# Discovery of dormancy associated antigens of Mycobacterium tuberculosis : novel targets for the development of post-exposure or therapeutic tuberculosis vaccines

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# Chapter 3

## Identification of CD4+ and CD8+ T cell responses to *Mycobacterium tuberculosis* DosR regulon encoded dormancy antigens and mapping of HLA class I and II restricted peptide-epitopes.

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## Abstract

Effective global anti-tuberculosis (TB) strategies involves development of better vaccines, including preventive as well as post-exposure -or therapeutic- TB vaccines. The latter aim at preventing TB reactivation in already infected individuals, particularly since the widely used BCG-vaccine fails to protect against reactivation TB. Over 2 billion people are thought to be latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). Target antigens suitable for post-exposure vaccination need to be expressed by *M. tuberculosis* during clinical latency, i.e. late stage infection. We previously reported human T cell activity against *M. tuberculosis* DosR regulon encoded antigens, which are strongly up-regulated during the *M. tuberculosis* stress response upon intracellular infection. Human T cell responses to *M. tuberculosis* DosR regulon encoded antigens were associated with control of latent TB infection, but the nature of the response remains unidentified. Protective cellular immunity to *M. tuberculosis* thrives on both $CD4^+$ and $CD8^+$ T cell mediated responses. Here, we identify the presence of both $CD4^+$ and $CD8^+$ T cell responses towards a series of immunodominant *M. tuberculosis* DosR regulon encoded antigens in *M. tuberculosis* responsive individuals. We further show that *M. tuberculosis* DosR regulon encoded antigens contain both $CD4^+$ and $CD8^+$ T cell epitopes, which are recognized in the context of HLA-class II or class I molecules, respectively. Proliferative responses were visualized using a CFSE based assay, allowing simultaneous detection of $CD4^+$ as well as $CD8^+$ proliferative T cell responses to the same antigens and peptides. These results significantly increase our understanding of the human immune response to *M. tuberculosis* phase/stage specific antigens, and may help in designing *M. tuberculosis* DosR regulon encoded antigen or peptide based vaccination approaches to TB.

## Introduction

Annually, TB claims over 1.5 million lives and causes active (infectious) TB disease in over 9 million cases. It is estimated that over 2 billion people are latently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis (TB). This vast reservoir of latently infected individuals forms a major source of new TB cases. One in every 10 *M. tuberculosis* infected individuals will eventually develop active TB during their lifetime whereas the remainder is able to contain the bacilli without developing any clinical symptoms. Current control of the TB pandemic is seriously impeded by the strong increase in TB morbidity and mortality during active HIV co-infection, and the continuous rise of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains (38).

*Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is today's only available TB vaccine. BCG protects against severe forms of TB in young children and against leprosy, but does not efficiently and consistently protect against pulmonary TB in adults (8,13). Effective anti-TB strategies not only require better preventive vaccines, but also vaccines exerting post-exposure/therapeutic activity (25,40) since BCG is ineffective in latently infected individuals. Post-exposure TB vaccines should aim at preventing reactivation of TB infection in latently infected individuals by inducing robust immunity to antigens that are expressed by persisting *M. tuberculosis* bacilli during latent infection. Such immune responses are considered essential in controlling, or even better, eradicating persisting bacilli. This likely requires induction of adequate $CD4^+$ and $CD8^+$ T-cell responses against these dormancy associated *M. tuberculosis* antigens. Recently, we showed that genes of the *M. tuberculosis* DosR (Rv3133c) regulon encode antigens that can induce significant T cell responses in *M. tuberculosis* infected individuals (latently or actively infected)(18). The 48-gene DosR regulon is expressed by tubercle bacilli during in vitro exposure to hypoxia, low dose nitric oxide and carbon monoxide, conditions thought to be encountered by *M. tuberculosis* in vivo when persisting in immuno-competent hosts (36). Approximately half of the *M. tuberculosis* DosR regulon is also expressed over prolonged periods of time in the recently proposed enduring hypoxia response (EHR) model (30). Immunity to *M. tuberculosis* DosR regulon encoded antigens seems to be associated with control of latent TB infection, since several DosR regulon encoded antigens were (preferentially) recognized by individuals with latent TB infection (LTBI) (10,18,29). We therefore hypothesized that immune responses to these antigens might contribute to the control of persistent *M. tuberculosis* infection.

However, the exact nature of these human T cell responses to *M. tuberculosis* DosR regulon encoded antigens has not been studied in detail. Both $CD4^+$ and $CD8^+$ T cells participate in the optimal protective response to *M. tuberculosis*. Here, we describe a number of relevant and highly recognized *M. tuberculosis* DosR antigens that induce both $CD4^+$ and $CD8^+$ human T cell responses in mycobacteria primed individuals. Responses were mapped to a series of *M. tuberculosis* DosR regulon encoded peptides, which were recognized in the context of HLA class I and HLA class II molecules. Detailed epitope mapping offers the potential to elucidate possible subdominant epitopes; these might be exploited in designing improved vaccines by inducing

immunity to a broader epitope repertoire than would be seen following natural infection or protein vaccination (22,24).

## Materials and Methods

### Study subjects

Buffy coats from twenty-one in vitro PPD responsive ($PPD^+$) healthy anonymous, HLA typed blood bank donors were included in this study. No prior selection was made concerning HLA type. No clinical information is available from these anonymous healthy donors concerning TB infection/exposure, TB contact history or BCG vaccination, except that they were healthy and had no chronic viral infections. However, given the low TB incidence in the Netherlands, and the lack of a national BCG vaccination policy, none of these parameters would be expected to be of high significance in this cohort. Additionally, PBMC from 5 TB patients and 8 tuberculin skin test (TST) converters were studied. Blood of these 13 donors were obtained by venapuncture after written informed consent was obtained. The study protocol was approved by the Institutional Review Board of the Leiden University Medical Centre (LUMC). PBMC were isolated using Ficoll density gradient centrifugation and stored in liquid nitrogen until use.

### *M. tuberculosis* antigens and peptides

Recombinant proteins were produced as described previously (14). Briefly, selected *M. tuberculosis* H37Rv genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway Technology (Invitrogen, San Diego, CA). Proteins were over-expressed in *Escherichia coli* strain BL21(DE3) and purified as described (14). All recombinant proteins were subjected to quality control assays including sequencing, size and purity check, residual endotoxin levels, non-specific T-cell stimulation and potential cellular toxicity (19). Purified protein derivative (PPD, batch RT49) was purchased from Statens Serum Institute, Denmark.

Synthetic peptides were synthesized as previously described (17). Peptides from *M. tuberculosis* DosR antigens Rv1733c, Rv2029c, Rv2031c, Rv2627c and reference antigen Ag85B were 20-mers peptides with 10 amino acids (aa) overlap except peptides 20-22 of Ag85B which were 15-mers with 10 aa overlap. The 20-mer peptides of Rv1733c, Rv2029c and Rv2627c were elongated with two lysine (K) residues at the C-terminal to improve solubility. Candidate HLA-A2 epitopes consisted of 9-mers and candidate HLA-DR3 epitopes were 15 or 16-mers. The HLA-A*0201-restricted, HIV-1 p17 $Gag_{77–85}$ epitope (SLYNTVATL) was used as control peptide (34).

### Generation of $CD4^+$ T cell lines

T cell lines were generated as previously described (2). In brief, PBMC were incubated at 1-2x$10^6$ cells/well in 24-well plates (Nunc, Roskilde, Denmark) in the presence of *M. tuberculosis* lysate (5 μg/ml) in IMDM supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Breda, The Netherlands) and 10% pooled human serum; further addressed as culture medium. After 6 days, IL-2 (25 U/ml) (Cetus,

Emeryville, CA, USA) was added and cultures were continued for another 2 to 3 weeks in presence of IL-2. T-cells were frozen and stored in liquid nitrogen until use.

**Generation of CD8+ T cell lines**

$CD8^+$ T cell lines were generated by using (autologous) dendritic cells (DC) as antigen presenting cell (APC) and three *M. tuberculosis* DosR regulon encoded candidate HLA-A2 epitopes. PBMC from a HLA-$A2^+$ TB patient (HLA-A2, A3, and DR11) were used: at day -7, $CD14^+$ cells were isolated by using MACS beads (Miltenyi, Germany) and plated in 6-wells plates at $3x10^6$ $CD14^+$ cells in RPMI, 10% FCS, containing 80 ng/ml GM-CSF (Biosource) and 500 U/ ml rIL-4 (Peprotech) further referred to as DC culture medium, at 37°C and 5% $CO_2$ to induce immature DCs (iDC) (35). $CD8^+$ T cells were isolated from the $CD14^-$ fraction. Both $CD8^+$ and $CD8^-$ fraction were frozen and stored in liquid nitrogen until use. iDCs were matured on day -1 in DC culture medium, supplemented with 50 ng/ml LPS. At day 0, mature DCs (mDCs) were harvested and resuspended in RPMI with 1% pooled human serum in the presence of 25 μg/ml per HLA-A2 candidate epitope and 3 μg/ml β2-microglobulin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 4 hours at 37°C and 5% $CO_2$ with hourly resuspension of cells. After washing, peptide loaded mDCs were irradiated at 2500 rad. $CD8^+$ and $CD8^-$ T cell fractions were thawed and mixed in a 10:1 ratio and resuspended at $3x10^6$ cells in 1 ml in a 24-wells plate, forming the responding population. To the responders cells 1 ml stimulation culture (irradiated, peptide pulsed mDCs) was added (in a ratio 10:1 respectively) in culture medium supplemented with 10 ng/ml IL-7 and 50 pg/ml IL-12. At day 7, 1 ml medium was refreshed with culture medium and IL-7 and IL-2 in final concentration of 20 ng/ml and 50 U/ml respectively. At day 12, $4x10^6$ autologous PBMC were seeded at $4x10^6$ cells/ml in IMDM in a 24-wells plate. After two hours, non-adherent cells were washed away and 20 μg/ml per HLA-A2 candidate epitope and 3 μg/ml β2-microglobulin were added for an additional 4 hours in a volume of 0.5 ml/well. The responder CTL culture was resuspended at $1.5x10^6$ cells/ml in culture medium. Medium was removed from the peptide pulsed adherent cells and 1 ml CTL culture was added. At day 14, culture medium was added and IL-7 and IL-2 in final concentration of 20 ng/ml and 50 U/ml respectively. The CTL culture was restimulated every 7-8 days as described above, two days following restimulation, IL-7 and IL-2 were added as described above.

**Lymphocyte stimulation assays**

*Proliferation based on CFSE dilution:*

PBMC were thawed, washed and labeled at $10^7$ cells/ml with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C, dark in PBS/0.5% bovine serum albumin (BSA, Sigma-Aldrich, Zwijndrecht, The Netherlands). Labelling was stopped by adding 10% heat-inactivated fetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands). Cells were washed in PBS/0.5% BSA and resuspended in culture medium. Cells were added to 96-well U-bottom microtiter plates (Nunc) at $1.5x10^5$ cells/well and stimulated in duplicate with antigens at 37°C and 5% $CO_2$. Antigens were tested in the following concentrations: DosR antigens, Ag85B and their respective single peptides, control peptide (HIV-1 p17 $Gag_{77-85}$): all at 10 μg/ml; PPD at 5 μg/ml; phytohaemagglutinin

(PHA, Remel, UK) at 2 μg/ml (positive control), and medium only as negative control. Cells of duplicates were pooled and washed with PBS with 0.1% BSA for evaluation by flow cytometry. Similar proliferation and readout assays were performed for the *M. tuberculosis* HLA-A2 candidate epitopes but with minor changes: cells were tested at $1x10^5$ cells/well (six-fold) in culture medium for either six or ten days. At day six, supernatants were harvested followed by staining cells for flow cytometry as described below. Ten day cultures included addition of recombinant IL-7 (5 ng/ml, AMDS, Benelux) on day 0 and recombinant IL-2 (10 U/ml, Cetus, Emeryville, CA, USA) on day seven; harvesting of cells occurred at day 10.

*CD4 T cell line stimulation:*

T cells from *M. tuberculosis* antigen specific-T cell lines ($1.5 \times 10^4$/well) were cultured in triplicate in culture medium with autologous or HLA-DR matched/mismatched PBMC as APC ($5 \times 10^4$/well, irradiated (2000 rad) in 96-wells flat-bottomed microtiter plates in the presence or absence of antigen and cultured at 37°C and 5% $CO_2$. At day three, supernatants were collected for the detection of IFNγ (2).

**Flow Cytometry**

Cells were stained with anti-CD3-PerCP, anti-CD4-APC and anti-CD8-PE (BD Biosciences, Erembodegem, Belgium) for 30 minutes at 4°C. Cells were washed in PBS/0.1% BSA and fixed in1% paraformaldehyde (Pharmacy LUMC, The Netherlands) and analyzed on a FACS Calibur (BD Biosciences). Analysis was done using CellQuest Pro software (BD Biosciences).

**Analysis of proliferating lymphocytes (CFSE)**

Cells gated on live lymphocytes combined with gating on $CD3^+$ T cells were analyzed for CFSE proliferation. The Δ geometric mean was used as a measure of proliferation and calculated as follows: Δ geometric mean = geometric mean (non-proliferated cells cells) - geometric mean (total cells). The Δ geometric mean was then used to calculate the relative proliferation which is in fact the percentage of the maximal proliferation (PHA), corrected for spontaneous proliferation (HIV-1 p17 $Gag_{77-85}$): ((Δ geometric mean sample- Δ geometric mean control peptide)/(Δ geometric mean PHA- Δ geometric mean control peptide))*100% = % of maximal proliferation. The cut off for a positive proliferation was set at 10% relative proliferation. This arbitrary high threshold value was chosen in order to limit the number of candidate epitopes to be evaluated in subsequent experiments.

**IFNγ ELISA**

The concentration IFNγ in the supernatants was measured by ELISA (U-CyTech, Utrecht, The Netherlands) according to the manufacturer's instructions. The detection limit of the assay is 20 pg/ml IFNγ. Samples were tested in duplicate; the mean value of unstimulated cultures was subtracted from the mean value of the stimulated cultures. An IFNγ response ≥ 100 pg/ml was considered positive for PBMC and for *M. tuberculosis* antigen specific T cell lines when IFNγ response was ≥ 50 pg/ml unless indicated otherwise.

### Chromium release assay

As target cells, human EBV-BLCL JY (HLA-A*0201, -B7, -Cw7), EBV-BLCL JY-Rv1733c-GFP (EBV-BLCL JY transduced with an expression vector encoding the *M. tuberculosis* DosR gene Rv1733c and the GFP marker (31,33)) and EBV-BLCL JY-GFP (EBV-BLCL JY transduced with an empty control vector encoding only GFP) were incubated at 37°C for 1 hr with 0.1 mCi $Na_2{}^{51}CrO_4$ (Amersham, United Kingdom), washed, and plated in triplicate in 96-well round-bottom plates (2500 cells/well). Effector $CD8^+$ T cells were added in different effector-to-target (E:T) ratios, 12:1, 25:1 and 50:1 together with either medium, peptide (25 μg/ml), or 5% Triton X-100 and with of without pan HLA-class I or HLA-class II antibodies. After six hours the supernatants were harvested, and the percentage lysis was calculated as follows:
[(release - spontaneous release)/(maximum release- spontaneous release)] * 100%.

## Results

### Selection of recombinant *M. tuberculosis* proteins and corresponding peptides

For this study we selected four *M. tuberculosis* DosR regulon encoded antigens previously shown to be prominently recognized in humans, in order to identify potential $CD4^+$ and $CD8^+$ T cell responses as well as to identify peptide epitopes recognized in the context of specific HLA-class I and class II molecules. The immunodominant, early phase secreted antigen Ag85B was taken along as reference antigen. *M. tuberculosis* DosR antigens Rv1733c, Rv2029c and Rv2627c were chosen on the basis of T cell recognition profiles in *M. tuberculosis* infected individuals (TB patients and tuberculin skin test (TST) converters) from our previous work (18): all three antigens ranked among the top 10 most frequently recognized antigens in the *M. tuberculosis* infected groups. *M. tuberculosis* DosR antigen Rv2031c (HspX, hsp16, a-crystallin) was included since many studies have addressed the role of this heat-shock protein in anti-mycobacterial immunity (15,39,41). Recombinant proteins (Table 1) and overlapping sets of peptides of these five antigens were prepared (see supplementary file 1 for all peptide sequences).

**Table 1. Selected *Mycobacterium tuberculosis* antigens tested in present study**

| | Rv number | Gene name [a] | Molecular mass (kDa)[a] | Product [a] | References |
|---|---|---|---|---|---|
| DosR genes | Rv1733c | | 22,4 | conserved transmembrane protein | 6, 10 |
| | Rv2029c | *pfkB* | 35,4 | phosphofructokinase PfkB | 6, 10, 14 |
| | Rv2031c | *hspX* | 16,3 | heat shock protein HspX (alpha-crystallin) | 6, 9, 10, 14, 21-23 |
| | Rv2627c | | 46,3 | conserved hypothetical protein | 6, 10, 14 |
| reference gene | Rv1886c | *fbpB* | 34,6 | secreted antigen 85-B fbpB (mycolyltransferase 85B, Ag85B) | 25 |

[a] Annotations are from www.tbdb.org and http://genolist.pasteur.fr/TubercuList/

**Identification of *M. tuberculosis* induced antigen specific CD4⁺ and CD8⁺ T cell responses based on CFSE proliferation**

We used CFSE based proliferation of PBMC, since this method allows tracking of both CD4⁺ and CD8⁺ T cell subset responses in the same cell population (20). Figure 1 demonstrates typical CFSE based CD4⁺ and CD8⁺ T cell response profiles to *M. tuberculosis* antigens and control conditions when assaying PBMC from a PPD responsive donor. Following stimulation of cells with PPD, significant CD4⁺ and to a lesser extent also CD8⁺ T cell responses could be observed, underlining the significant advantage of this assay allowing responding subpopulations to be phenotyped and characterized. No response was seen to the negative control medium or to the irrelevant control peptide HIV-gag$_{77–85}$ . Following stimulation of PBMC with *M. tuberculosis* DosR regulon encoded antigen Rv1733c or its corresponding peptides proliferative CD4⁺ T cell responses were observed (Figure 1a).

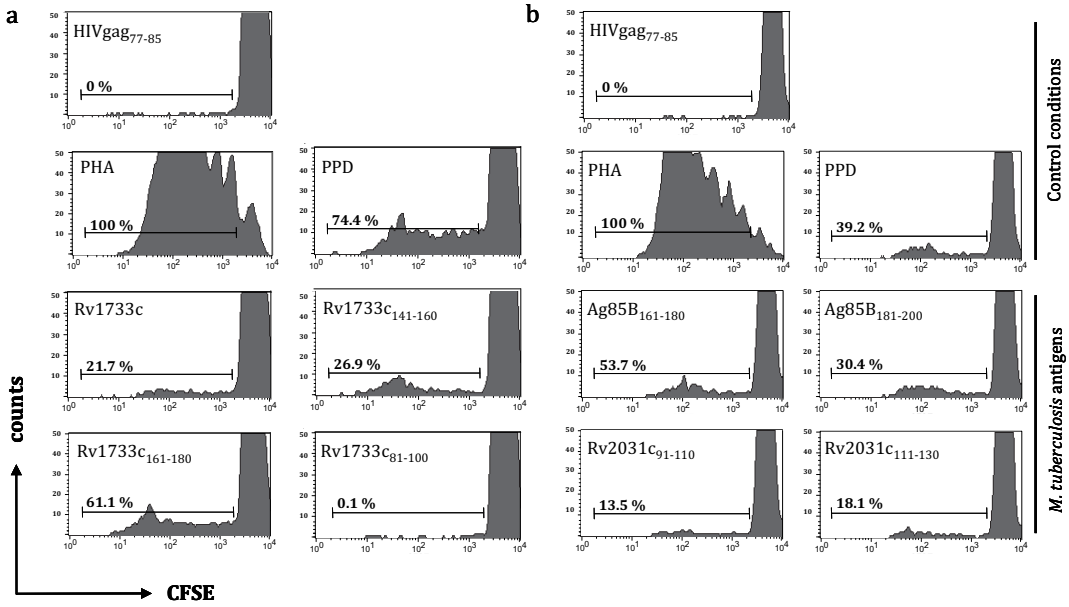



***Figure 1. Proliferative T cell response profiles to M. tuberculosis antigens compared to control conditions.*** *PBMC from donor 9 (Table 2, HLA-A2, -A24, -B35, B57, -C4, C6, DR-7, DR-14) were stimulated with PHA or HIVgag$_{77-85b}$ as positive and negative controls, respectively, or with M. tuberculosis test antigens. After 6 days of culturing CFSE proliferation of CD4⁺ (a) or CD8⁺ T cells (b) was measured: CD4⁺ or CD8⁺ T cells were gated from a CD3⁺ T cell gate combined with a live lymphocyte gate. Individual histogram plots show the % relative proliferation ((Δ geometric mean sample - Δ geometric mean control peptide)/ (Δ geometric mean PHA- Δ geometric mean control peptide))*100% = % of maximal proliferation. PBMC were stimulated with Rv1733c and corresponding peptides, some of which induced CD4⁺ T cell proliferation while others did not (a) PBMC were also stimulated with four 20-mer peptides of Ag85B and Rv2031c encoding identified and published HLA-A2 restricted epitopes (see text) to which indeed CD8⁺ T cell proliferation was observed in this HLA-A2 positive donor (b). A relative proliferation ≥ 10% was considered positive.*

We wanted to establish whether our method was able to confirm already published epitopes of Ag85B and Rv2031c (HspX, α-crystallin), in order to further validate the CFSE based proliferation method. For the mature Ag85B protein, five HLA-A*0201 epitopes have been described ($Ag85B_{p45-53}$ GLPVEYLQV, $Ag85B_{p166-175}$ SMAGSSAMIL, $Ag85B_{p183-192}$ FIYAGSLSAL, $Ag85B_{p198-206}$ GMGPSLIGL and $Ag85B_{p239-247}$ KLVANNTRL (16)). We therefore included 20-mer peptides containing these five HLA-A*0201 restricted epitopes in our study. Four out of the five 20-mers were indeed recognized by $CD8^+$ T cells from this donor (Figure 1b) as well as by other HLA-$A2^+$ individuals (data not shown).
Also for Rv2031c three HLA-A*0201 restricted epitopes have previously been identified: $Rv2031c_{p21-29}$ LFAAFPSFA (6), $Rv2031c_{p91-105}$ SEFAYGSFVRTVSLP (15) and $Rv2031c_{p120-128}$ GILTVSVAV (4,6). Our data show that 20-mers encoding epitopes $Rv2031c_{p91-105}$ and $Rv2031c_{p120-128}$ (but not $Rv2031c_{p21-29}$ in this donor) were both recognized by several HLA-$A2^+$ individuals. Figure 1b demonstrates proliferative $CD8^+$ T cell responses following stimulation with several of the above mentioned 20-mer *M. tuberculosis* peptides.
Also peptides encoding previously published, HLA class II presented epitopes were recognized in this assay. These included: Rv2031c peptide 4 encoding $Rv2031c_{p31-50}$ (LRPTFDTRLMRLEDEMKEGR, HLA-DRB1*0301) (9,15,21) and Rv2031c peptides 3, 9, and 10 which encode several epitopes recognized in the context of HLA-DRB1*1501 (1,5) (data not shown). Taken together, the results from the CFSE assay agree well with results from other assays, including the ability to identify peptide epitopes recognized by $CD4^+$ and $CD8^+$ T cells.

**Immunogenicity of *M. tuberculosis* DosR regulon encoded antigens based on CFSE proliferation and flow cytometry**

CFSE labelled PBMC from randomly selected, healthy, PPD-responsive blood bank donors were stimulated with *M. tuberculosis* lysate; PPD; *M. tuberculosis* DosR regulon encoded proteins Rv1733c, Rv2029c, Rv2627c, Rv2031c; Ag85B protein; and all respective single peptides from each of the 5 recombinant antigens. Table 2 shows the HLA class-A, –B, -C and -DR genotypes and the corresponding *in vitro* PPD reactivity of the donors. IFNγ responses to PPD exceeding 100 pg/ml were considered positive, as used in our previous studies (2,18,19).
Immunogenic regions could be identified within the sequences of these *M. tuberculosis* DosR regulon encoded antigens (Table 3a-c). Table 3a and 3b show the comprehensive recognition profiles of $CD4^+$ and $CD8^+$ T cells of the tested antigens and their respective single peptides in all donors studied here. As anticipated, responses to the whole recombinant proteins were mostly confined to the $CD4^+$ T cells. In several cases $CD4^+$ T cell responses were accompanied by $CD8^+$ T cell responses (e.g. in donor 1), while isolated $CD8^+$ T cell responses to the recombinant proteins were not observed.
As expected, there were substantial inter-individual differences in antigen and peptide recognition, since some donors responded to many *M. tuberculosis* DosR regulon encoded proteins and corresponding peptides whereas others only responded to few proteins or peptides. Nevertheless, all 15 $PPD^+$ donors responded to at least one of the recombinant proteins and/or peptides investigated.

**Table 2. HLA-class A, -B, -C and –DR genotypes and IFNγ responses to *M. tuberculosis* PPD of donors studied in current study**

| Donor | HLA type | | | | PPD[a] |
|---|---|---|---|---|---|
| number | HLA-A | HLA-B | HLA-C | HLA-DR | pg/ml |
| 1 | 1 | 7,8 | 7 | 2,3 | + |
| 2 | 2,31 | 8,44 | 5, 7 | 3,4 | + |
| 3 | 11,30 | 18,27 | 1, 5 | 3,4 | + |
| 4 | 2,29 | 45,50 | 3, 6 | 4,7 | + |
| 5 | 2 | 44,39 | 5, 7 | 7,15 | +++ |
| 6 | 2,3 | 51,7 | * | 15,11 | + |
| 7 | 2 | 7,8 | 7 | 2,3 | + |
| 8 | 3,31 | 56,35 | 1, 4 | 1,4 | ++ |
| 9 | 2,24 | 35,57 | 4, 6 | 7,14 | ++ |
| 10 | 3,24 | 14,18 | * | 1,4 | +++ |
| 11 | 1,31 | 51,52 | * | 11,15 | + |
| 12 | 2,68 | 44,51 | 1, 5 | 11,14 | + |
| 13 | 3 | 7,51 | 7, 4 | 1,15 | ++ |
| 14 | 3,24 | 7,62 | 7, 9 | 11,13 | + |
| 15 | 3,11 | 15,38 | 1, 12 | 3,13 | + |

*, HLA-C type unknown.

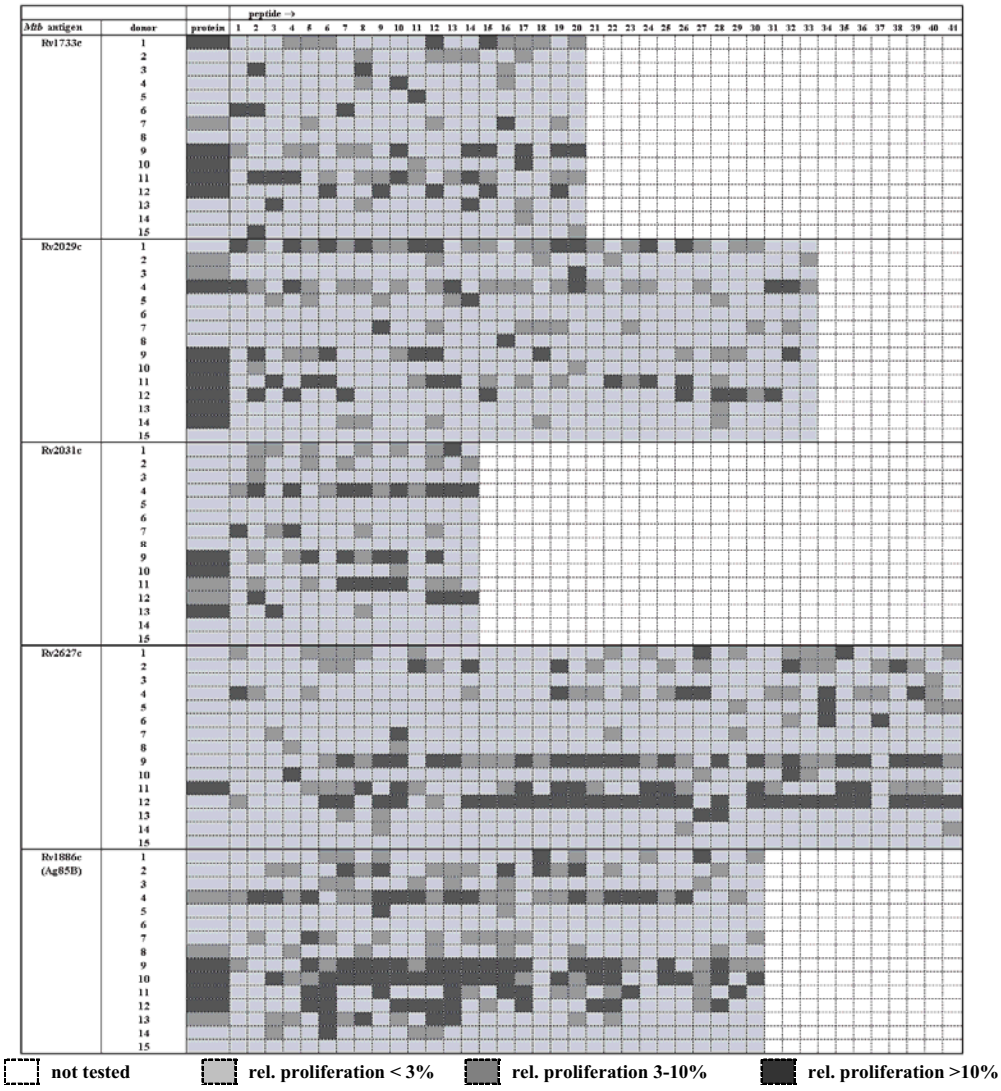
[a], Responses to PPD: +, 100-500 pg/ml; ++, 500-1000 pg/ml; +++, >1000 pg/ml.

In this series of donors, recombinant antigens Rv1733c, Rv2029c and Rv1886c (Ag85B) were recognized most efficiently: 7/15 $PPD^+$ donors recognized Rv2029c, 5/15 recognized Rv1733c and 4/15 recognized Ag85B. This contrasted to Rv2627c, which was recognized by only a single donor. In line with our previous observations, Rv2031c/hspX/*acr* was recognized by a minority of the donors (18).

Peptides in several instances appeared to be more able to induce $CD4^+$ or $CD8^+$ T cell responses when compared to recombinant proteins; this might be due to their preprocessed nature, facilitating high efficiency antigen presentation, and/or to their higher molarity, since proteins and peptides all were tested at 10 microgram/ml concentrations, regardless of molecular mass.

Importantly, a substantial number of immunogenic peptides recognized by $CD4^+$ T cells, $CD8^+$ T cells or both could be identified using this approach. Some peptides were recognized by $CD4^+$ T cells from at least one third of the donors, e.g. Ag85B peptides 9, 13 (6 donors), Ag85B peptides 5, 6 and Rv2627c peptide 19 (5 donors). Several other peptides were recognized by $CD4^+$ T cells from 4/15 donors, such as Rv1733c peptide 2, Rv2627c peptides 10, 19, 32, 34 and 35, and Ag85B peptides 10, 12, 16 and 22. A large number of peptides was recognized by at least three donors. Various $CD8^+$ T cell responses were detected as well, although the numbers of peptides recognized overall was lower than that for $CD4^+$ T cells. This might be related partly to the length of the peptides, since HLA class I alleles typically prefer 9-11-mer peptides for binding, whereas those assayed here were 20-mers. Nevertheless, particularly for Rv1733c and Ag85B, there were several peptides recognized by $CD8^+$ T cells from 4-5 donors like Rv1733c peptides 17 (5 donors), 2 and 19 (both 4 donors) and Ag85B peptides 5 and 13 (both 4 donors). Multiple peptides were recognized by at least 3 donors, still representing 20% of the panel tested.

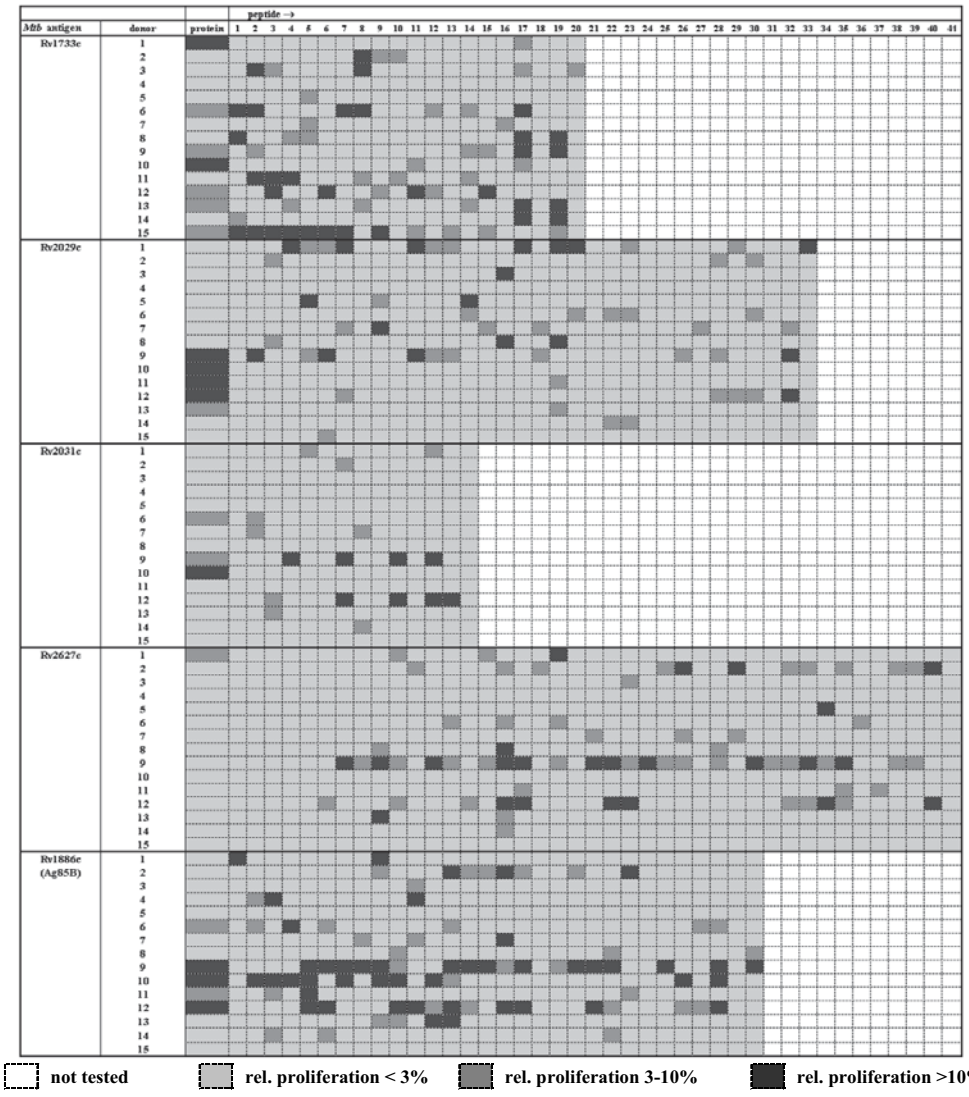
**Table 3a. Proliferative CD4+ T cell response to *M. tuberculosis* peptides among all donors (n=15, numbers corresponding with donors in Table 2). A relative proliferation ≥ 10% was considered positive.**




*Mtb, M. tuberculosis*

Table 3c shows the cumulative recognition results for both CD4+ and CD8+ T cells to all tested antigens and peptides for all donors tested. The results show that many proliferative responses to the *M. tuberculosis* DosR regulon encoded antigens can be identified. This also underlines the immunogenicity of *M. tuberculosis* DosR regulon encoded antigens. Interestingly, several peptides were recognized by both CD4+ and CD8+ T cells (Rv1733c: peptide 2; Ag85B peptides 5 and 13). This issue is commonly not addressed in epitope mapping studies, since read-outs are mostly biased towards detecting CD4+ T cell responses. Furthermore, the results suggest some enrichment of

**Table 3b. Proliferative CD8⁺ T cell response to *M. tuberculosis* peptides among all donors (n=15, numbers corresponding with donors in Table 2). A relative proliferation ≥ 10% was considered positive.**




*Mtb*, *M. tuberculosis*

epitopes in particularly immunogenic regions, for example Rv1733c peptides 1-3, 17-19, and Ag85B peptides 5-13.

Taken together, we applied the cell tracking dye CFSE to visualize and simultaneously quantify both CD4⁺ and CD8⁺ T cell responses to specific *M. tuberculosis* antigens and individual peptides. We identify a large series of new possible peptide epitopes located on *M. tuberculosis* DosR regulon encoded antigens, which are recognized with high efficiency by CD4⁺ and/or CD8⁺ T cells.

**Table 3c. Cumulative CD4$^+$ and CD8$^+$ T cell response profiles to *M. tuberculosis* proteins and their respective peptides among PPD responsive donors (n=15).**

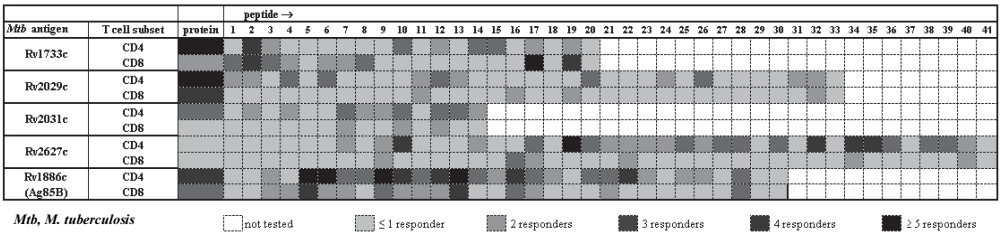
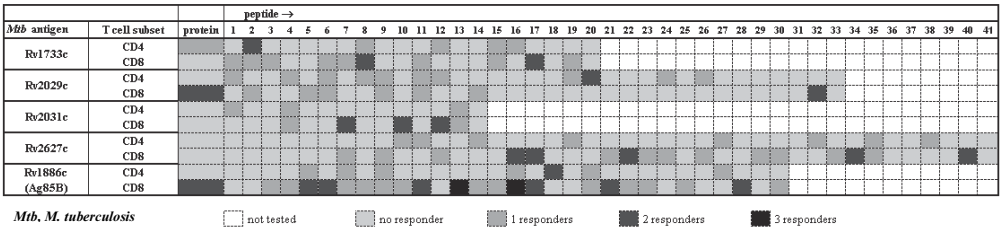



**Table 3d. Cumulative CD4$^+$ and CD8$^+$ T cell response profiles to *M. tuberculosis* proteins and their respective peptides among heterozygous HLA-DR3$^+$ (n=5, CD4$^+$ T cell responses) and HLA-A2$^+$ (n=7, CD8$^+$ T cell responses) PPD responsive donors.**




## Identification of *M. tuberculosis* DosR regulon encoded peptide epitopes in the context of genetic HLA-A2 and HLA-DR3 polymorphism

The above described *M. tuberculosis* DosR regulon encoded peptide epitopes were recognized by different donors with varying HLA types. Many of the *in vitro* responses matched with *in silico* epitope motif searches for the relevant HLA types (26)(data not shown). This suggests that responses to *M. tuberculosis* DosR regulon encoded antigens can naturally occur in a wide range of HLA types. In order to better understand the molecular mechanisms of DosR regulon encoded epitope presentation, we examined peptide recognition in the context of HLA class I and class II polymorphism. Given the relatively limited number of individuals included in the panel, we decided to focus on 2 highly frequent alleles, HLA-A*0201 and HLA-DRB1*0301 (DR3/17) (frequencies are ~50% and ~20%, respectively among Caucasians)(23). Among the panel of 15 PPD$^+$ blood bank donors, 5 HLA-DR3 positive and 7 HLA-A2$^+$ donors were identified. T cell responses (CD4$^+$) to *M. tuberculosis* DosR regulon encoded peptides among the 5 HLA-DR3$^+$ donors were found in 2/5 donors, including to Rv1733c peptides 8 and 2, Rv2029c peptide 20 and Rv2627c peptide 19 (Table 2 and 3a). Many peptides were also recognized by (CD8$^+$ T cells from) HLA-A2$^+$ donors. Some peptides were recognized by up to 4/7 HLA-A2$^+$ donors such as Rv2627c peptides 19 and 34. A number of other peptides induced responses in 3/7 HLA-A2$^+$ donors: Rv2029c peptide 32, Rv2627c peptides 10, 26, 32, 38-40 and Rv2031c peptides 4, 7, 10 and 12 (Table 2 and Table 3b). Interestingly, Rv2627c protein was recognized by CD4$^+$ T cells of one donor whereas the single peptides of Rv2627c were recognized by many HLA-A2$^+$ and -DR3$^+$ donors implying that using peptides may be more sensitive in antigen discovery than using intact whole antigens.

**Table 4a. List of *M. tuberculosis* DosR regulon encoded candidate HLA-DR3 epitopes**

| *M. tuberculosis* DosR antigen | epitope sequence[a] | start aa | end aa | score *in silico* prediction[a] |
|---|---|---|---|---|
| **Rv1733c** | TFRLRLPCRTILRVF | 15 | 29 | 26 |
| | ATMITFRLRLPCRTI | 11 | 25 | 19 |
| | VGIWVDSAGQLVDEPA | 141 | 156 | 19 |
| **Rv2029c** | TEKMRCGAPRYDPGG | 35 | 49 | 11 |
| | GINVARIVHVLGGCS | 51 | 65 | 12 |
| | YRFVLPGPSLTVAEQ | 114 | 128 | 15 |
| | KASVRELRECVGSEL | 194 | 208 | 18 |
| | VVSLGSQGALLATRH | 231 | 245 | 14 |
| | SIPMTAVSGVGAGDA | 252 | 266 | 13 |
| **Rv2031c** | TLPVQRHPRSLFPEF | 4 | 18 | 26 |
| | SVAVSEGKPTEKHIQ | 125 | 139 | 12 |
| **Rv2627c** | VAAGLEFYGNRRWLEK | 93 | 108 | 17 |
| | MGRAPLDLAVFRAWK | 186 | 200 | 13 |
| | RHTVKMAEPIGRMIS | 316 | 330 | 13 |

[a], Epitope motif scores and candidate epitope sequences are obtained from www.syfpeithi.de

**Table 4b. List of *M. tuberculosis* DosR regulon encoded candidate HLA-A2 epitopes**

| *M. tuberculosis* DosR antigen | epitope sequence[a] | start aa | end aa | score *in silico* prediction[a] | $K_D$ <IC50> mM[b] | ELISA response[c] | CFSE proliferation[d] |
|---|---|---|---|---|---|---|---|
| Rv1733c | TRHPATATV | 81 | 89 | 18 | > 250 | - | - |
| | HVYAHQAQT | 73 | 81 | 11 | 5,5 | + | - |
| | QAQTRHPAT | 78 | 86 | 10 | > 250 | + | - |
| | GLWLSVAAV | 170 | 178 | 29 | 0,11 | + | - |
| | AALGLWLSV | 167 | 175 | 24 | 3 | - | - |
| | IADAALAAL | 161 | 169 | 23 | 2.8 - 34 | + | + |
| | ALLALTRAI | 181 | 189 | 26 | 0,42 | + | + |
| | LTRAILIRV | 185 | 193 | 20 | 53 | + | - |
| | AILIRVRNA | 188 | 196 | 20 | 2,8 | + | + |
| Rv2029c | ELAAEPTEV | 314 | 322 | 21 | 1,8 | + | - |
| | TEVGQDQYV | 320 | 328 | 12 | > 250 | - | - |
| Rv2031c | AYGSFVRTV | 94 | 102 | 19 | 13 | - | - |
| | GSFVRTVSL | 96 | 104 | 18 | 59 | + | - |
| | RTVSLPVGA | 100 | 108 | 12 | 2,5 | + | - |
| Rv2627c | TANNREYAL | 159 | 167 | 17 | 4,4 | - | - |
| | GSQRWLSYT | 151 | 159 | 9 | 209 | - | - |
| | GLACAILGV | 286 | 294 | 29 | 0,0041 | - | + |
| | ILGVPVADL | 291 | 299 | 29 | 0,72 | - | - |
| | SLEEGLACA | 282 | 290 | 25 | 0,24 | - | - |
| | PIGRMISPL | 324 | 332 | 19 | 200 | - | - |
| | LSLTPLVPM | 332 | 340 | 15 | 9 | - | - |
| | SLTPLVPMP | 333 | 341 | 19 | 49 | - | + |
| | PMPGRFIYA | 339 | 347 | 11 | 8,6 | + | - |
| | AALEQSGLL | 394 | 402 | 20 | 3 | + | - |
| | GLLDAPRTQ | 400 | 408 | 16 | 26 | + | - |

[a], Epitope motif scores and candidate epitope sequences are obtained from www.syfpeithi.de.
[b], control peptide (cold peptide) in binding assay had a $K_D$ of 0.006
[c], positive IFNγ response to candidate epitope: +, positive responses exclusively found in HLA-A2$^+$ (heterozygous) donors (n=11);
-, positive responses found in donors with different HLA class A alleles (n=5).
[d], CD8$^+$ T cell proliferation following stimulation with candidate epitope (% proliferating CD8$^+$ cells): +, positive responses exclusively found in HLA-A2$^+$ (heterozygous)donors. When % proliferating CD8$^+$ cells was > [medium + 3*SD] it was considered positive.

Table 3d presents the cumulative $CD4^+$ and $CD8^+$ T cell response profiles to *M. tuberculosis* proteins and respective peptides among PPD responsive, heterozygous HLA-$DR3^+$ and HLA-$A2^+$ donors.
Next we further analyzed the *M. tuberculosis* DosR regulon encoded peptide epitopes in the context of HLA-DR3 and HLA-A2 presentation. First, we examined responses to control antigens, as above. Indeed, HLA-$A2^+$ donors recognized Ag85B peptides 13, 15, 16, 20, and Rv2031c peptides 10, 12, which all contained previously published HLA-A*0201 restricted epitopes. Similarly, Rv2031c peptide 4 that carries a HLA-DR3 epitope was indeed recognized by HLA-$DR3^+$ positive donors (Figure 1 and Table 3d), thus confirming once more that the method we use here is able to identify peptide specific responses.
A substantial number of peptides was recognized by the selected HLA-$DR3^+$ and HLA-$A2^+$ donors. These peptides were next subjected to further in silico minimal epitope motif searches (26) in the context of HLA-A2 or HLA-DR3 to select the precise epitopes embedded in the recognized 20-mers. Despite the limited number of individuals investigated, we were able to identify 14 *M. tuberculosis* candidate DosR regulon encoded HLA-DR3 presented epitopes (Table 4a) and 25 *M. tuberculosis* candidate DosR regulon encoded HLA-A2 presented epitopes (Table 4b).

**In vitro verification of candidate *M. tuberculosis* DosR regulon encoded epitopes in the context of HLA-DR3**

All fourteen candidate HLA-DR3 *M. tuberculosis* DosR regulon encoded epitopes were next synthesized as 15-mer peptides (Table 4a), and assessed for recognition by PBMC from two newly tested PPD responsive donors (#18: HLA-DR3, 5; #19: HLA-DR3, 2), both of which were not included in Table 2. Both donors were found to recognize a substantial number of these *M. tuberculosis* candidate HLA-DR3 presented epitopes (10 and 4, respectively) as shown in Figure 2a, whereas mycobacterium naïve donors 20 (HLA-DR1, 3) and 21 (HLA-DR3, 7) did not recognize any of the epitopes. Most importantly, these results show that responsiveness to *M. tuberculosis* candidate HLA-DR3 epitopes are clearly observed in individuals exposed/infected with mycobacteria but not in mycobacteria naïve, PPD negative donors.
Next, *M. tuberculosis* DosR regulon encoded antigens and selected epitopes were presented to a HLA-$DR3^+$ $CD4^+$ T cell line (TCL) which had been generated from PBMC of a treated TB patient (HLA-DR3, 15) by stimulation with *M. tuberculosis* lysate. Antigens were presented via (homozygous) HLA-DR3 matched or mismatched APC. For all antigens, except Rv1733c, both protein and peptide responses could be measured. For Rv1733c responses were seen against peptide-epitopes $Rv1733c_{p15-29}$ and $Rv1733c_{p141-156.}$ This result is compatible with the notion that peptide stimulation can visualize also a "subdominant" epitope repertoire which may not be visible following suboptimal stimulation with whole protein. Notably, no responses were found to peptide-epitope $Rv1733c_{p11-25}$ despite its large overlap with $Rv1733c_{p15-29;}$ this points to a C-terminal location of essential residues involved in either HLA-DR3 binding and/or TCR ligation.
In this TCL, responses to 11 out of the 14 *M. tuberculosis* candidate HLA-DR3 presented epitopes were observed. No or very low responses were observed when antigens or peptides were presented via HLA-DR3 mismatched APC (Figure 2b-e).

Only for peptide-epitope Rv2627cp$_{316-330}$ a remaining response, albeit strongly reduced, was observed when presented by HLA-DR3 mismatched APC. This could indicate that this peptide might be presented also by other HLA-class II molecules than HLA-DR3.

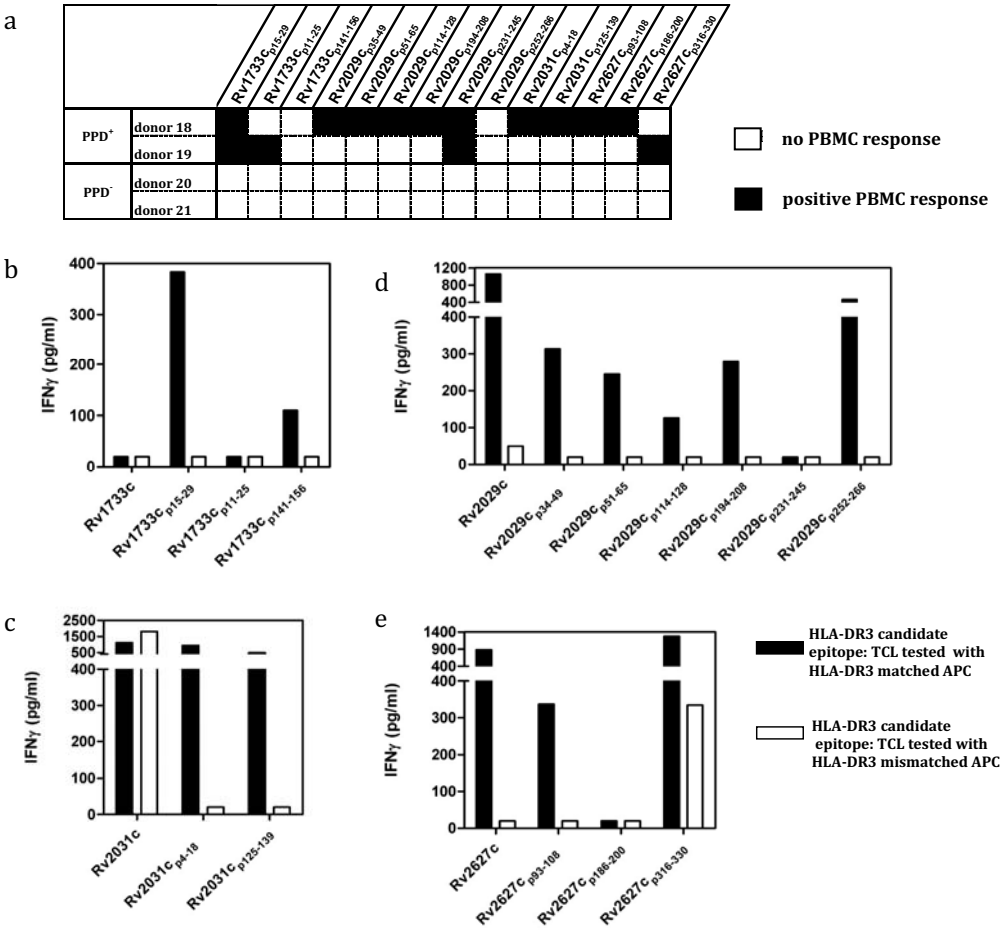



***Figure 2. Recognition of candidate HLA-DR3 epitopes by assessment of IFNγ responses in PBMC and in CD4⁺ T cell lines****. PBMC from PPD⁺ HLA-DR3⁺ (heterozygous) donors (donor 18 and 19) both recognized a substantial number of candidate HLA-DR3 epitopes, in contrast to PPD⁻ HLA-DR3⁺ (heterozygous) donors (black box, response, white box, no response)* ***(a).*** *Immunodominant M. tuberculosis dosR antigens and their respective candidate HLA-DR3 epitopes were tested, using a CD4⁺ M. tuberculosis lysate induced T cell line (TCL) with HLA-DR3 matched (homozygous) APC (black bars)* ***(b-e)****. For Rv2029c* ***(d)****, Rv2031c* ***(c)*** *and Rv2627c* ***(e)*** *responses to both protein and candidate epitopes were observed. For Rv1733c (b) only responses to its respective candidate epitopes were observed (see text for discussion). When the TCL was tested with DR3 mismatched APC (white bars), no or strongly reduced responses were observed* ***(b-e)****. An IFNγ response ≥ 100 pg/ml was considered positive for PBMC and for M. tuberculosis antigen specific T cell lines when IFNγ response was ≥ 50 pg/ml.*

Confirming the above observations, responses to the longer 20-mer peptides of the *M. tuberculosis* DosR regulon encoded antigens containing the 15-meric HLA-DR3 candidate epitopes were also observed (data not shown). Thus, taken together these results strongly suggest that a series of *M. tuberculosis* DosR regulon encoded epitopes is presented to CD4+ T cells by HLA-DR3 molecules.

### In vitro verification of candidate *M. tuberculosis* DosR regulon encoded epitopes in the context of HLA-A*0201

Taking a similar approach, 25 *M. tuberculosis* DosR regulon encoded HLA-A*0201 restricted candidate 9-meric epitopes were synthesized. First, their binding to HLA-A2 was measured (Table 4b). Several of the in silico predicted candidate HLA-A2 epitopes had intermediate or high binding affinity (IC50 <10 μM) for HLA-A*0201 molecules. Other candidate HLA-A2 epitopes, despite high prediction scores and in vitro CD8+ T cell recognition of the corresponding 20-mer peptide, had low (IC50>10 μM) or no measurable (IC50>100 μM) affinity for HLA-A*0201 molecules.

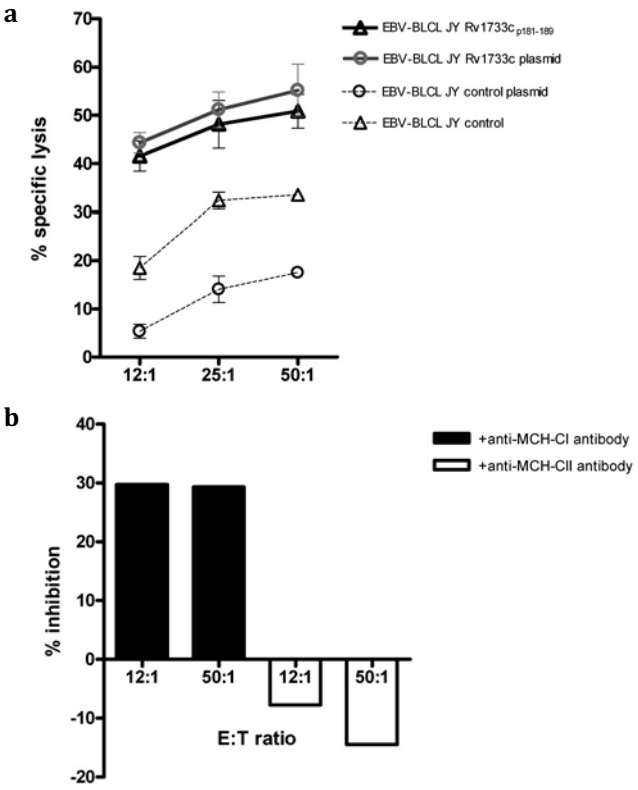



***Figure 3. Cytotolytic activity of a CD8+ polyclonal T cell line against the HLA-A2 restricted M. tuberculosis epitope Rv1733c$_{p181-189}$.*** *The results show specific recognition of peptide epitope Rv1733c$_{p181-189}$ by CD8+ T cells when peptide was loaded onto EBV-BLCL JY target cells (compared to medium control). CD8+ T cells also recognized EBV-BLCL JY target cells that had been transduced with an expression plasmid encoding Rv1733c, thus facilitating endogenous expression and presentation of Rv1733c in the context of HLA-A*0201. In contrast EBV-BLCL JY target cells which were transduced with an empty plasmid* ***(a)*** *were killed to a much lesser extent. Specific lysis of EBV-BLCL JY target cells loaded with Rv1733c$_{p181-189}$ could be inhibited partly by addition of anti-HLA-class-I but not anti-HLA-class-II antibody* ***(b)****. The % specific lysis was calculated as follows: [(release-spontaneous release)/ (maximum release-spontaneous release)] x 100%.*

All 25 candidate HLA-A2 epitopes were tested in a lymphocyte stimulation test in 11 HLA-A2$^+$ and 5 HLA-A2$^-$ individuals (PPD$^+$ blood bank donors (either from Table 2 or newly tested), TST converters or TB patients) to examine their recognition in the context of HLA-A2 molecules. Five HLA-A2 candidate peptides were recognized solely by HLA-A2$^+$ but not HLA-A2$^-$ donors as assessed by CFSE proliferation (Table 4b). A polyclonal CD8$^+$ T cell line was generated to explore possible functional properties of CD8$^+$ T cells directed to these *M. tuberculosis* DosR regulon encoded epitopes. PBMC from a TB patient (HLA-A2, -A3, and -DR11) were stimulated with a combination of the candidate HLA-A2 restricted epitopes Rv1733c$_{p181-189}$, Rv2031c$_{p96-104}$ and Rv2627c$_{p151-159}$. All three peptides were recognized by this donor (data not shown). Effector CD8$^+$ T cells showed specific peptide dependent lysis of EBV-BLCL JY target cells (HLA-A*0201) when pulsed with peptide Rv1733c$_{p181-189}$ (but not with Rv2627c$_{p151-159}$ or Rv2031c$_{p96-104}$). Killing was observed at different effector-to-target ratios (E: T) (Figure 3a). As expected, the use of allogeneic EVB-BLCL target cells in combination with polyclonal effector cells resulted invariably in some background activity, even in such short term assays (5-35 % lysis of control targets). Nevertheless, peptide dependent lysis was clearly evident. CD8$^+$ T cells also recognized and lysed EBV-BLCL JY target cells that had been transduced with a plasmid encoding Rv1733c, while not recognizing EBV-BLCL JY target cells similarly transduced with control plasmid (Figure 3a).
Specific peptide dependent lysis of EBV-BLCL JY target cells loaded with HLA-candidate epitopes Rv1733c$_{p181-189}$ could be inhibited partly (~30 %) by the addition of a pan anti-HLA-class I antibody whereas no decrease in lysis was observed following addition of pan anti-HLA-class II antibody (Figure 3b).
Collectively these results show that CD8$^+$ T cells can recognize *M. tuberculosis* DosR antigen encoded peptide epitopes presented by HLA- A*0201 restricted. Furthermore, peptide specific CD8$^+$ T cells were capable of lysing peptide loaded- and endogenously processed antigen loaded- target cells in the context of HLA-A*0201 molecules. These results further document the presence of functional *M. tuberculosis* DosR regulon encoded epitopes for human CD8$^+$ T cells in the context of HLA class I molecules.

## Discussion

Here we describe for the first time CD4$^+$ as well as CD8$^+$ T cell responses to a series of *M. tuberculosis* DosR regulon encoded antigens. These responses were detectable in mycobacteria primed (PPD$^+$) individuals and were HLA class II and class I restricted, respectively. A number of selected peptide epitopes was fine mapped in the context of the prevalent HLA alleles HLA-A*0201 and HLA-DR3. Responses to the here identified *M. tuberculosis* DosR regulon encoded antigenic peptides include CD4$^+$ and CD8$^+$ T cell proliferation, production of IFNγ (data not shown) and in the case of CD8$^+$ T cells also cytolytic activity.
We have hypothesized that *M. tuberculosis* DosR regulon encoded antigens (18) which are expressed during conditions mimicking intracellular stress as encountered during infection, represent rational targets for TB vaccination since immune responses to *M.*

*tuberculosis* DosR regulon encoded antigens will be activated during latent infection. Indeed, immune responses to *M. tuberculosis* DosR regulon encoded antigens have been shown to be associated with control of latent TB infection (18,29). It is against this background that we set out to identify *M. tuberculosis* DosR regulon encoded antigen specific CD4+ and CD8+ T-cell responses and to identify relevant epitopes.
We selected four *M. tuberculosis* DosR antigens, Rv1733c, Rv2029c, Rv2031c (HspX, α-crystallin) and Rv2627c, previously found to be prominently recognized by human T cells, for detailed CD4+ and CD8+ T-cell response profiling and epitope identification (Table 1). The use of overlapping peptides to examine T cell responses has been applied before (28,32). Although HLA-class I presented peptides are typically 8-11 amino acids long, and HLA-class II ligands can be between 10-25 amino acids (12,26,27), we nevertheless find efficient CD8+ T cell responses using 20-mers, next to using 9-mer peptides. We tested 20-mer peptides with 10 aa overlap to limit the total numbers of peptides needed to allow us to visualize both CD4+ and CD8+ T cell responses in human samples (11). Our 6-10 day incubation period may have allowed internalization and processing of peptides for HLA-class I presentation, or allowed cross-presentation via alternative antigen presentation pathways (3,37). We were able to validate this strategy first by identification of CD4+ as well as CD8+ T cell responses to 20-mer peptides that contained already known epitopes (notably on Ag85B and Rv2031c (4,6,9,15,16,21)) (Figure 1b). We conclude that the use of 20-mer peptides in combination with CFSE based proliferation in human PBMC samples is a powerful and efficient approach to (i) visualize and quantitate both CD4+ and CD8+ T cell responses to the same antigen; and (ii) to identify and fine-map peptide epitopes for both CD4+ and CD8+ T cells simultaneously against the same peptides.
Peptides in several instances appeared to be more able to detect CD4+ or CD8+ T cell responses compared to recombinant proteins; this at first sight counter-intuitive observation, however, is readily explained by the preprocessed nature op peptides, facilitating high efficiency antigen presentation, as well as by their much higher molarity, since proteins and peptides all were tested at 10 microgram/ml concentrations, regardless of molecular mass. The lack of competition with otherwise dominant epitopes for processing when using whole recombinant proteins may also have permitted the identification of subdominant epitopes using peptides, which might have escaped detection otherwise.

A striking observation was the wealth of epitopes that could be identified in *M. tuberculosis* DosR regulon encoded antigens, following confirmation of known epitopes for CD4+ and CD8+ T cells in Ag85B and Rv2031c/hspX. This underscores the significant immunogenicity of *M. tuberculosis* DosR regulon encoded antigens in a wide variety of HLA class I and class II backgrounds. Moreover, within several *M. tuberculosis* DosR regulon encoded antigens highly immunogenic regions could be identified (Table 3a-c). Of further interest was that a substantial number of peptides elicited both CD4+ and CD8+ T cell responses.
We further investigated responses against a subset of epitopes, notably those that were most likely recognized in the context of HLA-A2 and HLA-DR3 molecules. Peptide presentation via HLA-DR3 matched vs. mismatched antigen presenting cells showed that indeed the majority of the predicted epitopes (Table 4a) was presented

to $CD4^+$ T cells by HLA-DR3 molecules. In parallel, HLA-A2 restriction of peptide recognition was verified by using a $CD8^+$ T cell line specific for the Rv1733c epitope $Rv1733c_{p181-189}$. The T cell line's cytotoxic activity towards target cells that expressed HLA-A*0201 could be inhibited partly by anti-HLA-class I antibody. Interestingly, these $CD8^+$ T cells also were able to recognize endogenously processed antigen, since they killed target cells that were transduced with Rv1733c.
Although *in silico* prediction is a valuable tool in epitope identification, our results showed a not very strict correlation between the *in silico* epitope prediction scores, *in vitro* affinity or the magnitude of the observed T cell responses (proliferation, IFNγ production, target cell killing). Using the functional methods described here, we were able to identify a set of *M. tuberculosis* DosR regulon encoded epitopes in the context of HLA-A2 and -DR3, some of which were investigated in detail. We intend to further characterize immune responses to these epitopes (e.g. using multiplex functional assays and polychromatic flow cytometry). Eventually, the protective efficacy of these epitopes need to be assessed in relevant in vivo models, including e.g. HLA transgenic mice (7).
In conclusion, we have identified $CD4^+$ and $CD8^+$ T cell responses to several immunodominant *M. tuberculosis* DosR regulon encoded antigens and corresponding HLA-class I and class II restricted peptide epitopes. These responses could be detected by applying the cell tracker dye CFSE, allowing simultaneous detection of $CD4^+$ and $CD8^+$ T cell responses to the same antigens and peptides. These results significantly increase our understanding of the human immune response to *M. tuberculosis* specific antigens, and may help in designing *M. tuberculosis* DosR regulon encoded antigen and/or peptide based vaccination approaches for TB.

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## Supplementary file 1.

*Table Ia-e. Overlapping peptide sets of antigens selected for this study. Table Ia-d, overlapping peptide sets from DosR antigens Rv1733c, Rv2029c, Rv2031c and Rv2627c, respectively. All DosR peptides are 20 aa long with 10 aa overlap. All DosR derived peptides, with exception of peptides derived from DosR antigen Rv2031c were elongated with two lysine (K) residues at the C-terminal to improve solubility. Table Ie shows the overlapping peptide set of reference (non-DosR) antigen Ag85B. Ag85B peptides are also 20 aa long with 10 aa overlap with exception of Ag85B peptide numbers 20-22 which were 15 aa long with 10 aa overlap (see text in Table Ie).*

**Table I-a. Overlapping peptide set of DosR antigen Rv1733c**

| peptide | DosR antigen Rv1733c | position |
|---|---|---|
| number | aa sequence | start-end |
| 1 | MIATTRDREGATMITFRLRLKK | 1-20 |
| 2 | ATMITFRLRLPCRTILRVFSKK | 11-30 |
| 3 | PCRTILRVFSRNPLVRGTDRKK | 21-40 |
| 4 | RNPLVRGTDRLEAVVMLLAVKK | 31-50 |
| 5 | LEAVVMLLAVTVSLLTIPFAKK | 41-60 |
| 6 | TVSLLTIPFAAAAGTAVQDSKK | 51-70 |
| 7 | AAAGTAVQDSRSHVYAHQAQKK | 61-80 |
| 8 | RSHVYAHQAQTRHPATATVIKK | 71-90 |
| 9 | TRHPATATVIDHEGVIDSNTKK | 81-100 |
| 10 | DHEGVIDSNTTATSAPPRTKKK | 91-110 |
| 11 | TATSAPPRTKITVPARWVVNKK | 101-120 |
| 12 | ITVPARWVVNGIERSGEVNAKK | 111-130 |
| 13 | GIERSGEVNAKPGTKSGDRVKK | 121-140 |
| 14 | KPGTKSGDRVGIWVDSAGQLKK | 131-150 |
| 15 | GIWVDSAGQLVDEPAPPARAKK | 141-160 |
| 16 | VDEPAPPARAIADAALAALGKK | 151-170 |
| 17 | IADAALAALGLWLSVAAVAGKK | 161-180 |
| 18 | LWLSVAAVAGALLALTRAILKK | 171-190 |
| 19 | ALLALTRAILIRVRNASWQHKK | 181-200 |
| 20 | IRVRNASWQHDIDSLFCTQRKK | 191-210 |

**Table I-b. Overlapping peptide set of DosR antigen Rv2029c**

| peptide | DosR antigen Rv2029c | position |
|---|---|---|
| number | aa sequence | start-end |
| 1 | MTEPAAWDEGKPRIITLTMNKK | 1-20 |
| 2 | KPRIITLTMNPALDITTSVDKK | 11-30 |
| 3 | PALDITTSVDVVRPTEKMRCKK | 21-40 |
| 4 | VVRPTEKMRCGAPRYDPGGGKK | 31-50 |
| 5 | GAPRYDPGGGGINVARIVHVKK | 41-60 |
| 6 | GINVARIVHVLGGCSTALFPKK | 51-70 |
| 7 | LGGCSTALFPAGGSTGSLLMKK | 61-80 |
| 8 | AGGSTGSLLMALLGDAGVPFKK | 71-90 |
| 9 | ALLGDAGVPFRVIPIAASTRKK | 81-100 |
| 10 | RVIPIAASTRESFTVNESRTKK | 91-110 |
| 11 | ESFTVNESRTAKQYRFVLPGKK | 101-120 |
| 12 | AKQYRFVLPGPSLTVAEQEQKK | 111-130 |
| 13 | PSLTVAEQEQCLDELRGAAAKK | 121-140 |
| 14 | CLDELRGAAASAAFVVASGSKK | 131-150 |
| 15 | SAAFVVASGSLPPGVAADYYKK | 141-160 |
| 16 | LPPGVAADYYQRVADICRRSKK | 151-170 |
| 17 | QRVADICRRSSTPLILDTSGKK | 161-180 |
| 18 | STPLILDTSGGGLQHISSGVKK | 171-190 |
| 19 | GGLQHISSGVFLLKASVRELKK | 181-200 |
| 20 | FLLKASVRELRECVGSELLTKK | 191-210 |
| 21 | RECVGSELLTEPEQLAAAHEKK | 201-220 |
| 22 | EPEQLAAAHELIDRGRAEVVKK | 211-230 |
| 23 | LIDRGRAEVVVVSLGSQGALKK | 221-240 |
| 24 | VVSLGSQGALLATRHASHRFKK | 231-250 |
| 25 | LATRHASHRFSSIPMTAVSGKK | 241-260 |
| 26 | SSIPMTAVSGVGAGDAMVAAKK | 251-270 |
| 27 | VGAGDAMVAAITVGLSRGWSKK | 261-280 |
| 28 | ITVGLSRGWSLIKSVRLGNAKK | 271-290 |
| 29 | LIKSVRLGNAAGAAMLLTPGKK | 281-300 |
| 30 | AGAAMLLTPGTAACNRDDVEKK | 291-310 |
| 31 | TAACNRDDVERFFELAAEPTKK | 301-320 |
| 32 | RFFELAAEPTEVGQDQYVWHKK | 311-330 |
| 33 | TEVGQDQYVWHPIVNPEASPKK | 318-339 |

**Table I-c. Overlapping peptide set of DosR antigen Rv2031c**

| peptide | DosR antigen Rv2031c | position |
|---|---|---|
| number | aa sequence | start-end |
| 1 | MATTLPVQRHPRSLFPEFSE | 1-20 |
| 2 | PRSLFPEFSELFAAFPSFAG | 11-30 |
| 3 | LFAAFPSFAGLRPTFDTRLM | 21-40 |
| 4 | LRPTFDTRLMRLEDEMKEGR | 31-50 |
| 5 | RLEDEMKEGRYEVRAELPGV | 41-60 |
| 6 | YEVRAELPGVDPDKDVDIMV | 51-70 |
| 7 | DPDKDVDIMVRDGQLTIKAE | 61-80 |
| 8 | RDGQLTIKAERTEQKDFDGR | 71-90 |
| 9 | RTEQKDFDGRSEFAYGSFVR | 81-100 |
| 10 | SEFAYGSFVRTVSLPVGADE | 91-110 |
| 11 | TVSLPVGADEDDIKATYDKG | 101-120 |
| 12 | DDIKATYDKGILTVSVAVSE | 111-130 |
| 13 | ILTVSVAVSEGKPTEKHIQI | 121-140 |
| 14 | SVAVSEGKPTEKHIQIRSTN | 125-144 |

**Table I-d. Overlapping peptide set of DosR antigen Rv2627c**

| peptide | DosR antigen Rv2627c | position |
|---|---|---|
| number | aa sequence | start-end |
| 1 | MASSASDGTHERSAFRLSPPKK | 1-20 |
| 2 | ERSAFRLSPPVLSGAMGPFMKK | 11-30 |
| 3 | VLSGAMGPFMHTGLYVAQSWKK | 21-40 |
| 4 | HTGLYVAQSWRDYLGQQPDKKK | 31-50 |
| 5 | RDYLGQQPDKLPIARPTIALKK | 41-60 |
| 6 | LPIARPTIALAAQAFRDEIVKK | 51-70 |
| 7 | AAQAFRDEIVLLGLKARRPVKK | 61-80 |
| 8 | LLGLKARRPVSNHRVFERISKK | 71-90 |
| 9 | SNHRVFERISQEVAAGLEFYKK | 81-100 |
| 10 | QEVAAGLEFYGNRRWLEKPSKK | 91-110 |
| 11 | GNRRWLEKPSGFFAQPPPLTKK | 101-120 |
| 12 | GFFAQPPPLTEVAVRKVKDRKK | 111-130 |
| 13 | EVAVRKVKDRRRSFYRIFFDKK | 121-140 |
| 14 | RRSFYRIFFDSGFTPHPGEPKK | 131-150 |
| 15 | SGFTPHPGEPGSQRWLSYTAKK | 141-160 |
| 16 | GSQRWLSYTANNREYALLLRKK | 151-170 |
| 17 | NNREYALLLRHPEPRPWLVCKK | 161-180 |
| 18 | HPEPRPWLVCVHGTEMGRAPKK | 171-190 |
| 19 | VHGTEMGRAPLDLAVFRAWKKK | 181-200 |
| 20 | LDLAVFRAWKLHDELGLNIVKK | 191-210 |
| 21 | LHDELGLNIVMPVLPMHGPRKK | 201-220 |
| 22 | MPVLPMHGPRGQGLPKGAVFKK | 211-230 |
| 23 | GQGLPKGAVFPGEDVLDDVHKK | 221-240 |
| 24 | PGEDVLDDVHGTAQAVWDIRKK | 231-250 |
| 25 | GTAQAVWDIRRLLSWIRSQEKK | 241-260 |
| 26 | RLLSWIRSQEEESLIGLNGLKK | 251-270 |
| 27 | EESLIGLNGLSLGGYIASLVKK | 261-280 |
| 28 | SLGGYIASLVASLEEGLACAKK | 271-290 |
| 29 | ASLEEGLACAILGVPVADLIKK | 281-300 |
| 30 | ILGVPVADLIELLGRHCGLRKK | 291-310 |
| 31 | ELLGRHCGLRHKDPRRHTVKKK | 301-320 |
| 32 | HKDPRRHTVKMAEPIGRMISKK | 311-330 |
| 33 | MAEPIGRMISPLSLTPLVPMKK | 321-340 |
| 34 | PLSLTPLVPMPGRFIYAGIAKK | 331-350 |
| 35 | PGRFIYAGIADRLVHPREQVKK | 341-360 |
| 36 | DRLVHPREQVTRLWEHWGKPKK | 351-370 |
| 37 | TRLWEHWGKPEIVWYPGGHTKK | 361-380 |
| 38 | EIVWYPGGHTGFFQSRPVRRKK | 371-390 |
| 39 | GFFQSRPVRRFVQAALEQSGKK | 381-400 |
| 40 | FVQAALEQSGLLDAPRTQRDKK | 391-410 |
| 41 | AALEQSGLLDAPRTQRDRSAKK | 394-413 |

**Table I-e. Overlapping peptide set of early secreted antigen Ag85B (Rv1886c)**

| peptide | antigen Ag85B | position |
|---|---|---|
| number | aa sequence | start-end* |
| 1 | FSRPGLPVEYLQVPSPSMGR | 41-60 |
| 2 | LQVPSPSMGRDIKVQFQSGG | 51-70 |
| 3 | DIKVQFQSGGNNSPAVYLLD | 61-80 |
| 4 | NNSPAVYLLDGLRAQDDYNG | 71-90 |
| 5 | GLRAQDDYNGWDINTPAFEW | 81-100 |
| 6 | WDINTPAFEWYYQSGLSIVM | 91-110 |
| 7 | YYQSGLSIVMPVGGQSSFYS | 101-120 |
| 8 | PVGGQSSFYSDWYSPACGKA | 111-130 |
| 9 | DWYSPACGKAGCQTYKWETF | 121-140 |
| 10 | GCQTYKWETFLTSELPQWLS | 131-150 |
| 11 | LTSELPQWLSANRAVKPTGS | 141-160 |
| 12 | ANRAVKPTGSAAIGLSMAGS | 151-170 |
| 13 | AAIGLSMAGSSAMILAAYHP | 161-180 |
| 14 | SAMILAAYHPQQFIYAGSLS | 171-190 |
| 15 | QQFIYAGSLSALLDPSQGMG | 181-200 |
| 16 | ALLDPSQGMGPSLIGLAMGD | 191-210 |
| 17 | PSLIGLAMGDAGGYKAADMW | 201-220 |
| 18 | AGGYKAADMWGPSSDPAWER | 211-230 |
| 19 | GPSSDPAWERNDPTQQIPKL | 221-240 |
| 20 | NDPTQQIPKLVANNT # | 231-245 |
| 21 | QIPKLVANNTRLWVY # | 236-250 |
| 22 | VANNTRLWVYCGNGT # | 241-255 |
| 23 | VANNTRLWVYCGNGTPNELG | 241-260 |
| 24 | CGNGTPNELGGANIPAEFLE | 251-270 |
| 25 | GANIPAEFLENFVRSSNLKF | 261-280 |
| 26 | NFVRSSNLKFQDAYNAAGGH | 271-290 |
| 27 | QDAYNAAGGHNAVFNFPPNG | 281-300 |
| 28 | NAVFNFPPNGTHSWEYWGAQ | 291-310 |
| 29 | THSWEYWGAQLNAMKGDLQS | 301-320 |
| 30 | YWGAQLNAMKGDLQSSLGAG | 306-325 |

# 15-mers, 10 aa overlap

* sequence Ag85B consists of 325 aa. First 40 aa is leader sequence; not tested in study. Here, start of aa at position 41