Immunogenicity and Protective Efficacy of Cocktail Subunit Vaccines against Tuberculosis in the Mouse Model

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the world's three major infectious diseases, along with AIDS and malaria. An effective vaccine against TB would be the most cost effective way of controlling the disease. Given the controversial efficacy of BCG vaccine against TB in adults, it has become urgent that we develop new TB vaccines, which can replace BCG and overcome the inhibitory effect shown by environmental mycobacteria upon vaccine effectiveness. In an effort to develop subunit vaccines against TB, the recombinant proteins Antigen 85A (Ag85A), ESAT-6, 16 kDa, and 38 kDa of *M. tuberculosis* were examined for protective efficacy in a mouse model. In addition, four adjuvants, namely, alum, MPL-SE, DDA, FIA, and a combination of MPL-SE and DDA were evaluated in term of their synergistic effects on immune response to the recombinant antigens.

C57BL/6 mice were immunized three times subcutaneously with individual antigen or cocktail antigens that consisted of two to four antigens

including Ag85A as a key antigen. Cellular and humoral immune responses were measured four weeks after the last immunization, and IgG isotypes were determined. Protective efficacy was evaluated by bacterial counts in lungs and spleens four or eight weeks after aerosol challenge with *M. tuberculosis* H37Rv.

IFN-γ production was consistently elevated in mice immunized with cocktail vaccines consisting of two or three TB antigens compared to mice immunized with a single TB antigen. Interestingly, however, no significant increase in IFN-γ was observed production in mice that received a cocktail vaccine consisting of four TB antigens.

When mice were immunized with an individual antigen, there was no significant protection against *M. tuberculosis*. However, significant reductions in bacterial count in the lungs and spleens were observed when mice were immunized with cocktail subunit vaccines consisting of two or three TB antigens. Cocktail subunit vaccines consisting of Ag85A + ESAT-6, Ag85A + 38 kDa, and Ag85A + ESAT-6 + 16 kDa rendered greater protection than other antigen combinations. However, no significant reduction in bacterial load was found in mice immunized with a cocktail vaccine consisting of four TB antigens, probably because of reduced amounts of individual antigens.

This study demonstrates that cocktail antigens containing to two or three antigens are more effective than a single antigen for the protection of

mice against *M. tuberculosis* infection. Cocktail subunit vaccines consisting of Ag85A + 38 kDa and Ag85A + ESAT-6 + 16 kDa were found to be most promising as vaccines against TB. Further study needs to be undertaken to determine the proper doses of individual antigens in order to maximize protective efficacy.

Key Words : tuberculosis, *Mycobacterium tuberculosis*, Ag85A, protective efficacy, cocktail subunit vaccines

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I. INTRODUCTION

1. Current status of tuberculosis

Tuberculosis (TB) is a chronic necrotizing infection caused by a characteristic bacillus *Mycobacterium tuberculosis,* which was discovered by Robert Koch in 1882.[1] The disease appears to be as old as humanity itself; skeletal remains of prehistoric humans in Germany dating back to 8000 BC, show clear evidence of the disease.[2,3] In addition, Egyptian skeletons dating from 2500 to 1000 BC show evidence of Pott's disease of

the spine,[4] and ancient Hindu and Chinese writings have also documented the presence of the disease.

M. tuberculosis is an acid-fast bacillus and grows only slowly, reproducing itself every 17 to 18 hours under optimal conditions,[5] and is often in a dormant state. It usually produces a chronic disease, frequently involving periods of apparent good health, and has a tendency to reactivate many years after the initial infection.

TB is a major global health problem, especially in developing countries. The World Health Organization (WHO) estimates that one third of the world's population has been infected with *M. tuberculosis*, and about 8 million new TB cases and 2.8 million cases of TB-related deaths occur globally each year.[6] The incidence of TB is increasing dramatically in many countries, significantly in parallel with the acquired immune deficiency syndrome (AIDS) epidemic and the emergence of multi-drug resistant tuberculosis (MDR-TB). Outbreaks of MDR-TB in institutional settings in the United States and Europe in the early 1990s[7] brought international attention to bear on the growing global threat of drug-resistant TB. The majority of individuals identified in these outbreaks have been dually infected with human immunodeficient virus (HIV) and *M. tuberculosis*. TB in these patients is associated with a very high mortality, ranging from 72% to 89% and with a median interval between the diagnosis of TB and death of 4 to 14 weeks.[8]

Drug-resistant and MDR-TB now affects nearly all countries and all regions. In a recent study in 35 countries, organized by the WHO and the International Union against Tuberculosis and Lung Disease (IUATLD), upon the resistance of *M. tuberculosis* strains to first-line drugs (isoniazid, rifampicin, streptomycin, and ethambutol), the median prevalence of *M. tuberculosis* mono-resistance in previously untreated patients was 9.9% (range, 2% - 41%). MDR-TB occurred in a median of 1.4% of individuals (range, 0% - 14.4%). Among patients with histories of treatment of 1 month or less, the prevalence of resistance to any one of the four drugs was 36% (range, 5.3% - 100%), and MDR-TB occurred in 13% of these subjects (range, 0% - 54%).[9] Thus, TB retains its position as a major cause of disease and death in the world.

2. BCG vaccine

Current approaches to the prevention of TB are badly outmoded. Bacillus Calmette-Guérin (BCG), first used about 80 years ago, and is still a widely used vaccine with a well-established efficacy for the prevention of TB meningitis and miliary TB in infants.[10] Despite high coverage with this vaccine in many countries, there is little evidence that BCG vaccination has had a significant effect on the global epidemiology of TB,[11] and it is evident that it offers highly variable protection against pulmonary TB, the most

common form of TB in adults. The protective efficacy of BCG vaccine ranged from 0% to 81% in 21 large case-control and randomized clinical trials of various BCG vaccines.[12] Moreover, one recent meta-analysis by Colditz *et al*.[9] concluded that BCG vaccines offer 50% protection against the development of all types of TB. Another meta-analysis found that the protective effect of BCG in terms of preventing pulmonary TB was so heterogeneous that no summary measure of protection could be calculated.[10] The highest protective efficacy of BCG against TB was observed in trials conducted in populations in the northern latitudes. This makes the development of new vaccines and improved vaccines against TB an urgent matter, which has been given very high priority by WHO.

3. New TB vaccines research

The ideal vaccine against TB would be safe and inexpensive, requires only a single administration, and it should have a demonstrated efficacy in all age groups and geographic areas. From the perspective of a developed country, it would be desirable if the vaccine did not convert the purified protein derivative (PPD) skin test. Several vaccine strategies are currently under investigation, which include vaccines based on bacterial DNA, culture filtrate proteins, recombinant BCG vaccines, avirulent mycobacteria as

vectors to overexpress *M. tuberculosis* proteins, and auxotrophic mycobacterial vaccines.[13]

A. DNA vaccines

DNA vaccines utilize DNA sequences of *M. tuberculosis* proteins introduced via plasmids into mice against *M. tuberculosis* infection. This method was first used by Silva and Lowrie,[14] who successfully vaccinated mice with a tumor-derived macrophage cell line transfected with the hsp60 gene in 1994. Subsequently, plasmid-based vectors encoding for Antigen 85A (Ag85A), or *Pst*S-3 were successfully used to induce protection against *M. tuberculosis* challenge in mice.[15,16]

B. Live attenuated vaccines

Two different and mutually exclusive approaches have been taken in the development of live vaccines against TB. The first involves the enhancement of the immunogenicity of BCG by producing recombinant strains that express cytokines (TNF-α, IL-2)[17] or that overexpress potentially protective antigens (Ag85B, 19 kDa lipoprotein).[18,19] The second approach to live vaccine development taken by McAdam *et al*.[20] involved the generation of attenuated strains of *M. tuberculosis* by the disruption of

specific genes (leucine and methonine auxotrophic mutants) associated with *M. tuberculosis* replication or virulent factors.[21,22]

C. Subunit vaccines

A subunit vaccine is one that contains a few key molecules of *M. tuberculosis* that are capable of inducing protective immunity.[23] These vaccines have substantial advantages over BCG or other vaccines, since the subunit vaccine consists of only a few selected molecules (e.g., proteins) rather than the thousands of different molecules (proteins, lipids, glycolipids, liposaccharides, nucleic acids, etc), and is more likely to be safe. Moreover, it might induce a stronger protective immune response than a whole-bacterium vaccine since a subunit vaccine can be constructed so as to eliminate irrelevant or even immunosuppressive components of the whole bacterium. Although a DNA vaccine encodes specific antigens like subunit vaccines, it needs many plasmids to induce an adequate immune response and protective efficacy, because DNA vaccines are less strong than other vaccines. Finally, a subunit vaccine can be rigorously standardized, allowing the production and use of a consistent preparation, and hence meaningful predictions to be made regarding its efficacy and safety.

Many attempts to define protective mycobacterial substances have been made, and from 1950 to 1970, several investigators reported the induction of

resistance to *M. tuberculosis* in animal models after experimental vaccination.[19,24-26] Since increased resistance against challenge with virulent *M. tuberculosis* is generated efficiently only by immunization with live bacilli, interest has been focused on proteins actively secreted by live bacilli during growth.[27,28] Secreted proteins have been key protective antigens, and have led to acquired resistance against *M. tuberculosis*, and among the native antigens, culture filtrate proteins (CFP) have been the most widely explored, and have been shown to be effective at reducing the bacterial loads in the lungs and spleens of mice and guinea pig TB models.[19,24-26]

Recently, in parallel with developments in biotechnology, proteomic approaches have been used to identify to such protective antigens in CFP.[29-32] Daugelat *et al*.[29] and Weldingh *et al*.[30] and used two-dimensional electrophoresis to analyze for *M. tuberculosis* CFP and found many proteins, about 2,000 spots in two-dimensional gel electrophoresis of CFP. Rosenkrands *et al*.[31] and Covert *et al*.[32] attempted to screen and purify the immunologically active proteins from CFP.

Several secreted proteins which gave partial protection were identified and included; antigen 85 (Ag85) complex (30-32 kDa),[26] 38 kDa,[33] and 16, 23, 24 and 71 kDa.[26] In some animal models, immunization with secreted protein belonging to the Ag85 complex provided protective immunity.[26] More recently, immunization with plasmid DNA encoding either Antigen 85A[15] or 65 kDa heat shock protein[16] was shown to protect mice against

challenge with *M. tuberculosis*, and importantly the Ag85 complex has been the main protective antigen against *M. tuberculosis* until now.

A major portion of the secreted protein in *M. tuberculosis* and BCG culture filtrate is accounted for the Ag85 complex, a 30- to 32-kDa family of three proteins (Ag85A, Ag85B, and Ag85C),[34-37] which all possess mycolyltransferase enzyme activity, which is required for the biogenesis of cord factor,[38] a dominant structure necessary for maintaining cell wall integrity.[39] Ag85 complex induces strong T-cell proliferation and IFN-γ production in most healthy individuals infected with *M. tuberculosis*[34] and in the BCG-vaccinated,[39] making it a promising candidate as a protective antigen.[36,37,40]

The immunodominance of the 16 kDa antigen both in the murine and human system has been recognized for over 10 years.[41-44] This knowledge has acquired greater significance in the light of more recent findings that this antigen has a crucial role in the ability of *M. tuberculosis* to survive in a state of non-replicating persistence within the host's hostile intracellular environment.[45-47] When subject to anaerobic stress, *M. tuberculosis* induces a massive up-regulation of this protein, which is associated with a thickening of the cell envelope. The antigen may stabilize cell structure during long-term survival and permit the bacilli to survive within the low-oxygen environment of the granuloma. Although classified as a heat shock protein, which are a widely distributed class of proteins, the 16 kDa antigen

has not been detected outside BCG or *M. tuberculosis*,[46,47] which is consistent with its unique role in the physiology of *M. tuberculosis*.

The mycobacterial antigen ESAT-6 can be isolated from a highly stimulatory low-molecular-mass fraction of short-term-culture filtrate (ST-CF), and this antigen is strongly recognized in TB patients,[48,49] in cattle infected with *M. bovis*,[50] and in several strains of TB-infected mice.[51] Because ESAT-6 is such a broadly and strongly recognized antigen in several species, its was has been suggested in future vaccine against TB. In addition ESAT-6 showed protective efficacy against TB when it was emulsified in MPL-DDA adjuvant.[52]

A 38 kDa lipoprotein has been identified in *M. tuberculosis* by detergent phase separation and metabolic labeling.[53] This glycosylated lipoprotein has been found both intracellularly and secreted in the extracellular culture supernatant.[53,54] This lipoprotein shares a 30% sequence identity with *Pst*S and PhoS, the later of which is a periplasmic protein of *E. coli*, suggesting that it has a role in phosphate transport.[55] This lipoprotein was recognized by murine[56] and human T-cells from PPD-positive healthy subjects and tuberculosis patients.[57,58]

No a specific protective immune response as potent as that achieved by BCG has been achieved by experimental vaccines. While *in vitro* protective immune responses to a number of *M. tuberculosis*-derived proteins have been demonstrated,[59-61] relatively little is known about *M. tuberculosis*

proteins in terms of inducing protective immune responses, particularly when more than one antigen is administered to mice. Although individual proteins, like those mentioned above, have given protective efficacy in animal models, it may be necessary to use more than one antigen in order to enhance protection against *M. tuberculosis* infection, and to examine the synergistic or additive effects of such cocktail subunit vaccines on immunogenicity and protective efficacy.

In this study, therefore, four *M. tuberculosis* antigens, namely Ag85A, ESAT-6, 16 kDa, and 38 kDa were used as components of cocktail subunit vaccines. These vaccines were then investigated for their protective efficacy against *M. tuberculosis* infection in the mouse model, and the results obtained were compared to those of the individual antigens. In addition, immunological parameters, such as, IFN-γ production, IgG isotypes and adjuvant effects on subunit vaccination were investigated.

II. MATERIALS AND METHODS

1. Mice

Specific pathogen-free female C57BL/6 mice were purchased from Japan SLC, Inc., Shijuoka, Japan. The mice were 6-7 weeks of age at the beginning of the experiments, and were maintained under barrier conditions in a BL-3 biohazard animal room at Yonsei University Medical Research Center. Animals were fed a sterile commercial mouse diet and water *ad libitum*.

2. Bacteria

Mycobacterium tuberculosis H37Rv (ATCC 27294) and *M. bovis* BCG (Pasteur strain 1173P2) were used in this study, were grown for about 10 days at 37 °C as a surface pellicle on Sauton medium enriched with 0.4% sodium glutamate and 3.0% glycerine. 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 °C until used. After thawing, viable organisms were counted by plating serial dilutions on Middlebrook 7H11 agar (Difco, Detroit, MI, USA). For inoculation of *M. tuberculosis* into mice, bacterial suspensions were

sonicated briefly in a sonic bath and diluted with PBS to reach the desired numbers.

3. Recombinant proteins and mycobacterial antigens

Recombinant proteins of *M. tuberculosis* were prepared by cloning and expressing target genes in *E. coli* using the pQE30 expression vector system. The expressed proteins were purified using a His-tag column and this was followed by a re-naturation process. Recombinant proteins prepared are listed in Table 1. The SDS-PAGE profiles of the recombinant proteins are shown in Fig. 1.

Table 1. List of recombinant proteins used in this study

Molecular weight	Name
6 kDa	ESAT-6 protein
16 kDa	*HspX*, alpha-crystalline protein
32 kDa	Antigen 85A
38 kDa	PhoS

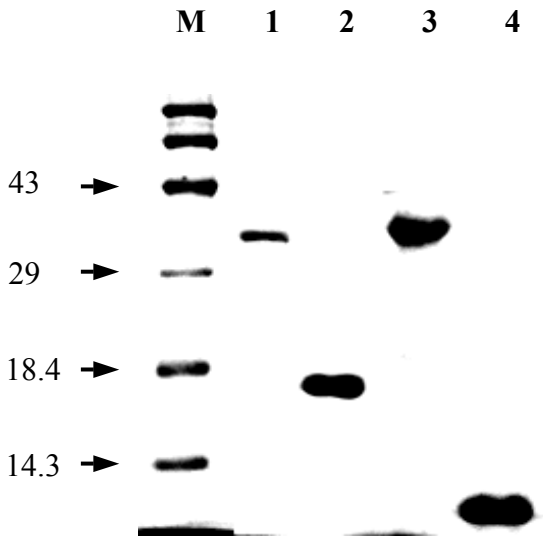

Fig. 1. SDS-PAGE analysis of purified recombinant *M. tuberculosis* antigens. M, low molecular weight marker (kilodaltons); lane 1, recombinant Ag85A; lane 2, recombinant 16 kDa; lane 3, recombinant 38 kDa; lane 4, recombinant ESAT-6. Protein bands were visualized by Coomassie blue staining.

Culture filtrated protein (CFP) was purified from *M. tuberculosis* as previously described.[62] *M. tuberculosis* bacteria (2×10^6 CFU/mL) were grown in modified Sauton medium without Tween 80 on an orbital shaker for 7 days. The culture supernatants were sterile filtered and concentrated, by ultrafiltration, over an Amicon ultrafiltration stirred cell (Amicon, Danvers, MA, USA) fitted with a PM 10 membrane (Millipore, Bedford, MA, USA). Samples were further concentrated to a final volume of about 5 mL by centrifugation in a Savant Speed-Vac (Savant Instruments, Holbrook, NY, USA). Protein content was determined by using the commercial bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), according to the manufacturer's instruction. These preparations were aliquoted into 1 mL samples and stored at –20 °C.

4. Immunization

Mice were immunized three times at 3-week intervals subcutaneously (s.c.) in the back with experimental vaccines containing 40 μg of recombinant proteins or CFP per injection with adjuvants. The following adjuvants were used with antigens in this study. Alum (Alum; Pierce, Rockford, IL, USA) was diluted 1:2 with antigen in sterile saline, according to the manufacturer's instructions. The resulting vaccines consisted of 2.2 mg of aluminum hydroxide plus 40 μg of antigen per 200 μL injection.

Monophosphoryl lipid A in stable emulsion (MPL-SE, Corixa Co., Seattle, WA, USA) was diluted with antigen in sterile saline according to the supplier's guidelines. Prior to use, MPL-SE was warmed to room temperature and vortexed briefly. MPL-SE was then diluted 1:9 with antigen in saline. The mixture contained 10 μg of MPL-SE plus 40 μg of antigens per 200 μL injection. The incomplete Freund's adjuvant (IFA; Sigma, St. Louis, MO, USA) was emulsified with equal parts of aqueous antigen with a syringe. Dimethyl dioctadecyl ammonium bromide (DDA; Sigma, St. Louis, MO, USA) was prepared as previously described.[25] Briefly, a 2.5 mg/mL suspension in sterile, pyrogen-free water was heated at 80 °C for 10 min to achieve a homogenous suspension. After cooling to room temperature, DDA was diluted with antigen. Each vaccine consisted of 50 μg of DDA plus 40 μg of antigen per 200 μL injection.

At the time of the second subunit immunization, one group of mice received a single dose of BCG (1×10^5 CFU/mouse) injected s.c. at the base of the tail. Negative controls were injected s.c. with 200 μL of sterile, pyrogen-free saline or 200 μL of adjuvants.

5. Experimental infections and bacterial enumeration in organs

Mice were challenged by aerosol exposure with *M. tuberculosis* H37Rv using an inhalation device (Glas-Col, Terre Haute, IN, USA) calibrated to deliver about 200 bacteria into the lungs. Mice were challenged 4 weeks after the last immunization, and sacrificed 4 weeks or 8 weeks after challenge. The numbers of viable bacteria in the lungs and spleens were determined by plating serial dilutions of whole organ homogenates on Middlebrook 7H11 agar (Difco, Detroit, MI, USA). Colonies were counted after incubating for 3-4 weeks at 37 °C. The resulting values are presented as means of $\log_{10}$CFU ± standard deviation per organ.

6. IgG isotype detection by enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assays (ELISA) were performed previously as described.[52] Ninety-six-well enzyme immunoassay (EIA) plates (Costar, Cambridge, MA, USA) were coated with recombinant proteins (2 μg/mL) in polycarbonate buffer (pH 9.2) overnight at 4 °C. Plates were then aspirated and blocked with PBS containing 5% (vol/vol) goat normal serum (PBST-NGS) for 2 h at 37 °C, and washed in PBS containing 0.1% Tween 20 (PBST). A dilution of serum (1/100 for total IgG,

IgG1; 1/20 for IgG2a, IgG2b) in PBST-NGS was added to the wells, the plates were incubated overnight at 4 °C, and then washed four times with PBST. For isotype analysis, bound antibodies were detected with affinity-purified biotinylated, rat anti-mouse antibodies (immunoglobulin G [IgG], 1/14,000; IgG1, 1/8,000; IgG2a, 1/2,000, and IgG2b, 1/2,000 [SEROTEC Ltd., Oxford, UK]). The plates were then washed four times in PBST, incubated with avidin-horseradish peroxidase conjugate (1/2,000 dilution; Pharmingen, San Diego, CA, USA) for 30 min, washed seven times in PBST and incubated with σ-phenylenediamine (OPD; Sigma, St. Louis, MO, USA) substrate for 15 min. The reaction was stopped by adding 1 N sulfuric acid, and plates were read at 490 nm with an Molecular Device plate reader (EL311; Biotek Instruments, Hyland Park, VA, USA).

7. Lymphocyte cultures

Lymphocytes were obtained by preparing single-cell suspension from spleens by dispersing tissue with sterilized slide glasses. Erythrocytes were lysed with a solution containing 155 mM of ammonium chloride and 10 mM of potassium bicarbonate buffer, and cells were thoroughly washed. Isolated cells were cultured in 96-well cell culture plates (Nunc, Roskilde, Denmark), each well contained 2×10^5 lymphocytes in a volume of 200 μL of RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 1% penicillin-

streptomycin, 1 mM glutamine and 10% (vol/vol) fetal calf serum. The recombinant protein or mycobacterial antigens were all used at a final concentration of 10 μg/mL. Concavalin A (Sigma, St Louis, MO, USA) was used in all experiments as a positive control for cell viability at a concentration of 2.5 μg/mL. The T-cell co-receptors CD4 or CD8 were blocked by adding monoclonal antibody L3T4 (anti-CD4;) or Ly-2 (anti-CD8; Pharmingen, San Diego, CA, USA) directly into cultures at 5 μg/mL. Supernatants were harvested from cultures after one or 6 days incubation for interleukin-10 (IL-10) or IFN-γ, respectively.

8. Cytokine assays

Cytokines present in culture supernatants were quantified by sandwich ELISA. IL-10 and IFN-γ were determined using a mouse IL-10 and IFN-γ OptEIATM Set (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. In brief, EIA plates (Costar, Cambridge, MA, USA) were coated overnight with 2 μg/mL of monoclonal anti-mouse IL-10 or IFN-γ in polycarbonate buffer (pH 9.2) at 4 °C. Free binding sites were blocked with PBST-NGS, and plates were washed with PBST. Culture supernatants were tested in duplicate. IL-10 or IFN-γ was detected with the

appropriate biotin-labeled anti-mouse monoclonal antibodies. Recombinant IL-10 and IFN-γ were used as standards.

9. Statistical analysis

Differences between the experimental group means were analyzed using the Student *t*-test. Differences were considered significant when *P* was <0.05.

III. RESULTS

1. Influence of adjuvants on production of IFN-γ by splenocytes

Since IFN-γ is an indicator of protective immunity against TB,[63] it may be useful to identify adjuvant(s) that enhance IFN-γ production by lymphocytes when used in subunit vaccines. Mice were immunized three times with cocktail antigen consisting of four antigens mixed with a series of adjuvants to compare the effects of adjuvants on IFN- γ production. Spleen cells were prepared and re-stimulated with CFP and cocktail antigen to evaluate IFN-γ production.

When spleen cells were stimulated with CFP, IFN-γ production was enhanced significantly in mice immunized with cocktail antigen mixed with MPL-SE, DDA, IFA, and MPL-SE + DDA (Fig. 2). A synergistic effect was observed when MPL-SE and DDA adjuvants were used in combination. By comparison, when spleen cells were stimulated with the cocktail antigen, strong IFN- γ production was observed in mice given DDA, IFA, and MPL-SE + DDA and moderate IFN- γ production was observed in mice given MPL-SE. Overall, MPL-SE + DDA was associated with the highest level of IFN- γ production.

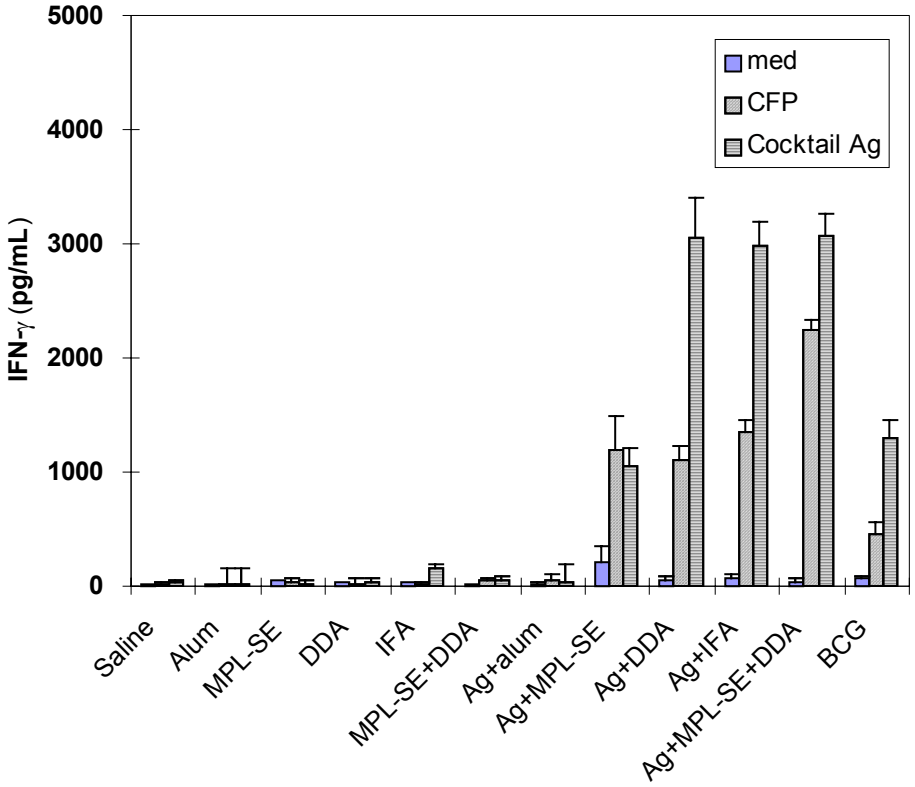

Fig. 2. IFN-γ production by splenocytes from mice immunized with subunit vaccines mixed with various adjuvants. Spleen cells were prepared from mice 5 weeks after last vaccination and re-stimulated *in vitro* with CFP and cocktail antigens. The data shown are means of triplicate analysis ± standard errors.

2. Influence of adjuvants on isotypes of antigen-specific antibodies

The IgG1 subclass responses are known to reflect Th2 cell stimulation, and IgG2a and IgG2b responses to reflect Th1 cell reactivity.[64,65] The induction of specific antibodies of these subclasses during immune response, therefore, reflects the overall Th1/Th2 balance. In order to identify adjuvant(s) that give a favorable Th1 response, cocktail antigen-specific IgG isotype antibodies were determined in sera from mice administered antigen mixed with various adjuvants. Strong total IgG and IgG1 responses to the cocktail antigen in mice immunized with antigen mixed with all adjuvants included in this study were observed (Fig. 3). Cocktail antigen-specific IgG2a responses were, however, very weak compared to IgG1 responses regardless of the nature of adjuvants. Interestingly, strong IgG2b antibody responses were noted in mice immunized with cocktail antigen mixed with MPL-SE, DDA, and MPL-SE + DDA, but only weak or null responses were noted in mice with IFA or alum, respectively. As expected, no cocktail antigen-specific IgG antibody response was evident in sera from mice immunized with saline or adjuvant alone. The results indicate that all adjuvants under investigation in this study induced strong IgG1 subclass responses to the cocktail antigen probably by stimulating Th2 cells. Only MPL-SE and DDA seemed to stimulate Th1 cells, which resulted the in generation of IgG2b subclass responses.

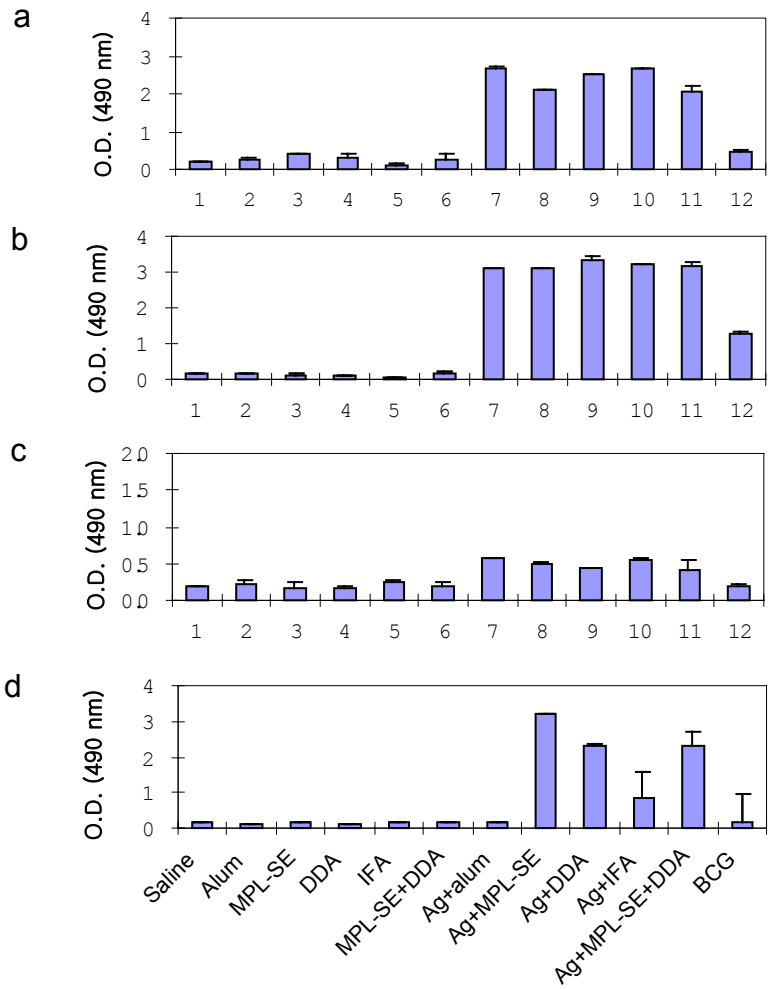

Fig. 3. IgG isotypes induced by experimental vaccines. Mice were immunized three times with a cocktail antigen emulsified in different adjuvants or received a BCG vaccination. Five weeks after the last immunization, serum samples from three mice in each group were collected, and cocktail antigen-specific a) total IgG, b) IgG1, c) IgG2a and d) IgG2b levels were determined by ELISA. Values shown indicate O.D. readings at 490 nm. The results are expressed as means of three mice ± standard errors.

3. Influence of adjuvant on protective efficacy induced by the subunit vaccines

Reduction in growth of *M. tuberculosis in vivo* has been one of the major parameters of the protective efficacy of new vaccine candidates. In order to examine the effects of adjuvants on the protective efficacy of cocktail subunit vaccines, mice were immunized three times with the cocktail antigen emulsified in a series of adjuvants and then challenged with *M. tuberculosis* H37Rv by inhalation. Bacterial loads in the lungs and spleens of mice were determined 5 weeks post-challenge. Bacterial counts were significantly reduced in the lungs and spleens of mice immunized with cocktail antigens in MPL-SE (P=0.04, Student t-test), DDA (P=0.0008), and IFA (P=0.015) versus the control mice given saline (Table 2). No significant difference in bacterial reduction was found between mice immunized with the cocktail antigen in DDA and in MPL-SE + DDA adjuvants. Interestingly, bacterial reduction in the lungs of mice immunized with the cocktail antigen in MPL-SE + DDA was as great as BCG vaccine. No significant protection was found when mice were immunized with cocktail antigen in alum adjuvant. The results indicate that the adjuvants MPL-SE, DDA, and IFA are effective at stimulating immune responses in mice treated with the cocktail antigen, which confer protection against *M. tuberculosis* infection.

Table 2. Effects of adjuvants on protective efficacy of subunit vaccine

Immunized with[b]	$Log_{10}CFU$[a]	
	Lung	Spleen
Saline	5.65 ± 0.34	4.87 ± 0.16
Alum	5.70 ± 0.23	5.01 ± 0.24
MPL-SE	5.89 ± 0.12	4.98 ± 0.34
DDA	5.84 ± 0.17	5.00 ± 0.31
IFA	5.65 ± 0.48	4.79 ± 0.39
MPL-SE+DDA	5.49 ± 0.03	4.46 ± 0.13 *
Ag[c] + Alum	5.71 ± 0.32	5.19 ± 0.57
Ag + MPL-SE	5.07 ± 0.32 *	4.31 ± 0.37 *
Ag + DDA	4.57 ± 0.23 **	3.62 ± 0.40 **
Ag + IFA	4.85 ± 0.25 *	3.70 ± 0.12 **
Ag + MPL-SE + DDA	4.38 ± 0.20 **	3.80 ± 0.12 **
BCG	4.46 ± 0.47 **	3.65 ± 0.41 **

[a] Mean $log_{10}CFU$ ± standard error. Bacterial counts from the lungs and spleens were done 5 weeks post-challenge.

[b] Each group consisted of five mice.

[c] Cocktail antigen composed of four antigens including Ag85A, ESAT-6, 16 kDa, and 38 kDa.

* $P < 0.05$, ** $P < 0.01$, Student *t*-test.

4. Comparsion of cocktail subunit vaccines in the production of IFN-γ by splenocytes

Multi-antigen cocktail subunit vaccines consisting of two to four antigens were prepared including Ag85A as the key antigen, ESAT-6, 16 kDa, and 38 kDa. Cocktail subunit vaccines used to immunize mice with MPL-SE as adjuvant and their stimulatory effects on IFN-γ production by splenocytes was evaluated. This is a well-known parameter of protective immune response in mice. The IFN-γ production in mice immunized with the cocktail subunit vaccines was then compared with that of mice immunized with individual antigen-based subunit vaccines.

When splenocytes were stimulated *in vitro* with cocktail antigen composed of four-antigens, splenocytes from mice immunized with Ag85A + 38 kDa gave the strongest IFN-γ production, followed by those from mice with Ag85A + 16 kDa, Ag85A + ESAT-6 + 38 kDa, and Ag85A + 16 kDa + 38 kDa (Fig. 4). Mice immunized with Ag85A + ESAT-6 + 16 kDa, Ag85A + ESAT-6 gave a weak but significant increase in IFN-γ production. Interestingly, mice immunized with Ag85A alone also gave a significant increase in IFN-γ production by splenocytes stimulated with cocktail antigen. However, no significant level of IFN-γ production was observed in mice

immunized with any other single antigen or with any four-antigen cocktail subunit vaccine. This suggests that immunization with more than three antigens may not stimulate IFN-γ production if the total amount of antigen is fixed at 40 μg per injection per mouse.

When each of Ag85A, ESAT-6, 16 kDa, and 38 kDa antigens was used to stimulate splenocytes, a significant level of antigen-specific IFN-γ production was observed by splenocytes from mice immunized with multi-antigen cocktail vaccine containing the relevant antigen. For an example, strong Ag85A-specific IFN-γ production was noted in mice immunized with Ag85A alone, Ag85A + 16 kDa, and Ag85A + 38 kDa (Fig. 5A). In addition, weak IFN-γ production was found in mice immunized with Ag85A + ESAT-6 + 38 kDa and Ag85A + ESAT-6 + 16 kDa. However, IFN-γ production levels were not significantly increased in mice immunized with Ag85A + ESAT-6, Ag85A + 16 kDa + 38 kDa, and all four antigens, for unknown reasons. Likewise, ESAT-6 (Fig. 5A), 16 kDa (Fig. 5B), and 38 kDa (Fig. 5B) antigens caused strong IFN-γ production in mice immunized with multi-antigen cocktail subunit vaccines containing the respective antigen.

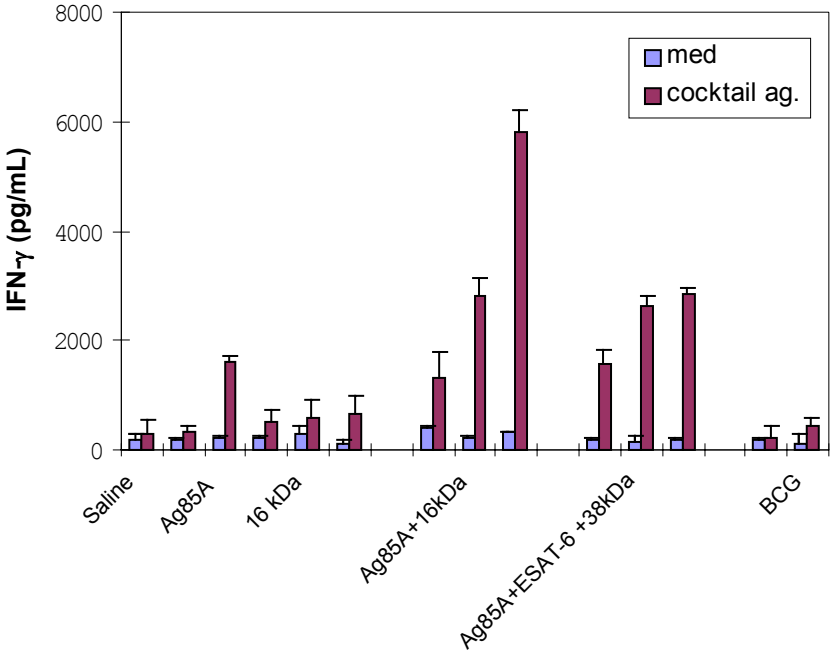

Fig. 4. IFN-γ production by splenocytes stimulated with cocktail antigen. Spleen cells were prepared from mice 5 weeks after last vaccination and re-stimulated *in vitro* with cocktail antigen. The data shown are means of triplicate analysis ± standard errors.

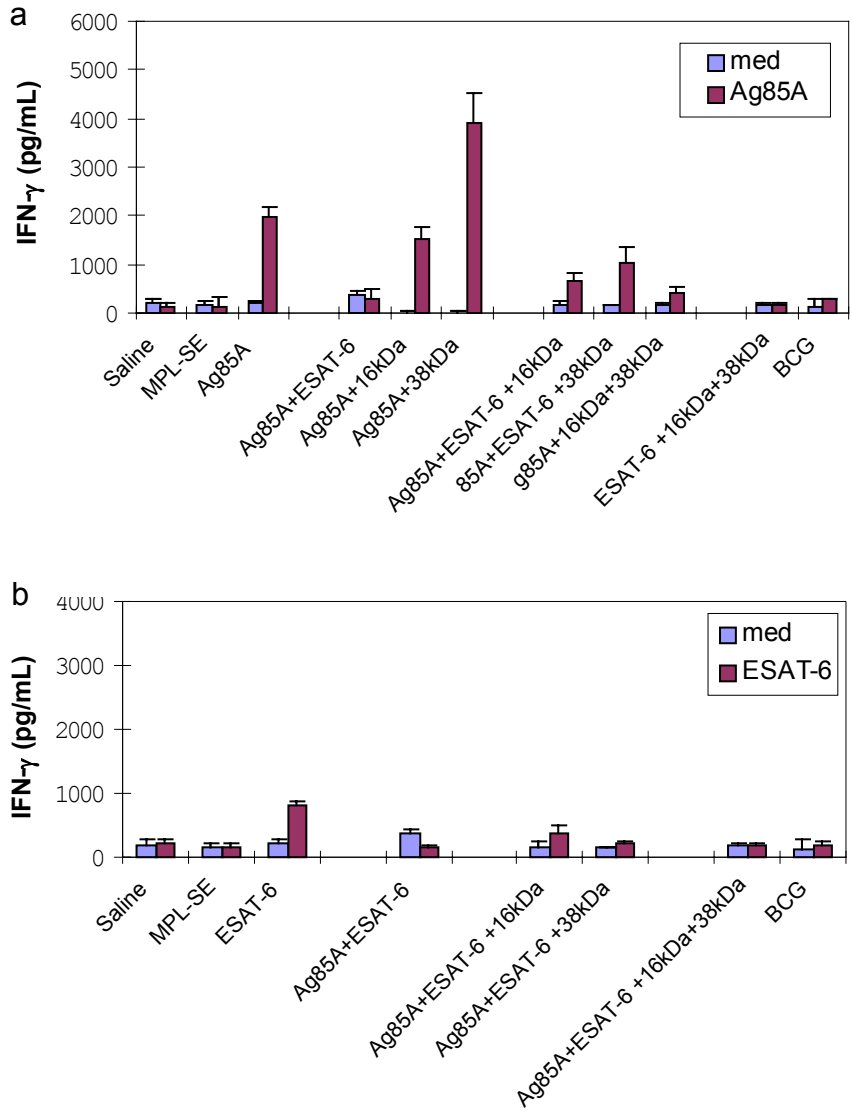

Fig. 5A. IFN-γ production by splenocytes stimulated with Ag85A and ESAT-6. Spleen cells were prepared from mice 5 weeks after last vaccination and re-stimulated *in vitro* with Ag85A (a) or ESAT-6 (b) antigens. The data shown are means of triplicate analysis ± standard errors.

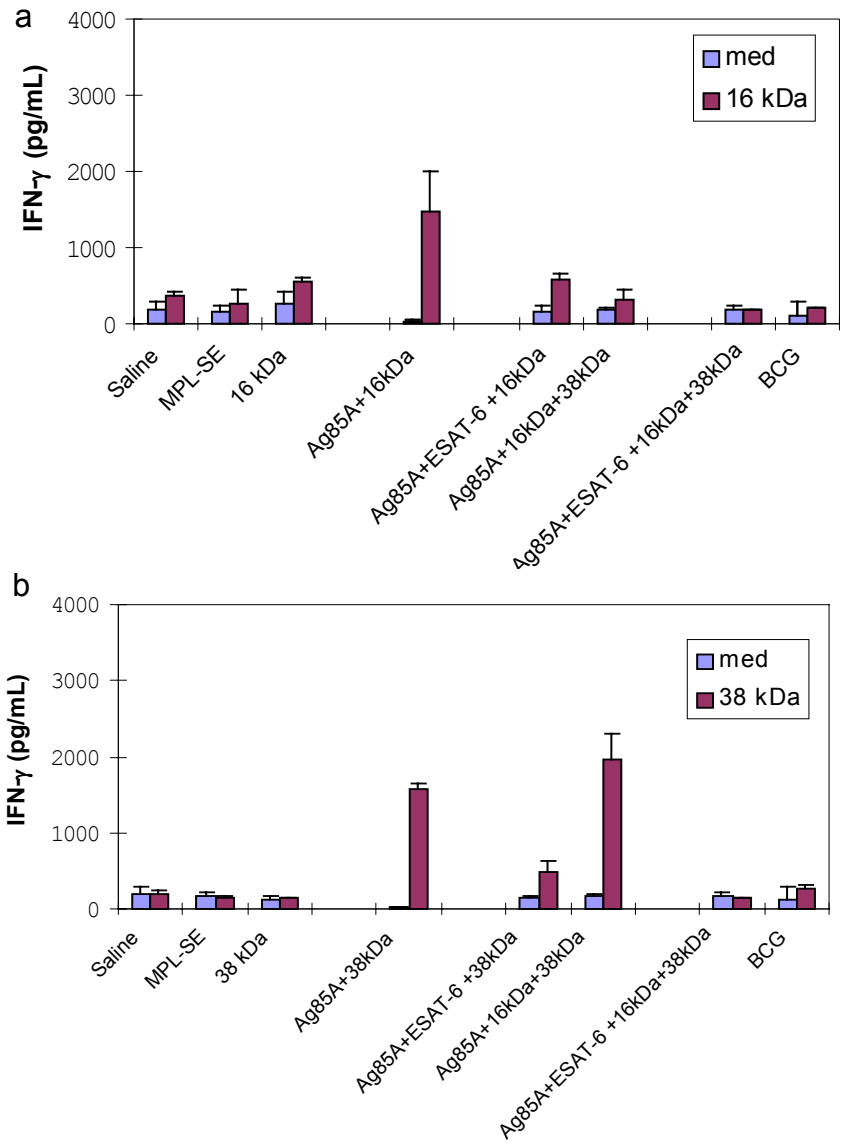

Fig. 5B. IFN-γ production by splenocytes stimulated with 16 kDa and 38 kDa. Spleen cells were prepared from mice 5 weeks after last vaccination and re-stimulated *in vitro* with 16 kDa (a) or 38 kDa (b) antigens. The data shown are means of triplicate analysis ± standard errors.

5. Analysis of T cell subset and other cytokines that contribute to protective immune responses by cocktail subunit vaccines

Cell-mediated immunity is important for protection against intracellular infections, including mycobacteria.[26,66,67] It has been accepted that protection is mainly due to antigen-specific CD4 T helper type 1 (Th1) cells, which produce IFN-γ to activate macrophages, which in turn kill the mycobacterium during phagocytosis.[68,69] However, the function of $CD8^+$ T cells against TB remains controversial.

In order to identify T cell subsets that are important for IFN-γ production, splenocytes were stimulated with *M. tuberculosis* antigens in the presence of antibodies to CD4 or CD8 markers, as blocking reagents, and examined for IFN-γ production. As shown in Fig. 6, antigen-specific IFN-γ production was reduced by more than 79% when spleen cells were incubated with anti-CD4 antibodies indicating that $CD4^+$ T cells produce the majority of IFN-γ. There were also indications of IFN-γ production by $CD8^+$ T cells, but their contribution to overall IFN-γ production was weak.

Th2 type cytokines including IL-4 and IL-10 have been implicated in the pathogenesis of TB by interfering with the host protective immune responses.[65,70,71] The culture supernatants of splenocytes from mice immunized with various cocktail subunit vaccines were also analyzed for the presence of IL-4 and IL-10, which are known as Th2 cytokines, however,

both IL-4 and IL-10 levels were under the detection limit of the assay kits used (data not shown).

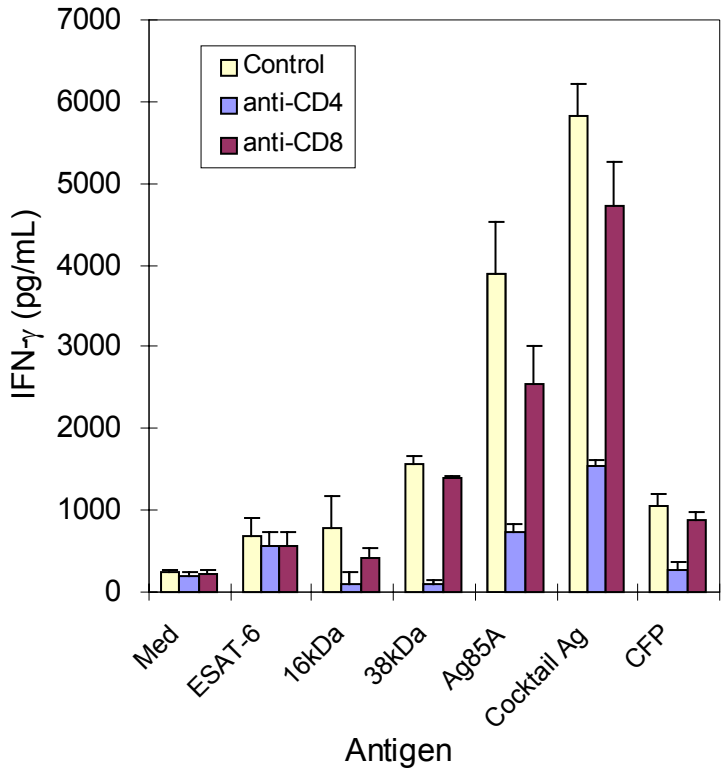

Fig. 6. Comparison of IFN-γ production by splenocytes treated with anti-CD4 and anti-CD8 antibodies. Splenocytes were prepared from mice immunized with cocktail subunit vaccine containing Ag85A and 38 kDa antigen 5 weeks after last vaccination. Splenocytes were re-stimulated *in vitro* with TB antigens with or without anti-CD4 or anti-CD8 antibodies. The data shown are means of triplicate analyses ± standard errors.

6. Humoral responses in mice immunized with cocktail subunit vaccines

Although cell mediated immunity is responsible for protection against *M. tuberculosis* infection, antigen specific IgG antibodies were detected in serum samples in order to confirm the immunization status of mice after the administration of cocktail subunit vaccines and individual recombinant proteins in this study. In addition, IgG isotypes were assessed to determine whether there was any indication of Th1 contributing to isotype switching, which can be translated to IgG2b isotype.

Serum samples were obtained from mice 5 weeks after the last treatment of cocktail subunit vaccines or individual proteins, and antigen-specific IgG and its subclass antibodies were determined by ELISA. Strong IgG reactivity to Ag85A, 16 kDa, and 38 kDa antigens was observed in the sera of mice immunized with the antigens or with cocktail antigens containing the respective testing antigen in ELISA (Fig. 7A & 7B). However, IgG responses to the ESAT-6 antigen were relatively low in all mice including those mice immunized with the ESAT-6 protein alone, for unknown reasons (Fig. 7A). The results demonstrate that mice were immunized sufficiently with Ag85A, 16 kDa, and 38 kDa antigens

individually or in combination, including four antigens cocktail, but that this was not case for ESAT-6 protein.

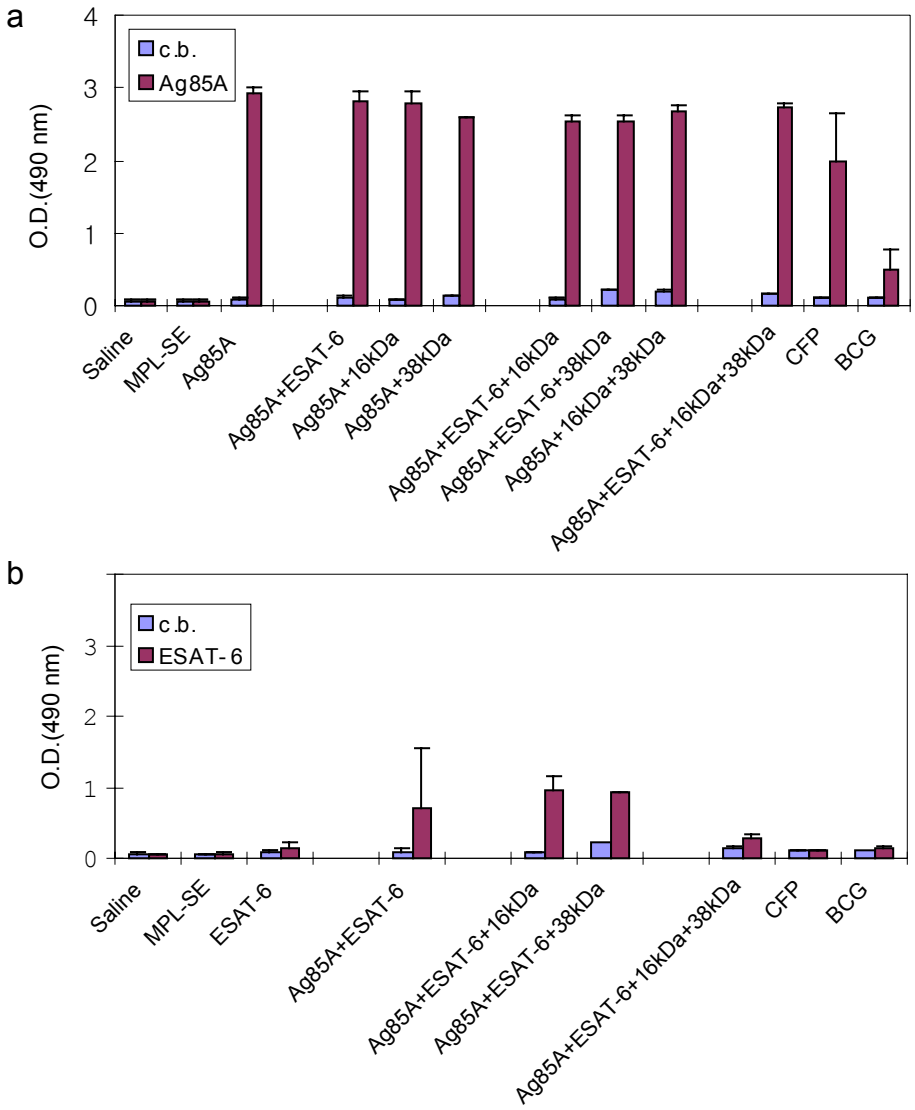

Fig. 7A. Ag85A and ESAT-6-specific IgG1 isotypes induced by cocktail subunit vaccines. Mice were immunized three times with cocktail subunit vaccines or received a BCG vaccination. Five weeks after the last immunization, serum samples from three mice in each group were collected and tested using Ag85A (a) and ESAT-6 (b) antigens. Values are indicated as O.D. readings at 490 nm, the results are expressed as the means of three mice ± standard errors.

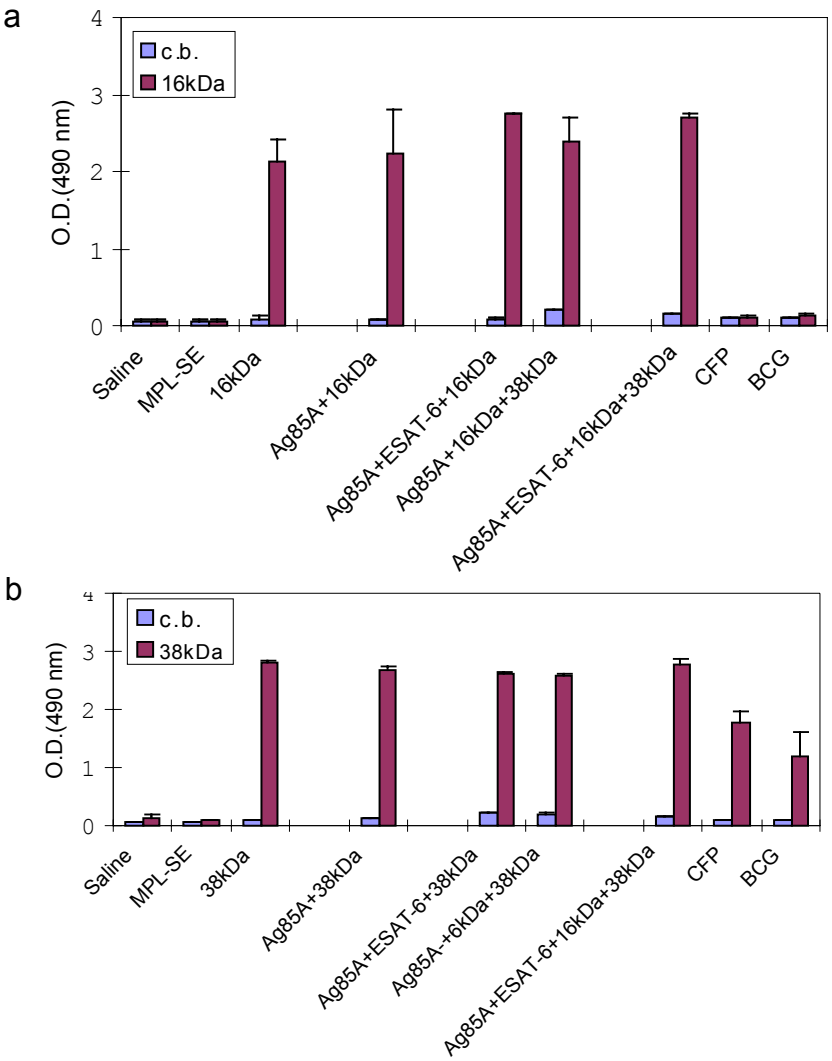

Fig. 7B. 16 kDa and 38 kDa-specific IgG1 isotypes induced by cocktail subunit vaccines. Mice were immunized three times with cocktail subunit vaccines or received a BCG vaccination. Five weeks after the last immunization, serum samples from three mice in each group were collected and tested using 16 kDa (a) and 38 kDa (b) antigens. Values were indicated as O.D. readings at 490 nm, and the results are expressed as the means of three mice ± standard errors.

7. Protective efficacy induced by cocktail subunit vaccines

One of the main objectives of this study was to identify cocktail subunit vaccines, which confer protection to mice against *M. tuberculosis* infection, as expressed by a reduced bacterial count in vaccinated mice. As described above, groups of mice were immunized with individual proteins and cocktail antigens, and then challenged with virulent *M. tuberculosis* H37Rv by inhalation. Bacterial counts were determined in the lungs and spleens of mice immunized with subunit vaccines 5 and 8 weeks after challenge, and the results were then compared to those of control mice.

When bacterial counts were determined 5 weeks post-challenge, significant reductions in the tubercle bacilli counts in the lungs of mice immunized with Ag85A + ESAT-6 ($P < 0.05$, Student *t*-test), Ag85A + 38 kDa ($P < 0.01$), Ag85A + ESAT-6 + 16 kDa ($P < 0.01$), and BCG ($P < 0.01$) as a positive control were noted. No significant bacterial reductions were observed in the lungs of mice immunized with other cocktail antigens, including four-antigen cocktails and with individual antigens 5 weeks after challenge. When bacterial counts were taken 8 weeks after challenge, significant reductions in the bacterial loads in the lungs of mice immunized with the cocktail subunit vaccines, Ag85A + ESAT-6 ($P < 0.01$), Ag85A + 38 kDa ($P < 0.01$), and Ag85A + ESAT-6 + 16 kDa ($P < 0.01$) were observed. In addition, mice immunized with a single antigen, i.e., Ag85A

($P < 0.05$), ESAT-6 ($P < 0.01$), or 16 kDa ($P < 0.05$) also gave a significant level of protection against M. tuberculosis infection in mice. In the spleens, however, mice given only Ag85A + 38 kDa showed a significant reduction in bacterial counts both 5 and 8 weeks after challenge ($P < 0.05$). These data indicate that cocktail subunit vaccines consisting of Ag85A + ESAT-6, Ag85A + 38 kDa, and Ag85A + ESAT-6 + 16 kDa provide a significant level of protection in the lungs of mice against *M. tuberculosis* infection. Of the three cocktail subunit vaccines, however, only Ag85A + 38 kDa gave consistent protection in lungs and spleens of mice.

Table 3. Protective efficacy of the cocktail subunit vaccines in mice against *M. tuberculosis* infection

Immunization with[b]	$Log_{10}CFU$[a]			
	Week 5		Week 8	
	Lung	Spleen	Lung	Spleen
Saline	6.27±0.03	5.05±0.23	6.29±0.08	5.26±0.28
MPL-SE	6.35±0.16	4.94±0.06	6.16±0.10	5.20±0.17
Ag85A	6.04±0.17	4.79±0.36	6.00±0.24*	5.32±0.16
ESAT-6	6.05±0.17	4.96±0.37	6.08±0.06**	5.12±0.17
16 kDa	6.37±0.22	5.25±0.13	4.99±0.31**	4.25±0.27*
38 kDa	6.10±0.21	5.01±0.43	6.23±0.09	5.22±0.11
Ag85A+ESAT-6	5.91±0.31*	5.17±0.13	5.85±0.20**	5.19±0.16
Ag85A+16kDa	6.11±0.32	5.37±0.51	6.10±0.13*	5.36±0.24
Ag85A+38kDa	5.89±0.07**	4.78±0.14*	5.29±0.41**	4.86±0.11*
Ag85A+ESAT-6 +16kDa	5.65±0.17**	4.63±0.25*	5.82±0.21**	5.12±0.22
Ag85A+ESAT-6 +38kDa	6.10±0.57	4.96±0.24	6.29±0.13	5.31±0.28
Ag85A+16kDa+38kDa	6.23±0.19	5.13±0.31	6.14±0.18	5.14±0.35
Ag85A+ESAT-6 +16kDa+38kDa	6.30±0.11	5.04±0.23	6.26±0.09	5.24±0.35
CFP	6.29±0.19	4.92±0.15	5.94±0.18*	5.13±0.12
BCG	5.37±0.37**	4.39±0.34**	5.58±0.28**	4.86±0.22*

[a] Mean $log_{10}CFU$ ± standard errors. Bacterial numbers are given as log_{10} CFU of *M. tuberculosis* isolated from the lungs and spleens of mice.

[b] Each group consisted of five mice.

* $P< 0.05$, ** $P< 0.01$, Student *t*-test.

IV. DISCUSSION

Subunit vaccines have been widely as one of new vaccine candidates aginst tuberculosis along with recombinant vaccines, DNA vaccines, and attenuated vaccines. Both native and recombinant proteins have been studied as subunit vaccines. In this study, cocktail subunit vaccines consisting of two to four recombinant protein antigens of *M. tuberculosis* were investigated for their efficacy against *M. tuberculosis* infection in a mouse model. In addition, five adjuvants were examined their enhancing effects on the immune responses to the recombinant antigens. The results obtained indicate that multi-antigen cocktail subunit vaccines consisting of two or three antigens are more effective than single-antigen based subunit vaccines at inducing protective immune responses and protective effects against TB even though the amount of each antigen in cocktail subunit vaccine is reduced.

For an example, a cocktail subunit vaccine consisting of Ag85A and ESAT-6 enhanced both IFN-γ production and protective efficacy compared to Ag85A or ESAT-6 antigen alone. The subunit vaccine ESAT-6 alone did not show any significant reduction in bacterial loads in the lungs and spleens. This was in contrast with the results of a previous study, in which ESAT-6 was found to give significant protection against TB.[52] The only difference between the two studies was the type of adjuvant mixed with the

ESAT-6 antigen; MPL-DDA in the previous study[52] and MPL-SE or DDA in this study. Moreover, Lise *et al*. reported that the combination of Ag85B and ESAT-6 enhanced protective efficacy against TB compared to the single antigen.[40] These results imply that ESAT-6 has an additive or synergistic effect with Ag85A or Ag85B even though the protective efficacy offered by ESAT-6 per se is weak.

The combination of Ag85A and 38 kDa also enhanced the production of IFN-γ by lymphocytes and protective efficacy. In this case, IFN-γ and bacterial reduction were well correlated. This was found to be the most protective combination among cocktail subunit vaccines composing two antigens. However, no additive effects were observed in cocktail subunit vaccines containing the 38 kDa antigen with other antigens, except for the 38kDa antigen with Ag85A.

Although IFN-γ production by splenocytes was induced by the cocktail subunit vaccine composed of Ag85A and 16 kDa was higher than that of Ag85A and ESAT-6, the cocktail subunit vaccine consisting of Ag85A and 16 kDa did not induce a significant bacterial reduction compared to saline in control mice. This tendency was noted also in cocktail subunit vaccines consisting of three TB antigens, and no significant difference was found in IFN-γ production for the subunit vaccines composing of three TB antigens. The cocktail subunit vaccine consisting of Ag85A, ESAT-6, and 16 kDa induced lower levels of IFN-γ than the other three antigen-based cocktail

subunit vaccines. However, there was a significant bacterial reduction in the lungs and spleens of mice immunized with cocktail subunit vaccine consisted of Ag85A, ESAT-6, and 16 kDa. In this case, there was also a weak correlation between IFN-γ production by lymphocytes and bacterial reduction in the organs of mice. This indicated that IFN-γ is not an absolute but relative protective indicator as demonstrated by Agger and Anderson.[72]

In mice immunized with single antigen based subunit vaccine, no significant bacterial reduction was shown at 5 weeks after challenge with *M. tuberculosis*, but bacterial reductions were shown 8 weeks after challenge. Interestingly, mice immunized with the 16 kDa antigen showed the greatest protective efficacy for single antigen based subunit vaccines. However, this result should be confirmed, because the 16 kDa antigen did not show protection at 5 weeks and also because it did not show any additive effect in mice immunized with Ag85A + 16 kDa compared to with Ag85A + ESAT-6 or with Ag85A + 38 kDa.

Interestingly, no IFN-γ production or protection against TB was observed in mice immunized with a cocktail subunit vaccine containing all four TB antigens, perhaps due to an insufficiency of each antigen, which suggests that each antigen has a minimum dose in terms of inducing protective immunity. MPL-SE used in this cocktail subunit study is known to be a weak adjuvant.[73,74] If stronger adjuvants like DDA or a combination of MPL-SE and DDA are used with cocktail antigens, cocktail subunit

vaccines composing more than three antigens would show strong protective effects against TB.[52]

These findings suggest an additive, perhaps even a synergistic effect of multiple epitopes on the development of the immune responses elicited by antigen cocktails. However, it is difficult to increase the number of antigens in antigen cocktails without limitation, because a large amount of antigens could to induce the anergy state of immune response. Therefore, it seems important to increase the numbers of antigens in cocktail subunit vaccines, but to a level less than that required to induce the anergy state. Moreover, the number of antigens in the antigen cocktails may be dependent on function and effect of the adjuvant. Effective adjuvant may enable hosts to induce protective immune responses even if the amount of antigen in the cocktail antigens is low.

In this study, therefore, we used a series of well-characterized adjuvants to stimulate immune response in mice to TB antigen from *M. tuberculosis*. The adjuvants used in this study included aluminum hydroxide, which is the most widely used adjuvant in practical vaccination, and experimental adjuvants,[75] which have been reported to be good stimulators of antibody-mediated immunity. Although Freund's complete or incomplete adjuvant is one of the most effective adjuvants known,[73] it is highly reactogenic and cannot be used in human vaccines.[76] In contrast, the adjuvant dimethyl diocadecylammonium bromide (DDA) has been found to have a low toxicity

and to induce strong cell-mediated immunity responses.[55,77,78] In addition, this adjuvant was previously used successfully for TB vaccines based on culture filtrate antigens[78] and more recently for vaccines against *M. bovis*.[79] Recently, Lise B. *et al.*[40] also found that DDA as adjuvant gave a very potent immune response to a subunit vaccine of Ag85B and ESAT-6 of *M. tuberculosis* antigens in mice, when monophosphoryl lipid A (MPL-TDM) adjuvant was used as a co-adjuvant. When cocktail antigens were emulsified in alum, there were no significant levels of IFN- γ production and the IgG2b isotype was observed, and little protection against *M. tuberculosis* infection was noted in the mice group. DDA and IFA adjuvants induced higher IFN-γ levels and protective effects than MPL-SE against TB. Furthermore, the combination of DDA with MPL-SE was found to enhance immunogenicity and protective efficacy versus DDA. However, the combination DDA and MPL-SE could not induce significantly additive or synergistic effects even though combination DDA and MPL-SE was associated with slightly higher IFN-γ production and bacterial reduction in the lungs than DDA only. In the adjuvant study, antigen-specific IgG2b isotypes expressions tended to be correlated with IFN-γ production. Since antigen-specific IgG2a isotypes were not detected, as reported by Martin and Lew,[64] whether or not IgG2b is an indicator of Th1 response in C57Bl/6 mice remains to be determined.

Interestingly, cocktail antigens emulsified in DDA or in combination of DDA and MPL-SE induced a protective efficacy that was comparable to or

greater than that of BCG in the present study. The results demonstrate that subunit vaccines could be developed as new vaccines against TB, which can replace BCG, currently the only vaccine available. With more information about relevant genomics and proteomics, the development of subunit cocktail vaccines looks promising.

V. CONCLUSION

Tuberculosis is one of the three most prevalent infectious diseases in the world, along with AIDS and malaria. Given the existing controversy concerning the efficacy of BCG vaccine against TB among adults, a new TB vaccine is urgently required to replace BCG. In an effort to develop a subunit vaccine against TB, cocktail subunit vaccines composed of combinations of recombinant proteins, such as, Ag85A, ESAT-6, 16 kDa, and 38 kDa of *M. tuberculosis* were examined in the present study to determine their protective efficacies in a mouse model of TB. In addition, four adjuvants including alum, MPL-SE, DDA, FIA, and combinations of MPL-SE and DDA were evaluated for their enhancing effects on immune responses to the recombinant antigens. The results of the study show that:

1. Th1 response, such as IFN-γ production was induced when mice were immunized with antigens emulsified in MPL-SE, DDA, or IFA adjuvants. Slightly higher IFN-γ production was induced when mice were immunized with antigen in combination with MPL-SE and DDA as opposed to the other adjuvants.

2. Antigen-specific total IgG, IgG1 titers were not different for alum, MPL-SE, DDA, and IFA. However, antigen-specific IgG2b titers were

induced strongly by MPL-SE followed DDA, combination of MPL-SE and DDA, and IFA, but alum gave no antigen-specific IgG2b response.

3. Protective efficacy was enhanced when mice were immunized with cocktail antigen emulsified in a combination of MPL-SE and DDA rather than DDA. Bacterial reduction of tissue from mice immunized with cocktail antigen in DDA and in a combination of MPL-SE with DDA was comparable to that of BCG vaccinated mice.

4. Recombinant proteins Ag85A, ESAT-6, 16 kDa, and 38 kDa induced protective immune responses more effectively when given to mice in combination rather than as single antigens.

5. Antigen-specific antibody responses were noted even though the amounts of antigens were reduced according to the numbers of antigen in the cocktail vaccines.

6. Cocktail subunit vaccine composed of Ag85A and 38 kDa, and that of Ag85A, ESAT-6, and 16 kDa gave more effective protection than other combinations of antigens against TB.

7. BCG vaccine still induced a significant level of protection against TB even though antibody titer and IFN-γ production to the antigens used in this study were lower than that observed for other experimental vaccines. These results indicate that other antigens are also involved in protective immune response against TB.

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국문요약

마우스 모델에서의 결핵에 대한 Cocktail Subunit 백신의 면역성 및 방어효과

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결핵은 AIDS, 말라리아와 함께 세계 3 대 주요 전염성 질환의 하나이다. 결핵에 대한 백신으로 BCG 가 전세계적으로 사용되고 있으나, 가장 문제가 되는 성인의 폐결핵에 대하여 BCG 백신의 백신효과가 일정하지 않아 BCG 백신을 대체할 수 있는 새로운 결핵백신의 개발이 절실한 실정이다. 결핵에 대한 subunit 백신을 개발하기 위해 결핵의 재조합 단백질인 Antigen 85A (Ag85A), ESAT-6, 16 kDa, 그리고 38 kDa을 이용하여 마우스 모델에서 결핵에 대한 방어효과를 조사하였다. 그리고 alum, MPL-SE, DDA, IFA, 그리고 MPL-SE과 DDA를 조합한 adjuvants를 사용하여 재조합 항원에 대한 면역반응과 방어효과를 증가시키고자 하였다.

C57BL/6 마우스에 결핵항원을 단독 또는 조합하여 피하로 3 주 간격으로 3 회 면역을 실시한후 4 주후에 세포성 및 항체매개 면역반응을 측정하였다. 결핵에 대한 subunit 백신의 방어능을 측정하기 위하여 *M. tuberculosis* H37Rv 결핵균을 공기감염시키고 4 주 또는 8 주 후에 폐와 비장에서 결핵균수를 측정하였다.

두 세가지 항원으로 조합된 cocktail subunit 백신으로 면역시킨 마우스에서 단독항원으로 면역시킨 경우보다 결핵에 대한 세포성 면역이 증가하였다. 하지만 4 가지 결핵항원을 조합하여 면역시킨 마우스에서는 결핵에 대한 세포성 면역반응이 미약하였다. 그러나 항체매개 면역은 두 가지 이상의 항원을 조합하여 면역시킨 마우스에서 모두 증가하였다.

결핵항원을 단독으로 면역시킨 마우스에서는 결핵에 대한 유의성 있는 방어능이 나타나지 않았지만, 둘 또는 세 가지 항원을 조합하여 면역시킨 마우스에서 폐와 비장에서 유의성 있게 결핵균수가 감소하였다. Ag85A에 ESAT-6 또는 38 kDa 항원을 조합한 cocktail subunit 백신과 Ag85A에 ESAT-6와 16 kDa을 조합한 cocktail subunit 백신에서 다른 조합으로 된 cocktail subunit 백신보다 높은 방어능을 나타내었다. 하지만 4 가지 결핵항원으로 조합한 cocktail subunit 백신에서는 유의성 있는 결핵균수의 감소가 관찰되지 않았다.

본 연구에서 한 가지 항원으로 면역시키는 것보다 둘 또는 세 가지 항원을 조합하여 면역시킬 때에 결핵에 대하여 백신효과가 증가하는 것을 관찰할 수 있었다. Ag85A에 16 kDa 또는 38 kDa 항원을 조합한 cocktail subunit

백신과 Ag85A에 ESAT-6와 16 kDa 항원을 조합한 cocktail subunit 백신이 결핵에 대한 백신후보로서의 가능성이 시사된다. 나아가 백신의 효과를 최대화하기 위하여 각 항원의 적절한 용량에 대한 연구가 필요할 것으로 사료된다.

핵심되는 말 : 결핵, *Mycobacterium tuberculosis*, Ag85A, 방어능, cocktail subunit 백신