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Brief Communication

RpoB$_{127-135}$ Peptide Derived from *Mycobacterium tuberculosis* is Processed and Presented to HLA-A*0201 Restricted CD8+ T Cells via an Alternate HLA-I Processing Pathway

Jang-Eun Cho[1], Sang-Nae Cho[2] and Sungae Cho[3,†]

[1]Department of Biomedical Laboratory Science, Daegu Health College, Daegu 702-722, Korea
[2]Department of Microbiology and [3]Institute of Immunology and Immunological Diseases, College of Medicine, Yonsei University, Seoul 120-752, Korea

Mycobacterium tuberculosis (MTB) resides and replicates inside macrophages. In our previous report, we reported that CD8+ T cell-mediated immune responses specific for the peptide derived from MTB RNA polymerase beta-subunit (RpoB$_{127-135}$) could be induced in TB patients expressing HLA-A*0201 subtype. In order to examine whether RpoB$_{127-135}$ specific CD8+ T cells can recognize MTB infected macrophages *in vitro*, CD8+ T cell lines specific for RpoB$_{127-135}$ peptide were generated from peripheral blood mononuclear cells (PBMCs) of healthy HLA-A*0201 subjects by *in vitro* immunization technique. In this study, we observed RpoB$_{127-135}$ specific CD8+ T cells could recognize and destroy macrophages infected with MTB for 2 to 4 days. RpoB$_{127-135}$ specific CD8+ T cell immune response was inducible from PBMC of healthy subjects expressing HLA-A*0206 subtype, one of HLA-A2 supertype members. Next, we investigated the HLA-I processing mechanism of RpoB$_{127-135}$ peptide in MTB infected macrophages. As a result, the presentation of the MTB derived epitope peptide, RpoB$_{127-135}$, to CD8+ T cells was not inhibited by the treatment with brefeldin-A (ER-Golgi transport inhibitor) or lactacystin (proteasome inhibitor), which blocks the classical HLA-I processing pathway. However, RpoB$_{127-135}$ specific CD8+ T cell activity was blocked either by the blocking agent for the endocytosis (cytochalasin D) or by the blocking antibody (W6/32) for HLA-I molecules. Therefore, the RpoB$_{127-135}$ peptide may be processed by accessing the alternate HLA-I processing pathway. Understanding the processing and presentation mechanisms of the MTB derived proteins will help to improve the efficacy of vaccines and the efficiency of therapeutic agents for TB.

Key Words: *Mycobacterium tuberculosis*, RpoB$_{127-135}$ peptide, CD8+ T cells, Macrophages, HLA-I processing pathway

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) was discovered by Robert Koch in 1882 (Koch R, 1882). MTB is a facultative intracellular pathogen and acid fast bacillus that replicates within human macrophages. It grows only slowly, reproducing itself every 17 to 18 hours under optimal conditions. In addition, MTB is often in a dormant state and usually produces a chronic disease which is reactivated many years after the initial infection. TB is a major global health problem, especially in developing countries (Wayne, 1982). The World Health Organization (WHO) estimates that one third of the world's population has been infected with MTB, and 9.3 million new TB cases and 1.3 million cases of TB-related deaths occurs globally each year (WHO, 2010).

CD8+ T cells have significant roles in the protective mechanism against MTB infection by releasing IFN-γ and

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†Corresponding author: Sungae Cho. Institute of Immunology and Immunological Diseases College of Medicine, Yonsei University Seoul 120-752, Korea.
Tel: +82-2-2228-2545, Fax: +82-2-392-9310
e-mail: sungaecho@gmail.com

by cytotoxicity. CD8+ T cell lines and clones can recognize MTB antigens *in vitro* and lyse MTB infected macrophages in an antigen-specific manner, and constrain the MTB growth in macrophages (Tascon RE et al., 1996; Turner J et al., 1996; Tan JS et al., 1997). TAP (transporter associated with antigen processing) or β_2m (β_2 microglobulin) deficient mice that do not express major histocompatibility complex (MHC)-I molecules succumb to MTB infection, implying that MHC-I restricted CD8+ T cells are essential in the immune responses against MTB infection (Sousa et al., 2000). However, the mechanism by which antigens from MTB gain access to the MHC-I-restricted presentation pathway has not been comprehended completely. Experimental evidence has shown that phagocytosed mycobacterium can be processed and presented in both cytosolic and non-cytosolic (vacuolar alternate) pathways for MHC-I restricted CD8+ T cells. In cytosolic processing pathway, mycobacterial antigens are processed by proteasome, transported into endoplasmic reticulum-Golgi (ER-Golgi) by TAP and loaded onto MHC-I molecules. This conventional pathway is both proteasome-dependent and ER-Golgi transport pathway-dependent (or TAP-dependent). On the other hand, MTB antigens can also be processed by the proteasome-dependent pathway, but which is not transported through the ER-Golgi pathway (TAP-independent) (Harriff et al., 2012). In non-cytosolic pathway, the mycobacterial proteins such as 19 kD protein and particulate antigens can be processed to combine with MHC-I molecules inside phagosome (Chefalo et al., 2003; Tobian et al., 2003).

Electron microscopic studies on intracellular localization of MTB have suggested that MTB do not need to escape to the cytosol of the infected macrophages for the cytosolic MHC-I processing pathway (Hariff et al., 2012). MTB may have developed its own machinery to release antigenic substances into the cytosol of macrophage which can be sensed by the host immune system. Many factors such as nature of MTB antigens, infectivity and type of infected cells seem to control the MHC-I processing pathway of intracellular MTB. For instances, previous data suggests that MTB soluble or secreted antigens are processed by the cytosolic pathway (Grotzke et al., 2010). Therefore, to further understand the mechanism of mycobacterial antigen processing pathway for MHC-I presentation, we aimed to examine the processing pathway of the $RpoB_{127-135}$ peptide derived from RNA polymerase subunit B (RpoB) since RpoB is highly expressed in infected macrophages (Mariani et al., 2000) and its epitope for HLA-A*0201 restricted CD8+ T cells was defined in our previous study (Cho et al., 2000).

To begin the experiment, a HLA-A*0201-restricted CD8+ T cell-specific epitope peptide was synthesized at the Korea Basic Science Institute (Seoul, Korea). Sequences were confirmed by mass spectrometry analysis. Sequences of $RpoB_{127-135}$ used for the assays is MTYAAPLFV (IC50 = 13.8 nM), derived from RNA polymerase-subunit B. Study subjects expressing HLA-A2 type were selected 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). Only two different HLA-A*02 alleles (A*0201 and 0206) were used in this study. As a target cell line for CD8+ T cells derived from HLA-A*0201 subtype, .221A2 cell line (Cho et al., 2000) was used, which is an Epstein-Barr virus (EBV)-transfected B-cell line mutagenized and selected for loss of HLA antigens, then transfected with HLA-A*0201. To generate a target cell line for CD8+ T cells derived from HLA*0206 subtype, B lymphoblastoid cell line (B-LCL) was generated by transforming 5×10^5 peripheral blood mononuclear cells (PBMCs) from healthy subjects with EBV-containing supernatants from B-95.8 cell line. PBMCs from heparinized venous blood were isolated by density gradient centrifugation over Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden).

To demonstrate that CD8+ T cell responses specific for MTB derived epitope peptide, $RpoB_{127-135}$, can be induced in healthy subjects expressing HLA-A*0201 and A*0206 subtype, *in vitro* CD8+ CTL induction experiments were performed. PBMCs from HLA-A*0201 and HLA-A*0206 healthy subjects were pulsed with 50 μg/mL of $RpoB_{127-135}$ 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/mL) and keyhole limpet hemocyanin (5 μg/mL, 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/ mL every 3~4 days (Cho et al., 2000). 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. As shown in Fig. 1, CD8+ T cell lines specific for $RpoB_{127-135}$ were generated, showing cytotoxic activity for $RpoB_{127-135}$ pulsed target cells.

Next, we performed kinetic studies of cytotoxic activity

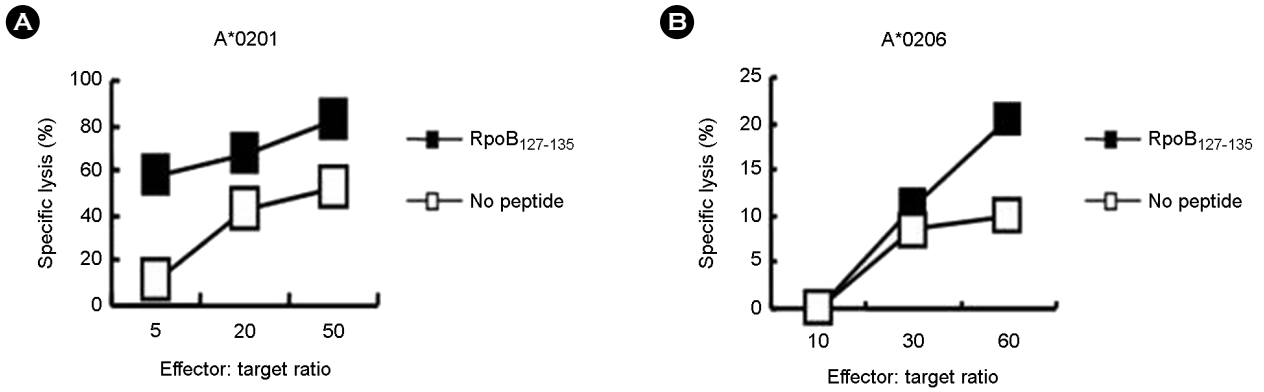

Fig. 1. Generation of CTL lines from healthy HLA-A2 subjects by *in vitro* immunization PBMCs from HLA-A* 0201 (A) and HLA-A*0206 (B) subjects were pulsed with 50 μg/ml of peptide at 3×10^6 cells/well in 10% pooled human serum with rhIL-7 (10 ng/ml) and keyhole limpet hemocyanin (5 μg/ml). Cultures were re-stimulated weekly with peptide-pulsed and irradiated autologous monocytes and cell culture was supplemented with rhIL-2 at 10 units/ml 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). Specific lysis (%) was calculated by using the formula 100 × (experimental release-spontaneous release)/ (maximal release-spontaneous release). Maximal release was determined from supernatants of target cells lysed by the addition of 1% Triton X-100. Spontaneous release was determined from supernatants of target cells incubated with media only. For cytotoxicity experiments, 1×10^6 target cells 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 $5\sim7 \times 10^3$ cells/well. CD8+ T cell lines were added at various effector to target ratios.

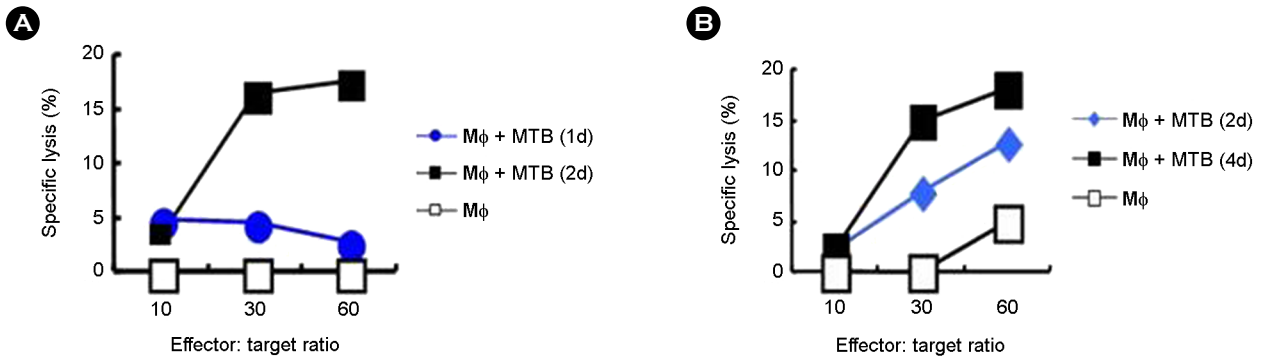

Fig. 2. Kinetic studies of MTB $RpoB_{127-135}$ peptide processing inside macrophages. Macrophages were generated by culturing adherent monocytes in RPMI 1640 with 10% FBS for 3~4 days. These macrophages were then infected with MTB (H37Rv) at a multiplicity of infection (moi) of 3 for 1~2 days (A) or 2~4 days (B). Extracellular nonphagocytosed MTB was removed by washing. For cytotoxicity experiments, 1×10^6 macrophages were labeled with 100 μCi 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 generated from PBMC of HLA-A*0201 subjects were added at various effector to target ratios. Specific lysis (%) was determined as described above.

of $RpoB_{127-135}$ specific CD8+ T cell lines for MTB 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 MTB (H37Rv, ATCC 27294) at multiplicity of infection (MOI) 3 for 4 h. After culturing for 1~4 days, the infected cells were used as targets in CTL assays. Infectivity of macrophages was more than 70% when measured by Ziehl-Neelsen method (Data is not shown). As shown in Fig. 2, $RpoB_{127-135}$ specific CD8+ T cell lines generated from both HLA-A*0201 and A*0206 subjects showed the specific lysis for MTB infected macrophages. This result indicates that MTB $RpoB_{127-135}$ peptide is processed and presented from intracellular MTB to these CD8+ T cells. In addition, RpoB protein was processed and presented more efficiently when the infection period was increased longer (Fig. 2).

Lastly, to examine the processing pathway of $RpoB_{127-135}$ peptide, macrophages were pretreated with metabolic inhibitors (brefeldin A 3 μg/mL, Sigma, St. Louis, MO, USA), cytochalasin D (10 μg/mL, Sigma) or lactacystin (40 μM, Sigma) one hour before the infection with MTB. After 18 h of co-incubation with MTB, macrophages were used as target cells for CTL assays. Processing of RpoB protein for the presentation of $RpoB_{127-135}$ to HLA-I restricted CD8+ T cells was insensitive to brefeldin A (ER-Golgi transport inhibitor) and lactacystin (proteasome inhibitor) treatment. Conversely, the recognition of MTB infected cells by $RpoB_{127-135}$ specific CD8+ T cells was inhibited by anti-HLA class I blocking antibody (W6/32) or cytochalasin D (phagocytosis inhibitor) (Fig. 3). Therefore, this data imply that the processing of $RpoB_{127-135}$ requires phagocytosis of MTB and the peptide is presented as a HLA-I restricted manner. However, the processing was not inhibited by lactacystin, suggesting that RpoB antigen may not be processed by proteasomal degradation (Fig. 3C). In addition,

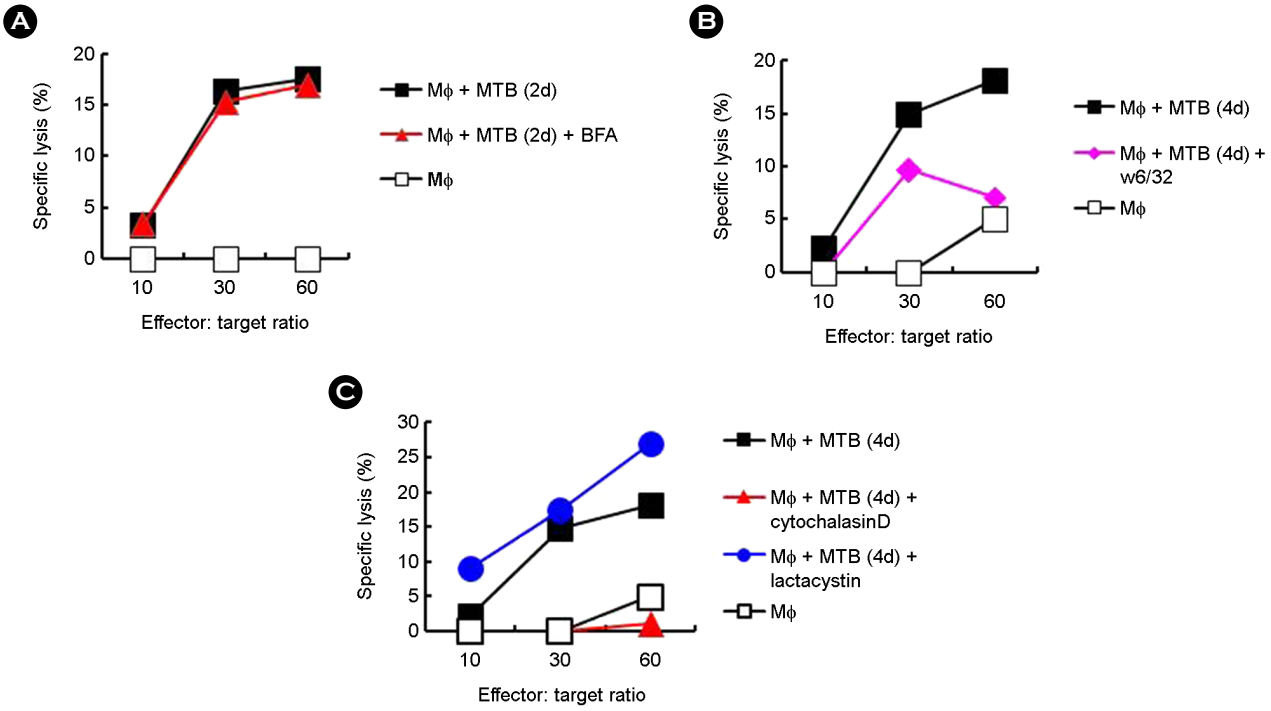

Fig. 3. Effect of metabolic inhibitors on the presentation of MTB-derived $RpoB_{127-135}$ peptide. CTL lines from healthy HLA-A*0201 subjects by *in vitro* immunization were generated. $RpoB_{127-135}$ specific CD8+ CTL lines were stimulated with macrophages that had been pre-incubated with metabolic inhibitors, brefeldin A (Golgi-ER transport; 3 μg/ml), lactacystin (proteasome, 40 μM) or cytochalasin D (phagocytosis; 10 μg/ml) for 1 h before the addition of MTB (A & C). Anti-MHC class I blocking antibody (W6/32, 10 μg/ml) was added to target cells 1 h before CTL assay (B).

the absence of inhibition by brefeldin A demonstrated that the antigen processing may bypass the ER-Golgi pathway (Fig. 3A). The failure of brefeldin A to inhibit antigen presentation suggests that processing of RpoB peptide does not require ER-Golgi transport and thus the antigen presenting structure is not transported to the cell surface by conventional cytosolic MHC-I processing pathway. This data suggests that RpoB antigen may be processed by alternate MHC-I processing pathway even though the precise mechanism by which such presentation occurs is yet to be investigated.

MTB has evolved a number of mechanisms to invade and persist within macrophages. While antigenic peptide presentation by MHC 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-I restricted CD8+ T cell-mediated immune protective mechanisms. It was reported that MTB derived antigens can gain access to MHC-I processing pathway via either cytosolic or vacuolar pathway (Tobian et al., 2004). Even though recent findings have shown that intracellular MTB antigens are mainly processed by cytosolic pathway (proteasome dependent and TAP dependent) for MHC-I restricted antigen presentation, there have been reports showing that MTB antigens can be processed and presented diversely for MHC-I presentation pathway (Grotzke et al., 2010). The apoptotic blebs generated by intracellular MTB can be engulfed by surrounding macrophages or dendritic cells, and presented to CD8+ T cells by the acidification dependent but proteasome independent processing pathway (Schaible et al., 2003). In addition, earlier studies demonstrated that non-classically restricted T cells comprised the majority of MTB specific CD8+ T cells in two latently infected subjects (Heinzel et al., 2002). Other intracellular bacterial infections and experiments using exogenous antigens such as ovalbumin have shown that an alternate MHC-I processing pathway can be used for phagosomal antigens (Mazzaccaro et al., 1998).

In conclusion, we showed that one of soluble MTB proteins, RNA polymerase subunit B (RpoB), may be processed by alternate MHC-I processing pathway and recognized by cytotoxic CD8+ T cells. Since RpoB is a big protein (129 kDa) and the RNA polymerase core protein composed of five subunits (2α, β, β' and ω) is estimated as about 400 kDa, RpoB can be processed as a particulate antigen in phagosome (Borukhov and Nudler, 2003 and Banerjee et al., 2014). The mechanism that MTB protein in phagosome is processed by TAP-independent and ER-Golgi independent pathway needs to be investigated furthermore to understand CD8+ T cell mediated immune responses for MTB infection and to develop protein vaccines for TB.

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