Transcriptional Response of Respiratory Epithelium to Nontuberculous Mycobacteria

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Abstract

The incidence of pulmonary nontuberculous mycobacteria (NTM) disease is increasing, but host responses in respiratory epithelium infected with NTM are not fully understood. In this work, we aimed to identify infection-relevant gene expression signatures of NTM infection of the respiratory epithelium. We infected air-liquid interface (ALI) primary respiratory epithelial cell cultures with *Mycobacterium avium* subsp. *avium* (MAC) or *Mycobacterium abscessus* subsp. *abscessus* (MAB). We used cells from four different donors to obtain generalizable data. Differentiated respiratory epithelial cells at the ALI were infected with MAC or MAB at a multiplicity of infection of 100:1 or 1,000:1, and RNA sequencing was performed at Days 1 and 3 after infection. In response to infection, we found down-regulation of ciliary genes but upregulation of genes associated with cytokines/chemokines, such as IL-32, and cholesterol biosynthesis. Inflammatory response genes tended to be more upregulated by MAB than by MAC infection. Primary respiratory epithelial cell infection with NTM at the ALI identified ciliary function, cholesterol biosynthesis, and cytokine/chemokine

production as major host responses to infection. Some of these pathways may be amenable to therapeutic manipulation.

Keywords: air-liquid interface method; pulmonary nontuberculous mycobacteria disease; RNA-seq

Clinical Relevance

Pulmonary nontuberculous mycobacteria (NTM) disease has increased over the past several decades. Although respiratory epithelium is clearly affected by nontuberculous mycobacterial infection, the transcriptional response of the epithelium to NTM has not been characterized. We identified ciliary function, cholesterol biosynthesis, and cytokine/chemokine production as major functions and pathways affected by NTM infection. These pathways and functions provide new targets to elucidate the pathophysiology of NTM and develop possible treatments.

Nontuberculous mycobacteria (NTM) are increasingly important causes of pulmonary morbidity and mortality worldwide, especially in industrialized countries (1, 2). Pulmonary NTM (PNTM) disease typically develops in individuals without clearly

recognized host immune defects, and especially in slender, postmenopausal women, suggesting that both environmental exposure and host susceptibility are required for the establishment of PNTM disease.

Several genetic diseases have been associated with PNTM, including cystic fibrosis and primary ciliary dyskinesia (3, 4). PNTM is seen mostly in the setting of bronchiectasis, but the reasons for this remain elusive. In addition, high rates of

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cystic fibrosis transmembrane conductance regulator (*CFTR*) heterozygosity and decreased ciliary beat frequency have been associated with PNTM (5, 6). Whole-exome sequencing identified more low-frequency, protein-affecting variants in immune, *CFTR*, ciliary, and connective-tissue genes in patients with PNTM than in controls (7). It is likely that some of these genes and their relative burdens play important roles in human respiratory and immune cell functions.

Recently, transcriptome analyses such as RNA sequencing (RNA-seq) and microarray have enabled detailed investigations of host responses to NTM infection (8–10). In these studies, human macrophages were infected with NTM bacteria before transcriptome analyses were performed. In macrophages infected with *Mycobacterium avium*, responses involving apoptosis and inflammation were observed (8, 9). In the case of macrophages infected with *Mycobacterium abscessus*, type I interferon (IFN) and proinflammatory cytokine responses were detected (10).

However, the host responses of human respiratory epithelial cells are incompletely characterized. We tried to model NTM infection of human respiratory epithelium by culturing normal primary human bronchial epithelial (NHBE) cells at the air-liquid interface (ALI), and analyzed transcriptomic responses to the PNTM *Mycobacterium avium* subsp. *avium* (MAC) and *Mycobacterium abscessus* subsp. *abscessus* (MAB). We found transcriptional signatures in response to NTM infection that may provide novel and testable targets for elucidating the pathogenesis of these morbid and persistent infections and developing therapies to treat them.

Methods

A detailed description of the materials and methods used in this work is provided in the data supplement.

Table 1. Details of the Normal Primary Human Bronchial Epithelial Cells Used for RNA Sequencing

Materials from LONZA	Lot Number	Sex	Age	Race	Smoking Status
CC-2540	408030	Female	57	Caucasian	Negative
CC-2540	348757	Male	75	Caucasian	Negative
CC-2540	365445	Female	69	Caucasian	Negative
CC-2540	501936	Female	42	Hispanic	Negative

Culture and Differentiation of NHBE Cells

Commercially available primary NHBE cells (Lonza) were cultured under ALI conditions. Primary cells cultured for 3 weeks after transfer to ALI were used in this study. For RNA-seq, we used NHBE cells from four different donors: a 57-year-old woman (57F), a 75-year-old man (75M), a 69-year-old woman (69F), and a 42-year-old woman (42F). Detailed information is shown in Table 1.

Infection with Mycobacteria

Strains of MAC (ATCC 35717) and MAB (ATCC 19977) were used. In addition, they were transformed with a GFP plasmid (pMSP12) (11) to allow for visualization by confocal microscopy. NHBE cells were cultured at the ALI for 3 weeks and then exposed to either MAB or MAC for 3 days (multiplicity of infection [MOI] = 100:1 or 1,000:1). On Days 1 and 3 after infection, samples were harvested. Infected cells in each Transwell from each donor were lysed in sterile water supplemented with 0.05% Triton X-100 at Days 1 and 3 after infection, and appropriate serial dilutions were seeded on Middlebrook 7H11 agar plates to count CFU.

Immunofluorescence Staining and Confocal Microscopy

At Days 1 and 3 after infection with either MAC or MAB, epithelium-containing Transwell inserts were fixed and permeabilized. Next, the epithelium-containing inserts were probed with a rabbit anti- β -tubulin monoclonal antibody labeled with Alexa Fluor 594 (Cell Signaling Technology), and F-actin was labeled with Alexa Fluor 647 phalloidin (Molecular Probes). Subsequently, cell membranes were cut out of the Transwell insert and mounted using VECTASHIELD mounting medium (Vector Laboratories)

with DAPI. Then, confocal microscopy was performed.

RNA Extraction and Sample Preparation

Total RNA was extracted using the RNeasy Mini kit (Qiagen) and treated with DNase I. RNA quantity and quality were ascertained using a Qubit 2.0 fluorometer (Life Technologies) and an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies). All samples had RNA integrity numbers greater than 8.5.

Library Preparation, Sequencing, and Differential-Expression Analysis

In total, 40 strand-specific RNA libraries for high-throughput sequencing were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina). Samples were derived from four different donors, as stated above, for each condition. The R package DESeq2 (12) was used to perform differential-expression tests and generate the log₂ fold change values. Genes were identified as differentially expressed (DE) if they had an adjusted *P* value (Benjamini–Hochberg false discovery rate [FDR] method for multiple testing correction) of 0.05 or less. The data are available under GEO series accession number GSE93526.

Functional Enrichment Analysis and Pathway Analysis of DE Genes

GO terms enriched in the DE genes were identified using the ToppGene Suite (<http://toppgene.cchmc.org>) (13). An adjusted *P* value (Benjamini–Hochberg FDR method for multiple testing correction) of ≤ 0.05 was used as the cutoff criterion. Biological pathways enriched in the data were identified with Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) using Fisher's exact test (*P* value threshold of ≤ 0.05).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Data comparisons among the experimental groups were performed using a paired *t* test, two-way ANOVA, or one-way ANOVA followed by *post hoc* tests. *P* values < 0.05 were considered to be statistically significant.

Results

NTM Bacteria–Cell Association

We used confocal microscopy to localize GFP-producing MAC and MAB in this culture system. Differentiation of NHBE cells was confirmed by cilia movement with a high-speed camera. Infected cells were labeled with phalloidin, which binds F-actin, outlining the cells, and anti- β -tubulin, which identifies cilia and DAPI (nuclear stain). Figures 1A and 1B show confocal images of infected NHBE cultures from two different donors (57F and 75M, respectively). Other donors are shown in Figures E1A and E1B (69F and 42F, respectively) in the data supplement. Cells exposed to MAC and MAB (both at MOIs of 100 and 1,000) were studied on Days 1 and 3 after infection. When cells exposed to MAC and MAB (at an MOI of 1,000) were studied on Day 8 after infection by confocal microscopy, most of the cells were dead and many bacteria were observed in MAB-infected respiratory epithelium (data not shown). Therefore, we decided to focus on infection at Days 1 and 3 for further analysis. At 1 day of infection, both MAC and MAB at either MOI tended to be bound to both ciliated and nonciliated cells (Figures 1 and E1). By 3 days of infection, the NTM localization varied by the NHBE donor (Figures 1 and E1). For example, most of the NTM existed in the cytoplasm at Day 3, especially at an MOI of 1,000 in the epithelium derived from 57F (Figure 1A). However, most of the NTM were still on the epithelial surface, even at an MOI of 1,000, in the epithelium derived from 75M (Figure 1B).

We assessed the number of bacteria associated with respiratory epithelium at Days 1 and 3 after infection (Figure 1C). At 1 day of infection, the number of bacteria at MOI 1,000 was significantly higher than at MOI 100 among both MAC-infected and MAB-infected respiratory epithelia. In addition, infected epithelium showed very similar numbers of MAC and MAB bacteria at MOIs of 100 and 1,000. By contrast, at 3 days after infection, there were differences between MAC and MAB infections at either MOI, with extensive donor-to-donor variability.

The percent cell association (% cells with bound or ingested bacteria) was measured by confocal microscopy on Days 1 and 3 after infection. MAB at MOI 1,000 at

Figure 1. NTM associated with NHBE cells at the air-liquid interface. (A and B) Infected NHBE cells were fixed with paraformaldehyde, immunofluorescently labeled, and analyzed by confocal microscopy. Immunostaining: F-actin (magenta), β -tubulin (red). MAC and MAB: GFP (green). Cell nuclei: DAPI (blue). Extracellular (white arrows) and intracellular (yellow arrows) bacteria are shown across z-stacks. Scale bars: 5 μ m. (A) Confocal images from a 57-year-old woman (57F). (B) Confocal images from a 75-year-old man (75M). MAC 100 and MAC 1,000: MAC at an MOI of 100 and 1,000; MAB 100 and MAB 1,000: MAB at an MOI of 100 and 1,000. (C) Mycobacteria growth in the Transwell at Days 1 and 3 after inoculation of 5×10^6 CFU (MOI = 100) or 5×10^7 CFU (MOI = 1,000) of MAC or MAB. The results are expressed as CFU per Transwell from three donors. *Significant difference between an MOI of 100 and an MOI of 1,000 at 1 day after infection ($P < 0.05$).

Table 2. Top 10 Upregulated and Downregulated Differentially Expressed Genes (FDR < 0.05) for MAC- or MAB-infected Respiratory Epithelium versus Uninfected Control Respiratory Epithelium as Ranked by Log₂ Fold Change at Days 1 and 3 after Infection

D1MAB100			D1MAB1000			D1MAC100			D1MAC1000		
Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj
CCL20	0.74	3.4E-10	VNN3	1.80	8.2E-10	ICAM1	0.92	9.5E-18	CXCL2	1.18	9.0E-20
RGPD5	0.66	2.2E-08	ANGPTL4	1.79	1.4E-15	IL32	0.87	4.2E-15	IL32	1.03	1.4E-21
CXCL3	0.64	6.4E-07	C1QTNF1	1.78	1.1E-07	SLC26A4	0.80	2.6E-09	ICAM1	1.03	4.8E-17
ICAM1	0.61	3.4E-10	EBI3	1.72	2.2E-06	SOD2	0.80	1.3E-15	SOD2	0.93	7.2E-18
VNN3	0.56	3.5E-05	KDR	1.66	6.3E-09	C15orf48	0.77	2.7E-12	PGLYRP4	0.92	3.8E-14
CXCL2	0.55	5.7E-05	ZC3H12A	1.61	2.8E-08	ZC3H12A	0.76	1.0E-08	CXCL8	0.88	1.2E-13
NFKBIZ	0.53	1.8E-06	DUSP5	1.59	3.9E-07	VNN3	0.75	1.5E-07	VNN3	0.87	2.5E-09
ZC3H12A	0.53	6.3E-05	THEMIS2	1.54	1.8E-10	TNFAIP3	0.74	7.5E-17	CSF3	0.87	1.2E-09
BCL9L	0.52	2.3E-07	NFKBIZ	1.53	1.5E-07	TNFAIP2	0.72	4.1E-20	TNFAIP3	0.84	2.3E-22
TNFAIP3	0.51	1.1E-08	HCG4B	1.51	6.3E-09	CXCL3	0.71	1.2E-06	CXCL3	0.83	2.3E-08
HLA-DPA1	-0.44	0.0057	NRSN1	-1.50	4.7E-05	CYP2F1	-0.50	0.0025	ATP6V1B1	-0.62	1.6E-07
RGPD6	-0.39	0.0023	EGFL6	-1.49	2.5E-07	ANPEP	-0.46	0.0007	KLRD1	-0.60	0.0002
CAPS	-0.34	0.0236	ADH1C	-1.44	1.4E-09	PTPRZ1	-0.45	6.5E-05	SPAG17	-0.59	3.9E-05
N/A	N/A	N/A	PTPRZ1	-1.38	1.3E-15	DNAI1	-0.44	0.022	FAM216B	-0.58	1.4E-05
N/A	N/A	N/A	LRRC26	-1.23	5.7E-06	POU2F1	-0.43	0.012	PTPN4	-0.55	0.0003
N/A	N/A	N/A	USP2	-1.22	0.0001	MUC5B	-0.43	0.000	CYP2F1	-0.55	0.0005
N/A	N/A	N/A	GAS1	-1.17	0.0005	VWA3A	-0.42	0.036	ALOX15	-0.54	2.3E-08
N/A	N/A	N/A	LMCD1	-1.14	0.0031	ZMYND10	-0.42	0.020	CDHR3	-0.53	3.9E-05
N/A	N/A	N/A	RPTN	-1.13	0.0016	CFAP46	-0.42	0.038	ENKUR	-0.53	0.0001
N/A	N/A	N/A	ADGRF5	-1.13	0.0011	CC2D2A	-0.41	0.013	CYP4B1	-0.52	0.0006

D3MAB100			D3MAB1000			D3MAC100			D3MAC1000		
Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj	Gene	Log ₂ FC	Padj
CCL20	1.90	1.1E-64	IL32	1.35	4.4E-07	WDR12	0.78	3.5E-20	PI3	1.15	8.2E-14
UBD	1.24	2.1E-17	FN1	1.31	4.4E-07	C15orf48	0.77	4.1E-26	CXCL3	1.05	5.1E-12
PDGFB	1.24	1.0E-11	TREM1	1.18	4.5E-06	IL32	0.76	2.3E-09	TNFAIP3	1.04	5.1E-18
IL36G	1.21	5.2E-09	TNFAIP3	1.15	2.6E-06	UBD	0.75	3.7E-09	IL32	1.03	1.2E-12
CXCL8	1.16	1.1E-33	GLIPR1	1.09	5.1E-05	TNFAIP3	0.71	3.7E-09	IL1A	1.03	1.3E-16
ANGPTL4	1.11	5.2E-13	EDN1	1.08	0.0002	CXCL6	0.71	3.7E-09	CXCL6	1.00	5.2E-18
CXCL2	1.07	7.1E-09	IL23A	1.04	0.0011	PLAU	0.67	8.0E-20	INHBA	0.93	2.6E-09
ICAM1	1.03	8.7E-15	LIF	1.01	0.0010	CXCL2	0.61	5.9E-06	OR211P	0.92	3.5E-09
C15orf48	0.99	1.1E-16	OR211P	0.99	0.0019	C1S	0.60	4.4E-06	C15orf48	0.89	5.1E-18
OR211P	0.98	5.1E-06	SLC2A6	0.96	0.0013	CXCL3	0.58	2.4E-05	ABCA13	0.87	1.2E-12
CYP2F1	-1.16	1.3E-10	DEGS2	-1.61	4.3E-10	CYP2F1	-0.48	0.001	CYP4B1	-0.87	2.7E-12
ADH7	-0.87	8.0E-11	LRRC26	-1.46	3.6E-07	IQCG	-0.45	3.7E-05	DEGS2	-0.80	1.2E-06
GSTA2	-0.84	2.4E-07	DNAH6	-1.45	2.2E-07	GSTA2	-0.41	0.014	DNAH6	-0.79	8.6E-10
DEGS2	-0.80	0.0007	SCGB3A1	-1.43	6.3E-08	CFAP161	-0.40	0.013	MSA48	-0.68	2.4E-05
IGSF10	-0.76	0.0019	C6	-1.41	6.8E-07	IFIT1	-0.40	0.017	CFAP52	-0.64	2.3E-05
ADH1C	-0.71	5.0E-07	WDR49	-1.36	1.4E-06	VPS41	-0.39	6.8E-05	STATH	-0.63	0.0001
KLK7	-0.66	8.2E-05	PLPPR3	-1.35	2.8E-06	STATH	-0.39	0.025	NWD1	-0.61	1.3E-07
CRIP1	-0.66	0.011	CYP4B1	-1.32	2.2E-07	PDS5B	-0.38	0.001	COL21A1	-0.60	0.0003
FAM46C	-0.65	0.002	KNDC1	-1.26	1.2E-05	WDR49	-0.38	0.027	ATP6V1B1	-0.60	0.0002
SMN1	-0.64	0.019	CCDC78	-1.26	4.6E-06	RNF25	-0.38	0.027	CFAP161	-0.60	0.0004

Definition of abbreviations: FC = fold change; FDR = false discovery rate; MAC = *Mycobacterium avium* subsp. *avium*; MAB = *Mycobacterium abscessus* subsp. *abscessus*; N/A = not applicable; Padj = adjusted *P* value.

1 day after infection showed only an ~20% cell association, and MAC at MOI 1,000 at 1 day after infection showed an ~25% cell association. MAB at MOI 1,000 3 days after

infection showed an ~50% cell association, whereas MAC at MOI 1,000 3 days after infection showed only an ~20% cell association (Figure 1D). Measurements of

lactate dehydrogenase as a marker for cytotoxicity showed no significant increases in the basolateral medium at Days 1 and 3 after infection (Figure E2), indicating that

Figure 1. (Continued). two-way ANOVA followed by *post hoc* tests). Data are expressed as the mean $\pm$ SEM. (D) Percent of respiratory epithelial cells with associated bacteria. Tiled images of infected respiratory epithelium were analyzed and the percentage of infected cells was determined as the total number of cells associated with bacteria. Data are expressed as the mean $\pm$ SEM from four donors. No statistically significant changes were observed (two-way ANOVA followed by *post hoc* tests). CFU = colony-forming unit; MAB = *Mycobacterium abscessus* subsp. *abscessus*; MAC = *Mycobacterium avium* subsp. *avium*; MOI = multiplicity of infection; NHBE = normal primary human bronchial epithelial; NTM = nontuberculous mycobacteria.

the viability of the respiratory epithelium was intact even when it was infected with NTM at an MOI of 1,000.

Gene Expression Analysis

To investigate the differential expression of mRNA transcripts upon infection with MAC and MAB, we performed a DESeq2 analysis using RNA-seq data obtained on Days 1 and 3 after infection. To focus on DE genes that were affected by the bacteria in a consistent manner across donors, we used a paired analysis model in DESeq2 that took into account donor-to-donor variability. We determined DE genes for each condition compared with uninfected controls at Days 1 and 3 after infection, respectively, for FDR-adjusted *P* values of 0.05. The top 10 upregulated and downregulated genes (ranked by log₂ fold change) that were expressed in respiratory epithelium infected with MAC or MAB at Days 1 and 3 after infection are shown in Table 2 (all DE genes are shown in Table E1). The log₂ fold change values were at most 2, even at an MOI of 1,000, suggesting an overall low responsiveness of lung epithelial cells to bacterial challenge. Genes involved in inflammatory responses were upregulated at both Days 1 and 3 after infection, including C-C motif chemokine ligand 20 (*CCL20*), colony stimulating factor 3 (*CSF3*), C-X-C motif chemokine ligand 8 (*CXCL8*), and IL-32 (Table 2). Genes related to cilia were significantly downregulated at Days 1 and 3 after infection, including dynein axonemal intermediate chain 1 (*DNAI1*), sperm-associated antigen 17 (*SPAG17*), and dynein axonemal heavy chain 6 (*DNAH6*) (Table 2).

The DE genes that were common to all four conditions (MAB and MAC at MOIs of 100 and 1,000) are listed in Table E2 and consisted of 16 and 39 genes at Days 1 and 3 after infection, respectively (Figure E3). These genes included those related to chemokines and immune functions. Among them, IL-32, NFKB inhibitor α (*NFKBIA*), TNF- α -induced protein 3 (*TNFAIP3*), and peptidoglycan recognition protein 4 (*PGLYRP4*) were upregulated under all four conditions at both Days 1 and 3 after infection. To validate the RNA-seq data, we measured the expression of these four genes by real-time quantitative PCR (qPCR) and examined the correlation expression measurements based on RNA-seq. RNA-seq and qPCR confirmed a statistically significant Pearson correlation across samples for each gene (Figure E4).

Because genes related to cytokines and chemokines were upregulated in NTM-infected respiratory epithelium, we analyzed the protein production of CCL20, CSF3, and CXCL8. Reflecting the RNA-seq results, these three proteins were higher in infected epithelium supernatants than in uninfected ones at Days 1 and 3 after infection (Figure 2). However, high donor-to-donor variability was observed.

To visualize gene expression under various conditions, we created a heatmap using the mean log₂ fold change of the 50 most upregulated and 50 most downregulated genes in respiratory epithelium infected with MAC at an MOI of 1,000 on Day 1 after infection, because MAC PNTM infection is

more common than MAB infection (Figure 3). Both MAC and MAB induced genes related to chemokines, cytokines, and oxidative stress, including IL-32, vanin 3 (*VNN3*), *TNFAIP2*, *CCL20*, and dual oxidase 2 (*DUOX2*). One day after infection, the magnitude of gene induction in this gene set by MAB at an MOI of 1,000 was generally greater than that observed with MAC at the same MOI. In contrast, both MAC and MAB infections decreased the expression of genes related to cilia, including cilia- and flagella-associated protein 43 (*CFAP43*), *CFAP45*, radial spoke head 4 homolog A (*RSPH4A*), dynein axonemal intermediate chain 1 (*DNAI1*), dynein axonemal heavy chain 12 (*DNAH12*), and radial spoke head 1 homolog (*RSPH1*).

Figure 2. Basolateral cytokine and chemokine production. Production of CCL20 (A), colony-stimulating factor 3 (CSF3) (B), and CXCL8 (C) was measured in infected and uninfected cultures at Days 1 and 3 after infection. The same samples that were analyzed by RNA-seq were used. *Significant difference between infected and uninfected respiratory epithelium at Days 1 and 3 after infection ($P < 0.05$, paired *t* test). Data are expressed as the mean $\pm$ SEM from four donors. RNA-seq = RNA sequencing.

Figure 3. Genes related to chemokines, cytokines, oxidative stress, and cilia were highly altered compared with uninfected controls. Hierarchical clustering based on the mean log₂ fold change of the 50 most upregulated and 50 most downregulated genes in MAC-infected (MOI = 1,000) respiratory epithelium at Day 1 after infection. FC = fold change.

The down-regulation of these genes by MAB at an MOI of 1,000 was greater than that observed with MAC at the same MOI on Day 1 after infection, as well as on Day 3.

To verify that the immune response genes detected in cultures were not due to residual leukocytes, we looked for CD45 staining by flow cytometry and found none (Figure E5), confirming that the immune gene expression responses we detected were not coming from differentiated leukocytes.

Functional Categorization of DE Genes and IPA

We performed a functional categorization of DE genes using the ToppGene Suite (<http://toppgene.cchmc.org>) at Days 1 and 3 after infection to identify enriched Gene Ontology (GO) terms, including those from biological processes, molecular functions, and cellular components (Tables E3 and E4). Table 3 shows the top 10 significantly enriched GO terms for either up- or downregulated genes across all four

conditions. At Day 1 after infection, upregulated DE genes across all four conditions were significantly enriched in GO categories related to inflammation and apoptosis, such as inflammatory response, programmed cell death, and apoptotic processes. In addition, the number of genes related to GO categories associated with inflammation and apoptosis was much higher in response to MAB at an MOI of 1,000 compared with other conditions (Table 3).

In contrast, on Day 1 after infection, the downregulated genes from MAB at an MOI of 1,000 were predominantly enriched in GO categories related to mitochondria (Table 3). Moreover, in respiratory epithelium infected with MAC at an MOI of 100 or 1,000, the GO categories related to cilia (including cilium organization, cilium assembly, and cilium) were notable (Table 3).

At Day 3 after infection, the upregulated DE genes derived from each condition were predominantly enriched in GO categories related to inflammation and cell motility

(Table 3). The downregulated genes in respiratory epithelium infected with MAC at MOI 1,000 and MAB at either MOI 100 or 1,000 were predominantly enriched in GO terms related to cilia, such as cilium movement, cilium assembly, cilium organization, and cilium (Table 3). Taken together, these results suggest that both MAC and MAB infections downregulate cilia-related gene expression and upregulate chemokine gene expression.

Given the observed down-regulation of cilia-related genes, we measured the percentage of ciliated cells by confocal microscopy after MAB or MAC infection at an MOI of 1,000. Because there was high heterogeneity in terms of ciliation in this ALI culture, we obtained a large tiled image to robustly measure the percentage of ciliated cells (Figure E6). The percentage of ciliated cells was lower in NTM-infected respiratory epithelium than in uninfected respiratory epithelium (Figure 4).

IPA was also used to identify the canonical pathways that were enriched for DE genes at Days 1 and 3 after infection (Table E5). Table 4

Table 3. The Most Significantly Enriched GO Terms for Upregulated or Downregulated Genes at Days 1 and 3 after Infection

	GO Name	D1MAB100		D1MAB1000		D1MAC100		D1MAC1000	
		Padj	Number of Genes	Padj	Number of Genes	Padj	Number of Genes	Padj	Number of Genes
Upregulated	Response to lipopolysaccharide	1.7E-07	11	2.5E-08	43	2.0E-08	17	1.1E-16	33
	Inflammatory response	7.8E-07	13	1.3E-14	85	5.4E-13	29	7.7E-19	49
	Response to bacterium	9.3E-07	12	5.0E-08	60	1.3E-07	20	4.3E-16	41
	Programmed cell death	3.4E-04	15	8.4E-15	166	3.7E-03	26	3.7E-10	63
Downregulated	Apoptotic process	3.0E-04	15	5.5E-15	165	3.2E-03	26	2.1E-10	63
	Cilium	N/A	N/A	N/A	N/A	N/A	N/A	3.8E-07	17
	Cilium organization	N/A	N/A	N/A	N/A	2.2E-02	4	4.7E-06	12
	Cilium assembly	N/A	N/A	N/A	N/A	2.2E-02	4	4.9E-06	12
	Mitochondrion	N/A	N/A	8.3E-09	108	N/A	N/A	N/A	N/A
	Mitochondrial part	N/A	N/A	1.8E-08	71	N/A	N/A	N/A	N/A

	GO Name	D3MAB100		D3MAB1000		D3MAC100		D3MAC1000	
		Padj	Number of Genes	Padj	Number of Genes	Padj	Number of Genes	Padj	Number of Genes
Upregulated	Regulation of cell motility	4.3E-21	51	2.4E-11	36	6.7E-09	28	9.5E-20	63
	Cell migration	5.6E-16	56	2.4E-10	44	6.7E-09	36	1.9E-19	81
	Defense response	4.3E-14	60	2.2E-05	39	2.1E-12	48	3.8E-23	100
	Inflammatory response	8.2E-17	43	1.2E-04	22	9.1E-11	30	1.1E-21	63
Downregulated	Cilium	N/A	N/A	1.4E-22	59	N/A	N/A	2.0E-07	28
	Cilium movement	3.5E-02	4	1.6E-19	22	N/A	N/A	1.2E-03	8
	Cilium assembly	N/A	N/A	4.0E-16	37	N/A	N/A	6.1E-04	17
	Cilium organization	N/A	N/A	1.2E-15	36	N/A	N/A	3.9E-03	15

Definition of abbreviations: GO = Gene Ontology; MAC = *Mycobacterium avium* subsp. *avium*; MAB = *Mycobacterium abscessus* subsp. *abscessus*; N/A = not applicable; Padj = adjusted *P* value.

Figure 4. The percentage of ciliated cells decreased in NTM-infected respiratory epithelium. (A) Representative images of NHBE cells derived from a 67-year-old woman (67F). Infected or uninfected NHBE cells were fixed with paraformaldehyde, immunofluorescently labeled, and analyzed by confocal microscopy. Immunostaining: F-actin (magenta), β -tubulin (red). MAC and MAB: GFP (green). Scale bars: 100 μ m. (B) Tiled images of infected or uninfected respiratory epithelium were analyzed and the percentage of ciliated cells was determined as the total number of cells high in β -tubulin staining. Data are expressed as the mean $\pm$ SEM (three different donors [67F, 44F, and 67M], two fields of view per each donor). *Significant difference between infected and uninfected epithelium at Days 1 and 3 after infection ($P < 0.05$, one-way ANOVA followed by *post hoc* tests).

shows the top 10 significantly enriched canonical pathways across all four conditions. One day after infection, the majority of pathways induced by both MAC and MAB infection were related to immunobiological functions (Tables 4 and E5), such as Toll-like receptor (TLR) signaling and IL-17 activity. In addition to immune functions, cholesterol biosynthesis genes were also significantly upregulated under most circumstances at Day 1 after infection (Table 4 and Figure E7).

At Day 3 after infection, the major canonical pathways observed under all

conditions were related to immunobiological functions such as TLR signaling and the IL-17 signaling pathway (Tables 4 and E5). As shown in Figure E8, the canonical pathway for TLR signaling was significantly enriched at Day 3 after infection in MAC-infected respiratory epithelium at an MOI of 1,000. Figure 5A also shows the canonical pathway for IL-17A signaling in airway cells under the same condition. The downstream targets within the IL-17 signaling pathway were elevated, although IL-17A itself was not upregulated. In fact, no expression of IL-17A

was detected in infected or uninfected respiratory epithelium at Days 1 and 3 by real-time qPCR. The value of cycle threshold (Ct) was undetectable. When MAC-infected respiratory epithelia were cotreated with exogenous IL-17A, additive induction of CCL20 and human β defensin 2 (HBD-2) was observed (Figures 5B and 5C).

Discussion

The normal respiratory epithelium is a complex milieu of ciliated, nonciliated, and immune cells existing at the ALI. Differentiation of NHBE cells by growth at the ALI is known to affect function, as evinced by particle exposure in ALI and submerged cells (14). Therefore, we developed NHBE cells at the ALI to study NTM infection.

We found that large inocula of bacteria were required for MAC and MAB infections. At an MOI of 5 for 1 day, we could not detect bacteria in differentiated NHBE cells by confocal microscopy (not shown). Previous studies found that only small numbers of MAC bacteria adhered to intact mucosa in organ cultures, despite the use of large inocula (15, 16). Moreover, epithelial damage, which exposes extracellular matrix, and poor mucus clearance both predispose to MAC infection (16). Consistent with these findings, the rates of infection were only at most $\sim 50\%$. Also, the log₂ fold change values were at most 2 from RNA-seq data, even at an MOI of 1,000.

In contrast to the NTM in this study, some bacterial pathogens, such as *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, and *Mycoplasma pneumoniae* specifically target ciliated cells for adherence and can infect respiratory epithelium with smaller MOIs over shorter incubations (17). In our study, MAC and MAB adhered to respiratory epithelium regardless of the ciliation of the cell. In addition, a day was needed to complete NTM infection. Taken together, and consistent with the clinical reality of older patients who have been exposed to NTM for a long time before developing overt disease, our results indicate that NTM infection of the respiratory epithelium is markedly different from infections caused by other bacteria.

Previous reports have shown that ciliary defects are important for susceptibility to PNTM (6, 7). It is therefore notable that genes related to cilia were

Table 4. The Most Significantly Enriched Canonical Pathways at Days 1 and 3 after Infection

Canonical Pathway	D1MAB100		D1MAB1000		D1MAC100		D1MAC1000	
	Pval	% Genes	Pval	% Genes	Pval	% Genes	Pval	% Genes
Superpathway of cholesterol biosynthesis	N/A	N/A	5.9E-05	32.1	3.0E-05	14.3	3.0E-10	32.1
IL-17A signaling in fibroblasts	2.1E-03	5.71	1.9E-03	22.9	2.1E-02	5.71	1.3E-13	34.3
Role of IL-17A in psoriasis	9.1E-09	30.8	N/A	N/A	7.2E-05	23.1	4.0E-12	61.5
Acute phase response signaling	3.5E-04	2.34	2.9E-06	17.2	1.2E-04	4.14	2.5E-11	11.2
Role of IL-17A in arthritis	2.4E-07	7.25	N/A	N/A	9.3E-04	5.97	7.8E-09	16.4
IL-17A signaling in airway cells	1.8E-05	5.06	N/A	N/A	1.6E-03	5.19	3.7E-07	13
IL-17A signaling in gastric cells	1.6E-05	12	N/A	N/A	N/A	N/A	4.0E-05	20
Toll-like receptor signaling	4.2E-04	4.05	3.7E-04	18.9	1.3E-03	5.41	2.5E-06	12.2

Canonical Pathway	D3MAB100		D3MAB1000		D3MAC100		D3MAC1000	
	Pval	% Genes	Pval	% Genes	Pval	% Genes	Pval	% Genes
Role of IL-17A in psoriasis	1.6E-08	46.2	N/A	N/A	7.4E-06	30.8	7.9E-12	69.2
Acute phase response signaling	6.0E-10	10.1	3.0E-03	7.1	1.1E-05	5.92	3.8E-07	11.2
Role of IL-17F in allergic inflammatory airway diseases	4.4E-05	13.6	N/A	N/A	8.7E-05	11.4	3.4E-06	20.5
Toll-like receptor signaling	1.4E-06	12.2	2.2E-04	12.2	1.1E-05	9.46	7.6E-06	14.9
Role of IL-17A in arthritis	3.8E-09	16.4	4.0E-02	7.46	2.9E-08	13.4	1.1E-04	13.4
IL-10 signaling	6.5E-05	10.3	6.2E-04	11.8	4.9E-07	11.8	2.1E-05	14.7

Definition of abbreviations: MAC = *Mycobacterium avium* subsp. *avium*; MAB = *Mycobacterium abscessus* subsp. *abscessus*; N/A = not applicable; % genes = percentage of pathway genes that hit against total genes of the pathway; Pval = *P* value.

downregulated in respiratory epithelium infected with NTM. Similarly, and with important consequences, respiratory syncytial virus infection causes epithelial damage and loss of cilia (18). Given the overall importance of cilia for respiratory tract defense, and the clear and severe consequences of their impairment in primary ciliary dyskinesia and secondary cilia-impairing diseases (e.g., cystic fibrosis), finding NTM impairment of cilia gene expression is consistent with what we know about conditions that predispose to PNTM disease.

The major upregulated gene that was common to both MAC- and MAB-infected respiratory epithelium was IL-32, a proinflammatory cytokine that is involved in inflammation, cancer, and infections (19, 20), including *Mycobacterium tuberculosis* (21, 22). Bai and colleagues reported a significant elevation of IL-32 in epithelial cells and macrophages in resected lung tissue from patients with PNTM (23). They found that IL-32 was associated with apoptosis in a bronchial epithelial cell line, as well as with restricted intracellular MAC growth. IL-32 was our top-ranked upregulated gene in response to both MAC and MAB infection of differentiated NHBE cells at Days 1 and 3. However, whether IL-32 functions to control NTM or to induce immunopathology is unclear.

Microbes are first recognized by pathogen-recognition receptors (24), such as TLRs, at the plasma membrane of the epithelium or immune cells (e.g., macrophages) (24, 25). TLRs are known to be important pathogen-recognition receptors in mycobacterial infection (25). Consistent with these observations, we found that the TLR signaling pathway was significantly enriched.

The role of IL-17 in regulating intracellular pathogens, such as mycobacteria, is not fully understood (26). Previous reports have suggested that IL-17-producing Th17 cell-mediated immunity is important for the development of protective responses against MAC lung disease (27, 28). We found that downstream targets within the IL-17 signaling pathway were elevated in both MAC- and MAB-infected respiratory epithelium, although IL-17 itself was not upregulated. In addition, no expression of IL-17A was detected by real-time qPCR in this culture system. Therefore, apparently using factors other than IL-17 itself, such as TLR signaling, NTM induced expression of the downstream targets within the IL-17 signaling pathway in the respiratory epithelium. However, these findings indicate that the infected respiratory epithelium was behaving as if it were

stimulated by IL-17. The induction of nearly all IL-17 target genes is enhanced by the synergistic effects of IL-17 with TNF- α , IFN- γ or IL-1 β (29). In our study, IL-17A and MAC infection additively induced the expression of targets in the IL-17 signaling pathway, such as CCL20 and HBD-2, in the respiratory epithelium. Therefore, exogenous IL-17 from immune cells might be associated with inflammatory consequences in the respiratory epithelium in PNTM disease.

The superpathway of cholesterol biosynthesis is an upregulated canonical pathway in both MAC- and MAB-infected respiratory epithelium. Cholesterol is required for *M. tuberculosis* intracellular adaptation, and its dysregulation can impair the host immune response to that organism (30–32). In addition, a transcriptome analysis of pulmonary alveolar epithelial cells infected with *M. tuberculosis* also showed upregulation of the superpathway of cholesterol biosynthesis, with some tuberculosis strain-specific effects (33). Taken together, these findings identify the cholesterol biosynthesis pathway as a common component of the host response to mycobacteria.

At Day 3 after infection, we generally found more MAB than MAC associated

A **B** **C**

Figure 5. IL-17 signaling pathway in NTM-infected respiratory epithelium. (A) The canonical pathway for IL-17A signaling in airway cells at Day 3 after infection in MAC-infected respiratory epithelium at an MOI of 1,000. Red indicates genes that were more highly expressed in infected cells than in uninfected controls, and white indicates no gene expression. No genes were downregulated in this pathway. (B and C) IL-17A and MAC bacteria provoked additive induction of CCL20 and HBD-2 in respiratory epithelium at Day 3 after infection. IL-17A (10 ng/ml) was added to basolateral media at the time of infection. Total RNA was harvested from MAC-infected (MOI = 1,000) respiratory epithelium 3 days after infection and IL-17A addition. The mRNA levels for CCL20 (B) and HBD-2 (C) were evaluated by real-time quantitative PCR and normalized to GAPDH levels. Cotreatment with IL-17A and MAC additively upregulated mRNA expression of CCL20 and HBD-2. Triplicate Transwells were used for each experiment, and experiments were repeated with cultures derived from a different NHBE cell donor. *Significant difference compared with cotreatment ($P < 0.05$, two-way ANOVA followed by *post hoc* tests). Data are expressed as the mean $\pm$ SEM.

with respiratory epithelial cells. At Day 1 of infection, similar numbers of MAB and MAC were associated with the respiratory epithelium. However, by Day 3, more MAB grew in the Transwell culture system than MAC, which may simply reflect the fact that MAB grow more rapidly than MAC. To be less confounded by burden and more sensitive to intrinsic differences between MAC and MAB, we compared their responses on Day 1 of infection. In addition, because the number of DE genes in NTM-infected respiratory epithelium at an MOI of 100 was much smaller than that observed at an MOI of 1,000, we thought that an MOI of 100 was not sufficient to complete infection at Day 1 after infection. Thus, we decided to use an MOI of 1,000 to compare MAB-infected epithelium with MAC-infected epithelium. The magnitude of gene induction of chemokines and cytokines by MAB was generally greater than that observed for MAC at an MOI of 1,000 (Figure 3). In addition, the number of genes associated with GO categories related to inflammation was much higher in response to MAB than to MAC at an MOI of 1,000 (Table 3). In a previous report, MAB induced higher cytokine and chemokine responses than MAC in human peripheral blood mononuclear cells (34). Interestingly, heat-killed MAB still elicit more cytokine responses than heat-killed MAC, indicating that the different inflammatory factors in MAB and MAC do not require replication or metabolism and are heat stable (34). It is interesting and possibly relevant for future experimental models that similar differences are observed between peripheral

blood and infected respiratory epithelium in NTM infection.

RNA-seq analysis in respiratory epithelium infected with nontypeable *Haemophilus influenza* at the ALI showed neither upregulation of IL-32 nor the superpathway of cholesterol biosynthesis, indicating that what we observed was not due to a nonspecific inflammatory response (35). The lack of upregulation of IL-32 or cholesterol biosynthesis suggests a possible specificity for mycobacterial infection, which can be further tested.

The relatively low number of human subjects is a significant limitation of this study. Although we used four donors and multiple time points and NTM strains, resulting in a total of 40 RNA-seq samples, we observed a high degree of donor-to-donor variability, indicating that future studies would benefit from an increased sample size. Furthermore, we observed divergent localization of NTM in different subjects (as shown in Figures 1A, 1B, E1A, and E1B). Although a subject-specific variation in localization and its effect on gene expression would be an interesting topic for a future study, the current study was not designed to address this issue, which would require more subjects or replicates. Finally, the NHBE cells we used were collected from a site near the bifurcation of the trachea. The human trachea, bronchi, and bronchioles are primarily lined by a pseudostratified epithelium whose surface is dominated by ciliated cells (36). However, in this ALI system, the percentage of ciliated cells depends on how large an area is included

(Figure E6). As a result, the absolute % of ciliated cells is small (at most ~4%) compared with the absolute % of ciliated cells *in vivo*. Therefore, although this culture system approximates physiological conditions more than any other *ex vivo* culture system (including the typical submerged culture system), it does not perfectly reflect the real human airway *in vivo*.

Our comprehensive transcriptome analysis using normal respiratory epithelium infected with MAC or MAB revealed that genes related to cilia were downregulated by both MAC and MAB infection, overlapping with the known role that ciliary dysfunction plays in PNTM susceptibility. In contrast to the downregulation of ciliary genes, several pathways were upregulated, especially around inflammation. Further, MAB induced more inflammatory responses in respiratory epithelium than MAC. These data form the foundation for further mechanistic explorations of the interaction of NTM with the respiratory epithelium, and identify several tractable targets for therapeutic investigations in PNTM disease. ■

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