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Cross-Talk of Inflammatory Mediators and Airway Epithelium Reveals CFTR as a Major Target

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Summary: Disease-specific inflammatory cytokines induce airway mucus hyperproduction, changes in chloride channels/transporters and modulate epithelial differentiation.

Abstract

Airway inflammation, mucus hyperproduction and epithelial remodelling are hallmarks of many chronic airway diseases, including asthma, Chronic Obstructive Pulmonary Disease and Cystic Fibrosis. While several cytokines are dysregulated in these diseases, most studies focus on the response of airways to IL-4 and IL-13, which were shown to induce mucus hyperproduction and shift the airway epithelium towards a hypersecretory phenotype.

We hypothesised that other cytokines might induce the expression of chloride (Cl^-) channels/transporters, regulate epithelial differentiation and mucus production. To this end, fully-differentiated human airway basal cells (BCi-NS1.1) were treated with cytokines identified as dysregulated in those diseases, namely interleukins-8, 1β, 4, 17A, 10, 22, and tumour necrosis factor-α (TNF-α).

Our results show that CFTR is the main Cl^- channel modulated by inflammation, in contrast to TMEM16A, whose levels only changed with IL-4. Furthermore, we identified novel roles for IL-10 and IL-22 by influencing epithelial differentiation towards ciliated cells and away from pulmonary ionocytes. Contrarily, IL-1β and IL-4 reduced the number of ciliated cells while increasing club cells. Interestingly, while IL-1β, IL-4 and IL-10 upregulated CFTR expression, IL-4 was the only cytokine that increased both its function and the number of CFTR-expressing club cells, suggesting that this cell-type may be the main contributor for CFTR function. Additionally, all cytokines assessed increased mucus production through a differential upregulation of MUC5AC and MUC5B transcript levels.

Altogether, this study reveals a novel insight into differentiation resulting from the cross-talk of inflammatory mediators and airway epithelial cells, which is particularly relevant for chronic airway diseases.

Introduction

The human airway epithelium is a barrier that maintains fluid homeostasis, clears particles/pathogens, and recruits immune cells during infection [1]. These functions are achieved by a heterogeneous population of epithelial cells, namely ciliated cells, which drive clearance through ciliary beating; goblet cells that secrete mucus to trap pathogens; club cells that produce protective surfactants and antiproteases; and basal cells that differentiate into the other cell-types [2].

Efficient mucociliary clearance (MCC) depends on a regular airway surface liquid (ASL) that is regulated by epithelial ion channels and transporters [3]. Proper airway hydration is achieved by apical sodium (Na^+) absorption and chloride (Cl^-) secretion, with water flowing according to the osmotic gradient. Apical Cl^- secretion is driven by the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), the Transmembrane protein 16A (TMEM16A, also Anoctamin1/ANO1) and exchangers of the solute carrier family 26 (SCL26A9 and SLC26A4, also Pendrin), while the epithelial sodium channel (ENaC) mediates Na^+ absorption [3]. Additionally, several Cl^- channels/transporters are also permeable to bicarbonate (HCO_3^-), essential for mucus release and expansion [4].

Upon infection, airway epithelial cells are exposed to inflammatory mediators that act as signals to increase mucus and fluid secretion [5]. Previous studies established a link between inflammation and ion transport. However, the focus has been on the response to interleukins (IL)-4 and -13, which upregulate CFTR and TMEM16A while inhibiting ENaC activity [6],[7]. Regarding other inflammatory mediators, the results are contradictory. For example, IL-1β and TNF-α are described to upregulate [8],[9] and downregulate [10],[11] CFTR, whereas no information is available about other Cl^- channels/transporters. Furthermore, it remains unknown how certain cytokines modulate the airways, namely chemokines (e.g., IL-8), which recruit immune cells to the site of infection [12], and anti-inflammatory cytokines (e.g., IL-10) [13].

Inflammatory mediators also regulate tissue homeostasis, modulating the distribution of airway epithelial cell-types. Airway remodelling is a key feature of airway diseases, including Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD) and asthma. Although several cytokines are unbalanced in these conditions [14],[15], most reports focus on IL-4 and IL-13, which increase goblet cells at the expense of ciliated cells [16],[17]. Notably, there is no information about the potential effect of inflammation on pulmonary ionocytes, a rare cell-type

highly enriched in CFTR expression [18], although their contribution to CFTR function is controversial [19].

This study aimed to determine the effect of different inflammatory mediators on airway epithelial cells in Cl^- channels/transporters, epithelial differentiation and mucus production. To this end, we assessed cytokines that are secreted upon infection (IL-8, IL-1β, TNF-α, IL-17A) [20],[21], anti-inflammatory (IL-10) [13] or tissue-protective (IL-22) [22]. IL-4 was also included as its effect on mucus production and epithelial Cl^- transport is well described [7],[23].

Our data, using a human respiratory basal cell line (BCi-NS1.1) with the ability to multi-differentiate into the various airway epithelial cell types [24], demonstrate that CFTR is the main Cl^- channel modulated by inflammation, with all cytokines increasing its expression, except for TNF-α. Remarkably, some cytokines also regulate the percentage of ciliated (IL-1β, IL-4, IL-10, IL-22), club cells (IL-1β, IL-4) and ionocytes (IL-10, IL-22). Furthermore, we observed that IL-4 upregulated CFTR activity, which may be explained by the increase in the number of CFTR-expressing club cells and/or TMEM16A, an enhancer of CFTR function [25]. All cytokines upregulated mucus production by increasing the transcript levels of MUC5AC (IL-4, IL-10, IL-22), MUC5B (IL-1β, TNF-α, IL-17A) or both (IL-8). Finally, we grouped the different cytokines according to their overall effect on the airway epithelium.

Materials and Methods

Cell Culture

Basal Cell Immortalised Non-Smoker 1.1 (BCi-NS1.1) cells [24] were cultured in Pneumacult-Ex medium (Stemcell Technologies, Vancouver, Canada) and differentiated under air-liquid interface (ALI) as described [26], using 6.5- or 12-mm diameter-size transwells with 0.4 µm pore polyester membrane inserts (Corning, New York, USA) previously coated with human type IV collagen (Sigma-Aldrich, St. Louis, USA).

Exposure to cytokines

BCi-NS1.1 cells (ALI day 27) were exposed to 10 ng/mL IL-8 (R&D Systems, Minneapolis, USA), IL-1β, TNF-α, IL-17A, IL-10, IL-22 (Stemcell Technologies, Vancouver, Canada) and IL-4 (Thermo Fisher Scientific, Waltham, USA) from the basolateral media for 72h.

qRT-PCR

mRNA expression was quantified as described [26], using the primer pairs: MUC5AC (fwd:5'-CCTGAGGGGACGGTGCTT-3',rv:5'-ACGAGGTGCAGTTGGTGC-3'), MUC5B (fwd:5'-GTGAGGAGGACTCCTGTCAAGT-3',rv:5'-CCTCGCAGAAGGTGATGTTG-3'), SLC26A9 (fwd:5'-GACTACATCATTCCTGACCTGC-3',rv:5'-AGGAGTAGAGGCCATTGACTG-3'), GAPDH (fwd:5'-ATGGGGAAGGTGAAGGTCG-3',rv:5'- GGGGTCATTGATGGCAACAATA-3'). Mean relative levels of expression were calculated for the two target genes using the ΔΔCT method, where fold-change = $2^{(-\Delta\Delta CT)}$.

Primary Antibodies

The primary antibodies used were anti-CFTR [596,570] (CFF), anti-TMEM16A [SP31], anti-SLC26A4, anti-GAPDH, anti-βTubulinIV (Abcam, Cambridge, UK), anti-DNAI1, anti-FOXI1 (Sigma-Aldrich, St. Louis, USA), anti-CC16 (BioVendor, Brno, Czech Republic) and anti-Calnexin (BD Biosciences, San Jose, USA).

Western Blot

Protein lysates were prepared as described [26] and loaded in acrylamide gels for electrophoresis. Membranes were blocked with 5% non-fat milk (NFM)-PBST and probed with anti-SLC26A4, anti-DNAI1 (1:1000), anti-CC16 (1:500), anti-GAPDH and anti-Calnexin (1:10000) diluted in blocking buffer. Membranes probed for TMEM16A, CFTR and FOXI1 were

blocked with 1% NFM-TBST and incubated with respective antibodies (1:500) diluted in blocking buffer. Incubation with secondary antibodies was performed as described [26].

Immunofluorescence

Immunofluorescence was performed as described [26] using primary antibodies at a 1:100 dilution (anti-CFTR [570], anti-βTubulin IV, anti-CC16, anti-FOXI1). Nuclei were stained with Hoechst 33,342 solution (200 ng/mL, Sigma-Aldrich, St. Louis, USA). Transwells were imaged with a Leica TCS SP8 confocal microscope. Cell-types were quantified by averaging values obtained from at least five different images in each transwell, using the Fiji software with maximum intensity projections and confirming co-staining with xz images. Nuclei were quantified in maximum X-Y image projections using the StarDist plugin in Fiji [27].

Ussing Chamber

Monolayers of BCi-NS1.1 cells were analysed under open-circuit conditions in a micro-Ussing chamber as described [26]. 30 µM amiloride was added apically to block ENaC. CFTR activity was measured by co-applying 2 µM Forskolin and 100 µM IBMX, followed by 30 µM CFTR-inh172. All compounds from Sigma-Aldrich, St. Louis, USA.

Data Analyses

Data is mean ± SEM. Statistical analysis was performed with unpaired t-tests (p-value < 0.05).

Heatmap was generated in Rstudio (1.2.5042), using the heatmap.2 function (package gplots) and rescaling data between 0 and 1.

Results

Effect of inflammatory mediators on airway epithelial Cl^- channels/transporters

Fully-differentiated BCi-NS1.1 cells were exposed to different cytokines, and the CFTR, TMEM16A and SLC26A4 protein levels were analysed by Western blot (WB). SLC26A9 expression was analysed by qRT-PCR, as no WB signal was detected with two different antibodies (data not shown).

Our results show that all cytokines upregulate CFTR expression, except for TNF-α, which had the opposite effect (Fig.1A,D). In contrast, IL-4 was the only cytokine increasing TMEM16A expression, which is undetectable in control BCi-NS1.1 cells (Fig.1B,D), as previously described [26]. Moreover, SLC26A4 expression was significantly upregulated by IL-17A and downregulated by IL-22 (Fig.1C,D). Contrarily, these cytokines did not affect the SLC26A9 transcript levels, which increased with IL-8, IL-1β and IL-4 (Fig.1E).

Furthermore, the contribution of each protein for total Cl^- channels/transporters expression was calculated by dividing the fold-change of each channel's protein/mRNA expression levels (Fig.1D-E) by the sum of all (Fig.1F). IL-4 was the only cytokine inducing TMEM16A (Fig.1B,D), accounting for ~36% of total Cl^- channels/transporters expression (Fig.1F). CFTR contribution was highest (~61%) in IL-10-treated cells, while SLC26A4 and SLC26A9 had a maximal expression with IL-17A and IL-1β (~58% and 39%, respectively) (Fig.1F).

Effect of inflammatory mediators on epithelial cell differentiation

~~We previously observed that BCi-NS1.1 cells express CFTR in ciliated, club cells and ionocytes (Simões and Amaral lab, unpublished data).~~ Previous studies identified CFTR expression in ciliated, club cells and ionocytes [16],[18],[27]. Thus, we next asked whether these cytokines change differentiation into these cell-types, as inflammatory mediators were reported to regulate epithelial differentiation [16],[17].

To this end, we compared protein expression of markers for club cells (CC16), ciliated (DNAI1) and ionocytes (FOXI1). WB results demonstrate that while IL-1β downregulated CC16 expression, IL-4 significantly increased (~3-fold) (Fig.2A,D). Moreover, IL-1β and IL-4 decreased DNAI1 levels, contrasting IL-10 and IL-22, which increased its expression by ~2.5-fold (Fig.2B,D). Regarding pulmonary ionocytes, IL-10 and IL-22 were the only cytokines downregulating FOXI1 protein levels (Fig.2C,D).

To determine whether changes in protein levels correspond to equivalent differences in the percentage of ciliated, club cells and ionocytes, we performed immunofluorescence with antibodies against βTubulin-IV (another ciliated-cell marker) CC16 and FOXI1, co-staining each marker with CFTR to determine its distribution under different inflammatory conditions.

Indeed, we observed matching differences in the percentage of ciliated, club cells and ionocytes relative to the expression of cell-type markers (Fig.2,3). In agreement with previous studies, we also detected CFTR expression in ciliated, club cells and pulmonary ionocytes resulting from differentiation of BCi-NS1.1 cells (Fig.4), similarly to those cells present in the native epithelium. Moreover, co-staining of βTubulin-IV and CFTR demonstrated that, although IL-10 and IL-22 increased ciliated cells (~1.5-fold), the total number of CFTR-expressing βTubulin-IV$^+$ cells did not change (Fig.~~3A,D,~~4A,D). Contrarily, IL-1β and IL-4 decreased ciliated cells by half and reduced its contribution to total CFTR expression (Fig.~~3A,D,~~4A,D). Interestingly, co-labelling of CFTR and CC16 showed that IL-4 increased the percentage of club cells and the number of CFTR-expressing club cells (Fig.~~3B,D,~~4B,D), suggesting that the IL-4-induced CFTR upregulation (Fig.1A,D) occurs in club cells. Contrarily, IL-1β did not change the percentage of cells co-labelled with CFTR and CC16, despite the decrease in club cells (Fig.~~3B,D,~~4B,D). Furthermore, the IL-10 and IL-22-induced downregulation of FOXI1 protein levels (Fig.2C,D) was also associated with a significant decrease in the percentage of pulmonary ionocytes, although the number of CFTR-expressing FOXI1$^+$ cells did not change (Fig.~~3C,D,~~4C,D).

Modulation of CFTR activity by IL-1β, IL-4 and IL-10

While IL-1β, IL-4 and IL-10 upregulated CFTR expression (Fig.1A,D), they showed ~~opposite~~ different effects in ciliated, club cells and pulmonary ionocytes (Fig.2A-D). Thus, by comparing CFTR function with these cytokines, we can conclude which cell-type contributes more to total CFTR function. Ussing chamber results demonstrated that IL-4 was the only cytokine that significantly increased the cAMP-activated currents (Fig.~~4–~~5A,C), elicited by IBMX and Forskolin. These data suggest an appreciable contribution of club cells to CFTR function, as this cell-type was induced by IL-4 (Figs.2,3). Additionally, we observed that IL-1β and IL-4 significantly decreased the amiloride-sensitive Na^+ absorption currents (Fig.~~4–~~5A,B), downregulating ENaC function.

Regulation of mucin gene expression by inflammatory mediators

Next, we assessed MUC5AC and MUC5B transcript levels, the two main mucins in human airway mucus [29]. As these mucins are essential components of the MCC machinery, it is

relevant to determine the ratio between them to define how mucus properties change with inflammation.

Our qRT-PCR results demonstrate that all cytokines increased MUC5AC and/or MUC5B transcripts, albeit at differential levels, resulting in different ratios. IL-8, IL-4, IL-10 and IL-22 upregulated MUC5AC transcripts (Fig.~~5~~6A), with IL-4 inducing the greatest unbalance as it had no significant effect on MUC5B (~12-fold in MUC5AC vs MUC5B). Contrarily, MUC5B levels were higher with IL-1β (~8-fold), followed by IL-8, TNF-α and IL-17A (Fig.~~5~~6B).

Additionally, the relative expression of each mucin was calculated by dividing the MUC5AC or MUC5B fold-change expression levels by the sum of the two (Fig.~~5~~6C). While IL-8 significantly increased the MUC5AC and MUC5B mRNA expression levels (Fig.~~5~~6A,B), the relative percentage of each mucin is similar to control. In contrast, IL-4, IL-10 and IL-22 mainly upregulated MUC5AC, whereas MUC5B represents over 60% of mucin mRNAs with IL-1β, TNF-α or IL-17A (Fig.~~5~~6C).

Summary of the effect of different cytokines on Cl^- channels/transporters, epithelial differentiation and mucus production

The observed effects on the expression of Cl^- channels/transporters, epithelial differentiation and mucus production (Figs.1,2,~~5~~6) are summarised in a heatmap (Fig.~~6~~7) where cytokines are clustered according to their effect on the airway epithelium properties. Altogether, we observed that IL-4 induced the most significant changes in all these parameters. Moreover, IL-10 and IL-22 showed similar effects by upregulating CFTR, DNAI1 and MUC5AC, while decreasing FOXI1. IL-1β and IL-8 are in the same cluster as both increased CFTR, SLC26A9 and MUC5B expression. Finally, IL-17A and TNF-α were the cytokines with fewer effects on BCi-NS1.1 cells (Fig.~~6~~7).

Discussion

Airway inflammation is a hallmark of many airway obstructive diseases, occurring with mucus hyperproduction and epithelial remodelling. Under these conditions, there is a complex cross-talk between airway epithelial cells and the immune system to ensure efficient MCC. When MCC fails, e.g. due to ASL dehydration, mucus plugging and airway clogging arise, followed by progressive lung failure. To fully understand the pathogenesis caused by inflammation, it is essential to characterise how airway epithelial cells respond to inflammatory stimuli. Although several cytokines are dysregulated upon disease [14],[15], most studies focus on the response of airway epithelial cells to IL-4 and IL-13 due to their involvement in asthma [17],[28]. In this study, we screened a panel of seven cytokines that are dysregulated in CF, COPD and/or asthma – IL-8, IL-1β, TNF-α, IL-4, IL-17A, IL-10, IL-22 [14],[15] – and determined their effect on airway epithelial cells, using BCi-NS1.1 cells that, like primary cells, differentiate into a mucociliated epithelium, but with an extended number of passages [24].

Increasing evidence supports the concept that inflammatory stimuli modulate transepithelial Cl^- transport, which is essential to regulate the ASL, maintaining the epithelium barrier's homeostasis and host defence [5]. Therefore, we first evaluated the expression of CFTR, TMEM16A, SCL26A4 and SLC26A9. Overall, our results show that CFTR is the primary Cl^- channel modulated by inflammation, with all cytokines increasing its expression, except for TNF-α (Fig.1A,D). Interestingly, while these observations agree with previous studies [5], they also establish novel roles for IL-8, IL-10 and IL-22 as regulators of CFTR protein expression. Interestingly, previous studies showed that *wt*-CFTR suppresses IL-8 secretion from airway epithelial cells [30], here we observed that IL-8 also has an autocrine effect, upregulating CFTR and SLC26A9 (Fig.1A,D,E).

Furthermore, although all cytokines increased mucus production (Fig.5), IL-4 was the only one upregulating TMEM16A (Fig.1B,D,F). This observation may shed some light on the current controversy on whether TMEM16A is required for mucus production [31]. Indeed, we have previously shown that IL-4-mediated TMEM16A upregulation does not drive mucus hyperproduction, but instead, both events result from an increase in cell proliferation [26].

Besides regulating Cl^- channels/transporters, we also show that cytokines modulate epithelial differentiation. Particularly, our results demonstrate that while IL-1β and IL-4 decrease ciliated cells, they have the opposite effect on club cells (Figs.2;3A,B,D). These effects should be considered when designing therapies to reduce mucus production in chronic airway diseases,

as they might not be sufficient if the airways lack ciliated cells to achieve proper MCC. This is particularly relevant for CF, a disease characterised by mucus plugging and stasis, in which IL-1β is the main cytokine driving mucus hyperproduction [32]. Contrarily, IL-10 and IL-22 biased the airway epithelium towards ciliated cells and away from ionocytes (Fig.2,3), suggesting that these cytokines might support mucosal defence by directly regulating MUC5AC production (Fig.~~5~~6) and ciliated-cell differentiation (Fig.2,3), ensuring efficient MCC. Moreover, the reduction in pulmonary ionocytes induced by IL-10 and IL-22 (Fig.3C,D) may help understand this new cell-type function.

Interestingly, our results showed that although IL-1β, IL-4 and IL-10 upregulated CFTR expression (Fig.1A,D), IL-4 was the only cytokine significantly increasing its function (Fig.~~4~~5A,C). These results may be explained by opposite changes in ciliated, club cells and ionocytes (Fig.2,3). Indeed, IL-4 increased the percentage of CFTR-expressing club cells (Fig.~~3E~~4B,D), suggesting that this cell-type is the main contributor to CFTR function in IL-4-treated cells. This conclusion is supported by other studies describing the contribution of club cells to CFTR expression/function [32],[33]. Additionally, this effect may also be explained by TMEM16A upregulation, an enhancer of CFTR function [25]. Furthermore, whereas IL-1β decreased club cells, it did not affect this cell-type contribution for CFTR expression (Fig.~~3~~4) or function (Fig.~~4~~5A,C). Our results on IL-10-treated cells also show that pulmonary ionocytes do not necessarily mediate CFTR function, as this cytokine decreased their percentage (Fig.3C,D) while not affecting cAMP-activated currents (Fig.~~4~~5A,C).

Inflammatory cytokines are among the main factors inducing mucin hyperproduction [34]. Here, all cytokines increased mucus production by upregulating mRNA expression of either MUC5AC (IL-4, IL-10, IL-22), MUC5B (IL-1β, TNF-α, IL-17A) or both (IL-8) (Fig.~~5~~6). These results corroborate previous reports describing a differential induction of MUC5AC and MUC5B due to activation of different transcription factors and signalling pathways [35],[36]. These mucins play distinct roles in maintaining lung homeostasis: MUC5B is essential to regulate MCC and avoid infection, while MUC5AC is considered an "acute response mucin", and its levels rapidly increase after an allergic inflammatory challenge [29]. Furthermore, MUC5AC is a key controller of mucus transport by anchoring MUC5B-containing strands secreted from submucosal glands [37]. Thus, the differential induction of both mucins will likely affect mucus transport. These effects are particularly relevant for CF, where MUC5B-mucus strands remain tethered to submucosal glands, leading to mucus stasis [38]. Thus, by increasing MUC5B mRNA expression, IL-8, IL-1β, TNF-α, and IL-17A may worsen mucus stasis in CF. Nonetheless, the

overall cytokine-mediated mucus hyperproduction likely occurs as a protective response of airway epithelial cells to ensure proper clearance by trapping the inhaled pathogens in the secreted mucus. However, when CFTR – a key channel ensuring ASL hydration – is deficient, the effect of mucus hypersecretion is noxious.

While it is widely established that pro-inflammatory mediators stimulate mucus production [34], less information is available about anti-inflammatory cytokines. In this study, MUC5AC transcript levels were also upregulated by IL-10 (Fig.~~5~~6), a potent anti-inflammatory cytokine [13]. Interestingly, IL-10 was identified to regulate MUC2 expression, the prominent intestinal mucin [39], through the activation of the transcription factors STAT1 and STAT3, the latter being also involved in MUC5AC transcription [40]. Moreover, IL-22 induced a similar effect (Fig.~~5~~6), a cytokine that maintains the mucosal cell barrier by promoting tissue regeneration [22]. IL-22 belongs to the IL-10 family, and both cytokines activate similar pathways, including STAT3 [22]. Thus, further studies are required to determine how IL-10 and IL-22 regulate MUC5AC gene expression and ultimately address whether this effect is translated into an increase in mucin protein expression and percentage of secretory cells.

In summary, by assessing Cl^- channels/transporters, epithelial differentiation status and mucus production, we grouped these cytokines according to their effect on the airway epithelium (Fig.~~6~~7). Overall, we observed that IL-4 induced the most extensive modifications, upregulating CFTR, TMEM16A and SLC26A9 (Fig.1), club-cell differentiation (Fig.2,3), MUC5AC hyperproduction (Fig.~~5~~6A) and decreasing the number of ciliated cells (Fig.3A,D). Therefore, as IL-4 also increased CFTR function (Fig.~~4~~–5A,C), it would be interesting to elucidate the mechanisms behind this effect, which may lead to the identification of novel strategies to increase CFTR expression and function in CF cells.

Taking together all data in this study, we propose a model for the effect of each cytokine on airway epithelial cells. For simplicity, cytokines grouped on the same cluster are represented together (Fig.~~6,7~~7,8). In summary, these models describe the effects of the different cytokines on the differential induction of MUC5AC and MUC5B; the percentage of different airway epithelial cell-types; and expression of Cl^- channels/transporters. Overall, these results will help to understand the impact of inflammatory mediators on airway epithelium properties. Further studies are required to fully characterise the signalling pathways activated by these cytokines that may improve MCC so as to identify druggable targets for the treatment of chronic pulmonary diseases while reducing the impact of inflammation. Additionally, this knowledge may

also allow predicting a specific individual's pathophysiology according to the circulating levels of these cytokines.

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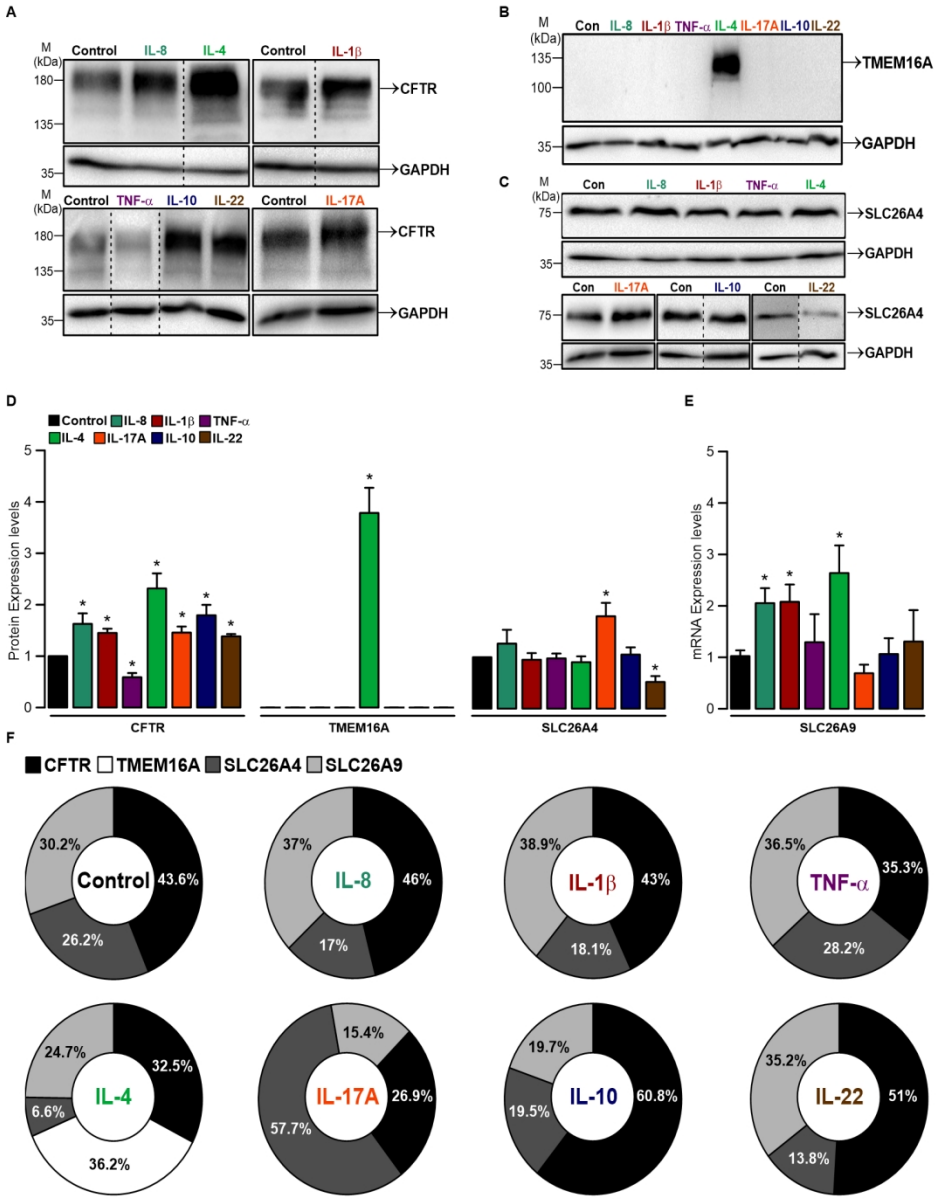
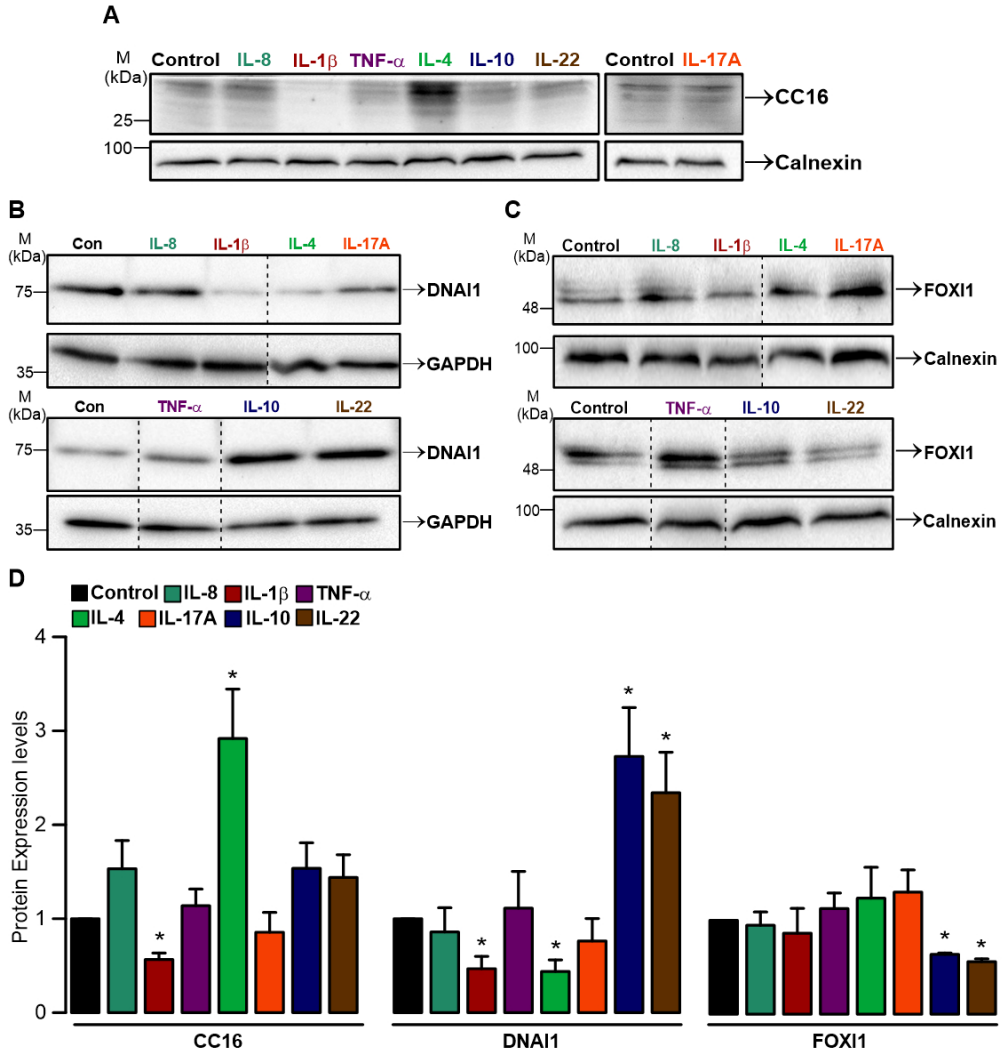


Figure 1. Effect of inflammatory mediators on the expression of airway chloride channels and transporters in differentiated BCi-NS1.1 cells. Western blot (WB) of endogenous (A) CFTR (~180 kDa), (B) TMEM16A (~120 kDa) and (C) SLC26A4 (~85 kDa) expression following exposure to cytokines. GAPDH (~36 kDa) was used as a loading control. For each cytokine, representative blots are shown. The dashed line indicates lanes run on the same gel but noncontiguous. (D) CFTR, TMEM16A and SLC26A4 protein expression levels were detected by WB, quantified by densitometry and normalised to the loading control and control untreated cells. Data are shown as mean ± SEM (n = 3 - 4). For each protein, asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test). (E) SLC26A9 mRNA expression levels following exposure to cytokines, quantitatively measured by qRT-PCR and normalised to the housekeeping gene GAPDH. Fold-change values are mean ± SEM, relative to control untreated cells (n = 3 - 4). Asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test). (F) Contribution of CFTR (black), TMEM16A (white), SLC26A4 (dark grey) and SLC26A9 (light grey) for total chloride channels/transporters expression following exposure to each cytokine. Values were calculated by dividing the fold change of each channel's protein/mRNA expression levels shown in (D) and (E) by the sum of all.



Effect of inflammatory mediators on cell-type markers specific for ciliated, club cells and pulmonary ionocytes. Western blot (WB) comparing the endogenous expression of the (A) club-cell marker CC16 (~25 kDa), (B) ciliated-cell marker DNAI1 (~75 kDa) and (C) pulmonary ionocyte marker FOXI1 (~40 kDa) following exposure to cytokines. GAPDH (~36 kDa) and Calnexin (~90 kDa) were used as loading controls. For each cytokine, representative blots are shown. The dashed line indicates lanes run on the same gel but noncontiguous. (D) DNAI1, CC16 and FOXI1 protein expression levels were detected by WB, quantified by densitometry and normalised to the loading control and control untreated cells. Data are shown as mean ± SEM (n = 3 - 4). For each protein, asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test).

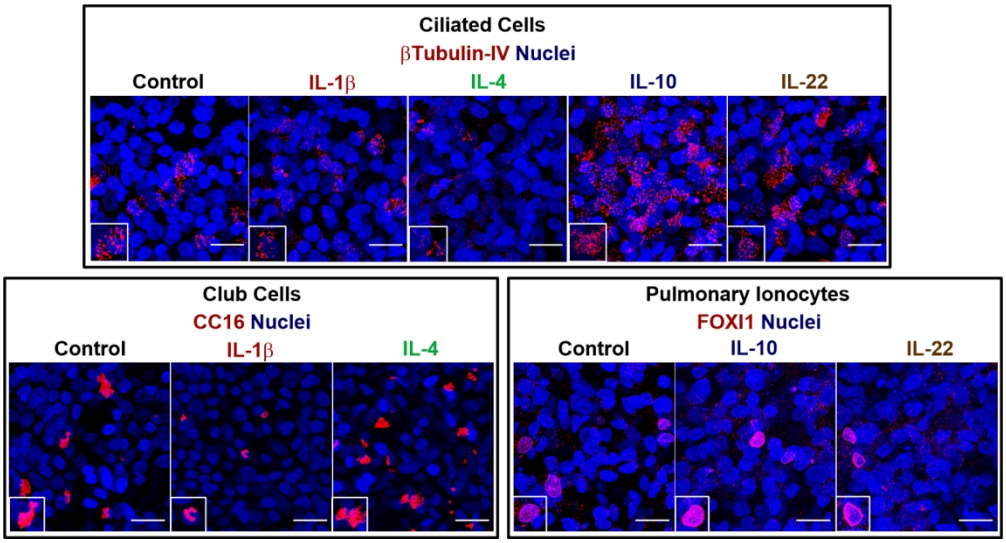
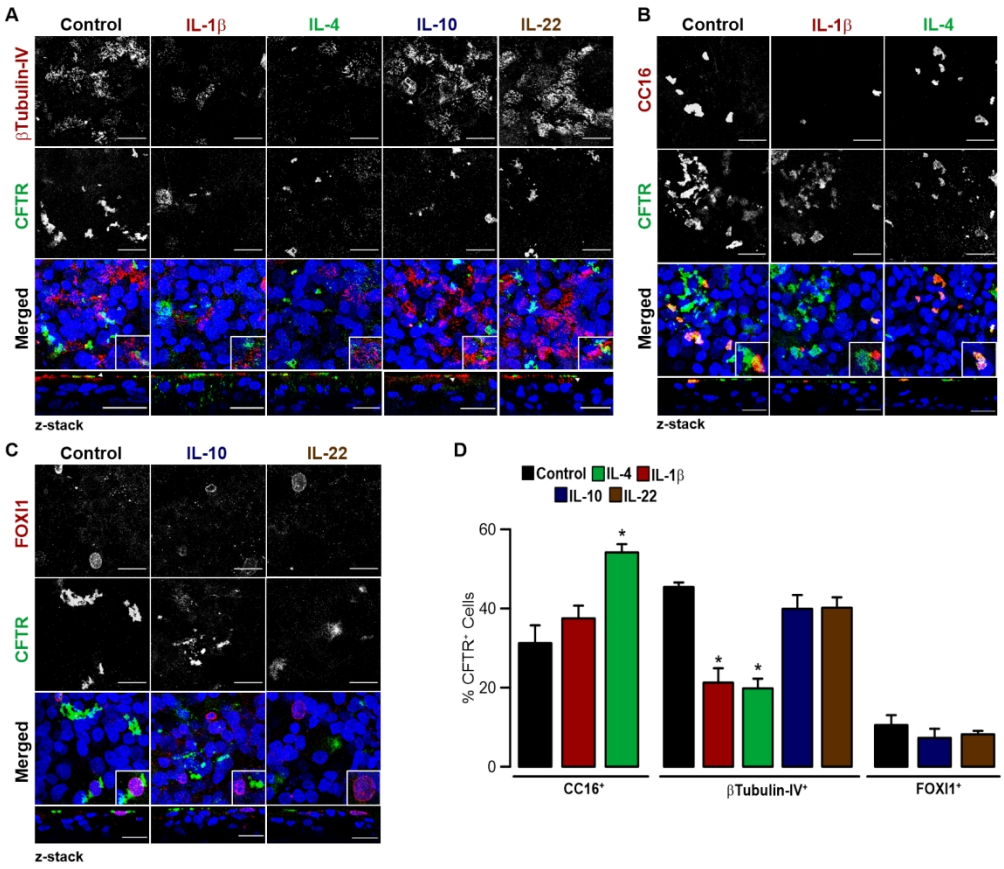


Figure 3. Modulation of epithelial differentiation towards ciliated, club cells and pulmonary ionocytes by IL-1β, IL-4, IL-10 and/or IL-22 in BCi-NS1.1 cells. Confocal immunofluorescence microscopy images showing (A) ciliated cells (labelled with βTubulin-IV), (B) club cells (labelled with CC16) or (C) pulmonary ionocytes (labelled with FOXI1) in permeabilised fully-differentiated BCi-NS1.1 cells (ALI day 30) exposed to IL-1β, IL-4, IL-10 or IL-22. Nuclei were stained with Hoechst. . Images are maximum projections (and insets) of an amplified region. Scale bar = 20 μm. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63 x oil, NA 1.4). (D) Quantification of the % of ciliated, club cells and pulmonary ionocytes (stained with the βTubulin-IV, CC16 and FOXI1 antibodies shown in A-C, respectively). The number of each cell-type was normalised to the total number of nuclei.



CFTR distribution under different inflammatory conditions. Confocal immunofluorescence microscopy images showing CFTR (green) and (A) ciliated cells (labelled with βTubulin-IV in red), (B) club cells (labelled with CC16 in red) or (C) pulmonary ionocytes (labelled with FOXI1 in red) in permeabilised fully-differentiated BCi-NS1.1 cells (ALI day 30) exposed to IL-1β, IL-4, IL-10 or IL-22. Nuclei were stained with Hoechst. Images are maximum projections (and insets) or z-stacks of an amplified region. Scale bar = 20 µm. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63 x oil, NA 1.4). White arrows in z-stack images (A) show cells that are co-stained with CFTR and β-Tubulin IV. (D) Quantification of the % of cells co-stained with CFTR and CC16, βTubulin-IV or FOXI1 normalised to the total number of CFTR+ cells. Quantifications were performed in 15 images randomly acquired in three independent experiments (5 images/experiment). Values are mean ± SEM (n = 3). For each cell-type, asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test).

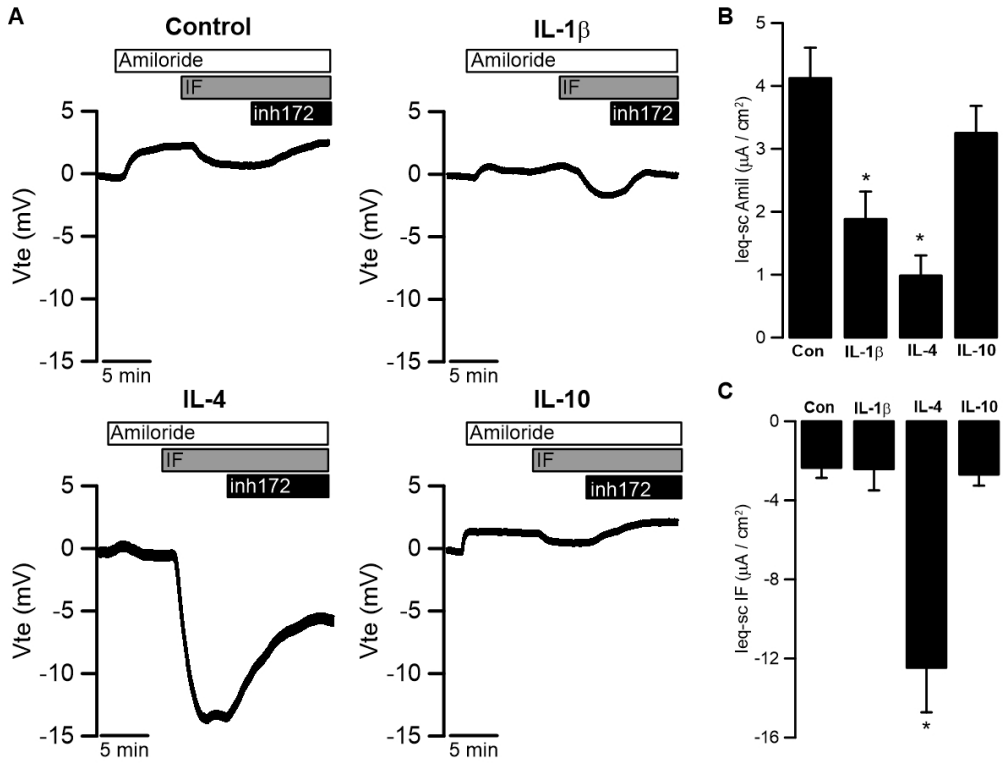


Figure 5. Effect of IL-1β, IL-4 and IL-10 on the cAMP-activated and amiloride-sensitive currents in polarised BCi-NS1.1 cells. (A) Representative Ussing chamber tracings of transepithelial voltage measurements (Vte) for untreated BCi-NS1.1 cells (control, black) and treated with IL-1β (red), IL-4 (green) and IL-10 (blue). A positive deflection is observed after stimulation with the epithelial Na+ channel (ENaC) inhibitor, amiloride (30 μM), which is followed by a negative deflection after stimulation with apical IBMX (100 μM) and Forskolin (2 μM). This effect was reversed upon the addition of the CFTR inhibitor-172 (30 μM). (B) Summary of the Isc-eq (μA/cm2) calculated for the response to amiloride (Amil). (C) Summary of the Isc-eq (μA/cm2) calculated for the response to IBMX + Forskolin (IF). Values are mean ± SEM (n = 3 - 5). Asterisks indicate significant differences vs control. (p-value < 0.05, unpaired t-test).

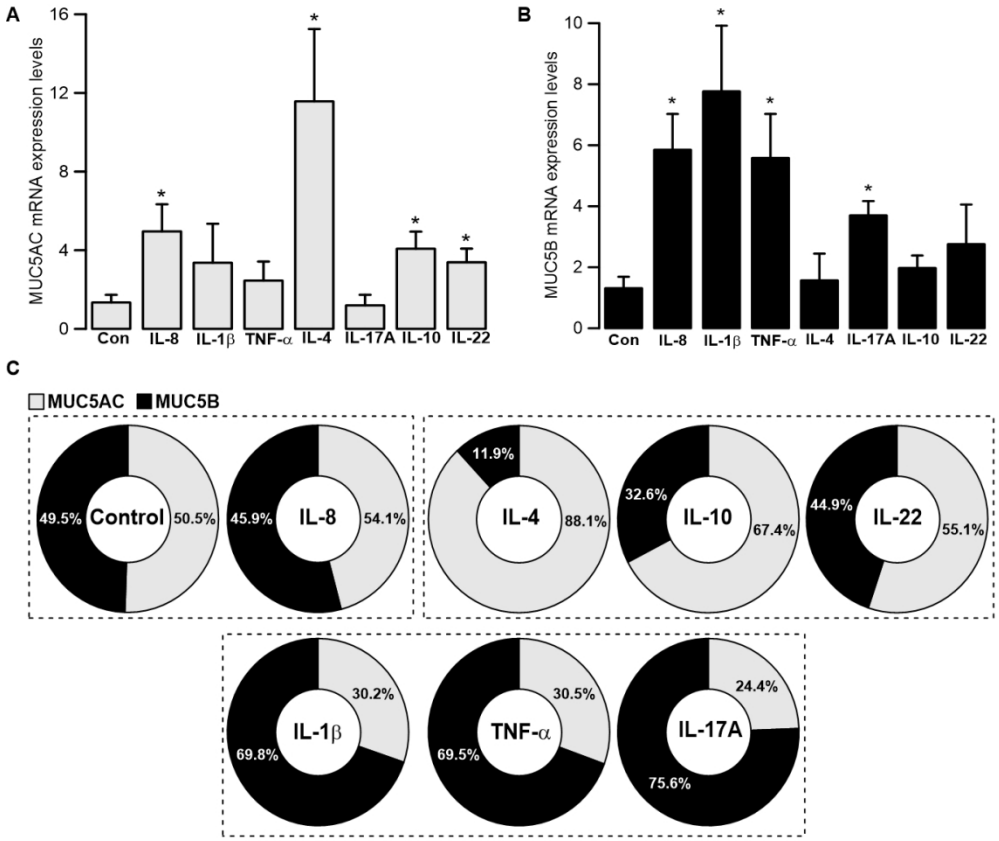


Figure 6. MUC5AC and MUC5B mRNA expression in differentiated BCi-NS1.1 cells following exposure to cytokines. (A) MUC5AC and (B) MUC5B mRNA expression levels following exposure to cytokines, quantitatively measured by qRT-PCR and normalised to the housekeeping gene GAPDH. Fold-change values are mean ± SEM, relative to control untreated cells (n = 3 - 5). Asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test). (C) Contribution of MUC5AC (grey) and MUC5B (black) for mucin expression. Values were calculated by dividing each mucin's fold-change expression levels by the sum of the MUC5AC and MUC5B expression levels shown in (A) and (B).Dashed lines indicate groups of treatments that have matching contributions of MUC5AC or MUC5B expression.

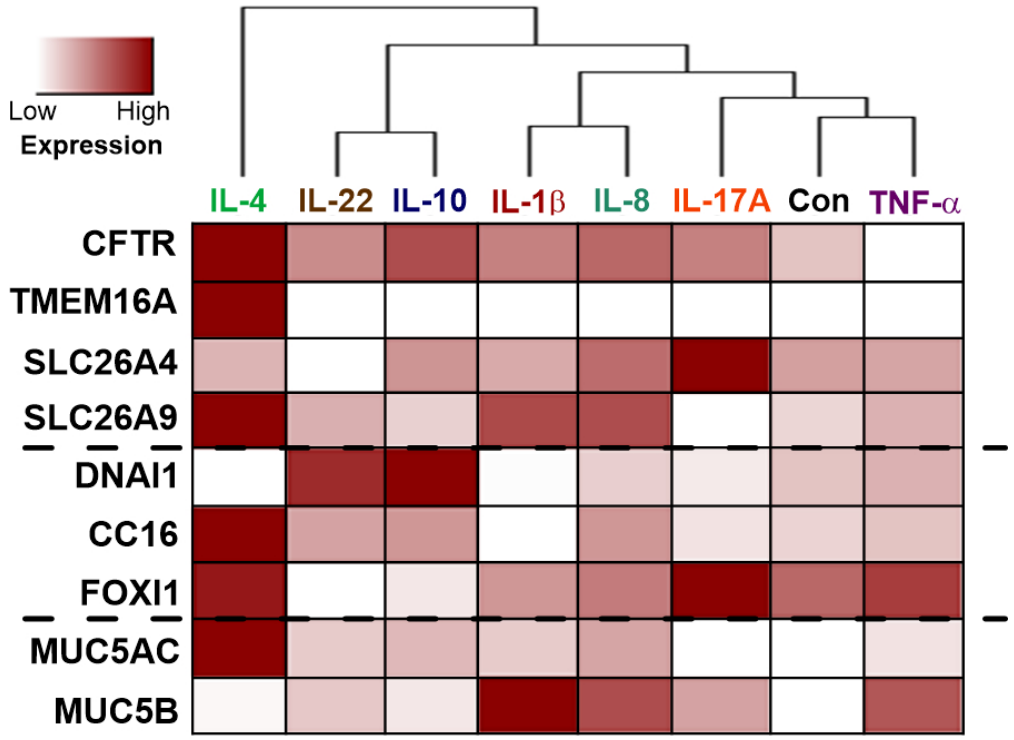


Figure 7. Summary of the observed changes in different chloride channels/transporters expression and differentiation markers upon exposure to cytokines. The heatmap was generated based on the results shown in Figs. 1, 2 and 5. Data was rescaled between 0 and 1, with lower and higher expression levels represented in white and red, respectively. Inflammatory mediators are grouped into clusters according to the relative expression levels of all the proteins analysed. Dashed lines separate the chloride channels/transporters, differentiation markers and mucins.

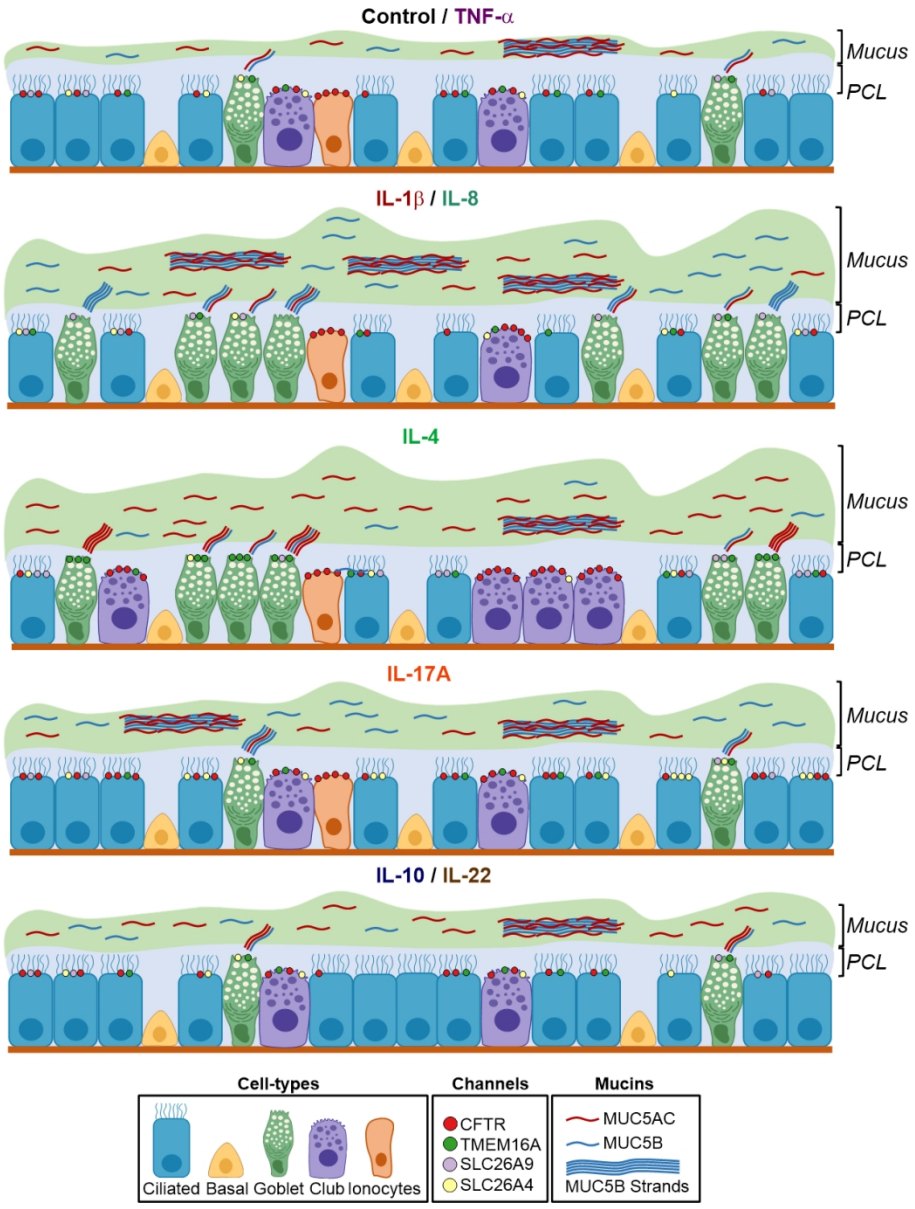


Figure 8. Models for the impact of distinct cytokines on the airway epithelium in terms of differentiation status, mucus production and expression of chloride channels/transporters. Cytokines are represented based on the heatmap clusters (Fig.6) – IL-8 and IL-22 induced similar effects as IL-1β and IL-10, respectively; TNF-α is not shown as it was grouped with control untreated cells. Mucus hyperproduction is represented by a higher thickness of the mucus gel layer (green) above the periciliary layer (PCL, blue), resulting from the upregulation of MUC5AC (red threads) or MUC5B (blue threads). MUC5B strands are also shown, usually secreted from submucosal glands and coated in the surface epithelium by MUC5AC. MUC5AC hyperproduction was prevalent in IL-4 and IL-10-treated cells, while IL-1β and IL-17A increased MUC5B. The cytokine's effects on epithelial differentiation are displayed by changes in the number of ciliated cells (higher in IL-10, lower in IL-1β and IL-4), club cells (higher in IL-4, lower in IL-1β) and pulmonary ionocytes (lower in IL-10). The number of CFTR, TMEM16A, SLC26A4 and SLC26A9 channels/transporters vary according to the observed differences following exposure to cytokines (Fig.1) and have a cell-type distribution matching what is described for the human airway epithelium.