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

Yun-Gyoung Hur,†,‡ Amelia C. Crampin,§ Christina Chisambo,∥ James Kanyika,∥ Rein Houben,§∥ Richard Ndhlovu,∥ Themba Mzembe,∥ Maeve K. Lalor,†,‡ Jacky Saul,§ Keith Branson,§ Carolyrne Stanley,∥ Bagrey Ngwira,∥ Neil French,∥ Tom H. Ottenhoff,§ Hazel M. Dockrell,† Patricia Gorak-Stolinska†

Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom; Department of Infectious Disease Epidemiology, Faculty of Epidemiology & Population Health, London School of Hygiene & Tropical Medicine, London, United Kingdom; Karonga Prevention Study, Chilumba, Karonga District, Malawi†; Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom∥; Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands§

A positive gamma interferon (IFN-γ) response to Mycobacterium tuberculosis early secretory antigenic target-6 (ESAT-6)/culture filtrate protein-10 (CFP-10) has been taken to indicate latent tuberculosis (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 Mycobacterium avium and Mycobacterium kansasii. Archived culture supernatant samples from 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 for 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 enzyme-linked immunosorbent assays (ELISAs) on supernatants from diluted whole-blood assays, IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays, QuantiFERON TB Gold-In-Tube tests, and multiplex bead assays were performed. More than 45% of the 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, interleukin 5 (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 differentiated the strong responders to M. tuberculosis ESAT-6 from those who preferentially responded to M. kansasii and M. avium ESAT-6. Thus, it has been suggested that an IFN-γ response to ESAT-6 and the 10-kDa culture filtrate protein-10 (CFP-10) on its own was not sufficient to detect M. tuberculosis infection in areas where both M. tuberculosis and environmental NTM or other pathogenic mycobacteria are endemic. To develop a more specific immunodiagnostic test for the detection of latent TB infection (LTBI), studies were designed to identify additional biomarkers and alternative tests to differentiate host immune responses to the M. tuberculosis ESAT-6 and CFP-10 proteins from those against their homologues in environmental mycobacteria.
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 in the Karonga Prevention Study (KPS) in Chilumba, Malawi (12, 13). The immune responses of the infants were followed up between 2002 and 2006 at 3 months, 12 months, and 3 years post-BCG vaccination, and 13.6% (13/98) of the infants tested at 3 years postvaccination responded to M. tuberculosis ESAT-6/CFP-10 (14). Such a result might suggest that the 13 infants who showed positive IFN-γ responses to M. tuberculosis ESAT-6/CFP-10 were infected with M. tuberculosis. However, none showed symptoms of clinical disease; an alternative explanation might be that the response shown was 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 species from the M. avium-intracellulare complex as most common, and Mycobacterium gordonae, Mycobacterium terrae, M. kansasi, and Mycobacterium malmoense were also isolated (15).

In this study, we hypothesized that the positive IFN-γ responses to M. tuberculosis ESAT-6/CFP-10 observed in these 13 children at 3 years post-BCG vaccination might not reliably indicate M. tuberculosis infection but can 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. tuberculosis ESAT-6 and those who responded more strongly to ESAT-6 derived from NTM. To test these two hypotheses, we chose M. avium subsp. avium and M. kansasi, which have ESAT-6 homologues, among the species frequently found in the sputa of TB patients in northern Malawi. We measured the immune responses of the children at 8 years post-BCG vaccination in Malawi and assessed the cross-reactive responses between M. tuberculosis ESAT-6 and ESAT-6 homologues of M. avium and M. kansasi to identify how these responses related to the positive IFN-γ responses to M. tuberculosis ESAT-6/CFP-10 at 3 years post-BCG vaccination. In addition, we analyzed the cytokine/chemokine signatures in response to M. tuberculosis ESAT-6/CFP-10 and M. tuberculosis purified protein derivative (PPD) to identify potential biomarkers which can discriminate M. tuberculosis infection from the cross-reactive response to ESAT-6 homologues of M. avium and M. kansasi.

MATERIALS AND METHODS

Ethical permissions. Authorization for 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 months, 12 months, and 3 years postvaccination was granted by the NHSRC (approval 01/38) and the London School of Hygiene & Tropical Medicine (LSHTM) ethics committee (approval 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 (approval 5929) and the NHSRC in Malawi (approval 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 the samples and questionnaires. Forms with the ethics application, the research proposal, consent forms, and information sheets were reviewed by the ethics committees of the LSHTM and the 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 the previous results that showed that 15 of 98 infants tested had positive IFN-γ responses to M. tuberculosis ESAT-6/CFP-10 fusion protein in a whole blood assay (WBA) at 3 years postvaccination, archived culture supernatants from 13 IFN-γ responders to M. tuberculosis ESAT-6/CFP-10 and 11 nonresponders were retrieved and transported to the LSHTM laboratory to determine their cytokine/chemokine profiles. Samples which had been stimulated with M. tuberculosis PPD (batch RT49, lot 204; Statens Serum Institut [SSI], Copenhagen, Denmark), M. tuberculosis ESAT-6/CFP-10 (Bill and Melinda Gates Foundation Grand Challenge 6 [BMGF GC6] project, batch 040010) (16, 17), phytohemagglutinin-M (PHA-M) (Sigma-Aldrich, Poole, United Kingdom), and culture medium (RPMI 1640; Sigma-Aldrich) were analyzed further from each of the selected study participants. To test the sample quality following extended storage since 2006, 4 additional archived samples were stimulated with 19 different antigens: PHA-P, M. tuberculosis 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 (DoxR) regulon encoded antigens such as M. tuberculosis Rv0081, Rv1737C, Rv1812C, Rv2006, Rv2625C, Rv3132C, Rv3133C, and Rv0574C (BMGF GC6 project) in addition to RPMI medium were also retrieved and transported to the LSHTM laboratory for IFN-γ enzyme-linked immunosorbent assays (ELISAs).

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. tuberculosis ESAT-6/CFP-10 were traceable under the demographic KPS in Malawi (55% male and 45% female), and they were recruited for the new follow-up study at 8 years postvaccination (14). As a control group, 11 nonresponders at 3 years postvaccination who were also confirmed to be traceable were recruited. In addition, because of the high possibility of individuals converting from nonresponder to responder during the 5 years after the last follow-up visit, 18 additional ESAT-6/CFP-10 nonresponders at 3 years post-BCG vaccination were randomly selected and recruited (59% male and 41% female). All of the recruited responders and nonresponders had a BCG vaccination within 1 week after birth. To act as a positive-control population for ESAT-6 responses, 15 TB patients were recruited from the Karonga District Hospital and the Chilumba Rural Hospital. As laboratory-confirmed cases of TB in children are rare in the Karonga District, at diagnosis or within the first 3 months of treatment we recruited adult TB patients between the ages of 18 and 50 years (40% male and 60% female). TB was confirmed by smear/culture of sputa, and the TB patients were not eligible if they were 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 the extended storage period. The IFN-γ ELISA protocol for this test followed a 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 heparinized tube (170 IU of sodium heparin; BD Vacutainer, Plymouth, United Kingdom). Four hundred and fifty microliters
is used for a diluted WBA, and the remaining blood was used for peripheral blood mononuclear cell (PBMC) isolation. PBMCs were prepared by density gradient centrifugation using Ficoll (Sigma-Aldrich). The cells were diluted to 2.5 × 10⁵ cells/180 μl in AIM-V growth medium (Fisher Scientific) and 180 μl of resuspended cells were added into enzyme-linked immunosorbent spot (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% i-glutamine (1 in 5; Invitrogen, Paisley, United Kingdom), and 100 μl was added into each well with 100 μl each of M. tuberculosis PPD (RT50, lot 219; SSI) at a final concentration of 5 μg/ml, ESAT-6/CFP-10 (BMGF GC6 to 74 project, batch 040101) at a final concentration of 10 μg/ml (16, 17), PHA (lot 017K4029; Sigma-Aldrich) at a final concentration of 5 μg/ml, and RPMI 1640 supplemented with 1% i-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 an ELISA (19). A positive response in an IFN-γ ELISA was defined as >62.5 pg/ml, which is twice the limit of detection of the assay (19). The concentrations of IFN-γ above 4,000 pg/ml were set to be 4,000 pg/ml.

**ESAT-6 overlapping peptides derived from M. tuberculosis, 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. tuberculosis ESAT-6 and ESAT-6 homologues of NTM in Malawian children and TB patients. The protein sequences between M. tuberculosis ESAT-6 and ESAT-6 homologues of M. avium and M. kansasii are >90% identical to M. kansasii but only 27% identical to M. avium (see [http://blast.ncbi.nlm.nih.gov/Blast.cgi?report=genpept][GenBank accession no. CAE55648.1], [http://www.ncbi.nlm.nih.gov/protein/CAE55648.1?report=genpept][NCBI reference sequence ZP_05218333.1], and [http://www.ncbi.nlm.nih.gov/protein/gb%7CAGCG70856.1][GenBank accession no. ACG70856.1]). The positions of predominantly recognized epitopes are scattered throughout the ESAT-6 protein sequence, and the multiple T-cell responses are recognized different according to the population (20–22). Based on published papers and the SYFPEITHI program used to predict epitope sites (23), 14 overlapping peptides, including 15 mers with predicted epitopes for major histocompatibility complex (MHC) type II binding, were designed using the full-length ESAT-6 amino acid sequence from M. tuberculosis and M. avium subsp. avium (see Fig. S1 in the supplemental material). The ESAT-6 amino acid sequences are identical from M. tuberculosis 55-95, M. avium 55-95, and M. kansasii 55-95 (see Fig. S1).
were significantly greater in TB patients than in children (M. tuberculosis both ranging from 324 to 4,000 pg/ml. The median IFN-0.001 and H9253 the median IFN- responses to PHA (Fig. 2A). Twelve out of the 15 TB patients responded to M. tuberculosis ESAT-6/CFP-10 (median concentration, 245 pg/ml), while only 3 of 40 children at 8 years post-BCG vaccination responded (median response, 15 pg/ml). IFN-γ responses to PHA (Fig. 2A). Of the 3 IFN-γ-nonresponding TB patients (<62.5 pg/ml) was a patient at diagnosis, and two were patients on treatment. The IFN-γ responses to M. tuberculosis PPD were positive in all of the TB patients recruited, and the median IFN-γ response was 2,691 pg/ml, with IFN-γ responses ranging from 324 to 4,000 pg/ml. The median IFN-γ responses to both M. tuberculosis ESAT-6/CFP-10 and M. tuberculosis PPD were significantly greater in TB patients than in children (P < 0.001 and P < 0.01, respectively), while there was no difference in the median IFN-γ responses to PHA (P = 0.15) (Fig. 2A). Interestingly, only two subjects (103278 and 103738) at 8 years post-BCG vaccination had very marked increases in IFN-γ, which were >10 times greater (4,000 and 1,958 pg/ml, respectively) than the IFN-γ responses to M. tuberculosis ESAT-6/CFP-10 in the same children at 3 years post-BCG vaccination (Fig. 2B), while most previous nonresponders did not show positive IFN-γ responses to M. tuberculosis 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. tuberculosis ESAT-6 and its homologues. To determine the level of cross-reactivity of IFN-γ responses between M. tuberculosis ESAT-6 and ESAT-6 homologues of M. avium and M. kansasii were synthesized (see Fig. S1 in the supplemental material), and the quantification of T cells producing IFN-γ in response to the ESAT-6 peptide antigens was measured ex vivo using PBMCs from 40 children and 15 TB patients. Among the 40 children, only 5% (2/40; subjects 103738 and 104043) showed positive IFN-γ-producing cells in response to the M. tuberculosis ESAT-6/CFP-10 fusion protein and either M. tuberculosis1–55 or M. tuberculosis55–95 ESAT-6 peptides (Table 1). Two more subjects (subjects 103604 and 104041) showed positive IFN-γ-producing cells in response to M. tuberculosis55–95 ESAT-6, although they did not show positive responses to the M. tuberculosis ESAT-6/CFP-10 fusion protein, and they were not counted as responders to M. tuberculosis 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 greater IFN-γ-positive responses to M. tuberculosis ESAT-6 in the ELISpot assay (Table 1) and in the IFN-γ ELISA (Fig. 2). The level of cross-reactivity of IFN-γ responses between M. tuberculosis ESAT-6 and ESAT-6 homologues of M. avium and M. kansasii is shown in Table 2. The two responders to M. tuber-
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Between the IFN-\(\gamma\) ELISA and the ELISPOT assay was high (kappa, 0.7872, \(P < 0.01\)). In the 40 children only, the concordance between the IFN-\(\gamma\) ELISA and the ELISPOT assay was also high (kappa, 0.7872, \(P < 0.01\)), while the agreement between the QFT-IT test and both the IFN-\(\gamma\) ELISA and the ELISPOT assay was low (kappa, 0.2188 and 0.2857, \(P > 0.05\) and \(P < 0.05\), 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, an IFN-\(\gamma\) ELISA was performed, and the level of IFN-\(\gamma\) production was compared with previous data obtained in 2006. The levels of IFN-\(\gamma\) measured from the archived samples collected from 4 infants at 3 years post-BCG vaccination were similar to those in the previous data from 2006 and slightly higher in some of the supernatant aliquots than the IFN-\(\gamma\) detection in the past (see Fig. S2A in the supplemental material). However, no significant differences were found in the IFN-\(\gamma\)-concentrations of 19 culture supernatant samples from each of 4 subjects (indicated by lab number) when measured in 2006 and again in 2010 (subject 38289 \([P = 0.49\], subject 38290 \([P = 0.14\]), subject 38291 \([P = 0.36\], and subject 38633 \([P = 0.50\) by the Wilcoxon signed rank test) (see Fig. S2A). In addition, the Spearman correlation coefficient calculated using the IFN-\(\gamma\) data obtained from all 76 samples was 0.9808 \((P < 0.0001)\), indicating a strong correlation between the IFN-\(\gamma\) values obtained in 2006 and 2010 (see Fig. S2B in the supplemental material). Based on the IFN-\(\gamma\)-production in response to M. tuberculosis ESAT-6/CFP-10, 17 cytokines and chemokines were analyzed among the IFN-\(\gamma\)-responders and nonresponders. 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. tuberculosis ESAT-6/CFP-10 stimulation differentiated 13 IFN-\(\gamma\) responders from 11 nonresponders using 5 cytokines and chemokines, IL-1α, IL-10, MIP-1α, IP-10, and GM-CSF, with median responses showing a difference of >5-fold in the two groups. Furthermore, IL-5, IL-9, IL-13, and IL-17 were not produced in most of those tested, irrespective of whether they were IFN-\(\gamma\) responders or nonresponders (23/24) (Fig. 3). In response to PHA, the median concentration of most cytokine and chemokine responses measured was high apart from those of IL-2 and IL-4, which were below levels of detection (data not shown).

Comparison of cytokine/chemokine signatures between 3 and 8 years post-BCG vaccination. To examine how the immune responses had changed over the 5 years since the vaccinees had been studied and to determine if the cytokine responses other than IFN-\(\gamma\) may differentiate between two strongly positive IFN-\(\gamma\) responders to M. tuberculosis ESAT-6/CFP-10 (subjects 103278 and 103738) and nonresponders at 8 years post-BCG vaccination, the

### TABLE 2 Cross-reactivity of IFN-\(\gamma\)-responses between M. tuberculosis ESAT-6 and ESAT-6 homologues of M. avium and M. kansasii

| No. of responders to M. tuberculosis ESAT-6/total no. | No. of responders to the indicated ESAT-6 peptide (% of cross-reactivity) |
|------------------------------------------------------|-------------------------------------------------------------------------|
| Children \((n = 40)\)                                | TB patients \((n = 15)\)                                                | M. avium\(_{2–59}\) | M. avium\(_{57–97}\) | M. kansasii\(_{2–59}\) | M. kansasii\(_{57–95}\) |
| 2/40 children                                      | 2 (100)                                                               | 1 (50)             | 2 (100)             |                                    |
| 11/15 TB patients                                  | 5 (45.5)                                                             | 3 (27.3)           | 7 (63.6)           |                                    |

\(^a\) Cross-reactivity of IFN-\(\gamma\)-responses between M. tuberculosis ESAT-6 and ESAT-6 homologues of M. avium and M. kansasii was measured in the positive IFN-\(\gamma\) responders in 2 children and 11 TB patients. Two children showed IFN-\(\gamma\)-producing cells in response to both M. avium\(_{2–59}\) and M. kansasii\(_{57–95}\). In 11 TB patients who showed positive responses to M. tuberculosis ESAT-6, >45% of the responders showed cross-reactivity with M. avium\(_{2–59}\) and M. kansasii\(_{57–95}\) ESAT-6.

### TABLE 1 Detection of positive antigen-reactive T cells producing IFN-\(\gamma\) in Malawian children and TB patients

| Antigen                        | Children \((n = 40)\) | TB patients \((n = 15)\) |
|-------------------------------|-----------------------|--------------------------|
|                               | No. of positive samples | Positivity (%) | No. of positive samples | Positivity (%) |
| M. tuberculosis ESAT-6/CFP-10 | 2                     | 5.0 (11)               | 73.3 (11)               |
| M. tuberculosis ESAT-6         | 3                     | 7.5 (8)                | 60.0 (8)                |
| M. avium\(_{2–59}\) ESAT-6    | 3                     | 7.5 (6)                | 40.0 (6)                |
| M. tuberculosis ESAT-6         | 4                     | 10.0 (8)               | 53.3 (8)                |
| M. avium\(_{57–97}\) ESAT-6   | 2                     | 5.0 (3)                | 20.0 (3)                |
| M. kansasii\(_{57–95}\) ESAT-6| 3                     | 7.5 (7)                | 46.7 (7)                |
| M. tuberculosis PPD            | 30                    | 75.0 (14)              | 93.3 (14)               |
| Anti-human CD3                 | 39                    | 97.5 (15)              | 100.0 (15)              |

\(^a\) The number of samples which showed positive responses to each antigen and the rate of positivity in the IFN-\(\gamma\) ELISPOT assay are shown. IFN-\(\gamma\)-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-\(\gamma\)-producing cells that responded to M. tuberculosis ESAT-6/CFP-10. About half of the patients showed IFN-\(\gamma\)-responses to ESAT-6 homologues of M. avium\(_{2–59}\) or M. kansasii\(_{57–95}\). Positive IFN-\(\gamma\)-responses were defined as detailed in Materials and Methods.
cytokine and chemokine responses at 3 and 8 years post-BCG vaccination were compared in 11 of the previous 13 responders who showed positive IFN-γ responses to *M. tuberculosis* ESAT-6/CFP-10 at 3 years post-BCG vaccination. From the 11 subjects, only two subjects (103278 and 103738) showed strong positive IFN-γ responses (Fig. 2B) at 8 years post-BCG vaccination (see Fig. 4, marked in red). The production of IL-12p70, IL-1α, IL-10, IP-10, MDC, and GM-CSF in response to *M. tuberculosis* ESAT-6/CFP-10 was greater in the two IFN-γ responders than in the other subjects (Fig. 4). IL-17 and the Th2 type cytokines IL-5, IL-9, and IL-13 were not produced in response to *M. tuberculosis* ESAT-6/CFP-10 at 3 years post-BCG vaccination, and 9 of the 11 previous IFN-γ responders still showed low levels of those cytokines 5 years later (Fig. 4). However, the two IFN-γ responders at 8 years post-BCG vaccination showed increases in IL-17, IL-5, IL-9, and IL-13 in response to *M. tuberculosis* ESAT-6/CFP-10 (Fig. 4), and one of the two responders (103738) also showed large increases in IL-5, IL-9, and IL-13 in response to *M. tuberculosis* PPD since 3 years postvaccination (data not shown). No remarkable differences in the cytokine and chemokine responses to *M. tuberculosis* ESAT-6/CFP-10 were observed between 3 and 8 years post-BCG vaccination.
PPD and PHA were found between the two IFN-γ responders and others at 8 years post-BCG vaccination (data not shown).

Cytokine/chemokine signatures between IFN-γ responders to M. tuberculosis ESAT-6 and those responding to ESAT-6 homologues of M. avium and M. kansasii. Cytokine and chemokine signatures were compared between the subject who showed a higher frequency of IFN-γ-producing cells to M. tuberculosis ESAT-6 peptides (subject 103738) and those who responded more strongly to M. avium or M. kansasii ESAT-6 (subjects 103278 and 104041) in the ELISpot assay (Fig. 5A). In response to M. tuberculosis ESAT-6/CFP-10, subject 103738, who had stronger IFN-γ responses to M. tuberculosis ESAT-6 peptides, showed about 10-fold-greater production of IFN-γ, sIL-2Rα, IL-17, IL-5, IL-13, and sCD40L than an individual who showed positive responses to M. avium and M. kansasii (subject 104041) and the subject who showed a strong response to M. kansasii (subject 104043) in the IFN-γ ELISpot assay (Fig. 5B; see also Fig. S3 in the supplemental material). However, sIL-2Rα and sCD40L were also highly produced in some other subjects who did not respond to M. tuberculosis ESAT-6/CFP-10 (data not shown). In the M. tuberculosis ESAT-6 responder (subject 103738), TNF-α, IL-9, IL-10, IL-12p70, MDC, and GM-CSF were also highly produced, and IL-9 and IL-12p70 production was still greater in response to M. tuberculosis PPD, while the other cytokines that were exclusive to subject 103738 in response to M. tuberculosis ESAT-6/CFP-10 did not show significant differences in response to M. tuberculosis PPD (data not shown). We noticed that the level of MCP-1 in the background without stimulation with M. tuberculosis antigens was very high in two IFN-γ responders (subjects 103738 and 103278) compared with those in the others.

DISCUSSION
This study provides preliminary evidence that multiple cytokine/chemokine signatures may identify potential biomarkers for a better diagnosis of M. tuberculosis infection in children and supports the observation that IFN-γ on its own is not sufficient for diagnosing M. tuberculosis infection based upon M. tuberculosis 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. tuberculosis ESAT-6/CFP-10 in IFN-γ ELISAs after a 6-day WBA compared to those of the original 13 responders 5 years earlier. In the ELISpot assay, >50% of the IFN-γ responders to M. tuberculosis 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. tuberculosis ESAT-6 were greater than those to ESAT-6 homologues of M. avium and M. kansasii. These data indicate that an IFN-γ response to M. tuberculosis ESAT-6 alone cannot differentiate M. tuberculosis infection from infection with NTM in this setting, as shown in a 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. tuberculosis ESAT-6/CFP-10 were exclusively restricted to the two strong M. tuberculosis ESAT-6 IFN-γ responders, while the IFN-γ nonresponders and the one weakly positive responder did not produce these cytokines at 8 years post-BCG vaccination. In addition, these cytokines differentiated the IFN-γ responder to M. tuberculosis 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 vac-
ination and 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 >2 weeks, weight loss, or
hemoptysis.

All of the 13 previous IFN-γ responders to *M. tuberculosis*
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. tuberculosis*
ESAT-6/CFP-10 at 8 years post-BCG vaccination. The production
of IL-1α, IFN-γ, IP-10, MIP-1α, and GM-CSF, which was highly
detected in previous IFN-γ responders at 3 years post-BCG vac-
nination, was also highly detected in all of the IFN-γ responders at 8
years post-BCG vaccination regardless of the preferential IFN-γ
responses to *M. tuberculosis* ESAT-6 or ESAT-6 homologues of *M.
avium* and *M. kansasi* 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 have been cross-
reactive responses with ESAT-6 homologues of environmental
NTM. However, it is also possible that the 11 nonresponders who
showed positive responses at 3 years post-BCG vaccination might
have been transiently infected with *M. tuberculosis* which had been
cleared during the subsequent 5 years.

Previous and recent reports showing cytokine and chemokine
production in latent and active TB disease support the findings
observed in this study, i.e., greater production of IL-17, IL-5, IL-9,
and IL-13 upon *M. tuberculosis* antigen stimulation in positive
IFN-γ responders (25–30). The proportions of CD4+ T cells ex-
pressing IFN-γ, IL-17, and IL-22 were observed to be significantly
greater upon mycobacterial antigen stimulation in both latent and
active TB disease than in healthy controls (25). 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 (26), suggesting a protective
role for IL-17 in disease progression to active TB. In humans, IL-13
mRNA and IL-4 mRNA were significantly expressed in TB patients
compared with those in the controls (27), while higher levels of IL-13 and sCD40L were also observed in TB patients who
quickly responded to anti-TB therapy than in slow responders
(28). In contrast, it was also reported that the production of IL-4
and IL-5 is associated with progression to active disease (29). 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 produc-
tion in response to *M. tuberculosis* PPD and ESAT-6/CFP-10 in
WBAs was significantly greater in tuberculin skin test-positive in-
dividuals in a West African cohort (30).

The peptides of *M. kansasi* ESAT-6 used in this study were
derived from the amino acid positions 55 through 95, which in-
clude two different amino acids than those in *M. tuberculosis*
ESAT-6 (see http://www.ncbi.nlm.nih.gov/protein/67CACG7
0856.1 [GenBank accession no. ACG70856.1]). The small differ-
ence in only two amino acids between *M. tuberculosis* and *M.
kansasi* ESAT-6 may not indicate that the peptides would act as an
epitope that is specific to *M. kansasi*, as we showed a high percent-
age of cross-reactivity between the *M. tuberculosis* ESAT-6 and *M.
kansasi* 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 (31). In cattle, *M. bovis* ESAT-6 (which is
identical to *M. tuberculosis* ESAT-6) and *M. kansasi* ESAT-6 were
differentially recognized by bovine T cells depending on their
MHC types (8). The IFN-γ ELISA after a 6-day WBA, the IFN-γ ELISpot assay,
and the QFT-IT test showed low discordance measured by the
kappa statistic (0.56 < kappa = 0.71, P < 0.01). Any discordances
among the tests are derived from the fact that different parameters
are measured in each assay. The IFN-γ ELISA and multiplex bead
assay measured the magnitude of IFN-γ production following a
6-day culture of whole blood with *M. tuberculosis* ESAT-6/CFP-
10, while the ELISpot and QFT-IT assays measured overnight re-
sponses. The ELISpot assay measures the frequency of IFN-γ-
producing cells, and the QFT-IT test measures secreted cytokines;
effector T cell function is measured in the ELISpot and QFT-IT
assays, while the WBA measures the memory recall responses.
Compared with an ELISpot assay which uses a fixed number of
isolated PBMCs, the QFT-IT test uses a whole-blood sample and
may have a greater variability in results depending on the lympho-
cyte count.

The current study was derived from a cohort study with a
larger adequately powered group of infants recruited in 2002 and
examined the 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 main-
tenance 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. tuberculosis* ESAT-6/CFP-10
at 3 years (13 among 98 tested) and 8 years (3 among 40 tested,
including the initial 11 responders) post-BCG vaccination, a
much larger sample size than that of 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. tuberculosis* region of difference 1-encoded antigens, while no
studies have been published regarding biomarkers to distinguish
*M. tuberculosis* 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,
and IL-17) to distinguish between LTBI and exposure to *M.
avium* and *M. kansasi* (Fig. 6). These findings, although preliminary
in nature due to the small number of subjects involved, contrib-
ute knowledge to the ongoing development of novel diagnostic
tests with higher specificities to predict *M. tuberculosis* 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 infection
to validate the diagnostic value of the suggested cytokine signa-
ture.

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