Mycobacterium tuberculosis-associated synthetic mycolates differentially exert immune stimulatory adjuvant activity

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Mycolic acids (MAs) are highly hydrophobic long-chain α-alkyl β-hydroxy fatty acids present in the cell wall of *Mycobacterium tuberculosis* (*Mtb*) as a complex mixture of molecules with a common general structure but with variable functional groups in the meromycolate chain. In this study, we addressed the relationship between the MA molec-ular structure and their contribution to the development of T-cell immune responses. Hereto, we used the model antigen ovalbumin and single synthetic MAs, differing in oxygenation class and *cis* versus *trans* proximal cyclopropane configuration, as immune stimulatory agents. Subcutaneous delivery of liposome-formulated MAs with a proximal *cis* cyclopropane elicited antigen-specific Th1 and cytotoxic T-cell immune responses, whereas intratracheal immunization elicited pulmonary Th17 responses. These immune stimulatory activities depended not only on the *cis* versus *trans* proximal cyclopropane configuration but also on the MA oxygenation class. Our study thus shows that both the presence and nature of the functional groups in the meromycolate chain affect the immune stimulatory adjuvant activity of *Mtb* mycolates and suggests that *Mtb* bacilli may impact on the host protective immune response by modulating the *cis* versus *trans* stere-ochemistry of its mycolates as well as by altering the oxygenation class of the meromy-colate functional group.

Keywords: Adjuvant biolipids · *Mycobacterium tuberculosis* · Mycolic acid · T-cell responses

Introduction

Mycolic acids (MAs) are highly hydrophobic long-chain α-alkyl β-hydroxy fatty acids present in the outer membrane of *Corynebacterineae*, a distinct group of bacteria including the lung

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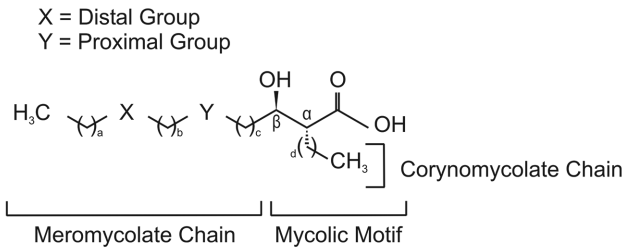
pathogen *Mycobacterium tuberculosis* (*Mtb*). Natural MAs are present in the bacterial cell membrane either as membrane-bound penta-arabinose tetramycolates or as noncovalently bound mycolate esters such as trehalose dimycolate (TDM). The MAs synthesized by *Mtb* present a complex mixture of molecules with a common general structure but with a large variability in chain lengths and functional groups. While the shorter α-alkyl MA chain, i.e. the corynomycolate chain, does not contain any functional groups, the longer β-hydroxy MA chain, i.e. the meromycolate chain, contains two functional groups. Based on the oxygenation of the distal functional group, three major MA classes can be distinguished, namely α-mycolic acids (αMAs), methoxy-mycolic acids (mMAs), and keto-mycolic acids (kMAs). The proximal functional group consists mainly of a cyclopropane that can either have a *cis* or *trans* configuration in the oxygenated keto- and methoxy-MAs and almost always occurs predominantly in the *cis* configuration in the nonoxygenated αMAs [1, 2].

The chemical synthesis of MAs with a defined molecular structure identical to naturally occurring MAs [3–7] has enabled the discovery of MA features related to their structural complexity. In a previous report by Vander Beken *et al.*, analysis of MA-elicited lung inflammation revealed a strong dependence of the innate inflammatory response on MA stereochemistry and functional groups. Whereas αMA was inert, oxygenated methoxy-MA and keto-MA with *cis* cyclopropane orientation elicited solid to mild inflammatory responses respectively. In contrast herewith, the oxygenated MAs with *trans* cyclopropane orientation showed reduced inflammatory potential or even exerted anti-inflammatory activity [8]. The differential outcome of MA structure on innate immune activation in the lung led us to compare the relationship between the molecular structure of the MAs and their ability to drive adaptive immune response in the mouse host when co-formulated with the model protein antigen, ovalbumin (OVA). While MAs have already been described as lipid antigens in cellular and humoral anti-mycobacterial immune responses [9, 10], this is the first study to explore their role as modulators of these adaptive immune responses.



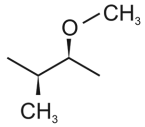
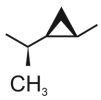
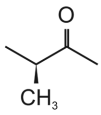
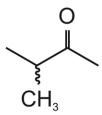
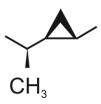
	Distal Group	Proximal Group	Chain Lengths a, b, c, d
cis αMA			19, 14, 11, 23
cis mMA			17, 16, 17, 23
trans mMA			17, 16, 18, 23
cis kMA			17, 18, 15, 23
trans kMA			17, 18, 16, 23

Figure 1. Chemical structure of *Mycobacterium tuberculosis*-associated synthetic mycolic acids (MAs). The general structure of an MA consists of a mycolic motif that contains a corynomycolate chain without functional groups and of a meromycolate chain with two functional groups. The proximal and distal functional group of the meromycolate chain is depicted for all compounds with its stereochemistry. In the nomenclature *cis* or *trans* refers to the stereochemistry of the proximal cyclopropane group. αMA, mMA, and kMA represent MAs with a distal functional group consisting of a cyclopropane, a methoxy, or a ketone group, respectively. The length of the carbon chains is represented by a, b, c, d.

Results and discussion

Synthetic mycolic acids are formulated in phosphatidyl choline liposomes for in vivo delivery

In this study we addressed the immunomodulatory properties of single synthetic mycolic acid isomers, representing all three naturally occurring mycolic acid classes, varying in *cis* versus *trans* cyclopropane configuration (Fig. 1). The αMA contains two cyclopropane groups in *cis* configuration (*cis* αMA). The methoxy-MA has a distal (S,S)-α-methyl-methoxy group and a proximal cyclopropane group that occurs either in *cis* or *trans* configuration (*cis* and *trans* mMA). The keto-MA either consists of a distal (S)-α-methyl-ketone group and of a proximal *cis*-cyclopropane (*cis* kMA) or of a mixture of both epimers of the distal α-methyl-ketone group with S- and R-stereochemistry and of a proximal *trans*-cyclopropane (*trans* kMA).

As MAs are highly hydrophobic and are not soluble in aqueous solutions, they were formulated into phosphatidyl choline liposomes together with the model antigen ovalbumin (OVA).

Subcutaneous mycolic acid immunization elicits an antigen-specific Th1 and cytotoxic T-cell immune response

In a first instance, the variant antigen formulations were delivered by subcutaneous route. Either we performed a single immunization or applied a prime-boost immunization strategy consisting of two injections separated by a 3-week interval. One week after immunization, the number of OVA-specific IFN-γ-producing $CD8^+$ T-cells and $CD4^+$ T-cells in the spleen were measured by ELISPOT analysis and compared to the numbers found in mice immunized with liposome-formulated OVA. The

different MAs were also compared with AddaVax™ as a gold standard. AddaVax™ is a squalene-based oil-in-water emulsion which is currently used as adjuvant in various influenza vaccines [11]. Immunization with PBS served as a negative control and resulted in IFN-γ-secreting $CD8^+$ and $CD4^+$ cell numbers below $25/10^6$ splenocytes throughout all experiments (not shown).

Immunization with *cis* αMA resulted in a robust $CD8^+$ and $CD4^+$ IFN-γ response already 1 week after a single immunization as compared to OVA formulated in liposomes alone (Fig. 2A, B). At this time point, no $CD8^+$ or $CD4^+$ T-cell responses were seen in splenocytes of mice immunized with AddaVax™ or any of the other MAs. Pronounced CD4 and CD8 T-cell responses following immunization with AddaVax™ were observed only after a second immunization, thus requiring a conventional prime-boost schedule. In contrast, a second immunization with *cis* αMA did not exert a boost effect; eliciting CD4 and CD8 T-cell recall responses in the same range as elicited by the prime immunization (Fig 2A, B). The swift induction by *cis* αMA of an effector immune response already after a primary immunization is remarkable as in a previous report this particular MA did not induce innate inflammatory responses in the lung or peritoneal cavity [8]. The ability of *cis* αMA to induce $CD8^+$ and $CD4^+$ IFN-γ responses already after a primary immunization and in the absence of an innate inflammatory response makes it a promising candidate for vaccine adjuvant development. However, the most pronounced $CD4^+$ and $CD8^+$ IFN-γ responses after a prime-boost immunization, comparable to those seen in mice immunized with AddaVax, were observed with OVA-liposomes co-formulated with *cis* kMA (Fig. 2A, B). Here, a high number of IFNγ-producing cells were observed also in the absence of OVA peptide restimulation. Likely, this response results from $CD4^+$ and $CD8^+$ T-cells which are still highly activated 1 week after the boost immunization. Modest T-cell responses were seen mostly with *cis* mMA, *trans* mMA, and *trans* kMA as immune stimulatory biolipid. Combined, these data show that the most pronounced immune stimulatory activity was elicited with *cis* αMA and *cis* kMA. When co-formulated with OVA antigen, the biolipids promoted strong effector T-cell responses after prime immunization or strong memory T-cell responses after prime-boost immunization, respectively. Strikingly, MAs possessing a distal methyl-methoxy functional group (mMA) or having a proximal cyclopropane that occurs in *trans* configuration (*trans* kMA, *trans* mMA) showed a much lower potency in exerting immune stimulatory adjuvant activity. This dependence on MA class and stereochemistry of eliciting IFN-γ-driven host immune responses against accompanying antigens suggests that *Mtb* may control the strength of the host IFN-γ immune response by modifying the nature of the functional groups in the MA meromycolate chain.

Analysis of the humoral immune response after prime-boost immunization revealed a quite different pattern. None of the mycolates co-formulated with OVA-liposomes significantly increased the levels of OVA-specific IgG1 and IgG2c above the levels already elicited by the liposomal formulation of OVA alone (Fig. 2C). Thus, whereas select MAs promoted cellular immunity, none of the MAs stimulated antigen-specific antibody production.

Intratracheal mycolic acid immunization elicits a pulmonary Th17 response

Aside from a central role of IFN-γ-producing Th1 and cytotoxic T cells in primary *Mtb* infection, a role for Th17 cells has also been documented [12, 13]. As the pulmonary route constitutes the natural route of infection of *Mtb*, we verified to what extent intratracheal (i.t.) administration of the different MA formulations could promote a Th1/Th17 immune response in the lungs against the co-formulated OVA. AddaVax™ was not included in this experiment, as this adjuvant is only suited for parenteral application. One week after i.t. prime-boost immunization, the numbers of IFN-γ- and IL-17-producing $CD4^+$ T cells in the lungs were measured by flow cytometry-based cell counting. Also here, *cis* kMA led to a significant increase in IFN-γ-producing $CD4^+$ T cells as compared to liposome-formulated OVA (Fig. 3A). Whereas this response was rather weak, a pronounced increase in IL-17-producing $CD4^+$ T-cells was observed with *cis* kMA but also with *cis* mMA as adjuvant. Thus, c*is* mMA and especially *cis* kMA here emerge as *Mtb* biolipids capable of exerting immune stimulatory adjuvant activity promoting the induction of Th1 and Th17-driven cellular immune defenses of importance in the protective immune response against pulmonary *Mtb* infection [14–17].

Concluding remarks

Our findings identify MAs with a proximal *cis*-cyclopropane and belonging especially to the keto oxygenation class as modulators of T-cell-mediated cellular immunity, enabling the induction of Th1, Th17, and cytotoxic T-cell immune responses against accompanying protein antigens in the absence of a humoral antibody response. When extrapolated to *Mtb* infection, this differential immune stimulatory adjuvant activity, dependent on the nature and stereochemistry of the functional group in the MA meromycolate chain, suggests that the structural complexity of the biolipids may play a role in determining the strength and nature of the host immune response to infection with *Mtb* bacilli.

Materials and methods

Mice

C57BL/6 female mice were purchased from Janvier Labs (St. Berthevin, France) and were housed under specific pathogen-free conditions in individually ventilated cages in a controlled day-night cycle and given food and water ad libitum. Mice were 8 weeks at the start of the experiments. All experiments were approved by the animal ethics committee of Ghent University

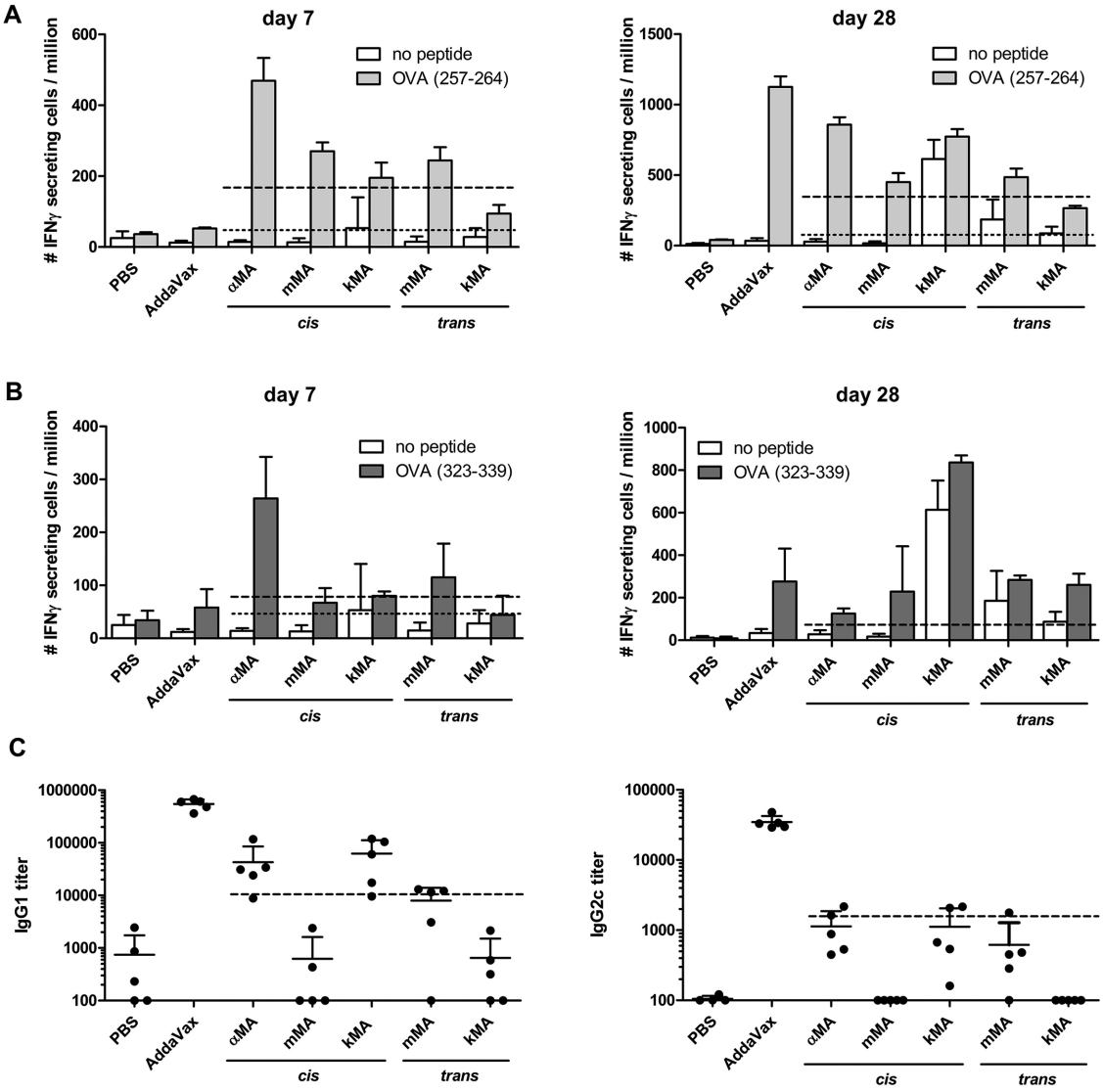


Figure 2. Antigen-specific IFN-γ-secreting $CD8^+$ and $CD4^+$ T cells in the spleen and IgG1 and IgG2c serum titers after MA immunization. Mice were immunized s.c. with OVA or OVA emulsified with AddaVax or formulated in liposomes with or without MAs. Splenocytes were cultured in the presence or absence of (A) MHCI-restricted OVA(257-264) peptide or (B) MHCII-restricted OVA(323-339) peptide. The number of (A) IFN-γ-secreting $CD8^+$ T cells and (B) IFN-γ-secreting $CD4^+$ T-cells per million splenocytes was measured by ELISPOT analysis at day 7 (left panels) and day 28 (right panels) post prime immunization. The number of IFN-γ-secreting $CD8^+$ and $CD4^+$ T-cells of mice immunized with OVA formulated in liposomes without MAs is depicted by a dashed (stimulation with relevant peptide) or a dotted (no peptide stimulation) line. (C) The titer of OVA-specific IgG1 and IgG2c was measured in the serum at day 28 post prime immunization by ELISA. The IgG1 and IgG2c serum titers of mice immunized with OVA formulated in liposomes without MAs are depicted by a dashed line. Samples depicted with a titer of 100 represent samples falling below the detection limit of the assay. Data are mean ±SD, $n = 5$ mice per group. For the ELISPOT analyses, spleens were pooled per group of five mice and analyzed in quadruplicate. The results show one representative experiment out of three independent experiments with for each experiment five mice per group.

(accreditation nr. LA1400091, Belgium), in accordance with European guidelines.

Vaccine preparation

Chemically defined MA isomers were synthesized as described previously [3–7]. The MAs were incorporated into phosphatidyl choline liposomes as described previously [8, 18, 19]. Briefly, per mouse 25 μg of MA was mixed with 4,5 μL phosphatidyl choline from egg yolk (Sigma, St. Louis, MO, USA) dissolved at 100 mg/mL in chloroform. The chloroform was evaporated and 10 μg of OVA grade VII (Sigma; 0,048 EU endotoxin/10 μg OVA) dissolved in 70–100 μL of endotoxin free PBS (Lonza, Basel, Switzerland) was added to the lipids. To obtain liposomes the suspension was vortexed for 10 s and put in a sonication bath at 60–70°C for 10 min. This was repeated four times. AddaVax (Invivogen, San Diego, CA, USA) was used according to the manufacturer's instructions and mixed at a 1:1 ratio with 10 μg OVA grade VII per mouse in endotoxin-free PBS.

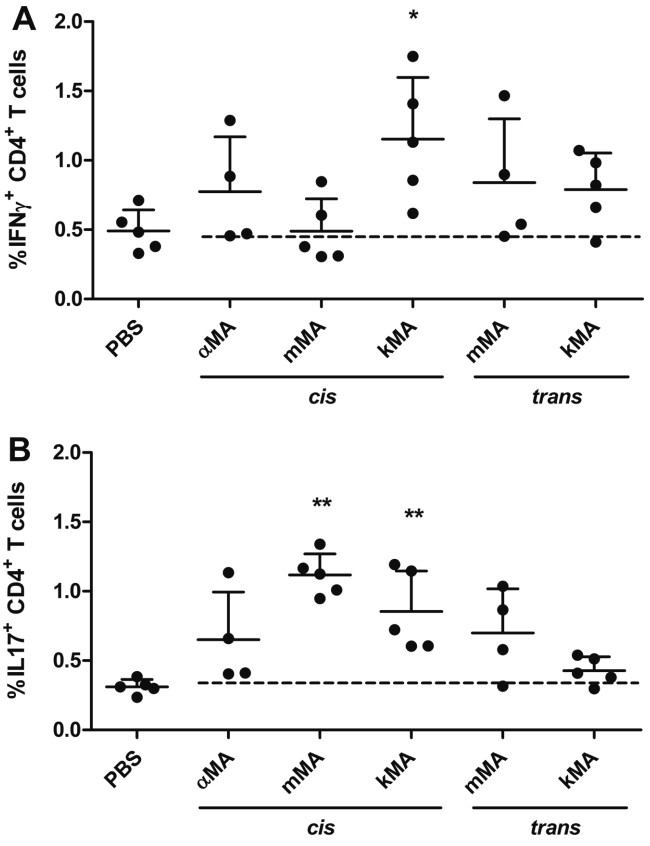


Figure 3. Number of IFN-γ- and IL-17-producing $CD4^+$ T cells in lungs after pulmonary MA immunization. Mice were immunized i.t. with PBS or with OVA formulated in liposomes with or without MAs. (A-B) At day 28, lung cells were stimulated with anti-CD3 ex vivo. The number of IFN-γ- (A) and IL-17-producing (B) $CD4^+$ T cells was determined by intracellular cytokine staining and flow cytometry analysis. Dashed line represents the number of IFN-γ- and IL-17-producing $CD4^+$ T-cells in the lungs of mice immunized with OVA formulated in liposomes without MAs. Data are mean ±SD, $n = 5$ mice per group. Statistical analysis between two groups was performed using a Mann–Whitney test. Significant p-values were ranked as $p < 0.05$ (*) and $p < 0.01$ (**). The data are derived from one independent experiment.

Immunization

Mice were immunized according to a prime-boost vaccination strategy consisting of two immunizations with a 3-week interval. For subcutaneous (s.c.) immunization, mice were injected with 100 μL of vaccine formulation at the tail base. For intracheal (i.t.) immunization, mice were sedated with ketamine/xylazine and instilled with 70 μL vaccine formulation.

Detection of IFN-γ-producing cells by ELISPOT assay

Spleens were pooled per group and passed through a 70 μm cell strainer to obtain a single cell suspension. Red blood cells were lysed using ACK lysing buffer (Lonza) and 2×10^5 cells were cultured on a PVDF plate coated with mIFN-γ capture antibody (Diaclone, Besançon, France) in the presence of 10 μg/mL MHCI-restricted OVA(257-264) peptide or 15 μg/mL MHCII-restricted OVA(323-339) peptide (AnaSpec, EGT Group, Freemont, CA, USA). For each group, cells were plated in quadruplicate. After 24 h, the plate was developed according to the manufacturer's instructions.

Measurement of OVA-specific serum IgG1 and IgG2c levels

OVA-specific IgG1 and IgG2c levels in serum were determined by ELISA. Briefly, plates were coated with OVA (grade V, Sigma) and incubated with a four-fold serial dilution of the serum samples. The OVA-bound IgG1 and IgG2c antibodies were detected with an HRP-conjugated rat anti-mouse IgG1 or IgG2c detection antibody (Southern Biotech, Birmingham, AL, USA). The plate was developed with TMB substrate (BD Biosciences, San Jose, CA, USA) and measurements were made at 450/655nm using a microplate reader after stopping the reaction with sulphuric acid. The dilution factor of the serum at a specific absorbance value was calculated and used as a measure of OVA-specific IgG1 or IgG2c antibody levels in the serum.

Intracellular cytokine staining of lung cells

Lungs were cut into small pieces, digested for 20 min at 37°C with 1 mg/mL collagenase type IV (Sigma) and 150 U/mL DNaseI (Roche Life Science, Basel, Switzerland) and passed through a 70 μm cell strainer to obtain a single cell suspension. Red blood cells were lysed with ACK lysis buffer and 2×10^6 cells were cultured in a 24-well plate coated with 1 μg anti-CD3 per well to restimulate the cells. After 1 h, 1 μL/mL brefeldinA (Golgi Plug BD Biosciences) was added and cells were harvested 6 h later. To allow for differential cell counting, cells were first labeled for the surface markers CD45 (30-F11) and CD4 (RM4-5) (both from BD Biosciences) together with CD3 (17A2) and a fixable live/dead marker (both from eBioscience, San Diego, CA, USA). Cells were labeled with the antibodies at 4°C in the presence of anti-CD16/32 (2.4G2) (Fc block, BD Biosciences) to block nonspecific binding. After staining, the cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer's instructions, after which they were stained for the intracellular cytokines IL-17 (TC11-18H10) and IFN-γ (XMG1.2) or their isotype control IgG1 κ (R3-34) conjugated to the respective fluorophores (all from BD biosciences). Measurements were performed on an LSRII flow cytometer (BD Biosciences) and analysis was done with FACS Diva software (BD Biosciences).

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Abbreviations: **MA**: mycolic acid · ***Mtb***: *Mycobacterium tuberculosis* · **OVA**: ovalbumin