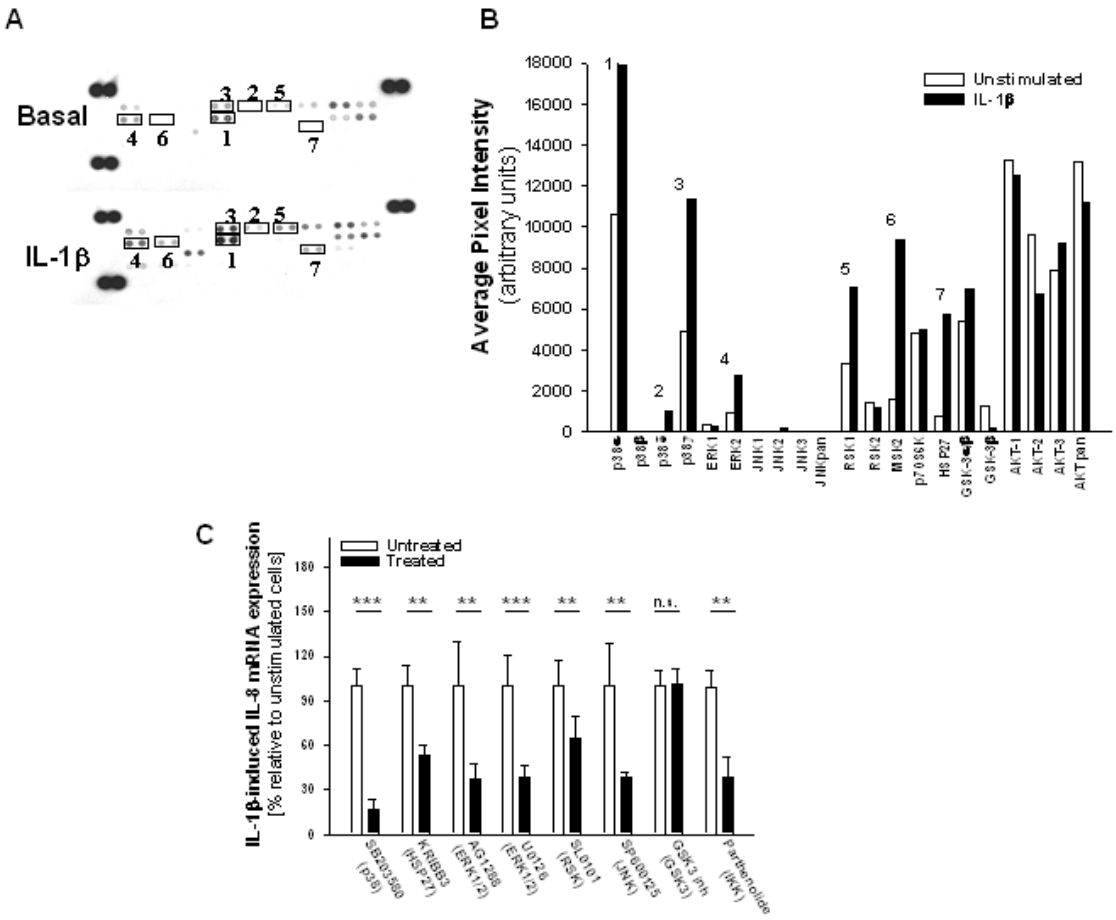

Figure 7

Phosphorylation pattern of kinases in cells stimulated with IL-1β. *IB3-1 cells were exposed to IL-1β (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 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 IL-1β for 4 h. SB203580 (10 μM) was added 1 h before infection; KRIBB3 (1 μM) 1 h before infection; AG1288 (200 μM) 2 h before infection; U1026 (10 μM) 1 h before infection; SL0101 (2 μM) 1 h before infection; SP600125 (3 μM) 1 h before infection; GSK3 inhibitor II (100 nM) 1 h before infection; and parthenolide (10 μM) 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 ± 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's t test.*

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-1β–dependent IL-8 gene transcription (Fig. 7C). 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. 7C). On the contrary, GSK3 inhibitor did not reduce IL-8 gene transcription elicited by IL-1β (Fig. 7C). Finally, parthenolide strongly inhibited IL-8 gene transcription (Fig. 7C).

To study a completely MyD88-independent signaling pathway of regulation of IL-8 gene transcription, we exposed IB3-1 cells to TNFα, which has been reported to transduce p38 and JNK signaling through TNFR-associated factor protein activation [Hoffmann, 2002; Bouwmeester, 2004]. Additionally, HSP27 has been reported to be activated after TNFα stimulation through TAK1 kinase [Alford, 2007]. As shown in Fig. 8A and 8B, TNFα induced phosphorylation of MAPK p38 isoform δ but not α, β, and γ. Additionally, a 2-fold induction of HSP27 was found (Fig. 8A, 8B). We observed phosphorylation of JNK1 and JNK2 phosphokinases (Fig. 8A, 8B). Interestingly, TNFα inhibited to different extents the basal phosphorylation of several substrates, such as AKT-1 and AKT-3, ERK 1/2, MSK2, and GSK3β. As shown in Fig. 8C, 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 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. 8C). Furthermore, the GSK3 inhibitor partially reduced the transcription of IL-8 gene (Fig. 8C). Finally, IKK inhibition by parthenolide showed a potent decrease of IL-8 mRNA expression mediated by TNFα (Fig. 8C).

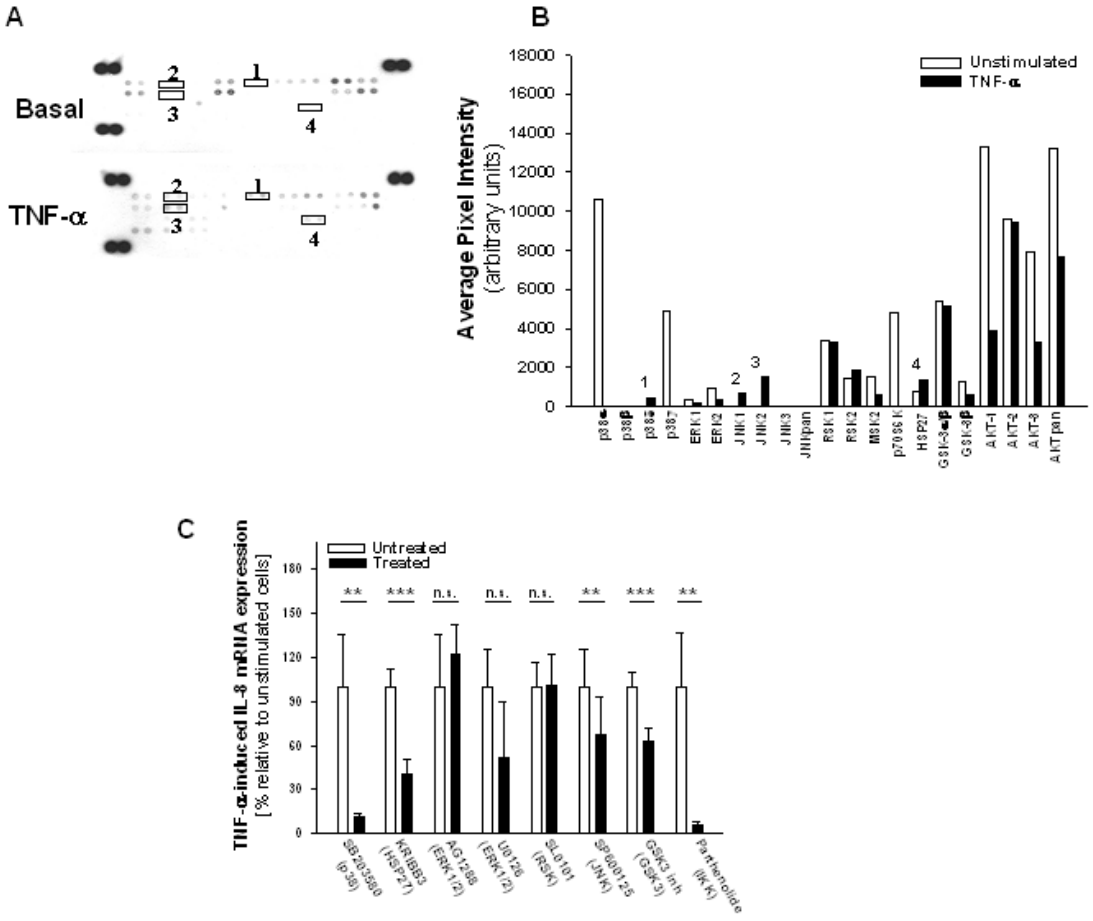

Figure 8

Phosphorylation pattern of kinases in cells stimulated with TNFα. *IB3-1 cells were exposed to TNFα (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 TNFα for 4 h. SB203580 (10 μM) was added 1 h before infection; KRIBB3 (1 μM) 1 h before infection; AG1288 (200 μM) 2 h before infection; U1026 (10 μM) 1 h before infection; SL0101 (2 μM) 1 h before infection; SP600125 (3 μM) 1 h before infection; GSK3 inhibitor II (100 nM) 1 h before infection; and parthenolide (10 μM) 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 ± 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's t test.*

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-1β 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 TNFα–induced pathway does not activate ERK1/2, but it does activate MAPK JNK and only the isoform d of MAPK p38.

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

Despite the fact that NF-κB and AP-1 are widely established as TFs implicated in the expression of IL-8 gene in different cell models [Hoffmann, 2002; Bezzerri, 2008], other TFs have been recently reported as possible regulators of IL-8 gene transcription, such as the TF CHOP in T-cells and in human bronchial IB3-1 cells [Cucinotta, 2008; Vij, 2008], the TF CREB in U937 monocytic cells and in A549 cells [Hisatsune, 2008; Venza, 2009], and the TF NF-IL6 in human conjunctival and bronchial cells induced by *P. aeruginosa* [Venza, 2009]. 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 2180 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. The figure 9 summarizes the major TFs which 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. 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 [Bezzerri, 2008]. In addition to the sequences identified for known TFs, the sequences localized between -163/ -128 and -71/-22 bp were termed intermediate sequences A 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-κB, NF-IL6, AP-1, CHOP, and CREB, nuclear extracts of stimulated cells were produced and preincubated with each TF decoy ODN and run by EMSA. As a source of TFs, IB3-1 cells were stimulated with IL-1β, which

induces abundant amounts of TFs in the unfractionated nuclear preparation. The results shown in Fig. 10 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 TNFα, in which 95% inhibition of DNA/ protein interactions was obtained.

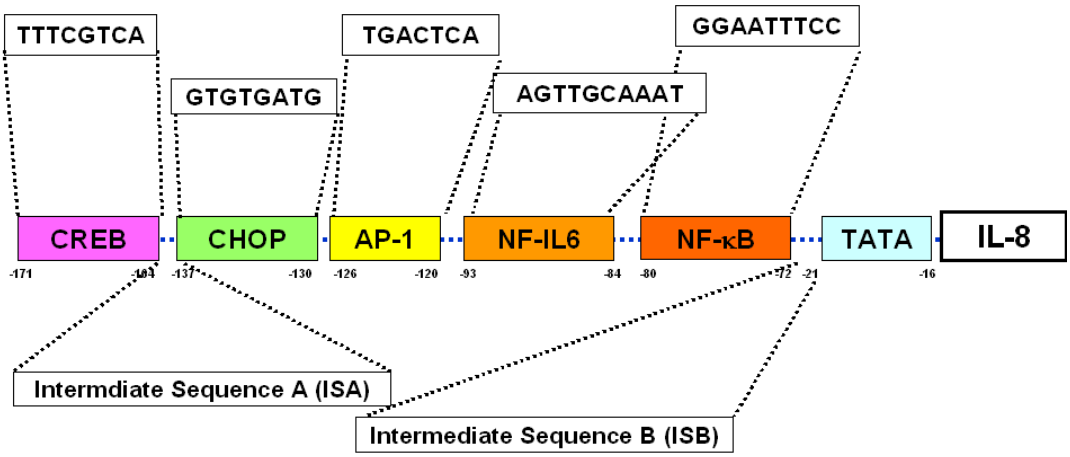

Figure 9

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-κB. A previously reported CHOP consensus sequence was included (30). ISA, ISB, and TATA box are reported.*

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 non-active or exhibiting a significantly lower activity. This is shown in Fig. 11 (courtesy of Prof. Roberto Gambari and Dr. Monica Borgatti, University of Ferrara), which reports the results of a competitive EMSA

experiment in which 100 ng cold competitor decoy NF-κB, NF-IL6, AP-1, CHOP, and CREB oligonucleotides were incubated for 20 min with nuclear extracts before addition of the [^{32}P]-labeled EMSA probes. Fig. 11B clearly shows that NFIL6, AP-1, CHOP, and CREB double-stranded oligonucleotides do not compete with the NF-κB probe for binding to nuclear extracts.

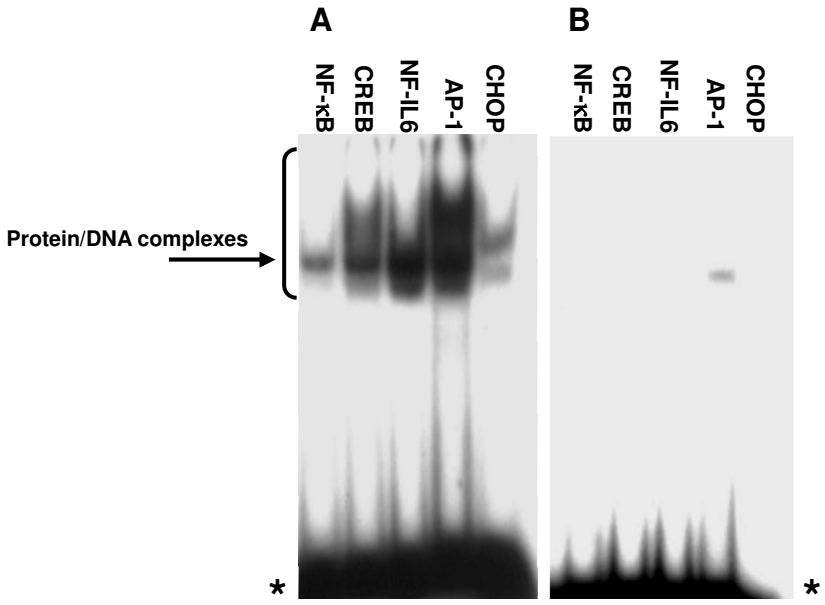

Figure 10

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-κB, CREB, NFIL6, AP-1, and CHOP identified in the IL-8 promoter, to TFs isolated from IB3-1 cells induced with IL-1β. [^{32}P]-labeled dsDNA molecules were incubated for 40 min in the presence of 2 μg 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-κB, CREB, NF-IL6, AP1, 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 μg) 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. Arrow indicates complexes between proteins and target molecules, and asterisks indicate the free [^{32}P]-labeled probe (courtesy of Prof. Roberto Gambari and Dr. Monica Borgatti, University of Ferrara).*

As expected, full suppression of the interactions between the [^{32}P]-labeled NF-κB probe and nuclear extracts was observed when a cold NF-κB double-stranded ODN competitor was employed. Similarly, no cross-competition was observed using [^{32}P]-labeled CREB (Fig. 11C), NF-IL6 (Fig. 11D), AP-1 (Fig. 11E), and CHOP (Fig. 11F), with the exception of the CREB oligonucleotide, which to some extent competes with AP-1 (but with lower efficiency, as expected; see Fig. 11E). The competitive EMSA analysis reported in Fig. 11 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. Toward this end, further chromatin immunoprecipitation (ChIP) experiments need to be performed.

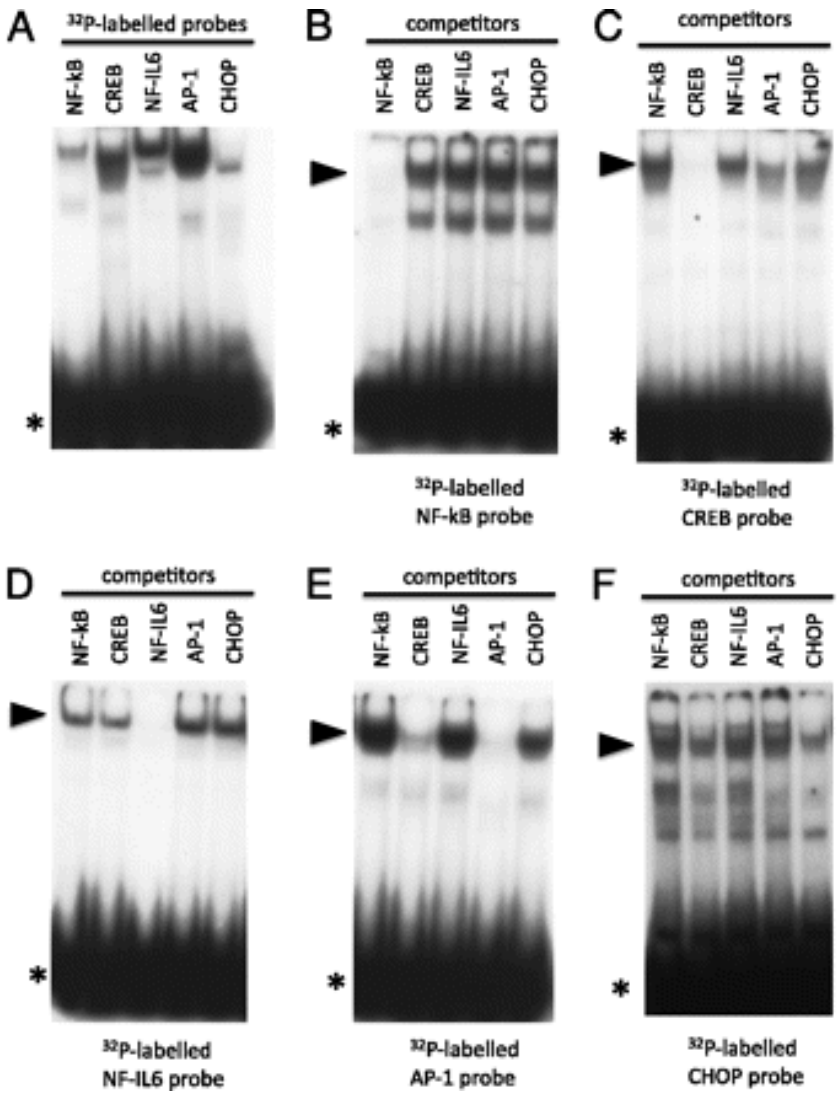

Figure 11

Competitive EMSA assay. *EMSA assays were performed using [^{32}P]-labeled NF-κB, CREB, NF-IL6, AP-1, and CHOP probes (*A–F*). Crude nuclear extracts (2 μg) were incubated for 20 min in the presence of 200 ng cold NF-κB, CREB, NF-IL6, AP-1, and CHOP competing dsDNA molecules, as indicated, and then incubated with [^{32}P]-labeled NF-κB (*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-κB, CREB, NF-IL6, AP-1, and CHOP in the absence of competitors is shown (courtesy of Prof. Roberto Gambari and Dr. Monica Borgatti, University of Ferrara).*

4.5 Effect of TF decoy ODNs on IL-8 expression in differentially stimulated bronchial epithelial cells

IB3-1 cells were transfected with IL-8 NF-κB, 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 24 h before exposure to *P. aeruginosa*, IL-1β, or TNFα for further 4 hours. As shown in Fig. 12, *P. aeruginosa*-dependent IL-8 transcription in bronchial epithelial cells was significantly inhibited using decoy ODNs against the TFs NF-κB, 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-1β was diminished after pre-incubation of decoy ODNs interfering with TFs NF-κB, NF-IL6, AP-1, CHOP, and CREB. Third, TNFα–mediated IL-8 gene transcription was reduced only by pre-incubation of decoy ODNs against NF-κB, NFIL6, and AP-1. These results overall suggest a differential interference of TF decoy ODNs depending on the pro-inflammatory stimulus in that the TNFα pathways seem independent of the participation of the TFs CHOP and CREB. Focusing again on *P. aeruginosa*, we previously demonstrated the strong inhibitory effect of TF NF-κB decoy ODNs on the expression of IL-8 gene in CuFi-1 and Calu-3 cells [Bezzerri, 2008]. 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. 13, 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-1β, or TNFα. The lack of inhibition of IL-8 transcription always observed with ISA and ISB ODNs, based on the sequence localized from -163 to -138 and from -71 to -22 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 the TFs NF-κB, NF-IL6, AP-1, CHOP, and CREB.

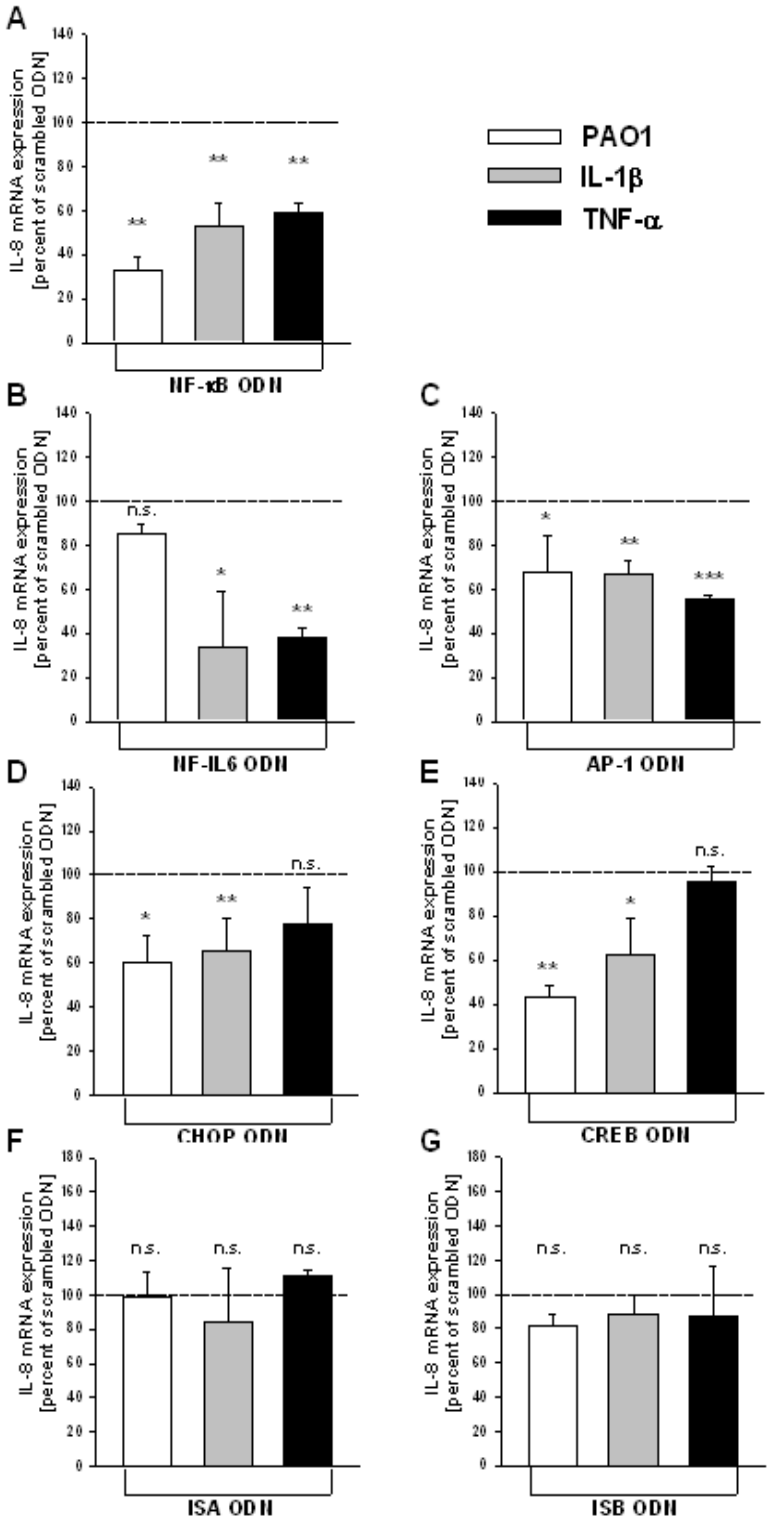

Figure 12

Effect of TF decoy ODNs on IL-8 gene transcription induced by P. aeruginosa, IL-1β, and TNFα in IB3-1 bronchial epithelial cells. *IB3-1 cells were transfected from 24 h before infection with TF decoy ODNs against NFkB (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 pre-incubation period, cells were challenged with P. aeruginosa (PAO1, 100 CFU/cell), IL-1β (10 ng/ml), and TNFα (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 by transfection with scrambled ODN. Data shown are means (± SEM) of three independent experiments performed in duplicate. *p< 0.05, **p < 0.01, ***p < 0.001 by Student's t test.*

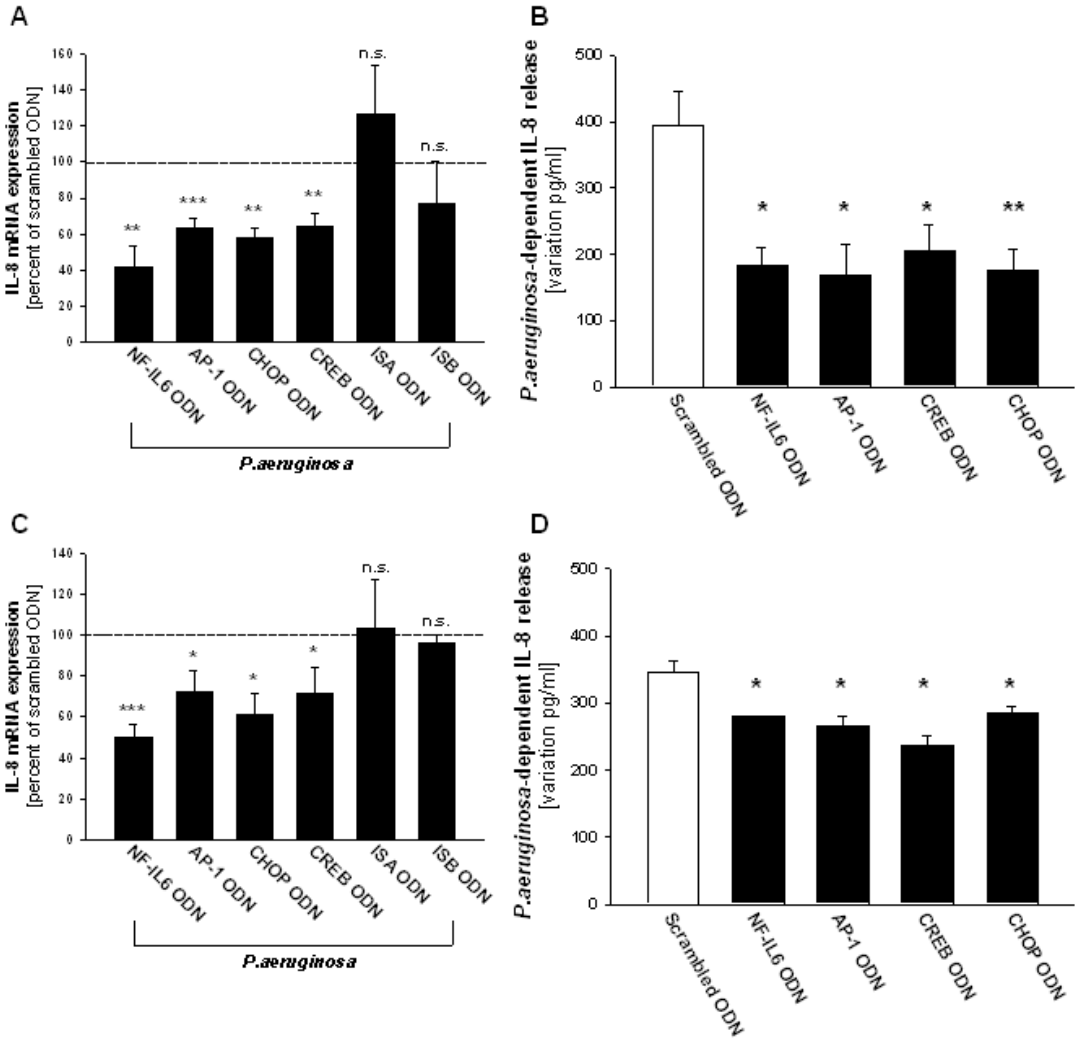

Figure 13

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 24 h before infection with TF decoy ODNs against NF-κB, NF-IL6, AP-1, CHOP, CREB, or with sequences ISA and ISB complexed with Lipofectamine 2000. After this preincbation period, cells were challenged with P. aeruginosa (PAO1, 100 CFU/cell) for further 4 h and IL-8 gene expression was measured by qRT-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 (± SEM) of three independent experiments performed in duplicate. *p< 0.05, **p< 0.01, ***p< 0.001 by Student's t test.*

4.6 Silencing of PLCB3 gene reduces the expression of IL-8 in bronchial epithelial cells exposed to *P. aeruginosa* and flagellin

PLCβ isoforms are implicated in signal transduction by receptors for hormones, growth factors, neurotransmitters and other ligands involved in regulation of different cellular processes, including the immune response. Although human bronchial epithelial cells express, albeit at different levels, the transcripts of all the PLCβ isoforms, we focused our attention on PLCB3, because it is the most highly expressed within the β isoforms (see fig. 14).

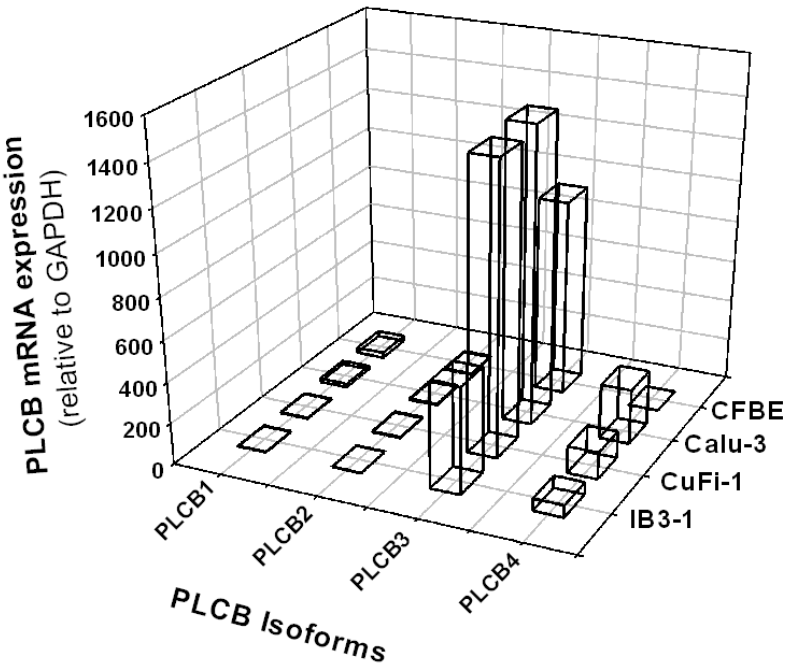

Figure 14

Comparison of PLCB gene isoforms expression in different bronchial epithelial cells. *CF bronchial epithelial cells lines IB3-1 and CuFi-1, primary bronchial epithelial cells derived from a CF patient (CFBE) and non CF cell line Calu-3 were lysed and total RNA was extracted in order to quantify PLCB1, PLCB2, PLCB3 and PLCB4 mRNA expression by qRT-PCR.*

To understand whether PLCB3 could be relevant in the induction of IL-8 in respiratory cells exposed to bacterial infection, we studied the transcription and release of IL-8 after silencing the expression of endogenous PLCB3 with siRNA oligonucleotides in human bronchial epithelial cells from CF patients exposed to *P. aeruginosa*. Transfection of two different duplexes PLCB3 siRNA reduced significantly, albeit partially, the levels of expression of PLCB3 mRNA (Fig. 15A) and protein (Fig. 15 panels B–E), as detected by quantitative RT-PCR and confocal immunofluorescence, respectively. No significant reduction of transcript levels of PLC isozymes β1, β2, and β4 was observed with PLCB3 siRNA in IB3-1 cells. Infection with *P. aeruginosa* did not change significantly the levels of PLCB3 mRNA. In the same experimental model, partial silencing of PLCB3 produced a parallel reduction of IL-8 transcription and release in CF bronchial epithelial IB3-1 and CuFi-1 cells exposed to *P. aeruginosa* (Fig. 16 panels A,B and D) without changing the basal IL-8 mRNA levels in uninfected cells. Silencing PLCB3 did reduce the IL-8 transcription and release induced by flagellin, a component of *P. aeruginosa* interacting with TLR5, but did not affect the TNFα–dependent IL-8 expression (Fig. 16 panels C-D), suggesting that PLCB3 may have a role in downstream signaling of TLR5 but not TNFRs. The effect of silencing PLCB3 gene seems mainly restricted to IL-8, because the expression of other genes induced by *P. aeruginosa* in bronchial epithelial cells, such as ICAM-1, growth-related oncogene (GRO) γ, IL-6, and TNFα, is not reduced (Fig. 17). These results provide, to our knowledge, the first evidence that PLCB3 could be one of the components of a signaling network involved in the expression of IL-8 in human bronchial epithelial cells exposed to *P. aeruginosa*.

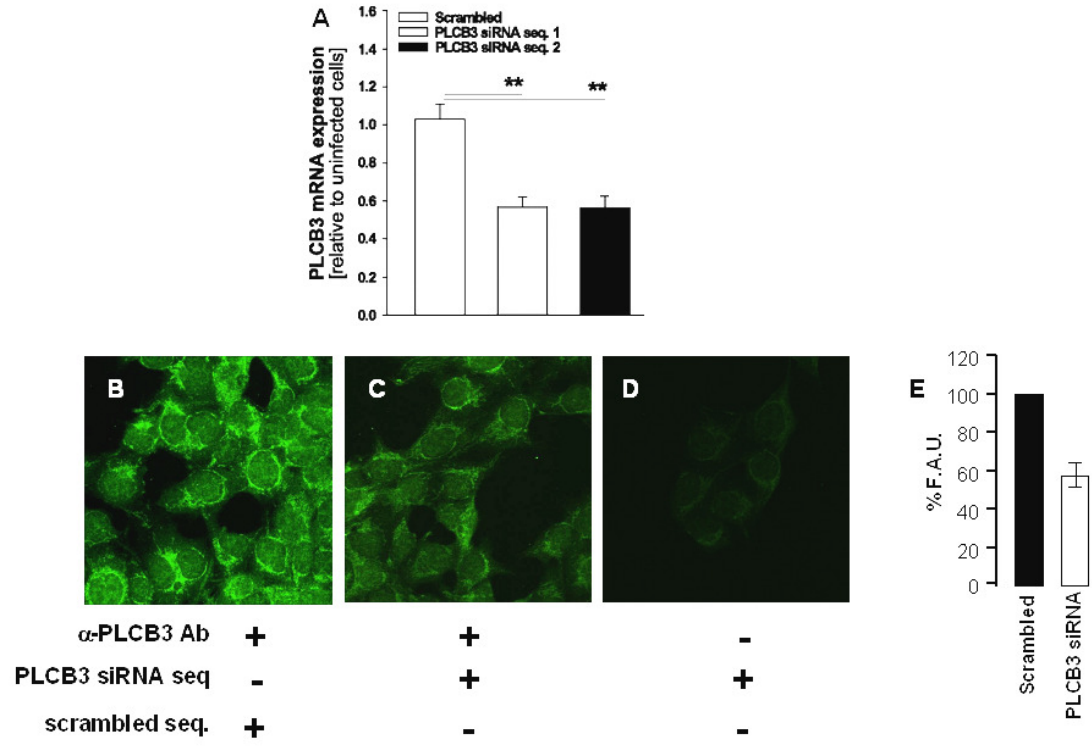

Figure 15

Silencing of PLCB3 gene reduces P. aeruginosa-dependent expression and release of IL-8 in human bronchial epithelial IB3-1 cells. *A, Quantitative expression of PLCB3 mRNA by quantified real-time PCR after transfection with PLCB3 siRNA or scrambled oligonucleotides sequences 1 and 2 for 24 h and subsequent infection with PAO1 (100 CFU/cell) for an additional 4 h. The mRNA expression reported in y-axis is relative to scrambled-treated uninfected cells. Mean ± SEM of eight independent experiments performed in duplicate. Immunofluorescence signal of PLCβ3 protein in IB3-1 cells transfected with scrambled oligonucleotide sequence 1 (B) or PLCB3 siRNA oligonucleotide sequence, in the presence of primary anti-PLCβ3 Ab (C) or irrelevant Ab (D). E, Quantification of the fluorescence signal as percentage of fluorescence arbitrary units (F.A.U.) related to the expression of PLCβ3 protein of IB3-1 cells treated with scrambled versus PLCB3 siRNA oligonucleotides. **p< 0.01, by Student's t test.*

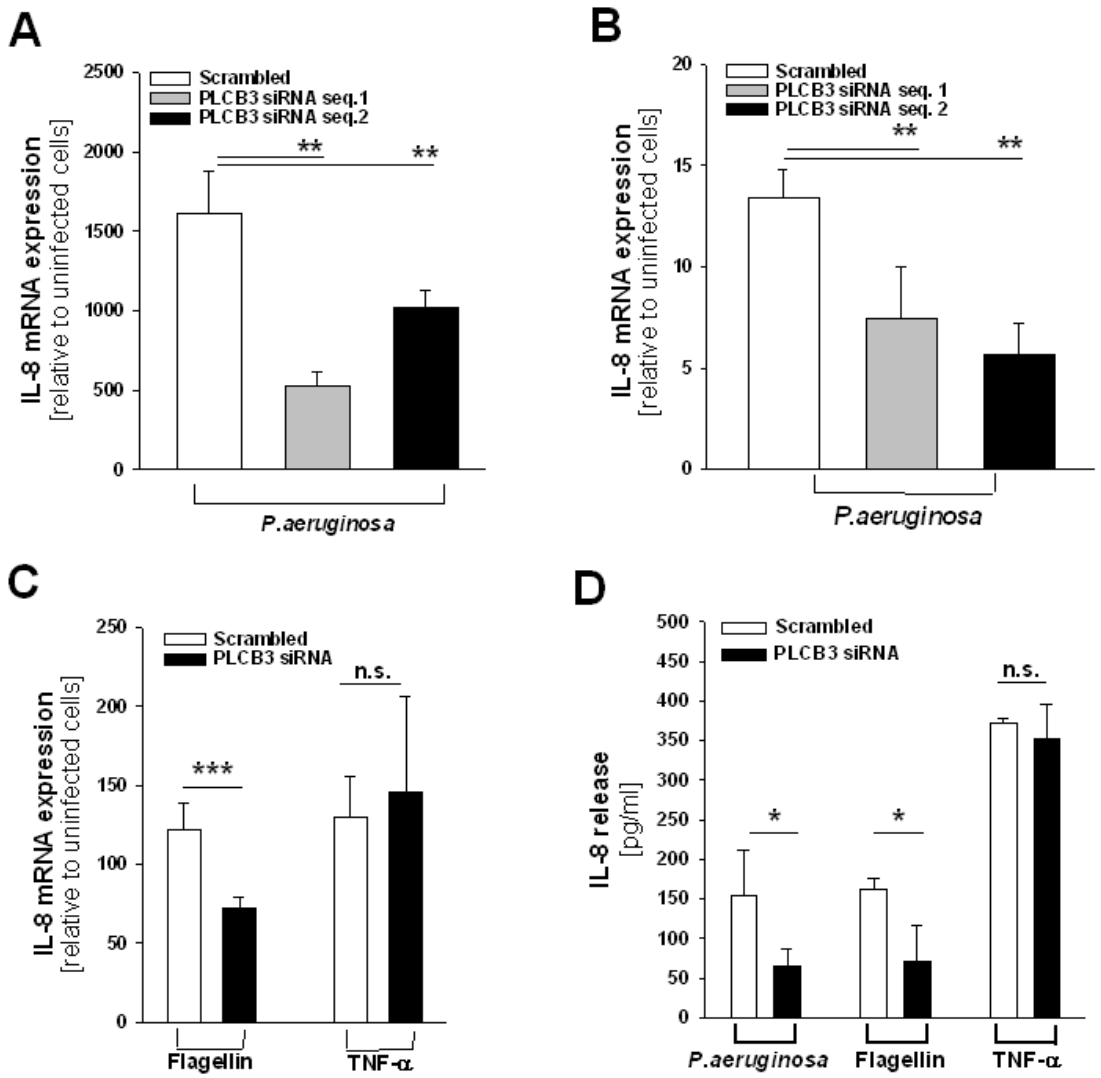

Figure 16

Silencing of PLCB3 gene reduces P. aeruginosa-dependent expression and release of IL-8 in human bronchial epithelial IB3-1 cells. *A Quantitative expression of IL-8 mRNA after transfection with PLCB3 siRNA sequences 1 and 2 or scrambled oligonucleotides in IB3-1 cells. B Quantitative expression of IL-8 mRNA after transfection with PLCB3 siRNA sequences 1 and 2 or scrambled oligonucleotides in CuFi-1 cells. Mean ± SEM of five independent experiments performed in duplicate. C, Effect of PLCB3 siRNA (sequence 1) on IL-8 transcription induced by flagellin (10 μg/ml) and TNFα (50 ng/ml). Mean ± SEM of three independent experiments performed in duplicate. D, Effect of PAO1 (100 CFU/cell), flagellin (10 μg/ml), and TNFα (50 ng/ml) on IL-8 protein release, treated as in A with PLCB3 siRNA (sequence 1), or scrambled oligonucleotide. Mean ± SEM of three independent experiments performed in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test.*

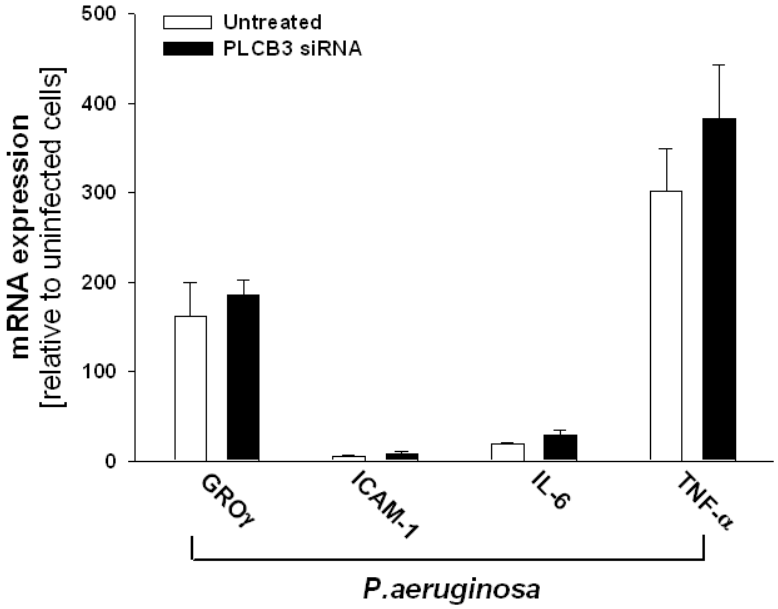

Figure 17

Pro-inflammatory gene expression in PLCB3 knocked down IB3-1 cells upon exposure to P. aeruginosa infection. *Effect of PAO1 (100 CFU/cell) in IB3-1 cells transfected with PLCB3 siRNA (sequences 1 or 2) on ICAM-1, GROγ, IL-6, and TNFα mRNA transcription. Mean ± SEM of three independent experiments performed in duplicate.*

4.7 PLCB3 is implicated in Ca^{2+}-related signaling

As with the other PLC isoforms, PLCB3 catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to generate two second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which in turn activate intracellular calcium transients [Katan, 1998; Suh, 2008]. Notably, it has been recently shown that PLCB3 is a critical regulator of intracellular Ca^{2+} in murine macrophages [Roach, 2008]. In collaboration with Prof. Paolo Pinton's team (University of Ferrara), here we show how the exposure of IB3-1 cells to *P. aeruginosa* induced a sustained increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) as measured using Fura-2 technique (Fig. 18A). Conversely, buffering the increase of $[Ca^{2+}]c$ with the intracellular Ca^{2+}-chelator BAPTA reduced significantly the induction of IL-8 mRNA (Fig. 18B), as already reported by other investigators [Ratner, 2001; Adamo, 2004]. BAPTA further reduced the IL-8 mRNA expression in cells silenced for PLCB3 (Fig. 18C), but in BAPTA-loaded cells PLCB3, gene silencing did not reduce IL-8 expression below the level detected in cells treated with the control siRNA.

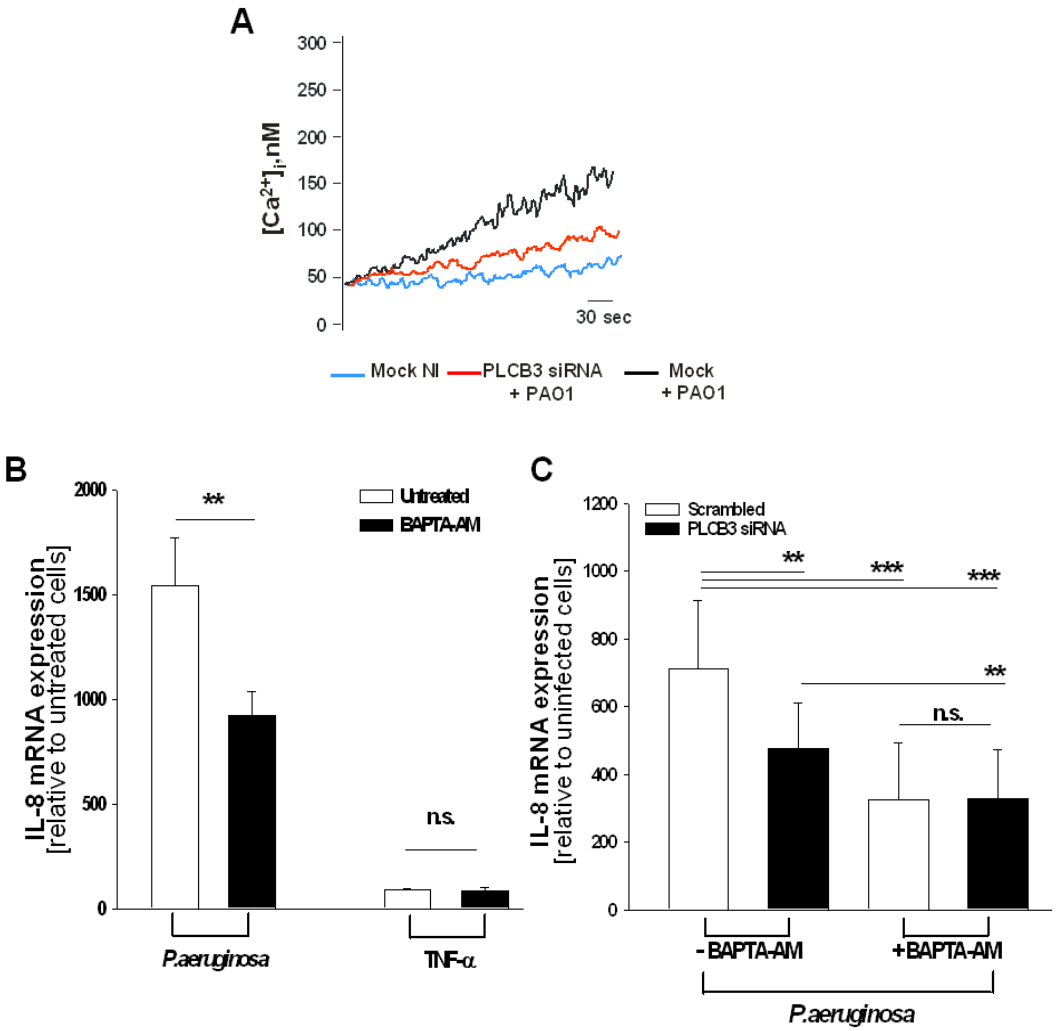

Figure 18

Implication of PLCB3 in P. aeruginosa-dependent calcium signaling. *A) Cytosolic free Ca^{2+} concentration as measured with Fura-2/AM assay in IB3-1 cells transfected with scrambled duplex and exposed to PAO1 (black trace), or with tissue culture medium alone (blue trace), or after transfection of PLCB3 siRNA and a further infection with PAO1 (red trace) (courtesy of Prof. Paolo Pinton and Dr. Alessandro Rimessi, University of Ferrara). B) The intracellular Ca^{2+}-chelator BAPTA-AM (5 μM) was pre-incubated with IB3- 1 cells for 30 min at 37°C. Cells were exposed to PAO1 (100 CFU/cell) or TNFα (50 ng/ml) for a further 4 hrs. C) After pre-incubating IB3-1 cells with PLCB3 siRNA or scrambled oligonucleotides for 24 h, BAPTA-AM (5 μM) was added for 30 min at 37°C. Infection with PAO1 strain was performed for an additional 4 hrs. Data shown are means (± SEM) of three independent experiments performed in duplicate. **p , 0.01, ***p , 0.001 by Student's t test.*

These findings suggest two set of conclusions. First, that in our experimental model, calcium signaling is not completely mediated by PLCB3. Notably, bronchial epithelial cells express different PLC isoforms besides the -β3 ones, in particular PLCB1 and PLCB4 (Fig. 14). Second, that silencing of PLCB has an effect only because it reduces calcium signaling, because in cells in which calcium is chelated by BAPTA, no further reduction of IL-8 expression is induced by PLCB3 siRNA. The $[Ca^{2+}]c$ increase promoted by *P. aeruginosa* was reduced in IB3-1 cells preincubated with PLCB3 siRNA (Fig. 18A), although not completely, possibly as a result of a parallel partial reduction of PLCB3 expression. These results indicate that PLCB3 plays a relevant role in triggering free calcium transients induced by *P. aeruginosa* in human bronchial epithelial cells.

4.8 Extracellular ATP is not sufficient for IL-8 expression but acts in synergy with TLRs

Interaction of *P. aeruginosa* with ASGM1R, co-localized with TLR5, is known to promote the release of nucleotides from epithelial cells, activating an autocrine loop with P2Y2 receptors [McNamara 2001; Adamo, 2004]. Interestingly, PLCB3 has been shown to be selectively coupled to the P2Y2 receptor-dependent activation of intracellular Ca^{2+} transients in recombinant CHO cells [Strassheim, 2000]. The role of different ligands on the expression of IL-8 has been tested preliminarily. IL-8 transcription was induced, albeit at different extents, after exposing IB3-1 cells to intact *P. aeruginosa* bacteria of strains PAO1 and PAK, to the purified *P. aeruginosa* bacterial components flagellin and pilin, and to the proinflammatory cytokine TNFα (Fig. 19A). PAK FliC, a recombinant *P. aeruginosa* PAK strain lacking expression of flagellin, induces IL-8 expression at a level lower than that observed with PAK (Fig. 19A), suggesting a strong contribution of bacterial flagellum in this signaling pathway. On the contrary, no significant induction was obtained with classical ligands of TLR4 and purinergic receptor Y2 (P2Y2R), such as LPS and ATP/UTP, respectively (Fig. 19A). Because ATP-dependent induction of IL-8 in bronchial epithelial cells has been previously described only in association with ligands activating TLRs [Fu, 2007], we tested the effect of ATP on the expression of IL-8 upon stimulation with flagellin, which interacts with TLR5. We observed that ATP is not sufficient by itself to induce IL-8 expression, but it is able to potentiate the flagellininduced one (Fig. 19B). To verify that *P. aeruginosa* induces an autocrine loop of release of nucleotides, we tested the effect of the ectonucleotidase apyrase in our model system. system.

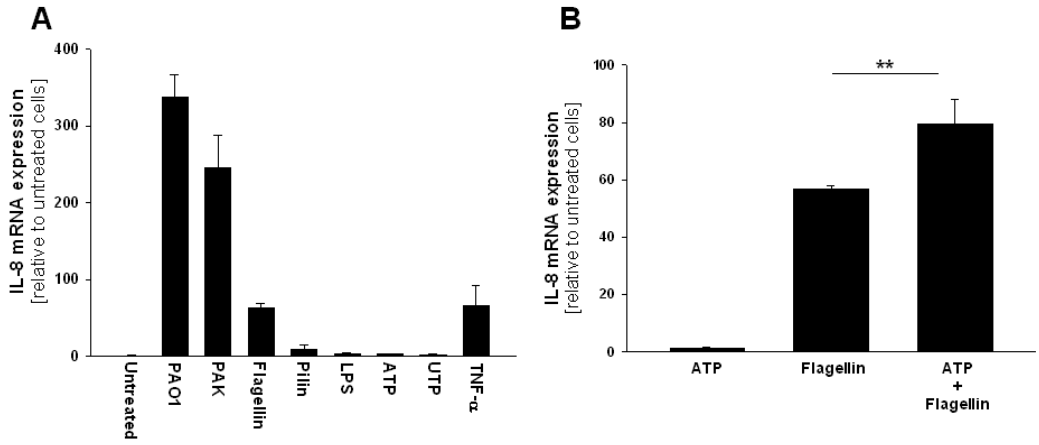

Figure 19

ATP is not sufficient to activate IL-8 mRNA transcription but acts in synergy with TLR-dependent signaling. *A, IB3-1 cells were exposed to the P. aeruginosa laboratory strains PAO1, PAK, or PAK FliC (100 CFU/cell), to flagellin (10 μg/ml) and pilin (10 μg/ml) purified from PAK recombinant cells, to LPS (10 μg/ml), to ATP and UTP (1 mM), or to TNFα (50 ng/ml) for 4 h before extraction of total RNA and measurement of IL-8 mRNA. B, Similarly to A, IB3-1 cells were exposed to ATP or flagellin alone, or to both stimulants together, for 4 h before extraction of total RNA and measurement of IL-8 mRNA. Mean ± SEM of three independent experiments performed in duplicate. **p< 0.01, by Student's t test.*

Pre-incubation of IB3-1 cells with apyrase before exposure to *P. aeruginosa* PAO1 strain, reduced the sustained increase of $[Ca^{2+}]c$ (Fig. 20 A, kindly provided by Prof. Paolo Pinton and Dr. Alessandro Rimessi, University of Ferrara) and, more relevantly, the IL-8 mRNA transcription and release of IL-8 protein (Fig. 20 B-C). Interestingly, after gene silencing of PLCB3, apyrase does not further reduce the *P. aeruginosa*-dependent IL-8 expression (Fig. 20 D), suggesting that the contribution of the purinergic receptor-dependent IL-8 expression is mainly mediated its coupling with PLCB3. This confirms a role of extracellular nucleotides, released upon interaction of *P. aeruginosa* with IB3-1 bronchial epithelial cells, in the pro-inflammatory signaling leading to the expression and secretion of IL-8 in our model.

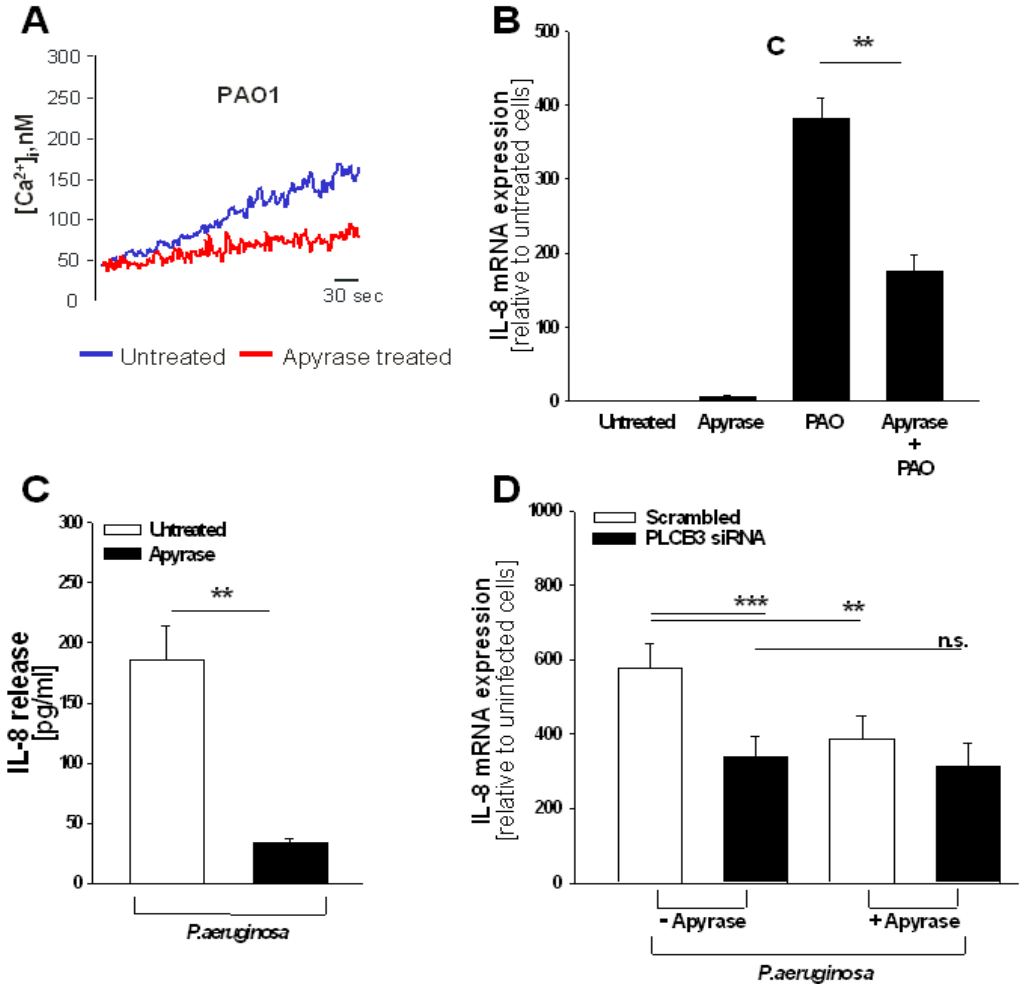

Figure 20

The ectonucleotidase apyrase affects calcium signaling and IL-8 expression induced by P. aeruginosa. *A, Cytosolic Ca^{2+} transients by Fura-2/AM assay in IB3-1 cells exposed to PAO1 treated with apyrase (red trace) or solvent alone (untreated, blue trace) - (courtesy of Prof. Paolo Pinton and Dr. Alessandro Rimessi, University of Ferrara). B, IB3-1 cells exposed to PAO1 treated with either apyrase (3 UI/ml) or solvent alone. C, IL-8 protein was measured by ELISA in IB3-1 cells treated as for B. C, Apyrase (3 UI/ml) was incubated for 2 hrs at 37°C after a pre-incubation of 24 hrs with PLCB3 siRNA or scrambled oligonucleotides in IB3-1 cells. An additional 4-hrs infection with PAO1 strain was performed. Data shown are means (± SEM) of four independent experiments performed in duplicate. **p< 0.01, ***p< 0.001 by Student's t test.*

4.9 Role of PLCB1 and PLCB4 on *P. aeruginosa*-dependent IL-8 expression

As it has been shown that *P. aeruginosa*-mediated calcium signaling is not completely mediated by PLCB3 isoform and that PLCB1 and PLCB4 are also expressed by bronchial epithelia (Fig. 14) we hypothesized a partial involvement of PLCB1 and PLCB4 on *P. aeruginosa*-dependent IL-8 expression. In order to investigate the role of these PLC isoforms in our experimental model, we performed several experiments of gene silencing. Transfection of two different siRNA molecules reduced significantly, albeit partially, the levels of expression of PLCB1 and PLCB4 mRNA (Fig. 21 A-B), as detected by quantitative RT-PCR. Silencing of PLCB1 and PLCB4 genes did reduce the IL-8 transcription (Fig. 21 C-D) and protein release (Fig. 21 E) induced by *P. aeruginosa* infection. Moreover, simultaneous silencing of PLCB1, PLCB3 and PLCB4 further reduced *P. aeruginosa*-dependent IL-8 mRNA expression in IB3-1 cells (Fig. 22A) and CuFi-1 cells (Fig. 22B) upon acute infection. Taken together, these data suggest that PLCB1 and PLCB4 may have a redundant role together with PLCB3 on pro-inflammatory signal transduction in bronchial epithelial cells. In order to check the possible presence of cross-reactivity among the siRNAs molecules against other PLCB isoforms we measured the transcripts levels of each PLCB isoform after each incubation of siRNA molecules. No cross-reactivity of siRNAs with unspecific PLCB isoform was detected (Fig. 23A-C).

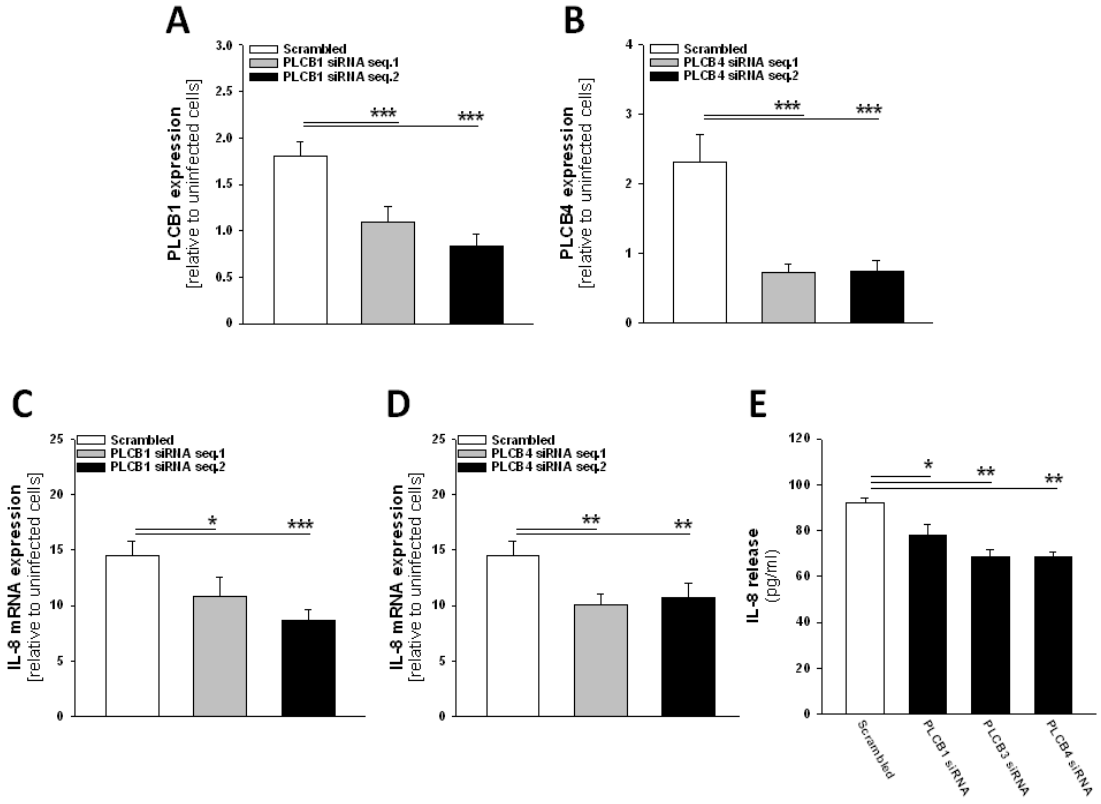

Figure 21

Silencing of PLCB1and PLCB4 genes reduces P. aeruginosa-dependent expression and release of IL-8 in human bronchial epithelial cells. *Quantitative expression of PLCB1 (A) and PLCB4 (B) mRNA by real-time PCR after transfection with PLCB1 and PLCB4 siRNA or scrambled oligonucleotides (2 different siRNA sequences) for 24 h in IB3-1 cells and subsequent infection with PAO1 (100 CFU/cell) for an additional 4 h. The mRNA expression reported in y-axis is relative to scrambled-treated uninfected cells. C, quantitative expression of IL-8 mRNA after transfection with PLCB1 siRNA oligonucleotides in IB3-1 cells. D, quantitative expression of IL-8 mRNA after transfection with PLCB4 siRNA oligonucleotides in IB3-1 cells. Effect of PAO1 (100 CFU/cell) on IL-8 protein release, treated as in A with PLCB1, PLCB3 and PLCB4 siRNA (sequence 1), or scrambled oligonucleotides. Mean ± SEM of four independent experiments performed in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test.*

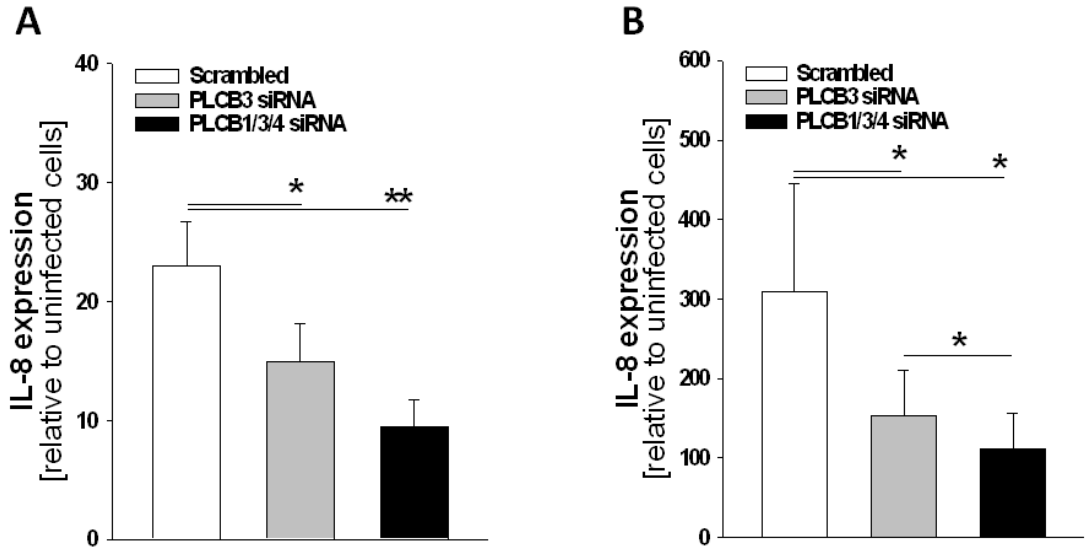

Figure 22

Comparison of the effect of simultaneous gene silencing of PLCB1, PLCB3 and PLCB4 with PLCB3 gene silencing alone, on P. aeruginosa-dependent IL-8 expression in bronchial epithelial cells. *Quantitative expression of IL-8 mRNA by real-time PCR after transfection with PLCB1, PLCB3 and PLCB4 siRNA or scrambled oligonucleotides for 24 h in IB3-1 cells (A) or CuFi-1 cells (B). Data shown are means (± SEM) of four independent experiments performed in duplicate. *p , 0.05, **p , 0.01, by Student's t test.*

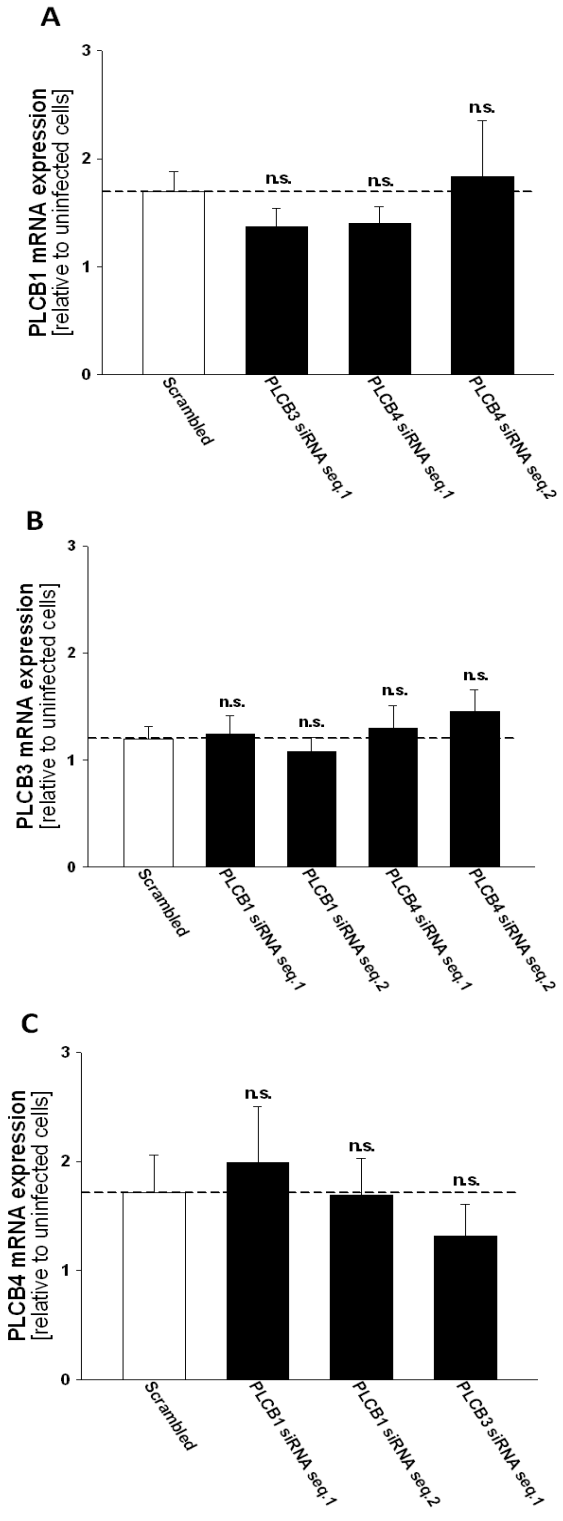

Figure 23

Check of cross-reactivity of siRNA molecules against PLCB isoforms. *Quantitative expression of PLCB1 (A) PLCB3 (B) and PLCB4 (C) mRNA by real-time PCR after transfection with PLCB1, PLCB3 and PLCB4 siRNA or scrambled oligonucleotides for 24 h in IB3-1 cells. Data shown are means (± SEM) of three independent experiments performed in duplicate.*

4.10 PLCG2 is not involved in *P. aeruginosa*-depenent IL-8 gene expression in bronchial epithelial cells.

Bronchial epithelial cells do express different isoforms of PLC that could regulate intracellular calcium homeostasis and, in particular, IB3-1 and CuFi-1 cells express detectable transcript levels of PLCG2, besides PLCB isoforms (Fig. 24). Activation of PLC-γ and -ε isoforms is known to be dependent on tyrosine-kinase–coupled receptors, of the PLC-δ isoforms on elevation of cytosolic calcium, of the PLC-β isoforms on seven-membrane spanning domain receptors through GTP-binding proteins [Suh, 2008]. As far as we know, among the surface receptors expressed in bronchial epithelial cell that are engaged by *P. aeruginosa*, TLRs and ASGM1R have not been described as coupled to GTP-binding proteins [Wettschureck, 2005]. However, it has been previously shown that the interaction of *P. aeruginosa* with bronchial epithelial cells induces the release of ATP in the extracellular milieu, which binds to the seven-membrane spanning P2Y2 purinergic receptors [McNamara, 2001; Adamo, 2004]. To investigate the role of other PLC isoforms which could be activated in our experimental model, we transfected three different siRNA molecules against the isoform PLCG2 in bronchial epithelial cells in order to silence the PLCG2 gene expression. As shown in figure 25A, two of these three siRNA molecules were able to reduce PLCG2 expression in IB3-1 cells. However, the PLCG2 gene silencing has not effect on *P. aeruginosa*-dependent IL-8 expression (Fig. 25B). These results seems to indicate that the *P. aeruginosa*-dependent calcium signal transduction occurs via ATP-P2Y2 pathways which in turn elicits PLCB specific activation.

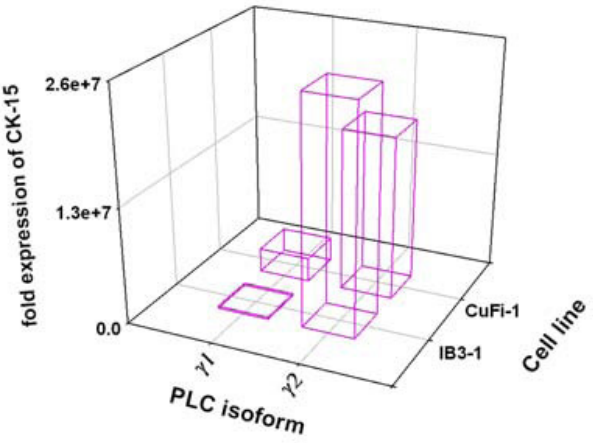

Figure 24

Relative transcript expression levels of PLCγisoforms in CF bronchial epithelial IB3-1 and CuFi-1 cell lines. *Expression of PLCγmRNA was quantified by qRT-PCR relative to the levels of expression of the housekeeping gene cytokeratin (CK)-15.*

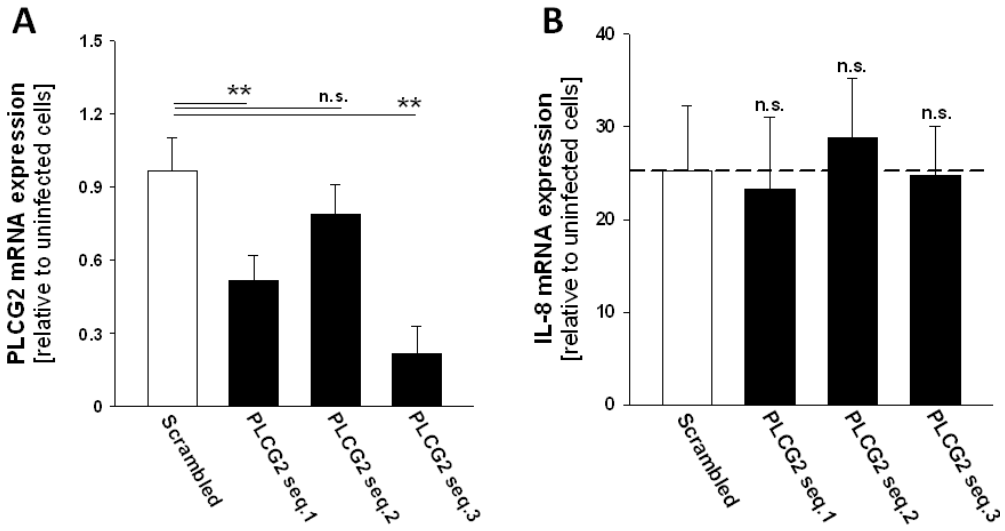

Figure 25

Effect of PLCG2 gene silencing on P. aeruginosa-dependent IL-8 expression. *A, quantitative expression of PLCG2 mRNA by real-time PCR after transfection with PLCG2 siRNA or scrambled oligonucleotides for 24 h in IB3-1 cells. Data shown are means (± SEM) of three independent experiments performed in duplicate. B, expression of IL-8 mRNA by real-time PCR in IB3-1 cells transfected with PLCG2 siRNA or scrambled oligonucleotides for 24 h and infected with P. aeruginosa for further 4 h. **p< 0.01 by Student's t test.*

4.11 PLCB3 gene silencing reduces *P. aeruginosa*-dependent NF-κB activation in bronchial epithelial cells.

It has been previously shown that *P. aeruginosa* PAO1 strain activates a Ca^{2+}-dependent activation of the transcription factor NF-κB, which is critical in the regulation of IL-8 gene transcription also in human airway epithelial cells [Ratner, 2001]. Therefore, we tested the role of PLCB3 on the activation of NF-κB p65 induced by PAO1 in IB3-1 cells, with a time course preceding the lapse of time of 4 hours chosen to measure IL-8 mRNA levels. We confirm that PAO1 progressively activates NF-κB to translocate into the nucleus of infected IB3-1 cells, and that silencing PLCB3 significantly reduces the *P. aeruginosa*-induced activation of NF-κB p65 (Fig. 26). However, this assay measured only the total activation of NF-κB into the nucleus but did not formerly confirm the increased binding of NF-κB on IL-8 gene promoter. In order to address this issue, a Chromatin Immuno-precipitation (ChIp) need to be planned. Nevertheless, the assay performed indicates a partial role of PLCB3 on NF-κB pathway and highlights the potential therapeutic target of PLCB3 in CF lung inflammation.

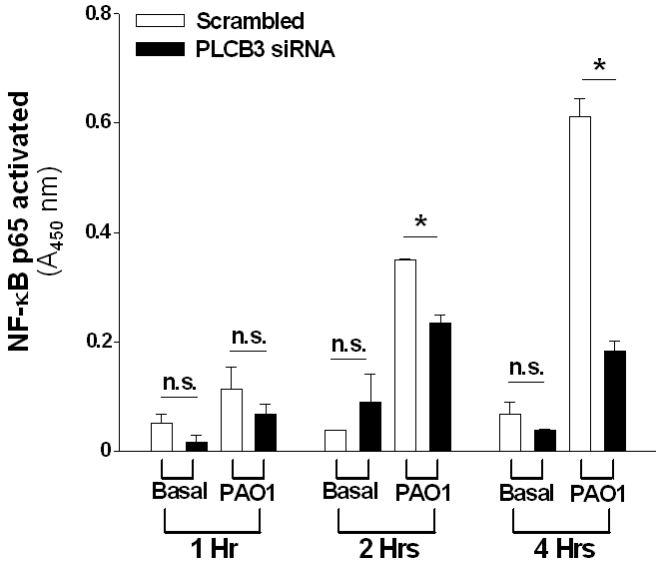

Figure 26

Effect of PLCB3 gene silencing on P. aeruginosa-dependent NF-κB activation in IB3-1 cells. *Activation of NF-κB p65 in IB3-1 cells transfected with either PLCB3 siRNA sequence 1 or scrambled oligonucleotide for 24 h before exposure to PAO1 (100 CFU/cell) or solvent alone in a lapse of time ranging from 1 to 4 h. Absorbance at 450 nm wavelength is proportional to the activation of NF-κB p65, as performed with the TransAM NF-κB p65 Activation Assay kit. Data are mean ± SEM of four independent experiments performed in duplicate. *p< 0.05 by Student's t test.*

5. CONCLUSIONS AND DISCUSSION

Excessive inflammation in the lungs of patients affected by CF is considered a major cause of the lung tissue damage leading to respiratory insufficiency. Therefore, a thorough understanding of the molecular pathways regulating the chronic lung inflammation in CF is highly relevant to design novel approaches to reduce the progressive destruction of the pulmonary tissue. As the reported excessive recruitment of neutrophils, the chemokine IL-8, is considered a key therapeutic target. It is already known that *P. aeruginosa*, upon interaction with PRRs expressed in respiratory epithelial cells, induces a pro-inflammatory signaling involving the participation of the MAP kinases p38 and ERK, and of the nuclear transcription factors NF-κB, NF-IL6 and AP-1, via MyD88-dependent cascades and intracellular calcium [McNamara, 2006; Fu, 2007; Boncoeur, 2008]. In this thesis, these findings are basically confirmed and further implication of RSK, GSK3, HSP27, and the transcription factors CREB and CHOP are proposed.

To investigate the signaling cascade of kinases activated by *P. aeruginosa*, here it has been performed a phosphokinase assay. Results confirm that *P. aeruginosa* induces phosphorylation of the MAPK p38 pathway and, specifically, of the p38 isoforms alpha, delta and gamma and of their downstream effector HSP27. The role of p38 pathway in IL-8 expression is confirmed by the pharmacological inhibition of both p38 and HSP27 with SB203580 and KRIBB3, respectively. The p38 MAPK and its downstream target, HSP27, were the only kinases which was activated by all the three pro-inflammatory challenges (*P. aeruginosa*, IL-1β and TNFα) and here are both reported to partecipate always in IL-8 mRNA expression. It has been also observed a direct *P. aeruginosa*-dependent phosphorylation of ERK1/2 in CuFi-1 cells, as reported by other investigators [McNamara, 2006], although this data seem controversial in IB3-1 cells (Fig. 5). However, results shown a clear increase of phosphorylation of RSK1 and MSK2, which are substrates of ERK1/2 and, in parallel, an inhibitory effect of *P. aeruginosa*-dependent IL-8 transcription with the inhibitors AG1288 (ERK1/2) and SL0101 (RSK) (Fig. 6). All this considered, we are in favour of a role of ERK pathway in *P. aeruginosa*-dependent expression of IL-8. The results obtained here with three pro-inflammatory challenges, could provide suggestions on the signaling related to MyD88-dependent

cascades involved in the regulation of transcription IL-8. Both *P. aeruginosa* and the pro-inflammatory cytokine IL-1β are known to transduce intracellular signals through a MyD88/TIR-dependent cascade, whereas TNFα utilizes alternative pathways [Bouwmeester, 2004]. As expected from a MyD88 activation, both *P. aeruginosa* and IL-1β activate MAPK p38 and ERK (Fig. 7), whereas TNFα involves p38 and JNK, but not ERK pathway (Fig. 8). It has been widely reported that NF-κB and AP-1 have a key-role in IL-8 mRNA expression induced by pro-inflammatory stimuli, including *P. aeruginosa* [Hoffman, 2002; Hisatsune, 2008; Sun, 2008; Vij, 2008]. In collaboration with Prof. Roberto Gambari's team (University of Ferrara), we observed that *P. aeruginosa* and IL-1β promoted activation of the TFs NF-κB, NF-IL6, AP-1, CREB and CHOP, as observed by EMSA (Fig. 10). However, TF decoy ODNs against TFs NF-κB, NF-IL6, AP-1, CREB and CHOP, resulted in inhibition of IL-8 transcription induced by *P. aeruginosa* and IL-1β (Figs. 12-13) . Furthermore, it has been proposed a role for CHOP in the activation of transcription of IL-8 induced by IL-1β and PGE_2 in the same IB3-1 bronchial epithelial cells [Vij, 2008]. Here we confirm the involvement of CHOP after exposure of IB3-1 cells to both IL-1β and *P. aeruginosa* by TF decoy approach (Figs. 12-13). MAPK p38/HSP27 pathway has been related to CHOP activation in melanoma cells [Sarkar, 2002]. Consistently to this hypothesis, we also observed phosphorylation of MAPK p38 and a very relevant phosphorylation of HSP27 after testing those pro-inflammatory stimuli in which CHOP seems involved, namely *P. aeruginosa* and IL-1β (Figs. 6-7). CREB has been previously related to IL-8 transcription in U937 monocytic cells exposed to VacA toxin [Hisatsune, 2008], but never in bronchial epithelial cells challenged with *P. aeruginosa* and IL-1β as shown here. As RSK has been reported as a potential activator of CREB [Roux, 2004] and we showed that RSK1 was phosphorylated after exposure to *P. aeruginosa* and IL-1β, our transcription factor decoy approach seem to confirm the relation between the activations of RSK and CREB, in our experimental model. In parallel, the lack of inhibition of CREB by TF decoy ODNs in IB3-1 cells stimulated with TNFα is consistent with the suppression of CREB activity by TNFα, previously reported in a murine Leydig tumor cells [Arai, 2005]. In addition, here we observe

the involvement of RSK1 and MSK2 in the MyD88 pathway and of HSP27 in the common p38-related pathway. It is already known that interaction of *P. aeruginosa* with bronchial epithelial cells induces the release of ATP, as danger signal, which in turn activates the heterotrimeric G-coupled P2Y purinergic receptor [McNamara, 2006; Lazarowski, 2009]. Thus, as G-alpha or G-beta/gamma subunits activate phospholipase C beta, leading to intracellular calcium mobilization, the beta/gamma subunit activates phosphoinositide-3 kinase (PI3K)/AKT [Santiago-Perez, 2001; Lazarowski, 2009]. PLCs have been shown to be implicated in different cellular responses, due to their role in intracellular calcium homeostasis (for review see Katan, 1998). As far as its role in inflammatory processes is concerned, PLCB3 has been investigated in the context of leukocyte chemotaxis [Bach, 2007]. PLC beta2- and beta3-dependent rise in intracellular calcium has been shown to regulate T lymphocyte chemotaxis [Li, 2000]. Because T lymphocytes infiltrate the bronchial walls of CF patients, these early reports established already a possible link between PLCB3 and the progression of CF lung disease. Our findings strengthen this notion implicating PLCB3 in regulation of IL-8 expression by bronchial epithelial cells and hence neutrophil recruitment into the airways. Bronchial epithelial cells do express different isoforms of PLC that could regulate intracellular calcium homeostasis and, in particular, IB3-1 and CuFi-1 cells express detectable transcript levels of PLC beta 1, beta 3, beta 4 (Fig. 14) and PLC gamma 2 (Fig 24). It has been previously shown that the interaction of *P. aeruginosa* with bronchial epithelial cells induces the release of ATP in the extracellular milieu, which binds to the seven-membrane spanning P2Y2 purinergic receptors [Adamo, 2004; McNamara, 2006]. In this thesis, silencing experiments reducing cytosolic calcium increase confirm the involvement of PLCB3 in the Ca^{2+} pathway activated by *P. aeruginosa* (Fig. 18). Thus, these results are consistent with the coupling of P2Y2 purinergic receptors with PLCB3, which is known to involve $Ga_{q/11}$ heterotrimeric GTPase protein [Prince, 2006; Fu, 2007; Lazarowski, 2009]. Because PLC beta 1 and 4 are also able to interact with seven-membrane spanning receptors, we can not definitely restrict to PLCB3 the role to modulate *P. aeruginosa*-dependent calcium transients in bronchial epithelial cells. Indeed, silencing of both PLCB1 and PLCB4 isoforms reduced the *P. aeruginosa*-dependent IL-8 expression (Fig. 21). Moreover, contemporary silencing of PLCB1,

PLCB3 and PLCB4 further reduced the *P. aeruginosa*-mediated IL-8 expression compared to the PLCB3 gene silencing alone in our cell models (Fig. 22).

However, also the contemporary silencing of PLCB1, PLCB3 and PLCB4 reduced only partially the *P. aeruginosa*-dependent expression of IL-8 (Fig. 22). This is not surprising at the light of the partial efficiency of PLCB isoforms silencing and of the evidence that *P. aeruginosa* activates the inflammatory response due to its capability to interact with multiple receptors, including TLRs and ASGM1Rs [Prince, 2006; McNamara, 2006]. Thus the ATP–P2Y2R autocrine loop that generates intracellular Ca^{2+}-signaling should be considered only one of the pathways regulating IL-8 expression, in parallel with those elicited by TLRs via MyD88-dependent signals. We observed a significant reduction of IL-8 mRNA expression with the intracellular Ca^{2+} chelator BAPTA (Fig. 18C), whereas direct stimulation of IB3-1 cells with P2Y2 ligands, such as ATP or UTP, that are known to stimulate directly cytosolic Ca^{2+} transients, were not sufficient to induce transcription of IL-8 mRNA (Fig. 19A), as previously observed by other investigators [Fu, 2007]. This apparent discrepancy can be explained observing that the addition of ATP to the TLR5/2 and ASGM1R ligand flagellin increases IL-8 mRNA expression (Fig. 19B), thus suggesting that the intracellular calcium signaling triggered by purinergic receptors upon release of ATP, albeit not sufficient by itself to completely activate the transcription machinery for IL-8 expression, works in synergy with TLRs-mediated signalling. As a further evidence that Ca^{2+} - signaling mediated by PLCB3 is indeed relevant to regulate IL-8 expression, we observed that silencing of PLCB3 significantly reduced the activation of the transcription factor NF-κB (Fig. 26), which plays a critical role in the induction of IL-8 transcription [Bezzerri, 2008]. Based on these and previous findings [Adamo, 2004; McNamara, 2006], we conclude that in the CF airway tract chronically infected with *P. aeruginosa*, the Ca^{2+}-dependent pathway induced by the release of nucleotides, through binding to P2Y2R, activates PLCB1, PLCB3 and PLCB4 amplifying the innate defense signalling based upon TLRs and ASGM1R. The cartoon reported in Fig. 27 summarizes our working hypothesis.

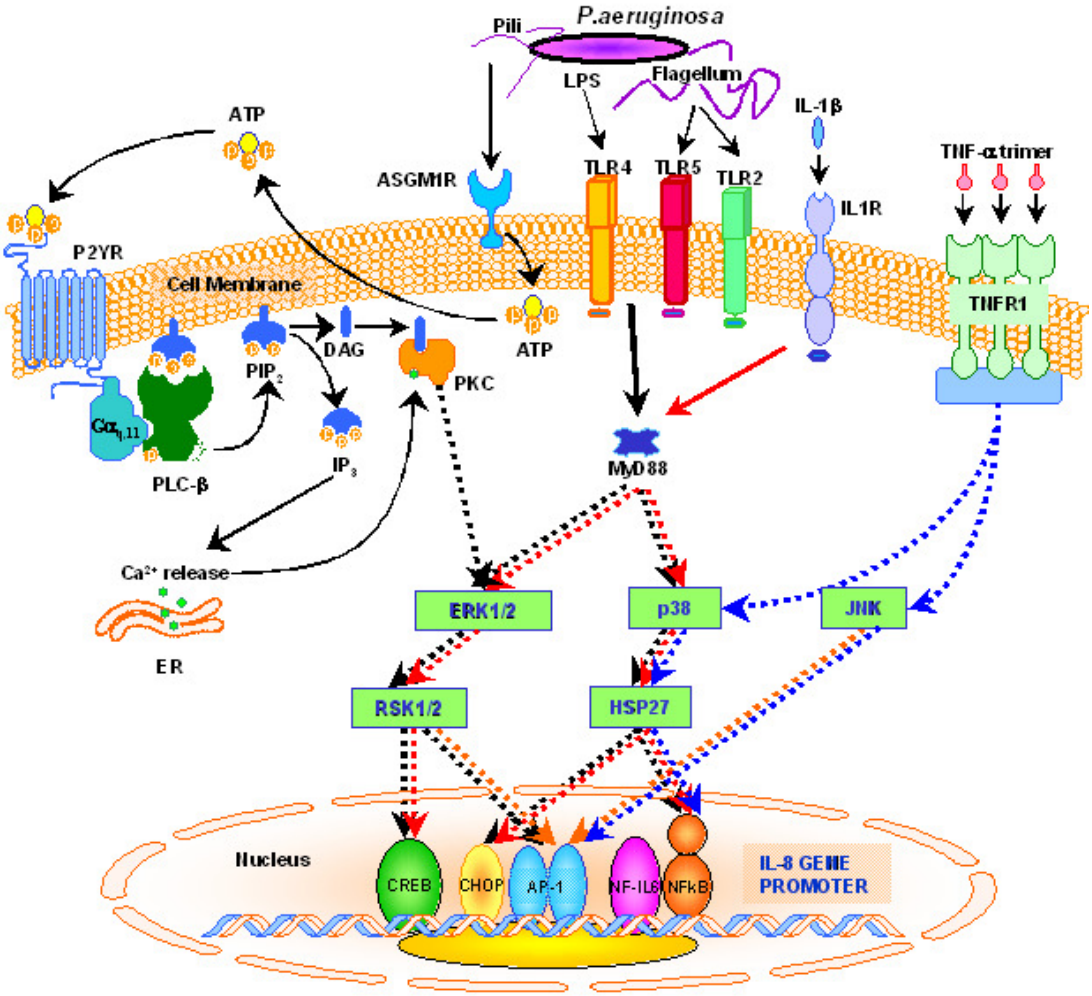

Figure 27

The pro-inflammatory signal transduction within the lung pathology observed in cystic fibrosis. *The illustration depicts the signaling pathways elicited by TLRs/MyD88 and P2Y2R/PLCB, based on previous reports from other investigators and the results presented in this thesis. Binding of P. aeruginosa surface components (flagellin, LPS and pilin) with TLR5, TLR4 and TLR2 triggers a MyD88-dependent proinflammatory signaling cascade, eventually leading to nuclear translocation of NF-κB, which is a critical transcription factor for the expression of IL-8 gene, together with NF-IL6, AP-1, CREB and CHOP. TLRs/MyD88 pathway is sufficient to promote transcription of IL-8 gene. Besides exerting this direct effect, P. aeruginosa induces the extracellular release of ATP, possibly via a cooperative interaction of TLR5, TLR2, and ASGM1R. Extracellular ATP binds to P2Y2R that activates PLC-β through the Gαq,11 heterotrimeric GTPase protein. By degrading phosphatidylinositol 4,5-biphosphate, PLC-β promotes IP3 release and DAG formation. IP3 triggers Ca^{2+} release from intracellular stores which ultimately cooperate in activation of NF-κB. The P2Y2R/PLC-β pathway is not sufficient to induce IL-8 transcription by itself but strongly act in synergy with the TLR/MyD88 signaling cascade.*

In conclusion, the present work widens the horizon of the different steps of activation of transcription of IL-8, particularly in bronchial epithelial cells. A thorough understanding of the activatory pathways of RSK, GSK3, HSP27 and of the transcription factors CHOP and CREB requires further investigation, as well as the PLCB-mediated signal transduction, which relevantly regulates the extracellular nucleotide-cytosolic Ca^{2+} signaling axis potentiating the Toll-like Receptors signaling cascade. This study revealed novel pharmacological targets which could be useful to attenuate the excessive recruitment of neutrophils without completely abolishing the inflammatory response observed in cystic fibrosis.

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