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

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

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## ABSTRACT

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

## INTRODUCTION

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

Since there is a high frequency of TB in the households of index TB cases in Malawi (11), children are vulnerable and are at high risk of becoming infected by adults with TB. IFN-γ responses to mycobacterial antigens have been extensively studied in cohort studies in Malawian infants at the Karonga Prevention Study (KPS) in Chilumba, Malawi (12-13). The immune responses of the infants were followed up at 3, 12 months and 3 years post BCG vaccination between 2002 and 2006, and 13.6% (13/98) of infants tested at 3 years post vaccination responded to *M. tb* ESAT-6/CFP-10 (14). Such a result might suggest that the 13 infants who showed positive IFN-γ responses to *M. tb* ESAT-6/CFP-10 were infected with *M. tb.* However, none showed symptoms of clinical disease, and an alternative explanation might be that the response shown is due to cross reactivity with ESAT-6 homologues from other NTM which are endemic in the area, as *M. leprae* infection is now uncommon in Malawi. The major slow growing NTM found in the sputa of TB patients in Northern Malawi have been identified as being species from the *M. avium-intracellulare complex* as most common and *M. gordonae, M. terrae, M. kansasii, M. malmoense* were also isolated (15).

In this study, we hypothesized that the positive IFN-γ responses to *M. tb* ESAT-6/CFP-10 observed in these 13 children at 3 years post BCG vaccination might not reliably indicate *M. tb* infection but could be derived from cross reactive responses to ESAT-6 homologues of environmental NTM, and that cytokine/chemokine signatures may distinguish between the subjects who showed stronger IFN-γ responses to *M. tb* ESAT-6 and those who responded more strongly to ESAT-6 derived from NTM. To test these two hypotheses, we chose *M. avium subspecies avium* and *M. kansasii* which have ESAT-6 homologues, among the species frequently found in the sputa of TB patients in Northern Malawi. We followed up the immune responses of the children at 8 years post BCG vaccination in Malawi and assessed the cross reactive responses between *M. tb* ESAT-6 and ESAT-6 homologues of *M. avium* 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*.

## MATERIALS AND METHODS

*Ethical permissions*

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

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

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

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

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

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

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

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

*IFN-γ ELISA to test archive sample quality*

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

*Blood collection and PBMC isolation*

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

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

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

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

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

peptides were put together into 5 peptide pools i.e. *M. tb* $_{1-57}$, *M. tb* $_{55-95}$, *M. avium* $_{2-59}$, *M. avium* $_{57-97}$, *M. kansasii* $_{55-95}$ (Fig. S1).

*IFN-γ ELISpot assay*

The IFN-γ ELISpot assay was carried out as previously described (18). The final concentration of *M. tb*, *M. avium* and *M. kansasii* peptides was 10 µg/mL each and the concentration of *M. tb* PPD was 5 µg/mL. Anti-human CD3 mAb was used at 0.1 µg/mL (Mabtech, Nacka Strand, Sweden). Spots were counted using an ELISpot reader (AID GmbH 4.0; San Diego, CA). The positive responses to each antigen were measured by an empirical rule (ER) 1 which defines a positive response as at least twofold increase of spot number in the experimental wells over the background with a minimum threshold of 5 spots per 100,000 PBMCs in the experimental wells (28). A *M. tb* ESAT-6 specific positive response was defined if spots appeared in *M. tb* ESAT-6/CFP-10 and either *M. tb* ESAT-6 $_{1-57}$ or *M. tb* ESAT-6 $_{55-95}$ stimulation alongside the positive response to anti-CD3 antibody.

*Quantiferon TB Gold-In Tube Test*

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

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

*19plex and 42plex bead assays*

Culture supernatants from cells stimulated with *M. tb* ESAT6/CFP10, *M. tb* PPD, PHA-M, RPMI1640 were selected from the archive at 3 years post BCG vaccination for a multiplex bead assay with 19 different cytokines and chemokines: IL-1α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IFN-γ, TNF-α, IFN-α, GM-CSF, MIP-1α (CCL3), IP-10 (CXCL10), MDC (CCL22), and MCP-3 (CCL7). The beads in the 42plex kit for the samples from newly recruited subjects are a combination of a premixed 39 bead mix including IL-1α, IL-1ra, IL-1β, IL-2, sIL-2Rα, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IFN-α2, IFN-γ, TNF-α, TNF-β, sCD40L, MIP-1α (CCL3), MIP-1β (CCL4), Gro-α (CXCL1), IL-8 (CXCL8), IP-10 (CXCL10), MCP-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), TGF-α, G-CSF, GM-CSF, IL-3, IL-7, Eotaxin, FGF-2, Flt-3L, Fractalkine (CX3CL1), EGF, VEGF plus the addition of 3 chemokines; PDGF-AA, PDGF-AB/BB and RANTES (CCL5) which were added as 70 µL each from 3 individual vials after sonicating and vortexing of the beads. Beads were diluted 1 in 2 and the detailed protocol of the 42plex bead assay followed the manufacturer's protocol (no. MPXHCYTO60KPMX42; MILLIPLEX®$_{MAP}$ Kit, Millipore, Billerica, MA, USA) as described in a previous study (13). The range of standard curve was from 3.2 to 10000 pg/mL and the values below 3.2 pg/mL were set to 1.6. Considering cost restraints, samples showing the values above the 10000 pg/mL could not be retested with dilution but the values were set to 15000 pg/mL (13).

*Statistical analysis*

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

## RESULTS

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

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

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

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

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

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

*IFN-γ responses by QFT-IT test*

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

*Agreement between the results from different assays*

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

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

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

In order to examine if the cytokine proteins in the archived samples still remained intact, IFN-γ ELISA was performed and the level of IFN-γ production was compared with previous data obtained in 2006. The levels of IFN-γ measured from the archived samples collected from 4 infants at 3 years post BCG vaccination were similar to the previous data from 2006 and slightly higher in some of the supernatant aliquots than the IFN-γ detection in the past (Fig. S2A). However, no significant difference was found in IFN-γ concentrations of 19 culture supernatant samples from each of 4 subjects (indicated by lab number) when measured in 2006 and again in 2010 [38289 (P=0.49), 38290 (P=0.14), 38291 (P=0.36) and 38633 (P=0.50) by Wilcoxon signed rank test; Fig. S2A]. In addition, the Spearman correlation coefficient calculated using the IFN-γ data obtained from the total 76 samples was 0.9808 (P<0.0001), indicating a strong correlation between IFN-γ values obtained in 2006 and 2010 (Fig. S2B). Based on the IFN-γ production in response to *M. tb* ESAT-6/CFP-10, 17 cytokines and chemokines were analysed between IFN-γ responders and non-responders. IL-4 and IL-15 were excluded from this analysis as they were produced at levels below the limit of detection of the assay. *M. tb* ESAT-6/CFP-10 stimulation was able to differentiate 13 IFN-γ responders from 11 non-responders using 5 cytokines and chemokines; IL-1α, IL-10, MIP-1α, IP-10 and GM-CSF with median responses showing a difference of more than 5 fold 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-1α, 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-2Rα, IL-17, IL-5, IL-13 and sCD40L when compared with both an individual who
388 showed a positive responses to *M. avium* $_{57\text{-}97}$ and *M. kansasii* $_{55\text{-}95}$ (104041) and the subject
389 who showed strong response to *M. kansasii* $_{55\text{-}95}$ (104043) in the IFN-γ ELISpot assay (Fig.
390 5B, Fig. S3). However, sIL-2Rα 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.

## DISCUSSION

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

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

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

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

The peptides of *M. kansasii* ESAT-6 used in this study were derived from the amino acid positions 55-95, which includes two different amino acids compared with *M. tb* ESAT-6 (23). The small difference in only two amino acids between *M. tb* and *M. kansasii* ESAT-6 may not indicate that the peptides would act as an epitope which is specific to *M. kansasii* as we showed a high percentage of cross-reactivity between the *M. tb* ESAT-6 and *M. kansasii* ESAT-6 peptides. However, changing a single residue in a 20-mer amino acid peptide can result in a lack of MHC binding and may lead to a loss of recognition by T cells that were specific for the wild type peptide (36). In cattle *M. bovis* ESAT-6 (which is identical to *M. tb* ESAT-6) and *M. kansasii* ESAT-6 were differentially recognised by bovine T cells depending on their MHC types (8).

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

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

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

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

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

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637 **FIGURE LEGENDS**

638

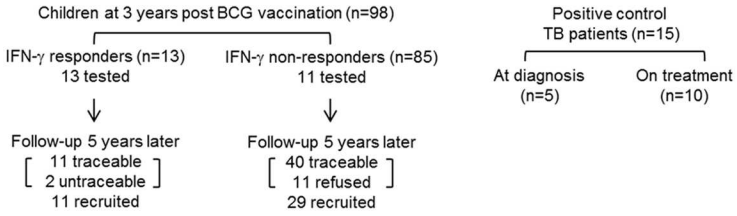
639 **Fig. 1. Collection of archived samples and recruitment of study subjects.** In a previous
640 study cohort, 13 of 98 children at 3 years post BCG vaccination showed positive IFN-γ
641 response to *M. tb* ESAT-6/CFP-10. In this study, 24 sets of archived culture supernatant
642 samples from the 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.

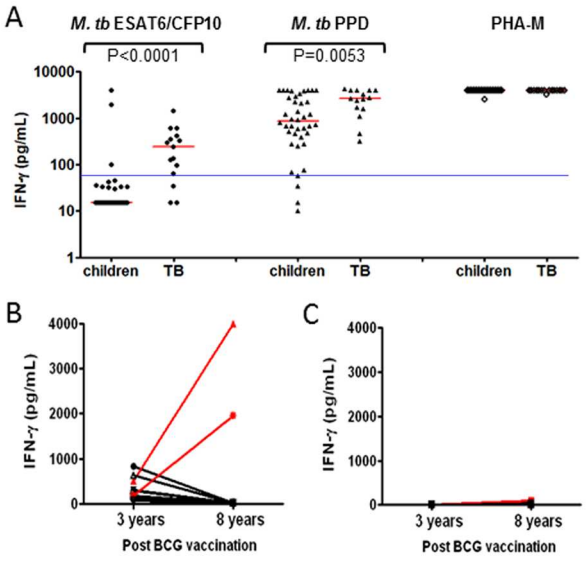
650

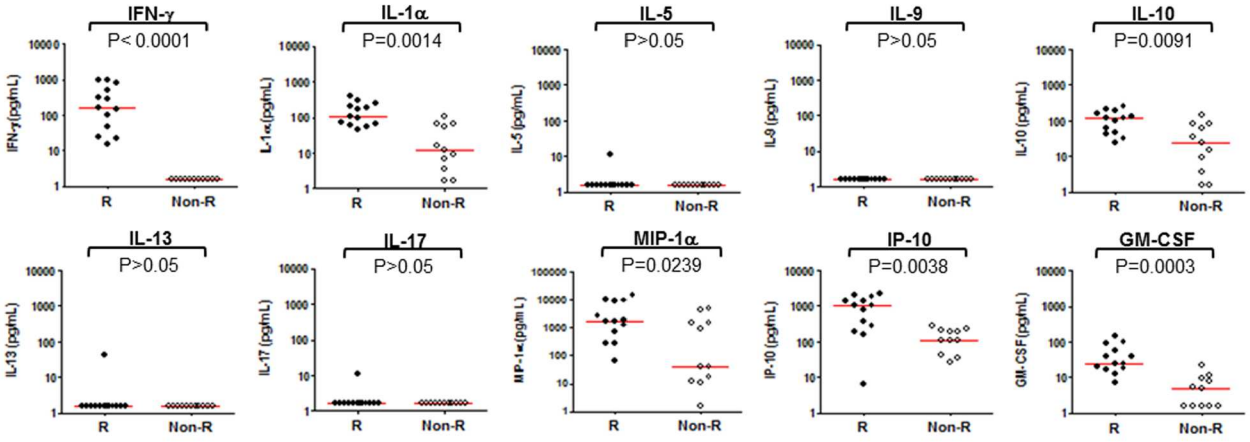
651 **Fig. 2. IFN-γ responses to *M. tb* ESAT-6/CFP-10, *M. tb* PPD and PHA in 40 children at 8**
652 **years post BCG vaccination and 15 TB patients.** *A*. TB patients showed significantly
653 higher IFN-γ production in response to *M. tb* ESAT-6 (P<0.0001) and *M. tb* PPD (P=0.013)
654 compared with 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

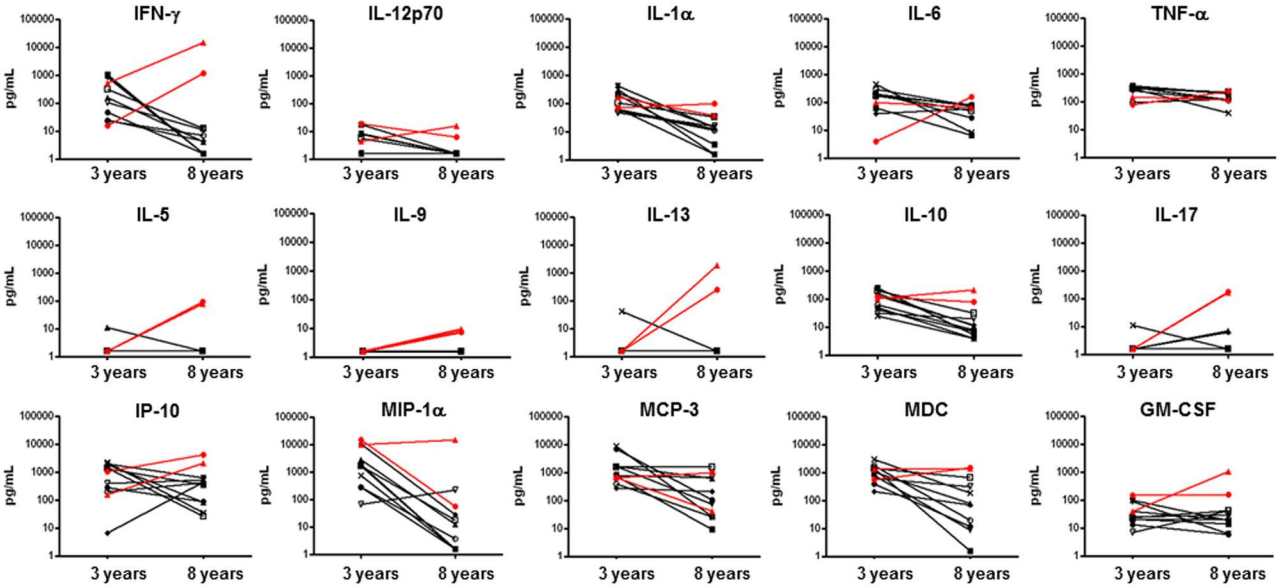
661 marked in red. Two of 11 previous responders had a marked increase in IFN-γ in response to
662 *M. tb* ESAT-6/CFP-10. In the previous non-responder group, IFN-γ was increased to 101
663 pg/mL in one subject.
664
665 **Fig. 3. Cytokine/chemokine responses in archived samples from children at 3 years 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.*

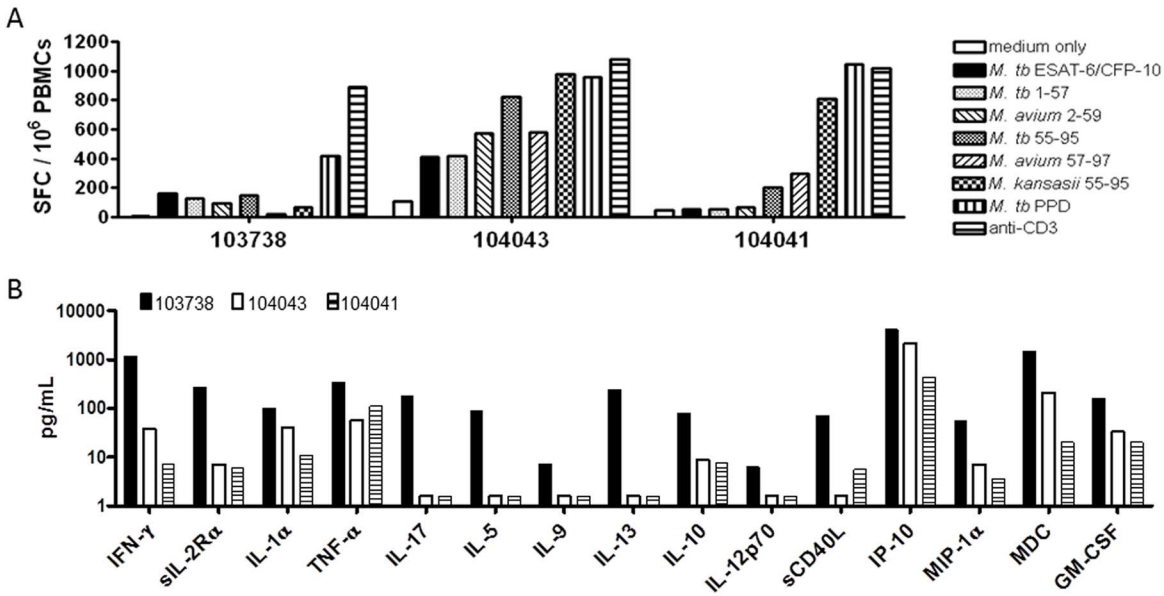
686 *kansasii* ESAT-6 peptides while 104043 showed a much higher number of SFC in response
687 to *M. kansasii* ESAT-6 compared with the number of SFC in response to *M. tb* ESAT-6.
688 Another subject, 104041 showed higher numbers of SFC in response to *M. avium* $_{57\text{-}97}$
689 compared to *M. 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*.
694
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).
701
702

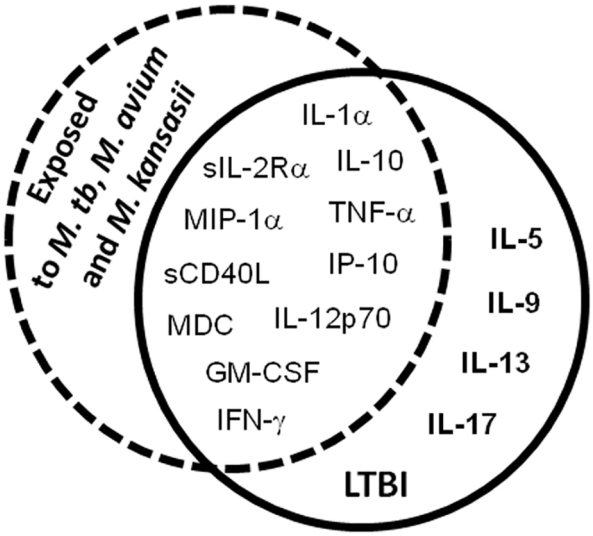












## TABLES

| antigen | Children (n=40) | | TB patients (n=15) | |
|---|---|---|---|---|
| | number of positive samples | Positivity (%) | number of positive samples | Positivity (%) |
| *M. tb* ESAT6/CFP10 | 2 | 5.0% | 11 | 73.3% |
| *M. tb* $_{1-57}$ ESAT-6 | 3 | 7.5% | 9 | 60.0% |
| *M. avium* $_{2-59}$ ESAT-6 | 3 | 7.5% | 6 | 40.0% |
| *M. tb* $_{55-95}$ ESAT-6 | 4 | 10.0% | 8 | 53.3% |
| *M. avium* $_{57-97}$ ESAT-6 | 2 | 5.0% | 3 | 20.0% |
| *M. kansasii* $_{55-95}$ ESAT-6 | 3 | 7.5% | 7 | 46.7% |
| *M. tb* 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 *M. tb* ESAT-6 / total number | number of responders to each ESAT-6 peptide (% of cross reactivity) | | |
|---|---|---|---|
| | *M. avium* $_{2-59}$ | *M. avium* $_{57-97}$ | *M. kansasii* $_{55-95}$ |
| 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.