



Defining Discriminatory Antibody Fingerprints in Active and Latent Tuberculosis

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Tuberculosis (TB) is among the leading causes of death worldwide from a single infectious agent, second only to COVID-19 in 2020. TB is caused by infection with *Mycobacterium tuberculosis* (Mtb), that results either in a latent or active form of disease, the latter associated with Mtb spread. In the absence of an effective vaccine, epidemiologic modeling suggests that aggressive treatment of individuals with active TB (ATB) may curb spread. Yet, clinical discrimination between latent (LTB) and ATB remains a challenge. While antibodies are widely used to diagnose many infections, the utility of antibody-based tests to diagnose ATB has only regained significant traction recently. Specifically, recent interest in the humoral immune response to TB has pointed to potential differences in both targeted antigens and antibody features that can discriminate latent and active TB. Here we aimed to integrate these observations and broadly profile the humoral immune response across individuals with LTB or ATB, with and without HIV co-infection, to define the most discriminatory humoral properties and diagnose TB disease more easily. Using 209 Mtb antigens, striking differences in antigen-recognition were observed across latently and actively infected individuals that was modulated by HIV serostatus. However, ATB and LTB could be discriminated, irrespective of HIV-status, based on a combination of both antibody levels and Fc receptor-binding characteristics targeting both well characterized (like lipoarabinomannan, 38 kDa or antigen 85) but also novel Mtb antigens (including Rv1792, Rv1528, Rv2435C or Rv1508). These data reveal new Mtb-specific immunologic markers that can improve the classification of ATB versus LTB.

Keywords: active and latent tuberculosis, antibodies, HIV, biomarkers, diagnostics

INTRODUCTION

Infection with *Mycobacterium tuberculosis* (Mtb) affects one quarter of the world's population (WHO report 2020) and causes approximately 1.5 million deaths annually (WHO global TB report). While the majority of infected individuals control the infection for life, in a state referred to as latent tuberculosis infection (LTB) (1, 2), approximately 5–10% of the infected individuals lose control of the bacterial infection. Consequently, these individuals progress to active tuberculosis (ATB) disease, and if untreated contribute centrally to dissemination of the infection and may ultimately succumb to death (3–5). Moreover, in the setting of HIV co-infection, the risk of developing ATB is 20 to 30 times higher, and without treatment the mortality rate of ATB reaches nearly 100% (3). Thus, diagnostic tools are urgently needed to help guide clinical care across populations.

Current diagnostic tests include assays of bacteriological presence (microscopic or genetic), supplemented by the analysis of symptoms, radiological evidence and tests that involve the detection of recall-memory to Mtb antigens based on the tuberculin skin test (TST) or the ex vivo detection of memory Mtb-specific T-cells based on the interferon- γ (IFN- γ) release assay (IGRA) (6, 7). While TST and IGRA clearly capture Mtb exposure, current diagnostics distinguish ATB and LTB poorly, rendering clinical management of TB disease a major challenge in curbing deaths and disease spread. Thus, improved TB diagnostics are urgently needed to reduce morbidity and mortality.

Pathogen-specific antibodies represent critical markers of infection and disease across many infectious disorders (8–10). Since 1983, antibody responses have been studied for the serodiagnosis of TB (11–16). In addition, studies on HLA-linked immune response highlighted association between sputum smear-positive TB and expression of Class II HLA genes HLA-DR2, with HLA-DR2 being strongly correlated with antibody titers against 38-kDa Mtb protein (17), thus further motivating the identification of antibody-diagnostics to guide TB clinical care (18, 19). However, like many infectious diseases, including COVID-19 (20, 21), antibody titers to Mtb increase with pathogen burden (22–25). Because antigen load can vary drastically across subjects, antibody magnitude may be an incomplete marker of disease progression. However, emerging data strongly suggest that antibodies differ across ATB and LTB, both with respect to antigen-specificities targeted selectively across disease states, but also with respect

to differences in the quality of the Mtb-specific humoral immune response (isotype, subclass, and Fc-glycosylation) (26–29). Thus far, studies aiming at developing antibody-based diagnostics for ATB and LTB have focused on a small number of Mtb antigens including lipoarabinomannan (LAM), purified protein derivative (PPD), ESAT6/CFP10, Ag85A/B and MPT64 (26, 27, 30). Even in the setting of HIV, which is associated with CD4 T cell suppression and altered humoral immunity (4), humoral immune responses to Mtb have been shown to discriminate between ATB and LTB (30). However, whether additional Mtb antigen specificities and antibody qualities could provide clearer resolution of LTB and ATB remains unclear. Thus, in this study we comprehensively profiled the humoral immune response across 209 Mtb antigens, predicted to be highly enriched in the lung under hypoxic conditions as would be present in a granuloma (31, 32). Using a systems serology approach, we identified novel classes of antigen-specific antibody profiles able to resolve and discriminate ATB and LTB, independent of HIV status.

MATERIALS AND METHODS

Study Subjects

Plasma samples from 4 groups of adults were included in this study: ATB/HIV+ (n = 12), LTB/HIV+ (n = 22), ATB/HIV- (n = 21), and LTB/HIV- (n = 22) (**Table 1**). All subjects were recruited from Cape Town, South Africa. LTB was defined as the absence of TB symptoms, no previous history of TB diagnosis or treatment and presence of IFN- γ based on an IFN- γ release assay (IGRA). ATB was defined as a positive culture for *Mycobacterium tuberculosis* (Mtb) growth or positive sputum smear microscopy. Blood from ATB individuals was obtained between 0 and 7 days of standard course anti-TB treatment following South African National Health Guidelines. None of the patients included in the study were on antiretroviral therapy (ART) at the time of enrollment. For each participant, whole blood was collected in sodium heparin Vacutainer tubes (BD Biosciences). Plasma samples were isolated by centrifugation under 500g after 5 minutes, within 4 hours of collection. Prior to the study, a written and informed consent was given by all study participants. Consents were approved by the Human Research Ethics Committee of the University of Cape Town and the Western Cape Department of Health, as well as the study institutional review board at Massachusetts General Hospital and Partners Healthcare.

TABLE 1 | Demographic data and HIV-associated parameters.

	HIV+/ATB	HIV+/LTB	HIV-/ATB	HIV-/LTB
Total number	12	22	21	22
Mean age (years \pm SD)	34,8 \pm 7.6	32,8 \pm 7.4	40,2 \pm 10.5	28,6 \pm 8.6
Gender (Females)	6 (50%)	17 (77%)	8 (38%)	9 (41%)
Viral load mean (copies/ml \pm SD)	127722,5 \pm 280219,9	38225,3 \pm 62464,65		
CD4+ T cell count mean (cells/mm³ \pm SD)	206,2 \pm 183,6	506,8 \pm 295,0		

Antigens

Two hundred and nine Mtb antigens were used in this project. Purified LAM was received from BEI Resources and purified protein derivative (PPD) was obtained from the Statens Serum Institute. The remaining 207 proteins were recombinantly expressed Mtb antigens received from Dr. Tom Ottenhoff and Kees Franken (Table S1), and prepared as described previously (33, 34). These antigens were selected based on their immunogenicity and their discriminatory potential for TB diagnosis (33, 35, 36).

Luminex Beads Coupling and Antigen-Specific Immunoglobulin Quantification

Antigen-specific antibody subclass and isotype levels present in the plasma of individuals with TB were measured using a custom multiplexed Luminex assay, as previously described (37). This customized Luminex platform has been used extensively across diseases (38–41). Mtb antigens were coupled to magnetic carboxylated fluorescent Luminex beads (Luminex Corporation) by carbodiimide-NHS ester coupling, with one bead region per antigen. Before the coupling with the antigens, beads were activated in an activation buffer containing 100 mM monobasic sodium phosphate (pH 6.2), in addition to 50 mg/ml N-hydroxysulfosuccinimide (Sulfo-NHS; Pierce) resuspended in H₂O and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide-HCl (EDC; Pierce) resuspended in activation buffer. After a 30-minute incubation at room temperature (RT), beads were washed in coupling buffer (50 mM morpholineethanesulfonic acid (MES; pH 5.0)), then incubated with Mtb antigens for 2 hours at RT. Beads were then blocked during a 30 minute incubation at RT in phosphate buffered saline (PBS)-TBN (0.1% bovine serum albumin [BSA], 0.02% Tween 20, and 0.05% azide [pH 7.4]). Finally, beads coupled to proteins were washed in PBS-Tween (0.05% Tween 20) and stored in PBS with 0.05% sodium azide at 4°C. As LAM is a glycolipid, a modification by COOH-4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) was required before the coupling to Luminex beads, as described previously (42). For this protocol, 2 µl of DMTMM (200 mg/ml; Sigma-Aldrich) were used for 25 µg of LAM. After 1 hour of incubation at RT, the excess of DMTMM was removed by using Sephadex G-25 PD-10 desalting columns (GE Healthcare). LAM was then added to the beads and incubated overnight at RT. The next day, LAM-coupled beads were washed in PBS then stored in PBS with 0.05% sodium azide at 4°C.

Antigen-coupled beads were added to plasma samples from TB individuals at 1:100 dilution in PBS and incubated at 4°C for 18 hours of shaking. Beads were then washed 3 times with PBS-Tween (0.05% Tween 20) and incubated with phycoerythrin (PE)-conjugated mouse anti-human IgG1, IgG3, IgA1 or IgM (Southern Biotech) at 1.3 µg/ml. After 1 hour of incubation at RT with shaking at 800 rpm, beads were washed 3 times with PBS-Tween (0.05% Tween 20) and resuspended in sheath fluid (Luminex Corporation). Each plasma sample was tested in duplicate, and PE median fluorescence intensity (MFI) levels were measured *via* the iQue screener plus (Intellicyt) analyzer.

Antigen-Specific Fcγ-Receptor Binding

Fcγ-receptors (FcγRs) were purchased from Duke Human Vaccine Institute to study the relative binding levels of Mtb-specific antibodies to individual FcγRs (43). Avi-tagged FcγR2AR, FcγR2B, FcγR3AV and FcγR3B were biotinylated with a BirA biotin-protein ligase (BirA500; Avidity) and the excess of biotin was removed with Zeba spin desalting columns (7K MWCO; Thermo Fisher Scientific).

Diluted plasma samples (1:100) were added to antigen-coupled beads to form immune complexes as described above, and after 18 hours of incubation at 4°C, beads were washed 3 times with PBS-Tween (0.05% Tween 20). At the end of the incubation, streptavidin-R-phycoerythrin (ProZyme) was added to each biotinylated FcγRs in a 4:1 molar ratio during an incubation time of 20 minutes at RT. Fluorescent labeled FcγRs (1 µg/ml in 0.1% BSA-PBS) were then added to immune complexes and incubated for 1 hour at RT. Finally, beads were washed 3 times with PBS-Tween (0.05% Tween 20), then resuspended in sheath fluid (Luminex Corporation) and the median PE intensity was measured *via* the iQue screener plus (Intellicyt) system. Samples were tested in duplicate.

Statistical and Computational Analysis

Data analysis was performed using R version 4.0.2 (2020-06-22). Comparisons between active and latent individuals were performed using a Mann-Whitney U-test followed by a Benjamini-Hochberg (BH) correction for multiple comparisons.

A multivariate approach, combining a least absolute shrinkage and selection operator (LASSO) for feature selection and classification using partial least square discriminant analysis (PLS-DA) with the LASSO-selected features was used to define feature that discriminated active and latent antibody profiles. Prior to analysis, mean fluorescence (MFI) values were first log-transformed and all data were normalized using z-scoring. Models were built using the R package “ropls” version 1.20.0 (44) and “glmnet” version 4.0.2. Model accuracy was assessed using five-fold cross-validation. For each test fold, LASSO-based feature selection was repeated 100 times, features selected at least 90 times out of 100 were identified as selected features. A PLS-DA classifier was applied to the training set using the selected features, and a prediction accuracy was recorded. The model was also validated *via* permutation testing, where the performance of the selected model was evaluated against randomly shuffled active-latent labels. Selected features were ordered according to their Variable Importance in Projection (VIP) score, and the first two latent variables (LVs) of the PLS-DA model were used to visualize the samples.

RESULTS

A Limited Mtb Antigen Panel Highlights Differences Across ATB and LTB but Does Not Fully Discriminate Across Disease Status

To explore humoral responses in individuals with LTB and ATB, we began by profiling isotype and subclass levels across 8

common *Mtb* antigens (LAM, PPD, ESAT6/CFP10, Ag85A and 85B, groES and PSTS3) in ATB and LTB individuals that were HIV positive or negative. As previously described, HIV negative ATB individuals exhibited higher levels of IgG1, IgG3 and IgA1 responses to particular antigens, including LAM, PPD, and groES (**Figures 1A, B**). Conversely, an opposite profile was observed in HIV positive individuals, marked by significantly elevated humoral immune responses, and particularly IgM responses to Ag85A and PSTS3 among HIV positive LTB (**Figures 1A, C**). However, these features did not result in complete discrimination of ATB and LTB patients across HIV serostatus (**Figures 1D, E**) (cross-validation accuracy of 62.8% for HIV-; 67.6% for HIV+), suggesting that common antigen-specific antibody profiles provide only a modest level of discrimination across TB infection and disease states.

An Expanded *Mtb* Antigen Panel Identifies Unappreciated *Mtb*-Specific Antibody Differences Between ATB and LTB Across HIV Infection Status

Despite the lack of differences across a limited number of common *Mtb* antigens (**Figure 1**), *Mtb* can express up to 4000 distinct antigens (45, 46), many of which may serve as additional antibody targets. Thus, we next elected to deeply profile the humoral immune response across LAM, PPD as well as 207 *Mtb* antigens (**Table S1**), transcriptionally enriched in *in vitro* or *in vivo* infection models, including lung specimens

(36). Specifically, IgG1, IgG3, IgA1 and IgM levels were profiled across all antigens in ATB and LTB among HIV positive and HIV negative individuals (**Figure 2**). The data highlighted generally expanded IgG1 and IgG3 responses across HIV negative and positive ATB compared to LTB, with some sporadic elevated IgG1/IgG3 responses in HIV-positive LTB (**Figure 2A**). IgA responses were significantly elevated in HIV negative ATB compared to LTB, despite expanded IgA immunity in HIV-positive LTB (**Figure 2A**). IgM responses were more diffuse across HIV negative groups, despite the presence of a few high IgM responses in ATB compared to HIV negative LTB. Conversely, the IgM response was distinct in the setting of HIV co-infection, marked by significant expansions of particular *Mtb*-specific IgM responses that were either enriched in ATB or were enriched in LTB (**Figure 2A**). Thus, using this expanded antigen set, striking differences were noted in the evolution of the humoral immune response across HIV-serostatus, pointing to the presence of particular antigen-specific humoral immune responses that may discriminate between TB disease states.

Minimal Sets of *Mtb*-Specific Antibody Responses Discriminate Between ATB and LTB Across HIV Status

To define the minimal set of specific antigens that were differentially targeted across the groups, we began by quantifying the number of antigens that were differentially targeted by each subclass or isotype of antibodies (**Table S2**).

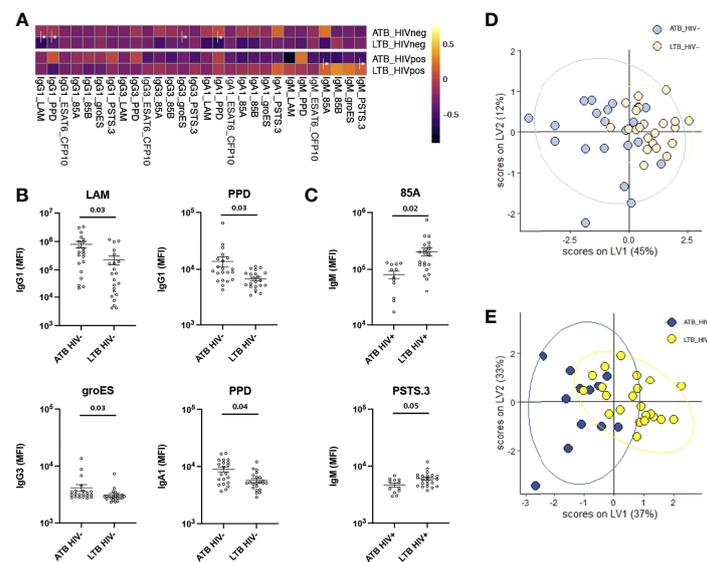


FIGURE 1 | Antibody levels against common *Mtb* antigens differ between ATB and LTB patients. Relative levels of IgG1, IgG3, IgA1 and IgM against 8 common *Mtb* antigens (LAM, PPD, ESAT6/CFP10, Ag85A and 85B, groES and PSTS3) were quantified via Luminex in the plasma of ATB ($n=21$) and LTB ($n=22$) patients in the HIV negative group and ATB ($n=12$) and LTB ($n=22$) patients in the HIV positive group. **(A)** For the heatmap, Z-score transformation of the MFI values was performed, and the median of each group was graphed. Antibody levels that are significantly different between ATB and LTB are graphed separately for all individuals in the HIV negative **(B)** and the HIV positive **(C)** groups. A Mann-Whitney U-test followed by a Benjamini-Hochberg (BH) correction for multiple comparisons was used to test for statistically significant differences ATB and LTB. Multivariate analysis using LASSO and PLS-DA model shows antibody levels comparisons between ATB and LTB in HIV negative **(D)** and positive **(E)** populations. Cross-validation accuracy for **(D, E)** was 62.8% and 67.6%, respectively.

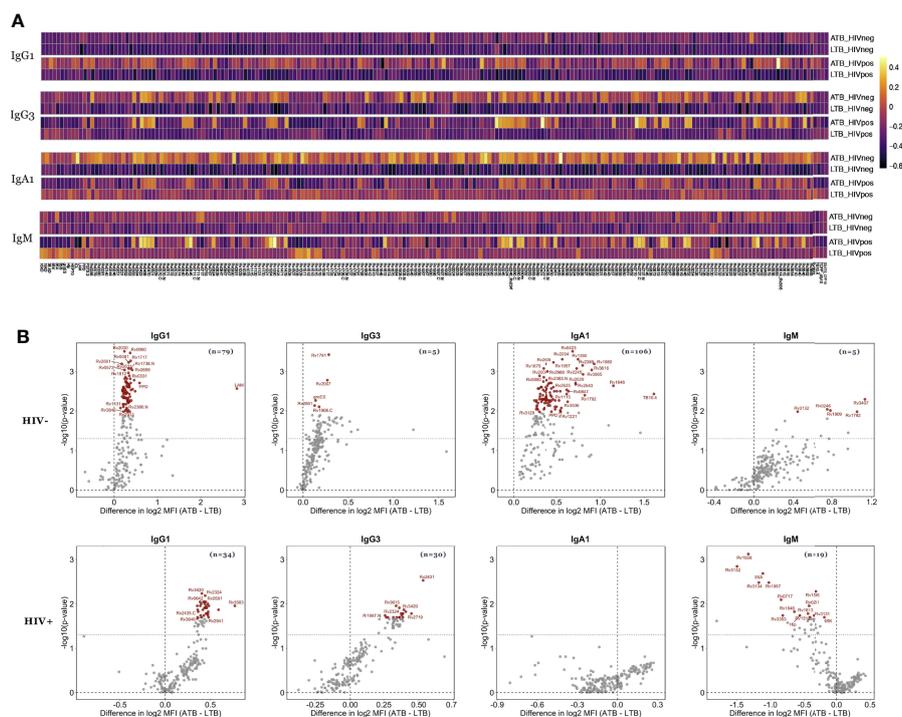


FIGURE 2 | Mtb-specific antibody levels differ between ATB and LTB in HIV negative and HIV positive populations. Relative levels of IgG1, IgG3, IgA1 and IgM against 209 Mtb antigens were quantified *via* Luminex in the plasma of ATB ($n=21$) and LTB ($n=22$) patients in the HIV negative group and ATB ($n=12$) and LTB ($n=22$) patients in the HIV positive group. **(A)** Heatmaps illustrate the median of Z-scored MFI data for each group indicated. **(B)** The volcano plots characterize the magnitude (\log_2 fold change of ATB/LTB) and the significance (p values) of antibody levels between ATB and LTB. Values above black dashed lines are statistically different between ATB and LTB ($p < 0.05$). For adjusted p values, significant data are shown in red, non-significant differences are in black.

Specifically, in HIV negative individuals, 79 Mtb antigens were differentially targeted by IgG1, 5 antigens were differentially targeted by IgG3, 106 were differentially targeted by IgA1, and 5 antigens were targeted distinctly by IgM (**Figure 2B** and **Table S2**). Conversely, in HIV positive individuals, fewer statistically significant differences were observed after multiple correction but included: 34 Mtb antigens that were targeted statistically differently by IgG1, 30 that were differentially targeted by IgG3, and 19 antigens were targeted uniquely by IgM across the two groups (**Figure 2B**). Interestingly, for most comparisons, IgG1, IgG3, and IgA1 levels were higher in ATB individuals, in accordance with published data and likely reflective of the enhanced inflammatory state and bacterial burden observed in ATB infection among HIV positive individuals (22, 23). However, Mtb-specific IgM responses were targeted differently in HIV positive and negative individuals, with higher Mtb-specific IgM responses in ATB in the HIV negative group, but higher Mtb-specific IgM responses in LTB in the HIV positive population, reflecting a shift in B cell response profiles in the setting of HIV. Yet, collectively, these data highlight the striking differences in Mtb-specific humoral immune responses in the setting of HIV infection, but still associated to the persistence of differential humoral immune responses across ATB and LTB.

Differential Mtb-Specific Antibody Binding to Fc γ -Receptors Across Mtb Disease States

Beyond overall changes in antibody subclass and isotype responses to Mtb, emerging data point to significant differences in the inflammatory state of Mtb-specific antibodies across disease states (26, 29). These changes are induced by alterations in antibody-Fc-glycosylation, aimed at deploying antibody effector functions required for enhanced clearance and control of the pathogen (47). Because these Fc-glycosylation changes result in altered binding to Fc γ -receptors (FcR), we next examined whether Mtb-specific FcR binding differences (Fc γ R2AR, Fc γ R2B, Fc γ R3AV, and Fc γ R3B) existed against LAM, PPD and 207 Mtb antigens (**Table S1**) in HIV positive and negative individuals with ATB or LTB (**Figure 3**).

Thirty-nine Mtb antigens were differentially recognized by Fc γ R2AR binding antibodies in HIV negative LTB populations compared to ATB (**Figure 3B**). Moreover, 54, 31 and 50 Mtb antigens were differentially recognized by Fc γ R2B, Fc γ R3AV and Fc γ R3B-binding antibodies, respectively, with higher binding observed in ATB (**Figures 3A, B** and **Table S2**). Conversely, FcR binding antibodies were globally shifted in HIV positive individuals, marked by disproportionately higher

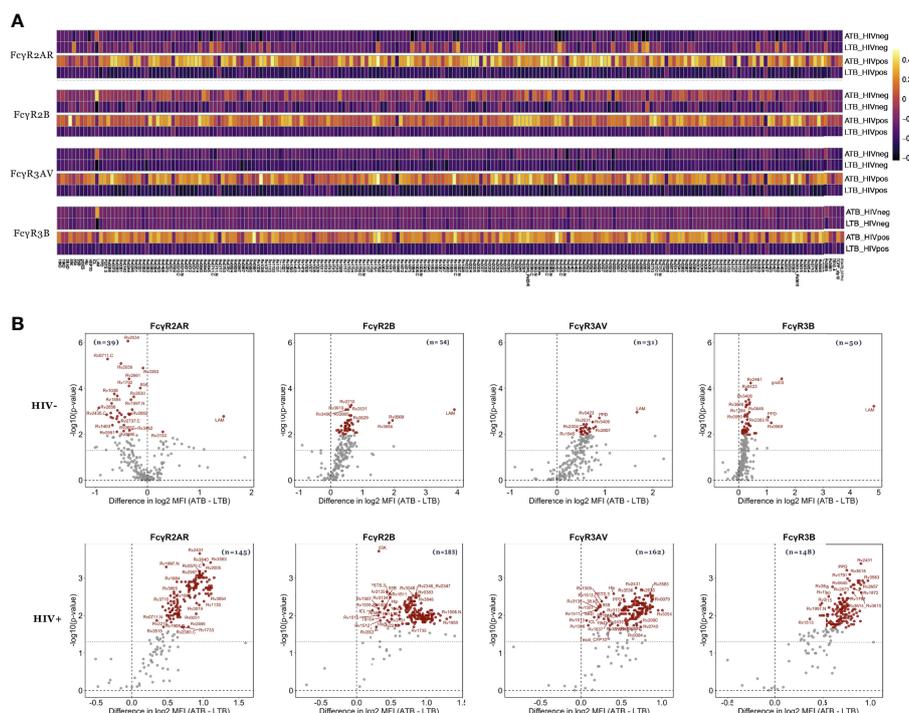


FIGURE 3 | Mtb-specific FcR binding distinguishes ATB from LTB among HIV negative and positive individuals. The ability of antibodies to bind to FcγR2AR, FcγR2B, FcγR3AV and FcγR3B was measured in the plasma of ATB (n=21) and LTB (n=22) patients without HIV infection as well as ATB (n=12) and LTB (n=22) patients with HIV. **(A)** Heatmaps show median values of FcR binding after Z-score transformation to each of the 209 Mtb antigens. **(B)** Fold change between FcR binding in ATB and LTB as well as the significance (p values) of differences are plotted the volcano plots. Values above black dashed lines are statistically different between ATB and LTB ($p < 0.05$). For adjusted p values, significant data are shown in red, non-significant differences are in black.

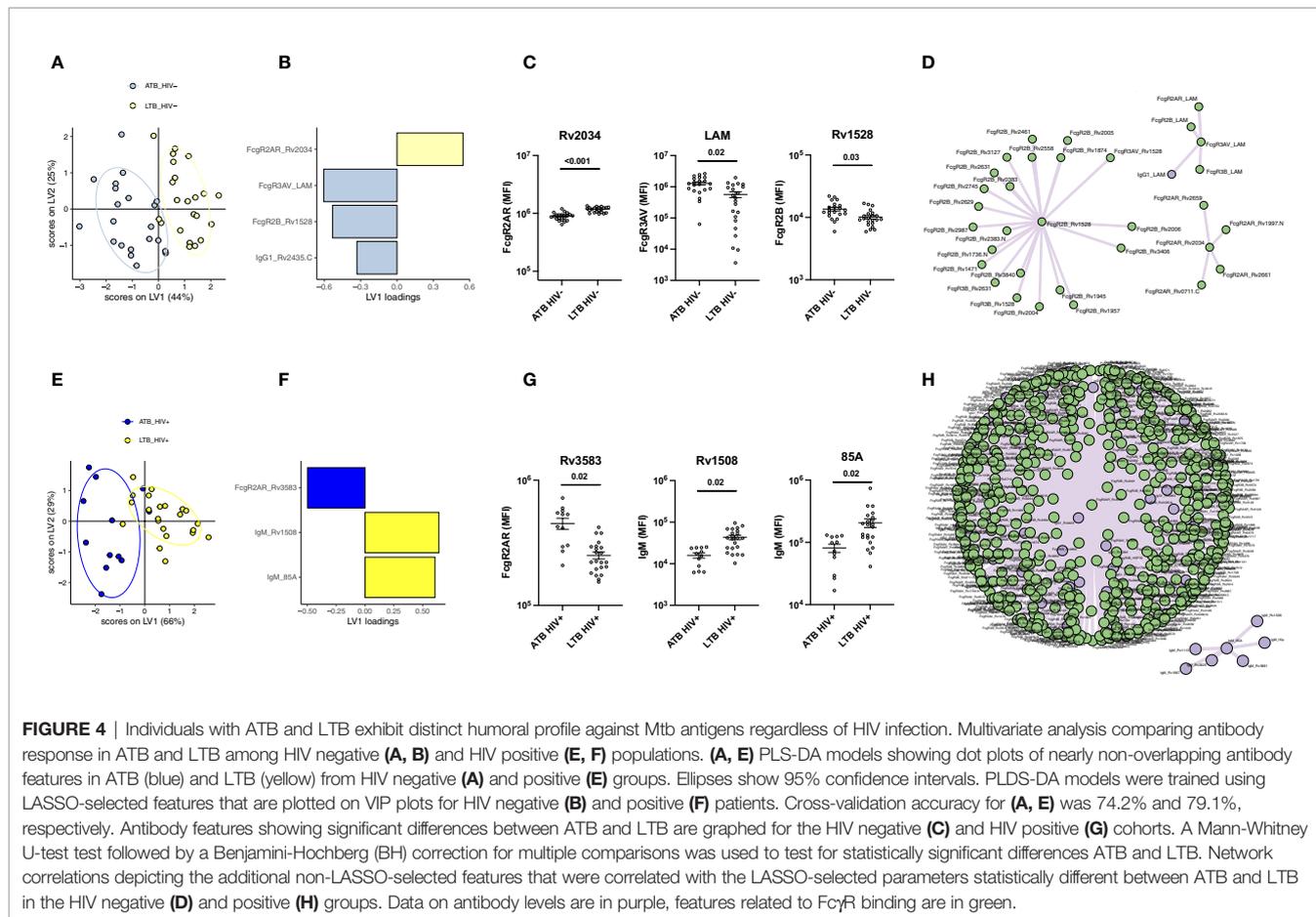
levels of FcγR2AR, FcγR2B, FcγR3AV and FcγR3B binding antibodies in HIV+ ATB compared to LTB (**Figure 3**). Specifically, 145, 183, 162 and 148-Mtb-antigens were targeted more robustly by FcγR2AR, FcγR2B, FcγR3AV and FcγR3B binding antibodies, respectively, in ATB compared to LTB (**Table S2**). Thus, beyond alterations in overall antibody subclass/isotype binding profiles across TB disease status, alterations are observed with FcR binding, that are amplified in the setting of HIV co-infection, again pointing to the possibility for antibody-based discrimination across LTB and ATB across HIV status.

A Minimal Set of Antibody Biomarkers Can Discriminate LTB and ATB Irrespective of HIV Status

Given the significant differences in isotype, subclass, and FcR binding differences across LTB and ATB in both HIV positive and negative individuals, we next aimed to identify a minimal set of potential candidate antigens that could be used to discriminate between ATB and LTB. A computational analysis that combined LASSO feature down-selection and PLS-DA classification was conducted in the HIV negative (**Figures 4A–D**) and positive (**Figures 4E–H**) groups. Out of the 1680 antigen-specific antibody features included in the analysis, only 4 features were

sufficient to separate HIV negative ATB and LTB (**Figures 4A, B**): RV2034-specific FcγR2AR binding, which was higher in LTB, as well as LAM-specific FcγR3A binding antibodies, RV1528-specific FcγR2B binding antibodies, and RV2435c IgG1 levels that were enriched in ATB (**Figure 4B**). Three of the LASSO selected features were significantly differentially targeted across the groups, including RV2034-specific FcγR2AR binding, LAM-FcγR3A binding and RV1528-specific FcγR2B binding antibodies (**Figure 4C**). Given that LASSO selects a minimal set of features that account for the greatest variance across the groups, the LASSO-selected features may represent groups of correlated antibody features that diverge across TB groups. Thus, to gain enhanced insights into the global changes across groups, a co-correlate analysis was built on the LASSO-selected features highlighting the presence of a large, expanded FcγR2B network in ATB, as well as two smaller networks of LAM-biomarkers and FcγR2AR binding biomarkers enriched in HIV negative ATB individuals (**Figure 4D**).

Similar to the HIV negative LTB/ATB individuals, as few as 3 of the 1680 analyzed features were sufficient to separate LTB and ATB individuals with HIV (**Figures 4E, F**). Specifically, RV3583 FcγR2AR binding was enriched in ATB individuals, and RV1508 and Ag85A IgM levels were enriched among LTB (**Figures 4E, F**). All features were significantly different across the groups



(**Figure 4G**). Interestingly, co-correlates analysis revealed that the single ATB feature was tightly linked to nearly all IgG/FcR binding levels that were globally expanded in ATB (**Figure 4H**). Additionally, a smaller network of IgM features emerged, marking the unique expansion of IgM responses among HIV positive LTB.

Finally, we aimed to estimate the classification accuracy of the LASSO-selected antibody features across ATB and LTB. An area under the receiver operating characteristic (ROC) curve (AUC) was calculated for each feature (**Figures 5A, B**). Importantly, the combination of the selected antibody features gave an AUC close to 1 (AUC = 0.98 for HIV-; AUC = 0.92 for HIV+). However, even using individual antibody features, classification accuracies of 0.96, 0.8, 0.71 and 0.74 were observed with Rv2034-specific binding to FcγR2AR, LAM-specific binding to FcγR3AV, Rv1528-specific binding to FcγR2B and Rv2435C-specific IgG1 levels, respectively, among HIV negative individuals (**Figure 5A**). Similarly, in HIV positive individuals, individual feature AUCs reached 0.81, 0.86 and 0.84 for Rv1508-specific IgM levels, binding of Rv3583-specific antibodies to FcγR2AR and Ag85A-specific IgM levels, respectively (**Figure 5B**). Our results showed that 4 parameters were systematically enriched in ATB compared to LTB among HIV negative and positive populations, which are IgG1 levels against Rv2435.C, as well as Rv3583-, Rv1528- and LAM-binding to FcγRAR, FcγR2B and FcγR3AV, respectively (**Figure 5C**). Thus, a

minimal set of largely novel Mtb-specific humoral biomarkers may provide a unique opportunity to help discriminate between LTB and ATB. Given the ease of generation of rapid point-of-care antibody-based diagnostics, these data point to a simple opportunity to develop rapid point-of-care tests for the diagnosis of Mtb disease state.

DISCUSSION

The lack of rapid and specific point-of care diagnostic tools has impeded the control of tuberculosis, particularly among HIV positive individuals (WHO report 2020; (4, 48, 49). Serum antibody-based tests represent promising alternatives to the current medically intensive diagnostic approaches associated to sputum collection for culture or non-specific tests like TST or IGRA to diagnose active TB. However, previous work with strict quantitative measurements of canonical antigen-specific antibody levels has failed, in the past, to identify Mtb-specific humoral biomarkers able to discriminate ATB and LTB (50). We thus aimed to broaden the scope of biomarker discovery, integrating both qualitative differences in humoral immune profiles as well as the breadth of antigen-specific antibody responses across individuals with/without TB among HIV positive and negative adults. In addition to the 8 common Mtb

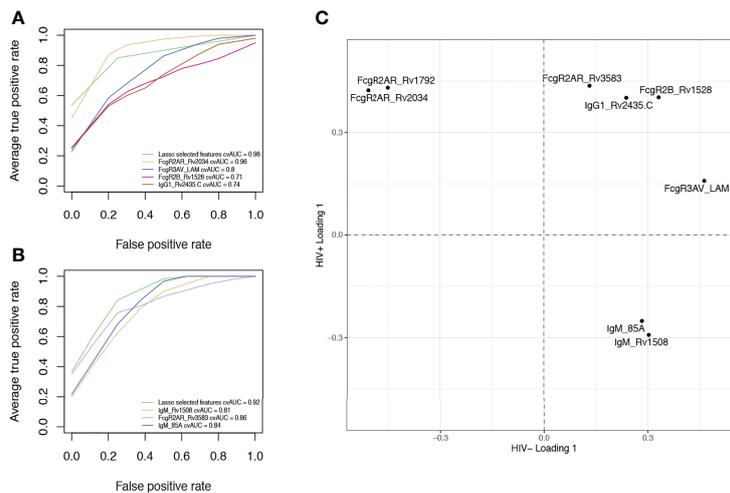


FIGURE 5 | Discriminatory features between ATB and LTb. ROC curves showing the sensitivity and the specificity of potential biomarkers discriminating ATB and LTb in HIV negative (A) and positive (B) populations. (C) Loading plot showing LASSO-selected features enriched in ATB, both among HIV negative and HIV positive individuals (upper right quadrant) versus those enriched in LTb among HIV negative and HIV positive groups (low-left quadrant). Features in the upper left quadrant are increased in ATB compared to LTb among HIV+ but are enriched in LTb among HIV-. Features in the low right quadrant are enriched in LTb compared to ATB among HIV+ but are higher in ATB among HIV-.

antigens (LAM, PPD, ESAT6/CFP10, Ag85A and 85B, groES and PSTS3), 201 additional novel Mtb antigens were included, collectively providing significant resolution of ATB and LTb in HIV negative populations, including higher levels of IgG1 against LAM and PPD in ATB individuals, as well as increased groES-specific IgG3 and PPD-specific IgA1 levels (Figures 1, 2). Conversely, while HIV positive ATB/LTB were also resolvable, the antigen-specific antibody profiles used to discriminate these groups shifted to include higher 85A- and PSTS3-specific IgM response in LTb due to expected HIV induced alterations in immunity. This immune dysregulation in ATB patients with HIV might in part be attributable to lower CD4⁺ T cell counts and higher viral loads, resulting in altered B cell responses and elevated inflammation (Table 1). However, collectively, a set of antigen-specific antibody profiles were defined in both HIV negative and positive populations that provide antigen-specific resolution that could guide TB diagnostic development, providing a unique opportunity to build point-of-care diagnostic tools that would improve TB medical care.

Increased IgG and IgA responses have been previously observed in ATB individuals (22, 24, 30). Studies have shown that high titers are correlated with bacillary load in patient sputum (51). Interestingly, only a weak separation was observed between ATB and LTb when using a limited list of common Mtb antigens (accuracy: 62.8%) (Figure 1). Similarly, other studies have shown that antibodies targeting known antigens including Ag85, ESAT6, and CFP10 have limited diagnostic accuracy. Moreover, conflicting reports have demonstrated that the sensitivity of Ag85-specific IgG levels reaches 67.5% for the diagnosis of ATB (50), while others suggested that Ag85-specific IgG levels can provide up to 84.1% (52). Conversely, here the expansion of antigens to additional targets that may be expressed more abundantly in the lung

during Mtb infection (36), improved diagnostic accuracy to 98% in HIV negative individuals and 92% in HIV positive subjects (Figure 5). Whether these antigen-specific antibodies contribute to the immune response to Mtb remains unclear but may reflect the presence of a more robust host (antibody) response to highly expressed antigens, that may more sensitively resolve individuals with differential control of Mtb.

Surprisingly, HIV co-infection abrogated the differences in IgG and IgA observed in ATB compared to LTb among HIV negative individuals. In accordance with our observations, data published previously reported that total amounts of LAM IgG were higher in ATB/HIV- compared to ATB/HIV+ individuals (30). Also, the loss of IgM observed in ATB individuals with HIV points to a significant shift in the humoral immune response upon co-infection (53). Moreover, given our emerging appreciation for the importance of IgM in Mtb control (54), it is plausible that the loss of IgM may compromise anti-microbial control in HIV/Mtb co-infection, contributing to disease progression. IgM is known for its antimicrobial function, inducing opsonophagocytosis, complement deposition and agglutination (55). Studies in mice have highlighted the main role that IgM plays in the control of Mtb infection by regulating immune response inside granulomas, leading to improved survival of infected mice during the chronic phase of the disease (56, 57). Here we observed that HIV positive individuals with LTb had higher IgM levels against particular Mtb antigens compared to individuals with ATB, including responses against Ag85A and PSTS3, potentially pointing to specific IgM targets that may represent critical Mtb therapeutic targets (Figures 1, 2).

In addition to isotype differences across ATB/LTB, Mtb-specific FcR binding profiles were significantly differentiated across the groups, both in HIV positive and negative populations (Figure 3).

Changes in FcR binding are attributable to both alterations in subclass distribution and Fc-glycosylation (58, 59), both of which shift rapidly in response to inflammatory cues that arise during infection. Thus, FcRs may act as sensitive markers of inflammatory changes in antibodies (47). Similarly, previous studies have highlighted striking differences in Fc-glycosylation across HIV negative LTB and ATB (26, 29). Moreover, despite the robust general Fc-inflammatory changes associated with HIV (60, 61), Fc-glycosylation changes still likely occur in the setting of HIV co-infection, marked by striking, and previously unappreciated highly significant increases in all FcR binding in the setting of ATB infection across most Mtb antigens (**Figure 3**). Interestingly, while the differences are not as pronounced in HIV negative ATB/LTB, Mtb-specific FcR measurements were still key to resolving the groups. FcR binding differences are likely attributed to significant changes in Fc-glycosylation, that can be detected using Mass-spectrometry, but may be more readily quantified, in a simpler point-of-care approach using FcR detection.

Specific antigen-dependent antibody responses were key to discriminating ATB and LTB. In HIV negative individuals, IgG1 levels against Rv2435.C as well as Fc γ R2AR, Fc γ R3AV and Fc γ R2B binding associated with Rv2034, LAM and Rv1528 respectively were sufficient for the separation of the groups (**Figure 4B**). In coinfecting individuals, only 3 parameters were enough for ATB versus LTB discrimination: Rv3583-specific binding to Fc γ R2AR in addition to Rv1508- and Ag85A-specific IgM levels (**Figure 4F**). Other than LAM and Ag85A, the additional targets are less well defined. Rv2034 has been described as a transcriptional regulator from the ArsR family (62) and shown to be expressed during pulmonary infection, inducing a strong T cell response (63), and clearly - now - also a robust antibody response. Little is known about Rv1529 and Rv2435.C, the former thought to be a polyketide-associated acyltransferase involved in lipid metabolism (64), and Rv2435.C has been defined as an adenyl cyclase essential for Mtb survival (65, 66). In HIV positive individuals, antibodies to Rv3583, a transcriptional regulator (67), were among the most discriminatory Mtb responses. Finally, IgM responses to Ag85A and Rv1508, the latter involved in cell surface structures and transport (68), were highly discriminatory. Interestingly, 4 features were similarly enriched in ATB compared to LTB in both HIV positive and negative groups, including RV2435c IgG1 levels as well as Rv3583-, Rv1528- and LAM-binding antibodies to Fc γ RAR, Fc γ R2B and Fc γ R3AV, respectively (**Figure 5C**). While it remains unclear how BCG-vaccination alters these antibody responses across populations, these data suggest that by including a set of 7 antigens and 3 Fc-readouts in a multiplexed diagnostic test, it may be possible to develop a single set of antigens and Fc-detectors to distinguish LTB and ATB across HIV positive and negative populations.

Collectively, this work identifies a number of novel antibody targets in ATB and LTB that may help guide diagnostic development. Specifically, these data point to novel combinations of Mtb antigen specificities and antibody Fc-qualities in discriminating between ATB/LTB. Indeed, in addition to LAM, RV2435c, Rv3583 and Rv1528 were also

essential for the discrimination of ATB and LTB regardless of HIV status. While several combinations of inflammatory cytokine or transcriptomic biomarkers have been proposed as new exploratory approaches to help discriminate between ATB and LTB, the development of a simple Mtb-antigen-specific antibody point of care diagnostic would provide simplicity and ease of diagnosis. Moreover, while a single Mtb-antigen antibody Fc feature that could discriminate across ATB/LTB in both HIV negative and positive individuals would be desirable, a single solution may not be possible. However, with the advent of novel, simple lateral flow diagnostics that can multiplex antigen and detectors, the combination of the 7 antigens with unique detectors could be easily developed, providing a simple diagnostic to rapidly determine ATB/LTB status in any particular population. Whether additional antigen-specificities, not probed here, could add additional resolution is possible, and warrants additional analysis. Moreover, further discriminatory antibody profile discovery in IGRA-negative individuals living in TB-endemic areas, among BCG-vaccinated individuals living outside of endemic regions, and analysis of LTB and ATB from disparate geographical regions will provide a better insight into the robustness of these antibody signatures. Additionally, future functional and Fc-glycosylation profiling may reveal whether these unique humoral signatures also track with differential control of the infection, which might lead to the identification of features in LTB individuals that are associated with high risk of progression to ATB. While the ultimate development of a TB diagnostic will require extensive validation across multiple populations globally, overall, our data expand the current knowledge of Mtb antigen-specific humoral immunity and highlights the need to deeply understand how humoral changes may be leveraged both in vaccine and diagnostic development.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of the University of Cape Town and the Western Cape Department of Health, and the study institutional review board at Massachusetts General Hospital and Partners Healthcare. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GA and NN designed and conceived the research study. NN and KF performed the experiments. DC, EL, WJ, and NN analyzed the data. MK and WH contributed with study cohort

administration and implementation. NN and GA wrote the manuscript, with contributions from DC, LD, BF, KF, CD, and TO. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.856906/full#supplementary-material>

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Conflict of Interest: GA is a founder and equity holder in Systems Seromyx and Leyden Labs. GA's interests were reviewed and managed by MGH and Partners HealthCare in accordance with their conflict of interest policies.

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