

ORIGINAL ARTICLE

N-acetylcysteine and azithromycin affect the innate immune response in cystic fibrosis bronchial epithelial cells in vitro

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ABSTRACT

Background and objective. We have previously reported that N-acetylcysteine (NAC), ambroxol and azithromycin (AZM) (partially) correct the chloride efflux dysfunction in cystic fibrosis bronchial epithelial (CFBE) cells with the $\Delta F508$ homozygous mutation in vitro. **Methods.** In the present paper, we further investigated possible immunomodulatory effects of these drugs on the regulation of the innate immune system by studying the expression of the cytosolic NOD-like receptors NLRC1 and NLRC2, and interleukin (IL)-6 production in CFBE cells. **Results.** Under basal conditions, PCR and Western Blot data indicate that the NLRC2 receptor has a reduced expression in CF cells as compared to non-CF (16HBE) cells, but that the NLRC1 expression is the same in both cell lines. AZM significantly upregulated NLRC1 and NLRC2 while NAC upregulated only NLRC2 receptor expression in CF cells. Reduced basal IL-6 production was found in CF cells as compared to non-CF cells. MDP (an NLRC2 agonist), NAC and AZM, but not Tri-DAP (an NLRC1 agonist), increased IL-6 production in CF cells, indicating that in CF cells IL-6 upregulation is independent of NLRC1, but involves the activation of NLRC2. **Conclusion.** Overall, the results indicate that NAC and AZM not only can correct the chloride efflux dysfunction but also have a weakly strengthening effect on the innate immune system.

KEYWORDS Cystic fibrosis, chloride efflux activators, NOD-like receptors

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a protein that predominantly functions as a chloride (Cl^-) channel in epithelial cells. The most important clinical symptom of the disease is chronic obstructive airway disease, due to the fact that the defect in CFTR results in decreased epithelial Cl^- efflux, reduced airway surface liquid (ASL) volume, delayed mucus clearance, and increased mucus adhesion to the airway surface. This increased mucus adhesion eventually leads to chronic and recurrent

microbial infections mainly with *Staphylococcus aureus* and *Pseudomonas aeruginosa* which leads to hyper-inflammation and progressive lung function decline [1–4]. The airway epithelium plays a key role in the defense of the respiratory system by provoking the innate immune system [5]. Dysregulation of the innate immune system of the airway epithelium has been observed in a number of diseases, such as CF, chronic obstructive pulmonary disease (COPD), and asthma that are associated with compromised immunity and chronic inflammation of the lungs [6].

Host-directed immunomodulatory therapies are one potential approach to initiate or increase protective antimicrobial immunity, and to reduce the extent of inflammation-induced tissue injury. NLRC1 and NLRC2 (formerly known as NOD1 and NOD2) belong to the NOD-like receptor (NACHT and LRR (leucine-rich repeat) domain containing proteins;

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NLR) family that forms an important subfamily of cytoplasmic innate immunity proteins [7, 8]. NLRC1 and NLRC2 are expressed in human oral, nasal, lung, and intestinal epithelial cells [9–12]. NLRC1 detects *m*-DAP (L-Ala- γ -D-Glu-*m*-diaminopimelic acid), an amino acid found in most Gram-negative and some Gram-positive bacteria, whereas NLRC2 recognizes muramyl dipeptide (MDP) present in both Gram-negative and Gram-positive bacteria [7]. This recognition results in activation of the nuclear factor- κ B (NF- κ B) and mitogen activated protein kinases (MAPK) signaling pathway and ultimately in production of proinflammatory cytokines, including interleukin (IL)-6, and in this way initiates an appropriate innate immune response [13, 14].

In CF, attempts have been made to correct the defective Cl⁻ efflux by drugs [15]. Previously, our group has published that N-acetylcysteine (NAC), ambroxol, and azithromycin (AZM) increased Cl⁻ efflux from CF airway epithelial cells with Δ F508 homozygous mutation, the most prevalent and first identified CF-causing mutation among Caucasians [2, 16–18]. CF is a monogenetic disease that has been clearly linked to a mutation in CFTR. However, CF cells show many differences from their non-CF counterparts, such as altered expression of epithelial Na⁺ channel (ENaC) subunits, decreased expression of inducible nitric oxide synthase (iNOS), altered expressions of mucins, altered rates of wound healing, and the relation between these changes and the mutation in CFTR is unclear [19]. In vivo, CF airways show differences from healthy airways (viscous mucus, hyperinflammation, frequent bacterial infection), of which the relation with the mutation in CFTR sometimes has been established, but sometimes not (yet).

NAC has been shown to be useful in the treatment of CF as a mucolytic and anti-oxidant drug [20]; the anti-oxidant action of this drug may involve its conversion to glutathione (GSH), which is interesting in view of the effects of another potential drug for CF, namely S-nitrosoglutathione [21,22]. Ambroxol and AZM have also been shown to improve clinical parameters in CF patients [23–26]. AZM is prescribed to CF patients even without *Pseudomonas* infection and considered as a potential part of medication in CF care [4, 6, 26]. Recently, an immunomodulatory effect of AZM on lipopolysaccharide tolerance in mice has been reported [27]. Whereas the primary use of NAC and ambroxol is as mucolytic, antioxidant, and anti-inflammatory drugs, AZM is an antibiotic used to treat CF patients with hyper-inflammation. However, a potential immunomodulatory effect of NAC, ambroxol, and

AZM on the expression and function of NLRs in CF airway epithelial cells before microbial infection and inflammation has not, so far, been investigated.

Both airway epithelial cells and immune cells contribute to the pulmonary inflammation [28], but studies on immortalized CF airway epithelial cells have shown reduced TLR4 expression, reduced pro-inflammatory cytokine (IL-6, IL-8) production [29–31], reduced antimicrobial (psoriasis) expression [32], and diminished type I interferon signaling [33]. However, these findings are controversial [34–36], which may be because of differences in cell model, experimental conditions, analytical tools, and/or CFTR genotype [28, 37]. Also here, the relation with the mutation in CFTR has not been clearly established. If there is a connection, which would be a reasonable assumption, it would not be illogical that (some) drugs that would correct the error in Cl⁻ transport, also would correct other abnormalities in CF airways.

In vivo, the CF lung environment is comprised of immune cells along with epithelial cells due to which it is very difficult to determine whether pro-inflammatory cytokines and/or chemokines are secreted only from immune cells or from both cell types [37]. It would therefore appear simpler to investigate this in an in vitro model. The current study is an extension of our previously published studies in order to investigate: (1) whether NLRC1 and NLRC2 are differently expressed in CF airway epithelial cells compared to non-CF airway epithelial cells, and (2) whether NAC, ambroxol, and AZM in addition to partially correcting the Cl⁻ efflux defect in CF cells, also can strengthen the innate immune response by affecting NLRC1 and NLRC2 expression, and IL-6 production in CF airway epithelial cells.

MATERIALS AND METHODS

Cell Lines

CF bronchial epithelial cells (CFBE, homozygous for the Δ F508 mutation) and non-CF bronchial epithelial cells [38] were a kind gift of Dr D. Gruenert (San Francisco, CA, USA). The cells were cultured in adherent flasks (Sarstedt, Landskrona, Sweden) in Medium 199 (Invitrogen/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% O₂. The medium was changed twice weekly.

Treatment with N-acetylcysteine, Ambroxol, and Azithromycin (AZM)

Cells were grown to confluence on 6-well plates (Sarstedt). When the cells were confluent, the M199 medium was replaced with 2 mL fresh medium plus 10 mM N-acetylcysteine (NAC), 100 μ M ambroxol, or 40 μ M AZM (all from Sigma—Aldrich, St. Louis, MO, USA) for 4 hours.

RNA Preparation and cDNA Synthesis

Total RNA was isolated from CF and non-CF cells using an RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, RNA from approximately 4×10^6 cells was eluted in 50 μ L RNase-free water and stored at -80°C . The total RNA concentration and the quality of the isolated RNA were assayed with an Agilent Bioanalyzer 2100 and RNA 6000 Nano Assay Kit (Agilent Technology, Santa Clara, CA, USA) according to the manufacturer's protocol. For first-strand cDNA synthesis a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions. Briefly, 0.5 μ g of total RNA was used to produce first-strand cDNA with dNTP in a final volume of 20 μ L RNase-free water and stored at -20°C .

Real-time Reverse Transcriptase-Polymerase Chain Reaction (Real-time RT-PCR)

Real-time RT-PCR was performed using the thermal cycler TaqMan7500 Fast Real Time PCR System (Applied Biosystems) with 7500 Fast Sequence Detection and Absolute Quantification software packages. For detection of NLRC1 and NLRC2 receptors, probes were purchased from Applied Biosystems, and used according to the manufacturer's instructions. The thermal cycling conditions were as follows: step one: 95°C for 2 minutes, and step two: 95°C for 3 seconds and 60°C for 30 seconds (step 2 repeated 30 times). PCR reactions were performed in 15 μ L using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and 1.5 μ L cDNA. The mRNA expression of the samples was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Validation of GAPDH as an internal control was performed by calculating the fold change by $2^{-\Delta\Delta\text{Ct}}$ [39]; the expression of GAPDH remained constant. Samples were run as singleplex in triplicate wells.

Western Blot Analysis

For differential expression of basal NLRC1 and NLRC2 protein expression in CF and non-CF cells Western blotting was used. Furthermore, the effect of 10 mM NAC, 100 μ M ambroxol, or 40 μ M AZM on NLRC1 and NLRC2 protein expression in CF cells after 4-hour treatment was determined. The cells were lysed in cold radioimmunoprecipitation assay (RIPA) buffer (1 mM Tris, 15 mM NaCl, 0.2 mM EDTA pH 7.4, 0.125% Triton-X100), containing protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by the DC protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) at an absorbance of 750 nm on a Multiscan Ascent (Thermo Labsystems, Stockholm, Sweden). 10 μ g protein was loaded using a 5X protein loading buffer pack (Fermentas Life Sciences, Burlington, Canada). Proteins were separated on any kDTM Mini-Protean[®] TGXTM Precast Gel and transferred to PVDF membranes (Bio-Rad), for 1 hour. Membranes were blocked with 5% bovine serum albumin (BSA) (Bio-Rad) in PBS-0.1% Tween buffer and incubated either with NLRC1 goat-polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with NLRC2 rabbit polyclonal antibodies (Santa Cruz Biotechnology) overnight, at 4°C . Blots were washed in PBS-Tween (Sigma-Aldrich) and incubated with secondary anti-bodies, donkey anti-goat IgG-horse radish peroxidase (HRP) and goat anti-rabbit IgG-HRP respectively, for NLRC1 and NLRC2, at room temperature for 1 hour. After a final wash in PBS-Tween, blots were detected using LuminataTM Forte Western HRP substrate (Merck Millipore, Billerica, MA, USA). Images of bands were determined by Molecular ImagerH (ChemiDocTM XRS, Bio-Rad), as described in the manufacturer's instructions. Band density was quantified using Quantity One[®] version 4.6.3 software (BioRad). The band density of each protein was normalized by stripping and reprobing the respective membranes with the primary antibody of β -tubulin.

Cytokine Determination Using Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6 was determined in CF and non-CF cells under basal conditions, and in the CF cells treated with the indicated doses of NAC, ambroxol, and AZM, as well as with the NLRC1 agonist L-Ala- γ -D-Glu-mDAP (Tri-DAP) or the NLRC2 agonist muramyl dipeptide (MDP) (both Invivogen, San Diego, CA, USA). After 4 hours, the culture supernatant was collected, centrifuged, and stored at -80°C . The concentration of

IL-6 was determined using a commercially available OptEIA Set for enzyme-linked immunosorbant assay (BD Biosciences).

Statistical Analysis

All data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests and an unpaired *t*-test using Graphpad (San Diego, CA, USA) Prism version 5.02.

RESULTS

NLRC2 but not NLRC1 mRNA and Protein Levels Are Reduced in CF Airway Epithelial Cells Compared to Non-CF Cells

We first investigated the mRNA expression of NLRC1 and NLRC2 receptors in CF (CFBE) and non-CF (16HBE) bronchial epithelial cells under basal conditions. RT-PCR revealed that there was no significant difference in the level of NLRC1 between these two cell lines (Figure 1A). There was, however, a significant ($P < .001$) reduction in the expression of NLRC2 in CFBE cells compared to 16HBE cells (Figure 1A).

Western blot analysis, using the anti-NLRC1 antibody, showed a distinct band at 108 kDa in both CF cells and non-CF cells (Figure 1B). The anti-NLRC2 antibody revealed a distinct band in the non-CF cells, but a less intense band in the CF cells at 91 kDa (Figure 1B). These results were supported by quantitative analysis of the blots (Figure 1C).

Azithromycin Upregulates mRNA Expression of Both NLRC1 and NLRC2, but N-Acetylcysteine Upregulates Only mRNA Expression of NLRC2 in CF Cells

An investigation of the effect of NAC, ambroxol, or AZM on the mRNA expression of NLRC1 and NLRC2 revealed that AZM significantly ($P < .01$) increased the expression of NLRC1 mRNA in CF cells, whereas neither NAC nor ambroxol had this effect on NLRC1 expression (Figure 2A). On the other hand, both NAC and AZM, but not ambroxol, caused a significant increase in the mRNA expression of NLRC2 in CF cells as compared to untreated CF cells (Figure 2B).

N-acetylcysteine, Ambroxol, and Azithromycin Have no Effect on Protein Expression of NLRC1 and NLRC2 in CF Cells

To verify the effect of NAC, ambroxol, or AZM on the mRNA levels (Figure 2A, B), the protein expression of NLRC1 and NLRC2 in CF cells was studied. The data indicate that NAC, ambroxol, or AZM do not have a significant effect on NLRC1 and NLRC2 protein expression in CF cells (Figure 2C, D).

Reduced IL-6 Production in CF Cells as Compared to non-CF Cells Under Basal Conditions

In order to determine the functional significance of the NLRC1 and NLRC2 receptors in CF cells, we measured the secretion of the pro-inflammatory cytokine IL-6. Using ELISA, we measured the basal concentration of IL-6 in the culture supernatant of CF and non-CF cells. IL-6 was constitutively secreted both by untreated CF as well as non-CF cells. However, the levels of IL-6 secreted by non-CF cells were much higher ($P < .01$) compared to those secreted by CF cells after 4 hours (Figure 3A).

IL-6 Production in CF cells is Upregulated by MDP But Not by Tri-DAP

In order to determine whether the NLRC1 and/or the NLRC2 receptor mediate IL-6 production, the cells were treated with Tri-DAP (10 $\mu\text{g}/\text{mL}$), the known agonist of NLRC1, and MDP (10 $\mu\text{g}/\text{mL}$), the known agonist of NLRC2. Whereas MDP was found to upregulate IL-6 production in CF cells, Tri-DAP was not (Figure 3B).

IL-6 Production in CF cells is Upregulated by N-acetylcysteine, Ambroxol, and Azithromycin

The CF-cells were treated with NAC, ambroxol, or AZM to find out whether these drugs have any effect on the IL-6 production in CF cells. NAC, ambroxol, or AZM upregulated IL-6 production in CF cells (Figure 3C).

DISCUSSION

In the present study, the expression of NOD-like receptors in CF (CFBE) and non-CF (16 HBE) airway epithelial cells was determined under basal conditions, knowledge that is of relevance for the understanding of the clinical course of CF.

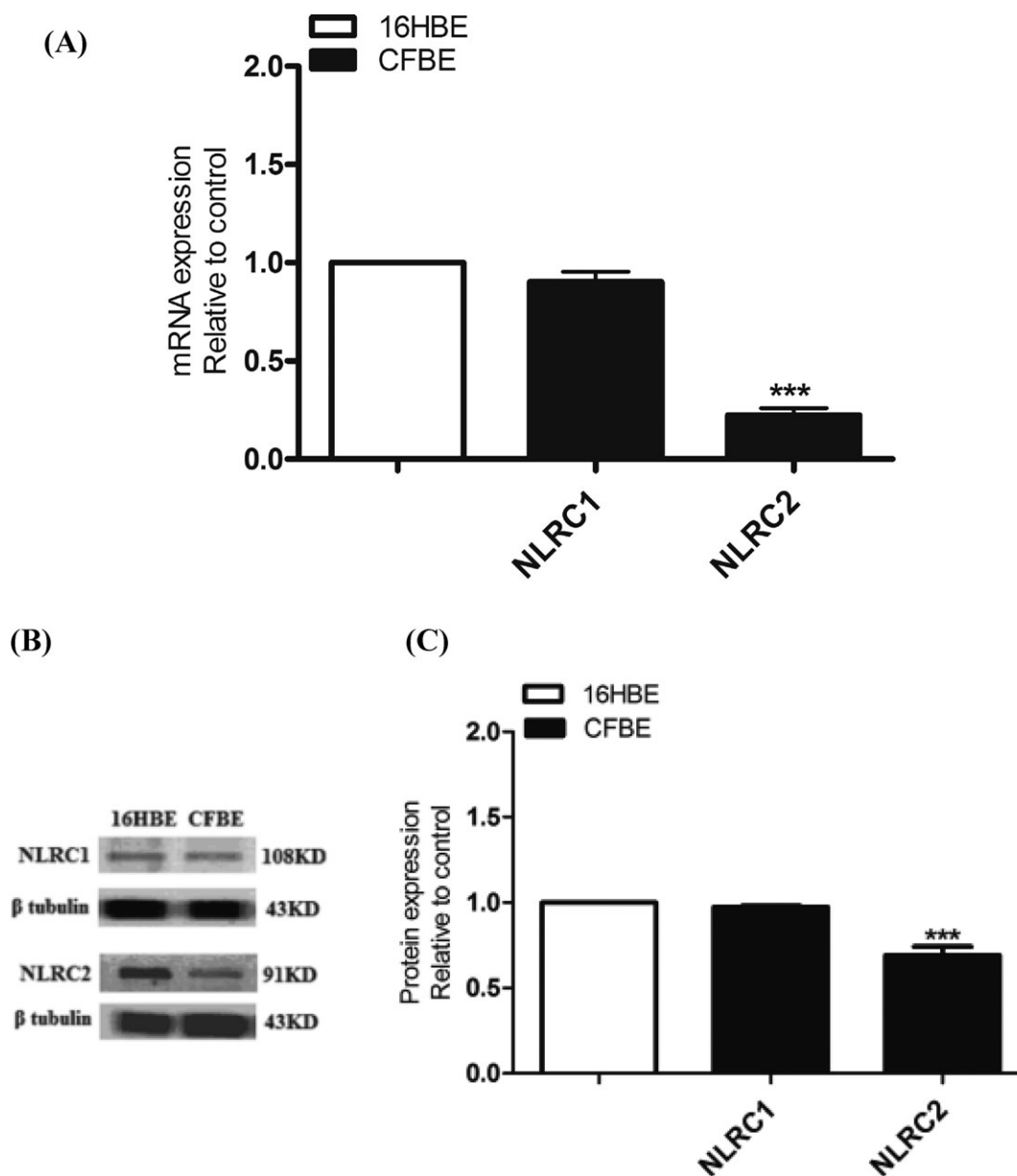


FIGURE 1. The gene and protein expression of NLRC1 and NLRC2 in bronchial epithelial cells under basal conditions. The mRNA and protein expression of NLRC1 and NLRC2 were examined in CF (CFBE) and non-CF (16HBE) bronchial epithelial cells by (A) QRT-PCR and (B) (C) Western Blot. Results are expressed relative to the expression levels in non-CF cells and expressed as mean \pm standard error of the mean (S.E.M.). One way analysis of variance and Tukey's post-test were used to compare experimental and control groups ($***P < .001$). PCR results are from three experiments, Western Blot results in (B) are from one representative experiment of three experiments. (C) is a quantitative analysis of the Western blot data from the same three experiments.

Airway epithelium is the first barrier against invasive micro-organisms entering the body via the air. Pattern-recognition receptors (PRRs) such as NLRC1 and NLRC2, part of the innate immune system, participate in bacterial clearance by initiating IL production and antimicrobial activity [13]. In the current study we found that both CF and non-CF

airway epithelial cells expressed the same levels of NLRC1, but that the level of NLRC2 was decreased in CF cells, compared to non-CF cells. The decreased expression of NLRC2 observed in CF cells may point to a dysregulated innate immune response and partly explain the compromised immunity found in CF [6]. In addition, the fact that also reduced

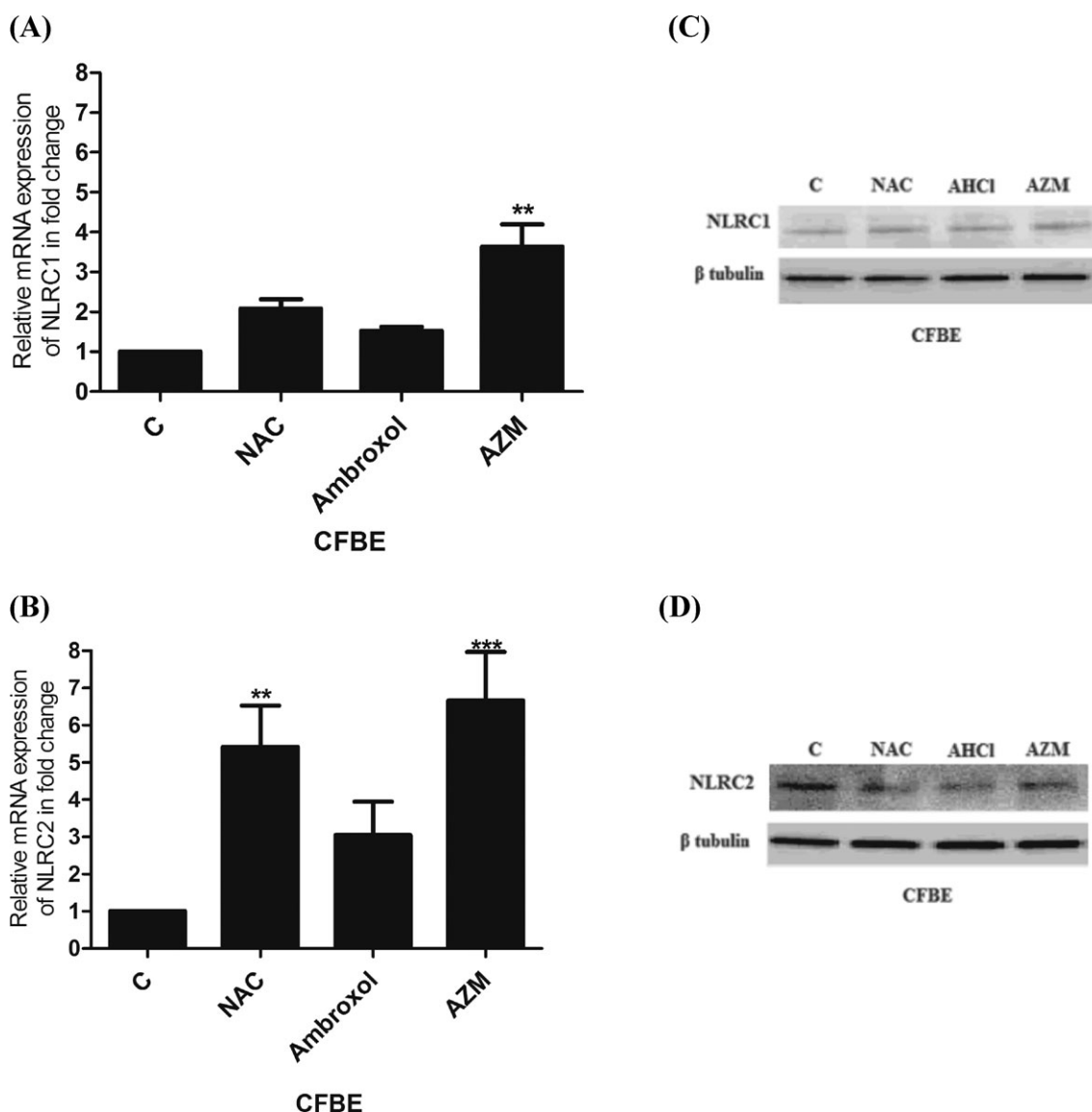


FIGURE 2. The gene and protein expression of NLRC1 and NLRC2 in CF bronchial epithelial cells after N-acetylcysteine (NAC), ambroxol, and azithromycin (AZM) treatment. The CF (CFBE) cells were treated with 10 mM NAC, 100 μ M ambroxol, or 40 μ M AZM for 4 hours and then the expression of NLRC1 (A) mRNA and (C) protein as well as NLRC2 (B) mRNA and (D) protein was determined. PCR results are expressed relative to the expression levels in untreated CF cells (c) and expressed as mean \pm standard error of the mean (S.E.M.). One way analysis of variance and Tukey's post-test were used to compare experimental and control groups (** $P < .01$, *** $P < .001$). PCR results are from three experiments and Western Blot results are from one representative experiment of two experiments.

expression of an antimicrobial protein, psoriasin, in CFBE cells has been found [32], further supports our finding of weak resistance to bacterial infection in CFBE cells. We suggest that specific therapeutic measures to increase the levels of NLRC2 may improve the innate immune response to bacteria in CF patients. We also found bands for the NLRC1 protein at 108 kDa as predicted, but not for NLRC2 at the predicted 115 kDa, but at nearly 91 kDa. It has been reported that NLRC2 RNA transcripts are alterna-

tively spliced into at least 8 putative NLRC2 variants in leukocytes and in buccal epithelial cells; data that support the assumption that NLRC2 protein size is affected by splice variants in CF cells [40].

Previously we have investigated the effect of NAC, ambroxol, and AZM on the Cl^- efflux from CFBE cells and found that NAC and ambroxol increased not only the amount of CFTR protein in the CF cells, but also the Cl^- efflux from these cells [17, 18]. AZM increased the Cl^- efflux from these cells, although no

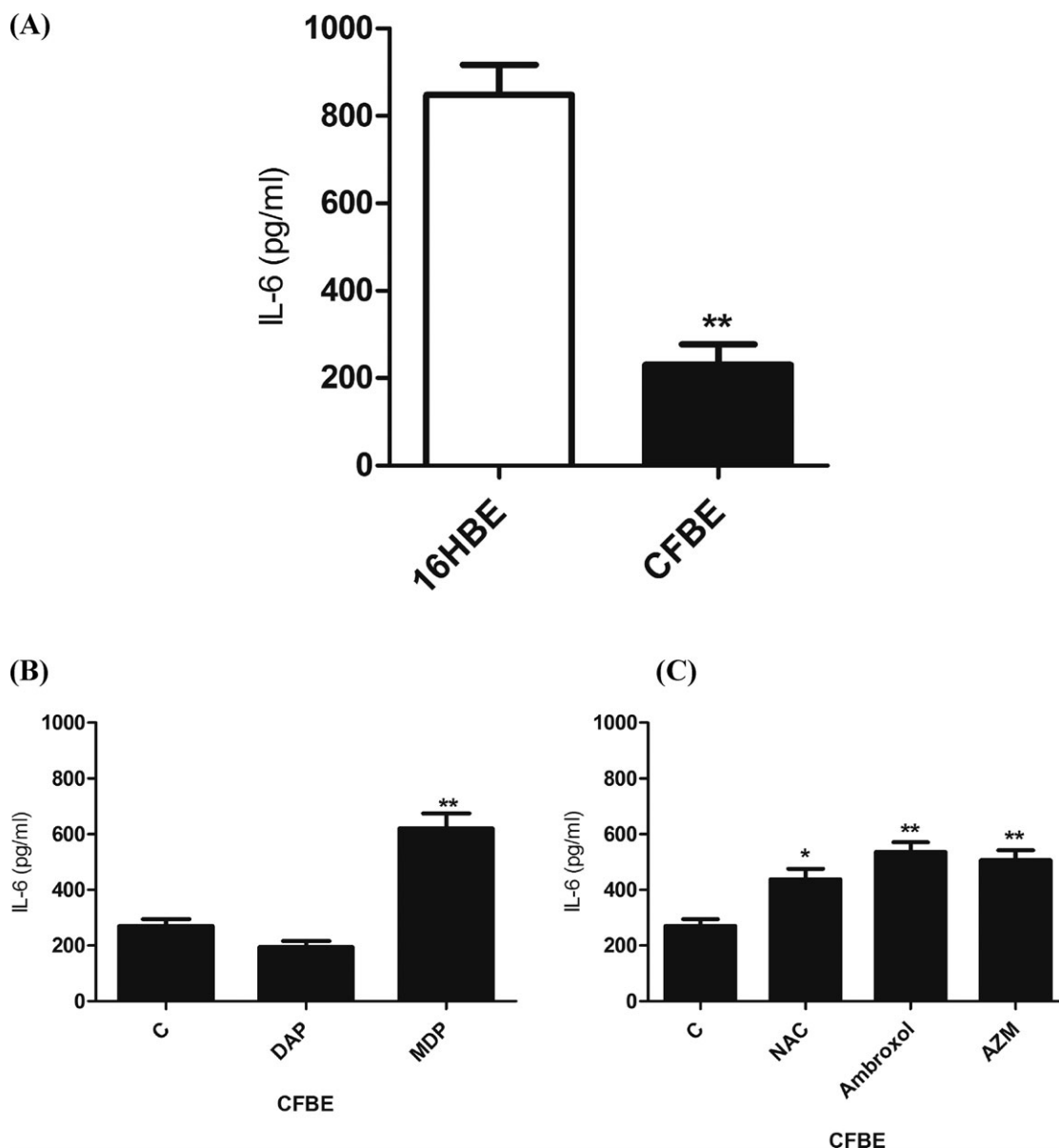


FIGURE 3. The production of IL-6 in bronchial epithelial cells under basal conditions and after Tri-DAP, MDP, N-acetylcysteine (NAC), ambroxol, and azithromycin (AZM) treatment. (A) The production of IL-6 was examined in CF (CFBE) and non-CF (16HBE) bronchial epithelial cells under basal condition and compared with expression level in non-CF cells. (B) The production of IL-6 in CF cells in response to Tri-DAP (NLRC1 agonist) and MDP (NLRC2 agonist), (C) and in response to NAC, ambroxol, and AZM (concentrations as in the legend to FIGURE 2) treatment for 4 hours and compared to the expression levels in untreated CF cells (c). IL-6 levels in the supernatant were determined with a commercial ELISA kit. Un-paired t-test, One way analysis of variance and Tukey's post-test were used to compare experimental and control groups (* $P < .05$, ** $P < .01$). The results are from three experiments and are presented as the mean \pm standard error of the mean (S.E.M.).

significant increase in CFTR protein could be ascertained [16]. Since not only the Cl^- efflux, but also the innate immune system is compromised in CF airway epithelial cells [30], we investigated whether these drugs not only corrected the Cl^- transport, but also the innate immune system, focusing on the NLRC1 and NLRC2 expression. The CF cells were

treated with NAC, ambroxol, or AZM in order to study whether these drugs upregulate NLRC1 and/or NLRC2 mRNA and protein expression in CF cells and thus may strengthen CF innate immunity. QRT-PCR data showed that AZM caused a 3.6-fold increase in NLRC1 and a 6.6-fold increase in NLRC2 mRNA expression in CF cells as compared to the

CF control, while NAC increased only the NLRC2 mRNA expression (5.4-fold) in CF cells. On the other hand, Western blot data did not show any significant upregulation in NLRC1 and NLRC2 protein expression after NAC, ambroxol, or AZM treatment in CF cells. This lack of correlation between mRNA and protein expression may be because of dynamic imbalance among the regulatory processes of post-transcriptional, translational and protein degradation after mRNA synthesis [41].

Furthermore, we investigated whether stimulation of NLRC1 or NLRC2 receptors had an effect on the secretion of a multifunctional pro-inflammatory cytokine, i.e., IL-6, in CF cells. IL-6 has a dual effect, both as a defense mechanism and/or as a pro-inflammatory cytokine [42] and it also plays an important role in the regulation of immune responses, such as induction of antibody production, hematopoiesis, and inflammation [43]. IL-6 has a vital role during the transition from innate to acquired immunity [44]. Usually, dysregulated persistent IL-6 production has been reported in the development of various auto-immune, chronic inflammatory diseases [44] and in CF [45]. In vivo, CF airways have higher levels of IL-6 than normal [46], but whether this is due to the epithelial cells or to the leukocytes of the airways is difficult to ascertain in vivo, and, in addition, in vivo the epithelial cells are exposed to bacteria, which is another factor adding to the uncertainty whether increased IL-6 levels are an intrinsic property of CF airway epithelial cells per se, or an infection-induced effect. In the present study, we found reduced basal IL-6 levels in CF cells as compared to non-CF cells, which is in agreement with similar findings reported by another group in the same cell line [30].

In order to study whether IL-6 is regulated by NLRC1 and/or NLRC2 receptors, CF cells were treated with Tri-DAP (NLRC1 agonist) and MDP (NLRC2 agonist). MDP increased IL-6 production to (nearly) two-fold in CF cells, whereas the NLRC1 agonist Tri-DAP had no effect on IL-6 production. In CF cells, IL-6 upregulation is hence independent of the NLRC1 receptor but rather depends on NLRC2. Upon treating the CF cells with NAC, ambroxol, and AZM it was found that these drugs increased IL-6 production to about two-fold in CF cells. This means that NLRC2 is functionally expressed in CFBE cells, even if at a lower level than normal, and can mediate IL-6 production in these cells elicited by NAC and AZM. NAC and AZM are considered to be anti-inflammatory drugs in vivo and one might therefore expect that IL-6 production by CFBE cells would be lowered by these drugs. However, as outlined above, the situation in cell cultures is fundamentally different

from the in vivo situation, and it can be questioned whether the cell cultures are an entirely adequate model to study inflammation. Enhancement or suppression of immune response is a useful therapeutic strategy including prevention and treatment of infections in diseases like cancer [8]. AZM, which is classically considered as an anti-inflammatory drug, increases the expression of both NLRC1 and NLRC2, while NAC only increases the expression of NLRC2 mRNA. This may indicate a novel immunomodulatory effect of these drugs in CF. Taken together our results show that NAC and AZM in airway epithelial cells expressing the $\Delta F508$ mutation may increase host defence response to bacteria through the up-regulation of NLRC2 expression and IL-6 production.

In conclusion, AZM upregulates NLRC1 mRNA in CFBE cells, NAC and AZM upregulate NLRC2 mRNA in these cells, and NAC, AZM, and ambroxol upregulate IL-6 production by CFBE cells. Hence, in addition to their effect on chloride transport, these compounds have, albeit to a different extent, a weak effect on the innate immune system.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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