

1 **Profile of *Mycobacterium tuberculosis*-specific CD4 T cells at the site of disease and blood**
2 **in pericardial tuberculosis.**

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33 **Running title:** Mtb-specific T cell response in pericardial tuberculosis

34 **ABSTRACT**

35

36 Our understanding of the immune response at the site of disease in extra-pulmonary
37 tuberculosis (EPTB) is still limited. In this study, using flow cytometry, we defined the
38 pericardial fluid (PCF) cellular composition and compared the phenotypic and functional
39 profile of *Mycobacterium tuberculosis* (Mtb)-specific T cells between PCF and whole blood in
40 16 patients with pericardial TB (PCTB). We found that lymphocytes were the predominant cell
41 type in PCF in PCTB, with a preferential influx of CD4 T cells. The frequencies of TNF- α
42 producing myeloid cells and Mtb-specific T cells were significantly higher in PCF compared to
43 blood. Mtb-specific CD4 T cells in PCF exhibited a distinct phenotype compared to those in
44 blood, with greater GrB expression and lower CD27 and KLRG1 expression. We observed no
45 difference in the production IFN γ , TNF or IL-2 by Mtb-specific CD4 T cells between the two
46 compartments, but MIP-1 β production was lower in the PCF T cells. Bacterial loads in the PCF
47 did not relate to the phenotype or function of Mtb-specific CD4 T cells. Upon anti-tubercular
48 treatment completion, HLA-DR, Ki-67 and GrB expression was significantly decreased, and
49 relative IL-2 production was increased in peripheral Mtb-specific CD4 T cells. Overall, using a
50 novel and rapid experimental approach to measure T cell response *ex vivo* at site of disease,
51 these results provide novel insight into molecular mechanisms and immunopathology at site of
52 TB infection of the pericardium.

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55 **Keywords:** Pericardial tuberculosis, site of disease, CD4 response, treatment response.

56 INTRODUCTION

57

58 Tuberculosis (TB) causes more deaths than any other bacterial disease. The WHO estimated
59 that there were 1.2 million TB deaths in HIV uninfected individuals and 208 000 HIV/TB co-
60 infected deaths in 2019 ¹. Extra-pulmonary TB (EPTB) contributes 15% of the global TB
61 incidence and presents diagnostic and therapeutic challenges ¹. Meta-analyses of post-mortem
62 studies have shown that in HIV/TB co-infected cases dissemination is frequent, with a pooled
63 summary estimate of 87.9% of all TB cases ³. Importantly, 45.8% (95% CI 32.6–59.1%) of TB
64 cases in HIV-1 infected persons were undiagnosed at time of death, highlighting the urgent
65 need for improved rapid diagnostic tools for EPTB ³.

66 An important extra-pulmonary manifestation is pericardial TB (PCTB): the most common
67 cause of pericardial disease in Africa, associated with debilitating complications and high
68 mortality ². PCTB disproportionately affects HIV-1 coinfecting persons who are at high risk of
69 hematogenous dissemination of TB. Although PCTB is a severe form of EPTB, our
70 understanding of the broad range of clinicopathological PCTB phenotypes has not advanced
71 since its first descriptions in the 1940s and 1960s ⁴⁻⁶. Moreover, studies of the immune response
72 at the site of TB disease are limited.

73 Current understanding is of a spectrum of disease. At one extreme, pericardial fluid (PCF)
74 contains a high bacillary load and represents a failure of immune control. At the other end of
75 the spectrum, PCF can be paucibacillary with low yield of culture and polymerase chain
76 reaction (PCR)-based tests. In this latter situation, a presumptive diagnosis of PCTB maybe
77 made using a limited number of biomarkers with suboptimal specificity.

78 Despite the broad spectrum of disease phenotypes, the treatment approach is uniform and likely
79 sub-therapeutic, as key sterilizing anti-tuberculosis drugs do not penetrate the pericardial space
80 at sufficient concentrations to inhibit Mtb ⁷. This is of particular concern as both high bacillary
81 load and a CD4 count <200 cells/mm³ in HIV-1 infected patients are predictors of mortality in
82 PCTB ⁸. Interventions targeting both control of Mtb and HIV-1 are thus critical in HIV-1
83 coinfecting persons with PCTB. However, regardless of ATT and anti-retroviral treatment
84 (ART), severe complications such as re-accumulation of pericardial effusion after
85 pericardiocentesis, compromised cardiac function due to tamponade and chronic pericardial
86 inflammation leading to constriction (pericardial thickening with fibrosis) and death remain
87 frequent ⁹. Thus, with few validated diagnostic, prognostic, and treatment monitoring
88 biomarkers to guide clinicians in the management of PCTB, a better understanding of the
89 immune response to PCTB represents an urgent unmet research priority to identify potential
90 new clinical assessment tools ¹⁰.

91 Mtb control relies on a highly orchestrated immune response at the site of infection, and both
92 innate and adaptive responses act synergistically to restrict Mtb growth. CD4 T cells, and in
93 particular intact Th1 cellular responses are essential for control of Mtb¹¹. We have previously
94 shown that a simple whole blood-based assay can be utilized to measure functional and
95 phenotypic cellular markers that correlate with Mtb bacterial load, clinical disease severity and
96 treatment response in PTB patients, regardless of HIV-1 status^{12, 13}.
97 Considering the importance of functional Mtb-specific CD4 T cells in Mtb control, in this
98 study, we compared the frequency, polyfunctional capacity and phenotypic profile of Mtb-
99 specific CD4 T cells in blood and PCF of patients with PCTB. We assessed whether the cellular
100 profile associated with Mtb bacillary load and defined the effect of ATT on the peripheral Mtb-
101 specific CD4 T cell response.

102 RESULTS

103

104 Study population

105 A total of 16 participants were included in the study, of which 9 were male, with a median age
106 of 34 (Interquartile range (IQR): 28-43 years). Clinical characteristics of the study participants
107 are listed in **Table 1** and data for each individual patient presented in **Supplemental Table 1**.

108 The majority (87.5% [14/16]) were HIV-1 infected, and 71.4% [10/14] had either not
109 commenced or had defaulted ART at the time of enrolment into the study. The median
110 peripheral CD4 count of the HIV-1 infected participants were 141 cells/mm³ (IQR: 45-188) and
111 the median HIV-1 viral load was 47,907 mRNA copies/ml (IQR: 1,756-178,520). All
112 participants had large pericardial effusions with the median volume of PCF aspirated being 950
113 ml (IQR: 480-1200). The PCF of 9 participants (56.2%) returned Mtb culture positive results,
114 with a median time to culture positivity of 22 days (IQR: 13-26). All aspirated effusions were
115 exudates, with high protein content and elevated adenosine deaminase (ADA) and lactate
116 dehydrogenase (LDH). The majority showed a predominance of mononuclear cells (median:
117 81.7%) over polymorphonuclear cells (median: 18.3%) by PCF cell count. The Tygerberg
118 Diagnostic Index Score (TBH DI Score) is a weighted score incorporating clinical symptoms
119 and laboratory values in the diagnosis of TB pericarditis (sensitivity 86%, specificity 84%)¹⁴.
120 The median TBH DI Score in our study was 10, with all participants scoring above the
121 diagnostic cut-off of 6 for TB pericarditis. No difference in any of the measured clinical
122 parameters were observed between PCTB patients with a positive PCF Mtb culture and PCTB
123 patients with a negative PCF Mtb culture (**Supplemental Table 2**).

124

125 Cellular composition of pericardial fluid in PCTB

126 First, we examined the cellular composition of pericardial fluid (PCF) samples using flow
127 cytometry (**Figure 1A**). As characteristically observed in PCTB^{14,15}, we found lymphocytes to
128 be the most abundant cell type in PCF (median 67%), while granulocytes/myeloid cells
129 accounted for a median of 24% of the total live cell population (**Figure 1B**). However, it is
130 notable that in six of the 16 studied participants, lymphocytes represented less than 50% of the
131 total cell population. While not statistically significant, these participants tended to have a
132 lower blood CD4 count and higher plasma HIV viral load compared to those with dominant
133 lymphocytosis (median CD4: 92 vs 177 cells/mm³ and median HIV VL: 31,540 vs 131,600
134 mRNA copies/ml, respectively, data not shown). Further analysis comparing lymphocytic
135 populations showed that the proportion of CD4 T cells was significantly higher in PCF
136 compared to blood (median: 28.6 vs 20.8%, $P = 0.029$, respectively), while fewer CD3 negative

137 cells were found in PCF compared to blood (median: 16.2 vs 28.5%, $P = 0.049$, respectively)
138 (**Figure 1C**). This suggests a preferential recruitment of CD4 T cells into the pericardium
139 during PCTB, a finding which is further supported by a significantly higher CD4/CD8 ratio in
140 PCF compared to blood (median: 0.8 vs 0.43, $P = 0.006$, respectively) (**Figure 1D**).
141 We next compared the phenotype of total CD4 and CD8 T cells between blood and PCF. CD4
142 T cells in PCF showed higher expression of Ki-67 and HLA-DR (median: 9.5% vs 4.4%, $P =$
143 0.0006 and 15.3% vs 7.4%, $P = 0.044$, respectively) and lower expression of GrB and KLRG1
144 (median: 1.8% vs 8%, $P = 0.006$ and 0.7% vs 6.5%, $P < 0.0001$, respectively) compared to
145 peripheral CD4 T cells (**Figure 1E**, left panel). Similarly, CD8 T cells in PCF expressed less
146 GrB and KLRG1 compared to peripheral CD8 T cells (median: 38.9% vs 63.5%, $P = 0.001$ and
147 27.4% vs 54.8%, $P < 0.0001$, respectively). Moreover, in PCF, CD8 T cells showed higher
148 expression of CD27 compared to blood (median: 75.8% vs 59.5%, $P = 0.002$) (**Figure 1E**, right
149 panel). The lower expression of KLRG1 on T cells found at the disease site is likely related to
150 the poor ability of these cells to migrate to site of infection, as previously demonstrated in
151 murine models ^{16, 17}. Overall, these results suggest that infiltrating CD4 T cells were more
152 activated and exhibited a more differentiated memory profile compared to peripheral cells,
153 while CD8 T cells in PCF had a lower cytotoxic potential and displayed features of an earlier
154 differentiated phenotype compared to their blood counterparts.

155

156 **Comparison of the Mtb-specific Immune response in paired blood and PCF**

157 To measure the Mtb-specific immune response, we used an approach where *ex vivo*
158 unprocessed whole blood or pericardial fluid samples were freshly stimulated for a short time
159 (5h) using an Mtb peptide pool combined with γ -irradiated Mtb (H37Rv) in the presence of
160 protein transport inhibitors (added at the onset of the stimulation). The combination of peptides
161 and whole bacteria allows the simultaneous detection of the Mtb-specific T cell response and
162 toll like receptor (TLR)-dependent innate responses. Moreover, this type of assay has the
163 advantage of preserving all cell subsets and soluble components present *in vivo*, thus
164 maintaining a more physiologic environment ¹⁸.

165 First, although the flow cytometry panel used in this study was not designed to measure the
166 innate immune response in detail, we were able to compare Mtb-induced production of TNF- α
167 by peripheral and PCF granulocytes/myeloid cells (**Figure 2A**). Figure 2B shows that while
168 Mtb stimulation led to TNF- α production in granulocytes/myeloid cells from both
169 compartments, the frequencies of TNF- α producing cells was ~10 fold higher [IQR: 3.3 - 20.2]
170 at the site of TB disease compared to blood (median: 1.1% vs 10.2%, respectively, $P < 0.0001$).
171 TNF- α production was observed almost exclusively in the HLA-DR+ population, suggesting

172 that Mtb-responding cells are likely classical (CD14⁺⁺CD16⁻) or intermediate
173 (CD14⁺⁺CD16⁺) monocytes¹⁹. No difference in the frequency of TNF- α responding
174 granulocytes/myeloid cells in PCF was observed between patients with a positive or a negative
175 PCF Mtb culture (**Supp Figure 1A**).

176 Next, to compare the adaptive immune response between blood and PCF, we measured IFN- γ ,
177 TNF- α and IL-2 expression in CD4 T cells in response to Mtb antigens (**Figure 3A**). Only one
178 participant had no detectable Mtb-specific CD4 response in blood. The frequency of Mtb-
179 specific CD4 T cells detected in PCF was significantly higher (~ 5-fold) than in blood for all
180 measured cytokines (**Figure 3B**). There was no correlation between the frequency of Mtb-
181 specific CD4 T cells in blood and PCF ($P = 0.59$, $r = 0.14$, **Figure 3C**). Moreover, we did not
182 find any association between the frequency of Mtb-specific CD4 T cells and the number of
183 absolute CD4 T cells in blood, nor between the frequencies of Mtb-specific CD4⁺ T cells and
184 the percentage of total CD4 T cells in PCF ($P = 0.11$, $r = -0.40$ and $P = 0.32$, $r = -0.26$,
185 respectively). Despite the major difference in magnitude of the Mtb-specific CD4 response
186 between compartments, we did not observe any significant differences in polyfunctional
187 capacity (i.e. expression of IFN- γ , TNF- α or IL-2) of Mtb-specific CD4 T cells between blood
188 and PCF. Mtb-specific CD4 T cells exhibited a highly polyfunctional profile with a sizable
189 proportion (median: 58% for blood and 55% for PCF) of cells co-expressing IFN- γ , TNF- α and
190 IL-2 (**Figure 3D**). Additionally, Mtb culture positivity in PCF did not associate with the
191 magnitude or polyfunctional capacities of Mtb-specific CD4 T cells in blood or in PCF (**Supp**
192 **Figure 1B&C**).

193 Detailed phenotyping was performed to define and compare the activation and maturation
194 profile of Mtb-specific CD4 T cells from blood and PCF. Firstly, irrespective of the studied
195 compartments, Mtb-specific CD4 T cells exhibited high expression of HLA-DR (ranging from
196 48 to 97%) and Ki67 (ranging from 3.6 to 79%) and low expression of CD27 (ranging from 9 to
197 46%); profiles characteristic of active TB disease, as previously reported²⁰⁻²³ (**Figure 4A**).

198 Paired comparison of blood and PCF revealed that Mtb-specific CD4 T cells in PCF had
199 distinct characteristics compared to their peripheral counterparts, with elevated expression of
200 GrB (medians: 36.6% vs 16%, respectively, $P = 0.017$) and lower expression of MIP-1 β (36.6%
201 vs 16%, $P = 0.003$), CD27 (16.2% vs 20%, $P = 0.03$) and KLRG1 (0.5% vs 3.8%, $P = 0.002$)
202 (**Figure 4A**). However, despite differences in the level of expression of GrB and KLRG-1
203 between blood and PCF, the phenotype of Mtb-specific CD4 T cell in the blood associated with
204 the profile of PCF Mtb-specific CD4 T cells for GrB, KLRG-1, CD153, Ki-67, and HLA-DR,
205 with the strongest correlations being observed for GrB ($P = 0.0034$, $r = 0.74$) and CD153 ($P =$

206 0.0034, $r = 0.74$) (**Figure 4B**). Additionally, the phenotypic profile of PCF Mtb-specific CD4 T
207 cells was comparable regardless of PCF culture status (**Figure 4C**).

208 We also investigated the frequency and phenotype of Mtb-specific CD8 T cells in these
209 participants (**Supp Figure 2**). As previously described in TB patients²⁴, a CD8 T cell response
210 was not observed in all participants. Mtb-specific CD8 T cells were detected in blood from 5
211 out of 16 patients (32%), while 8 of 16 patients (50%) had a detectable CD8 T cell response in
212 PCF (**Supp Figure 2B**). As in the case of CD4 responses, we did not find any phenotypic
213 differences between blood and PCF CD8 responses (**Supp Figure 2C**). Of note, compared to
214 Mtb-specific CD4 T cells, CD8 T cells exhibited limited capacity to produce IL-2, expressed
215 significantly higher level of GrB and Mip-1 β ; with CD153 expression being undetectable
216 (**Supp Figure 2D**). The activation profile (defined with HLA-DR and Ki67 expression) were
217 comparable between Mtb-specific CD4 and CD8 T cell responses in PCF and blood (**Supp**
218 **Figure 2D**).

219

220 **Impact of ATT on blood Mtb-specific CD4 T cell responses**

221 In the context of pericardial TB (and extra-pulmonary TB, in general), the monitoring of the
222 response to treatment is particularly challenging due to the lack of sensitive and specific tools.
223 In this study, we defined the impact of ATT on the frequency, polyfunctional and phenotypic
224 profile of peripheral Mtb-specific CD4 T cells pre- and post-ATT (at week 24 or 52, depending
225 on sample availability). While successful ATT did not alter the magnitude of Mtb-responding
226 CD4 T cells (**Figure 5A**), it significantly modified their functional capacity, with the proportion
227 of IL-2 and TNF- α dual producing CD4 T cells becoming significantly expanded post-ATT,
228 counterbalanced by a decrease of IFN- γ and TNF- α dual producing CD4 T cells (**Figure 5B**).
229 More importantly, ATT led to major changes in the phenotype of the peripheral Mtb-specific
230 CD4 response (**Figure 5C&D**). Post-ATT, HLA-DR, Ki67 and GrB expression by Mtb-
231 specific CD4 T cells were significantly reduced compared to pre-treatment (medians: 15.7 vs
232 70% for HLA-DR; 2.8 vs 35% for Ki67 and 0.9 vs 10.9% for GrB, respectively), while no
233 changes were observed for CD153, CD27 or Mip-1 β expression (**Figure 5D**). Overall, these
234 results suggest that assessing the activation profile of Mtb-specific CD4 T cells could aid
235 monitoring of treatment response in extra-pulmonary TB.

236 DISCUSSION

237

238 Pericardial TB is an understudied and severe form of extra-pulmonary TB¹⁰. Only two previous
239 published studies have used flow cytometry to assess the cellular profile of PCF in PCTB^{15, 25}.

240 Here, we demonstrate that a simple laboratory methodology (derived from a previously
241 described whole blood assay¹³) can successfully be applied to PCF, revealing differences and
242 similarities between the two compartments. Using this approach, we investigated the overall
243 cellular profile of PCF, assessed whether PCF Mtb culture positivity associate with the Mtb
244 specific cellular response and evaluated the evolution of peripheral Mtb-specific CD4 T cell
245 responses in relation to ATT.

246 In accordance with previous reports, we found a significantly higher frequency of lymphocytes
247 in PCF compared to blood, whereas neutrophils predominated in blood¹⁴. Additionally, we
248 observed significantly higher frequencies of CD4 T cells in PCF compared to blood, whereas
249 preferential recruitment was not observed for CD8 T cells. This effect is likely to be even more
250 pronounced in cohorts comprised of higher numbers of HIV-1 uninfected participants, given the
251 findings by Reuter *et al.* showing lower frequencies of CD4 T cells and higher frequencies of
252 CD8 T cells in PCF of HIV-1 co-infected compared to HIV-1 uninfected patients with PCTB¹⁵.

253 Here, we report that Mtb-specific CD4 T cells are more abundant in PCF than in blood in HIV-
254 1 infected individuals, similar to what has been reported in studies of other TB disease sites
255 (e.g. lymph nodes, broncho-alveolar lavage and pleural fluid)²⁶⁻³¹. A previous study comparing
256 PCF and blood of PCTB patients only observed increased PCF CD4 T cell responses in HIV-1
257 uninfected individuals through examining numbers of spot forming cells in response to ESAT-6
258 detected by IFN- γ ELISpot²⁵. This difference is likely attributable to the use of different
259 laboratory assays (ELISpot versus flow cytometry), sample type (cryopreserved cells versus *ex*
260 *vivo* whole blood or whole pericardial fluid) and stimuli (ESAT-6/CFP-10 versus Mtb300).

261 Despite markedly higher frequencies of cytokine producing Mtb-specific CD4 T cells in PCF
262 compared to blood, we did not observe any significant differences in their functional capacity,
263 in keeping with previous reports^{26, 32}. Similarly, in a prior study, we did not observe significant
264 differences in cytokine production between ESAT-6/CFP-10 specific CD4 T cells in PCF and
265 blood of pericardial patients²⁵. The frequency of Th1 cytokine producing Mtb-specific CD4 T
266 cells may play an important role for Mtb control, as demonstrated by a study involving low
267 dose Mtb infection of non-human primates which compared T cell cytokine production at
268 granuloma level in the lung to peripheral blood³³. They observed significant variability in
269 cytokine production between different granulomas in the same animal, with sterile granulomas
270 comprising a higher frequency of cytokine (IL-17, TNF and any Th1 cytokine [IFN- γ , IL-2, or

271 TNF)] producing T cells than non-sterile granulomas. It is thus likely that the predominance of
272 Mtb-specific CD4 T cells in PCF over blood reflects homing of these cells to the pericardium to
273 attempt contribution to Mtb control.

274 We hypothesized that the presence of a higher Mtb load, as denoted by positive Mtb culture
275 status, would enhance T cell proliferation (thus frequency), and may promote further
276 differentiation and activation of responsive T cells^{20,34}. However, we did not observe any
277 major differences in the Mtb-specific T cell profile according to PCF Mtb culture status. It
278 should be borne in mind that PCF Mtb culture is of poor sensitivity to diagnose PCTB¹⁰.
279 Nonetheless, this finding warrants further investigation, especially since time to culture
280 positivity in PCF significantly predicts mortality in PCTB and considering the important
281 established role of an efficient Th1 response in protecting against Mtb⁸. Future studies of
282 pericardial biopsy or pericardiectomy samples could potentially shed further light on this by
283 analyzing T cell responses and Mtb load at the granuloma level.

284 Growing evidence points towards the necessity to elicit a balanced immune response to Mtb,
285 with the predominance of either pro- or anti-inflammatory mediators being detrimental in the
286 containment and elimination of Mtb^{33,35}. IFN- γ and TNF- α are examples of essential pro-
287 inflammatory cytokines that are required in the immune response to Mtb³⁶, especially through
288 their capacity to activate infected macrophages to eliminate Mtb³⁷, but in excess, these
289 cytokines may also exacerbate immunopathology³⁸⁻⁴⁰. Excessive TNF- α can induce
290 mitochondrial reactive oxygen species that lead to necrotic cell death and activate matrix
291 metalloproteinases both of which have been associated with lung tissue damage during PTB³⁹,
292⁴¹. We found PCF granulocytes to have significantly higher capacity to produce TNF- α in
293 response to Mtb compared to blood granulocytes. Whilst we found no significant difference in
294 frequency of Mtb-specific granulocytes producing TNF- α in culture positive compared to
295 negative PCF, it remains to be investigated whether higher frequencies of TNF- α producing
296 granulocytes are associated with enhanced immune pathology and complications such as
297 pericardial fibrosis.

298 The clinical management of pericardial TB is complicated owing to the lack of sensitive and
299 specific diagnostic and treatment monitoring tools. A definitive diagnosis of PCTB relies on the
300 detection of Mtb in PCF or in pericardial tissue, and thus requires invasive sampling. The
301 sensitivity of conventional PCF culture techniques is between 50 - 65%, whilst Xpert MTB/RIF
302 of PCF has 66 - 66.7% sensitivity when assessed against a composite reference^{42,43}. Whilst
303 Xpert MTB/RIF yields rapid results, Mtb culture can take up to 4-6 weeks to yield results. The
304 diagnosis thus rests on the presence of a lymphocyte predominant effusion with elevated levels
305 of the biomarker ADA. Measurement of IFN- γ in PCF and the use of IFN- γ release assays

306 (IGRAs) have produced variable, but promising results as diagnostic tests, but is currently
307 limited to the research setting pending further optimization, head-to-head comparison with
308 current composite reference standards and studies evaluating its scalability in low resource
309 settings^{44, 45}. Few, if any, advances have recently been made in efforts to monitor treatment
310 efficacy in EPTB. Indeed, PCTB treatment response is only assessed clinically owing to the
311 impracticality of invasive sampling of the pericardium, contrasting with PTB where follow up
312 sputums can be obtained to monitor Mtb clearance. We and others have previously shown that a
313 simple, whole blood-based assay assessing the activation profile of Mtb-specific CD4 T cells
314 can be used as a non-sputum-based method to track both disease severity and response to ATT
315^{13, 20, 21, 46, 47}. Here, we build on these findings showing that 1) in PCTB patients, the activation
316 profile of peripheral Mtb-specific CD4 T cells is comparable to those observed in active PTB
317 and 2) successfully treated PCTB is associated with significantly decreased expression of HLA-
318 DR, Ki-67 and GrB and expansion of IL-2 producing Mtb-specific CD4 T cells compared to
319 baseline. HLA-DR has been shown to be a robust biomarker to discriminate latent TB infection
320 from PTB^{13, 20, 21, 47} and EPTB²³, and to monitor PTB treatment response^{13, 20, 46, 47}. Here, we
321 show that HLA-DR expression on peripheral Mtb-specific CD4 T cells significantly decreases
322 between baseline and completion of ATT in PCTB, thus extending the potential utility of this
323 biomarker for the monitoring of treatment response in PCTB.

324 Our study was limited by small numbers of HIV-1 uninfected participants. However, we have
325 previously shown that our panel of phenotypic and functional T cell markers perform equally
326 well regardless of HIV-1 status¹³. Moreover, this study would have benefited from the
327 inclusion of patients with non-tuberculous pericardial effusion to further ascertain the
328 specificity of HLA-DR expression on Mtb-specific T cells as potential TB diagnostic
329 biomarkers. In this study, our objective was not to investigate immune correlates of adverse
330 outcomes such as pericardial constriction, other complications or mortality, with these
331 remaining important research priorities for future studies. The performance of multiple PCF
332 cultures, or the additional testing of Xpert MTB/RIF with reporting of cycle threshold values
333 may have increased the sensitivity of our estimate of Mtb burden.

334 Nonetheless, in view of the dearth of knowledge on immune responses at TB disease site, our
335 study demonstrates a novel and rapid experimental approach to measure T cell response *ex vivo*
336 at site of disease. This technique allowed us to define key differences and similarities between
337 the Mtb immune response in PCF and blood and may aid to the diagnostic and/or treatment
338 monitoring of PCTB patients. The latter will be of particular importance as pharmacodynamic
339 readout in ongoing trials aiming to optimize treatment regimens for PCTB, with the current
340 standard of care ATT lacking sufficient concentrations in the pericardial space to inhibit Mtb⁷.

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342 study. P.H., A.J. and E.d.B. recruited the study participants. S.R., E.d.B. and C.R. performed
343 the whole blood and PCF assay. E.d.B. and C.R. performed the flow experiments. C.R.
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361

362 **Conflict of interest:** The authors declare no conflict of interest.

363 **METHODS**

364

365 **Study population**

366 Participants with suspected PCTB recruited from the Grootte Schuur Hospital Cardiology Unit.
367 Only adults (≥ 18 years of age) who were undergoing pericardiocentesis as part of the routine
368 management of their pericardial effusion and who had received no more than 3 doses of ATT
369 prior were included. Pregnancy, severe anemia (Hemoglobin ≤ 7 g/dL), multi-drug resistant TB
370 and severe concurrent opportunistic infection were exclusion criteria. All participants provided
371 written informed consent and the study was approved by the University of Cape Town Human
372 Research Ethics Committee (HREC: 050/2015, DMID protocol 15-0047). At the time of
373 pericardiocentesis the study team collected PCF and paired blood samples for analysis. Only
374 participants with definite (Mtb culture positive) or probable PCTB were included in the study.
375 Probable PCTB was defined per criteria from Mayosi *et al.* where there was evidence of
376 pericarditis with microbiologic confirmation of Mtb-infection elsewhere in the body and/or an
377 exudative, lymphocyte predominant pericardial effusion with elevated adenosine deaminase
378 (≥ 35 U/L)⁴⁸. Study participants were followed up over the course of their ATT and up to one
379 year.

380

381 **Pericardial fluid, blood collection and stimulation assay**

382 Pericardial fluid was obtained at the time of pericardiocentesis, placed in sterile Falcon tubes
383 and transported to the laboratory at 4°C. Blood was collected in sodium heparin tubes at the
384 time of pericardiocentesis. Both blood and pericardial fluid were processed within 3 hours of
385 collection. The whole blood or whole PCF assay were adapted from the protocol described by
386 Hanekom *et al.*⁴⁹. Briefly, 0.5 ml of whole blood or 1 ml of whole PCF were stimulated with a
387 pool of 300 Mtb-derived peptides (Mtb300, 2 μ g/ml)⁵⁰ combined with γ -irradiated Mtb
388 (H37Rv, 100 μ g/ml, obtained through BEI Resources, NIAID, NIH) at 37°C for 5 hours in the
389 presence of the co-stimulatory antibodies, anti-CD28 and anti-CD49d (1 μ g/ml each; BD
390 Biosciences, San Jose, CA, USA) and Brefeldin-A (5 μ g/ml; Sigma-Aldrich, St Louis, MO,
391 USA) and Monensin (5 μ g/ml, BD Biosciences, San Jose, CA, USA). The combination of Mtb
392 peptides and inactivated whole bacteria allows the simultaneous detection of Mtb-specific T
393 cell response and toll like receptor (TLR)-dependent innate response. Unstimulated cells were
394 incubated with co-stimulatory antibodies and Brefeldin-A only. Red blood cells were then lysed
395 in a 150 mM NH₄Cl, 10 mM KHCO₃, 1 mM Na₄EDTA solution. Cells were then stained with a
396 Live/Dead Near-InfraRed dye (Invitrogen, Carlsbad, CA, USA) and then fixed using a
397 Transcription Factor Fixation buffer (eBioscience, San Diego, CA, USA), cryopreserved in

398 freezing media (50% fetal bovine serum, 40% RPMI and 10% dimethyl sulfoxide) and stored in
399 liquid nitrogen until use.

400

401 **Cell staining and flow cytometry**

402 Cryopreserved cells were thawed, washed and permeabilized with a Transcription Factor
403 perm/wash buffer (eBioscience). Cells were then stained at room temperature for 45 minutes
404 with the following antibodies: CD3 BV650 (OKT3; Biolegend, San Diego, CA, USA), CD4
405 BV785 (OKT4; Biolegend), CD8 BV510 (RPA-T8; Biolegend), CD27 PE-Cy5 (1A4CD27;
406 Beckman Coulter, Brea, CA, USA), HLA-DR BV605 (L243; Biolegend), Ki67 PerCPcy5.5.
407 (B56, BD), Granzyme B (GrB) BV421 (GB11, BD), Killer cell Lectin-like Receptor G1
408 (KLRG1) APC (13F12F2, eBioscience), IFN γ BV711 (4S.B3; Biolegend), TNF α PEcy7
409 (Mab11; Biolegend), IL-2 PE/Dazzle (MQ1-17H12, Biolegend), Mip-1 β Alexa Fluor 488
410 (#24006, R&D systems, Minneapolis, MN, USA) and CD153 (R&D116614, R&D). Samples
411 were acquired on a BD LSR-II and analyzed using FlowJo (v9.9.6, FlowJo LCC, Ashland, OR,
412 USA). The granulocytes/monocytes population was defined based on their FSC/SSC
413 characteristics. A positive cytokine response was defined as at least twice the background of
414 unstimulated cells. To define the phenotype of Mtb300-specific cells, a cut-off of 30 events was
415 used.

416

417 **Statistical analyses**

418 Statistical tests were performed in Prism (v9.1.2; GraphPad, San Diego, CA, USA).
419 Nonparametric tests were used for all comparisons. The Kruskal-Wallis test with Dunn's
420 Multiple Comparison test was used for multiple comparisons and the Mann-Whitney and
421 Wilcoxon matched pairs test for unmatched and paired samples, respectively.

422 **LEGENDS**

423 **Table 1: Clinical characteristic of study patients.**

424 *: Median and Interquartile range. M: Male, F: Female, PCF: Pericardial Fluid, ADA: adenosine
425 deaminase, LDH: lactate dehydrogenase, TBH DI: Tygerberg Diagnostic Index Score (TBH DI
426 Score) is a weighted score incorporating clinical symptoms and laboratory values in the
427 diagnosis of TB pericarditis (sensitivity 86%, specificity 84%, ref: 14). CRP: C-reactive
428 protein, VL: viral load.

429

430 **Figure 1: Comparison of the distribution of immune cells in whole blood and pericardial**
431 **fluid (PCF) in patients with pericarditis. (A)** Representative example of the distribution and
432 phenotype of T cells in whole blood (red) and PCF (blue) using flow cytometry. **(B)** Proportion
433 of lymphocyte (Lympho) and neutrophils (Neutro) defined according to their FSC/SSC profile
434 and expressed as a percentage of total live cells in patients with pericarditis (n = 16). Triangles
435 depict HIV-uninfected participants (n = 2). **(C)** Proportion of CD3-, CD3+CD4-CD8-,
436 CD3+CD4+ and CD3+CD8+ cells, expressed as a percentage of total live lymphocytes. **(D)**
437 CD4 to CD8 ratio in whole blood and PCF. **(E)** Expression of CD27, GrB, HLA-DR, Ki67 and
438 KLRG1 in CD4 T cells (left panel) and CD8 T cells (right panel) from whole blood and PCF.
439 Bars represent medians. Statistical comparisons were performed using a Wilcoxon rank test.

440

441 **Figure 2: Comparison of TNF α production by granulocytes/myeloid cells in response to**
442 **Mtb stimulation between whole blood and PCF. (A)** Representative example of TNF α -
443 producing cells in responses to Mtb antigen stimulation. Granulocytes/myeloid cells were
444 identified based on their FCS/SSC profile. **(B)** Frequency of TNF α -producing
445 granulocytes/myeloid cells in unstimulated (Uns) and Mtb-stimulated samples (Mtb). The
446 triangles depict HIV-uninfected participants (n = 2). Bars represent medians. Statistical
447 comparisons were performed using a Wilcoxon rank test for paired samples or a Mann-Whitney
448 test for unpaired samples.

449

450 **Figure 3: Comparison of the functional profile of Mtb-specific CD4 T cells between whole**
451 **blood and PCF. (A)** Representative example of IFN γ , IL-2, and TNF α production by CD4 T
452 cells in unstimulated (unstim) and Mtb-stimulated whole blood and PCF samples. Number
453 represents cytokine positive cells expressed as a percentage of total CD4 T cells. **(B)** Magnitude
454 of Mtb-specific CD4 T cells in whole blood (red) and PCF (blue). The triangles depict HIV-
455 uninfected participants (n = 2). Bars represent medians. Statistical comparisons were defined
456 using a Wilcoxon rank test. **(C)** Correlation between the frequency of Mtb-specific CD4 T cells

457 in blood and at site of disease (PCF). The triangles depict HIV-uninfected participants ($n = 2$).
458 Correlation was tested by a two-tailed non-parametric Spearman rank test. **(D)** Polyfunctional
459 profile of Mtb-specific CD4 T cells in whole blood (red) and PCF (blue). The x-axis displays
460 all possible cytokine combinations (flavors), the composition of which is denoted with a dot for
461 the presence of IL-2, IFN γ and TNF α . The proportion of each flavor contributing to the total
462 Mtb-specific CD4 response per individual is shown. The median (black bar) and interquartile
463 ranges (box) are shown. Each flavor is color-coded, and data are summarized in the pie charts,
464 representing the median contribution of each flavor to the total Mtb response. No statistically
465 significant differences were observed using a Wilcoxon rank test to compare response patterns
466 between groups and a permutation test to compare pies.

467

468 **Figure 4: Comparison of the phenotypic profile of Mtb-specific CD4 T cells in blood and**
469 **PCF. (A)** Expression of GrB, CD27, HLA-DR, Ki67, KLRG1, CD153 and MIP1 β in Mtb-
470 specific CD4 T cells (i.e. producing any measured cytokine IFN γ , IL-2, and TNF α). Only
471 paired samples with Mtb-specific responses >30 events are depicted ($n=14$). Bars represent
472 medians. The triangles depict HIV-uninfected participants ($n = 2$). Statistical comparisons were
473 performed using a Wilcoxon rank test. **(B)** Spearman correlation r values between indicated
474 Mtb-specific CD4 T cell phenotype in blood and PCF. P values are indicated for each
475 comparison. **(C)** Comparison of the phenotypic profile of Mtb-specific CD4 cells from PCF
476 between patients who are PCF Mtb culture negative (PCF/Mtb Cult-, light blue, $n=7$) or PCF
477 Mtb culture positive (PCF/Mtb Cult+, dark blue, $n=9$). Bars represent medians. Statistical
478 comparisons were performed using the Mann-Whitney test.

479

480 **Figure 5: Evolution of the magnitude, polyfunctional capacity and phenotypic profile of**
481 **Mtb-specific CD4 T cells response in whole blood from baseline (pre-ATT initiation) to**
482 **ATT completion (24 or 52 weeks post-ATT). (A)** Frequency of Mtb-specific CD4 T cells pre-
483 ATT (Baseline, red) and post ATT (24 weeks, orange or 54 weeks, light blue). The triangles
484 depict HIV-uninfected participants ($n = 2$). Bars represent medians. Statistical comparisons
485 were performed using the Mann-Whitney test. **(B)** Polyfunctional profile of Mtb-specific CD4
486 T cells pre-ATT (BL) and post ATT (W24 or W52). The median and interquartile ranges are
487 shown. A Wilcoxon rank test was used to compare cytokine combination between groups and a
488 permutation test to compare pies. **(C)** Representative flow plots of HLA-DR, Ki67, GrB, CD27,
489 KLRG1, CD153 and MIP1 β expression in Mtb-specific CD4 T cells pre- and post-ATT in one
490 patient. **(D)** Summary graph of the expression of phenotypic markers measured. Only paired
491 samples are depicted ($n=9$). Red symbols correspond to Baseline samples, orange to W24

492 samples and blue to W52 samples. The triangles depict HIV-uninfected participants (n = 2).
493 Bars represent medians. Statistical comparisons were performed using the Wilcoxon rank test.

494
495

496 **SUPPLEMENTARY MATERIAL**

497

498 **Supp Table 1: Clinical characteristics in each studied patient (n=16).**

499 PCF: Pericardial Fluid, M: Male, F: Female, TTP: time to positivity, ADA: adenosine
500 deaminase, LDH: lactate dehydrogenase. CRP: C-reactive protein, VL: viral load. Nd: not done,
501 na: not applicable.

502

503 **Supp Table 2: Comparison of the clinical characteristics between patients with a PCF 504 positive Mtb culture (n=9) and patients with a PCF negative Mtb culture (n=7).**

505 *: Medians and Interquartile ranges. M: Male, F: Female, PCF: Pericardial Fluid, ADA:
506 adenosine deaminase, LDH: lactate dehydrogenase, TBH DI score: Tygerberg Diagnostic Index
507 Score is a weighted score incorporating clinical symptoms and laboratory values in the
508 diagnosis of TB pericarditis (sensitivity 86%, specificity 84%, ref: 14). CRP: C-reactive
509 protein, VL: viral load. Statistical comparisons were performed using the Mann-Whitney test.

510

511 **Supp Figure 1: Comparison of the frequency and polyfunctional profile of immune**

512 **responses in blood and PCF, stratify based on PCF Mtb culture status.** (A) Frequency of
513 TNF α + Neutrophils in response to Mtb stimulation in whole blood and PCF from PCF Mtb
514 culture negative (n = 7) and PCF Mtb culture positive (n = 9) patients. (B) Frequency of Mtb-
515 specific CD4+ T cells in whole blood and PCF from patients who are PCF Mtb culture negative
516 or PCF Mtb culture positive. (C) Polyfunctional profile of Mtb-specific CD4+ T cells.

517 Statistical comparisons were performed using the Wilcoxon rank test for paired samples or the
518 Mann-Whitney test for unpaired samples.

519

520 **Supp Figure 2: Frequency and phenotypic profile of Mtb-specific CD8+ T cell response in**

521 **Blood and PCF.** (A) Representative example of flow cytometry plots showing the expression
522 of TNF α and IFN γ in CD8+ T cells from blood (top) and PCF (bottom) in one PCTB patient.
523 (B) Comparison of the frequency of Mtb-specific CD8+ T cells in whole blood and PCF (n =
524 16). The proportion of participant exhibiting a detectable Mtb-specific CD8+ T cell response is
525 indicated at the top of the graph. Bars represent medians of responders. (C) Comparison of the
526 phenotypic profile of Mtb-specific CD8+ T cells in whole blood (n = 5) and PCF (n = 8). (D)

527 Comparison of the phenotypic profile of Mtb-specific CD4+ and CD8+ T cells in whole blood
528 (left panel and PCF (right panel). Bars represent medians. Statistical comparisons, performed
529 using a Mann-Whitney test, are reported at the top of the graph.

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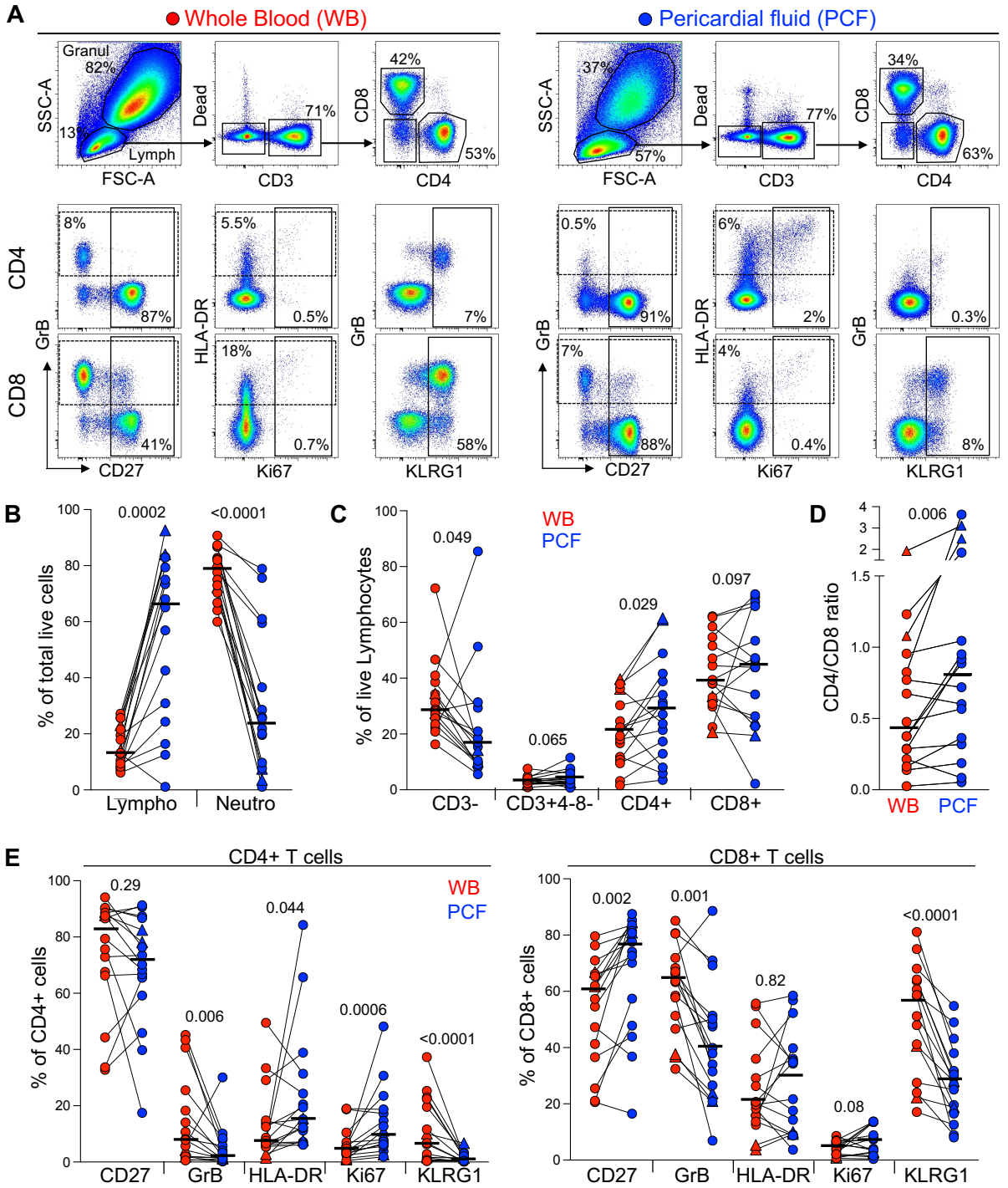
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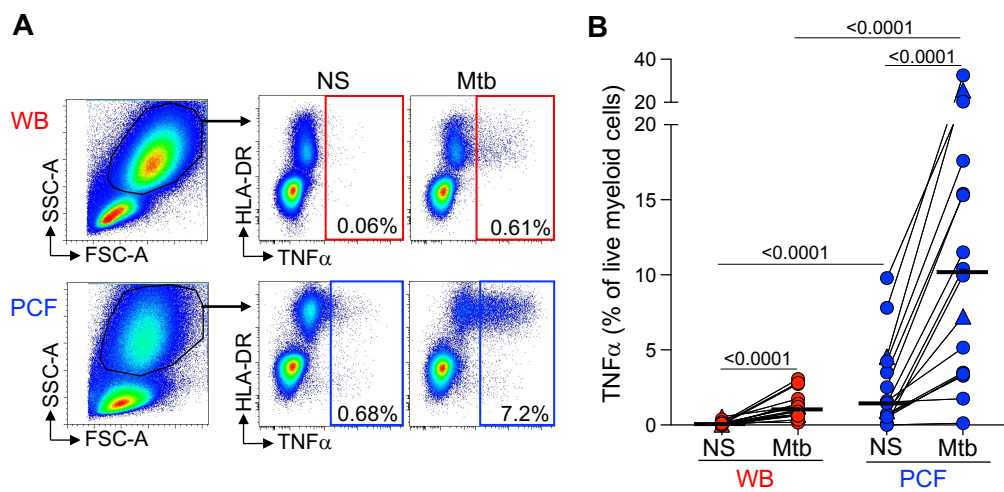
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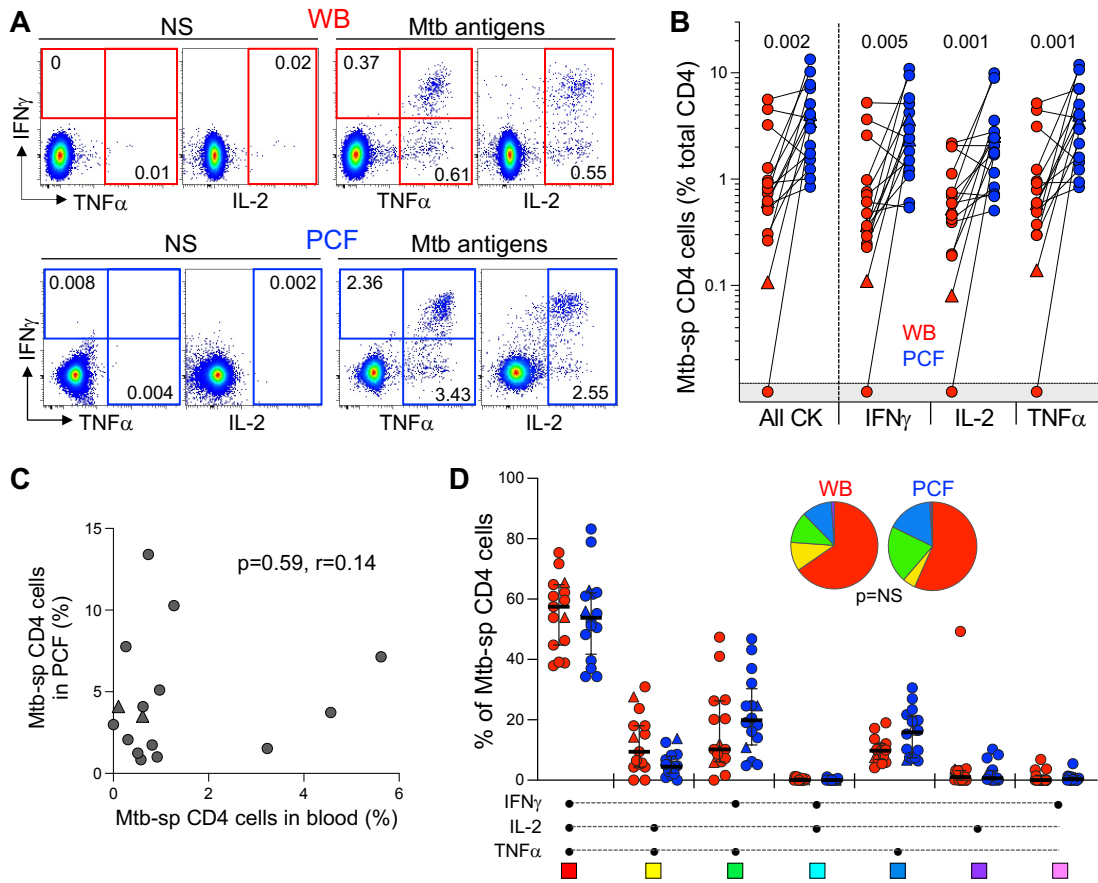
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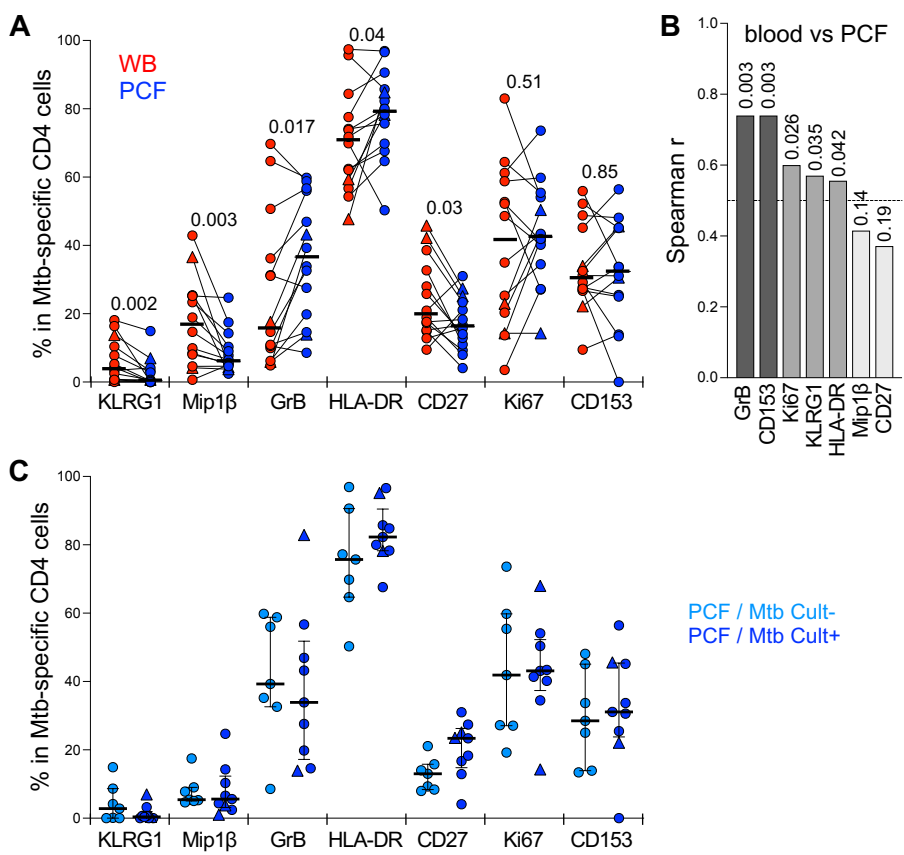
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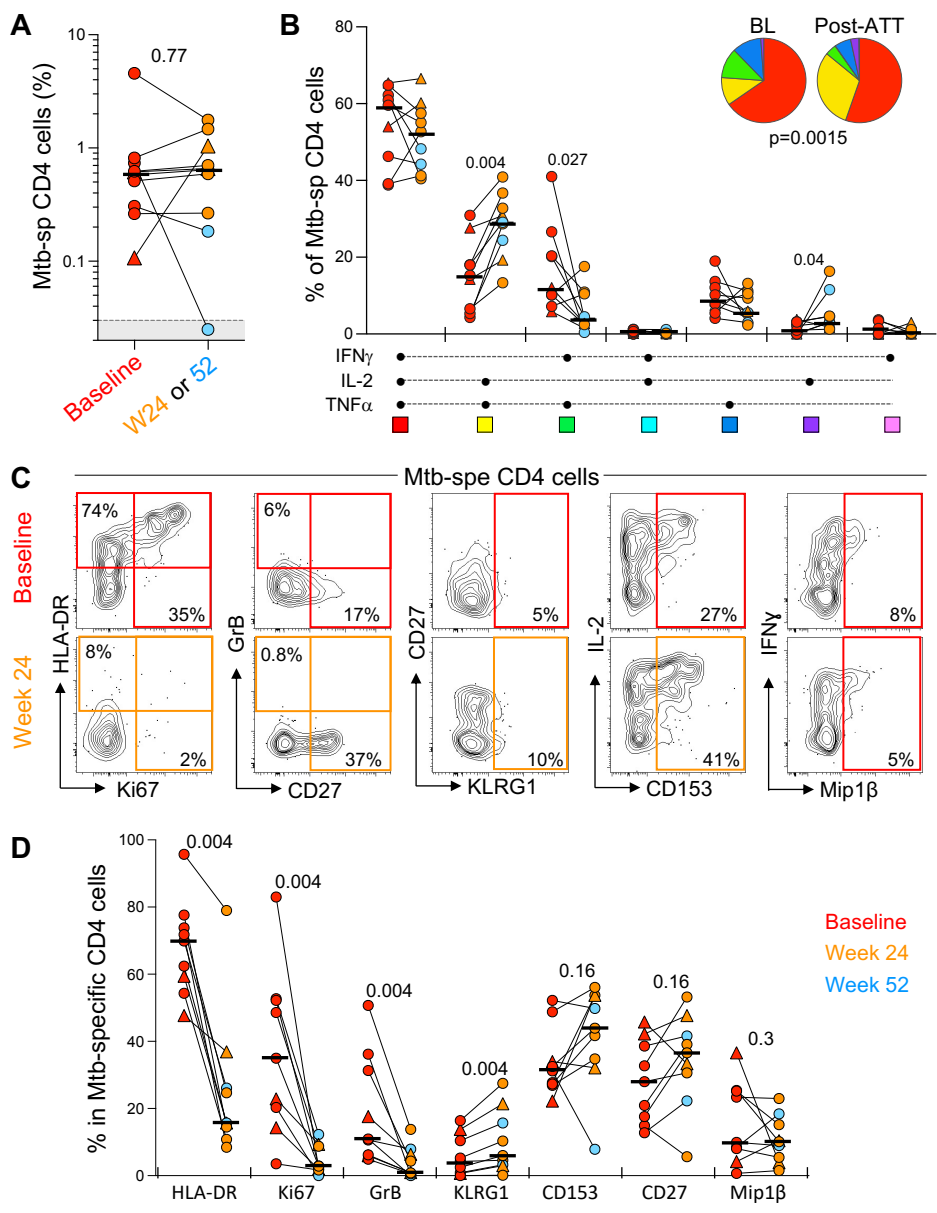
N	16
Age*	34 [28-43]
Gender (M/F)	9/7
HIV infected (%)	87.5% (14/16)
Vol PCF aspirated (ml)*	950 [480-1200]
Positive Mtb culture in PCF (%)	56.2% (9/16)
Time to culture positivity (days)*	22 [13-26]
ADA (U/l) in PCF*	51.2 [40-58]
LDH (IU/ml) in PCF*	753 [525-1025]
Protein (g/l) in PCF*	65 [58-66]
Mononuclear cells (%) in PCF*	81.7 [58-89]
TBH DI score	10 [9-10]
Plasma CRP ($\mu\text{g/ml}$)*	138 [88-205]
CD4 count (cells/ mm^3) in blood*	141 [45-188]
Plasma VL (mRNA copies/ml)*	47,907 [1,756-178,520]











PID	Sex	Age	HIV	PCF					Plasma		
				Mtb culture	TTP (days)	PCF ADA (U/L)	PCF LDH (IU/mL)	PCF protein (g/L)	Plasma CD4 count	Plasma Log ₁₀ HIV VL	Plasma CRP (µg/mL)
EN-3017	F	40	Pos	Neg	>46	nd	nd	nd	437	1.49	201
EN-3013	M	35	Pos	Neg	>46	222	4324	52	171	3.33	177
EN-3005	M	45	Pos	Neg	>46	51.2	603	66	199	4.68	112
EN-3016	M	32	Pos	Neg	>46	9.8	1025	nd	50	5.29	211
EN-3008	M	32	Pos	Neg	>46	57.8	845	58	14	5.46	286
EN-3015	F	29	Pos	Neg	>46	40.7	937	72	30	6.05	104
EN-3012	M	39	Pos	Neg	>46	64.8	970	51	120	6.42	172
EN-3014	M	40	Pos	Pos	11	157.6	3946	65	140	1.3	80
EN-3019	F	34	Pos	Pos	22	58.5	706	65	260	2.76	320
EN-3002	F	28	Pos	Pos	25	39.9	443	66	142	3.35	188
EN-3018	F	26	Pos	Pos	35	55.2	439	74	170	4.18	60
EN-3011	M	42	Pos	Pos	24	40.1	418	65	94	4.68	207
EN-3001	F	33	Pos	Pos	18	38.9	753	66	21	4.96	103
EN-3009	F	47	Pos	Pos	15	55.5	1811	65	135	5.24	86
EN-3006	M	21	Neg	Pos	12	75.3	986	58	nd	na	95
EN-3007	M	20	Neg	Pos	27	41.6	607	58	nd	na	164

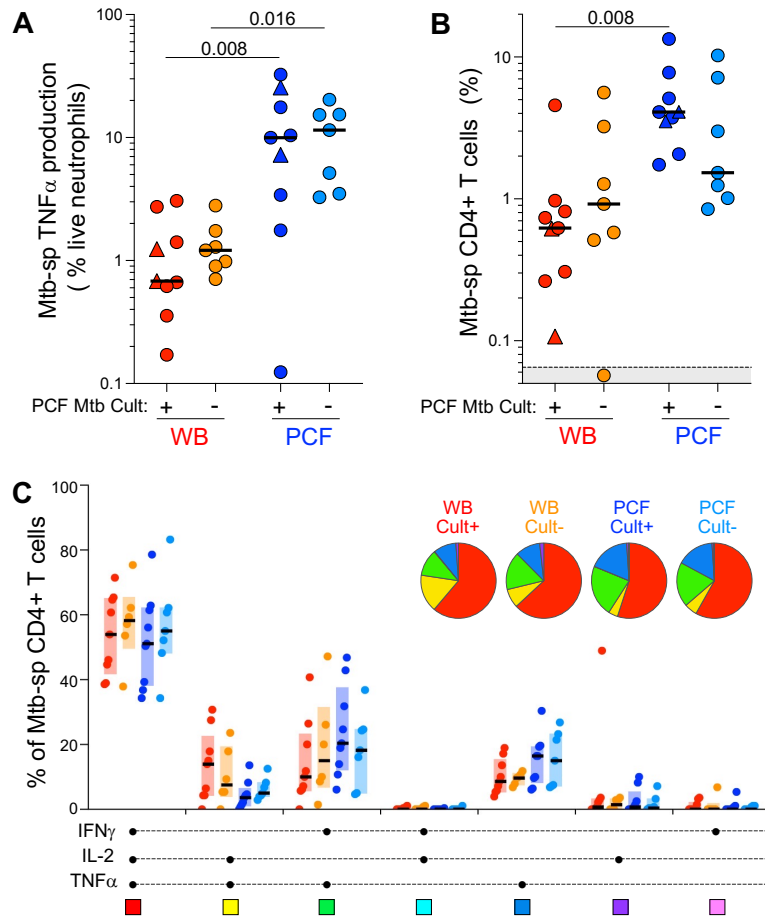
Supp Table 1: Clinical characteristics in each studied patients (n=16).

PCF: Pericardial Fluid, M: Male, F: Female, TTP: time to positivity, ADA: adenosine deaminase, LDH: lactate dehydrogenase. CRP: C-reactive protein, VL: viral load. Nd: not done, na: not applicable.

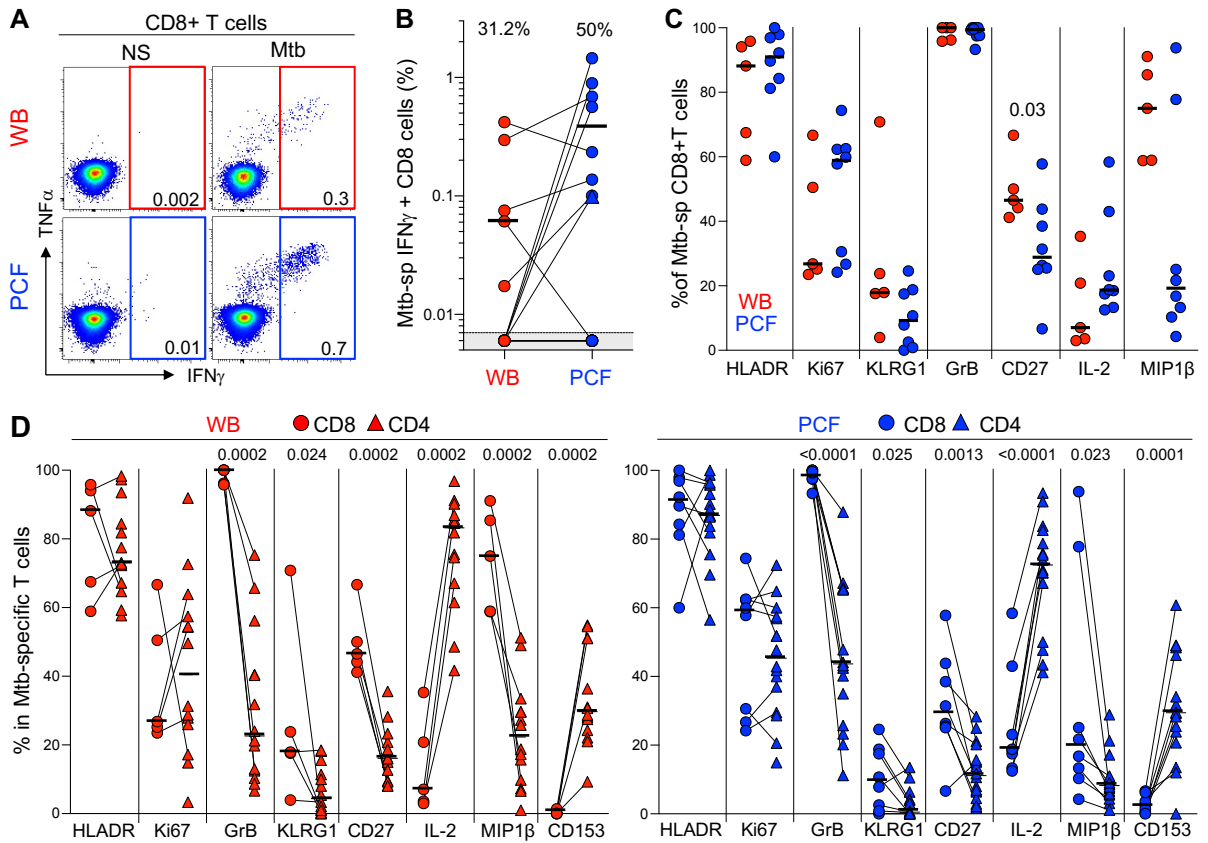
	Positive Mtb cult in PCF	Negative Mtb cult in PCF	<i>P</i> values
N	9	7	
Age*	33 [24-45]	35 [32-40]	0.84
Gender (M/F)	4/5	5/2	na
HIV positive (%)	77.8% (7/9)	100% (7/7)	na
Vol PCF aspirated (ml)*	1040 [455-1275]	835 [460-1140]	0.71
Time to culture positivity (days)*	22 [13-26]	na	na
ADA (U/l) in PCF*	55 [40-68]	46 [31-99]	0.60
LDH (IU/ml) in PCF*	301 [202-525]	324 [226-432]	0.46
Protein (g/l) in PCF*	65 [61-66]	64 [55-69]	0.59
Mononuclear cells (%) in PCF*	81.7 [48-89]	82.3 [71-88]	0.74
TBH DI score*	10 [8.5-10]	10 [8.5-10]	0.91
Plasma CRP (µg/ml)*	103 [83-197]	177 [104-211]	0.41
CD4 count (cells/mm ³) in blood*	140 [94-170]	171 [30-199]	0.80
Plasma VL (mRNA copies/ml)*	15,223 [569-90,452]	109,051 [2,151-287,630]	0.21

Supp Table 2: Comparison of the clinical characteristics between patients with a PCF positive Mtb culture (n=9) and patients with a PCF negative Mtb culture (n=7).

*: Medians and Interquartile ranges. M: Male, F: Female, PCF: Pericardial Fluid, ADA: adenosine deaminase, LDH: lactate dehydrogenase, TBH DI score: Tygerberg Diagnostic Index Score is a weighted score incorporating clinical symptoms and laboratory values in the diagnosis of TB pericarditis (sensitivity 86%, specificity 84%, ref: 14). CRP: C-reactive protein, VL: viral load. Statistical comparisons were performed using the Mann-Whitney test.



Supp Figure 1: Comparison of the frequency and polyfunctional profile of immune responses in blood and PCF, stratified based on PCF Mtb culture status. (A) Frequency of TNF α + Neutrophils in response to Mtb stimulation in whole blood and PCF from PCF Mtb culture negative (n = 7) and PCF Mtb culture positive (n = 9) patients. (B) Frequency of Mtb-specific CD4+ T cells in whole blood and PCF from patients who are PCF Mtb culture negative or PCF Mtb culture positive. (C) Polyfunctional profile of Mtb-specific CD4+ T cells. Statistical comparisons were performed using a Wilcoxon rank test for paired samples or a Mann-Whitney test for unpaired samples.



Supp Figure 2: Frequency and phenotype of Mtb-specific CD8+ T cell response in Blood and PCF. (A) Representative example of flow cytometry plots showing the expression of TNF α and IFN γ in CD8+ T cells from blood (top) and PCF (bottom) in one PCTB patient. (B) Comparison of the frequency of Mtb-specific CD8+ T cells in whole blood and PCF (n = 16). The proportion of participant exhibiting a detectable Mtb-specific CD8+ T cell response is indicated at the top of the graph. Bars represent medians of responders. (C) Comparison of the phenotypic profile of Mtb-specific CD8+ T cells in whole blood (n = 5) and PCF (n = 8). (D) Comparison of the phenotypic profile of Mtb-specific CD4+ and CD8+ T cells in whole blood (left panel and PCF (right panel). Bars represent medians. Statistical comparisons, performed using a Mann-Whitney test, are reported at the top of the graph.