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An Unbiased Peptide-Wide Discovery Approach to Select *Mycobacterium tuberculosis* Antigens that Target CD8+ T Cell Response During Infection

Mark J. Cayabyab^{1,2,†}, Lizeng Qin^{1,†}, Suely S. Kashino¹, Angelo Izzo³, and Antonio Campos-Neto^{1,*}

¹Global Infectious Disease Research Center, The Forsyth Institute, 245 First Street, Cambridge, MA 02142

²Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston MA 02115

³Department of Immunology and Pathology, Mycobacteria Research Laboratories, Colorado State University, Fort Collins, CO, USA

Abstract

Accruing data strongly support the possible role of CD8+ T cells in immunity against tuberculosis (TB). Multivalent vaccines against *Mycobacterium tuberculosis* (*Mtb*) that incorporate CD8+ T cell antigens with those that elicit CD4+ T cells are therefore highly desirable. To screen for potential CD8+ T cell antigens that are produced by *Mtb* during infection, we isolated pathogen-derived peptides that bound to MHC Class I molecules expressed in adherent splenocytes obtained from *Mtb*-infected mice. Mass spectroscopy analysis revealed the following four nonamer peptides that had 100% homology with *Mtb* proteins: DGYVGAPAH (MT_0401), TTMLPLFAD (MT_1164), RSGAATPVR (MT_2160.1) and LAAVVGVL (MT_0078). The gene MT_0401 codes the protein 5-Phosphoribosylglycinamide transformylase 2 and the other three genes code for hypothetical proteins with unknown function. The NCBI/Blast analysis showed that among the four peptides DGYVGAPAH had the highest maximum alignment score and lowest E value (number of alignments expected by chance). Therefore, we assessed whether MT_0401 expressed in two genetic vaccine formulations was capable of stimulating CD8+ T cell response that is specific to DGYVGAPAH peptide. When mice were immunized with a recombinant plasmid DNA and an E1/E3-deleted Adenovirus 5 expressing MT0401 protein, using both homologous and heterologous prime-boost protocols, they developed strong DGYVGAPAH-specific CD8+ T cell response as well as antibody and CD4+ specific T cell response to the full length MT0401 protein. Equally important was the observation that mice infected with *Mtb* developed

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*Corresponding author: Global Infectious Disease Research Center, Forsyth Institute, 245 First Street, Cambridge, MA 02142. Phone: (617) 892-8393. Fax: (617) 892-8610. acampos@forsyth.org.

[†]These authors contributed equally to this work

Two of the authors' affiliations have changed since the completion of this study:

- Lizeng Qin: Novartis Corporation, Cambridge, MA
- Suely Kashino: Adolpho Lutz Institute, São Paulo, SP, Brazil

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DGYVGAPAH-specific CD8+ T cell responses in both spleen and lungs. These results demonstrate that *Mtb* antigens that are processed and presented via MHC Class I machinery can be readily identified by the described approach and may be useful candidate antigens to stimulate specific CD8+ T cell responses in vaccine development programs.

Keywords

Tuberculosis; CD8 associated antigen; MT0401; immunogenicity

INTRODUCTION

Despite the availability of specific anti-tuberculosis drugs and the worldwide administration of the bacille Calmette–Guérin (BCG) vaccine, the TB pandemic continues to have a devastating toll on human lives with 2.2 billion people currently infected with the causative agent, *Mycobacterium tuberculosis* (*Mtb*), and an alarming 1.4 million deaths each year [1]. Clearly, better preventive measures that block *Mtb* transmission are needed, including vaccines that prevent infection or prevent further development of disease into active state.

The development of an effective vaccine against TB has proved to be difficult mainly because of our limited knowledge of the protective immunity that is needed to clear the infection as well as the lack of identification of antigens that when targeted by the immune response will result in protection against disease development. TB vaccine candidates should elicit cellular immune responses that are important in controlling *Mtb* and T lymphocytes are generally believed to mediate immunity against TB based both on animal models of infection and human data. CD4+ T+ cells and antigen-specific CD8+ T cells clearly play an important role in immunity against TB [2–7]. The CD8+ T cell mediated resistance is MHC class I restricted [8], granzymes A and B and granulysin dependent and perforin independent [9].

Numerous studies suggest that *Mtb* efficiently induces Ag-specific CD8+ T cells to various MHC I-restricted epitopes [10–15], and *Mtb*-specific CD8+ T cells are important in protection to mice in secondary *Mtb* infection [16;17].

In this study, we utilized a unique strategy to directly identify CD8+ T cell antigens by eluting *Mtb*-specific peptides bound to MHC Class I molecules from adherent spleen cells of infected mice. Through the utilization of this approach we described and discussed herein the identification of several CD8+ T cell antigen candidates and the construction and immunogenicity of two delivery systems that prime CD8+ T cell responses, i.e., a unique plasmid DNA and a non-replicative adenoviral vector expressing a selected vaccine candidate antigen.

MATERIALS AND METHODS

Identification of MHC I binding *Mtb* peptides

MHC Class I-molecules were purified from adherent spleen cells of C57BL/6 mice infected intravenously for 10–14 days with 10^7 CFU of *Mtb* H37Rv. Splenic adherent cells were obtained and lysed with CHAPS detergent (Boehringer Mannheim Corp., Ridgefield, CT) and MHC class I molecules were isolated by affinity purification on a protein G sepharose column linked to anti-H-2Kb/Db monoclonal antibody (ATCC HB-11). Peptides were acid eluted from the purified MHC class I molecules as described [18;19] and molecules smaller than 5 kDa were collected by ultra-filtration over a Millipore Ultrafree-CL 5 kDa cutoff. The low molecular weight material was fractionated by microbore reverse phase HPLC (Delta-

Pak C18 column, 300 Å pore size, 5µ particle) and peptide peaks were analyzed by microcapillary liquid chromatography coupled with electrospray ionization mass spectrometry (LC-MS) to determine the molecular mass and abundance. Eluted peptides were introduced into the LCQ-Ion Trap mass spectrometry (Finnigan, CA) by electrospray ionization interface (Cytopeia, Seattle, WA) and analyzed by data dependent MS and MS/MS scan. The collision induced dissociation spectra generated during the experiment was searched against mouse and *Mtb* protein databases using Sequest software to identify possible sequence matches.

Mice

8–12 week old female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and kept under specific pathogen-free conditions. All animal procedures were carried out under the guidelines of the Institutional Animal Care and Use Committee at the Forsyth Institute.

Mtb antigens and peptides

The MT0401 gene was subcloned into pET14b expression vector (Novagen-EMD Chemicals, Gibbstown, NJ), expressed in BL21(DE3)pLysS *E. coli* host (Invitrogen, Carlsbad, CA) and the over-expressed recombinant protein was purified by affinity chromatography as we have previously described [20]. The MHC I restricted peptide DGYVGAPAH of MT0401 was synthesized by New England Peptide (Gardner, MA).

Generation of recombinant DNA and adenovirus

The VRC8400 expression plasmid (donated by Dr. Gary Nabel, NIH) was constructed as described [21]. The MT_0401 gene was cloned from *Mtb* genomic DNA by PCR into the PCR2.1-TOPO plasmid (Invitrogen) and subsequently inserted into the VRC8400 plasmid.

Recombinant E1/E3-deleted Ad5 construct containing the MT_0401 gene was generated by a previously described method [22]. Briefly, the MT_0401 was inserted into the pAdBgIII containing a CMV promoter, enhancer and the bovine growth hormone polyadenylation signal. The adenovirus contained a deletion in E1 to render the vector replication defective and a partial deletion/substitution in E3, which disrupts the coding sequences for the E3 proteins [22]. The Ad5-MT_0401 vector was produced and amplified in 293 cells. Viruses were purified using the AdEasy™ Virus Purification and Viral Titer Kits (Stratagene, LaJolla, CA), respectively. Viral titer was calculated as infectious units (IFU) per ml.

Western Blot

293A cells (Invitrogen) were transfected with DNA-MT_0401 using a calcium phosphate transfection protocol (Promega) or infected with 10⁹ IFU rAd5-MT_0401. The rMT0401 protein expressed in the DNA-transfected or rAd5-infected cell lysates were resolved in 4–20% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Millipore, Medford, MA). Blot was blocked with 1% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and subsequently incubated with rabbit anti-rMT0401 overnight at 4°C. After rinses with TBS-T, goat anti-rabbit IgG labeled with horseradish peroxidase (Thermo Scientific Pierce, Rockford, IL) was added. Bound conjugates were detected using ECL enhanced chemiluminescence system (Amersham/GE Healthcare, Piscataway, NJ) and proteins were visualized by autoradiograph (Kodak BioMax, Rochester, NY).

Antibody ELISA

IgG1 and IgG2a antibodies raised against rMT0401 were titrated by standard ELISA [23].

Intracellular cytokine staining analysis (ICS)

Spleen cells were obtained using standard procedures. Mononuclear lymphoid cells infiltrating the lung tissues were obtained as follows: lungs from individual mice were removed and minced in 5ml solution of RPMI 1640 containing 1 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO). After incubation under gentle shaking at 37°C for 30 minutes the preparations were centrifuged at 1,700 rpm, washed with RPMI 1640 containing 5% fetal calf serum, and passed through pre-separation filters (Miltenyi Biotec, Auburn, CA). Splenocytes and lung mononuclear cells were cultured at 37°C in a 5% CO₂ environment for 6h in the presence of RPMI-1640/10% fetal calf serum alone (unstimulated), or with 5µg/ml of synthetic nonamer peptides, or with 10 µg/ml rMT0401. All cultures contained Monensin (GolgiStop; BD Biosciences). The cultured cells were cell-surface stained with the following monoclonal antibodies purchased from BD Biosciences: anti-CD3-FITC (145–2C11), anti-CD4-allophycocyanin-Cychrome7 (GK1.5), anti-CD8 - peridinin chlorophyll protein-Cychrome 5-5 (53–6.7). After fixing with Cytofix/Cytoperm solution (BD Biosciences), cells were permeabilized and stained with anti-IFN- / allophycocyanin (XMG1.2). Labeled cells were fixed in 1% formaldehyde-PBS. Samples were collected on a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Approximately 500 000–1 000 000 events were collected per sample. Doublets were excluded by forward scatter-area versus forward scatter-height. The CD4+ and CD8+ T cells were determined by their expression of CD3, CD4 or CD8. Functional analysis was performed by plotting the expression of each cytokine molecule against another, and a Boolean combination of single functional gates was generated using FlowJo software (version 7.6.3; Tree Star). The frequency of cells producing IFN- was determined using FlowJo (Tree Star). All values used for analysis are background subtracted. Responses were considered positive when the percentage of total cytokine-producing cells was at least twice that of the background.

RESULTS

Identification of *Mtb* antigens presented via MHC Class I molecules

Using the protocol described in Material and Methods to identify MHC Class I molecules associated peptides approximately 500 peptide sequences were identified by mass spectroscopy. As expected, most sequences had identical sequence homologies with those of mouse proteins. Importantly, four peptide sequences that had no known homologies with mouse proteins had identical sequence homologies with the deduced sequences of four different *Mtb* proteins. Table 1 depicts these peptides. Overall, these findings suggest that the protein donors of these peptides are *Mtb* antigens produced during the disease and processed within the MHC Class I machinery of antigen processing and presentation.

These proteins (Table 1) include a *Mtb* 5'-Phosphoribosylglycinamide transformylase 2 (MT0401), and three other hypothetical proteins of unknown function (MT_0078, MT_1164 and MT2160.1). The SYFPEITHI epitope prediction algorithms (<http://www.syfpeithi.de/index.html>) for both H2-Db and H2-Kb molecules confirmed that the sequences of three out the four discovered peptides provided significant binding scores for these MHC Class I alleles (Table 2).

Moreover, the NCBI/Blast analysis of the selected peptide sequences revealed Maximum Alignment Scores ranging from 31.2–28.6 and E values (number of alignments expected by chance) ranging from 3.3–22. The highest Maximum Alignment Score (31.2) and lowest E value (3.3) was attributed to the peptide DGYVGAPAH. In addition, at 100% homology identity this peptide was found to be present only in *Mtb*, which strongly validates MT0401 as the peptide donor protein. Although the Max Score and E value were also relatively

strong for the other three peptides their protein donors are apparently more ubiquitous and present in other organisms than *Mycobacterium* (Table 1).

Therefore, the present study concentrates on the evaluation of MT0401 antigen as a potential vaccine candidate for TB that prime and stimulates CD8⁺ T cells.

Construction of recombinant DNA and Adenovirus expressing MT_0401

The MT_0401 gene was PCR cloned from purified *Mtb* H37Rv genomic DNA into pCR2.1 plasmid and subsequently shuttled into the plasmid DNA VRC8400 expression vector as well as in the replication deficient Adenovirus 5 (Ad5). We used the VRC8400 because this vector was previously shown to express vaccine antigens at significantly higher levels and was demonstrated to induce higher magnitude of T cell responses compared to first-generation DNA vaccine vectors [24–27]. Additionally, a highly immunogenic recombinant E1/E3-deleted Adenovirus serotype 5 [28;29] expressing the MT0401 antigen was constructed.

Prior to immunogenicity studies, we assessed, by Western blot analysis, the expression of the *Mtb* MT0401 antigen in the DNA or rAd5 vector in the 293 cell line. The MT0401 antigen was expressed in both DNA-MT0401-transfected and rAd5 MT0401-infected cells (Fig. 1, lanes 2 and 4) but not in sham DNA-transfected nor sham rAd5-infected cells (Fig. 1, lanes 1 and 3). A rMT0401 with a molecular weight of approximately 45 kDa was included as a positive control in the Western blot analysis (Fig. 1, lane R). These data show that both DNA and rAd5 vaccine constructs express the MT0401 antigen.

Prime-boost immunization with recombinant DNA-MT0401 and rAd5-MT0401 elicited rMT0401-specific CD4⁺ T cell and DGYVGAPAH-specific CD8⁺ T cell responses

To begin the immunogenicity studies we evaluated the antibody responses induced by homologous or heterologous prime/boost immunizations, mice received different immunization protocols. The following groups were tested: Group 1, Saline; Group 2, Sham DNA (40µg) - 3x, four weeks interval; Group 3, Sham Ad5 (5×10⁸ PFU); Group 4, DNA-MT_0401 (40µg) - 3x, four weeks interval; Group 5, rAd5-MT_0401 (5×10⁸ PFU); and Group 6, Priming with DNA-MT_0401 (40ug - 2x, four weeks interval) followed by one boost with Ad5-MT_0401 (5×10⁸ PFU). Both IgG1 and IgG2a antibody responses were evaluated by ELISA using specific anti-mouse isotype antibodies. Sera isolated from the groups 1–3 (controls) had little or undetectable antibodies of either isotype. In contrast, immunization with DNA-MT0401 (group 4), rAd5-MT0401 (group 5) or a combination of both (group 6) induced **high titers (1/360)** of IgG1 and IgG2a antibody responses to rMT0401 (not shown). We next evaluated the generation of CD8⁺ T cell and CD4⁺ T cell responses in C57BL/6 mice sensitized using the same genetic immunization protocol described above. Mice were sacrificed 30 days after the last immunization and lung and spleen mononuclear cells were isolated and stimulated *in vitro* with either DGYVGAPAH or with the full length rMT0401. CD8⁺ as well as CD4⁺ T cell responses were measured by ICS (IFN- γ) in cultures stimulated with the peptide DGYVGAPAH or with rMT0401, respectively. *In vitro* stimulation of splenocytes (Fig. 2) or lung mononuclear cells (Fig. 3) with the peptide DGYVGAPAH induced the production of substantial amounts of IFN- γ by CD8⁺ T cells comparable to non-stimulated cells. Conversely, when both cell populations (lung and spleen) were stimulated with the rMT0401 substantial amounts of IFN- γ was produced by CD4⁺ T cells comparable to non-stimulated cells. Taken together, these results show that the VRC8400 DNA and rAd5 vaccine vectors can deliver the MT0401 protein to antigen presenting cells, which process and present the DGYVGAPAH peptide for the priming of *Mtb* antigen specific-CD8⁺ T cells *in vivo*.

CD8 T cells of *Mtb*-infected mice recognized the MT0401 DGYVGAPAH epitope

Although immunogenicity evaluation of vaccine candidates is critical in vaccine development against infectious diseases, the actual recognition of the selected vaccine candidate by the immune system of the infected host is also critical. To test this requirement, we next evaluated the recognition of MT0401 by the immune system of mice infected with *Mtb*. C57BL/6 mice were infected i.v. with 2×10^6 CFU of *Mtb* strain 18b and six weeks later they were sacrificed and spleen cells and mononuclear lung cells were obtained and assayed for recognition of rMT0401 and DGYVGAPAH peptide by ICS. Fig. 4 shows that CD4⁺ T cells obtained from both spleen and lungs of infected mice strongly recognized the recombinant protein. Importantly, CD8⁺ T cells from both spleen and lungs also recognized the DGYVGAPAH peptide. These results strongly confirm that the infectious process caused by *Mtb* results in recognition and processing of the selected antigen in both MHC Class I and MHC Class II pathways and hence supporting its subsequent validation in vaccine experiments.

DISCUSSION

The identification of protective tuberculosis antigens and the efficient delivery of these antigens *in vivo* is a *sine qua non* condition for the development of TB vaccines [30]. Because CD8⁺ T cell responses are important mediators of immunity to tuberculosis, we identified *Mtb* proteins that are involved in stimulating these cells during the infectious process *in vivo*. We used a direct approach to identify *Mtb* peptides associated with MHC Class I molecules on adherent spleen cells obtained from infected C57BL/6 mice. Sequence analysis of the eluted peptides revealed that four peptide sequences showed high homology with *Mtb* proteins (Table 1). Because the intracellular biology of the *Mtb* infectious process appears to be same in both mouse and human cells, it is reasonable to expect that a *Mtb* protein that reaches the MHC Class I compartments of the antigen presentation pathway in mouse cells also will reach this destination in infected human cells. Consequently, despite the fact that an MHC class I associated peptide found in murine cells may not have the right motif to bind to human MHC Class I molecules, it is very likely that the peptide donor protein should have one or more other motifs that can bind to the human MHC Class I molecules. Indeed, epitope prediction analysis revealed that the discovered four proteins have a high number of epitopes that can potentially bind to most human MHC Class I alleles. This conclusion was reached using the MHC Class I peptide binding prediction algorithm from “The Immune Epitope Database and Analysis Resource” (<http://tools.immuneepitope.org/main>). This approach clearly indicate that the proteins coded for by MT_0401 and MT_0078 genes contain large numbers of epitopes (~3–15) that were predicted to bind with high affinity to majority of the human MHC Class I alleles of both HLA A and HLA B haplotypes. Although the proteins coded for by the genes MT_2160.1 and MT_1664 have smaller MW than those coded by MT_0401 and MT_0078, they have 1–5 such epitopes per molecule (not shown).

To begin the validation of these antigens as vaccine candidates in the mouse model of TB we chose the antigen coded for by MT_0401 gene because this molecule is the donor of the nonamer peptide DGYVGAPAH, which has very significant binding scores as predicted by the SYFPEITHI epitope prediction algorithm for both H2-Db and H2-Kb molecules of the mouse MHC and its gene is conserved within *Mycobacterium*. We generated recombinant DNA and Ad5 vectors expressing MT0401 protein and evaluated the immunogenicity of these constructs in mice using various homologous and heterologous prime boost regimens. Both homologous DNA prime boost regimen as well as heterologous DNA prime-Ad5 boost regimens elicited antibody as well as CD4 and CD8 T cell responses better than Ad5 alone. Importantly, DGYVGAPAH-specific CD8⁺ T cells were elicited in vaccinated mice.

Interestingly, the homologous DNA prime boost regimen was the best immunization protocol for eliciting IgG1 and IgG2a titers, consistent with the observation that the optimized VRC8400 DNA vaccine is a strong inducer of CD4+ T helper cells, which is needed for optimal antibody responses [31–33].

Because the DGYVGAPAH peptide was eluted from MHC Class I of adherent splenocytes isolated from infected mice, it was important to verify if infected mice produced DGYVGAPAH epitope-specific CD8+ T cells. Indeed, CD8+ T lymphocytes isolated from lung and splenocyte of *Mtb*-infected mice clearly responded to DGYVGAPAH peptide stimulation. These results confirm that during infection the MT0401 antigen is processed and stimulates specific CD8+ T cells. Moreover, MT0401-specific CD4+ T cells were also found in the lungs and spleen of *Mtb*. From these observations we conclude that during the infectious process, MT0401 protein is being produced by the *Mycobacterium*, processed and presented via the MHCI and MHCII pathway to prime antigen-specific T cells.

In conclusion, the antigen discovery strategy used in this study to directly unravel *Mtb* antigens associated with MHC Class I molecules of adherent spleen cells of mice infected with this pathogen is a reliable approach to identify the pathogen's molecules that stimulate CD8+ T cell responses during infection. In addition, consistent with previous observations, **both plasmid DNA and heterologous prime/boost immunizations constitute excellent procedures** to effectively stimulated IgG2a antibody response (Th1 response) and potent CD8+ T cell response. Protection studies to evaluate the preclinical efficacy of prime-boost strategies using the DNA-MT0401 and rAd5-MT0401 vaccine constructs are forthcoming.

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HIGHLIGHTS

- We describe a direct approach to discover MHC Class I associate peptides of Mtb
- Prime/boost immunization induced strong CD8+ T cell response to discovered peptide
- CD8+ T cells from lungs/spleen of Mtb-infected mice recognize discovered peptide

Figure 1. Expression of MT0401 in recombinant DNA and recombinant Ad5 vaccine vectors
 293A cells were transfected with 50 μ g of DNA [DNA sham (DNA, lane 1) or DNA-MT0401 (DNA, lane 2)] or infected with 10^9 IFU rAd5 [rAd5 sham (Ad5, lane 1) or rAd5-MT0401 (Ad5, lane 2)]. The lysates of the DNA-transfected or rAd5-infected cell were resolved in 4–20% SDS-PAGE gels and transferred to PVDF membrane. Presence of MT0401 on the membrane was determined using rabbit anti-rMT0401 followed by goat anti-rabbit IgG labeled with horseradish peroxidase. Bound conjugates were detected using ECL enhanced chemiluminescence system and proteins were visualized by autoradiography. Arrow points to the bands of ~45kDa that is recognized by the specific anti-rMT0401 on lanes DNA 2, Ad5 2 as well as in lane R (purified recombinant MT0401 from *E. coli*). Note that the migration of the purified recombinant protein is slightly higher than the molecular mass of the molecules produced by the 293A cells. This minor difference is expected because of the addition of the His tag sequence and a thrombin site added to the recombinant protein to facilitate its purification. The rMT0401 antigen produced by the 293A cells do not contain the His tag sequence and thrombin site.

Figure 2. Recognition of DGYVGAPAH and rMT0401 by CD8+ and CD4+ T cells from spleens of genetically immunized mice

C57BL/6 mice were either immunized with saline (A) or immunized i.m. with DNA-MT0401 (40 μ g) three times (4 weeks interval) (B), or immunized once with Ad5-MT0401 (5 $\times$ 10⁸ PFU) (C). **In D, mice were primed with DNA-MT_0401 (40 μ g - 2x, four weeks interval) followed by one boost with Ad5-MT_0401 (5 $\times$ 10⁸ PFU).** Four weeks after the last immunization mice were sacrificed and spleens were removed. Mononuclear cell suspensions were prepared and stimulated for six hours with either medium, the peptide DGYVGAPAH, or with rMT0401 protein. Production of IFN- γ by CD8+ T cells (cultures stimulated with DGYVGAPAH) and by CD4+ T cells (cultures stimulated with rMT0401) was assayed by intra-cellular cytokine staining. Results are expressed as the percentage of IFN- γ positive CD8+ or CD4+ T cells. **This is a representative experiment of three experiments with the same results.**

Figure 3. Recognition of DGYVGAPAH and rMT0401 by CD8+ and CD4+ T cells from lungs of genetically immunized mice

C57BL/6 mice were either immunized with saline (A) or immunized i.m. with DNA-MT0401 (40 μ g) three times (4 weeks interval) (B), or immunized once with Ad5-MT0401 (5 $\times$ 10⁸ PFU) (C). **In D, mice were primed with DNA-MT_0401 (40 μ g - 2x, four weeks interval) followed by one boost with Ad5-MT_0401 (5 $\times$ 10⁸ PFU).** Four weeks after the last immunization mice were sacrificed and lungs were removed. Mononuclear cell suspensions were prepared and stimulated for six hours with either medium, the peptide DGYVGAPAH, or with rMT0401 protein. Production of IFN- γ by CD8+ T cells (cultures stimulated with DGYVGAPAH) and by CD4+ T cells (cultures stimulated with rMT0401) was assayed by intra-cellular cytokine staining. Results are expressed as the percentage of IFN- γ positive CD8+ or CD4+ T cells. **This is a representative experiment of three experiments with the same results.**

Figure 4. Recognition of DGYVGAPAH and rMT0401 by CD8+ and CD4+ T cells from mice infected with *M. tuberculosis*

C57BL/6 mice were infected i.v. with 2×10^6 CFU of *M. tuberculosis* strain 18b. Six weeks after infection mice were sacrificed and both spleen and lungs were removed. Mononuclear cell suspensions were prepared from these organs and stimulated for six hours with either medium, the peptide DGYVGAPAH, or with rMT0401 protein. Production of IFN- γ by CD8+ T cells (cultures stimulated with DGYVGAPAH) and by CD4+ T cells (cultures stimulated with rMT0401) was assayed by intra-cellular cytokine staining. Results are expressed as the percentage of IFN- γ positive CD8+ or CD4+ T cells. **This is a representative experiment of two experiments with the same results.**

Table 1
NCBI/Blast of Selected Peptides Isolated from MHC Class I Molecules of Adherent Spleen Cells of *M. tuberculosis*-Infected Mice.

Peptide	Peptide Sequence	Donor Protein	Max Score	E value	Source	Max Ident %	MW	TIGR annotation
1	DGYVGAPAH	5 -PHOSPHORIBOSYL-GLYCINAMIDE- TRANSFORMYLASE 2	31.2	3.3	<i>M. tuberculosis</i>	100	43,678	MT_0401
2	TTMPLFAD	HYPOTHETICAL PROTEIN	29.9	6.9	<i>M. tuberculosis</i> <i>Acinetobacter baumannii</i>	100	17,722	MT_1664
3	RSGAATPVR	HYPOTHETICAL PROTEIN	29.9	7.6	<i>M. tuberculosis</i> <i>Neosartorya fischer</i> <i>Aspergillus fumigatus</i>	100	6,014	MT_2160.1
4	LAADVGVVL	CONSERVED HYPOTHETICAL PROTEIN	28.6	22	<i>M. tuberculosis</i> <i>Natrialba aegyptia</i> <i>Streptomyces sp</i>	100	36,410	MT_0078

Table 2Binding Scores of Selected Peptides to MHC Class I Molecules of Mice ^{*}.

Peptide Sequence	MHC Class I Binding Scores	
	H2-Kb	H2-Db
DGYVGAPAH	7	5
TTMPLFAD	1	0
RSGAATPVR	9	2
LAAVVGCVL	12	16
SYFPEITHI ^{**}	9	15

^{*} Defined by the SYFPEITHI epitope prediction algorithms (<http://www.syfpeithi.de/index.html>)

^{**} Binding scores for the reference peptide of the algorithm. Added for comparison purpose only.