ORIGINAL ARTICLE
COPD

Azithromycin treatment modifies airway and blood gene expression networks in neutrophilic COPD

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ABSTRACT Long-term, low-dose azithromycin reduces exacerbation frequency in chronic obstructive pulmonary disease (COPD), yet the mechanism remains unclear. This study characterised genome-wide gene expression changes in patients with neutrophilic COPD following long-term, low-dose azithromycin treatment.

Patients with neutrophilic COPD (>61% or >162×10^4 cells per mL sputum neutrophils) were randomised to receive either azithromycin or placebo for 12 weeks. Sputum and blood were obtained before and after 12 weeks of treatment. Gene expression was defined using microarrays. Networks were analysed using the Search Tool for the Retrieval of Interacting Gene database.

In sputum, 403 genes were differentially expressed following azithromycin treatment (171 downregulated and 232 upregulated), and three following placebo treatment (one downregulated and two upregulated) compared to baseline (adjusted $p<0.05$ by paired t-test, fold-change >1.5). In blood, 138 genes were differentially expressed with azithromycin (121 downregulated and 17 upregulated), and zero with placebo compared to baseline (adjusted $p<0.05$ by paired t-test, fold-change >1.3). Network analysis revealed one key network in both sputum (14 genes) and blood (46 genes), involving interferon-stimulated genes, human leukocyte antigens and genes regulating T-cell responses.

Long-term, low-dose azithromycin is associated with downregulation of genes regulating antigen presentation, interferon and T-cell responses, and numerous inflammatory pathways in the airways and blood of neutrophilic COPD patients.

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This study is registered at www.anzctr.org.au with identifier number ACTRN 12609000259246. Data sharing is not in place for this trial.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common illness that poses a major global health burden [1, 2]. It is characterised by airway neutrophilia, persistent airflow obstruction and exacerbations. The prevention of exacerbations in COPD is of great importance given their association with lung function decline, impairment of quality of life, and mortality risk [3–5].

Azithromycin is a macrolide antibiotic used in the treatment of acute, infectious exacerbations of COPD. Long-term use of low-dose azithromycin has been shown to reduce the number of exacerbations in a range of respiratory diseases, including asthma [6], COPD [7–9], cystic fibrosis [10, 11] and non-cystic fibrosis bronchiectasis [12, 13]. Additional benefits include improved lung function in diffuse panbronchiolitis [14] and bronchiolitis obliterans syndrome [15], and a reduced rate of lung function decline in cystic fibrosis [11]. While such clinical outcomes are promising for the future management of these diseases, the underlying mechanism remains unclear.

Macrolides, in addition to their antimicrobial properties, have numerous anti-inflammatory and immunomodulatory effects. Using microarrays to define how macrolides affect gene expression across the whole genome will facilitate the understanding of how they impact airway inflammation, and potentially identify targets for future drug development that do not have the same issues with antibiotic resistance [16].

We hypothesised that 12 weeks of azithromycin treatment would modify gene expression profiles in the airway and blood of patients with neutrophilic COPD. This study characterised these gene expression changes using microarrays to identify the underlying molecular mechanisms responsible for the clinical effects of long-term, low-dose azithromycin in COPD.

Materials and methods

Study design and population

This randomised controlled trial was conducted between April 2009 and December 2011, and clinical and inflammatory results have been published previously [8]. Recruitment for this study targeted adults with doctor diagnosed symptomatic COPD, as described in detail previously [8], with stable persistent neutrophilic inflammation, defined by a sputum differential cell count demonstrating >61% or $>162\times10^4$ cells per mL neutrophils on two separate occasions (with at least one being the screening visit). These cut-offs, as well as inclusion and exclusion criteria have been discussed previously [8].

Eligible participants (n=30) were randomly allocated (1:1) to receive oral azithromycin 250 mg daily or placebo for 12 weeks. In addition to the screening visit, participants attended four visits at monthly intervals with the final study visit conducted 4 weeks after the end of treatment.

Ethics statement

Participants gave written informed consent. The Hunter New England Area Health Service and University of Newcastle Research Human Ethics Committees approved the study (06/12/13/3.08 and H-2008-0272) and it was registered with the Australian New Zealand Clinical Trials Registry ACTRN 12609000259246.

Study protocol

At screening (visit 1) demographics, pre- and post-bronchodilator spirometry, skin-prick testing, medication history, smoking status, and exhaled carbon monoxide were assessed.

At visit 2 (baseline randomisation) mucus hypersecretion, St George's Respiratory Questionnaire [17], symptom visual analogue scores, Clinical COPD Questionnaire [18], and modified Medical Research Council dyspnoea scale were performed. Induced sputum and peripheral blood (PAXgene Blood RNA tubes; PreAnalytix, Hombrechtikon, Switzerland) were collected at the baseline randomisation visit and after 12-weeks of azithromycin or placebo treatment, for gene expression profiling.

Sputum induction and analysis

Sputum was induced at baseline and after the 12-week treatment period with hypertonic saline (4.5%), as previously described [19]. Selected sputum was dispersed using dithiothreitol for differential cell counts or stored by placing directly into RLT buffer for RNA extraction. Total cell counts of leukocytes and their viability were performed on filtered suspensions. Cytospins were prepared, stained (May-Grunwald Geimsa) and a differential cell count obtained from 400 non-squamous cells. Following centrifugation, supernatant was stored at −80°C.

Whole-genome gene expression microarrays

RNA was extracted from induced sputum using RNeasy Mini Kits (Qiagen, Hilden, Germany), whereas peripheral blood collected in PAXgene RNA tubes was extracted using the PAXgene Blood RNA Kit

(Qiagen) according to the manufacturers' instructions. RNA was quantitated using Quant-iT RiboGreen RNA Quantitation Assay Kits (Molecular Probes Inc, Invitrogen, Eugene, OR, USA). A total of 500 ng RNA was reverse-transcribed into cRNA and biotin-uridine-5′-triphosphate labelled using Illumina TotalPrep RNA Amplification Kits (Ambion, Austin, TX, USA). A total of 750 ng cRNA was hybridised to Illumina Sentrix HumanRef-8 Version 2 Expression BeadChips and scanned using an Illumina Bead Station and captured using BeadScan 3.5.11 (Illumina, San Diego, CA, USA). Samples and gene profiling results were included in the analysis if the sample was of suitable purity (optical density 260 nm/280 nm: 1.7–2.1) and was successfully amplified (sufficient cRNA generated) and hybridised (95th percentile of fluorescence score >500) and the data passed quality controls in GeneSpring GX (correlation coefficients and principle component analysis plots; Agilent Technologies, Santa Clara, CA, USA).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [20] and are accessible through GEO Series accession number GSE119314 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119314).

Network analysis

We used the Search Tool for the Retrieval of Interacting Genes (STRING) v9.1 (http://string-db.org/) to identify gene networks amongst our differentially expressed genes. STRING is an online protein–protein interaction database curated from the literature that predicts associations from systemic genome comparisons. All network diagrams presented here are based on high confidence scores (>0.7), to retain the highest possible stringency, as well as at least one network consisting of a node with more than three neighbours.

Statistical methods

Statistical analysis was performed using Stata 11 (StataCorp, College Station, TX, USA). Results are presented as mean±SD or median (interquartile range) with t-tests for parametric data and Wilcoxon rank sum tests for nonparametric data. Paired data were analysed using the Wilcoxon signed rank test. Categorical data were compared using the Chi-squared or Fisher's exact test as appropriate. p-values <0.05 were considered significant.

Microarray data were exported using Genome Studio (Illumina) and analysed using GeneSpring GX12.5 (Agilent Technologies). Data were log transformed and quantile normalisation performed. Data were filtered on expression level, and only genes with a raw fluorescence of >250 in all samples were included in further analysis. Hierarchical clustering analysis was performed using the Euclidean algorithm with Ward's linkage. The Euclidian algorithm takes the standard sum of squared distance between two entities. Ward's linkage, based on the ANOVA approach, computes the sum of squared errors around the mean for each cluster, then the two clusters are joined to minimise the increase in error. Differential gene expression before and after azithromycin treatment or placebo was determined using paired t-tests, and further filtered on a volcano plot, whereby those genes with adjusted p<0.05 (Benjamini–Hochberg) and fold-change >1.5 (sputum) or >1.3 (blood) were included. Gene ontology categories and pathways that were overrepresented in differentially expressed gene lists were determined by using GeneSpring GX12.5 where an adjusted p<0.05 was considered significant.

Results

Sample details

All clinical and inflammatory data has been published previously [8]. Briefly, the mean±SD age was 70±7.6 years, 63.3% were female, and 73.3% were ex-smokers with a mean±SD smoking history of 46.11±36.11 pack-years. The mean±SD forced expiratory volume in 1 s (FEV_1) was 53.69±13.74 % predicted and the mean±SD FEV_1/FVC was 57.79±11.24. There were no significant differences in any clinical or inflammatory parameters between the placebo and azithromycin groups.

Microarray analysis was performed on blood (n=11 azithromycin group; n=12 placebo group) and sputum (n=11 azithromycin group; n=13 placebo group) in a subset of patients who had paired samples from baseline and post-treatment visits, as well as sufficient RNA available. Regarding blood samples, five patients did not have paired samples, and a further two did not have sufficient RNA available for analysis. Regarding sputum samples, three patients did not have paired samples, and a further three did not have sufficient RNA available for analysis.

Exacerbation data has been published in detail previously [8]. Of the subset from the original study that were analysed, six (46.2%) out of 13 in the placebo group and three (21.4%) out of 14 in the azithromycin group experienced an exacerbation. The median (quartile 1, quartile 3) (range) number of exacerbations experienced were 0 (0, 1) (0–2) and 0 (0, 0) (0–1) for the placebo and azithromycin groups, respectively.

Differential gene expression in sputum after azithromycin or placebo treatment

Following azithromycin treatment, the expression of 1877 cRNAs was significantly altered ($p<0.05$ paired t-test) compared to baseline. Further filtering on a volcano plot (adjusted $p<0.05$ paired t-test, fold-change >1.5) resulted in 667 cRNAs that were differentially expressed. This corresponded to 403 genes that were differentially expressed (with unannotated, predicted targets and duplicated probes removed) (171 downregulated, 232 upregulated). Gene ontologies for biological processes that were significantly altered with azithromycin treatment were identified (table 1). Of interest, there were 35 differentially expressed genes involved in immune system processes and nine genes involved in antigen processing and presentation (all genes involved in antigen presentation were also involved in immune system processes) (table 2). Gene expression patterns are detailed in figure 1.

Following treatment with placebo, the expression of 227 cRNAs was significantly altered ($p<0.05$ paired t-test) compared to baseline. Further filtering on a volcano plot (adjusted $p<0.05$ paired t-test, fold-change >1.5 fold) resulted in only three genes being differentially expressed. These were *RASA4P* (RAS p21 protein activator 4, non-coding RNA, 1.56-fold upregulated), *ATP6V0D1* (ATPase, H+ transporting, lysosomal 38 kDa, V0 subunit d1 mRNA, 1.56-fold downregulated) and *HLA-H* (Major histocompatibility complex, class I, H (pseudogene), non-coding RNA, 1.62-fold upregulated). As the expression of these three genes were significantly altered following treatment with both azithromycin and placebo, the altered regulation could not be confidently attributed to azithromycin and they were removed from further analysis.

Differential gene expression in peripheral blood after azithromycin or placebo treatment

Following azithromycin treatment, the expression of 2398 cRNAs was altered ($p<0.05$ paired t-test) compared to baseline. Further filtering using a volcano plot (adjusted $p<0.05$, fold-change >1.5) resulted in 25 cRNAs that were differentially expressed. This corresponded to 22 differentially expressed genes (with unannotated, predicted targets and duplicated probes removed), far fewer than the number seen in sputum. All genes were downregulated (supplementary table S1). There were no significant gene ontologies due to the small number of differentially regulated genes. The use of an identical fold-change cut-off in blood and sputum was, however, deemed impractical as the concentration of azithromycin is often 10-fold lower in plasma than in tissues due to its high cellular penetration [21]. Consequently, the fold-change cut-off was lowered to 1.3 to more accurately reflect this difference, resulting in 166 significantly altered cRNAs (138 genes). 48 of these genes were involved in immune system processes (table 3). Figure 2 shows the shift in gene expression profile after azithromycin treatment.

Following treatment with placebo, the expression of 1374 cRNAs was altered ($p<0.05$ paired t-test) compared to baseline. However, none of these genes reached significance on filtering using the volcano plot (adjusted $p<0.05$ paired t-test, fold-change >1.3).

Network analysis of differentially expressed genes in sputum and blood following azithromycin

To investigate potential pathways underlying the differential gene expression the STRING database was used. From the 35 genes that were differentially expressed in sputum with a fold-change of >1.5 and involved in immune system processes and antigen processing and presentation, 33 genes were recognised by the STRING database. One key network was found that involved 14 genes (figure 3). Key nodes were

TABLE 1 Gene ontology (GO) biological processes altered in the airways with azithromycin treatment

GO biological process	Number of differentially expressed genes	Adjusted p-value
Translational elongation	28	1.25×10^{-17}
Translation	39	7.99×10^{-18}
Immune response	31	0.004
Energy coupled proton transmembrane transport, against electrochemical gradient	5	0.012
Cellular macromolecule biosynthetic process	74	0.016
Immune system processes	36	0.017
Macromolecule biosynthetic process	74	0.017
Antigen processing and presentation	9	0.017
Actin filament-based process	15	0.035
Antigen processing and presentation of peptide antigen	6	0.042
Cellular biosynthetic process	85	0.044

TABLE 2 Differentially expressed genes in sputum after azithromycin treatment involved in immune system processes

Gene symbol	Gene name	p-value	Fold change	Regulation	Probe ID	Accession
HLA-H	Major histocompatibility complex, class I, H (pseudogene), non-coding RNA	0.038	2.3	down	1980592	NR_001434.1
HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	0.045	2.0	down	510079	NM_021983.4
CD83	CD83 molecule, transcript variant 1	0.047	2.0	down	6620026	NM_004233.3
HLA-A	Major histocompatibility complex, class I, A	0.038	1.9	down	3400438	NM_002116.5
HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1	0.049	1.9	down	1190039	NM_033554.2
GBP1	Guanylate binding protein 1, interferon-inducible, 67 kDa	0.039	1.9	down	2190148	NM_002053.1
CFD	Complement factor D (adipsin)	0.043	1.9	down	1240152	NM_001928.2
CTSC	Cathepsin C, transcript variant 1	0.039	1.8	down	5270367	NM_001814.2
HLA-E	Major histocompatibility complex, class I, E	0.041	1.8	down	1030747	NM_005516.4
HLA-B	Major histocompatibility complex, class I, B	0.038	1.8	down	5310168	NM_005514.5
ARHGDIB	Rho GDP dissociation inhibitor beta	0.045	1.7	down	1570193	NM_001175.4
HLA-DRA	Major histocompatibility complex, class II, DR alpha	0.040	1.7	down	2570564	NM_019111.3
CYBB	Cytochrome b-245, beta polypeptide	0.038	1.7	down	840168	NM_000397.2
MSN	Moesin	0.042	1.7	down	730332	NM_002444.2
CCL23	Chemokine (C-C motif) ligand 23, transcript variant CKbeta8	0.038	1.6	down	6110343	NM_145898.1
IFI30	Interferon, gamma-inducible protein 30	0.045	1.6	down	2000300	NM_006332.3
CD14	CD14 molecule, transcript variant 1	0.047	1.6	down	7000369	NM_000591.2
CKLF	Chemokine-like factor, transcript variant 6	0.043	1.6	down	2760519	NM_001040139.1
GBP2	Guanylate binding protein 2, interferon-inducible	0.041	1.6	down	1940162	NM_004120.3
IFITM3	Interferon induced transmembrane protein 3 (1-8U)	0.044	1.6	down	6650242	NM_021034.2
ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	0.045	1.5	down	7200156	NM_000211.1
CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen), transcript variant 1	0.038	1.5	down	4040022	NM_175862.2
BCAP31	B-cell receptor-associated protein 31	0.038	1.5	down	6450138	NM_005745.6
CCL5	Chemokine (C-C motif) ligand 5	0.038	1.5	down	620717	NM_002985.2
DBNL	Drebrin-like, transcript variant 2	0.049	1.5	down	2140671	NM_001014436.1
DCLRE1C	DNA cross-link repair 1C (PSO2 homolog, *Saccharomyces cerevisiae*), transcript variant b	0.038	2.0	up	6860647	NM_022487.2
FCAR	Fc fragment of IgA, receptor for, transcript variant 9	0.049	2.0	up	4250193	NM_133279.1
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	0.043	2.0	up	3370128	NM_005118.2
ZNF160	Zinc finger protein 160, transcript variant 2	0.0378	1.9	up	6980474	NM_198893.1
SPN	Sialophorin, transcript variant 1	0.0378	1.8	up	10358	NM_001030288.1
TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14, transcript variant 2	0.048	1.7	up	1170673	NM_172014.1
DCLRE1C	DNA cross-link repair 1C (PSO2 homolog, *S. cerevisiae*), transcript variant c	0.0388	1.7	up	430044	NM_001033858.1
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a), transcript variant 1	0.0378	1.6	up	4050039	NM_000569.6
LAX1	Lymphocyte transmembrane adaptor 1	0.0378	1.6	up	580411	NM_017773.2
KIR2DS5	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 5	0.0379	1.5	up	2070561	NM_014513.1

determined by the number of connections with other differentially expressed genes. Those with ⩾3 connections are shown in supplementary table S2.

From the 138 genes that were differentially expressed in blood with a fold-change of >1.3, 135 were recognised by the STRING database. One key network was found that involved 46 genes (figure 4). Key nodes were determined by the number of connections with other differentially expressed genes. Those with ⩾3 connections are shown in supplementary table S3.

Discussion

This study examined the underlying mechanisms of long-term, low-dose azithromycin in neutrophilic COPD. Patients with stable, neutrophilic COPD were administered either low-dose azithromycin or placebo

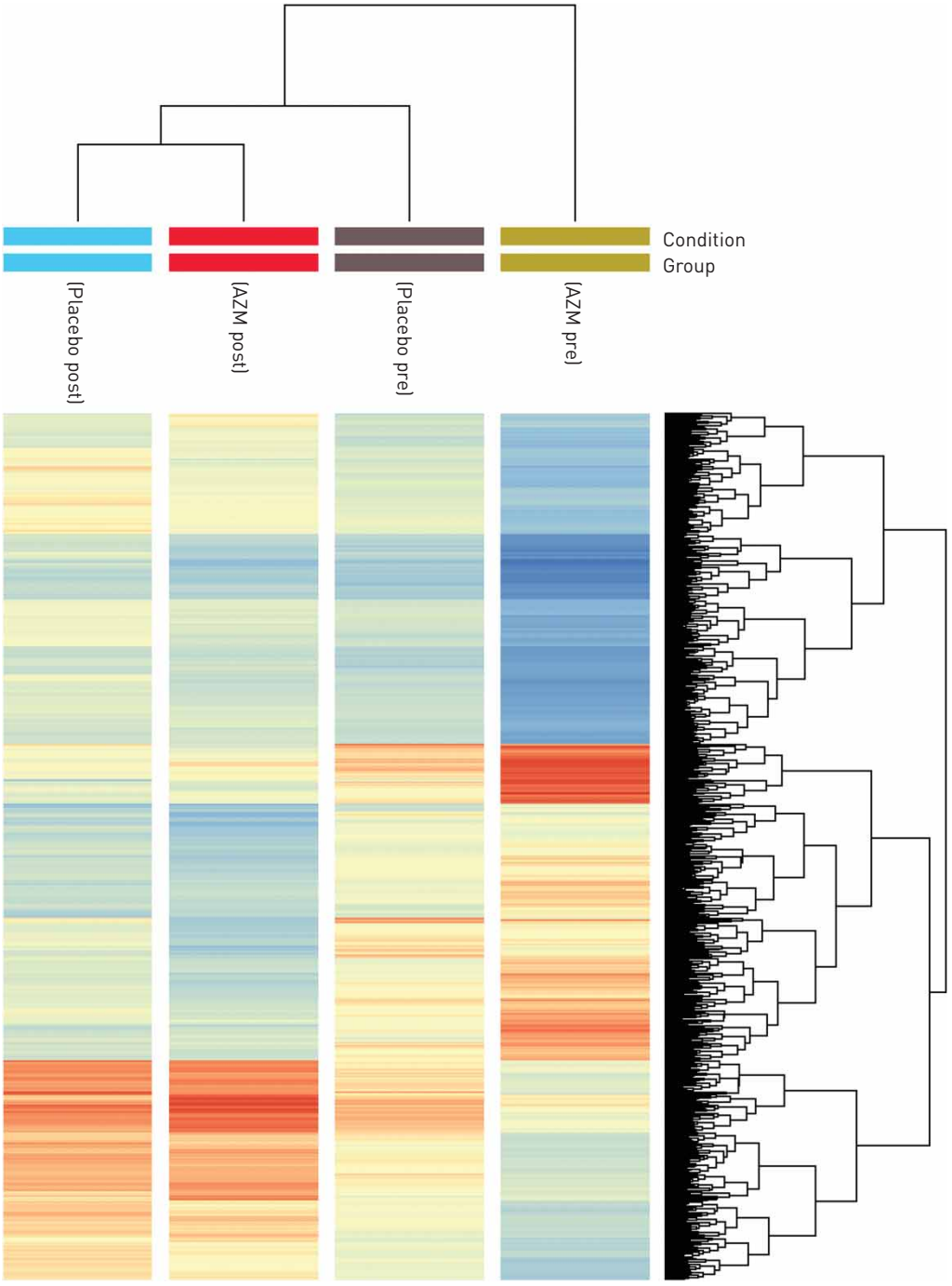

FIGURE 1 Microarray clustering of differentially expressed genes in sputum. Genes are clustered in rows, with red indicating high expression, yellow intermediate expression and blue low expression. Treatment groups are clustered in columns. Groups are clustered to reflect similarities and differences in gene expression (as per the Euclidean algorithm with Ward's linkage). AZM: azithromycin.

over 12 weeks. Treatment with azithromycin resulted in significant changes in gene profiles in sputum and blood, including the downregulation of genes associated with antigen presentation, inflammation, and interferon and T-cell responses. Gene profiles showed only minor changes following treatment with placebo.

COPD exacerbations can be triggered by infectious and non-infectious agents, and the mechanism by which azithromycin prevents exacerbations is probably complex and multi-factorial. Previous research has demonstrated that long-term, low-dose azithromycin suppresses pro-inflammatory cytokine production, enhances macrophage phagocytosis [22, 23] and the expression of anti-inflammatory cytokines [24], and

TABLE 3 Differentially expressed genes in blood after azithromycin treatment involved in immune system processes

Gene symbol	Gene name	p-value	Fold change	Regulation	ProbeID	Accession
SYK	Spleen tyrosine kinase	0.038	1.8	down	110685	NM_003177.3
HLA-DRB3	Major histocompatibility complex, class II, DR beta 3	0.041	1.8	down	1770504	NM_022555.3
GBP5	Guanylate binding protein 5	0.003	1.7	down	1510364	NM_052942.2
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64), transcript variant 1	0.015	1.6	down	2710709	NM_001017986.1
OAS2	2′-5′-oligoadenylate synthetase 2, 69/71 kDa, transcript variant 1	0.039	1.6	down	7320561	NM_016817.2
OAS1	2′,5′-oligoadenylate synthetase 1, 40/46 kDa, transcript variant 3	0.004	1.6	down	1090390	NM_001032409.1
IL4R	Interleukin 4 receptor, transcript variant 1	0.024	1.5	down	5910609	NM_000418.2
GBP1	Guanylate binding protein 1, interferon-inducible, 67 kDa	0.024	1.5	down	2190148	NM_002053.1
GBP2	Guanylate binding protein 2, interferon-inducible	0.003	1.5	down	1940162	NM_004120.3
TAP1	Transporter 1, ATP-binding cassette, sub-family B	0.020	1.4	down	7330392	NM_000593.5
FYN	FYN oncogene related to SRC, FGR, YES, transcript variant 2	0.039	1.4	down	4860050	NM_153047.1
OASL	2′-5′-oligoadenylate synthetase-like, transcript variant 2	0.044	1.4	down	7650097	NM_198213.1
IL1RN	Interleukin 1 receptor antagonist, transcript variant 1	0.036	1.4	down	2470601	NM_173842.1
IFI16	Interferon, gamma-inducible protein 16	0.021	1.4	down	3870594	NM_005531.1
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	0.038	1.4	down	520086	NM_000566.2
HLA-H	Major histocompatibility complex, class I, H (pseudogene), non-coding RNA.	0.045	1.4	down	5870521	NR_001434.1
LILRB2	Leukocyte immunoglobulin-like receptor, subfamily B, member 2, transcript variant 2	0.017	1.4	down	540671	NM_001080978.1
PTPRC	Protein tyrosine phosphatase, receptor type, C, transcript variant 4	0.020	1.3	down	6180288	NM_080923.2
IRF7	Interferon regulatory factor 7, transcript variant b	0.004	1.3	down	1470382	NM_004029.2
IL7R	Interleukin 7 receptor	0.040	1.3	down	3830349	NM_002185.2
IGSF6	Immunoglobulin superfamily, member 6	0.041	1.3	down	3850202	NM_005849.1
ILF2	Interleukin enhancer binding factor 2, 45 kDa	0.038	1.3	down	5690437	NM_004515.2
OAS1	2′,5′-oligoadenylate synthetase 1, 40/46 kDa, transcript variant 3	0.047	1.3	down	7040035	NM_001032409.1
HLA-F	Major histocompatibility complex, class I, F	0.035	1.3	down	7330053	NM_018950.1
PTPRC	Protein tyrosine phosphatase, receptor type, C, transcript variant 2	0.018	1.4	down	2600408	NM_080921.2
HLA-G	HLA-G histocompatibility antigen, class I, G	0.044	1.3	down	2070088	NM_002127.3
BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	0.032	1.4	up	2640192	NM_004331.2

modulates interferon [25–29] and T-cell responses [30, 31]. Importantly, these functions are not universal to all macrolides (reviewed in [32]), with slight structural differences affecting antimicrobial and immunomodulatory properties [28].

Our research demonstrated that azithromycin was associated with a downregulation of several human leukocyte antigen (HLA) molecules in the airways (*HLA-A*, *HLA-B*, *HLA-DPA1*, *HLA-DRA* and *HLA-DRB4*). HLA-A and HLA-B are subtypes of major histocompatibility complex (MHC) class I surface receptors, while HLA-DPA1, HLA-DRA and HLA-DRB4 are subtypes of MHC class II surface receptors. MHC class I and II receptors are involved in the presentation of antigens to $CD4^+$ and $CD8^+$ T-cells, respectively. *TAP-1* expression, which is essential for the formation of MHC-I-peptide complexes (reviewed in [33]) and therefore, for effective antigen presentation to $CD8^+$ T-cells [34], was also downregulated following treatment with azithromycin. Antigen presentation is a critical step in T-cell activation, and therefore for the initiation of the adaptive immune responses in general. Consequently, azithromycin may suppress inflammatory responses in the airways by impairing antigen presentation and therefore limiting the immune system's capacity to identify and respond to antigen. The mechanism, however, is unclear.

Azithromycin was also associated with the downregulation of several interferon-stimulated genes in blood (*EIF2AK2*, *EPSTI*, *GBP1*, *GBP2*, *GBP5*, *HERC5*, *IFI16*, *IFIT1*, *MX1*, *OAS1*, *OAS2*, *OASL* and *UBE2L6*) and sputum (*GBP1*, *GBP2*, *IFI30* and *IFITM3*). This may be explained by the downregulation of several genes that are involved in the induction of interferon expression (*TRIM25* and *IRF7*) and interferon signalling (*STAT1* and *IRF9*). TRIM25 regulates the polyubiquitination of RIG-I, a pattern recognition receptor that responds to viral dsRNA. This process results in the induction of a signalling cascade, which leads to the

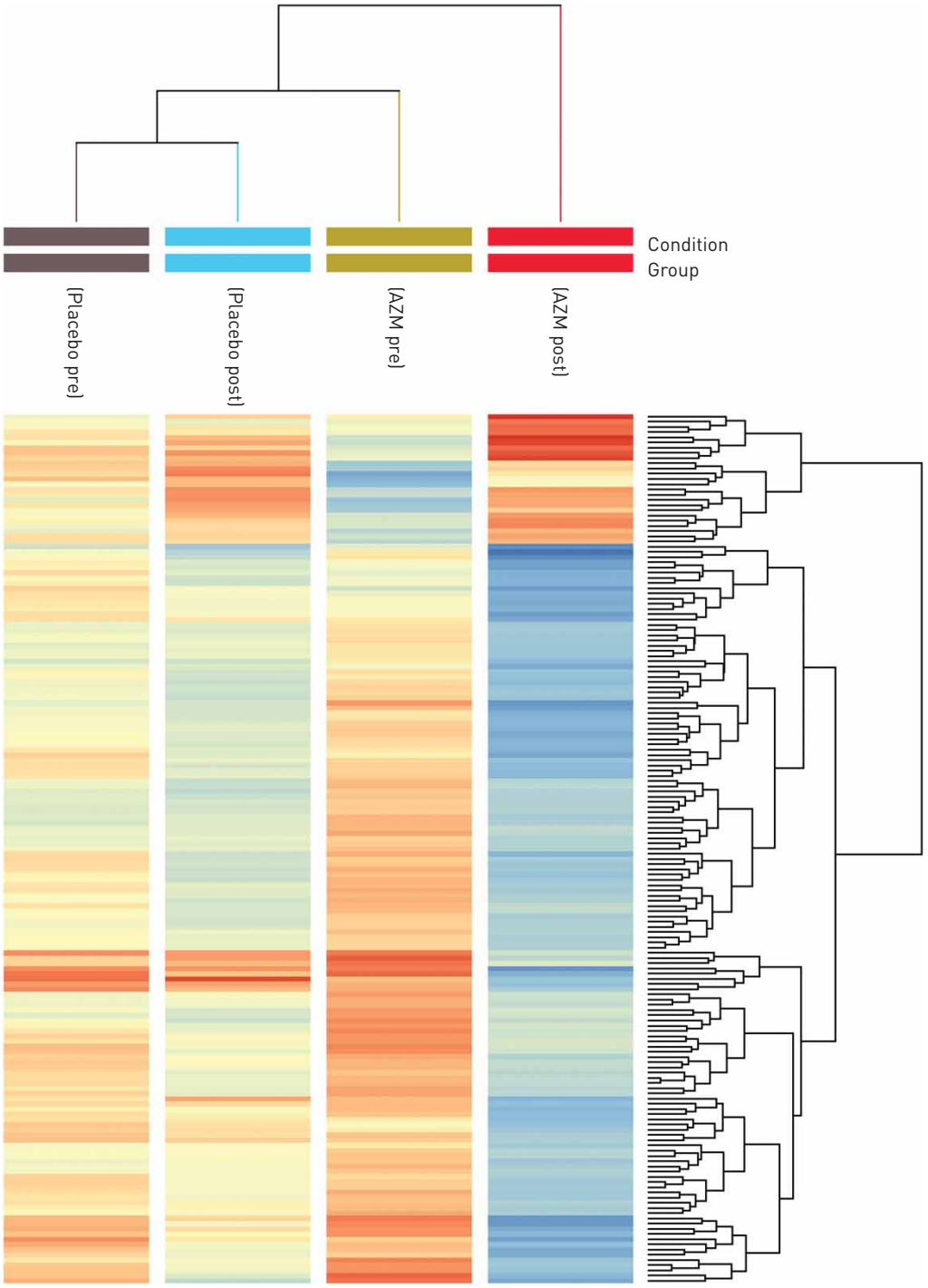

FIGURE 2 Microarray clustering of differentially expressed genes in blood. Genes are clustered in rows, with red indicating high expression, yellow intermediate expression and blue low expression. Treatment groups are clustered in columns. Groups are clustered to reflect similarities and differences in gene expression (as per the Euclidean algorithm with Ward's linkage). AZM: azithromycin.

activation of IRF-7, a transcription factor that induces the expression of type-I interferons (IFN-α and IFN-β). Once interferons are expressed, they are released from the cell and bind to interferon receptors on the surface of neighbouring cells. Interferon receptor activation induces a signalling cascade that culminates in the expression of interferon-stimulated genes. Two key transcription factors involved in this signalling cascade, *STAT1* and *IRF9*, were downregulated following azithromycin treatment. STAT1 forms a STAT1–STAT1 homodimer to induce the expression of IFN-γ-associated genes, and a STAT1–STAT2 heterodimer that complexes with IRF9 for the expression of IFN-α- and IFN-β-associated genes (reviewed

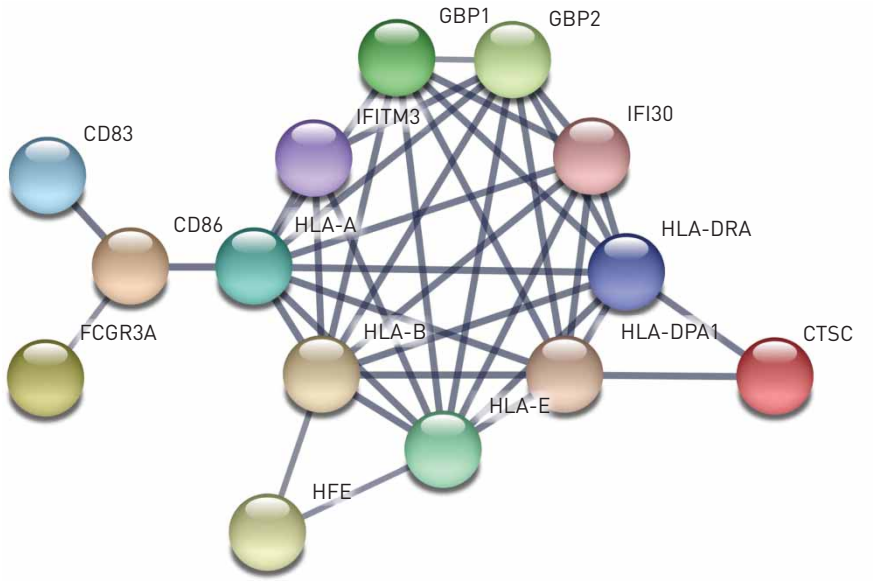

FIGURE 3 STRING network of differentially expressed genes in sputum. Connections reflect protein interactions (either physical or functional).

in [35]). Consequently, interferon responses appear to be suppressed at multiple stages following azithromycin treatment.

As interferon pathways are involved in the induction of inflammatory responses, azithromycin may reduce exacerbation frequency by limiting the inflammation associated with otherwise benign viral infections. In addition, as interferons induce MHC-I and -II expression, suppression of interferon pathways may also explain the observed downregulation of these molecules following azithromycin treatment.

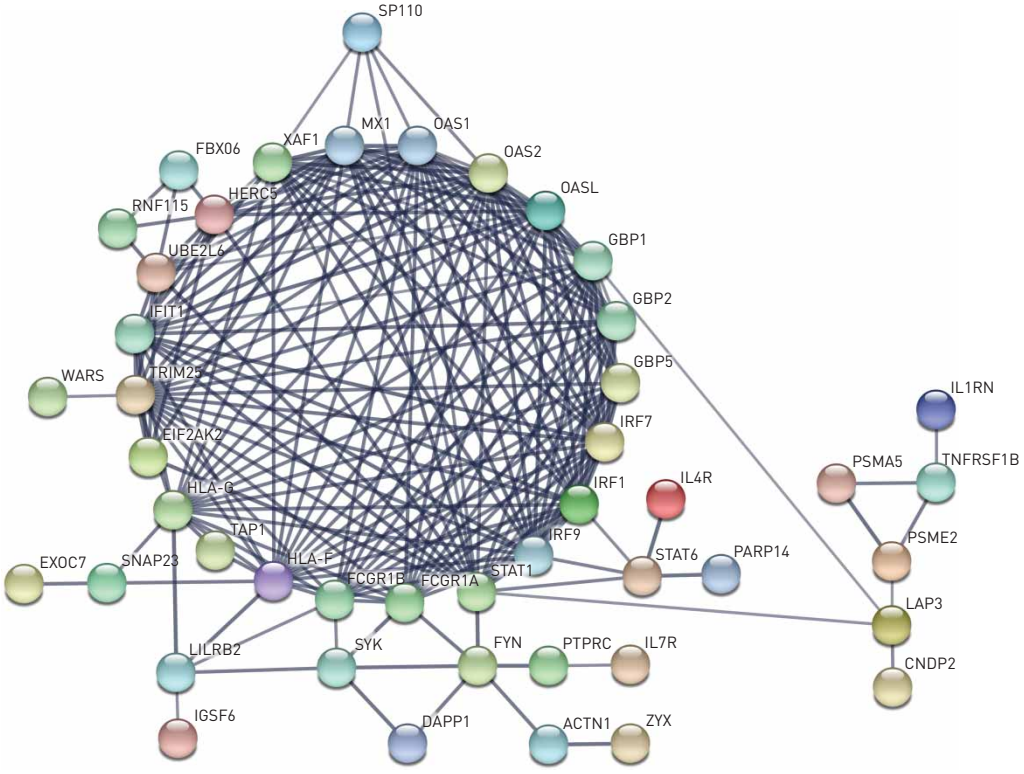

FIGURE 4 STRING network of differentially expressed genes in blood. Connections reflect protein interactions (either physical or functional).

However, as the induction of interferon responses is typically associated with infection, it is unclear whether these changes are a direct effect of azithromycin (*i.e.* through modulation of interferon signalling pathways) or secondary to a reduced microbial load from anti-microbial activity. While a trend towards a reduction in bacterial load was observed in these patients, it was not statistically significant [8]. The original study was, however, insufficiently powered to adequately explore this relationship, and so the delineation of azithromycin's immunomodulatory and antimicrobial properties remains unclear.

Previous studies have demonstrated that azithromycin increases viral-induced interferon responses [25, 28, 29]. In the absence of acute infection, however, azithromycin did not affect interferon expression [25, 28, 29], leading PORTER *et al.* [28] to suggest that macrolides such as azithromycin may work by "priming" interferon pathways. Our results indicate that in the absence of acute viral infection, azithromycin acts as a suppressor of interferon and other inflammatory pathways. Alternatively, a long-term reduction in viral loads by azithromycin may also explain these results. While azithromycin treatment has been shown to induce interferons independent of active viral infection, the effect was transient and lost by 24 h [26]. Our results, taken after 12 weeks of azithromycin treatment, support the notion that this effect is not sustained. Consequently, it is unlikely that infection-independent induction of interferons plays a role in the efficacy of long-term azithromycin treatment.

In the airways, azithromycin was associated with the downregulation of the surface receptor *CD86*, as well as the cytokines *CCL5*, *CCL23* and *CKLF*. Through its interaction with CD28, CD86 (B7-2) is a co-stimulatory molecule required for the activation and continued survival of T-cells. CCL5 (RANTES) [36], CCL23 (MPIF-1) [37] and CKLF [38] are chemokines involved in the chemotaxis of numerous leukocytes including T-cells, neutrophils and monocytes. These results support previous studies that have shown that azithromycin supresses T-cell activation [31] and suggest that it may also limit T-cell migration.

Azithromycin was also associated with the downregulation of several other genes involved in immune signalling, including *TNFRSF1B* (tumour necrosis factor-α receptor), *FCGR1a* and *FCGR1b* (constituents of the IgG receptor), *ILF2* (transcription factor required for the expression of interleukin (IL)-2 by T-cells) and *SYK* (a tyrosine kinase involved in B-cell receptor, T-cell receptor and Fc receptor signalling). Most striking, however, were the effects of azithromycin on the IL-4 signalling pathway, which is critical for T-helper (Th)2 differentiation. The IL-4 receptor (*IL4R*) was downregulated, as were *STAT6*, a transcription factor activated by the IL-4 receptor that forms STAT6–STAT6 homodimers to induce the expression of IL-4 stimulated genes, and *PARP14*, a polymerase that promotes STAT6-dependent transcription [39, 40]. *IRF1*, a key transcription factor for Th1 differentiation, was also downregulated. These results further support the notion that azithromycin suppresses T-cell responses.

In this study, azithromycin treatment resulted in larger fold-changes in gene expression in sputum than in blood. This is probably due to the high cellular penetration of azithromycin, which can result in tissue concentrations 10-fold higher than plasma concentrations [21]. These differences are also expected due to the nature of sputum. As the cells of the airways are exposed to more foreign stimulants than those in blood, the expression of inflammatory genes should be higher in general. Consequently, when baseline gene expression is higher, there is a greater capacity for a reduction in gene expression (and therefore a larger fold-change).

Limitations of this study include the use of mixed cell populations in sputum and blood, which reduced the ability to pinpoint a possible mechanism of azithromycin, and a small sample size, which underpowered the study in general. Furthermore, as all patients had neutrophilic COPD, it is not possible to extrapolate our findings to other respiratory diseases. However, the modulation of universal inflammatory pathways, such as those seen in this study, may explain why azithromycin demonstrates such broad efficacy across a range of respiratory diseases.

In summary, this study indicates that long-term, low-dose azithromycin treatment is associated with the downregulation of genes involved in antigen presentation, interferon and T-cell responses, and numerous inflammatory pathways. These changes may contribute to the capacity of azithromycin to reduce exacerbation frequency in COPD, and if so, suggest that it achieves this clinical outcome through broad modulation of the immune system. This study improves our understanding of azithromycin as an immunomodulatory agent and provides potential novel targets for future drug development. Further research is required to determine how azithromycin induces these changes, and whether they can be achieved without the associated risks of long-term antibiotic use.

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