CD8+ T cell responses and antigen presentation during *Mycobacterium tuberculosis* infection in humans

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CD8+ T cell responses and antigen presentation during *Mycobacterium tuberculosis* infection in humans

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"우리가 알거니와 하나님을 사랑하는 자 곧 그 뜻대로 부르심을 입은 자들에게는 모든 것이 합력하여 선을 이루느리라" (로마서 8:28)

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조 장 은 씀

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

TB, tuberculosis
MTB, *Mycobacterium tuberculosis*
AIDS, acquired immune deficiency syndrome
MDR-TB, multidrug-resistant tuberculosis
MHC, major histocompatibility complex
HLA, human leukocyte antigen
TAP, transport associated with antigen processing
β_2m, β_2-microglobulin
BCG, Bacillus Calmette-Guerin
CTL, cytotoxic T lymphocyte
PPD, purified protein derivative
PBMCs, peripheral blood mononuclear cells
PFMNCs, pleural fluid mononuclear cells
EBV, Epstein-Barr virus
DCs, dendritic cells
GM-CSF, granulocyte-macrophage-colony-simulating factor
MOI, multiplicity of infection
ER, endoplasmic reticulum
LMP, low-molecular-weight polypeptide
JAK, Janus kinase
GAS, gamma-activated sequence
IRF, IFN regulatory factor
RT-PCR, reverse transcriptase polymerase chain reaction

ABSTRACT

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Cell-mediated immune responses are a major protective mechanism against *Mycobacterium tuberculosis* infection. These cells mediated immune responses are composed of T cells and macrophages. In order to understand the immune mechanism for tuberculosis (TB), three aspects of the immune responses regarding the major histocompatibility complex **(**MHC) class I-restricted CD8+ T cells and the processing of *M. tuberculosis* antigens were researched. The three aspects included : CD8+ T cell responses to *M. tuberculosis*-derived peptides, the antigen processing mechanism of *M. tuberculosis* somatic antigen, and the effect of *M. tuberculosis* on interferon-

gamma (IFN-γ)-induced genes.

First, $ThyA_{30-38}$, $RpoB_{127-135}$, $PstA1_{75-83}$, and $85B_{15-23}$ were previously identified as *M. tuberculosis*-derived peptides specific for HLA-A*0201-restricted CD8+ T cells. This study characterized these peptide-specific CD8+ T cells in individuals that were either actively (TB patients) or latently infected with *M. tuberculosis* (PPD+). CD8+ T cell responses to these peptides were induced in all study groups (PPD+ healthy subjects, pulmonary TB patients, and TB pleurisy patients), except the PPD- subjects. However, the *M. tuberculosis* antigen-specific CD8+ T cell immunity appeared to be depressed in patients with advanced stages of TB. These *M. tuberculosis*-derived peptides also induced CD8+ T cell responses in subjects expressing the subtypes HLA-A*0203, A*0206, and A*0207, suggesting that these epitopes are A2 supertype peptides. Among these four peptides, the immunodominant peptide that induced the highest number of IFN-γ secreting CD8+ T cells differed depending on the subjects. Short-term cell lines specific for these peptides proliferated *in vitro* and secreted IFN-γ upon antigenic stimulation in PPD+ subjects. HLA-A*0201 dimer assays indicated that the $PstA1_{75-83}$-specific CD8+ T cell population in PPD+ healthy subjects was functionally heterogeneous, since only one-half or one-fourth of the cells produced IFN-γ upon peptide stimulation. In addition, by assaying cytotoxic T lymphocyte (CTL) activities, we observed that CTL responses specific for these *M. tuberculosis*-derived peptides could be induced in Bacille Calmette-Guerin **(**BCG)-vaccinated subjects. This result suggests that CD8+ T cells may be involved in controlling TB in BCG-vaccinated or PPD+ healthy people.

Secondly, *M. tuberculosis* resides and replicates inside macrophages. In our previous publication, CD8+ T cell-mediated immune responses specific for the peptide $RpoB_{127-135}$, which was derived from the RNA polymerase beta-subunit of the *M. tuberculosis* protein, could be induced in TB patients. In order to demonstrate that CD8+ T cells can recognize $RpoB_{127-135}$ that was processed by *M. tuberculosis*-infected macrophages, CD8+ T cell lines specific for the $RpoB_{127-135}$ peptide were generated from the peripheral blood mononuclear cells **(**PBMCs) of healthy HLA-A*0201 and A*0206 subjects, using *in vitro* immunization techniques. These CD8+ T cell lines specifically recognized and destroyed *M. tuberculosis* infected-macrophages. In addition, the presentation of the *M. tuberculosis*-derived epitope peptide, $RpoB_{127-135}$, to CD8+ T cells did not seem to be inhibited by brefeldin-A treatment, which blocks the classical MHC class I-restricted antigen presentation pathway in macrophages. Therefore, the $RpoB_{127-135}$ peptide may be processed by accessing the alternative MHC class I processing pathway, which was previously suggested as the processing pathway for the cytoplasmic proteins of *M. tuberculosis*. Since the RpoB gene of *M. tuberculosis* was reported to be actively expressed inside macrophages, the RpoB protein or derived peptides may be useful for the development of TB vaccines. This study also suggests that not only secreted but also somatic proteins of *M. tuberculosis* need to be screened for TB vaccines and therapeutic agents.

Lastly, the effect of *M. tuberculosis* infection on the expression of IFN-γ induced genes involved in MHC class I-restricted antigen processing pathway of the host cells was investigated. IFN-γ is a principal mediator of the bactericidal activation of

macrophages. *M. tuberculosis* is either resistant to the IFN-γ responsive microbicidal mechanisms of macrophages or alternatively may block the macrophage response to IFN-γ. This study demonstrates that the *M. tuberculosis* infection selectively affected the transcription of IFN-γ responsive genes. While the transcription of CD64 decreased, IFN-γ responsive genes involved in the MHC class I Ag processing pathway were either unaffected or induced by *M. tuberculosis*. Further studies are needed to elucidate the underlying mechanisms whereby *M. tuberculosis* inhibits cellular responses to IFN-γ.

Key words : *Mycobacterium tuberculosis*, CD8+ T cells, Peptide epitopes, HLA-A2, IFN-γ, Macrophage, RpoB $_{127\text{-}135}$ peptide, IFN-γ responsive gene

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I. INTRODUCTION

1. Current problems of tuberculosis

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* was discovered by Robert Koch in 1882[1]. *M. tuberculosis* is a facultative intracellular pathogen and acid fast bacillus that replicates within human macrophages. The World Health Organization (WHO) estimates that one-third of the world's population has been infected with *M. tuberculosis*, only 10% of infected people break down with the disease, 90% of infected people remains clinically latent. Therefore, *M. tuberculosis* is often in a dormant state in infected hosts, usually produces a chronic disease[2] and

has a tendency to reactivate many years after the initial infection. TB is a major global health problem, especially in developing countries. In addition, 8 million new TB cases and two to 2.5 million cases of TB-related deaths occurs annually[3].

The introduction of rifampicin, pyrazinamide and ethambutol in recent years offered in an ear of short-course treatment. But, the advent of HIV infection, the acquired immune deficiency syndrome (AIDS) pandemic in the 1980s, increases the risk of developing TB. Strains of *M. tuberculosis* resistant to both isoniazid and rifampicin with or without resistance to other drugs have been termed multidrug-resistant strains[4]. Multidrug-resistant tuberculosis (MDR-TB) is among the most worrisome elements of the pandemic of antibiotic resistance because TB patients that fail treatment have a high risk of death.

2. Immune responses of CD8+ T cells to *M. tuberculosis* infection

A. T cell-mediated protective immunity to *M. tuberculosis*

Clinical and experimental evidence suggests that immunity against *M. tuberculosis* is mainly controlled by the cell-mediated immune responses, involving T cells and macrophages. Studies on humans and animal models have demonstrated that CD4+ T cells are essential to the immune response to *M. tuberculosis* infection. In mouse models, CD8+ T cells have been reported to play significant roles in the containment of TB. In the mouse model, for example, β_2-microglobulin (β_2m)-

deficient, transporter associated with antigen processing (TAP)-deficient and CD8+ T cell-deficient mice were more susceptible to *M. tuberculosis* infection than normal control mice[5-7]. Recent study using a mouse model and reactivation suggests that CD8+ T cells may be even more important than CD4+ T cells in controlling the latent phase of TB infection[8].

Three different roles of the CD8+ T cells have been defined : the release of IFN-γ, the lysis of the infected targets and the direct antimicrobial activity. IFN-γ secretion from T cells induces both antigen presentation and the bactericidal activity of macrophages where bacilli mainly reside and replicate. A CD8+ T cell adoptive transfer experiment demonstrated that increased immunity to *M. tuberculosis* infection was the result of increased levels of IFN-γ secreting CD8+ T cells[9]. In a mouse model of a low dose aerosol infection, the IFN-γ production by CD8+ T cells was significantly involved in the protection against chronic *M. tuberculosis* infection [8, 10].

While CD8+ T cells are considered critical for the control of *M. tuberculosis* infection in the mouse model, its role in human infections remains less well understood. In humans, CD8+ T cells exhibit cytotoxic activity or growth inhibition against intracellular *M. tuberculosis* by releasing granules such as granulysin or direct contact via Fas (CD95), respectively[11, 12]. Perforin molecule does not seem to play a role in early protective response against *M. tuberculosis*, but only in the late phases on infection. There are two pathways of cytotoxicity. One is granule exocytosis and the other is Fas-Fas ligand (FasL) pathways. Both induce apoptosis in the target cells. The

granule exocytosis pathway involves directed and regulated secretion of the lytic granule constituents, including perforin (a Ca2+ dependent, pore forming protein related to the membrane attack complex of complement) and granzymes (serine esterases that activate the caspase cascade).

B. Epitope-based approaches to vaccination

Several laboratories have identified the *M. tuberculosis* peptides that are presented by human major histocompatibility complex (MHC) class I molecules to T cells as well as the role these T cells play in containing infection. For instance, peptides derived from a 19-kDa protein (membrane-bound lipoprotein) and an esat-6 protein (early secretory protein) have been identified as being immunogenic for MHC class I-restricted CD8+ T cells[13, 14]. In our previous study, four *M. tuberculosis*-derived epitopes for HLA-A*0201-restricted CD8+ T cells were also defined[15]. Three of these were derived from the somatic *M. tuberculosis* proteins, ThyA, RpoB, PstA1 and one was derived from antigen 85B, one of the major secreted proteins. Somatic antigens seem to be mainly derived from protein turn-over of bacilli during the late phase of infection. In contrast, secreted antigens from mycobacteria are produced by metabolically active organisms and related to the early or active phase of infection.

These *M. tuberculosis* peptide-specific CD8+ T cells could release IFN-γ upon the recognition of *M. tuberculosis*-infected cells and reduce the number of intracellular bacilli, suggesting that *M. tuberculosis* somatic antigens can be processed

and recognized by MHC class I-restricted CD8+ T cells for the protective immunity in humans.

The definition of epitope peptides contributes not only to the study of the functional implication of CD8+ T cell on TB but also to the development of a vaccine for TB. Indeed, the development of a new TB vaccine is increasingly needed since the efficacy of Bacillus Calmette-Guérin (BCG), which is the only currently available vaccine for TB, is estimated to be 0-80 % effective. To develop universal subunit vaccines, it is essential to identify whether these newly defined epitopes are A2 supertype peptides. Human leukocyte antigen (HLA)-A2 is one of the most frequent HLA-A specificity in the human population with an allele frequency of 10-40% in different ethnic groups[16-18]. HLA-A2 supertype is a family of HLA subtypes that share specificities for a degenerate ligand with HLA-A*0201. Molecules of the A2 supertype are characterized by preferences for peptides of 9 or 10 residues in length bearing small or alipathic hydrophobic residues (A, I, V, L, M, or T) in position 2 and at the peptide C-terminus. On the basis of these studies, the A2 supertype minimally includes A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*6802 and A*6901. The A2 supertype includes the A*0201, A*0202, A*0203, A*0204, A*0205, A*0206 and A*0207, A*6802 and A*6901 types, and A2 supertype epitope peptide can bind to the HLA-A molecules of supertype members. Therefore, the part one of this study was performed to determine if these epitope peptides are the A2 supertype peptides.

C. Immune responses of CD8+ T cells in TB patients

It has been known that IFN-γ responses in PBMCs is decreased in many TB patients, a demonstrated by reports showing that the IFN-γ therapy was effective for treating TB patients. On the contrary, latently infected people with *M. tuberculosis* usually react positive to *M. tuberculosis* purified protein derivatives (PPD) skin test, and the disease can be contained because their protective immunity is effective. Recent studies have demonstrated that the *M. tuberculosis* protein specific CD8+ T cells are in fact highly induced in healthy household TB contacts or PPD+ healthy individuals[19]. Therefore, in order to investigate the role for CD8+ T cells in protective immunity and to design an effective vaccine for TB, it is necessary to identify the correlations of the CD8+ T cell immune responses in latently infected subjects and in chronically infected TB patients. The part two of the study was subsequently designed to compare the distribution of these epitope-specific IFN-γ secreting CD8+ T cells in latently infected subjects and active TB patients expressing HLA-A2 supertype.

In addition, these CD8+ T cells specific for *M. tuberculosis*-derived peptides were further examined to determine if they are also induced in the pleural effusion from TB pleurisy patients. Tuberculous pleuritis usually manifests as lymphocytic exudate pleural effusions[20]. This local immune response is orchestrated by T cells and represents the protective immune response responsible for controlling the spreading of *M. tuberculosis* infection. CD4+ T cells play an important part in the development of tuberculous pleural effusion by cell-mediated immunity, inducing strong protective

immunity by concentrating a high level of IFN-γ in the localized disease sites[21]. Since the importance of CD8+ T cells in tuberculous pleurisy has not been defined yet, these *M. tuberculosis* peptide-specific CD8+ T cells were examined to determine if are present in a pleural effusion from TB pleurisy patients.

3. MHC class I antigen processing pathway of *M. tuberculosis* somatic antigens

In addition to cytotoxic activity, it is known that CD8+ T cells have significant roles in the protective mechanism against *M. tuberculosis* infection by releasing IFN-γ. However, little is known about the mechanism by which antigens from *M. tuberculosis* gain access to the MHC class I-restricted presentation pathway. Previous studies using BCG-activated CD8+ CTL have shown that proteosome and Golgi inhibitors reduced CTL activity to zero[22]. These data imply that the majority of CD8+ T cells against mycobacteria are restricted by MHC-class I. Recently, it was reported that BCG could activate CD8+ CTL by the MHC class I-restricted presentation pathway which is both proteasome-dependent and Golgi-endoplasmic reticulum (ER) transport pathway-dependent[22]. On the other hand, it was shown that *M. tuberculosis*-specific CD8+ T cells could recognize an antigen which was processed by the proteasome-dependent pathway, but which was not transported through the Golgi-ER pathway. The immune responses of these *M. tuberculosis*-reactive CD8+ T cells were not inhibited by brefeldin A, while inhibited by MHC class I blocking antibody. *M. tuberculosis*-derived antigen presentation was found to require proteasomal

processing, but to be presented in a manner that was brefeldin A and hence TAP independent[23]. In this report, we present the result that one of cytosolic *M. tuberculosis* proteins, RNA polymerase subunit B (RpoB) is processed by the alternative MHC class I presentation pathway and recognized by MHC class I-restricted CD8+ T cells. Alternative MHC class I processing pathway may permit the processing of phagosomal antigens. This pathway seems to be preferentially utilized by particulate antigens, including bacteria. *M. tuberculosis* induces apoptosis of host cells and the formation of apoptotic blebs. These blebs could be engulfed by macrophages or dendritic cells and presented in a TAP-dependent fashion to CD8+ T cells[24]. The mechanism of this Golgi-ER independent presentation pathway needs to be investigated furthermore.

4. Gene expression profiles of components of the MHC class I antigen processing machinery following infection with *M. tuberculosis*

Infection with *M. tuberculosis* induces a cellular immune response including CD4+ and CD8+ T cells that secrete IFN-γ. IFN-γ, the predominant activator of microbicidal functions of macrophages, is detectable at sites of *M. tuberculosis* infection, but is unable to stimulate macrophages to kill *M. tuberculosis*. IFN-γ acts by causing changes in gene expression through both transcriptional and posttranscriptional regulation with most genes being transcriptionally regulated by IFN-γ[25]. Moreover, the effect of *M. tuberculosis* on the response to IFN-γ at the

molecular level and the aspects of regulation that are affected remain poorly understood. *M. tuberculosis* interferes with cellular signal transduction pathways that are activated by IFN-γ and thereby avoids being killed within macrophages. *M. tuberculosis* decreases IFN-γ stimulated mRNA amount of the IFN-γ-regulated genes, Fcγ receptor I (CD64) and class II transactivator[26, 27]. Binding of IFN-γ to cell surface receptors results in activation of the tyrosine kinase Janus kinases 1 (JAK1) and JAK2, leading to phosphorylation of cytoplasmic signal transducers and activators of transcription 1 (STAT1)[26]. Tyrosine-phosphorylated STAT1 homodimerizes through interaction of the Src homology-2 (SH2) domain on one molecule with phosphotyrosine on another and translocates to the nucleus. In the nucleus, STAT1 homodimers activate transcription of specific genes that possess γ-activation sequences (GAS; consensus sequence is TTNCNNNAA) in the promoters of IFN-γ stimulated genes. Human genes that contain GAS include Fcγ receptor I (CD64), guanylate binding protein 2, class II transactivator, indoleamine-2,3-dioxygenase, TAP-1[28-30].

In addition, IFN-γ alters proteasome activity qualitatively. Vertebrate have three IFN-γ inducible β subunits (LMP2, LMP7, and LMP10), the former two being encoded in the MHC. Each inducible subunit is homologous with a constitutive catalytic subunit (LMP2/Y, LMP7/X, and LMP10/Z) and can replace its homologue during proteasome assembly. IFN-γ inducible proteasome subunits LMP2, LMP7 and LMP10 are called ‘immunosubunits’. The TAP2, LMP7, LMP10 and PA28 promoters contain different elements, but in all cases the IFN-consensus sequences[31], suggesting

a distinct regulation of their constitutive expression and a coordinated regulation upon IFN-γ treatment. In the ER, different chaperones, like tapasin, calnexin, calreticulin stabilize the MHC class I molecules during their folding and assembly or facilitate their loading with peptides[32-34]. The promoter of most chaperons has not been identified so far. Presumably, these seem to be additional factors regulating the expression of MHC class I, TAP, LMP and chaperone which are important for efficient MHC class I antigen processing and presentation.

It is not known whether regulation of interferon regulatory factor 1 (IRF-1) expression in response to IFN-γ is solely transcriptional, because IRF-1 expression can be regulated posttranscriptionally. GAS and IRF-1 regions are important for the induction of TAP1 expression in response to IFN-γ. IRF-1 is known as a key factor in the induction of type I IFN gene expression during *M. tuberculosis* infection[35]. IFN-γ increases TAP dependent peptide transport more rapidly than HLA class I molecule expression. IFN-γ-activated STAT1α/STAT1α binds to a GAS in the promoter of TAP1 and the promoter of the transcription factor IRF-1, which mediates the delayed response of HLA class I promoter. Infection with *M. tuberculosis* does not inhibit STAT1 tyrosine or serine phosphorylation, dimerization, nuclear translocation, or recognition of specific DNA sequences[27]. Infection with *M. tuberculosis* inhibits IFN-γ responses by directly or indirectly disrupting the essential interaction of STAT1α with the transcriptional coactivators CBP and p300[27].

To further understand the mechanisms whereby *M. tuberculosis* inhibits cellular responses to IFN-γ, the effect of *M. tuberculosis* infection on the expression of IFN-γ

responsive genes involved in MHC class I-restricted antigen processing pathway was investigated in this study.

II. MATERIALS AND METHODS

1. Immune responses of CD8+ T cells to *M. tuberculosis* infection

A. Study subjects

The HLA-A types of 60 healthy blood subjects, 84 chronically infected pulmonary TB patients and 38 TB pleurisy patients were determined. These subjects were previously vaccinated with BCG. Before blood sampling, informed consent was obtained from individuals after explaining the goals and methods of this project. Healthy PPD+ and PPD- subjects were selected randomly. The patients with mild, moderate and far advanced pulmonary TB from the Ewha woman's university hospital (Seoul, Korea) or the National Masan tuberculosis hospital (Masan, Korea) were classified based on a chest X-ray radiograph (CXR) (Table 2). The subject was defined as being PPD positive (+) if the hypersensitivity skin reaction with 2 T.U. tuberculin/0.1ml (Statens Serum Institut, Copenhagen S, Denmark) was > 6 mm at three days after the tuberculin injection.

B. HLA-A2 typing

To define HLA-A2 type, flow cytometry analysis was performed by using anti-HLA-A2 monoclonal antibody (mAb); BB7.2 (BD-Pharmingen, San-Jose, CA, USA).

HLA-A2 subtypes from each individual were subsequently identified by direct DNA sequence analysis of the polymorphic exons 2 and 3 of the HLA-A gene at the DNA Sequencing Facility at Hallym University (Ahnyang, Korea).

C. Peptide sequences and synthesis

Five HLA-A*0201-restricted CD8+ T cell-specific peptides were synthesized at the Korea Basic Science Institute (Seoul, Korea). Sequences were confirmed by mass spectrometry analysis. Sequences of the peptides used for the assays are as follows: $ThyA_{30-38}$ is RLPLVLPAV (IC50 = 5.1 nM), derived from thymidylate synthase, $RpoB_{127-135}$ is MTYAAPLFV (IC50 = 13.8 nM), derived from RNA polymerase-subunit, $85B_{15-23}$ is LMIGTAAAV (IC50 = 79.0 nM), derived from antigen 85B, and $PstA1_{75-83}$ is SLYFGGICV (IC50 = 10.6 nM), derived from putative phosphate transport system permease protein A-1 of *M. tuberculosis*. A*0201-binding epitope peptide derived from the influenza A virus matrix protein (Flu_{58-66}: GILGFVFTL) was used as the positive control.

D. Cell lines and culture media

.221A2 (provided by Robert DeMars, University of Wisconsin-Madison) is an Epstein-Barr virus (EBV)-transfected B-cell line mutagenized and selected for loss of HLA antigens, then transfected with HLA*0201, were used as target for the recall

CTL response. To generate B lymphoblastoid cell line (B-LCL), 5×10^5 peripheral blood mononuclear cells (PBMCs) from healthy subjects were transformed by EBV-containing supernatants from B-95.8 in RPMI 1640 (GIBCO, Grand Island, NY, USA) that had been supplemented with 2 mM L-glutamine, 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin (GIBCO, Grand Island, NY, USA) and 10% heat-inactivated fetal bovine serum (JBI, Taegu, Korea). After 14 days, the transformed cells were expanded in RPMI containing 10% FBS. Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY, USA) was used *in vitro* immunization. Hank's balanced salt solution (HBSS : Sigma, St Louis, MO, USA) was used to wash PBMCs.

E. Cytokines and monoclonal antibodies

Recombinant human (rh) IL-2, rhIL-7, rhGM-CSF and rhIL-4 were purchased from R&D systems Inc. (Minneapolis, MN, USA). And rhIFN-γ was purchased from Pierce Endogen (Rockford, USA). Monoclonal antibodies (mAb) specific for CD1a, CD83 and CD28 were supplied by BD Pharmingen (San-Jose, CA, USA). For the analysis of cell surface antigens, the following mAb specific for PerCP-conjugate CD3, FITC-conjugate CD8, FITC conjugate CD14, FITC conjugate HLA-DR and intracellular mAb specific PE conjugate IFN-γ and IL-4 (BD, Mountain View, CA, USA). W6/32 hybridoma producing Ab specific for human class I MHC was obtained from American Type Culture Collection (ATCC). Human IFN-γ elispot pair (purified anti-human IFN-γ and biotinylated anti-human IFN-γ), streptavidin-horseradish

peroxidase conjugate and anti-perforin Ab were purchased from BD Pharmingen (San-Jose, CA, USA). Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-mouse IgG1 were purchased from Molecular probes (Eugene, OR, USA). And anti-granulysin DH2 Ab was provided from Alan Krensky (Stanford Univ., USA).

F. Bacteria culture

Mycobacteria tuberculosis H37Rv (ATCC 27294) and *M. bovis* BCG (Pasteur strain 1173P2) used in this study was grown for about 10 days at 37℃ as a surface pellicle on Sauton medium enriched with 0.4% sodium glutamate and 3.0% glycerol. The surface pellicles were collected and disrupted with 6 mm glass beads by gentle vortexing. After clumps had settled out, the upper suspension was collected and aliquots were stored at -70℃ until used. After thawing, viable organisms were counted by plating serial dilutions on Middlebrook 7H11 agar (Difco, Detroit, MI, USA).

G. PBMCs separation

PBMCs derived lymphocytes from heparinized venous blood or pleural fluid mononuclear cells (PFMNCs) from pleural effusion were isolated by density gradient centrifugation over Ficoll-Paque^{TM} PLUS (Amersham Biosciences, Uppsala, Sweden). The cells were washed twice with culture medium and once with 1X HBSS.

H. Intracellular cytokine staining of short-term cell lines (STCLs)

STCLs were generated by stimulating the PBMCs of the healthy PPD+ subjects with each peptide for 10 days in the presence of IL-2 (10 units/ml) and IL-7 (10 ng/㎖) at 37°C in a CO_2 incubator. For induction of recall CD8+ T cell responses, the cultures were re-stimulated for an additional week with irradiated (3000 rad) autologous monocytes pulsed with each peptide. Subsequently, the EBV lines were stimulated with each peptide overnight. Next day, cells were incubated with 1 ㎍/㎖ brefeldin A (Sigma, St Louis, MO, USA) for 10 h. The cells were stained with anti-CD3 mAb PerCP-conjugate and anti-CD8 mAb FITC-conjugate. Intracellular cytokine staining for IFN-γ or IL-4 was performed using the Cytofix/Cytoperm kit (BD-Pharmingen, San-Jose, CA, USA) according to the manufacturer's instructions. The cells were incubated in fixation solution for 15 min at RT. The cells were washed and incubated with Perm/Wash solution and anti-IFN-γ mAb PE-conjugate, anti-IL-4 mAb PE-conjugate for 15 min at RT. Subsequently cells were fixed with 2% paraformaldehyde (PFA, Sigma, St Louis, MO, USA) and analyzed by a FACScalibur (Becton Dickinson, San Jose, CA, USA); $>10^5$ events were acquired for each sample.

I. *Ex vivo* IFN-γ elispot analysis of CD8+ T cells-specific for epitope peptides

Ninety-six-well polyvinylidene difluoride (PVDF)-backed plates (Millipore, Molsheim, France) were precoated with anti-IFN-γ mAb. The PBMCs or PFMNCs

derived lymphocytes were depleted of the CD4+ T cell population using magnet beads (Dynal Biotech ASA, Oslo, Norway), and plated with each synthetic peptide (10 ㎍/㎖) in 96 well plates in the presence of rhIL-2 (10 units/㎖) and anti-CD28 mAb for approximately 2 days in a CO_2 incubator at 37˚C. Subsequently the plates were washed ten times with phosphate buffered saline (PBS)/0.05% Tween 20 to remove cells and were incubated for 24 h with biotinylated anti-IFN-γ mAb. Streptavidin-horseradish peroxidase conjugate was added for 2 h in room temperature (RT) and subsequently chromogenic 3-amino-9-ethylcarbazole (AEC) peroxide substrate (Sigma, St Louis, MO, USA) was added. After 15 min, the colorimetric reaction was terminated by washing the plate with tap water. The immuno-spots-specific for single cells secreting IFN-γ were developed according to the manufacture's instruction and counted using an automatic elispot reader system (Zeiss, Germany).

J. *In vitro* induction of recall CTL responses from healthy subjects

PBMCs were resuspended in RPMI 1640 medium plus 10% pooled human serum and plated in a 24 well plate at 3×10^6 cells/well. Synthetic peptides were added to the PBMC cultures at a final concentration of 10 ㎍/㎖. On days 3 and 6, rhIL-2 was added to each well at a concentration of 10 units/㎖. On day 8, the cultures were re-stimulated with irradiated (3000 rad) autologous monocytes that were pulsed with peptide in the presence of 3 ㎍/㎖ of β_2m (Sigma, St Louis, MO, USA) for 2h. On

days 10 and 13, 10 units/㎖ of rhIL-2 was added into each well. The cytolytic activity of cultured PBMCs was tested on day 14 or 15.

K. CTL assay

Cytolytic activity was measured by a standard 4 h ^{51}Cr release assay. Approximately 2×10^6 target cells (.221A2 cells or macrophages) were pulsed with peptide (10 ㎍/㎖) and β_2m (3 ㎍/㎖) overnight. These cells were then labeled with 100 uCi $Na^{51}CrO_4$ (NEN, Boston, MA, USA) for 1h at 37℃. After washing, target cells were incubated with different ratios of effector cells in a 200 ㎕ total reaction volume of RPMI supplemented with 10% FBS in a 96 well plate. After 4 h incubation, 100 ㎕ of supernatant was removed from each well, and its radioactivity was counted in a γ-counter. Specific lysis (%) was calculated by using the formula 100 x (experimental release-spontaneous release)/(maximal release-spontaneous release). Maximal release was determined from supernatants of target cells lyses by the addition of 1% Triton X-100. Spontaneous release was determined from supernatants of target cells incubated with media only. Spontaneous release was < 20% of the maximum release in all assays.

L. Dimer staining

Dimeric complexs (BD-Pharmingen, San-Jose, CA, USA) were prepared by mixing

HLA-A*0201 : Ig protein with specific peptide at 640 molar excess at 37℃ overnight incubation. Peptide-specific STCLs were incubated with 10 ㎕ human serum at RT for 10 min. Cells were incubated with 1-2 ㎍ of peptide loaded HLA-A*0201 : Ig protein for each sample for 60 min at 4℃. Cells were washed with FACS buffer (PBS with 0.1% BSA and 0.02% sodium azide). Cells were given 10 ㎕ of human serum, then incubated for 10 min at RT. Subsequently, cells were incubated at 4℃ for 30 min in FACS buffer containing anti-CD8 mAb, anti-CD3 mAb and anti-mouse IgG1 (BD-Pharmingen, San-Jose, CA, USA). Then cells were fixed with 2% PFA and analyzed by FACScalibur.

M. Immunocytochemistry of CD8+ CTL lines

Slides were coated for 30 minutes with poly-L-lysin solution (Sigma, St Louis, MO, USA). Peptide-specific STLCs were plated for 30 min to allow cells to stick to slide, then washed three times in PBS. Slides were fixed for 10 min at RT with 4% PFA and then washed in PBS. Slides were added with Perm/Block solution (10% human serum, 10% goat serum, 0.01% saponin, 0.1% Triton X, 1% dry milk in PBS) and incubated for 1 h at RT. Then slides were washed in PBS then added antibody to perforin in phosphate-buffered saline (PBS) with dilution buffer (2% goat serum, 0.01% saponin and 0.5% milk) or nonbinding isotype-matched IgG2b control. Subsequently cells were incubated for 1 h, then washed with dilution buffer and incubated with Alexa Fluor 488 goat anti-mouse IgG in dilution buffer for 1 h at RT. Subsequently, samples

were stained with either anti-granulysin DH2 or IgG1 control, followed by additional wash with dilution buffer and incubated with Alexa Fluor 588 goat anti-mouse IgG in dilution buffer for 1 h at RT. Samples were fixed in 4% PFA for 5 min. For confocal microscopy, samples were mounted on glass slides in DAKO fluorescent mounting medium (DAKO, Carpinteria, CA, USA).

N. Statistical analysis

A two-sample Student *t*-test for the means was performed to determine if there is a statistically significant difference in the number of IFN-γ secreting CD8+ T cells in response to *M. tuberculosis*-derived peptides between groups.

2. MHC class I antigen processing pathway of *M. tuberculosis* somatic antigens

A. Generation of CTL lines from healthy HLA-A*0201 and A*0206 subjects by *in vitro* immunization

PBMCs from HLA-A*0201 and HLA-A*0206 healthy subjects were pulsed with 50 ㎍/㎖ of peptide at 3×10^7 cells/well in IMDM at 37℃ for 90 min. These cells were washed and plated at 3×10^6 cells/well in 10% pooled human serum with rhIL-7 (10 ng/㎖) and keyhole limpet hemocyanin (5 ㎍/㎖, Sigma, St Louis, MO, USA). Cultures were re-stimulated weekly with peptide-pulsed and -irradiated autologous

monocytes, and supplemented with rhIL-2 at 10 units/㎖ every 3-4 days [11]. After four to five cycles of re-stimulation, the cytotoxic activity of the CD8+ T cells was determined by using the chromium release CTL assay (target cell : .221A2 cell lines).

B. Cytotoxicity of CTL lines for *M. tuberculosis*-infected macrophages

Macrophages were generated by culturing adherent monocytes in antibiotics-free RPMI 1640 containing 10% FBS for 3-4 days. These macrophages were then infected with *M. tuberculosis* (H37Rv) at a multiplicity of infection for 4 h. Extracellular non-phagocytosed *M. tuberculosis* was removed by three times washing. The infected cells were cultured for 1-4 days before use as targets in CTL assays. The cell viability of macrophage populations was > 90% and about 70% of the cells were infected according to Ziehl-Neelson method for acid-fast bacteria. For cytotoxicity experiments, 1×10^6 macrophages were labeled with 100 μCi of $^{51}CrNa_2O_4$ for 1 h at 37℃, and then added to the wells of 96 well U-bottom plates at $5\text{-}7 \times 10^3$ cells/well. CD8+ T cell lines were added at various effector to target ratios. Specific lysis (%) was determined as described above.

C. Metabolic inhibition of antigen presentation of *M. tuberculosis*-infected macrophages

One hour before the infection of macrophage with *M. tuberculosis* (3MOI), brefeldin

A (3 ㎍/㎖, Sigma, St Louis, MO, USA), cytochalasin D (10 ㎍/㎖, Sigma, St Louis, MO, USA) or lactacystin (40 uM, Sigma, St Louis, MO, USA) were added to the culture medium. After 18 h of coincubation with *M. tuberculosis*, macrophages were used as target cells for the assays.

3. Gene expression of components of the MHC class I antigen processing machinery following infection with *M. tuberculosis*

A. Generation and infection of DCs with *M. tuberculosis*

DCs were generated from human monocytes by culturing with rhGM-CSF (800 U/ml) and rhIL-4 (500 U/ml) for 3 days in 6 well plates. Cells were then infected with *M. tuberculosis* for 4 h at a multiplicity of infection of 10. Extracellular bacteria were removed by 3 times washing with RPMI medium. The percentage of infection was estimated by staining aliquots of cells by the Ziehl-Neelson method. Routinely, the infectivity of DCs was approximately 70%. After one day, FACS analysis of cell surface markers for DCs were performed using Abs against MHC class II (anti-DR-FITC), MHC class I (anti-W6/32), B7.1 (anti-CD80), B7.2 (anti-CD86), anti-CD83, and anti-CD1a. All staining procedures were performed in FACS buffer for 30 min at 4℃. Cell were fixed with 2% PFA and analyzed by FACScaliber.

B. Reverse transcriptase PCR (RT-PCR) analysis

Macrophages and DCs were cultured under various experimental conditions as shown in results. Then total RNA was isolated from cultured cells by lysis with Trizol (Gibco Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized by reverse transcription with 2 μg total RNA, 0.25 μg of random hexamer (Gibco Invitrogen, Carlsbad, CA, USA) and 200 unit of Murine Molony Leukemia Virus Reverse Transcriptase (MMLV-RT, Gibco Invitrogen, Carlsbad, CA, USA). Subsequent PCR amplification using 0.2 units of Taq polymerase (Takara, Seoul, Korea) was performed in a thermocycler (PerkinElmer, USA) for 25-30 cycles (1 min at 94℃, 1 min at 54-58℃, 1 min at 72℃). An aliquot of the PCR products was electrophoresed in 1% agarose gel in Tris-borate buffer. Bands were visualized by ethidum bromide staining and photographed. The appropriate forward and reverse primer sequences for genes involved in MHC class I antigen processing pathway are shown in table 1[34].

Table 1 . Sequences of primers used for RT-PCR

Gene	Primer sequence	Temp.(℃)
TAP1	5'-CTC TGA GTG AGA ATC TGA GC-3'	57
gi:24797159	5'-GAG ACA TCT TGG AAC TGG AC-3'	
TAP2	5'-CGC CTT CTT CTT CCT TGT CC-3'	54
gi:32880154	5'-CTG AGC ATG AAG CCA TAC AG-3'	

LMP2	5'-CAT CTA CTG TGC ACT CTC TG-3'	57
gi:23110930	5'-CAG CTG TAA TAG TGA CCA GG-3'	
LMP7	5'-CGA ACA CGA ACA TGA CAA CC-3'	58
gi:34334013	5'-GCC ACA TGA GTG TCT TAC TG-3'	
LMP10	5'-CAA GAG CTG CGA GAA GAT CC-3'	54
gi:23110923	5'-GTA GCG GCC AGA CCT CTT CA-3'	
X	5'-GAC GGT GAA GAA GGT GAT AG-3'	58
gi:558525	5'-TTG ACT GCA CCT CCT GAG TA-3'	
Y	5'-CGC CAA TCG AGT GAC TGA CA-3'	58
gi:558527	5'-AAG CGA GAG CAT TGG CAG TG-3'	
Z	5'-CGG CTG TGT CGG TGT ATG CT-3'	58
gi:1531532	5'-CTC ACA CCT GTA CCG GCC AA-3'	
calnexin	5'-GGA AGT GGT TGC TGT GTA TG-3'	54
gi:27502676	5'-TTC ACA TAG GCA CCA CCA CA-3'	
calreticulin	5'-AAG TTC TAC GGT GAC GAG GA-3'	54
gi:5921996	5'-CTC TCC GTC CAT CTC TTC AT-3'	
PA28α	5'-GGA GCC AGC TCT CAA TGA AG-3'	54
gi:30581139	5'-GCA TCA CCA CGC TCA GAG AA-3'	
PA28β	5'-GGA GGT CTT CAG GCA GAA TC-3'	54
gi:23110923	5'-ATA GGC TGC CTC ATC TCG CT-3'	
HLA-A2	5'-GAG AAG GCC CAC TCA CAG A-3'	57
gi:34334013	5'-TAT CTG CGG AGC CAC TCC AC-3'	
HLA-DR	5'-GCT CCA ACT CTC CGA TC-3'	54
gi:18641378	5'-CCA CGT TCT CTG TAG TCT CTG G-3'	
CD64	5'-ATG GCA CCT ACC ATT GCT CAG G-3'	54
gi:21619685	5'-CCA AGC ACT TGA AGC TCC AAC TC-3'	
GAPDH	5'-CGG GAA GCT TGT GAT CAA TGG-3'	55
gi:182860	5'-GGC AGT GAT GGC ATG GAC TG-3'	

III. RESULTS

The significant roles of MHC class I-restricted CD8 + T cells in protective immunity for *M. tuberculosis* have been recognized in recent years. In order to understand the protective immune mechanism mediated by MHC class I-restricted CD8+ T cells, we have performed the following three different approaches ; 1) Characterization of HLA-A*0201-restricted CD8+ T cells specific for *M. tuberculosis* epitope peptide 2) MHC class I antigen processing pathway of *M. tuberculosis* somatic antigens 3) Gene expression of components of the MHC class I antigen processing machinery following infection with *M. tuberculosis.*

1. Characterization of HLA-A*0201-restricted CD8+ T cells specific for *M. tuberculosis* epitope peptides

A. HLA-A*02 allele types of the study subjects

Among 182 HLA-A typed individuals, 91 appeared to carry one of the HLA-A*02 alleles. As showed in Table 2, 63.6% of healthy subjects carries HLA-A*02 alleles. Among these, 30.3% expresses a HLA-A*0201 and the remaining 33.3% expresses other HLA-A*02 alleles. In pulmonary TB patients, 51.2 % of them were HLA-A*02 positive. Among these, 26.2% carries HLA-A*0201 and remaining 25% carries other HLA-A*02 alleles. Only HLA-A*0201, 0203, 0206 and 0207 of HLA-A*02 alleles

were identified in the Korean population in our study. In contrast to pulmonary TB patients, only 26.3% of TB pleurisy patients were HLA-A*02 positive. This is about 50% less than pulmonary TB patients. Even though only 38 TB pleurisy patients were typed in this study, this result implies that HLA-A*02 positive individuals may be more efficiently protected from TB pleurisy than HLA-A*02 negative individuals. Among the subjects expressing HLA-A2, 12 healthy PPD+ subjects, 7 PPD- subjects, 15 patients with mild or moderate (mild-moderate) TB, 5 patients with far-advanced TB and 3 TB pleurisy patients were selected in this study (Table 3).

Table 2. HLA-A genotype frequencies

HLA-A type	Healthy subjects	TB patients
A*0201	30.3% (18)	26.2% (22)
A*0203	3.3% (2)	1.2% (1)
A*0206	16.7% (10)	15.5% (13)
A*0207	13.3% (8)	8.3% (7)
Total	63.6% (38/60)	51.2% (43/84)

Table 3. Demography of the study subjects participated in this study

Group	N*	Age range	M / F
PPD+ healthy subjects	12	23-52 (33)	10 / 2
PPD- healthy subjects	7	23-45 (28)	6 / 1
Mild-moderate TB	12	18-72 (35.4)	10 / 2
Far-advanced TB	5	33-58 (45.7)	5 / 0
TB pleurisy	3	17-84 (52)	1 / 2

*N designates number of participants in each group. The number in parenthesis indicates the mean ages of subjects. M and F designate male and female, respectively.

B. CD8+ STCL generation from HLA-A*0201 PPD+ healthy subjects

STCLs (short term cell lines) were generated by stimulating PBMCs for 12-14 days with each peptide in the presence of IL-2. Cells were subsequently re-stimulated with each peptide overnight and stained for intracellular cytokine production for either IFN-γ or IL-4 (Fig 1). We observed each peptide-specific CD8+ STCL was all generated from PBMCs of PPD+ healthy subjects and produced IFN-γ upon peptide stimulation; however, these cells were not able to produce IL-4 indicating that these cells are Tc1 type cells. Flu_{58-66} peptide-specific for HLA-A*0201-restricted CD8+ T

cells was previously found to be able to bind at least four alleles of the HLA-A2 supertype including A*0201, A*0203, A*0206 and A*0207[36].

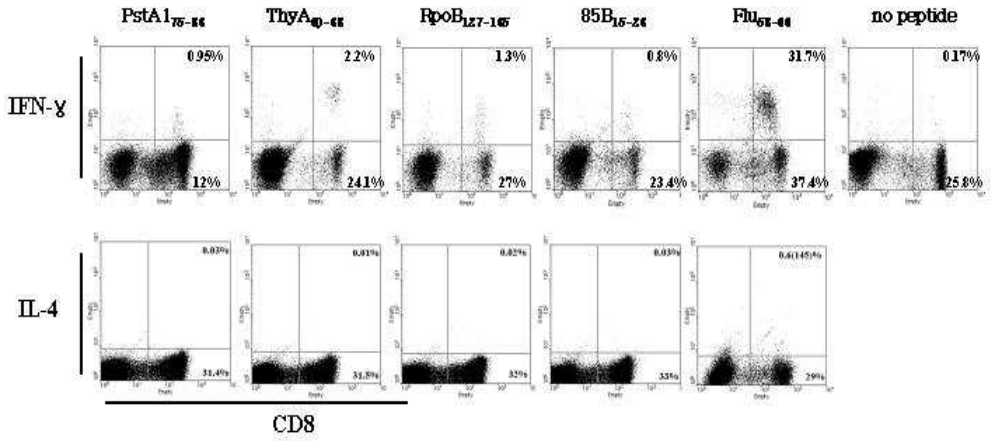


Fig. 1. Intracellular cytokine staining for HLA-class I-restricted CD8+ T cell populations specific for M. tuberculosis peptides in HLA-A*0201 subjects. STCL from PPD+ subjects expressing HLA-A*0201 were stimulated with each peptide overnight (PstA1$_{75-83}$, ThyA$_{30-38}$, RpoB$_{127-135}$, 85B$_{15-23}$, and Flu$_{58-66}$). On next day, STCLs were treated with brefeldin A and stained with IFN-γ, CD3+ and CD8+ specific mAb labeled with PE, PerCP and FITC, respectively. For the intracellular staining of IL-4, anti-IL-4 mAb labeled with PE was used in the assay. The frequencies of the peptide-specific IFN-γ or IL-4 secreting CD8+ T cell population were measured from the CD3-gated lymphocyte population.

C. Detection of IFN-γ producing CD8+ T cells from PPD+ subjects expressing HLA-A2 supertype

To identify whether these newly defined epitopes are A2 supertype peptides, the four peptides-specific for CD8+ T cell-mediated immune responses were examined using STCL generated from PBMCs of A*0203, A*0206 and A*0207 subjects (Fig 2). Using an intracellular IFN-γ staining method, we observed that CD8+ T cells specific for each peptide produced IFN-γ upon stimulation with each peptide in PPD+ healthy subjects expressing HLA-A2 supertype (A*0203, A*0206 and A*0207). However, $PstA1_{75-83}$ and $RpoB_{127-135}$ peptide-specific CD8+ T cell response was not induced in HLA-A*0203 subjects although only two healthy subjects were screened. It may be necessary to screen more subjects to explain whether $PstA1_{75-83}$ and $RpoB_{127-135}$ peptide-specific CD8+ T cell responses can be induced in HLA-A*0203 subjects. It is known that > 50% of the East-Asian population expresses the HLA-A*0201, A*0203, A*0206 or A*0207 subtype. Therefore, these peptides may be useful as effective vaccine components for the prevention of TB in East-Asia, one of the TB endemic areas in the world.

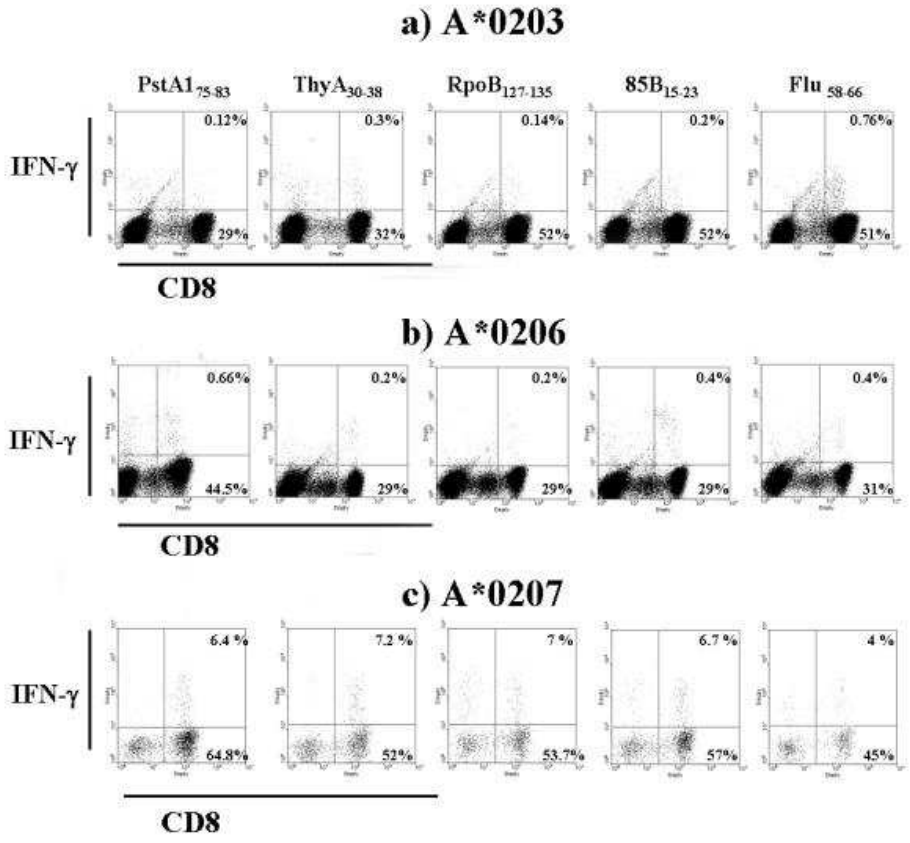


Fig. 2. Intracellular IFN-γ staining for HLA-class I-restricted *M. tuberculosis* peptide reactive CD8+ T cell populations in PPD+ subjects expressing HLA-A2 supertype. STCLs from PPD+ subjects expressing the HLA-A2 supertype (A*0203, A*0206 and A*0207) were stimulated with each peptide overnight ($PstA1_{75-83}$, $ThyA_{30-38}$, $85B_{15-23}$, $RpoB_{127-135}$ and Flu_{58-66}). On next day, each STCL was treated with brefeldin-A and stained with IFN-γ, CD3+ and CD8+ specific antibodies labeled with PE, PerCP and FITC, respectively. The frequencies of peptide-specific IFN-γ secreting CD8+ T cell population were measured from the CD3-gated lymphocyte population.

D. Quantification of the CD8+ T cell frequencies specific for M. tuberculosis-derived peptides from subjects expressing HLA-A2 supertype

To determine the correlations of the CD8+ T cell immune responses in latently infected subjects and in chronically infected TB patients, the frequency of CD8+ T cells specific for each peptide in PBMCs from PPD+ and PPD- healthy subjects and TB patients was quantified using IFN-γ elispot assay. To induce the IFN-γ production from peptide-specific CD8+ T cells after depletion of CD4+ T cells, rhIL-2 and anti-CD28 mAb were included in the assay. These peptide-specific CD8+ T cell responses were detected in subjects expressing the A*0201, A*0203, A*0206 and A*0207 subtypes. Again, $PstA1_{75-83}$-specific CD8+ T cell responses were low in A*0203 subjects. In the IFN-γ elispot assay, there was no hierarchy of the frequencies or spot sizes based on the subtypes (Fig. 3), which suggests that these peptides may bind to any of the subtypes with similar affinities in this assay system except $PstA1_{75-83}$ peptide. The presence of the peptide reactive CD8+ T cell responses suggests that these peptides were processed and presented to the T cells during a natural infection in latently or actively infected individuals.

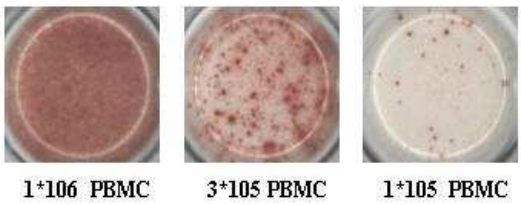


Fig. 3. Photomicrograph displaying IFN-γ specific elispot formation from CD8+ T cells.

The frequencies of the CD8+ T cell populations specific for these peptides ranged from 1 to 40 in the 5.5 x 10^5 circulating PBMCs in the study subjects. Among the four peptides tested, the frequencies of the CD8+ T cells specific for $ThyA_{30-38}$, $RpoB_{127-135}$ or $85B_{15-23}$ peptides were higher in the PPD+ healthy subjects than either of the PPD- healthy subjects or in the far advanced TB patients. There were no statistically significant differences among the four groups regarding the CD8+ T cell response to the $PstA1_{75-83}$ peptide. The frequency of the $RpoB_{127-135}$-specific CD8+ T cell population in the PPD+ subjects was significantly higher than that in the PPD- subjects (*p=0.0001*), mild-moderate TB patients (*p=0.001*) or the far-advanced TB patients (*p=0.001*). The statistical differences were also observed in the frequencies of the $RpoB_{127-135}$ (*p=0.049*) and $85B_{15-23}$ (*p=0.05*)-specific CD8+ T cell population by comparing the epitope-specific frequency of CD8+ T cells from the patients with mild-moderate TB as well as that from the patients with far-advanced TB. In the mild-moderate TB patients, no significant difference in the CD8+ T cell frequency was observed compared with the PPD+ healthy subjects except in the $RpoB_{127-135}$-specific CD8+ T cells. Therefore, the immune response of the CD8+ T cells for these *M. tuberculosis* antigens appears to be induced in both the PPD+ healthy subjects and patients with mild-moderate TB for the protective immunity to *M. tuberculosis*. On the other hand, the CD8+ T cell-mediated immune responses for some of these *M. tuberculosis* antigens appeared to be decreased in the far-advanced TB patients. However, this depression of the CD8+ T cell responses may depend on the *M. tuberculosis* antigens, as shown in Fig. 4. The frequencies of the control peptide,

Flu_{58-66}, specific CD8+ T cell populations were similar among the four groups.

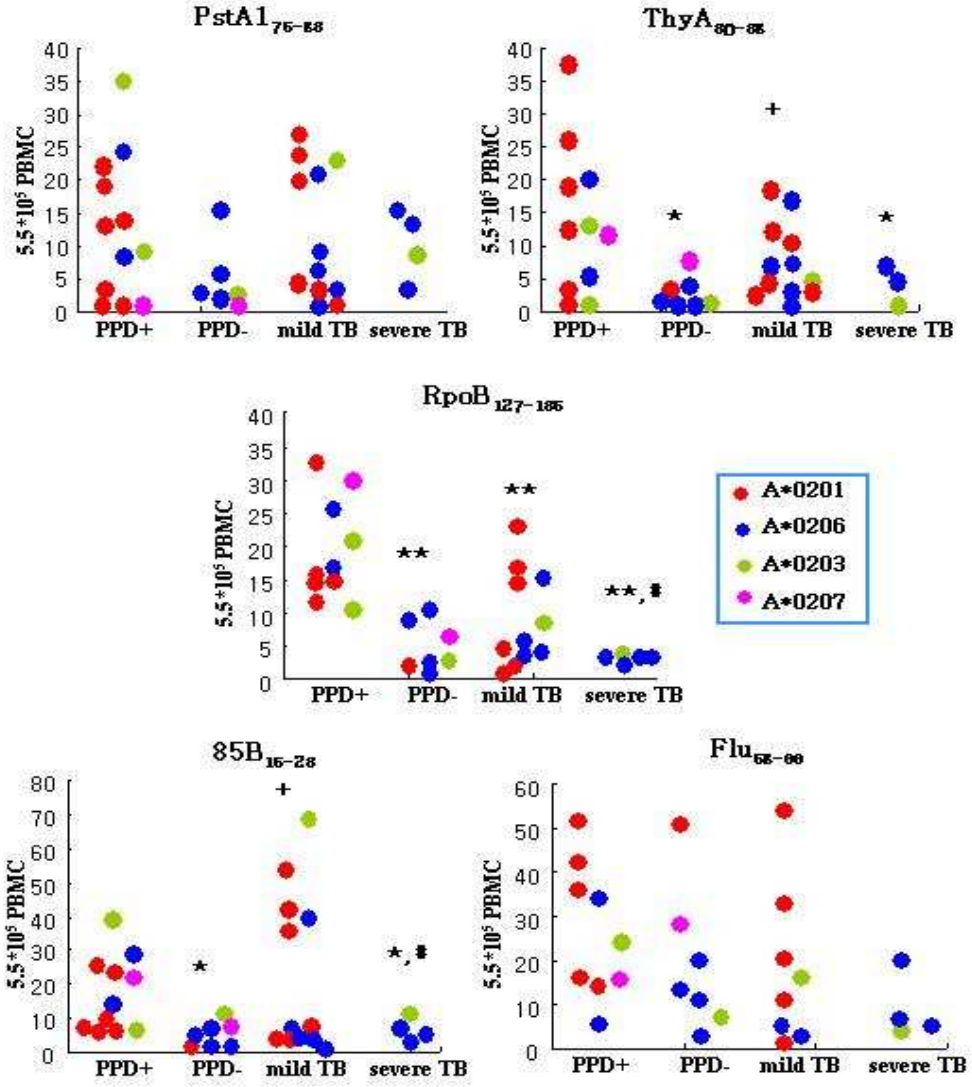


Fig. 4. CD8+ T cell-mediated responses to M. tuberculosis-specific epitope peptides in TB patients and healthy subjects using ex-vivo IFN-γ elispot assay. An *ex-vivo* IFN-γ elispot assay was used to quantify the frequency of the circulating epitope-specific IFN-γ secreting CD8+ T cells in healthy subjects (PPD+ and PPD-), patients with mild or moderate TB (mild TB) and patients with far-advanced TB

(severe TB). Each dot designates the number of IFN-γ secreting CD8+ cells per $5.5x10^5$ PBMCs for one person (A*0201: red, A*0206: blue, A*0207: green and A*0203: pink dots). The frequencies of the SFCs (spot forming cells) were calculated as a mean of two duplicate wells and the values are expressed as number of spots per $5.5x10^5$ PBMCs after subtracting the number of spots in the un-stimulated PBMCs. The significant difference compared with PPD+ (*P<0.05 and **P<0.001), a significant difference compared with mild-moderate TB (# P<0.05) and a significant difference compared with PPD- ($^+$P<0.05) was statistically calculated using two-sample Student *t*-test for means. a) $PstA1_{75-83}$, b) $ThyA_{30-38}$, c) $RpoB_{127-135}$, d) $85B_{15-23}$ and e) Flu_{58-66} peptides were used for IFN-γ elispot assay.

E. Quantification of the frequencies of the CD8+ T cells specific for the *M. tuberculosis*-derived peptides in a pleural effusion from TB pleurisy patients

Pleural tuberculosis is a localized disease where the protective immune responses are active. Therefore, an IFN-γ elispot assay was performed to examine if these epitope-specific CD8+ T cell immune responses are concentrated in the pleural effusion from TB pleurisy patients (Fig. 5). Interestingly, the epitope-specific release of IFN-γ by CD8+ T cells was observed in all three patients (two patients : A*0201 and one patient : A*0206). This suggests that these peptide-specific immune responses are involved in the protective immunity at the site of disease. The frequencies of CD8+ T cells specific to the *M. tuberculosis* peptides in TB pleural effusion were equivalent to those in the PBMCs.

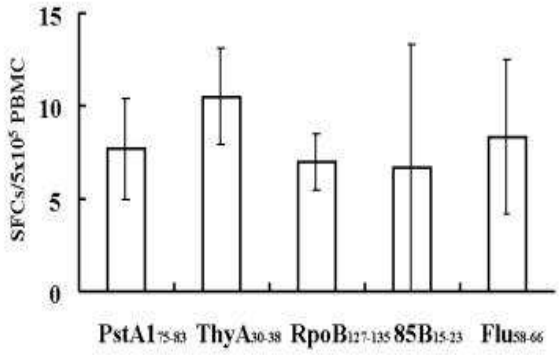
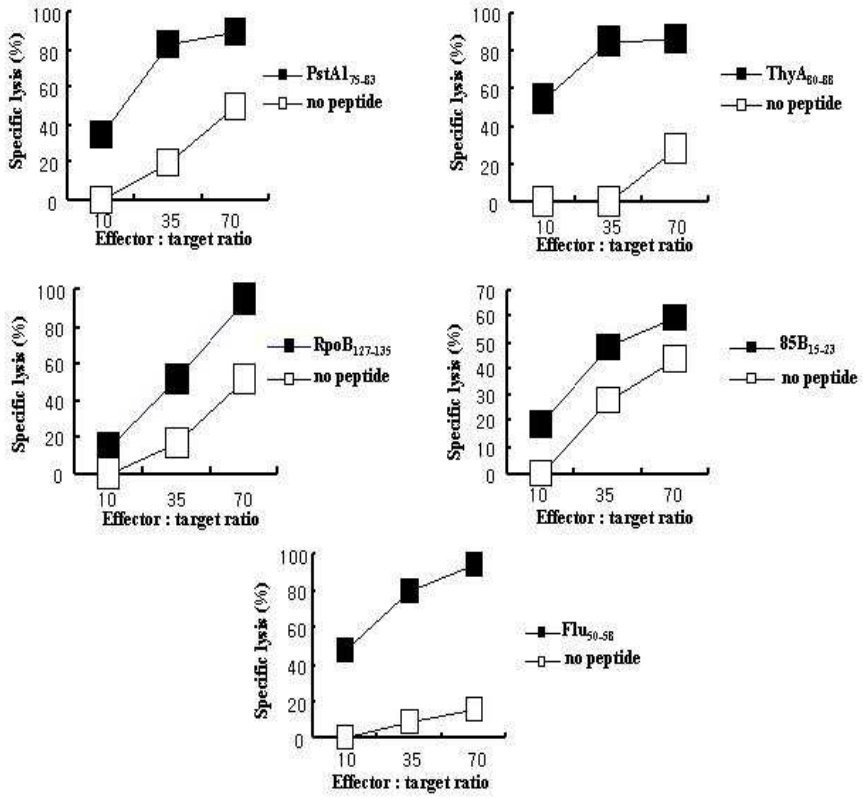


Fig. 5. CD8+ T cell-mediated responses to *M. tuberculosis*-specific epitope peptides in TB pleurisy patients using ex-vivo IFN-γ elispot assay. The values are expressed as a number of spots per $5.5x10^5$ pleural effusion derived cells after subtracting the number of spots in the un-stimulated cells. The data is expressed as a mean ± standard deviations. Flu_{58-66} is used as a positive control.

F. *In vitro* induction of recall CTL responses from healthy subjects expressing HLA-A2 supertype

Not only IFN-γ production but also cytotoxic T cell responses by CD8+ T cells in human as an important function to control *M. tuberculosis* infection. Therefore, we observed the IFN-γ production specific these peptides in healthy BCG vaccinated subjects. Recall responses measure memory cells but not necessarily effector T cells. memory T cells generally have poor cytotoxicity without *in vitro* restimulation[37]. Thus, we *in vitro* stimulated PBMCs from BCG vaccinated subjects and tested the CTL activities. Recall CTL activities specific for $PstA1_{75-83}$, $RpoB_{127-135}$, $ThyA_{30-38}$ and $85B_{15-23}$ peptides were all observed in healthy BCG vaccinated subjects expressing HLA-A*0201 type (Fig. 6). Moreover, $PstA1_{75-83}$, $ThyA_{30-38}$ and $85B_{15-23}$ peptides stimulated CD8+ T cell lines showed CTL activity in healthy BCG vaccinated subjects who express HLA-A*0206 subtype.

A) HLA-A*0201


Specific lysis (%)
Effector : target ratio
PstA1$_{75-83}$
no peptide
ThyA$_{80-88}$
no peptide
RpoB$_{127-135}$
no peptide
85B$_{15-23}$
no peptide
Flu$_{50-58}$
no peptide

B) HLA-A*0206

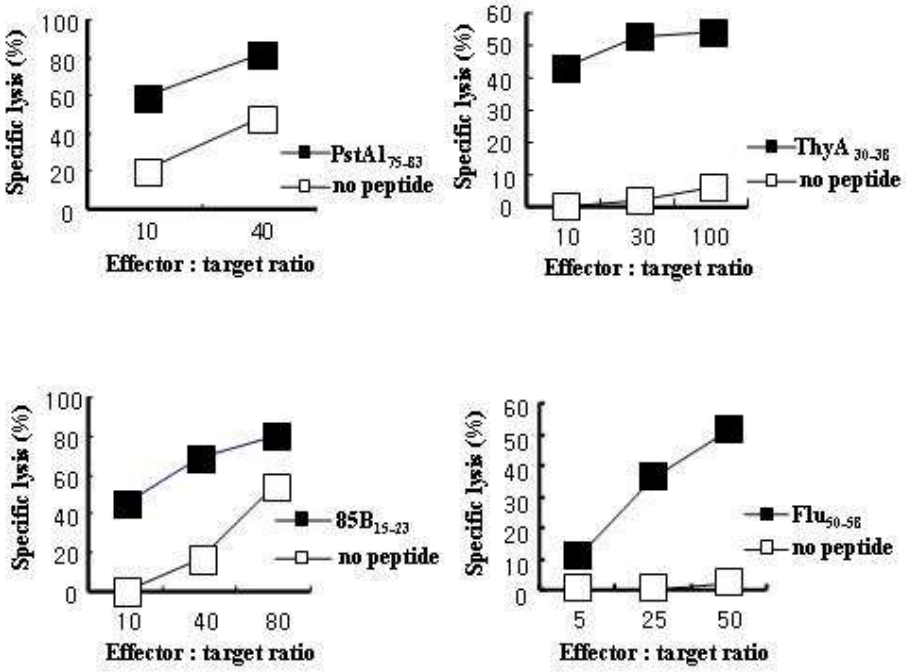


Fig. 6. Recall CTL response from healthy subjects expressing HLA-A2 supertype. Synthetic peptides were added to the PBMCs cultures at a final concentration of 10 ㎍/㎖. On days 3 and 6, rhIL-2 was added to each well at a concentration of 10 units/㎖. On day 8, the cultures were re-stimulated with irradiated (3000 rad) autologous monocytes that were pulsed with peptide in the presence of 3 ㎍/㎖ of β_2m for 2 h. On days 10 and 13, 10 units/㎖ of rIL-2 was added into each well. The cytolytic activity of cultured PBMCs was tested on day 14. Cytolytic activity was measured by a standard 4 h ^{51}Cr release assay.

G. Enumeration of frequencies of peptide-specific CD8+ T cell populations using HLA-A*0201 dimer complexes

The HLA-A*0201-restricted CD8+ T cell specific peptides were used to synthesize A*0201 dimer complexs to stain the peptide-specific CD8 + T cells from PBMCs. To determine the specificity of each dimer, fresh blood PBMCs were obtained from HLA-A*0201 subjects. Dimer staining revealed that the frequency of circulating $PstA1_{75-83}$-specific CD8+ T cells was higher when measured by dimer staining compared with IFN-γ intracellular staining (Fig. 7). It indicated that $PstA1_{75-83}$-specific CD8+ T cell population in PPD+ healthy subjects is functionally heterogeneous since one-half or one-fourth of $PstA1_{75-83}$-specific CD8+ T cell population in PPD+ healthy subjects produced IFN-γ upon peptide stimulation. The ratio of CD8+ T cell population specific for IFN-γ production and A*0201-dimer was not distinctly different in other peptide-specific T cell population (Table 4).

Table 4. Frequency of peptide-specific CD8+ T cells detected by IFN-γ intracellular staining and HLA-A*0201 dimer staining

Donors	$PstA1_{75-83}$	$ThyA_{30-38}$	$RpoB_{127-135}$	$85B_{15-23}$	Flu_{58-66}
	ICS / dimer (%)	ICS / dimer (%)	ICS / dimer (%)	ICS /dimer (%)	ICS /dimer (%)
1	0.4 / 0.8	0.3 / 0.1	0.2 / 0.3	0.1 / 0.08	1.3 / 3.5
2	0.2 / 0.8	0.2 / 0.2	0.4 / 0.2	0.2 / 0.4	0.2 / 0.4
3	0.2 / 0.8	0.2 / 0.2	0.4 / 0.2	0.2 / 0.3	0.2 / 0.4
4	0.22 / 0.67	0.15 / 0.13	0.2 / 0.07	0.1 / 0.05	0.2 / 0.97
5	0.87 / 1.47	0.42 / 0.76	0.54 / 0.64	1.2 / 1.33	0.93 / 1.6

*ICS : intracellular staining, dimer : HLA-A*0201 dimer

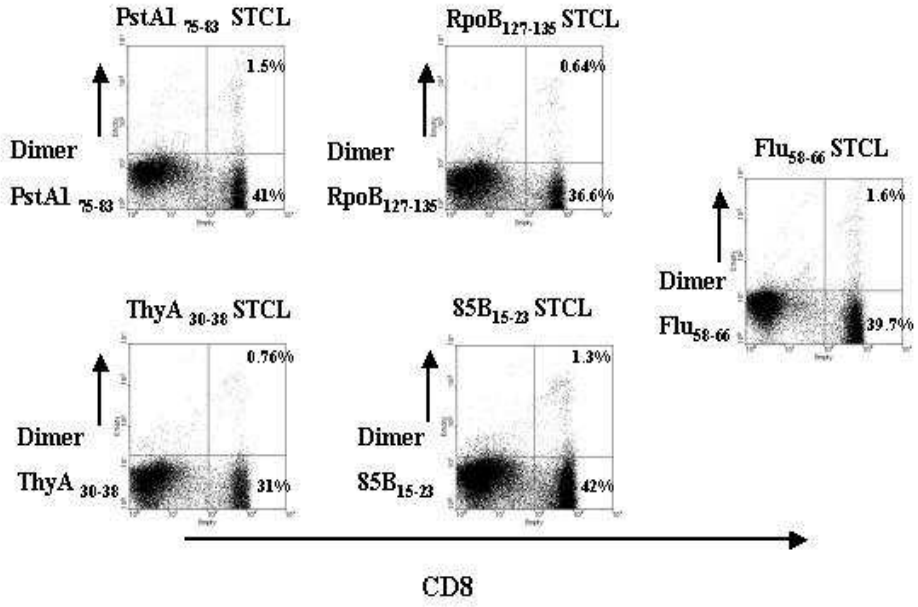


Fig. 7. HLA-A*0201 dimer staining for M. tuberculosis peptide-specific STCL generated from healthy subjects. Dimeric complexs were prepared by mixing HLA-A*0201 : Ig protein with specific peptide at 640 molar excess at 37℃ overnight incubation. Peptide-specific STCLs were incubated with 10 ㎕ pooled human serum at RT for 10 min. Cells were incubated with 1-2 ㎍ of peptide loaded HLA-A*0201 : Ig protein to each sample for 60 min at 4℃. Subsequently, cells were incubated at 4℃ for 30 min in FACS buffer containing anti-CD8 mAb, anti-CD3 mAb and anti-mouse IgG1.

H. Immunocytochemistry for perforin and granulysin expression in *M. tuberculosis* peptide-specific CD8+ CTL lines

Granulysin is a protein present in cytotoxic granules of CTL and natural killer (NK) cells, which are released upon antigen stimulation. CD8+ CTLs lyses *M. tuberculosis* infected macrophages by a granule-dependent mechanism that results in killing of the intracellular pathogen. Moreover, granulysin directly killed extracellular *M. tuberculosis* changing the membrane integrity of the bacillus and in conjunction with perforin, decreased the viability of intracellular *M. tuberculosis*[11]. Confocal microscopy analysis showed that cytotoxic granules, granulysin and perforin, are colocalized in $PstA1_{75\text{-}83}$, $ThyA_{30\text{-}38}$, $RpoB_{127\text{-}135}$, $85B_{15\text{-}23}$, and $Flu_{58\text{-}66}$-specific CD8+ CTLs. This data indicates that these peptide-specific cell lines released perforin and granulysin may be lead to kill *M. tuberculosis* (Fig. 8).

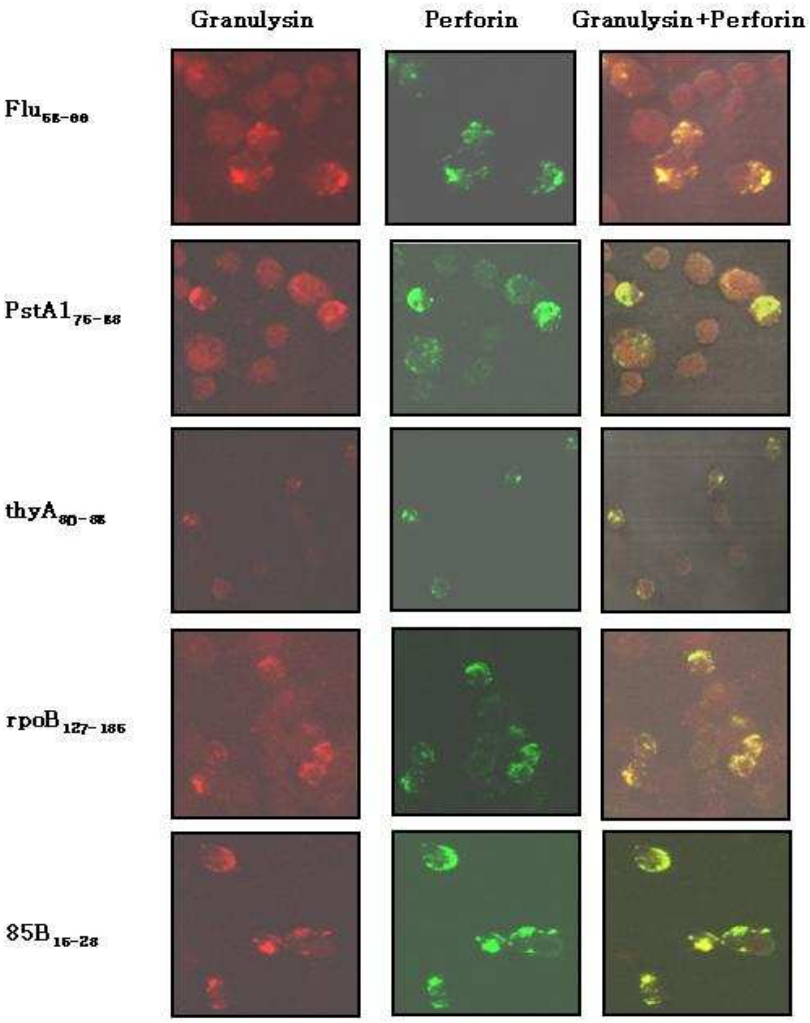


Fig. 8. Detection of perforin and granulysin in CTLs by confocal laser microsopy. Fluorescent confocal images were obtained for granulysin expression (red, first panel of each row) and perforin expression (green, second panel of each row). The two images were then superimposed (third panel of each row) to show vesicles expressing both perforin and granulysin (yellow).

2. MHC class I antigen processing pathway of *M. tuberculosis* somatic antigens

A. Generation of CD8+ T cell lines for *M. tuberculosis* somatic antigens

To demonstrate that CD8+ T cell response against epitopes-derived from somatic antigens can be induced in healthy subjects expressing HLA-A*0201 and A*0206 type, *in vitro* CD8+ CTL induction experiments were performed. PBMCs from healthy HLA-A*0201 and A*0206 subjects were stimulated weekly for 4-5 weeks with autologous PBMCs pulsed with $RpoB_{127-135}$ peptide. CD8+ T cell lines specific for $RpoB_{127-135}$ peptide were generated. These CTL lines showed cytotoxic activity for $RpoB_{127-135}$ peptide pulsed target cells as shown in Fig. 9.

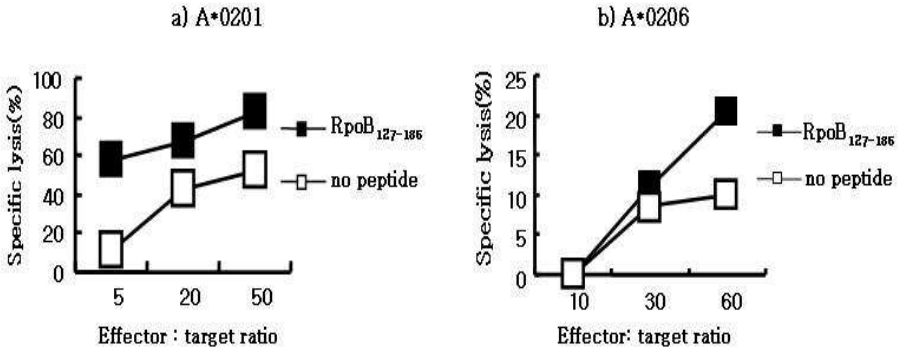


Fig. 9. Generation of CTL lines from healthy HLA-A2 subjects by *in vitro* immunization PBMCs from HLA-A*0201 and HLA-A*0206 subjects were pulsed with 50 ㎍/㎖ of peptide at 3×10^6 cells/well in 10% pooled human serum with rhIL-7 (10 ng/㎖) and keyhole limpet hemocyanin (5 ㎍/㎖). Cultures were re-stimulated weekly with peptide-pulsed and irradiated autologous monocytes and cell culture was supplemented with rhIL-2 at 10 units/㎖ every 3-4 days. After four to five cycles of re-stimulation, the cytotoxic activity of the CD8+ T cells was determined by using the chromium release CTL assay (A*0201 target cell : .221A2 cell lines, A*0206 target : EBV-transformed line from A*0206 PBMC).

B. Kinetics of cytotoxic activities of $RpoB_{127-135}$-specific CD8+ T cells for *M. tuberculosis*-infected macrophages

The kinetic studies of specific killing of *M. tuberculosis*-infected (3 MOI) macrophages by $RpoB_{127-135}$-specific CD8+ T cells suggest that RpoB protein is processed more efficiently when the infection period is increased longer (Fig 11). Infectivity of macrophages was >70% by Ziehl-Neelsen method (Fig. 10).

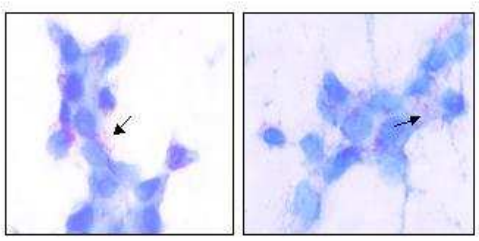


Fig. 10. AFB staining. Human monocyte-derived macrophages were infected with H37Rv at 3MOI for 4 h. After three times washing, cells were fixed and stained with Ziehl-Neelsen method (infectivity >70%).

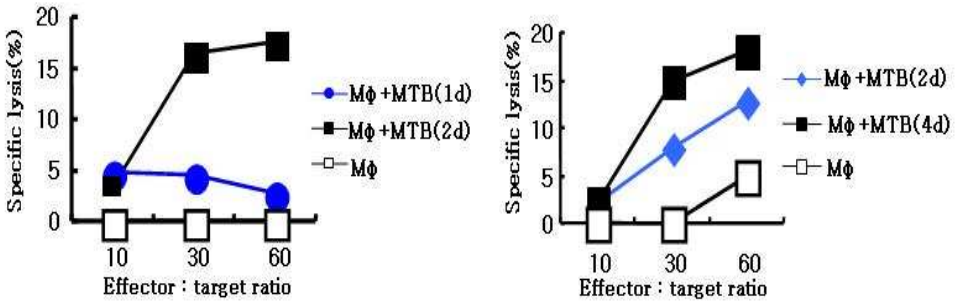
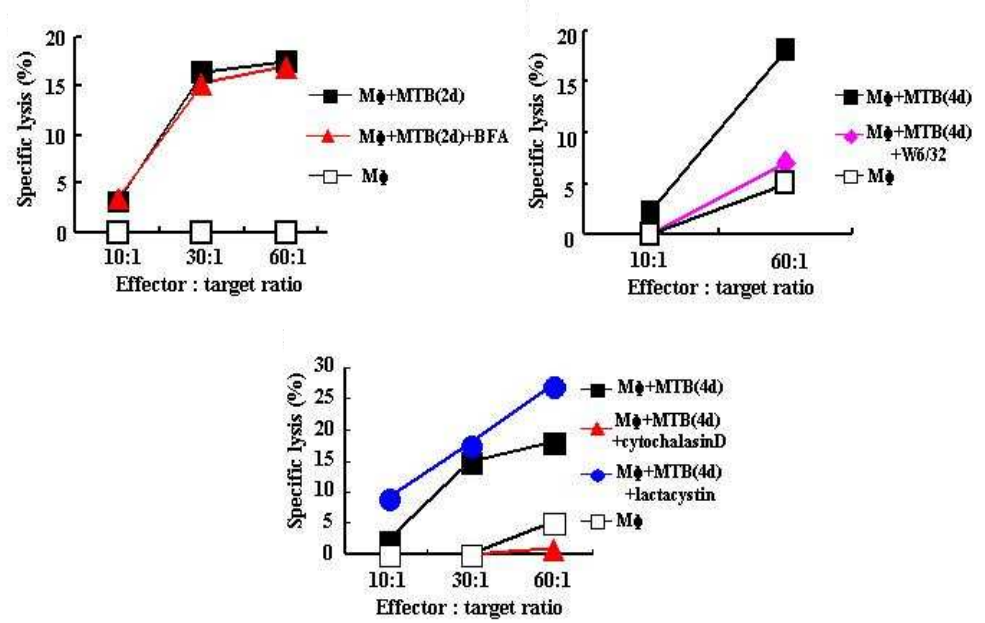


Fig. 11. Kinetics of *M. tuberculosis* RpoB$_{127-135}$ peptide processing inside macrophages. Macrophages were generated by culturing adherent monocytes in RPMI 1640 with 10% FBS for 3-4 days. These macrophages were then infected with *M. tuberculosis* (H37Rv) at a multiplicity of infection of 3 for 1-4 days. Extracellular non-phagocytosed *M. tuberculosis* was removed by washing. For cytotoxicity experiments, 1×10^6 macrophages were labeled with 100μCi of ^{51}Cr for 1 hour at 37℃, and then added to the wells of 96 well U-bottom plates at 5×10^3 cells/well. CD8+ T cell lines were added at various effector to target ratios. Specific lysis (%) was determined as described above.

C. $RpoB_{127-135}$ peptide presentation requires phagocytosis and bypass Golgi-ER transport in *M. tuberculosis*-infected macrophages

$RpoB_{127-135}$ peptide-specific CD8+ T cell lines generated from both HLA-A*0201 and A*0206 subjects showed the specific lysis for *M. tuberculosis*-infected macrophages. Processing of RpoB (RNA polymerase β-subunit) protein of *M. tuberculosis* for the presentation of $RpoB_{127-135}$ peptide to MHC class I-restricted CD8+ T cells was insensitive to brefeldin-A (Golgi-ER transport inhibitor) and lactacystin (proteasome inhibitor) treatment, although the recognition of *M. tuberculosis*-infected cells by $RpoB_{127-135}$-specific CD8+ T cells was inhibited by anti-MHC class I blocking antibody (W6/32) or cytochalasin D (phagocytosis inhibitor). This result indicates that *M. tuberculosis* $RpoB_{127-135}$ peptide is processed and presented from intracellular MTB antigen to these CD8+ T cells. It requires phagocytosis of the bacteria, with antigen entry to the cytoplasm. However, the absence of inhibition by brefeldin A demonstrates that the antigen processing may bypass the Golgi-ER pathway. In addition, the processing seems to be not inhibited by lactacystin. This result demonstrated RpoB antigen was not processed by proteasomal degradation. This suggests that the RpoB peptide may be processed by the alternative MHC class I-restricted presentation pathway (Fig. 12).

A) Cytotoxic assay using CTL line generated from HLA-A*0201 PBMCs



B) Cytotoxic assay using CTL line generated from HLA-A*0206 PBMCs

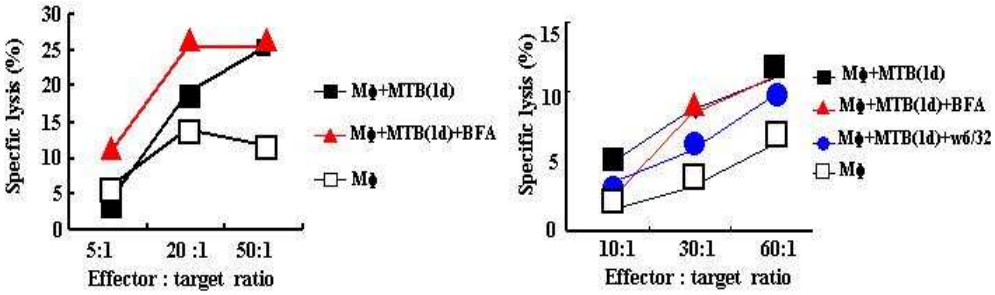


Fig. 12. Effect of metabolic inhibitors on presentation of *M. tuberculosis*-derived RpoB protein. $RpoB_{127-135}$-specific CD8+ CTL lines were stimulated with macrophages that had been preincubated with metabolic inhibitors, brefeldin A (Golgi-ER transport; 3 ㎍/㎖), lactacystin (proteasome, 40μM) or cytochalasin D (phagocytosis; 10 ㎍/㎖) for 1 h before the addition of *M. tuberculosis*. Anti-MHC class I blocking antibody (W6/32, 10 ㎍/㎖) was added to target cells 1 h before CTL assay.

3. Gene expression of components of the MHC class I antigen processing machinery following infection with *M. tuberculosis*

It has been known that *M. tuberculosis* infection affected the expression of enormous genes in host cells. *M. tuberculosis* infects and replicates mainly in either macrophages or dendritic cells. In our next experiment, therefore, we analyzed the profile of the gene expression of MHC class I antigen processing components either in *M. tuberculosis*-infected macrophages or dendritic cells.

A. Effect of IFN-γ on the expression of MHC molecules of macrophages

Monocytes-derived macrophages were treated with various concentrations of rhIFN-γ for 18 h. The highest expression of MHC class I (W6/32) and MHC class II (DR) in human macrophages was observed when the concentration of IFN-γ was 20 ng/ml (Fig. 13).

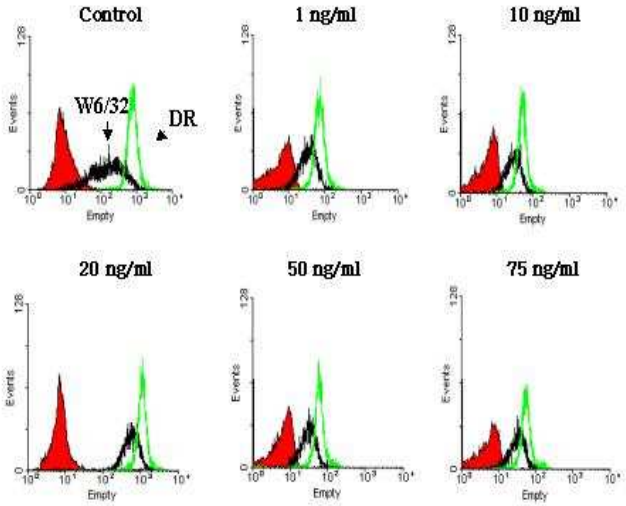


Fig. 13. Effect of IFN-γ concentrations in the expression of human MHC molecules. Human monocyte-derived macrophages were treated with various concentrations of rhIFN-γ for 18 h. Cells were stained with isotype control Ab (solid histograms), anti-class I Ab (W6/32 : black histograms) or class II Ab (DR : grey histograms) and then analyzed by flow cytometry.

B. The kinetics of IFN-γ induced gene expression of MHC class I Ag processing pathway affected by *M. tuberculosis* infection

After treating macrophages with various concentrations of rhIFN-γ (20 ng/㎖), the expression of genes involved in MHC class I processing machinery were examined by RT-PCR analysis. The expression of TAP2 gene by IFN-γ induction was down-regulated by *M. tuberculosis* infection at 24 h, while the expression of TAP1 gene by IFN-γ induction was increased maximally by *M. tuberculosis* infection at 12 h (Fig. 14A). The mRNA expression of LMP2, TAP2 and LMP10 genes by IFN-γ induction was not increased at the first 4 h after IFN-γ treatment. In addition, the mRNA expression of TAP2 and LMP2 genes by IFN-γ induction was increased in a time dependent manner and it is maximized at 24 h (Fig. 14A). *M. tuberculosis* infection inhibited the IFN-γ induction of LMP2 gene expression at 24 h (Fig. 14A). The expression of CD64 gene by IFN-γ induction was maximal at 8 h (Fig. 14B). Up to 8-12 h, the expression of PA28α gene by IFN-γ induction was steadily maintained and its expression decreased at 24 h. But the expression of PA28β by IFN-γ induction was maintained up to 24 h (Fig 14B). The expression of other genes was not nearly changed throughout the whole incubation period with IFN-γ and *M. tuberculosis* infection (Fig 14B, C). Therefore, this result indicates that MHC class I processing genes were regulated differently during the time periods after IFN-γ or *M. tuberculosis* infection.

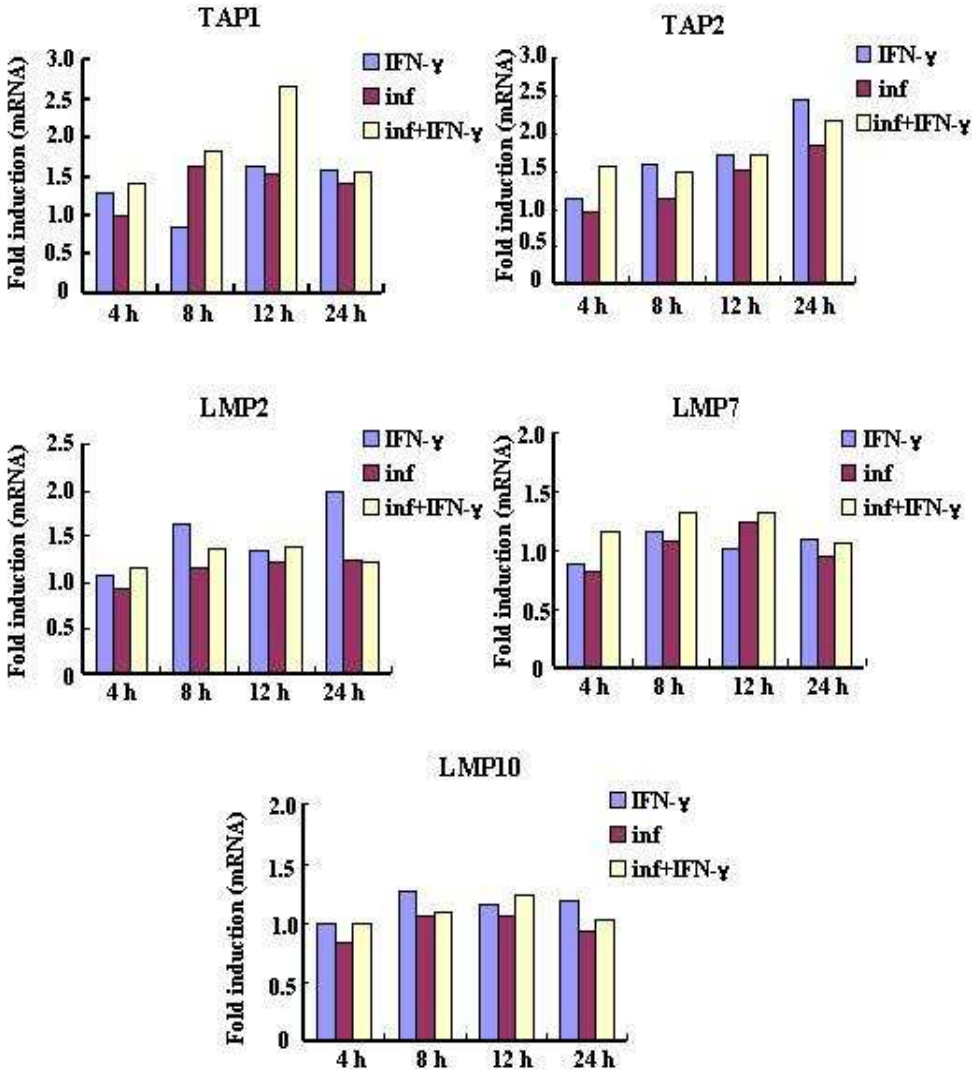


Fig. 14A. The kinetics of MHC class I gene expression in response to IFN-γ treatment and M. tuberculosis infection. Densitometric quantitation of the relative induction of MHC class I processing genes were normalized with the amount of GAPDH mRNA in human macrophages. The fold induction was calculated as (ratio of target gene/GAPDH intensity in study group) / (ratio of target gene/GAPDH intensity in control). The detailed information of primers is described in Table 1.

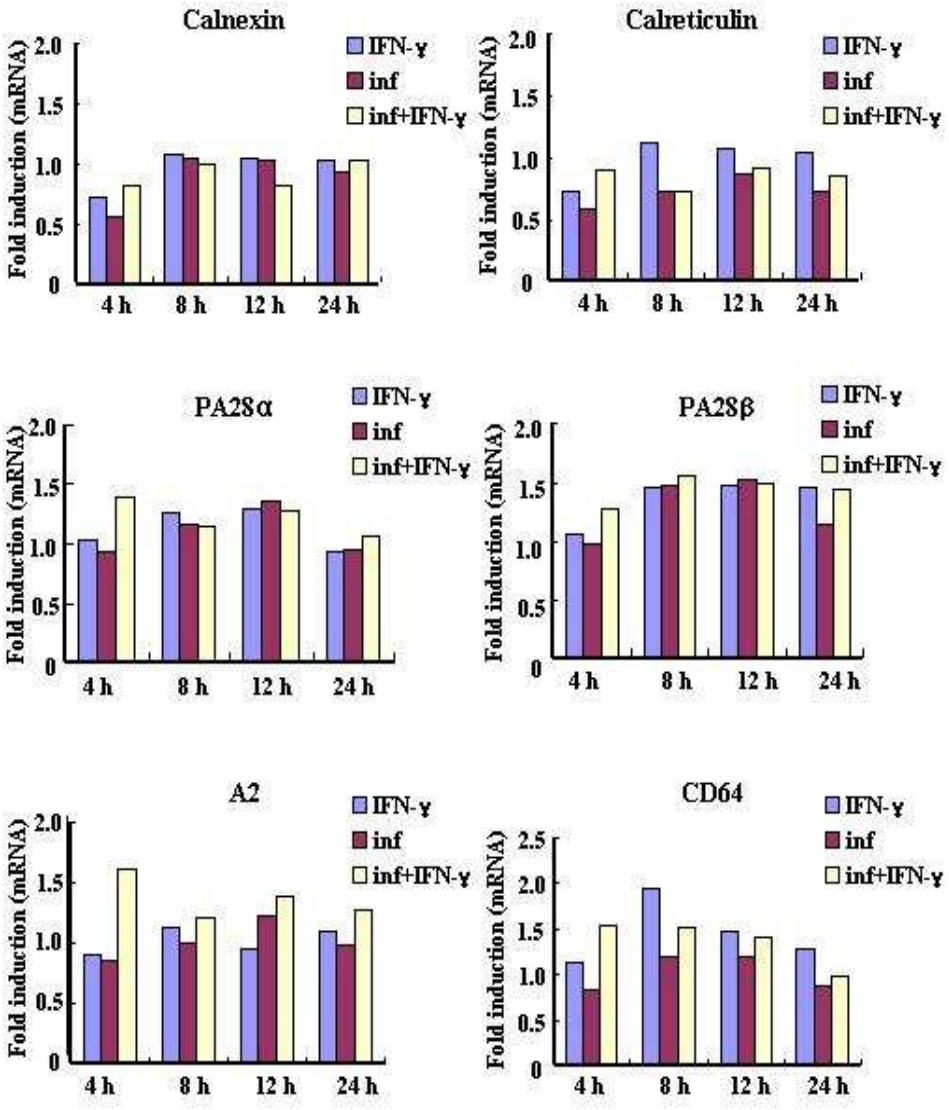


Fig. 14B. The kinetics of MHC class I gene expression in response to IFN-γ treatment and *M. tuberculosis* infection. Densitometric quantitation of the relative induction of MHC class I processing genes were normalized with the amount of GAPDH mRNA in human macrophages. The fold induction was calculated as (ratio of target gene/GAPDH intensity in study group) / (ratio of target gene/GAPDH intensity in control).

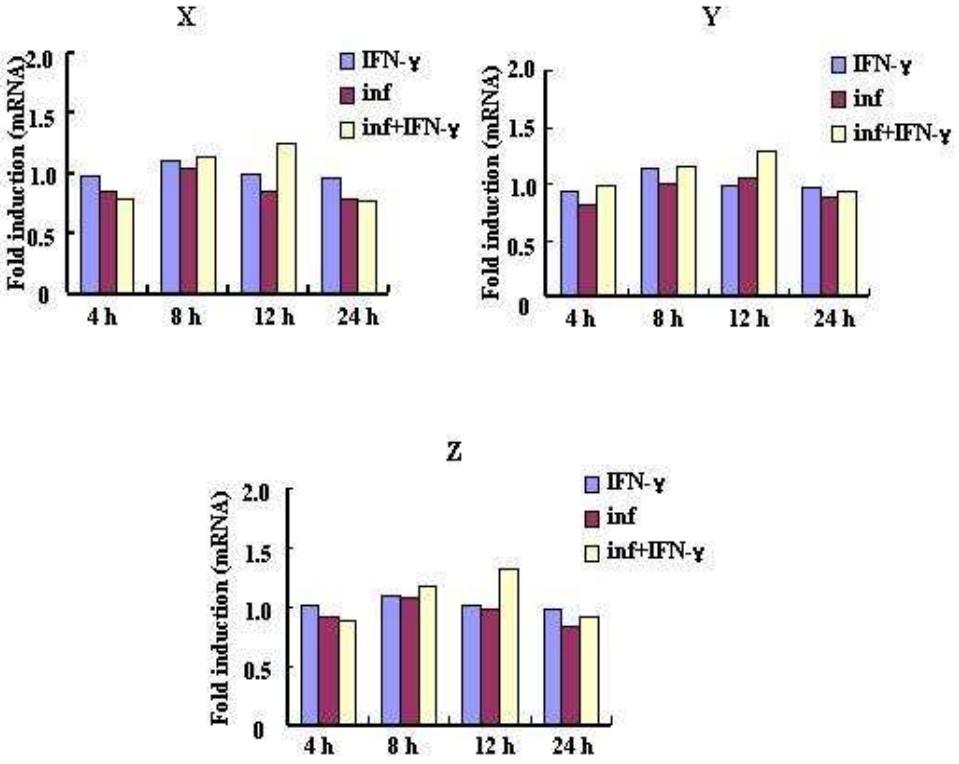


Fig. 14C. The kinetics of MHC class I gene expression in response to IFN-γ treatment and *M. tuberculosis* infection. Densitometric quantitation of the relative induction of MHC class I processing genes were normalized with the amount of GAPDH mRNA in human macrophages. The fold induction was calculated as (ratio of target gene/GAPDH intensity in study group) / (ratio of target gene/GAPDH intensity in control).

C. Isolation and characterization of human immature DCs from peripheral blood derived adherent cell cultures.

We generated DCs from adherent PBMCs cells by culturing with rhGM-CSF and rhIL-4. After 3-4 days of culture, analysis of cell surface markers was performed and it was demonstrated that cells expressed DCs specific marker, CD1a and CD83. These cells expressed high levels of MHC class I and MHC class II molecules as well as the co-stimulatory molecules such as CD80 and CD86. These DCs appear to have the phenotype and functional characteristics of immature DCs which are very effective at antigen capturing and processing [38, 39] (Fig. 15).

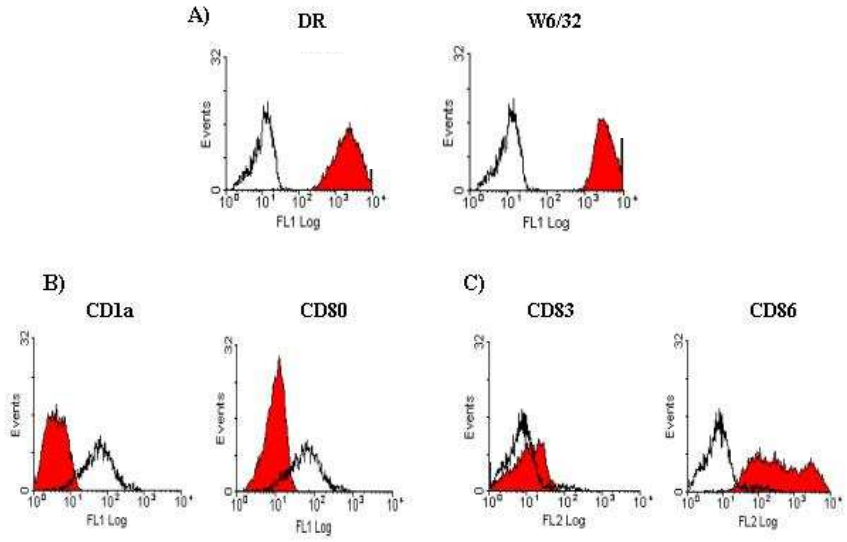


Fig. 15. Cell surface molecule expression of human immature dendritic cells. Human monocyte-derived immature DCs were treated with rhGM-CSF and rhIL-4 for 3 days. Cells were stained with IgG isotype control, or FITC conjugated Abs against DR, CD1a, CD80, PE conjugated Abs against CD83 and CD86 and subsequently examined by flow cytometry. A) DR and W6/32 (solid histograms), isotype control (open histograms), B) CD1a and CD80 (open histograms), isotype control (solid histograms), C) CD83 and CD86(solid histograms), isotype control (open histograms).

D. The effect of IFN-γ treatment and *M. tuberculosis* infection on the expression of genes involved in MHC class I antigen processing

The expression of the genes involved in MHC class I antigen processing, TAP, proteasome subunits, proteasome activators, different chaperones and MHC class I antigens were analyzed by RT-PCR. After treating human monocyte-derived macrophages with IFN-γ (20 ng/㎖), mRNA expression levels of TAP-1, TAP-2, LMP2, LMP10, PA28α, PA28β and HLA-DR were increased (Fig. 17, 19). IFN-γ regulated differentially gene expression of proteasome inducible components, LMP10, PA28α, PA28β and constitutive subunits X, Y, Z[40]. The promoter regions of TAP2, LMP7, LMP10 and PA28 all contain the IFN-consensus sequences[31, 41], suggesting a distinct regulation of these genes upon IFN treatment. But mRNA of LMP7 after IFN-γ treatment was not nearly increased in their experiment (Fig. 17, 19). In addition, *M. tuberculosis* infection inhibited IFN-γ induction of proteasome component LMP2 and TAP2 gene of macrophage and DCs by 10-20% (Fig. 17, 18). In addition, the IFN-γ induction of LMP10 was slightly reduced in macrophage and DCs by MTB infection (Fig 17, 19).

Interestingly, IFN-γ increased the expression of TAP1 gene on *M. tuberculosis* infected DCs and macrophages 1.2 fold (Fig 17, 19). On the contrary, *M. tuberculosis* infection inhibited IFN-γ induction of TAP2 gene of macrophage and DCs by 10-20% (Fig 17-20). Interestingly, BCG infection inhibited IFN-γ induction of TAP1 and TAP2 genes of macrophages. Moreover, the *M. tuberculosis* infection affected

differently mRNA expression of HLA-DR depending on cell types. Namely, infection of macrophages either with *M. tuberculosis* or BCG inhibited IFN-γ induction of DR, but infection of DCs with *M. tuberculosis* accelerated IFN-γ induction of DR expression. The inhibitory effect by *M. tuberculosis* and BCG infection on the induction of gene expression by IFN-γ seems to be various depending on the responsive genes. The gene expression levels involved in MHC class I processing pathway were different dependent on antigen presenting cells. Infection with *M. tuberculosis* inhibited IFN-γ signaling on CD64 gene in macrophages and DCs, similar to previously reported data. Moreover, infection with BCG inhibited IFN-γ signaling on CD64 gene in macrophage. The gene expression of chaperons such as calnexin, calreticulin, x, y and z was not changed according to IFN-γ treatment, *M. tuberculosis* infection or BCG infection. These results suggest that cellular signal transduction pathways which are activated by IFN-γ might be differentially interfered depending on gene with *M. tuberculosis* and BCG Infection. Accordingly, *M. tuberculosis* might survive within macrophages and DCs by evading protective immune mechanism of host cells.

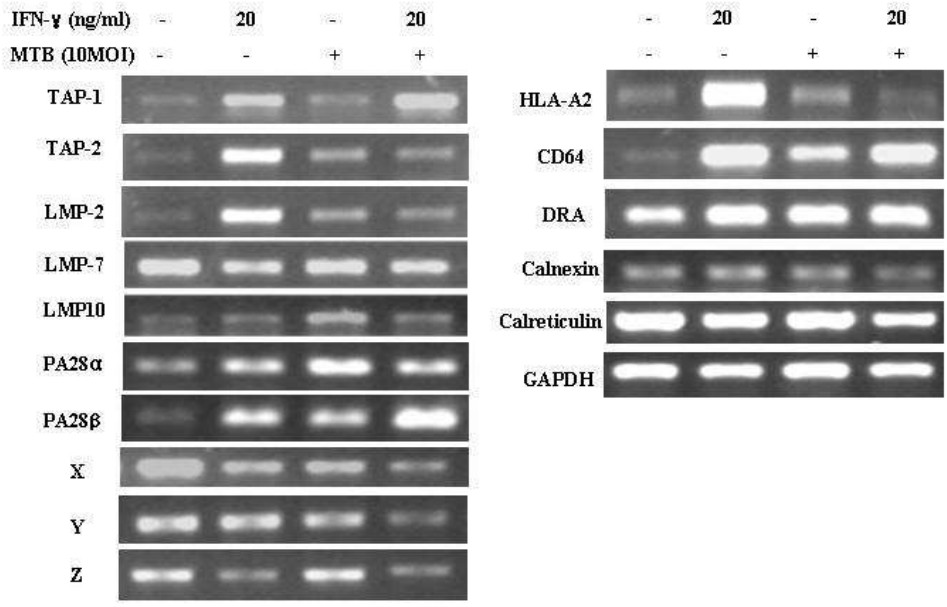


Fig. 16. RT-PCR analysis for MHC class I antigen processing genes in human monocyte-derived dendritic cells (DCs). Total cellular RNA from human DCs infected with *M. tuberculosis* (10MOI) and treated with 20 ng/㎖ IFN-γ for 8 h was extracted and used for RT-PCR analysis. GAPDH is used as an internal control of RNA amount.

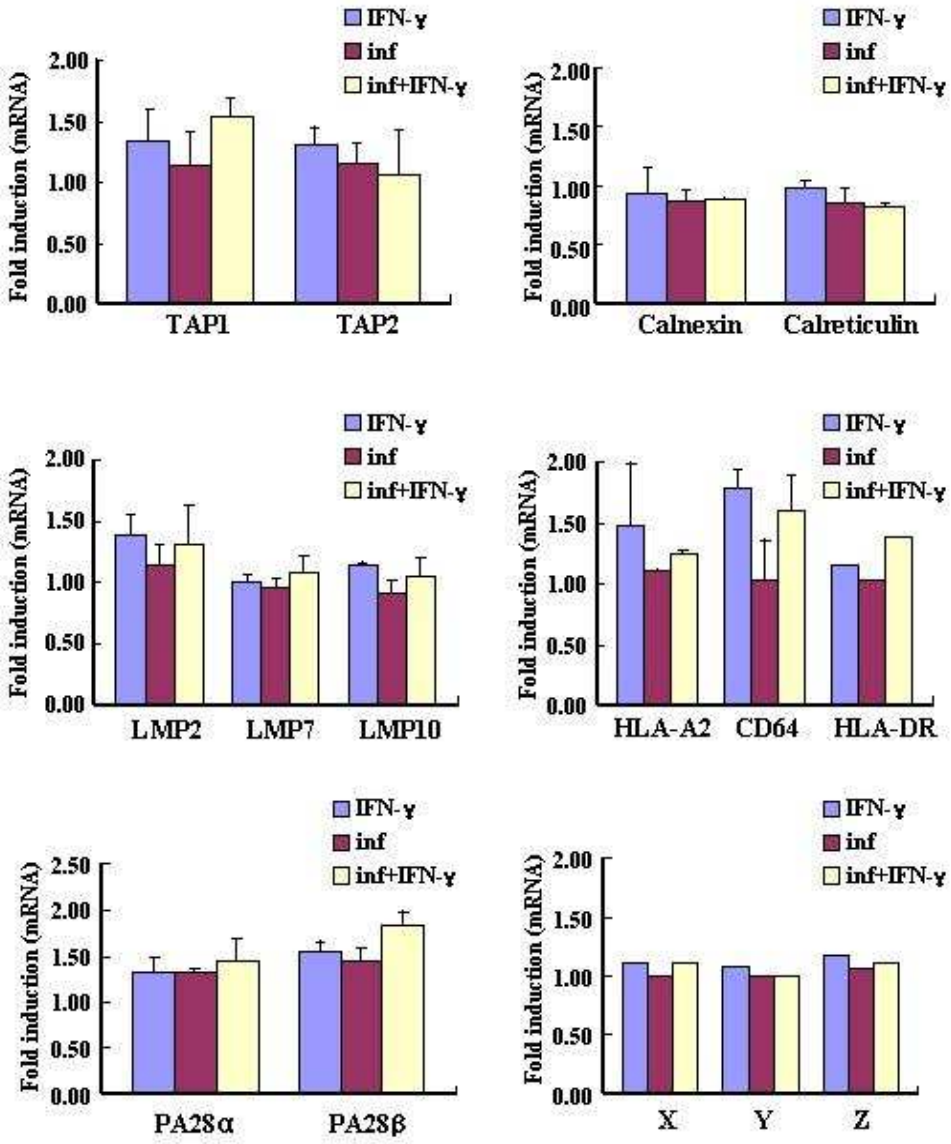


Fig. 17. Densitometric quantitation of the relative induction of genes involved in MHC class I antigen processing. Results were normalized for the amount of GAPDH mRNA in human monocyte-derived dendritic cells after IFN-γ and *M. tuberculosis* infection. The fold induction was calculated as (ratio of target gene/ GAPDH intensity in study group)/(ratio of target gene / GAPDH intensity in control). Data shown are representative of three experiments and are expressed as mean ± S. D.

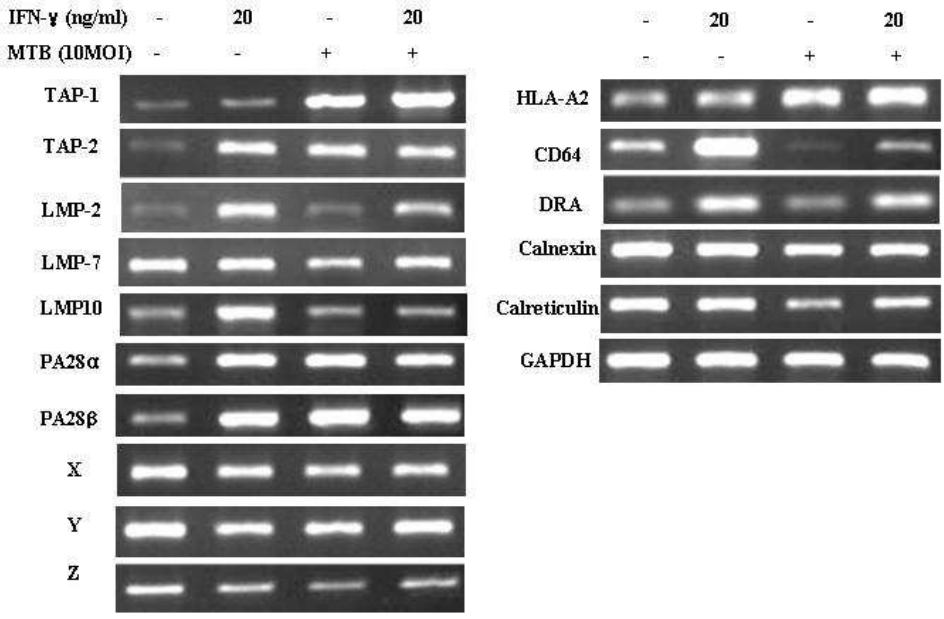


Fig. 18. RT-PCR analysis to exam the mRNA expression pattern of genes involved in MHC class I antigen processing in macrophages. Total cellular RNA from human macrophages infected with *M. tuberculosis* for 24 h and subsequently treated with 20 ng/㎖. IFN-γ for 8h was extracted and used for RT-PCR analysis. GAPDH is used as an internal control of RNA amount in each sample.

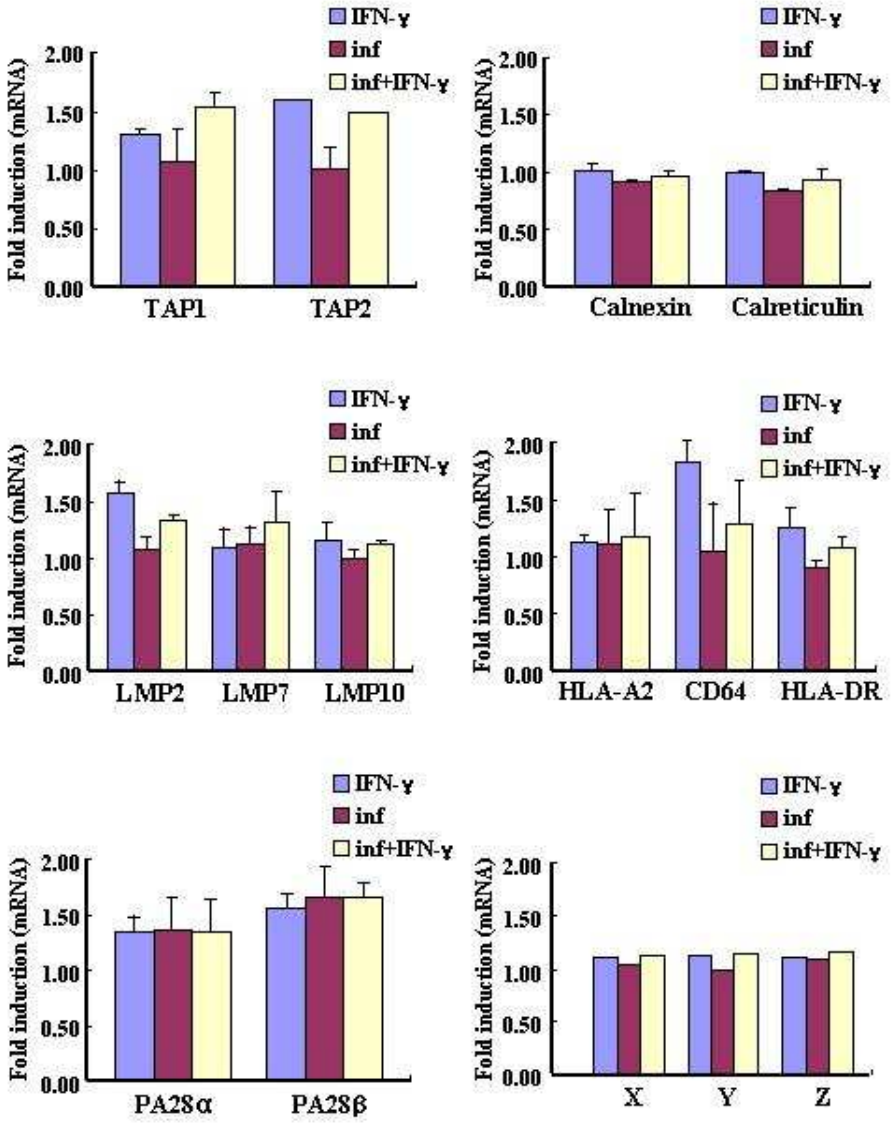


Fig. 19. Densitometric quantitation of the relative induction of genes involved in MHC class I antigen processing. Results were normalized for the amount of GAPDH mRNA in human macrophages after IFN-γ and *M. tuberculosis* infection. The fold induction was calculated as (ratio of target gene / GAPDH intensity in study group)/(ratio of target gene / GAPDH intensity in control). Data shown are representative of three experiments and are expressed as mean ± S. D.

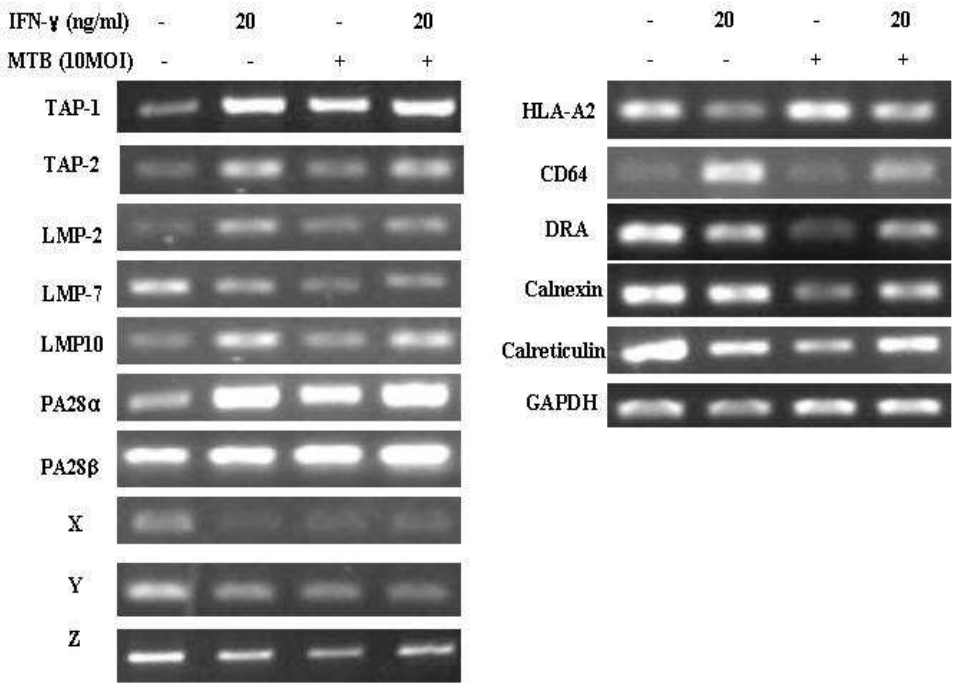


Fig. 20. RT-PCR analysis for genes involved MHC class I antigen processing in human monocyte-derived macrophages. Total cellular RNA from human macrophages infected with BCG (10MOI) and treated with 20 ng/㎖ IFN-γ for 8h was extracted and used for RT-PCR analysis. GAPDH is used as an internal control of RNA amount.

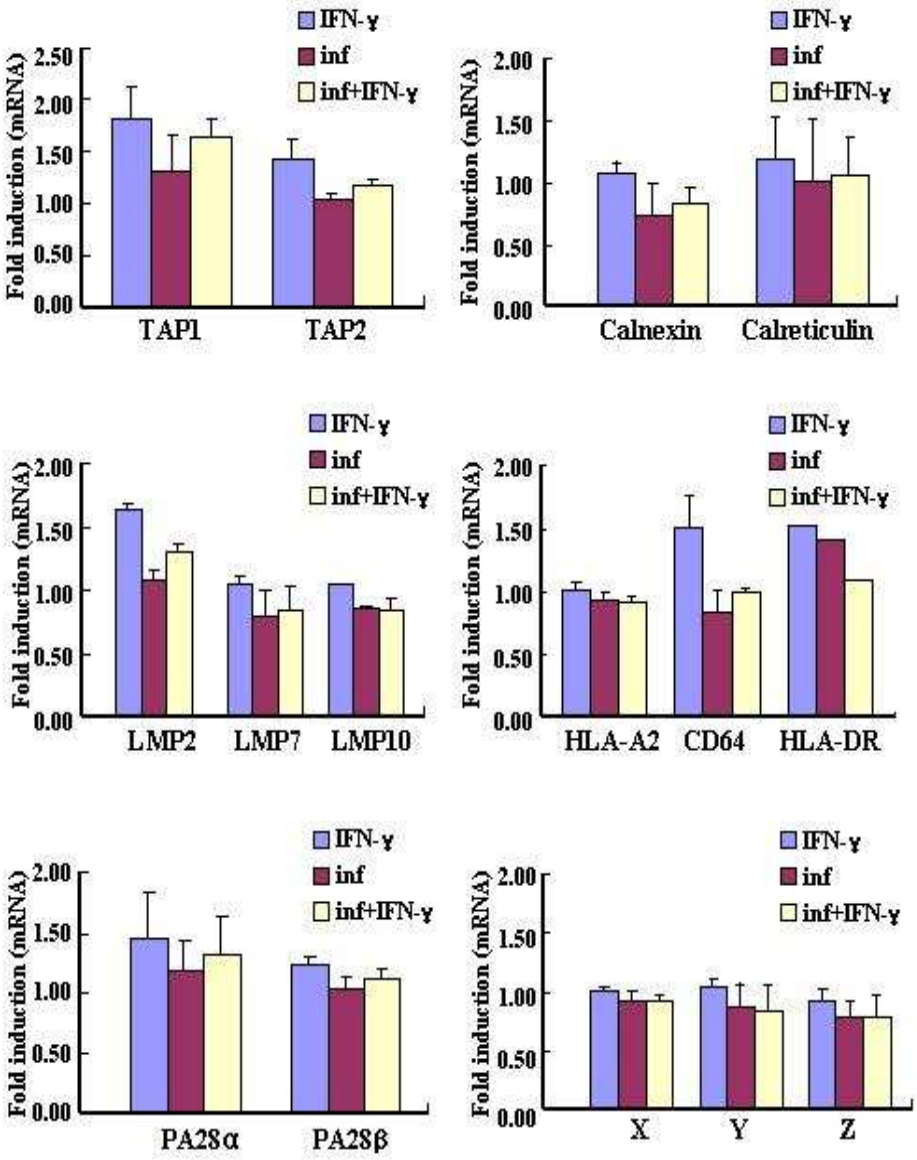


Fig. 21. Densitometry quantitation of the relative induction of genes involved in MHC class I antigen processing. Results were normalized for the amount of GAPDH mRNA in human macrophages after IFN-γ and BCG infection. Fold induction calculated as (ratio of target gene / GAPDH intensity in study group)/(ratio of target gene / GAPDH intensity in control group). Data shown are representative of two experiments and are expressed as mean ± S. D.

IV. DISCUSSION

M. tuberculosis is a highly successful intracellular pathogen that is able to survive and cause disease despite the induction of innate and adaptive immune responses in the host. The incidence of TB has been increasing since the emergence of multi-drug resistant strains of *M. tuberculosis* and HIV co-infection. So, the development of effective TB vaccine is urgently required. It is well known that CD4+ T cells are important in the protection against infectious disease caused by *M. tuberculosis*. But the contribution of CD8+ T cells has been less well established in this respect. Recently, human studies have demonstrated that MHC class I-restricted CD8+ T cells recognized *M. tuberculosis*-infected cells and can release IFN-γ, lyse infected target cells, and kill intracellular bacteria[14, 42-44]. These evidences indicate that MHC class I-restricted CD8+ T cell immune responses are one of the protective immune mechanisms in human. In addition, there has been a continuous effort to define *M. tuberculosis* epitopes that can be presented by MHC class I molecules to CD8+ T cells. CD8+ T cell responses against these epitopes can also be induced in healthy HLA-A*0201 positive subjects by prolonged stimulation with peptide *in vitro*, which indicates that both can sensitize naïve CD8+ T cells and as a consequence, enable the generation of peptide-specific CTL lines *in vitro*[36].

In a previous study, four *M. tuberculosis*-derived peptide epitopes-specific for the HLA A*0201-restricted CD8+ T cells were defined[15]. While the selection of immunodominant peptides for vaccines or therapeutic agents is allowed for the search

for the HLA allele specific epitope peptides, this approach has had limited applications because the selected peptides can induce immune responses in the population expressing suitable HLA types. In order to overcome this limitation, a supertype has been recently defined as a group of HLA alleles that share a similar binding motif. Among the four major HLA supertypes (A2, A3, B7, B44), the HLA-A2 supertype is one of the most prevalent HLA supertype. These subtypes differ among each other by one or a few amino acids, and these amino acid changes have much effect on the peptide binding characteristic as well as on the fine specificity of many HLA-A2-restricted cytotoxic T lymphocyte clones[45, 46]. Although different peptides among the members of A2 supertype are known to cross-react at different levels, these peptide-specific immune responses were induced efficiently in circulating PBMCs of the PPD+ healthy subjects expressing the HLA-A2 supertype alleles including A*0203, A*0206 and A*0207 (Fig 1, 2). HLA-A*0206 and A*0207 molecules express identical amino acid sequences as A*0201 molecule except one amino acid and A*0203 molecule has three amino acids differences from A*0201 molecule in ligand binding domain of HLA-A molecule. In addition, these peptides did not induce the type 2 cytokine, IL-4, production from CD8+ T cells (Fig 1), whereas the HLA-A*0201-specific peptides from the 19 kDa protein of *M. tuberculosis* induced IL-4 production[47]. Therefore, these peptides may be useful as components for a protective vaccine for TB.

To estimate the frequency of the *M. tuberculosis* peptide-specific CD8+ T cells in PBMCs from the subjects expressing HLA-A2 supertype, IFN-γ elispot assay was

used in this study. The frequency of CD8+ T cells specific for the tested peptides were rather higher in PPD+ healthy subjects than either TB patients or PPD- healthy subjects (Fig. 4). Considering that *M. tuberculosis* is replicating actively in an infected host, the antigenic load from the bacilli was expected to be higher in the TB patients than in the PPD+ healthy subjects[48]. In this approach, however, high frequencies of $RpoB_{127-135}$, $85B_{15-23}$ or Thy_{30-38}-specific CD8+ T cells were observed in the PPD+ people or mild–moderate TB, but not in advanced TB (Fig 4). This data can be explained in two ways. First, it is possible that an increase in the *M. tuberculosis* antigens in far-advanced TB patients may exhaust the CD8+ T cell immune responses. For example, it was suggested that the protective CD8+ T cells can be exhausted by deletion or anergy by chronic viral infection if the immunogenic antigens are overloaded in a virally infected host, while viral replication can be contained in latently infected host[8]. Similarly, CD8+ T cells specific for *M. tuberculosis* antigens may be deleted or functionally become anergic due to the heavy increase of *M. tuberculosis* antigens in far-advanced TB patients. Secondly, it is also possible that the pattern of *M. tuberculosis* gene expression inside host macrophages may be different between latent infection and active infection with TB. For instance, it was reported that the RpoB gene is not transcribed in activated macrophages while it does in resting macrophages. We also propose that some of *M. tuberculosis* proteins may not be actively turned over and processed for antigenic presentation inside activated macrophages of TB patients. Therefore, dominant *M. tuberculosis* antigens inducing CD8+ T cells may change depending on patient clinical status.

In addition, it was shown that different mechanisms could depress the CD8+ T cells depending on the viral epitopes from the same virus[48]. These findings suggest that MHC class I-restricted CD8+ T cells specific for *M. tuberculosis* antigens induce immune protection in the latently infected healthy subjects (Fig 4). Lastly, the presence of a low *M. tuberculosis*-specific T cell frequency in the periphery in TB patients has been suggested due to the migration of *M. tuberculosis*-specific T cells into the disease sites. However, the frequency of the CD8+ T cells specific for the *M. tuberculosis* peptides in a TB pleural effusion were comparable to that in PBMCs, suggesting that CD8+ T cells specific for *M. tuberculosis* are not sequestered in the pleural TB (Fig 5). In summary, the reduced immune response of the CD8+ T cells specific for the *M. tuberculosis* antigens in TB patients appears to be one of the immune depressions induced by TB infection.

This study also suggests that the search for immunogenic antigens should be focused not only on the culture filtrate proteins but also on the total proteins produced by *M. tuberculosis* including the somatic proteins. Accordingly, an appreciation of the epitope peptide-specific immune responses may help to understand the disease progress, and the immunodominant epitope peptides, which do not induce the immune unresponsiveness, can be selected as candidate peptides for both diagnostic agents and future vaccines for TB.

CD8+ T cells play an important role during memory immune response in intracellular bacterial infection with *Listeria monocytogenes* and *Chlamydia pneumoniae*[49, 50]. CD8+ T cells in the recall response to *M. tuberculosis* challenge

may be important in rational of vaccine development and design. The memory CD8+ T cells gathered rapidly to the lungs during secondary *M. tuberculosis* infection and became activated to an extent similar to that of CD4+ T cells[51]. Likewise, we observed recall CTL responses specific for these *M. tuberculosis*-derived peptides could be induced in BCG vaccinated subjects. This result suggests that CD8+ T cells may be involved in the control of TB in BCG vaccinated people.

The development of antigen-specific T cell staining using tetrameric or dimeric peptide/MHC complexs as well as intracellular cytokine staining has made it possible to visualize antigen-specific T cells[52, 53] Most of the previous studies with human cells have used tetramers for tumor or viral peptide epitopes[54-56]. Interestingly, higher frequencies of peptide-specific CD8+ T cells were seen by dimer staining when compared with frequencies for IFN-γ protecting CD8+ T cells. Detection of peptide-specific CD8+ T cells using such dimer complexes is not dependent on T cell function. Previous studies have also found higher frequencies of virus and tumor-specific CD8+ T cells by tetramer staining method than by limiting dilution or elispot assay. In this study, HLA-A*0201 dimer assays indicated that 10-50% of $PstA1_{75-83}$-specific CD8+ T cell population in PBMCs and PFMNCs could produce IFN-γ upon peptide stimulation (Fig 7 and Table 4), suggesting the presence of functional heterogeneity within the CD8+ T cell population in PBMCs and PFMNCs. It is unknown how phagosomally derived antigens interact with the MHC class I processing machinery, which primarily presents endogenously derived antigens, or those derived from pathogen which resides in the cytoplasm.

Earlier studies demonstrated that non-classically restricted T cells comprised the majority of *M. tuberculosis*-specific CD8+ T cells in two latently infected subjects[57]. In this study, processing of RpoB protein of *M. tuberculosis* for the presentation of antigenic peptides to MHC class I-restricted CD8+ T cells was insensitive to brefeldin A, but inhibited by anti-MHC class I blocking antibody and cytochalasin D (Fig 13). Therefore, the failure of brefeldin A to inhibit antigen presentation suggests that processing of RpoB peptide does not require Golgi-ER transport and thus the antigen presenting structure is not transported to the cell surface by the same pathway as conventional MHC class I molecules. But, the precise mechanism by which such presentation occurs is unclear. From other intracellular bacterial infections, and model systems using exogenous antigens such as ovalbumin, it appears that there is an alternative MHC class I processing pathway for phagosomal antigens[42]. The mechanism that *M. tuberculosis* protein in phagosomes is processed needs to be investigated furthermore to understand pathogenesis and immune responses of TB.

M. tuberculosis has evolved a number of mechanisms to invade and persist within macrophages, earlier studies established that *M. tuberculosis* can interfere with IFN-γ mediated activation and IFN-γR signaling in human macrophages[27]. To kill the intracellular *M. tuberculosis*, macrophage has to be activated by IFN-γ and TNF-α released by neighboring T cells. IFN-γ acts primarily through regulation of gene expression[58], and induces macrophages to kill intracellular pathogen, including *Toxoplama, Leishmania, Legionell*a, and *Chlamydia in vitro*[59, 60]. But IFN-γ cannot induce either monocyte-derived macrophages or alveolar macrophages to kill *M.*

tuberculosis in human *in vitro*[61, 62]. According to previous reports, the expression of IFN-γ inducible genes are down-regulated in macrophages infected with mycobacteria[27, 63, 64]. While antigenic peptide presentation by HLA-molecules is an important factor in the development of CD8+ T cell responses, proteasomal processing, TAP binding, and the T cell repertoire all play important roles in MHC class I-restricted CD8+ T cell-mediated immune protective mechanisms.

In this study, therefore, the regulation of IFN-γ induced gene expression in antigen presenting cells infected with *M. tuberculosis* was examined. In addition, macrophages have a superior phagocytic and degradative activity over DCs, whereas DCs are better equipped for T cell priming and stimulation due to abundant surface expression of MHC and costimulatory molecules. Therefore, we compared the effect of *M. tuberculosis* infection on the gene expression level between macrophages and DCs.

The mRNA expression of calnexin, calreticulin remained nearly unchanged after IFN-γ treatment, *M. tuberculosis* or BCG infection. As expected, the expression of IFN-γ inducible subunits, LMP2 and LMP10 were increased after IFN-γ treatment. The mRNA expression of TAP2 and LMP2 genes by IFN-γ induction was increased in a time dependent manner. Infection of both macrophages and DCs with *M. tuberculosis* inhibited the IFN-γ induction of LMP2, LMP10 and TAP2 gene expression, while increased the IFN-γ induction of TAP1 and LMP7 gene expression (Fig. 17, 19). But, the expression of constitutive subunits Y and Z of the proteasome was not changed by either *M. tuberculosis* infection or IFN-γ treatment (Fig 17, 19).

Consequently, *M. tuberculosis* regulated differently mRNA gene expression of TAP1 and TAP2 induced by IFN-γ treatment. *M. tuberculosis* regulated differently mRNA gene expression of LMP2, LMP7, LMP10 by IFN-γ induction as well. But infection of macrophages with BCG also inhibited the IFN-γ induction of TAP1, TAP2, LMP2, LMP7 and LMP10 gene expression (Fig. 20, 21). *M. tuberculosis* exerts gene selective inhibition of transcriptional responses to IFN-γ without inhibiting STAT1 function : while transcription of CD64 and class II transactivator were decreased, certain other IFN-γ-responsive genes either were unaffected or were increased by *M. tuberculosis*[65].

Thus, IFN-γ signaling in *M. tuberculosis* and BCG infected cells may be differently modulated depending on the type of both genes and host cells. In murine and human macrophages, *M. tuberculosis* or its 19-kDa lipoprotein inhibits IFN-γ stimulated MHC class II Ag presentation through TLR-2[66, 67]. Inhibition of IFN-γ induced CIITA by *M. tuberculosis* 19-kDa lipoprotein may allow *M. tuberculosis* to evade detection by CD4+ T cells. Therefore, the ability of *M. tuberculosis* to block macrophage responses to IFN-γ is likely to be an important feature by the bacteria in response to the development of cell-mediated immunity. Overcoming this blocking may allow the immune system to eliminate *M. tuberculosis* and may be valuable therapies for latent and active TB.

V. CONCLUSION

1. Among the Korean population, HLA-A typing results of healthy subjects showed HLA-A*0201, 30.3%; A*0206, 16.7%; A*0207, 13.3%, and A*0203, 3.3%. However, the HLA-A typing results for TB patients were HLA-A*0201, 26.2%; A*0206, 1.2%; A*0207, 8.3%, and A*0203, 1.2%.

2. In *ex vivo* IFN-γ elispot assay, four epitope peptide-specific T cell populations were observed in the PPD+ healthy subjects who expressed the HLA-A*0201, A*0206, or A*0207 subtypes. The IFN-γ specific SFCs (spot-forming cells), specific for any of these four epitopes, were almost undetectable in PPD- healthy subjects who expressed the HLA-A*0201, A*0206, or A*0207 subtype.

3. Upon peptide stimulation, these epitope-specific CD8+ T cell lines released IFN-γ, not IL-4, indicating that these cell lines are Tc1 type cells. These epitope-specific CD8+ T cell-mediated immune responses can be induced in subjects expressing HLA-A2 supertype molecules (A*0201, A*0203, A*0206, and*0207), as demonstrated by the IFN-γ intracellular staining method.

4. The frequencies of the CD8+ T cells specific for $ThyA_{30-38}$, $RpoB_{127-135,}$ or $85B_{15-23}$ peptides were higher in the PPD+ healthy subjects than in either of the PPD- healthy subjects or the severely progressed TB patients. There were no differences among the

four groups regarding CD8+ T cell response to the $PstA1_{75-83}$ peptide. In the mild-moderate TB patients, no significant difference in the CD8+ T cell frequency was observed when compared to the PPD+ healthy subjects, except in the $RpoB_{127-135}$-specific CD8+ T cells. The difference in frequencies of the CD8+ T cell population, specific for each peptide, may depend on the type of *M. tuberculosis* antigens.

5. Epitope ($PstA1_{75-83}$, $RpoB_{127-135}$, $ThyA_{30-38}$, $85B_{15-23}$)-specific CD8+ T cell-mediated immune responses can be induced in the PFMNCs of pleural TB patients expressing HLA-A2 supertype molecules (A*0201 and A*0206), as demonstrated by *ex-vivo* IFN-γ ELISPOT assay.

6. Recall CTL activities specific for $PstA1_{75-83}$, $RpoB_{127-135,}$ and $ThyA_{30-38}$ peptides were observed in healthy subjects expressing the HLA-A*0201 type. Moreover, $ThyA_{30-38}$ peptide-specific CD8+ T cell lines showed strong CTL activity in healthy subjects who expressed the HLA-A*0206 subtype.

7. $RpoB_{127-135}$ peptide-specific CD8+ T cell lines, generated from both the HLA-A*0201 and A*0206 subjects, showed the specific lysis of the *M. tuberculosis*-infected macrophages.

8. Processing of the RpoB (RNA polymerase β-subunit) somatic protein of *M. tuberculosis* for the presentation of the $RpoB_{127-135}$ peptide to MHC class I-restricted

CD8+ T cells was insensitive to brefeldin-A and lactacystin treatment, although the recognition of *M. tuberculosis*-infected cells by $RpoB_{127-135}$-specific CD8+ T cells was inhibited by anti-MHC class I blocking antibody and cytochalasin D. This result indicates that the *M. tuberculosis* $RpoB_{127-135}$ peptide may be processed by the alternative MHC class I- restricted antigen presentation pathway.

9. The kinetic study of the specific killing of *M. tuberculosis*-infected macrophages by $RpoB_{127-135}$-specific CD8+ T cells suggests that the RpoB protein is processed more efficiently when the infection period is increased.

10. This study demonstrates that the inhibition of transcribing IFN-γ responsive gene by *M. tuberculosis* and BCG differed, depending on the genes and cell types. While transcription of CD64 decreased, those of calnexin, calreticulin, and the x, y, z genes were unaffected. Infection of both macrophages and immature DCs with *M. tuberculosis* induced IFN-γ responses of TAP1 and LMP7 genes, but decreased IFN-γ induction of TAP2, LMP2, and LMP10 genes. In addition, infection of the macrophage with BCG inhibited IFN-γ induction of TAP1, TAP2, LMP2, LMP7, and LMP10 genes.

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ABSTRACT (in Korean)

사람의 결핵균감염에서 일어나는 CD8+ T 세포 면역반응과 항원제시

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조장은

결핵에 대한 보호면역반응은 세포매개면역반응으로 T 세포와 대식세포가 중요한 역할을 한다. 결핵에 대한 면역기전을 이해하기 위해 MHC class I 에 제한적인 CD8+ T 세포와 결핵항원의 처리과정에 대한 면역반응을 세가지 측면, 즉 결핵균에서 유래된 펩티드에 대한 CD8+ T 세포반응, 결핵균 균체 (somatic) 항원의 처리과정, 결핵균이 IFN-γ에 의해 유도되는 유전자에 미치는 영향에 대해 연구하였다. 첫번째로 $ThyA_{30-38}$, $RpoB_{127-135}$, $PstA1_{75-83}$, $85B_{15-23}$이 HLA-A*0201 에 제한적인 CD8+ T 세포에 특정한 결핵균에서 유도된 펩티드임이 이미 보고된바있다. 활동성 결핵환자와 PPD+ 인 사람에서 이 펩티드에 특정한 CD8+ T 세포의 특징을 알아보고자 하였다. 위의 펩티드는 HLA-A*0203, A*206, A*0207 type 을 가진 사람에서 CD8+ T 세포반응을 유도하였고, 따라서 항원결정기가 A2 supertype 펩티드임을 제시하였다. 펩티드에 대한 CD8+ T 세포 면역반응은 PPD+ 건강인, 폐결핵환자, 흉막성 결핵환자에서 유도되었다. 그러나 결핵 항원에 특정한 CD8+ T 세포 면역반응은 결핵이 심하게 진전된 환자

에서 감소되었다. 이러한 4 개의 펩티드중에서 많은 양의 IFN-γ의 분비를 유도하는 면역우세 (immunodominant) 펩티드는 사람마다 달랐다. 펩티드에 특정한 IFN-γ를 분비하는 단기세포주 (short term cell line)는 *in vitro* 에서 증식하였고, 항원자극 후 PPD+ 인 사람에서 IFN-γ를 분비하였다. 또한 HLA-A*0201 dimer assay 를 수행한 결과, PPD+ 건강인에서 $PstA1_{75-83}$ 에 특정한 CD8+ T 세포 집단의 1/2 에서 1/4 만이 IFN-γ를 생성함을 확인하였고, PPD+ 건강인에서 IFN-γ를 생성하는 $PstA1_{75-83}$ 에 특정한 CD8+ T 세포집단이 다양함을 알수있었다. 또한 BCG 백신을 맞은 사람에서 결핵균 유래펩티드에 특정한 기억독성면역 (recall CTL) 반응을 알아보았다. 이 결과는 CD8+ T 세포가 BCG 백신을 맞은 사람 들에서 결핵에 대한 보호면역반응을 유도할 수 있음을 제시한다.

두번째로 결핵균은 대식세포에서 살고 증식한다. 결핵균 단백의 RNA polymerase 로부터 유래한 $RpoB_{127-135}$ 펩티드에 특정한 면역반응을 매개하는 CD8+ T 세포는 결핵환자에서 유도되었다. 이는 CD8+ T 세포가 결핵균이 감염된 대식세포에서 처리된 $RpoB_{127-135}$ 를 인지하는지 알아보기 위해 $RpoB_{127-135}$ 펩티드에 특정한 CD8+ T 세포를 *in vitro* immunization 방법으로 정상인 HLA-A*0201 와 A*0206 type 을 가진 정상인의 말초혈액을 사용하여 만들었다. 이렇게 만들어진 CD8+ T 세포는 결핵균이 감염된 대식세포를 특이적으로 인지하여 파괴하였다. 또한 결핵균에서 유도된 $RpoB_{127-135}$ 펩티드를 CD8+ T 세포에 제시하는 기전은 brefeldin A 처리 후에 억제되지 않았다. 따라서 결핵균에서 유도된 $RpoB_{127-135}$ 펩티드는 결핵균의 세포질단백의 처리방법으로 제시된 alternative MHC class I 경로

로 처리되어 면역세포에 전달되고 인식된다고 사료된다. 결핵균의 RpoB 유전자가 대식세포안에서 잘표현되었고, 따라서 RpoB 단백질이나 RpoB 단백질에서 유도된 펩티드는 결핵백신개발에 유용할 것으로 기대된다.

마지막으로 IFN-γ는 대식세포를 활성화시키는 주된 매개자이고, 결핵균은 대식세포에서 IFN-γ에 의한 세균억제기전에 저항하거나 IFN-γ에 의한 대식세포의 활성화를 막을 수 있을 것이다. 이번 연구는 결핵균으로 감염된 대식세포와 수상돌기세포에서 IFN-γ에 반응하는 MHC class I 처리와 제시에 관여하는 유전자의 전사에 대한 억제와 유도가 유전자마다 다른것을 증명하였다. 따라서 다음 단계에서는 결핵균이 IFN-γ의 세포반응을 억제하는 기전을 이해하는 것이 필요하고, 이런 연구는 결핵에 대한 백신과 치료제 개발에 기여하리라 사료된다.

핵심되는 말 : 결핵균, CD8+ T 세포, 펩티드 항원 결정기, HLA-A2, IFN-γ, 대식세포, $RpoB_{127-135}$ 펩티드