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"Tuberculosis in advanced HIV infection is associated with increased expression of IFNγ and its downstream targets"

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Abstract

Background: Tuberculosis (TB) is the major cause of death in Human Immunodeficiency Virus (HIV)-infected individuals. However, diagnosis of TB in HIV remains challenging particularly when HIV infection is advanced. Several gene signatures and serum protein biomarkers have been identified that distinguish active TB from latent infection. Our study was designed to assess if gene expression signatures and cytokine levels would distinguish active TB in advanced HIV.

Methods: We conducted a case-control study of whole blood RNA-Seq and plasma cytokine/chemokine analysis in HIV-infected with CD4$^+$ T cell count of ≤ 100 cells/μl, with and without active TB. Next, the overlap of the differentially expressed genes (DEG) with the published signatures was performed and then receiver operator characteristic (ROC) analysis was done on small gene discriminators to determine their performance in distinguishing TB in advanced HIV. ELISA was performed on plasma to evaluate cytokine and chemokine levels.

Results: Hierarchical clustering of the transcriptional profiles showed that, in general, HIV-infected individuals with TB (TB-HIV) clustered separately from those without TB. IPA indicated that the TB-HIV signature was characterized by an increase in inflammatory signaling pathways. Analysis of overlaps between DEG in our data set with published TB signatures revealed that significant overlap was seen with one TB signature and one TB-IRIS signature. ROC analysis revealed that transcript levels of *FcGR1A* (AUC = 0.85) and *BATF2* (AUC = 0.82), previously reported as consistent single gene classifiers of active TB irrespective of HIV status, performed successfully even in advanced HIV. Plasma protein levels of IFNγ, a stimulator of *FcGR1A* and *BATF2*, and CXCL10, also up-regulated by IFNγ, accurately classified active TB (AUC = 0.98 and 0.91, respectively) in advanced HIV. Neither of these genes nor proteins distinguished between TB and TB-IRIS.

Conclusions: Gene expression of *FcGR1A* and *BATF2*, and plasma protein levels of IFNγ and CXCL10 have the potential to independently detect TB in advanced HIV. However, since other lung diseases were not included in this study, these final candidates need to be validated as specific to TB in the advanced HIV population with TB.

Keywords: Tuberculosis, HIV, Biomarker, TLR, Inflammation, IRIS, Transcriptomics, Cytokines, CXCL10, IFNγ, *FcGR1A*, *BATF2*

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Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 2 of 13

Background

Tuberculosis (TB) is the leading cause of death in HIV-infected persons. The majority (74%) of TB-HIV coinfected persons reside in Sub-Saharan Africa [1]. Overall, HIV-infected are 20–30 times more likely to develop active TB than those without HIV infection, with an annual risk as high as 5–15% per year [2, 3]. The transition from latent TB infection (LTBI) to active disease in HIV-infected depends on host immunity and on the level of immunosuppression [4, 5]. The CD4+ T cell count is only an approximate measure of the degree of immunosuppression at the population level and thus an inaccurate marker of the likelihood of reactivation in a HIV-infected host [6–8].

Along the spectrum of infection, individuals with asymptomatic active disease (subclinical TB) [9] are clinically unrecognized and often progress to active symptomatic TB disease [10]. In a high prevalence setting, 8.5% of asymptomatic, HIV-infected persons had positive sputum cultures for *Mycobacterium tuberculosis* (Mtb) [11]. Even when sputum culture is used to exclude active TB, asymptomatic cases may be misclassified particularly in the immunosuppressed where disseminated disease may predominate [12]. HIV-infected with occult TB are at risk of unmasking TB-IRIS, which has significant morbidity and mortality [7, 13]. Therefore, new approaches to the diagnosis of TB in this group are imperative.

Transcriptional profiling studies have identified several different biosignatures that can differentiate active TB from LTBI [14–20] and from other related inflammatory diseases [16, 17, 21]. Two of these, the 27-gene signature identified in the study by Kaforou et al., [16], and the 251-gene signature reported by Dawany et al., [22], performed well even in HIV-infected individuals. The biosignatures derived from these multiple cohorts do not show significant overlap. However, publically available databases provide a wealth of material for meta-analysis and derivation of a biomarker signature that can potentially function across cohorts. Using this approach, a targeted Real Time-PCR (RT–PCR) array was designed by Maertzdorf and colleagues [23] based on two of their microarray datasets [24, 25] and then applied to a new cohort from India. This strategy generated a 4-biomarker set (*GBP1*, *IFITM3*, *P2RY14* and *ID3*) that performed satisfactorily even in HIV-coinfected individuals. Subsequently, a 3-gene set consisting of *GBP5*, *CD64* and *GZMA* was identified that accurately separated TB from OPD (other pulmonary diseases) [26]. Other studies have shown that *CD64*, *FcGR1B* and *LTF* are differentially expressed in TB versus other lung diseases [17, 27, 28]. Another meta-analysis [29], using a total of 14 datasets identified a different set of three genes (*GBP5*, *DUSP3*, and *KLF2*) that were highly

diagnostic for active tuberculosis in HIV infected and uninfected populations. Validation studies confirmed that this 3-gene set separated active tuberculosis from healthy controls, latent tuberculosis and other diseases. The usefulness of this gene set in monitoring treatment response is under-scored by the observation that the expression of the 3-gene set declined during treatment of patients with active tuberculosis [29]. In another multi-site study [30], a targeted approach was used to validate genes identified in single-site populations. This study found that *FcGR1A* was the most reliable classifier of active TB and its expression was not confounded by HIV status or ethnic background. The performance of *FcGR1A* was not compromised even in advanced HIV [30]. Similarly, another recent study analyzed new and previously published data on blood transcriptional profiles and found that elevated transcript levels of *BATF2* can also robustly discriminate active TB from healthy controls [31]. However, the study found elevated *BATF2* levels even in the absence of TB in HIV negative individuals who presented with various other infectious diseases. The authors concluded that *BATF2* transcript may have better value as a test for ruling out TB. We therefore tested the accuracy of *FcGR1A*, which was not confounded by HIV status, and *BATF2*, which had better negative predictive value for TB, in discriminating active TB in the context of coinfection with HIV.

Biomarkers for tuberculosis have also been identified through serum proteomic profiles. A comparison of TB cases to healthy controls identified fibrinogen degradation product (FDP) [32] and Orosomucoid (ORM) as potential biomarkers [33]. Another study found that levels of serum amyloid A, transthyretin, C-reactive protein, and neopterin discriminated patients with TB from other respiratory disorders (ORD) and inflammatory diseases [34]. In comparison to LTBI and ORD, serum samples from TB patients expressed a biomarker panel of 8 proteins [35]. This study also found that in the HIV coinfected TB patients, the composition of the host protein biomarker panel was slightly different and consisted of 10 host proteins [35]. Other metabolomic profiling studies have also provided additional biomarker panels that discriminate TB patients from healthy controls [36–38]. In a recent study using Somascans, an aptamer-based proteomic platform, De Groote and colleagues measured over 4000 host proteins and identified a 6-marker signature for active TB that included SYWC, kallistatin, C9, gelsolin, testican-2, and aldolase C [39].

Thus, the aim of this study was to determine if gene expression signature and plasma protein biomarkers would distinguish active TB in severely immunosuppressed TB-HIV participants prior to initiation of TB treatment or anti-retroviral therapy (ART).

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 3 of 13

Methods

Study design and participant recruitment

Inpatients from Mulago National Tertiary Referral Hospital and outpatients from the Infectious Diseases Institute at Makerere University College of Health Sciences with serologically confirmed HIV-1 infection, who were $\geq$ 18 years of age with a $CD4^+$ T cell count ≤ 100 cells/μL, were recruited to a prospective cohort study between March and December 2013. The participants were included on the basis of a $CD4^+$ T cell count documented in the chart. $CD4^+$ T cell counts were rechecked at baseline for 2 participants, and were found to be slightly above 100 cells/μl. They were still included in the study. A targeted medical history as well as socio-demographic information was collected. Patients were systematically screened for TB at the Infectious Diseases Institute. HIV status was gathered either from the patient's chart or was retested, if unknown. During follow-up, the one participant who was HIV positive, on ART and developed TB was reclassified as such. None of the included patients had any other documented opportunistic infection. Participants were assigned to one of 3 groups: new diagnosis of smear-positive or microbiologically confirmed TB and ART-naïve; smear or microbiologically-confirmed TB within 180 days of initiating ART (unmasked TB); no signs or symptoms of TB and ART-naïve. Participants were excluded if they had received more than 2 days of anti-TB medication within the previous 60 days. Blood samples were only collected at baseline. Participants were only followed to determine 6 month outcome and to verify that they did not have unmasking TB or immune reconstitution inflammatory syndrome (IRIS).

Blood sample collection and processing

Whole blood was collected into PAXgene Blood RNA tubes (supplied by Qiagen, catalog # 762125) and plasma was frozen at -80 °C at the time of enrollment. Whole blood was also collected into Becton Dickinson vacutainer acid citrate dextrose (ACD) tubes for collection of peripheral blood mononuclear cells (PBMC). PAXgene Blood RNA tubes were stored at -80 °C and PBMC were stored in liquid nitrogen until further use.

RNA sequencing (RNA-Seq) and data analysis

RNA was isolated from whole blood using the PAXgene Blood RNA Kit (Qiagen, catalog # 762164). Total RNA input was amplified using MessageAmp™ II aRNA Amplification Kit (ThermoFisher Scientific). 100 ng of amplified RNA was used to prepare the library. Following standard instructions for fragmentation, purification, 3′ and 5′ adapter ligation and reverse transcription, PCR amplicons were purified using AMPure XP beads and the library was quantified. This library was used for RNA Sequencing on Illumina Hiseq2500. On average 49–50 million basepair (bp) reads were produced per sample. Quality check of reads was performed with the software FastQC. Low quality reads and adapters were trimmed with Cutadapt [40]. Trimmed reads were mapped to the human genome GRCh38/hg19 with STAR [41], and the expression level for each gene was counted with HTSeq [42] according to gene annotations from Ensembl. The Bioconductor DESeq package in R [43] was used to normalize the counts and call differential expressions. Principal Component Analysis (PCA) was used for data visualization. Hierarchical clustering was performed with the gplots package in R. The ROC curve was plotted with the pROC package in R. Functional and network analyses of differentially expressed genes were performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, https://www.qiagen-bioinformatics.com/products/ingenuity-pathway-analysis/, Redwood City, CA).

Multiplex immunoassays

Pre-coated 10-spot MULTI-SPOT® plates with capture antibodies were purchased [catalog # K15054D-2; Meso Scale Discovery (MSD), Rockville, MD, USA]. The assays are based on the principle of electrochemiluminescence (ECL) sandwich ELISA. Briefly, plasma samples were centrifuged for 20 min at 2000 g and diluted 2-fold for cytokine analytes and 4-fold for chemokine targets prior to using. Calibrator controls, detection antibody mix and read buffer were prepared as per manufacturer's instructions. The prepared samples and calibrators were added to the plates and incubated overnight at 2–8 °C. Plates were then washed and incubated with detection antibody solution at room temperature with shaking for 2 h. After washing, 2X Read Buffer was added to each well and plates were analyzed on MESO Quickplex SQ120. The calculations to establish calibration curves and determine analyte concentrations were carried out using the MSD DISCOVERY WORKBENCH® analysis software. Protein levels of IFN-γ, IL-6, IL-8, TNF-α, Eotaxin, MCP-1, MCP-4, MIP-1α, MIP-1β, CCL17, IL-12/IL-23p40, IL-15, IL-16, IL-17A, VEGF and CXCL10 were assessed. The software generated calibration curves by fitting the signals from the calibrators to a 4-parameter logistic model with a $1/Y^2$ weighting. Analyte concentrations were determined from the ECL signals by back-fitting to the calibration curves.

Statistical analysis

Descriptive statistics were used to characterize the study population. Patient baseline characteristics were compared by Fischer's exact for categorical data and Wilcoxon rank sum test for continuous variables. Non-parametric analysis of plasma cytokine levels was done using Mann Whitney U-test.

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 4 of 13

Results

Participant demographics and baseline characteristics

Sixteen HIV-infected participants with smear (+) or microbiologically confirmed TB (TB-HIV), 15 HIV-infected participants (HIV) that had no clinical symptoms of TB and 2 TB-HIV participants undergoing treatment with ART (unmasked TB) were studied. Table 1 shows the demographic characteristics and outcomes for 32 participants, except for one TB-HIV for whom data was not available. Most of the study subjects had a CD4$^+$ T cell count ≤ 100 cells/μl. Greater than 90% of participants with HIV alone were reported alive after 6 months from their initial admission, but almost 60% participants with TB-HIV diagnosis did not survive; 5 of the 16 TB-HIV died within 16 days of enrollment. None of the HIV-infected (no TB) had been treated with ART prior to study. Blood samples were collected prior to start of TB treatment in all participants, except one patient who had received 2 days of TB treatment.

Distinct blood transcriptional profile distinguishes TB and HIV coinfected from HIV alone

Whole blood RNA-Seq analysis was performed on all 33 participants. Hierarchical clustering of the transcriptional profiles based on logarithmic plotting of normalized counts showed two clustering patterns that were non-random, that is, within cluster variance looks smaller than between cluster variance (Fig. 1a). The top 50 DEG are listed in Additional file 1: Figure S1. Differential expression of genes between TB-HIV and HIV is reported in the volcano plot (Fig. 1b). Eleven of the 16 HIV participants with active TB (TB-HIV) clustered separately (Cluster 1) from HIV-infected participants (HIV) that were asymptomatic, with no clinical or microbiological evidence of TB (Cluster 2). The remaining 5 TB-HIV segregated with Cluster 2 and the 3 HIV segregated with Cluster 1. The 2 TB-HIV being treated with ART also segregated with Cluster 1 (Fig. 1a). The data were

further analyzed using PCA. Consistent with the heat map, plot of PC1 vs. PC2 also corroborated that the participants segregated into two main groups (Fig. 1c). The 11 TB-HIV and 3 HIV that belonged to Cluster 1 in the global expression analysis also segregated as one group by PCA and included the 2 TB-HIV that had previously received treatment with ART and then developed unmasked TB. Additionally, both clustering and PCA resulted in the same 5 TB-HIV patients separating out from the majority grouping of TB-HIV patients (Fig. 1c). Although some participants with TB-HIV did not survive, PCA and RNA-Seq patterns seen previously were not indicative of a mortality-related clustering (Fig. 2). Of note, the only subject that died in the HIV group was in fact one of the 3 HIV that segregated with Cluster 1.

Data mining using IPA reveals enrichment of innate inflammatory pathway genes in TB-HIV coinfected participants

Network, function, and pathway analyses were generated using IPA (Qiagen). Differential expression gene list comparing TB-HIV samples to HIV only, with *p*-value < 0.01 and a log fold change greater than 2, was generated and uploaded to IPA. Analysis showed that the top five canonical pathways contributing to overexpression of genes related to the following signaling pathways: Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis; IL-10, p38 MAPK and TLR signaling, Hepatic Fibrosis/Hepatic Stellate Cell Activation (Table 2). Predicted upstream regulators of this signature were IL-1, Immunoglobulin, PGR, lipopolysaccharide, CSF2 (Table 2). The top five canonical pathways contributing to down-regulated expression of genes related to EIF2, Primary Immunodeficiency, B cell development, Granzyme A and Regulation of eIF4 and p70S6K signaling pathways (Table 2). Predicted upstream regulators of these pathways were IL-15, MYCN, Alefacept (a genetically

Table 1 Demographic Characteristics and Participant Outcomes

	Total[a] (n = 32)	TB-HIV (n = 17)	HIV (n = 15)	*p*-value
Female	62% (20/32)	47% (8/17)	80% (12/15)	0.08^
Median Age (range)	32 [18–53]	30 [18–52]	36 [24–53]	0.43^^
Initial CD4$^+$ (cells/μL)	50 [4–105][b]	23 [4–105]	67 [11–96]	0.15^^
Time to ART start (days)	14 [6–80][c]	14.5 [12–80]	12 [6–51]	0.08^^
IRIS	20% (5/25)	45% (5/11)	0% (0/14)	0.13^
Alive at 6 months	75% (24/32)	59% (10/17)	93% (14/15)	0.03^

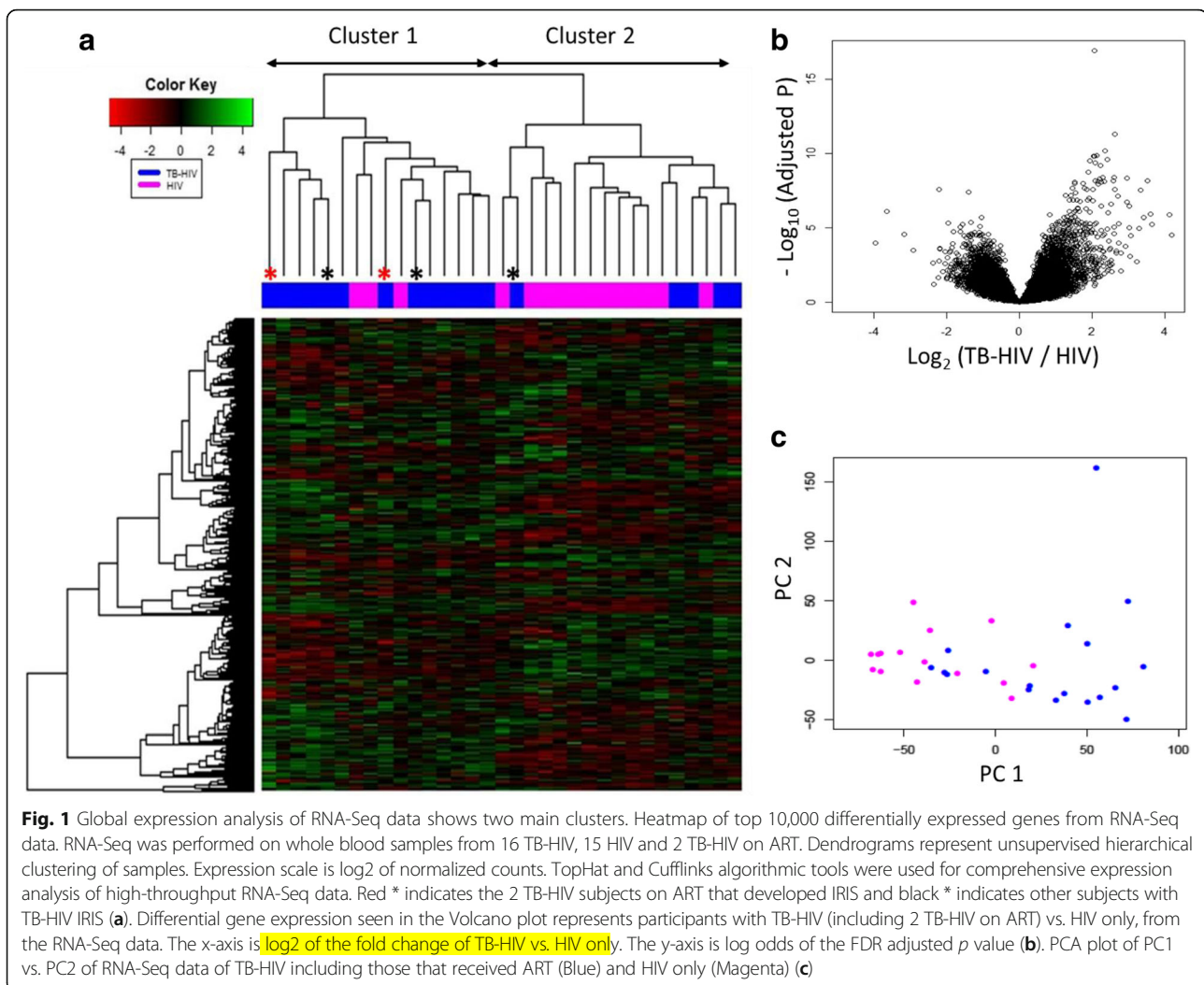[a]Demographic data available for 32 study participants. Whole blood RNA-Seq analysis done for 33 participants
[b]The initial CD4 count for 2 participants was 103 and 105 cells/μl; however when repeated for the study, the counts were less than 100 cells/μl
[c]19 observations
^p-value calculated by Fischer's exact
^^p-value calculated by Wilcoxon rank sum test
Age given is median age, range is entire range of ages. CD4$^+$ range is entire range of CD4$^+$ T cell counts

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 5 of 13

Fig. 1 Global expression analysis of RNA-Seq data shows two main clusters. Heatmap of top 10,000 differentially expressed genes from RNA-Seq data. RNA-Seq was performed on whole blood samples from 16 TB-HIV, 15 HIV and 2 TB-HIV on ART. Dendrograms represent unsupervised hierarchical clustering of samples. Expression scale is log2 of normalized counts. TopHat and Cufflinks algorithmic tools were used for comprehensive expression analysis of high-throughput RNA-Seq data. Red * indicates the 2 TB-HIV subjects on ART that developed IRIS and black * indicates other subjects with TB-HIV IRIS (**a**). Differential gene expression seen in the Volcano plot represents participants with TB-HIV (including 2 TB-HIV on ART) vs. HIV only, from the RNA-Seq data. The x-axis is log2 of the fold change of TB-HIV vs. HIV only. The y-axis is log odds of the FDR adjusted *p* value (**b**). PCA plot of PC1 vs. PC2 of RNA-Seq data of TB-HIV including those that received ART (Blue) and HIV only (Magenta) (**c**)

engineered anti-inflammatory drug), T Cell Receptor and lipopolysaccharide (Table 2).
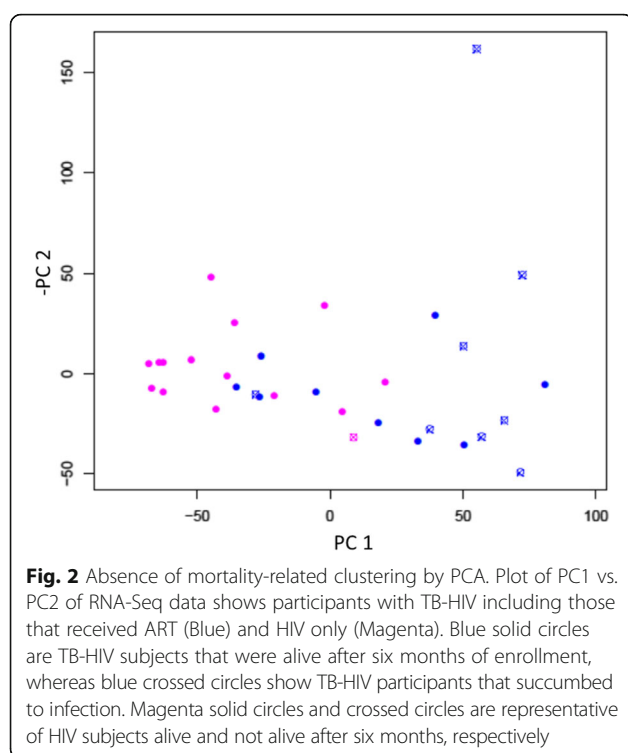
Overlap of differentially expressed genes in TB-HIV versus HIV with existing TB signatures

A 27 [16] and a 251-gene [22] signature have been reported to accurately segregate TB-HIV coinfected from HIV only. We therefore tested whether the 749 differentially expressed genes in our dataset overlapped significantly with published gene signatures. As shown in Table 3, there was significant overlap with the 27-gene signature, however significant overlap was not observed with the 251-gene signature. IPA pointed to an enrichment of innate inflammatory pathway genes in TB-HIV coinfected participants in our cohort, similar to that reported by Lai et al. [44]. Here, a 43-transcript signature characterized by innate Toll-like receptor and inflammasome signaling predicts the development of immune reconstitution inflammatory syndrome (IRIS) in TB-HIV [44]. We found that the DEG that were over-expressed

in Cluster 1 had significant overlap with this 43-transcript signature (Table 3). We also found significant overlap with the 16-gene blood transcriptional signature that predicts risk of progression to TB disease [45].

Cytokine levels in plasma

IPA predicted up-regulated expression of innate inflammatory genes in TB-HIV. We therefore quantified the levels of 16 pro-inflammatory mediators in the plasma of TB-HIV and HIV to determine if a cytokine/chemokine signature would distinguish the two groups. Significantly higher levels of IFNγ and CXCL10 were seen in TB-HIV compared to HIV ($p < 0.0001$) (Fig. 3a and d). IL-6, IL-8 and TNF were also significantly up-regulated in the TB-HIV ($p < 0.0001$) (Fig. 3a). Levels of key leukocyte chemotactic agents MIP-1α and MIP-1β were up-regulated as well in TB-HIV, compared to those with HIV only (Fig. 3b). Interestingly, the 3 HIV-infected participants whose transcriptional profile was different from the others with HIV alone exhibited pro-inflammatory

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 6 of 13

Fig. 2 Absence of mortality-related clustering by PCA. Plot of PC1 vs. PC2 of RNA-Seq data shows participants with TB-HIV including those that received ART (Blue) and HIV only (Magenta). Blue solid circles are TB-HIV subjects that were alive after six months of enrollment, whereas blue crossed circles show TB-HIV participants that succumbed to infection. Magenta solid circles and crossed circles are representative of HIV subjects alive and not alive after six months, respectively

cytokine levels equivalent to the other HIV-infected patients (Fig. 3). Also noteworthy were the 5 TB-HIV participants that clustered away from the parent TB-HIV Cluster 1 in RNA-Seq, yet showed cytokine levels comparable to their parent group (Fig. 3). Together, these data indicate that: i) soluble markers of immune activation are elevated in TB-HIV compared to HIV and ii) the TB-HIV and HIV who did not segregate with their fellow members in their transcriptional profile, nonetheless had cytokine profiles similar to their fellow members. Although there were no biases due to gender, age or other evaluated parameters, it was not clear why some samples were misclassified in RNA-Seq.

FcGR1A and *BATF2* transcript levels, and IFNγ and CXCL10 plasma protein levels singly classify active TB

A successful diagnostic test requires minimal number of markers, and consequently several studies have pursued this goal to come up with 3–10 genes as sufficient for successfully classifying active TB from controls [20, 23, 29]. As shown in Table 4, we obtained AUC scores for these signatures to determine how well they would perform in discriminating TB in advanced HIV. The AUC score for the 3-gene transcriptional signature from the study by Sweeney et al. [29] was 0.89. The 4-gene signature from the Maertzdorf study [23] and the 10-gene signature from the Sambarey study [20] performed well, with AUC scores of 0.91 and 0.92, respectively (Table 4).

FcGR1A is part of the discriminatory gene set in a number of biomarker studies [14, 15, 46] and as a single marker it fairly discriminates active TB from latent infection regardless of HIV status or genetic background [30]. Given that *FcGR1A* is differentially expressed in our study between TB-HIV and HIV (Fig. 4a), we performed ROC analysis to determine if *FcGR1A* was also a good classifier of active TB in advanced HIV. We found that *FcGR1A* correctly classified 85% of the patients [AUC = 0.85; 95% CI: 0.7095–0.9905] (Fig. 4b). A recent report found *BATF2* transcript was also an accurate classifier of active TB in both HIV-uninfected and infected individuals [31]. In our advanced HIV cohort, *BATF2* transcript levels discriminated active TB with an AUC score of 0.82 [95% CI: 0.6642–0.9692] (Fig. 4a and c).

IFNγ is a potent stimulator of *FcGR1A* and *BATF2* expression and its protein levels are significantly upregulated in TB-HIV (Fig. 3). We therefore obtained ROC AUC of 0.98 for the diagnostic potential of IFNγ protein expression in our dataset [95% CI: 0.9402–1] (Fig. 5a). Next, we also performed similar ROC analysis to determine the performance of CXCL10 since its plasma levels were upregulated in TB-HIV (Fig. 3) and its expression is also induced by IFNγ. CXCL10 accurately classified TB with an AUC score of 0.91 [95% CI: 0.8078–1] (Fig. 5b).

Development of IRIS

The cohort was followed for 6 months. Five of the TB-HIV coinfected (including the two that were undergoing treatment with ART at enrollment) developed IRIS (Table 1). The participants who developed IRIS segregated with Cluster 1 and had comparable levels of IFNγ and CXCL10 with respect to the TB-HIV cases that did not develop IRIS (Additional file 2: Figure S2). These findings indicate that in our cohort, participants with TB showed a high innate inflammatory signature irrespective of whether they developed IRIS or not.

Discussion

The significant outcome of this study is the finding that differential transcript levels of *FcGR1A* and *BATF2* and protein expression levels of IFNγ and CXCL10 robustly classified active TB in advanced HIV, including the 3 HIV-infected and 5 TB-HIV individuals whose gene expression pattern was not consistent with their fellow members. *FcGR1A* is expressed on most myeloid cells [47] and it activates a number of effector functions, including phagocytosis, antigen-presentation, the production of cytokines and reactive oxygen species and antibody-mediated cellular cytotoxicity (reviewed in [48]). The study by Sutherland and colleagues [30] found that *FcGR1A* could correctly segregate 75% of the HIV participants into active TB disease or not. In our study,

Table 2 IPA analysis enlisting top five canonical pathways and upstream regulators in TB-HIV and HIV gene sets

Top pathways (upregulated)	*p*-value	Overlap	Overlapping Genes
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	6.01E-06	6.3% (18/287)	SOCS3,ICAM1,TLR8,CEBPD, IRAK3,IL1R1, CREB5, PDGFC, FCGR1A, IL18R1, IL18RAP, IL1R2,FOS, NFKBIA,MAPK14,TLR5,TLR1,TCF7L2
IL-10 Signaling	3.24E-05	11.8% (8/68)	IL1R2,FOS, SOCS3, IL4R,NFKBIA, MAPK14,IL1R, IL18RAP
p38 MAPK Signaling	5.71E-05	8.5% (10/117)	IL1R2, MAPK14,TIFA,DUSP1, MKNK1, IRAK3,IL1R1, CREB5, IL18RAP, HSPB1
Toll-like Receptor Signaling	3.68E-04	9.6% (7/73)	FOS,NFKBIA,MAPK1,TLR5, TLR1,TLR8, IRAK3
Hepatic Fibrosis / Hepatic Stellate Cell Activation	5.09E-04	6.1% (11/181)	IL1R2,IL4R, ICAM1,HGF, FGFR1,IGF1R, COL4A2,IL1R, IFNAR2, PDGFC, IL18RAP
Upstream Regulators (upregulated)	*p*-value of overlap		
Immunoglobulin	1.51E-13		
PGR	3.23E-08		
Lipopolysaccharide	4.31E-08		
CSF2	4.81E-07		
IL-1	4.98E-07		
EIF2 Signaling	3.40E-08	7.5% (13/173)	RPS7,RPS6, RPL4, RPLP1, RPL23A, RPL22L1, RPL37,EIF4A2, RPL23,RPS17, RPS4,RPL13A,RPS11
Primary Immunodeficiency Signaling	6.06E-06	13.6% (6/44)	RFX5,LCK, ZAP70, CD8A,UNG, IGHD
B Cell Development	1.67E-04	14.8% (4/27)	SPN,CD79B, HLA-DMB,IGHD
Granzyme A Signaling	6.82E-04	17.6% (3/17)	PRF1,H1FX, APEX1
Regulation of eIF4 and p70S6K Signaling	7.64E-04	4.9% (7/143)	RPS7,RPS6, EIF4A2, RPS17,RPS4X, RPS11,ITGA4
Upstream Regulators (downregulated)	*p*-value of overlap		
MYCN	2.72E-09		
Alefacept	5.84E-08		
TCR	2.59E-06		
Lipopolysaccharide	2.89E-06		
IL-15	9.25E-06		

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 8 of 13

Table 3 Overlap of DEGs with existing gene signatures

Gene list source	Genes in Signature	Overlap with DE genes (Current study)	Common	Overlap *p*-value
Kaforou et al. (PMID: 24167453)	27	CD79B, DUSP3, FAM20A, FLVCR2, FCGR1A, ANKRD22	6	0.004707
Dawany et al. (PMID: 24587128)	251	ZNF516, SMAD7, ITGA4, FLVCR2, MSRB2, C9orf91, IFNAR2, PPBP, ARL4C, GK HLA-DMB	11	0.6178
Lai et al. (PMID: 26399326)	43	MAPK14, SIPA1L2, CDK5RAP2, ANXA3, BCL2A1, DOK3, ACSL1, TPST1, PFKFB3, BASP1, GPR97, TLR5	12	5.39E-08
Zak et al. (PMID: 27017310)	16	FCGR1A, ANKRD22, BATF2	3	0.0001189

FcGR1A performed better by correctly classifying 85% of the advanced HIV participants. Treatment of TB results in a significant reduction of *FcGR1A* expression [49] suggesting that monitoring *FcGR1A* may provide a useful tool to monitor treatment response in advanced HIV.

Basic leucine zipper transcription factor ATF-like (BATF) 2, is a transcription factor that belongs to the activator protein 1 family of transcription factors and contains the basic leucine zipper domain. *BATF2* mediates pro-inflammatory responses by associating with Interferon regulatory factor 1 (IRF1). Of note, *BATF2* gene expression is induced by type I IFNs [50] and by LPS, IFNγ and Mtb in macrophages [51]. Based on data from

multiple study cohorts, Roe and colleagues proposed the application of *BATF2* as a robust discriminator of active TB from healthy individuals [31]. Their study also demonstrated that the classification accuracy was not compromised by HIV infection. Performance of *BATF2* in our cohort (AUC 0.82) was equivalent to that reported by Roe et al. (AUC 0.84).

Expression of *FcGR1A* is induced by IFNγ [52] and consistent with this we found IFNγ levels to accurately segregate TB-HIV patients from HIV. In the seminal study of O'Garra and colleagues [14], an IFN signature was associated with TB. However, it was surprising to see a strong IFNγ response in patients with CD4+
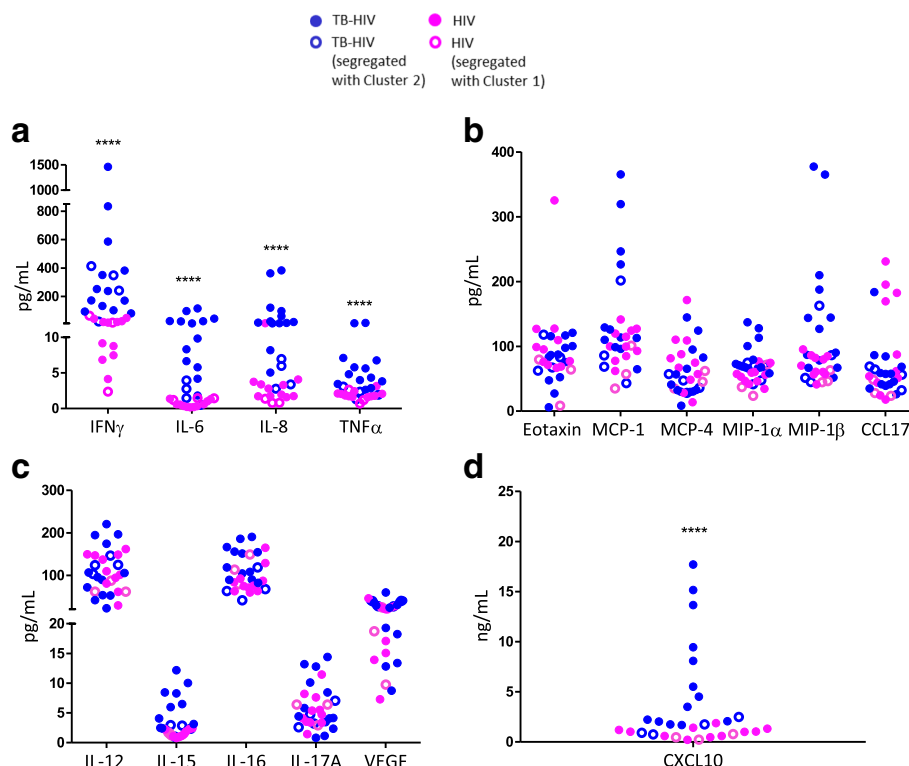

Fig. 3 Increased levels of inflammatory mediators in plasma of TB-HIV. Plasma samples from 31 participants were analyzed for cytokines and chemokines using a multi-analyte detection system (Meso Scale Discovery, Rockville, MD, USA). Plasma samples from 1 individual from each of the TB-HIV and HIV were not available. Data is represented as absolute value for each participant for a given cytokine or chemokine (**a**-**d**). Significance was determined by Mann Whitney U test. ****$p < 0.0001$

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 9 of 13

Table 4 Receiver Operator Characteristic AUC scores of different signatures to discriminate active TB from controls

Gene list source	HIV	Genes in signature	AUC	95% CI
Sweeney et al. (PMID: 26907218)	yes	DUSP3, GBP5, KLF2	0.89	0.771–1
Laux de Costa et al. (PMID: 26025597)	no	GBP5, FcGR1A, GZMA	0.87	0.7575–1
Maertzdorf et al. (PMID: 26682570)	yes	GBP1, IFITM3, P2RY14, ID3	0.91	0.8001–1
Sambarey et al. (PMID: 28065665)	no	FcGR1A, HK3, RAB13, RBBP8, IFI44L, TIMM10, BCL6, SMARCD3, CYP4F3, SLPI	0.92	0.8453–1

counts < 100. Other innate cell types, including γδ T cells and NK cells secrete IFNγ during TB infection (reviewed in [53]). It would be interesting to investigate the cell type(s) producing IFNγ in advanced HIV since it may provide opportunities for developing flow cytometry-based diagnostic tools. Even if the specificity of such an assay is low, it would serve as a potential point-of-care triage test. CXCL10 is selectively up-regulated in TB pleural effusions compared with malignant effusions [54–56] and in lateral flow based test it showed promise as a diagnostic tool for pleural TB [57]. CXCL10 is also one of the seven-marker serum protein biosignature for the diagnosis of active TB disease in African primary healthcare clinic attendees with signs and symptoms suggestive of TB

[58]. In our study plasma levels of CXCL10 accurately classified TB in 91% of the TB-HIV patients, indicating the potential of this analyte as a diagnostic biomarker.

A study from Lai and colleagues found a consistent over-abundance of 43 genes encompassing innate immune mediators of the TLR and inflammasome signaling pathways in TB-IRIS participants, 2 weeks before they developed IRIS when compared to non-IRIS controls [44]. It is not clear why the TB-HIV participants in our cohort who did not progress to IRIS also had a high innate inflammatory signature. One possibility is the timing of the blood sample collection for transcriptomics. The blood samples in our study were obtained from participants prior to anti-TB treatment, whereas in
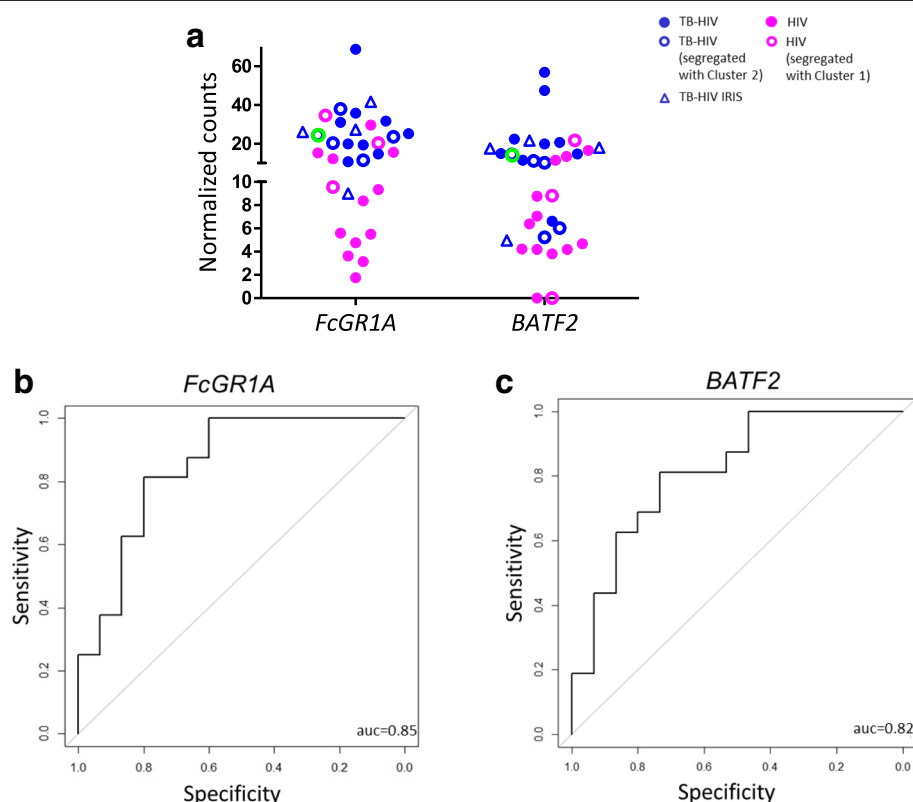

Fig. 4 Classification of active TB in advanced HIV using *FcGR1A* and *BATF2*. Normalized counts obtained from RNA-Seq analysis for all 33 participants are plotted for *FcGR1A* and *BATF2* (**a**). Green open circle is representative of sample from one TB-HIV participant, who segregated with Cluster 2 and also developed IRIS (**a**). ROC AUC score for each target was obtained using the pROC package in R.Functional (**b** and **c**)

Verma *et al. BMC Infectious Diseases* (2018) 18:220
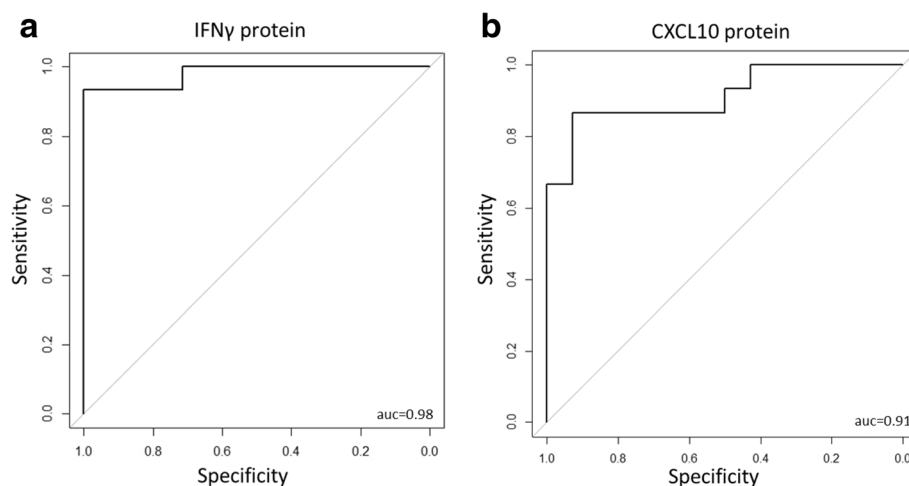
Page 10 of 13

Fig. 5 Classification of active TB in advanced HIV using IFNγ and CXCL10. Protein concentrations from patient plasma samples were used to determine ROC AUC scores for IFNγ and CXCL10 (**a** and **b**). AUC scores were obtained using the pROC package in R.Functional

the Lai study [44] they were collected following approximately 1 month of anti-TB therapy. Given that even 2 weeks of anti-TB treatment is sufficient to significantly down-modulate the TB gene signature [59], the baseline samples collected prior to ART, but post anti-TB treatment, from TB-HIV coinfected participants may have a lower expression of inflammatory genes. Therefore in the Lai study, using these baseline samples as comparator, the increase in inflammatory genes associated with TLR and inflammasome signaling could be detected as early as 0.5 week following ART in only those progressing to IRIS. The mechanisms leading to enhanced inflammation in untreated TB versus in those developing IRIS may be different, but since the mediators of inflammation are non-specific, the gene signature for TB in the HIV-infected and that for IRIS may overlap. Thus, our findings would suggest that use of gene transcript sets as biomarkers for predicting IRIS should take into account the TB treatment status of the TB-HIV subjects.

Although the sample size in this study is small, nonetheless, pathway analysis (through IPA) of the transcriptional data provides a few clues to the underlying mechanisms leading to the enhanced inflammatory response in the TB-HIV group. For example, TLR signaling, p38 MAPK signaling and role of macrophages, fibroblasts and endothelial cells in Rheumatoid Arthritis were some of the top upregulated pathways. Interleukin-1β, one of the upregulated upstream regulators is clearly essential for anti-mycobacterial immunity but excessive amounts of the cytokine is pro-inflammatory and can cause tissue damage (reviewed in [60]). Granulocyte-macrophage colony-stimulating factor (GM-CSF), also referred to as colony-stimulating factor 2 (CSF2), another upregulated upstream regulator, also contributes to anti-mycobacterial immunity [61]. However, in the

context of autoimmune disease, GM-CSF is a strong inducer of tissue inflammation and plays a critical role in disease progression (reviewed in [62]). EIF2 signaling and primary immunodeficiency signaling were the top downregulated pathways. Phosphorylation of Eukaryotic initiation factor 2 (eIF2) is activated by diverse stress responses and results in repression of global translation, except for the translation of select genes, including *ATF4* (activating transcription factor 4). ATF4 is a master regulator of the integrated stress response [63, 64]. Interestingly, treating macrophages with the drug Guanabenz increased the phosphorylation level of eIF2α and resulted in elevated mRNA levels of IL-6 and GM-CSF [65]. This leads to the conjecture that the decreased EIF2 signaling may be related to the enhanced inflammatory response seen in TB-HIV. Granzyme A signaling was another downregulated pathway. Since Granzyme-A is expressed by NK cells and CD8+ cytotoxic T cells and is a tryptase that induces a caspase-independent cell death [68], its downregulation is suggestive of functional defects in the two immune cell types. Alefacept, is a dimeric fusion protein made up of the extracellular CD2-binding portion of the human leukocyte function–associated antigen 3 (LFA-3) linked to the Fc portion of human IgG1 [66]. Alefacept preferentially targets CD4+ and CD8+ effector memory T cells [66] and therefore in the context of HIV fits as one of the upstream regulators that was found to be downregulated. IL-15, a cytokine critical for NK cell proliferation and memory CD8+ T cell development, was another upstream regulator that was downregulated [67]. Overall, the IPA data point to upregulated and downregulated pathways as well as regulators that could together result in increased inflammation in TB-HIV. This is further reflected in the increased levels of inflammatory biomarkers in plasma.

Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 11 of 13

Conclusion

Overall, we found that plasma protein levels of IFNγ and CXCL10 and gene expression of *FcGR1A* and *BATF2* have the potential of independently detecting TB in advanced HIV. However, a limitation of this study is the small sample size and lack of inclusion of other relevant "conditions" in HIV-infected. Future endeavors should include other inflammatory and infectious diseases. Additional focus should be given to longitudinal cohort studies to determine whether measure of FcGR1A, *BATF2*, IFNγ and CXCL10 could be developed into an accurate and rapid diagnostic test for subclinical TB disease in advanced HIV, particularly in countries where both infections are endemic.

Additional files

Additional file 1: Figure S1. A heatmap of top 50 genes differentially expressed between TB-HIV and HIV. Heat map showing differential gene expression of top 50 genes using largest value of adjusted fold change and *p* = 0.01 in TB-HIV as compared to HIV-only group. The x-axis is log2 of the fold change between the two groups. The y-axis is log odds of the FDR adjusted *p* value. (PPTX 297 kb)

Additional file 2: Figure S2. Progression to IRIS does not alter plasma cytokine/chemokine levels in TB-HIV participants. Plasma samples obtained from TB-HIV and TB-HIV with IRIS were analyzed for cytokines and chemokines using the Meso Scale Discovery 30-plex multi-analyte detection system. Data is represented as absolute value for each participant for a given cytokine or chemokine. Significance was determined by Mann Whitney U test. (PPTX 121 kb)

Abbreviations

ACD: Acid citrate dextrose; ART: Anti-retroviral therapy; AUC: Area under the curve; BP: Basepair; CI: Confidence intervals; DEG: Differentially expressed genes; HIV: Human Immunodeficiency Virus; IFN: Interferon; IPA: Ingenuity pathway analysis; LPS: Lipopolysaccharide; LTBI: Latent TB infection; Mtb: *Mycobacterium tuberculosis*; OPD: Other pulmonary diseases; ORD: Other respiratory disorders; PBMC: Peripheral blood mononuclear cells; PCA: Principal component analysis; ROC: Receiver operator characteristic; TB: Tuberculosis; TB-IRIS: TB-immune reconstitution inflammatory syndrome

Authors' contributions

PS, JJE and YCM contributed to the study design. SV, PD and PS contributed towards data acquisition. SV, PD, PS, JJE and YCM contributed equally towards data interpretation. DN, SH, JB, LN and YCM were responsible for patient recruitment, sample collection and analysis of clinical data. SV, PD, PS, JJE and YCM contributed equally to writing of the manuscript. All authors read and approved the final manuscript.

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Verma *et al. BMC Infectious Diseases* (2018) 18:220

Page 12 of 13

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Page 13 of 13

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