Title

A High Throughput Whole Blood Assay for Analysis of Multiple Antigen-Specific T Cell Responses in Human Mycobacterium tuberculosis Infection

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Abstract

Antigen (Ag)-specific CD4 and CD8 T cells are important components of the immune response to *Mycobacterium tuberculosis* (Mtb), yet little information is currently known regarding how the breadth, specificity, phenotype and function of Mtb-specific T cells correlate with Mtb infection outcome in humans. To facilitate evaluation of human Mtb-specific T cell responses targeting

[3]Membership of the TBRU-ASTRa Study Group is provided at the end of the paper.

multiple different Ags, we sought to develop a high throughput and reproducible T cell response spectrum assay (RSA) requiring low blood sample volumes. We describe here the optimization and standardization of a microtiter plate-based, diluted whole blood stimulation assay utilizing overlapping peptide pools corresponding to a functionally diverse panel of 60 Mtb Ags. Using IFN-γ production as a readout of Ag specificity, the assay can be conducted using 50μl of blood per test condition and can be expanded to accommodate additional Ags. We evaluated the intra- and inter-assay variability, and implemented testing of the assay in diverse cohorts of Mtb-unexposed healthy adults, foreign-born adults with latent Mtb infection (LTBI) residing in the U.S., and TB household contacts with LTBI in a TB-endemic setting in Kenya. The Mtb-specific T cell RSA further enhances the immunological toolkit available for evaluating Mtb-specific T cell responses across different states of Mtb infection, and can be readily implemented in resource limited settings. Moreover, application of the assay to longitudinal cohorts will facilitate evaluation of treatment- or vaccine-induced changes in the breadth and specificity of Ag-specific T cell responses, as well as identification of Mtb-specific T cell responses associated with Mtb infection outcomes.

Introduction

The vast majority of individuals infected with *Mycobacterium tuberculosis* (Mtb) never develop signs or symptoms of active tuberculosis (TB) disease, thus providing compelling evidence that there are host immune responses that are capable of containing the infection. Both innate and adaptive immunity are essential components of the immune response to Mtb infection, with an important role for CD4 T cells demonstrated by animal models of Mtb infection (1–3) and human studies of individuals co-infected with HIV (4, 5). Induction and/or boosting of Mtb-specific CD4 T cell responses has been a central focus of novel TB vaccine candidates (6, 7). Despite the importance of CD4 T cells in Mtb infection, the antigen specificity, phenotype, and function of Mtb-specific CD4 T cells that correlate with immune control or risk of TB disease have not been well defined. Immunological tools that enable identification of individuals with latent Mtb infection (LTBI) who are at highest risk for development of active TB disease are currently lacking.

The Mtb genome contains over 4.4 million base pairs, encoding approximately 4,000 genes (8), thus imposing a significant challenge to conducting genome-wide characterization of Mtb-specific CD4 T cell responses. Several studies have measured IFN-γ secretion to evaluate T cell responses to panels of recombinant Mtb proteins in cross-sectional cohorts of individuals with LTBI and active TB disease, in an effort to identify novel vaccine candidates as well as Ags associated with different states of Mtb infection (9–16). However, such studies have generally not determined CD4 and CD8 T cell reactivity or further defined specific T cell epitopes, and have also not been applied to longitudinal cohort studies to identify changes in Mtb Ag recognition during the course of Mtb infection or treatment.

Although the Mtb-specific CD4 T cell correlates of protection are not well-defined, differential CD4 T cell responses that underlie or correlate with Mtb infection states may consist of different T cell phenotypes, functions, trafficking capabilities, activation states, and/or antigen specificities (17, 18). Tracking Mtb-specific CD4 T cells on an epitope-

specific level requires additional, in-depth screening with peptides, including synthesis of overlapping peptides spanning the full sequence of an Ag, or synthesis of specific epitope peptides according to HLA binding predictions. Studies conducting comprehensive screening of Mtb-specific CD4 T cell responses in individuals with LTBI have been conducted recently using large panels of peptides corresponding to epitopes predicted to bind to a panel of HLA DR, DP, and DQ class II alleles (19, 20). These studies have further underscored the heterogeneity of CD4 T cell immunity in LTBI, and have identified antigenic islands of epitope specificity, which are largely focused on Ags related to bacterial secretion systems (20). However, it remains unknown whether distinct populations of Mtb-specific T cells have differential contributions to mediating control of Mtb infection. Moreover, it is currently unclear how the breadth and specificity of Mtb-specific T cell responses changes over time within the same individual in the setting of immunotherapeutic interventions, progression to TB disease, and antimicrobial treatment for either LTBI or active TB disease.

To further enhance the toolkit for evaluating Mtb-specific T cell responses in multi-site and longitudinal studies of individuals with distinct states of Mtb infection, we sought to develop a high throughput and reproducible assay requiring low blood sample volumes to evaluate Mtb-specific T cell responses to multiple Mtb Ags simultaneously. We describe here the optimization of a microtiter plate-based, diluted whole blood stimulation assay utilizing overlapping peptide pools corresponding to a functionally diverse panel of Mtb antigens; furthermore, we describe implementation of the assay in the US and a field site in Kenya, as well as application to longitudinal cohort studies of Mtb-infected individuals in a TB-endemic setting.

Materials and Methods

Study participants and sample collection

Blood samples were collected in sodium heparin Vacuette® tubes (Greiner Bio-One) from LTBI individuals and uninfected, healthy control adults enrolled at the DeKalb County Board of Health Refugee Clinic and the Emory Vaccine Center in metropolitan Atlanta, GA, USA and the KEMRI (Kenya Medical Research Institute) Clinical Research Center in Kisumu, Kenya. Healthy control adults with a negative tuberculin skin test (TST) and/or a negative QuantiFERON-TB Gold (QFT) test and no history of exposure to active TB were enrolled only in Atlanta, whereas individuals with LTBI were enrolled both in Atlanta and Kenya. A total of 20 healthy control individuals were enrolled in Atlanta (65% Black, 20% White, 10% Asian, and 5% Hispanic); 44 individuals with LTBI were enrolled in Atlanta (66% Black, 2% White, and 32% Asian); and 18 individuals with LTBI were enrolled in Kenya (100% Black). All individuals with LTBI included in the study met the following inclusion criteria: asymptomatic adults ≥ 18 years of age; a positive QFT result; seronegative for HIV antibodies (Abs); no previous history of diagnosis or treatment for active TB disease; no previous history of treatment for LTBI; and a normal chest X-ray. Individuals with LTBI who were enrolled in Kenya were household contacts of a sputum smear-positive active TB patient. All subjects provided written informed consent for participation in the study, which was approved by the Institutional Review Boards at Emory University, the

Georgia Department of Public Health, and the KEMRI Scientific and Ethics Review Unit (SERU). The U.S. Centers for Disease Control and Prevention reviewed the protocol and chose to rely on the oversight of KEMRI SERU.

Antigens

Initial optimization experiments were conducted using pooled, overlapping 15- or 16-mer peptides corresponding to the sequences of CFP-10, ESAT-6, and TB10.4 (BEI Resources, NIAID, NIH: Peptide Array, *M. tuberculosis* CFP-10 Protein, NR-34825; Peptide Array, *M. tuberculosis* ESAT-6 Protein, NR-34824; Peptide Array, *M. tuberculosis* TB10.4 Protein, NR-34826). 18-mer peptides (overlapping by 11 amino acids) corresponding to the 60 Mtb Ags listed in Table 1 were synthesized on a 10mg scale using Fmoc chemistry (Synthetic Biomolecules, San Diego, CA) according to the H37Rv genome sequence. Peptides were pooled by Ag (10 – 121 peptides per pool, with a median of 39 peptides per pool); peptide pools were numbered consecutively according to Rv gene numbers (Table 1). After pooling the peptides for each Ag, pools were lyophilized, followed by reconstitution in DMSO at a final centration of 1mg/ml per peptide. Peptide pools were further diluted in RPMI-1640 medium supplemented with 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin, to prepare working stocks for use in the stimulation assays described below. Phytohemagglutinin-M (PHA; Sigma-Aldrich) was used a positive control.

Whole blood stimulation

Heparinized whole blood was transported to the respective laboratories at the Emory Vaccine Center and KEMRI, and processed within 2 hours of collection. In initial studies, blood was diluted at varying dilutions up to 5-fold with RSA media, consisting of RPMI-1640 medium supplemented with 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin. Diluted blood was added in a volume of 100μl to each well of a sterile 96-well round bottom, tissue-culture treated plate (Corning). Each well contained 100μl of either RSA media alone (negative control), RSA media with Mtb peptide pools, or RSA media with PHA (positive control). The final volume in the assay was 200μl per well, with the final blood dilutions ranging from 1:2 to 1:10. 200μl of sterile PBS was added to the empty wells on the outer edge of the 96-well plate (Fig 1). Plates were incubated in a 37°C incubator with 5% CO_2 for 7 days. On day 7, plates were centrifuged at 2,000 rpm for 5 minutes, and 150μl of plasma supernatant was removed from each well and transferred to a v-bottom 96-well plate (Corning). Plasma supernatants were either used immediately or stored at −80°C until use in ELISAs.

IFN-γ ELISAs

IFN-γ ELISAs were conducted according to manufacturer's instructions (Human IFN-γ Uncoated Ready-SET-Go! ELISA kit, eBiosciences). 50μl of plasma supernatant from the whole blood stimulations was diluted with 50μl of assay diluent for use in the IFN-γ ELISA. ELISA plates were read at 450 nm using a Molecular Diagnostics spectrophotometer.

Data analysis and statistics

IFN-γ ELISA data were analyzed using SoftMax Pro v6.3 software (Molecular Devices). Background IFN-γ production for each individual was determined by calculating the average concentration of IFN-γ in the 6 negative control wells, and the mean background IFN-γ production was subtracted from the Ag-stimulated wells. A maximum concentration of quantifiable IFN-γ was set at 1,000 pg/ml, corresponding to the concentration of the highest standard of recombinant human IFN-γ protein in the ELISA. IFN-γ concentrations below the level of detection by the ELISA standard curve were set to 0 pg/ml.

Statistical testing was done using GraphPad Prism v7.0b and R software programs. Paired comparisons were evaluated using the Wilcoxon matched-pairs signed rank test. Correlations were evaluated using the Pearson correlation, with statistical significance evaluated using the Kendall rank correlation coefficient. P values <0.05 were considered significant.

Results

Selection of a panel of Mtb antigens for the T cell response spectrum assay (RSA)

To further enhance the evaluation of Mtb-specific T cell responses in cohorts of individuals across different states of Mtb infection, we sought to develop a high throughput, reproducible and transportable Ag-specific T cell response spectrum assay (RSA) in which we could measure Mtb-specific T cell responses to multiple Ags within the same individual using small quantities of blood. Mtb infection in humans generates a heterogeneous T cell response targeting highly conserved epitopes across a broad range of Ags (20–22). We selected a panel of 60 Mtb Ags for further evaluation that have been previous confirmed for recognition by T cells in humans with different states of Mtb infection (9, 20, 23, 24), and that represent a diverse range of functional categories, bacterial cell fractions, and gene families with specific characteristics (Table 1). Overlapping 18-mer peptides were synthesized according to the full-length H37Rv sequence of each Ag; peptides were pooled by Ag.

Blood dilutions and kinetics of IFN-γ production in whole blood stimulation assays

Previous studies have measured cytokine production in 1:5 and 1:10 diluted whole blood cultures stimulated for 7 days with mitogens (25) and recombinant Mtb proteins expressed in *E. coli* (10, 12, 14, 15). To determine the optimal blood dilutions and the kinetics of IFN-γ production for our panel of Mtb peptide pools, blood was collected from individuals with LTBI and stimulated with peptide pools corresponding to the immunodominant Mtb Ags CFP-10, ESAT-6, and TB10.4. Blood was used undiluted (200μl blood/well), or diluted with RSA medium at 1:2, 1:5, and 1:10 (in a final volume of 200μl of diluted blood/well). Peptide pools were stimulated in duplicate wells, with 4 replicate plates set up for harvesting supernatants on days 1, 3, 5, and 7. For all three Mtb peptide pools in both individuals tested, IFN-γ production, as measured by ELISA, was low to undetectable in 1:10 diluted cultures, compared with undiluted, 1:2, and 1:5 diluted blood (Fig 2).

Harvesting of supernatants from each of the indicated blood dilutions at multiple time points indicated IFN-γ production was detectable as early as day 1, with peak detection by day 5 (Fig 2). Given the similarities in concentrations of IFN-γ between days 5 and 7, combined with the logistical consideration of harvesting supernatants on weekdays and not weekends, we opted to proceed with harvesting of diluted blood supernatants on day 7.

The data above indicated diminished detection of Mtb-specific IFN-γ production in 1:10 diluted blood cultures, thus 1:10 diluted blood was excluded from further testing. In addition, we excluded undiluted blood, which would not be feasible given the large volumes of blood that would be required to test the full panel of 60 Mtb Ags and controls. Therefore, we proceeded with the next phase of optimization comparing 1:2 and 1:4 diluted blood.

Selection of Ag concentrations in the T cell RSA

We next evaluated the concentrations of Mtb peptide pools and PHA for use in the T cell RSA. Stimulation of 1:10 diluted blood for 7 days with peptide pools at a final concentration of 1μg/ml per peptide has been previously described (16). Using 1:2 diluted blood from individuals with LTBI, we measured IFN-γ production by ELISA in cultures stimulated for 7 days with 2μg/ml, 1μg/ml, and 0.5μg/ml of CFP-10, ESAT-6, and TB10.4 peptide pools (Fig 3). For the three Mtb peptide pools tested, IFN-γ production in response to 1μg/ml of peptide pools was not inferior to 2μg/ml of peptide pools (Fig 3A–C). However, IFN-γ production was lower following stimulation with 0.5μg/ml ESAT-6 peptide pool, compared with 2μg/ml and 1μg/ml (Fig 3B). Therefore, to increase the potential of detecting low magnitude Mtb-specific T cell responses, and to economize use of peptides synthesized for large cohort studies, we opted to proceed with a final peptide pool concentration of 1μg/ml for use in the T cell RSA.

To demonstrate that the IFN-γ production measured following stimulation of diluted whole blood is indeed Ag-specific, we stimulated 1:2 diluted blood from healthy QFT⁻ adults with CFP-10, ESAT-6, and TB10.4 peptide pools. In contrast to QFT⁺ individuals with LTBI, significant IFN-γ production was not detected to any of the three Mtb peptide pools, at any of the concentrations tested (Fig 3A–C).

Lastly, we determined optimal concentrations of the positive control (PHA) in the T cell RSA. In both QFT⁺ and QFT⁻ individuals, stimulation of 1:2 diluted blood with 1μg/ml PHA induced significantly lower levels of IFN-γ, compared with 5μg/ml PHA (Fig 3D). All individuals tested had a positive IFN-γ response to stimulation with 5μg/ml PHA, thus this concentration was selected for use in subsequent assays.

Evaluation of 1:2 and 1:4 diluted blood following 7-day stimulation with a panel of 20 Mtb peptide pools

The initial optimization experiments described above were conducted using a small panel of immunodominant Mtb peptide pools: CFP-10, ESAT-6, and TB10.4. A major goal in establishing the T cell RSA was to enable testing of a large panel of Ags while minimizing the amount of blood required. To address these issues, our next set of experiments incorporated stimulation of 1:2 and 1:4 diluted blood with 20 Mtb peptide pools. In order to select 20 Ags in an unbiased manner, we chose every third Ag from our consecutive list of

Mtb Ags in Table 1. Using heparinized blood samples collected from a cohort of 15 QFT[+] individuals, we first evaluated the performance of 1:2 and 1:4 diluted blood in the negative (no Ag) and positive control (PHA) conditions. There was no difference in IFN-γ production in direct comparisons of 1:2 and 1:4 diluted blood for the negative and positive controls (Fig 4A). Next, we correlated IFN-γ production in 1:2 versus 1:4 diluted blood stimulated with the selected panel of 20 Mtb peptide pools. There was a significant positive correlation between IFN-γ production to Mtb peptide pools following stimulation with 1:2 and 1:4 diluted blood from QFT[+] individuals (Fig 4B).

To evaluate intra-assay variability, each of the 20 Mtb peptide pools was tested in triplicate wells of the 96-well plate. Of the Mtb peptide pools eliciting a response greater than 50pg/ml of IFN-γ, the median coefficient of variation (CV) of IFN-γ across the triplicate wells was similar when comparing 1:2 and 1:4 diluted blood (25% and 29%, respectively, Fig 4C). The intra-assay variability was significantly lower than inter-individual variation in IFN-γ responses to the 20 Mtb peptide pools in this cohort of QFT[+] individuals (Fig 4C), thus indicating heterogeneity in the pattern of Mtb T cell Ag recognition in individuals with LTBI.

We next evaluated whether 96-well plates containing Mtb peptide pools and PHA could be prepared in batch and frozen at −80°C prior to use. There were no differences in IFN-γ production in the negative controls, PHA, or Mtb peptide pool stimulations when adding 1:2 or 1:4 diluted blood to freshly prepared Ag plates, or Ag plates that had been previously prepared in batches, frozen, and then thawed just prior to blood collection (Fig 5 and data not shown). Taken together, these results indicate that 1:4 diluted blood correlates well with, and is not inferior to, 1:2 diluted blood when evaluating IFN-γ production following 7-day stimulation with a panel of 20 Mtb peptide pools. Moreover, large quantities of 96-well plates containing Mtb peptide pools and PHA can be pre-prepared in batch and frozen prior to addition of the diluted blood, thus further reducing intra-assay variability and facilitating on-site assay standardization for large cohort studies.

Reproducibility of the T cell RSA

From the experiments described above, we concluded that 1:4 diluted blood could be used to measure T cell IFN-γ production to Mtb peptide pools with an intra-assay variability that is substantially lower than the inter-individual variability. One of the intended applications of the T cell RSA is in longitudinal cohort studies. Thus, in order to evaluate the reproducibility of the T cell RSA, we collected blood from 15 QFT[+] individuals at three separate time points, spaced at weekly intervals. The T cell RSA was conducted using 1:4 diluted blood collected at each of the three study visits, and stimulated with the panel of the 20 Mtb peptide pools depicted in Fig 4. IFN-γ production to these 20 Mtb peptide pools was highly consistent across each of the three time points (Fig 6).

Application of the T cell RSA to evaluate Mtb-specific T cell responses in cross-sectional and longitudinal cohorts

We next initiated testing of the T cell RSA using the full panel of 60 Mtb peptide pools in cohorts recruited at study sites in the US and in a TB-endemic setting in Kisumu, Kenya. A

schematic overview of the fully optimized T cell RSA is shown in Fig 1. In a cohort of Mtb-unexposed, QFT⁻ adults from the metropolitan Atlanta area, the median IFN-γ production to stimulation with our panel of 60 Mtb peptide pools ranged from 0 – 41 pg/ml, with 75% of the Mtb peptide pools eliciting a median IFN-γ response of 0 pg/ml (Fig 7A). All Mtb-unexposed individuals had a maximal IFN-γ response to PHA (>1,000 pg/ml) in this assay.

We next evaluated the performance of the T cell RSA in a cohort of refugees with LTBI who had recently arrived in the US and were resettled in the metro-Atlanta area. In contrast with the healthy, uninfected individuals in Atlanta, multiple Mtb peptide pools elicited high concentrations of IFN-γ in the refugee LTBI cohort, with many individuals displaying maximal responses >1,000 pg/ml to individual Mtb peptide pools (Fig 7B). Ag85A (pool 57) elicited the highest median concentration of IFN-γ in this cohort, followed by ESAT-6 (pool 59).

We also evaluated the feasibility of establishing the T cell RSA in a TB-endemic region in Kisumu, Kenya. Multiple Mtb peptide pools elicited a positive IFN-γ response in the Kenyan LTBI cohort, although the responses were generally lower in magnitude than the refugee LTBI cohort in Atlanta. EspI (pool 60) elicited the highest median concentration of IFN-γ, followed closely by Ag85A (pool 57); all individuals in the Kenyan LTBI cohort elicited a maximal IFN-γ response (>1,000 pg/ml) to stimulation with PHA (Fig 7C).

Lastly, we evaluated the performance of the T cell RSA in a longitudinal cohort of 10 QFT⁺ household contacts of a smear⁺ active TB patient in Kisumu, sampled every 6 months over the course of a year (Fig 8). As indicated in the heat map in Fig 8, Mtb peptide pool stimulation induced IFN-γ production consistently across the three time points in these individuals. For example, PPE26 (pool 26) and PPE51 (pool 52) elicited a maximum IFN-γ response (>1,000 pg/ml) in donor 12 at enrollment (baseline), month 6, and again at month 12, whereas pool 30 (Rv1954c) elicited strong IFN-γ responses in this same donor at baseline and month 6, but decreased by month 12 (Fig 8). Together these data indicate that the T cell RSA can be applied at several diverse study sites, and can be reliably implemented in longitudinal cohort studies to monitor the spectrum of Mtb-specific T cell responses over time; such tools are particularly useful in monitoring individuals during treatment for LTBI or active TB disease, and monitoring changes in T cell responses over time that are associated with or predict Mtb infection outcome.

Discussion

Infection with Mtb induces a broad and heterogeneous Ag-specific T cell response, yet many questions remain to be addressed before comprehensive T cell signatures of successful immune control of Mtb infection can be defined. Among these knowledge gaps is an incomplete understanding of the relationship between the breadth and specificity of the Mtb-specific T cell response and infection outcome, and how this response changes with TB disease progression, anti-TB treatment, or vaccination. Previous studies on immunogenicity of multiple Mtb Ags in Mtb-infected individuals have utilized either diluted whole blood stimulation assays with IFN-γ secretion detected by ELISA (9, 10, 12, 15, 16), or PBMC-based IFN-γ ELISpot assays (19, 20). We have established a reproducible and high

throughput diluted whole blood assay to further refine the immunological tools for monitoring multiple Mtb-specific T cell responses simultaneously that could be applicable to large cohort studies, multi-site studies, and longitudinal studies of either natural history of infection, vaccination, or treatment interventions.

Important initial considerations in development of an assay for measurement of T cell responses to a spectrum of Mtb Ags were sample source and volume. In order to minimize the amount of sample processing and operator variability, we opted to utilize a diluted whole blood-based assay, initially described as a tool for measuring human cytokine production profiles following mitogen stimulation of 1:10 diluted blood cultures (25). Similar assays have subsequently been conducted for measurement of immunogenicity of recombinant Mtb Ags in 7-day, 1:10 diluted blood stimulation assays (10, 12, 14, 15). While assays utilizing 1:10 diluted blood have the advantage of using minimal amounts of blood, the potential loss of detection IFN-γ-secreting Mtb-specific T cells with increasing blood dilutions had not been systematically evaluated in these previous reports. By comparing levels of IFN-γ secretion to three Mtb peptide pools across multiple blood dilutions from the same individual, we determined that detection of IFN-γ in culture supernatants was substantially diminished following stimulation of 1:10 diluted blood with Mtb peptide pools, compared with 1:5, 1:2, and undiluted blood. Expanding our analysis to a broader panel of 20 different Mtb peptide pools, we determined that 1:4 diluted blood was not inferior to 1:2 diluted blood in our ability to detect Mtb-specific T cell IFN-γ secretion. In an assay volume of 200μl per well of a 96-well plate, this corresponds to 50μl of blood per test condition in 1:4 diluted blood assays, versus 100μl of blood in 1:2 diluted blood assays. Using a final blood dilution of 1:4 enables evaluation of 60 Mtb Ags, as well as controls, with a total volume of 3.5ml blood. Assays evaluating Ag-specific T cell responses to multiple Ags simultaneously, using small amounts of blood and no prior cell processing, are highly advantageous in pediatric studies in which blood volumes are limited, as well as resource limited settings that lack the capacity to isolate and store cryopreserved PBMCs.

In addition to sample type and volume, another key consideration in establishment of this assay was the nature of the Mtb Ag used to stimulate T cells. Our goal is to define the Ag specificity of Mtb-specific T cell responses down to the epitope level, thus we opted to pursue assay optimization using 18-mer peptides overlapping by 11aa, and pooled by Ag. Most previous studies using 1:10 diluted blood assays to identify immunogenic Mtb Ags have stimulated with recombinant Mtb proteins, expressed in *E. coli* (9, 12, 15, 26). However, this approach does not directly facilitate further downstream identification of specific T cell epitopes targeted; moreover, bacterial products from the protein expression process may also elicit responses by cells other than Mtb-specific T cells. Previous studies utilizing synthetic peptides to screen Mtb-specific CD4 T cell responses have utilized HLA binding prediction algorithms to synthesize predicted epitopes (19, 20), although this approach primarily detects epitopes that bind to multiple HLA class II alleles. To facilitate use of the assay in diverse international settings and cohorts for which HLA types may not already be well-defined, we synthesized 18-mer overlapping peptides spanning the length of each Ag to increase the likelihood of detecting any Mtb-specific T cell response within a given Ag. Large peptide libraries consisting of 15-mer peptides overlapping by 9aa have

been used successfully to comprehensively define Ags and epitopes targeted by human Mtb-specific CD8 T cells (27–29).

A critical component of any assay for measurement of Ag-specific T cell responses is intra- and inter-assay variability. Testing our panel of 60 Mtb Ags and controls in a 96-well plate format necessitated that each Mtb peptide pool be tested in a single well. During the optimization phase, we measured IFN-γ concentrations for a subset of 20 Mtb peptide pools tested in triplicate wells, which indicated an intra-assay CV of 29%. Using the same subset of 20 Mtb peptide pools tested in triplicate wells for intra-assay variability, we also conducted repeat assays at three separate time points, spaced at weekly intervals, which also indicated a high level of reproducibility and low inter-assay variability. Previous studies screening T cell responses to broad panels of Mtb Ags have compared groups of individuals with different states of Mtb infection in a cross-sectional manner (9, 10, 12, 15, 16, 19), and thus intra-assay variability and assay reproducibility had not been previously well established.

In contrast with commercially available IGRAs, which contain a pool of CFP-10 and ESAT-6 peptides (30), or the mega-pool approach, which pools hundreds of peptides from dozens of different mycobacterial Ags in a single peptide pool (31), the T cell RSA provides information on responses to individual Mtb Ags, which can be tracked and monitored on a per-Ag basis over time. We therefore anticipate that the T cell RSA will be advantageous as a tool to test new hypotheses, including the hypothesis that T cell responses to individual Mtb Ags vary in distinct and discordant ways that may depend on the clinical or microbiological state of infection. In support of this hypothesis, a recent study reported that CD4 T cells targeting ESAT-6 and Ag85B have distinct phenotypic and functional profiles, and differential capacity to mediate protection against Mtb (32). Moreover, evaluating T cell responses to Mtb Ags associated with different phases of infection, such as latency-associated Ags or resuscitation-promoting factor-like proteins, may enable differentiation of individuals across a range of Mtb infection states, (12–14), and may facilitate identification of individual Mtb Ags that have prognostic value in predicting progression to TB disease in prospective longitudinal cohort studies.

Using cohorts of Mtb-unexposed US adults, foreign-born refugees with LTBI who were re-settled in the US, and a population of Kenyan adults with LTBI who are household contacts of an active TB case, we have applied this T cell RSA to begin to identify differences in the pattern of Ag recognition by Mtb-specific T cell responses across these diverse cohorts. Our preliminary findings indicate that differences in the breadth and magnitude of Mtb-specific T cell responses are evident in populations of individuals with LTBI from diverse geographical regions. Our preliminary findings are consistent with previous studies screening T cell responses to multiple Mtb Ags in geographically diverse populations worldwide (10, 16, 19). Differences in the spectrum of T cell Ag recognition in Mtb infection are likely driven by multiple factors, including host genetics, environmental mycobacteria exposure, microbiome composition, nutritional status, and co-infection with other pathogens. Further studies incorporating HLA typing and TCR sequencing will be important for defining host factors influencing the patterns of Mtb T cell Ag recognition and immunodominance in human Mtb infection (33, 34).

Several limitations of our diluted whole blood T cell RSA should be considered. First, by utilizing 50μl of whole blood per stimulation condition, it is possible that the frequency of a given Ag-specific T cell population may be below the threshold of detection using this small volume of blood. However, the stimulation period of 7 days allows for proliferation of Ag-specific T cells, thus increasing the likelihood that sufficient IFN-γ will be secreted during the course of the assay to be detectable above background in the ELISA. Secondly, as with previous Mtb Ag screening assays, the functional readout is IFN-γ secretion, thus Mtb-specific T cells that produce cytokines other than IFN-γ will not be detected. Sufficient supernatants are harvested from each well to store for future analyses using multiplex bead-based arrays to detect a much broader panel of cytokines and chemokines secreted by Ag-specific T cell populations. Third, the ability to precisely quantify IFN-γ concentrations in supernatants from individuals with very strong Mtb-specific T cell responses is blunted by the dynamic range of the standard curve in the ELISA. The IFN-γ ELISAs in this study were conducted using 2-fold diluted RSA supernatants to perform a qualitative evaluation of IFN-γ production. However, individuals with high Mtb-specific T cell IFN-γ production (>1,000pg/ml) will require the ELISA to be repeated at higher dilutions of supernatants in order for these IFN-γ concentrations to be within the dynamic range of the standard curve for accurate and precise quantification. Additionally, there is a high degree of homology between Mtb and other mycobacterial species, including *M. bovis* bacillus Calmette-Guerin (BCG) vaccine strains, and a high level of sequence conservation in T cell epitopes in Mtb and BCG (21, 35). Given that 57 of the 60 Mtb Ags we tested are expressed by both Mtb and BCG, and that universal childhood BCG vaccination is recommended in over 150 countries worldwide (36), it is possible that this assay will detect T cell responses primed by BCG vaccination, as well as Mtb infection. However, previous studies evaluating mycobacteria-specific T cell responses indicate significantly higher frequency and magnitude of Ag-specific T cell responses in individuals with LTBI, compared with Mtb-uninfected individuals who have been vaccinated with BCG (19), thus suggesting T cell responses primed by BCG do not predominate over Ag-specific T cell responses detectable in the setting of natural infection with Mtb.

In summary, we describe here the development of a standardized diluted whole blood-based assay platform for evaluation of multiple Ag-specific T cell responses, with conditions optimized to incorporate additional Ags, and the ability to be conducted in resource limited settings, using small blood volumes. The intra-assay variation is lower than the inter-assay variation, thus allowing conclusions to be drawn in terms of biological variability that may reveal novel information on the breadth and spectrum of Mtb Ag recognition that is associated with protection from progression to TB disease. We anticipate that this assay will be applicable in multiple settings, including cross-sectional studies across multiple study sites and longitudinal cohort studies to monitor Ag-specific T cell responses associated with Mtb infection outcomes, treatment interventions, vaccination, and possibly assessment of Mtb exposure and/or re-infection.

Acknowledgments

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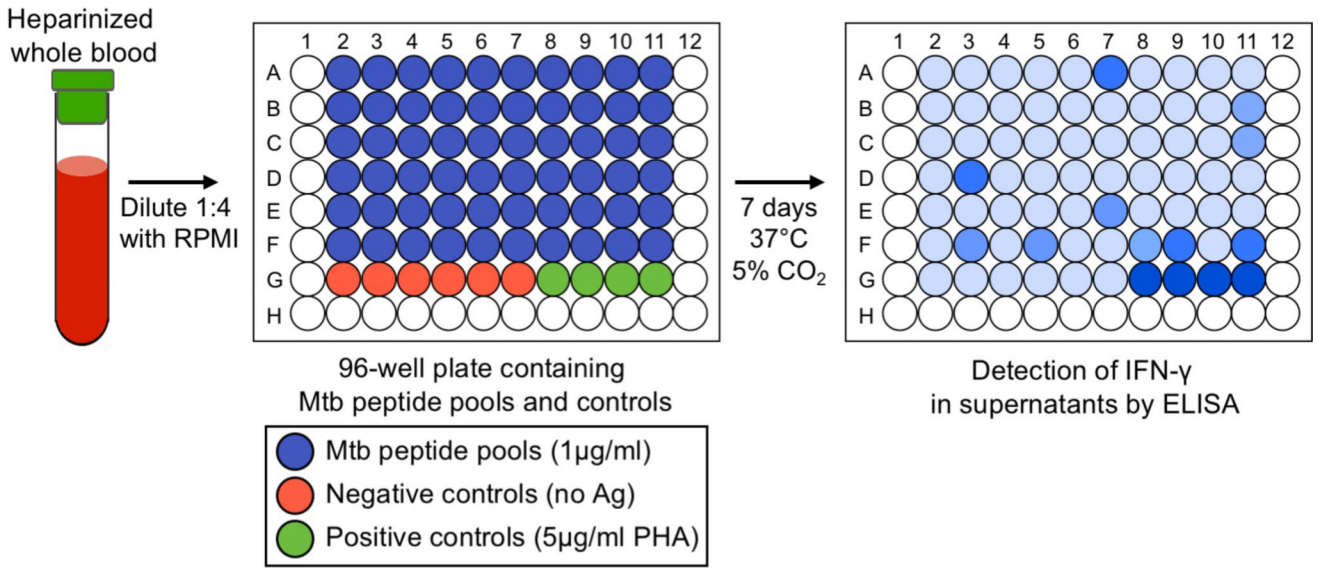
Figure 1. Schematic summary of the T cell RSA

Fresh, heparinized whole blood is diluted 1:2 with RPMI-1640; 100μl of 1:2 diluted blood is added to each well containing 100μl of RPMI-1640 containing Mtb peptide pools (blue wells), negative controls containing media alone with no antigen (red wells) and positive controls containing PHA (green wells). The final dilution of blood in each well is 1:4. The plates are incubated at 37°C in a 5% CO_2 incubator for 7 days. Plates are then centrifuged and plasma supernatants are removed for measurement of IFN-γ production by ELISA.
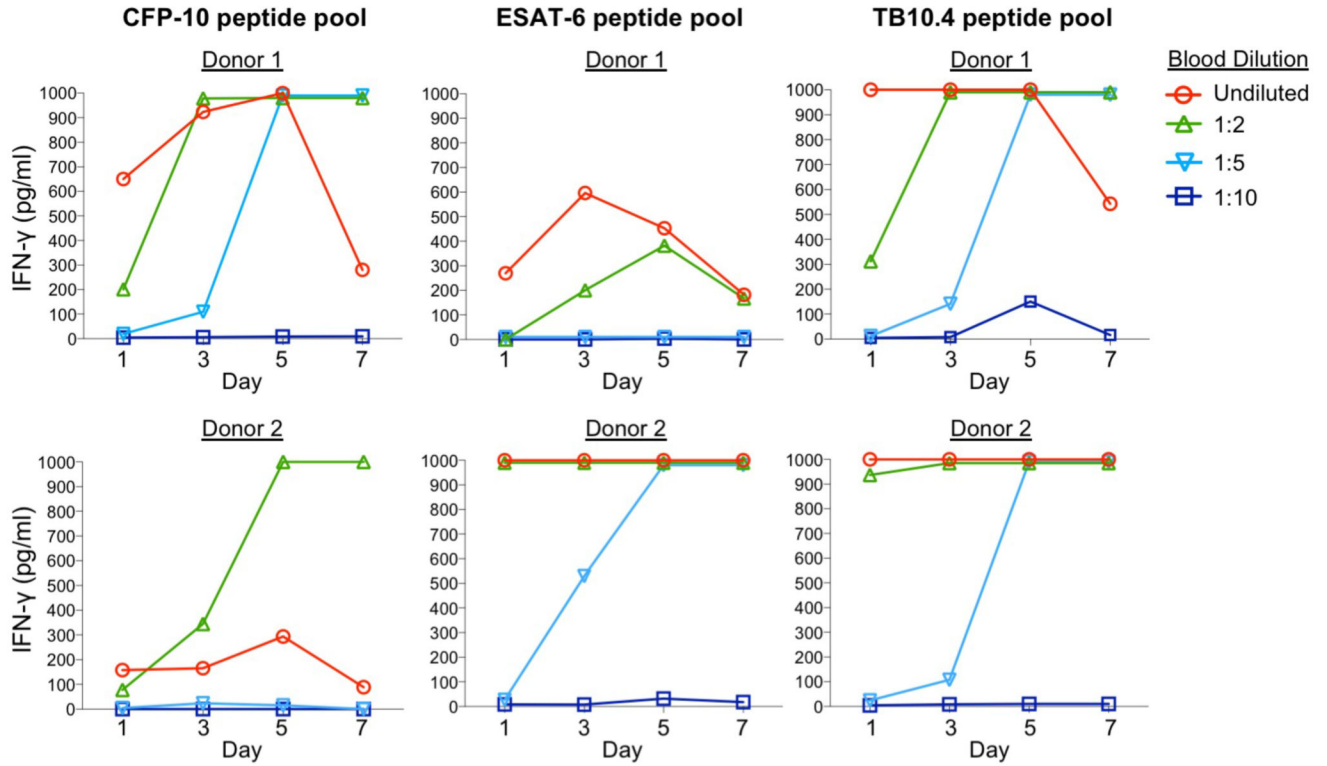
Figure 2. Whole blood dilutions and kinetics of IFN-γ production following stimulation with Mtb peptide pools

Fresh whole blood from two QFT[+] individuals was used either undiluted (red circles), or diluted 2-fold (green triangles), 5-fold (light blue triangles), and 10-fold (dark blue squares) with RPMI-1640. CFP-10, ESAT-6, and TB10.4 peptide pools were added to each dilution in 96-well plates, and the plates incubated at 37°C for the indicated number of days. On days 1, 3, 5, and 7, plates were centrifuged and plasma was removed for measurement of IFN-γ in supernatants by ELISA. Data from the first donor is shown in the top row; data from the second donor is shown in the bottom row.
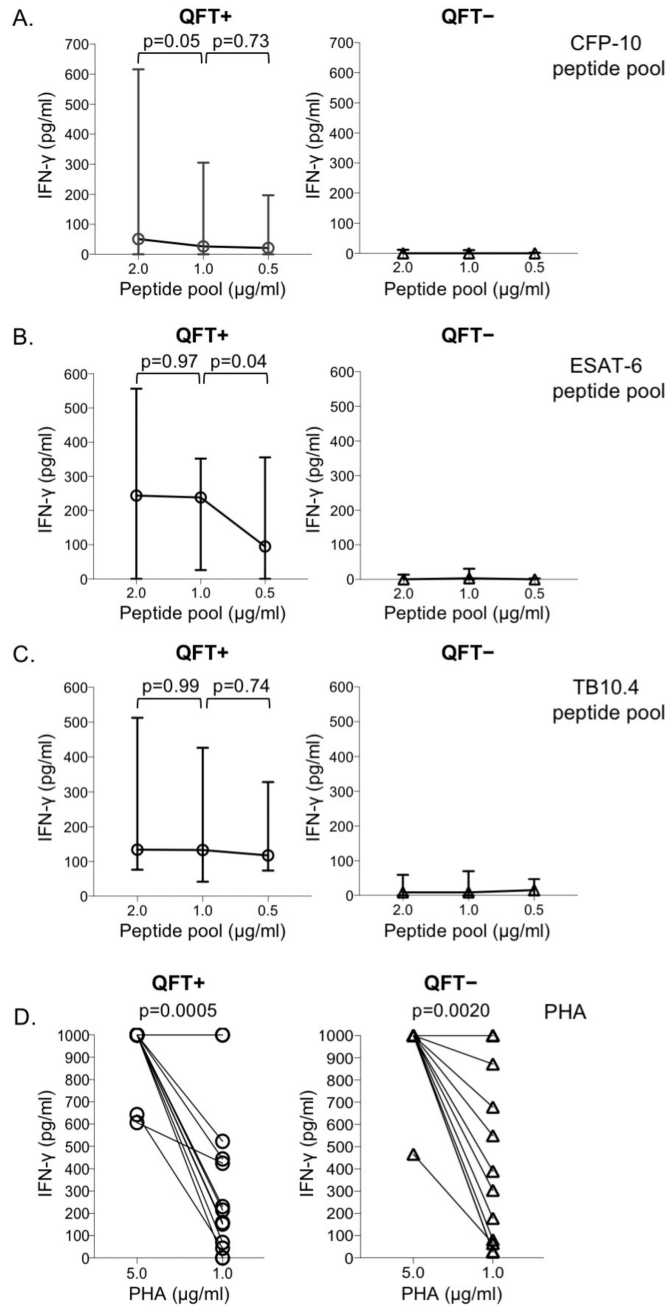
Figure 3. Optimization of antigen concentration in the whole blood stimulation assay
Fresh whole blood was collected from 15 QFT[+] and 13 QFT[−] individuals. Blood was diluted 2-fold with RPMI-1640 and incubated with the indicated concentrations of CFP-10 (A), ESAT-6 (B), and TB10.4 (C) peptide pools, and with PHA (D), for 7 days. Plasma supernatants were harvested on day 7 for measurement of IFN-γ in supernatants by ELISA. The median and IQR are shown in panels A – C. Differences in IFN-γ production between antigen concentrations were determined using the Wilcoxon matched-pairs signed rank test.
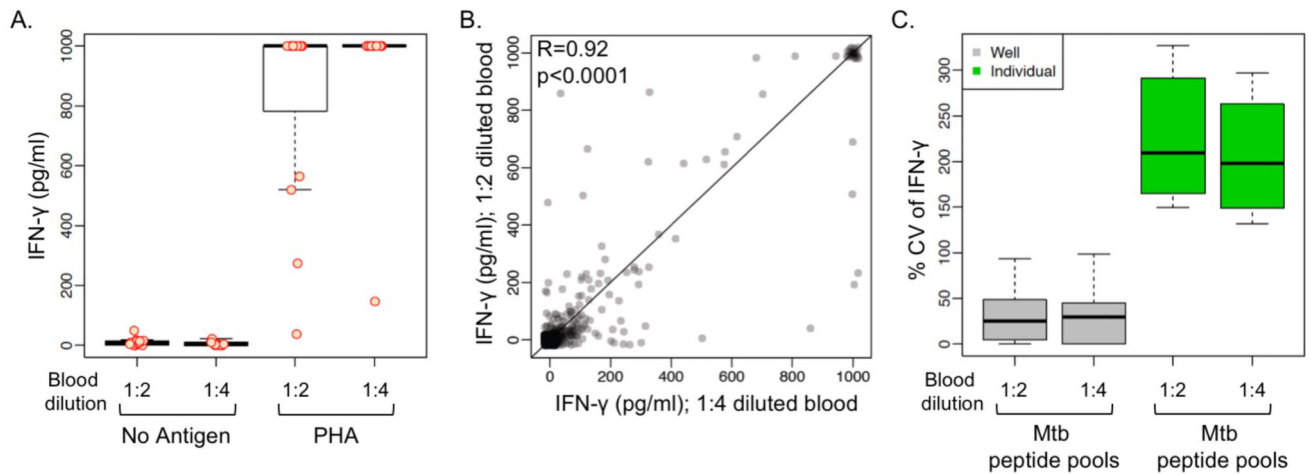
Figure 4. Comparison of IFN-γ production in 1:2 and 1:4 diluted whole blood multiple antigen RSAs

Fresh whole blood was diluted 2-fold and 4-fold with RPMI-1640. Diluted blood from 15 QFT[+] individuals was incubated with 20 different Mtb peptide pools and PHA for 7 days. Each peptide pool was measured individually in triplicate wells. Plasma supernatants were harvested on day 7 for measurement of IFN-γ in supernatants by ELISA. (A) Comparison of IFN-γ production in 1:2 and 1:4 diluted blood in the negative control wells (no antigen) and positive control wells (5μg/ml PHA). Box plots represent the median and IQR; outliers are shown as individual points. (B) Pearson's correlation between IFN-γ production in 2-fold and 4-fold diluted to 20 Mtb peptide pools. Each peptide pool was tested in triplicate, and the average IFN-γ production of the triplicate wells is shown for 2-fold and 4-fold diluted blood from the same individuals. The Kendall rank correlation coefficient was used to determine the p-value. (C) Coefficient of variation (CV) of IFN-γ production to 20 different Mtb peptide pools across triplicate wells of each peptide pool (intra-assay variation, grey box plots), and across individual donors (inter-individual variation, green box plots). The average IFN-γ production in the triplicate wells for each peptide pool in each individual was used to calculate the inter-individual CV of IFN-γ production.
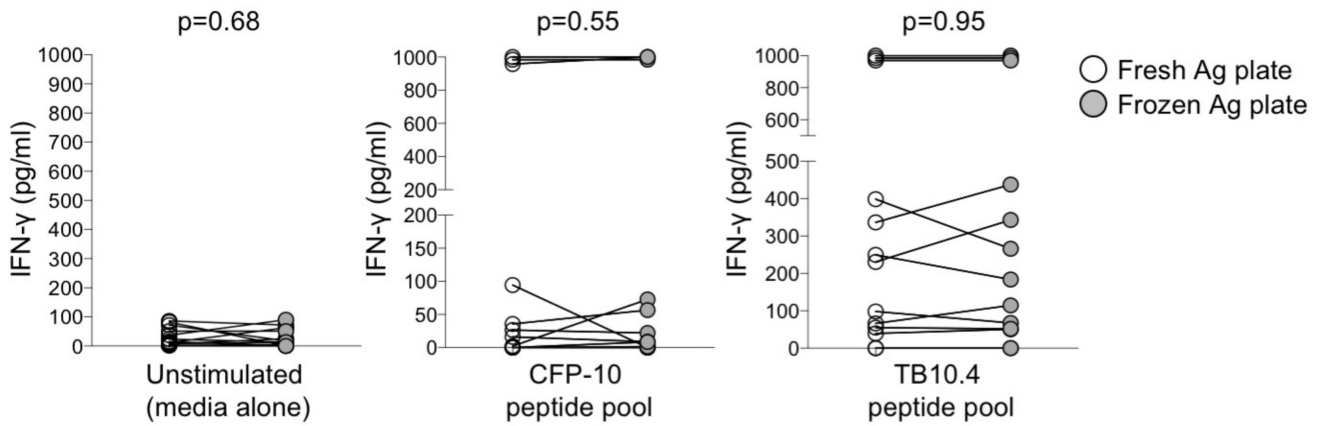
Figure 5. Comparison of IFN-γ production in frozen and freshly prepared RSA Ag plates
96-well plates containing Mtb peptide pools (CFP-10 and TB10.4) and PHA in RPMI-1640 were prepared and frozen at −80°C. On the day of blood draw, an identical set of 96-well plates were freshly prepared with Mtb peptide pools and PHA in RPMI-1640. Fresh blood was diluted 1:4 in RPMI-1640 and added to the 96-well plates that had been previously prepared ('Frozen Ag plate', open circles; plates were thawed at room temperature on the day of blood collection), as well 96-well plates that had been prepared with Ags just prior to blood collection ('Fresh Ag plate', filled circles). Plasma supernatants were harvested on day 7 for measurement of IFN-γ in supernatants by ELISA. Comparisons of IFN-γ production in the fresh and frozen antigen plates were done using the Wilcoxon matched-pairs signed rank test.
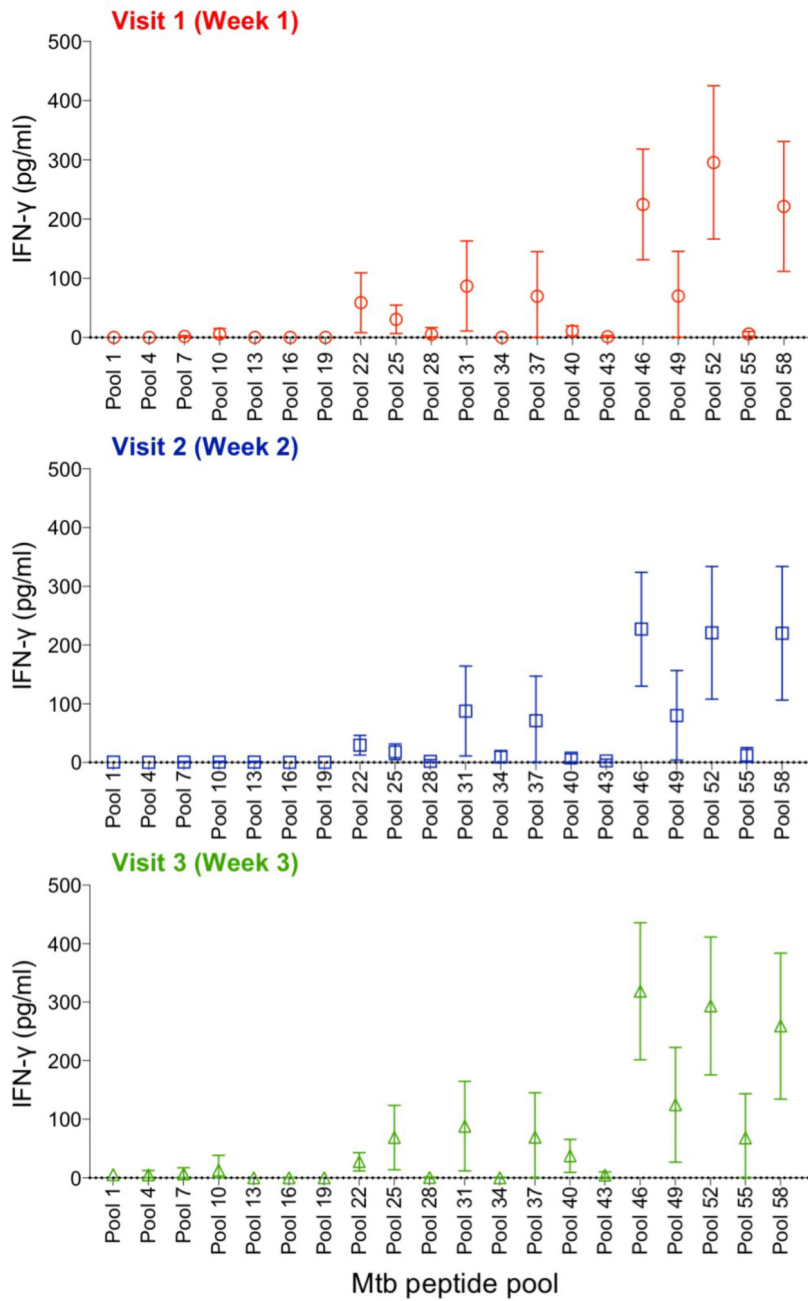
Figure 6. Reproducibility of the T cell RSA across three study visits
Blood was collected from 15 QFT[+] individuals at three time points, spaced at weekly intervals (Visit 1, Visit 2, and Visit 3). Blood was diluted 1:4 in RPMI-1640 and stimulated for 7 days with 20 Mtb peptide pools and PHA. Background IFN-γ production in the negative control wells was subtracted from the Ag-stimulated wells. The mean and 95% confidence level of IFN-γ production are shown for each peptide pool at each study visit for 15 QFT[+] individuals.
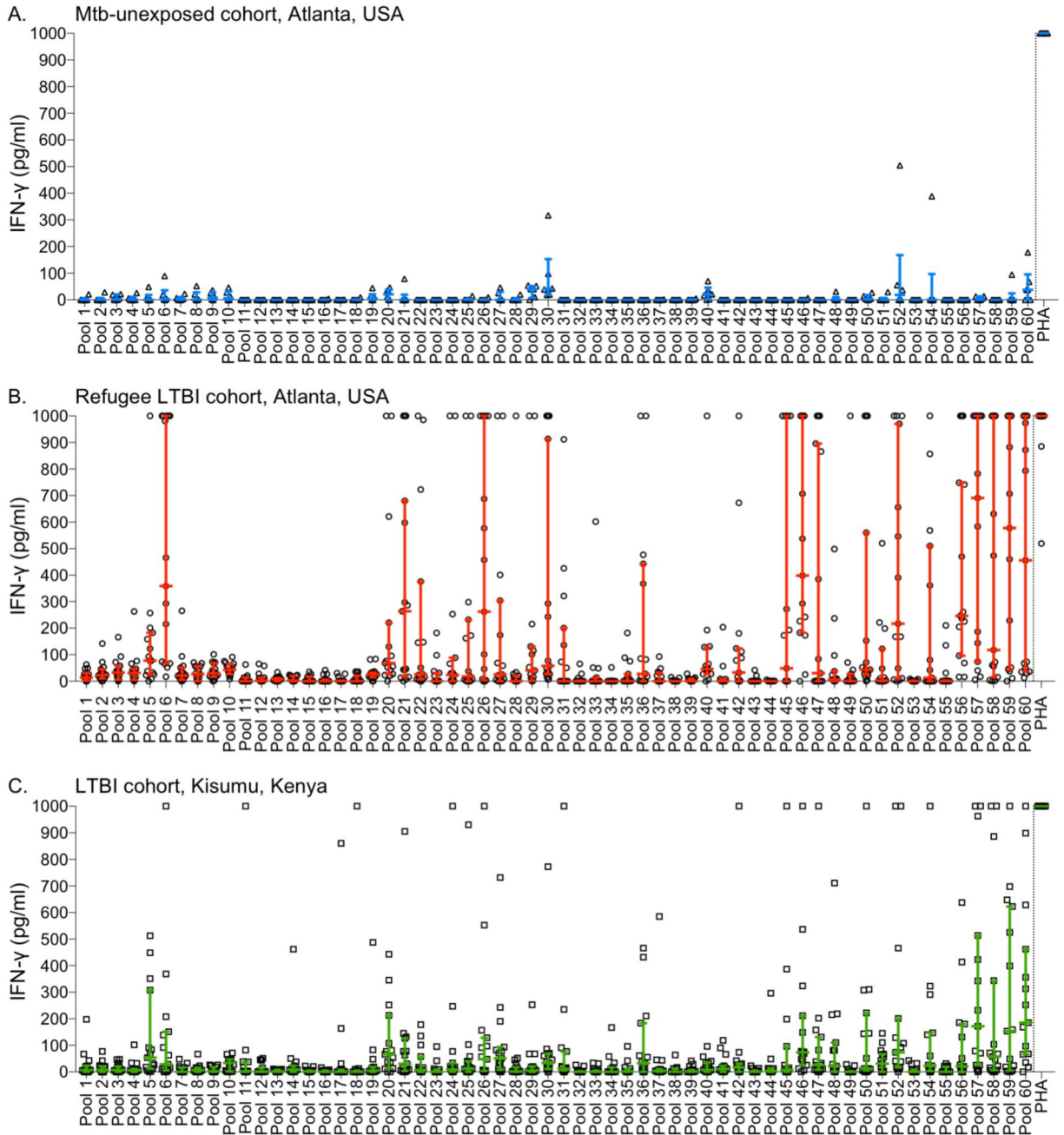
Figure 7. Application of the T cell RSA to Mtb-unexposed and LTBI cohorts across international study sites

The whole blood multiple antigen response spectrum assay was conducted with 1:4 diluted blood from 6 Mtb-unexposed healthy donors enrolled in Atlanta, GA (A), 15 QFT[+] individuals enrolled in a refugee cohort in Atlanta, GA (B), and 15 QFT[+] individuals enrolled in Kisumu, Kenya (C). None of the healthy donors have been vaccinated with BCG. Of the 15 QFT[+] individuals enrolled in Atlanta, 10 have been vaccinated with BCG, 3 have not been vaccinated, and 2 are unsure if they have received the BCG vaccine. All QFT[+] individuals enrolled in Kenya have been vaccinated with BCG. Diluted blood was stimulated with 60 Mtb peptide pools and PHA. Results are shown after subtraction of background

IFN-γ production in the negative control wells. Horizontal bars represent the median; vertical bars represent the interquartile range.
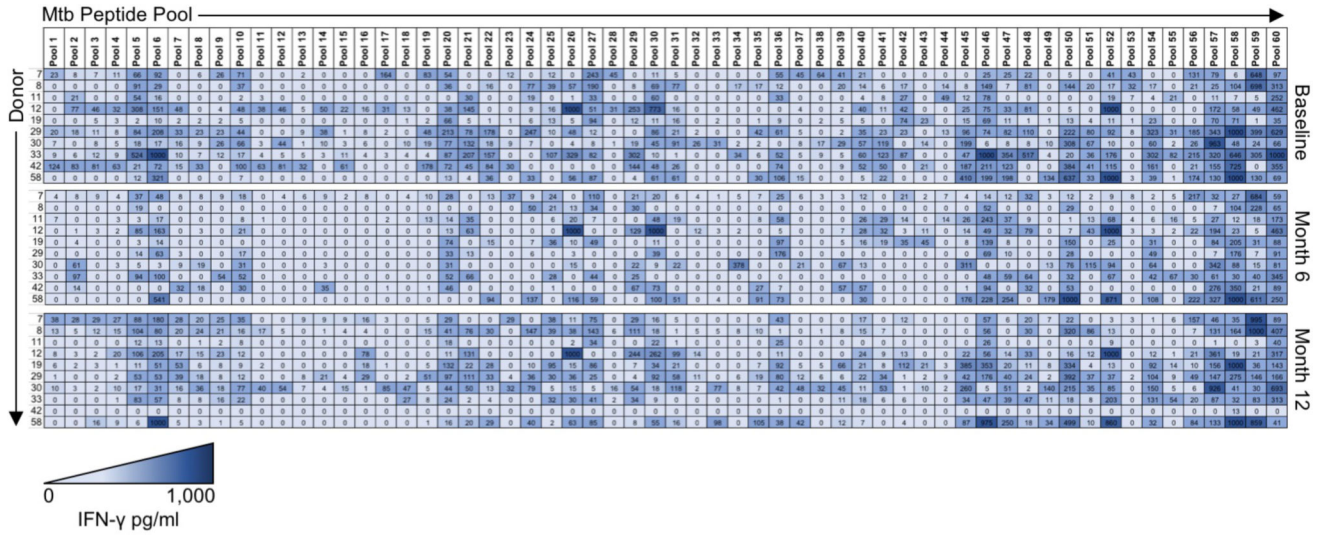
Figure 8. Application of the T cell RSA to longitudinal cohorts of individuals with LTBI in a TB-endemic setting in Kisumu, Kenya

The whole blood multiple antigen RSA was conducted with 4-fold diluted blood from 10 QFT[+] individuals with LTBI enrolled in Kisumu, Kenya, at three time points: enrollment (Baseline, top row), month 6 (middle row) and month 12 (bottom row). All individuals have been vaccinated with BCG. Diluted blood was stimulated with 60 Mtb peptide pools and PHA; the numbers in each cell represent the concentration of IFN-γ after subtraction of the background IFN-γ production in the negative control wells. Data are shown as a heat map, with low IFN-γ levels shown in light blue, and increasing concentrations of IFN-γ indicated by darker blue cells. The 60 Mtb peptide pools are shown in the columns; the same 10 individuals are shown consecutively in the same order in each row for the three time points.

Table 1

M. tuberculosis T cell antigen panel

Peptide Pool Number	Gene	Protein	Functional category[*]	Bacterial fraction
Pool 1	Rv0010c	Rv0010c	1	Membrane
Pool 2	Rv0012	Rv0012	1	Secreted
Pool 3	Rv0062	CelA1	2	Secreted
Pool 4	Rv0093c	Rv0093c	1	Membrane
Pool 5	Rv0287	EsxG	1	Secreted
Pool 6	Rv0288	EsxH/TB10.4	1	Secreted
Pool 7	Rv0289	EspG3	1	Unknown
Pool 8	Rv0290	EccD3	1	Membrane
Pool 9	Rv0291	MycP3 protease	2	Membrane
Pool 10	Rv0292	EccE3	1	Membrane
Pool 11	Rv0293c	Rv0293c	6	Cytoplasm
Pool 12	Rv0294	Tam	2	Cytoplasm
Pool 13	Rv0298	Rv0298	3	Cytoplasm
Pool 14	Rv0299	Rv0299	6	Unknown
Pool 15	Rv0690c	Rv0690c	2	Unknown
Pool 16	Rv0985c	MscL	1	Membrane
Pool 17	Rv0987	Rv0987	1	Membrane
Pool 18	Rv0995	RimJ	4	Cytoplasm
Pool 19	Rv1172c	PE12	PE family	Membrane
Pool 20	Rv1195	PE13	PE family	Cytoplasm
Pool 21	Rv1196	PPE18	PPE family	Membrane
Pool 22	Rv1198	EsxL	1	Secreted
Pool 23	Rv1366	Rv1366	2	Cytoplasm
Pool 24	Rv1471	TrxB1	2	Cytoplasm
Pool 25	Rv1788	PE18	2	Unknown
Pool 26	Rv1789	PPE26	PPE family	Membrane
Pool 27	Rv1791	PE19	PE family	Unknown
Pool 28	Rv1872c	LldD2	2	Cell wall, membrane
Pool 29	Rv1886c	Ag85B	5	Secreted
Pool 30	Rv1954c	Rv1954c	6	Unknown
Pool 31	Rv1955	HigB	3	Unknown
Pool 32	Rv1957	Rv1957	6	Cytoplasm
Pool 33	Rv2022c	Rv2022c	6	Membrane
Pool 34	Rv2024c	Rv2024c	6	Unknown
Pool 35	Rv2031c	HspX	3	Cytoplasm, membrane
Pool 36	Rv2345	Rv2345	1	Membrane

Peptide Pool Number	Gene	Protein	Functional category[*]	Bacterial fraction
Pool 37	Rv2714	Rv2714	1	Membrane
Pool 38	Rv2719c	Rv2719c	1	Predicted membrane
Pool 39	Rv2823c	Rv2823c	6	Membrane ± secreted
Pool 40	Rv2853	PE-PGRS48	PE-PGRS family	Unknown
Pool 41	Rv2874	DipZ	2	Membrane
Pool 42	Rv2875	MPT70	1	Secreted
Pool 43	Rv2996c	SerA1	2	Membrane
Pool 44	Rv3012c	GatC	4	Membrane
Pool 45	Rv3015c	Rv3015c	4	Cytoplasm
Pool 46	Rv3018c	PPE46	PPE family	Unknown
Pool 47	Rv3019c	EsxR (TB10.3)	1	Predicted secreted
Pool 48	Rv3020c	EsxS	1	Predicted secreted
Pool 49	Rv3024c	TrmU	4	Unknown
Pool 50	Rv3025c	IscS	2	Membrane
Pool 51	Rv3135	PPE50	PPE family	Unknown
Pool 52	Rv3136	PPE51	PPE family	Membrane
Pool 53	Rv3221c	Tb7.3	5	Secreted
Pool 54	Rv3330	DacB1	1	Cell wall
Pool 55	Rv3418c	GroES	3	Cytosol, cell wall
Pool 56	Rv3615c	EspC	1	Membrane
Pool 57	Rv3804c	Ag85A	5	Secreted
Pool 58	Rv3874	EsxB (CFP-10)	1	Secreted
Pool 59	Rv3875	EsxA (ESAT-6)	1	Secreted
Pool 60	Rv3876	EspI	1	Membrane

[*] Functional categories: 1, cell wall and cell processes; 2, intermediary metabolism and respiration; 3, virulence, detoxification, adaptation; 4, information pathways; 5, lipid metabolism; 6, conserved hypothetical