Mucosal and systemic antigen-specific antibody responses correlate with protection against active tuberculosis in nonhuman primates

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Summary

Background Increasing evidence supports that antibodies can protect against active tuberculosis (TB) but knowledge of potentially protective antigens, especially in the airways, is limited. The main objective of this study was to identify antigen-specific airway and systemic immunoglobulin isotype responses associated with the outcome of controlled latent *Mycobacterium tuberculosis* (*Mtb*) infection (LTBI) versus uncontrolled infection (TB) in nonhuman primates.

Methods In a case–control design, using non-parametric group comparisons with false discovery rate adjustments, we assessed antibodies in 57 cynomolgus macaques which, following low-dose airway *Mtb* infection, developed either LTBI or TB. We investigated airway and systemic IgG, IgA, and IgM responses in paired bronchoalveolar lavage and plasma samples prior to, two-, and 5-6-months post *Mtb* infection using an antigen-unbiased approach with *Mtb* glycan and proteome-wide microarrays.

Findings Macaques that developed LTBI ($n = 36$) had significantly increased airway and plasma IgA reactivities to specific arabinomannan (AM) motifs prior to *Mtb* infection compared to those that developed TB ($n = 21$; $p < 0.01$, $q < 0.05$). Furthermore, LTBI macaques had higher plasma IgG reactivity to protein MTB32A (Rv0125) early post *Mtb* infection ($p < 0.05$) and increasing airway IgG responses to some proteins over time.

Interpretation Our results support a protective role of pre-existing mucosal (lung) and systemic IgA to specific *Mtb* glycan motifs, suggesting that prior exposure to nontuberculous mycobacteria could be protective against TB. They further suggest that IgG to *Mtb* proteins early post infection could provide an additional protective mechanism. These findings could inform TB vaccine development strategies.

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Introduction

A full understanding of correlates of protection against active tuberculosis (TB), a deadly, transmissible, predominantly respiratory disease, remains a major gap in the field. Fueled by the COVID-19 pandemic, TB cases and related mortality have increased globally to an estimated 10.6 million cases with around 1.3 million associated deaths in 2022,[1] emphasizing the need for

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Research in context

Evidence before this study

While T cell responses have an established role in the defense against active tuberculosis (TB), many individuals with intact cell-mediated immunity develop TB and vaccines targeting this arm of the adaptive immunity lack high efficacy. In the past years, several studies have shown that serum antibodies from humans exposed to or latently infected with *Mycobacterium tuberculosis* (*Mtb*; LTBI) have protective *in vitro* and *in vivo* functions against *Mtb*. We have shown that polyclonal serum IgG to the mycobacterial glycan arabinomannan (AM) and related lipoarabinomannan (LAM) from humans exposed to *Mtb* or with LTBI is protective against TB. However, knowledge of the range of antigen-specific isotype responses associated with control of *Mtb* infection, especially at the airway level, is limited. Recently, two independent studies showed that mucosal airway or intravenous BCG vaccination of rhesus macaques protected against TB; in addition to cell-mediated responses, protection was associated with increased anti-mycobacterial airway IgA in the former and airway and plasma IgG and IgA responses in the latter, respectively. Another recent study showed that ongoing exposure of BCG vaccinated mice to nontuberculous mycobacteria (NTM) resulted in enhanced anti-*Mtb* systemic and airway IgG and IgA responses and provided superior protection against TB than BCG alone. While these studies investigated antibody responses to multi-antigen mycobacterial mixtures, a follow-up study analysing antibody responses in rhesus macaques BCG-vaccinated by various routes found an association between the presence of plasma IgM to antigens LAM, PstS1, and Apa and BALF IgA, IgG, and IgM to LAM and PstS1 with reduced *Mtb* burden. However, this study focussed on a set of 5 selected *a priori* antigens and unbiased approaches to investigate airway and systemic antigen-specific isotype responses associated with control of *Mtb* infection are lacking.

Added value of this study

Nonhuman primate models allow for the investigation of immune responses before and after *Mtb* infection, including in the airways, which would not be feasible in humans for whom the time of infection is typically unknown. We studied cynomolgus macaques (CMs), because compared to rhesus macaques that develop mostly TB after low dose airway *Mtb* infection, CMs develop LTBI and TB in about equal proportions. They are thus an excellent model for studying immune correlates for outcomes of natural *Mtb* infection in humans, who to the majority can control their infection. We used an antigen unbiased approach with unique mycobacterial glycan and proteome-wide microarrays to investigate antibody isotype profiles associated with the outcome of controlled infection (LTBI) versus uncontrolled infection (TB). We show that CMs which developed LTBI have significantly increased 1) airway and plasma IgA to specific AM glycan motifs prior to *Mtb* infection, 2) plasma IgG reactivity to the protein MTB32A (Rv0215) early post *Mtb* infection, and 3) airway IgG responses to some proteins after *Mtb* infection. These data provide new insights into humoral immune correlates that could determine the outcome of *Mtb* infection in humans. Importantly, they suggest a role of pre-existing airway and systemic IgA to specific *Mtb* AM/LAM epitopes in the protection against TB, indicating that prior exposure to NTM could influence the outcome of a later *Mtb* infection. These findings could inform TB vaccine development and immunization routes.

Implications of all the available evidence

Consistent with other studies, our results support a protective role of antibodies in the defense against *Mtb* and highlight the importance of antigen-specific antibody isotypes, especially IgA in the airways. They further provide insights into potentially protective effects of prior NTM exposure against *Mtb*. Collectively with other recent data, our findings provide a more holistic view of correlates of immunity against TB. They suggest that different airway and systemic antibody isotypes reactive with the mycobacterial surface glycans AM and LAM and partly overlapping other mycobacterial proteins, could provide various protective mechanisms against *Mtb*. These findings require further mechanistic investigations and could inform TB vaccine development strategies.

better control measures. The only licensed TB vaccine, given intradermally (i.d.) to newborns in TB endemic regions, is based on the attenuated strain of *Mycobacterium bovis*, Bacillus Calmette-Guerin (BCG). It protects against disseminated TB in children but has limited protection against pulmonary TB during childhood or TB later in life.[2,3] Its effect on reduction of initial infection with *Mycobacterium tuberculosis* (*Mtb*) is controversial,[4–6] with recent data suggesting that i. d. BCG revaccination in adolescents can reduce the rate of sustained new IGRA conversion.[7,8] Nevertheless, around a quarter of the world's population is estimated to be infected with *Mtb*.[9] Although most of these individuals can control their infection (termed latent infection), 5–10% develop disease over their lifetime, with the highest risk within the first 1–2 years following infection.[10–13] The lifetime risk for TB is much higher in certain immunocompromised groups, such as those who are HIV co-infected, emphasizing the known importance of cell-mediated immunity against *Mtb* (reviewed in[14,15]). Still, most individuals with TB have no overt immune impairment. A more comprehensive knowledge of all the immunologic components that protect against TB could inform the development of more effective vaccines and immunotherapies against TB.

Increasing evidence supports a role for humoral immunity in the protection against TB (reviewed in[16–24]). Several studies show that serum antibodies from individuals exposed to or latently infected with *Mtb*, in contrast to those from patients with TB, have protective *in vitro* and *in vivo* functions against *Mtb*.[10,15,25] Most of these studies were performed with polyclonal total serum IgG or IgG to multi-antigen preparations, such as tuberculin purified protein derivative (PPD), and little is known about protective antigens. We have recently shown that serum IgG to the mycobacterial surface polysaccharide arabinomannan (AM), also a component of the glycolipid lipoarabinomannan (LAM), has protective functions against *Mtb in vitro* and *in vivo*.[25,26] The protective functions were observed with AM-specific IgG from asymptomatic individuals who were BCG vaccinated and *Mtb* exposed or latently infected but not from patients with TB and were associated with reactivity to certain AM motifs.[25,26] Murine passive transfer studies with monoclonal antibodies (mAbs) support the protective efficacy of IgG to AM and LAM but also suggest roles of IgG as well as IgA to certain protein antigens (reviewed in[16–24,27]). Nevertheless, because of the tremendous heterogeneity of human antibody responses to *Mtb*, identification of significantly different antigen-specific antibody responses in individuals with controlled latent *Mtb* infection (LTBI) compared to uncontrolled infection in TB has been challenging and has provided few insights.[28,29]

The predominant route of initial *Mtb* infection is through the lungs but the spectrum of airway mucosal antibodies and their association with the outcome of infection remains poorly understood. A recent study showed that mucosal airway vaccination with BCG prior to low dose *Mtb* airway challenge protected rhesus macaques against the development of TB.[30] The protection was associated with polyfunctional T-helper type 17 cells, interleukin (IL)-10, and increased IgA in the bronchoalveolar lavage fluid (BALF) but *Mtb* antigen-specific IgA reactivities were not determined. In another recent study, intravenous (i.v.) BCG given prior to low dose *Mtb* airway challenge also strongly protected rhesus macaques against TB and induced significantly higher levels of PPD-antigen-responsive CD4+ and CD8+ T cells and IgG and IgA titers against whole cell *Mtb* lysate in both the BALF and plasma compared to other vaccination routes.[31] However, PPD, whole cell lysates, and other mycobacterial fractions contain mixtures of hundreds of mycobacterial proteins and glycolipids.[32] In a systems analysis approach, the presence of plasma IgM to antigens LAM, PstS1, and Apa and BALF IgA, IgG, and IgM to LAM and PstS1 correlated with reduced *Mtb* burden in rhesus macaques that were BCG vaccinated by various routes.[33] While this report selected five antigens previously observed to induce antibodies in humans during *Mtb* infection *a priori*, nonbiased approaches are needed to capture the breadth and heterogeneity of the antibody response that is associated with protection to such a complex pathogen like *Mtb*.

Nonhuman primates (NHPs) reflect the heterogeneity of human immune responses and outcomes in *Mtb* infection and have been key models for understanding human *Mtb* infection ([34,35], and reviewed in[36–39]). Because the exact time of infection is known, animal model studies provide important information. Following low-dose *Mtb* airway infection, around 90% of rhesus macaques progress to TB but cynomolgus macaques (CMs) develop LTBI and TB in equal proportions.[34,38,39] The CM model provides a unique opportunity to examine immunologic factors that influence the outcome of *Mtb* infection, particularly around the early events. Our primary objective was to investigate the local airway and systemic antigen-specific IgG and IgA responses before and early after *Mtb* infection of CMs using an antigen-unbiased approach. Our secondary objectives were to compare the mucosal and systemic antibody signatures, and to evaluate the dynamics of the antibody responses over time. Our overarching hypothesis was that mycobacterial antigen-specific antibody signatures before and during early infection are associated with the control of *Mtb* infection (LTBI).

Methods

Non-human primate samples and clinical definition of outcome

We used a case–control design to investigate antibody isotype profiles associated with the outcome of controlled infection (LTBI) versus uncontrolled infection (TB). Samples used for this study consisted of all available archived paired BALF and plasma samples from adult Chinese CMs (*Macacca fasicularis*) (66% of whom were male, median age of 7.1 years [IQR 6.2–8.0]) from prior *Mtb* studies[34,40–42] (Supplementary Table S1a and b). Although the animals were dedicated to different experiments, they all underwent airway infection with low dose *Mtb* (Erdman, ~25 CFU) via bronchoscopic installation. Prior to infection, all animals were clinically well and screened for potential co-morbidities (e.g. SIV, *Mtb*, parasites), and all animals completed follow-up for as long as they lived. The BAL procedure was performed as previously described via direct visualization of the airway and lavage of ~ 40 ml normal saline (0.9%).[40] Plasma and BALF were simultaneously obtained around a median of one month prior to infection, and 2–3 months (early) and 5–6 months (late) after *Mtb* infection. All samples were aliquoted and frozen at—80 °C the day they were obtained. Animal protocols (# 0906,877, 1,105,870, 0602,370, 1,003,622, 0601,229, 0703,399, 0508,791, 0802,011, 0808,244, 1,011,342) and procedures were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee before the collection of samples.

Similar to human testing, all animals in this study had confirmed *Mtb* infection demonstrated by conversion from a negative to positive TST and/or IGRA.[34,40–42] Clinical classification of animals was also based on human clinical definitions as previously published.[34,40] In brief, CMs with TB developed abnormal chest X-rays, TB-related symptoms (e.g. weight loss, cough), elevated erythrocyte sedimentation rate (marker of inflammation) and/or positive *Mtb* growth by gastric aspirate or BAL after 2 months post *Mtb* infection. Animals were classified as having LTBI if they had no *Mtb* growth after 2 months post infection and remained asymptomatic with no evidence of inflammation and normal chest X-rays after 6 months.[34,40–42]

BALF processing and standardization

Archived BALF was kept frozen at –80C, thawed and then filtered through 0.22 μm centrifugal filters (ThermoFisher F2519-E) twice under BSL3 conditions to sterilize the samples. To assure that all BALF samples had the same dilution and because urea freely diffuses between the blood and airways,[43,44] we measured the urea concentration as per manufacturers instruction (BioAssay Systems) in the timely paired BALF and plasma samples. Two urea standards were included to represent a known high concentration (50 mg/dL) and low concentration (5 mg/dL) of urea (BioAssay Systems). Two reference samples made up of pooled plasma or pooled BALF from biologically independent NHP samples (assessed in preliminary studies) were included in each assay to assure we maintained an inter-assay coefficient of variability of less than 15%. Standards and samples were set up in duplicates on all 96 well plates and assays were repeated on two separate days. To estimate the original airway fluid volume diluted by the BAL, we applied the following formula[43,44]: [Urea] = (OD_{sample}—OD_{blank})/($OD_{standard}$—OD_{blank}) * df * [STD] (mg/dL). OD_{sample}, $OD_{standard}$, and OD_{blank} are optical densities at 520 nm of the sample, standard, and blanks; df is the dilution factor; [STD] is the concentration of the standard (either 50 mg/dL or 5 mg/dL). Using Amicon Ultra Centrifugal filter units (Millipore ACS505024), we then concentrated all BALF samples accordingly to a 1:4 dilution using the ratio [urea in plasma]/[urea in BALF]. The person processing and standardizing the BALF was blinded to the outcome of the animals.

Glycan array

We used our recently developed glycan microarray comprised of 63 synthetically generated OS to profile the reactivity of NHP airway and systemic antibodies to motifs of *Mtb* surface glycans.[45] The glycans, printed in triplicates, were comprised of 30 motifs from AM/LAM with the remaining motifs from six other *Mtb* surface glycans (α-glucan, trehalose mycolates, lipooligosaccharides (LOSs), phenolic glycolipids (PGLs), phosphatidyl-*myo*-inositol mannosides (PIMs) and glycopeptidolipids (GPLs). Each array contained BSA spots printed in triplicates as negative controls. Slides with 8 arrays on each were blocked with 3% BSA (GEMINi Bio-Products, standard grade) in PBS at 4 °C overnight, washed with 0.1% PBS-T, and then incubated with BALF (1:8) or plasma (1:100), diluted in 0.1% PBST with 3% BSA, for 4 h at 37 °C. We included sera from two human subjects with known high IgG and IgA anti-AM titers and diverse glycan reactivity as positive controls and reference samples. After 6 washes with 0.1% PBS-T, slides were incubated with Cy3 Goat Anti-Human Serum IgA (Jackson ImmunoResearch Labs Cat# 109-165-011, RRID:AB 2337721), Alexa 647 Goat Anti-Human IgG (Jackson ImmunoResearch Labs Cat# 109-605-008, RRID:AB_2337882, DyLight™ 649 Conjugated anti-human IgM (Rockland, 609-143-007); diluted at 1:300 in 1% BSA in PBS) for 2 h at 37 °C. After further washing steps, the slides were dried by centrifugation (200 x G, 20 °C, 5 min) and scanned at 560 nm and 480 nm with the GenePix 4000 Microarray scanner system (Molecular Devices, CA). We used GenePix Pro 7.3.0.0 to analyze the images and measure the median pixel intensity (MPI) and neighboring background pixel intensity (BPI) of individual spots. The person performing the work with the glycan arrays was blinded to the outcome of the animals.

Glycan data processing and statistical analysis

Glycan array reference samples and non-linear normalization. One of the two reference samples in each run (average of 6 slides per day) was used for a non-linear, locally weighted regression method, previously described for DNA microarrays, to normalize the glycan array data.[46] Briefly, we constructed two 8 × 8 scatter plot matrices and loess curves to observe the day-to-day variation of the reference samples on the glycan microarrays. This predicted the conditional mean of each glycan fragment (S1–S63) for the reference sample from one experiment as a function of the same reference sample from a different experiment. For all sample-pairs in each plot, after assuring assumption for Pearson were met, we calculated a Pearson correlation between the loess predicted value and raw value. We designated the reference sample that had the highest average Pearson correlation to be the standard reference sample used to adjust all data points. We based the individual adjustment of all glycan array fragments (S1–S63) from all NHP samples on the differences in the locally estimated mean intensity between the standard reference sample and the reference sample set up on the same day of the experiment.

Glycan array filtering data

To reduce multiple comparisons, we filtered for glycans with the highest reactivity and coefficient of variance in

at least 10% of all the samples per isotype and body fluid. This was an unsupervised filtering without considering infection status (LTBI or TB). Only glycans with the highest reactivity and most variability in 10% of the samples were kept in the analysis. To calculate filtering criteria for each isotype and to filter out glycans with lower reactivity in our data set, we averaged the values from the reference sample from eight experiments and plotted their frequency distribution. Sixteen bins were tabulated in a histogram, and we used the median + IQR in the first bin to calculate the filtering criteria for IgA, IgG, and IgM, respectively. Due to the exploratory nature of our studies, no reference samples for BALF were available. To account for the higher background observed for BALF samples, we calculated the ratio of the median background of the BALF samples and divided that by the median background of plasma samples for IgA, IgG, and IgM, respectively. The filtering criteria for the BALF were multiplied by this ratio to adjust for the differences in background between BALF and plasma. We analyzed each sample type and isotype as four independent datasets with four different MFI filtering criteria (BALF IgA: 1404.9, BALF IgG: 2653.6, BALF IgM: 375 Plasma IgA: 1080.7, and Plasma IgG: 1200.7, Plasma IgM: 938). To keep multiple comparisons limited in each dataset, we included only those glycan motifs in the analysis of a dataset if at least 10% of the samples had values above the filtering criteria for that dataset. These criteria were also used for feature selection for partial least-squares discriminant analysis (PLS-DA). A separate cutoff to determine reactivity above the background signal was calculated using the average background of all the samples for BALF and plasma. Cut-off values for BALF reactivity were MFI of 438 (IgA), 1210 (IgG), 279 (IgM). Cut-off values for plasma were MFI of 197 (IgA), 456 (IgG), and (685).

Nucleic Acid Programmable Protein Array (NAPPA)

To screen for antibody responses to the *Mtb* proteome, we used our recently developed high-density nucleic acid programmable microarray (HD-NAPPA).[47] In this second-generation NAPPA platform, planar glass surfaces are replaced with etched silicon wafers to provide nanowells. The utilization of this platform increases the density of features that can be analyzed on a single microscope slide-sized array.[48] Briefly, 3295 genes from H37Rv and 437 genes CDC1551 were cloned in a pANT7-cGST expression vector, which encodes a C-terminal fusion partner for the target gene of Glutathione-S-Transferase (GST). Three distinct genes were printed into one well allowing for initial high-throughput screening for antibody reactivity in the NHP cohort samples which was then followed by deconvolution array screening as described.[47] The person performing the work with the NAPPA arrays was blinded to the outcome of the animals.

Initial (triple genes) HD-NAPPA. Each slide contained 4 full *Mtb* proteome arrays and was processed as described.[47] Briefly, initial steps included blocking and protein expression using a human cell-free expression system (In Vitro Transcription and Translation coupled system; IVTT; Thermo Fisher Scientific). When expressed, plasmids containing open reading frame (ORF) cDNAs encoding the desired proteins create a chimeric protein of the targeted ORF fused to a Glutathione S-Transferase (GST) tag partner. Proteins are captured through an anti-GST tag murine monoclonal Ab (mAb; Cell signaling technology, Danvers, MA) co-printed with the plasmid, followed by detection with Alexa 647-labeled Goat anti-mouse IgG (H + L) secondary Ab (Molecular Probes Cat# A-21235, RRID:AB_2535804) to confirm capture and expression of proteins. Negative controls included several spots containing master mix (plasmid encoding GST without any fusion partner) or empty spots without plasmid. Positive controls included plasmid encoding human and murine IgA and IgG and 7 viral genes from Epstein–Barr virus (EBV), Influenza, and Human cytomegalovirus HCMV[47] to which most humans have high antibody titers. A pooled sample of sera from 3 HIV−, TB + human subjects with known strong reactivity to various *Mtb* proteins was used in each experiment as a reference sample.[17,47]

Blocked microarray slides were incubated with BALF (1:35) or plasma (1:100) diluted in phosphate buffered saline with 5% skim milk and 0.2% tween 20 (PBST) for 16 h at 4 °C. After washing with 5% skim milk and 0.2% tween 20 (PBST) (3 × 5 min), slides were incubated with Cy3 Goat Anti-Human Serum IgA (Jackson ImmunoResearch Labs Cat# 109-165-011, RRID:AB_2337721) and Alexa 647 Goat Anti-Human IgG (Jackson ImmunoResearch Labs Cat# 109-605-008, RRID:AB_2337882; diluted at 1:200 in 5% skim milk and 0.2% tween 20 (PBST)) for 2 h at 4 °C. After washing with PBST (3 × 5 min) to remove unbound secondary Ab, slides were dried by centrifugation and scanned at 635 nm and 535 nm with a Tecan PowerScanner, and images were quantified with the ArrayPro Analyzer Software (Media Cybernetics, Inc.). Spots were considered reactive if the ratio of the median fluorescent intensity (MFI) of the gene was greater than 1.4 × the median of all the genes.[47]

Deconvolution (single gene) NAPPA. Individual genes of reactive spots were reprinted on a new microarray containing only single gene per well if the reactive triple gene spots met either of the two criteria[1]: a ratio ≥1.4 in at least 20% of the LTBI or 20% of the TB group samples at pre-infection or early post infection time points (representing antibody reactivity to proteins specific to LTBI or TB groups); or[2] a ratio ≥2.0 in the LTBI group (representing very high antibody reactivity to proteins only in the LTBI group). Arrays were basically performed as described.[47] In the deconvolution step, 17

human proteins (Supplementary Table S14) were included as additional negative controls. These proteins were selected based on their negative reactivity with human antibodies (unpublished data) and were included so, rather than using the median reactivity to all gene spots, the MFIs of the single *Mtb* gene spots could be divided by the median of the negative human gene controls to quantify reactivity.

NAPPA data processing

NAPPA reference samples and non-linear normalization. We used the same pooled reference samples in each run (average of 6 slides per day) and normalization process as described for glycan arrays. As described above, we used with the pool sample with the highest average Pearson correlation among all runs for a non-linear, locally weighted regression method to normalize the NAPPA data.[46] We based the individual adjustment of all proteins from all NHP samples on the differences in the locally estimated mean intensity between the standard reference sample and the reference sample set up on the same day of the experiment. To keep multiple comparisons limited in each dataset, we included only those proteins in the analysis if at least 10% of the NHP samples had reactivity values above the ratio of 1.4.

Statistical analysis

Because we had previously identified glycan motifs associated with protective serum IgG functions in humans with LTBI, we set a more stringent significance level at $p \leq 0.01$ combined with a false discovery rate (FDR) below 5% (presented as q values) for all glycan array comparisons. However, this was an exploratory study regarding protein data. We therefore kept a $p < 0.05$ significance level for the NAPPA data. Because the antibody reactivity data were not normally distributed, we used the nonparametric Mann–Whitney U test for two group comparisons. Pearson correlations were used to assess the covariance of BALF, plasma, IgG, and IgA to individual glycan motifs. For paired data, the non-parametric Friedman and Wilcoxon matched pairs signed rank tests were used for three and two group comparisons, respectively. PLS-DA models were implemented using the mixOmics package.[49] All statistical analysis and graphing were completed using RStudio Version 1.2.5042 and Prism Version 9.1.2 (GraphPad, Boston, MA, USA).

Role of funders

The funding sources for this project played no role in the study design, data collection, analysis, interpretation, writing, or editing of the manuscript.

Results

In a case–control design, we investigated the airway mucosal and systemic IgA and IgG responses to mycobacterial antigens in adult CMs before and 2–3 months (early) and 5–6 months (late) after low dose *Mtb* airway infection to determine whether specific antibody responses are associated with control of *Mtb* infection and the outcome of LTBI (Fig. 1). All available archived and paired samples of serial BALF and concurrent plasma from 57 CMs (LTBI = 36, TB = 21 TB) airway infected with low dose (~ 25 CFU *Mtb*) were included. Not all animals had samples from all time-points, especially those with active TB at late post-infection time points because these did not survive that long without intervention. However, most CMs had pre-infection and almost all animals had paired BALF and plasma samples from early post infection time points available (Table 1 & Supplementary Table S1).

Mtb OS motifs induce strong and diverse airway and systemic antibody reactivities. Concentrated and normalized BALF and diluted plasma samples were tested for antibody isotype reactivities to 63 OS motifs of the mycobacterial glycans arabinomannan/lipoarabinomannan (AM/LAM), α-glucan, trehalose mycolates and lipooligosaccharides (LOS), phenolic glycolipids (PGLs), phosphatidyl-myo-inositol mannosides (PIMs), glycopepitdolipids (GPLs) using *Mtb* glycan[45] Our unsupervised analysis to filter out OS motifs with low isotype reactivity per body fluid resulted in a subset of 22, 21, 43, and 44 glycan motifs that were compared between the LTBI and TB groups for BALF IgA, plasma IgA, BALF IgG, and plasma IgG, respectively (Supplementary Table S2). Overall, there was more diverse glycan recognition compared to *Mtb* protein reactivity; 70% of all glycan motifs were highly reactive in at least 10% of all CMs compared to 13% of all *Mtb* proteins tested (Supplementary Tables S2 and S9). We identified significantly higher IgA reactivity to several glycan motifs (all within AM/LAM) prior to *Mtb* infection in the animals who developed LTBI versus TB ($p < 0.01$ [Mann–Whitney]; Fig. 2) but did not find significantly higher IgA nor IgG to any of the glycans in the LTBI group at post infection time-points. Because of recent data showing an association of airway and systemic IgM to LAM with the protection against TB in BCG vaccinated rhesus macaques,[33] we further investigated IgM reactivity. Due to our limited sample volumes, we focused on investigating airway and systemic IgM to AM/LAM motifs only. IgM reactivity to AM motifs was diverse and increased during early infection but was not significantly different between the LTBI and TB group (Supplementary Tables S7 and S8).

IgA reactivity to specific glycan motifs prior to Mtb infection is significantly associated with LTBI. IgA reactivities to specific AM motifs, two (S56 and 57) in the BALF and four (S3, 4, 7, and 56) in the plasma, were significantly higher in the LTBI compared to mostly absent reactivity in the TB group (Fig. 2). These met our significance criteria of a more stringent $p < 0.01$, and, in addition, were within a false discovery rate (FDR) of less

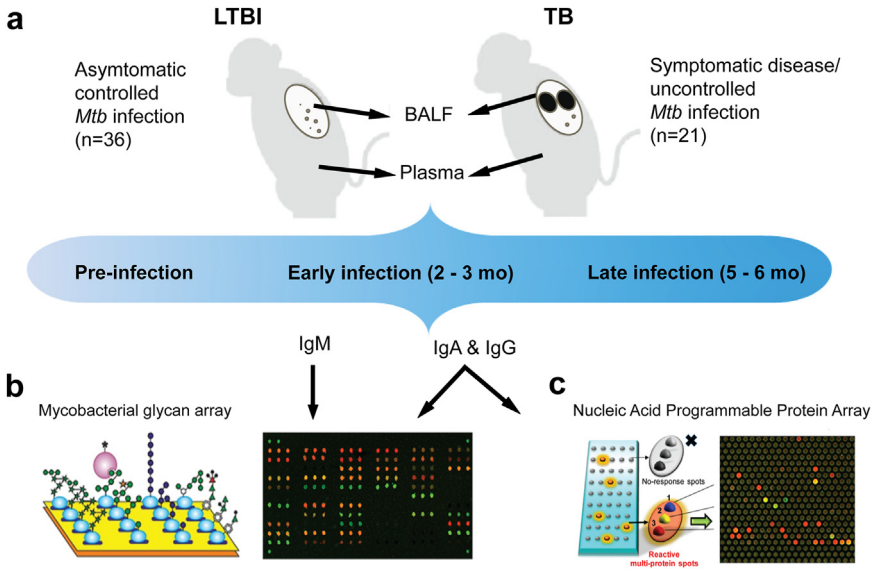

***Fig. 1: Mtb* antigen-unbiased approach to investigating airway and systemic antibodies associated with the outcome of *Mtb* infection.** **(a)** Cynomolgus macaques stratified by outcome of interest into latent *Mtb* infection (LTBI) and active tuberculosis (TB). Schematic demonstration for investigating bronchoalveolar lavage fluid (BALF) and plasma IgA and IgG against **(b)** 63 OS motifs from mycobacterial glycans printed in triplicates on microarrays, and **(c)** 3732 *Mtb* protein genes printed as three genes per spot on multiplex High-density Nucleic Acid Programmable Protein Array (HD-NAPPA) followed by deconvolution HD-NAPPA of reactive spots with testing samples against 321 single *Mtb* genes. IgM reactivity to 30 AM/LAM OS motifs was tested with pre-infection and early post infection BALF and plasma samples.

than 5%. Among these five glycan motifs, S3, S4, and S7 are from the mannose-capped terminal LAM (ManLAM) group and share four arabinofuranose (Ara*f*) residues in the same configuration, β-(1 → 2), α-(1 → 5), α-(1 → 5), which are further functionalized with additional mannose, and, for S7, also a 5-methylthio-xylofuranose (MTX) residue. The other two motifs, S56 and S57, are components of the mannan core and share mannose attached to five Ara*f* residues in the same α configuration.

IgA reactivity to AM/LAM motifs pre-infection correlated strongly within but not between BALF and plasma. Within the BALF obtained prior to *Mtb* infection, IgA reactivity between S56 and S57 correlated strongly and significantly ($r \geq 0.96$; $p < 0.001$ [Pearson]; Supplementary Figure S1), suggesting cross-reactivity to the α-Ara_5 motif shared by both S56 and S57. Similarly, within pre-infection plasma, IgA reactivity between S3, S4, and S7 correlated strongly and significantly ($r \geq 0.96$; $p < 0.001$ [Pearson]) suggesting cross-reactivity between these capped ManLAM motif, which all share a β-Ara_4 motif. While still significant, the correlations between IgA reactivity to S3, S4, or S7 and S56 within plasma were weaker (r range = 0.62–0.76; $p < 0.001$ [Pearson]), suggesting less cross-reactivity. The reactivity between the glycan motifs in the BALF (S56 and S57) and the plasma (S3, S4, S7), however, did not strongly correlate (r range = 0.34–0.51 [Pearson]), and neither did plasma and BALF reactivity to either of the capped ManLAM motifs S3, S4, S7 (r range = 0.30–0.35 [Pearson]; Supplementary Figure S2), suggesting that the pre-infection IgA signatures in the BALF and plasma are distinct and induced through different oral versus airway exposures. This hypothesis was supported by an increase in BALF IgA to S56 concomitant with a significant plasma IgA decrease to this OS motif after airway *Mtb* infection ($p < 0.01$ [Friedman]; Fig. 3a and c).

Airway and systemic antibody reactivities to mycobacterial glycan motifs change over time in most CMs with LTBI. In a secondary analysis, we tested in a subset of LTBI CMs with samples available from all three time points (n = 19) whether isotype reactivities to *Mtb* glycan motifs (those remaining after filtering) could be boosted by airway *Mtb* infection and changed significantly at a $p < 0.01$ over time. While a rise in antibody reactivity to glycan motifs over time did not meet our stringent significance criteria, we saw an increase in the BALF

Time point	LTBI n = 36		TB n = 21	
	Plasma	BALF	Plasma	BALF
Pre-infection (%)	26 (72)	30 (83)	15 (75)	17 (85)
Early post-infection (%)	34 (94)	36 (100)	20 (95)	20 (95)
Late post-infection (%)	22 (61)	25 (69)	11 (55)	11 (55)

[a]Pre-infection samples were collected at a median of one month prior to *Mtb* infection, post-infection samples were obtained 2–3 months (early) and 5–6 months (late) post *Mtb* infection. BALF: Bronchoalveolar lavage fluid; LTBI: Latent *Mtb* infection; TB: active tuberculosis.

***Table 1:* Cynomolgus macaque samples investigated and stratified by *Mtb* infection outcome of LTBI versus TB.[a]**

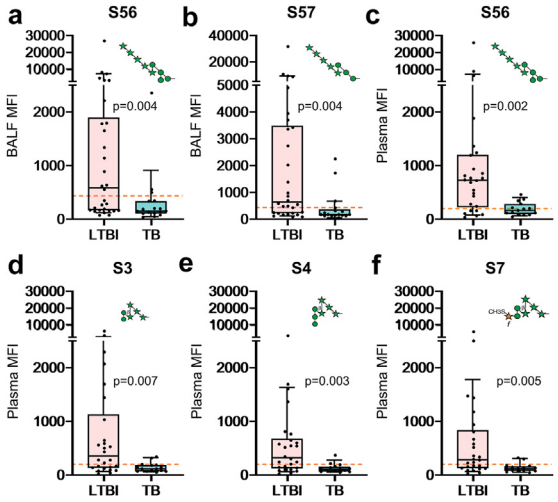

Fig. 2: **Airway and plasma IgA reactivities to specific glycan motifs are significantly higher prior to airway *Mtb* infection in CMs who develop LTBI versus TB**. IgA reactivity in pre-infection **(a** and **b)** bronchoalveolar lavage fluid (BALF) to glycan motifs S56 and S57 and **(c–f)** plasma to glycan motifs S56, S3, S4, and S7. Symbol representation of glycan motifs with green stars for arabinofuranose, green circles for mannose, and orange star for α-(1 → 4)-linked methylthio-D-xylofuranose (MTX). Glycan motif bonds are alpha unless noted with a beta (β) symbol. Box plots show median fluorescent intensity [MFI] with interquartile range, whiskers show 10th and 90th percentile values. Data is shown for those motifs meeting our significance criteria of a $p < 0.01$ [Mann–Whitney U test] and an FDR of 5% [$q < 0.05$]. The orange dotted line represents the cut-off for reactivity above the background (438 for BALF IgA and MFI 197 for plasma IgA).

IgA reactivity to S56 and/or S57 2–3 months post *Mtb* infection in 58% (11/19) of the LTBI animals (Fig. 3), indicating that the lung mucosal IgA responses to the AM/LAM motifs associated with LTBI can be boosted. However, only 17% (1/6) animals in the TB group showed such an increase early post *Mtb* infection. On the other hand, we saw a significant decrease of plasma IgA to S56 over time in the LTBI animals ($p < 0.01$ [Friedman]), providing further evidence that the antibody responses at the airway mucosal and systemic level are distinct. Due to limited remaining sample volumes, data for IgM were only available for AM motifs in pre- and early post infection samples. IgM reactivities to several AM motifs increased significantly between pre and early post infection but without significant differences between the TB and LTBI group.

Anti-Mtb glycan-specific IgA antibody signatures can discriminate LTBI from TB. We used partial least squares discriminant analysis (PLSDA)[49] to quantify pre and early post infection BALF and plasma anti-glycan IgA profile differences between LTBI and TB. We show that OS specific anti-*Mtb* glycan IgA reactivity profiles can distingush between CMs that develop LTBI versus TB (Fig. 4a). The corresponding Receiver Operating Characteristic (ROC) using classification error rate for cross-validation had an area under the curve (AUC) of 0.88 showing the predictive value of IgA reactivities to OS glycans for LTBI (Fig. 4b).

Antibody responses to Mtb proteins are most diverse for plasma IgG. Using a similar method to that employed in the glycan array studies, we filtered out proteins with low antibody reactivity; this led to a subset of 2, 21, 8, 39 proteins that were compared between the LTBI and TB groups for BALF IgA, plasma IgA, BALF IgG, and plasma IgG, respectively (Supplementary Table S7). We saw the greatest breadth of antibody reactivities to *Mtb* protein antigens in the plasma IgG of CMs whereas BALF IgG and BALF and plasma IgA reactivities to *Mtb* proteins were considerably less (Supplementary Table S9). Because of the highly exploratory analysis, we did not control for FDR in the antibody reactivities to proteins. As anticipated, BALF and plasma IgG recognition of a few protein antigens were significantly higher from the TB group in early and late post-infection (Supplementary Tables S12 and S13). These could have biomarker value but were not the focus of the current studies. As for the glycans, our analysis focused on identifying antibody responses that were significantly higher in the LTBI compared to the TB group. In contrast to the observed differences in antibody reactivities to surface glycan motifs prior to *Mtb* infection, there was no significant difference between IgG or IgA reactivities to *Mtb* proteins between the LTBI and TB groups prior to *Mtb* infection. We did, however, observe reactivities to proteins that were higher in the LTBI compared to TB CMs early post *Mtb* infection.

Plasma IgG reactivity to MTB32A is higher during early infection in CMs with LTBI. Early (2–3 months) after *Mtb* infection, plasma IgG to MTB32A (Rv0125) but no other protein was significantly higher in the LTBI compared to the TB group ($p < 0.05$ [Mann–Whitney]; Fig. 5a). Pre-infection plasma IgG reactivity to MTB32A had a trend towards higher levels in the LTBI versus TB group ($p = 0.057$ [Mann–Whitney]; Supplementary Table S13), and further increased significantly from pre-to early post-infection ($p = 0.03$ [Mann–Whitney]; Fig. 5b). Interestingly, in the CMs with LTBI, IgG reactivity to MTB32A in the BALF at early infection was lower than in plasma and reactivities above cut-off of 1.4 did not correlate significantly between the BALF and plasma ($r = 0.08$ [Pearson]).

Airway and systemic antibody reactivities to mycobacterial proteins change over time in some CMs with LTBI. Similar to antibody reactivities to mycobacterial glycans, we tested in a secondary analysis in a subset of LTBI CMs with samples available from all three time points ($n = 19$) whether isotype reactivities to *Mtb* proteins (those remaining after filtering) changed significantly at a $p < 0.01$ over time. IgG reactivities to three *Mtb* proteins, specifically α-crystallin (Rv2031c) and an uncharacterized protein (MtbXG006680-D12) in the BALF, and

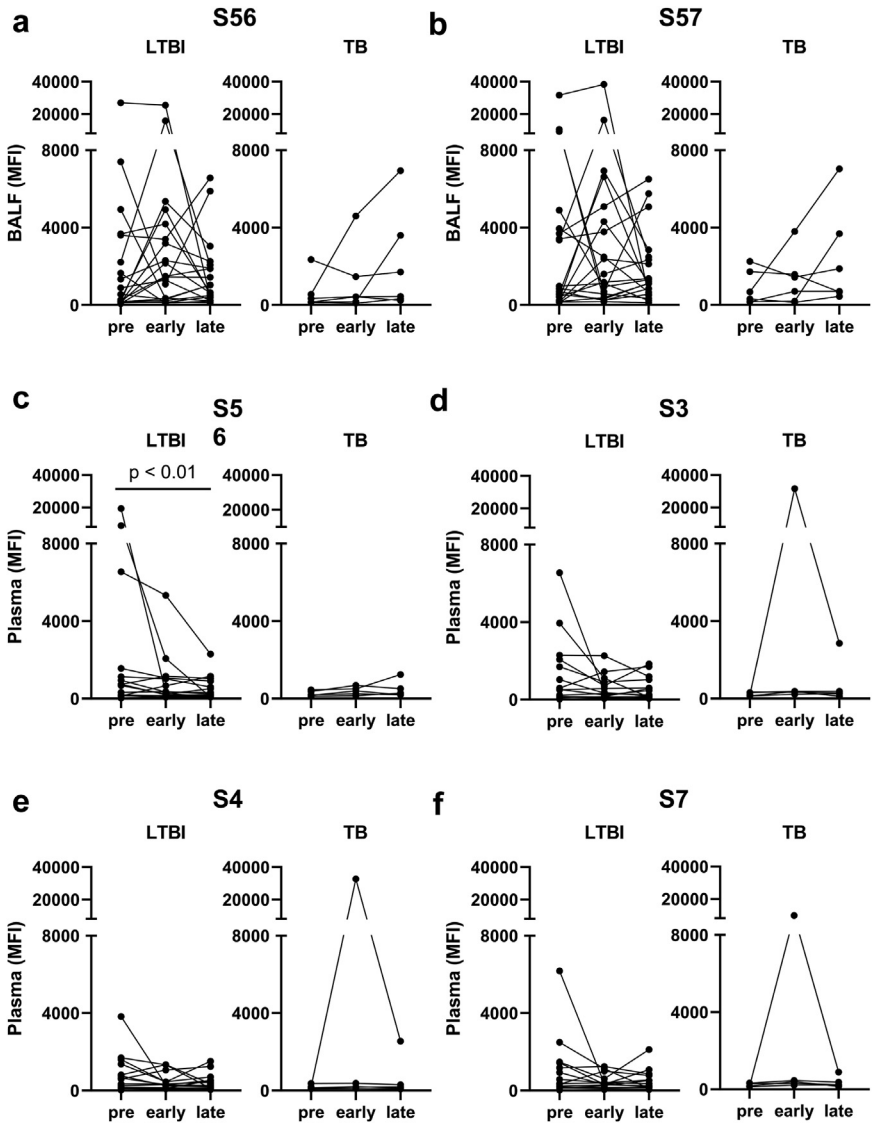

Fig. 3: **IgA reactivity in bronchoalveolar lavage fluid (BALF) and plasma over time from pre-infection to late infection.** IgA reactivity for **(a** and **b)** BALF to glycan motifs S56 and S57 and **(c–f)** plasma to glycan motifs S56, S3, S4, and S7 [Friedman test]. LTBI (n = 19) and TB (n = 6) for paired BALF, and LTBI (n = 15) and TB (n = 6) for paired plasma.

ESX-1 secretion-associated protein A (Rv3616c) in the plasma showed a significant increase from pre-to post-infection levels in animals with LTBI ($p < 0.01$ [Friedman]; Fig. 6).

Mucosal and systemic antigen-specific antibody signatures in CM pre- and early post Mtb infection are associated with LTBI versus TB outcome

To compare antibody reactivities associated with LTBI in individual animals, we illustrated reactivities in LTBI CMs (n = 24) with available matching pre-infection and early post infection samples. Over half of the LTBI CMs (13/24 [54%]) had pre-infection IgA to AM OS 56/57 in the BALF with or without plasma IgA to AM OS 3, 4, 7 and/or 56 (Fig. 7). When combining these with the additional animals that had only pre-infection serum IgA reactivity to AM OS 3, 4, 7 and/or 56, 18/24 (75%) of animals, and when combining with IgG reactivity to MTB32A early post-infection, most animals had mucosal and/or systemic antibody signatures (21/24 [88%]) associated with protection against TB. These data highlight the heterogeneity of antibody responses significantly associated with LTBI, suggest a combination of mechanisms, and further illustrate the in part distinct mucosal airway and systemic antibody responses in CMs with LTBI.

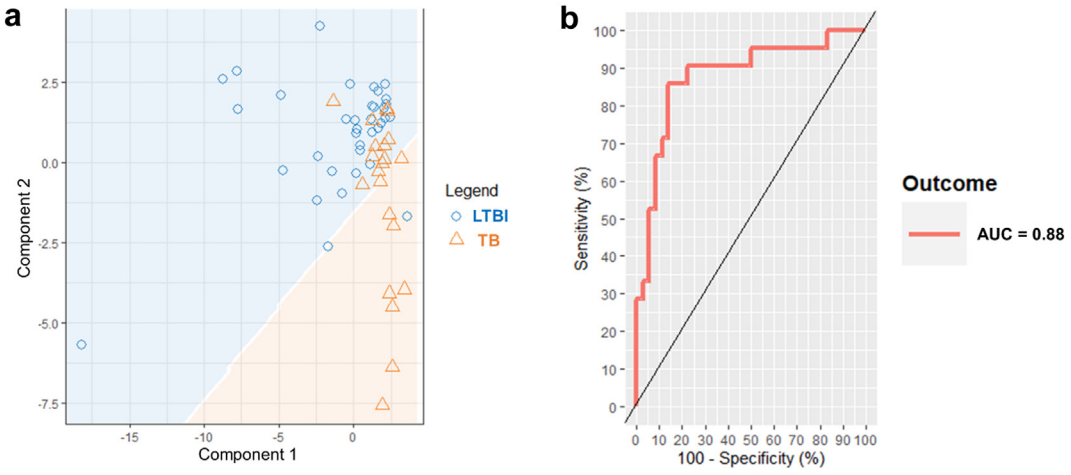

Fig. 4: **PLSDA identifies combinations of IgA reactivities to *Mtb* glycan OS that distinguish between LTBI and TB and can predict the outcome of *Mtb* infection. (a)** Prediction background generated by pre and early post infection BALF and plasma samples from CMs with LTBI (n = 36) and TB (n = 21). Symbols representing individual animals with LTBI (blue circle) or TB (orange triangle). **(b)** ROC curve and AUC from PLSDA on the IgA glycan dataset using classification error rate for cross-validation to predict the outcome LTBI. Each component groups the pre-infection and early post-infection BALF and plasma IgA glycan reactivity to separate LTBI group from the TB group. Each component is plotted to estimate the distance between LTBI and TB using both dimensions.

Discussion

Our findings enhance the understanding of antibody correlates of protection against TB. Using an antigen-unbiased screening approach with high-throughput *Mtb* surface glycan and proteome-wide HD-NAPPA microarrays, we show here that CMs that developed LTBI had significantly increased airway and plasma IgA to specific AM glycan epitopes prior to *Mtb* infection compared to those that had uncontrolled infection and developed TB. Our results support a protective role of pre-existing lung mucosal and systemic IgA to specific *Mtb* surface glycan epitopes, provide insights into

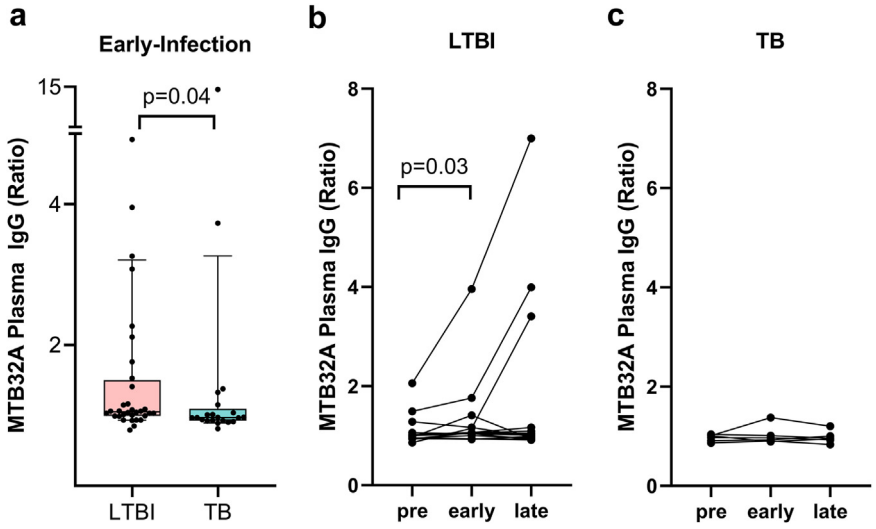

Fig. 5: **IgG to MTB32A is higher in LTBI plasma during early infection and significantly increases in early compared to pre-infection levels. (a)** Plasma IgG reactivity to MTB32A in LTBI (n = 33) compared to TB (n = 20) [Mann–Whitney U test]. **(b)** Paired plasma IgG reactivities to MTB32A in CMs with LTBI (n = 19 for animals with samples from all time-points) and **(c)** paired plasma IgG of TB (n = 6 for animals with samples from all time-points) from pre-infection, early infection, and late infection showing significant increase from pre-to early post infection in LTBI [Wilcoxon matched pairs signed rank test]. Ratios were calculated using the median fluorescent intensity (MFI) of the antibody reactivity to a protein divided by the median of all negative control proteins. Cut-off for reactivity was a ratio of 1.4.

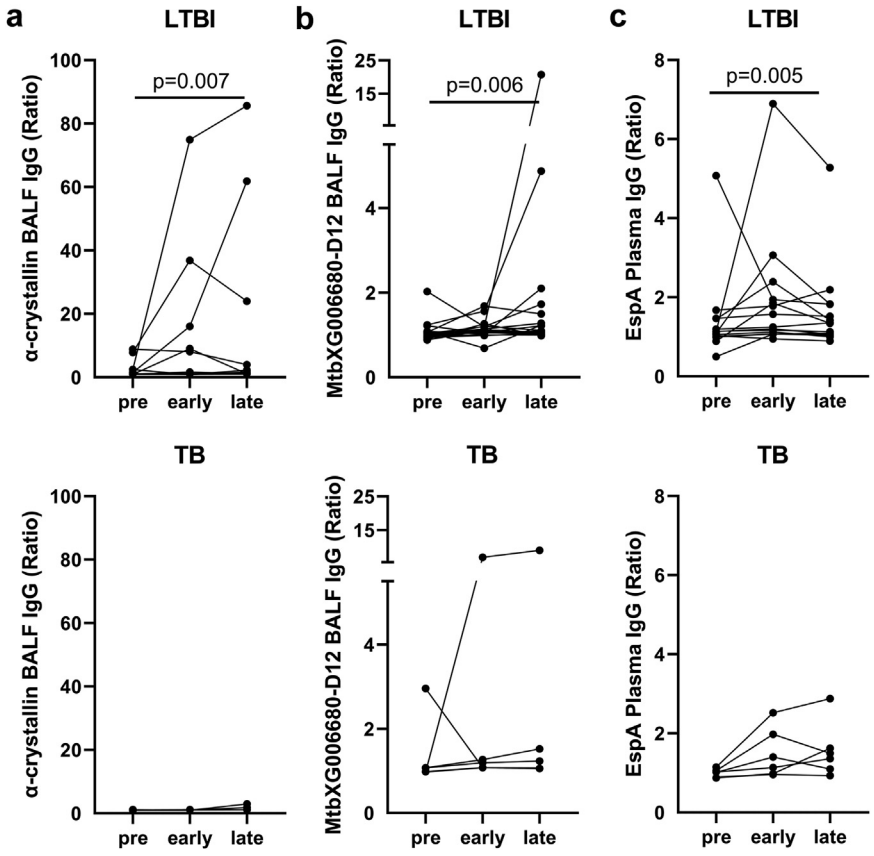

Fig. 6: **IgG reactivity in BALF and plasma to three *Mtb* proteins significantly changes over time from pre-infection to late infection in LTBI. (a)** α-crystallin (Rv2031c), **(b)** uncharacterized protein (MtbXG006680-D12), and **(c)** EspA (Rv3616c). LTBI (n = 19) and TB (n = 6) for paired BALF, and LTBI (n = 15) and TB (n = 6) for paired plasma [Friedman test]. Ratio is calculated using the median fluorescent intensity (MFI) of the gene divided by the median of all negative control genes. Cut-off for reactivity was a ratio of 1.4.

determinants of LTBI, and could inform strategies for TB vaccine development and routes of immunization.[50]

Capsular AM and cell wall/membrane LAM are immunomodulatory mycobacterial surface glycans that facilitate the establishment of *Mtb* infection (reviewed in[51,52]). Human serum IgG to AM/LAM has been associated with and shown to be protective against TB,[25,26,53–56] but data on IgA to these immunogenic glycans are limited. Our data are in line with other studies showing that IgA is associated with improved outcomes of mycobacterial infection in vaccination studies and animal models. IgA deficient mice were found to be more susceptible to BCG infection,[57] and plasma IgA levels have also been associated with protection from leprosy in humans.[58] Importantly, protection against TB after mucosal BCG vaccination of NHPs was significantly associated with increased lung mucosal IgA to purified protein derivative (PPD), a mixture of 100–200 mycobacterial proteins that also contains LAM.[30,59] In a separate NHP study, protection after i.v. BCG vaccination was associated with significantly higher IgA, IgG, and IgM responses against *Mtb* whole cell lysate and PPD in the BALF and plasma.[31,33] A follow-up study which selected five antigens a priori, including LAM, showed that plasma anti-LAM IgM titers post BCG vaccination correlated with lower *Mtb* burden in the lungs of *Mtb* infected rhesus macaques.[33] These studies, in contrast to ours, did not identify antigen-specific polyclonal IgA. At the monoclonal level, intranasal administration of some but not all IgA mAbs to the mycobacterial protein α-crystallin reduced the lung colonization of *Mtb* in mice.[60–62] Collectively, these data support a role of IgA in the defense against TB and underscore the importance of our findings for the field.

Immune responses prior to and during the first few weeks of *Mtb* infection are major drivers of clinical outcomes.[63,64] The airway and plasma IgA levels to specific AM motifs prior to *Mtb* infection in CMs raised outdoors were likely induced by inhalation and/or ingestion of environmental nontuberculous mycobacteria (NTM). These higher levels could be protective through various mechanisms.[65,66] For example, secretory

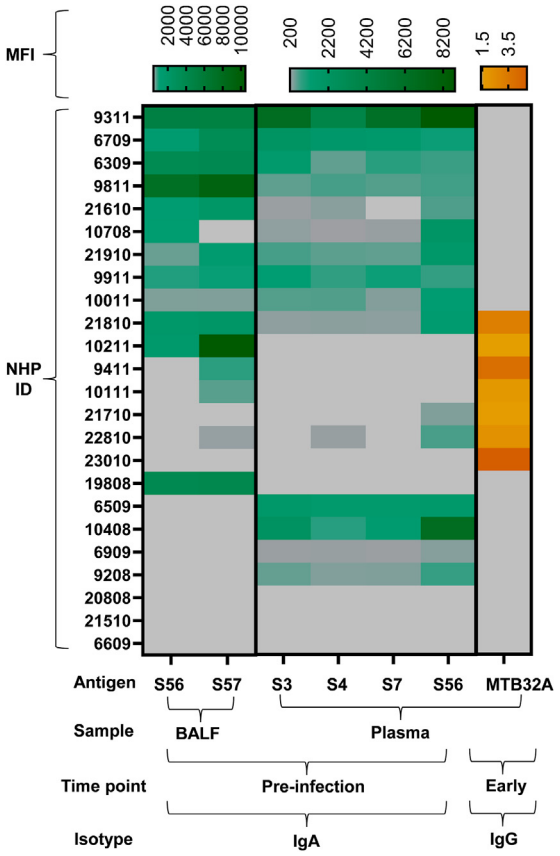

Fig. 7: **Mucosal and systemic antigen-specific antibody signatures in CM with LTBI versus TB outcome**. Heatmap showing antibody characteristics and reactivities associated with protection in BALF and plasma samples prior to and early after (2–3 months) airway *Mtb* infection for individual CMs with LTBI. IgA glycan reactivity to ManLAM motifs (S3, S4, and S7) and Core mannan motifs (S56 and S57) from AM/LAM and IgG reactivity to MTB32A are compared. Cut-off for positive reactivity was median fluorescent intensity [MFI] of 438, 197, and 1.4 for BALF IgA, Plasma IgA, and NAPPA IgG, respectively.

IgA has a crucial role in the innate immune response and prevents infections by immune exclusion at mucosal sites.[65,67] In TB, this is supported by a study showing that IgA mAbs binding to *Mtb* and the heparin-binding hemagglutinin protein HBHA reduce *Mtb* uptake in lung epithelial cells.[50] IgA complexes can further be eliminated through recognition by poly immunoglobulin receptors (pIgR) and excretion by transcytosis from epithelial cells.[65,67] Moreover, FcαRI receptors on innate immune cells in humans can lead to enhanced uptake and clearing of IgA opsonized mycobacteria further contributing to the containment of infection.[66] While the specific IgA-mediated mechanisms against *Mtb* need to be investigated, it is therefore conceivable that airway IgA to AM/LAM epitopes could bind to *Mtb* and prevent infection of lung epithelial cells. This, in turn, could reduce *Mtb* burden in the lungs, allowing innate and early adaptive immune responses to control the infection more effectively.

The impact of specific structural LAM features on biological functions in *Mtb* infection is not well understood,[51] but their mannose residues have been shown to interact with various host cell receptors.[45] Of the 63 motifs from seven mycobacterial surface glycan classes investigated, the significantly higher mucosal and systemic IgA reactivities associated with LTBI targeted five specific glycan motifs. Three of the motifs (S3, S4, S7) belong to capped ManLAM and overlapped with those identified by us to correlate significantly with protective serum IgG functions against *Mtb* in humans with LTBI and recent BCG vaccination.[25,26] The other two motifs to which airway and systemic IgA reactivity was significantly higher in LTBI versus TB were S56 and S57, both components of the AM/LAM mannan core. Little is known about the immunogenicity of S56/S57. None of the anti-AM/LAM mAbs generated from animals immunized with *Mtb*, BCG or *M. leprae* are reported to react with them.[68] We, however, have shown that polyclonal serum IgG from humans with LTBI and TB reacts with S56/S57,[26] as does polyclonal serum IgG from humans infected with *M. abscessus* and *M. avium* (data unpublished), supporting their immunogenicity. In addition, we have recently isolated an mAb from an asymptomatic *Mtb*-infected human adult subject, shown that it reacts with several AM/LAM motifs, including ManLAM caps and S56/S57, and demonstrated that as human IgG1 it is protective *in vitro* and *in vivo*.[69] Studies with IgA mAbs reactive with diverse and distinct AM/LAM epitopes, including ManLAM caps and S56/S57, are now needed to investigate their functions and mechanisms against *Mtb*.

Little is known about the protective effects of antibodies in response to prior NTM exposure.[70] Because few other pathogens contain the glycans AM and LAM,[68] the airway and plasma IgA levels to specific AM motifs associated with protection prior to *Mtb* infection were likely induced by exposure to environmental NTMs which are ubiquitous in soil and water. In studies of both humans and mice, there are mixed reports on the effect of NTM exposure on BCG vaccination efficacy and the protection against TB.[6,70–72] While the results of these studies are difficult to interpret due to differences in the species, dose, route, and duration of NTM exposure, a recent study showed that continuous exposure of BCG vaccinated and unvaccinated mice to NTM via drinking water induced strong airway and systemic IgG and IgA responses to *Mtb* lysates and enhanced survival of mice after *Mtb* infection.[73] Collectively with our results, these data support that NTM exposure can have protective effects against TB.

Overall, we observed higher and more diverse antibody reactivities to *Mtb* glycans than to proteins. While pre-infection IgA to AM/LAM OS motifs was associated

with LTBI outcome, we saw little IgA reactivity to *Mtb* proteins at any time-point, especially in the BALF. Early (2–3 months) post-*Mtb* infection, we did, however, observe higher plasma IgG reactivity to the protein MTB32A (Rv0215) in CMs with LTBI compared to TB. Moreover, the reactivity to this protein increased significantly between pre and early post-infection plasma in the LTBI but not the TB group. MTB32A is a known T cell antigen with a key role in the protection against TB.[74,75] It is one of two antigens that make up M72, a fusion protein in the *Mtb* subunit vaccine M72/AS01E that recently completed a phase 2b clinical trial.[76] This vaccine provided approximately 50% protection against pulmonary TB in subjects with LTBI, and, in addition to polyreactive CD4+ T cells, induced persistently elevated IgG titers to MTB32A. These data raise the hypothesis that early protein-specific systemic IgG responses could have an additional role in influencing the outcome of *Mtb* infection but warrant further investigations.

Little is known about antibody dynamics throughout *Mtb* infection. Such dynamics could enhance our understanding of immune components associated with disease progression or protection. Protective responses would be anticipated to occur before or early after *Mtb* infection, while those arising later are more likely biomarkers for disease progression. Moreover, antibodies induced during early infection, especially to protein antigens, could be involved with the *Mtb*-specific T cell response that takes a few weeks to occur after infection. When investigating changes in antibody responses over time, we observed significant increases of IgG to three mycobacterial proteins in the LTBI group. Of these, the changes in magnitude were most pronounced for BALF IgG reactivity to α-crystallin, a small heat-shock protein (also known as HspX) with critical roles in *Mtb* survival during LTBI.[77] Passive transfer of IgA to HspX reduced bacterial burden in the lungs of transgenic mice.[60] Moreover, IgG3 to HspX was higher in patients who had TB once compared to those with recurrent TB.[78] These data support the relevance of our findings and suggest that several mechanisms are involved in antibody-mediated protection against TB. Collectively, they highlight the need for a better understanding of the dynamics of airway and systemic antigen-specific isotypes and their mechanisms to elucidate the additional role these could have in the ongoing control of *Mtb*.

We saw tremendous heterogeneity in antibody responses among the CMs, irrespective of LTBI or TB group, consistent with other NHP and human studies profiling antibody responses to large numbers of *Mtb* proteins.[32,47,79] In both NHPs and humans, this heterologous immunity to *Mtb* is likely due to a combination of the large numbers of differentially expressed *Mtb* antigens,[42,80] differences in granuloma formation and immune activation,[37,41] prior BCG vaccination and/or exposure to environmental mycobacteria.[81] The resulting antibody heterogeneity contributes to the challenges of identifying immunodominant protective antigens. Moreover, differences in our results compared to other studies are likely due to variations in study design, methods, and NHP species. For example, in a different NHP study, plasma anti-LAM IgM titers post BCG vaccination (i.v. and intradermal) correlated moderately but significantly with lower *Mtb* burden in the lungs of *Mtb* infected rhesus macaques that are more susceptible to TB than CM.[33,34,38,39] In our study with CM, that develop TB and LTBI in about equal proportions after *Mtb* infection,[34,38,39] we observed IgM increases to several AM/LAM glycan motifs without significant difference between the groups.

To the best of our knowledge, this is the largest study to date characterizing airway and systemic antigen-specific antibody responses associated with LTBI. Although we standardized BALF dilutions, applied stringent data normalization and filtering criteria to best capture potential antigens of interest, we faced challenges in managing the tremendous antibody heterogeneity. We applied a stringent FDR of 5% combined with a two-tailed alpha of 0.01 for the mycobacterial glycan motifs investigated. However, due to the exploratory nature of the HD-NAPPA approach, our study was limited by not applying the same criteria to adjust for multiple comparisons for the isotype responses to proteins. Furthermore, paired BALF and plasma samples were not available for each animal at all three time points. Nevertheless, we still had samples for all three time-points for the majority of CMs with LTBI (BALF from 19 and plasma from 15), allowing us to draw conclusions about antibody dynamics over time in LTBI.

In conclusion, our findings suggest a role of pre-existing airway and systemic IgA to specific *Mtb* AM/LAM epitopes in the protection against TB in NHPs, indicating that prior exposure to NTM could influence the outcome of a later *Mtb* infection to the benefit of the host. Our data further suggest that different isotypes could mediate a range of protective mechanisms that could distinct between the airways and the blood and complementary in the protection against TB. We highlight the need for a more holistic knowledge of the heterogeneity of the immune response to *Mtb* to better understand how all immune arms contribute to the protection against TB. This includes the dynamics of antibody isotypes to several different protective antigens and their likely diverse mechanisms prior to and early after *Mtb* infection, especially in the lungs. Our findings enhance understanding of correlates of protection against TB and could inform TB vaccine development and immunization strategies.

Contributors

E.I., P.L.L., and J.M.A conceptualized and designed the study; E.I. and J.M.A analyzed and interpreted all data, performed literature review, and wrote the manuscript; E.I, D.T.C, T.C., Y.L, T.M.R. curated data and managed the project and resources; E.I., R.S.K., and J.M.A. conducted

formal analysis; T.C., L.S., D.M.M., T.L.L., P.L.L., J.M.A supervised experiments, reviewed and edited final manuscript; J.L., D.M.M, T.L.L., P.L.L. and J.M.A. developed methodology and/or provided resources, and T.L.L., P.L.L., and J.M.A. acquired funding. More than one author, in our study E.I and J.M.A., verified the underlying data. All authors critically read and commented on the manuscript and approved the final version for submission.

Data sharing statement

Glycan array and NAPPA data were deposited in the NCBI Gene Expression Omnibus (GEO) database and are available under GSE243663. Further information and requests for reagents may be directed to the corresponding author Jacqueline M. Achkar (jacqueline.achkar@einsteinmed.edu).

Declaration of interests

Data in this paper were included in a thesis (EI) submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Biomedical Sciences, Albert Einstein College of Medicine (ProQuest TX 9-135-714). Otherwise, all authors declare no competing interests.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104897.

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