Supporting Information Appendix for

Rational design of new adjuvants ligands of the C-type lectin Mincle

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This PDF file contains the SI Materials and Methods, Figures S1 to S13 and references.

SI MATERIALS AND METHODS

Purification of natural mycobacterial lipids

Lipids were purified from *M. tuberculosis* H37Rv and characterized as previously described (1-3).

Mincle reporter cell lines experiments

The HEK-Blue™ hMincle and HEK-Blue™ mMincle cell lines (InvivoGen), derivatives of HEK293 cells that stably express the human or murine Mincle gene along with a NF-κB-inducible reporter system (secreted alkaline phosphatase) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% Fetal Bovine Serum (FBS, Gibco) 4.5 g/l glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma) and 100 µg/ml zeocin, 200 µg/ml hygromycin, 10 µg/ml blasticidin, 1 µg/ml puromycin and 50 µg/ml mofetil (all from Invivogen).

Generation of human monocyte-derived dendritic cells and macrophages

PBMC were isolated from Buffy coat by standard density gradient centrifugation on lymphocyte separation medium (Eurobio). Monocytes were isolated from PBMC by magnetic cell sorting using anti-CD14-coated beads (according to manufacturer recommendations, Miltenyi Biotec). For dendritic cells generation, monocytes ($3x10^6$ cells) were cultured in 1.5 ml of RPMI 1640 (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol (Invitrogen) as well as 500 IU/ml of each GM-CSF and IL-4 (Miltenyi Biotec). On day 2, 0.5 ml of fresh medium containing GM-CSF and IL-4 was added. Immature DCs were used on day 5.

To obtain human monocyte-derived macrophages, monocytes (2×10^6 cells) were cultured for 2 h in 0.5 ml RPMI 1640 and 2 ml of RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin as well as 500 IU/ml of M-CSF (Miltenyi Biotec) were then added. On day 3, 0.5 ml of fresh medium containing M-CSF was added. Monocyte-derived macrophages were used on day 5.

Cells were primed with IFN-γ1b (Miltenyi Biotec, 10 ng/ml) for 3 h prior to the addition of the compounds.

Generation of murine bone marrow derived dendritic cells and macrophages

Bone marrow cells were flushed from the tibias and femurs of C57BL/6 mice (Janvier) with 5 ml of cold DMEM. For macrophage generation, the resulting cell suspension was centrifuged for 10 min at 300 g and 4°C, resuspended in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and cultured for a period of 4h on cell culture dishes in order to remove already differentiated cells. Non-adherent cells distributed in 6-well plates at a density of $2x10^6$ cells/well and incubated in 2 ml DMEM supplemented with 10% FBS and 1% L929 cell conditioned medium (as a source of M-CSF). On day 4 after seeding, 0.5 ml medium containing M-CSF was added and on day 7 the medium was renewed. On day 10, the BMDMs were primed with IFN-γ (Miltenyi Biotec, 10 ng/ml) for 3 h prior to the addition of the compounds. For DCs differentiation, the cell suspension was cultured at a density of 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM, Lonza) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 10% J558 cell conditioned medium (as a source of GM-CSF). On day 3, fresh medium containing GM-CSF was added and on day 6 one half of the medium was renewed. BMDC were harvested on day 8 and used without priming.

Receptor-dependence of primary cells activation

To investigate Mincle and TLR2 dependence, cells were pre-incubated for 30 min at 37°C before stimuli addition with 5 µg/ml of anti-TLR2 monoclonal antibody (clone T2.5, InvivoGen), anti-Mincle antibody (anti-hMincle clone 2B12 and anti-mMincle clone 7B7, InvivoGen) or isotype control (mIgG1, mIgG2a, eBiosciences and rIgG2b, Biolegend).

Flow cytometry

Single cell suspensions were stained with anti-Mincle antibodies (anti-hMincle clone 2B12 or anti-mMincle clone 7B7, InvivoGen) or isotype control (mIgG2a, eBioscience and rIgG2b, Biolegend), followed by PE-conjugated anti-mIgG or FITC-conjugated anti-rIgG antibodies (eBioscience), in PBS for 30 min at 4 °C. The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Toxicity assay

TDB or GlcC14C18 were added to 96-well plates in serial dilutions from 0 to 100 µg/ml and incubated with THP-1 or moDCs cells ($5x10^4$/well) in 100 µl RPMI 1640 supplemented with 10% FBS. After 24 h, 25 µL of a 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue; Sigma) solution in RMPI was added and the cells were further incubated for 4 h at 37°C. Then, 100 µL of the medium were removed and 200 µL of DMSO were added to each well. After thorough pipetting to dissolve the dark blue crystals, O.D. was read at 540 nm. Cell mortality percentage was calculated as (1 – O.D. of treated cells / O.D. of non-treated cells) x 100.

Binding of hMincle-Fc

Lipids (1 µg/well) were coated in isopropanol on 96-wells Maxisorp plates (Nunc). hMincle-Fc, a soluble form of the human Mincle receptor, was constructed by fusing the C-terminal extracellular domain of human Mincle (aa 41-219) to the C-terminus of an engineered human IgG1 Fc domain with a 10 amino acid linker containing the TEV protease recognition sequence (ENLYFQGSGS). A soluble form of the murine Dectin-1 receptor fused to the same human IgG1 Fc domain was used as a non-relevant protein control (IgG1 Fc control). hMincle-Fc and IgG1 Fc control proteins were expressed in CHO cells and purified by G protein affinity chromatography. Mutations in the C-terminal extracellular domain of human Mincle were introduced by synthesis of EcoRI-AvrII fragments and confirmed by sequencing. The mutated and wild-type control proteins were produced with Expi293 Expression System (Thermo Fisher scientific) according to the manufacturer's protocol and purified by G protein affinity chromatography.

Human IgG1 Fc control or hMincle-Fc fusion proteins (1 µg/ml in PBS, 1mM $CaCl_2$, 1% BSA) were pre-incubated or not with 20 mM EDTA or 40 mM trehalose (Sigma) and were allowed to react with lipids for 2h at RT (in 50 µl). Wells were washed once with PBS and the bound Fc fusion proteins were detected using biotin-conjugated anti-human IgG Fcγ specific antibodies (eBioscience) and avidin-horseradish peroxidase (eBioscience).

Computational procedures

All MD simulations were carried out using the AMBER 11 suite of programs (4). The molecular all-atom ff03 (5, 6) and carbohydrate GLYCAM06 (7) force fields were used for Mincle and the ligands, respectively. The starting models were derived from the high-resolution crystal structures of bovine Mincle in complex with trehalose (PDB code: 4KZV) (8). After a preliminary energy minimization procedure, models of the complexes with various ligands

were then subjected to 10 ns MD simulations carried out in explicit TIP3P water molecules at constant temperature (303K) and pressure (1 bar) conditions. The temperature and pressure were controlled using Langevin (9) thermostat and Berendsen barostat (10). The time step of the simulations was 2.0 fs and the SHAKE algorithm was used to constrain the lengths of all chemical bonds involving hydrogen atoms to their equilibrium values (11). The resulting trajectories were analyzed and clustered using the Ptraj module of the AMBER 11 package.

Preparation and biophysical characterization of adjuvant liposomes

The liposomes were prepared by the thin film method as described previously (12). Briefly, weighed amounts of DDA (Sigma) and glycolipid (25:1 mol/mol) were dissolved in chloroform/methanol (9:1, v/v) and the organic solvent was removed using a gentle stream of N_2, forming a thin lipid film on the bottom of the test vial. The lipid film was dried overnight under vacuum to remove trace amounts of the organic solvent. The lipid films were hydrated in 10 mM Tris HCl pH 7.4 to a final concentration of 2.5 mg/dose of DDA by heating for 30 min at 60 °C and vortexing 30 s every 5 min. *M. tuberculosis* antigen Ag85A, recombinantly produced in *E. coli* (13), was added to a final amount of 10 µg/dose. The size of liposomes was determined by dynamic light scattering. Size and zeta potential measurements (data are shown in Fig. S13) were performed on samples diluted 1:50 (N = 3) at 25 °C using a DynaPro Nanostar (Wyatt Technology) and a NanoZS (Malvern Instruments) respectively. For viscosity and refractive index, the value of pure water, i.e. 1.0, was used.

Immunization of mice

Female C57BL/6 mice, 8 to 10 weeks old, were obtained from Janvier. All mice were housed under specific pathogen-free conditions, had access to food and water *ad libitum*, and were treated according to the Centre National de la Recherche Scientifique guidelines for housing

and care of laboratory animals. *Clec4e*$^{-/-}$ backcrossed more than 10 times to C57BL/6J (kindly provided by Dr. Robert Ashman and Dr. Christine Wells) (14) and WT controls established from recent matings of *Clec4e*$^{+/-}$ mice were bred at the CNIC in specific pathogen-free conditions. All protocols were reviewed and approved by the Regional Ethics Committee of Midi-Pyrénées (France) for Animal Experimentation (authorization no. MP/03/38/06/08) and the Local Ethics Committee at CNIC. Mice were injected intradermally (i.d.) at the base of the tail three times with a 2-week interval between the injections (0.1 ml/dose as described above). Spleens were harvested 21 days after the last immunization. Individual splenocytes cultures were obtained using gentleMACS dissociator (Miltenyi Biotec) or by manually mashing the spleens through a 70 μm cell strainer. Cultures were performed in triplicate in round-bottomed microtiter wells (Nunc) containing $2.5x10^5$ cells in a volume of 200 μl RPMI supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. Ag85A for re-stimulation was used in a concentration of 4 μg/ml. Wells containing medium only or 4 μg/ml of ConA (C5275, Sigma) were included as negative and positive controls respectively. Culture supernatants were harvested from parallel cultures after 24 h and 72 h of incubation in the presence of antigen, and the amounts of IL-2, IL-17A and IFN-γ were determined by ELISA using commercially available kits (eBioscience, BD Biosciences, BD Pharmingen). Antibody titers were determined by ELISA as previously described (12). Plates were coated with Ag85A (0.5 μg /well) in PBS and individual mouse sera were analyzed in 3-fold dilutions. Biotin-conjugated anti-mouse IgG, IgG1 and IgG2b antibodies (Biolegend) diluted 1/2000 in PBS containing 1% BSA were added, and Ag85A specific antibodies were detected by avidin-HRP. Antibody titers were then defined as the serum dilution which gives an absorbance value 2.5 fold higher than that of the background.

M. tuberculosis challenge of mice

Mice were immunized as described above. TDB was used at an optimal concentration (ratio DDA/TDB, 10:1). Three weeks after the last inoculation, mice were challenged with virulent *M. tuberculosis* H37Rv. One week before the challenge, mice were transferred to biohazard facilities and housed in cages contained within a laminar flow safety enclosure. A frozen ampoule of *M. tuberculosis* suspension was thawed and vortex-mix vigorously. The suspension was diluted to get 3,000 CFU in 40 µL. Intranasal infection was performed on isoflurane-anesthetized mice by placing 40 µL of mycobacterial suspension on the mouse nares and allowing animals to inhale. Three mice were sacrificed after 3 days to confirm the infection status. 4- and 8-weeks after the challenge, the bacterial burden was assessed in the lungs. 6 mice per group were euthanized and the organs were remove aseptically and homogenized separately in 5 mL of 0,02% Tween 80-PBS using a gentleMACS-dissociator and -M tubes (Miltenyi Biotec). The homogenates were serially diluted serially in 0,02% Tween 80-PBS and 100 µL aliquots were plated on Middlebrook 7H11 agar (Difco laboratories) containing glycerol and 10 % OADC enrichment. The number of CFU was determined after 21 days of incubation at 37°C.

Statistical analysis

Results are expressed as mean ± SEM and were analyzed using One-Way analysis of variance followed by Tukey test to determine significant differences between samples.

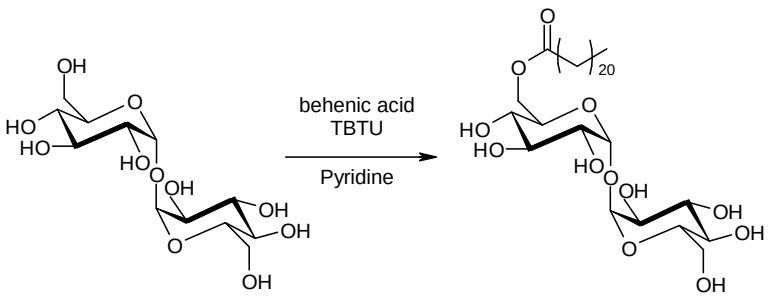
General synthesis methods

All reactions were run in anhydrous solvents under a dry nitrogen atmosphere using commercial solvents as

received. THF was dried by distillation from sodium benzophenone and dichloromethane was distilled from P_2O_5. Dry DMF and dry pyridine were used as purchased. Mycolic acids were obtained from *M. tuberculosis* $H_{37}Rv$ strain, by saponification of purified arabinogalactan complex. Flash chromatography was carried out on silica gel 60. Analytical thin layer chromatography was conducted on aluminium backed 254 mm thin silica with fluorescent indicator and products were visualized with 5% H_2SO_4/EtOH. Proton nuclear magnetic resonance spectra (^{1}H NMR, 300 or 400 MHz) and proton-decoupled carbon magnetic resonance spectra (^{13}C NMR, 100 MHz) were obtained in deuterochloroform ($CDCl_3$), methanol-*d4* (CD_3OD) or DMSO-*d6* on Bruker AV300, AV400 and AV400HD. Residual protonated solvents or solvent carbon signals were used as internal standards. Abbreviations for multiplicity are: s: singlet, d: doublet, t: triplet, m: multiplet. HR-Mass spectra were obtained from the mass spectrometry service of the Institut de Chimie de Toulouse (Toulouse, France).

Synthesis of trehalose analogues

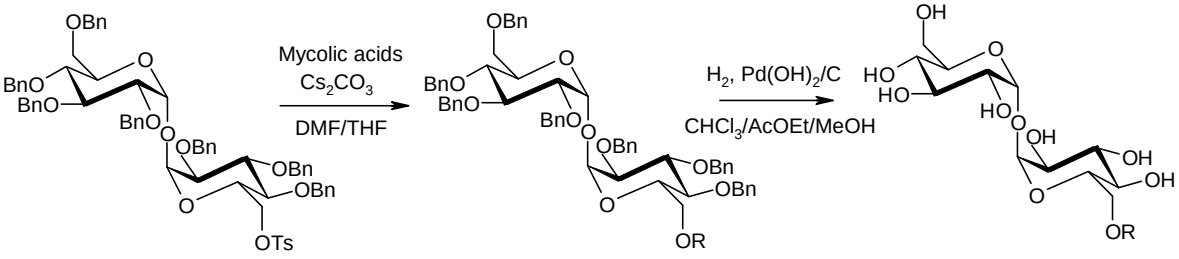
Trehalose 6-monobehenate (TMB)



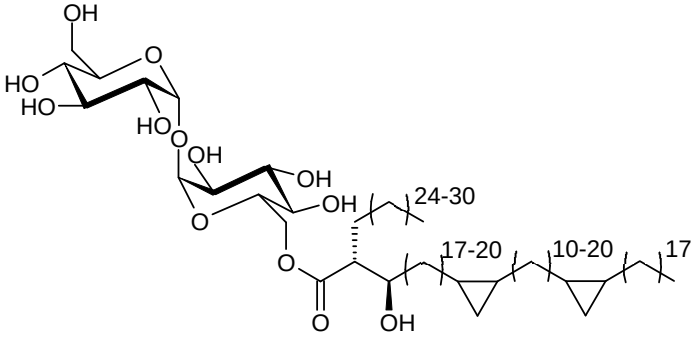
In an oven-dried round bottomed flask equipped with a magnetic stir bar, behenic acid (0.325 g, 2.9 mmol) and *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU, 0.93 g, 2.9 mmol) were dissolved in anhydrous pyridine (5 mL), and the resulting

mixture was stirred at rt for 30 min under a nitrogen atmosphere. A solution of trehalose (1 g, 2.6 mmol) in dry pyridine (5 mL) was then injected into the reaction mixture *via* syringe, and stirring was continued at rt for 5 days. Pyridine was removed under vacuum and the product was purified using silica gel column chromatography with elution using a gradient of 5−25% methanol in EtOAc−CH_2Cl_2 (1:1) (0.17 g, yield = 9%). R_f = 0.16 (CH_3OH/CH_2Cl_2/EtOAc = 1:2:2); ^{1}H NMR (400 MHz, DMSO-*d6*): δ 0.86 (t, J = 7.2 Hz, 3H), 1.24−1.28 (m, 36H), 1.51 (m, 2H), 2.27 (t, J = 7.6 Hz, 2H), 3.10−3.16 (m, 2H) 3.14 (m, 2H), 3.47 (dd, J = 5.2 Hz, 12 Hz, 1H), 3.63−3.67 (m, 4H), 3.90 (ddd, J = 8.4 Hz, 5.2 Hz, 2.8 Hz, 1H), 4.04 (dd, J = 5.6 Hz, 11.6 Hz, 1H), 4.23 (dd, J = 2.0 Hz, 11.6 Hz, 1H), 4.84 (d, J = 3.6 Hz, 1H) 4.87 (d, J = 3.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.43, 22.56, 24.93, 28.95, 29.17, 29.22, 29.28, 29.47, 29.49, 31.76, 33.99, 61.23, 63.55, 70.10, 70.56, 71.93, 72.03, 73.06, 73.29, 93.69, 93.79, 173.28; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{34}H_{64}O_{12}Na$, 687.4295; found, 687.4285

Trehalose 6-monomycolate (TMM)

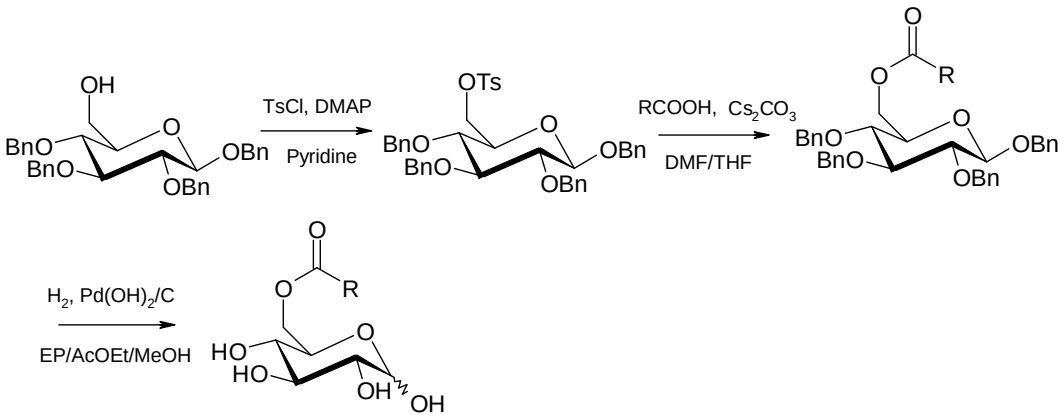


Trehalose-6-mycolate

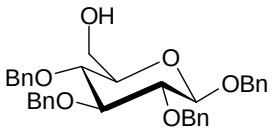


Mycolic acids from *M. tuberculosis* H_{37}Rv (97 mg, 0.074 mmol) were dissolved in a mixture of THF and DMF (THF–DMF 4:1, 2.5 mL). To this solution were added 2,2',3,3',4,4',6-hepta-*O*-benzyl-6'-*O*-tosyl-α-D-trehalose (70 mg, 0.06 mmol) and dry cesium carbonate (59 mg, 0.18 mmol). The mixture was brought to 70 °C and left at this temperature for 2.5 days. The reaction mixture was then cooled to room temperature, diluted with EtOAc, and the organic phase was washed twice with water. The product was purified using column chromatography on silica, petroleum ether/EtOAc 7:1, dissolved in a mixture of petroleum ether, EtOAc and CH_3OH (5 mL of a 5:4:1 v/v/v mixture) and treated under a hydrogen atmosphere with a catalytic amount of 20% palladium hydroxide on charcoal. The mixture was stirred for one day at room temperature and centrifuged (10 min, 1000 *g*). The clear supernatant was removed and the black solid residue was taken up in $CHCl_3$–CH_3OH, shaken and centrifuged as above two more times. After pooling the organic phase and evaporation of the solvents, the residue was filtered on silica (CH_2Cl_2–CH_3OH 8:2) to give TMM (28 mg, yield = 26%); R_f = 0.57 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (400 MHz, $CDCl_3$): δ −0.30 (cis-cyclopropane, myco.), 0.54 (d, CH–CH_3 myco.), 0.65 (m, CH_2–CH_3 myco.), 0.90 (m, 6H, 2xCH_3), 1.13–1.45 (m, 100H, CH_2 myco.), 1.68 (m, 1H), 2.53 (m, CH–CO myco.), 2.98 (m, CH-OMe, myco.), 3.10−3.15 (m, 2H), 3.47 (dd, 1H), 3.56 (s, OCH_3 myco.),3.62−3.67 (m, 4H), 3.91 (ddd, 1H), 4.06 (dd, 1H), 4.22 (dd, 1H), 4.84 (d, 1H) 4.87 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 17.86, 24.69, 25.10, 25.91, 30.24, 30.58, 30.99, 31.66, 33.29, 52.03, 57.44, 64.14, 64.95, 70.16, 70.97, 71.65, 72.86, 73.20, 74.28, 95.50, 95.53, 173.37

Synthesis of glucose derivatives

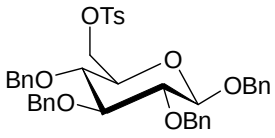


Benzyl 2,3,4-tri-O-benzyl-β-D-glucopyranoside



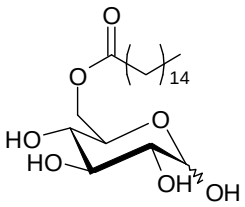
Benzyl 2,3,4-tri-*O*-benzyl-β-D-glucopyranoside was synthesized as reported. All spectral data were in good accordance with reported data (15).

Benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside



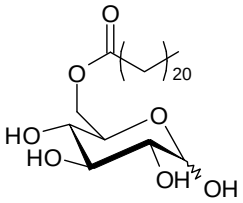
Benzyl 2,3,4-tri-*O*-benzyl-6-*O*-tosyl-β-D-glucopyranoside was synthesized as reported. All spectral data were in good accordance with reported data (16).

6-O-palmitoyl-D-glucose (GlcC$_{16}$)



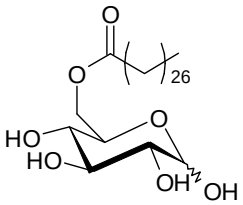
Palmitic acid (4.9 mg, 0.019 mmol) was dissolved in a mixture of THF and DMF (THF–DMF 4:1, 0.5 mL). To this solution were added benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (10 mg, 0.015 mmol) and dry cesium carbonate (15.3 mg, 0.047 mmol). The mixture was brought to 45 °C and left at this temperature for 2.5 days. The reaction mixture was then cooled to room temperature, diluted with EtOAc, and the organic phase was washed twice with water. The product was purified using column chromatography on silica, petroleum ether/EtOAc 7:1, dissolved in a mixture of petroleum ether, EtOAc and CH_3OH (5 mL of a 5:4:1 v/v/v mixture) and treated under a hydrogen atmosphere with a catalytic amount of 20% palladium hydroxide on charcoal. The mixture was stirred for one day at room temperature and centrifuged (10 min, 1000*g*). The clear supernatant was removed and the black solid residue was taken up in $CHCl_3$–CH_3OH, shaken and centrifuged as above two more times. After pooling the organic phase and evaporation of the solvents, the residue was filtered on silica (CH_2Cl_2–CH_3OH 8:2) to give GlcC16 (3.8 mg, yield = 95%); R_f = 0.31 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (400 MHz, CD_3OD): δ 0.85 (t, J=7Hz, 3H), 1.21-1.28 (m, 26H), 1.53-1.64 (pent, J=7Hz, 2H), 2.28-2.34 (t, J=7Hz, 2H), 3.26-3.34 (m, 2H), 3.40 (dd, J = 12.5 Hz, 5Hz, 1H), 3.95 (ddd, J = 12.5 Hz, 6.5Hz, 3Hz, 1H), 4.27 (d, J = 6.5Hz, 1H), 4.30 (d, J = 3Hz, 1H), 5.12 (d, J = 5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.43, 22.56, 24.94, 28.90, 29.16, 29.34, 29.46, 29.50, 31.75, 33.93, 37.40, 47.75, 52.35, 64.30, 69.60, 70.61, 70.99, 72.65, 73.32, 73.96, 92.75; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{22}H_{42}O_7Na$, 441.2828; found, 441.2829

6-O-behenoyl-D-glucose (GlcMB/GlcC$_{22}$)



The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (10 mg, 0.015 mmol) and behenic acid (6.5 mg, 0.019 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (1mg, yield = 13%); R_f = 0.32 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (400 MHz, CD_3OD): δ 0.85 (t, J=7Hz, 3H), 1.21-1.28 (m, 36H,), 1.53-1.64 (pent, J=7Hz, 2H), 2.28-2.34 (t, J=7Hz, 2H), 3.26-3.34 (m, 2H), 3.40 (dd, J = 12.5 Hz, 5Hz, 1H), 3.95 (ddd, J = 12.5 Hz, 6.5 Hz, 3 Hz, 1H), 4.27 (d, J = 6.5Hz, 1H), 4.30 (d, J = 3Hz, 1H), 5.12 (d, J = 5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.05, 22.93, 23.50, 28.11, 29.11, 29.39, 31.02, 31.95, 34.93, 37.49, 48.99, 51.33, 63.19, 70.85, 71.28, 72.70, 73.98, 75.54, 95.35

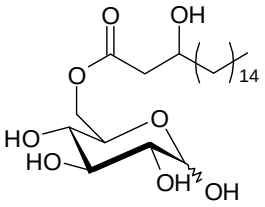
6-O-octacosanoyl-D-glucose (GlcC$_{28}$)



The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (10 mg, 0.015 mmol) and octacosanoic acid (8 mg, 0.019 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (0.7mg, yield = 5.9%); R_f = 0.33 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (300 MHz, CD_3OD): δ 0.86 (t, J=7Hz, 3H), 1.21-1.29 (m, 42H,), 1.45-1.55 (m, 2H), 2.24-2.35 (m, 2H), 3.23-3.35 (m, 2H), 3.39 (m, 1H), 3.97 (m, 1H), 4.25 (d, 1H), 4.31 (d, 1H), 5.07 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.45, 22.53,

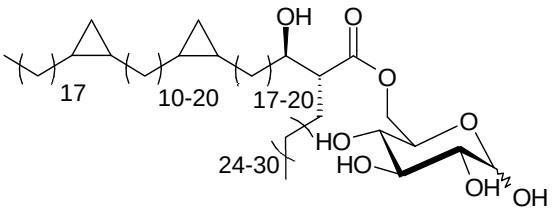
24.62, 29.29, 31.91, 33.88, 35.05, 37.34, 47.69, 52.32, 66.13, 70.04, 70.70, 72.23, 73.42, 74.88, 76.67, 93.56; ; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{34}H_{66}O_7Na$, 609.4701; found, 609.4707

6-O-(3'(R,S)-hydroxyoctadecanoyl)-D-glucose (Glc3OHC18)



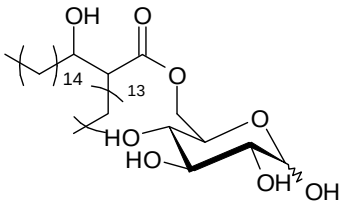
The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (15 mg, 0.022 mmol) and 3-hydroxyoctadecanoic acid (8.1 mg, 0.027 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (2.8mg, yield = 22.5%); R_f = 0.15 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (400 MHz, DMSO-*d6*) δ 0.86 (t, 3H), 1.24 (m, 26H), 1.46 (m, 2H), 2.35 (m, 2H), 3.16 (m, 2H), 3.43 (m, 1H), 3.95 (m, 1H), 4.27 (d, J = 6.5 Hz, 1H), 4.32 (d, J = 3 Hz, 1H), 4.90 (d, J = 5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.42, 22.55, 25.54, 29.16, 29.46, 29.50, 31.75, 37.30, 42.93, 64.48, 67.50, 69.59, 73.33, 73.99, 75.15, 76.88, 92.74, 97.34, 173.53; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{24}H_{46}O_8Na$, 485.3090; found, 485.3093

6-O-Mycoloyl-D-glucose (GlcMM)



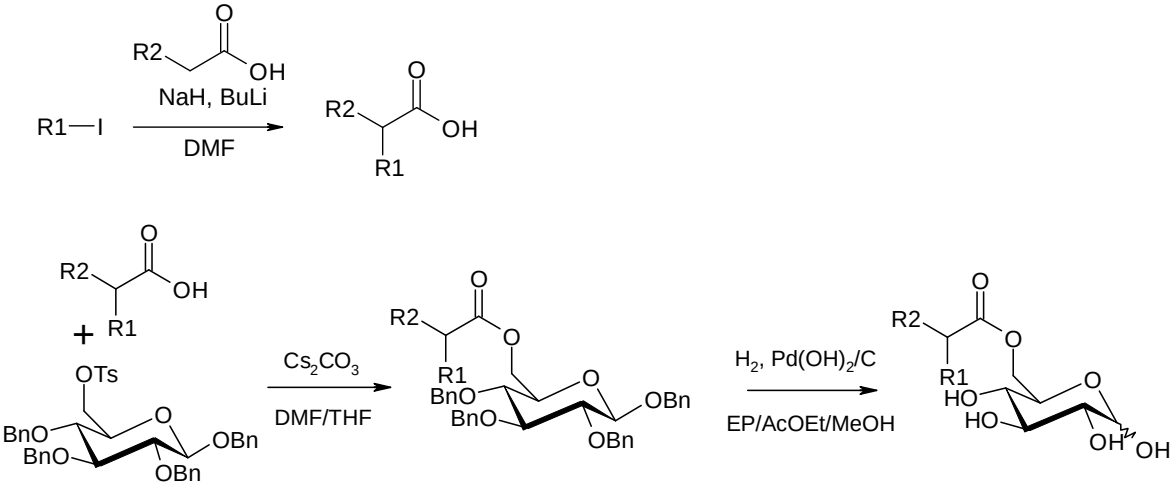
6-*O*-Mycoloyl-D-glucose was synthesized as reported. All spectral data were in good accordance with reported data (16).

6-O-(2'(R,S),3'(R,S)-corynomycolyl)-D-glucose (GlcMCM)

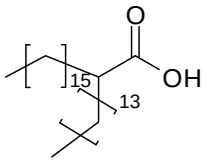


The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-□-D-glucopyranoside (10 mg, 0.015 mmol) and corynomycolic acid (9.4 mg, 0.019 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (1.4 mg, yield = 12%); R_f = 0.16 (CH_3OH/CH_2Cl_2 = 1:9); ^1H NMR (300 MHz, DMSO-*d6*): δ 0.86 (t, 6H), 1.23 (m, 50H), 1.42 (m, 2H), 1.61 (m, 1H), 2.19-2.27 (m, 1H), 3.05-3.16 (m, 2H), 3.42 (m, 1H), 3.86-3.96 (m, 1H), 4.36 (d, 1H), 4.60 (d, 1H), 5.09 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*): 14.27, 22.54, 28.91, 29,17, 29.48, 31.75, 33.50, 36.96, 47.32, 51.91, 51.96, 56.51, 69.56, 70.35, 70.72, 71.41, 73.30, 74.19, 75.09, 76.19, 77.14, 92.72, 97.24, 174.70; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{38}H_{74}O_8Na$, 681.5281; found, 681.5269

Synthesis of glucose derivatives with ramified fatty acids

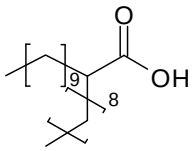


2-tetradecyloctadecanoic acid



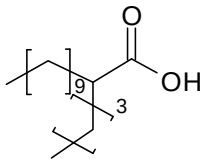
To dry THF (2 ml) under nitrogen at -78°C were added diisopropylamine (310 µl, 2.1 mmol) and butyllithium (900 µl, 1.95 mmol). The mixture was stirred for 5 min at -78°C to obtain LDA. To a suspension of stearic acid (0.5 g, 1.75 mmol) and NaH (80 mg of 60% dispersion in mineral oil, 1.95 mmol) in dry THF (10 ml) at 0°C was added LDA (2 ml) and the mixture was stirred for 30min at RT. 1-iodotetradecane (630 mg, 2.1 mmol) was added and the reaction mixture was stirred 3h at 45°C. The mixture was quenched with HCl 1N and extracted twice with diethylether. The product was purified using column chromatography on silica, petroleum ether/EtOAc 20:1 to 13:1 (116.7 mg, yield =14%). All spectral data were in good accordance with reported data (17).

2-nonyldodecanoic acid



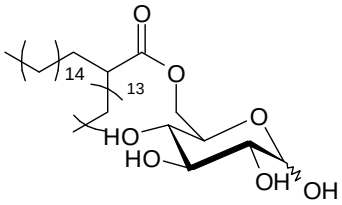
The title compound was prepared from 1-iodobutane (0.74 g, 4 mmol) and dodecanoic acid (0.5 g, 2.5 mmol) according to a procedure similar to that described for 1-iodotetradecane (1.7 mg, yield = 13%); R_f = 0.23 (petroleum ether/EtOAc = 13:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88-0.92 (t, 6H), 1.28 (m, 30H), 1.45-1.52 (m, 2H), 1.59-1.68 (m, 2H), 2.32-2.40 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.93, 14.12, 22.47, 22.63, 22.69, 27.38, 29.33, 29.47, 29.55, 29.57, 29.60, 31.91, 32.21, 45.39, 181.93; HRMS (m/z): $[M]^-$ calcd. for $C_{21}H_{41}O_2$, 325.3107; found, 325.3105

2-butyldodecanoic acid



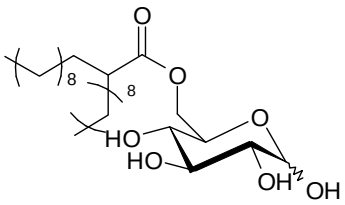
The title compound was prepared from 1-iodobutane (1 g, 13.5 mmol) and dodecanoic acid (0.5 g, 2.5 mmol) according to a procedure similar to that described for 1-iodotetradecane (5.1 mg, yield = 5%); R_f = 0.2 (petroleum ether/EtOAc = 13:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88-0.94 (m, 6H), 1.28-1.34 (m, 20H), 1.45-1.53 (m, 2H), 1.59-1.69 (m, 2H), 2.33-2.43 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.92, 14.11, 22.63, 22.69, 27.38, 29.33, 29.47, 29.54, 29.57, 29.60, 31.91, 32.19, 45.48, 182.57; HRMS (m/z): $[M]^-$ calcd. for $C_{16}H_{31}O_2$, 255.2324; found, 255.2332

6-O-(2'(R,S)-tetradecyloctadecanoyl)-D-glucose (GlcC14C18)



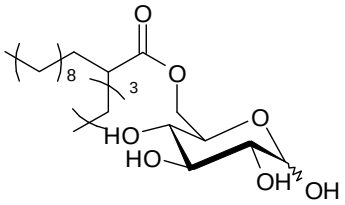
The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (40 mg, 0.06 mmol) and 2(R,S)-tetradecyloctadecanoic acid (40 mg, 0.072 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (16.1 mg, yield = 41%); R_f = 0.14 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.81 (t, 6H), 1.20-1.28 (m, 52H), 1.40 (m, 2H), 1.51 (m, 2H), 2.30-2.37 (m, 1H), 3.21-3.34 (m, 2H), 3.40 (m, 1H), 3.72-3.78 (m, 1H), 4.25 (d, 1H), 4.35 (d, 1H), 5.18 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.27, 22.57, 26.97, 29.23, 29.45, 29.54, 29.56, 31.81, 31.89, 45.06, 49.05, 63.64, 64.08, 69.63, 70.69, 70.87, 72.67, 73.33, 74.11, 75.14, 76.88, 92.76, 97.36, 175.76; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{38}H_{74}O_7Na$, 665.5332; found, 665.5320

6-O-(2'(R,S)-nonyldodecanoyl)-D-glucose (GlcC9C12)



The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (40 mg, 0.06 mmol) and 2(R,S)-nonyldodecanoic acid (20 mg, 0.06 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (7.4 mg, yield = 50%); R_f = 0.14 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (300 MHz, CDCl3): δ 0.90 (m, 6H), 1.22-1.46 (m, 30H), 1.49 (m, 2H), 1.62 (m, 2H), 2.38-2.45 (m, 1H), 3.30-3.44 (m, 2H), 3.54 (m, 1H), 3.84 (m, 1H), 4.31 (d, 1H), 4.52 (d, 1H), 5.30 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.43, 22.56, 27.11, 29.13, 29.28, 29.42, 31.75, 32.29, 33.87, 45.23, 52.27, 70.64, 72.59, 72.71, 74.13, 75.31, 76.94, 77.50, 94.10, 97.38, 178.35

6-O-(2'(R,S)-butyldodecanoyl)-D-glucose (GlcC4C12)

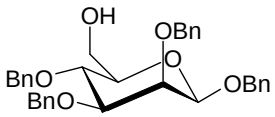


The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-glucopyranoside (40 mg, 0.06 mmol) and 2(R,S)-butyldodecanoic acid (5.1 mg, 0.0066 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (1.1 mg, yield = 41%); R_f = 0.13 (CH_3OH/CH_2Cl_2 = 1:9); ^{1}H NMR (400 MHz, CD_3OD): δ 0.90 (m, 6H), 1.22-1.46 (m, 20H), 1.47 (m, 2H), 1.60 (m, 2H), 2.35-2.49 (m, 1H), 3.30-3.40 (m, 2H), 3.50 (m, 1H), 3.96 (m, 1H), 4.19 (d, 1H), 4.47 (d, 1H), 5.10 (d, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 13.35, 22.41, 29.12, 29.39, 31.72, 47.33, 47.54, 47.75, 47.97, 48.18, 48.39, 48.60, 77.37, 77.69, 78.01,

92.68, 97.42, 174.53; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{27}H_{52}O_7Na$, 511.3611; found, 511.3606

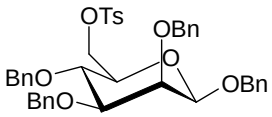
Synthesis of mannose derivatives

Benzyl 2,3,4-tri-O-benzyl-β-D-mannopyranoside



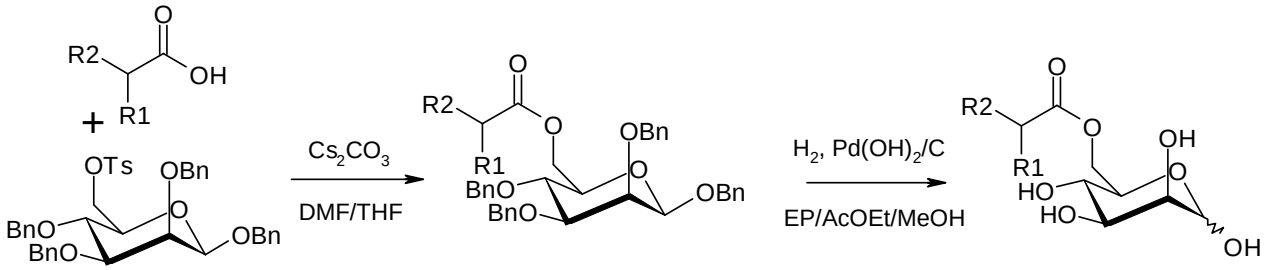
Benzyl 2,3,4-tri-*O*-benzyl-β-D-mannopyranoside was synthesized as reported. All spectral data were in good accordance with reported data (15).

Benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-mannopyranoside

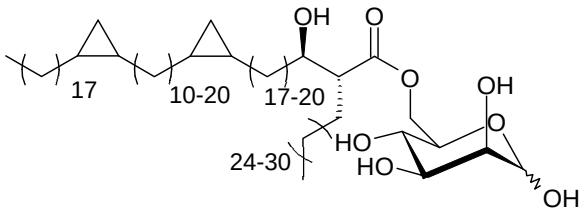


Benzyl 2,3,4-tri-*O*-benzyl-β-D-mannopyranoside (100 mg, 0.18 mmol) was dissolved in dichloromethane (3 mL) and the solution was cooled to 0 °C. Pyridine (0.7 mL), DMAP (9 mg, 0.074 mmol) and tosyl chloride (140 mg, 0.74 mmol) were sequentially added at 0 °C. The mixture was warmed to room temperature and was stirred overnight. The mixture was diluted with EtOAc and hydrolyzed with 3 M HCl (5 mL). The organic phase was decanted and washed twice with saturated $NaHCO_3$ and brine. The product was purified using column chromatography on silica, petroleum ether/EtOAc 95/5 to 80/20 (0.12 g, quantitative); $R_f = 0.33$ (petroleum ether/EtOAc = 8:3); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.40 (s, 3H, C*H*$_3$-tosyl), 3.45–3.52 (m, 3H), 3.78 (m, 1H), 4.14 (dd, J = 10 Hz, 7 Hz, 1H), 4.35 (dd, J = 10 Hz, 2 Hz, 1H,), 4.39–4.56 (m, 5H), 4.85–4.95 (m, 4H), 7.22–7.78 (m, 24H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.64, 69.36, 70.73, 71.45, 73.68, 73.97, 74.16, 75.00, 76.72, 82.09, 99.88, 127.70, 127.75,

127.81, 128.07, 128.15, 128.16, 128.31, 128.41, 128.45, 128.49, 128.59, 129.77, 137.16, 137.84, 138.52, 144.70; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{41}H_{42}O_8NaS$, 717.2498; found, 717.2501

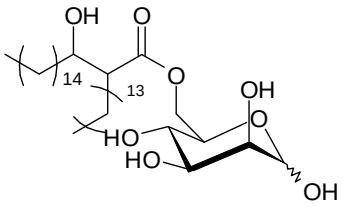


6-O-mycoloyl-D-mannose (ManMM)



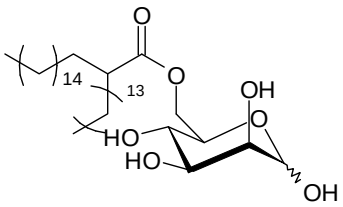
The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-mannopyranoside (20 mg, 0.03 mmol) and mycolic acids from *M. tuberculosis* H37Rv (50 mg, 0.074 mmol) according to a procedure similar to that described for 6-O-mycoloyl-D-glucose (1.9 mg, yield = 3.6%); $R_f = 0.1$ (CH_3OH/CH_2Cl_2 = 5:95); ^1H NMR (400 MHz, $CDCl_3$): −0.31 (cis-cyclopropane, myco.), 0.55 (d, CH–CH_3 myco.), 0.67 (m, CH_2–CH_3 myco.), 0.90 (m, 6H, 2xCH_3), 1.11–1.45 (m, 100H, CH_2 myco.), 1.43 (m, 2H), 1.68 (m, 1H), 2.51 (m, CH–CO myco.), 2.98 (m, CH-OMe, myco.), 3.56 (s, OCH_3 myco.), 3.68–3.77 (m, 3H), 4.02 (m, 1H), 4.33 (d, 1H), 4.82 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 10.92, 12.95, 14.53, 15.54, 22.98, 24.07, 25.53, 29.32, 33.91, 35.01, 52.31, 58.29, 63.51, 69.84, 70.60, 70.99, 71.30, 73.35, 74.12, 85.56, 92.50, 97.04, 177.25

6-O-2'(R,S),3'(R,S)-corynomycoloyl-D-mannose (ManMCM)



The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-mannopyranoside (20 mg, 0.03 mmol) and corynomycolic acid (20 mg, 0.036 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (1.2 mg, yield = 5.1%); R_f = 0.13 (CH_3OH/CH_2Cl_2 = 5:95); ^{1}H NMR (400 MHz, CD_3OD): 0.90 (t, 6H), 1.30 (m, 52H), 1.45 (m, 2H), 1.53 (m, 2H), 1.78 (m, 2H), 2.43 (m, 1H), 3.46–3.82 (m, 3H), 3.94 (m, 1H), 4.21 (d, 1H), 4.46 (d, 1H), 5.07 (d, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.42, 22.56, 29.17, 29.35, 29.51, 31.76, 33.94, 37.40, 52.35, 66.20, 70.76, 71.85, 73.67,73.95, 74.85, 75.72, 77.43, 77.64, 94.64, 174.88; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{38}H_{74}O_8Na$, 681.5281; found, 681.5287

6-O-(2'(R,S)-tetradecyloctadecanoyl)-D-mannopyranose (ManC14C18)



The title compound was prepared from benzyl 2,3,4-tri-O-benzyl-6-O-tosyl-β-D-mannopyranoside (20 mg, 0.03 mmol) and 2-tetradecyloctadecanoic acid (20 mg, 0.036 mmol) according to a procedure similar to that described for 6-O-palmitoyl-D-glucose (4.5 mg, yield = 23%); R_f = 0.11 (CH_3OH/CH_2Cl_2 = 5:95); ^{1}H NMR (400 MHz, $CDCl_3$): 0.90 (t, 6H), 1.27 (m, 52H), 1.48 (m, 2H), 1.62 (m, 2H), 2.46 (m, 1H), 3.34–3.68 (m, 3H), 3.92-3.99 (m, 1H), 4.25 (d, 1H), 4.66 (d, 1H), 5.31 (m, 1H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 14.39, 22.53, 29.13,

29.42, 31.73, 33.76, 52.15, 70.94, 71.85, 72.31, 73.82, 73.98, 74.47, 76.58, 77.75, 79.51, 94.47, 176.16; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{38}H_{74}O_7Na$, 665.5332; found, 665.5316

SI FIGURES

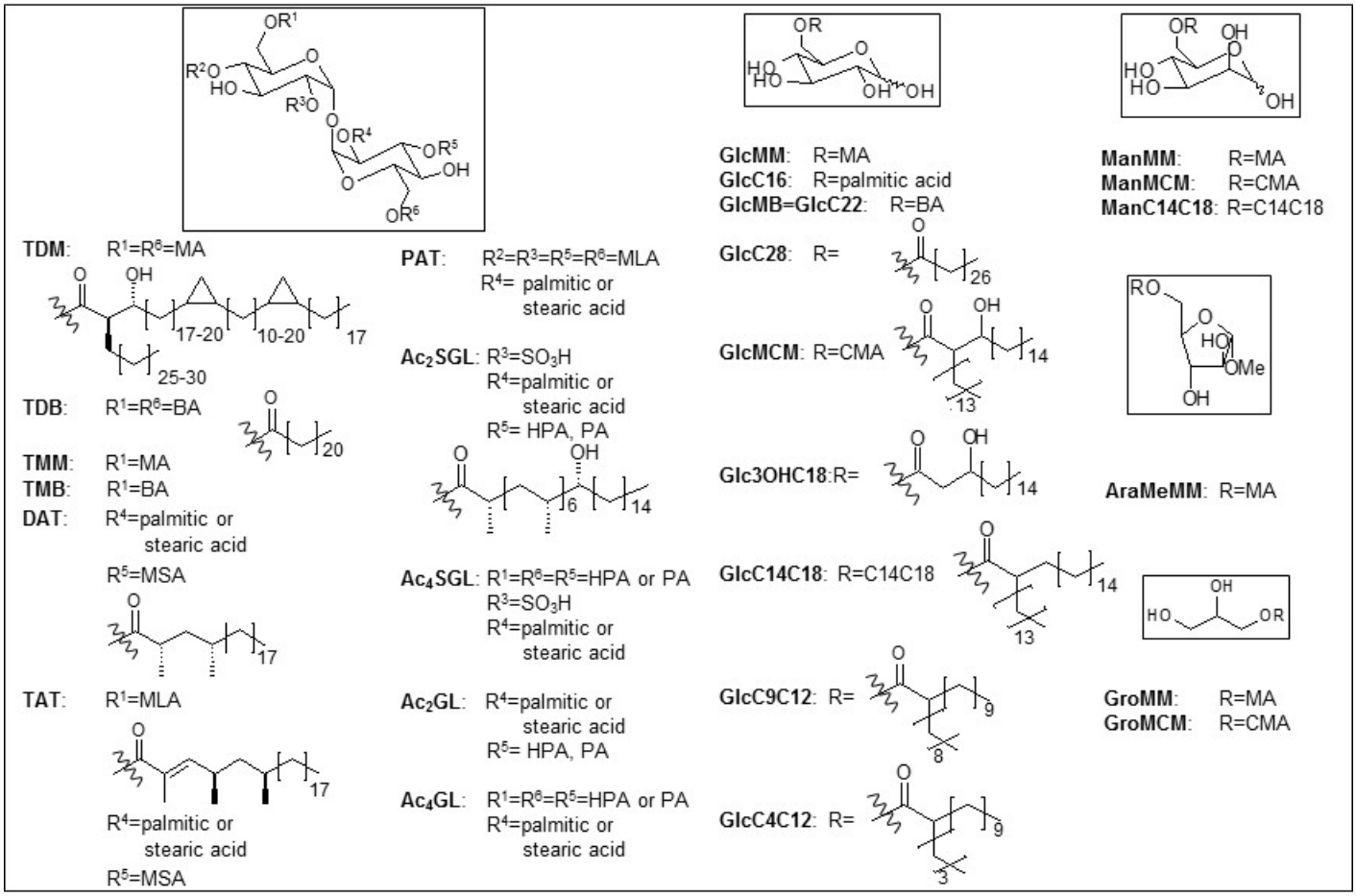


Fig. S1: Chemical structures of the natural and synthetic lipids evaluated.

BA, behenic acid; CMA, corynomycolic acid; HPA, hydroxyphtioceranoic acid; MA, mycolic acids (forms containing cyclopropyl groups are shown as an example); MLA, mycolipenic acid; MSA, mycosanoic acid; PA, phtioceranoic acid.

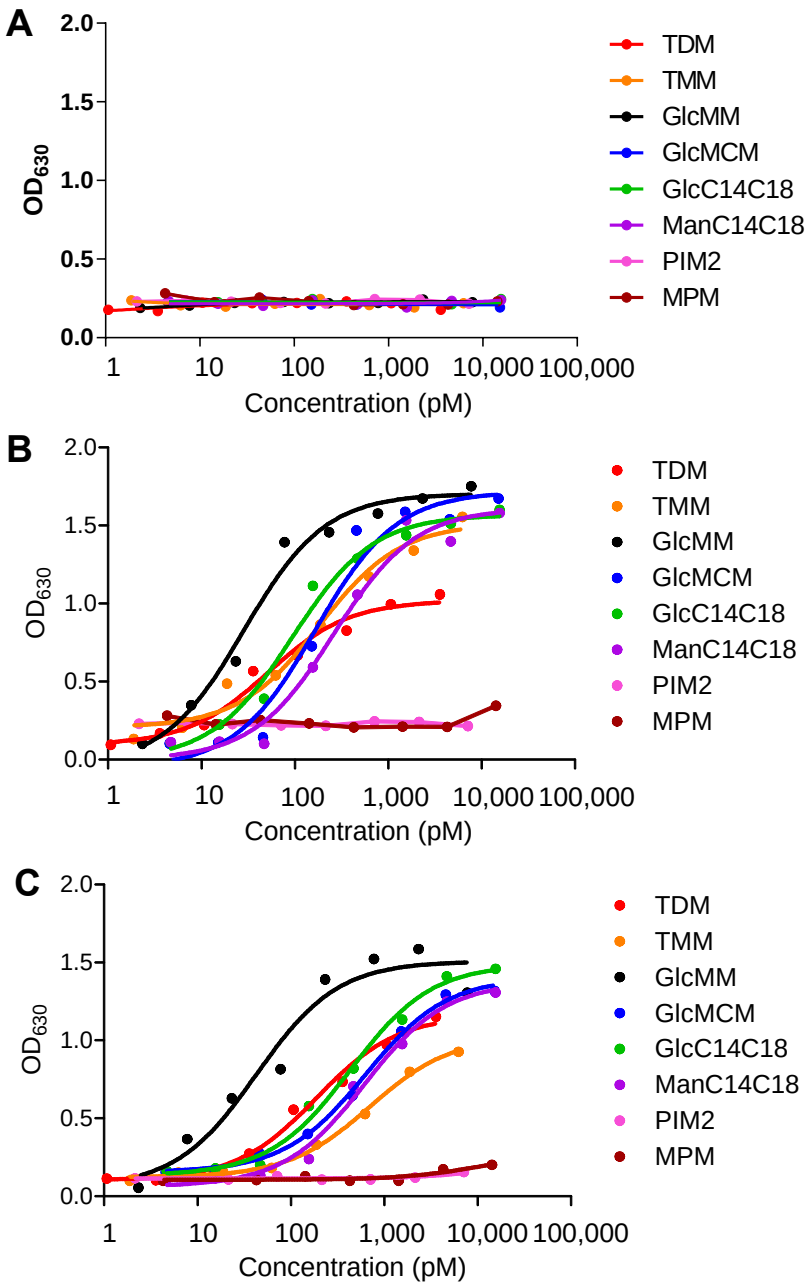


Fig. S2: NF-κB activation induced in parent HEK (A), HEK-hMincle (B) and HEK-mMincle (C) cells by different lipids.

HEK cells (5×10^4) expressing human (HEK-hMincle) or murine (HEK-mMincle) Mincle or no receptor and a NF-κB-inducible reporter system were stimulated with plate-bound lipids for 24h. NF-κB activation was determined by measuring alkaline phosphatase activity and reading O.D. at 630 nm after mixing of 20 µl of the culture supernatant with 180 µl of Quanti-Blue™ (InvivoGen). EC_{50} values were determined (as shown in Fig. 1) and remained constant with cell stimulation time from 12 to 24 h and Quanti-Blue™ incubation time from 10 to 180 min.

Synthetic MPM (18) was kindly provided by Dr Ruben P. van Summeren and Prof Adrian Minaard (University of Groningen, The Netherlands).

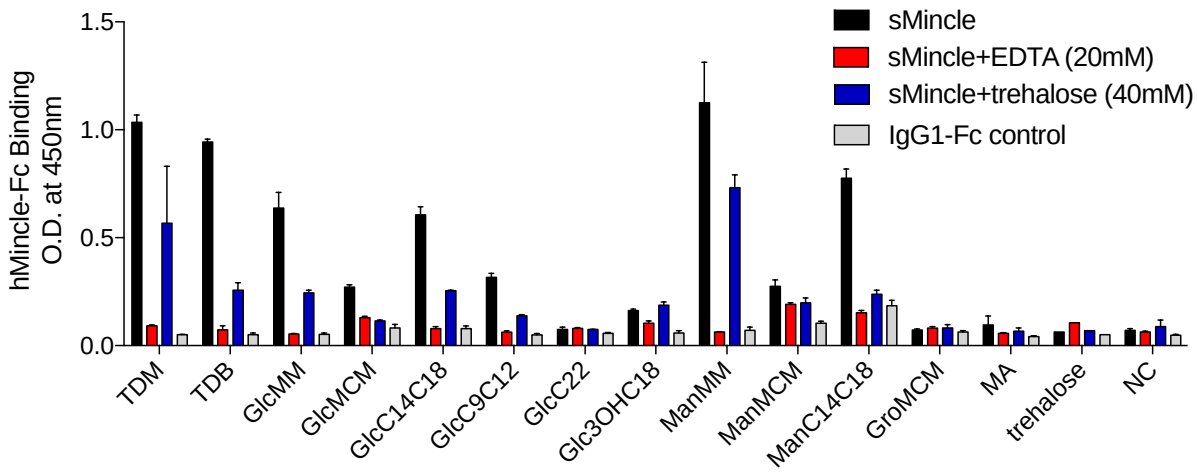


Fig. S3: **Binding of hMincle-Fc to lipids.**

Plate-bound lipids (1 µg/well) were tested for binding to hMincle-Fc (1 µg/ml in PBS, 1mM $CaCl_2$, and 1% BSA), a soluble form of the human Mincle receptor fused with an IgG1 Fc domain. Human IgG1 Fc protein was used as control. Binding was detected using biotin-conjugated anti-human IgG Fcγ-specific antibodies and avidin-HRP. Samples were read at an O.D. at 450 nm. Compound structures are described in Figs. 1A and S1.

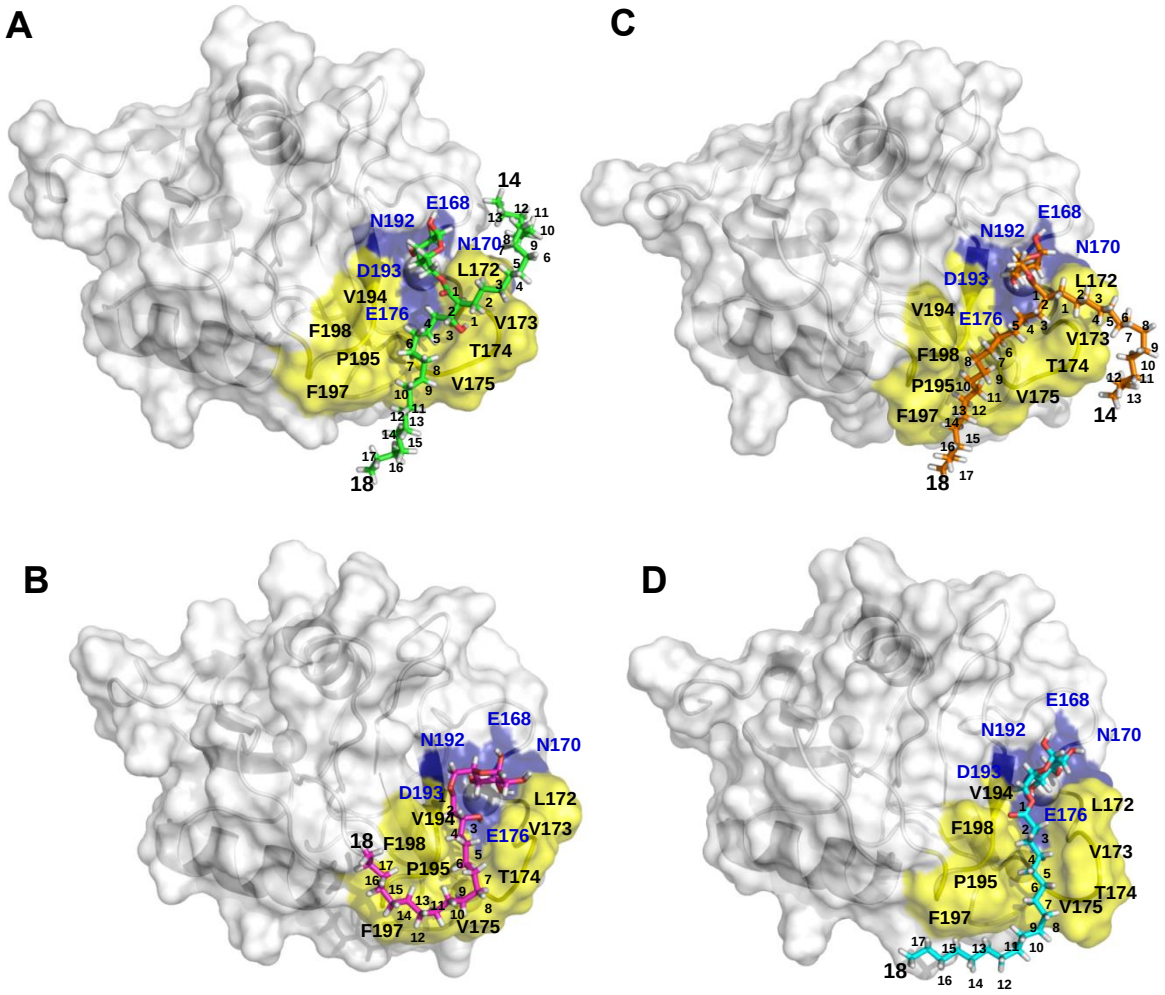


Fig. S4: **Selected binding modes of GlcMCM (A), Glc3OHC18 (B), GlcC14C18 (C), and GlcC18 (D) with Mincle inferred by MD simulations.**

Numbering of alkyl chains is shown for reference. Amino acid residues in blue are involved in polar interactions with glucose or Ca^{2+}. Amino acid residues in yellow are involved in van der Waals interactions with alkyl chains from ligands.

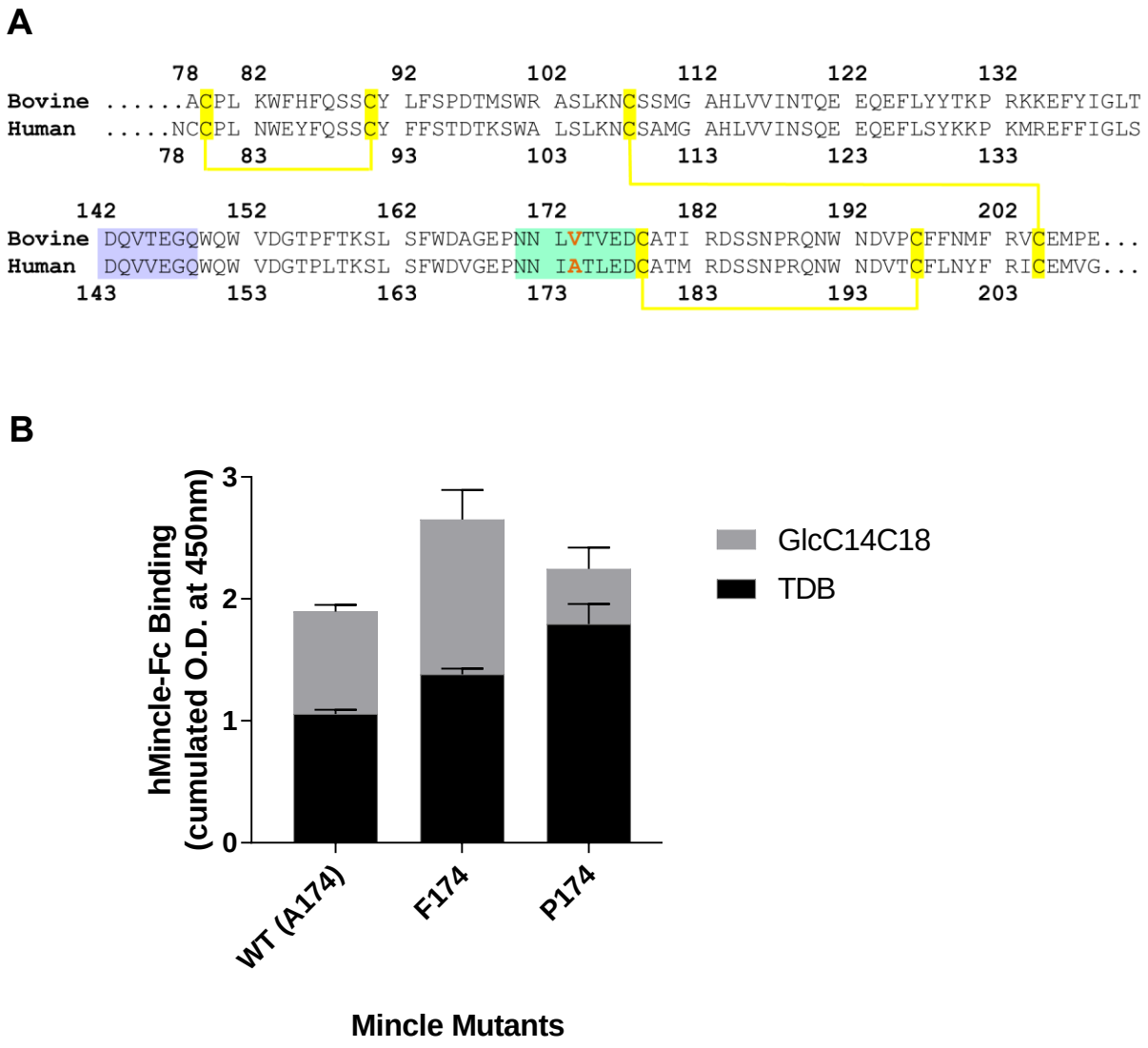


Fig. S5: **Sequence alignment of bovine and human Mincle proteins (A) and binding of mutant hMincle-Fc to GlcC14C18 or TDB (B).**

A) Three disulfide bonds are highlighted in yellow. The two flexible regions, D142-Q148 and N170-D177, are colored respectively in purple and green. In orange bold is shown the amino-acid position targeted by mutagenesis.

B) Binding of wild-type (WT) and mutant hMincle-Fc proteins to GlcC14C18 (bearing a single fatty acid with a 2-alkyl chain) or TDB (bearing two linear C22 fatty acids). See the structures in Fig. S1.

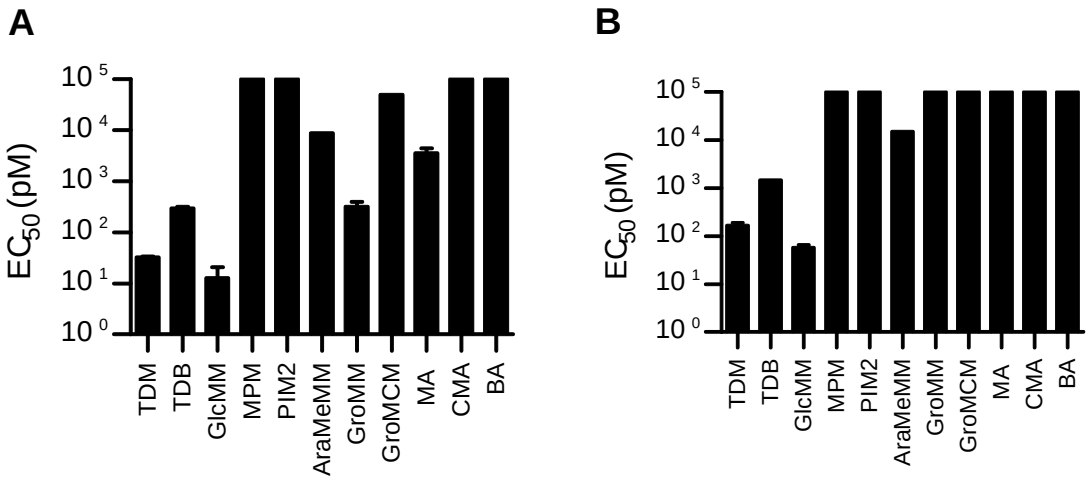


Fig. S6: EC_{50} values for different lipids.

EC_{50} values determined from the activation curves of HEK-hMincle (A) and HEK-mMincle (B). The EC_{50} values are presented as mean ± SEM calculated from at least three independent experiments. Compound structures are described in Figs. 1 and S1.

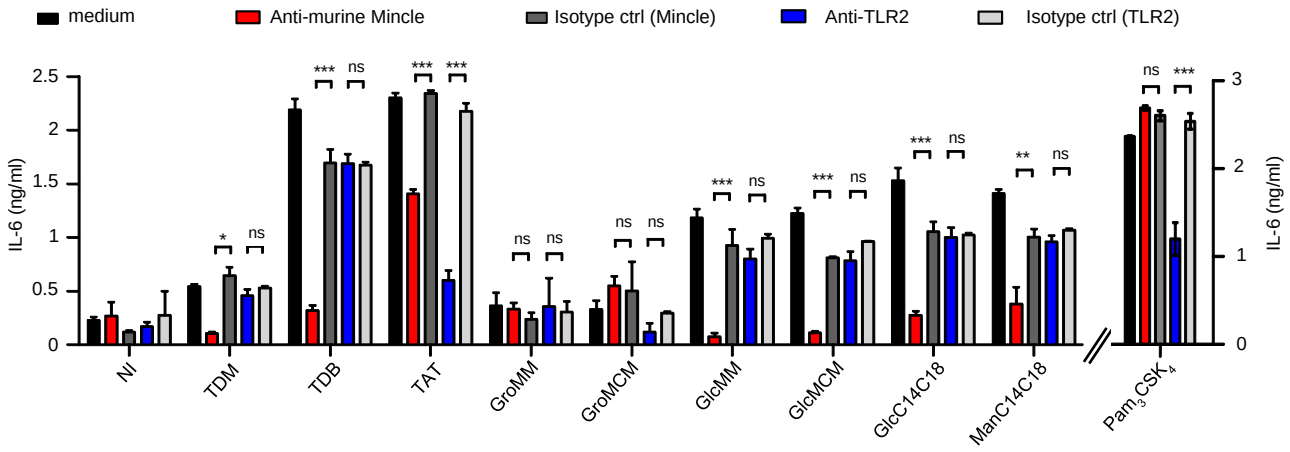


Fig. S7: IL-6 production by BMDCs stimulated by Mincle ligands.

Cells were stimulated with 1 µg of plate-coated lipids for 18 h and IL-6 release in the culture supernatant was determined by ELISA. Mincle and TLR2 dependence was investigated by pre-incubating cells for 30 min at 37°C with 5 µg/ml of anti-mMincle, anti-hMincle, anti-TLR2 or isotype control antibody before the addition of the lipids. The Pam_3CSK_4 lipopeptide was used as a control TLR2 ligand at a concentration of 100 ng/ml. Data show mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IL-6 production by BMDMs and moMΦ stimulated by Mincle ligands except TAT was barely detectable at the concentrations used. IL-6 production by TAT was fully dependent on TLR2.

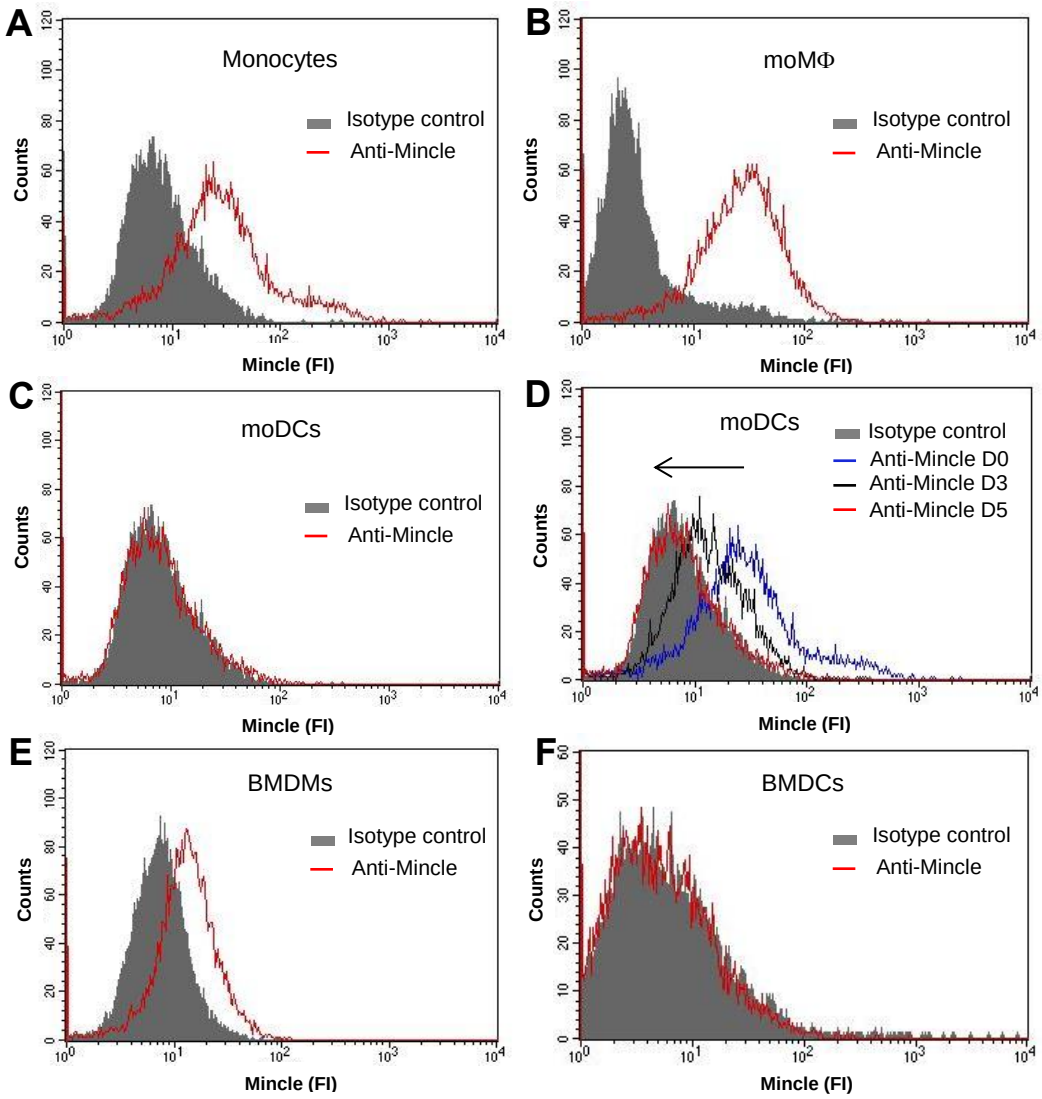


Fig. S8: Cell surface expression of Mincle on human monocytes (A), moMΦ (B), moDCs (C, D), BMDMs (E), or BMDCs (F).

Cells were stained with anti-Mincle antibodies (anti-hMincle clone 2B12 or anti-mMincle clone 7B7) or an isotype control. FI, fluorescence intensity.

As previously reported, cell surface expression of Mincle was detected on murine bone marrow-derived macrophages (BMDM) (14), but not on murine bone marrow-derived dendritic cells (BMDC) (19). Human monocytes and monocyte-derived macrophages (moMΦ) also expressed Mincle. However, Mincle expression was lost during the differentiation of human monocytes (J0) into moDCs (J5) (panel D).

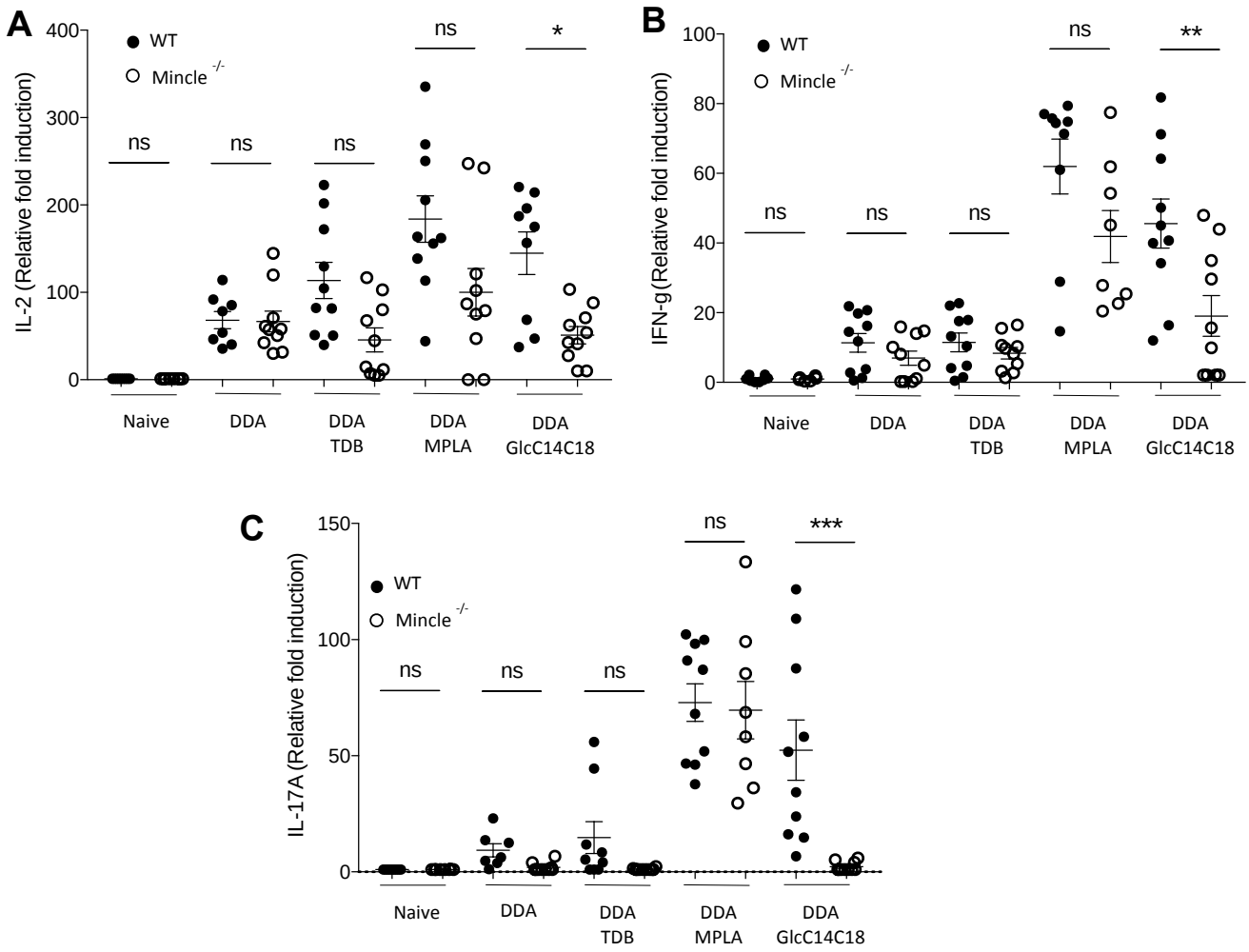


Fig. S9: GlcC14C18 adjuvanticity *in vivo* is Mincle-dependent.

WT and Mincle$^{-/-}$ mice were immunized intradermally three times with 10 µg of the *M. tuberculosis* antigen Ag85A in DDA, DDA/TDB, DDA/MPLA, or DDA/GlcC14C18. The immune response was monitored 3 weeks after the last immunization. IL-2 (A), IFN-γ (B) and IL-17A (C) release in the culture supernatant of splenocytes after restimulation with Ag85A were determined by ELISA. Results show the individual data and mean ± SEM of two pooled independent experiments. *, P <0,05; **, P <0,01; ***, P <0,001. The increased production of cytokines induced by GlcC14C18 was abrogated in Mincle$^{-/-}$ mice, while the effect of MPLA (Monophosphoryl Lipid A), a ligand of TLR4, was independent of Mincle.

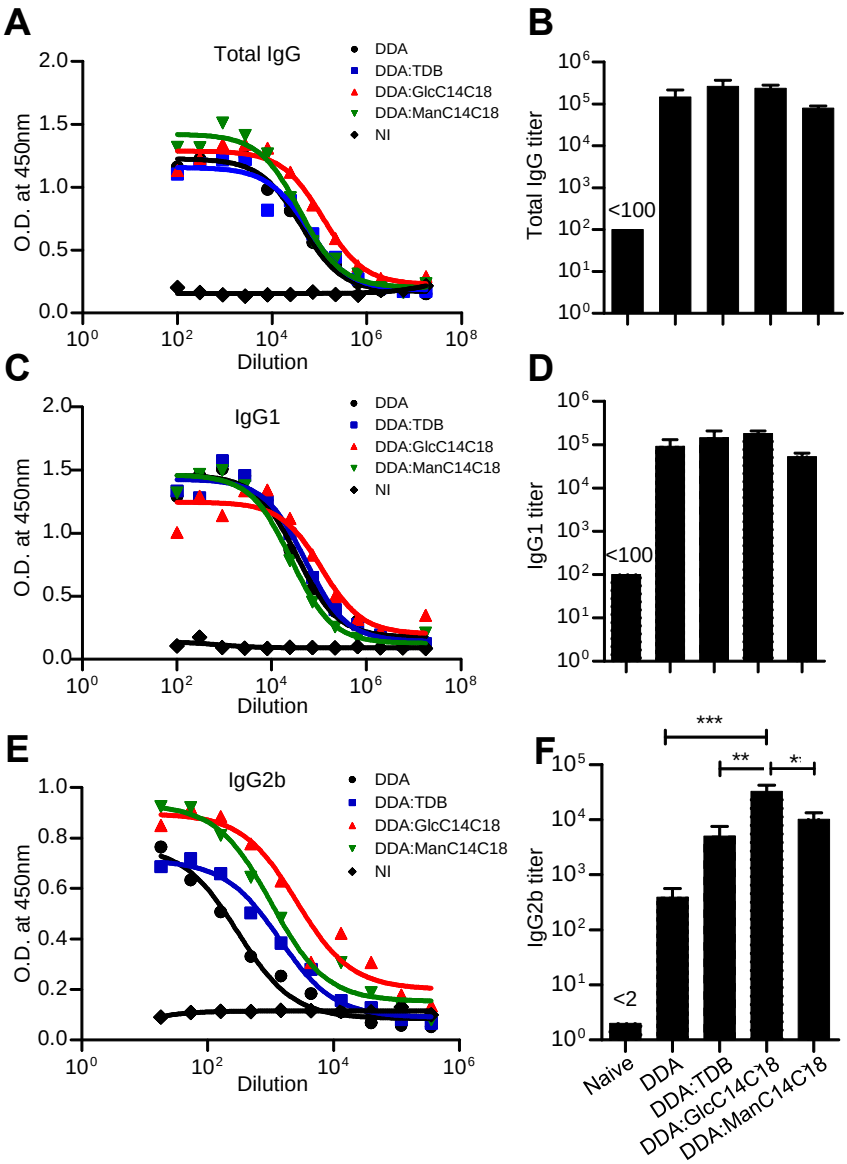


Fig. S10: Ag85A-specific antibody titers in mouse serum.

Dilution curves and calculated titers are shown for total IgG (A, B), IgG1 (C, D) and IgG2b (E, F). Plates were coated with Ag85A (0.5 µg /well) in PBS and individual mouse sera were probed in 3-fold dilutions with biotin-conjugated anti-mouse IgG, IgG1, and IgG2b antibodies. Antibody titers were defined as the serum dilution that gave an absorbance value 2.5 fold higher than that of the background. Data in panel F are also shown in Fig. 4D. The IgG2a titer, a widely used indicator of a Th1-type of immune response, was not measured since the gene is deleted in C57Bl/6 mice (12, 20). **, $P < 0.01$; ***, $P < 0.001$.

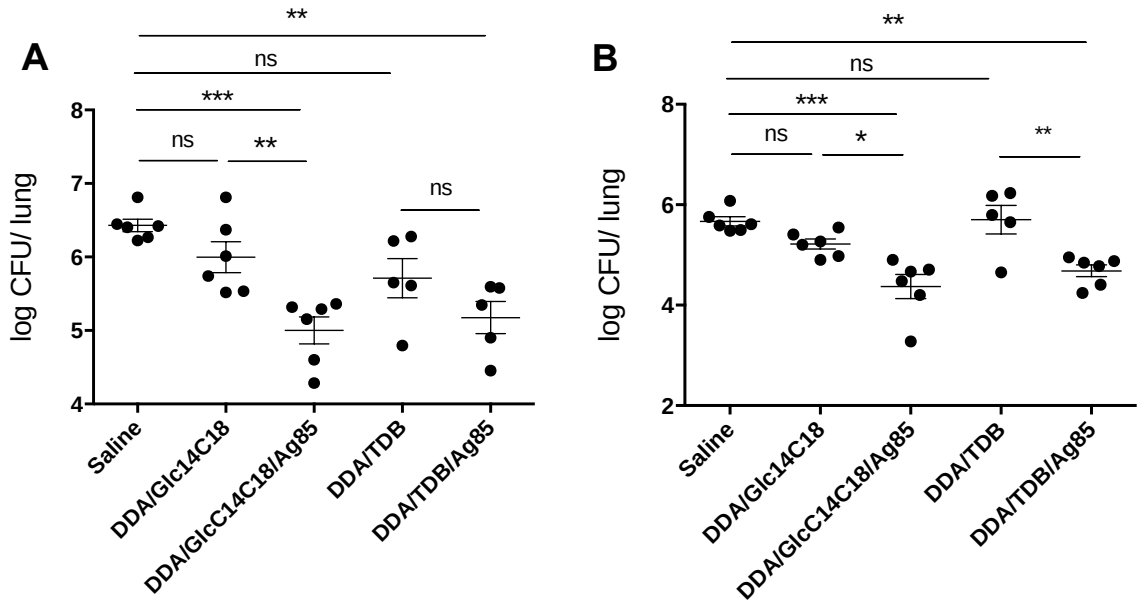


Fig. S11: Induction of protective immunity to *M. tuberculosis* infection by GlcC14C18 adjuvant.

Mice (N=6) were immunized intradermally three times with or without 10 µg of the *M. tuberculosis* antigen Ag85A in DDA/TDB (optimal ratio 10:1 mol/mol) or DDA/GlcC14C18 (ratio 25:1 mol/mol) and then infected intranasally with 3000 CFU of *M. tuberculosis*, resulting in a lung implantation of ~100 CFU. After 28 (A) and 56 (B) days, mice were killed and the bacterial load was determined in the lungs. Data show the mean ± SEM. *, P <0,05; **, P <0,01; ***, P <0,001.

GlcC14C18 induced a protective immunity, which was observed at an earlier time point than the one induced by TBD, likely due to higher IFN-γ and IL-2 responses elicited by GlcC14C18 upon re-stimulation, when compared to TDB (see Fig. 4).

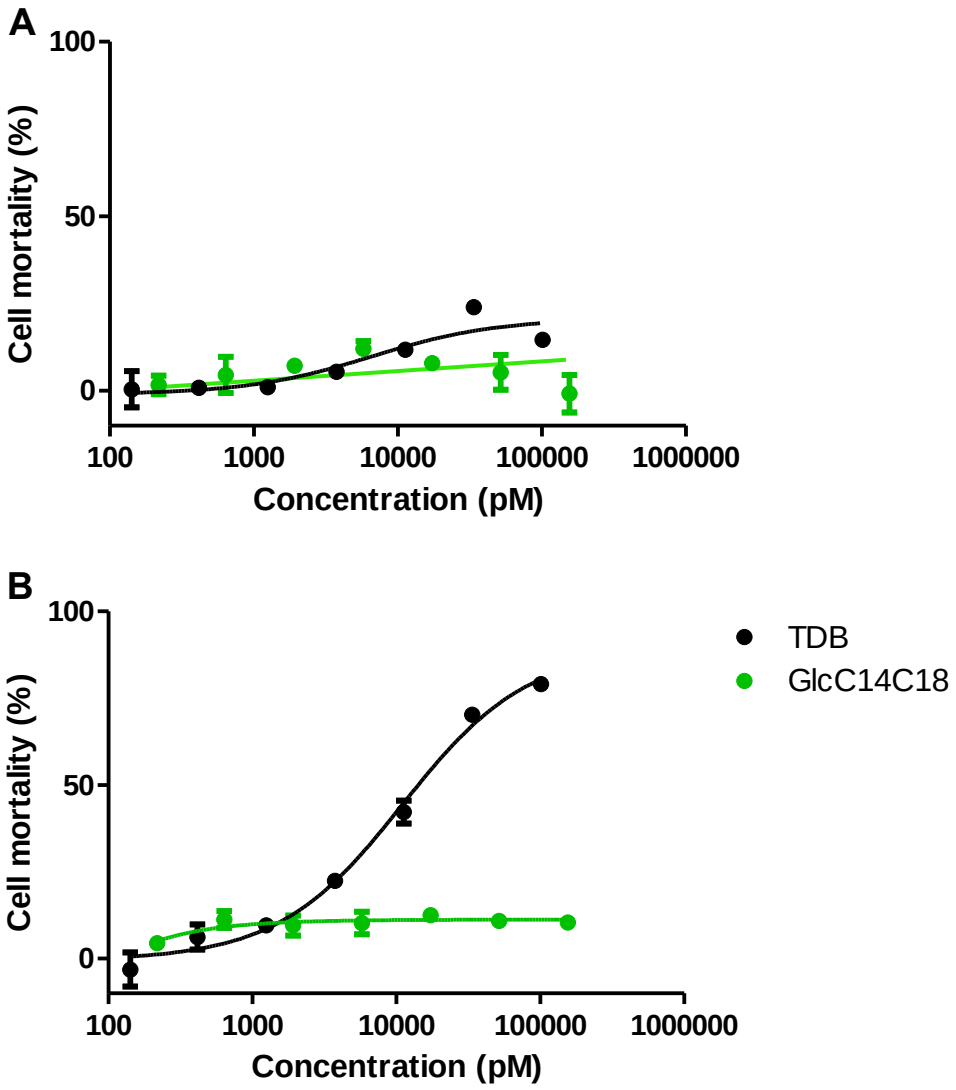


Fig. S12: Toxicity of TDB and GlcC14C18 on human monocyte THP-1 cell line (A) and moDCs (B).

TDB or GlcC14C18 were added to 96-well plates in serial dilutions from 0 to 100 µg/ml and incubated with THP-1 or moDCs cells (5 x 10^4/well) in RPMI 1640 supplemented with 10% FBS. After 24 h, cell viability was determined by a MTT-based assay. Data show the mean ± SEM.

		size	zeta potential
-Ag85A	DDA	284 +/-28 nm	68 +/- 1 mV
	DDA:TDB	232 +/- 5 nm	78 +/- 4 mV
	DDA:GlcC14C18	357 +/- 12 nm	68 +/- 0,3 mV
	DDA: ManC14C18	329 +/- 15 nm	66 +/- 0,4 mV
+Ag85A	DDA	405 +/- 20 nm	54 +/- 1 mV
	DDA:TDB	320 +/- 8 nm	68 +/- 2mV
	DDA:GlcC14C18	414 +/- 14 nm	56 +/- 3 mV
	DDA: ManC14C18	462 +/- 27 nm	57 +/- 1 mV

Fig. S13: Size and zeta potential of liposomes.

Liposomes were prepared by the lipid hydration method and analyzed in 10 mM Tris HCl buffer, pH 7.4. Data show mean ± SD.

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