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Azithromycin fails to reduce inflammation in cystic fibrosis airway epithelial cells

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ABSTRACT

Cystic fibrosis is a hereditary disease caused by a mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene that encodes a chloride (Cl^-) channel. Cystic fibrosis pulmonary pathophysiology is characterised by chronic inflammation and bacterial infections. Azithromycin, a macrolide antibiotic, has shown promising anti-inflammatory properties in some inflammatory pulmonary diseases. Moreover, all clinical studies have presented an improvement of the respiratory condition of cystic fibrosis patients, but the molecular and cellular mechanisms remain unknown. The aim of this study was to investigate, in bronchial epithelial cells, the effects of azithromycin on inflammatory pathways involved in cystic fibrosis. We have analysed the effects of azithromycin on cystic fibrosis and non-cystic fibrosis bronchial epithelial cell lines but also in non-immortalized non-cystic fibrosis human glandular cells. To create an inflammatory context, cells were treated with Tumor Necrosis Factor (TNF)-α or Interleukin (IL)1-β. Activation of the NF-κB pathway was investigated by luciferase assay, western blotting, and by Förster Resonance Energy Transfer imaging, allowing the detection of the interaction between the transcription factor and its inhibitor in live cells. In all conditions tested, azithromycin did not have an anti-inflammatory effect on the cystic fibrosis human bronchial epithelial cells and on CFTR-inhibited primary human bronchial glandular cells. More, our data showed no effect of azithromycin on IL-1β– or TNF-α–induced IL-8 secretion and NF-κB pathway activation. Taken together, these data show that azithromycin is unable to decrease *in vitro* inflammation in cystic fibrosis cells from airways.

1. Introduction

Cystic fibrosis is the most common genetic disorder in Caucasian populations. Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene is a member of the "ATP-binding cassette" superfamily of transporters, that encodes a chloride (Cl^-) channel (O'Sullivan and Freedman, 2009). Lung disease is the foremost cause of morbidity and mortality in cystic fibrosis patients due to an exaggerated inflammation and to abnormally thick mucus resulting in chronic infections. In cystic fibrosis airways, the classical origin of inflammation supports the concept of an increase in the transcription Nuclear Factor-κB (NF-κB) and the Mitogen-Activated Protein kinases (MAP kinases)/Activator Protein (AP)-1 activities that leads to an increase in interleukin (IL)-8 production and neutrophil recruitment (Muselet-Charlier et al., 2007; Perez et al., 2007; Raia et al., 2005; Srivastava et al., 2006; Tabary et al., 2006).

To treat this inflammation many therapeutic strategies have been tested like corticosteroids and macrolides. Macrolides are antibiotics that have shown anti-inflammatory effects in some pulmonary diseases and on bronchial epithelial cells through the inhibition of NF-κB and/or MAP kinases pathways (Cigana et al., 2006, 2007; Shinkai et al., 2006). Macrolides, such as erythromycin and azithromycin, have been used to successfully treat patients with diffuse panbronchiolitis (DPB), which exhibits some similarities with cystic fibrosis (Brugiere et al., 1996; Sakito et al., 1996). Because macrolides can increase survival among DPB patients, they have been tested in cystic fibrosis patients. These studies have reported improvement of the clinical parameters in cystic fibrosis and summarized in a meta-analysis (Florescu et al., 2009). Thus, the authors demonstrated that azithromycin is clearly associated with improvement of lung parameters with a reduced rate of decline of lung function by an unknown mechanism.

This study investigates the effects of macrolides on inflammatory pathways in CFTR-sufficient and CFTR-deficient *in vitro* bronchial epithelial cells.

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2. Materials and methods

2.1. Materials

CFTR inhibitor, CFTR inhibitor (inh-172, 10 μM), clarithromycin and erythromycin were purchased from Sigma-Aldrich (both macrolides were used at a 10 μg/ml-concentration). Azithromycin (10 μg/ml) was a kind gift from Pfizer. This concentration is physiologically relevant and concordant with data obtained by Di Paolo *et al.* who have shown that a 9,17 μg/ml concentration was found in the lungs of non-cystic fibrosis patients treated with a 500 mg/day oral dose of azithromycin (Di Paolo et al., 2002). TNF-α and IL-1β were purchased from R&D Systems (R&D Systems, Lille, France) and used at the final concentration of 10 ng/ml as previously described (Muselet-Charlier et al., 2007).

2.2. Cell culture

IB3-1 is a bronchial epithelial cell line derived from a cystic fibrosis patient with a F508del/W1282X CFTR genotype. It was used in conjunction with the S9 cell line that was derived from IB3-1 cells after stable transduction with wild-type *CFTR* (CFTR-corrected cells) (Zeitlin et al., 1991). Both cell lines were purchased from the American Type Culture Collection (LGC Promochem SARL, Strasbourg, France) and cultured as previously described (Roque et al., 2008). It has been shown that CFTR Cl^- channel dysfunction in cystic fibrosis tracheal submucosal gland cells leads to abnormal transepithelial salt and fluid secretion. Based on these observations it is reasonable to postulate that intrinsic abnormalities in these cells related to CFTR deficiency could lead early inflammation in cystic fibrosis airways. More, a recent study comparing the genome-wide expression profile of tracheal and bronchial human airway epithelia *in vivo* to the expression profile of primary cultures of human airway epithelia has shown that the use of primary cultures is important to recapitulate airway epithelia biology (Pezzulo et al., 2011). Therefore, non-immortalized Human Bronchial Glandular (HBG) cells from non-cystic fibrosis adult patients were also used, and exhibited *in vitro* characteristics of bronchial secretory glandular epithelial cells as previously described (Tabary et al., 1998). The protocol was approved by the local ethics committee of Broussais Hospital (Paris, France). Briefly, cells isolation were obtained by enzymatic digestion from bronchial submucosal and grown onto type I collagen coated culture flasks in a DMEM/Ham's F12 mixture (50/50%, v/v) supplemented with 1% Ultroser G (Pall Corporation, Saint Germain en Laye, France) (Kammouni et al., 1997). After 4 weeks, third-passage HBG cells has proliferated and exhibited characteristics of homogeneous submucosal epithelial and secretory cells (Tabary et al., 1998). For this study, cells were treated for 72 h with a CFTR inhibitor (Inh-172) or with vehicle alone (dimethyl sulfoxide) before macrolide treatment.

2.3. CFTR activity

The activity of CFTR protein was assessed by I^- quenching of halide-sensitive YFP using Premo Halide sensor technology (Invitrogen, Villebon sur Yvette, France). Assays were performed using a FluoStar fluorescence plate readers (BMG Labtechnologies, Champigny sur Marne, France). As previously described, each well of a 96-well plate, containing the cells, was washed three times with PBS (200 μl/wash), leaving 50 μl of PBS. CFTR conductance was stimulated by an agonist mixture (forskolin, 3-isobutyl-1methylxanthine, apigenin). After 15 min, the 96-well plates were transferred to a plate reader for fluorescence assay. Each well was assayed individually for CFTR-mediated I^- efflux by recording fluorescence continuously (400 ms/point) for 2 s (base line), then 50 μl of a 140 mM I^- solution. Iodide rather than chloride is used because of strong YFP-H148Q/I152L quenching by iodide, and because CFTR is permeable to iodide (Verkman and Galietta, 2009).

2.4. ELISA IL-8

Cells were pre-treated with the macrolides or the vehicle for 30 and TNF-α, IL-1β, or LPS solutions were added for 16 h before assays. Culture supernatants were collected, centrifuged to remove the debris and stored at −80 °C until IL-8 protein concentration was determined using the human IL-8 Duo Set kit (R&D Systems, France), following the manufacturer's instructions.

2.5. 7-plex phosphoprotein assay

After treatment, proteins were extracted using Bio-Plex cell lysis kit (Bio-Rad, Marnes-la-Coquette, France) following the manufacturer's instructions and proteins were quantified. To detect the chosen phosphoproteins (P-p65-NF-κB, P-p38 MAP kinase, P-JNK, P-ERK, P-STAT3, P-STAT6, P-CREB), the Phosphoprotein Detection Kit was used (Bio-Rad, France) and fluorescently labelled streptavidin reporter was added to wells before the phosphoproteins were quantified with the Bio-Plex Array Reader.

2.6. NF-κB and AP-1 luciferase reporter gene assay

Cells were grown to 70% confluence on 12-well plates and were transiently transfected with 0.5 μg of one of the luciferase expression vector (NF-κB-Luc or AP-1-Luc; Stratagene, Montigny-le-Bretonneux, France), along with 0.5 μg of pRL-TK vector (Promega, Lyon, France) as an internal control, using the Exgen 500 reagent (Euromedex, Souffelweyersheim, France) following the protocol previously published (Roque et al., 2008).

2.7. SDS-PAGE and immunoblotting

Equal amounts of total protein (20 μg) from cultured cystic fibrosis and non-cystic fibrosis airway epithelial cells were loaded on 10% SDS-polyacrylamide gels. Specific antibody against IκB-α (Abcam, Paris, France) and β-actin (Sigma, St. Quentin Fallavier, France) was performed and quantified on a LAS 1000 densitometer; the intensities of the bands were compared on a basis of adjusted volume (mean optical density x area).

2.8. Förster Resonance Energy Transfer (FRET) experiments

FRET experiments were performed with YFP-p65/NF-κB and IκBα-CFP plasmids as previously described (Tabary et al., 2006).

2.9. Data analysis

The data are expressed as mean ± S.E.M. of at least five different experiments in duplicate. The n in the figure legend indicates the number of experiments. Statistical differences were determined using either a paired or an unpaired, one-tailed *t* test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. IL-1β and TNF-α, but not LPS from P. aeruginosa, induce IL-8 secretion by cystic fibrosis and non-cystic fibrosis cell lines

To determine CFTR activity in our cells, we have performed experiments using the iodide-sensitive fluorescent protein method (Verkman and Galietta, 2009). Fig. 1A illustrates screening in a 96-well format with normal or corrected cells (HBG and S9 cells) and CFTR deficient cells (IB3-1, S9) and HBG treated with CFTR inhibitor, CFTR (Inh-172). In S9 and HBG cells, we observed a strong decrease of YFP fluorescence that was inhibited in IB3-1 and in S9 or HBG cells cultured with the CFTR inhibitor.

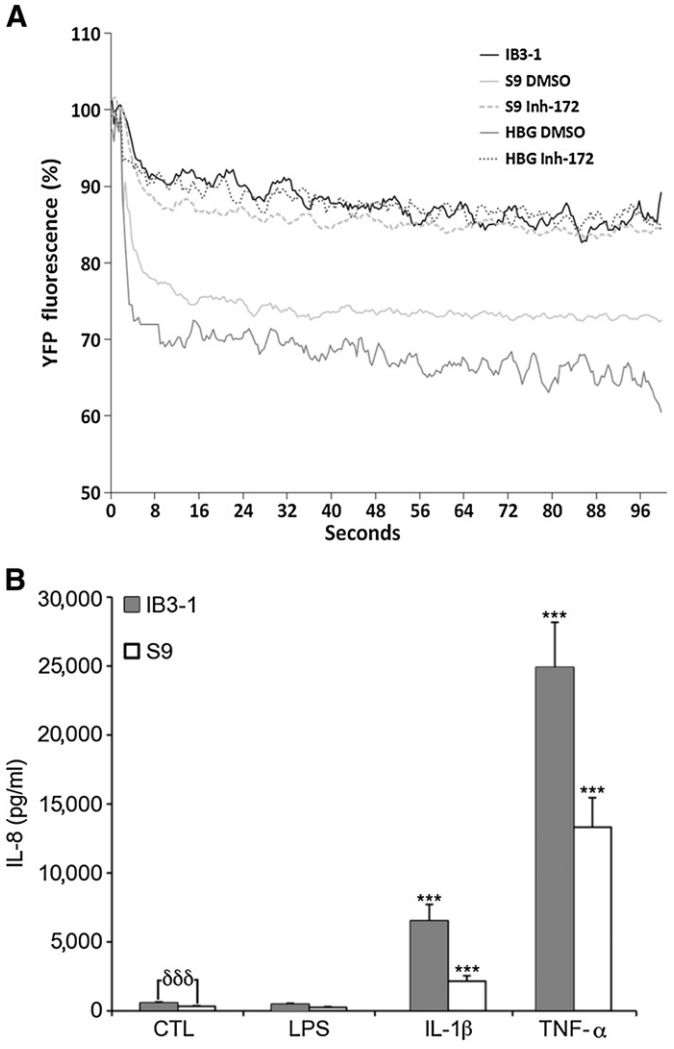

Fig. 1. CFTR activity and effect of LPS, IL-1β and TNF-α on IL-8 secretion. Representative current traces showing CFTR activity of IB3-1, S9 and HBG cells (A). CFTR activities were determined by CFTR halide conductance in cells expressing an YFP-H148Q/I152L/F46L indicator stimulated by CFTR agonist mixture of CFTR. Iodide efflux is measured by YFP fluorescence. S9 and HBG were pre-treated with vehicle (DMSO) or the inhibitor of CFTR (Inh-172). IB3-1 and S9 cells were treated with LPS, IL-1β or TNF-α for 16 h. Media were collected, centrifuged, and stored at −80 °C until IL-8 concentration was measured by ELISA assay (B). The data are shown as mean ± S.E.M. (n = 20-; δδδ $P<0.001$; *** $P<0.001$ *versus* CTL).

We have also analysed IL-8 secretion of cells cultured with different pro-inflammatory agents. Increased levels of pro-inflammatory cytokines IL-1β and TNF-α have been found in bronchoalveolar lavage (BAL) from cystic fibrosis patients (Levine, 1995). In order to induce IL-8 secretion, which is the main pro-inflammatory cytokine found in the lung of cystic fibrosis patients, we cultured cystic fibrosis and non-cystic fibrosis bronchial epithelial cells with IL-1β or TNF-α for 16 h as previously described (Muselet-Charlier et al., 2007). In basal conditions, cystic fibrosis cells secreted more IL-8 than non-cystic fibrosis cells (576.28 ± 72.36 *versus* 332.52 ± 45.04 pg/ml). IL-1β and TNF-α respectively induced an 11-fold and a 43-fold increase in IL-8 secretion by cystic fibrosis cells (Fig. 1B).

3.2. Azithromycin, but not erythromycin and clarithromycin, is able to decrease TNF-α–induced IL-8 secretion in non-cystic fibrosis but not in cystic fibrosis cells

To study the effects of macrolides on TNF-α- and IL-1β-induced IL-8 secretion, cystic fibrosis and non-cystic fibrosis cells were pre-incubated for 30 min with the macrolide followed by treatment with TNF-α or IL-1β for an additional 16 h period. Neither azithromycin, erythromycin nor clarithromycin treatment were able to reduce IL-1β-induced IL-8 secretion (Fig. 2A). Azithromycin, however, was the only macrolide that decreased significantly TNF-α–induced IL-8 secretion. This anti-inflammatory effect was observed in non-cystic fibrosis (S9) but not in CFTR-deficient bronchial epithelial cells (IB3-1 or S9 treated with the CFTR inhibitor Inh-172, Fig. 2B and C). To confirm these results, we used primary culture of normal Human Bronchial Glandular cells that were treated with Inh-172 or the vehicle (dimethyl sulfoxyde, DMSO) for 3 days, as previously described (Perez et al., 2007). As shown in Fig. 2 C and D, azithromycin was able to decrease TNF-α-induced IL-8 secretion only when the CFTR chloride channel was functional (Fig.1A).

3.3. Azithromycin decreases TNF-α–induced IL-8 secretion through NF-κB pathway only in non-cystic fibrosis bronchial epithelial cells

To establish which signalling pathway could be affected in non-cystic fibrosis cells' response to azithromycin, we performed a phosphoproteins-multiplex assay that allowed a screening of seven different pathways in the same sample. Results showed that azithromycin was able to decrease TNF-α-induced p65 NF-κB phosphorylation in non-cystic fibrosis cells (TNF-α: 8.47 ± 2.40 *versus* azithromycin + TNF-α: 6.69 ± 2.19) whereas it had no effect on cystic fibrosis cells (TNF-α: 9.26 ± 3.60 *versus* azithromycin + TNF-α: 9.91 ± 3.20). To confirm these results, luciferase plasmids containing either AP-1 or NF-κB binding sites were transfected into cystic fibrosis and non-cystic fibrosis bronchial epithelial cells. Results showed that TNF-α was not able to modulate AP-1 transcriptional activity significantly (Fig. 3A) but induced an increase in NF-κB activity in both cell lines (Fig. 3B). Moreover, azithromycin decreased NF-κB transcriptional activity in non-cystic fibrosis cells but not in IB3-1 or S9 cells treated with CFTR inhibitor (Inh-172) (Fig. 3B). These results were also confirmed on primary culture of normal HBG cells treated with Inh-172 or the vehicle (DMSO) (Fig. 3C).

Next, we analysed IκB-α expression, the cytoplasmic inhibitor of NF-κB. The expression of IκB-α was measured by Western blotting and results showed a strong decrease in IκB-α expression following TNF-α exposure in both cell lines. Only in S9 cells azithromycin partially reversed TNF-α induced decrease in IκB-α expression (Fig. 4A). Under the same conditions, we observed a slight non-significant decrease in IκB-α expression in cystic fibrosis cells after azithromycin treatment.

Finally, the *in situ* dynamics of the interaction between NF-κB and its inhibitor IκB-α was assessed by FRET using live cell imaging. This method allowed us to analyse the dissociation between the p65 subunit of NF-κB and IκB-α in living epithelial cells (Tabary et al., 2006). These experiments confirmed the involvement of NF-κB in non-cystic fibrosis cells response to azithromycin treatment as shown in Fig. 4B, which is representative of 7 independent experiments, and in Fig. 4C that summarizes the data from at least 35 transfected cells per condition in 7 independent experiments. In the corrected cells, TNF-α induced a 35% FRET decrease, reflecting the dissociation of p65-NF-κB from its inhibitor IκB-α and thereby an increase in the activation of the transcription factor. We observed that azithromycin was able to abolish the TNF-α-induced FRET decrease in non-cystic fibrosis cells only, meaning that NF-κB dissociation from its inhibitor was prevented by azithromycin. The macrolide was, however, not able to suppress p65/IκB-α dissociation in cystic fibrosis cells after TNF-α treatment (Fig. 4B and C).

4. Discussion

In cystic fibrosis patients, airway inflammation is disproportionally increased and participates to the lung dysfunction suggesting

that inflammation remains a major stake to treat cystic fibrosis patients (Jacquot et al., 2008). Anti-inflammatory treatments can be very useful when used in conjunction with aggressive antibiotic therapy and chest physiotherapy techniques to augment mucus clearance. Attention has focused primarily on the therapeutic potential of corticosteroids and non-steroidal anti-inflammatory drugs. Oral corticosteroids may be beneficial; however, unacceptable adverse effects limit long-term use. Inhaled corticosteroids are under investigation as a safer alternative (Balfour-Lynn and Welch, 2009). Recently, macrolide antibiotics are receiving increasing attention for their therapeutic benefits in the treatment of cystic fibrosis patients. Azithromycin has been successfully recommended as a treatment

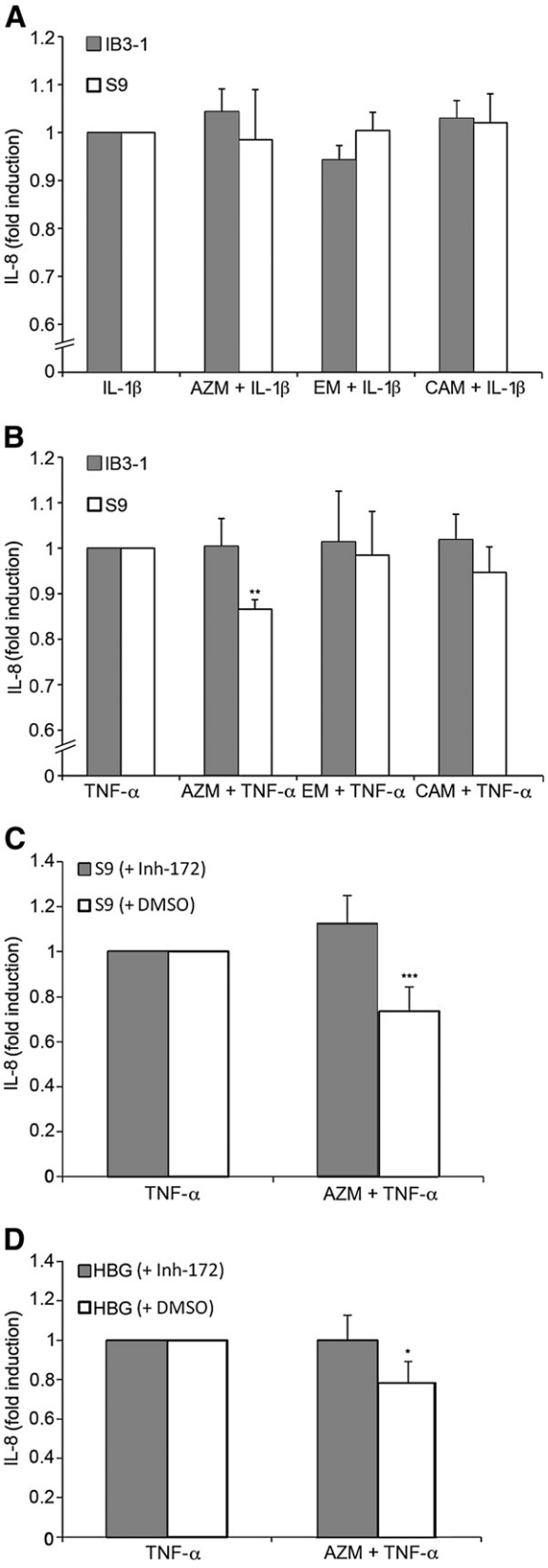

Fig. 2. Effect of Azithromycin (AZM), Erythromycin (EM), and Clarithromycin (CAM) on IL-1β- and TNF-α-induced IL-8 secretion. IB3-1 and S9 cells were pre-treated for 30 min with the macrolide followed by a treatment with IL-1β (A) or TNF-α (B) for an additional 16 h. Normal S9 (C) and HBG (D) cells were treated with the CFTR inhibitor CFTR (Inh-172) or with the vehicle (DMSO) for 3 days and then pre-treated with azithromycin for 30 min and with TNF-α for additional 16 h. After the incubation, supernatants were collected, centrifuged, and stored at −80 °C until the measurement of IL-8 concentration by ELISA. Data are shown as mean ± S.E.M. of at least five independent experiments (**P<0.01 *versus* IL-1β or TNF-α alone).

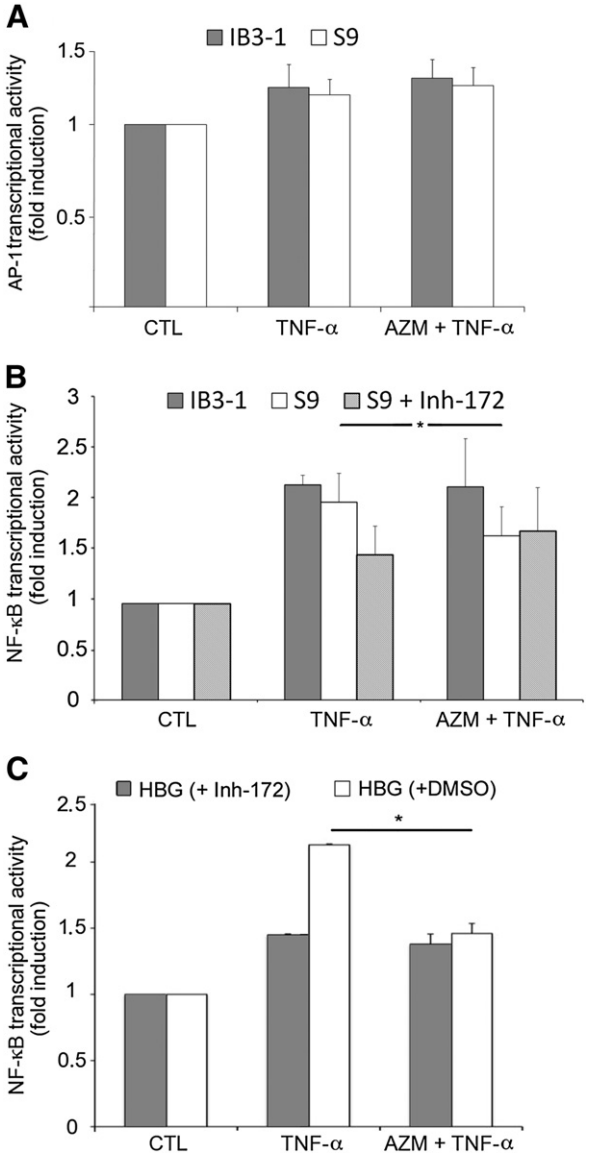

Fig. 3. Effect of azithromycin (AZM) on AP-1 and NF-κB transcriptional activity. IB3-1 and S9 cells were transiently transfected with AP-1 (A) or p65-NF-κB (B) reporter constructs together with a Renilla luciferase internal control plasmid. 24 h later, cells were pre-incubated with the inhibitor of CFTR (Inh-172) for 24 h and/or azithromycin for 30 min and TNF-α was added for an additional 8 h. In the same manner, HBG cells (C) were transiently transfected with the p65-NF-κB construct, incubated with either Inh-172 or the vehicle (DMSO) for 3 days and then pre-treated with azithromycin for 30 min followed by a 8 h TNF-α treatment. Data are normalised to the Renilla luciferase internal control and expressed as mean ± S.E.M. (n = 8 **P<0.01).

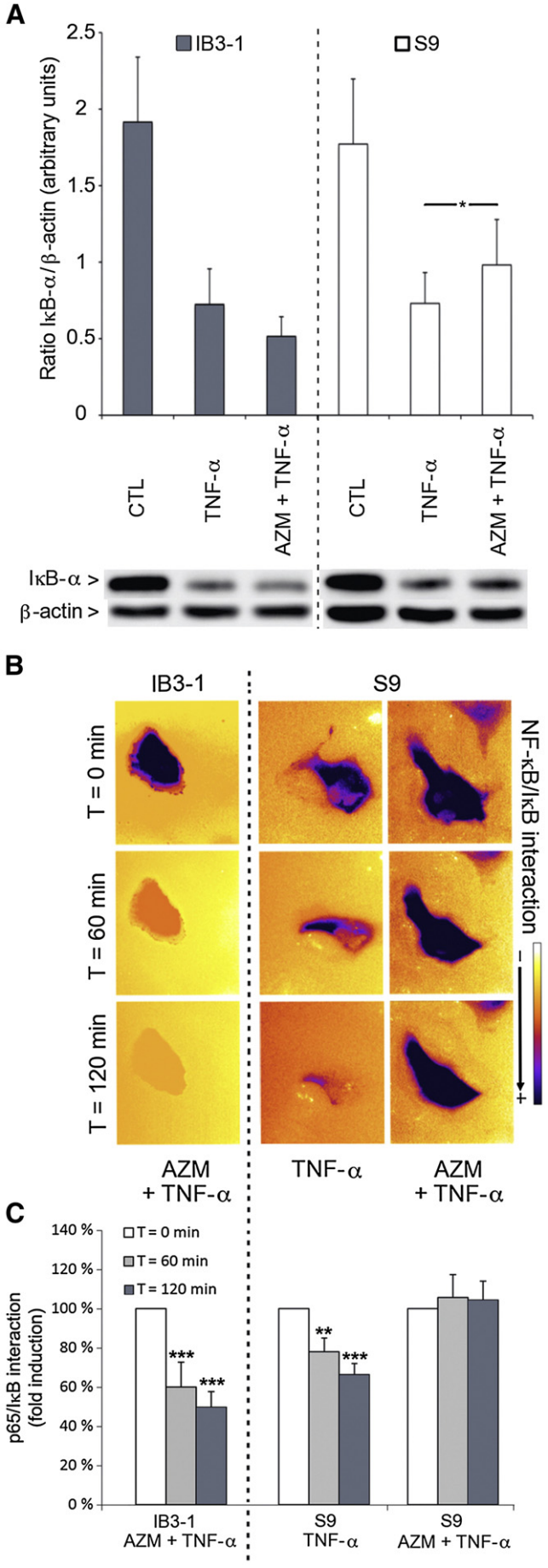

Fig. 4. Effects of azithromycin on TNF-α–induced NF-κB signalling pathway activation. IκB-α expression levels were quantified by Western blotting (A). The representative immunoblot indicates the IκB-α and β-actin protein levels in each group. The histogram represents the mean ± S.E.M. of the ratio of IκB-α/β-actin of seven independent experiments (*P<0.05). Interaction between YFP-p65-NF-κB and IκB-α-CFP was measured and calculated by FRET method and represent in pseudo-colors (B). Pictures shown are representative of measurements performed on 35 different cells from 7 independent experiments. C. The bar graph shows the quantification (mean ± S.E.M., n = 7, ** pP<0,01 ; *** P<0,001 *versus* control) of the calculated FRET fluorescence Ft/F0 of 7 independent experiments.

but the cellular and molecular mechanisms underlying its effects are still unclear (Florescu et al., 2009). Whereas an anti-inflammatory effect of the drug was clearly shown for non-cystic fibrosis bronchial epithelial cells (Shinkai et al., 2008), the results on cystic fibrosis cells remain not very clear. Therefore the aim of this work was to clarify whether azithromycin could decrease inflammation in cystic fibrosis bronchial epithelial cells.

Under our conditions, we have demonstrated that, in contrast to other macrolides like erythromycin or clarithromycin, azithromycin was able to reduce IL-8 secretion, but only in non-cystic fibrosis cells or in primary human bronchial glandular. IL-8 inhibition in non-cystic fibrosis cells was mainly due to the inhibition of the NF-κB pathway as demonstrated by different complementary approaches using IκB-α expression level, luciferase activity, and FRET experiments. In all conditions tested, azithromycin failed to reduce IL-8 secretion and NF-κB activity in cystic fibrosis bronchial epithelial cells. Moreover, we have also found that azithromycin fails to inhibit AP-1 activity in both cystic fibrosis and non-cystic fibrosis cells. Our results were strengthened by some previous work. Blau *et al.* demonstrated that moxifloxacin, a synthetic fluoroquinolone antibiotic, but neither ciprofloxacin nor azithromycin, inhibits IL-8, IL-6, JNK MAP kinase, and NF-κB activation in cystic fibrosis bronchial epithelial cell lines (Blau et al., 2006). Moreover, azithromycin failed to inhibit IL-8 and IL-6 cytokine secretion after stimulation with TNF-α, IL-1β, or LPS challenge in both cystic fibrosis- and non-cystic fibrosis cells (Shinkai et al., 2006). The absence of inhibition of IL-8 secretion by azithromycin was observed in non-cystic fibrosis primary well-differentiated human airway cells activated with supernatant of mucopurulent material from cystic fibrosis patient's airways (Ribeiro et al., 2009). This absence of azithromycin effects on NF-κB pathway in cystic fibrosis bronchial epithelial cells may be due to the constitutive hyperactivation of this pathway which has been often described (DiMango et al., 1998; Tabary et al., 1998, 1999, 2000).

Despite the absence of anti-inflammatory effects demonstrated in azithromycin clinical trials (Saiman et al., 2003; Wolter et al., 2002), Melotti's group, in their own conditions, described an anti-inflammatory effect in cystic fibrosis cell lines with a reduction of IL-8 and TNF-α mRNA and protein expression. These reductions of IL-8 secretion were correlated with an inhibition of NF-κB, SP1 and AP-1 transcription factors (Cigana et al., 2006, 2007). Possible reasons for theses inconsistencies include different culture conditions, variation between cell lines and primary cultures, different stimuli, and differing measurements of the inflammatory response. azithromycin could partly modulate inflammatory pathways by interfering with neutrophil recruitment or macrophage activation as demonstrated in cystic fibrosis mice (Legssyer et al., 2006).

The exciting results data observed in cystic fibrosis patients suggest a potential role for long-term macrolide therapy in patients with cystic fibrosis (Florescu et al., 2009). Different mechanisms have been proposed to explain the beneficial effects of azithromycin in patients with cystic fibrosis. It has been suggested that azithromycin could act on tight junction proteins (Asgrimsson et al., 2006), lipid gene and cell cycle (Ribeiro et al., 2009), mucin expression (Lu et al., 2011; Shimizu et al., 2003), autophagy (Stamatiou et al., 2009), neutrophil degranulation and apoptosis (Tsai et al., 2004). Recent articles

have demonstrated that macrolides such as azithromycin are able to modulate ion mobilisation and to have an effect on the lung pathophysiology in cystic fibrosis patient. Thus, Lu *et al.* showed that macrolides inhibit stimulated mucus released by inhibiting calcium entry in swine submucosal mucous gland cells (Lu et al., 2011) and confirmed previous results showing that erythromycin inhibits muscle contraction through the inhibition of calcium influx and the modulation of intracellular calcium movement (England and Nicholls, 2004). The modulation of ion mobilisation was also observed on chloride efflux and could explain beneficial effect observed on cystic fibrosis patients. We, and others have demonstrated that azithromycin is able to restore chloride efflux on cell lines but also on cystic fibrosis primary cell cultures (Oliynyk et al., 2009; Saint-Criq et al., 2011). The stimulation of Cl^- efflux could be partly explained for the clinical improvement seen among the patients.

In conclusion, our study shows that azithromycin does not have an anti-inflammatory effect in cystic fibrosis bronchial epithelial and glandular cells. A functional CFTR Cl^- channel is required in order for this macrolide to decrease the activation of the NF-κB pathway leading to a reduction of the secretion of the main pro-inflammatory cytokine in cystic fibrosis. The present findings support the hypothesis that there must be alternative molecular mechanisms induced by azithromycin that improve cystic fibrosis patients' pulmonary pattern.

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