

Tricks to translating TB transcriptomics

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Abstract: Transcriptomics and other high-throughput methods are increasingly applied to questions relating to tuberculosis (TB) pathogenesis. Whole blood transcriptomics has repeatedly been applied to define correlates of TB risk and has produced new insight into the late stage of disease pathogenesis. In a novel approach, authors of a recently published study in *Science Translational Medicine* applied complex data analysis of existing TB transcriptomic datasets, and *in vitro* models, in an attempt to identify correlates of protection in TB, which are crucially required for the development of novel TB diagnostics and therapeutics to halt this global epidemic. Utilizing latent TB infection (LTBI) as a surrogate of protection, they identified IL-32 as a mediator of interferon gamma (IFN γ)-vitamin D dependent antimicrobial immunity and a marker of LTBI. Here, we provide a review of all TB whole-blood transcriptomic studies to date in the context of identifying correlates of protection, discuss potential pitfalls of combining complex analyses originating from such studies, the importance of detailed metadata to interpret differential patient classification algorithms, the effect of differing circulating cell populations between patient groups on the interpretation of resulting biomarkers and we decipher weighted gene co-expression network analysis (WGCNA), a recently developed systems biology tool which holds promise of identifying novel pathway interactions in disease pathogenesis. In conclusion, we propose the development of an integrated OMICS platform and open access to detailed metadata, in order for the TB research community to leverage the vast array of OMICS data being generated with the aim of unraveling the holy grail of TB research: correlates of protection.

Keywords: Biomarker; tuberculosis (TB); protective immunity; correlates of risk; microarray; systems biology

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Introduction

In 2013, an estimated 9.0 million people developed tuberculosis (TB) and 1.5 million died from the disease (1). Despite ongoing research efforts and ever-increasing knowledge of how *Mycobacterium tuberculosis* (*M.tb*) interferes with the human immune response, we are still far from developing the diagnostic and therapeutic approaches that would reduce the severity of this global epidemic. Vaccines form the cornerstone of potential eradication strategies of TB, yet the biological factors that provide protection from disease progression (so-called biological correlates of protection), which would assist design and testing of new vaccine candidates are lacking. A better

understanding of natural protective immunity to TB would facilitate these attempts. Following inhalation of *M.tb* bacilli, latent TB infection (LTBI) is the most common outcome. It is considered that in LTBI the growth of the bacilli is contained by the coordinated host innate and adaptive immune response, preventing disease progression, but there is failure to completely eradicate all organisms, such that an underlying asymptomatic infection persists. This makes LTBI a particularly useful model system for the discovery of protective correlates.

In the last 8 years, 15 transcriptomic studies have been published that use whole-genome gene expression microarrays in an effort to gain a broader understanding of the human response to *M.tb* infection, during various

stages of pathogenesis and treatment (*Figure 1*). These studies have generally characterized the whole blood response, although the papers by Berry *et al.* and Bloom *et al.* (5,12) also investigated expression profiles on separated cell populations to delineate the effect of varying cell number and cell activation on the whole blood response. These studies provide evidence for correlates of risk of active TB, and have been remarkably concordant in their findings. In particular, they reveal an important role for type-I interferon signaling and neutrophil influx in disease pathogenesis, driving new areas of TB research. Moreover, they provide evidence that LTBI actually represents a spectrum of disease states: the whole blood signature of some LTBI cases clustered with those with active TB, suggesting these participants may be at risk of developing disease. The microarray data on which these studies are based have mostly been deposited in public databases (*Figure 1*), but generally the associated patient metadata is incomplete or unavailable. This significantly limits the utility of the data as the primary research can often not be reproduced. Without such detailed metadata, it can be challenging to meaningfully combine datasets in meta-analyses, even once the challenge of combining data from different platforms has been overcome. Moreover, while these studies provide evidence for blood biomarkers to diagnose active TB and monitor treatment, they have shed less light on correlates of protection against disease.

In a recent paper (17) published in *Science Translational Medicine*, Montoya *et al.* used an interesting combination of informatics and additional *in vitro* experiments to do just that. The novel strategy employed by the authors consisted of three components. Firstly, an idealized monocellular *in vitro* system was studied using transcriptomic approaches. The authors identified genes that correlate with defense response in differentiating macrophages, whose phenotype was previously associated with *M.tb* control (18,19). Hypotheses resulting from the first section were then tested in a second set of *in vitro* experiments. The final step involved further informatics analyses of existing human TB transcriptomic datasets to identify genes up-regulated in LTBI cases or which are decreased during active TB and increase during TB therapy. The three components were then integrated by determining the overlap of the genes sets from the *in vitro* and *in vivo* transcriptomic analyses in order to identify genes representing potential biomarkers of protective immunity. Via this method Montoya *et al.* identified IL-32 as a mediator of interferon gamma (IFN γ)-vitamin D mediated antimicrobial activity, and a marker of LTBI.

The importance of sample and patient characterization

While this novel approach yielded interesting candidate biomarkers of LTBI, there are a number of factors which must be taken into account to ensure this method yields translatable outcomes for understanding protective immunity (*Table 1*). Montoya *et al.*'s approach critically depends on how they differentiated and purified their *in vitro* cell populations, the differing proportions of circulating cell subsets between patient groups, and, critically, how they (or rather the authors of the original datasets) classified individuals as having LTBI.

Diagnosis of LTBI relies on the measurement of the adaptive response by tuberculin skin test (TST) or interferon-gamma release assay (IGRA), performed on whole blood samples. Thus, these tests only inform us about immune memory, not current infection status; they cannot differentiate whether an individual eradicated the infection, either via the innate response or with help from the adaptive response. It has become clear that reliance on TST or IGRA-based classification approaches to define LTBI can result in very specific patient cohorts with different, and limited, disease phenotypes. Moreover, studies use different combinations of these tests to define LTBI (*Figure 1*). Thus better methods for defining LTBI are required; in particular, a marker for current *M.tb* infection, not based on immune sensitization. PET/CT imaging is one such approach that holds promise (20). When using LTBI as a model of protective immunity, it is therefore imperative that results are interpreted in the context of how patient groups were actually defined, particularly when an immune measure is used for classification; otherwise findings may be biased towards a specific phenotype. Moreover, outcomes need to be framed in the context of their derivation and not given a broad implication until validated on additional patient cohorts.

The cellular composition of samples from which RNA was extracted for these analyses is the second vital component to interpreting the results from transcriptomic studies. Whole-blood signatures are highly influenced by differing proportions of circulating cell populations, which obviously differ by disease states. Many of the differences in T cell transcripts identified between individuals with TB and healthy controls, identified by Berry *et al.* (5), were ascribed to decreased circulating lymphocyte numbers in TB patients, rather than functional differences in cells. TB is known to be a

Studies					Classes: <i>in vivo</i>	Inclusion criteria and cutoffs										N							
Study	Sample	Platform	GEO/ArrayExpress ID	Country	Classes	HIV-1 status	Prior TB	Contact with active TB	TST	IGRA-QFT	IGRA-other/not specified	Compatible symptoms and signs	Sputum smear	Sputum/BAL culture +	Radiographic disease	Non-respiratory sample culture	Alternative diagnosis	Histology					
Mistry <i>et al.</i> (2) 2007	Blood	Two custom arrays: 50,000 clones (Invitrogen) and 8,000 genes	Not deposited	South Africa	Active PTB	-								+						10			
					LTBI	-	-		>15 mm												10		
					Cured TB (1 st episode, disease free ≥18 months)	-											-						10
					Recurrent TB (2-3 episodes, disease free ≥18 months)	-											-						10
Jacobsen <i>et al.</i> (3) 2007	PBMC	Custom Agilent (8,033 genes)	GSE6112	Germany	Active PTB (<6 weeks of rx)	-								+	+	±				9			
					LTBI	-		+	>10 mm													9	
Kim <i>et al.</i> (4) 2010	Tissue	Affymetrix Human XP3	GSE20050	South Africa	PTB granuloma (prob MDR)														+	5			
					Healthy lung																+	2	
Berry <i>et al.</i> (5) 2010	Blood	Illumina HT12 v3	GSE19439 GSE19444 GSE19435	UK	Active PTB									+							34		
					LTBI (TST and IGRA)				≥6 mm/ ≥15 mm*	+												38	
					Healthy				≤6 mm/ ≤15 mm*	-													24
	Purified leukocytes	GSE19442	South Africa	Active PTB	-									+							20		
				LTBI (IGRA)	-				+												31		
				Active PTB												+						7	
GSE19443	UK	Active PTB																		4			
		Healthy					≤6 mm/ ≤15 mm*	-															
Maertzdorf <i>et al.</i> (6) 2011	Blood	Agilent Human 4x44k	GSE28623	Gambia	Active PTB	-								+		+				46			
					LTBI	-		+	≥10 mm												25		
					Healthy	-			0 mm													37	
Maertzdorf <i>et al.</i> (7) 2011	Blood	Agilent 2 colour	GSE25534	South Africa	Active PTB	-									±	+	+			33			
					LTBI	-		+	+						-	-	-				34		
					Healthy	-			-													9	
Lesho <i>et al.</i> (8) 2011	PBMC	Affymetrix HG 133 Plus 2.0	Not deposited	USA	Active PTB	-								+	+	+				5			
					LTBI (documented conversion)	-			≥15 mm												6		
					Healthy BCG vaccinated	-			<15 mm													5	
					Healthy BCG unvaccinated	-			0 mm													7	
Bloom <i>et al.</i> (9) 2012	Blood	Illumina HT12v4	GSE40553	South Africa	Active PTB	-								+					29				
				UK	LTBI (IGRA)	-				+										38			
Ottenhoff <i>et al.</i> (10) 2012	Blood	IlluminaRef-8 v3	GSE56153	Indonesia	Active PTB	-							+	+	+				23				
					Not active PTB	-										-	-			23			
Maertzdorf <i>et al.</i> (11) 2012	Blood	Agilent Human 4x44k	GSE34608	Germany	Active PTB	-														8			
					Sarcoidosis	-															18		
					LTBI (IGRA)	-					+										4		
					Healthy control	-																14	
Bloom <i>et al.</i> (12) 2013	Blood, purified leukocytes	Illumina HT12v4	GSE42834	UK, France	Active PTB	-								+						35			
					Sarcoidosis	-										+			±		61		
					Pneumonia	-					+					+					14		
					Lung cancer	-											+			±		16	
					Healthy control	-																113	
Cliff <i>et al.</i> (13) 2013	Blood	Affymetrix U133 Plus 2.0	GSE31348, GSE36238	South Africa	Active PTB	-							+		+				36				
Kaforou <i>et al.</i> (14) 2013	Blood	Illumina HT12v4	GSE37250	South Africa, Malawi	Active PTB	-								+		+				97			
					Active PTB	+										+					97		
					LTBI (TST and IGRA)	-			≥10 mm	+	-	-										83	
					LTBI (TST and IGRA)	+			≥25 mm	+	-	-										84	
					OD	-															±		83
Cai <i>et al.</i> (15) 2014	PBMC	Affymetrix U133 Plus 2.0	GSE54992	China	Active PTB									±	±	±	±			9			
					LTBI (IGRA)																	6	
Anderson <i>et al.</i> (16) 2014 [†]	Blood	Illumina HT12v4	GSE39941	South Africa, Malawi, Kenya	Active TB culture +	-								+	±	±	±	±		97			
					Active TB culture +	+											+	±	±	±		52	
					Active TB culture -	-															±		27
					Active TB culture -	+												+			±		17
					LTBI (TST and/or IGRA)	-			+	+			+	-									71
					OD	-																±	
OD	+																±		97				

Figure 1 Human sample types and patient group classification criteria in studies publishing novel TB transcriptomic data (2-16). [†], this study exclusively recruited children <15 years; all other studies recruited adults >18 years. *, if BCG vaccinated. LTBI data subsets highlighted in red were used in the study by Montoya *et al.* -, negative test result; +, positive test result; ±, test not essential for inclusion. BAL, bronchoalveolar lavage; IGRA, interferon-gamma release assay; LTBI, latent TB infection; MDR, multi-drug resistant TB; OD, other disease; PBMC, peripheral blood mononuclear cells; PTB, pulmonary tuberculosis; rx, treatment; TST, tuberculin skin test.

Table 1 Considerations for interpreting transcriptomic outputs based on inputs

Patient classification
Diagnostic criteria
TB diagnosis: sputum culture or smear, CXR, empiric
Latent TB: TST cut-off, IGRA, TB contact
Healthy controls: no TB exposure, asymptomatic, negative TST and IGRA
Population of origin
Genetic diversity
Environmental exposures (NTM, co-morbidity)
Differences in demographics (age, sex)
Disease severity
Duration of symptoms
Extent of disease
Pulmonary vs. extrapulmonary or disseminated
Treatment regimen
Duration of treatment prior to sampling
Sample composition
Single cell populations
Method of isolation
Method of differentiation
Measure of purity
Whole blood
Cellular composition
Separated cell subsets
Time and season of blood draw
<p>TB, tuberculosis; CXR, chest X-ray; TST, tuberculin skin test; IGRA, interferon gamma release assay; NTM, non-tuberculous mycobacteria.</p>

disease of relative lymphopenia and neutrophilia, thus, it may be predictable that whole-blood from TB patients is dominated by a neutrophil-driven transcriptomic response. Moreover, extrapolating observations in blood to processes of disease pathogenesis occurring at the site of disease remain problematic. Although this comparison does identify biomarkers of TB disease, in comparison to someone who is healthy, what is actually required is a disease-specific signature. Thus, a more rigorous comparison should include a third group of individuals with similar symptoms but differing etiology. Three such studies have performed this (*Figure 1*), two comparing TB and sarcoidosis patients (11,12), another granulomatous

lung disease, and one comparing TB patients and respiratory symptomatics with other diagnoses (14). In their study, Bloom *et al.* found the signature of TB and sarcoidosis to cluster together, when compared to pneumonia and lung cancer, and both to be dominated by interferon-inducible transcripts. Thus, one must be extremely careful when conducting any transcriptomic study to ensure the interpretation of the differentially expressed genes are contextualized with regard to the disease states of the individuals, the effect of the disease state on the peripheral blood compartment and how it may relate to processes at the site of disease.

Finally, not only is it vital to acknowledge the effect of differing proportions of cell types in differing disease states, during a whole-blood comparison, but also the purity of an isolated cell population from *in vitro* or *ex vivo* analyses, when results are to be extrapolated to a particular cell type. Montoya *et al.* made particular note of this latter point, in relation to describing unexpected gene expression results from purified CD14⁺ monocyte cultures to the presence of potentially contaminating CD8 T cells. There must be consistency in how results are interpreted. If unexpected results are attributed to an unknown percentage of contaminating cells it can become difficult to ascribe other observations from the same data solely to the predominant cell population.

The paper by Montoya *et al.* is novel in its approach and is rich in data. But, to interpret the relevance of their findings, it is necessary to carefully examine the methods, both *in vitro* and the complex informatics that form the backbone of the authors' findings. Immunologically, the *in vitro* methods are logical and easy to follow; based on their previous finding that IL-15 differentiates monocytes into macrophages exhibiting an M1-like phenotype with antimycobacterial properties (18,19), they extracted RNA for microarrays (GEO accession GSE59184) from CD14⁺ selected monocytes (90% purity) derived from adherent peripheral blood mononuclear cells (PBMC) from healthy donors treated for 6 and 24 hours with IL-15, IL-4 and IL-10 (to generate M2-like macrophages) or media control. Weighted gene co-expression network analysis (WGCNA) was then applied to the data and forms the crux of understanding all informatics analyses conducted in this study. This systems biology approach has the potential to elucidate novel pathways of cellular interaction which is vital to our understanding the complex disease pathogenesis of TB; but to correctly interpret its output, an understanding of the methodology is required.

Translating modular analyses

The WGCNA algorithm identifies modules of co-expressed genes whose transcript abundance co-varies across samples by correlation and clustering analysis. For each module, a module eigengene (ME) is determined; this represents the first principal component of the expression data matrix for the module (a genes \times b samples). This ME summarizes the expression of the module in a single number, it does not represent one gene in the module, but all genes. Then the matrix of module eigengenes (m modules \times by b samples) and the matrix of phenotype variables (b samples \times p phenotypes) are correlated with a resulting m \times p correlation matrix. This matrix provides information on the association between modules and phenotype. In the Montoya *et al.* paper, only a single module (*MEblack*) is highlighted as biologically relevant, being the most positively correlated with IL-15 treatment; all other modules are excluded in further discussion. The black module is enriched for the gene ontology (GO) term “defense response” (48 genes out of 802 probes), and contains *IL32*. Next, 36 “myeloid defense genes” are defined, characterized as any of these 48 defense response genes expressed in resting myeloid cells, as determined from a preexisting transcriptional dataset (21). Interestingly the authors define *IL32* as myeloid-derived despite lymphoid cells being the predominant cells expressing it in their comparison of 24 gene-sets from resting cells. They further support the myeloid origin of *IL32* by reference to studies identifying its expression by monocytic cells following IFN γ , TLR4 or NOD2 activation; an alternate interpretation of this data is that *IL32* expression is induced by stimulation and not abundant in resting myeloid cells. Other “defense-response” genes were labeled myeloid-derived based on their informatics approach alone and it highlights the need for consistency in the interpretation and selection of candidate genes which become the focus of further analyses. The top interactions for these myeloid-defense genes were then visualized using the network visualization and analysis tool VisANT and consequently *IL32* was linked to the vitamin D antimicrobial pathway, via correlation with *CYP27B1* induction, which encodes the final enzyme needed to activate vitamin D.

Avoiding selective bias

While the authors elegantly demonstrate with subsequent *in vitro* silencing experiments that IL-32, reliant on vitamin D, is a downstream gene in the IFN γ antimicrobial pathway,

it would have been of interest to have more information about the module in which *IL32* was identified, including the gene list for *IL15black*, the fold-change of *IL32*, direction of induction of and its position in the list of genes regulated by IL-15. It is clear *IL32* is an important gene in this module, but there are others, which may also pose as potential correlates of protection. This is a fundamental issue researchers working with transcriptomic data now face; given a list of 100 interesting genes, what is the basis for further investigation? Supervised selection of genes may bias outcome and more high-throughput methods for robust validation and confirmation using *in vivo* systems is required. When researchers are selective, they must ensure their approach is transparent and reproducible. They must provide all gene lists which guided their selective approach, to ensure their outcomes are not biased and their findings are placed within the context of the greater gene regulation network(s).

The final section of the paper focuses on mining existing transcriptomic datasets from patients with active TB, LTBI or healthy controls, utilizing four of the 15 available studies (5,9,11,14). The overall approach is to identify genes that are more highly expressed in individuals with LTBI (diagnosed using a combination of TST, Quantiferon Gold and in-house IGRA assays) compared to those with either active TB or healthy controls (asymptomatic IGRA-negative). One of these studies (9) also investigated the transcriptional response during TB treatment, and was used to identify genes highly expressed in LTBI, which are low at TB diagnosis and increase during treatment. The previously identified *IL15black* module from the macrophage microarray experiments was then overlapped with the LTBI-high genes and with the one module from the response to treatment analysis, which was selected on the basis that it contains IL-32. Four other modules from the TB treatment data set actually show a more significant ME for genes which increase during treatment, but these do not contain IL-32 and are therefore not followed-up. Thus there remain many uninvestigated but interesting modules, which may provide further potential correlates of protection, but which were deprioritized in a somewhat self-fulfilling approach.

While it is clear that *IL32* has a role in antimicrobial immunity, there are two methodological issues which may arise in any TB OMICS study, which should heed caution to it being defined as a correlate of protective immunity, until further validation. Firstly, the immunological basis of LTBI diagnosis, secondly, the differences in cell populations

between healthy and disease individuals. As the LTBI group used in this study was defined by IGRA positivity, and thus their greater ability to produce IFN γ , it is potentially self-fulfilling that transcripts associated with the IFN- γ pathway, i.e., *IL32*, were identified to be more abundant in latent *vs.* healthy individuals. Moreover, *IL32*, which is predominantly expressed by lymphoid cells, is less abundant in active TB, a state of peripheral lymphopenia, and the increase in *IL32* during therapy is coincident with peripheral lymphocyte reconstitution. Thus, the lack of the marker in TB may merely be a reflection of the peripheral blood state of disease and not necessarily a functional defect resulting in loss of protective immunity. These issues highlight the importance of using non-immunological criteria for patient group classification and the need to adjust for differences in the cellular composition of samples compared between groups.

The future of TB OMICS

Translational Medicine increasingly relies on high-throughput data to inform hypothesis generation and system descriptions. While the generation of such data is becoming routine; increasingly complex informatics approaches are being utilized in order to optimally extract information from the data. Based on our interpretation, it is not clear that the approach used to identify correlates of protection from active TB was truly unsupervised, but rather semi-supervised and it appears that the study conclusions were to some extent self-fulfilling. Despite these limitations, such systems approaches to identify correlates of protection using existing data sets are useful, as is the use of transcriptomics to identify novel pathways of protection from *in vitro* cultures. For the informatics approaches to work best, higher-quality patient-level metadata is required for published transcriptomic datasets. Such information is crucial to account for differences in populations, disease presentation, *M.tb* exposure and underlying co-existent conditions (infections and non-communicable diseases). Ownership of such data and authorship of future manuscripts using this data may become complex. However, if we are to move towards utilizing the wealth of data accumulated over the last decade, in order to provide the crucial insights needed to advance TB research, this is a step researchers need to tackle.

To fully utilize the information from high-throughput OMICS approaches, there is the need to develop a platform for data integration. This should systematically capture all

information from transcriptomics, proteomics and detailed clinical phenotype data. There should be an emphasis on shared relationships and common associations in order to develop a multiscale model of the biology of the host response to TB, constrained by unbiased, high-throughput observations. We therefore propose the time is ripe for the field of TB-OMICS to convene a combined workshop for all groups who have deposited large OMICS data sets to define how to proceed to look for translational outcomes for these data, specifically markers of protection, diagnostics and treatment monitoring.

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