

Arabino-mycolates derived from cell-wall skeleton of *Mycobacterium bovis* BCG as a prominent structure for recognition by host immunity

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ABSTRACT: Arabino-mycolates are components of the cell-wall skeleton of *Mycobacterium bovis* BCG (BCG-CWS). It is known that synthesized arabino-mycolates induce the production of tumor necrosis factor alpha (TNF- α) in murine macrophage cell lines at an intensity similar to that of BCG-CWS. However the immunological activity of natural arabino-mycolates isolated from BCG has not been investigated, probably due to the complexity of the molecule. In this paper, we investigated the immunostimulatory activity of arabino-mycolates isolated from BCG-CWS by acid hydrolysis. Arabino-mycolates obtained by acid hydrolysis from the originally prepared CWS (SMP-105) of *M. bovis* BCG Tokyo 172 strain consisted mainly of mono-arabinose mono-mycolate, penta-arabinose tetra-mycolate and hexa-arabinose tetra-mycolate fractions. Arabino-mycolates significantly induced TNF- α production with an intensity comparable to that of CWS and enhanced delayed type hypersensitivity (DTH) reactions against inactivated tumor cells. Arabino-mycolates-induced TNF- α production was completely dependent on TLR2 and MyD88 pathways. These findings indicate that isolated natural arabino-mycolates possess potent adjuvant immunostimulatory activity.

Keywords: Arabino-mycolates, TLR2, BCG-CWS, SMP-105

1. Introduction

The cell-wall skeleton (CWS) of *Mycobacterium bovis* BCG (BCG-CWS), a microbial adjuvant, has intensively been investigated for decades, and has consequently been shown to possess promising activity as a cancer immunotherapeutic agent (1-4). Toll-like receptors (TLRs) have been characterized as pattern-recognition receptors that recognize microbial components (5,6). Stimulation of macrophages and dendritic cells *via* TLRs induces production of cytokines and chemokines, and creates bridges to establish acquired immunity. Recent studies suggest that BCG-CWS induces cytokines and antitumor activity through TLR2 (7,8). However, due to the complexity of BCG-CWS, it is not clear which motif is essential for stimulating immunity or TLR2 agonistic activity.

BCG-CWS has a high molecular weight and is reported to be a mycoloyl-arabinogalactan-peptidoglycan complex. We have tried to shed light on the arabino-mycolate moiety of BCG-CWS and analyze its structure and biological activity. GC-MS and NMR of cell wall arabinogalactan oligosaccharide fragments revealed that the arabinogalactan contains a homogalactan segment of alternating 5-linked α -galactofuranosyl (Gal β) and 6-linked β -Gal β residues, and arabinan segments consisting of two major domains: a linear 5-linked arabinofuranosyl (α -Araf) unit with branching introduced by 3,5- α -Araf and branched oligosaccharides [β -Araf-(1-2)-Araf]₂-3,5- α -Araf at the nonreducing termini (9). Separately, synthesized mycolic esters of the arabinan in the terminal lipo-arabinan motif have been shown to induce production of TNF- α (10). Although some glycolipids that do not constitute BCG-CWS, such as trehalose 6,6'-dimycolate (TDM), lipoarabinomannans, and phosphatidylinositol mannosides, have energetically been studied (11-15), arabino-mycolates that constitute BCG-CWS have not been investigated. We therefore were interested in isolating BCG-CWS arabino-mycolates and investigating their structural properties and immunostimulatory activity. Uenishi *et al.* have developed a unique method

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for isolation of arabino-mycolates derived from the cell-wall skeleton of *M. bovis* BCG Tokyo (SMP-105) (16). By making use of this method, particularly the isolated fraction consisting mainly of mono-arabinose mono-mycolate, tetra-arabinose tetra-mycolate, penta-arabinose tetra-mycolate and hexa-arabinose tetra-mycolate (Figure 1), we proceeded with our structural and biological investigation of BCG-CWS arabino-mycolates.

In this paper, we demonstrate that arabino-mycolates isolated from CWS activate macrophages *via* the TLR2-MyD88 pathway *in vitro* and have potent adjuvant activity *in vivo*. The mechanism of cell-mediated immunity activation by a giant insoluble BCG-CWS will be addressed in the context of digestive degradation reported in our previous paper (17).

2. Materials and Methods

2.1. Preparation of cell-wall skeleton (CWS) and arabino-mycolates

CWS (SMP-105) was prepared from *M. bovis* BCG Tokyo 172 strain as previously described (18), and arabino-mycolates were isolated as indicated elsewhere (16). In brief, a CWS suspension in toluene/0.1 mol/L hydrochloric acid (1:1, v/v) was heated at 100°C for about 1.5 hours. The toluene layer, taken as the portion containing the arabino-mycolates was washed with water and then evaporated. The residual arabino-

mycolates were developed on silica gel HPTLC (Silica gel 60, 10 × 10 cm, Merck Ltd., Darmstadt, Germany) with a solvent containing chloroform/acetone/acetic acid/methanol (90:6:1:10, v/v) for further purification, which revealed multiple spots. The major spots, called A, B, B1, C and D, were deduced to be penta-arabinose tetra-mycolate, hexa-arabinose tetra-mycolate, mono-arabinose mono-mycolate, hepta-arabinose tetra-mycolate, and octa-arabinose tetra-mycolate, respectively. The A, B and B1 spots were selected as arabino-mycolates for investigation of biological activity. The yield of refined arabino-mycolates was approximately 17.9%.

2.2. Trehalose 6,6'-dimycolate (TDM)

TDM was prepared as described previously (4). In brief, lipids were extracted from heat-killed *M. bovis* BCG Tokyo 172 strain with chloroform/methanol (2:1, v/v). After the lower phase of two phases containing major glycolipids was collected, the solvent was evaporated in a rotary evaporator. Total lipids were then separated by thin-layer chromatography (TLC) on silica plates (Uniplate; Analtech, DE, USA), and TDM was recovered from the plate immediately after the iodine color had disappeared by passing through a small glass column. TDM was further purified by repeated TLC until a single spot was obtained.

2.3. Reagents

Pam3CSK4 was purchased from Calbiochem (Merck, Tokyo, Japan). *Escherichia coli* J5 lipopolysaccharide (LPS) was purchased from LIST Biological Laboratories (Campbell, CA, USA), and further purified using a phenol extraction method (19-21).

2.4. Animals

C57BL/6J female mice were purchased from Japan SLC (Shizuoka, Japan). TLR2 (22)-, TLR4 (23)- and MyD88 (24)-deficient mice were obtained from Oriental Bio Service (Kyoto, Japan). All mice were maintained under specific pathogen-free conditions. All animal experiments were conducted according to the guidelines of the Animal Care and Use Committee at Dainippon Sumitomo Pharma.

2.5. Cell lines

Murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA, USA), and was maintained in DMEM (Sigma-Aldrich, MO, USA) supplemented with 10% FCS, 2 mM L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin. Lewis lung carcinoma (3LL) was obtained from the Cancer Institute for the Japanese

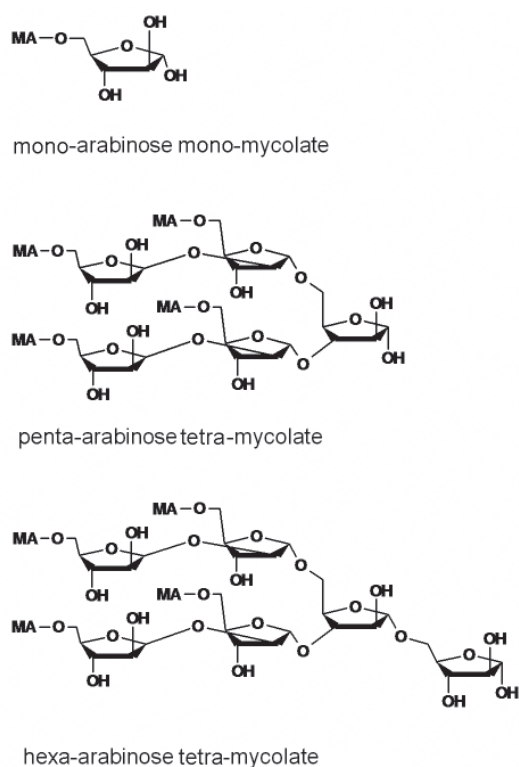


Figure 1. Structure of arabino-mycolate fragments obtained from CWS of *M. bovis* BCG Tokyo 172. MA, micolic acid.

Foundation for Cancer Research (Tokyo, Japan). 3LL cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 50 µg/mL of streptomycin, and 50 U/mL of penicillin. To prepare inactivated 3LL, 3LL cells were incubated at 37°C for 20 min at 1×10^7 cells/mL in a culture medium containing 200 µg/mL of mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan), followed by repeated washing with sufficient culture medium.

2.6. Preparation of mouse peritoneal exudate cells

Thioglycollate-elicited peritoneal exudate cells (TG-PEC) were prepared from mice 5 days after intraperitoneal injection of 3% thioglycollate medium (Difco; Becton Dickinson Japan, Tokyo, Japan), and seeded at 5×10^5 cells/well in a 96-well plate. After removal of non-adherent cells by washing, adherent macrophages were treated with 1 ng/mL of recombinant mouse IFN- γ (R&D Systems, MN, USA) for 2 hours.

2.7. TNF- α induction assay

For experiments using RAW264.7 cells, CWS and arabino-mycolates were suspended in hexane/ethanol (9:1, v/v) and dispensed into 96-well polypropylene microplates before the solvent was evaporated in a clean bench. RAW264.7 cells were then seeded at 5×10^4 cells/well and cultured overnight.

For TG-PEC, a suspension of CWS or arabino-mycolates was prepared in saline containing 0.01% polysorbate 80 using a Potter-type homogenizer at 1,200 rpm for 5 min. TG-PEC was cultured overnight with the suspension of CWS or arabino-mycolates. The concentrations of TNF- α in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems; Minneapolis, MN, USA).

2.8. Delayed type hypersensitivity (DTH) reaction

Inactivated 3LL cells (3×10^4 cells) in an oil-in-water emulsion of vehicle, CWS (12.5 µg), or arabino-mycolates (12.5 µg) were intradermally administered twice with a 7-day interval between injections into the left flank region of C57BL/6J mice.

Seven days after the second administration, inactivated 3LL cells (10^5 cells in 50 µL Hanks' Balanced Salt Solution) were inoculated into the left hind footpads of mice. Just before and 24 hours after inoculation, the thickness of the left footpad was measured using a dial gauge (Mitsutoyo Corp., Kanagawa, Japan). Percentage swelling of the footpad was calculated according to the following equation:

Footpad swelling (%) = $(\text{thickness of post-injected footpad (mm)} - \text{thickness of pre-injected footpad (mm)}) / \text{thickness of pre-injected footpad (mm)}$

The emulsion was prepared as follows; A four hundred micro liter aliquot of CWS or arabino-mycolates suspension dispersed in chloroform at 2 mg/mL was taken in a Potter-type homogenizer and 5.7 µL of squalane was added, followed by evaporation of the chloroform. Next, 0.66 mL of 5.1% (w/v) mannitol solution containing 1% (w/v) polysorbate 80 was added, and the mixture was homogenized at 2,000 rpm for 10 min with a Potter-type homogenizer. The composition of the vehicle was the same except for CWS or arabino-mycolates.

2.9. Statistical analysis

Results from all experiments are expressed as mean \pm standard deviation (S.D.). Significant differences in DTH reaction were assessed using Dunnett's multiple comparison. Statistical analyses were carried out using SAS software (SAS Institute; Cary, NC, USA).

3. Results

3.1. Arabino-mycolates induce production of TNF- α in RAW264.7 cells

To confirm that arabino-mycolates activate macrophages, we investigated the ability of arabino-mycolates isolated from CWS to induce TNF- α production in RAW264.7 cells. Arabino-mycolates dose-dependently induced TNF- α production in RAW264.7 cells with an intensity similar to that of CWS (Figure 2a). This finding indicates

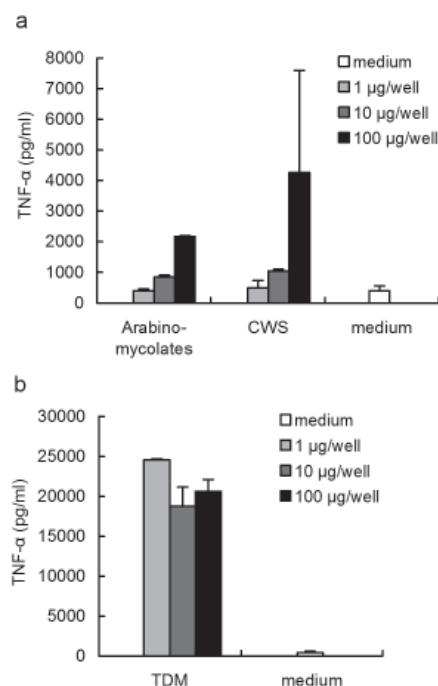


Figure 2. CWS and arabino-mycolates induced production of TNF- α in RAW264.7 cells. RAW264.7 cells were cultured in microplates absorbing CWS or arabino-mycolates (a) or TDM (b) overnight. TNF- α in the supernatant was determined by ELISA. Values are expressed as the means \pm S.D. of triplicate determinations.

that arabino-mycolates stimulate innate immunity. Like CWS and arabino-mycolates, TDM, a mycolic ester of saccharides contained in the cell wall of mycobacteria, also induced TNF- α production in RAW264.7 cells (Figure 2b). The amount of TNF- α induced by TDM was greater than that induced by CWS or arabino-mycolates.

3.2. Arabino-mycolates activate TG-PEC in a TLR-2-dependent manner

To evaluate the effects of arabino-mycolates on TLRs, we next examined the ability of TG-PECs derived from TLR2, TLR4, or MyD88 knockout mice to induce TNF- α production. CWS and Pam3CSK4 (two TLR2 agonists), and LPS (a TLR4 agonist) were used as controls. As expected, CWS and Pam3CSK4 induced TNF- α production in TG-PEC derived from WT and TLR4 knockout mice, but not in TG-PEC derived from TLR2 or MyD88 knockout mice. LPS on the other hand induced TNF- α production in TG-PEC derived from WT and TLR2 knockout mice, but not in TG-PEC derived from TLR4 or MyD88 knockout mice. As in RAW264.7 cells, arabino-mycolates induced TNF- α production in TG-PEC derived from wild type mice, but not in TG-PEC derived from TLR2 or MyD88 knockout mice (Figure 3). Arabino-mycolates also induced TNF- α production in TG-PEC derived from TLR4 knockout mice with an intensity comparable to that found in wild type mice. These results indicate that the mycolyl-arabino structure activates TG-PEC in a TLR-2-/MyD88- dependent manner.

3.3. Arabino-mycolates enhance a DTH reaction

We next evaluated the ability of arabino-mycolates to stimulate innate immunity using a DTH reaction

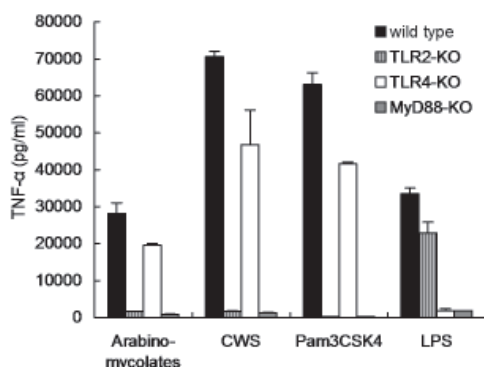


Figure 3. Arabino-mycolates induced production of TNF- α in TG-PEC derived from different mice. TG-PEC derived from wild type mice (black bar), TLR2 knockout (KO) mice (vertical stripes), TLR 4 KO mice (white bar), or MyD88 KO mice (gray bar) were cultured in the presence of 1 ng/mL of recombinant mouse interferon- γ with a medium control, CWS (100 μ g/mL), arabino-mycolates (100 μ g/mL), Pam3CSK4 (1 μ g/mL) or LPS (1 μ g/mL). After 18 hours, each supernatant was collected and the concentration of TNF- α was determined by ELISA. Values are expressed as the means \pm S.D. of triplicate determinations.

in mice. C57BL/6J mice were twice (once weekly) sensitized with mitomycin C treated 3LL tumor cells as antigen and CWS or arabino-mycolates as adjuvant. The mice were elicited one week after the 2nd sensitization by administration of the antigen only into the hind footpad. The DTH reaction was accessed by footpad swelling 24 hours after elicitation. CWS significantly enhanced the DTH reaction in mice (Figure 4). Arabino-mycolates on the other hand moderately enhanced DTH, which was significantly evoked with the vehicle. These findings indicate that arabino-mycolates induce establishment of T cell immunity. We think that the DTH results are consistent with the results of arabino-mycolates induction of TNF- α production *in vitro*.

4. Discussion

To our knowledge, this study is the first to demonstrate the immunostimulatory activity of arabino-mycolates isolated from CWS, although CWS itself has previously been reported to induce TNF- α in murine TG-PEC (8), and synthesized arabino-mycolates have been shown to induce TNF- α in RAW264.7 cells (10). Using a recently reported method for isolation of mycolic esters of arabinan (10,16), we isolated fractions containing the mono-arabinose mono-mycolate, penta-arabinose tetra-mycolate, and hexa-arabinose tetra-mycolate (Figure 1) and used them to investigate arabino-mycolates immunological activity. We found that arabino-mycolates activate macrophages *via* the TLR2-MyD88 pathway *in vitro*, and possess potent adjuvant activity in a DTH model.

Arabino-mycolates induced TNF- α production in RAW264.7 cells with an intensity similar to that of

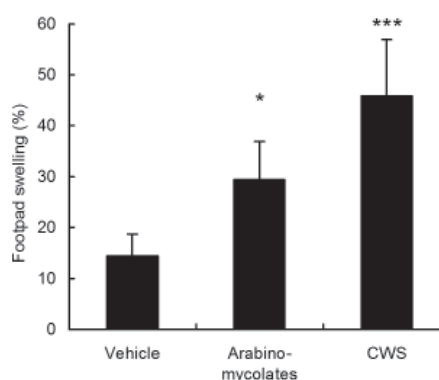


Figure 4. Effects of arabino-mycolates from CWS on delayed-type hypersensitivity reaction against 3LL tumor cells. Inactivated 3LL cells (3×10^4 cells) in an oil-in-water emulsion of vehicle, CWS (12.5 μ g) or arabino-mycolates (12.5 μ g) were intradermally administered twice with a 7-day interval between injections into the left flank region of C57BL/6J mice. Seven days after the second administration, inactivated 3LL cells (10^5 cells) were inoculated into the left footpads and swelling was monitored by measuring the thickness of the footpads 24 hours after inoculation, * $p < 0.05$; *** $p < 0.001$ (parametric Dunnett's test for multiple comparisons; compared to vehicle). Values are expressed as the means \pm S.D. of six mice.

CWS. This finding indicates that arabino-mycolates stimulate innate immunity (Figure 2a). Uenishi *et al.* have shown that CWS from *M. bovis* BCG Tokyo 172 contains 38.8% mycolic acid and 23.0% arabinose (18). Considering that the mycolate and arabinose constitute over half of the CWS, the amount of TNF- α produced by CWS seems to be consistent with the arabino-mycolate moiety.

We have previously reported that CWS activates the immune response in a TLR2-/MyD88- dependent manner (8). Accordingly, in this study arabino-mycolates induced TNF- α production in RAW264.7 cells as well as in TG-PEC derived from wild type mice, but not in TG-PEC derived from TLR2 or MyD88 knockout mice (Figure 3). In addition, and as expected, arabino-mycolates induced TNF- α production in TG-PEC derived from TLR4 knockout mice with an intensity comparable to that in wild type mice. Because neither mycolate nor arabinose can induce production of TNF- α (10), we postulate the mono-arabinose mono-mycolate as the smallest binding motif for TLR2. It is also likely that TLR2 dimerizes with different TLRs, *i.e.* TLR1 or TLR6, to recognize arabino-mycolates (25,26). Therefore, it is believed that the linkage region between mycolate- and arabinose-residues constitutes a crucial structure for binding to TLR2, which may render a plausible explanation as to why TDM is not recognized by TLR2 in spite of its similar structure to arabino-mycolates (27-29). Recently Ishikawa *et al.* have shown that macrophage inducible C-type lectin (Mincle) is an essential receptor for TDM in Mincle-deficient mice (30). Thus, the different signaling between arabino-mycolates and TDM can explain the difference of the amount of TNF- α induced (Figures 2a and 2b).

There is a commonly-held view that TLR agonists affect antigen-presenting cells by inducing an innate immune response and subsequently activating the adaptive immune system. In addition a number of studies have shown that stimulation of innate immunity with CWS and TDM leads to establishment of antigen-specific T cell immunity by the DTH reaction (17,31). We accessed in this study arabino-mycolate activity and used CWS as a control for the DTH reaction. Our results show that arabino-mycolates evoke significant swelling of the footpad (Figure 4). The swelling of footpad induced by CWS was also significant and almost comparable to that induced by arabino-mycolates. There is so far no report showing that isolated natural arabino-mycolates stimulate innate immunity *in vivo*, although synthesized arabino-mycolates have been shown to induce TNF- α production in RAW264.7 cells *in vitro* (10). In this study, we show for the first time that isolated natural arabino-mycolates stimulate innate immunity *in vivo*. This finding indicates that arabino-mycolate structure is important as it has a direct effect on adjuvant activity in CWS.

In this study, we show that arabino-mycolates stimulate macrophages to produce TNF- α and that this

effect is dependent on the TLR2-MyD88 pathway *in vitro*. We also show that arabino-mycolates enhance establishment of T cell-mediated immunity *in vivo*. We have previously reported that CWS is engulfed into dendritic cells and distributed in lysosomes, and that activation of dendritic cells is blocked by inhibition of phagocytosis, addressing digestive fragments of CWS that may function as TLR2 ligands (17). Now that we have shown that arabino-mycolate fragments prepared by acid hydrolysis can stimulate TLR2, it is highly likely that CWS is engulfed by phagocytosis and transferred into lysosomes that undergo acid hydrolysis by digestive enzymes to produce fragments capable of interacting with TLR2 directly.

In conclusion, arabino-mycolates produced by acid hydrolysis of BCG-CWS have been shown to have potent TLR2 ligand activity and to constitute an important antigen determinant of *M. bovis* BCG. Further investigation of other multilateral players in immunity would be applauded.

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