Potentiation of Th1-Type Immune Responses to *Mycobacterium tuberculosis* Antigens in Mice by Cationic Liposomes Combined with De-O-Acylated Lipooligosaccharide

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**Introduction**

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. Bacillus Calmette-Guérin (BCG) vaccine is the only TB vaccine currently available, but it is not sufficiently effective in preventing active pulmonary TB or adult infection. With the purpose of developing an improved vaccine against TB that can overcome the limitations of the current BCG vaccine, we investigated whether adjuvant formulations containing de-O-acylated lipooligosaccharide (dLOS) are capable of enhancing the immunogenicity and protective efficacy of TB subunit vaccines. The results revealed that the dLOS/dimethyl dioctadecyl ammonium bromide (DDA) adjuvant formulation significantly increased both humoral and Th1-type cellular responses to TB subunit vaccines that are composed of three antigens, Ag85A, ESAT-6, and HspX. The adjuvanted TB vaccine also effectively induced the Th1-type response in a BCG-primed mouse model, suggesting a potential as a booster vaccine. Finally, the dLOS/DDA-adjuvanted TB vaccine showed protective efficacy against *M. tuberculosis* infection in vitro and in vivo. These data indicate that the dLOS/DDA adjuvant enhances the Th1-type immunity and protective efficacy of the TB subunit vaccine, suggesting that it would be a promising adjuvant candidate for the development of a booster vaccine.

**Keywords:** *Mycobacterium tuberculosis*, adjuvant, de-O-acylated lipooligosaccharide, booster vaccine
elicit sufficient immune responses to prevent *M. tuberculosis* infection [6]. To effectively induce strong cell-mediated immune responses, TB subunit vaccines are co-administered with adjuvants [6]. Adjuvant combinations consisting of two or more classical adjuvants with different modes of action are more effective in promoting both humoral and cellular immune responses to various vaccine antigens [7, 8]. Several adjuvant systems, including AS01, IC31, and CAF01, have been shown to significantly increase Th1-type cellular responses and, thus, the protective efficacy of TB subunit vaccines in mouse models and human clinical trials [9–13].

De-O-acylated lipooligosaccharide (dLOS) is a TLR4 agonist derived from an *Escherichia coli* lipooligosaccharide containing a short carbohydrate moiety [14, 15]. In a previous study, we demonstrated that dLOS activated innate immunity and induced Th1-, Th2-, and Th17-type immune responses [15]. In addition, dLOS-based adjuvants were effective in enhancing both humoral and cellular immune responses to various bacterial and viral vaccine antigens [14, 16–22]. The combination of dLOS with alum increased antigen-specific antibody responses and Th1-type cellular immune response to human papillomavirus (HPV) L1 virus-like particles and anthrax protective antigen, and maintained immune responses up to 24 weeks after immunization [20, 21]. Furthermore, the dLOS/alum-adjuvanted influenza vaccine showed increased protective efficacy against a lethal influenza virus without inducing harmful inflammatory responses following viral infection in vaccinated animals [22]. Subsequently, the safety and immunogenicity of the dLOS/alum-adjuvanted HPV vaccine was demonstrated in a Phase I clinical trial.

In this study, we investigated the adjuvanticity of two dLOS-based adjuvant formulations to recombiant TB antigens. The results showed that cationic liposome combined with dLOS increased not only humoral and Th1-type cellular immune responses to TB antigens but also protective efficacy against *M. tuberculosis* infection.

## Materials and Methods

### Materials

*M. tuberculosis* strain BCG was kindly provided by Prof. Eun-Kyong Jo at Chungnam National University, Korea. Aluminum hydroxide (alum) and calcium phosphate (CAP) were purchased from Brenntag Biosector (Denmark). Cationic lipid dimethyl dioctadecyl ammonium bromide (DDA) was obtained from Sigma-Aldrich (USA). Goat anti-mouse IgG and IgG2b antibodies were purchased from Jackson ImmunoResearch (USA) and Serotec (UK). Cell culture media and antibiotics were purchased from Gibco/Invitrogen (USA). Interferon (IFN)-γ cytokine ELISA and ELISpot kits were purchased from R&D Systems (USA) or BD Biosciences (USA).

### Preparation of TB Antigens and Adjuvants

Three recombinant TB antigens, Ag85A, ESAT-6, and HspX proteins, were expressed in *E. coli* and purified by ion-exchange chromatography. The purified recombinant proteins were quantified using a BCA protein assay kit (Pierce, USA). Residual endotoxin levels of the recombinant proteins were less than 7 EU/μg protein as determined using the LAL-based assay Endosafe-PTS (Charles River Laboratories, USA). The TB subunit vaccine was prepared by mixing Ag85A, ESAT-6, and HspX proteins at a ratio of 2:1:2.

**dLOS** was prepared from an *E. coli* LPS mutant strain as previously described by Cho et al. [14], quantified using the 2-keto-3-deoxyoctonate assay [23], and visualized on a silver-stained SDS-polyacrylamide gel. Cationic DDA liposome was prepared by an aqueous heating method as described by Holten-Andersen et al. [24]. The TB vaccine mixed with the liposome formulation was used for immunization immediately after preparation, whereas the TB vaccine mixed with the alum or CAP formulation was incubated on ice for 2 h before use for immunization. The adjuvant doses used for immunization were as follows: 25 μg/mouse for alum and CAP, 0.5 μg/mouse for dLOS, and 25 or 50 μg/mouse for DDA.

### Immunization of Mice

Specific pathogen-free 6-week-old female C57BL/6 mice were purchased from SLC (Japan) and randomly assigned into experimental groups consisting of six mice. The mice were immunized intramuscularly three times at 2-week intervals with the TB subunit vaccine alone or in combination with various adjuvants. For a BCG priming-boosting model, the mice were immunized with either the TB vaccine containing various adjuvants or the BCG vaccine (5 × 10⁷ colony forming units (CFUs)/mouse) (Japan BCG Laboratory, Japan), and 2 weeks later, boosted twice at a 2-week interval with the adjuvanted TB vaccine. The mice were sacrificed 3 or 4 weeks after the last immunization, and immune responses to TB antigens were assessed. All animal experiments were reviewed and approved by the Animal Care and Welfare Committees of Sejong University and Yonsei University.

### Determination of Antigen-Specific Serum Antibody Titers

TB antigen-specific antibody titers in the sera of individual mice were measured by endpoint-dilution ELISA. A 96-well ELISA plate was coated with 1 μg/ml of Ag85A, ESAT-6, or HspX, or a mixture of the three antigens, and blocked with 1% BSA in PBS for 1 h at 37°C. The plate was washed five times with PBS containing 0.05% Tween 20 and incubated for 2 h at 37°C with mouse sera that was 2-fold serially diluted with 1% BSA in PBS. Bound antibody was detected with HRP-conjugated goat anti-mouse IgG or IgG2b antibody, and color development with 3,3′,5,5′-
tetramethylbenzidine substrate. A volume of 1 N H₂SO₄ (100 μl) was added to terminate the reaction, and the absorbance at 450 nm was measured using an Infinite M200 microplate reader (Tecan, Switzerland). The endpoint titer was defined as the highest serum dilution with an absorbance value two times greater than the non-immune serum absorbance value, with the cut off value set at 0.1. The geometric mean titers (GMT) were calculated from individual log₁₀-transformed titers and expressed as GMT ± standard deviation (SD) of six individual sera for each experimental group.

Measurement of IFN-γ Cytokine Levels

Single cell suspensions of mouse spleens and lungs were prepared as described by Sable et al. [25]. Cells were seeded at a density of 1 x 10⁶ or 5 x 10⁶ cells/well in a 96-well culture plate and were stimulated with 10 μg/ml of a mixture of the three TB antigens or BCG at 37°C in a CO₂ incubator for 72 h. Culture media were collected, and secreted IFN-γ levels were determined by sandwich ELISA.

IFN-γ ELISpot Assay

The numbers of IFN-γ-secreting cells were determined by ELISpot assays as previously described by Han et al. [20], with slight modifications. Splen and lung cells were stimulated with 10 μg/ml of Ag85A, ESAT-6, or HspX or a mixture of the three antigens, for 24 h. The numbers of spots in the developed plates were counted using a CTL-ImmunoSpot S5UV Micro Analyzer (Cellular Technology, USA).

In Vitro M. tuberculosis Growth Inhibition Assay

In brief, bone marrow cells were isolated from the femurs of C57BL/6 mice and cultured in the presence of 10% L-929 cell culture supernatant for 7 days to differentiate into bone marrow macrophages. The bone marrow macrophages were seeded at a density of 1 x 10⁶ cells/ml in a 24-well culture plate and infected with M. tuberculosis strain Erdman. Mice were sacrificed 4 or 8 weeks post-infection, and the lungs and spleens were collected. The numbers of live bacteria in organ samples were determined by plating serial dilutions of organ homogenates on Middlebrook 7H10 Bacto Agar containing 10% OADC and amphotericin and incubating at 37°C for 4 weeks. Results are presented as the geometric means ± SD of the values obtained from five mice in each group.

Statistical Analysis

SPSS 18.0 software (IBM, USA) was used to perform statistical analysis. Differences among experimental groups were analyzed using one-way ANOVA with Tukey’s multiple comparison test. p-Values < 0.05 were considered significant.

Results

Increase in Immune Responses against TB Antigens by dLOS-Based Adjuvants

To investigate whether a TB subunit vaccine combined with adjuvants, including CAP, dLOS, or DDA, effectively induces immune responses, mice were immunized with the TB subunit vaccine alone or together with different adjuvants. Three weeks after the last immunization, total serum IgG and IgG2b antibody titers against Ag85A, ESAT-6, HspX, or a mixture of the three were determined. All of the groups immunized with the adjuvanted TB vaccine, except for the CAP-adjuvanted vaccine group, showed significant increase in both total IgG and IgG2b antibody titers compared with the non-adjuvanted vaccine (Fig. 1). Among the adjuvants, the two dLOS-based formulations were more effective than dLOS alone.

Next, to evaluate Th1-type cellular immune responses induced by the adjuvants, we collected splenocytes from the mice administered the non-adjuvanted or adjuvanted vaccines, and assessed for the number of IFN-γ-secreting cells upon stimulation with TB antigens by ELISpot assay. The mice administered CAP- or dLOS-adjuvanted TB vaccines were not any higher in the number of IFN-γ-secreting cells compared with the non-adjuvanted vaccine.
Both groups immunized with dLOS/CAP- or dLOS/DDA-adjuvanted TB vaccines exhibited higher numbers of TB-specific IFN-γ-secreting cells, but the dLOS/DDA-adjuvanted vaccine group yielded better results. The levels of IFN-γ secreted into the culture media were determined by cytokine ELISA and found to be consistent with the results obtained by IFN-γ ELISpot assay (data not shown). These data suggest that both dLOS-based adjuvant systems enhance humoral and cellular immune responses to recombinant TB antigens.

In Vitro M. tuberculosis Growth Inhibition by the Adjuvanted TB Vaccine

Next, we tested whether immune splenocytes are able to inhibit the growth of intracellular M. tuberculosis (Fig. 3). For this study, we employed the M. tuberculosis in vitro growth inhibition assay. BCG-infected macrophages were co-cultured with splenocytes from mice immunized with the non-adjuvanted or adjuvanted TB vaccines for 7 days, after which live bacteria in the infected macrophages were determined. All of the groups immunized with adjuvanted TB vaccines were able to inhibit bacteria growth, but the
CAP- and dLOS/DDA-adjuvanted vaccines, in particular, were more effective in inhibiting the growth, decreasing the bacteria number by 60% and 65%, respectively, as compared with the PBS control group, although the differences among the groups were not statistically significant.

**Induction of Th1-Type Cellular Immune Responses by the dLOS/DDA-Adjuvanted TB Vaccine**

We next compared the ability of the dLOS-based adjuvants to induce Th1-type immune responses to TB antigens in naïve and BCG-primed mouse models. Mice were immunized with either the BCG vaccine or TB subunit vaccine in the absence or presence of the dLOS-based adjuvants. Two weeks later, the mice were given two booster injections of the non-adjuvanted or adjuvanted TB vaccines. Four weeks after the last immunization, spleen and lung cells were collected for assays. As shown in Fig. 4, IFN-γ responses to TB antigens were significantly higher in the spleen cells from the mice immunized with the dLOS/DDA-adjuvanted TB vaccine, irrespective of BCG-priming, than in those from the mice immunized with the dLOS/alum- or dLOS/CAP-adjuvanted TB vaccines. In contrast, in the lung cells, the IFN-γ response induced by the dLOS/DDA-adjuvanted vaccine was far greater in naïve mice than in BCG-primed mice. BCG stimulation also elicited 2–2.5-times higher levels of IFN-γ secretion from the splenocytes of the adjuvanted vaccine groups in BCG-primed mice but not in naïve mice, although differences among the groups were not statistically significant. We also determined the levels of IL-5 in the culture media of the splenocytes and found that the level was negligible in all experimental groups, which suggested that the immune responses induced by the dLOS/DDA-adjuvanted vaccine was predominantly Th1-type biased.

To determine proliferative T-cell responses upon stimulation with TB antigens, splenocytes from immunized mice were labeled with CFSE and incubated with recombinant TB antigens. After 3 or 7 days, CFSE-labeled cells were stained with anti-CD4 and anti-CD8 mAbs, and then proliferative responses were analyzed by monitoring the progressive decrease of fluorescence with each cell division. Overall, mice administered the dLOS-based adjuvanted vaccines exhibited increased proliferative responses of both CD4+ and CD8+ T-cells compared with these given the non-adjuvanted vaccine, but higher responses were observed in the dLOS/DDA-adjuvanted vaccine groups than the dLOS/alum- and dLOS/CAP-adjuvanted vaccine groups (Fig. 5). In addition, both CD4+ and CD8+ T-cell responses were generally greater in BCG-primed mice than in naïve mice. Taken together, these results demonstrate that dLOS-based adjuvants, particularly dLOS/DDA, increase Th1-type immune responses and have a potential to boost cellular immune responses after BCG vaccination.

**Protective Efficacy of TB Vaccines Adjuvanted with dLOS-Based Adjuvant Systems against M. tuberculosis Infection in Mice**

Next, we evaluated the protective efficacy of the adjuvanted TB vaccines in a mouse challenge model. Mice were immunized three times with 10 μg of the TB subunit vaccine in the absence or presence of the dLOS-based adjuvants. As controls, groups of mice were immunized with PBS, dLOS/DDA, or BCG vaccine. Four weeks after the last immunization, the mice were challenged with M. tuberculosis, and protective efficacy was assessed by determining the bacterial load in the lungs and spleens (Fig. 6). Among the experimental groups, mice receiving the BCG vaccine exhibited the highest protective efficacy in both spleens and lungs, followed by those receiving the dLOS/DDA-adjuvanted vaccine. It is of note, however, that mice administered the BCG vaccine exhibited an increased bacterial burden at week 8 post-infection as compared with that at week 4, both in the spleen and lung \((p < 0.05)\) and \((p < 0.005)\), respectively, whereas mice immunized with the dLOS/DDA-adjuvanted vaccine showed a decreased or unchanged bacterial burden. These data suggest that long-term follow-up may show a higher bacterial burden in the
Bacterial DNA vaccination, composed of various vaccine antigens [17, 19, 21, 22]. These dLOS-adjuvant systems were shown to promote cellular immune responses as well as antibody-mediated immune responses. In the present study, we evaluated the adjuvant activity of dLOS-based adjuvant systems toward recombinant TB antigens. Our findings demonstrate that vaccination of mice with TB subunit vaccine adjuvanted with dLOS-based adjuvant formulations, particularly with the dLOS/DDA adjuvant, significantly elevated TB antigen-specific IFN-γ responses by de-O-acylated lipooligosaccharide (dLOS)-based adjuvants.

Mice were immunized i.m. either with the BCG vaccine (5 × 10⁴ CFU) or TB vaccine (2.5 μg/mouse) alone or in combination with dLOS-based adjuvants and, 2 weeks later, boosted twice at a 2-week interval with the non-adjuvanted or adjuvanted TB vaccine. Four weeks after the last immunization, spleen (A and C) and lung (B and D) cells were collected and stimulated with BCG or a mixture of three TB antigens. The numbers of IFN-γ-secreting cells (A and B) and the levels of IFN-γ secreted into the culture media (C and D) were determined by ELISpot assay and sandwich ELISA, respectively. Data are expressed as the mean ± SD of values obtained from three samples that used two organs each. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001 as compared with the group administered the non-adjuvanted TB vaccine. #p < 0.05; ##p < 0.01 as compared with the group administered BCG vaccine only.

**Discussion**

TB, caused by infection with *M. tuberculosis*, continues to be a serious worldwide scourge that requires a new generation of TB vaccines [1]. BCG, the only TB vaccine currently available, has critical limitations, especially with respect to preventing active adult pulmonary TB and latent TB infections [2]. Despite considerable progress in recent decades, new TB vaccines are still under the developmental stage [4]. Subunit vaccines for TB have shown better safety and stability profiles, as well as the ability to boost prior BCG vaccination [5]. However, when used as a stand-alone vaccine, these antigens are unable to protect against *M. tuberculosis* infection, but instead require adjuvants to elicit a strong immune response [8].

In our previous studies, we developed dLOS-based adjuvant systems composed of dLOS and alum, a bacterial DNA fragment, and various vaccine antigens [17, 19, 21, 22]. These dLOS-adjuvant systems were shown to promote cellular immune responses as well as antibody-mediated immune responses. In the present study, we evaluated the adjuvant activity of dLOS-based adjuvant systems toward recombinant TB antigens. Our findings demonstrate that vaccination of mice with TB subunit vaccine adjuvanted with dLOS-based adjuvant formulations, particularly with the dLOS/DDA adjuvant, significantly elevated TB antigen-specific IFN-γ responses by de-O-acylated lipooligosaccharide (dLOS)-based adjuvants.

Mice were immunized i.m. either with the BCG vaccine (5 × 10⁴ CFU) or TB vaccine (2.5 μg/mouse) alone or in combination with dLOS-based adjuvants and, 2 weeks later, boosted twice at a 2-week interval with the non-adjuvanted or adjuvanted TB vaccine. Four weeks after the last immunization, spleen (A and C) and lung (B and D) cells were collected and stimulated with BCG or a mixture of three TB antigens. The numbers of IFN-γ-secreting cells (A and B) and the levels of IFN-γ secreted into the culture media (C and D) were determined by ELISpot assay and sandwich ELISA, respectively. Data are expressed as the mean ± SD of values obtained from three samples that used two organs each. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001 as compared with the group administered the non-adjuvanted TB vaccine. #p < 0.05; ##p < 0.01 as compared with the group administered BCG vaccine only.
It has been reported that CD4+ T-cells provide protective immunity against TB through secretion of IFN-γ, and thus are essential in controlling the initial TB infection [26, 27]. CD8+ T-cells are also associated with protective immunity against TB [28, 29]. They produce several types of cytokines, including IL-2, IFN-γ, and TNF-α, during M. tuberculosis infection and kill M. tuberculosis-infected cells via granule-mediated cytotoxicity or Fas-induced apoptosis [29, 30]. We observed that the dLOS/DDA adjuvant promoted the secretion of IFN-γ in response to TB antigens mainly by CD4+ T-cells (data not shown). In addition, administration of the dLOS/DDA-adjuvanted vaccine induced both CD4+ and CD8+ T-cell proliferative responses to TB antigens. Taken together, these data indicate that the dLOS/DDA adjuvant is able to promote cellular immune responses associated with protective immunity in response to co-administered TB antigens, suggesting that this combination is suitable for TB subunit vaccines.

IL-17 is also a critical part of the defense against specific Th1-type immune responses as well as serum IgG antibody titers.

Fig. 5. CD4+ and CD8+ T-cell proliferative responses to TB antigen.

Splenocytes from the mice that were immunized as described in Fig. 4 were labeled with CFSE and stimulated with a mixture of 3 recombinant TB antigens (10 μg/ml). Three or seven days later, CFSE-labeled cells were stained for CD4 (A) or CD8 (B) and subjected to flow cytometry. CD4- or CD8-positive cells were gated, and CSFE intensity was analyzed. Data are expressed as the mean fluorescence intensity (MFI) ± SD of values obtained from three samples that used two spleens each. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001 as compared with the group administered the non-adjuvanted TB vaccine; ###p < 0.001 as compared with the group administered BCG vaccine only.

Fig. 6. Protective efficacy by adjuvanted TB vaccines against M. tuberculosis infection.

Mice (n = 5) were immunized three times at 3-week intervals with the non-adjuvanted or adjuvanted TB vaccines. Control mice were given PBS or dLOS/DDA adjuvant alone. A BCG vaccine control group was also included. Four weeks after the last immunization, the mice were infected by an aerosol challenge with M. tuberculosis. Four or eight weeks post-infection, the numbers of live bacteria in the spleen (A) and the lung (B) of the infected mice were determined as described in Materials and Methods. Results are represented as the geometric mean ± SD of values obtained from five individual mice in each group. Statistical differences were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. *p < 0.05; ***p < 0.001 as compared with the group administered the non-adjuvanted TB vaccine.
extracellular bacterial pathogens [31]. It provides protective immunity against the hypervirulent M. tuberculosis by promoting chemokine expression in the lung and recruiting CD4+ T-cells, which inhibit the bacterial infection by producing IFN-γ [31, 32]. In the present study, we observed that significantly higher levels of IL-17 were induced in response to TB antigens in the spleen and lung cells collected from the mice administered the dLOS/DDA-adjuvanted vaccine (data not shown). This may further contribute to the protective immunity of the vaccine against M. tuberculosis infection.

In this study, we evaluated the immune responses elicited by administration of a mixture of three recombinant TB antigens, Ag85A, ESAT-6, and HspX, with dLOS-based adjuvants. The dLOS-based adjuvants, particularly dLOS in combination with DDA, enhanced antibody responses and cellular immune responses to recombinant TB antigens. Ag85A and ESAT-6 are promising antigen candidates for TB subunit vaccines because they induce a strong Th1 immune response and demonstrate high protective efficacy in an animal model [33]. Recent studies have shown that latency-associated proteins such as HspX and Rv2660c are also related to protective effects exerted at the stationary or latent stage of M. tuberculosis infection [34]. The “prime-boost” strategy, involving additional inoculation with a subunit vaccine to increase the immune response previously induced by BCG, is a further application of such recombinant TB antigens [35]. Additional vaccination of adolescents and/or adults with subunit vaccines can provide a strong immune response and long-term immunity. In recent preclinical trials, prime-boost vaccines have been reported to protect against M. tuberculosis infection more effectively than BCG vaccine alone [36]. In our experiment using a BCG prime-boost model, the dLOS/DDA-adjuvanted vaccine was able to promote IFN-γ secretion and proliferative responses of both CD4+ and CD8+ T-cells. These data suggest that the adjuvanted vaccine may be used after BCG vaccination as a boosting vaccine to prevent TB infection in adolescents and adults.

In conclusion, the dLOS-based cationic liposome adjuvant system induced high levels of TB antigen-specific IFN-γ, indicating induction of a strong cellular immune response to TB subunit vaccine. It also enhanced the protective efficacy of the adjuvanted TB vaccine against M. tuberculosis in vitro and in vivo. These findings obtained in this study indicate that the dLOS/DDA-adjuvanted TB subunit vaccine is a promising candidate as a booster vaccine to compensate for the limitations of the current BCG vaccine, and warrants further preclinical and clinical evaluation.

Acknowledgments

We thank Prof. Eun-Kyeong Jo of Chungnam National University for kindly providing the M. tuberculosis BCG strain. This study was supported by a grant from the Technological Innovation R&D Program of Small and Medium Business Administration (No. S2052272) and a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare (No. HI14C2664).

Conflict of Interest

Shin Ae Park, Kwang Sung Kim, and Yang Je Cho are inventors of EyeGene-owned patents for the dLOS adjuvant and employees of EyeGene. Na Gyong Lee is an inventor of the EyeGene-owned patents and a scientific advisor to EyeGene.

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