Published in final edited form as:

Tuberculosis (Edinb). 2008 November ; 88(6): 560–565. doi:10.1016/j.tube.2008.04.002.

The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface

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Abstract

By labeling surface carbohydrates we found that a pool of lipoglycans, cell wall associated, is exposed at the cell surface of mycobacteria and thus, most probably, inserted in the outer leaflet of the outer membrane. In contrast, plasma membrane anchored lipoglycans are not accessible to surface labeling. This result supports the role of lipoglycans as key immunomodulatory molecules but raises the question of their transport from the plasma membrane, where they are synthesized, to the outermost layers of the envelope, where they can act as modulins. The data are discussed in term of consequences for cell envelope organization.

Keywords

Mycobacterium; lipoglycan; localization; cell envelope; transporter

The lipoglycans, lipoarabinomannan (LAM) and lipomannan (LM), are major immunomodulatory molecules of the mycobacterial cell envelope^{1–4}. Mannose-capped LAM (ManLAM) is a potential ligand for the entry of *Mycobacterium tuberculosis* into phagocytic cells via the host C-lectins⁵. In addition, purified ManLAM reproduces several properties of *M. tuberculosis* that may contribute to the inhibition of the host defense response and define ManLAM as a major virulence factor of the pathogen⁵. In contrast, phosphoinositol-capped LAM (PILAM) and LM stimulate innate immunity *via* signaling through Toll-like receptor 2 (TLR2)⁵. Lipoglycans are delivered from infected macrophages, via exosomes or apoptotic vesicles, to non-infected bystander dendritic cells^{6–8} and thus can modulate the functions of the latter, *via* binding C-lectins⁹ or TLR-2, even though they are not necessarily receptor ligands on the whole bacterium¹⁰. Nevertheless, their role as mycobacterial cell surface adhesins or as soluble molecules released by phagocytic cells implies that they are exposed to

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the cell surface or, at least, located in the outermost part of the cell envelope. However, their precise localization remains unclear. They are not covalently attached to the cell envelope, but they have never been found in culture supernatants or in the surface-exposed material obtained by gentle mechanical treatment of cells with glass beads and/or detergent treatment ^{11,12}, suggesting that they are instead imbedded in the cell wall.

In the present study, the exposition of lipoglycans to the cell surface of mycobacteria was investigated by cell labeling with biotin. The validity of this approach relies on the assumption that labeling is indeed restricted to surface components. BCG cells were grown as surface pellicles in Sauton's medium for 20–25 days. We assumed that these cells had an intact envelope since the culture supernatant was found to be devoid of the cytosolic heat shock protein 65 and of traces of the cell wall polysaccharide, arabinogalactan (AG). Cells were thus submitted to periodate oxidation to generate aldehydic functions in surface exposed-carbohydrates, by incubation for 20 min, at 4°C in the dark with 0.1 M sodium acetate buffer pH 5.5 (buffer A) containing 15 mM sodium metaperiodate (Merck). Oxidized cells were then labeled for 2 h at room temperature in buffer A containing 5 mM of biotin-hydrazide (Sigma) ^{6,13}. Bacteria retained around 98% viability and electron microscopy examinations showed that the whole bacilli morphology (Figures 1B and C) as well as cell wall organization (not shown) of labeled cells were undistinguishable of those of untreated control cells. Moreover, AG, known to be imbedded in the cell wall, was not labeled by biotin as determined by dot-blot and alkaline phosphatase-conjugated streptavidin (AP-streptavidin) detection (Figure 1A, lane f) whereas the crude ethanol/water extract (lane c) of treated cells gave an intense response. Altogether these data led us to conclude that biotin labeling did not affect the integrity of the bacilli and that it was indeed restricted to cell surface-exposed molecules.

Analysis of the lipoglycan fractions

We previously set up a purification protocol where a first lipoglycan fraction, tentatively called “parietal” is obtained directly by ethanol/water extraction of the delipidated cells (Figure 1A), whereas a second one, termed “cellular”, can be obtained only after sonication of the resulting cells ¹⁴. Both fractions, prepared from labeled cells, gave a positive response when probed with AP-streptavidin (Figure 1A, lanes a and b) suggesting that, in contrast to our first hypothesis, molecules contained in these fractions do not differ by a particular localization in the cell envelope but rather by the strength of their association to cell wall material after delipidation, the molecules recovered in the “cellular” fraction being more firmly attached, possibly as a result of a higher acylation degree ^{15,16}. It is well-known that cell wall purification requires SDS extraction steps to remove molecules, including lipoglycans, that remain strongly associated to mAGP complex after cell lysis.

Further analyses were performed on the total lipoglycan pool obtained by water/ethanol extraction of the cells disrupted immediately after delipidation ¹³ (Figure 1A, lane c). Contaminating proteins, glucan and nucleic acids were enzymatically removed yielding a fraction enriched in glycans and lipoglycans, which were further resolved by hydrophobic interaction chromatography (HIC) ¹³. As expected, the glycan fraction, which contains the surface-exposed arabinomannan (AM) and mannan ^{11,12}, was labeled (Figure 1A, lane e). More interestingly, the lipoglycan fraction, found by SDS-PAGE and silver staining to contain only ManLAM and LM, was also biotin positive (Figure 1A, lane d). As previously reported ¹³, its analysis by western blot with AP-streptavidin detection revealed two bands that unambiguously corresponded to biotin-labeled ManLAM and LM. Similar results were obtained with *Mycobacterium xenopi* ¹³ and *Mycobacterium smegmatis* (see further), indicating that ManLAM and LM are exposed to the cell surface of these mycobacteria, where they are accessible to small reagent molecules such as biotin, and thus possibly to host immune receptors.

Lipoglycan localization and transport within the mycobacterial cell envelope

The mycobacterial cell envelope is composed of a plasma membrane, a wall and a capsule¹². The wall contains an outer membrane (OM) where the inner leaflet is made of arabinogalactan-bound mycolic acids and the outer leaflet contains the extractable lipids^{17–22}. In this context, three hypotheses have been proposed regarding the localization of LAM. LAM molecules could be secreted and have no permanent position¹ or could be inserted through their lipidic anchor, i) in the outer leaflet of the OM among other lipids²³, and/or ii) in the plasma membrane^{24,25}. The later scenario has been favored in the past years¹ owing to the relatively strong conditions needed to extract LAM and recently reinforced by the finding that glycosyltransferases involved in the last steps of LAM biosynthesis are membrane-associated^{26–28}. Accordingly, upon subcellular fractionation, LAM has been detected in BCG membranes by ELISA²⁹. Similarly, by SDS-PAGE and silver nitrate staining, we detected apparently mature LAM and LM in BCG and *M. smegmatis* (Figure 2B) membrane fractions after protein digestion (Figure 2A). They represented around 5–10% of the total lipoglycans of the cells and showed the same migration pattern as cell wall associated lipoglycans; LAM reacted with CS35 antibody (Figure 2D) and BCG LAM had mannose caps as revealed by mild acid hydrolysis and capillary electrophoresis analysis (not shown). The plasma membrane might be the primary location for lipoglycans since they are also found in actinomycetes species that do not produce mycolic acids, and thus do not harbor an OM^{21,22}, such as *Amycolaptosis sulphurea*³⁰, *Turicella otitidis*³¹ or *Saccharothrix aerocolonigenes*³². However, in contrast to cell wall associated lipoglycans, membrane anchored lipoglycans from labeled cells were not positive for biotin tagging (Figure 2C), indicating that they do not reach the cell surface. This is in agreement with the estimated size of LAM. Indeed, BCG ManLAM in solution forms micelles about 30 nm in diameter³³. Recent electron microscopy analyses on unperturbed cells revealed the thickness of the cell wall plus the plasma membrane to be around 40 nm^{21,22}. Depending on the mycobacterial species, the thickness of the capsule is estimated to be 20 to 100 nm^{12,19,34,35}. Thus, LAM, with an estimated “length” of 15 nm, is not large enough to cross the cell envelope completely (Figure 3). This implies that lipoglycans accessible from the outside, and associated to cell wall, constitute a second pool of these molecules that might be anchored in the OM, as proposed by Rastogi²³ (Figure 3). This later population of lipoglycans is likely to be the one involved in host-pathogen interactions, acting as adhesins in C-type lectin-mediated phagocytosis or trafficking inside phagocytic cells after their release from the mycobacterial cell surface^{6,7}. The present results are not necessarily contradictory with the fact that lipoglycans, contrary to their lipid-free counterparts AM and mannan, have never been found in the surface-exposed material recovered after gentle treatment of mycobacterial cells with glass beads and/or detergent^{11,12}. They probably only reflect the fact that, although not covalently-bound to the cell envelope, lipoglycans are sufficiently strongly attached to the cells not to be extracted by these gentle treatments.

The presence of a second pool of lipoglycans exposed at the cell surface and cell wall associated raises the question of their transport from the plasma membrane, where they are synthesized, to the outermost layers of the envelope. Recent studies have shown that different families of proteins are involved in the transport of lipids/glycolipids to the cell surface^{36,37}. In *Escherichia coli*, LPS transport from the inner membrane (IM) to the OM requires^{38–40}: i) an ABC transporter at the IM, presumably involved in providing the energy to extract LPS from the IM, ii) a periplasmic chaperone that escorts LPS through the periplasm, and iii) an OM assembly protein complex, whose supposed function is to receive LPS at inner leaflet of the OM and to flip it across the membrane. It is tempting to envision that translocation of mycobacterial lipoglycans could be realized by analogous machinery. We thus investigated the possible involvement of a putative polysaccharide ABC transporter encoded by the genes *Rv3781* and *Rv3783*⁴¹ by disrupting by homologous recombination⁴² the ortholog of *Rv3783* in *M. smegmatis*. The lipoglycans of the mutant were, however, as efficiently tagged

by biotin as in its wild-type parent. Moreover, the mutant secreted similar amounts of glucan as well as the LAM- and LM-derived glycans, AM and mannan. Thus, the existence of lipoglycan translocation machinery still remains an open but crucial question, since the virulence of bacilli may depend on the export of immunomodulatory molecules to the cell surface, as recently shown for phthiocerol dimycocerosates⁴³.

Acknowledgements

We thank Dr Mamadou Daffé for helpful discussions and sharing unpublished data. We gratefully acknowledge Dr Patricia Constant for expert advice with cell culture and Ms Lucile Puntil and Anne-Claire Jolivet for helpful technical assistance. Profs G. Marchal (Paris) and A. Basten (Sydney) are thanked for the generous gift of the anti-Apa and HAT5 antibodies respectively. CS-35 monoclonal anti-LAM antibody was obtained from Colorado State University (NIH contract NIAID No-1-AI-40091). This work was supported by grants from CNRS, Agence Nationale de la Recherche (ANR-05-MIIM-038-04), the National Institute of Allergy and Infectious Diseases/National Institutes of Health (grant 5R01AI064798-3) and the IFCPAR (project 2903-3).

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Figure 1. Biotin labeling of various BCG sub-fractions (A) and scanning electron microscopy of control (B) and biotin-hydrazide labeled (C) BCG cells

A) 1 µg of each fraction (10 µg for arabinogalactan) were dot-blotted and probed with AP-streptavidin. 1, control cells; 2, biotinylated cells. HIC, hydrophobic interaction chromatography.

B) Bacteria were fixed with 2% glutaraldehyde (EMS, Washington PA) in 0.1 M cacodylate buffer pH 7.4 during 1 hour at 4°C. Fixed bacteria were washed in 0.2 M cacodylate buffer (pH 7.4), postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h and dehydrated in graded ethanol series. After dehydration samples were critical point dried with an emscope CPD 750 apparatus, mounted on stubs, coated with gold-palladium alloy with a JEOL JFC

1100 ion sputtering apparatus and examined with a Hitachi S-450 scanning electron microscope at an accelerating voltage of 15 kV. Bars, 0.5 μm .

Figure 2. LAM from cell wall but not plasma membrane fractions is labeled with biotin

The cell wall (cw) and plasma membrane (pm) fractions were prepared from *M. smegmatis* cells as described in A) and submitted to α -amylase and protease digestions. Lipoglycans were recovered after HIC and LAM (5 μ g) was analyzed by: B) SDS-PAGE revealed by periodic acid-silver nitrate staining; C) western blot probed with AP-streptavidin; D) western blot probed with anti-LAM CS35 antibody. 1, LAM from non-labeled control cells; 2, LAM from biotin-hydrazone labeled cells; Sc, *in vitro* biotinylated *M. smegmatis* total LAM standard; Sd, *M. smegmatis* total LAM standard.

Figure 3. LAM localization in the cell envelope

All the constituents are tentatively presented to scale and the drawing is based on the recent electron microscopy analyses on unperturbed cells ^{21,22}. Lipoglycans are synthesized at the level of the plasma membrane where they have not access to cell surface. The hydrophilic part of the molecule is most probably located in the periplasmic space, which has now been clearly evidenced ^{21,22}, or may protrude through the cell wall skeleton via the pores made by cross-linked peptidoglycan strands ²⁰. After synthesis, a part of or all the lipoglycans are transferred, possibly via a transporter(s) that still remains to be discovered, to the outer layers of the cell envelope. At this stage, they are exposed at the cell surface and most probably inserted among other lipids in the outer layer of the outer membrane and can play their roles of modulins and adhesins. Action of an endogenous α -endomannosidase might convert a portion of lipoarabinomannan (LAM) and lipomannan molecules into their lipid free glycan counterparts, arabinomannan (AM) and mannan respectively that can be subsequently released into the culture medium.