Proinflammatory Cytokine Secretion is Suppressed by TMEM16A or CFTR Channel Activity in Human Cystic Fibrosis Bronchial Epithelia

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Abbreviations: ALC, air-liquid culture; ASL, airway surface liquid; CaCC, Ca2+-activated chloride channel; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; cpt-cAMP, 8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphate; Dox, doxycycline; ECM, extracellular matrix; GPCR, G-protein coupled receptor; HBE, human bronchial epithelia; IB, immunoblot; IBMX, 3-Isobutyl-1-methyl-xanthine, IL-8, interleukin-8, Inh172, CFTR inhibitor 172; LLC, liquid-liquid culture; MOI, multiplicity of infection; P2YR, P2Y receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; PM, plasma membrane; TLR, toll-like receptor.

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Abstract

Cystic fibrosis (CF) is caused by the functional expression defect of the CF transmembrane conductance regulator (CFTR) chloride channel at the apical plasma membrane. Impaired bacterial clearance together with hyperactive innate immune response are hallmarks of the CF lung disease, yet the existence of and mechanism accounting for the innate immune defect that occurs prior to infection remain controversial. Inducible expression of either CFTR or the calcium-activated chloride channel TMEM16A attenuated the proinflammatory cytokines IL-6,
IL-8 and CXCL1/2 in two human respiratory epithelial models under air-liquid, but not liquid-liquid interface culture. Expression of wild-type but not the inactive G551D-CFTR indicate that secretion of the chemoattractant IL-8 was inversely proportional to CFTR channel activity in cfrΔF508/ΔF508 immortalized and primary human bronchial epithelia. Likewise, direct but not P2Y receptor-mediated activation of TMEM16A attenuated IL-8 secretion in respiratory epithelia. Thus augmented proinflammatory cytokine secretion caused by defective anion transport at the apical membrane may contribute to the excessive and persistent lung inflammation in CF and, perhaps in other respiratory diseases associated with documented downregulation of CFTR (e.g. chronic obstructive pulmonary disease). Direct pharmacological activation of TMEM16A offers a potential therapeutic strategy to reduce the inflammation of CF airway epithelia.

**Introduction**

Cystic fibrosis (CF) is caused by mutations that impair the biosynthesis, function and/or stability of the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel (Riordan et al., 1989; Welsh and Smith, 1993; Zielenski and Tsui, 1995). Attenuated chloride and bicarbonate secretion across the apical plasma membrane (PM) of CF airway epithelia lead to dehydration and increased viscosity of the airway surface liquid (ASL) as well as impaired mucociliary clearance, and bacterial colonization in concert with excessive inflammatory response of the CF respiratory epithelia (Derichs et al., 2011; Matsui et al., 1998; Tarran et al., 2001). The augmented release of proinflammatory cytokines promotes neutrophil chemotaxis and degranulation, further compromising mucociliary clearance and enhancing inflammation (Berger, 2002; Downey et al., 2009; Elizur et al., 2008). This positive feed-back loop, which culminates in irreversible tissue damage and respiratory insufficiency, represents the major cause of mortality in CF (Ratjen and Doring, 2003; Rowe et al., 2005). A major unresolved question is whether the hyperinflammatory state of airway epithelia is initiated by the
CFTR functional expression defect and precedes infection or is merely the consequence of impaired mucociliary clearance and bacterial colonization (Machen, 2006; Ratjen, 2006).

An early onset of increased proinflammatory cytokine secretion into the bronchoalveolar lavage in CF infants has been reported, but whether this occurs prior to infection remains controversial (Armstrong et al., 2005; Muhlebach and Noah, 2002). Impaired bacterial clearance and hyperinflammation was proposed to be a consequence of lung infection in the CF mouse, pig and ferret (Stoltz et al., 2010; Sun et al., 2010; van Heeckeren et al., 2006). Intriguingly, the pancreas in newborn CF pigs shows hallmarks of increased immune and inflammatory response without apparent infection (Abu-El-Haija et al., 2011). Elevated interleukin-8 (IL-8) production has been reported in CF cell lines and primary cultures both constitutively and following activation of Toll-like or tumor necrosis factor alpha receptors, implying a CFTR-dependent alteration in the innate immune response (Ribeiro et al., 2005; Roussel et al., 2011; Vandivier et al., 2009). However, this could not be reproduced in some cellular models (Fulcher et al., 2009; Hybiske et al., 2007; Pizurki et al., 2000), while in others CFTR appeared to have a permissive role in IL-8 secretion (John et al., 2011; John et al., 2010). Resolving this fundamental and long-lasting controversy is hampered by the modification of the epigenome configuration following repeated infection and inflammation, which could modulate the innate immune response in freshly isolated airway epithelial cells and primary cultures (Adcock et al., 2007; Brigati et al., 2010). Furthermore, whether CFTR expression/signalling (Bensalem et al., 2005; Estell et al., 2003; Hallows et al., 2006; Mehta, 2007) or its transport function (Hunter et al., 2010; Perez et al., 2007; Vega-Carrascal et al., 2011) is necessary and sufficient to suppress proinflammatory cytokine secretions remains an unanswered question.

The recently identified TMEM16A channel (also called Ano1) confers calcium activated chloride conductance to the PM of respiratory epithelia (Caputo et al., 2008; Schroeder et al.,
2008; Yang et al., 2008). Transient mobilization of intracellular Ca\(^{2+}\) by G-protein coupled receptor (GPCR) signalling is widely used to promote TMEM16A activation (Caputo et al., 2008; Yang et al., 2008) and P2Y receptor (P2YR) mediated activation of TMEM16A has been explored as a potential therapeutic approach in CF (Accurso et al., 2011; Yerxa et al., 2002).

We examined the impact of CFTR variants and TMEM16A expression on proinflammatory cytokine secretion by human airway epithelia models lacking endogenous CFTR. Inducible expression systems were developed to tightly control the anion channel expression and minimize the impact of genetic heterogeneity, epigenetic variation and phenotypic drift of epithelia. Here we show that functional CFTR expression attenuates IL-8 secretion from \(cfr^{\Delta F508/\Delta F508}\) immortalized and primary human bronchial epithelia under air-liquid culture (ALC), but not liquid-liquid culture (LLC). Likewise, direct but not P2YR-mediated activation of TMEM16A suppressed IL-8 secretion. Taken together, these findings provide a novel link between transepithelial anion transport and IL-8 secretion and suggest that constitutive activation of TMEM16A may be a useful therapeutic target for anti-inflammatory treatment in CF.

Results

Cellular model with inducible CFTR expression to investigate the innate immune response of human bronchial epithelia

We selected CFBE41o- (CFBE) cells, a well-characterized CF airway cell line, to examine the consequence of CFTR expression on proinflammatory cytokine secretion. CFBE cells were originally derived by immortalizing human bronchial epithelial cells from a patient with \(cfr^{AF508/AF508}\) genetic background and have no detectable expression of the mutant protein (Ehrhardt et al., 2006). To develop cells with adjustable CFTR expression, CFBE cells were sequentially transduced with lentiviral particles encoding a tetracycline-controlled transactivator (Gossen and Bujard, 1992) and with wild-type (wt) CFTR bearing an extracellular 3HA tag.
(Sharma et al., 2004). The inducible CFTR expression system minimized genetic and epigenetic heterogeneity caused by clonal selection (Babnigg et al., 2000) and the possibility of phenotypic drifts during the propagation of the cells.

Wt CFTR-3HA expression was undetectable by immunoblot (IB) in CFBE cells, when the transactivator was expressed alone (TetON+) or in combination with wt CFTR-3HA (iCFTR-) without doxycycline (dox) induction (Fig.1A). Increasing concentrations of dox induced progressively higher accumulation of the complex-glycosylated form of CFTR, as measured by IB with anti-HA or anti-CFTR antibody (Ab) after 3 days induction (Fig. 1A, B). At maximal dox-induction the CFTR expression level was comparable to that seen for endogenous CFTR in Calu-3 cells (see Fig. 2D). CFTR expression reached a steady-state level after three days of dox induction (Fig.S1A). A comparable kinetics and dox concentration dependence was found for CFTR appearance at the PM, determined by cell surface ELISA (Fig. 1C and S2A) (Okiyoneda et al., 2010).

Indirect immunostaining and laser confocal fluorescence microscopy confirmed that wt CFTR was predominantly confined to the apical PM in filter-grown, polarized CFBE cells (Fig. 1D).

Short-circuit current measurement ($I_{sc}$) verified that the CFTR-3HA channels were active. Transactivator transduced (TetON+) CFBE cells had negligible cAMP-stimulated chloride transport activity ($0.03 \pm 0.4 \mu A/cm^2$). CFTR chromosomal integration (iCFTR-) however, was sufficient to result in a small forskolin-inducible $I_{sc}$ ($4.9 \pm 0.4 \mu A/cm^2$) in the absence of dox-induction. This could be attributed to induction independent low level of transcriptional and translational activity of the transgene. Remarkable, dox induced a ~30 fold increase in the $I_{sc}$ ($139 \pm 9 \mu A/cm^2$) (Fig. 1E). The cAMP-dependent protein kinase (PKA) activated current was sensitive to inhibitor-172 (Inh172), a CFTR blocker (Ma et al., 2002).
CFTR expression attenuates the proinflammatory cytokines IL-8, IL-6 and CXCL1/2 in CFBE14o- epithelia under ALC

The CF lung inflammation is characterized by elevated secretion of proinflammatory cytokines, including the chemokine IL-8 with a pivotal role in recruiting the excessive number of neutrophils into the airway lumen (Downey et al., 2009; Elizur et al., 2008; Ratjen and Doring, 2003). To examine whether re-establishing CFTR function would lead to a decrease of the innate immune response of CF epithelial cells in the absence of infection, the secretion profile of 42 cytokines from CFBE epithelia was determined. Induction of CFTR expression significantly attenuated basolateral secretion of the proinflammatory cytokines IL-8, IL-6, CXCL1-3 by CFBE cells grown on permeable filter supports under ALC, mimicking the native environment of the respiratory epithelia (Fig. 1F) (Dvorak et al., 2011). Remarkably, the CFTR suppressive effect on proinflammatory cytokine secretion was absent in epithelia grown under LLC (Fig. S1B). While we cannot rule out that CFTR may influence the biogenesis, trafficking and/or PM fusion of secretory vesicles containing proinflammatory cytokines (Stanley and Lacy, 2010), these possibilities are unlikely since CFTR induction significantly decreased the mRNA level of IL-8, IL-6, CXCL1 and CXCL2 in CFBE epithelia kept under ALC (Fig.1G-J). CFTR expression did not significantly decrease CXCL3 mRNA (Fig. S1D). To generate a second expression model, the human papillary adenocarcinoma cell line NCI-H441 (H441) of Clara cell origin (Gazdar et al., 1990) with no detectable PKA-stimulated channel activity was transduced with inducible wt CFTR (Fig. 2D and S2C). Similar to CFBE epithelia, inducing CFTR expression in H441 monolayers attenuated basolateral IL-8, IL-6 and CXCL1-3 secretion under ALC, but had little effect on cells grown in LLC (Fig. S1D).

In subsequent experiments we focused on the secretion of the chemokine IL-8, a potent stimulant for neutrophil recruitment. Consistent with the reduction of mRNA levels shown in Fig.
basolateral IL-8 secretion determined by ELISA was attenuated in CFTR expressing CFBEE cells grown under ALC. The suppressive effect was absent in epithelia under LLC and within the first two hours after transferring the monolayers from LLC to ALC (Fig. 2A, B). CFTR PM density and constitutive channel function was only slightly affected by shift from LLC to ALC. Returning the epithelia to LLC completely abrogated the suppressive effect of CFTR in IL-8 secretion (Fig. S1E, F).

As little as ~10% of the maximum CFTR expression (at 5 ng/ml dox) was sufficient to suppress IL-8 secretion (Fig. 2C, also see Fig. 1B, C). Importantly, while CFTR suppression of IL-8 secretion was detected in most cell clones, the absolute amount of IL-8 secretion and its fractional attenuation by CFTR differed considerably (Fig. S1G). Thus the integration site of the virus cDNA and/or genetic and epigenetic variability of the host cell may influence the regulation of IL-8 secretion in clonally isolated cell populations. To avoid potential bias introduced by clonal selection, all subsequent studies used mixtures of at least hundred individual clones.

**CFTR channel function is required to suppress IL-8 secretion**

To assess whether CFTR apical PM expression is involved as a signalling platform (Li and Naren, 2005; Mehta, 2007) or as an anion translocation pathway, the effect of the non-functional G551D CFTR mutant expression on IL-8 secretion was established. G551D and wt CFTR have similar biosynthetic and endocytic processing, but the G551D CFTR cannot be activated by PKA stimulation (Anderson and Welsh, 1992; Barriere et al., 2009). The concentration- and time-dependencies of G551D and wt CFTR expression in CFBEE cells were similar (Fig. 2E and S2A). As expected, G551D CFTR failed to confer PKA-stimulated PM iodite conductance in the absence of potentiator in both, CFBEE and H441 epithelia (Fig. S2B, C). Induction of wt, but not G551D CFTR, significantly attenuated IL-8 secretion under ALC, but not under LLC in both
model systems (Fig. 2F, G), strongly suggesting that CFTR channel activity is indispensable for suppressing IL-8 secretion in these polarized respiratory epithelia.

If CFTR channel activity plays a critical regulatory role in IL-8 secretion, CFTR inhibition should enhance IL-8 secretion similar to that observed in non-induced CFBE epithelia. This was indeed the case. Both, the robust forskolin-activated and the small constitutive $I_{sc}$ current detected in the absence of activator were inhibited by three structurally distinct CFTR blockers; Inh172, the pyrimido-pyrrolo-quinoxalinedione PPQ102 (Tradtrantip et al., 2009) and the benzopyrimido-pyrrolo-oxazinedione compound BPO27 (Snyder et al., 2011) (Fig. 3A-C). PPQ102 and BPO27 abolished the suppressive effect of wt CFTR on IL-8 secretion in iCFTR+ CFBE monolayers. These inhibitors had no influence on the IL-8 production by TetON or iCFTR- cells, ruling out non-specific effects (Fig. 3D). In contrast, Inh172 produced a two-fold CFTR-independent activation of IL-8 secretion, conceivable by eliciting reactive oxygen species production in mitochondria (Kelly et al., 2010) (Fig. S2D).

To suppress IL-8 secretion from iCFTR- CFBE cells, a panel of PKA agonists was tested. The low amount of constitutively translated CFTR was activated by forskolin (adenylate cyclase activator), 3-Isobutyl-1-methyl-xanthine (IBMX, inhibitor of phosphodiesterase) or 8-(4-Chlorophenylthio)-adenosine-3’,5’-cyclic monophosphate (cpt-cAMP), a cell permeant cAMP analogue. According to $I_{sc}$ measurements the residual activity represents only ~3% of the maximally induced CFTR channel activity (Fig. 3E). All three PKA agonists with distinct pharmacological targets were able to suppress IL-8 secretion in iCFTR- CFBE cells without interfering with the CFTR-independent IL-8 secretion of TetON+ cells (Fig. 3F). Together these results suggest that maximal activation of a small number of CFTR channels or the constitutive partial activity of a large number of CFTR channels are equally efficacious in suppressing the proinflammatory cytokine secretion.
Non cell-autonomous mechanism of CFTR-induced suppression of IL-8 secretion in CFBE and primary human bronchial epithelia (HBE)

Based on the discovery that ALC is necessary for CFTR-induced suppression of the IL-8 secretion, it was plausible to assume that CFTR may influence the ion composition and physico-chemical properties of the ASL via a non cell-autonomous mechanism. This means that CFTR expression in only a subset of cells would suppress the IL-8 secretion of the entire epithelial monolayer. To test this hypothesis, the effect of increasing proportions of CFTR expressing cells (iCFTR+) in a mixed cell populations that contained iCFTR+ and TetON cells at ratios of 1:9 and 1:4 on IL-8 secretion was assessed (Fig. 4A-C). After 5 days on filter supports these ratios resulted in a mixed cell population, containing ~10% or ~20% of CFTR expressing cells, respectively, as determined by immunostaining and cell surface ELISA (Fig. 4A, B). This was sufficient to suppress the basolateral IL-8 secretion by 34% and 77%, respectively, relative to that of monolayers consisting only of CFTR expressing cells. This indicates a significantly higher IL-8 attenuation than would be expected for a cell-autonomous mechanism that predicts linear correlation between the number of CFTR expressing cells and the amount of IL-8 suppression (Fig. 4C).

To confirm the results obtained with inducible epithelial models primary HBE cells isolated from lung tissue of $cfr^{AF508/AF508}$ CF individuals (Fulcher et al., 2005) were transduced with lentiviral particles (multiplicity of infection (MOI) 4 and 8) conferring constitutive wt or G551D CFTR expression. IB and immunostaining indicated that wt or G551D CFTR were expressed in ~10% of the epithelial cells after 15 days culture on filter supports (Fig. 4D, E). While the basolateral IL-8 secretion varied considerably between individuals, normalization with control cells that had been transduced with lentiviral particles containing empty vector showed that wt CFTR reduced the mean IL-8 secretion by 24% (Fig. 4F). In contrast, G551D CFTR
expression had no effect (Fig. 4F). These results support the relevance of CFTR modulation of the innate immune response regulation in primary airway epithelia and substantiate the notion that CFTR-mediated ion transport of a subpopulation of cells is sufficient to modulate the inflammatory state of the monolayer, likely through influencing ASL properties.

**Endogenous TMEM16A activation fails to suppress IL-8 secretion by CFBE monolayers**

Based on the suppressive effect of CFTR channel activity on IL-8 secretion, we hypothesized that TMEM16A-mediated anion transport may similarly attenuate IL-8 production in respiratory epithelia. This was tested by modulating the activity of endogenous TMEM16A in CFBE epithelia.

TMEM16A activity was monitored by $I_{sc}$ upon P2YR activation with ATP or UTP following the channel inhibition or activation by three methods. 1) The Ca$^{2+}$-dependent chloride secretion caused by the P2YR agonists (ATP or UTP) was profoundly reduced by shRNA TMEM16A (Fig. 5A and S3A). Gene silencing decreased the TMEM16A mRNA by >70% (Fig. S3D) and unmasked K$^+$ secretion that was blocked with TREM34, an inhibitor of the Ca$^{2+}$-activated potassium channel K$_{Ca}$3.1 (Fig. S3A). 2) Buffering cytosolic Ca$^{2+}$ concentration transients with BAPTA-AM was equally efficient in inhibiting ATP- or UTP-induced Ca$^{2+}$-activated chloride and potassium secretion (Fig. 5A and S3B). 3) As a complementary approach, TMEM16A was activated by ATP or UTP alone, or following long-term IL-4 treatment to stimulate TMEM16A transcription (Caputo et al., 2008) (Fig. 5A and S3C, E).

Inhibition of TMEM16A by BAPTA-AM, shRNA or depleting nucleoside triphosphates with extracellular apyrase all failed to stimulate IL-8 secretion in TetON CFBE cells and did not changed the differential IL-8 secretion in presence or absence of CFTR (Fig. 5B, C and S3F). Likewise, IL-8 secretion remained unaltered upon agonist-dependent activation of TMEM16A without or with IL-4 treatment (Fig.5D and S3G). These results suggest that endogenous
TMEM16A activity confers negligible chloride conductance relative to that of CFTR in CFBE epithelia, therefore it is not sufficient to modulate IL-8 secretion. To enhance the PM anion transport activity, the impact of TMEM16A overexpression on IL-8 secretion was evaluated next.

**TMEM16A is localized at the apical and lateral membranes of polarized epithelia**

To establish whether the localization of heterologously expressed TMEM16A mimics its endogenous counterpart, a prerequisite for anion secretion, we used immunohistochemistry, domain specific biotinylation and I$_{sc}$ measurements. Inducible overexpression of TMEM16A in CFBE epithelia was accomplished by using the splice isoform (abc) containing a partial internal Flag epitope (DYKDDDK) close to the N-terminus (Caputo et al., 2008) (Fig. 6A). For immunostaining a myc-epitope was attached to the C-terminus. Dox-induced TMEM16A-myc was expressed with the predicted molecular mass and was confined to the apical and lateral PM membrane in stably transduced CFBE epithelia (Fig. 6A, B), as well as in transiently transduced MDCK cells (Fig. 6C), an established model for epithelial polarity.

Polarized distribution of TMEM16A was determined by domain-specific PM biotinylation on filter grown CFBE epithelia (Sargiacomo et al., 1989). Quantitative IB analysis revealed that ~65% of TMEM16A were confined to apical PM, while 35% were localized to the basolateral PM in parental or non-induced cells (Fig. 6D). Overexpression of TMEM16A at low dox level (~70-fold increased over endogenous level at 5 ng/ml dox) modestly shifted the apical-to-basolateral expression ratio to 50:50%. Strong TMEM16A overexpression (~200-fold over endogenous level at 500 ng/ml dox) reverted the apical-to-basolateral ratio to 40:60% (Fig. 6D and Fig. S4B).

Inducible overexpression of TMEM16A led to a dox-dependent increase in ATP or UTP stimulated Ca$^{2+}$-activated chloride secretion that paralleled the TMEM16A protein expression in CFBE cells (Fig. S4A, B). To evaluate the polarized distribution of TMEM16A functionally, the
basolateral or the apical PM was permeabilized with nystatin prior to the Isc measurement. After apical permeabilization the ATP-stimulated basolateral current was sensitive to the TMEM16A inhibitor A01, but not to NKCC, Na+/K+-ATPase or K+ channel inhibition, indicating TMEM16A mediated chloride transport (Fig. S4D). Based on domain-specific current densities, the apical-to-basolateral PM conductance ratio was found to be 2:1 for both endogenous and leaked TMEM16A (Fig. 6E). The functional distribution of the highly overexpressed channel was decreased to 1.5:1 ratio (Fig. 6E). Jointly, these biochemical and functional assays confirmed the immunohistochemical localization results and showed that TMEM16A is expressed at the apical and lateral PM of polarized respiratory monolayers.

**TMEM16A overexpression attenuates proinflammatory cytokine secretion of CFBE epithelia under ALC**

The effect of TMEM16A overexpression on proinflammatory cytokine secretion was evaluated next. TMEM16A induction attenuated basolateral secretion of the proinflammatory cytokines IL-8, IL-6, CXCL1-3 and CCL2 in CFBE cells grown on permeable filter supports under ALC, but had little effect on cells in LLC (Fig. S5A). This was confirmed by measuring the relative mRNA abundance of cytokines (Fig. S5B-H). Similar to the mRNA levels, IL-8 secretion was attenuated proportionally with TMEM16A expression and reached 50% inhibition of parental (TetON) or non-induced (iTMEM16A-) cells, resembling the suppressive effect of CFTR (Fig. 7A, B and also see Fig 2A). The TMEM16A mediated attenuation was abrogated under LLC and in cells loaded with the high affinity Ca2+-chelator, 5,5’-dimethyl-BAPTA-AM (Kd 40 nM for Ca2+-binding) (Fig. 7A-B).

The use of A01, a TMEM16A specific blocker (Namkung et al., 2011a) or the Ca2+-chelator 5,5’-dimethyl-BAPTA-AM verified that elevated chloride secretion in dox-induced TMEM16A expressing CFBE epithelia was independent of ligand-induced P2YR activation (Fig. 7C, D).
The $I_{sc}$ generated by constitutively active TMEM16A was ~50% that of detectable in CFTR overexpressing cells in the absence of exogenous PKA stimulation (see Fig. 3B, C). Consistent results were obtained by measuring the halide conductance with cytosolic YFP-H148Q/I152L/F46L, a halide-sensitive fluorescent protein (Ferrera et al., 2009) (Fig. 7E, F and S5I). The increased halide conductance of CFBE epithelia exposed to dox-induction was sensitive to both 5,5’-dimethyl-BAPTA-AM and AO1 (Fig. 7E-F). These observations strongly suggest that constitutive activity of overexpressed TMEM16A is responsible for IL-8 suppression by compensating for the loss of CFTR transport activity in CFBE cells. Combining the data, an inverse correlation is apparent between the magnitude of IL-8 secretion relative to TetON cells and the $I_{sc}$ carried by the TMEM16A or CFTR channels at various dox-induction and inhibition levels (Fig. 7G).

**Direct, but not P2YR-dependent TMEM16A activation suppresses IL-8 secretion**

Since P2YR-dependent activation of the endogenous TMEM16A was unable to attenuate IL-8 secretion of CFBE epithelia, we examined whether TMEM16A overexpression could confer P2YR agonist-mediated regulation of IL-8 secretion. To this end, overexpressed TMEM16A was activated by ATP, UTP or the non-hydrolysable analogues, ATPγS or UTPγS that stimulated TMEM16A with comparable efficiency (Fig. S5J). To our surprise neither ATP nor ATPγS activation of P2YRs potentiated the TMEM16A suppressor effect on IL-8 secretion in dox-induced CFBE epithelia (Fig. 8A-B). Apical exposure to UTP exerted a modest, but significant inhibition on IL-8 secretion independently of TMEM16A expression (Fig. 8A also see Fig. 5D). This was not observed with UTPγS (Fig. 8B), suggesting that UTP or its hydrolytic intermediate may trigger a signalling cascade that attenuates IL-8 secretion independently of TMEM16A.
$I_{sc}$ measurements of both endogenous and overexpressed TMEM16A activity revealed that P2YR agonist-induced chloride secretion was transient and coupled to rapid inactivation in monolayers (Fig. 8C, S3A-C and S4A). The inactivation kinetics were independent of the TMEM16A expression levels, implying that basolateral chloride influx was not rate limiting (Fig. 8C). ATP stimulation for 15 min led to near complete homologous desensitization of P2YRs, indicated by the marginal chloride secretion elicited by repeated ATP exposure (Fig. 8G and insert in panel D). Resensitization after the removal of extracellular ATP was slow with 50% recovery of the ATP-stimulated $I_{sc}$ requiring ~30 min (Fig. 8D). Homologous desensitization likely occurs upstream of PLC activation, considering the limited heterologous desensitization of the TMEM16A-mediated $I_{sc}$, activated by histamine and the muscarinic receptors in the presence of ATP (Fig. 8E-G). In accord, cytosolic Ca$^{2+}$ mobilization was inhibited only by 20-25% for carbachol or histamine stimulation after ATP pre-treatment, while it was attenuated by 75-80% for a second ATP or UTP exposure (Fig. 8H-J). Thus, rapid inactivation of P2YR explains the inability of receptor agonist to suppress IL-8 secretion despite the preserved responsiveness of TMEM16A to activation by other Ca$^{2+}$-mobilizing GPCRs in CFBE monolayers.

In sharp contrast to the ligand-induced transient stimulation of the P2YRs, the Ca$^{2+}$-signalling independent activator F (Namkung et al., 2011b) led to a significantly more sustained activation of TMEM16A (Fig. 9A-B). As a consequence, activator F was able to attenuate IL-8 secretion in CFBE cells with endogenous or low heterologous TMEM16A channel expression (Fig. 9C-D). Similar results were obtained for H441 cells with inducible overexpression of TMEM16A (Fig. 9E and S5K). This effect is specific, since it was diminished by the shRNA mediated ablation of endogenous TMEM16A expression in CFBE (Fig. 9D), suggesting that constitutive activation of endogenous TMEM16A may reduce the innate immune response and tissue damage of the CF lung.
Discussion

The question if hyperinflammation is a sole consequence of ASL dehydration and bacterial colonization or whether these processes can be primed by the CFTR functional expression defect has been extensively investigated by a number of laboratories, but remains controversial (Fulcher et al., 2009; Hybiske et al., 2007; Pizurki et al., 2000; Ribeiro et al., 2005; Roussel et al., 2011; Vandivier et al., 2009). Several factors could account for the conflicting results.

The expression of proinflammatory cytokines might be modulated by the epigenome configuration that is influenced by repeated infection and inflammation in CF. The hypoxic environment associated with infection and inflammation induces the production of hypoxia-inducible factor 1 (HIF-1) (Brigati et al., 2010; Frede et al., 2007) that downregulates the histone deacetylase 2 (HDAC2) (Charron et al., 2009), leading to epigenetic modifications of proinflammatory cytokine genes. The inflammatory response was indeed amplified by shifting the relative activity of histone-acetylase (HAT) and HADC in asthma, as well as in chronic obstructive pulmonary disease (COPD) (Barnes et al., 2005; Ito et al., 2005).

A number of studies have been performed on clonally isolated CF and non-CF cell lines. These cell lines were obtained by either complementation of CF cells with wt CFTR (e.g. the IB-3 and C38 cell line pair) or by CFTR inactivation in non-CF cells (e.g. 16HBEo− sense and antisense cells). Isolation of individual clones from heterologous populations, however, may favor unrepresentative phenotypes as clonal variations can exceed the underlying differences in the regulated IL-8 secretion (as illustrated in Fig. S1G) or regarding Ca2+ signalling (Babnigg et al., 2000). To bypass these potentially confounding mechanisms and evaluate the direct effect of CFTR loss on proinflammatory cytokine secretion, CFTR and TMEM16A chloride channels were inducibly expressed in the human respiratory epithelial cell lines CFBE and H441.
Our results indicate that inducible expression of CFTR suppressed the expression and release of the proinflammatory cytokines IL-8, IL-6 and CXCL1/2. Focusing on IL-8, compelling evidence supports the notion that the transport function rather than CFTR signalling is required to inhibit the tonic cytokine secretion by these cell lines. 1) Expression of CFTR as low as ~10 % of that in Calu-3 cells was sufficient to suppress IL-8 secretion by ~50%. 2) The functionally inactive, but normally processed G551D variant cannot substitute for wt CFTR in suppressing IL-8 release. 3) Inhibiting the constitutive activity of wt CFTR with PPQ102 or BPO27 (Snyder et al., 2011) curtailed the suppressive effect on IL-8 secretion. 4) Concordantly, PKA activation by forskolin, IBMX or cpt-cAMP was sufficient to suppress IL-8 secretion by stimulating the transport function of the low copy number CFTR at the apical PM even without dox-induction. 5) Overexpression of TMEM16A could substitute for the suppressive role of CFTR in IL-8 secretion. 6) Finally, these results were confirmed by the suppressive effect of wt, but not G551D CFTR expression on IL-8 secretion by primary HBE cells isolated from cftr ΔF508/ΔF508 CF individuals.

The regulatory cascade that triggers IL-8 secretion in CF respiratory epithelia in the absence of infection remains to be deciphered. Considering that CFTR suppressor effect was restricted to respiratory epithelia cultured under ALC but not LLC, it is conceivable that besides ionic or compositional changes, physico-chemical alterations (e.g. surface tension, osmotic and oxido-reductive state) of the ASL and/or the apical PM may constitute upstream signalling for IL-8 secretion. Among other factors signal transduction pathways involved could be modulated by ion channel, receptor protein kinase or phosphatase functions affected by changes in the membrane potential and pH (Bocharov et al., 2008; Sandoval et al., 2011), as well as the oxido-reductive state of the ASL (Blanchetot et al., 2002). The supra-proportional effect of few individual wt CFTR expressing cells on the IL-8 secretion by CFBE cells in mixed populations, as well as in
transduced primary HBE cultures suggest that an average reduction in the anion transport capacity at the apical membrane lead to global changes in signalling of epithelial sheets. This notion is strengthened by the proportionality between IL-8 suppression and CFTR-mediated chloride conductance. These results, collectively, link the loss of CFTR-mediated ion transport to the hyperinflammation observed in CF and provide credence to the suggestion that the basic defect in CF can modulate the innate immune response of airway epithelia in the absence of infection. A similar conclusion was favored by the augmented lung inflammation of mice overexpressing the epithelial sodium channel (ENaC) β-subunit. Increased sodium absorption was associated with increased secretion of the macrophage inflammatory protein-2 (MIP-2), the mouse orthologue of IL-8, and keratinocyte chemoattractant (KC) by bronchial epithelia, as well as profound lung infiltration with neutrophils in the absence of bacterial infection (Mall et al., 2004).

The critical role of apical PM anion conductance in reducing the inflammatory state of CF bronchial epithelia was underscored by the impact of TMEM16A activity on IL-8 secretion. The sub-cellular localization of TMEM16A is cell type dependent. In acinar cells of the submandibular and salivary glands the channel is predominantly apical (Huang et al., 2009; Yang et al., 2008), while in cholangiocytes TMEM16A is localized at the apical and lateral PM (Dutta et al., 2011). The latter pattern coincides with the preferentially apical and lateral distribution of TMEM16A in polarized CFBE and MDCK cells. Thus maintained activity of apical TMEM16A could compensate, in principle, for the loss-of-function of CFTR channels in CF.

Our in vitro data using TMEM16A inhibition or induction of TMEM16A expression by IL-4 treatment alone as well as in combination with P2YR agonists indicate that endogenous TMEM16A activity is normally insufficient to modulate IL-8 secretion in human respiratory
epithelia. In contrast, tonic activity of overexpressed TMEM16A significantly reduced IL-8, IL-6 and CXCL1-3 secretion under ALC. Additional nucleotide stimulation was ineffective in further attenuating IL-8 release consistent with the conclusion that P2YR-induced cytoplasmic Ca^{2+} signalling is transient and the coupled TMEM16A activation is too short-lived to effect cytokine release. The transient activation of TMEM16A by apical nucleotides provokes rapid fluid secretion, but the increased ASL height returns to baseline within one hour of stimulation (Tarran et al., 2001). These observations, jointly, may provide a plausible explanation for the limited efficacy of denufosol, a P2Y_{12}R agonist, in a recent phase three clinical trial (Accurso et al., 2011).

Non-hydrolysable nucleoside triphosphate analogues and repeated agonist stimulations showed that TMEM16A inactivation cannot be attributed to nucleotide breakdown, but rather to rapid de-sensitization of the P2YR signalling. Agonist-induced phosphorylation of the P2YR promotes the recruitment of β-arrestin-2 to and subsequent internalization of the receptor (Flores et al., 2005; Hoffmann et al., 2008; Morris et al., 2011). Heterologous desensitization of other GPCRs was modest, indicating that desensitization occurred at the P2YR level. As an alternative approach to GPCR activation, local administration of duramycin, a Ca^{2+} ionophore, was attempted to stimulate CaCCs in respiratory epithelia (Cloutier et al., 1993; Grasemann et al., 2007). Elevated cytosolic Ca^{2+} concentrations, however, promote proinflammatory cytokine secretion and, therefore, may counteract the beneficial effect of ion transport correction (Gewirtz et al., 2000; Sakamoto et al., 2007). These limitations could be overcome by the activator F that probably activates TMEM16A allosterically without triggering cytosolic Ca^{2+} singalling (Namkung et al., 2011b). Activator F was able to attenuate IL-8 secretion via endogenous TMEM16A in CFBE, substantiating the proposed functional link between apical PM ion transport capacity and the innate immunity defect of CF respiratory epithelia.
In a broader context our results suggest that the proinflammatory cytokine secretion is regulated among other factors by the anion transport capacity of the apical PM in airway epithelia. This mechanism may contribute to excessive lung inflammation not only in cystic fibrosis, but perhaps in other chronic lung diseases (e.g. cigarette smoke-induced COPD), where CFTR down-regulation has been documented (Cantin et al., 2006; Clunes et al., 2011). Direct activation of TMEM16A may provide therapeutic benefit in these conditions if the channel activation is restricted to respiratory epithelial cells.
Materials and methods

Reagents and antibodies. CFTR inhibitors PPQ102 and BPO27 (Snyder et al., 2011; Tradtrantip et al., 2009), and the TMEM16A inhibitor A01 and activator F have been described (Namkung et al., 2011a; Namkung et al., 2011b). ATPγS tetralithium salt, UTPγS trisodium salt, CFTRinh 172, TRAM 34 were purchased from Tocris Bioscience. BAPTA acetomethylester (BAPTA-AM) and 5,5'-dimethyl BAPTA acetomethylester (5,5'-dimethyl BAPTA-AM) were obtained from Invitrogen. Human interleukin-4 (IL-4) was obtained from R&D Systems and all other chemicals were purchased from Sigma-Aldrich.

The following antibodies were used: monoclonal mouse anti-CFTR (L12B4, Chemokine), monoclonal mouse anti-HA (MMS101R, Covance), monoclonal mouse anti-myc (9E10, Santa Cruz Biotechnology), monoclonal mouse anti-flag (M2, Sigma-Aldrich), monoclonal rabbit anti-TMEM16A (SP31, Abcam), monoclonal rat anti-HSPA4 (1B5, Stressgen), monoclonal mouse anti-Na⁺/K⁺-ATPase (H3, Santa Cruz Biotechnology), polyclonal rabbit anti-occludin (Zymed laboratories), monoclonal rat anti-ZO1 (MAB1520, Chemicon international) and monoclonal mouse anti-Ecadherin (3B8, a kind gift from Dr. W. Gallin, University of Alberta, Edmonton, Canada).

Cell culture and stable cell line generation. The human CF bronchial epithelial cell line CFBE, with a cfrΔF508/ΔF508 genotype (a generous gift from Dr. D. Gruenert, University of California, San Francisco, CA) was maintained in minimal essential medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine and 10 mM HEPES. For propagation the cells were cultured in plastic flasks coated with an extracellular matrix (ECM mix) consisting of 10 µg/ml human fibronectin (EMD), 30 µg/ml PureCol collagen preparation (Advanced Biomatrix) and 100 µg/ml bovine serum albumin (Sigma-Aldrich) diluted in LHC basal medium (Invitrogen). Madine-Darby Canine Kidney (MDCK) cells were
grown in DMEM medium containing 10% FBS and 10 mM HEPES and H441 cells (ATCC) were cultured in RPMI-1640 medium supplemented with 10% FBS and 10 mM HEPES.

CFBE and H441 cell lines containing inducible CFTR or TMEM16A were generated using the Lenti-X TetON Advanced Inducible Expression System (Clontech). Briefly, wt or G551D CFTR harbouring an extracellular 3HA tag (Sharma et al., 2004) were subcloned into pLVX-tight-puro using a NotI / blunt-end strategy. A myc-tag coding sequence was fused in frame to the C-terminus of TMEM16A splice variant abc containing a partial internal flag epitope (Caputo et al., 2008) by PCR and cloned into the NotI / MluI restriction sites. PCR amplified constructs were verified by sequencing. For the generation of stable knockdown cell lines shRNAmir constructs for TMEM16A (V2LHS_155384) and SCNN1A (V2HS_93913) were purchased from OpenBiosystems and transferred into a modified pGIPZ vector (Open Biosystem) containing a hygromycin selection cassette. Lentiviral particles were produced in the HEK293T cells with the Lenti-X Packaging System (Clontech) following the manufacturer’s instructions. The cell lines were generated by consecutive transduction with viral particles containing the cDNA for the tetracycline-controlled transactivator, inducible CFTR or TMEM16A and shRNAmir followed by selection with G418 (200 µg/ml, Invivogen), puromycin (3 µg/ml, Invivogen) and hygromycin B (200 µg/ml, Invivogen), respectively.

Isolation and culture of HBE cells. Human lung tissues were obtained from 8 cftrΔF508ΔF508 CF individuals after lung transplantation under the protocol and consent form approved by the Institutional Review Board of the Research Ethics Office of McGill University. Isolation, culture and differentiation of HBE were adapted from procedures previously described (Fulcher et al., 2005). Briefly, CF airway epithelial cells were isolated from bronchial tissue by enzyme digestion and were cultured in bronchial epithelial growth medium on type I collagen-coated plastic flasks (Vitrogen 100, PureCol, Advanced BioMatrix), then trypsinized, counted, and
cryopreserved. Passage 1 (P1) or cryopreserved P1 cells were transduced with lentiviral particles mediating cDNA transfer of wt, G551D CFTR harbouring an extracellular 3HA tag or empty TranzVector (Tranzyme, (Wu et al., 2000)) at a MOI of 4 or 8 and subsequently transferred onto collagen IV-coated 6mm Transwell filters (Corning) in air-liquid interface medium at a density of 1.2x10^5 cells/filter. During the first 5 days, the air-liquid interface medium was changed every day and then the apical media was removed and the cells were grown at an air-liquid interface for an additional 10 days before use. The isolation and growth media were complemented with antibiotics that were adapted according to recent patient antibiograms however only penicillin and streptomycin were added to the air-liquid interface medium.

**Screening of human cytokines and IL-8 assay.** CFBE or H441 cells were plated on ECM-mix coated 12 mm Transwell filters (Corning) at a density of 1 x 10^5 cells/cm^2 under LLC. Protein expression was induced 24 h after plating (day 1) with 500 ng/ml dox unless otherwise specified and medium was changed to serum-free Opti-MEM (Invitrogen) supplemented with 2 mM L-glutamine at day 4 to avoid cross-contaminating of samples with bovine serum cytokines. Epithelia were switched to ALC at day 5 and the basolateral medium was conditioned for the indicated times. The secretion of 42 cytokines into the basolateral medium was determined using a antibody array (human cytokine antibody array 3, RayBiotech) following the manufacturer’s protocol except for the detection that was performed with a IRDye 800 streptavidin conjugate (Licor Biosciences) followed by imaging and quantification with an Odyssey Infrared Imaging System (Licor Biosciences). IL-8 levels were determined by ELISA (eBioscience) following the manufacturer’s instructions except for the final detection with SuperSignal ELISA Femto luminescence substrate (Pierce) on a VICTOR Light plate reader (PerkinElmer).

**I_sc measurement.** CFBE cells were plated on ECM-mix coated 12 mm Snapwell filters (Corning) at a density of 1 x 10^5 cells/cm^2. Polarized epithelia (≥ 5 days post confluence) were
mounted in Ussing chambers, bathed in Krebs-bicarbonate Ringer (KBR, ion composition: 140 mM Na\(^+\), 120 mM Cl\(^-\), 5.2 mM K\(^+\), 25 mM HCO\(_3\)\(^-\), 2.4 mM HPO\(_4\)\(^2-\), 0.4 mM H\(_2\)PO\(_4\), 1.2 mM Ca\(^{2+}\), 1.2 mM Mg\(^{2+}\), 5 mM glucose, pH 7.4) and continuously bubbled with 95% O\(_2\) and 5% CO\(_2\). To impose a chloride gradient NaCl was replaced by Na\(^+\)-gluconate or with K\(^+\)-gluconate to establish a potassium gradient in the absence of chloride. To functionally isolate the apical or basolateral membranes, the opposite PM domain was permeabilized with 100 µM amphotericin B (for stimulated CFTR activity measurements). Measurements were performed under I\(_{sc}\) conditions at 37°C, recorded with the Acquire and Analyze package (Physiologic Instruments) and expressed as current/cm\(^2\). Unless otherwise noted measurements were performed after imposing an apical-to-basolateral chloride gradient in presence of 100 µM amiloride.

**Apical and basolateral PM functional channel density measurement.** To determine the functional density of TMEM16A at the apical or basolateral PM by I\(_{sc}\) measurement the opposite PM (basolateral and apical, respectively) was permeabilized with 100 µM nystatin. Efficient apical PM permeabilization was confirmed by the BaCl\(_2\)-sensitive increase of the basolateral potassium current (Rochat et al., 2004) (also see Fig. S4C). ATP-stimulated changes in I\(_{sc}\) were measured in presence of an outward directed chloride gradient and peak current quantification was performed to calculate the apical to basolateral current ratios.

**Immunostaining and confocal microscopy.** Polarized epithelial monolayers were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 or 0.1% saponin followed by blocking with 1% BSA in PBSCM (phosphate buffered saline with 0.1 mM CaCl\(_2\) and 1 mM MgCl\(_2\), pH 7.4). CFTR-3HA expressing CFBE cells were co-stained with anti-HA and anti-ZO1 antibodies. To detect TMEM16A-myc localization, the channel was visualized by indirect immunostaining using primary anti-myc antibody, while tight junctions were stained with anti-occludin or anti-ZO1 antibodies. Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary
antibodies (Invitrogen) were employed. Co-staining of TMEM16A and E-cadherin was accomplished using sequential incubation, first with anti-myc antibody and Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen). The free epitopes of the anti-myc antibodies were blocked with goat anti-mouse Fab fragment (Jackson ImmunoResearch). Finally anti-E-cadherin antibody incubation was followed by Alexa Fluor 488-labelled goat anti-mouse antibody (Invitrogen). Nuclei were stained with DAPI. Images were obtained on a LSM710 microscope (Carl Zeiss) equipped with a Plan-Apochromat 100x/1.40 Oil DIC objective. Typically 20-30 optical xy-sections were acquired and reconstituted using the Zen 2009 software package and representative xz-sections are shown. For better visibility the gamma setting for ZO1, occludin, E-cadherin and DAPI stainings were adjusted.

**CFTR cell surface density measurement.** The PM density of wt and G551D CFTR was determined by cell surface ELISA (Okiyoneda et al., 2010). Cells were seeded in 96-well plates at a density of 2 x 10^4 cells/well and induced for CFTR expression with the indicated dox concentrations for 3 days. The extracellular 3 x HA tag was detected by incubation with anti-HA antibody followed by incubation with HRP-conjugated secondary antibody in PBSCM containing 0.5% bovine serum albumin on ice. Excess antibody was removed by extensive washing and specific binding was determined with Amplex Red (Invitrogen) HRP-substrate. The fluorescence intensity was measured at 544 nm excitation and 590 nm emission wavelengths using a POLARstar OPTIMA (BMG labtech) fluorescence plate reader and values determined from parental cells were subtracted as background.

**Cell surface biotinylation to determine TMEM16A apical-to-basolateral ratios and IB analysis.** CFBE cells with or without induced TMEM16A expression with 0, 5 or 500 ng/ml dox were grown on 24 mm polyester Transwell filters for at least 3 days post-confluence. Basolateral or apical PM proteins were biotinylated for 15 min on ice with 1 mg/ml EZ Link sulfo-SS-NHS-
biotin (Thermo Fisher Scientific) in buffer H (154 mM NaCl, 3 mM KCl, 10 mM HEPES, 1mM MgCl₂, 0.1 mM CaCl₂, 10 mM glucose, pH 7.8) followed by excess biotin reagent quenching with 100 mM glycine in PBSCM. Cells were lysed in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate, pH 8.0) containing proteases inhibitors and postnuclear lysates were collected after centrifugation. The biotinylated proteins were isolated on streptavidin-agarose beads (Invitrogen) at 4°C with end-over-end rotation for 1 h. Proteins were eluted with 2x Laemmlı sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane. IBs were probed with rabbit anti-TMEM16A, mouse anti-Na⁺/K⁺-ATPase or rat anti-HSPA4 antibodies followed by IRDye 680 anti-rabbit, IRDye 800 anti-mouse or IRDye 680 anti-rat antibodies (Licor Biosciences). IBs were imaged and quantified with the Odyssey Infrared Imaging System (Licor Biosciences).

**Q-PCR.** Total RNA was extracted from polarized CFBE grown on Transwell filters using the miRNeasy kit (Qiagen) and reverse transcription of 1µg total RNA was performed with the QuantiTect reverse transcription kit (Qiagen) following the manufacturer’s recommendations. The abundance of transcripts was determined using the SYBR advantage qPCR premix (Clontech) with an Mx3005P real-time cycler (Stratagene). Primer sequences were either retrieved from the PrimerDepot (http://primerdepot.nci.nih.gov) or designed using the PearlPrimer software (Marshall, 2004) and are summarized in Table 1. PCR data was analyzed by efficiency-corrected comparative quantification (Pfaffl, 2001) and reported as relative change in mRNA abundance.

**Cytosolic calcium measurements.** CFBE cells seeded into black 96-well microplates at a density of 2 x 10⁴ cells/well were cultured for 3 days post-confluence. Cytosolic calcium levels were determined using the Fluo-4 NW calcium assay kit (Invitrogen) following the manufacturer’s protocol. The Fluo-4 fluorescence was measured at 488 ±5 nm excitation and
516 ±6 nm emission wavelengths in an Infinite M1000 fluorescence plate reader (Tecan) equipped with syringe pumps. Fluorescence values from non-dye loaded cells were subtracted as background and the relative changes in cytosolic calcium levels are expressed as percent of basal calcium signals.

**Halide permeability determination of the PM.** The halide sensor YFP-F46L/H148Q/I152L (Namkung et al., 2010) was amplified by PCR and cloned into the XhoI / BamHI restriction sites of the pLVX-IRES-Hyg lentiviral vector (Clontech). Lentiviral particles were produced as described above and CFBE cells containing the transactivator alone or in combination with inducible TMEM16A were transduced, followed by selection with hygromycin B (200 µg/ml). YFP expressing cells or controls were seeded to 96well microplates at a density of 2 x 10^4 cells/well and induced for TMEM16A expression with the indicated dox concentrations for 3 days. For the YFP quenching assay cells were incubated in 100µl/well PBS-chloride (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5mM KH_2PO_4, 1.1 mM MgCl_2, 0.7 mM CaCl_2 and 5 mM glucose, pH 7.4) followed by well-wise injection of 100µl PBS-iodide in which NaCl was replaced with NaI. The fluorescence was monitored over 50 seconds with a 5 Hz acquisition rate at 485 nm excitation and 520 nm emission wavelengths using a POLARstar OPTIMA (BMG labtech) fluorescence plate reader. For analysis the fluorescence of non-YFP expressing cells was subtracted and the quenching curves were fitted to the following exponential equation:

\[
Fluorescence = F_q + (F_1 \times e^{-R_1t}) + (F_2 \times e^{-R_2t})
\]

Where \(F_q\) is the residual fluorescence after complete YFP quenching, \(t\) is time, \(R_1\) and \(R_2\) are quenching rates and \(F_1\) and \(F_2\) are the relative contributions of \(R_1\) and \(R_2\) to fluorescence quenching, respectively. The overall quenching rate was determined by:

\[
Rate = \frac{(F_1 \times R_1) + (F_2 \times R_2)}{(F_1 + F_2)}
\]
Statistical analysis. Results are presented as mean ± SEM for the indicated number of experiments. Statistical analysis was performed by two-tailed Student's t-test with the means of at least three independent experiments and the 95% confidence level was considered significant.

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We thank H. Salah’s contribution to the initial phase of the study, D. Gruenert for the parental CFBE14o- cell line and W. Gallin for the anti-E-cadherin antibody.

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References


Table 1. Primers used for qPCR

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Figure 1. Inducible CFTR expression in CF human bronchial epithelia (CFBE) attenuates the proinflammatory cytokines IL-8, IL-6 and CXCL1/2. (A) IB of CFBE transduced with transactivator (TetON) or in combination with inducible CFTR carrying an extracellular 3HA tag (iCFTR) with (+) or without (-) dox (500 ng/ml) induced expression for 3 days. (B, C) Dox concentration dependence of CFTR cellular expression determined by IB (B) or by cell surface
ELISA (C). Densitometric analysis of the complex-glycosylated CFTR expression is expressed as % of the maximum (lower panel of B). (D) Apical localization of CFTR (green) in polarized CFBE cells by confocal microscopy. ZO1 was used as tight junction marker (red) and nuclei were stained with DAPI (blue). Ap, apical; b, basal; bar, 5 µm. (E) Maximal CFTR current (Isc) was measured through stimulation with 10 µM forskolin followed by inhibition with 20µM Inh172 (quantification depicted in bar chart) after basolateral permeabilization with amphotericin B (100 µM). (F) Antibody array comparing the basolateral cytokine secretion of polarized CFBE with or without induced CFTR expression. The basolateral media were conditioned for the time period 4-8 h after switch to ALC, the dotted line represents 2fold background and the pre-spotted positive control was used to normalize between arrays. (G-J) CFTR expression dependent mRNA levels of IL-8 (G), IL-6 (H), CXCL1 (I) and CXCL2 (J) in CFBE cells kept under LLC or switched for 8h to ALC determined by qPCR as described in materials and methods. Values show means ± SEM from three independent experiments (B, E, G-J), means ± SD of two independent experiments (F) or means ± SD of one representative experiment (C). kDa, kiloDalton; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 2. Functional CFTR expression attenuates IL-8 secretion in human bronchial epithelia under ALC. (A) CFTR expression dependent basolateral IL-8 secretion of polarized CFBE cells subjected for 24 h to ALC or kept under LLC. (B) Time dependence of the basolateral IL-8 secretion following the switch from LLC to ALC. (C) CFTR expression level dependence of IL-8 secretion of polarized CFBE epithelia kept under ALC. (D) IB of CFBE or H441 cells transduced with inducible wild-type (iCFTR) or G551D (iG551D) CFTR with (+) or without (-) dox in comparison to the endogenous CFTR expression in Calu3 cells. (E) The dependence of wt and G551D CFTR PM densities on dox concentration as determined by cell surface ELISA. (F,
G) WT or G551D expression dependent basolateral IL-8 secretion of polarized (F) CFBE or (G) H441 cells switched to ALC or kept under LLC for the indicated times. IL-8 levels were determined by ELISA. Values represent means ± SEM from three (A, D, F, G) or two (C) independent experiments or means ± SD of one representative experiment (B, E). kDa, kiloDalton; n.s., not significant; *, P < 0.05; ***, P < 0.001.
**Figure 3.** CFTR function is required to suppress IL-8 secretion. (A) PPQ102 (25 µM) or BPO27 (25 µM) inhibit CFTR activity as measured by $I_{sc}$ after stimulation with 10 µM forskolin. (B) The constitutive activity of CFTR in iCFTR+ CFBE14o- cells was unmasked by CFTR inhibitor 172 (Inh172, 20 µM), PPQ102 (25 µM) or BPO27 (25 µM). Measurements were performed in intact monolayers. (C) Quantification of the maximal inhibition as determined in B. (D) Basolateral IL-8 secretion of polarized CFBE cells after channel inhibition with PPQ102 or BPO27. Ratios between basolateral IL-8 secretion with or without induced CFTR expression are depicted and significance was tested using the ratios derived from three independent experiments.
(E) CFTR channel activation in CFBE epithelia with leaked expression of CFTR with forskolin (1 µM), IBMX (0.5 mM) or cpt-cAMP (0.5 mM) determined by $I_{sc}$. (F) Basolateral IL-8 secretion of polarized CFBE cells under ALC after CFTR channel activation with 1 µM forskolin, 0.5 mM IBMX or 0.5 mM cpt-cAMP. Numbers indicate the ratio between cells with or without induced CFTR expression. Values show means ± SEM from three (C-E) or two (F) independent experiments. *, $P < 0.05$. 
Figure 4. A limited number of wt CFTR expressing cells suppresses the overall IL-8 secretion of CFBE epithelia and primary HBE. (A, B) CFTR expression monitored by confocal microscopy (A) or CFTR PM density (B) in polarized CFBE TetON or iCFTR cells as well as 9:1 or 4:1 mixtures of both after 5 days induction with dox. (C) Basolateral IL-8 secretion of polarized CFBE TetON, iCFTR cells or mixtures of both. The theoretical values estimating a linear correlation between the number of CFTR expressing cells and IL-8 secretion are depicted for comparison and were used for significance testing. (D, E) IB (D) and confocal microscopy pictures (E) of primary CF HBE transduced with lentiviral particles transferring wt, G551D
CFTR (green) or empty vector cDNA at a MOI of 4 or 8. ZO1 was used as tight junction marker (red). (F) Basolateral IL-8 secretion from CF HBE (n = 8) transduced at a MOI of 4 and grown on filter supports for 15 days. The left panel shows absolute IL-8 values, the right panel depicts the percentage change in comparison to empty vector. Values indicate means ± SEM from two (B) three (C) or eight (F) independent experiments. kDa, kiloDalton; n.s., not significant; **, P < 0.01; ***, P < 0.001; bar, 50 µm.
Figure 5. Altering the endogenous TMEM16A activity in CFBE epithelia by shRNA, inhibitor or transcriptional activation cannot modulate IL-8 secretion. (A) ATP or UTP (each 100 μM) stimulated peak current quantification in CFBE cells in combination with TMEM16A shRNA expression, BAPTA-AM (10 μM, 30 min) or IL-4 (10 ng/ml, 24 h) pre-treatment (B, C) CFTR expression dependent basolateral IL-8 secretion of polarized CFBE cells under ALC in combination with (B) 10 μM BAPTA-AM treatment or (C) shRNA expression targeting TMEM16A or SCNN1A. (D) IL-8 secretion in CFBE epithelia with or without induced CFTR expression, or with shRNA silencing of TMEM16A in combination with IL-4 treatment to increase endogenous TMEM16A expression and channel activation with 100 μM ATP or UTP. Significance tested in comparison to untreated cells. Values indicate means ± SEM from three to five independent experiments. *, P < 0.05; ***, P < 0.001.
Figure 6. TMEM16A is localized at the apical and lateral PM of polarized epithelia. (A) Schematic depiction of the TMEM16A proteins and IB of inducible expression in CFBE cells. (B, C) Apical and lateral localization of TMEM16A (red) in (B) polarized CFBE or (C) MDCK cells by confocal microscopy. Occludin or ZO1 were used as tight junction marker (green), lateral membranes were labelled with E-cadherin (green) and nuclei were stained with DAPI.
(blue). Ap, apical; b, basal; bars, 10 μm. (D) Expression of TMEM16A in the apical and basolateral membranes of polarized CFBE probed by cell surface biotinylation as described in materials and methods. (E) ATP stimulated change (100 μM) in apical or basolateral chloride currents after permeabilization of the basolateral or apical membranes with nystatin (100 μM) in presence of an outward directed chloride gradient. Values represent means ± SEM from three to five independent experiments. kDa, kiloDalton; IP, immunoprecipitation.
Figure 7. The constitutive activity of overexpressed TMEM16A suppresses IL-8 secretion in CFBE epithelia. (A) CFBE cells transduced with transactivator alone (TetON) or in combination with inducible TMEM16A with (+) or without (-) dox (500 ng/ml) induced expression were subjected to ALC or kept under LLC. Basolateral IL-8 secretion was determined by ELISA. (B) IL-8 secretion of polarized CFBE cells at different TMEM16A expression level (0, 5 or 500 ng/ml dox) or after lowering of cytosolic Ca$^{2+}$ with 5,5'-dimethyl BAPTA-AM (10 µM). (C) Constitutive TMEM16A activity was determined by $I_{sc}$ measurements in intact CFBE
monolayers with (5 or 500 ng/ml dox) or without TMEM16A expression following the addition of the A01 inhibitor (100 µM). (D) Bar graph summarizes the constitutive TMEM16A activity with or without pre-treatment with 10 µM 5,5’-dimethyl BAPTA-AM. (E, F) Maximal fluorescence quenching rate after addition of iodide buffer to CFBE cells expressing the halide-sensitive YFP and TMEM16A induced with the indicated dox concentrations. As controls TMEM16A activity was reduced by 10 µM 5,5’-dimethyl BAPTA-AM (E) or using A01 (F). (G) Correlation between IL-8 secretion and constitutive I_{sc} of CFBE epithelia expressing different levels of CFTR or TMEM16A in combination with channel activation or inhibition. Values show means ± SEM from three or four independent experiments. BAPTA, 5,5’-dimethyl BAPTA-AM; frk, forskolin; cAMP, cpt-cAMP; **, P < 0.01.
Figure 8. P2YR activation cannot potentiate the suppressive effect of TMEM16A on IL-8 secretion. (A, B) TMEM16A expression level dependent IL-8 secretion under ALC in polarized CFBE in combination with channel activation with (A) 100 µM ATP or UTP or (B) 100 µM stable nucleoside triphosphate analog ATPγS or UTPγS. (C) Time-dependent desensitization of...
100 µM ATP stimulated peak $I_{sc}$ measured in polarized CFBE epithelia expressing different levels of TMEM16A. (D) Re-sensitization of ATP-stimulated TMEM16A activity. After initial stimulation (100 µM ATP, 5 min) ATP was removed and the cells were allowed to re-sensitize for the indicated period before restimulation with 100 µM ATP. Insert: representative traces of one experiment. (E, F) Activation of TMEM16A by stimulation with histamine receptor agonist (100 µM histamine, E) or muscarinic receptor agonist (100 µM carbachol (CCh), F) in presence or absence of 100 µM ATP (15min pre-treatment), 10 µM BAPTA-AM (1 h pre-treatment) or 10 µM A01 (5 min pre-treatment) determined by $I_{sc}$ measurement. (G) Quantification of the peak $I_{sc}$ after 15 min pre-treatment with ATP. (H, I) Cytoplasmic calcium mobilization measured by Fluo-4 fluorescence. CFBE cells with or without ATP pre-treatment (100 µM, 15 min) were stimulated with 100 µM ATP (H) or 100 µM histamine (I). (J) Quantification of peak Fluo-4 fluorescence after 15 min pre-treatment with ATP. Bar charts depict means ± SEM from three to five independent experiments as percent of controls. *, P < 0.05.
Figure 9. Activator F results in prolonged stimulation of TMEM16A and decreases IL-8 secretion in CFBE and H441 expressing low TMEM16A cannal density. (A, B) Sample traces (A) and inactivation kinetics (B) of TMEM16A in polarized CFBE stimulated with 100 µM ATP or 20 µM activator F. I_{sc} was determined in intact epithelia. (C-E) IL-8 secretion in polarized CFBE (C, D) or H441 (E) with different levels of TMEM16A expression (induced with 0, 5 or 500 ng/ml dox (C, E)), endogenous TMEM16A or shRNA silencing (D) with or without channel activation by activator F (20 µM, added at 0 h ALC). Values show means ± SEM from three independent experiments. **, P < 0.01; ***, P < 0.001.