Supporting Information Appendix for

Mycobacterium tuberculosis inhibits human innate immune responses *via* the production of TLR2 antagonist glycolipids

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This PDF file contains the SI Materials and Methods, Table S1, Figures S1 to S4 and references.

SI MATERIAL AND METHODS

Mutant library screening

50 µl of 3-week old *M. tuberculosis* Beijing GC1237 mutant culture from the previously used library (1) were two-fold diluted in PBS and seeded into a 96-well plate (Nunc), and 20 µl of the diluted suspension were subsequently transferred into a 384-well assay plate (Greiner, 781091) using the BioTek Precision TM XS sample processor. Mutants from three 96-well plates were transferred into columns 4 to 21 of one 384-well plate. The entire library was thus formatted into forty-four 384-well plates. Column 3 was harvested with cells only (negative control, not induced) and column 22 was harvested with LPS (used as a positive control inducing NF-κB activation) at 1 µg/mL (A22-D22), LPS at 100 ng/mL (E22-H22), *M. tuberculosis* GC1237 wild-type strain (MOI 5, I22-L22; MOI 1 M22-P22). Subsequently, 30 µl of the THP-1-Blue^{TM} cells (Invivogen) suspension at 6.7×10^5 cells/ml (2×10^4 cells/well) in HEK-Blue^{TM} Detection medium (Invivogen) were distributed into columns 3 to 22 of the 384-well assay plates using the AquaMax DW4 liquid handling device (Molecular Devices). After 16 h incubation at 37°C under 5% CO_2, NF-κB activation was monitored by reading O.D. at 625 nm using a Victor3 (Perkin Elmer). Plate by plate quality check was performed by determination of *Z'* (negative control *vs* LPS at 1 µg/mL). Bacterial titer of each mutant in 96-well plates was quantified by reading at O.D. at 595 nm. Mutants for which the titer was lower than 0.1 (corresponding to a too low MOI) were removed from the analysis. 64 Hits with a Z score value < -1.45 or > 2 were then selected for amplification and mapping of the transposon insertion sites by ligation-mediated PCR (2).

Bacterial growth

M. tuberculosis GC1237 wild-type and selected transposon mutant strains were grown for 2 months on Sauton's medium as surface pellicle. Bacteria were dissociated by gentle shaking for 30 s with 4-mm glass beads and numbered with a Thoma cell counting chamber.

Purification of natural mycobacterial lipids - Preparation of synthetic analogs - TLR2 and NOD2 ligands

Natural sulfoglycolipids, Ac_2SGL and Ac_4SGL, were purified and analyzed by MALDI-TOF mass spectrometry, and synthetic Pam_2SGL (i.e. 2,3-dipalmitoyl-2′-sulfate-α-α′-D-trehalose; SGL1), unsulfated SGL and Trehalose sulfate prepared as previously described (3-6). Lipoproteins were purified from *M. tuberculosis* GC1237 strains by a phenol/water extraction and quantified as previously described (7, 8). Lipoproteins were labeled with FITC (Fluorescein isothiocyanate isomer I, Sigma) according to manufacturer's instructions. FITC-labeling did not alter their ability to stimulate HEK-TLR2 cells. LM was purified from *M. tuberculosis* H37Rv (9). Lipoglycans were purified from *M. tuberculosis* GC1237 strains and quantified by monosaccharide analysis relatively to proteins as previously described (7). Pam_3CSK_4, FSL-1 and MDP were from Invivogen.

THP-1 cell experiments

THP-1-Dual™ cells (Invivogen), derivatives of THP-1 monocyte/macrophage human cells that stably express a NF-κB-inducible reporter system (secreted alkaline phosphatase), were used according to the manufacturer's instruction. The different stimuli and competitors were added, at MOI or concentrations indicated in the figures, in 96-wells plates and cells were then distributed at 10^5 cells per well in 200 µl RPMI 1640 culture medium (Lonza). After 18 h, NF-κB activation was measured by mixing 20 µl of the culture supernatant and 180 µl of Quanti-

Blue™, and reading O.D. at 630 nm, and IL-8 was assayed in the culture supernatant by sandwich ELISA using commercially available kits (eBioscience). To investigate TLR2 dependence, cells were pre-incubated for 30 min at 37°C, before stimuli addition, with 10 µg/ml of anti-TLR2 monoclonal antibody (clone T2.5, InvivoGen) or isotype control (IgG1, eBioscience). For monitoring CD40 expression, cells were harvested and resuspended in Dubelco's PBS, 0.5 % BSA and labelled with CD40-PE conjugated antibody (Beckman Coulter). Cells were subjected to flow cytometry analysis by using the CellQuest software on a flow cytometer (FACSCalibur; Becton Dickinson).

HEK-TLR2 cell experiments

HEK cell lines were all derivatives of the HEK-Blue™ Null1 cell line (InvivoGen). HEK-Blue Null1 cells express a secreted alkaline phosphatase reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. First, HEK-Blue Null1 was transfected with a blasticidin resistant plasmid pUNO1-hTLR2 (HEK-TLR2) or a hygromycin resistant plasmid pVITRO2-hTLR2-hCD14 (HEK-TLR2-CD14). Then, co-receptors were introduced into HEK-TLR2 by transfection with hygromycin resistant plasmids pUNO3-hCD36 and pUNO3-hMARCO to generate HEK-TLR2-CD36 and HEK-TLR2-MARCO respectively. Finally, HEK-TLR2-CD14 was transfected with a blasticidin resistant plasmid pUNO1-hSRAI to obtain HEK-TLR2-CD14-SRA. All plasmids are mammalian expression vectors from InvivoGen. HEK cell lines 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) 100 µg/ml zeocin (InvivoGen). The cell culture medium was supplemented with 10 µg/ml blasticidin for HEK-TLR2 cells, with 200 µg/ml hygromycin for HEK-TLR2-CD14 cells, and with 200 µg/ml hygromycin and 10 µg/ml blasticidin for the other cell lines. The different stimuli and

competitors were added, at concentrations indicated in the figure legends, in 96-wells plates and cells were then distributed at 5 x 10^4 cells per well in 200 µl DMEM culture medium. Alkaline phosphatase activity was measured after 18 h, as described above.

Statistical analysis

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

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Mutant ID	Gene	Putative function	Variation of NF-κB activation (%) [a]
P003H04	*rv1068c-rv1069c* [b]	Unknown, PE-PGRS family protein - Unknown, conserved protein	-31 ±16
P037D01	*rv1510*	Unknown, probable membrane protein	-28 ± 11
P069F01	*rv3109 (moaA1)*	Molybdenum cofactor biosynthesis	-27 ± 16
P088D08	*rv0032 (bioF2)*	Biotin biosynthesis	-22 ± 23
P102B07	*Rv2067c*	Unknown, conserved protein	+26 ± 10
P110E03	*rv3350c*	Unknown, PPE family protein	-10 ± 3
P116E09	*rv3112 (moaD1)*	Molybdenum cofactor biosynthesis	+31 ± 3
P117C11	*rv3112 (moaD1)*	Molybdenum cofactor biosynthesis	+21 ± 2
P118B01	*rv3823c (mmpl8)*	Sulfoglycolipid biosynthesis	+18 ± 7
P119B10	*rv0032 (bioF2)*	Biotin biosynthesis	-26 ± 12

Table S1 : List of confirmed mutants that induce an altered NF-κB activation.

[a] corresponds to the mean ± S.D. of the variation of NF-κB activation as compared to the wild-type strain at MOI = 30; calculated as (OD_{630nm}(mutant) - OD_{630nm}(wild-type)) / OD_{630nm}(wild-type) x 100, from at least three independent experiments.

[b] the transposon was inserted in the intergenic region.

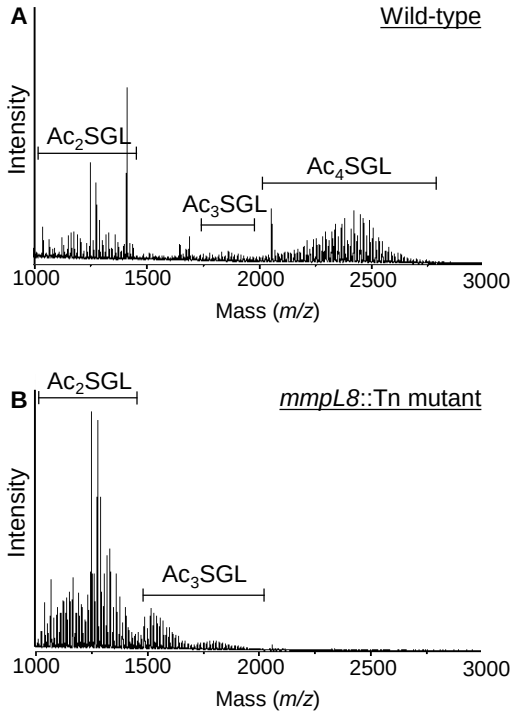

Fig. S1 : *mmpL8*::Tn mutant is impaired for Ac_4SGL production.
The total bacterial lipids obtained by chloroform/methanol extraction were partitioned by acetone precipitation. Lipids contained in the acetone-soluble fraction were analyzed by MALDI-TOF mass spectrometry in the negative-ion mode. Negative mass spectra recorded for wild-type (A) and *mmpl8*::Tn mutant (B) strains are shown.

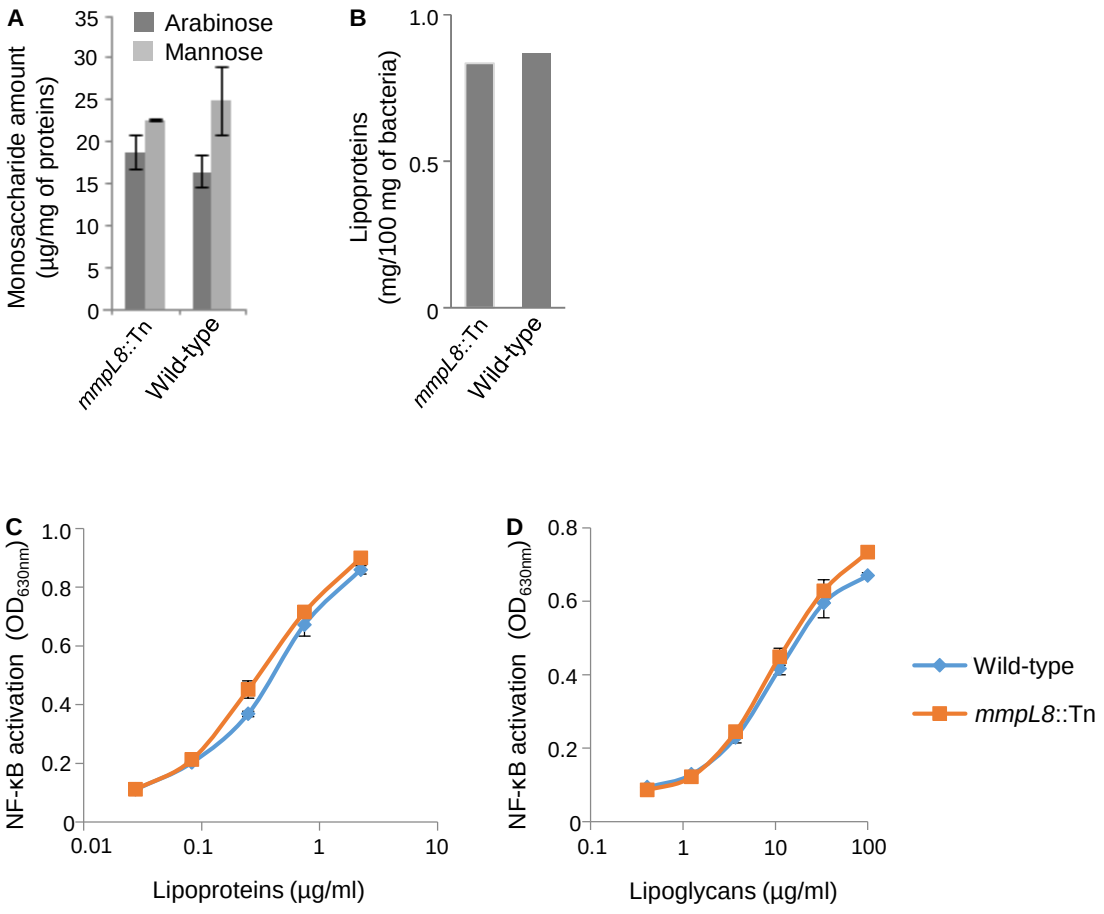

Fig. S2: Lipoproteins or lipoglycans are not overproduced in the *mmpL8*::Tn mutant strain (A, B) and show the same capacity than those from the wild-type strain to induce NF-κB activation in THP-1 cells (C, D).

A) Lipoglycan amount was determined by the quantification of arabinose and mannose. These monosaccharides were quantified from an equivalent amount of 1 µg of proteins by capillary electrophoresis after total acid hydrolysis. B) Lipoprotein amount was obtained by weighing after extraction from bacteria by a phenol/water partition. C, D) THP-1 cells were stimulated with lipoproteins (C) or lipoglycans (D) at the indicated concentrations. After 16h, NF-κB activation was determined by measuring alkaline phosphatase activity in the culture supernatant and reading O.D. at 630 nm.

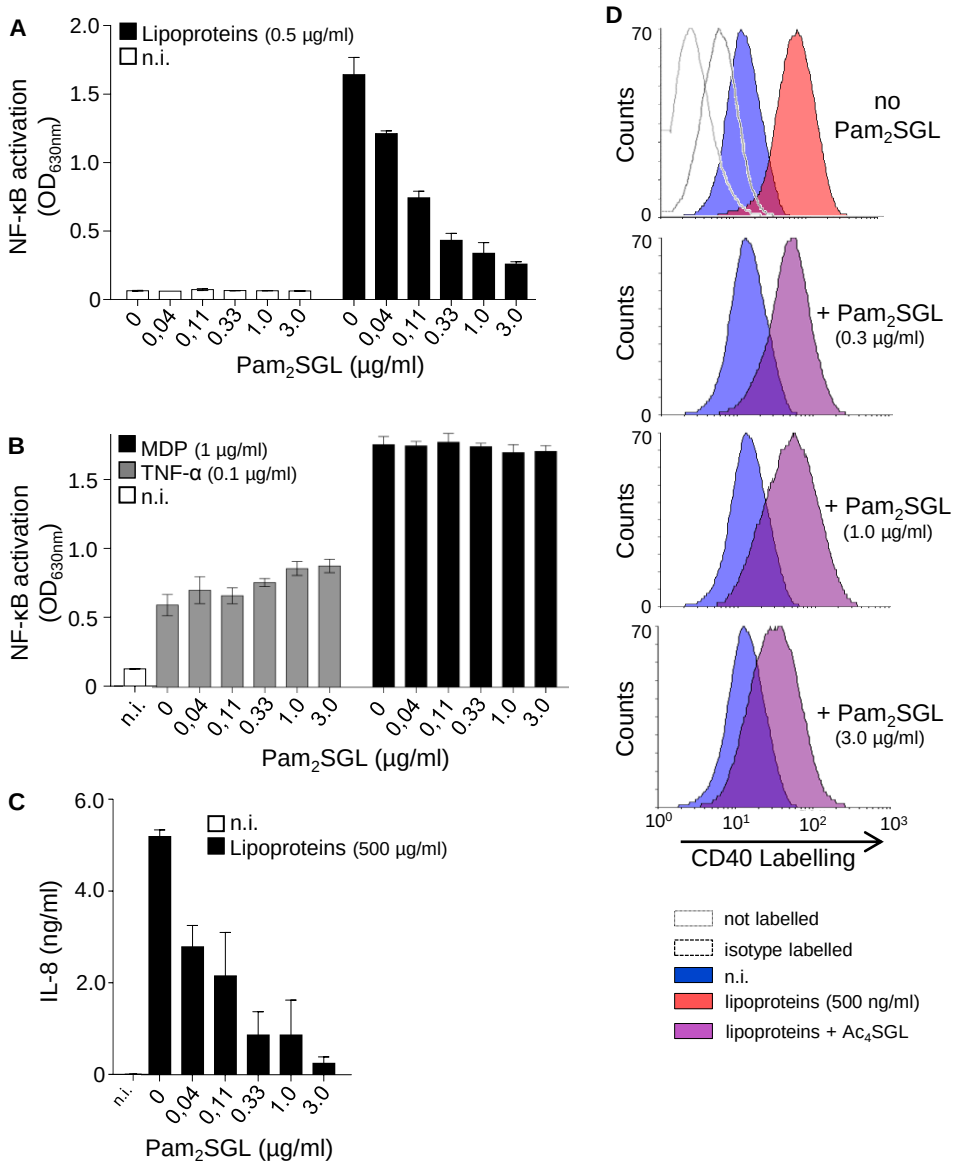

Fig. S3 : Pam_2SGL inhibits activation of TLR2 signaling.

THP-1 cells were stimulated with various stimuli in the presence or not of Pam_2SGL at the indicated concentrations. After 16h, NF-κB activation (A, B), IL-8 in the culture supernatant (C) and CD40 expression (D) were measured. A, B) NF-κB activation was determined by measuring alkaline phosphatase activity in the culture supernatant and reading O.D. at 630 nm. C) IL-8 release in the culture supernatant was determined by sandwich ELISA. D) CD40 expression was monitored by flow cytometry. Data show mean ± SEM. MDP, muramyl dipeptide, a ligand of NOD2; n.i., not induced.

It is noticeable that Pam_2SGL, as Ac_4SGL (Fig. 2B), slightly increased NF-κB activation triggered by MDP. Yet, the underlying mechanisms are unknown.

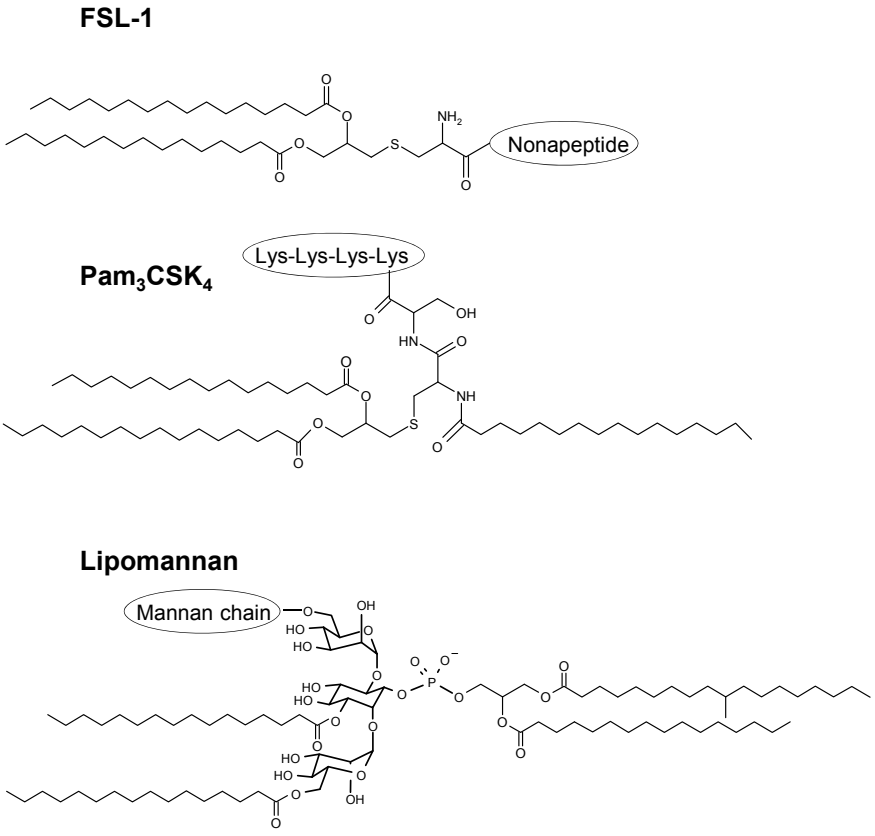

Fig. S4 : Structure of the different TLR2 ligands used in the study.
FSL-1 and Pam_3CSK_4 are synthetic lipopeptides based on an acylated cysteine lipid anchor. Mycobacterial lipoproteins are mainly tri-acylated. Mycobacterial lipomannan is based on a mannosyl-phosphatidyl-*myo*-inositol anchor, which is essentially tri- or tetra-acylated.