Azithromycin Downregulates Gene Expression of IL-1β and Pathways Involving TMPRSS2 and TMPRSS11D Required by SARS-CoV-2

To the Editor:

At the time of this report, more than 20 million people have been infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Disease pathophysiology suggests the virus initially enters the nasal cavities (1) and then infects the ciliated airway epithelium (2). Often, there is an excessive inflammatory response to the virus mediated by overexpressed TNF-α (tumor necrosis factor-α), IL-6, and IL-1β (3), which leads to significant damage to the integrity and function of the lung parenchyma, causing death in the most vulnerable populations (4). To date, additional treatments against SARS-CoV-2 infections remain needed.

An interesting drug candidate against SARS-CoV-2 is azithromycin, a drug with recognized antiinflammatory (5) and epithelial repair effects (6) already being used in the treatment of chronic obstructive pulmonary disease and cystic fibrosis (7). However, its role in the regulation of the lung parenchyma, causing death in the most vulnerable populations (4). To date, additional treatments against SARS-CoV-2 infections remain needed.

Interestingly, Gene Ontology (GO) pathways were also demonstrated by a significant downregulation of Hallmark and Gene Ontology canonical inflammatory response pathways (NES = −2.0.729, P = 0.0005 and NES = −2.0.569, P = 0.0020, respectively) together with IFN-γ and IFN-α pathways (NES = −2.1717, P = 0.0005 and NES = −2.1484, P = 0.0005, respectively). Moreover, downregulation of key IL signaling pathways, including IL-2, IL-6, and IL-8, was also seen.

Interestingly, Gene Ontology’s sterol biosynthetic process and Hallmark’s cholesterol homeostasis were upregulated (NES = 3.0991, P = 0.0002 and NES = 3.0543, P = 0.0005, respectively). Selected significant pathways are presented in Figure 1A and summarized in Table E1. A full table of all significantly modulated canonical pathways are presented in Table E3.

Differential Gene Expression of cultures treated with 10 μg of azithromycin demonstrated a significant downregulation of IL-1β (fold change = −1.411, P = 0.00094) and NDST-1 (fold change = −1.345, P = 0.0276). Interestingly, within the lipid and cholesterol biosynthesis pathways, most of its individual genes were significantly upregulated. A display of selected genes is found in Figure 1B and Table E2. A full table of all tested genes are presented in Table E4.

Methods

Briefly, three previously enrolled patients who were part of a larger descriptive study were asked to participate in this pilot study. These patients had a diagnosis of chronic rhinosinusitis according to the published American Association of Otorhinolaryngology - Head and Neck Surgery guidelines and were scheduled for endoscopic sinus surgery. A nasal biopsy at the level of the anterior ethmoid bulla was taken at the time of surgery. Three male patients with age 41, 49, and 53 years with no significant comorbidities other than chronic obstructive pulmonary disease in the latter were the sources of the nasal biopsies. No patient had received oral corticosteroids or topical or systemic antibiotic therapy in the preceding 5 days. All subjects had ceased topical intranasal corticosteroids 14 days before surgery.

Primary airway nasal epithelial cells were isolated from biopsies of the anterior ethmoid bulla and cultured according to a modified protocol from Maille and colleagues (8). Through immunohistochemistry, the freshly isolated cell suspension was characterized to be composed of basal (cytokeratine 13–positive cells), ciliated (BIV-tubulin–positive cells), and goblet (MUC5AC–positive cells) nasal epithelial cells (Figure E1 in the data supplement). These cell types have all been described as expressing ACE2 and harbor the potential of sustaining a SARS-CoV-2 infection (9). To obtain a uniform and consistent cell population during our experiments with azithromycin treatment, this cell suspension was then expanded for 5–7 days, leading to a homogenous cell culture, predominantly composed from progenitor basal cells.

Based on previous azithromycin toxicity studies on human bronchial airway epithelial cells, the plate was treated with 10 μg/ml of azithromycin diluted in DMEM (Sigma-Aldrich) or a mock.

RNA was extracted from these cultures treated with azithromycin or mock. Then, samples for microarray studies were prepared using the Illumina RNA Amplification TotalPrep kit from Ambion (Life Technologies) and collected with the Illumina Bead Array Reader (Illumina). Raw gene expression data was preprocessed, and pathway analysis was performed using the gene set enrichment analysis. Differential Gene Expression was then performed using the LIMMA package from Bioconductor (10). For a more detailed Methods section, refer to the data supplement.

Results

Pathway analysis using gene set enrichment analysis showed that cultures treated with 10 μg of azithromycin demonstrated a significant downregulation in serine hydrolase activity pathway (normalized enrichment score [NES] = −1.8720, P = 0.00020) together with endocytosis (NES = −1.6866, P = 0.00020) and receptor-mediated endocytosis pathway (NES = −1.5139, P = 0.00124). This is particularly interesting because the strongest associated genes included TMPRSS2 and TMPRSS11D.

Azithromycin’s antiinflammatory properties were also demonstrated by a significant downregulation of Hallmark and Gene Ontology canonical inflammatory response pathways (NES = −2.0.729, P = 0.0005 and NES = −2.0.569, P = 0.0020, respectively) together with IFN-γ and IFN-α pathways (NES = −2.1717, P = 0.0005 and NES = −2.1484, P = 0.0005, respectively). Moreover, downregulation of key IL signaling pathways, including IL-2, IL-6, and IL-8, was also seen.
With this study, we provide some evidence that azithromycin downregulates key pathways involving genes **TMPRSS2** and **TMPRSS11D**, which code for two serine proteases required by SARS-CoV-2 for its activation (2) and cell-to-cell transmission (11), respectively.

Furthermore, downregulating **IL-1β** and **NDST-1** (12) together with associated inflammation and leukocyte recruitment pathways may help reduce the characteristic excessive respiratory epithelial inflammation, a key feature of SARS-CoV-2 infection.

Finally, the unexpected upregulation of multiple genes involved in cholesterol biosynthesis is believed to be a process known as drug-induced phospholipidosis, which may decrease cholesterol in cell membrane lipid rafts (5). This may hinder SARS-CoV-2 infection, as **in vitro** studies demonstrated that depletion of cholesterol in the cell membrane resulted in decreased SARS-CoV-1 cell infection (13, 14). Moreover, our data are in line with a previous **in vitro** study in which azithromycin upregulated lipid and cholesterol pathways while decreasing...
important proinflammatory cytokines in differentiated human bronchial epithelial cell cultures (15).

Our study should, however, be interpreted with caution because it is limited by its small sample size, the inclusion of only a male population, and the lack of experiments validating that the observed changes in gene expression had an impact on protein levels. Nevertheless, our findings harbor significant information to better orient larger in vivo or clinical studies on future treatments against SARS-CoV-2 infections.

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