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Recognition of CD8+ T-cell epitopes to identify adults with pulmonary tuberculosis

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To the Editor:

Tuberculosis (TB), the disease caused by *Mycobacterium tuberculosis* (Mtb), remains a leading cause of morbidity and mortality. Current tools to identify Mtb-infected individuals, specifically interferon-γ release assays (IGRAs) and the tuberculin skin test (TST), cannot distinguish between asymptomatic Mtb-infected individuals (latent Mtb infection (LTBI)) and those with TB [1]. Advancement of TB diagnostics and their application in TB-endemic settings requires an assay that distinguishes between individuals with LTBI and TB. In this pilot study, we compared the ability of three $CD8^+$ T-cell-based assays to distinguish Ugandan adults with: confirmed pulmonary TB (HIV-uninfected and HIV-infected), LTBI (HIV-uninfected only), and those who are TST-negative (HIV-uninfected only).

Participants were recruited from the National TB Treatment Center at Mulago Hospital, hospital staff and the community surrounding Kampala, Uganda, between 2001 and 2014, into four cohorts: culture-confirmed pulmonary TB/HIV-uninfected (TB cohort); culture-confirmed pulmonary TB/HIV-infected (TB/HIV cohort; CD4 count >150 cells·μL^{-1}); LTBI/HIV-uninfected (LTBI cohort; TST ⩾10 mm); and TST negative/HIV-uninfected (TST-negative cohort; TST<10 mm). Assignment to the LTBI and TST-negative cohorts was based

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on TST result and absence of signs or symptoms of TB (prolonged cough, haemoptysis, fever, weight loss and night sweats). This study received Institutional Review Board approval from all sponsoring institutions and written, informed consent was obtained from all participants.

A total of 170 participants were enrolled: 43 TB cohort, 42 TB/HIV cohort, 46 LTBI cohort, and 39 TST-negative cohort. Whole blood was drawn and peripheral blood mononuclear cells (PBMC) isolated and cryopreserved at the time of cohort assignment, and prior to TB treatment (if indicated). Three assays were used to quantify Mtb-specific $CD8^+$ T-cells: intracellular cytokine staining (ICS) assay to measure $CD8^+$ T-cell interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin-2 (IL-2) production in response to ESAT6 and CFP10 peptide pools (ESAT6/CFP10 ICS assay); ICS assay to measure $CD8^+$ T-cell IFN-γ, TNF-α, and IL-2 production in response to a pool of Mtb-specific epitopes (epitope-pool ICS assay); and a flow cytometry assay to measure $CD8^+$ T-cell binding to a tetramer-pool (tetramer-pool assay).

We hypothesised that eight minimal epitopes from four Mtb-specific genes, previously identified and validated by our group to be recognised by $CD8^+$ T-cells from Mtb-infected individuals, would elicit strong $CD8^+$ T-cell responses from individuals with confirmed TB regardless of their HIV status, when pooled for use in an immune-based assay [2–4]. The gene, location and restriction alleles are: $CFP10_{2–9}$/HLAB45, $CFP10_{3–11}$/HLAB08, $CFP10_{2–11}$/HLAB44, $CFP10_{49–58}$/HLAB35, $CFP10_{75–83}$/HLAB15, $Mtb8.4_{33–41}$/HLAB15, $EsxG_{3–11}$/HLAA02 and $EsxJ_{24–34}$/HLAB57. To test this hypothesis, we used this pool of eight epitopes in our ICS epitope-pool assay. We observed significant differences in the proportion of individuals with a positive IFN-γ response to the epitope-pool among cohorts (figure 1a). When comparing the magnitude and pattern of cytokine production, we also observed significant differences in the frequency of $CD8^+$ T-cells productive of either IFN-γ or TNF-α in the TB as compared with the LTBI cohort (figure 1b). Individuals in the TB cohort were also more likely to have $CD8^+$ T-cells productive of both IFN-γ and TNF-α than individuals with LTBI, and those with TB/HIV (figure 1b). These findings contrast to those obtained in our ESAT6/CFP10 ICS assay, where there were no significant differences in the proportion of individuals with positive cytokine responses among the TB, TB/HIV and LTBI cohorts (68.3%, 61% and 64.3%, respectively).

We sought to determine if a tetramer-based assay could distinguish individuals with confirmed TB (TB and TB/HIV cohorts) from individuals without TB disease (LTBI and TST-negative cohorts). To investigate, we used the identical pool of eight epitopes in a tetramer-pool assay. Significant differences in the proportion of $CD8^+$ T-cells that bound the tetramer-pool between cohorts were observed (figure 1c). The frequencies of $CD8^+$ T-cell tetramer-pool binding were also significantly different between TB and LTBI, and TB and TB/HIV cohorts (figure 1d). Spearman's correlation assessed associations between donor responses to the tetramer-pool and epitope-pool ICS assays; however, correlation between production of either cytokine and tetramer-pool binding was low (r_s=0.349 for IFN-γ and r_s=0.368 for TNF-α; $p<0.001$ both comparisons). We identified two TB/HIV co-infected individuals who bound the tetramer-pool, but did not produce IFN-γ or TNF-α in the epitope-pool ICS assay, and upon further investigation noted that $CD8^+$tetramer$^+$ T-cells

from these discordant individuals demonstrated high expression (11.4% and 6.6%) of the exhaustion marker PD-1 (median $CD8^+tetramer^+PD\text{-}1^+$ T-cells from TB/HIV donors was 1.99%; interquartile range 1.04–10.2%). Conversely, 11 individuals (five TB cohort; four HIV/TB cohort; and two LTBI cohort) who produced IFN-γ in response to the epitope-pool failed to bind the tetramer-pool. We suspect that human leukocyte antigen (HLA)-mismatching between these donors and the HLA-restricting alleles used to generate the tetramers limited the strength of the relationship between epitope-pool ICS and tetramer-pool assays.

Several groups have used Mtb-specific $CD8^+$ T-cell functional or phenotypic responses to distinguish individuals with LTBI from those with TB [5–11]. Unlike some prior reports, we could not detect significant differences in the proportion of $CD8^+$ T-cells that produced pro-inflammatory cytokines in response to ESAT6/CFP10 peptide pools between individuals with LTBI and TB [6, 9, 10]. However, when using an epitope-pool as a stimulus in our ICS assay, $CD8^+$ T-cell cytokine responses were significantly more common among adults with TB compared to those with LTBI. Activation of Mtb-specific $CD4^+$ T-cells can lead to indirect activation of $CD8^+$ T-cells, as has been shown in a murine model of TB [12]. We hypothesise that the use of minimal epitopes limited amplification of Mtb-specific $CD4^+$ T-cell responses in donors with LTBI, and only elicited cytokine production from Mtb-specific $CD8^+$ T-cells in individuals with TB who had a high bacillary burden. Thus, the use of a pool of minimal epitopes in an ICS assay provided a gain in specificity for individuals with TB.

Our $CD8^+$ T-cell tetramer-pool assay was also highly specific for individuals with TB. Tetramer-based assays have several technical advantages compared to ICS-based assays, and can be analysed using a flow cytometer with limited laser capacity. Tetramers detect low affinity T-cell receptor/HLA interactions and identify antigen-specific T-cells with limited capacity to mount a cytokine response, as we observed among two TB/HIV participants who exhibited tetramer pool binding but no cytokine production in response to the epitope pool. A major biological restriction of tetramer-based assays is the requirement for HLA-compatibility for tetramer-binding to occur. Nonetheless, in this pilot study, we have shown the feasibility of such an approach.

Our study was limited by the lack of a LTBI cohort with HIV-infection (LTBI/HIV). Individuals with LTBI/HIV are a heterogeneous population, with advanced imaging revealing evidence for subclinical, active disease among asymptomatic individuals with normal chest radiographs [13]. Thus, as some individuals with LTBI/HIV harbour subclinical, active disease, they may have a bacillary-load sufficient to elicit a Mtb-specific $CD8^+$ T-cell response in our assays. We hypothesise that $CD8^+$ T-cell responses to both our assays would be quite heterogeneous among individuals with LTBI/HIV, and that this population should be included in future studies.

In summary, $CD8^+$ T-cell recognition of a pool of eight immunodominant Mtb-specific epitopes was highly selective for subjects with pulmonary TB, including individuals with TB/HIV co-infection. Once validated, a $CD8^+$ T-cell epitope-pool assay may show promise

to distinguish Mtb-infected individuals without TB disease, from those individuals suffering from active TB.

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Conflict of interest: C. Lancioni has nothing to disclose. G.M. Swarbrick is an employee of ViTi, Inc, a company that may have commercial interest in the results of this research. This potential conflict has been reviewed and managed by OHSU. B. Park has nothing to disclose. M. Nyendak reports other from ViTi, Inc., outside the submitted work; Required language from OHSU: OHSU and M. Nyendak have a financial interest in ViTi, a company that may have a commercial interest in the results of this research and technology. These potential individual and institutional conflicts of interest have been reviewed and managed by OHSU. M. Nsereko has nothing to disclose. H. Mayanja-Kizza has nothing to disclose. M.D. Null is an employee of ViTi, Inc, a company that may have commercial interest in the results of this research. This potential conflict has been reviewed and managed by OHSU. M.E. Cansler is an employee of ViTi, Inc, a company that may have commercial interest in the results of this research. This potential conflict has been reviewed and managed by OHSU. R.B. Duncan has nothing to disclose. J. Baseke has nothing to disclose. K. Chervenak has nothing to disclose. L. Malone has nothing to disclose. E.G. Heaphy has nothing to disclose. W.H. Boom reports grants from NIH/NIAID, during the conduct of the study. D.M. Lewinsohn reports grants from NIH (HHSN272200900053C), grants from NIH (NO1-AI-95383 and HHSN266200700022C/NO1-AI-70022), other from Papé Family Research Institute, during the conduct of the study; other from ViTi Inc., outside the submitted work; OHSU and D.M. Lewinsohn have a financial interest in ViTi, a company that may have a commercial interest in the results of this research and technology. These potential individual and institutional conflicts of interest have been reviewed and managed by OHSU. D.A. Lewinsohn reports grants from NIH (HHSN272200900053C), grants from NIH (NO1-AI-95383 and HHSN266200700022C/NO1-AI-70022), other from Papé Family Research Institute (OHSU institutional funds), during the conduct of the study; other from ViTi Inc., outside the submitted work; OHSU and D.A Lewinsohn have a financial interest in ViTi, a company that may have a commercial interest in the results of this research and technology. These potential individual and institutional conflicts of interest have been reviewed and managed by OHSU.

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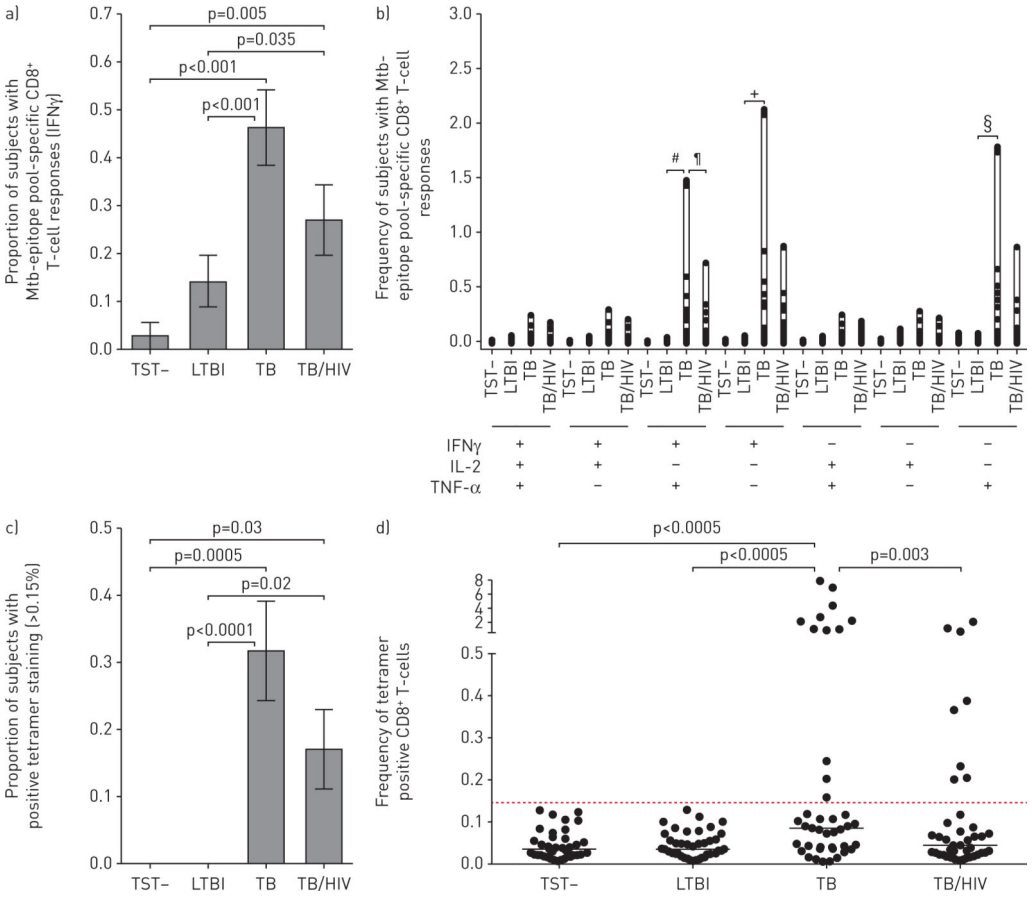

FIGURE 1.

CD8$^+$ T-cell cytokine and tetramer responses to *Mycobacterium tuberculosis* (Mtb)-epitope pool distinguishes latent tuberculosis infection (LTBI) from tuberculosis (TB). a) The proportion of subjects with detectable CD8$^+$ T-cell responses to a pool of eight Mtb-specific epitopes, as measured by interferon (IFN)-γ production in an intracellular cytokine staining (ICS) assay is shown (error bars indicate SEM). A positive response is defined as having at least 0.05% of CD8$^+$ T-cells expressing IFN-γ after subtraction of background (no stimulation) responses. The proportion of positive responses between groups were compared using Chi-squared tests. b) The frequency of Mtb-specific CD8$^+$ T-cells based on their production of either IFN-γ, tumour necrosis factor (TNF)-α and/or interleukin (IL)-2 is shown. The combinations of different cytokine functions are shown on the x-axis, with the frequency of distinct cytokine-producing cells within the Mtb-epitope pool responsive CD8$^+$ T-cell compartment on the y-axis. The frequency of Mtb-specific CD8$^+$ T-cells productive of each cytokine were compared using Kruskal–Wallis test, following adjustment for multiple comparisons using the Dwass, Steel, and Critchlow-Fligner method. c) The proportion of subjects with detectable CD8$^+$ T-cell binding to a pool of eight Mtb-specific tetramers, as measured by flow cytometry, is shown (error bars indicate SEM). A positive response is defined as having at least 0.15% of CD8$^+$ T-cells staining positive for tetramer. Statistical analysis with Chi-squared followed by pairwise comparison (Bonferroni adjustment). d) The frequencies of CD8$^+$tetramer$^+$ T-cells in different cohorts are shown. Statistical analysis with Kruskal–Wallis test followed by pairwise comparison (Bonferroni adjustment). Statistical

Analysis System v9.4 (SAS Institute Inc., Cary, NC, USA) was used for analysis. [#]: p=0.02; [¶]: p=0.0009; [+]: p=0.012; [§]: p=0.006.