ORIGINAL RESEARCH

Transient Receptor Potential Ankyrin 1 Channels Modulate Inflammatory Response in Respiratory Cells from Patients with Cystic Fibrosis

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Abstract

Pseudomonas aeruginosa colonization, prominent inflammation with massive expression of the neutrophil chemokine IL-8, and luminal infiltrates of neutrophils are hallmarks of chronic lung disease in patients with cystic fibrosis (CF). The nociceptive transient receptor potential ankyrin (TRPA) 1 calcium channels have been recently found to be involved in nonneurogenic inflammation. Here, we investigate the role of TRPA1 in CF respiratory inflammatory models *in vitro*. Expression of TRPA1 was evaluated in CF lung tissue sections and cells by immunohistochemistry and immunofluorescence. Epithelial cell lines (A549, IB3-1, CuFi-1, CFBE41o^{-}) and primary cells from patients with CF were used to: (*1*) check TRPA1 function modulation, by Fura-2 calcium imaging; (*2*) down-modulate TRPA1 function and expression, by pharmacological inhibitors (HC-030031 and A-967079) and small interfering RNA silencing; and (*3*) assess the effect of TRPA1 down-modulation on expression and release of cytokines upon exposure to proinflammatory challenges, by quantitative RT-PCR and 27-protein Bioplex assay. TRPA1 channels are expressed in the CF pseudostratified columnar epithelium facing the bronchial lumina exposed to bacteria, where IL-8 is coexpressed. Inhibition of TRPA1 expression results in a relevant reduction of release of several cytokines, including IL-8 and the proinflammatory cytokines IL-1β and TNF-α, in CF primary bronchial epithelial cells exposed to *P. aeruginosa* and to the supernatant of mucopurulent material derived from the chronically infected airways of patients with CF. In conclusion, TRPA1 channels are involved in regulating the extent of airway inflammation driven by CF bronchial epithelial cells.

Keywords: cystic fibrosis; transient receptor potential ankyrin 1; IL-8; *Pseudomonas aeruginosa*; inflammation

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Clinical Relevance

Innovative antiinflammatory therapy tailored to the specific pathophysiology of cystic fibrosis (CF) lung disease, an unmet need, will help reduce the progressive tissue damage affecting these patients. The present data outline how transient receptor potential ankyrin 1 channels can represent a potential molecular target to be investigated by repositioning to CF the most recent pharmaceutical inhibitors that are presently under investigation in preclinical and clinical trials.

Although cystic fibrosis (CF) is a multiple-organ disease, the lung pathology is the major cause of morbidity and mortality (for review, *see* Ref. 1). CF transmembrane conductance regulator (CFTR) dysfunction, which leads to altered ion transport in bronchial epithelia, causes dehydration of the airway surface liquid, impairing mucociliary clearance (2–4). In this condition, mucus accumulates on the bronchial surface, promoting recurrent bacterial infection and ultimately chronic colonization by *Pseudomonas aeruginosa*. Bacterial–host interactions amplify the release of cytokines and the exuberant recruitment of polymorphonuclear neutrophils in the bronchial lumina, the latter being mainly driven by the potent neutrophil chemokine, IL-8 (5). In this site, neutrophils are unable to clear infection and their massive presence ultimately contributes to lung tissue damage because of the release of proteases (e.g., elastase) and reactive oxygen species (6, 7). To limit the side effects of the excessive lung inflammation in patients with CF, multitarget antiinflammatory drugs, such as ibuprofen and corticosteroids, are currently used in daily clinical practice. Their limited efficacy and adverse effects stress the need for finding novel CF-tailored antiinflammatory molecular targets and drugs (7) to be associated with the CFTR modulators (correctors and potentiators), and more effective antibacterial drugs in those adult patients with CF who already exhibit chronic lung infection and inflammation.

The transient receptor potential (TRP) ankyrin (TRPA) 1 (8), which belongs to the family of TRP channels (for review, *see* Ref. 9), was found to be expressed by a subset of nociceptors, where it conveys nociceptive signals and contributes to hyperalgesia in models of inflammatory and neuropathic pain (10). As regards inflammation, TRPA1 channels expressed by airway sensory nerves mediate the early inflammatory response to cigarette smoke in rodents (11), inflammatory cell infiltration, and hyperresponsiveness evoked by allergen exposure (12). Thus, neuronal TRPA1 has been proposed to contribute to the mechanism of chronic obstructive pulmonary disease and asthma. More recently, our laboratory and those of others have reported that mouse and human pulmonary cells express functional TRPA1, and that channel activation promotes the release of IL-8 (13). It is therefore possible that extraneuronal TRPA1 calcium-transporting channels cooperate with the neuronal channels to drive chronic inflammation. This is particularly evocative of a potential role in CF lung inflammation, as we previously found a synergy between intracellular calcium homeostasis and the *P. aeruginosa*–dependent proinflammatory signaling regulating IL-8 and IL-1β release in CF bronchial epithelial cells (BECs) (14, 15).

To verify the hypothesis that TRPA1 calcium channels are involved in CF respiratory inflammation, we first assessed the expression and localization of TRPA1 in CF lung tissue. We further modulated TRPA1 expression and function in CF BECs coexpressing TRPA1 and inflammatory cytokines upon exposure to CF-specific proinflammatory challenges.

Material and Methods

Tissue Collection

Formalin-fixed and paraffin-embedded bronchial sections of lung specimens (3- to 5-μm thick) from patients with CF were obtained from Gaslini Institute (Genova, Italy; Drs. L. Galietta and L. Ferrera). The use of human bronchi, obtained from patients undergoing lung transplant, was approved by the Ethics Committee of the Gaslini Institute, following the guidelines of the Italian Ministry of Health. Each patient provided written informed consent to the study using a form that was also approved by the Ethical Committee (approval no. 13). Sections of non-CF diseased individuals were purchased from U.S. Bio Corp. (Rockville, MD).

Immunohistochemical Staining

Immunohistochemical analysis was performed as previously described (13).

In Situ Hybridization

In situ hybridization assay was performed on serial sections of CF bronchi using the RNA scope 2.0 HD Reagent Kit Brown (catalog no. 310035) with the probes for Hs–IL-8 (cat no. 310381), Homo sapiens peptidylprolyl isomerase B (cyclophilin B) (PPIB) (positive control; catalog no. 313901), and dihydrodipicolinate reductase (DapB) (negative control; catalog no. 310043), according to the protocol provided by Advanced Cell Diagnostics (Hayward, CA). Serial tissue sections were scanned by using the D-sight 2.0 System (Menarini Diagnostics, Firenze, Italy).

Cell Culture

Human A549 alveolar type II–derived epithelial cells, IB3-1 cells (bronchial epithelial cell line from a CF patient with genotype F508del/W1282X, transformed with adenovirus 12/SV40), CuFi-1 cells (bronchial epithelial cell line from a CF patient with genotype F508del/F508del and transformed by reverse transcriptase component of telomerase, hTERT, and human papillomavirus type 16 E6 and E7 genes), CFBE41o^{-} cells (bronchial epithelial cell line from a CF patient with genotype F508del/F508del, immortalized with psVori plasmid) cells, and primary cultures derived from columnar epithelia of human bronchi, obtained from the American Tissue Culture Collection (Manassas, VA) or kindly donated by collaborators, were cultured using standard procedures (*see* the online supplement).

Calcium Imaging

Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were determined using the standardized method of wavelength ratio of 340:380 nm, recorded with a dynamic image analysis system (*see* the online supplement).

Immunofluorescence Assay and Confocal Analyses

Immunofluorescence experiments and confocal analyses were performed as described in the online supplement.

Transient Silencing of TRPA1 Channel Expression

Transient silencing was performed with TriFECTa RNAi Kit (Integrated DNA Technologies, Coralville, IA) accordingly to the manufacturer's instructions (*see* the online supplement).

Stimulation with *P. aeruginosa*, heat-killed laboratory strain PAO1 of *P. aeruginosa* (HKPAO) and supernatant of mucopurulent material (SMM)

Stimulation with PAO1, HKPAO, and SMM was performed as described in the online supplement.

Quantitative RT-PCR

For real-time PCR studies, cells were treated as described in the online supplement.

Bioplex Assay and Analysis

Cytokines released in culture medium were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA; *see* the online supplement).

Statistical Analysis

Data were analyzed using two-tailed Student's *t* test and ANOVA followed by Bonferroni's *post hoc* test for comparisons between multiple groups. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Differences were considered significant for *P* values less than 0.05.

Results

IL-8 Is Colocalized with TRPA1 in Bronchial Columnar Epithelial Cells of CF Lung

To test the hypothesis that TRPA1 calcium channels are involved in nonneurogenic lung inflammation in CF, we first assessed the localization of TRPA1 protein in the respiratory tissue of patients with CF undergoing lung transplantation, a condition characterized by advanced infection, inflammation, and lung pathology. Serial sections from different areas of the respiratory tract, including the surface columnar epithelia of the conductive airways, submucosal glands, and alveoli of lungs explanted from patients with CF, were analyzed to detect TRPA1 expression. Different lung areas were identified by staining sections with hematoxylin and eosin. Figure 1A shows surface bronchial mucosa with the pseudostratified epithelium and extensive submucosal infiltrates of inflammatory cells, typical of lungs from adult patients with CF with chronic bacterial infection and inflammation. Submucosal glands, both serosal and mucosal, are shown in Figure 1B. Alveolar areas are shown in Figure 1C. Expression of TRPA1 protein was evaluated by immunohistochemistry by brown peroxidase staining in separate 3- to

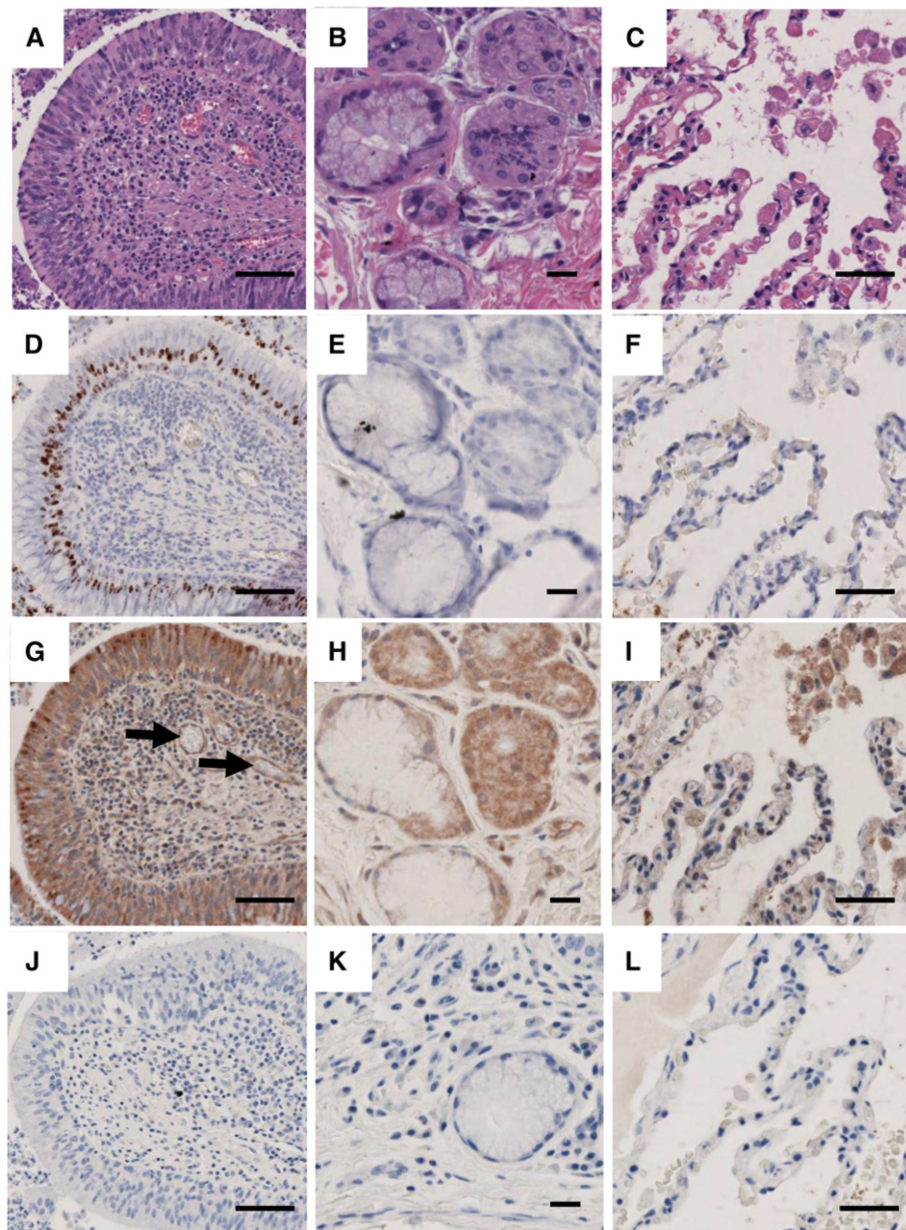

Figure 1. IL-8 is colocalized with transient receptor potential ankyrin (TRPA) 1 in bronchial columnar epithelial cells of cystic fibrosis (CF) lung. Different 3- to 5-μm serial sections of human CF bronchi were stained with hematoxylin and eosin (*A–C*) for IL-8 mRNA by *in situ* hybridization (*D–F*), TRPA1 protein by immunocytochemistry (*G–I*) and secondary antibody only (*J–L*). (*A*, *D*, and *G*) Bronchial epithelial cells (BECs) show colocalization of IL-8 (*D*) and TRPA1 (*G*) in the ciliated pseudostratified columnar epithelium. (*G*) Expression of TRPA1 is present in columnar epithelial cells, in the endothelium of blood vessels (*arrows*) and in some mononucleated cells. (*B*, *E*, and *H*) Submucosal gland sections display expression of TRPA1 mainly in serosal cells (*H*). (*C*, *F*, and *I*) Alveolar sections exhibit TRPA1 expression mainly in pneumocytes (*I*) and no IL-8 mRNA signal (*F*). (*J–L*) Control slides stained with secondary antibody only. *Scale bars*, 100 μm (*A*, *C*, *D*, *F*, *G*, *I*, *J*, and *L*) and 20 μm (*B*, *E*, *H*, and *K*).

5-μm-thick seriated sections of different lung areas, including the surface columnar epithelia of the conductive airways, submucosal glands, and alveoli (TRPA1 antibody, Figures 1G–1I; secondary antibody only, Figures 1J–1L). As shown in Figure 1, TRPA1 protein was evidenced in different epithelial cells, namely, the ciliated pseudostratified columnar epithelium facing the bronchial lumen with prevalent accumulation of the signal in the apical portion of the columnar cells (Figure 1G), in the serous cells of the submucosal glands (Figure 1H), and pneumocytes of alveoli (Figure 1I). As expected from previous reports, TRPA1 staining was also observed in the endothelium of blood vessels (*see arrows*) and in some mononucleated cells infiltrating the submucosa (Figure 1G). TRPA1 staining was also confirmed in lung sections of individuals without CF, as shown in Figure E1 in the online supplement. CF lungs are known to produce a huge amount of the neutrophil chemokine, IL-8 (16), a hallmark of CF disease. To localize the cells expressing IL-8, mRNA *in situ* hybridization was performed in the seriated sections and detected with brown peroxidase staining. As shown in Figure 1, IL-8 mRNA signal was clearly detectable in the columnar pseudostratified epithelium, mainly, but not exclusively, in the basal cells (Figure 1D). Alveolar epithelia were consistently negative (Figure 1F), whereas serous submucosal cells showed a faint IL-8 mRNA staining only in some cases (data not shown), as reported in previous studies (17). In summary, the images in Figure 1 indicate that the chemokine, IL-8, is strongly and prevalently expressed in the BECs lining the lumina of the bronchi, the anatomical site principally exposed to the *P. aeruginosa* bacterial infection in the patients with CF with advanced stages of the disease, confirming an important role of these epithelial cells in CF lung pathology. Notably, these cells coexpress TRPA1 protein, which provides a preliminary hint to investigate the potential role of TRPA1 in the transcriptional regulation of IL-8, and possibly other soluble inflammatory mediators.

TRPA1 Channels Are Expressed and Functional in BEC Models

To study the involvement of TRPA1 in proinflammatory signaling in airway epithelial cell models, we first verified whether the expression of TRPA1 was conserved in *in vitro* cultures by immunofluorescence. We analyzed several respiratory epithelial cells, namely, the neoplastic alveolar type II–derived A549 cells, immortalized human BECs (HBECs) derived from patients with CF with CFTR mutated genotype, such as IB3-1, CuFi-1, CFBE41o^{-} cells, and primary HBECs from wild-type (WT) CFTR individuals (WT HBEC) and from F508 del CFTR homozygous patients (CF HBEC) (Table 1). Cells were labeled for TRPA1 channels (anti-TRPA1 antibody, green signal), the epithelial-specific marker, cytokeratin (CK; anti-pan CK antibody, red signal), and the cell nuclei (4′,6-diamidino-2-phenylindole nuclear staining, blue signal) (Figure 2). Signals from TRPA1 and CK were merged to address colocalization, as shown in the *third panel* of each cell type. As shown in Figure 2, TRPA1 protein is detected in all these cells, being located both on the plasma membrane and in the cytoplasm. To further ascertain the plasma membrane localization of TRPA1, confocal images were taken from CF HBEC slides (Figure 2, last micrograph of *bottom panels*). Segment analysis showed higher accumulation of TRPA1 protein in the plasma membrane compared with the cytoplasmic region of CF HBECs. The specificity of the signal was tested with preabsorption of TRPA1 antibody with its immunogenic peptide. The green signal was almost abolished, confirming the specificity of the green signal for TRPA1 (Figure E2). The results obtained on protein expression (Figures 1 and 2) strongly confirm that TRPA1 is expressed in CF respiratory epithelial cells of the conductive districts, not only in immortalized cell lines, but also in primary cells derived from CF bronchi and cultured *in vitro* without genomic manipulation.

The recent report of TRPA1 transcript splicing variants opens the possibility of different regulation of activation within TRPA1 isoforms (18). To verify that the TRPA1 channels expressed in these cells are functional, we exposed A549, IB3-1, CuFi-1, and CFBE41o^{-}, and CF HBECs, and WT HBECs, to different TRPA1-selective agonists, namely acrolein (ACR) and allyl isothiocyanate (AITC). In this way, we evaluated the increase of $[Ca^{2+}]_i$ as an effect of TRPA1-mediated intracellular influx. Upon stimulation with ACR, an endogenously produced TRPA1-selective agonist, and with AITC, an exogenous TRPA1 agonist contained in *Brassica*, a sharp increase in $[Ca^{2+}]_i$ was observed in all cell lines, as shown in Figures 3A, 3D, 3G, 3J, 3M, and 3P. Importantly, the effect of both TRPA1 agonists was invariably prevented by the preincubation of the cells with two different selective TRPA1 antagonists, HC-030031 (HC03) and A-967079 (A96), as shown in Figures 3B, 3C, 3E, 3F, 3H, 3I, 3K, 3L, 3N, 3O, 3Q, and 3R. The observation that AITC and ACR induce a sharp calcium response, which is strongly abated by TRPA1 antagonists, confirms that TRPA1 channels expressed in these cells are functional and that the calcium response observed with the agonists is mostly mediated by TRPA1 stimulation. These findings strengthen the

Table 1. Characteristics of Human Bronchial Epithelial Cell Donors

Name	Genotype	Sex	Age* (*Yr*)	Pathogens
HBEC 43/3	ΔF508/ΔF508	M	29	*P. aeruginosa*
HBEC 49/3	ΔF508/ΔF508	M	22	*P. aeruginosa*
HBEC 63/3	CFTR wt	F	63	NO
HBEC 73/3	ΔF508/ΔF508	F	34	*S. aureus*
HBEC 91/3	ΔF508/ΔF508	M	36	*P. aeruginosa*, *S. aureus*
HBEC 51/3	CFTR wt	F	19	NO

Definition of abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; F, female; HBEC, human bronchial epithelial cell; M, male; NO, no pathogens; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*; wt, wild type.

Genotypic characteristics and resident pathogens in lungs of patients from which HBECs were established. These cells were used for immunofluorescence, mRNA quantification, and cytokine release quantification experiments. HBEC 63/3 refers to an individual affected by idiopathic fibrosis, and HBEC 51/3 refers to a healthy donor without apparent lung pathology.

*Age at the time of lung transplantation.

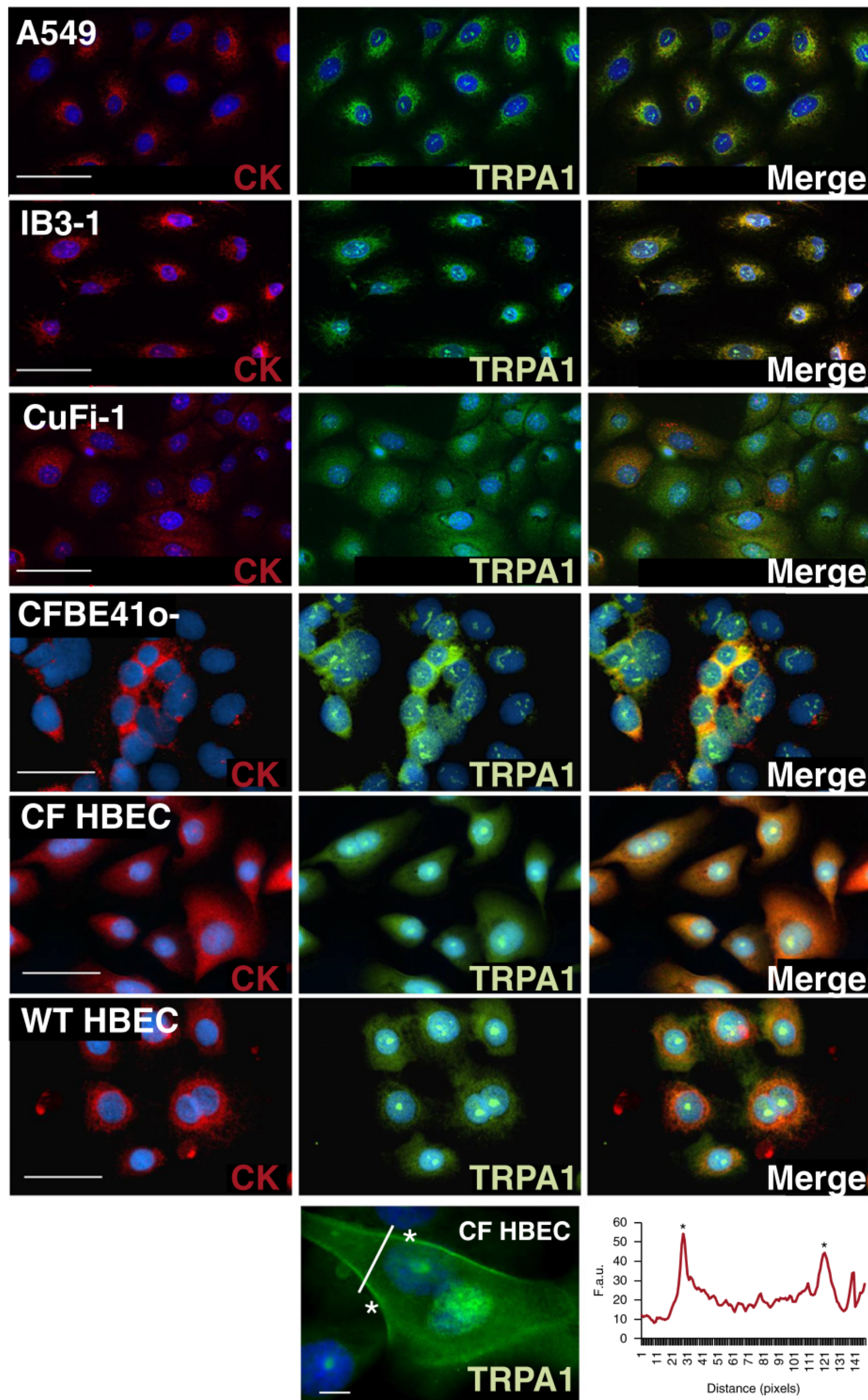

Figure 2. TRPA1 channels are expressed in BEC models. Immunolabeling for cytokeratin (CK) and TRPA1 channels (TRPA1) was performed in A549, IB3-1, CuFi-1, CFBE41o^{-} (*see* Material and Methods section), CF human BECs (HBECs), and wild-type (WT) HBECs. The *bottom panels* represent the confocal image and the fluorescence intensity analysis of CF HBECs. The *graph* shows the quantitation of TRPA1 fluorescence intensity (fluorescence arbitrary units [F.a.u.]) along the *diagonal line* crossing the cell (*thick white line* in the micrograph). The two *asterisks* laying above the two main fluorescence peaks refer to the two *asterisks* subtended by the *diagonal line* in the micrograph. Cells were fixed, permeabilized, and stained for CK (*red*), TRPA1 (*green*), and nuclei (*blue*). The *far right panel* of each cell type shows merging of the two signals (*Merge*), indicating the areas of colocalization of TRPA1 and CK. *Scale bars*, 20 μm and 10 μm for the last confocal image.

rationale of exploring the potential TRPA1 involvement in CF respiratory inflammation in those cells lining the bronchial surface, which are known to express soluble inflammatory mediators in response to bacterial challenge–induced calcium mobilization.

Pharmacological Inhibition of TRPA1 Function Reduces *P. aeruginosa*–Dependent Cytokine Transcription

To explore a potential link between TRPA1 channel function and *P. aeruginosa*–dependent transcription of IL-8 and other major proinflammatory cytokines released in the inflammatory milieu of CF lungs, namely, IL-1β, IL-6, and TNF-α, we challenged airway epithelial A549 and CuFi-1 cells with *P. aeruginosa* (PAO1 laboratory strain) for 4 hours. Cells were preincubated with the TRPA1-specific inhibitors, HC03 or A96, 1 hour before bacterial exposure. As shown in Figure 4, preincubation with TRPA1 inhibitors markedly reduced the transcription of these proinflammatory genes in the airway epithelial cells exposed to *P. aeruginosa*. The effect of the A96 inhibitor appeared more efficacious and was always statistically significant. These results suggest that functional inhibition of TRPA1 channels, obtained with the pharmacological agents HC03 or A96, blunts *P. aeruginosa*–dependent transcription of four major soluble mediators of CF lung inflammation involved in proinflammatory signaling.

Silencing the Expression of TRPA1 Channels Reduces IL-8 Gene Transcription upon Different Proinflammatory Challenges

To exclude that the reduction of transcription of proinflammatory genes obtained with the pharmacological inhibitors, HC03 or A96 (Figure 4), could be partly mediated by off-target effects, we first tested the transcription of IL-8 upon silencing TRPA1 channel expression. Transfection with TRPA1 small interfering RNA (siRNA) was performed for 72 hours before proinflammatory challenge in A549, IB3-1 cell lines, and in the primary BECs derived from explanted respiratory tissue of patients with CF before lung transplantation (CF HBECs). TRPA1 silencing experiments were performed in parallel with either a TRPA1 siRNA or a scrambled siRNA oligonucleotide, which

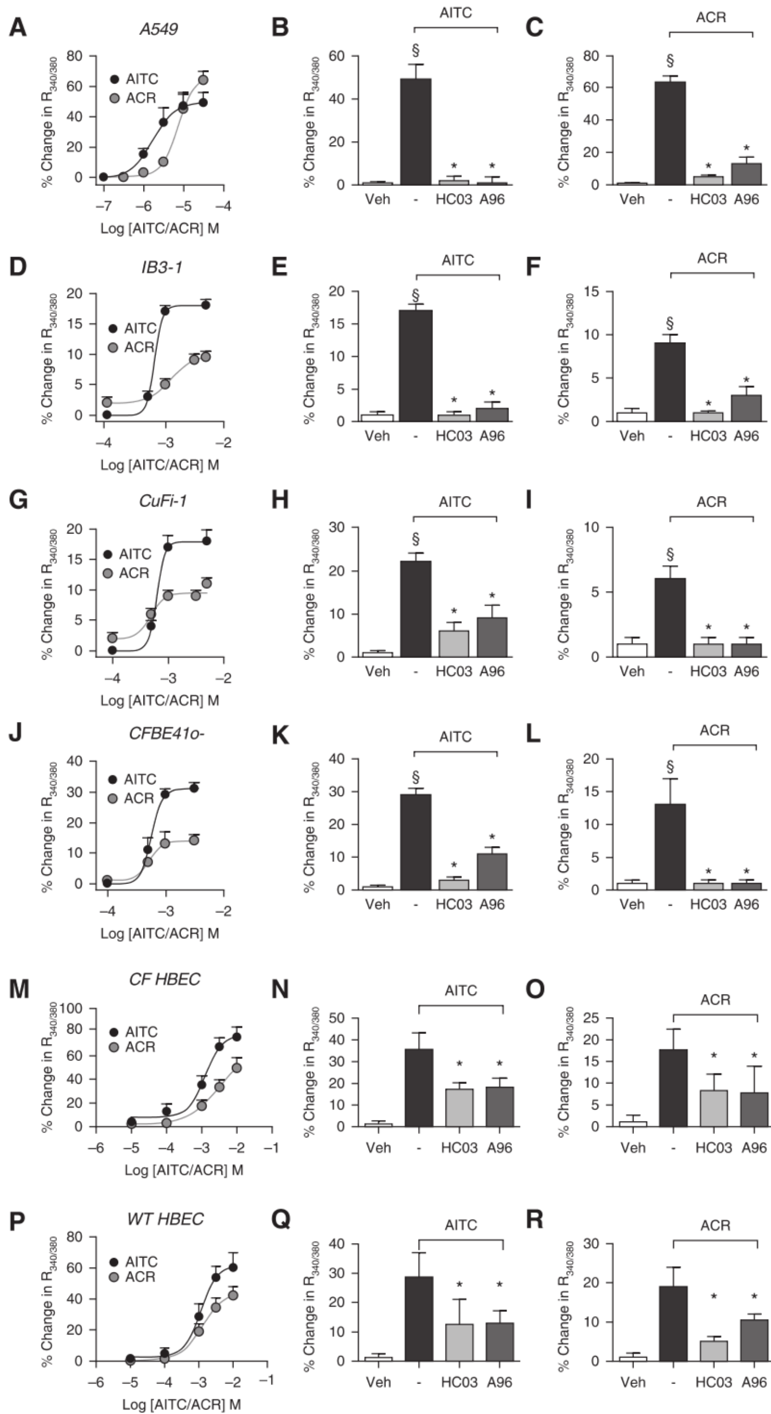

Figure 3. TRPA1 channels are functional in BEC models: A549, IB3-1, CuFi-1, CFBE41o$^-$, CF HBECs, and WT HBECs (*see* Material and Methods section). Intracellular calcium response as percent change in Fura-2 fluorescence (excitation wavelengths ratio of 340:380 nm [$R_{340/380}$]) was used to assess agonist-induced TRPA1 activation in A549, IB3-1, CuFi-1, CFBE41o$^-$, CF HBECs, and WT HBECs. The selective TRPA1 agonists (from 10^{-2} to 10^{-7} M), allyl isothiocyanate (AITC) shown in *black circles*, and acrolein (ACR) depicted in *gray circles* evoke a concentration-dependent intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response in all different cell types (*A*, *D*, *G*, *J*, *M*, and *P*). TRPA1 activation was inhibited with HC-030031 (HC03) at 30 μM and A-967079 (A96) at 10 μM (*B*, *C*, *E*, *F*, *H*, *I*, *K*, *L*, *N*, *O*, *Q*, and *R*). Vehicles (Veh) is a combination of vehicles of HC03 and A96. ANOVA and Bonferroni's *post hoc* tests were performed for statistical significance. Values are mean ± SD of $n > 25$ cells. $^§P < 0.05$ versus Veh and $^*P < 0.05$ versus AITC or ACR.

served as internal control to test transfection toxicity (Figure 5). Silencing efficiency was measured through TRPA1 residual mRNA expression that was, on average, decreased to a residual expression of 20–40% of the constitutive expression levels (Figure E3). To investigate the effect of TRPA1 silencing in a time lapse of at least 24 hours, these cells were first challenged with the HKPAO to avoid the cell toxicity frequently observed after prolonging the exposure of the living PAO1 bacteria to *in vitro*–cultured cells for more than 6–8 hours. As shown in Figures 5A–5C, TRPA1 silencing causes a significant decrease of IL-8 transcription upon stimulation with HKPAO for 24 hours in all these different cell models. Exposure of respiratory epithelial cells to the planktonic form of *P. aeruginosa*, both living and heat-inactivated, simulates more closely the acute bacterial infection moiety occurring in the lungs of patients with CF, either in the early phases of the disease or during the recurrent bacterial exacerbations in adult life. To investigate the role of TRPA1 when epithelial cells are exposed to the inflammatory milieu that chronically overlays the surface of CF bronchi, we challenged CF HBECs with the SMM obtained from a pool of respiratory excretions of patients with CF chronically infected with *P. aeruginosa* clinical isolates (19). As shown in Figure 5D, silencing TRPA1 also blunts SMM-induced transcription of IL-8. The results observed after TRPA1 siRNA silencing are consistent with those obtained with pharmacological inhibitors (Figure 4) and support a role for TRPA1 in the transcription of IL-8 gene, the major neutrophilic chemokine in CF lungs. Moreover, these observations suggest that TRPA1 is functionally important for IL-8 gene under conditions simulating both CF acute bacterial exacerbations and CF chronic infection/inflammation, based on the data with HKPAO planktonic bacteria and SMM, respectively.

Silencing the Expression of TRPA1 Has a Broad Inhibitory Effect on Several Cytokines Released from CF Primary BECs

The role of TRPA1 channels in the model of CF infection/inflammation driven by BECs was extended by investigating the effect of TRPA1 silencing on the release of a larger panel of cytokines. Based on the results shown in Figures 4 and 5, we chose to use

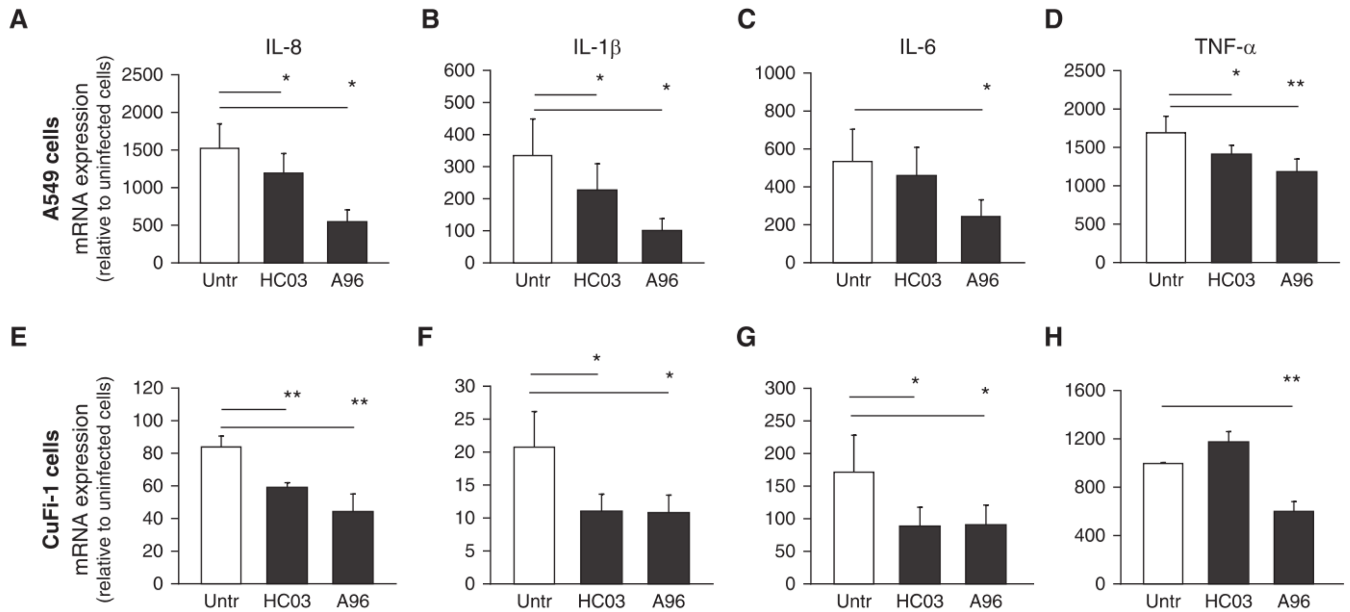

Figure 4. Pharmacological inhibition of TRPA1 channels reduces *Pseudomonas aeruginosa*–dependent cytokine transcription. A549 (*A–D*) and CuFi-1 cells (*E–H*) have been preincubated with 50 μM HC03 or A96 1 hour before exposure to *P. aeruginosa* (PAO1 laboratory strain) for a further 4 hrs. Untreated (Untr) refer to cells infected with PAO1 without any pharmacological inhibition. IL-8 (*A* and *E*), IL-1β (*B* and *F*), IL-6 (*C* and *G*), and TNF-α (*D* and *H*) mRNAs were quantified by quantitative RT-PCR (qRT-PCR). Data are expressed relative to uninfected cells. Data are mean ± SEM of four independent experiments performed in duplicate. Student's *t* test for unpaired data (*$P < 0.05$ and **$P < 0.01$) was performed.

the BEC model more closely related to the lungs of patients with CF, the CF HBEC. HBEC from three and four different patients with CF were cultured, silenced, and challenged with either inactivated planktonic bacterium (HKPAO) or SMM, respectively. Both HKPAO and SMM significantly induced the release of the major neutrophilic chemokine, IL-8, and the proinflammatory cytokines, IL-1β, IL-6, and TNF-α (Figures 6A and 6B). As expected, IL-8 was the most potently induced cytokine, with average stimulated levels at least above one order of magnitude (Figures 6A and B). The comparative potency of the two stimuli was variable, SMM being two orders of magnitude more potent than HKPAO in inducing the release of IL-1β (Figures 6A and 6B). Upon both stimuli (HKPAO and SMM), silencing TRPA1 significantly reduced the release of IL-8, IL-1β, and TNF-α, but not IL-6 (Figures 6A and 6B).

We extended the analysis of cytokine release to other families of soluble mediators. Several chemokines involved in the recruitment of mononucleated cells of the immune response are released from BECs upon exposure to HKPAO such as monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-β), regulated on activation, normal T cell expressed and secreted (RANTES), and interferon γ-induced protein 10 (IP-10) or upon exposure to supernatant of mucopurulent material (SMM) such as MIP-1β, although the absolute values of induction are much lower than those of the neutrophil chemokine, IL-8 (Figures 6A–6D). Notably, silencing TRPA1 showed some reduction effect for monocyte chemoattractant protein 1 (MCP-1) and MIP-1β (heat killed *P. aeruginosa*, HKPAO, Figure 6C). Within cytokines inhibiting the immune response, interleukin-1 receptor antagonist (IL-1ra) was strikingly induced by one order of magnitude by SMM (Figure 6D), which parallels the huge two orders-of-magnitude induction of IL-1β by SMM (Figure 6B). TRPA1 silencing reduces IL-1β and halves IL-1ra induced by SMM (Figures 6B and 6D), making the interpretation of the role of TRPA1 in IL-1 regulation intriguing, considering the opposite biological effects of IL-1β and of IL-1ra. Within the family of cytokines intervening in the innate-adaptive cross-talk of the immune response, HKPAO and SMM did not promote striking induction of expression and release in terms of absolute amounts in the CF HBECs, although some statistically significant induction could be observed for IL-7 (HKPAO and SMM, Figures 6C and 6D), IL-12 (SMM, Figure 6D), and IFN-γ (HKPAO and SMM, Figures 6C and 6D). Overall, TRPA1 silencing did not lead to significant decreases in this group of cytokines (IL-2, IL-4, IL-7, IL-9, IL-12, IL-13, IL-15, IL-17), except for IFN-γ, the basal induction of which was doubled by both HKPAO and SMM, and this effect was partially abolished by TRPA1 silencing (Figures 6C and 6D).

Our analyses also included four paradigmatic growth factors involved in angiogenesis and lineage maturation, namely, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), granulocyte colony–stimulating factor (CSF), and granulocyte/macrophage CSF (GM-CSF) (Figures 6C and 6D). Interestingly, granulocyte CSF is strikingly induced by HKPAO (Figure 6C), and, in contrast, significantly reduced by SMM (Figure 6D). These findings could be interpreted as a consistent positive effect on granulocyte proliferation and release in the bloodstream during acute host–pathogen interactions (HKPAO, Figure 6C) and a consistent shutdown signal during chronic infection/inflammation (SMM, Figure 6D). TRPA1 does not seem to intervene on this

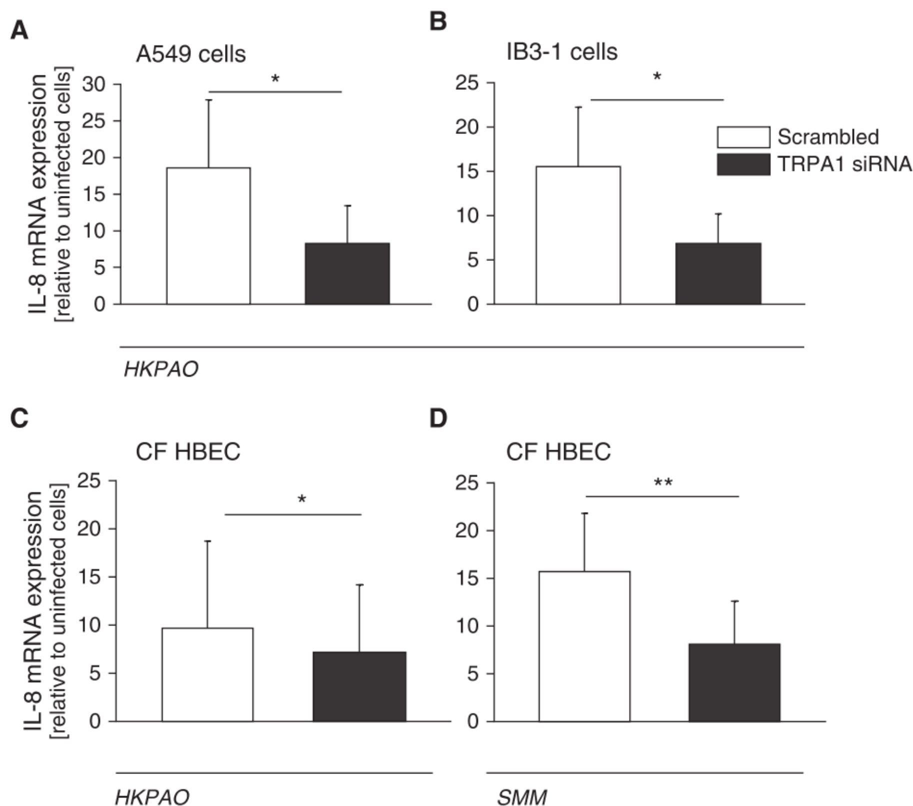

Figure 5. Silencing of TRPA1 channels reduces IL-8 mRNA levels in respiratory models due to heat-killed laboratory strain PAO1 of *P. aeruginosa* (HKPAO) and supernatant of mucopurulent material (SMM) stimulation. IL-8 mRNA levels were measured by qRT-PCR in A549 cells (*A*) and IB3-1 cells (*B*) with HKPAO. CF HBEC primary cells derived from three patients with CF were challenged with HKPAO (*C*) or SMM (*D*). *Open bars* represent scrambled treatment; *solid bars* show TRPA1-silenced cells. Silencing efficiency was measured through TRPA1 residual mRNA expression that was, on average, 20–40% (*see* Figure E3). Data are expressed relative to uninfected cells (no stimulation either with HKPAO or SMM). Data are mean ± SEM of at least four different experiments. Student's *t* test for paired data (*$P < 0.05$ and **$P < 0.01$). siRNA, small interfering RNA.

signaling (Figures 6C and 6D). In parallel, PDGF and VEGF, intervening in regulating the angiogenesis during tissue remodeling in chronic inflammation, are robustly induced by SMM (Figure 6D), and TRPA1 silencing blunts the effect SMM on VEGF.

In synthesis, the analysis of the release of soluble mediators of the innate and adaptive immune response confirms that primary BECs from patients with CF are strong producers of IL-8, the proinflammatory cytokines, IL-1β, IL-6, and TNF-α, and the growth factors, PDGF and VEGF, that play a role in tissue remodeling. However, differences in the production of these soluble mediators can be observed, depending on whether the infectious stimulus mimics acute exacerbations or chronic airway infection/inflammation. Within this scenario, TRPA1 channels appear to be mainly involved in the regulation of IL-8, IL-1β, and TNF-α.

Discussion

The TRP channel family has been implicated in the pathogenesis of relevant chronic respiratory diseases (for review, *see* Ref. 20). In particular, TRPA1 channels have been found to be expressed in different components of the respiratory mucosa, such as sensory nerves, B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes (20), as well as in small airway epithelial cells, bronchial *muscolaris* smooth muscle cells, and bronchial fibroblasts (13). Expression of TRPA1 channels is not limited to the respiratory tissue derived from patients with CF, as shown by our results (Figure E1). Moreover, TRPA1 channels were found to play a role in chronic obstructive pulmonary disease and asthma (11, 12), possibly by mediating nerve activation in response to irritants and allergen-induced airways inflammation (for review, *see* Ref. 20). To date, although no previous studies have reported a role for TRPA1 in CF airway inflammatory disease, transient receptor potential canonical channel 6 (TRPC6) channels have been found to mediate an abnormally elevated Ca^{2+} influx in CF BECs (21). Although this abnormal response has been coupled to the CFTR defect, as CFTR correctors normalize TRPC6 function (21), the role of TRPC6 in CF respiratory inflammation was not explored. This is worthy of consideration, as Ca^{2+} influx is amplified by the chronic inflammatory state dependent on chronic infection. For instance, exposure of primary cultures of bronchial epithelia to SMM produces an expansion of endoplasmic reticulum (ER) Ca^{2+} stores, which mediates a Ca^{2+}-dependent hyperinflammatory phenotype (19). In the present study, we found that TRPA1 channels modulate the inflammatory response of CF bronchial epithelia induced by exposure to planktonic bacterium or SMM, resembling acute or chronic infection by *P. aeruginosa.*

We previously reported that direct activation of TRPA1 with agonists is sufficient to induce the release of IL-8 in small airway epithelial cells (13). In parallel, we found that the phospholipase C (PLC) β–driven signaling pathway, which triggers the release of Ca^{2+} from the ER, is not sufficient for the expression of IL-8, but requires the activation of Toll-like receptor 5/myeloid differentiation primary response 88 (TLR5/MyD88) signaling (14). Here, we strengthen the role of intracellular Ca^{2+} transients in CF epithelial inflammation, because blocking TRPA1 expression/function results in a reduction of the calcium signals associated with the inflammatory responses (Figures 4–6). This could lead to the question of why the Ca^{2+} increase operated by extracellular entry (e.g., via TRPA1) is a sufficient proinflammatory mechanism, whereas the Ca^{2+} increase regulated by the release from the ER (e.g., via inositol 1,4,5-trisphosphate) produced by PLC is only synergic, but not sufficient. One possible explanation could be that the kinetics of cytosolic Ca^{2+} increase, in terms of onset, peak level, and prolongation, is different in the two conditions, making extracellular Ca^{2+} entry via TRPA1 able to activate independently of MyD88 signaling those

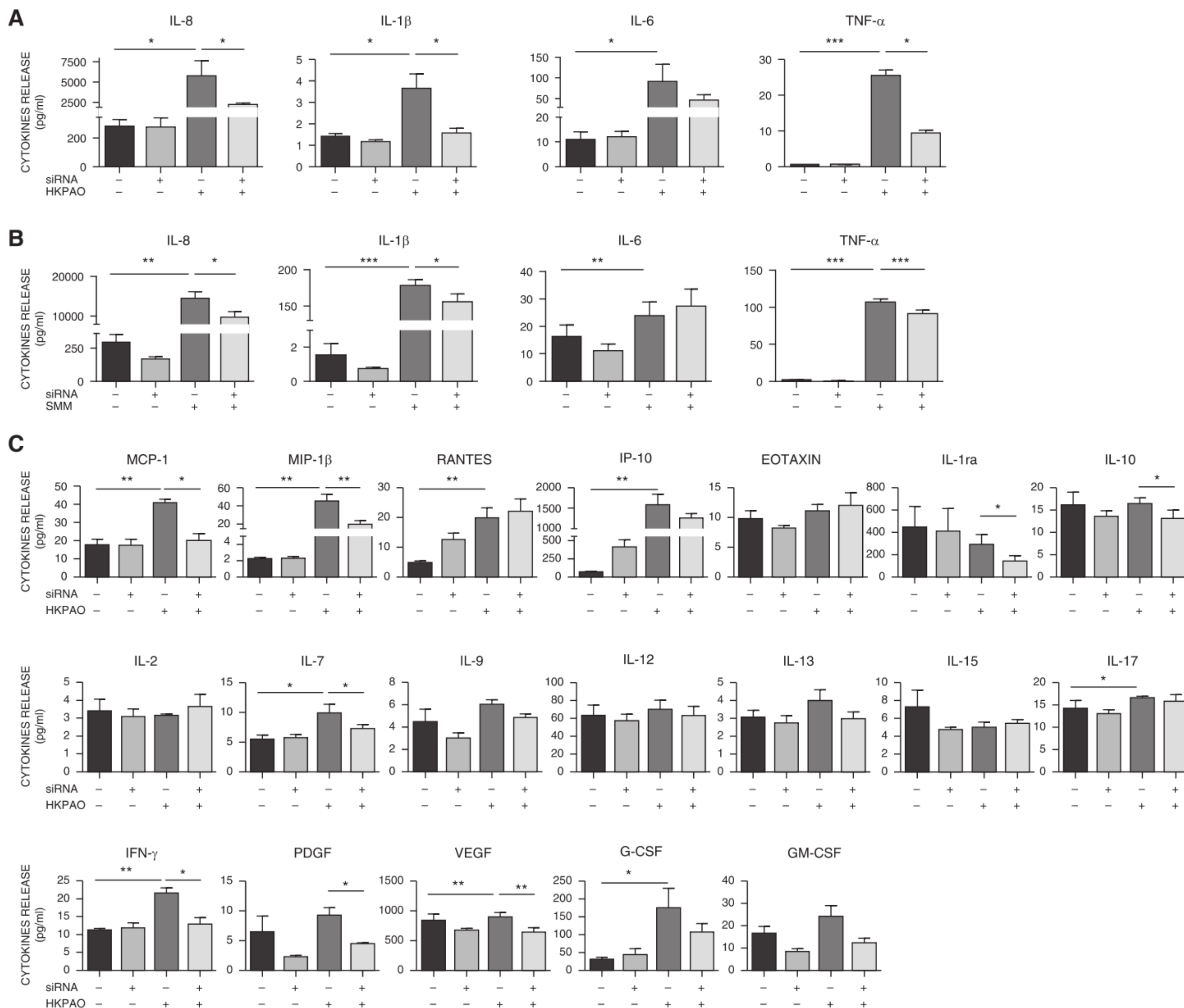

Figure 6. Silencing TRPA1 channels produces a generalized reduction in the release of proinflammatory cytokines from CF primary BECs exposed to *P. aeruginosa* or to SMM. HBEC primary cells from patients with CF were transfected with TRPA1 siRNA (+) or scrambled (−) oligonucleotides for 72 hours. Cells were subsequently exposed to HKPAO for a further 24 hours (*A* and *C*) or SMM for a further 4 hours (*B* and *D*). Supernatants were collected, and a panel of 27 human cytokines was analyzed by Bioplex multiplex assay (*A–D*). (*A* and *B*) Four major cytokines (IL-8, IL-1β, IL-6, and TNF-α) are shown. Four relative conditions are reported for each of them (i.e., scrambled untreated, siRNA untreated, scrambled stimulated with HKPAO or with SMM, and siRNA stimulated

nuclear transcription factors required to start the transcription of IL-8 gene (22). In this respect, TRPA1 has been found to favor interesting interplays with both ER and mitochondria in regulating intracellular Ca^{2+} buffering. In particular, TRPA1 activation has been demonstrated to negatively regulate stromal interaction molecule 1-ORAI calcium release-activated calcium modulator 1 (STIM1-Orai1) association (23) and to participate in mitochondria mobility in a Miro protein–dependent fashion (24). An alternative explanation takes into account the microdomains, where the Ca^{2+} increase intervenes in these different stimulatory conditions, considering the intracellular location of the different calcium channels involved, namely, the plasma membrane and the ER, where calcium waves originate (for reviews, *see* Refs. 25, 26). This is particularly interesting, both in general terms and specifically for CF epithelial cells, where intracellular Ca^{2+} signaling was found to be dysregulated (27–30). Therefore, more in-depth analyses on the intracellular calcium microdomains regulated by TRPA1 and, broadly, TRP channels, together with the specific calcium-dependent responses in polarized BECs upon infective challenges (28), could provide further insight on TRPA1 in the CF context. This could be further extended to

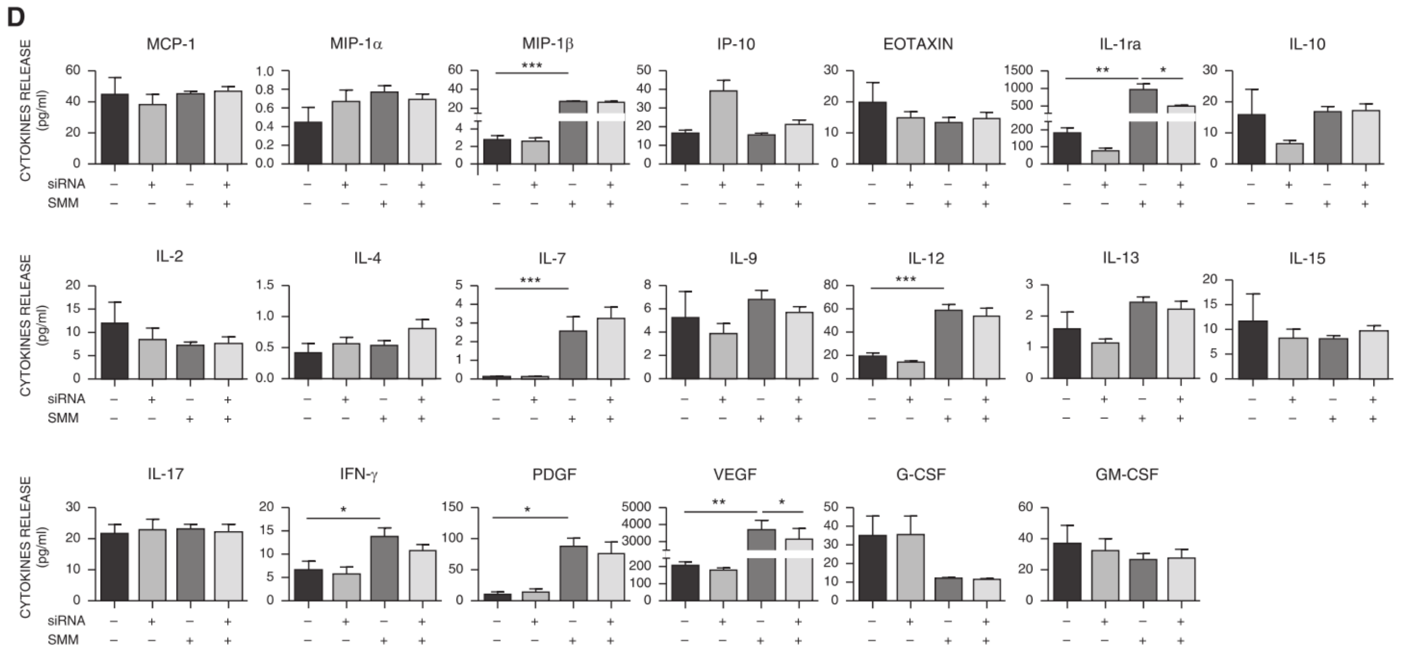

Figure 6. (Continued). with HKPAO or SMM). (*C*) Data of a further 19 cytokines after HKPAO stimulation are shown with the exclusion of macrophage inflammatory protein 1-α (MIP-1α), IL-4, IL-5, and fibroblast growth factor (FGF), which were under the threshold of detectability. (*D*) Data of a further 20 cytokines after SMM challenge are reported, with exclusion of RANTES (regulated upon activation, normal T cell expressed and secreted), IL-5, and FGF, which were under the limit of detection. Cytokine release was measured in HBECs from three and four CF individuals assayed in duplicate for HKPAO and SMM, respectively. Data are mean ± SEM. Student's *t* test for paired data (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$) was performed. EOTAXIN, eosinophil chemotactic proteins; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1ra, IL-1 receptor antagonist; IP-10, interferon γ-induced protein 10; MCP, monocyte chemoattractant protein; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

the immune cells expressing TRPA1 (e.g., T and B lymphocytes), which intervene in the immune response of the whole respiratory mucosa.

We observed here that inhibition of TRPA1 calcium transport by selective antagonists and transient reduction of expression by gene silencing significantly reduce the transcription and release of different cytokines, including IL-8, IL-1β, and TNF-α (Figures 4–6). This implies that, in our experimental conditions, TRPA1 becomes activated in its function (for review, *see* Ref. 31). TRPA1 channel function is known to be triggered by exogenous oxidants and intracellular oxidative stress (32, 33). In this regard, exposure of CF BECs to *P. aeruginosa* increases the production of hydrogen peroxide by dual oxidase 2 (Duox2) enzyme (34) and mitochondrial oxidation (15), thus making plausible that, in our models, TRPA1 can be activated by oxidation of cysteine residues, as described previously (32). The oxidated status of TRPA1 can also be considered particularly relevant in the bronchial mucosa of patients with CF, as the abundant amount of infiltrating neutrophils is known to release huge amounts of superoxide anions and other reactive oxygen species (for review, *see* Ref. 35). PLC activation, through G protein–coupled receptors, has been claimed to positively regulate TRPA1 channel function (36, 37), although the precise mechanisms are controversial, also depending on the different nonepithelial cell models used. PLC is known to transform phosphatidylinositol-4,5-biphosphonate (PIP2) residing in the plasma membrane into diacylglycerol and inositol 1,4,5-trisphosphate, which activates protein kinase C isoforms and the release of Ca^{2+} from the ER, respectively. PIP2 has been found either to activate (9) or inhibit (38) TRPA1 channel transport. The TRPA1-related transient receptor potential vanilloid channel 1 (TRPV1) channel was shown to be activated by protein kinase C–dependent phosphorylation; however, this mechanism was not confirmed for TRPA1, suggesting that PLC is positively acting by depleting PIP2 from plasma membrane, but not through its downstream effector, protein kinase C (38). The possibility that the Ca^{2+} transients induced by the PLC–diacylglycerol signaling pathway activate TRPA1 directly has been considered (36), and the role of Ca^{2+} released from the ER subsequently ascertained by specific investigations defining both the threshold of TRPA1 activation (in the 900-nM [Ca^{2+}] range) and the domain EF of TRPA1 as the binding site for Ca^{2+} (39, 40). Although the mechanisms of activation of TRPA1 in BECs have not been clarified so far, the signaling cascade induced by *P. aeruginosa* (14, 41) makes reasonable the presence of activated TRPA1 channels in our experimental models. In particular, an interesting TRPA1–PLC β interplay could be involved, although the precise TRPA1 functional regulation in CF epithelial cells will require future investigation.

In conclusion, the results presented here suggest considering TRPA1 as a druggable target to control the excessive inflammation reported in the lungs of patients with CF, reducing tissue damage without completely blunting the immune response. In this respect, recent announcements have been forwarded on a first phase I clinical trial with the TRPA1 antagonist, CB-189625 (structure not disclosed) by HydraCubist Pharmaceuticals

(Lexington, MA) and Hydra Biosciences (Cambridge, MA). In addition, Glenmark Pharmaceuticals (Mumbai, India) selected the compound GRC-17536 (structure not disclosed) as a clinical candidate after successful completion of preclinical studies. GRC-17536 has shown efficacy in animal models of inflammatory and neuropathic pain (available from http://www.evaluatepharma.com), and was well tolerated in a phase I clinical study just completed. Whether pharmacological inhibition of TRPA1 channels in nonneuronal cells could provide significant amelioration of CF lung inflammation warrants further investigation, and could pave the way to testing new pharmacological molecules targeting this channel. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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