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1 Identification of Immunological Biomarkers Which May Differentiate Latent TB from
2 Exposure to Environmental Nontuberculous Mycobacteria in Children

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5 Yun-Gyoung Hur,^{a##} Amelia C Crampin,^{b,c} Christina Chisambo,^c James Kanyika,^c Rein
6 Houben,^{b,c} Richard Ndhlovu,^c Themba Mzembe,^c Maeve K Lalor,^{a*} Jacky Saul,^b Keith
7 Branson,^b Carolynne Stanley,^a Bagrey Ngwira,^c Neil French,^{c,d} Tom H Ottenhoff,^c Hazel M
8 Dockrell,^a Patricia Gorak-Stolinska^a

9

10 Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases,
11 London School of Hygiene & Tropical Medicine, London, UK^a; Department of Infectious
12 Disease Epidemiology, Faculty of Epidemiology & Population Health, London School of
13 Hygiene & Tropical Medicine, London, UK^b; Karonga Prevention Study, Chilumba, Karonga
14 District, Malawi^c; Institute of Infection and Global Health, University of Liverpool,
15 Liverpool, UK^d; Department of Infectious Diseases, Leiden University Medical Center,
16 Leiden, The Netherlands^c

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19 Running Head: Biomarkers for LTBI in Regions Endemic for TB and NTM

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22 #Address correspondence to Yun-Gyoung Hur, hur1225@gmail.com.

23 *Present address: Yun-Gyoung Hur, Yonsei University, Seoul, South Korea; Maeve K Lalor,

24 Public Health England, London, UK

25 **ABSTRACT**

26 A positive IFN- γ response to *M. tuberculosis* ESAT-6/CFP-10 has been taken to indicate
27 latent TB infection but it may also be due to exposure to environmental nontuberculous
28 mycobacteria in which ESAT-6 homologues are present. We assessed the immune responses
29 to *M. tuberculosis* ESAT-6 and cross-reactive responses to ESAT-6 homologues of *M. avium*
30 and *M. kansasii*. Archived culture supernatant samples of children at 3 years post BCG
31 vaccination were tested for cytokine/chemokine responses to *M. tuberculosis* antigens.
32 Furthermore, the IFN- γ responses to *M. tuberculosis* antigens were followed up in 40 children
33 at 8 years post BCG vaccination and 15 TB patients were recruited as a control group for the
34 *M. tuberculosis* ESAT-6 response in Malawi. IFN- γ ELISAs on supernatants from diluted
35 whole blood assays, IFN- γ ELISpot assays, QuantiFERON TB Gold-In Tube tests and
36 multiplex bead assays were performed. More than 45% of responders to *M. tuberculosis*
37 ESAT-6 showed IFN- γ responses to *M. avium* and *M. kansasii* ESAT-6. In response to *M.*
38 *tuberculosis* ESAT-6/CFP-10, IL-5, IL-9, IL-13 and IL-17 differentiated the stronger IFN- γ
39 responders to *M. tuberculosis* ESAT-6 from those who preferentially responded to *M.*
40 *kansasii* and *M. avium* ESAT-6. A cytokine/chemokine signature of IL-5, IL-9, IL-13 and IL-
41 17 was identified as a putative immunological biosignature to differentiate latent TB infection
42 from exposure to *M. avium* and *M. kansasii* in Malawian children, indicating this signature
43 might be particularly informative in areas where both TB and exposure to environmental
44 nontuberculous mycobacteria are endemic.

45 INTRODUCTION

46 Around half a million children worldwide aged from 0 to 14 years became ill with
47 tuberculosis (TB) in 2011, resulting in approximately 64,000 deaths (1). Furthermore, 10
48 million children became orphans due to parental deaths from TB in 2009 (1). Despite the fact
49 children are at higher risk of developing TB disease once infected and more susceptible to
50 death, paediatric TB often goes undiagnosed in children aged from birth to 15 years old (2).
51 This is because access to health services and diagnostics are often severely limited, clinical
52 signs and symptoms of TB in children are non-specific and current diagnostic tests lack
53 sensitivity (2). This highlights the need to develop a more accurate test for TB infection than
54 the tuberculin skin test which lacks specificity and sensitivity due to cross reactivity induced
55 by BCG vaccination or exposure to environmental nontuberculous mycobacteria (NTM) (3-
56 4). An IFN- γ release assay based on detection of the specific IFN- γ release from Ag-specific
57 activated T cells that are incubated *ex vivo* with peptides from *Mycobacterium tuberculosis*
58 (*M. tb*) antigens such as early secretory antigenic target-6 (ESAT-6), and antigen TB7.7 has
59 been considered as these putative *M. tb* specific antigens are genetically deleted from all *M.*
60 *bovis* BCG strains (5). However, ESAT-6 homologues or ESAT-6 like proteins are present in
61 *M. leprae* as well as some environmental NTM which exist in water and soil such as *M.*
62 *kansasii*, *M. marinum*, *M. szulgai*, and *M. avium* (5-9). Thus it was suggested that an IFN- γ
63 response to ESAT-6 and 10 kDa culture filtrate protein (CFP-10) on its own was not
64 sufficient to detect *M. tb* infection in the areas where both *M. tb* and environmental NTM or
65 other pathogenic mycobacteria are endemic (10). To develop a more specific
66 immunodiagnostic test for detection of latent TB infection, studies were designed to identify
67 additional biomarkers and alternative tests to differentiate the host immune responses to *M. tb*
68 ESAT-6 and CFP-10 proteins from those against their homologues in environmental NTM,
69 particularly in regions where both TB and environmental NTM are endemic.

70 Since there is a high frequency of TB in the households of index TB cases in Malawi (11),
71 children are vulnerable and are at high risk of becoming infected by adults with TB. IFN- γ
72 responses to mycobacterial antigens have been extensively studied in cohort studies in
73 Malawian infants at the Karonga Prevention Study (KPS) in Chilumba, Malawi (12-13). The
74 immune responses of the infants were followed up at 3, 12 months and 3 years post BCG
75 vaccination between 2002 and 2006, and 13.6% (13/98) of infants tested at 3 years post
76 vaccination responded to *M. tb* ESAT-6/CFP-10 (14). Such a result might suggest that the 13
77 infants who showed positive IFN- γ responses to *M. tb* ESAT-6/CFP-10 were infected with *M.*
78 *tb*. However, none showed symptoms of clinical disease, and an alternative explanation might
79 be that the response shown is due to cross reactivity with ESAT-6 homologues from other
80 NTM which are endemic in the area, as *M. leprae* infection is now uncommon in Malawi.
81 The major slow growing NTM found in the sputa of TB patients in Northern Malawi have
82 been identified as being species from the *M. avium-intracellulare complex* as most common
83 and *M. gordonae*, *M. terrae*, *M. kansasii*, *M. malmoense* were also isolated (15).
84 In this study, we hypothesized that the positive IFN- γ responses to *M. tb* ESAT-6/CFP-10
85 observed in these 13 children at 3 years post BCG vaccination might not reliably indicate *M.*
86 *tb* infection but could be derived from cross reactive responses to ESAT-6 homologues of
87 environmental NTM, and that cytokine/chemokine signatures may distinguish between the
88 subjects who showed stronger IFN- γ responses to *M. tb* ESAT-6 and those who responded
89 more strongly to ESAT-6 derived from NTM. To test these two hypotheses, we chose *M.*
90 *avium subspecies avium* and *M. kansasii* which have ESAT-6 homologues, among the species
91 frequently found in the sputa of TB patients in Northern Malawi. We followed up the
92 immune responses of the children at 8 years post BCG vaccination in Malawi and assessed
93 the cross reactive responses between *M. tb* ESAT-6 and ESAT-6 homologues of *M. avium*
94 and *M. kansasii*, to identify how these responses related to the positive IFN- γ responses to *M.*

95 *tb* ESAT-6/CFP-10 at 3 years post BCG vaccination. In addition, we analysed
96 cytokine/chemokine signatures in response to *M. tb* ESAT-6/CFP-10 and *M. tb* purified
97 protein derivative (PPD) to identify potential biomarkers which can discriminate *M. tb*
98 infection from the cross reactive response to ESAT-6 homologues of *M. avium* and *M.*
99 *kansasii*.

100 **MATERIALS AND METHODS**

101

102 *Ethical permissions*

103 Authorisation of the exportation of archives in Malawi was granted by the National Health
104 Sciences Research Committee (NHSRC). Ethical permission for the previous studies to look
105 at immune response in infants at 3, 12 months and 3 years post vaccination was granted by
106 NHSRC (01/38) and the London School of Hygiene and Tropical Medicine (LSHTM) ethics
107 committee (745A) in 2001. Ethical permission for a follow-up study to determine the immune
108 responses of 40 children at 8 years post BCG vaccination and 15 TB patients was granted by
109 the LSHTM ethics committee (5929) and NHSRC in Malawi (866) in 2011.

110 Consent forms and information sheets, including translation into local languages were
111 prepared for the parents/guardians of children from the previous cohort study group and TB
112 patients. Appropriate informed written consent was obtained from adult TB patients and from
113 the parents or guardians of the children recruited into the study. All of the study participants
114 had the study explained to them and were given the opportunity to ask questions.
115 Confidentiality was ensured by using unique study numbers and blood sample numbers on
116 samples and questionnaires. Forms of the ethics application, research proposal, consent forms
117 and information sheets were reviewed by ethics committees in LSHTM and NHSRC in
118 Malawi.

119

120 *Selection of the archived samples at 3 years post BCG vaccination*

121 Previously collected culture supernatant samples obtained from Malawian infants at 3 years
122 post BCG vaccination, who participated in a vaccination cohort study, were retrieved from
123 the archive at the laboratories of the KPS (14). Based on previous results that 13 of 98 infants
124 tested had positive IFN- γ responses to *M. tb* ESAT6/CFP10 fusion protein in the whole blood

125 assay (WBA) at 3 years post vaccination, archived culture supernatants from 13 IFN- γ
126 responders to *M. tb* ESAT-6/CFP-10 and 11 non-responders were retrieved and transported to
127 the LSHTM laboratory to determine cytokine/chemokine profiles. Samples which had been
128 stimulated with *M. tb* PPD [batch RT49 Lot 204; Statens Serum Institut (SSI), Copenhagen,
129 Denmark], *M. tb* ESAT6/CFP10 [Bill and Melinda Gates Foundation Grand Challenge 6
130 (BMGF GC6) project; batch 040101] (16-17), Phytohaemagglutinin (PHA)-M (Sigma-
131 Aldrich, Poole, UK) and culture medium (RPMI1640; Sigma-Aldrich) were analysed further
132 from each of the selected study participants. To test the sample quality following extended
133 storage since 2006, 4 additional archived samples which were stimulated with 19 different
134 antigens: PHA-P, *M. tb* PPD, *M. avium* PPD, *M. bovis* BCG (SSI), Tetanus toxoid, Antigen
135 85A, Soluble egg antigen, Streptokinase streptodornase antigen, ESAT-6, TB10 (Rv0288),
136 PHA, and Dormancy survival regulator (DosR) regulon encoded antigens such as *M. tb*
137 Rv0081, Rv1737C, Rv1812C, Rv2006, Rv2625C, Rv3132C, Rv3133C, Rv0574C (BMGF
138 GC6 project) in addition to RPMI medium were also retrieved and transported to the LSHTM
139 laboratory for IFN- γ ELISA.

140

141 *Recruitment of children at 8 years post BCG vaccination and TB patients*

142 It was confirmed that 11 of the 13 subjects who previously showed positive IFN- γ responses
143 to *M. tb* ESAT-6/CFP-10 were traceable under the demography study at KPS in Malawi (55%
144 of males and 45% of females) and they were recruited for the new follow-up study at 8 years
145 post vaccination (14). As a control group, 11 non-responders at 3 years post vaccination who
146 were also confirmed to be traceable were recruited. In addition because of the high possibility
147 of individuals converting from non-responders to responders during the 5 years since the last
148 follow up visit, 18 additional ESAT-6/CFP-10 non-responders at 3 years post BCG
149 vaccination were randomly selected and recruited (59% of males and 41% of females). All of

150 the recruited responders and non-responders had BCG vaccination within one week after
151 birth. To act as a positive control population for ESAT-6 responses, 15 TB patients were
152 recruited from Karonga District Hospital and Chilumba Rural Hospital. As laboratory
153 confirmed cases of TB in children are rare in Karonga District we recruited adult TB patients
154 aged between 18 and 50 years (40% of males and 60% of females), at diagnosis or within the
155 first three months of treatment. TB patients were confirmed by smear/culture of sputa and
156 they were not eligible if HIV positive, taking immunosuppressant medication, suffering from
157 cancer or diabetes, pregnant, a prisoner or unable to give consent (Fig. 1).

158

159 *IFN- γ ELISA to test archive sample quality*

160 The production of IFN- γ was retested in archived samples from 4 different individuals to
161 determine if the archived samples had retained their integrity following an extended storage
162 period. The IFN- γ ELISA protocol for this test followed the previous protocol (12, 14) using
163 a standard sigmoid curve fit.

164

165 *Blood collection and PBMC isolation*

166 A total of 10 mL of blood was collected in a heparinised tube (170 international units of
167 Sodium Heparin, BD Vacutainer, Plymouth, UK). Four hundred and fifty μ L was used for a
168 diluted WBA and the remaining blood was used for PBMC isolation. PBMCs were prepared
169 by density gradient centrifugation using Ficoll (Sigma-Aldrich) (18). The cells were diluted
170 to 2.5×10^5 cells/180 μ L in AIM-V growth medium (Fisher Scientific) and 180 μ L of
171 resuspended cells were added into ELISpot plate wells which contained 20 μ L of each
172 peptide antigen or controls.

173

174 *Diluted whole blood assay and measurement of IFN- γ*

175 Blood was diluted in RPMI supplemented with 1% L-glutamine (Invitrogen, Paisley, UK; 1
176 in 5), and 100 μ L was added into each well with 100 μ L of each *M. tb* PPD (RT50, Lot219;
177 SSI) at a final concentration of 5 μ g/mL, ESAT-6/CFP-10 (BMGF GC6-74 project; batch
178 040101) at a final concentration of 10 μ g/mL (16-17), PHA (Lot017k4029; Sigma-Aldrich) at
179 a final concentration of 5 μ g/mL and RPMI 1640 supplemented with 1% L-glutamine
180 (Sigma-Aldrich). After a 6-day incubation at 37°C, the culture supernatant was harvested and
181 the production of IFN- γ was measured in 50 μ l of culture supernatant by ELISA (19). A
182 “positive” response in an IFN- γ ELISA was defined as being > 62.5 pg/mL which is twice the
183 limit of detection of the assay (19). The concentrations of IFN- γ above 4000 pg/mL were set
184 to be 6000 pg/mL.

185

186 *ESAT-6 overlapping peptides derived from M. tb, M. avium and M. kansasii*

187 *M. avium* and *M. kansasii* which contain homologous ESAT-6 sequences were selected to
188 examine the cross-reactivity between *M. tb* ESAT-6 and ESAT-6 homologues of NTM in
189 Malawian children and TB patients. The protein sequences between *M. tb* ESAT-6 and
190 ESAT-6 homologues of *M. avium* and *M. kansasii* are more than 90% identical to *M. kansasii*
191 but only 27% identical to *M. avium* (20-23). The positions of predominantly recognized
192 epitopes are scattered throughout the ESAT-6 protein sequence and the multiple T cell
193 epitopes recognised are different depending on the population (24-26). Based on published
194 papers and the SYFPEITHI program used to predict epitope sites (27), 14 overlapping
195 peptides including 15-mers with predicted epitopes for MHC type II binding were designed
196 using the full length ESAT-6 amino acid sequence from *M. tb*, and *M. avium subspecies*
197 *avium* (Fig. S1). The ESAT-6 amino acid sequences are identical from amino acid 1 to 62
198 between *M. tb* and *M. kansasii* and only two overlapping peptides including different
199 sequences were synthesized for ESAT-6 homologues of *M. kansasii*. The overlapping

200 peptides were put together into 5 peptide pools i.e. *M. tb*₁₋₅₇, *M. tb*₅₅₋₉₅, *M. avium*₂₋₅₉, *M.*
201 *avium*₅₇₋₉₇, *M. kansasii*₅₅₋₉₅ (Fig. S1).

202

203 *IFN-γ* ELISpot assay

204 The IFN- γ ELISpot assay was carried out as previously described (18). The final
205 concentration of *M. tb*, *M. avium* and *M. kansasii* peptides was 10 $\mu\text{g}/\text{mL}$ each and the
206 concentration of *M. tb* PPD was 5 $\mu\text{g}/\text{mL}$. Anti-human CD3 mAb was used at 0.1 $\mu\text{g}/\text{mL}$
207 (Mabtech, Nacka Strand, Sweden). Spots were counted using an ELISpot reader (AID GmbH
208 4.0; San Diego, CA). The positive responses to each antigen were measured by an empirical
209 rule (ER) 1 which defines a positive response as at least twofold increase of spot number in
210 the experimental wells over the background with a minimum threshold of 5 spots per 100,000
211 PBMCs in the experimental wells (28). A *M. tb* ESAT-6 specific positive response was
212 defined if spots appeared in *M. tb* ESAT-6/CFP-10 and either *M. tb* ESAT-6₁₋₅₇ or *M. tb*
213 ESAT-6₅₅₋₉₅ stimulation alongside the positive response to anti-CD3 antibody.

214

215 *Quantiferon TB Gold-In Tube Test*

216 The QFT-IT test is a commercially available diagnostic assay to measure cell-mediated
217 immune response to *M. tb* specific antigens using ESAT-6, CFP-10, and TB7.7 (4). For the
218 test, 1 mL of blood was collected directly into each of two QFN TB Gold tubes (Nil and *M.*
219 *tb* Ag tube; ESAT-6, CFP-10 and TB7.7 peptide Ags, Cellestis, Valencia, CA). The tubes
220 containing blood were incubated upright at 37°C for 24 hours and plasma was harvested for
221 IFN- γ ELISA. Plasma samples were stored at -80°C until the recruitment of all study subjects
222 was complete. The plasma samples were assayed using an IFN- γ ELISA according to the
223 manufacturer's protocol (QuantiFERON-TB Gold, Cellestis). The data was analysed using

224 the QuantiFERON-TB Gold IT Analysis Software (Cellestis) and the results were expressed
225 as positive, negative and intermediate responses.

226

227 *19plex and 42plex bead assays*

228 Culture supernatants from cells stimulated with *M. tb* ESAT6/CFP10, *M. tb* PPD, PHA-M,
229 RPMI1640 were selected from the archive at 3 years post BCG vaccination for a multiplex
230 bead assay with 19 different cytokines and chemokines: IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-9,
231 IL-10, IL-12p70, IL-13, IL-15, IL-17, IFN- γ , TNF- α , IFN- α , GM-CSF, MIP-1 α (CCL3), IP-
232 10 (CXCL10), MDC (CCL22), and MCP-3 (CCL7). The beads in the 42plex kit for the
233 samples from newly recruited subjects are a combination of a premixed 39 bead mix
234 including IL-1 α , IL-1ra, IL-1 β , IL-2, sIL-2R α , IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-
235 12p70, IL-13, IL-15, IL-17, IFN- α 2, IFN- γ , TNF- α , TNF- β , sCD40L, MIP-1 α (CCL3), MIP-
236 1 β (CCL4), Gro- α (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MCP-1 (CCL2), MCP-3
237 (CCL7), MDC (CCL22), TGF- α , G-CSF, GM-CSF, IL-3, IL-7, Eotaxin, FGF-2, Flt-3L,
238 Fractalkine (CX3CL1), EGF, VEGF plus the addition of 3 chemokines; PDGF-AA, PDGF-
239 AB/BB and RANTES (CCL5) which were added as 70 μ L each from 3 individual vials after
240 sonicating and vortexing of the beads. Beads were diluted 1 in 2 and the detailed protocol of
241 the 42plex bead assay followed the manufacturer's protocol (no. MPXHICYTO60KPMX42;
242 MILLIPLEX[®]_{MAP} Kit, Millipore, Billerica, MA, USA) as described in a previous study (13).
243 The range of standard curve was from 3.2 to 10000 pg/mL and the values below 3.2 pg/mL
244 were set to 1.6. Considering cost restraints, samples showing the values above the 10000
245 pg/mL could not be retested with dilution but the values were set to 15000 pg/mL (13).

246

247 *Statistical analysis*

248 The Wilcoxon signed rank test and Spearman's rank correlation test were used to compare
249 the IFN- γ concentrations measured in 2006 and 2010 in 4 different archived samples
250 including the culture supernatants stimulated with 19 antigens in each sample. The IFN- γ
251 concentration measured by IFN- γ ELISA was compared by Mann Whitney test between 40
252 children and 15 TB patients. Mann Whitney test was used to compare cytokine responses
253 measured by multiplex bead assay between 13 IFN- γ responders and 11 non-responders to *M.*
254 *tb* ESAT-6 as well. Agreement of the results obtained from different assays such as IFN- γ
255 ELISA, IFN- γ ELISpot assay, and QFT-IT test was assessed by kappa statistics.

256 **RESULTS**

257

258 *IFN- γ responses to M. tb ESAT-6/CFP-10, PPD and PHA*

259 IFN- γ responses to *M. tb* ESAT-6/CFP-10, *M. tb* PPD and PHA-M were investigated by IFN-
260 γ ELISA after a 6 day culture of diluted whole blood isolated from 40 children at 8 years post
261 BCG vaccination and 15 TB patients at diagnosis or on treatment for less than 3 months.
262 Among the 40 children recruited at 8 years post BCG vaccination, 3 children showed positive
263 IFN- γ responses to *M. tb* ESAT-6/CFP-10 (> 62.5 pg/mL) while 36 of 40 children showed
264 positive IFN- γ responses to *M. tb* PPD (> 62.5 pg/mL). The median response of IFN- γ was
265 much higher in response to *M. tb* PPD (853 pg/mL) compared with *M. tb* ESAT-6/CFP-10
266 (15 pg/mL), and all of the children recruited responded to PHA (Fig. 2A). Twelve out of the
267 15 TB patients responded to *M. tb* ESAT-6/CFP-10 with a median concentration of 245
268 pg/mL while only 3 of 40 children at 8 years post BCG vaccination responded with a median
269 response of 15 pg/mL of all (Fig. 2A). One of the 3 IFN- γ non-responding TB patients ($<$
270 62.5 pg/mL) was a patient at diagnosis and two were patients on treatment. The IFN- γ
271 responses to *M. tb* PPD were positive in all of the TB patients recruited and the median IFN- γ
272 response was 2691 pg/mL with IFN- γ ranging from 324 to 4000 pg/mL. The median IFN- γ
273 responses to both *M. tb* ESAT-6/CFP-10 and *M. tb* PPD were significantly higher in TB
274 patients compared with children ($P < 0.001$, $P < 0.01$ respectively) while there was no
275 difference in median IFN- γ responses to PHA ($P = 0.15$) (Fig. 2A). Interestingly, only two
276 subjects (103278, 103738) at 8 years post BCG vaccination had a very marked increase in
277 IFN- γ which was more than 10 times higher (4000 and 1958 pg/mL respectively) compared
278 with the IFN- γ responses to *M. tb* ESAT-6/CFP-10 in the same children at 3 years post BCG
279 vaccination (Fig. 2B) while most previous non-responders did not show positive IFN- γ

280 responses to *M. tb* ESAT-6/CFP-10 and one subject (104043) showed a weak positive IFN- γ
281 response (101 pg/mL) at 8 years post BCG vaccination (Fig. 2C).

282

283 *Cross-reactivity between M. tb ESAT-6 and its homologues*

284 To determine the level of cross-reactivity of IFN- γ responses between *M. tb* ESAT-6 and
285 ESAT-6 homologues of *M. avium subspecies avium* and *M. kansasii*, 5 overlapping peptide
286 antigen pools derived from *M. tb* ESAT-6 and ESAT-6 homologues of *M. avium* and *M.*
287 *kansasii* were synthesized (Fig. S1) and the quantification of T cells producing IFN- γ in
288 response to the ESAT-6 peptide antigens was measured *ex vivo* using PBMC from 40
289 children and 15 TB patients. Among the 40 children, only 5% (2/40; 103738 and 104043)
290 showed IFN- γ producing cells in response to *M. tb* ESAT-6/CFP-10 fusion protein and either
291 *M. tb* ₁₋₅₇ or *M. tb* ₅₅₋₉₅ ESAT-6 peptides (Table 1). Two more subjects (103604, 104041)
292 showed positive IFN- γ producing cells in response to *M. tb* ₅₅₋₉₅ ESAT-6 while these did not
293 show positive responses to *M. tb* ESAT-6/CFP-10 fusion protein and they were not counted
294 as a responder to *M. tb* ESAT-6 (data not shown). In all, 5 children responded to *M. avium*
295 ESAT-6 peptides and 3 children showed positive responses to *M. kansasii* ESAT-6 peptides
296 (Table 1). In response to PPD, IFN- γ producing cells were detected in 75% (30/40) of the
297 tested children. Compared with the proportion of positivity in children, TB patients showed
298 much higher IFN- γ positive responses to *M. tb* ESAT-6 in the ELISpot assay (Table 1) as
299 well as in the IFN- γ ELISA (Fig. 2). The level of cross reactivity of IFN- γ responses between
300 *M. tb* ESAT-6 and ESAT-6 homologues of *M. avium* and *M. kansasii* is shown in Table 2.
301 The two responders to *M. tb* ESAT-6/CFP-10 antigens and *M. tb* ESAT-6 peptides also
302 showed positive IFN- γ responses to both *M. avium* ₂₋₅₉ and *M. kansasii* ₅₅₋₉₅. In 11 TB patients
303 who showed positive IFN- γ producing cells to *M. tb* ESAT-6, more than 45% and 60% of the
304 responders showed cross-reactivity with *M. avium* ₂₋₅₉ (5/11 patients) and *M. kansasii* ₅₅₋₉₅

305 ESAT-6 (7/11 patients), respectively. In summary, more than half of the responders who
306 showed positive IFN- γ producing cells in response to *M. tb* ESAT-6 had cross reactive IFN- γ
307 responses to *M. avium* 2-59 and *M. kansasii* 55-95 ESAT-6 peptides with higher cross reactivity
308 with *M. kansasii* ESAT-6.

309

310 *IFN- γ responses by QFT-IT test*

311 Forty children at 8 years post BCG vaccination and 15 TB patients were tested by QFT-IT
312 IFN- γ ELISA. Positive IFN- γ responses were detected in 4 of 40 (10%) children. Among the
313 4 positive responders in the children, only one subject (103738) matched with the results of
314 IFN- γ ELISA after WBA and IFN- γ ELISpot assay while the other 3 positive responders by
315 QFT-IT test were not found to be positive by IFN- γ ELISA and ELISpot assay. On the other
316 hand, greater agreement of results of the three tests was found in the TB patient group.
317 Thirteen of 15 TB patients showed positive IFN- γ responses by QFT-IT test, 12 and 11
318 patients showed positive responses to *M. tb* ESAT-6/CFP-10 by IFN- γ ELISA and ELISpot
319 assay respectively. Among the 13 QFT-IT responders, all of the 5 TB patients at diagnosis
320 were positive by QFT test and 8 of 10 patients who were on treatment showed positive IFN- γ
321 responses.

322

323 *Agreement between the results from different assays*

324 In this study, 3 different methodologies, IFN- γ ELISA, IFN- γ ELISpot assay and QFT-IT test
325 were used to measure IFN- γ responses in 40 children at 8 years post BCG vaccination and in
326 15 TB patients. The outcome of the different assays was discordant and kappa statistics was
327 applied to quantify this. The concordance of ELISA and ELISpot assay was highest with 89%
328 agreement (Kappa 0.7130, $P < 0.01$). The agreement between ELISpot assay and QFT-IT was
329 86% (Kappa 0.6358, $P < 0.01$), and 82% between IFN- γ ELISA and QFT-IT (Kappa 0.5600,

330 P<0.01). In the 40 children only, the concordance between the IFN- γ ELISA and the ELISpot
331 assay was also high (Kappa 0.7872, P<0.01) while the agreement between QFT-IT and both
332 IFN- γ ELISA and ELISpot assay was low (Kappa 0.2188, P>0.05 and 0.2857, P<0.05,
333 respectively).

334

335 *Cytokine/chemokine signatures in children at 3 years post BCG vaccination*

336 In order to examine if the cytokine proteins in the archived samples still remained intact,
337 IFN- γ ELISA was performed and the level of IFN- γ production was compared with previous
338 data obtained in 2006. The levels of IFN- γ measured from the archived samples collected
339 from 4 infants at 3 years post BCG vaccination were similar to the previous data from 2006
340 and slightly higher in some of the supernatant aliquots than the IFN- γ detection in the past
341 (Fig. S2A). However, no significant difference was found in IFN- γ concentrations of 19
342 culture supernatant samples from each of 4 subjects (indicated by lab number) when
343 measured in 2006 and again in 2010 [38289 (P=0.49), 38290 (P=0.14), 38291 (P=0.36) and
344 38633 (P=0.50) by Wilcoxon signed rank test; Fig. S2A]. In addition, the Spearman
345 correlation coefficient calculated using the IFN- γ data obtained from the total 76 samples was
346 0.9808 (P<0.0001), indicating a strong correlation between IFN- γ values obtained in 2006
347 and 2010 (Fig. S2B). Based on the IFN- γ production in response to *M. tb* ESAT-6/CFP-10,
348 17 cytokines and chemokines were analysed between IFN- γ responders and non-responders.
349 IL-4 and IL-15 were excluded from this analysis as they were produced at levels below the
350 limit of detection of the assay. *M. tb* ESAT-6/CFP-10 stimulation was able to differentiate 13
351 IFN- γ responders from 11 non-responders using 5 cytokines and chemokines; IL-1 α , IL-10,
352 MIP-1 α , IP-10 and GM-CSF with median responses showing a difference of more than 5 fold
353 in the two groups. Furthermore, IL-5, IL-9, IL-13 and IL-17 were not produced in most of

354 those tested, irrespective of whether they were IFN- γ responders or non-responders (23/24;
355 Fig. 3). In response to PHA, the median concentration of most cytokine and chemokine
356 responses measured was high apart from IL-2 and IL-4 which were below levels of detection
357 (data not shown).

358

359 *Comparison of cytokine/chemokine signatures between 3 and 8 years post BCG vaccination*

360 To examine how the immune responses had changed over the 5 years since the vaccinees had
361 been studied and to determine if the cytokine responses other than IFN- γ may differentiate
362 between two strong positive IFN- γ responders to *M. tb* ESAT-6/CFP-10 (103278, 103738)
363 and non-responders at 8 years post BCG vaccination, the cytokine and chemokine responses
364 at 3 and 8 years post BCG vaccination were compared in 11 of the previous 13 responders
365 who showed positive IFN- γ responses to *M. tb* ESAT-6/CFP-10 at 3 years post BCG
366 vaccination. From the 11 subjects, only two subjects (103278, 103738) showed strong
367 positive IFN- γ responses (Fig. 2B), at 8 years post BCG vaccination (marked in red; Fig. 4).

368 The production of IL-12p70, IL-1 α , IL-10, IP-10, MDC and GMCSF in response to *M. tb*
369 ESAT-6/CFP-10 was higher in the two IFN- γ responders compared with the other subjects
370 (Fig. 4). IL-17 and the Th2 type cytokines IL-5, IL-9 and IL-13 were not produced in
371 response to *M. tb* ESAT-6/CFP-10 at 3 years post BCG vaccination and 9 of the 11 previous
372 IFN- γ responders still showed low levels of those cytokines 5 years later (Fig. 4). However,
373 the two IFN- γ responders at 8 years post BCG vaccination showed an increase in IL-17, IL-5,
374 IL-9 and IL-13 in response to *M. tb* ESAT-6/CFP-10 (Fig. 4) and one of the two responders
375 (103738) also showed a large increase in IL-5, IL-9 and IL-13 in response to *M. tb* PPD since
376 3 years post-vaccination (data not shown). No remarkable differences in the cytokine and
377 chemokine responses to *M. tb* PPD and PHA were found between the two IFN- γ responders
378 and others at 8 years post BCG vaccination (data not shown).

379

380 *Cytokine/chemokine signatures between IFN- γ responders to *M. tb* ESAT-6 and those*
381 *responding to ESAT-6 homologues of *M. avium* and *M. kansasii**

382 Cytokine and chemokine signatures were compared between the subject who showed a higher
383 frequency of IFN- γ producing cells to *M. tb* ESAT-6 peptides (103738) and those who
384 responded more strongly to *M. avium* or *M. kansasii* ESAT-6 (104043, 104041) in the
385 ELISpot assay (Fig. 5A). In response to *M. tb* ESAT-6/CFP-10, 103738 who had stronger
386 IFN- γ responses to *M. tb* ESAT-6 peptides showed about 10-fold higher production of IFN- γ ,
387 sIL-2R α , IL-17, IL-5, IL-13 and sCD40L when compared with both an individual who
388 showed a positive responses to *M. avium*₅₇₋₉₇ and *M. kansasii*₅₅₋₉₅ (104041) and the subject
389 who showed strong response to *M. kansasii*₅₅₋₉₅ (104043) in the IFN- γ ELISpot assay (Fig.
390 5B, Fig. S3). However, sIL-2R α and sCD40L were also highly produced in some other
391 subjects who did not respond to *M. tb* ESAT-6/CFP-10 (data not shown). In the *M. tb* ESAT-
392 6 responder 103738, TNF- α , IL-9, IL-10, IL-12p70, MDC and GM-CSF were also highly
393 produced and IL-9 and IL-12p70 production was still higher in response to *M. tb* PPD while
394 the other cytokines which were exclusive to 103738 in response to *M. tb* ESAT-6/CFP-10 did
395 not show significant differences in response to *M. tb* PPD (data not shown). It was noticed
396 that the level of MCP-1 in the background without stimulation with *M. tb* antigens was very
397 high in two IFN- γ responders (103738, 103278) compared with others.

398 **DISCUSSION**

399 This study provides preliminary evidence that multiple cytokine/chemokine signatures may
400 identify potential biomarkers for better diagnosis of *M. tb* infection in children and supports
401 the observation that IFN- γ on its own is not sufficient to diagnose *M. tb* infection upon *M. tb*
402 ESAT-6/CFP-10 stimulation in this setting. At the 8 year follow up only two children showed
403 strong positive IFN- γ responses to *M. tb* ESAT-6/CFP-10 in the IFN- γ ELISA after a 6-day
404 WBA compared to the original number of 13 responders five years earlier. In the ELISpot
405 assay, more than 50% of IFN- γ responders to *M. tb* ESAT-6 showed positive IFN- γ
406 producing T cells to *M. avium* ESAT-6 or *M. kansasii* ESAT-6 as well, while the magnitudes
407 of IFN- γ responses to *M. tb* ESAT-6 were higher compared with those to ESAT-6
408 homologues of *M. avium* and *M. kansasii*. These data indicate that an IFN- γ response to *M. tb*
409 ESAT-6 alone cannot differentiate *M. tb* infection from infection with NTM in this setting as
410 shown in the report by Arend and colleagues (7). The analysis of multiple
411 cytokine/chemokine signatures demonstrated that the signatures of IL-17, IL-5, IL-9 and IL-
412 13 in response to *M. tb* ESAT-6/CFP-10 were exclusively restricted to the two strong *M. tb*
413 ESAT-6 IFN- γ responders while the IFN- γ non-responders and the one weak positive
414 responder did not produce these cytokines at 8 years post BCG vaccination. In addition, these
415 cytokines discriminated the IFN- γ responder to *M. tb* ESAT-6 from those who showed
416 stronger responses to ESAT-6 homologues of *M. avium* and *M. kansasii* although it was not
417 possible to determine the statistical significance of these findings due to the small sample
418 size. None of the 40 children recruited at 8 years post BCG vaccination, as well as none of
419 the 13 IFN- γ positive responders at 3 years post BCG vaccination had any clinical symptoms
420 suggestive of active TB disease such as coughing for more than 2 weeks, weight loss or
421 haemoptysis.

422

423 All of the 13 previous IFN- γ responders to *M. tb* ESAT-6/CFP-10 at 3 years post BCG
424 vaccination showed limited production of IL-17, IL-5, IL-9 and IL-13 while the cytokine
425 levels increased in the two IFN- γ responders to *M. tb* ESAT-6/CFP-10 at 8 years post BCG
426 vaccination. The IL-1 α , IFN- γ , IP-10, MIP-1 α and GM-CSF production, which were highly
427 detected in previous IFN- γ responders at 3 years post BCG vaccination, were also highly
428 produced in all of the IFN- γ responders at 8 years post BCG vaccination regardless of the
429 preferential IFN- γ responses to *M. tb* ESAT-6 or ESAT-6 homologues of *M. avium* and *M.*
430 *kansasii* in the ELISpot assay (Fig. 5). These data suggest that most of the positive IFN- γ
431 responses observed in children at 3 years post BCG vaccination may be cross reactive
432 responses with ESAT-6 homologues of environmental NTM. However, it is also possible that
433 the 11 non-responders who showed positive responses at 3 years post BCG vaccination might
434 be transiently infected with *M. tb* which has been cleared during the subsequent 5 years.

435

436 The previous and recent reports to show cytokine and chemokine production in latent and
437 active TB disease support the findings observed in this study i.e. higher production of IL-17,
438 IL-5, IL-9 and IL-13 upon *M. tb* antigen stimulation in positive IFN- γ responders (30-35).
439 The proportion of CD4⁺ T cells expressing IFN- γ , IL-17 and IL-22 were observed to be
440 significantly increased upon mycobacterial antigen stimulation in both latent and active TB
441 disease compared with healthy controls (30). Another report demonstrated that IL-17
442 production was significantly increased in household contacts while it was decreased in TB
443 cases in response to mycobacterial antigen stimulation (31), suggesting the protective role of
444 IL-17 in disease progression to active TB. In humans, IL-13 and IL-4 mRNA was
445 significantly expressed in TB patients compared with the controls (32) while the higher levels
446 of IL-13 and sCD40L were also observed in TB patients who quickly responded to anti-TB
447 therapy compared with slow responders (33). In contrast, it was also reported that production

448 of IL-4 and IL-5 is associated with progression to active disease (34). The enhanced
449 production of both IFN- γ and IL-13 in our study is consistent with the previous finding that
450 IL-13 and IFN- γ production in response to *M. tb* PPD and ESAT-6/CFP-10 in WBA was
451 significantly higher in tuberculin skin test positive individuals in a West Africa cohort (35).

452

453 The peptides of *M. kansasii* ESAT-6 used in this study were derived from the amino acid
454 positions 55-95, which includes two different amino acids compared with *M. tb* ESAT-6 (23).

455 The small difference in only two amino acids between *M. tb* and *M. kansasii* ESAT-6 may
456 not indicate that the peptides would act as an epitope which is specific to *M. kansasii* as we
457 showed a high percentage of cross-reactivity between the *M. tb* ESAT-6 and *M. kansasii*
458 ESAT-6 peptides. However, changing a single residue in a 20-mer amino acid peptide can
459 result in a lack of MHC binding and may lead to a loss of recognition by T cells that were
460 specific for the wild type peptide (36). In cattle *M. bovis* ESAT-6 (which is identical to *M. tb*
461 ESAT-6) and *M. kansasii* ESAT-6 were differentially recognised by bovine T cells
462 depending on their MHC types (8).

463

464 The IFN- γ ELISA after a 6-day WBA, IFN- γ ELISpot assay and QFT-IT test showed low
465 discordance measured by Kappa statistic ($0.56 \leq \kappa \leq 0.71$, $P < 0.01$). Any discordance among the
466 tests is derived from the fact that different parameters are measured in each assay. The IFN- γ
467 ELISA and multiplex bead assay measured magnitude of IFN- γ production following a 6-day
468 culture of whole blood with *M. tb* ESAT-6/CFP-10 while the ELISpot and QFT-IT assays
469 measured overnight responses. The ELISpot measures frequency of IFN- γ producing cells
470 and QFT-IT measures secreted cytokine; effector T cell function is measured in ELISpot and
471 QFT-IT assays while WBA measures the memory recall responses. Compared with an
472 ELISpot assay which uses a fixed number of isolated PBMCs, a QFT-IT assay uses a whole

473 blood sample and may have higher variability in the result depending on the lymphocyte
474 count.

475

476 The current study was derived from a cohort study with a larger, adequately powered group
477 of infants recruited in 2002, which examined expression of genetic markers and immune
478 responses in 590 infants at 3 months and 552 infants at 12 months post BCG vaccination. A
479 group of 113 children at the 3 year follow-up time point was recruited to look at the
480 maintenance of the immune response between 3 months and 3 years post BCG vaccination
481 and the study group was adequately powered for that purpose. However, based on the
482 proportion of positive IFN- γ responders to *M. tb* ESAT-6/CFP-10 at 3 years (n=13 among 98
483 tested) and 8 years (n=3 among 40 tested including the initial 11 responders) post BCG
484 vaccination, a much larger sample size than the initial study with 590 children would be
485 needed in this setting to validate these findings. Alternatively these potential biomarkers
486 could be validated in another setting with a higher incidence of LTBI in children than is
487 present in Karonga, Malawi.

488

489 There have been many studies to address T cell responses to *M. tb* region of difference 1
490 encoded antigens while no studies have been published regarding biomarkers to distinguish
491 *M. tb* infection from the exposure to environmental NTM which can affect the diagnosis of
492 TB or LTBI. The results from this study suggested putative biomarkers (IL-5, IL-9, IL-13,
493 IL-17) to distinguish between LTBI and exposure to *M. avium* and *M. kansasii* (Fig. 6).
494 These findings, although preliminary in nature due to the small number of subjects involved,
495 contribute knowledge to ongoing development of novel diagnostic tests with higher
496 specificity to predict *M. tb* infection in children. However, taking the small number of
497 potential LTBI cases into consideration, further studies using these candidate biomarkers

498 should be taken forward in a larger study population or cohorts with higher incidence of
499 childhood latent TB to validate the diagnostic value of the suggested cytokine signature.

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637 **FIGURE LEGENDS**

638

639 **Fig. 1. Collection of archived samples and recruitment of study subjects.** In a previous
640 study cohort, 13 of 98 children at 3 years post BCG vaccination showed positive IFN- γ
641 response to *M. tb* ESAT-6/CFP-10. In this study, 24 sets of archived culture supernatant
642 samples from the children at 3 years post BCG vaccination were collected for 19plex bead
643 assay. For the new follow-up study at 8 years post BCG vaccination, 55 subjects including 40
644 children at 8 years post BCG vaccination and 15 TB patients were recruited; the 40 healthy
645 children included 11 previous IFN- γ responders to ESAT-6/CFP-10 and 29 non-responders
646 from the previous study. Fifteen TB patients were recruited as a positive control for IFN- γ
647 response to *M. tb* ESAT-6/CFP-10 and included those at diagnosis or on treatment for less
648 than 3 months. The blood samples obtained from the 55 participants were used for IFN- γ
649 ELISA, IFN- γ ELISpot and 42plex bead assay.

650

651 **Fig. 2. IFN- γ responses to *M. tb* ESAT-6/CFP-10, *M. tb* PPD and PHA in 40 children at 8**
652 **years post BCG vaccination and 15 TB patients.** *A.* TB patients showed significantly
653 higher IFN- γ production in response to *M. tb* ESAT-6 ($P < 0.0001$) and *M. tb* PPD ($P = 0.013$)
654 compared with children at 8 years post BCG vaccination while all of the children and TB
655 patients had positive IFN- γ response to PHA-M ($P = 0.82$) in the IFN- γ ELISA. The median
656 levels of IFN- γ are indicated in red and the cut-off for positivity (> 62.5 pg/mL) is marked in
657 blue. Values above 4000 pg/mL were considered to be 6000 pg/mL. *B-C.* IFN- γ responses to
658 *M. tb* ESAT-6/CFP-10 were measured from 11 previous responders (B) and 29 previous non-
659 responders (C) at 3 years post BCG vaccination. The subjects who showed positive IFN- γ
660 responses (> 62.5 pg/mL) at a follow-up time point of 8 years post BCG vaccination are

◇

661 marked in red. Two of 11 previous responders had a marked increase in IFN- γ in response to
662 *M. tb* ESAT-6/CFP-10. In the previous non-responder group, IFN- γ was increased to 101
663 pg/mL in one subject.

664

665 **Fig. 3. Cytokine/chemokine responses in archived samples from children at 3 years post**
666 **BCG vaccination.** The levels of cytokines and chemokines in response to *M. tb* ESAT-
667 6/CFP-10 measured by multiplex bead assay were compared between positive IFN- γ
668 responders (marked as R; black circle) and non-responders (marked as NR; white circle); 6 of
669 19 different cytokines and chemokines were highly produced in IFN- γ responders compared
670 with non-responders with more than a 5 fold difference in median responses. The significance
671 of difference of immune responses (P values) between IFN- γ responders and non-responders
672 is marked on each graph. The median levels of each cytokine are indicated in red.

673

674 **Fig. 4. Cytokine/chemokine responses to *M. tb* ESAT-6/CFP-10 at 3 and 8 years post**
675 **BCG vaccination.** Among the 11 subjects who had positive IFN- γ responses to *M. tb* ESAT-
676 6/CFP-10 at 3 years post BCG vaccination, only two subjects showed positive IFN- γ
677 responses to *M. tb* ESAT-6/CFP-10 at 8 years post vaccination (marked in red; 103738 and
678 103278). Compared with 9 IFN- γ non-responders at 8 years post BCG vaccination, 2 IFN- γ
679 responders (marked in red) showed greater increase of cytokine and chemokine responses, in
680 particular, the production of IL-17 and Th2 cytokines such as IL-5, IL-9 and IL-13.

681

682 **Fig. 5. Comparison of cytokine and chemokine signatures between the subject who**
683 **responded to *M. tb* ESAT-6 and those who responded to ESAT-6 homologues of *M.***
684 ***avium* and *M. kansasii*.** A. The subject, 103738 had a higher number of SFC in response to
685 *M. tb* ESAT-6 peptides compared with the number of SFC in response to *M. avium* or *M.*

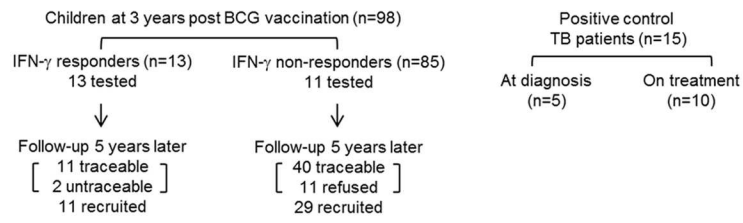
686 *kansasii* ESAT-6 peptides while 104043 showed a much higher number of SFC in response
687 to *M. kansasii* ESAT-6 compared with the number of SFC in response to *M. tb* ESAT-6.
688 Another subject, 104041 showed higher numbers of SFC in response to *M. avium* ⁵⁷⁻⁹⁷
689 compared to *M. tb* ESAT-6 and strong positive response to *M. kansasii* ESAT-6 peptide. *B.*
690 The analysis of 42 cytokine and chemokine signatures to *M. tb* ESAT-6/CFP-10 showed that
691 IL-17 and Th2 cytokines such as IL-5, IL-9 and IL-13 were produced in greater quantities in
692 the IFN- γ responder to *M. tb* ESAT-6 (103738) compared with the IFN- γ responders to *M.*
693 *avium* and *M. kansasii*.

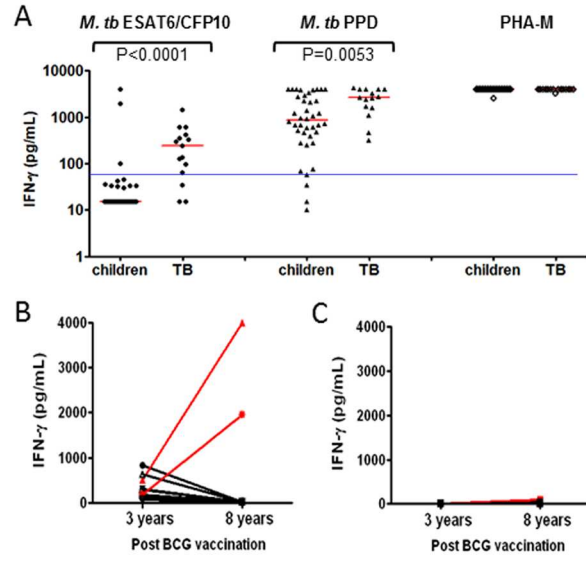
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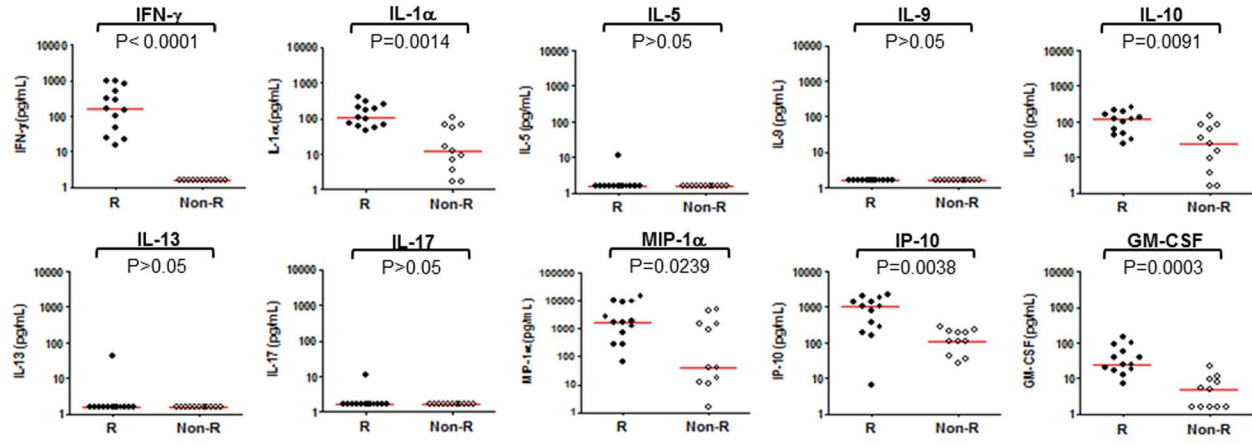
695 **Fig. 6. The cytokines induced by *M. tb* ESAT-6/CFP-10 in 3 categorised groups.** This
696 diagram shows how the cytokine production following *M. tb* ESAT-6/CFP-10 stimulation can
697 improve diagnosis of latent TB. Among the cytokines which were tested in children at 3 and
698 8 years post BCG vaccination, only 4 cytokines (IL-17, IL-5, IL-9, IL-13) were able to
699 distinguish the responders to *M. tb* ESAT-6 (103278, 103738) from those to ESAT-6
700 homologues of *M. avium* and *M. kansasii* (104041, 104043).

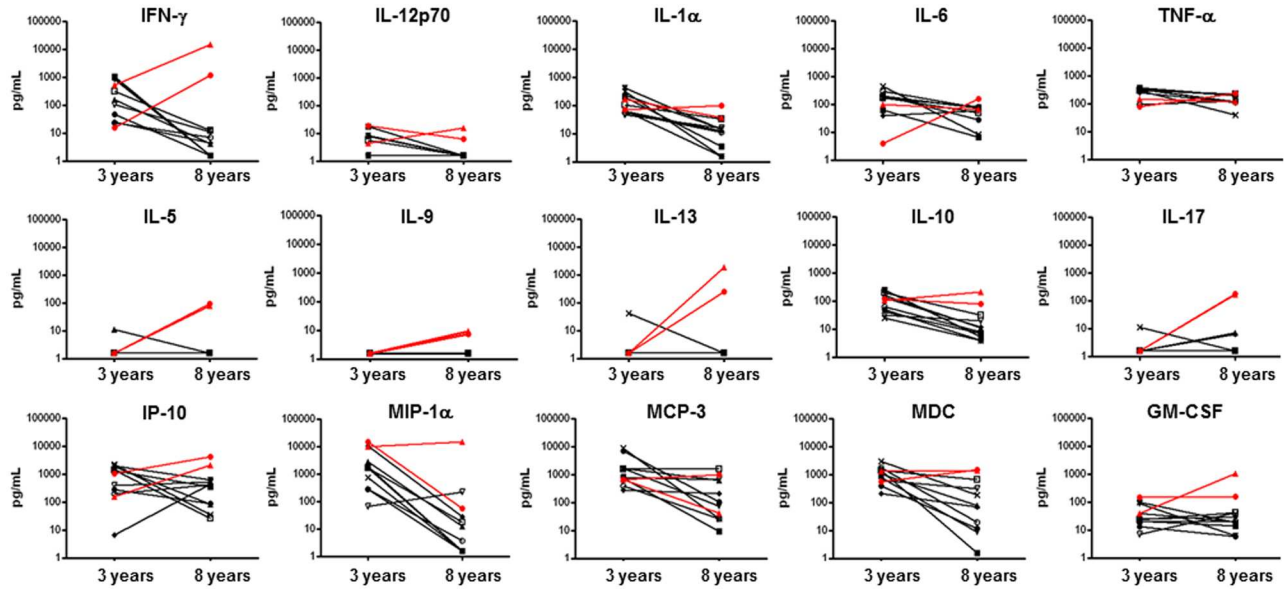
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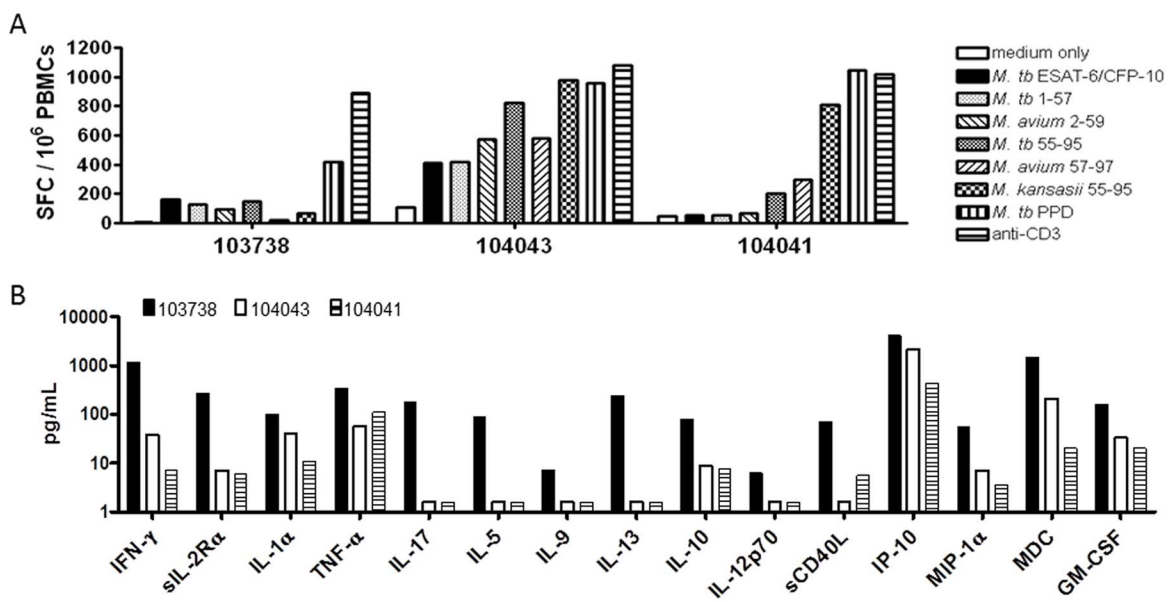
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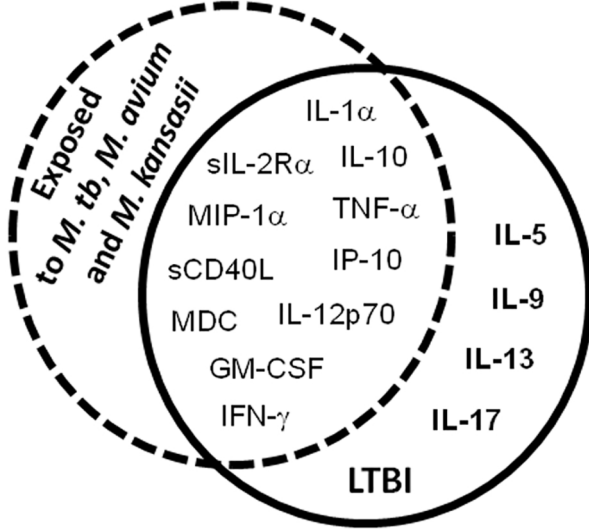












TABLES

antigen	Children (n=40)		TB patients (n=15)	
	number of positive samples	Positivity (%)	number of positive samples	Positivity (%)
<i>M. tb</i> ESAT6/CFP10	2	5.0%	11	73.3%
<i>M. tb</i> ₁₋₅₇ ESAT-6	3	7.5%	9	60.0%
<i>M. avium</i> ₂₋₅₉ ESAT-6	3	7.5%	6	40.0%
<i>M. tb</i> ₅₅₋₉₅ ESAT-6	4	10.0%	8	53.3%
<i>M. avium</i> ₅₇₋₉₇ ESAT-6	2	5.0%	3	20.0%
<i>M. kansasii</i> ₅₅₋₉₅ ESAT-6	3	7.5%	7	46.7%
<i>M. tb</i> PPD	30	75.0%	14	93.3%
anti-human CD3	39	97.5%	15	100.0%

Table 1. Detection of positive antigen reactive T cells producing IFN- γ in Malawian children and TB patients. The number of samples which showed positive responses to each antigen and the rated of positivity in the IFN- γ ELISpot assay are shown. IFN- γ responses to ESAT-6 homologues of *M. avium* or *M. kansasii* were detected in only 2 or 3 subjects. Eleven of 15 TB patients showed IFN- γ producing cells that responded to *M. tb* ESAT-6/CFP-10. About half of the patients showed IFN- γ responses to ESAT-6 homologues of *M. avium* ₂₋₅₉ or *M. kansasii* ₅₅₋₉₅. Positive IFN- γ responses were defined as detailed in Materials and Methods.

number of responders to <i>M. tb</i> ESAT-6 / total number	number of responders to each ESAT-6 peptide (% of cross reactivity)		
	<i>M. avium</i> 2-59	<i>M. avium</i> 57-97	<i>M. kansasii</i> 55-95
2 / 40 children	2 (100%)	1 (50%)	2 (100%)
11 / 15 TB patients	5 (45.5%)	3 (27.3%)	7 (63.6%)

Table 2. Cross reactivity of IFN- γ responses between *M. tb* ESAT-6 and ESAT-6 homologues of *M. avium* and *M. kansasii*. Cross reactivity of IFN- γ responses between *M. tb* ESAT-6 and ESAT-6 homologues of *M. avium* and *M. kansasii* was measured in the positive IFN- γ responders in 2 children and 11 TB patients. Two children showed IFN- γ producing cells in response to both *M. avium* 2-59 and *M. kansasii* 55-95. In 11 TB patients who showed positive responses to *M. tb* ESAT-6, more than 45% of the responders showed cross-reactivity with *M. avium* 2-59 and *M. kansasii* 55-95 ESAT-6.