TITeL:
The secreted protein Rv1860 of Mycobacterium tuberculosis stimulates human polyfunctional CD8⁺ T cells.

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Running title: Human CD8⁺ T cells specific to MTB Rv1860 protein.

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We previously reported that Rv1860 protein from *Mycobacterium tuberculosis* (MTB) stimulated CD4+ and CD8+ T cells secreting IFN-\(\gamma\) in healthy PPD-positive individuals and protected guinea pigs immunized with a DNA vaccine and a recombinant poxvirus expressing Rv1860 from a challenge with virulent MTB. We now show Rv1860-specific polyfunctional T (PFT) cell responses in blood of healthy latently MTB-infected individuals dominated by CD8+ T cells, using a panel of 32 overlapping peptides spanning the length of Rv1860. Multiple subsets of CD8+ PFT cells were significantly more numerous in healthy latently infected volunteers (HV) than in TB patients (PAT). The responses of PBMCs from TB patients to peptides of Rv1860 were dominated by TNF-\(\alpha\) and IL-10 secretion, the former coming predominantly from non T cell sources. Notably, the pattern of T cell response to Rv1860 was distinctly different from the widely studied MTB antigens ESAT-6, CFP-10, Ag85A and B which elicited a CD4+ T cell-dominated response as previously reported in other cohorts. We further identified a peptide spanning amino acids 21 to 39 of the Rv1860 protein with potential to distinguish latent TB infection from disease due to its ability to stimulate differential cytokine signatures in HV and PAT. We suggest that a TB vaccine carrying these and other CD8+ T cell stimulating antigens has the potential to prevent progression of latent MTB infection to TB disease.
INTRODUCTION

Although only 5-10% of those infected with Mycobacterium tuberculosis (MTB) progress to disease depending upon their HIV status, the annual incidence of new cases of tuberculosis runs into millions due to more than two billion infected individuals worldwide (1). The life time risk of an MTB-infected HIV-negative individual progressing to TB disease is a mere 10%, testifying to the success of immune mechanisms in containing the tubercle bacteria and maintaining them in a persistent state (2). The prophylactic management and control of tuberculosis has relied for the past several decades on the live attenuated strain of Mycobacterium bovis, Bacille Calmette-Guerin or BCG which showed variable efficacy in trials worldwide in the post second world war era (3). However, despite extensive investigations into the immunology of tuberculosis, the requirements for protective immunity in the host and the bacterial components that trigger such protective immune responses are poorly understood (4-6), which in turn has stalled the development of efficacious new vaccines.

The need to find improved vaccines for TB has become all the more pressing following the discouraging results from human phase 2b trials of MVA85A (7-10), the most advanced among the twelve candidate vaccines that are undergoing human clinical trials, despite encouraging results in animal models. Development of effective TB vaccines is constrained by lack of immune correlates of protection in humans and reliable animal models. While interferon gamma (IFN-γ) was for long believed to be a correlate of protective immune responses against MTB based primarily on the increased susceptibility to TB of mice lacking IFN-γ (4, 11), several recent publications have demonstrated the absence of such a correlation (12-17). Polyfunctional T (PFT) cells capable of simultaneously secreting multiple TH1 cytokines were shown to confer protection against TB in the mouse model (18, 19); however, studies in humans revealed conflicting results. Several studies showed that human CD4+ PFT
specific to the secreted antigens ESAT-6, CFP-10, 16kDa protein, Ag85A and Ag85B correlated with bacterial antigen burden (20-23); not only did these responses wane with chemotherapy (21, 23), but T cell responses to Rv3873 and Rv3878 from within the RD1 locus in fact predicted progression to active disease (24). These observations led to the suggestion that PFT cells may at best serve as a useful biomarker of active tuberculosis and cure and may have no role in protection. However in a South African cohort, PFT were more numerous in healthy latently infected individuals compared to TB patients and six months of chemotherapy led to a resurgence of PFT (25). The contrasting results were attributed to differences in methodology, antigens tested and cohort characteristics. The majority of these investigations with the exception of the S. African study (25) queried only CD4+ and did not include CD8+ T cell responses. Clearly, correlates of protective immunity against TB in humans which are useful in reliably identifying potential vaccine candidates are yet to be discovered.

CD8+ T cells are increasingly being recognized for their contribution to TB control in mice (26-28), non-human primates (29) and humans (30-32). The increased susceptibility to TB of rheumatoid arthritis and ankylosing spondylitis patients under infliximab treatment to neutralize TNF-α was shown to be due to loss of the CD8+ TEMRA cell subset which displayed antimicrobial activity against MTB (33). This CD8+ TEMRA subset was in fact reported to be deficient in TB patients, in contrast to latently infected healthy controls (34). However, very few antigens of MTB with ability to stimulate human CD8+ T cells have been identified (30). We initially identified Rv1860 from a screen of twenty four recombinant proteins obtained from a genomic DNA expression library of MTB (35), for its ability to elicit proliferation and IFN-γ secretion from both CD4+ and CD8+ T cells of healthy latently infected individuals and for its ability to protect guinea pigs against a challenge with virulent field strain of MTB (36 and unpublished observations). Rv1860 is a well characterized secreted glycoprotein of MTB.
M. bovis-BCG; the BCG homologue was first identified as a proline-rich culture filtrate protein (37, 38) immunogenic in infected guinea pigs. Elegant analysis of the glycosylation moieties of the purified 45 kDa culture filtrate-derived MTB Rv1860 protein revealed that the threonine residues at positions 10, 18, 27 and 277 were glycosylated and the attached carbohydrates were single mannose, mannobiose, or mannotriose units strung together by α1-2 linkages (39, 40). We earlier reported that the glycosylated form of Rv1860 inhibited T cell polarizing functions of mouse bone marrow derived dendritic cells (41). In this study we report that peptides derived from the sequence of Rv1860 stimulated human PFT cell responses which were dominated by CD8+ T cells in contrast to the CD4+ T cell-dominated response to the well-studied antigens ESAT-6, CFP-10, Ag85A and B. Several subsets of Rv1860-specific polyfunctional CD4+ and CD8+ T cells were significantly more numerous in HV than in PAT, in contrast to the reported superior CD4+ T cell response to ESAT-6, CFP-10, Ag85A and B in TB patients (21-23, 42). Our results suggest that Rv1860, by virtue of its capacity to stimulate CD8+ T cells may serve as a useful candidate for inclusion in a TB vaccine with potential to prevent reactivation of latent MTB infections which accounts for up to 80% of TB cases in some countries (43). We also identified a peptide spanning amino acids 21 to 39 of the Rv1860 protein sequence that gave rise to a mutually exclusive proliferation and cytokine signature from stimulated PBMC of HV and PAT, revealing potential for its use in evaluating new therapeutics and for monitoring progression from TB latency to disease.

MATERIALS AND METHODS

Study subjects. Individuals presenting at the outpatient department of M.S. Ramiah Hospital, Bangalore, India and diagnosed with pulmonary tuberculosis based on presence of culturable acid-fast bacilli in sputum were recruited to participate in this study and included 17 males (mean age, 47 ± 8 years, mean weight, 54 ± 6 Kg) and 3 females (mean age, 37 ± 6
years, mean weight, 45 ± 5 Kg; Supplementary Table S2). Diagnosis of pulmonary TB was routinely confirmed by sputum culture on Lowenstein-Jensen slants at the National Tuberculosis Institute. TB patients (PAT) were bled once before chemotherapy was initiated. 30 healthy PPD-positive individuals, judged to be latently-infected with MTB (HV) based on release of \( \geq 0.7 \) IU of IFN-\( \gamma \) (1 IU of IFN-\( \gamma \) = 50 pg/ml) following 24 hrs. stimulation of whole blood with a pool of peptides covering the amino acid sequences of ESAT-6 and CFP-10, with minimum induration reading of 9 mm in the Mantoux test [1 tuberculin unit of RT-23 from Statens Serum Institute, Copenhagen (44)] were drawn from Indian Institute of Science and National Tuberculosis Institute, Bangalore, India. Unstimulated control blood samples did not produce detectable IFN-\( \gamma \). 28 individuals completed the study (HV; 19 males; mean age, 41 ± 7 years, mean weight, 57 ± 7 Kg and 9 females; mean age, 39 ± 6 years, mean weight, 47 ± 4 Kg; Supplementary Table S2). Exclusion criteria comprised fever, malaise, clinical symptoms of TB (verified by chest roentgenogram and sputum smears for acid-fast bacilli) and positive tests for HIV and hepatitis B. A group of 5 healthy purified protein derivative (PPD)-negative (induration diameter below 5 mm) volunteers with no IFN-\( \gamma \) response to ESAT-6 + CFP-10 peptides were available as controls. This study was approved by the Institutional Human Ethics Committees of IISc and the Ethical Review Board of MSR hospital. Signed informed consent was obtained from all individuals prior to enrolment in the study after the purpose and consequences of the study were fully explained.

Isolation of PBMC and T cell proliferation assays. 1.5 \( \times \) 10^5 peripheral blood mononuclear cells (PBMC) isolated from heparinized blood by the Ficoll-Hypaque density gradient method (45) were cultured per well in triplicate with peptides at a concentration of 2 \( \mu \)g/ml for 5 d. Stimulation with the mitogen phytohemagglutinin A (PHA, Sigma, St. Louis, MO at 10 \( \mu \)g/ml) was carried out for 3 d. Lymphocyte proliferation was measured by the incorporation of \(^3\)H-thymidine (NEN, Dupont, Boston, MA) added at 0.5 \( \mu \)Ci per well for the
final 18-20 hours of culture. Incorporated radiolabel in harvested cells was quantitated and
the proliferative response was expressed as stimulation index [SI = (mean counts per minute
(46) of test antigen-stimulated cultures) / (mean counts per minute of unstimulated cultures in
triplicate wells)] as described earlier (36).

A positive response to the Rv1860 peptides was scored on the criterion that the SI was
equal to or greater than 2.5 and the average cpm obtained on stimulation with recombinant
antigen was 500 or more along with a significant increase in cpm induced by the test antigen
over unstimulated control as ascertained by the Student t test. We recovered comparable
numbers (1-2 × 10^6 PBMC per ml) of PBMC from blood of TB patients and PPD-positive
healthy donors.

**Antigens:** Peptides of 20 mer length spanning the entire length of the Rv1860
protein and overlapping with each other by 10 amino acids were obtained from Genemed
Synthesis Inc. TX, USA (Supplementary Table S1). ESAT-6 (NR-14868), CFP-10 (NR-
14869), Ag85A (NR-14871) and Ag85B (NR-14870) were obtained from the Biodefense and
Emerging Infections (BEI) Resources of the American Type Culture Collection.

**Quantitation of cytokines by ELISA.** IFN-γ, IL-2, IL-10 and TNF-α were
quantitated by ELISA using commercially available antibody pairs (R&D systems) according
to manufacturer’s instructions. IFN-γ was measured in 72 h PBMC culture supernatants while
all other cytokines were measured using 48 h culture supernatants. The lower limit of
detection for all the cytokines was 15 pg/ml.

**Intracellular cytokine detection.** Whole, heparinized (sodium heparin) blood was
diluted 1:1 with RPMI 1640 and 1 ml aliquots were stimulated with peptides, each at a
centration of 2.5 µg/ml in 13-ml tubes for 18 hours as described (47). Data were acquired
on a BD-FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA). For details of protocol and analysis, refer to Supplementary Methods.

Small lymphocytes were collected by gating on forward versus side scatter and then gated on singlet cells, followed by CD3^high T lymphocytes (Supplementary Figure S1A). We excluded dead cells (verified by separate live/dead staining to be less than 0.01% of CD3^+ T cells) by avoiding cells high on SSC and those close to the Y axis, which was feasible as all staining was done on fresh PBMC. CD3-high/CD8^- cells were considered as CD4^+ T cells.

For each analysis, a minimum of 100,000 CD4^-/CD8^- T cell subsets were acquired and data was analyzed using FlowJo, (Treestar) PESTLE and SPICE (Mario Roederer, NIH, USA) software. Positive staining was affirmed by comparing the dot-plots of antibody-stained unstimulated and antigen-stimulated cells and a minimum number of 50 events was used as cut-off for positive response. Gates were positioned to ensure that the percentages of fully stained unstimulated cells were \( \leq 0.01 \) % of total CD4^- or CD8^- T cells for IFN-\( \gamma \), TNF-\( \alpha \) and IL-2 secreting T cells, while it was \( \leq 0.05 \) for MIP-1\( \beta \) secreting T cells.

**HLA Restriction analysis.** Alleles of *HLA*-\*A*, *HLA*-\*B* and *HLA*-\*DRB1* loci were studied using Polymerase chain reaction followed by hybridization with sequence specific oligonucleotide probes (SSOPs) as described earlier (48) using a bead-based technology (Luminex, Austin, Tx) following manufacturer’s instructions (Labtype SSO kit from One Lambda, Canoga Park, USA). The latest nomenclature for the *HLA* system was used to designate the alleles of the three loci studied (49). Acquired data were analyzed using Labtype software (One Lambda) for analysis of HLA alleles.

**Statistical analysis.** Results of lymphoproliferative assays with human PBMC are represented as mean SI values from triplicate wells of a 96 well plate. Results of ELISAs are expressed as pg/ml, means of duplicate wells. Cytokine (IL-2, IL-6, IL-10, IFN-\( \gamma \) and TNF-\( \alpha \)) and S.I. values (Figure 2) were compared between HV and PAT using the Students t test and
the Mann Whitney test using the GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA). Bonferroni’s correction applied for these six comparisons, gave a critical value for each test of $0.05/6 = 0.0083$. Where significant differences are shown, both tests confirmed this result and the more conservative values are indicated. Age, weight and sex trends for SI and cytokine responses were analysed using logistic regression and were found not to significantly influence the outcome. Age and weight of males and females were compared using the 2 tailed Student’s t test (Supplementary Table S2). For comparing percentages of polyfunctional T cell subsets between HV and PAT, the non-parametric Wilcoxon test available within SPICE was used on log transformed data. We then applied Bonferroni’s correction for multiple comparisons between HV and PAT or between CD4+ and CD8+ T cells, of the fifteen different CD4+ or CD8+ T cell subsets secreting different combinations of the four different cytokines IL-2, IFN-γ, MIP-1β and TNF-α, giving a calculated critical value for each individual comparison of $0.05/15 = 0.0033$; p values below this were considered significant. For analysis of association of HLA alleles and cytokine gene polymorphisms, Fisher’s exact test or chi-square test were used wherever suitable. The central limit theorem for proportions and the Fisher’s exact test were used to compare proportions of men and women between HV and PAT groups (Supplementary Table S2) as well as to compare the frequency of specific HLA alleles between the two groups.
Rv1860 peptides stimulate recall T cell responses in MTB-infected individuals. In our initial screen of 24 recombinant MTB proteins, we observed proliferation and IFN-γ secretion by human PBMC in response to Rv1860 (36). We therefore investigated in detail the proliferative and cytokine response of human PBMC to a panel of 32 20mer peptides spanning the complete sequence of Rv1860 (shown at the bottom of Supplementary Table S1) and overlapping by 10 amino acids (Supplementary Table S1). 28 healthy latently infected individuals (HV; Supplementary Table S2) out of the recruited 30 volunteers were tested for proliferative response of PBMC to the 32 overlapping peptides from Rv1860 (Figure 1A). 2 volunteers did not show proliferative response to any of the peptides. One individual responded to only 3 peptides while 2 responded to only 4 peptides. PBMC from all other latently infected healthy individuals showed proliferative response to multiple peptides while one individual unusually responded to 23 out of the 32 overlapping peptides from Rv1860. Of the total 203 proliferative responses obtained, the majority had SI values between 2.5 and 5, while 38 responses had SI values between 5 and 20, 13 of which were to peptide 1803. Out of 20 TB patients (PAT; Supplementary Table S2 and Figure 1B) tested, 16 responded by proliferation of PBMC to at least 3 peptides each. Only two patients had SI values between 5 and 10 to eight different peptides while all other responses fell between 2.5 and 4.5; all 20 patients, however produced cytokines. Figure 1 summarises the frequency of proliferative responses to each peptide among the 28 HV and 20 PAT tested. PBMC from all volunteers secreted substantial levels (between 100 and 2000 pg/ml) of one or more cytokines IFN-γ, TNF-α, IL-10 or IL-6 in response to at least one peptide, measured by ELISA; the cytokine profiles in patients were dominated by TNF-α and IL-10 (Figure 2).

Peptides 1803, 1821 and 1826 (Supplementary Table S1) elicited the largest frequency of proliferative responses in both HV and PAT (Figure 1). Analysis of the cytokine
responses to these 3 peptides revealed a distinct pattern of response to each peptide (Figure 2). While 1803 stimulated significantly greater proliferative (circles, 5.511 ± 0.7699 for HV and squares, 2.390 ± 0.4215 for PAT; p=0.0003) and IFN-γ responses in HV (336.6 ± 90.43 for HV and 77.78 ± 33.72 for PAT; p=0.001), it elicited significantly more TNF-α (245.2 ± 31.92 for HV and 954.7 ± 220.6 for PAT; p=0.003) and IL-10 (232.7 ± 59.02 for HV and 680.9 ± 105.8 for PAT; p=0.0006) responses in PAT (Figure 2A). Peptide 1821 stimulated significantly more TNF-α secretion from PAT (Figure 2B). In response to 1826, however, HV secreted significantly greater levels of IFN-γ while PAT secreted significantly greater amounts of TNF-α and IL-10 (Figure 2C). PPD-negative controls did not secrete measurable levels of cytokines although we detected low levels of IL-10 in one sample in response to peptide 1803 (data not shown). IL-2 and IL-6 did not display significant differences between HV and PAT (data not shown). Thus, response to peptide 1803 and 1826 were capable of distinguishing latently infected healthy individuals from TB patients.

**Rv1860 peptides stimulate human polyfunctional T cells.** We then queried the subset of T cells in HV and PAT, responsible for the secretion of the cytokines observed above using the gating strategy for flow cytometry shown in Supplementary Figure S1A. A mixture of the three immunodominant peptides 1803, 1821 and 1826 from Rv1860 stimulated robust PFT cells secreting all combinations of IFN-γ, IL-2, TNF-α and MIP-1β, in HV (Supplementary Figure S1B and Figure 3) and also in PAT (Figure 4). The striking feature of the Rv1860 T cell response was the dominant role for CD8+ T cells (right bar in each pair) secreting a variety of cytokine combinations both in HV (Figure 3) and PAT (Figure 4), with several subsets reaching statistical significance. After Bonferroni’s correction, while the difference in seven different T cell subsets remained statistically significant in HV (symbol * in Figure 3), only the subset secreting a combination of IL-2, IFN-γ, and TNF-α showed significantly greater frequency of CD4+ T cells in PAT (symbol * in Figure 4), perhaps due to the overall
lower responses observed. The pie chart (Figure 3) revealed that in HV, Rv1860-specific memory T cells secreting IL-2 alone made up a greater proportion of CD8+ populations, a feature missing in PAT (Figure 4). This feature is also evident in the pie chart comparing the CD8+ T cell subsets between HV and PAT (Supplementary Figure S3). Single TNF-α and IFN-γ secretors made up a larger proportion of CD8+ T cells in PAT than in HV while single IL-2 and MIP-1β secretors made up a larger proportion of CD8+ T cells in HV than in PAT (Pie chart in Supplementary Figure S3). However, with the exception of IFN-γ, the frequencies of the other three single cytokine-secreting CD8+ T cells along with those secreting a combination of IL-2 and MIP-1β were significantly more in HV (symbol * in Supplementary Figure S3). Overall, several cytokine secreting combinations were significantly over represented in HV compared to PAT in both CD4+ and CD8+ T cell subsets (Supplementary Figures S2 and S3, respectively) and support our previously reported predominance of T cell responses specific to Rv1860 in healthy latently infected individuals compared to TB patients (36). It is to be noted that the high levels of TNF-α in stimulated PBMC culture supernatants from PAT that was detected by ELISA was not borne out by the flow cytometric analysis of T cells, pointing to myeloid cells, presumably neutrophils and monocyte/macrophages as the major source of this inflammatory cytokine in PAT, corroborating the neutrophil-dependent inflammatory signature reported in TB patients (50).

As a comparison, we also carried out PFT analysis in our cohort in response to the well-studied antigens ESAT-6, CFP-10, Ag85A and Ag85B of MTB. As evident from Figure 5 and Supplementary Figures S4, S5 and S6, the latter two showing average values from all four antigens, for the subsets where cytokine secreting pattern differed significantly between CD4+ and CD8+ T cells, both T cell types were equally represented, in contrast to the CD8+ T cell-dominated response to Rv1860 peptides. However, following Bonferroni’s correction for multiple comparisons, only three of these p values remained significant.
**HLA alleles associated with response to Rv1860.** We carried out typing for the alleles of HLA-A, HLA-B and HLA-DRB1 loci for all HV and PAT (Supplementary Tables S3A and S3B). We found HLA-DRB1-*15:02 allele in 9 of the 30 HV (0.3) initially recruited for the study while only one of the 20 PAT (0.05) carried this allele (highlighted in Supplementary Tables S3A and S3B; p=0.03). No data on epitopes from Rv1860, validated by immunoassays for binding to HLA alleles were available in the immune epitope database (http://www.iedb.org/); however, there were several epitopes predicted to bind several HLA class I and II alleles with low IC50 (nM) values. We additionally used the epitope prediction softwares SYFPEITHI and BIMAS to predict epitopes binding to HLA class I and II alleles from the 3 immunodominant peptides 1803, 1821 and 1826 as well as full length Rv1860 protein. All three prediction methods identified the same epitopes from both 20 mer peptide and full Rv1860 protein sequences and the predictions broadly concurred with one another. As expected, additional 15 mer MHC class II epitopes were predicted from the full length protein that were not available in the peptides since the overlap between any two consecutive peptides was only 10 aminoacids.

We compared the predicted scores for HLA allele binding to epitopes from the 3 immunodominant peptides to the experimentally detected PBMC responses. We saw no correlation with proliferation (S.I. values) but strong correlations were evident with secreted cytokines. SYFPEITHI scores below 20 did not show strong correlations to experimentally observed responses and were not used for analysis. While MHC molecules are extremely polymorphic, alleles with similar peptide binding pockets may be clustered in one group called supertypes, such that HLA molecules in one supertype may present similar peptides (51). The A02 supertype (51) correlated with strong response to peptide 1803 (amino acids 21 to 40 of Rv1860), to which a very high proportion (22/28 HV) responded as shown by either SI and/or cytokine secretion. The decamer AMASALSVT (amino acid position 25-34)
22 to 31 of Rv1860) with predicted SYFPEITHI score of 30 for binding to HLA-A*02:01 along with other nonamers overlapping with this sequence and predicted to bind this HLA allele with scores of 21 and 22, seems to have contributed to this immunodominant response. Additionally, HLA-B*51:01 (B07 supertype) was predicted to bind the nonameric M A S A S L V T V in peptide 1803 (amino acid position 23 to 31 of Rv1860) with score of 25 along with 2 other epitopes with score of 21. Nineteen of the 22 HV for whom complete data from proliferation and cytokine ELISA to each of the 32 individual peptides, along with ICC data for the 3 immunodominant peptides were available, responded to peptide 1803. Ten of these nineteen HV carried the HLA-A02 supertype and thirteen HV carried the HLA-B07 supertype while eight HV carried both supertype alleles. The levels of cytokines IFN-γ (400 to 2900 pg/ml) and TNF-α (200-700 pg/ml) were 7 to 15 fold higher in the 10 individuals who carried a HLA-A02 supertype allele, compared to those with HLA-B*51:01 and other B07 supertype alleles (80 to 200 pg/ml IFN-γ and 50-100 pg/ml TNF-α). Interestingly, BIMAS scores for HLA A02 binding were between 5 and 10 while that for HLA-B*51:01 binding were between 110 and 170 for nonamers from peptide 1803.

LAT3 with HLA-A*02:11; 68:01 and HLA-B*51:01; 40:04 (Supplementary Table S3A) responded strongly to peptide 1803 and weakly to peptide 1826, with no response whatsoever to any other peptide, confirming that HLA-A02 supertype restricted strong response to peptide 1803. LAT4 with HLA- A*1101 (A03 supertype) but lacking both A02 and B07 supertype alleles had weak response to peptide 1803, in keeping with the predicted score of 20 for HLA-A*11:01 binding to decamer epitope A S L V T V A V P A (aa 26 to 35 of Rv1860) within peptide 1803. The above predictions matched with alleles predicted by IEDB; all top 20 alleles with percentile rank 0.2 to 3.0 predicted to bind 10 or 9 mers from the 1803 peptide sequence belonged either to supertype A02 or B07, similar to SYFPEITHI and BIMAS. 11 out of top 17 had IC50 (nM) values between 3 and 49 while 6 values were
between 55 and 957nM. Thus, peptides from 1803 had very high predicted binding affinity
for their cognate alleles; IC50 (nM) values for peptides predicted from 1821 and 1826 were
higher and reflected the much lower levels of IFN-γ, IL-2, TNF-α and IL-6 secreted by
PBMC in response to peptides 1821 and 1826.

The response to peptide 1821 (amino acids 201 to 220 of Rv1860) was also
dominated by the HLA-A02 supertype alleles. HLA-A*02:01 was predicted to bind the
nonamer R I N Q E T V S L (amino acid 204-212 of Rv1860) with SYFPEITHI score of 25
but had poor scores in BIMAS, whereas for binding of HLA-B*27:05 to the decamer
T R I N Q E T V S L (amino acid 203-212 of Rv1860), with a score of 24 in SYFPEITHI, the
BIMAS score was 200. HLA-B27 supertype was poorly represented in our cohort with only
one individual (LAT1) carrying a B27 supertype allele, who did not experimentally respond
to peptide 1821. Peptide 1821-specific responses were weaker than that to 1803 and 1826,
reflecting the lower SYFPEITHI scores for binding of A02 supertype alleles to epitopes from
the sequence of 1821. In the IEDB predictions, HLA-A*68:02 (A02 supertype) and HLA-
A*32:01 (A01 supertype) occupied the top 3 percentile ranks of 0.15, 0.4 and 0.8 with IC50
values between 4 and 33 nM, neither of which alleles were present in our cohort. Although 5
HLA B alleles figured in the top 10, all except one were poor binders with IC50 values
between 2000 and 14000.

The response to peptide 1826 (amino acids 251 to 270 of Rv1860) was predicted to be
solely due to presentation by HLA-B alleles. The predicted binding of HLA-B*07:02 to the
decamer P P Q R W F V V W L (aa 255-264) with score 23, of HLA-B*51:01 (B07
supertype) to the nonamer G P P Q R W F V V (aa 254 to 262) with score 22 and octamer
P P Q R W F V V (aa 255-262) with score 23, HLA-B*35:01 (B07 supertype) binding to
decamer P P Q R W F V V W L (aa 255 to 264) with score 22, HLA-B*53:01 binding to
decamer A P D A G P P Q R W (aa 250-259) with score 25, explained the experimentally
observed response to 1826. However, secreted cytokine levels were higher in volunteers with HLA-B*35 and HLA-B*07 alleles compared to HLA-B*51 alleles.

Of the 13 HV with B07 supertype alleles, 12 showed cytokine response to peptide 1826 (30 to 350 pg/ml of IFN-\(\gamma\) and TNF-\(\alpha\)) as predicted by the 3 different methods used. However, the average levels of cytokines were 5 to 10 fold less than that seen for peptide 1803. LAT12 and LAT21 with HLA-B*07:05 secreted the highest levels of IFN-\(\gamma\) and TNF-\(\alpha\) (500 to 1200 pg/ml) in response to peptide 1826; LAT9 with HLA-B*35:03 had a much stronger cytokine response to peptide 1826 compared to LAT-11 and LAT-30 with HLA-B*35:01 whereas LAT-17 with HLA-B*35:04 had no detectable response to this peptide. LAT-25 with HLA-B*07:18 and 51:04 [both alleles with one exact and one key residue pocket match in the B07 supertype; see (51)] had a weak TNF-\(\alpha\) response to peptide 1826. LAT10 with HLA B*51:06 [one exact and one key residue pocket match in the B07 supertype; see (51)] showed no response whatsoever to peptide 1826. Thus we observed graded cytokine responses depending on the exact B07 supertype allele present in the volunteer. 3 HV who did not carry any HLA-B07 supertype allele also responded weakly to peptide 1826. The HLA-B*53:01 allele (B07 supertype) which scored the highest for binding epitopes from peptide 1826 using all 3 prediction methods was not represented in our cohort. Thus, broadly for class I alleles, prediction scores correlated with magnitude of experimentally observed response. Scrutiny of the flow cytometry data further confirmed that in keeping with the predictions, cytokines were secreted by CD8\(^+\) T cells. Thus, Rv1860 appears to sport multiple stretches with HLA class I binding propensity, consistent with its observed strong human CD8\(^+\) T cell stimulating property.

In contrast, we observed poor correlation between prediction scores and observed responses for HLA class II alleles. None of the 4 volunteers with HLA DRB1-0301 responded with SI or cytokines to peptides 1816 or 1819, which carried 15 mer epitopes with
predicted high binding SYFPEITHI scores of 34 and 29 (P P P V A N D T R I V L G R L; amino acids 156 to 170 of Rv1860 and A A R L G S D M G E F Y M P Y; amino acids 186 to 200 of Rv1860), respectively. Thorough analysis of the flow cytometry data revealed distinct but low cytokine secretion by CD4+ T cells in response to peptide 1826 in individuals carrying the HLA-DRB1*0701 allele, which was however not supported by prediction scores.

DISCUSSION

A meaningful understanding of the immunology of tuberculosis has the potential to favourably impact global health through the development of an efficacious vaccine superior to the currently available sole vaccine BCG as well as through informed use of immune parameters as surrogates for monitoring new prophylactics and therapeutics. Towards this end, we have attempted to identify proteins of MTB that stimulate a stronger TH1-dominated CD4+ and CD8+ T cell response in healthy latently infected individuals than in TB patients with the simple premise that a measurable response that is greater in healthy people living in TB endemic areas, but absent or lower in TB patients is highly likely to contribute towards preventing the latently infected state from progressing to disease. We show in this study that peptides derived from the sequence of Rv1860, a secreted antigen of MTB which we previously reported to differentially stimulate IFN-γ-secreting CD4+ and CD8+ T cells in healthy MTB-exposed volunteers better than in TB patients (36), stimulated human PFT cells secreting various combinations of the four cytokines IFN-γ, IL-2, TNF-α and MIP-1β, predominantly by CD8+ T cell subsets. In striking contrast, the human PFT response to the well-studied secreted proteins ESAT-6, CFP-10, Ag85A and Ag85B of MTB was primarily from CD4+ T cells in multiple studies of cohorts from several continents (21-23, 25, 42), a feature common to our cohort also. Additionally, these responses were significantly greater in HV, in contrast to the superior PFT response in TB patients to the secreted proteins ESAT-6,
CFP-10, Ag85A and Ag85B of MTB, that waned with chemotherapy (21, 23). Interestingly, soluble cytokine measurements from stimulated PBMC revealed an abundance of TNF-α and IL-10 in TB patients, the former not corroborated by the PFT analysis, in keeping with the reported neutrophil-dependent inflammatory signature in TB patients (50). The simultaneous secretion of IL-10 by PAT would result in a much smaller TH1/TH2 ratio in PAT as reported by other investigators (52).

To the best of our knowledge, Rv1860 represents the first reported protein of MTB capable of eliciting a dominant CD8⁺ human T cell response and constitutes a welcome addition to the highly restricted panel of CD4⁺ T cell eliciting proteins that include ESAT-6, CFP-10, Ag85A and Ag85B, using which all conclusions about the contribution of MTB-specific T cells to protection/exacerbation/inflammation in TB disease were hitherto drawn. As a secreted protein of MTB, Rv1860 perhaps gains efficient access to the infected cell cytosol from the MTB-containing phagosome, allowing for efficient cross presentation on MHC class I to CD8⁺ T cells (53, 54). The loss of anti-tuberculosis immunity in rhesus macaques depleted of CD8⁺ T cells suggests an important role for this T cell subset in human immunity to TB (29), facilitated by the expanded repertoire of CD1 molecules in primates, that present epitopes along with MHC class I to this subset of T cells. We first identified Rv1860 as reactive to TB patient sera from a genomic DNA expression library of a field strain of MTB (35) and it represented one of only two human T cell stimulatory proteins among twenty four random proteins that we screened. Rv1860, also called APA due to the repeating alanine-proline-alanine motifs was first reported as a secreted culture filtrate protein of BCG, capable of stimulating both a delayed type hypersensitive (DTH) (37) and an antibody response in guinea pigs immunized with live, but not killed *Mycobacterium bovis* BCG (38). The MTB homolog coding for a 50-55 kDa, 325 amino acid long Rv1860 protein (55), was subsequently cloned and expressed both in *M. smegmatis* and *E. coli* (56).
Multiple subsets of predominantly CD8+ and some subsets of CD4+ T cells specific to Rv1860 were significantly over represented in HV compared to PAT. CD8+ T cells were shown to be required for controlling latent phase of MTB in mice (28) and their depletion resulted in a 10 fold increase in lung bacterial burden during latent phase of infection, while having no effect on the acute phase. We may therefore surmise that human Rv1860-specific CD8+ T cells (along with other subsets of CD8+ T cells with other specificities) perhaps contributed to preventing reactivation of latent MTB in the latently infected volunteers. In fact, CD8+ T cells specific to MTB antigens were also previously reported to be significantly more in BCG vaccinees relative to TB patients (57). Several studies have reported the protective potential of human MTB-specific CD8+ T cells (30-33) by virtue of killing MTB-infected cells or MTB directly. Despite this, neither their role in protective immunity against TB nor the identities of MTB antigens that elicit CD8+ T cells have been investigated in depth. One study (34), again using the well-studied proteins ESAT-6 and CFP-10, reported CD8+ TEMRA cells significantly over represented in latently infected human volunteers relative to TB patients whose CD8+ T cells were TEM. CD4+ T cells with the dual IFN-γ plus IL-2 cytokine profile were earlier shown in an exhaustive and elegant longitudinal study to progressively increase during TB chemotherapy and dominate the immune response post cure (58). It has been suggested that T cells of this phenotype belong to the effector-memory population (59-61). We found Rv1860-specific CD8+ memory T cells with this dual cytokine secretion profile (along with other IL-2 and IFN-γ secreting profiles) to be more abundant in HV than PAT (Supplementary Figure S3). The ability of these CD8+ T cells to secrete IL-2, a well-established T cell growth factor, would help in their self-perpetuation and IFN-γ, a cytokine that activates macrophages to eliminate MTB would be crucial for protection against TB (4, 11, 62). Rv1860-specific memory CD8+ T cells secreting only IL-2 also occupied a higher proportion of the total CD8+ response in HV than in PAT (Supplementary Figure S3).
The critical role of Treg cells elicited by IL-2 in TB resistance in the rhesus macaque model of pulmonary TB (63) further reinforces the importance of IL-2 secreting T cells in TB control. Of the 4 subsets of CD8+ T cells significantly overrepresented in HV than in PAT, 2 detected at reasonably high frequency were single cytokine producers (symbol *, Supplementary Figure S3).

Several reports have pointed to the role of T cells in TB pathogenesis (reviewed in (64). The lung tissue destruction leading to enhanced TB transmission associated with cavitary TB has been directly correlated with CD4+ T cell numbers in HIV-TB co-infected individuals (65). Again, the exacerbated pulmonary inflammation suffered by PD1 knockout mice infected with MTB was alleviated by CD4+ T cell depletion (66). However, unlike CD4+ T cells, we have not encountered reports of deleterious effect of CD8+ T cells in TB pathology.

All volunteers, both HV and PAT responded to at least one peptide by both proliferation and secretion of at least one cytokine. We observed strong correlations between predicted binding of epitopes from the three immunodominant peptides to HLA class I A and B alleles and the measured levels of secreted cytokines from PBMC of volunteers carrying those alleles. Scrutiny of the flow cytometry data further confirmed cytokine secretion from CD8+ T cells. Thus, peptides from Rv1860 must be presented by a wide array of HLA alleles, as predicted with high scores by epitope prediction softwares such as IEDB, SYFPEITHI and BIMAS, suggesting that a vaccine carrying Rv1860 would have wide population coverage. Flow cytometry analysis of single peptide-stimulated whole blood samples revealed both CD4+ and CD8+ T cell responses in several individuals, revealing both MHC class I and II-binding epitopes, within single peptides.
The results from the recently concluded human phase 2b trial of MVA85A (7-10) revealed that currently used mouse and guinea pig animal models cannot reliably predict vaccine efficacy in humans. It also suggests that IFN-γ secretion and ability to stimulate PFT in humans do not correlate with protective efficacy against TB. Transcriptional signatures have been recently suggested as a potentially more reliable biomarker of efficacy for vaccines and therapies (67). While our observations need validation from a larger human cohort study, the capacity of Rv1860 to elicit a memory CD8+ T cell-dominated PFT profile, combined with the significantly greater response in HV over PAT that we observed in this pilot study, suggests that vaccines including this antigen may have the ability to protect against progression of latent MTB infection to TB disease. Additionally, the significantly superior proliferation along with significantly reduced IL10 and TNF-α secretion by PBMC in response to Rv1860-derived peptide 1803 in HV over PAT, suggests a potential use for this peptide in monitoring the efficacy of TB vaccines and TB therapy as well as progression of latently infected individuals to TB disease.

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Figure 1. Percentage of volunteers whose PBMC showed proliferative response to individual 20 mer peptides derived from Rv1860 among 28 latently MTB-infected healthy volunteers (HV) (A) and 20 TB patients (PAT) (B). The three most immunodominant peptides 1803, 1821 and 1826 are shown highlighted in A.

Figure 2. Proliferation and cytokine secretion by PBMC in response to immunodominant peptides from Rv1860. 1803 (A), 1821 (B) and 1826 (C). 28 HV and 20 PAT were compared. p values for significant difference between the two groups were computed using Mann Whitney test; p values that did not remain significant after Bonferroni’s correction are indicated as ns i.e. not significant.

Figure 3. Polychromatic flow cytometry analysis of cytokine production profiles of Rv1860-derived peptide-specific T cells from latently MTB infected volunteers (HV). Whole blood ICC samples stimulated with a mixture of peptides 1803, 1821 and 1826 were stained and analysed as described in methods. Percentage of CD4+ (left bar in each pair) were compared with CD8+ (right bar in each pair) T cells secreting the different combinations of cytokines IFN-γ, IL-2, TNF-α and MIP-1β indicated below the pairs of bars as +/-, in healthy volunteers (HV). Boxes represent the 25th and 75th percentile values while bars denote median. Dots represent individual values. P values computed by the non-parametric Wilcoxon test for significant difference between CD4+ and CD8+ are given above the bars (#). Significant p values below the Bonferroni-corrected critical value of 0.05/15 = 0.0033 are indicated (*).

Figure 4. Polychromatic flow cytometry analysis of cytokine production profiles of Rv1860-derived peptide-specific T cells in TB patients (PAT). Whole blood ICC samples stimulated with a mixture of peptides 1803, 1821 and 1826 were stained and analysed as described in
methods. Percentage of CD4⁺ (left bar in each pair) were compared with CD8⁺ (right bar in each pair) T cells secreting the different combinations of cytokines IFN-γ, IL-2, TNF-α and MIP-1β indicated below the pairs of bars as +/-. Boxes represent the 25th and 75th percentile values while bars denote median. Dots represent individual values. P values computed by the non-parametric Wilcoxon test for significant difference between CD4⁺ and CD8⁺ (#) are given above the bars. Significant p values below the Bonferroni-corrected critical value of 0.05/15 = 0.0033 are indicated (*).

Figure 5. Polyfunctional human T cell response to secreted antigen ESAT-6. The plots show comparison of frequency of CD4⁺ (left bar in each pair) with CD8⁺ (right bar in each pair) T cells in latently infected HV (HV; upper panel) and TB patients (PAT; lower panel) secreting the different combinations of cytokines IFN-γ, IL-2, TNF-α and MIP-1β indicated below the pairs of bars as +/-. p values for significant difference between HV and PAT computed by the non-parametric Wilcoxon test available within SPICE, are shown above indicated populations (#). None of the values were significant following Bonferroni’s correction for multiple comparisons.