



Mycobacterial cell-wall skeleton as a universal vaccine vehicle for antigen conjugation

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ABSTRACT

Mycobacterial cell-wall skeleton (CWS) is an immunoactive and biodegradable particulate adjuvant and has been used for immunotherapy in patients with cancer. The CWS of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-CWS) was studied as a universal vaccine vehicle for antigen conjugation, to develop potentially effective and safe vaccines. Here, we describe experiments in which protein antigens, such as keyhole limpet haemocyanin (KLH), ovalbumin (OVA) and bovine serum albumin (BSA) were highly efficiently coupled to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide (EDC/NHS)-activated carboxyl groups of BCG-CWS, and tested the immunogenicity of OVA-conjugated BCG-CWS vaccine. We found that a strong immune response was induced in mice immunised with OVA-conjugated BCG-CWS, which was similar to the enhancement of the immune responses in mice immunised with OVA and complete Freund's adjuvant. Covalent conjugation of OVA to BCG-CWS was essential for Th1-skewed immune responses, with prominent expression of IFN- γ . Furthermore, antigen-conjugated BCG-CWS vaccine is simple to manufacture, safe, and easy to use. Our results suggest that mycobacterial CWS as a universal vaccine vehicle for conjugation of a wide variety of antigens constitutes a breakthrough for development of the most promising vaccines for infections, allergic diseases, and cancer.

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1. Introduction

Adjuvants have been used to enhance immunogenicity at least since the early 20th century [1]. Recently, various vaccine vehicles for antigen delivery have been studied in an attempt to improve vaccine uptake and efficacy [2,3]. Despite a plethora of options, aluminium adjuvants are the most commonly used adjuvants in human and animal vaccines around the world [4,5] because of their direct stimulation of antigen-presenting cells (APCs) and the formation of an antigen depot at the site of injection [6]. However, there is an urgent need for new adjuvants and vaccine vehicles, especially for weak immunogens or antigens with limited availability. In an effort to develop more effective and safer vaccines, many new microbial and tumor vaccines are currently under investigation [7,8]. To achieve this goal, novel adjuvants or vaccine vehicles

must be developed [1,9].

The mycobacterial cell wall strongly stimulates host inflammatory responses, leading to granuloma formation, the up-regulation of antigen presentation and inflammatory cytokines, and subsequent increases in immune responses [10,11]. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), the current live attenuated vaccine for tuberculosis, has been administered to approximately three billion people since 1921 and is one of the safest and most widely used vaccines [12–14]. In particular, the cell wall skeleton of BCG (BCG-CWS); mycobacterial components such as muramyl dipeptide (MDP), trehalose dimycolate (TDM), and methanol extractable residue (MER); and heat-killed mycobacterial cells in Freund's adjuvant are known to be strong activators of innate immunity [15,16]. Therefore, mycobacterial components have been frequently used as an adjuvant for the induction of antibody production and activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.

The BCG-CWS is made up of several immunoactive components: a covalently linked mycolic acid, arabinogalactan, and a peptidoglycan complex [17–19]. The insoluble peptidoglycan complex consists of alternating units of *N*-acetylglucosamine and modified muramic acid residues, which are cross-linked

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by tetrapeptide (L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine) side chains [17–19]. Theoretically, the free carboxyl groups of the glutamate and meso-diaminopimelic acid (DAP) in the murein tetrapeptides of the BCG-CWS could be coupled to the free amino groups of vaccine candidate antigens.

The BCG-CWS acts as a potent immune adjuvant sufficient to promote antibody production, induces CTL proliferation, and exerts anti-tumor activity in immunoadjuvant therapy [11,20]. The clinical efficacy of the BCG-CWS as a tumor immunotherapeutic agent has been recognized in many studies [21,22]. Several reports have described the use of recombinant BCG as a vaccine delivery vehicle; its ability to promote the expression of foreign antigens makes it attractive for this purpose [23–25]. As Toll-like receptor (TLR) 2 and TLR4 are the principal putative BCG-CWS uptake receptors on dendritic cells (DCs), the mechanism of BCG-CWS-mediated host immune activation is due to at least in part to DC maturation [16,26]. Hence, the BCG-CWS may induce DC activation by TLR signalling [26].

The BCG-CWS offers potential advantages both as an innately immunogenic adjuvant and a vaccine vehicle. In this study, we successfully established a large-scale preparation method for the BCG-CWS and a highly efficient method of coupling protein antigens to the BCG-CWS. We also evaluated the adjuvant activity of the BCG-CWS and the immunogenicity of an ovalbumin (OVA)-conjugated BCG-CWS, which was chosen as an immunisation model.

2. Materials and methods

2.1. Reagents

All reagents and chemicals used were of analytical or pharmaceutical grade. Keyhole limpet hemocyanin (KLH), OVA, bovine serum albumin (BSA), alum (aluminium hydroxide), complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA) were purchased from Pierce (Rockford, IL). Concanavalin A (Con A) and lipopolysaccharide (LPS) were obtained from Fluka (Milwaukee, WI).

2.2. Autoclaved wet cell mass of BCG

M. bovis BCG Pasteur 1173P2 was used in this experiment. The bacilli were grown as a surface pellicle on Sauton's medium at 37 °C for six weeks. The surface pellicle was harvested by passage through a stainless mesh screen (pore size, 1 mm), suspended in an equal volume of saline, and autoclaved. The bacterial cells were pelleted by centrifugation at 15,000 × g for 30 min.

2.3. Preparation of the BCG-CWS

The BCG-CWS was prepared according to an optimised and modified method based on delipidation and deproteinisation using detergents and sonication [27]. Briefly, the autoclaved wet cell mass of BCG was suspended in five volumes of 2% (w/v) Triton X-100, sonicated for 5 min using a VCX 750 ultrasonic processor (Sonics & Materials, Inc., Newtown, CT), heated for 3 h in a boiling water bath, and then pelleted by centrifugation at 15,000 × g for 30 min. The above procedure for delipidation and deproteinisation was repeated twice with 2% (v/v) Triton X-100, and then three times with 2% (w/v) sodium dodecyl sulfate (SDS). The resulting BCG-CWS pellet was washed twice with five volumes of 10% 2-propanol by vigorous mixing and centrifugation at 15,000 × g for 30 min, and then washed three times with 100% acetone. To remove the remaining detergents and nonconstituent soluble proteins and lipids of the BCG-CWS, the pellets were washed thoroughly with 10% 2-propanol until the absorbance of the supernatant at 280 nm was <0.05.

Finally, the washed BCG-CWS pellet was suspended in 100% 2-propanol at a concentration of 50 mg/ml (dry wt.) and stored at –20 °C until use. To measure the dry weight, 0.5 ml of the BCG-CWS suspension in 100% 2-propanol was transferred to a 2.0-ml screw-cap microtube, pelleted for 5 min at 14,000 rpm, and then dried overnight at 50 °C. To evaluate the purity of the prepared BCG-CWS, its protein content was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce). The measurements for five samples were averaged. The average content of protein per milligram of the prepared BCG-CWS was 97.5 ± 0.9 µg (9.75% of the dried BCG-CWS); the quality was sufficient for the coupling experiments because the purified CWS itself also contained about 10.6% of constituent amino acids, including alanine (2.8%), glutamic acid (3.6%), and diaminopimelic acid (4.2%) by chemical analysis [28]. We used the same BCG-CWS preparation throughout the study to promote consistency of the results.

2.4. Coupling of protein antigens to the BCG-CWS

KLH, OVA, and BSA were covalently coupled to carboxyl groups in the BCG-CWS by a two-step NHS-enhanced EDC-mediated coupling procedure. Briefly, 25 mg of the BCG-CWS was resuspended vigorously in 1.0 ml of an activation solution (20 mM EDC and 50 mM NHS dissolved in 100% 2-propanol), mixed end-to-end for 30 min at room temperature, and then washed three times with 1.5 ml of 100% 2-propanol. The EDC/NHS-activated BCG-CWS was conjugated with 0.5 ml each of KLH, OVA, and BSA in 0.1 M PBS (pH 7.2) at an increasing concentration (1–8 mg/ml) by mixing end-to-end overnight at 4 °C. The unbound protein was removed by washing three times with 1.5 ml of 0.01 M PBS (pH 7.2) containing 0.02% Tween 80 and 1% ethanol (suspension buffer). The resulting antigen-conjugated BCG-CWS pellet was resuspended vigorously in suspension buffer for protein assays, immunisations and *in vitro* experiments.

The amount of protein antigen conjugated to the BCG-CWS was determined by a BCA assay (Pierce) and calculated after subtracting the protein content of unconjugated BCG-CWS. The coupling efficiency was calculated by dividing the total amount of antigen bound to the BCG-CWS by the amount of antigen used in the coupling reaction.

2.5. Animals

Six-week-old male BALB/c mice were purchased from KOATECH (Pyung-Teck, Korea) and acclimated to their new environment for one week prior to use. The mice were maintained under standard laboratory conditions under a 12:12-h light/dark cycle, with free access to food and water. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University.

2.6. Immunisation studies

Eleven groups of mice (five per group) received three immunisations at two-week intervals subcutaneously at the tail base with 100 µl of the following formulations: OVA antigen dissolved in PBS, OVA admixed in alum, OVA admixed with CFA and then with IFA consecutively, OVA admixed with decreasing doses of BCG-CWS, or OVA-conjugated BCG-CWS admixed with decreasing doses of BCG-CWS and bound OVA. In this study, we used lower doses of BCG-CWS as an adjuvant, whether mixed or antigen-conjugated, because the BCG-CWS itself at higher doses often provoked severe granulomatous reactions. The group receiving OVA alone was immunised with 20 µg of OVA antigen only. The OVA + alum group was immunised with 20 µg of OVA mixed with an equal volume of alum. The OVA + FA group was immunised with 20 µg of OVA

Table 1

Comparison of coupling efficiency and total amount of conjugated antigens to the EDC/NHS-activated BCG-CWS.

Antigen concentration for coupling reaction ^a	Amount of conjugated antigen per milligram of BCG-CWS (coupling efficiency ^b , %)		
	Proteins		
	KLH	OVA	BSA
1.0 mg/ml	18.8 ± 0.1 µg(94.0%)	19.9 ± 1.3 µg (99.5%)	18.2 ± 2.1 µg(91.0%)
2.0 mg/ml	32.5 ± 0.4 µg(81.3%)	37.7 ± 3.6 µg (94.3%)	22.3 ± 2.5 µg(55.7%)
4.0 mg/ml	42.9 ± 0.6 µg(51.1%)	60.7 ± 6.3 µg (75.9%)	24.4 ± 2.7 µg(31.4%)
8.0 mg/ml	53.5 ± 0.9 µg(33.4%)	104.8 ± 6.6 µg(65.5%)	25.1 ± 4.5 µg(15.2%)

KLH, keyhole limpet hemocyanin; OVA, ovalbumin; BSA, bovine serum albumin.

^a 25 mg (dry wt.) of EDC/NHS-activated BCG-CWS was coupled with each of KLH, OVA, and BSA antigens at the concentration of 1, 2, 4 and 8 mg/ml in the reaction volume of 0.5 ml.^b The coupling efficiency was calculated by dividing the total amount of bound antigen to BCG-CWS by the amount of antigen used for coupling reaction.

emulsified with an equal volume of CFA for primary immunisation, and then with IFA for the second and third immunisations. The four OVA + CWS groups – OVA + CWS (100 µg), OVA + CWS (50 µg), OVA + CWS (25 µg), and OVA + CWS (10 µg) – were immunised with 20 µg of OVA mixed with decreasing doses of the BCG-CWS suspension in PBS – 100, 50, 25, and 10 µg, respectively. The four OVA-conjugated BCG-CWS groups were immunised in a similar manner. The OVA–CWS (10.4–100 µg), OVA–CWS (5.2–50 µg), OVA–CWS (2.6–25 µg), and OVA–CWS (1.04–10 µg) groups were immunised with 100, 50, 25, and 10 µg of BCG-CWS covalently conjugated with 10.4, 5.2, 2.6, and 1.04 µg of OVA antigen, respectively.

2.7. Measurement of OVA-specific antibody

The endpoint titres of OVA-specific IgG, IgG1, and IgG2a isotype antibodies in sera were determined by an indirect enzyme-linked immunosorbent assay (ELISA), as described previously [29]. The ELISA endpoint titres were defined as the reciprocal of the antibody dilution that showed 0.1 optical density (OD) units more than the background (preimmunisation serum well). The OD values of the preimmunisation sera were averaged. This value was subtracted from the OD of all wells containing the titration of the pooled serum samples.

2.8. Splenocyte proliferation assay

Splenocyte proliferation was assayed as described previously [30]. Splenocytes were seeded into a 96-well flat-bottom plate (BD Biosciences, Bedford, MA) at 1.0×10^6 cells/ml in 100 µl of complete medium [RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY), 1 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin], after which Con A (5 µg/ml), LPS (5 µg/ml), OVA (5 and 20 µg/ml), or medium was added. The plates were incubated at 37 °C in a humidified atmosphere under 5% CO₂ for 48 h. Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan).

2.9. Cytokine measurement by ELISA

Splenocyte suspensions (2.5×10^6 cells/ml) were prepared in 48-well plates, (Nunc, Roskilde, Denmark) and 250 µl/well was mixed with equal volumes of Con A (5 µg/ml), LPS (5 µg/ml), OVA (5 and 20 µg/ml), or medium. The culture supernatants were collected after 48 or 72 h of incubation. Cytokine assays for the measurement of interleukin (IL)-2, IL-4, IL-10, IL-12p40, and interferon (IFN)-γ were performed using the BD OptEIA set (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions.

2.10. Statistical analysis

All data obtained from independent experiments are presented as the mean ± SD. The data were analysed using Student's *t*-test with Bonferroni's adjustment or ANOVA for multiple comparisons. Differences were considered significant at *P* < 0.05.

3. Results

3.1. Antigen coupling to the EDC/NHS-activated BCG-CWS

To increase the coupling efficiency, the purified BCG-CWS was chemically activated in a mixture of EDC and NHS dissolved in 100% 2-propanol. Table 1 shows the total amount of protein bound to the EDC/NHS-activated BCG-CWS and the coupling efficiency [the ratio (percentage) was calculated by dividing the total amount of antigen bound to the BCG-CWS by the total amount of antigen used in the coupling reaction]. The coupling efficiencies for KLH, OVA, and BSA peaked at 94.0, 99.5, and 91.0%, respectively. These results indicate that most protein antigens could be efficiently coupled to the EDC/NHS-activated carboxylic groups of the BCG-CWS by a peptide bond. Although the coupling efficiency gradually decreased as the antigen concentration in the coupling reaction increased (KLH, 33.4%; OVA, 65.5%; and BSA, 15.2% with an antigen concentration of 8 mg/ml), the total amount of antigen bound to the activated BCG-CWS was greatly increased. Surprisingly, the amount of bound OVA per milligram of BCG-CWS was greatly increased from 19.98 ± 1.25 µg at an OVA concentration of 1 mg/ml to 104.8 ± 6.56 µg at an OVA concentration of 8 mg/ml. These data show that protein antigens could be efficiently coupled to the immunoactive BCG-CWS by our two-step NHS-enhanced EDC-mediated coupling reaction.

3.2. Measurement of OVA-specific end-point IgG titres by indirect ELISA

To investigate the immunogenicity of the antigen-conjugated BCG-CWS, we chose OVA-conjugated BCG-CWS (OVA–CWS) as a representative vaccine since it showed the highest coupling efficiency. Pooled sera from each group (five per group) were obtained at two weeks after each immunisation and used to determine the OVA-specific end-point IgG titres. As shown in Fig. 1, most groups showed a gradual increase in end-point titre after each immunisation (at two-week intervals) and peaked after the third immunisation. However, the OVA + FA group was characterised by an abrupt and strong increase in the IgG end-point titre (1:256,000) as early as just after the first immunisation. The IgG end-point titre in the other groups ranged from 1:62.5 for the OVA–CWS (1.04–10 µg) group to 1:2000 for the OVA–CWS (10.4–100 µg) group. Both the OVA + FA and OVA + CWS (100 µg) groups showed the highest IgG end-point peak titre (1:2,048,000), which was

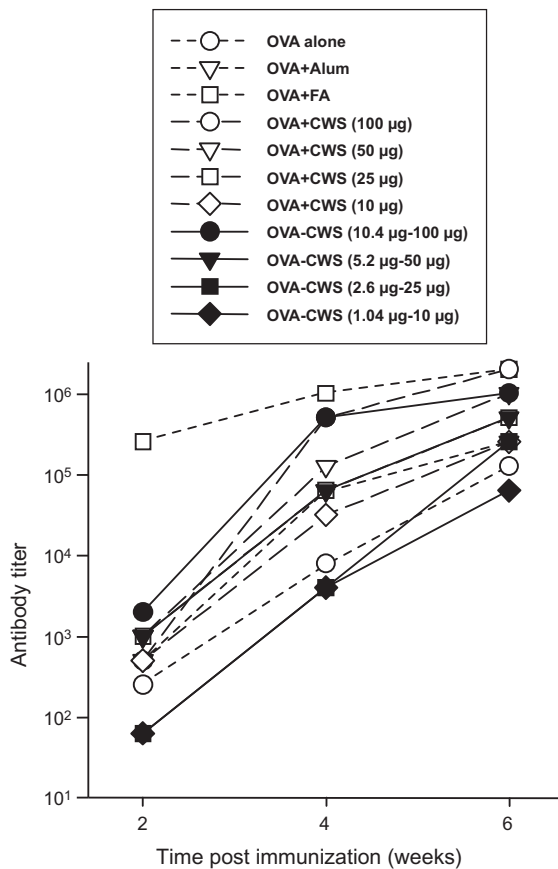


Fig. 1. Comparison of OVA-specific IgG antibody responses in BALB/c mice after each immunisation with different antigen formulations. Groups of five mice were immunised subcutaneously with 20 µg of OVA alone or 20 µg of OVA plus alum, FA, BCG-CWS (10, 25, 50, or 100 µg), or OVA-conjugated CWS (10.4–100, 5.2–50, 2.6–25, or 1.04–10 µg). OVA-specific IgG antibodies in pooled serum from each group were detected by indirect ELISA. The antibody titre was calculated in reciprocal dilutions that gave an optical density >0.1. OVA, ovalbumin; alum, aluminium hydroxide; FA, complete and incomplete Freund's adjuvant; CWS, BCG-cell wall skeleton.

eight-fold higher than that in the OVA + alum group (1:256,000). The OVA-CWS (10.4–100 µg) group also reached a high IgG end-point peak titre (1:1,024,000), which was four-fold higher than that in the OVA + alum group (1:512,000). The OVA-CWS (2.6–25 µg) group showed the same IgG end-point peak titre (1:512,000) as the OVA + alum group.

Table 2
IgG1 and IgG2a isotype analysis after third immunisation with different formulations of OVA antigen.

Groups of antigen formulations ^a	End-point titer ^b		IgG1/IgG2a ratio
	IgG1	IgG2a	
20 µg OVA alone	2.56×10^5	20	128,800
20 µg OVA + alum	5.12×10^5	<10	>51,200
20 µg OVA + FA	4.096×10^6	1.6×10^4	256
20 µg OVA + CWS (100 µg)	2.048×10^6	2.5×10^2	819.2
20 µg OVA + CWS (50 µg)	2.048×10^6	<10	>204,800
20 µg OVA + CWS (25 µg)	1.024×10^6	<10	>102,400
20 µg OVA + CWS (10 µg)	5.12×10^5	<10	>51,200
OVA-CWS (10.4–100 µg)	1.024×10^6	3.2×10^4	32
OVA-CWS (5.2–50 µg)	5.12×10^5	8.0×10^3	64
OVA-CWS (2.6–25 µg)	5.12×10^5	4.0×10^3	128
OVA-CWS (1.04–10 µg)	1.28×10^5	1.0×10^3	128

OVA, ovalbumin; alum, aluminium hydroxide; FA, complete and incomplete Freund's adjuvant; CWS, BCG-cell wall skeleton.

^a Each of five mice from eleven groups were immunised subcutaneously with 20 µg OVA alone, 20 µg OVA plus alum, FA, BCG-CWS (10, 25, 50 or 100 µg), or OVA-conjugated CWS (10.4–100, 5.2–50, 2.6–25, or 1.04–10 µg).

^b OVA-specific IgG1 and IgG2a isotype antibodies in each pooled serum from mice of eleven groups were determined by an indirect ELISA. The antibody titre was calculated in reciprocal dilutions of that gave an optical density higher than 0.1.

3.3. IgG1 and IgG2a isotype responses

In general, the development of a predominant Th1 or Th2 immune response can impact vaccine efficacy. Therefore, we analysed serum antibody isotypes to determine the effects of the OVA-conjugated BCG-CWS on the type of humoral immune response generated. Pooled serum samples from each group of mice (five per group) were collected two weeks after the third immunisation and analysed for end-point titres of OVA-specific IgG1 and IgG2a by indirect ELISA. The groups of mice co-administered OVA with either alum or CWS as an adjuvant showed a strong Th2-biased response with a predominant IgG1 antibody isotype response (Table 2). However, the OVA + FA and OVA-CWS groups showed a low IgG1/IgG2a ratio that was skewed mostly toward a Th1 response. Surprisingly, all of the OVA-CWS groups had lower IgG1/IgG2a ratios (32–128) than the OVA + FA group (256). In particular, even though the IgG peak titre in the OVA-CWS (10.4–100 µg) group was two-fold lower than that in the OVA + FA group, the IgG2a peak titre in the OVA-CWS (10.4–100 µg) group was two-fold higher than that in the OVA + FA group (Table 2). These data suggest that a low IgG1/IgG2a ratio, which reflects a Th1-biased response, may be related to the effect of the OVA-conjugated BCG-CWS (OVA-CWS), which strongly induces Th1 cytokines via APC activation. Interestingly, even though the OVA + FA, OVA + CWS, and OVA-CWS groups showed high IgG peak titres, measurement of their OVA-specific end-point IgG titres by indirect ELISA showed that the IgG isotype profile and IgG1/IgG2a ratio in these groups were completely different from one another (Table 2).

3.4. Lymphoproliferation of mouse splenocytes in response to OVA stimulation

Thirteen groups of mice that were given three injections of the respective antigen formulations were tested for lymphoproliferative responses to OVA. Splenocytes from each group were harvested, pooled, and assayed for lymphoproliferation to OVA antigen two weeks after the third immunisation. Both the OVA-CWS (10.4–100 µg) and OVA-CWS (5.2–50 µg) groups showed a significant increase in lymphoproliferation (Fig. 2), but the other groups were indistinguishable in terms of their lymphoproliferative response. Interestingly, the lymphoproliferative activities of the OVA-CWS group paralleled the expression levels of IL-2, IL-12p40, and IFN-γ (Figs. 3–5). In contrast, in spite of the strong IgG antibody responses (Fig. 1), splenocytes collected

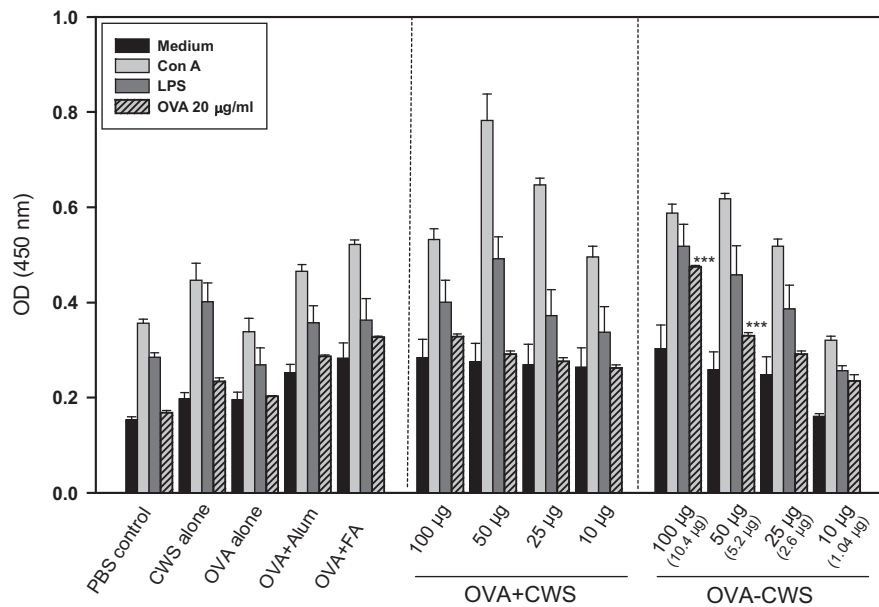


Fig. 2. Effect of OVA-conjugated BCG-CWS immunisation on lymphoproliferation. Splenocytes were collected from three mice from each group two weeks after the third immunisation. The ability of the cells to proliferate in response to 5 µg/ml Con A, 5 µg/ml LPS, and 20 µg/ml OVA was evaluated using CCK-8; the absorbance was measured at 450 nm. The data are expressed as the mean $\pm$ SD in triplicate. $P < 0.05$ was considered statistically significant. ** and *** indicate significant differences from the OVA + alum group ($P < 0.01$ and $P < 0.001$, respectively). Con A, concanavalin A; LPS, lipopolysaccharide; FA, complete and incomplete Freund's adjuvant; CWS, BCG-cell wall skeleton.

from the OVA+FA and OVA+CWS groups did not show significant proliferative responses to OVA when compared to that of the OVA + alum group. The mean proliferative responses stimulated by Con A and LPS were increased in all groups immunised with antigen formulations containing the BCG-CWS, including the groups receiving CWS alone, OVA + CWS, and OVA-CWS, indicating that the BCG-CWS itself activated lymphocytes *in vivo* and caused an increased lymphoproliferative in response to Con A or LPS mitogen *in vitro*. These data also suggest that OVA strongly induces specific lymphocyte proliferation *in vivo* when covalently coupled to the BCG-CWS.

3.5. Effect of OVA-conjugated BCG-CWS immunisation on IL-2, IL-12p40, and IFN- γ

The different results obtained for the OVA + CWS and OVA-CWS groups in terms of their IgG isotype responses (Table 2) might be explained by differences in the cytokine milieu evoked by OVA administration. To examine this possibility, the Th1 cytokines IL-2, IFN- γ , and IL-12p40, produced in splenocyte cultures from each group, were examined by indirect ELISA. In particular, the levels of cytokines from the OVA-CWS groups were compared with those from the OVA + alum group (Figs. 3–5).

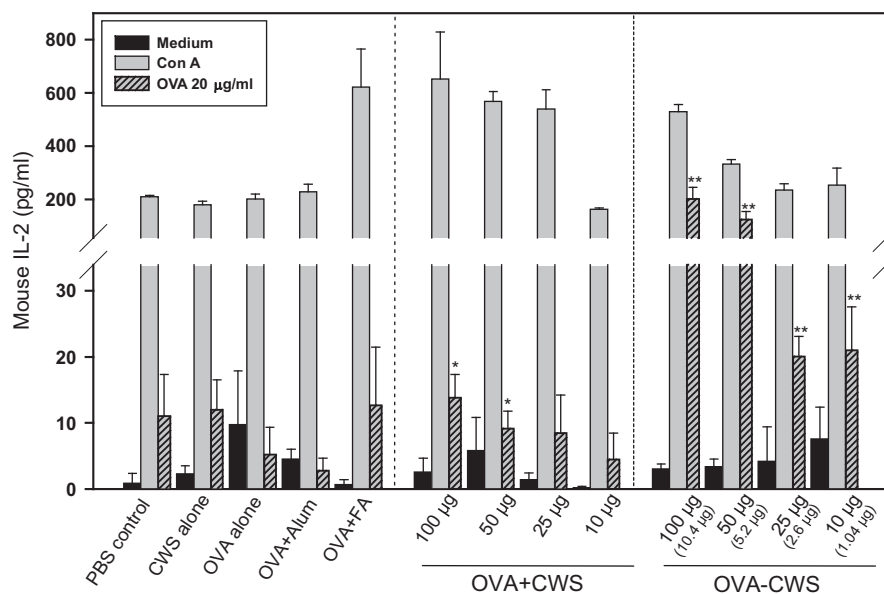


Fig. 3. Effect of OVA-conjugated BCG-CWS immunisation on IL-2 production. Splenocytes were collected from three mice per group two weeks after the third immunisation and cultured unstimulated or stimulated with 5 µg/ml Con A or 20 µg/ml OVA for 48 h. The data are presented as the mean $\pm$ SD. $P < 0.05$ was considered statistically significant. * and ** indicate significant differences from the OVA + alum group ($P < 0.05$ and $P < 0.01$, respectively). Con A, concanavalin A; FA, complete and incomplete Freund's adjuvant; OVA, ovalbumin; CWS, BCG-cell wall skeleton.

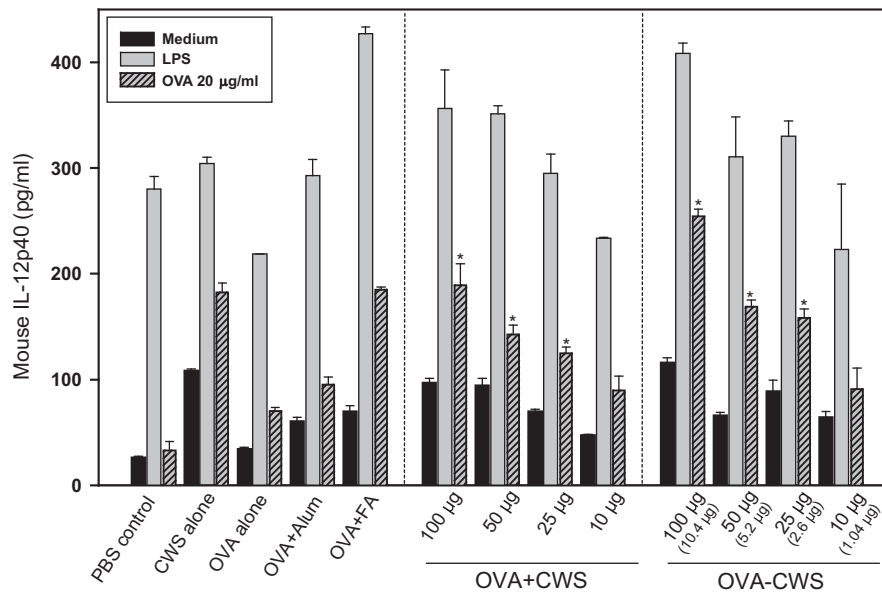


Fig. 4. Effect of OVA-conjugated BCG-CWS immunisation on IL-12p40 production. Splenocytes were collected from three mice per group two weeks after the third immunisation and cultured unstimulated or stimulated with 5 µg/ml LPS or 20 µg/ml OVA for 48 h. The data are presented as the mean ± SD. $P < 0.05$ was considered statistically significant. * indicates results significantly different from those in the OVA + alum group ($P < 0.05$). LPS, lipopolysaccharide; FA, complete and incomplete Freund's adjuvant; OVA, ovalbumin; CWS, BCG-cell wall skeleton.

As shown in Fig. 3, all of the OVA-CWS groups immunised with OVA-conjugated BCG-CWS produced a significantly greater amount of IL-2 compared to the OVA + alum group ($P < 0.01$). Interestingly, IL-2 production in the OVA-CWS (10.4–100 µg) group was 15.9-fold (200.2 ± 44.6 pg/ml) that in the OVA + FA group (12.6 ± 8.9 pg/ml) and 21.8-fold that in the OVA + CWS (100 µg) group. Antigen covalently coupled to the BCG-CWS induced the expression of IL-2 much more strongly than antigen simply mixed with the BCG-CWS. In this study, the CWS alone, OVA + FA, and most of the OVA + CWS and OVA-CWS groups produced significantly higher levels of IL-12p40 than the OVA + alum group (Fig. 4). These results suggest that antigen formulations containing mycobacterial CWS either mixed or covalently conjugated strongly activate APCs

to induce IL-12. This finding is in accordance with the production of the Th1 cytokines IL-2 and IFN-γ (Figs. 3 and 5). As shown in Fig. 5, IFN-γ expression was significantly higher in the OVA + CWS and OVA-CWS groups than in the OVA + alum group. Three of the OVA + CWS groups produced significantly greater amounts of IFN-γ in response to *in vitro* OVA stimulation compared to the OVA + alum group ($P < 0.05$, $P < 0.01$, and $P < 0.05$). All of the OVA-CWS groups produced significantly greater amounts of IFN-γ compared to the OVA + alum group ($P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.01$). Surprisingly, IFN-γ production in the OVA-CWS (10.4–100 µg) group was 241-fold (10 and 130 pg/ml) that in the OVA + alum group (42 pg/ml), and 80-fold that in the OVA + FA group (126 pg/ml). The strong increase in IFN-γ could explain the Th2-to-Th1 shift

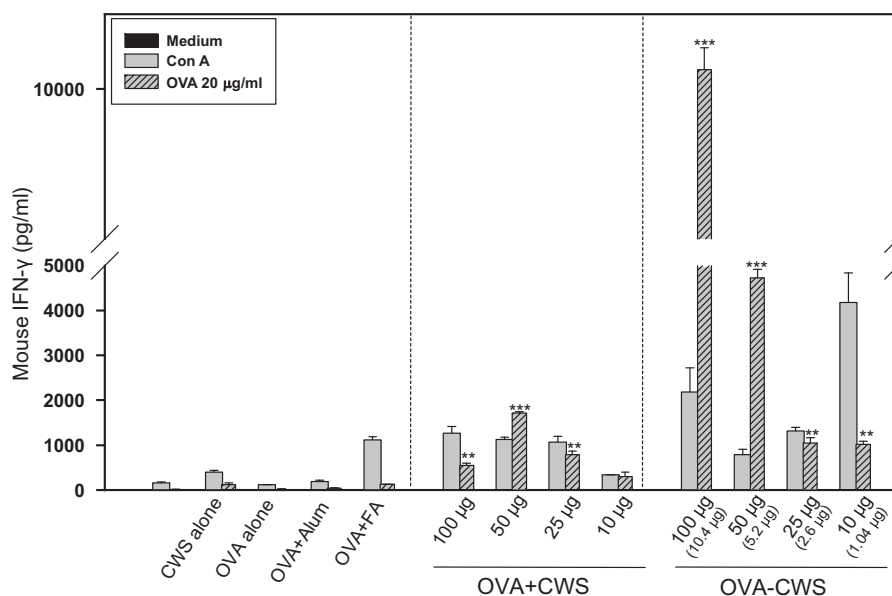


Fig. 5. Effect of OVA-conjugated BCG-CWS immunisation on IFN-γ production. Splenocytes were collected from three mice per group two weeks after the third immunisation and cultured unstimulated or stimulated with 5 µg/ml Con A or 20 µg/ml OVA for 72 h. The data are presented as the mean ± SD. $P < 0.05$ was considered statistically significant. ** indicates results significantly different from those in the OVA + alum group ($P < 0.01$). Con A, concanavalin A; FA, complete and incomplete Freund's adjuvant; OVA, ovalbumin; CWS, BCG cell-wall skeleton.

with decreased IgG1/IgG2a ratio observed in the OVA–CWS groups (Table 2). These findings suggest that antigen-conjugated BCG–CWS induces a much higher level of IFN- γ than does antigen plus BCG–CWS.

4. Discussion

In this study, we developed a simple and efficient procedure to purify BCG–CWS that is suited for large-scale production and evaluated BCG–CWS as a universal vaccine vehicle and adjuvant. In particular, we tested the efficiency of our two-step EDC/NHS coupling method in conjugating the free carboxyl groups of the glutamate and DAP in the murein tetrapeptides of BCG–CWS with the free amino groups of representative protein antigens. As expected, excellent coupling efficiencies were obtained for all tested antigens – KLH, OVA, and BSA – using EDC/NHS-activated BCG–CWS.

Although a number of adjuvants evoke strong immune responses and are widely used in research, their toxic side effects, such as allergic and auto-immune responses, make them unsuitable for use in human and animal vaccines [4,5]. The only adjuvants approved for human use worldwide for modern vaccines are Th2-promoting aluminium-based compounds [30,31]. This has led vaccinologists to focus on the development of safe Th1-inducing adjuvants for human use [32]. Although many adjuvants have been evaluated, not much progress has been achieved. To overcome the scarcity of available adjuvants, we tested the immunogenicity of OVA-conjugated BCG–CWS vaccines, which were prepared by the covalent coupling of OVA to EDC/NHS-activated BCG–CWS. Interestingly, immunisation with OVA-conjugated BCG–CWS (OVA–CWS groups) strongly promoted OVA-specific Th1 isotype antibody responses, whereas OVA mixed with BCG–CWS (OVA + CWS groups) strongly induced Th2-driven anti-OVA antibody responses (Table 2). The OVA-conjugated BCG–CWS, which contained approximately 2.6 μ g of bound OVA (one-eighth the amount in the OVA + alum group), was sufficient for the induction of appropriate Th1-driven immunity. The peptidoglycan portion of the BCG–CWS is recognized by TLR2 and TLR4 and is the main portion responsible for Th1 cytokine induction [16,26]. Therefore, we hypothesised that efficient covalent coupling of antigens such as proteins or peptides in BCG–CWS may induce a Th1 response through TLRs.

Mycobacteria and various mycobacterial preparations, including CWS, were found to generate strong specific immune responses against a range of different antigens. Mycobacterial preparations have been shown to exert their effects through several TLRs and have long been recognized as an important source of adjuvant [33]. These immune responses are characterised by the elevated production of Th1 antibodies and cytokines, including IL-12 and IFN- γ , and the efficient maintenance of immunological memory [34,35]. Our findings indicate that IL-12 and IFN- γ were significantly increased in mice immunised with OVA antigen with BCG–CWS (Figs. 4 and 5). Furthermore, IL-2, IL-12, and IFN- γ productions were increased by the OVA-conjugated BCG–CWS compared with the other adjuvants. IL-12 is expressed in monocyte-derived DCs (APCs), and it acts as a crucial factor in IFN- γ production on lymphocytes [10,36]. BCG–CWS is efficiently taken up by immature DCs, activates DCs from an immature to a mature form, and induces the expression of various costimulatory molecules and cytokines [16,26]. Thus, DC maturation induced by BCG–CWS, in concert with varying mixtures of other cytokines, which may critically affect the adjuvant function for vaccines, would be important in establishing efficient anti-tumor and infectious immunity.

Our data show that Th2 OVA antigen covalently coupled to the BCG–CWS induced greater expression of IL-2, IL-12p40, and IFN- γ than antigen mixed with the BCG–CWS. IFN- γ induced by the BCG–

CWS likely contributes to the immunostimulatory effect through various mechanisms, such as enhancing NK cell activity and inducing T cell activation [37,38]. BCG–CWS has pleiotropic effects on the immune system through the activation of APCs [37,39]. In the current experiment, the BCG–CWS showed a strong Th1-inducing effect, which makes it useful as an immunological adjuvant to vaccines in cancer immunotherapy, allergy, and microbial vaccination [21,37,39].

In summary, we found that the OVA-conjugated BCG–CWS produced by a two-step EDC/NHS-coupling method showed not only high coupling efficiency but also strong Th1-skewed immune responses. Although the exact mechanism of the T-cell response to antigen-conjugated CWS vaccines is not fully explained by our results, this report demonstrates new possibilities for the use of mycobacterial CWS as a vaccine vehicle.

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