Impact of azithromycin treatment on macrophage gene expression in subjects with cystic fibrosis

Theodore J. Cory a,b, Susan E. Birke a,c, Brian S. Murphy d, Don Hayes Jr. e, Michael I. Anstead f, Jamshed F. Kang a, Robert J. Kuhn a, Heather M. Bush g, David J. Feola a,*

a Department of Pharmacy Practice and Science, University of Kentucky College of Pharmacy, 789 S. Limestone, Lexington, KY 40536, United States
b Department of Pharmacy Practice, University of Nebraska College of Pharmacy, 986000 Nebraska Medical Center, Omaha, NE 68198, United States
c Department of Medicine, University of Alabama at Birmingham, 1918 University Blvd., Birmingham, AL 35294, United States
d Department of Internal Medicine, University of Kentucky College of Medicine, 138 Leader Avenue, Lexington, KY 40506, United States
e Section of Pulmonary Medicine, Department of Pediatrics, The Ohio State University, Nationwide Children's Hospital, 700 Children's Dr., Columbus, OH 43205, United States
f Department of Pediatrics, University of Kentucky, 138 Leader Avenue, Lexington, KY 40506, United States
g Department of Biostatistics, University of Kentucky College of Medicine, 725 Rose Street, Lexington, KY 40536, United States

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Abstract

Background: Azithromycin treatment improves clinical parameters in patients with CF, and alters macrophage activation from a pro-inflammatory (M1) phenotype to a pro-fibrotic, alternatively activated (M2) phenotype. The transcriptional profile of cells from patients receiving azithromycin is unknown.

Methods: Gene expression in association with macrophage polarization, inflammation, and tissue remodeling was assessed from sputum samples collected from patients with CF. Transcriptional profiles and clinical characteristics, including azithromycin therapy, were compared.

Results: Expression of NOS2 and TNFα was decreased in subjects receiving azithromycin, whereas expression of M2-associated genes was unaffected. Principal component analysis revealed gene expression profiles consistent with M1- (MMP9, NOS2, and TLR4) or M2-polarization (CCL18, fibronectin, and MR1) in select subject groups. These expression signatures did not significantly correlate with clinical characteristics.

Conclusions: Pro-inflammatory gene expression was low in subjects receiving AZM. Genes were stratified into groupings characteristic of M1- or M2-polarization, suggesting that overall polarization status is distinct among patient groups.

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1. Introduction

Patients with cystic fibrosis (CF) endure a progressive pulmonary pathology governed by a chronic, exaggerated inflammatory response [1]. Defects in the cystic fibrosis transmembrane regulator (CFTR) gene result in lungs with thickened mucus that are culture positive for a wide variety of bacteria, most commonly Pseudomonas aeruginosa. Progression of the disease is characterized by repeated acute bacterial flares during which an influx of neutrophils and macrophages occurs [2], causing a decline in pulmonary function over time [1]. Azithromycin (AZM) is a macrolide antimicrobial agent commonly used in this patient population for its anti-inflammatory properties. Chronic AZM therapy improves clinical outcome measures in CF patients including delayed lung function decline, time to acute pulmonary exacerbation, and requirement for antimicrobial treatment [3–7]. Our previous work demonstrated that AZM polarizes macrophages...
away from the pro-inflammatory classically activated (M1) phenotype and toward the anti-inflammatory, pro-fibrotic alternatively activated (M2) phenotype [8]. This results in decreased neutrophil influx and blunted pulmonary injury in mice infected with *P. aeruginosa* [9]. In a previous study of 48 CF patients that evaluated M1 and M2 polarization biomarkers, we showed heightened expression of the M2 protein mannose receptor (MR) on alveolar macrophages as well as an inverse correlation between both MR expression and M2-effector protein arginase expression as pulmonary function declines [10]. These results suggest that M2 function has a role in the pathophysiology of CF as the disease progresses and the protective effect of AZM may be related to its ability to induce M2 polarization.

Here we investigated the impact of chronic AZM therapy and other clinical parameters including positive bacterial culture and other antibiotics received on gene expression associated with macrophage polarization and fibrosis development in cells isolated from sputum samples from patients with CF. We utilized principal component analysis (PCA) to group gene expression values in order to describe the macrophage gene expression signature in these patients.

2. Methods

2.1. Clinical design

The study was approved by the Institutional Review Board at the University of Kentucky Medical Center. Patients were recruited from the pediatric and adult CF clinics during routine visits. Patients diagnosed with CF who were not acutely ill as determined by the treating pulmonologist were included, although if a subject had completed a course of therapy for an acute exacerbation, they were eligible for inclusion. Patients were ineligible if they had a decline in pulmonary function measured as the forced expiratory volume in one second (FEV1)% predicted compared to baseline measurements or if acute antibiotic therapy was required. Additional exclusion criteria included other active infectious processes (both pulmonary and systemic), malnutrition, HIV infection, and cancer.

2.2. Sample collection

Subjects provided a spontaneous sputum sample during routine clinic visits. Samples were subjected to a digestion process with the addition of 0.1% DL-dithiothreitol (Promega, Madison, WI) plus 50 units/ml deoxyribonuclease I (Sigma Aldrich, St. Louis, MO) to decrease viscosity. The process was repeated once if the sample was deemed to be overly viscous. The entire sample was utilized for later analysis, as no enrichment for macrophages was performed. Cells were placed in RNAlater (Ambion, Austin, TX) and frozen at −80 °C.

2.3. Generation of cDNA

RNA was isolated using commercially available RNeasy Mini Kits (Qiagen, Valencia, CA) according to a standard protocol and quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Reverse transcription was performed using materials from Applied Biosystems (Foster City, CA). A set amount of isolated mRNA was incubated with a reverse transcriptase cocktail with random hexamers.

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on the samples for a group of genes that are associated with macrophage polarization, inflammatory responses, and fibrosis using TaqMan gene probes (Applied Biosystems, Foster City, CA). The genes for inducible nitric oxide synthase (NOS2), Toll-like receptor 4 (TLR4), tumor necrosis factor alpha (TNFα), interleukin (IL)-1α, IL12α, and IL12β were selected as genes produced by the M1 phenotype in the context of inflammation. M2 and anti-inflammatory genes selected included arginase 1 (ARG1), arginase II (ARG2), chemokine (C–C motif) ligand 18 (CCL18), MR1, IL10, and transforming growth factor beta (TGFβ). Matrix metalloprotease 9 (MMP9), collagen 1A1 (COL1A1), and fibronectin were chosen as representative genes for tissue remodeling. It is important to note that many of the inflammatory and fibrotic genes are expressed in a variety of the immune cells found in the lungs. Thus, this analysis provides a general picture of the inflammatory and fibrotic status of the lungs of these individuals. Samples were run in triplicate and analyzed using an ABI 7900HT system (Applied Biosystems, Foster City, CA). Gene expression was calculated as the relative expression between the gene of interest and the housekeeping gene GAPDH.

2.5. Statistical analyses

PCA was performed as a method of data reduction utilizing the statistical software package SPSS (IBM, Chicago, IL). PCA creates a new set of uncorrelated variables comprised of combinations of the original variables [11]. The resulting new variables were weighted using the correlations between the various genes. The first generated component explains the highest amount of the total variance between the initial genes; the second explains the next highest amount of total variance, and so forth. Components with an eigenvalue greater than 1 were deemed to explain a significant amount of the total variance. Additional statistical analysis was performed utilizing GraphPad Prism (GraphPad Software, La Jolla, CA). Comparisons between groups were made using t-tests or the Kruskal–Wallis test for non-parametric distributions for continuous variables and by Fisher’s exact test for nominal data.

3. Results

3.1. Subject recruitment

Thirty patients were recruited for the study, with a total of 65 samples collected from September 2009 to December 2010. Of these, 30 were original samples, and 18, 12, 4, and 1 were first, second, third, and fourth follow-up samples, respectively, collected during subsequent routine visits. While the median age of the recruited population was 16 years (range 8–48), a majority were under the age of 18 years (66%). A wide range of
disease severity was observed, indicated by both FEV1% predicted (median of 55%, range 23–116%) and forced vital capacity (FVC)% predicted (median of 72%, range of 33–133%). Importantly, 76% of the cohort was chronically treated with AZM, and 40% was being treated with other chronic antimicrobial therapies. Among the population, 66% were culture positive for *Staphylococcus aureus* and 53% for *P. aeruginosa*.

Age, gender, and markers of disease severity were stratified among subjects based upon AZM treatment and positive colonization with *P. aeruginosa* or *S. aureus* (Table 1). The mean age of subjects with *P. aeruginosa* was significantly higher than that of culture negative subjects, while age stratification based on positive *S. aureus* cultures showed the opposite result (p = 0.052). Positive culture for *P. aeruginosa* was associated with a lower baseline FEV1% predicted and a trend toward a lower FVC% predicted. AZM treatment status trended similarly as positive culture for *P. aeruginosa*. Positive culture status for *S. aureus* had no impact upon these measurements.

### 3.2. Effect of AZM treatment on gene expression

Fig. 1 shows an expression of genes as stratified into subsets based upon AZM treatment from both initial and follow-up sputum samples. Expression of the pro-inflammatory genes TLR4, TNFα, IL1α, NOS2, and surprisingly the M2-associated gene Arg2 was decreased in subjects receiving AZM. No significant effects were observed in the other M2-associated genes, although there was a trend of an increase in CollA1 expression with AZM therapy.

### 3.3. Principal component analysis and gene grouping

The heterogeneous gene expression pattern in the cells isolated suggested that a multifactorial evaluation scheme would be more effective in classifying a molecular phenotype. Our cohort was stratified into molecular signature subsets based upon AZM treatment status when patients with CF were grouped by molecular phenotype. Subset 3 was comprised of 5 subjects and represents relatively high expression levels of the genes in the restricted set. Set 2 was comprised of 6 subjects and represents relatively high expression of a more inflammatory, or M1, overall phenotype. Subset 3 was comprised of 5 subjects and represents an overall gene expression signature that was polarized to an M2-like profile.

Gene expression associations were confirmed through an examination of the correlation between each pair of genes identified from the restricted gene set (Fig. 2C). While PCA was performed only on data from first visits, correlations were obtained using data from initial and follow-up visits. Genes grouped within PC1 were all highly correlated with one another, while the genes that were grouped together in PC2 in general also showed high levels of correlation. Exceptions included significant correlations between MMP9 and CCL18 expression, TLR4 and CCL18 expression, and MR1 and TLR4 expression.

Comparisons were then made in individual biomarker expression when patients with CF were grouped by molecular phenotype. Fig. 3 demonstrates the significant differences observed in Sets 2 and 3 compared to the expression values for the low-level gene expression Set 1. Significantly higher expression was observed in subjects from Set 2 for TGFβ and MMP9, with trends toward differences between these sets in their expression of ARG2, NOS2, and TLR4. For Set 3, significantly higher expression was demonstrated for the M2-associated genes CCL18, fibronectin,

### Table 1

<table>
<thead>
<tr>
<th>Subject Demographics</th>
<th>Azithromycin treatment</th>
<th>P. aeruginosa culture positive</th>
<th>S. aureus culture positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>p-Value</td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.8</td>
<td>15.5</td>
<td>0.231</td>
</tr>
<tr>
<td>Gender (percent male)</td>
<td>26.1</td>
<td>57.1</td>
<td>0.136</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>58.58</td>
<td>78.57</td>
<td>0.058</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>72.64</td>
<td>87.57</td>
<td>0.128</td>
</tr>
</tbody>
</table>
and MR1, with non-significant increases in Col1A1 and IL12β. Interestingly, the pro-inflammatory cytokines TNFα and IL1α were both significantly higher in Set 3 compared to Set 1.

3.4. Correlation between molecular phenotype and clinical characteristics

Next, we assessed the clinical characteristics of subject groups as defined by the molecular signatures using PCA (Table 2). AZM therapy did not influence the gene expression profile as stratified by PCA (p = 0.422, Chi-square test). We also assessed the bacterial infection status among our PCA-stratified subject groups. There was no significant difference in bacterial infection between the 3 groups for either *P. aeruginosa*, *S. aureus*, or both organisms. Chronic, non-AZM antibiotic use was also examined among the 3 groups. While no difference was observed for treatment with inhaled tobramycin, the use of other oral or intravenous antibiotics was predictive of a gene signature placement into Set 1 (p = 0.027, Chi-square test). We found that patients with M2-biased expression displayed a trend of an increase in age as compared to the other 2 sets. There were no differences in the mean FEV1% and FVC% predicted between the 3 groups; however, subjects in the M2-biased group were more tightly clustered around a high FVC% predicted mean value of >77%.

4. Discussion

In this study we describe the transcriptional profile obtained from macrophages and other cells isolated from patients with CF. Differences in gene expression were evaluated based upon chronic AZM therapy and other clinical parameters. As pulmonary function gradually declines in patients with CF, a shift to a net Th2 polarization occurs [13]. The role of the disposition of macrophages, however, has not been clearly defined. We have identified a number of genes expressed primarily in macrophages which are altered in subjects treated with AZM. Results revealed that AZM did not increase M2 polarization as much as it decreased inflammatory gene expression and genes related to M1 macrophages. Whether these changes in expression are due primarily to the change in T cell characteristics or other causes related to disease progression is a topic of interest for future study. Higher level statistical analyses revealed patterns of gene expression that showed gene expression profiles consistent with M1- or M2-polarization in select subjects.
We observed a significantly lower expression profile in several pro-inflammatory genes in subjects receiving AZM. There was very little expression of NOS2 in any of the samples obtained from subjects receiving AZM. This is consistent with our previous in vitro and in vivo results [8–10]. NOS2 plays an important role in the oxidative burst in M1 macrophages needed to kill internalized microorganisms [14,15]. Excessive NOS2 gene expression can be detrimental, especially in the context of an overly-inflammatory M1 response [16], leading to lung damage and a decline in pulmonary function [17]. Conversely, ARG1 and ARG2 play an important role in tissue remodeling after an acute flare of CF and other obstructive lung diseases [9,15,18,19]. Our data supports the notion that at rest, the dominant impact of AZM is likely on reducing inflammatory mediators, as a majority of the gene expression differences were in the M1-associated subset.

The first gene grouping identified by PCA consisted of NOS2, TLR4, TGFβ, and MMP9. NOS2 and TLR4 expression is associated with the M1 phenotype. MMP9, a matrix metalloprotease, is associated with cleavage of fibrotic proteins and is responsible for the degradation of the extracellular matrix as part of the tissue remodeling process. This protein has also been previously linked to the M1 phenotype [19,20] and appears to be up-regulated in patients with CF [21–23]. We recently published data showing that AZM increases the expression of MMP9 in both in vitro and in a mouse model of P. aeruginosa infection [24]. These experiments, however, examined animals in the acute phase of the response to infection, as opposed to the subjects in this study who were not undergoing an exacerbation. MMP9 may be a key regulator in the ability of AZM to exert its protective effects in the acute phase and is a topic of ongoing investigation.

TGFβ expression was grouped along with the genes in component 1. Activation of TGFβ by proteases leads to fibrogenesis [25]. Correlation with up-regulated gene expression during stable disease again may indicate that in this subset of patients with CF, some level of tissue remodeling is taking place. Studies have suggested that higher concentrations of plasma TGFβ are associated with positive culture for P. aeruginosa and worsening clinical outcomes [26]. The placement of this gene in the M1-based component further suggests that a bias toward the M1 phenotype may be detrimental. TGFβ is excreted from cells in an inactivated state and binds to the extracellular matrix. Once a part of this matrix, it can be cleaved into its active form by proteases including MMP9 [25,26]. As the molecule is excreted in an inactive form, it is difficult to correlate mRNA expression of TGFβ to the activation of the protein.
The second principal component grouped strong weightings for CCL18, fibronectin, IL12b, and MR genes. CCL18, fibronectin, and MR are prominently associated with the M2 phenotype \([12,27]\). In a clinical study of patients with a variety of non-CF lung conditions, CCL18 production by cells collected by alveolar lavage was significantly increased\([28]\). This was associated with an increase in collagen production in these patients, as well as with a shift toward an M2-dominant macrophage phenotype\([29]\). Our results show that up-regulation of CCL18 corresponds to like up-regulation in other M2-associated genes.

We sought to determine whether the differences in gene expression signature were predictive of clinical characteristics. Long-term AZM treatment is beneficial to those with positive cultures for \textit{P. aeruginosa} \([3,5,7]\). Our previous work that suggests AZM can induce an M2-like polarization in the acute phase of inflammation indicates that macrophage alterations are likely involved\([9,10,24]\). The differences observed for positive bacterial cultures may illustrate that a shift toward the M2 phenotype could contribute to increased susceptibility to S. aureus or other pathogens. It is also possible that bacterial infection may affect macrophage phenotype. Rates of subjects with positive cultures for \textit{S. aureus} and other pathogens have been greatly increasing in subjects with CF in recent years, and the etiology for this is unknown \([30]\).

Our ability to correlate these patterns to clinical parameters was on the whole unsuccessful. This could be due to the relatively small sample size: because most subjects were receiving AZM, comparisons based on its chronic therapy were difficult. The amount of variability inherent in this patient population is significant in terms of other disease complications, other drug therapies, etc. Moreover, data generated at the gene expression level may be inadequate, as stronger correlations were previously established when examining protein expression in immune cells isolated from the sputum of subjects with CF \([9]\). We also did not perform differential cell counts. By doing so we would have been able to further distinguish if gene expression that we observed was from macrophages, or from other immune cells in the sputum samples. Furthermore, we did not enrich samples for macrophages. By not enriching the samples we believe we are getting a better general view of the total inflammatory/fibrotic status of the lungs of these individuals; however, by enriching collected samples for macrophages we may have been able to see more significant differences in markers for macrophage polarization. Additionally, we only assessed gene expression in this project and did not assess protein concentrations. However, we are currently analyzing protein data in the sputum samples of these subjects in the context of tissue remodeling. This data will show whether the gene expression signatures then produce a protein expression profile that is similar.

Treatment with AZM is a commonly utilized component of cystic fibrosis therapy. This study shows that at baseline, AZM primarily impacts pro-inflammatory gene expression, and that the ability of AZM to polarize macrophages to an M2-like expression pattern may be restricted to the acute inflammation stage. Studies are ongoing to both characterize expression at the protein level in individuals at baseline, as well as the longitudinal study of gene and protein expression in subjects as they advance in the status of their disease. It will be important to increase our understanding of macrophage function in patients with CF, as the ability of AZM to improve outcomes in these conditions is not yet fully understood.
individuals supports future evaluation of therapeutic target development involving effector proteins and functional aspects of alternative macrophage activation.

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**References**


