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HLA-A*0201-restricted Cytotoxic T-cell Epitopes in Three PE/PPE Family Proteins of *Mycobacterium tuberculosis*

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Abstract

$CD8^+$ T cells are thought to play an important role in protective immunity against tuberculosis. We report the identification of three peptides derived from Rv1818c, Rv3812 and Rv3018c proteins of *Mycobacterium tuberculosis* that bound to HLA-A*0201 molecules and their ability to induce *in vitro* T-cell response in peripheral blood lymphocytes from HLA-A*0201-positive healthy individuals (PPD+) and patients with TB. The peptide-specific cytotoxic T lymphocytes (CTL) generated were capable of recognizing peptide pulsed targets. Three 9-mer peptides bound with high affinity to HLA-A*0201 and displayed low dissociation rates of the bound peptide from HLA. Epitope-specific recognition was demonstrated by the release of perforin and γ-interferon. Overall, our results demonstrate the presence of HLA class I-restricted $CD8^+$ CTL against proteins from PE and PPE proteins of *M. tuberculosis* and identify epitopes that are strongly recognized by HLA-A*0201-restricted $CD8^+$ T cells in humans. These epitopes thus represent potential subunit components for the design of vaccines against tuberculosis.

Introduction

Tuberculosis, caused by the intracellular pathogen *Mycobacterium tuberculosis,* is one of the leading causes of morbidity and mortality worldwide [1]. The bacilli Calmette–Guerin (BCG) vaccine is an attenuated strain of *Mycobacterium bovis* and represents the world's most widely used live vaccine, with more than two billion vaccinations performed to date [2]. BCG protects against miliary tuberculosis in children [3], but fails to consistently protect against pulmonary tuberculosis in adults, the most prevalent form of the disease [4], with variability of 0–80% [5].

Effective cell-mediated immunity is essential to control infection with *M. tuberculosis.* Many studies have shown that major histocompatibility complex (MHC) class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) contribute to the control of *M. tuberculosis* infection by mediating specific effector functions, which include lysis of *M. tuberculosis* infected cells, killing of intracellular pathogens and release of Th1 cytokines that activate anti-mycobacterial mechanisms in macrophages [6]. In humans, the MHC class I-restricted $CD8^+$ T cells specific for immunodominant epitopes derived from *M. tuberculosis* proteins lyse infected cells and also release IFN-γ upon recognition of their targets [7, 8]. However, only a small number of MHC class I-restricted CTL epitopes have been identified within a few mycobacterial proteins. As the antigen specificity of the human T-cell response is known to be strongly controlled by HLA polymorphism [9], the immunogenic potential of candidate vaccines needs to be defined in the context of major HLA polymorphism. HLA-A2 is the most common HLA-A allele in most of the populations, particularly in the Asian population with an estimated frequency of >40% [10]. As tuberculosis affects many parts of Asia, identification of HLA-A*0201-restricted CTL epitopes of *M. tuberculosis* proteins is an important step towards vaccine design. The prediction of epitopes that have the potential of eliciting a CTL response has been greatly facilitated by the identification of binding motifs for different MHC class I alleles.

Completion of the *M. tuberculosis* genome sequence paved a way for the identification of many new candidate

antigens for protective vaccine against tuberculosis. This includes the discovery of two multigene families of proteins PE and PPE. Members of PE and PPE protein families are characterized by highly conserved N-terminal domains and the C-terminus, which, however, show considerable variation in the number of residues as well as in the sequence [11]. Till date, little is known about the functional role of the proteins of PPE or PE family in the biology of *M. tuberculosis*. Recently, it has been shown that rv1818c protein is loosely associated with the cell wall and is localized on the outer membrane [12] and the protein is released into the extracellular compartment through exosomes secreted by dendritic cells or macrophages infected by *M. tuberculosis* [13]. It has also been shown that the protein is taken up by T cells inducing apoptosis [13].

In an earlier work, employing immuno-informatics approach, we had identified a set of HLA class I-binding peptides from these proteins. Further, their binding abilities for HLA class I were ascertained using independent methods such as molecular modelling and structural analysis [14]. This study has led to the identification of potential T-cell epitopes from PE and PPE proteins. We have shown that one of the PE and PPE proteins, Rv1818c protein induces good T-cell response in mouse models [15]. We have also shown that Rv3812 (a PE_PGRS) and Rv3018c (a PPE protein) are good T-cell antigens [16].

In this work, we have employed four peptides derived from three PE/PPE proteins predicted to bind to HLA-A*0201 molecule with high affinity. Four peptides, thus, identified to bind to HLA-A*0201 *in silico* were experimentally tested for their ability to bind to the HLA-A*0201 molecule *ex vivo*, and the ability of HLA-A*0201-binding peptides to provoke *in vitro* T-cell responses in peripheral blood mononuclear cell (PBMC) preparations from HLA-matched healthy donors as well as patients with tuberculosis. Our findings show that the CTL generated in PBMC can elicit an antigen-specific, HLA-A2-restricted response, effectively killing peptide-pulsed T2 cells.

Materials and methods

HLA typing. Peripheral blood was collected from nine people with HLA-A*0201. Five were healthy, BCG vaccinated and were all PPD positive. Four were suffering from tuberculosis, as established by the presence of clinical symptoms of TB, positive results of tuberculin (PPD) skin testing and signs visible on chest radiography. PBMC from patients with TB were obtained from Dr Ambedkar Medical College Hospital, Bangalore, India. PBMC were HLA typed by molecular typing using HLA typing kit (Dynal Biotech Ltd, Olso, Norway) and HLA-A*0201 sub-typing was accomplished by polymerase chain reaction amplification technique using Sequence Specific Oligonucleotide Primers. Formal consent was obtained from all patients and the human experimentation guidelines of our institution were followed in the conduct of clinical research.

Peptides. Peptides with high predicted half-time of dissociation ($t_{1/2}$) as predicted by BIMAS algorithm [17] were purchased from Peptron (Peptron Inc., Daejeon, South Korea) (Table 1). Two peptides which do not have any binding motif for HLA-A*0201 were chosen as negative controls, one peptide from Rv3812 $_{490}$NYIPQQLAL$_{498}$ and one peptide from Rv3018c $_{71}$AYVPYVAWL$_{79}$. As a positive control for HLA-A*0201 binding ability, HLA-A*0201-restricted HTLV-tax peptide $_{11}$LLFGYPVYV$_{19}$ [18] was used.

Cell lines. Human transporter associated with antigen processing (TAP)-deficient T2 cell line (kind gift from Dr François Lemonnier, Unité Cellulaire Antivirale, Institut Pasteur, Paris) transfected with HLA-A*02 was obtained and grown in RPMI-1640 medium supplemented with 10% FCS (RPMI/10% FCS). The EBV-transformed B-cell line JY (HLA type: A*0201, B7,

Table 1 The relative affinity of putative peptides for stable HLA-A*0201 molecules at the cell surface and the ability of the peptide to stabilize the empty MHC class I molecules on cell surface of TAP deficient cells.

Protein	Sequence	AA position	Stability HLA-A*0201 DT_{50} (h)	IC_{50} (μM)	Predicted $t_{1/2}$ (min)
Rv1818c	ALGGGATGV	385–393	7	1.4	69
Rv1818c	TIPEALAAV	6–14	6	2.8	90
Rv3018c	AQLLTEFAI	254–262	5	25	22
Rv3812	NLLVTGFDT	260–268	7	2.4	159
Rv3812	NYIPQQLAL	490–498	1	32	<1
Rv3018c	AYVPYVAWL	71–79	1	28	<1

Jurkat cells were treated with acid for 5 min. Cells were immediately buffered with RPMI medium, re-suspended in the presence of 1.5 μg/ml β2-microglobulin and mixed with 150 nM of an FL-labelled reference peptide plus test peptide at a range of concentrations. After 12 h at 4 °C, cells were washed and analysed by flow cytometry. The mean fluorescence (MF) values obtained with the FITC-labelled peptide in the absence of competitor peptide was taken as the maximal binding and equated to 0% inhibition. The MF value obtained without FITC-labelled peptide was taken as being 100% inhibition. Relative binding affinity of the peptide was expressed as the peptide concentration needed to inhibit 50% of the binding of the reference-labelled peptide (IC_{50}). $t_{1/2}$ represents the estimated half-time of dissociation of peptide as predicted by BIMAS algorithm.

Cw7, kind gift from Dr Linda Sherman, TSRI, La Jolla, CA, USA) was grown in RPMI-1640 medium supplemented with 10% FCS.

*HLA-A*0201 peptide binding and complex stability assay.* To determine weather synthetic peptides could bind to HLA-A*0201 molecules, peptide-induced HLA-A*0201 upregulation on T2 cells was examined as described earlier [19]. Briefly, T2 cells were incubated at 25 °C for 16 h, and further incubated with 100 μM of peptides for 1 h at 25 °C and 2 h at 37 °C. Cells were then washed to remove unbound peptides and incubated at 37 °C for 0, 2, 4, 6 and 12 h after peptide withdrawal. Subsequently, the expression of HLA-A*0201 on T2 cells in the absence of exogenous peptide was measured by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody BB7.2 (eBioscience, San Diego, CA, USA). The increase in HLA-A*0201 expression on T2 cells reflects the stabilization of MHC by the addition of exogenous peptides and for each time point, peptide-induced HLA-A*0201 expression was calculated as (mean fluorescence of peptide-pre-incubated T2 cells mean fluorescence of T2 cells treated in similar conditions in the absence of peptide). DC_{50} (dissociation complex, DC) was defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at $t = 0$. The definitive DC_{50} value for each peptide was determined from at least three independent experiments.

A second, competition-based HLA-A2 peptide binding assay using JY-EBV cells was performed as described previously [20]. In short, HLA molecules on JY-EBV cells were stripped via a mild acid treatment. Stripped cells were subsequently incubated with various concentrations of peptides for 24 h at 4 °C in the presence of 150 nM of a fluorescein (FL)-labelled reference peptide [FLPSDC(-FL)FPSV] and 1.5 μg/ml human β2-microglobulin, and analysed by flow cytometry. The binding capacity of a peptide is expressed as the concentration required for inhibiting 50% of the binding of the FL-labelled reference peptide (IC_{50}).

Generation of CTL in PBMC donors. To generate polyclonal CTL, 2×10^6 PBMC from HLA-A2-positive donors were isolated from whole blood by Ficoll/Hypaque (Sigma, St Louis, MO, USA) density gradient centrifugation. The PBMC were co-cultivated for 7 days with irradiated (5000 rad) autologous PBMC prepulsed with synthetic peptides (10 μg/ml). On every third day, IL-2 was added (100 U/well; eBioscience). After 2 weeks of culture, the cells were employed for further experiments.

Intracellular IFN-γ measurement using flow cytometry. Peripheral blood mononuclear cells (2×10^6 per ml) from donors were cultured in 96-well tissue culture plates in the presence of 20 μg of protein/ml for 1 or 3 days. Brefeldin A (10 μg/ml; Sigma) was added to the cultures for the last 5 h to prevent secretion of the intracellular cytokine. One million cells from each group were first incubated with FITC-conjugated anti-CD8 Ab (clone RPA-T8; eBioscience) for 30 min at 4 °C. Cells were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.1% saponin. Cells were incubated with PE conjugated anti-IFN-γ Ab (clone 4S.B3; eBioscience) for 30 min at 4 °C, washed, and acquired on a cytofluorometer (FACSCALIBUR; BD, Mountain View, CA, USA). An analysis was carried out using Cell Quest software.

Human IFN-γ ELISPOT assay. An ELISPOT analysis for IFN-γ secretion using human PBMC was carried out as previously described [21].

CTL assay. A cytotoxic T lymphocyte assay was performed employing a non-radioactive method, based on the release of LDH from target cells as described earlier [22]. Briefly antigen-specific CTL were collected from *in vitro* expanded culture and washed once with RPMI-1640. Viable cells were purified by ficoll density gradient centrifugation and re-suspended in RPMI medium. T2 target cells were incubated with the peptides (10 μg/ml) for 2 h at 37 °C. After washing, 5×10^3 cells/100 μl were added to 100 μl of various numbers of effector cells that had been plated in 96-well plates to get a target-to-effector ratio of 1:20, 1:40 and 1:100. The medium or the target cells alone were kept as low control (Spontaneous LDH release). For the high control (maximum LDH release), 2% of Triton X 100 was added to the target cells .The cells were incubated for 12 h. Afterwards, 100 μl of culture the culture supernatant is collected cell-free and assayed for LDH release by Cytotoxicity Detection Kit-LDH (Roche Applied Science, Mannheim, Germany). The percentage cell-mediated cytotoxicity was determined by the following equation:

$$\text{Cytotoxicity}\,(\%) = \left[\frac{(\text{effector target cell mix} - \text{effector cell control}) - \text{lowcontrol}}{\text{high control} - \text{low control}}\right] \times 100$$

Statistical analysis. The statistical significance of differential findings between experimental groups was determined by Student's *t*-test. Data were considered statistically significant at $P < 0.05$.

Results

Identification of HLA-A2-binding peptides within PE and PPE proteins

To identify potential HLA-A2-restricted epitopes within Rv3812 and Rv3018c, the amino acid sequence was analysed by a computer program designed to predict HLA-binding peptide, based on an estimation of the half-time of dissociation of the HLA-peptide complex. Four peptides earlier identified [14] to bind to HLA-A*0201 allele employing predictive algorithms were chosen (Table 1). These peptides were then analysed for their capacity to stabilize HLA-A*0201 molecules on the surface of the antigen-processing deficient cell line T2. The T2 cells were incubated with the relevant peptide and the resultant increase in the cell surface expression of HLA-A*0201 was measured. In this assay, a nonamer peptide from HTLV-Tax protein ($_{11}$LLFGYPVYV$_{19}$), which has a high binding affinity for the HLA-A*0201 molecule [19], was used as positive control. This peptide resulted in a sixfold increase in cell surface HLA-A*0201 stabilization (Fig. 1).

The highest level of stabilization was observed with Rv3812 Peptide$_{260–268}$, Rv1818c Peptide$_{385–393}$ and Rv1818c Peptide$_{6–14}$ for more than 6 h. The two negative control peptides could not stabilize the peptide–MHC complex for more than 1 h (Table 1).

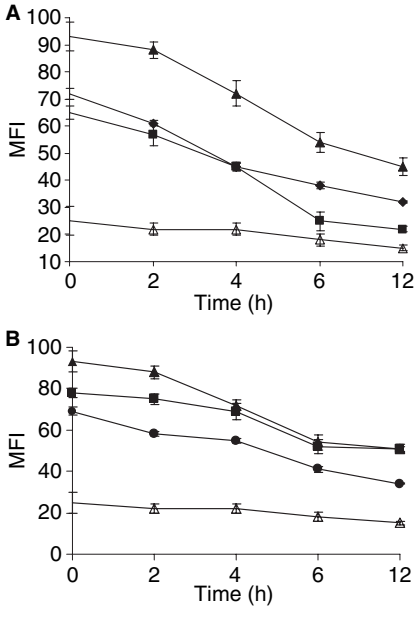

Figure 1 Stability of peptide bound to major histocompatibility complex class I (HLA-A*0201) molecules on T2 cells. (A) (●) Rv3812 Peptide$_{260–268}$, (■) Rv3018c Peptide$_{254–262}$, (▲) Positive control peptide-LLFGYPVYV, (Δ) No Peptide. (B) (●) Rv1818c Peptide$_{6–14}$, (■) Rv1818c Peptide$_{385–393}$ (▲) Positive control peptide-LLFGYPVYV, (Δ) No peptide. The results are expressed as mean fluorescence intensity. T2 cells were incubated for 16 h at 25 °C, washed and incubated with peptides for different time points. The cells were then washed and stained with FITC-conjugated MoAb directed against HLA-A*0201. Results are representative of three independent experiments. Data represent mean ± SD.

The relative affinity of all the peptides bearing the motif for HLA-A*0201 molecule was then determined using a competition-based HLA class I-binding assay [20]. In this assay, the lymphoblastoid cell line JY, was used after acid treatment, aimed to remove the peptides from HLA molecules present on its surface. These stripped cells were then incubated with a mixture of a fixed amount of a fluoresceinated reference peptide, and different concentrations of the four peptides under test. Peptides with affinity for the HLA-A*0201 molecule will compete for the binding of the fluoresceinated reference peptide. Three peptides showed high IC_{50} < 5 mM (Table 1). Although the T2 stabilization assay measures the stability of binding, the peptide binding competition assay determines those peptides which bind faster and compete better.

Stimulation of PE and PPE peptide-specific T cells *in vitro*

We evaluated the ability of four PE and PPE-derived peptides, which showed reproducibility of binding to HLA-A*0201 molecules as judged by two independent methods, for their ability to elicit antigen-specific T-cell response. To achieve this, PBMC from healthy and tuberculosis-infected HLA-A*0201 individuals were isolated and stimulated *in vitro* with peptides. Three of the peptides that displayed relatively high binding affinity for HLA-A*0201 molecules were able to induce peptide-specific stimulation of PBMC both in healthy donors and patients with TB (Fig. 2A and B). At the same time, the non-HLA-A*0201-restricted negative control peptides induced little or no stimulation of PBMC (Fig. 2). However, upon stimulation with the peptides, the *in vitro* proliferation of patient PBMC was lesser compared with healthy control PBMC.

Cytotoxic activity of peptide-specific T cells

We investigated whether these peptides would trigger specific and functional CTL responses in HLA-A*0201-positive donors, both healthy and patients with TB. PBMC from donors were isolated and stimulated *in vitro* with peptides. The cytotoxic activity of the responding cells was measured after two consecutive stimulations against T2 cells pulsed with the peptides used in the stimulations. In both healthy patients and TB HLA-A*0201-positive donors tested, peptide-specific $CD8^+$ T cells were generated that specifically lysed peptide-pulsed T2 cells (Table 2). But the specific lysis of the target cells by PBMC from healthy individuals was significantly more than that induced by PBMC from patients with TB (P = 0.003). The peptide specificity of the target cells was clearly demonstrated as target cells loaded with

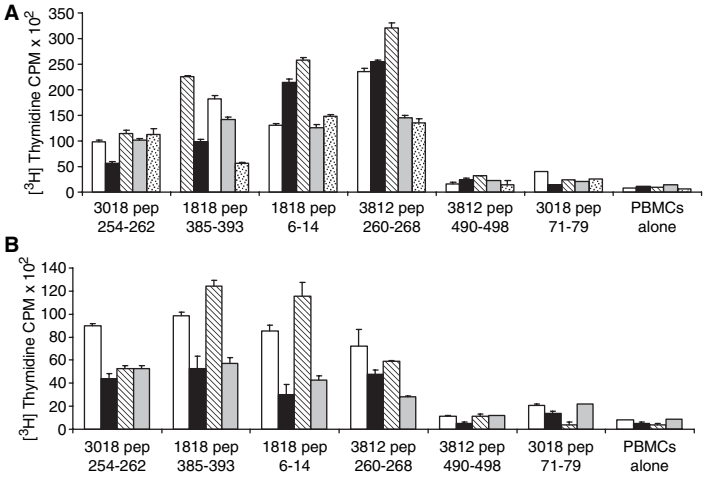

Figure 2 *In vitro* T-cell proliferative response specific for HLA-A*0201-binding peptides. (A) HLA-A*0201-typed human PBMC were *in vitro* re-stimulated with synthetic peptides (10 μg/ml) pulsed T2 cells (1×10^4) for 96 h and tested for the T-cell proliferation. Results are presented as mean ± standard deviations of pooled response of three independent experiments. Each bar represents T-cell response to each HLA-A*0201 individual. (A) PPD-positive healthy controls. (B) Patients with TB.

irrelevant peptides were not lysed. HLA restriction was demonstrated by the lack of lysis of HLA-A*0201-mismatched target cells (data not shown). Moreover, the peptide-specific cytolysis of targets was inhibited by anti-MHC class I MoAb, thus corroborating the MHC class I restriction and CD8 dependency of these effector cells.

Effector function of antigen-specific T cells

Production of Th1 type of cytokines is an essential effector function of T cells responding to bacterial infections in humans and mice [22]. We tested whether peptide-specific CTL can release IFN-γ, from *in vitro* expanded PBMC from HLA-A*0201-positive individuals when challenged with the peptides by an ELISPOT assay. The predominant response was seen for Rv3812 Peptide$_{260–268}$, with a much reduced response to Rv1818c Peptide$_{6–14}$ and Rv1818c Peptide$_{385–393}$ and very low response to Rv3018c Peptide$_{254-262}$. The response was HLA-A*0201 mediated as shown by the inhibition of response by anti HLA-A2 antibodies (Table 3). The frequencies of cytokine-producing CD8$^+$ T cells among effectors were determined by intracellular cytokine staining. The frequency of epitope-specific CD8$^+$ T cells positive for IFN-γ was the highest for Rv3812 Peptide$_{260–268}$ ranging from 8% to 12% and Rv1818c Peptide$_{385-393}$ ranging from 8% to 14% in different individuals (Table 4). All the *in vitro* expanded peptide-specific T cells, more by healthy donors, induced a good number of CD8$^+$ T cells secreting INF-γ on stimulation with the corresponding peptides, which followed the pattern of CTL assay. The negative control Rv3812 Peptide$_{490–498}$ Rv3018c Peptide$_{71–79}$ induced peptide-specific CD8$^+$ T cells positive for IFN-γ ranging from 1.2% to 3.8% and from 2% to 2.8% respectively.

Table 2 Cytotoxic response of antigen-specific T cells.

	Patients with TB	PPD-positive healthy donors
1818pep6–14	32 ± 8	68 ± 14
1818pep385–393	57 ± 14	77 ± 11
3812pep260–268	28 ± 11	68 ± 13
3018pep254–262	55 ± 10	66 ± 14
3812pep490–498	18 ± 5	17 ± 8
3018pep71–79	22 ± 9	24 ± 9

PBMC from HLA-A*0201 donors were *in vitro* expanded in the presence of the peptides (10 μg/ml). The ability to induce CTL was tested by performing cytotoxicity assay against different numbers of target cells, antigen-stimulated T2 cells. % of specific lysis was measured for different effectors and target ratio. % lysis of unpulsed T2 cells by PBMC was the negative control. Results are representative of three independent experiments. Data represent mean ± SD. The specific lysis by T-cell lines from healthy controls was significantly higher than that induced by T-cell lines from patients with TB (P = 0.003).

Discussion

Cellular immunity mediated by CD4$^+$ T cells as well as MHC class I-restricted CD8$^+$ T cells are important for protective immunity against tuberculosis as mice lacking functional CD8$^+$ T cells were more susceptible to tuberculosis, β2-microglobulin deficiency are more susceptible to MTB [23–25]. In this work, we have employed PE/PPE protein-derived epitopes with high affinity for HLA-A*0201 molecules identified, using computational methods and verified by MHC peptide binding assays. Four peptides from Rv1818c, Rv3812 and Rv3018c of PE/PPE family were recognized by T cells from HLA-A*0201-typed PPD-positive healthy donors and patients with TB. The peptide-specific responses were studied *in vitro* by testing CTL responses of T cells from HLA-A*0201-matched healthy donor PBMC. CTL generated in response to peptides from the chosen three proteins secreted IFN-γ and killed target cells in peptide-specific and HLA-A*0201-restricted manner. Peptides derived from PE/PPE proteins could induce significant CTL responses in PBMC from healthy human donors compared with patients with TB,

Table 3 Functional activity of peptide-specific human A*0201-typed T-cell lines.

	SFC (per 10^6 cells) (ELISPOT), patients with TB		SFC (/10^6 cells) (ELISPOT), PPD-positive healthy donors	
	Antigen	Anti-HLA-A2 MoAb	Antigen	Anti-HLA-A2 MoAb
1818pep6–14	88 ± 12	22 ± 8	125 ± 31	122 ± 22
1818pep385–393	187 ± 44	27 ± 10	324 ± 47	85 ± 14
3812pep260–268	202 ± 27	49 ± 10	342 ± 54	54 ± 9
3018pep254–262	147 ± 32	51 ± 11	250 ± 34	47 ± 11
3812pep490–498	65 ± 28	64 ± 12	72 ± 18	62 ± 35
3018pep71–79	42 ± 12	42 ± 15	112 ± 21	92 ± 22

Production of IFN-γ in response to stimulation with PE/PPE peptides. Human PBMC were *in vitro* expanded in the presence of synthetic peptides (10 μg/ml) Primed T cells (4×10^5 per well) were *in vitro* expanded in the presence of T2 (1×10^5 per well) pulsed with peptides as APC and in the presence of monoclonal antibodies to HLA-A2. After 24 h of stimulation, spot forming cells were counted (SFC) by an ELISPOT. Data represent mean ± SD.

Table 4 Intracellular IFN-γ staining of antigen-specific T cells from five HLA-A*0201-typed PBMC of healthy controls and patients with TB.

	IFN-γ-secreting $CD8^+$ T cells (%)								
	HD1	HD2	HD3	HD4	HD5	TP1	TP2	TP3	TP4
Un-stimulated	0.11 ± 0.04	0.22 ± 0.02	2.3 ± .05	0.44 ± 0.04	0.88 ± 0.04	0.3 ± 0.05	0.13 ± .05	0.2 ± 0.04	1.3 ± .05
Rv1818 pep6–14	6.5 ± 1.2	5.8 ± 2.7	11.2 ± 0.8	9 ± 2.8	2.4 ± 0.34	3.5 ± 0.45	2.3 ± 0.35	2.6 ± 1.4	2.4 ± 3.2
Rv1818 pep385–393	8.5 ± 1.8	8.8 ± 2.3	14 ± 1.4	11.2 ± 2.2	12.2 ± 1.6	4.1 ± 1.1	4.3 ± 1.2	8.8 ± 3.5	5.7 ± 1.5
Rv3812 pep260–268	8.2 ± 2.1	11.9 ± 1.4	9.4 ± 2.7	12.2 ± 3.1	9.5 ± 1.1	5.7 ± 1.5	5.3 ± 1.2	3.8 ± 1.1	2.3 ± 0.2
Rv3018c pep254–262	7.9 ± 1.3	3.5 ± 1.4	6.4 ± 0.9	9.5 ± 2.4	3.5 ± 1.5	2.7 ± 1.7	1.3 ± 0.4	2.2 ± 1.5	1 ± 0.23

Percentage of $CD8^+$ T cells secreting IFN-γ was determined by doing a double staining for surface CD8 and intracellular IFN-γ using FACSscan. Results are representative of three independent experiments. HD, healthy donors; TP, patients with TB.

suggesting that CTL precursor populations against these mycobacterial peptides exist at a higher frequency within the T-cell repertoire of healthy individuals. Reduced CTL activity, IFN-γ production shown by peptide-specific T-cell lines derived from patients with TB in comparison with peptide-specific T-cell lines from healthy donors strongly suggests that the $CD8^+$ T-cell epitopes of the three proteins may have a role in protective immunity. Our results are in agreement with the earlier reported observations of preferential T-cell responses to Ag85 complexes [26, 27] and Apa protein of *M. tuberculosis* [28]. CTL reactivity is considered to be protective in the control of *M. tuberculosis* growth. Using an intracellular assay for assessing IFN-γ production to measure peptide-specific $CD8^+$ T-cell frequencies, we demonstrated that $CD8^+$ T-cell responses to the chosen peptides are detected *ex vivo* in PBMC, which indicates that the $CD8^+$ T-cell response to these peptides may be elicited during the course of *M. tuberculosis* infection *in vivo*.

One of the possible obstacles of any epitope-based approach to vaccination in humans is HLA polymorphism because choice of epitopes is allele specific. However, this obstacle might be overcome using appropriate mixtures of synthetic peptide epitope or by constructing vectors to express polypeptides in which the relevant epitope sequences are linearly joined together. More recently, work in a murine model has also shown that each of several CTL epitopes combined in a polyepitope construct was capable of eliciting a CTL response *in vivo* in animal models [29]. In the long term, it may be possible to combine CTL epitopes from the PE and PPE proteins with other mycobacterial antigens generating a chimeric protein that fuses the important immunogenic determinants from different types of antigens to design an effective vaccine. It is important to mention here that although the result from the present work does not lead to any firm conclusions on the efficacy of a PE/PPE epitope-based vaccine in humans, it does clearly show that CTL epitopes from these two classes of proteins can be used as immunogens to induce an efficient CTL response *in vivo*. Earlier it has been shown that HLA-A*0201-binding nonamer peptides derived from the acr/16-kDa antigen (Rv2031c), 19-kDa lipoprotein (Rv 3763), superoxide dismutase (Rv3846), alanine dehydrogenase (Rv2780) and glutamine synthetase (Rv2220) of *M. tuberculosis* elicit cytotoxic $CD8^+$ T cells from patients with TB [30–33]. Epitope-specific recognition was demonstrated by the lysis of both *M. tuberculosis*-infected and peptide-pulsed macrophages, the release of cytotoxic granules and IFN-γ production.

In conclusion, our study has identified novel HLA-A*0201-restricted, immunogenic T-cell epitopes of Rv1818c, Rv3812 and Rv3018c proteins of PE/PPE

family of *M. tuberculosis*. These newly identified epitopes may help in the design of a better vaccine candidate against tuberculosis.

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