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Identification of antibody responses to the serotype-nonspecific molecular species of glycopeptidolipids in *Mycobacterium avium* infection

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ABSTRACT

Glycopeptidolipids (GPLs) comprise a major surface glycolipid of *Mycobacterium avium* complex (MAC), and their unique oligosaccharide extensions are known to define MAC serotypes. Beside the mature form of "serotype-specific" GPLs (ssGPLs), those that share the backbone structure but lack the oligosaccharide extensions exist as abundantly in all MAC serotypes, but the presumption was that antibody responses might not be directed to these "serotype-nonspecific" GPLs (nsGPLs) due to the lack of the sugar chain epitope. Here, we show that IgG responses to nsGPLs indeed occur in MAC-infected guinea pigs. The pool of anti-nsGPL antibodies was distinct from that of anti-ssGPL antibodies in terms of requirements for the oligosaccharide and acetylation for their target recognition. Because nsGPLs are shared in virtually all MAC strains, but totally absent in *Mycobacterium tuberculosis*, this study suggests that detecting serum anti-nsGPL antibodies can potentially be useful for differential diagnosis of MAC infection and tuberculosis.

Mycobacterium avium complex (MAC) comprises a group of acid-fast bacteria that are ubiquitous in the environment [1]. The organisms occasionally infect into humans to cause a disease that resembles tuberculosis. Although less virulent than *Mycobacterium tuberculosis*, MAC is now one of the most important opportunistic pathogens in patients infected with human immunodeficiency virus-1, and also, the incidence of clinically overt MAC infection even in immunocompetent individuals has increased significantly in recent years. Because of the multi-drug resistance evolved by the microbes, MAC infection is often very difficult to clear with anti-tuberculosis agents, and therefore, differential diagnosis of these two clinically important mycobacterial infections is crucial for efficient control of the diseases.

Both MAC and *M. tuberculosis* possess lipid-rich cell walls in which mycobacteria-specific long-chain fatty acids, termed mycolic acids, are densely packed in close association with a variety of surface exposed glycolipids, thereby forming the hydrophobic structure that primarily accounts for their acid-fast properties. One of the most striking differences between the two groups of mycobacteria is the presence of the surface exposed glycolipid, glycopeptidolipids (GPLs), in MAC that is totally absent in *M. tuberculosis* [2]. GPLs contain a tetrapeptide backbone (D-phenylalanine-D-*allo*-threonine-D-alanine-L-alaniol), in which *O*-methylated rhamnose (Rha) is linked to the hydroxyl group of the C-terminal alaniol residue and the long chain fattyacyl (C26–C34) is attached to the N-terminal D-phenylalanine. Further, the hydroxyl group of the D-*allo*-threonine is modified with the acetylated (mono-acetyl or di-acetyl) 6-deoxytalose (dTal), on which variable oligosaccharide sequences are elaborated. Structural variations of these oligosaccharide extensions are known to account for serological differences among MAC strains, and therefore, the mature molecular species of GPLs with the fully extended oligosaccharide have been referred to as "serotype-specific" GPLs (ssGPLs) (Fig. 1A). Besides ssGPLs, GPL species that share the backbone structure, but lack the oligosaccharide extensions also exist as abundantly in virtually all MAC strains. These immature GPL species, which are presumably precursors in the biosynthetic pathway of ssGPLs, have been designated as "serotype-nonspecific" GPLs (nsGPLs) because of the lack of the serotype determinants (Fig. 1B).

It has been thought that antibody responses directed against nsGPLs are not elicited during MAC infection [2–4]. We now show that IgG responses to nsGPLs with acetylated dTal are indeed mounted in MAC-infected guinea pigs. Identification of antibodies against a microbial component that is shared in all MAC strains, but absent in *M. tuberculosis*, is of significant medical implications, including differential serodiagnosis of MAC infection and tuberculosis.

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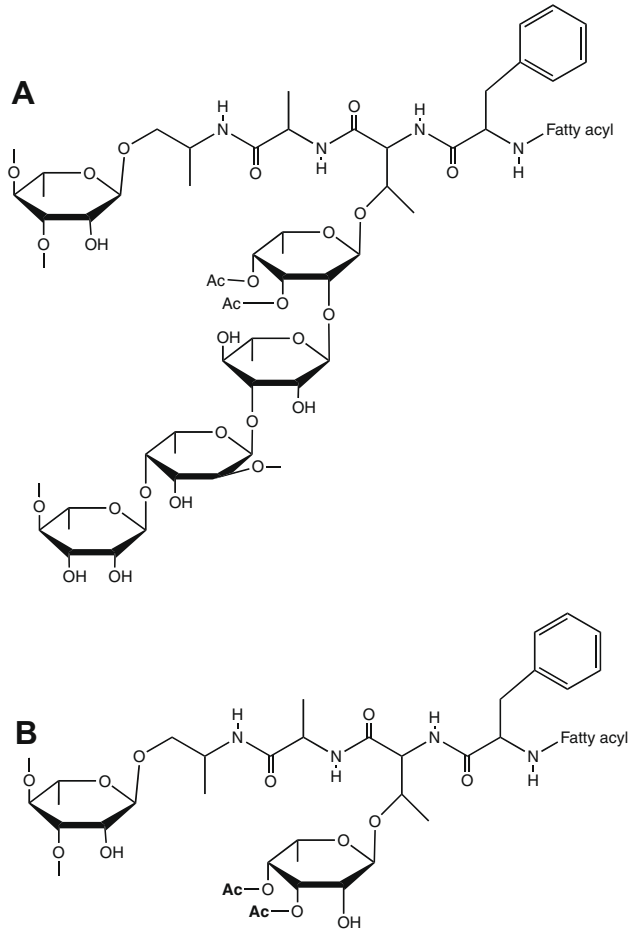

Fig. 1. The basic structure of MAC serovar 4-specific ssGPL (A) and nsGPL (B).

Materials and methods

Bacteria and chemical reagents. *Mycobacterium avium* ATCC 35767 (serovar 4) was obtained from American Type Culture Collection (Manassas, VA). *M. avium* JATA-51-01 (serovar 2) and KK41-445 (serovar 1) were kindly provided from Research Institute of Tuberculosis (Tokyo, Japan). Bacteria were grown in Middlebrook 7H9 broth media (Becton Dickinson, Franklin Lakes, NJ) supplemented with the albumin-dextrose-catalase (ADC) enrichment (Becton Dickinson) and 0.05% Tween 80. For determination of colony-forming units (CFU) of the bacteria preparations, the bacteria were grown on 7H10 agar plates supplemented with the oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson). Chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated.

Infection of guinea pigs with MAC. Four- to six-week-old female inbred strain 2 guinea pigs were purchased from Japan SLC, Inc. (Shizuoka, Japan), and housed under specific pathogen-free conditions. *M. avium* serovar 4 (5×10^7 CFU) was suspended in phosphate-buffered saline (PBS) and injected intradermally in the upper hind legs. Mock infection was done in parallel by injecting an equal volume of PBS alone. Sixteen weeks after infection, sera were collected and the aliquots were stored frozen until assays were performed. In some experiments, booster infection was done 4 weeks after the primary infection, and the hyperimmune sera were collected 3 weeks after the second infection. All animal experiments were performed according to the institutional guidelines on animal welfare and humane treatment of laboratory animals.

GPL purification. Total lipids were prepared from each MAC strain as described previously [5]. The lipids (~10 mg) were dissolved in 1 ml of chloroform:methanol (C/M, 2:1, vol/vol), and 30 ml of ice-cold acetone was added. After incubation on ice for 20 min and centrifugation at 1,500g for 15 min at 0 °C, the supernatant, referred to as the acetone-soluble fraction, was carefully collected, which contained nsGPLs, ssGPLs and phospholipids, but not trehalose mycolates. Lipids in the acetone-soluble fraction were dried under a nitrogen gas flow and then dissolved in a small amount of chloroform, followed by loading onto a silica gel column (Alltech, Newark, DE; 23 ml bed volume) equilibrated with chloroform. After washing the column with 40 ml of chloroform, nsGPLs were eluted with 40 ml of acetone and subsequently, ssGPLs and phospholipids were eluted with 50 ml of methanol. The eluted nsGPL species were visualized as two clusters of spots on a silica gel G thin-layer chromatography (TLC) plate (Analtech) developed with a solvent of C/M (95:5), and therefore, the upper cluster of spots with the larger *Rf* values and the lower cluster of spots with the smaller *Rf* values were purified separately from the TLC plate. The eluted ssGPL species were resolved as two major spots with a couple of additional minor spots on a silica gel TLC plate developed with a solvent of C/M/water (90:15:1, vol/vol/vol), and the two major species of ssGPLs were purified from the TLC plate and used for experiments. The nsGPL and ssGPL preparations thus obtained represented their native forms that were not subjected to deacetylation during the purification processes, and therefore called "intact" GPLs in this study.

For artificial deacetylation of GPLs by mild alkaline hydrolysis, the GPL preparations were treated with 0.2 M NaOH in methanol at 37 °C for 1 h. After neutralization with HCl, 2 vol of chloroform and 1 vol of water were added, and the organic layer containing deacetylated GPLs was collected. To isolate the serovar 4-specific oligosaccharide, β-elimination reaction for ssGPLs was performed for ssGPLs as described previously [6]. Briefly, ssGPLs were dissolved in 1 ml of ethanol and 1 ml of $NaBH_4$ (10 mg/ml in 0.5 N NaOH) was added. After overnight incubation at 60 °C, 2 ml of methanol was added and then, the solution was neutralized with Dowex 50W-X8 (Bio-Rad Laboratories, Inc., Hercules, CA). After centrifugation, the supernatant was collected and dried under a nitrogen gas flow. The residue was dissolved in 2 ml of C/M (2:1) and an equal volume of water was added. The aqueous phase was collected and used as the oligosaccharide preparation.

Mass spectrometry. Mass spectrometric analysis of the GPL preparations was carried out, using an electrospray-ion trap-time of flight mass spectrometer (Shimadzu LCMS-IT-TOF, Shimadzu Co. Ltd., Kyoto, Japan). Samples were dissolved in C/M/water (10:85:5, vol/vol/vol) containing 0.1 mM sodium acetate and directly infused into the mass spectrometer with a flow rate of 2 μl/min. Mass ions were detected in the positive mode with the spray voltage at 1.5 kV and the capillary temperature at 200 °C. Collisionally-induced dissociation-mass spectrometry (CID-MS) was performed with 70–80% of the maximum collision energy. The trapping of product ions was performed with a *q* value of 0.25.

TLC immunostaining. Fifty microgram of the acetone-soluble fractions from each MAC strain were applied onto a silica gel TLC plate (Polygram Sil G, Macherey-Nagel GmbH & Co., Düren, Germany) and developed with C/M (9:1). Simultaneously, the replica was also made for visualization of all the lipids by spraying with 50% sulfuric acid. After drying, the plate was incubated with PBS containing 5% bovine serum albumin at 4 °C overnight and washed with PBS containing 0.05% Tween 20 (PBS-T). Subsequently, the plate was incubated for 2 h at room temperature with the MAC-infected guinea pig serum that was diluted at 1:500 in PBS-T. After washing 3 times with PBS-T, the plate was incubated for 1 h at room temperature with horseradish peroxidase-conjugated antibodies against the Fc portion of the guinea pig IgG (Nordic

Immunological Laboratories, Tilburg, The Netherlands) at a dilution of 1:2,000 in PBS-T. After washing 3 times with PBS-T, the labeled lipids were visualized by incubation with diaminobenzidine (0.6 mg/ml) in the presence of hydrogen peroxide.

Enzyme-linked immunosorbent assay (ELISA). The GPL preparations were dissolved in hexane/isopropanol (3:2, vol/vol), placed into wells of Nunc Immuno plates (Roskilde, Denmark) at 50 μl per well (~390 pmol of GPLs, a 500 ng equivalent of the intact upper cluster nsGPL, per well), and air-dried until solvents were evaporated completely. The wells were then blocked with 200 μl of the assay diluent (Becton Dickinson) for at least 1 h at room temperature and washed with PBS-T. Fifty microliters of the sera from MAC-infected guinea pigs were added to the wells at indicated dilutions. After incubation for 2 h at room temperature, the wells were washed with PBS-T, and 50 μl of PBS-T containing the peroxidase-labeled anti-guinea pig IgG Fc antibodies (1:2000, Nordic) were added. After 1 h incubation, the wells were washed with PBS-T, and bound antibodies were detected with the TMB immuno detection kit (Thermo Scientific, Rockford, IL). The detection reaction was performed at room temperature for 15 min. Absorbance at 450 nm and at 570 nm (background) was measured by an ELISA plate reader (VersaMax-W, Molecular Device Corp., Sunnyvale, CA). In the neutralization experiments with oligosaccharides, the diluted guinea pig sera were preincubated for 30 min at room temperature with an excess amount of the serovar 4-specific oligosaccharide at a molar ratio of 200:1, followed by ELISA as described above.

Results and discussion

It has been thought that antibody responses to GPLs are directed solely to the oligosaccharide portion of ssGPLs, but not to nsGPLs that lack the sugar chain [2–4]. This presumption, however, has been based on experimentation using artificially deacetylated species of GPLs purified after mild alkaline treatment. Considering the possibility that deacetylation might result in apparent loss of antibody reactivity to nsGPLs, we prepared native acetylated species of GPLs and antibody reactivity to these "intact" GPL species was examined. Lipids in the acetone-soluble fraction obtained from different MAC strains were resolved on a silica gel TLC plate, and visualized with 50% sulfuric acid. As shown in Fig. 2A, intact ssGPLs derived from serovar 1 (lane 1), serovar 2 (lane 2), and serovar 4 (lane 3) MAC strains were detected as spots with highly variable R*f* values, reflecting their distinct size and molecular composition of the sugar chain. On the other hand, intact nsGPLs were detected as a cluster of spots with similar R*f* values in all lanes, consistent with their being shared in all MAC strains. To examine their antibody reactivity, the replica plate was generated, and immunolabeling was performed with the immune sera that were obtained from guinea pigs infected twice with the serovar 4 MAC strain (Fig. 2B). As expected, the sera labeled ssGPLs from MAC serovar 4, but not those from other MAC strains. In sharp contrast, nsGPLs from all the three MAC strains were labeled similarly throughout the lanes, suggesting the presence of specific antibodies against the serotype-nonspecific molecular species of GPLs.

To determine the molecular identity of the nsGPL species, the nsGPL cluster was purified from MAC serovar 4, and analyzed by mass spectrometry. Three discrete species were separated on a TLC plate developed with a solvent of C/M (9:1) (Fig. 3A). Mass spectrometric analysis revealed several ion peaks that differed by 14 mass units, likely to represent nsGPL species with different numbers of the methylene unit (Fig. 3B). CID-MS for the dominant signal with *m/z* 1295 was consistent with sodium adduct of nsGPL species containing di-acetyl-dTal, di-*O*-methyl-Rha, and the hydroxy fatty acid with C32:2 (Fig. 3C). CID-MS analysis for each of the other signals detected in Fig. 3B confirmed that most of the fragment ions were derived from the nsGPL molecular species mentioned above that only differed in the length of the acyl chain. Some of the fragment ions did not match precisely with the theoretical fragmentation patterns of this nsGPL. Therefore, we performed deacetylation of the nsGPL preparation and further purification of each deacetylated molecular species by TLC. MS analysis of these deacetylated molecular species revealed that this preparation contained at least 3 other molecular species; namely, nsGPL species containing either mono-*O*-methyl-di-acetyl-dTal and di-*O*-methyl-Rha, mono-*O*-methyl-mono-acetyl-dTal and di-*O*-methyl-Rha, or mono-*O*-methyl-mono-acetyl-dTal and mono-*O*-methyl-mono-acetyl-Rha (data not shown). These structural variations on the sugar residues were consistent with those reported in previous studies [7]. Thus, the current study has provided evidence for the first time that specific antibody responses to the serotype-nonspecific molecular species of GPLs are induced by MAC infection.

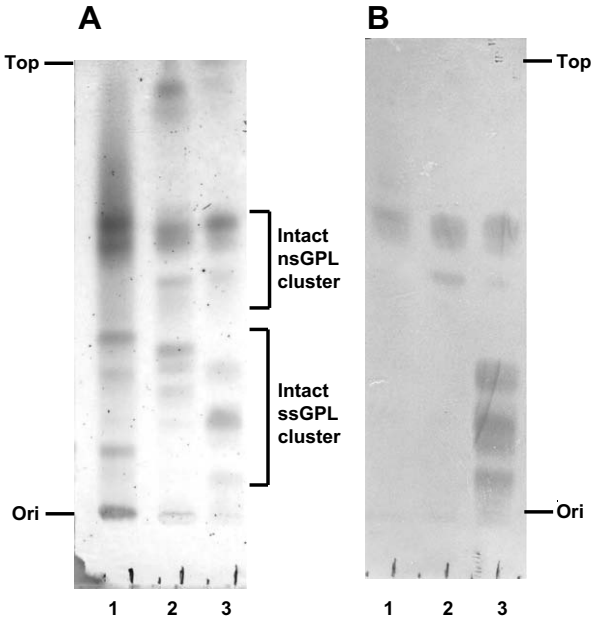

Fig. 2. Immune recognition of intact nsGPL species. (A) Acetone-soluble lipid fractions from MAC serovar 1 (lane 1), serovar 2 (lane 2) and serovar 4 (lane 3) were developed on a TLC plate. Lipids were visualized by spraying with sulfuric acid and baking. Clusters of spots corresponding to intact nsGPLs and ssGPLs are indicated with brackets. (B) The replica plate was immunostained with the immune sera that were obtained from a guinea pig infected twice with the MAC serovar 4 strain. No signals were detected when non-immune sera obtained from mock-infected guinea pigs were used (data not shown).

To gain further insight into the molecular basis for the GPL immune recognition, antibody reactivity to ssGPLs and nsGPLs was compared in two different sets of experiments. First, the oligosaccharide consisting of the four hexose sugar residues was prepared from MAC serovar 4 and tested for its capacity to neutralize the antibody reactivity. The guinea pig sera obtained 16 weeks after the primary infection with MAC serovar 4 showed antibody reactivity to both ssGPLs and nsGPLs in the absence of the oligosaccharide (Fig. 4A). When the sera were pretreated with the oligosaccharide, the reactivity to ssGPLs was reduced to the baseline, confirming that the sugar chain was the serotype determinant. In sharp contrast, no significant effect of the oligosaccharide was detected on the reactivity to nsGPLs (Fig. 4A). Secondly, we examined the reactivity of the sera to either intact or deacetylated species of GPLs (Fig. 4B). The sera showed

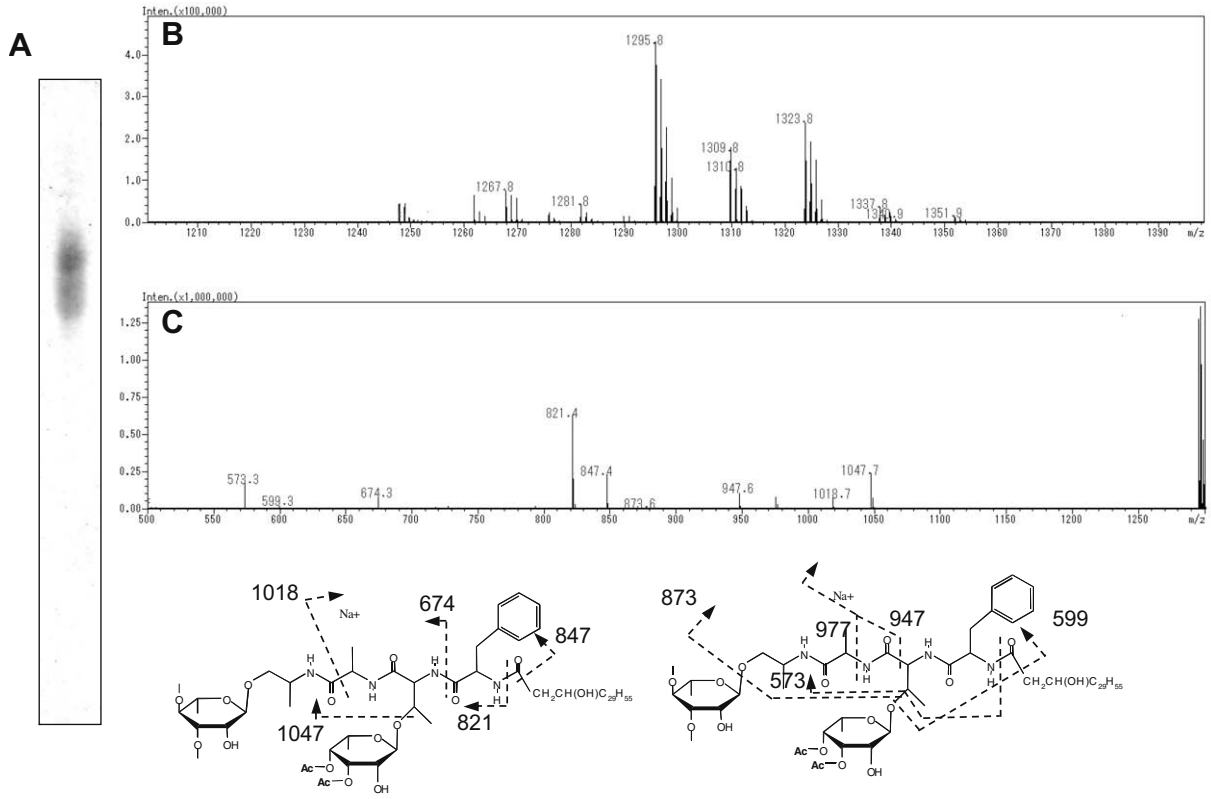

Fig. 3. Molecular analysis of purified nsGPL upper cluster. (A) The thin layer chromatogram of the purified nsGPL cluster, which separated at least 3 distinct molecular species. (B) Mass spectrum of the purified nsGPL cluster. (C) CID-mass spectrum of *m/z* 1295, and schematic interpretation of fragmentation patterns.

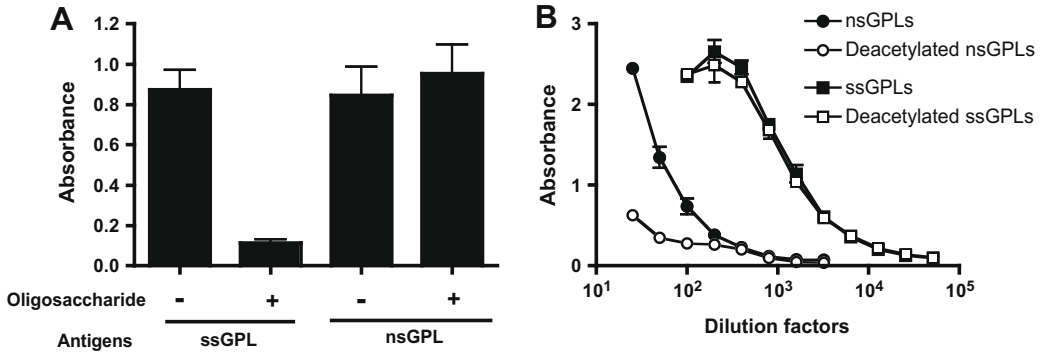

Fig. 4. Detection of anti-GPL antibodies by ELISA. (A) Guinea pig sera obtained 16 weeks after the primary infection with MAC serovar 4 were diluted either at 1:150 (for detection of anti-nsGPL antibodies) or at 1:4000 (for detection of anti-ssGPL antibodies). The diluted samples were incubated with or without excess amounts of the serovar 4-specific oligosaccharide, and then, the reactivity to ssGPLs (with either monoacetylated or diacetylated dTal) and nsGPLs were measured by ELISA as described in Materials and Methods. The results are shown as the means ± standard deviation of triplicate samples. (B) The sequentially diluted guinea pig sera were tested for their reactivity to intact (closed circles) and deacetylated (open circles) nsGPL species as well as intact (closed squares) and deacetylated (open squares) ssGPL species by ELISA.

almost identical reactivity to intact (closed squares) and deacetylated (open squares) ssGPLs, as was the results reported previously [8,9]. On the other hand, the reactivity to nsGPLs was greatly decreased after deacetylation (closed circles for intact nsGPLs and open circles for deacetylated nsGPLs). Taken together, these results emphasize that the pool of anti-nsGPL antibodies is clearly distinguished from that of anti-ssGPL antibodies in terms of requirements for the oligosaccharide and acetylation for their GPL recognition.

Most of previous studies aiming at detecting humoral immune responses to GPLs were carried out by using deacetylated GPLs as the target antigens [4,10–13], resulting in demonstration of only the antibody reactivity to ssGPLs, but not that to nsGPLs. The present study has now identified the antibody response to the purified "intact" GPL species. Given that nsGPLs are shared in all MAC strains, but are totally absent in *M. tuberculosis*, this study immediately raises the possibility that detection of anti-intact nsGPL antibodies in the sera will be useful for differential serodiagnosis of MAC infection and tuberculosis, the two most important mycobacterial infections in humans.

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