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IMMUNOLOGICAL ASPECTS

Immune response to *Mycobacterium tuberculosis* specific antigen ESAT-6 among south Indians

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SUMMARY

The 6-kDa early secreted antigenic target (ESAT-6) is a T-cell antigen recognized by individuals infected with *Mycobacterium tuberculosis*. The aim of the study was to identify "protective epitopes" of ESAT-6 protein in the south Indian population. Proliferative and Interferon gamma (IFN-γ) responses to ESAT-6 peptides were studied by flow cytometry and Enzyme linked immunosorbent assay (ELISA). Healthy household contacts (HHC) recognized Esp1 (10/17) and Esp6 (9/17) peptides. Among pulmonary tuberculosis patients (PTB), Esp1 (3/11) and Esp6 (5/11) were recognized. Maximal response (7/10) was found for Esp1 and Esp8 in treated patients (TR). Median values for the responding subjects gave the following results: Esp1 (76 pg/ml), Esp6 (64 pg/ml), induced IFN-γ production in HHC; PTB gave low IFN-γ responses for the peptides. TR responded to the peptides Esp1 (141 pg/ml), Esp8 (102 pg/ml). The proliferation of CD4 cells was similar in both PTB and TR for all peptides; but HHC showed an increase for Esp1 ($p < 0.05$) and Esp6 ($p < 0.01$). Esp1 (amino acids aa 1–20) and Esp6 (aa 51–70) were the immunogenic peptides recognized by the alleles HLA DRB1*04 and HLA DRB1*10 among HHC. But the association of the alleles with ESAT-6 peptide presentation needs to be confirmed in a large cohort of subjects. We speculate that ESAT-6 can be used along with other immune-eliciting proteins for vaccine design strategies in south Indian population.

1. Introduction

Tuberculosis (TB) remains one of the major causes of death worldwide. It is the major opportunistic infection in individuals with Human immunodeficiency virus (HIV) infection.[1] The spread of multi-drug resistant TB and extensively drug resistant TB are the other factors which threaten the public health.[2] The vaccine administered for TB is Bacille Calmette Guerin (BCG). It does not protect against adult forms of TB and is not safe for those with HIV infection.[3]

Much of the published reports assign a protective role for secreted antigens of *Mycobacterium tuberculosis* (*M. tuberculosis*).[4] Among the secreted proteins, the 6-kDa early secreted antigenic target (ESAT-6) gains importance. ESAT-6 is present in many virulent mycobacterial strains, but absent in BCG and most of the environmental mycobacteria.[5] ESAT-6 antigen is strongly recognized by T lymphocytes and is protective in animal models.[6–8] In humans, it is a predominant target for cell-mediated immunity and induces IFN-γ release in TB patients.[9–11] The recognition of ESAT-6 peptides by T lymphocytes in humans has been studied in many populations.[9–13]

It has been demonstrated that a strong Th1 activity is required for successful containment of *M. tuberculosis* infection.[14] In a region like south India, where BCG vaccine itself is a failure,[15] immune recognition of the protein ESAT-6 will be of interest. The study was aimed to identify "protective epitopes" of ESAT-6 protein in the south Indian population. In order to achieve this aim, IFN-γ secretion and proliferative response to ESAT-6 protein and its overlapping 20-mer peptides were compared in patients with tuberculosis and their healthy household contacts.

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2. Materials and methods

2.1. Study participants

The study was approved by the Institutional Ethics Committee. Informed consent was obtained from the subjects before drawing blood.

2.2. Healthy house hold contacts of TB patients ($n = 23$)

This study group was recruited from families where there was at least one sputum positive PTB patient (index case) living in the same household, sharing the kitchen and bathroom, for at least 3 months immediately preceding the start of treatment of the index case, who were sure to be infected.[16] Their age ranged from 27 to 54 years. The male:female ratio was 13:10. During recruitment, the subjects were clinically evaluated for symptoms of tuberculosis and chest radiographs were taken. None of the HHC presented with clinical symptoms or lesions in chest X-rays excluding the possibility of TB disease. Sputum smears and cultures were also negative in this group. After recruitment and drawing blood, they were followed up for a period of 6 months and none broke down with tuberculosis, which confirmed their disease free status at the time of participation in the study.

2.3. Tuberculosis patients ($n = 34$)

2.3.1. Newly diagnosed pulmonary tuberculosis patients (PTB) ($n = 21$)

Their age ranged from 28 to 52 years and the male:female ratio was 17:4. All the PTB patients were positive for sputum smears by microscopy and culture. The subjects of this group were naive for anti tuberculous treatment. Examination of smear and culture was done according to the methods established in our centre.[17] Two spot and 1 overnight sputum specimens were collected from each patient. Acid Fast Bacilli (AFB) staining was done for smears and they were examined under fluorescence microscope. For culture, sputum was concentrated and inoculated onto Lowenstein–Jensen (LJ) media and incubated for up to 8 weeks at 37 °C and checked for positivity.

2.3.2. Treated tuberculosis patients (TR) ($n = 13$)

This group comprised of subjects who have completed 6 months course of anti tuberculous therapy (ATT). The presence of active TB in this study group was excluded by chest X-ray and smear microscopy. The age range was 26–50 years and the male:female ratio was 10:3. The TR were recruited after a period range of 7–36 months of start of their treatment. The drug regimen followed for these patients was $2EHRZ_3/4RH_3$.

All subjects were seronegative for HIV as evidenced by two enzyme immunoassays (EIAs) Tridot (J.Mitra & Co., India) and Retroquic (Qualprodiagnostics, India) in serum.

PPD (purified protein derivative) skin testing was not done in our study subjects as it is neither sensitive nor specific. It has been observed that >70% of females and >80% of males turn positive for PPD by 25 years due to exposure to *M. tuberculosis* as well as other environmental mycobacteria. Thus PPD positivity in our population can mean infection not only with *M. tuberculosis* but also with other environmental mycobacteria. The more sensitive, Quantiferon test uses antigens unique for *M. tuberculosis*, the test identifies infected subjects and BCG vaccination or exposure to environmental mycobacteria does not affect the test results. Quantiferon TB-Gold (in-tube) test was performed for all the study subjects and all were found to be positive. This test was done after subject recruitment and was not used for preselecting positive subjects.

2.4. Quantiferon TB-Gold in tube assay

The TB infection status was ascertained by an ELISA based Quantiferon TB-Gold kit (Cellestis Inc., Victoria, Australia) as per the manufacturer's instructions. Blood was collected in tubes labeled nil control, mitogen and TB antigen (ESAT-6 and CFP-10 overlapping peptides and a TB 10.4 peptide). The tubes were incubated at 37 °C for 16–24 h and then plasma was collected from the tubes. The cut-off point was set as 0.35 IU/ml as per the manufacturer's instructions.

2.5. Recombinant proteins and synthetic peptides

Recombinant protein ESAT-6 was provided by Dr. Pawan Sharma, New Delhi and has been prepared as described previously.[18] ESAT-6 peptides (20-mers with 10-amino acid overlap) (kind gift from Dr. Thomas B Nutman, USA) (Esp1-Esp9) covered the entire length of the protein. Protein estimation was done using BCA protein assay kit (Pierce, Rockford, IL, USA). Lyophilized peptides were reconstituted in dimethyl sulphoxide (DMSO), aliquoted and stored at −80 °C until use.

The peptides used for *in vitro* studies were not identified based on *in silico* analysis. All the peptides of ESAT-6 covering the entire sequence of the protein were tested in all *in vitro* experiments. Individual peptides were used for all the experiments and the peptides were not pooled for testing the activity.

2.6. Intracellular cytokine staining for IFN-γ, IL-4

Whole blood was diluted 1:2 with Roswell Park Memorial Institute medium (RPMI) (Sigma–Aldrich Corporation, St. Louis, MO, USA) to which costimulatory molecules CD49d (1 μg/ml), CD28 (1 μg/ml), (BD biosciences, San Diego, CA, USA) were added. To the culture plates, overlapping peptides of ESAT-6/ESAT-6 protein were added at pre-fixed optimal concentrations. The plate was incubated for 18 h in the presence of Brefeldin A (5 μg/ml) (BD Biosciences, San Diego, CA, USA) during the last 16 h (Costar, Corning Inc., NY, USA) at 37 °C in 5% CO_2 atmosphere. After stimulation, cells were washed and labeled with fluorochrome-conjugated specific antibodies anti-CD4 APC, CD8 PE-Cy5, IFN-γ PE and CD4 APC, CD8 PE-Cy5, IL-4 PE in separate tubes (BD Biosciences, San Diego, CA, USA). Fixing and permeabilising of cells were done using Cytofix/cytoperm buffer (BD Biosciences, San Diego, CA, USA), washed with Perm/wash buffer (BD Biosciences, San Diego, CA, USA) and then stained for cytokines. After washing, cells were fixed using 4% paraformaldehyde and analyzed using a FACSCalibur Flow cytometer (BD Biosciences, San Diego, CA, USA). Percentage of cytokine secreting CD4 and CD8 cells were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA, version 7.1.1).

2.7. BrdU (Bromo deoxy uridine) incorporation assay

PBMCs were separated from venous blood using Histopaque (Sigma–Aldrich Corporation, St. Louis, MO, USA). To the PBMC, peptides, recombinant proteins of ESAT-6 and *M. tuberculosis* culture filtrate antigen (CFA) were added at the optimum concentration and incubated at 37 °C in 5% CO_2 for 5 days. BrdU incorporation assay was carried out using a BrdU flow kit as per the manufacturer's instructions (BD Biosciences, San Diego, CA, USA).

The cells were fixed and analyzed on a FACSCalibur Flow cytometer (BD Biosciences, San Diego, CA, USA). Data were collected and analyzed using FlowJo software (Tree Star Inc., San Carlos, CA, version 7.1.1).

2.8. IFN γ ELISA

Five day culture supernatants from Peripheral Blood Mononuclear Cells (PBMCs) were assayed for the presence of IFN γ by ELISA (BD Biosciences, San Diego, CA, USA) as per manufacturer's instructions. The detection limit of the assay ranged from 4.7 to 300 pg/ml. The lowest detection limit of the kit was 1 pg/ml.

2.9. MHC typing

DNA was extracted from polymorphonuclear cells as well as PBMCs by a salting out method as described elsewhere.[19] DRB typing was performed by PCR with Sequence specific primers (SSP). Low resolution PCR-SSP was performed for DRB1 alleles.[20]

2.10. In silico prediction of potential binding regions in ESAT-6

Epitope prediction was done for full length ESAT-6 and individual 20-mer sequences of ESAT-6 as used in our *in vitro* studies. MHC binding peptides (9-mers) as potential epitopes were predicted using Propred available at http://www.imtech.res.in/raghava/propred/ [21] for ESAT-6. The default threshold value 3 was used and the HLA screening was restricted to 41 HLA subtypes (of the alleles expressed by our study subjects) out of 51 available in the ProPred.

2.11. Statistical analysis

The results were analyzed using GraphPad Prism (Graphpad Software 4.0, San Diego, USA). The differences among the groups in IFN-γ secretion and proliferation were assessed by one-way analysis of variance (ANOVA) with Bonferroni's correction. A $p < 0.05$ was considered significant.

The number of subjects recruited for the study was relatively small. With 95% confidence interval and 80% power, the total sample size for meaningful results will be 175. Since power calculations (done for hypothesis testing and clinical trials) are not mandatory for a laboratory study like this, it was not performed before subject recruitment.

The IFN-γ responders to individual peptides were calculated and expressed as actual numbers and percentages. Percent responders among various groups were calculated by taking 100 pg/ml cut-off. This cut-off was arrived at by considering mean +3SD of unstimulated culture in each group which was ~100 pg/ml. Many earlier studies have used such a cut-off for ELISA.[22,23] Those values above the cut-off were considered positive. This cut-off was also used for calculating peptide positivity for various alleles in different groups. The IFN-γ response by all the subjects was calculated using Median. Due to variation in IFN-γ response values, Median was calculated for all the groups, rather than arithmetic or geometric mean. Intracellular IFN-γ and IL-4 response in PTB and HHC groups were analyzed by Student's *t* test. Initially, lymphocytes were gated in the forward/side scatter and within which CD4+ and CD8+ cell positivity for cytokines was studied.

For BrdU incorporation assays, the cut-off was set by considering Mean + 3SD of unstimulated culture of each subject.

3. Results

3.1. Percentage responders and IFN-γ levels

The cut-off was set at 100 pg/ml to ascertain the positives. Unstimulated culture values were subtracted from the stimulated values. The response to peptides Esp1, Esp6, Esp2, Esp3, Esp7, Esp8, ESAT-6 protein and CFA by HHC, PTB and TR has been shown (Figure 1).The responders for CFA and ESAT-6 protein among PTB were 14/21 (67%) and 11/21 (52%) respectively (Figure 1).

The peptide responders were then studied out of 11 responders for ESAT-6 protein. Three out of eleven (27%) of ESAT-6 protein responders reacted to Esp1 and 5/11 (45%) to Esp6. The results for rest of the peptides were 3/11 (27% – Esp2), 5/11 (45% – Esp3,7,8) (Figure 1), 3/11 (27% – Esp9), 3/11(27% – Esp5) and 2/11 (18% – Esp4) (Data not shown).

The responders to CFA among HHC were 19/23 (83%) and for ESAT-6 protein, 17/23 (74%). It was observed that 10/17 (59%) and 9/17 (53%) of those reacting to ESAT-6 were positive for Esp1 and Esp6 respectively. For the other peptides, responders were 7/17 (41% – Esp7), 4/17 (24% – Esp8), 4/17 (24% – Esp3), and 4/17 (24% – Esp2)

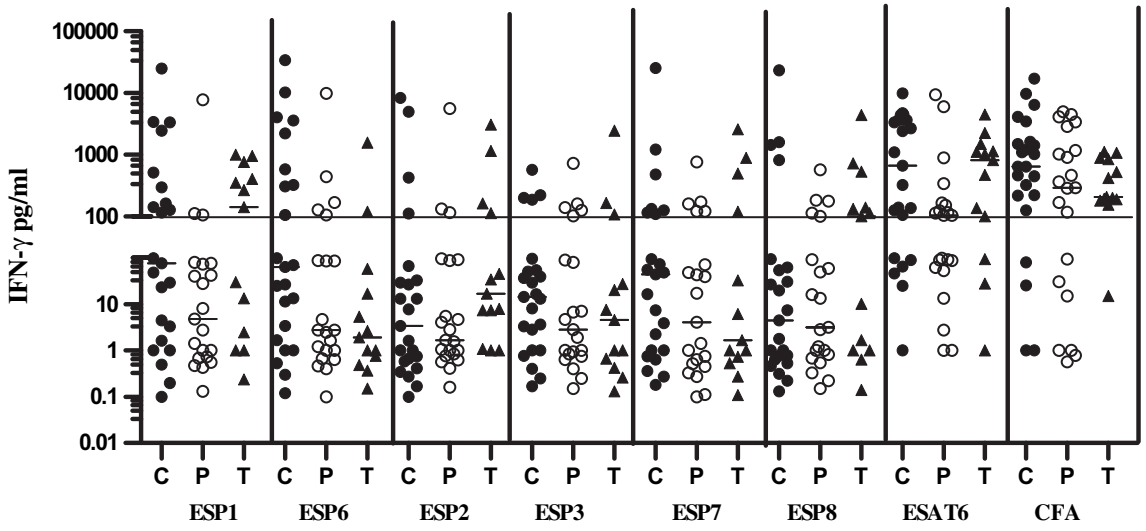

Figure 1. IFN-γ response to peptides Esp1, Esp6, Esp2, Esp3, Esp7, Esp8, whole ESAT-6 and CFA. Response was determined by subtracting the unstimulated culture values from peptide response values. Significance between groups was analyzed by one way ANOVA with Bonferroni's post test. No significance was observed between groups. Each dot represents an individual and the horizontal bar in each group represents the median. The background cut-off is shown as a line at 100 pg/ml. ESAT-6 (Early secreted antigenic target-6) represents whole protein. C-HHC, P-PTB, T-TR.

(Figure 1). The data for other peptides: 4/17 (24% – Esp9), 4/17 (24% – Esp4), 4/17 (24% – Esp5) are not shown.

In TR, 12/13 (92%) responded for CFA and 10/13 (77%) responded to ESAT-6. Among ESAT-6 responders, 7/10 (70%) reacted to Esp1 and Esp8, 4/10 (40%) reacted to Esp2 and Esp7. Other peptide responses were at 3/10 (30%), for Esp3, and Esp6 (2/10 – 20%) (Figure 1). For Esp9 (3/10, 30%), and for Esp4 and Esp5 (1/10, 10%) the response data was not shown in the figure.

Esp-1 was predominantly recognized, whereas Esp4 and 5 were least recognized by all the groups.

The median was calculated for all the groups after subtracting unstimulated from the stimulated culture values. When compared to peptides, ESAT-6 protein and CFA generated higher (median range 290–827 pg/ml) IFN-γ secretion in all the groups. It was also observed that peptides Esp1 and Esp6 induced an increased median IFN-γ (76 and 64 pg/ml) production among HHC (Figure 1). In PTB group, the median IFN-γ response ranged from 1–5 pg/ml for all the peptides.

Differences between the groups were calculated and the response between the groups was not significant. The fold increase in IFN-γ secretion was more for Esp1 (15.7) and Esp6 (23.28) on comparison with PTB. For Esp7 also 10.7 fold increase was observed in HHC, as compared to PTB (Table 1).

Of all the peptides, Esp1 (141 pg/ml), Esp8 (102 pg/ml) and Esp2 (17 pg/ml) induced the maximal response among TR. The response was 1–5 pg/ml for rest of the peptides.

3.2. Intracellular IFN-γ and IL-4

The cytokine positive cells were calculated within CD4+ and CD8+ cells. IFN-γ positive CD4 cells in response to ESAT-6 protein were significantly elevated in HHC, when compared to PTB ($p < 0.01$). Esp1 and Esp6 induced significant increase in IFN-γ positive CD4 cells in HHC, when compared to PTB ($p < 0.01$). The ESAT-6 protein increased the number of IFN-γ positive CD8 cells both in HHC and PTB. Esp1 and Esp6 caused a significant ($p < 0.01$) increase in HHC group than PTB.

The number of IFN-γ secreting CD4 and CD8 cells increased in HHC as well as PTB when compared to IL-4 secreting CD4 and CD8 cells (Table 2). Due to non-availability of samples, the IFN-γ/IL-4 ratio was not assessed in TR.

Esp6 showed the highest IFN-γ/IL-4 ratio when compared to other peptides and the mean ratio was significantly higher ($p < 0.05$) in HHC compared with PTB. Esp1 also showed an increased IFN-γ/IL-4 ratio, but the difference in mean values between HHC and PTB was not significant (Data not shown).

3.3. Proliferative responses to ESAT-6 peptides

Representative graphs on proliferation (BrdU incorporation assay) are given in Figures 2 and 3.

Table 1
Fold increase in interferon gamma response for stimulants.

Stimulants	HHC	PTB	Fold increase
	Median	Median	
Esp1	75.65	4.82	15.7
Esp6	63.55	2.73	23.28
Esp2	3.36	1.56	2.15
Esp3	14.25	2.81	5.07
Esp7	43.63	4.08	10.69
Esp8	4.42	3.17	1.39
ESAT-6	670.8	104.4	6.43
CFA	649.2	291.4	2.23

ESAT-6 represents Early secreted antigenic target-6 protein; CFA stands for culture filtrate antigen.
HHC – healthy household contacts; PTB – pulmonary tuberculosis patients.

For ESAT-6 protein, CD4 cells of 15/23 HHC responded. Among peptides, highest proliferation was observed for Esp1 (16/23), Esp6 (13/23), Esp7 (11/23), Esp8 (7/23) in HHC (Table 3a). In PTB, 9/21 subjects responded to ESAT-6. The response to Esp7 (4/21), Esp6 (3/21) were the highest. Esp2, Esp5 and Esp7 (3/13) were the peptides which showed high responses in TR group.

The HHC response for CD8 cell proliferation to ESAT-6 protein was 12/23. Highest peptide responses were observed for Esp8 (11/23), Esp1 (10/23), Esp7 (10/23), Esp6 (6/23), Esp9 (6/23) in HHC (Table 3b). Among PTB, CD8 proliferation to ESAT-6 was found for 8/21 subjects. An increased proliferation was found for peptides Esp6 and Esp7 (4/21), as well as Esp2 and Esp3 (2/21). The CD8 proliferative response to ESAT-6 was found for 2/13 TR individuals.

3.4. Esp1 and Esp6 and MHC class II alleles

3.4.1. Typing of the subjects for HLA DRB1 gene yielded the following results

Esp1 (aa1-20) produced high IFN-γ levels by HHC of different HLA DRB1 alleles. MHC typing of the 4 high IFN-γ producers showed that HLA DRB1 *04 and *10 were the alleles to which peptides were predominantly bound. The 4 subjects who responded to Esp6 (51–70), had the alleles HLA DRB1 *04, HLA DRB1 *10 and HLA DRB1 *15. (Table 4).

The PTB exhibited predominance of HLA DRB1*15. But, an increased response was not observed for any of the peptides for the alleles studied. The responding alleles in the TR group were HLA DRB1*12 and HLA DRB1*14 and the peptides which gave response were Esp1, Esp2, Esp7 and Esp9.

After subtraction from stimulated culture, 10 and 9 HHC subjects were positive for IFN-γ for the peptides Esp1 and Esp6. But of these IFN-γ positive subjects, HLA results were available only for 4 subjects each. Among treated subjects, 7 and 4 were positive for IFN-γ for the peptides Esp1 and Esp2. Out of these IFN-γ positive subjects, HLA results were available only for 3 and 1 subjects respectively for the peptides. In the case of Esp7 and Esp9, IFN-γ positivity was observed for 4 and 3 subjects. Of these positives, HLA results were available for 2 subjects for each peptide. HLA results were not available for rest of the responders.

3.5. Bioinformatics analyses of putative epitopes in ESAT-6 protein

Potential HLA-DRB1 binding peptides present in the ESAT-6 were predicted using ProPred. Nanomer **L**QNLARTIS (69–77) was predicted to be a promiscuous epitope since it was found to be a binder with 28 HLAs out of 41 selected (all the subtypes for the alleles in our present study). Further, 16 HLAs were predicted to recognize a nanomer from Esp2 (**I**QGN**V**TSIHSLLD:18–26) while 11 HLAs were predicted to bind to two different nanomers (**W**N**F**AGIEAAAS: 6–16) from Esp1 (Table 5).

4. Discussion

In any subunit vaccine development, the important issue to be addressed is the immune response to antigens in genetically heterogeneous populations. This study attempted to investigate the immune response among south Indians (in whom BCG failed to offer protection) to ESAT-6 antigen.

The ESAT-6 has been a protein of considerable interest because it is a predominantly recognized antigen early during infection.[24–26] This antigen was known to induce T-cells to secrete IFN-γ in *M. tuberculosis* infected mice[24,25,27] and humans.[9,11] In the present study, immune responses to overlapping peptides of ESAT-6 were studied in PTB, HHC and TR.

Table 2
Intracellular IFN-γ and IL-4 response for CD4 and CD8 cells in PTB and HHC.

S. No.	Stimulants	IFN-γ				IL-4			
		CD4		CD8		CD4		CD8	
		PTB	HHC	PTB	HHC	PTB	HHC	PTB	HHC
1	Unstimulated	0.48 ± 0.09	0.83 ± 0.24	0.58 ± 0.10	1.07 ± 0.24	0.33 ± 0.11	0.79 ± 0.67	0.42 ± 0.07	1.31 ± 0.57
2	Esp1	0.68 ± 0.18	2.84 ± 0.27*	0.67 ± 0.13	2.95 ± 0.36*	1.88 ± 0.48	0.62 ± 0.44	0.42 ± 0.07	1.44 ± 0.57
3	Esp2	0.09 ± 0.03	0.90 ± 0.38	0.40 ± 0.09	1.56 ± 0.29	0.89 ± 0.31	1.23 ± 0.77	1.26 ± 0.71	0.35 ± 0.07
4	Esp3	0.45 ± 0.23	0.73 ± 0.36	0.31 ± 0.09	1.34 ± 0.51	0.83 ± 0.22	0.84 ± 0.66	0.76 ± 0.37	1.21 ± 0.54
5	Esp4	0.55 ± 0.22	0.89 ± 0.65	0.32 ± 0.06	1.10 ± 0.37	0.92 ± 0.36	1.48 ± 1.32	0.35 ± 0.08	0.44 ± 0.10
6	Esp5	0.89 ± 0.28	0.66 ± 0.37	0.37 ± 0.06	1.51 ± 0.29	1.26 ± 0.45	1.34 ± 0.95	0.42 ± 0.05	0.58 ± 0.30
7	Esp6	0.94 ± 0.35	2.45 ± 0.36*	0.30 ± 0.07	2.27 ± 0.47*	1.09 ± 0.32	1.11 ± 0.98	0.77 ± 0.33	1.00 ± 0.43
8	Esp7	0.80 ± 0.24	1.25 ± 0.39	0.64 ± 0.25	1.91 ± 0.59	1.00 ± 0.47	1.08 ± 0.72	0.53 ± 0.16	0.86 ± 0.52
9	Esp8	0.95 ± 0.44	1.61 ± 0.11	0.28 ± 0.08	1.43 ± 0.42	1.06 ± 0.39	0.88 ± 0.44	0.48 ± 0.12	0.71 ± 0.5
10	Esp9	0.69 ± 0.21	0.81 ± 0.50	0.23 ± 0.04	1.56 ± 0.54	1.07 ± 0.45	0.98 ± 0.70	0.40 ± 0.02	1.46 ± 0.58
11	ESAT-6 ptn.	0.58 ± 0.11	2.90 ± 0.25*	1.68 ± 0.18	3.28 ± 0.36	0.92 ± 0.51	1.76 ± 0.46	1.25 ± 0.27	1.63 ± 0.24

Percentage of IFN-γ and IL-4 positive CD4 and CD8 cells were ascertained by flow cytometry. Comparison was made between PTB and HHC groups by Student's *t* test.
*$p < 0.01$.

HHC were considered as "protected" group because they do not develop the disease even after being continuously exposed to *M. tuberculosis*, thus protective responses are worth studying in this group.

Immunological mechanisms of protection against TB are not yet fully understood and evidence shows the dependence of antigen specific T lymphocytes and their ability to stimulate anti-mycobacterial activity of macrophages through release of IFN-γ. The central role of IFN-γ in the control of TB is demonstrated by experiments which show that the disruption of IFN-γ gene in mice[28] and mutation of IFN-γ receptor gene in humans results in increased susceptibility to TB infection.[29] Thus the ability to stimulate T-cell release of IFN-γ has been used as a critical criterion for identification of protective antigens of TB. Multiple studies provide evidence that antigens recognized by "protected group" but not active TB patients can be considered for vaccine development strategies using IFN-γ response as the protective correlate.[30–32] Although variation exists in antigen recognition and magnitude of response between individuals, this approach is a highly viable method for identifying protective antigens.[33] Hence, we have used IFN-γ as a correlate for protection in screening the ESAT-6 peptide responses.

A generalized low response was observed for ESAT-6 whole protein and all peptides among patients in this study. Although there are reports on high level of IFN-γ in PTB, few studies have shown the low response in PTB than HHC.[10,34–37] There may be a genetic predisposition to inadequate IFN-γ production in some individuals following tuberculosis infection which may lead to failure of macrophage activation and in such individuals infection may progress to active disease. Many earlier studies have shown that tuberculosis patients have a suppressed cell-mediated immunity and this has been reported to be associated with advanced stages of the disease.[38–41] It has been hypothesized that in such advanced stages, cells are recruited to sites of infection leading to paucity of cells in periphery and decreased IFN-γ responses.[42–44] Many other mechanisms have been proposed for the decreased IFN-γ viz., inhibition of T cell responses by inhibitory cytokines (TGF-β) from infected macrophages or defect in antigen presenting cell functions.[45]

In our study, TR has been included as a group to ascertain whether the immune responses are restored or have been modulated due to chemotherapy. The IFN-γ response in TR was increased when compared to PTB and HHC. This might be due to restoration of the immune response after effective tuberculous chemotherapy as shown by earlier reports.[46–48]

In previous studies, IFN-γ response to ESAT-6 peptides has been studied by ELISPOT and ELISA. In a study using ELISPOT,[13] it has been demonstrated among Bombay and Oxford residents that PTB as well as healthy subjects respond to the peptide sequence ESAT-6(aa1–15), ESAT-6(aa6–20), ESAT-6(aa51–65), and ESAT-6 (aa56–70).

In studies in German[9] and Ethiopian & Danish[10] populations, ELISA was used to study IFN-γ response. In the former study, ESAT-6 peptides aa1–20 and aa10–30 have been recognized by PTB and not by healthy subjects, for whom the response was not observed for

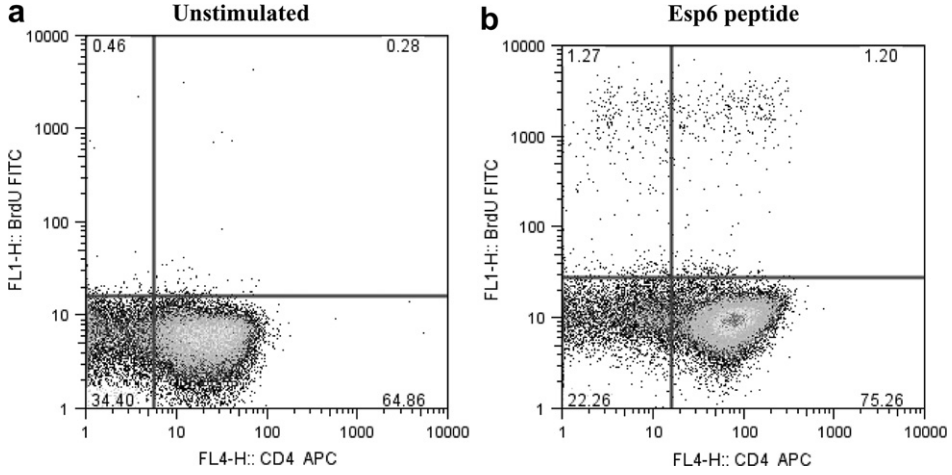

Figure 2. For flow cytometric analysis of proliferating cell population, lymphocyte population was gated in Forward scatter/Side scatter plot and within the gate; BrdU positive CD4 cells were calculated. CD4 cell responses: unstimulated culture (a) and Esp6 peptide (b) by a house hold contact.

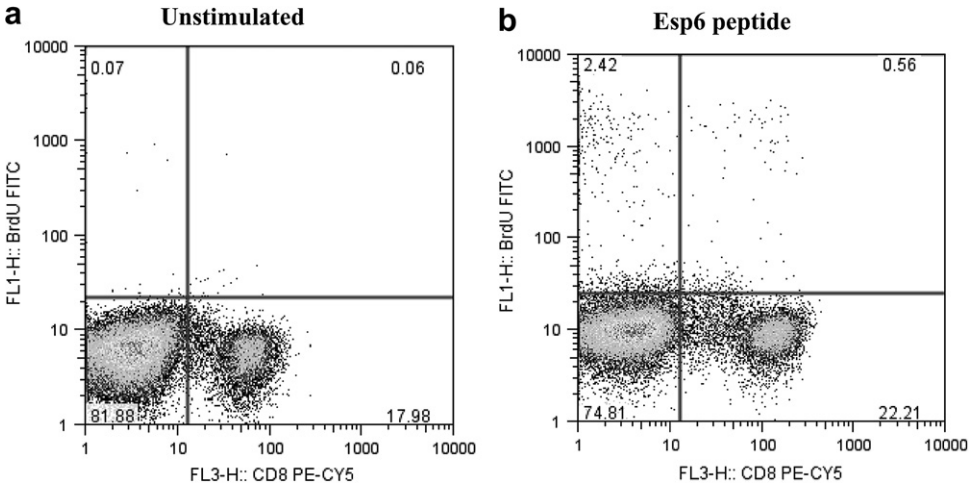

Figure 3. For flow cytometric analysis of proliferating cell population, lymphocyte population was gated in Forward scatter/Side scatter plot and within the gate; BrdU positive CD8 cells were calculated. CD8 cell responses: unstimulated culture (a) and Esp6 peptide (b) by a house hold contact.

any peptide. In the latter study, the groups were healthy volunteers and PTB from Denmark and HHC and PTB from Ethiopia. They have shown that N-terminal aa1–30 and aa 42–75 region to be immunogenic. In a study from Kuwait using ELISA,[49] the peptide sequence aa 72–95 has been shown to induce potent IFN-γ responses in PTB. In this study, the peptide Esp6(51–70) was recognized by PTB. Like the Kuwait study,[49] ESAT-6 aa 72–95 (Esp8 in our study) was recognized in our present study in PTB.

For the 20-mers used in the present study, CD4+ mediated response is expected because of the size of the peptide. But in our study, the 20-mer peptides elicited response (proliferation as well as intracellular IFN-γ and IL-4) in CD8+ cells also. This may due to the 20-mer peptides being processed and binding class I molecules in endocytic compartments or at the cell surface after regurgitation of processed peptides. In a study by Eberl et al.,[50] it has been shown that a 69-mer synthetic polypeptide (a malarial parasite circumsporozoite antigen) is presented to MHC class I restricted CD8 cells. The study involved an *in vivo* system (a mouse model) as well as *in vitro* system (cell lines) and has suggested that serum components such as β_2 microglobulin and proteases may allow the processing and loading of exogenous polypeptides (classically which do not enter cytosolic processing pathway) onto empty cell surface class I MHC molecules for presentation to CD8 cells.

In our study, human AB serum was used in the cell culture medium whose components might have played a role in enhancing the presentation of breakdown products of 20-mer peptides to CD8 cells and activating it.

Other possible mechanisms suggested for presentation of exogenous peptides to CD8 cells are as follows:

Macropinocytosis of phagocytosis of polypeptide by a particular set of macrophages followed by pinosome or phagosome disruption.

Pinocytosis or endocytosis of antigens bound to proteins for which receptors exist on cell surface followed by lysosomal processing, escape into cytosol or regurgitation to the cell surface.

In cell proliferation experiments, the same peptides Esp1, Esp6, Esp7, Esp8 which induced an increased IFN-γ response among HHC

Table 3a
CD4 proliferative response in HHC group.

Subjects	Peptides									
HHC	Esp1	Esp2	Esp3	Esp4	Esp5	Esp6	Esp7	Esp8	Esp9	ESAT-6
1						+				
2						+				
3	+		+		+	+	+			+
4	+									
5				+						+
6	+						+	+		
7	+	+	+	+	+	+	+	+	+	+
8	+							+		+
9	+	+					+	+	+	+
10						+				+
11			+	+		+			+	
12	+					+	+			
13	+				+	+	+			
14	+					+				+
15	+					+	+	+		+
16	+					+	+	+		+
17	+							+		+
18	+	+	+				+			+
19	+					+				+
20	+									+
21	+					+	+		+	+
22							+			+
23										
Total responders	16	3	4	3	3	13	11	7	4	15

Table 3b
CD8 proliferative response in HHC group.

Subjects	Peptides									
HHC	Esp1	Esp2	Esp3	Esp4	Esp5	Esp6	Esp7	Esp8	Esp9	ESAT-6
1	+			+						+
2										+
3	+	+	+	+	+	+	+	+	+	+
4	+							+		+
5	+						+	+	+	+
6						+				
7			+	+						
8								+		
9	+			+			+	+	+	+
10	+	+			+	+	+	+	+	+
11							+			
12	+	+		+	+	+		+		+
13										
14	+		+				+			
15						+				
16	+	+	+					+	+	+
17							+	+		
18							+			+
19						+				
20	+		+					+	+	+
21							+	+		
22		+					+			+
23										
Total responders	10	5	5	5	3	6	10	11	6	12

than PTB, also induced an enhanced proliferation of CD4 and CD8 cells. In PTB, Esp6 and Esp7 elicited proliferation of CD4 and CD8 cells. In TR, Esp1 and Esp8 peptides which gave an increased median IFN-γ production did not induce an increase in proliferation. This discrepancy may be due to the differences in epitope recognition by T cell subsets primed for IFN-γ production or proliferation.[9]

For a protective response, it is not enough to have a higher Th1 response, but also a lower Th2 response. Since IL-4 is a key Th2 cytokine, we measured its level in response to the peptides. A lowered IL-4 response has been indicated for protection in animals as well as humans.[51,52] In Balb/c mice, with IL-4 gene knock out, it has been observed that absence of IL-4 led to diminished growth of *M. tuberculosis* which indicate that IL-4 absence afforded protection.[51] Human data suggest that those subjects (TB healthcare workers) who had pre-existing IL-4 responses had a higher rate of progression to active TB.[52] Because of the importance of IL-4 and Th1 cytokine IFN-γ in tuberculosis and protection, this issue was addressed in the present study. It has earlier been observed by Demissie et al.,[53] in Ethiopian subjects (a TB endemic population) that an increase in IL-4 in subjects correlate with a higher risk of developing tuberculosis by the subjects.[53] While RT-PCR was used by Demissie et al.,[53] for quantifying cytokines IFN-γ and IL-4, we have used flow cytometry for quantification of the cytokine positive CD4 and CD8 cells. Our present study is the first one in concordance with this earlier finding[53] in south Indian population, wherein we have found an increase in IL-4 positive CD4 and CD8 cells in PTB to ESAT-6 as in their study. On studying intracellular IFN-γ/IL-4 ratio, the peptide Esp6 $_{51\text{-}60}$ was the one for which a higher ratio was observed among HHC. In our population, the inclusion of this region in a vaccine would give better outcome than other regions in ESAT-6.

Although the assay for secreted IFN-γ showed positivity for the other peptides (Apart from Esp1, Esp6, Esp7, Esp8) when compared to intracellular IFN-γ, the intracellular staining assay and ELISA have differences. The intracellular staining assay incubation was of lesser duration (18 h) whereas for ELISA it was 5 days. During 18 h time point, effector cells contribute to IFN-γ production and at 5 days, the secretion is attributable to effector as well as memory cells turned into effectors. This might be a reason for response to other peptides in ELISA. Moreover, other cell types can also contribute to IFN-γ secretion in ELISA which might be a reason for response to other peptides.

Many earlier reports have suggested the association between HLA and tuberculosis.[54] It is reported that HLA-DR molecules are

Table 4
HLA typing results of HHC and TR.

HHC	Δ IFN- γ (pg/ml)*	HLA-DRB1
Esp1$_{1\text{-}20}$		
HHC1	49572.57	DRB1***04*10**
HHC2	2079.71	DRB1***04***11
HHC3	296.57	DRB1***04*10**
HHC5	517.2	DRB1*15***10**
Esp6$_{51\text{-}70}$		
HHC1	32518.2	DRB1***04*10**
HHC3	583.52	DRB1***04*10**
HHC6	106.93	DRB1***15***07
HHC7	3567.35	DRB1***15***07
TR		
Esp1$_{1\text{-}20}$		
TR1	269.26	DRB1*15***14**
TR2	671.74	DRB1*15***14**
TR7	961.17	DRB1***12***14
Esp2$_{11\text{-}30}$		
TR7	1165.03	DRB1***12***14
Esp7 $_{61\text{-}90}$		
TR2	398.47	DRB1*15***14**
TR7	897.8	DRB1***12***14
Esp9$_{76\text{-}95}$		
TR1	579.96	DRB1*15***14**
TR2	541.61	DRB1*15***14**

HHC stands for Household contacts, TR for Treated TB patients. The table shows the available HLA results from HHC and TR donors who are IFN-γ responders. The alleles showed in bold are those for which the peptide values were positive. The response to each allele was studied by taking mean for each allele as positives and regarding the mean of all other alleles as negatives. Only the responses to responding peptide regions and the alleles in HHC and TR group are shown. HLA results were not available for many donors.
The bold face alleles represent the positivity when compared to other alleles.
* The IFN-γ values were initially subtracted from the unstimulated culture values (Delta IFN-γ).

Table 5
Potential epitopes in ESAT-6 specific for HLA-DRB1 alleles from South Indian Population.

No.	20-mers	Peptide sequence	*In vitro* response	No. and % HLA-DR alleles predicted *in silico*	MHC-class_II restriction using ProPred
1	1–20	MTEQQ**W**N**F**AGIEAAASAIQG	Yes	11/41 (26.8%)	Nanomer starting with 6th residue (W) predicted as binding peptide with HLA DRB1_0305,DRB1_0401, DRB1_0426, DRB1_1114, DRB1_1323. Nanomer starting with 8th residue (F) DRB1_0401, DRB1_0405, DRB1_0408, DRB1_0426, DRB1_1101, DRB1_1128, DRB1_1305, DRB1_1307.
2	11–30	IEAAASA**I**QGN**V**TSIHSLLD	No	16/41 (39.02%)	Nanomer starting with 18th residue (I) DRB1_0306, DRB1_0307, DRB1_0308, DRB1_0311, DRB1_0401, DRB1_0402, DRB1_0404, DRB1_0405, DRB1_0408, DRB1_0410, DRB1_0421, DRB1_0423, DRB1_0426, DRB1_1107, DRB1_1304, Nanomer starting with 22nd residue (V) DRB1_0405, DRB1_0410, DRB1_1321
3	21–40	N**V**TSIHSLLDEGKQSLTKLA	No	3/41 (7.31%)	Nanomer starting with 22nd residue (V) DRB1_0405, DRB1_0410, DRB1_1321
4	31–50	EGKQSLTKLAAAWGGSGSEA	No	0%	–
5	41–60	AA**W**GGSGSEA**Y**QGVQQKWDA	No	6/41 (14.63%)	Nanomer starting with 43rd residue (W) DRB1_0401, DRB1_0421, DRB1_0426, DRB1_0701, DRB1_0703, Nanomer starting with 51st residue (Y) DRB1_1321
6	51–70	**Y**QGVQQKWDATATELNNALQ	Yes	1/41 (2.43%)	Nanomer starting with 51st residue (Y) DRB1_1321
7	61–80	TATELNNA**L**QNLARTISEAG	Yes	28/41 (68.29%) Promiscuous epitope	Nanomer starting with 69th residue (L) DRB1_0301, DRB1_0305, DRB1_0306, DRB1_0307, DRB1_0308, DRB1_0309, DRB1_0311, DRB1_1101, DRB1_1102, DRB1_1104, DRB1_1106, DRB1_1107, DRB1_1114, DRB1_1120, DRB1_1121, DRB1_1128, DRB1_1301, DRB1_1302, DRB1_1304, DRB1_1305, DRB1_1307, DRB1_1311, DRB1_1321, DRB1_1322, DRB1_1323, DRB1_1327, DRB1_1328, DRB1_1506.
8	71–90	NLARTISEAGQAMASTEGNV	Yes	0%	–
9	76–95	ISEAGQAMASTEGNVTGMFA (76–95)	No	0%	–

Individual 20-mers of ESAT-6 protein (as used in our experiments) overlapping by 10 amino acids were submitted for epitope prediction. Potential HLA-DRB1 binding peptides were predicted by ProPred. In our study population, HLA typing was carried out only at low resolution for HLA-DRB1*03, 04, 07, 11, 13, 15 by low resolution. Therefore, the subtypes of these HLAs alone were screened for 9-mers in the ProPred. The letters shown in bold are the starting/anchoring residues and the underlined region in each peptide sequence shows the minimal peptide(s) predicted by Propred. HLA-DRB1 *10, 12, 14 and 16 were not done because these alleles are not included in the Propred.

the main presenters of mycobacterial antigens to T cells.[55–58] The HLA profiles have also been related to vaccine efficiency because different HLA molecules present peptides to T cells with variable efficiencies.[59] HLA-based vaccines need to be designed for different geographic areas and even for specific ethnic groups.

The HLA-DRB1 gene is expressed five times higher than its paralogues DRB3, DRB4 and DRB5 and DRB1 is present in all individuals. In the present study, we have found a predominance of HLA DRB1*04 and DRB1*10 among HHC and DRB1*15 in PTB and the response to ESAT-6 peptides suggest that these HLA molecules are efficient peptide presenters. In south India, the association of HLA DRB1*10 and DRB1*15 alleles with resistance and susceptibility to tuberculosis infection respectively has been studied.[60] However, HLA-DP and DQ alleles have also been studied in tuberculosis[60,61] and are known to present epitopes. They are also responsible for immune effector functions. In this study, HLA-DRB1 a more polymorphic allele has been typed but not other alleles.

ESAT-6 protein has already been studied by *in silico* methods. MHC binding and experimental evaluation of the predicted regions of ESAT-6 and its peptides in T-cell assays has been performed.[62,63] In the study by Vincenti et al.,[62] the identification of ESAT-6 epitopes was done for all the available HLA class II alleles in the databases. The study by Mustafa & Shaban[63] revealed a promiscuous epitope at 69–77 both by *in silico* and *in vitro* methods and in addition P3(31–55) and P4 (46–70) as promiscuous by *in vitro* studies. We have found in the present study, that the peptide sequence Esp1 (1–20) as immunodominant *in vitro*. This result was supported by our *in silico* analysis which predicted peptides ranging from 6–14, 8–16 and 18–26 as HLA binding peptides. Like the Mustafa & Shaban[63] study, we have identified the epitope 69–77 (Esp7) as a promiscuous epitope by *in silico* for the alleles of our population. Mustafa et al.,[64] have performed MHC-restriction analysis using HLA-DR-typed autologous and allogeneic antigen presenting cells and ESAT-6-specific T-cell lines. But in their study, peptide responsiveness was studied to HLA type expressed by the pulmonary TB patients of Kuwait. Our present study was directed against studying the probable ESAT-6 epitopes for the HLA molecules of the south Indian population.

Initially, epitope prediction by *in silico* was performed for the whole ESAT-6 sequence for the alleles of our population. A potential demerit exists if the prediction is done for individual 20-mers as used for our *in vitro* studies. The immunogenic epitope may be present in any region of the protein sequence. On using 20-mers, the epitopes present in a region between any two different peptide sequences would be missed from identification in any of the peptide sequence. For example, the peptides Esp6(51–70) and Esp7(61–80) were found to be immunodominant *in vitro* in this study. Since ten amino acids are shared beween these peptides, the complete sequence analysis will help in predicting the minimal epitope (nanomer) that is present at 69–77 (the amino acid position being present in Esp6 and Esp7). Epitope prediction was done by using ESAT-6 sequence as well as individual 20-mers in the present study.

In vitro response was observed for Esp1, 6, 7 and 8 in the present study. Using ProPred, potential HLA-DRB1 binding peptides were predicted. The immune response was observed *in vitro* for Esp6 and Esp7. Among all, the nanomer **L**QNLARTIS (69–77) was predicted by ProPred to be a promiscuous epitope and this result is in accordance with the *in vitro* results wherein Esp7 was found to be immunogenic *in vitro*.

Further, only one HLA was predicted to recognize a nanomer (51–59) from Esp6 while an enhanced immune response was observed for the Esp6 *in vitro*; on the other hand the predicted promiscuous epitope **L**QNLARTIS (69–77) has the binding residue (anchor site) in the 69th position that is present in both Esp6 and

Esp7. The presence of the anchor residue (69L) in both the peptides might be the reason for the *in vitro* response against Esp6 and Esp7. Therefore, further investigation is required to identify the minimal epitope within the region of 51–80, causing immune response against Esp6 and Esp7 in the south Indian population. Further, *in vitro* immune response observed against Esp1 correlated with *in silico* analysis. Out of 41 HLA alleles restricted in ProPred in accordance with our HLA typing, 11 (26.8%) HLA alleles were predicted to bind with nanomers from Esp1 while, 16 (39.02%) HLA alleles were predicted to bind with nanomers from Esp2. Further investigations would be required to confirm whether any promiscuous epitopes are present in Esp1 or Esp2.

The study has a few drawbacks. HLA typing which shows the binding of specific epitopic regions of ESAT-6 to HLA-DRB1 alleles has not been confirmed using peptide-binding assays. Moreover, the number of responding subjects is also small in HLA-DR studies, so larger number of responders will be needed to establish the observed results. Also, HLA results were not available for all the donors. Although only HLA-DRB1 has been typed in the present study, the involvement of other HLA class II molecules (DP, DQ) in peptide presentation also deserve attention, which has not been performed in this study. Recently, instead of single cytokine secreting population, the focus has moved towards poly functional T cells (IFN-γ^+TNF-α^+IL-2^+). Although IFN-γ and IL-4 responses were focused for protection in this study, understanding of poly functional and regulatory T cell responses would provide more detailed insight into protection.

From our present study on ESAT-6 in south India, we report that certain regions ($Esp1_{1-20}$, $Esp6_{51-70}$) are more commonly recognized by the HLA DRB1 alleles *04 and *10 by the ''protected population'' (HHC). We observed IFN-γ responses, proliferation and a decreased IL-4 response for these regions. These results suggest that in a region where even BCG vaccine was a total failure, certain immunogenic regions of ESAT-6 are recognized by the ''protected HHC population''. Further investigation of this protein along with other immunogenic proteins in a larger cohort of subjects will aid in designing an effective vaccine for this region.

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