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TRPA1 channels modulate inflammatory response in respiratory cells from cystic fibrosis patients

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TRPA1 channels and 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 Cystic Fibrosis (CF) patients. The nociceptive Transient Receptor Potential Ankyrin 1 (TRPA1) calcium channels have been recently found involved in non-neurogenic inflammation. Here, we investigated 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 by immunofluorescence. Epithelial cell lines (A549, IB3-1, CuFi-1, CFBE41o$^-$) and primary cells from CF patients were utilized to a) check TRPA1 function modulation, by Fura-2 calcium imaging, b) down-modulate TRPA1 function and expression, by pharmacological inhibitors (HC-030031 and A-967079) and siRNA silencing, and c) assess the effect of TRPA1 down-modulation on expression and release of cytokines upon exposure to pro-inflammatory challenges, by qRT-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 co-expressed. Inhibition of TRPA1 expression results in a relevant reduction of release of several cytokines, including IL-8 and the pro-inflammatory 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 CF patients. In conclusion, TRPA1 channels are involved in regulating the extent of airway inflammation driven by CF bronchial epithelial cells.

Keywords

Cystic Fibrosis, TRPA1, Interleukin - 8 , *Pseudomonas aeruginosa*, inflammation

Introduction

Although Cystic fibrosis (CF) is a multiple-organ disease, the lung pathology is the major cause of morbidity and mortality (for review see (1)). CF Transmembrane conductance Regulator (CFTR) dysfunction, which leads to altered ion transport in bronchial epithelia, causes dehydration of the airway surface liquid, impairing the mucociliary clearance (2-4). In this condition, mucus accumulates on 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 Interleukin (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 CF patients, multi-target anti-inflammatory drugs, such as ibuprofen and corticosteroids, are currently utilized in daily clinical practice. Their limited efficacy and adverse effects stress the need of finding novel CF-tailored anti-inflammatory molecular targets and drugs (7), to be associated with the CFTR modulators (correctors and potentiators) and more effective antibacterial drugs in those adult CF patients that exhibit already chronic lung infection and inflammation.

The Transient Receptor Potential Ankyrin 1 (TRPA1) (8), which belongs to the family of TRP channels (for review see (9)), was found 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 hyper-responsiveness

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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, other and our lab 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 extra-neuronal 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 pro-inflammatory signaling regulating IL-8 and IL-1β release in CF bronchial epithelial cells (14, 15).

To verify the hypothesis that TRPA1 calcium channels are involved in CF respiratory inflammation, we firstly assessed the expression and localization of TRPA1 in CF lung tissue. We further modulated TRPA1 expression and function in CF bronchial epithelial cells co-expressing TRPA1 and inflammatory cytokines, upon exposure to CF-specific pro-inflammatory challenges.

Material and Methods

Tissue collection

Formalin-fixed and paraffin-embedded bronchial 3-5 µm-thick sections of lung specimens from CF patients were obtained from Gaslini Institute (Drs. L. Galietta and L. Ferrera). The use of human bronchi, obtained from patients undergoing lung transplant, was approved by the Ethical Committee of the Gaslini Institute (Genova, Italy), 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 #13). Sections of non-CF diseased individuals were purchased from US Bio Corp (MD, USA).

Immunohistochemical (IHC) staining

IHC analysis was performed as previously described (13).

In situ hybridization (ISH)

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

Cell culture

Human A549 alveolar type II-derived epithelial cells, IB3-1 cells, CuFi-1 cells, CFBE41o- cells and primary cultures derived from columnar epithelia of human bronchi obtained from the American Tissue Culture Collection (ATCC) or kindly donated by Collaborators were cultured using standard procedures (see Supplementary Material).

Calcium Imaging

Changes in intracellular Ca^{2+} concentration were determined using the standardized method of $Ratio_{340/380}$ ($R_{340/380}$) recorded with a dynamic image analysis system (see Supplementary Material).

Immunofluorescence assay and confocal analyses

Immunofluorescence experiments and confocal analyses were carried out as described in Supplementary Material.

Transient silencing of TRPA1 channels expression

Transient silencing was carried out with TriFECTa RNAi Kit (Integrated DNA technologies, Coralville, Iowa, IA) accordingly to the manufacturer's instructions (see Supplementary Material).

Stimulation with P. aeruginosa, heat killed P. aeruginosa (HKPAO) and Supernatant of Mucopurelent Material (SMM)

AJRCMB Articles in Press. Published on 09-June-2016 as 10.1165/rcmb.2016-0089OC

Stimulation with PAO1, HKPAO and SMM was performed as described in Supplementary Material.

Quantitative RT-PCR

For Real-Time PCR studies, cells were treated as described in Supplementary Material.

Bioplex assay and analysis

Cytokines released in culture medium were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA) (see Supplementary Material).

Statistical analysis

Data were analyzed using two-tailed Student's t test and ANOVA followed by Bonferroni post-hoc test for comparisons between multiple group. Statistical analysis was performed using the GraphPad Prism Software (La Jolla, CA). Differences were considered significant for $P < 0.05$.

Results

IL-8 is co-localized with TRPA1 in bronchial columnar epithelial cells of CF lung.

To test the hypothesis that TRPA1 calcium channels are involved in non-neurogenic lung inflammation in CF, we first assessed the localization of TRPA1 protein in the respiratory tissue of CF patients 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 CF patients were analyzed to detect TRPA1 expression. Sections stained with haematoxylin and eosin allowed to identify the different lung areas. Fig. 1, panel A, shows surface bronchial mucosa with the pseudostratified epithelium and extensive submucosal infiltrates of inflammatory cells, typical of lungs from adult CF patients with chronic bacterial infection and inflammation.

Submucosal glands, both serosal and mucosal glands are shown in Fig. 1 panel B. Alveolar areas are shown in panel C. Expression of TRPA1 protein was evaluated by immunohistochemistry by brown peroxidase staining in separate 3-5 μm thick seriated sections of different lung areas, including the surface columnar epithelia of the conductive airways, submucosal glands and alveoli (TRPA1 Ab Fig. 1 G-I, secondary Ab only Fig. 1 J-L). As shown in Fig. 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 (panel G), in the serous cells of the submucosal glands (panel H) and pneumocytes of alveoli (panel I). As expected from previous reports, TRPA1 staining was observed also in the endothelium of blood vessels (see arrows) and in some mononucleated cells infiltrating the submucosa (panel G). TRPA1 staining was confirmed also in lung sections of non-CF individuals, as shown in Supplementary Fig E1. 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 Fig. 1, IL-8 mRNA signal was clearly detectable in the columnar pseudostratified epithelium, mainly but not exclusively in the basal cells (panel D). Alveolar epithelia were consistently negative (panel F), whereas serous submucosal cells showed a faint IL-8 mRNA staining only in some cases (not shown), as reported in previous studies (17). In summary, the images of Fig. 1 indicate that the chemokine IL-8 is strongly and prevalently expressed in the bronchial epithelial cells lining the lumina of the bronchi, the anatomical site principally exposed to the *P.aeruginosa* bacterial infection in the CF patients with advanced stages of the disease, confirming an important role of these epithelial cells in CF lung pathology. Notably, these cells co-express TRPA1 protein, which provides a preliminary hint to investigate the potential role

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of TRPA1 in the transcriptional regulation of IL-8 and possibly other soluble inflammatory mediators.

TRPA1 channels are expressed and functional in bronchial epithelial cell models.

In order to study the involvement of TRPA1 in pro-inflammatory signaling in airway epithelial cell models, we firstly 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 bronchial epithelial cells derived from CF patients with CFTR mutated genotype, such as IB3-1, CuFi-1, CFBE41o- cells and primary human bronchial epithelial cells (HBECs) from wild type CFTR individuals (WT HBEC) and from F508del CFTR homozygous patients (CF HBEC) (Table 1). Cells were labelled for TRPA1 channels (anti-TRPA1 antibody, green signal), the epithelial specific marker cytokeratin (CK; anti-pan Cytokeratin antibody, red signal) and the cell nuclei (DAPI nuclear staining, blue signal). Signals from TRPA1 and CK were merged to address co-localization, as shown in the third panel of each cell type. As shown in Fig. 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 (bottom panels Fig 2). Segment analysis shows higher accumulation of TRPA1 protein in the plasma membrane compared to the cytoplasmic region of CF human bronchial epithelial cells. The specificity of the signal was tested with pre-absorption of TRPA1 antibody with its immunogenic peptide. The green signal was almost abolished, confirming the specificity of the green signal for TRPA1 (Supplementary Fig. E2). The results obtained on protein expression (Figs. 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, CFBE41o- , CF HBEC and WT HBEC cells to different TRPA1 selective agonists, namely acrolein (ACR) and allyl isothiocyanate (AITC). In this way, we evaluated the increase of intracellular Ca^{2+} concentration, 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 intracellular Ca^{2+} concentration was observed in all cell lines, as shown in Fig. 3, panels A, D, G, J, M and P. 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 Fig. 3 panels B, C, E, F, H, I, K, L, N, O, Q and R. The observation that AITC and ACR induce a sharp calcium response, that 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 rationale to explore 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.

In order to explore a potential link between TRPA1 channel function and *P. aeruginosa*-dependent transcription of IL-8 and other major pro-inflammatory 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 lab strain) for 4 hours. Cells

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were pre-incubated with the TRPA1 specific inhibitors HC-030031 or A-967079 one hour before bacterial exposure. As shown in Fig. 4, pre-incubation with TRPA1 inhibitors markedly reduced the transcription of these pro-inflammatory genes in the airway epithelial cells exposed to *P. aeruginosa*. The effect of the A-967079 inhibitor appeared more efficacious and was always statistically significant. These results suggest that functional inhibition of TRPA1 channels, obtained with the pharmacological agents HC-030031 or A-967079, blunts *P. aeruginosa*-dependent transcription of four major soluble mediators of CF lung inflammation involved in pro-inflammatory signaling.

Silencing the expression of TRPA1 channels reduces IL-8 gene transcription upon different pro-inflammatory challenges.

To exclude that the reduction of transcription of pro-inflammatory genes obtained with the pharmacological inhibitors HC-030031 or A-967079 (Fig. 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 siRNA was performed for 72 h before pro-inflammatory challenge in the A549 and IB3-1 cell lines, and the primary bronchial epithelial cells derived from explanted respiratory tissue of CF patients before lung transplantation, the CF Human Bronchial Epithelial Cells (CF HBEC). TRPA1 silencing experiments were carried out in parallel with either a TRPA1 siRNA or a scrambled siRNA oligonucleotide, which served as internal control to test transfection toxicity (Fig. 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 (Supplementary Fig E3). To investigate the effect of TRPA1 silencing in a time-lapse of at least 24 h, these cells were first challenged with the heat-killed laboratory strain PAO1 of *P.aeruginosa* (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 hrs. As shown in Fig. 5 (panels A-C), TRPA1 silencing causes a significant decrease of IL-8 transcription upon stimulation with HKPAO for 24 h 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 CF patients, either in the early phases of the disease or during the recurrent bacterial exacerbations in the 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 HBEC with the supernatant of mucopurulent material (SMM) obtained from a pool of respiratory excretions of CF patients chronically infected with *P. aeruginosa* clinical isolates (19). As shown in Fig. 5 (panel D), 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 (Fig. 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 bronchial epithelial cells.

The role of TRPA1 channels in the model of CF infection/inflammation driven by bronchial epithelial cells was extended by investigating the effect of TRPA1 silencing on the release of a larger panel of cytokines. Based on the results from Figs. 4 and 5, we chose to utilize the bronchial epithelial cell model more closely related to the lungs of CF patients, the CF HBEC. HBEC from 3 different CF patients were cultured, silenced and challenged with either inactivated planktonic bacterium (HKPAO) or SMM. Both HKPAO and SMM

AJRCMB Articles in Press. Published on 09-June-2016 as 10.1165/rcmb.2016-0089OC

significantly induce the release of the major neutrophilic chemokine IL-8 and the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (Fig 6 panels A and B). As expected, IL-8 is the most potently induced cytokine, with average stimulated levels at least above one-order of magnitude (Figs. 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 (Fig. 6A and B). Upon both stimuli, silencing TRPA1 significantly reduced the release of IL-8, IL-1β and TNF-α, but not IL-6 (Fig. 6A and B).

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 bronchial epithelial cells upon exposure to HKPAO (MCP-1, MIP-1β, RANTES, IP-10) or SMM (MIP-1β), although the absolute values of induction are much lower than those of the neutrophil chemokine IL-8 (FIG. 6 panels A-D). Notably, silencing TRPA1 showed some reduction effect for MCP-1 and MIP-1β (panel C, HKPAO). Within cytokines inhibiting the immune response, IL-1ra was strikingly induced by one-order of magnitude by SMM (Fig. 6D), which parallels the huge two-orders of magnitude induction of IL-1β by SMM (Fig. 6B). TRPA1 silencing reduces IL-1β and halves IL-1ra induced by SMM (panels B and D), 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 HBEC, although some statistically significant induction can be observed for IL-7 (HKPAO and SMM, Figs. 6C and D) and IL-12 (SMM, Fig. 6D) and IFN-γ (HKPAO and SMM, Fig. 6 C and D). 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, which basal induction is doubled by both HKPAO and SMM and this effect is partially abolished by TRPA1 silencing (Fig. 6C and D).

Our analyses also included four paradigmatic growth factors involved in angiogenesis and lineage maturation, namely PDGF, VEGF, G-CSF and GM-CSF (Fig. 6C and D). Interestingly, G-CSF is strikingly induced by HKPAO (Panel C) and, in contrast, significantly reduced by SMM (Panel D). These findings could be interpreted as a consistent positive effect on granulocyte proliferation and release in the bloodstream during acute host-pathogen interactions (HKPAO, panel C) and a consistent shut down signal during chronic infection/inflammation (SMM, panel D). TRPA1 does not seem to intervene on this signaling (Figs. 6C and D). In parallel, PDGF and VEGF, intervening in regulating the angiogenesis during tissue remodeling in chronic inflammation, are robustly induced by SMM (Fig. 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 bronchial epithelial cells from CF patients are strong producers of IL-8, the pro-inflammatory 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 (20)). In particular, TRPA1 channels, have been found expressed in different components of the respiratory mucosa, such as sensory

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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 CF patients, as shown by our results (Fig. E1). Moreover, TRPA1 channels were found to play a role in Chronic Obstructive Pulmonary Disease (COPD) and asthma (11, 12), possibly by mediating nerve activation in response to irritants and allergen-induced airways inflammation (for review see (20)). To date, while no previous studies have reported a role for TRPA1 in CF airway inflammatory disease, TRPC6 channels have been found to mediate an abnormally elevated Ca^{2+} influx in CF bronchial epithelial cells (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 worth 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) beta-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/MyD88 signaling (14). Here we strengthen the role of intracellular Ca^{2+} transients in CF epithelial inflammation, since blocking TRPA1 expression/function results in a reduction of the calcium signals associated with the inflammatory responses (Figs. 4-6). This could open the question on why the Ca^{2+} rise operated by extracellular entry, e.g.

via TRPA1, is a sufficient pro-inflammatory mechanism whereas the Ca^{2+} rise 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 kinetic of cytosolic Ca^{2+} rise, 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 nuclear transcription factors required to start the transcription of IL-8 gene (22). In this respect, TRPA1 has been found to favour interesting interplays with both ER and mitochondria in regulating intracellular Ca^{2+} buffering. In particular, TRPA1 activation has been demonstrated to negatively regulate 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+} rise 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 review see (25, 26)). This is particularly interesting both in general terms and specifically for CF epithelial cells, where intracellular Ca^{2+} signaling was found dysregulated (27-30). Therefore, more deep analyses on the intracellular calcium microdomains regulated by TRPA1 and broadly TRP channels, together with the specific calcium-dependent responses in polarized bronchial epithelial cells upon infective challenges (28), could provide further hints on TRPA1 in the CF context. This could be further extended to 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 that transient reduction of expression by gene silencing significantly reduce the transcription and release of different cytokines, including IL-8, IL-1β and TNF-α (Figs 4-6). This implies that in our experimental conditions TRPA1 becomes activated in its function (for review see (31)). TRPA1 channel function is known to be triggered by

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exogenous oxidants and intracellular oxidative stress (32, 33). In this regards, exposure of CF bronchial epithelial cells to *P.aeruginosa* increases the production of hydrogen peroxide by Duox2 enzyme (34) and mitochondrial oxidations (15), thus making plausible that, in our models, TRPA1 can be activated by oxidation of cysteine residues, as described (32). Oxidated status of TRPA1 can be also considered particularly relevant in the bronchial mucosa of CF patients, since the abundant amount of infiltrating neutrophils are known to release huge amounts of superoxide anions and other reactive oxygen species (for review see (35)). Phospholipase C (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 non-epithelial cell models utilized. PLC is known to transform phosphatidylinositol-4,5-biphosphonate (PIP2) residing in the plasma membrane into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), 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) the TRPA1 channel transport. The TRPA1-related 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-DAG signaling pathway activate directly TRPA1 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 bronchial epithelial cells have not been cleared 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 beta 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 CF patients, 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 and Hydra Biosciences. In addition, Glenmark Pharmaceuticals 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 [http://www.evaluatepharma.com], and was well-tolerated in a Phase I clinical study just completed. Whether pharmacological inhibition of TRPA1 channels in non-neuronal cells could provide significant amelioration of CF lung inflammation warrants further investigation and could pave the way to test new pharmacological molecules targeting this channel.

AJRCMB Articles in Press. Published on 09-June-2016 as 10.1165/rcmb.2016-0089OC

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Table 1

Characteristics of HBEC donors

NAME	GENOTYPE	GENDER	AGE	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

Genotypic characteristics and resident pathogens in lung patients from which HBEC cells 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 to a healthy donor without apparent lung pathology.

M= male; F= female; Age = years at the time of lung transplantation;*P.aeruginosa* = *Pseudomonas aeruginosa*, *S.aureus*= *Stafilococcus aureus*

Figure legends

Fig 1: **IL-8 is co-localized with TRPA1 in bronchial columnar epithelial cells of CF lung.** Different 3-5 μm serial sections of human CF bronchi were stained for H&E (A, B and C), for IL-8 mRNA by *in situ* hybridization (D, E and F), TRPA1 protein by immunocytochemistry (G, H and I) and secondary antibody only (J, K and L). Panels A, D and G: bronchial epithelial cells show co-localization of IL-8 (D) and TRPA1 (G) in the ciliated pseudostratified columnar epithelium. In panel G expression of TRPA1 is present in columnar epithelial cells, in the endothelium of blood vessels (arrowed) and in some mononucleated cells. Panels B, E and H: submucosal glands sections display expression of TRPA1 mainly in serosal cells (H). Panels C, F and I: alveolar sections exhibit TRPA1 expression mainly in pneumocytes (I) and no IL-8 mRNA signal (F). Panels J, K and L were control slides stained with secondary antibody only. Scale bar (black line) is 100 μm (panels A, C, D, F, G, I) or 20 μm (panels B, E, H) .

Fig 2: TRPA1 channels are expressed in bronchial epithelial cell models. Immunolabelling for cytokeratin (CK) and TRPA1 channels (TRPA1) was performed in A549, IB3-1, CuFi-1, CFBE41o$^-$, CF HBEC and WT HBEC cells. The two last bottom panels represent the confocal image analysis of CF HBEC. The graph shows the quantitation of TRPA1 fluorescence intensity along a line crossing the cell (thick white line in the micrograph). Cells were fixed, permeabilized, and stained for CK (red), TRPA1 (green) and nuclei (blue). The third panel of each cell type shows merging of the two signals (Merge), indicating the areas of co-localization of TRPA1 and CK. Scale bar (white line) is 20 μm, or 10 μm for the last confocal image.

Fig 3: TRPA1 channels are functional in bronchial epithelial cell models.

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Intracellular calcium response as % change in Fura-2 fluorescence (excitation wavelengths ratio $R_{340/380}$) was used to assess agonist-induced TRPA1 activation in A549, IB3-1, CuFi-1, CFBE41o-, CF HBEC and WT HBEC cells. The selective TRPA1 agonists (from 10^{-2} to 10^{-7} M), allyl isothiocyanate (AITC, black circles) and acrolein (ACR, grey circles), evoke a concentration-dependent $[Ca^{2+}]i$ response in all different cell types (panels A, D, G, J, M and P).

TRPA1 activation was inhibited with HC-030031 (HC03, 30 μM) and A-967079 (A96, 10 μM) (panels B, C, E, F, H, I, K, L, N, O, Q and R). Veh is a combination of vehicles of HC03 and A96. ANOVA and Bonferroni *post hoc* tests were performed for statistical significance. Values are mean ± SD of n > 25 cells. § $p< 0.05$ *vs*. Veh and * $p< 0.05$ *vs.* AITC or ACR.

Fig 4: Pharmacological inhibition of TRPA1 channels **reduces *P.aeruginosa*-dependent cytokine transcription.** A549 (A-D) and CuFi-1 cells (E-H) have been pre-incubated with 50 μM HC-030031 (HC03) or A-967079 (A96) 1 hour before exposure to *P.aeruginosa* (PAO1 lab strain) for further 4 hrs. Untreated (Untr) refer to cells infected with PAO1 without any pharmacological inhibition. IL-8 (A,E), IL-1β (B,F), IL-6 (C,G), and TNFα (D,H) mRNAs were quantified by qRT-PCR. Data are expressed relative to uninfected cells. Data are mean ± SEM of 4 independent experiments performed in duplicate. Student's t test for unpaired data (* $p<0.05$, ** $p<0.01$) was performed.

Fig 5: **Silencing of *TRPA1 channels reduces IL8 mRNA levels in respiratory models due to HKPAO (Heat Killed Pseudomonas aeruginosa) and SMM (Supernatant of Mucopurulent Material) stimulation.*** IL-8 mRNA levels were measured by qRT-PCR in A549 cells (A) and IB3-1 cells (B) with Heat Killed PAO1 (HKPAO). CF HBEC primary cells derived from 3 CF patients were challenged with HKPAO (C) or SMM (D). White bars

represent scrambled treatment, black bars show TRPA1 silenced cells. Silencing efficiency was measured through TRPA1 residual mRNA expression that was, on average, 20-40% (see Supplementary Fig 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 (* $p<0.05$).

Fig 6: Silencing TRPA1 channels produce a generalized reduction in the release of pro-inflammatory cytokines from CF primary bronchial epithelial cells exposed to P. aeruginosa or to SMM. HBECs primary cells from CF patients were transfected with TRPA1 siRNA (+) or scrambled (-) oligonucleotides for 72 hrs. Cells were subsequently exposed to heat-killed *P.aeruginosa* lab strain PAO1 (HKPAO) for further 24 h (A, C) or SMM for further 4hrs (B, D). Supernatants were collected and a panel of 27 human cytokines was analyzed by Bioplex multiplex assay (A-D). In panels A and B, 4 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 with HKPAO or SMM. In panel C, data of further 19 cytokines after HKPAO stimulation are shown with the exclusion of MIP-1α, IL-4, IL-5 and FGF, which were under the threshold of detectability. In panel D, data of further 20 cytokines after SMM challenge are reported, with exclusion of RANTES, IL-5 and FGF which were under the limit of detection. Cytokines release was measured in HBECs from 3 and 3 CF individuals assayed in duplicate for HKPAO and SMM respectively. Data are mean ± SEM. Student's t test for paired data (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) was performed.

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FIG 1

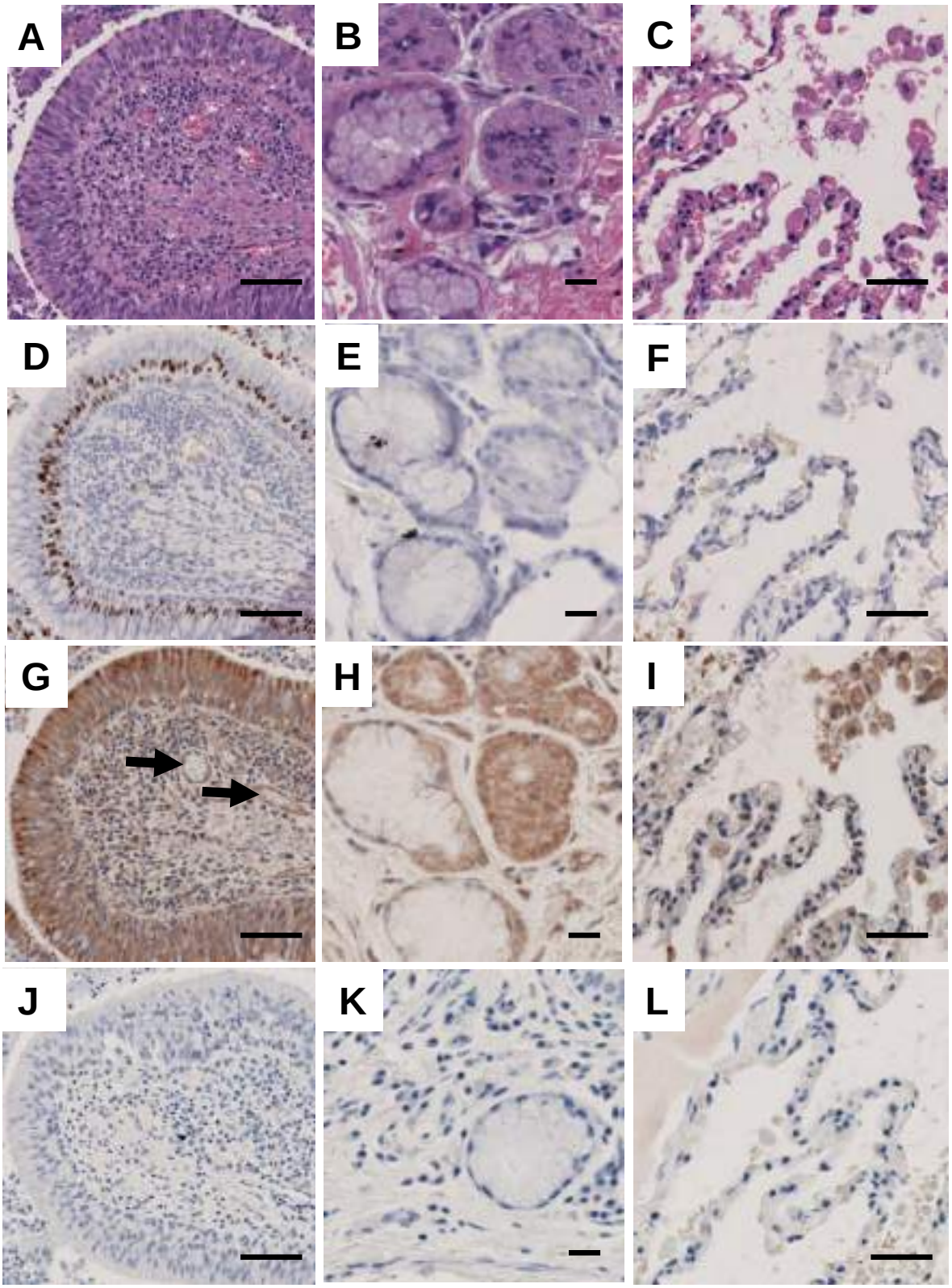
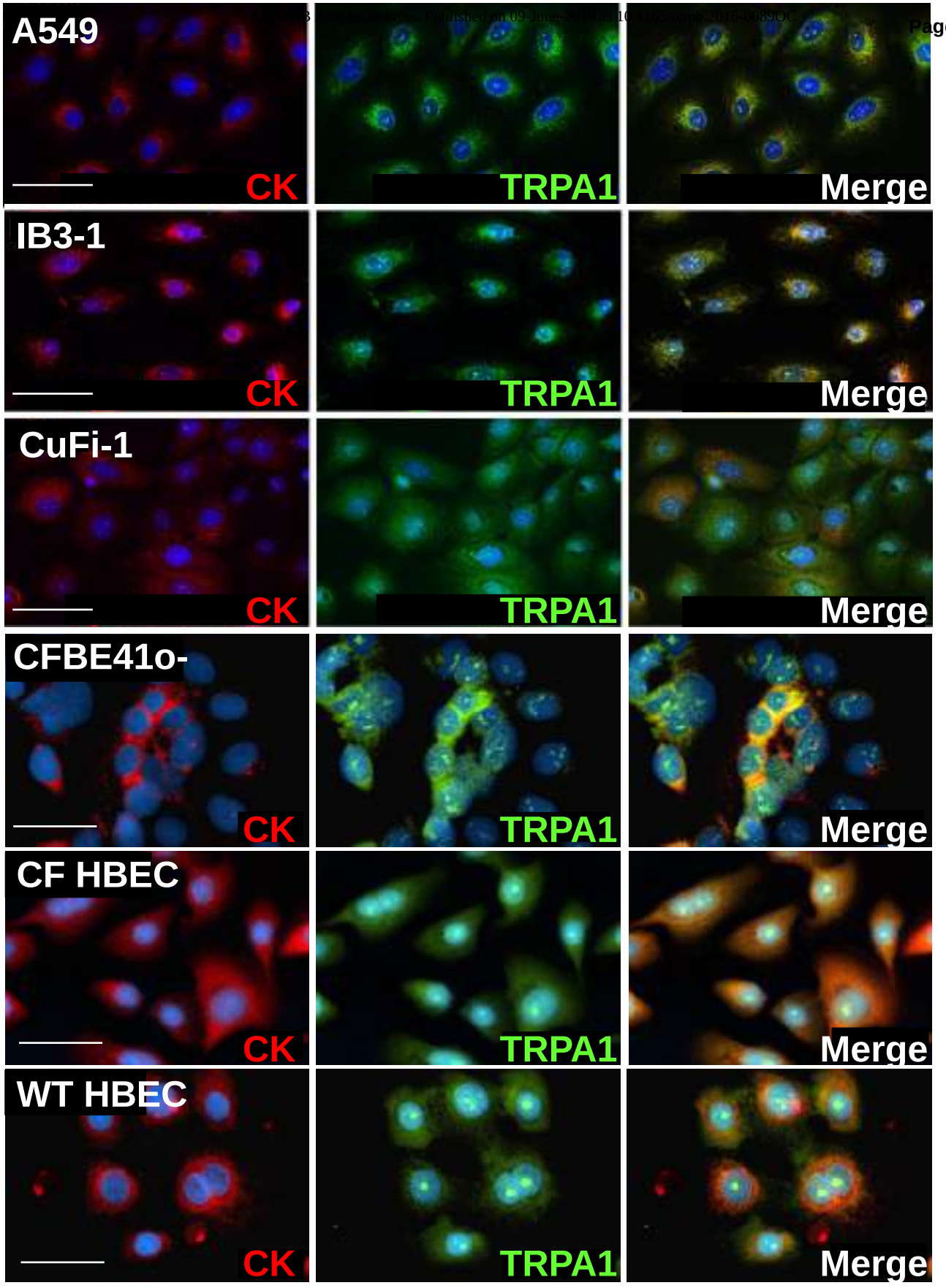
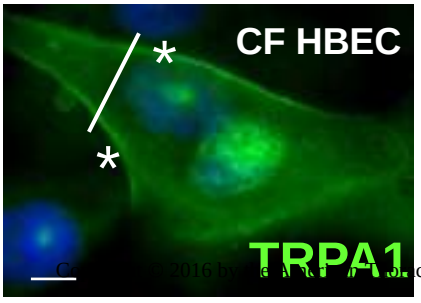


Figure 1





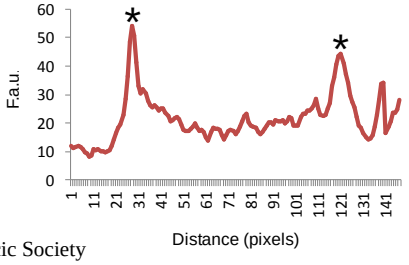


Figure 2

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FIG 3

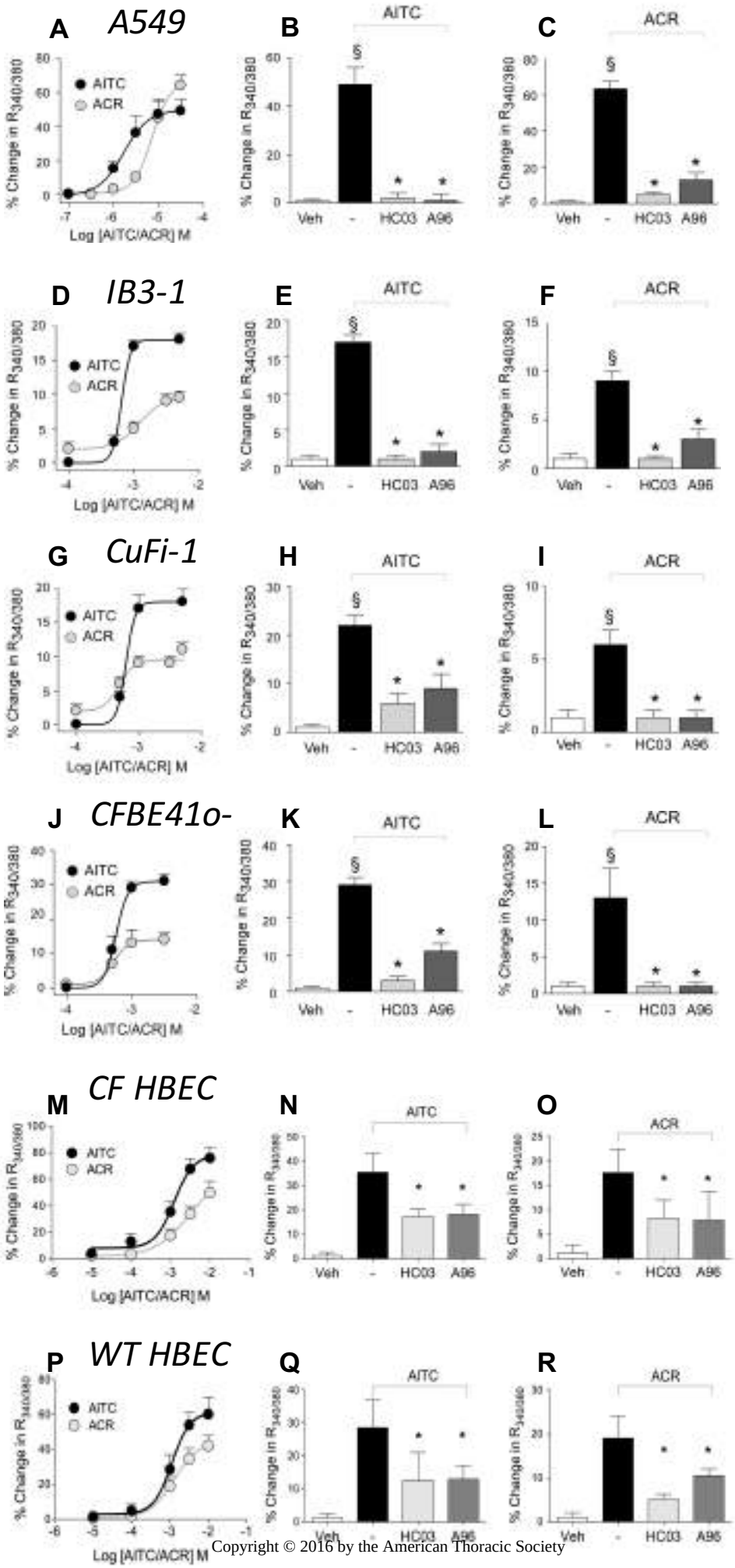

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FIG 4

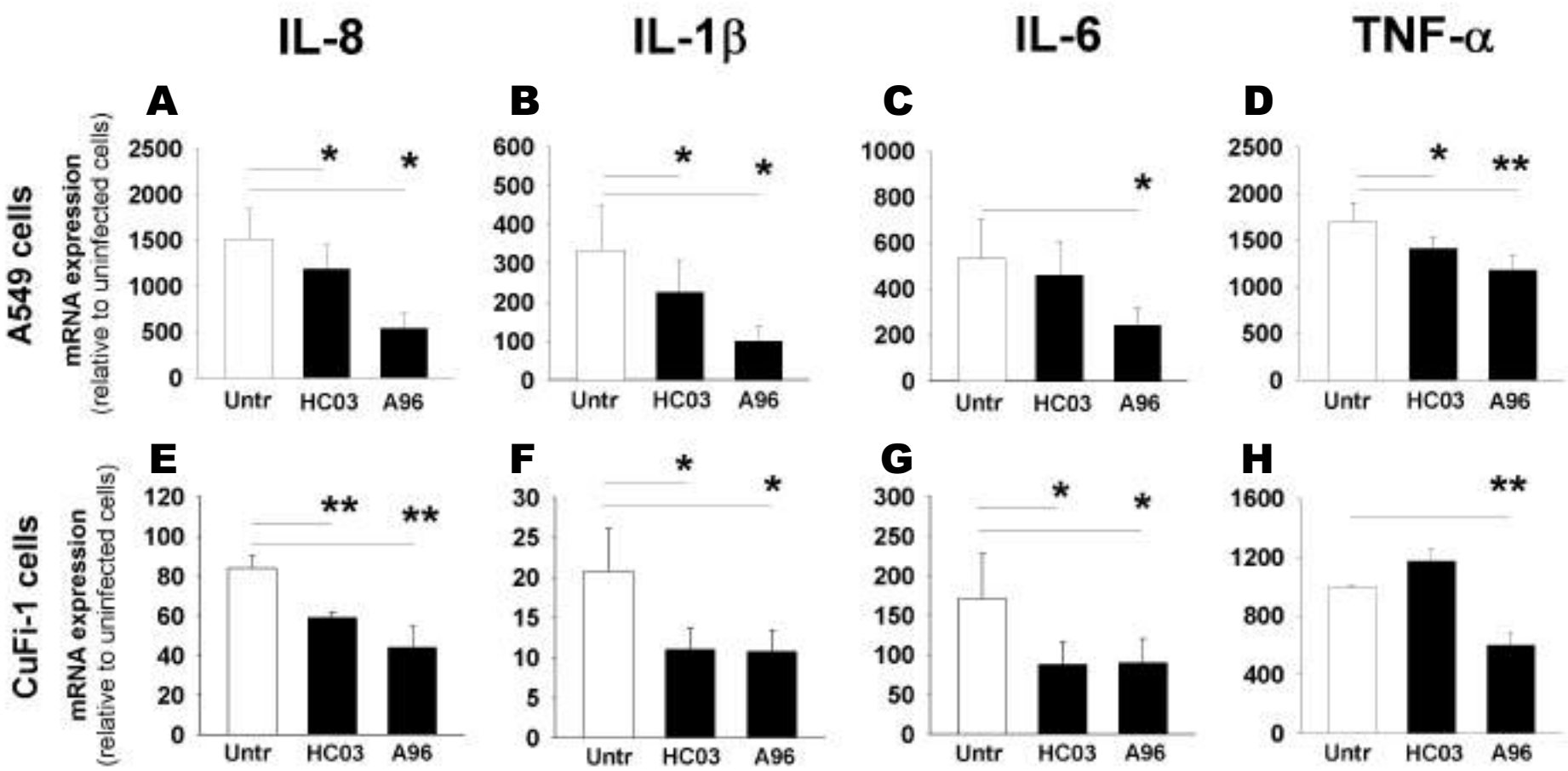

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FIG 5

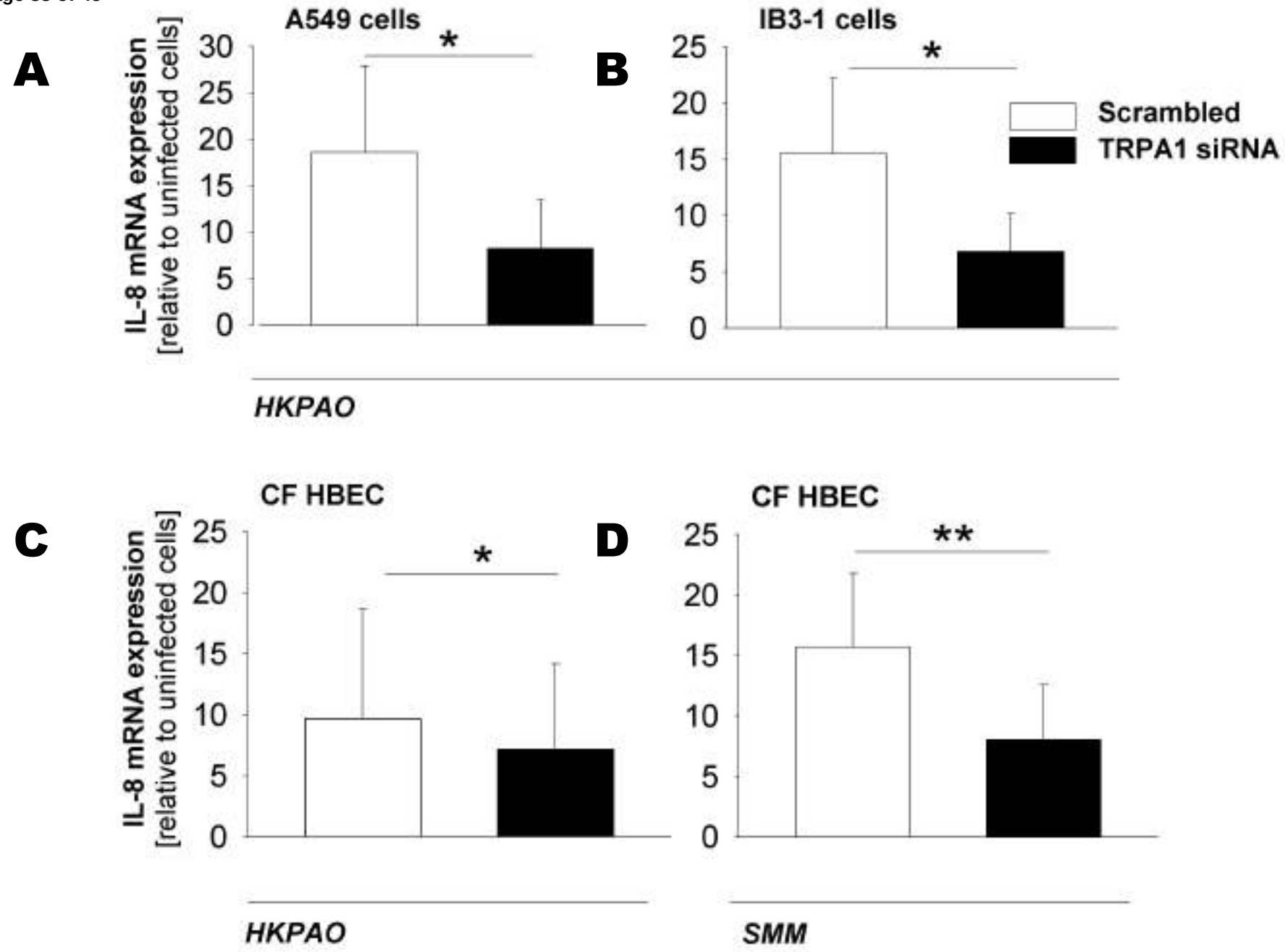
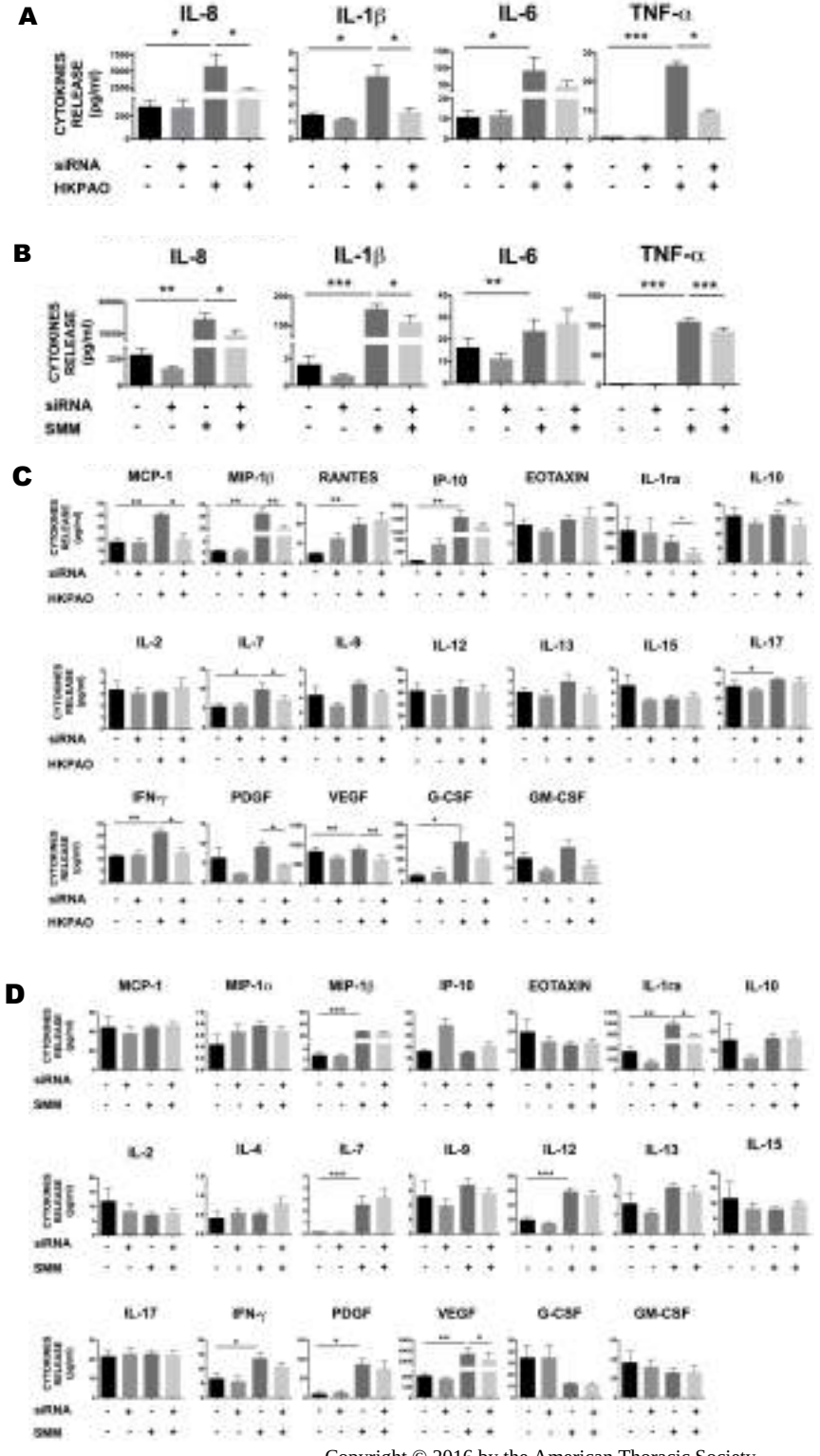


FIG 6



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ONLINE DATA SUPPLEMENT

Manuscript title:

TRPA1 channels modulate inflammatory response in respiratory cells from cystic fibrosis patients

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Cell culture

Human A549 alveolar type II-derived epithelial cells (American Type Culture Collection, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% low-endotoxin fetal bovine serum (FBS; Bio-Whittaker, Walkerswille, MD, USA). IB3-1 cells (LGC Promochem Europe, Teddington, UK) were

grown in Laboratory of Human Carcinogenesis (LHC)-8 basal medium (Biofluids, Rockville, MO, USA) 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, USA), 1 μg/ml BSA (Sigma-Aldrich, St. Luis, MO, USA) and 1 μg/ml human fibronectin (BD Biosciences). CuFi-1 cells, kindly donated by A. Klingelhutz, P. Karp and J. Zabner (University of Iowa, Iowa City, IA), were grown on human placental collagen type IV (Sigma-Aldrich)-coated flasks in bronchial epithelial growth medium (Cambrex Biosciences, Walkersville, MD, USA). CFBE41o- cells, kindly donated by Dr J. P. Clancy (University of Cincinnati, Children's Hospital Medical Center, Cincinnati, Ohio, USA), were grown in Eagle's minimal essential medium (MEM) (Euroclone, Milan, Italy), supplemented with 10% fetal bovine serum and L-glutamine, in plastic flasks coated with an extracellular matrix containing fibronectin, collagen and bovine serum albumin. Primary cultures of human bronchial epithelial cells (Italian Cystic Fibrosis Foundation, Primary Culture Core Facility) were grown in LHC9-RPMI (ratio 1:1) medium without serum, in plastics coated with rat collagen.

Calcium Imaging

Plated cells were loaded with 5 μM Fura-2AM-ester (Alexis Biochemicals; Lausen, Switzerland) added to the buffer solution (37 °C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After loading (40 min), cells were washed and transferred to a chamber on the stage of a Nikon Eclipse TE-2000U microscope for recording. Cells were excited alternatively at 340 nm and 380 nm to indicate relative intracellular calcium changes by the $Ratio_{340/380}$ ($R_{340/380}$) recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCSoftware, Florence, Italy). To evoke a TRPA1-dependent calcium response, cells were challenged with TRPA1 selective agonists, allyl isothiocyanate (AITC)

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and acrolein (ACR). Buffer solution containing 0.3 % dimethylsulfoxide (DMSO) was used as vehicle. Cells were pre-exposed (10 min) to selective TRPA1 antagonists HC-030031 and A-967079 or their vehicle (0.3% DMSO) before the acute addition of the TRPA1 agonists. Results are expressed: as the percentage of the increase in $R_{340/380}$ over baseline, normalized to the maximum effect induced by ionomycin (5 µM) added at the end of each experiment (% Change in $R_{340/380}$).

Immunohistochemical (IHC) staining

IHC analysis was performed on the section immediately following the one used for (H&E). Serial sections of 3-5 µm thickness were cut from formalin-fixed, paraffin-embedded samples. The sections were deparaffinized, subjected to antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with H_2O_2/3% for 10 minutes. After blocking with Protein Block serum-free (Dako), sections were incubated overnight at 4°C with TRPA1 antibody 1:2500 (Novus Biologicals, Cambridge, UK) and immune complexes were detected using the Bond Polymer Refine Detection Kit according to the manufacturer's protocol (Leica Biosystems, Wetzlar, Germany). Negative control was performed without primary antibody. Histological analyses were performed by an expert pathologist (E.G.). The images are representative of two CF patients and two normal controls.

Immunofluorescence assay and confocal analysis

Cells were grown in medium on autoclaved coverslips with the required coating at a density of 3×10^5 cells/mL. They were then fixed in ice-cold methanol/Acetone for 5 min at -20 °C, washed with PBS and blocked with BSA and NGS (1mg/ml, 10%) in PBS for 1h RT. The cells were then incubated with the primary antibodies to TRPA1 (Novus Biologicals, CO, USA, NB110-40763), and pan cytokeratin (Abcam, Cambridge, USA, ab6401), diluted 1:600 and 1:300 in blocking buffer respectively, for 1 hour at room

temperature. After 3 washes with PBS the cells were incubated for 2 hours with a goat polyclonal secondary antibody to rabbit IgG and goat polyclonal secondary antibody to mouse IgG respectively conjugated to Alexa Fluor® 594 and Alexa Fluor® 488 (R37121, R37116,ThermoFisher Scientific, Waltham, MA, USA,) diluted according to the manufacturer's recommendations (typically diluted 1:600). After extensive washes in PBS the coverslips were mounted using Fluoroshield™ with DAPI (Sigma Aldrich, F6057). The cells were visualized and digital images were captured using an Olympus BX51.

Confocal images were recorded using a digital imaging system based on laser-scanning confocal Zeiss LSM-510. The data were acquired and processed using the ImageJ analyzing program, and plotted as quantitation of TRPA1 fluorescence intensity along a line crossing the cell (thick white line in the micrograph of figure).

Transient silencing of TRPA1 channels expression

Transient silencing was carried out with TriFECTa RNAi Kit (Integrated DNA technologies, Coralville, IA, USA) accordingly to the manufacturer's instructions. Human bronchial epithelial cells were transfected with specific small-interfering RNA for TRPA1 duplexes complexed with cationic liposomes Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA,USA). TRPA1 siRNA or scrambled duplexes (20 nM) were added and incubated for 6 hours. Cells were then washed twice with culture medium and left at 37°C/5% CO_2 for a further 66 hours before stimulation with PAO1, HKPAO and SMM.

Stimulation with P. aeruginosa, heat killed P. aeruginosa (HKPAO) and Supernatant of Mucopurelent Material (SMM)

The well-characterized motile non-mucoid laboratory strain of *P. aeruginosa* PAO1 has been kindly donated by A. Prince (Columbia University, New York, NY,USA). Bacteria colonies from overnight cultures on trypticase soy agar plates (Difco, Detroit, MI, USA) were grown in 20 ml trypticase soy broth (Difco) at 37°C until an OD (A660 nm

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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 were added to cells at the concentration of about 100 CFU per cell. Heat killed PAO1 was obtained by growing *P. aeruginosa* for 24 hrs in 100 ml trypticase soy broth at 37°C and then by water-bath heating at 60°C for 30 minutes. Two aliquotes from this process were taken and both plated and inoculated in the required colture medium to test the success of bacteria killing. Aliquotes of HKPAO were then stored at -80°C and used just once. Supernatant of Mucopurulent Material was obtained from filtered supernatants of mucopurulent material (SMM) harvested from the airway lumens of excised human CF lungs infected by *P.aeruginosa* and *S.aureus*. All stimulation were carried out for 4 hrs, after that cells were harvested and lysed with Pure Link™ RNA Minikit (Ambion, ThermoFisher Scientific, MA, USA) and RNA were isolated and purified following the manufacturer's instructions.

Quantitative RT-PCR

After 4 hrs of SMM (supernatant of mucopurulent material) stimulation and after 24 hrs HKPAO challenge, the total RNA from different cell lines was isolated using the Pure Link™ RNA Minikit (Ambion), and 500 ng of RNA was reverse-transcribed to cDNA using the High Capacity cDNA Archive kit with random primers (Applied Biosystems, ThermoFischer Scientific). Reverse transcription reactions without the enzyme (RT-) were also performed to evaluate genomic DNA contamination. The relative gene expression quantification was performed using 7900 HT Fast Real-Time PCR systems (Applied Biosystems) and the following TaqMan assays were used: TRPA1 (Hs00929057_m1), IL-8 (Hs00174103_m1), GAPDH (Hs02758991_g1). IL-1β, IL-6, TNF-α, IL-8 and ACTB transcripts amplification was performed with Sybrgreen Master Mix (Applied Biosystems) and QuantiTect R primer assays: IL1B_1_SG QT00021385, IL6_1_SG QT00083720, TNF_3_QT01079561, IL8_1_SG QT00000322 and ATCB_2_QT01680476. Each sample

was amplified in triplicate and when the replicates deviated by more than 0.2 Ct from the mean were excluded from further analysis. ΔΔCt method was applied to perform relative quantification (Applied Biosystems User Bulletin 2) using GAPDH and ACTβ gene as normalizing genes for Taqman and Sybrgreen quantification, respectively. The ratios of relative expression of IL-8 mRNA over GAPDH or β-Actin, obtained in cells exposed to stimulation by different agents (PAO1, HKPAO or SMM), were then rescaled and calculated as fold changes over uninfected samples (considered equal to 1).

Bioplex assay and analysis

Cytokines released in culture medium were measured by Bio-Plex cytokine assay (Bio-Rad Laboratories, Hercules, CA). The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 µl of sample. In our experiments, we used the premixed multiplex beads of the Bio-Plex human cytokine Human 27-Plex Panel (Bio-Rad, Cat. n.o. 171-A11127), which included twenty-seven cytokines [IL-1β; IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(P70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFNγ, IP-10, MCP1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF]. Briefly, 50 µl of cytokine standards or samples (supernatants from treated cells) were incubated with 50 µl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking. Plates were then washed three times with 100 µl of Bio-Plex wash buffer using the Bio-plex Pro Wash Station (Bio-Rad Laboratories, Hercules, CA, USA), 25 µl of diluted detection antibody was added, and plates were incubated for 30 min at room temperature with shaking. After three washes, 50 µl of streptavidin-phycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Finally, plates were washed three times, beads were suspended in Bio-Plex assay buffer, and samples were

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analyzed on Bio-Rad 96 plate reader using the Bio-Plex suspension array system and Bio-Plex manager software (Bio-Rad Laboratories).

SUPPLEMENTARY FIGURES

Fig E1: **TRPA1 is expressed in bronchial columnar epithelial cells of normal lung.** Different 3-5 μm serial sections of human normal bronchi were stained for H&E (A, B and C), and TRPA1 protein (D, E and F) by immunocytochemistry. Expression of TRPA1 is positive in columnar epithelial cells (D), in submucosal glands sections, mainly in serosal cells (E) and in pneumocytes (F). Scale bar (black line) is 100 μm (panels A, C, D, F) or 20 μm (panels B, E).

Fig E2: Pre-absorption of TRPA1 antibody with immunogen peptide.

Antibody for TRPA1 was pre-absorbed with its immunogen peptide (TRPA1+PEP) and immunofluorescence was performed in A549, IB3-1, CuFi-1, CFBE41o-, CF HBEC and WT HBEC cells. Cells were fixed, permeabilized, and stained for CK (red), TRPA1+PEP (green) and nuclei (blue). The second panel of each cell type shows an almost abolished green signal for each cell type, indicating that TRPA1 green signals observed in Fig 2 are specific for TRPA1 channels. The third panel of each cell type shows merging of the two signals. Scale bar (white line) is 20 μm.

Fig E3: **Silencing of *TRPA1 channels in human bronchial epithelial cell models***

A549 and CF HBEC primary cells were transfected with TRPA1 and scrambled oligonucleotides. White bars represent scrambled treatment, black bars show TRPA1 silenced cells. TRPA1 mRNA levels were measured for A549 (A) (n= 4 experiments) and CF primary cells HBEC (B) (n= 4 individuals). Silencing efficiency was measured through

TRPA1 residual mRNAs expression that was, on average, 40% and 20% for A549 and HBEC respectively, relative to constitutive expression. Data are expressed relative to scrambled cells**.** Data are mean ± SEM of at least four experiments. Student's t test for paired data (** $p<0.01$, ***$p<0.001$) was performed.

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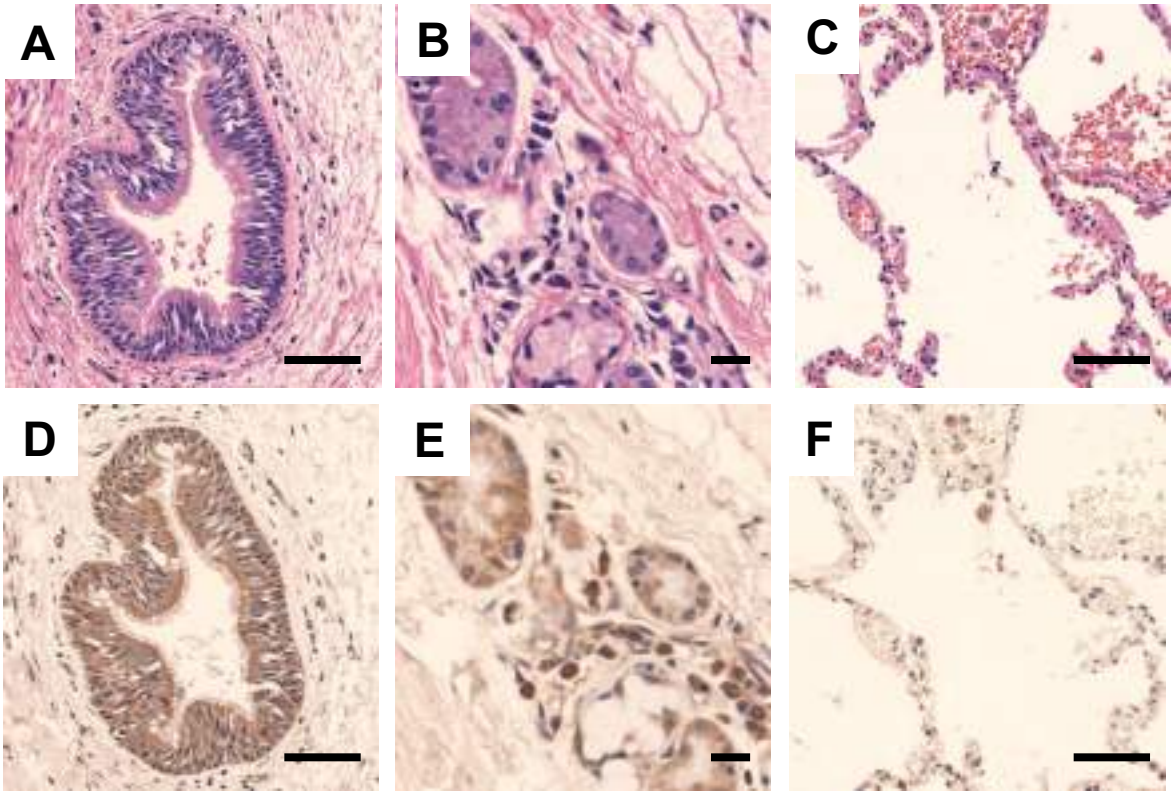
SUPPLEMENTARY FIG E1



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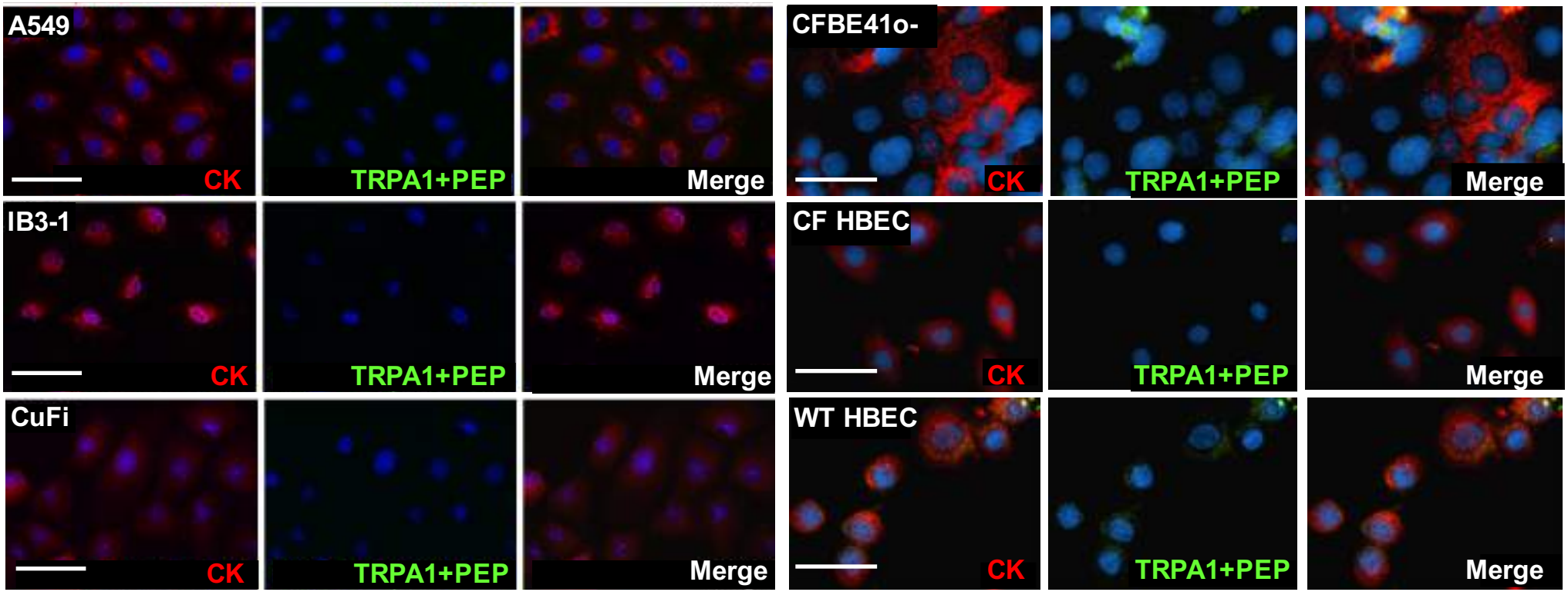
SUPPLEMENTARY FIG E2



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SUPPLEMENTARY FIG E3