Mycobacterium tuberculosis infection associated immune perturbations correlate with antiretroviral immunity

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Highlights

- In people with HIV-1 (PWH), MTB infection is associated with a perturbation of the PBMC transcriptome independently of re-exposure to MTB specific antigen.

- MTB infection is associated with a shift away from Type 1 Interferon towards a trained immunity type phenotype.
- The perturbations associated with MTB infection correlate with improved retroviral control and are lost during progression to active tuberculosis.

Abstract

Infection with *Mycobacterium tuberculosis* (MTB) remains one of the most important opportunistic infections in people with HIV-1 (PWH). While it is well accepted that active Tuberculosis (TB) leads to rapid progression of immunodeficiency in PWH, the interaction between MTB and HIV-1 during the asymptomatic phase of both infections remains poorly understood.

Here, we show in a well-matched cohort of PWH with suppressed HIV-1 viral load that MTB infection is associated with a baseline transcriptional profile away from a Type 1 Interferon towards a proinflammatory cytokine response. The transcriptional changes are conserved during HIV-1 viremia and are inversely correlated with HIV-1 viral load, suggesting that MTB infection-associated perturbations of the innate immune system improve HIV-1 control. In line with these findings, the transcriptional profile of individuals with MTB infection overlaps with that of HIV-1 long term non-progressors. Conversely, during active TB, the transcriptional pattern is reversed, indicating loss of immune control and suggesting a shared immunological mechanism of control and progression for both HIV-1 and MTB infection.

In conclusion, our data show that MTB infection is associated with a shift in the activation state of the immune system. This shift contributes to lower HIV-1 viral load in MTB/HIV-1 co-infection and is lost during disease progression.

Significance statement

Mycobacterium tuberculosis (MTB) affects 23% of the human population, but little is known about its impact on human biology. MTB infection is associated with better HIV-1 control and fewer opportunistic infections, but with an increased risk of non-communicable diseases such as diabetes. To understand why this is the case, we studied individuals co-infected with HIV-1 and MTB infection and found that MTB infection affects the innate immune system, improving

91 the control of HIV-1 replication. Animal and BCG vaccination studies also support the idea that
92 MTB affects the innate immune system and provides immunity against other diseases. Overall,
93 this study advances our understanding of how MTB infection can have unexpected effects on
94 host responses and sheds light on its complex nature.
95
96

97 Introduction

98 Globally, HIV-1 remains one of the most important risk factors for the development of active
99 Tuberculosis (TB)(1, 2). Individuals with HIV-1 and active TB suffer from earlier TB
100 dissemination, faster progression and increased risk for TB drug resistance(2).
101 However, active TB is only an extreme of the spectrum of *Mycobacterium tuberculosis* (MTB)
102 infection. While the exact numbers remain uncertain, it is well accepted that less than 10% of
103 MTB-infected HIV-uninfected individuals ever develop active disease(1). Similarly, only the
104 minority of people with HIV-1 (PWH) exposed to MTB will develop active disease(2, 3).
105 Until now, the sole method of verifying exposure to MTB in the absence of symptomatic TB
106 has been through the identification of MTB specific T cells in the bloodstream. However, this
107 approach has a significant limitation. The presence of MTB specific T cells could indicate one
108 of three possibilities. Firstly, it could suggest the presence of bacteria that are in a state of
109 reversible latency and have the potential to cause disease. Secondly, it could indicate bacteria
110 that are in a state of practically "irreversible" latency, requiring an excessive and unsustainable
111 amount of immune suppression to become active again (which is incompatible with the host's
112 survival). Lastly, it could signify a memory T cell response following the eradication of the
113 infection(4). Additionally, these tests rely on functional T cells, which can be compromised in
114 their function due to the replication of HIV-1.
115 Priming of an MTB specific T cell response requires substantial exposure to the pathogen,
116 irrespective of whether these bacteria are re-activated sometimes in the future or not. For
117 example, a positive TB spot is defined as 5 or more spots out of 250,000 cells producing IFN-
118 γ when exposed to ESAT-6/CFP-10, antigens encoded in the MTB region of difference 1 locus.
119 The human body is estimated to harbor $2x10^{12}$ lymphocytes. The detection of 5 MTB specific
120 spots suggests that approximately 40 million lymphocytes (ranging from $1.5x10^{7}$ to $9.9x10^{7}$)
121 are committed to two MTB specific antigens (ESAT-6 and CFP-10) (Figure 1A). This estimate
122 is conservative since it has been observed that during active TB, MTB specific T cells are
123 recruited to the infection site and disappear from peripheral circulation(5). Although the extent

124 of recruitment during asymptomatic infection is still uncertain, it is reasonable to assume that
125 maintaining an MTB specific T cell response in peripheral blood involves a commitment of 10
126 to 100 million lymphocytes to MTB antigens.
127
128 In this work, we hypothesized that exposure to MTB does not only induce an antigen specific
129 T cell immune response but also changes the activation state of the innate immune system. This
130 hypothesis originated from data we generated in an *in vivo* model of asymptomatic MTB
131 infection and from observations we made from a large clinical cohort(6, 7). The small animal
132 model of asymptomatic MTB infection ("contained MTB infection" (CMTB) model) shows
133 profound changes in the innate immune system: low-grade cytokinemia results in the activation
134 of myeloid cells, which in turn leads to profound heterologous effects against unrelated
135 challenges, such as infection with *Listeria monocytogenes*(6). In people with HIV-1, MTB
136 infection but not active TB was associated significantly reduced HIV-1 viral set point and with
137 a lower frequency of the most common opportunistic infections(7). In sum, these observations
138 support the idea of an asymptomatic MTB infection acting as "universal adjuvant" altering
139 systemic immunity both in a small animal model and in humans. In line, BCG, the 100 year old
140 MTB vaccine, does profoundly alter the immune response and induces heterologous immunity
141 via the induction of "trained immunity"(8).
142 In analogy, we hypothesized that asymptomatic mycobacterial infection induces systemic
143 immune activation, including changes in the transcriptional programs of circulating innate cells.
144 Hence, we should be able to observe these changes in the peripheral blood transcriptome by
145 comparing PWH with and without MTB infection. Also, if these changes are causally linked to
146 the observed heterogeneous effects(7) we should be able to detect correlations between genes
147 associated with MTB infection and the heterologous effects. Specifically, in the current work,
148 we investigated the potential interaction between MTB infection associated changes in the
149 immune system and HIV-1 viral set point, given the striking and highly consistent effects on a
150 population level(7).
151
152

153 Material and Methods

154 <u>Selection and matching of study population</u>

155 We obtained peripheral blood mononuclear cells (PBMC) from 36 PWH enrolled in the Swiss
156 HIV Cohort Study (SHCS)(9). The SHCS is a prospective, longitudinal, multicentre,

157 representative cohort study that collects demographic and clinical data from people with HIV-
158 1 twice a year and stores plasma and PBMC samples in a biobank(9). MTB positive is defined
159 as at least one of the tuberculosis (TB) test results (IGRA or TST or both) being positive while
160 not being diagnosed with either pulmonary or extrapulmonary tuberculosis. We included only
161 samples from patients with an unambiguous TB test result. These samples were categorized
162 into four different groups based on the combination of *Mycobacterium tuberculosis* infection
163 (MTB) and HIV infection status, which are: *i)* MTB positive and viremic (MTB+ HIV^{vir}; n=7),
164 *ii)* MTB positive and suppressed (MTB+ HIV^{supp}; n=11), *iii)* MTB uninfected and viremic
165 (MTB- HIV^{vir}; n=7), and *iv)* MTB uninfected and suppressed (MTB- HIV^{supp}; n=11). For each
166 blood sample taken for each individual, the closest TB test to the sampling time is used, leading
167 to 78% of the samples being taken at the exact time of the TB test. Regarding HIV status,
168 suppressed PWH were defined as having an HIV viral load below 500 copies/ml for the
169 respective blood test, whereas viremic PWH had a viral load above/equal this threshold. Only
170 blood samples taken after the year 2000 were included to minimize degradation of live cells
171 over time. Individuals with opportunistic infections were excluded (full list in Supplementary
172 Table 1). Practical constraints also limited the number of PWH selected for our analysis, such
173 as the number of aliquots per sample and whether the sample contained viable PBMCs.
174 MTB- and MTB+ PWH were matched within each group of HIV-suppressed and viremic PWH,
175 given their sex, ethnicity, and CD4 cell counts. For each individual, we first identified all the
176 other candidates with the same sex and same ethnicity who could be a potential match and then
177 chose the individuals with the closest CD4 cell count. After the pair was determined, they were
178 removed from the pool of potential candidates to avoid any duplication in matching
179 (Supplementary Figure 1).
180
181 <u>Cell stimulation</u>
182 0.5 x 10^6 or 0.2 x 10^6 PBMCs for FACS and transcriptome analysis respectively were plated
183 out and stimulated with 150 ng/ml phorbol myristate acetate (PMA)/Ionomycin
184 (ThermoFischer, 00-4975-93) or not stimulated and cultured in RPMI-1640 medium with 1-
185 glutamine, 10% heat-inactivated FBS and 1% P/S at 37°C, 5% CO_2. For flow cytometric
186 analysis, protein transport inhibitor (ThermoFischer, 00-4980-03) was added according to
187 manufacturer's instructions. Cells were stimulated for 6 hours and subsequently analysed with
188 flow cytometry or prepared for RNA sequencing.
189

190 Flow cytometric analysis

191 Cells were washed twice with PBS to remove stimulation compounds and subsequently surface
192 proteins were stained for 30 min at 4°C with NIR Zombie dye APC-Cy7 (Biolegend, Cat No.
193 423105), CD20 PE (Biolegend, Cat No. 302306), CD3 AF700 (Biolegend, Cat No. 300324),
194 CD69 FITC (Biolegend, Cat No. 310904), CD56 BV510 (Biolegend, Cat No. 304608) CD16
195 PE-Dazzle (Biolegend, Cat No. 302054), HLA-DR BV605 (Biolegend, Cat No. 307640),
196 CD11b PE-Cy7 (Biolegend, Cat No. 301322), CD14 BV421 (Biolegend, Cat No. 301829).
197 Afterwards, cells were washed with PBS and fixed with Cytofix/Cytoperm buffer (BD
198 Biosciences, Cat No. 554722) for 20 min at 4°C. Cells were washed and resuspended in PBS.
199 Flow cytometry analysis was performed with the High throughput sampler (HTS) LSR II
200 Fortessa 4L (Supplementary Figure 2) and data was analysed with FlowJo version 10.0.8r1 and
201 R.

202

203 RNA isolation and sequencing

204 After stimulation, cells were centrifuged for 5 min, at 600 g and RNA was isolated with the
205 RNeasy Micro Kit (Qiagen) according to manufacturer's instructions either manually or with
206 the QIAcube (Qiagen). RNA was quantified on a TapeStation (Agilent) using a high sensitivity
207 RNA ScreenTape and RNA sequencing (RNAseq) was performed using 20 ng total RNA as
208 input using the SmartSeq2 library preparation with 1x100 bp single-end read (Illumina) with a
209 minimum read depth of 30 million reads per sample on the NovaSeq6000 (Illumina) according
210 to manufacturer's instructions at the Functional Genomics Centre Zurich (FGCZ).

211

212 RNAseq analysis

213 The RNA sequencing raw reads were first cleaned by removing adapter sequences, trimming
214 low quality ends, and filtering reads with low quality (phred quality <20) using Fastp (Version
215 0.20)(10). Sequence pseudo alignment of the resulting high-quality reads to the human
216 reference genome (build GRCh38.p13) and quantification of gene level expression (gene
217 models based on GENCODE release 37) was carried out using Kallisto (Version 0.46.1)(11).
218 Differential expression was computed using the generalized linear model as implemented in the
219 Bioconductor package DESeq2 (R version: 4.2.0, DESeq2 version: 1.36.0)(12). Genes showing
220 altered expression with adjusted (Benjamini and Hochberg method) p-value <0.05 were
221 considered differentially expressed. Using the k-means function in the R stats package, we
222 performed k-means clustering on the normalized gene counts belonging to the HIV suppressed

223 (HIV^{supp}) PWH. Genes with a differential gene expression (DEG) p-value below 0.01 are used
224 for the analysis. Number of clusters are set to 4, since data used for this analysis belongs to 4
225 different groups (cells from MTB+ patients unstimulated, cells from MTB- patients stimulated,
226 cells from MTB+ patients stimulated with PMA/Ionomycin, cells from MTB- patients
227 stimulated with PMA/Ionomycin). For data visualisation using heatmaps we applied an
228 additional batch correction step by using the removeBatchEffect() function implemented in the
229 limma package (v3.54.1)(13). Leading genes from the GSEA analysis with a p-value below
230 0.05 are selected for visualization. A Z-score normalization is performed on the log2 of the
231 normalized read counts across samples for each gene. For comparison, the same genes in the
232 dataset from Xu et al., 2014[14] are filtered for the respective DEG heatmap, where a Z-score
233 normalization is performed on the microarray samples for each gene, but without a logarithmic
234 transformation.
235 We compared the distributions of correlation coefficients between log10 transformed viral
236 RNA and log10 transformed normalized gene expression values for conserved and not
237 conserved transcripts for (abs(LogFC)>1 and p-value<0.01 and visualized the distributions in
238 the form of boxplots, and performed a t-test to quantify the significance of the difference
239 between the mean values. Furthermore, we visualized the correlations and calculated the
240 correlation coefficients between the log10 transformed viral RNA and log10 transformed
241 normalized gene expression values for each of the previously detected genes mentioned above.
242 Functional annotation was performed by mapping the transcripts to the human protein atlas(15-
243 17).
244
245 Integration and comparison to other datasets
246 For validation of our results two independent datasets were used: first a cohort of HIV-1
247 progressors and long term non-progressors(14) and second a cohort of PWH with MTB
248 infection or active TB (PWH MTB+, PWH developing active TB)(18).
249 Genome-wide analysis of gene expression in whole blood from HIV-1 progressors and long
250 term non-progressors data from Xu et al., 2014[14] was downloaded from NCBI GEO
251 (GSE56837), and the DEG values were calculated using the data of the long term non-
252 progressors over the progressors for the genes with an expression level above the median
253 expression for each sample. These DEG values are used to identify the genes that are present
254 in both studies with similar transcriptional profiles, and by using random sampling, to test

255 whether the observed number of genes are significant enough to support our hypothesis on the
256 similarity of the MTB infection and HIV control status.

257 Background-subtracted quantile-normalized whole blood microarray expression data from
258 Kaforou et al.(18) was downloaded from NCBI GEO (GSE37250) and log2 transformed. Two-
259 tailed t-tests were used to test whether genes of interest were significantly changed between
260 PWH with MTB infection (PWH MTB+) and active TB (PWH developing active TB). A
261 signature score was computed by summing the transformed expression values of the genes and
262 t-tests used to check for changes in signature scores separately in Malawi and South African
263 PWH MTB+ and PWH developing active TB samples.

264

265

266 ## Results

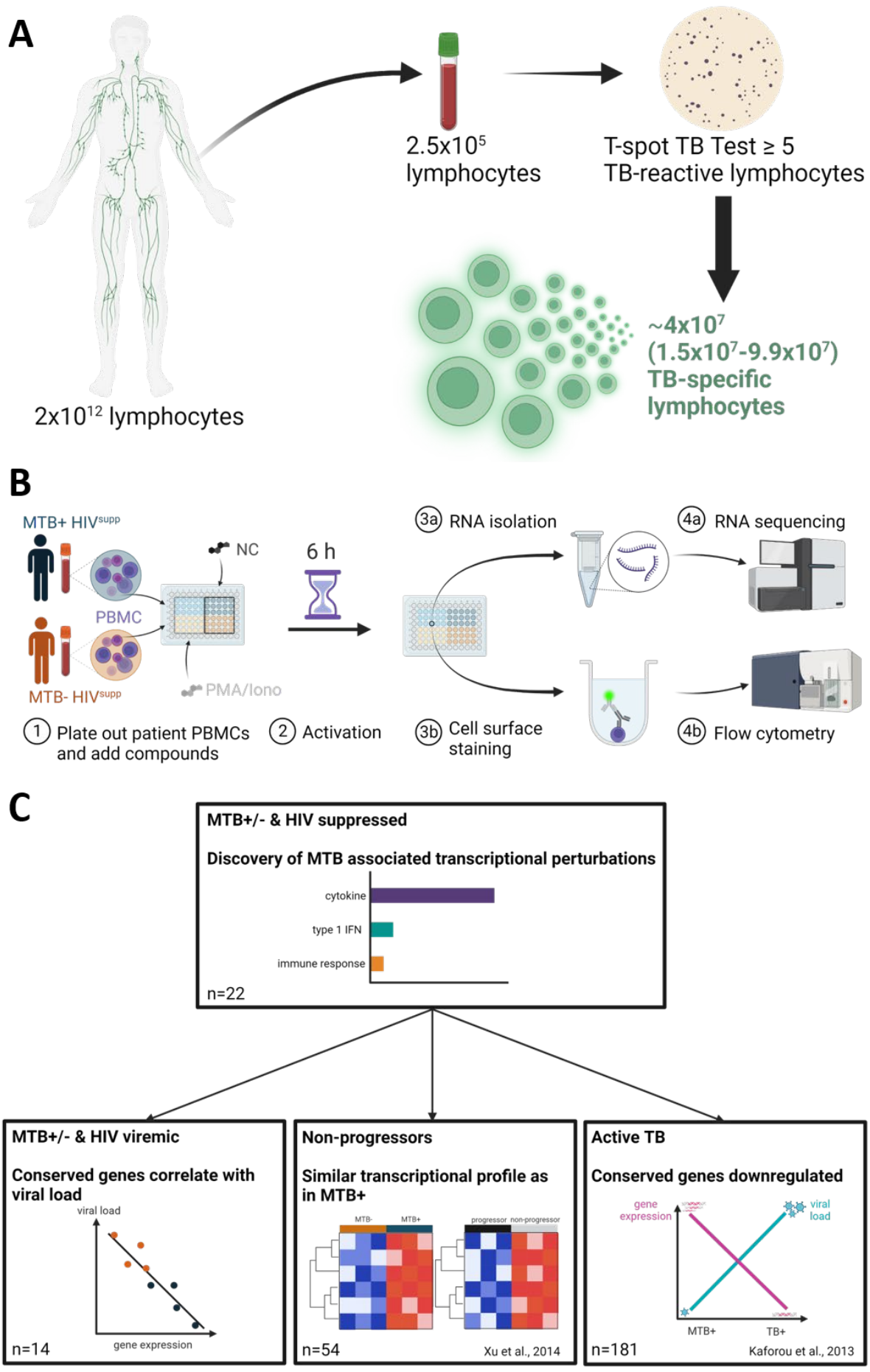
267 MTB infection perturbs the transcriptome of PWH with suppressed viral load

268 To assess potential differences in the peripheral transcriptome in HIV-1 suppressed PWH with
269 and without MTB infection (MTB+ HIV^{supp} and MTB- HIV^{supp}), we used peripheral blood
270 mononuclear cells (PBMC). To exclude potential confounding factors, 22 PWH were matched
271 1:1 according to CD4 cell count, ethnicity and sex (Table 1). PBMC of matched PWH were
272 either activated with PMA and Ionomycin or not activated. After 6 hours detection of cell
273 surface markers via flow cytometry and RNA isolation and sequencing was performed (Figure
274 1B). We then aimed to validate our findings using independent cohorts (Figure 1C).

275 Transcriptome clustering after dimensionality reduction separated the different groups,
276 including MTB+ HIV^{supp} and MTB- HIV^{supp} samples in not activated PBMCs (Figure 2A). We
277 then applied gene-set enrichment analysis (GSEA) to identify biological processes and gene
278 sets specifically associated with MTB infection. GSEA identified 663 leading edge genes, i.e.
279 genes that largely contribute to the enrichment of the functional pathways. Out of these 663
280 genes, 203 genes were differentially expressed when comparing MTB+ HIV^{supp} to MTB-
281 HIV^{supp} ($p < 0.05$), of which 177 were downregulated and 26 were upregulated (Figure 2B,
282 Supplementary Table 2). Enriched functional pathways all play a role in immune response
283 (Figure 2C). Interestingly, cytokine activity is upregulated in MTB+ HIV^{supp}, while all other
284 pathways are downregulated in MTB+ HIV^{supp}, namely regulation of immune response, innate
285 immune response, type 1 interferon signaling, immune response and defense response to virus.

286 To determine whether a specific PBMC subpopulation was responsible for the changes in the
287 transcriptome, the frequency of major cell populations was measured by flow cytometry. We

288 did not detect significant differences in CD16+ NK cell (MTB+ HIV^{supp}: 1.62 (1.14, 1.97),
289 MTB- HIV^{supp}: 2.13 (1.05, 4.16), median (IQR), p=0.3), B cell (MTB+ HIV^{supp}: 4.55 (3.32,
290 5.46), MTB- HIV^{supp}: 4.42 (3.22, 6.97), median (IQR), p=1) or T cell (MTB+ HIV^{supp}: 50.5
291 (36.6, 59.8), MTB- HIV^{supp}: 55.7 (48.4, 58.4), median (IQR), p=1) abundance (Supplementary
292 Figure 3). Hence, changes observed in transcriptome cannot be explained by shifts in major cell
293 populations.


A
2x10^12 lymphocytes
2.5x10^5 lymphocytes
T-spot TB Test ≥ 5
TB-reactive lymphocytes
~4x10^7
(1.5x10^7-9.9x10^7)
TB-specific lymphocytes
B
MTB+ HIV^supp
PBMC
MTB- HIV^supp
NC
PMA/Iono
6 h
3a RNA isolation
4a RNA sequencing
3b Cell surface staining
4b Flow cytometry
1 Plate out patient PBMCs and add compounds
2 Activation
C
MTB+/- & HIV suppressed
Discovery of MTB associated transcriptional perturbations
cytokine
type 1 IFN
immune response
n=22
MTB+/- & HIV viremic
Conserved genes correlate with viral load
viral load
gene expression
n=14
Non-progressors
Similar transcriptional profile as in MTB+
MTB-
MTB+
progressor
non-progressor
n=54
Xu et al., 2014
Active TB
Conserved genes downregulated
gene expression
viral load
MTB+
TB+
n=181
Kaforou et al., 2013

294

295 **Figure 1: Study design. A)** Approximately $2x10^{12}$ lymphocytes are present in the human body. For a
296 well in the T-spot TB test, 250'000 lymphocytes are used. The detection of 5 spots is indicative of
297 approximately $4x10^7$ ($1.5x10^7$– $9.9x10^7$) MTB specific lymphocytes in the entire body under the
298 assumption that the cells are equally distributed. **B)** Workflow depicting RNA sequencing and flow
299 cytometry analysis of PBMCs. PBMCs from suppressed PWH with either MTB infection (MTB+ HIV^{supp})
300 or no MTB infection (MTB- HIV^{supp}) are plated out and activated with PMA and Ionomycin (PMA/Iono)
301 or not activated (NC) for 6 hours. RNA isolation and subsequent RNA sequencing or cell surface staining
302 and flow cytometry analysis are performed. **C)** Summary figure showing how we used different cohorts
303 for discovery and confirmation steps.
304
305 **Table 1: Characteristics of HIV-1 suppressed PWH.**

		MTB+ HIV^{supp}	**MTB- HIV^{supp}**	**all HIV^{supp}**	**p-value**
		11	11	22	
CD4 (median [IQR])		480 [458, 742]	482 [404, 749]	481 [425, 839]	0.948
Viral load (median [IQR])		0 [0, 0]	0 [0, 0]	0 [0, 0]	
TB test type (%)	TST	2 (18.2)	3 (27.3)	5 (22.7)	0.631
	IGRA	9 (81.8)	8 (72.7)	17 (77.3)	
Sex (%)	male	9 (81.8)	9 (81.8)	18 (81.8)	1
	female	2 (18.2)	2 (18.2)	4 (18.2)	
Ethnicity (%)	white	9 (81.8)	9 (81.8)	18 (81.8)	1
	black	1 (9.1)	1 (9.1)	2 (9.1)	
	hispano-american	1 (9.1)	1 (9.1)	2 (9.1)	

306 Shown are median and interquartile range or % for MTB+ HIV^{supp} (n=11), MTB- HIV^{supp} (n=11) and all
307 HIV^{supp} (n=22) with corresponding p-values from two sample t-test. TST= Tuberculin skin test, IGRA=
308 Interferon gamma release assay.

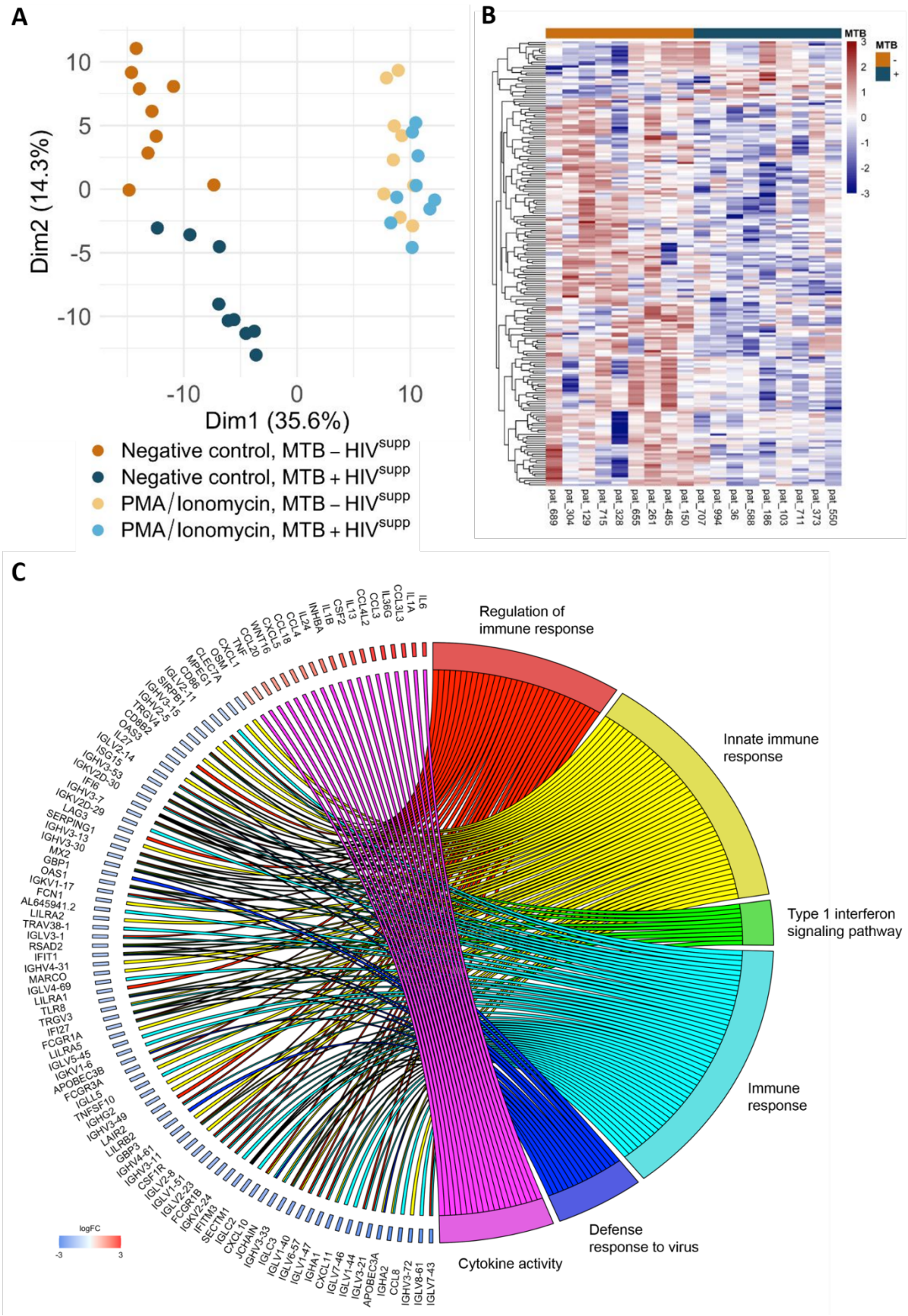


Figure 2: Differences in MTB+ versus MTB- in HIV suppressed PWH. A) Principal component analysis of transcription profile of activated (PMA/Ionomycin) and not activated (Negative control)

312 PBMCs from MTB+ HIV^{supp} (dark/light blue) and MTB- HIV^{supp} (brown/yellow) PWH with a cut-off p-
313 value<0.01. **B)** Heatmap showing leading edge genes for not activated samples with a cut-off p-
314 value<0.05 for HIV suppressed PWH (grey). Heatmaps are Z-score adjusted. MTB- in orange, MTB+ in
315 blue. Red color shows upregulated genes, while blue color shows downregulated genes. List of genes
316 are given in Supplementary Table 2. **C)** Gene set enrichment analysis for genes with a cut-off of
317 abs(logFC)>1 for HIV suppressed PWH. Red color shows upregulated genes, while blue color shows
318 downregulated genes. Detected six sets of gene are shown in different colors.
319

320 "Conserved transcripts" correlate with increased viral control

321 Since we hypothesized that MTB infection associated immune perturbations provide
322 heterologous immunity against HIV-1, we investigated the impact of MTB infection on HIV
323 viral load in HIV viremic PWH. A total of 14 viremic PWH with and without MTB infection
324 (MTB+ HIV^{vir}, MTB- HIV^{vir}) were randomly selected and matched according to CD4 cell
325 count, ethnicity and sex (Supplementary Figure 1). Strikingly, HIV-1 viral load was almost one
326 log lower in MTB+ HIV^{vir}, recapitulating our previous finding that MTB infection is associated
327 with lower HIV-1 viral load(7) (Table 2).
328 This spread of HIV-1 viral load also facilitated identifying genes that correlate with HIV viral
329 load across MTB+ and MTB- PWH. We defined "conserved transcripts" as genes detected to
330 be different in MTB+ HIV^{supp} versus MTB- HIV^{supp} and that were found to differ similarly in
331 MTB+ HIV^{vir} versus MTB- HIV^{vir} (Figure 3A). Thus, according to our hypothesis, the
332 conserved transcripts would reflect an improved antiretroviral immune state. Therefore, we
333 tested the correlations between HIV-1 viral loads with differentially expressed gene abundance
334 ($p<0.01$, with three different abs(logFC) values: (abs(LogFC)>1 n=97, abs(LogFC)>1.5 n=53,
335 abs(LogFC)>2 n=30) as identified in HIV-1 suppressed PWH across both MTB infected and
336 uninfected individuals (Figure 3B). As we increased the abs(logFC) cut-off, i.e., choosing genes
337 with a higher differential expression, we observed a higher and more significant negative
338 correlation with the viral load, underscoring the robustness of our finding.
339 Out of the 97 genes with abs(LogFC)>1 and p-values<0.01, 32 showed conserved
340 transcriptional profile and from those, we detected 20 conserved transcripts negatively
341 correlated to viral load. Transcriptional profile between HIV^{supp} and HIV^{vir} PWH was highly
342 correlated for these conserved transcripts (grey areas), while for not conserved transcripts this
343 was not the case (Figure 3C, Supplementary Table 3).
344 Mapping of the 20 conserved transcripts to The Human Protein Atlas(15-17) showed that
345 expression of these genes is associated with immune cells; specifically, 50% are expressed in

346 innate immune cells (Figure 3D). Again, we were not able to detect significant differences on
347 a cell population level by flow cytometry analysis (Supplementary Figure 4).
348
349
350 **Table 2: Characteristics of HIV-1 viremic PWH.**

		MTB+ HIV^{vir}	**MTB- HIV^{vir}**	**All HIV^{vir}**	**p-value**
		7	7	14	
CD4 (median [IQR])		453 [373, 656]	453 [373, 653]	453 [355, 677]	0.93
Viral load (median [IQR])		33'643 [18'461, 93'725]	120'000 [21'782, 700'000]	33'872 [18'343, 472'678]	0.258
TB test type (%)	IGRA	7 (100.0)	7 (100.0)	14 (100.0)	
Sex (%)	male	6 (85.7)	6 (85.7)	12 (85.7)	1
	female	1 (14.3)	1 (14.3)	2 (14.3)	
Ethnicity (%)	white	7 (100.0)	7 (100.0)	14 (100.0)	

351 Shown are median and interquartile range or % for MTB+ HIV^{vir} (n=7), MTB- HIV^{vir} (n=7) and all HIV^{vir}
352 (n=14) with corresponding p-values from two sample t-test. IGRA= Interferon gamma release assay.
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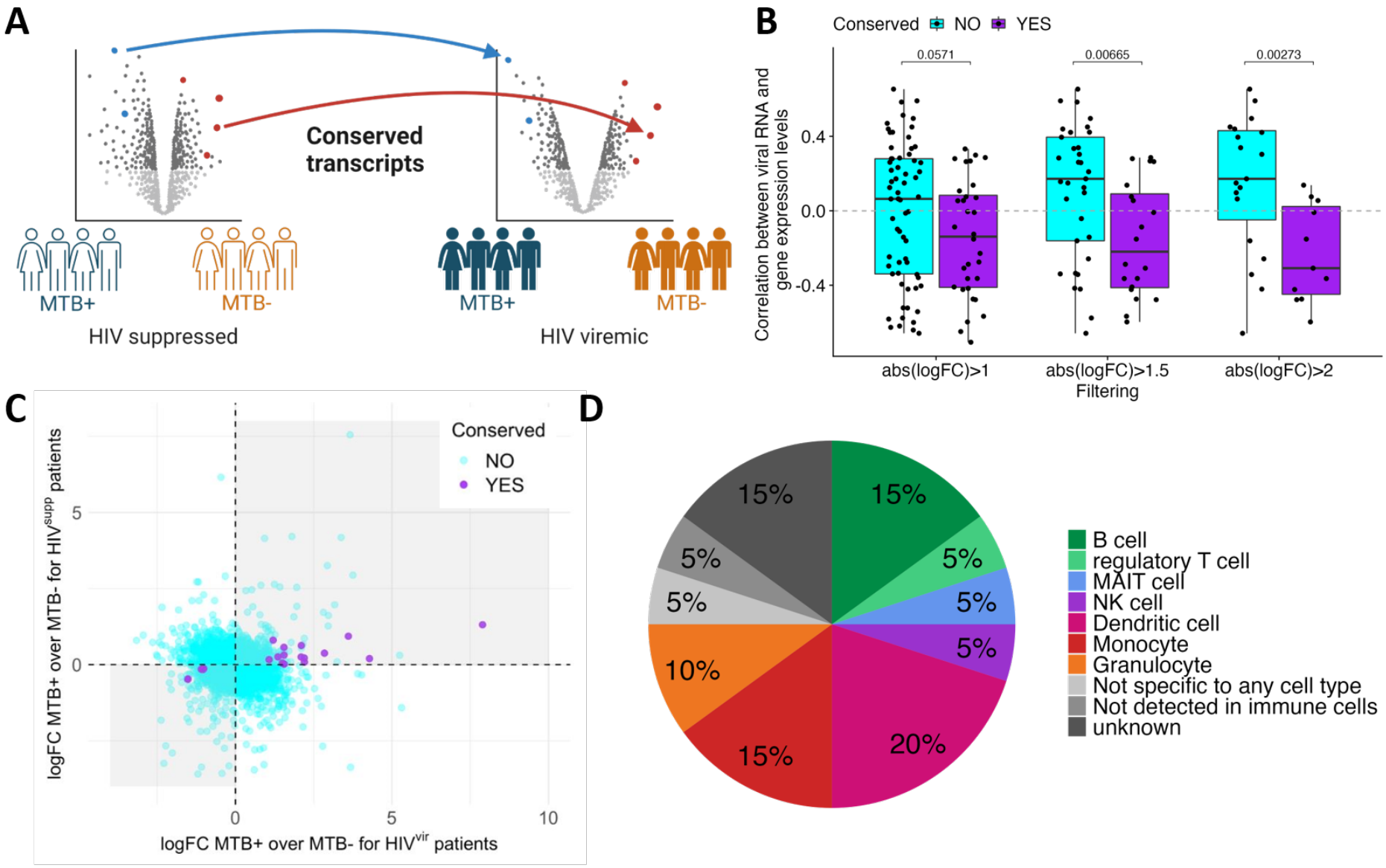

A
Conserved transcripts
MTB+
MTB-
HIV suppressed
MTB+
MTB-
HIV viremic
B
Conserved NO YES
0.0571
0.00665
0.00273
Correlation between viral RNA and gene expression levels
0.4
0.0
-0.4
abs(logFC)>1
abs(logFC)>1.5
abs(logFC)>2
Filtering
C
Conserved
NO
YES
logFC MTB+ over MTB- for HIV^supp patients
5
0
0
5
10
logFC MTB+ over MTB- for HIV^vir patients
D
15%
15%
5%
5%
5%
5%
10%
15%
20%
B cell
regulatory T cell
MAIT cell
NK cell
Dendritic cell
Monocyte
Granulocyte
Not specific to any cell type
Not detected in immune cells
unknown

367

368 **Figure 3: Conserved transcripts are associated with increased viral control. A)** Concept of conserved transcripts: differentially expressed transcripts
369 between MTB+ HIV^{supp} compared to MTB- HIV^{supp} are detected also as differentially expressed transcripts between MTB+ HIV^{vir} compared to MTB- HIV^{vir} with the
370 same directionality (up-regulated in HIV suppressed and up-regulated in HIV viremic (red) or down regulated in HIV suppressed and down regulated in HIV viremic
371 (blue)). **B)** Correlation of gene expression and viral load for conserved transcripts (purple) and not conserved transcripts (cyan) with a p-value<0.01 and
372 abs(logFC)>1 (n=97, p=0.0571), abs(logFC)>1.5 (n=53, p=0.00665) and abs(logFC)>2 (n=30, p=0.00273). **C)** Correlation of log2 fold change of differentially
373 expressed transcripts from HIV suppressed and HIV viremic PWH, with an abs(logFC)>1 and a p-value<0.01. Transcripts in grey areas show same transcriptional
374 direction in HIV suppressed as well as HIV viremic PWH. Conserved transcripts (purple) and not conserved transcripts (cyan). **D)** Percentages of different immune
375 cells expressing the conserved transcripts with a negative correlation to viral load (n=20), according to the human protein atlas(15-17).

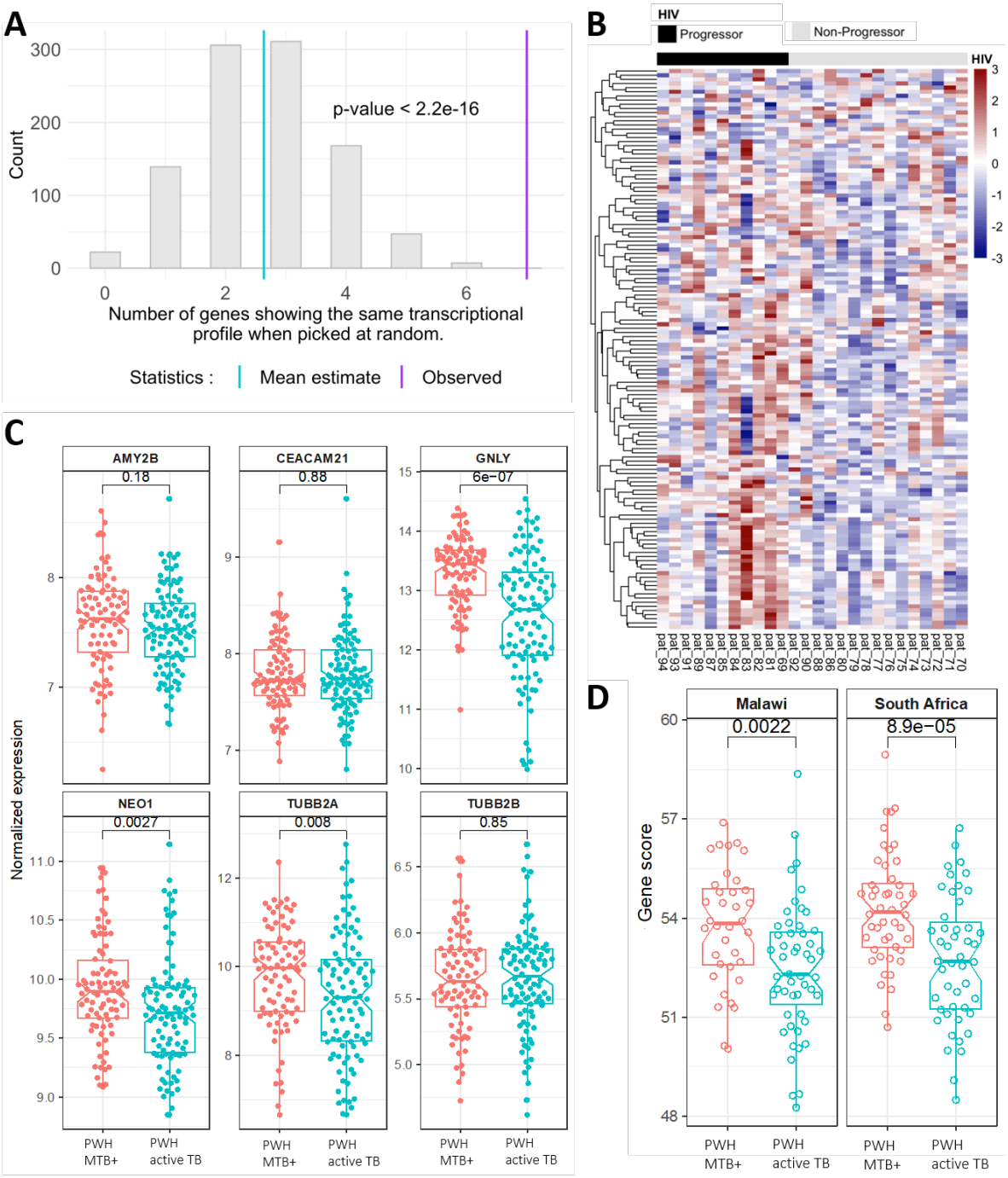
376 Transcriptional profiles of MTB infected PWH and HIV-1 long term non progressors share
377 basic properties

378 Next, we tested whether we could validate these MTB-induced HIV-protective gene expression
379 patterns in other clinically relevant phenotypes of increased HIV-1 immunity. Therefore, we
380 chose a cohort of ART-naive PWH comprising both disease progressors [n=15] (decline in
381 CD4+ T cells of >50 cells/mm3/year) and long term non-progressors (non-progressors) [n=11]
382 (no decline in CD4+ T cells, viral load <5,000 copies/ml)(14) (Figure 1C). If the MTB-induced
383 immune state we identified in PWH is generalizable and contributes to antiretroviral immunity
384 in a broader sense, we hypothesized that our MTB induced gene expression profiles associated
385 with reduced HIV viral load may be reproducible in HIV long term non-progressors. To test
386 this hypothesis, we first identified the differentially expressed genes that are present in both
387 datasets [abs(logFC)>1, p-value<0.01] which correlate negatively with the HIV viral load in
388 our study. Based on those criteria there are 40 genes, 15 of which belong to the 20 conserved
389 transcripts identified in our study. 7 of these 15 conserved transcripts show the same
390 transcriptional profile in our study as well as in the external cohort, which is significantly more
391 than what would be expected if 15 genes from the intersecting pool of 40 genes of two cohorts
392 were to be randomly sampled ($p<2.2x10^{-16}$) (Figure 4A). Furthermore, conserved transcripts in
393 long term non-progressors showed the same transcriptional profile as MTB+ PWH (Figure 2B,
394 4B). In total, 74.8% of the genes show the same transcriptional profile between the two datasets.
395

396 The specific transcriptional patterns are lost during active tuberculosis

397 We and others have shown that active TB is associated with increased HIV-1 viral load(2, 7,
398 19). We therefore hypothesized that the improved antiretroviral state is lost during active TB.
399 We tested this hypothesis by defining the following conditions: genes that (1) are upregulated
400 in asymptomatically MTB infected and (2) negatively correlated with HIV-1 viral set point
401 should be down regulated in active TB. We selected the 8 genes from our 20 conserved
402 transcripts showing the strongest negative correlation with HIV-1 viral load (Supplementary
403 Table 3) and assessed their changes in an independent cohort of PWH with MTB infection
404 (PWH MTB+) or active TB (PWH developing active TB) from Malawi and South Africa(18).
405 Six out of the eight genes (AMY2B, CEACAM21, GNLY, NEO1, TUBB2A, TUBB2B) were
406 detected in the database and showed increased expression in PWH MTB+ compared to PWH
407 developing active TB (Figure 4C, Supplementary Table 4, Supplementary Figure 5). We
408 calculated a gene score averaging expression across all six genes for samples from Malawi and

South Africa separately, showing higher expression of genes in PWH MTB+ compared to PWH developing active TB (Figure 4D). In addition, PWH developing active TB show an opposing gene expression pattern with Type 1 Interferon genes being upregulated (Figure 4E). In sum, these data support our hypothesis that losing control over HIV-1 and MTB infection is a parallel process pointing to shared immunological mechanisms.



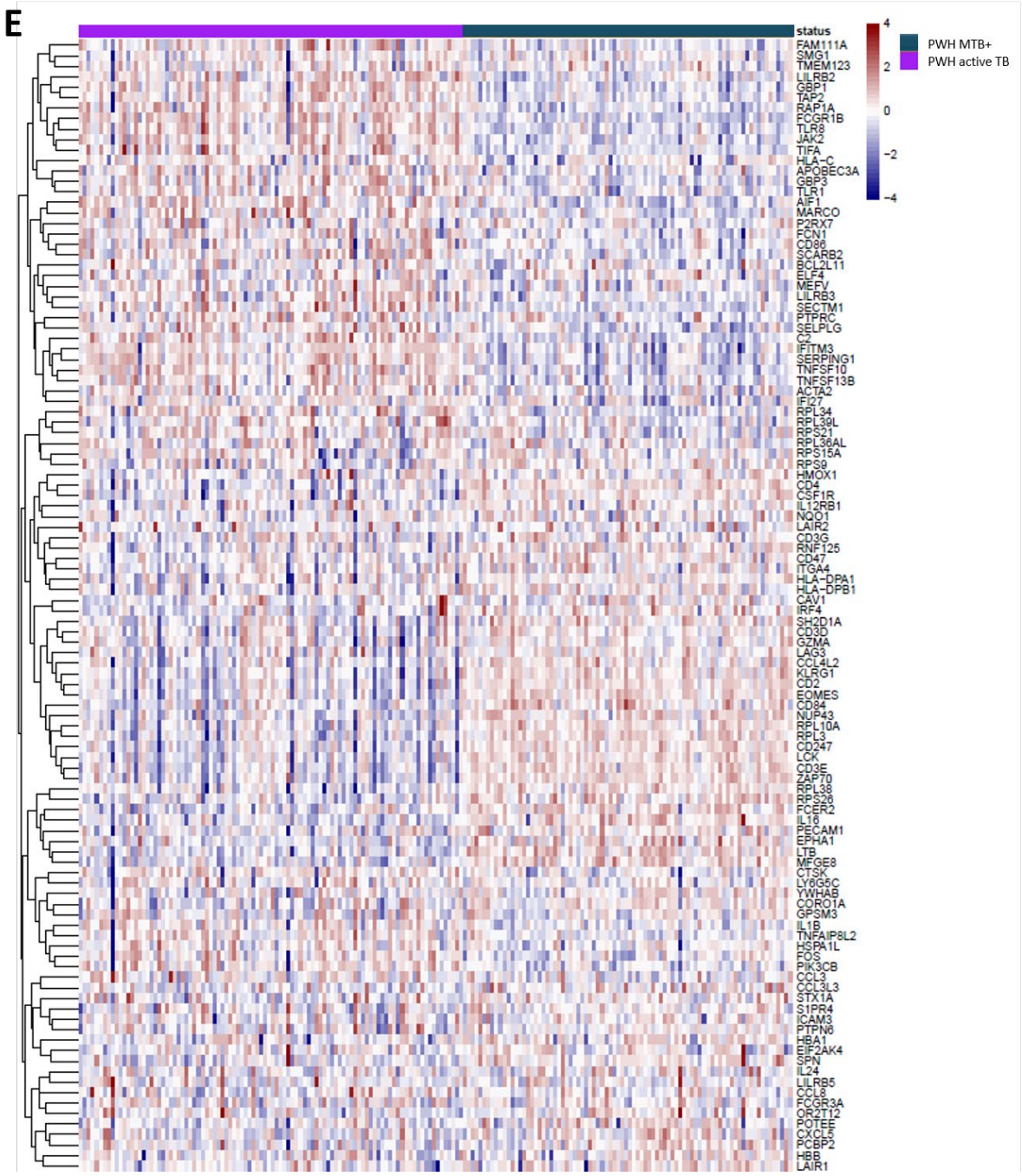


Figure 4: External cohorts show same transcriptional profile and high expression of conserved transcripts in long term non-progressors versus progressors and in PWH with MTB infection versus PWH developing active TB. A) Random sampling analysis showing number of genes with the same transcriptional profile in our study and in Xu et al., 2014(14) . The blue line represents the mean value of the number of genes with the same transcriptional profile in both studies when genes are picked at random for 1000 iterations, and the purple line represents the observed number of genes with the same transcriptional profile in both studies, which are significantly different ($p<2.2x10^{-16}$) **B)** Heatmap showing leading edge genes with a cut-off p-value<0.05 for HIV-1 long term non-progressors (non-progressors) (grey) and progressors (black) from Xu et al., 2014(14). Heatmaps are Z-score adjusted. Red color shows upregulated genes, while blue color shows

426 downregulated genes. **C)** Normalized gene expression of 6 genes (AMY2B, CEACAM21, GNLY, NEO1,
427 TUBB2A, TUBB2B) in PWH MTB+ (red) and PWH developing active TB (blue) from the Kaforou et al.,
428 2013(18) dataset. **D)** Gene score over all 6 genes (AMY2B, CEACAM21, GNLY, NEO1, TUBB2A, TUBB2B)
429 for Malawi and South Africa samples separately for PWH MTB+ (red) and PWH developing active TB
430 (blue) from the Kaforou et al., 2013(18) dataset. **E)** Heatmap showing leading edge genes with a cut-
431 off p-value<0.05 for PWH MTB+ (MTB+ Active TB-) (blue) and PWH developing active TB (MTB+ Active
432 TB+) (purple) from Kaforou et al., 2013(18). Heatmaps are Z-score adjusted. Red color shows
433 upregulated genes, while blue color shows downregulated genes.
434
435

436 ## Discussion

437 In this study, we demonstrated that a detectable MTB specific T cell response in peripheral
438 blood is correlated with systemic transcriptional changes that are also evident in PWH with a
439 suppressed viral load on ART. MTB infection associated transcriptional changes observed in
440 virologically suppressed PWH on ART were also found in HIV-1 viremic PWH on ART and
441 correlated inversely with HIV-1 viremia, pointing towards a potential causal effect between
442 MTB infection and increased control of HIV-1 replication.
443 To investigate natural immunity against HIV-1, most studies in humans compared, for example,
444 elite controllers and long term non-progressors and found significantly different immune
445 responses(20). However, conclusions regarding cause and effect have been very limited. Our
446 study setting is a significant advance over these previous approaches since we defined our genes
447 of interest by identifying a candidate protective phenotype in the absence of replicating HIV-1:
448 asymptomatic MTB infection, known to offer broad heterologous protection, and using it to
449 generate testable predictions which we then validated in three different clinical phenotypes in
450 PWH. Despite these efforts, our signal remains an association without proving direct causality.
451 As with every human cohort study, our set up remains susceptible to selection bias: for example,
452 it remains possible that a genetic predisposition for maintenance of an MTB specific T cell
453 response in peripheral blood is present. In humans, the only conceivable way to overcome this
454 bias is to perform a randomized controlled trial. Since human challenge studies with HIV-1 and
455 MTB are not possible, we believe that our careful approach of defining genes of interest in the
456 absence of both active TB and HIV-1 viremia followed by testing in different, independent
457 cohorts comes as close as it can get to a mechanistic study investigating interactions of co-
458 infections with two of the deadliest human pathogens.

459 Our analysis reveals a shift in MTB infected PWH to a pro-inflammatory (IL-6, IL-1)
460 transcriptional baseline with a relatively blunted Type 1 Interferon antiviral response. The
461 relative decrease of Type 1 Interferons together with the generic GSEA "anti-viral responses"
462 cluster seems counterintuitive at the first sight given that these individuals are able to control
463 HIV-1 better, and not worse. However, the double-edged role of Type 1 Interferons in chronic
464 infections and, specifically HIV-1 and MTB infection, has been the subject of intense
465 investigation. The duality of Type 1 Interferons in HIV-1 has been summarized in various
466 reviews: they appear to contribute to viral control during acute infection but are detrimental
467 during the chronic stage(21, 22). Likewise, the progression to active TB is associated with an
468 increased Type 1 Interferon response(23, 24), while impaired Type 1 Interferon signaling
469 increased resistance(25).

470 We add a novel aspect by demonstrating that successful containment of MTB infection in
471 humans induces an inflammatory state of the innate immune system that resembles clinical
472 phenotypes associated with control of HIV-1 replication. The proposition that MTB infection
473 is not merely an adaptive phenotype but also impacts the innate part of the immune system is
474 biologically plausible considering the substantial numbers of MTB specific T cells that are
475 required to detect an MTB specific response in peripheral blood. This observation raises
476 intriguing questions about the potential benefits of mycobacterial infection and for the control
477 of retroviral infections. Also, MTB infection is an "acquired" phenotype of HIV-1 resistance
478 resembling the phenotype of "inherited" resistance in long term non-progressors, which is
479 partly explained by variations in HLA(26). Therefore, the potential "acquired" mechanism
480 mediated by mycobacterial infection could lead to therapeutic interventions to improve anti-
481 retrovirus immunity.

482 The shift of the base line transcriptional profile poised towards IL-1 and IL-6 is reminiscent of
483 a "trained immunity" phenotype described after Bacillus Calmette-Guérin (BCG) vaccination
484 which was associated with increased control of yellow fever vaccine virus(27). While a shift of
485 the immune system towards a "trained immunity" phenotype might be beneficial for the control
486 of systemic viral infections, it carries the drawback of increased risk of non-communicable and
487 autoimmune diseases driven by increased inflammation. The prototypical disease, Type 2
488 Diabetes mellitus (DM), is an inflammasome driven disease mediated through dysregulated IL-
489 1 signaling(28). Consistent with this, we have previously shown that mycobacterial infection
490 substantially increases the risk for DM in PWH(29).

491 A large study on MTB has recently described an increase in NK cell frequency associated with
492 MTB infection(30) which we were unable to detect. These differences might be due to technical

differences between flow cytometry and mass spectrometry based approaches or biological differences of HIV-1 uninfected and HIV-1 infected but suppressed individuals. Also, we matched our individuals according to CD4 cell count, which is usually not done in clinical cohorts.

Our study has limitations: we included only highly selected PWH recruited from a longitudinal cohort study in Switzerland. While we tested our predictions and hypotheses in independent test cohorts, we cannot exclude selection biases. Also, our bulk RNAseq approach might miss differences detectable in single cell methods. However, the clinical samples are limited in cell numbers and preclude long protocols with significant cell loss, which is why we opted for a bulk RNA sequencing experimental design.

The strengths of our study are our ability to very carefully select and match PWH with an extra-ordinarily well characterized clinical phenotype and our study design that allows us to test predictions across independent cohorts.

In sum, our study reveals novel aspects of the interaction between two of the world`s deadliest pathogens, HIV-1 and MTB, which might lay the foundations for novel diagnostic and therapeutic approaches.

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Conflict of interest statements.

HFG has received honoraria for DSMB or advisory board membership from Merck, Gilead Sciences, ViiV healthcare, GSK, Janssen, Johnson and Johnson and Novartis and a travel grant from Gilead Sciences. JN received honoraria for DSMB or advisory board membership from Gilead, ViiV healthcare and a travel grant from Menarini. The institution of HF received educational grants from Merck, Gilead Sciences, ViiV healthcare, GSK, Janssen, and Abbvie. KJM has received travel grants and advisory board honoraria from Gilead Sciences and ViiV; and the University of Zurich received research grants from Gilead Science and Novartis for studies that KJM serves as principal investigator. DLB reports honoraria payed to himself for advisory boards from the companies Abbvie, MSD, Gilead & ViiV outside of the submitted work.

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