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Research paper

Immunogenicity of late stage specific peptide antigens of *Mycobacterium tuberculosis*

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ABSTRACT

Global burden of latent TB infection comprises one-third of the world population. Identifying potential *Mycobacterium tuberculosis* (Mtb) latency associated antigens that can generate protective immunity against the pathogen is crucial for designing an effective TB vaccine. Usually the immune system responds to a small number of amino acids as MHC Class I or Class II peptides. The precision to trigger epitope specific protective T-cell immune response could therefore be achieved with synthetic peptide-based subunit vaccine.

In the present study we have considered an immunoinformatic approach using available softwares (ProPred, IEDB, NETMHC, BIMAS, Vaxijen2.0) and docking and visualizing softwares (CABSDOCK, HEX, Pymol, Discovery Studio) to select 10 peptides as latency antigens from 4 proteins (Rv2626, Rv2627, Rv2628, and Rv2032) of DosR regulon of Mtb. As Intracellular IFN-γ secreted by T cells is the most essential cytokine in Th1 mediated protective immunity, these peptides were verified as potential immunogenic epitopes in Peripheral Blood Mononuclear Cells (PBMCs) of 10 healthy contacts of TB patients (HTB) and 10 Category I Pulmonary TB patients (PTB).The antigen-specific CD4 and CD8 T cells expressing intracellular IFN-γ were analyzed using monoclonal antibodies in all subjects by multi-parameter flow cytometry.

Both, PTB and HTB individuals responded to DosR peptides by showing increased frequency of IFN-γ^+CD4 and IFN-γ^+CD8 T cells. The T-cell responses were significantly higher in PTB patients in comparision to the HTB individuals. Additionally, our synthetic peptides and pools showed higher frequencies of IFN-γ^+CD4 and IFN-γ^+CD8 T cells than the peptides of Ag85B.

This pilot study can be taken up further in larger sample size which may support the untapped opportunity of designing Mtb DosR inclusive peptide based post-exposure subunit vaccine.

1. Introduction

Tuberculosis has become a global health crisis evident by the data that 2 billion world population is exposed to the deadly pathogen out of which approximately 1.5 million deaths occur annually. The scenario is challenging for endemic countries especially India and Africa where 40% of the TB patients go undiagnosed (WHO, 2016). Recently, with the evolving new strains of the pathogen resistant to the potent drug-regimen, control of tuberculosis infection has further become cumbersome.

Understanding the mechanism of host cell immune response along with the antigenic structure of pathogen is extremely crucial in vaccine development studies. Availability of entire Mtb genome sequence led to expedited research for identifying the specific antigens which could activate and mount a protective Th1 type immune response (Geluk et al., 2014). Various early phase secreted whole-protein antigens of Mtb such as Hsp60,Ag85A,Ag85B,ESAT-6,CFP-10 have already hit the clinical trials for their ability to be recognized by TLRs and stimulate Th1 type cells. But, these whole-protein based vaccines provide limited protection as they are only expressed during early stages of active TB

Abbreviations: TB, Tuberculosis; BCG, Bacillus Calmette-Guerin; Mtb, *Mycobacterium tuberculosis*; DosR, Dormancy survival regulon; PTB, Pulmonary TB patients; HTB, Healthy contacts of TB patients; PBMC, Peripheral Blood Mononuclear Cell; IL-2, Interleukin-2; FCS, Fetal Calf Serum; IFN-γ, Interferon gamma

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infection (Kaufmann et al., 2017). Whole protein based subunit vaccine unnecessarily increases the immunogenic load and may lead to allergic responses in the host (Li et al., 2014). Also, neither whole pathogen nor the pathogen whole protein is immunogenic; only specific amino acid fragments within the pathogen acts as potential immunogen and are sufficient to mount protective immune response in the host. This rationale leads to designing of 'peptide based vaccine' which incorporates only specific immunogenic peptide epitopes. Identification of all the potential immunogenic epitopes within the pathogen genome can be done using Reverse vaccinology approach with the help of available softwares. Incorporation of antigens expressed during both early and late phase of TB infection with or without BCG could be an ideal approach to design an effective vaccine against Mtb. Thus, entirely synthetic peptide based vaccines holds a promising future for vaccination (Skwarczynski and Toth, 2016).

The dormancy survival regulator or DosR regulon consists of 48 genes (1.3% of the Mtb genome) classified into 9 functional categories and plays an important role in Mtb adaptation in adverse conditions (Peddireddy et al., 2016; Singh et al., 2014). Immuno-informatic approach based on scanning of vital Mtb proteins like DosR proteins for their immunogenicity can be crucial in understanding the pathogenesis of TB infection. Mtb pathogenesis initially starts with recognition of pathogen associated molecular patterns (PAMPs) by receptors on immune cells especially macrophages and dendritic cells (Kornfeld et al., 1999). After recognition, antigens are processed into smaller peptide fragments and mounted on Major Histocompatibility Complex (MHC) molecules. MHC class II loaded with peptide epitope prime the $CD4^+$ helper T cells which further activates the $CD8^+$ cytotoxic T cells. The T-cell subsets are known to be crucial in host mediated immune response to pathogen (Stenger, 2001), (Kaufmann, 2002). Macrophage activation by T cells appears to be a central step of acquired resistance against Mtb (Gideon and Flynn, 2011). T lymphocytes are known to secrete different types of interleukins and pro-inflammatory cytokines like Interferon- γ (IFN-γ) and IL-2 involved in the activation of macrophages (Kaufmann, 1991). IFN-γ activates macrophages and stimulates them to ingest and kill mycobacterium more effectively (Philips and Ernst, 2012). The role of $CD4^+$ T cells as chief mediators of anti-tuberculous immunity has been reported through many studies (Mogues et al., 2001; Orme, 1987). The importance of cytokine mediated activation of macrophages and control of bacterial growth is well studied in mouse models (Cooper et al., 1993; Flynn et al., 1993). $CD8^+$ T cells also have a cytolytic function which results from recognition of mycobacterium antigens presented by MHC Class I molecules on the surface of infected macrophages (Sud et al., 2006).

The present study is focused on investigating the immunogenicity of some of these DosR proteins and characterizing the immunodominant T-cell peptide epitopes within these proteins. Immunoinformatic studies predicted a large number of promiscuous peptide epitopes within 4 DosR genes namely Rv2626, Rv2627, Rv2628, and Rv2032 as probable antigens; strongly binding the alleles of MHC class I and II. *Ex vivo* results with 10 of these promiscuous synthetic peptides on PBMC of 10 PTB patients and 10 HTB subjects elicited a heightened T-cell population of IFN-γ^+ CD4 and IFN-γ^+ CD8 T cells as compared with the unstimulated PBMC.

2. Materials and methods

2.1. Selection of T cell epitopes within the proteins by Bioinformatic prediction

For MHC Class II, 9 and 15 amino acid long peptides were selected using software ProPred and IEDB respectively. ProPred and IEDB have been extensively used as immunological softwares for predicting MHC class II restricted CD4+ T cell binding epitopes (Fleri et al., 2017; Mustafa and Shaban, 2006). For ProPred, top scoring peptides with high class II binding affinities were chosen by setting a threshold of > 1%. In case of IEDB, SMM-align method was employed to obtain potential MHC Class II binders. SMM align is a stabilization matrix algorithm methodology that allows for relevant prediction of peptide:MHC binding affinities (Nielsen et al., 2007). Cut-off value of IC50 within 250 nM was chosen for the predicted binders. The antigenic score for each promiscuous epitope was generated using Vaxijen2.0 (Doytchinova and Flower, 2007). A threshold of 0.4 was set and score of > 0.4 was considered antigenic. Depending on the promiscuity and the antigenic scores, we selected 10 of these peptide epitopes for further study. MHC Class I restricted CD8 T cell epitopes of these DosR encoded proteins has already been reported by us (Pandey et al., 2016).

2.2. Peptide MHC docking by CABS dock and Hex

The binding of top scoring antigenic promiscuous peptides to their respective class II alleles was investigated by docking. One MHC Class II allele, HLA-DRB1*01:01 (PDB ID-4MCZ) was used to carry out docking because of its global presence. Firstly, the docking was carried out by the CABS dock server where the HLA allele and peptides were subjected to energy minimization (Kurcinski et al., 2015). The linear peptide and MHC molecule were separated from the docked structure and docked again using Hex to obtain an energy score (Macindoe et al., 2010). The control peptide was a rheumatoid arthritis epitope "GVYATRSSAVRLR". A thorough analysis of binding energy (Kcal/mol) of the test and control peptide with the HLA allele was performed. The docked structures obtained from both the software were visualized using Discovery Studio Visualizer 4.1 (Accelyrs Inc., USA). The binding sites were also analyzed for the hydrogen bonds and hydrophobic interactions between the amino acid residues of peptide and the HLA molecule. The docking of promiscuous peptides with MHC class I alleles has already been reported by us (Pandey et al., 2016).

2.3. Custom synthesis of peptides

The 10 bio-informatically selected peptides were custom synthesized as per sequence given below (BioChain Incorporated).

Peptide 1- YMREHDIGA- Rv2626.
Peptide 2- GLDPNTATA- Rv2626.
Peptide 3- AAAGTTANV-Rv2032.
Peptide 4- IYYVDANAS- Rv2626.
Peptide 5-LIGLNGLSL-Rv2627.
Peptide 6- SAFRLSPPV- Rv2627.
Peptide 7- YAGIADRLV- Rv2627.
Peptide 8- WHPRKVQSA- Rv2628.
Peptide 9- YAIGEHLSV- Rv2628.
Peptide 10- QRPRHSGIR- Rv2628.

Three peptide pools were also designed. Pool A and Pool B constituted of 4 peptides each according to their promiscuity. Pool P constituted of all the 10 synthetic peptides. Two peptides of 30kD-secretory protein antigen 85B (Ag85B) namely peptide 17-SMGRDIKVQFQSGGN-31 (Ag85B P5) and peptide 21DIKVQFQSGGNNSPA-35 (Ag85B P6) were selected to be positive controls as Ag85B is a well-established immunogenic early phase Mtb secretory protein. Both peptides have a good vaxijen score of 1.531 and 1.451 respectively and were obtained from BEI resources.

Pool A- Peptide 1, 2, 3, 4.
Pool B- Peptide 4, 5, 6, 9.
Pool P- All ten peptides.

2.4. Study subjects

In our study, we have included 10 category 1 pulmonary TB patients (PTB) from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis Hospital (RBTB), Mahatma Gandhi Marg, GTB Nagar, Delhi, India. PTB patients were all sputum and chest X-ray positive with other symptoms like fever, coughing and weight loss (age range

18–52 yrs). Ten (10) healthy contacts of TB patients (HTB) individuals were selected from Mycobacteriology Laboratory, Vallabhbhai Patel Chest Institute (VPCI), University of Delhi (age range 25–53 yrs).

All the subjects were BCG vaccinated. The donors provided informed consent in writing. All the human blood related work was strictly conducted in accordance with the ethical guidelines for biomedical research on human subjects as contained in Declaration of Helsinki and the protocol was ethically approved by Human Ethical Committee of RBTB Hospital.

2.5. HLA typing of the subjects

Peripheral blood (2 ml) was collected from all the 20 subjects. Using standard protocol from the manufacturer (HiYield Real™ Genomic DNA Mini Kit) the genomic DNA was isolated from the peripheral blood of each subject.HLA typing was carried out using HISTO TYPE SSP test kits for tissue typing of HLA alleles (Class I: HLA-A and B and Class II: HLA-DR).Gel electrophoresis was done followed by gel visualization using BIORAD Gel Doc™ XR + with Image Lab™Software. For Interpretation of the data, software BAG HISTO MATCH Version 2.6.5.0 was used.

2.6. PBMC isolation and peptide stimulation

Peripheral blood (10 ml) was collected from all the subjects. Peripheral Blood Mononuclear Cells (PBMC) were isolated by density gradient centrifugation on Ficoll-hypaque at 400 $\times g$ for 30 min at 22 °C. Interface cells (PBMC) were recovered and cultured at 1×10^5 cells/ml in 12 well culture plates and treated with Interleukin-2 (IL-2) 30 IU. The culture plates were left for 24 h rest and incubated at 37C in 5% CO2 humidified air.

Kinetic studies were performed and two-time points *i.e.* 2 h and 4 h for stimulation with 10 μg of each Peptide/ Peptide pool were found to be optimum for IFN-γ production. Along with the peptide stimulation, 10μg/ml Brefeldin A (Biolegend, USA) was added for studying the intracellular expression of IFN-γ.

2.7. Flow cytometry to detect peptide specific CD4 and CD8 T cells expressing intracellular IFN-γ

After 2–4 h of stimulation, cells were harvested, washed in PBS supplemented with 0.5% BSA and stained for cell surface markers $CD4^+$ and $CD8^+$ with anti-CD4-PE-Cy7 (clone: A161A1), and anti-CD8-PE (clone:SK1) respectively. Cells were washed with PBS and permeabilized for 20 min with permeabilization buffer (1 ×, Biolegend, USA). Cells were washed with PBS and stained with anti human-IFN-γ-APC (clone: 4S·B3, Biolegend, USA) for 30 min, washed in PBS and acquired in BD Accuri C6 flow cytometer and analyzed using BD Accuri C6 software.

3. Results

3.1. Selection of T cell epitopes within the proteins by bioinformatic prediction

Among all the 4 DosR proteins, Rv2627 was the largest in size generating a total of 404 nonamers with a step size of one amino acid residue. Rv2628 was the smallest with a total of 111 overlapping nonamers. Out of 52 class II HLA DR alleles present in ProPred, Rv2627 and Rv2032 showed binding affinity for 96% of the alleles. A total of 15 HLA DR alleles are covered under IEDB using SMM align algorithm. Rv2032, Rv2626c, and Rv2627 showed binding affinity for approximately 80% of them. Promiscuity means a given MHC molecule can bind numerous different peptides and a promiscuous peptide motif is recognized by several different MHC molecules (Brusic et al., 1998). For MHC Class II, peptides which bound to 5 alleles in both ProPred and IEDB were considered promiscuous. The binding profile for all the selected proteins along with their evaluation parameters have been enlisted in Table 1. Highest vaxijen score of 2.16 was observed for Rv2628 followed by 1.90 for Rv2627. Rv2626 and Rv2032 gave a vaxijen score of 1.7. Rv2627 generated 49 promiscuous epitopes which was much higher than the 16 promiscuous epitopes generated by Rv2032. Rv2626 generated a total of 8 promiscuous epitopes and Rv2628 gave only 1 promiscuous epitope binding to class II MHC alleles. The binding affinity of 4 proteins to Class I MHC alleles using BIMAS and NETMHC3.4 is already published by us (Pandey et al., 2016).

Table 1
Prediction of $CD4^+$ T cell binding epitopes of the selected DosR Proteins.

Software	Parameter	Rv2626c	Rv2627	Rv2628	Rv2032
ProPred	Overlapping nonamer	134	404	111	322
	Total number of binding epitopes	8	32	4	16
	Number of alleles bound	42	50	14	50
	Number Of promiscuous epitopes	2	10	1	11
IEDB	Overlapping nonamer	134	404	111	322
	Total number of binding epitopes	56	226	41	179
	Number of alleles bound	12	12	4	13
	Number Of promiscuous epitopes	6	39	0	5
Vaxijen 2.0	Antigenic score	1.70	1.90	2.16	1.77

Using ProPred: Rv2627 and Rv2032 bound to largest number and Rv2628 to least number of alleles. A total of 24 promiscuous epitopes were found in all the 4 genes. Using IEDB: Rv2032 bound to maximum alleles but generated a total of 5 promiscuous epitopes. Rv2627 bound to 12 alleles but gave highest number of 39 promiscuous epitope. Rv2628 gave no promiscuous epitopes but bound to 4 different MHC II alleles.

Ten of these peptides were then shortlisted based on their promiscuity, antigenicity, and affinity for either class I or class II MHC alleles or both. At least one peptide from each protein Rv2626c, Rv2627, Rv2628, and Rv2032 of DosR was selected. Peptide 6 'SAFRLSPVV' of Rv2627 showed binding affinity for maximum number of 33 HLA class I and 9 HLA class II alleles with highest vaxijen score of 1.9157. Peptide 9 'YAIGEHLSV' of Rv2628 showed binding affinity for 20 HLA class I and 5 HLA class II alleles and a vaxijen score of 1.08. Peptide 1, 2, and 3 showed binding affinities for only class I MHC alleles. The antigenicity and binding profile of these peptides are listed in Table 2.

3.2. Peptide MHC docking by CABS dock and Hex

All the ten shortlisted peptide epitopes were docked with HLA-DRB1*01:01(PDB ID- 4MCZ) and their energy scores were generated using Hex 8.0.0. The maximum energy score of −564.94 was observed for peptide 9 'YAIGEHLSV' of Rv2628. The next highest energy score of −508.11 was observed for peptide 6 "SAFRLSPPV" of Rv2627. The energy score of control peptide was −696.0. Peptide 9 formed five hydrogen bonds and Peptide 6 formed four hydrogen bonds at various positions with the given MHC allele(Fig. 1, Table S1). These peptide epitopes have hydrophobic AA at the fourth and sixth position as these residue help in binding the peptide into the MHC groove as reported (Reizis et al., 1996; Wieczorek et al., 2017)

3.3. Flow cytometry to detect peptide specific CD4 and CD8 T cells expressing intracellular IFN-γ

PBMC's were gated on $IFN\gamma^+$ CD4 and $IFN\gamma^+$ CD8 cells and threshold for positive staining was set using FMO (Fluorescence minus one) controls (Fig.S1). FMO controls are necessary to negate the flow data caused due to multiple fluorochromes in a given panel. Among 10 PTB individuals, stimulation with Peptide 4,6,8,9, and Pool B showed a

Table 2
Antigenicity and Binding profile of ten shortlisted peptides of DosR proteins, their pools, and peptides of Ag85B.

Peptide ID	Sequence	Vaxijen score	Alleles bound
P1	YMREHDIGA	1.2034	A*02:03, A*02:06, A*02:11, A*02:12, A*02:50
P2	GLDPNTATA	1.2741	A*01:01, A*02:01, A*02:11, A*02:12, A*02:16, A*02:50
P3	AAAGTTANV	1.8645	A*02:03, A*02:19, A*02:50, A*68:02, A *69:01, B*51:01, B*51:02, B*51:03
P4	IYYVDANAS	1.1663	A*24:03, DRB1*03:05,DRB1*03:09,DRB1*04:01,DRB1*04:04,DRB1*04:08, DRB1*04:21,DRB1*04:23,DRB1*04:26,DRB3*,DRB1*13:02,DRB1*01:01
P5	LIGLNGLSL	1.2084	A*02:50, B*07:02, B*07:07, B*08:01,B*14:02,B*15:17, DRB1*07:01,DRB1*15:01, DRB1*15:06, DRB1*01:01, DRB1*01:02
P6	SAFRLSPPV	1.9157	A*02:16,A*30:01,A*32:07,A*32:15,A*68:23,A*69:01, B*27:20,B*39:01,B*04:03,B*46:01, B*51:01 DRB1*51:01,DRB1*51:02,DRB1*51:03,DRB1*52:01,DRB1*01:01,DRB1*01:02,DRB1*03:01,DRB1*03:05,DRB1*03:06,DRB1*03:07,DRB1*03:08,DRB1*03:09,DRB1*03:11,DR- B1*04:01,DRB1*04:08,DRB1*04:21,DRB1*04:26,DRB1*07:03,DRB1*08:02,DRB1*08:13,DRB1*11:01,DRB1*11:07,DRB1*11:14,DRB1*11:20,DRB1*11:28,DRB1*13:02,DRB1*13:05,- DRB1*13:07,DRB1*13:23,DRB1*04:04,DRB1*09:01,DRB1*07:01,DRB1*04:01,DRB1*03:01,DRB1*04:05, DRB5*01:01
P7	YAGIADRLV	1.2044	B*51:01, B*51:02, B*51:03, DRB1*01:01, DRB1*07:01, DRB1*09:01, DRB1*11:20, DRB1*11:28, DRB1*13:02, DRB5*01:01
P8	WHPRKVQSA	1.2193	B*14:02,B*15:09, DRB1*04:02,DRB1*08:13,DRB1*08:01, DRB1*08:02, DRB1*07:01
P9	YAIGEHLSV	1.082	A*02:03,A*02:06,A*02:11,A*02:01,A*02:16,A*02:19,A*02:50,A*25:01,A*21,A*32:15,A*66:01,A*68:23,A*69:01, B*15:17, B*35:01, B*39:01, B*40:13, B*46:01, B*51:01, B*51:02, B*51:03 B*53:01 DRB1*01:01,DRB1*07:01,
P10*	QRPRHSGIR	0.9367	B*27:05
Pool A	P1 + P2 + P3 + P4	–	A*01:01, A*02:01,A*02:03, A*02:06, A*02:11, A*02:12, A*02:16, A*02:19, A*24:03,A*02:50,A*68:02,A*69:01, B*51:01,B*51:02,B*51:03, DRB1*03:05,DRB1*03:09,DRB1*04:01,DRB1*04:08,DRB1*04:21,DRB1*04:06, DRB3*
Pool B	P4 + P5 + P6 + P9	–	A*02:16,A*02:50,A*30:01,A*32:07,A*32:15,A*68:23,A*69:01,A*24:03, B*27:20,B*39:01,B*40:13,B*46:01,B*51:01,B*07:02,B*08:01,B*14:02,B*15:07 DRB1*51:01,DRB1*51:02,DRB1*51:03,DRB1*52:01,DRB1*01:01,DRB1*01:02, DRB1*03:01,DRB1*03:05,DRB1*03:06,DRB1*03:07,DRB1*03:08,DRB1*03:09, DRB1*03:11,DRB1*04:01,DRB1*04:08,DRB1*04:21,DRB1*04:26,DRB1*07:03, DRB1*08:02,DRB1*08:13,DRB1*11:01,DRB1*11:07,DRB1*11:14,DRB1*11:20, DRB1*11:28,DRB1*13:02,DRB1*13:05,DRB1*13:07,DRB1*13:23,DRB1*14:17, DRB1*15:01,DRB1*15:06,DRB1*04:04,DRB1*09:01,DRB1*07:01,DRB1*04:01, DRB1*03:01,DRB1*04:05, DRB5*01:01, DRB3*
Ag85B P5	SMGRDIKVQFQSGGN	1.531	B*27:05, DRB1*03:01,DRB1*03:05,DRB1*03:06,DRB1*03:07,DRB1*03:08,DRB1*03:09, DRB1*03:11,DRB1*11:07
Ag85B P6	DIKVQFQSGGNNSPA	1.4515	B*27:05, DRB1*04:01

*Although Peptide 10 is not promiscuous but it was selected for having a good vaxijen score.
On the basis of promiscuity, antigenicity, and affinity for either class I or class II MHC alleles or both, 10 peptides were shortlisted. At least one peptide from each protein Rv2626c, Rv2627, Rv2628, and Rv2032 of DosR were selected. Peptide 6 'SAFRLSPVV' of Rv2627 showed binding affinity for maximum number of 33 HLA class I and 9 HLA class II alleles. Peptide 1, 2, and 3 showed binding affinities for only class I MHC alleles. Pools of respective peptides bound a wide range of MHC alleles. Two peptides of Ag85B were included as positive control. Antigenicity defined by the vaxijen score for each peptide was also highest for peptide 6 followed by peptide 3 and peptides of Ag85B. A threshold value of 0.4 was setup and values ≥ 0.4 is considered to be antigenic.

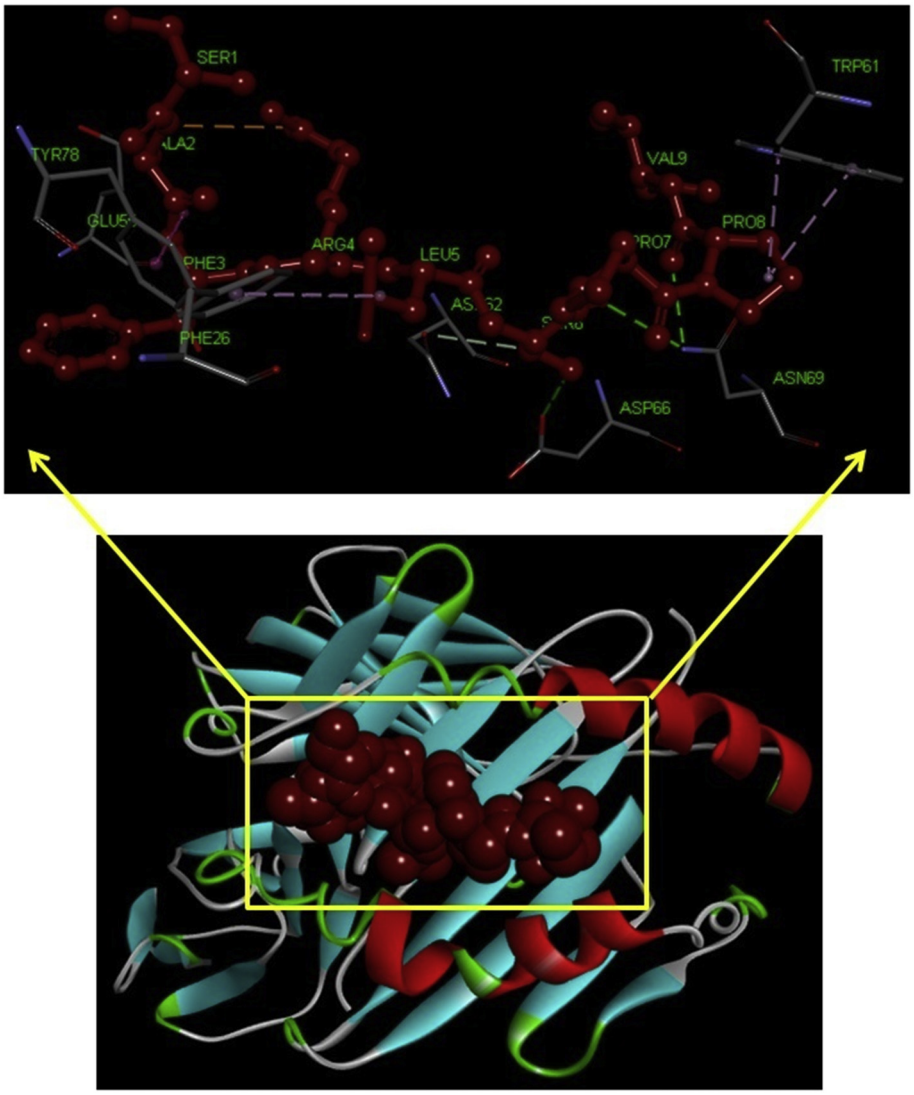

Fig. 1. Docking of representative antigenic promiscuous peptide of Rv2627 with MHC class II HLA DRB1*01:01. Ball and stick model of epitope "SAFRLSPPV" (cherry red) docked onto the DRB1*01:01 (4MCZ). The inset shows the detailed interactions of epitope (cherry red) with MHC residue (grey). Potential bonds formed between the two are shown as dotted lines (green-hydrogen, orange-electrostatic, purple-hydrophobic). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

statistically significant (*$P < .05$ **$P \leq .01$) increased frequency in range of 0.01–5.9% IFNγ^+CD4T cells and of 0.01–6.7% IFNγ^+CD8 T cells as compared to the un-stimulated PBMC. In addition peptide 5 and Pool A also showed a statistically significant(*$P < .05$) increased frequency in range of 0.02–3% IFN-γ^+CD8 T cells as compared to the un-stimulated PBMC.

Among 10 HTB subjects, stimulation with almost all the peptides and their pools showed increased frequency in range of 0.01–1.11% IFN-γ^+ CD4 and IFN-γ^+CD8$^+$ T cells as compared to the un-stimulated PBMC (Fig. 2(i) and 2(ii)).

Experimentally, subjects were categorized as 'good responders' if the frequency of IFN- γ^+ CD4 and IFN- γ^+CD8T cells in the peptide stimulated samples were higher than the mean of un-stimulated sample. Subjects were considered as 'high responders' if the frequency of IFN-γ^+ CD4 and IFN- γ^+ CD8 T cells in the peptide stimulated samples were higher than the 95% Confidence limit (mean + 2SD)of the un-stimulated sample. The MHC binding affinity of 10 shortlisted promiscuous peptide epitopes was matched with the HLA profile of the subjects and the number of predicted responders was observed (Table 3a and b) and it was found that the subjects showed a predicted binding to maximum 3 to 10 of the chosen peptides.

There were no predicted responders producing IFN- γ^+ CD4 T cells in response to peptide 1,2,3, and 10 since these peptides had no binding affinity for MHC class II alleles. *Ex vivo* results showed 30–80% subjects as good responders and 20% subjects as high responders among PTB patients producing IFN-γ^+ CD4 T cells on stimulation with peptide 1,2,3, and 10. *Ex vivo* results among HTB contacts showed 10–30% good responders producing IFN-γ^+ CD4 T cells on stimulation with peptide 1,2,3, and 10. A maximum of 10% HTB subjects were found to be high responders when stimulated with peptide 2 producing IFN-γ^+ CD4 T cells (Fig. 3 and Table S2).

Among PTB patients, 10–100% predicted responders were observed with peptide 4, 5,6,8,9, Pool A and Pool B for both T cell subsets however, *ex vivo* results gave 30–60% good responders and 10–40% high responders. Peptides1,2,3,7 and 10 showed a predicted binding in 10–60% PTB subjects while stimulation with these peptides showed 20–30% good responders and 20% high responders with IFN-γ^+ CD8 T cells among PTB subjects. Peptide 7 showed 10% PTB subjects as predicted responder and *ex vivo* stimulation showed 30% good responders and 10% high responders with IFN-γ^+ CD4 T cells. Peptides of Ag85B showed predicted binding in 10–40% PTB subjects and stimulation with these peptides showed 20–30% good responders and 10–20% high

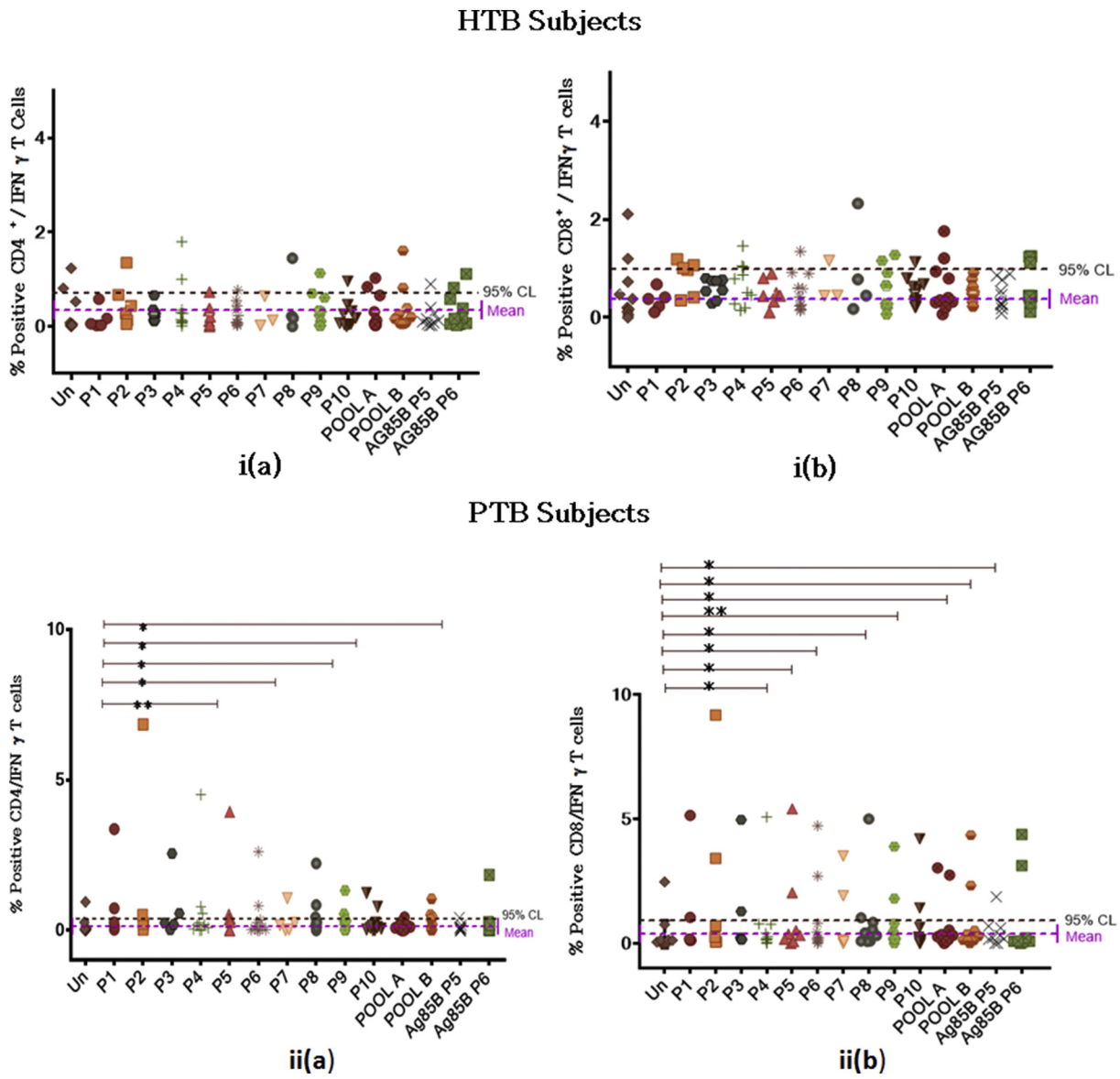

Fig. 2. Estimation of i(a,b)CD4$^+$IFN-γ$^+$and CD8$^+$IFN-γ$^+$cells of HTB individuals and ii(a,b)CD4$^+$IFN-γ$^+$and CD8$^+$ IFN-γ$^+$ cells of PTB patients following stimulation with selected promiscuous peptide epitopes of DosRregulon proteins. PBMCs were stimulated for 2 to 4 h. with respective synthetic peptides. Flow cytometry was done for IFN-γ$^+$CD4 and IFN-γ$^+$CD8 T cells. Statistical analysis was done between un-stimulated and peptide stimulated samples with non-parametric Wilcoxon matched-pair signed-rank test. Result were represented as Mean with 95% Cl. $*P < .05$ $**P \leq .01$.

responders among PTB subjects for both T cell subsets (Fig. 3 and Table S2).

Among HTB subjects, 10–100% predicted responders were observed with peptide 4, 5,6,8,9, Pool A and Pool B producing both T cell subsets however, *ex vivo* results gave 10–70% good responders and 10–50% high responders. There were 10–40% good HTB responders with IFN-γ$^+$ CD4 T cells while 30–60% good HTB responders with IFN- γ$^+$ CD8 T cells on stimulation with peptide 6, 7, 9, and 10. *Ex vivo* results also showed 10–50% high responders producing IFN-γ$^+$ CD8 T cells on stimulation with peptide 6, 7, 9, and 10. Stimulation with Pool A and Pool B gave 30–70% good responders and 10–40% high responders for both T cell subsets. Peptides of Ag85B showed 10–60% predicted HTB responders for both T cell subsets. However, *ex vivo* stimulation showed 20–80% good responders and 10–30% high responders with peptides of Ag85B (Fig. 3 and Table S2).

4. Discussion

A total of 48 genes in Mtb, encoded within the DosR regulon, are late phase expressing proteins responsible for persistence of pathogen in dormant state(Hu et al., 2006; Voskuil et al., 2003). Many of these DosR proteins have been extensively studied for their imunogenicity and as markers to discriminate between active and latent TB infection(Meier et al., 2018; Rakshit et al., 2017; Roupie et al., 2007; Zvi et al., 2008). DosR proteins have been reported to be specifically recognized by T-cells in Mtb exposed individuals. Immune response against DosR antigens is indicative of the protective strategy of host cell to suppress the Mtb pathogen (Demissie et al., 2006; Leyten et al., 2006). T cell mediated immune response plays a central role by inducing protective immunity against intracellular pathogen including Mtb(Canaday et al., 2001; Cooper, 2009; Gideon and Flynn, 2011). The subsets of T lymphocytes including CD4 and CD8 T cells have gained attention for their key role of secreting IFN-γ. At present IFN-γ is considered to be one of the prominent surrogate markers of protective immunity against Mtb

Table 3
HLA type of a) HTB individuals and b) PTB patients showing predicted binding profile with different peptides.

a)

PTB subjects	HLA type	Predicted peptide binding with
A	A*29,A*11,A*32,B*55,B*27,DRB1*01,DRB1*15	P4,P5,P6,P7,P9,PoolA,PoolB, Ag85B P5, Ag85B P6
B	A*11, A*24, B*07,B*40,DRB1*15,DRB1*16, DRB5*	P4,P5,P6,P7,P9,PoolA,PoolB
C	A*01, A*33, B*37,B*44,DRB1*07,DRB1*11, DRB3*, DRB4*	P2,P4,P5,P6,P7,P8,P9, PoolA, PoolB, Ag85B P5
D	A*02, A*03,A*24, B*44,B*52,DRB1*07,DRB1*15, DRB5*	P1, P2, P3, P4, P5,P6, P7,P8, P9, PoolA, PoolB
E	A*24,A*74,B*07,B*08,B*35,DRB1*03,DRB1*07,DRB3*,DRB4*	P4, P5,P6, P7,P8, P9, PoolA, PoolB,Ag85B P5
F	A*03,A*33,A*30,B*35,B*44,DRB1*13,DRB1*07,DRB3*,DRB4*	P4, P5,P6,P7, P8, P9, PoolA, PoolB
G	A*03, A*25, A*24, B*07, B*15, DRB1*11, DRB1*12, DRB4*	P4,P5,P6,P7,P8,P9, PoolA, PoolB, Ag85B P5
H	A*01, A*31, A*74,B*37,B*51,DRB1*04,DRB1*10	P2, P3, P4, P6, P7, P8, P9, PoolA, PoolB, Ag85B P6
I	A*02, A*11,B*27,B*52,DRB1*15,DRB1*13, DRB3*,DRB4*	P1, P2, P3, P4, P5, P6,P7,P9,P10, PoolA, PoolB
J	A*01, A*33,B*37,B*58,DRB1*03,DRB1*11, DRB3*	P2, P4, P6,P7, PoolA, PoolB, Ag85B P5

b)

HTB subjects	HLA type	Predicted peptide binding with
K	A*24,A*68,B*08,B*40,DRB1*03,DRB1*14, DRB3*	P3,P4,P5,P6,P9,PoolA,PoolB,Ag85B P5
L	A*01, A*32, B*51, B*52, DRB1*04, DRB4*	P2,P3,P4,P6,P7,P8,P9,PoolA,PoolB, Ag85B P6
M	A*02,A*11,B*15,B*48,DRB1*15,DRB1*12, DRB3*,DRB5*	P1,P2,P3,P4,P5,P6,P7,P8,P9, Pool A, Pool B,
N	A*01, A*02, B*08, B*58, DRB1*03, DRB3*	P1,P2,P3,P4,P5,P6,P9,Pool A,PoolB, Ag85B P5
O	A*11, B*57, B*52, DRB1*15, DRB1*07, DRB4*,DRB5*	P4,P5,P6,P7,P8,P9,PoolA,PoolB,
P	A*03, A*24, B*57,B*35, DRB1*14, DRB1*11, DRB3*	P4,P6,P7,P9,Pool A, Pool B, Ag85B P5
Q	A*02, A*11, B*07, B*35, DRB1*10, DRB1*14, DRB3*	P1,P2,P3,P4,P5,P6,P9,PoolA,PoolB,
R	A*26, B*44, B*51, DRB1*11, DRB1*13, DRB3*	P3,P4,P6,P7,P9,PoolA,PoolB,Ag85B P5
S	A*01,A*02,B*52,B*40,DRB1*09,DRB1*11,DRB3*, DRB4*	P1, P2, P3, P4, P5, P6,P7, P9, PoolA, Pool B, Ag85B P5
T	A*01,A*03,B*07, B*50, DRB1*15, DRB1*03, DRB3, DRB5*	P4,P5,P6,P7,Pool A, Pool B, Ag85B P5

All the study subjects were MHC typed for HLA- A, B, and DRB. The predicted binding profile of the shortlisted peptides was matched with the known HLA of the subjects and the binding predictability was observed.

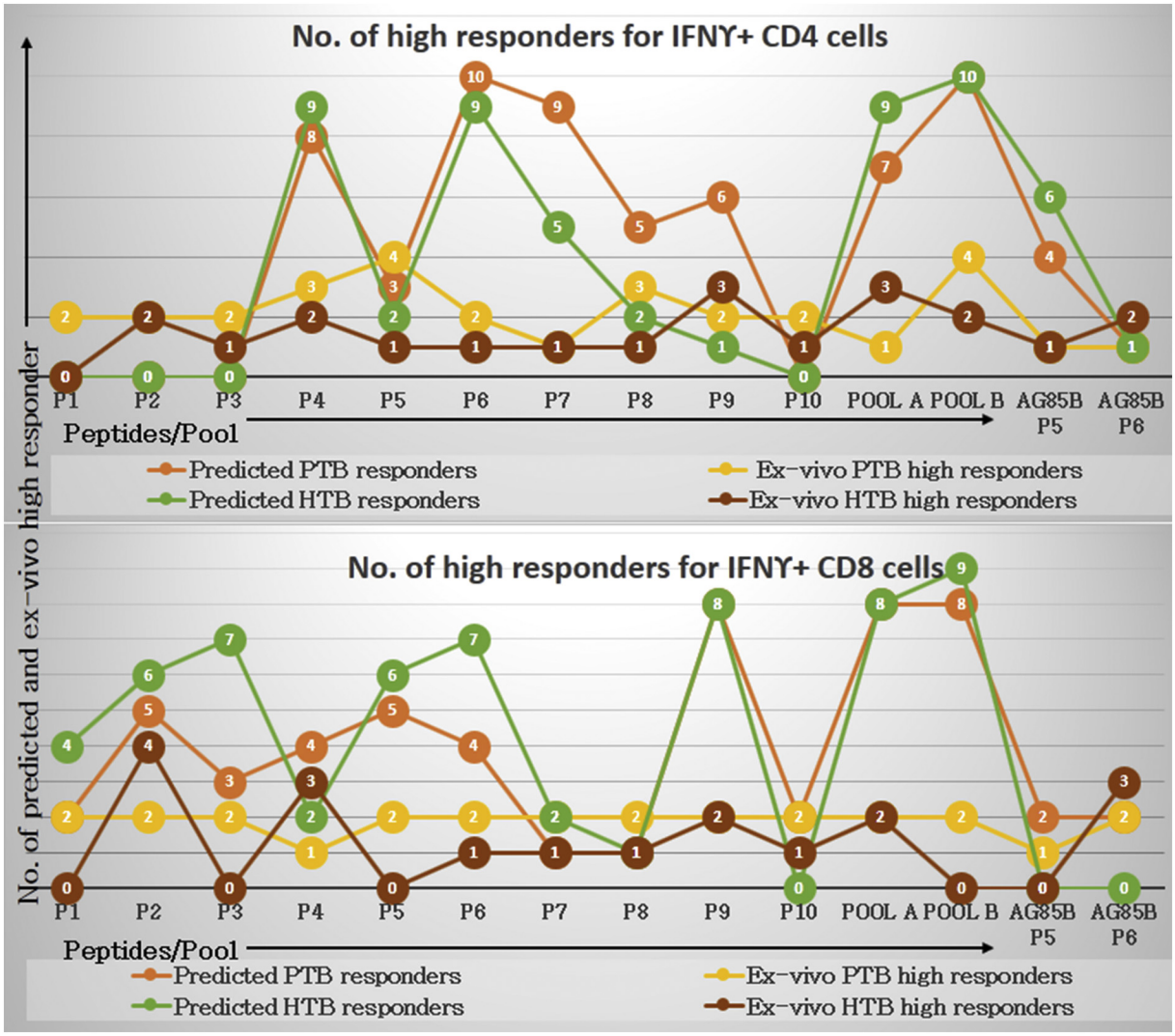

Fig. 3. Total number of predicted responders and experimental high responders on the y-axis inducing IFN-γ^+CD4 and IFN-γ^+CD8 T cells for each synthetic peptide on the x-axis.

On the basis of HLA profile of the subjects and the binding affinity of our synthetic peptide/pool for different MHC alleles, total number of predicted responders for specific peptide/pool was observed. On the basis of *ex vivo* stimulation of PBMC, subjects were considered to be high responders if the frequency of IFN-γ^+CD4 and IFN-γ^+CD8 T cells in the peptide stimulated samples were above the mean + 2SD of the un-stimulated sample.

(Cowley and Elkins, 2003);(Flynn et al., 1993; Yuk and Jo, 2014). The use of intracellular cytokine staining (ICS) in detection of antigen induced cytokine production helps in studying the major players of the immune system in infectious diseases (Prezzemolo et al., 2014).

We have already reported that sixteen DosR regulon proteins can be sudied as potential vaccine candidates (Singh et al., 2014). In another publication we predicted the immunogenic potenial of six such DosR proteins Rv2626, Rv2627, Rv2628, Rv2029, Rv2032, and Rv2031 binding to class I MHC alleles using bio-informatic analysis(Pandey et al., 2016).

The present study highlighted the MHC class II restricted CD4 T cell epitopes within 4 DosR proteins namely Rv2626c, Rv2627, Rv2628, and Rv2032 and validated the immunogenicity of these peptides in an *ex vivo* system using PBMC of 20 BCG vaccinated Indian subjects (10 PTB patients and 10 HTB contacts).

In silico analysis through softwares ProPred and IEDB predicted an enormous repertoire of epitopes that could be identified in just four of the selected Mtb DosR-regulon-encoded proteins. These four proteins were found to be highly immunogenic and can be ranked on the basis of number of promiscuous epitopes generated by them (Leyten et al., 2006; Roupie et al., 2007) Rv2627 was found to be highly immunogenic followed by Rv2032, Rv2626, and Rv2628. The reason for highest immunogenicity of Rv2627 can be attributed to the large size of this protein generating maximum number of nonamers and its binding affinity for a wide range of HLA-DRB alleles. ProPred has a total of 52 alleles out of which epitopes of Rv2627 showed binding to 50 of them. Out of a total of 15 HLA-DRB alleles documented in IEDB, epitopes of Rv2627 were able to bind to 12 of them. Surprisingly, the vaxijen or the antigenic score for Rv2628 was 2.16 which was highest among all the 4 proteins followed by 1.90 for Rv2627. The fact that a pathogen protein is processed into smaller epitopes and only some of them are potential antigens, accomplishes both promiscuity and immunogenicity as more accurate parameters to analyse the potential of any given antigenic epitope.

Individual *in silico* analysis of each of the 10 selected peptide epitopes showed peptide 6 'SAFRLSPPV' of Rv2627 to bind a maximum number of MHC alleles. Peptide 6 has been reported to be highly immunogenic and binds a wide array of MHC alleles (Pandey et al., 2016) and hence, is predicted to bind maximum number of subjects. Computational analysis depicted peptide 6 of Rv2627 to be the most immunogenic among the 10 selected peptides. Docking of peptide 6 with HLA-DRB1*01:01 gave a high energy score which can be attributed to

the 5 hydrogen bonds that the peptide forms with the MHC allele. Hydrogen bonding secures the peptide's N- and C- terminus independent of peptide sequence and serves as a bridge between peptide and the hydrophilic MHC residue (Fan and Wiley, 1999). Surprisingly, the energy score of peptide 9 was the highest which can be attributed to 5 hydrogen and 9 hydrophobic bonds formed between the peptide and the MHC molecule.

We validated the immunogenicity of these immuno-informatically selected peptides by performing *ex vivo* stimulation of PBMC's. PBMC of all the 10 PTB and 10 HTB subjects when stimulated with our 10 selected synthetic peptides and some of the peptide pools showed some interesting results. In our study the length of peptides was kept to be 9 amino acids which is ideal for both class I and II MHC binding (Dimitrov et al., 2010; Paul et al., 2013; Vani et al., 2006).

Ag85B induces a strong T-cell proliferation and IFN-γ secretion in most healthy individuals exposed to *M. tuberculosis*, and in BCG-vaccinated mice and humans (Huygen et al., 1996; Lozes et al., 1997). Therefore, we have included two peptides of Ag85B having a high vaxijen score as positive control in our *ex vivo* studies.

Interestingly, the HTB individuals and PTB patients showed even a higher percentage of IFN-γ^+ CD4 and IFN-γ^+CD8 T cells on stimulation with some of our peptides/pool as compared with the antigenic peptides of Ag85B. Stimulation with some of our synthetic peptides 4,5,6,8, and Pool B showed more subjects as high responders for IFN-γ^+CD4 T cells in PTB subjects and IFN-γ^+ CD8T cells in HTB subjectsas compared to the peptides of Ag85B.

The findings of our study indicates the responsiveness for 10 synthetic DosR peptides in varied stages of Mtb exposure and infection. Statistically significant increased popultaion of IFN-γ secreting CD4 and CD8 T cells on stimulation with peptide 4,6,8,9 and pool B in PTB subjects shows the expression of these late phase associated antigenic epitopes even during active TB infection. Additional peptides such as peptide 5, pool A, and pool B showed significant increased population of specific IFN-γ^+ CD8 T cells indicating the immunogenicity of our peptides slightly skewed towards cytotoxic T cell mediated immune response. PTB subjects I and J were consistently high responding individuals with almost all the peptide/pool stimulating IFN-γ production from both the T cell subsets. However, PTB patient B and D were high responders only for IFN-γ^+ CD4 T cells with these peptides. Six of the HTB subjects (L,N,O,P,S, and T) were among high responding individuals with many of our peptides stimulating higher frequency of IFN-γ^+ CD8 T as compared to that of IFN-γ^+ CD4 T cells.

We found some interesting observations by comparing the results of *in silico* and *ex vivo* analysis. Peptide 6 of Rv2627 as predicted in ProPred and IEDB was the most immunogenic among the 10 shortlisted peptide epitopes with a high docking energy score. *Ex vivo* experiments with peptide 6 also gave significant T cell response in PTB subjects and at least 40% good responders among the subjects. Peptide 9 of Rv2628 with highest docking energy score also gave significant increased frequency of T cell subsets secreting IFN-γ in PTB subjects and at least 30% good responders among the subjects. Additionally, some of the peptides like P1,P2, P3, and P10 having either no or limited binding affinity for MHC class II and hence, no predicted responders still showed some good responders within subjects in *ex vivo* experiments. These observations signifies the importance of considering both *in silico* and *ex vivo* results to reach a conclusion.

Experimental methodologies, such as the source of T cells, the incubation period and the use of T-cell stimulatory cytokine *i.e.* IL-2 can affect the sensitivity of intracellular IFN-γ assays (Smith et al., 2015). These results very well explain the diversified results of the T cell profiles within 20 subjects observed in our study. Sample size of only 10 PTB and 10 HTB subjects can be one of the limitations of this study. The HTB subjects in this study were not tested using IGRA or Tuberculin skin test but because of their high probability of getting exposed to pathogen are considered to be close contacts of TB patients.

In conclusion, our study showed prominent priming and activation of IFN-γ secreting T cell responses specific to Mtb DosR peptide antigens which were significantly higher for PTB patients than HTB subjects. We hypothesize these peptide antigens to be associated with cell mediated immune response and thus, can control TB infection in all stages. These immunogenic DosR peptide epitopes therefore would be taken up further for post-exposure vaccine designing studies with larger sample size comprising of normal BCG vaccinated individuals, active TB patients, and latently infected individuals. We also wish to study the poly-functionality of T cells in terms of cytokine production.These results significantly enhance our understanding of the human immune response to phase-dependent antigens in long-term control of infection, and pave the way for designing Mtb DosR antigen inclusive post-exposure peptide-based vaccine.

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Conflict of interest

The authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103930.

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