Surface-Exposed Glycopeptidolipids of *Mycobacterium smegmatis* Specifically Inhibit the Phagocytosis of Mycobacteria by Human Macrophages. Identification of a Novel Family of Glycopeptidolipids.

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The abbreviations used are:

Ab, antibody; a.m.u, atomic mass units; CR3, Complement Receptor 3; FITC, Fluorescein Isothiocyanate; GC, Gas Chromatography; GC/MS, Gas Chromatography/Mass Spectrometry; GPL, glycopeptidolipid; MALDI-TOF, Matrix-Assisted Laser-Desorption/Ionization – Time Of Flight; MDMs, Macrophages Derived from Monocytes; MS/MS, Electrospray ionization/Tandem Mass Spectrometry; NMR, nuclear magnetic resonance; nsGPL, non-serovarspecific glycopeptidolipid; PIM, Phosphatidylinositolmannoside; SEM, Surface Exposed Material; TLC, Thin-Layer Chromatography.
ABSTRACT

Phagocytosis by macrophages represents the early step of the mycobacterial infection. It is governed both by the nature of the host receptors used and the ligands exposed on the bacteria. The outermost molecules of the non-pathogenic *Mycobacterium smegmatis* were extracted by a mechanical treatment and found to specifically and dose-dependently inhibit the phagocytosis of both *M. smegmatis* and the opportunistic pathogen *M. kansasii* by human macrophages derived from monocytes. The inhibitory activity was attributed to surface lipids because i) it is recovered only in the organic solvent phase of a water/chloroform partition of the surface-exposed material and ii) it is dramatically reduced by alkaline hydrolysis, but not by a protease treatment. Fractionation of surface lipids by adsorption chromatography indicated that the major inhibitory compounds consisted of phospholipids and glycopeptidolipids (GPLs). Mass spectrometry and nuclear magnetic resonance spectroscopy analyses, combined with chemical degradation methods, demonstrated the existence of a novel family of GPLs that consists of a core composed of the long-chain tripeptidyl aminoalcohol with a di-O-acetyl-6-deoxytalosyl unit substituting the allo-threoninyl residue and a 2-succinyl-3,4-di-O-CH₃-rhamnosyl unit linked to the alaninol end of the molecules. These compounds, as well as diglycosylated GPLs at the alaninol end and de-O-acylated GPLs, but not the non-serovar specific di-O-acylated GPLs, inhibited the phagocytosis of *M. smegmatis* and *M. avium* by human macrophages at a few nanomolar concentration without affecting the rate of zymosan internalization. At micromolar concentrations, the native GPLs also inhibit the uptake of both *M. tuberculosis* and *M. kansasii*. De-O-acylation experiments established the critical roles of both the succinyl and acetyl substituents. Collectively, these data provide evidence that surface-exposed mycobacterial glycoconjugates
are efficient competitors of the interaction between macrophages and mycobacteria and, as such, could represent pharmacological tools for the control of mycobacterial infections.
INTRODUCTION

*Mycobacterium* spp. are responsible for several pathologies. For instance, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and numerous other opportunistic mycobacterial pathogens can cause respiratory infections (1,2). Bacilli enter the organism by inhalation. Once in the lung, they infect alveolar macrophages where they survive and multiply (3). After host cell lysis, bacilli released in the extracellular environment infect other macrophages. This cyclic infection is usually under the control of the immune system which confines bacilli in multi-cellular structures, called granulomas, where mycobacteria can stay in latency for years. However, reactivation of the infectious cycle may occur under some immunocompromised situations, leading to an active pathology (4). Therefore, elucidation of the mechanisms involved in the interaction between macrophages and mycobacteria could help to develop new pharmacological strategies to prevent macrophage infection.

Pathogenic mycobacteria have the ability to persist in macrophages (5) that are usually meant to kill microorganisms. This successful parasitism involves mycobacterial strategies to protect themselves from potent host anti-microbial processes, such as restriction of lysosomal fusion with their phagosomes (6-8). The early process of recognition and internalization of mycobacteria in macrophages is essential in the outcome of infection. In human macrophages derived from monocytes (MDMs), we have demonstrated that the early step of infection by opportunistic mycobacteria did not evoke microbicidal responses, inducing neither a production of superoxide anions nor a fusion of phagosomes with a sub-population of lysosomes (9). Interestingly, phagocytosis of non-pathogenic mycobacteria, such as *M. smegmatis*, did not either elicitate bactericidal responses (9). Finally, at the early step of infection, mycobacteria do not actively control their host cell since phagosomes containing
live or heat-killed bacteria are indistinguishably refractory to fusion with lysosomes (10). In sharp contrast, when mycobacteria were serum opsonized, their phagocytosis was associated with an oxidative response (9) and a maturation of phagosomes towards a fusion with lysosomes (6,11,12). This led us to propose that, during the initial steps of the infection, mycobacteria have developed a common strategy, which consists in the use of receptors of non-opsonic phagocytosis uncoupled to bactericidal responses. In agreement with this proposal, we recently demonstrated that the mannose receptor, which efficiently participates in binding and internalization of pathogenic and non-pathogenic mycobacteria, was not coupled to bactericidal functions in human macrophages (9). Similarly, binding and internalization of the opportunistic pathogen *M. kansasii* through the complement receptor type 3 (CR3) did not activate the NADPH-oxidase in macrophage cell lines (13).

The mycobacterial envelope is composed of a plasma membrane surrounded by a complex cell wall, which in turn is recovered by a superficial layer composed of proteins, carbohydrates and, to a lesser extent, lipids (14-16). This outermost structure, also called capsule in the case of pathogenic species, represents a privileged interface between bacilli and their host cells. Some of its components have been implicated in the interaction with macrophages (17). The major carbohydrate constituent of the capsule, a glycogen-like glucan (14), has been shown to inhibit the binding of *M. tuberculosis* to CR3-expressing CHO cells (18). The purified mannose-capped lipoarabinomannan (Man-LAM), a cell envelope-associated glycoconjugate, interacts with the mannose receptor of macrophages (19). Others components of the mycobacterial cell envelope, *e. g.* sulfatides, phenolic glycolipids, glycopeptidolipids (GPLs), phosphatidylinositolmannosides (PIMs) induce various host cell responses (for a comprehensive review, see (20)). Some of these biologically active molecules have been precisely localized in the outermost layer of the cell envelope (15,21), but the precise location of others is still a matter of debate.
We extracted mycobacterial surface-exposed compounds by gentle surface abrasion with glass beads (15) and tested their role in the interactions between mycobacteria and MDMs to identify the most active bacterial partners of the phagocytic process. As we have demonstrated that pathogenic and non-pathogenic strains use the same phagocytic receptors to infect cells (9,10,22,23), this work was performed with the non-pathogenic species, *M. smegmatis*. Using this approach, we demonstrated that surface exposed C-type GPLs inhibited the non-opsonic phagocytosis of GPL-containing mycobacteria and to a lower extent those of other mycobacteria.

EXPERIMENTAL PROCEDURES

*Bacterial Strains and Growth Conditions —* *M. smegmatis* ATCC 607, *M. tuberculosis* H37Rv ATCC 27294 and *M. kansasii* ATCC 124478 were grown as surface pellicles on liquid Sauton’s medium at 37°C without agitation; *M. avium* (serovar 4) was cultured under shaking at 250 rpm in Middlebroock 7H9 medium supplemented with 10% albumin-dextrose-catalase enrichment (Difco). The growth curves were determined as previously described (24).

Single cell suspensions were prepared with late log-phase cultures (6 days for *M. smegmatis*, 3 weeks for *M. kansasii*, *M. tuberculosis* and *M. avium*), as previously described (23). When specified, *M. smegmatis* were isolated from early log phase culture (J3). Briefly, pellicles (*M. smegmatis* and *M. kansasii*) were harvested by pouring off the medium, dispersed by gentle shaking for 30s with 5 g of glass beads (4-mm diameter) and resuspended in PBS, pH 7.4. For *M. tuberculosis*, this procedure was repeated to further desaggregate the pellicles. In the case of *M. avium*, the culture was recovered by centrifugation at 10,000 x g for 10 min. To remove the remaining clumps, the bacterial suspensions were sedimented for 10 min. The supernatants were collected and then centrifuged for 10 min at 200 x g. Up to
90% of mycobacteria were individualized, the remaining formed small aggregates containing two or three bacilli. The viability of mycobacterial cells was assessed by i) quantifying the level of isocitrate dehydrogenase, an indicator of autolysis, in the culture filtrates (24), ii) labelling cells with both propidium iodide and fluorescein diacetate (10), iii) serial dilutions and plating on Middlebroock 7H10 medium. The percentage of viable mycobacteria averaged 85%. When specified, mycobacteria were labelled with FITC, as previously described (23).

Surface-Exposed Material (SEM) Preparation – Surface pellicles of mycobacteria grown on Sauton’s medium were treated with 10 g of glass beads (4-mm diameter) for 1 min and resuspended in distilled water (50 ml per flask). Bacilli were removed by filtration through a 0.2-µm-pore-size sterile filter (Nalgene). The crude filtrate, which contains the surface-exposed material (SEM), was then extensively dialyzed against distilled water with cellulose membrane (1000 Da) (Spectra/Por 7, Spectrum). When specified, dialysis was performed using a 100-Da or 6000-8000-Da molecular weight cut-off membrane. The protein content of the sterile SEMs were determined using the Bradford method (Bio-Rad). Surface extracts were treated at 37°C with either 0.1 M NaOH for 4 hours or 0.1 mg of pronase or proteinase K per mg of proteins overnight in the presence of chloramphenicol and gentamicin (100 µg per ml). Proteases were inactivated at 100°C for 15 min. For both treatments, SEMs were then dialyzed and the pH was restored to 7.

Portions of the SEMs were extracted with chloroform and methanol according to the Bligh and Dyer procedure (25). After drying, the organic phases were washed. The aqueous phases were re-extracted with chloroform, and the interphase was rinsed with a mixture of CHCl₃/H₂O (1:1 v/v). The three phases were then dried under vacuum, weighed and resuspended in a volume of sterile water equal to the initial volume of the crude surface extract. The lipid components of SEMs were identified by thin-layer chromatography (TLC) as previously described (26) and further fractionated as described below.
Extraction and Purification of Mycobacterial Lipids – Lipids were purified both from whole cells and SEMs. In the former case, wet cells were extracted firstly with CHCl₃/CH₃OH (1:2, v/v) and then with CHCl₃/CH₃OH (1:1, v/v) at least three times. SEMs and pooled whole cell lipid extracts were separately dried under vacuum and partitioned between water and chloroform (1:1, v/v). The organic phases were extensively washed with distilled water and evaporated to dryness. Portions of the chloroformic phases were used directly for biological assays. The remaining portions were dried and lipids were resuspended in a minimal volume of chloroform and precipitated by trickling methanol. After standing for two hours at 4°C, methanol soluble lipids were recovered by centrifugation at 4°C for 20 min (8000 x g). These lipids were then chromatographed on a Florisil (60-100 mesh) column (1.5 x 25 cm) irrigated with chloroform and then with a stepwise gradient of increasing concentrations of methanol and water in chloroform. GPLs and phospholipids, which were coeluted in polar fractions with a mixture of CHCl₃/CH₃OH/H₂O (65:25:4, v/v/v), were further separated using an anion-exchange QMA-silica gel (Chromabond SB, Macherey-Nagel). GPLs were eluted with CHCl₃/CH₃OH/H₂O (65:25:4, v/v/v), whereas phospholipids were eluted using 0.1 and 0.2 M ammonium acetate in CHCl₃/CH₃OH (1/2, v/v) and 0.2 M ammonium acetate in methanol. All the purification steps were monitored by TLC on silica-Gel 60-precoated plates (0.25-mm thickness; Merck) developed with CHCl₃/CH₃OH (90:10, v/v). Sugar-containing compounds were visualized by spraying plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating, whereas the Dittmer-Lester reagent was used to detect phosphorus-containing substances (27).

Analytical procedures – Three different chemical degradation methods were applied to the purified GPLs (Fig. 1) (28), and the resulting products were analysed by MALDI-TOF mass spectrometry: i) de-O-acylated and both de-O-acylated and β-eliminated GPLs were obtained from treatment of native GPLs with 0.5 M sodium methanolate for 2 h at 37 °C; after
neutralisation with glacial acetic acid, the aqueous phase was extracted with chloroform, ii) perdeuteriomethylation of GPLs was carried out according to the method described by Blakeney and Stone (29) with trideuteriomethyl iodide (ICD₃) as methylating agent, iii) the N-acyl-phenylalanyl moiety of GPLs was produced as methyl ester from native or perdeuteriomethylated GPLs by methanolysis with anhydrous 1.5 M CH₃OH/HCl for 16 h at 80°C; a portion of the native GPLs was further acetylated in 1:1 Ac₂O/pyridine (100°C, 1h) or its double bound was cleaved by a permangante-periodate oxidation (30), iv) the partially methylated alditol acetates were obtained from perdeuteriomethylated GPLs after hydrolysis with 2 M trifluoroacetic acid (100°C, 2 h), reduction with NaBH₄ and acetylation with 1:1 Ac₂O/pyridine (100°C, 1h).

**Characterization of the succinyl group** – Five mg of GPLs III were treated by 0.5 ml of 0.5 M sodium methanolate for 2 h at 37°C. After neutralisation with glacial acetic acid, the mixture was dried under a stream of N₂, dissolved in water and desalted by filtration over a cation-exchange resin (Dowex 50, H⁺ form) column. After evaporation to dryness in the presence of CH₃OH, the residue was methylated by diazomethane (CH₂N₂) for Gas chromatography Mass Spectrometry (GC/MS) analysis.

**Spectrometric methods** – Matrix-Assisted Laser-Desorption/Ionization – Time Of Flight (MALDI-TOF) mass spectrometry analysis of lipids was performed as previously described (31). Sample solutions were prepared in chloroform at a concentration of 1 mM and were directly applied onto the sample plate as 1 µl droplet, followed by the addition of 0.5 µl of the matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in CHCl₃/CH₃OH 1:1, v/v). Samples were then allowed to crystallize at room temperature. MALDI-TOF spectra were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) equipped with a pulsed nitrogen laser emitting at 337 nm and were analyzed in the Reflectron mode using an extraction delay time set at 100 ns and an accelerating voltage operating in positive ion mode.
of 20 kV. To improve the signal-to-noise ratio, 150 single shots were averaged for each mass spectrum and, typically, four individual spectra were accumulated to generate a summed spectrum. An external mass spectrum calibration was performed using the calibration mixture 1 of Sequazyme Peptide Mass Standards Kit (PerSeptive Biosystems), including known peptide standards in a mass range from 900 to 1600 Da.

ElectroSpray Ionization Mass Spectrometry (ESI) and ESI/Tandem Mass Spectrometry (MS/MS) analyses were done by using a FinniganMat TSQ 700 triple quadrupole (ThermoFinnigan San Diego, USA). Lipids were dissolved in 1 % acetic acid in methanol. The mass spectra were obtained by direct infusion using a syringe pump (Harvard Apparatus, South Natick, MA USA) at a flow rate of 3 µl min\(^{-1}\). Full scan spectra (positive mode, spray potential 4.6 kV) were acquired in the ion peak centroid or profile modes over the mass/charge range of 200-2000 at 3 s. MS/MS experiments were performed by conducting collision-induced dissociation in the radio frequency only collision cell of the triple quadrupole at a collision energy of 80 ev. Argon was used as a collision gas in the range of 1.5 - 2.5 mtorr. At least 15 scans were accumulated and averaged.

Gas Chromatography/Mass Spectrometry (GC/MS) analyses were performed on a Hewlett-Packard 5890 series II gas chromatograph, fitted with an OV1 fused-silica capillary column (12 m x 0.30 mm) and connected to a Hewlett-Packard 5989X mass spectrometer in Electron-Impact (EI) mode with an ionization potential of 70 eV. The temperature programs used were 100 °C (delay 3 min) to 290° C at 8 °C/min or isotherm at 40 °C for alditol acetates or short acids methyl esters analysis respectively.

One- and two-dimensional \(^1\)\(^{1}\)H-NMR spectra were recorded on a Bruker AMX-500 spectrometer using standard pulse sequences available in the Bruker software. The chemical shifts were expressed in parts per million relative to acetone as internal standard (δ\(_H\) 2.22).
De-O-acylation of GPLs – GPLs were de-O-acylated with 0.1 N NaOH in CHCl₃/CH₃OH (50:50, v/v) for 30 min at 37°C. After neutralization by glacial acetic acid, the mixture was dried under a stream of N₂, then the de-O-acylated GPLs were extracted with chloroform and extensively washed with water. The effectiveness of the de-O-acylation was checked by MALDI-TOF mass spectrometry analysis.

Isolation and Culture of Human Macrophages Derived from Monocytes (MDMs) – Human peripheral blood monocytes were isolated as previously described (9) and cultured on sterile glass coverslips in 24-well tissue culture plates (5x10⁵ cells/well) containing RPMI medium with 10% heat-inactivated FCS and antibiotics, for 6 to 7 days at 37°C in 5% CO₂. The culture medium was renewed at the third day. Before use, MDMs were washed twice with fresh RPMI medium and equilibrated for 20 min at 37°C in 5% CO₂.

Opsonization of Bacteria and Zymosan – Fluorescein Isothiocyanate (FITC)-stained mycobacteria or zymosan (Z) were incubated with pooled human sera for 25 min at 37°C, washed twice and suspended in phosphate buffered saline (PBS), pH 7.4 (9,23).

Infection of Adherent Macrophages and Phagocytosis Assay – When specified, MDMs were pretreated for 15 min at 37°C with either the crude SEMs, the different phases obtained after phase partition or the purified lipid fractions resuspended in sterile apyrogenic water and sonicated for 10 min. All dilutions were performed in RPMI medium. MDMs were then put in contact with bacilli for 1 hr and washed twice with fresh medium to remove unbound particles. Phagocytosis of FITC-stained bacteria was determined as previously described (22). Briefly, MDMs were fixed with 3.7% paraformaldehyde in PBS containing 15 mM sucrose, pH 7.4 for 20 min at room temperature. After neutralization with 50 mM NH₄Cl, extracellular mycobacteria were labelled with rabbit polyclonal antibodies (Abs) directed against mycobacteria (Camelia Ab, 1/100) (22), revealed by a TRITC-conjugated secondary Ab. MDMs containing at least one FITC-stained mycobacterium were counted out of 100 cells in
duplicate samples. MDMs having engulfed either BSA-coated latex beads and Z for 1 hour (50 particles per cell) or serum-opsonised zymosan (OZ) for 30 min (50 particles per cell) were permeabilized in methanol for 6 min at -20°C and washed in PBS containing 0.1% Tween 20. Phagosomes containing particles were stained with a lysosomal membrane marker, CD63, revealed with a fluorescent-conjugated secondary Ab as previously described (9).

**Statistics** – Data are presented as the mean ± standard error of the mean (sem) of the indicated number of experiments (n) performed in duplicate. The significance of the differences was determined by the paired or unpaired Student’s test.

**RESULTS**

**Inhibitory Activity of Surface-Exposed Material from M. smegmatis on Mycobacterial Phagocytosis** – We first checked that the surface-exposed material (SEM) of *M. smegmatis* readily affected the ability of macrophages to internalize bacilli. The crude SEM was arbitrary dialyzed using a 100-Da molecular weight cut-off membrane, to eliminate small molecules from the culture medium. The amount of SEM to be used, corresponding to bacilli in contact with MDMs (50 particles/cell), was rationalized by using a final concentration of 150 ng protein/ml SEM. Under these conditions, SEM isolated from early log-phase grown *M. smegmatis* (day 3) decreased by 43 ± 2 % (n=2) the rate of phagocytosis of 3-day old bacteria. Similarly, SEM isolated from late log-phase grown *M. smegmatis* (day 6) decreased by 41 ± 10 % (n=4) internalization of 6-day old bacteria. It was thus concluded that SEM exhibited an inhibitory effect on the phagocytosis of *M. smegmatis* that was independent from growth conditions.

**Chemical Nature of the Inhibitory Compounds from the Surface-Exposed Material** – We estimated the approximate size of these molecules by dialysis. While the dialysis of SEM...
using a cut-off of 1000-Da did not affect its inhibitory effect (52 ± 4 %, n=9) (Fig. 2), no inhibition was observed with SEM dialyzed at a 6-8 kDa cut-off (4 ± 1 % inhibition, n=3). These data indicated that the inhibitory molecules possess a low molecular weight (between 1000 and 8000 Da).

Accordingly, the subsequent work was performed with SEM dialyzed with a 1000-Da molecular weight cut-off membrane and isolated from 6-day old cultures of *M. smegmatis* to get enough biological material for further fractionation experiments. Under these conditions, SEM from *M. smegmatis* inhibited the uptake of both heat-killed *M. smegmatis* (data not shown) and the opportunistic pathogen *M. kansasii* (Fig. 2). In sharp contrast, the internalization of mycobacteria opsonized with human serum was not affected (Fig. 2). Furthermore, SEM did not either modify phagocytosis of various inert particles such as latex beads and opsonized or non-opsonized zymosan (Fig. 2). It was thus concluded that SEM contained inhibitory molecules that specifically decrease the non-opsonic phagocytosis of mycobacteria but do not affect internalization of other particles.

An overnight incubation of SEM with non-specific/broad spectrum proteases, pronase and proteinase K, did not influence its inhibitory effect on *M. smegmatis* internalization (Fig. 3a), suggesting that the observed inhibition was not due to proteins. In contrast, when SEM was treated with alkali, a treatment that hydrolyses ester bounds, a pronounced decrease of its inhibitory activity was observed (Fig. 3a), implying that a significant portion of inhibitory molecules was alkali-labile. Based on the occurrence of ester linkages in most of the mycobacterial lipids, these molecules were isolated by partitioning SEM between chloroform, methanol and water. TLC analysis of the organic phase showed the presence of GPLs, 6-monomycoloyltrehalose, PIMs, phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG) (data not shown) as previously reported (21). The interphase and the aqueous phase contained mostly carbohydrates and proteins. As depicted on Fig. 3b, the
organic phase exhibited the highest inhibitory effect on phagocytosis of \textit{M. smegmatis}. Although some inhibition was observed with the aqueous phase (Fig. 3b), an extensive extraction with organic solvents to eliminate residual lipids abolished this effect (data not shown). The inhibitory effect of the interphase was negligible (Fig. 3b). Altogether, these data indicated that most of the inhibitory activity of SEM was attributable to its lipid constituents.

\textit{Purification and Identification of the Inhibitory Substances of \textit{M. smegmatis} – Lipids}

from the SEM were fractionated by adsorption chromatography on a Florisil column. They were eluted with chloroform, increasing concentrations of methanol in chloroform and finally with a mixture of chloroform, methanol and water. Five lipid fractions were obtained and tested at 100 µg lipid/ml for their ability to inhibit \textit{M. smegmatis} phagocytosis. Only two of them exerted an inhibitory activity. The most polar fraction showed the highest inhibitory activity comparable to that of the organic phase (50 ± 6 and 47 ± 1 %, n=3, respectively). TLC analysis (data not shown) demonstrated that this fraction contained glycolipids that shared with GPLs the same “string-of-beads” aspect and green-blue coloration with anthrone, and phospholipids such as PIMs, PI, PG and PE, suggesting that both surface-exposed GPLs and phospholipids possessed inhibitory activities. The second fraction that inhibited phagocytosis of \textit{M. smegmatis} by 26 ± 4 %, (n=3) contained GPL-like molecules but not phospholipids (data not shown).

As GPL-like substances were present in the two inhibitory fractions, we next focused on these compounds. Because GPLs are present in both the outermost layer of mycobacteria cell envelope and the whole bacteria (26,32), they were isolated from whole bacterial lipids in order to obtain more material. The extract was first enriched in GPL-like molecules by methanol precipitation, a procedure known to concentrate phospholipids and mycoloylated glycolipids in the methanol-insoluble fraction. As expected, the extract enriched in GPL-like was able to inhibit the internalization of \textit{M. smegmatis} (Fig. 4). At concentrations ranging
from 1 pg to 100 µg lipid/ml, GPLs induced a biphasic effect on phagocytosis of *M. smegmatis*. The inhibitory effect increased from 1 to 100 pg lipid/ml, with a maximal effect at 100 pg lipid/ml that remained stable up to 10 ng lipid/ml and then progressively decreased (Fig. 4). This phenomenon possibly reflects the critical aggregatory concentration of lipid compounds. Therefore, a concentration of 10 ng lipid/ml GPLs was used for subsequent experiments. Lipids from the methanol-soluble extract were fractionated on a Florisil column into 21 fractions. As shown in Fig. 5a, inhibitory activities of fractions increased with the polarities of the solvent mixtures used. TLC analysis showed that fractions S8 to S20 contained GPL-like compounds while S21 fraction was composed of both GPL-like compounds (Fig. 5b) and some phospholipids (data not shown).

To firmly establish the nature of the active compounds, S21 was chromatographed again on an anion-exchange column. The purified GPL-like compounds from S21 decreased by more than 60 % the internalization of *M. smegmatis* (from 35 ± 7 to 13 ± 6 %, n=3). In contrast, the phospholipid fraction eluted from the ion-exchange column with ammonium acetate in CHCl₃/CH₃OH reduced the internalization of bacteria by only 30 % (from 35 ± 7 to 23 ± 8 %, n=3). This led us to conclude that GPL-like molecules of S21 were the main compounds involved in inhibition of *M. smegmatis* phagocytosis. We finally checked by TLC that the various GPL-like molecules isolated from the whole lipid extract of *M. smegmatis* were also present in the SEM and showed by MALDI-TOF mass spectrometry that they were composed of the same species (Fig. 6).

Because GPLs are species-specific (33,34), the purified GPL-like compounds from S21 of *M. smegmatis* were tested in competition with *M. avium*, a species containing GPLs (33), and *M. tuberculosis* and *M. kansasii*, two species devoid of GPLs (16). As depicted in Fig. 7, pre-treatment of MDMs with 10 ng lipid/ml significantly decreased the rate of phagocytosis of *M. avium* (from 29 ± 4 to 20 ± 4 %, n = 4, p<0.01) while it did not
significantly affect the internalization of *M. tuberculosis* and *M. kansasii* (from 55 ± 4 to 45 ± 9 % and from 31 ± 9 to 31 ± 8 %, n = 4, respectively). However, when lipid concentration was increased from 10 ng/ml to 10 µg/ml, phagocytosis of *M. tuberculosis* progressively decreased (data not shown). At 10 µg/ml the inhibitory effect was similar to that obtained with 10 ng lipid/ml on *M. smegmatis* phagocytosis (Fig. 7). As a control, we checked that 10 µg lipid/ml from *M. smegmatis* decreased to the same extent the phagocytosis of *M. kansasii* (from 27 ± 1 to 16 ± 2 %, n = 2), while they did not interfere with the internalization of zymosan (Fig. 7). This led us to propose that the GPL-like molecules that composed S21 specifically inhibited the non-opsonic phagocytosis of mycobacteria. As these compounds exhibited TLC mobilities similar to those of GPL-like substances found in fractions S13-18 (Fig. 5b), we postulated that subtle structural features might exist between the isolated compounds.

*Structural features of GPL-like substances* – The structure of the various constituents of S8-21 was determined by NMR, MALDI-TOF mass spectrometry and analysis of chemical degradation products according to the procedures shown in Fig. 1. Preliminary MALDI-TOF mass spectrometry analysis has led us to choose fractions S8, S13 and S20 as representative of three types of GPL-like compounds. Their one- (Fig. 8) and two-dimensional 1H-NMR spectra were very similar to those of the previously characterized C-type GPLs (33) and confirmed the presence of i) phenylalaninyl, threoninyl, alaninyl and alaninol residues, ii) deoxysugar units, iii) β-hydroxylated long-chain, iv) double bonds, and v) methoxyl groups in these compounds (35). Molecules in fractions S8, S13 and S20 were thereafter called GPLs I, II and III, respectively (Fig. 9). In addition, signals at 2.62 and 2.70 p.p.m were seen in the 1H-NMR spectrum of S20 (GPLs III) (Fig. 8b) and absent from those of the other fractions (Fig. 8a). Strong acid methanolysis (CH3OH/HCl 1.5M, 16 h at 80°C) of S8-S20 (Fig. 1), followed by the analysis of the resulting *N*-acyl-phenylalaninyl methyl ester by MALDI-TOF
mass spectrometry, demonstrated the occurrence of major pseudomolecular ion \([M + Na]^+\) peaks corresponding to a mixture of hydroxylated C\(_{28}\), C\(_{30}\) and C\(_{32}\) and methoxylated C\(_{29}\), C\(_{31}\) and C\(_{33}\) saturated and unsaturated fatty acyl-phenylalanyl methyl esters. The presence of methoxylated homologues in the mixture was further ascertained by the analysis of the MALDI-TOF mass spectra of both the perdeuteriomethylated and peracetylated products (Fig. 1). While perdeuteriomethylation of hydroxylated compounds induced a 34 atomic mass unit (a.m.u.) shift of their \([M + Na]^+\) values, those of methoxylated substances were shifted by only 17 a.m.u. (a CD\(_3\) on the nitrogen atom of the phenylalanyl residue). Similarly, peracetylation of the mixture of fatty acyl-phenylalaninyl methyl esters (Fig. 1) induced a 42 a.m.u. shift of hydroxylated compounds whereas no change was observed for methoxylated homologues. Oxidative cleavage of the double bond occurring in the fatty acyl-phenylalanyl moiety, followed by methanolysis and GC/MS analysis, led to the identification of C\(_{15}\)-C\(_{17}\) fatty acid methyl esters. MALDI-TOF mass spectrometry analysis of the native and perdeuterioacetylated phenylalanyl diacid methyl esters resulting from the oxidative cleavage showed that the hydroxyl and methoxyl groups were located on the phenylalanyl diacid moiety of the unsaturated molecules. It followed then that fractions S8-20 contained all the structural features found in C-type GPLs (Fig. 9).

Structures of the various GPLs of *M. smegmatis* – The MALDI-TOF mass spectrum of the native S8 (Fig. 10a) showed a series of major pseudomolecular ion \([M + Na]^+\) peaks corresponding to the previously described apolar non-serovar-specific GPLs (nsGPLs or GPLs I) of *M. smegmatis* (36). The \([M + Na]^+\) species corresponded to substances differing from one another by the degrees of \(O\)-methylation of either the rhamnosyl unit or the hydroxyl group of the fatty acyl substituent (36-38). In addition, ion peaks differing by 14 a.m.u., i.e. one methylene unit, were observed and reflected the variability in the chain length
of the fatty acyl substituent which contains C$_{28}$ to C$_{32}$ (36) (Fig. 9). The complexity of the 
mass spectra was further enhanced by the variable occurrence of a double bound in the fatty 
acid chain (36), resulting in the presence of pseudomolecular [M + Na]$^+$ ions differing from 
those of saturated chains by 2 a.m.u. Perdeuteriomethylation of GPLs I followed by acid 
hydrolysis and analysis of partially methylated partially acetylated alditol derivatives by 
GC/MS (Fig. 1) led to the identification of i) 1,5-di-O-acetyl-2,3,4-tri-O-CD$_3$-6-deoxytalitol, 
ii) 1,5-di-O-acetyl-2,3,4-tri-O-CH$_3$-rhamnitol and iii) 1,5-di-O-acetyl-2-O-CD$_3$-3,4-di-O-CH$_3$-
rhamnitol in a 1:0.7:0.3 ratio. The general structure of these substances is depicted in Fig. 9.

The mass spectra of fractions S9 to S15 (GPLs II) contained two series of 
pseudomolecular ion peaks of similar intensity. The $m/z$ values of the major [M + Na]$^+$ peaks 
were 84 a.m.u. lower than those of GPLs I, i.e. at 1199.7 and 1213.7 $m/z$ rather than 1283.8 
and 1297.6 for GPLs I. This series corresponded to GPLs I devoid of the two acetyl groups 
(de-O-acetylated GPLs I or GPLs IIa sub-family). The other series of [M + Na]$^+$ peaks, 174 
a.m.u. higher than those observed for GPLs I, were also present in the mass spectra of 
fractions S9-15 (hyperglycosylated GPLs I or GPLs IIb sub-family; data not shown). Acid 
hydrolysis of these fractions, followed by GC and GC/MS analysis of the sugar derivatives 
(Fig. 1), identified as alditol acetates 6-deoxytalosyl, 3,4-di-O-CH$_3$-rhamnosyl and 2,3,4-tri-
O-CH$_3$-rhamnosyl residues. Based on our previous observations (36), our data suggested that 
fractions S9 to S15 contained de-O-acylated GPLs I (GPLs IIa, Fig. 9), composed of a 6-
deoxytalosyl residue linked to allo-threonyl and either a 3,4-di-O-CH$_3$-rhamnosyl or 2,3,4-tri-
O-CH$_3$-rhamnosyl residue attached to the alaninol residue. The other components of fractions 
S9-15 were hyperglycosylated GPLs I (GPLs IIb, Fig. 9) in which a diglycosyl units 
composed of two 3,4-di-O-CH$_3$-rhamnosyl residues modified the alaninol end of GPLs. While 
this work was in progress, this last class of compounds has been identified in *M. smegmatis* 
(39).
The most active fractions S17-21 contained GPL-like molecules (called GPLs III) exhibiting mobilities on TLC similar to those of GPLs II (Fig. 5b). This observation was unexpected since S17-21 were eluted from the gel only when water was added to the mixture of chloroform and methanol. When these fractions were analyzed by MALDI-TOF mass spectrometry, however, the observed \([M + Na]^+\) peaks (Fig. 10b) were clearly different from those of both native and deacylated GPLs I. The major pseudomolecular \([M + Na]^+\) ion peaks of GPLs III were seen at 1355.1, 1357.1, 1383.1 and 1385.1 \(m/z\) (Fig. 10b, GPLs IIIa sub-family). A minor series of peaks was observed at higher (174 a.m.u.) \(m/z\) values at 1515.1, 1517.1, 1543.1, 1545.1, 1557.1 and 1559.1 (Fig. 10b, GPLs IIIb sub-family). Treatment of GPLs III with 0.5 M sodium methanolate (Fig. 1) resulted in a 184 a.m.u. downshift of the \([M + Na]^+\) peaks of both series (Fig. 10c), indicating the occurrence of an alkali-labile substituent on native GPLs III in addition to the acetyl groups esterifying the 6-deoxytalosyl unit. The alkaline treatment (Fig. 1) also induced the \(\beta\)-elimination of the sugar unit linked to the allo-threonyl residue, \textit{i.e.} the 6-deoxytalosyl unit, with the additional loss of 174 a.m.u. The \([M + Na]^+\) peaks due to the remaining molecules were observed for the major series at 1008.9, 1036.9 and 1064.9 \(m/z\) (Fig. 10c). Perdeuteriomethylation of GPLs III followed by MALDI-TOF mass spectrometry analysis (Fig. 1), showed that hydroxylated compounds incorporated 9 CD\(_3\) (including 4 on the peptide core) whereas only 8 CD\(_3\) were incorporated in methoxylated molecules (data not shown). Acid hydrolysis of the perdeuteriomethylated products (Fig. 1) and analysis of the partially-\(O\)-methylated partially \(O\)-acetylated alditol derivatives by GC-MS led to the identification of i) 1,5-di-\(O\)-acetyl-2,3,4-tri-\(O\)-CD\(_3\)-6-deoxytalitol, ii) 1,5-di-\(O\)-acetyl-3,4-di-\(O\)-CH\(_3\)-2-\(O\)-CD\(_3\)-rhamnitol and iii) 1,2,5-tri-\(O\)-acetyl-3,4-di-\(O\)-CH\(_3\)-rhamnitol in a 1:0.4:0.3 ratio. These data demonstrated that GPLs III were composed of a 6-deoxytalosyl unit linked to the allo-threoninyl residue and either a 3,4-di-\(O\)-
CH₃-rhamnosyl unit (for the GPLs IIIa sub-family; Fig. 9) or a disaccharide constituted of two 3,4-di-O-CH₃-rhamnosyl units (for GPLs IIIb sub-family; Fig. 9) attached at the alaninol end of the molecules. The proposed structure was consistent with the observed 14 a.m.u. downshift of the mass values of the major series of de-O-acylated GPLs III when compared to those of the de-O-acylated GPLs I (data not shown), i.e. the replacement of the 2,3,4-tri-O-CH₃-rhamnosyl residue of GPLs I by a 3,4-di-O-CH₃-rhamnosyl unit in GPLs III. Likewise, the observed difference of 174 a.m.u. between the two sub-families of GPLs III, in the MALDI-TOF mass spectra of both the native and alkali-treated GPLs III (Fig. 10b,c), suggested the presence of a disaccharidyl residue composed of two 3,4-di-O-CH₃-rhamnosyl units in the minor series of GPLs III (Fig. 9).

The chemical nature of the O-acyl residue that substituted the 3,4-di-O-CH₃-rhamnosyl unit was guessed from the ¹H-NMR spectrum of the GPLs III (Fig. 8). Two proton resonances at 2.62 and 2.70 p.p.m were present in the spectrum of GPLs III and absent from those of both GPLs I and their de-O-acylated forms (GPLs II). These deshielded resonances were attributed to methylene located near carboxylic functions. Furthermore, analysis of the two-dimensional homonuclear spectrum of GPLs III showed a correlation peak between the two resonances and no correlation with other resonances (data not shown). Based on the mass value deduced from the MALDI-TOF mass spectrum (101 a.m.u.) the O-acyl residue was postulated to be a succinyl group. Final identification of the O-acyl residue was performed by GC/MS analysis of the short-chain compounds released by alkaline methanlysis of GPLs III. These products were first desalted on a Dowex H⁺ column and the putative carboxylic acids were methylated. GC analysis of the methyl esters using an isotherm program at 40°C showed the presence of a peak whose mass spectrum gave an intense ion peak at 115 m/z that corresponded to the loss of a methoxyl group [M-31] and a fragment ion peak at 87 m/z attributed to the loss of a carboxyl methyl ester group [M-59]. Based on the structures
proposed for GPLs I and III (Fig. 9), the only possible location for the additional O-acyl residue that accounts for 101 a.m.u. would be position 2 of the terminal di-O-CH₃-rhamnosyl unit. This location was supported by the analysis of the MS/MS spectra on two [M + Na]⁺ of the native GPLs III at 1382.9 and 1557.8 m/z, respectively. The fragmentation pattern of the ion at 1382.9 m/z, representative of the major series of GPLs IIIa, showed a prominent peak at 1110.3 m/z that corresponded to the loss of succinylated anhydro-3,4-di-O-CH₃-rhamnosyl residue. A similar fragmentation pattern was observed in the spectrum of the species at 1557.8 m/z, representative of the minor series of GPLs IIIb. The MS/MS spectrum showed two intense peaks at 1283.8 and 1109.7 m/z resulting from the loss of succinylated anhydro-3,4-di-O-CH₃-rhamnosyl and anhydro-3,4-di-O-CH₃-rhamnosyl-(1->2)-3,4-di-O-CH₃-rhamnosyl residue, respectively.

**Role of the succinyl and acetyl substituents** - GPLs III, the most inhibitory compounds of the family (Fig. 5), are characterized by the presence of a succinyl group on the terminal rhamnosyl unit. GPLs II, which were slightly less effective, differ from the non-active GPLs I, by the absence of the acetyl substituents (GPLs IIa) or the presence of a second rhamnosyl unit (GPLs IIb). GPLs I from S8 and GPLs III from S21 (Fig. 5) were de-O-acylated and analyzed for their inhibitory effect on *M. smegmatis* phagocytosis. The efficiency of the de-O-acylation procedure was checked by MALDI-TOF analysis. The alkaline treatment of GPLs I and GPLs III resulted in a 84 a.m.u. downshift (loss of two acetyl groups) and a 184 a.m.u. downshift (loss of two acetyl plus a succinyl group) of the [M + Na]⁺ peaks, respectively (data not shown). Compared to the native compounds, the inhibitory activity of the de-O-acetylated GPLs I was dramatically increased (8.67 ± 0.09 fold, n = 3). In contrast, the loss of the succinyl and acetyl substituents from GLPs III led to a decrease of the inhibitory effect (1.78 ± 0.05 fold, n=3). Therefore, the succinyl substituent on the terminal rhamnosyl unit of GPLs
III plays an essential role in their inhibitory activity as did the presence of free hydroxyl groups on positions 3 and 4 of the 6-deoxytalosyl moiety.

DISCUSSION

The outermost layer of mycobacteria represents the interface between bacilli and their host macrophages and probably specifies the intracellular fate of bacteria. Mycobacterial surface-exposed molecules are recognized by cognate cell surface receptors that enable bacteria to reach their habitable environment where they survive and multiply. A model of the outer leaflet proposed that glycolipids are embedded in a capsular layer of polysaccharides and proteins (40). A few purified molecules have been shown to play a role in the interaction between mycobacteria and host cells (40) but their surface location and their accessibility to their cell partners remain an unsolved question. To identify the nature of the putative mycobacterial partners of the phagocytic process, we used a step by step isolation procedure and were able to isolate surface-exposed molecules on the cell envelope of *M. smegmatis* that specifically prevent interactions between bacilli and macrophages. Among them, we identified phospholipids, including PIMs, and C-type glycopeptidolipids (GPLs) which were the most active substances. The surface exposure of GPLs (32) and the recent demonstration of the impact of the absence of GPLs on the phagocytosis of *M. smegmatis* by human macrophages (26) are consistent with the implication of these molecules in the internalization of some non-tuberculous mycobacteria.

C-type GPLs are a family of species-specific lipids that typifies many non-tuberculous mycobacterial species (16,33). In particular, they have been identified on the surface of *M. smegmatis* and *M. avium* but not on *M. tuberculosis* or *M. kansasii* (32). This observation certainly explains why nanomolar concentrations of GPLs from *M. smegmatis* decreased the internalization of *M. avium* while much higher concentrations are necessary to affect
phagocytosis of *M. tuberculosis* and *M. kansasii*. The four mycobacteria species tested herein have been previously shown to use common receptors, such as mannose receptors and CR3, to infect macrophages (unpublished data and (9,10,19,41). Therefore, one can assume that mycobacteria express distinct ligands that can be recognized by a same receptor but with various ranges of affinity, a matter that remains to be addressed.

C-type GPLs share a common lipopeptidyl core composed of a long-chain fatty acyl linked to a tripeptide and terminated by alaninol. In all known C-type GPLs the alaninol residue is substituted by a O-CH₃-rhamnosyl or dirhamnosyl residue (33). In *M. smegmatis*, a di-O-acylated-6-deoxy-talosyl residue is linked to the allo-threoninyl residue. These simplest forms are found in all GPL-containing mycobacterial species and, accordingly, are called nsGPLs or apolar GPLs. The allo-threoninyl-linked sugar unit of nsGPLs may be further glycosylated in some GPL-containing mycobacteria, such as *M. avium* (33,34). These discrete modifications confer to GPLs variable TLC patterns and antigenic properties (20,33,34). Detailed chemical analysis of the various purified GPLs that occur in *M. smegmatis* demonstrated the existence of a novel class of these molecules passed unnoticed in previous studies (Fig. 9). A combination of mass spectrometry, NMR and chemical degradation techniques established the composition of the main constituents (GPLs IIIa) of this new family as a mixture of molecules possessing a core composed of the long-chain tripeptidyl aminoalcohol with a di-O-acetyl-6-deoxytalosyl unit substituting the allo-threoninyl residue and a 2-succinyl-3,4-di-O-CH₃-rhamnosyl unit linked to the alaninol end. Although various degrees of acetylation, methylation or glycosylation exist in GPLs III, all these molecules have in common the presence of a succinyl substituent.

Structure-function relationship analysis indicated that two structural features of GPLs play a critical role in their inhibitory activity. Firstly, since the 3,4-de-O-acetylation of GPLs I led to an inhibitory effect, part of the GPLs II activity was attributable to the presence of free
hydroxyl groups on the 6-deoxytalose moiety. Secondly, an alkali-treatment of GPLs III, which removes the O-acetyl groups from the 6-deoxytalosyl residues and the succinyl group from the rhamnosyl unit, dramatically abolished the inhibition. This implies that the O-succinylation is the critical structural feature of the inhibition. This agrees with the absence of inhibition with GPLs I which lack the succinyl substituent on the terminal rhamnosyl residue and are 3,4-O-acetylated on the 6-deoxytalosyl moiety. Such a dramatic phenotypical change following the succinylation of a glycoconjugate is not without precedent. For instance, strains of *Rhizobium meliloti*, which have lost their succinyl substituents on exopolysaccharides, became defective in alfalfa nodule invasion (42). Other succinylated glycoconjugates, such as a succinylated arabinomannan identified in *M. tuberculosis* envelope (21) could share receptors with GPLs III. This would explain the inhibitory effect of *M. smegmatis* GPLs III on *M. tuberculosis* at high concentration. However, the new GPLs characterized in *M. smegmatis* exhibit other structural variations (Fig. 9) than succinylation, such as the presence of the second rhamnosyl unit. Therefore, a more detailed structure-function relationship study should be carried out in order to elucidate the precise role of these variations in biological activity of GPLs.

The mechanisms by which surface-exposed GPLs participate in the binding and phagocytosis of mycobacteria deserve consideration. GPLs from *M. avium*, essentially through its lipopeptide fragment, have been reported to disturb cell membrane ultrastructure and to change the expression of surface receptors of murine macrophages (43). In addition, mycobacterial GPLs are able to get inserted into phospholipid monolayers (44) and to disturb its properties (45). Such an insertion of molecules may alter interactions of mycobacteria with their host cell. However, while all GPLs share the same lipid core, only GPLs II and III were active. In addition, GPLs have no effect on the internalization of control particles such as zymosan, suggesting a specific recognition of structurally defined molecules. Taken together,
these observations ruled out a non-specific effect of GPLs under our experimental conditions. Further studies are clearly needed to elucidate the precise mechanisms by which GPLs affect the internalization of mycobacteria, either directly as ligands of receptor and/or indirectly as modulators of a specific receptor function.

In conclusion, the outermost layer of *M. smegmatis* contained new classes of C-type GPLs and phospholipids that efficiently inhibited the non-opsonic phagocytosis of mycobacteria. As such, these molecules may help to design pharmacological drugs with a new therapeutic strategy, consisting in the inhibition of mycobacterial multiplication by preventing their cyclic internalization into macrophages. Mycobacteria are internalized by several receptors into human macrophages. Some of them are pattern recognition receptors, which can elicit different intracellular signals depending on the ligands used (13). Therefore, identification of surface exposed mycobacteria ligands should also help to dissect the signalling pathways of receptors already known to internalize mycobacteria, as well as to discover new receptors involved in the infection of macrophages by mycobacteria.
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LEGENDS OF FIGURES

Fig. 1: Schema of the chemical degradation procedures used to establish the structure of the GPL-like compounds. All the products shown in brackets have been analyzed by MALI-TOF mass spectrometry or GC/MS. R, CH$_3$-(CH$_2$)$_n$-CH=CH-(CH$_2$)$_m$ (m+n = 24~28); DHAB, dehydroaminobutirlyl.

Fig. 2: *M. smegmatis* surface-exposed material specifically inhibits the non-opsonic phagocytosis of mycobacteria. MDMs were incubated at 37°C for 15 min with or without *M. smegmatis* dialysed (1000 kDa cut-off) surface-exposed material (SEM) adjusted to 150 ng protein per ml and then allowed to ingest particles. Macrophages were challenged for 45 min with FITC-stained mycobacteria (25 particles/cell): non-opsonized *M. smegmatis* (*Msm*) and *M. kansasii* (*Mka*) or human serum-opsonized *M. smegmatis* (OS). Cells were then washed, fixed in paraformaldehyde and the remaining extracellular bacteria were revealed by rabbit anti-mycobacterial serum and TRITC-conjugated anti-rabbit IgG. Macrophages were also challenged with 50 particles/cell BSA-coated latex beads (LB) for 1 h, human serum opsonized zymosan (OZ) for 30 min or non-opsonized zymosan (Z) for 1h. Intracellular particles were visualized by staining phagosomal membranes with a lysosomal marker, CD63, revealed with a fluorescent-conjugated secondary Ab. Phagocytosis was determined by fluorescence microscopy as the percentage of macrophages having engulfed at least one particle. The inhibitions are expressed as the percentages of effect when compared to response under control conditions. Data are presented as the mean ± sem of 3 to 11 experiments performed in duplicate. Significance of SEM effect was assessed by a paired Student’s t-test. *, P< 0.05; **, P< 0.01 when compared to untreated conditions.
Fig. 3: The inhibitory property of *M. smegmatis* surface-exposed material is attributable to its lipid content. MDMs were incubated at 37°C for 15 min with *a*) 150 ng protein/ml of *M. smegmatis* surface-exposed material (SEM) treated overnight at 37°C with either 0.1 mg pronase (*P*) or proteinase K (*K*) per mg protein or with 100 mM NaOH for 4 h at 37°C or *b*) 150 ng/ml protein equivalent of the phases obtained after SEM partition: organic phase (*OP*), interphase (*IP*) and aqueous phase (*AP*). Cells were then challenged for 45 min at 37°C with *M. smegmatis* (25 particles/cell) and phagocytosis was determined by fluorescence microscopy. The inhibitions are expressed as the percentages of effect when compared to response under control conditions. Data are presented as the mean ± sem of 3 to 4 (a) and 4 to 8 (b) independent experiments performed in duplicate. Significance was assessed by an unpaired Student’s t-test. *, *P* < 0.05; **, *P* < 0.01 when compared to the effects of untreated SEM.

Fig. 4: The enriched-GPL-like fraction induces a dose-dependent inhibition of *M. smegmatis* phagocytosis. MDMs were preincubated for 15 min with the methanol supernatant (CH₃OH SN) of extractable lipids at concentrations ranging from 1 pg/ml to 100 µg/ml and put in contact with *M. smegmatis* (25 particles per cell) for 45 min at 37°C. The inhibitions are expressed as the percentages of effect when compared to response under control conditions. One experiment representative of 3 independent experiments is shown.

Fig. 5. GPL-like compounds of *M. smegmatis* inhibit its phagocytosis. Methanol-soluble lipids extracted from *M. smegmatis* were separated according to their polarity by adsorption chromatography; elution was carried out with a stepwise gradient of increasing concentrations of methanol and water in chloroform (from 100:0:0 to 65:25:4). *a*) The 21 fractions (noted S1 through S21) obtained after elution of a Florisil column were preincubated at 10 ng/ml with
MDMs for 15 min at 37°C. Cells were then challenged for 45 min at 37°C with *M. smegmatis* (25 particles /cell) and the percentage of phagocytosis was determined. The inhibitions are expressed as the percentages of effect when compared to response under control conditions. Data are presented as the mean ± sem of 3 independent experiments. Significance was assessed by a paired Student’s t-test. *, P< 0.05; **, P< 0.01 when compared to phagocytosis under untreated conditions.

*Fig. 6: MALDI-TOF mass spectra of unfractionated GPL-like compounds.* MALDI-TOF mass spectra of GPL-like-enriched methanol soluble lipids prepared from *a*) whole cells or *b*) surface-exposed material of *M. smegmatis*.

*Fig. 7: Polar GPL-like compounds specifically inhibit the non-opsonic phagocytosis of mycobacteria.* MDMs were incubated for 15 min at 37°C with 10 nM (grey bar) or 10 µM (white bar) purified GPLs from fraction S21. Cells were then challenged for 45 min at 37°C with *M. smegmatis* (*Msm*), *M. avium* (*Mav*), *M. tuberculosis* (*Mtu*), *M. kansasii* (*Mka*) or zymosan (*Z*) (25 particles /cell) and the percentage of phagocytosis was determined. The inhibitions are expressed as the percentages of effect compared to control values. Data are presented as the mean ± sem of 4 independent experiments, except for effects of 10 µM GPLs on *M. kansasii* (n=2). Significance was assessed by a paired Student’s t-test. **, P< 0.01 when compared to phagocytosis under untreated conditions.

*Fig. 8: Compared 1H-NMR spectra of *a*) GPLs I and *b*) GPLs III.* Spectra were obtained in CDCl₃ with a GPL concentration of 10 mg/ml.
Fig. 9: Structures of the GPLs found in *M. smegmatis*. R1, acetyl, excepted for the GPLs IIa where R1=H.

Fig. 10: MALDI-TOF mass spectra of purified GPLs. MALDI-TOF mass spectra of a) apolar GPL I, b) polar GPL III (a and b sub-families) and c) GPL III treated with 0.5 M Na methanolate: 1) pseudomolecular ions (M+Na)$^+$ of the de-O-acylated and $\beta$-eliminated GPL IIIa sub-family, 2) and 3) pseudomolecular ions (M+Na)$^+$ of de-O-acylated GPL IIIa and GPL IIIb sub-families, respectively.
Fig. 1
Fig. 2

Phagocytosis inhibition (%)

Msm  Mka  OS  LB  Z  OZ

0  10  20  30  40  50  60

**  **
Fig. 3

Phagocytosis inhibition (%)

SEM  P  K  NaOH

Phagocytosis inhibition (%)

SEM  OP  IP  AP

Fig. 3
Fig. 4

Phagocytosis inhibition (%) vs. log [CH$_3$OH SN] (mg/ml)
Fig. 5

(a) Phagocytosis inhibition (%) for different CHCl₃/CH₃OH/H₂O compositions:
- CHCl₃/CH₃OH/H₂O 100/0/0
- CHCl₃/CH₃OH/H₂O 65/25/4

(b) [Image of gel showing samples S8, S11, S13, S15, S18, S20, S21]
Fig. 6
Fig. 7

Phagocytosis inhibition (%)

-10  10  30  50  70

Msm  Mav  Mtu  Mka  Z

**  **  **  

Fig. 7
Fig. 8

H$_\alpha$ Phe
aromatic H
-H$_\beta$ Phe
-CH=CH-

-OCH$_3$

-CO-CH$_2$CH$_2$COOH

-CH$_2$-

-CH$_3$

anomeric H

H$_\alpha$ Phe
H$_\beta$ Phe

CH$_3$-CO-

-OCH$_3$
Fig. 9
Fig. 10

a)  

b) Major series of GPLIIla

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b) Minor series of GPLIIlb

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c)  

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Fig. 10
Surface-exposed glycopeptidolipids of mycobacterium smegmatis specifically inhibit the phagocytosis of mycobacteria by human macrophages. Identification of a novel family of glycopeptidolipids

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