ORIGINAL RESEARCH

Whole-Blood Gene Expression in Pulmonary Nontuberculous Mycobacterial Infection

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Abstract

The factors predisposing toward the development of pulmonary nontuberculous mycobacterial (pNTM) disease and influencing disease progression remain unclear. Impaired immune responses have been reported in individuals with pNTM disease, but data are limited and inconsistent. In this study, we sought to use gene expression profiling to examine the host response to pNTM disease. Microarray analysis of whole-blood gene expression was performed on 25 subjects with pNTM disease and 27 uninfected control subjects with respiratory disease. Gene expression results were compared with phenotypic variables and survival data. Compared with uninfected control subjects, pNTM disease was associated with downregulation of 213 transcripts enriched for terms related to T cell signaling, including *IFNG*. Reduced *IFNG* expression was associated with more severe computed tomography changes and impaired lung function. Mortality was associated with the expression of transcripts related to the innate immune response and inflammation, whereas transcripts related to T and B cell function were associated with improved survival. These findings suggest that pNTM disease is associated with an aberrant immune response, which may reflect an underlying propensity to infection or result from NTM infection itself. There were important differences in the immune response associated with survival and mortality in pNTM disease.

Keywords: nontuberculous mycobacteria; gene expression profiling; interferon-γ

Clinical Relevance

These findings suggest that pulmonary nontuberculous mycobacterial disease is associated with an altered immune response that may contribute to both the development of disease as well as its severity. The expression of genes identified in this study warrant further investigation as potential markers of disease activity and suggest targets for potential therapeutic intervention.

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Pulmonary nontuberculous mycobacterial (pNTM) disease is an increasingly common and challenging infection. Although ubiquitous in the environment, disease usually occurs in the context of an existing pulmonary disease, such as bronchiectasis or chronic obstructive pulmonary disease (COPD); however, in a subgroup of patients, disease occurs in the absence of any known risk factors. The disease is associated with a high mortality, ranging between 12.5% and 41.1% at 5 years (1–5). The clinical course of disease is variable and unpredictable; although some individuals develop progressive disease, some may remain stable without treatment (1, 6, 7). The factors governing the acquisition of disease and its subsequent clinical course are poorly understood.

Disseminated NTM disease occurs in the context of immunodeficiency, such as acquired immunodeficiency syndrome or inherited and acquired defects of the IFN-γ/IL-12 pathway, and several studies have also demonstrated functional defects in the host immune response of subjects with pNTM disease (8–13). A recent study employing exome sequencing revealed an excess of variants affecting genes implicated in the immune response in subjects with pNTM disease compared with both the control population and unaffected family members (14). These findings suggest that defective immune responses may play a role in predisposing individuals to pNTM disease. The aims of this study were to investigate the host response to pNTM disease using global gene expression profiling of peripheral blood and to explore the relationship between gene expression and clinical outcomes.

Methods

Individuals with pNTM disease were recruited from the Royal Brompton Hospital and Chelsea and Westminster Hospital between September 2012 and November 2013. All participants met American Thoracic Society 2007 criteria, and were unanimously deemed by two clinicians with expertise in pNTM disease (authors M.R.L. and R.W.) to have active disease requiring treatment. Control subjects with respiratory disease (bronchiectasis or COPD), but no radiological or microbiological evidence of mycobacterial disease, were recruited from the same departments. Written consent was obtained from all participants and the study was approved by the local research ethics committee (reference 12/LO/1034).

Participants underwent clinical assessment and pulmonary function testing. Peripheral blood was taken for RNA extraction and clinical testing, and spontaneously expectorated sputum was collected for phenol auramine microscopy and bacterial, mycobacterial, and fungal cultures. If not performed within the previous 6 months, a high-resolution computed tomography (CT) was performed. For pNTM cases, CT scoring was performed by a specialist radiologist blinded to clinical details, as described previously (15), and a composite score calculated by summing the scores for individual features. Further methods are provided in the data supplement.

Gene Expression Analysis

Peripheral blood was collected into PAXgene RNA tubes (Becton Dickinson Co.) and RNA extracted using the PAXgene Blood RNA Kit (PreAnalytiX). Extracted RNA was amplified to complementary DNA using the Ovation Pico WTA System V2 (NuGEN Technologies), then cDNA was fragmented and labeled using the Encore Biotin Module (NuGEN Technologies) and immediately hybridized to Affymetrix Human Gene 1.1 ST array plates and scanned using the GeneTitan instrument (Affymetrix). Samples were randomized before amplification and again before fragmentation, labeling, and hybridization.

Outliers were identified using the arrayQualityMetrics package (16) in the R environment version 3.1.2, and removed. Raw probe-level data from .CEL files passing quality control were summarized, quantile normalized, and $\log_2$ transformed using the Affymetrix Power Tools package. Expression data were reimported into R, where low-expressed and unannotated probes were removed and all downstream analyses performed.

Differentially expressed genes were identified using significance analysis of microarrays (SAM) (17) applying a false discovery rate (FDR) of 5% for significance. Gene ontology analyses were performed using the WebGestalt tool (http://www.webgestalt.org) using the Gene Ontology (GO) Consortium and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. All expressed genes were used as the reference set, and an adjusted P value of 0.05 was deemed significant.

Weighted gene coexpression gene network analysis (WGCNA) was performed using the WGCNA package (18) with the default-recommended parameters. All expressed genes were entered into the analysis; however, only genes with a connectivity in the highest tertile were used to construct the topological overlap matrix.

Correlation between gene (or gene module) expression and clinical variables was performed using the Pearson or Spearman correlation for continuous variables according to their distribution, and polyserial correlation for categorical variables. The t test and ANOVA were used to compare expression values between groups. The log-rank test was used to compare differences in survival curves.

Results

A total of 52 subjects (25 pNTM disease cases and 27 control subjects) matched for age, sex, and ethnicity was studied. Clinical characteristics of the study cohort are detailed in Table 1, with further details provided in Tables E2 and E3 of the data supplement. The proportion of subjects with a diagnosis of bronchiectasis was lower (although not significantly so) in the group of pNTM cases due to the inclusion of subjects with "Lady Windermere" syndrome. These individuals ($n = 7$) had no underlying lung disease before their NTM infection, although, at the time of enrolment, bronchiectasis was present in all subjects on high-resolution CT.

Gene Expression and pNTM Disease

A total of 213 transcripts was identified as differentially expressed between cases and control subjects, with an FDR of 5% (for full list, *see* Table E4 and Figure E1). All transcripts were downregulated in pNTM disease. The top 25 genes with the highest fold change are detailed in Table 2.

One of the top differentially expressed genes in pNTM disease cases compared with control subjects was IFN-γ (*IFNG*) (Figure 1). IFN-γ is the primary effector cytokine of the T-helper cell type 1 pathway, which plays a vital role in mycobacterial immunity. In light of this, it is notable that other differentially expressed genes included nuclear factor of activated T cells 2 (*NFATC2*), cytotoxic and regulatory T cell molecule (*CRTAM*), and X-C motif chemokine ligands 1 and 2

Table 1. Clinical Characteristics of the Study Population

Characteristics	pNTM Cases (*n* = 25)	Controls (*n* = 27)
Age, yr	66.9 (±10.3)	65.4 (±7.5)
Male sex, *n* (%)	9 (36)	10 (37)
Ex- or current smoker, *n* (%)	14 (56)	14 (52)
Underlying diagnosis, *n* (%)		
COPD	7 (28)	7 (26)
Bronchiectasis	11 (44)	20 (74)
No underlying lung disease	7 (28)	0
FEV_1 % predicted	48.64 (±31.42)	71.27 (±25.98)
FVC % predicted	73.78 (±39.2)	102.25 (±20.87)
Prophylactic antibiotics, *n* (%)	6 (24)	8 (29)
Systemic corticosteroids, *n* (%)	3 (12)	2 (7)
Inhaled corticosteroids, *n* (%)	12 (48)	17 (63)
NTM treatment, *n* (%)	9 (36)	—
NTM species, *n* (%)		
MAC	14 (56)	—
M. abscessus	4 (16)	—
M. xenopi	3 (12)	—
M. kansasii	2 (8)	—
M. malmoense	1 (4)	—
M. fortuitum	1 (4)	—

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; *M.* = *Mycobacterium*; MAC = *M. avium* complex; NTM = nontuberculous mycobacterial; pNTM = pulmonary nontuberculous mycobacterial. Continuous variables are stated as means (±SD).

(*XCL1*, *XCL2*). *NFATC2* encodes NFATc2, a member of the NFAT family of transcription factors. NFATc2 has been shown to be an *in vitro* regulator of both IFN-γ (19, 20) and TNF-α (21, 22) release. The product of *CRTAM* also promotes IFN-γ release from $CD8^+$ T cells (23–27) and natural killer (NK) cells (28, 29). *XCL2* encodes a chemokine that is chemotactic for $CD8^+$ and $CD4^+$ T cells (30, 31), and its homolog, *XCL1*, encodes a chemokine associated with the T-helper cell type 1 response, the silencing of which has been shown to lead to reduced IFN-γ production and disordered granuloma formation in tuberculosis-infected mice (32, 33).

GO analysis of the set of differentially expressed transcripts revealed enrichment for one biological process, negative regulation of α-β T cell proliferation (enrichment ratio, 36.01; adjusted $P = 0.049$) and a further 10 GO cellular components. In addition, KEGG analysis revealed enrichment for the pathway T cell receptor signaling (enrichment ratio, 6.12; adjusted $P < 0.001$), with nine genes in the pathway (*IFNG*, *AKT3*, *CD247*, *MAPK9*, *CBLB*, *NFATC2*, *ICOS*, *RASGRP1*, and *ITK*) being significantly underexpressed in pNTM disease cases. Full details are given in Table E5.

To examine the influence of systemic steroid use and NTM treatment on the observed difference in gene expression, multiple linear modeling was performed using these as additional explanatory variables in addition to pNTM disease. The expression of 168 of the 213 genes (79%) remained significantly associated with pNTM disease (*see* Table E6).

The 213 transcripts were compared with the 380 gene "meta-signature" of active tuberculosis identified by Blankley and colleagues (34). There was an overlap of only nine genes (*CDK5RAP2*, *EPHA4*, *FAIM3*, *FCRL3*, *GPR183*, *GZMK*, *KLF12*, *NELL2*, and *SMA4*), most of which were also downregulated in tuberculosis, except *CDK5RAP2* and *SMA4*, which showed increased expression. Comparison with studies of sarcoidosis, another granulomatous disease, revealed that 11 of the 213 transcripts (*AKT3*, *CBLB*, *CCND2*, *CD247*, *ICOS*, *IFNG*, *IL2RB*, *ITK*, *MAPK9*, *NFATC2*, and *RASGRP1*) are also present in the 31-gene sarcoid signature described by Zhou and colleagues (35), representing significant ($P < 0.001$) enrichment. However, none of the 20 genes in the experimentally derived "unbiased" signature from this study were present, and there was little or no overlap with other reported gene signatures in sarcoidosis (36, 37).

To identify modules associated with pNTM disease, WGCNA was performed using all 13,360 expressed transcripts from the 52 subjects, resulting in 10 modules being identified (detailed in Table E7 and E8). Three modules showed significantly ($P \leq 0.05$) different expression in pNTM disease cases compared with control subjects. These were enriched for multiple GO terms, including those relating to T cell selection, differentiation, activation, and signaling, and others associated with IFN-γ activity, such as NK cell–mediated cytotoxicity, antigen processing and presentation, and major histocompatibility complex class II protein complex (Table 3). In all cases, the expression of genes within each module was lower in subjects with pNTM disease. Of the 213 genes identified to be differentially expressed in pNTM disease cases compared with control subjects, 114 (53.5%) were present in one of the three modules that showed association with pNTM disease.

IFNG Expression and Clinical Outcomes

Given the essential role of IFN-γ in the immune response to mycobacteria, the relationship between *IFNG* expression in pNTM disease cases and clinical variables was explored further.

The expression of *IFNG* was significantly positively correlated with several measures of lung function, including forced expiratory volume in 1 second % predicted (Pearson's $r = 0.43$, $P = 0.05$), forced vital capacity % predicted (Pearson's $r = 0.51$, $P = 0.019$), and total lung capacity % predicted (Pearson's $r = 0.48$, $P = 0.016$). No significant correlations, however, were seen for transfer factor for carbon monoxide or the transfer factor corrected for alveolar volume.

Expression of *IFNG* was strongly negatively correlated with combined CT score (Figure 2A; Pearson's $r = -0.53$, $P = 0.007$). When individual CT features were examined (*see* Figure E2), *IFNG* expression was significantly lower in the presence of moderate or severely extensive bronchiectasis compared with a limited extent ($P = 0.017$ and $P = 0.029$, respectively).

In addition, *IFNG* expression was associated with other markers of disease severity: it was positively correlated with serum albumin (Spearman's $\rho = 0.50$, $P = 0.011$), and negatively correlated with C-reactive protein (Spearman's $\rho = -0.42$, $P = 0.034$) and neutrophil count (Pearson's $r = -0.52$, $P = 0.007$). Interestingly, *IFNG* was also negatively correlated with time

Table 2. The Top 25 Differentially Expressed Genes between Pulmonary Nontuberculous Mycobacterial Cases and Control Subjects

Rank	Gene Name	Entrez ID	Fold Change	*d*-Statistic	Name
1	*FLJ45825*	100505530	1.657	2.646	Uncharacterized LOC100505530
2	*GZMK*	3003	1.61	2.857	Granzyme K (granzyme 3; tryptase II)
3	*TARP*	445347	1.609	2.619	TCR γ alternate reading frame protein
4	*TARP*	445347	1.609	2.619	TCR γ alternate reading frame protein
5	*XCL2*	6846	1.553	3.269	Chemokine (C motif) ligand 2
6	*A2M*	2	1.507	2.293	α-2-macroglobulin
7	*CRTAM*	56253	1.465	3.57	Cytotoxic and regulatory T cell molecule
8	*PMS2P1*	5379	1.453	4.053	Postmeiotic segregation increased 2 pseudogene 1
9	*FCRL3*	115352	1.446	3.26	Fc receptor-like 3
10	*PZP*	5858	1.435	2.275	Pregnancy-zone protein
11	*TIGIT*	201633	1.4	3.333	T cell immunoreceptor with Ig and ITIM domains
12	*PPIH*	10465	1.393	2.897	Peptidylprolyl isomerase H (cyclophilin H)
13	*AK5*	26289	1.39	2.515	Adenylate kinase 5
14	*MUC12*	10071	1.39	2.388	Mucin 12, cell surface associated
15	*IFNG*	3458	1.382	2.622	IFN, γ
16	*FAHD2A*	51011	1.364	3.021	Fumarylacetoacetate hydrolase domain containing 2A
17	*SMA4*	11039	1.361	3.602	Glucuronidase, β pseudogene
18	*VSIG1*	340547	1.356	2.784	V-set and immunoglobulin domain containing 1
19	*SAMD3*	154075	1.35	2.367	Sterile α motif domain containing 3
20	*XCL1*	6375	1.348	2.247	Chemokine (C motif) ligand 1
21	*IL2RB*	3560	1.347	2.242	IL-2 receptor, β
22	*PSPH*	5723	1.339	2.232	Phosphoserine phosphatase
23	*LDHB*	3945	1.336	2.312	Lactate dehydrogenase B
24	*NELL2*	4753	1.323	2.276	NEL-like 2 (chicken)
25	*NFATC2*	4773	1.322	2.804	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2

Definition of abbreviations: ITIM = immunoreceptor tyrosine-based inhibition motif; TCR = T-cell receptor.
Genes are ranked by fold change; all displayed decreased expression in pulmonary nontuberculous mycobacterial cases.

since diagnosis (Figure 2B; Spearman's $\rho = -0.60$, $P = 0.005$), although there was no correlation between the combined CT score and disease duration (Spearman's $\rho = 0.24$, $P = 0.287$).

No significant differences were seen in mean *IFNG* expression between survivors and nonsurvivors, although, when subjects were dichotomized into low and high *IFNG* expression, there was a trend toward reduced survival in the low-expression group. This, however, did not reach significance (log-rank test $P = 0.06$).

For disease control subjects, no significant associations between any measures of lung function or markers of disease severity and *IFNG* expression were observed (Table E9), suggesting that the changes were specific to pNTM disease, and not merely due to disease severity in general.

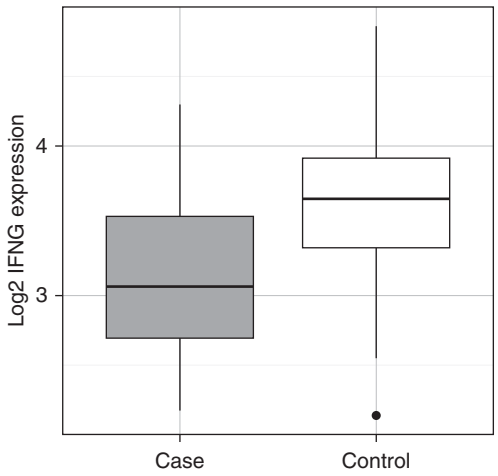

Figure 1. Boxplots of *IFNG* expression in pulmonary nontuberculous mycobacterial (pNTM) disease cases (gray) and control subjects (white). The central line, box boundaries, and whiskers represent the median, first and third quartiles, and the lowest and highest values lying within 1.5 times the interquartile range, respectively.

Gene Expression and Survival in pNTM Disease

Survival data were available for 24 of the pNTM disease cases who had a mortality rate of 33% (eight deaths) during the study. No deaths were observed in the control group. Survival analysis was performed on these 24 cases using SAM with an FDR of 5%; 215 genes were found to show association with decreased survival and 1,131 genes with increased survival. The top enriched GO terms in relation to survival are given in Table 4 (for full results of the analysis, please *see* Tables E10–E12 and Figure E3).

Table 3. Gene Modules Identified as Significantly Associated with Nontuberculous Mycobacterial Disease by Weighted Gene Coexpression Gene Network Analysis

Module Name	No. of Genes	Correlation with pNTM Disease	*P* Value	Relevant Enriched GO Terms
Cyan	1,139	−0.31	0.02	Thymic T cell selection T cell receptor signaling pathway Antigen processing and presentation
Greenyellow	85	−0.32	0.02	Cellular defense response Positive regulation of immune response T cell activation T cell receptor complex Natural killer cell mediated cytotoxicity CCR1 chemokine receptor binding
Lightcyan	37	−0.27	0.05	B cell activation B cell receptor complex MHC class II protein complex

Definition of abbreviations: GO = gene ontology; MHC = major histocompatibility complex; pNTM = pulmonary nontuberculous mycobacterial.

The top genes most strongly associated with reduced survival included genes forming part of the inflammasomes (absent in melanoma 2 [*AIM2*], caspase 1 [*CASP1*], and NLR family caspase recruitment domain [CARD] domain containing 4 [*NLRC4*]), or related to them (caspase 4 [*CASP4*] and CARD family member 16 and 17 [*CARD16*, *CARD17*]). The set of genes associated with reduced survival was enriched for 50 GO terms, many of which were related to the innate immune response and inflammation, including the ice protease-activating factor and AIM2 inflammasome complexes. In addition, the genes were enriched for six KEGG pathways, including Toll-like receptor (TLR) signaling pathway.

The top genes most strongly associated with increased survival included *CD4*, the T-helper cell coreceptor; *CD28*, a coreceptor which mediates T cell activation; and *IL2RB*, the β subunit of the receptor for IL-2, which plays a key role in the regulation of lymphocyte proliferation and activation, and is also of importance in NK cell function through its roles as a subunit of the IL-15 receptor. The set of genes associated with improved survival was enriched for 26 GO terms, the majority of which were related to T cell and B cell function, and four KEGG pathways, including "antigen processing and presentation."

WGCNA was repeated focusing only on the pNTM disease cases to investigate disease survival. Nine modules were identified, three of which showed significant differential expression in nonsurvivors compared with survivors (Table 5 and Tables E13 and E14). Of the 215 genes associated with reduced survival by SAM, 74 (34.6%) were present in either the "white" or "grey60" modules, whereas 666 (58.9%) of the 1,131 genes associated with increased survival were present in the "darkorange" module.

Of note is the enrichment of the grey60 module for the GO term "RAGE receptor binding." RAGE (the receptor for advanced glycation endproducts) is a pattern recognition receptor that has been implicated in neutrophilic inflammation in tuberculosis (38, 39). Although grey60 module itself was not significantly correlated with neutrophil count (Pearson's r = 0.29, $P = 0.152$), many of the genes associated with RAGE were found to be significantly correlated with neutrophilia (*HMBG2*: Pearson's $r = 0.5$, $P = 0.011$; *S100A8*: Pearson's $r = 0.56$, $P = 0.003$; *S100A9*: Pearson's $r = 0.56$, $P = 0.003$; *S100A12*: Pearson's $r = 0.53$, $P = 0.006$).

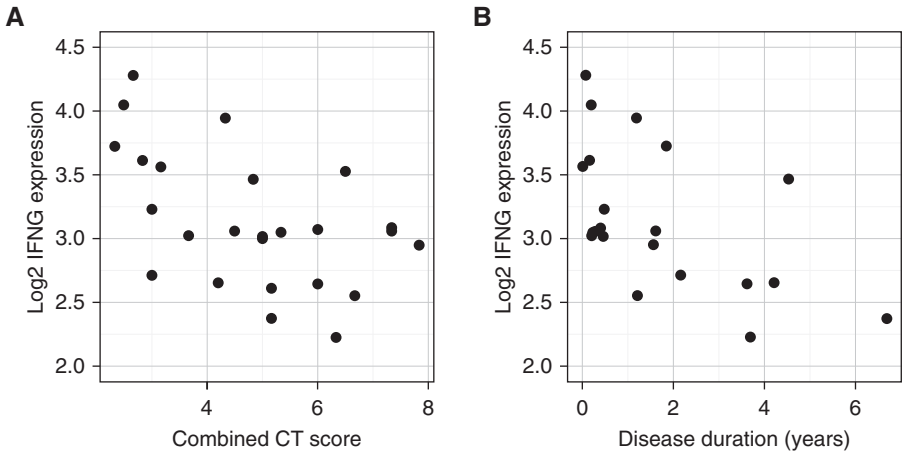

Figure 2. (*A*) Expression of *IFNG* and combined computed tomography (CT) score in pNTM cases. (*B*) Expression of *IFNG* and duration of pNTM disease.

Discussion

In this study, we have shown that pNTM disease is associated with the differential expression of over 200 genes. These were characterized by the reduced expression of many genes associated with cellular immunity, some of which have previously been identified to be important in the host response to mycobacterial infection. One of the top differentially expressed genes was *IFNG*, which plays an essential role in antimycobacterial immunity. Not only was *IFNG* expression lower in subjects with pNTM disease, but, within this group, reduced *IFNG* expression was significantly associated with more severe radiological disease and impaired lung function. There was also a trend toward an increased mortality in subjects with lower *IFNG* expression.

These data are consistent with experimental evidence from *in vitro* studies, which have demonstrated diminished type 1 cytokine responses in patients with pNTM disease, showing reduced IL-12 (8–10) and IFN-γ (8–13) release after stimulation with both mitogen and NTM. These studies have employed varying methodologies (different cell types, stimuli, and read-outs), and not all such studies have detected a difference in the immune response in subjects with pNTM disease (40–42). The evidence from the present study therefore provides valuable,

Table 4. The Top Five Most Significantly Enriched Gene Ontology Terms in Genes Associated with Decreased and Increased Survival in Nontuberculous Mycobacterial Disease

Top GO Terms	GO Accession Number	Enrichment Ratio	Adjusted *P* Value
Associated with decreased survival			
Response to other organism	GO:0051707	3.16	0.001
Response to biotic stimulus	GO:0009607	3.00	0.001
Activation of innate immune response	GO:0002218	5.56	0.001
Detection of molecule of bacterial origin	GO:0032490	30.71	0.001
Regulation of innate immune response	GO:0045088	4.23	0.001
Associated with increased survival			
T cell selection	GO:0045058	6.19	1×10^4
Lymphocyte differentiation	GO:0030098	2.37	6×10^4
Thymic T cell selection	GO:0045061	6.97	6×10^4
Immune system development	GO:0002520	1.74	0.002
Leukocyte differentiation	GO:0002521	2.01	0.002

Definition of abbreviation: GO = gene ontology.

unbiased evidence for an impaired IFN-γ response in subjects with pNTM disease, and is the first study to link this impairment with adverse clinical features.

There is evidence that immunotherapy with IFN-γ may be beneficial in mycobacterial disease. Recombinant IFN-γ has been shown to improve sputum culture conversion in pulmonary tuberculosis infection (43), and has been used in refractory cerebral tuberculosis (21). Case reports have reported benefit from IFN-γ administration in NTM infection both in the presence (23, 25–27) and absence (28) of immunodeficiency. Two randomized, controlled trials have been conducted in pNTM disease, with one finding no benefit with inhaled IFN-γ (30) and the other finding a significant increase in treatment response with the addition of intramuscular IFN-γ (32).

These results were supported by the networks analysis, which identified gene modules significantly underexpressed in pNTM disease that were enriched for several terms linked to IFN-γ activity. In light of reports of immunodeficiency due to anti-cytokine autoantibodies (44), it is also interesting to note that one module was enriched for genes regulating thymic T cell selection.

The 215 genes associated with reduced survival were significantly enriched for multiple terms relating to innate immunity and pattern recognition receptors, including TLR signaling. Although TLR2 may have a protective role in murine models of NTM infection (45–49), and has been reported to display reduced expression in humans with pNTM disease (8), the proinflammatory response triggered by TLR2 may also be deleterious in NTM infection. The "rough" variant of Mycobacterium *abscessus* has been shown to trigger a hyperinflammatory response via TLR2 signaling (50), which may play a role in the virulence associated with this phenotype (51).

Table 5. Gene Modules Identified as Significantly Associated with Decreased and Increased Survival in Nontuberculous Mycobacterial Disease

Module Name	No. of Genes	Correlation with Mortality	*P* Value	Relevant Enriched GO Terms
darkorange	1,413	−0.5	0.01	T cell receptor signaling pathway Thymic T cell selection
grey60	490	0.55	0.004	RAGE receptor binding
White	71	0.46	0.02	None

Definition of abbreviations: GO = gene ontology; RAGE = receptor for advanced glycation endproducts.

Interestingly, genes associated with inflammasome formation and pyroptosis were also overrepresented in the genes associated with reduced survival. Inflammasome activation has been reported in tuberculosis (52, 53), and has been proposed as a mechanism for the immune reconstitution inflammatory syndrome seen in human immunodeficiency virus (54). To date, only one report has demonstrated inflammasome activation by NTM (55), and little is known about its role in NTM infection.

Network analysis identified an association between mortality and the expression of genes related to RAGE, including the proteins S100A8 and S100A9 (which, together, form calprotectin, an important proinflammatory mediator) (56). In a murine model, expression of RAGE in lung tissue has been shown to increase in response to tuberculosis infection, and gene knockout caused an increase in weight loss and mortality (57). The expression of both *S100A8* and *S100A9* have been associated with neutrophilic inflammation in tuberculosis (38, 39), with the expression of *S100A9* being suggested as a potential tuberculosis biomarker (58).

Improved survival was associated with 1,131 genes, which were enriched for several GO terms predominantly relating to T cell differentiation and activation. Despite the trend toward higher expression in surviving pNTM cases, *IFNG* was not identified by SAM as being significantly associated with survival, although it was related to many of the enriched functional terms. Again, the results of network analysis were also supportive of these findings and identified a gene module associated with survival that was enriched for terms associated with T cell development and signaling.

Taken together, these findings suggest that pNTM disease is associated with the downregulation of genes involved in the T cell response, including *IFNG*, which is consistent with the results of *in vitro* studies in subjects with NTM infection. In subjects in whom disease has developed, the expression of genes involved in the T cell response is associated with a better prognosis, perhaps reflecting the preservation of an appropriate response to NTM infection. Conversely, a poor prognosis is associated with an upregulation of genes involved in innate immunity and inflammation, which may potentially

mediate tissue damage and, thus, the progressive radiological changes observed.

Infection with Mycobacterium tuberculosis has been reported to downregulate *in vitro* IFN-γ release (59); thus, it is not clear whether the differences in gene expression represent a predisposing defect in host response or are a result of immunomodulation after NTM infection. The reduction in *IFNG* seen as disease duration increases may point toward the latter. Similarly, the question remains as to whether the upregulation of the innate immune response associated with poor survival is in some way contributory to pathogenesis, perhaps due to excessive inflammation and tissue damage, or is merely a response to an increased burden of mycobacterial infection in those with severe disease.

Pulmonary NTM disease is a heterogeneous condition containing a variety of clinical phenotypes, the most clinically relevant distinction being between fibrocavitary and nodular-bronchiectatic disease. It is likely that the transcriptional response differs between these groups. Given the small sample size, no comparison was made between different radiological patterns; however, it is notable that *IFNG* expression strongly correlated with composite CT score, and was lower (although not significantly so) in individuals with severe cavitation. Another notable subgroup of individuals with pNTM disease is that which demonstrates stable, nonprogressive disease in the absence of treatment (1, 6, 7), and it is also likely that the gene expression profiles of those subjects differ from those seen in the individuals with active infection included in the present cohort.

Several studies have been conducted examining gene expression in tuberculosis, and, recently, Blankley and colleagues (34) characterized a 380-gene "meta-signature" of active tuberculosis showing consistent differential expression in at least 9 datasets. There was little overlap between this signature and the genes associated with pNTM disease in the current study, and, in contrast to pNTM disease gene expression in tuberculosis, was characterized by an upregulation of IFN-γ–regulated genes (although not *IFNG* itself). This difference may relate to the infecting organism, or reflect a more appropriate host response to tuberculosis in a generally younger population with fewer comorbidities, as opposed to the potentially aberrant response seen in individuals with pNTM disease. There was, however, a point of similarity in that genes related to T cells were consistently downregulated in both tuberculosis and pNTM disease.

There was no overlap with experimentally derived expression signatures of sarcoidosis (35–37). However, the 213 transcripts associated with pNTM disease were significantly enriched for genes in the 31-gene sarcoid signature described by Zhou and colleagues (35). These genes were chosen *a priori*, as they are part of the T cell receptor, Janus kinase-signal transducer and activator of transcription, and cytokine–cytokine receptor signaling pathways implicated in sarcoidosis, and were able to differentiate sarcoidosis from control subjects with 82.2% accuracy. In the current study, 11 (35%) of these genes were also associated with pNTM disease, all of which were regulated in the same direction (down) in sarcoidosis. This is particularly interesting, given the hypothesis that sarcoidosis may be driven by an aberrant immune response to mycobacterial antigens (60).

This study has some limitations. The selection of subjects with definite active disease was necessary to provide a cohort with a well defined phenotype, but has limited the sample size, and therefore the statistical power, of the study. This may therefore limit the ability to generalize these findings to the wider NTM population. Even with such selection, the pNTM study population remains highly heterogeneous, further limiting the power to detect differences in expression. This may explain the relatively modest differences in expression seen between groups in contrast to studies of more homogenous populations, such as those seen in tuberculosis. The use of inhaled or systemic steroids may have an effect on gene expression; however, the proportions were similar between pNTM cases and control subjects, and the exclusion of these subjects would necessitate the exclusion of most subjects with COPD, an important group at risk of pNTM disease, and would further limit the ability to generalize the findings.

Although being matched for basic demographics, important phenotypic differences still remained between cases and control subjects, such as the high mortality seen in cases, systemic steroid use, and the treatment given for NTM. These differences may potentially contribute to the observed differences in gene expression. However, intervening to reduce the differences between groups by altering treatment would not have been feasible on ethical grounds, and the mortality in our cohort is in keeping with the known high mortality associated with pNTM disease reported in other cohorts (1–5). Furthermore, the expression of the majority of the differentially expressed transcripts (including *IFNG*) was significantly associated with pNTM disease, independent of systemic steroid use or NTM treatment. Similarly, several other factors were observed to be associated with survival (such as the presence of mycetoma), and the association seen between gene expression and survival may therefore be confounded by these factors. Unfortunately, the sample size precludes multivariate analyses to investigate them further.

Conclusions

This is the first study of gene expression associated with NTM infection. The expression of many genes mediating cellular immunity was depressed in pNTM disease, including *IFNG*, which correlated negatively with markers of disease severity. Although requiring further validation in independent cohorts, these findings are mechanistically plausible and consistent with previous experimental data. Mortality in pNTM disease was associated with reduced expression of genes related to cellular immunity and increased expression of genes related to innate immunity, suggesting that disease progression may be driven by immune dysregulation. To our knowledge, this is the first time the immune response to NTM has been linked to differences in disease outcomes. The expression of genes identified in this study warrants further investigation as potential markers of disease activity, and suggests targets for potential therapeutic intervention. ■

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