**Inflammatory properties and adjuvant potential of synthetic glycolipids homologous to mycolate esters of the cell wall of *Mycobacterium tuberculosis*.**

Hermann Giresse TIMA (1), Juma'a Raheem AL DULAYYMI (2), Olivier DENIS (1), Pauline LEHEBEL (1), Klarah Sherzad BAOLS (2), Mohsin Omar MOHAMMED (2), Laurent L'HOMME (3), Mohaned Mohammed SAHB (2), Georges POTEMBERG (1), Sylvie LEGRAND (3), Roland LANG (4), Rudi BEYAERT (5), Jacques PIETTE (3), Mark Stephen BAIRD (2)#, Kris HUYGEN (1)#, Marta ROMANO (1)*#.

(1): Scientific Service Immunology, O.D. Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), B-1180 Brussels, Belgium

(2): School of Chemistry, Bangor University, Bangor, Gwynedd LL57 2UW, UK

(3): Laboratory of Virology and Immunology, GIGA-Signal Transduction, University of Liege, B-4000 Liege, Belgium

(4): Mikrobiologisches Institut, Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen, 91054 Erlangen, Germany

(5): Inflammation Research Center VIB and Department for Biomedical Molecular Biology, Ghent University, B-9052 Ghent, Belgium

**Short title: "Inflammatory properties and adjuvant potential of synthetic mycolate esters"**

**#These authors share senior authorship**

***Corresponding author:** Marta ROMANO, Scientific Service Immunology, O.D. Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), 642 rue Engelandstraat, B-1180 Brussels, Belgium

Phone: +32 2 373 33 25

Fax: +32 2 373 33 67

e-mail: marta.romano@wiv-isp.be

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**Abstract**

The cell wall of mycobacteria is characterized by glycolipids composed of different classes of mycolic acids (MAs) (alpha-, keto- and methoxy-) and sugars (trehalose, glucose and arabinose). Studies using mutant *Mtb* strains have shown that the structure of MAs influences the inflammatory potential of these glycolipids. As mutant *Mtb* strains possess a complex mixture of glycolipids, we analyzed the inflammatory potential of single classes of mycolate esters of *Mtb* cell wall using 38 different synthetic analogues. Our results show that synthetic trehalose dimycolates (TDMs) and trehalose-, glucose- and arabinose-monomycolates (TMMs, GMMs and AraMMs) activate BMDCs in terms of production of pro-inflammatory cytokines (IL-6 and TNF-α) and ROS, upregulation of costimulatory molecules and activation of NLRP3 inflammasome by a mechanism dependent on Mincle. These findings demonstrate that Mincle receptor can also recognize pentose esters and seem to contradict the hypothesis that production of GMM is an escape mechanism used by pathogenic mycobacteria to avoid recognition by the innate immune system. Finally, our experiments indicate that TMM and GMM as well as TDM can promote Th1 and Th17 responses in mice in an OVA immunization model and that further analysis of their potential as novel adjuvants for subunit vaccines is warranted.

**Introduction**:

*Mycobacterium tuberculosis* (*Mtb)*, the causative agent of tuberculosis (TB) belongs to the single genus *Mycobacterium* of the *Mycobacteriaceae* family. Bacteria from this family are characterized by the presence in their cell wall of high molecular weight C-60 to C-90 2-alkyl-3-hydroxy fatty acids called mycolic acids (MA). Mycolic acids are the major lipids constituting the mycobacterial cell wall [1,2], present as free mycolic acids [3] or esterified to different sugars. They are predominantly bound to the cell wall with arabinogalactan as penta-arabinose tetramycolates, but they are also present as non-bound solvent extractable esters, such as trehalose mono- and dimycolate (TMM and TDM), glucose monomycolate (GMM) and glycerol monomycolate (Gro-MM) [1,2].

Among these components, trehalose 6,6'-dimycolate (TDM) - also known as cord factor – is the major and most studied mycolate ester. Indeed, in mouse models TDM can mimic certain aspects related to *Mtb* infection, including the production of pro-inflammatory cytokines [4,5], the induction of granuloma and pro-coagulant activity [6–8]. In addition, TDM can delay phagolysosome fusion [9], is involved in tissue damage and necrosis, and possesses adjuvant properties [10]. However, natural TDM used so far in these different studies was isolated from mycobacteria and consisted of a complex mixture of structurally related compounds composed of mycolates from different classes. Indeed, based on the functional group(s) present on their meromycolate chain, mycolic acids are subdivided in different classes. The principal classes are the alpha-, keto- and methoxy- mycolic acids. These classes differ considerably between mycobacterial species and strains [11], and appear to influence their virulence. For example, the attenuated vaccine strain *M. bovis* BCG-Pasteur lacks methoxy-mycolic acids [12] and saprophytic mycobacteria such as *M. smegmatis* fail to produce oxygenated (keto- and methoxy-) mycolic acids [2]. In contrast, highly pathogenic mycobacteria such as *Mtb* and *M. leprae* produce oxygenated mycolic acids. Genetic inactivation of enzymatic pathways involved in the synthesis of oxygenated mycolic acids resulted in *in vivo* attenuated *Mtb* strains [13,14]. Therefore, the use of synthetic analogues can be useful to discriminate the contribution of different types of TDM or of mycolate esters in general to the pathogenicity of mycobacterial infection and to the inflammatory potential of these cell wall components. Indeed, the contribution of other mycolate esters found in the cell wall of *Mtb* such as TMM, GMM and arabinose mycolates in the activation of innate immune cells following mycobacterial infection is poorly defined.

TMM is known as a key precursor for the biosynthesis of TDM and has been reported to have some similarity with TDM in terms of inflammatory potential [4,15,16]. GMM has been demonstrated to be an antigen that is presented by CD1b molecules to T cells [17], but the activation of innate immune cells by GMM from *Mtb* is controversial. Indeed, it was reported that Mincle (macrophage inducible C-type lectin receptor) does not recognise GMM obtained by trehalase treatment of TDM [18], but that GMM of corynebacteria (C-32 mycolates in corynebacteria versus C-60 to C-90 mycolates in mycobacteria) does bind to Mincle and activates cells [19]. Mincle and MCL (macrophage C-type lectin) have been shown to be essential receptors for recognition of TDM [18,20] and TMM [21] and hence crucial for stimulation of cord factor induced innate immune responses. On the other hand, arabino-mycolate ester (a complex mixture of mono-arabinose monomycolates, tetra-arabinose tetra-mycolates, penta-arabinose tetra-mycolates and hexa-arabinose tetra-mycolates) obtained by acid hydrolysis of BCG cell wall skeleton (CWS) was reported to induce production of TNF-α by mechanisms dependent on TLR-2 / MyD88 pathway [22]. Finally, it has been shown that trehalose dibehenate (TDB), a short-acyl-chain structural analogue of TDM, activates the intracellular multiprotein complex called NLRP3 inflammasome [23] and that this activation is essential for the Th17 responses induced by TDB [24]. For the moment, it is still unknown whether TMM, GMM and arabinose mycolates can activate the inflammasome as well.

To address the relationship between the structure and the inflammatory power of mycolate esters and the signalling pathways involved, we have assayed in this study thirty-eight single synthetic mycolate ester isomers of high purity and known stereochemistry [25–28]. The mycolate esters tested here vary in terms of their carbohydrate moiety (trehalose, glucose or arabinose) and in terms of the number, nature and class of mycolic acids bound to it. The selected mycolate esters are composed of mycolic acids that are representative of the major classes found in the cell wall of *Mtb*, namely alpha-, methoxy- and keto- mycolates of either *cis* or *trans* configuration and with different chain lengths. In addition, also synthetic mycolate esters composed of alpha-mycolates containing an alkene group and homologous to those found in non-pathogenic mycobacteria (such as *M. smegmatis*) have been tested here for comparison purposes. Using murine bone marrow derived dendritic cells, we examined *in vitro* the ability of these compounds to induce production of pro-inflammatory cytokines (TNF-α, IL-6, IL-12 and IL-1β) and we identified the pathways involved in their inflammatory potential. We also confirmed their *in vivo* inflammatory potential and tested

their adjuvant capacity to promote adaptive immune responses in an ovalbumin vaccination model.

## Material and Methods

### Mice

C57BL/6 $^{wt/wt}$, MyD88$^{-/-}$ and MALT1$^{-/-}$ mice were bred and kept at the experimental animal facilities of WIV-ISP (Ukkel site, Brussels), complying with the Belgian legislation that transposes European Directive 2009/41/EC, repealing Directive 90/219/EC (EC, 2009). For some experiments, female C57BL/6 mice aged 6-8 weeks were purchased from Janvier-labs. Breeding pairs of MyD88$^{-/-}$ mice were kindly provided by C. Desmet (Cellular and Molecular Physiology, GIGA-Research, ULg, Belgium). MALT1$^{-/-}$ mice were described [29]. Bones (femurs and tibia) of Mincle$^{-/-}$, FcR$\gamma^{-/-}$ and NLRP3$^{-/-}$ mice were provided by R. Lang (University Clinics Erlangen, Germany) and B. Ryffel (CNRS, Orleans, France).

### Preparation of glycolipid coated plates

Stocks of synthetic glycolipids (synthetized at Bangor University [25–28,30,31], TDB (Invivogen) and natural TDM from *Mtb* (Adipogen) were solubilised in chloroform-methanol solution (9:1) at 5mg/mL. The different glycolipids were then dissolved in isopropanol and coated on flat bottomed culture plates (Greiner). After evaporation of the solvent, the plates were used directly or stored at -20°C. Final concentrations are indicated in the figure legends and results section. For the experiments performed at equimolar concentration, molarity of natural TDM (a mix) was calculated using an estimated molecular weight of 2754 g mol-1. A possible negative effect of the tested glycolipids on cell viability was tested by Alamar blue assay and no increased mortality was observed under the test conditions.

### Generation of BMDCs

BMDCs were generated as previously described [32]. Briefly, murine bone marrow from femur and tibia was flushed with PBS and red blood cells were lysed with Sigma's lysing buffer. After lysis, cells were cultured (5% $CO_2$ at 37°C) in T75 flask in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10 ng/mL recombinant murine GM-CSF (Immunotools), $5 \times 10^{-5}$ M 2-mercaptoethanol, 100 µg/mL gentamycin (GIBCO), 2mM glutamine, 1 mM sodium pyruvate, non-essential amino acids (Thermo Fisher Scientific) and 10% fetal calf serum (FCS, Greiner). Cells were differentiated for 6-7 days and the medium was replaced every 3 days. 90 % of CD11c positive cells were obtained after differentiation.

### In vitro stimulation with glycolipids and analysis of samples

BMDCs were harvested after 7 days using a cell scraper, counted and seeded in glycolipid-coated plates at a concentration of $10^6$ cells/ mL for different time periods. Culture supernatants were collected after 24h and the content of pro-inflammatory cytokines TNF-α, IL-12p40, IL-1β and IL-6 was determined by ELISA (eBiosciences).

Expression of co-stimulatory molecules was analysed after 48h of incubation with the different compounds. Cells were labelled with anti-mouse CD80, anti-mouse CD86 and anti-mouse MHC class II monoclonal antibodies (eBiosciences). Fluorescence was analysed using a FACSCalibur flow cytometer and analysed with CELL-QUEST software (BD Biosciences).

**HEK-blue mMincle stimulation**

Human embryonic kidney (HEK)-blue mMincle cells (Invivogen) stably transfected with murine C-type lectin receptor *mincle* gene, as well as the genes of Mincle-NF-κB signalling pathway and secreted alkaline phosphatase (SEAP) under the control of NF-κB activation, were used to analyse the activation of Mincle receptor by the compounds. HEK-blue mMincle cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5g/L glucose and supplemented with 10% FCS, selective antibiotics and 2mM glutamine. Cells were passed in T75 flask when 70-80% confluence was reached. For the stimulation, cells were seeded in 96 well plates at the density of $5.10^4$ cells/ well for 24 h. Quanti-blue substrate (Invivogen) was used for the detection of SEAP in the supernatant according to the manufacturer's instructions.

**Inflammasome assays**

BMDCs at $1.10^6$ cells/ mL were primed for 3 h with 1µg/mL of *E. coli* K12 ultra-pure LPS (Invivogen) prior to stimulation with glycolipids (on coated plates) and controls. Cells were stimulated in triplicate with 5mM ATP (for 1h) or 10 µg/mL of mycolic acid esters (for 5h). For the inhibition studies, Glibenclamide (50 µM - Sigma), Ebselen (10 µM - Sigma), Cytochalasin D (1 µM - Sigma), CA-074 methyl ester (10 µM - Sigma) and Z-VAD-FMK (10 µM - Invivogen) were added 1h prior to glycolipid stimulation. The effect of these inhibitors on cell viability was tested by Alamar blue or MTT assay and no increased mortality was observed under the test conditions. IL-1β cytokine production in cell-free supernatant was evaluated by ELISA (eBiosciences).

For the detection of activated caspase-1 in supernatants, cells were stimulated in FCS-free medium. Triplicate samples were pooled and the proteins were concentrated by methanol-

chloroform precipitation. Briefly, 500 µl of supernatant was mixed with 500 µl of methanol and 125 µl of chloroform, vortexed and centrifuged. The aqueous phase was collected, washed with methanol, mixed and centrifuged. The supernatant was discarded and the pellet was dried, reconstituted in SDS sample buffer (75mM Tris-HCl, pH 6.8, 100mM DTT, 2% SDS, 0.01% bromophenol blue and 10 % glycerol) and heated for 5 min at 95°C. Proteins were separated by electrophoresis for 1h30 at 100 V in 15% Tris-Glycine Gel and transferred to a nitrocellulose membrane using a semi-dry system (Amersham bioscience). 3% BSA and TBS-Tween 20 were used respectively as blocking and washing buffer. Monoclonal mouse anti-caspase-1 p20 Casper-1 (Adipogen) was used as primary antibody and polyclonal goat anti-mouse IgG HRP (R&D systems) as a secondary antibody. The revelation was done by applying ECL substrate (GE Healthcare Life Sciences) on the membrane and pictures were taken using an ImageQuant LAS 4000 device.

**ROS assay**

Reactive oxygen species (ROS) were analysed with cell-permeant 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) assay according to the manufacturer's protocol (Thermo Fisher Scientific). Briefly, BMDCs ($10^6$ cells/mL) were pre-incubated for 30 minutes in PBS to allow the dye to enter the cells, washed and seeded in 24-well coated plates for 4 hours. ROS levels were analysed by flow cytometry. Results were analysed using a FACSCalibur flow cytometer and analysed with CELL-QUEST software (BD Biosciences).

**Footpad immunisation with Ovalbumin**

Water in oil in water emulsions (w/o/w) were prepared by minor modification of a previously described protocol [33]. Briefly, glycolipids were dissolved in 30% of Incomplete Freund's Adjuvant (IFA) and vortexed vigorously. Next, PBS 0.1M was added and vortexed again vigorously to make a water in oil emulsion and finally PBS containing 0.2 % of Tween 80 containing the needed amount of ovalbumin was added to the water in oil preparation to make a water in oil in water emulsion. Groups of 4-5 mice were injected subcutaneously in both hind footpads with 25 µl of the emulsion containing 30% IFA, 5µg of glycolipids and 25µg of ovalbumin (Invivogen). Footpad swelling in individual mice was measured with a caliper before and at several time points after injection.

**Analysis of local footpad inflammation**

One week after administration of the w/o/w samples, the mice were sacrificed and the tissues of the hind footpads were collected and digested using collagenase and DNAse (Sigma). Cells were collected and strained with a 100 -µm nylon cell strainer (BD). Total RNA was isolated from footpad cells with Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was prepared using the GoScript Reverse Transcriptase system (Promega). qRT-PCR was performed on Stratagene 3000p using GoTaq qPCR Master mix (Promega) according to the manufacturer's instructions. Forward and reverse primers used are described below. GAPDH was used as reference housekeeping gene for the normalization. Additionally, footpad cells were incubated with Ly6G-Ly6C PE and viability dye V450 (eBiosciences) and the fluorescence was analysed with a FACSverse device.

**Primers**

CXCL1 forward: 5'-CCGAAGTCATAGCCACACTCAA-3'
CXCL1 reverse: 5'-GCAGTCTGTCTTCTTTCTCCGTTAC-3'
IL-1β forward: 5'-TTGACGGACCCCAAAAGAT-3'
IL-1β reverse: 5'-AGCTGGATGCTCTCATCAGG-3'
IL-12p35 forward: 5'-CCTCGGCATCCAGC-3
IL-12p35 reverse: 5'-TCTGGCCGTCCTCA-3'
TNF-α forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3'
TNF-α reverse: 5'-TGGGAGTAGACAAGGTACAACCC-3'
GAPDH forward: 5'-TCGGCCTTGACTGTGCCGTT-3'
GAPDH reverse: 5'-TCCCAGCCTCGTCCCGTAGAC-3'

**Analysis of adaptive immune responses**

One week after subcutaneous administration of the different w/o/w preparations, mice were sacrificed and popliteal and inguinal lymph nodes were removed and passed through a 100 -µm nylon cell strainer (BD). Cells were counted and stimulated in RPMI medium supplemented with $5 \times 10^{-5}$ M 2-mercaptoethanol, antibiotics and 10% FCS in round-bottom 96 well plates with 5µg/mL of ovalbumin, culture medium as a negative control and Concanavalin A (Sigma) (4µg/mL) as a positive control. Cell free culture supernatants were harvested after 24h and 72h of incubation at 37°C-5% CO2. Levels of IL-2 were measured in 24h supernatants by ELISA (eBiosciences). Levels of IFN-γ and IL-17A were determined in 72h supernatants by ELISA (respectively BD Pharmingen and eBiosciences).

**Statistical analyses**

Statistical analyses were performed using Graphpad Prism 6 software (Graphpad Software, La Jolla, CA, USA). For the statistical analysis of the *in vitro* experiments, data from three to four independent experiments were pooled and tested for Gaussian distribution with

D’Agostino Pearson test followed by pairwise comparisons performed by Mann-Whitney test. For the analysis of the *in vivo* experiments, pairwise comparisons were performed by Mann-Whitney test. For all analyses, $p<0.05$ was considered as statistically significant.

## Results

### 1. Synthetic trehalose di- and mono-mycolates are inflammatory

To study the influence of the class of mycolates and the number of mycolate chains on the inflammatory potential of trehalose mycolate esters, 17 trehalose dimycolates and 13 trehalose monomycolates were synthetized (Table1, TDM and TMM columns) [25–28,30,31]. These compounds vary in terms of their MA class (alpha-, methoxy-, keto-), the configuration of their cyclopropanation (*cis*- versus *trans*-) and the number of MAs bound to trehalose (dimycolates versus monomycolates). Bone marrow derived dendritic cells (BMDCs) were incubated for 24h with the different synthetic TMMs, TDMs, evaporated isopropanol (ISO) as a negative control, trehalose-6,6-dibehenate (TDB) or TDM isolated from *Mtb* (TDM natural mix) as positive controls. The production of pro-inflammatory cytokines IL-6 and TNF-α was evaluated by ELISA in culture supernatants and results obtained for 10 TDMs and 9 TMMs at equimolar concentrations are shown in Figure 1, while results obtained at 10µg/ml for 16 TDMs and 11 TMMs are shown in supplementary Table S1. All the tested synthetic TMMs and TDMs stimulated the production of TNF-α and IL-6. Interestingly, when dose-response analysis was performed with pairs of TMM and TDM composed of the same mycolate moiety, we observed that the number of mycolate chains bound to trehalose influenced the level of TNF-α and IL-6 produced. Indeed, as shown in Figure 1A and 1B, at equimolar concentrations the *cis*-methoxy TMM KB51 (monomycolate) induced TNF-α and IL-6 levels that were 2-3 fold lower than those achieved by stimulation with the corresponding *cis*-methoxy TDM KB52 (dimycolate). TNF-α levels induced by KB51 were comparable to those induced by trehalose 6,6 dibehenate (TDB), while IL-6 levels induced by KB51 were lower than those obtained by TDB. This influence of the number of mycolate chains on the intensity of the inflammatory response was observed for a total of 9 TMM / TDM pairs representative of the different classes and configurations of mycolates present in mycobacteria (Fig. 1C). Indeed, statistically significant differences in levels of TNF-α (Fig. 1C) and IL-6 (data not shown) were observed when comparing the data obtained for a given TDM with the data obtained for the corresponding TMM (same mycolate moiety). In addition, the majority of the tested TDM compounds induced significantly more TNF-α compared to TDB (statistical analysis not shown). Only the dialkene TDM (ST198) and the cis-keto TDM (AD132) induced TNF-α levels comparable to those induced by TDB (Fig. 1C). Concerning the influence of the class of MAs on the levels of inflammatory responses induced by TDM, 8

synthetic TDMs representative of the different classes and configurations of mycolates present in mycobacteria were selected and a dose response analysis was performed. All the tested synthetic compounds induced significant TNF-α production already at the lowest 0.001 µM concentration tested and a plateau of response was observed at 0.1 to 1 µM (Supplementary Fig.S1). To compare responses induced with TDMs from the same class but of different configuration (*cis*- versus *trans*-), data obtained in 3 independent experiments at 1µM with alpha-, keto- or methoxy-TDMs of *cis*- or *trans*-configuration were pooled and differences analyzed statistically (Fig. 1D). . Results indicated that the tested *trans*-alpha-TDM (MMS139) was more inflammatory than the tested *cis*-alpha-TDM (MH175), while for the oxygenated mycolates *cis*- compounds were more inflammatory than *trans*- compounds (Fig. 1D). When results obtained for *cis*- compounds are statistically compared, *cis*-methoxy-compound (KB52) induces higher TNF-α levels as compared to *cis*-alpha TDM (MH175) or *cis*-keto TDM (AD132).

**2. Synthetic arabinose and glucose mono-mycolates are inflammatory**

We next assessed the importance of the sugar moiety on the inflammatory potential of synthetic glycolipids. For that purpose, levels of pro-inflammatory cytokines produced by a *cis*-methoxy mycolate bound to trehalose (TDM-KB52 or TMM-KB51), or to glucose (GMM-SMP73) or arabinose (AraMM-MOD16) were evaluated. As shown in Figure 2A-D, significant TNF-α, IL-6, IL-12p40 and IL-1β levels were measured in BMDC culture supernatants with all the tested compounds after 24h of incubation. At the highest concentration tested (1 µM), TDB induced significantly more IL-6, IL-1β, TNF-α and IL-12p40 than *cis*-methoxy AraMM-MOD16 and TMM-KB51, whereas GMM-SMP73 - compared to TDB - induced comparable levels of IL-1β and TNF-α but less IL-12p40 and IL-6. Finally, TDM-KB52 induced similar levels of IL-6, IL-1β and IL-12p40 but higher TNF-α levels as compared to levels achieved by TDB. Overall, these data indicate that AraMM-MOD16 is the weakest inducer of pro-inflammatory cytokines in comparison to TDM-KB52, TMM-KB51 and GMM-SMP73. In an additional experiment, we also compared the inflammatory potential of 5 synthetic GMMs and 3 AraMMs composed of mycolates from different classes and configuration (supplementary fig. S2). Notably, all the tested GMMs induced TNF-α levels at least comparable to those obtained with TDB (Fig.S2). In contrast, the TNF-α levels induced with the three different synthetic AraMMs were lower than those induced with TDB or the tested GMMs. Thus AraMMs are the weakest inducers of pro-

inflammatory cytokines and TDMs the strongest inducers, while TMMs and GMMs induce responses comparable in magnitude to those observed after stimulation with TDB.

As a second read-out for the activation of BMDCs, we measured surface expression of co-stimulatory molecules CD86 and CD80, major histocompatibility complex class II molecules (MHC-II) and production of reactive oxygen species (ROS) induced by incubation with the synthetic glycolipids. Expression of CD86, CD80 and MHC-II was increased after 48h of incubation with TDM-KB52, TMM-KB51, GMM-SMP73, AraMM-MOD16 and LPS and TDB controls as compared to non-stimulated BMDCs (Fig 2E). AraMM-MOD16 (and LPS) stimulated CD86 expression to a lesser extent that the other mycolate esters. Expression of CD80 and MHC class II was increased by all four compounds to the same level as by TDB. TDM-KB52, TMM-KB51, and GMM-SMP73 all significantly induced the production of intracellular ROS, while the weakest response was again observed for the synthetic AraMM tested (Fig. 2F).

### 3. Synthetic arabinose and glucose mono-mycolates activate BMDCs by mechanisms dependent on C-type lectin receptor Mincle

Several receptors have been reported to interact with mycolate esters. For example, recognition of natural arabinose mycolate esters has been described to be dependent on MyD88 and TLR-2 [22], while C-type lectin receptor Mincle and MCL receptor have been associated with recognition of cord factor, its structural analogue TDB, TMM and GMM from corynebacteria [18–21,34]. In order to identify the signaling pathways involved in the inflammatory responses observed with the synthetic compounds, BMDCs were generated from C57BL/6 WT, Myd88$^{-/-}$, Mincle$^{-/-}$, FcRgamma$^{-/-}$ and MALT1$^{-/-}$ mice. MALT1 and FcR gamma are two proteins involved in Mincle-NF-κB activation pathway. As shown in Fig. 3A, TNF-α production induced by LPS was dependent on Myd88 but was independent of Myd88 for the synthetic TDM-KB52, TMM-KB51, GMM-SMP73, TDB and natural TDM mix, and also for AraMM-MOD16, in contrast to what was reported for natural AraMM isolated from BCG [22]. TNF-α production induced by LPS was, as expected, independent of Mincle, FcRgamma and MALT1, while complete inhibition of TNF-α production was observed in BMDCs from Mincle$^{-/-}$, FcRgamma$^{-/-}$ and MALT1$^{-/-}$ mice after stimulation with synthetic TDM-KB52, TMM-KB51, GMM-SMP73, TDB, natural TDM mix and with AraMM-MOD16 (Fig. 3B, 3C). Similar results were obtained when piceatannol was used as an inhibitor of spleen tyrosine kinase, a protein involved in NF-κB activation by Mincle (data not

shown). In addition, we confirmed that synthetic glucose and arabinose monomycolates are Mincle agonists, using HEK-Blue murine Mincle reporter cell line (Invivogen) (Fig. 3D). Comparable levels of NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) were induced by TDM-KB52, TMM-KB51, GMM-SMP73 and TDB at concentrations as low as 0.001 µM, while one-hundred fold higher concentrations of AraMM-MOD16 were needed to stimulate comparable SEAP levels, suggesting a weaker interaction with Mincle. Similar results were obtained with all the tested synthetic TDMs, TMMs, GMMs and AraMMs (Fig.S3 for the TDMs' results).

In summary, these results indicate that synthetic GMMs and AraMMs activate BMDCs by mechanisms that are independent of the Myd88 pathway but dependent on Mincle-FcRγ-Syk MALT1 pathway, demonstrating that the C-type lectin Mincle receptor can also recognize mycolic acids from mycobacteria bound to glucose and arabinose.

### 4. Glucose and arabinose mono-mycolates activate the NLRP3 inflammasome

It was previously shown that the production of IL-1β induced by natural TDM (mix) and TDB is associated with activation of the NLRP3 inflammasome [23]. The inflammasome is a multiprotein platform which mediates the maturation of caspase-1. Mature caspase-1 cleaves pro- IL-1β and pro-IL-18 and induces the secretion of mature IL-1β and IL-18 [35]. The activation of the inflammasome by TDB is dependent on Mincle and has been shown to be essential for its ability to induce IL-17 responses [24]. As we found that synthetic GMMs and AraMMs also bind to Mincle and induce the production of ROS species, we next analysed whether these compounds could also activate the inflammasome. A selected series of 4 mycolate esters composed of the same *cis*-methoxy mycolate were tested, i.e. TDM-KB52, TMM-KB51, GMM-SMP73 and AraMM-MOD16. BMDCs were primed with LPS 3h prior to incubation for 5h with the tested compounds and controls. As shown in Figure 4A, synthetic *cis*-methoxy TDM-KB52, TMM-KB51, GMM-SMP73 and AraMM-MOD16 all stimulated IL-1β secretion by LPS-primed BMDCs to levels comparable to those achieved by natural TDM and TDB. Because the secretion of IL-1β is dependent on the maturation of caspase-1, we also analysed the secretion of the active caspase-1 p20 subunit in the supernatant and we found that stimulation of LPS-primed BMDCs by TDM-KB52, TMM-KB51, GMM-SMP73 or AraMM-MOD16 was sufficient to induce caspase-1 maturation (Fig. 4B and 4C). Blocking of caspase activity with the pan-caspase-inhibitor z-VAD-FMK strongly reduced the secretion of IL-1-β (Figure 4D), while, as expected, levels of secreted

TNF-α were only marginally reduced following z-VAD-FMK addition (Fig.4E). Overall, these results indicate that also synthetic TMM, GMM and AraMM can induce in BMDCs a caspase-1/inflammasome dependent secretion of IL-1β. Using BMDCs derived from NLRP3$^{-/-}$ KO and caspase1/11$^{-/-}$ mice, we observed that IL-1β induction by the tested compounds was completely abrogated in the absence of NLRP3 and significantly reduced in the absence of caspase1/11$^{-/-}$ (Fig.4F), demonstrating that synthetic TMM, GMM and AraMM induce production of IL-1β by NLRP3 dependent mechanisms (Fig. 4F). The fact that only levels of IL-1β but not of TNF-α were affected by the genetic deletion of NLRP3 or caspase1/11 confirms that these deletions only impact inflammasome activation (Fig.4G). As ROS production, potassium efflux and cathepsin B activity are involved in inflammasome activation by TDB [23], we analysed whether similar mechanisms are also involved in BMDCs activation by synthetic mycolate esters. To this end, production of IL-1β following incubation of BMDCs with ROS scavenger (Ebselen) and synthetic TDM-KB52, TMM-KB51, GMM-SMP73 and AraMM-MOD16 was measured. A significant reduction in IL-1β secretion was observed (Supplementary Figure S4A) following incubation with the different compounds in the presence of Ebselen. The inhibition of IL-1β secretion was also observed when Glibenclamide was used as an inhibitor of potassium efflux pump or when a culture medium rich in potassium was used to inhibit potassium efflux (Fig. S4B). Finally, blocking cathepsin B activity with pharmacological inhibitor CA-074 and blocking actin polymerization with cytochalasin D significantly reduced the production of IL-1β by TDB and synthetic TDM-KB52, TMM-KB51, GMM-SMP73 and AraMM-MOD16 mycolate esters without affecting the level of IL-1β induced with the positive control ATP (Fig. S4C and S4D). In summary, these data indicate that ROS production, potassium efflux, cathepsin B activity and phagocytosis are mechanisms involved in IL-1β induction by synthetic mycolate esters.

### 5. Synthetic mycolate esters demonstrate inflammatory and adjuvant properties *in vivo*

Our *in vitro* results indicated that synthetic trehalose di- and monomycolates, glucose monomycolates and to a lesser extent arabinose mono-mycolates had an inflammatory potential. To confirm these findings *in vivo* and to explore their possible use as novel adjuvants for sub-unit vaccines, water in oil in water emulsions (w/o/w) composed of incomplete Freund's adjuvant (IFA) as a vehicle, synthetic mycolate esters and ovalbumin as a test antigen were prepared and injected once in the two hind footpads of C57BL/6 mice.

Footpad swelling was monitored for 1 week as a read-out of local inflammation; at day seven we analysed the type of cells recruited in the footpad and the upregulation of pro-inflammatory chemokines and cytokines in these cells. Finally, OVA-specific immune responses were analysed by stimulating cells isolated from the lymph nodes draining the injection site 1 week after injection. Results obtained after injection of w/o/w emulsions composed of 30% IFA and *cis*-methoxy TDM-KB52, TMM-KB51, GMM-SMP73 or AraMM-MOD16 (10µg /mouse) and ovalbumin (50µg /mouse) are shown in Figure 5 and Figure 6. Responses were compared with those obtained with a vehicle control (30% IFA + ovalbumin) or TDB w/o/w emulsions (30% IFA, TDB 10 µg/mouse and ovalbumin 50 µg/mouse). As shown in Figure 5A, neither AraMM-MOD16 nor 30% IFA vehicle control induced any footpad swelling, while TDM-KB52, TMM-KB51 and GMM-SMP73 induced a footpad swelling of 1-2 mm over 7 days, comparable to the one observed for a w/o/w emulsion with TDB. Interestingly, GMM-SMP73 induced a significantly weaker footpad swelling than TDB. Cells recruited to the site of injection were analysed by flow cytometry. The analysis of cells recruited to the footpad showed a high infiltration of granulocytes ($Ly6C^+$ and $Ly6G^+$) for TDB, natural TDM, TDM-KB52, TMM-KB51, GMM-SPM73 but not for AraMM-MOD16 (Fig.5B). qRT-PCR analysis confirmed that all the positive compounds (in terms of footpad swelling and granulocyte infiltration) induced also increased expression of pro-inflammatory cytokines (IL12p35, IL-1β, and TNF-α) and chemokine (CXCL1) as compared to vehicle control and AraMM-MOD16 (Fig.5C-F). No significant difference was observed between TDB, natural TDM, TDM-KB52, TMM-KB51 and GMM-SMP73. Draining lymph node cell number increased twofold in animals injected with these five compounds, while the cell number was comparable in lymph nodes from animals injected with vehicle control or the AraMM-MOD16 group (Fig. 6A). Production of OVA-specific IL-2, IFN-γ and IL-17A was measured in draining lymph node cell cultures stimulated *in vitro* with ovalbumin. Formulation with AraMM-MOD16 stimulated low levels of OVA-specific IL-2, IFN-γ and no IL-17A production, whereas formulation with TDM-KB52, TMM-KB51 and GMM-SMP73 induced elevated levels of these cytokines, comparable to those achieved by TDB (Fig.6B-D).

Our analysis of the *in vitro* inflammatory properties of the synthetic mycolate esters had shown that the class of mycolate bound to a given sugar exerted only a small influence on the magnitude of the inflammatory response (Figure 1). Nevertheless, there was a trend of the tested synthetic methoxy-TDM to induce more TNF-α than TDB or the alpha- and keto-

compounds. We therefore compared *in vivo* responses of w/o/w emulsions composed of *cis*-alpha TDM-MH175 or TMM-MH176 with responses obtained with w/o/w emulsions composed of *cis*-methoxy TDM-KB52 or TMM-KB51. No differences between the *cis*-alpha and *cis*-methoxy mycolate esters could be observed in terms of footpad swelling and OVA-specific IFN-γ and IL-17A responses (Supplementary Figure S5).

## DISCUSSION

The cell wall of mycobacteria is characterized by components that interact with different arms of the immune system in case of infection. Several of these components have been associated with virulence and capacity to modulate host immune responses. In this work, we were interested in analyzing the role of esters of mycolic acids using pure synthetic compounds. Mycolic acids are high molecular weight C-60 to C-90 2-alkyl-3-hydroxy fatty acids typical of mycobacteria. Different classes of mycolic acids are present in mycobacteria, mainly alpha-, keto- and methoxy- mycolic acids but also alkene and diene mycolic acids. In addition, mycolic acids can exist in different isomeric forms (e.g., *cis*- and *trans*-stereochemistry). In the mycobacterial cell wall, mycolic acids are present as free mycolic acids or esterified to different sugars (trehalose, glucose or arabinose). Trehalose dimycolate (TDM) or cord factor is probably the most abundant and best studied of the non- cell wall-bound mycolate esters and several studies have reported that changing the classes of MA in TDM influences its inflammatory properties. Indeed, TDM from the *Mtb ΔcmaA2* mutant, lacking *trans*-cyclopropanation of MAs, is hyperinflammatory for macrophages compared to TDM extracted from wild type *Mtb* [36]. Likewise, the cord factor isolated from *Mtb ΔmmaA4,* lacking oxygenated MA classes, induces *in vitro* more TNF-α and IL-12 in macrophages than TDM from wild type *Mtb* [4]. On the other hand, TDM isolated from *Mtb ΔpcaA* (an enzyme required for α-mycolate cyclopropanation) is hypoinflammatory in macrophages [5]. In this study we have analysed the inflammatory power of 17 pure synthetic TDMs along with 13 TMMs, 5 GMMs and 3 AraMMs. Our results only partially confirm the data previously obtained using these mutant *Mtb* strains [4,5,36]. Indeed, the monoalkene TDM and particularly the dialkene TDM, lacking one and respectively two cyclopropanes, show a lower inflammatory potential, confirming the findings of Rao *et al* [5]. However, we found that the class of mycolate (alpha- versus keto- versus methoxy-) and the *cis*- versus *trans*-conformation only poorly influences the intensity of the inflammatory responses induced by these compounds. *Cis*-methoxy- mycolate esters seem to be more inflammatory compared to *cis*-alpha- and *cis*-keto- mycolate esters *in vitro*, but this observation was not confirmed *in vivo*. It is important to mention that the naturally isolated TDM preparations from *Mtb* mutants were composed of a complex mixture containing diverse mycolates in terms of class and chain length (C-60 to C-90), while the synthetic compounds tested in our study were of a particular class and generally restricted in chain length from C-74 to C-84. Synthetic compounds with chain lengths varying in a wider range between C-60 to C-90 need to be

tested to make definitive conclusions. Moreover, Ostrop and colleagues have shown that natural TDM induces a different profile of pro-inflammatory cytokines and chemokines compared to TDB in primary human APCs [37]. These results suggest a potential role of chain length or presence of chemical groups in the activation of primary human cells. To elucidate that, it would be of great interest to compare the inflammatory potential of the synthetic compounds of our study in primary human APCs.

In addition, with this study we were able to demonstrate that besides TDMs and TMMs, GMMs and AraMMs are also inflammatory mycolate esters. Their ability to induce inflammatory responses is linked to their interaction with the C-type lectin receptor Mincle. Using BMDC of Mincle$^{-/-}$ mice and human reporter cells expressing murine Mincle, we show that long-chain mycobacterial GMMs - similarly to corynebacterial GMMs – can indeed interact with Mincle [19]. These results are different from what previously reported by Ishikawa *et al.*, who showed that GMM isolated by trehalose treatment of natural TDM does not interact with mouse Mincle receptor [18]. Possibly, this difference in results is linked to the use of products of enzymatic treatment as compared to the use of pure synthetic compounds in our study and in the study by van der Peet *et al.*. In addition, Matsunaga *et al*., reported that shortly after infection, mycobacteria have a down-regulated production of TDM and an up-regulated production of GMM [38]. This reciprocal regulation is caused by competitive substrate selection by antigen 85A. The results reported by Matsunaga *et al*., coupled to the results of Ishikawa *et al.*, led to the hypothesis that up-regulation of GMM production is an escape mechanism of pathogenic mycobacteria to avoid recognition by the innate immune system [18,38]. Our results suggest that even if *in vivo* a down-regulation of TDM and an up-regulation of GMM occur, innate immune cells remain able to recognize GMM via Mincle.

Regarding AraMMs, our results differ from what was described for natural AraMM purified from *M. bovis* BCG, for which a dependence on TLR-2 / Myd88 was reported [22]. However, in the Miyauchi paper, arabino-mycolate esters obtained by acid hydrolysis of BCG cell wall skeleton were used, which consist of a complex mixture of mono-arabinose monomycolates, tetra-arabinose tetra-mycolates, penta-arabinose tetra-mycolates and hexa-arabinose tetra-mycolate. The relative proportion of these different compounds in the preparation was not quantified; we can therefore not exclude that mono-arabinose monomycolates signal via Mincle while the other arabinose mycolates would signal via TLR-2/MyD88. Noteworthy, to our knowledge, this is the first study demonstrating that the C-type receptor Mincle can

recognize pentose-based glycolipids, even if the intensity of the induced downstream signal was lower than the signal induced by trehalose- and glucose-based glycolipids.

In addition, our study demonstrates that like TDMs, TMMs and GMMs also AraMMs induce the activation of the NLRP3 inflammasome. It should be noted that, even if the tested synthetic AraMMs were not as inflammatory as the synthetic TDMs, TMMs and GMMs in the *in vitro* and *in vivo* experiments performed, in an *in vitro* model involving an LPS priming of BMDCs, a level of IL-1β secretion was observed for all the tested AraMMs comparable to the levels observed for synthetic TDMs, TMMs and GMMs. This indicates that synthetic AraMMs have a potential comparable to the other synthetic mycolate esters to induce NLRP3 inflammasome activation and hence pro-IL-1β processing. It is tempting to speculate that combining synthetic AraMM with a TLR-agonist could lead to the development of adjuvant formulations with Th1 and Th17 promoting potential. Indeed, the TLR agonist could trigger the production of pro-Th1 cytokines along with pro-IL-1β (similar to the LPS priming in our experimental set-up) and synthetic AraMM could lead to pro-IL-1β maturation by NLRP3 activation resulting in induction of Th17 responses [24,39].

The *in vivo* analysis of the tested synthetic mycolate esters in IFA formulations with ovalbumin confirmed the inflammatory properties of synthetic TDMs, TMMs and GMMs as measured by footpad swelling following subcutaneous administration. No difference in terms of footpad swelling or OVA-specific T cell responses could be observed when comparing the *cis*-alpha TDM-MH175 or TMM-MH176 compounds to the *cis*-methoxy TDM-KB52 or TMM-KB51 compounds, indicating that also *in vivo* the class of mycolate does not influence the inflammatory power of mycolate esters. Interestingly, Huber and colleagues recently reported that trehalose monoesters with short acyl chains (C12, 14, 16, 18, 20) induce significantly lower Th1 and Th17 responses compared to trehalose diester analogues. These results demonstrated that the adjuvant property of simple fatty acids requires two acyl chains. In contrast to that study, we have observed comparable levels of Th1 and Th17 immune responses between TDM-KB52 and TMM-KB51 or between TDM-MH175 and TMM-MH176 suggesting that with a longer acyl chain, TMMs can *in vivo* also trigger strong immune responses [40]. Differently from what was reported by Shenderov *et al*., purified peptidoglycan was not needed and formulation of these synthetic mycolate esters in IFA was sufficient in our hands to trigger antigen-specific Th17 responses along with Th1 responses [39]. Differences in formulation and read-outs could account for this discrepancy. Our results indicate that synthetic TDMs, TMMs and GMMs should be further evaluated as novel

adjuvants for subunit vaccines to be used against infectious diseases for which Th1 and Th17 CD4+ T cell responses correlate with protection. However, it is known that natural TDM extracted from *Mtb* can be toxic *in vivo* [41]. Nevertheless, this toxicity is linked to the nature of the acyl chain bound to trehalose. Indeed, TDM from *Rhodococcus sp.4306* with shorter chain length (C34-38) induces lower granulomatogenicity compared to TDM from *Mtb* [15]. In addition, using natural TDMs extracted from different mycobacteria species that varies in terms of the classes and proportions of mycolates present in their cell wall, Fujita and co-workers showed that the classes of MA bound to trehalose can influence the toxicity of the molecule. Thus, toxicity of the synthetic compounds with an adjuvant potential described in this study should be assessed *in vivo* to down-select non-toxic compounds.

GMMs from mycobacteria are also protective non-protein antigens recognized by CD1b restricted T cells. Hence, given this dual ability of GMMs to stimulate both innate and adaptive responses, it is tempting to speculate that administration of IFA formulations of synthetic GMMs with no additional adjuvant could stimulate protective immune responses. In future experiments it will be of interest to evaluate such formulations in animal models such as the guinea pigs or human group 1 CD1 transgenic mice [42]. In addition, as already analyzed for TMM [21] and in order to simplify the synthesis process, it would be interesting to determine the minimal carbon chain length necessary for the adjuvant potential of GMM and for its antigenic potential in TB vaccines. More importantly, this study raises an interesting possibility that GMM through Mincle and CD1b may contribute to both innate (induction of pro-inflammatory cytokines) and acquired (induction of Th1/Th17- CD1b restricted T cell responses) immunity against mycobacteria. This fact provides an interesting possibility in the TB vaccine field to target both classical and non-classical T cell responses by combining synthetic GMM with a protective antigen.

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**Legend to the figures:**

**Figure 1. Synthetic trehalose mono- and dimycolate esters stimulate the *in vitro* production of TNF-α and IL-6 by Bone Marrow derived Dendritic Cells (BMDCs).**

**A-B**: BMDCs derived from C57BL/6 mice were stimulated for 24 h in triplicate cultures with increasing concentrations of plate-coated synthetic *cis*-methoxy TMM-KB51 (grey triangles), or *cis*-methoxy TDM-KB52 (black triangles) or TDB (white diamonds). The supernatants were harvested from separate wells and the amount of pro-inflammatory cytokines (TNF-α and IL-6) was determined by sandwich ELISA. Results are expressed as mean pg/ml of cytokines ± SD of three independent experiments: TNF-α (A) or IL-6 (B).

**C**: Separate BMDC cultures derived from C57BL/6 mice were stimulated for 24h in triplicate with 9 pairs of synthetic trehalose di- versus corresponding monomycolate esters at 0.1µM, with evaporated isopropanol as negative control and TDB or natural TDM at the same concentration as positive controls. The supernatants were harvested and the amount of TNF-α was determined by sandwich ELISA. Results are expressed as mean pg/mL of TNF-α ± SD and data of 4 representative experiments have been pooled. Statistical analysis of results obtained for the TDM/TMM pairs was performed by Mann-Whitney test: ns= not significant, * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$.

**D**: Separate BMDC cultures derived from C57BL/6 mice were stimulated for 24h in triplicate with single TDMs representative of the different classes and configurations (D) of mycolates present in mycobacteria at 1µM, with evaporated isopropanol as negative control and TDB or natural TDM at the same concentration as positive controls. The supernatants were harvested and the amount of TNF-α was determined by sandwich ELISA. Results are expressed as mean pg/mL of TNF-α ± SD and data of 3 representative experiments have been pooled. Statistical analysis of the results of the different groups was performed by Mann-Whitney test: ns= not significant, * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. Isopropanol (stars), TDM natural mix (crossed circles), TDB (white diamonds), *cis*-alpha TDM-MH175 (black circles), *trans*-alpha TDM-MMS139 (white circles), *cis*-keto TDM-AD132 (black squares), *trans*-keto TDM-RT82F2 (white squares), *cis*-methoxy TDM-KB52 (black triangles) and *trans*-methoxy TDM-RT126 (white triangles).

**Figure 2. Synthetic glucose monomycolates and arabinose monomycolates activate BMDCs.**

**A-D**- BMDC cultures from C57BL/6 mice were stimulated for 24 h in triplicate with escalating concentrations of plate-coated synthetic mycolate esters composed of a same *cis*-methoxy mycolate bound twice to trehalose (TDM-KB52) or once to trehalose (TMM-KB51) or glucose (GMM-SMP73) or arabinose (AraMM-MOD16) or controls (natural TDM mix, TDB or evaporated isopropanol (ISO)). The supernatants were harvested and the amounts of pro-inflammatory cytokines (IL-6 (A), IL-1β (B), TNF-α (C) and IL-12p40 (D)) were determined by ELISA. Results are expressed as mean pg/ml of cytokines ± SD (n=four independent experiments). Statistical analysis on results obtained for synthetic compounds and natural TDM mix compared to TDB was performed by Mann Whitney test: ns= not significant, * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$

**E**- Up-regulation of co-stimulatory molecules (CD86 and CD80) and MHC class II was evaluated by flow cytometric analysis after 48 h of stimulation with 10µg/mL of synthetic mycolate esters composed of a same *cis*-methoxy mycolate bound twice to trehalose (TDM-KB52) or once to trehalose (TMM-KB51) or glucose (GMM-SMP73) or arabinose (AraMM-MOD16) and controls (unstimulated, LPS (100 ng/mL ) or TDB (10µg/mL)). Results are means ± SD and representative of at least three independent experiments. Statistical analysis on results obtained for synthetic compounds, TDB or LPS compared to unstimulated cells was performed by Mann Whitney test: ns= not significant, * for $p<0.05$.

**F**-BMDCs were treated for 30 minutes with 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) and stimulated for 4h with 10µg/mL of synthetic mycolate esters composed of a same *cis*-methoxy mycolate bound twice to trehalose (TDM-KB52) or once to trehalose (TMM-KB51) or glucose (GMM-SMP73) or arabinose (AraMM-MOD16) and controls (unstimulated, PMA (100ng/mL or TDB (10µg/mL)). The production of ROS by the tested compound (black line) was measured by flow cytometry and compared to unstimulated cells (grey shade).

**Figure 3. Synthetic GMM and AraMM activate BMDCs by Mincle dependent mechanisms.**

**A, B** and **C**- BMDCs from C57BL/6 WT, MyD88$^{-/-}$, FcRγ$^{-/-}$ , Mincle$^{-/-}$ and MALT1$^{-/-}$ mice were stimulated in triplicate with 10µg/mL of different synthetic mycolate esters, TDB, natural TDM mix or negative control (evaporated isopropanol) for 24 h. The supernatants were analysed for their content in pro-inflammatory cytokines (TNF-α) by ELISA. Results are expressed as mean pg/mL of TNF-α ± SD and are representative of at least 4 independent experiments.

**D**- HEK293 reporter cell expressing murine Mincle were used to evaluate the binding to Mincle receptor. SEAP release after NF-κB activation was evaluated by colorimetric assay using Quantie-blue substrate and by measuring the OD at 655 nm. Isopropanol (stars), TDM natural mix (crossed circles), TDB (white diamonds), *cis*-methoxy TDM-KB52 (black triangles), *cis*-methoxy TMM-KB51 (grey triangles), *cis*-methoxy GMM-SMP73 (white triangles) and *cis*-methoxy AraMM-MOD16 (white and black triangles).

**Figure 4. Synthetic glucose and arabinose mycolate esters activate the NLRP3 inflammasome**

**A**: BMDCs prepared from C57BL/6 mice were primed for 3 h with 1µg/mL of ultrapure LPS and stimulated next for 5 h with 10µg/mL of synthetic mycolate esters, with evaporated isopropanol as negative control and TDB or natural TDM mix at the same concentration as positive controls. The supernatants were harvested and the amount of IL-1β in the supernatants was determined by ELISA

**B**: The secretion of active caspase-1-p20 was detected by Western blot in the supernatant after methanol-chloroform precipitation

**C**- Quantification of Caspase-1 p20 in the supernatant. Relative intensity of Caspase-1 p 20 was determined relative to a non-specific (NS) band. NS band was used as loading control in the supernatant.

**D-E** BMDCs from C57BL/6 mice were primed with 1µg/mL of ultrapure LPS for 2 h, incubated with 10 µM of Z-VAD-FMK (grey) or control medium (black) for 1 h and stimulated with 10µg/mL of synthetic mycolate esters and controls for 5h. Secretion of IL-1β (D) and TNF-α (E) was determined by ELISA in the supernatants

**F-G** BMDCs were prepared from WT (black), NLRP3$^{-/-}$ (grey) and Caspase1/11$^{-/-}$ (white) C57BL/6 mice and primed for 3 h with 1µg/mL of ultrapure LPS before stimulation for 5 h with 10µg/mL of synthetic mycolate esters and controls. The amount of IL-1β (F) and TNF-α (G) in the supernatants was determined by ELISA. Results represent mean ± SD of triplicate cultures and are representative of at least two independent experiments.

**Figure 5. *In vivo* inflammatory properties of synthetic mycolate esters.**

C57BL/6 mice were injected s.c in the two hind footpads with w/o/w emulsions composed of 30% Incomplete Freund's Adjuvant, 10µg/mouse of specified synthetic mycolate esters and

50 µg/mouse of ovalbumin. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 10 µg/mouse of TDB replaced the synthetic glycolipids.

**A**: Mean footpad size (mm) of five mice tested individually was measured with a caliper on the day of injection and 1, 3 and 7 days after injection.

**B**: Percentage of granulocytes ($Ly6G^+$ and $Ly6C^+$) was determined in cells isolated from footpad by flow cytometry 7 days post administration.

**C-F:** The relative expression of cytokines and chemokine were determined by qRT-PCR in the footpad cells. Fold change was determined in comparison to vehicle.

Footpad swelling and percentage of granulocytes induced by synthetic mycolate esters were statistically analysed in comparison to TDB using Mann-Whitney test: ns= not significant, * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$.

**Figure 6. *In vivo* adjuvant potential of synthetic mycolate esters in an experimental OVA model.**

**A**: C57BL/6 mice were injected s.c in the two hind footpads with w/o/w emulsions composed of 30% Incomplete Freund's Adjuvant, 10µg/mouse of specified synthetic mycolate esters and 50 µg/mouse of ovalbumin. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 10 µg/mouse of TDB replaced the synthetic glycolipids. Mice were sacrificed 7 day post immunisation and cells from pooled popliteal and inguinal lymph nodes were collected, counted (A) and stimulated with culture medium (RPMI) or with 5µg/ml of ovalbumin (OVA) for 24h or 72h. Supernatants were harvested and the concentration of IL-2 (**B**) and IFN-γ/IL-17A (**C** and **D**) was determined by ELISA.

Results are means ± SD of five mice tested individually and representative of at least four independent experiments. ns= not significant, *, **, ***= statistically significant, as determined by Mann-Whitney test, $p<0.05$, $p<0.01$ or $p<0.001$.

**Table 1. Structures and code names of the tested synthetic mycolate esters.**

**Table S1. All the tested synthetic trehalose mycolate esters induce the production of TNF-alpha and IL-6 by Bone Marrow derived Dendritic Cells (BMDCs)**

BMDCs derived from C57BL/6 mice were stimulated for 24 h in triplicate with 10 µg/mL of plate-coated synthetic trehalose mycolate esters or controls (TDB, TDM natural mix or evaporated isopropanol (ISO)). The supernatants were harvested individually and the amount

of pro-inflammatory TNF-alpha and IL-6 was determined by sandwich ELISA. Results are expressed as mean pg/mL of cytokines ± SD of triplicate cultures and representative of at least three independent experiments.

**Figure S1: Synthetic trehalose dimycolate esters of different classes and configuration stimulate the *in vitro* production of TNF-α by Bone Marrow derived Dendritic Cells (BMDCs).**

BMDCs cultures derived from C57BL/6 mice were stimulated for 24h in triplicate with single TDMs representative of the different classes and configurations (*cis* vs *trans*) of mycolates present in mycobacteria at escalating doses of 0.001µM (black and white bars), 0.01µM (white bars), 0.1 µM (grey bars) or 1µM (black bars), with evaporated isopropanol as negative control and TDB and a natural mix of TDM at the same concentrations as positive controls. The supernatants were harvested and the amount of TNF-α was determined by sandwich ELISA. Results are expressed as mean pg/mL of TNF-α ± SD and data of 4 representative experiments have been pooled.

**Figure S2. Synthetic glucose monomycolates and arabinose monomycolates activate BMDCs.**

BMDCs from C57BL/6 mice were stimulated for 24 h in triplicate with 1µM of plate-coated synthetic GMMs, AraMMs or controls (TDB or evaporated isopropanol (ISO)) Supernatants were harvested and the amount of TNF-α was determined by ELISA. Results are expressed as mean pg/mL of TNF-α ± SD and data of 3 representative experiments have been pooled.

**Figure S3. TDMs activate BMDCs by Mincle dependent mechanisms.**

BMDCs from C57BL/6 WT, FcR$\gamma^{-/-}$ , and Mincle$^{-/-}$ mice were stimulated in triplicate with 10 µg/mL of different synthetic trehalose dimycolates, TDB, natural TDM mix or negative control (evaporated isopropanol) for 24 h. The supernatants were analyzed for their content in pro-inflammatory cytokines (TNF-α) by ELISA. Results are expressed as mean pg/mL of TNF-α ± SD of triplicate cultures and data are representative of at least 4 independent experiments.

**Figure S4. Mechanisms involved in the activation of inflammasome by GMM and AraMM**

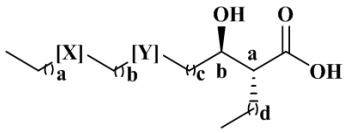
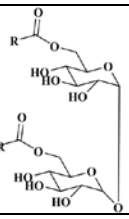
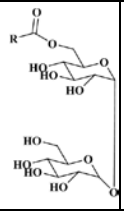
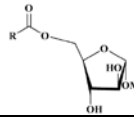
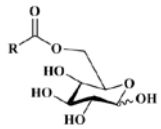
**A-D:** BMDCs from C57BL/6 mice were primed with 1µg/mL of ultrapure LPS for 2 h, pretreated 1 h or not (black bars) with 10 µM of Ebselen (A-grey bars), with 50 µM of glibenclamide (B-grey bars) or 50mM of KCl (B-white bars) or with 10µM of CA-074 (C-grey bars) or 1 µM of cytochalasin D (D-grey bars) and stimulated 5 h with 10µg/mL of synthetic mycolate esters and controls. The level of IL-1β was determined in the supernatant by ELISA. Results are means ± SD of triplicate cultures and representative of at least three independent experiments.

**Figure S5.** ***In vivo*** **inflammatory properties of synthetic mycolate esters and adjuvant potential.**

C57BL/6 mice were injected s.c in the hind footpads with w/o/w emulsions composed of 30% Incomplete Freund's Adjuvant, 10µg/mouse of specified synthetic mycolate esters and 50 µg/mouse of ovalbumin. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 10 µg/mouse of TDB replaced synthetic glycolipid in the emulsion**.**

**A:** footpad swelling measured with a caliper on the day of injection and 1, 3 and 7 days after injection. B: cell counts in the lymph nodes draining the injection site 7 days post administration

**C** and **D:** IFN-γ and IL-17A concentrations measured by ELISA on 72h supernatants of pooled popliteal and inguinal lymph node cells stimulated with culture medium (RPMI) or with 5 µg/mL of ovalbumin (OVA).

| class of mycolic acid | Structure of mycolic acid | TDM | TMM | GMM | AraMM |
|---|---|---|---|---|---|
| |  |  |  |  |  |
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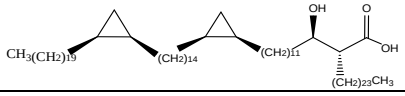
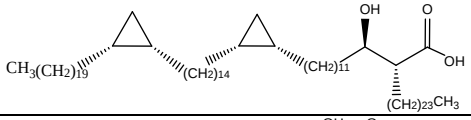
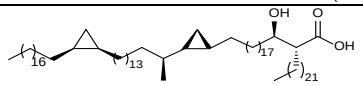
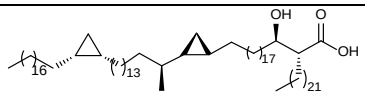
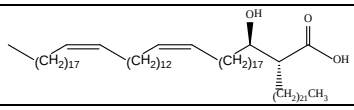
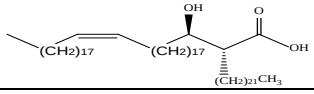
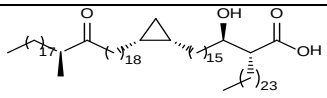
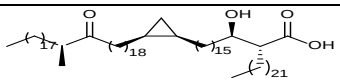
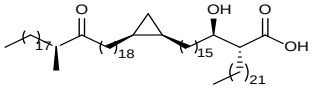
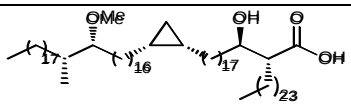
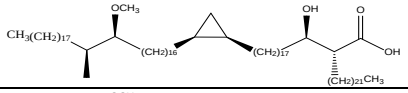
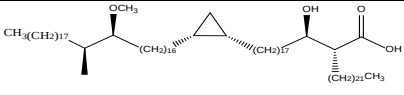
| α |  | JRMM337 [27] | JRMM336 [27] | | |
| α |  | MH175 [27] | MH176 [27] | SMP74 [26] | |
| α |  | MMS144 [28] | MMS146 [28] | | |
| α |  | MMS139 [28] | MMS140 [28] | | |
| α-diene |  | ST198 [27] | ST196 [27] | | |
| α-alkene |  | ST192 [27] | ST165 [27] | | |
| **keto mycolic acids** | | | | | |
| cis-keto |  | AD132 [27] | AD134 [27] | | |
| cis-keto |  | | RT136 [27] | | |
| cis-keto |  | RT137 [31] | | | |
| trans-keto |  | RT82 [31] | RT86 [31] | | |
| trans-keto |  | | | SMP75 [26] | MOD23 [25] |
| **methoxy mycolic acids** | | | | | |
| cis-methoxy |  | AD104 [27] | AD118 [27] | | |
| cis-methoxy |  | KB52 [31] | KB51 [31] | SMP73 [26] | MOD16 [25] |
| cis-methoxy |  | KB50 [31] | KB49 [31] | SMP70 [26] | |

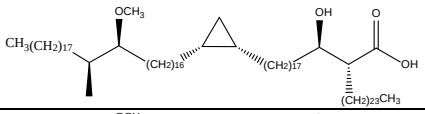
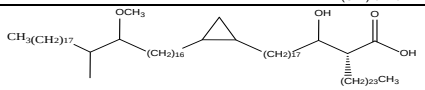
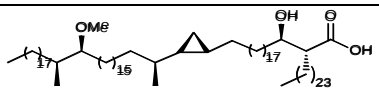
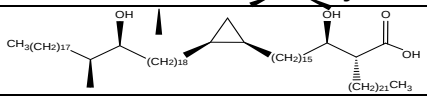
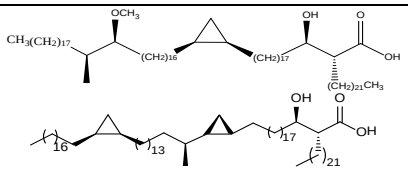
| | | | | | |
|---|---|---|---|---|---|
| cis-methoxy |  | KB91 [27] | | SMP71 [26] | |
| cis-methoxy |  | JRMM324 [27] | JRMM319 [27] | | MOD18 [25] |
| trans-methoxy |  | RT126 [27] | | | |
| **hydroxy mycolic acids** | | | | | |
| hydroxy |  | RT141 [27] | | | |
| **mixed mycolic acids** | | | | | |
| Mixed methoxy and α mycolates |  | KB55 [31] | | | |

Table 1. Structure, class, code names and reference to synthesis of the tested synthetic mycolate ester