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XIX CICLO

ROLES OF AZITHROMYCIN OTHER THAN BACTERICIDAL: RELEVANCE FOR THERAPY OF CYSTIC FIBROSIS

S.S.D. MED/04 - PATOLOGIA GENERALE

Coordinatore: Prof. **MARCO CASSATELLA**

Tutor: Dott./ssa **PAOLA MELOTTI**

Dottorando: Dott./ssa **CRISTINA CIGANA**

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INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population. It affects one in about 2500 live births. It occurs due to recessive mutations of a gene that encodes for a transmembrane chloride channel (Cystic Fibrosis Transmembrane conductance Regulator, CFTR) expressed in the epithelium of multiple organs.

Impairment of the pulmonary defence system, the major cause of morbidity and mortality in CF disease, is characterized by chronic infection, particularly with *Pseudomonas aeruginosa*, and neutrophil-dominated inflammation that lead to airway obstruction and lung destruction.

However CF is a complex disease that affects several organs. Indeed other manifestations are exocrine pancreatic insufficiency (characterized by maldigestion, fat and fat-soluble vitamins malabsorption, and finally by pancreatitis and diabetes mellitus), meconium ileus, distal intestinal obstruction syndrome, male infertility, focal biliary cirrhosis, excessive salt loss from the sweat glands and osteoporosis [1].

CFTR gene

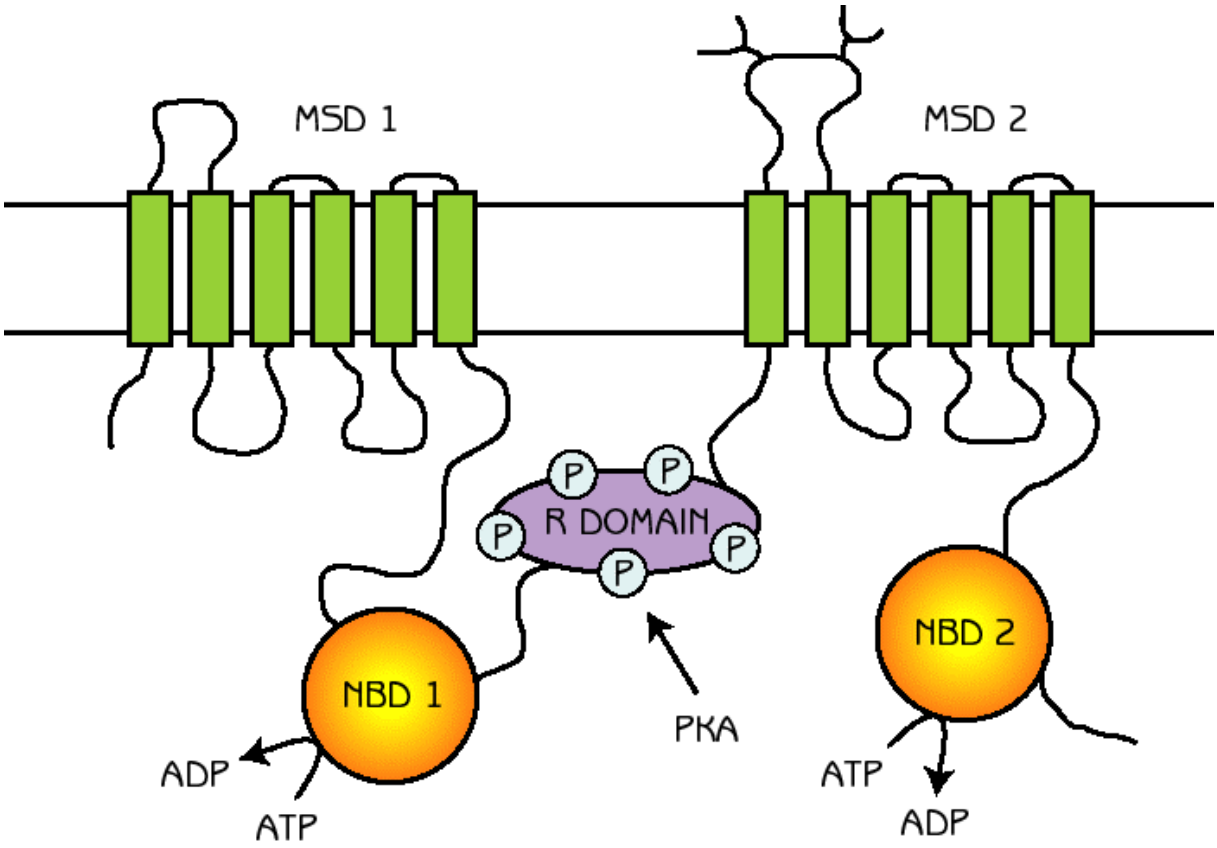
The CFTR gene contains 27 exons and its locus encompasses about 250 kb of DNA on chromosome 7. Its mature message RNA of 6.5 kb encodes a 1480 amino acids long protein with a molecular mass of 168 kDa [2].

CFTR protein

CFTR belongs to the ABC (ATP-binding cassette) transporters superfamily. All ABC proteins contain two nucleotide-binding domains (NBDs), which share high sequence similarity among different members, and two membrane-spanning domain (MSDs). The NBDs bind and hydrolyze ATP. The energy produced in this reaction allows active transmembrane transport and conformational changes in the MSDs. Most MSDs are composed of six transmembrane domains and all of them confer specific transport function to the channel.

The regulatory (R) domain contains several phosphorylation sites for PKA, PKC e cGMP-kinase and many charged amino acids [3].

CFTR is a chloride channel. However, it has been demonstrated that CFTR can transport other substrates as water, urea and glutathione [4]. It is still debated if it could be also permeable to ATP or it regulates an ATP channel.



CFTR structure (by physpharm.ohsu.edu)

Intracellular ATP is required for gating of phosphorylated CFTR Cl^- channels. Although ATP interacts with both NBDs are not functionally equivalent but they cooperate [5]. ATP binding at NBD1 is a prerequisite for channel opening, while ATP hydrolysis at NBD2 is essential for channel closure.

Regulation of channel activity is precisely controlled by the balance of kinase and phosphatase activity within cells. The most important kinase responsible for CFTR Cl^- channel activity regulation is the cAMP-dependent protein kinase (PKA), even if several isoforms of protein kinase C (PKC), type II cGMP-dependent protein kinase (cGKII) and the tyrosine kinase $p60^{c\text{-}src}$ can phosphorylate and activate CFTR Cl^- channels. The removal or inactivation of a kinase causes the decrease of channel activity by dephosphorylation. On the other hand, protein phosphatases 1, 2A and 2C (PP1, PP2A, PP2C) play an important role in dephosphorylating CFTR [1].

It is emerging that the R domain can have both inhibitory and stimulatory effects on CFTR channel according to serines and threonines phosphorylation or dephosphorylation respectively which facilitate interaction of ATP with the NBDs or prevent it by steric inhibition.

Finally, in addition to the main function of Cl^- channel, CFTR can regulate the function of several other membrane proteins as the Outwardly-Rectifying Cl^- Channel (ORCC), the Epithelial Na^+ Channel (ENaC), the ATP-sensitive K^+ channel and the Cl/HCO_3 Exchanger [1].

Molecular mechanisms of CFTR dysfunction.

Mutations in the gene encoding CFTR lead to 5 mechanisms of CFTR dysfunction.

Class I mutations: absent protein production. Splicing abnormalities, frame-shifts due to insertions or deletions, and nonsense mutations belong to this class of severe mutations. They can generate an unstable mRNA and no detectable protein. In other cases, a truncated or aberrant protein may be produced. Such proteins are often unstable and are degraded rapidly, as for mutations G542X and R553X.

Class II mutations: defective protein processing. Newly synthesized CFTR is initially glycosylated in the endoplasmic reticulum (ER). From there it traffics to the Golgi complex, where further glycosylation occurs, and then on to the apical surface of epithelial cells. The class II mutations are all severe and cause protein misfolding during biosynthesis. The consequence is that CFTR is ubiquinated and degraded by the proteasome, so it fails to traffic to the cell membrane.

Several missense mutations as ΔF508 and N1303K belong to this class.

Class III mutations: defective regulation. These mutations are associated with several rates of severity in CF disease. They affect the NBDs leading to alteration in the channel function. On the contrary mutations in the R domain are rare. The missense mutation G551D is an example.

Class IV mutations: defective conduction. These are mild missense mutations that alter CFTR conductance. These mutations, as R117H, R334W and R347P, have been identified in the MSDs. Even if the protein are correctly processed and delivered to the apical membrane, they generate reduced Cl^- current.

Class V mutations: reduced protein production. They reduce, but not eliminate, the amount of functional protein by causing abnormal or alternative splicing, as it happens for the mutations 3849+10kb C>T and A455E.

Pathogenesis of CF disease in airway epithelia

Defective electrolyte and liquid transport

CF airway epithelia manifest defects in two membrane transport processes. First, the absence of CFTR Cl^- channels markedly reduces the apical membrane Cl^- permeability and abolishes the response to cAMP agonists. Second, the activity of apical Na^+ channels is approximately twice that in non-CF epithelia. Under basal conditions, the epithelium absorbs liquid due to active Na^+ absorption through ENaC. Cl^- can follow Na^+ passively via a transcellular or a paracellular route. It appears that a significant fraction of the Cl^- that follows Na^+ passes through the cells via CFTR Cl^- channels at the apical membrane.

Although many groups have tried to study transepithelial electrolyte transport by airway epithelia, current knowledge of the composition of the airway surface liquid (ASL) is inadequate.

It is known that defective ion transport gives rise to augmented liquid absorption and subsequent CF mucus dehydration. Furthermore the high sulfation of glycoproteins in CF [6] results in the increase of ASL viscosity. Because of the concentration and thickening of the mucus, the effectiveness of ciliary beating is impaired leading to reduced mucociliary clearance.

Airway infections.

Recurrent infections of both the upper and lower airways are proper to CF pulmonary disease. Different hypotheses to explain the link between CFTR defect and the propensity to infection have been proposed.

First of all, the submucosal glands and surface epithelium secrete in the ASL several antimicrobial peptides and proteins as lysozyme, lactoferrin, human beta defensins 1 and 2 and NO [7,8]. Their activity is inhibited by the increased ASL NaCl concentration [7], thus predisposing to repeated airway infections. As a result, macrophages and neutrophils may be required to clear bacteria, thereby initiating or worsening the inflammatory cascade observed in CF airways.

Secondly, CF airway epithelial cells bind more to *P. aeruginosa* than non-CF cells because of the increased expression of the *P. aeruginosa* receptor asialoganglioside 1 (aGM1) on the

apical surface of CF cells [9]. In fact the loss of CFTR function leads to an abnormal pH in vesicles targeted to the apical surface and consequent defective sialylation of apical membrane glycoproteins and glycolipids. The result is that less fully sialylated cell surface serves as a better receptor for *P. aeruginosa*.

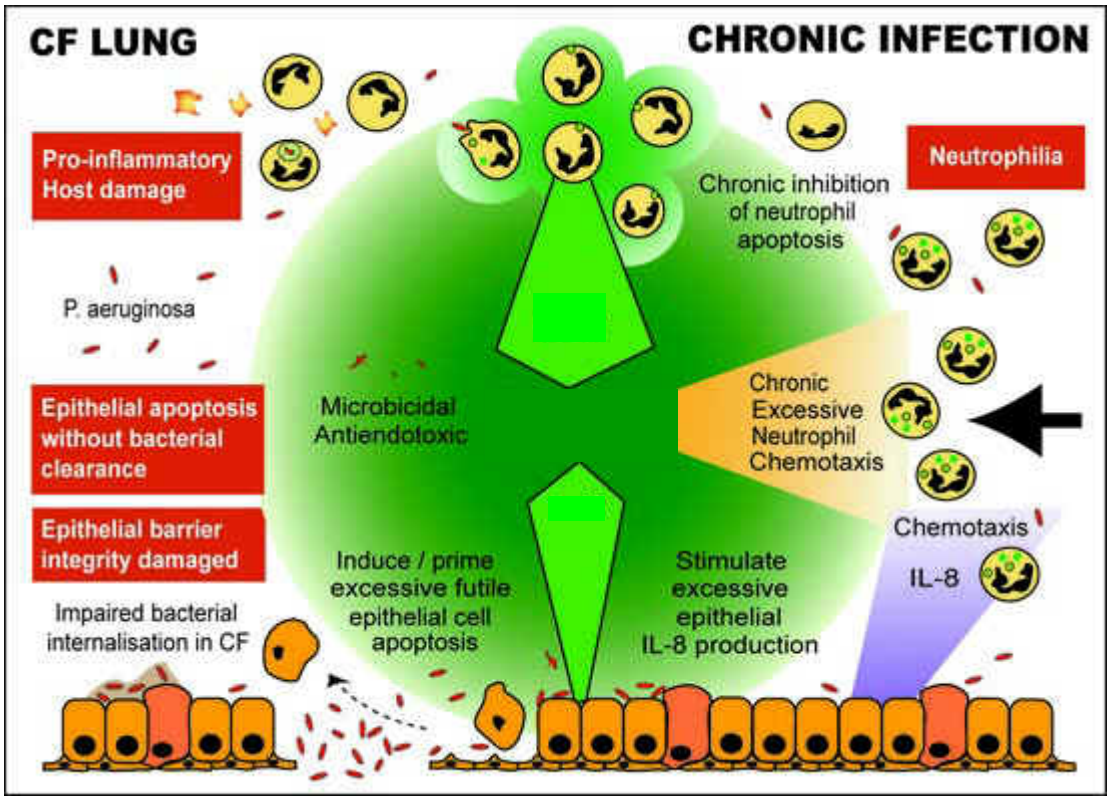
Moreover it has been proposed that CFTR serves not only as a receptor for *P. aeruginosa* binding but also for its subsequent phagocytosis. CFTR defect causes impairment in this process [10].

Airway inflammatory response.

The inflammatory response in CF airways plays a central role in the disease progression and eventual lung destruction. It is characterized not only by great amounts of neutrophils in the airways, but also by high concentration of inflammatory mediators. Among these molecules, over-expression of pro-inflammatory cytokines as tumor necrosis factor (TNF)-α [11-14], interleukin (IL)-1β, IL-6, and chemokines as IL-8, leukotriene (LT) B4 and RANTES have been reported in CF airways fluids [15-18].

It still remains controversial whether there is an intrinsic hyperinflammatory state arising directly from a lack of functional CFTR [14,19,20] or if inflammation is only a consequence of infection. The hypothesis of primary inflammation is based firstly on clinical observations of inflammation preceding detectable infection in CF neonates and young children, but also on experimental reports. Newborn infants and very young children with CF have elevated numbers of neutrophils and strikingly increased concentrations of pro-inflammatory cytokines in their bronchoalveolar lavage fluid (BALF), even when not facing pathogen challenge [21-23]. Higher intraluminal concentrations of IL-8 and increased subepithelial leukocytes have been observed in CF human fetal tracheal grafts compared to non-CF grafts independently of any infection [24]. Mice overexpressing an epithelial Na^+ channel, which mimics the increased NaCl concentrations found in CF airway surface fluid [25,26], show an inflammatory response characterized by neutrophilic infiltration and increased levels of macrophage inflammatory protein 2 and a murine analogue of IL-8 even in the absence of lung infection [27], supporting the hypothesis that dysregulated ion transport can provoke inflammation in the airway epithelium before infection. *In vitro* findings show that bronchial gland epithelial cells exposed to hypertonic NaCl solution release significantly higher IL-8 than healthy control cells in the same experimental conditions [25,26]. Furthermore, recent evidence suggests that in CF airways inflammatory response to bacterial and other infectious agents is exaggerated or prolonged [28]. CF patients had greater amounts of neutrophils and

IL-8 in BAL than patients without CF in response to similar levels of infection [29,30]. CF mice had an excessive pulmonary inflammatory response and higher mortality compared with normal animals after airway infection with comparable quantities of *P. aeruginosa* [31]. Finally not only CF airway epithelial cell lines produced higher quantities of IL-8 in comparison with stably cftr-corrected cells in response to IL-1β and tumor necrosis factor (TNF)-α [13,32-34] and to bacterial stimulation [13,31,35,36], but they have been also reported to produce large quantities of IL-8 and IL-6 after sustained and durable stimulation well after non-CF cells have ceased or slowed down the production of those cytokines or even after bacteria have been eradicated [37].



Airway cystic fibrosis disease (by cir.med.ed.ac.uk)

Glutathione (GSH) deficiency.

GSH (γ-glutamyl-cysteinyl-glycine) is the predominant low-molecular-weight thiol in animal cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes). With the exception of bile acid, extracellular concentrations of GSH are relatively low. Because of the cysteine residue, GSH is readily oxidized non-enzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species). The GSSG efflux from cells contributes to a net loss of intracellular GSH. Cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions. The [GSH]:[GSSG] ratio, which is often used as an indicator of the cellular redox state, is >10 under normal physiological conditions [38].

Glutathione participates in many cellular reactions. First of all, GSH effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) directly, and indirectly through enzymatic reactions. Second, GSH reacts with various electrophiles, physiological metabolites (e.g., prostaglandins and leukotrienes), and xenobiotics (e.g., bromobenzene) to form mercapturates. These reactions are initiated by glutathione-*S*-transferase (a family of Phase II detoxification enzymes). Third, GSH conjugates with NO to form an *S*-nitroso-glutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO. Moreover, not only GSH is required for the conversion of prostaglandin H_2 (a metabolite of arachidonic acid) into prostaglandins D_2 and E_2 by endoperoxide isomerase but also for glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) [38].

CFTR maintains a cellular homeostatic balance of ions, including sodium, chloride and GSH. Normal levels of GSH in the epithelial lining fluid (ELF) of the lung are 150 times higher than other tissues [39] where it serves as an essential antioxidant that protects the tissue from inhaled toxins.

CF is characterized by systemic GSH deficiency that progresses over time. This may result from both the high oxidative burden in CF [40] and the deficiency in CFTR-dependent GSH transport into the alveolar lumen. Low levels of GSH lead to inflammation, a hallmark of CF, and oxidative stress that can lead to damage to cell membranes, cellular proteins and DNA. In support of the causative influence of ROS in CF, the patients frequently have higher levels of lipid peroxidation byproducts [41].

Cellular GSH deficiency has been associated with an increase in transcription of NF-κB, which participates in the regulation of the inflammatory cytokines [39]. In type II alveolar epithelial cells, GSH-enhancing agents downregulated the proinflammatory cytokines IL-1β, IL-6, and TNF-α [42].

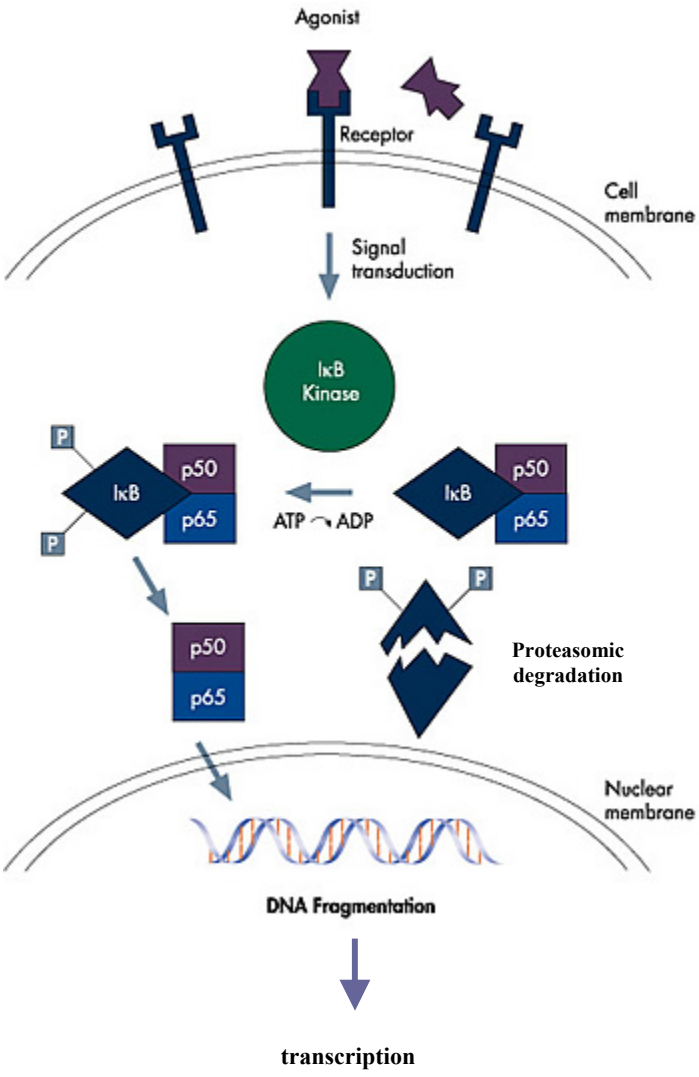
Nuclear factor kB (NF-kB)

The transcription factor NF-kB belongs to a family of proteins that have a key role in the regulation of expression of genes involved in immunity, cell proliferation and apoptosis. The mammalian Rel/NF-kB family is comprised of five structurally related and evolutionarily conserved polypeptides, Rel (c-Rel), RelA (p65), RelB, NF-kB1 (p50 and its precursor p105), and NF-kB2 (p52 and its precursor p100), which associate to form transcriptionally competent homo- or heterodimers. Of these dimers, the p50/p65 heterodimer is the most abundant and biologically active. All these proteins share a highly conserved 300 amino acid long N-terminal Rel homology domain (RHD) responsible for DNA binding, dimerization, and association with the IkB inhibitory proteins. In resting cells, most NF-kB/Rel dimers are bound to IkBs and retained in the cytoplasm. All members of the IkB family contain an ankyrin repeat domain required for both association with NF-kB and inhibitory activity. In the N-terminus region of IkB-α, IkB-β and IkB-ε there are two serines whose phosphorylation leads to proteosomic degradation. In the N-terminus there is also a polyubiquitination site characterized by a lysine. Interaction of IkB-α with an NF-kB dimer prevents the nuclear uptake of the DNA-binding subunits through the masking of nuclear localization signals (NLS) [43-44].

The IKK (IkB kinase) complex enables the activation of NF-kB. It is composed of the two catalitic subunits IKKα and IKKβ, and the regulatory subunit IKKγ. As a consequence of several stimuli as TNF-α, IL-1 or lipopolysaccharide (LPS), the subunit IKKβ is activated and phosphorylates the two serines in the N-terminus of IkB. This event triggers its ubiquitination process that leads to its degradation by the proteasome 26S and consequently the NF-kB is free to enter the nucleus [43-44].

NF-kB regulates the transcription of most pro-inflammatory molecules among which adhesion molecules as Intercellular Adhesion Molecule 1 (ICAM-1), enzymes as cyclooxygenase-2 (COX-2) or inducible NO synthetase, several cytokines as IL-1β, TNF-α or IL-6, and chemokines as IL-8 [45]. NF-kB can be induced by microorganisms as *P. aeruginosa* or their products as LPS, and by cytokines as TNF- α and IL-1β [45-47]. The

phosphorylation of the inhibitory subunits IkBs leads to the release of NF-kB that localizes in the nucleus. There it binds the consensus sequence of the genes encoding for the pro-inflammatory molecules already mentioned to initiate the transcription process.



NF-kB activation (by www1.qiagen.com)

Specificity protein 1 (Sp1)

With the recent advances of the human genome sequencing projects, eight Sp factors have been found. The assignment of factors to the Sp subfamily is based on homology and chromosomal localization [48]. Within the Sp factors, Sp1, Sp2, Sp3, and Sp4 form a subgroup based on their similar modular structure. They contain three zinc fingers close to the C-terminus and glutamine-rich domains adjacent to serine/threonine stretches in their N-terminal region. The 81 amino acids C2H2-type zinc finger region that represents the DNA-binding domain is the most highly conserved part of the proteins.

Sp1 was the first transcription factor identified and cloned. It is ubiquitously expressed and it is shown to be a sequence-specific DNA-binding protein that activated a broad and diverse spectrum of mammalian and viral genes. It mediates the expression of many housekeeping genes, tissue-specific genes, and genes involved in the regulation of physiological function and in the maintenance of homeostasis. However, the involvement of Sp1 in virus-mediated induction of TNF-α gene expression has been observed [49] demonstrating that a protein associated with the regulation of constitutively expressed house-keeping genes is recruited in an inducible fashion to the promoter of an immediate early-response cytokine gene.

Sp1 protein recognises GC/GT boxes and interacts with DNA through three C_2H_2-type zinc fingers located at the C-terminal domain. Based on results of crystal structure and NMR studies, each of the three zinc fingers in Sp1 recognises three bases in one strand, and a single base in the complementary strand of the GC-rich elements where the consensus Sp1 binding site is 5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3′ [50].

Like many other transcription factors, regulation of Sp1-dependent transcription may be conveyed by changes in DNA binding activity, by association with other transcription factors, by changes in Sp1 abundance or in transactivation activity owing to biochemical modification. Although the available amount of Sp1 protein is regulated to a large extent at the mRNA level, there are also other control mechanisms. Sp1 undergoes proteasome-dependent degradation under conditions of nutrient starvation and adenylate cyclase stimulation. Initiation of this process is thought to be determined by a low glycosylation state of Sp1 and consists of an endoproteolytic cleavage triggered by an N-terminal region of Sp1 [48]. The two major types of post-translational modifications that are thought to be involved in transcription regulation by Sp1 are glycosylation and phosphorylation. The glycosylation has been related to the nuclear localization, the stability and/or the transactivation potential of Sp1. Glucose deprivation in combination with adenylate cyclase stimulation results in

reduced glycosylation of Sp1, associated with an increased susceptibility to proteasome-dependent degradation. Glycosylated proteins are also phosphoproteins, and there is some evidence indicating that both types of modification may be reciprocally regulated in Sp1. Sp1 becomes phosphorylated at its N-terminus by DNA-dependent protein kinase upon binding to DNA. The C-terminus of Sp1 can also be phosphorylated; this has been linked to cell cycle progression from G_0 to G_1 [48]. Sufficient evidence suggests that Sp1 phosphorylation may represent a means for regulating transcriptional initiation [51]. Furthermore it is known that its association with NF-kB is important in the regulation of cytokine expression [52].

ABC proteins and CF.

Multidrug Resistance-associated Protein 1 **(**MRP1) is a member of the ABC transporter superfamily sharing a close structural homology with CFTR [53]. It has been considered as a potential modifier gene in CF since a more severe phenotype seems to be associated to low levels of MRP1 mRNA expression [54]. MRP1 is involved in the regulation of chloride transport [55,56] and it can also mediate the extrusion of glutathione-S conjugates (GSH). GSH amount is decreased in the plasma and bronchoalveolar lavage fluid from CF patients and in the apical fluid from CF airway epithelial cells [40,57]. Low levels of GSH may play a critical role in the pathogenesis of CF by increasing the susceptibility of the airway to oxidative damage during chronic inflammation. Furthermore cellular GSH deficiency is related directly to activation of Nuclear Factor kB (NF-kB) pathway [58,59], which has been already reported to be altered in CF [13]. MRP1 could complement CFTR functions also because it has a great affinity for leukotriene C4 [60], a potent mediator of the inflammatory response.

MDR1 is the first human ABC transporter cloned and characterized through its ability to confer a multidrug resistance phenotype to cancer cells. It has been demonstrated to be a promiscuous transporter of hydrophobic substrates and drugs as well as lipids, steroids, xenobiotics and peptides. In addition, MDR1 has been reported to play a role in the regulation of cell volume activated chloride channels and to have chloride channel activity itself [61,62]. MDR1 is also suggested to be involved in ATP-dependent pathways [63,64]. Even if it was initially thought to transport ATP by itself, it is clear now that MDR1 regulates another ATP channel [65], as it has been described for CFTR [66].

Furthermore several authors have demonstrated a complementary pattern of CFTR and the Multidrug Resistance Protein 1 (MDR1) expression, suggesting a co-ordinated regulation of these genes [67-69].

Azithromycin (AZM) and CF

Clinical reports demonstrated that the macrolides antibiotics improve pulmonary function, and decrease morbidity and mortality in patients with diffuse panbronchiolitis (DPB) [70,71], a disease with many similarities to CF. Macrolides decrease not only mucus hypersecretion but also the amounts of proinflammatory cytokines and neutrophils in the BALF of DPB patients, therefore protecting the airway epithelium from damage [72]. Following this success in DPB, these antibiotics have received increasing attention for their possible therapeutic benefits in the treatment of CF. Recently, several multicenter reports have supported the possibility that macrolides lead to clinical beneficial effects in CF by improving lung function [73-78]. CF patients treated with AZM experienced improvement in the viscoelasticity of the sputum [79], decreased content of mucoid *P. aeruginosa* in sputum samples [77], decline in the number of pulmonary exacerbations [76,80] and significant increases in respiratory function parameters such as FEV1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) [75-77,80,81]. AZM was chosen over other macrolides because of ease of administration and its accumulation in sputum and tissues. Its plasma half-life is considerably longer than that of other macrolides. It also accumulates in alveolar macrophages, which could represent a delivery vehicle to transport it to affected sites.

Despite a wide clinical use, the mechanisms of the efficacy of macrolides are still unclear, even if several possible explanations have been proposed among which the possibility to alter airway epithelial chloride transport, to decrease the production of virulence factors by *P. aeruginosa*, the most common microorganism causing chronic endobronchial infection in CF, and to have anti-inflammatory properties [82-84].

AIMS

In this study we aimed to evaluate the non-bactericidal effects of AZM potentially relevant for therapy of CF.

AZM has beneficial clinical effects in CF treatment. Even if its mechanisms of action are still debated, several hypotheses arise including other than antibacterial effects as anti-inflammatory properties and complementation of CFTR defect by ABC proteins [7-10,32,36].

First we aimed to investigate the expression of ABC members in CF cell lines and their regulation by AZM as a possible mechanism of complementation of the CFTR defect in CF. In particular we studied the constitutive expression of MRP1 and MDR1 in CF cells in comparison to non-CF cells and the effects of AZM on this expression.

CF airway inflammation occurs very early in CF patients [21,22,24]. Furthermore, some reports pointed out that it is exaggerated both in the absence and in the presence of stimuli [13,30,33,36], even if this statement is still controversial [23,50].

We aimed to evaluate not only whether a differential TNF-α expression could be detected between CF and non-CF cells, but also whether AZM could reduce these levels in CF cells. We investigated also the molecular mechanisms of AZM effect on these cells studying the DNA-binding activity of Nuclear Factor-kB (NF-kB) and Specificity protein 1 (Sp1) transcription factors before and after incubation with AZM, as both these proteins are involved in the regulation of TNF-α gene expression.

To evaluate the hypothesis of exaggerated inflammatory response to stimuli, we studied whether LPS derived by *P. aeruginosa* could induce excessive inflammation in CF cells in comparison to non-CF cells and the effects of AZM on this response by evaluating the mRNA expression of IL-8, IL-6, TNF-α and ICAM1.

The beneficial effects of AZM were characterized by high variability among CF patients. We have been considering genetic factors as possible mechanisms (85).

Furthermore we aimed to investigate the effects of this macrolide on the inflammatory response to specific stimuli. To this aim we studied the AZM effects on TNF-α mRNA expression induced in CF cells by supernatants collected by different *P. aeruginosa* clinical strains cultures.

MATERIALS AND METHODS

Cell cultures.

IB3-1 cells, an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (ΔF508/W1282X), and C-38 cells, the rescued cell line which expresses a plasmid encoding a copy of functional CFTR, were a kind gift from Pamela Zeitlin (Johns Hopkins University, Baltimore, MD, USA); the non-CF isogenic cell line S9, corrected by transfection with wild-type adeno-associated viral CFTR, was purchased from LGC Promochem (Teddington, UK) [86,87]. Cells were grown in LHC-8 media (Biosource, Camarillo, CA, USA) supplemented with 5% foetal bovine serum (FBS) (Cambrex Bio Science, Verviers, Belgium).

2CFSMEo- cells, a SV40-transformed tracheobronchial submucosal gland epithelial cell line derived from a CF patient (ΔF508/unknown), obtained from D. Gruenert (University of California, San Francisco, CA, USA) [88], were grown in Eagle's MEM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% FBS and 1% L-glutammine (Cambrex Bio Science).

All culture flasks and plates are coated with a solution of LHC-basal medium (Biosource) containing 35 μg/ml bovine collagen (BD Biosciences, Bedford, MA, USA), 1 μg/ml bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) and 10 μg/ml human fibronectin (BD Bio Science) as described [87].

Collection of bacterial supernatants.

PAO1, a prototypic laboratory strain, the isogenic mucoid PDO 300, and clinical isolates of *P. aeruginosa*, kindly provided A. Bragonzi (San Raffaele, Milan, Italy), were inoculated onto trypticase soy agar (Difco, BD Biosciences) plates and allowed to grow at 37°C overnight. They were then inoculated into modified Vogel-Bonner medium (MVBM) and incubated overnight with continuous agitation. To prepare MVBM [89], $MgSO_4$ 3.3 mM (Sigma), citric acid 10 mM, $NaNH_4HPO_4$ 28 mM (Sigma), K_2HPO_4 37 mM (Sigma) were dissolved in apirogen water. After autoclaving, filter sterilized potassium D-gluconate 214 mM (Sigma) was added. The final pH was 7.2.

The day after, *P. aeruginosa* cells were diluted in MVBM at a concentration of 1 x 10^8 cfu/mL (OD of 0.1 at 600 nm) with or without 8 μg/ml AZM (Pfizer, Roma, Italy). These concentrations, which are in the sub-MIC range for *P. aeruginosa*, are consistent with those

described in lungs of patients treated with AZM [90]. In the presence of AZM for 24 hrs at 8 μg/ml the cell viability was >95% as determined by Tripan Blue exclusion test while at higher concentrations starting from 16 μg/ml the viability was <95%. The cultures were incubated at 37°C for 16 hours with continuous agitation in aerobiosis or in an anaerobic jar in microaerophilic conditions by adding a sachet containing ascorbic acid as active component (Oxoid, Basingstoke, UK).

The cultures were then normalized to an optical density of 0.2 OD at 600 nm by adding MVBM. Supernatants from normalized bacterial cultures were collected by centrifugation (4000g, 20 min, 4°C) and filtered through a 0.22-μm filter to remove any remaining bacteria and stored at -80°C.

Cells treatments.

Cells were seeded in a concentration of 4.5 x 10^5 cells/cm^2 for 4 hours experiments, 2.5 x 10^5 cells/cm^2 for 24 hours experiments and 1 x 10^5 cells/cm^2 for 72 hours experiments. After 24 hours, cells were exposed to 8 μg/ml AZM and/or JM (Yamanouchi Pharmaceutical, Japan) to study constitutive gene expression. For long-lasting experiments cells were split whenever they were confluent and every time AZM was added to the culture medium. For studies concerning stimulated gene expression, 24 hours after the sedimentation cells were pre-incubated with AZM 8 μg/ml for 2 hours; after that LPS derived by *P. aeruginosa* 10 μg/ml (Sigma) or 10 % of *P. aeruginosa* strains' supernatants were added to the cell cultures. AZM has been utilized also at lower concentrations up to 0.25 μg/ml and for 6 or 72 hrs obtaining no statistically significant effects.

RNA isolation, reverse transcription and quantification.

Cells, treated as described above, were washed with PBS and lysed.

Total RNA was extracted using High Pure RNA Isolation Kit (Roche, Mannheim, Germany), following the Supplier's instructions. In particular, cells are resuspended in a lysis buffer containing guanidine-HCl and Triton X-100 in a filter, and then centrifuged and the flowthrough liquid discarded. After incubation with a DNase solution for 20 min, the filter was washed firstly with a wash buffer containing guanidine-HCl and then with a wash buffer containing NaCl. Finally, after discarding the flowthrough liquid, the RNA was eluted with nuclease-free double-distilled water.

Reverse transcription (RT) was performed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA): 1 μg of total RNA was reverse transcribed in the presence of random hexamers 1X, dNTPs 2.5X, RNAse Inhibitor 100 U, Multiscribe Reverse Transcriptase 250 U and Reverse Transcriptase Buffer 1X in a total volume of 100 μl, for 10 min at 25°C and 2 hours at 37°C. As negative control, the reaction was also performed in the absence of RNA.

Relative quantification of gene expression was performed by real time quantitative PCR analysis as described by the manufacturer (Applied Biosystems User Bulletin 2). The cDNA (5 μl) was amplified using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Grand Island, NY, USA) in the ABI Prism 5700 sequence detection system. Human MRP1, MDR1, TNF-α, IL-6, IL-8, ICAM1, GAPDH and CK-15 sequences were amplified in separate tubes. The primers (Sigma-Genosys, St. Louis, MO, USA), selected by Primer Express Software (Applied Biosystems), are indicated in table 1. The PCR thermal protocol consisted of 2 min at 50°C, a denaturation step at 95°C for 2 min followed by 50 cycles of a 15 sec 95°C denaturation step and a 30 sec annealing/extension step at 60°C. The real-time PCR reactions were performed in duplicates for both target and normalizer genes. Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems User Bulletin 2). Changes in mRNA expression level were calculated following normalization to GAPDH or CK-15. Results were expressed as mean ± standard deviation (SD).

Table 1. Primer sequences utilized in the quantitative PCR analysis

gene		sequence (5'>3')	accession number	primer concentration*
MRP1	FW	GGGCCTCTCAGTGTCTTACTCATT	NM_004996	25
	RV	ACATCCGAACCAGCCAGTT		25
MDR1	FW	AGCTTAGTACCAAAGAGGCTCTGGA	NM_000927	450
	RV	TCCAAAAGGAAACTGGAGGTATACTT		450
TNF-α	FW	GGACCTCTCTCTAATCAGCCCTC	NM_000594	25
	RV	TCGAGAAGATGATCTGACTGCC		25
IL-6	FW	CGGTACATCCTCGACGGC	NM_000600	25
	RV	CTTGTTACATGTCTCCTTTCTCAGG		450
IL-8	FW	GACCACACTGCGCCAACA	AF385628.2	150
	RV	GCTCTCTTCCATCAGAAAGTTACATAATTT		150
ICAM1	FW	TATGGCAACGACTCCTTCTCG	NM_000201	150
	RV	CTCTGCGGTCACACTGACTGA		150
GAPDH	FW	GTGGAGTCCACTGGCGTCTT	J04038	25
	RV	GCAAATGAGCCCAGCCTTC		150
CK-15	FW	GGCTGGCTGCGGACG	AF202320	150
	RV	GCAGGGCCAGCTCATTCTC		150

*concentration used for FW/RV primers (nM)

Flow cytometry.

To fix and permeabilize cells, we used the Cytofix/Cytoperm kit (BD Biosciences Pharmingen, Palo Alto, CA, USA) according to the manufacturer's instructions. Cells were detached in the presence of EGTA, then washed and resuspended. Cytofix/Cytoperm solution was added for 20 min at 4°C. After two washings with a buffer containing saponin as permeabilizing agent, cells were stained with the phycoerythrin conjugated mouse monoclonal antibody anti-MRP1 QCRL-1 (Santa Cruz Biotechnology, CA, USA); permeabilization was assessed by staining of β–actin as cytoplasmic positive control. Previously the primary mouse anti-MRP1 MRPm6 monoclonal antibody (MP Biomedicals, Irvine, CA, USA) and a secondary phycoerythrin conjugated antibody were utilized with similar results. The phycoerythrin conjugated mouse monoclonal antibody anti-MDR1 from clone 17F9 (Santa Cruz Biotechnology) was utilized for MDR1 staining. Irrelevant isotypic phycoerythrin conjugated antibodies were utilized as negative controls. Cytofluorimetric analysis was performed using FACScan (BD).

Gene reporter studies.

Cells were transiently cotransfected using FUGENE 6 (Roche) according to the manufacturer's instructions with the reporter vector including the 5' flanking region of MRP1 driving the expression of luciferase as reporter gene (kindly provided by Dr. Deeley, Queen's University, Ontario, Canada) and a beta-galactosidase expressing vector utilised for normalisation. In particular 3 μl of Fugene and 2 μg of the combination of vectors were added to the serum-free culture medium and mixed. After at least 15 min the solution was added to the cells.

The day after the transfection cells were incubated for 24 h with or without AZM 8 μg/ml. Luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. After the wash with PBS and the addition of lysis buffer, cells were scraped, collected in a tube and put at –80° for 15 min to increase the signal. After that, the lysate was vortexed and centrifuged (max speed for 15 sec). The supernatant was collected and stored at –80°C. To analyse βgal activity, the cell lysate was incubated in a solution containing Mg^{2+}, ONPG (o-nitrophenyl-beta-d-galactopyranoside) and Na+ phosphate. Within 30 min, in the presence of the yellow colour, the reaction was stopped by adding Na_2CO_3. After that, the solution was read at 420 nm. To evaluate luciferase activity, the luciferase assay solution was added to the cell lysate and the

mix was read at the luminometer. Luciferase activity in cells transfected with the insert-less reporter vector was almost undetectable. We assessed that our method is able to detect variations, as described [91], since we measured significantly different levels of luciferase activity using a reporter vector including a region of the IL-8 promoter inducible by TNF-α.

TNF-α secretion.

Growth media were collected at the end of the experiments above described, centrifuged and the supernatants were stored at –80°C. Released TNF-α was determined using an ELISA kit, the TNF-α EASIA kit (Bender MedSystems, Wien, Austria), according to the manufacturer's instructions. Briefly, samples, standards and blanks were inoculated in microwells coated with polyclonal antibody (rabbit) to human TNF-α, then biotin-conjugate was added. After 2 hours with shaking, wells were washed four times and streptavidin-HRP was added to all wells. After 1 hour with shaking, all wells were washed 4 times and then incubated with TMB substrate solution (tetramethylbenzidine and H_2O_2) for about 10 minutes. The reaction was stopped by adding the stop solution containing phosphoric acid when the highest standard developed a dark blue colour. After that, the absorbance at 450 nm was read on a spectro-photometer. The limit of detection was 3.83 pg/ml.

Measurements were performed at least in duplicate. Values were normalized to 10^6 cells; results were expressed as mean ± SD.

DNA binding activity studies.

DNA binding activity of NF-kB and Sp1 was measured in nuclear extracts using TransAM kit (Active Motif, Belgium) and Mercury Transfactor Profile kit (Clontech, USA), respectively, according to the manufacturer's instructions.

To evaluate NF-kB DNA binding, binding buffer was added to the wells coated with an oligonucleotide containing the NF-kB consensus site 5'-GGGACTTTCC-3'. Then 5 μg of nuclear extracts diluted in lysis buffer were added to the sample wells. Blank wells were inoculated with lysis buffer and positive control wells with Jurkat nuclear extracts. After 1 hour with mild agitation, wells were washed and anti-NF-kB p65 antibody was added for 1 hour. After washing anti-IgG HRP-conjugated antibody was added. After 1-hour incubation and washing, developing solution was added for 2-10 min till medium to dark blue colour was developed. Then stop solution was added and the plate was read at 450 nm.

A 96-well plate coated with an oligonucleotide containing the Sp1 consensus binding site 5'-GGGGCGGGG-3'was utilized to evaluate Sp1 DNA binding. TransFactor blocking buffer was added to all wells for 15 min. After removing it, 5 μg of nuclear extracts diluted in TransFactor blocking buffer were added. Nuclear extracts obtained from MCF-7 cells were utilized as positive control. After 1 hour, wells were washed and incubated with the primary anti-Sp1 antibody for 1 hour. After washing three times, the secondary anti-mouse IgG-HRP antibody was added for 30 min. After 4 washing four times, wells were incubated with TMB solution for 10 min. When blue colour developed in the positive control wells, reaction was stopped by adding H_2SO_4 and absorbance was read at 450 nm.

Measurements were performed at least in duplicate; results were expressed as mean ± SD.

Experiments were performed following incubation of cells with AZM 8 μg/ml for 4 or 24 hrs obtaining very similar results.

Statistical analysis.

Statistical calculations and tests were performed using Student's t test considering $p \leq 0.05$ as limit of statistical significance. All data were expressed as mean ± standard deviation (SD).

RESULTS

Possible induction of complementation by ATP binding cassette superfamily proteins by AZM treatment.

Constitutive MRP1 and MDR1 transcript levels in CF versus non-CF cells.

Real-time qPCR was utilised to evaluate the expression of MRP1 and MDR1 mRNAs. A 2.5 and 3.2 folds higher levels of MRP1 mRNA were detected in CF cells IB3-1 versus non-CF cells C38 and S9 ($p<0.01$ and $p<0.001$ respectively, n=4, Fig 1A). IB3-1 cells expressed 66% MDR1 mRNA respect to C38 (n=4, not significant) and 13% compared with S9 cells ($p<0.01$, n=4) (Fig 1B).

Constitutive MRP1 and MDR1 protein levels in CF versus non-CF cells.

MRP1 and MDR1 protein expression was evaluated by flow cytometry in order to establish if the mRNA regulation could have a functional relevance. The results of the MRP1 and MDR1 analysis are shown in Fig. 2A and 2B, respectively. The analysis revealed no differences of MRP1 and MDR1 protein expression levels among all CF and non-CF isogenic cell lines.

Effects of AZM on MRP1 and MDR1 transcript expression.

To address the question whether AZM could modulate MRP1 and MDR1 expression, we performed a time course experiment in which IB3-1 cell line had been exposed to 8 μg of AZM for 1, 3, 7 and 14 days. Figure 3 shows the results obtained from MRP1 and MDR1 quantification in IB3-1 cells by real-time qPCR. We observed a less than 30% increase in transcript level of MRP1 after 1 and 3 days of treatment ($p<0.01$ and $p<0.01$ respectively, n=4), which was not maintained after 7 and 14 days (Fig 3A). AZM decreased MDR1 expression of less than 30% after 14 days of incubation ($p<0.01$, n=4) (Fig 3B). A different CF cell line as 2CFSMEo- was then tested in the same conditions, in order to establish whether the changes in MRP1 and MDR1 transcript expression detected with IB3-1 could be reproduced. In 2CFSMEo- cells we found no regulation in MRP1 (Fig 4A) and MDR1 (Fig 4B) expression by AZM treatment at all times tested.

Effects of AZM on MRP1 and MDR1 protein expression.

Even if AZM does not appear to consistently modulate MRP1 and MDR1 transcript levels, we investigated whether protein expression might be modulated at post-transcriptional level. We performed time course experiments using IB3-1 and 2CFSMEo- cell lines exposed to 8 μg of AZM for a minimum of 24 h to a maximum of 4 weeks. MRP1 and MDR1 protein expression was evaluated by flow cytometry. No differences between treated and untreated

cells after 24 h, 72h, 1 week, 2 weeks and 4 weeks of treatment were detected. Representative results of MRP1 and MDR1 protein analysis after 24 h and 4 weeks of treatment of IB3-1 and 2CFSMEo- cells are shown in Fig. 5A and 5B, respectively.

Effects of AZM on MRP1 promoter activity.

We proceeded to assess whether AZM may affect at least transcription of MRP1 in our models. We then analysed by gene reporter studies the effects of AZM (8 μg/ml for 24 h) on the MRP1 promoter activity in IB3-1 and 2CFSMEo- cell lines. We did not detect statistically significant differences in the reporter activity in treated versus untreated IB3-1 and 2CFSMEo- cells as shown in Fig. 6 consistently with our findings previously reported in another CF airway epithelial cell line [85].

Possible anti-inflammatory properties of AZM.

Effects of AZM on constitutive TNF-α mRNA expression.

We measured the expression levels of TNF-α gene. All cell lines constitutively expressed TNF-α mRNA, however the level of basal expression in CF cells was significantly higher than in isogenic non-CF cells (see Fig 7). We confirmed this differential TNF-α expression using cells at different passages as well as after 96 hours from sedimentation (data not shown). Following exposure of CF cell lines to 8 μg of AZM for 24 hours we found that this macrolide reduced TNF-α mRNA of about 35% in IB3-1 ($n=5$, $p<0.001$, Fig 7A), approximately to the levels of untreated isogenic non-CF cells C38. A 30% reduction of TNF-α mRNA was detected in 2CFSMEo- cells after AZM treatment ($n=5$, $p<0.01$, Fig 7B). The macrolide JM, known to lack clinical anti-inflammatory properties, had no significant effects on TNF-α mRNA expression in all cell lines (data not shown).

Effects of AZM treatment on constitutive IL-6 mRNA expression.

In terms of IL-6 mRNA expression we found no statistically significant differences between CF cell lines and isogenic non-CF cells (see Fig 8). We then exposed CF cell lines to 8 μg of AZM for 24 hours. AZM had no statistically significant effects on IL-6 mRNA expression in IB3-1 and 2CFSMEo- cells (Fig 8A and 8B respectively).

Effects of AZM on constitutive TNF-α protein levels.

We confirmed the higher TNF-α expression in CF cells in comparison to isogenic non-CF cells also at protein level (see Fig 9). Treatment with 8 μg of AZM was effective in reducing TNF-α protein levels of 45% in IB3-1 treated for 24 hours ($n=3$, $p<0.05$, Fig 9A), to the

levels observed in untreated isogenic non-CF cells C38. Furthermore AZM reduced TNF-α protein expression of 40% in 2CFSMEo- (n=3, $p<0.01$, Fig 9B).

Effects of AZM on constitutive NF-kB DNA binding activity.

We found that IB3-1 cells showed twofold-higher constitutive NF-kB DNA binding levels than isogenic non-CF cells C38 (n=3, $p<0.01$, Fig 10A). Following exposure of CF cell lines to 8 μg/ml AZM for 24 hours the NF-kB DNA binding activity in IB3-1 cell line was reduced of 40%, nearly to the levels of untreated C38 cells. A 45% reduction of NF-kB DNA binding activity was detected in 2CFSMEo- cells after AZM treatment (n=3, $p<0.05$, Fig 10B). Furthermore, JM had no effects on NF-kB DNA binding activity (data not shown).

Effects of AZM on constitutive Sp1 DNA binding activity.

We decided to evaluate whether AZM could affect the levels of Sp1 DNA binding. We did not detect statistically significant differences in the constitutive Sp1 DNA binding levels in IB3-1 cells versus isogenic non-CF C38 cells, as shown in Fig. 11A. After 24 hours of treatment with 8 μg of AZM, 60% and 65% Sp1 DNA binding activity reductions were detected in IB3-1 and 2CFSMEo- cells respectively (n=3, $p<0.01$ and $p<0.05$ respectively, Fig 11B). JM was not effective also on Sp1 DNA binding activity (data not shown).

Effects of AZM on IL-8, TNF-α and IL-6 mRNA induction by LPS stimulation.

We measured the mRNA levels of IL-8, TNF-α and IL-6 genes. Treatment with 10 μg/ml of LPS derived by *P. aeruginosa* for 4 hours or the use of lower concentrations had no effect on the abovementioned genes expression (data not shown). On the contrary, the induction of expression of IL-8, TNF-α and IL-6 genes in CF cells IB3-1 treated with 10 μg/ml of LPS for 24 hours was significantly higher than in isogenic non-CF cells C38 (see Fig 12A). The incubation of CF cells with 8 μg of AZM from 2 hours before the addition of LPS and for all the treatment reduced IL-8 and IL-6 mRNA of about 30% and TNF-α mRNA of about 40% in IB3-1 cells ($p<0.01$, Fig 12A). A 55% reduction of IL-8 and IL-6 mRNA and a 35% reduction of TNF-α mRNA induced by LPS were detected in 2CFSMEo- cells after AZM treatment (n=5, $p<0.001$, $p<0.001$ and $p<0.05$ respectively, Fig 12B). JM had no significant effects on IL-8, TNF-α and IL-6 mRNA induction after LPS treatment in both cell lines (data not shown).

Effects of AZM on ICAM1 mRNA induction by LPS stimulation.

We then measured the mRNA levels of ICAM1 after treatment with LPS derived by *P. aeruginosa* for 24 hours. The induction of expression of ICAM1 gene in CF cells IB3-1 treated with 10 μg/ml of LPS for 24 hours was significantly higher than in isogenic non-CF

cells C38 (see Fig 13A). The incubation of CF cells with 8 μg of AZM from 2 hours before the addition of LPS and for all the treatment had no inhibitory effects on ICAM1 mRNA induced by LPS nor in IB3-1 cells (Fig 13A) neither in 2CFSMEo- cells (Fig 13B). JM was not effective on ICAM1 mRNA induction after LPS treatment in both cell lines (data not shown).

Effects of AZM on TNF-α mRNA induction by stimulation with *P. aeruginosa* strains' supernatants.

We measured the levels of TNF-α mRNA after we treated cell lines with 10% of supernatants derived by *P. aeruginosa* strains for 4 hours. The supernatants derived from different strains (indicated below the graph) lead to differential amounts of induction of TNF-α mRNA both in IB3-1 (Fig 14A) and in 2CFSMEo- cells (Fig 14B). The results ranged from absence of induction for both cell lines to about two times of TNF-α mRNA induction in IB3-1 (Fig 14A) and about 3.5 times of induction in 2CFSMEo- cells (Fig 14B). Similar results were obtained for IL-8, ICAM1 and IL-6 mRNA (data not shown). The incubation of CF cells with 8 μg of AZM from 2 hours before the addition of the supernatants and for all the treatment had inhibitory effects on TNF-α mRNA induced by the supernatants of only some strains in both cell lines (Fig 14) with an inhibition range from 25% to 35%. Similar results were obtained also for IL-8 and IL-6 mRNA mRNA (data not shown). On the contrary, ICAM1 mRNA induction by supernatants was not reduced by AZM treatment in any case (data not shown).

Fig. 1

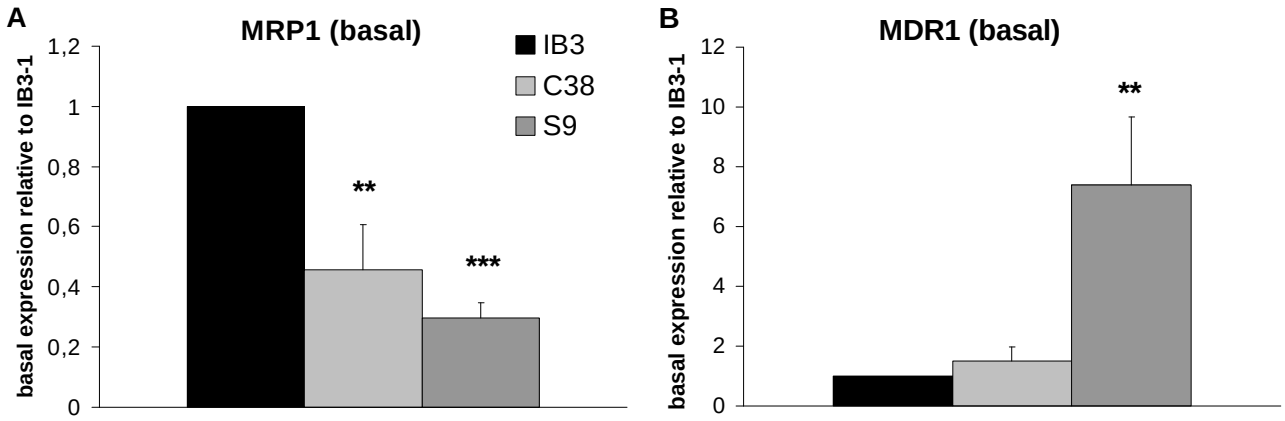


FIGURE 1. Constitutive expression of MRP1 and MDR1 mRNA in CF cells IB3-1 and isogenic non-CF C38 and S9 cell lines. Total RNA was extracted and retrotranscribed. The results of MRP1 (A) and MDR1 (B) mRNA quantification are based on real-time q-PCR analysis as described in materials and methods. The values represent the expression levels relative to IB3-1 (means ± SD). The experiment was repeated four times. **p<0.01, ***p<0.001.

Fig. 2

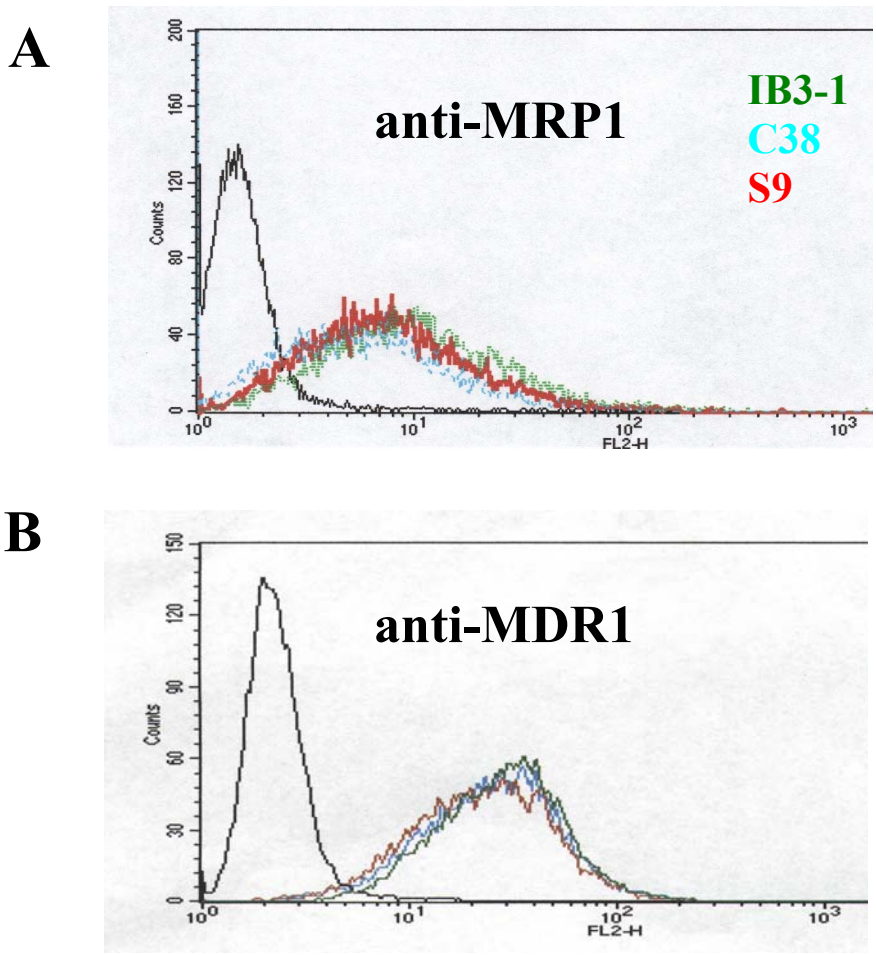


FIGURE 2. Constitutive expression of MRP1 and MDR1 proteins in CF cells IB3-1 and isogenic non-CF C38 and S9 cell lines. Expression of MRP1 and MDR1 proteins analyzed by flow cytometry in IB3-1 (CF), C38 and S9 (both non-CF) cell lines stained with the specific antibody anti-MRP1 (A) and anti-MDR1 (B). The black histogram is the negative isotypic control and is representative for all cell lines. In the presence of the specific antibodies, histograms for each cell line are shown in different colours according to the legend. Experiments are representative of at least three performed with very similar results.

Fig. 3

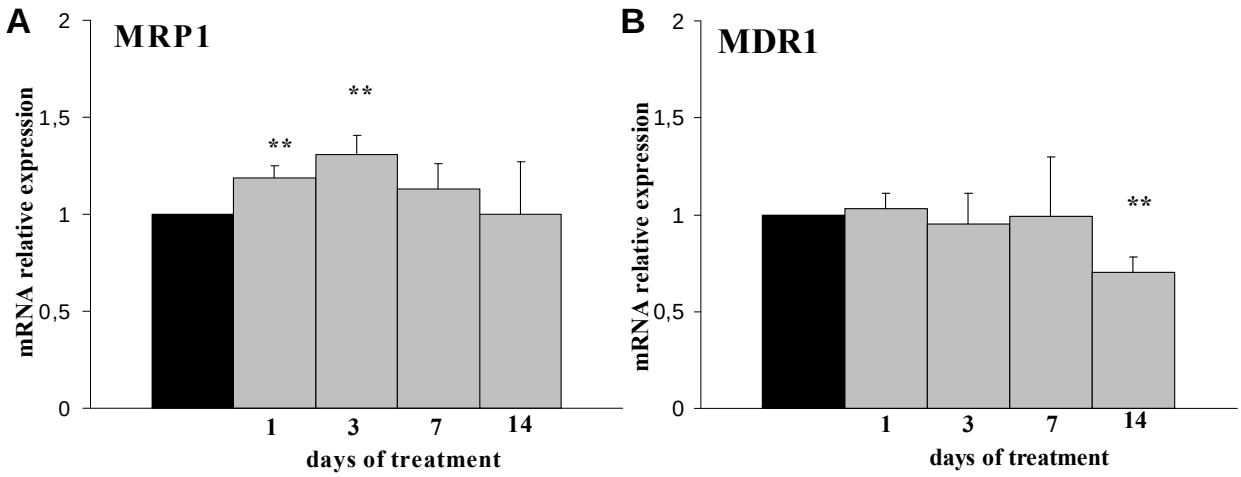


FIGURE 3. Time course analysis of MRP1 (A) and MDR1 (B) mRNA expression in IB3-1 cells. Total RNA was extracted and retrotranscribed. Real-time q-PCR was performed as described in materials and methods. The values represent the expression levels relative to untreated cells (means ± SD). The experiment was repeated four times. **$p<0.01$.

Fig. 4

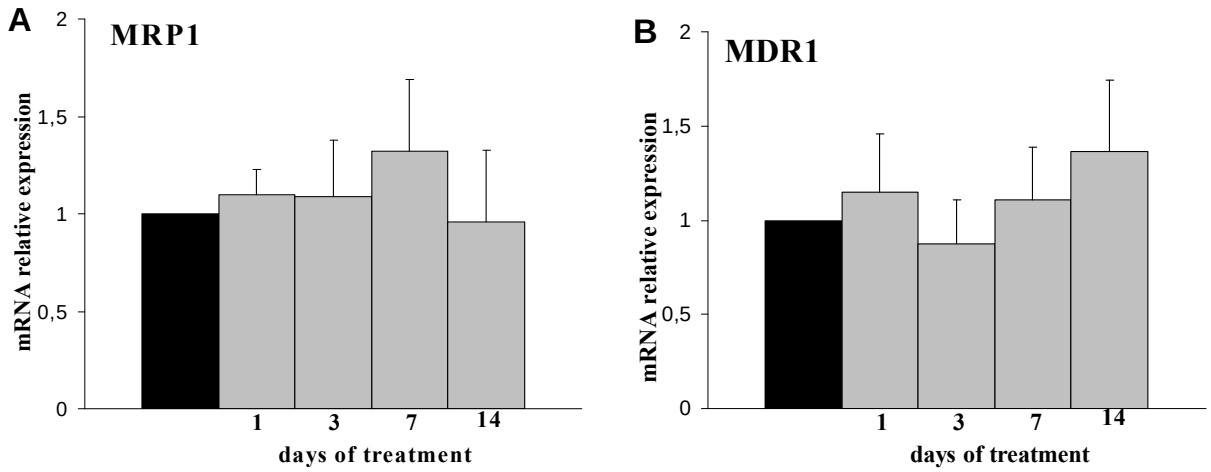


FIGURE 4. Time course analysis of MRP1 (A) and MDR1 (B) mRNA expression in 2CFSMEo- cells. Total RNA was extracted and retrotranscribed. Real-time q-PCR was performed as described in materials and methods. The values represent the expression levels relative to untreated cells (means ± SD). The experiment was repeated four times.

Fig. 5

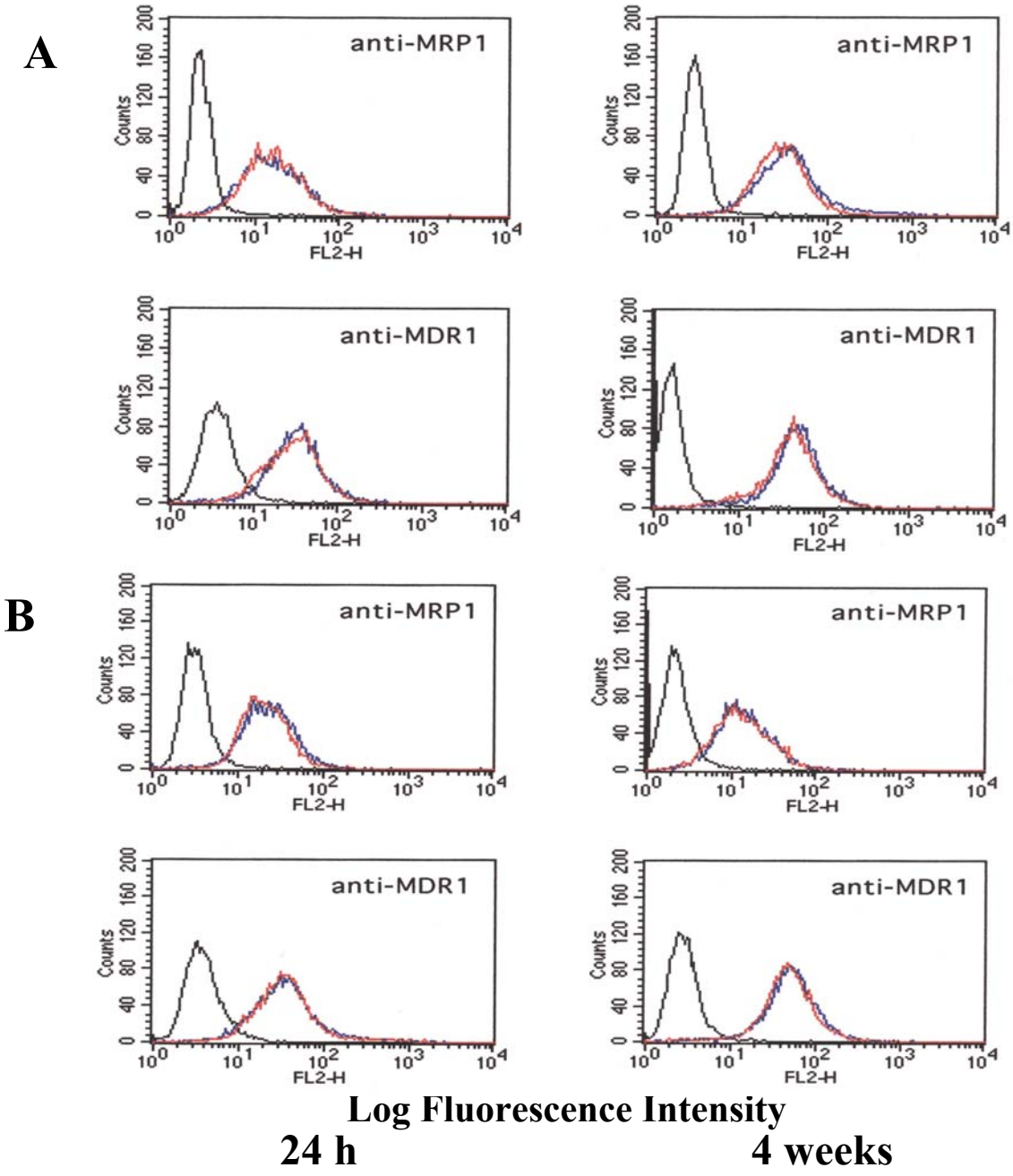


FIGURE 5. Evaluation of AZM effects on expression of MRP1 and MDR1 proteins. Following treatment with AZM for 24 h or 4 weeks, flow cytometry was performed in IB3-1 (A) and 2CFSMEo- cells (B) stained with the PE-conjugated specific antibody anti-MRP1 or anti-MDR1. Black histograms represent the negative isotypic control superimposable in all conditions while red and blue histograms correspond to treated and untreated cells, respectively, stained with the specific antibody indicated in each panel. Logarithmic fluorescence intensity is indicated on the y-axes. Experiments are representative of at least three, performed with very similar results.

Fig. 6

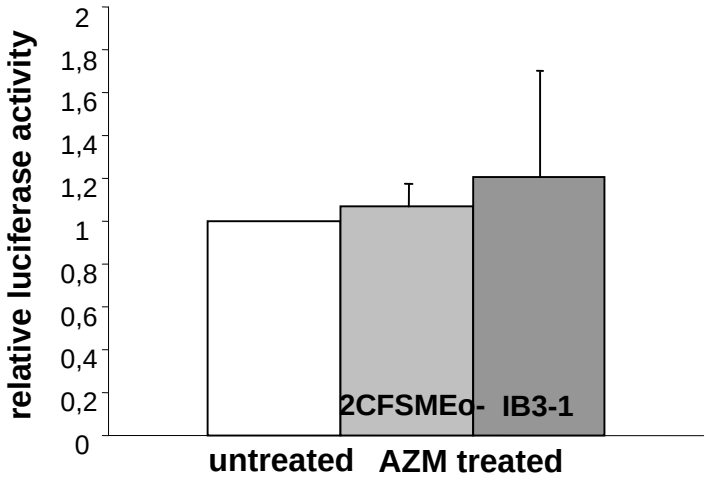


FIGURE 6. MRP1 promoter activity. MRP1 promoter activity was analysed by gene reporter assay following AZM treatment. Luciferase activity was normalised to beta-galactosidase activity in 2CFSMEo- and IB3-1 cells treated for 24 h with AZM and indicated on y axis relative to untreated cells as described in materials and methods (means ± SD). Both cell lines were assayed three times.

Fig. 7

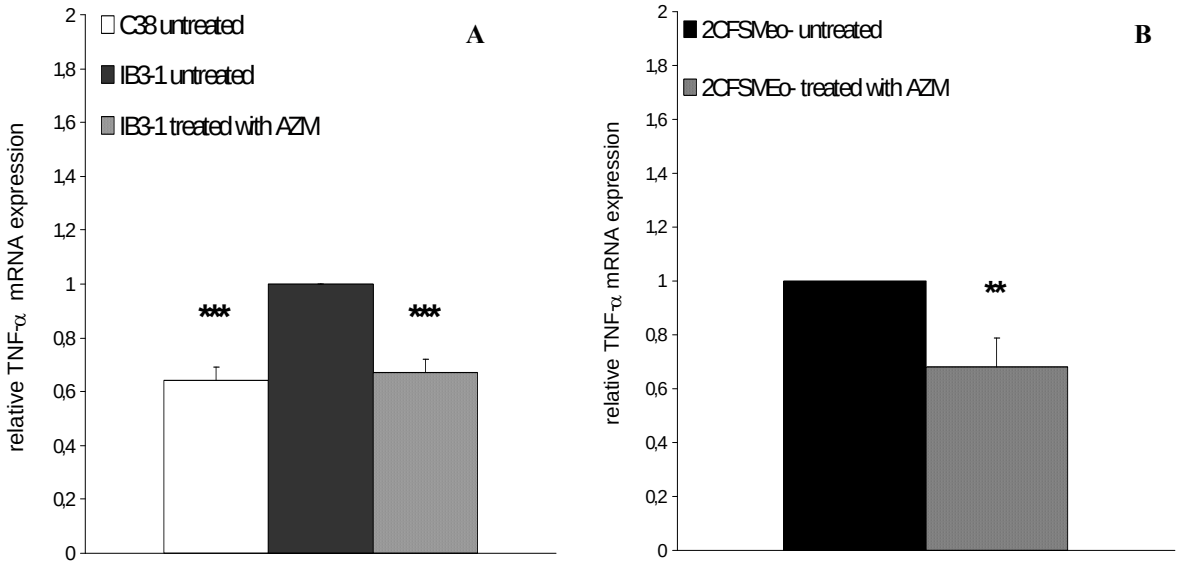


FIGURE 7. TNF-α mRNA expression. Expression of TNF-α mRNA in (A) non-CF C38 cells and isogenic CF IB3-1 cell line and (B) 2CFSMEo- cells at constitutive level and after treatment with AZM. Total RNA was extracted and retrotranscribed. The values of TNF-α mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) IB3-1, and (B) 2CFSMEo- cells (means ± SD). The experiment was repeated five times. **p<0.01, ***p<0.001.

Fig. 8

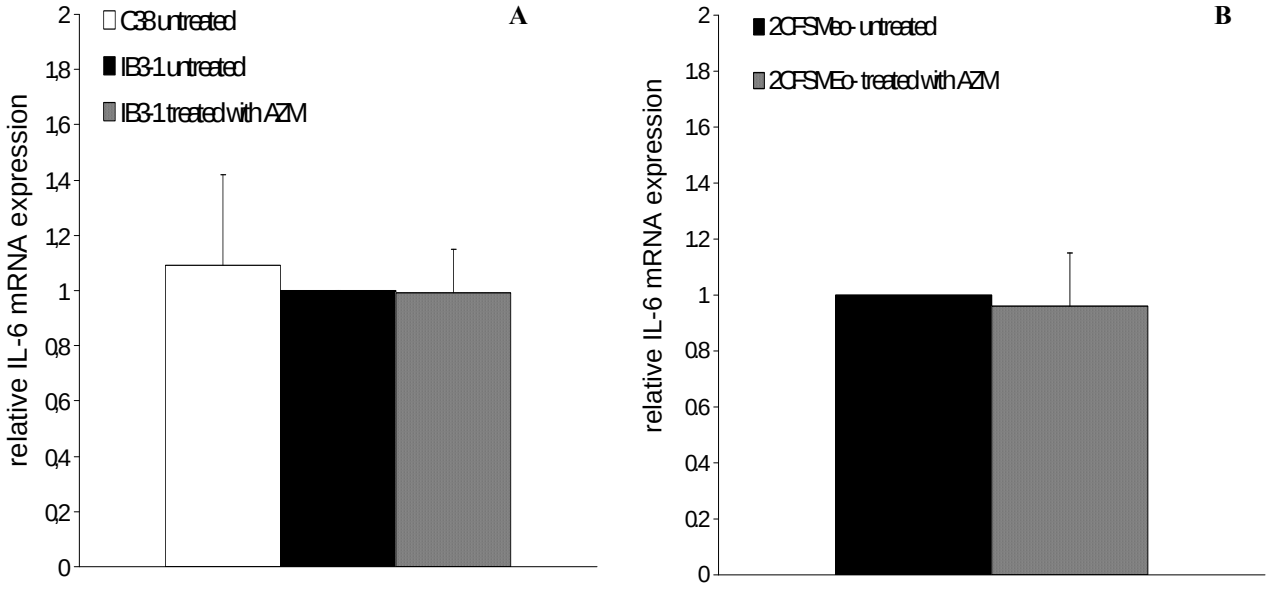


FIGURE 8. IL-6 mRNA expression. Expression of IL-6 mRNA in (A) non-CF C38 cells and isogenic CF IB3-1 cell line, and (B) 2CFSMEo- cells at constitutive level and after treatment with AZM. Total RNA was extracted and retrotranscribed. The values of IL-6 mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) IB3-1 and (B) 2CFSMEo- cells (means ± SD). The experiment was repeated five times.

Fig. 9

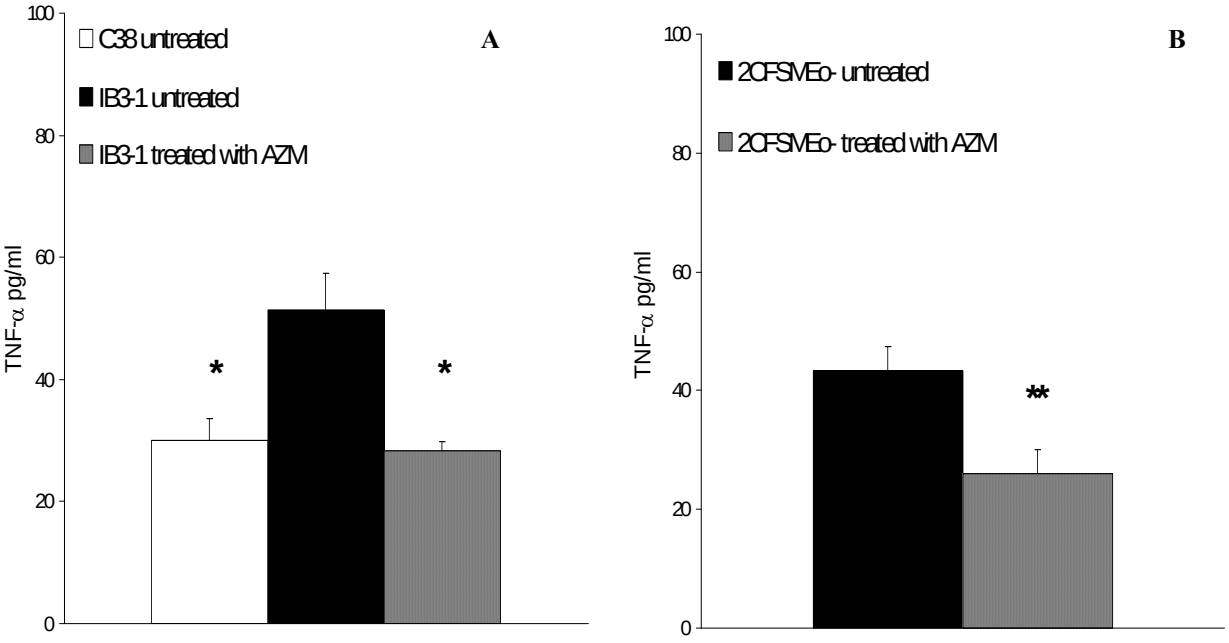


FIGURE 9. TNF-α protein release. Secretion of TNF-α in (A) non-CF C38 cells and isogenic CF IB3-1 cell line, and (B) 2CFSMEo- cells at constitutive level and after treatment with AZM and JM. TNF-α protein levels were measured by commercial ELISA kit. The values represent the secretion levels relative to untreated (A) IB3-1 and (B) 2CFSMEo- cells (means ± SD). The experiment was repeated three times. *$p<0.05$, **$p<0.01$.

Fig. 10

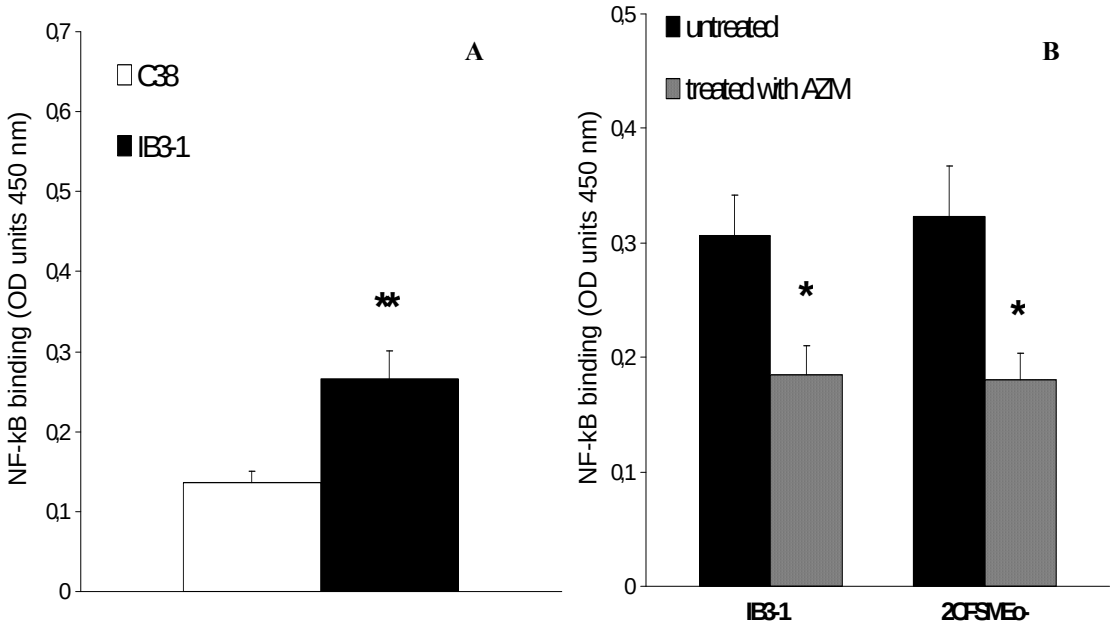


FIGURE 10. DNA binding of NF-kB. (A) Constitutive binding to the DNA of NF-kB in non-CF C38 cells and isogenic CF IB3-1 cell line. (B) Effect of the treatment with AZM on the DNA binding of NF-kB in CF cells (IB3-1 and 2CFSMEo-). NF-kB DNA binding activity was analysed using a commercial kit following the manufacturer's instructions. The experiment was repeated three times (means ± SD). *$p<0.05$, **$p<0.01$.

Fig. 11

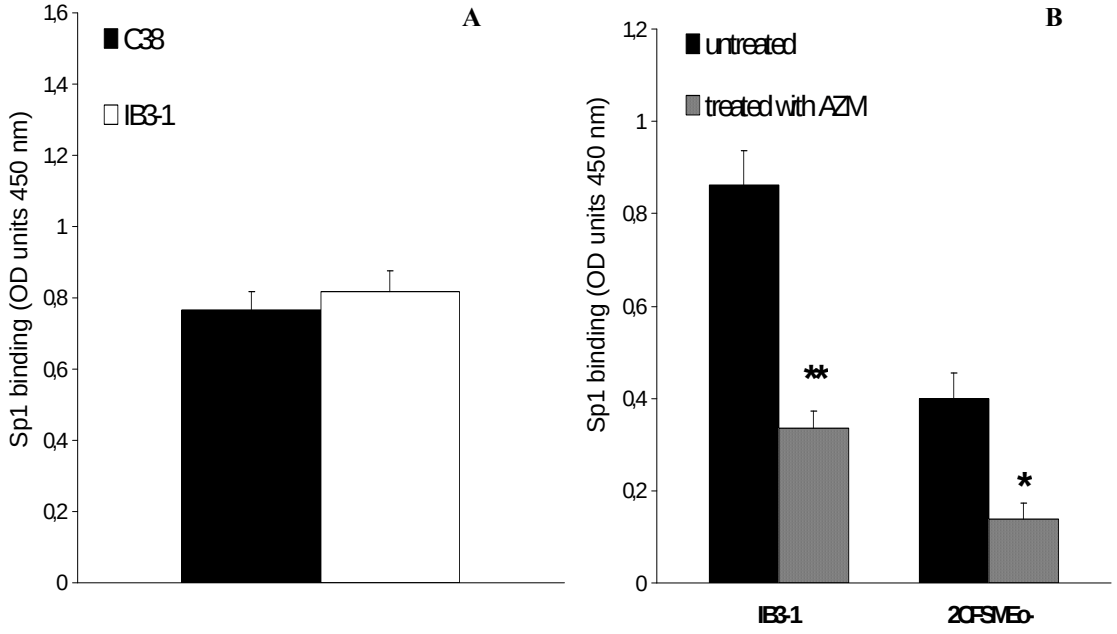


FIGURE 11. DNA binding of Sp1. (A) Constitutive binding to the DNA of Sp1 in non-CF C38 cells and isogenic CF IB3-1 cell line. (B) Effect of the treatment with AZM on the DNA binding of Sp1 in CF cells (IB3-1 and 2CFSMEo-). Sp1 DNA binding activity was analysed using a commercial kit following the manufacturer's instructions. The experiment was repeated three times (means ± SD). *$p<0.05$, **$p<0.01$.

Fig. 12

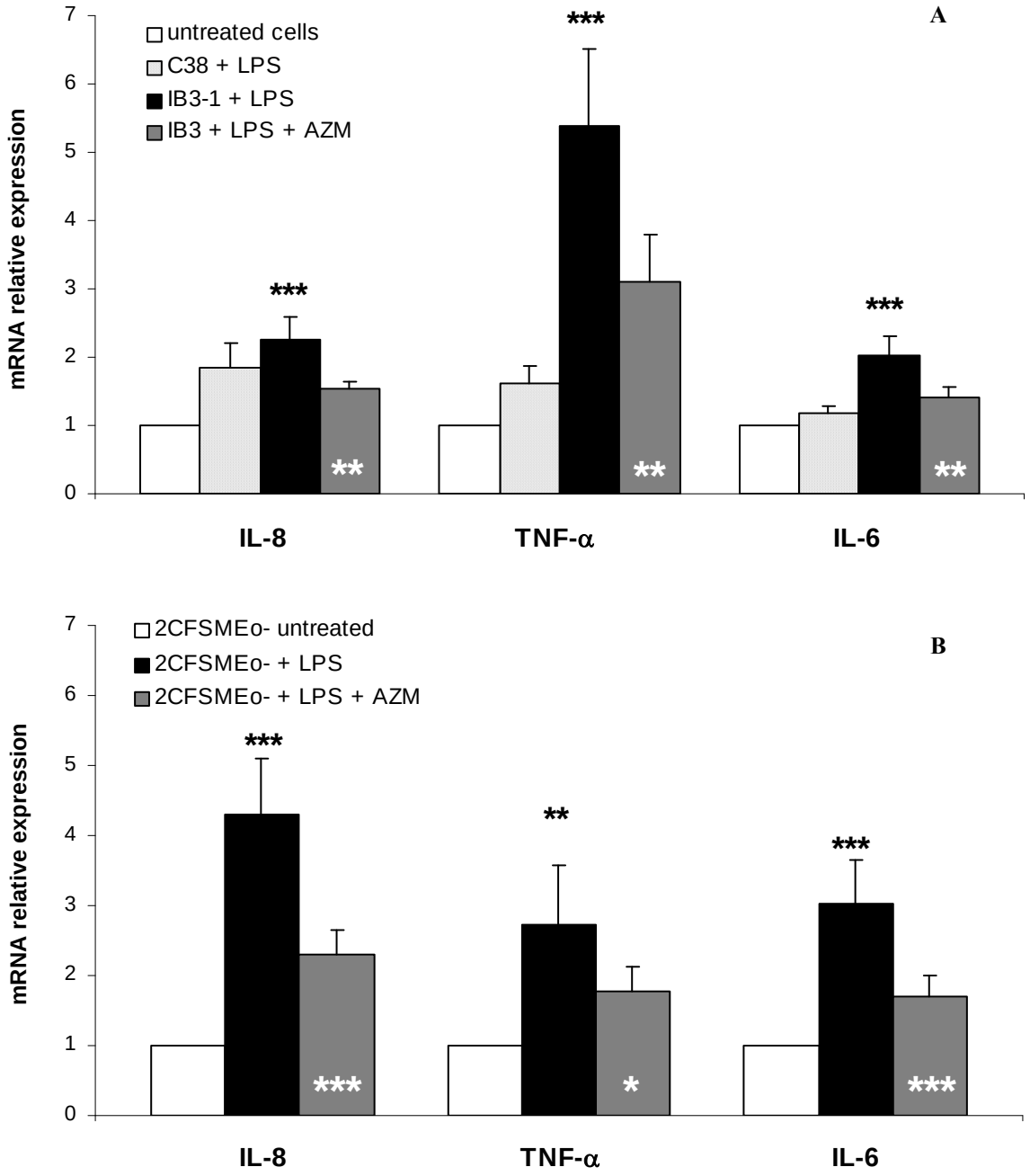


FIGURE 12. IL-8, TNF-α and IL-6 mRNA expression. Expression of IL-8, TNF-α and IL-6 mRNA in (A) non-CF C38 cells and isogenic CF IB3-1 cell line and (B) 2CFSMEo- cells after treatment with 10 μg/ml of LPS for 24 hours and with or without treatment with AZM 8 μg/ml. Total RNA was extracted and retrotranscribed. The values of IL-8, TNF-α and IL-6 mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) both C38 and IB3-1, and (B) 2CFSMEo- cells (means ± SD). The experiment was repeated five times. The significance inside the columns considering AZM treatment is referred to the column preceding without AZM treatment. The significance above the columns is referred to the untreated cells. *p<0.05, **p<0.01, ***p<0.001.

Fig. 13

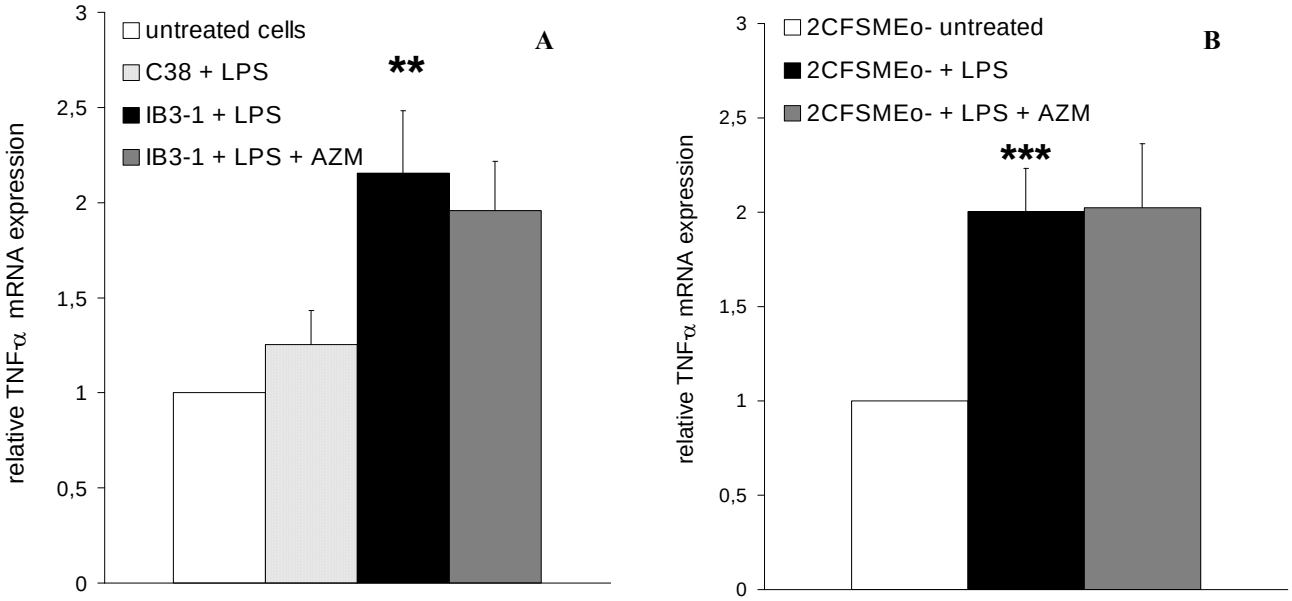


FIGURE 13. ICAM1 mRNA expression. Expression of ICAM1 mRNA in (A) non-CF C38 cells and isogenic CF IB3-1 cell line and (B) 2CFSMEo- cells after treatment with 10 μg/ml of LPS for 24 hours and with or without treatment with AZM 8 μg/ml. Total RNA was extracted and retrotranscribed. The values of ICAM1 mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) both C38 and IB3-1, and (B) 2CFSMEo- cells (means ± SD). The experiment was repeated five times. The significance inside the columns considering AZM treatment is referred to the column preceding without AZM treatment. The significance above the columns is referred to the untreated cells. **p<0.01, ***p<0.001.

Fig. 14

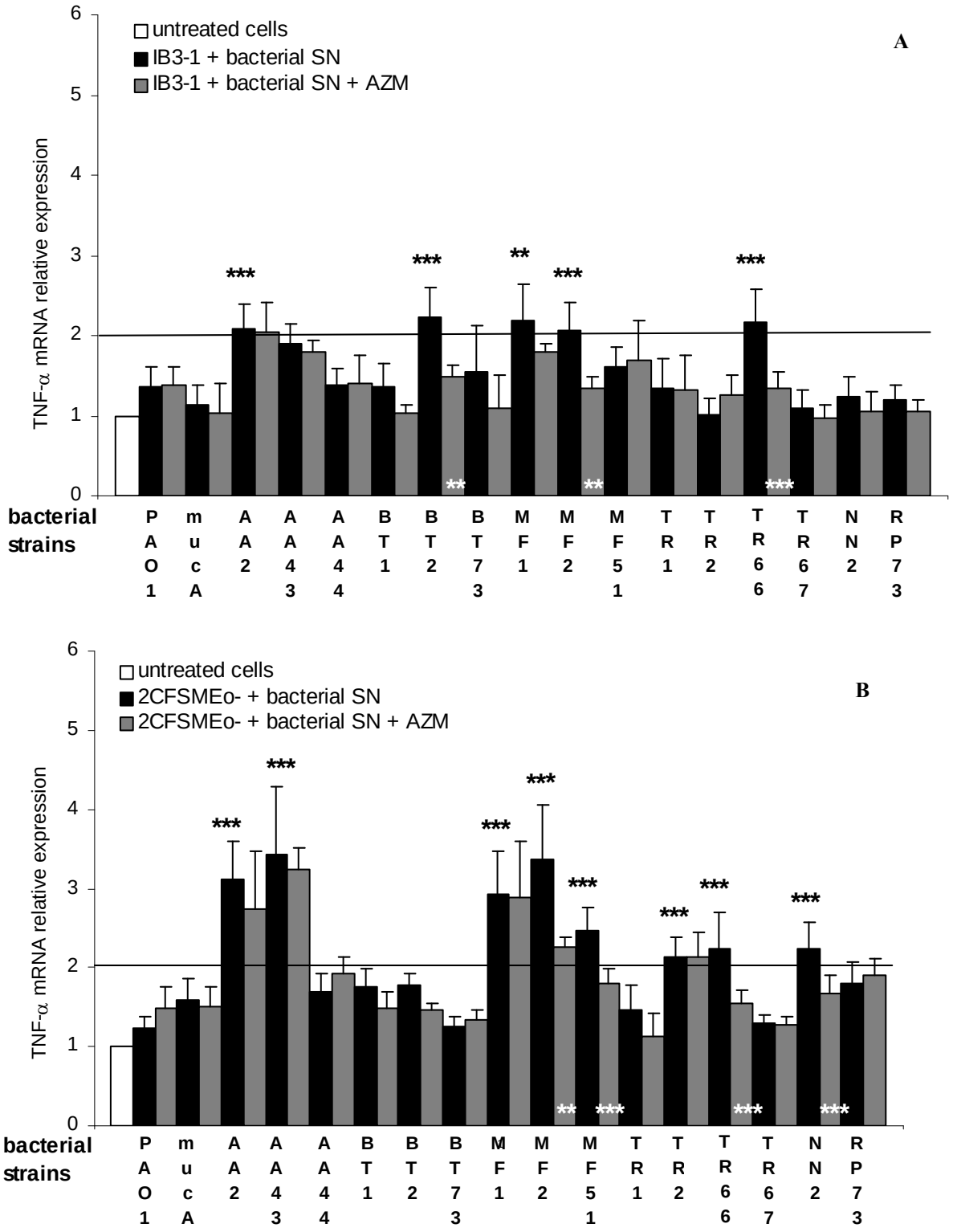

A
untreated cells
IB3-1 + bacterial SN
IB3-1 + bacterial SN + AZM
TNF-α mRNA relative expression
bacterial strains
PAO1
mucA
AA2
AA43
AA44
BT1
BT2
BT73
MF1
MF2
MF51
TR1
TR2
TR66
TR67
NN2
RP73
B
untreated cells
2CFSMEo- + bacterial SN
2CFSMEo- + bacterial SN + AZM
TNF-α mRNA relative expression
bacterial strains

FIGURE 14. TNF-α mRNA expression. Expression of TNF-α mRNA in (A) IB3-1 cell line and (B) 2CFSMEo- cells after treatment with 10% of supernatants derived by *P. aeruginosa* strains for 4 hours and with or without treatment with AZM 8 μg/ml. Total RNA was extracted and retrotranscribed. The values of TNF-α mRNA are based on real-time PCR analysis. The values represent the expression levels relative to untreated (A) IB3-1, and (B) 2CFSMEo- cells (means ± SD). The experiment was repeated five times. The significance inside the columns considering AZM treatment is referred to the column preceding. The significance above the columns is referred to the untreated cells. **$p<0.01$, ***$p<0.001$.

DISCUSSION

Long-term treatment with the macrolide antibiotic azithromycin (AZM) has been reported to determine improvement of lung disease in CF patients [73-75,77,80,81]. This macrolide may exhibit different effects on epithelial cells and on *P. aeruginosa*, which is a predominant cause of decreasing pulmonary function in CF patients [92]. However, the role of AZM has not been fully understood. Among various possible mechanisms as effects on tight junctions, bacterial growth and virulence factors [92-95], AZM has been suggested to modulate the expression of ABC transporters in the airways, thus complementing the CFTR defect, and/or to have anti-inflammatory properties.

The first premise of our work was related to the possibility that the Multidrug Resistance-associated Protein 1 (MRP1) and the Multidrug Resistance Protein 1 (MDR1), two ABC proteins, could have a role in AZM beneficial effects in CF patients.

The Multidrug Resistance-associated Protein 1 (MRP1) and the Multidrug Resistance Protein 1 (MDR1), as well as Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), the channel defective in CF, are members of the ATP Binding Cassette superfamily. These proteins share related functions supporting complementation among them.

Lallemand et al [96] reported improvement in lung function and clearance of *P.aeruginosa* infection in a CF patient following chemotherapy for a fibrosarcoma. Because cancer chemotherapy can induce overexpression of MRP and MDR, they hypothesized that CFTR could be complemented by these close ABC proteins. MDR and MRP mRNAs were detected in nasal epithelial cells of this patient but were absent in a CF patient never exposed to chemotherapics. Therefore a functional complementation by these ABC transporters could explain improvement in lung function in the reported case. Altschuler raised the hypothesis that AZM could improve lung function in colonised *P. aeruginosa* CF patients by inducing the expression of ABC transporters [97]. This possibility was corroborated by Pradal et al who studied the effects of 4 weeks of treatment with AZM in CF patients showing an improvement of chloride transport associated to increased MRP1 mRNA expression [98].

We aimed to evaluate whether AZM may affect the expression of ATP Binding Cassette superfamily members *in vitro.*

At first we investigated the constitutive expression of MRP1 and MDR1 in respiratory epithelial cell lines. A co-ordinated mRNA expression among homologous genes in our cellular models would be consistent with similar findings relative to CFTR and MDR1 in other cell types [67-69]. We found that, although CF cells expressed more MRP1 mRNA and

less MDR1 mRNA versus non-CF isogenic cell lines, there were no differences at protein level.

Furthermore, our results indicate minimal sporadic variations in both MRP1 and MDR1 gene transcription under AZM treatment but, again, protein levels were unaffected. To rule out the possibility that the effect could still occur at the transcription level but that RNA could be rendered more unstable by the treatment in our model, we studied the effects of AZM on the MRP1 promoter activity by gene reporter assays. AZM did not affect the reporter activity in treated CF cells.

Considering the possibility of regulation of a different pattern of ABC family members *in vitro* we have been focusing on possible targets of AZM other than MRP1 and MDR1. We found that AZM was not effective in regulating mRNA expression of other ABC proteins as ABCA1, ABCA13, MRP3, MRP5, CFTR, MDR7 and MDR10 (data not shown). The lack of effects in our models *in vitro* might be due to still unknown mechanism(s) causing also the high variability of beneficial effects among patients described by many authors. We have investigated the possible relevance of a MRP1 polymorphic site in the response to AZM [85]. Work is still in progress in order to identify an eventual MRP1 allele associated to the response to AZM and/or to the severity of disease. No statistically significant effects have been detected also when we have been trying to overcome the possible variability among models by using a nasal CF respiratory epithelial cell line (data not shown). Analysis of gene regulation by macrolides have also been performed using microarrays including DNA sequences of tens of thousands genes in airway epithelial cells [99], but to our knowledge there are no evidences of the regulation of ABC members by AZM to date. However variability among experimental models are arising also when other effects of AZM are being studied. Experimental models based on primary cell cultures revealed pro-inflammatory effects of AZM [100] while anti-inflammatory effects have been described *in vivo* in animal models [101] as well as in patients [74].

Nevertheless the data supporting the hypothesis of complementation of CFTR by MRP1 and MDR1 following AZM treatment [96-98,102], macrolides effects on ion transport are still unclear. Chloride transport through the apical membrane of airway epithelial cells measured as nasal potential differences (NPDs) was unchanged in CF mice following treatment with clarithromycin or AZM as well as in CF patients treated with clarithromycin [103]. Equi et al [78] reported essentially unchanged NPDs as well as CFTR and MDR mRNA levels in nasal epithelial cells in CF patients treated with AZM. Even if erythromycin induced the up-

regulation of the MDR1 expression in the liver of rhesus monkeys [104], this macrolide has been reported to inhibit the interferon-gamma induced activity of an outwardly rectifying chloride channel in a human bronchial epithelial cell line [105]. Moreover Tagaya et al [106] showed that erythromycin decreased the chloride diffusion potential difference across rabbit tracheal mucosa and that clarithromycin treatment decreased sputum production in patients affected by chronic bronchitis or bronchiectasis. They suggested that 14-membered macrolides could reduce chronic airway hypersecretion probably by inhibiting chloride secretion and the resultant water secretion. Furthermore mucus secretion volume was decreased in rhinitis patients after treatment with clarithromycin [107].

Our data do not support the hypothesis of induction of ABC transporters by AZM. Perhaps additional conditions *in vivo* or specific genetic background(s) might allow for complementary expression at protein levels, as suggested [97].

We then focused our attention on the hypothesis that AZM could have anti-inflammatory properties.

Several studies indicate that inflammation occurs very early in the lungs of CF patients and often seems to precede clear signs of infection [21,22,24]. Moreover, CF airway inflammation may manifest as disproportionately increased or prolonged in relation to the level of stimuli [13,30,33,36]. However whether a dysregulation of inflammation exists in CF patients is debated [23,108].

We aimed to establish whether differential expression of a relevant inflammatory marker as TNF-α could be detected in our CF cell models since it plays a relevant role in the pathogenesis of CF. We also wanted to establish whether AZM could reduce this excessive inflammation.

Data from literature regarding of AZM effects on TNF-α expression are scarce and contradictory. Although AZM has been reported to inhibit TNF-α expression in both *in vivo* in animal models and *in vitro* [109,110], in healthy human subjects sera TNF-α protein concentration was unaffected by a 24 hours-long treatment with AZM [83]. In this regard the experimental model seems to be critical and in particular differences between CF and non-CF models are relevant.

We saw not only that TNF-α constitutive expression was significantly higher in CF versus isogenic non-CF cells both at mRNA and protein level, but also that this finding was not dependent on cells passage or period from sedimentation. Moreover, we found no differential IL-6 mRNA expression between CF and non-CF cell lines.

The association between increased inflammatory markers and CFTR mutations is however controversial. Aldallal et al [33] found higher IL-8 expression in CF versus non-CF cells in cell line models but not in primary cultures, revealing a considerable variability in airway epithelial cell inflammation among different individuals and cell models. Becker MN et al [111] found no differential IL-8 and IL-6 expression between CF and non-CF cell lines and primary cultures respectively. These contradictory results could be due not only to the choice of the cell model and its origin but also to different culture conditions. Our experimental model consists of two human CF cell lines derived from different airway cell types and one of them has been compared to its isogenic non-CF cell line. This experimental model was appropriate for reproducing anti-inflammatory effects of AZM described *in vivo* [74,101].

As we already pointed out, AZM is receiving increasing attention for its clinical beneficial effects in the treatment of CF that might derive from the synergism of different effects, including inhibition of *P. aeruginosa* bacterial growth [92,94], decreased expression of bacterial virulence factors [92,93] modulation of inflammatory response [101], ion transport [98] and tight junctions [95].

We found that AZM reduced TNF-α expression at both transcript and protein levels in both our CF cell lines, bringing it to the levels of untreated isogenic non-CF cells. Conversely, IL-6 mRNA expression was not significantly affected by AZM treatment. As we found higher expression of TNF-α, but not of IL-6, in CF versus non-CF cells, we can speculate that AZM may be effective towards those proinflammatory molecules induced in the constitutive inflammation. The specificity of the results is warranted by the observation that JM, a macrolide known to lack clinical anti-inflammatory properties [112], was ineffective.

The possibility that AZM may act at the transcriptional level was tested by measuring the DNA binding activity of two transcription factors relevant in the regulation of TNF-α gene, NF-kB [113] and Sp1 [114].

We found that in the presence of AZM NF-kB DNA binding activity in CF cells was reduced approximately to the levels detected in isogenic non-CF cells. Also Sp1 DNA binding was reduced following treatment with AZM, while activity of this transcription factor was not significantly different in CF and non-CF cells. Once again the inhibitory effect was peculiar to AZM, as JM had no effect on NF-kB and Sp1 DNA binding activity. Assays of transcription factors binding to DNA do not rule out an effect on their activation. This approach has been utilized in order to establish whether NF-kB and Sp1 could be considered

as targets of AZM potentially involved in the regulation of TNF-α transcription by this macrolide.

Increased NF-kB activation in CF versus isogenic non-CF specimens was observed in several studies, both in absence and in presence of stimulation [13,36,46,115] in different experimental models. Furthermore, our results are consistent with previous studies describing higher NF-kB activation in CF versus non-CF cells in a cellular model utilized in this study [13,36].

Furthermore, it is of note that therapeutic inhibition of NF-kB has been proposed for treatment of inflammatory and immune diseases [116,117]. Decreased levels of TNF-α and IL-8, two NF-kB-regulated genes, could reduce the recruitment of neutrophils, which are considered responsible for epithelial damage in CF airways [101].

Sp1 can functionally cooperate with NF-kB to elicit maximal promoter activation of inflammatory genes [118]. Investigating the effects of AZM on Sp1 was considered relevant as this transcription factor has been described to regulate several inflammatory genes including the chemokine Macrophage Inflammatory Protein-2, heparanase and TNF-α [114] and therefore its inhibition could influence inflammatory responses. This approach was considered appropriate for investigating a possible mechanism of regulation of TNF-α by AZM. Inhibition of Sp1 activity by AZM seems to be a novel effect of this macrolide.

We obtained very similar results to those described for regulation of TNF-α when we analyzed the effects of AZM on IL-8 expression in CF airways epithelial cells [119]. In that model transcription factors as NF-kB and AP1 were affected by AZM suggesting possible mechanisms of regulation of IL-8 expression. These data are consisting in supporting anti-inflammatory effects of AZM.

Several reports suggest that CF airways are characterized by exaggerated or prolonged inflammation even in response to infectious agents [13,28-37].

We then decided to determine whether LPS derived by *P. aeruginosa*, a well-known cause of chronic infection in CF patients, could induce exaggerated inflammatory response in CF cells.

We saw that CF cells showed higher expression not only of TNF-α but also of IL-8, IL-6 and ICAM1 mRNA in comparison to isogenic non-CF cells, after exposition to LPS for 24 hours.

It is still debated whether CF cells show an exaggerated inflammatory response following treatment with different stimuli. Some reports demonstrate similar inflammatory parameters

between CF and non-CF cells after stimulation [33,63,111]. The choice of the cell model, its origin and the culture conditions could be the cause of contradictory results also in this case.

Then we found that AZM reduced LPS-induced expression of TNF-α, IL-8 and IL-6 mRNA in both our CF cell lines, while it was ineffective in ICAM1 transcript levels. The awareness that these effects are peculiar to AZM is warranted again by the observation that JM was ineffective. We found scarce data from literature regarding of AZM inhibition of induced inflammation *in vitro* [109,120]. More evidences can be found on AZM inhibition of inflammatory markers, as neutrophils and macrophages influx or cytokines and chemokines concentration, both in patients and in animal models *in vivo* [74,83,101,110,121,122].

Finally, we wanted to evaluate AZM effect on inflammatory markers induced by *P. aeruginosa*. As AZM is an antibiotic, to avoid its parallel antibacterial effect on *P. aeruginosa*, we used the supernatants collected by different *P. aeruginosa* strains cultures to stimulate our CF cell lines. These supernatants contain various virulence factors and other bacterial proteins that might induce inflammation as confirmed by proteomic analysis in progress in collaboration with the group of dr. Pierluigi Mauri at the Institute for Biomedical Technologies (ITB-CNR), Milan. This analysis might allow us to propose specific bacterial factors as pro-inflammatory stimuli as well as targets of AZM.

We found that not only supernatants collected from different *P. aeruginosa* strains cultures stimulated at different degrees the expression of TNF-α mRNA, but also that AZM could reduce these expressions only for some strains in both CF cell lines. Similar results were obtained in regards of other cytokines as IL-6 and IL-8, while once again there was no reduction in ICAM1 mRNA expression (data not shown).

The identification of bacterial strains able to induce a pro-inflammatory response sensitive to AZM is helping us to establish a murine model of infection appropriate for assessing the effects of AZM *in vivo*, in collaboration with dr. Alessandra Bragonzi at the Institute for Experimental Treatment of Cystic Fibrosis, Scientific Institute H.S. Raffaele, Milan.

CONCLUSIONS AND PERSPECTIVES

Several hypotheses on the mechanisms of action of AZM have been suggested [92-95]. Our data do not support the hypothesis of induction of ABC transporters by AZM. In fact we show that AZM had no effects on MRP1 and MDR1 expression.

Whether inflammation in CF is exaggerated is still controversial [13,23,30,33,36,108]. We indicated that CF cells show higher expression of the cytokine TNF-α in comparison to non-CF cells both in the absence and in the presence of stimuli.

Our results show that the antibiotic AZM has the features of an anti-inflammatory drug as it reduces the constitutive expression of TNF-α in our CF cell lines. We suggested also that NF-kB and Sp1 transcription factors, responsible for TNF-α transcriptional regulation, are relevant targets whose inhibition might contribute to ameliorate the excessive inflammatory response in CF.

Finally we found that AZM can reduce the expression of TNF-α, IL-8 and IL-6 mRNAs after stimulation not only with LPS derived by *P. aeruginosa*, but also with some supernatants collected from different *P. aeruginosa* clinical strains cultures.

We grew *P. aeruginosa* strains in the presence and absence of AZM to evaluate its effect on the production of bacterial proteins, but also both in aerobiosis and in microaerophilic conditions to point out the relevance of oxygen on the bacterial growth and proteic production as CF airways are known to have low oxygen concentration. The proteomic studies in collaboration with the group of dr. Mauri indicate bacterial proteins, regulated in these different conditions, that could be relevant in CF airways inflammation not only as stimuli but also as targets of AZM. Moreover the differential sensitivity of these strains to AZM could partially explain the high variability in clinical outcomes among patients treated with AZM. Therefore it might suggest an approach for selecting patients to be treated with this macrolide.

In collaboration with dr. Alessandra Bragonzi a mouse model for endobronchial infection has been established using a non-mucoid strain of *P. aeruginosa*, PAO1 [123] (Fig 15). The strain was embedded in agar beads and used to infect C57Bl/6 mice by intratracheal injection. One or two days following intratracheal bacterial challenge (top and bottom, respectively) mice were euthanized and lungs removed. Lung histology of infected mice showed that the agar beads, deposited in the bronchial lumen, contained bacterial cells

macrocolonies surrounded by polymorphonuclear cells. The infected mice developed persistent and stable *P. aeruginosa* colonization over 1 month resembling that of CF patients.

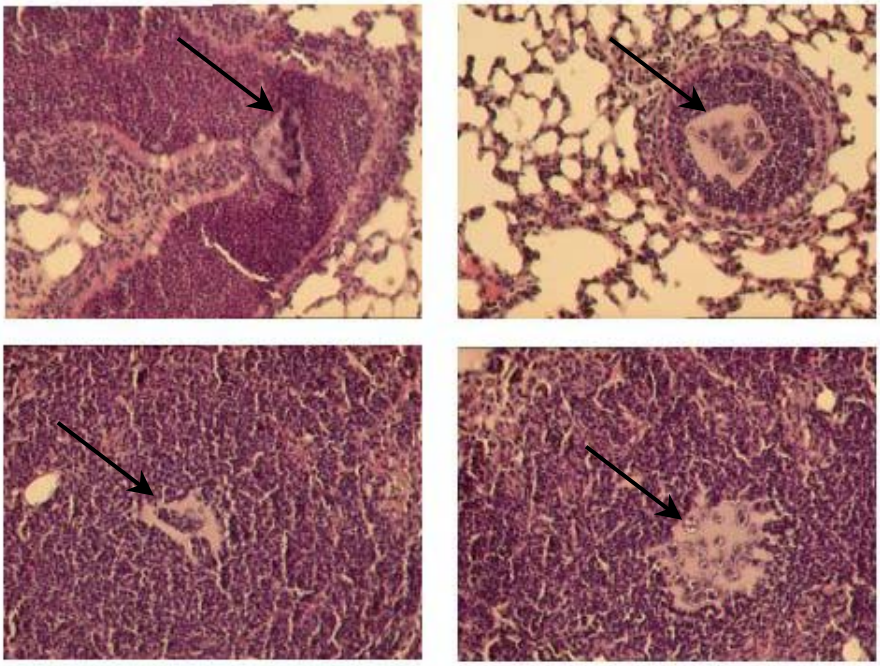


Figure 15. *Ematoxylin/eosin staining of formalin embedded murine lungs.* Agar beads containing *P. aeruginosa* (arrows) were deposited in the bronchial lumen and resulted in neutrophil influx 1 (upper) or 2 days (bottom) after intratracheal administration.

In future, we aim to use a clinical strain in this murine model. We would choose the strain in accordance not only with the modulation of its proteic products by AZM but also with the efficacy of this macrolide to inhibit the strain induced inflammation in CF cells.

In conclusion, we indicated differential transcription factors DNA-binding activity and subsequent cytokines expression in CF versus non-CF cells in the absence and the presence of stimulation as pathogenetic mechanisms relevant in CF. Furthermore we pointed out the role of bacterial products in inducing inflammation in CF airways. Finally we established the AZM inhibitory effects on constitutive as well as induced inflammation in CF cell lines.

Our results could help to optimize AZM use for CF therapy and to suggest molecular outcomes.

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DIPARTIMENTO DI PATOLOGIA
Sezione di Patologia Generale
Facoltà di Medicina e Chirurgia
UNIVERSITA' DEGLI STUDI DI VERONA
Strada Le Grazie, 8 - 37134 Verona (Italia)

Tel. +39 (045) 8027120
Fax. +39 (045) 8027127

Verona 9 Gennaio, 2007

Oggetto: Profilo della dottoranda, Dr.ssa Cristina Cigana

La dott.ssa Cristina Cigana ha iniziato a frequentare il Laboratorio di Patologia Molecolare del Centro Fibrosi Cistica dell'Azienda Ospeadaliera di Verona nel 2002 occupandosi fin dall'inizio dell'infiammazione in fibrosi cistica (FC). La supervisione della sua attività scientifica è stata da allora affidata alla dott.ssa Paola Melotti. Le principali aree di interesse hanno riguardato l'inibizione del fattore di trascrizione Nuclear Factor (NF)-kB come approccio anti-infiammatorio per la terapia della FC e la definizione dei meccanismi d'azione del macrolide azitromicina (AZM) per il quale diversi meccanismi, tra i quali quelli anti-infiammatori, sono stati indagati ed identificati. In particolare la candidata ha studiato con diverse metodiche l'attivazione di fattori di trascrizione (NF-kB, AP-1, Sp1) alcuni dei quali sono risultati indotti da proteine adenovirali rilevanti per approcci di terapia genica, oppure costitutivamente attivati in cellule di epitelio respiratorio con fenotipo fibrocistico o infine bersagli dell'AZM. Si è dedicata allo studio della regolazione della trascrizione genica da AZM e da interleuchina 10 procedendo anche a validazione con QPCR di dati ottenuti mediante microarrays cDNA nell'ambito di uno studio collaborativo.

La dott.ssa Cigana è stata impegnata presso gli Istituti Scientifici San Raffaele in collaborazione con il gruppo della dott.ssa Alessandra Bragonzi nella messa a punto di un modello di infiammazione polmonare murina rilevante per la FC in quanto conseguente ad infezione da Pseudomonas aeruginosa (Pa). La dott.ssa Cigana ha poi sviluppato presso il Centro Fibrosi Cistica di Verona un modello di valutazione in vitro della capacità di diversi ceppi di Pa di indurre l'espressione di molecole pro-infiammatorie; il metodo è utile anche per stabilire la sensibilità all'AZM della risposta infiammatoria. Per approfondire i meccanismi di induzione della risposta infiammatoria e del danno epiteliale da Pa la dott.ssa Cigana partecipa molto attivamente a studi basati anche sull'analisi proteomica in collaborazione con il gruppo del dott. Pierluigi Mauri, CNR Segrate-MI. Ha presentato oralmente diversi studi da lei condotti nell'ambito di congressi anche internazionali ed è stata prima autrice di diverse pubblicazioni su riviste peer reviewed. Si sta tuttora applicando con buona autonomia alla stesura di diversi manoscritti.

Complessivamente la dott.ssa Cigana ha dimostrato spiccata capacità di coniugare l'interesse per mettere a punto modelli originali alla prontezza a cogliere spunti teorici da ambiti diversi. La maturità professionale raggiunta le permette di formulare ipotesi con la capacità di gestirne la verifica sperimentale. La sua capacità organizzativa le permette di partecipare molto attivamente a diversi progetti senza perdere di vista gli obiettivi principali. Ha fornito e continua a fornire con le sue iniziative personali un importante ausilio nella gestione dei progetti, in particolare di quelli collaborativi. Per valutazione unanime del Collegio dei Docenti la Dr.ssa Cigana è matura per il conseguimento del titolo di Dottore di Ricerca.

Il Coordinatore del Dottorato
Prof. Marco Cassatella

Il relatore della tesi:
Dr.ssa. Paola Melotti

PUBLISHED ARTICLES

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- Cigana C, Nicolis E, Pasetto M, Assael BM, Melotti P.
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The GCC repeat length in the 5'UTR of *MRP1* gene is polymorphic: a functional characterization of its relevance for cystic fibrosis

Elena Nicolis, Matteo Pasetto, Cristina Cigana, Ugo Pradal, Baroukh M Assael and Paola Melotti*

Address: Cystic Fibrosis Center, Azienda Ospedaliera di Verona, Piazzale Stefani 1, 37126 Verona, Italy

Email: Elena Nicolis - elenni@libero.it; Matteo Pasetto - biomatteo@tin.it; Cristina Cigana - cristinacgn@libero.it; Ugo Pradal - ugo.pradal@mail.azosp.vr.it; Baroukh M Assael - baroukh.assael@unimi.it; Paola Melotti* - paolamelotti@libero.it

* Corresponding author

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Abstract

Background: Among the members of the ATP binding cassette transporter superfamily, MRPs share the closest homology with the CFTR protein, which is defective in CF disease. *MRP1* has been proposed as a potential modifier gene and/or as novel target for pharmacotherapy of CF to explain the clinical benefits observed in some CF patients treated with the macrolide AZM. The 5'UTR of the *MRP1* gene contains a GCC triplet repeat that could represent a polymorphic site and affect the activity of the promoter.

Methods: The *MRP1* 5' flanking region was amplified by PCR from 36 CF patients and 100 non-CF subjects and the number of GCC triplets of each allele was determined by sequence and electrophoretic analysis. We performed gene reporter studies in CF airway epithelial cells 16HBE14o-AS3, in basal conditions and in the presence of AZM.

Results: We found that the GCC repeat is polymorphic, ranging from 7 to 14 triplets either in CF or in non-CF subjects. Our data are preliminary and have to be confirmed on a larger population of CF subjects. The transcriptional activity of the proximal *MRP1* 5' regulatory region revealed no statistically significant correlations between the number of repeats and treatment with AZM.

Conclusion: We identified a novel polymorphism in the 5'UTR of *MRP1* gene that provides multiple alleles in a gene relevant for multidrug resistance as well as for CF, determining that this region is transcriptionally active and that this activity does not appear to be influenced by AZM treatment.

Background

CF is an autosomal recessive disease primarily manifested in the lung, which leads to respiratory failure. CF is caused by mutations of the *CFTR* gene whose product acts as a cAMP-activated chloride channel that is permeable to organic anions, including GSH [1,2]. A wide range of disease severities has been described, even among CF patients harbouring the same mutation. Therefore endogenous factors, that modulate or complement CFTR function, must exist. Because of the structural homology between CFTR and MRP1, a functional complementation of CFTR defect by MRP1 has been hypothesized [3]. Induction of MRP1 expression has been suggested to be responsible for improvement in lung function in a CF

Table 1: *MRP1* (GCC)n alleles frequencies in non-CF and CF subjects

Number of GCC triplets	Alleles in non-CF subjects (%)	Alleles in CF patients (%)
7	32	29
10	3	0
11	16	21
12	13	10
13	31	38
14	5	2

patient following chemotherapy with cyclophosphamide and epirubicin [4]. Low levels of MRP1 transcripts have been associated with more severe CF phenotype [5], while increased expression of the *MRP1* gene has been associated with restored chloride conductance in a group of CF patients following treatment with AZM, giving rise to clinical beneficial effects [6,7]. *MRP1* has been considered as a potential modifier gene [5] and/or as a target for therapy of CF in subjects responsive to AZM [8].

The *MRP1* was originally cloned in a drug-selected lung cancer cell line [9]. MRPs are transmembrane proteins, whose mRNAs are detectable in most human tissues [10]. They share the closest homology with the CFTR protein, which belongs to the same C subfamily of the ABC transporter superfamily [11]. MRP1 confers resistance to chemotherapeutic drugs as well as to heavy metal oxyanions. It transports reduced glutathione conjugates, cysteinyl leukotriene LTC_4, steroid glucoronides and bile salt derivatives in human cells, agents involved in the transcription factor NF-kB activation pathway, which has been reported to be altered in CF [12,13]. Therefore a functional link exists between genes involved in GSH metabolism and MRP1. NF-kB regulates expression of GSTP1 and polymorphisms of the anti-oxidizing enzymes GSTP1 and GSTM3 are associated with severity of CF [14]. The *GSTM1* gene, encoding an enzyme that forms glutathione adducts, is deleted in most severe CF patients [15]. CF patients carrying a wild type allele for both GSTM1 and GSTT1 may be at reduced risk of severe lung disease. The abnormal reduced glutathione transport caused by CFTR mutation seems to play a critical role in the pathogenesis of CF and this parameter might also be complemented by MRP1 in CF patients. MRP1 has been also involved in the regulation of endogenous channels as chloride channels [16,17]. The physiological functions of MRP1 are the subject of many current investigations: the available evidence indicates that it is involved in detoxification, drug resistance, oxidative stress and inflammation [18,19]. An impaired response to inflammatory stimuli has been described in MRP1(-/-) knockout mice [20], even if *MRP1* has not been demonstrated to be a disease-causing gene.

The sequence of the *MRP1* gene has been reported [9]. The 5' flanking region is GC rich (88%) while the 5'UTR contains a GCC triplet repeat that has been hypothesized to represent a polymorphic site [12]. The influence of the number of triplets in the 5'UTR on MRP1 mRNA transcription, stability and translational efficiency has not been determined.

We searched a genetic marker for *MRP1* with potential functional relevance aiming to establish: 1) whether the GCC repeat in the *MRP1* 5'UTR is polymorphic, 2) if the GCC triplets length could affect the activity of the promoter in CF airways epithelial cells either in basal conditions or upon AZM treatment.

Methods

Patients and controls

Genomic DNA from 100 unrelated non-CF subjects and 36 CF patients was collected. Nucleic acid was extracted from peripheral blood leukocytes using the salting out method [21]. Diagnosis of CF was based on clinical, biochemical and genetic data. The most common CF causing mutation, deltaF508, was detected in 24 patients, 7 of which were homozygous. This study was approved by the local institutional Ethic Committee.

Genotyping

Specific primers were designed based on the nucleotide sequence of the 5' regulatory region of *MRP1* gene (GenBank:U07050) (sequences are available upon request). PCRs were performed in an Applied Biosystems 9700 Thermal Cycler, using a final volume of 50 μl. Due to the high GC levels of the PCR products, 7-deaza-dGTP (Roche) was utilized instead of dGTP in the dNTPs mix. PCR-1 started with 350 ng of genomic DNA; 2.5 μl of PCR-1 were subjected to further amplification (PCR-2) and a third round of amplification (PCR-3) was performed with 2.5 μl of PCR-2. Amplifications were carried out starting with 10 min template denaturation/AmpliTaq Gold (Applied Biosystems) activation step at 95°C, followed by 40 cycles (20 cycles for PCR-3) of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension

Table 2: Genotypes frequencies of the *MRP1*(GCC)n alleles in the control group and in the CF patients

Genotype	% in non-CF subjects	% in CF subjects
7/7	23	25
13/13	15	6
7/13	12	0
11/12	10	11
11/13	9	0
12/13	7	28
7/11	5	6
12/12	4	3
11/11	3	0
14/14	3	0
10/11	2	8
10/13	2	0
10/14	2	0
13/14	2	2
7/14	1	0
12/14	0	8
7/12	0	3

at 72°C for 30 s. As a final product the fragment from -337 nt to -43 nt, starting from the translational starting site of the *MRP1* 5' gene, was obtained.

PCR products were purified with the NucleoSpin Extract kit (Macherey-Nagel). Samples were then sequenced using Thermo Sequenase II Dye Terminator Cycle Sequencing Premix Kit (Amersham Biosciences) and the automated 373A DNA Sequencer (Applied Biosystems).

PCR-3 was carried out in the presence of a primer conjugated to FAM dye (5-carboxyfluorescein) (Applied Biosystems) that generated labelled amplicons suitable for electrophoresis and Genescan analysis.

Genescan analysis Software (Applied Biosystems) was used to determine the length of the PCR products. In order to validate our method we utilized a vector including the *MRP1* (GCC)7 allele (kind gift from Roger Deeley, Queen's University, Kingston, Ontario, Canada) as control. A proof-reading enzyme was utilized and at least duplicates of each sample and control vector were performed to exclude polymerase slippage.

Gene reporter studies

The *MRP1* 5' regulatory region containing 7 or 14 GCC triplets, obtained as PCR-3 product, was cloned in the pGL3 vector (Promega). The constructs were checked by restriction, sequence and Genescan analysis and named MRP1(GCC)7-luc or MRP1(GCC)14-luc, according to the number of triplets. The CF airway epithelial cell line 16HBE14o-AS3 (kind gift from Pamela Davis, Case Western Reserve University School of Medicine, Cleveland, OH, USA) was transiently co-transfected using FUGENE (Roche) with the reporter vector MRP1(GCC)7-luc or MRP1(GCC)14-luc and a beta-gactosidase expressing vector, utilized for normalization. The day after the transfection, cells were incubated for 24 hrs with or without AZM 8 μg/ml (Pfizer Italia). This concentration is consistent with that achieved in the lung of CF patients treated with AZM [22]. Luciferase activity was determined using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activity in insert-less (pGL3) transfected cells was almost undetectable.

Results

The MRP1 5' flanking region is polymorphic

The *MRP1* 5' flanking region was amplified by PCR from 36 CF patients and 100 non-CF subjects and the number of triplets of each allele was determined by sequencing and electrophoretic analysis.

In non-CF subjects the number of triplets ranged from 7 triplets to 14. Six distinct alleles consisting of 7, 10, 11, 12, 13 and 14 GCC triplets were found. The frequencies of these *MRP1*(GCC)n alleles, where n indicates the number of triplets, is shown in Table 1. The genotypes frequencies of the GCC alleles are indicated in Table 2.

Assessment of transcriptional activity

In order to establish whether the length of the GCC repeat can affect the transcriptional activity we performed gene reporter studies in CF airway epithelial cells 16HBE14o-AS3. This region has been previously demonstrated to be transcriptionally active by gene reporter studies [9]. Since the clinical status as well as the response to AZM has been associated to the levels of *MRP1* mRNA in CF patients, we tested the transcriptional activity of *MRP1* in our model by gene reporter assays in the presence or absence of the drug. The levels of luciferase activity measured in the con-

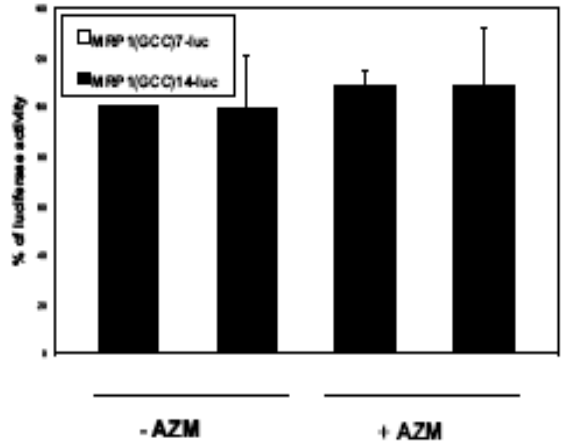

Figure 1
Comparison of the transcriptional activity of MRP1 alleles containing 7 or 14 GCC triplets. 16HBE14o-AS3 cells were transfected with reporter vectors including the *MRP1* 5'UTR region with 7 [MRP1(GCC)7-luc] or 14 triplets [(GCC)14-luc] driving the luciferase gene. 16HBE14o-AS3 cells transfected with MRP1(GCC)7-luc and not treated with AZM were considered as calibrator. Percentages of luciferase activity normalized with beta galactosidase are shown. Specificity of the signal was assessed by determining the luciferase activity in insert-less (pGL3) transfected cells, which was almost undetectable. Experiments were performed in triplicate (n = 3, $p > 0.05$ for all conditions, paired T-Test; SEMs are indicated by bars).

structs containing 7 or 14 GCC triplets of the *MRP1* 5' regulatory region were not significantly modified ($p > 0.05$) either in basal conditions or in the presence of AZM (Figure 1).

Discussion

This study demonstrates that the genomic region from -337 nt to -43 nt of the *MRP1* 5' regulatory region includes a polymorphic site and possesses transcriptional activity. We also demonstrate that the *MRP1*(GCC)7 and *MRP1*(GCC)14 alleles (chosen as representative of the shortest and longest triplet length we identified) do not show any statistically significant differences in transcriptional activity when tested in CF airway epithelial cells *in vitro*. We chose these two alleles since we hypothesized that the length of the GCC repeat could influence *MRP1* transcriptional activity as suggested by other authors [9]. Since we have been focusing to a partial region of the 5'UTR of the *MRP1* gene, our approach was not aimed to test the influence of upstream regulatory sites eventually associated with specific GCC(n) alleles, as a recently described G/C single nucleotide polymorphism [23].

The number of alleles described in our limited group of CF subjects does not allow for searching genotype-phenotype correlation. Efforts are in progress in order to perform this analysis. Our data are preliminary and have to be confirmed on a larger population of CF subjects.

We show that AZM treatment does not affect reporter gene activity in either of the tested alleles. Therefore, at least under the experimental conditions we utilized in our assays, the sequence responsible for variable levels of MRP1 detected in other studies [5,8] does not appear to be included in the DNA sequence we analyzed. Previous studies reported that multiple Sp-1 binding domains are located close to *MRP1* transcriptional start sites near the GCC repeat and might participate in the modulation of transcriptional activity. Zhu and Center [9] suggested a variable length of the GCC repeat in the *MRP1* 5' regulatory region and proposed the influence of this sequence on the *MRP1* mRNA transcription. In support of this hypothesis, it has been reported that a triple GCC repeat within the 5' flanking region contributes to the regulation of interleukin 1 alpha expression [24]. As Slapak and colleagues demonstrated that *MRP1* gene amplification is not sufficient for explaining drug resistance [25], the polymorphism we identified might be utilized for investigating whether the number of GCC triplets could be associated with this feature. However, we still cannot exclude a functional relevance of this polymorphism on *MRP1* mRNA transcription, stability and/or translational efficiency in its native context.

Conclusion

In summary we established that: 1) the GCC repeat in the *MRP1* 5'UTR is polymorphic, 2) the GCC triplets length do not affect *in vitro* the activity of the promoter in CF airways epithelial cells either in basal conditions or upon AZM treatment.

We propose our experimental for testing whether other molecules potentially relevant for therapy of CF have the capability to influence *MRP1* gene expression acting on this promoter region.

Finally, the polymorphic length of the GCC repeat we described can be exploited as a genetic marker, possibly linked to additional sequences involved in regulation of constitutive or induced MRP1 expression.

List of abbreviations

AZM azithromycin

CF cystic fibrosis

CFTR cystic fibrosis transmembrane conductance regulator

GST glutathione S transferase

MRP multidrug resistance-associated protein

UTR untranslated region

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

EN designed and provided the genotyping method and contributed to the redaction of the manuscript. MP performed the gene reporter analysis and contributed to genotyping, CG participated in the molecular genetic studies. UP collected the clinical data and provided clinical material. BA was involved in supervision of the work. PM designed, coordinated the study and drafted the manuscript.

All authors read and approved the final manuscript.

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Anti-inflammatory effects of azithromycin in cystic fibrosis airway epithelial cells

Cristina Cigana, Elena Nicolis, Matteo Pasetto, Baroukh Maurice Assael, Paola Melotti *

Cystic Fibrosis Center, Azienda Ospedaliera di Verona, Piazzale Stefani 1, 37126 Verona, Italy

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Abstract

We aimed at identifying molecular mechanisms for anti-inflammatory effects of azithromycin (AZM) suggested by clinical evidences. IL-8 expression and DNA binding activity of two key pro-inflammatory transcription factors (TF), NF-κB and AP-1, were investigated in cystic fibrosis (CF) and isogenic non-CF airway epithelial cell lines. AZM reduced about 40% of IL-8 mRNA and protein expression ($n = 9$, $p = 0.02$, and $n = 4$, $p = 0.00011$) in CF cells reaching the levels of non-CF cells. In the presence of AZM we found about 50% and 70% reduction of NF-κB and AP-1 DNA binding, respectively ($n = 3$, $p = 0.01$, and $n = 3$, $p = 0.0017$), leading to levels of non-CF cells. The relevance of NF-κB and AP-1 in regulating IL-8 promoter transcriptional activity was demonstrated by gene reporter assays ($n = 4$, $p = 8.54 \times 10^{-7}$, and $n = 4$, $p = 6.45 \times 10^{-6}$). Our data support the anti-inflammatory effects of AZM in CF cells, indicating inhibition of transcription of pro-inflammatory genes as possible mechanism, thus providing a rationale for the possible use of specific TF inhibitors for therapy.

Keywords: Cystic fibrosis; Azithromycin; IL-8; NF-κB; AP-1

There are increasing evidences from clinical trials about beneficial effects of the macrolide azithromycin (AZM) in cystic fibrosis (CF) patients [1]. Properties relevant for therapy of CF other than bactericidal activity have been proposed for this macrolide, including effects on ion transport, tight junctions, bacterial growth, virulence factors, and inflammation [2–6].

Progressive lung damage in CF is a consequence of exaggerated and chronic pro-inflammatory processes which are considered intrinsic in this disorder [7–9]. The basal inflammatory imbalance contributes to develop severe mucosal damage, paving the way for bacterial colonization [10].

AZM has been reported to ameliorate airway inflammation in CF patients [11].

Anti-inflammatory activity as well as modulation of *Pseudomonas aeruginosa* (Pa) virulence factors were suggested as mechanisms for the beneficial effects since macrolides were below the minimum inhibitory concentration required for anti-bacterial activity versus Pa and independent of Pa infection [12].

It has been reported that AZM is capable of reducing IL-8 serum levels in healthy human subjects [13] and IL-8 in nasal polyps [14].

It is controversial whether inflammation is constitutive or is a response to infection in CF. There are evidences about exaggerated constitutive activation of the transcription factor (TF) nuclear factor κB (NF-κB) in CF [15,16]. Activating Protein 1 (AP-1) and NF-κB are known to be involved in activating the transcription of many pro-inflammatory molecules [17] including IL-8, the major polymorphonuclear chemokine in the lung. AZM down-regulates activation of both these factors in human vascular smooth muscle cells [18].

In this study we have investigated the ability of AZM to regulate the constitutive IL-8 expression in CF bronchial epithelial cells as well as the activation of TFs relevant for its transcription such as NF-κB and AP-1.

* Corresponding author. Fax: +39 045 8072042.
E-mail address: paolamelotti@libero.it (P. Melotti).

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Materials and methods

Cell cultures. 16HBE14o-AS3 and 16HBE14o-S1 cells (kind gift of P. Davis, Case Western Reserve University School of Medicine, Cleveland, OH), with CF and non-CF phenotypes [19], were cultured in E-MEM supplemented with 10% fetal bovine serum in the presence of the selective agent G418 200 μg/ml (Sigma, St. Louis, MO) on coated surfaces as previously described [19]. The passage numbers of cells ranged from 18 to 37 for 16HBE14o-AS3 and from 27 to 44 for 16HBE14o-S1. The results were confirmed at different cell passages.

Drug treatment. In the presence of AZM (Pfizer, Roma, Italy) or josamycin (JM) (Yamanouchi, Tokyo, Japan) for 24 h at 8 μg/ml the cell viability was >95% as determined by Trypan blue exclusion test while at higher concentrations starting from 16 μg/ml the viability was <95%.

Serial dilutions (0, 0.125, 0.5, 2, and 8 μg/ml) of the macrolides were utilized in dose–response experiments. These concentrations are consistent with those described in lungs of patients treated with AZM [20].

IL-8 and ICAM-1 mRNA quantification. The real-time quantitative PCR (qPCR) was performed to quantify IL-8 and ICAM-1 transcripts. Total RNA was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). Reverse transcription was performed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and random primers, in a total volume of 100 μl, following the manufacturer's instructions.

The cDNA (2 μl) was amplified using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Grand Island, NY) in the ABI Prism 5700 sequence detection system. The Primer Express Software (Applied Biosystems, Foster City, CA) was used to select the specific primers. Primer sets (Sigma-Genosys, St. Louis, MO, USA) are shown in Table 1. The PCR thermal protocol consisted of 2 min at 50 °C, a denaturation step at 95 °C for 2 min followed by 50 cycles of a 15 s 95 °C denaturation step and a 30 s annealing/extension step at 60 °C. The real-time qPCRs were performed in duplicate for both target and normalizer gene.

Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer's User Bulletin 2 (Applied Biosystems, Foster City, CA). Changes in mRNA expression level were calculated following normalization to GAPDH. The ratios obtained following normalization were expressed as folds of change over untreated samples.

IL-8 secretion analysis. IL-8 secretion was determined in supernatants collected from the cell cultures above described by Enzyme Amplified Sensitivity Immunoassay using the IL-8 EASIA kit (Biosource Camarillo, CA) according to the manufacturer's instructions. Measurements were performed at least in duplicate. Values were normalized to 10^6 cells.

Gene reporter studies. We utilized reporter vectors in which the proximal region of IL-8 promoter including wild type or mutated NF-κB or AP-1 binding sites is driving the β-luciferase gene (kind gifts of Dr. A.R. Brasier, University of Texas Medical Branch, Galveston, TX) [21].

16HBE14o-AS3 cells were transiently co-transfected using FUGENE (Roche, Mannheim, Germany) with a reporter and a β-galactosidase expressing vector, utilized for normalization of transfection efficiency. The day after the transfection, the medium was replaced and 24 h later the cells were harvested. Luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

Experiments were performed in triplicate. Luciferase activity in insertless vector transfected cells was almost undetectable.

Transcription factors activity assays. DNA binding activity of NF-κB and AP1 was measured by multi-well colorimetric assays [22] in nuclear extracts using TransAM kits (Active Motif, Rixensart, Belgium) and Mercury Transfactor Profile kit (Clontech, Mountain View, California), respectively, according to the manufacturer's instructions.

Statistical analysis. Statistical calculations and tests were performed using Student's t test. The limit of statistical significance was defined as $p \leqslant 0.05$. Data were expressed as means and standard deviation (SD).

Results

Regulation of IL-8 mRNA

We analyzed the basal levels of IL-8 mRNA in the 16HBE14o-AS3 cell line with CF phenotype and in the isogenic 16HBE14o-S1 non-CF cells in order to assess the specific constitutive pro-inflammatory state of CF airway epithelial cells.

CF cells express higher levels of IL-8 mRNA than isogenic non-CF cells ($n = 7$, $p = 0.024$) (Fig. 1A). We then investigated whether AZM was able to reduce this differential expression. In order to address this question 16HBE14o-AS3 were incubated in the presence of AZM for 24 h at sub-inhibitory concentrations [23].

We found that 8 μg/ml AZM specifically reduced the IL-8 mRNA to those detected in the 16HBE14o-S1 non-CF cell line, almost abolishing the differential expression observed between the isogenic cells ($n = 9$, $p = 0.02$) (Fig. 1A); lower concentrations reduced the levels of IL-8 transcript without statistically significant effects (not shown). After 6 h of incubation with AZM we detected 20% of inhibition of IL-8 mRNA expression (not shown) which was not statistically significant. The basal IL-8 expression in non-CF cells is not significantly affected by the macrolide (data not shown). In order to assess the specificity of the effect of AZM we utilized another macrolide, JM, which has been described to be lacking clinical anti-inflammatory effects. This molecule did not significantly regulate expression of IL-8 mRNA ($n = 4$, $p = 0.07$) (Fig. 1A).

In order to establish whether IL-8 was a specific target of AZM, the expression of another pro-inflammatory molecule was analyzed. We found that ICAM-1 mRNA expression was not significantly affected by AZM ($n = 6$, $p = 0.45$) nor was it significantly different between CF and non-CF isogenic cells ($n = 5$, $p = 0.11$) (Fig. 1B).

Table 1
Real-time qPCR primer sequences

Primer	Sequence (5′–3′)	Accession No.	nM
IL 8-F	GACCACACTGCGCCAACA	AF385628.2	15
IL 8-R	GCTCTCTTCCATCAGAAAGTTACATAATTT		15
ICAM-1-F	TATGGCAACGACTCCTTCTCG	NM_000201	15
ICAM-1-R	CTCTGCGGTCACACTGACTGA		15
GAPDH-F	GTGGAGTCCACTGGCGTCTT	J04038	2.5
GAPDH-R	GCAAATGAGCCCAGCCTTC		15

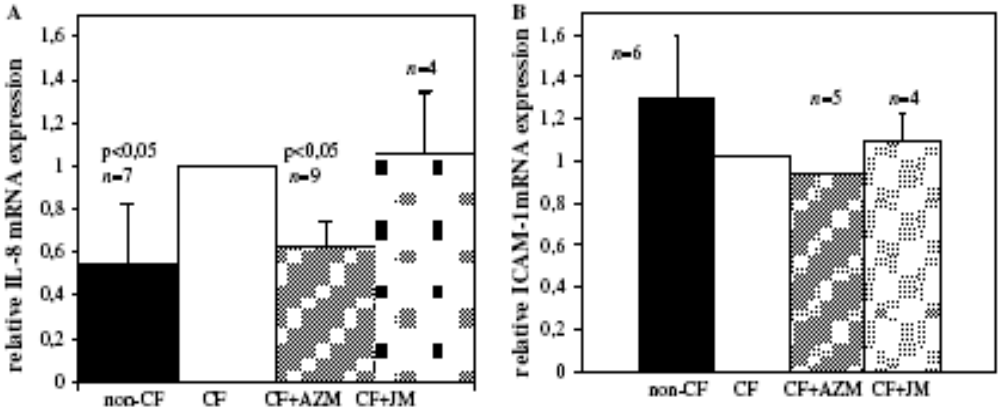

Fig. 1. Effects of AZM on IL-8 and ICAM-1 mRNA levels. Constitutive expression of IL-8 (A) and ICAM-1 mRNA (B) in 16HBE14o-S1 (non-CF) cells and isogenic 16HBE14o-AS3 cells (CF) cells untreated and after treatments with AZM or JM for 24 h. Total RNA was extracted and retrotranscribed. The values of transcripts levels are based on real-time qPCR analysis as described in Materials and methods. The values represent expression levels relative to untreated 16HBE14o-AS3 (means ± SD). Experiments were performed at least four times.

Regulation of IL-8 secretion

We analyzed the secretion of IL-8 in the same experimental conditions described above. The protein levels of IL-8 were also specifically decreased of about 50% in CF cells in the presence of AZM at 8 μg/ml for 24 h ($n = 4$, $p = 0.00011$), approximately to the levels constitutively detected in the isogenic non-CF cell line16HBE14o-S1 ($n = 3$, $p = 0.01$) (Fig. 2). Lower concentrations of AZM did not exert statistically significant effects (not shown). The basal IL-8 expression in non-CF cells is not significantly affected by the macrolide (data not shown). JM did not significantly affect IL-8 secretion ($n = 4$, $p = 0.88$) (Fig. 2).

Regulation of transcription factors activity

We investigated the role of NF-κB and AP-1 in the regulation of IL-8 promoter transcriptional activity in 16HBE14o-AS3 cells utilizing reporter vectors including wild type or mutated NF-κB or AP-1 binding sites in the proximal region of IL-8 promoter.

In the presence of mutated NF-κB or AP-1 binding sites the transcriptional activity of the proximal region of IL-8 promoter was strongly inhibited in 16HBE14o-AS3 cells ($n = 4$, $p = 8.54 \times 10^{-7}$ and $p = 6.45 \times 10^{-6}$ respectively) (Fig. 3). These results indicate that both TFs play important roles in the regulation of IL-8 promoter transcriptional activity in our CF model.

Since we have found that AZM decreases IL-8 mRNA and protein expression we addressed the question whether this macrolide affects the activity of these transcription factors in 16HBE14o-AS3 cells. We determined the DNA

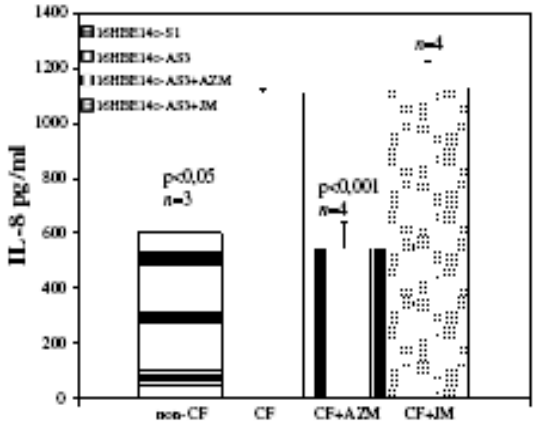

Fig. 2. Regulation of IL-8 secretion by AZM. IL-8 protein levels were detected in supernatants of cultures in the same conditions described in Fig. 1. Analysis was performed by ELISA as described in Materials and methods. The values correspond to means ± SD. Experiments were performed at least four times.

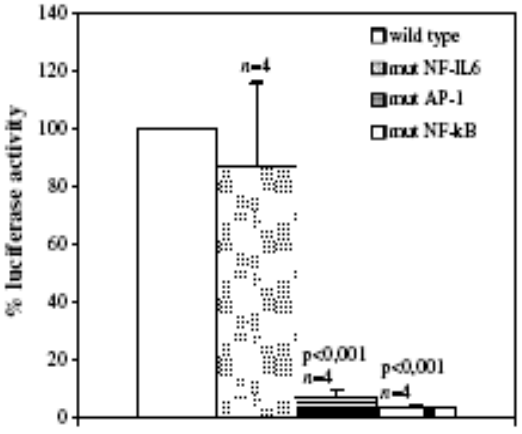

Fig. 3. Relevance of NF-κB and AP-1 binding for transcriptional activity of IL-8 promoter. The activity of IL-8 wild type promoter was compared to that detected in the presence of mutated NF-κB, AP-1 or NF-IL6 binding sites. Luciferase activity normalized to β-galactosidase was detected by gene reported studies performed as described in Materials and methods. Values (means ± SD) on the y axis are expressed as percentages of the normalized activity measured in 16HBE14o-AS3 cells transfected with the wild-type gene reporter construct. Experiments were performed four times.

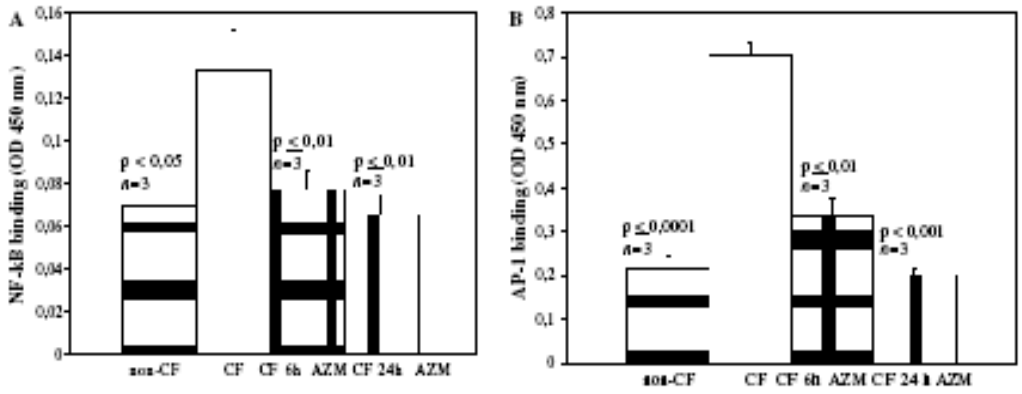

Fig. 4. Effects of AZM on DNA binding activity of NF-κB and AP-1. DNA binding activity of NF-κB p65 (A) and AP-1 c-jun (B) was measured as reported in Materials and methods in 16HBE14o-S1 (non-CF, column with horizontal lines) cells and isogenic 16HBE14o-AS3 (CF) cells untreated (white column) and after treatments with AZM (column with squares) or JM (column with vertical lines) for 24 h. Values on they axis are expressed as means ± SD obtained by three experiments.

binding activity of the NF-κB p65 subunit and of the AP-1 c-jun subunit following treatment of CF cells with AZM for 24 h. AZM reduced NF-κB DNA binding of 50% ($n = 3$, $p = 0.01$) (Fig. 4A) and AP-1 DNA binding of 70% ($n = 3$, $p = 0.0017$) (Fig. 4B) in 16HBE14o-AS3, almost neutralizing these TFs' differential DNA binding between CF and non-CF cells ($n = 3$, $p = 0.02$ and $p = 0.03$ respectively for NF-κB and AP-1) (Fig. 4). Similar results for both TFs were obtained following 6 h of treatment with AZM (Fig. 4). JM did not regulate DNA binding activity of these TF (not shown), suggesting a specific ability of the macrolide AZM to regulate the activity of NF-κB and AP-1. FosB DNA binding activity was not significantly affected by AZM (not shown), indicating that NF-κB and AP-1 are specific targets of AZM.

Discussion

Novel strategies for treatment of lung inflammation are required since no satisfactory anti-inflammatory treatment is available at present for clinical use in CF [24].

Accumulation of mistrafficked CFTR, the Cl^- channel mutated in CF, as well as defective glutathione transport, leading to exaggerated oxidative stress, have been proposed as possible mechanisms of NF-κB activation in CF [25] determining over-expression of IL-8.

The clinical efficacy of AZM is widely accepted [1]. Non-bactericidal effects relevant for therapy of CF have been suggested for AZM [2–6].

Anti-inflammatory activity of AZM seems to have been less investigated than those of 14-membered macrolides such as erythromycin and clarithromycin [26]. In mice AZM reduced the levels of keratinocyte-derived chemokine and blocked the neutrophils' recruitment in Pa endobronchial infection [27]. Airways of CF patients are characterized by both chronic inflammation and Pa colonization and therefore long-term AZM therapy could be promising for limiting the severe lung damage in CF.

In this study we investigated the ability of this macrolide to reduce the expression of the pro-inflammatory chemokine IL-8 in CF airway epithelial cells and regulation of TFs as possible mechanisms of anti-inflammatory effects. We observed a constitutively higher expression of IL-8 as well as NF-κB and AP-1 activation in 16HBE14o-AS3 compared with isogenic non-CF cells.

Our results are consistent with higher activation of NF-κB in CF versus non-CF cells previously described [7,28–32].

The observation that AP-1 DNA binding is increased in CF cells compared with non-CF cells suggests another mechanism to explain a constitutive pro-inflammatory state in CF bronchial epithelial cells.

IL-8 differential expression and secretion between CF and non-CF bronchial epithelial cells could represent relevant therapeutic targets for AZM in CF airways as in this work they are almost abolished by treatment with this macrolide but are unaffected by JM. Instead, ICAM-1 mRNA expression was not significantly affected by both macrolides indicating that we have identified a specific effect.

Among CF patients high variability in the response to the drug has been reported and genetic factors have been investigated in this regard [33]. Contradictory results about the effects of macrolides on inflammation have been reported by several authors and could be due to the choice of the cell model. Our experimental model has been appropriate for reproducing anti-inflammatory effects of AZM described *in vivo* [27,34]. On the contrary in primary airway cell cultures pro-inflammatory effects have been described [35].

AZM is receiving increasing interest for its therapeutic benefits in the treatment of CF. Equi et al. reported promising clinical effects without significant changes of IL-8 and neutrophil elastase levels in sputa of CF patients following treatment with AZM [36]. However our results are consistent with the reduction of IL-8 release described after lung transplantation, supporting anti-inflammatory properties of this macrolide [37–40].

The molecular basis of this effect has been more thoroughly investigated. Since we found that AZM decreases IL-8 mRNA and protein expression we investigated the relevance of both transcription factors in the regulation of IL-8 transcription. The finding that in the presence of mutated NF-κB or AP-1 binding sites the transcriptional activity of the proximal region of IL-8 promoter is strongly inhibited indicates that NF-κB and AP-1 play important roles in regulating IL-8 transcription in CF airway epithelial cells. Furthermore we determined the capability of AZM in inhibiting the activity of both TFs suggesting a wide pattern of possible other targets of this macrolide. In fact, NF-κB and AP-1 regulate the expression of a large variety of genes involved in the inflammatory process [17,41].

This study shows the ability of AZM to regulate NF-κB and AP-1 activity providing possible mechanisms of inhibition of IL-8 expression in CF cells. Our results suggest novel mechanisms for therapeutic anti-inflammatory effects of AZM in CF, improving the rationale for its use for therapy.

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Azithromycin Selectively Reduces Tumor Necrosis Factor Alpha Levels in Cystic Fibrosis Airway Epithelial Cells[▽]

Cristina Cigana, Baroukh Maurice Assael, and Paola Melotti*

Cystic Fibrosis Center, Azienda Ospedaliera di Verona, Verona, Italy

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Azithromycin (AZM) ameliorates lung function in cystic fibrosis (CF) patients. This macrolide has been suggested to have anti-inflammatory properties as well as other effects potentially relevant for therapy of CF. In this study, we utilized three CF (IB3-1, 16HBE14o- AS3, and 2CFSMEo-) and two isogenic non-CF (C38 and 16HBE14o- S1) airway epithelial cell lines to investigate whether AZM could reduce tumor necrosis factor alpha (TNF-α) mRNA and protein levels by real-time quantitative PCR analysis and an enzyme-linked immunosorbent assay (ELISA), respectively. We studied the effects on the DNA binding of NF-κB and specificity protein 1 (Sp1) by an ELISA. Non-CF cells express significantly lower TNF-α mRNA and protein levels than an isogenic CF cell line. In CF cells, AZM treatment causes a 30% reduction of TNF-α mRNA levels ($P < 0.05$) and a 45% decrease in TNF-α secretion ($P < 0.05$), reaching approximately the levels of the untreated isogenic non-CF cells. In CF cells, NF-κB and Sp1 DNA binding activities were also significantly decreased (about 45 and 60%, respectively; $P < 0.05$) after AZM treatment. Josamycin, a macrolide lacking clinically described anti-inflammatory effects, was ineffective. Finally, AZM did not alter the mRNA expression levels of interleukin-6, a proinflammatory molecule not differentially expressed in CF and isogenic non-CF cells. The results of our study support the anti-inflammatory activities of this macrolide, since we show that AZM reduced the levels of TNF-α and propose inhibitions of NF-κB and Sp1 DNA binding as possible mechanisms of this effect.

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene encoding a transmembrane chloride channel.

Neutrophil-dominated inflammation is a hallmark of the airway disease in CF. Uncontrolled release of neutrophilic cytotoxic contents leads to mucus hypersecretion and progressive lung damage, ultimately contributing to morbidity and mortality in CF patients.

It remains controversial as to whether there is an intrinsic hyperinflammatory state arising directly from a lack of a functional CFTR (10, 24). The hypothesis of primary inflammation preceding detectable infection is based first on clinical observations of inflammation in CF neonates and young children (25, 34) but also on experimental reports (12, 40). Other evidences suggest that CF airway inflammatory response to infectious agents is exaggerated and/or prolonged (33). CF patients have been shown to exhibit larger amounts of neutrophils and interleukin-8 (IL-8) in bronchoalveolar lavage fluid than non-CF subjects in response to similar levels of infection (28). Furthermore, CF airway epithelial cell lines produced larger quantities of IL-8 than CFTR-corrected cells in response to IL-1β and tumor necrosis factor alpha (TNF-α) (1, 43) and to bacterial stimulation (16, 43).

Clinical studies have shown that macrolides have beneficial effects on lung function in CF patients (18, 36). CF patients treated with azithromycin (AZM) experienced improvement in the viscoelasticity of the sputum (4), decreased content of mucoid *Pseudomonas aeruginosa* in sputum samples (19), a decline in the number of pulmonary exacerbations (30, 35), and significant increases in respiratory function parameters such as FEV1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) (19, 30, 35).

High concentrations of TNF-α (8, 43), a proinflammatory cytokine able to induce the production of secondary mediators by epithelial cells, including cytokines (e.g., IL-8), have been observed in CF airway fluids.

The aim of this work was to evaluate whether AZM could reduce TNF-α levels in CF cells and whether CF cells express larger amounts of this cytokine than non-CF cells. Finally, we investigated the molecular mechanisms of AZM effects on these cells by studying the transcriptional activities of NF-κB and specificity protein 1 (Sp1) transcription factors before and after incubation with AZM, as both of these proteins are involved in the regulation of TNF-α gene expression.

MATERIALS AND METHODS

Cell cultures. IB3-1 and isogenic C38 cells, with CF and non-CF phenotypes, respectively, obtained from P. Zeitlin (Johns Hopkins University, MD) (46), were grown in LHC-8 medium (Biosource, Rockville, MD) supplemented with 5% fetal bovine serum (FBS) (Cambrex Bio Science, Verviers, Belgium). IB3-1 cells were derived from the bronchial epithelium of a CF patient, and the isogenic rescued C38 cell lines express a plasmid-encoded functional CFTR (46).

16HBE14o- AS3 and the isogenic 16HBE14o- S1 cell lines, with CF and non-CF phenotypes, respectively (32), obtained from P. Davis (Case Western Reserve University, OH), were grown in Eagle's minimal essential medium (Cambrex Bio Science) supplemented with 10% FBS, 1% L-glutamine (Cambrex Bio Science), and 0.4% G418 sulfate (Calbiochem, CN Biosciences, La Jolla, CA). 16HBE14o- AS3 cells lack CFTR expression following transfection with an antisense CFTR sequence, while isogenic 16HBE14o- S1 cells transfected with a sense CFTR sequence express wild-type CFTR (32).

The CF cell line 2CFSMEo-, a kind gift of D. Gruenert (University of California) (13), was derived from submucosal tracheobronchial glands of a CF patient and grown in Eagle's minimal essential medium supplemented with 10% FBS and 1% L-glutamine.

Epithelial respiratory cell lines were cultured at 37°C in a humidified atmo-

* Corresponding author. Mailing address: Cystic Fibrosis Center-Azienda Ospedaliera di Verona, piazzale Stefani, 1-37126 Verona, Italy. Phone: 39 045 8123419. Fax: 39 045 8122042. E-mail: paola.melotti@azosp.vr.it.

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TABLE 1. Primer sequences utilized in the quantitative PCR analysis

Gene	Primer*	Sequence (5′→3′)	Accession no.	Primer concn (nM)
TNF-α gene	FW	GGACCTCTCTCTAATCAGCCCTC	NM_000594	25
	RV	TCGAGAAGATGATCTGACTGCC		25
IL-6 gene	FW	CGGTACATCCTCGACGGC	NM_000600	25
	RV	CTTGTTACATGTCTCCTTTCTCAGG		450
GAPDH gene	FW	GTGGAGTCCACTGGCGTCTT	J04038	25
	RV	GCAAATGAGCCCAGCCTTC		150

* FW, forward; RW, reverse.

sphere with 5% CO_2. Cells were seeded in a concentration of 1.5×10^5 cells/cm^2, and after 24 h, they were exposed to up to 8 μg/ml AZM (Pfizer, Italy) and/or josamycin (JM; Yamanouchi Pharmaceutical, Japan) for 24 h. These concentrations, which are in the sub-MIC range for *P. aeruginosa*, are consistent with those observed in lungs of patients treated with AZM (17, 23).

In the presence of 8 μg/ml AZM for 24 h, cell viability was >95%, as determined by a trypan blue exclusion test, while at higher concentrations starting from 16 μg/ml, viability was <95%.

Serial dilutions (0, 0.125, 0.5, 2, and 8 μg/ml) of the macrolides were utilized in dose-response experiments. We observed no statistically significant effects on TNF-α expression after treatment with AZM concentrations lower than 8 μg/ml (data not shown).

RNA isolation, quantification, and reverse transcription. Cells were lysed. Total RNA was extracted with a total RNA isolation kit (Roche, Germany) and converted to cDNA using a high-capacity cDNA archive kit (Applied Biosystems). The reaction mixture was then incubated at 25°C for 10 min and at 37°C for 2 h.

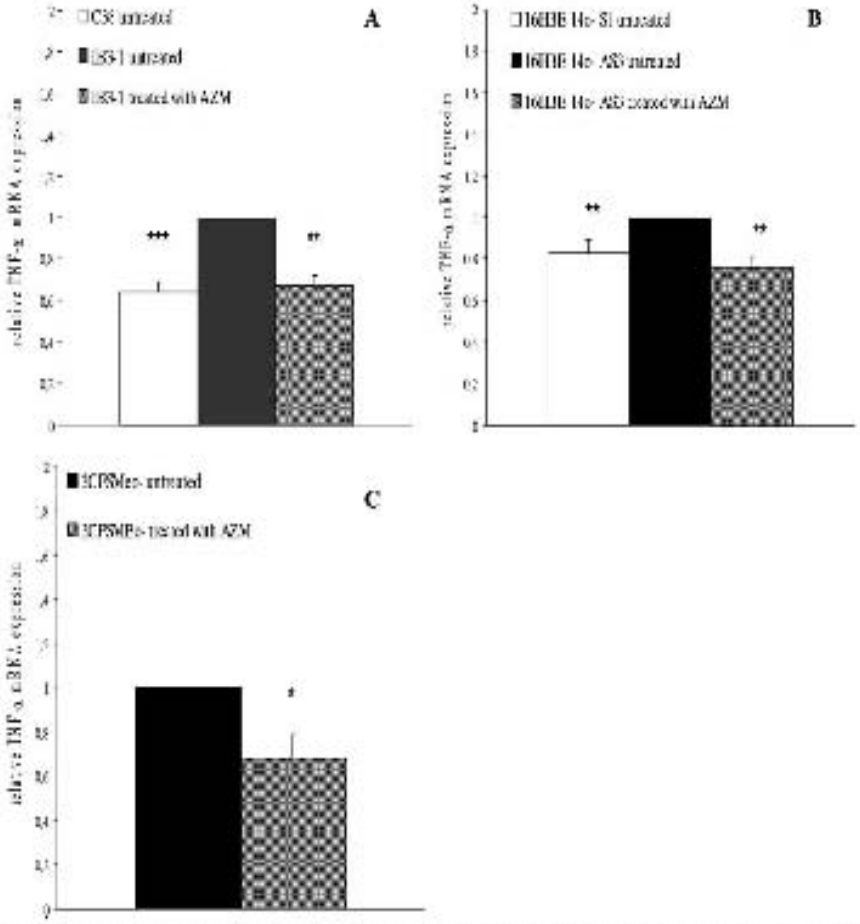

FIG. 1. TNF-α mRNA expression. Expression of TNF-α mRNA in (A) non-CF C38 cells and an isogenic CF IB3-1 cell line, (B) non-CF 16HBE14o- S1 cells and an isogenic CF 16HBE14o- AS3 cell line, and (C) 2CFSMEo- cells at a constitutive level and after treatment with AZM. Total RNA was extracted and retrotranscribed. The values of TNF-α mRNA are based on real-time PCR analysis. The values represent the expression levels relative to those of untreated (A) IB3-1, (B) 16HBE14o- AS3, and (C) 2CFSMEo- cells (means ± SD). The experiment was repeated five times. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

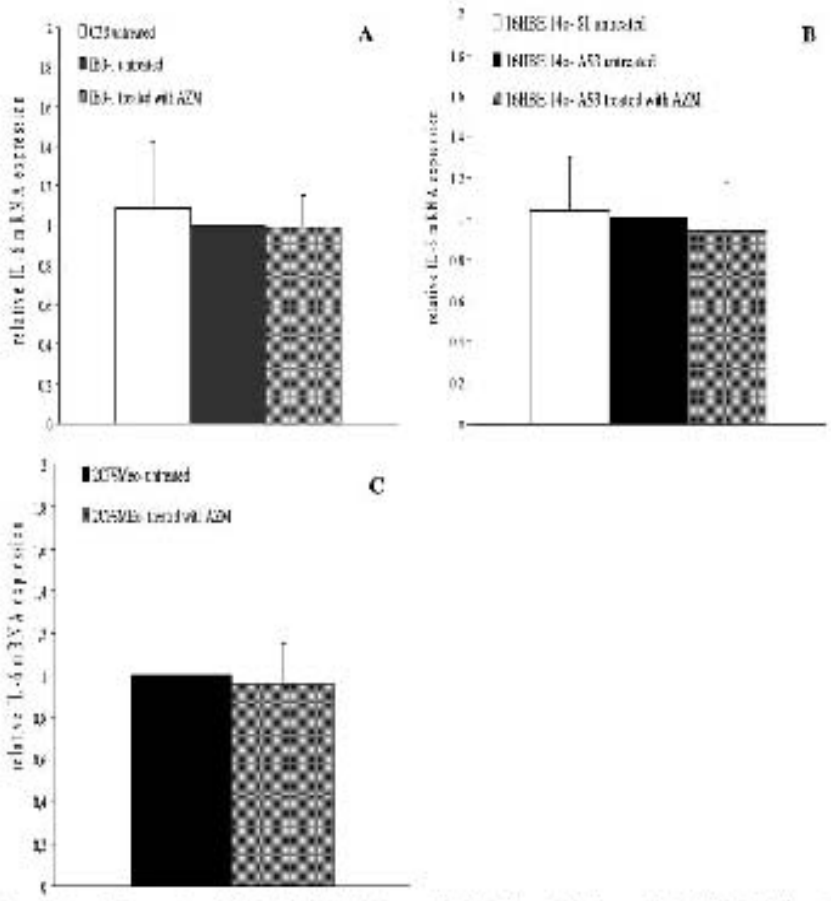

FIG. 2. IL-6 mRNA expression. Expression of IL-6 mRNA in (A) non-CF C38 cells and an isogenic CF IB3-1 cell line, (B) non-CF 16HBE14o- S1 cells and an isogenic CF 16HBE14o- AS3 cell line, and (C) 2CFSMEo- cells at a constitutive level and after treatment with AZM. Total RNA was extracted and retrotranscribed. The values of IL-6 mRNA are based on real-time PCR analysis. The values represent the expression levels relative to those of untreated (A) IB3-1, (B) 16HBE14o- AS3, and (C) 2CFSMEo- cells (means ± SD). The experiment was repeated five times.

RNA quantification. Relative quantification of gene expression was performed by real-time quantitative PCR analysis as described by the manufacturer (Applied Biosystems User Bulletin 2). The cDNA (2 μl) was amplified using Platinum SYBR green quantitative PCR supermix-UDG (Invitrogen, Grand Island, NY) in the ABI Prism 5700 sequence detection system. Primer Express Software (Applied Biosystems) was used to select the specific primers. The primer sets (Sigma-Genosys, St. Louis, MO) are shown in Table 1. The PCR thermal protocol consisted of 2 min at 50°C, a denaturation step at 95°C for 2 min, followed by 50 cycles of a 15-s 95°C denaturation step, and a 30-s annealing/extension step at 60°C. The real-time PCRs were performed in duplicate for both target and normalizer genes. Changes in mRNA expression level were calculated following normalization to GAPDH (glyceraldehyde-3-phosphate). The degree of variation of the target gene/normalizer gene ratios in the experiments was less than 8% in each cell line. Results were expressed as means ± standard deviations (SD).

Protein quantification. TNF-α secretion in supernatants from the cell cultures described above was determined by an enzyme-amplified sensitivity immunoassay using a TNF-α EASIA kit (Bender MedSystems, Austria) according to the manufacturer's instructions. The limit of detection was 3.83 pg/ml. Measurements were performed at least in duplicate. Values were normalized to 10^6 cells; results were expressed as means ± SD.

DNA binding activity studies. DNA binding activities of NF-κB and Sp1 in nuclear extracts were measured using TransAM kits (Active Motif, Belgium) and a Mercury TransFactor profiling kit (Clontech), respectively, according to the manufacturers' instructions. Measurements were performed at least in duplicate; results were expressed as means ± SD.

Statistical analysis. Statistical calculations and tests were performed using the Friedman test for comparison between non-CF and CF cells and the Mann-Whitney U test for the evaluation of treatments with macrolides of the CF cell line.

The limit of statistical significance was defined as a P value of ≤ 0.05. All data were expressed as means ± SD.

RESULTS

Regulation of TNF-α mRNA expression by AZM treatment. In this study, first of all, we measured the expression levels of the TNF-α gene. All cell lines constitutively expressed TNF-α mRNA; however, the level of basal expression in CF cells was significantly higher than in isogenic non-CF cells (Fig. 1). We confirmed this differential TNF-α expression by using cells at different passages as well as after 96 h from sedimentation (data not shown). Following exposure of CF cell lines to 8 μg of AZM for 24 h, we found that this macrolide reduced TNF-α mRNA levels of about 35% and 25% in IB3-1 and 16HBE14o- AS3 cells, respectively ($n = 5$; $P < 0.01$) (Fig. 1A and B), approximately to the levels of untreated isogenic non-CF cells C38 and 16HBE14o- S1, respectively. A 30% reduction of TNF-α mRNA levels was detected in 2CFSMEo- cells after AZM treatment ($n = 5$; $P < 0.05$) (Fig. 1C). The macrolide JM, known to lack clinical anti-inflammatory properties, had

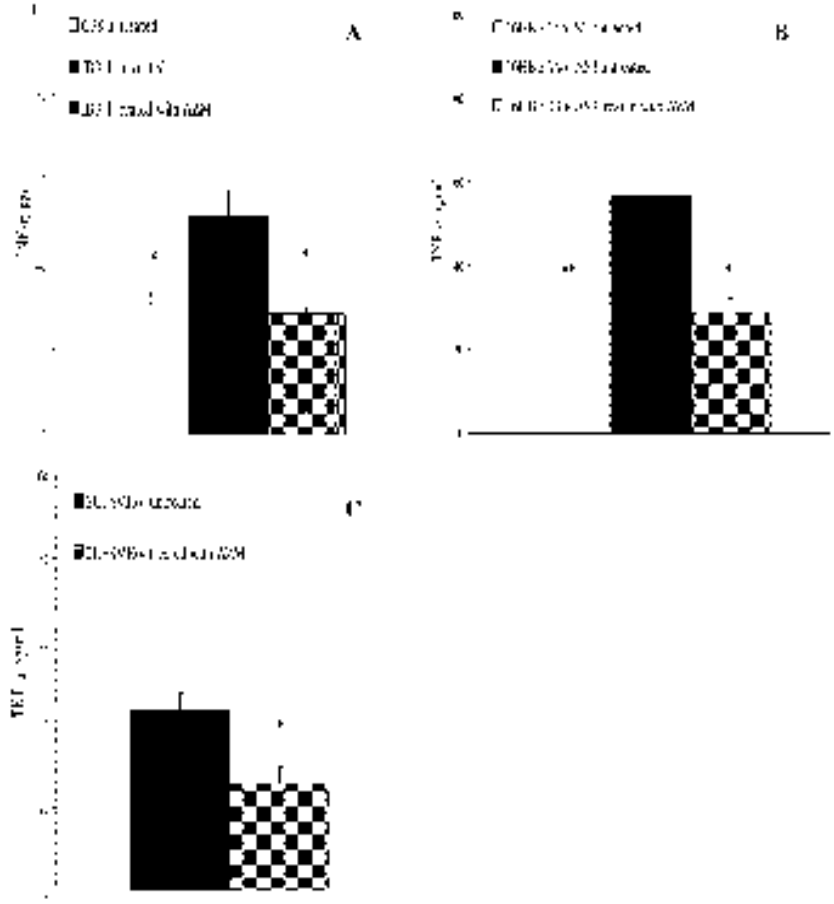

FIG. 3. TNF-α protein release. Secretion of TNF-α in (A) non-CF C38 cells and an isogenic CF IB3-1 cell line, (B) non-CF 16HBE14o- S1 cells and an isogenic CF 16HBE14o- AS3 cell line, and (C) 2CFSMEo- cells at a constitutive level and after treatment with AZM and JM. TNF-α protein levels were measured by a commercial enzyme-linked immunosorbent assay kit. The values represent the secretion levels relative to those of untreated (A) IB3-1, (B) 16HBE14o- AS3, and (C) 2CFSMEo- cells (means ± SD). The experiment was repeated three times. *, $P < 0.05$; **, $P < 0.01$.

no significant effects on TNF-α mRNA expression in all cell lines (data not shown).

Effects of AZM treatment on IL-6 mRNA expression. In terms of IL-6 mRNA expression, we found no statistically significant differences between CF cell lines and isogenic non-CF cells (Fig. 2). We then exposed CF cell lines to 8 μg of AZM for 24 h. AZM had no statistically significant effects on IL-6 mRNA expression in IB3-1, 16HBE14o- AS3, and 2CFSMEo- cells (Fig. 2A, B, and C, respectively).

Regulation of TNF-α protein levels by AZM treatment. We confirmed the higher TNF-α expression in CF cells than in isogenic non-CF cells at the protein level (Fig. 3). Treatment with 8 μg of AZM was effective in reducing TNF-α protein levels of 45% and 50% in IB3-1 and 16HBE14o- AS3 cells treated for 24 h, respectively ($n = 3$; $P < 0.05$) (Fig. 3A and B), to the levels of untreated isogenic non-CF cells. Furthermore AZM reduced TNF-α protein expression of 40% in 2CFSMEo- cells ($n = 3$; $P < 0.05$) (Fig. 3C).

Effects of AZM on NF-κB DNA binding activity. We found that IB3-1 and 16HBE14o- AS3 cells showed twofold-higher constitutive NF-κB DNA binding levels than isogenic non-CF cell lines C38 and 16HBE14o- S1 ($n = 3$; $P < 0.01$ and $P < 0.05$, respectively) (Fig. 4A). Following exposure of CF cell lines to 8 μg/ml AZM for 24 h, NF-κB DNA binding activity in IB3-1 and 16HBE14o- AS3 cells was reduced from 40% and 45%, respectively, nearly to the levels of untreated C38 and 16HBE14o- S1 cells. A 45% reduction of NF-κB DNA binding activity was detected in 2CFSMEo- cells after AZM treatment ($n = 3$; $P < 0.05$) (Fig. 4B). Furthermore, JM had no effects on NF-κB DNA binding activity (data not shown).

Effects of AZM on Sp1 DNA binding activity. We decided to evaluate whether AZM could affect the levels of Sp1 DNA binding. We did not detect statistically significant differences in the constitutive Sp1 DNA binding levels in IB3-1 and 16HBE14o- AS3 cells versus those of isogenic non-CF cell lines C38 and 16HBE14o- S1, respectively, as shown in Fig. 5A. After 24 h of treatment with 8 μg of AZM, 60%, 64%, and 65% Sp1 DNA binding activity reductions were detected in IB3-1, 16HBE14o- AS3, and 2CFSMEo- cells, respectively ($n = 3$; $P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) (Fig. 5B). JM was also not effective on Sp1 DNA binding activity (data not shown).

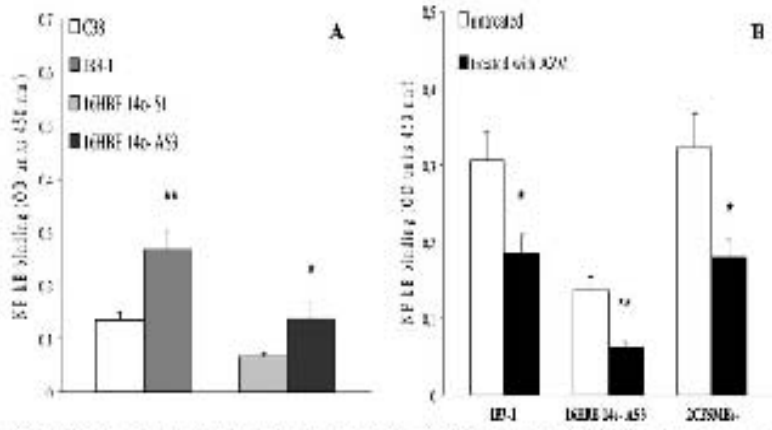

FIG. 4. DNA binding of NF-κB. (A) Constitutive binding to the DNA of NF-κB in non-CF C38 cells and an isogenic CF IB3-1 cell line and in non-CF 16HBE14o- S1 cells and an isogenic CF 16HBE14o- AS3 cell line. (B) Effect of the treatment with AZM on the DNA binding of NF-κB in CF cells (IB3-1, 16HBE14o- AS3, and 2CFSMEo-). NF-κB DNA binding activity was analyzed using a commercial kit following the manufacturer's instructions. The experiment was repeated three times (means ± SD). *, $P < 0.05$; **, $P < 0.01$. OD, optical density.

DISCUSSION

Defective expression or function of the CFTR channel in airway epithelial cells leads to persistent and overwhelming infection and inflammation. Several studies indicate that inflammation occurs very early in the lungs of CF patients and often seems to precede clear signs of infection (25, 34, 40). Moreover, CF airway inflammation may manifest as disproportionately increased or prolonged in relation to the level of stimuli (1, 16, 28, 43). However, whether a dysregulation of inflammation exists in CF patients is debated (2, 15).

Data from literature regarding AZM effects on TNF-α expression are scarce and contradictory. Although AZM has been reported to inhibit TNF-α expression both in vivo in animal models and in vitro (20, 21), in healthy human subjects serum TNF-α protein concentration was unaffected by a 24-h-long treatment with AZM (14). In this regard, the experimental model seems to be critical and, in particular, differences between CF and non-CF models are relevant.

In this work, we first aimed to establish whether differential expression of a relevant inflammatory marker such as TNF-α could be detected in our CF cell models since it plays a relevant role in the pathogenesis of CF. We showed not only that its constitutive expression was significantly higher in CF cells than in isogenic non-CF cells at both mRNA and protein levels but also that this finding was not dependent on cells' passage or period from sedimentation. Moreover, we found no differential IL-6 mRNA expression between CF and non-CF cell lines. We determined that exposition to lipopolysaccharide derived from *P. aeruginosa* for 24 h induced higher expression of TNF-α mRNA in CF cells than in non-CF cells in our models (data not shown), confirming an exaggerated inflammatory response in CF cells in the presence as well in the absence of stimuli.

The association between increased inflammatory markers and CFTR mutations is, however, controversial. Aldallal et al. (1) found higher IL-8 expression in CF cells than in non-CF cells in cell line models but not in primary cultures, revealing a

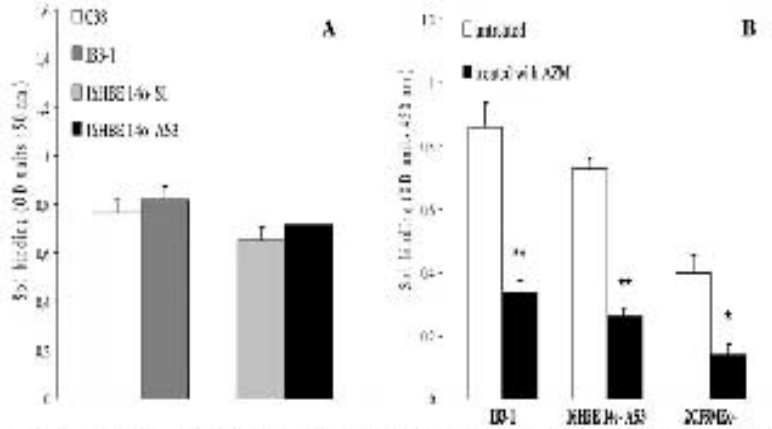

FIG. 5. DNA binding of Sp1. (A) Constitutive binding to the DNA of Sp1 in non-CF C38 cells and an isogenic CF IB3-1 cell line and in non-CF 16HBE14o- S1 cells and an isogenic CF 16HBE14o- AS3 cell line. (B) Effect of the treatment with AZM on the DNA binding of Sp1 in CF cells (IB3-1, 16HBE14o- AS3, and 2CFSMEo-). Sp1 DNA binding activity was analyzed using a commercial kit following the manufacturer's instructions. The experiment was repeated three times (means ± SD). *, $P < 0.05$; **, $P < 0.01$. OD, optical density.

considerable variability in airway epithelial cell inflammation among different individuals and cell models. Becker et al. (5) found no differential IL-8 and IL-6 expression between CF and non-CF cell lines and primary cultures, respectively. These contradictory results could be due not only to the choice of the cell model and its origin but also to different culture conditions. Our experimental model consists of several human CF cell lines derived from different airway cell types, and two of them have been compared to their isogenic non-CF cell lines. This experimental model was appropriate for reproducing the anti-inflammatory effects of AZM described in vivo (41, 45).

Macrolide antibiotics are receiving increasing attention for their possible therapeutic benefits in the treatment of CF. AZM was chosen over other macrolides because of its ease of administration and its accumulation in sputum and tissues. Its plasma half-life is considerably longer than those of other macrolides. It also accumulates in alveolar macrophages, which could represent a delivery vehicle to transport it to affected sites. Finally, the results of several clinical trials (19, 30, 35) encourage clinicians to subject CF patients to long-term treatment with AZM, although certain heterogeneity in the response has been reported. However, the mechanisms of its efficacy are still unclear. Clinical beneficial effects of AZM might derive from the synergism of different effects, including inhibition of *P. aeruginosa* bacterial growth (29, 44), decreased expression of bacterial virulence factors (39, 44), modulation of the inflammatory response (41), ion transport (31), and tight junctions (3).

We found that AZM reduced TNF-α expression at both transcript and protein levels in all of our CF cell lines, bringing it to the levels of untreated isogenic non-CF cells. Conversely, IL-6 mRNA expression was not significantly affected by AZM treatment. As we found higher expression of TNF-α, but not of IL-6, in CF cells than in non-CF cells, we can speculate that AZM may be effective towards those proinflammatory molecules induced in the constitutive inflammation. The specificity of the results is warranted by the observation that JM, a macrolide known to lack clinical anti-inflammatory properties (38), was ineffective.

The possibility that AZM may act at the transcriptional level was tested by measuring the DNA binding activities of two transcription factors relevant in the regulation of the TNF-α gene, NF-κB (27) and Sp1 (42).

We found that in the presence of AZM, NF-κB DNA binding activity in CF cells was reduced approximately to the levels detected in isogenic non-CF cells. Also, Sp1 DNA binding was reduced following treatment with AZM, while the activities of this transcription factor were not significantly different in CF and non-CF cells. Once again, the inhibitory effect was peculiar to AZM, as JM had no effect on NF-κB and Sp1 DNA binding activities. Assays of transcription factors binding to DNA do not rule out an effect on their activation. This approach has been utilized in order to establish whether NF-κB and Sp1 could be considered targets of AZM potentially involved in the regulation of TNF-α transcription by this macrolide. We are focusing on the ability of AZM to affect the activation of NF-κB, Sp1, and AP-1 at different levels (11, 12).

Increased NF-κB activation in CF versus isogenic non-CF specimens was observed in several studies, both in the absence and in the presence of stimulation (9, 16, 22, 43) in different experimental models. Furthermore, our results are consistent with previous studies describing higher NF-κB activation in CF cells than in non-CF cells in a cellular model utilized in this study (16, 43).

Furthermore, it is of note that therapeutic inhibition of NF-κB has been proposed for the treatment of inflammatory and immune diseases (7, 37). Decreased levels of TNF-α and IL-8, two NF-κB-regulated genes, could reduce the recruitment of neutrophils which are considered responsible for epithelial damage in CF airways (41).

Sp1 can functionally cooperate with NF-κB to elicit maximal promoter activation of inflammatory genes (26). Investigating the effects of AZM on Sp1 was considered relevant, as this transcription factor has been described to regulate several inflammatory genes, including the chemokine macrophage inflammatory protein-2, heparanase, and TNF-α (42), and therefore, its inhibition could influence inflammatory responses. This approach was considered appropriate for investigating a possible mechanism of regulation of TNF-α by AZM. Inhibition of Sp1 activity by AZM seems to be a novel effect of this macrolide.

At present, no satisfactory anti-inflammatory treatments are available for clinical use in CF because of limited efficacy and/or undesired effects (6, 24). Therefore, the identification of novel therapeutic targets is required to develop novel strategies for the treatment of lung inflammation in CF. Our results indicate that the antibiotic AZM has the features of an anti-inflammatory drug and that NF-κB and Sp1 transcription factors are relevant targets whose inhibition might contribute to ameliorate the excessive inflammatory response in CF.

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EFFECTS OF THE MACROLIDE AZITHROMYCIN ON THE EXPRESSION OF ATP BINDING CASSETTE TRANSPORTERS IN CYSTIC FIBROSIS AIRWAY EPITHELIAL CELLS

Cristina Cigana, Elena Nicolis, Matteo Pasetto, Baroukh Maurice Assael and Paola Melotti*
Cystic Fibrosis Center, Azienda Ospedaliera di Verona, Piazzale Stefani 1, 37126 Verona, Italy

*Corresponding author. Mailing address: Cystic Fibrosis Center - Azienda Ospedaliera di Verona, piazzale Stefani, 1 - 37126 Verona, Italy. Phone: +39 045 8123741. Fax: +39 045 8122042. E-mail: paolamelotti@azosp.vr.it

ABSTRACT

Induction of ATP Binding Cassette (ABC) proteins involved in the chloride transport has been proposed as possible mechanism of beneficial effects of azithromycin (AZM) in cystic fibrosis (CF) patients. This study focused on the effects of AZM on mRNA and protein expression of Multidrug Resistance-associated Protein 1 (MRP1) and Multidrug Resistance Protein 1 (MDR1) by real-time quantitative PCR, flow cytometry and gene reporter assays in two CF and two isogenic non-CF airway epithelial cell lines. We detected higher levels of MRP1 and lower levels of MDR1 mRNA in CF versus non-CF cells while both proteins were not differentially expressed. Following AZM treatment we found modest differences of MRP1 and MDR1 mRNA expression while protein levels were unaffected. The ability of AZM to regulate MRP1 promoter transcriptional activity was excluded by gene reporter assays. Our data do not support the hypothesis of induction of ABC transporters by AZM.

Keywords: cystic fibrosis; azithromycin; MRP1; MDR1; ABC.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene which encodes for a cAMP-regulated chloride channel.

Chronic airway inflammation and subsequent progressive lung damage characterise lung dysfunction, the major cause of death in CF disease. CF patients, harbouring the same mutations show, however, a wide range of disease severity. An explanation could come from the presence of endogenous and/or exogenous factors which may complement CFTR function (1). As CFTR belongs to the ATP Binding Cassette (ABC) transporter superfamily and members of the same family share related functions, a functional complementation of CFTR defect by other ABC proteins could be hypothesised (2). Several authors have demonstrated a complementary pattern of CFTR and the Multidrug Resistance Protein 1 (MDR1) expression, suggesting a co-ordinated regulation of these genes (3,4). Among other functions, MDR1 has been reported to play a role in the regulation of cell volume activated chloride channels and to have chloride channel activity itself (5,6). Moreover MDR1 is involved in regulation of an ATP channel (7), as well as CFTR (8).

Long-term treatment with the macrolide antibiotic azithromycin (AZM) has been reported to determine improvement of lung disease in CF patients (9-14), but the mechanisms of action of this antibiotic are still undefined. Lallemand et al reported improvement in lung function and clearance of *Pseudomonas aeruginosa* infection in a CF patient following chemotherapy for a fibrosarcoma (2). Because cancer chemotherapy can induce overexpression of MRP (Multidrug Resistance-associated Protein) and MDR, they hypothesised that CFTR could be complemented by these close ABC proteins. MDR and MRP mRNAs were detected in nasal epithelial cells of this patient but were absent in a CF patient never exposed to chemotherapics. Therefore a functional complementation by these ABC transporters could explain improvement in lung function in the reported case. Altschuler raised the hypothesis that AZM could improve lung function in colonised *P. aeruginosa* CF patients by inducing the expression of ABC transporters (15). This possibility was corroborated by Pradal et al who studied the effects of 4 weeks of treatment with AZM in CF patients showing an improvement of chloride transport associated to increased MRP1 mRNA expression (16). MRP1 shares a close structural homology with CFTR (17). MRP1 is involved in the regulation of chloride transport (18) and in the extrusion of glutathione-S conjugates (GSH). GSH amount is decreased in the plasma and bronchoalveolar lavage fluid from CF patients

and in the apical fluid from CF airway epithelial cells (19,20). Low levels of GSH may play a critical role in the pathogenesis of CF by increasing the susceptibility of the airway to oxidative damage during chronic inflammation. Furthermore cellular GSH deficiency is related directly to activation of Nuclear Factor kB pathway (21,22), which has been already reported to be exaggerated in CF (23).

In this study we aimed to investigate the expression of ABC members in CF cell lines and their regulation by AZM as a possible mechanism of complementation of the CFTR defect in CF.

MATERIALS AND METHODS

Cell cultures. The parental IB3-1 cell line from the bronchial epithelium of a CF patient and the isogenic non-CF C38 cell line were a kind gift from Pamela Zeitlin (Johns Hopkins University, Baltimore, MD, USA); the non-CF isogenic cell line S9 was purchased from LGC Promochem (Teddington, UK) (24,25). Cells were grown in LHC-8 media (Biosource, Camarillo, CA, USA) supplemented with 5% foetal bovine serum (FBS) (Cambrex Bio Science, Verviers, Belgium). The epithelial respiratory cell line 2CFSMEo- was obtained from D. Gruenert (University of California, San Francisco, CA, USA) (26), and grown in Eagle's MEM (Cambrex) supplemented with 10% FBS and 1% L-glutammine (Cambrex). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2. 24 h after sedimentation, cells were exposed to 8 μg/ml AZM (Pfizer, Rome, Italy) for 1, 3, 7, 14 and 28 days. This concentration is consistent with those described in lungs of patients treated with AZM (27). Lower concentrations were utilised obtaining similar results by flow-cytometry. Moreover, in the presence of AZM at 8 μg/ml the cell viability was >95% as determined by Tripan Blue exclusion test while at higher concentrations starting from 16 μg/ml the viability was <95%.

Real-time qPCR. Total RNA was extracted with the Total RNA Isolation kit (Roche, Mannheim, Germany) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) and random primers, following the manufacturers instructions.

The cDNA was amplified using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Grand Island, NY, USA). Primer sets (Sigma-Genosys, St. Louis, MO, USA) are shown in Table 1. The PCR thermal protocol consisted of 2 min at 50°C, a denaturation step at 95°C for 2 min followed by 50 cycles of a 15-s 95°C denaturation step and a 30-s annealing/extension step at 60°C. The real-time qPCR reactions were performed in duplicates for both target and normaliser gene.

Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer's User Bulletin 2 (Applied Biosystems). Changes in mRNA expression level were calculated following normalisation to GAPDH. The results obtained following normalisation were expressed as folds of change over untreated samples.

Flow cytometry. Following fixation and permeabilization using the Cytofix/Cytoperm kit (BD Biosciences Pharmingen, Palo Alto, CA, USA) according to the manufacturer's

instructions, cells were stained with the phycoerythrin conjugated mouse monoclonal antibody anti-MRP1 QCRL-1 (Santa Cruz Biotechnology, CA, USA); permeabilization was assessed by staining of β−actin as cytoplasmic positive control. Previously the primary mouse anti-MRP1 MRPm6 monoclonal antibody (MP Biomedicals, Irvine, CA, USA) and a secondary phycoerythrin conjugated antibody were utilised with similar results. The phycoerythrin conjugated mouse monoclonal antibody anti-MDR1 from clone 17F9 (Santa Cruz Biotechnology) was utilised for MDR1 staining. Irrelevant isotypic phycoerythrin conjugated antibodies were utilised as negative controls. Cytofluorimetric analysis was performed using FACScan (BD).

Gene reporter studies. Cells were transiently cotransfected using FUGENE (Roche) according to the manufacturer's instructions with the reporter vector including the 5' flanking region of MRP1 driving the expression of luciferase as reporter gene (kindly provided by Dr. Deeley, Queen's University, Ontario, Canada) and a beta-galactosidase expressing vector utilised for normalisation. The day after the transfection cells were incubated for 24 h with or without AZM 8 μg/ml. Luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity in cells transfected with the insert-less reporter vector was almost undetectable. We assessed that our method is able to detect variations, as described (28), since we measured significantly different levels of luciferase activity using a reporter vector including a region of the interleukin 8 promoter inducible by tumor necrosis factor alpha.

Statistical analysis. Statistical calculations and tests were performed using Student's t test considering $p \leq 0.05$ as limit of statistical significance. Data were expressed as means and standard deviation (SD).

RESULTS

MRP1 and MDR1 transcript levels in CF versus non-CF cells.

Real-time qPCR was utilised to evaluate the expression of MRP1 and MDR1 mRNAs. A 2.5 and 3.2 folds higher levels of MRP1 mRNA were detected in CF cells IB3-1 versus non-CF cells C38 and S9 ($p=0.009$ and $p=0.0005$ respectively, n=4, Fig 1A). IB3-1 cells expressed 66% MDR1 mRNA respect to C38 (n=4, not significant) and 13% compared with S9 cells ($p=0.0013$, n=4) (Fig. 1B).

MRP1 and MDR1 protein levels in CF versus non-CF cells.

MRP1 and MDR1 protein expression was evaluated by flow cytometry in order to establish if the mRNA regulation could have a functional relevance. The results of the MRP1 and MDR1 analysis are shown in Fig. 2A and 2B, respectively. The analysis revealed no differences of MRP1 and MDR1 protein expression levels among all CF and non-CF isogenic cell lines (data not shown).

Regulation of MRP1 and MDR1 transcript expression by AZM treatment.

To address the question whether AZM could modulate MRP1 and MDR1 expression, we performed a time course experiment in which IB3-1 cell line had been exposed to 8 μg of AZM for 1, 3, 7 and 14 days. Figure 2 shows the results obtained from MRP1 and MDR1 quantification in IB3-1 cells by real-time qPCR. We observed a less than 30% increase in transcript level of MRP1 after 1 and 3 days of treatment ($p=0.009$ and $p=0.008$ respectively, n=4), which was not maintained after 7 and 14 days (Fig 2A). AZM decreased MDR1 expression of less than 30% after 14 days of incubation ($p=0.009$, n=4) (Fig 2B). A different CF cell line as 2CFSMEo- was then tested in the same conditions, in order to establish whether the changes in MRP1 and MDR1 transcript expression detected with IB3-1 could be reproduced. In 2CFSMEo- cells we found no regulation in MRP1 and MDR1 expression by AZM treatment at all times tested (data not shown).

Regulation of MRP1 and MDR1 protein expression by AZM treatment.

Even if AZM does not appear to consistently modulate MRP1 and MDR1 transcript levels, we investigated whether protein expression might be modulated at post-transcriptional level. We performed time course experiments using IB3-1 and 2CFSMEo- cell lines exposed to 8 μg of AZM for a minimum of 24 h to a maximum of 4 weeks. MRP1 and MDR1 protein expression was evaluated by flow cytometry. No differences between treated and untreated cells after 24 h, 72h, 1 week, 2 weeks and 4 weeks of treatment were detected.

Representative results of MRP1 and MDR1 protein analysis after 24 h of treatment of IB3-1 and 2CFSMEo- cells are shown in Fig. 3A and 3B, respectively.

Effects of AZM on MRP1 promoter activity.

Considering the data reported *in vivo* about regulation of MRP1 mRNA levels by AZM we proceeded to assess whether AZM may affect at least transcription of MRP1 in our models. We then analysed by gene reporter studies the effects of AZM (8 μg/ml for 24 h) on the MRP1 promoter activity in IB3-1 and 2CFSMEo- cell lines. We did not detect statistically significant differences in the reporter activity in treated versus untreated IB3-1 and 2CFSMEo- cells as shown in Fig. 4 consistently with our findings previously reported in another CF airway epithelial cell line (29).

DISCUSSION

The aim of our study was to evaluate whether AZM could affect the expression of ABC transporters. Controlled clinical studies have reported that AZM is beneficial in CF patients. This macrolide may exhibit different effects on epithelial cells and on the production of virulence factors by *Pseudomonas aeruginosa* which is a predominant cause of decreasing pulmonary function in CF patients (30). However, the role of AZM has not been fully understood. Among various possible mechanisms AZM has been suggested to modulate the expression of MRP1 and MDR in the airways.

At first we investigated the constitutive expression of MRP1 and MDR1 in respiratory epithelial cell lines. A co-ordinated mRNA expression among homologous genes in our cellular models would be consistent with similar findings relative to CFTR and MDR1 in other cell types (3,4). We found that, although CF cells expressed more MRP1 mRNA and less MDR1 mRNA versus non-CF isogenic cell lines, there were no differences at protein level.

Furthermore, our results indicate minimal sporadic variations in both MRP1 and MDR1 gene transcription under AZM treatment but, again, protein levels were unaffected. To rule out the possibility that the effect could still occur at the transcription level but that RNA could be rendered more unstable by the treatment in our model, we studied the effects of AZM on the MRP1 promoter activity by gene reporter assays. AZM did not affect the reporter activity in treated CF cells.

Considering the possibility of regulation of a different pattern of ABC family members *in vitro* we have been focusing on possible targets of AZM other than MRP1 and MDR1. We found that AZM was not effective in regulating mRNA expression of other ABC proteins as ABCA1, ABCA13, MRP3, MRP5, CFTR, MDR7 and MDR10 (data not shown). The lack of effects in our models *in vitro* might be due to still unknown mechanism(s) causing also the high variability of beneficial effects among patients described by many authors. We have investigated the possible relevance of a MRP1 polymorphic site in the response to AZM (29). Work is still in progress in order to identify an eventual MRP1 allele associated to the response to AZM and/or to the severity of disease. No statistically significant effects have been detected also when we have been trying to overcome the possible variability among models by using a nasal CF respiratory epithelial cell line (data not shown). Analysis of gene regulation by macrolides have also been performed using microarrays including DNA

sequences of tens of thousands genes in airway epithelial cells (31), but to our knowledge there are no evidences of the regulation of ABC members by AZM to date.

MRP1 and MDR1 are closely related to CFTR, both for structure and function. Data supporting the hypothesis of complementation of CFTR by MRP1 and MDR1 following AZM treatment have been proposed as rationale of this study (1,2,15,16). Nevertheless macrolides effects on ion transport are still unclear. Chloride transport through the apical membrane of airway epithelial cells measured as nasal potential differences (NPDs) was unchanged in CF mice following treatment with clarithromycin or AZM as well as in CF patients treated with clarithromycin (32). Equi et al (33) reported essentially unchanged NPDs as well as CFTR and MDR mRNA levels in nasal epithelial cells in CF patients treated with AZM. Even if erythromycin induced the up-regulation of the MDR1 expression in the liver of rhesus monkeys (34), this macrolide has been reported to inhibit the interferon-gamma induced activity of an outwardly rectifying chloride channel in a human bronchial epithelial cell line (35). Moreover Tagaya et al (36) showed that erythromycin decreased the chloride diffusion potential difference across rabbit tracheal mucosa and that clarithromycin treatment decreased sputum production in patients affected by chronic bronchitis or bronchiectasis. They suggested that 14-membered macrolides could reduce chronic airway hypersecretion probably by inhibiting chloride secretion and the resultant water secretion.

It is evident that the mechanisms of action of macrolides are debated and the reports concerning their effects are still controversial. Our data do not support the hypothesis of induction of ABC transporters by AZM. Perhaps additional conditions *in vivo* or specific genetic background(s) might allow for complementary expression at protein levels, as suggested (15). Defining these conditions would provide interesting therapeutic targets and could be critical for improving the use of AZM for treatment of CF.

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FIGURE LEGENDS

FIGURE 1. *Constitutive expression of MRP1 and MDR1 mRNA in CF cells IB3-1 and isogenic non-CF C38 and S9 cell lines.* Total RNA was extracted and retrotranscribed. The results of MRP1 (A) and MDR1 (B) mRNA quantification are based on real-time q-PCR analysis as described in materials and methods. The values represent the expression levels relative to IB3-1 (means ± SD). The experiment was repeated four times.

FIGURE 2. *Time course analysis of MRP1 (A) and MDR1 (B) mRNA expression in IB3-1 cells.* Real-time q-PCR was performed as described in materials and methods. The values represent the expression levels relative to untreated cells (means ± SD). The experiment was repeated four times.

FIGURE 3. *Evaluation of AZM effects on expression of MRP1 and MDR1 proteins.* Following treatment with AZM for 24 h, flow cytometry was performed in IB3-1 (left) and 2CFSMEo- cells (right) stained with the PE-conjugated specific antibody anti-MRP1 or anti-MDR1. Black histograms represent the negative isotypic control superimposable in all conditions while red and blue histograms correspond to treated and untreated cells, respectively, stained with the specific antibody indicated in each panel. Logarithmic fluorescence intensity is indicated on the y-axes. Experiments are representative of at least three, performed with very similar results.

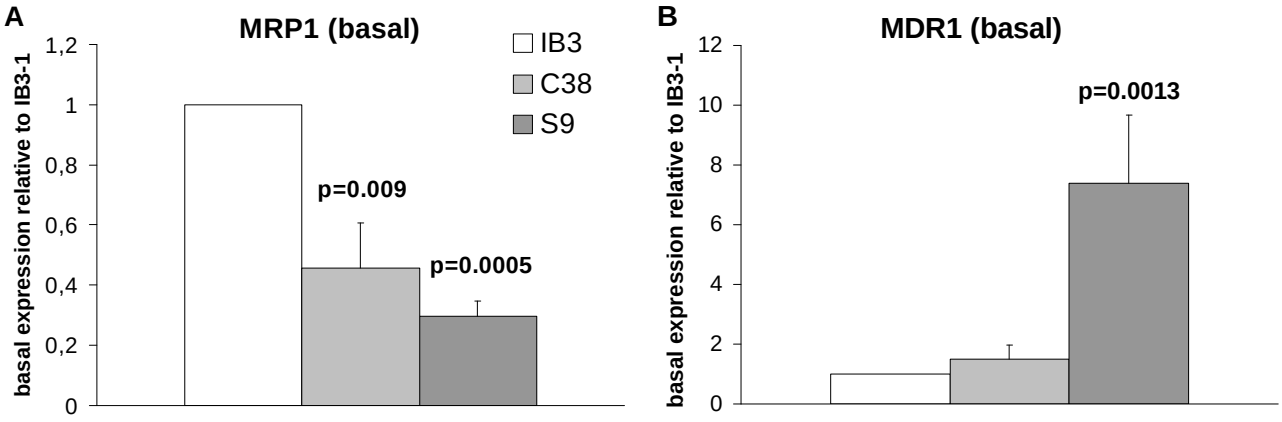
FIGURE 4. *MRP1 promoter activity analysed by gene reporter assay following AZM treatment.* Luciferase activity was normalised to beta-galactosidase activity in 2CFSMEo- and IB3-1 cells treated for 24 h with AZM and indicated on y axis relative to untreated cells as described in materials and methods. Both cell lines were assayed three times.

Table 1

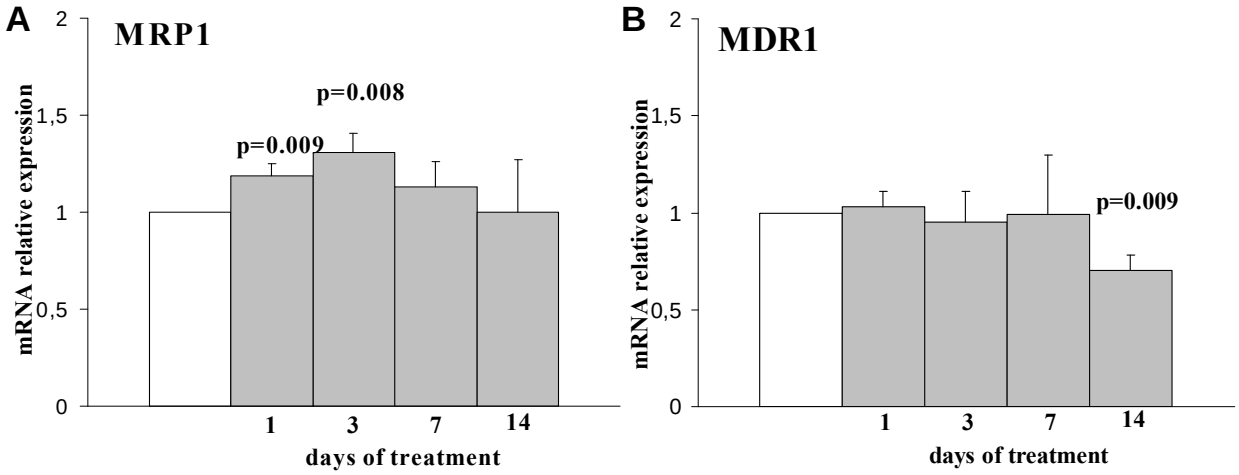
Real-time primers

gene		sequence (5'>3')	accession number	primer concentration (nM)	normaliser gene
MRP1	FW	GGGCCTCTCAGTGTCTTACTCATT	NM_004996	25	GAPDH
	RV	ACATCCGAACCAGCCAGTT		25	
MDR1	FW	AGCTTAGTACCAAAGAGGCTCTGGA	NM_000927	450	CK-15
	RV	TCCAAAAGGAAACTGGAGGTATACTT		450	
GAPDH	FW	GTGGAGTCCACTGGCGTCTT	J04038	25	
	RV	GCAAATGAGCCCAGCCTTC		150	
CK-15	FW	GGCTGGCTGCGGACG	AF202320	150	
	RV	GCAGGGCCAGCTCATTCTC		150	

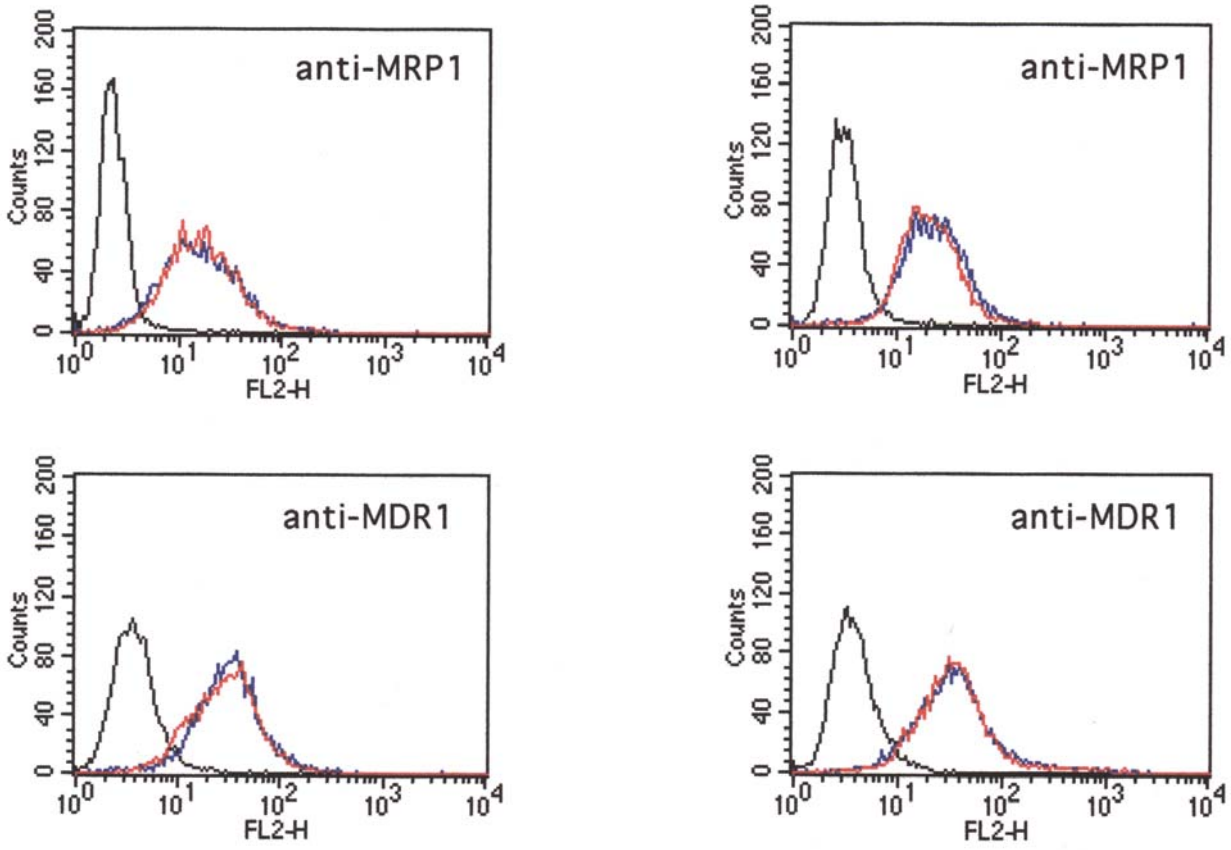
Cigana C, Fig. 1

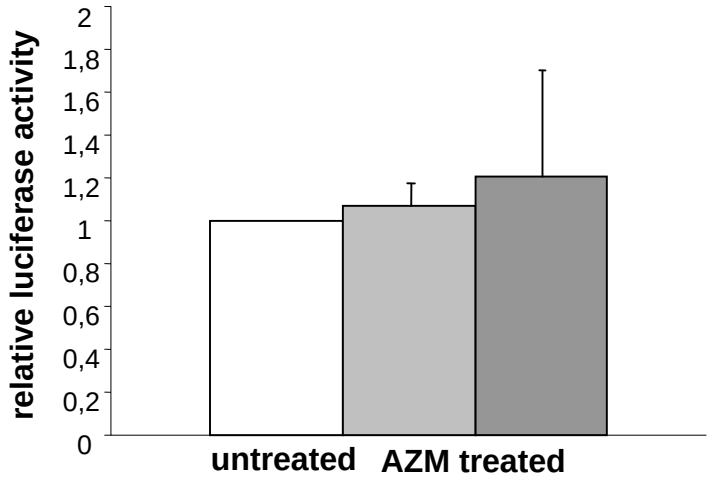


Cigana C, Fig. 2



Cigana C, Fig. 3




relative luciferase activity
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
untreated
AZM treated