Differential Recognition of *Mycobacterium tuberculosis*–Specific Epitopes as a Function of Tuberculosis Disease History

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Abstract

Rationale: Individuals with a history of tuberculosis (TB) disease are at elevated risk of disease recurrence. The underlying cause is not known, but one explanation is that previous disease results in less-effective immunity against *Mycobacterium tuberculosis* (Mtb).

Objectives: We hypothesized that the repertoire of Mtb-derived epitopes recognized by T cells from individuals with latent Mtb infection differs as a function of previous diagnosis of active TB disease.

Methods: T-cell responses to peptide pools in samples collected from an adult screening and an adolescent validation cohort were measured by IFN- γ enzyme-linked immunospot assay or intracellular cytokine staining.

Measurements and Main Results: We identified a set of “type 2” T-cell epitopes that were recognized at 10-fold-lower levels in Mtb-infected individuals with a

history of TB disease less than 6 years ago than in those without previous TB. By contrast, “type 1” epitopes were recognized equally well in individuals with or without previous TB.

The differential epitope recognition was not due to differences in HLA class II binding, memory phenotypes, or gene expression in the responding T cells. Instead, “TB disease history-sensitive” type 2 epitopes were significantly ($P < 0.0001$) more homologous to sequences from bacteria found in the human microbiome than type 1 epitopes.

Conclusions: Preferential loss of T-cell reactivity to Mtb epitopes that are homologous to bacteria in the microbiome in persons with previous TB disease may reflect long-term effects of antibiotic TB treatment on the microbiome.

Keywords: T-cell epitopes; *Mycobacterium tuberculosis*; tuberculosis; adaptive immunity; microbiome

Mycobacterium tuberculosis (Mtb) infection in humans spans acute, latent (defined as Mtb-specific T-cell response without clinical manifestations of tuberculosis

[TB] disease), and active disease, and, if antibiotic therapy is successful, cure. Individuals infected with Mtb remain at risk of developing active TB disease

throughout their lifetime (1), although with a 79% reduced risk of disease upon reinfection, compared with uninfected individuals (2). Immune responses induced

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At a Glance Commentary

Scientific Knowledge on the

Subject: Although tuberculosis (TB) is one of the leading causes of mortality worldwide, current understanding of the effects of previous active disease on mycobacteria-specific immunity is still incomplete.

What This Study Adds to the

Field: We demonstrate that *Mycobacterium tuberculosis*-derived T-cell epitopes are differentially recognized as a function of active TB disease history. Interestingly, in individuals with a history of active disease, T-cell reactivity was preferentially lost to *M. tuberculosis* epitopes with homology to bacterial peptides in the microbiome. These data suggest a long-term effect of antibiotic TB treatment on the microbiome.

by established Mtb infection, therefore, appear to control bacterial growth very effectively. However, individuals with previous active TB disease have several fold higher risk of developing disease again (3–5). Although not well understood, one explanation may be that previous disease results in different, less-effective immunity against Mtb.

Host control of Mtb infection requires CD4 T cells and IFN- γ /tumor necrosis factor (TNF)- α production (6–8). Immunological factors required to control Mtb during initial infection, progression from infection to disease, or relapse-free

elimination after anti-TB therapy are not completely understood. In addition, it is not known if different subpopulations of Mtb-specific T cells differ in their ability to control Mtb infection (9). Another factor may be the antigens targeted by the specific T-cell response; it has been proposed that, although T cells specific for certain Mtb antigens may be protective, other antigens might act as decoys or even promote disease spread (10).

Mtb is known to express different proteins in different stages of infection, which may give rise to stage-specific immune responses (11–13). Moreover, murine studies have suggested distinct patterns of antigen expression, where reduced mRNA expression of several Mtb antigens coincided with onset of adaptive immunity and slowing of bacterial replication (14, 15). Mtb selectively down-regulates some antigens (i.e., Mtb antigen 85B), but not others (early secretory antigenic target 6 kDa [ESAT-6]), during chronic infection, and it is unclear how this would enable persistence of Mtb (16–18).

We hypothesized that IFN- γ release assay (IGRA)-positive individuals will exhibit differential recognition of T-cell epitopes, depending on TB disease history. A previous study characterized the epitopes recognized in a cohort of healthy, Mtb-infected, IGRA-positive individuals from South Africa (19). Here, we have identified a set of epitopes that are differentially recognized in healthy IGRA-positive individuals who had a previous diagnosis of active TB disease and those who lack evidence of prior TB disease, and investigated the likely causes of this differential recognition. Some of the results of these studies have been previously reported in the form of an abstract (20).

Methods

Ethics Statement

This research was performed in accordance with approval from the Human Research Ethics Committee of the University of Cape Town (Cape Town, South Africa). All participants provided written informed consent before participation. Adolescents provided written informed assent, with a parent or legal guardian providing written informed consent.

Study Subjects

Three different cohorts from the Worcester region of South Africa were investigated (Table 1): screening, validation, and active cohorts. The screening and validation cohorts (IGRA positive, HIV negative) were identical to the cohorts investigated in a previous study (19). Here, we divided each healthy cohort into groups based on no history of active TB (non-PostTB) or self-reported history of active TB (PostTB), including year of active TB diagnosis as either more or less than 6 years ago (see Figure E2 in the online supplement). The 6-year cutoff was somewhat arbitrary, based on our screening cohort where the majority of individuals were diagnosed 5–6 years previously.

For the active TB cohort, we retrieved cryopreserved peripheral blood mononuclear cells from 16 adults from the Worcester region with sputum XpertMTB/RIF (Cepheid, Sunnyvale, CA)-positive TB disease. Venous blood was collected in heparin-containing tubes within 1 week of TB diagnosis and initiation of TB treatment.

Peptides

Epitopes (19) were segregated based on the ratio of their response in IGRA-positive

Table 1. Demographic Characteristics of Enrolled Participants

Characteristics	Screening Cohort		Validation Cohort		Active Cohort: Active TB at Diagnosis
	Non-PostTB	PostTB	Non-PostTB	PostTB	
Total participants enrolled, n	42	21	30	27	16
Time after active TB diagnosis, median (range), yr	—	6 (1–19)	—	11 (1–17)	—
Male, n (%)	18 (43)	4 (19)	18 (60)	12 (44)	N/A
Median age (range), yr	23 (20–26)	23 (20–25)	16 (14–20)	17 (14–19)	>18
Colored (mixed race), n (%)	40 (95)	19 (90)	25 (83)	19 (70)	N/A
Black African, n (%)	2 (5)	1 (5)	5 (17)	8 (30)	N/A
White, n (%)	0 (0)	1 (5)	0 (0)	0 (0)	N/A
HIV seropositive, n	0	0	Not tested	Not tested	N/A
QuantiFERON-TB Gold In-Tube positive, n (%)	42 (100)	20 (95)	30 (100)	27 (100)	N/A

Definition of abbreviations: N/A = not analyzed; Non-PostTB = individuals without a history of active TB; PostTB = individuals with a history of active TB; TB = tuberculosis.

individuals with and without previous TB disease (*see RESULTS*): epitopes recognized by both groups (i.e., non-PostTB/PostTB ratio < 2 [“type 1” epitopes]), and epitopes recognized preferentially in those without previous TB (non-PostTB/PostTB > 2 [“type 2” epitopes]; Table E1 and Figure E1).

RNA Sequencing Analysis

Gene expression values were quantile normalized and pairwise comparisons between pool-specific memory CD4⁺ T cells were performed using DESeq (Bioconductor) (21).

BLOSUM Score

We used the BLOSUM62 (block substitution matrix built using sequences with more than 62% similarity) matrix (22) to quantify the maximum level of amino acid sequence similarity of type 1 and 2 epitopes to sequences from *Mtb*, nontuberculous mycobacteria (NTM), or the microbiome. This score is based on the relative frequency of amino acids and their substitution probability,

and yields log-odds scores for each of the 20 possible amino acid substitutions.

Differential Antigen Recognition

Epitopes were mapped to antigens, and, for every antigen, we calculated the proportion of the total T-cell response accounted for by peptides homologous to microbiome species or that were mycobacteria specific.

All statistical analyses are described in the online supplement, and were performed using Prism 7 software (GraphPad Software, La Jolla, CA). The remainders of all experimental procedures are described in detail in the SUPPLEMENTAL MATERIALS and METHODS.

Results

Lower Total Epitope Reactivity in Individuals with Previous TB Disease

We recently described T-cell reactivity in 63 IGRA-positive adults to peptides from a wide

range of *Mtb* antigens (19). A total of 21 of these individuals had a history of previous active TB (median, 6 yr; range, 1–19 yr) before study enrolment (Table 1, screening cohort). The remaining 42 individuals had no record of prior active TB disease (Table 1).

Here we compared reactivity to all epitopes recognized (total) or a subset of 66 epitopes accounting for 80% of the total response (top epitopes) between the PostTB and non-PostTB individuals. PostTB individuals had lower responses than those without a history of TB disease, by both IFN- γ enzyme-linked immunospot assay (Figure 1A) and intracellular cytokine staining (Figure 1B). Interestingly, responses to heat-killed *Mtb* lysate were not different between the two groups (Figure 1B).

These lower responses in PostTB individuals appeared to be largely restricted to IFN- γ -expressing CD4 T cells; with the exception of IL-2-expressing CD4 T cells (top epitopes), no significant differences were observed when comparing frequencies of IL-2-, TNF- α -, or IL-22-expressing CD4 T cells between the groups (Figure 1B).

Figure 1. Total epitope reactivity reveals differential reactivity in individuals with latent *Mycobacterium tuberculosis* (*Mtb*) infection with (PostTB) and without (non-PostTB) previous tuberculosis disease in the screening cohort. (A) Number of IFN- γ -expressing cells in response to a single peptide pool of 300 *Mtb* epitopes (Total MTB300) or a subset of 66 peptides previously shown to comprise 80% of the total response (Top Epitopes), expressed as the total magnitude of response (spot-forming cells [SFC]) per donor. Each dot represents one donor, orange bars indicate non-PostTB individuals ($n = 42$), and blue bars indicate PostTB individuals ($n = 21$). Two-tailed Mann-Whitney test was used. (B and C) Frequencies of cytokine-expressing CD3⁺CD4⁺ T cells (B) and CD3⁺CD8⁺ T cells (C) in response to the pool of top 66 epitopes or all epitopes, as well as heat-killed H37Rv lysate (*Mtb*). Each dot represents one donor; median and interquartile range are indicated. Non-PostTB ($n = 17$) is indicated by dashed light-colored bars; PostTB ($n = 15$) is indicated by solid colored bars. The numbers above the brackets are P values determined by one-tailed Mann-Whitney test (comparing cytokine responses in peptide pools) and two-tailed Mann-Whitney test (*Mtb*); bold P values indicate a significant difference. TNF = tumor necrosis factor.

Furthermore, no significant differences in IFN- γ -, IL-2-, or TNF- α -expressing CD8 $^{+}$ T cells were detected between the groups (Figure 1C). Thus, the difference observed between PostTB and non-PostTB individuals was predominantly mediated by IFN- γ^{+} CD4 $^{+}$ T cells.

Differential Epitope Response as a Function of Disease History

We hypothesized that these comprehensive sets of peptides could be comprised of discrete epitope subsets associated with differential, disease history-associated responses.

Type 1 epitopes were recognized equally well, irrespective of disease history, whereas responses to type 2 epitopes were 10-fold higher in those without previous active TB compared with those with previous disease (Figure 2A). Thus, disease history influenced the overall magnitude

Figure 2. Differential epitope response as a function of tuberculosis (TB) disease history. (A and B) Total magnitude of response against type 1 or type 2 epitopes, or total response (sum of spot-forming cells [SFC] type 1 and type 2) expressed per donor in the screening cohort. Each dot represents one donor. (A) Individuals without previous TB disease (non-PostTB; orange bars; n = 42) and individuals with previous active TB (PostTB; n = 21; blue bars). (B) Non-PostTB (n = 42; orange bars), PostTB less than 6 years after diagnosis (n = 17; green bars), and PostTB greater than 6 years after diagnosis (n = 4; blue bars). (C) Total magnitude of response against *Mycobacterium tuberculosis* lysate 300 (MTB300), type 1, and type 2 epitope pools in the validation cohort. Each dot represents one donor. Non-PostTB (n = 30; orange bars), PostTB less than 6 years after diagnosis (n = 11; green bars), and PostTB over 6 years after diagnosis (n = 16; blue bars) are shown. (D) Percentage of IFN- γ detected from memory CD4 $^{+}$ T cells in response to the pools of type 1 and type 2 epitopes. Each dot represents one donor. Non-PostTB (n = 8; orange bars), PostTB less than 6 years after diagnosis (n = 5; green bars), and PostTB over 6 years after diagnosis (n = 5; blue bars) are shown. (E) Total magnitude of response against MTB300, type 1, and type 2 epitope pools. Each dot represents one donor. Non-PostTB (n = 30; orange bars; same as in Figures 2A and 2B for reference) and active TB at diagnosis (n = 16, pink bars) are shown. (F) Percentage of total memory CD4 $^{+}$ T cells, T memory cells expressing central memory (T_{CM}), T memory cells expressing effector memory (T_{EM}), T memory cells expressing terminally differentiated effector (T_{EMRA}), or naive T memory cells (T_{Naive}) detected in unstimulated samples from non-PostTB (n = 8; orange bars), PostTB less than 6 years after diagnosis (n = 5; green bars), and PostTB over 6 years after diagnosis (n = 5; blue bars). (G) Percentage of memory IFN- γ^{+} cell subsets detected in response to type 1 (white bars) and type 2 (gray bars) epitopes in non-PostTB individuals (n = 8). (A–G) Median and interquartile range are indicated. The numbers above the brackets are P values determined by two-tailed (solid lines) and one-tailed (dashed lines) Mann-Whitney test; bold P values indicate a significant difference. (H) Gene expression of differentially expressed genes in response to type 1 (white bars) and type 2 (gray bars) epitopes in non-PostTB individuals (n = 3).

of the response and the repertoire of epitopes recognized.

As predicted, the type 1 epitopes were recognized equally well, irrespective of disease history and time since diagnosis, whereas responses to the type 2 epitopes were 10-fold lower only in those with previous active TB less than 6 years previously (Figure 2B). No difference was observed between individuals without previous active TB and those who had active TB over 6 years previously (Figure 2B).

Independent Validation of Differential Epitope Responses

To determine reproducibility of this finding, we studied a separate cohort of 57 IGRA-positive South African adolescents (Table 1, validation cohort). A total of 27 of these individuals had previous active TB 1–17 years before sampling (Figure E2), whereas 30 had no previous diagnosis of active TB disease.

Peptide pools corresponding to type 1 and type 2 epitopes were generated, and the previously described MTB300 pool (19) was used as control. As described previously here, type 1-specific T-cell responses were not different by disease history in the validation cohort (Figure 2C). By contrast, individuals who had a TB diagnosis within the previous 6 years had significantly lower responses to type 2 epitopes compared with individuals without history of TB, or those who had TB more than 6 years before. These results were validated using intracellular cytokine staining assays (Figure 2D), which again showed lower responses in IGRA-positive individuals PostTB less than 6 years previously, compared with those without prior active TB.

If the differential epitope recognition were driven by the disease process, it would be expected that patients with active TB would have lower responses than IGRA-positive individuals with or without a past TB diagnosis. We therefore examined responses to the peptide pools in samples from 16 patients with newly diagnosed active TB (Table 1, active cohort). Patients with active TB had higher overall responses to MTB300 than non-PostTB individuals, possibly reflecting higher antigen load associated with active disease (Figure 2E). Interestingly, this elevated response was almost entirely associated with the type 1 epitopes, whereas responses to the type 2 epitopes in patients with active TB were not different from those in IGRA-positive individuals (Figure 2E).

In conclusion, T-cell reactivity to type 1 “persistent” epitopes was increased during active TB. By contrast, reactivity to type 2 “PostTB-sensitive” epitopes was not increased during active TB, and reactivity in individuals with a history of active TB less than 6 years prior was lower, but gradually regained more than 6 years after TB disease.

Similar Phenotypes of T Cells

Recognizing Type 1 and 2 Epitopes

We next examined whether T cells recognizing PostTB-sensitive (type 2) or persistent (type 1) epitopes were associated with different phenotypes. Frequencies of CD4 T memory cells and proportions of these cells expressing central memory, effector memory, terminally differentiated effector, or naive phenotypes were not different between individuals with or without previous TB (Figure 2F). In addition, because individuals without prior disease responded to both epitope pools, phenotypic differences could be investigated between type 1 and type 2 epitope-specific T cells. The majority of IFN- γ -producing memory CD4⁺ T cells were T memory cells expressing effector memory, and T cells responding to the two peptide pools were not different (Figure 2G). Moreover, no significant differences in proportions of IFN- γ -producing memory CD4⁺ T cells expressing CD127, programmed cell death protein 1, or CD27 were detected between type 1- and 2-stimulated samples (Figure 2G).

To examine responding T cells in more detail, we isolated epitope-specific T cells by IFN- γ -capture assay and performed transcriptomic profiling by RNA sequencing. Only nine genes, *ARID5B*, *BIRC3*, *HLA-A*, *MLL5*, *GLTSCR2*, *SET*, *SUN2*, *SYNE2*, and *SELPLG*, were identified as differentially expressed (adjusted $P < 0.05$, all below twofold expression levels) between type 1- and 2-stimulated T cells (Figure 2G). Of these nine genes, *HLA-A* is involved in presentation of epitopes to CD8 T cells, *SUN2* plays a role in T cell proliferation and HIV infection of CD4 cells (23), and *SELPLG* is differentially expressed between patients with active TB and control subjects with latent Mtb infection (24). To avoid a bias toward IFN- γ production, we also sequenced RNA from cells isolated based on the activation markers CD25 and OX40 (25). Only one gene, *MFS2A*, was differentially

expressed. In comparison, overall differences between CXCR3⁺CCR6⁺ T helper cells, which contain the majority of Mtb-specific T cells, and other T helper cell subsets revealed 518 differentially expressed genes with twofold or greater change (26). These data suggest that the peptide-specific CD4⁺ memory T cells for type 1 and 2 epitopes had similar phenotypes.

Differential Reactivity Is Not Due to Differential Binding to HLA Class II Molecules

Next, we examined whether the two different epitope types may be associated with differential breadth or affinity of binding to HLA class II molecules. Binding predictions for a panel of 27 DR, DP, and DQ HLA alleles (27) indicated no significant differences between type 1 and 2 epitopes (Figure 3A). As expected (19), in both epitope sets, approximately 80% of the epitopes were predicted to be promiscuous. Furthermore, no difference was observed in predicted HLA class II binding at the level of specific HLA alleles (Figure 3B). Thus, differential HLA class II binding could not explain differential recognition of type 1 and 2 epitopes in these donors.

Persistent Recognition of Mtb Epitopes Is Inversely Correlated with Homology to the Human Microbiome

We hypothesized that factors driving these observations may not be Mtb specific, but rather homologous cross-reactive antigens encoded by bacteria in the human microbiome. This was supported by a recent report that demonstrated that Mtb epitopes share significant homology to bacteria found in the microbiome (28). To investigate the homology of epitopes to bacterial species in the microbiome, Mtb and NTM, we calculated epitope similarity to antigens using a BLOSUM (block substitution matrix) matrix-based score. Strikingly, type 2 epitopes were significantly more homologous to bacterial sequences in the human microbiome than type 1, to which responses persisted after TB disease (Figure 4A), suggesting that the differential response between epitope types are linked to cross-reactivity with microbiome species.

Homology of Mtb Epitopes in NTMs versus the Human Microbiome

Certain Mtb-derived epitopes share significant homology with NTMs (29).

Figure 3. No difference in predicted HLA class II binding between type 1 and 2 epitopes. (A) Proportion of epitopes that are predicted to bind the indicated number of HLA class II alleles (percentile rank < 10). Type 1 epitopes (white dots) and type 2 epitopes (gray dots) are presented. (B) Proportion of epitopes that are predicted to bind each allele (percentile rank < 10). Type 1 epitopes (white bars) and type 2 epitopes (gray bars) are shown.

We considered the hypothesis that type 1 epitope recognition relative to disease history might be associated with a continued stimulation from environmental exposure to NTM. As expected, both epitope sets were highly conserved within Mtb complex species (Figure 4B). Although type 1 epitopes were significantly less homologous with NTM than type 2 epitopes (Figure 4C), the difference between the two epitope sets was smaller than that observed for the human microbiome. These data further support that homology to species in the microbiome, and not only NTM homology, underlie the differences in immune reactivity observed between type 1 and 2 epitopes.

Differential Epitope Homology Maps to Distinct Recognition of Specific Antigens

We next examined whether epitopes with high microbiome homology (BLOSUM > 0.75 for microbiome) and epitopes more exclusively found in mycobacteria

(BLOSUM < 0.75) map to particular Mtb antigens. In several instances, multiple epitopes mapped to the same antigen. Nine antigens were identified as mycobacteria specific (including ESAT-6 and 10-kDa culture filtrate antigen; i.e., >50% of the total reactivity accounted for by epitopes with BLOSUM scores < 0.75 to the microbiome; Figure 5A). In contrast, 44 antigens were identified as microbiome homologous (i.e., >50% of the total reactivity accounted for by epitopes with BLOSUM scores > 0.75; Figure 5A).

Thus, these analyses indicate that differential antigen, as well as epitope, recognition is linked to TB disease history and microbiome homology.

Immunogenicity of Mycobacteria-Specific and Not Microbiome-Homologous Antigens Is Associated with Their Expression in Cultured Mtb

Our results suggest that Mtb-specific epitopes/antigens are influenced by their expression in Mtb, whereas the responses to microbiome-homologous epitopes/antigens

may also be influenced by the microbiome. This implies that recognition of Mtb-specific type 1 antigens should be associated with their expression levels in Mtb. By contrast, a weaker association or no association between T cell recognition and expression levels would be expected for microbiome-homologous type 2 antigens. To test this hypothesis, we compared mRNA expression levels in Mtb culture (15) of the mycobacteria-specific and microbiome-homologous antigens.

Indeed, immunogenicity of mycobacteria-specific antigens correlated directly with Mtb transcript levels, suggesting that, for these antigens, expression levels drive the magnitude of immune reactivity (Figure 5B). By contrast, reactivity to microbiome-homologous antigens did not correlate with their mRNA expression levels in Mtb (Figure 5C). These data further suggest that *in vivo* recognition of type 2 epitopes (and antigens) is largely driven by sensitization of microbiome-derived antigens in addition to those expressed by Mtb.

Figure 4. Differential recognition of *Mycobacterium tuberculosis* (Mtb) epitopes correlates with conservation in the microbiome and less so in nontuberculous mycobacteria (NTM). Conservation of type 1 ($n = 113$; white dots) and type 2 ($n = 123$; gray dots) epitopes in (A) the human microbiome, (B) Mtb complex strains, and (C) NTM strains is shown. Each dot represents the maximum BLOSUM score per individual peptide (i.e., the highest homology found for each peptide within the human microbiome, Mtb complex strains, or NTM strains). Median and interquartile range are indicated. The numbers above the brackets are P values determined by two-tailed Mann-Whitney test.

Discussion

We identified two sets of Mtb-derived epitopes and antigens that display distinct immune reactivity in IGRA-positive individuals with and without a prior history of TB disease. T cell responses to type 2 “disease history-sensitive” epitopes with considerable homology to bacteria in the microbiome were lower in individuals with previous TB. By contrast, T cell responses to type 1 “persistent” epitopes with high specificity for Mtb and low homology to microbiome species were not influenced by TB disease history. We speculate that the differential reactivity to the epitope sets represents long-lasting effects of anti-TB therapy. We found that type 1-specific T cell responses persisted, whereas type 2-specific responses were lost after anti-TB treatment. We propose that this is likely due to dysbiosis and/or loss of cross-reactive microbiome species. There are at least two potential implications of this finding that appear to affect type 1 and 2 epitopes differently. First, specific Mtb antigens may persist, despite sterilization of Mtb, which would lead to persistent antigen exposure and preferential maintenance of type 1-specific T cells, although it is unlikely that this would persist for many years after cure.

Second, type 2-specific responses may be induced and/or maintained by antigens expressed by cross-reactive microbiome species, which are lost or decrease upon dysbiosis during and after anti-TB therapy. This could imply that either the type 2-specific T cell responses are not exclusively Mtb specific or, alternatively, the antigens recognized have a different pattern or kinetic of persistence in the context of the bacteriostatic or bactericidal effects of anti-TB therapy. Indeed, it has been shown that individual antigens persist to different degrees during the course of Mtb infection in mice (16–18).

The differences in reactivity to these distinct epitopes were not associated with phenotypic or transcriptomic characteristics of the responding T cells, although this was assessed in a small number of individuals.

Standard treatment for drug-sensitive TB disease is a minimum of 2 months of intensive therapy, followed by a continuation phase for 4 more months (1). The long-term effects of TB treatment on antigen-specific responses are unclear. Some studies reported decreases or undetectable T cell responses during and after treatment, whereas others have described increased or persistent responses (30–33).

Our data suggest a more nuanced picture. Preferential loss of reactivity to Mtb epitopes that are homologous to bacteria in the microbiome may be influenced by long-term effects of antibiotic treatment for TB on the microbiome. In addition, it is currently unclear which microbiome species are modulated by anti-TB therapy. We hypothesize that antibiotics may influence even low-abundance bacteria, resulting in dysbiosis and immunological consequences for the host. A study in mice has shown that microbiome dysbiosis resulted in a decline in IFN- γ -producing CD4 T cells (34).

It is intriguing that immunological effects were detectable even years after TB disease and treatment. Broad-spectrum antibiotic treatment was reported to cause marked shifts in microbiome composition that last for years, with highly heterogeneous restoration of the baseline composition (35). Specifically, significant and long-lasting changes in respiratory and intestinal microbiota after Mtb infection have been reported (36–39). The majority of antibiotics against Mtb is specific to *Mycobacterium* species and, therefore, will likely affect NTMs that may have colonized the host and are part of the normal commensal population. However, exposure to environmental NTM should not be affected by treatment, thus favoring the hypothesis that the microbiome is the main driver of the differences observed. We recognize that NTM species may also be present in the microbiome, and these may be implicated in the microbiome dysbiosis. More work is required to better understand the mechanisms that underlie the findings presented here. In light of our data, it will be important to longitudinally study immune responses to type 1 and 2 epitopes in persons during TB treatment, while also tracking the abundance of microbial species in mucosal microbiomes. This will allow ascertainment of influences of interindividual microbiome composition, which are also dependent on geography, age, dietary habits, and disease (40). Previous studies have shown that Mtb-specific T cell phenotypes are altered by treatment, which was interpreted to reflect effects of changes in bacterial load (8, 41).

Current measures of treatment success evaluate detection of culturable Mtb from sputum samples. However, a recent study (4) detected Mtb nucleic acids in some patients up to 1 year after clinical cure of active TB. Long-term Mtb persistence also

Figure 5. Immunogenicity of mycobacteria-specific antigens is associated with *in vitro* mRNA expression in cultured *Mycobacterium tuberculosis* (Mtb). (A) Proportion of total magnitude of response to indicated antigens. Responses for mycobacteria-specific antigens (black bars) and microbiome-homologous antigens (gray bars). (B and C) Correlation between immunogenicity (y-axis) and mean mRNA expression levels in Mtb cultures (x-axis) for proteins that correspond to mycobacteria-specific (B) and microbiome-homologous (C) antigens. Correlation is indicated by Spearman's r and associated two-tailed P value. R/G = channel 2 RNA/channel 1 DNA.

clearly occurs in healthy individuals, because viable bacteria can be cultured from apparently healthy tissues from individuals without evidence of disease (42). In addition, reactivation of latent Mtb can occur decades after initial infection in patients with rheumatoid arthritis from nonendemic settings (43). Finally, studies in low-prevalence settings report positive IGRA results that persist 6–12 months after

diagnosis, suggesting that immune responses can be maintained (44, 45). Regardless, we studied individuals from a high TB burden setting, and therefore cannot exclude effects of Mtb reinfection on our results.

Mtb-derived epitope sequences are generally hyperconserved (46), strongly suggesting that Mtb does not rely on antigenic variation as a mechanism to

evade T cell recognition. We have previously shown that epitopes conserved among Mtb and NTM are frequently targeted by T cell responses, and that this response reactivity correlates with the pattern of reactivity in the general population (29). In addition, the host microbiome plays a profound role in shaping and modulating host reactivity and immune functioning (47). Moreover, recent

results indicate that pathogen-derived epitopes with high homology to microbiome sequences may be perceived as self from the immune system, and thereby be tolerogenic, resulting in the elimination of potentially reactive T cells (28). Taken together, these data suggest that the reactivity against type 2 epitopes in individuals with a history of active TB within 6 years of diagnosis is likely modulated by the microbial environment.

Our findings may also have implications for mycobacterial vaccines. A considerable proportion of the microbiome-homologous, type 2 epitopes are also expressed by bacillus Calmette-Guérin, raising the possibility that sensitization to microbiota before bacillus Calmette-Guérin

vaccination may play a role in the blocking and/or masking effects commonly attributed to NTM sensitization (48, 49).

The unique epitope pools used here, encompassing several antigens, enable us to capture the majority of Mtb-specific responses. A study by Kim and colleagues (50) evaluated whether previous TB history had a long-term effect on ESAT-6 and 10-kDa culture filtrate antigen responses after anti-TB treatment. They also found significantly higher reactivity in individuals with latent Mtb infection compared with those with TB 1–59 years before, but, in this study, different epitope repertoires were not resolved.

In conclusion, this study provides evidence for differentially recognized

epitopes as a function of history of active disease and an intriguing association with epitope homology with the microbiome. These results illustrate a novel interplay between Mtb, the human host, and the microbiome, and indicate that distinct responses to different epitope categories should be studied further to fully characterize the dynamics of Mtb immune response. ■

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