Lipoarabinomannan, and its related glycolipids, induce divergent and opposing immune responses to *Mycobacterium tuberculosis* depending on structural diversity and experimental variations

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In memoriam. Stefan Svenson, who was the architect of much of the reflections presented herein, passed away unexpectedly before this manuscript could be published. We dedicate this paper to his memory.

**Keywords:**
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Mycobacterium
Glycolipids
Lipoarabinomannan

**SUMMARY**

Exposure to *Mycobacterium tuberculosis* (Mt) may lead to active or latent tuberculosis, or clearance of Mt, depending essentially on the quality of the host’s immune response. This response is initiated through the interaction of Mt cell wall surface components, mostly glycolipids, with cells of the innate immune system, particularly macrophages (Mφs) and dendritic cells (DCs). The way Mφs and DC alter their cytokine secretome, activate or inhibit different microbicidal mechanisms and present antigens and consequently trigger the T cell-mediated immune response impacts the host immune response against Mt.

Lipoarabinomannan (LAM) is one of the major cell wall components of Mt. Mannosyl-capped LAM (ManLAM), and its related cell wall-associated types of glycolipids/lipoglycans, namely phosphatidylinositol mannosides (PIMs) and lipomannan (LM), exhibit important and distinct immunomodulatory properties. The structure, internal heterogeneity and abundance of these molecules vary between Mt strains exhibiting distinct degrees of virulence. Thus ManLAM, LM and PIMs may be considered crucial Mt-associated virulence factors in the pathogenesis of tuberculosis. Of particular relevance for this review, there is controversy about the specific immunomodulatory properties of these distinct glycolipids, particularly when tested as purified molecules in vitro. In addition to the variability in the glycolipid composition conflicting reports may also result from differences in the protocols used for glycolipid isolation and in vitro experiments including immune cell types and procedures to generate them.

Understanding the immunomodulatory properties of these cell wall glycolipids, how they differ between distinct Mt strains, and how they influence the degree of Mt virulence, is of utmost relevance to understand how the host mounts a protective or otherwise pathologic immune response. This is essential for the design of preventive strategies against tuberculosis. Thus, since clarifying the controversy on this matter is crucial we here review, summarize and discuss reported data from in vitro stimulation with the three major Mt complex cell wall glycolipids (ManLAM, PIMs and LM) in an attempt to conciliate the conflicting findings.
**Introduction**

*Mycobacterium tuberculosis* (MtB) is the causative agent of tuberculosis (TB). While infection with MtB may result in active disease, in most cases the bacteria persist in the infected host without open signs of disease [1]. The different outcomes of exposure to MtB, active TB, latent TB or no disease, are known to depend to a large extent on the interplay between the invading MtB and the host immune system.

Both innate and acquired branches of the immune response are essential in the battle against MtB [2,3]. Importantly, the very first steps of the innate immune response are crucial to modulate the overall innate as well as the acquired immune response [4]. MtB cell wall components exhibit important specific pathogen-associated molecular patterns (PAMPs); the innate immune response is initiated upon recognition of these molecules by pattern recognition receptors (PRRs), expressed mostly by macrophages (Mφs) and dendritic cells (DCs). Several classes of PRRs have been implicated in the recognition of MtB, including Toll-like receptors (TLRs) and C-type lectin receptors (CLR), such as DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) [5]. There are several TLR and non-TLR PRRs involved in activation of Mφs and DCs by MtB. Among these, an impressive body of data shows the importance of TLR2, while reports on the involvement of other TLRs are still controversial [6–9]. Via TLRs and/or nucleotide-binding oligomerization domain receptors, intracellular signals are induced in Mφs and DCs, initiating a local immune response leading to cytokine production and increased numbers of Mφs and DCs in the infected tissue and draining lymph nodes. Following activation by cytokines and innate receptor agonists, infected Mφs elicit direct bactericidal effector functions. DCs are known to phagocytose the bacteria in infected tissues, migrate to draining lymph nodes, and initiate the adaptive immune response by priming naïve T and B lymphocytes. Here, the maturation/activation state of DCs has been shown to play an important role in their response to infection and in the subsequent acquired immune response [10].

Several cell wall associated mycobacterial glycolipids/lipoglycans play a crucial role in the immunomodulation induced by MtB by eliciting distinct immune responses and activating/repressing different immune cascades [11]. In particular lipooarabinomanann (LAM) has been studied for its immunomodulatory properties by numerous researchers. In addition to LAM, its precursors lipomannan (LM) [12–14] and phosphatidyl-mylo-inositol mannosides (PIMs) [12–15] have been shown to have potent modulatory effects in vitro on cells of the immune system (Figure 1). LAM, LM and PIMs are all prevalent components of the mycobacterial cell wall [16]. In addition to the LAM/LM/PIM family of molecules the cell wall is composed of other lipids and glycolipids such as trehalose containing and phenolic glycolipids, as well as glyco- and lipo-proteins, which also contribute to the immune response. However these will not be the focus of this review.

In 1989 the major cell wall associated glycolipid, manno-capped LAM (ManLAM) from MtB was reported to stimulate the release of pro-inflammatory cytokines such as tumour necrosis factor (TNF) from human and murine Mφs [17]. However, in subsequent publications, the reported effects of ManLAM on Mφs and DCs were not consistent, in respect to activation and release of pro-inflammatory cytokines, such as interleukin (IL)-12 and IL-6 (Table 1), and expression of co-stimulatory (CD80, CD86) and antigen presenting molecules (MHC class II) (Table 2). In summary, over the last three to four decades many conflicting results on the immunomodulatory effects of ManLAM and other LAMS and their precursor molecules have accumulated, leaving the scientific community in confusion.

These and other complex and sometimes contradictory reported actions of MtB associated cell wall glycolipids prompted us to analyse the possible reasons underlying those discrepant results. A deep analysis of the literature revealed that several factors contribute to such diverse effects and also to the lack of consistency. On one hand there are very important differences between the immunomodulatory abilities of the different glycolipids (PIM, LM, LAM and ManLAM and potentially others) present in the MtB cell wall [18]; the analysis of the different molecules separately is essential if one aims to understand how MtB glycolipids modulate the host immune response. On the other hand, the same glycolipid may vary in composition between distinct MtB complex strains. In addition to this inherent glycolipid diversity, differences in the protocols used to perform the experiments might also have contributed to the lack of consistency in the published results. Differences include variables associated with distinct protocols to grow the bacteria (which have a strong influence on the composition of the bacterial cell wall glycolipids); diverse strategies to isolate the distinct glycolipids (which have an effect on the composition/structure of the isolated glycolipids) compounded by the amphiphilic/amphipathic nature of LM and LAM; differences in the immune cells used (Mφs, monocytes and DC; mouse or human origin); and on the protocols used to isolate/stimulate/differentiate...
Figure 1. Schematic figure of proposed structures of ManLAM, LM and PIMs based on current data [29,42,88–92]. There is some question as to the exact attachment points of the arabinan portions to the LM core in LAM, and the number of Ara residues and their arrangement can vary.

Table 1
Cytokine production by monocytes/Mϕs and DCs exposed to M. tuberculosis complex ManLAM.

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<tr>
<th>Publication/year</th>
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<th>Strains</th>
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<th>TNF</th>
<th>IL-6</th>
<th>IL-12</th>
<th>IL-10</th>
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<td>H37Rv</td>
<td>Inhouse</td>
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<td>H37Rv</td>
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<td>Nacalai Tesque Co</td>
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<td>CNRS</td>
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<td>[66] 2014</td>
<td>BMDC mouse</td>
<td>Mtb strain not specified</td>
<td>Not stated</td>
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</table>

Abbreviations used: AM — alveolar Mϕs; BMM — bone marrow derived Mϕs; BMDC — bone marrow derived DC; CNRS — Centre National de la Recherche Scientifique; CSU — Colorado State University; KI — Karolinska Institutet; OSU — Ohio State University; PM — peritoneal Mϕs.

Symbols used: ↑ Increased production; ↓ Decreased production; ▼ No difference.

* Brackets around arrows mean weak response.

1 Not clear which preparation was used in which experiment.

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these cells (which impact on the activation/maturation status of the cells, receptor (PPR) equipment and signalling pathways that are active). To all of these variables in the protocols used by distinct laboratories one needs still to consider the use of preparations of allegedly pure specific glycolipids that have been shown to be contaminated with other glycolipids or even other components [18].

In an attempt to bring some clarification to this important issue we here review the reported effects of the three major mycobacterial cell wall glycolipids, ManLAM, LM and PIMs on the maturation and cytokine profiles of murine and human M0s and DCs, dissecting the differences associated with the structure/composition of these molecules as well as the discrepancies that might result from experimental conditions.

Although this review focuses on the effects of glycolipids on M0s and DCs it is of relevance to stress that these molecules also exert effects on other important cells in the immune response against Mtb. This is the case for neutrophils that in combination with alveolar M0s are recognized as the first line of defence against pulmonary TB. Several studies show how the very initial response of neutrophils, in particular the ability to trigger apoptosis, influences the overall subsequent immune response [19]. Most of these studies address how neutrophils respond to whole bacteria, which seems necessary to trigger apoptosis of neutrophils [20]. However, purified glycolipids, in particular LAM, but not PIM, have been shown to be sufficient to induce other functions, namely TRAIL release by neutrophils [21]. T cells have been mostly investigated as the main players of the acquired immune response triggered by Mtb components presented in the context of antigen presenting molecules. However, purified glycolipids are also known to directly influence T cells. LAM and/or more specifically mannose capped lipoarabinomannan (ManLAM), have been shown to directly inhibit the activation/proliferation of T cells [22–24] and to specifically modulate the cytokine profile released by these cells [25].

LAMs are found in the cell wall of all mycobacterial species [26]. Generally they present a tripartite structure composed of an acylated mannosylphosphatidylinositol (MPI) anchor, attached to a poly-mannosyl backbone with arabian branches, and different capping motifs [16,27] (Figure 1). ManLAM is most abundant in the virulent laboratory strain Mtb H37Rv [37]. Strains of the Mtb Beijing lineage, defined by specific RD deletions, are differentially recognized by TLRs and trigger different immune responses [36]. The mechanism responsible for these strain-related differences is most likely multi-factorial, but may in part be dependent on the different nature and relative amounts of the cell wall-associated glycolipid molecules produced by different mycobacterial strains. Small but
important differences in glycolipid structure between individual Mtb-complex strains may account for important differences in the immune response [14,33,39]. It has, for example, been shown that ManLAMs from H37Rv, Mtbe Erdman and BCG differ in structural aspects [28] such as mannosyloligosaccharide capping content [40], which might be important for biological function. Mtb-complex strains containing truncated and more branched forms of mannose-capped ManLAM result in their low association with the Mₙ mannose receptor [39]. Thus LAM from Mtbe H37Rv and BCG were reported to be heterogeneous with respect to arabinan and mannan domains and to differ in abundance of acyl-isofoms [41]. Variations in mannose-capped terminal arabinan motifs have been observed in LAMs from clinical isolates of Mtb [42], which may well be responsible for the different biological activities. Cytokine expression is influenced by the acylation state of LM ligands [43] and there is a relationship between the overall charge of the ManLAM molecule and its capacity to stimulate the production of interferon-γ (IFN-γ) in different CD1b-restricted T-cell lines [44]. There are also differences in the ability of LAM from different Mtb strains (Erdman, H37Rv and H37Ra) to mediate adherence to Mφs [45].

Culture conditions of the Mtb-complex strains have been shown to affect the composition of the isolated glycolipids [46,47]. Hence, in order to dissect the role of mycobacteria derived molecules on the innate immune responses not just the choice of mycobacterial strain for isolation of the glycolipids is of utmost importance but also the protocol used to grow the bacteria and isolate the glycolipids (discussed in more detail ahead).

**Mₙ activation/maturation and cytokine production upon exposure to ManLAM from various sources**

ManLAM purified from Mtb complex were, in early studies in the late 1980s early 1990s, found to stimulate the production of pro-inflammatory cytokines, in particular TNF, by both human [17,48] and mouse [17,49–51] Mφs (Table 1). However, the reported magnitude of the TNF secretion varied greatly between studies. ManLAM has also been reported to affect other Mₙ functions, such as inhibition of various IFN-γ mediated microbicidal and tumoricidal activities [52–54], but it has also been reported that ManLAM can trigger the microbicidal activity of IFN-γ-primed Mφs [50].

In the report by Moreno et al. [17] in 1989 treatment of the ManLAM with alkali greatly reduced the TNF-secreting activity, suggesting that O-acyl groups were involved. A few years later ManLAM derived from the “virulent” laboratory Mtb strain Erdman was reported to be a weak stimulator of a pro-inflammatory response in respect to TNF production [49] and stimulation of NF-κB [55] in mouse Mφs compared to LAM isolated from the so called “avirulent” H37Ra laboratory strain. Some of the discordant information about the effect of ManLAM on the induction of a pro-inflammatory response by Mφs apparently stem from these early papers.

**Erdman ManLAM induces low/no pro-inflammatory response**

ManLAM prepared from the Mtbe Erdman strain [56] was used in many of the early studies analyzing Mₙ activation (Table 2) and cytokine production (Table 1). Erdman ManLAM preparations were repeatedly reported to either fail to induce the production of TNF in mouse [49,50,57] Mφs, or to produce very low amounts of TNF (Table 1), not only compared to LAM (ArAALAM, see ahead) prepared from the so called “avirulent” H37Ra laboratory strain [49–51,57] (see ahead) but also compared to ManLAM from H37Rv [51]. When ManLAM preparations from the strains H37Rv and Erdman were examined side by side [51], H37Rv ManLAM was found to induce higher amounts of TNF compared to that from Erdman [51] in murine peritoneal Mφs. ManLAM from Erdman also failed to induce IL-12p40 in mouse Mφs [58]. Reports suggest that IFN-γ activation is needed to stimulate TNF production through stimulation of peritoneal Mφs with Erdman ManLAM [50], while in other studies bone marrow derived cells were unresponsive to Erdman ManLAM despite IFN-γ activation [57].

**The ArAALAM that in early papers was said to be from H37Ra was from a rapid growing strain**

Part of the discordant results from the early in vitro experiments [13,49,50] stems from the fact that the ArAALAM (without mannose cap, and a potent inducer of TNF) that in these early papers was said to stem from the “avirulent” attenuated H37Ra strain in fact came from a rapidly growing mycobacteria species, presumably *M. smegmatis* [28,29], in which LAM is not mannose-capped (while LAMs from both H37Rv and H37Ra are mannose capped) [29]. In general, LAM lacking mannose capping (ArAALAM/PILAM) stimulates higher TNF production than ManLAM from Mtb-complex strains [59], indicating that the amount of mannose capping may be responsible for the pro-inflammatory capacity of a particular strain. Indeed the Erdman Mtb strain is reported to be significantly more mannose-capped, compared to H37Rv [40] and H37Ra [29,40], which may be part of the explanation for its low/pro-inflammatory activity. However, although the loss of LAM mannose caps in a mutant of BCG induced a somewhat higher production of TNF in Mφs, as compared to the wild type strain, it did not affect the production of TNF in DCs [60]. Neither did the lack of the mannose cap in a Mtb mutant affect its virulence in mice [60] an observation that deserves attention in the future.

**ManLAM from Mtbe H37Rv drives maturation/activation of human DCs**

The interaction of ManLAM with DCs, in particular with human DCs, is less well studied than the interaction with Mφs [61–63]. It was demonstrated that a highly purified H37Rv ManLAM was a potent activator of human DCs showing several similarities with LPS in respect to DC cytokine production and cell maturation profile [18] (Table 2). These observations are in agreement with the findings by Dulphy et al. [64] where DCs were activated with ManLAM from H37Rv, displaying a dose-dependent maturation phenotype, in terms of CD83 and CD86 expression but in contrast to the findings by Geijtenbeek et al. [63] and Wu et al. [65] where ManLAM did not induce DC maturation. The reason for this discrepancy is not clear but it could be hypothesized that also here strain-associated differences in the relative abundance of molecular forms of ManLAM or/and degree of purity of the preparation used could contribute to the observed divergent effects on DC maturation.

**Cytokine response of DCs stimulated by ManLAM**

ManLAM from H37Rv was shown to induce a strong pro-inflammatory cytokine response in human DCs, manifested by TNF, IL-6, and IL-12 release [18] (Table 1). Similar results were reported in a recent study using mouse cells, where ManLAM activated bone-marrow-derived DC were shown to produce pro- and anti-inflammatory cytokines via a C-type lectin receptor, Dectin-2 [66]. Thus ManLAM induces the production of TNF and IL-6 as well as the anti-inflammatory cytokine IL-10. In a study by Dulphy et al. [64], ManLAM-activated DCs produced the pro-inflammatory cytokines IL-6 and IL-8 but at a lower amount compared with cells...
stimulated by LPS, as well as low amounts of IL-10 and the functional IL-12p70 heterodimer.

In an earlier study by Nigou et al. different types of ManLAM obtained from BCG were reported to have different stimulatory capacity [62]. While the cell wall-associated preparation, “parietal” ManLAM, was shown to induce TNF and IL-8 production, the cell membrane-associated “cellular” ManLAM preparation did not induce a clear production of TNF or IL-8 [62]. The “parietal” type was reported to be uniquely O-acylated and Man-capped to a larger extent than the “cellular” type [62].

Considering its TNF stimulatory effect, the H37Rv ManLAM preparation investigated by Mazurek et al. [18] behaved as the “parietal” ManLAM used by Nigou et al. [62]: however, one should keep in mind that more than 90% of ManLAM preparations from BCG consists of “cellular” ManLAM [62]. A potential reason for the discrepant results in terms of pro-inflammatory responses by ManLAM may be differences in the strains from which the ManLAM was isolated. Thus ManLAM derived from M. bovis was shown to induce pro-inflammatory cytokines in DCs at a much lower level than ManLAM from H37Rv [18]. The ManLAM used by Nigou et al. was from BCG [62], while the ManLAMs used by Mazurek et al. [18] and Dulphy et al. [64] were from H37Rv (Table 1). Yet another reason could be differences in the type of DCs used and protocols to prepare these cells.

ManLAM has been reported to be unable to trigger IL-10 release from human blood monocytes [59]. However, ManLAM preparations from H37Rv induced IL-10 from human DCs in the study by Mazurek et al. [18] as well as in a study using mouse DC [66]. This was hardly surprising given that IL-10 is secreted in response to TLR ligation as well as to high levels of TNF and IL-6 [67] in a negative feedback manner.

Effect of ManLAM on LPS-activated Moś and DCs

The pro-inflammatory molecule LPS [68] is known to drive maturation of Moś [69] and DCs, and triggers production of pro-inflammatory cytokines [70] such as TNF, IL-6, and IL-12. For this reason the interaction between ManLAM and LPS has been studied, and also here the activity of various ManLAM preparations varies in different experimental setups (Table 3). In early studies Erdman ManLAM was found to block subsequent LPS-induced Moś activation [50]. On the other hand a more recent study showed that H37Rv ManLAM potentiated the LPS-induced expression of CD80, but not the expression of CD86, or MHC II, and stimulated a moderate but significant increase of cytokine production (TNF, IL-6, and IL-12) in human DCs over that induced by LPS alone [18]. Similar observations for IL-12p35, IL-12p40, IL-6 and IL-10 were made by Gringhuis et al. [71] who found that the production of cytokines was increased when cells were treated with LPS together with ManLAM as compared to cells exposed to LPS alone [71]. The findings that H37Rv ManLAM provides additional stimuli to the LPS-induced cytokine secretion, resulting in the augmented cytokine output, suggests that different PRRs on human DCs may be involved in the ManLAM as compared to LPS-driven cytokine responses.

In contrast with these results a paper by Nigou et al. reported that the “cellular” ManLAM preparation from BCG inhibited IL-12 secretion by human DCs when stimulated with LPS [61]. A similar negative effect was reported by Knutson et al. [72] making use of a monocyte cell line. Also reporting a negative effect the study by Geijtenbeek et al. [63] showed that Mtb ManLAM inhibited LPS-induced DC maturation.

Immunomodulation induced by LM and PIMs

LMs from different mycobacterial species have been reported to exhibit contradictory activities, both pro-inflammatory and anti-inflammatory responses through TLR2-dependent and -independent pathways [7,43,73] (Table 4). LM, including purified from H37Rv has been shown to induce IL-12 production in a TLR-2 dependent manner [42] and also inhibit IL-12 production in a TLR-independent manner [7]. LM (from BCG and Mtb H37Rv) has been reported to be a strong inducer of TNF through a TLR2 dependent pathway [74], but LM from H37Rv has been also reported to block TNF biosynthesis [75]. LM purified from M. chelonae and Mycobacterium kansasi has been described to induce TNF and IL-8 secretion through a CD14-TLR2-dependent mechanism [73]. The LM activity appears to depend on the acylation degree [44], on the length of the mannan chain building the mannopyranosyl backbone of LM [74] and to be determined by binding to TLR2 [74]. PIM2 and PIM6 are the two most abundant classes of PIMs found in Mtb and BCG [76]. In earlier studies non-fractionated PIMs from H37Ra [15,77] and PIM6 from BCG [14] were reported to stimulate the production of TNF through TLR2 signalling in Moś [14,15] (Table 5). According to the authors this effect depended on the presence of the lipid component of the molecule as de-acylation of PIM6 abrogated the TNF production [14]. By contrast, in a recent report, PIM2 and PIM6 from BCG were reported to present anti-inflammatory activities, inhibiting LPS-induced TNF, IL-12, IL-6, but also inflammatory activities by inhibiting IL-10 production in mouse Moś through a TLR2-independent mechanism [76].

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Table 3
Effect of ManLAM on LPS-induced cytokine production and maturation by Moś and DCs.

<table>
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<th>Publication/year</th>
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<td>↓</td>
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<tr>
<td>[72] 2004</td>
<td>Human monocyte derived cell line THP-1</td>
<td>Erdman</td>
<td>CSU contract N01 AI-25147</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>[61] 2001</td>
<td>DC human</td>
<td>BCG</td>
<td>CNRS</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>[63] 2003</td>
<td>DC human</td>
<td>Mtb strain unknown</td>
<td>CSU contract N01 AI-73520</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>[94] 2005</td>
<td>Raw cell line</td>
<td>H37Rv</td>
<td>CSU</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>[71] 2009</td>
<td>DC human</td>
<td>Mtb strain unknown</td>
<td>CSU</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>[18] 2012</td>
<td>DC human</td>
<td>H37Rv</td>
<td>KI</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>[95] 2013</td>
<td>DC human</td>
<td>BCG</td>
<td>Not stated</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

Abbreviations used: BMM — bone marrow derived Moś; CNRS — Centre National de la Recherche Scientifique; CSU — Colorado State University; KI — Karolinska Institutet; PM — peritoneal Moś.
Symbols used: ↑ Increased production; ↓ Decreased production; • No difference.
* Brackets around arrows mean weak reactions.

1 “Cellular” ManLAM, not parietal.
Effects were reported to depend on the acylation degree; di- and triacylated PIMs were strongly inhibitory, while tetra-acylated were less inhibitory, and mono-acylated PIMs were non inhibitory [76].

The observations by Doz et al. of a prominent anti-inflammatory action of PIMs on murine Mφs [76] were corroborated and extended by Mazurek et al. using human DCs [18]. In these studies PIM2 and total PIM, isolated both from Mtb H37Rv and BCG, were extended by Mazurek et al. using human DCs [18]. In these studies PIM2 analogues were also shown to inhibit TNF and IL-12p40 reporter gene activity similar to that of PIMs from Mtb H37Rv [18]. This is consistent with the fact that the two differently acylated isoforms of PIM2 and PIM6 are simultaneously present in both Mtb H37Rv and BCG [78]. More recently synthetic PIM1 and PIM2 analogues were also shown to inhibit TNF and IL-12p40 expression induced by TLR2 or TLR4 pathways in murine Mφs [76,79]. PIM1 and PIM2 inhibited LPS-induced TNF release by a CD14-dependent pathway, while IL-12p40 inhibition was CD14 independent.

In conclusion there is clear evidence that certain PIMs from Mtb H37Rv, M. bovis and BCG are potent anti-inflammatory agents that inhibit the pro-inflammatory activity of both LPS and ManLAM.

**Experimental factors potentially relevant for reported differences in immunomodulation of LAM, LM and PIM glycolipids**

The immune response elicited in vitro by glycolipids is strongly dependent on the experimental conditions.

The type and activation state as well as the origin of cells (human/animal) chosen for in vitro stimulation experiments may profoundly affect the final outcome. Bradbury and Moreno [51] examined the action of ManLAM on murine peritoneal and bone marrow derived Mφs and found that only the former produced TNF in response to H37Rv ManLAM. In accordance, several preparations of ManLAM from Mtb H37Rv, M. bovis and BCG are potent anti-inflammatory agents that inhibit the pro-inflammatory activity of both LPS and ManLAM.
Molecules of the LAM/LM/PIM family have been reported to bind to and signal through various PRRs on host cells. Some of these molecules bind to TLR2 and/or other TLRs [6–8], while others, such as ManLAM, are thought to bind DC-specific C-type lectin DC-SIGN/CD209 and mannose receptors and to deliver negative signals that interfere with TLR-mediated signalling [61]. Mycobacterial lipoglycans of the LAM/PIM family show a varying degree of TLR2-dependent pro-inflammatory activities [74]. Thus, since PPR expression is strongly influenced by the cells used and the protocol applied to prepare them, one needs to pay special attention to this information when analyzing the data generated.

Other experimental factors that may influence results are e.g. time of exposure and amounts of ManLAM used in the treatment of cells as well as its presentation. Thus for example augmentation of LPS-induced TNF production was found after short stimulation (1–4 h) of MoS with ManLAM [59,83], while it was not observed after prolonged exposure [83]. The stimulation of Dectin-2 receptor with ManLAM induced the production of both pro- and anti-inflammatory cytokines according to its presentation (soluble/plate coated) [66].

Glycolipid purification procedures impact on the elicited immune response

Several procedures have been described by different laboratories for the purification of LAM and other mycobacterial glycolipids [74,84]. Generally, these procedures rely on the initial extraction from dried bacterial cells or cell walls with a lipophilic organic solvent/water system, followed by hydrophobic interaction and size exclusion chromatography in the presence of a detergent. These purification methods may result in complex glycolipid fractions of varying compositions that are not limited to mannosyl-terminated glycolipid species alone. In addition, in many instances the purification procedure used, and most importantly the methods used to control the purity of the preparations are not described.

ManLAM preparations from Colorado State University (CSU) have over the years been used by many investigators, and are regarded as the gold standard. During recent years they have been produced under NIH contracts, as TB Vaccine Testing and Research Materials (TBVTRM). We recently found that some TBVTRM ManLAM preparations were contaminated with other potent immunomodulatory molecules that might affect the biological activity of the preparations [18]. Of two TBVTRM ManLAM lots investigated both were shown to contain PIMs and also some unidentified high molecular weight materials in addition to ManLAM. One TBVTRM lot tested showed a very high content of glucose suggesting contamination with mycobacterial glucans. One fraction was most likely arabinomannan (AM), devoid of most or completely lacking the lipid (acyl) part of ManLAM [18] (Figure 1).

Altogether the findings of contaminating materials (glucans, PIMs) and potential lack of acylation of the ManLAM in the TBVTRM ManLAM preparation described previously [18] may explain some observations where TBVTRM ManLAM preparations have been used. Thus mycobacterial glucans have been reported to suppress IL-12 production in monocyte-derived DCs [85]. The degree of acylation of mycobacterial glycolipids is important for e.g. the regulation of pro-inflammatory cytokines [14,43,86] so non-acylated or only partially acylated LAM in a ManLAM preparation might exert a competitive/inhibitory effect on the ManLAM. High levels of contaminating anti-inflammatory PIMs may also compete with or inhibit the effects induced by a particular ManLAM lot (see above).

It must also be remembered that all of these studies have in common the introduction of exogenous isolated ManLAM or other glycolipid ligands into essentially hydrophilic in vitro assays; yet, these are all highly amphipathic with varying degrees of aqueous compatibility, an important consideration in explaining contradictory outcomes. Now, with the availability of mutants of Mtb devoid of key structural/immunogenic aspects of LAM and LM, a whole bacterium approach can be applied to these questions [87].

Conclusion

In conclusion, we here review the profound and divergent effects of the major family of lipoglycans ManLAM, LM and PIMs, on MoS and DCs with regard to cell maturation and cytokine responses, and try to reconcile conflicting reports about the specific properties of these glycolipids. We summarize data showing that the described discrepancies are associated on one hand with differences in glycolipids themselves and/or their preparation, and on the other hand with differences in the cells used in the experiments.

With respect to the glycolipids one should keep in mind the variability in their composition, discrete differences in structure, the use of glycolipids from different bacterial strains and also differences in the protocols used for glycolipid isolation. Part of the discordant views of LAM stem from early experiments where AraLAM (without mannose cap, and a potent inducer of TNF) that in early papers [11,36,37] was said to stem from the “avirulent” attenuated H37Ra strain in fact came from a rapidly growing strain. Furthermore in some instances the contradictions may have been caused by the use of LAM preparations that were not sufficiently pure, containing, in addition to LAM, other related biologically active molecules.

ManLAM presents varying degrees of pro-inflammatory capacity, depending on the strain it comes from. ManLAM from the laboratory strain Mtb Erdman shows extremely low or no pro- and anti-inflammatory activity, compared to ManLAM from Mtb H37Rv. Some of the low pro-inflammatory activities reported for certain ManLAM preparations may depend on contamination with e.g. PIMs as a result of the difficulty in separating ManLAM and PIM during the isolation process of ManLAM.

A point seldom recognized is that LAM of whatever flavour, and LM, as we know them chemically, consist of a heterogeneous population and the structures presented (e.g. Figure 1) are average representations. Hence, in order to dissect the role of mycobacteria derived molecules in innate immune responses the choice of mycobacterial strain for isolation of the glycolipids is of utmost importance.

It is conceivable that different LAM/PIM ratios in the cell wall of different bacterial strains, and during distinct stages of Mtb infection [47], may be a crucial factor in determining the differential Mtb stimulation or inhibition of the immune system and thereby be decisive for the emergence and outcome of the disease [7,16,18].

Some of the discrepant results may be explained by differences in the cells that were used, such as differences in maturation (resting or already activated) or receptor equipment (TLR, DC-SIGN, mannose receptor etc). One early publication by Bradbury and Moreno [51] clearly illustrates this, where they examined the action of ManLAM on murine peritoneal and bone marrow derived MoS and found that only the former produced TNF in response to H37Rv ManLAM.

Thus, unless head to head experiments are performed, using the same cells, definitive conclusions are difficult to achieve regarding quantitative and even qualitative differences in maturation and cytokine expression.

It will not be possible or even desirable to achieve full consistency in experiments utilizing lipoglycans and immune cells within
this large scientific field. However we think that it is important to be aware of the discrepancies described here to be able to critically compare data from distinct sources, to evaluate the relevance of individual experiments and to design properly future research on LAM and its related molecules. To be able to do so it is important that the glycolipids and cells used in the studies are well characterized and that the methods and protocols for preparation of glycolipids and cells are carefully documented.

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References

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