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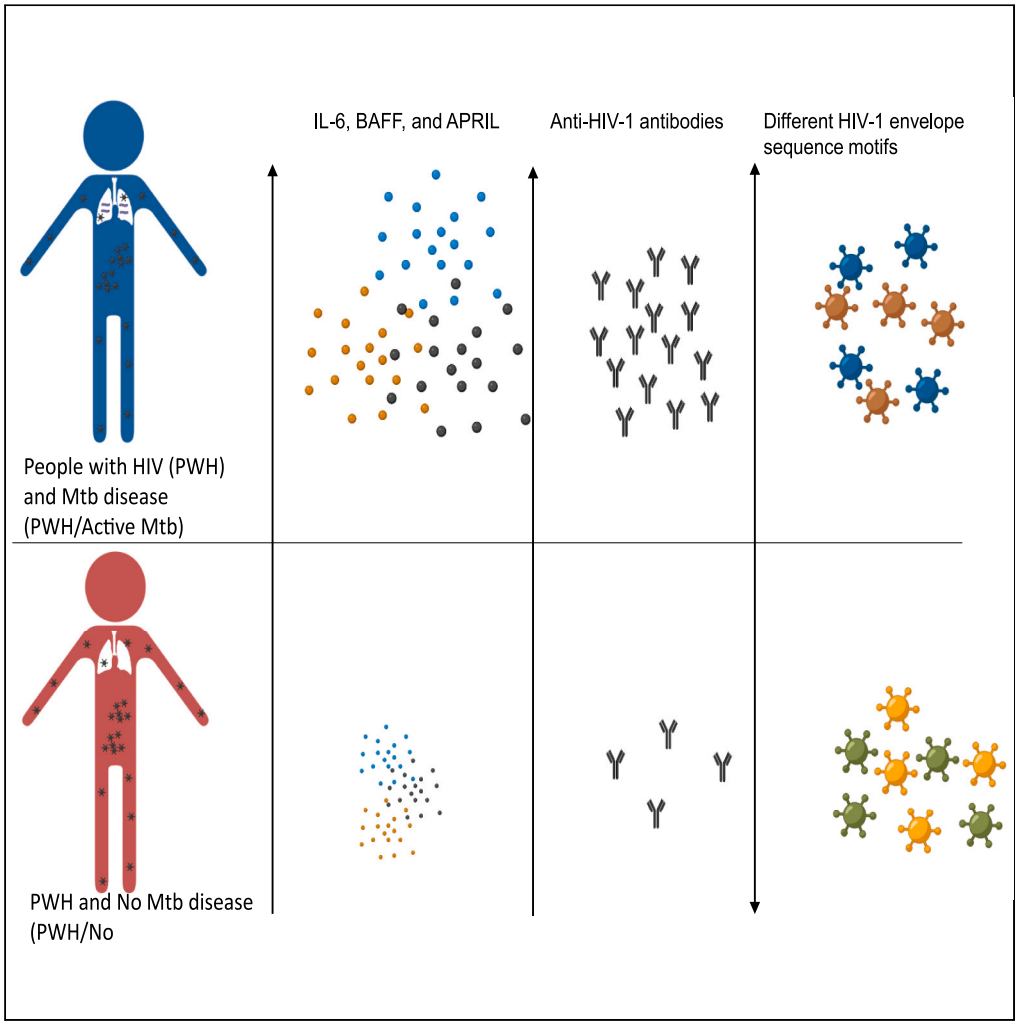

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Highlights

Mycobacterium tuberculosis (Mtb) disease enhances HIV-1 antibody responses

HIV-1 motifs associated with antibody resistance differ in the presence of Mtb

Mediators important for B cell and antibody development are elevated with Mtb

Mtb does not induce HIV-1 cross-reactivity or global antibody increase in PWH

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Mycobacterium tuberculosis disease associates with higher HIV-1-specific antibody responses

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SUMMARY

***Mycobacterium tuberculosis* (Mtb) is the most common infection among people with HIV (PWH). Mtb disease-associated inflammation could affect HIV-directed immune responses in PWH. We show that HIV antibodies are broader and more potent in PWH in the presence as compared to the absence of Mtb disease. With co-existing Mtb disease, the virus in PWH also encounters unique antibody selection pressure. The Mtb-linked HIV antibody enhancement associates with specific mediators important for B cell and antibody development. This Mtb humoral augmentation does not occur due to cross-reactivity, a generalized increase in all antibodies, or differences in duration or amount of antigen exposure. We speculate that the co-localization of Mtb and HIV in lymphatic tissues leads to the emergence of potent HIV antibodies. PWH's Mtb disease status has implications for the future use of HIV broadly neutralizing antibodies as prophylaxis or treatment and the induction of better humoral immunity.**

INTRODUCTION

Heterologous immunity describes immune responses induced against a pathogen or an antigen by an unrelated stimulus.[1] For instance, a previous viral infection can elicit beneficial or pathologic cross-reactive responses after exposure to another related but different virus.[2,3] Similarly, viral infections can also induce autoimmunity because of cross-reactivity between a virus epitope and a self-antigen.[4] Beyond cross-reactivity, heterologous immunity can occur through a phenomenon termed "trained immunity" when one exposure, classically mycobacterial infections, induces epigenetic changes that enhance innate immune responses against a subsequent unrelated infection.[5,6] Mycobacteria, specifically *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), induced trained immunity provides protection against secondary influenza, yellow fever virus, and malaria infections in human clinical trials.[5,7,8]

Other mycobacteria, such as *Mycobacterium tuberculosis* (Mtb), may stimulate immune responses against unrelated pathogens by other means besides trained immunity, such as bystander cell activation. Mtb infection imparts diffuse immune activation, which subsequently influences pathways associated with antibody production, potency, and functionality.[2] For example, heat-inactivated Mtb in oil, termed complete Freund's adjuvant (CFA), constitutes one of the most powerful activators of the humoral immune response.[9,10] CFA promotes antigen uptake and processing in antigen-presenting cells. This triggers a cytokine storm that impacts cellular and antibody responses.[10,11] CFA's use is banned in humans and highly restricted in animals, and thus there is limited ability to examine its impact in a prospective manner. Interestingly, individuals with chronic human immunodeficiency virus type 1 (HIV-1) infection and prior Mtb exposure have lower plasma virus levels as compared to those with no Mtb infection, possibly implying an immune interaction.[12] While immunization with Mtb and BCG antigens has been shown to promote higher antibody levels against HIV-1 and simian immunodeficiency virus (SIV) proteins in animal models,[13,14] there is limited human evidence for the impact of Mtb on HIV-1 humoral responses.

Two billion people in the world are infected with Mtb,[15] and Mtb is the most common co-infection in people with HIV-1 (PWH).[16] While the majority of Mtb-infected individuals have latent "inactive" infection, untreated HIV-1 accelerates the development of Mtb disease.[17] Furthermore, antiretroviral treatment (ART) initiation can restore anti-Mtb immune responses and "unmask" previously hidden Mtb disease.[18]

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While there are extensive studies of the impact of HIV-1 and ART on Mtb, surprisingly, the influence of Mtb on HIV-1 immune responses remains poorly characterized. We and others have previously demonstrated that PWH and Mtb disease have a unique inflammatory profile that can potentially impact the humoral response.[19,20] For instance, increased interleukin (IL)-6 observed in PWH and Mtb disease can drive the secretion of IL-21 in naive and memory T cells to promote antibody production.[21] Mycobacteria and HIV-1 can also co-exist in the same anatomic region, such as lymph nodes, and even in the same cell, such as macrophages,[16,22,23] and this co-localization may also impact the subsequent immune response. Based on CFA's known actions, unique Mtb-induced inflammation, and Mtb-HIV-1 co-localization, we hypothesized that Mtb disease may augment humoral immune responses against HIV-1.

Here, we show that PWH with as compared to those without active Mtb disease have broader and more potent HIV-1 humoral responses. The augmented HIV-1 antibody response is associated with specific plasma mediators known to be important for antibody production. Importantly, the enhanced antibody response does not occur from the non-specific induction of all antibodies or cross-reactivity. These observations have implications for using HIV-1 broadly neutralizing antibodies (bnAbs) for prophylaxis or therapeutics and future strategies aimed at enhancing humoral responses in PWH.

RESULTS

PWH and Mtb disease have higher HIV-1-specific neutralization breadth and potency

We first compared HIV-1 neutralization responses in ART-naive PWH who had confirmed Mtb disease (PWH/Active Mtb, n = 15) to PWH with no diagnosed or suspected active Mtb (PWH/No Mtb, n = 37, Figure S1). Prior to any ART, all the PWH/Active Mtb samples were from individuals enrolled in an Mtb diagnostic trial in Uganda.[20,24] The PWH/No Mtb individuals were from the Mtb diagnostic trial (n = 16) and the AIDS Clinical Trial Group (ACTG) 5274 study (n = 21).[25] The ACTG 5274 study compared the efficacy of isoniazid prophylaxis versus empiric Mtb treatment for decreasing mortality in therapy-naive PWH starting ART. At enrollment, the ACTG 5274 and the Uganda PWH/No Mtb individuals had no confirmed or probable Mtb disease. They were not evaluated for the presence of latent Mtb infection. The PWH/No Mtb as compared to PWH/Active Mtb (Table S1) and the Uganda as compared to ACTG 5274 (Table S2) individuals had significant differences in baseline characteristics, such as the absolute CD4 T cell count. Thus, we used multivariate analyses to account for these diverse demographics.

We examined responses against a panel of twelve different heterologous HIV-1 envelope glycoproteins (Envs) using the TZM-bl neutralization assay to compare HIV-1 neutralizing antibodies (nAbs) among the two groups.[26] Neutralization against these twelve Envs recapitulates responses against over 200 globally isolated HIV-1 variants.[27] HIV-1 neutralizing responses were compared using a breadth and potency (BP) score as described previously.[28] Briefly, the neutralization BP score consists of an average of the log normalized percent neutralization against each virus at the highest tested plasma concentration (1:50 dilution). BP scores range from a minimum of 0 indicating no neutralization capacity to 1, which represents the ability to neutralize all twelve variants. Importantly, percent neutralization observed at the highest tested plasma dilution strongly correlates with both area under the neutralization curve and the plasma dilution required to achieve half-maximal inhibitory concentration (ID_{50}) calculated using a series of diluted plasma.[28–30] Furthermore, the neutralization BP score strongly correlates with the neutralization breadth, defined as the percent of the global Env panel neutralized at greater than 50% at the highest tested plasma dilution (Figure S2).

Prior to any ART, PWH/Active Mtb as compared to the PWH/No Mtb had significantly higher neutralization BP scores ($p < 0.0001$, Figure 1A). In multivariate linear regression analysis, PWH/Active Mtb as compared to PWH/No Mtb had around 0.26-unit higher BP score (95% confidence interval [CI] 0.15–0.37, $p < 0.0001$) after adjusting for gender, $\log_{10}$ plasma virus level, absolute CD4 count, and age (Table 1). Neutralization fingerprints were compared among the PWH/Active Mtb and PWH/No Mtb groups to highlight potential differences in antibody specificities among the groups. Prior to starting ART, heatmap analysis showed that most plasma samples separated into three clusters with 100% bootstrap support (Figure 1B). Cluster 3 contained only PWH/No Mtb samples, and these samples demonstrated less than 50% neutralization against nearly all Env variants except subtype A X398F1. Neutralization capacity against eight other Envs also differentiated PWH/Active Mtb and PWH/No Mtb (Figure S3).

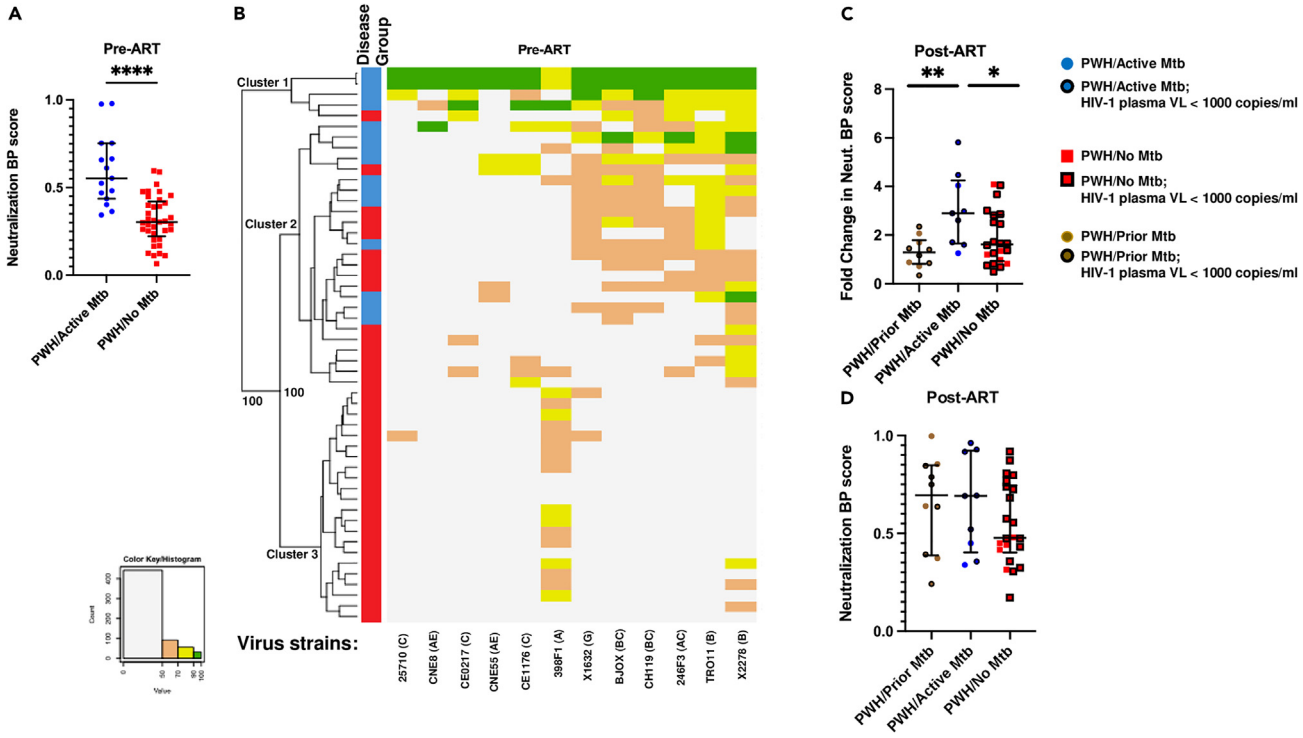

Figure 1. Mtb disease enhances the HIV-1 antibody response

(A and D) Neutralizing breadth potency (BP) score among (A) pre-ART and (D) post-ART plasmas.

(B) Heatmap showing clustering of PWH/Active Mtb and PWH/No Mtb plasma samples before ART. Rows are individual plasma samples, and columns are the 12 different Env variants. The left-hand side shows the clustering with bootstrap support. The bars to the left of the heatmap indicate either PWH/Active Mtb (blue) or PWH/No Mtb (red) plasma samples. The color histogram on the bottom left shows levels of neutralization: green 90–100%, lime green 70–90%, peach 50–70%, and light gray 0–50%. Below the heatmap, the column labels indicate the Env name and subtype in bracket.

(C) Fold change of neutralization BP score at follow-up relative to baseline among post-ART plasmas. In all graphs, PWH/Treated Mtb (brown circles), PWH/Active Mtb (blue circles), and PWH/No Mtb (red squares). In (C & D), the brown, blue, and red symbols with black boundaries represent those with post-ART plasma virus levels below 1000 copies per milliliter. Lines and error bars show median and interquartile ranges. Asterisks (*), (**), and (****) denote p values <0.05, <0.005, and less than 0.00005, respectively, and are calculated based on an unpaired t-test with Welch's correction (A) and Mann-Whitney test (D). See also Figures S3, S4, S14, and S15.

After enrollment, all participants initiated ART, and follow-up samples around six months later were available from 41 of the 52 individuals (Figure S1). PWH/Active Mtb at baseline also started Mtb treatment based on national guidelines, and follow-up samples were collected after Mtb therapy completion (PWH/Prior Mtb, n = 10, Table S3). Nine of the PWH/No Mtb at entry had diagnosed or suspected Mtb disease a median of 140 (range 105–169) days after starting ART. These nine were either assigned to isoniazid prophylaxis (n = 4) or empiric Mtb treatment (n = 5) per ACTG 5274 protocol. These individuals were categorized as post-ART PWH/Active Mtb. None of the remaining PWH/No Mtb at baseline that were subsequently on ART alone (n = 11), or also on isoniazid prophylaxis (n = 3), or empiric Mtb treatment

Table 1. Predictors of neutralization BP score prior to ART initiation

	Estimate (β)	95% confidence interval	p value
(Intercept)	0.002	−0.59 to 0.59	0.99
Mtb disease	0.26	0.15 to 0.37	<0.0001
Age	0.001	−0.004 to 0.006	0.62
Male	−0.03	−0.13 to 0.08	0.54
CD4 count (cells/mm^3)	0.0003	−0.0001 to 0.0007	0.16
Log_{10} plasma virus (copies/ml)	0.05	−0.05 to 0.14	0.33

See also Tables S1 and S2.

(n = 8) were diagnosed or suspected to have Mtb disease at follow-up (post-ART PWH/No Mtb). The post-ART PWH/Active Mtb group had fewer men with lower absolute CD4 T cell counts compared to the other two groups although these differences were not statistically significant (Table S3). After ART initiation, PWH/Active Mtb had a significantly higher ratio of follow-up to baseline neutralization BP score (median ratio 2.9, range 1.2–5.8) compared to PWH/Prior Mtb (median ratio 1.3, range 0.4–2.3) and PWH/No Mtb (median ratio 1.6, range 0.5–6.8) ($p = 0.01$, one-way ANOVA, Figure 1C). In multivariate linear regression analysis, developing Mtb disease after starting ART associated with around 1.6-fold higher follow-up to baseline neutralization BP ratio (95% CI 1.0–2.6, $p = 0.05$) after accounting for absolute CD4 cell count, plasma virus level, and gender. This suggests that active Mtb that develops after starting ART also associates with higher HIV-1 nAbs. Furthermore, after ART initiation, neutralization BP score was relatively stable for PWH/Prior Mtb after finishing Mtb treatment and for the PWH/No Mtb individuals (Figures 1C and 1D). In contrast to the pre-ART analysis, post-ART PWH/Active Mtb samples did not have significant separation in a neutralization heatmap compared to the other two groups (Figure S4).

Mtb disease in ART-experienced PWH associates with a higher antibody-dependent celluar cytotoxicity (ADCC)

Besides neutralization, antibodies can induce effector functions through Fc domains, such as natural killer (NK) cell-mediated ADCC. We and others have previously demonstrated that ADCC and neutralization responses are poorly correlated, which implies that they are independent functions.[30–33] We examined ADCC against ten of the reference panel Envs among the groups before and after ART initiation using an assay that measures the killing of infected cells only and not of uninfected cells with bound shed Envs.[32] An ADCC BP score was estimated in a similar manner as neutralization BP with 0 and 1 indicating no and 100% ADCC against all the strains in the panel, respectively.[30] Prior to starting ART, the ADCC BP score was not significantly different between the PWH/Active Mtb and PWH/No Mtb in both univariate (Figure 2A) and multivariate analysis. After starting ART, PWH and active Mtb had a significantly higher ADCC BP score ($p = 0.006$, one-way ANOVA, Figure 2B) and a higher ratio of follow-up to baseline ADCC BP ($p = 0.0005$, one-way ANOVA, Figure 2C) compared to PWH/Prior Mtb and PWH/No Mtb. In multivariable analysis, those with active Mtb disease after ART initiation had ADCC BP ratio around 0.18 units higher (95% CI 0.06 to 0.31, $p = 0.004$) after accounting for absolute CD4 count, gender, and plasma virus level (Table S4). The follow-up to baseline ADCC BP score was relatively stable in the PWH/Prior Mtb (median ratio 1.1, range 0.9–1.2) and PWH/No Mtb (median ratio 1.1, range 0.8–1.4) groups.

In contrast to our prior observations, neutralization and ADCC BP scores were moderately correlated prior to ($r = 0.52$, $p < 0.0001$, Figure 2D) but not after starting ART ($r = 0.28$, $p = 0.07$, Figure 2E). This pre-ART correlation was stronger in those with Mtb disease ($r = 0.72$, $p = 0.003$) as compared to the PWH/No Mtb ($r = 0.32$, $p = 0.06$). Thus, active Mtb disease associates with higher ADCC but primarily after starting ART. Furthermore, neutralization and ADCC BP correlate primarily among those with active Mtb prior to ART initiation.

Factors associated with antibody BP

We next aimed to understand if the enhanced HIV-1 antibody responses observed in those with active Mtb disease were associated with previously identified factors deemed important for humoral breadth. Broad and potent HIV-1 antibody responses primarily develop because of a high level of virus replication, longer duration of infection, and exposure to a greater number of Env variants.[34–36] High plasma virus levels and low absolute CD4 counts serve as rough surrogates for greater virus replication and longer duration of HIV-1 infection, respectively. Our previous multivariable linear regression analysis showed that these factors did not independently associate with neutralization BP score after accounting for Mtb disease status (Table 1). Individuals in the different groups were matched initially on plasma virus levels, and thus this characteristic was not significantly different among those with or without Mtb (Tables S1–S3). Both longitudinal and cross-sectional studies, however, have shown that Mtb disease increases HIV-1 replication.[37,38] Increased rounds of virus replication generate a larger number of genetic variants. Thus, Mtb disease may augment humoral responses by increasing the diversity of the HIV-1 Env antigen encountered by antibody-producing cells. We isolated 341 pre-ART Env sequences from a subset of the PWH/Active Mtb and PWH/No Mtb individuals to examine the impact of Env diversity on neutralizing responses. We did not analyze samples after ART initiation because ART distorts Env representation. Similar number of individuals and Envs per PWH were examined from PWH/Active Mtb and (n = 14, median Envs per subject 11, range 3–19) and PWH/No Mtb (n = 15, median Envs per subject 12, range 3–27, $p = 0.32$). Env sequences from

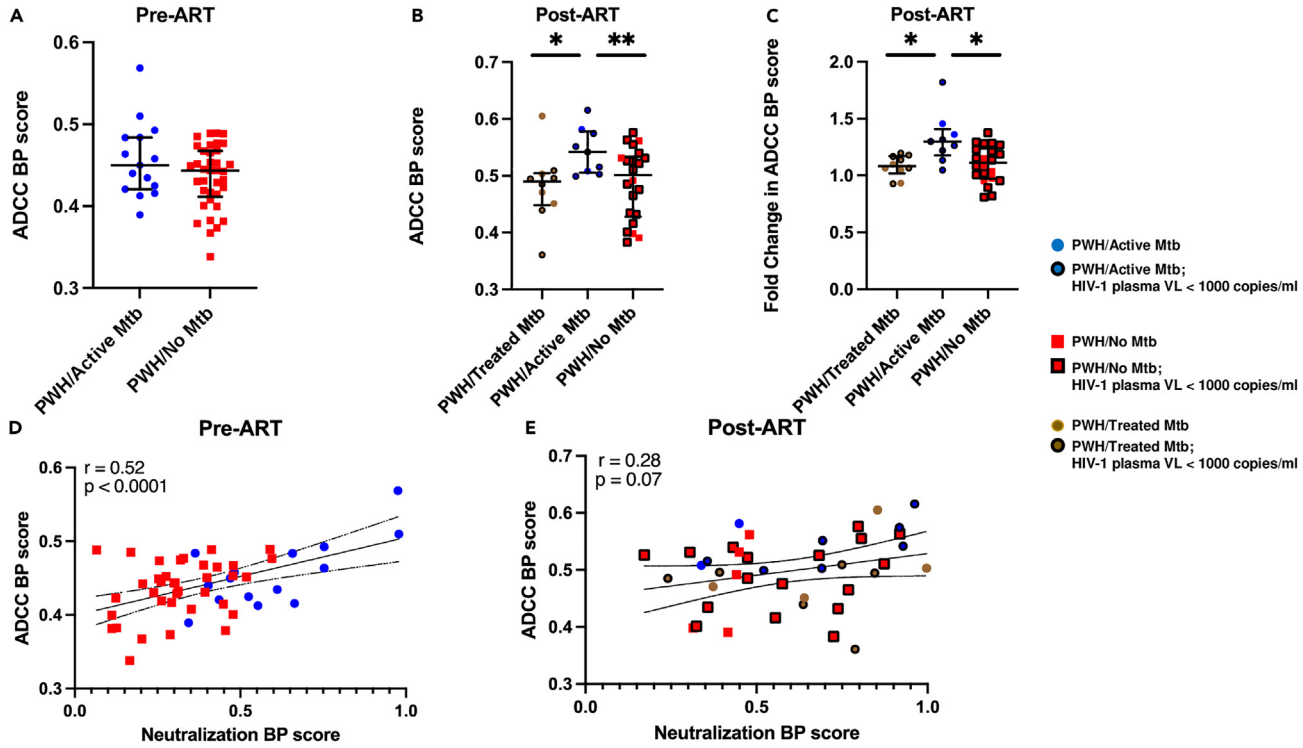

Figure 2. Plasma effector function activity associates with neutralizing activity

(A and B) ADCC breadth potency (BP) score among (A) pre-ART and (B) post-ART plasmas.

(C) Fold change of ADCC BP score at follow-up relative to baseline. In (A – C) plasma from PWH/Treated Mtb (brown circles), PWH/Active Mtb (blue circles), and PWH/No Mtb (red squares). Lines and error bars show median and interquartile ranges. Asterisks (*) and (**) denote p values <0.05 and <0.005, respectively, calculated based on an unpaired t-test with Welch's correction.

(D and E) Pearson's correlation plots depict associations for (D) pre-ART ADCC BP score with neutralization BP score and (E) post-ART ADCC BP score with neutralization BP score. In (B, C, & E), the symbols with boundaries represent those with post-ART plasma virus levels below 1000 copies per milliliter. Lines on correlation plots show the 95% confidence bands of linear regression.

different individuals clustered independently, although one PWH/No Mtb had sequences in more than one cluster suggesting a possible super-infection[39] (Figure 3A). There was no significant difference in Env genetic diversity among the PWH/Active Mtb and PWH/No Mtb (Figure 3B). Furthermore, Env genetic diversity did not correlate with pre-ART neutralization or ADCC BP scores (Figures 3C and 3D). Around 40%, 15%, 25%, and 20% of the individuals had subtype A, C, D, and circulating recombinant HIV-1 Envs. The clade distribution was not different among PWH/Active Mtb and PWH/No Mtb ($p = 0.98$, chi-squared test). These analyses suggest that characteristics previously associated with HIV-1 antibody BP do not account for the enhanced humoral responses observed in PWH and Mtb disease.

Mtb disease confers unique antibody selection pressure

BnAbs target conserved structures on the HIV-1 Env, which accounts for their BP.[40] Changes at specific sites that affect this conserved structure confer bnAb neutralization resistance. We hypothesized that escape from the differential antibody pressure will yield HIV-1 Envs with unique amino acid signatures among PWH/Active Mtb as compared to PWH/No Mtb. We used the GenSig tool (https://www.hiv.lanl.gov/content/sequence/GENETICSIGNATURES/gs.html) to identify amino acids differentially present in the PWH/Active Mtb and PWH/No Mtb Env sequences. This analysis compares the proportion of each single amino acid in the PWH/Active Mtb and the PWH/No Mtb sequences with a phylogenetic correction to minimize false positives due to lineage effects.[41] We used a false discovery rate (FDR) of $Q < 0.1$ to account for the multiple testing. Overall, there were thirty amino acids and eight predicted asparagine (N) linked glycosylation sites (PNGS) present at different frequencies in the PWH/Active Mtb and PWH/No Mtb sequences (Tables S5 and S6). Among these differentially expressed motifs, thirteen sites have previously been determined to influence sensitivity to various HIV-1 bnAbs (Table S7). Four of these thirteen differentially present

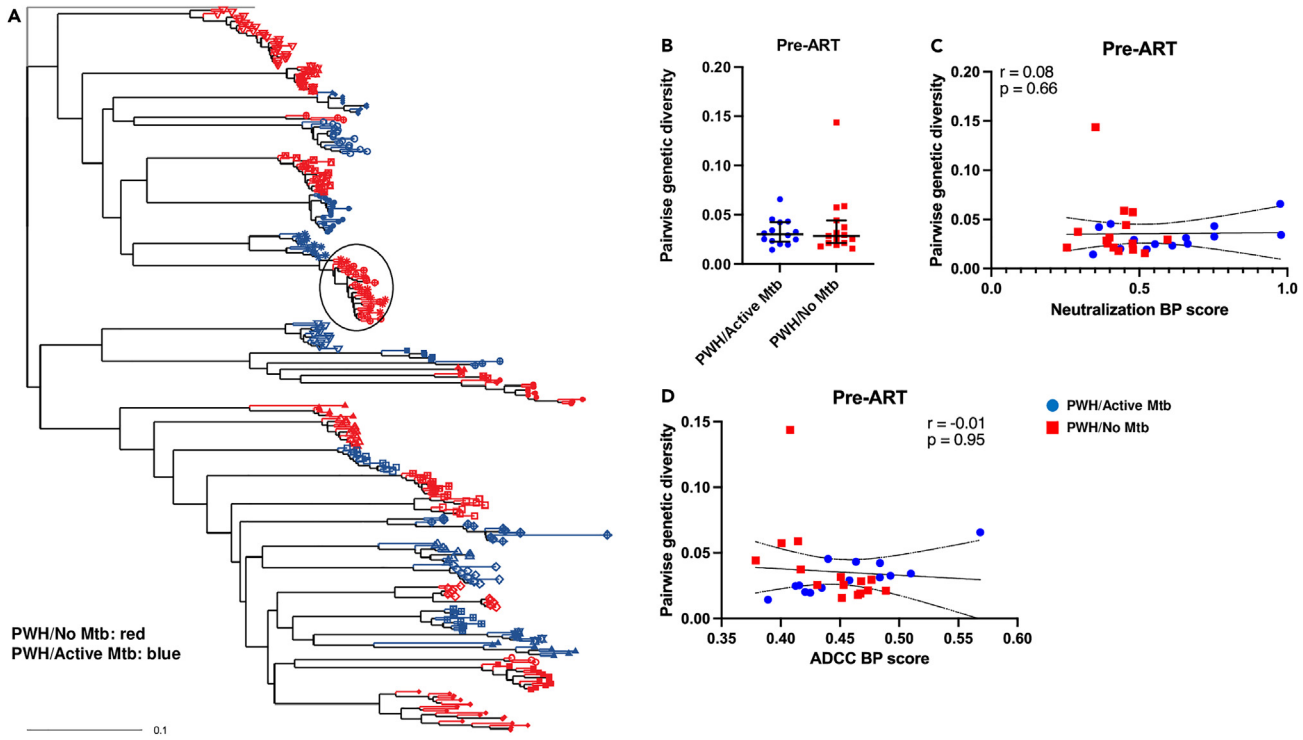

Figure 3. Viral Env diversity does not associate with neutralization and ADCC BP scores

(A) Maximum likelihood phylogenetic tree for PWH/Mtb (blue) and PWH/No Mtb (red). Different symbols at the tips indicate sequences from different individuals, and the circle shows interspersed sequences from two PWH/No Mtb.

(B) Pairwise genetic diversity of PWH/Active Mtb (blue circles) as compared to PWH/No Mtb (red squares). Lines and error bars show median and interquartile ranges.

(C and D) Correlation plots depict associations for (C) neutralization BP score and (D) ADCC BP score with pairwise genetic diversity in the PWH/Active Mtb (blue circles) and PWH/No Mtb (red squares). The r and p values indicate Spearman correlation statistic and, lines depict linear regression with a 95% confidence interval.

amino acids have been previously associated with both increased sensitivity and resistance depending on the Env context and antibody under consideration. PWH/Active Mtb and PWH/No Mtb sequences were enriched for seven and two of the remaining nine amino acid changes associated with resistance to bnAbs, respectively ($p = 0.06$, Fisher's exact test). Interestingly, the seven changes enriched in the PWH/Active Mtb sequences and associated with increased resistance to various bnAbs were all present in the Env surface unit (gp120). In contrast, the two resistance-inducing amino acids present at higher frequency in the PWH/No Mtb sequences were in the transmembrane membrane domain (gp41). Env variable loop length and the number of glycosylation sites also influence neutralization.[41,42] There was no significant difference in the variable loop lengths and the number of predicted glycosylation sites in the PWH/Active Mtb and PWH/No Mtb sequences.

We next used the bnAb-Resistance Predictor (bnAb-ReP) to estimate susceptibility to various bnAbs for the 341 different Envs.[43] BnAb-Rep is an *in-silico* algorithm that uses machine learning to generate a resistance probability (ranging from 0 to 1) for different bnAbs based on Env sequences only. Envs are deemed resistant to a specific bnAb if they have a predicted probability below 0.5. Importantly, this algorithm has high prediction accuracy relative to *in vitro* results. None of the 33 bnAbs assessed was predicted to neutralize all 341 Envs (Table S8). We focused on the few bnAbs targeting different Env epitopes that were both predicted to neutralize the majority of the 341 different Envs and also had a previously published prediction performance area under the curve above 0.85 (Tables 2 and S8).[43] Envs from PWH/Active Mtb as compared to PWH/No Mtb showed significant differential predicted susceptibility to various bnAbs (Table 2). PWH/Active Mtb had Envs with more than 2.5-fold greater chance of predicted resistance to the CD4-binding site bnAb, VRC01 (Table 2), which is in advanced clinical testing for preventing HIV infection[44] and treatment.[45,46] In general, the PWH/Active Mtb individuals were predicted to have more Envs resistant to

Table 2. Predicted bnAb susceptibility among PWH/Active Mtb as compared to PWH/No Mtb

bnAb	Class	Prediction Accuracy	Median resistance probability in the 341 Envs (median range among 29 individuals)[a]	Chance of predicted resistance in PWH/Active Mtb versus PWH/No Mtb Envs (95% confidence interval, p value)
VRC01	CD4-binding site	0.92	0.54 (0.24–0.81)	2.68 (1.68–4.26, <0.001)
3BNC117		0.90	0.87 (0.06–0.99)	1.61 (0.95–2.72, 0.08)
10–1074	V3	0.94	0.82 (0.06–0.96)	0.54 (0.34–0.85, 0.008)
PGT128		0.86	0.75 (0.02–0.99)	0.53 (0.34–0.84, 0.007)
VRC26.08	V1-V2	0.85	0.61 (0.09–0.93)	1.81 (1.17–2.80, 0.007)
VRC26.25		0.87	0.71 (0.19–0.93)	3.02 (1.88–4.87, <0.001)
2F5	MPER	0.95	0.93 (0.02–0.98)	0.25 (0.15–0.42, <0.001)
>1	multiple	N/A	1 (0–3)[b]	1.08 (0.70–1.68, 0.72)

bnAb: Broadly neutralizing antibody.
MPER: membrane proximal external region variable region.
V: Env variable region.
N/A: not applicable.
See also Tables S8 and S9.
[a]Probability less than 0.5 considered resistant.
[b]Indicates median number of the 4 bnAb classes with predicted resistance.

CD4-binding site and V1-V2-directed bnAbs and lower number with less sensitivity to V3 loop and membrane proximal external region bnAbs. Predicted resistance to more than one of the four assessed bnAb classes was similar in PWH/Active Mtb and PWH/No Mtb (Tables 2 and S9). This differential enrichment for the sequence motifs associated with bnAb resistance among PWH/Active Mtb and PWH/No Mtb further suggests unique antibody selection pressure in the presence of Mtb disease.

All or non-HIV-1 antibodies are not elevated in Mtb disease

We hypothesized that Mtb disease does not enhance the production of all antibodies, but rather it is HIV-1 specific in PWH. First, we examined total antibody levels to assess if Mtb disease associates with a generalized antibody increase. Total immunoglobulin G (IgG) levels were not significantly higher among those with active Mtb both before and after starting ART (Figures 4A and 4B). Furthermore, different immunoglobulin isotypes were not significantly different among those with and without Mtb disease both before and after ART (Figures S5A–S5L). In prior studies, HIV-1-specific neutralizing BP has also been associated with elevated total IgG levels,[47] but there was no correlation between total IgG levels and neutralization BP score, both before and after ART (Figures S6A and S6B). In previous studies from our group and others, a higher IgG/IgA ratio associates with a greater ADCC.[30,31,48–50] Similarly, in multivariable linear regression analysis, the pre-ART IgG/IgA ratio positively correlated with ADCC after accounting for baseline demographics (Table S10).

We next examined levels of antibodies that likely preexist in PWH prior to the development of Mtb disease. Antibodies against tetanus and herpes simplex virus ½ (HSV-1/2) are commonly detected in HIV-1 seropositive adults.[51,52] In contrast to the HIV-1 humoral responses, PWH/Active Mtb had lower anti-HSV-1 IgG titers prior to starting ART although this was not statistically significant after accounting for demographic differences in multivariable analysis (Figure 4C). There was no difference in anti-HSV-1 IgG after starting ART among the three groups (Figure 4D). Anti-tetanus IgG titers were similar in PWH/Active Mtb and PWH/No Mtb prior to ART (Figure 4E). The PWH/Prior Mtb had higher anti-tetanus IgG compared to those with active Mtb after ART, but this was not statistically significant in multivariate analysis. (Figure 4F).

Finally, we assessed the levels of antibodies that are potentially generated *de novo* during acute disease. Numerous infections have been associated with the emergence of autoantibodies.[53,54] We estimated autoantibody levels using Luminex-based mean fluorescence intensity (MFI) against beads coated with seventeen different autoantigens. We also used ELISA to measure anti-cardiolipin phospholipid IgG levels. Before and after ART, some autoantibody levels were different among the groups in univariate analysis (Figures 4G and 4H), but no group was consistently higher or lower. The presence of Mtb disease also

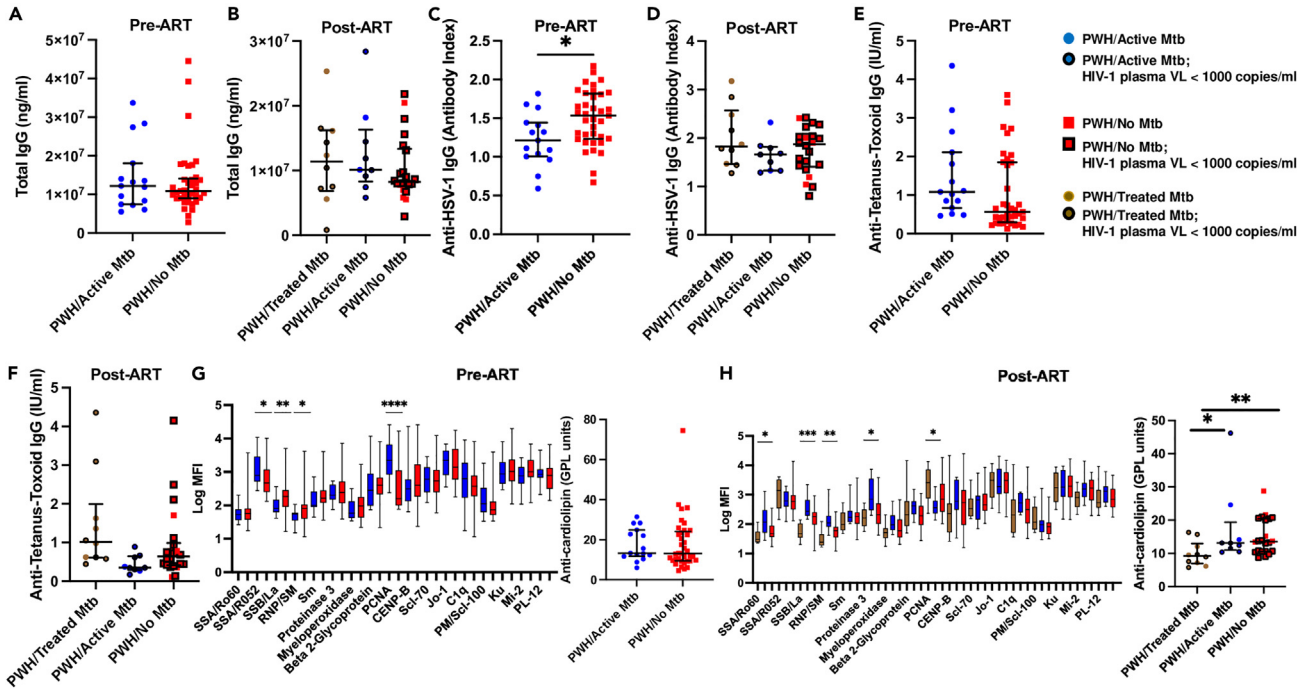

Figure 4. Mtb disease does not affect the levels of immunoglobulins and non-HIV-1 antibodies

(A–F) Concentrations of total IgG, anti-HSV-1 IgG, and tetanus-toxoid specific IgG (A, C, & E) before and (B, D, & F) after ART. Lines and error bars show median and interquartile ranges.

(G and H) Mean fluorescent intensity (MFI) of autoantibodies and phospholipid IgG (GPL) levels of anti-cardiolipin (G) before and (H) after ART. In (G & H), lines and whiskers show median and minimum to maximum range, respectively. Graphs show PWH/Active Mtb (blue circles), PWH/No Mtb (red squares), and PWH/Treated Mtb (brown circles), and the symbols with black boundaries represent those with post-ART plasma virus levels with less than 1000 HIV-1 copies/ml. Asterisks (*), (**), (***), (****) denote p values <0.05, <0.005, <0.0005, and <0.00005, respectively, calculated based on an unpaired t-test with Welch's correction. See also Figures S5 and S6 and Table S10.

did not have a significant association with any of the assessed autoantibody levels after accounting for multiple comparisons. None of the autoantibodies associated with either neutralization or ADCC BP score before or after ART in multivariable linear regression analysis after accounting for multiple comparisons.

Mtb infection does not elicit cross-reactive responses

Mtb disease association with enhanced HIV-1 neutralizing response in the absence of a generalized increase in antibodies may result from cross-reactivity. A previous study suggested that Mtb and HIV-1 share some epitope similarities.[55] We assessed HIV-1 plasma neutralization activity in 32 HIV-1 seronegative participants, some with a previous history of Mtb infection to examine possible cross-reactivity. Of these 32 participants, eight were healthy control, sixteen had latent Mtb, four had active Mtb disease, and four had just finished treatment for active Mtb (Table S11).[56] No individual's plasma demonstrated greater than 50% inhibition against any of the five tested reference panel Envs (246F3, BJOX, CE1176, 25710, and CNE8) (Figure S7). Thus, Mtb infection and disease do not elicit nAbs that cross-react with HIV-1 Envs.

Active Mtb disease is associated with a unique inflammatory profile

In the absence of cross-reactivity and a generalized antibody increase, we hypothesized that Mtb-mediated systemic inflammation may enhance HIV-1 antibody response as a bystander effect. Both the development of broad and potent neutralizing activity and Mtb disease have been associated with immune activation.[57,58] We measured a diverse set of analytes deemed important for lymph node germinal center (GC) formation, B cell development, and antibody production using a human B cell multiplex panel. Only seven IL-6, C–X–C motif chemokine 10 (CXCL10), chemokine motif ligand 5 (CCL5, RANTES), IL-10, a proliferation-inducing ligand (APRIL), B cell activating factor (BAFF), and soluble CD40 ligand (sCD40L)) of the fifteen measured analytes deemed important for B cells and antibodies yielded values above the limit of detection (LOD) for the majority of samples. Prior to any treatment, IL-6, APRIL, and

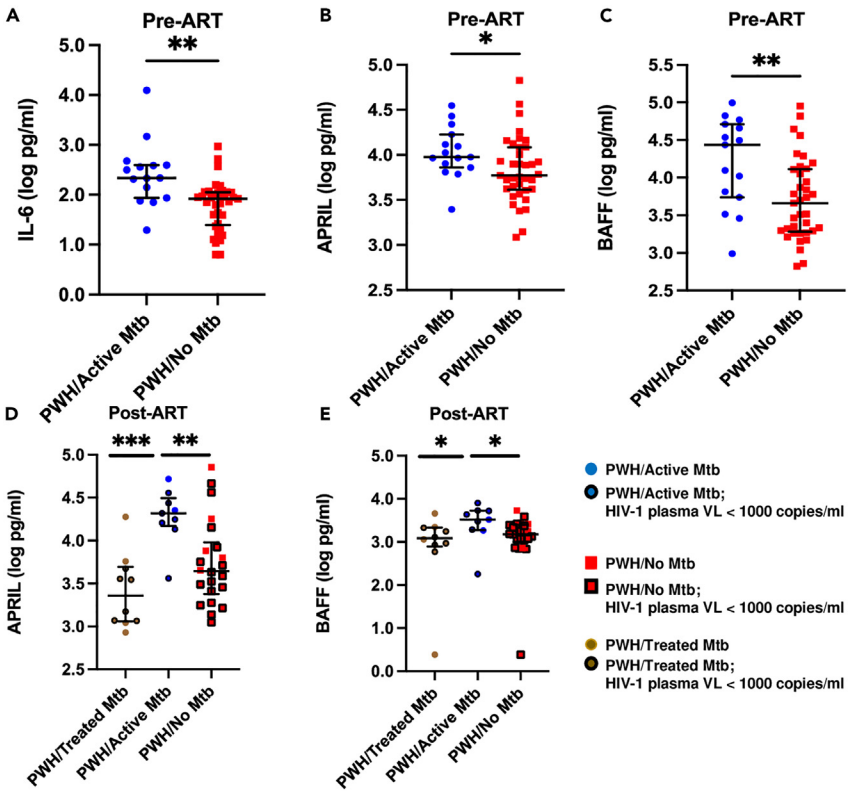

Figure 5. Plasma markers of germinal center activity associate with neutralizing activity

(A–C) Pre-ART (A–C) and post-ART concentrations (D–E) of IL-6 (A), APRIL (B & D), and BAFF (C & E) for PWH/Active Mtb (blue circles), PWH/No Mtb (red squares), and PWH/Treated Mtb (brown circles). In (D & E), the symbols with boundaries represent those with post-ART plasma virus levels below 1000 copies per milliliter. Lines and error bars show median and interquartile ranges. Asterisks (*), (**), and (***) denote p values <0.05, <0.005, and <0.0005, respectively, and are calculated based on an unpaired t-test with Welch's correction (A–D) and Mann-Whitney test (E).

BAFF were significantly higher in PWH/Active Mtb as compared to PWH/No Mtb (Figures 5A–5C), but only the IL-6 difference was statistically significant after accounting for multiple comparisons. Log_{10} IL-6 level ($\beta = 0.12$, 95% CI 0.04–0.22, $p = 0.007$) and Log_{10} BAFF level ($\beta = 0.11$, 95% CI 0.02–0.21, $p = 0.01$) also predicted neutralization BP score after accounting for age, gender, absolute CD4 count, and log_{10} plasma virus level in multivariable linear regression analysis. As expected, only Mtb disease status and not IL-6 nor BAFF levels predicted pre-ART neutralization BP score in a multivariable linear regression model (Table S12) because IL-6 (Figure 5A) and BAFF (Figure 5C) associate with Mtb disease. Thus, IL-6, BAFF, and Mtb disease status are not independent.

After starting ART, APRIL ($p = 0.0006$, one-way ANOVA, Figure 5D) and BAFF ($p = 0.03$, Kruskal-Wallis test, Figure 5E) were significantly higher in PWH/Active Mtb as compared to the other two groups. Similar to our previous observations after ART initiation, the majority of individuals had plasma IL-6 levels below the LOD preventing us from doing IL-6 comparisons.[20] Log_{10} APRIL levels ($\beta = 1.0$, 95% CI 0.2–1.9, $p = 0.02$) but not BAFF levels significantly associated with the post-ART to pre-ART neutralization BP ratio after adjusting for gender, plasma virus level, and post-ART CD4 count. Similar to the pre-ART analysis as expected, APRIL did not predict the follow-up to baseline neutralization BP ratio if Mtb disease status was included in a multivariable linear regression model because APRIL and presence of Mtb disease are inter-dependent (Figure 5D). In aggregate, this implies that Mtb disease associates with higher IL-6, BAFF, and APRIL levels and this increase subsequently predicts HIV-1 neutralization BP.

DISCUSSION

The existence of mycobacteria-mediated heterologous protection against different pathogens has been extensively documented in humans.[5,7,59] Despite the high burden of HIV-1 and Mtb co-infection, the

impact of Mtb infection and disease on the humoral response against HIV-1 has not been investigated. Our study suggests that active Mtb disease associates with the emergence of a broad and more potent anti-HIV-1 neutralization response. After ART initiation, Mtb disease associates with both broader and more potent nAbs and ADCC. Moreover, greater antigen exposure, higher level of virus replication, longer duration of HIV-1 infection, generalized antibody increase, and cross-reactivity do not account for the augmented antibody responses in PWH/Active Mtb. Rather from our data, we speculate that Mtb disease alters cytokines important for antibody production in lymphoid tissues that harbor both pathogens, and this accounts for enhanced HIV-1 humoral immunity. Although broad and potent HIV-1 humoral responses do not provide virologic control or improve disease outcomes in ART-naive PWH,[35,36,60] our observations have implications both for using bnAbs as therapeutics and preventing HIV-1 acquisition and for developing strategies to induce optimal immunity in PWH.

We provide multiple independent lines of evidence that Mtb disease associates with enhanced HIV-1 nAbs. First, we observed that PWH/Active Mtb had around 0.26-unit higher neutralization BP score as compared to the PWH/No Mtb prior to ART initiation. This increase is similar to or greater than the difference between some second- as compared to first-generation HIV-1 bnAbs.[28] In general, the second- as compared to first-generation bnAbs are more potent and can inhibit a larger diversity of HIV-1. Thus, the observed difference in neutralization capacity among PWH/Active Mtb as compared to PWH/No Mtb group is biologically meaningful. Second, we show that, after starting ART, PWH and active Mtb disease have a greater relative increase in neutralization BP compared to PWH and prior Mtb or no Mtb disease. This suggests that developing Mtb disease after ART also associates with greater neutralization capacity. Third, the Mtb disease impact on HIV-1 humoral immunity is not transient because, although antibodies can increase soon after ART initiation,[61] the PWH with either prior-treated Mtb or no Mtb had relatively stable humoral responses over time. Notably, the PWH and no Mtb had the lowest antibody responses both prior to and after ART. Fourth, our sequence analysis suggests PWH/Active Mtb have differential enrichment for sequence motifs associated with bnAb resistance. In general, HIV-1 variants evolve to escape nAbs,[42,62,63] and the presence of differentially susceptible strains implies that the virus population in individuals with Mtb disease is under unique antibody selection pressures. In aggregate, active Mtb disease associates with higher HIV-1 nAb response both prior to and after ART initiation.

Not all individuals with chronic HIV-1 infection develop broad and potent neutralization responses, and the reasons that only a minority develop bnAbs remain uncertain.[34,64] Previous studies have shown that factors important for bnAb development include exposure to higher amounts and longer duration of diverse viruses. Mtb disease increases HIV-1 plasma copies and leads to lower CD4 T cells.[37,38] The Mtb-associated neutralization increase, however, did not correlate with plasma virus level, absolute CD4 count, or envelope genetic diversity, which are surrogate markers for the antigen levels, duration of infection, and epitope variation, respectively. In the absence of HIV-1, individuals with latent, active, or treated Mtb also did not have demonstrable HIV-1 antibody responses, and thus the observed results are not due to cross-reactivity. Three independent lines of evidence also imply that the increase in HIV neutralization capacity in the presence of Mtb disease does not merely reflect a generalized increase in antibody production. First, although chronic HIV-1 infection is associated with hypergammaglobulinemia and derangements in the IgG isotype proportions,[65,66] in general, there was no difference in total IgG, IgA, or IgM levels in the PWH/Active Mtb and PWH/No Mtb groups. Second, there was no difference in antibody levels that most likely pre-existed prior to Mtb disease, such as those against tetanus and HSV-1/2. Third, antibodies that target different autoantigens, which are often generated *de novo* after infection, were neither consistently higher nor lower in the groups. In aggregate, this implies Mtb infection specifically enhances HIV-1 antibodies. We assessed antibody levels for the non-HIV-1 antibodies, while we quantified antibody functionality (neutralization and ADCC) for the HIV-1 responses. HIV-1 nAb BP correlates with anti-Env IgG-binding titers,[47] and thus we would predict that total HIV-1 anti-Env antibody levels are also higher in the PWH and active Mtb disease. We concentrated on neutralization and ADCC because these are biologically more relevant than Env-specific binding titers. In summary, the usual factors associated with higher and broader HIV-1 nAbs do not account for the Mtb-linked enhanced HIV-1 antibody response.

We speculate that there are likely novel mechanisms for the Mtb-associated increase in HIV-1 antibody BP. During Mtb disease and chronic HIV-1 infection, both microorganisms can be found in lymph nodes, which are the primary site for B cell development and antibody production. Furthermore, both organisms may be present in granulomas formed during Mtb disease,[22,23] and granulomas are often classified as secondary

lymphoid structures.[67,68] Mtb is known to engage pathogen recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and these are present on the lymphoid tissue-resident follicular dendritic cells (FDCs), $CD4^+$ T follicular helper (TFH), and B cells.[69] Mtb engagement of these receptors may influence antigen presentation and protein production by the adjacent HIV-1-laden FDCs and TFH.[70] This may increase the amount of HIV-1 Env sampled by B cells in the lymph nodes that harbor both HIV-1 and Mtb. Longer and greater antigen presentation is important for the generation of HIV-1 bnAbs.[71] Furthermore, Mtb disease likely increases tissue levels of soluble factors important for antibody production, such as IL-6, BAFF, and APRIL, as indicated by our plasma analysis. Elevated IL-6 levels may promote B-TFH cell interactions, which are important for somatic hypermutation (SHM).[21,72] The majority of HIV-1 bnAbs display extensive SHM,[73] and Mtb-mediated IL-6 elevation may promote SHM among the HIV-1 antibodies that are being generated in the lymphoid tissue that contain both HIV-1 and Mtb. Furthermore, elevated levels and correlations observed with BAFF/APRIL suggest that Mtb may help promote the survival and maintenance of B cells, especially some that may be autoreactive.[74] The BAFF/APRIL cascade has been associated with the presence of autoimmune antibodies, and self-reactivity is deemed important in bnAb emergence.[74] The multivariate linear regression analysis suggests that these cytokines may drive neutralization BP after they are induced by Mtb disease. This possible model argues that HIV-1 antibody response may be enhanced in a bystander fashion; Mtb disease elevates cytokine levels in co-infected lymphoid tissues, and this local IL-6, BAFF, and APRIL augmentation drives the production of potent HIV-1 antibodies. Examining this potential biological mechanism will require the development of a co-infection model or lymphoid tissue from PWH and Mtb disease.

Similar to neutralization, ADCC BP was also higher in those with active Mtb disease, albeit only after starting ART. In addition, ADCC moderately correlated with nAb response, primarily in those with Mtb. ADCC requires antibody-binding (Fab) portion attaching to the antigen and the antibody crystallizable (Fc) fragment engaging with Fc receptors (FcRs) on effector cells. Mtb disease may influence both antibody binding to HIV-1 Env on infected cells and antibody Fc compositions. While broad and potent HIV-1 neutralization responses are mediated via bnAbs rather than a panoply of different antibodies,[75] the antibody characteristics responsible for HIV-1 ADCC BP have not been defined. Similar to bnAbs, ADCC BP could potentially result from antibodies that target conserved Env epitopes. On the other hand, Mtb disease inflammation, in the presence as compared to the absence of ART, may impact Fc properties important for potent ADCC, such as afucosylation and galactosylation.[76,77] Active Mtb disease has been associated with Fc glycan changes.[78] Isolating and characterizing individual monoclonal antibodies from PWH and Mtb disease will provide further insights. We did not have peripheral blood mononuclear cells (PBMCs) to isolate HIV-1-specific memory B cells and characterize whether the nAbs and the antibodies that mediate cellular cytotoxicity target the same Env epitopes and have similar Fc properties.

The inclusion of a relatively large number of individuals from multiple cohorts representing diverse geographical sites is a strength of the study. This study, however, is limited by the lack of PBMCs and tissue samples, and participant dropout at follow-up. Furthermore, in this investigation, we cannot decipher if the HIV-1 infection preceded active Mtb or vice versa. Although the order of infections is not known, our longitudinal observations suggest that Mtb disease development with prior documented HIV-1 infection is associated with HIV-1 nAb and ADCC enhancement. It remains uncertain if latent Mtb infection in the absence of active disease is also associated with similar changes in neutralization capacity and ADCC. It could be speculated that PWH and latent Mtb infection potentially also have more potent immune responses because PWH and latent Mtb have lower set point HIV-1 plasma virus levels.[12] In general, however, antibodies do not have a significant impact on the level of viremia.

Previous studies have demonstrated that broad and potent nAb responses do not impact HIV-1 disease progression among individuals who do not have suppressed virus levels.[35,36,60] Neutralization escape variants emerge quickly with the ongoing virus replication, and antibodies are ineffective against neutralization-resistant strains.[63] We observed that PWH with current Mtb disease potentially harbor more bnAb-resistant strains, especially to some antibodies under advanced clinical investigation, such as VRC01. These observations have implications for using HIV-1 antibodies as therapy and preventing acquisition.[44–46,79] We speculate that future bnAb use to suppress viremia and prevent transmission will likely be less effective among populations with high burden of Mtb disease because the PWH that are likely to either transmit the virus or be a candidate for bnAb treatment will have a greater chance of having neutralization-resistant strains.

It is well known that Mtb disease worsens morbidity and mortality in PWH even if they are on suppressive ART.[16] In addition, BCG vaccination is strictly contraindicated in HIV-1-infected children regardless of disease stage or treatment. Thus, mycobacterial infections cannot be used to enhance HIV-1 immune responses. Our results, however, could provide the scientific basis for future investigations aimed at understanding how Mtb disease may yield broad and potent HIV humoral responses, besides the usual unmodifiable factors, namely prolonged duration of infection and high plasma virus level.[34–36] Extrapolation of our observations would suggest that individuals with suppressed virus levels who undergo an active immunization intervention that mimics aspects of Mtb disease may develop a broad and potent HIV-1 nAb and ADCC response. If the augmented nAbs and ADCC are effective against the autologous strains, it could change the size and nature of the residual HIV-1 latent reservoir as long as virus suppression prevents the emergence of escape variants. Examining larger cohorts, quantifying responses against the autologous strains, and assessing impact of HIV-1 latent reservoir will be required to understand these issues in detail. Similar strategies to boost the immune response against autologous strains are being pursued with bnAb infusion treatments.[79] In contrast, novel insights gleaned from understanding the mechanism for Mtb antibody enhancement could be more practical for the majority of HIV-1-infected individuals worldwide.

Limitations of the study

This study demonstrates that PWH have broader and more potent HIV-1 antibody responses in the presence as compared to the absence of active Mtb disease. Examination of other cohorts could provide further confirmation for these findings. PWH/Active Mtb as compared to PWH/No Mtb have different proportion of predicted HIV-1 Env bnAb resistance. While this further implies differential humoral pressure, antibody susceptibility has not been quantified *in vitro* and monoclonal antibodies were not generated from those with and without Mtb. PWH/Active Mtb as compared to PWH/No Mtb have higher levels of mediators important for B cell and antibody development, and these increases correlate with greater neutralization capacity. Furthermore, the Mtb-associated antibody enhancement does not occur because of cross-reactivity or a generalized increase in all antibodies. These observations hint at but do not provide definitive mechanisms for the observed association of Mtb disease with greater HIV-1 antibody responses.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106631.

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AUTHOR CONTRIBUTIONS

MS conceived and designed the study. BA, YM, AJO, EN, and MZ conducted the experiments. LN, YCM, AG, MCH, JK, and KRJ provided clinical samples, data, and critical input. BA and MS analyzed the data and conducted statistical analyses. BA and MS wrote the manuscript with editorial assistance from the co-authors. The AIDS Clinical Trials Group A5274 (REMEMBER) Study Team provided samples, and Table S13 lists the investigators.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper self-identifies as a gender minority in their field of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Tuberculosis diagnostic center	Kampala, Uganda	N/A
ACTG 5274 trial	Multicenter	NCT01380080
Tuberculosis Clinic or the Immigrant and Refugee Health Program Clinic	Boston Medical Center (BMC)	N/A
Critical commercial assays		
LEGENDplexTM Custom Human 12plex Panel	BioLegend	Item # 900000352
LEGENDplexTM Human IL-4	BioLegend	Item # 740042
LEGENDplexTM Human IL-6	BioLegend	Item # 740044
LEGENDplexTM Human IL-17A	BioLegend	Item # 740546
LEGENDplexTM Human IL-17F	BioLegend	Item # 740718
Melon Gel IgG Spin Purification Kit	ThermoFisher	Cat # 45206
Bio-Plex Pro Human IgG Total Isotyping Assay	Bio-Rad	Cat# 171A3103M
Bio-Plex Pro Human Isotyping Panel, 6-plex	Bio-Rad	Cat# 171A3100M
HSV-1 IgG ELISA	Calbiotech	Cat #H1029G
Human Anti-Tetanus Toxin/Toxoid IgG ELISA	Alpha diagnostics	Cat # 930-100-TTG
Human Autoimmune Auto-antibody Magnetic Bead Panel	Millipore Sigma	Cat # HAIAB-10K-20
Quanta Lite ACA IgG III	Werfen	Part # 708625
Deposited data		
HIV-1 envelope sequences	This study	GenBank: OK513840 - OK514180
Software and algorithms		
Prism (Version 8.0)	GraphPad software	https://www.graphpad.com
HIV align tool	Los Alamos HIV Database	https://www.hiv.lanl.gov/content/sequence/VIRALIGN/viralign.html
PhyML interface	Los Alamos HIV Database	https://www.hiv.lanl.gov/content/sequence/PHYML/interface.html
Rainbow tree tool	Los Alamos HIV Database	https://www.hiv.lanl.gov/content/sequence/RAINBOWTREE/rainbowtree.html
GenSig	Los Alamos HIV Database	https://www.hiv.lanl.gov/content/sequence/GENETICSIGNATURES/gs.html
Heatmap tool	Los Alamos HIV Database	https://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html
bnAb-ReP algorithm	(Rawi et al., 2019)[43]	https://github.com/RedaRawi/bNAb-ReP

RESOURCE AVAILABILITY

Lead contact

Further information and reasonable requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Manish Sagar (msagar@bu.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

HIV-1 envelope sequences generated in this study have been deposited into GenBank under accession numbers OK513840 - OK514180. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study participants

Samples were obtained from three different cohorts. The first set of samples were from consecutively enrolled individuals newly diagnosed with HIV-1 infection who were part of a Mtb diagnostic study in Kampala, Uganda as described previously.[20,24] Briefly, the presence and absence of Mtb disease was assessed with GeneXpert MTB/RIF test and two Mtb liquid and one solid culture on two expectorated sputum samples. PWH who had confirmed Mtb disease (PWH/Active Mtb, n = 15) had a positive GeneXpert MTB/RIF result and at least one sputum sample culture positive for the presence of Mtb. PWH with no diagnosed or suspected active Mtb (PWH/No Mtb, n = 16) had negative results with all Mtb tests. PWH/No Mtb were matched to the PWH/Active Mtb based on age, absolute CD4 count, and plasma virus levels. PWH/Active Mtb initiated standard six-month anti-Mtb therapy immediately, and ART was started approximately two weeks later. PWH/No Mtb began ART after HIV-1 diagnosis. All treatments were based on national and World Health Organization guidelines. Blood samples were also obtained from participants that returned for a follow-up visit around six months after the initial enrollment visit. At this point, none of the PWH/Active Mtb had ongoing TB disease, and they were classified as PWH/Prior Mtb (n = 10). After starting ART, none of the PWH/No Mtb had evidence of active Mtb over follow up (n = 11). The age and gender information for the participants is provided in Tables S1 and S2.

The second set of samples was from outpatient research clinics in South Africa, Haiti, Kenya, and India that were part of the ACTG 5274 trial (NCT01380080), which compared isoniazid as compared to empiric Mtb treatment for preventing mortality in PWH after starting ART.[25] We acquired samples from this trial to both increase our sample size and have blood from individuals that were diagnosed with active Mtb around six months after starting ART. We first identified eighteen individuals that developed Mtb disease while on ART. Eighteen other individuals that did not develop Mtb disease over follow up were matched based on age, gender, absolute CD4 count, plasma virus level, and geographic location. Enrollment (n = 21) and follow up (n = 20) samples were available from some but not all identified participants. At enrollment and prior to ART, none of the PWH in the ACTG 5274 trial had confirmed or probable Mtb disease (n = 21). All individuals started ART, and they were randomized to isoniazid prophylaxis or empiric Mtb treatment. At follow up, nine individuals were diagnosed with or presumed to have Mtb disease based on national standards and testing, including acid-fast bacilli smears, chest radiography, ultrasound, mycobacterial culture, and GeneXpert. These individuals were classified as post-ART PWH/Active Mtb (n = 9). Eleven individuals did not have confirmed or probable Mtb at the follow-up visit. In both the Uganda and A5274 cohort, none of the PWH/No Mtb were evaluated for the presence of latent Mtb at any time. The age and gender information for the participants is provided in Table S2.

The final set of samples was from 32 HIV-1 uninfected individuals who either had diagnosed latent Mtb (n = 16), active Mtb disease (n = 4), recovered from active Mtb after treatment (n = 4), or no detectable Mtb exposure (n = 8) as described previously.[56] The age and gender information for the participants is provided in S11.

Study approval

The institutional review boards at Boston University, Infectious Disease Institute, Johns Hopkins University, and Joint Clinical Research Center in Kampala Uganda approved the studies. The ACTG 5274 samples were obtained after new works concept sheet approval. All participants gave written, informed consent for sample use.

METHOD DETAILS

Virus stocks and cell lines

Twelve reference Envs were obtained from the NIH AIDS reagent program.[27] Of the twelve, ten reference Envs (TRO11, X2278, X1632, 246F3, BJOX, CE1176, 25710, 398F1, CNE8, and CNE55) were incorporated into replication-competent viruses,[80,81] and two reference Envs (CH1119 and CE0217) were pseudotyped

using protocols detailed previously.[42] All viral stocks generated were stored at −80°C and titers were determined on TZM-bl cells.

TZM-bl and HEK293T cells were obtained from the NIH AIDS reagent program. NK (CD16+KHYG-1) and MT4-CCR5-Luc cells were obtained and propagated as described previously.[31,32] TZM-bl and HEK293T cells were maintained in complete Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. MT4-CCR5-Luc cells were maintained in complete Roswell Park Memorial Institute (RPMI) medium (Invitrogen) containing 10% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), 100U/mL of penicillin, and 100 μg/mL of streptomycin. NK (CD16+KHYG-1) cells were sustained in complete RPMI supplemented with 0.1 mg/mL Primocin (InvivoGen), 25 mM HEPES (Invitrogen), 1 μg/mL cyclosporine (CsA) (Sigma), and 10U/mL interleukin-2 (IL-2).[32]

Neutralization assay

We performed all neutralization assays in duplicate at least three independent times. All pre-ART plasma samples were heat-inactivated at 56°C for 1 h, and total IgG was isolated from all post-ART plasma samples using the Melon gel IgG spin purification kit (Thermo Scientific) according to the manufacturer's protocol. A 1:50 plasma dilution or equivalent quantity of isolated antibodies was used in the TZM-bl assay. A simian immunodeficiency virus (SIV) Env deleted pseudotyped with vesicular stomatitis virus (VSV) G protein Env was used as a negative control in the neutralization assays. Briefly, heat-inactivated plasma or isolated IgG was incubated with around 1000 infectious particles of a viral strain at 37°C for 1 h. Approximately $1X10^6$ TZM-bl cells were added to each well with a plasma-virus mix. We estimated the number of infectious virus after two days by quantifying the luminescence in each well using Bright Glo Luciferase Assay System (Promega). Differences in relative luciferase units (RLUs) in the presence or absence of plasma or antibody were used to calculate percent neutralization after subtracting background RLU (TZM-bl cells and growth medium only). Table S14 shows the means and standard errors for each plasma – virus combination.

Antibody-dependent cellular cytotoxicity assay

All ADCC assays were performed in duplicate at a minimum of three independent times. ADCC capacity was assessed against MT4-CCR5-Luc cells infected with different replication-competent variants. Briefly, MT4-CCR5-Luc cells were infected with different virus stocks for four to seven days, and the infected cells were used as targets when RLUs were at least 10-fold over that observed in the MT4-CC5-Luc cells not exposed to a virus. Heat-inactivated 1:50 dilution plasma samples or an equivalent amount of isolated antibodies were added to infected $1X10^5$ MT4-CCR5-Luc cells at 37°C. After 15 min, $5X10^4$ of NK ($CD16^+$ KHYG-1) cells were added at 1:1 dilution to each well.[32] After 24 h, wells were assessed for luminescence using Bright Glo Luciferase Assay System (Promega) per manufacturer's instructions. Differences in relative luciferase units (RLUs) in the presence or absence of plasma or antibody and growth medium were used to calculate percent ADCC after subtracting background RLU (uninfected MT4-CCR5-Luc cells and $CD16^+$KHYG-1 cell in RPMI). Table S15 shows the means and standard errors for each plasma – virus combination.

Cytokine levels

All assays were performed in duplicate. Plasma levels of fifteen cytokines including IL-6, CXCL10, IL-17A, IL-17F, IL-4, IL-2, IL-13, IP-10, CCL5, RANTES, TNF-β, TNF-α, IL-10, IL-12p70, APRIL, BAFF, and sCD40L were measured using the human B cell premix kit from Biolegend Legendplex using manufacturer instructions. All analytes were measured on samples that were not previously thawed.

Immunoglobulins, anti-HSV, and anti-tetanus-toxoid levels

All assays were performed in duplicate. We measured absolute concentrations of IgG, IgG1, IgG2, IgG3, IgG4, IgA, and IgM using a Luminex magnetic-based assay according to the manufacturer's instructions (Bio-Rad). Plasma levels of Herpes simplex virus ½ (HSV-1/2) and *Clostridium tetani* (tetanus)-toxoid-specific IgG were captured and measured with ELISA kits from Calbiotech and Alpha diagnostics according to manufacturers' protocols.

Auto-antibody levels

All assays were performed in duplicate. Plasma levels of antibodies reacting to seventeen self-antigens including β-2-Glycoprotein, C1q, CENP-B, Jo-1, Ku, Mi-2, Myeloperoxidase, Proteinase 3, PCNA, PL-12, PM/Scl-100, RNP/SM, Scl-70, Sm, SSA/Ro52, SSA/Ro60, and SSB/La were determined using a magnetic bead panel (Millipore) and quantified on Magpix (Luminex) instrument containing xPONENT 4.2 software (Boston University Analytical Core Facility). Anti-cardiolipin antibody levels were measured by ELISA (Inova Diagnostics) and quantified on BioTek Synergy HT plate reader at 450 nm absorbance.

Single genome amplification (SGA)

Intracellular RNA from participants' blood samples stabilized in PAXgene BRTs was isolated and purified with PAXgene Blood RNA IVD kit (Qiagen) according to the manufacturer's instructions. Isolated RNA was reverse transcribed with OFM19 (5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3') primer to make viral cDNA. The cDNA was diluted to ensure a maximum of 30 out of 96 PCRs yielded positive reactions using primer sets as protocol as described previously.[82,83] Amplicons from positive PCR reactions were cleaned using ExoSAP IT (Affymetrix) as previously described.[29,31] Viral Envs were sequenced by Sanger-based sequencing (Genewiz) and edited on the Sequencher program (Gene Codes).

Envelope sequence analyses

HIV-1 Env sequences from all pre-ART participants were aligned using Los Alamos HIV sequence database HIV align tool (https://www.hiv.lanl.gov/content/sequence/VIRALIGN/viralign.html). Sequence alignments were done using the HMM alignment model. A maximum phylogenetic tree was generated using the general-time-reversible (GTR) distance model on the PhyML interface (https://www.hiv.lanl.gov/content/sequence/PHYML/interface.html). The color and symbol-coded phylogram was generated using the rainbow tree tool in LANL (https://www.hiv.lanl.gov/content/sequence/RAINBOWTREE/rainbowtree.html). The mean pairwise Env genetic diversity was estimated based on a GTR substitution model with optimized equilibrium frequencies on DIVEIN as previously described.[84] All sequences have been deposited in GenBank (OK513840 - OK514180).

Differential amino acid frequency among PWH/Active Mtb and PWH/No Mtb sequences were determined using the LANL GenSig tool (https://www.hiv.lanl.gov/content/sequence/GENETICSIGNATURES/gs.html). This is a phylogenetically corrected analysis to minimize false positive due to lineage effects. Briefly, the algorithm presents the odds ratio for the differential amino acid prevalence in PWH/Active Mtb as compared to PWH/No Mtb sequences relative to the ancestral state in a phylogenetic tree. We used a false discovery rate ($q < 0.1$) to correct for multiple comparisons. Among the identified sites, we only considered positions that have previously been associated with changes to broadly neutralizing antibodies (bnAb) susceptibility.

The bnAb-ReP algorithm was obtained from https://github.com/RedaRawi/bNAb-ReP, and the packages were run on the Boston University Shared Computing Cluster (SCC).[43] Input included the predicted amino acid sequence for the 341 Envs in fasta format. The output was probabilities from 0 to 1 with higher values indicating neutralization sensitivity against the 33 different bnAbs.

Heatmap

The heat maps were generated to highlight neutralization fingerprints in the study using the Los Alamos HIV sequence database heatmap tool (https://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html). Euclidean distance method was used to create hierarchical clustering and bootstrap to calculate cluster stability on the heatmap.

BP score and bnAb-Rep estimation

Breadth and potency (BP) scores for percent neutralization and percent ADCC were estimated as previously described.[28] Briefly, BP score consists of an average of the $\log_2$ transformed % neutralization +1 or $\log_2$ transformed % ADCC +1.

For the bnAb-Rep analysis, each Env – bnAb pair was classified as sensitive or resistant based on the predicted probability above versus below 0.5 respectively. A generalized estimating equation (GEE) population-averaged model was used to evaluate differences in chance of sensitivity versus resistance in the

PWH/Active Mtb versus PWH/No Mtb Envs for each of the selected bnAbs. The GEE model used a binomial distributed dependent variable (sensitive = 1 and resistant = 0), and the data was grouped according to subject to account for the multiple Envs from single individuals. Within each subject, the Envs were deemed to have an independent correlation structure. Any Env with a bnAb-Rep prediction below 0.5 for all the assessed bnAbs in a class (CD4: VRC01 and 3BNC117; V3: 10–1074 and PGT128; V2: VRC26.08 and VRC26.25; MPER: 2F5) were deemed to have class resistance.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Stata (version 17.0) and GraphPad Prism 9.4. Summary data for each cohort are depicted as boxes and whiskers with the median and interquartile range indicated. Unpaired t-tests with Welch's correction or Mann-Whitney tests were used to compare normally and non-parametric distributed estimates between two groups respectively. Comparisons among the three post-ART groups were done either with ANOVA or Kruskal Wallis tests for normally and nonparametric distribution respectively. Fisher's exact and chi-square tests were used to assess proportion differences among groups. A non-parametric Spearman or parametric Pearson correlation test was used to determine associations based on distribution of the data. In the different multivariate linear regression models, only prior-deemed important disease and demographic factors were included to limit the number of independent variables. Pre-ART multivariable model independent variables included Mtb status (categorical variable), gender, $\log_{10}$ plasma virus level, absolute CD4 count, and age at enrollment. Post-ART multivariable models included active Mtb disease versus no disease (either prior Mtb or no Mtb), gender, plasma virus level post-ART, and absolute CD4 count post-ART start as independent variables. The geographic origin of the samples was not included in the multivariable analysis because of the large number of countries. In all evaluations, we considered two-sided p values of less than or equal to 0.05 as statistically significant. The Bonferroni correction was used to account for multiple comparisons.