Disruption of mycobacterial AftB results in complete loss of terminal β(1→2) arabinofuranose residues of lipoarabinomannan

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SUPPLEMENTARY METHODS

Chemicals, reagents and enzymes. All chemicals and solvents were from Sigma-Aldrich, Bio-Rad and Fisher Chemicals unless otherwise stated, and were of AnalR grade or equivalent. All restriction enzymes and Phusion DNA polymerase enzyme were sourced from New England Biolabs. Bioline quick ligation kit was used to perform ligation reactions. Oligonucleotides were from MWG Biotech Ltd and PCR fragments were purified using the QIAquick gel extraction kit (Qiagen). Plasmid DNA was purified using the QIAprep purification kit (Qiagen).

Bacterial strains and culture conditions. All cloning steps were performed using *E. coli* TOP10 strain (Invitrogen), which was grown at 37 °C in Luria-Bertani broth (Difco). *M. smegmatis* mc^2155 was grown at 37 °C in either tryptic soy broth (TSB; Difco) supplemented with 0.05 % (v/v) Tween 80 or minimal medium [1] supplemented with 0.05 % (v/v) Tween 80 and 0.2 % (w/v) glucose; solid media included 1.5 % (w/v) agar. The concentrations of antibiotics were 50 μg mL^{-1} of kanamycin and 150 μg mL^{-1} of hygromycin for *E. coli* and 25 μg mL^{-1} of kanamycin, 100 μg mL^{-1} of hygromycin, and 0.2 % (w/v) of acetamide for *M. smegmatis*.

Determination of viable cell counts. For determining numbers of CFU, aliquots of culture were serially diluted in TSBT (10-fold dilutions). The appropriate dilutions were spotted on TSB agar supplemented with 0.2 % acetamide and plates were incubated at 37 °C for 72 h before CFU were counted.

Extraction and purification of lipoglycans. Dried cells were resuspended in water and disrupted by sonication; an equal volume of ethanol was added and the mixture refluxed, followed by centrifugation and the supernatant recovered. The extraction process was repeated five times and the combined supernatants were dried, subjected to hot-phenol treatment, and dialyzed against water. The retentate was dried, resuspended in water, and digested with α-amylase, DNase, RNase, chymotrypsin, and trypsin. The retentate was dialyzed and subjected to hydrophobic and size exclusion chromatography [2,3]. Eluates were collected, dialyzed, concentrated, and analyzed by SDS–PAGE using Pro-Q emerald glycoprotein stain (Invitrogen).

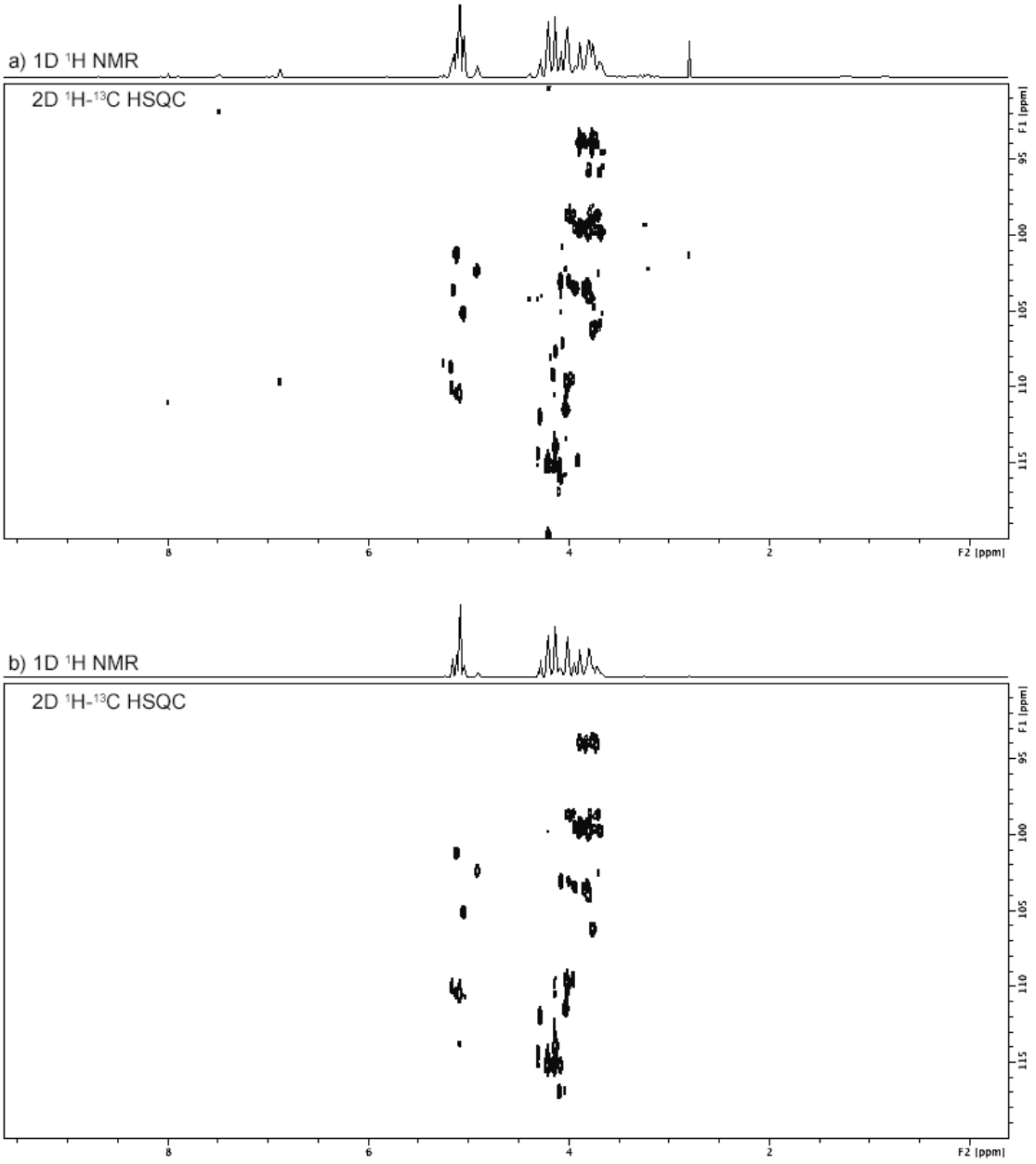
Immunoblot assay. [^{14}C]-Lipoglycans were extracted as described above and samples were subjected to an SDS-PAGE analysis and subsequently immunostained after electroblotting on a PVDF membrane (Millipore). F30-5 mAb of the IgM class, which recognizes the branches of the arabinan domain of LAM, was used [4,5]. F30-5 was generated using a three-step immunization protocol (two rounds of immunization followed by one booster) in BALB/c mice with *M. leprae* (intact as well as sonicated cells) and Freud's incomplete adjuvant followed by hybridoma technology as described previously [4]. F30-5 was provided by Prof. B. J. Appelmelk, VU University Medical Center, Amsterdam, the Netherlands. The PVDF membrane was incubated with 0.5 % (w/v) blocking reagent (Roche) for 1 h at 37 °C, followed by another incubation of 2 h with the mAb diluted (1:1000) in the blocking buffer and PBS supplemented with 0.05 % (v/v) Tween-80 (PBST). After washing with PBST, the membrane was incubated with alkaline phospatase (AP)-labeled goat anti-mouse IgM (1:2000; Santa Cruz Biotech, sc-2978, lot # G0611) in PBST with 0.5 % (v/v) normal goat serum. After washing, the lipoglycan bands were visualized using SIGMA FAST™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; 1 tablet per 10 ml of dH_2O) system.

NMR spectroscopic analysis of lipoglycans. 2-D NMR spectra were recorded at 900 MHz using Bruker 900 AVANCE III. All samples were exchanged in D_2O (D, 99.97% from Sigma), with intermediate lyophilization, and then dissolved in 0.5 mL D_2O and analyzed at 313 K. The ^{1}H and ^{13}C NMR chemical shifts were referenced relative to the solvent signal D_2O at δ 4.79. All details concerning NMR sequences and the experimental procedures used were described previously [6,7].

Cell culture. THP-1-Dual^{TM} cells (InvivoGen), derivatives of THP-1 monocyte/macrophage human cells that stably express a NF-κB-inducible reporter system (secreted alkaline phosphatase) and the HEK-BlueTM-2/HEK_BlueTM-4 cell lines (InvivoGen), derivatives of HEK293 cells that stably express human TLR2 or TLR4 along with a NF-κB-inducible reporter system, were grown according to the manufacturer's instructions.

SUPPLEMENTARY FIGURES

Figure 1.



Supplementary Figure 1. One-dimensional ^{1}H NMR and two-dimensional ^{1}H-^{13}C HSQC spectra in D_2O at 313 K of *M. smegmatis* LAM extracted from the *aftB* conditional mutant in the presence (a) and absence (b) of AftB.

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