Differential proteomics approach to identify putative protective antigens of *Mycobacterium tuberculosis* presented during early stages of macrophage infection and their evaluation as DNA vaccines

Shingar Sharma1,2, , RS Rajmani2, , Arun Kumar2, , Ashima Bhaskar2, , Amit Singh2, , Venkatasamy Manivel2, , Anil K Tyagi1 & Kanury VS Rao2,*

1Department of Biochemistry, University of Delhi South Campus, New Delhi-110 021, India
2Immunology group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110 067, India

Received 10 February 2015; revised 22 April 2015

Unsatisfactory performance of the existing BCG vaccine, especially against the adult pulmonary disease, has urged the need for an effective vaccine against tuberculosis (TB). In this study, we employed differential proteomics to obtain a list of antigens as potential vaccine candidates. Bacterial epitopes being presented at early stages on MHC class I and class II molecules of macrophages infected with *Mycobacterium tuberculosis* (*M. tb*) were identified using iTRAQ labelling and reverse phase LC-MS/MS. The putative vaccine candidates, thus identified, were tested as plasmid DNA vaccines in mice to ascertain their protective efficacy against the aerosolized *M. tb* challenge, based on their ability to reduce the bacterial load in the lungs of infected mice. Here, we observed that 4 out of the 17 selected antigens imparted significant protection against the challenge of *M. tb*. The four shortlisted antigens were further assessed in a more stringent guinea pig model, where too, they demonstrated significant protection. It concludes that combining a proteomics approach with the *in vivo* assessment of vaccine candidates in animal models can be valuable in identifying new potential candidates to expand the antigenic repertoire for novel vaccines against TB.

**Keywords**: BCG vaccine, Early antigens, MHC, Pulmonary disease, Tuberculosis (TB)

With an estimated 9 million new TB cases and 1.5 million deaths in 2013 tuberculosis (TB) continues to be a scourge of mankind1. Due to the problems of multidrug resistance, HIV-TB co-infections and socio-economic issues, the chances of controlling the global spread of TB look bleak without an effective vaccine2. The apparent failure of bacillus Calmette-gerin (BCG), the only TB vaccine approved for human use, to protect against adult pulmonary TB is well-documented3-9. BCG shows highly variable protective efficacy ranging from 0 to 80%8-10 for which a number of hypotheses have been put forward including differences in BCG strains, genetic heterogeneity between vaccinated populations, exposure to environmental mycobacteria and co-infection with helminths4,5. Moreover, BCG is potentially pathogenic in immuno-compromised hosts6,7 and hence, not recommended for use in HIV-infected individuals11.

Considering these challenges, concerted efforts are being made to develop TB vaccines that not only impart superior protection than BCG but also have a better safety profile in immunocompromised individuals. Currently, more than a dozen vaccine candidates are being evaluated in human clinical trials12. One of the basic approaches adopted towards the development of novel TB vaccines involves priming with BCG and boosting with another vaccine. DNA vaccination represents an attractive method for boosting the immunity of BCG because of the ability of DNA vaccines to stimulate both MHC class-I and class-II antigen presentation pathways13, and this approach has been used for many TB vaccine candidates14-17.

The development of an effective TB vaccine requires identification of target immunogens and/or strategies for their efficient presentation to induce the desired Th-1 responses. In the case of natural
infection, most individuals are able to mount a vigorous initial immune response that effectively controls the infection. In some individuals though, *M. tb* infection of macrophages leads to down regulation of surface expression of MHC-II levels, co-stimulatory (B7) molecules and gamma interferon (IFN-γ) receptor, the macrophages eventually becoming desensitized to any activation by antigen-specific T cells.

We based our present study on the hypothesis that greater success may be obtained if the early stage of infection is targeted and the initial immune response against the pathogen is strengthened. We surmised that a kinetic profiling of the antigens presented by macrophages soon after the uptake of *M. tb* would help to identify the subset of early stage *M. tb*-specific proteins.

In this study, we exploited techniques of quantitative proteomics such as isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labelling and mass spectrometry to capture early-stage antigens during intra-macrophage growth of *M. tb*. The antigens selected in this manner were evaluated as DNA vaccines for imparting protection against aerosolized *M. tb* challenge in the mouse model. Shortlisted candidates from *in-vivo* protection studies in mice were evaluated further in the more stringent guinea pig model. This study demonstrates the rationale of our strategy, that differential proteomics approach can be successfully applied to find new TB vaccine candidates.

**Materials and Methods**

*Bacterial strains—* *M. tb* H37Rv (donated by Dr. David Sherman, Seattle Biomedical Research Institute, Seattle) and *M. bovis* BCG (Danish strain, BCG laboratories, Chennai, India) were grown in Middlebrook (MB) 7H9 medium supplemented with 0.5% glycerol, 0.2% Tween-80 and 10% ADC and in MB 7H11 solid agar supplemented with 0.5% glycerol and 10% OADC (Difco).

*Cell culture—*THP-1 (ATCC® TIB-202™) and Mono Mac 6 (MM6, German Collection of Microorganisms and Cell Cultures, DSMZ ACC 124) cells were grown in RPMI 1640 (GIBCO Laboratories, NY, USA) supplemented with 10% foetal calf serum (Hyclone, Logan, UT, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. The medium for MM6 cells additionally contained 1 mM oxaloacetic acid and 9 μg/ml bovine insulin (Sigma, St. Louis, MO, USA).

*Infection of cells—*THP-1 cells were differentiated with 20 ng/ml PMA (phorbol-12-myristate-13-acetate) for 24 h. MM6 cells constitutively express phagocytic properties. Single cell suspension from *M. tb* H37Rv cultures was prepared by sonication and the bacteria were added to the macrophages at a multiplicity of infection (MOI) of 10 bacilli per macrophage. Infected cells were harvested by centrifugation at 6, 12 and 24 h after infection.

*Extraction of MHC class-I bound peptides from THP-1 cells—*Peptides were separated from MHC class-I molecules by mild acid elution. Pellets of infected THP-1 cells were incubated with citrate-phosphate buffer (pH 3) followed by centrifugation at 1000 rpm for 15 min at 4 °C. Cell-free supernatant containing extracted peptides was concentrated on a SepPak C18 cartridge (Millipore, Billerica, MA, USA). Bound peptides were eluted by 60% acetonitrile (ACN:H₂O) followed by 80% acetonitrile, pooled and dried. The dried peptides were then reconstituted in water containing 0.1% formic acid and passed through 3 kDa cutoff Centricon spin-column (Millipore). The filtrate was concentrated in a SpeedVac and stored at −80 °C until use. Uninfected THP-1 cells serving as a control underwent the same treatment as for the infected sample.

*Extraction of MHC class-II bound peptides from MM6 cells—*The extraction of MHC class-II bound peptides was carried out by an immuno-affinity matrix consisting of Protein-A Sepharose beads (Sigma) covalently linked with mAb HLA-DR/DP/DQ/DX (sc-53302, Santa Cruz Biotechnology, Santa Cruz, CA, USA). MM6 cells were used since THP-1 cells are relatively inherently deficient in MHC class II surface expression. Pellets of infected MM6 cells were lysed in Triton X-100 detergent (Sigma, 0.1% in 20 mM Tris-saline pH 8.0) containing protease inhibitors (Boehringer Mannheim, Germany) for 30 min on ice. After centrifugation at 13000 rpm for 20 min at 4 °C, supernatant was pre-cleaned by incubating with Sepharose beads for 30 min and centrifuged again. Supernatant was collected and incubated with the immuno-affinity matrix at 4 °C for 4 h followed by centrifugation at 1000 rpm at 4 °C. The matrix beads were then incubated with 0.1% trifluoroacetic acid in ddH₂O for 30 min at 37 °C followed by centrifugation at 2000 rpm for 5 min, and supernatant was passed through 10 kDa cutoff spin-column
The filtrate obtained was concentrated in SpeedVac at 25 °C and stored at −80 °C until use. The uninfected control cells underwent the same treatment as for the infected sample.

*iTRAQ labelling of peptides*—Peptide mixtures (100 μg) each from control cells and infected cells were labelled using iTRAQ Reagent (Applied Biosystems Inc., Foster City, CA, USA) 4-Plex kit following the manufacturer’s protocol with a minor modification by excluding the trypsin digestion. The peptides were processed and mixed with their respective isobaric tags (Supplementary Data 1 and 2) followed by incubation at room temperature in dark for 2 h. The labelled peptides from all samples were combined and concentrated in SpeedVac. The samples were then purified by performing strong cation exchange (SCX) chromatography using the cation exchange system provided in the iTRAQ Method Development Kit. Eluted fraction having labelled peptides was desalted using SepPak C18 cartridges and dried.

**HPLC tandem mass spectrometry on QSTAR XL**—HPLC separation was performed on 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). Dried SCX fractions (10 μg of each sample) were dissolved in mobile phase A (3% ACN/H2O/0.1% formic acid), injected onto a reverse phase capillary column (0.075×150 mm, Zorbax C18, 300 Å pore, 3.5 μm particle) and eluted at a flow rate of 250 nl/min using a linear gradient going to 60% of mobile phase B (90%ACN/H2O/0.1% formic acid) over 80 min. Mass data was acquired on QSTAR XL in an information-dependent acquisition (IDA) mode. While in the MS mode, ions were analyzed from m/z 350–1500 Da for a 1.0 s acquisition cycle, and MS/MS data was screened from m/z 100–1500 Da for the three most abundant ions with charge states between 1 and 2. The TOF region of the instrument was calibrated by using Glu-Fib (Sigma) to <5 ppm. Peptide identification was done by the Mascot Database (Matrix Science) using NCBI nr (version: 16.02.2007). The data analysis parameters were as follows: Sample type, iTRAQ (peptide labeled); Cys Alkylation, MMTS; Digestion, None; Instrument, QSTAR ESI; Species, *Mycobacterium tuberculosis* complex.

**Generation of DNA vaccine construct**—Experiments involving genetic manipulation were approved by IBSC as per guidelines (Project#136/2009; BT/17/06/96-PID). The expression vector pVRM was constructed by introducing additional cloning sites in the eukaryotic DNA vaccine vector pVR1020 (Vical Inc., San Diego, CA, USA) between the BamHI and BgII sites. The additional sites (for BstBI, NheI, XbaI and SpII) were introduced by PCR using forward primer 5’-CGGGATCCTTTCGAAGCTAGCTCTAGA CCTACGGATATCTGCCTGACGCTGCTGTGGCCTTTCTTAGTT-3’ and reverse primer 5’-CTGCGAGAACCAGGGACGGTGACACCGGACAGGCTGTGTCAC-3’. Gene fragments encoding about 100-300 residue polypeptides were designed that included the sequence presented on the MHC molecules and flanking regions, which were added to help overcome restriction of immune response to short length sequences. Synthesis of gene fragments and codon optimization for expression in mammalian cells was carried out by GeneArt (Regensburg, Germany). The synthesized gene fragments were then inserted at the appropriate cloning sites in pVRM to prepare the DNA vaccine constructs (Fig. 1). Recombinant plasmids were maintained in *Escherichia coli* DH5α strain grown in LB broth or on LB agar supplemented with kanamycin (25 μg/ml).

---

**Fig. 1**—Schematic diagram of the modified plasmid VRM (pVRM) showing the orientation of the CMV promoter, the tPA signal sequence, the additionally introduced multiple cloning site (MCS) and the position of the inserted synthesized gene sequences (between BstBI and XbaI restriction sites) to create the DNA vaccine constructs.
In vitro gene expression—NIH 3T3 cells were transfected with the vaccine constructs using Lipofectamine® (Invitrogen, Carlsbad, CA, USA). The transfected cells were maintained in DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 10% foetal calf serum and 2 mM glutamine, at 37 °C and 5% CO₂ for 48 h. RNA isolation for real-time PCR was carried out using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Gene expression was confirmed by performing real-time PCR on the CFX96 detection system (Bio-Rad, CA, USA). For vaccination experiments, the DNA vaccine constructs were purified from large volume cultures (4-6 L) with the EndoFree plasmid Giga purification kit (Qiagen) according to the manufacturer’s protocol.

Immunization and challenge in mice—C57BL/6 female mice, 6-8 wk old, were housed in Tuberculosis Aerosol Challenge Facility, ICGEB, New Delhi (biosafety level 3 facility) under specific-pathogen-free conditions and provided with food and water ad libitum. Mice were divided into groups of 4-5 for each vaccine candidate and the control groups. Each mouse was immunized intramuscularly with 100 μg DNA. The immunizations were carried out thrice at day 0, 21 and 42. The positive control groups received 1×10⁶ CFU BCG per animal subcutaneously on day 0. The negative control groups were immunized with saline or 100 μg of the vector alone (pVRM). Four weeks after the third immunization, mice were challenged with M. tb in the Madison Aerosol Chamber with an estimated aerosol challenge dose of 100-130 bacilli per animal. Mice were euthanized at 60 days post-infection. Whole lungs were aseptically removed and homogenized in 2 ml sterile saline solution. Ten-fold serial dilutions of the homogenate were plated onto MB 7H11 agar plates. Plates were incubated at 37 °C and colonies were counted after 3 wk.

Protection study in guinea pigs—Guinea pigs (Dunkin Hartley strain, 200-300 gm) were purchased from National Center for Laboratory Animal Science (NCLAS), Hyderabad, India and maintained in Tuberculosis Aerosol Challenge Facility. Protection studies in guinea pigs were carried out similarly as described for mice. Briefly, guinea pigs in groups of six were immunized using the same protocol as for mice, infected with aerosolized M. tb (20-50 bacilli per animal) and euthanized at 60 days post-infection. Lungs and spleens were aseptically removed and homogenized in 5 ml sterile saline solution. Bacterial counts were enumerated by plating 10-fold serial dilutions of the homogenate onto MB 7H11 agar and incubating the plates at 37 °C for 3 wk.

For histopathological analysis, portions of lung and spleen were aseptically removed at the time of sacrifice and fixed in 10% neutral buffered formalin. The tissue was embedded in paraffin wax and cut into five-micron sections using a microtome. Sections were then stained with hematoxylin and eosin (H&E) stain and subjected to histopathological analysis. Evaluation of tissue sections and scoring of granulomas was done by a pathologist blinded to the experimental groups. Granuloma scores were assigned as follows: (a) Granuloma with necrosis given a score of 5; (b) Granuloma without necrosis given a score of 2.5; (c) Granuloma with fibrous connective tissue given a score of 1. Total granuloma score was calculated by multiplying number of granulomas of each type by the score and then adding them up to obtain a total granuloma score for each sample.

Ethics statement—Animal experiments were carried out with the approval of the Institutional Animal Ethics Committee of International Centre for Genetic Engineering and Biotechnology, New Delhi (IAEC Approval number IMM-20;IMM-23/2011).

Statistical analysis—Data was analysed using both St de nt’s t-test and one-way ANOVA to compare the mean values of all the groups and P <0.05 was considered significant.

Results

Selection of vaccine candidate antigens through differential mass spectrometry—Antigenic epitopes of M.tb presented during early stages (within 24 h) of macrophage infection were identified using iTRAQ labelling and RP-LC-MS/MS technique. The mass spectra were submitted to MASCOT search engine for identification of the peptides and corresponding proteins (Supplementary data 1 and 2). The data was compiled from three experiments and antigens that are not already subjects of intensive research were picked out. Five MHC class-I restricted and twelve MHC class-II restricted peptides were selected (Table 1) for testing as vaccine candidates. The data for antigens detected in peptide search but excluded from the present study is provided in Supplementary data 3 and 4. Most of the selected antigens represent conserved hypothetical proteins and a few belong to the PE and PPE gene families.
Table 1—Selected antigens (17) with predicted gene function and their MHC class restriction.

<table>
<thead>
<tr>
<th>H37Rv Antigen (Rv#)</th>
<th>Experiment code</th>
<th>Name of gene/ Predicted function</th>
<th>MHC presentation</th>
<th>Mascot Ion Score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0143c</td>
<td>2D</td>
<td>Chloride channel gene</td>
<td>class I</td>
<td>10</td>
<td>TTGGAVLS</td>
</tr>
<tr>
<td>0694</td>
<td>4D</td>
<td>Probable lld1 protein, L-lactate dehydrogenase</td>
<td>class II</td>
<td>25</td>
<td>NHGGGNLDGTPASIRA</td>
</tr>
<tr>
<td>0874c</td>
<td>6D</td>
<td>Conserved hypothetical protein</td>
<td>class II</td>
<td>24</td>
<td>GADPSTGSIIEDEVVQVATMQ</td>
</tr>
<tr>
<td>0894</td>
<td>8D</td>
<td>Hypothetical protein, possible transcriptional regulator, luxR family</td>
<td>class II</td>
<td>22</td>
<td>VRPEQTLCATVQWSHAL</td>
</tr>
<tr>
<td>1087</td>
<td>10D</td>
<td>Hypothetical glycine protein</td>
<td>class I</td>
<td>10</td>
<td>PGQDGGPGG</td>
</tr>
<tr>
<td>1159</td>
<td>12D</td>
<td>Conserved hypothetical transmembrane protein, probable mannosyltransferase</td>
<td>class II</td>
<td>21</td>
<td>RVTSPPARTRAQAV</td>
</tr>
<tr>
<td>1384</td>
<td>14D</td>
<td>Carbamoyl phosphate synthase</td>
<td>class I</td>
<td>14</td>
<td>TEVERVAEASG</td>
</tr>
<tr>
<td>2490c</td>
<td>16D</td>
<td>PE-PGRS-43</td>
<td>class I</td>
<td>12</td>
<td>AGGAAPAGQV</td>
</tr>
<tr>
<td>3345c</td>
<td>20D</td>
<td>PE-PGRS-50</td>
<td>class I</td>
<td>12</td>
<td>TTGGAGAA</td>
</tr>
<tr>
<td>3296</td>
<td>24D</td>
<td>ATP-dependent helicase</td>
<td>class II</td>
<td>24</td>
<td>DSAPMLTAEPAIEFTDA</td>
</tr>
<tr>
<td>3388</td>
<td>26D</td>
<td>PE-PGRS-52</td>
<td>class I</td>
<td>35</td>
<td>QLIGNGNGGGGGGNGTGDG</td>
</tr>
<tr>
<td>1548c</td>
<td>28D</td>
<td>PPE-21</td>
<td>class II</td>
<td>22</td>
<td>NVTGSMGLFNSHGTNTGS</td>
</tr>
<tr>
<td>1797</td>
<td>30D</td>
<td>Conserved hypothetical protein, probable membrane protein</td>
<td>class II</td>
<td>24</td>
<td>TRLPKEVAAGNRNLTGRQLA</td>
</tr>
<tr>
<td>2432c</td>
<td>32D</td>
<td>Hypothetical unknown protein</td>
<td>class II</td>
<td>20</td>
<td>VRAEHCRCAGGCDECPSTM</td>
</tr>
<tr>
<td>2474c</td>
<td>34D</td>
<td>Conserved hypothetical protein</td>
<td>class II</td>
<td>23</td>
<td>VRPDDLSVAARSLAHLATTD</td>
</tr>
<tr>
<td>2744c</td>
<td>36D</td>
<td>Conserved 35kDa alanine rich protein</td>
<td>class I</td>
<td>18</td>
<td>KMQEQVSLRSMSELAL</td>
</tr>
<tr>
<td>3876</td>
<td>38D</td>
<td>Conserved hypothetical Pro- Ala- rich protein</td>
<td>class II</td>
<td>27</td>
<td>RPHEGMEAPDQMAAQ</td>
</tr>
</tbody>
</table>

<sup>a</sup>Peptides were selected based on individual peptide mass ion score instead of Mowse score since a reasonably high Mowse score could only be achieved if there were several peptides representing a single complete protein. However, the antigenic peptide presentation by macrophages does not allow such possibility. Thus, ion mass score for the selected antigens varied from 10 to 35.

Synthesis of gene fragments—The synthesis of codon-optimized gene fragments containing the sequences encoding the selected peptides and flanking sequences was carried out by GeneArt (Supplementary data 5).

Construction of DNA vaccines and in vitro gene expression—Vector pVRM was constructed by modifying the eukaryotic expression vector pVR1020 as described earlier, and the synthesized gene fragments were cloned (Fig. 1). For immunization experiments, the plasmids carrying the gene inserts (referred to by the Rv numbers of the genes to which the corresponding synthesized fragments belong) were purified from large volume cultures to obtain endotoxin free preparations. NIH 3T3 cells were transfected with the vaccine constructs using Lipofectamine® (Invitrogen), RNA isolated 48 h later and gene expression was confirmed by real time PCR (representative data in Supplementary data 6).

Protective efficacy against M. tb challenge in mice—Mice were immunized with 100 μg of DNA vaccine candidates, a total of three times at 3-wk intervals. Four weeks after the final dose, all mice were aerosol challenged with M. tb and the CFU counts in the lungs were determined 60 days after infection. Antigens Rv0143c and Rv1384 exhibited protection comparable to BCG with a significant reduction of approximately, 0.8 log<sub>10</sub> to over 1.0 log<sub>10</sub> in the bacterial CFU counts as compared to the vector control pVRM (Fig. 2). Rv0694 and Rv2490c also showed protection, with a CFU count reduction of approximately, 0.3 log<sub>10</sub> to 0.6 log<sub>10</sub> compared to the
Fig. 2—Protective efficacy of vaccine candidates in mice. Bacillary load in the lungs (CFU counts) at 60 days post-infection in mice immunized with (a) 17 vaccine candidates; and (b) Rv0694, Rv2490c, Rv3296, Rv0143c and Rv1384 in repeat experiment. [The 17 vaccine candidates were divided into 4 groups at random, each group having both positive (BCG) and negative (empty vector VRM) controls. One group also included a saline control to ensure that there were no non-specific protective effects. Rv3296 was selected at random for the repeat experiment in order to ascertain that the non-protective effect of certain vaccine candidates was not merely by chance. It possibly acts as a second negative control in the repeat experiment. C57BL/6 mice were immunized three times with 100 μg DNA at three-week intervals. Four weeks after the last immunization, mice were aerosol challenged with M. tb. Bars indicate the mean ± S.E. (n=4). Statistical analysis was carried out by one-way ANOVA and Student’s t-test. *P < 0.05 compared to vector control VRM was considered significant.]

Fig. 3—Protective efficacy of shortlisted vaccine candidates in guinea pigs. Bacterial load in (a) lungs and (b) spleen of guinea pigs in various groups at 60 days post-infection. [Vaccine candidates Rv0143c, Rv0694, Rv1384 and Rv2490c were evaluated for protective efficacy in guinea pigs. Guinea pigs were immunized thrice with 100 μg DNA at 3-wk intervals and rested for 4 wk followed by aerosol challenge with M. tb. The data shown is from one out of two similar experiments. Bars indicate the mean ± S.E. (n=6). Statistical analysis was carried out by one-way ANOVA and Student’s t-test. Level of significance: *P <0.05, **P <0.005, ***P <0.0005 compared to vector control VRM.]

vector control. Four antigens (Rv2744c, Rv2474c, Rv3876 and Rv1548c) however, seemed to exacerbate the bacterial burden. The remaining nine vaccine candidates (Rv0874c, Rv1087, Rv1159, Rv3345c, Rv3296, Rv3388, Rv0894, Rv1797 and Rv2432c) did not show any significant reduction in the bacterial counts compared to the vector control.

Protective efficacy in guinea pigs—Four vaccine candidates (Rv0143c, Rv0694, Rv1384 and Rv2490c) were shortlisted from mice experiments and tested further for protective efficacy in guinea pigs employing the same protocol as in mice. CFU counts in lungs and spleen were enumerated 60 days post-infection. All four candidates displayed statistically significant protection as compared to the vector control pVRM, although none protected as good as BCG. CFU counts in lungs were reduced by approximately, 0.5 log_{10} to 1.0 log_{10} (Rv0694 and Rv2490c, respectively) as compared to the vector group (Fig. 3a). The bacillary load in lungs of guinea pigs immunized with BCG was reduced by nearly 2 log_{10}. The CFU counts in spleens were lowered by approximately, 0.6 log_{10} to more than 1.0 log_{10} in comparison to the vector control whereas BCG immunized animals showed a decline in CFU count by nearly 2.5 log_{10} (Fig. 3b).
**Assessment of pathological changes**—Visual examination of infected organs of guinea pigs revealed markedly reduced gross lesions in lungs and spleens of animals immunized with vaccine candidates compared to organs of the vector-immunized animals, which clearly showed numerous discrete tubercles (Fig. 4). The gross pathological changes observed were substantiated by histopathological examination of infected tissues. Animals immunized with Rv0143c and Rv2490c exhibited significantly reduced histopathological damage compared to all other groups except BCG. BCG immunized animals showed minimum gross or histopathological lesions (Fig. 5). The total granuloma scores for lungs and spleen of various groups are presented in Table 2.

### Discussion

Of all *M. tb*-infected individuals, only 5-10% develop active disease, clearly indicating that strong natural protective immunity against TB exists. However, the exact immune mechanisms responsible for effective protection against TB and the events occurring during earliest stages of *M. tb* infection are not yet fully understood. The T cell mediated immune response plays a central role in protection against TB. Hence, identification of relevant T cell epitopes from *M. tb* proteins has become an important approach for selecting novel vaccine candidates to develop more potent TB vaccines than BCG. Various methodologies can be employed to identify MHC-bound epitopes from protein targets. Multiplexed quantitative proteomic analysis by using iTRAQ reagents has proved to be an efficient strategy to identify and quantify proteins in a variety of samples ranging from *E.coli* to human saliva.

In this study, we used iTRAQ labelling and tandem mass spectrometry to identify and select the antigens presented on MHC molecules during the early stages of *M. tb* infection of macrophages, and tested the selected antigens for protective efficacy in the mouse model.

---

**Table 2—Granuloma Scores of H&E stained (a) lung and (b) spleen sections of guinea pigs**

<table>
<thead>
<tr>
<th>Group</th>
<th>Granulomas with necrosis Score = 5</th>
<th>Granulomas without necrosis Score= 2.5</th>
<th>Granulomas with fibrosis Score = 1</th>
<th>Total granuloma score</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Lungs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VRM</td>
<td>8</td>
<td>21</td>
<td>0</td>
<td>92.5</td>
</tr>
<tr>
<td>BCG</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>7.5</td>
</tr>
<tr>
<td>Rv0143c</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>Rv0694</td>
<td>5</td>
<td>15</td>
<td>0</td>
<td>62.5</td>
</tr>
<tr>
<td>Rv1384</td>
<td>3</td>
<td>12</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Rv2490c</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>(b) Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VRM</td>
<td>0</td>
<td>&gt;30</td>
<td>0</td>
<td>&gt;75</td>
</tr>
<tr>
<td>BCG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rv0143c</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>Rv0694</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>Rv1384</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>Rv2490c</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>37.5</td>
</tr>
</tbody>
</table>
Fig. 5—Representative images of H&E stained tissue sections of (a) lung; and (b) spleen sections from guinea pigs at 60 days after infection. [Tissues were harvested, fixed and processed as described in Materials and Methods. Images at 100X are shown in left panel and 400X magnification on corresponding right panel. G, Granuloma; L, Lymphocytes; FC, Foamy histiocytic cells; AS, Alveolar spaces; and E, Epithelioid cells.]
Immunization with Rv0143c, Rv1384, Rv0694 and Rv2490c significantly reduced lung CFU counts as compared to the vector control. Three of these (Rv0143c, Rv0694 and Rv1384) are cell membrane proteins and are constituents of important physiological processes\textsuperscript{33}. Rv0143c is a conserved transmembrane protein, possibly a chloride ion channel. Rv0694 is L-lactate dehydrogenase (cytochrome), a flavin mononucleotide binding enzyme involved in respiration and the conversion of lactate into pyruvate. Rv1384 is carbamoyl phosphate synthase, involved in the arginine and pyrimidine biosynthesis pathways. The fourth, Rv2490c belongs to the PE-PGRS family of genes. Nearly 10\% of the \textit{M. tb} genome is dedicated to encoding the glycine-rich PE and PPE proteins, and although their exact function remains unknown, it has been suggested that they could be a source of antigenic variation or serve to disrupt immune response against the bacterium by inhibiting antigen processing\textsuperscript{33}. Immune response to PE and PE-PGRS proteins has been reported in literature\textsuperscript{34,35}.

It is interesting to note that except Rv0694, the rest three antigens are presented on MHC class-I molecules. The role of CD8+ T cells in TB immunity has not yet been fully explored. However, it has been demonstrated that MHC class-I knockout mice are highly susceptible to TB, thus clearly underlining the importance of cytotoxic T cell response in protection against the disease\textsuperscript{36-38}. In accordance, our data shows that immunization with antigens presented early during infection to CD8+ T cells can effectively reduce bacillary load in the lungs of infected mice.

The above-mentioned four vaccine candidates were further tested in guinea pigs, which is used as the next step in the hierarchy of animal models to evaluate protective efficacy. Being more stringent than the mouse model, many new vaccine candidates do not necessarily show protection in the guinea pig model. However, all four vaccine candidates demonstrated significant protection in guinea pigs as evidenced by decreased bacillary count in lungs and spleens of infected animals as well as lowered gross and histopathological damage. The levels of protection observed are comparable to those conferred by other successful DNA vaccine candidates tested in guinea pigs\textsuperscript{39,40}.

Our approach also helped us to identify several antigens that are not yet well characterized. Some of these, when used for immunization, appeared to enhance the bacterial load in the mice lungs compared to the vector control. The reason behind such observation remains unclear, but these particular antigens could possibly play an immunosuppressive role and their expression may represent the pathogen’s attempt to subvert the host immune responses during early infection. It must also be noted that unless CD4+ or CD8+ T cells recognize an epitope presented on the MHC molecules, no immune response is likely to develop against it. The antigens identified in this study are mainly not secretory proteins and as such it can be speculated that these are probably not immunodominant, but subdominant antigenic epitopes may provide us with an insight into the complex host-pathogen interactions and serve to be a valuable addition to the vaccine antigen repertoire.

The levels of protection induced by DNA vaccines alone are usually lower than those induced by BCG but immunization strategies comprising BCG priming and boosting with DNA have been reported to enhance protection against TB\textsuperscript{25,39}. Thus, combinations of plasmid DNA with BCG can possibly serve as a promising approach for potent vaccination against TB.

In conclusion, in the present study we have shown that combining proteomics with \textit{in vivo} experiments for plasmid-based screening of identified early-presented antigenic epitopes can be an effective strategy to shortlist promising TB vaccine candidates, which can further be evaluated for protective efficacy in more discriminatory animal models.

\textbf{Acknowledgments}

This work was supported by Centre of Excellence grant (Grant DB/01/COE/05/06) from the Department of Biotechnology (DBT), Government of India to KVSR and AKT. One of authors, SS acknowledges UGC, New Delhi, India for the award of fellowship. We are grateful to Dr. David Sherman (SBRI, Seattle, USA) for \textit{M. tb} H37Rv strain. Experiments requiring BSL-3 containment were carried out in DBT supported Tuberculosis Aerosol Challenge Facility (TACF) at ICGEB, New Delhi.

\textbf{References}


