의학석사 학위논문

# Recombinant *Mycobacterium smegmatis* transfected with a pMyong2 vector expressing Human Immunodeficiency Virus Type I Gag can induce enhanced virus-specific immune responses in mice

# pMyong2 벡터 시스템을 이용한 Human Immunodeficiency Virus Type I Gag 단백 발현 재조합 *Mycobacterium smegmatis*의 면역 증진 효과

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A thesis of the Degree of Master

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# ABSTRACT

Recently, number of patient co-infected HIV-1 and TB is increased. The circumstance has pushed novel medicine for treating both HIV-1 and TB at one go. In the situation, a novel *Mycobacterium-Escherichia coli* shuttle vector system was developed using pMyong2, a linear plasmid from *Mycobacterium yongonense*. The novel vector system provides an enhanced expression of heterologous genes in recombinant *Mycobacterium smegmatis* (rSmeg), compared to the pAL5000 derived vector system. To investigate the usefulness of rSmeg using pMyong2 in vaccine application, I transformed *M. smegmatis* with pMyong2 system expressing Human Immunodeficiency Virus Type I (HIV-1) Gag p24 antigen (rSmeg-pMyong2-p24) and examined its cellular and humoral immune responses against HIV-1 gag protein.

I found that rSmeg-pMyong2-p24 expressed higher levels of Gag protein in infected macrophage cell line (J774A.1) and mouse bone marrow derived dendritic cells (BMDCs) compared to rSmeg strains using two other vector systems, pAL5000 derived vector (rSmeg-pAL5000-p24) and the integrative plasmid, pMV306 (rSmeg-pMV306-p24). Immunization of mice with rSmeg-pMyong2-p24 elicited more effective immunity, compared to the other

two rSmeg strains, as evidenced by higher levels of HIV-1 Gag-specific CD4 and CD8 T lymphocyte proliferation, interferon gamma ELISPOT cell induction, and antibody production. Furthermore, rSmeg-pMyong2-p24 showed a higher level of cytotoxic T cell response against target cells expressing Gag p24 proteins. My data suggest that *Mycobacterium-Escherichia coli* shuttle vector system with pMyong2 may provide an advantage in vaccine application of rSmeg over other vector systems.

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Keywords : pMyong-2, Mycobacterium, Mycobacterium yongonense, Human immunodeficiency virus, recombinant mycobacterium vaccine
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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AEC | 3-amino-9-ethylcarbazole |
| BM | Bone Marrow |
| BMDCs | Bone Marrow derived Dendritic Cells |
| CFSE | Carboxylfluorescein Succinimidyl Ester |
| CTL | Cytotoxic T Lymphocyte |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FBS | Fetal Bovine Serum |
| HAART | Highly Active Antiretroviral Treatment |
| HIV-1 | Human Immunodeficiency Virus type 1 |
| HSP65 | Heat Shock Protein 65 gene |
| IPTG | Isopropyl β-D-Thiogalactoside |
| MHC | Major Histocompatibility Complex |
| MLR | Mixed Lymphocyte Response |
| M.O.I | Multiplicity Of Infection |
| NTM | Non Tuberculosis Mycobacteria |
| OADC | Oleate-Albumin-Dextros e-Catalse |
| PBST | PBS containing 0.05% Tween-20 |
| PMA | Phorbol 12-Myristate 13-Acetate |
| rSmeg | recombinant *M. smegmatis* |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SFUs | Spot Forming Units |
| S.C. | Subcutaneously |
| TB | Tuberculosis |
| TNF-α | Tumor Necrosis Factor-α |
| WT | Wild Type |

# INTRODUCTION

The emergence of human immunodeficiency virus type 1 (HIV-1) has aggravated pervasive cases of tuberculosis (TB) worldwide. TB makes people to impair to easy infection with all phases of HIV and is leading killer of HIV positive patients. It's widely accepted that HIV causes a depletion of CD4 T cells, which is likely to contribute to the susceptibility of co-infection persons to TB, as the CD4 T cell subset is also important to cure of TB. As result depletion of CD4 T cell cannot down-regulate CD8 T cell response. To overcome the condition and to develop more effective human immunodeficiency virus Type 1 (HIV-1), I think to make a tool to elicit virus-specific neutralizing antibodies and cytotoxic T lymphocyte (CTL) responses. Although an immunogen that induces antibodies that can neutralize diverse HIV type 1 (HIV-1) isolates has not yet been defined, a number of strategies including plasmid DNA vaccine, adenovirus serotype 5, and pox-vectored vaccine are being developed for generating HIV-1-specific CTL [1]. However, there are problems associated with each of these approaches with regard to eliciting CTL, which could limit their practical uses.

*Mycobacterium* have features that make them attractive as potential HIV-1

vaccine vectors. *Mycobacterium bovis* BCG (BCG), currently the most widely administered vaccine in the world, is a live attenuated vaccine used to protect against tuberculosis and leprosy [2-5]. It demonstrates excellent adjuvant properties, induces long lasting immunity and has a low production cost [6-8]. It also has many properties that make it one of the most attractive live vectors for the development of recombinant vaccines in murine models against various infectious agents, including *Borrelia burgdorferi*, *Streptococcus pneumoniae, Bordetella pertussis*, rodent malaria, leishmania, and measles virus [9-14]. *Mycobacterium smegmatis,* a non-pathogenic member of the genus *Mycobacterium*, grows rapidly and can be transformed effectively with many genes in vitro [15]. These properties make this bacterium an ideal vaccine vector [16-19]. It has been reported that recombinant *M. smegmatis* (rSmeg) engineered to express human immunodeficiency virus type 1 (HIV-1) Env elicits HIV-1 envelope-specific CD8 T-cell responses [16]. Unlike other mycobacterial species, such as BCG that can survive in host cells by inhibiting phagosome maturation, *M. smegmatis* is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells [20, 21], facilitating the rapid uptake of expressed antigens in bacteria and cross-presentation of antigen into T cells. Furthermore, *M. smegmatis* can induce cytokine production by macrophages

better than pathogenic mycobacterial species and can activate and induce the maturation of dendritic cells better than BCG by the upregulation of major histocompatibility complex (MHC) class I and costimulatory molecules [22-24]. *M. smegmatis* can also access the MHC class I pathway for presentation of mycobacterial antigens more efficiently than BCG, suggesting it has an advantage in inducing CTL response, which is necessary in HIV vaccine [25, 26].

Despite the intrinsic trait of mycobacteria in inducing CTL response, there is one pitfall in the application of recombinant mycobacteria into vaccine application, which is the lack of stability and the levels of heterologous expression of a foreign gene. Thus, there is an urgent need for the development of a novel *Mycobacterium-Escherichia coli* shuttle vector system which can improve upon conventional systems. Recently, Lee et al have introduced a novel *Mycobacterium-Escherichia coli* shuttle vector system using pMyong2, a linear plasmid of the slowly growing *Mycobacterium yongonense* DSM 45126$^{T}$ strain. Of note, Lee et al found that rSmeg with the pMyong2 vector system increased the a copy number of human macrophage migration inhibitory factor (hMIF) gene approximately 37-fold and increased the protein expression of hMIF approximately 50-fold compared to rSmeg with the pAL5000 vector system, the

most widely used vector for heterologous expression of foreign genes in mycobacteria, demonstrating the potential utility of the pMyong2 vector system in heterologous gene expression in rSmeg [27].

The aim of the present study is to investigate the usefulness of rSmeg with pMyong2 in HIV vaccine application. To this end, I constructed the rSmeg with pMyong2 system expressing HIV-1 p24 Gag antigen (rSmeg-pMyong2-p24) and examined its cellular and humoral immune responses against HIV Gag proteins in vaccinated mice compared with rSmeg strains transfected with 2 other vector systems, an episomal plasmid, pAL5000 derived vector (rSmeg-pAL 5000-p24) and an integrative plasmid, pMV306 (rSmeg-pMV306-p24).

# MATERIALS AND METHODS

## 1. Cell culture and Transfection

Cells of the mus musculus mastocytoma line p815 (H-$2^d$) were used for target cell of CTL assay. The cells were grown in DMEM (biowest) supplemented with penicillin streptomycin, and 10% bovine serum; and maintained in air with 5% $CO_2$ at 37℃. Twenty-four hours prior to transfection, the cells were plated at $4\times10^5$ in 6-well plates. Opti-MEM (Gibco BRL) was used for starvation and incubation, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect plasmid DNA.

## 2. Mice and immunization procedures

I purchased female BALB/c and C57BL/6 mice (~25g, 7 weeks old) from Orient-Bio (Seoul, Korea) and used for experiments at 8 weeks of age. Mice were randomly divided into four groups with five mice in each group. I immunized Mice subcutaneously with wild type and recombinant *M. smegmatis* strains (rSmeg-pMV306-p24, rSmeg-pAL5000-p24, and rSmeg-pMyong2-p24) twice at 2-week intervals at the bottom of tail. Two weeks after the final

immunization, mice were euthanized by $CO_2$ inhalation and their spleens were removed and used for immunological assays. Animal studies were approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Resources, Seoul National University (SNU-160118-2-1).

### 3. Construction of *Mycobacterium-E. coli* shuttle vectors for expression of human immunodeficiency virus Type Ⅰ (HIV-Ⅰ) p24 antigen Gag

To generate *Mycobacterium-Escherichia coli* shuttle vectors expressing of Human Immunodeficiency Virus Type I (HIV-1) Gag p24 antigen (p24), the heat shock protein 65 gene (*hsp65*) promoter region and DNA sequences encoding Gag p24 antigen were amplified by overlapping PCR of *M. bovis* BCG genomic DNA and pNL4-3-deltaE-EGFP vector (NIH AIDS Reagent Program, Germantown, MD, USA) respectively. [42]

The forward primer sequence for *hsp65* promoter of *M. bovis* BCG and reverse primer sequence were used for polymerase chain reaction (PCR). The forward primer sequence and reverse primer sequence for p24 gene for PCR to amplified p24 gene. Two PCR products were conjugated by overlapping PCR using the *hsp65* promoter forward primer and p24 gene reverse primer. The

overlapping sequence (phsp-p24) was digested with *KpnI* and *XabI* (NEB, Ipswich, MA, USA) and cloned into *Mycobacterium-E. coli* shuttle vector pMV306 [28] with T4 ligase (TaKaRa, Kyoto, Japan). The pMV306-p24 construct was also used as a template for amplification of phsp-p24 to clone into pAL5000-TOPO and pMyong2-TOPO vectors [27]. The sequence of phsp-p24 was amplified again from the pMV306-p24. PCR product was also digested with *EcoRV* and *XbaI*, and cloned into the plasmids, pAL5000 and pMyong2-TOPO. The studies using recombinant *M. smegmatis* strains were approved by the Seoul National University Institutional Biosafety Committee. (SNUIBC-p151223-1)

| Name | direction | Sequence |
|---|---|---|
| *hsp*65 promoter | Forward | 5’-TTGGTACCGGTGACCACAACGACGCGC-3’ *(kpn1)* |
| | Reverse | 5’-CTGCACTATAGGCATTGCGAAGTGATTCCT -3’ |
| p24 | Forward | 5’-AGGAATCACTTCGCAATGCCTATAGTGCAG -3’. |
| | Reverse | 5’-AATCTAGACTACAAAACTCTTGCCTTATGGC CAGG-3’*(Xba1)* |
| phsp-p24 | Forward | 5’-TTGATATCGGTGACCACAACGACGCGC-3’(*Eco RV*) |
| | Reverse | 5’-AATCTAGACTACAAAACTCTTGCCTTATGGC CAGG-3’ *(EcoRV)* |

Table 1. PCR primer set

## 4. Production of recombinant Ag85B and p24 proteins from *E. coli*

Recombinant Ag85B and p24 proteins were purified from *E. coli* as previously described [43] with minor modification. For the expression and purification of fusion protein, *E. coli* BL21 strain (RBC Bioscience, Taipei City, Taiwan) was transformed with pET23a-Ag85B or pET23a-p24. Bacteria were grown in LB broth containing ampicillin (100 μg/ml; Duchefa Biochemia, Haarlem, The Netherlands). Protein expression was induced by adding 0.4 mM isopropyl β-D-thiogalactoside (IPTG, Duchefa Biochemie). Bacterial cells were harvested and disrupted by sonication on ice for 10 min. Sonicated lysates were centrifuged at 1600×g for 20 min at 4°C, and the pellets containing Ag85B and p24 proteins were resuspended in binding buffer containing 4M urea (Sigma Aldrich), sonicated and centrifuged at 1600×g for 30 min. The proteins were purified using Ni-NTA His binding resin (Novagen), and eluted with elution buffer (300mM NaCl, 50mM sodium phosphate buffer, 250mM imidazole) containing 4M urea. Purified proteins were dialyzed serially against the elution buffer to remove imidazole, urea and residual salts.

### 5. Generation of rSmeg strains expressing HIV-1 p24 Gag protein

To generate three different types of rSmeg strains expressing HIV-1 p24 Gag, rSmeg with pMyong2-p24 plasmid (designated as rSmeg-pMyong2-p24), rSmeg with pAL5000-p24 plasmid (rSmeg-pAL5000-p24), and rSmeg with pMV306-p24 plasmid (rSmeg-pMV306-p24), the three constructed plasmids,

pMV306-p24, pAL5000-p24 and pMyong2-p24 were electroporated into competent *M. smegmatis* $mc^2$ 155 strain using the Gene Pulser II electroporation apparatus (Bio-Rad, Hercules, CA, USA) [44]. Transformants were selected on Middlebrook 7H10 medium (Difco Laboratories, Detroit, MI, USA) supplemented with OADC containing 100 μg/ml of kanamycin. Typically, the selected colonies of transformants from the plates were transferred into 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% glycerol, 0.05% Tween-80, 10% ADC and kanamycin were cultured for 3days. The growth of rSmeg strains was determined by optical density (OD) at 600 nm.

**6. Determination of the p24 Gag expression levels from rSmeg strains**

To determine the p24 Gag expression levels of the rSmeg strains, I performed Western blot and enzyme-linked immunosorbent assay (ELISA) analysis to quantify p24 concentration. Total proteins from each recombinant *M. smegmatis* were prepared as follows. First, the pellets of cultured recombinant *M. smegmatis* were suspended in B-PER buffer (Thermo Scientific, Rockford, IL, USA) supplemented with lysozyme (100μg/ml), DNase (5U/ml) and proteinase inhibitor. The suspensions were sonicated for 5 min (pulse: 0.3sec, stop: 0.7sec) on ice and centrifuged at 13,000 rpm, 4°C for 15min. Aqueous phage protein samples were quantified by Quick Start™ Bradford Protein Assay (Bio-Rad, USA). The same amount of proteins in the aqueous phase was used for Western blot and ELISA analysis. Protein samples from recombinant *M. smegmatis* strains were mixed with sample buffer and boiled at 100℃ for

5min. The samples were separated by electrophoresis on 12% SDS-PAGE gels and transferred to nitrocellulose (NC) membranes. The membranes were blocked with 5% skim milk in TBST for 1 hr at room temperature. Mouse anti-p24 monoclonal antibody (Abcam, Cambridge, USA; 1:1,000 dilution) was added and incubated overnight at 4°C. After incubation, the membranes were treated with the HRP-conjugated goat anti-mouse secondary antibody (Abcam, Cambridge, USA; 1:2,000 dilution) for 1hr at room temperature. After each step, the membrane was washed with TBST (0.05% Tween-20). The immune-reactive signals were detected using a WEST-one™ Western blot Detection System (iNtRON, Kyungkido, Republic of Korea) with LAS-3000 (Fujifilm, Tokyo, Japan). The same amount of proteins was used for detection of p24 levels with the p24 ELISA kit (ABL, Rockville, USA) according the manufacturer's instructions [45].

**7. Generation of bone marrow derived dendritic cell (BMDC) from mice**

Bone marrow derived dendritic cells (BMDCs) were generated from the bone marrow (BM) of 6- to 12-week-old BALB/c and C57BL/6 mice as previously described [46]. Briefly, the BM cells were flushed out of the femurs and tibias with serum-free Iscove's modified Eagle medium (IMDM; Gibco Invitrogen, UK). The single cell suspension was filtered through a nylon cell strainer (70 $\mu$m Nylon mesh; SPL, Korea), washed twice with complete IMDM supplemented with 10% FBS (Gibco Invitrogen, UK), recombinant mouse GM-CSF (1.5 ng/ml, PeproTech, Rocky Hill, NJ, USA) and mouse IL-4 (1.5ng/ml;

PeproTech, USA), penicillin (100 units/ml; Gibco Invitrogen, UK), streptomycin (100 $\mu$g/ml; Gibco Invitrogen, UK), gentamicin (50 $\mu$g/ml; Gibco Invitrogen, UK), L-glutamine (2mM; Gibco Invitrogen, UK), and $\beta$-mercaptoethanol (50 nM; Gibco Invitrogen, UK), and seeded at a concentration of $1 \times 10^6$ cells per well in a 24-well plate in final volume of 2ml of complete IMDM. Half of the medium was replaced every other day with an equal volume of complete IMDM for 6days. The immature BMDCs generated were infected with wild-type Smeg or the three different rSmeg strains expressing p24 or induced to mature by treating the cells with 1 $\mu$g/ml lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, MO, USA) for 18 hours.

**8. Determination of the p24 Gag expression levels from BMDCs and J774A.1 cell infected with rSmeg strains**

The murine macrophage cell line, J774A.1 cell (American Type Culture Collection, ATCC TIB-67) was maintained at 37°C and 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific, Rockford, IL, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, and essential amino acids. BMDCs were generated from mouse bone marrow as described above. For rSmeg strains infection, J774A.1 cell and BMDCs were seeded $5\sim10\times10^5$cells per well (24-well plate) and cultured for 18hr. The cells were infected with the three different rSmeg strains at a multiplicity of infection (M.O.I.) of 10. The macropages were incubated for 2hr to allow phagocytosis of the bacteria, and the extracellular bacteria were removed by

washing with PBS three times. Infected J774A.1 cells and BMDCs were incubated for 24hr. To analysis p24 expression in cells, total proteins of cell pellets were prepared by suspension in RIPA lysis buffer and used to determine levels of p24 using the p24 ELISA kit.

**9. CFUs assay from BMDCs and J774A.1 infected with wild type *M. smegmatis* and rSmeg strains.**

Infected BMDCs and J774A.1 were prepared as described above. and the extracellular bacteria were removed by washing with PBS three times. Infected J774A.1 cells and BMDCs were incubated for 24hr. Infected cells were lysated for CFU plating using lysis buffer (0.1% Tween 80, w/v in $H_2O$) for 5min. Lysates were 1:10 serial dilution and plate out of chosed diltions on 7H10 agar plate as described above. At 3days, remove the plates from incubator and count colonies. CFU calculation are done.

**10. p24 Specific CD4 or CD8 T cell proliferation assay**

To conduct T cell proliferation assay, CD4 and CD8 T cells from p24 protein immunized C57BL/6 mice and BMDCs infected with wild type and rSmeg strains were used. First, mice were injected intravenously with p24 protein. After 7 days, spleens were isolated from the p24 injected mice and homogenized into single cells. Single cells were washed with ice-cold FACS

buffer (PBS containing 1% bovine serum albumin (BSA) and 1mM EDTA) and blocked on ice for 30min with super block solution containing 10% rat sera (Sigma Aldrich), 10% goat sera (Gibco Invitrogen), 10% mouse sera (Sigma Aldrich), and 2.4G2 monoclonal antibody (10ug/ml; BD Pharmingen). The cells were subsequently stained with BV421-conjugated anti-CD4 (Clone GK1.5, BD Horizon) and PE-conjugated anti-CD8a (Clone 53-6.7, eBioscience) for 30 min at 4°C and washed three times with ice-cold FACS buffer. FACS AriaIII instrument (BD Biosciences) was used to sort CD4 and CD8 T cell populations. Naive BMDCs were also infected with the wild- type or the three rSmeg strains (rSmeg pMV306-p24, pAL5000-p24 and pMyong2-p24) at an MOI of 10 for 24 hours. Infected cells were washed twice with PBS, and resuspended in complete IMDM ($4 \times 10^5$ cells/100 $\mu$l). Proliferation assays were conducted using the fluorescent cytoplasmic tracking dye Carboxylfluorescein succinimidyl ester (CFSE) (Invitrogen, UK) as previously described [29]. Sorted CD4 and CD8 T cells were stained with CFSE 5 $\mu$M for 4min at 37°C and for 4min in ice. Labeled cells were washed twice and resuspended in complete IMDM ($4 \times 10^5$ cells/100 $\mu$l). Labeled T cells and infected BMDCs were co-cultured for 4 days. Co-cultured cells were blocked on ice for 30min with super block solution and stained with CD4 BV421-conjugated anti-CD4 (Clone GK1.5, BD Horizon) and PE-conjugated anti-CD8a (Clone 53-6.7, eBioscience) for 30 min at 4 °C. Thereafter, cells were washed three times with ice-cold FACS buffer. The cell cycle profiles were determined using FACS LSRFortessa (BD Biosciences), and analyzed using Flowjo software. All the experiments were conducted in triplicate.

## 11. Interleukin-2 (IL-2) enzyme-linked immunosorbent assay (ELISA) assay

The amounts of murine IL-2 released in the supernatants of the above co-cultured T cell proliferation assay were also determined by ELISA according to the manufacturer's instructions (BioLegend, USA). Briefly, co-culture supernatants were analyzed immediately after removing cell debris by centrifugation at 500×g for 5min at 4°C. Supernatants were placed into 96-well microtiter plates, previously coated with the appropriate mAb according to manufacturer's directions. After incubation and washing, a second biotinylated Ab was added, followed by avidin peroxidase or streptavidin peroxidase and substrate (hydrogen peroxidase and chromogen) (Genzyme). A NanoQuant Infinite M200 reader (TECAN, Männedorf, Switzerland) was used for measurement.

## 12. Enzyme-linked Immunospot (ELISPOT) assay

Splenocytes from mice immunized with wild- type and rSmeg strains were used to conduct ELISPOT assay as previously described [47]. In brief, 96- well PVDF membrane ELISPOT plates were coated with mouse IFN-γ (3 μg/ml, clone AN-18) capture antibody (BD-Biosciences, San Diego, CA, USA) in PBS and incubated overnight at 4°C. After discarding the capture antibody, the plates were washed with PBS containing 0.05% Tween-20 (PBST) and PBS (3 times each), and the plates were blocked with 200μl of RPMI 1640 medium with 10% FBS for 3hours at 37°C. After blocking, $5\times10^5$ splenocytes from

vaccinated mice were loaded into each well. For each treatment group, cells were stimulated in triplicate with 5 μg/ml of purified p24 antigen or medium alone in a total volume of 200 μl. The plate was incubated at 37°C for 24 hours. As a positive control, cells were stimulated with 5 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, USA) and 500 ng/ml of ionomycin (Sigma-Aldrich). After washing with PBST and PBS (3 times each), biotin-labeled mouse IFN-γ (3 μg/ml, clone XMG1.2) detection antibody (BD-Biosciences) was added to each well and the plates were incubated overnight at 4 °C. The wells were washed again and horseradish peroxidase (HRP)-conjugated streptavidin was added to each well. The HRP reaction was developed with 3-amino-9-ethylcarbazole (AEC) substrate reagent (BD Biosciences). The number of spot forming units (SFUs) per well was counted automatically using an ELISPOT reader (AID ELISPOT Reader, Strassberg, Germany).

**13. Determination cytokine production from mice immunized with rSmeg strains loaded**

Splenocytes from immunized mice were adjusted to a concentration of $1\times10^6$ cells/well (96 well microplate, 200μl volume) in RPMI 1640 medium with 10 % FBS and purified p24 protein was added at a concentration of 5μg/ml for in vitro stimulation. The cells were cultured and the supernatants were harvested for determination of IL-2 (BioLegend, San Diego, CA, USA), TNF-α (eBioscience, San Diego, CA, USA) (24 hr incubation each), IL-10 (R&D

Systems, Minneapolis, MN, USA) and IFN-γ (BioLegend) (72 hr incubation each) using ELISA kits.

**14. Serum antibody detection**

To detect the serum antibody ratio, serum samples were collected from the immunized mice by heart puncture after euthanasia by $CO_2$ hyperventilation. Plates (96-well) were coated with purified p24 protein (5μg/ml) in 0.05M carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. Plates were washed three times with PBST and PBS, and blocked at room temperature (RT) for 1 hour with 5% bovine serum albumin (BSA, in PBST). Serum samples were diluted at a ratio of 1:10 in PBS and 100 μl was added to each well. Plates were incubated for 2 hours at RT, washed three times with PBST and PBS, and incubated for 1hour with mouse IgG2a and IgG1 antibody (BD Biosciences, 1:1,000 dilution). Thereafter, the plates were washed again and incubated with HRP conjugated streptavidin for 30 min at RT, and reacted with BD OptEIA substrate (BD Biosciences) for 10 min before stopping the reaction with 1N $H_2SO4$. Optical density (OD) was determined by spectrophotometry at 450nm [48].

**15. Cytotoxic T lymphocyte (CTL) assay**

Induced CTL responses were determined as previously described [49] with

minor modification. In brief, P815 cells (H-$2^d$) were transfected with the plasmid containing p24 or Ag85B-ESAT-6 (pcDNA3.3-p24 or pcDNA3.3-Ag85B) with lipofectamine 2000 (Invitrogen, Carlsbad, USA), and used as target cells ($5\times10^4$ cells) for cytotoxic T lymphocyte (CTL) assay. The expression of p24 or Ag85B was detected by immunoblot assay. Non-transfected cells were used as negative controls. Splenocytes ($5\times10^6$ cells/well) from vaccinated mice were co-cultured in RPMI 1640 medium (Biowest, Nuaille, France) containing 10% FBS with p24 or Ag85B antigen (5 $\mu$g/ml). Splenocytes were co-cultured with antigens at 37 °C in 5% $CO_2$ incubator for 6 days and used as effector cells for CTL responses. Cell cytotoxicity was evaluated with lactate dehydrogenase (LDH) assay in U bottom 96-well plates according to the manufacturer's protocol (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega, Madison, USA). In brief, effector cells (splenocytes stimulated with antigens) were added to target cells (transfected P815 cells) in triplicate at different effector/target (E/T) ratios (10:1, 20:1 to 50:1) for 6 hours,. From the cultured supernatants, released LDH values were measured by spectrophotometry at 490 nm. The percentage of specific cell lysis was calculated with the following formula: [(Experimental − Effector spontaneous − Target spontaneous)/(Target maximum − Target spontaneous)] × 100 (%).

**16. Statistical analysis**

All of the results from ELISA and cell proliferation assays in this study

were representative of more than three independent experiments, and the results were expressed as percentages, means ± SD, or as medians (range). Differences between categorical variables were analyzed using Fisher's exact test or $\chi^2$ test. For continuous variables, Student's t test was used when the data showed a normal distribution, or the Mann–Whitney U test was used when the data were not normally dis-tributed. The value of $P < 0.05$ (two-tailed) was considered to be statistically significant. The SPSS version 18.0 software (Professional Statistic, Chicago, IL, USA) was used for the performance of all statistical analyses.

# RESULTS

**The rSmeg-pMyong2-p24 strain elicited low CFU and enhanced HIV-1 p24 Gag expression in bacteria and in infected murine macrophage cell line and BMDCs.**

To explore the usefulness of pMyong2 vector system in the generation of rSmeg strains for HIV-1 p24 Gag vaccination, I generated a total of 3 types of rSmeg strains expressing p24, rSmeg-pMyong2-p24, rSmeg-pAL5000-p24 and rSmeg-pMV306-p24 using different types of *Mycobacterium-E. coli* shuttle vectors, pMyong2-TOPO [27], pAL5000-TOPO [27], and pMV306 [28], respectively (Fig. 1). When the growth rates of the 3 rSmeg strains in 7H9 broth (with 100 μg/ml of kanamycin) for 5 days were compared with each other, rSmeg- pMyong2-p24 strains showed retardation in growth in the interval from 6 hrs to 48 hrs. But, after 48 hrs, all the 3 strains showed almost the same growth rate (Fig. 2). But Transformed three vectors have seriously effect on CFU in murine macrophage (J774A.1) and BMDC. I think pMyong-p24 plasmid makes *M. smegmatis* attenuated compared to other plasmid. (Fig. 3) [30] I don't know exactly why, but low CFU (colony forming unit) strains could be good for vaccine candidate because of effectively inducing enhanced HIV-1 specific immune responses. And to compare the levels of HIV-1 p24

protein expression in bacteria among the 3 rSmeg strains, I conducted p24 ELISA analysis (Fig. 4A) and Western blot (Fig. 4B) against p24 after lysis of cultured bacteria. All the rSmeg strains could express the p24 protein. But, differences in p24 expression levels were found among the 3 strains, particularly between rSmeg-pMyong2-p24 and the other 2 strains. The rSmeg-pMyong2-p24 produced approximately five to ten times higher levels of p24 than other strains. The rSmeg-pAL5000-p24 produced slightly higher level of p24 than rSmeg-pMV306-p24 (Fig. 4A and 4B). To determine whether the difference in p24 expression among the 3 strains could be recapitulated in infected macrophages and infected BMDCs, I checked the p24 level by ELISA in infected J774A.1 cells and mouse BMDCs. (Fig. 5A and 5B) Generally, similar trends noted in lysed bacteria were also observed in infected cells, but the differences were more pronounced. Taken together, my data indicated that rSmeg-pMyong2-p24 showed an increased production of p24 in infected macrophages and BMDC as well as in bacteria itself, compared to the other two rSmeg strains, rSmeg-pAL5000-p24 and rSmeg-pMV306-p24. This finding suggests that the former has the potentials to elicit an enhanced p24 antigen specific immune response in vaccinated animals or, in advanced, humans by loading more p24 antigen into APC (antigen presenting cells), compared to the

latter two strains. And much more p24 antigen loading to APC such as dendritic cell induced stronger immune response to HIV-1 to cure AIDS.

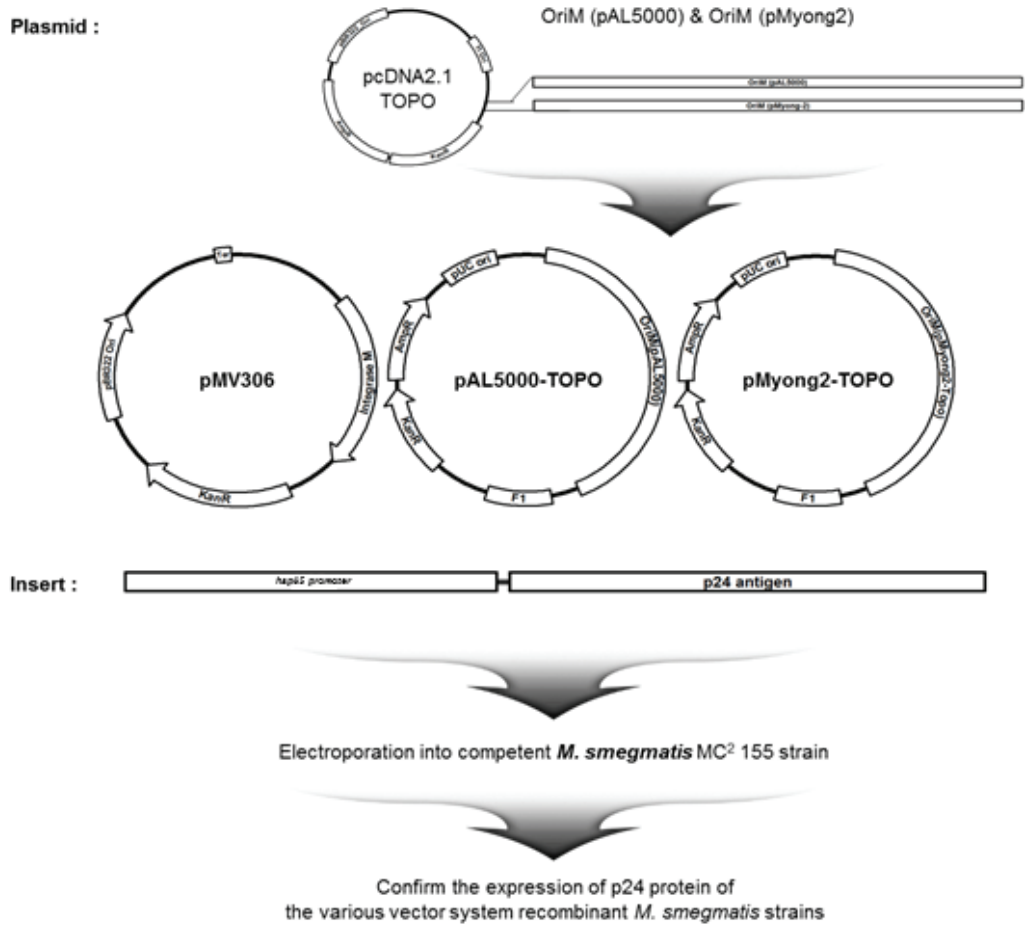



**Figure 1. Maps of p24 expression vectors and schematic flow of the study.**

Maps of the constructed p24 expression *Mycobacterium-E. coli* shuttle vectors. pMV306-p24, pAL5000-p24 and pMyong2-p24 vectors expressed p24 under control of the *hsp65* promoter from *M. bovis* BCG. Additionally, the schematic flow of this study is summarized.

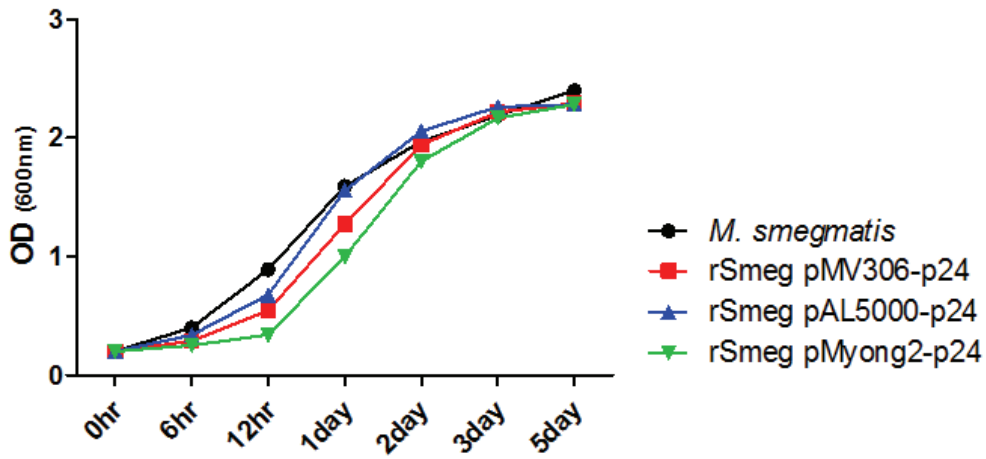



**Figure 2. Growth curve of p24 recombinant *M. smegmatis* strains in 7H9 broth supplemented with ADC and 100 ug/ml of kanamycin**.

In the case of wild type *M. smegmatis* culture, kanamycin was excluded from 7H9 broth. To establish the growth curve, culture aliquots were taken at each time point and the OD at 600nm was measured over a period of 5 days.

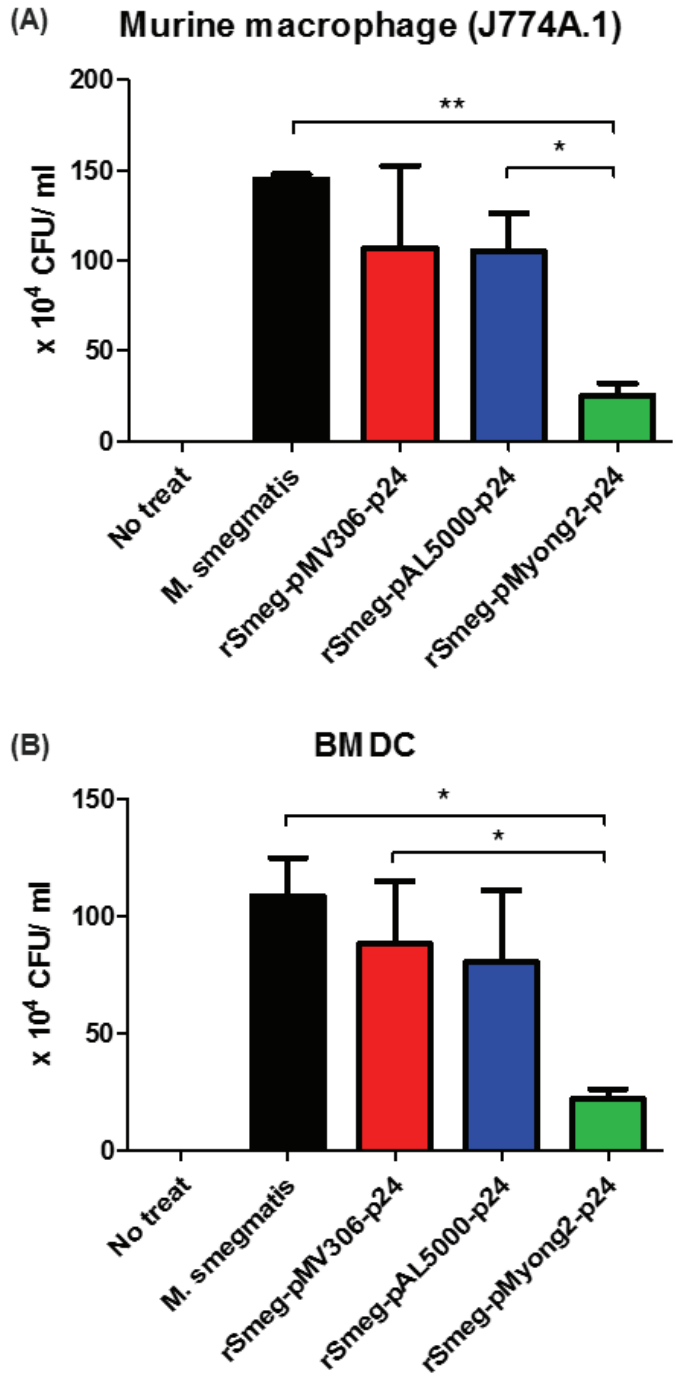

(A)
Murine macrophage (J774A.1)
x 10^4 CFU/ ml
200
150
100
50
0
**
*
No treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24
(B)
BMDC
x 10^4 CFU/ ml
150
100
50
0
*
*
No treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

**Figure 3. Levels of CFUs of *M. smegmatis* and rSmeg strains in infected murine macrophages and infected BMDCs.**

(A) CFUs of *M. smegmatis* and rSmeg strains in infected murine macrophages. (B) CFUs of *M. smegmatis* and rSmeg strains in infected BMDCs. Cells were infected for 2 hrs and washed cell for three times after then cells were incubated 24hrs in media. All the statistical significance were calculated comparing with values of rSmeg-pMyong2-p24. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

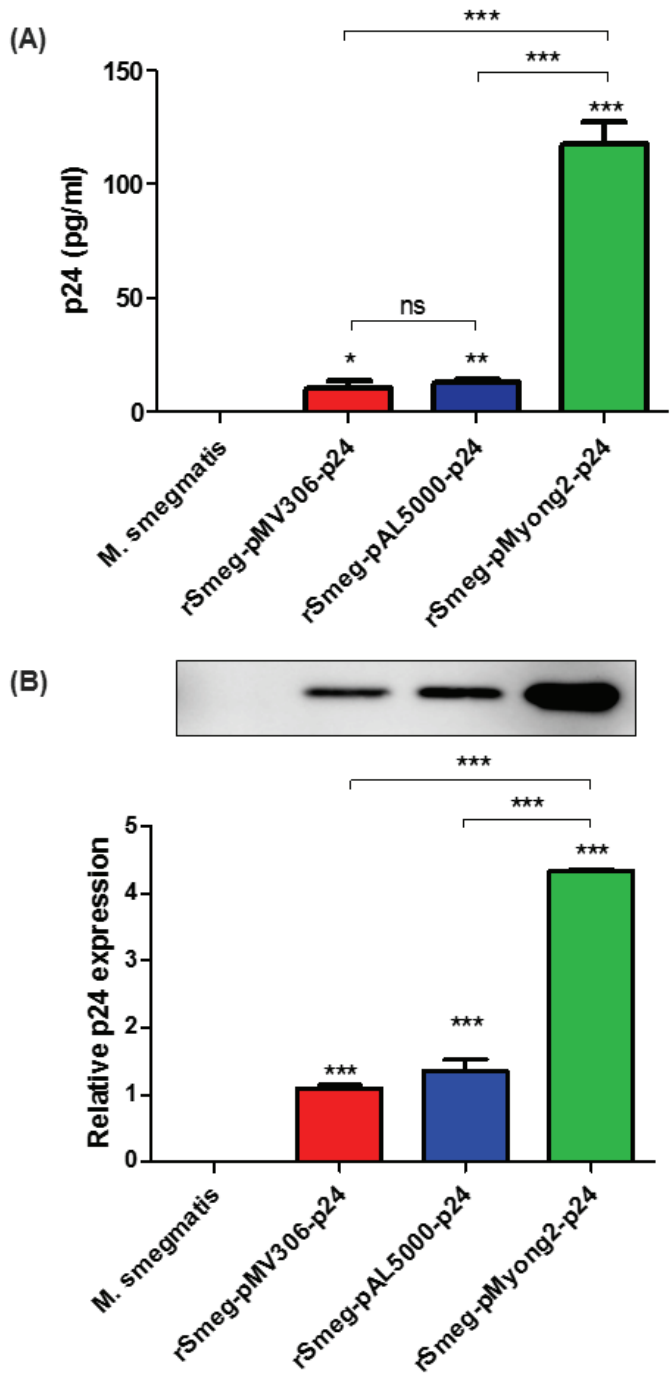
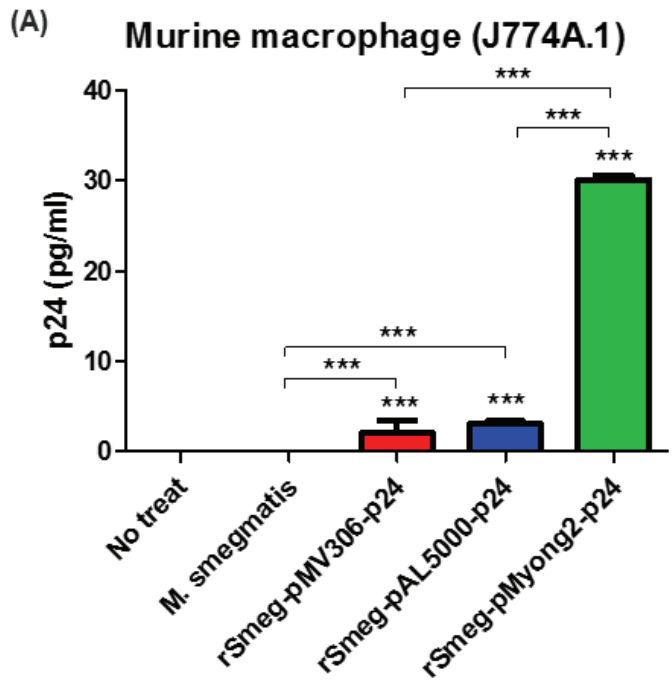

(A)
p24 (pg/ml)
150
100
50
0
***
***
***
ns
*
**
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24
(B)
Relative p24 expression
5
4
3
2
1
0
***
***
***
***
***
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

**Figure 4. Relative levels of p24 expression by wild type *M. smegmatis* and rSmeg strains in p24 ELISA and western blot.**

(A) Confirmation of p24 expression in recombinant *M. smegmatis* by ELISA. (B) Confirmation of p24 expression in recombinant *M. smegmatis* by Western blot. Proteins were extracted from wild- type *M. smegmatis* (lane 1) and rSmeg strains. The expression levels of p24 are plotted. All the statistical significance were calculated comparing with values of one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.


(A)
Murine macrophage (J774A.1)
p24 (pg/ml)
40
30
20
10
0
***
***
***
***
***
***
No treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

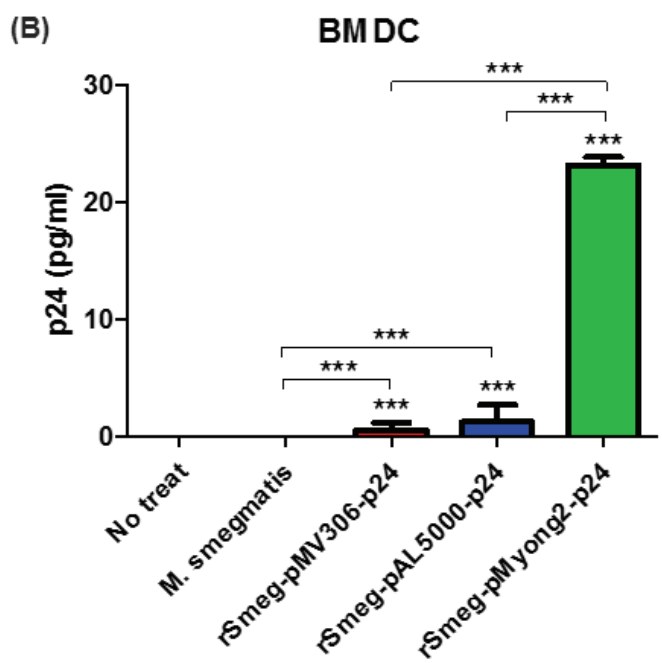

(B)
BM DC
p24 (pg/ml)
30
20
10
0
***
***
***
***
***
***
No treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

**Figure 5. Levels of p24 protein in murine macrophages and BMDCs infected with wild type *M. smegmatis* and rSmeg strains.**

Levels of p24 expression after infection of the murine macrophage cell line J774A.1 (A) and BMDCs (B) with wild type *M. smegmatis* and rSmeg strains (rSmeg-pMV306-p24, rSmeg-pAL5000-p2, rSmeg-pMyong2-p24). All the statistical significance were calculated comparing with values of one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

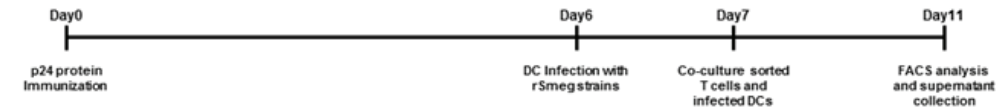
**BMDCs infected with rSmeg-pMyong2-p24 strain elicited enhanced CD4 and CD8 T cell proliferation from murine splenocyte immunized by HIV-1 Gag p24**

To test whether rSmeg-pMyong2-p24 enhanced p24 protein production has improved T cell proliferation capacity, I conducted a T cell proliferation assay of infected BMDCs with three different types of rSmeg strains, rSmeg-pMyong2-p24, rSmeg-pAL5000-p24 and rSmeg-pMV306-p24 and a wild-type strain as a control measuring CFSE dye dilution in a mixed lymphocyte reaction (MLR) assay [29]. The schematic schedule for T cell proliferation assay is described in Fig. 6A.

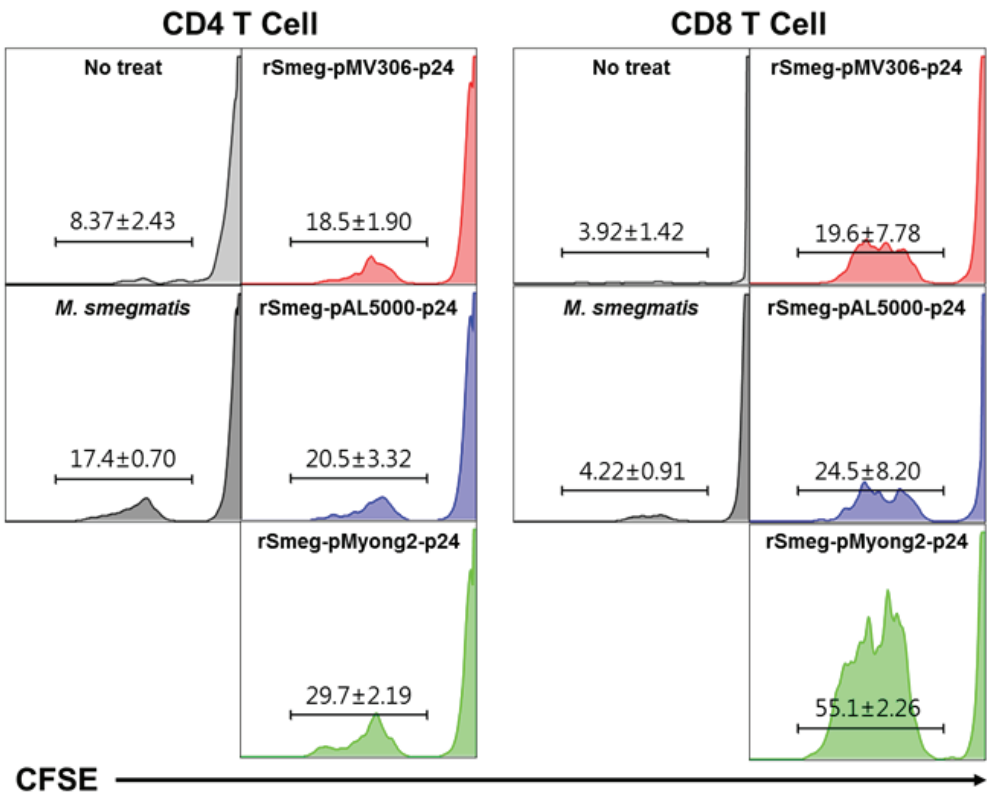
All the BMDCs infected with the 4 Smeg strains (one wild-type and three rSmeg strains) induced significantly higher levels of CD4 T cell proliferation, compared to BMDCs not infected with *M. smegmatis* strains. BMDCs infected with rSmeg-pMyong2-p24 induced significantly higher levels of CD4 and CD8 T cell proliferation compared to the other two rSmeg strains as well as the wild-type strain. Of note proliferation of CD8 T cell that is concern to cure viral infection disease was dramatic increased. I think two factors make higher growth of CD4 and CD8 T cell. first rSmeg-pMyong2-p24 was easily degradation in APC. (Fig. 3A and B) Low CFUs make me to infer induced p24 antigen specific presentation to CD4 and CD8 T cells.

Second. pMyong2-p24 make *M. smegmatis* increase expression of p24 protein. It step antigen presentation up. No significant difference between the rSmeg strains rSmeg-pAL5000-p24 and rSmeg-pMV306-p24 could be found. Even in CD4 T cell proliferation, BMDCs infected with rSmeg-pAL5000-p24 or rSmeg-pMV306-p24 showed similar proliferation levels to that of the wild- type (Fig. 6C and 6D). Comparison of IL-2 amounts from stimulated CD4 and CD8 T cells also showed similar trends as shown in T cell proliferation assays. The result showed that BMDCs infected with rSmeg-pMyong2-p24 induced significantly higher levels of secreted IL-2 from both CD4 and CD8 T cells, compared to BMDCs infected with the other 2 rSmeg strains and wild-type strain (Fig. 7). Taken together, my data indicated that rSmeg-pMyong2-p24 induced increased T cell proliferation, maybe due to enhanced antigen loading into antigen presenting cells such as DCs, resulting in potentiating vaccine efficacy.

(A)


Day0
Day6
Day7
Day11
p24 protein Immunization
DC Infection with rSmeg strains
Co-culture sorted T cells and infected DCs
FACS analysis and supernatant collection

(B)


CD4 T Cell
CD8 T Cell
No treat
rSmeg-pMV306-p24
8.37±2.43
18.5±1.90
3.92±1.42
19.6±7.78
M. smegmatis
rSmeg-pAL5000-p24
17.4±0.70
20.5±3.32
4.22±0.91
24.5±8.20
rSmeg-pMyong2-p24
29.7±2.19
55.1±2.26
CFSE

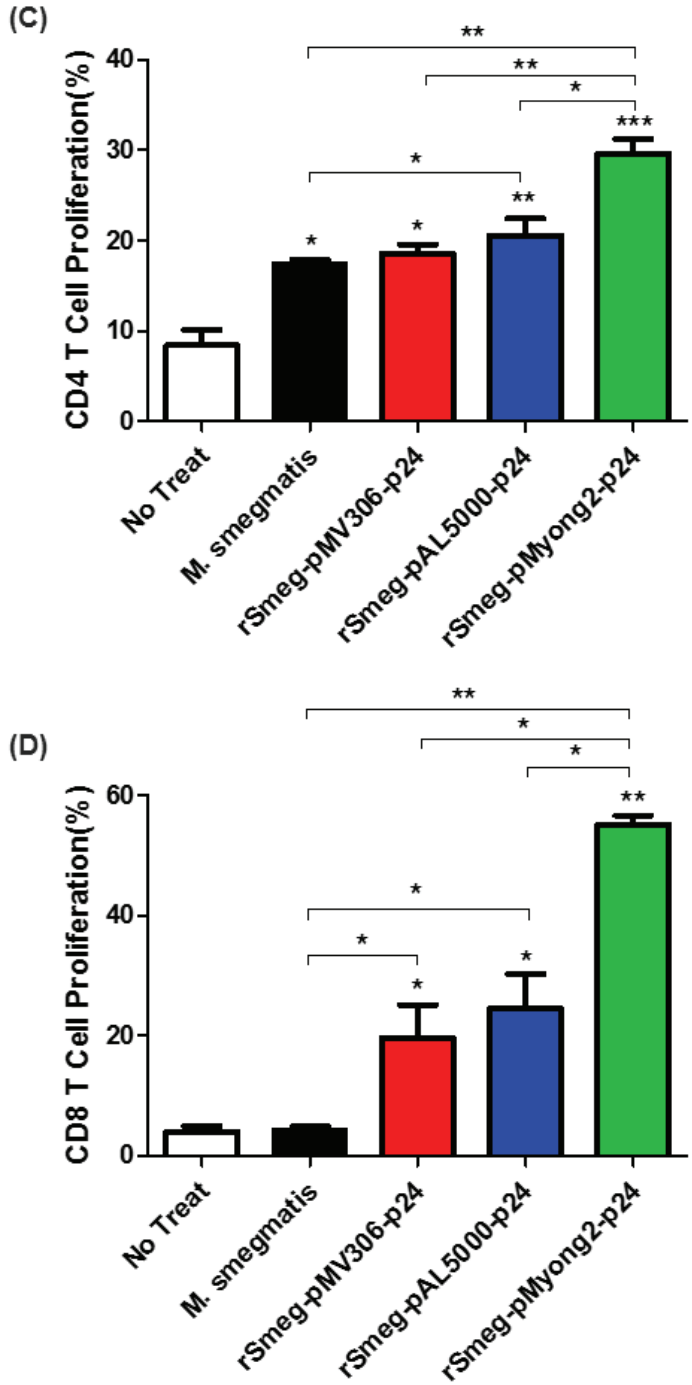

(C)
CD4 T Cell Proliferation(%)
40
30
20
10
0
**
**
*
***
*
*
*
**
No Treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24
(D)
CD8 T Cell Proliferation(%)
60
40
20
0
**
*
*
**
*
*
*
No Treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

**Figure 6. Levels of p24 specific T cell proliferation induced by BMDCs infected with wild type *M. smegmatis* and p24 rSmeg strains.**

(A) Schematic schedule for the T cell proliferation assay. (B and C) Raw data of flow cytometric analysis of the proliferation of CFSE-labeled CD4 and CD8 T cells due to BMDCs infected with p24 recombinant *M. smegmatis* strains. (C and D) Levels of CD4 and CD8 T cell proliferations were made graph. All the statistical significance were calculated comparing with values of one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

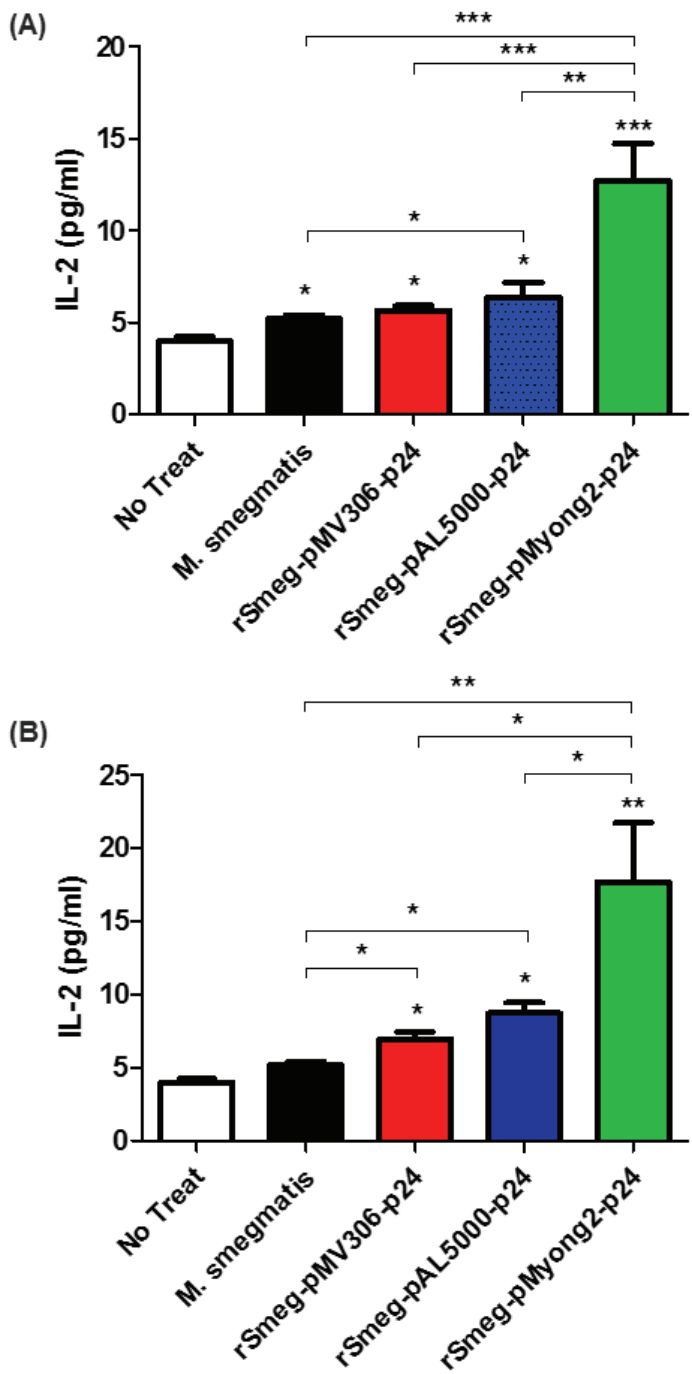

(A)
IL-2 (pg/ml)
20
15
10
5
0
***
***
**
***
*
*
*
*
No Treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24
(B)
IL-2 (pg/ml)
25
20
15
10
5
0
**
*
*
**
*
*
*
No Treat
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

**Figure 7. Levels of IL-2 produced by BMDCs infected with wild type *M. smegmatis* and p24 rSmeg strains during CD4 and CD8 T cell proliferation.**

ELISA determines concentration of IL-2 released in the supernatants of CD4 (A) and CD8 (B) cells in a MLR assay. Data are representative of 3 independent experiments. All the statistical significance were calculated comparing with values of one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

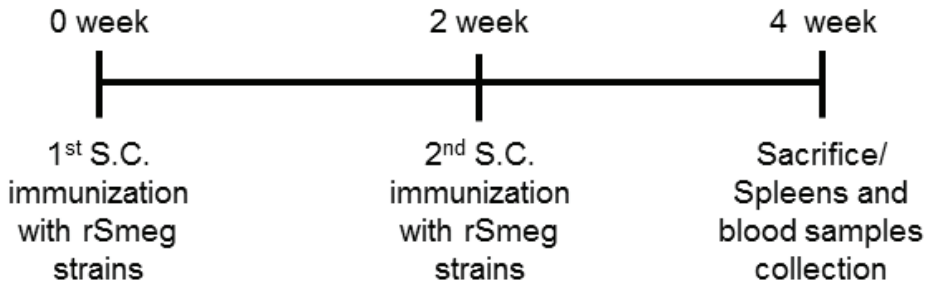
**The rSmeg-pMyong2-p24 strain elicits an enhanced HIV-1 p24 Gag-specific IFN-γ spot forming cells (SFC) in mice spleens generated by subcutaneous immunization**

To test whether rSmeg-pMyong2-p24 has improved T cell response after vaccination, splenocytes were isolated from spleens of BALB/c mice subcutaneously (s.c.) immunized with three different types of rSmeg strains, rSmeg-pMyong2-p24, rSmeg-pAL5000-p24 and rSmeg-pMV306-p24 (~$10^6$ CFU) (Fig. 8A) and a wild- type strain as a control and assayed for HIV-1 p24 Gag-specific T cell responses using IFN-$\gamma$ ELISPOT assays. Splenocytes from s.c. immunized mice with two rSmeg strains,

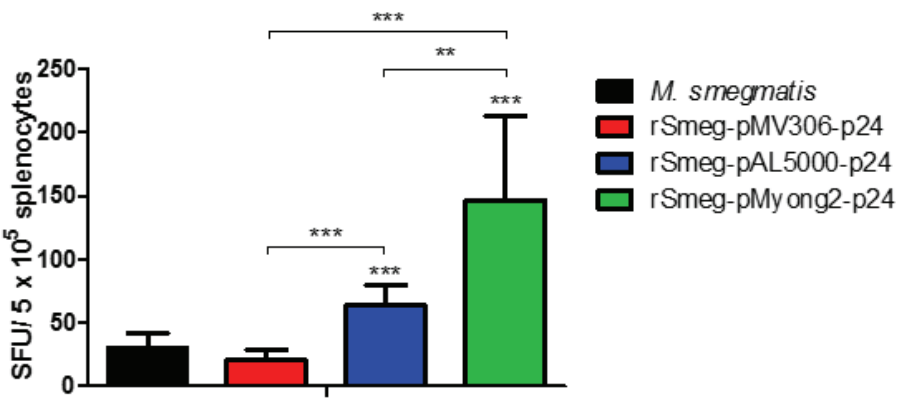
rSmeg-pMyong2-p24, and rSmeg-pAL5000-p24 yielded significantly higher SFUs than those of wild-type and rSmeg-pMV306-p24 strains. Of note, splenocytes from mice immunized with rSmeg-pMyong2-p24 (146.33 ± 66.91 SFUs/5 × $10^5$ splenocytes) yielded significantly higher SFUs than those of the two other rSmeg strains, rSmeg-pAL5000-p24 (63.63 ± 15.72 SFUs/5 × $10^5$ splenocytes) and rSmeg-pMV306-p24 (24.74 ± 11.91 SFUs /5 × $10^5$ splenocytes) (Fig. 8B). Interestingly, a significant difference in SFC numbers was also observed between the two rSmeg strains rSmeg-pAL5000-p24 and

rSmeg-pMV306-p24, which showed no or little difference in T cell proliferation or p24 expression. Taken together, my data indicated that rSmeg-pMyong2-p24 elicited an improved effector T cell function in vaccinated animals. I think much induced immune response come from low CFUs and high expression of p24 protein. These factors make synergy effects in mice and they could influence T cell proliferation and effector T cell.

(A)


0 week
2 week
4 week
1st S.C. immunization with rSmeg strains
2nd S.C. immunization with rSmeg strains
Sacrifice/ Spleens and blood samples collection

(B)


SFU/ 5 x 10^5 splenocytes
250
200
150
100
50
0
***
**
***
***
***
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

(C)

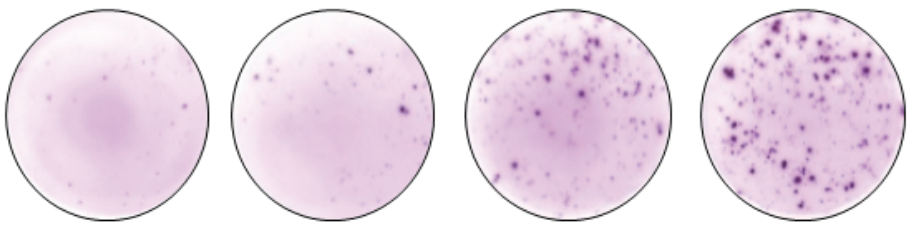

<M. smegmatis>
<rSmeg-pMV306-p24>
<rSmeg-pMV306-p24>
<rSmeg-pMV306-p24>

**Figure 8. In vivo immune responses induced by p24 recombinant *M. smegmatis* strains.**

(A) Schematic immunization schedule for in vivo immunological assays. (B) Levels of IFN-γ secretion levels by in vitro stimulated splenocytes from vaccinated mice with p24 recombinant *M. smegmatis* strains were detected with ELISPOT analysis. Representative images of ELISPOT membrane in each group are shown below the graph. All the statistical significance were calculated comparing with values of one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

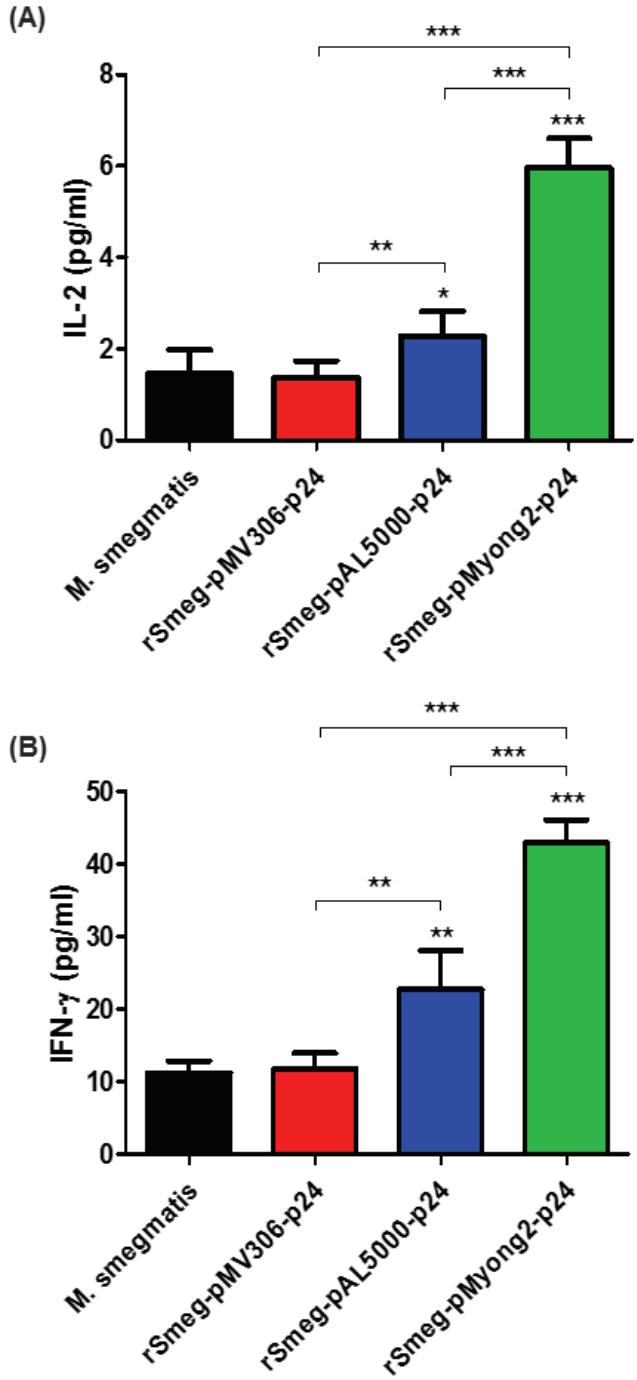
**The rSmeg-pMyong2-p24 strain increased production of various cytokines in re-stimulated splenocyte from immunized C57BL/6 mice**

Cytokine production during protective responses was the subject under study. The Th1-type cytokines (IL-2, IL-12, TNF-α and IFN-γ) and the Th2-type cytokines (IL-10) predominantly was induced by the cellular and humoral arms of the immune responses, respectively. IL-12 work for activation of NK cells and cytotoxic T cells secreting type Ⅱ interferon (IFN-γ) production from Th and CTL cells. IL-12 has been found to induce Th1-type immune responses, to promote NK activity, and to activate cytotoxic T lymphocytes. [41] As a result, Th1-type cytikines important in viral infection and its immunity.

To investigate production of cytokine of immunized mice, splenocytes obtained two weeks after the second immunization with wild type *M. smegmatis* and rSmeg strains (Fig. 9) were stimulated in vitro with purified p24 protein (5μg/ml), and the induced production of IL-2, IFN-γ, TNF-α and IL-10 cytokines were measured in the cell culture supernatants. For the detection of IL-10 and TNF-α cytokines, splenocytes were stimulated with p24 for 24 hr; for IFN-γ and IL-2, splenocytes were incubated with p24 for 72 hr. In the case of rSmeg-pMyong2-p24 immunized splenocytes, the levels of all the Th1 immune response related cytokines (IL-2 for rSmeg-pMV306-p24 vs.

rSmeg-pAL5000-p24 vs. rSmeg-pMyong2-p24: 1.37 ± 0.37 vs. 2.27 ± 0.54 vs. 5.95 ± 0.66 pg/ml; IFN-γ: 11.78 ± 2.16 vs. 22.67 ± 5.44 vs. 42.95 ± 3.21 pg/ml and TNF-α: 16.00 ± 2.26 vs. 21.08 ± 3.77 vs. 28.92 ± 3.41 pg/ml, respectively) were significantly higher than those of wild- type or rSmeg strains. (Fig. 9 A-C)

The rSmeg-pMyong2-p24 strain also increased the level of IL-10 release slightly; however, all the strains showed similar levels (rSmeg-pMV306-p24 vs. rSmeg-pAL5000-p24 vs. rSmeg-pMyong2-p24: 43.88 ± 3.87 vs. 52.12 ± 3.85 vs. 55.72 ± 3.12 pg/ml) (Fig. 9D) Increase of IL-10 was naturally followed by pressing induced immune response. And I think that it was not for anti inflammatory response.


(A)
IL-2 (pg/ml)
0
2
4
6
8
***
***
***
**
*
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24
(B)
IFN-γ (pg/ml)
0
10
20
30
40
50
***
***
***
**
**
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

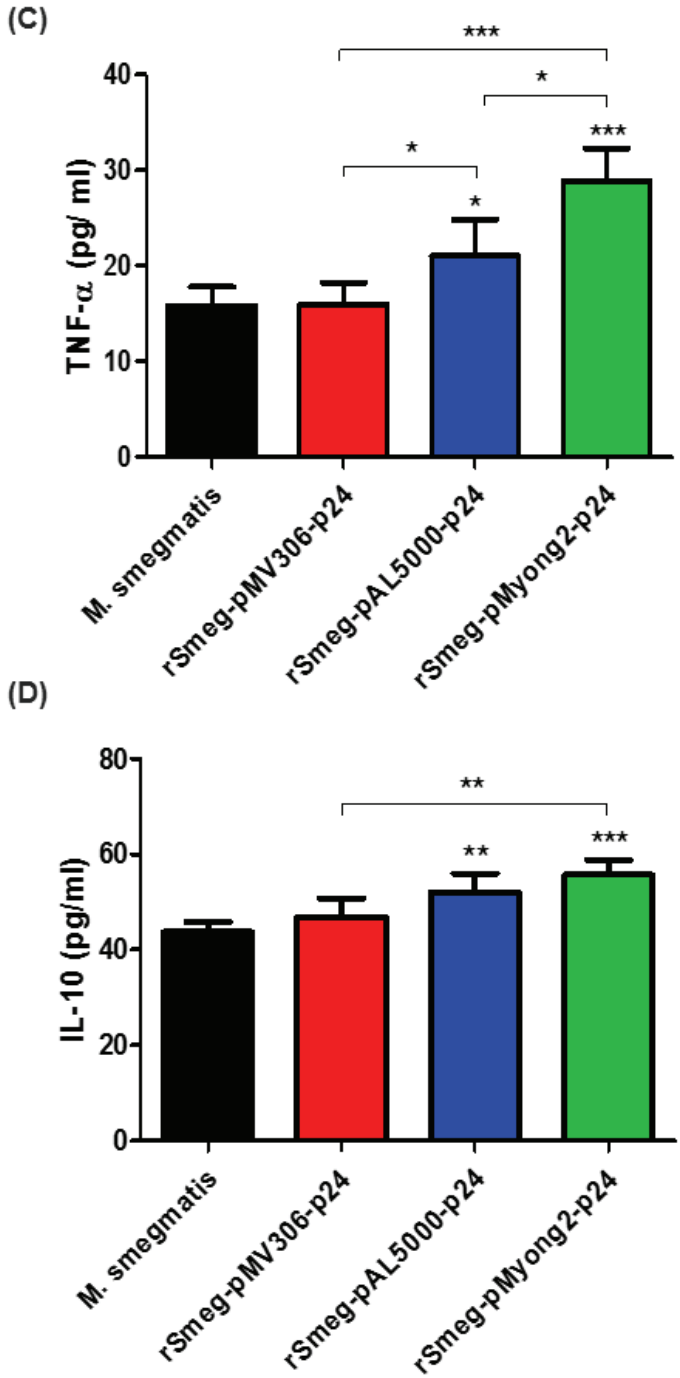

(C)
TNF-α (pg/ ml)
0
10
20
30
40
***
*
*
***
*
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24
(D)
IL-10 (pg/ml)
0
20
40
60
80
**
**
***
M. smegmatis
rSmeg-pMV306-p24
rSmeg-pAL5000-p24
rSmeg-pMyong2-p24

**Figure 9. Level of cytokines in re-stimulated splenocytes induced by p24 recombinant *M. smegmatis* strains.**

(A) Levels of IL-2, IFN-γ, TNF-α, and IL-10 cytokines by in vitro stimulated splenocytes with p24 from mice vaccinated with wild type *M. smegmatis* and rSmeg strains were detected with ELISA analysis. For the detection of IL-10 and TNF-α cytokines, splenocytes were stimulated with p24 for 24 hr; for IFN-γ and IL-2, splenocytes were incubated with p24 for 72 hr. All the statistical significance were calculated comparing with values of one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

**The rSmeg-pMyong2-p24 strain elicits a HIV-1 p24 Gag-specific Th1-biased humoral response in immunized mice**

Th1-biased humoral response in developing novel vaccine platform is important in modern vaccine technology. [7] In mice, high titers of the IgG1 antibody isotype denote Th2 immunity, while high titers of the IgG2a antibody isotype reflect Th1 immunity. To measure titers of antigen specific antibodies, I used indirect ELISA. [25]

To test whether rSmeg-pMyong2-p24 elicits a Th1-biased humoral response in immunized mice, I analyzed the levels of HIV-1 p24 Gag-specific IgG2a and IgG1, which are known as markers for Th1 and Th2 responses, respectively [30-32]. The sera of BALB/c mice s.c. immunized with 3 different types of rSmeg strains, rSmeg-pMyong2-p24, rSmeg-pAL5000-p24 and rSmeg-pMV306-p24 and a wild- type strain as a control were analyzed. As shown in Fig. 10, both rSmeg-pMyong2-p24 and rSmeg-pAL5000-p24, but not rSmeg-pMV306-p24 elicited significantly higher levels of IgG2a isotype than wild type.

With regard to the IgG1 isotype, rSmeg-pMyong2-p24 elicited a lower level of IgG1 than rSmeg-pAL5000-p24; however, it does not reach statistical

significance (P = 0.146). Collectively, the IgG2a/IgG1 ratio, of which a higher level indicates more Th1- biased humoral immune response [31], was the highest in sera immunized by rSmeg-pMyong2-p24 (Value = 1.21), compared to those immunized by other types of *M. smegmatis* (wild type = 1.05; rSmeg-pAL5000-p24 = 1.03; rSmeg-pMV306-p24 = 0.97) (Fig. 10), suggesting that rSmeg-pMyong2-p24 strain can elicit an enhanced HIV-1 p24 Gag-specific Th1-biased humoral response in immunized mice.

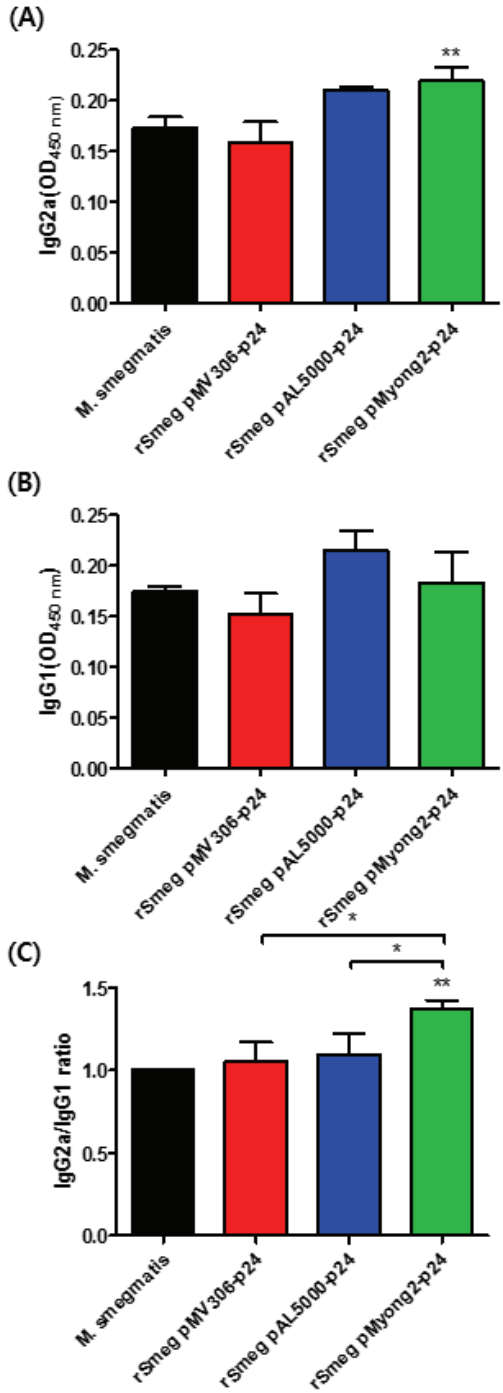

(A)
IgG2a(OD450 nm)
0.25
0.20
0.15
0.10
0.05
0.00
**
M. smegmatis
rSmeg pMV306-p24
rSmeg pAL5000-p24
rSmeg pMyong2-p24
(B)
IgG1(OD450 nm)
0.25
0.20
0.15
0.10
0.05
0.00
M. smegmatis
rSmeg pMV306-p24
rSmeg pAL5000-p24
rSmeg pMyong2-p24
(C)
IgG2a/IgG1 ratio
1.5
1.0
0.5
0.0
*
*
**
M. smegmatis
rSmeg pMV306-p24
rSmeg pAL5000-p24
rSmeg pMyong2-p24

**Figure 10. Level of Th1- or Th2- antibodies in serum induced by p24 recombinant *M. smegmatis* strains.**

(A and B) p24 specific immunoglobulin subtypes (IgG2a and IgG1) were detected by ELISA at 450 nm. OD values mean for levels of IgG2a and IgG1 subtypes (C) The ratio of IgG2a/IgG1 were compared. All the statistical analyses were calculated by comparisons with the values for rSmeg-pMyong2-p24. Means ± SD are shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

**The rSmeg-pMyong2-p24 strain elicits an enhanced HIV-1 p24 Gag-specific cytotoxic T lymphocyte response in immunized mice**

Continuous efforts to develop efficacious vaccines against HIV-1 were partly built on the rule that the HIV-1 specific antiviral cytotoxic T lymphocyte (CTL) response is important for immune control of viral replication [55]. So effect of CTL were count for developing HIV-1 vaccine. I do the test to compare CTL response using rSmeg-pMyong with wild type *M. smegmatis* and others rSmeg strains.

To test whether rSmeg-pMyong2-p24 elicits an enhanced HIV-1 p24 Gag-specific CTL responses in immunized mice, I analyzed CTL activity of splenocytes immunized with four different types of Smeg strains, 3 rSmeg, rSmeg-pMyong2 -p24, rSmeg-pAL5000-p24 and rSmeg-pMV306-p24 and wild type via an LDH cytotoxicity assay.

The immunized procedure is described in Fig 8A. The P815 cells (H-$2^d$) transfected with the plasmid transfecting p24 or Ag85B-ESAT-6 fusion genes (pcDNA3.3-p24 or pcDNA3.3-Ag85B-ESAT-6) were served as target cells and the effector/target ratios were 10:1, 20:1, and 50:1. As shown in Fig.11, at the E:T ratio of 50:1, the CTLs from mice immunized with rSmeg-pMyong2-p24

could elicit a significant higher level of HIV-1 p24 Gag specific target cell lysis, compared to those of Ag85B-ESAT6 (Fig 11A). But, no significant difference in Ag85B specific CTL killing between 4 strains was not found. But my data indicated that rSmeg-pMyong2-p24 strain can elicits an enhanced HIV-1 Gag p24-specific CTL response in immunized mice compared to other strains including wild type. (Fig 11B)

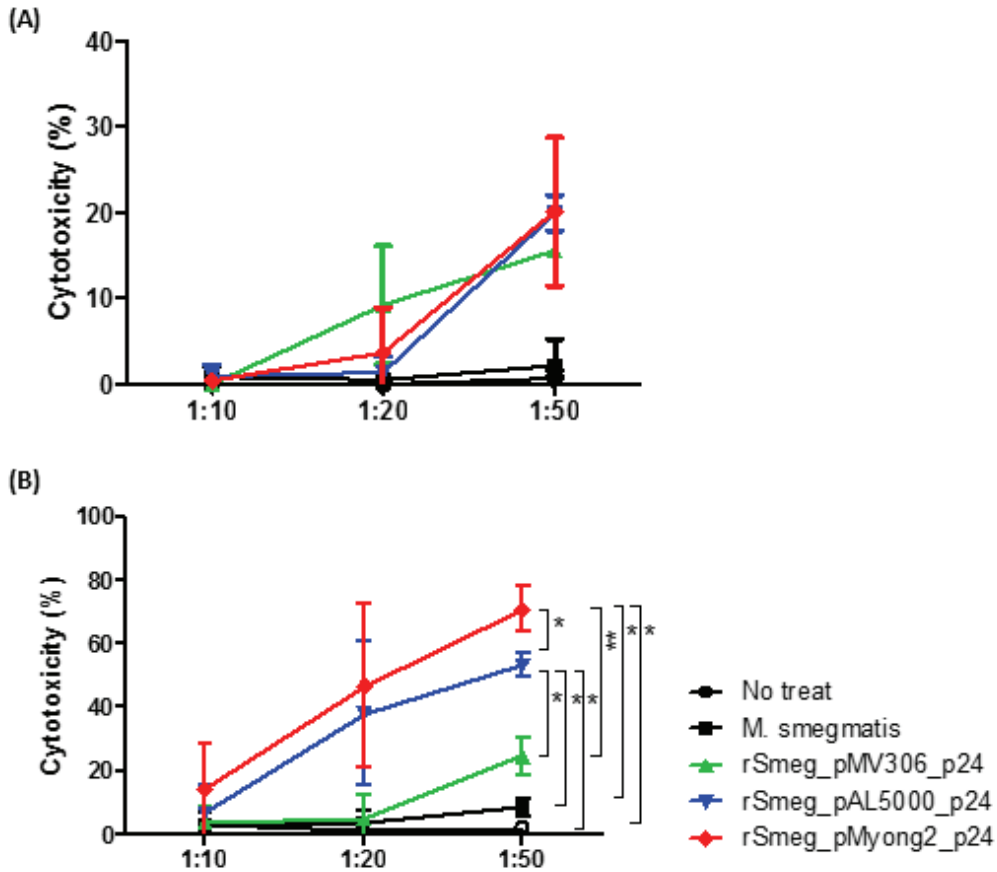



**Figure 11. Cytotoxic T lymphocyte responses from immunized mice with recombinant *M. smegmatis* strains.**

(A) To measure CTL response against Ag85B-ESAT6, CTL responses due to the reaction of in vitro stimulated splenocytes with Ag85B and Ag85B transfected P815 cells (B) To measure CTL response against HIV-1 Gag p24, CTL responses due to the reaction of in vitro stimulated splenocytes with p24

and p24 transfected P815 cells. Data are representative of two independent experiments. All the statistical analyses were calculated by comparisons with the values for one another. Means ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

# DISCUSSION

For vaccine development against diseases such as AIDS and tuberculosis, attention has focused on developing strategies for the vaccine induction of cellular immunity, particularly CTL [1]. Studies of laboratory animals and early-phase clinical trials with humans have shown that live recombinant vectors can generate CD4 and CD8 T-lymphocyte responses to a variety of pathogenic microorganisms [33-38]. Of note, the most-effective strategies for the elicitation of cellular immune responses are heterologous prime/boost regimens. Recently, the rSmeg strain of live recombinant vectors has been shown to be useful as priming vectors in prime/boost vaccination regimens for the induction of cellular immune responses against HIV-1 infection by potentiating a vigorous secondary immune response following boosting, particularly expanding a large pool of competent CTLs. It is mainly due to the capacity of Smeg to induce differentiation into antigen specific memory CD8 T cells [39]. However, there is a potential limitation of rSmeg vector that induces a small number of antigen-specific CD8 T cells in comparison to those elicited by other vectors. This limitation may be in part a consequence of the in vivo expression of only small amounts of antigen by rSmeg or in part due to the limited access of

antigen to the cytosol of infected phagocytes, preventing an efficient MHC class I presentation [39]. Thus, to improve efficacy of rSmeg as priming vectors in prime/boost vaccination regimens, a proper *Mycobacterium* vector system that is particularly efficient in directing transgene products into MHC class I processing pathways should be selected. The simplest approach for this purpose ensures that rSmeg can maintain robust levels of transgene expression, which, mainly depends on the nature of the used vector, such as its copy number and expression capacity of transgene at the transcriptional or translational level.

Therefore, to search a proper *Mycobacterium* vector system facilitating the vaccine efficacy of rSmeg, I compared the HIV-1 p24 expression levels obtained with rSmeg strains using three different vector systems (two episomal vectors, pAL5000 derived vector with 2-6 copies per cell and pMyong2 derived vectors with copy numbers approximately 37 times higher than pAL5000 vector and one integrating vector, pMV306) under the control of a mycobacterial *hsp65* promoter. my results demonstrate that the best expression was achieved using rSmeg-pMyong2-p24 with the pMyong2 vector (Figs. 4). Furthermore, the more pronounced difference in p24 expression was found in infected phagocytes (Fig. 5), providing a mechanistic basis regarding the enhanced p24 specific T

cell proliferation of BMDCs (Fig. 6 and 7), T cell effector function (Fig. 8 - 11), particularly in CTLs (Fig 11), and Th1- biased humoral immune response (Fig. 10) of rSmeg-pMyong2-p24.

Comparison of the growth rate of 3 rSmeg strains in 7H9 broth showed that there was growth retardation during the interval between 0 and 48 hrs for the rSmeg-pMyong2-p24 strain compared to the other rSmeg strains (Fig. 2). This difference may be attributed into the pressure of maintaining a high copy number of the pMyong2 vector system. This result is consistent with the previous report that BCG or Smeg strains transfected with the pMyong2 derived vector were much slower in colony formation in 7H10 agar than those transfected with the pAL5000 derived vector [27]. This finding hints that rSmeg-pMyong2-p24 may be more attenuated in macrophages or in vivo mice infection than other rSmeg strains. Actually, I found that after infection of macrophages and BMDC, rSmeg-pMyong2-p24 formed colony forming units (CFUs) 2-3 times less than those of rSmeg-pAL5000-p24 strain or rSmeg-pMV306-p24 strain. Given the previous finding that attenuated Smeg elicits stronger immune responses than wild-type strain by presenting more antigens to phagocytes [40,41], it can provide rSmeg-pMyong2-p24 an additive advantage in its vaccine application.

In the current study, I have demonstrated that rSmeg-pMyong2-p24 with pMyong2 shuttle vector system elicited higher levels of HIV-1 p24 Gag protein expression and can deliver more p24 antigens into phagocytes, compared to other rSmeg strains using pAL5000 or pMV306 derived system. I also showed that the strain could enhance T cell proliferation capacity of infected BMDCs and elicit improved T cell effector function and Th1 biased humoral immune response in vaccinated mice. These findings suggest that rSmeg-pMyong2-p24 may be an effective candidate vaccine for HIV-1 or co-infection with both HIV-1 and tuberculosis.

In the current study, I have demonstrated that rSmeg-pMyong2-p24 using pMyong2 shuttle vector system elicited higher level of HIV-1 p24 Gag protein expression and can deliver more p24 antigens into phagocytes, compared to other rSmeg strains using pAL5000 or pMV306 derived system. I also proved that the strain could enhance T cell proliferation capacity of infected BMDC and elicit improved T cell effector function and Th1 biased humoral immune response in vaccinated mice. These findings suggest that rSmeg-pMyong2-p24 may be a meaningful candidate vaccine for HIV-1 or co-infection of both HIV-1 and tuberculosis.

rSmeg-pMyong2-p24 has ‘one more thing’ that is advantage for patients

infected HIV with TB. TB and AIDS are a fatal disease, however recently they are not curable but chronic disease. For example patients infected HIV-1 are treated highly active antiretroviral treatment (HAART). It makes their life expectancy improved. Though novel treatments are developed, AIDS patients' immunocompetence go down as a necessity and it increase chance of opportunistic infection. When someone have experienced infection of TB or Untreated latent TB infection, AIDS is lethal infection because the immune system is already weakened.

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# 국문초록

## pMyong2 벡터 시스템을 이용한 Human Immunodeficiency Virus Type I Gag 단백 발현 재조합 *Mycobacterium smegmatis*의 면역 증진 효과

**공 정 렬**

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의과학과 의과학전공

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Human immunodeficiency virus (HIV)에 감염된 감염자 수는 매년 지속적으로 증가하고 있다. 하지만 이에 대한 치료제는 아직 개발 되지 않고 있으며, 현재까지는 만성 질환으로써 감염이 더 이상 진행되지 않게 하는 치료를 진행하고 있다. 이를 극복하기 위해 plasmid DNA vaccine, adnovirus serotype 5 and pox-vectored vaccine등 다양한 방법으로 예방 혹은 치료용 백신을 개발하고 있다.

최근 본 연구자가 속한 연구실에서 신종 균주로 보고한 *Mycobacterium yongonense*에서 발견된 선형 pMyong-2 플라스미드를 이용하여 미코박테리움-대장균간의 셔틀 벡터 시스템을 개발하였다. 이 시스템을 이용하면 기존의

미코박테리움-대장균간의 셔틀 벡터로 알려진 pAL5000 시스템에 비하여 이질 단백질의 발현이 mRNA, Protein수준에서 증가함을 확인 하여 보고하였다.

본 연구자는 이 pMyong-2 미코박테리움-대장균간의 셔틀 벡터 시스템과 *hsp65* promoter를 이용하여 HIV-1의 항원으로 알려진 p24 이질 단백질을 발현하는 플라스미드를 유전자 재조합 하였으며 이를 비결핵성 마이코박테리움인 *Mycobacterium smegmatis* (*M. smegmatis*)에 유전자 재조합 하였다. 이렇게 만들어진 균주를 기존의 미코박테리움-대장균간의 셔틀 벡터 시스템인 pAL5000과 인테그레이션 벡터 시스템인 pMV306과 p24 이질 단백질의 발현량을 비교하였다. Western blot과 ELISA를 이용하여 균 자체에서 발현하는 p24 이질 단백질 양과 감염된 대식세포와 수지상 세포 내에서 발견되는 p24의 발현량을 비교했을 때 기존의 pMV306, pAL5000 시스템에 비해서 pMyong2를 이용한 시스템이 월등히 높은 발현량을 보였다.

단순히 균내에서 발현량이 높아지는 현상에 그치지 않고 이를 이용해서 같은 양의 균을 이용해서 HIV-1 항원 p24에 대한 특이적인 면역반응을 비교해 보았다. 첫 번째로, 마우스의 골수에서 분화시킨 수지상세포에 3가지 시스템 (pMV306, pAL5000, pMyong-2)을 이용하여 만든 재조합 *M. smeg*와 야생형 *M. smeg*를 수지상 세포에 감염 시킨 뒤, CFSE로 염색한 CD4/CD8 T 림프구와 함께 배양한다. 이후 T 세포의 증식 양상을 보면 같

은 기간 내에 pMyong-2 시스템을 이용한 *M. smeg*에 감염된 수지상 세포군에서 상대적으로 더 많은 T세포가 증식함을 확인하였고, 이에 중요한 역할을 하는 것으로 알려진 Interleukin-2의 분비량 또한 T 세포의 증식과 유사하게 증가함을 확인 할 수 있었다.

두 번째로 체외에서의 림프구의 증식과 함께 마우스 체내에서 반응을 살펴보기 위해서 마우스의 피하로 2주 간격으로 2회 야생형과 3종류의 유전자 변이 균주를 접종하였다. 이후 비장세포를 추출하여 비장세포를 이용하여 IFN-$\gamma$을 분비하는 세포의 양을 비교하는 실험을 진행하였을 때, 앞에서와 마찬가지로 pMyong-2을 이용한 벡터 시스템에서 IFN-$\gamma$를 분비하는 세포가 더 많음을 확인하였다. 또한 비장 세포를 체외에서 5일간 p24로 자극하고 이를 p24를 발현하는 세포와 함께 배양해서 세포독성 T 세포의 세포독성이 어느 정도 인지 확인하는 실험에서도 pMyong-2을 이용한 벡터 시스템이 앞선 결과들과 마찬가지로 세포독성이 증가하는 결과를 확인 할 수 있었다. 마지막으로 면역된 마우스의 비장 세포를 p24 항원으로 재자극하여 염증성 Cytokine(IL-2, TNF-$\alpha$, IFN-$\gamma$)과 항염증성 Cytokine (IL-10)의 양을 확인 하였을 때 두 경우 모두 pMyong-2을 이용한 벡터 시스템에서 더 많이 증가하였음을 확인할 수 있었다. 또한 면역된 마우스의 혈액에서 혈청을 분리하여 혈청 속에 항체의 역가를 확인 하는 실험에서 Th1에 의해서 분화된 B세포가 분비하는 것으로 알려진 IgG2a의 항체의 역가가 증가함을 확인 했

다.

위의 실험을 통해서 pMyong-2 벡터 시스템에서 이질 단백질의 증가는 단순히 발현량의 증가뿐만 아니라 기존의 발현 시스템에 비해서 효과적으로 이질 단백에 대한 면역능을 항진 시킬 수 있음을 확인하였고, 앞으로 효과적으로 목표 항원에 대한 면역반응을 항진 할 수 있는 보편적인 시스템이 될 수 있음을 시사한 결과를 얻었다. 이와 같은 결과를 봤을 때 앞으로 많은 감염성 질환의 치료제를 개발하는데 효과적인 도구가 되기를 기대한다.

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