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

Running title: Pleiotropic mechanisms of azithromycin suggested by gene expression profiling may be useful against SARS-CoV-2

Axel E. Renteria, MD, MSc[1,2], Leandra Endam Mfuna, MSc[1], Damien Adam, PhD[1,3]; Ali Filali-Mouhim, PhD[1]; Anastasios Maniakas, MD, MSc[4,5]; Simon Rousseau, PhD[6,7]; Emmanuelle Brochiero, PhD[1,3]; Stefania Gallo, MD[8]; Martin Desrosiers, MD, FRCSC[1,2]

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montréal, QC, Canada; [2]Département d'oto-rhino-laryngologie et chirurgie cervico-faciale du centre hospitalier de l'Université de Montréal (CHUM), Montréal, QC, Canada; [3]Département de Médecine, Université de Montréal, Montréal, QC, Canada; [4]Division d'oto-rhino-laryngologie et chirurgie cervico-faciale du CIUSSS de l'Est-de-l'île-de-Montréal associé à l'Université de Montréal; [5]Division of Experimental Surgery, McGill University, Montreal, QC, Canada; [6]Division of Experimental Medicine, McGill University, Montreal, QC, Canada; [7]Research Institute of McGill University Healthcare Centre (MUHC), Montréal, QC, Canada; [8]Clinica Otorinolaringoiatrica, ASST Sette Laghi e Università dell'Insubria, Varese, Italy

AR: conception of study, data analysis, literature review, wrote manuscript, **LME:** study design, data collection, patient recruitment, in vitro experiments, **DA:** immunohistochemistry data collection and results, **AFM**: Gene and pathway expression data processing and analysis, **AM:** conception of study, **SR**: conception of study, study design, **EB:** conception of study, study design, **SG:** study design, patient recruitment, in vitro experiments, **MD**: conception of study, study design
All co-authors contributed to the correction and revision of the manuscript

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Disclosures: This material has never been published and is not currently under evaluation in any other peer-reviewed publication. The authors have not received funding for this study, and there are no conflicts of interest.

Corresponding author:

Martin Desrosiers, MD, FRCSC

University of Montreal Hospital Center (CHUM) and Research Center (CRCHUM)

1051 Sanguinet Street, Montréal, Quebec, CANADA, H2X 3E4

Tel: (514) 890-8000 ext. 14170

Email: desrosiers_martin@hotmail.com

Keywords: azithromycin, SARS-CoV-2, COVID-19, TMPRSS2, TMPRSS11D, INSIG-1, IL-1β, IL-6, NDST1, Cholesterol

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

To the editors,

At the time of this report, more than 20 million people have been infected with SARS-CoV-2. Disease pathophysiology suggests the virus initially enters the nasal cavities (1) to then infects the ciliated airway epithelium (2). Often, there is an excessive inflammatory response to the virus mediated by overexpressed TNF-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 COVID-19 is azithromycin, a drug with recognized anti-inflammatory (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 TMPRSS2, ACE2 and TMPRSS11D genes, coding for proteins necessary for SARS-CoV-2 activation, infection, and transmission, respectively (2), remains to be further investigated.

Methods

Briefly, three previously enrolled patients part of a larger descriptive study were asked to participate in this pilot study. These patients had a diagnosis of chronic rhinosinusitis (CRS) according to the published AAO-HNS guidelines and were scheduled for endoscopic sinus surgery (ESS). A nasal biopsy at the level of the anterior ethmoid bulla was taken at the time of surgery. Three male patients of age 41, 49 and 53 years with no significant co-morbidities other than chronic obstructive pulmonary disease (COPD) 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 30 days. All subjects had ceased topical intranasal corticosteroids 14 days prior to surgery.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Primary airway nasal epithelial cells were isolated from biopsies of the anterior ethmoid bulla and cultured according to a modified protocol from Maillé *et al*. (8). Through immunohistochemistry, the freshly isolated cell suspension was characterized to be composed of basal (cytokeratine 13 positive cells), ciliated (βIV-tubulin positive cells) and goblet (MUC5AC positive cells) nasal epithelial cells (Figure S1). These cell types have all been described expressing ACE2 and harbor the potential of sustaining a SARS-CoV-2 infection (9). To obtain an uniform and consistent cell population during our experiments with azithromycin treatment, this cell suspension was then expanded for 5 to 7 days, leading to an 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 10µg/ml of azithromycin diluted in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON, Canada) 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, Burlington, ON, Canada) and collected with the Illumina Bead Array Reader (IIlumina, San Diego, CA, USA). Raw gene expression data was pre-processed and pathway analysis was performed using the gene set enrichment analysis completed (GSEA). Differential Gene Expression (DGE) then was performed using the LIMMA package from Bioconductor (10). For a more detailed materials and methods section refer to the online supplemental section.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Results

Pathway analysis using GSEA showed that cultures treated with 10μg of azithromycin demonstrated a significant downregulation in serine hydrolase activity pathway (NES= -1.8720, P=0.0020), along with endocytosis (NES= -1.6866, P=0.0020) and receptor-mediated endocytosis pathway (NES= -1.5139, P=0.0124). This is particularly interesting because the strongest associated genes included TMPRSS2 and TMPRSS11D.

Azithromycin's anti-inflammatory properties were also demonstrated by a significant downregulation of Hallmark and GeneOntology canonical inflammatory response pathways (NES= -2.0729, P=0.0005 and NES= -2.0569, P=0.0020, respectively) along 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 interleukin signaling pathways including IL-2, IL-6 and IL-8 was also seen.

Interestingly, GeneOncology' sterol biosynthetic process and Hallmark's cholesterol homeostasis were upregulated (NES= 3.0991, P=0.0020 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.

DGE of cultures treated with 10μg of azithromycin demonstrated a significant downregulation of IL-1β (FC: -1.411, P=0.0094) and NDST-1 (FC= -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 gene is found in Figure 1b and Table E2. A full table of all tested genes are presented in Table E4.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

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

Finally, the unexpected upregulation of multiple genes involved in cholesterol biosynthesis is thought to be a process known as drug-induced phospholipidosis (DLP) 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 were azithromycin upregulated lipid/cholesterol pathways, while decreasing important pro-inflammatory 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 proteins levels. Nevertheless, our findings harbor significant information to better orient larger *in vivo* or clinical studies on future treatments against SARS-CoV-2 infections.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Figures

Figure 1. Host transcriptional response to azithromycin in basal nasal epithelial cells. a) Gene Set Expression Analysis (GSEA) comparing differential expression in selected significant pathways from MsigDB, Hallmark, C2 and C5 gene set collections. All pathways present have an FDR<0.05. Data is presented as normalized enrichments scores (NES) where values > 0 represent upregulation and values < 0 represent downregulation when comparing azithromycin treated cell culture with mock treated cell cultures. **b)** Heatmap of selected differentially expressed genes between cell cultures treated with azithromycin and mock treated cell cultures. Selected genes were based on biological relevance and a nominal P<0.05.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

References

1. Lechien JR, Chiesa-Estomba CM, De Siati DR, Horoi M, Le Bon SD, Rodriguez A, et al. Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. European Archives of Oto-Rhino-Laryngology. 2020:1.

2. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020.

3. Simoes e Silva A, Silveira K, Ferreira A, Teixeira M. ACE2, angiotensin-(1-7) and M as receptor axis in inflammation and fibrosis. British journal of pharmacology. 2013;169(3):477-92.

4. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. JAMA. 2020.

5. Parnham MJ, Haber VE, Giamarellos-Bourboulis EJ, Perletti G, Verleden GM, Vos R. Azithromycin: mechanisms of action and their relevance for clinical applications. Pharmacology & therapeutics. 2014;143(2):225-45.

6. Haydar D, Cory TJ, Birket SE, Murphy BS, Pennypacker KR, Sinai AP, et al. Azithromycin polarizes macrophages to an M2 phenotype via inhibition of the STAT1 and NF-κB signaling pathways. The Journal of Immunology. 2019;203(4):1021-30.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

7. Cigana C, Nicolis E, Pasetto M, Assael BM, Melotti P. Anti-inflammatory effects of azithromycin in cystic fibrosis airway epithelial cells. Biochemical and biophysical research communications. 2006;350(4):977-82.

8. Maillé E, Trinh NTN, Privé A, Bilodeau C, Bissonnette É, Grandvaux N, et al. Regulation of normal and cystic fibrosis airway epithelial repair processes by TNF-α after injury. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2011;301(6):L945-L55.

9. Sungnak W, Huang N, Bécavin C, Berg M, Queen R, Litvinukova M, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. Nature Medicine. 2020:1-7.

10. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with Bioconductor. Nature methods. 2015;12(2):115.

11. Bertram S, Glowacka I, Müller MA, Lavender H, Gnirss K, Nehlmeier I, et al. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. Journal of virology. 2011;85(24):13363-72.

12. Zuberi RI, Ge XN, Jiang S, Bahaie NS, Kang BN, Hosseinkhani RM, et al. Deficiency of endothelial heparan sulfates attenuates allergic airway inflammation. The Journal of Immunology. 2009;183(6):3971-9.

13. Baglivo M, Baronio M, Natalini G, Beccari T, Chiurazzi P, Fulcheri E, et al. Natural small molecules as inhibitors of coronavirus lipid-dependent attachment to host cells: a possible

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

strategy for reducing SARS-COV-2 infectivity? Acta Bio-medica: Atenei Parmensis. 2020;91(1):161-4.

14. Wang H, Yang P, Liu K, Guo F, Zhang Y, Zhang G, et al. SARS coronavirus entry into host cells through a novel clathrin-and caveolae-independent endocytic pathway. Cell research. 2008;18(2):290-301.

15. Ribeiro CMP, Hurd H, Wu Y, Martino ME, Jones L, Brighton B, et al. Azithromycin treatment alters gene expression in inflammatory, lipid metabolism, and cell cycle pathways in well-differentiated human airway epithelia. PloS one. 2009;4(6).

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Figure 1a

1202x842mm (72 x 72 DPI)

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

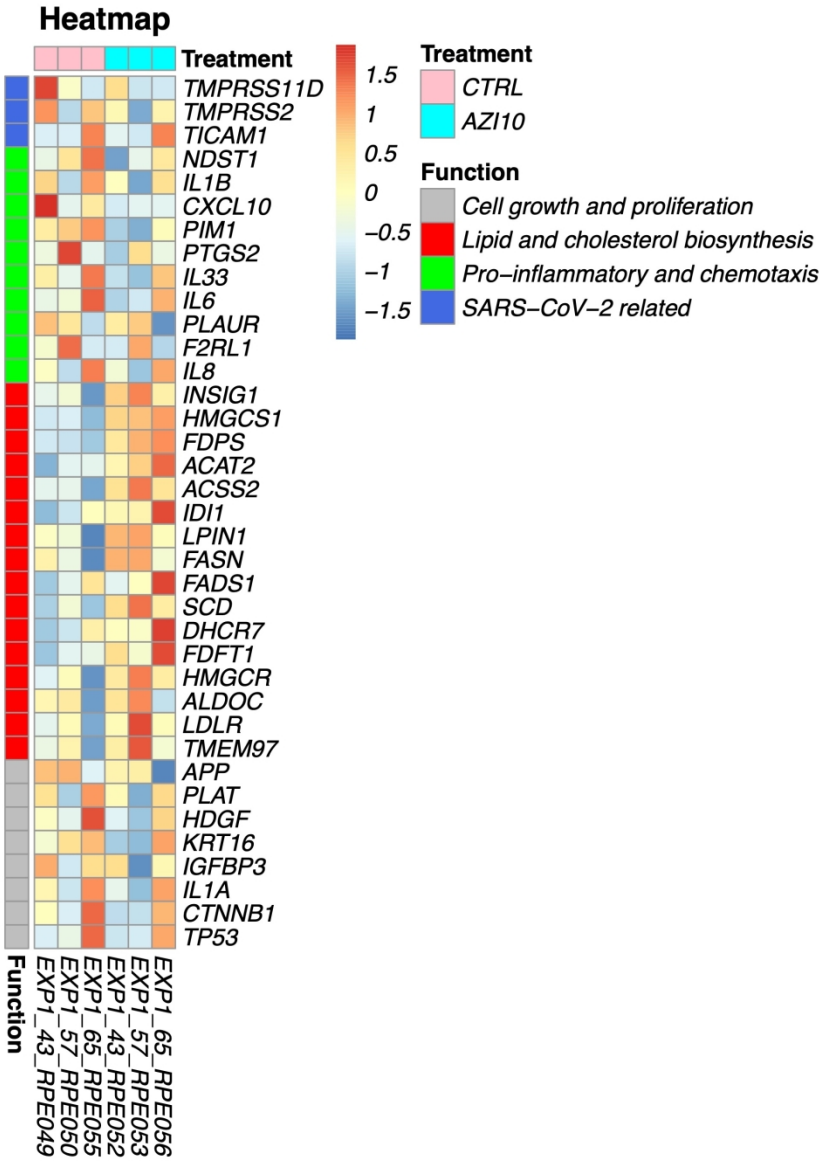

Figure 1b

736x995mm (72 x 72 DPI)

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

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

Running title: Novel pleiotropic mechanisms of azithromycin suggested by gene expression profiling may be useful against SARS-CoV-2

Axel E. Renteria, MD, MSc[1,2], Leandra Endam Mfuna, MSc[1], Damien Adam, PhD[1,3]; Ali Filali-Mouhim, PhD[1]; Anastasios Maniakas, MD, MSc[5,5]; Simon Rousseau, PhD[6,7]; Emmanuelle Brochiero, PhD[1,3]; Stefania Gallo, MD[8]; Martin Desrosiers, MD, FRCSC[1,2]

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montréal, QC, Canada; [2]Département d'oto-rhino-laryngologie et chirurgie cervico-faciale du centre hospitalier de l'Université de Montréal (CHUM), Montréal, QC, Canada; [3]Département de Médecine, Université de Montréal, Montréal, QC, Canada; [4]Division d'oto-rhino-laryngologie et chirurgie cervico-faciale du CIUSSS de l'Est-de-l'île-de-Montréal associé à l'Université de Montréal; [5]Division of Experimental Surgery, McGill University, Montreal, QC, Canada; [6]Division of Experimental Medicine, McGill University, Montreal, QC, Canada; [7]Research Institute of McGill University Healthcare Centre (MUHC), Montréal, QC, Canada; [8]Clinica Otorinolaringoiatrica, ASST Sette Laghi e Università dell'Insubria, Varese, Italy

Disclosures: This material has never been published and is not currently under evaluation in any other peer-reviewed publication. The authors have not received funding for this study, and there are no conflicts of interest.

Corresponding author:

Martin Desrosiers, MD, FRCSC

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

University of Montreal Hospital Center (CHUM) and Research Center (CRCHUM)

1051 Sanguinet Street, Montréal, Quebec, CANADA, H2X 3E4

Tel: (514) 890-8000 ext. 14170

Email: desrosiers_martin@hotmail.com

Keywords: azithromycin, SARS-CoV-2, COVID-19, TMPRSS2, TMPRSS11D, INSIG-1, IL-1β, IL-6, NDST1, Cholesterol

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Extended materials and methods

Enrolled Patients

This study was approved and supervised by the institutional review board of the Centre de recherche du Centre Hospitalier de l'Université de Montreal (CRCHUM). Briefly, three previously enrolled patients part of a larger descriptive study were asked to participate in this pilot study. These patients with known CRS and scheduled for endoscopic sinus surgery had a nasal biopsy taken at the time of surgery. All patients participated voluntarily and signed an informed consent.

Materials

Azithromycin were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Cell Cultures

Primary airway nasal epithelial cells were isolated from nasal biopsy samples obtained during endoscopic sinus surgery (ESS) performed for management of chronic sinusitis (CRS). In all cases, biopsy samples were obtained at the level of the anterior ethmoid bulla to ensure maximal repeatability across disease conditions. The patients with CRS had a diagnosis of CRS with nasal polyps according to published AAO-HNS guidelines (1), and have failed at least one course of maximal medical therapy. Patients with immune deficiencies, systemic disorders affecting immunity such as diabetes or renal insufficiency taking systemic immunosuppressive drugs, or with cystic fibrosis were excluded. No patient had received oral corticosteroids or topical or systemic antibiotic therapy in the preceding 30 days. All subjects had ceased topical intranasal corticosteroids 14 days prior to surgery. Nasal epithelial cells were cultured according to a

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

modified protocol from Maillé *et al*. (2). Briefly, nasal biopsy samples were immediately placed on a moist sterile compress in a sterile plastic container. The tissues from each patient were incubated separately overnight in MEM medium with 1% protease-DNAse at 4°C. Nasal epithelial cells were isolated by gently scraping each sample with a scalpel. Cells from each sample were cultured in different T25 coated with Purecol with Bronchial Epithelial Growth Medium (BEGM) (Lonza, Walkersville, MD) culture medium and supplemented with growth factors SingleQuots (Lonza) without gentamicin, 50 units/ml penicillin G, 50 µg/ml streptomycin, 25 ng/ml hEGF and retinoic acid $5x10^{-8}$M. Nasal epithelial cells were maintained at 37°C, 5% CO_2, 100% humidity. Once confluency was reached (5 to 7 days of primary culture), cells were trypsinized and the cell suspension was seeded into two wells of a 6-well plate (for each patient's primary nasal epithelial sample). BEGM medium supplemented with retinoic acid was added. Based on previous azithromycin toxicity studies on human bronchial epithelial cells (3, 4), the plate was treated with 10µg/ml of azithromycin (n=3) or mock (n=3). Briefly, azithromycin was received as a 5mg of powder and was diluted in DMSO to reach a stock concentration of 5mg/mL. 4µL of this stock solution was added to 2mL plates to reach a final concentration of 10µg/mL of azithromycin. Cells were then incubated at 37°C, 5% CO_2 atmosphere, 100% humidity during 24 hours.

Cell characterization

Freshly isolated nasal epithelial cells were cytocentrifuged (at 750 rpm) on slides. They were the fixed with 4% paraformaldehyde and blocked for an hour with a solution of PBS containing 10% FBS (Saradign, USA) and 10% BSA (Sigma-Aldrich). Slides were incubated overnight at 4°C with anti-cytokeratin-13 (basal cells) mouse monoclonal (1:1000, IgG1 clone KS-1A3, Sigma

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Aldrich), anti-MUC-5AC (goblet cells) mouse monoclonal (1:100, IgG1 clone 45M1, ThermoFisher) or anti-tubulin-βIV (ciliated cells) mouse monoclonal (1:1000, IgG2b clone KMX-1, Chemicon). The day after, they were blocked again with PBS 10% FBS, 10% BSA, incubated with Alexafluor 488 conjugated goat anti-mouse secondary antibody (1:200, Life technologies, USA) for one hour, followed by a DAPI staining (1:1000, Sigma) before mounting with Prolong Gold (Invitrogen, Thermo Fisher Scientific). Photographs were taken with an Exiqua camera (QImaging, Canada) under an inverted fluorescence microscope (Olympus, Canada) at 400x (NA = 0.75) and analyzed with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

RNA Extraction

Total RNA was extracted using RNeasy Mini Kit (Qiagen). The cells were rinsed three times with PBS. 350µl extraction buffer RLT was added on the cells and they were scraped with a rubber policeman. The cells were homogenized by passing the lysate 5 times though a 20 gauge (G) needle fitted to a syringe and deposited on a collection tube. 350µl of 70% ethanol were added to the cells, mixed well and transferred to RNeasy mini columns. Then the columns were centrifuged for 15 seconds at 10000 rpm. 350µl of RW1 buffer was added to the RNeasy columns and these were centrifuged as above. The column was transferred to a new tube and 500µl of RPE buffer was added on the column and the column was centrifuged as above. The procedure was repeated with buffer RPE, the column was centrifuged for 2 min at 10000 rpm. The column was transferred to a new 1.5ml collection tube and was centrifuged for 1 min at 15000 rpm. Finally, 30uL of RNAse-free water was added to the middle of the membrane and centrifuged for 1 min at 15000 rpm. RNA samples were stored at -40 °C.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Microarray Gene Expression

RNA integrity values were assessed on an Agilent 2100 Bioanalyzer Nanochip (Agilent, Palo Alto, CA, USA). Total RNA preparations with RNA ratio [28s/18s] ratio between 1.51–2.53 and RNA integrity number ≥ 9.4 with enough quantity were used for the expression analysis. The labeling was done with the Illumina® RNA Amplification TotalPrep™ kit from Ambion (Life Technologies, Burlington, ON, Canada). Labeled cRNA (750 ng) was hybridized 18h at 58°C to Illumina HumanRef-8 v3.0 BeadChip Array (IIlumina, San Diego, CA, USA) then washed and developed using fluorolink streptavidin-Cy3 (GE Healthcare). BeadChips were then scanned using the Illumina Bead Array Reader (IIlumina, San Diego, CA, USA).

Gene Expression Data Analysis

Raw Illumina probe data were exported from BeadStudio and screened for quality. Pre-processing and statistical analysis was conducted using the R statistical language (5) and software packages from Bioconductor as described by Huber *et al*. (6). Quantile normalization was applied, followed by a log2 transformation.

Pathway Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed using the Bioconductor's package FGSEA (7). Tested pathways and gene sets came from the Molecular Signature Database (MsigDB, http://www.broad.mit.edu/gsea/msigdb), Hallmark (h.all.v5.0.symbols.gmt), C2 (C2.all.v6.2.symbols.gmt) and C5 (C5.all.v6.2.symbols.gmt) collections. P values associated to the obtained pathways following GSEA were adjusted for multiple test correction with False Discovery Rate (FDR) cut-offs significance of 0.05.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

GSEA was performed to assess whether a known biological pathway or sets of individual genes were significantly enriched among the genes ranked by the moderated t-test following the differential gene expression analysis between 10μg of azithromycin and mock treated samples.

Differential Gene Expression

The LIMMA package from Bioconductor (6) was used to fit a linear model to each probe and to perform a moderated t-test comparing 10μg of azithromycin and mock treated samples. P values from the resulting comparison were adjusted for multiple testing according to the method of Benjamini and Hochberg (8). This method controlled the false discovery rate (FDR), which was set to 0.05. Gene up- or downregulation was considered significant only when they had an adjusted P value (FDR) <0.05 and an absolute fold change >1.3.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

References

1. Benninger MS, Ferguson BJ, Hadley JA, Hamilos DL, Jacobs M, Kennedy DW, et al. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngology-Head and Neck Surgery. 2003;129(3):S1-S32.

2. Maillé E, Trinh NTN, Privé A, Bilodeau C, Bissonnette É, Grandvaux N, et al. Regulation of normal and cystic fibrosis airway epithelial repair processes by TNF-α after injury. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2011;301(6):L945-L55.

3. Cigana C, Nicolis E, Pasetto M, Assael BM, Melotti P. Anti-inflammatory effects of azithromycin in cystic fibrosis airway epithelial cells. Biochemical and biophysical research communications. 2006;350(4):977-82.

4. Menzel M, Akbarshahi H, Bjermer L, Uller L. Azithromycin induces anti-viral effects in cultured bronchial epithelial cells from COPD patients. Scientific reports. 2016;6(1):1-11.

5. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria URL https://www/ R-project org. 2017.

6. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with Bioconductor. Nature methods. 2015;12(2):115.

7. Korotkevich G, Sukhov V, Sergushichev A. Fast gene set enrichment analysis. bioRxiv. 2019:060012.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

8. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological). 1995;57(1):289-300.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Supplementary figures

Figure E1. Identification of cell specific epithelial markers within nasal epithelial samples. Freshly isolated nasal epithelial cells were cytocentrifuged and then stained with anti-cytokeratin 13 (basal cells), MUC5AC (goblet cells) or βIV-tubulin (ciliated cells) antibodies (left columns). Nuclei were counterstained with DAPI (middle column). Merged images are also shown on the right. Enlargement (x400). Bar scale: 10 μm.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Supplementary tables

Table E1. Custom selection normalized enrichment scores in major gene pathways of biological interest between azithromycin and mock treated cells cultures

Canonical pathways	NES✧	P value*	Associated genes of interest
Viral associated pathways			
GO - Response to virus	-1.8970	0.0020	IL-6, IL-33, CXCL-10, F2RL1, TICAM1
GO - Serine hydrolase activity	-1.8720	0.0020	TMPRSS2, TMPRSS11D, MMP10, PLAT
GO - Regulation of viral genome replication	-1.8402	0.0044	IL-8
GO - Defense response to virus	-1.8351	0.0020	IL-6, IL-33, CXCL-10, F2RL1, TICAM1
GO - Positive regulation of viral process	-1.7039	0.0056	TMPRSS2
GO - Endocytosis	-1.6866	0.0020	APP, TMPRSS2
GO - Regulation of viral entry into host cell	-1.5870	0.0827	TMPRSS2
GO - Viral life cycle	-1.5634	0.0020	SERPIN B3, ITG-β1, ACE2
GO - Receptor mediated endocytosis	-1.5139	0.0124	TMPRSS2, IL-8
Mantovani viral GPCR signaling upregulation	-1.4979	0.0562	MYC
Inflammation and immune response			
Hallmark - TNFα signaling via NFκB	-2.7675	0.0005	SERPIN B2, IL-1β, IL-6, PLAUR
Schoen NFκB signaling	-2.7147	0.0017	IL-1β, IL-1α, IL-8, MMP9, PTGS-2
GO - Positive regulation of leukocyte chemotaxis	-2.4674	0.0020	IL-1β, IL-6, IL-8, CXCL-10, F2RL1
Hallmark - Coagulation	-2.4113	0.0005	PLAT, SERPIN B2, MMP10
GO - Acute phase response	-2.3681	0.0020	IL-1β, IL-1α, IL-6, STAT3
GO - Positive regulation of macrophage activation	-2.3511	0.0020	IL-33
Biocarta - IL-1R pathway	-2.2232	0.0017	IL-1β, IL-1α, IL-6, TGF-β2
Hallmark - IFN-γ response	-2.1717	0.0005	PIM-1, IL-6, CXCL-10
Hallmark - IFN-α response	-2.1484	0.0005	CXCL-10
GO - Positive regulation of IL-6 production	-2.1113	0.0020	IL-1β, IL-6, IL-33, F2RL1, TICAM1
Hallmark - Inflammatory response	-2.0729	0.0005	IL-1β, IL-6, IL-8, PLAUR, CXCL-10
GO - Inflammatory response	-2.0569	0.0020	NDST1, IL-1β, IL-6

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

KEGG - Nod-like receptor signaling pathway	-2.0493	0.0017	IL-1β, IL-6, IL-8
Hallmark - IL-2 mediated STAT5 signaling	-2.0426	0.0005	PIM-1, CXCL-10
Hallmark - IL-6 mediated JAK-STAT3 signaling	-1.9318	0.0008	PIM-1, IL-1β, IL-6, CXCL-10, STAT3
GO Positive regulation of IL-8 production	-1.8612	0.0067	IL-1β, F2RL1
Lipid and cholesterol biosynthesis			
GO - Sterol biosynthetic process	3.0991	0.0020	INSIG1, SQLE, IDI-1, HMGCS1, FDPS, FDFT1, HMGCR
Hallmark - Cholesterol homeostasis	3.0543	0.0005	SCD, SQLE, ACSS2, ACAT2, HMGCS1, FDPS, FASN, HMGCR, ALDOC, TMEM97, LDLR
Reactome - Cholesterol biosynthesis	3.0158	0.0017	SQLE, IDI-1, DHCR7, HMGCS1, FDPS, FDFT1, HMGCR
KEGG - Steroid biosynthesis	2.7429	0.0017	SQLE, DHCR7, FDFT1
Hallmark - mTORC1 signaling	2.4584	0.0005	INSIG1, SCD, SQLE, IDI-1, HMGCS1, HMGCR, TMEM97, FADS1, FADS2
GO - Fatty acyl-CoA metabolicprocess	2.3055	0.0020	SCD, FASN
GO - Acetyl-CoA biosynthetic process	2.2227	0.0020	ACSS2
GO - Lipid biosynthetic process	2.1309	0.0021	INSIG1, SCD, SQLE, ACSS2, IDI-1, LPIN1, HMGCS1, FDPS, FASN, FDFT1, HMGCR
Hallmark - Fatty acid metabolism	2.0170	0.0005	ACAT2, IDI-1, HMGCS1, FASN, IDH1
GO - Phospholipid biosynthetic process	1.8238	0.0021	IDI-1, LPIN1, FDPS
GO - Sterol homeostasis	1.7594	0.0080	LDLR
GO - Fatty acid metabolic process	1.7354	0.0021	SCD, ACSS2, LPIN1, FASN
GO - Phospholipid transport	1.3385	0.1847	SCP2
GO - Lipid localization	1.2628	0.1152	SCP2
GO - Regulation of sterol transport	1.0856	0.4635	SCP2
Cell cycle, growth and proliferation			
Hallmark - K-Ras signaling	-2.3247	0.0005	PLAT, IL-1β, IGFBP3, IL33, MMP10, PLAUR, CXCL-10, PTGS2
Hallmark - p53 pathway	-2.1169	0.0005	APP, IL-1α, TP53

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Hallmark - Apoptosis	-2.0919	0.0005	IL-1β, IL-1α, IL-6, PLAT, APP, CTNN-β1
Hallmark - Myc targets version 2	-1.9591	0.0008	MYC
Hallmark - Wnt/β-catenin signaling	-1.9017	0.0023	MYC, TP53, CTNN-β1
KEGG - Apoptosis	-1.7831	0.0029	IL-1β, IL-1α, TP53
ST - FAS signaling pathway	-1.9341	0.0040	IL-8, IL-1α, TP53
GO - Apoptotic signaling pathway	-1.6066	0.0020	IL-1β, IL-1α, IL33, TICAM1, TP53
GO - Cell proliferation	-1.6090	0.0020	PIM-1, IL-6, KRT16, CTNN-β1

✧NES: Normalized enrichments score. Values > 0 represent upregulation and values < 0 represent downregulation when comparing azithromycin treated cell culture with BEGM treated cell cultures

*Represents FDR values using GSEA

Abbreviations: ACAT2: acetyl-CoA acetyltransferase 2; ACE2: angiotensin I converting enzyme 2; ACSS2: acyl-CoA synthetase short chain family member 2; ALDOC: aldolase, fructose-bisphosphate C; APP: amyloid beta precursor protein; CTNN-β1: Catennin beta 1; CXCL10: C-X-C motif chemokine ligand 10; DHCR7: 7-dehydrocholesterol reductase; F2RLI: F2R like trypsin receptor 1; FADS: fatty acid desaturase 1; FASN: fatty acid synthase; FDFT: farnesyl-diphosphate farnesyltransferase 1; FDPS: farnesyl diphosphate synthase; HDGF: heparin binding growth factor; GO: GeneOntology; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; IDI-1: isopentenyl-diphosphate delta isomerase 1; IGFBP3:insulin like growth factor binding protein 3; IL-1β: interleukin 1 beta, IL-6: interleukin 6; IL-8: interleukin 8; IL-33: interleukin 33; INSIG-1: insulin induced gene 1; KRT-16: keratin 16; LDLR: low density lipoprotein receptor; LPIN-1: lipin 1; MMP-9: matrix metalloprotease 9; MMP-10: matrix metalloprotease 10; MYC: MYC proto-oncogene; NDST1: N-deacetylase and N-sulfotransferase 1; PIM-1: Pim-1 proto-oncogene, serine/threonine kinase; PLAT: plasminogen activator, tissue type; PLAUR: plasminogen activator, urokinase receptor; PTGS2: prostaglandin-endoperoxide synthase 2; SCD: stearoyl-CoA desaturase; SCP2: sterol carrier protein 2; TGF-β2: tranforming grwoth factor beta 2; TICAM: Toll-like receptor adaptor molecule 1; TMEM97: transmembrane protein 97; TMPRSS2: transmembrane serine protease 2; TMPRSS11D: transmembrane serine protease 11D; TP53: tumour protein 53

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Table E2. Custom selection of differentially expressed genes of biological interest expression between azithromycin and mock treated cells cultures

Genes	FC✧	Unadjusted P value*	Adjusted P value*
SARS-CoV-2 related			
TMPRSS11D	-1.262	0.00805	0.5734
TMPRSS2	-1.260	0.00032	0.1663
TICAM1	-1.153	0.00218	0.3787
Pro-inflammatory and chemotaxis			
NDST1	-1.411	<0.00001	**0.0094**
IL-1β	-1.345	0.00001	**0.0276**
CXCL-10 (IP-10)	-1.334	0.01256	0.6476
PIM-1	-1.333	0.00006	0.0644
PTGS2 (COX-2)	-1.251	0.02132	0.7198
IL-33	-1.241	0.00030	0.1633
IL-6	-1.227	0.00009	0.0755
PLAUR (uPAR)	-1.203	0.00133	0.3155
F2RL1 (PAR-2)	-1.178	0.00494	0.4861
IL-8	-1.165	0.00094	0.2717
Lipid and cholesterol biosynthesis			
INSIG-1	1.940	<0.00001	**0.0054**
HMGCS1	1.755	<0.00001	**0.0148**
FDPS	1.733	0.00001	**0.0230**
ACAT2	1.725	0.00000	**0.0094**
ACSS2	1.697	0.00001	**0.0168**
IDI-1	1.696	0.00000	**0.0139**
LPIN-1	1.668	0.00000	**0.0145**
FASN	1.570	0.00002	**0.0337**
FADS1	1.548	0.00017	0.1109
SCD	1.506	<0.00001	**0.0054**
DHCR7	1.455	0.00003	**0.0499**
FDFT1	1.417	0.00002	**0.0408**
HMGCR	1.415	0.00004	0.0515
ALDOC	1.386	0.00007	0.0688
LDLR	1.325	0.00018	0.1120
TMEM97	1.301	0.00009	0.0755
Cell growth and proliferation			
APP	-1.409	0.00003	**0.0434**
PLAT (tPA)	-1.390	0.00001	**0.0192**
HDGF	-1.362	0.00004	0.0515

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

KRT-16	-1.361	0.00004	**0.0499**
IGFBP3	-1.302	0.00033	0.1663
IL-1α	-1.187	0.00256	0.3795
CTNN-β1	-1.126	0.00981	0.6106
TP53	-1.115	0.01239	0.6454

✧FC: Fold change. A negative value represents downregulation in the azithromycin treated cells compared to BEGM treated cells and vice-versa

*Using FGSEA

Abbreviations: ACAT2: acetyl-CoA acetyltransferase 2; ACE2: angiotensin I converting enzyme 2; ACSS2: acyl-CoA synthetase short chain family member 2; ALDOC: aldolase, fructose-bisphosphate C; APP: amyloid beta precursor protein; CTNN-β1: Catennin beta 1; CXCL10: C-X-C motif chemokine ligand 10; DHCR7: 7-dehydrocholesterol reductase; F2RLI: F2R like trypsin receptor 1; FADS: fatty acid desaturase 1; FASN: fatty acid synthase; FDFT1: farnesyl-diphosphate farnesyltransferase 1; FDPS: farnesyl diphosphate synthase; GO: GeneOntology; HDGF: heparin binding growth factor; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; IDI-1: isopentenyl-diphosphate delta isomerase 1; IGFBP3:insulin like growth factor binding protein 3; IL-1β: interleukin 1 beta, IL-6: interleukin 6; IL-8: interleukin 8; IL-33: interleukin 33; INSIG-1: insulin induced gene 1; KRT-16: keratin 16; LDLR: low density lipoprotein receptor; LPIN-1: lipin 1; MMP-9: matrix metalloprotease 9; MMP-10: matrix metalloprotease 10; MYC: MYC proto-oncogene; NDST1: N-deacetylase and N-sulfotransferase 1; PIM-1: Pim-1 proto-oncogene, serine/threonine kinase; PLAT: plasminogen activator, tissue type; PLAUR: plasminogen activator, urokinase receptor; PTGS2: prostaglandin-endoperoxide synthase 2; SCD: stearoyl-CoA desaturase; SCP2: sterol carrier protein 2; TGF-β2: tranforming grwoth factor beta 2; TICAM: Toll-like receptor adaptor molecule 1; TMEM97: transmembrane protein 97; TMPRSS2: transmembrane serine protease 2; TMPRSS11D: transmembrane serine protease 11D; TP53: tumour protein 53

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

Table E3. Complete list of major pathways between azithromycin and mock treated cells cultures.

NES: Normalized enrichments score. Values > 0 represent upregulation and values < 0 represent downregulation when comparing azithromycin treated cell culture with mock treated cell cultures

Pval: unadjusted P value

Padj: adjusted P value

Leading Edge: Represents the key genes involved in the given pathway

This table is available as an excel file.

Table E4. Complete list of differentially expressed gene between azithromycin and mock treated cells cultures

FC: Fold change. A negative value represents downregulation in the azithromycin treated cells compared to mock treated cells and vice-versa

P.Value: unadjusted P value

adj.P.Val: adjusted P value

logFC: Log transformation of fold change

t: moderated T-test

Code: Probe ID

This table is available as an excel file.

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE

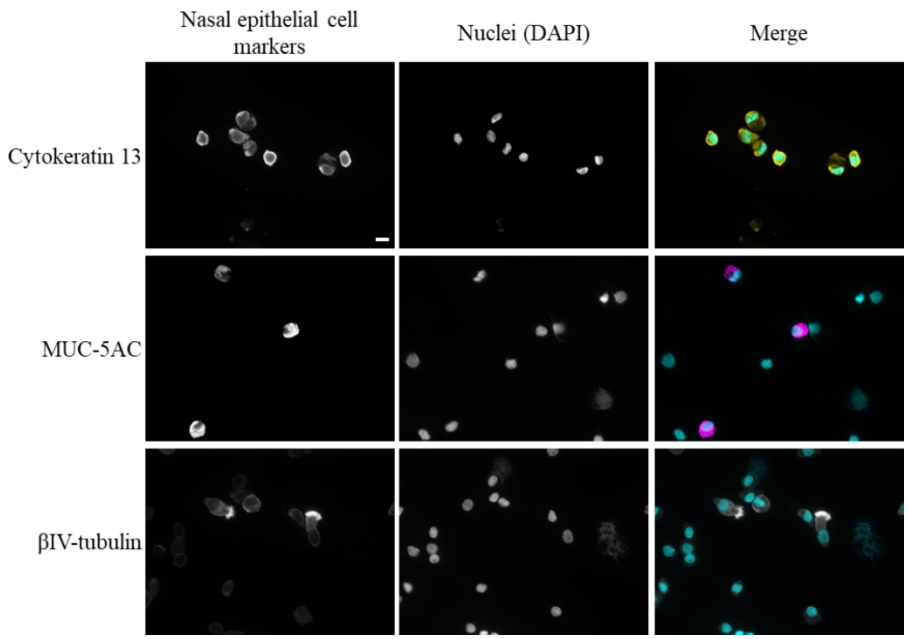

Figure S1

1188x757mm (72 x 72 DPI)

AJRCMB Articles in Press. Published August 28, 2020 as 10.1165/rcmb.2020-0285LE