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Immune profiling enables stratification of patients with active TB disease or *M. tuberculosis* infection

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Summary: We tested TruCulture, an immunomonitoring tool, to identify active disease from latent *Mtb* infection. TruCulture showed improved discrimination of TB cases from LTBI as compared to Quantiferon. TB stratification could be further improved by the *Mtb* Ag/BCG IFNγ ratio.
Abstract

Background: Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb) infection and is a major public health problem. Clinical challenges include the lack of a blood-based test for active disease. Current blood-based tests, such as QuantiFERON (QFT) do not distinguish active TB disease from asymptomatic *Mtb* infection.

Methods: We hypothesized that TruCulture, an immunomonitoring method for whole blood stimulation, could discriminate active disease from latent *Mtb* infection (LTBI). We stimulated whole blood from active TB patients and compared to LTBI donors. *Mtb*- specific antigens and live bacillus Calmette-Guerin (BCG) were used as stimuli, with direct comparison to QFT. Protein analyses were performed using conventional and digital ELISA, as well as Luminex.

Results: TruCulture showed discrimination of active TB cases from LTBI (p < 0.0001 AUC = 0.81) as compared to QFT (p = 0.45 AUC = 0.56), based on an IFNγ readout after *Mtb* antigen stimulation. This result was replicated in an independent cohort (AUC = 0.89). In exploratory analyses, TB stratification could be further improved by the *Mtb* Ag/BCG IFNγ ratio (p < 0.0001 AUC = 0.91). Finally, the combination of digital ELISA and transcriptional analysis showed that LTBI donors with high IFNγ clustered with TB patients, suggesting the possibility to identify sub-clinical disease.

Conclusions: TruCulture offers a next-generation solution for whole blood stimulation and immunomonitoring with the possibility to discriminate active and latent infection.

Keywords: Tuberculosis, Immune profiling, Patient stratification, cytokines, biomarkers
Introduction

Tuberculosis (TB) is a global public health problem, with an estimated 1.7 billion persons latently infected by *Mycobacterium tuberculosis* (*Mtbc*) \(^1\), \(^2\). Most newly infected individuals mount an effective immune response that controls infection, however the host response does not fully eliminate the bacteria resulting in a clinically asymptomatic state \(^3\). An estimated 5-10% of individuals with chronic infection progress to active disease at some point in their life, translating into approximately 10 million progressing to TB disease annually \(^3\).

Due to the burden of infected persons in endemic regions and the high risk of reinfection, treatment strategies typically prioritize patients with active disease with the goal to limit transmission. As a result, there is a critical need to diagnose active disease and to distinguish it from latent infection. Diagnosis of active TB disease can be achieved by microscopy, PCR or culture-based detection of *Mtbc* presence in sputum. However, many TB patients cannot produce sputum and it is preferable to utilize blood-based clinical assays, but available methods cannot reliably stratify active disease from latent infection. Whole blood assays can distinguish infected from uninfected persons, based on stimulation with *Mtbc* antigens, followed by an IFNγ assay. Such assays include the QuantiFERON–TB Gold (QFT) which utilizes *Mtbc*-specific antigens ESAT-6, CFP-10, and TB7.7 to stimulate immune cells in a blood collection tube with IFNγ secretion measured by ELISA, and T-SPOT.TB which uses similar antigens with IFNγ measured by ELISPOT. Potential sources of technical variability with QFT may include the range of blood volumes (0.8-1.2mL) and incubation times (16-24 hours) permitted in the manufacturer’s protocol, and recent studies have addressed these sources of variance \(^4\), \(^5\). Despite these improvements, QFT has limitations for use as a diagnostic in TB endemic countries, although it has been recently used to measure the efficacy end-point in a prevention of *M.tb* infection Phase II vaccine trial \(^6\) and is used to assess inclusion/exclusion criteria \(^7\), \(^8\).

We have previously described use of TruCulture® (TruC) devices, a syringe based whole blood collection and incubation system that allows immunomonitoring in response to diverse immune agonists, using proteomic \(^9\) or transcriptional \(^10\), \(^11\) assays. Specifically, we demonstrated greater reproducibility in multi-centre studies, as compared to PBMC stimulation \(^12\). Given these findings in healthy donors, we evaluated whether TruC is applicable for immunomonitoring of TB patients. As shown herein, we demonstrated the ability to more accurately classify patients with active disease and latently infected persons using TruC.
Methods

Participant groups

25 healthy adults with asymptomatic, latent *Mtb* infection (LTBI), defined by a positive QuantiFERON-TB Gold In-Tube (QFT+) assay (Qiagen, Germany), and 25 HIV-negative adults with TB disease (TB), defined by a positive sputum XpertMTB/RIF test (Cepheid, United States) were identified and recruited at the South African Tuberculosis Vaccine Initiative (SATVI), Worcester, South Africa. For the replication cohort: TB patients (n=51) enrolled in the PREDICT trial (Clinicaltrials.gov NCT02821832) at SATVI, were co-enrolled into this biomarker study. LTBI controls (n=9), recruited at SATVI and healthy donors (n=10), recruited in Paris, France, were also included. The TB clinical studies, protocols and informed consent forms were approved by the Human Research Ethics Committee of the University of Cape Town (ref: 234/2015). Written informed consent was obtained from all study participants. Additional cohort details are provided in the supplemental methods and Table 1.

Whole blood stimulations

TruC tubes (Myriad RBM) were batch-prepared and maintained at -20°C until time of use. To prepare TruC TB antigen tubes, 3 QFT TB antigen tubes (the QFT Gold In-tube system was used, as the study was performed prior to introduction of the QFT Gold Plus) were rinsed with 2mL of TruC media and the media transferred into empty TruC tubes to maintain the same concentration of *Mtb* antigens as found in QFT. Live bacillus Calmette-Guerin (BCG; Connaught strain, Sanofi Pasteur) tubes were prepared to have a final concentration of $10^5$ bacteria/mL.

Multi-analyte protein profiling

Supernatants from QFT and TruC tubes were analyzed for IFNγ by standard ELISA (Qiagen) and values were expressed in IU/mL, calculated by subtraction of values from the relevant non-stimulated controls and normalized for the dilution factor. Luminex xMAP technology was used to measure 32 proteins in the same samples (Myriad RBM). To detect low concentrations of IFNγ a homebrew Simoa ELISA was developed as previously described and detailed in the supplemental methods.
Nanostring transcriptional analysis

Nanostring gene expression analysis (Human Immunology V2 panel plus 30 TB related genes listed in Table S1) was performed following extraction of RNA from Trizol stabilized TruC cell pellets as previously described and detailed in the supplemental methods 10.

Results

Improved discrimination of patients using TruC TB Ag stimulation

To enable comparison between TruC and QFT, we transferred *Mtb* antigens from QFT into TruC tubes as described (see Methods). We sampled blood from active TB patients and LTBI persons, and measured induced IFNγ production utilizing ELISA. Confirming previous reports 15, QFT assays did not stratify TB and LTBI groups (Fig 1a, p=0.45). By contrast, TruC using the same *Mtb* antigens and IFNγ readout, showed a significantly higher response in TB patients as compared to LTBI controls (p < 0.0001, Fig 1b). Inclusion criteria for defining LTBI cases was based on historical QFT positivity (IFNγ >0.35 IU/mL), confirmed upon re-testing (Fig 1a). Indicating distinct parameterization between the two assays, when this pre-defined cut off was applied to the TruC results, only 9 LTBI cases and 17 TB patients scored positive (Fig 1b).

All patients with active disease were treated, and 18 agreed to retesting 12-18 months later all of whom had a successful treatment outcome. We also retested 19 LTBI controls after a similar 12-18 month time interval. At this time point no differences were observed between the LTBI and treated TB patients with either QFT or TruC systems (Fig 1c-d). When the effect of treatment on TB patients was directly examined, both QFT and TruC assays showed significant differences (pre- vs. post-treatment, paired T test, Fig 1e-f). Paradoxically, patients showed an increased IFNγ response in QFT (p=0.001) when comparing post- vs. pre-treatment cytokine levels; whereas the majority of patients showed the expected decrease in IFNγ responses as measured by TruC (p=0.01). TruC pre-treatment results had an area under the ROC curve (AUC) of 0.814 (95% CI: 0.69-0.93), in comparison to 0.563 (95% CI: 0.40-0.72) for QFT (Fig 1g). A bootstrap test between the ROC curves showed a statistically significant improvement for TruC compared to QFT (p=0.04). To replicate the TruC result we recruited an independent cohort of actively infected TB patients (n=51), healthy, LTBI controls (n=9), and healthy non-endemic donors (n=10) to test the ability of TruC *Mtb* Ag stimulation to correctly classify active disease. In this blinded study TruC *Mtb* Ag induced IFNγ had an AUC = 0.89 (95% CI: 0.82-0.97) for identification of patients with TB disease (Fig 1h).
**Multiple cytokine responses stratify active TB and latent infection after TruC Mtb Ag stimulation**

To assess the value of measuring additional inflammatory cytokines, we performed Luminex multi-analyte profiling on all supernatants. This identified 12 proteins that were differentially (q<0.01) expressed between TB and LTBI groups (Fig 2a, c, Table S2) in the *Mtb* antigen TruC supernatants, whereas only IL-2 was different in the respective QFT assays with this stringent cut-off (Fig 2b, Table S2). A heat map representation of the TruC results illustrates 10 proteins with higher responses (IFNγ, IL-18, IL-1RA, IL-2, IL-6, IL-8, CCL3, CCL4, TNFα) and 2 with lower responses (CCL11, Factor VII) in active TB as compared to LTBI patients (Fig 2a), with no discernible pattern observed in the QFT stimulations for these cytokines (Fig 2b). Individual plots of protein concentrations are depicted for differential cytokines observed with TruC (Fig 2c) and QFT (Fig S1). Following successful treatment of the TB group there were no significant differences between treated TB patients and LTBI controls (Fig 2d). This analysis indicated that TruC stimulation could reveal multiple immune perturbations in active TB disease.

**QFT negative control tubes have high non-specific cytokine activation**

To examine underlying differences between TruC and QFT, we considered the non-specific activation using the Null control conditions. To avoid potential artefacts caused by outlier measurements, we performed pre-filtering based on variance (σ/σ_{max} = 3.25x10^{-5}), which led to removal of 9 proteins that showed low variance across all conditions. Analysis of the remaining 23 proteins revealed significant differences (null conditions QFT vs. TruC q<0.01) with all proteins showing higher concentrations in the QFT tubes (Fig 3a, b, Table S3, S4). IL-6, IL-1β and CCL2 were the 3 most differentially expressed proteins (Fig 3c). Notably these differences were independent of disease status, as all proteins remained significantly different after regressing for patient status (TB or LTBI).

To further validate and provide interpretability for this analysis, we performed additional experiments in healthy, *Mtb*-uninfected European donors. The same QFT and TruC stimulations were performed as described above. Additionally, we investigated the hypothesis that the QFT tube or the TruC media might account for the observed variability between the Null conditions. We tested conditions in which blood collection was performed in the TruC tubes followed by transfer of the blood/media mixture into a QFT Null tube; as well as the converse, blood collection and mixing in QFT tubes followed by transfer into a TruC Null tube containing media in the absence of stimuli. A comparison between QFT and TruC negative control tubes and *Mtb* Ag tubes, showed similar results to the TB patients and LTBI controls, with significantly higher levels of innate cytokines in QFT (IL-6, IL-1β and CCL2 shown for comparison with prior results in Fig 3). Strikingly, in both of the tube transfer conditions, cytokine levels reflected the TruC condition and indicated that the TruC media
minimized non-specific innate cell activation observed when using QFT tubes. Unexpectedly, 1 donor showed elevated IFNγ responses in both stimulation systems (Fig 4a), however the fold change of the \textit{Mtb} Ag over the Null response was 4-fold in QFT, and 16-fold in TruC, illustrating the improved signal-to-noise achievable for induced antigen specific immune responses using TruC. This particular donor was also an outlier for other cytokine responses (e.g., IL-6, IL-1β, CCL2) in \textit{Mtb} Ag TruC stimulations (Fig 4b, c, d), but further clinical and radiological investigations ruled out TB disease. These combined results removed possible confounding factors due to \textit{Mtb}-infection and demonstrated that TruC media and the conditions reported facilitate an improved method for immune stimulation and immune monitoring.

\textit{TruC BCG stimulation revealed additional immune response differences and improved patient classification}

Given its use as a TB vaccine and its ability to trigger an innate response in whole blood\textsuperscript{16}, we explored the use of BCG as an additional TruC stimulation condition in our comparison study of TB and LTBI groups. Of the 22 proteins that were induced by BCG, 10 were differentially expressed (q<0.01) between the two groups (Fig 5a, b, Table S5). In contrast to TB Ag responses, the BCG-induced differences were mostly higher in the LTBI group (except for IL-18 and IL-1RA), with 4 of the most differentially expressed proteins being IL-1 family members (IL-1α, IL-1β, IL-1RA, and IL-18) (Fig 5b). Interestingly, IFNγ was only nominally higher in the LTBI group (p=0.02) (Fig 5c). Again, the immune responses in TB patients normalized after treatment to those seen in LTBI controls (Fig. S2).

Given that the pattern of BCG stimulation was inverse to that observed using \textit{Mtb} antigen (i.e. higher in LTBI compared to TB), we predicted that BCG-induced cytokines could be leveraged for improving the stratification of patient groups. We therefore calculated a composite index, the ratio of \textit{Mtb} Ag and BCG induced IFNγ response, which showed a >10-fold difference between the two patient groups (p=0.002, Fig 5d), and an AUC of 0.918 (95% CI: 0.84-0.98) (Fig 5e). Notably, this AUC was superior to those achieved for the individual tests: TruC \textit{Mtb} Ag (AUC 0.814, 95% CI: 0.69-0.93), or BCG (AUC 0.697, 95% CI: 0.54-0.84); and the QFT \textit{Mtb} Ag (AUC 0.563, 95% CI: 0.40-0.72), and a bootstrap test for correlated ROC curves revealed statistically significant improvements over both TruC \textit{Mtb} Ag, (p=0.02) and QFT \textit{Mtb} Ag (p<0.0001). These findings demonstrate the potential advantage of combining peptide antigen and complex stimuli for improved patient classification.
Differential IFNγ responses to TB Ag and BCG stimulation.

Finally, to investigate further the differential responses observed between TB Ag and BCG induced IFNγ (Fig 6a-b) we tested whether this reflected differing numbers of circulating antigen-specific T cells. For this we examined previously published intracellular cytokine flow cytometry data from the same donors\cite{13}. No significant differences in the total numbers (Fig 6c, d) or frequencies (Fig S3a) of IFNγ+ CD4+ and CD8+ T cells after TB Ag stimulation were observed between LTBI and TB groups. However, the BCG results again contrasted with those of TB Ag stimulation, with significantly (q=0.001) higher numbers (Fig 6d) and frequencies (Fig S3b) of circulating BCG specific IFNγ+ CD4+ and CD8+ T cells in LTBI donors.

Given that the higher levels of secreted IFNγ protein in TB patients in the TB Ag stimulation condition were not due to T cell differences, we next examined the transcriptional IFNγ response using nanostring assays on the stimulated whole blood cell pellet. This showed a significant difference (q=0.02) between the two groups (Fig 6e) but to a lesser degree than the protein response, suggesting either differential kinetics or post-transcriptional regulatory mechanisms. For BCG, the transcriptional response (Fig 6f) mirrored the protein and cellular data, supporting the conclusion that TB patients have lower numbers of circulating BCG specific T cells resulting in a reduced IFNγ response.

To further investigate why some LTBI and TB patients did not secrete detectable levels of IFNγ in the TruCulture system, we developed and applied a Simoa digital ELISA with a limit of detection (LOD) of 11 fg/ml. This technique identified secretion of IFNγ from all TB patients and 17/24 LTBI donors (Fig 6g). Using this ultrasensitive readout we examined the correlation between RNA transcription and protein secretion. LTBI donors showed a strong correlation (Rs=0.78), while for TB patients it was significant but weaker (Rs=0.57) (Fig 6h), again suggesting possible altered post-transcriptional regulation. In contrast, the RNA-protein correlation for both groups after BCG stimulation was strong (Rs>0.76) (Fig S3c).

Finally, we examined how this ultrasensitive digital ELISA readout applied to TruC stimulation would classify LTBI and TB patients. Utilizing the QFT equivalent cut-off of 14 pg/ml, we created four groups; LTBI IFNγ<sub>low</sub>, LTBI IFNγ<sub>high</sub>, TB IFNγ<sub>high</sub>, and TB IFNγ<sub>low</sub> (Fig 6h). To test the potential biological relevance of this new classification, we performed unsupervised hierarchical clustering analysis based on the 50 most differential genes after TB Ag stimulation between LTBI and TB. This analysis showed that 4/5 LTBI IFNγ<sub>high</sub> individuals clustered with the active TB group when stimulated with TB Ag (Fig 6i), but not after BCG stimulation (Fig S3d). Therefore, this combined approach of standardized whole blood stimulation and digital ELISA may allow identification of additional stages within the spectrum of Mtb infection and disease, which is now recognized to include incipient and subclinical TB\cite{17}.
We conclude that the use of TruC may provide considerable advantages if further developed as a method for immunomonitoring in TB clinical studies and patient management strategies.

**Discussion**

Blood-based immunomonitoring is increasingly used in clinical studies due to the ease of sampling and the possibility of longitudinal measurements during medical interventions. TB is a relevant example of how such an approach can be applied to monitor functional immune responses in clinical applications, and this approach has been extended to cytomegalovirus infection and transplantation settings\(^{18}\). However, the use of QFT blood-based tests in TB endemic countries has been limited by their poor ability to discriminate active TB disease from asymptomatic *Mtb* infection. Such stratification is required for proposed TB control strategies that focus on preventive treatment to reduce risk for disease progression, thus diminishing the chance for *Mtb* transmission. This may potentially be achieved by TruCulture stimulation with heparin-binding hemagglutinin adhesin (HBHA), a mycobacterial antigen which has been shown to induce IFNγ preferentially in LTBI donors\(^ {19}\).

We demonstrated here a clear advantage of utilizing an alternative immunomonitoring tool, TruCulture, for the analysis of induced immune responses in TB disease. TruC showed significant differential IFNγ responses in patients with active disease and controls with LTBI, differences that have not been achieved using the QFT test\(^ {15}\). Stimulation with BCG yielded a unique signature, with higher expression of multiple cytokines in LTBI as compared with active disease. This was at least partly explained by a reduced number of circulating BCG-specific T cells as revealed by flow cytometry. Combining the *Mtb* Ag and BCG-induced responses improved classification of active versus LTBI individuals significantly. Use of TruC also revealed differential induction of other cytokines, representing both innate and adaptive immune responses. Importantly, we show that such immune response differences may be obscured in QFT by cytokines that are activated non-specifically, most likely from myeloid cells in the absence of a liquid media.

The high concentrations of multiple cytokines in the control QFT tube was a striking observation. The elevated non-specific immune responses were reminiscent of our previously reported non-specific activation of myeloid cells\(^ {12}\). To minimize such issues in clinical applications of QFT, decision making is restricted to IFNγ responses, with the non-stimulated control being subtracted from the *Mtb* Ag stimulation\(^ {5}\). The manufacturer’s instructions for QFT-TB Gold and QFT-Plus permit up to 8 IU/mL of IFNγ within the unstimulated control before assigning the test as
indeterminate, which is a high level of “background” response. While this delivers meaningful information about antigen-specific adaptive responses, our study illustrates the high value of reducing the overall background biological noise due to the method of stimulation\textsuperscript{20}, as it revealed previously unappreciated differences in antigen-specific signals that discriminate active TB and asymptomatic infection. We compared our results with published studies that utilized QFT stimulations and Luminex technology\textsuperscript{22,23}. The ranges reported and those observed in our study were of a similar magnitude (e.g. for IL-1RA, CXCL10\textsuperscript{22}, IL-1\(\alpha\), IL1\(\beta\), IL-2, IL-6, CCL2, CCL3, TNF\(\alpha\)\textsuperscript{24}). Notable differences were observed in the levels of IL-8 and CCL4, which were an order of magnitude lower than those observed in our study. This may be explained by differences in antibodies used or the populations studied.

While an obvious caveat of our study is the relatively small sample sizes, we highlight that the high effect size seen between LTBI and TB infected persons was great enough to observe a statistically significant difference, which replicated in an independent cohort. The strong effect size also highlights the importance of assay standardization: lower variability obtained from more robust sampling can facilitate the powering of scientific questions, especially those with smaller study sizes. Additional studies are planned to identify clinical questions that would benefit from TruC based immunomonitoring. Given recent advances in \textit{ex vivo} blood transcriptomic signatures for diagnosing subclinical or active TB disease\textsuperscript{26,27}, the requirement for an incubation step may represent a barrier to near-patient testing. Despite the stated limitations, we believe that there is sufficient justification for testing TruC as next generation immunomonitoring tools in TB clinical studies.

In summary, given the numerous challenges still present in the TB field and the critical need for better tools, novel robust and adaptable immunomonitoring tools may support ongoing efforts to combat TB worldwide.
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Conflict of interest

Matthew Albert is an employee of Insitro. DD reports other support from Myriad Rules Based Medicine, outside the submitted work. GW reports two issued patents on TB diagnostic markers issued (PCT/IB2013/054377, Host markers in whole blood culture supernatants for diagnosis for active TB) and (PCT/IB2013/054377 (USA), host markers in whole blood culture supernatants); a patent on a method for diagnosing TB pending (AP/P/2016/009427 (ARIPO), 201580023042.X (China), 15755681.2 (Europe), 201617030869 (India), F/P/2016/258 (Nigeria), 2016/06324 (South Africa)); and a patent on serum host biomarkers for tuberculosis disease pending (PCT/IB2017/052142, due for national filing). All other authors have nothing to declare.
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**Figure Legends**

**Figure 1. IFNγ Mtb Ag response.** IFNγ response following Mtb Ag stimulation and subtraction of the Null control in LTBI and TB patients in (a) QFT tubes pre-treatment, (b) TruC tubes pre-treatment, (c) QFT tubes after successful antibiotic treatment in TB patients, (d) TruC tubes after successful antibiotic treatment in TB patients. (a, b n=25/25; c,d n=19/18 LTBI/TB, Mann Whitney), bars represent the median values, dotted line is QFT positive cut off at 0.35 IU/ml). Paired IFNγ responses following Mtb Ag stimulation and subtraction of the Null control in TB patients pre and post treatment in (e) QFT tubes and (f) TruC tubes (Paired T test). (g) ROC curve analysis of the IFNγ response to classify active disease following Mtb Ag stimulation in TruC (black line) or QFT (dash line) tubes in the initial cohort, (f) ROC curve analysis of the IFNγ response to classify active disease following Mtb Ag stimulation in TruC in a blinded independent replication study (n=80). TB: black squares, LTBI: open triangle, TB post-treatment: black triangles.

**Figure 2. Differential cytokine responses in Mtb infection versus TB disease.** Heat maps of relative expression levels for 12 differential cytokines (LTBI v TB groups, Mann Whitney q<0.01) segregated by patient group (LTBI: black square, TB: open triangles) after Mtb Ag stimulation in TruC (a) or QFT (b) tubes prior to treatment, and (d) TruC Mtb Ag stimulation after successful antibiotic treatment of the TB patient group (TB post Tx: closed triangles). (c) Dot plot representations of the differential cytokine concentrations between LTBI and TB groups (q<0.01) in TruC tubes prior to treatment. (a, b, c, n=25/25; e n=19/18 latent/active, bars represent the median values, q value: FDR corrected Mann Whitney test).

**Figure 3. Differential cytokines in QFT and TruC Null tubes.** Heat maps of relative cytokine expression levels segregated by patient group (LTBI: black square, TB: open triangles) in (a) QFT and (b) TruC Null tubes for 22 out of 32 cytokines measured, selected based on variance (σ/σ_{max} = 0.138), (c) Concentrations of IL-6, IL-1β, CCL2 in QFT and TruC Null tubes in LTBI and TB patients. (n=25/25, bars represent the median values)

**Figure 4. Cytokine responses in donors from a non-endemic TB region.** Concentrations of (a) IFNγ (b) IL-6 (c) IL-1β and (d) CCL2 in QFT Null, QFT TB Ag, QFT Mitogen, TruC Null, TruC TB Ag, TruC BCG, and mixed cultures of TruC-QFT and QFT-TruC Null conditions in healthy donors.
from a non-endemic region (n=10, bar represents the median values, Friedman test with Dunn’s multiple comparisons test).

**Figure 5. BCG induced immune responses in *Mtb* infection versus TB disease.** (a) Heat map of relative cytokine expression levels segregated by patient group (LTBI: black square, TB: open triangles) after BCG TruC stimulation, and identification of differential proteins after FDR adjusted Mann Whitney tests between LTBI and TB groups. (b) Dot plot representations of the cytokine concentrations of differential proteins between LTBI and TB groups: GMCSF, TNFα, IL-1β, IL-1α, IL-12p40, CCL2, IL-3, IL-17, IL-18, IL-1RA. (c) IFNγ BCG response and (d) ratio (IU/ml) of *Mtb* Ag/BCG stimulation for LTBI and TB patients. (e) ROC curve analysis of IFNγ ratio to *Mtb* Ag/BCG stimulation (black), IFNγ concentrations of TruC *Mtb* Ag (blue), TruC BCG (green), and QFT *Mtb* Ag (red) stimulations. (n=25/25, bars represent the median values, q value: FDR corrected Student T test).

**Figure 6. Differential IFNγ responses to TB Ag and BCG stimulations.** IFNγ protein levels measured by Luminex in TruCulture supernatants after TB Ag (a) and BCG (b) stimulations. Total numbers of IFNγ+ CD4+ and IFNγ+ CD8+ T cells measured by flow cytometry in TB Ag (c) and BCG stimulated whole blood (d). IFNγ mRNA levels measured by Nanostring in TruCulture cell pellets after TB Ag (e) and BCG (f) stimulations. (g) IFNγ levels measured by Simoa digital ELISA in TruCulture supernatants after TB Ag stimulation. Classification in four different groups according to disease status and IFNγ levels: LTBI IFNγlow (black), LTBI IFNγhigh (green), TB IFNγhigh (dark blue) and TB IFNγlow (light blue). (h) Correlation plot between IFNγ protein levels measured by Simoa and IFNγ mRNA total counts measured by Nanostring, after TB Ag stimulation (Pearson correlation). (i) Heatmap showing the 50 most differentially expressed genes between TB and LTBI for the TB Ag stimulation (unsupervised hierarchical clustering). Individuals are coded according to disease status and levels of IFNγ secretion, as illustrated in (g). Solid lines depict median. Comparisons of LTBI/TB groups within the same stimulation were performed using unpaired Mann Whitney tests; comparisons between Null and stimulated conditions within the LTBI/TB groups were performed using a Wilcoxon test. Correction for multiple comparisons was then applied. (LTBI: black square, TB: open triangles).
| Initial Cohort | TB patients | LTBI controls | p value |
|----------------|------------|---------------|---------|
| Age (median years, IQR) | 33 (25-40) | 33 (24-41) | 0.89 |
| Sex (% female) | 24% | 24% | >0.99 |
| Ethnicity (% Cape Mixed ancestry*) | 72% | 76% | >0.99 |
| Household TB contact (% yes) | 56% | 40% | 0.26 |
| BMI (median, IQR) | 20 (19-21) | 26 (24-28) | <0.0001 |

| Smoking (n) | Smoker | Ex-smoker | Non-smoker |
|-------------|--------|-----------|------------|
|             | 14     | 7         | 4          |
|             | 13     | 2         | 10         |
| Visit 1 (n° donors) | 25 | 25 | NA |
| Visit 2 (n° donors) | 18 (after treatment) | 19 | NA |

* Referred to as coloured in South Africa

Table 1. Patient characteristics.
Figure 1
Figure 2

(a) TruC Mtb Ag stimulation (pre-treatment)

(b) QFT Mtb Ag stimulation (pre-treatment)

(c) TruC Mtb Ag stimulation (pre-treatment)

(d) TruC Mtb Ag stimulation (post-treatment)
Figure 3
Figure 5
Figure 6