MicroReview

Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response

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Summary

The cell wall component lipoarabinomannan (Man-LAM) from Mycobacterium tuberculosis is involved in the inhibition of phagosome maturation, apoptosis and interferon (IFN)-γ signalling in macrophages and interleukin (IL)-12 cytokine secretion of dendritic cells (DC). All these processes are important for the host to mount an efficient immune response. Conversely, LAM isolated from non-pathogenic mycobacteria (PILAM) have the opposite effect, by inducing a potent proinflammatory response in macrophages and DCs. LAMs from diverse mycobacterial species differ in the modification of their terminal arabinose residues. The strong proinflammatory response induced by PILAM correlates with the presence of phospho-myoinositol on the terminal arabinose. Interestingly, recent work indicates that the biosynthetic precursor of LAM, lipomannan (LM), which is also present in the cell wall, displays strong proinflammatory effects, independently of which mycobacterial species it is isolated from. Results from in vitro assays and knock-out mice suggest that LM, like PILAM, mediates its biological activity via Toll-like receptor 2. We hypothesize that the LAM/LM ratio might be a crucial factor in determining the virulence of a mycobacterial species and the outcome of the infection. Recent progress in the identification of genes involved in the biosynthesis of LAM is discussed, in particular with respect to the fact that enzymes controlling the LAM/LM balance might represent targets for new antitubercular drugs. In addition, inactivation of these genes may lead to attenuated strains of M. tuberculosis for the development of new vaccine candidates.

Introduction

Mycobacteria are extraordinarily successful pathogens with the remarkable ability to persist within the host's tissues even in the presence of an intact immune system. Pathogenic mycobacteria are predominantly intracellular parasites capable of replicating within the normally hostile environment of macrophages. In this location, the bacillus is protected from many of the immune mechanisms that normally eliminate bacterial invaders. One major challenge that the intracellular bacteria face is overcoming cell-mediated mechanisms of immunity that detect signals originating from infected cells. An important key to the success of pathogenic mycobacteria is likely to be their unusual cell wall structure and its interactions with the immune system. This cell envelope consists of a highly complex array of distinctive lipids, glycolipids and proteins. It has been intensely scrutinized as a potential effector in the interaction of Mycobacterium tuberculosis with the human host (Glickman and Jacobs, 2001; Russell et al., 2002; Brennan, 2003; Flynn and Chan, 2003).

Lipoarabinomannan (LAM) as well as its related precursors, lipomannan (LM) and phosphatidyl-myoinositol mannosides (PIMs), are found interspersed in the mycobacterial cell wall. PIMs, LM and LAM are major lipoglycans that are non-covalently attached to the plasma membrane through their phosphatidyl-myoinositol anchor and extend to the exterior of the cell wall (Besra and Brennan, 1997; Belanger and Inamine, 2000; Nigou et al., 2003). These complex molecules are believed to play important roles in the physiology of the bacterium as well as in the modulation of the host response during infection. For example, LAM is an important modulator of the
immune response in the course of tuberculosis and leprosy (Chatterjee and Khoo, 1998; Nigou et al., 2002) and a key ligand in the interaction between \textit{M. tuberculosis}, macrophages and dendritic cells (DCs) (Schlesinger et al., 1994; Maeda et al., 2003). In addition, recent studies highlight the potential role of LM in mycobacterial virulence via its strong proinflammatory and apoptosis-inducing activity.

A thorough investigation of the roles of PIMs, LM and LAMs in mycobacterial virulence has been hampered by a lack of defined mutants that fail to synthesize these specific cell surface components. Recently, advances in the genetic manipulation of mycobacteria and related actinomycetes, together with the sequencing of the \textit{M. tuberculosis} genome, have allowed several lipoglycan mutants with defined envelope deficiencies to be generated. Progress in the study of mycobacterial glycolipid biosynthesis bears the promise of identifying enzymes that might be essential for the viability and/or virulence of \textit{M. tuberculosis} and targets for future drug development.

This review article reports the advances made in the current understanding of PIMs, LM and LAM biosynthesis and will describe only briefly the structural organization of the different domains comprising these complex molecules as this has been the subject of many excellent reviews (Chatterjee and Khoo, 1998; Brennan, 2003; Nigou et al., 2003). We also discuss recent observations relating to the immunomodulatory functions of LAM and its precursors, in addition to their receptors and intracellular signalling pathways. The role of these lipoglycans as antigens presented by the CD1 system, the host's lipid antigen-presenting molecule, has been reviewed recently (Porcelli and Besra, 2003).

**Structure of mycobacterial LAM and related lipoglycans**

PIMs and their multiglycosylated counterparts, LM and LAM, are complex lipoglycans that are found ubiquitously in the envelopes of all mycobacterial species. PIMs, LM and LAM all share a conserved mannosyl-phosphatidylinositol (MPI) that is presumably used to insert these structures into the plasma membrane (Hunter and Brennan, 1990), suggesting that they are metabolically related (Besra and Brennan, 1997). In addition to the MPI, LAM possesses a mannan core with a branched arabinan polymer and, in some cases, cap motifs decorate the termini of the branched arabinan (Nigou et al., 2003) (Fig. 1).

The mannan core consists of an \(\alpha 1,6\)-linked Man\(_p\) backbone, which is substituted at C-2 by single Man\(_p\) units in numerous species, including \textit{M. tuberculosis}, \textit{Mycobacterium leprae}, \textit{Mycobacterium kansasi} and \textit{Mycobacterium smegmatis}, and at C-3 by single Man\(_p\) units in \textit{Mycobacterium chelonae} (Guerardel et al., 2002).

The size and the degree of branching of the mannan core are species dependent. The arabinan polymer of LAM consists of a linear \(\alpha(1\rightarrow5)\)-linked arabinofuranosyl backbone punctuated with branched hexa-arabinofuranosides (Ara\(_n\)) and linear tetra-arabinofuranosides (Ara\(_m\)) (Chatterjee et al., 1991; 1993) (Fig. 1).

LAM can be classified into three major structural families according to the capping motifs present on the non-reducing termini of the arabinosyl side-chains. The arabinan termini in the pathogenic strains \textit{M. tuberculosis}, \textit{M. leprae}, \textit{Mycobacterium avium} and \textit{M. kansasii} are modified with caps consisting of a single Man\(_p\), a dimannoside or a trimannoside, with dimannosides predominating (Nigou et al., 1997; Vercellone et al., 1998; Khoo et al., 2001; Guerardel et al., 2003), resulting in molecules designated ManLAM. ManLAM contains about 50 Man\(_p\) and 60 Ara\(_f\) units. A general picture of the \textit{M. tuberculosis} ManLAM structure is proposed in Fig. 1. In the fast-growing non-pathogenic species \textit{M. smegmatis}, \textit{Mycobacterium fortuitum} and in an unidentified species, branches of the terminal arabinan are terminated by inositol phosphate caps (Khoo et al., 1995), characterizing the PILAM family. A third LAM family, designated AraLAM, recently identified in \textit{M. chelonae}, comprises a LAM molecule devoid of both the manno-oligosaccharide and inositol phosphate caps (Guerardel et al., 2002).

**Biogenesis of PIMs, LM and LAM**

Understanding the biosynthesis of PIMs, LM and LAM has been the focus of recent genetic and biochemical studies (Nigou et al., 2003). Enzymes that clearly participate in the elaboration of these complex lipoglycans are represented in Fig. 1.

**Biogenesis of PIMs**

Several mannosyltransferases involved in the mannosylation steps can be distinguished with respect to the mannosyl donor they use (either GDP-Man\(_p\) during early steps in PIM biosynthesis, or C\(_{18}\)/C\(_{19}\)-P-Man\(_p\) later in LM synthesis from PIM precursors). PIM biosynthesis is initiated by two distinct mannosyltransferases that use GDP-Man\(_p\) as the sugar donor. The first step involves the transfer of a mannose residue from GDP-Man\(_p\) to the 2-position of the myo-inositol ring of phosphatidyl-myo-inositol (PI) to form phosphatidyl-myo-inositol monomannoside (PIM\(_{1m}\)). This reaction is catalysed by the \(\alpha\)-mannosyltransferase PimA (Kordulakova et al., 2002). The \(pimA\) gene of which is essential, demonstrating that PIM\(_{1m}\), and presumably higher mannosylated PIMs, are required for cell growth. Interestingly, \(pimA\) is the fourth gene in an operon of five genes that are all potentially involved in PIM biosynthesis (Kordulakova et al., 2002). The first gene in this cluster
encodes a protein of unknown function, while the second encodes PgsA1, the PI synthase that catalyses the condensation of inositol and the diglyceride of CDP-diacylglycerol (Jackson et al., 2000). The third gene (Rv2611c) of this operon encodes a protein with high similarity to bacterial acyltransferases. This protein has been shown to be responsible for the acylation of the 6-position of the Manp residue linked to position 2 of the myo-inositol in PIM₁ and PIM₂, with the mono-mannosylated lipid acceptor being the primary substrate of the enzyme (Kordulakova et al., 2003). In contrast to pimA or pgsA1, which are both essential, the acyltransferase Rv2611c is dispensable in M. smegmatis, although its disruption induces dramatic changes in the PIM content and a severe growth defect (Kordulakova et al., 2003). The last gene of the PIM cluster, Rv2609c, encodes a putative GDP-Manp hydrodrolase that awaits further characterization.

The second mannosylation step, catalysed by PimB, allows the transfer of another Manp residue to the 6-position of the Manp residue linked to position 2 of the myo-inositol ring of PIM₁, leading to PIM₂ (Schaeffer et al., 1999). A third Manp unit is finally introduced on to the growing molecule to form PIM₃ in a reaction carried out by the product of the pimC gene identified in M. tuberculosis CDC1551 (Kremer et al., 2002). How-

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**Fig. 1.** General structure of ManLAM from M. tuberculosis and structural relationship between PIMs, LM and LAM. PIM₁ is a precursor of the highly mannosylated LM molecule, which is further extended by the arabinan domain to form LAM. In both LM and LAM, an α1,6-linked Manp backbone substituted at C-2 by single Manp units constitutes the mannan domain. The arabinan polymer is a linear α(1→5)-linked arabinofuranosyl backbone punctuated with branched hexa-arabinofuranosides: β-D-Araf(1→2)α-D-Araf(1→3)α-D-Araf(1→5)α-D-Araf→. The mannosic caps, which terminate the arabinan domain, consist of a single Manp residue, a dimannoside (α-Manp(1→2)α-Manp→) or a trimannoside (α-Manp(1→2)α-Manp(1→2)α-Manp→). R₁, R₂ and R₃ are fatty acyl chains. C₃₅/C₅₀-P-Manp represents a polyrenyl monophosphomannose. The a, b, c and d values are species specific. Arrows indicate enzymes confirmed to participate in the biosynthesis of these lipoglycans. PimC was found to be present in M. smegmatis CDC1551 but absent from M. tuberculosis H37Rv. Classification of the glycosyltransferases by their CAZY family is indicated in brackets.
ever, inactivation of \textit{pimC} in \textit{Mycobacterium bovis} BCG did not affect cell growth and did not alter the PIM/LM/LAM composition of the mutant. This suggests the presence of an alternative synthesis pathway present in \textit{M. bovis} BCG and \textit{M. tuberculosis} CDC1551, a hypothesis that is supported by the fact that \textit{pimC} is not found in \textit{M. tuberculosis} H37Rv (Kremer et al., 2002).

The mannose unit at the position 6 of \textit{PIM}\textsubscript{3} is then further elongated with mannose residues to generate \textit{PIM}s\textsubscript{4,6}. However, mannosyltransferases participating in this elongation process have not been identified.

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\textit{Biogenesis of the arabinan domain in LAM}
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The 'mature' LM is then subsequently glycosylated with arabinan to form LAM. Until very recently, little was known about the genetics of arabinan biosynthesis. Two forms of arabinans are found in the mycobacterial cell wall: one is part of the heteropolysaccharide arabinogalactan (AG) and the other is part of LAM. The two forms of \(\alpha\)-arabinan differ in that mycolic acids esterify arabinan in AG, thus constituting the basis of the lipid barrier of mycobacteria.

In contrast, in \textit{M. tuberculosis} LAM, the arabinan moiety is further capped with mannose residues responsible for some of its biological functions. Therefore, arabinan represents a valid target for the generation of antimycobacterial drugs because blocking of its biosynthesis would lead to dual disruption of both the mycolyl-AG–peptidoglycan cell wall complex and LAM.

As mentioned above, the arabinan domain consists of a linear \(\alpha(1\rightarrow5)\)-Araf backbone substituted by two kinds of arrangements, linear tetra-arabinofuranosides (Ara\(_4\)) and hexa-arabinofuranosides (Ara\(_6\)). In both cases, the non-reducing end is characterized by the disaccharide unit \(\beta-D\text{-Ara}(1\rightarrow2)\alpha-D\text{-Araf}(1\rightarrow)\) (Besra and Brennan, 1997; Brennan, 2003).

The only Araf sugar donor identified so far is the \(C_{50}\) polyprenyl monophosphoarabinose (\(C_{50}/C_{50}\)-P-Araf) (Wolucka et al., 1994), which is synthesized from 5-phospho-D-ribose pyrophosphate (Scherman et al., 1996). Initially identified as the major target of ethambutol (an effective antimycobacterial drug) in \textit{M. avium} (Belanger et al., 1996) and \textit{M. tuberculosis} (Telenti et al., 1997), the two homologue proteins EmbA and EmbB have been reported to participate in the formation of the proper Araf\(_6\) motif in AG (Escuyer et al., 2001). These two proteins have been proposed to catalyse \(\alpha\)-3-arabinosyltransferase activity in the arabinan of AG. \textit{M. smegmatis} mutants lacking \textit{embA} or \textit{embB} are viable, probably because the two gene products partially compensate for each other. Although arabinosylation of AG was dramatically diminished, arabinosylation of LAM remained unaffected in these mutants (Escuyer et al., 2001).

In \textit{M. tuberculosis}, the Emb proteins are encoded by a cluster of three genes, \textit{embC}, \textit{embA} and \textit{embB} (Cole et al., 1998). Zhang et al. (2003) found recently that inactivation of the remaining \textit{embC} gene in \textit{M. smegmatis} abolished arabinosylation of LAM, but not AG. The three Emb proteins are predicted to contain 13 membrane-spanning segments in their N-terminal region and a globular C-terminal domain. It has been proposed that the N-
terminus of EmbC participates in the recognition of the LM as a precursor of LAM and that the C-terminus is responsible for arabinosylation (Zhang et al., 2003). The transmembrane segments of the Emb proteins are very likely to be involved in translocating the arabinan component across the plasma membrane. However, whether the C-terminal domain is able to synthesize full-length arabinan is not known. It remains possible that arabinan motifs might be preassembled on carrier molecules, polymerized and attached to the LM acceptor molecule, a scenario that would suggest the requirement of numerous arabinosyltransferases.

EmbR belongs to the Streptomyces coelicolor antibiotic regulatory protein (SARP) family (Wietzorrek and Bibb, 1997), known to regulate genes involved in the synthesis of secondary metabolites. Belanger et al. (1996) proposed that EmbR influences the expression of the M. avium embAB operon. M. smegmatis membranes carrying the M. avium embAB and embR genes retain significantly more arabinosyltransferase activity than membranes originating from M. smegmatis carrying only the embAB cluster, when treated with similar amounts of ethambutol (Belanger et al., 1996). The M. avium embR gene is located immediately upstream of embAB, while the embR gene of M. tuberculosis is elsewhere in the genome (Telenti et al., 1997; Cole et al., 1998). It was demonstrated recently that PknH, a newly described Ser/Thr kinase from Streptomyces coelicolor, phosphorylates EmbR through recognition of a FHA (forkhead-associated) domain (Molle et al., 2003). Arg-312, Ser-326 and Asn-348 in the EmbR FHA domain participate in the regulatory phosphorylation of EmbR by PknH through preferential recognition of Ser/Thr and a role in the transcriptional regulation of the embCAB cluster in ethambutol-resistant M. tuberculosis. Whether the PknH/EmbR pair regulates the arabinosyltransferase activity of EmbC in vivo, ultimately leading to arabinan synthesis of LAM, is currently under investigation.

**Biogenesis of the mannose cap**

LAM is modified further by either manno-oligosaccharides or phospho-inositol caps, according to the species, resulting in ManLAM or PILAM respectively. It is noteworthy that, although ethambutol was shown to affect the complete elaboration of the arabinan in PILAM from an ethambutol-resistant M. smegmatis mutant (Khoo et al., 1996), it has also been suggested that ethambutol inhibits the extent of mannose capping of ManLAM in M. tuberculosis strains grown in the presence of subminimal inhibitory drug concentrations (Khoo et al., 2001). As mannose capping is a major structural entity engaged in receptor binding and subsequent immunopathogenesis, inhibition of this motif may directly affect the biological functions associated with ManLAM. In this regard, it is conceivable that inhibitors such as ethambutol may modulate the immune interactions of M. tuberculosis with the host, although this remains to be demonstrated further. Genes participating in the synthesis of these caps have not been reported, and the identification of mannosyltransferases involved in this reaction remains a challenge. Pathak et al. (2004) reported the synthesis of two α(1→6)- and α(1→2)-linked Manp-Manp disaccharides as photoaffinity probes for active-site labelling studies. Photoaffinity probe technology offers new avenues for the identification of putative mannosyltransferases involved in the synthesis of the α(1→6)-mannan core and mannose caps.

All known sequences of glycosyltransferases have been gathered into 69 families in the CAZY database (http://afmb.cnrs-mrs.fr/CAZY/). It was reported recently that M. tuberculosis H37Rv contains 37 putative glycosyltransferases, but the precise reaction catalysed by most of them has not been determined experimentally. Classification of glycosyltransferases with functions that have been confirmed shows that they belong to the GT-2 (Ppm1), GT-4 (PimA, PimB, PimC) and GT-53 (EmbC) CAZY family (Wimmerova et al., 2003). Although glycosyltransferases share little sequence similarity, they are proposed to adopt only two different folds, BGT and SpsA, according to the first structure solved in each case. For instance, Ppm1 has been proposed to contain an SpsA fold, and PimA and PimB a BGT fold (Wimmerova et al., 2003).

**Modulation of the immune response by PIM/LM/LAM**

**Inflammatory-inducing activity**

Historically, most studies analysing the effect of LAM on the induction of an inflammatory response by macrophages or DCs have been performed using ManLAM from M. tuberculosis or M. bovis BCG and PILAM from an unidentified, fast-growing mycobacterial species (previously named AraLAM) that is structurally very similar to PILAM of M. smegmatis. Results from these studies demonstrated that treatment of macrophages with PILAM induced the secretion of various cytokines [interleukin (IL)-8, IL-12, tumour necrosis factor (TNF)-α] and apoptosis, whereas ManLAM did or did so only weakly (Chatterjee et al., 1992; Roach et al., 1993; Zhang et al., 1995; Riedel and Kaufmann, 1997; Yoshida and Koide, 1997; Ghosh et al., 1998). These observations led to the hypothesis that the presence of mannose caps on LAM (such as in ManLAM) inhibits its proinflammatory activity. Unfortunately, uncapped LAM was not included in these studies for direct comparison of the biological effects of ManLAM and PILAM. Therefore, some of the biological effects associated with PILAM could also be attributed to their phospho-myco-inositol caps. The recent isolation and characterization of LAM (AraLAM) from the facultative patho-
genic M. chelonae revealed that it lacks both the manno-oligosaccharide and phosphoinositol caps on its terminal arabinose residues (Guerardel et al., 2002). Interestingly, only PILAM, but not ManLAM or AraLAM, significantly induces IL-12 expression and apoptosis (Dao et al., 2004). PILAM, but neither ManLAM nor AraLAM, consistently induces the secretion of the proinflammatory cytokines IL-8 and TNF-α (Guerardel et al., 2002; Vignal et al., 2003). These results support the hypothesis that mannose caps do not inhibit the proinflammatory activities of LAM, but rather that the phosphoinositol caps of PILAM are potent proinflammatory constituents. However, this does not diminish the potential importance of mannose caps with respect to their capacity to inhibit proinflammatory signals engaged by other ligands, as discussed below, which is most likely to be an important activity in the context of infection of macrophages or DCs. The availability of AraLAM makes it feasible to address this hypothesis experimentally.

Characterization of LAM from the facultative pathogenic mycobacteria M. kansasi and M. chelonae enabled us to analyse the effects of their precursors on the induction of proinflammatory cytokines and apoptosis in macrophages. Interestingly, whereas neither ManLAM from M. kansasi nor AraLAM from M. chelonae had any activity, the addition of LAM from either species induced potent secretion of IL-8 and TNF-α (Vignal et al., 2003) and significant expression of IL-12 and apoptosis (Dao et al., 2004). LM purified from M. smegmatis, M. tuberculosis and M. bovis BCG also induced proinflammatory responses (Dao et al., 2004). Moreover, LM but not the corresponding LAM induced macrophage activation characterized by cell surface expression of CD40 and CD86, as well as NO secretion (Quesniaux et al., 2004).

Therefore, LMs of mycobacteria in general are strong proinflammatory factors and, as LAM and LM are part of the cell wall, one could argue that it is important for virulent mycobacteria to minimize the amount of LM present in the cell wall in order to reduce the host's proinflammatory response. Consequently, one might expect a direct correlation between mycobacterial virulence and a high LAM/LM ratio. Analysis of the LAM/LM ratio in the cell walls of different virulent, facultative pathogenic and non-pathogenic mycobacteria would address this hypothesis. Alternatively, differences in the structural organization of the cell wall between bacteria may also lead to different accessibility of LM for its interaction with TLR-2 on infected macrophages.

The arabinan domain of LAM inhibits the proinflammatory activity of LM on macrophages, presumably by masking the mannan core of LAM (Fig. 1). Consistently, gradual chemical reduction in the amount of arabinan domain of the M. kansasi ManLAM correlated with increased proinflammatory cytokine expression of the truncated LAM molecules, thus revealing the proinflammatory activity of the LM core (Vignal et al., 2003).

Deciphering the complex molecular basis of LAM/LM activities could greatly benefit from the increasing characterization of new structural LAM variants. Lipoglycans related to mycobacterial LAM have been described in several actinomycetes, including Rhodococcus (Garton et al., 2002), Corynebacteria (Sutcliffe, 1995), Gordonia (Flaherty and Sutcliffe, 1999) and Amycolatopsis (Gibson et al., 2003b). The LAM-like molecule from the intracellular pathogen Rhodococcus equi consists of a linear (α-1-6)-mannan backbone substituted by 2-linked single Manp residues (Garton et al., 2002). In contrast to mycobacterial LAM, there are no extensive arabinan domains but single terminal α-D-Aral residues capping the 2-linked α-D-Manp. This ‘simpler’ LAM molecule, which resembles an LAM-like molecule, was found to induce an early macrophage proinflammatory response (Garton et al., 2002), supporting the notion that an extended arabinan domain may hinder the LM-dependent inflammatory response.

LAM from Tsukamurella paurometabola was recently demonstrated to induce the secretion of TNF-α in murine and human macrophages (Gibson et al., 2004). Interestingly, this activity was dramatically increased after removal of the arabinan chains by mild acidic treatment, which exposed the LM core. These observations are consistent with the results analysing mycobacterial LM/LAM, and therefore reinforce our hypothesis that the LM-mediated proinflammatory activity is obstructed by the arabinan chains in the native LAMs.

As a consequence, enzymes modifying the LM core by the addition of arabinose residues should be important targets for the creation of attenuated strains of M. tuberculosis and for the discovery of new antitubercular drugs. One attractive gene candidate for inactivation in M. tuberculosis is embC, which has been shown to participate in the arabinosylation of LM in M. smegmatis (Zhang et al., 2003). Deletion of this gene should strongly increase the amount of LM in the cell wall and should affect the virulence of this mutant.

Several reports demonstrated that PIMs isolated from M. tuberculosis are able to induce TNF-α and IL-8 secretion by human and murine macrophages (Barnes et al., 1992; Zhang et al., 1995; Jones et al., 2001). Highly purified PIMp and PIMe were also found to induce similar but very low levels of TNF-α secretion (Gilleron et al., 2003). In contrast, a number of studies failed to detect significant induction of IL-8, IL-12 and TNF-α secretion and found no increased induction of apoptosis upon treatment of macrophages with PIMs isolated from M. tuberculosis, M. kansasi or M. chelonae compared with treatment of cells with equal molar amounts of PILAM or LM (Guerardel et al., 2002; 2003; Vignal et al., 2003; Dao et al., 2004). Interestingly, the two studies (Barnes et al., 1992; Zhang...
et al., 1995) reporting the strongest activity of PIMs on cytokine secretion used either primary human peripheral blood mononuclear cells or primary human alveolar macrophages respectively. In contrast, the activity of PIMs on cytokine secretion, reported by Jones et al. (2001) and Gilleron et al. (2003), was modest compared with the activity of PILAM, LM or LPS and was conducted using murine macrophages. Therefore, it appears that PIMs display a residual proinflammatory activity, which becomes more or less apparent depending on the sensitivity of the target cells (primary human cells versus murine cells) and the detection assay [reverse transcription polymerase chain reaction (RT-PCR) versus enzyme-linked immunosorbent assay (ELISA)] used. In addition, the purity of the PIM fraction is critical as a crude preparation of PIM would contain ‘higher’ PIMs with multiple mannose residues (such as PIM5), which may explain their biological activity as these structures start to resemble LM.

Inhibition of cellular responses

The first demonstration of the capacity of LAM to inhibit a host response involved in defence against bacterial infection was conducted by Sibley et al. (1988), who reported the inhibition of the interferon (IFN)-γ response of macrophages by ManLAM. Subsequently, live M. tuberculosis infection was shown to inhibit IFN-γ signalling, as demonstrated by the reduction in the IFN-γ-mediated cell surface expression of MHC class II and receptors for the Fc portion of IgG after infection of macrophages with M. tuberculosis (Hmama et al., 1998; Hussain et al., 1999; Ting et al., 1999; Pai et al., 2003). Furthermore, ManLAM from M. tuberculosis inhibited the M. tuberculosis infection-induced apoptosis of macrophages (Rojas et al., 1997; Rojas et al., 2000) and the secretion of IL-12 induced by lipopolysaccharide (LPS) in DCs (Nigou et al., 2001) and macrophages (Knutson et al., 1998). The activity of ManLAM reflects the capacity of whole M. tuberculosis bacteria to inhibit infection-induced apoptosis (Keane et al., 2000) and IL-12 secretion of macrophages (Giacomini et al., 2001; Hickman et al., 2002; Li et al., 2002). Contradictory results show that, in DCs, M. tuberculosis seems either to induce secretion of IL-12 (Giacomini et al., 2001) or to inhibit IL-12 production (Johansson et al., 2001; Demangel et al., 2002). One of the hallmarks of the host–pathogen interaction between macrophages and M. tuberculosis is the ability of M. tuberculosis to inhibit the fusion of phagosomes with lysosomes (Armstrong and Hart, 1971). Lysosomes have a low pH and contain a multitude of lytic enzymes that are meant to lyse any bacterial or parasitic invaders that have been phagocytosed by the macrophages. Therefore, the capacity of M. tuberculosis to inhibit the fusion of its phagosome with lysosomes is crucial for its intracellular survival. Latex beads coated with ManLAM inhibit phagosome–lysosome fusion (Fratti et al., 2001; 2003), suggesting that ManLAM is an important mediator of the inhibition of phagosome maturation in the context of infection with live bacteria.

Receptors involved in inhibition and activation processes

Toll-like receptors are important initiators of the innate immune response that are specific for pathogen-associated molecular patterns, such as CpG-oligodeoxy-nucleotides, lipoteichoic acid, peptidoglycan and flagellin (Kopp and Medzhitov, 2003). Interaction of agonists with TLR-2 induces IL-12 secretion and apoptosis by the cell. PILAMs purified from rapidly growing mycobacteria, but not ManLAM from M. tuberculosis, have been shown to interact with TLR-2 (Heldwein and Fenton, 2002). Interestingly, LM isolated from M. kansasii, M. chelonae or M. tuberculosis all interact with TLR-2, but not with TLR-4, as determined by TLR-induced CD25 expression in transfected Chinese hamster ovary cells (Dao et al., 2004). These results were also confirmed in in vitro assays on bone marrow-derived macrophages isolated from TLR-2+/+ or TLR-4−/− mice, showing that LM had no activity in the former but had normal cytokine-inducing activity in the latter (Quesniaux et al., 2004). Moreover, macrophage activation by LM was also found to be mediated through the adaptor protein myeloid differentiation factor 88 (MyD88), but independent of either TLR-4 or TLR-6 recognition (Quesniaux et al., 2004). PIMs were shown to be TLR-2 agonists, which may explain their biological activity observed by some investigators (Jones et al., 2001; Gilleron et al., 2003).

Two receptors have been implicated to date in the inhibitory activity of ManLAM. ManLAM can inhibit the LPS-induced IL-12 secretion of human DCs (Nigou et al., 2001). This activity was abolished by enzymatic removal of the mannose caps or by treatment with antimannose receptor (MR) antibodies, and was mimicked by the addition of mannan from Saccharomyces cerevisiae, a known agonist of the MR, suggesting that the MR is the receptor that mediates the inhibition. Nevertheless, subsequent studies showed that anti-MR antibodies did not block binding of ManLAM to DCs, in contrast to antibodies directed against DC-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2003; Tailleux et al., 2003). Furthermore, the binding of ManLAM to DC-SIGN on DCs induced the secretion of IL-10, a known inhibitor of IL-12 secretion (Geijtenbeek et al., 2003). Thus, DC-SIGN appears as a major mediator of IL-12-inhibition by ManLAM on DCs.

Intracellular mediators of inhibition

Very little is known about the signalling components that
connect DC-SIGN and/or the MR after binding of ManLAM to the intracellular effectors that have been reported to be triggered by ManLAM binding. We propose that the *M. tuberculosis*-mediated inhibition of the increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{c}\)) (Fig. 2), which is usually associated with phagocytosis of bacteria, is a central mediator of the inhibition of three important macrophage responses to infection: phagosome maturation, macrophage apoptosis and the IFN-\(\gamma\) signalling (Fig. 3).

First, how does *M. tuberculosis* or ManLAM inhibit the cellular [Ca\(^{2+}\)]\(_{c}\) response? Recent work demonstrated that live, but not dead, *M. tuberculosis* inhibit sphingosine kinase 1 (SK1) activity (Malik *et al*., 2003) (Fig. 2). This enzyme converts sphingosine to sphingosine-1-phosphate (S1P). Increased concentrations of S1P induce an increase in [Ca\(^{2+}\)]\(_{c}\), levels through the release of Ca\(^{2+}\) from the endoplasmic reticulum by an unknown mechanism that is independent of the inositol triphosphate pathway (Malik *et al*., 2003). It remains to be established whether this activity of *M. tuberculosis* on SK1 can also be reproduced using purified ManLAM. The comparison between live and dead (heat killed or irradiated) bacteria suggests that the inhibition of SK1 activity is mediated through a factor (protein/lipid/glycolipid) that has a fast turnover and therefore requires the continuous bacterial transcription and translation machinery. Alternatively, the difference between live and dead bacteria may result from the requirement for specific genes that are only induced during phagocytosis of the bacteria by the macrophage. In either case, the molecular mechanism by which live *M. tuberculosis* mediate the inhibition of SK1 activation remains to be established.

How does *M. tuberculosis* - or ManLAM-mediated inhibition of the cellular [Ca\(^{2+}\)]\(_{c}\) response arrest the phagosome maturation? Initially, the inhibition of [Ca\(^{2+}\)]\(_{c}\) by live *M. tuberculosis*, but not dead *M. tuberculosis*, was reported as important only for inhibiting phagosome maturation (Malik *et al*., 2000). Further characterization of the signalling pathway demonstrated that phagosomes containing live *M. tuberculosis* contained less of the [Ca\(^{2+}\)]\(_{c}\)-dependent effector protein calmodulin (CaM) compared with phagosomes containing dead *M. tuberculosis* (Malik *et al*., 2001). This results in lower activation of the CaM-dependent protein kinase II (CaMKII) on the phagosome membrane. Interestingly, the same characteristics could also be attributed to phagosomes containing ManLAM-coated latex beads compared with uncoated beads (Fratti *et al*., 2001; 2003; Vergne *et al*., 2003a). The lack of activated CaMKII seems to decrease the recruitment of phosphoinositol-3-kinase (PI3K) on the phagosome, thereby inhibiting the increase in phosphoinositol-3-phosphate (PI3P) in the membranes (Vergne *et al*., 2003b). The amount of PI3P is crucial for recruitment of early endosomal antigen 1 (EEA1) to phagosomes (Fratti *et al*., 2001; Vergne *et al*., 2003b). Furthermore, beads coated with ManLAM, but not PIMs, inhibited the recruitment of the intracellular markers syntaxin 6 and cathepsin D to the engulfing phagosome as a result of inhibition of EEA1 recruitment (Fratti *et al*., 2003). The importance of the lipid composition of the phagosome membrane for its intracellular trafficking has been clearly demonstrated by Anes *et al*., 2003). In an elegant *in vitro* assay, these authors characterized various lipids that either accelerated or inhibited phagosome maturation (Anes *et al*., 2003). Furthermore, the addition of these lipids to cells infected with *M. tuberculosis* had the same effect on phagosome maturation, which subsequently resulted in either accelerated killing or prolonged survival of the intracellular bacteria (Anes *et al*., 2003). These studies were the first to demonstrate the relationship between phagosomal lipid composition, intracellular trafficking and the survival of mycobacteria within this compartment.

Regulation of programmed cell death via calcium fluxes has been reviewed recently (Mattson and Chan, 2003; Orrenius *et al*., 2003), and one report provides evidence of a possible link between the activity of ManLAM in inhibiting infection-induced apoptosis and its capacity to inhibit
[Ca\(^{2+}\)]\(_{c}\); accumulation in macrophages (Rojas et al., 2000). Effector mechanisms by which [Ca\(^{2+}\)]\(_{c}\); accumulation might lead to apoptosis include the induction of increased membrane permeability of the mitochondria, which leads to cytochrome C release into the cytosol (Fig. 3). Increased cytosolic cytochrome C leads to the formation of the apoptosis complex. In caspases are activated. Next, activated caspases and nucleases finalize the apoptosis process by digesting proteins and DNA respectively (Mattson and Chan, 2003; Orrenius et al., 2003).

The connection between the Ca\(^{2+}\)-CaM pathway and IFN-\(\gamma\)-mediated upregulation of MHC II on macrophages was first demonstrated using a calmodulin antagonist (W7) that inhibited MHC II expression, whereas an inhibitor of the protein kinase C had no effect (Ina et al., 1987; Koide et al., 1988). Furthermore, CaMKII is known to mediate phosphorylation of residue S727 of Stat1, a critical event in IFN-\(\gamma\)-induced gene activation (Nair et al., 2002), presumably because phosphorylation of Stat1 at this position allows its interaction with the transcription factors CBP and p300 (Fig. 3). Thus, an important part of the inhibition of IFN-\(\gamma\) signalling by M. tuberculosis is mediated through the inhibition of [Ca\(^{2+}\)]\(_{c}\).

Finally, ManLAM can probably mediate inhibition of cellular responses in addition to inhibition of the cellular [Ca\(^{2+}\)]\(_{c}\) response. Indeed, ManLAM directly activates the Bad/Akt signalling pathway and thus promotes cell survival (Maiti et al., 2001). In addition, ManLAM increases the activity of the Src homology 2-containing tyrosine phosphatase 1 (SHP-1) (Knutson et al., 1998), which inhibits IFN-\(\gamma\) signalling by inducing dephosphorylation of the IFN-\(\gamma\) receptor-associated JAK kinases (Starr and Hilton, 1999). Moreover, the ability of ManLAM to inhibit apoptosis of macrophages is absent from macrophages isolated from mice deficient in SHP-1 expression (Rojas et al., 2002). In addition, SHP-1 activity might also be involved in the inhibition of the [Ca\(^{2+}\)]\(_{c}\) response usually associated with complement receptor 3 (CR3)-mediated phagocytosis by inducing dephosphorylation of tyrosine kinases that are important for the signal transduction upon binding of mycobacteria to CR3 (Fig. 3).

### Conclusion

Considerable strides have been made in identifying and characterizing genes that are required for PIMs, LM and LAM biosynthesis, but there is still much to be learned. Genetic strategies have shown that genes involved in the early steps of PIM biosynthesis are essential for mycobacterial growth. Recent work demonstrated that it is now feasible to generate LAM-deficient strains of C. glutamicum or M. smegmatis, and that LAM, in contrast...
to PIM, is not a requisite for in vitro growth. This also suggests that it will be possible to generate similar mutants in *M. tuberculosis* in the near future, which will be essential in order to establish the biological importance of LM/LAM in mycobacterial virulence, persistence and replication in the infected host. Such a genetic approach will demonstrate a causal relationship between the multitudes of biological activities attributed to isolated LAM and LM and the effect of bacterial infection on macrophages and DCs. In view of the vast array of effects mediated by LAM, some of these mutant strains should be strongly attenuated in animal models of tuberculosis and might therefore be interesting vaccine candidates. These mutants will also help to define targets for new tuberculosis drug developments.

Acknowledgements

G.S.B. acknowledges support as a Lister Institute–Jenner Research Fellow, from the Medical Research Council and the Wellcome Trust. L.K. is supported by INSERM. V.B. is supported by NIH grant AI51696-01, and S.A.P. by NIH grants AI48933 and AI45889. We would like to thank Dr David J. Kusner for critical reading of the manuscript and helpful comments.

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