

Increasing Cellular Immune Response in Liposomal Formulations of DOTAP Encapsulated by Fusion Protein Hspx, PPE44, And Esxv, as a Potential Tuberculosis Vaccine Candidate

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Abstract

Background: Due to the ineffectiveness of the BCG vaccine, especially in adult pulmonary tuberculosis (TB), and variable efficacies against childhood forms of TB, developing an effective TB vaccine is a major priority in controlling this disease. The aim of this study was to evaluate the immunogenicity of a DOTAP liposome formulation containing a fusion protein (FP) containing *Mycobacterium tuberculosis* HspX, PPE44, and EsxV.

Methods: The FP was expressed in *E. coli* BL21 cells and confirmed by SDS-PAGE and Western blots. The FP was then encapsulated in various liposomal formulations. Afterwards, liposomal size, zeta potential, and encapsulation efficiency were evaluated. Mice were subcutaneously vaccinated on days 0, 14, and 28 with liposomes containing the FP. Two weeks after the last injection, IFN- γ , IL-4, IL-17, and IL-12 in spleen cell culture supernatants, and IgG2a, IgG1, and IgG2b titers in sera were measured.

Results: The FP concentration was 1mg/ml. The encapsulation efficiency of the liposomes varied from 69% in Lip (DOTAP/TDB/CHOL/FP) to 80% in Lip (DOTAP/CHOL/FP). The greatest IFN- γ and IL-12 levels were observed in BCG-primed mice that were boosted with Lip (DOTAP/CHOL/FP). In addition, IL-17 production was significantly greater in all groups than controls except in those that received histidine buffer and FP. The IgG2a/IgG1 ratios were greater in the Lip (DOTAP/TDB/CHOL/FP), Lip (DOTAP/CHOL/FP), Lip (DOTAP/CHOL), and BCG-primed and Lip (DOTAP/CHOL/FP)-boosted groups than in the other groups, indicating a cellular immune response.

Conclusions: The liposomes containing DOTAP combined with the fusion protein induced a Th1 response. The mice that first received BCG and then Lip (DOTAP/CHOL/FP), produced the most IFN- γ and IL-12, indicating a strong Th1 response.

Keywords: DOTAP, Vaccines, Fusion Protein, Liposomes, *Mycobacterium tuberculosis*, Trehalose 6, 6'-Dibehenate.

Introduction

Tuberculosis (TB), an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) is

a major cause of death worldwide. In 2016, 10.4 million people had active TB, and the infection

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caused 1.3 million deaths, an unprecedented rate of death rate from infectious disease (1). Presently, bacillus Calmette-Guérin (BCG) is the only available vaccine against TB. Its efficacy against severe forms of childhood TB is proven, but its effectiveness against pulmonary TB in adults varies. Therefore, it is necessary to produce second-generation TB vaccines (2, 3). These second-generation TB vaccine efforts have focused on replacing BCG and using a new vaccine as booster for BCG. Recent investigations have included subunit, vector, and DNA vaccines (4). The most commonly studied TB vaccine candidates have been subunit vaccines, which include one or more immunodominant antigens combined with adjuvants in animal models (4, 5). HspX, PPE44, and EsxV have been examined in recent studies (6-9). Furthermore, simultaneous vaccination with multiple *Mtb* antigens may improve the protective effects against all forms of TB (4). For that reason, we used these three antigens in the form of a fusion protein. Previous studies showed antigens encapsulated in cationic liposomes induced stronger Th1 responses than antigen alone (10-12). Liposomes have also been used as adjuvants to boost the immunogenicity of peptide or protein antigens, and to produce Th1 and cytotoxic T lymphocyte (CTL) responses as well (13, 14). 1, 2-dioleoyl 3-trimethylammonium propane (DOTAP) is a cationic liposome-forming compound that has recently been used as an adjuvant in vaccine-based research. The aim of this study was to determine the most effective DOTAP liposome formulation for a subunit vaccine containing a fusion protein of *Mtb* HspX, PPE44, and EsxV and characterize its protective effect in mice.

Table 1. Liposome sizes, zeta potentials, polydispersity indexes (PDI), and fusion protein encapsulation efficiencies in the various liposomal formulations (means \pm SDs, n=3)

Formulation	Average size (nm)	Zeta potential (mV)	PdI	Encapsulation efficiencies (%EE)
Lip ^a (DOTAP ^b /CHOL ^c)	268.35 \pm 15.75	65.8 \pm 6.57	0.26 \pm 0.02	-
Lip (DOTAP/CHOL/FP ^d)	363.86 \pm 33.69	47.83 \pm 3.59	0.38 \pm 0.01	80%
Lip (DOTAP/TDB ^e /CHOL/FP)	503.63 \pm 32.12	40.5 \pm 16.42	0.5 \pm 0.03	69%

^aLiposome ^bDioleoyl-trimethylammonium-propane ^cCholesterol ^dFusion protein ^eTrehalose6,6'-dibehenate

Materials and methods

Fusion protein preparation

As we described previously, for fusion protein (FP) synthesis, HspX, PPE44, and EsxV gene sequences were derived from the NCBI site and their protein sequences from the Uniprot site. Two AEAAAKA linkers were inserted between the three protein sequences and the nucleotide sequence was optimized by for expression in *Escherichia coli* (*E. coli*) using JCat software. The sequence encoding the FP was then ligated into plasmid pET21b. *E. coli* Top10 BL21 cells were transformed with the plasmid, expression was induced, and the recombinant protein was purified by Ni-NTA chromatography (QIAGEN). The protein concentration was determined with a Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, USA)(15) and the purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (9).

Preparation of liposomal formulations

Various DOTAP liposome formulations (Table 1) were prepared by the thin film method. Briefly, cholesterol (CHOL) (4 μ mol/ml), DOTAP (4 μ mol/ml) and trehalose 6, 6'-dibehenate (TDB) (0.5 μ mol/ml) were dissolved in chloroform-methanol (2:1) and transferred into borosilicate glass tubes. The tubes were connected to a rotary evaporator to remove the organic solvent, leaving a thin lipid layer at the bottom of the tube. The lipid film was freeze-dried (16) and then hydrated in NaCl-histidine (140 mM NaCl containing 10 mM histidine, pH 6.5) with or without the FP (1 mg/ml) at 37 °C. After multiple vortexing, liposomes were sonicated for 5 min at 37 °C and extruded through 400, 200, and 100 nm polycarbonate membrane filters at 37 °C.

Liposome characterization

A dynamic light scattering instrument (Nano-ZS; Malvern, UK) was used to measure the liposomes' mean sizes and zeta potentials. Particle sizes and polydispersity indexes (PIs) were reported as means \pm standard deviations ($n = 3$). Zeta potentials were reported as means \pm zeta deviations ($n = 3$).

Indirect evaluation of protein encapsulation efficiency

To determine the amount of antigen encapsulation in the formulations, 100kDa Amicon® Ultra-4 Centrifugal Filter Units were used. The encapsulated formulations were centrifuged in Amicon filters at 2000 x g for 30 min and the protein percent encapsulation was calculated using the following formula:

$$\text{Encapsulation efficacy (EE\%)} = (\text{amount of total protein} - \text{amount of protein in the filtrate}) / \text{amount of total protein} \times 100$$

Immunization

Forty-eight 6-8-week-old female Balb/C mice were purchased from the Pasteur Institute of Iran (Tehran, Iran), divided into eight groups of six mice each, and immunized with the formulations shown in Table 2. Based on previous studies, each mouse received 50 μ g of antigen on days 0, 14, and 28 (17). Two groups of mice were primed with BCG on day 0 and then boosted with either Lipc (DOTAP/CHOL/FP) [BCG^a/Lipc (DOTAP/CHOL/FP)] or FP (BCG/FP) on days 14 and 28.

Cell culture

Three weeks after the last injection the mice were killed by cervical dislocation. The spleens were transferred to microtubes containing 1 ml of cold RPMI medium without antibiotics. The spleens

Table 2. Mouse injection groups and quantities

Injection groups	Injection quantity
Histidine Buffer	Histidine buffer (10 mM, pH 6.5)
FP ^a	FP 1 mg/ml
BCG ^b	1.5-6 \times 10 ⁶ cfu per mouse
BCG+ FP	1.5-6 \times 10 ⁶ +1 mg/ml FP
Lipc(DOTAPd/CHOLe)	DOTAP 4 μ mol/ml, CHOL 4 μ mol/ml
Lip (DOTAP/CHOL/ FP)	DOTAP 4 μ mol/ml, CHOL 4 μ mol/ml, FP 1mg/ml
Lip (DOTAP/TDBf/CHOL / FP)	DOTAP 4 μ mol/ml, TDB 0.5 μ mol/ml, CHOL 4 μ mol/ml, FP 1mg/ml
BCG+ Lip (DOTAP/CHOL/ FP)	1.5-6 \times 10 ⁶ cfu + DOTAP 4 μ mol/ml, CHOL 4 μ mol/ml, FP 1mg/ml

^aFusion Protein ^bBacillus Calmette Guerin ^cLiposome ^dDioleoyl-trimethylammonium-propane ^eCholesterol ^fTrehalose6, 6'-dibehenate

were transferred into falcon tubes with 4-5 ml of cold RPMI medium. The spleen cells were centrifuged at 300 x g for 5 min at 4 °C, the supernatants removed, and 10 ml of cold ammonium chloride buffer was added and slowly mixed with the cell pellet to lyse the red blood cells. The remaining cells were washed with 5 ml of cold RPMI and counted. To each well of a 24-well plate, 3 \times 10⁶ cells were added in 5 ml of complete RPMI media containing 10% FBS and 1% penicillin-streptomycin. Either 5 μ g/ml of the fusion antigen or 3% PHA as a positive control was added. The plates were incubated in a CO₂ incubator for 72 h, culture supernatants were collected and transferred to a sterile microtube, and centrifuged at 3000 x g for 2 min at 4 °C. The culture supernatants were stored at -80 °C until use.

ELISAs for IFN- γ , IL-12, IL-4, and IL-17 production

Enzyme-linked immunosorbent assays (ELISAs) were used to measure IFN γ , IL-12, IL-4 and IL-17 cytokine concentrations. In brief, 100 μ l of the diluted capture antibody with a coating buffer was added to the wells of 96-well plates and incubated overnight at 4 °C. After washing with 300 μ l of PBS buffer containing 0.05% Tween 20, blocking solution was added to the wells and the plates were incubated overnight at 4 °C. After washing, various dilutions of the standard and FP-stimulated cell supernatants were added to the wells. Then, 100 μ l of the diluted detection antibody was added to each well and the plates were incubated at room temperature with shaking for 2 h. After that, 100 μ l of diluted streptavidin-HRP was added to each well and the plate was incubated for 30 min at room

temperature. After washing, 100 μ l of TMB substrate solution was added per well and the plate was incubated in the dark at room temperature for 15 minutes. Finally, 50 μ l of stop solution was added to the wells and their optical densities (ODs) were measured on an ELISA reader at 450 nm with background subtraction at 630 nm.

Antibody isotype assay

Blood was collected before killing the mice, and their serum was isolated and stored at -20 °C. IgG2a, IgG2b, and IgG1 titers were measured by ELISAs according to the manufacturer's instructions (Invitrogen Inc., USA). A 96-well plate was coated with 100 μ l of PBS buffer containing 5 μ g of the FP per well and incubated overnight at 4 °C. After washing, the wells were blocked with 200 μ l of blocking buffer containing 2% BSA per well and incubated overnight at 4 °C. Serum samples diluted 1/200, 1/2,000, and 1/20,000 were added to the wells. After washing, 100 μ l of the detection antibodies were added to the wells and the plate was incubated at room temperature with shaking for 2 h. After washing, 100 μ l of TMB substrate solution was added to each well, the plate was incubated for 15 minutes at room temperature, and 50 μ l of stop solution was added to each well. Optical densities were measured on an ELISA reader at 450 nm with background subtraction at 630 nm.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the control and experimental groups, and the Tukey-Kramer test was used to compare each two groups using SPSS (version 16). $P < 0.05$ was considered significant.

Results

Fusion protein characterization

The expressed fusion protein contained 642 amino acids with an isoelectric pH of 4.74 and a molecular weight of about 70 kDa. After Ni-NTA purification and dialysis with 10 mM histidine buffer, the fusion protein concentration was determined to be 1 mg/ml via the BCA method.

Liposome characterization

The liposomes particle sizes, zeta potentials, and polydispersity indexes are shown in Table 1. The Lip (DOTAP/CHOL) empty control liposome size

was about 268 nm. However, fusion protein encapsulation increased the size significantly. This could be due to the negatively-charged nature of the fusion protein at pH 6.5 (pI 4.74), which electrostatically interacts with the positively-charged DOTAP liposomes from both inside and outside the liposome bilayer. Interaction from outside could mediate liposome aggregation.

The Lip (DOTAP/CHOL) empty control liposome zeta potential was 65.8 mV, which is due to the positively charged DOTAP in the formulation. Encapsulation of the fusion protein in the liposomes reduced the zeta potential. This reduction correlated with the percent encapsulation (Table 1). subtraction at 630 nm.

Liposomal encapsulation efficiency

The fusion protein encapsulation efficiencies in the Lip (DOTAP/CHOL/FP) and Lip (DOTAP/TDB/CHOL/FP) liposomal formulations were 80 and 69%, respectively (Table 1). This high efficiency is due to the negatively-charged fusion protein at pH 6.5 (pI 4.74) electrostatically interacting with the positively-charged DOTAP liposomes from both inside and outside the liposome bilayer.

Cytokine assay

The IFN- γ levels in all the vaccinated groups, except for the FP group, were significantly greater than that of the control histidine buffer group ($P < 0.05$). The greatest IFN- γ concentration was observed in the BCG-primed group that was boosted with Lip (DOTAP/CHOL/FP) [BCG /Lip (DOTAP/CHOL/FP)], Fig. 1A, lane 8). Although the IFN- γ concentration was greater in the mice that received the encapsulated fusion protein, the difference was not significant ($P > 0.05$). In addition, all groups except the FP group produced significantly more IL-12 than the group that received histidine buffer alone ($P < 0.05$, Fig. 1B). IL-17 production was significantly greater in all mouse groups than in the histidine buffer and FP control groups. The greatest IL-17 concentrations were observed in the Lip (DOTAP/CHOL/FP), Lip (DOTAP/TDB/CHOL/FP), and BCG /Lip (DOTAP/CHOL/FP) groups (Fig. 1C, lane 6,7 and 8). IL-4 was significantly greater in all the groups than in the histidine buffer control group ($P < 0.05$).

Furthermore, all the groups except for the Lip (DOTAP/CHOL) and Lip (DOTAP/CHOL/FP)

groups produced significantly more IL-4 than the FP group (Fig. 1D).

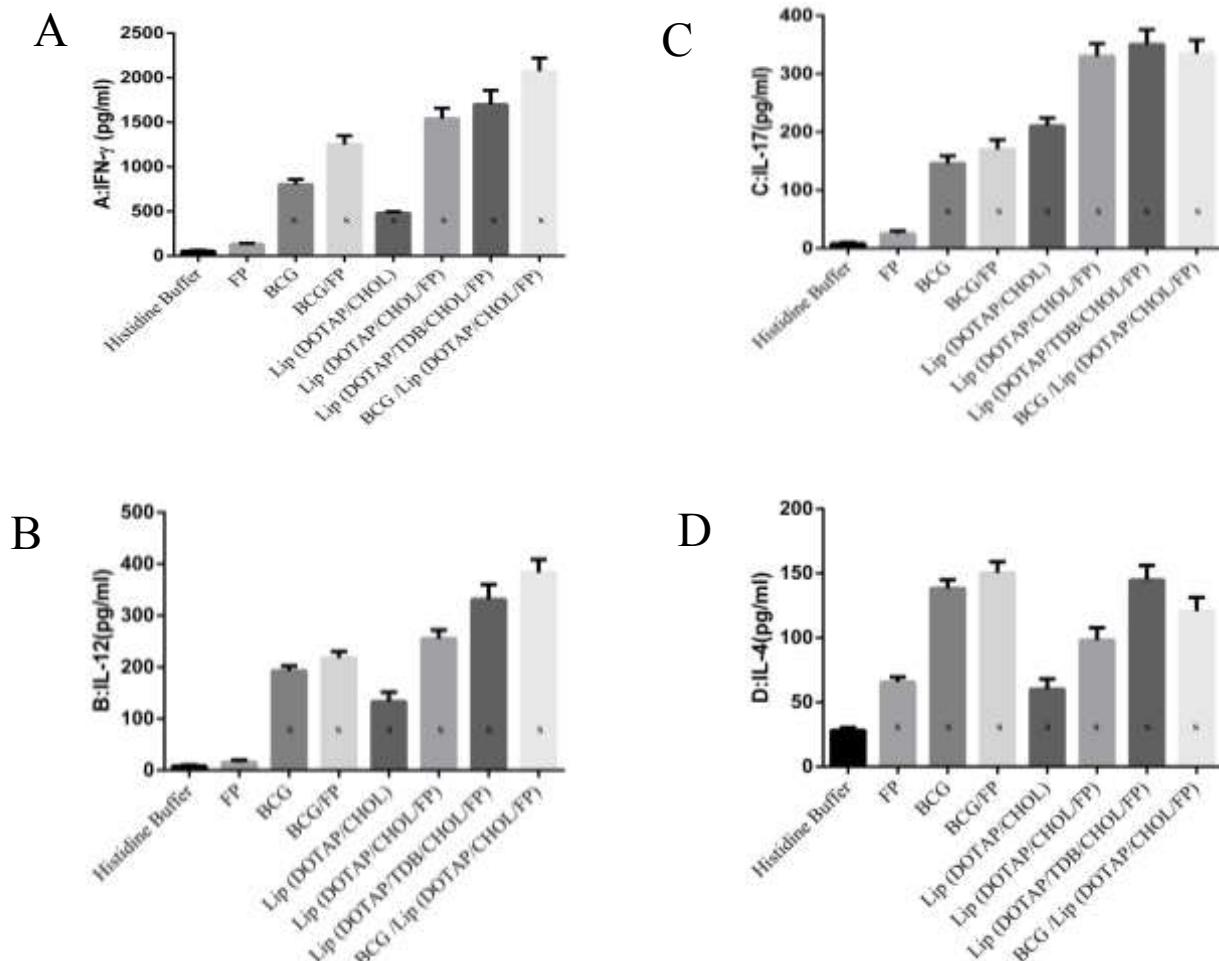


Fig. 1. Cytokine concentrations in immunized mice three weeks after the last injection. Mice were immunized three times at two-week intervals. Their spleens were removed and the splenocytes were cultured and stimulated in vitro with the HspX, PPE44, EsxV fusion protein (FP) (5 μ g/ml). IFN- γ (A), IL-17 (B), IL-12 (C), and IL-4 (D) concentrations in splenocyte supernatants were measured by sandwich ELISAs after 72 h of in vitro incubation. S: Significant difference with Histidine Buffer control group.
(DOTAP, Dioleoyl-trimethylammonium-propane; TDB, Trehalose6, 6'-dibehenate; CHOL, Cholesterol; FP, Fusion protein; BCG, Bacillus Calmette Guerin).

Antibody evaluation

To assess the immune responses of mice, besides measuring cytokines, we also measured IgG1 antibody associated with humoral immune responses, and IgG2a and IgG2b associated with cellular immune responses with serum dilutions of 1/200, 1/2,000, and 1/20,000 (Fig. 2). In this study, the IgG1 titers were significantly greater in all the groups than in the histidine buffer control group ($P < 0.005$, Fig. 2A). The greatest IgG2a antibody titers were observed in the Lip

(DOTAP/TDB/CHOL/FP) and BCG /Lip (DOTAP/CHOL/FP) (Fig. 2B, lane 7 and 8). IgG2a was also significantly greater in all the groups than in the histidine buffer control group ($P < 0.005$) (Fig. 2B). IgG2b was significantly greater in the BCG /Lip (DOTAP/CHOL/FP), Lip (DOTAP/TDB/CHOL/FP) and Lip (DOTAP/CHOL/FP) groups than in the histidine buffer and FP control groups (Fig. 2C, lane 6,7 and 8).

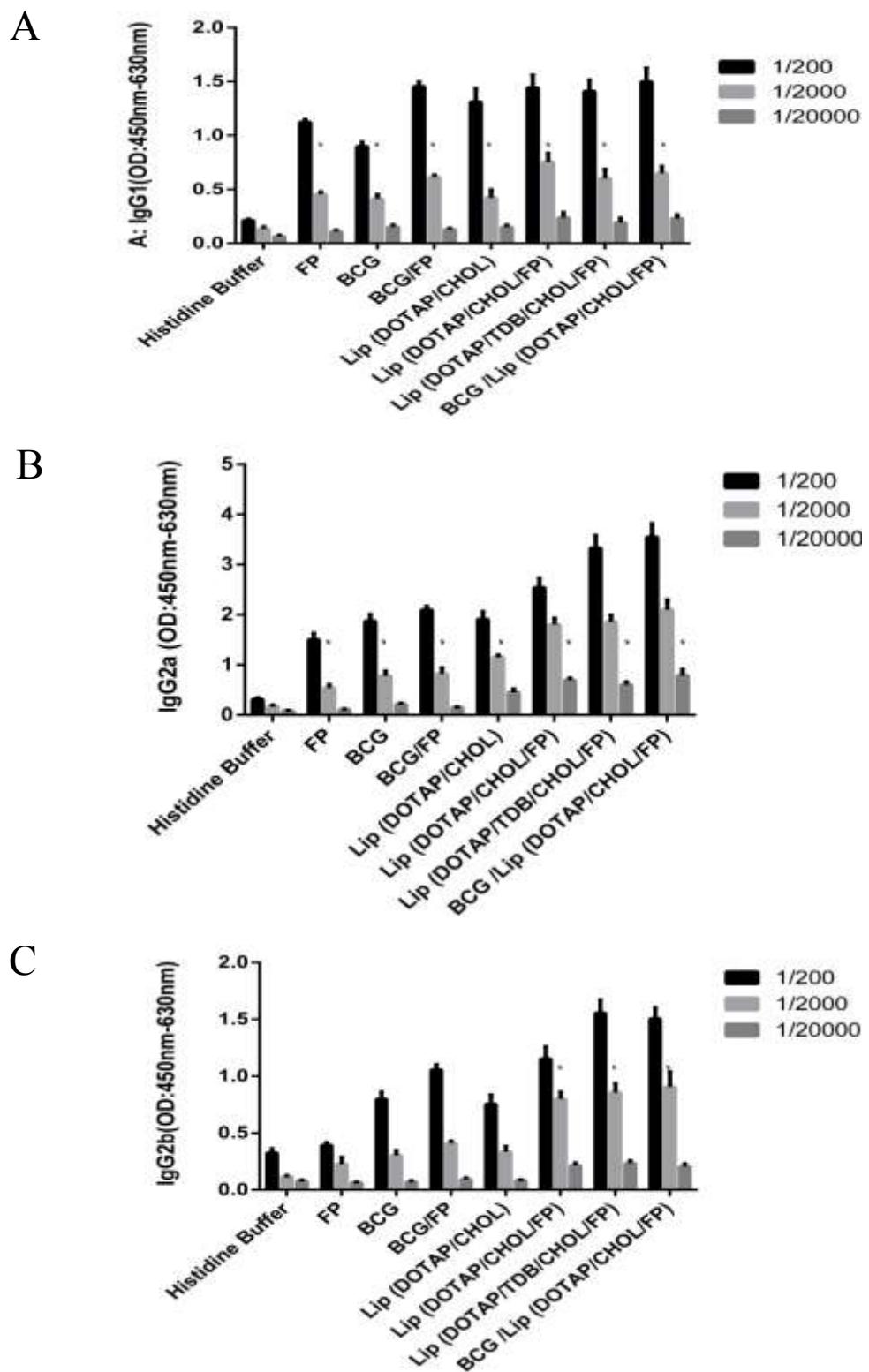


Fig. 2. IgG1 (A), IgG2a (B), and IgG2b (C) titers in sera of immunized BALB/c mice. Mice were immunized subcutaneously three times at two-week intervals. Blood samples were collected from the mice three weeks after the last injection. The IgG1, IgG2a, and IgG2b titers were determined by ELISAs. The assays were performed in triplicate at 200, 2,000 and 20,000 -fold dilutions for each serum sample. S: Significant difference with Histidine Buffer control group. (DOTAP, Dioleoyl-trimethylammonium-propane; TDB, Trehalose6, 6'-dibehenate; CHOL, Cholesterol; FP, Fusion protein; BCG, Bacillus Calmette Guerin).

No significant differences were seen between the other groups and these controls (Fig. 2C). The IgG2a/IgG1 ratio was greater in the Lip (DOTAP/TDB/CHOL/FP), Lip (DOTAP/CHOL/FP), Lip (DOTAP/CHOL), and

BCG/DOTAP/CHOL/FP groups than in the histidine buffer and FP controls, indicating a cellular immune response. The greatest IgG2a/IgG1 ratios at all three dilutions were observed in the BCG /Lip (DOTAP/CHOL/FP) (Table 3).

Table 3. IgG2a/IgG1 ratios of diluted serum from immunized mice (OD: 450 nm-630 nm).

Injection groups	1/200	1/2000	1/20000
Histidine Buffer	1.55	1.31	1.33
FP ^a	1.34	1.18	0.91
BCG ^b	2.08	1.9	1.4
BCG/FP	1.44	1.33	1.15
Lip ^c (DOTAP ^d /CHOL ^e)	1.46	2.73	3
Lip (DOTAP/CHOL/ FP ^d)	2.03	2.4	2.87
Lip (DOTAP /TDB ^f /CHOL / FP)	2.34	3	3.1
BCG + Lip (DOTAP/CHOL/ FP)	2.36	3.23	3.39

^aFusion Protein ^bBacillus Calmette Guerin ^cLiposome ^dDioleoyl-trimethylammonium-propane ^eCholesterol ^fTrehalose6, 6'-dibehenate

Discussion

Currently, the only effective TB vaccine is the BCG. BCG is an attenuated *Mycobacterium bovis* strain that can protect against many childhood forms of TB, but not against pulmonary TB (PTB) in adults (18). Therefore, to control the disease a more efficient TB vaccine is required. Vaccines under study include subunit vaccines, which contain *Mtb* immunodominant antigens (19). These vaccines may be BCG replacements or boosters (23). In addition, subunit vaccines can be used post-exposure to prevent active TB (20). In this study, a subunit TB vaccine was constructed using HspX, EsxV, and PPE44, which are expressed in the early and dormant stages of the infection. HspX is associated with latent tuberculosis infection (LTBI) and is produced during early log-phase growth. Therefore, HspX is a rational vaccine target antigen that can stimulate the immune system during early and latent infections (21).

EsxV is an acute phase antigen. ESX secretion system proteins are important members of the ESAT-6 family (22). This protein family may be important for vaccine purposes; for example EsxV and EsxW have been reported as T cell targets in TB patients (23). Furthermore, these proteins have been suggested as vaccine candidates for boosting BCG (24). PPE44, also an acute phase antigen, belongs to the Pro-Pro-Glu (PPE) protein family, and is less

abundant in the BCG vaccine than in H37Rv *Mtb*. Recent studies have examined the function, immunogenicity, and antigenic properties of PPE44 (7, 25). The purpose of this study was to evaluate the immunogenicity of a multistage tuberculosis vaccine using a combination of an early, latent, and acute phase antigens to target all TB stages. It has been shown that administration of the purified recombinant proteins alone does not sufficiently stimulate the immune system to provide protective long-term immunity. (26). It was previously shown that liposomes vaccine adjuvants have immunomodulatory effects (20). Cationic liposomes, containing DOTAP have been studied in combination with various immunomodulators including TDB (20). DOTAP has also been used as an adjuvant to enhance peptide or protein antigens immunogenicity and to produce Th1 and cytotoxic T lymphocyte (CTL) responses (27). This cationic lipid has been used in TB vaccine development (28-30).

In the present study, besides providing an optimal method to express and purify the recombinant fusion protein of *Mtb* (9), we investigated the immunogenicity of this protein with liposomes contain DOTAP with and without TDB adjuvants.

The encapsulation efficiency in two groups was high considering the positively charged liposome and the negatively charged protein; however, encapsulation efficiency was greater in the Lip (DOTAP/CHOL/FP) formulation than Lip (DOTAP/TDB/CHOL/FP) formulation. Thus, the positive charge on the liposome strongly enhances their uptake by macrophages and dendritic cells (10) Based on previous studies (10, 33), we kept the DOTAP concentration at 4 mol/ml (2.79 g/l) and the fusion protein concentration at 1mg/ml in the various liposomal formulations.

Increasing the liposome size and immune response in the Lip (DOTAP/TDB/CHOL/FP) group is due to the TDB adjuvant in this formulation. Therefore, the combination of DOTAP and TDB induces a strong immune response. Studies on animal and human models have shown that IFN- γ , produced by Th1 cells, is the most important cellular immune cytokine in stimulating a protective response against *Mtb*. In addition, IL-12, produced by macrophages and dendritic cells in response to *Mtb* infection, has a major role in driving naive T cells toward the Th1 phenotype (34). In our study, the highest IFN- γ and IL-12 concentrations were observed in the BCG-primed group that was boosted with Lip (DOTAP/CHOL/FP) [BCG /Lip (DOTAP/CHOL/FP)] (Figs. 1A and 1B).

In the BCG-primed group that was boosted with FP, (BCG/FP) ,a slight increase in the production of these two cytokines was seen. Therefore, it seems that the fusion protein alone is relatively ineffective as a booster.

In the present study, IL-17 production was high in all groups except for the histidine buffer and FP groups, especially in the Lip (DOTAP/CHOL/FP), Lip (DOTAP/TDB/CHOL/FP), and BCG /Lip (DOTAP/CHOL/FP) groups (Fig. 1C). The highest IL-17 level was observed in Lip (DOTAP/TDB/CHOL/FP) group. Therefore, it seems that TDB strongly stimulates antigen presenting cells (APCs) and subsequently induces potent Th1/Th17 responses (17). In addition, according to the study Becattini and co-workers, Th1 cells may be produced indirectly from Th17 cells (35). It has also been reported that IL-17 can act as an effector molecule against *Mtb* infection (36). Unlike Th1 and Th17 lymphocytes, Th2

lymphocytes suppress Th1 responses via IL-4 production; therefore, IL-4 can contribute to the spread of tuberculosis (37). However, the relatively low Th2 response seems to prevent immunopathological effects and balance the host's immune system after the infection. In this study, high IL-4 production was observed in the BCG-primed and Lip (DOTAP/TDB/CHOL/FP) groups. Our study and others indicate that the Th1 response is associated with a low Th2 response. (38, 39).

It is not yet clear whether humoral immunity is effective in protecting against TB. Lu et al. reported that IgG production in LTBI subjects is more effective in inhibiting TB than antibodies produced in individuals with active TB (40); therefore, it is likely that antibodies play at least some part in the immune response, such as preventing the development of TB or the conversion of latent into active TB. Additionally, antibodies protect against intracellular pathogens by immunomodulation via Fc receptor phagocytosis (40, 41) Considering that IgG1 is related to immune humoral, and IgG2a and IgG2b are related to cellular immunity, in our study the abundance and ratios of these antibodies indicate that the immune response is slanted toward the cellular arm.

Our results indicate that Lip (DOTAP/CHOL/FP) and Lip (DOTAP/TDB/CHOL/FP) liposomes alone or as boosters for BCG are suitable formulations for inducing the Th1 pathway. However, further research is needed. Lack of an animal challenge model is a limitation of the present study.

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