A NOVEL LIPOARABINOMANNAN
FROM THE EQUINE PATHOGEN RHODOCOCCUS EQUI:
structure and effect on macrophage cytokine production.

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Summary

*Rhodococcus equi* is a major cause of foal morbidity and mortality. We have investigated the presence of lipoglycan in this organism as closely related bacteria, notably *Mycobacterium tuberculosis*, produce lipoarabinomannans (LAM) that may play multiple roles as virulence determinants. The lipoglycan was structurally characterised by gas chromatography-mass spectrometry following permethylation, capillary electrophoresis after chemical degradation, $^1$H and $^{31}$P and 2D heteronuclear nuclear magnetic resonance studies. Key structural features of the lipoglycan are a linear $\alpha$(1-6) mannan with side chains containing one 2-linked $\alpha$-D-Manp residue. This polysaccharidic backbone is linked to a phosphatidylinositol mannosyl anchor. In contrast to mycobacterial LAM there are no extensive arabinan domains but single terminal $\alpha$-D-Araf residue capping the 2-linked $\alpha$-D-Manp. The lipoglycan binds Concanavilin A and mannose binding protein consistent with the presence of t-$\alpha$-D-Manp residues. We studied the lipoglycans ability to induce cytokines from equine macrophages, in comparison to whole cells of *R. equi*. These data revealed patterns of cytokine mRNA induction that suggest that the lipoglycan is involved in much of the early macrophage cytokine response to *R. equi* infection. These studies identify a novel LAM variant which may contribute to the pathogenesis of disease caused by *R. equi*. 
Introduction

*Rhodococcus equi* is a significant cause of disease in foals between the age of 1 and 5 months and is responsible for ca. 3% of global foal mortality (1). This organism has also emerged as an opportunistic human pathogen, notably of people with compromised immunity (2). *R. equi* is an intracellular pathogen of alveolar macrophages and infection is characterised by bronchopneumonia. The bacterium is known to enter macrophages primarily (but not exclusively) via the complement receptor type 3 (Mac-1) following complement component C3 deposition (2,3). Once within the macrophage the bacteria resist host killing mechanisms and multiply, eventually killing the macrophage. Specific bacterial factors which facilitate entry of the organisms into the macrophages, or which aid intra-macrophage persistence, have yet to be identified.

*R. equi* is a member of the mycolata, a supra-generic taxon including the extensively studied facultative intracellular pathogen *Mycobacterium tuberculosis* (4). Members of the mycolata have a characteristic cell envelope architecture, dominated by lipids, notably the high molecular weight branched-chain mycolic acids. The cell envelope profoundly affects the properties of these bacteria and its composition and organisation has been a major focus of mycobacterial research (5, 6). Lipoarabinomannan (LAM) is a complex mycobacterial cell envelope component that has been identified as a putative virulence factor of *M. tuberculosis* (7, 8). The structure of this macroamphiphile has been studied in detail and consists of a glycosyl-phosphatidylinositol (GPI) anchor unit which bears a branched D-mannan and D-arabinan heteropolysaccharide. However, many elaborations of this core structure have been described, including variations in the pattern of the lipid anchor acylation, the presence of succinate residues and capping motifs on the non-reducing termini of arabinan branches (7, 9-14). Some of these structural variations, notably the presence of particular capping motifs, may vary between the LAM of different
mycobacterial species in a species-specific manner (7). To date LAM have been classified into ManLAM (15) and PILAM (16), according to their small mannooligosaccharide or phosphoinositide cap structures, respectively. The former were found in slow growing mycobacterial species (as *Mycobacterium bovis* BCG, *M. tuberculosis*) while the latter were found in fast growing mycobacterial species (as *Mycobacterium smegmatis*).

ManLAM has been shown to have many properties which potentially influence the pathogenicity of *M. tuberculosis*. In the early stages of infection, ManLAM may facilitate the adherence of bacteria to alveolar macrophages, particularly to mannose receptors (8, 17-19). It has been demonstrated that mycobacterial internalisation via these receptors evades macrophage bactericidal mechanisms (20). LAM has also been reported to have powerful immunomodulatory properties, promoting distinctive patterns of macrophage cytokine induction which subsequently directs host immune responses (8, 21). Small differences in LAM structure can strongly influence these biological activities, demonstrating the value of detailed structural studies.

Lipoglycans apparently structurally related to LAM have been identified in representative organisms of other genera within the mycolata, including *Corynebacterium matruchotii* (22), *Dietzia maris* (23), *Gordonia rubropertincta* (24, 25) and *Rhodococcus rhodnii* (26). Although these lipoglycans display components typical of LAM, considerable variation in monosaccharide composition has been found. Thus, further detailed study of the lipoglycan components of these taxa is necessary. By analogy with mycobacterial ManLAM, we hypothesised that a lipoglycan may be involved in the pathogenic success of *R. equi*. This study describes the isolation, purification and structural characterisation of a *R. equi* lipoglycan. The potential role of this lipoglycan in *R. equi* virulence was assessed by comparing the early cytokine responses of equine macrophages to the lipoglycan and to infection with virulent *R. equi*. 
Experimental procedures

Growth and maintenance of organisms.

Three strains of *R. equi* were used in this study, *R. equi* 103+ (foal isolate) and *R. equi* 28+ (pig isolate) which are clinical isolates containing plasmids associated with increased virulence (27) and the attenuated type strain *R. equi* ATCC 6939. Stock cultures were maintained on slopes of brain-heart infusion (BHI) agar ('Oxoid', Unipath Ltd, Basingstoke, U.K.) at 4°C. Cultures were maintained by routine subculture onto BHI agar and growth at 37°C for 18h. Broth cultures were grown in BHI broth incubated at 37°C with shaking (200 r.p.m.) for 18h. Growth was then harvested, washed twice in PBS and lyophilised.

Extraction of lipoglycans.

Lyophilised cells were delipidated with chloroform : methanol (1:1 v/v; 50 mg/ml) at ambient temperature for 18 h (28). The cells were then recovered by centrifugation (4000 r.p.m. for 10 min) and washed twice with phosphate buffered saline (PBS). Cells were then permeabilised with mutanolysin (50 U/ml) and lysozyme (25 mg/ml) according to the method of Assaf and Dick (29). An equal volume of phenol (90% w/v) was then added to the cell suspension in lysozyme buffer and the mixture was incubated with shaking at 68°C for 1 h to extract lipoglycans, which were subsequently recovered into the aqueous phase formed on refrigerated centrifugation as described previously (30).

Purification of the lipoglycans.

The crude aqueous extract was purified using a modification of the hydrophobic interaction chromatography (HIC) method (22, 31). Briefly, the crude extract was taken up
into 6 ml equilibration buffer (100 mM sodium acetate buffer pH 4.5, containing 15% v/v n-propanol) and loaded onto a column (1.25 x 17 cm) of octyl-sepharose CL-4B (Amersham Pharmacia Biotech, Little Chalfont, UK). The column was eluted with 40 ml of this buffer prior to gradient elution with a 120 ml gradient of 15-65% v/v n-propanol in 100 mM sodium acetate buffer (pH 4.5). Fractions were assayed for carbohydrate using the method of Fox and Robyt (32). HIC fractions were also monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples (30 µL) of alternate fractions were prepared and electrophoresed. With consideration of SDS-PAGE analysis, carbohydrate-containing peak fractions were pooled, dialysed extensively and lyophilised. To remove a persistent protein contaminant from the lipoglycan, a 1 mg/ml aqueous solution of lipoglycan was treated with an equal volume of 90% phenol (w/v). The mixture was heated at 68°C for 1 h and then separated into distinct aqueous and phenol phases by centrifugation (30 min, 4000 r.p.m., 4°C). The upper aqueous phase was recovered and re-extracted with phenol. After further centrifugation, the aqueous phase was recovered and extensively dialysed before being lyophilised and stored at -20°C.

Lipoglycan-containing fractions (PK3) were pooled, dialysed and lyophilised. Contaminants were removed by gel filtration. Sample (16 mg) was dissolved in 0.2 M NaCl, 0.25% sodium deoxycholate (DOC) (w/v), 1 mM ethylenediaminetetra-acetic acid disodium salt (EDTA) and 10 mM Tris pH=8 to a final concentration of 200 mg/ml, incubated two days at room temperature and loaded on a gel permeation Bio-Gel P-100 column (52x3 cm) eluted with the same buffer at a flow rate of 5 ml/h.

Electrophoresis and Western Blotting Procedures.

SDS polyacrylamide gel electrophoresis was performed as described by Laemmli (33) using 15 % acrylamide resolving gels. The lipoglycan bands were characterized by silver
staining with polysaccharide-specific periodic acid oxidation according to Tsai and Frasch (34). Western blotting and lectin blotting using Concanavalin A were performed as described previously (23).

**Analysis of lipoglycan carbohydrate and fatty acid composition.**

Lipoglycan samples (1 mg) were acid hydrolysed with 2M trifluoroacetic acid (TFA, 250 µL) at 110°C for 2 h in a 8.5 ml polytetrafluoroethylene (PTFE) screw-capped tube. The hydrolysates were then neutralised in vacuo over sodium hydroxide pellets and the dried residue was taken up in distilled water (250 µL) and then lyophilised. The monosaccharides were derivatised as alditol acetates and analysed by gas-liquid chromatography (GLC) as previously described (23). Fatty acid composition of the lipoglycan samples (1 mg) was analysed by GLC of the acid hydrolysate following derivatisation to form fatty acid methyl esters as described previously (23). An Immunopure® recombinant mannose binding protein (MBP) column, (Pierce & Warriner Ltd., Chester, U.K) was used to demonstrate interaction of lipoglycan with MBP as described previously (23).

**Permethylation analysis of lipoglycan samples.**

Lipoglycan (2 mg) from *R. equi* strains 103+ and 28+ was deacylated according to the method of Beachey *et al.* (35). The deacylated lipoglycans were permethylated according to the method of Dell *et al.* (36). The permethylated samples were cleaned using a C18 Sep-Pak Cartridge (Waters Ltd, Watford, UK). Each sample was taken up into chloroform:water (1:1 v/v, 1 ml). The cartridge was conditioned by sequential washing with distilled water (5 ml), acetonitrile (MeCN, 5 ml) and distilled water (10 ml). The sample was then loaded onto the cartridge and distilled water (5 ml) and 2 ml each of 15%, 35%,
50 %, 75 % aqueous MeCN and MeCN (2ml) were used to elute the sample. Fractions collected at each step were assayed for carbohydrate as described previously and by thin layer chromatography (TLC) in MeCN:H₂O (85:15, v/v), visualising with α-naphthol. Fractions which were found to contain carbohydrate were pooled and evaporated under nitrogen.

The permethylated samples were hydrolysed with 2 M TFA at 110°C for 2h as above. The methylated monosaccharides were then reduced with sodium borodeuteride and acetylated according to the method of Saddler et al. (37). GC-MS analysis of the samples was performed on a Carlo-Erba 8060 MS gas chromatograph connected to a Micromass Trio 2000 mass spectrometer. Samples were injected with a split injector (split rate of 50:1). The injection port temperature was 250°C and the transfer line 250°C. The column was a 30 m x 0.32 mm internal diameter BPX-5 fused silica column with helium (50 kPa) as the carrier gas. The oven was programmed to hold 140°C for 1 min followed by a 10°C/min rise to 280°C and a 10 min hold. The mass spectrometer operated in electron ionisation mode (70 eV) and was set to scan from 20 to 650 amu.

**MALDI-TOF analysis.**

Analysis by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) was carried out on a Voyager DE-STR (PerSeptive Biosystems, Framingham, MA) using linear mode detection. All samples were irradiated with UV light (337 nm) from a N₂ laser and were analysed with the instrument operating at 20 kV in the negative-ion mode. The matrix used was 2,5-DHB (2,5-dihydroxybenzoic acid).
**Acetolysis procedure**

10 µg of samples were treated with 15 µl of a acetic anhydride : acetic acid : sulphuric acid (10:10:1, v/v/v) mixture for 3 h at 40°C. The reaction was quenched by addition of 40 µl of water. Acetolysis products were extracted twice with 40 µl of chloroform, and after drying, were deacetylated with 20 µl of a methanol:20% aqueous ammonia solution (1:1 v/v) at 37°C for 18 h. The reagents were removed under a stream of nitrogen. The samples were then submitted to APTS tagging (see below).

**APTS derivatization.**

Dried hydrolysis (complete hydrolysis: 2M TFA at 110°C for 2h, mild hydrolysis: 0.1M HCl at 110°C for 30 min) or acetolysis (38) products were mixed with 0.4 µl of 0.2 M 1-aminopyrene-3,6,8-trisulfonate (APTS) (Interchim, Montluçon, France) in 15% (v/v) acetic acid and 0.4 µl of a 1 M sodium cyanoborohydride solution dissolved in tetrahydrofuran (39). The reaction was performed for 90 min at 55°C and was quenched by addition of 20 µl of water. Dilutions of 1 to 5 µl of the APTS derivatives were prepared in 20 µl total water before analysis by capillary electrophoresis (CE).

**Capillary electrophoresis.**

The electropherograms were acquired and stored on a Dell XPS P60 computer using the System Gold software package (Beckman Instruments, Inc.). APTS derivatives were loaded by applying 0.5 psi (3.45 kPa) vacuum for 5 s (6.5 nl injected). Separations were performed using an uncoated fused-silica capillary column (Sigma, Division Supelco, Saint-Quentin-Fallavier, France) of 50 µm internal diameter with 40 cm effective length (47 cm total length). Analyses were usually performed on a P/ACE capillary electrophoresis system (Beckman Instruments, Inc.) with the cathode on the injection side and the anode
on the detection side (reverse polarity) (Figures 3a, c and d). They were carried out at a temperature of 25°C with an applied voltage of -20 kV and using acetic acid 1% (w/v), triethylamine 30mM in water, pH 3.5 as running electrolyte. For figure 3b, the electropherogram was recorded in normal mode, at a temperature of 25°C with an applied voltage of +25 kV and borate buffer (20 mM, pH 9.2) as running electrolyte.

Detection system consisted in a Beckman laser-induced fluorescence (LIF) equipped with a 4-mW argon-ion laser with the excitation wavelength of 488 nm and emission wavelength filter of 520 nm.

**NMR Spectroscopy.**

Prior to NMR spectroscopic analysis, fractions were exchanged in D₂O (99.9% purity, Eurisotop, Saint Aubin, France) at room temperature with intermediate freeze-drying, and then dissolved in 400 µl of D₂O or Me₂SO-d₆ (99.8% purity, Eurisotop, Saint Aubin, France). ReqLAM from strain 28⁺ (15 mg) and strain 103⁺ (3 mg) were analyzed in a 200 x 5 mm 535-PP NMR tubes at 313K on a Bruker DMX-500 500 MHz NMR spectrometer equipped with a double resonance (¹H/X)-BBi z-gradient probe head. Data were processed on a Bruker-X32 workstation using the xwinnmr program. Proton and carbon chemical shifts are expressed in ppm downfield from internal acetone ($\delta_{\text{H}}$/TMS 2.225 and $\delta_{\text{C}}$/TMS 34.00). The 1D phosphorus ($^{31}\text{P}$) spectra were measured at 202.46 MHz and phosphoric acid (85%) was used as the external standard ($\delta_{\text{P}}$ 0.0). All 2D NMR data sets were recorded without sample spinning and data were acquired in the phase sensitive mode using the time-proportional phase increment (TPPI) method (40). Four 2D Homonuclear Hartmann-Hahn (HOHAHA) spectra were recorded using MLEV-17 mixing sequences of 9 ms, 43 ms, 82 ms, 113 ms (41). The $^{1}\text{H}$-$^{13}\text{C}$ and $^{1}\text{H}$-$^{31}\text{P}$ single-bond correlation spectra (HMQC) were obtained using Bax's pulse sequence (42). The GARP
sequence (43) at the carbon or phosphorus frequency was used as a composite pulse decoupling during acquisition. The pulse sequence used for $^1$H-detected heteronuclear relayed spectra (HMOC-HOHAAHA) was that of Lerner and Bax (44) and for HMBC, that of Bax and Summers (45).

**Macrophage culture.**

Peripheral blood macrophages were isolated from a healthy adult horse using Percoll (Sigma, St Louis, MO) and resuspended at $5 \times 10^6$ cells/ml in Dulbecco-modified Eagle medium (DMEM; Gibco-BRL, Grand Island, NY) with 10% fetal calf serum (FCS) supplemented with penicillin (100 µg/ml), streptomycin (80 µg/ml) and gentamicin (20 µg/ml). 1 ml of the suspension was placed in each well of a 24-well tissue culture plate, with 10 wells used for each treatment. Following overnight (18 h) incubation at 37°C in a humidified atmosphere containing 5% CO$_2$, nonadherent cells were removed by washing the plate with warm DMEM-FCS with or without antibiotics, for *R. equi* and lipoglycan treatments, respectively. Following 4 to 6 h of culturing, $2.5 \times 10^6$ cells remained in each well and were used for either bacterial infection or lipoglycan stimulation.

**Bacterial infection and lipoglycan stimulation.**

Initial studies were done to optimize lipoglycan concentration. Infection of horse macrophages with *R. equi* was carried out as described by Giguère and Prescott (46). A multiplicity ratio of five bacteria per macrophage was used. After 40 min of incubation to allow phagocytosis, the macrophages were washed three times and then incubated in DMEM-FCS with antibiotics, in order to kill extracellular bacteria and to prevent further extracellular bacterial growth and reinfection of macrophages. For the time course study of lipoglycan stimulation, 10 µl of lipoglycan in phosphate buffered saline, pH 7.2 (PBS) with
200 µg/ml polymyxin were added to each well to yield a final lipoglycan concentration of 5 µg/ml. Macrophages were harvested for RNA extraction at 0.7, 4, 8, 12 and 24 h following lipoglycan stimulation or exposure to R. equi for infection. Thus, the first time point for the R. equi infected macrophages represents the time point at which infection was stopped by replacement with DMEM-FCS medium containing antibiotics. Untreated macrophages cultured under the same conditions were used as controls.

**Quantitation of cytokine mRNA expression by real time PCR.**

Macrophages harvested at the times described above were centrifuged at 400 x g for 5 min, washed with warm PBS, and total RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen Inc., CA). All RNA samples were treated with amplification grade DNAase I (Gibco, ON, Canada) to remove any traces of genomic DNA contamination. 1 µg of total RNA was used for cDNA synthesis in a volume of 25 µl using the Thermoscript RT-PCR System kit (Gibco). After cDNA synthesis by reverse transcription, the reaction was diluted to 80 µl. Gene specific primers and internal oligonucleotide probes for IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-18, TNF-α and IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were selected based on equine cDNA sequences (Table 3) using the Primer Express Software (Applied Biosystems, Foster City, CA). The internal probes were labeled at the 5’ end with the reporter dye 6-carboxyfluorescein, and at the 3’ end with the quencher dye 6-carboxytetramethyl-rhodamine. Amplification of 2 µl of cDNA was performed in a 25 µl PCR reaction containing 900 nM of each primer, 250 nM of Taqman probe and 12 µl of TaqMan Universal PCR Mastermix (Applied Biosystems). Amplification and detection were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with initial incubation steps at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each
sample was assayed in triplicate and the mean value was used for comparison. Samples without cDNA were included in the amplification reactions to determine background fluorescence and check for contamination. cDNA from 24 h Concanavallin A-stimulated equine blood mononuclear cells was used as positive control. To account for variation in the amount and quality of starting material, all the results were normalized to G3PDH expression. Relative quantitation between samples was achieved by the comparative threshold cycles method and is reported as the \( n \)-fold difference relative to cytokine mRNA expression in unstimulated macrophages (47).

**Results**

**Purification of the lipoglycan fraction of *R. equi*.**

In order to minimise contamination with extractable lipids (particularly phosphatidylinositol dimannoside, PIM\(_2\)) and to maximise yields, *R. equi* cells were delipidated and permeabilised prior to extraction with hot water-phenol (28, 48). The carbohydrate profile for the HIC purification of a crude hot water-phenol extract of *R. equi* 28\(^+\) is shown in Figure 1a. Similar results, with a reduced proportion of PK2, were obtained for the purification of extracts of *R. equi* 103\(^+\) and ATCC 6939.

Initial elution of the column with equilibration buffer removed hydrophilic contaminant material which includes nucleic acids, polysaccharides and proteins (PK1, Figure 1a). Two carbohydrate-containing peaks eluted within the propanol gradient (PK2 and PK3, Figure 1a). Each fraction was analysed by SDS-PAGE and modified silver staining which revealed PK3 to contain lipoglycan (ReqLAM). However, the final few fractions were contaminated with a low molecular weight mannose-containing lipid thought to be phosphatidylinositolmannosides (PIM) larger than PIM\(_2\). Moreover, the fractions
which composed PK2 were not revealed using this staining method and were subsequently shown to contain a very high molecular weight polysaccharide, the composition of which varied between strains, and no fatty acids. Consequently PK2 was thought to derive from capsular polysaccharide and as such was not studied further. Lipoglycan-containing fractions (PK3) were pooled, excluding those containing the low molecular weight contaminant. Following dialysis and lyophilisation, HIC purified lipoglycan typically represented 0.4% of the dry cell weight extracted.

Subsequently, NMR studies revealed that PK3 was still contaminated by small mannose-containing molecules. The PK3 lipoglycan was further purified by gel filtration in presence of sodium deoxycholate buffer. Gel filtration chromatographic profile shows two peaks, I and II (Figure 1b). Peak I was tentatively assigned to ReqLAM, based on its electrophoretic mobility on SDS-PAGE (Figure 2). However, ReqLAM shows an electrophoretic behaviour slightly different from those of *M. tuberculosis* and BCG ManLAM, in agreement with MALDI–TOF MS spectrum showing a broad peak centred at m/z 8000 (data not shown) indicating a molecular weight for the most abundant ReqLAM molecular species of 8 kDa. A molecular weight around 17 kDa was established for the BCG ManLAM (15, 38).

**Structural characterisation of *R. equi* LAM.**

**ReqLAM polysaccharidic backbone**

The ReqLAM (strain 28°) was first hydrolysed (2M TFA for 2h at 110°C) and the resulting monosaccharides were derivatized with APTS and analysed by CE (12). The electropherograms (Figures 3a and 3b) are dominated by one peak assigned to Man-APTS and a peak of lower intensity attributed to Ara-APTS in both running electrolytes used. From peak integration, the relative composition of the ReqLAM polysaccharide
backbone was 86 % Manp and 14 % Araf. Glycerol and inositol, components of a putative phosphatidylinositol anchor, were also detected by GC analysis. These data suggested that the *R. equi* lipoglycan may represent an unusual variant on the LAM archetype (ReqLAM).

Western blotting of the lipoglycan preparations with polyclonal anti-LAM antibody (raised against ManLAM from *M. tuberculosis*, strain H37Rv) demonstrated a weak positive cross-reaction only with material from *R. equi* 103+ (results not shown). This was further indication that the lipoglycan from *R. equi* represents a structural variant of the mycobacterial LAM.

In order to investigate the glycosidic linkages present in the ReqLAM, the sample was deacylated, permethylated, hydrolysed and derivatised as alditol acetates. The various methylated alditol acetates were routinely identified by GC-MS as terminal (t-), 2-, 2,6- and 6-O-linked mannopyranose residues in 13%, 12%, 27% and 32% respective abundance with traces of 4-O-linked mannose. The majority of the arabinose present (11%) determined as being in the furanose form, was detected as terminal residues. From these data and by analogy to the BCG and *M. tuberculosis* ManLAM structure, a backbone of 6-O-linked Manp can be tentatively advanced. In addition, the presence of 2-O-linked Manp suggested that the branches may contain 2-O-linked Manp residues. The t-Manp and t-Araf cap the 2-O-linked Manp or may be directly linked to the C-2 of linear 6-O-linked Manp. The presence of t-Manp residues was confirmed by lectin blotting of lipoglycan material using Concanavalin A (ConA) (not shown).

The absence of 5-O-linked Araf and consequently of mannose caps which typify the BCG and *M. tuberculosis* ManLAM was supported by the following experiments. ReqLAM was submitted to mild acidic hydrolysis (0.1M HCl for 30 min at 110 °C) followed by APTS derivatization and CE analysis (48). Mild hydrolysis leads to selective cleavage
of Araf links and consequently to the release of mannose caps with one Ara unit at the reducing end (12, 14, 38). The electropherogram (Figure 3c) showed mainly two peaks assigned to Ara-APTS and Man-APTS supporting, as expected, the absence of (t-Manp→Araf) sequence.

We then investigated whether t-Manp and t-Araf cap the 2-O-linked Manp or are directly linked to the C-2 of linear 6-O-linked Manp. ReqLAM was submitted to acetolysis, which allows preferential cleavage of 6-O-linked hexopyranose and the Araf linkages (38), followed by deacetylation, APTS derivatization and CE analysis. Three peaks of interest (Figure 3d) were observed assigned to Ara-APTS, Man-APTS and Manpα1→2Man-APTS derivatives in the relative abundance of 20 %, 45 % and 35 %. This result indicated the existence of side chains composed by a single Manp capped selectively by t-Araf and not by t-Manp; This implied that t-Manp is linked directly to the 6-O-linked backbone. Moreover, a percentage of branching of 44% was estimated by the ratio of Manp α1→2Manp-APTS / Manp-APTS + Manp α1→2Manp-APTS. This was in agreement with the value of 46% branching obtained from alditol acetate analysis as the ratio of 2,6-O-linked Manp (27%) / 2,6-O-linked Manp (27%) + 6-O-linked Manp (32%).

Purified ReqLAM from strain 28+ was then analysed by NMR. The 1D 1H NMR anomeric zone (Figure 4b) was composed of a multitude of signals, assignment of which required more sophisticated experiments. A complete NMR strategy, involving two-dimensional 1H-1H COSY, HOHAHA with different mixing times, ROESY, 1H-13C HMQC, HMQC-HOHAHA and HMBC, was undertaken in order to characterise the different spin systems which compose the ReqLAM (Table 1) and to determine the sequence of these monosaccharidic units. This strategy was realised through NMR analysis of the ManLAM (unpublished results) and ManAM (49) of the mycobacterial strain M. bovis BCG in D2O and ManLAM of BCG (13) and M. tuberculosis (14) in Me2SO-d6.
The different anomeric protons were characterised by $^1$H-$^1$H HOHAHA (Figure 5b) and $^1$H-$^{13}$C HMQC experiments, evidencing 9 spin systems, noted from I to IX in Table 1. The anomeric area of the $^1$H-$^{13}$C HMQC spectrum (Figure 5c) highlights 5 anomeric C/H pairs: at 104.2/5.21 (I$_1$), at 112.3/5.20 (II$_1$), at 101.3 with protons at 5.15 (III$_1$), 5.12 (IV$_1$), and 5.10 (V$_1$), at 105.2/5.06 (VI$_1$), at 102.4/4.96 (VII$_1$), and at 102.6 with protons at 4.93 (VIII$_1$) and 4.92 (IX$_1$).

The spin system II was unambiguously assigned to t-$\alpha$-Araf. The $\alpha$ anomeric configuration was based on the C-1 chemical shifts ($\delta$C-1 Araf, $\alpha$:109.2 / $\beta$:103.1). This spin system could be defined up to H-5/C-5. The different chemical shifts proved that this unit was terminal. The furanose ring was deduced from the C-4 chemical shift ($\delta$ 86.7) and from the intense cross peak observed on the $^1$H-$^{13}$C HMBC spectrum between the H-1 ($\delta$ 5.20) and C-4 ($\delta$ 86.7) and between C-1 ($\delta$ 112.3) and H-4 ($\delta$ 4.12) (not shown). The spin systems I, III and IV typified 2-O-linked-$\alpha$-Manp. Indeed, the C-2 were found at 80.4 for system I and at 81.7 ppm for system III and IV on the 2D $^1$H-$^{13}$C HMQC-HOHAHA spectrum (not shown). The pyranose ring of system I was confirmed by the cross peak observed on the $^1$H-$^{13}$C HMBC between the H-1 ($\delta$ 5.21) and C-5 ($\delta$ 76.3). Concerning systems III and IV, their $^{13}$C resonances were identical, highlighting a glycosylated C-6 position ($\delta$ 69.9). They were assigned to 2,6-O-linked $\alpha$-Manp. The chemical shifts of systems III and IV were found very similar to the ones described for the 2,6-O-linked $\alpha$-Manp of the mycobacterial AM (49). System V had approximately the same anomeric chemical shifts ($\delta$ C-1 at 101.3 and $\delta$ H-1 at 5.10) as systems III and IV (Table 1) and was then attributed to $\alpha$-Manp. However, the C-2 was not found around 82 ppm on the 2D $^1$H-$^{13}$C HMQC-HOHAHA spectrum but at 73.2 ppm showing that this spin system is not C-2 glycosylated. This chemical shift was similar to the one of the C-2 of system VI ($\delta$ 73.1) which was attributed to terminal $\alpha$-Manp (t-$\alpha$-Manp). Spin system V was then tentatively
attributed to the 4-O-linked α-Manp observed by the permethylation analysis. Then, systems VII, VIII, IX were found to correspond to 6-O-linked α-Manp by analogy to the mycobacterial LAM (unpublished results). Glycosylation in position C-6 was proved by the C-6 chemical shift (δ 68.7).

The glycosylated carbons were defined from the $^{13}$C chemical shifts. The next step was to determine the sequence of units from $^1$H-$^1$H ROESY and $^1$H-$^{13}$C HMBC experiments (data not shown). The saccharidic linear core was determined to consist of 6-O-linked α-Manp. The same types of correlations were observed concerning systems VII, VIII, IX in one side and systems III, IV in the other side: correlations between H-1 and H-6/6’ on the $^1$H-$^1$H ROESY spectrum and between H-1 and C-6 on the $^1$H-$^{13}$C HMBC spectrum. These correlations proved independent domains of 6-O-linked Manp and 2,6-O-linked Manp. T-Manp (VI) and 2-O-linked Manp (I) were observed in position 2 of the 2,6-O-linked Manp (IV) from correlations between H-1(VI)/H-2(IV) and H-1(I)/H-2(IV) on the ROESY spectrum and between H-1(VI)/C-2(IV), C-1(VI)/H-2(IV) and H-1(I)/C-2(IV), C-1(I)/H-2(IV) on the HMBC spectrum. Then, position 2 of 2-O-linked Manp (I) was shown to be glycosylated by t-α-Araf (II) by correlations between H-1(II)/C-2(I) on the HMBC spectrum.

In summary, the alditol acetate, permethylation, NMR and CE data taken together allow us to propose the three following structural features for the polysaccharidic backbone (Figure 7): i) a domain composed of a linear chain of 6-O-linked α-Manp, ii) linear 6-O-linked-α-D-Manp chains with side chains located at the C2 composed of a α-Manp unit and iii) linear 6-O-linked-α-D-Manp chains with side chains located at the C2 composed of a t-Arafα1→2Manp unit. Indeed, t-α-Araf was evidenced by NMR as only capping the 2-O-linked α-Manp in agreement with the permethylation data showing that the percentage of 2-O-linked Manp (12.3%) was similar to the percentage of t-Araf.
Moreover, the ratios Ara-APTS / Manpα1→2Man-APTS measured by CE (20/35) or (2-O-linked Manp / t-Manp+2-O-linked Manp) (12.3/12.6+12.3) allow to deduce that one side chain in two was capped with t-α-Araf. Moreover, the alditol acetate and permethylation data are consistent with the same structure being present in the ReqLAM from \textit{R equi} strain 103.

**Phosphatidyl-myo-inositol anchor acylation state**

The phosphatidyl-myo-inositol anchor structure was investigated from 1D and 2D phosphorus NMR. The 1D\(^{31}\)P spectrum of ReqLAM exhibited broad unresolved signals in D\(_2\)O (not shown) consistent with multi-acylated ReqLAM. Indeed, the 1D \(^1\)H NMR spectrum (Figure 4a) evidenced the presence of fatty acids by the signals at 0.88 and 1.30 ppm. This was consistent with the presence of fatty acids as analysed by GLC. The predominant fatty acids found were hexadecanoic acid (C16:0) (56%) and 10-methyl octadecanoic acid (tuberculostearic acid, t19:0) (19%), while heneicosanoate (C21:0), octadecenoic acid (C18:1), heptadecanoic acid (C17) and a C16-branched fatty acid (possibly 10-methylhexadecanoic acid) are present in smaller amounts (11%, 6%, 4% and 5%, respectively). The fatty acid composition of the lipoglycan reflected that of the whole bacterial cells (data not shown) although a reduction in the relative proportion of unsaturated fatty acids was noted, as has been observed previously for other LAM-like lipoglycans of the mycolata (22,25,26).

As ReqLAM exhibited broad unresolved signals in D\(_2\)O, no connectivities between phosphate and protons could be obtained by 2D \(^1\)H-\(^{31}\)P HMQC and HMQC-HOHAHA. Recently, it was shown that Me\(_2\)SO-d\(_6\) is a suitable solvent for recording high resolution 1D \(^{31}\)P NMR spectra of multi-acylated mycobacterial ManLAM (13). 1D \(^{31}\)P spectrum of ReqLAM dissolved in Me\(_2\)SO-d\(_6\) mainly showed three signals at δ 1.83, δ 3.50, and δ 4.05.
From their chemical shifts and by comparison with those of the $^{31}$P signals observed in the 1D $^{31}$P spectrum of the *M. bovis* BCG cellular ManLAM (13; Figure 6), two of these signals were tentatively assigned to P3 ($\delta$ 1.83) and P5 ($\delta$ 3.50).

The $^{31}$P resonance assignments were confirmed with help of 2D $^{1}$H-$^{31}$P NMR spectroscopy. The $^{1}$H-$^{31}$P HMQC-HOHAHA spectrum of ReqLAM (not shown) exhibited a complex panel of correlations. P3 showed correlations with downfield resonances at $\delta$ 5.13 in $F_2$ dimension, which were assigned to methine protons H-2 of di-acylated glycerol according to previous results (11, 13, 14). A similar downfield correlation was not observed for P5; instead the Gro H-2 resonance is superimposed with the Gro H-3/H-3', indicating lack of acylation of O-2 in this minor species. So, only P3 corresponds to 1,2-di-acyl-3-phospho-sn-glycerol unit, P5 corresponding to 1-acyl-2-lyso-3-phospho-sn-glycerol unit. The inspection of the different cross peaks (chemical shifts, multiplicity of the signals and $^{3}J_{HH}$ coupling constants), by comparison with the $^{1}$H-$^{31}$P HMQC-HOHAHA of *M. bovis* BCG cellular ManLAM (11,13,14) allowed their attribution to the different protons of glycerol and myo-inositol spin systems (Table 2). From these data, it can be proposed that P3 typified a di-acylated Gro anchor. Although the P5 signal was weak, we were able to deduce the absence of an acyl residue on the C2 of the Gro. P3 and P5 corresponded to phosphatidyl-my/o-inositol anchor devoid of fatty acid on their my/o-Ins unit.

The linkage of the backbone to the myo-inositol anchor was postulated by analogy to the mycobacterial ManLAM anchor structure. In the same way, $\alpha$-Manp unit was postulated in position 2 of the phosphatidyl-my/o-inositol anchor based on the characterization of PIM$_2$ by MALDI (Figure 8) as PIM$_2$ are considered as LAM precursors.

*Biological activities of the lipoglycan.*
ReqLAM showed a strong reaction on Western blotting with Hypermune-RE, an anti-\textit{R. equi} antiserum produced commercially for the prophylactic treatment of foals (data not shown) confirming the antigenicity of the lipoglycan \textit{in vivo}. Moreover, ReqLAM gave reactions of varying intensity with four out of four convalescent sera from foals that had recovered from \textit{R. equi} infection (data not shown). Mycobacterial LAM, lipomannan (LM) and PIM have been shown to interact with mannose binding protein (MBP) (50, 51). ReqLAM was applied to a column of immobilised MBP and retention of lipoglycan material was monitored by electrophoresis followed by blotting and probing with ConA (23). ReqLAM was retained by the MBP (Figure 9), eluting only after the application of buffer containing EDTA to disrupt calcium dependent interactions between the lipoglycan and the MBP carbohydrate recognition domain.

Further study of the biological activities of the ReqLAM focused on its ability to stimulate cytokine production by equine peripheral blood macrophages. Cytokines selected for study were representative of the major cytokines produced by macrophages and indicative of the development of different Th responses. In preliminary experiments, various concentrations (1, 5, 10 or 20 µg/ml) of ReqLAM were used to stimulate equine macrophages and TNF-\(\alpha\) and IL-12 p40 mRNA expression was quantitated. For both cytokines, ReqLAM at 5 µg/ml induced the highest level of mRNA expression (data not shown) and therefore this concentration was used in subsequent experiments. Treatment of 5 µg/ml ReqLAM with 200 and 1,000 µg/ml of polymyxin showed no inhibitory effect at either concentration on TNF-\(\alpha\) and IL-12 p40 mRNA cytokine production over that caused by polymyxin alone (data not shown). This confirmed that ReqLAM was not contaminated by endotoxin.

Subsequently we examined cytokine induction in horse macrophages by virulent \textit{R. equi} or by ReqLAM (5 µg/ml), at various time points. Quantitative expression of cytokine
mRNA at different times following infection or lipoglycan stimulation are shown in Figures 10 and 11. For all the representative inflammatory cytokines assayed, with the exception of TNF-α, ReqLAM induced a greater early response than the whole cells (Figure 10) but that at later time points the overall profile of cytokine induction was similar for both R. equi and ReqLAM, albeit with typically higher response to the R. equi whole cells. Likewise, for the representative regulatory cytokines assayed, ReqLAM again induced a greater early response compared to the whole cells (Figure 11). At later time points in the assay of the regulatory cytokines, the whole cells typically induced a greater response than the ReqLAM, with the exception of IL-18 which was not induced by either stimulant (Figure 11B). The ability of ReqLAM to induce production of both inflammatory and regulatory cytokines was also confirmed in preliminary experiments with ReqLAM from strain 103+ (data not shown).

Discussion

The R. equi lipoglycan ReqLAM has a structure related to, but clearly distinct from, that of mycobacterial ManLAM from BCG and M. tuberculosis (7). ReqLAM is smaller than ManLAM; MALDI-TOF mass spectrometry revealed the molecular mass of ReqLAM to be centred around 8 kDa. Using a similar method, Venisse et al. (15) measured the average size of ManLAM of M. bovis BCG as 17.4 kDa and that of LM as 6 kDa (unpublished data). The broad diffuse band observed by SDS-PAGE and the spread of molecular mass revealed by mass spectrometry indicate that like ManLAM, ReqLAM is heterogeneous in size.

The small size of ReqLAM as compared with ManLAM is consistent with the reduced arabinose content, approximately 11% of the carbohydrate moiety compared with 55% for BCG ManLAM (15). The α1-6 mannan backbones of ManLAM of M. tuberculosis
Erdman and *M. bovis* BCG were demonstrated to be highly branched although the degree of branching and size were heterogeneous (52, 53). Results of permethylation and CE analysis indicate that ReqLAM has a similar α1-6 mannan backbone, with approximately 44% of branching. Acetolysis and study of derivatised products by CE, revealed the branches to contain one 2-O-linked mannose residue either as t-α-Manp or capped by t-α-Araf. The majority of the arabinose residues were detected by permethylation analysis as t-αAraf in agreement with the absence of mannose caps which typify the BCG and *M. tuberculosis* ManLAM (12). A structural model of ReqLAM can be proposed here, consisting of a similar mannan backbone to that of ManLAM but in which single arabinose residues decorate the mannan backbone instead of elaborate arabinan branches (Figure 7). So, in agreement with its molecular weight of 8 kDa instead of 17 kDa for the ManLAM, the arabinan domain of the ReqLAM is restricted to single α-Araf capping the 2-O-linked α-D-Manp. This structural feature reveals that the biosynthesis of an extensive arabinan domain comparable to that of mycobacterial ManLAM (by addition of 5-O-linked α-Araf) does not occur in *R. equi*, probably due to the absence of a 5-arabinosyl transferase. The reduced arabinose content of the ReqLAM may explain the relatively weak or negative cross-reactions of the lipoglycan with a polyclonal anti-LAM antiserum as the arabinose residues appear to be the principal antigenic determinants in ManLAM (7, 54). However the ReqLAM does elicit antibody production in foals and adult horses, as judged by its reaction with hyperimmunune and convalescent antisera.

The presence of terminal mannose units within the ReqLAM structure (Figure 7) may have considerable relevance with respect to the biological significance of this lipoglycan, notably through interaction with components of the innate immune response. The interaction of ReqLAM with recombinant MBP (Figure 9) is consistent with the previously observed interaction of MBP with a variety of mannoconjugates including
mycobacterial ManLAM, LM and PIM (51) and the lipomannan of Micrococcus luteus (55). Binding of MBP by ReqLAM in vivo may activate complement C3b deposition onto R. equi via the lectin pathway (56) thereby helping promote the previously described (3) complement-receptor Mac-1 mediated uptake of R. equi into macrophages. ReqLAM may be able to bind other collectins as both LM and ManLAM have been identified as ligands for human pulmonary surfactant protein A (57) and human pulmonary surfactant protein D binds ManLAM (58), although only binding of the former surfactant promotes binding of M. tuberculosis to phagocytes. Equine pulmonary surfactant protein A binds mannose (59) and equine pulmonary surfactant protein D has been shown to bind both mannose and PI (60). Thus ReqLAM may bind either or both of these surfactants in the foal lung. LAM may also influence mycobacterial uptake into host cells by interacting with other receptors including macrophage mannose receptors (8, 17-19) and similar interactions may be possible for ReqLAM. Entry via some pathways, notably macrophage mannose receptors (20), may influence intracellular survival by circumventing the activation of macrophage antimicrobial responses. Consequently, the multifaceted potential of ReqLAM for promoting bacterial entry into host cells warrants further investigation.

We show here that the pro-inflammatory and immune cytokine response of equine macrophages to ReqLAM largely parallels the response to infection with live virulent R. equi. Activation of resident macrophages is one of the earliest responses to microbial invasion, with macrophage-derived cytokines playing a critical role in initiating the inflammatory response as well as in regulating the immune response. The results shown here suggest that much of the early macrophage cytokine response occurring after infection with R. equi can be attributed in part to its ReqLAM component. The effect of ReqLAM observed herein was not the result of contamination with LPS, since the effect could not be inhibited by use of polymyxin, which inactivates LPS. These findings extend
and complement the results of earlier studies with infected murine macrophages, which failed to demonstrate any cytokine response which could be specifically attributed to possession of the virulence plasmid (46).

The ManLAM of *M. tuberculosis* has been shown to produce a wide spectrum of immunomodulatory functions *in vitro*, but the biological implications of these effects are still largely undefined. These effects include suppression of T-cell proliferation, inhibition of interferon (IFN)-γ-induced functions including microbicidal activity, scavenging of cytotoxic free oxygen radicals and complement activation (7, 8, 61). However, only LAM lacking mannose caps (PILAM and AraLAM) has been found to induce significant TNF-α and IL-12 expression by human and murine macrophages (7, 8). Our studies with ReqLAM also show the marked effect of this lipoglycan on macrophage cytokine induction. It is intriguing that ReqLAM induces both pro-inflammatory cytokines (Figure 10) and macrophage deactivating cytokines (Figure 11). The lack of immediate TNF-α response to ReqLAM was a notable difference from the immediate TNF-α response to whole cells of *R. equi* (Figure 10D). Darrah and others (62) have shown that both TNF-α and IFN-γ are required to activate macrophages in order to kill *R. equi* by peroxynitrite.

Finally, the observation of a marked optimal dose effect of ReqLAM on selected cytokine mRNA transcription (data not shown) was unexpected. Further study is needed to support the intriguing possibility that ReqLAM could produce an immunosuppressive effect through an intracellular bacterial load effect on macrophage cytokine expression. Possible effects attributable to ReqLAM thus need to be considered in future studies of the cytokine responses of equine macrophages to infection with virulent and avirulent *R. equi*.

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References


Footnotes

1 The abbreviations used are:

APTS, 1-aminopyrene-3,6,8-trisulfonate; Araf, arabinofuranose; BHI, brain heart infusion; C₁₆:₀, hexadecanoate, tC₁₉:₀, tuberculostearate; CE, capillary electrophoresis; Con A, concanavalin A; COSY, correlated spectroscopy; DMEM, Dulbecco-modified Eagle medium; DOC, deoxycholate; FCS, 10% fetal calf serum; GC-MS, gas chromatography mass spectrometry; GLC, gas liquid chromatography; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Gro, glycerol; HIC, hydrophobic interaction chromatography; HMBC, heteronuclear multiple bound correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; IL, interleukin; INF, interferon; Ins, inositol; LAM, lipoarabinomannan; LM, lipomannan; LPS, lipopolysaccharide; MALDI-TOF, matrix assisted laser desorption ionisation-time of flight; Manp mannopyranose; ManLAM, LAM with mannosyl extensions; MBP, mannose binding protein; MHC, major histocompatibility complex; ¹H, ¹³C and ³¹P NMR, proton, carbon and phosphorous nuclear magnetic resonance; PBST, 5% (w/v) skimmed milk in phosphate buffered saline containing 0.05% (w/v) Tween 80; PI or Ac₂Pl, phosphatidylinositol; PILAM, LAM with phosphoinositide extensions; PIM, phosphatidylinositol mannosides; PIM₂, phosphatidylinositol dimannosides; Ac₃PIM₂ and Ac₃PIM₂, PIM₂ with 3 and 4 fatty acids appendage; PTFE, polytetrafluoroethylene; ReqLAM, lipoglycan of Rhodococcus equi; ROESY, Rotating frame Overhauser Effect spectroscopy; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; t, terminal; TFA, trifluoroacetic acid; TNF-α, tumour necrosis factor alpha.
Figure Legends

Figure 1. Purification of the lipoglycan fraction from *R. equi* 28+. (a) Crude phenol extract was loaded to the HIC column and washed through until fraction 10 to remove hydrophilic contaminant material (PK1). Gradient elution with increasing concentrations of propanol (15% to 65% v/v; fractions 10 to 39) was used to recover two peaks of interest (PK2 and PK3). Fractions 40 to 47 were eluted with 65% v/v propanol. Fractions (4.2 ml) were monitored by assay for carbohydrate (♦). (b) Lipoglycan-containing fractions (PK3) were pooled, dialysed, lyophilised and loaded on a Bio-Gel P-100 gel permeation column eluted with a DOC-containing Tris buffer. Fraction I contained ReqLAM while fraction II contained a contaminant.

Figure 2. SDS-PAGE analysis of ReqLAM and related *M. bovis* BCG lipoglycans.

Lane 1, LM from *M. bovis* BCG; Lane 2, ManLAM from *M. bovis* BCG; Lane 3, PK3 fraction (ReqLAM) from *R. equi* strain 28+. The gel was stained with a silver stain containing periodic acid.

Figure 3: CE-LIF electropherograms of monosaccharide and oligosaccharide APTS derivatives resulting from (a and b) total or (c) partial acid hydrolysis and (d) acetylation of ReqLAM from strain 28+. CE-LIF electropherograms of figures 3a, c and d were carried out with an applied voltage of -20 kV and using acetic acid 1% (w/v), triethylamine 30 mM in water (pH 3.5) as running electrolyte. CE-LIF electropherogram of figure 3b was recorded in normal mode, with an applied voltage of +25 kV and borate buffer 20 mM (pH 9.2) as running electrolyte. A, Ara-APTS; M, Man-APTS, MM, Manpα1→2Man-APTS. Peaks labelled with asterisks (*) arise from the APTS reagent.
Figure 4: Expanded regions of the 1D $^1$H spectra ($\delta^1$H, 0.5-7.0) (a) and anomeric zone ($\delta^1$H, 4.8-5.4) (b) of the ReqLAM strain 28$^+$ in D$_2$O at 313 K. The scan number was 128.

Figure 5: Expanded regions of the 1D $^1$H spectrum ($\delta^1$H, 4.85-5.30) (a), of the 2D 82 ms $^1$H-$^1$H HOHAHA spectrum ($\delta^1$H, 4.85-5.30 and 3.60-4.30) (b) and of 2D $^1$H-$^13$C HMQC spectrum ($\delta^1$H, 4.85-5.30 and $\delta^{13}$C, 95-115) (c) in D$_2$O at 313K of the ReqLAM, strain 28$^+$. I, 2-$\alpha$-Manp; II, t-$\alpha$-Araf; III, IV, 2,6-$\alpha$-Manp; V, 4-$\alpha$-Manp; VI, t-$\alpha$-Manp; VII, VIII and IX, 6-$\alpha$-Manp.

Figure 6: Expanded regions of the 1D $^{31}$P spectra ($\delta^{31}$P, 0-6) of the BCG ManLAM (a) and of the ReqLAM, strain 28$^+$ (b) in Me$_2$SO-$d_6$ at 343 K. Signals labelled with asterisk (*) correspond to non attributed signals.

Figure 7: Structural model of ReqLAM. Ins-P-Gro-Ac$_2$ = phosphatidyl-my-ino-sitol anchor, with Ac corresponding to palmitic and tuberculostearic acids (C$_{16}$/C$_{19}$ 7/3 for 103$^+$ ReqLAM and 6/2 for 28$^+$ ReqLAM). The diacyl form represents the major acyl form. The linkage of the backbone to the myo-inositol anchor was postulated by analogy to the mycobacterial ManLAM anchor structure. In the same way, $\alpha$-Manp unit was postulated in position 2 of the phosphatidyl-my-ino-sitol anchor based on the characterization of PIM$_2$ which are considered as LAM precursors.

NMR data indicate two domains, one corresponding to 6-O-linked $\alpha$-Manp and the other to 2,6-O-linked $\alpha$-Manp. From the molecular weight (8 kDa), the percentage of branching (44%) and the fact that one side chain in two was demonstrated capped with t-Araf, m and
n could be estimated to 6 and 8, respectively. X corresponds to the terminal unit, which could be assigned to either t-Araf or t-Manp.

**Figure 8: Negative MALDI mass spectrum of the lipid extract of R. equi strain 28⁺.**

The peaks correspond to Ac₂Pl acylated predominantly by C₁₆/C₁₇ (823), C₁₆/C₁₈ (837), C₁₆/C₁₉ (851) and C₁₇/C₁₉ (863), Ac₃PIM₂ acylated predominantly by 2C₁₆/C₁₇ (1385) and 2C₁₆/C₁₉ (1413) and Ac₄PIM₂ acylated predominantly by 3C₁₆,C₁₇ (1623) and 3C₁₆,C₁₉ (1651).

**Figure 9: Interaction of ReqLAM with recombinant mannose bindinding protein.**

ReqLAM from strain 103⁺ was loaded onto a commercial immobilised mannose binding protein column and washed with binding buffer. Material retained by the mannose binding protein was subsequently eluted with the manufacturers elution buffer. Fractions were analysed by SDS-PAGE followed by lectin blotting with concanavilin A. Lanes 1-4, fractions 1-4 eluted with wash buffer; Lanes5-8, fractions 1-4 from elution with elution buffer. The positions of the protein molecular weight standards (kDa) are marked at the right hand side.

**Figure 10: Inflammatory cytokine mRNA expression in equine macrophages infected with R. equi or stimulated with ReqLAM.** Equine macrophages were infected with virulent R. equi (solid bars) or incubated with 5 µg/ml of ReqLAM from strain 28⁺ (striped bars). The induction of (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF-α mRNA expression was quantitated by real-time PCR. Results are reported as the n-fold difference relative to cytokine mRNA expression in unstimulated macrophages.
Figure 11: Regulatory cytokine mRNA expression in equine macrophages infected with *R. equi* or stimulated with ReqLAM. Equine macrophages were infected with virulent *R. equi* (solid bars) or incubated with 5 μg/ml of ReqLAM from strain 28+ (striped bars). The induction of (A) IL-10, (B) IL-18, (C) IFN-γ and (D) IL-12p40 mRNA expression was quantitated by real-time PCR. Results are reported as the n-fold difference relative to cytokine mRNA expression in unstimulated macrophages.
Table 1. Proton and carbon chemical shifts of strain 28+ ReqLAM. Chemical shifts were measured at 313 K in D$_2$O and are referenced relative to internal acetone at $\delta_H$ 2.225 and $\delta_C$ 34.00.

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<tr>
<td>VII</td>
<td>6-O-linked $\alpha$-Manp</td>
<td>102.4</td>
<td>73.1</td>
<td>74.1</td>
<td>69.9</td>
<td>76.3</td>
<td>68.7</td>
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<td>4.96</td>
<td>4.15</td>
<td>4.05</td>
<td>4.05</td>
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<tr>
<td>VIII</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>
Table 2. $P_3 / P_5$ Gro and myo-Ins $^1$H chemical shifts of strain 28\textsuperscript{r} ReqLAM in Me$_2$SO-$d_6$ at 343K.

<table>
<thead>
<tr>
<th></th>
<th>myo-Ins</th>
<th></th>
<th>Gro</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-1</td>
<td>H-2</td>
<td>H-3</td>
<td>H-4</td>
</tr>
<tr>
<td>P-3</td>
<td>4.01</td>
<td>4.16</td>
<td>3.26</td>
<td>3.46</td>
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<tr>
<td>P-5</td>
<td>4.00</td>
<td>4.19</td>
<td>nd</td>
<td>nd</td>
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Table 3. Oligonucleotide primer and probe sequences for amplification of various equine cytokines and the G3PDH control.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer/probe</th>
<th>Sequence 5’-3’</th>
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</thead>
<tbody>
<tr>
<td>G3PDH</td>
<td>Forward</td>
<td>GGTGGAGGCCAAAAGGGTCTAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTCAGCCCAACTCAAAACAT</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TCTCTCGCTCTTCGCTGATGCC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>TGAAGGGCAGCTTCCAAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGAGAATTGAAGCTGGATGC</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TGGACCTCAGCTCCATTGGCGA</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>CCCCTGAACCAACTGCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGTTGTGTCTTCAGCCAACACTA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CCTGCTAGCTGAGCTGATTTCAGA</td>
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<tr>
<td>IL-8</td>
<td>Forward</td>
<td>CGGTGCCAGTGATCAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGCCCAACTCCTCAATACCTA</td>
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<tr>
<td></td>
<td>Probe</td>
<td>CGCACTCCAAACCTTTCAATCCAAACAT</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward</td>
<td>GTGGAGAGATGATCCAGTTTTACCTA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AGTTTCAGTGCTCCTGAGTCTCA</td>
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<tr>
<td></td>
<td>Probe</td>
<td>TGCCCCAGCAGTGACACCACG</td>
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<tr>
<td>IL-12p40</td>
<td>Forward</td>
<td>TGCTGTTCAACAAGCTCAATGATGA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GGGTGGGCTCTGGTTGATGA</td>
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<tr>
<td></td>
<td>Probe</td>
<td>CTACACCAGGCGCTCTGATCCAGG</td>
</tr>
<tr>
<td>IL-18</td>
<td>Forward</td>
<td>TGAAGCTGAAAAACCTTGGA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TTGGAGGTCCAATTTTGATGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CAGATTACTTTGGCAGGCTAATCCAAACTCTCA</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward</td>
<td>AAGTGAATCATAAGTGATGAATGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGAAATGGATCTGACTCCTTCA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TCGCCCAAGCTAATCCCTGAGAAGC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>GCTCCAGACGCTGTTTGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCGATACCCCAAGTGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TGTCGAGAGCCACAACGCT</td>
</tr>
</tbody>
</table>
Figure 1

Graph (a): Absorbance (492 nm) vs. Fraction number.

Graph (b): Refractive index (AU) vs. Time (min) with peaks labeled I and II.

Legend:
- PK1
- PK2
- PK3
Figure 2
Figure 3

(a) Migration time (min.)

(b) Fluorescence unit

(c) * * * A

(d) M M M M
Figure 4
Figure 5
Figure 6

a

P1
P3
P5
P4
P2

5.0  4.0  3.0  2.0  1.0 ppm

b

*  P3
P5

5.0  4.0  3.0  2.0  1.0 ppm
Figure 7

\[ X \rightarrow [6-\text{Man}p\alpha_1 \rightarrow 6-\text{Man}p\alpha_1]_m \rightarrow [6-\text{Man}p\alpha_1 \rightarrow 6-\text{Man}p\alpha_1]_n \rightarrow 6-\text{Ins-P-GroAc2} \]

\[
\begin{array}{ccc}
\text{Man}p\alpha & \text{t-Man}p\alpha & \text{t-Man}p\alpha \\
\uparrow & \uparrow & \uparrow \\
2 & 2 & 2 \\
\text{t-Ara/}\alpha & & \text{Ac}
\end{array}
\]
Figure 8
Figure 10

A

B

C

D
Figure 11
A novel lipoarabinomannan from the equine pathogen Rhodococcus equi: structure and effect on macrophage cytokine production

Natalie J. Garton, Martine Gilleron, Thérèse Brando, Han-Hong Dan, Steeve Giguère, Germain Puzo, John F. Prescott and Iain C. Sutcliffe

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