

Azithromycin fails to reduce increased expression of neutrophil-related cytokines in primary-cultured epithelial cells from cystic fibrosis mice

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Received 5 December 2008; received in revised form 11 February 2009; accepted 3 March 2009

Available online 3 April 2009

Abstract

Background: Beneficial effects of azithromycin in cystic fibrosis (CF) have been reported, however, its mechanism of action remains unclear. The present study aimed at investigating the effect of azithromycin on CF airway epithelial cells.

Methods: Primary cultures of purified tracheal epithelial cells from F508del and normal homozygous mice were established. Responses to lipopolysaccharide from *Pseudomonas aeruginosa* (LPS, 0.1 µg/ml) on mRNA expression of neutrophil-related chemokines, pro- and anti-inflammatory cytokines were investigated in the presence or the absence of azithromycin (1 µg/ml).

Results: CF airway epithelial cells showed upregulation of MIP-2 and KC responses to LPS, and azithromycin failed to downregulate these responses. In contrast, in CF cells, azithromycin increased KC and TNF-α expression under non-stimulated and LPS-stimulated conditions, respectively. In non-CF cells, the macrolide potentiated the LPS response on MIP-2 and on IL-10.

Conclusions: Airway epithelial cells contribute to the dysregulated immune processes in CF. Azithromycin rather stimulates cytokine expression in CF airway epithelial cells.

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Keywords: Cystic fibrosis; CFTR; Epithelial cells; Chemokines; Cytokines; Azithromycin

1. Background

Despite remarkable progress in understanding the pathogenesis of cystic fibrosis (CF), the clear mechanism connecting abnormal CF transmembrane conductance regulator (CFTR) protein function to chronic bacterial airway infection, including colonization with *Pseudomonas aeruginosa*, has not been fully elucidated. Inflammation and infection account for a progressive and chronic pulmonary disease, the major cause of morbidity and mortality in patients with CF. A pathobiologic feature of CF

pulmonary disease is a profound tissue neutrophil accumulation which is accompanied by elevated levels of pro-inflammatory mediators such as interleukin-(IL)-8, IL-6, tumor necrosis factor-(TNF)-α and by reduced levels of anti-inflammatory mediators, such as IL-10 [1–4]. Airway epithelial cells may also contribute to the inflammatory process in CF lung disease. Although recent studies have shown that epithelial cells are an important source of IL-8, a CXC chemokine acting as a neutrophil chemoattractant [5], the concept that inflammatory gene expression in airway epithelial cells is dysregulated in CF remains controversial.

Several clinical trials have demonstrated consistent beneficial effects of azithromycin, a macrolide antibiotic structurally modified from erythromycin, in respiratory function and quality

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of life of patients with CF [6–9]. The underlying mechanism of action of the macrolide is still unclear. Our previous *in vivo* study [10], performed in F508del homozygous mice [11], indicates that azithromycin exerts relevant anti-inflammatory properties in spontaneous and induced inflammatory responses. We hypothesized that airway epithelial cells participate to the exaggerated inflammation in CF and that azithromycin modulates inflammatory responses in these cells. In the present work, we analyzed the immune behavior and the effect of azithromycin in airway epithelial cells isolated and purified from CF mice comparatively to those corresponding cells obtained from wild-type animals.

2. Methods

2.1. Animal model

Female CF mice homozygous for the F508del mutation in the 129/FVB outbred background [11] and their wild-type littermates were housed in specific pathogen free conditions with free access to food and water. To prevent CF intestinal obstruction, Movicol® (55.24 g/L) (Norgine, Belgium) was administered in acidified drinking water. The mice were genotyped at 21 days of age, as previously described [10]. All studies and procedures were approved by the local Ethics Committee for Animal Welfare and conformed to the European Community regulations for animal use in research (CEE no. 86/609).

2.2. In vivo experiments

Bronchoalveolar lavage (BAL) was performed in animals treated or not with lipopolysaccharide from *P. aeruginosa* (LPS, 0.1 µg/ml; Sigma-Aldrich), as previously described [10].

2.3. Isolation of epithelial cells

Epithelial cells were isolated and purified from trachea of CF or wild-type mice. Animals were killed by subcutaneous injection of 24 mg sodium pentobarbital (Certa, Belgium). Trachea was excised from the proximal surface of the thyroid cartilage to the bifurcation of bronchi. Attached tissues were dissected and trachea was cut open lengthwise. Trachea was washed three times in PBS and incubated in 10 ml of a digestion solution containing HBSS medium, 1% penicillin–streptomycin to which 164 U/ml collagenase (Worthington Biochemical, Lakewood, NJ), 25 µg/ml liberase (Roche Diagnostics Meylan, France) or 1 mg/ml pronase (Sigma-Aldrich, Saint Louis, MO) were added. The quality of the enzymatic dissociation was

evaluated by monitoring the number of isolated viable cells by trypan blue exclusion and by checking cell morphology after Diff Quick staining. Different combinations of the three digestion enzymes with different incubation times (2 h or overnight) and incubation temperatures (37 °C or 4 °C) were performed. Digestion with pronase used at 1 mg/ml for 60 min at 37 °C provided the best results with a final cell density of 2.5×10^5 cells/mouse. Lower density was obtained with collagenase or liberase (2.0×10^5 cells/mouse).

At the end of pronase digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, Bedford, MA) in a 50 ml falcon tube. Cells were resuspended in 10 ml of an incubation medium composed of equal parts of DMEM and F-12 medium, spun for 10 min (120 g at 4 °C) and the pellet was resuspended in 3 ml of the incubation medium. Following 2 h adherence in 6-well culture plates at 37 °C in an atmosphere of 5% CO₂, macrophages and fibroblasts were depleted. Non-adherent cells were collected by an additional centrifugation (10 min at 120 g at 4 °C) and pellets were resuspended in BEG medium (Invitrogen) to reach 2×10^5 epithelial cells/ml. Cells ($2 \times 10^5/100$ µl) were seeded at 37 °C for 24 h in an atmosphere of 5% CO₂ in 96-well culture plates.

2.4. Immunohistochemistry

After 24 h plating, epithelial cells were identified based on their expression of cytokeratin and cadherin. For this purpose, immunostaining techniques were performed using rabbit antibodies against mouse pancytokeratin and e-cadherin (Santa Cruz, Heidelberg, Germany). Negative controls were performed by omitting the primary antibodies. Cells were fixed in methanol for 2 min, washed in PBS, and incubated in 3% H₂O₂ solution for 5 min. Cells were immersed in blocking solution (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA) for 15 min, then washed in PBS and incubated overnight at 4 °C with the primary antibody. Cell preparations were incubated for 30 min with a biotinylated secondary antibody (Anti Rabbit IgG; Vectastain) and with Vectastain Reagent for 30 min. Finally, diaminobenzidine tetrahydrochloride (DAB; Vectastain) stain was added and the specimens were analyzed using a microscope and an Axioplan 2 camera (Zeiss, Welwyn Garden City, UK).

2.5. Treatment of cultured cells

After 24 h seeding, inflammatory responses of tracheal epithelial cells were monitored after addition for 3 h of LPS to

Table 1
Forward and reward primers (Invitrogen) of amplified mRNA sequences of interest.

Forward	Reward	Gene
5'-CGG CTA CCA CAT CCA AGG AA-3'	5'-ATA CGC TAT TGG AGC TGG AAT TAC C-3'	18S RNA
5'-AGA GGG AAA TCG TGC GTG AC-3'	5'-CAA TAG TGA TGA CCT GGC CGT-3'	β-actin
5'-GCC TCT TCT CAT TCC TGC TTG-3'	5'-GGC CAT TTG GGA ACT TCT CA-3'	TNF-α
5'-GAC GGA CCC CAA AAG ATG AAG-3'	5'-CTC TTC GTT GAT GTG CTG CTG TG-3'	IL-1β
5'-CAG AAA ATT GTC CAA AAG ATG CTA AA-3'	5'-AGG ACC CTC AAA AGA AAT TGT ATA GTG-3'	KC
5'-CCA CCA ACC ACC AGG CTA C-3'	5'-GCT TCA GGG TCAA GGC AA-3'	MIP-2

cell cultures. The balance between the production of pro- and anti-inflammatory mediators was assessed by monitoring TNF- α , CCL-2 (Chemokine C-C motif, ligand 2), IL-1 β , NOS-2, KC (keratinocyte chemoattractant), MIP-(macrophage inflammatory protein)-2; and IL-10, respectively. Azithromycin (Pfizer, Belgium) 1 μ g/ml in <0.1% ethanol was applied to cell cultures at seeding (24 h) and during treatment with LPS (3 h).

2.6. RNA extraction and mRNA quantification

RNA from tracheal epithelial cells was extracted with Trizol[®] Reagent (Invitrogen), according to the manufacturer's instructions. RNA (122 ng–1 μ g) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with 350 picomoles random hexamers (Eurogentec, Belgium) in a final volume of 25 μ l. Resulting cDNA, from wild-type or CF cell cultures, was then diluted 10 \times in sterile distilled water and used as a template in subsequent RT-PCR. Sequences of interest were amplified using the primers shown in Table 1.

For amplification of mouse IL-10 and CCL-2 sequences, we had to resort to Taqman pre-designed primer (Applied Biosystem, Foster City, CA). A relative quantification of mRNA expression was performed on an ABI 7000 in the following conditions: 2 min 50 $^{\circ}$ C, 10 min 95 $^{\circ}$ C (15 s 95 $^{\circ}$ C, 1 min 60 $^{\circ}$ C) \times 40. Six serial 1:10 dilutions of a positive control sample of cDNA were used as standards in each reaction. Standards and samples (5 μ l) were amplified with 300 nM described primers using Power SYBR Green PCR Master Mix or with 900 nM of the IL-10 and CCL-2 primers using Taqman Universal Master Mix (Applied Biosystems) in a total volume of 25 μ l. PCR product specificity was checked by taking a dissociation curve and by agarose gel electrophoresis. Results were normalized to the amount of 18S RNA. RT-PCR analyses were performed in triplicate.

2.7. Cytokine assay

Mouse MIP-2 (Quantikine mouse MIP-2, R&D Systems) concentrations were measured in the supernatant of epithelial cell cultures or in BAL fluid samples, using a standard sandwich ELISA.

2.8. Statistics

Data are presented as means \pm SEM. Differences were evaluated using *t* tests with GraphPad InStat version 3.05 for Windows 95/NT (GraphPad Software, San Diego, CA). Statistical significance was considered at *P*<0.05.

3. Results

3.1. Purification of airway epithelial cells from CF and wild-type mice

In order to investigate the immune behaviour of CF airway epithelial cells, we first developed and validated a primary-cultured murine epithelial cell system. The efficiency of

different proteases (liberase, pronase and collagenase) and experimental protocols was compared (see Methods). The use of 1 mg/ml pronase at 37 $^{\circ}$ C for 60 min appeared to be the most appropriate protocol to release viable and purified airway epithelial cells. Cells stained with Diff Quick appeared, as classically observed for epithelial cells, uniform with a large cytoplasm and an undense nucleus (Fig. 1A). Cilia were observed (Fig. 1A, inset). The degree of cell purification of cultured epithelial cells was evaluated by immunocytochemical techniques by using antibodies against e-cadherin and pancytokeratin. Under these conditions, approximately 85% of

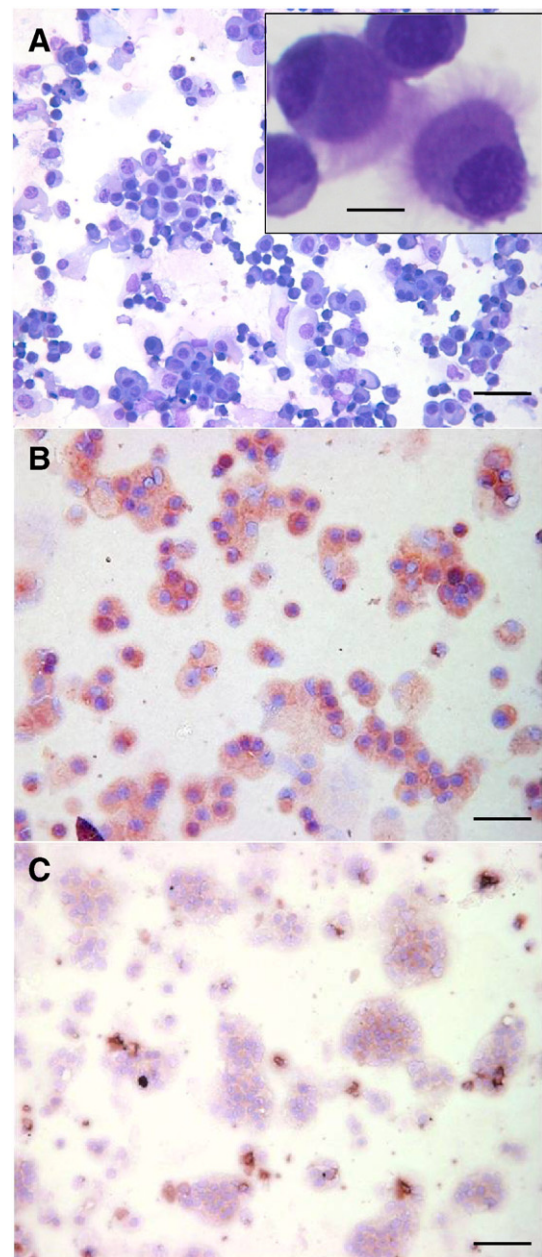


Fig. 1. Diff Quick staining of tracheal epithelial cell isolated from wild-type mice under pronase digestion; ciliated cells are shown in the inset (A). Immunostaining of isolated tracheal epithelial cells for pancytokeratin (B) and negative control (C). Calibration bars correspond to 50 μ m in panels A–C and to 10 μ m in the inset.

cultured tracheal epithelial cells were positively stained by antibody against pancytokeratine (Fig. 1B, C), and e-cadherine (data not shown).

3.2. Neutrophil related chemokine responses

To investigate whether CF epithelial cells may contribute to airway inflammation through expression of neutrophil chemoattractants, we analyzed, in CF and wild-type primary-cultured tracheal epithelial cells, basal and LPS-stimulated expression of MIP-2 and KC, two mouse neutrophil-chemoattractant mediators functionally homologs to the human IL-8.

Under non-stimulated conditions, no differential increase of the neutrophil-chemoattractant factors could be observed in mutant as compared to non-mutant cells, neither on mRNA expression nor on MIP-2 protein levels (Fig. 2A–B). However, under LPS stimulation, striking differences could be observed between CF and wild-type cells. Indeed, after LPS stimulation, MIP-2 and KC expression levels were significantly higher in CF cells. MIP-2 mRNA levels were increased by around 20-fold in wild-type and 100-fold in CF cells (Fig. 2A). KC levels were

increased by 12-fold in wild-type and 20-fold in CF cells (Fig. 2D). After LPS stimulation, MIP-2 and KC mRNA levels were found to be significantly higher in CF than in wild-type cells (Fig. 2A, D). An increase by 30% was observed in MIP-2 protein levels in LPS-stimulated CF cells (Fig. 2B). In CF BAL samples, MIP-2 protein levels were also increased as compared to samples obtained from wild-type animals (Fig. 2C).

These data indicate that F508del-CFTR mutation in airways epithelial cells is associated to exaggerated responses of neutrophil-chemoattractant factors.

3.3. Pro-inflammatory and anti-inflammatory immune response

To investigate whether CF epithelial cells may also contribute to airway inflammation in F508del-CF mice through the expression of pro- and anti-inflammatory cytokines, we monitored the mRNA expression levels of TNF- α , IL-1 β , NOS-2 and CCL-2, used as pro-inflammatory markers, and those levels of IL-10, used as anti-inflammatory marker. Under non-stimulated conditions, no differential increased mRNA expression could be observed on pro-inflammatory markers in CF as compared to wild-type cells (Fig. 3A–D). mRNA

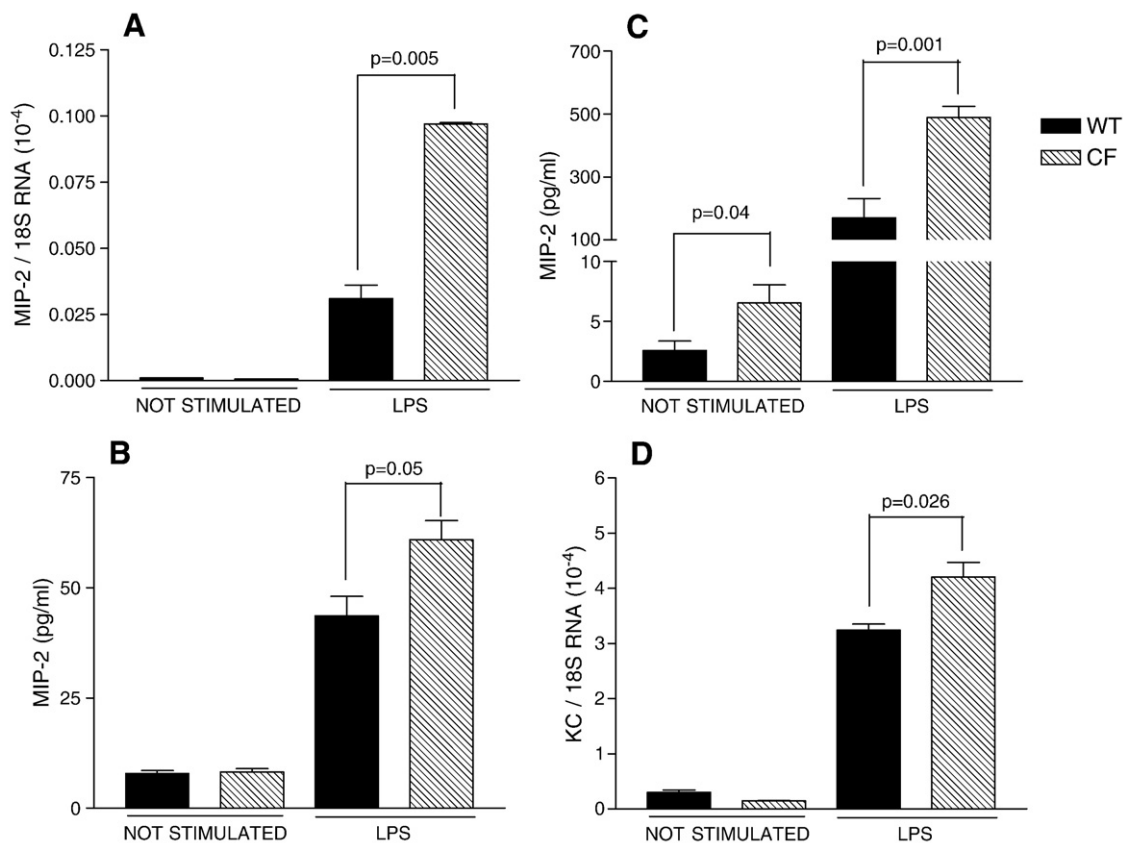


Fig. 2. mRNA expression of neutrophil-related chemokines [MIP-2 (A–C) and KC (D)] in purified tracheal epithelial cells obtained from F508del-CF (CF) and wild-type (WT) mice or in the fluid of bronchoalveolar lavage (BAL). Cells were cultured in non-stimulated conditions or under stimulation with 0.1 μ g/ml LPS. BAL was performed in non-stimulated conditions or 24 h after tracheal instillation of 10 μ g/20 g body weight of LPS from *Pseudomonas aeruginosa*, as described [10]. mRNA data normalized to the expression of 18S RNA. For cell culture experiments, bars represent means \pm SEM for 3–4 individual cell cultures obtained from tracheal cells isolated and pooled from 3–4 animals. For *in vivo* experiments, bars represent means \pm SEM for 6 animals per each group. Data illustrate one of 2 or 6 representative, independent experiments for cell cultures or BAL samples, respectively. *P* values denote levels of significance measured in CF vs that measured in the corresponding wild-type group.

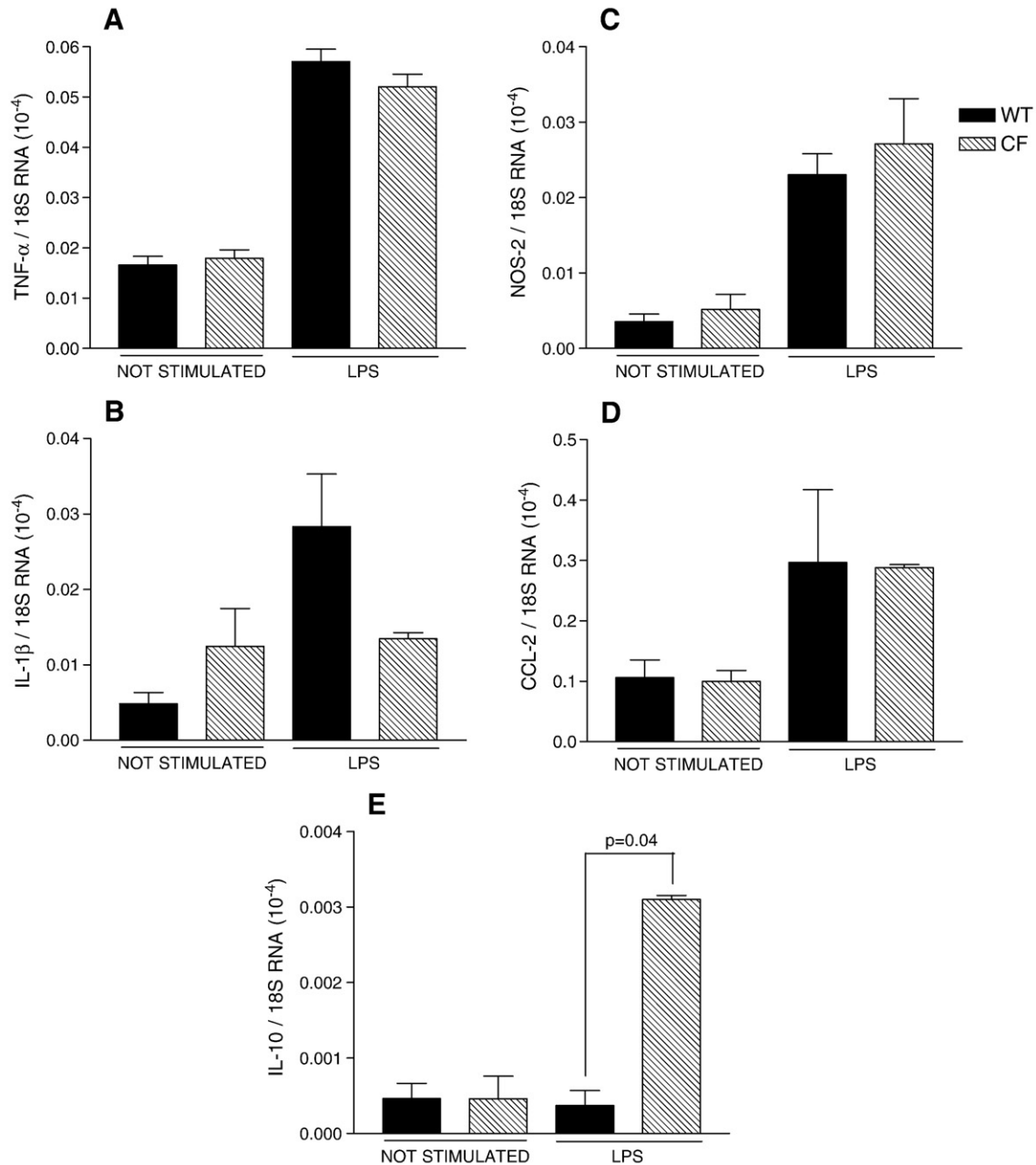


Fig. 3. mRNA expression of pro-inflammatory [TNF- α (A), IL-1 β (B), NOS-2 (C), CCL-2 (D)] and anti-inflammatory [IL-10 (E)] cytokines in purified tracheal epithelial cells obtained from F508del-CF (CF) and wild-type (WT) mice. Cells were cultured in non-stimulated conditions or under stimulation with 0.1 μ g/ml LPS. Data normalized to the expression of 18S RNA. Bars represent means \pm SEM for 3–4 individual cell cultures obtained from tracheal cells isolated and pooled from 3–4 animals. Data illustrate one of 2 representative, independent experiments. *P* values denote levels of significance measured in CF vs that measured in the corresponding wild-type cultured cells.

expression levels of pro-inflammatory cytokines, except to IL-1 β , were globally more pronounced under LPS-stimulated conditions. Indeed, an increase by 2- to 3-fold on mRNA expression levels was observed in CF and wild-type cells with comparable magnitude of responses in both cell genotypes (Fig. 3A–D).

LPS-stimulated epithelial cells expressed about 6-fold higher levels of IL-10 mRNA in CF than in non-CF cells (Fig. 3E). Without LPS stimulation, no difference was noticed between the two cell genotypes.

These findings indicate that F508del-CFTR is not associated to a different pattern of expression of pro-inflammatory cytokines in tracheal epithelial cells. Stimulation with LPS was associated to an increased anti-inflammatory IL-10 production by CF cells.

3.4. Effect of azithromycin

In order to investigate whether azithromycin can regulate the immune activity of airways epithelial cells, tracheal epithelial

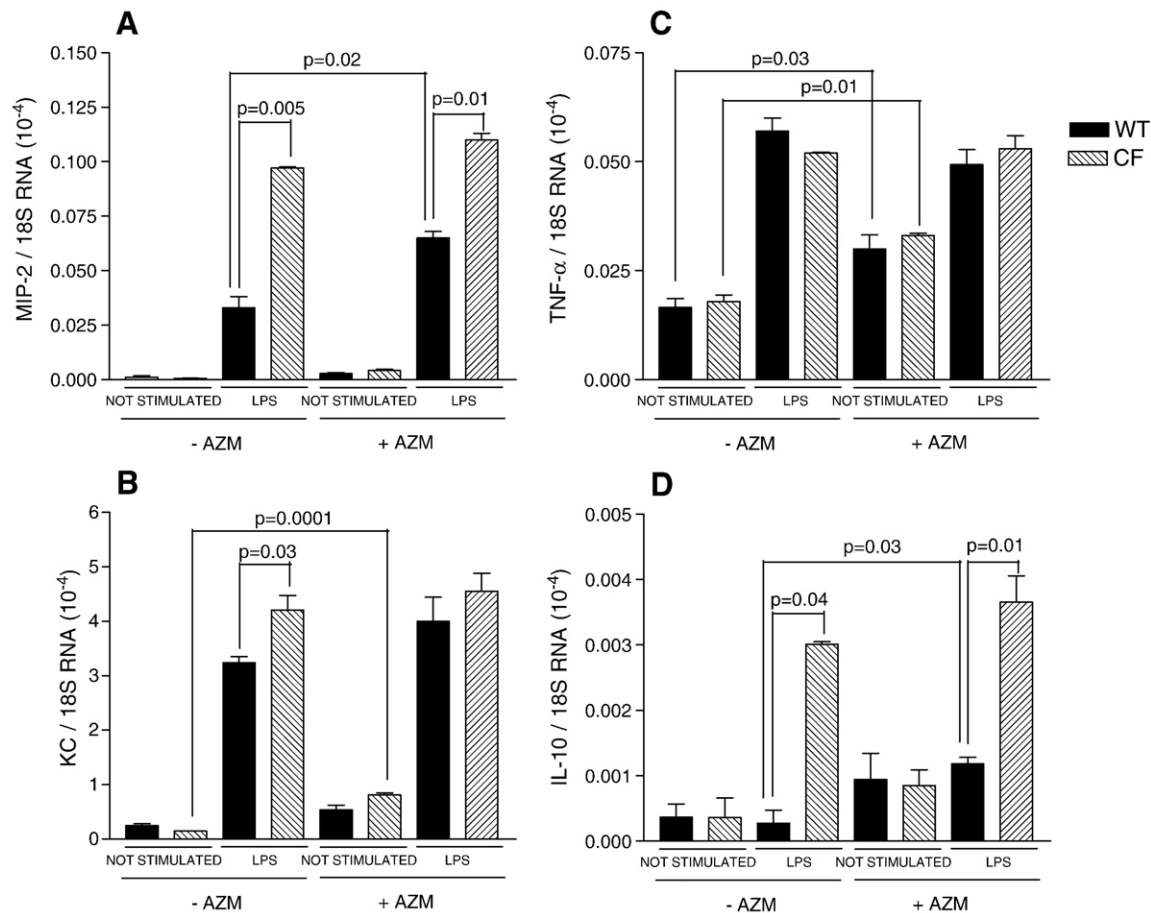


Fig. 4. Effect of azithromycin (AZM, 1 μ g/ml in <0.1% ethanol) on mRNA expression of chemokines [MIP-2 (A) and KC (B)], pro-inflammatory cytokine [TNF- α (C)] and anti-inflammatory marker [IL-10 (E)] in purified tracheal epithelial cells obtained from F508del-CF (CF) and wild-type (WT) mice. Cells were cultured in non-stimulated conditions and under stimulation with 0.1 μ g/ml LPS. Data normalized to the expression of 18S RNA. Bars represent means \pm SEM for 3–4 individual cell cultures obtained from tracheal cells isolated and pooled from 3–4 animals. Cell cultures not treated with azithromycin were exposed to culture medium containing <0.1% ethanol. Data illustrate one of 2 representative, independent experiments. *P* values denote levels of significance measured in azithromycin treated cells compared to the corresponding non-treated cells. Significant *P* values for comparison between CF vs the corresponding wild-type cells after LPS stimulation are also indicated.

cells were exposed with azithromycin at a dose of 1 μ g/ml, able to induce anti-inflammatory effects in macrophages from the same mouse model (unpublished data). After LPS stimulation, wild-type cells showed an increased MIP-2 response when treated with azithromycin as compared to not treated cells (Fig. 4A). The upregulating effect of azithromycin on gene expression of neutrophil-chemoattractant mediators was confirmed on KC, particularly in CF cells in the absence of LPS stimulation (Fig. 4B). The potentiating LPS response of azithromycin on MIP-2 was confirmed at the protein level: MIP-2 reached 77.1 ± 6.6 and 43.6 ± 7.2 pg/ml (*p*: 0.03) in LPS-stimulated non-CF cells treated or not with the macrolide.

The effect of azithromycin was also assessed on the mRNA expression of the pro-inflammatory cytokine TNF- α . In the absence of stimulation with LPS, treatment with azithromycin was followed by an increased expression of TNF- α in both mutant and non-mutant cells comparatively to the corresponding cell type not treated with the macrolide (Fig. 4C). No potentiating effect of azithromycin on TNF- α expression was observed when cell cultures were treated with LPS. The expression of other pro-

inflammatory cytokines assessed (IL-1 β and CCL-2) was not modified by azithromycin (data not shown).

The effect of azithromycin was also assessed on the expression of the anti-inflammatory cytokine IL-10. In the presence of LPS stimulation, wild-type cells showed increased responses to azithromycin on IL-10 expression as compared to those corresponding cells not treated with the macrolide (Fig. 4D).

Overall our *in vitro* results demonstrate that azithromycin was not efficient to downregulate the overexpression of chemokines and cytokines. In contrast, in the absence of external stimuli, the macrolide appears to act as a powerful inducer of gene expression of neutrophil-related mediators, notably KC and TNF- α . Our data also indicate that azithromycin potentiates the LPS response of MIP-2 and IL-10 expression in wild-type airway epithelial cells in culture.

4. Conclusions

While previous works [12–14] have established long-term proliferative cell cultures for studying transepithelial ion

transport properties, in this work we focused our attention on the development of a purified, functionally intact, short-term cell culture model for investigating immune properties and responses. It has been reported in primary-cultured human airway epithelia that release of pro-inflammatory chemokine IL-8 decreases with time after seeding [14]. The short-term, non-mitotic, well-differentiated, ciliated cell culture approach we developed here provides a high-fidelity *in vitro* model for evaluating expression and regulation of multiple key genes involved in immune processes.

We have previously reported that F508del-CF mice display a spontaneous inflammation characterized by basal and LPS-induced neutrophil and macrophage infiltrate [10]. We show here that LPS-induced chemoattractant factors, MIP-2 and KC, are upregulated in purified CF airway epithelial cells. These findings support the concept that inflammation in CF airways is dysregulated and that airway epithelial cells participate to the dysregulated inflammatory process in CF. The finding that IL-10 expression was not found, as it would be expected, decreased in CF epithelial cells in short-term cultures could be dependent on kinetics of cytokine responses. Indeed, in our *in vivo* experiments [10], IL-10 levels were found to be reduced in CF animals at 48 h after LPS challenge, whereas initial levels were similar or higher to those monitored in wild-type animals. Alternatively, it could be postulated that airway epithelial cells are not the major motor behind the pro-inflammatory phenotype and that other cell populations, such as alveolar macrophages, may essentially contribute to the pro- and anti-inflammatory imbalance we observed in our F508del-CF mice [10].

The precise biochemical events triggering and regulating exaggerated chemoattractant responses of CF airway epithelial cells could not be determined in this work. Studies on IL-8 secretion, performed with airway epithelial cells from CF patients or cell lines expressing mutant CFTR, have provided conflicting results varying from increased spontaneous or induced production to non-affected or even decreased levels [15–20]. It has been shown that CF airway epithelial cells can modulate IL-8 production following activation of surface toll-like receptors by cleaved fragments from neutrophil CXC receptors [21]. This cycle promotes further neutrophil infiltration into the airways and favors perpetuation of inflammation. IL-8 induction should ultimately require a combination of multiple mechanisms including transcriptional activation of the gene by nuclear factor-(NF)- κ B and activator protein-(AP)-1 pathways [22].

The growing interest in macrolide antibiotics as beneficial therapeutic agents in CF followed the success of long-term erythromycin for treating diffuse pan-bronchiolitis, a condition sharing common features with CF. Apart from its antibactericidal effects, a variety of possible mechanisms of action of azithromycin have been raised (for review see [23]). We have previously shown that azithromycin attenuates neutrophil and macrophage infiltration in airways of F508del-CF mice in both baseline and LPS-stimulated conditions and inhibits TNF- α and MIP-2 release in LPS-stimulated inflammation [10]. Consistent anti-inflammatory effects of macrolides have been demonstrated on neutrophils [24] and macrophages [25]. However,

fewer studies have investigated immune properties of azithromycin in airway epithelial cells. In the present work, we showed that, as in our *in vivo* data, azithromycin increases IL-10 levels in normal but not in CF airway epithelial cells. However, induction of neutrophil-chemoattractant gene expression (MIP-2 and KC) and potentiating effect on LPS-induced responses, particularly for MIP-2, were observed in this work. Our data, in addition to the finding that TNF- α expression was upregulated after azithromycin in CF and non-CF epithelial cells, are in keeping with a preliminary study in which it was reported that azithromycin induces a potentiating effect on IL-8 in human airway epithelia under exposure to a bacterial/inflammatory medium [28]. In this model, azithromycin was used at a dose 10- to 100-fold higher than that used in our work [28]. In contrast, at a dose 8 times higher than that used in this work, azithromycin inhibited transcription of pro-inflammatory genes (IL-8 and TNF- α) in CF airway epithelial cells [26,27].

The demonstration that macrolides time-dependently modulate pro-inflammatory cytokine secretion [29] may eventually account for differences among studies. A temporal transition with polyphasic responses spanning from an earlier pro-inflammatory to a later non-inflammatory effect of azithromycin on bronchial epithelial cells has been recently suggested [29]. In another recent report [30], azithromycin failed to inhibit IL-8 secretion in a CF bronchial cell line. It could be postulated that the transition from a pro- to a non-inflammatory status may vary according to cell type and culture conditions. It could also be proposed that the epithelial chemotactic and pro-inflammatory effect of azithromycin favors infiltration of inflammatory cells such as neutrophils and macrophages to the airways. Therefore, these infiltrating cells may account for the final *in vivo* anti-inflammatory effect of the macrolide.

Finally, we developed a high-fidelity well-differentiated *in vitro* epithelial cell model for evaluating expression and regulation of multiple key genes involved in immune processes. Our results clearly show that airway epithelial cells participate to the dysregulated inflammatory process in CF by over-expressing chemoattractant factors. Azithromycin induces a chemoattractant and pro-inflammatory gene expression in airway epithelial cells possibly explaining that, in some CF patients, azithromycin effect is ambiguous or inefficient.

Financial support

F.H. is a Research Associate with the Fonds National de la Recherche Scientifique (FNRS), Belgium.

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