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Original Article

ORIGINAL ARTICLE

Precision immunoprofiling to reveal diagnostic signatures for latent tuberculosis infection and reactivation risk stratification

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Abstract

Latent tuberculosis infection (LTBI) is estimated in nearly one quarter of the world's population, and of those immunocompetent and infected ~10% will proceed to active tuberculosis (TB). Current diagnostics cannot definitively identify LTBI and provide no insight into reactivation risk, thereby defining an unmet diagnostic challenge of incredible global significance. We introduce a new machine-learning-driven approach to LTBI diagnostics that leverages a high throughput, multiplexed cytokine detection technology and powerful bioinformatics to reveal multi-marker signatures for LTBI diagnosis and risk stratification. This approach is enabled through an individualized normalization procedure that allows disease-relevant biomarker signatures to be revealed despite heterogeneity in basal immune response. Specifically, cytokines secreted from antigen-challenged peripheral blood mononuclear cells were detected using silicon photonic sensor arrays and multidimensional data correlation of individually-normalized immune responses revealed signatures important for LTBI status. These results demonstrate a powerful combination of multiplexed biomarker detection technologies, precision immune normalization, and feature selection algorithms that revealed positively correlated multi-biomarker signatures for LTBI status and reactivation risk stratification from a relatively simple blood-based assay.

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Insight, innovation, integration

Tuberculosis (TB) is a global health crisis complicated by the high prevalence of latent tuberculosis infection (LTBI), an asymptomatic and quiescent disease state. Though LTBI is treatable, there are no accurate diagnostic tools to confidently identify those at the highest risk of reactivation and thus, most suitable for therapy. This study describes the development of a robust molecular diagnostic and bioinformatic workflow that reveals multiplexed biomarker signatures correlated with LTBI status. This method combines detailed characterization of immunological response of peripheral blood mononuclear cells and rapid multiplexed immunoassays to guide diagnostic assay development. This approach should further stratify LTBI subjects based on reactivation potential, thus providing a fundamentally new approach to identifying subjects most likely to benefit from therapeutic interventions.

INTRODUCTION

Tuberculosis (TB) is a leading cause of mortality worldwide with an estimated 2 billion individuals currently infected [1, 2]. Latent tuberculosis infection (LTBI), an asymptomatic and quiescent disease state, is the most common form of TB infection accounting for ~90% of the total infected population [3]. This heterogeneous population contains a majority who maintain a persistent lifelong asymptomatic infection and an estimated 10% of immunocompetent individuals who will progress to an active, highly infectious disease state [4, 5]. LTBI is treatable with prolonged antibiotic regimens, though potential side effects and increased antibiotic resistance place an impetus on developing diagnostic tools to identify patients at the highest risk of reactivation, and thus most likely to benefit from therapy while minimizing overtreatment [6]. As a path towards eradication, The World Health Organization has targeted a 75% reduction in TB deaths and 50% reduction in prevalence by 2025. These goals will be impossible to achieve without new diagnostically-guided approaches to disease management and targeted treatment of LTBI cases with the highest reactivation risk [7].

The tuberculin skin test (TST) and interferon gamma release assays (IGRAs) are commonly used for TB and LTBI diagnosis [8]. The TST provides a qualitative measure of adaptive immune response to non-specific TB antigens exposed intradermally. Though a good measure of TB exposure, the TST cannot distinguish LTBI from memory immune response, vaccine-initiated response, and non-tuberculous mycobacteria exposure. Moreover, the TST only offers a 1.5% positive predictive value for LTBI progression to active disease [2]. *In vitro* IGRAs, such as the Quantiferon®-TB Gold In-Tube test (QFT) or T-SPOT®.*TB* test, were developed to address the TST specificity issues by quantitatively measuring the release of the cytokine IFN-γ from circulating T-cells in patient blood during incubation with TB-specific antigens. IFN-γ is critical for innate and adaptive immunity against numerous infections, and therefore it is unsurprising that despite T-cell antigen specificity, IGRAs do not dramatically improve LTBI diagnostics [9]. IGRAs offer only a 2.7% positive predictive value for LTBI progression to active disease [2] and no enhanced prognostic power compared to the TST in high burden populations [10]. Overall, neither assay can differentiate TB patients and subjects with residual immunological memory from a completely cleared or well-contained infection from those latently infected and at risk of reactivation [11, 12].

While the TST and IGRA fail to clearly inform treatment strategies, both leverage the unique response of the immune system to TB antigens. Given the complex network of cytokines involved in the immune response in TB and subsequently released after T-cell antigen stimulation, we hypothesized that multiple-biomarker signatures may provide a more detailed, disease-specific glimpse into a patient's state of infection and likely outcome [13]. However, this deeper view into LTBI-specific immune function and memory can only be accessed via multiplexed technologies that provide robust and facile inflammatory biomarker profiles underlying nuanced host–pathogen responses.

An enabling array-based silicon photonic microring resonator detection technology is uniquely positioned to provide detailed immune profiling on account of high level multiplexing, high assay throughput, cost effective and scalable device fabrication, and robust assay performance [14]. These array-based sensors support resonant optical modes that are sensitive to changes in the local refractive index near the sensor surface. By monitoring spectral shifts in the resonance wavelengths of sensors functionalized with target-specific capture agents, biomolecules can be detected with high sensitivity and specificity (Fig. 1) [15]. Many of the key analytical capabilities of this technology have been previous reported in the context of detecting a range of protein biomarkers in complex matrices with detection limits at or below conventional immunoassays [16, 17].

Applied to the detection of cytokines, this technology uniquely supports rapid immunoprofiling under several TB-specific and non-specific antigen stimulation conditions to monitor TB-specific immune responses, identify multiple-biomarker LTBI signatures, and stratify LTBI+ subjects according to reactivation risk. This extensive immunological characterization allows for a more nuanced approach to understanding the broader role of personalized immune function for LTBI status and risk stratification. TB-independent immunomodulatory factors enforce unavoidable individual heterogeneities within basal immune function thereby limiting immune-based diagnostics. Using a precision medicine approach for personalized response normalization reveals previously obscured diagnostic signatures for LTBI status and reactivation risk potential.

The resulting high-density data streams generated by this approach are information rich, and advanced bioinformatic techniques are required to select those biomarker signatures correlated with LTBI status. Critical to bioinformatic-based decision-making are machine learning methods that operate by classifying diagnostically important variables particularly when *a priori* identification would not have otherwise been possible [18, 19]. The nature of this high-dimensional heterogeneous data presents unique analytical challenges because the number of features is orders of magnitude larger than the number of samples in the study [20, 21]. Likewise, the common univariate approaches for ranking feature relevance fail to capture complex multivariate relationships resulting in high false discovery rates and ureproducible predictive models [22, 23].

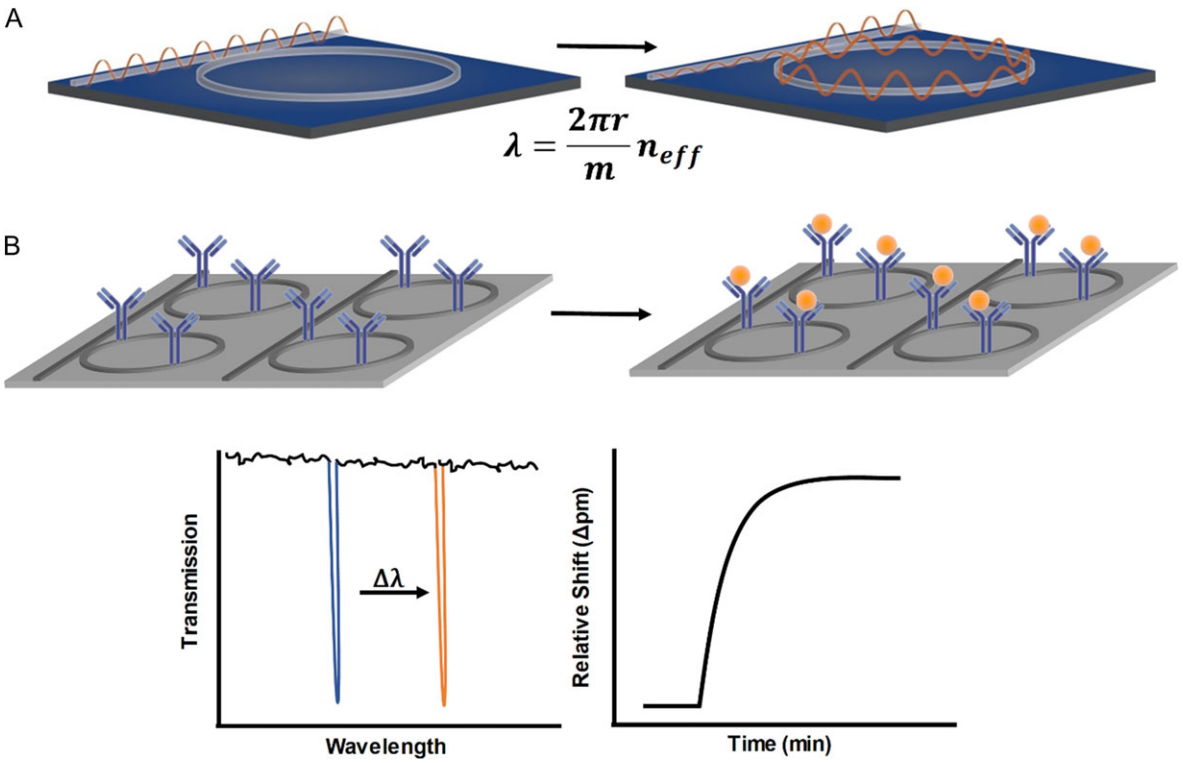

Figure 1. Operating principles for the microring resonator sensors. (A) The microring sensor detects changes in the local refractive index by coupling light from an external cavity diode laser into a linear waveguide via grating couplers. Light propagates down the linear waveguide via total internal reflection and couples into the microring when the resonance condition resulting in a dip in transmission past the microring. (B) The resonant wavelength shifts due to changes in refractive index near the surface of the ring depicted by a protein binding to a target-specific antibody. This binding can be monitored as a function of relative shift over time with the response proportional to the amount of captured target analyte.

In contrast, more promising feature selection approaches from the domain of machine learning demonstrate robustness and reproducibility and reduced selection bias [24]. Furthermore, they control overfitting even when many of the predictive features are noise and can be used when the number of features is significantly larger than the number of samples [25]. The aim of this study is to identify all relevant features related to the response (i.e. LTBI+ and risk assessment) providing the basis for mechanistic understanding beyond typical predictive model building [26, 27]. Overall, machine learning methods provide the basis for practical yet comprehensive biomarker selection and LTBI diagnostic signature development.

Herein, we present an analytical workflow to reveal multiplexed cytokine signatures for LTBI detection and reactivation risk stratification. Multiple cytokines secreted from peripheral blood mononuclear cells (PBMCs) following stimulation with both TB relevant- and control antigens were measured from a pool of 50 subjects recruited at Mayo Clinic including those with positive LTBI status and high reactivation risk. Cytokines selected for detection have separately, or in combination, been suggested to differentiate TB from LTBI and/or unexposed subjects [28–30]. The resulting cytokine levels were bioinformatically compared against established clinical classifiers revealing a set of biomarkers that, independent of additional clinical information, are promising for determining LTBI status. A richer signature was revealed through individualized normalization with off-target and control stimulation conditions correcting for population heterogeneities in basal immune function. This evidence strongly supports the central hypothesis that multiplexed biomarker detection, together with personalized immune normalization and feature selection, can reveal new diagnostic signatures to improve the positive prediction of LTBI diagnosis from a relatively simple blood-based assay. Patient risk stratification based on reactivation potential has substantial implications in terms of identifying LTBI positive subjects that are most likely to benefit from therapeutic intervention, and therefore this development suggests new biomarker-based strategies for targeted TB management.

MATERIALS AND METHODS

Sensor functionalization

Microring array sensor chips from Genalyte were first rinsed in acetone for 2 min to remove a protective coating. The chips were then silanized in a 1% APTES solution in acetone for 4 min followed by 2 min rinses in acetone and isopropyl alcohol, and then briefly rinsed with deionized water and dried under nitrogen. A 2 mM acetic acid solution containing 5 mM BS3 crosslinking reagent was then microspotted onto the chip. Antibody solutions of 0.25 mg/ml in 10 mM PBS and 5% glycerol were spatially arrayed onto clusters of four microring sensors. Each capture probe was redundantly spotted at different locations across the array for a total of 8 sensors per target in each fluidic channel (2-channels per chip) and then incubated in a humidity chamber for 1 h (Fig. S1). Chips were then coated in DryCoat and transferred to a desiccator at 4°C for storage. Antibody selection and validation is outlined in the Supplementary Information.

Immunoassay procedure

For the Maverick (M24) instrument (Fig. S2b), 12 chips were pre-assembled into a disposable plastic AutoArray with integrated fluidics allowing software-controlled reagent flow. Two samples were run simultaneously on a single chip (2 channels/samples per chip). The Maverick (M1) instrument was used exclusively for antibody validation. For both instruments, the reagents in the assay were diluted in running buffer (PBS-B: 10 mM PBS, 0.5% BSA), loaded into a 96-well plate, and delivered to the sensor surface under software control.

The assay consisted of the following steps: [1] PBS-B rinse (2 min) [2], sample (9 min) [3], PBS-B rinse (2 min) [4], biotinylated tracer antibody (9 min) [5], PBS-B rinse (2 min) [6], SA-HRP (9 min) [7], PBS-B rinse (2 min) [8], 4-cholornaphthol (8 min) [9], PBS-B rinse (2.5 min). Buffer rinse steps were completed with a 40 μl/min flow rate and all other steps were at 30 μl/min. The total assay time was 45.5 min, with two assays performed simultaneously.

Differences in resonance wavelength measured in buffer before and after the enzymatic signal enhancement step were used to quantify target levels in the supernatants of stimulated subject samples. Responses measured for a series of standards were fit to a logistic function to generate a calibration curve used for cytokine quantitation from supernatant samples. More details regarding microring sensor data processing and calibration can be found in the Supplementary Information (Tables S1–S2).

Clinical sample collection and stimulation

The study, which was part of a prospective and single-center study for LTBI immunodiagnostics, as previously described [31], was approved by the Mayo Clinic Institutional Review Board and Olmsted County Public Health Services. All study participants signed an informed written consent and were enrolled between October 2015 and March 2016. Study subjects included unexposed individuals and subjects with various risk for TB infection, including untreated LTBI patients and patients with prior LTBI therapy at low risk of reactivation. Risk factors for TB infection, TB progression, and/or TB reactivation were extracted through a validated questionnaire and review of medical records. LTBI diagnosis was made per the Center for Disease Control and Prevention (CDC) criteria and based on TB risk factors, and by prior TST and QuantiFERON®-TB (QFT) results [32–34]. The 'Online TST/IGRA calculator' was also used to estimate the cumulative risk of TB reactivation in all subjects [35]. Additional information about the incorporation of this assessment and a table describing the diagnosis criteria (Table S3) are including the Supplementary Information.

Blood samples were simultaneously obtained for QFT testing (Mayo Clinic Infectious Disease Serology Laboratory) and multiplexed cytokine immune reactivity profiling. PBMCs were separated by Ficoll gradient centrifugation within 2 h of blood sampling, and pellets frozen with 10% DMSO in liquid nitrogen until immune profiling in batches. Thawed PBMC pellets (viability ≥85%) were stimulated for 40–48 h with either TB-relevant antigens, including a mix of CFP-10/ESAT-6 peptides or purified protein derivative (PPD), or positive and negative controls, which included purified *Candida albicans* antigen (MyBioSource), anti-CD3 antibodies (Mabtech), or cell culture media, all in concentrations determined empirically via antigen titration and similarly to previously described study conditions [31]. Supernatants from stimulated PBMCs were stored at −80°C and shipped on dry ice for cytokine concentration measurements by individual ELISA and multiplexed microring resonator assays. To note, BCG vaccination and non-TB mycobacteria infection related responses should be excluded from positive CFP-10/ESAT-6 response (rendering it a TB-specific stimulation condition) minimizing false negative reactivity. Additionally, the stimulation time (40–48 h) reflects empirical optimization from commercially available IGRA assays.

Samples were thawed and vortexed prior to loading into the 96-well plate for microring assays and ELISA testing. Each sample was analyzed undiluted and after a 10-fold dilution in running buffer. ELISAs were performed to compare against microring-determined cytokine sample concentrations. QFTs was performed as recommended by the manufacturer (Qiagen GmbH). A cut-off level of IFN-γ ≥0.35 IU/ml and >50% above nil defined a QFT(+) test. Research laboratory personnel were blinded to the results of QFT.

Feature selection and statistical analysis

A Boruta feature selection model [26] was utilized to identify the biomarker features associated with LTBI diagnosis. This approach is more fully described in the Supplemental Information, but briefly Boruta is a modified Random Forest classification algorithm that iteratively removes features found statistically to be less relevant than random probes, known as shadow features (a more detailed description can be found in the Supplemental Information). Relevant features are identified as those that correlate to LTBI diagnosis bins and are statistically robust compared with the noise or shadow features, which are made up of original data but altered to destroy the relationship to the observations. The Boruta approach identifies the features that are important as well as those that it cannot label as unimportant (tentative). OriginPro 2017 was used to generate calibrations, box plots, and calculate Mann–Whitney and Kruskal–Wallis one-way ANOVA. Differences were considered statistically significant at $P < 0.05$. Box plots represent the 25th and 75th percentile (box), median (horizontal line), mean (square), 5th and 95th percentile (whiskers).

RESULTS

Immune cell stimulation and multiplexed cytokine profiling

We developed an experimental workflow to quantitatively measure multiplexed cytokines from stimulated PBMCs for the diagnosis of LTBI. The general workflow for cytokine profiling is outlined in Fig. 2. PBMCs isolated from whole blood were stimulated under five different conditions: CFP-10/ESAT-6 (TB-specific peptides), purified protein derivative (PPD–TST antigen), *Candida albicans* (off-target control), anti-CD3 antibody (pan-T-cell stimulating positive control), and cell media (negative control). Supernatants from the stimulated PBMCs were analyzed for cytokine concentrations on the microring resonator array platform by flowing across pre-functionalized sensor arrays (Figs S1 and S2).

A 45 min on-chip enzyme-enhanced, multiplexed sandwich immunoassay approach [16] (Fig. S3) begins with surface functionalized capture antibodies binding target protein from supernatant samples. The bound analyte is then recognized by target-specific biotinylated tracer antibodies and then streptavidin conjugated horseradish peroxidase enzyme. Enzymatic conversion of soluble 4-chloronaphthol to the insoluble precipitate 4-chloronaphthon then gives a dramatic enhancement in

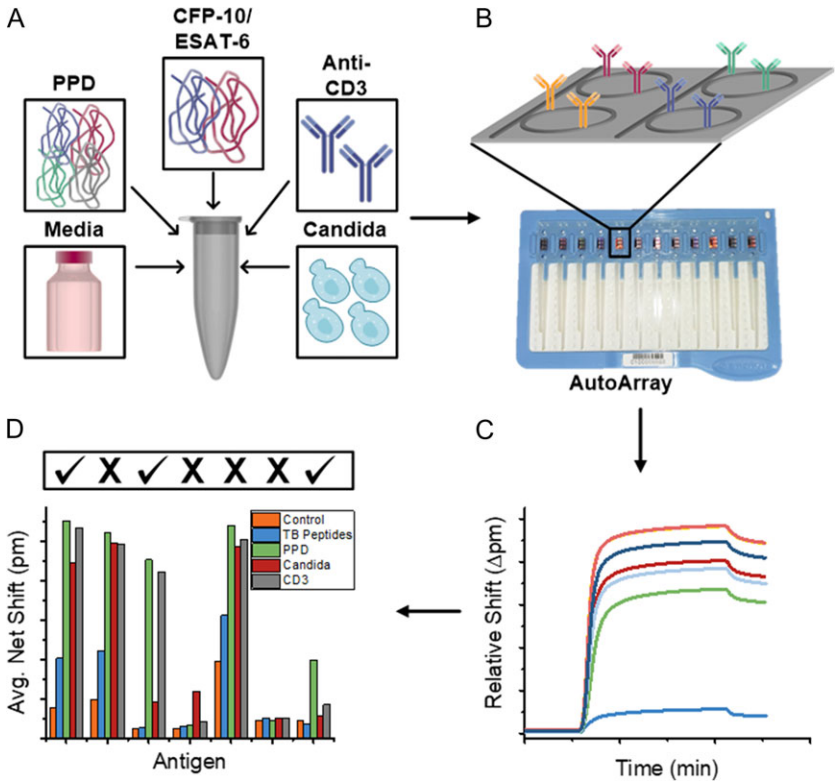

Figure 2. Multiplexed cytokine detection scheme toward LTBI diagnosis. (A) Patient PBMCs were stimulated with TB specific antigens (CFP-10/ESAT-6 or PPD) and the supernatant collected for analysis. (B) Supernatants were then flowed across a sensor array functionalized with target-specific capture probes. Twelve chips containing 24 distinct fluidic channels are contained in a single-use AutoArray. (C) Resonance wavelength shifts observed from an enzymatically enhanced immunoassay correspond to concentrations of each target. Error bars represent ± SD ($n \geq 3$ technical replicates). (D) Resonance wavelength shifts can be converted to concentrations and analyzed using feature selection algorithms to reveal those biomarkers most important for identifying LTBI.

resonance shift that is related to the concentration of the target in the supernatant solution. Concentrations of each cytokine were determined by comparing the signal response against a calibration curve generated for each target analyte.

Multiplexed assay development and characterization

Commercial antibody pairs for each target were selected and validated for optimal performance, and the response of each calibrated (Table S4). Recombinant proteins for each target were prepared as a cocktail, diluted from 50 ng/ml to 1.6 pg/ml (calibration range was protein dependent), and analyzed in running buffer. The calibration range was empirically determined to cover the saturation of the signal to below the limit of detection (LOD) when including the blank measurement (running buffer). LODs for each target ranged between 0.5 and 65 pg/ml and limits of quantitation from 2 to 200 pg/ml (Table S2). These values were calculated with the averaged calibration data ($N = 3$ chips) each containing redundantly functionalized microrings. To minimize variance, the assay calibration was reconfirmed throughout the study. Calibration curves for each target, averaged across three calibration processes collected throughout the study are shown in Fig. 3A with fitting parameters described in Table S3. To demonstrate assay precision, biomarker levels were determined using three different sensor chips each containing at least three redundantly spotted microrings per array. Results show consistent responses with coefficients of variation (CV) at or below 10% across multiple sensor chips, with redundantly spotted sensors on the same chip having CVs near 3% (Fig. 3b). For a subset of patients, the cytokine levels determined with the microring array were compared against multiple, single-analyte ELISAs and found to be in good agreement across all five stimulation conditions (Fig. S4).

Clinical sample analysis

The research subject pool (Table 1) consisted of 50 individuals who were screened for TB and evaluated for LTBI using standard of care diagnostic tests, including both TST and QFT. Positive TST and QFT results were reported for 38% and 10% of the subjects, respectively. These results were considered along with CDC guidelines allowing for individual classification according to LTBI diagnoses and risk of reactivation [32–34]. This process required careful evaluation of medical history, potential exposure to TB, chest x-rays and imaging, and clinical assay results. The LTBI designations included positive LTBI per the Center of Disease of Control and Prevention (CDC) recommendations (CDC+), and strict LTBI diagnosis (Strict+), which required either a positive QFT and a positive TST, or a positive TST conversion with a negative QFT along with other risk factors for TB infection (i.e. close TB contact or recent immigration from a location with high TB prevalence). The designations for risk of TB reactivation were defined as 'high-risk' or 'low-risk' with risk assessment determined using the online TST/IGRA interpreter multidimensional prediction tool (e.g. cumulative score of TB reactivation ≥2%) and other relevant clinical and/or laboratory conditions, including immunosuppression [35]. These designations were used to bin

the patients as follows: 20% CDC+, 24% Strict+, 18% low-risk, and 10% high-risk.

Stimulated PBMC supernatants were tested using the multiplexed microring detection panel, and concentrations for each biomarker determined as described above. In addition to the absolute target concentrations under each stimulation condition, measured target levels from the controls [baseline (media), an immunogenic off-target (*Candida*), and pan-activating positive (anti-CD3) supernatants] were each separately subtracted from TB-relevant stimulations (CFP-10/ESAT-6 and PPD) to control for different basal levels of immune response. This method of normalization was implemented to correct for differences in the basal immune state of each individual patient.

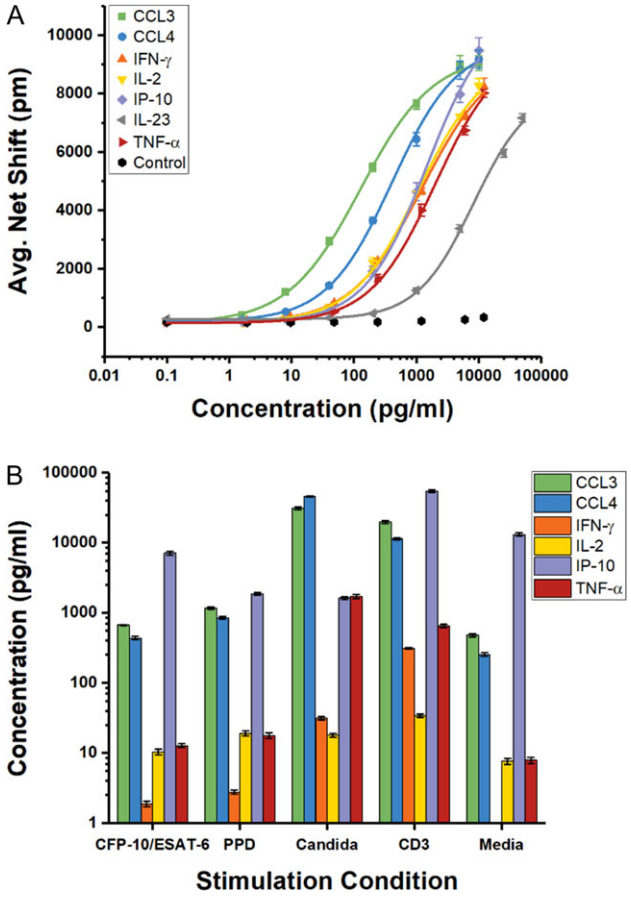

Figure 3. Calibration and technical replicates of the multiplexed sensor array. (A) Serial dilutions of recombinant protein standards for seven cytokine targets were used to assess the performance across approximately five orders of magnitude in 10 mM PBS/0.5% BSA buffer. The net shift from the enzymatic amplification was used as the readout and fit to a logistic function. The signal is an average of three separate calibration curves collected throughout the patient sample analysis time. Error bars represent ± SD ($n \geq 3$). (B) Chip array reproducibility using supernatant from stimulated patient PBMCs. The supernatant from PBMCs stimulated with five different conditions was measured with each measurement representing results from different sensor chips ($n = 3$) with functionalized sensors ($n \geq 3$) for each target. The error bars represent ± SD. CVs for all targets are ≤10%. IL-23 is not shown due to consistently low measured concentrations.

Machine learning feature selection analysis

Analysis with Random Forest based classification methods have found utility in computational biomedicine due to their ability to select features from high dimensional data streams on the basis of relevance to clinically-defined classifications [36, 37]. Boruta, a feature selection method, uses a Random Forest classification algorithm and shadow features, which are data that are random by design and act as a threshold above which real features that are truly important emerge [26]. This approach iteratively removes biomarkers that do not show any correlation with specific LTBI related diagnoses, while retaining all features with importance above the noise, thereby avoiding premature elimination of relevant features. However, features that are definitively important are discriminated from those that the model cannot label as unimportant. For this study, important features are indexed as 'important' while those provisionally important are notated as 'tentative'. In addition to Boruta analysis, each of the individually selected features were further statistically evaluated to first ensure their differential diagnostic capacity relative to LTBI status (+/–), and then orthogonality between absolute (e.g. 'CFP-10/ESAT-6') and/or normalized features from the same stimulation (e.g. 'CFP-10/ESAT-6 – Media').

Using the Boruta feature selection method, absolute biomarker levels from stimulated PBMCs were analyzed and feature selection revealed several stimulation-specific biomarker signatures that correlated with LTBI status (CDC+ and Strict+), as well as 'high' and 'low' reactivation risk, as shown in Fig. 4A. However, given the evaluation of multiple different TB-relevant and control conditions, the normalization of an individual's secreted cytokine levels using control stimulation levels allows for more nuanced signatures to be extracted. A separate feature selection analysis using normalized cytokine levels revealed a richer set of biomarker signatures for the aforementioned clinical classifications, as shown in Fig. 4B. Mann–Whitney and one-way ANOVA tests were employed to statistically evaluate the individual resulting features for preliminary verification that the features are non-redundant and potentially TB-relevant. An example of this analysis is shown for IP-10 in the main text (Fig. 4C–E), and for other biomarkers in Figs S5–15. For the case of IP-10, signatures from CFP-10/ESAT-6 stimulations were

Table 1. Demographic and clinical characteristics of study subjects.

Group	All	LTBI–	TST+	QFT+	CDC+	Strict+	High risk	Low risk
N (%)	50 (100)	35 (70)	19 (38)	10 (20)	10 (20)	12 (24)	5 (10)	9 (18)
Female, N (%)	33 (66)	23 (46)	11 (33)	6 (18)	6 (18)	8 (24)	3 (9)	6 (18)
Male, N (%)	17 (34)	12 (24)	8 (47)	4 (24)	4 (24)	4 (24)	2 (12)	3 (18)
HCW (%)	26 (52)	30 (60)	10 (38)	5 (19)	5 (19)	6 (23)	2 (8)	5 (19)
Age (Mean years ± SD)	57 ± 17	61 ± 15	58 ± 18	43 ± 16	43 ± 16	46 ± 17	42 ± 20	48 ± 16

Group: *N* (number), HCW (health care worker). Clinical classifications: LTBI– = negative for LTBI, TST+ = tuberculin skin test positive, QFT+ = QuantiFERON®-TB test positive, CDC+ = positive LTBI according to CDC recommendations, Strict+ = strict LTBI diagnosis, High Risk = LTBI positive with a high risk of reactivation, Low Risk = LTBI positive with a low risk of reactivation.

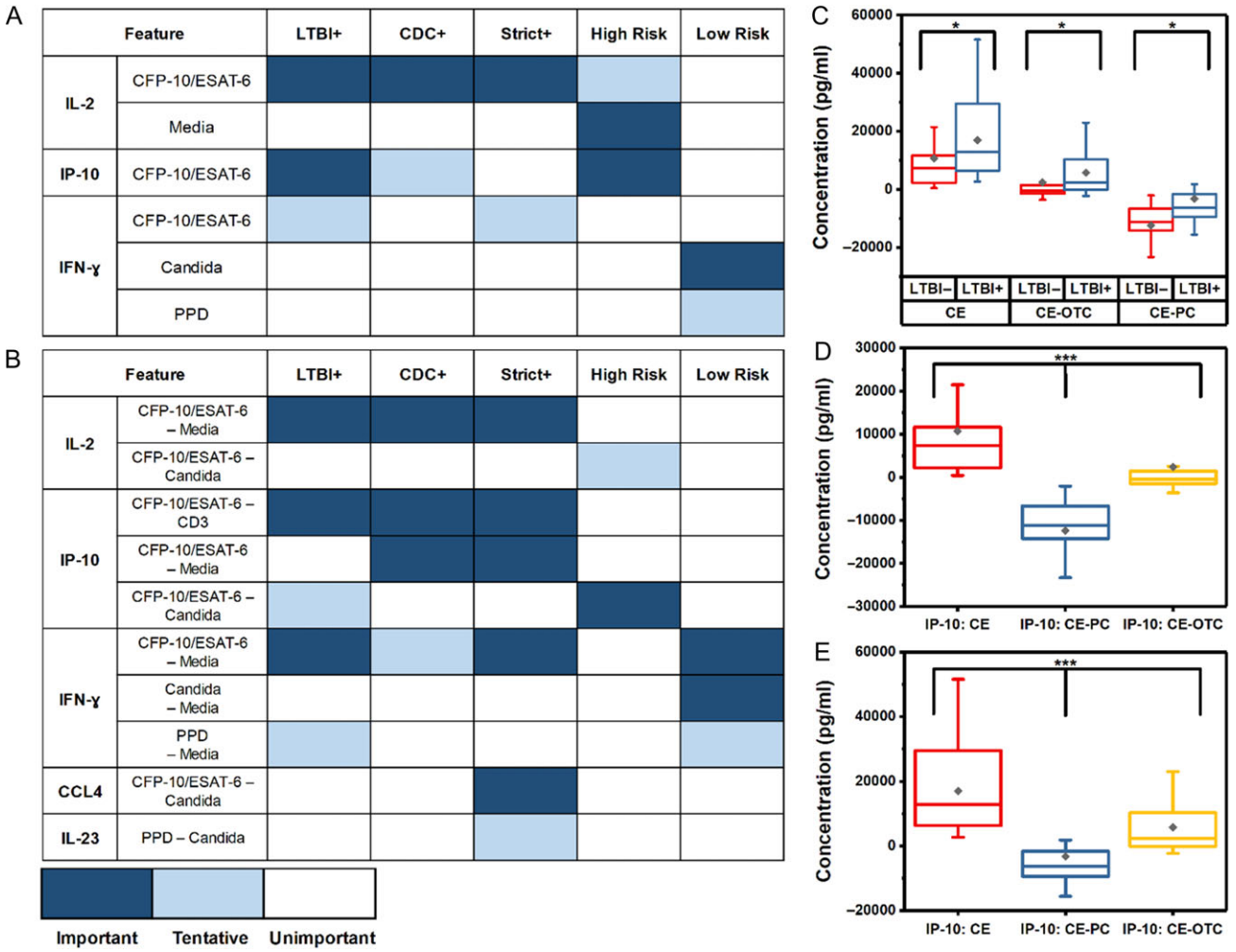

Figure 4. Boruta feature selection results for absolute value and normalized value features. (A) Feature selection results from post-stimulated PBMC supernatant for the absolute biomarker concentrations and (B) the control and off-target normalized biomarker concentrations. (C) Features associated with IP-10 were evaluated individually for differentiating LTBI status. Potential redundancy with IP-10 related features (both normalized and non-normalized) is evaluated for both (D) LTBI- ($n = 35$) and (E) LTBI+ ($n = 15$) populations demonstrating unique features. The clinical classifications are described as: LTBI- = negative for LTBI, CDC+ = positive LTBI according to CDC recommendations, Strict+ = strict positive LTBI diagnosis, High Risk = positive LTBI with a high risk of reactivation, Low Risk = positive LTBI with a low risk of reactivation. Stimulation conditions: CE: CFP-10/ESAT-6, CE-PC: [CFP-10/ESAT-6]-positive control (CD3), CE-OTC: [CFP-10/ESAT-6]-off-target control (Candida); $^{*}P < 0.05$, $^{***}P < 0.001$.

found to be statistically different for LTBI+ and LTBI- populations, and absolute and normalized features (by *Candida* and CD3) were statistically non-redundant (Fig. 4C). Features from other biomarkers showed varying levels of significance, but a majority of selected features were statistically robust for positive LTBI status by CDC criteria (Figs S7–8) and strict criteria (Figs S9–11), as well as high-risk (Figs S12–13) low-risk LTBI status (Figs S14–15).

When comparing absolute and normalized analyses for LTBI+ designations, there were consistencies for the important features described by IL-2 from CFP-10/ESAT-6 stimulation for CDC+ and Strict+ samples. Additionally, tentative features for IP-10 and IFN-γ from CFP-10/ESAT-6 stimulation from the absolute analysis aligned well with similar important features from the normalized analysis. This internal consistency for a subset of CFP-10/ESAT-6 related features was expected considering the stimulation condition should elicit a TB-specific immunological response and the CDC+ and Strict+ designations are nearly mutually inclusive [13]. Importantly, the personalized normalization with the off-target and control conditions revealed many additional important features for LTBI designations. The new features were predominantly TB-relevant stimulations, reinforcing the disease-specificity of these important features. Furthermore, CFP-10/ESAT-6 stimulated biomarkers separately normalized by *Candida* and CD3 stimulations response revealed unique important features for CDC+, Strict+, and high reactivation risk that would not have been otherwise identified from a less detailed stimulation assay, such as QFT. Feature selection from the precision normalized data input revealed an expected consistency between CDC+ and Strict+, with two additional important features for Strict+. Interestingly, the Strict+ designation contained two additional biomarkers, CCL4 (also known as MIP-1β) and IL-23, which were uniquely revealed only by personalized normalization and exclusive to this LTBI designation relative to all others.

The features correlating with high and low risk were generally consistent when using absolute or normalized cytokine levels, revealing IL-2, IP-10, and IFN-γ. Importantly, both analyses also displayed orthogonal features between high- and low-risk designations. Relative to the other LTBI- and TB- related

designations, the risk categories have the fewest important features; however, this is likely due to the limited number of subjects within these two subsets of the total LTBI+ population in this study. Nevertheless, the orthogonal nature of biomarker features for reactivation risk stratification offers high promise as it would help identify subjects most likely to benefit from LTBI therapy.

DISCUSSION

LTBI is a persistent global health problem that is currently not well diagnosed by the available clinical diagnostic methods to detect LTBI and assess potential risk for TB reactivation. To date, little work has been done to develop a robust integrated diagnostic workflow for the identification of new biomarker signatures for personalized reactivation risk assessment [9, 38, 39]. Recent studies have identified potential alternative biomarkers for discriminating active TB from LTBI, but this is often completed with traditional protein detection methods following QFT assays, which offer limited stimulation and sample conditions [40, 41]. Furthermore, there has been little success in stratifying patients according to reactivation potential, in part due to the limited capacity to profile panels of biomarkers from multiple stimulation conditions with sufficient throughput to access a more comprehensive immunological profile.

To this end, we developed a multiplexed cytokine detection assay based upon high density arrays of silicon photonic microring resonators. Using this platform, we developed and validated a 7-plex cytokine detection panel applied to LTBI. The microring resonator assay exhibited good dynamic range, limits of detection, and reproducibility, and correlated well with single-plex ELISA assays. This proof-of-concept study demonstrated that this diagnostic technology accurately detected and quantified concentrations of seven cytokines secreted from patient-derived PBMCs after stimulation with TB-relevant and control antigens and, when combined with a powerful feature selection approach, revealed individually-normalized immune signatures that correlated to LTBI status and risk of reactivation.

Using a Boruta feature selection method, absolute and normalized cytokine levels were found to be important for LTBI-relevant clinical designations under proper stimulation conditions. Of highest potential diagnostic relevance, IFN-γ, IP-10, and IL-2 correlated with Strict+, CDC+, and high-risk LTBI designations. Independently and as a subset of limited biomarker panels, each identified antigen has previously shown potential for diagnosing active TB and discriminating the active disease from LTBI [42–46]. In particular, while IFN-γ has been the sole biomarker for TB diagnostics [47, 48], IP-10 (chemokine ligand CXCL10) displays similar dynamics to IFN-γ and often found at higher concentrations yielding a promising increased detection rate for TB [42, 49–53]. This multiplexed biomarker approach provides improvement over current diagnostic strategies by potentially mitigating missing a false negative outcome due to occasional down-regulation of a single cytokine [54, 55].

A critical aim of this study was to determine whether a personalized approach to immunological normalization could help reveal new multi-biomarker signatures for LTBI diagnosis and stratification of reactivation risk. By employing a precision medicine approach to correct for immunological heterogeneities between individuals, a more diagnostically rich signature of the immunological landscape can be revealed. Furthermore, by identifying additional relevant features using machine learning methods, one might elucidate immunological changes that are specifically diagnostic of disease reactivation.

The potential for increased immunological nuance via normalized biomarker secretion is apparent by the increased number of important LTBI+ status features revealed through the Boruta feature selection for the normalized concentration analysis relative to the absolute concentration analysis. For example, when considering absolute cytokine concentrations IL-2 was the only feature identified as important for CDC+ and Strict+ designations, with IP-10 and IFN-γ found to be tentative for CDC+ and Strict+, respectively. By contrast, important features from all three of these markers were revealed for the same designations when considering precision-normalized cytokine levels. Additionally, tentative and important features for IL-23 and CCL4 were also identified for the Strict+ designation after normalization by off-target controls. CCL4 is of particular interest as increased levels of this chemoattractant have been correlated with TB+ contacts, suggesting it may be better correlated with exposure or latency rather than active TB [30, 56, 57].

Boruta feature selection for high- and low-reactivation-risk yielded equal numbers of important and tentative features using both absolute and normalized cytokine values, but interestingly both analyses for reactivation risk identified orthogonal signatures. This might suggest eventual differential diagnostic capabilities in identifying a subset of patients most likely to reactivate to active disease. Additionally, both absolute and normalized analysis identified features that were not from TB-relevant stimulation conditions. This is noteworthy because the mechanism for reactivation, while not well-defined, is thought to be affected by overall immune response. Therefore, basal immune response, such as elucidated from a non-TB, fungal pathogen, might provide insight into overall immune fitness that is somehow linked to risk of reactivation. The interplay between general immune response and reactivation risk is best illustrated by two characterized mechanisms for reactivation: co-infection with HIV and therapeutic neutralization of tumor necrosis factor. Both reactivation mechanisms result from compromised immune function and advantageous proliferation of TB [3, 5]. Therefore, immunoprofiling methods that extend beyond TB-related antigen response may be critical to provide a more comprehensive reactivation risk assessment.

CONCLUSION

We have developed and validated a multiplexed immunodiagnostic approach toward diagnosis of LTBI and stratification of reactivation risk that relies entirely upon secreted biomarker signatures from a simple *in vitro* assay. Multiplexed cytokine immunoprofiling using both TB-related and control antigen exposures of circulating immune cells was performed using a robust silicon photonic measurement platform. Machine learning feature selection approaches were used to reduce this data-dense sensor output to identify specific biomarker signatures correlated with LTBI status and reactivation risk. The biomarkers IFN-γ, IP-10, and IL-2 appear as particularly promising markers for assessing LTBI status and TB reactivation risk when considering comprehensive stimulation conditions and precision normalization for heterogeneities in basal immune response. Furthermore, orthogonal biomarker signatures were identified to stratify LTBI reactivation risk. Though this study is relatively sample-limited, the initial group of biomarkers will serve as the basis for building and evaluating a composite cytokine-based, multi-marker signature predictive for LTBI diagnosis and reactivation that will be expanded and applied to a larger research subject cohort. More generally, the initial success of this overall approach suggests that the integration of data-rich biosensing

technologies with powerful feature selection informatics may lead to new opportunities in inflammatory-based precision disease diagnostics.

SUPPLEMENTARY DATA

Supplementary data is available at *Integrative Biology Journal* online.

CONFLICT OF INTEREST STATEMENT

None declared.

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