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Research article

Adjuvant activity of *Mycobacteria*-derived mycolic acids

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ABSTRACT

Successful vaccination, especially with safe vaccines such as component/subunit vaccines, requires proper activation of innate immunity and, for this purpose, adjuvant is used. For clinical use, alum is frequently used while, for experimental use, CFA, containing Mycobacterial components, was often used. In this report, we demonstrated that mycolic acids (MA), major and essential lipid components of the bacterial cell wall of the genus *Mycobacterium*, has adjuvant activity. MA plus model antigen-immunization induced sufficient humoral response, which was largely comparable to conventional CFA plus antigen-immunization. Importantly, while CFA plus antigen-immunization induced Th17-biased severe and destructive inflammatory responses at the injected site, MA plus antigen-immunization induced Th1-biased mild inflammation at the site. MA induced dendritic cell activation by co-stimulatory molecule induction as well as inflammatory cytokine/chemokine induction. MA plus antigen-immunization successfully protected mice from tumor progression both in prevention and in therapy models. We thus submit that MA is a promising adjuvant candidate material for clinical purposes and for experimental purposes from a perspective of animal welfare.

1. Introduction

Vaccination is one of the most efficient methods for preventing and/or decreasing the severity of infectious diseases [1]. Successful development of vaccines against, for instance, diphtheria, pertussis, and tetanus, has significantly saved lives of infants. In addition, vaccination against other infectious diseases, including measles, rubella, and influenza, has not only saved individual lives but prevented transmission of pathogenic microorganisms among the population, thereby maintaining the public health of communities. Recent advances in analyses of diseases at molecular levels have helped identifying tumor (-related) antigens, which have been used as a candidate antigen(s) for development of cancer vaccines [2].

Although traditional vaccine approaches such as live-attenuated or inactivated viruses are highly effective in developing immunity against the pathogens, safety concerns have been raised [3]. Safer vaccines, such as subunit vaccines or component vaccines, therefore, have been developed. Unlike aforementioned traditional vaccines, subunit/component vaccines are less immunogenic due to lack of pathogen-associated molecular patterns (PAMPs), which stimulate innate immune receptors resulting in the successful activation of innate immunity followed by sufficient induction of acquired immunity [4, 5]. To circumvent this issue, adjuvants are usually included in the vaccine for stimulation of innate immunity and subsequent induction of memory type of immunity [4, 6]. Besides the primary purpose of adjuvants, that is to induce and increase the immune response to lower amounts of antigen, adjuvants are also used/selected to promote preferable types of immune responses, such as Th1, Th17 vs. Th2, humoral vs. cellular, and cytokine-mediated vs. cytotoxicity-mediated [4]. Various types of adjuvants, either licensed or experimental/preclinical, have been extensively studied for their biochemical features, mechanisms of immunostimulation, and principle immune response induced, such as antibodies, Th1/17/2, and CD8-mediated ones.

For clinical purposes, alum (hydroxy-aluminum) is frequently used. For experimental purposes, complete Freud adjuvant (CFA), which contains components of *Mycobacterium tuberculosis*, is widely used. While CFA is a very strong adjuvant, it cannot be used for human because it induces excessive inflammation and severe granuloma formation and/or

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ulceration at administrated areas. Even in mice or rats, use of CFA is limited and strictly regulated in terms of animal welfare.

In this study, we identified mycolic acids (MA) [7], a lipid component abundant in cell walls of mycobacterium, as a safe adjuvant candidate. It induced both humoral and cellular immunity when used with antigens for vaccination, as efficient as CFA, while inducing less severe inflammation and tissue damages than CFA. In tumor vaccination models, MA induced anti-tumor immune responses in prevention and also in therapeutic models by enhancing antigen-specific CTL activity. We would therefore like to submit that MA is a promising candidate for development of sufficient and safe adjuvant.

2. Materials and methods

2.1. Reagents

MA (JBL-104. Purity 95%) were kindly provided by Japan BCG Laboratory; preparation of MA from *M. bovis* BCG is as follows. Free MAs were isolated from heat-killed *M. bovis* BCG (Tokyo 172 strain). The BCG cells were suspended in 85% tetrahydrofuran (THF)/water solution under a nitrogen atmosphere, followed by reflux with stirring for 1 h. The cell suspension was filtrated under pressure and washed with 75% THF/water solution. The residue was resuspended in 75% THF/water solution under a nitrogen atmosphere, followed by reflux with stirring for 1h. The suspension was filtrated under pressure and washed with 75% THF/water solution three times and with methanol twice. Then, the bacterial cells were suspended in 50% 2-propanol/water solution containing 10% potassium hydroxide, followed by reflux with stirring for 2 h to complete alkaline hydrolysis of MA ester. After the refluxing, the suspension was cooled down on ice, and acidified with 6 M hydrochloric acid. The reaction mixture was extracted twice with n-heptane, and the n-heptane fraction was washed twice with water and then twice with 90% ethanol/water. Finally, the n-heptane fraction was concentrated in vacuo to obtain purified MAs. The product was applied for TLC (n-hexane:methyl tert-butyl ether: formic acid = 8/2/0.5, v/v/v) and MALDI-TOF mass spectrometry analyses to confirm the identity of MAs [8]. Provided MA were dissolved in chloroform at 1 mg/ml and then diluted with isopropanol to the working concentrations. For *in vitro* cell stimulation, the lipid solutions were added into the 96-well flat bottom plates at 20 μl/well and then the solvent was completely evaporated in a hood before plating macrophages, as described previously [9]. OVA was purchased from Sigma. Bayol F (mineral oil) was purchased from Serve Electrophoresis.

2.2. Mice

C57/BL6 mice were maintained in the animal facility at the Division of Biological Resources and Development, Analytical Research Center for Experimental Sciences, Saga University and female mice between 8-15 weeks were used in the experiments. Ethics in animal experimentation; experiments using animals (including usage of CFA) were performed under protocols reviewed and approved by Saga University Animal Care and Use Committee (Approval No. 26-043-0).

2.3. Immunization

Immunization of mice with antigen was performed as follows. OVA was prepared 200 μg/ml in PBS. MA was mixed with Bayol F, dissolved at 64 °C for 10–20 min and prepared as 200 μg/ml. OVA in PBS (750 μl) and MA in Bayol F (750 μl) were put and mixed at 25 °C for 5 min with a handy homogenizer (Handy ROUTER. RELIEF, Hyogo, Japan; 11,000rpm). OVA was emulsified in CFA similarly.

For immunization of mice with OVA, 50μl of OVA (OVA in PBS), OVA + MA, or OVA + CFA was injected subcutaneously or tail base of mice (Figure 1, left). On day 35 of immunization, blood samples were taken for antibody titers and spleen cells for cytokine production (See 2.4 Antigen-specific immune responses).

2.4. Histological examination and tissue sample preparation

For examination of local inflammation, mice were injected with OVA, OVA + MA or OVA + CFA intradermally at the ear. Injected sites in the ear were fixed and stained with hematoxylin and eosin for histopathological examination. For detection of inflammatory cytokine expression at the site of injection, mRNA expression in the tissue samples were examined as follow. RNAs were extracted using a Sepasol-RNA I Super G RNA-isolation kit (Nacalai Tesque). After the removal of DNA contamination by DNase I (Nippon Gene), the total RNA was reverse-transcribed with ReverTra Ace qPCR RT Master Mix (TOYOBO) to synthesize cDNA. Quantitative real-time PCR (qRT-PCR) was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and StepOnePlus (Thermo fisher scientific) for the presence of IL-1β/6/12α/17A, TNF-α, and MIP1/2.

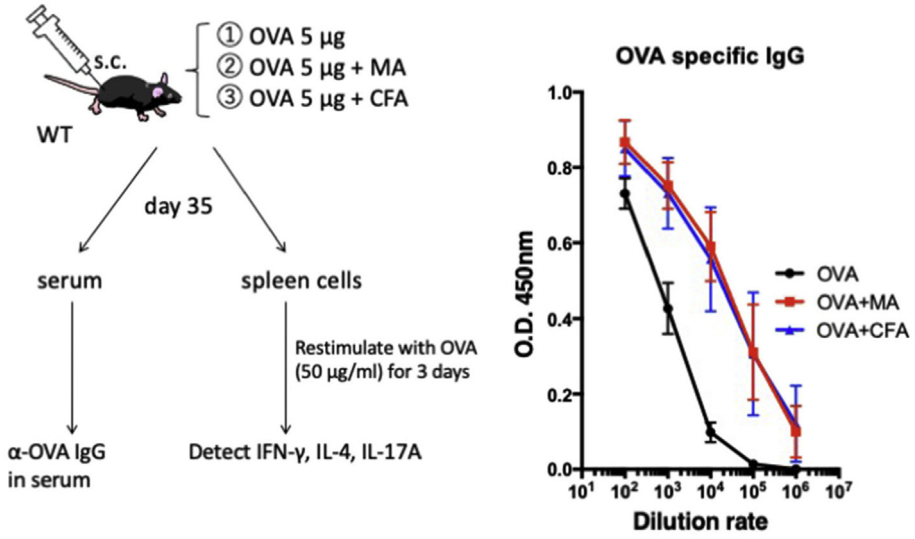


Figure 1. Induction of antibody responses by MA. Left; Schematic illustration of immunization and examination protocols. Right; anti-OVA antibody responses. Wild-type (WT) mice were immunized with OVA (black), OVA + MA (red), or OVA + CFA (blue). On day 35 of immunization, sera were examined for anti-OVA IgG responses. Mean ± SD are shown. Experiments were repeated 3 times with similar results.

2.5. Antigen-specific immune responses

Antigen (OVA)-specific antibody titers were evaluated according to a previous report [10] with slight modifications. Blood samples were prepared from tail vein of mice on day 35 of immunization and kept at 4 °C overnight. Serum was prepared by centrifuging at 800 x *g* for 15 min at 4 °C and kept at -30 °C until analysis. Anti-OVA antibodies were assayed by standard ELISA procedures. In short, MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with 50 μl of OVA (10 μg/ml in PBS) for overnight at 4 °C and washed with 0.1% Tween20/PBS three times. OVA-coated plate was blocked by 150 μl of 1%BSA/PBS for 2 h at 37 °C. After washing, serially diluted sera (0.5%BSA/PBS) were put in a 96-well plate coated with OVA. After washing, goat polyclonal anti-mouse IgG (H + L) conjugated to horseradish peroxidase (Jackson ImmunoResearch) were added and washed. Anti-OVA antibody titers were determined by TMB reagent (SUMILON). For antigen-specific cytokine responses, spleen cells were prepared from mice on day 35 of immunization. Whole spleen cells (10^6/ml) were stimulated with OVA (50 μg/ml) for 3 days in RPMI 1640 medium supplemented with 10% fetal bovine serum. IFN-γ, IL-17A, and IL-4 in the supernatants were examined using respective ELISA kits (Abcam).

2.6. Dendritic cell stimulation with MA

For dendritic cell (DC) stimulation with MA, bone marrow (BM)-derived DCs (BMDCs) were prepared from BM suspensions from femurs and tibias of mice as described elsewhere [11]. Briefly, bone marrow cells were cultured with 20 ng/ml murine GM-CSF (R&D Systems) and 10 ng/ml murine IL-4 (R&D Systems) for 7 days. DCs were purified by positive selection using MACS Separator system (Miltenyi Biotec) with anti-CD11c monoclonal antibody ($CD11c^+$ cells >90%). BMDCs were then cultured in the presence of plate-coated MA (0.01, 0.1, 1.0, and 10.0 μg/well) for 3 days and surface expression of CD80/86 and CD40 were examined by flow cytometry. Culture supernatants were examined for the production of cytokines and chemokine by ELISA.

2.7. Anti-tumor immunity assays

E.G7-OVA (ATCC, CRL-2113™), a mouse lymphoma cell line constitutively expressing OVA, was purchased from ATCC.

To determine induction of cellular cytotoxic immunity in a prophylactic model, mice were immunized with OVA, OVA plus MA or OVA plus CFA on day -21 and -14, at the tail base, or untreated (control). On day 0, mice were inoculated subcutaneously into the flank with 3×10^5 of E.G7-OVA. Tumor growth was scored by measuring perpendicular diameters. Tumor size was calculated as (A^2 x B/2), where A is the longest surface length and B is its perpendicular width.

To determine cellular cytotoxic immunity in a therapy model, mice were inoculated with E.G7-OVA (3×10^5) on day 0 and immunized on day 3, 7 and 10 with OVA, OVA plus MA, or untreated (control). Tumor size was monitored and calculated.

For CTL assay, spleens were isolated from three groups of mice (without vaccination, OVA vaccination and OVA + MA vaccination) on day 21 after tumor inoculation in a therapy model (3-time immunization after tumor inoculation). Single-cell suspensions were prepared, and cells were stimulated with irradiated (16,000 cGy) E.G7-OVA cells in the presence of 10 U/ml IL-2. After 5 days of culture, viable effector cells were separated by percoll gradient centrifugation, and cytotoxic T cell activity was measured against E.G7-OVA tumor cell targets using a standard 4-hour ^{51}Cr release assay. Briefly, the target cells were labeled with 100 μCi of ^{51}Cr for 2 h, washed, and then incubated for 4 h with effector cells at indicated effector/target ratio in triplicate. Spontaneous release and maximum release were determined by incubating target cells without effectors in medium alone or in HCl, respectively. Spontaneous release was always less than 20% of maximum. Radioactivity was counted in a liquid scintillation counter and the percentage of specific lysis was measured as the product of [(experimental release - spontaneous release)/(maximal release - spontaneous release)] x 100.

2.8. Statistics

Values were expressed as the mean ± SEM or the mean plus individual values. Differences among groups were analyzed using the ANOVA followed by the Student-Newman-Keuls analysis. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Adjuvant activity of MA

Lines of evidence have shown that immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors, including C-type lectin receptors (CLRs) and immunoglobulin superfamily receptors play critical roles in activation of innate immunity against various pathogens [12, 13, 14, 15]. Some of the receptor members, such as Dectin-1/2, and Mincle, are known to sense pathogen-derived material with PAMPs and induce activation of innate immunity [16, 17, 18, 19].

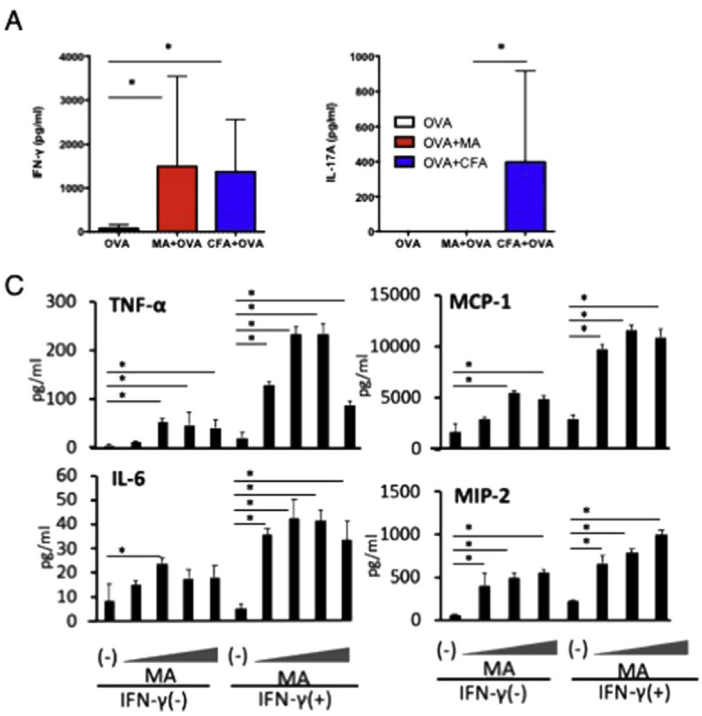
As described in the introduction, CFA shows strong adjuvant activity. It has been shown that the essential factor of the adjuvant activity of CFA is lipid components [20]; among them, trehalose dimycolate (TDM, cord factor), MA-containing glycolipids, has strong innate immunity-stimulating (adjuvant) activity [21, 22, 23]. TDM, however, also has severe toxicity *in vivo*, including lung granuloma formation and thymus atrophy [24]. While Mincle, a CLR, is responsible for the TDM-induced immune activation [9, 25], free MA (TDM devoid of sugar structure), which lacks Mincle-stimulating activity, still demonstrates innate immunity-stimulating activities in a Mincle-independent manner (Iizasa et al, manuscript submitted). With these reports and observations, we examined the adjuvant activity of MA in view of its efficiency and safety in comparison with experimentally used existing adjuvants.

First, we examined if MA had an adjuvant activity in a mouse model. To address this, we immunized mice with OVA only (OVA), OVA in MA (OVA + MA) or OVA in complete Freund's adjuvant (OVA + CFA) and OVA-specific antibody titers were evaluated on day 35 of immunization. As shown in Figure 1, while OVA induced a low level of antigen-specific Ig response, OVA + MA induced significantly higher antibody responses than that by OVA only. To our surprise, the antibody induction levels by OVA + MA was almost comparable with that by OVA + CFA.

We then examined antigen-specific cytokine responses. As shown in Figure 2A, cells from OVA + MA-immunized mice produced significantly elevated levels of IFN-γ while IFN-γ production by spleen cells from OVA-immunized mice was marginal. Production of IFN-γ by spleen cells from OVA + MA-immunized mice was again almost comparable to that in OVA + CFA-immunized mice. Production of IL-17A by cells from OVA + MA-immunized mice was almost undetectable while OVA + CFA induced significant levels of IL-17A production. Production of IL-4 by cells in the 3 types of immunization settings was only marginal and comparable among each other (data not shown). Along with data in Figure 1, these results clearly demonstrated promising adjuvant potential of MA and that adjuvant activity favors development of Th1 responses rather than Th2 or Th17 responses.

3.2. Activation of DCs by MA

There are a variety of different mechanisms of action, through which current adjuvant compounds exert their effects, such as, increasing the biological half-life of vaccines, activation of antigen-presenting cells (APCs), and induction of cytokines and chemokines. As TREM2, the putative receptor for MA, is expressed mainly (but not exclusively) on APCs (Iizasa et al., submitted), we hypothesized that MA activated APCs through the putative receptor. Figure 2B demonstrates the augmented expression of co-stimulatory molecules, CD80/86 and CD40 on BMDCs



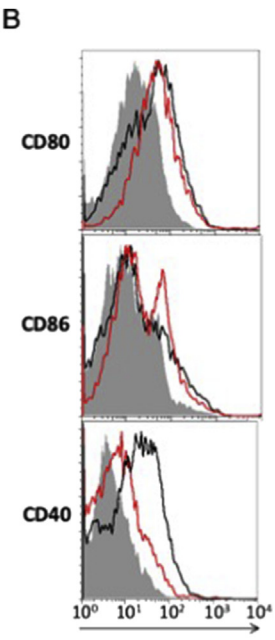


Figure 2. Cytokine production by cells from MA-immunized mice and *in vitro* activation of DCs by MA. A) Mice were immunized with OVA (black columns), OVA + MA (red columns), or OVA + CFA (blue columns). On day 35 of immunization, spleen cells were stimulated with OVA for 3 days and cytokines in the supernatants were examined. Mean +SD from representative data are shown. Experiments were repeated 3 times. *; $p < 0.05$. B) Bone marrow-derived DCs were stimulated with plate-coated MA (red. 1.0 μg/well), LPS (black. 1.0 μg/well) or left unstimulated (gray shade) for 3 days and examined for expression of CD80/86 and CD40. C) DCs were stimulated likewise with titrated doses of MA (0.01–10 μg/well) in the presence or absence of IFN-γ (10 ng/ml). Culture supernatants were examined for production of indicated cytokines/chemokines by respective ELISA. Experiments were repeated 3 times with similar results and representative data (Mean +SD) are shown. *; $p < 0.05$.

stimulated with plate-coated MA or LPS. While augmentation of CD80 expression was comparable between MA and LPS, that of CD86 was more by MA than by LPS. Contrary, augmentation of CD40 was more by LPS than by MA.

We then examined the cytokine/chemokine production by BMDCs stimulated with MA. Figure 2C shows that production of TNF-α, IL-6, MCP-1 and MIP-2 was augmented by MA in a dose-dependent manner. Augmentation was further enhanced in the presence of IFN-γ. These data clearly demonstrated that MA stimulated DCs for expression of co-stimulatory molecules and cytokine/chemokine production, through which MA exerted its adjuvant activity. We do not exclude other mechanisms of action, though.

3.3. Mild induction of local inflammation by MA

We then examined possible detrimental impacts of MA when used as an adjuvant. As known well, CFA shows a strong adjuvant activity and was widely used in animal experiments. CFA, however, also induces strong and excessive inflammatory responses at the site of injection, resulting in severe and sustained granuloma formation (sterile abscess) and ulcer formation. As such, use of CFA even in animals is regulated (USDA Category E) [26]; usually used only when necessary for the initial immunization. We therefore compared local inflammatory effects of MA with those of CFA. To address this issue, we intradermally injected mice with MA or CFA at their ear and examined pathology and cytokine production *in situ*.

As Figure 3A shows, OVA + MA injection caused cellular infiltration at the injected site on day 8, where macrophages were the major infiltrating cells (upper panels and inset). No ulcer formation was observed. In contrast, OVA + CFA injection induced massive cellular infiltration mainly with neutrophils (lower panels and inset). With cellular infiltration, swelling at the injected site was way more evident. Ulcer formation was also observed in some of the injected mice (data not shown). Given the less severe inflammation by OVA + MA injection as compared with OVA + CFA injection along with almost comparable adjuvant activities (Figures 1 and 2), we then examined cytokine expression levels at the injected sites. Figure 3B shows that local expressions of TNF-α and MIP-1/2 were only marginal and that those of IL-6, IL-1β, IL-12α, and IL-17A were undetectable in OVA mice (injected with OVA in PBS). In OVA + MA-injected mice, although some cytokines/chemokines expression appeared to be higher than those in OVA mice, there was no significant differences in these cytokine and chemokine expressions over OVA mice. Expression of MIP-2 in OVA + CFA-injected mice was significantly higher than in OVA + MA-injected mice. In addition, expressions of IL-1β, IL-6, IL-17A, and IL-12α in OVA + CFA-injected mice tended to be higher than in OVA + MA-injected mice, albeit no significance due to big variations among individual samples. This, along with higher production of IL-17A in OVA + CFA-injected mice (Figure 2A), may contribute to the aforementioned severer inflammation accompanied by neutrophil-dominant infiltration in OVA + CFA-injected mice (Figure 3A).

3.4. In vivo adjuvant activity of MA in in vivo and in vitro cellular cytotoxicity models

Given the substantial adjuvant effects of MA, we examined the efficacy of MA as an adjuvant in tumor models. In the prevention model, two serial vaccinations preceded tumor inoculation. As shown in Figure 4A, two-time immunization with OVA + MA (on day -21 and -14 of tumor inoculation) nicely prevented the growth of E.G7-OVA tumor. Virtually, no evident tumor growth was observed in OVA + MA-immunized mice, just like in CFA + OVA-immunized mice (data not shown), while OVA only showed no preventative effects. In a therapy model, in which tumor inoculation was followed by three serial vaccinations (on day 3, 7, and 10 of tumor inoculation), OVA + MA immunization again suppressed the growth of E.G7-OVA tumor (Figure 4B, left) while OVA only showed no therapeutic effects over untreated mice. In CTL assay using spleen cells from immunized mice, antigen-specific tumor-killing activity was significantly higher in OVA + MA-immunized (after tumor inoculation) mice than that in untreated or OVA-only-immunized mice (Figure 4B, right). These data showed adjuvant activity of MA in tumor-prevention/therapy models.

Mycolic acids are major and essential lipid components of the mycobacterial cell envelope of the genus *Mycobacterium*, with extremely long fatty acids (C_{60}–C_{90}). The structure and the biosynthesis pathway of MA have been extensively elucidated (For structural and biochemical features, see [7, 27]). As evident for CFA, mycobacterial components

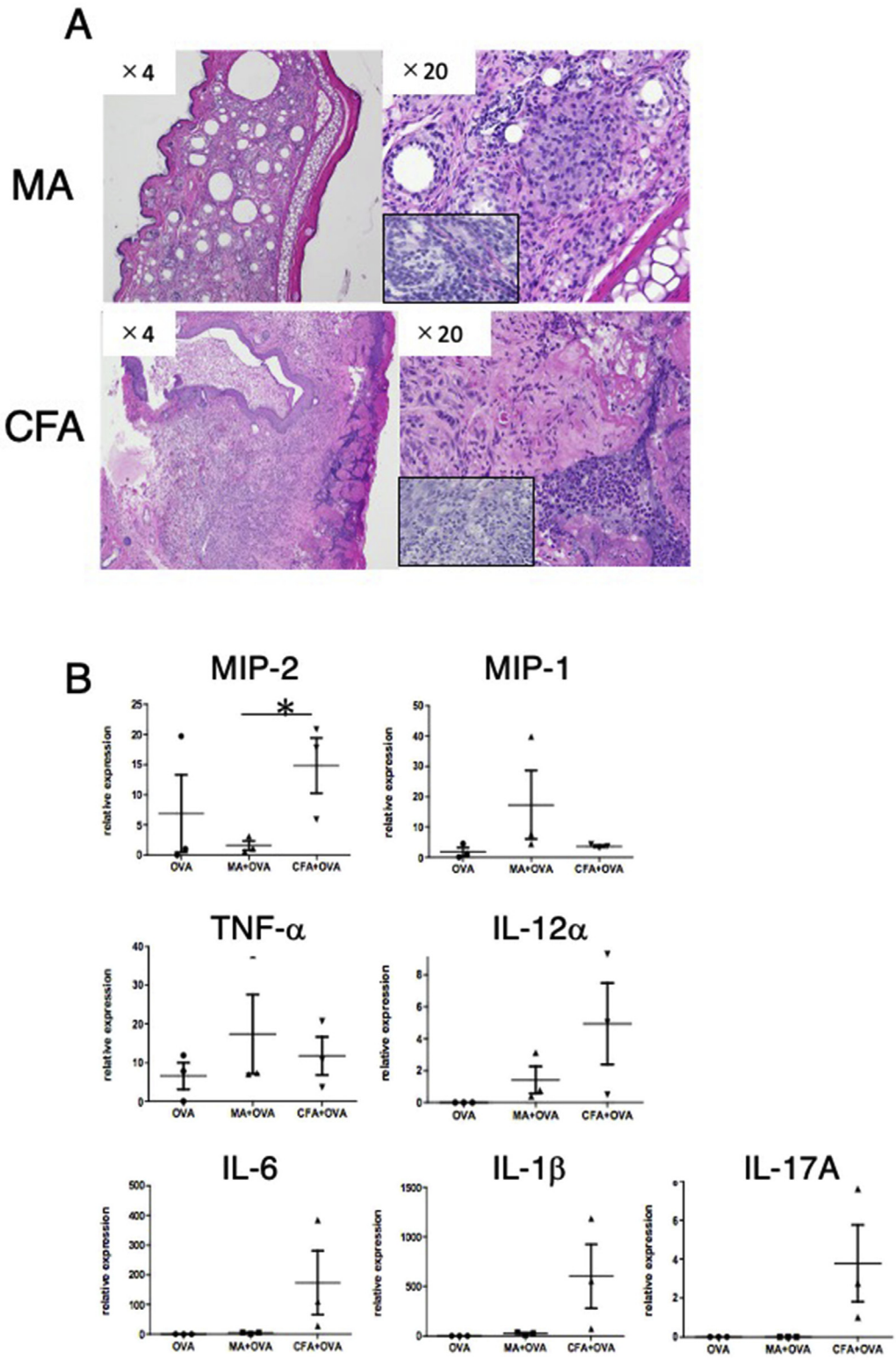


Figure 3. Less induction of tissue-destructing inflammation by MA. A) Mice were injected with OVA + MA (MA) or OVA + CFA (CFA) at the ear. On day 8, tissue samples were taken and fixed for histological examination. Insets are higher magnification views of infiltrating cells. B) Tissue samples were taken from OVA, OVA + MA (MA), or OVA + CFA (CFA)-injected mice and RNAs were extracted from the samples. Expression of indicated cytokines and chemokines were examined by realtime RT-PCR methods. Data from respective samples and Mean (horizontal bars) plus/minus SD are shown. *; $p < 0.05$.

show strong adjuvant activity, i.e., activity to stimulate innate immunity followed by subsequent activation of acquired immunity. Some of the MA-containing lipids from mycobacterial components, such as TDM, have been identified to show strong immunostimulatory activity. Importantly, Korf, et al. previously reported that MA shows typical properties of a PAMP, which triggers innate immune responses [28]. Although immunostimulatory activities of MA and MA-containing compounds have been shown, we in this study focused *in vivo* and *in vitro* adjuvant activity of purified MA.

While MA induced antibody responses comparable to CFA, it was less inflammatogenic than CFA. Tissue-destructive inflammation with neutrophil infiltration, induced in case of CFA injection, was not observed in MA-injected mice. In line with the observation that macrophages but not neutrophils were the dominant infiltrates in MA-injected areas, Th1 immune responses rather than Th17 was induced by MA

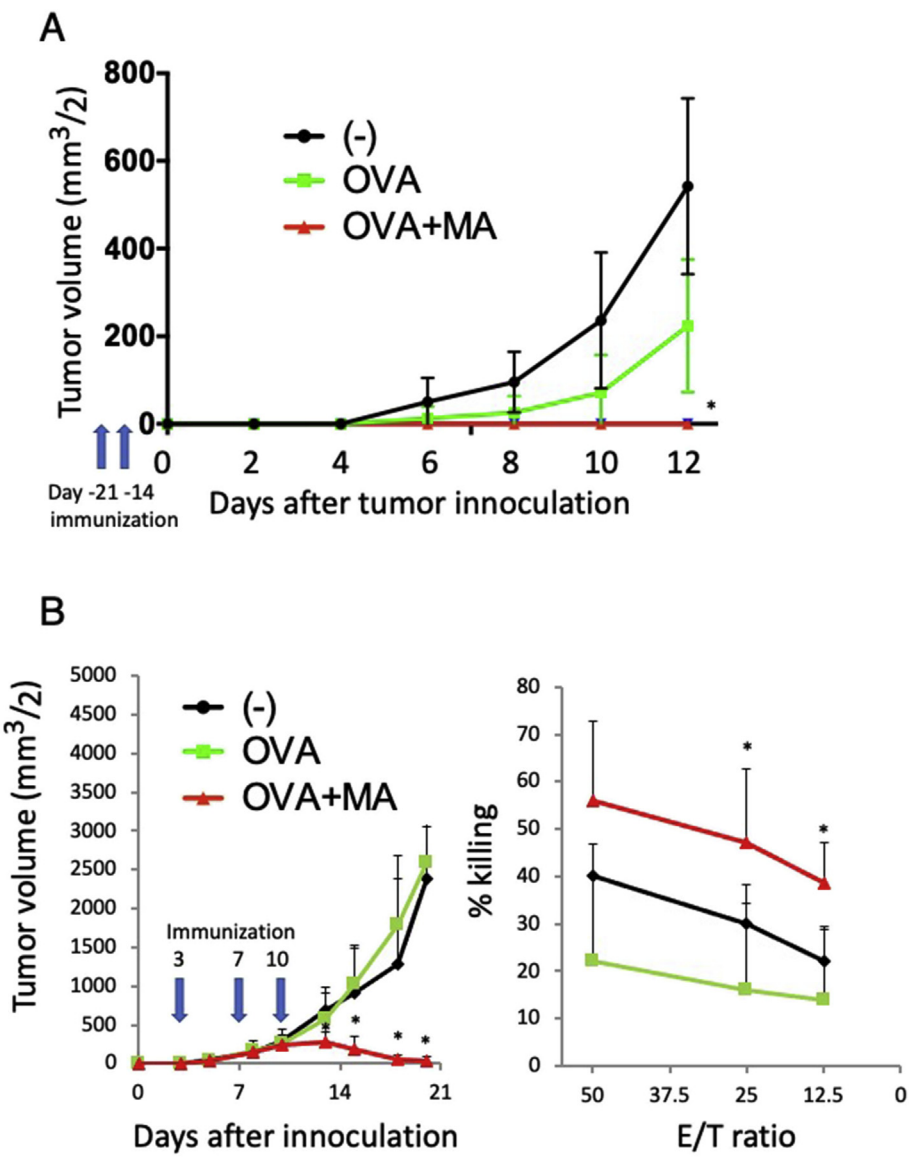


Figure 4. Induction of anti-tumor immunity by MA. A) In a prophylactic model, mice were immunized on days -21 and -14 with OVA (green), OVA plus MA (red), or untreated (black). On day 0, mice were inoculated with E.G7-OVA cells and tumor size was monitored. *(both OVA plus MA and OVA plus CFA as compared with untreated mice); $p < 0.05$. ($n = 5$ in each group. Experiments were repeated 3 times with similar results.) B) In a therapy model, mice were inoculated with E.G7-OVA cells on day 0 and immunized on days 3 and 7 with OVA (green), OVA plus MA (red), or untreated (black). Tumor size was monitored (left). *; $p < 0.05$ over untreated mice. ($n = 5$ in each group. Experiments were repeated 3 times with similar results.) On day 21 after tumor inoculation, CTL activity against E.G7-OVA cells was measured as described in materials and methods (right). E/T ratio; effector to target ratio (ratio of number of spleen cells from mice to that of ^{51}Cr-labeled E.G7-OVA cells). *; $p < 0.05$ over untreated mice. ($n = 5$ in each group. Experiments were repeated 3 times with similar results.)

(Figure 2A). Given the sufficient induction of antibody responses and anti-tumor immunity, MA will be a seed for a candidate material for development of new vaccine formulations.

Most of current vaccines provide immunity against pathogens through induction of humoral immunity [29]. Accordingly, most of adjuvants used and/or examined show antibody-inducing features, while each of them also has different properties to induce immune responses, i.e., Th1/17/2-mediated and CD8-mediated ones [4]. MA in the current study demonstrated enhancement of Th1 responses and augmentation of cytotoxicity-mediated tumor killing, in addition to increase in antibody titers. Induction of humoral responses by enhancing antibody production is a primarily important feature of an adjuvant for proper establishment of protective immunity against most pathogens. Th1 induction is also important and beneficial for protection against intracellular pathogens, such as *Mycobacterium*. Enhancement of cellular immunity by MA is of critical importance, since development of adjuvants that promote $CD8^+$ T cell response to soluble proteins is challenging; proteins in vaccine formulation are supposed to be phagocytosed by macrophages for MHC class II presentation while other mechanisms such as cross-presentation are needed for presentation of soluble proteins by MCH class I molecules, which is required for $CD8^+$ T cell responses. This property of MA to induce $CD8^+$ T cell responses is beneficial for development of tumor vaccines as well as vaccines against intracellular viral or bacterial pathogens.

Currently, the mechanisms of action of MA as an adjuvant is not clear. MA, however, activated bone marrow-derived DCs for co-stimulatory molecule induction and cytokine/chemokine production (Figure 2B, C). Interestingly, Vander Beken, et al. previously revealed structure-function relationships of MAs in that different types of MA, differing in properties of inter alia oxygenation and/or *cis*- vs. alpha-methyl-*trans*- cyclopropane chemistry, lead to differential innate immune responses in the lung [30]. Similarly, Smet, et al. reported that the presence and nature of the functional groups within the mycolate chain affected the immune responses [31]. Although we in the current study used purified mixture of MA as a whole, further analyses with different forms of MAs, either further purified or chemically synthesized, would demonstrate more specific adjuvant activities of MA and preferable forms of MA for induction of desired types of immune responses.

Given the different forms of MAs with differential immune-stimulating functions, it is important to identify the receptor(s) for MA for the adjuvant activity. In an effort to identify the cell-surface receptors for MA, we revealed that MA (same material used in this study) stimulated TREM2-expressing reporter cells and also macrophages for chemokine production in a TREM2-dependent manner (Iizasa et al.,

submitted). TREM2 (triggering receptor expressed on myeloid cells 2) is a member of immunoglobulin superfamily and is expressed on the cell surface of myeloid-derived innate immune cells as well as of microglial cells and has been implicated in various neurodegenerative diseases [32]. This would be in line with the previous report by Korf, et al. showing that MA stimulates innate immunity through receptor(s) distinct from Toll-like receptors [28]. Interestingly, Dkhar, et al. demonstrated that Testicular receptor 4 (TR4), a lipid-sensing nuclear receptor, is a receptor for *Mycobacterium*-derived keto-MA, shed from the bacteria inside the macrophages [33]. According to the report, keto-MA-TR4 axis is responsible for induction of foamy macrophages during granuloma formation. In contrast, TREM2 may be required for early induction of inflammatory responses upon encounter of innate immune cells with extracellular *Mycobacteria*. It is likely that distinct preparation of MAs (strains of *Mycobacterium*, purity, and ways to prepare antigen-in-adjuvant, etc.) may stimulate immune cells through distinct receptors, resulting in differential effects. For the adjuvant feature of MA, however, other mechanisms including depot effects, just like water-in-oil adjuvant, should be taken into consideration in addition to ligand/receptor interactions.

Even with the promising results in this study of the adjuvant activity of MA, further elucidation of the mode of action, features of induced immunity, and long-term safety needs to be taken into consideration before MA can be considered as an adjuvant for human vaccines. Also, natural MA is not a single component and may vary in composition from one isolated batch to another. For the safety and efficacy in human use, various adjuvants, including monophosphoryl lipid A (MPL®) [34] and MF59 [35] have been extensively studied and approved [36]. For experimental usage of adjuvants in animals, we may consider MA as an immediate candidate. The guiding principles underpinning the humane use of animals in scientific research require 3 Rs (Replace, Reduce, and Refine [37]). Given the less devastating inflammatory impacts of MA over CFA (Figure 3A) with substantially comparable adjuvant activity, replacement of CFA with MA may contribute to decrease the sufferings of animals and therefore provide a more refined approach to animal vaccination. We have to take the difference of immune responses induced by each adjuvant (Figure 2A), as well as the efficacy and safety of each material, into consideration, though.

In the current study, we prepared antigen in adjuvant by mixing and dissolving MA in Bayol F (mineral oil) followed by vigorous mixing with OVA solution, as performed with CFA. Immunization was performed either at the tail base or at the ear skin, which lead to induction of antigen-specific immune responses. Interestingly, however, Korf, et al. reported that MA incorporated into liposome resulted in the suppression of immune responses by induction of Foxp3+ regulatory T cells [38]. Alteration in macrophage activation status by interaction with MA-liposome was responsible for the Treg induction. While MA in liposome should be phagocytosed by macrophages and MA plays role intracellularly in this context (presumably interacting with nuclear receptor, TR4), MA in our study, injected in the skin, would initially interact with cell surface molecules (possibly TREM2). It is thus possible that the different methods of MA usage may lead to the different immune responses. However, we have to carefully examine the immune activating feature of MA in terms of dose, preparation, sites of injection, etc., in the future studies.

The adjuvant activity of MA in tumor prevention/therapy model is of significance. In our current study, MA as an adjuvant augmented antitumor activity in both prevention and therapy models (Figure 4A, B). Enhancement of antigen-specific CTL activity with MA is thus important, since only a couple of adjuvants have shown to induce CD8+ T cell responses so far [4]. Despite the results, it would be premature to implicate MA as an "anti-tumor vaccine adjuvant". Because the expression of model antigen (OVA) in parental EL4 lymphoma is artificial (driven by human β-actin promoter) [39] and should be high as compared with tumor (-associated) antigens in human cancers [40] In addition, there are various obstacles to overcome, including immune checkpoint molecules, for the tumor vaccine (plus adjuvant) to be effective *in vivo*. Suppression of tumor growth and/or tumor rejection in the current experimental settings is however evident. In addition to the enhancement of antigen-specific CTL activity, MA adjuvant may have facilitated the observed antitumor activity by other mechanisms such as induction of antibody mediated cell killing and immune orientation towards the Th1 mode.

As a conclusion, we have identified MA as a potent and safe adjuvant candidate in mice. Further studies on the required structure (for instance, minimum length of acyl group), mode of action, type of immunity induced, and long-term safety for various vaccination goals in different species of animals and man will add to the confidence to eventually take up MA as a general adjuvant for different commercial vaccines.

Declarations

Author contribution statement

H. Yoshida: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Kubota: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

E. Iizasa: Conceived and designed the experiments; Performed the experiments.

Y. Chuuma and H. Kiyohara: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

H. Hara: Conceived and designed the experiments; Analyzed and interpreted the data.

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Competing interest statement

Yasushi Chuuma and Hideyasu Kiyohara are employees of Japan BCG Laboratory. Other authors have no conflict of interest to disclose.

Additional information

No additional information is available for this paper.

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