*Iranian Biomedical Journal 11 (1): 23-31 (January 2007)*

# Identification of *Mycobacterium tuberculosis* CTL Epitopes Restricted by HLA-A*0201 in HHD Mice

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Received 29 October 2005; revised 21 June 2006; accepted 17 July 2006

## ABSTRACT

**Background:** $CD8^+$ T cells are thought to play an important role in protective immunity to tuberculosis. The major histocompatibility complex class I subtype HLA-A*0201 is one of the most prevalent class I alleles, with a frequency of over 30% in most populations. HLA-A*0201 transgenic, H-$2D^b$/mouse beta2-microglobulin double-knockout mice (HHD) which express human HLA-A*0201 but no mouse class I, was shown to provide a powerful model for studying induction of HLA-A*0201-restricted immune responses *in vivo*. **Methods:** HHD mice were immunized with plasmid DNA encoding MPB51 by using a gene gun, and IFN-γ production from the immune spleen cells was analyzed in response to a synthetic overlapping peptide library covering the mature MPB51 sequence. catatonic T lymphocytes (CTL) activity was measured using cytotoxicity assay and the three-color flowcytometry was used to reveal IFN-γ-producing immune spleen cells. **Results:** Our findings were shown that only one peptide, p51-70, appeared to stimulate the immune splenocytes to produce IFN-γ. Flow cytometric analysis with intracellular IFN-γ and the T-cell phenotype revealed that the p51-70 peptide contains an immunodominant $CD8^+$ T-cell epitope. Further analysis with computer-assisted algorithms permitted identification of a T-cell nona mer epitope, p54-62. Finally, we proved that the p54-62/HLA-A*0201 complex is strongly recognized by HLA class I-restricted $CD8^+$ MPB51-specific CTL cells. **Conclusion:** These results suggest that vaccination with MPB51 gene elicited MPB51-specific CTL. In addition, the P54-62 epitope thus represent potential subunit component for the design of vaccines against tuberculosis. *Iran. Biomed. J. 11 (1): 23-31, 2007*

*Keyword*: DNA vaccine, MPB51, HHD mice, Epitope

## INTRODUCTION

The world-wide problem of tuberculosis (TB) is increasing due to several factors, including multi-drug-resistant strains and coinfection with human immunodeficiency virus [1]. An attenuated strain of *Mycobacterium bovis* BCG is the only currently available anti-TB vaccine, still its efficacy, particularly its efficacy against pulmonary TB in adults, is controversial [2]. It is evident that there is an urgent need for a new and more reliable anti-TB vaccine [3].

Although the mechanisms of protection against TB have not been completely determined, cell-mediated immunity plays a pivotal role in the control of *Mycobacterium tuberculosis* infection. There is mounting evidence that $CD8^+$ cytotoxic T lymphocytes (CTL) contribute to disease resistance since susceptibility to *M. tuberculosis* is greater in mice deficient in $CD8^+$ T cells [4; 2]. In fact, mice with a β2-microglobulin ($\beta_2$m) deficiency are susceptible to *M. tuberculosis* infection [5, 6].

Many studies have indicated a prominently protective role for $CD4^+$ T cells [7], and several HLA class II-restricted epitopes have been identified on proteins of *M. tuberculosis* [8]. Since it was reported that $\beta_2$-$\beta_2$m-deficient mice, which lacks $CD8^+$ T cells, show increased susceptibility to experimental TB [5], the role of $CD8^+$ T cells has drawn increasing attention. The recent application of DNA vaccines to TB has provided evidence for MHC class I-restricted, $CD8^+$ T cell-mediated protection in mouse models of TB [9]. In humans, the existence of *Mycobacterium*-reactive MHC class I-restricted $CD8^+$ T cells has been demonstrated

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[ DOR: 20.1001.1.1028852.2007.11.1.5.1 ]

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[11], but very little is known about which antigens (Ag) are recognized by such T cells.

To better understand $CD8^+$ T cell responses during infection, it is important to identify epitopes that are recognized by $CD8^+$ T cells. Overlapping peptide libraries have widely employed for the identification of T cell epitopes. The libraries, consisting of 15-20 mer peptides overlapping by at least nine residues, span the whole protein of interest [12]. To design a new generation of vaccines, more information on the antigenic makeup of *M. tuberculosis* must be obtained in order to identify immunodominant proteins and epitopes. Secreted extracellular Ag are likely to be important in the induction of protective immunity [13], especially during the early phase of infection. Secreted and surface-exposed cell wall proteins seem to play a pivotal role in the induction of protective cellular immunity against TB [14]. MPB51 is recently known to be a major fraction of the secreted proteins [15] of *M. tuberculosis* which exhibits primary structure similarity (38 to 43%) to Ag 85 complex (Ag 85A, Ag 85B, and Ag 85C) [16, 17]. It is able to bind fibronectin [18], but the physiological role of MPB51 remains elusive. In fact, the Ag85 complex has been reported to induce Th1 cells and CTL in healthy individuals exposed to *M. tuberculosis* and in *M. bovis* BCG-infected mice [19-21]. Furthermore, vaccination of mice with plasmid DNA encoding Ag85A and Ag85B can induce strong cellular immune responses and confer protection against a challenge with *M. tuberculosis* [22, 23].

Since the Ag specificity of the human T cell response is known to be strongly controlled by HLA polymorphism [24], the immunogenic potential of candidate vaccines needs to be defined in the context of major HLA polymorphism. As HLA-A*0201 is one of the most prevalent class I allele in most populations, we used DNA vaccination of a humanized murine model for *in vivo* experiments, the HHD mice, which is both knockout for murine MHC class I and transgenic for HHD, a chimeric heavy chain containing HLA-A*0201 α1-α2 domains [25]. We report the identification of peptide-specific $CD8^+$, HLA-A*0201-restricted cytotoxic T cells in HLA-A*0201 transgenic, HHD, mice.

## MATERIALS ANS METHODS

***Mice.*** The homozygote HHD mice express a transgenic monochain class I molecule composed of the α1 and α2 domains of HLA-A0201 covalently linked to human β2m and α3, transmembrane and cytoplasmic domains from murine $H\text{-}2D^b$ on C57BL/6 background [26]. This allows the murine CD8 molecule on the murine $CD8^+$ T cells to interact with the syngeneic α3 domain of the hybrid MHC class I molecule. Expression of the HHD molecule and absence of $H\text{-}2D^b$ and $H\text{-}2K^b$ were confirmed by flowcytometric analysis using PE-conjugated antihuman HLA-A, B, C Ab, FITC-conjugated antimouse $H\text{-}2D^b$ Ab and FITC-conjugated antimouse $H\text{-}2K^b$ Ab [all Ab were purchased from Becton Dickinson (San Jose, CA, USA)]. The mice were kept under specific-pathogen-free conditions and fed autoclaved food and water ad libitum. Two-month-old female mice were used in all experiments. This study was approved by the Institutional Animal Committee and the Provincial Board.

***Construction of a plasmid DNA vaccine, pCI-MPB51.*** The DNA encoding the mature MPB51 molecule (Rv3803c) was amplified from a plasmid, pMB49 [15], by PCR with the following primers: forward primer with ATG starting codon, 5′-CCTCTAGA**ATG**GCCCATACGAGAACCTGA-3′ and reverse primer with stop codon, 5′-CAGGCTCTAGACATCGGCACCTGGC**TTA**GC-3′ (the underlined nucleotides are XbaI sites). The PCR fragment was digested with XbaI and inserted into the XbaI site located downstream of the cytomegalovirus immediate-early enhancer/ promoter region of expression plasmid pCI (Promega, Madison, Wis., USA). The integrity of the nucleotide sequence was validated by automated DNA sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, Foster City, CA, USA) using a dye primer cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

***Peptides.*** Peptides spanning the entire 266-amino-acid (aa) mature MPB51 sequence of *M. tuberculosis* were synthesized as 20-mers overlapping by 10 residues, except for the carboxyl-terminal 12-mer from aa 255 to 266. Lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA, USA), and the purity of the peptides was confirmed by mass spectrometry. To identify the potential HLA-A0201-restricted $CD8^+$ T-cell epitopes in the 20-mer peptides, computer-based programs were used with access through the Bio Informatics & Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions


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website (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) [27], the SYFPEITHI (a database of MHC ligands and peptide motifs) epitope prediction website (http://www.syfpeithi.de/) [28] and HLA Epitope binding prediction website (http://hlaligand.ouhsc.edu/prediction.htm). All peptides were dissolved in 5% dimethyl sulfoxide in RPMI 1640 medium to a concentration of 1 mM and were stored at -80°C.

***Immunization of mice.*** Mice were immunized with a plasmid DNA vaccine encoding the mature MPB51 molecule by using a gene gun system. For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, Calif., USA), a cartridge of DNA-coated gold particles was prepared according to the manufacturer's instructions. Finally, 0.5 mg of gold particles was coated with 1 μg of plasmid DNA, and the mice were inoculated twice with 0.5 mg of gold per shot. To immunize mice, the shaved abdominal skin was wiped with 70% ethanol. The spacer of the gene gun was held directly against the abdominal skin. Then, the device was discharged at a helium discharge pressure of 400 psi Mice were inoculated with 2 μg of plasmid DNA (by two shots immunization) four times at 1-week intervals.

***Preparation of splenocyte culture supernatants for evaluation of IFN-γ production.*** Two weeks after the last *M. tuberculosis* MPB51 DNA vaccination, mice were sacrificed and their spleens removed. Spleen cell suspensions ($1 \times 10^6$ cells/well) were cultured in RPMI/10FCS in 96-well plates in the presence of 5 μM peptide with 5% $CO_2$ at 37°C. Supernatants were harvested 24 h later and stored at -20°C until they were assayed for IFN-γ. The IFN-γ concentration was measured by a sandwich ELISA.

***Quantification of IFN-γ by a cytokine ELISA.*** IFN-γ production was measured by an ELISA. The 96-well ELISA plates (E.I.A./R.I.A. Plate A/2; Costar, Cambridge, Mass., USA) were coated with 2 μg/ml of capture antibody (anti-murine IFN-γ monoclonal antibody [mAb] R4-6A2; BD PharMingen, San Jose, CA, USA) at 4°C overnight, washed with PBS saline containing 0.05% Tween 20, and blocked with Block Ace (Dainippon Seiyaku, Tokyo, Japan) for 2 h at 37°C. After washing, the culture supernatant to be tested and a serially diluted IFN-γ standard was added to the plates and incubated at 4°C overnight. After washing, 0.5 μg/ml of biotin-labeled anti-murine IFN-γ mAb (XMG1.2; BD PharMingen, USA) was added to the plates, and they were incubated at room temperature for 1 h. After washing, 0.1 μg/ml of horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories, Inc., Burlingame, CA, USA) was added, followed by a 30 min incubation at room temperature. After washing, bound HRP-conjugated streptavidin was detected using the substrate 3,3′,5,5′-tetramethylbenzene dihydrochloride (Sigma-Aldrich Japan, Tokyo, Japan). After 5 min, the enzyme reaction was stopped by adding 2M $H_2PO_4$, followed by measuring the absorbance at 450 nm using an EZS-ABS microplate reader (Iwaki, Tokyo, Japan). All samples were analyzed in triplicate.

***Intracellular IFN-γ staining.*** An Ag-specific T-cell subset was also examined by simultaneous flow cytometric assessment of the T-cell phenotype and intracellular IFN-γ synthesis. Spleen cells from the immunized mice were treated with (ammonium chloride lysing) buffer at room temperature for 5 min to remove red blood cells and then washed twice with RPMI 1640 medium and resuspended in RPMI/10FCS at a concentration of $1 \times 10^7$ cells/ml. The cells (200 μl) were incubated for 4 h at 37°C in the presence or absence of 5 μM synthetic peptide with brefeldin A solution (BD PharMingen, USA) diluted 1:1,000. The cells were then washed twice with fluorescence-activated cell sorting (FACS) buffer (1% fetal calf serum and 0.1% $NaN_3$ in phosphate buffer solution), stained with FITC-conjugated anti-CD8 (53-6.7; BD PharMingen) and Cy-Chrome-conjugated anti-CD4 (RM4-5; BD PharMingen, USA) on ice for 30 min, and washed twice, and subsequently intracellular cytokine staining (ICS) was performed by using a Cytofix/Cytoperm kit (BD PharMingen, USA) according to the manufacturer's protocol. ICS for IFN-γ was performed with PE-conjugated anti-IFN-γ (clone XMG1.2; BD PharMingen, USA). Cells were washed twice and then resuspended in FACS buffer, and then were analyzed with an EPICS digital flow cytometer (EPICS XL; Beckman Coulter, Miami, Fla., USA).

***Cytotoxicity assay.*** One week after the last immunization, immune spleen cells were cocultured in 12-well plates at a density of $2 \times 10^7$ cells/well for 5 days with $2 \times 10^7$ syngeneic splenocytes per ml; the splenocytes had been treated with 100 μg/ml of mitomycin C (Kyowa Hakko, Tokyo, Japan) and pulsed with peptide for 2 h at 37°C. Each well also received 10 U/ml of human recombinant interleukin-2 (Hoffmann-La Roche, Nutley, N.J., USA).


[ DOR: 20.1001.1.1028852.2007.11.1.5.1 ]
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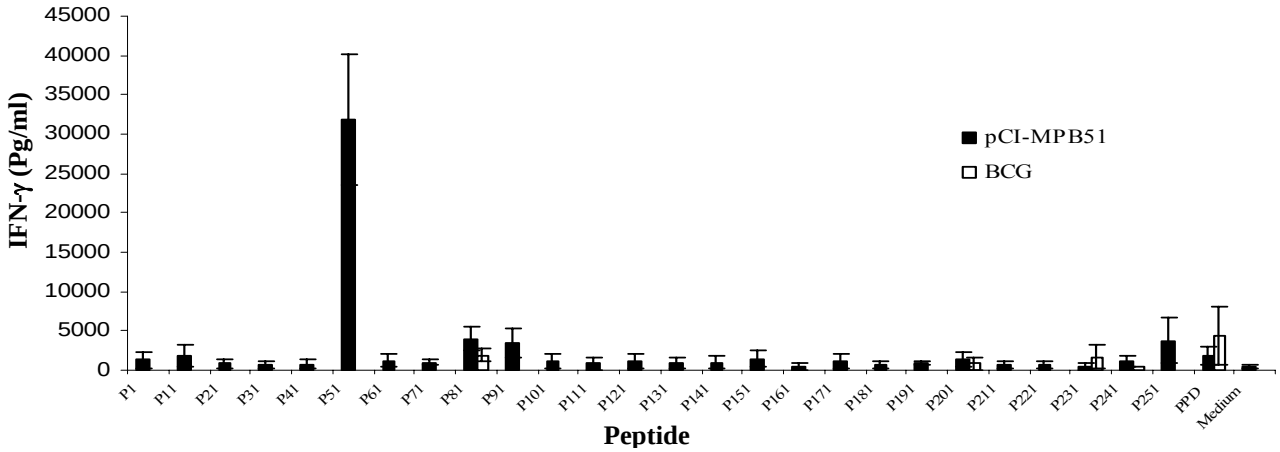


**Fig. 1.** IFN-γ production in response to restimulation with 5 μM synthetic 20-mer peptides (overlapping by 10 aa, spanning the whole nature MPB51 sequence) of spleen cells from HHD mice vaccinated with plasmid DNA encoding MPB51 molecule or medium alone. Splenocytes from naïve mice were also examined as controls. The data are representative of three independent expriments.

Cell-mediated cytotoxicity was measured by using a standard conventional $^{51}Cr$ release assay [29]. Briefly, HHD-transfected RMA-S cells (mouse lymphoma cell line) [25] were pulsed withrelevant (p54-62) or negative control ($^{571}$YLSGANLNL$^{579}$ of carcinoembryonic Ag) peptide at a concentration of 5 μM in FCS-free RPMI medium at 37°C for 1.5 h. Target cells at a concentration of $10^4$ cells/well were incubated for 5 h in duplicate at 37°C with serial dilutions of effector cells. Spontaneous- and total-release samples were prepared by adding the targets to wells containing only medium or medium plus 2 M $H_2SO_4$. The percentage of specific lysis was calculated as follows: (experimental release-spontaneous release)/(total release-spontaneous release) × 100. Experiments were preformed twice.

***Depletion of CD4$^+$ or CD8$^+$ T-cell subsets.*** CD4$^+$ or CD8$^+$ T-cell subsets of peptide-reactive T cells were determined by depletion studies with anti-murine CD4 mAb GK1.5 or anti-murine CD8α mAb 35-17-2 (Aichi Cancer Center, Nagoya, Japan). The mAb were purified from supernatants of the hybridomas by ammonium sulfate precipitation coupled with a PD-10 column (Amersham Biosciences, Tokyo, Japan). The immune spleen cells ($1 \times 10^7$ cells/ml) were suspended in cytotoxicity buffer (RPMI 1640 medium with 25 mM HEPES buffer and 0.3% BSA) containing the anti-CD4 or anti-CD8 mAb and incubated for 1 h at 4°C. Rabbit complement (Cedarlane, Hornby, Canada) was then added to the cell suspension, which was incubated for 1 h at 37°C and viable lymphocytes were purified on Lympholyte-M. Finally the cells were washed twice with RPMI 1640 medium, suspended in RPMI/10FCS, and used in cytokine induction experiments.

## RESULTS

***IFN-γ production in response to synthetic overlapping peptides from MPB51 in HHD mice.*** Splenocytes from HHD mice immunized with DNA vaccine encoding mature MPB51 were stimulated with the overlapping peptides for 24 h, and the IFN-γ concentrations in the culture supernatants were determined by ELISA. As shown in Figure 1, substantial IFN-γ production was observed after stimulation with peptide 51 (p51) (aa 51 to 70). As expected, spleen cells from HHD naïve mice showed no significant IFN-γ production in response to any of the peptides in the library (data not shown). In contrast to the robust IFN-γ responses observed in mice vaccinated with DNA, spleen cells from HHD mice vaccinated with *M. bovis* BCG produced no significant levels of IFN-γ. This is consistent with the previous observation that vaccination with DNA encoding the Ag85 complex induces a stronger and broader epitope repertoire than vaccination with BCG or infection with *M. tuberculosis* ([30; 31]. Further analysis by using CD4$^-$ or CD8$^-$ depletion revealed that only CD8$^+$ T cells produced significantly IFN-γ in response to p51 peptide (Fig. 2).

***Identification of a nine-mer CD8$^+$ T-cell epitope on p51 of MPB51.*** Since the CD8$^+$ T-cell epitopes presented by MHC class I molecules comprise 8 to

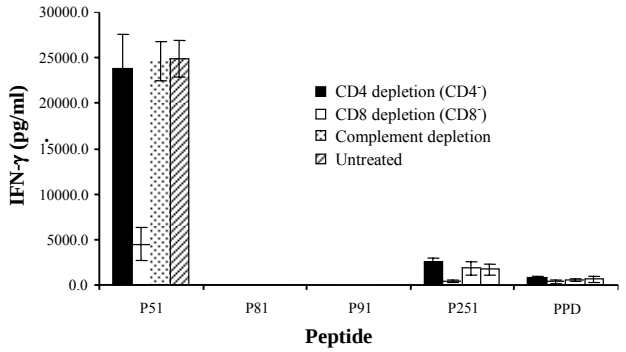


**Fig. 2.** IFN-γ production in response to restimulation with some synthetic 20-mer peptides (5 μM) of $CD4^-$ depleted ($CD4^-$) or $CD8^-$ depleted ($CD8^-$) spleen cells from HHD mice vaccinated with plasmid DNA encoding MPB51 molecule. The data are representative of three independent experiments.

10 aa (generally 9 aa), we tried to identify the fine $CD8^+$ T-cell epitope. Immunodominant epitopes often, but not always, display high-affinity binding for MHC molecules. Therefore, we predicted candidate peptides in the 20-mer peptides by using three computer-based programs, BIMAS HLA Peptide Binding Predictions [27], SYFPEITHI Epitope Prediction [28] and HLA Epitope binding prediction. We first synthesized three peptides: p52-62, p53-61, and p56-66, since p52-62 and p56-66 contain six HLA*0201-binding candidate epitopes and p53-61 exhibits a high binding score with HLA*0201. Three-color flow cytometric analysis of a T-cell subset and intracellular IFN-γ staining revealed that $CD8^+$ T cells, but not $CD4^+$ T cells, produced IFN-γ in response to p51 (Fig. 3A). It also indicated that p52-62 and p53-61, but not p56-66, could significantly stimulate the immune $CD8^+$ T cells to synthesize IFN-γ (Fig. 3A). Thus, we prepared two typical nine-mer candidate peptides: p54-62 and p57-65, which had higher theoretical binding stability (Table 1). As shown in Figure 3B, p54-62, but not p57-65, could induce vigorous intracellular IFN-γ synthesis in the immune $CD8^+$ T cells, indicating that the p54-62 nine-mer peptide is a bona fide $CD8^+$ T-cell epitope on the MPB51 molecule for HHD mice.

***Identification of an HLA-A*0201-restricted $CD8^+$ CTL in MPB51-immunized HHD mice.*** HHD mice represent a powerful model for the induction and examination of HLA-A*0201-restricted $CD8^+$ CTL responses *in vivo* ([32]. Since p54-62 was found to be a $CD8^+$ T-cell epitope for HHD mice, we tried to determine whether the p54-62-HLA-A*0201 complex was recognized by CTL. As shown in Figure 4, *in vitro*-stimulated splenic T cells from HHD mice immunized with MPB51 DNA vaccine appeared to lyse the peptide-pulsed RMA-S-HHD target cells substantially. Collectively, these data clearly indicate that the p54-62 peptide is an HLA-A*0201-restricted $CD8^+$ T-cell epitope.

## DISCUSSION

A greater understanding of the nature of protective immunity to TB would facilitate the development of a future vaccine. Although it is well known that

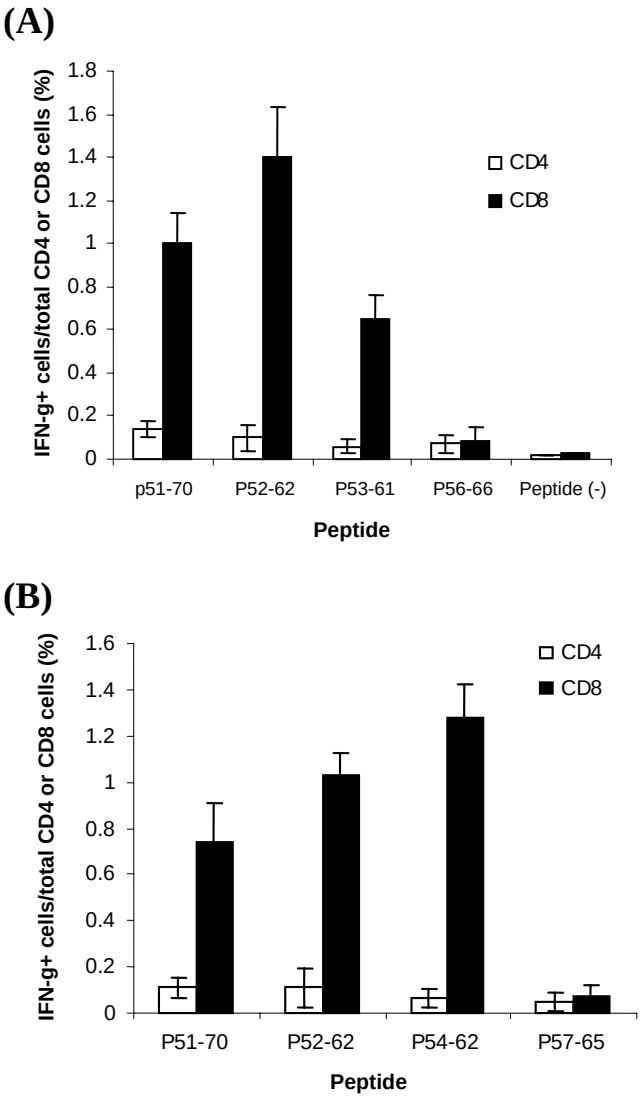


**Fig. 3.** Flow cytometric analyses of IFN-γ producing cells of transgenic HHD mice. **(A),** the percentages of $CD4^+$ or $CD8^+$ cells producing IFN-γ after 4 h of stimulation with peptides or Peptide (–), medium alone. P52-62, but not p57-65 in the p51-70 peptide contained the $CD8^+$ T-cell epitope. **(B),** p54-62 nine-mer peptide was a $CD8^+$ T-cell epitope on the MPB51 molecule.


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**Table 1.** Candidate T-cell epitopes on the P51 peptide of the MPB51 molecule.

| Peptide | Amino acid sequence | Estimated HLA-A*0201 binding scores | | |
|---|---|---|---|---|
| | | a* | b* | c* |
| P51 | MNTLAGKGISVVAPAGGAYS | | | |
| P52-62 | **NTLAGKGISVV** | | | |
| P53-61 | **T L A G K G I S V** | 69.5 | 6.8 | 152 |
| P54-62 | **L A G K G I S V V** | 1.56 | 6.5 | 100 |
| P57-65 | **K G I S V V A P A** | 0.26 | 7.1 | - |
| P58-66 | G I S V V A P A G | 0.01 | 6.07 | - |
| P61-69 | V V A P A G G A Y | 0.001 | 5.86 | - |
| P55-63 | A G K G I S V V A | 0 | 5.85 | - |
| P56-66 | **GKGISVVAPAG** | | | |
| P51-59 | M N T L A G K G I | 0.1 | 6.05 | - |
| P59-67 | I S V V A P A G G | - | 6.26 | - |

Boldface type indicates peptide sequences that were synthesized and used for experiments; a*, Scores of epitope binding predicted by SYPEITHI (http://www.syfpeithi.de); b*, Binding affinities estimated by BIMAS (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform); c*: http://hlaligand.ouhsc.edu/prediction.htm.

$CD4^+$ T cells are crucial in the protection against infectious disease caused by *M. tuberculosis* [7], there is now mounting evidence from murine models and human studies that $CD8^+$ T cells also play a pivotal role in the protective immunity against TB [4, 6]. However, the precise mechanism of MHCclass Ia-restricted $CD8^+$ T-cell-mediated protection is not known. IFN-γ, a potent activator of macrophages, is a crucial cytokine in antimycobacterial protection, as demonstrated in knockout mice, which have been genetically altered to eliminate IFN-γ production.

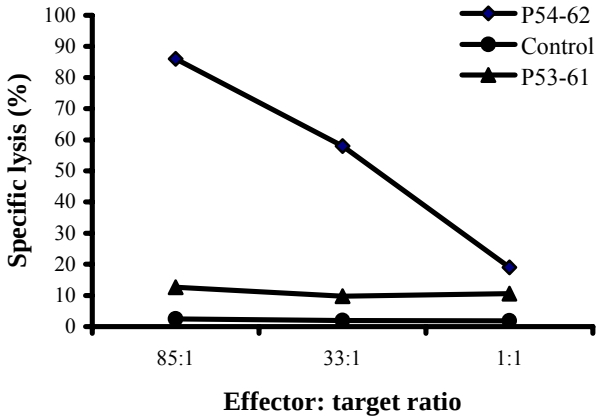


**Fig. 4.** Cytotoxity activity of splenocytes derived from MPB51 DNA-vaccinated HHD mice. P54-62 peptide-pulsed RMA-S-HHD cells (target cells) were efficiently lysed by immuned spleen cells. Immune spleen cells (effectors) were incubated with target cells by using the effector/target cell ratios (E/T ratio) indicated on the *x* axis. The peptide $^{571}$YLSGANLNL$^{579}$ of carcinoembryonic antigen used as nagative control.

DNA vaccination is a powerful tool for identifying T-cell epitopes, as previously reported [30]. In HHD mice, we identified an HLA-A*0201-restricted $CD8^+$ T-cell epitope, p54-62 (LAGKGISVV), on secreted-MPB51 protein by using a gene gun DNA vaccination and an overlapping peptide library with biometric analysis. IFN-γ responses were clearly seen in the spleen cells from mice immunized with plasmid DNA compared to the spleen cells from mice immunized with BCG. In addition, a computer-assisted algorithm is useful for identifying minimal epitopes after epitope mapping. In fact, p54-62, which we identified as a $CD8^+$ T-cell epitope, had a high binding score (half-time dissociation score), 100, for HLA-A*0201 in HLA Epitope binding prediction. However, various factors other than MHC-binding affinity are used to determine T-cell epitopes; such as (I) Ag processing (cleavage preference of the proteosome), (II) TAP-dependent peptide transport, and (III) the response to the T-cell repertoire. For example, p53-61 in p51-70, which was thought to contain a T-cell epitope(s), exhibits a high binding score for HLA-A*0201, 69.5 and 152, in BIMAS and HLA Epitope binding prediction. However, the p53-61 peptide failed to induce IFN-γ synthesis in spleen cells from immunized HHD mice. Three-color flow cytometric analysis demonstrated that the p54-62 peptide can induce intracellular IFN-γ synthesis in immune $CD8^+$ T cells, indicating that the p54-62 peptide is a $CD8^+$ T-cell epitope in HHD mice. We also demonstrated that the p54-62 peptide-HLA-A*0201 complex can be recognized by $CD8^+$ T cells producing IFN-γ and exhibiting CTL activity.

Recently, cytolytic and IFN-γ-secreting human CD8 T cells have been identified: these were directed against *M. tuberculosis* ESAT-6 (p69-76), in the context of HLA-B52 [33], or to the 19-kDa lipoprotein in the context of HLA-A*0201 [34].

It is especially noteworthy that a hydrophobic C-terminal residue is critical for the $CD8^+$ T-cell epitope since the immune $CD8^+$ T cells were able to recognize p52-62 and p54-62 but not p57-65. The importance of the C-terminal residue in the $CD8^+$ T-cell epitope was also observed in H2-$K^b$-restricted moloney murine leukemia virus [35], and in HLA-A*0201-restricted MAGE-A epitopes [36].

Although, previous studies have demonstrated less CD8 and lower HLA-A*0201 expression in the HHD mice compared to the A2.1/H-2$K^b$ mice [37] the absence of mouse class I resulted in both a quantitative and a qualitative difference in the HLA-A*0201 restricted CD8 cells generated after DNA vaccination. The HHD mice are, thus, useful for studying immunization strategies for inducing CD8 responses and identifing immunodominant epitope.

The level of epitope-specific IFN-γ production by $CD8^+$ T cells was below the detection level in BCG-vaccinated mice. This is consistent with the observation that in BALB/c mice, the level of Ag85-specific IFN-γ-producing T cells, which can be easily elicited by DNA vaccines, is below the detection level in BCG-vaccinated or TB-infected mice [30]. Since a DNA vaccine encoding MPB51 is capable of inducing $CD8^+$ T cells recognizing the p54-62 in the context of HLA-A*0201, it is possible that low-level expression of the $CD8^+$ T-cell epitope on *M. tuberculosis*-infected cells might be recognized by the DNA vaccine-induced $CD8^+$ T cells in HHD mice.

Also, because the epitope is presented by HLA-A*0201, not murine MHC molecules, and importantly, because HHD mice do not express any murine class I molecules, only the human HLA-A*0201 molecule, the protection must have been mediated by $CD8^+$ T cells restricted to the human HLA-A*0201 class I molecule.

Although the murine TCR repertoire is not identical with the human one, both are broad enough that CTL responses in HLA-A*0201-transgenic mice have been found to be predictive of human HLA-A*0201-restricted CTL responses [38], and the HHD strain makes a broader response to HLA-A*0201-restricted epitopes than A2.1/H-2$K^b$ mice [37], possibly because there is no competition from murine class I MHC molecules.

In conclusion, we identified one HLA-A*0201-restricted $CD8^+$ CTL epitope in HHD mice which is thought to play pivotal role in protection against *M. tuberculosis* infection. Identification of this T-cell epitope will be very useful for further elucidation of the role of MPB51-specific T cells in the protective immunity.

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[ DOR: 20.1001.1.1028852.2007.11.1.5.1 ]
[ DOI: - ]