1 **A Side-by-side Comparison of T cell Reactivity to Fifty-nine *M.***
2 ***tuberculosis* Antigens in Diverse Populations from five Continents**

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46 **Running title:** T cell reactivity to 59 MTB antigens

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Abstract

We compared T cell recognition of 59 prevalently recognized *Mycobacterium tuberculosis* (MTB) antigens in individuals latently infected with MTB (LTBI), and uninfected individuals with previous BCG vaccination, from nine locations and populations with different HLA distribution, MTB exposure rates, and standards of TB care. This comparison revealed similar response magnitudes in diverse LTBI and BCG-vaccinated cohorts and significant correlation between responses in LTBI from the USA and other locations. Many antigens were uniformly recognized, suggesting suitability for inclusion in vaccines targeting diverse populations. Several antigens were similarly immunodominant in LTBI and BCG cohorts, suggesting applicability for vaccines aimed at boosting BCG responses. The panel of MTB antigens will be valuable for characterizing MTB-specific CD4 T cell responses irrespective of ethnicity, infecting MTB strains and BCG vaccination status. Our results illustrate how a comparative analysis can provide insight into the relative immunogenicity of existing and novel vaccine candidates in LTBI.

67 **Keywords:** Tuberculosis; T cell antigen; vaccine; CD4; LTBI; BCG

68 **Abbreviations:**

69 MTB, *Mycobacterium tuberculosis*; LTBI, Latent tuberculosis infection; BCG, Bacillus
70 Calmette-Guérin; TB, tuberculosis; IGRA, Interferon gamma release assay; TST,
71 tuberculin skin test; SFC, spot-forming cells; NTM, non-tuberculous mycobacteria

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1. Introduction

The majority of MTB-infected individuals control the pathogen by mounting a successful, long-lived and protective immune response, leading to either resolution or a clinically latent TB infection (LTBI). Approximately 10% of LTBI individuals develop active TB [1, 2]. Established MTB infection provides considerable protection against TB disease from subsequent reinfection, compared with primary infection [3]. This suggests that immune responses introduced by MTB infection provide benefit in those who do not progress to TB disease immediately after they become infected. CD4 T cells secrete IFN γ , which is critical for immune control in MTB infected individuals [4, 5]. Therefore, it is important to identify the antigens that are consistently recognized by T cells in LTBI.

An issue of significant relevance in development of improved TB vaccines is which antigens should be selected. The recent disappointing results of the proof-of-concept trial of MVA85A have added urgency to the debate [6]. At least 13 vaccine candidates against TB are in clinical studies, most of which are aimed at enhancing immunity induced by BCG to prevent disease, but not to achieve sterile eradication or prevention of stable infection [7]. Most subunit vaccine candidates are based on one to four antigens, defined using traditional biochemical methods that are recognized by T cells from patients with LTBI or with cured TB [4, 8]. However, side-by-side comparisons of the frequency (immunodominance) and magnitude (immunogenicity) of immune responses have not been performed.

Significant issues to consider when studying different antigens are the pattern of antigen and epitope recognition in diverse populations associated with different HLA distributions, MTB exposure rates, BCG vaccine strains and circulating MTB strains,

variable exposure to environmental mycobacteria, and different standards of clinical care. Therefore, it is important to determine both how frequently and vigorously antigens are recognized overall, and how consistently they are recognized in different locations.

MTB is classified into seven main phylogenetic lineages, which are non-randomly distributed around the world, potentially due to migration with, and adaptation to, different human populations [9, 10]. The main MTB lineage in the Americas and Europe is Lineage 4, whereas, Lineages 1 and 3 are predominant in East Africa and India [10]. However, whether these differences influence the hierarchy of antigen recognition has not been addressed.

T cells recognize peptides bound to MHC molecules. Human (HLA) MHC molecules are extremely polymorphic and thousands of different variants are known [11]. However, at the population level almost 90% of each locus is covered by less than 50 HLA alleles [12] and promiscuous epitopes, recognized by multiple HLA variants, have been shown to represent a large fraction of total responses [13]. Whether promiscuous peptides can be used to monitor responses in multiple ethnicities remains to be experimentally demonstrated.

Another important issue in MTB antigen selection is the relationship between immunogenicity in MTB infection versus BCG vaccination. This is relevant since several TB vaccines currently considered rely on a BCGprime/TB vaccine boost concept [7]. Childhood vaccination with *M. bovis* BCG mostly protects against disseminated TB in young children. However, in non-endemic countries BCG vaccination is not performed due to the relatively low incidence of disease and variable effectiveness in preventing pulmonary TB in adults [14-16]. After its original development, BCG was distributed to

multiple countries worldwide leading to diversification of BCG into distinct sub-strains, which in turn lead to differential ability of BCG strains to induce specific immune responses [17, 18]. However, the results of clinical trials do not provide evidence for a correlation between the vaccine strain used and efficacy in preventing TB [19-21]. Accordingly, side-by-side comparison of the immunogenicity of different MTB antigens following BCG vaccination is also of high interest.

Here we describe reactivity to a set of antigens, previously identified in a non-biased genome-wide study[22], in donor cohorts derived from locations spanning the Americas, Europe, Asia, and Africa. As a result, we have identified a panel of antigens uniformly recognized by LTBI from different geographical locations. In addition, by comparing the relative ranking in LTBI and BCG-vaccinated subjects we identified several antigens that might be good targets for boosting BCG responses.

2.Methods

2.1.Study subjects

The different geographical locations, number of donors analyzed and general demographic and clinical characteristics of the populations are shown in Table 2. LTBI for all cohorts except Uganda was confirmed by a positive IGRA (QuantiFERON-TB Gold In-Tube, Cellestis or T-SPOT.TB, Oxford Immunotec). All the Ugandan LTBI subjects had a history of a positive tuberculin skin test (TST). All BCG vaccinated subjects from an area where BCG vaccination is prevalent were assumed to have been vaccinated. The BCG cohort from the USA were recruited based on self-reporting. The MTB-naïve donors did not endorse vaccination with BCG, were all born in countries where BCG vaccination is not used and were IGRA negative. None of the subjects studied

had physical exam and/or chest X-ray consistent with active TB, or evidence of HIV or Hepatitis B. Research conducted for this study was performed with approvals from respective Institutional Review boards. All participants, except anonymously recruited blood bank donors in India, provided written informed consent prior to participation in the study. The majority of the LTBI cohort (23 of 25 donors) from USA analyzed here was also utilized in our previous study[22].

2.2. Peptides

Sets of 15-mer peptides synthesized by A and A (San Diego) as crude material on a 1 mg scale were combined into pools of up to 20 peptides per antigen (median 18, 3-20 range, Supplementary Table 1). The IEDB submission number for previous described epitopes is 1000505 [22].

2.3. PBMC Isolation

PBMC were obtained by density gradient centrifugation, according to manufacturer's instructions. Cells were suspended in fetal bovine serum containing 10% dimethyl sulfoxide, and cryo-preserved in liquid nitrogen.

2.4. Ex vivo IFN γ ELISPOT Assay

PBMCs incubated at 2×10^5 cells/well were stimulated in triplicate with peptide pools (5 μ g/ml), PHA (10 μ g/ml) or medium containing 0.25% DMSO (percent DMSO in the pools, as a control) in 96-well plates (Immobilion-P; Millipore) coated with 5 μ g/ml anti-IFN γ (1-D1K; Mabtech). After 20 h incubation at 37°C, wells were washed with PBS/0.05% Tween 20 and incubated with 2 μ g/ml biotinylated anti-IFN γ (7-B6-1; Mabtech) for 2 h. The spots were developed using Vectastain ABC peroxidase (Vector

Laboratories) and 3-amino-9-ethylcarbazole (Sigma-Aldrich) and counted by computer-assisted image analysis (KS-ELISPOT reader, Zeiss). Responses were considered positive if the net spot-forming cells (SFC) per 10^6 were ≥ 20 , the stimulation index ≥ 2 , and $p \leq 0.05$ (Student's t-test, mean of triplicate values of the response against relevant pools vs. the DMSO control). All cohorts, except Brazil and India, were tested at La Jolla Institute for Allergy and Immunology. Samples tested in Brazil and India was tested in a manner analogous to the other cohorts. Samples were excluded if the viability was below 75%, as determined by trypan blue, and reactivity to PHA below 430 SFC/ 10^6 cells.

2.5. Factorial peptide pool testing

The number of cells available for each donor from Uganda, Brazil, Italy, and Russia was limiting and did not allow testing of all pools in the same donors. In these cases we tested each pool in the same number of donors but not all in the same individual donor.

3. Results

3.1. Definition of a set of MTB antigens and clinical sites to probe T cell reactivity

To evaluate CD4 T cell reactivity in LTBI donors from different geographical locations, we focused on 59 MTB antigens (Rv number and synonyms in Table 1). In our previous genome-wide study of MTB protein targets for CD4 T cell recognition [22], these antigens accounted for 77% of the total reactivity in LTBI donors recruited in San Diego, USA. With the exception of regulatory proteins, these antigens are representative of all protein categories described for the MTB proteome (Table 1), and include those used in the IFN γ release assay (IGRA) test, four currently being evaluated in human vaccines, and 25 previously described. Of interest, around half (28 out of 59) of these antigens were not

described as T cell antigens prior to the genome-wide study and are characterized in detail for the first time in the present study.

For each antigen, we assessed ex vivo IFN γ ELISPOT T cell reactivity using PBMC samples derived from nine different locations: USA, Central America, Peru, Brazil, South Africa, Uganda, India, Russia and Italy (Table 2), thereby spanning the Americas, Europe, Asia and Africa. We utilized peptide pools containing, for each antigen, all previously described epitopes and, to minimize the impact of different HLA distributions in different ethnicities, additional peptides selected on the basis of predicted promiscuous HLA class II binding capacity (Supplementary Table 1).

Table 2 lists the range of TB incidence in each respective location, ranging from 1.6 per 100,000 population for Puerto Rico to 860 per 100,000 for South Africa. In order to account for existing BCG immunity in the different cohorts, Table 2 lists whether BCG vaccination at birth is routinely administered to the general population [23]. It is generally expected that, with the exception of the USA and Italy, the individuals participating to the study were BCG-vaccinated at birth.

General epidemiological characteristics as well as composition of the various cohorts tested for each site are also detailed in Table 2. The average age for the various cohorts was 31 (ages 12 to 63), and the overall gender ratio was 52% male/48% female. Whenever possible we studied a cohort of LTBI and a control cohort of non-LTBI donors per site. On average each pool was tested in 17 individuals per cohort with a range of 8 to 30. Overall these data demonstrate that the clinical samples investigated span a significant range of location, gender, and age.

208 *3.2. Differential reactivity in the different LTBI cohorts and BCG-vaccinated MTB*
209 *uninfected cohorts*

210 The peptide pools representing each of the 59 antigens (Table 1) were tested for reactivity
211 in the various cohorts (Table 2). Fig. 1 shows the overall reactivity observed expressed as
212 Total SFC/individuals tested. As expected, significantly higher reactivity was noted for
213 LTBI as compared to BCG.

214 LTBI donors from the USA had similar overall reactivity to LTBI from most locations:
215 highest reactivity was seen in Indian LTBI, and reactivity in American LTBI was not
216 significantly different when compared to donors from Russia, Central America, South
217 Africa or Peru. Strikingly, the Italian, Ugandan and, to a lesser extent, Brazilian LTBI
218 cohorts, had lower reactivity than the other LTBI cohorts. The reasons for these
219 differences are not clear, but might include disparities in circulating MTB strains, local
220 standard of clinical care or cell isolation protocols.

221 The reactivity in LTBI was generally significantly higher than the reactivity in BCG-
222 vaccinated donors and MTB naïve controls (non-BCG vaccinated and non-LTBI). The
223 Russian BCG cohort had higher reactivity than any of the other BCG cohorts, which may
224 reflect the common practice of repeated BCG immunizations in Russia and likely in the
225 recruited cohort [23]. With the exception of the Russian BCG cohort, no significant
226 difference was noted when comparing BCG cohorts to each other. Although not
227 significant the Indian BCG cohort had lower reactivity than any of the other cohorts. The
228 reasons for this difference is not clear, but might be due to BCG strain used for
229 vaccination.

230 *3.3. Immunogenicity and immunodominance ranking of the antigen panel*

We next analyzed whether hierarchies of immunogenicity and immunodominance would be similar across the different cohorts. For this purpose, we considered both the relative frequency (e.g. immunodominance) and magnitude of responses (e.g. immunogenicity) detected in the cohorts against each antigen. For these analyses, the USA LTBI cohort that was originally used to define the top antigens was compared to each of the LTBI cohorts.

In all cases, this analysis demonstrated a significant correlation between USA and the other LTBI cohorts in terms of immunodominance (Spearman correlation 0.56 (Brazil) to 0.77 (Central America) $p < 0.0001$ in all cases, (Supplementary Figure 1)). Similarly, a significant correlation (Spearman correlation between 0.58-0.85, $p < 0.0001$) was detected in terms of immunogenicity (Supplementary Figure 2). These results demonstrate that despite significant variability in the overall response magnitude, the ranking of the various antigens is similar, suggesting that human immune recognition of the set of antigens is highly consistent in a diverse set of geographical locations.

Next, we calculated the relative immunodominance and immunogenicity ranking of the 59 antigens for each LTBI cohort, respectively, which was averaged to generate overall rankings. These two variables were highly correlated with each other (Spearman correlation $r = 0.98$, $p < 0.0001$, Fig. 2). The antigens that ranked highly for both parameters were from the cell wall and cell processes, as well as PE/PPE protein categories and included both IGRA proteins, previously known and novel T cell antigens (Fig. 2 and Table 3). Interestingly, some of the current vaccine candidates (Rv0288, Rv1196 and Rv3875) were highly ranked while others (Rv1886c and Rv3804c) were not.

3.4. Antigen selection based on uniformity of recognition

Uniform recognition in different geographical locations may be an important consideration in the selection of candidate vaccine antigens for worldwide use. Accordingly, the average ranking for each of the antigens was plotted against the associated standard deviation (SD), both in terms of immunogenicity and immunodominance (Fig. 3A and B, Table 3). The average ranking and associated standard deviation for each individual antigen is shown in Supplementary Table 2.

Interestingly, the correlation between immunodominance or immunogenicity and the associated SDs was rather poor. This highlights that selection of the most immunogenic antigens will not necessarily ensure uniform geographic and/or ethnic recognition. Nevertheless, the analysis identified 11 (including the IGRA antigen Rv3875) out of the top 20 antigens that ranked high for both immunogenicity and immunodominance, and had also low inter-cohort variation (i.e. were uniformly recognized in the LTBI cohorts; top right quadrant, Fig. 3A and B).

In terms of heterogeneity of responses each antigen were recognized by at least 3 (33%) of the 9 LTBI cohorts tested (Table 4). In fact, 47% were recognized by all of the LTBI cohorts tested and 88% of the antigens were recognized by more than half.

3.5. The relative ranking comparing BCG donors to LTBI

Differential reactivity in LTBI versus BCG vaccinees is a potentially important factor for vaccine and diagnostics development. Accordingly, immunogenicity and immunodominance of the 59 different MTB antigens in each of the BCG cohort were compared to the corresponding LTBI cohort.

A significant correlation was detected for immunodominance in all cases except for Brazil (Spearman correlations between 0.38-0.60 $p < 0.01$; Supplementary Figure 3). Interestingly, in all cases immunogenicity in the BCG and respective LTBI cohorts was significantly correlated (Spearman correlation between 0.27-0.62, $p < 0.05$; Supplementary Figure 4).

The overall relative immunogenicity and immunodominance rankings in LTBI and BCG vaccinees were also significantly correlated ($p < 0.0001$; Fig. 4). This analysis identified antigens with diagnostic potential (LTBI-specific) as well as promising antigen targets for boosting of BCG-induced responses (dominant antigens in both BCG and LTBI groups: Fig. 4 and Table 3). Interestingly, only two of the five antigens tested that are currently in vaccine trials were identified as dominant antigens in both BCG and LTBI cohorts.

4. Discussion

We report a side-by-side comparison of responses to dominant antigens previously identified in LTBI donors from the USA [22] and LTBI and BCG cohorts from multiple geographical locations representative of five continents and nine locations. The overall goal of this study was to define the antigens that are consistently recognized by LTBI donors across a wide-breadth of geographic locations, thus demonstrating their general applicability and potential usefulness worldwide. The analysis led to the identification of a set of 11 consistently recognized MTB antigens, which thereby represent potential candidates for vaccine development.

An important caveat to note within the study is that antigens recognized solely in specific populations are overlooked. The rationale for the employed design is based on the idea that epitopes and antigens of greatest interest are those commonly and uniformly

297 recognized by geographically diverse populations. Furthermore, it is important to note the
298 relatively small sample size for each of the sites, especially since the response frequency
299 is less than 50% for most antigens tested.

300 The study was designed to determine whether the antigens identified in the American
301 cohort were also recognized worldwide in populations that differ in several important
302 aspects. Indeed, the ethnic composition and genetic background of the donor population
303 is diverse between the various sites, and present an important diversity of HLA class II
304 alleles. Further, because of socioeconomic and geographic differences, the clinical
305 presentation, care and treatment of TB disease will vary, leading to potential differences
306 in disease severity and antigen exposure. Finally, differences in circulating MTB strains,
307 and whether childhood BCG vaccination is performed, may also impact antigen
308 recognition[9, 23, 24]. Our results showing remarkably consistent immunodominance and
309 immunogenicity amongst the 9 distinct human populations, suggest that CD4 T cell
310 recognition of MTB antigens is largely independent of the abovementioned covariates.
311 The disparity in correlation seen when comparing immunodominance to immunogenicity
312 highlights the importance of looking at both parameters when evaluating immune
313 responses to a group of antigens. Previous studies, using fewer antigens and whole blood
314 assays, have also seen remarkable similarities in antigen recognition by individuals across
315 diverse sites in Africa [25, 26].

316 This study included 5 of 11 antigens (Rv0288, Rv1196, Rv1886c, Rv3804c and Rv3875)
317 currently included in TB vaccine candidates [27]. Three of these antigens, Rv0288,
318 Rv1196 and Rv3875, were consistently recognized by persons with LTBI, while BCG-
319 vaccinated cohorts also recognized Rv0288 and Rv1196. As expected, the BCG donors

320 did not recognize Rv3875, since it is localized within the RD1 genomic region that is
321 missing in *M. bovis* BCG [28]. Interestingly, the closely related Rv1886c and Rv3804c,
322 together with Rv0129c part of the antigen 85 family, were not consistently recognized
323 despite being highly conserved amongst mycobacterial species and present in all BCG
324 strains [29]. Rv3804c is expressed by the MVA85A vaccine candidate, which was
325 developed as a heterologous boost for BCG [30, 31]. The recent landmark MVA85A trial
326 failed to show any additional efficacy against TB disease or MTB infection above that
327 afforded by BCG in infants [6]. A recent phylogenetic study of 12 BCG strains reported
328 an amino acid substitution in Rv1886c in all strains that affects protein structure and/or
329 stability [24]. This protein instability may underlie the differential T cell recognition
330 patterns of Rv1886c, and the largely homologous Rv3804c, between the different
331 settings, since relative contributions of BCG vaccination, exposure to MTB and
332 environmental mycobacteria will differentially influence T cell responses to these
333 antigens.

334 Our focus on LTBI individuals could also explain the lack of consistent recognition of the
335 antigen 85 family of proteins. It is well known that MTB has adapted to survival in the
336 immune host. Once LTBI has been established, it is thought that the bacteria transform
337 from a replicating metabolically active state to non-replicating persistence with low
338 metabolic activity [32, 33]. This process results in modification of the antigenic repertoire
339 where, for example, expression of Rv0129c, Rv1886c and Rv3804c are down-regulated
340 to very low levels [34-36]. Several human studies have reported differential immune
341 recognition of certain antigens by LTBI individuals as compared to patients with active
342 TB [26, 37-40].

It was recently found that T cell epitopes are hyperconserved, thus suggesting that T cell recognition may be beneficial to MTB and consistency in immune recognition may be undesirable for antigen selection for TB vaccines [41]. Conversely, 90% of individuals with LTBI do not develop TB disease [1, 2] and, importantly, a meta-analysis of risk of progression to active TB suggested that established LTBI provides 79% protection against disease upon re-infection [3]. Both support the idea that the immune response in LTBI, and thus selection of antigens that are immunodominant, is of significant benefit. Under the assumption that consistent T cell recognition of antigens is beneficial for MTB containment by the host, several antigens were identified as promising vaccine candidates. These include previously known T cell antigens (Rv0280, Rv0453, Rv3018c, Rv3020c, Rv3021c, and Rv3136) and the novel T cell antigens, Rv1788 and Rv2853. With the exception of Rv3020c, these antigens are from the large PE/PPE protein category, which are proteins believed to mostly be secreted by MTB and involved in antigenic variation and disease pathogenesis [42]. Rv1196 falls into the same protein category and is included in the M72 vaccine candidate, which has been successfully evaluated in preclinical models [43] and in phase I, I/II, and II trials in high endemic regions with promising results [44-49].

Here we detected high T cell reactivity in the Russian BCG cohort, possibly explained by multiple BCG vaccinations and/or non-tuberculous mycobacteria (NTM) exposure. A genomic comparison of predicted epitopes from MTB and BCG strains identified variations in the number of epitopes present in different BCG strains [24]. Interestingly, BCG Russia had fewer epitopes deleted than other BCG strains, which could also explain the higher and more frequent responses.

Our study underlines the need for an independent genomewide analysis of the antigens recognized following BCG vaccination. The majority (9 of 11) of the antigens consistently recognized in our study were recognized by both LTBI and BCG donors. Since we studied adolescent and adult populations who received BCG during childhood, and who likely have been exposed to NTM and/or MTB, it is not possible to infer how responses to different antigens are influenced by these different sources of sensitization. T cell responses to dominant antigens primed following BCG vaccination are therefore not yet known, which, along with antigens dominantly recognized in natural MTB infection, is important for the design of BCG prime-boosting vaccines. It is possible that differences in the immune repertoire between pathogenic infection and BCG may be associated with protection and vaccine targeting. However, at this point this is conjectural. An important caveat to note is that BCG, which contains these antigens, is not protective against the main pulmonary form of TB in adults. While the tested antigen targets may indeed be potentially protective, a suitable, yet unknown vaccine formulation remains the limiting factor.

While reactivity of patients from different geographical locations might differ in terms of actual epitopes recognized, the overall reactivity against each pool (protein) was anticipated to reflect the pattern of immunodominance at the antigen level. Due to the limited number of available cells the peptide pools were not deconvoluted to address whether different individual epitopes were recognized amongst the different cohorts.

Taken together, the results presented herein provide insight into the relative immunogenicity in LTBI cohorts of existing and novel vaccine candidates, across a variety of geographical locations with genetically, and environmentally diverse

389 populations. This type of comparative analysis is especially relevant for selection of new
390 TB vaccine candidates and also provides new information relevant to current candidates
391 in ongoing clinical trials.

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525 **Table 1. List of antigens**

Category	Rv no.	Synonym(s) ^a	Antigen classification ^b	Category	Rv no.	Synonym(s)	Antigen classification
Cell wall and cell processes	Rv0287	EsxG/TB9.8	Prev. known	Intermediary metabolism and respiration	Rv0291	-	Novel
	Rv0288	EsxH/CFP7/TB10.4	Vaccine		Rv0294	-	Novel
	Rv0289	-	Novel		Rv2874	-	Novel
	Rv0290	-	Novel		Rv2996c	serA/serA1	Novel
	Rv0292	-	Novel		Rv3025c	nifS/iscS	Novel
	Rv0985c	-	Novel	Lipid metabolism	Rv0129c	85C/fbpC/mpt45	Prev. known
	Rv0987	-	Novel		Rv1886c	85B/fbpB/mpt59	Vaccine
	Rv1198	EsxL/ES6_4/MTB9.9C	Prev. known	PE/PPE	Rv3804c	85A/fbpA/mpt44	Vaccine
	Rv2875	mpt70/mpb70	Prev. known		Rv0256c	PPE2	Prev. known
	Rv3019c	EsxR/ES6_9/TB10.3	Prev. known		Rv0280	PPE3	Prev. known
	Rv3020c	EsxS/PE28	Prev. known		Rv0453	PPE11	Prev. known
	Rv3330	-	Novel		Rv1172c	PE12	Novel
	Rv3615c	-	Prev. known		Rv1195	PE13	Novel
	Rv3874	EsxB/CFP10	IGRA		Rv1196	PPE18/mtb39a	Vaccine
	Rv3875	EsxA/ESAT-6	IGRA/Vaccine		Rv1387	PPE20	Prev. known
	Rv3876	-	Novel		Rv1705c	PPE22	Prev. known
	Rv0293c	-	Novel		Rv1788	PE18	Novel
Conserved hypotheticals	Rv0298	-	Novel		Rv1789	PPE26	Prev. known
	Rv0299	-	Novel		Rv1791	PE19	Novel
	Rv0690c	-	Novel		Rv1802	PPE30	Prev. known
	Rv1366	-	Novel		Rv1808	PPE32	Prev. known
	Rv2024c	-	Novel		Rv2123	PPE37	Prev. known
	Rv2823c	-	Prev. known		Rv2853	PE_PGRS48	Novel
	Rv3015c	-	Prev. known		Rv3018c	PPE46	Prev. known
Information pathways	Rv1317c	-	Novel		Rv3021c	PPE47	Prev. known
	Rv3012c	-	Novel		Rv3022c	PPE48	Novel
	Rv3024c	-	Novel		Rv3135	PPE50	Novel
Insertion sequences and phages	Rv1199c	-	Prev. known	Virulence, detoxification, adaptation	Rv3136	PPE51	Prev. known
	Rv3023c	-	Prev. known		Rv2031c	hspX	Prev. known
					Rv3418c	groES/cpn10/mpt57	Prev. known

526 ^aSynonyms as indicated in TubercuList [50].

527 ^bAntigen classification denotes antigens previously known as T cell antigens (Prev.

528 known) or novel T cell antigens (Novel) based on previously published work [22],

529 antigens contained in IGRA (Interferon gamma release assay) and antigens currently

530 included in vaccines in clinical trials (Vaccine).

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Table 2. Patient cohorts and characteristics

Location	TB Incidence per 100,000 population ^a	Childhood BCG vaccination	Cohort	No. individuals ^b	Age Median (range)	Gender M/F (%)
USA	3.3 (3.1-3.7)	No	LTBI	25	44 (20-65)	64/36
			BCG	12	32 (20-58)	58/42
			MTB naïve	30	31 (23-60)	73/27
India	171 (162-184)	Yes	LTBI	9	27 (20-57)	100/0
			BCG	11	N/A	N/A
Peru	124 (110-142)	Yes	LTBI	8	36 (20-49)	75/25
Russia	89 (82-100)	Yes	LTBI	9	41 (24-70)	17/83
			BCG	13	37 (23-60)	39/61
South Africa	860 (776-980)	Yes	LTBI	25	15 (13-18)	36/64
			BCG	23	16 (12-18)	43/57
Central America ^c	see footnote ^d	Yes	LTBI	20	N/A	N/A
			BCG	23	N/A	N/A
Brazil	46 (41-52)	Yes	LTBI	14	32 (18-55)	55/45
			BCG	25	30 (19-58)	31/69
Italy	5.7 (5.5-6.4)	No	LTBI	8	32 (14-63)	37/63
Uganda	166 (149-193)	Yes	LTBI	10	N/A	N/A
Average (range)				17 (8-30)	31 (12-63)	52/48

^aIncidence includes HIV+TB. Ranges in parenthesis represent uncertainty intervals

(WHO, March 2015, www.who.int/tb/data)

^bIndicates the number of different individuals each peptide pool was tested in

^cCentral America includes Jamaica, Haiti, Puerto Rico and Dominican Republic

^dJamaica: 6.5 (5.9-7.4), Haiti: 206 (179-231), Puerto Rico: 1.6 (1.4-1.8), Dominican

Republic: 60 (54-68)

Table 3. Antigens frequently recognized in all LTBI cohorts

Antigen characteristic	Rv#	LTBI Average ranking ($\pm$ SD)		Diagnostic	Uniform recognition in LTBI ^a	Shared dominant with BCG ^b
		Magnitude	Response frequency			
IGRA	Rv3874	7 ($\pm$ 12)	5 ($\pm$ 8)	Yes		
Vaccine / IGRA	Rv3875	9 ($\pm$ 7)	11 ($\pm$ 5)	Yes	Yes	
Prev. known	Rv3136	16 ($\pm$ 6)	13 ($\pm$ 10)		Yes	
Novel	Rv1788	15 ($\pm$ 6)	16 ($\pm$ 7)		Yes	Yes
Novel	Rv2853	20 ($\pm$ 8)	13 ($\pm$ 8)		Yes	Yes
Prev. known	Rv0280	14 ($\pm$ 6)	11 ($\pm$ 6)		Yes	Yes
Prev. known	Rv0453	11 ($\pm$ 4)	9 ($\pm$ 6)		Yes	Yes
Prev. known	Rv3018c	11 ($\pm$ 5)	9 ($\pm$ 8)		Yes	Yes
Prev. known	Rv3020c	10 ($\pm$ 8)	9 ($\pm$ 6)		Yes	Yes
Prev. known	Rv3021c	13 ($\pm$ 7)	12 ($\pm$ 10)		Yes	Yes
Vaccine	Rv0288	4 ($\pm$ 7)	7 ($\pm$ 7)		Yes	Yes
Vaccine	Rv1196	18 ($\pm$ 7)	14 ($\pm$ 7)		Yes	Yes
Novel	Rv1195	17 ($\pm$ 10)	14 ($\pm$ 9)			Yes
Prev. known	Rv0256c	14 ($\pm$ 11)	8 ($\pm$ 6)			Yes
Prev. known	Rv0287	13 ($\pm$ 17)	12 ($\pm$ 12)			Yes
Prev. known	Rv2123	16 ($\pm$ 12)	10 ($\pm$ 8)			Yes
Prev. known	Rv3019c	10 ($\pm$ 15)	11 ($\pm$ 14)			Yes
Novel	Rv1791	22 ($\pm$ 6)	17 ($\pm$ 10)			
Prev. known	Rv1387	13 ($\pm$ 11)	9 ($\pm$ 8)			
Prev. known	Rv3615c	21 ($\pm$ 20)	19 ($\pm$ 16)			

^aAntigens identified as uniformly recognized in all LTBI cohorts studied (Figure 3A and B).

^bAntigens identified as shared dominant between LTBI and BCG cohorts (Figure 4A and B).

547 **Table 4. Recognition of antigens in all LTBI cohorts tested**

Rv no.	LTBI cohorts responding (%) ^a	Antigen classification	Rv no.	LTBI cohorts responding (%) ^a	Antigen classification
Rv0129c	89%	Prev. known	Rv1802	100%	Prev. known
Rv0256c	100%	Prev. known	Rv1808	89%	Prev. known
Rv0280	100%	Prev. known	Rv1886c	100%	Vaccine
Rv0287	100%	Prev. known	Rv2024c	100%	Novel
Rv0288	100%	Vaccine	Rv2031c	100%	Prev. known
Rv0289	56%	Novel	Rv2123	100%	Prev. known
Rv0290	67%	Novel	Rv2823c	44%	Prev. known
Rv0291	56%	Novel	Rv2853	100%	Novel
Rv0292	78%	Novel	Rv2874	44%	Novel
Rv0293c	33%	Novel	Rv2875	100%	Prev. known
Rv0294	67%	Novel	Rv2996c	100%	Novel
Rv0298	44%	Novel	Rv3012c	78%	Novel
Rv0299	67%	Novel	Rv3015c	33%	Prev. known
Rv0453	100%	Prev. known	Rv3018c	100%	Prev. known
Rv0690c	78%	Novel	Rv3019c	100%	Prev. known
Rv0985c	67%	Novel	Rv3020c	100%	Prev. known
Rv0987	67%	Novel	Rv3021c	100%	Prev. known
Rv1172c	89%	Novel	Rv3022c	78%	Novel
Rv1195	100%	Novel	Rv3023c	56%	Prev. known
Rv1196	100%	Vaccine	Rv3024c	44%	Novel
Rv1198	100%	Prev. known	Rv3025c	67%	Novel
Rv1199c	78%	Prev. known	Rv3135	89%	Novel
Rv1317c	100%	Novel	Rv3136	100%	Prev. known
Rv1366	67%	Novel	Rv3330	78%	Novel
Rv1387	100%	Prev. known	Rv3418c	67%	Prev. known
Rv1705c	78%	Prev. known	Rv3615c	100%	Prev. known
Rv1788	100%	Novel	Rv3804c	67%	Vaccine
Rv1789	100%	Prev. known	Rv3874	100%	IGRA
Rv1791	89%	Novel	Rv3875	100%	IGRA/Vaccine
			Rv3876	44%	Novel

^a % of the 9 LTBI cohorts tested that respond to each antigen

Figure Legends:

Figure 1. Reactivity to selected antigens in all tested cohorts. Reactivity as total SFC/number of tested donors for each individual cohort, LTBI (grey bars), BCG (white bars) and MTB naïve (black bar, third from left). Median $\pm$ interquartile range is shown. Two-tailed Mann-Whitney test, ****, $p < 0.0001$, ***, $p < 0.001$, *, $p < 0.05$ and ns, no significant difference. Dashed lines indicate comparison between USA BCG and other BCG cohorts, solid lines indicate comparison between LTBI versus LTBI or LTBI versus BCG.

Figure 2. Correlation between immunodominance and immunogenicity. Correlation of average ranking of response frequency (x-axis) versus average ranking of magnitude (y-axis) calculated from all 9 LTBI cohorts. Previously known T cell antigens (grey dots), antigens currently in vaccine trials (blue dots), IGRA antigens (red dots) and novel T cell antigens (black dots). Box indicates top 20 antigens of the 59 selected. Correlation is indicated by Spearman r and associated two-tailed p -value.

Figure 3. Uniformity of antigen recognition. Correlation of average ranking of magnitude (A) or response frequency (B) (y-axis) versus standard deviation of ranking (x-axis). Top 20 antigens are above the horizontal dashed line. Uniform recognition SD < 10 and heterogeneous recognition SD > 10 indicated by vertical dashed line. Previously known T cell antigens (grey dots), antigens currently in vaccine trials (blue dots), IGRA antigens (red dots) and novel T cell antigens (black dots). Correlation is indicated by Spearman r and associated two-tailed p -value, ns, no significant correlation.

Figure 4. Shared dominant antigen recognition in BCG versus LTBI. Correlation of average ranking of response frequency (A) or magnitude (B) in BCG (y-axis) versus average ranking in LTBI (x-axis). Shared dominant antigens (average ranking > 20) are indicated by box (small dashes) and LTBI-specific antigens are indicated by dashed box. Previously known T cell antigens (grey dots), antigens currently in vaccine trials (blue dots), IGRA antigens (red dots) and novel T cell antigens (black dots). Correlation is indicated by Spearman r and associated two-tailed p -value.

Spearman $r=0.98$
 $p<0.0001$

A

B

A

B