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**Mycobacterium abscessus** Glycopeptidolipids Mask Underlying Cell Wall Phosphatidyl-myoinositol Mannosides Blocking Induction of Human Macrophage TNF-α by Preventing Interaction with TLR2

Elizabeth R. Rhoades,* Angela S. Archambault,† Rebecca Greendyke,‡ Fong-Fu Hsu,‡ Cassandra Streeter,§ and Thomas F. Byrd2†¶

*Mycobacterium abscessus* causes disease in patients with structural abnormalities of the lung, and it is an emerging pathogen in patients with cystic fibrosis. Colonization of the airways by nontuberculous mycobacteria is a harbinger of invasive lung disease. Colonization is facilitated by biofilm formation, with *M. abscessus* glycopeptidolipids playing an important role. *M. abscessus* can transition between a noninvasive, biofilm-forming, smooth colony phenotype that expresses glycopeptidolipid, and an invasive rough colony phenotype that expresses minimal amounts of glycopeptidolipid and is unable to form biofilms. The ability of this pathogen to transition between these phenotypes may have particular relevance to lung infection in cystic fibrosis patients since the altered pulmonary physiology of these patients makes them particularly susceptible to colonization by biofilm-forming bacteria. In this study we demonstrate that rough variants of *M. abscessus* stimulate the human macrophage innate immune response through TLR2, while smooth variants do not. Temperature-dependent loss or physical removal of glycopeptidolipid from the cell wall of one of the smooth variants leads to TLR2 stimulation. This response is stimulated in part through phosphatidyl-myoinositol mannosides that are present in the cell wall of both rough and smooth variants. Mannose-binding lectins bind to rough variants, but lectin binding to an isogenic smooth variant is markedly reduced. This suggests that glycopeptidolipid in the outermost portion of the *M. abscessus* cell wall masks underlying cell wall lipids involved in stimulating the innate immune response, thereby facilitating colonization. Conversely spontaneous “unmasking” of cell wall lipids may promote airway inflammation.


*Mycobacterium abscessus* is an important emerging pathogen causing a wide spectrum of disease in both immunocompetent and immunosuppressed patients. It is considered to be the most virulent rapidly growing mycobacterium and is responsible for most pulmonary infections due to rapidly growing mycobacteria (1). *M. abscessus* has also been identified as an important pulmonary pathogen in patients with cystic fibrosis (2).

Colonization of the lung airways before the development of invasive lung disease is a feature common to most nontuberculous mycobacteria (NTM) (3). In diseases in which there is an underlying component of bronchiectasis such as chronic obstructive pulmonary disease and cystic fibrosis, colonization can become chronic and contribute to disease morbidity. There is evidence that colonization of abnormal lung airways by pulmonary pathogens is facilitated by biofilm formation (4). In NTM, including *M. abscessus*, glycopeptidolipids (GPLs) found in the outermost portion of the cell envelope play an important role in biofilm formation (5, 6). GPLs are responsible for the smooth phenotype that occurs in species of NTM where there are smooth/rough colony variants (7, 8). *M. abscessus* can spontaneously change between a smooth form, which expresses GPL, and a rough form, lacking GPL. The smooth form has the ability to form biofilms and colonize surfaces but is unable to multiply in macrophages and cause persistent infection. Conversely, the rough variants lack GPL and are unable to form biofilms on inert surfaces, but they are able to multiply in macrophages and cause persistent infection (5).

In order for pathogenic bacteria to establish biofilms and colonize the lung airways, host defense mechanisms must be impaired. An important first-line lung defense mechanism is mucociliary clearance, which is impaired in patients with cystic fibrosis and primary ciliary dyskinesia, predisposing conditions for lung infection with biofilm-forming pathogens, including *M. abscessus* (9, 10). Another important first-line defense mechanism involves the innate immune response, which is mediated through TLRs present on cells within the lung airways. TLRs have been identified on alveolar macrophages, dendritic cells, and mucosal epithelial cells lining the lung (11, 12). TLRs recognize pathogen-associated molecular patterns, which are conserved motifs expressed by microorganisms, but not by higher eukaryotes (12). TLR interactions...
have been extensively studied in the case of Mycobacterium tuberculosis-derived ligands. An important group of TLR ligands from M. tuberculosis are the phosphatidyl-myooinositol mannosides (PIMs), which bind to TLR2 (13).

In this study we provide evidence for a novel mechanism whereby colonizing bacteria actively avoid host innate immune responses. We demonstrate that PIMs isolated from the cell wall of both M. abscessus smooth and rough variants stimulate macrophage TLR2, leading to TNF-α release. However, in the case of the M. abscessus smooth variant we demonstrate that expression of GPL masks underlying PIMs preventing recognition by macrophage TLR2. Thus, GPLs may facilitate M. abscessus colonization of the lung airways by promoting biofilm formation and by interfering with pathogen recognition by the host innate immune system. Conversely, spontaneous loss of GPL induces airway inflammation through M. abscessus engagement of TLR2.

Materials and Methods

Materials

Pooled human AB serum was purchased from Mediatech, anti-TLR2, anti-IL-8 ELISA BD OptEIA were from BD Biosciences. Iscove’s medium, yeast mannan from Saccharomyces cerevisiae, phosphatidylglycerol (β-oleoyl-α-palmitoyl (C18:1:16:0)), and methyl-α-mannoside were purchased from Sigma-Aldrich. Alexa 488-Con A was purchased from Invitrogen, and FITC-Galantius nivalis agglutinin (GNA) was purchased from United States Biological. Middlebrook 7H9 and 7H11 powders and OADC supplement were purchased from BD Biosciences. Polystyrene beads were obtained from Polysciences; however, their manufacturing process has since changed such that the hydrophobic form used for these experiments is no longer available. Enriched lipomannan and purified lipoorabominan from Mycobacterium smegmatis was obtained from the Tuberculosis Vaccine Testing and Research Materials Contract at Colorado State University.

Mycobacterial strains

The M. abscessus variants 390R, 390S, and 390V were used in these studies. The M. abscessus 390S variant has a smooth colony phenotype, while 390R and 390V variants have a rough phenotype. Briefly, 390R is a rough isolate from a patient that spontaneously disseminated to the smooth variant 390S when subcultured (14, 15). The variant 390V, an isogenic rough revertant, then arose from 390S on subculture (5). All three variants have stable phenotypes with low rates of spontaneous reversion (5, 14, 15). The rough variants are able to grow in human macrophages and cause persistent infection in mice, while the 390V variant lacks these capabilities (5, 16). Unlike the smooth variant the 390V variant does not express large amounts of GPL and is able to form biofilms (5). Note that assessing M. abscessus colony morphology alone is not sufficient for determining the presence of GPL. This is due to the fact that many clinical isolates have an indeterminate or ambiguous morphologic appearance with variable expression of GPL (our unpublished observations). Thus, identification of GPL in specific isolates also requires analysis by thin layer chromatography (TLC). In addition to the 390 variants, we obtained an isolate from Dr. R. Wallace designated MC-2643. This pulmonary isolate is from a cystic fibrosis patient with severe lung disease who underwent lung transplantation. When cultured it was found to consist of two distinct colony phenotypes: a rough phenotype and smooth phenotype, which were then subcultured separately; we refer to these as CF-S and CF-R. Bacteria were maintained as titered frozen stocks stored at ~70°C with intermittent passage for 3 days. Middlebrook 7H11 agar plates supplemented with Middlebrook OADC followed by flash freezing. Subcultures were grown on 7H11 agar or in 7H9 broth supplemented with OADC (7H9) as indicated.

Human macrophage culture

Human mononuclear cells were isolated from buffy coats purchased from United Blood Services as previously described (5), and cultured for 48 h in Teflon jars in Iscove’s 15% normal human serum (medium) at 37°C in 5% CO2 to facilitate monocyte maturation. Human monocytes were isolated by adherence to tissue culture wells (BD Falcon Primaria 24-well plate) followed by incubation for 24 h at 37°C in medium. Immediately before challenge with bacteria or beads, monocyte-derived macrophage (MDM) monolayers were washed twice to remove nonadherent cells.

Lipid purification, lipid analysis, and preparation of lipid-coated beads or liposomes

Cultures were extracted in CHCl3/CH3OH (2:1, v/v, 56°C) twice with sonication in a water bath for 20 min followed by extraction overnight at 4°C. Combined extracts were filtered through 0.22-μm polytetrafluoroethylene (Millipore), dried, and stored at ~20°C. To fractionate lipids, crude extracts were loaded onto a silica gel60 thin layer chromatography plate (EMD Chemicals) in CHCl3 and eluted stepwise with increasing concentrations of CH3OH. GPL and trehalose dimycolate (TDM) co-eluted with 9–10% CH3OH and were loaded onto a C18 reverse-phase column (Sep-Pak; Waters) in CH3OH. GPLs were isolated by virtue of their inability to be retained while TDM was eluted with 50–60% CHCl3. PIMs were eluted from the LC columns with 28–60% CH3OH. Preparative TLC in CHCl3/CH3OH/H2O (60:20:5, v/v) on Al-backed silica gel60 TLC plates (EMD Chemicals) was used to purify total PIMs, or the major monocyst and diacyl dimannosides (PIM5). Total lipids were visualized with ethanolic H2SO4 (50%) followed by heating with a heat gun until the characteristic colors of the heated lipids appeared. GPLs were selectively visualized by spraying with α-naphthol (10%) and heating. PIM5 were also purified from late-log cultures of Mycobacterium bovis bacillus Calmette-Guérin for use as a standard. The purities of the fractions were verified by two-dimensional TLC, and no endotoxin was detected using a Limulus amebocyte assay (Cambrex) (data not shown).

To verify that other mannolysolated glycolipids were not present, purified PIM5 and crude lipid extracts were analyzed by periodate oxidation. Briefly, samples were separated in a 15% SDS-PAGE gel, fixed (methanol/acidic acid, 10%; 5% periodic acid, 5 min), fixed (methanol/acidic acid, 5%/7%, 5 min followed by aqueous glutaraldehyde, 2%, 5 min), washed for 2 h, stained (dithiothreitol, 0.0025%, 5 min followed by silver nitrate, 0.1%, 5 min), and developed (sodium carbonate (3%) formaldehyde (3 drops)/200 ml water), 10 min).

Polystyrene beads were passively coated in an excess of purified lipids in a heated sonicator bath (56°C). Briefly, lipids (40 μg) were dried onto the walls of Eppendorf tubes. Beads (2.27 × 105; 2 μm in diameter) were added in endotoxin-free PBS, and they underwent multiple rounds of vortexing and sonication and were washed at 10,000 relative centrifugal force. To generate lipid vesicles, lipids were vortexed and sonicated in repeated rounds at 56°C in PBS in the presence of 25-μm diameter beads (200 μg of lipid/2.18 × 107 beads). The sample was chilled, washed in PBS at 10,000 relative centrifugal force, and the supernatant that contained most of the lipid vesicles was collected.

Surface removal of GPL from intact M. abscessus

Bacilli were grown in 7H9 for 6 days, washed in PBS/Tween 80 (0.01%), and divided among glass tubes. Bacterial wet pellets were extracted in isopropanol (or PBS as a negative control) for 5–15 min and then pelleted. Extracts were saved for TLC, and pellets were washed in PBS/Tween 20 to remove isopropanol residue. Isopropanol-extracted bacilli did not exhibit consistent viability as determined by plating on agar; therefore, isopropanol-extracted PBS-treated bacilli were used for LPS-assays. Beads were washed at 65°C for 1 h before addition to macrophage cultures. For TLC analyses, isopropanol-extracted and PBS-treated bacteria were subsequently extracted in CHCl3/CH3OH (2:1, v/v) to extract any remaining lipids. Extracts were resolved on TLC plates in CHCl3/CH3OH/H2O (100:14:0.8 (v/v) to resolve GPLs or 60:30:6 (v/v) to resolve PIMs).

Induction of macrophage TNF-α release (by M. abscessus or PIM-coated beads)

MDM monolayers in tissue culture wells received M. abscessus variants at a final concentration of ~2.5 × 107 to 2.5 × 108 bacteria per 500 μl (bacteria to macrophage ratio in the range of 0.1–10 to 1). Alternatively, 2-μm-diameter polystyrene beads coated in total PIMs were added at a final concentration of 0.053% solids (~2.5 × 107 beads). Monolayers were incubated at 37°C in 5% CO2, and supernatants were removed at various intervals after infection. Supernatants were filtered through 0.22-μm filter units and stored at ~70°C for later determination of TNF-α levels. Additionally, nucleic acid counts in replicate wells were determined at each time point, and TNF-α was standardized to 106 nuclei to account for any differences in macrophage number in monolayers subjected to different experimental treatments or conditions (5). We have found that this is critical for meaningful interpretation of results since it allows for standardization among different experimental groups. Additionally, there is substantial variation in tissue culture adherence of MDM from different donors, and thus this also allows for meaningful comparisons between experiments. Similarly, viability was analyzed in replicate infected wells by trypan blue exclusion at each time point to ensure that the
variants did not have a differential effect on macrophage viability independent of bacterial growth (5).

**Stimulation of TLR-transfected HEK-293 cells by M. abscessus variants**

HEK-293 cells stably transfected with human TLR2 gene or a control vector (InvivoGen) with IL-8 as the readout were cultured in 24-well plates in DMEM, 10% FBS. Before starting experiments with M. abscessus variants, cells were tested and found to be responsive to TLR2 agonists. Before specific treatment, medium was removed and replaced with Iscove’s medium, 5% normal human serum. Cells were challenged with 2.5 × 10⁵ M. abscessus CFU/mL supernatants, harvested 2 h after treatment, filtered through 0.22-μm filter units, and stored at −70°C for later determination of IL-8 levels. Additionally, nuclear counts in replicate wells were determined at each time point, and IL-8 was standardized to 10⁵ nuclei to account for any differences in variation of cell numbers between experiments. Viability was analyzed in replicate wells by trypan blue to ensure that variants did not have a differential effect on cell viability.

**Mass spectroscopy of the M. abscessus cell wall**

To analyze PIMs, total lipids were extracted from plate- or broth-grown, late-log cultures and analyzed in an unfractionated state as described previously (17, 18). MALDI-TOF MS analyses were performed in the negative ion mode on a Voyager DE STR mass spectrometer (PerSeptive Biosystems) equipped with a 337-nm nitrogen laser and delayed extraction. Analyses were conducted in the reflector mode at a mass range of m/z 1500–3000 with an accelerating voltage of 20 kV and a delay time of 30 ns. HABA (2-(p-hydroxyphenylazo)benzoic acid) at 10 μg/μl in 50% ethanol-1% trifluoroacetic acid was used as a matrix. The final mass spectra were from an average of 5–10 spectra, in which each spectrum is a collection from 200 laser shots. The structures of the major PIM species were confirmed by multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization. Purified TDM samples were submitted for analysis confirmed by multiple-stage quadrupole ion-trap mass spectrometry with TDM (82°C, 25 min). Labeled bacteria were washed in PBS/Tween 20, or labeled vesicles were washed in PBS. All were analyzed on a FACSCalibur flow cytometer (BD Biosciences) immediately. Nonlabeled bacteria or vesicles were used as nonfluorescent controls. Events were gated on nonclumped bacteria or a uniform vesicle size and analyzed using FlowJo software (Tree Star).

**Statistical analysis**

Statistical significance was analyzed with Student’s t test for unpaired data. Values of p < 0.05 were considered to be significant.

**Results**

**TNF-α release and TLR2 stimulation in response to M. abscessus GPL variants**

TNF-α is a powerful proinflammatory mediator of the innate immune response to mycobacteria. We compared the ability of our three M. abscessus variants grown at 37°C to induce TNF-α release from human MDM. The rough variants, 390R and 390V, induced substantially more TNF-α than did the 390S variant (Fig. 1A). The relative decrease in TNF-α release in response to 390S was not due to a suppressive effect of 390S on MDM function since simultaneously adding both 390S and 390V to MDM induced a similar amount of TNF-α release compared with 390V alone (data not shown). TNF-α levels were assessed at early time points out to 8 h to assess the innate immune response of human macrophages upon initially encountering M. abscessus and to avoid confounding variables: beyond 8 h, substantial bacterial multiplication occurs (in the case of variants 390R and 390V), or the process of bacterial degradation and clearance has begun (in the case of strain 390S) (5, 14).

Release of TNF-α can be induced by engagement of macrophage TLRs, and TLR2 has multiple mycobacterial ligands (19). The macrophage innate immune response to mycobacteria is mediated in part through TLR2. To determine whether TNF-α release induced by our M. abscessus rough variants is due to engagement of TLR2, we examined the ability of 390V to induce TNF-α release in the presence of blocking Ab to TLR2. At both 2 and 4 h there was a significant decrease in TNF-α release in the presence of anti-TLR2 Ab, but not isotype control Ab (Table I). TNF-α release was almost completely blocked at 2 h with some increase at 4 h, which could be due to dissociation of anti-TLR2 Ab from TLR2 receptor with increasing time in culture. For this reason, 2 h was chosen as the time point for assessment of TNF-α release in the presence of anti-TLR2 Ab in subsequent experiments. Additionally, since we used human monocytes as the source for our MDM, there is substantial variability in the number of cells in MDM monolayers. This is likely due to donor-specific differences in MDM adherence in cell culture. To allow for comparison of data across experiments, TNF-α release is corrected to 10⁵ macrophages (Table I), and data are presented in this format in subsequent experiments. In addition to 390V, the ability of 390R to induce TNF-α release in the presence of anti-TLR2 Ab was also significantly decreased (Fig. 1B). In contrast to anti-TLR2, anti-TLR4 Ab had no effect on TNF-α induction by either 390R or 390V (data not shown). LPS induced a strong TNF-α response from MDM, and this was partially blocked by anti-TLR4 Ab but was not affected by anti-TLR2 Ab (data not shown). These results indicate the specificity of the anti-TLR2 effect.

The specificity of the TLR2 response was also assessed by examining the interaction of M. abscessus 390 variants grown at 37°C with HEK-293 cells stably transfected with the human TLR2 gene with IL-8 expression as the readout for TLR2 stimulation. M. abscessus 390R markedly stimulated TLR2, compared with 390S (Fig. 1C). There was no release of IL-8 in response to either variant by HEK-293 cells transfected with vector alone (data not shown).

To determine whether our findings with the M. abscessus 390 variants are generalizable to other clinical isolates, we examined induction of TNF-α and stimulation of TLR2 by a smooth GPL-expressing variant (CF-S) and a rough non-GPL-expressing variant (CF-R) obtained from the same sputum sample produced by a patient with cystic fibrosis (MC-2643). These were grown at 37°C and, as with the 390 variants, the rough variant CF-R induced TNF-α release by MDM and stimulated TLR2, while the smooth variant CF-S did not (Fig. 1, D and E). Consistent with our 390 variant findings, CF-S expressed GPLs while CF-R did not (Fig. 1F).

In the course of our investigation we noted a change in the morphology of M. abscessus 390R bacterial colonies originally cultured on 7H11 plates at 37°C and then left at room temperature. The colonies developed a glistening, smooth appearance when cultured on 7H11 plates at 37°C and then left at room temperature. The colonies developed a glistening, smooth appearance when cultured on 7H11 plates at 37°C and then left at room temperature. The colonies developed a glistening, smooth appearance when cultured on 7H11 plates at 37°C and then left at room temperature.
response to temperature. To assess this possibility, we simultaneously cultured *M. abscessus* 390R from the same frozen stock on 7H11 plates for 7 days at 37°C and at 23°C. *M. abscessus* 390S and 390V from stocks created at the same time were also cultured at these temperatures for comparison. Gross examination of plates at 7 days showed 390R colonies at 23°C to be glistening and smooth. The variant 390R at 37°C had a rough appearance, while both 390S and 390V had their characteristic smooth and rough colony morphologies at both temperatures (data not shown). We next assessed the ability of *M. abscessus* 390 stock cultures grown...
respectively. and 390S at 23°C, respectively; lanes 3
Table I. Effect of Ab to TLR2 on TNF-α release from human MDM in response to challenge with M. abscessus 390V

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>MDM No. ($10^6$)</th>
<th>TNF-α (pg)</th>
<th>TNF-α (pg)/10^6 MDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
<td>2452 ± 29</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Anti-TLR2</td>
<td>3.9</td>
<td>3 ± 5</td>
</tr>
<tr>
<td></td>
<td>Isotype Ab</td>
<td>3.9</td>
<td>2444 ± 192</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>3.4</td>
<td>2766 ± 172</td>
</tr>
<tr>
<td></td>
<td>Anti-TLR2</td>
<td>3.5</td>
<td>490 ± 117</td>
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<tr>
<td></td>
<td>Isotype Ab</td>
<td>3.7</td>
<td>2315 ± 460</td>
</tr>
</tbody>
</table>

* Human MDM were preincubated with Ab to TLR2 or isotype control Ab and then challenged with M. abscessus 390V. Culture supernates were collected at 2 and 4 h after addition of bacteria and assayed by ELISA for TNF-α. Data represent the mean of triplicate determinations supernates ± SD. * p < 0.01 comparing 390V plus anti-TLR2 to 390V and 390V plus isotype control Ab at both 2 and 4 h.

at different temperatures to induce TNF-α release from MDM and stimulate TLR2 expressed by transfected HEK-293 cells. Whereas M. abscessus 390R grown at 37°C induced TNF-α release from MDM, 390R grown at 23°C did not (Fig. 2A). Similarly, 390R at 37°C stimulated TLR2 in HEK-293 cells, while 390R at 23°C did not (Fig. 2B). The 390V stocks grown at either temperature stimulated TLR2 to a similar extent (Fig. 2B). To assess whether differences in GPL expression were accounting for these phenotypic differences, we performed TLC on extracted lipids from our 390 variants grown at temperatures from 23°C to 37°C (data not shown). At 23°C 390R made the full complement of GPLs, identical to those found in 390S, while 390V primarily expressed the less polar GPLs (upper bands in Fig. 2C). As temperature increased, 390R gradually lost its ability to express GPL until there was little to no detectable GPL expressed at 37°C. At 37°C, 390V had also completely lost its ability to express the less polar GPLs. M. abscessus 390S expressed the full complement of GPLs over the range of all temperatures tested. These results suggest that when expressed, the full complement of M. abscessus GPL species confer a smooth colony phenotype and mask cell wall lipids that are involved in stimulating the innate immune response.

Effect of removal of GPL from M. abscessus 390S on macrophage TLR2 stimulation

To further test the hypothesis that surface GPLs mask underlying cell wall TLR2 agonists, we examined the ability of various solvents to selectively remove GPL from the M. abscessus 390S cell wall. Of those tested, isopropanol was the most selective (Fig. 3). While 390S cultures were nonclumping, isopropanol treatment generated GPL-depleted cultures that clumped similarly to 390R and 390V. This altered morphology is consistent with the idea that glycosylated GPLs lie on the bacterial outer surface and mask more hydrophobic lipids that would confer clumping or cording. MDM challenged with isopropanol-stripped M. abscessus 390S secreted TNF-α in amounts comparable to the rough variants and in amounts significantly higher than the 390S control (Fig. 4A). In contrast, isopropanol treatment had no significant effect on the ability of the 390R or 390V strains to induce TNF-α secretion from MDM (data not shown). Incubation with anti-TLR2 Ab almost completely blocked induction of TNF-α by isopropanol-treated 390S (Fig. 4B). These data demonstrate that removal of GPL is correlated directly with the ability of 390S to interact with TLR2. Experiments to coat GPL back onto isopropanol-treated 390S or onto native 390R were inconclusive because we could not adequately reconstitute GPL in the correct orientation, as indicated

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Influence of temperature-dependent GPL expression on induction of TNF-α and stimulation of TLR2. A. Human MDM were infected with M. abscessus variants: 390R grown at 37°C or 23°C. Culture supernatants were collected at 2 and 4 h after addition of bacteria and assayed by ELISA for TNF-α. Data are means ± SEM of two experiments done in duplicate. * p < 0.01 for 390R at 37°C compared with 390R at 23°C. B. HEK-293 cells stably transfected with the human TLR2 gene or control vector were challenged with M. abscessus variants: 390R, 390V, and 390S grown at 37°C or 23°C. Culture supernatants were collected at 2 h after addition of bacteria and assayed by ELISA for IL-8. Data are the means ± SEM for two experiments done in duplicate. * p < 0.01 for 390R at 37°C compared with media and 390R at 23°C. C. Equal weights of total lipid extracts from 390S, 390R, and 390V were resolved in CHCl3/CH3OH/H2O (65:25:4 or 100:14:0.8, by v/v) and visualized with 1-naphthol, which detects GPL. Lanes 1 and 2 show 390S at 37°C and 390S at 23°C, respectively; lanes 3 and 4, 390R at 37°C and 390R at 23°C, respectively; and lanes 5 and 6, 390V at 37°C and 390V at 23°C, respectively.
by failure to abolish clumping of the coated bacteria (data not shown).

**Mass spectral analysis of cell wall glycolipids of M. abscessus variants**

Mycobacterial PIMs are TLR2 ligands (20, 21). To compare the PIM content among the M. abscessus variants, we performed MALDI-TOF spectral analysis of their chloroform/methanol-extractable lipids. Mass spectrometry indicated that the PIM content of broth-grown 390R, 390S, and 390V was nearly identical, consisting primarily of monoacyl- and diacyl-PIM$_2$ with lesser proportions of monoacyl- and diacyl-PIM$_6$ or PIM$_3$ (Fig. 5). Phosphatidyl myo-inositol was also present in all extracts. The PIM content of plate-grown cultures was similar to broth cultures with a predominance of monoacyl-PIM$_2$ and phatidyl-myoinositol (data not shown). Mycobacterial lipomannan (LM) and lipoarabinomannan (LAM) are mannosylated glycoconjugates that have been reported to elicit TNF-$\alpha$ (22, 23). The extensive glycosylation of LM and LAM precludes their co-purification by our chloroform/methanol or isopropanol extraction methods. Nevertheless, we verified that no LM or LAM was present using periodate oxidation and TLC (supplemental data). TDM is another cell wall glycolipid that has been reported to be a potent inducer of TNF-$\alpha$ from macrophages (24). The MALDI-TOF spectra of trehalose mycolates from the strains of M. abscessus 390 were not noticeably different. Mono- and dimycolates of trehalose were expressed in similar proportions, exhibiting major [M$^+$/H$^+$/Na$^+$] ions of trehalose monomycolate and TDM between $m/z$ 2187 and 2631 (data not shown). Collectively, these data indicate that the complement of extractable monoacyl- and diacyl-PIMs and TDM did not differ noticeably between the M. abscessus 390 variants.

**Challenge of MDM with M. abscessus lipids coated onto polystyrene beads**

To explore a potential role for TDM or PIMs in macrophage TLR2 stimulation we purified these lipids and coated them onto hydrophobic polystyrene beads for challenge experiments using MDM. M. abscessus TDM purified from the 390R variant stimulated substantial TNF-$\alpha$ release when coated onto 90-, 25-, and 10- $\mu$m beads and incubated with MDM over a 6-h period, but it was not stimulatory when coated onto 2-$\mu$m beads (data not shown). A similar dependence on particle size for TDM has been reported by guest on August 31, 2017 http://www.jimmunol.org/ Downloaded from

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4 The online version of this article contains supplemental material.
previously for *M. bovis* bacillus Calmette-Guérin-derived TDM (25). Since the 2-µm bead size more closely approximates the size of an individual bacterium, it is likely that this particle size more accurately represents a bacterium in the initial bacteria-macrophage encounter than do the larger beads. As a result, we concluded that TDM was not the lipid responsible for rapid induction of TNF-α via TLR2. Hence, we assessed the ability of purified PIMs to stimulate TNF-α release from MDM. Two-micrometer beads were coated with a PIM fraction that contained mainly monoacyl and diacyl forms of PIM2, PIM3, and PIM6 from the different variants, and these beads were used to stimulate MDM. Rapid TNF-α secretion from MDM occurred within 2–6 h in response to 2-µm beads coated with PIM fractions from the 390R, 390V, and 390S variants, and release was comparable to that released by MDM when challenged with the intact rough variants (Fig. 6, A and B). Importantly, the induction of TNF-α by the PIM-coated 2-µm beads was completely abrogated by preincubation of macrophages with anti-TLR2 Ab (Fig. 6B). To further demonstrate the importance of the *M. abscessus* cell wall PIM fraction in stimulating macrophage TLR2, we assessed the interaction of purified GPL coated onto 2-µm beads with MDM. GPL-coated beads induced a low level of TNF-α release when compared with control; however, this was not blocked by anti-TLR2 Ab (Fig. 6C). Hence, the data are consistent with our finding that the *M. abscessus* PIM fraction contains the major lipids interacting with macrophage TLR2.

**FIGURE 5.** Mass spectrometric comparison of *M. abscessus* PIMs. Crude lipid extracts of *M. abscessus* (A, 390R; B, 390S; and C, 390V) were analyzed by tandem quadrupole ion MS. Major ions of phosphatidylinositol as well as the monoacyl and diacyl forms of PIM2, PIM3, and PIM6 were present in similar proportions in all variants as indicated.
FIGURE 6. Capacity of lipid-coated beads to trigger TNF-α release from human macrophages, and the effect of anti-TLR2 Ab. A, Human MDM were challenged with 2-μm beads coated with 390V or 390S PIM fractions (25 μg) or with PG-coated beads as a control. Culture supernatants were collected at 4 h after addition of beads and assayed by ELISA for TNF-α. Data are the means of duplicate determinations ± SD shown for PIM-coated beads. *, p < 0.05 comparing PIM-coated beads to PG-coated beads. B, Human MDM were preincubated with Ab to TLR2 or isotype control Ab and then challenged with 2-μm beads coated with the 390R PIM fraction (5 μg) or PG as a control. Culture supernatants were collected at 2 h after addition of beads and assayed by ELISA for TNF-α. Data are the means of triplicate determinations ± SD. The differences in TNF-α levels between the groups receiving GPL-coated beads alone or GPL-coated beads with Ab were not significant.

Accessibility of ligands for mannose-binding lectins on the surface of *M. abscessus* variants

PIMs are increasingly recognized as one of the immunostimulatory mannosylated lipoglyconjugates on the surface of the mycobacterial cell wall. To assay for the availability of PIMs on the surface the *M. abscessus* variants, we used fluorescently labeled mannose-binding lectins Con A and GNA. *M. abscessus* variants were incubated with either of the fluorescent lectins, and binding was measured by flow cytometry. A high percentage of 390R and 390V variants bound Con A or GNA, whereas a significantly smaller percentage of 390S variant bound the lectins (Fig. 7A). Preincubation with the inhibitors methyl-D-mannoside for Con A or yeast mannan for GNA (Fig. 7A). Preincubation with the inhibitors significantly reduced the percentage of lectin-labeled 390S variant bound the lectins (Fig. 7A). Specificity of lectin binding was demonstrated using the inhibitors methyl-D-mannoside for Con A or yeast mannan for GNA (Fig. 7B). Preincubation with the inhibitors significantly reduced the percentage of lectin-labeled 390R and 390V, but did not affect the proportion of lectin-labeled 390S. The median fluorescence for Con A-bound or GNA-bound 390R or 390V was higher compared with 390S, and preincubation with inhibitors diminished the degree of binding to 390R or 390V (Fig. 7, C and D, filled histograms). The variant 390V bound more of the mannose-binding lectins than did 390R, as indicated by brighter staining in the fourth decade. Significantly diminished binding of the lectins to 390S, despite the fact that this variant contains the same chro-}

In addition to PIMs, the mycobacterial cell wall contains other mannosylated lipoglyconjugates such as LM and LAM, which could be potential binding sites for the lectins. To determine whether Con A was able to bind to PIMs, we measured the binding of Alexa 488-Con A to liposomes of purified *M. abscessus* PIM2, GPL, a mixture of PIM2 and GPL, or PG. Alexa 488-Con A bound to PIM-containing liposomes or liposomes that contained a mixture of PIMs and GPL (Fig. 7E). Notably, the GPLs in the PIM plus GPL liposomes did not inhibit lectin binding, indicating that the orientation, rather than the presence, of GPLs is required to block PIM-lectin interactions. Labeling of PIM in the liposomes was inhibited by preincubation of the lectin with methyl-D-mannoside. The lectin inefficiently labeled GPL or PG in the liposomes. Similar results were collected using FITC-GNA (data not shown). Taken together, the data demonstrate that mannosylated moieties, including PIM2s, are accessible on the surface of rough variants of *M. abscessus*, but not on the GPL-expressing 390S.

**Discussion**

It has been increasingly recognized that mycobacterial cell wall components play an essential role in disease pathogenesis. Our results demonstrate that GPL present in the outermost portion of the cell wall of an *M. abscessus* smooth variant masks underlying PIMs involved in stimulation of the macrophage innate immune response mediated through TLR2. Thus, GPL may play a pivotal role in modulating the host response to this pathogen and perhaps other NTM.

Spontaneous loss of GPL expression, physical removal of GPL, or genetic manipulation resulting in absent or decreased GPL expression leads to altered physical properties of the bacterial surface. The most obvious manifestation of GPL expression is bacterial colony phenotype, which is generally smooth when GPL is expressed and rough when it is absent. This has been demonstrated in naturally occurring variants of both *M. abscessus* and the *Mycobacterium avium*-intracellulare complex (MAC) (5, 8, 14, 15). A study of *S. smegmatis* demonstrated that the hydrophobicity of this NTM is markedly increased in a GPL-deficient transposon.
mutagenesis-derived strain that exhibits a rough colony phenotype and forms large cellular aggregates when grown in liquid medium (26). Similarly, the *M. abscessus* 390R and 390V rough variants used in our study form macroscopic cellular aggregates and pellicles when grown in liquid medium. Microscopically, these bacterial aggregates consist of cords of mycobacteria that appear very similar to those seen in virulent *M. tuberculosis* strains (5). Additionally, removal of GPL from the *M. abscessus* 390S variant with isopropanol in our study resulted in bacterial aggregation and microscopic cordlike organization within the bacterial aggregates. These observations are consistent with our finding that TDM, the mediator of cord formation (27), is present in the three *M. abscessus* variants, and that the physical interaction of TDM molecules from adjacent bacteria is prevented by GPL in the *M. abscessus* 390S variant.

In our study we also report for the first time temperature-dependent expression of GPL by a mycobacterial species. Our 390R variant forms rough bacterial colonies on agar when grown at 37°C, which, when scraped and resuspended, do not readily disperse in aqueous medium. In contrast, when grown at 23°C, the colonies are smooth and hydrophilic in that they do readily disperse in aqueous medium. At 23°C, the 390R variant expresses the full complement of *M. abscessus* GPL molecules, which are absent when the bacteria are grown at 37°C. The 390S variant exhibits the full complement of *M. abscessus* GPL molecules at all temperatures. Interestingly, the 390V variant expresses the less polar GPL bands (higher retention factor values), but relatively little of the more polar GPL bands (lower retention factor values) at 23°C, with complete disappearance of all GPL bands at 37°C. Despite expression of these less polar bands at 23°C, the 390V variant retains a rough colony morphology at this temperature, and the bacterial colonies do not readily disperse in aqueous media (consistent with the greater hydrophobicity of less polar GPL species). Considering the behavior of 390V at both 23°C and 37°C in our assays, the data indicate that the more polar *M. abscessus* GPL species are the mediators of the GPL phenotype that we have observed. Whether temperature-dependent expression of GPL occurs in other *M. abscessus* isolates is currently being investigated in our laboratory.

Specific mycobacterial cell wall components have been demonstrated to elicit TNF-α release from macrophages. Interestingly, TDM isolated from *M. bovis* bacillus Calmette-Guérin is a potent stimulus for TNF-α release from murine macrophages when presented on 90 μm-diameter beads, but not 2 μm-diameter beads (25). This is also our observation using TDM purified from *M. abscessus*. There is evidence that the inflammatory response elicited by TDM is directly related to its presentation in a monolayer configuration (27), which would be approximated by 90-μm beads rather than by 2-μm beads. As a result, the release, accumulation, and association of TDM with lipids of the cell membranes from effector cells (10–100 μm in diameter) or cholesterol deposits within the granuloma may play a role in evoking an inflammatory response that leads to caseous necrosis (25, 27). Furthermore, the roles of TLR2 or TLR4 as ligands for TDM remain undefined (Ref. 25 and D. Bowdish, K. Sakamoto, M. Kim, M. Kroos, S. Mukhopadhyay, C. Leifer, K. Tryggvason, S. Gordon, and D. Russell, submitted for publication). Thus, TDM is likely more potent as a mediator of late inflammatory events in mycobacterial pathogenesis.

In contrast to TDM, PIMs have been implicated as one of the mediators of the immediate innate immune response to *M. tuberculosis*. A secretory product of *M. tuberculosis* in short-term culture filtrates, designated soluble tuberculosis factor, has been found to have TLR2-agonist activity. Subsequent studies demonstrated that the TLR2-agonist activity of soluble tuberculosis factor is due to PIMs (28). Our study demonstrates the presence of abundant PIM2 and lesser amounts of PIM4 and PIM6 in the chloroform/methanol extract of *M. abscessus*. Both PIM2 and PIM6 are the most abundant classes of PIM in the cell wall, demonstrate TLR2-agonist activity (21). The magnitude of the TNF-α response to our
intact \textit{M. abscessus} rough variants and iso-propanol-treated smooth variant is similar to that elicited by purified \textit{M. abscessus} PIMs on 2-µm polystyrene beads (approximating the size of an individual bacterium), suggesting that these may be the major TLR2 \textit{M. abscessus} ligands. While it is clear that the PIMs were stimulatory, it remains to be determined whether other mannosylated lipoglycans such as LM or LAM on the surface of \textit{M. abscessus} also possess TLR2-agonist activity. LAMs are a heterogeneous group of molecules in mycobacteria that demonstrate structure-based proinflammatory or antiinflammatory effects (29, 30, 31). In the case of \textit{M. tuberculosis}, the LAM molecule has a mannosic cap, ManLAM, and the host cell suppressive effect is thought to play a role in the pathogenesis of infection. Whereas in the case of an avirulent rapid grower such as \textit{M. smegmatis}, LAM is capped with phosphatidylinositol moieties (PILAM) that elicit proinflammatory cytokines. It is noteworthy that \textit{Mycobacterium chelonae}, a rapidly growing NTM species closely related to \textit{M. abscessus}, possesses a LAM molecule that, although lacking a mannosic cap, has several unique structural features that appear to render it incapable of stimulating a host cell inflammatory response (23).

Depending on their structure, GPLs have also been found to have immunostimulatory properties. The GPL molecule consists of a tripeptide-amino alcohol core with an amide-linked long chain fatty acid. This lipopeptide core is substituted with 6-deoxy-talose and \textit{O}-methylated rhamnose to generate the nonspecific core GPLs (nsGPLs) found in many species of NTM, including \textit{M. smegmatis}, \textit{M. chelonae}, \textit{M. abscessus}, and MAC (32, 33). These nonspecific GPLs are further glycosylated with a variable heptanic oligosaccharide at 6-deoxy-talose to produce serovar-specific GPLs (ssGPLs) in some mycobacterial species, notably MAC (34). While the nsGPLs of \textit{M. abscessus}, \textit{M. chelonae}, and \textit{M. smegmatis} are structurally identical (33), the variable heptanic oligosaccharide at 6-deoxy-talose present in MAC GPL forms the basis for serotyping MAC strains (34). It has been reported that ssGPLs from smooth variants of \textit{M. avium} serovars 1 and 2 stimulate macrophage TNF-\(\alpha\) release via TLR2, while nsGPLs do not (35). It is noteworthy, however, that rough variants of \textit{M. avium}, which do not express GPL, stimulate even greater amounts macrophage TNF-\(\alpha\) release than do \textit{M. avium} smooth variants (36). The \textit{M. avium} cell wall components stimulating TNF-\(\alpha\) release by the rough variants have not been identified. Since \textit{M. abscessus} possesses only nsGPL, this is consistent with an inability of \textit{M. abscessus} GPL-expressing smooth variants to induce significant TNF-\(\alpha\) release from macrophages, demonstrated in this study and previously reported (16). It is also consistent with our observation that low level TNF-\(\alpha\) induction by purified \textit{M. abscessus} GPL is not mediated through TLR2. Thus, from the standpoint of the macrophage innate immune response, \textit{M. abscessus} GPL is a relatively inert molecule.

It has been demonstrated that both rough and smooth variants of \textit{M. abscessus} occur as clinical isolates, with the rough phenotype predominating in patients with chronic pulmonary infection and the smooth phenotype predominating in wounds and contaminating environmental isolates (Ref. 37 and our unpublished data). We have postulated that initial lung airway colonization is facilitated by the biofilm-forming capability of \textit{M. abscessus} smooth variants, which can spontaneously give rise to rough variants possessing an inflammatory/invasive phenotype (5). Both rough and smooth phenotypes have been isolated from the same patient simultaneously and have been found to belong to a single strain, supporting this hypothesis (Ref. 38 and our unpublished data). The results of our study using previously characterized \textit{M. abscessus} 390 variants (5) and an \textit{M. abscessus} smooth and rough variant isolated from the sputum of a patient with cystic fibrosis support the hypothesis that GPL in the outermost portion of the \textit{M. abscessus} cell wall cloaks underlying PIMs involved in stimulating the innate immune response, thereby facilitating colonization. As far as we are able to determine, this is the first example of a pathogen gaining access to the host in a “cloaked” form, which likely allows it to colonize the lung airways without provoking an inflammatory response.

With spontaneous loss of the “cloaking” molecule, GPL, \textit{M. abscessus} becomes capable of evoking an inflammatory response and invading the lung parenchyma. Since NTM, as a group, are not highly virulent pathogens, this can result in a smoldering process without florid pneumonia. In the case of patients with cystic fibrosis, chronic airway inflammation leads to a progressive and irreversible loss of lung function, indicating that this process is particularly insidious.

When NTM are isolated from the sputum of a patient there is often a question as to whether the isolate signifies colonization or infection, and whether it should be treated. Our study suggests that isolation of an \textit{M. abscessus} rough variant from the sputum of a patient with underlying lung abnormalities due to a disease such as cystic fibrosis should be aggressively treated because of the potential for this morphotype to stimulate the host innate immune response and provoke airway inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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