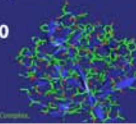

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Phospholipase C-β3 Is a Key Modulator of IL-8 Expression in Cystic Fibrosis Bronchial Epithelial Cells

Valentino Bezzerri,*[,1] Pio d'Adamo,[†,1] Alessandro Rimessi,[‡,1] Carmen Lanzara,[†] Sergio Crovella,[†] Elena Nicolis,* Anna Tamanini,* Emmanouil Athanasakis,[†] Maela Tebon,* Giulia Bisoffi,[§] Mitchell L. Drumm,[¶] Michael R. Knowles,[‖] Paolo Pinton,[‡,2] Paolo Gasparini,[†,2] Giorgio Berton,[#,2] and Giulio Cabrini*[,2]

Respiratory insufficiency is the major cause of morbidity and mortality in patients affected by cystic fibrosis (CF). An excessive neutrophilic inflammation, mainly orchestrated by the release of IL-8 from bronchial epithelial cells and amplified by chronic bacterial infection with *Pseudomonas aeruginosa*, leads to progressive tissue destruction. The anti-inflammatory drugs presently used in CF patients have several limitations, indicating the need for identifying novel molecular targets. To address this issue, we preliminarily studied the association of 721 single nucleotide polymorphisms from 135 genes potentially involved in signal transduction implicated in neutrophil recruitment in a cohort of F508del homozygous CF patients with either severe or mild progression of lung disease. The top ranking association was found for a nonsynonymous polymorphism of the phospholipase C-β3 (PLCB3) gene. Studies in bronchial epithelial cells exposed to *P. aeruginosa* revealed that PLCB3 is implicated in extracellular nucleotide-dependent intracellular calcium signaling, leading to activation of the protein kinase Cα and Cβ and of the nuclear transcription factor NF-κB p65. The proinflammatory pathway regulated by PLCB3 acts by potentiating the Toll-like Receptors' signaling cascade and represents an interesting molecular target to attenuate the excessive recruitment of neutrophils without completely abolishing the inflammatory response. ***The Journal of Immunology*, 2011, 186: 4946–4958.**

Cystic fibrosis (CF), an autosomal-recessive disease manifesting progressive respiratory insufficiency, defective exocrine pancreatic secretion, and elevated electrolyte concentration in the sweat, is related to loss-of-function mutations of the CF transmembrane conductance regulator (CFTR) gene encoding a chloride transporting protein (OMIM 219700).

The major cause of morbidity and mortality of CF patients is chronic lung disease. Innovative therapies to prevent the onset or reduce the pulmonary damage are under intensive preclinical and clinical investigation (1). Besides pharmaceutical approaches to correct the mutated CFTR protein and to potentiate its ion transport function, novel anti-inflammatory drugs will be particularly relevant to those adolescent and adult CF patients who have already developed lung pathology (2). However, current anti-inflammatory drugs, such as corticosteroids, show limitations in terms of efficacy or occurrence of adverse effects in CF patients, suggesting the need of identifying novel molecular targets and more effective anti-inflammatory drugs specifically tailored for CF pulmonary pathophysiology (2).

A hallmark of lung pathology in CF is a neutrophil-dominated inflammation, which is disproportionate to the bacterial infection, typically by *Pseudomonas aeruginosa.* Excessive infiltrates of neutrophils, releasing proteases upon continuous activation by bacterial products, are presently thought to play a major role in the progressive destruction of the CF lung tissue (3, 4). The chemokine IL-8 is the most abundant soluble mediator recruiting neutrophils in the bronchi of CF patients, being found in the airway secretions both in advanced and in early stages of the disease (5–7). Interestingly, IL-8 promoter variant alleles resulting in reduced expression of IL-8 protein are protective in respect to the severity of progression of lung disease in CF patients (8), supporting the concept that reduction of the excessive IL-8–driven neutrophil recruitment could be a relevant pharmacological objective to ameliorate CF pulmonary disease.

A major source of IL-8 release in CF lungs is the bronchial epithelial cells lining the conductive airways of CF patients (5, 0). Binding of bacterial components to pattern-recognition receptors

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Abbreviations used in this article: ASGM1R, asialo-GM1 receptor; BIM-I, bisindolylmaleimide-I; $[Ca^{2+}]_c$, cytosolic-free calcium concentration; $[Ca^{2+}]_{er}$, ER Ca^{2+} concentrations; $[Ca^{2+}]_i$, intracellular calcium concentration; $[Ca^{2+}]_m$, rise of $[Ca^{2+}]_c$ in the mitochondrial matrix; CF, cystic fibrosis; CTFR, cystic fibrosis transmembrane conductance regulator; DAG, 1,2-diacylglycerol; ER, endoplasmic reticulum; GMSG, Gene Modifier Study Group; IP_3, inositol 1,4,5-trisphosphate; KRB, Krebs Ringer's Buffer; LHC, Laboratory of Human Carcinogenesis; PLC, phospholipase C; PLCB, phospholipase C-β; P2Y2R, purinergic receptor Y2; siRNA, small-interfering RNA; SNP, single nucleotide polymorphism.

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expressed on epithelial cell surface, such as TLRs 2, 4, and 5, activates a series of kinases and adaptors, ultimately triggering nuclear translocation of transcription factors and expression of proinflammatory genes (10). In addition, interaction of *P. aeruginosa* with asialo-GM1 receptor (ASGM1R), which colocalizes with TLR5, promotes sustained release of nucleotides (11, 12). Extracellular ATP, through an autocrine binding to the seven-membrane spanning domain P2Y2 purinergic receptor, activates cytosolic calcium signaling and contributes, together with TLRs, to the expression of IL-8 (13).

Because of the high redundancy in receptors and downstream signaling components triggered by *P. aeruginosa* in bronchial epithelial cells, finding key targets to effectively reduce excessive inflammatory response represents a major challenge. To prioritize the investigation on key target molecule(s) intervening in this proinflammatory network, we performed a pilot genotype-phenotype association study between variants of genes involved in the innate immune response and the severity of lung disease in CF patients. So far, association studies have highlighted that the mannose-binding lectin 2, the TGF-β1 (TGFB1), and the IFRD1 genes can modulate the progression of lung disease in CF (14–16). Thus, to start identifying critical components modulating the inflammatory signaling cascade triggered by *P. aeruginosa* in bronchial epithelial cells, we studied the distribution of allelic variants of 721 single nucleotide polymorphisms (SNPs) from a panel of 135 genes of innate immunity in two groups of CF patients characterized by severe or mild progression of lung disease. By ranking the association of each SNP with the progression of lung disease severity, we found on the top a nonsynonymous polymorphism of the phospholipase C-β (PLCB)3 gene, leading to investigate first the role of the PLCB3 enzyme. Functional studies performed in human bronchial epithelial cells exposed to *P. aeruginosa* demonstrate that PLCB3, by regulating intracellular calcium transients, plays a relevant role in amplifying the expression and release of IL-8, the major chemokine recruiting neutrophils in CF airway lungs.

Materials and Methods

Materials

The calcium chelator BAPTA-AM was purchased from Molecular Probes (Eugene, OR), the broad protein kinase C (PKC) inhibitor bisindolylmaleimide-I (BIM-I) was purchased from Merck KGaA (Darmstadt, Germany), all the other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Subjects

DNA samples were collected from CFTR F508del homozygous patients enrolled by the University of North Carolina (Chapel Hill, NC) (M.R.K.) and by Case Western Reserve University (Cleveland, OH) (M.L.D.). CF patients were classified as "mild" ($n = 300$) or "severe" ($n = 208$) with respect to the progression of lung disease according to the forced expiratory volume at 1st minute measurements, according to the criteria reported previously by the North American Gene Modifier Study Group (GMSG) (15). The study was approved by the Institutional Review Boards of the participating institutions collecting the samples from patients affected by CF.

Selection of SNPs

Different types of SNPs have been chosen in candidate genes by using the HapMap database. In particular, two different types of polymorphisms have been chosen: 1) nonsynonymous coding SNPs that include a group of SNP having the highest impact on phenotype for their potential to directly affect the structure, function, and interactions of expressed proteins; and 2) Tag SNPs, which represent a subset of SNPs capturing most of the haplotype diversity of each haplotype block or gene-specific region. Tag SNPs have been selected using Tagger, a software implemented in Haploview (http://www.broad.mit.edu/mpg/haploview/). Genotype data in raw HapMap format have been uploaded to specify the genomic regions of interest within which tag SNPs to be picked. As output, Tagger produced a list of tag SNPs and corresponding statistical tests to capture all variants of interest and a summary coverage report of the selected tag SNPs.

Genotyping by Illumina technology

First, analysis of SNPs has been performed by Illumina technology. An overall number of 721 SNPs was selected within 135 genes and analyzed by using the "Custom Golden-Gate Genotyping" (http://www.illumina.com) assay on ILLUMINA platform. This genotyping system consisted of an initial allele-specific extension reaction, followed by PCR amplification. Amplified products were hybridized to an array matrix of bead-based probe sequences where each bead is coated with universal probes and represented multiple times for increased accuracy (average of 25 times).

Genotyping by TaqMan technology

The genotype of the SNPs ranking on top of statistical significance has been verified with TaqMan assays from Applied Biosystems. All reactions using TaqMan MGB probes were run under standard condition on an ABI PRISM 9700 HT system. Genotyping of phospholipase C (PLC) SNPs in the human bronchial epithelial cell lines IB3-1 and CuFi-1 was performed by both TaqMan technology and direct sequencing (see Supplemental Tables V, VI).

Human bronchial 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 (17). Cells were grown in Laboratory of Human Carcinogenesis (LHC)-8 basal medium (Biofluids, Rockville, MO) supplemented with 5% FBS. All culture flasks and plates were 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) as described previously. CuFi-1 cells were a gift from A. Klingelhutz, P. Karp, and J. Zabner (University of Iowa, Iowa City, IA). CuFi-1 cells were derived from human bronchial epithelia from a patient with CF (CFTR mutant genotype F508del/F508del) and transformed by reverse transcriptase component of telomerase, hTERT, and human papillomavirus type 16 E6 and E7 genes (18). These cells were grown on human placental collagen type IV (Sigma-Aldrich)-coated flasks in bronchial epithelial growth medium (Cambrex Bioscience, Walkersville, MD), as described previously (18). CuFi-1 cells were seeded onto cell culture inserts (pore size, 0.4 μm) in Falcon 24-well multitrays (BD Biosciences) at a density of 7×10^5 cells/insert and grown in bronchial epithelial growth medium for 15 d. Transepithelial electrical resistance was measured with an epithelial voltometer (World Precision Instruments, Sarasota, FL). The cell inserts were used for experiments when the cell monolayers reached a transepithelial electrical resistance $> 1000\ \Omega \times cm^2$.

Infection with P. aeruginosa bacterial strains

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

Expression of PLC isoforms and of IL-8

PLC and IL-8 transcripts were quantified real-time PCR as described previously (19). Briefly, total RNA from cells was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). Two microrgrams of total RNA was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit and random primers (Applied Biosystems, Foster City, CA) in a 100-μl reaction. The cDNA (2 μl) was then amplified for 40 PCR cycles using the SYBR Green PCR Master Mix (Applied Biosystems) in a 10-μl reaction using 7900HT Fast Real-Time PCR apparatus (Applied Biosystems, Foster City, CA). The quantified real-time PCRs were performed in duplicates for both target and normalizer genes. Primer sequences and concentration are shown in Supplemental Table IV. Primer sets were purchased from Integrated DNA Technologies (Tema Ricerca s.r.l., Bologna, Italy). Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems User Bulletin 2). Changes in mRNA

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Table I. Prevalence of polymorphic genotypes according to the severe or mild phenotype of CF patients

Gene SNP No.	Lung Function	Genotype CC Patients *n* (%)	Genotype CT Patients *n* (%)	Genotype TT Patients *n* (%)	Total Patients *n*	χ^2 Value	*p* Value
PLCB3	Severe	185 (92)	15 (8)	0	200	8.01	0.0046
rs35169799	Mild	260 (84)	45 (15)	3 (1)	308		

expression level were calculated after normalization to calibrator gene. The ratios obtained after normalization are expressed as fold changes over untreated samples for IL-8 mRNA and for the relative expression of the housekeeping gene human cytokeratin-15 for the isoforms of PLC. 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 −20°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. IL-8 protein release was measured with an ELISA. IB3-1 and CuFi-1 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.

Silencing PLCB3 gene

To perform silencing experiments of PLCB3 gene 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 PLCB3 (sequence 1, 5′-AGAUGAGGGACAAGCAUAAGAAGGA-3′; sequence 2, 5′-GCUCGAAAGAGGAACCGAAGCAUUUGUUCCU-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.

Activation of NF-κB

IB3-1 cell nuclear extracts were prepared as described previously (19). Briefly, IB3-1 cells were transfected with PLCB3 siRNA or scrambled duplexes for 24 h and stimulated with *P. aeruginosa*, PAO1 strain (100 CFU/cell) for an additional 1, 2, or 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. A quantitative NF-κB p65 subunit capture assay was performed by TransAM NF-κB p65 Activation Assay (Active Motif, Carlsbad, CA) using 2.5 μg nuclear extracts for each sample, according to the manufacturer's instructions. Briefly, nuclear extracts were added to wells coated with an oligonucleotide containing a NF-κB consensus binding site. The binding was revealed by subsequent addition of a primary Ab directed against p65 and a secondary Ab conjugated to HRP, and after the addition of the substrate, absorbance was read at a 450-nm wavelength.

Purification of flagellin and pilin from P. aeruginosa

Purification of flagellin was performed as previously described (20) starting from the PAK/NP recombinant *P. aeruginosa* strain lacking expression of pilin. Pilin proteins were isolated using the method of Castric (21), starting from the PAK/*fliC* recombinant *P. aeruginosa* strain lacking expression of flagellin.

Fura-2/AM measurements

The cytosolic free Ca^{2+} concentration was evaluated using the fluorescent Ca^{2+} indicator Fura-2/AM (Molecular Probes). Briefly, cells were incubated in medium supplemented with 2.5 μM Fura-2/AM for 30 min, washed with Krebs Ringer's Buffer (KRB) to remove the extracellular probe, supplied with preheated KRB (supplemented with 1 mM $CaCl_2$), and placed in a thermostated (37°C) incubation chamber of an LS50 Perkin Elmer fluorometer (Perkin Elmer, Beaconsfield, U.K.). Fluorescence was measured every 100 ms with the excitation wavelength alternating between 340 and 380 nm and the emission fluorescence being recorded at 510 nm. The intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated by the ratio method using the equation: $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R - R_{max}) \times Sf2/Sf1$, where K_d is the dissociation constant of Fura-2/AM for (Ca^{2+}) taken as 240 nM at 37°C, R is the ratio of fluorescence for Fura-2/AM at the two excitation wavelengths, F340/F380, R_{max} is the ratio of fluorescence in the presence of excess of calcium obtained by lysing the cells with 10 μM ionomycin (Sigma Aldrich), R_{min} is the ratio of fluorescence in the presence of minimal calcium obtained by lysing the cells and then chelating all the Ca^{2+} with 0.5 M EGTA, $Sf2$ is the fluorescence of Ca^{2+} free form of Fura-2/AM at 380-nm excitation wavelength, and $Sf1$ is the fluorescence of Ca^{2+}-bound form of Fura-2/AM at 380-nm excitation wavelength. IB3-1 cells were transfected and stimulated with *P. aeruginosa*, PAO1 strain (100 CFU/cell), as reported in the figures.

Aequorin measurements

The probes used (cytAEQ, erAEQmut, and mtAEQ) are chimeric aequorins targeted to the cytosol, endoplasmic reticulum (ER), and mitochondria, respectively (22). For the experiments with cytAEQ and mtAEQ, cells were incubated with 5 μM coelenterazine for 1–2 h in modified KRB supplemented with 1 mM $CaCl_2$. Then, the coverslip with transfected cells

Table II. Prevalence of polymorphic alleles according to the severe or mild phenotype of CF patients

Gene SNP No.	Lung Function	Allele	Allele n	Allelic Frequency	Minor Allele Odds Ratio (95% Confidence Interval)
PLCB3	Severe	C	385	385/400 = 0.962	Allele T in mild
rs35169799	Mild	C	565	565/616 = 0.917	CF patients
	Severe	T	15	15/400 = 0.037	2.297 (1.27–4.14)
	Mild	T	51	51/616 = 0.083	

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was placed in a perfused, thermostated chamber located in the close proximity of a low-noise photomultiplier with a built-in amplifier-discriminator. To reconstitute the erAEQmut with high efficiency, the luminal [Ca^{2+}] of the ER first had to be reduced. This was achieved by incubating the cells for 1 h at 4°C in KRB supplemented with 5 μM coelenterazine, the Ca^{2+} ionophore ionomycin, and 600 μM EGTA. After this incubation, cells were extensively washed with KRB supplemented with 2% BSA and then transferred to the perfusion chamber. All aequorin measurements were carried out in KRB supplemented with either 1 mM $CaCl_2$ (cytAEQ and mtAEQ) or 100 μM EGTA (erAEQmut). Agonist, as 100 μM histamine, and other drugs were added to the same medium, as shown in the figures. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-containing solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated offline into [Ca^{2+}] values, using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant aequorins. Chemicals and reagents were obtained from Sigma-Aldrich or from Merck, except for coelenterazine and coelenterazine n, which were obtained from Molecular Probes.

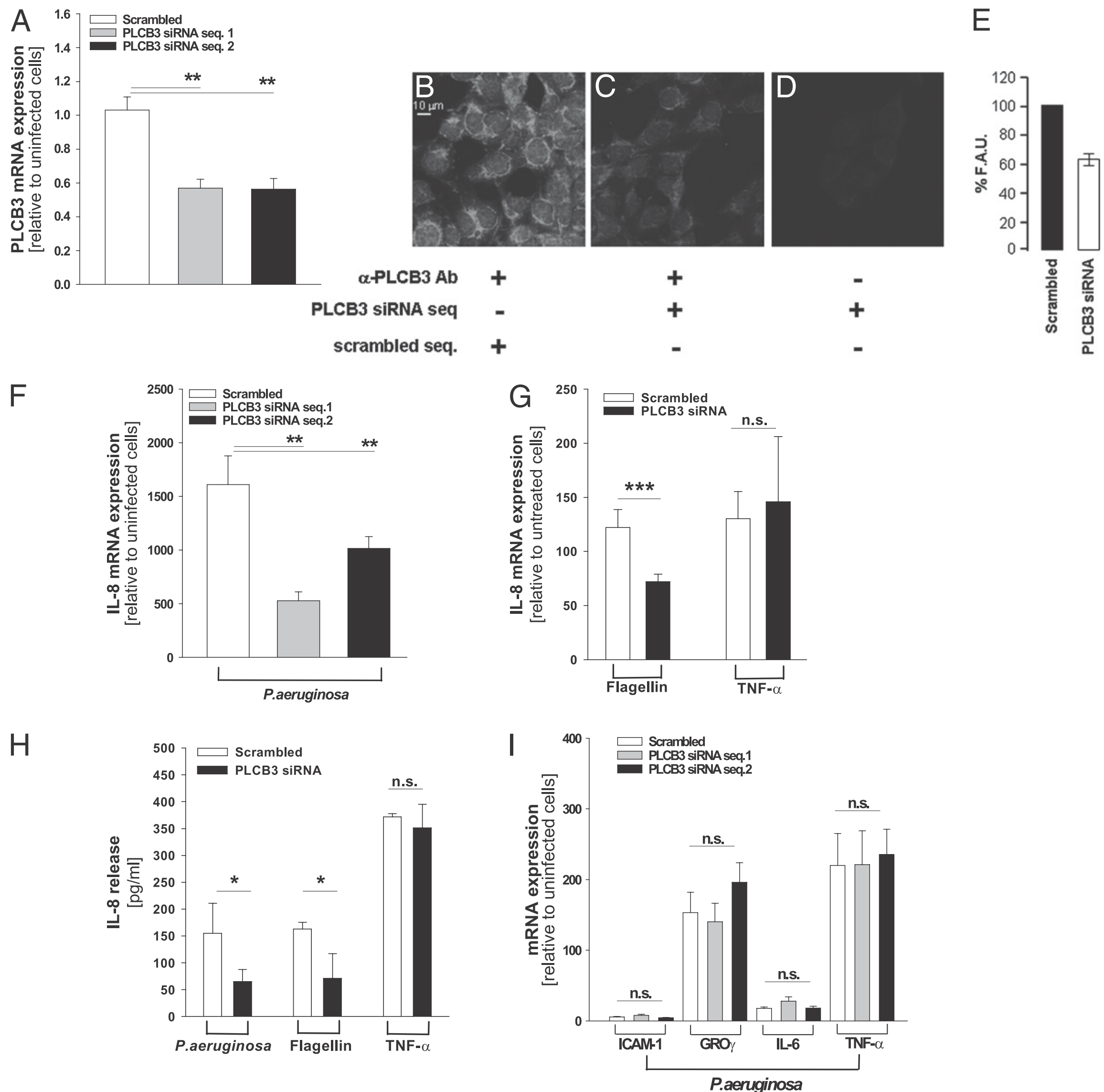

FIGURE 1. Silencing PLCB3 reduces *P. aeruginosa*-dependent expression and release of IL-8 in human bronchial epithelial IB3-1 cells. *A*, Quantitative expression of PLCB3 mRNA by quantified real-time PCR after transfection with PLCB3 siRNA or scrambled oligonucleotides sequences 1 and 2 for 24 h and 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 PLCB3 protein in IB3-1 cells transfected with scrambled oligonucleotide sequence 1 (*B*) or PLCB3 siRNA oligonucleotide sequence, in the presence of primary anti-PLCB3 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 PLCB3 protein of IB3-1 cells treated with scrambled versus PLCB3 siRNA oligonucleotides. Percentual reduction of 36.8 ± 6.6 of the fluorescent signal of 10 cells treated with PLCB3 siRNA and 10 cells treated with scrambled oligonucleotides, respectively. *F*, Quantitative expression of IL-8 mRNA after transfection with PLCB3 siRNA sequences 1 and 2 or scrambled oligonucleotides in IB3-1 cells as described for *A*. Mean ± SEM of five independent experiments performed in duplicate. *G*, Effect of PLCB3 siRNA (sequence 1) on IL-8 transcription induced by flagellin (10 μg/ml) and TNF-α (50 ng/ml). Mean ± SEM of three independent experiments performed in duplicate. *H*, Effect of PAO1 (100 CFU/cell), flagellin (10 μg/ml), and TNF-α (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. *I*, Effect of PAO1 (100 CFU/cell) in IB3-1 cells transfected with PLCB3 siRNA (sequences 1 or 2) on ICAM-1, growth-related oncogene γ, IL-6, and TNF-α mRNA transcription. Mean ± SEM of three independent experiments performed in duplicate.

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Microscopic analysis of PKC translocation

Images of PKC translocation were recorded using a digital imaging system based on a Zeiss Axiovert 200 fluorescence microscope. The data were acquired and processed using the MetaMorph analysis program (Universal Imaging). For computational deblurring, a stack of images through the z plane was acquired (200 ms/image; 20 plans 0.5 μm apart) and processed using the EPR software developed by the Biomedical Imaging group of the University of Massachusetts Medical School (Worcester, MA). Microscope analysis was performed 36 h after transfection. The medium was changed from LHC-8 basal medium plus 5% FCS to KRB. *P. aeruginosa* strain, and other drugs (400 nM phorbol ester PMA and 3 U/ml apyrase, respectively) were added to the buffer, as shown in the figures. The recruitment of the kinases are represented as plasma membrane translocation of different PKC–GFP chimeras, expressed as the increase in fluorescence ratio with respect to time 0 (calculated as the ratio of plasma membrane and cytosol average intracellular fluorescence, obtained from multiple regions inside the cytosol and on the cell membrane, measured on single cell). The graphs (Fig. 5) indicate the levels of PKC translocation, expressed as the fluorescence ratio, to plasma membrane (for PKCα, PKCβ, and PKCδ) or nucleus (for PKCζ), on the average of cytosolic fluorescence intensity.

Statistical methods

For association between disease and single SNPs, test for allelic association (which compares frequencies of alleles in cases versus controls) have been used. For these analyses, permutation tests (i.e., permuting the phenotypes) have been conducted to account for the large numbers of tests. Our analyses have been performed using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) and R (http://www.r-project.org/). After validation of Illumina genotype screening by TaqMan assay, allelic associations have been tested with χ^2 test. The Student *t* test has been applied to the experiments performed in human bronchial epithelial cells with statistical significance at $*p < 0.05$ or $**p < 0.01$ levels.

Results

Association of PLCB3 *gene with the progression of lung disease in CF*

A panel of genes of the innate immunity has been chosen as candidate modulators of the proinflammatory signaling elicited by *P. aeruginosa* in bronchial epithelial cells (Supplemental Table I). They included 135 genes, such as those encoding chemokines and adhesion molecules, pattern-recognition receptors sensing the presence of bacteria in the lumen of the conductive airways and several components of the signaling network acting downstream the pattern-recognition receptors. To choose in each gene those allelic variants most probably contributing to changes in expression or function of the encoded proteins, because of the large series of SNPs reported for each of these genes, first, we selected all those SNPs resulting in nonsynonymous changes in the coding regions and, second, those TagSNPs in intronic, 3′- and 5′-untranslated regions that mark the haploblocks composed of several single SNPs, for a total of 721 SNPs, as listed in the Supplemental Table II. DNA samples were from a representative cohort of 508 CF patients selected by the North American GMSG and classified as having either severe ($n = 200$) or mild ($n = 308$) progression of lung disease, according to the criteria described previously (15). Genotyping the 721 SNPs has been performed first by using Illumina platforms with individual 508 DNA samples. The top-ranking group of SNPs showing significant genotype-phenotype association was subsequently validated with TaqMan probe assays. By ranking the association between variant alleles and the severe or mild groups of CF patients, 26 SNPs showed a statistical significance with $p < 0.05$, as calculated by permutation test (Supplemental Table III). Interestingly, within the top-ranking list of the 721 SNPs, 5 of them were variants of PLC isoforms β, namely a nonsynonymous SNP of the PLC isoform β3 gene (PLCB3), three intronic Tag SNPs of the PLC isoform β1 gene (PLCB1), and one intronic Tag SNP of the PLC isoform β4 gene (PLCB4). The C2534T variant (rs35169799) of the PLCB3 gene was found on top of the rank of the 721 SNPs. The minor allele T was found significantly associated with the milder CF phenotype group (χ^2 test value = 8.01, permutation test; $p = 0.0046$) (Table I). Milder CF patients were associated with the minor 2534T allele with odds ratio = 2.297 (95 confidence interval = 1.27–4.14), as shown in Table II. The C2534T variant encodes a nonsynonymous serine to leucine change in the amino acid residue at position 845, which is localized in the C-terminal tail in close proximity to the C2 domain of the PLCB3 protein (23, 24). These two domains are known to be involved in the interaction with $G\alpha_{q/11}$ heterotrimeric GTPase protein and in the anchoring of PLCB3 to the plasma membrane through salt-like Ca^{2+} bonds, respectively (23, 24).

Silencing PLCB3 reduces the expression of IL-8 chemokine

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 (24). Although human bronchial epithelial cells express, albeit at different levels, the transcripts of all the PLC β isoforms, (Supplemental Figs. 1, 2), we focused our attention on PLCB3, because it is the most highly expressed within the β isoforms, and it is the top-ranking gene associated with clinical

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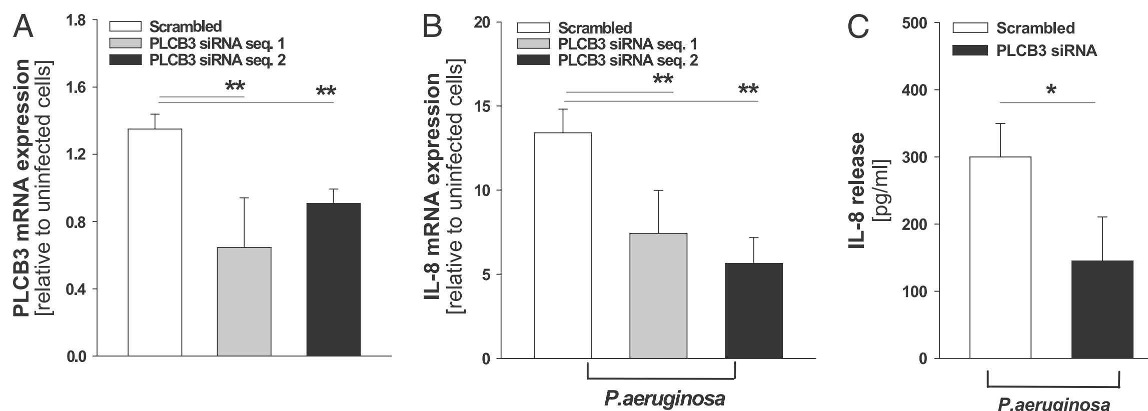

FIGURE 2. Silencing PLCB3 reduces *P. aeruginosa*-dependent expression and release of IL-8 in polarized human bronchial CF CuFi-1 cells. *A*, Quantitative expression of PLCB3 mRNA by quantified real-time PCR after transfection with PLCB3 siRNA sequences 1 and 2 or scrambled oligonucleotides for 24 h 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 four independent experiments performed in duplicate. *B*, Levels of IL-8 mRNA in the same experiments reported in *A*. *C*, Release of IL-8 protein in the same experiments reported in *A* with a PLCB3 siRNA sequence 1. IL-8 protein concentration refers to that collected from the apical side of the Transwell insert filter. Mean ± SEM of three separate experiments performed in duplicate.

phenotype. 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*. The bronchial cell lines used were all homozygous for the major allele of the PLCB3 C2534T variant (S845), as reported in Supplemental Table V. Transfection of two different duplexes PLCB3 siRNA reduced significantly, albeit partially, the levels of expression of PLCB3 mRNA (Fig. 1*A*) and protein (Fig. 1*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 (Supplemental Fig. 1). 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 IB3-1 cells exposed to *P. aeruginosa* (Fig. 1*F*, 1*H*) 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 TNF-α–dependent IL-8 expression (Fig. 1*G*, 1*H*), 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*

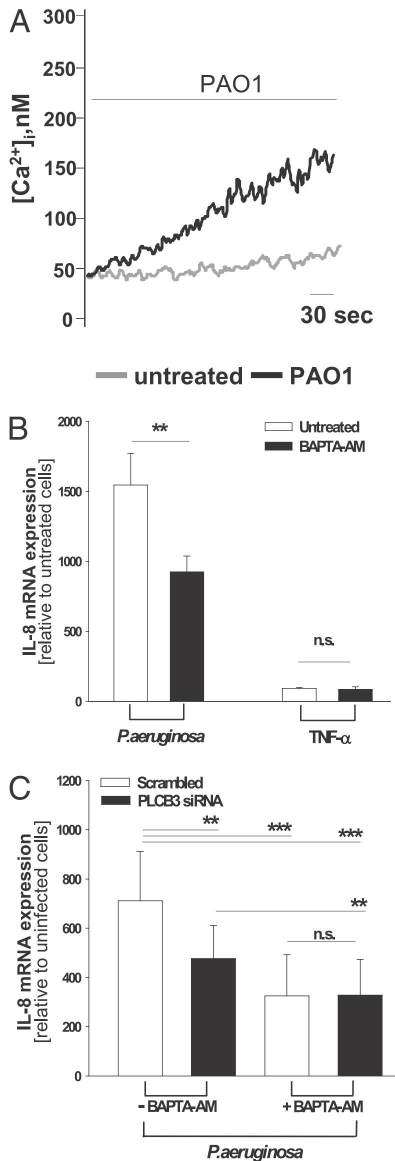

FIGURE 3. Intracellular calcium chelator BAPTA inhibits the transcription of IL-8 mRNA induced by *P. aeruginosa*. *A*, Cytosolic free Ca^{2+} concentration as measured with Fura-2/AM assay in IB3-1 cells exposed to PAO1 (black trace) or with tissue culture medium alone (gray trace). Traces are representative of eight independent experiments. *B*, The intracellular Ca^{2+}-chelator BAPTA-AM (5 mM) was preincubated with IB3-1 cells for 30 min at 37°C to obtain diesterification of the acetoxymethyl ester. Cells were exposed to PAO1 (100 CFU/cell) or TNF-α (50 ng/ml) for an additional 4 h before extraction of total RNA and quantified real-time PCR. Data are mean ± SEM of three separate experiments performed in duplicate. *C*, After preincubating IB3-1 cells with PLCB3 siRNA sequence 1 or scrambled oligonucleotides for 24 h, BAPTA-AM was added for 30 min at 37°C to obtain intracellular partition and diesterification of the acetoxymethy lester. Infection with PAO1 strain was performed for an additional 4 h, total RNA was extracted, and IL-8 mRNA was quantified by quantified real-time PCR. Data are mean ± SEM of four separate experiments performed in duplicate.

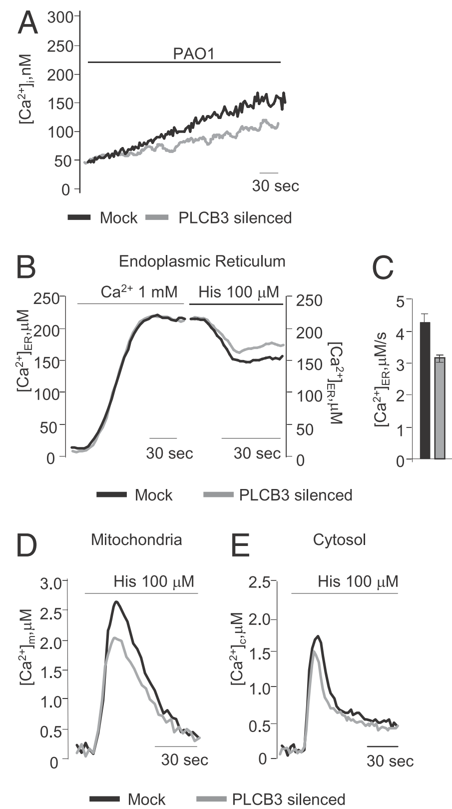

FIGURE 4. Silencing PLCB3 affects the release of calcium from the ER. IB3-1 cells were pretreated with PLCB3 siRNA sequence 1 (gray traces) or scrambled (black traces) oligonucleotides, respectively, for 24 h before measuring intracellular Ca^{2+} transients. *A*, intracellular free $[Ca^{2+}]_i$ with Fura-2/AM assay after addition of PAO1 (100 CFU/cell), $n = 8$. *B*, ER calcium concentration ($[Ca^{2+}]_{er}$) by ER-targeted aequorin in the presence of 1 mM extracellular calcium or with the addition of histamine (His, 100 mM) for the time indicated. Luminal ER Ca^{2+} concentration measurement was 212 ± 9.52 μM in mock versus 215 ± 5.51 μM in PLCB3 silenced cells, $n = 10$. *C*, Variation of ER calcium release kinetic upon histamine addition expressed as mM/s: in mock 4.26 ± 0.33 versus 3.31 ± 0.13, $p < 0.05$, $n = 10$ PLCB3-silenced cells, respectively. *D*, Mitochondrial calcium concentration ($[Ca^{2+}]_m$) by mitochondrial-targeted aequorin with the addition of histamine, $[Ca^{2+}]_m$: mock 2.58 ± 0.19 μM versus PLCB3 silenced 2.06 ± 0.23 μM, $n = 10$. *E*, cytosolic calcium concentration ($[Ca^{2+}]_c$) by native aequorin with the addition of histamine, $[Ca^{2+}]_c$: mock 1.65 ± 0.13 μM versus PLCB3 silenced 1.50 ± 0.11 μM, $n = 10$.

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in bronchial epithelial cells, such as ICAM-1, growth-related oncogene γ, IL-6, and TNF-α, is not reduced (Fig. 1*I*). The reduction of IL-8 expression after silencing PLCB3 was confirmed in the CF human bronchial epithelial cell lines CuFi-1 grown polarized on Transwell filters, after exposure to *P. aeruginosa* on the apical side (Fig. 2). 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*.

PLCB3 is implicated in Ca^{2+}-related signaling and activation of PKCα and β

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 (IP_3), which in turn activate intracellular calcium transients (23, 24). Notably, it has been recently shown that PLCB3 is a critical regulator of intracellular Ca^{2+} in murine macrophages (25). 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. 3*A*). Conversely, buffering the increase of $[Ca^{2+}]_c$ with the intracellular Ca^{2+}-chelator BAPTA reduced significantly the induction of IL-8 mRNA (Fig. 3*B*), as already reported by other investigators (12, 26). BAPTA further reduced the IL-8 mRNA expression in cells silenced for PLCB3 (Fig. 3*C*), but in BAPTA-loaded cells PLCB3, silencing did not reduce IL-8 expression below the level detected in cells treated with the control siRNA. These findings suggest two set of conclusions. First, that in our experimental model, calcium signaling is not completely mediated by PLCB3. Notably, IB3-1 and CuFi-1 bronchial epithelial cells express different PLC isoforms besides the β ones, in particular PLCγ_2, PLCδ_3, and PLCε_1, which could participate in activation of calcium transients (Supplemental Fig. 2). 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 role of PLCB3 in intracellular Ca^{2+} signaling upon interaction of *P. aeruginosa* with bronchial epithelial cells was

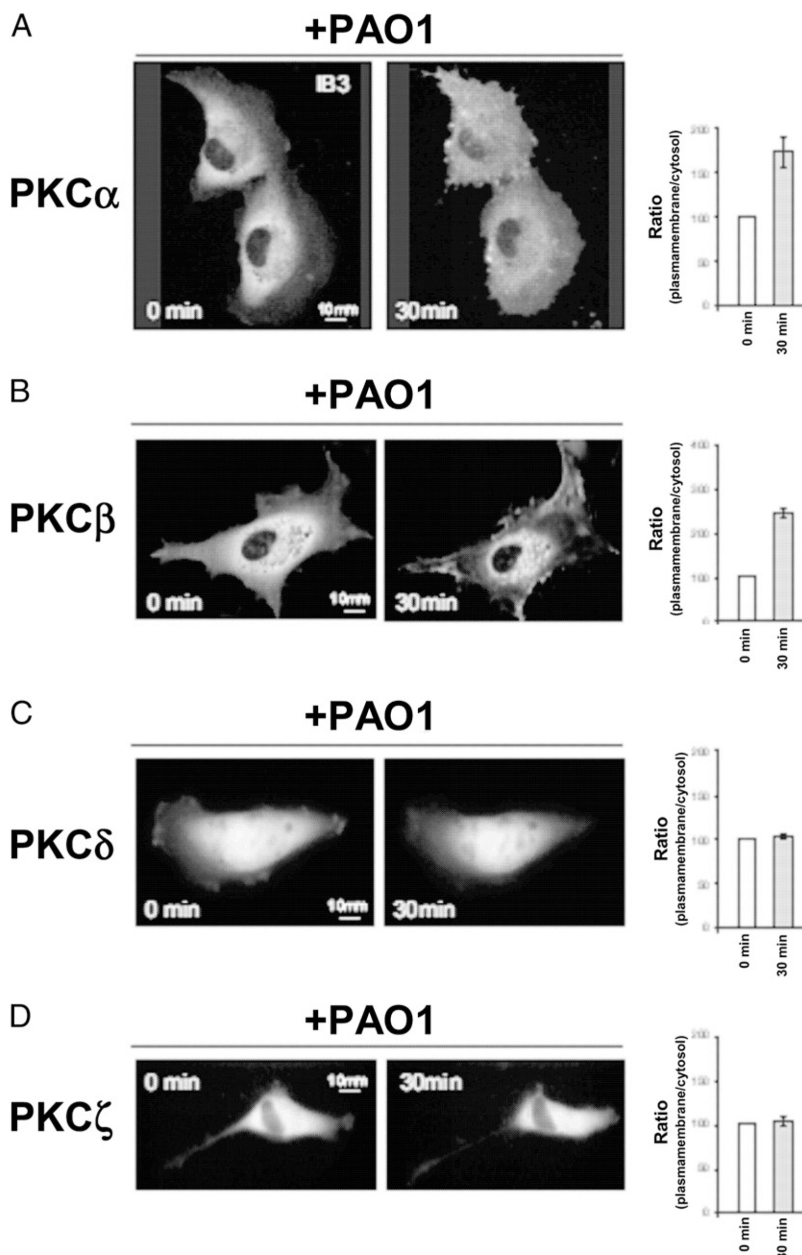

FIGURE 5. *P. aeruginosa* activates conventional PKC isoforms α and β. Fluorescence signal of GFP-tagged PKC isoforms *A*, α, *B*, β, *C*, δ and *D*, ζ before and after 30 min from the addition of PAO1 (100 CFU/cell) to IB3-1 cells. Histograms indicate the intracellular localization of PKC isoforms, as increase in fluorescence ratio with respect to time 0 (ratio of translocation from cytosol to plasma membrane or nucleus) as mean ± SEM for at least seven single cells (see *Materials and Methods*). Averaging of percent ratio: PKCα +76% ± 11.6; PKCβ +114% ± 11.4; PKCδ +5% ± 0.8; and PKCζ +4% ± 1.0.

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studied in some details. The $[Ca^{2+}]_c$ increase promoted by *P. aeruginosa* was reduced in IB3-1 cells preincubated with PLCB3 siRNA (Fig. 4*A*), although not completely, possibly as a result of a parallel partial reduction of PLCB3 expression. By inducing $[Ca^{2+}]_c$ transients with histamine (causing the generation of inositol 1,4,5 trisphosphate and the consequent release of Ca^{2+} from the ER) in IB3-1 cells expressing the intracellular organelle-targeted Ca^{2+}-sensitive aequorins, no difference in ER Ca^{2+} uptake was observed between silenced PLCB3 and control cells. Indeed, both groups of cells showed very similar luminal ER Ca^{2+} concentrations ($[Ca^{2+}]_{er}$) at rest (see left part of Fig. 4*B*). On the contrary, we found that silencing PLCB3 reduces the release of Ca^{2+} from the ER (right part of Fig. 4*B*, 4*C*). Accordingly, the transient rises of $[Ca^{2+}]_c$ and in the mitochondrial matrix ($[Ca^{2+}]_m$) elicited by the agonist were smaller in silenced PLCB3 cells compared with control cells (Fig. 4*D*, 4*E*). Signals that elevate intracellular Ca^{2+} and DAG, such as those induced by PLCs, are known to activate conventional PKC isoforms (27). Activation of PKC was studied by observing the translocation of the recombinant GFP-tagged Ca^{2+}-dependent conventional PKC isoforms α and β to the plasma membrane in IB3-1 cells infected with *P. aeruginosa*. Bacterial exposure induced activation of the conventional PKC isoforms α and β (Fig. 5*A*, 5*B*), but not of the novel δ isoform (Fig. 5*C*) and of the atypical ζ isoform (Fig. 5*D*), the most striking effect being observed for PKC β, where an average 56.1% of the cells showed membrane translocation. By silencing PLCB3 expression, the activation of PKC β was abrogated in the majority of the IB3-1 cells (Fig. 6*A*, 6*B*), a residual PKC β activation being observed only in an average 12.0% of cells exposed to *P. aeruginosa*. The role of PKC activation in the *P. aeruginosa*- but not TNF-α–dependent signaling of our experimental model has been also confirmed by the effect of the broad PKC inhibitor BIM-I (Fig. 6*C*). It has been previously shown that *P. aeruginosa* PAO1 strain activates a Ca^{2+}-dependent activation of the transcription factor NF-κB, which is

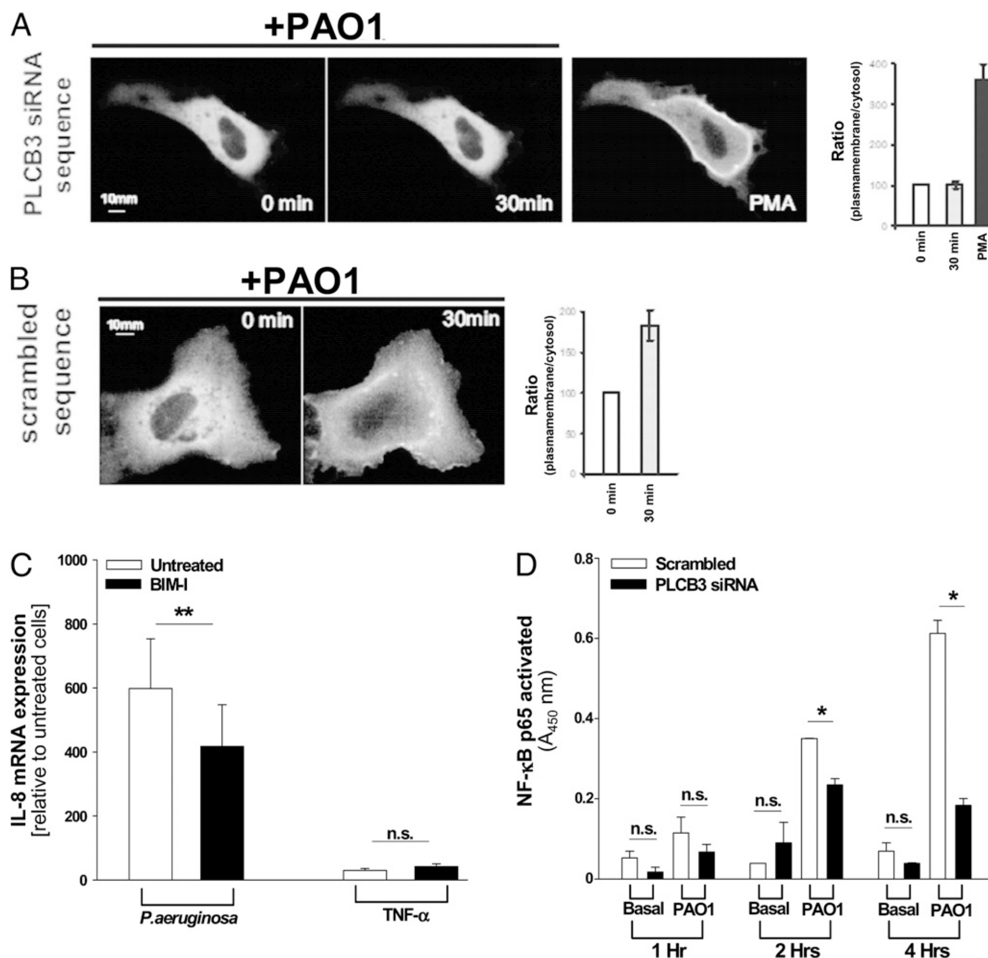

FIGURE 6. Silencing PLCB3 reduces the activation of PKC-β and of NF-κB p65 induced by *P. aeruginosa*. IB3-1 cells were transfected with PLCB3 siRNA sequence 1 (*A*) or scrambled oligonucleotides 24 h before the addition of PAO1 (100 CFU/cell) (*B*). Images refer to representative experiments of expression of GFP-tagged PKC isoform β before and 30 min after exposure to PAO1. The PKC activator PMA (400 nM) was added to cells not responding to PAO1-dependent translocation as an internal control. Translocation of PKCβ from cytosol to membrane was observed in 56.1% of the IB3-1 cells transfected with scrambled sequence (on 57 scrambled cells 32 cells presented PKCβ traslocation) and in 12.0% (on 25 PLCB3 siRNA cells only 3 cells presented PKCβ traslocation) of cells transfected with PLCB3 siRNA sequence. The histograms indicate the intracellular localization of PKCβ, expressed as ratio of translocation from cytosol to plasma membrane. Averaging of percent ratio in silenced cells: after 30 min to PAO +1% ± 10.3 and after treatment with PMA +275% ± 21.5 respect to time 0; in mock cells: after 30 min to PAO +78% ± 24.6. *C*, IB3-1 cells were preincubated with the PKCs inhibitor BIM-I (2 μM) for 30 min and infected with PAO1 or stimulated with TNF-α for an additional 4 h, before quantitation of IL-8 mRNA by quantified real-time PCR. Data are mean ± SEM of three separate experiments performed in duplicate. *D*, 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.

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critical in the regulation of IL-8 gene transcription also in human airway epithelial cells (26). 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 h chosen to measure IL-8 mRNA levels. We confirm that PAO1 progressively activates NF-κB and that silencing PLCB3 significantly reduces the *P. aeruginosa*-induced activation of NF-κB p65 (Fig. 6*D*). Collectively, these results indicate that PLCB3 plays a relevant role in triggering free calcium transients induced by *P. aeruginosa* in human bronchial epithelial cells, thus regulating the activation of the PKCs α and β and of the nuclear transcription factor NF-κB.

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

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 (11, 12). Interestingly, PLCB3 has been shown to be selectively coupled to the P2Y2 receptor-dependent activation of intracellular Ca^{2+} transients in recombinant CHO cells (28). The role of different ligands on the expression of IL-8 has been tested preliminarly. 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 TNF-α (Fig. 7*A*). 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. 7*A*), 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. 7*A*). Because ATP-dependent induction of IL-8 in bronchial epithelial cells has been previously described only in association with ligands activating TLRs (29), 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 flagellin-induced one (Fig. 7*B*). 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. Preincubation of IB3-1 cells with apyrase before exposure to *P. aeruginosa* PAO1 strain, reduced the sustained increase of $[Ca^{2+}]_c$ (Fig. 8*A*), the activation of PKC isoform β (Fig. 8*B*, 8*C*) and, more relevantly, the IL-8 mRNA transcription and release of IL-8 protein (Fig. 8*D*, 8*E*). Interestingly, apyrase does not further reduce the *P. aeruginosa*-dependent IL-8 expression (Fig. 8*F*), 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 proinflammatory signaling leading to the expression and secretion of IL-8 in our model system.

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, and therefore anti-inflammatory drugs are included within the therapeutic pipeline of the innovative therapies to treat or cure CF lung disease (1). The identification of novel molecular targets in the proinflammatory signaling, which is orchestrated first by the bronchial epithelial cells on the surface of the conductive airways, is presently of paramount importance (2).

To prioritize the relevance of specific molecules within the large series of different receptors, kinases, phosphatases, phospholipases, and adaptors regulating the expression of inflammatory genes, we chose to apply an association study between genes of the innate immunity and the clinical progression of CF lung disease, by a genetic ranking approach. In this respect, the aim of our genetic analysis was not to find the strongest modifier gene(s) for CF lung disease, a task that should be presently pursued by Genome-Wide Association Studies in much larger cohorts of affected individuals, but to obtain hints on the relative clinical relevance within a list of genes selected in a family with homogeneous pathophysiological role (e.g., the signaling network of the innate immune response to bacteria in respiratory epithelial cells). Therefore, although the association of PLCB3 with clinical progression of lung disease that we found here is quite modest, our genetic ranking approach allowed us to focus on PLCB3 to demonstrate its role in regulating the expression of IL-8 elicited by *P. aeruginosa* in bronchial epithelial cells.

PLCs have been shown to be implicated in different cellular responses, due to their role in intracellular calcium homeostasis (for review, see Ref. 24). In particular, it was initially proposed that

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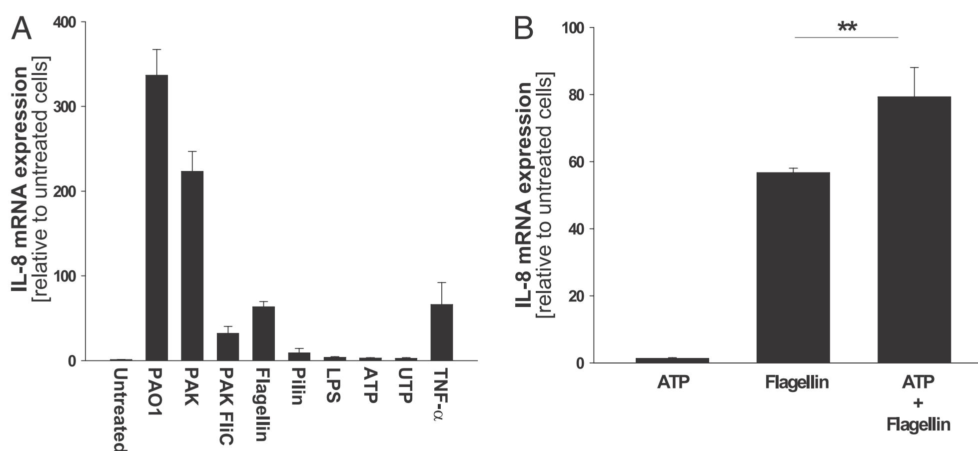

FIGURE 7. ATP is not sufficient to activate IL-8 mRNA transcription but acts in synergy with TLR-dependent signaling. *A*, IB3-1 cells were exposed to the *P. aeruginosa* laboratory strains PAO1, PAK, or PAK FliC (100 CFU/cell), to flagellin (10 mg/ml) and pilin (10 mg/ml) purified from PAK recombinant cells, to LPS (10 mg/ml), to ATP and UTP (1 mM), or to TNF-α (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.

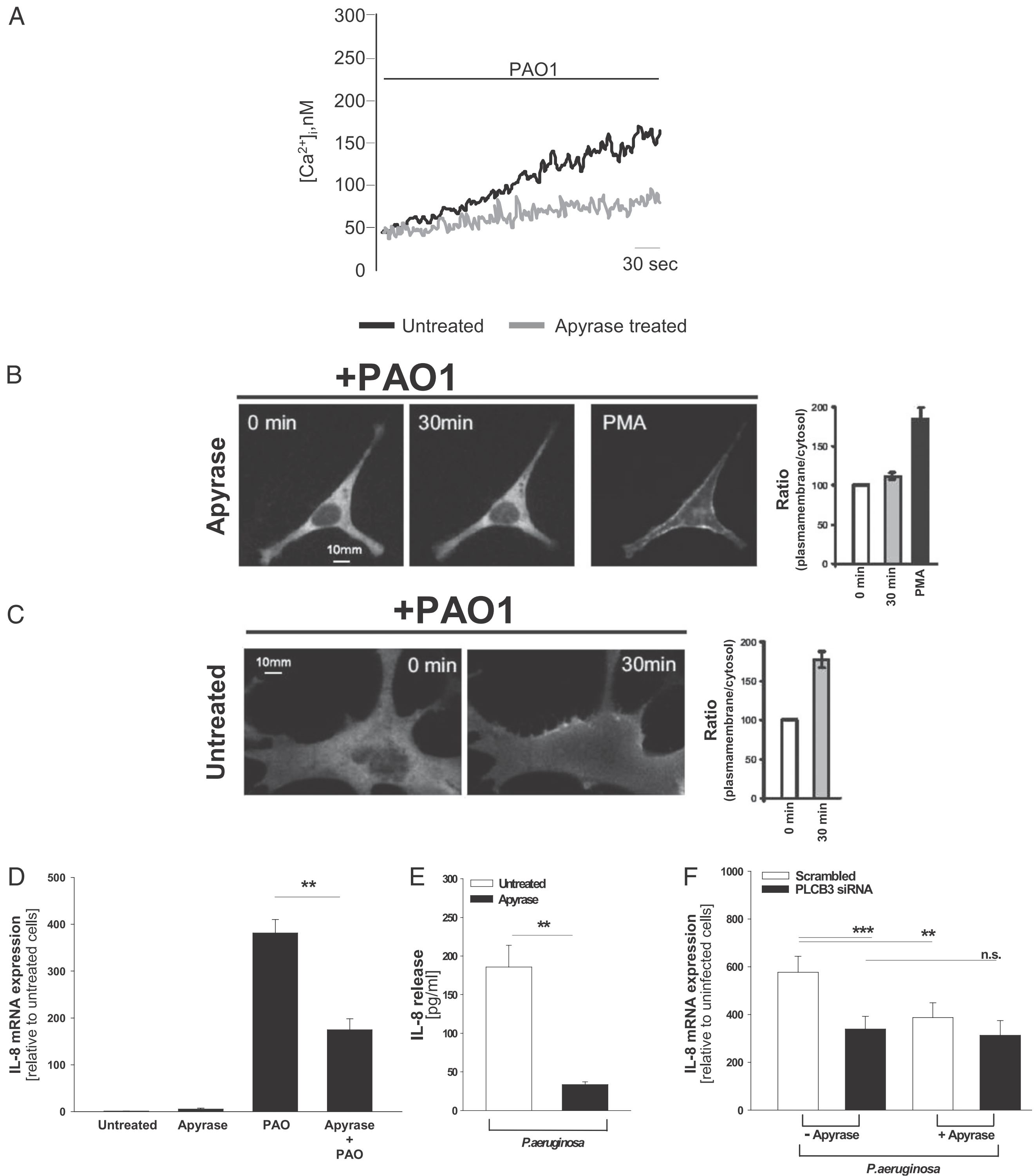

FIGURE 8. The ectonucleotidase apyrase affects calcium signaling and IL-8 expression induced by *P. aeruginosa*. The ectonucleotidase apyrase (3 U/ml) was added to IB3-1 cells 2 h before and again together with the addition of PAO1 for an additional 4 h. *A*, Cytosolic Ca^{2+} transients by Fura-2/AM assay in IB3-1 cells exposed to PAO1 treated with apyrase (gray trace) or solvent alone (untreated, black trace), $n = 8$. Activation of PKC isoform β in IB3-1 cells exposed to PAO1 treated with either apyrase (*B*) or solvent alone (untreated) (*C*). The PKC activator PMA (400 nM), a Ca^{2+}-independent DAG-like agonist, was added to cells not responding to PAO1-dependent translocation, as an internal control. Translocation of PKCβ from cytosol to membrane was observed in 56.1% of the IB3-1 cells mock treated and in 25.9% of cells treated with apyrase (on 27 pretreated apyrase cells only 7 cells presented PKCβ traslocation, after PAO1 exposition). Histograms show the intracellular localization of PKCβ isoform: averaging of percent ratio in apyrase-treated cells: after 30 min to PAO +8% ± 2.6 and after PMA +89% ± 16.0 respect to time 0; in untreated cells: after 30 min to PAO +61% ± 15.2. *D*, IL-8 mRNA transcription was quantitated in IB3-1 cells exposed to PAO1 treated with either apyrase or solvent alone. *E*, As for *C*, the release of IL-8 protein was measured by ELISA in IB3-1 cells treated with apyrase or solvent alone. Mean ± SEM of three independent experiments performed in duplicate. *F*, Apyrase (3 IU/ml) was incubated for 2 h at 37°C after a preincubation of 24 h with PLCB3 siRNA sequence 1 or scrambled oligonucleotides in IB3-1 cells. An additional 4-h infection with PAO1 strain was performed, and IL-8 transcription was quantified by quantified real-time PCR. Data are mean ± SEM of three separate experiments performed in duplicate.

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the PLCB3 isoform is involved in signal transduction triggered by hormones, growth factors, and neurotransmitters (30). As far as its role in inflammatory processes is concerned, PLCB3 has been investigated in the context of leukocyte chemotaxis (31). PLCB2- and PLCB3-dependent rise in intracellular calcium has been shown to regulate T lymphocyte chemotaxis (32). However, PLCB3-deficient neutrophils or macrophages, in which calcium transients were blunted, did not show reduction of chemotactic activity or migration (33, 34), suggesting that PLCB3 is required for the chemotaxis of T lymphocytes but not neutrophils or macrophages. 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 PLCB1 and PLCB4, PLCG2, PLCD3, and PLCE1, besides PLCB3 (Supplemental Fig. 2). How can we explain the specific contribution of PLCB3 in the proinflammatory signaling induced by *P. aeruginosa* in bronchial epithelial cells? Activation of γ and ε isoforms is known to be dependent on tyrosine-kinase–coupled receptors, of the δ isoforms on elevation of cytosolic calcium, of the β isoforms on seven-membrane spanning domain receptors through GTP-binding proteins (24). 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 (35). 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 (11, 12). Our results with the ectonucleotidase apyrase confirm a role of extracellular ATP in the $[Ca^{2+}]_c$ increase and report, to our knowledge, for the first time the activation of PKCs α and β by *P. aeruginosa* in an ATP-dependent manner (Fig. 8). Moreover, silencing experiments reducing cytosolic calcium increase and activation of PKC β confirm the involvement of PLCB3 in the Ca^{2+} pathway activated by *P. aeruginosa* (Figs. 4, 6). Thus, these results are consistent with the coupling of P2Y2 purinergic receptors with PLCB3, which is known to involve $G\alpha_{q/11}$ heterotrimeric GTPase protein (28, 35). Because PLCB1 and PLCB4 are also able to interact with seven-membrane spanning receptors, we cannot definitely restrict to PLCB3 the role to modulate *P. aeruginosa*-dependent calcium transients in bronchial epithelial cells. Interestingly, in murine macrophages, it has been shown that UDP can activate cytosolic calcium rise through purinergic receptors by using PLCB3 in parallel with PLCB4, whereas other ligands, such as C5a, use predominantly PLCB3 (25). Because, in our experimental model, selective silencing of PLCB3 resulted in a consistent, but not total, reduction of intracellular Ca^{2+}-dependent expression of IL-8, we cannot exclude a partial contribution of the other PLC β isoforms.

Silencing PLCB3 reduces only partially the *P. aeruginosa*-dependent expression of IL-8 (Figs. 1, 2). This is not surprising

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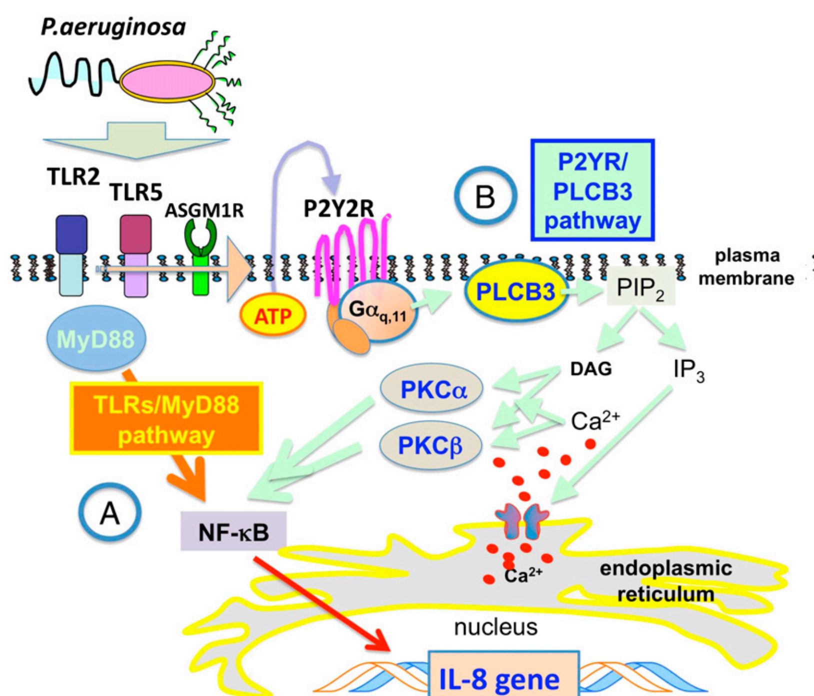

FIGURE 9. Model of cooperation of PLCB3 in the *P. aeruginosa*-dependent signaling in bronchial epithelial cells. The illustration depicts the signaling pathways elicited by TLRs/MyD88 (*A*) and P2Y2R/PLCB3 (*B*), based on previous reports from other investigators and the results presented in this paper. *A*, Binding of *P. aeruginosa* surface components (flagellin and pilin) with TLR5 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 AP-1 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. *B*, Extracellular ATP binds to P2Y2R that activates PLCB3 through the $G\alpha_{q,11}$ heterotrimeric GTPase protein. By degrading phosphatidylinositol 4,5-biphosphate, PLCB3 promotes IP_3 release and DAG formation. IP_3 triggers Ca^{2+} release from intracellular stores, and the rise of $[Ca^{2+}]_c$, together with DAG, promotes intracellular translocation of conventional PKC isoforms α and β, which ultimately cooperate in activation of NF-κB. The P2Y2R/PLCB3 pathway is not sufficient to induce IL-8 transcription by itself but strongly act in synergy with the TLR/MyD88 signaling cascade. CHOP, CREB-homologous protein transcription factor.

in light of the partial efficiency of PLCB3 silencing and of the evidence that *P. aeruginosa* activates the inflammatory response because of its capability to interact with multiple receptors, including TLRs and ASGM1Rs (36). 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. 3), 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.7), as previously observed by other investigators (29). 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. 7), 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 signaling. As 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 NF-κB (Fig. 6), which plays a critical role in the induction of IL-8 transcription (19, 29). On the basis of these and previous findings (11, 12), we conclude that in the CF airway tract chronically infected with *P. aeruginosa*, the Ca^{2+}-dependent pathway induced by the release of nucleotides activates, through binding to P2Y2R, PLCB3, and amplifies the innate defense signaling based on TLRs and ASGM1R. The illustration reported in Fig. 9 summarizes our working hypothesis.

The biological importance of intracellular calcium homeostasis in bronchial epithelial cells in the CF lung disease has been widely debated. For instance, it has been proposed that the defective chloride transport due to the mutated CFTR protein can be overcome by activating alternative Ca^{2+}-dependent chloride channels, such as those recently identified (37, 38). Independent of the presence of a constitutive inflammation in CF bronchial epithelial cells, in which alteration of Ca^{2+} homeostasis might not play a role (38), when chronic bacterial infection intervenes in the first decades of life, the Ca^{2+} signaling in the bronchial epithelial cells greatly amplifies the recruitment and transepithelial migration of leukocytes, first of all polymorphonuclear neutrophils (39), that are considered unwilling actors of the progressive destruction of CF lung tissue (2). Thus, in the advanced stages of CF lung disease characterized by chronic bacterial infection, intracellular Ca^{2+} signals are known to induce ER Ca^{2+} store expansion leading to an overt hyper-inflammatory phenotype, with inappropriate release of chemokines and cytokines, that amplifies the recruitment and activation of leukocytes (40).

The inexorable decline of the lung function in patients affected by CF is presently faced with different approaches directed toward the mutated CFTR protein, alternative chloride channels, novel anti-inflammatory and anti-infective drugs (1). Consensus is growing on that effective causative therapies, when available, should be initiated as soon as possible, to prevent the onset of pulmonary complications. However, in patients who already developed bacterial infection, a novel anti-inflammatory approach directed toward the specific pathophysiology of infected CF lungs should be taken into consideration. In this respect, PLCB3, which relevantly regulates the extracellular nucleotide-cytosolic Ca^{2+} signaling axis potentiating the TLRs signaling cascade, represents a novel pharmacological target to attenuate the excessive recruitment of neutrophils without completely abolishing the inflammatory response.

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Disclosures

The authors have no financial conflicts of interest.

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