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Review

Mycobacteria and their sweet proteins: An overview of protein glycosylation and lipoglycosylation in *M. tuberculosis*

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ABSTRACT

Post-translational modifications represent a key aspect of enzyme and protein regulation and function. Post-translational modifications are involved in signaling and response to stress, adaptation to changing environments, regulation of toxic and damaged proteins, proteins localization and host-pathogen interactions. Glycosylation in *Mycobacterium tuberculosis (Mtb)*, is a post-translational modification often found in conjunction with acylation in mycobacterial proteins. Since the discovery of glycosylated proteins in the early 1980's, important advances in our understanding of the mechanisms of protein glycosylation have been made. The number of known glycosylated substrates in *Mtb* has grown through the years, yet many questions remain.

This review will explore the current knowledge on protein glycosylation in *Mtb*, causative agent of Tuberculosis and number one infectious killer in the world. The mechanism and significance of this post-translational modification, as well as maturation, export and acylation of glycosylated proteins will be reviewed. We expect to provide the reader with an overall view of protein glycosylation in *Mtb,* as well as the significance of this post-translational modification to the physiology and host-pathogen interactions of this important pathogen.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011081 and 10.6019/PXD011081.

1. Tuberculosis and the tubercle bacilli

Despite increasing efforts of public health authorities, clinicians and researchers, Tuberculosis (TB) continues to be a relevant public health concern. TB was recognized in 2015 as the number one cause of death due to an infectious disease [1]. *Mycobacterium tuberculosis (Mtb),* a slow-growing bacillus with a characteristic waxy cell wall is the main causative agent of TB. Improvements in TB treatment and diagnosis, along with the development of an effective vaccine, are among the biggest challenges towards the successful control and eradication of this disease. Emergence of drug resistant strains, including multi-drug and extensively-drug resistant strains is on the rise [2], which further complicates control and treatment of TB. Understanding the physiology of *Mtb* and how the bacilli interact with the host is essential in order to design better diagnostics, treatments and improved vaccination strategies to control TB.

Mtb is an airborne facultative intracellular bacillus which upon entry into the respiratory airways, is phagocytized by lung macrophages and dendritic cells. These antigen presenting cells activate T cells which in turn activate and recruit additional macrophages. This cellular activity results in the formation of a granuloma, a multi-cell organized structure where the bacilli become dormant, only to reactivate in 5–10% of infected individuals. Several *Mtb* proteins and lipids have been identified as virulence factors. Some of these allow the bacteria to be recognized and persist inside the host, despite its detrimental environment. In addition, a number of proteins recognized as being virulence factors are decorated with post-translational modifications (PTM) essential for the correct function of the protein and/or recognition by the host cell.

PTMs are the result of a covalent linkage of a functional group to a protein or the modification of the protein via proteolysis by specific proteases and peptidases. PTMs are essential for the correct functioning of certain enzymes and proteins, and have a wide array of physiological consequences in mycobacteria. These range from regulation of cell functions and response to environmental stresses to the activation of host cellular responses. Here, we will discuss i) protein glycosylation of *Mtb* proteins; ii) provide an overview of acylation and export of glycosylated proteins in *Mtb*; and iii) we will present an in-depth review on

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the current knowledge of mycobacterial protein glycosylation mechanisms and pathways, their importance in both *Mtb* physiology and interactions with the host. We will conclude with some of the challenges and knowledge gaps for further research in this area.

2. Initial studies on mycobacteria protein glycosylation

Protein glycosylation is the covalent linkage of a mono- or polysaccharide to an amino acid residue, usually asparagine in *N*-glycosylation or serine and threonine in *O*-glycosylation. Glycoproteins were first described in eukaryotic cells and higher organisms and quickly became a point of interest due to their relevant physiological roles (i.e. immunoglobulins, hormones, cell receptors) (Reviewed many years ago in Refs. [3,4]). However, it was not until the early 80's that glycoproteins were identified as important immuno-dominant antigens and virulence factors in bacteria and viruses [5–8].

In Mycobacteria, the first indication of the existence of protein glycosylation was most likely presented by Schultz & Takayama in 1975. The authors carried out *in vitro* assays using *M. smegmatis* enzymatic preparations to demonstrate the transfer of radioactively labeled mannose to endogenous acceptors. These acceptors were then shown to be of proteic nature by digestion with proteases [9]. Later on, the existence of glycoproteins in *M. leprae* [10] and *Mycobacterium kansasii* [11] were demonstrated. In *M. leprae*, two putative glycoproteins, based on reaction with the Schiff's reagent were described in the early 80's. In *M. kansasii*, the existence of a presumed 50 KDa glycoprotein was described based on purification by lectin affinity chromatography, a technique that would become a paradigm for the identification of glycoproteins in mycobacteria. In fact, lectin binding was used to determine the possible existence of glycoproteins in *Mtb:* ConA-peroxidase labeling of culture filtrate proteins from H37Rv identified 3 major reactive bands at 38, 50 and 55 KDa. These bands were not only ConA reactive, but the reactivity was blocked by incubation with alpha-methyl D-mannoside, suggesting competition with mannoside residues [12]. Immunogenicity of these proteins was also demonstrated by interaction with serum from TB patients. To some extent, it was shown that serum interaction with the 38 KDa antigen was dependent on the carbohydrate moieties of the antigen. Even though this represented the first evidence of not only the existence, but also the potential role of glycoproteins in the immune response to *Mtb*, chemical characterization was still lacking. In fact, it was hypothesized that lectin reactivity could be due to heteropolysacharide-protein complexes or particular interactions of lipoarabinomannan with proteins.

Following a similar approach of ConA binding, the 19 KDa antigen was identified as being glycosylated in *M. bovis* [13] and later *Mtb* [14] in the early 1990s. In addition to ConA binding, Fifis and colleagues determined the carbohydrate composition of the putative glycosylated protein in *M. bovis* to be mannose as the primary carbohydrate with some minor amounts of arabinose [13].

Still, the chemical structure of these putative glycosyl modifications remained elusive until work by Dobos and colleagues in the mid 90's. The authors biochemically characterized the newly identified 45 KDa antigen (a.k.a Apa, Mpt32) [15], recognizing several of its glycosylated peptides via mass spectrometry analyses [16,17]. These studies resulted in the identification of a peptide in which *O*-glycosylation of threonine residues with up to two hexoses was demonstrated. Similar to what had been reported for the putative glycoproteins of *M. bovis*, carbohydrate analyses clearly revealed mannose as the primary sugar, with some evidence for potential glucose, galactose and arabinose residues. Amino acid analysis demonstrated the sequence of the 45 KDa Ag to be rich in proline and alanine, a trend that was also recognized in the *Mtb* 50–55 KDa antigen and 45–47 KDa antigen of *M. bovis* [18]. Since then, the enrichment of alanine-proline residues in glycosylated motifs has been accepted as a feature of mycobacteria glycoproteins. Dobos and colleagues also demonstrated the loss of hexoses after treatment with the jack bean alpha-D-mannosidase, providing further support of the mannosylation of the 45 KDa antigen [17]. Subsequent work by the same group identified additional peptides and glycosylation sites in the 45 KDa antigen and confirmed mannose to be the glycosyl residue bound to threonines by an α 1–2 mannobiose link [16]. Sugars other than mannose, which had been identified in these and previous studies were later shown to be a contaminant resulting from the various processes needed to purify the glycoproteins.

The next *Mtb* glycoprotein to be characterized was the 19 KDa antigen, also known as LpqH. Initially identified in M. bovis [13] and Mtb [14] as a potential glycosylated protein, site directed mutagenesis of LpqH's specific threonine residues resulted in marked reduction of ConA reactivity, confirming the glycosylation status of this protein [19]. Interestingly, non-glycosylated LpqH appeared to be more susceptible to proteolytic cleavage than the glycosylated form of the protein. This led to the hypothesis that glycosylation may inhibit proteolytic release of the protein from its lipid anchor in to the cell membrane [19]. Other studies seem to support the hypothesis that glycosylation may play a role in subcellular localization and protection against proteolysis [20].

While glycosylated threonine residues were readily identified during the first studies of glycosylation in *Mtb*, evidence for glycosylation of serine residues was not obtained until 2009. Sartain & Belisle characterized the glycosylation of serine residues in SodC using a combination of site directed mutagenesis and a variety of mass spectrometry analyses [20]. SodC is a membrane-associated lipoprotein with immunogenic characteristics [21] and had been previously identified as a potential lipoglycoprotein based on ConA reactivity [22]. Serine mannosylated residues in SodC were found to be located towards the *N*-terminus of the mature protein, similar to observations of threonine glycosylated proteins. Likewise, one to three α-mannose residues could be identified at each glycosylation site.

The discovery of mycobacterial glycoproteins opened the field to study the specific mechanisms of protein glycosylation. For instance, What are the enzymes involved in protein glycosylation? Is glycosylation required for survival and/or virulence? Are the glycosylation motifs important in host-pathogen interactions? If so, what are the host recognition mechanisms of glycosylated proteins? The following sections will explore the studies that provided answers to many of these questions and yet, opened another set of questions still waiting to be resolved.

3. Mechanistic events of protein glycosylation in mycobacteria

3.1. Rv1002c is a protein O-Mannosyl transferase

In the late 90's glycosyltransferase activity involved in the synthesis of mycobacterial cell wall polysaccharides and glycolipids was demonstrated via *in vitro* cell free assays with radiolabeled substrates [23,24]. Cooper and colleagues used a similar approach initially developed by Schultz and Takayama [9] to demonstrate the *in vitro* glycosylation of a synthetic peptide from the 45 KDa antigen when GDP-[14C]-mannose was added to subcellular fractions of *M. smegmatis* [25]. Glycosyltransferase activity was localized in the mycobacterial membrane and cell wall fractions, but was not present in the cytosolic fraction. This was consistent with previous studies on mycobacterial mannosyltransferases [23]. Moreover, the *in vitro* glycosyltransferase assay also demonstrated that the final mannose donor was a mannosyl-1-phosphoryl-deca/heptaprenol (C50PPM/C35PPM) [25].

With this evidence of protein glycosyltransferase activity a new question surfaced. What are the enzymes? Previous studies noted a similarity between protein glycosylation in mycobacteria and *O*-mannosylation in yeast [14,16]. Later on, VanderVen and colleagues identified Rv1002c as a *Mtb* homologue to the *Sacharomyces cerevisiae O*-mannosylatransferase (PMT) [26]. Using the cell-free assays previously developed to measure glycosyltransferase activity to synthetic peptides [9,25], the authors demonstrated that protein mannosylation occurred at much higher rates in membranes containing wild type Rv1002c,

while membranes containing Rv1002c mutated at the expected active site had a significant reduction in glycosyltransferase activity. These findings further supported the function of Rv1002c as the Protein Mannosyl Transferase (PMT) in *Mtb* [26].

Since then, homologues of Rv1002c in *Streptomyces lividans* and *Corynebacterium glutamicum* have been identified and their functions as a PMT have been confirmed [27,28]. Both of these species are able to produce glycosylated *Mtb* recombinant proteins [27,29].

Rv1002c has been shown to be important for virulence and *in vitro* growth in *Mtb* [30]. This mannosyl transferase recently has been shown to be expressed in higher quantities in the more virulent Beijing family when compared to other *Mtb* families [31], which supports its role in virulence. Interestingly, a *Mtb Rv1002c* Knock-Out (KO) mutant developed by Liu and colleagues, was impaired for growth in 7H9-dextrose broth and albumin enriched 7H11 solid media, but not in 7H10 supplemented with albumin, catalase and dextrose. In addition, this mutant was also impaired for intracellular persistence and proliferation in murine and human macrophages as well as for virulence in SCID mice. The authors concluded that *O*-mannosylation is an essential pathway for a successful host-pathogen interaction and the ability of *Mtb* to establish an infection [30].

Furthermore, our own proteomic studies comparing protein levels of the *Mtb* auxotrophic strain for panthotheonate and leucine (mc2 6206H37Rv ΔleuCDΔpanCD [32] and its counterpart carrying an additional deletion in Rv1002c (mc2 6206H37Rv ΔleuCDΔpanCDΔRv1002c [33] indicate that the lack of Rv1002c may indeed have broader implications in the physiology of the mycobacteria. Our findings provide evidence that the reduced virulence of the Rv1002c KO mutant observed by Liu and colleagues may result from a combination of decreased levels of immuno-dominant glycosylated proteins and also altered metabolism in the ΔRv1002c strain (Tables 1 and 2 and supplementary files 1, 2. Data have been deposited to the ProteomeXchange Consortium via the PRIDE [34] partner repository with the dataset identifier PXD011081 and 10.6019/PXD011081).

The decreased levels of immuno-dominant glycosylated proteins in the ΔRv1002c strain, many of which are known TLR-2 agonists (see section 4.2) may have direct implications in the ability of the host to recognize the pathogen and appropriately respond to infection. In addition, reduced levels of these proteins also support previous reports by VanderVen et al. and Herrmann et al. on the importance of protein glycosylation in secretion mechanisms [19,26]. Section 3.2 will offer additional details on this topic.

Our findings also suggest that the requirement of catalase for *in vitro* growth of the Rv1002c KO mutant may be due to a potential increase in oxidative damage in this strain. For instance, higher levels of SigH, the sigma factor involved in the response against oxidative stress [35,36] are found in whole cell lysates of the ΔRv1002c strain (Table 2). The ΔRv1002c also exhibited significantly decreased levels of cell associated proteins related to a variety of cellular pathways including enzymes involved in biosynthesis of amino acids, secondary metabolism and immunogenic proteins (Table 2). Rv2161c which is annotated as a potential enzyme involved in the synthesis of Lincomycin was also found in lower levels in the ΔRv1002c strain. Lincomycin is a macrolide class antibiotic for which biosynthesis is linked to that of the aromatic amino acid tyrosine. Interestingly, Crp (Cyclic AMP receptor protein) which in *Streptomyces* has been shown to regulate antibiotic production as well as other secondary metabolites [37] was also found in reduced levels in the ΔRv1002c mutant. Other enzymes involved in amino acid biosynthesis found in reduced levels in the ΔRv1002c strain included: IlvG, involved in Valine and Isoleucine biosynthesis; MetC, involved in methionine biosynthesis; and HisG, involved in histidine biosynthesis. In addition to amino-acid biosynthesis, the ΔRv1002c mutant also showed lower levels of enzymes involved in synthesis of fatty acids (HtdX and HtdY), translocation of PDIM (MmpL7) [38], and immunogenic proteins (Mpt70, Mpt64) among others (Tables 1 and 2). We now hypothesize that the broader effects of mannosyl transferase deficiency on bacterial cellular pathways, most notably in amino acid biosynthesis, reduction in dominant immunogenic glycoproteins, which may be essential for a successful interaction with the host and the alteration of secretion mechanisms impact the ability of the mycobacteria to thrive during infection.

One could argue that the reduced levels of lipoglycoproteins involved in export of PDIM and other lipids may result in a reduction of levels of these lipids in the Rv1002c KO mutant. However, a recent study by Alonso and colleagues showed that lack of mannosylation in the lipoglycoprotein LprG in a *Mtb* H37Rv ΔRv1002c mutant led to increased production of LAM, as well as an increased release of LAM-LprG complex into the extracellular milieu. These changes were further linked to an enhanced immune response in infected macrophages in a TLR-2 dependent manner [39]. As we will explore in section 3.4, there is an overlap in lipid and protein glycosylation. All of these support the complexity of the glycosylation pathway in *Mtb* with multiple downstream effects on substrates and their targets.

3.2. The role of Sec pathway in protein glycosylation

The majority of secreted proteins in bacteria are excreted from the cytoplasm through the Sec pathway [40]. This includes lipoproteins that remain in the cell envelope, and those that are fully secreted and deposited in the extracellular milieu. Proteins exported through the Sec pathway are recognized in the cytoplasm by SecA1 via the presence of a signal sequence at the secreted protein's *N*-terminus. SecA1 interacts with the channel forming proteins SecYEG and assist in the secreted protein's export through the channel in an ATP dependent manner [41]. Membrane proteins translocated to the cell membrane via the Sec pathway are recognized by their signal sequence through the ribonucleoprotein signal recognition particle or SRP [42] and delivered to FtsY as a ribosome-mRNA-nascent protein. FtsY then interacts with the SecYEG channel to complete the translocation of the protein into the cell membrane [43]. This process is also assisted by ATPase activity of SecA. SecA1 is an essential protein associated with the majority of secreted proteins, however, another ATPase, SecA2 recognizes a small subset of secreted proteins and directs them to the SecYEG channel for export [44–46]. While the signal sequence of proteins secreted via SecA1 do not differ from those secreted via SecA2, it appears that proteins which tend to undergo cytoplasmic folding as part of their physiological maturation are selectively recognized by SecA2. Signal sequences tend to have a net positive charge towards the *N*-terminus, a central hydrophobic domain and an uncharged hydrophilic domain towards the *C*-terminus which also includes the cleavage site [47]. In addition to these features, signal sequences from Sec-dependent lipoproteins contain a lipobox motif where the lipid moiety will be attached to a single cysteine [48]. After translocation, the signal sequence is cleaved by either type I or type II signal peptidases (LepB and LspA respectively) [49].

Glycoproteins are usually found in either the cell membrane, cell wall or as fully secreted proteins [17,26] which suggested a link between Sec translocation and glycosylation. The process of glycosylation was not directly linked to any secretion mechanisms until VanderVen and colleagues, in the same study that identified Rv1002c as *Mtb's* PMT, designed a clever experiment in which a mannosylated cassette of the 45 KDa antigen was fused to different secreted (both Sec-dependent and Sec-independent) and non-secreted proteins with and without signal sequences [26]. This study demonstrated that a Sec-dependent signal sequence was required for glycosylation. It also provided further confirmation by mass spectrometry that the glycosylation of the mannose cassette was composed of up to 3 hexoses [26]. Again, this study gave rise to additional questions. It pointed to a link between Sec translocation and glycosylatransferase activity, but the specific connection was not recognized. The authors hypothesized two potential scenarios. Proteins exported via the Sec-pathway are translocated to the membrane in an unfolded manner which may be required for the optimal

Table 1
Secreted proteins with significantly different levels (p-value < 0.05) in a mc2 6206H37Rv ΔleuCDΔpanCD ΔRv1002c vs. mc2 6206H37Rv ΔleuCDΔpanCD (Rv1002c WT).

Protein name[d]	Average WT[a]	SD WT[b]	Average KO[a]	SD KO[b]	Ratio (KO/WT)	Log ratio (KO/WT)	p-value[c]	Secreted	Functional Category[e]
Rv3627c	13.405	2.807	1.274	1.267	0.095	−1.022	0.0024	Yes	CH
Rv0907	8.897	4.473	1.026	1.776	0.115	−0.938	0.0472	No	CWCP
Rv1836c	12.548	4.611	1.451	1.546	0.116	−0.937	0.0168	No	CH
Rv0285	183.448	41.550	24.603	23.660	0.134	−0.873	0.0045	Yes	PE/PPE
EspJ	18.808	2.976	2.552	2.562	0.136	−0.868	0.0020	No	CWCP
Mmpl5	19.945	8.241	2.706	2.355	0.136	−0.867	0.0253	No	CWCP
GgtB	6.832	2.371	1.051	0.959	0.154	−0.813	0.0173	Yes	IMR
QcrC	5.237	2.167	0.807	1.399	0.154	−0.812	0.0410	No	IMR
Rv2223c	12.839	3.951	2.120	3.672	0.165	−0.782	0.0263	Yes	CWCP
Rv0064	10.860	2.931	1.855	1.626	0.171	−0.767	0.0096	No	CWCP
Rv0315	39.204	6.275	7.356	3.564	0.188	−0.727	0.0016	Yes	IMR
LpqB	16.035	4.253	3.050	1.221	0.190	−0.721	0.0071	Yes	CWCP
LpqL	17.945	2.210	3.528	4.133	0.197	−0.706	0.0060	Yes	CWCP
Rv0822c	22.796	5.992	4.484	2.580	0.197	−0.706	0.0083	No	CH
Rv3194	40.292	15.105	8.528	8.372	0.212	−0.674	0.0334	Yes	CWCP
LprQ	21.346	4.951	4.576	5.339	0.214	−0.669	0.0163	No	CWCP
Rv1096	13.820	5.188	4.169	1.919	0.302	−0.521	0.0391	No	IMR
Rv2091c	10.755	2.369	4.234	1.848	0.394	−0.405	0.0198	No	CWCP
MycP5	14.300	1.069	6.149	4.627	0.430	−0.366	0.0410	Yes	IMR
FecB	27.368	3.234	13.832	2.445	0.505	−0.296	0.0044	Yes	CWCP
Rv3705c	18.015	3.207	9.421	1.951	0.523	−0.282	0.0166	Yes	CH
Rv2721c	67.392	10.913	36.105	14.633	0.536	−0.271	0.0412	Yes	UK
Rv3312A	23.596	3.034	13.362	3.047	0.566	−0.247	0.0146	Yes	CWCP
Rv0398c	48.050	5.441	28.519	5.185	0.594	−0.227	0.0108	Yes	CWCP
MmpS5	16.144	0.644	10.297	3.446	0.638	−0.195	0.0446	Yes	UK
Mpt32	363.866	36.805	235.784	26.834	0.648	−0.188	0.0082	Yes	CWCP
PonA2	71.290	2.354	46.571	7.424	0.653	−0.185	0.0053	Yes	CWCP
PepD	88.100	5.178	57.898	7.547	0.657	−0.182	0.0046	No	IMR
GroES	489.510	97.684	763.410	12.726	1.560	0.193	0.0085	No	VDA
SecE2	18.421	1.016	34.522	3.325	1.874	0.273	0.0013	Yes	CWCP
Rv2716	15.306	3.564	29.889	4.741	1.953	0.291	0.0131	No	CH
Tkt	19.385	10.661	50.341	11.796	2.597	0.414	0.0280	No	IMR
FixB	7.569	3.964	20.284	2.358	2.680	0.428	0.0088	No	IMR
MenG	5.751	1.406	17.124	6.341	2.978	0.474	0.0387	No	RP
Rv0007	3.838	3.374	15.101	3.831	3.934	0.595	0.0188	No	CWCP
AceE	0.924	0.918	10.967	3.914	11.868	1.074	0.0124	No	IMR
TrxB1	0.306	0.530	6.137	2.430	20.062	1.302	0.0153	No	IMR
Frr	1.230	1.400	38.248	1.567	31.096	1.493	0.0000	No	IP
Rv0898	0.000	0.000	10.599	6.338	ABS WT	ABS WT	0.0443	No	**CH**

[a] Average of the Normalized Total Spectra of three biological replicates.
[b] Standard Deviation of the Normalized Total Spectra of three biological replicates.
[c] Significant statistical differences between strains for each protein were calculated using a Student's t-test of the average Normalized Total Spectra of each protein per strain. Values with p-value < 0.05 were considered significant.
[d] Protein names in bold correspond to known glycosylated proteins, all of them with decreased levels in the ΔRv1002c.
[e] Functional categories are as follows: Conserved Hypothetical (CH), Cell Wall and Cell Processes (CWCP), Intermediary Metabolism and Respiration (IMR), Regulatory Proteins (RP), Information Pathways (IP), Virulence, Detoxification and Adaptation (VDA), Unknown (UK), PE/PPE (PE (Proline-Glutamic) family of proteins).

activity by *Mtb's* PMT (Rv1002c) and may in part explain why Sec-independent secreted proteins are not glycosylated. The second possibility is that the Sec translocon and the PMT co-localize in the cell membrane, thus resulting in easy access to substrates by the PMT as has been observed in *N*− and *O*− glycosylation in eukaryotes [50,51]. Both of these possibilities remain untested in *Mtb*.

Findings from our proteomics study indicated that a large proportion of secreted proteins with a decreased abundance in the Rv1002c mutant were indeed known glycosylated and lipoglycosylated proteins (Table 1), supporting the idea that not only is Sec translocation required for glycosylation, as demonstrated by VanderVen et al. [26], but conversely, glycosylation is required for full export of the PMT substrate into the extracellular milieu.

Herrmann and colleagues proposed that lack of glycosylation may lead to increased proteolysis of lipoglycoproteins resulting in shedding of these proteins from the mycobacterial cell envelope. The authors based this hypothesis upon observations of a truncated form of recombinant non-glycosylated LpqH in the secreted fraction of *Mycobacterium vaccae* and *Mycobacterium smegmatis* expressing a *Mtb* LpqH construct that was mutated at the glycosylation sites [19]. Sartain and Belisle conducted similar studies on recombinant SodC expressed in *Mtb* and also observed a truncated version of rSodC in the non-glycosylated form of the recombinant protein. While neither study included a recombinant lipoprotein known to be non-glycosylated as a control, Sartain and Belisle noted some differences in the relative abundance of some of the constructs, supporting the conjecture of increased proteolysis in proteins with reduced glycosylation.

Based on these studies, two somewhat opposing results could be expected in the Rv1002c KO mutants. One, the shedding of the non-glycosylated protein from the membrane could result in increased amounts of protein into the extracellular milleu. Or conversely, the increased proteolysis may result in lower amounts of the non-glycosylated proteins in the extracellular milieu due to increased susceptibility to protease activity. Further studies utilizing targeted proteomics to measure the absolute quantification of these proteins in all subcellular fractions (secreted, cell membrane, cell wall, and cytosol) should provide additional information regarding the processing of these proteins in the presence and absence of glycosylation. It is also possible that increased proteolysis of LpqH and SodC may be entirely due to higher levels of expression usually seen in recombinant technology, leading to

Table 2
Proteins with significantly different levels (p-value < 0.05) in the whole cell lysate of a mc2 6206H37Rv ΔleuCDΔpanCD ΔRv1002c vs. mc2 6206H37Rv ΔleuCDΔpanCD (Rv1002c WT).

Protein name	Average WT[a]	SD WT[b]	Average KO[a]	SD KO[b]	Ratio (KO/WT)	Log ratio (KO/WT)	p-value[c]	Functional Category[d]	Function
Rv2510c	2.646	0.369	0.000	0.000	0.000	ABS KO	0.0002	CH	Conserved protein
PknF	2.025	0.331	0.000	0.000	0.000	ABS KO	0.0004	RP	Protein kinase, possibly involved in membrane transport
Mpt70	10.849	4.236	0.000	0.000	0.000	ABS KO	0.0114	CWCP	Lipoprotein antigen
CeoC	4.428	2.164	0.000	0.000	0.000	ABS KO	0.0239	CWCP	Trk, involved in potassium uptake
PepD	7.473	1.769	0.412	0.714	0.055	−1.258	0.0030	IMR	Serine protease pepD
FadD36	7.770	3.836	0.844	1.462	0.109	−0.964	0.0432	LM	Fatty acid CoA synthase
Rv1638A	8.955	1.084	1.123	1.946	0.125	−0.902	0.0037	CH	Similar to 35 KDa
MetC	8.564	1.691	1.125	1.949	0.131	−0.881	0.0075	IMR	Methionine biosynthesis
Cfp21	12.196	1.154	1.968	3.409	0.161	−0.792	0.0079	CWCP	Cutinase
IlvG	3.872	0.622	0.681	0.616	0.176	−0.755	0.0032	IMR	Valine and Isoleucine biosynthesis
EntC	5.810	2.556	1.049	0.915	0.181	−0.743	0.0385	IMR	Isochorismate synthase, possibly involved in enterobactin biosynthesis
Rv0241c	8.154	1.664	1.818	0.619	0.223	−0.652	0.0035	IMR	Probable 3-hydroxyacyl-thioester dehydratase HtdX
OtsA	2.462	0.392	0.563	0.975	0.229	−0.641	0.0352	VDA	Involved in osmoregulatory trehalose biosynthesis
Pca	17.222	2.215	4.348	5.214	0.252	−0.598	0.0170	IMR	Probable pyruvate carboxylase Pca (pyruvic carboxylase)
LipN	11.804	1.338	3.343	3.876	0.283	−0.548	0.0233	IMR	Lipase/esterase
Rv2161c	12.049	1.836	3.874	3.483	0.321	−0.493	0.0228	IMR	Potential lincomycin production
Rv2172c	22.710	6.401	7.502	6.693	0.330	−0.481	0.0467	CH	Unknown
Rv2159c	65.074	10.830	21.610	19.695	0.332	−0.479	0.0286	CH	Unknown
HisG	9.957	2.400	3.558	1.308	0.357	−0.447	0.0154	IMR	Involved in histidine biosynthesis
MurE	12.930	2.552	4.878	4.003	0.377	−0.423	0.0425	CWCP	Involved in peptidoglycan biosynthesis
Rv0458	26.112	3.343	10.352	3.997	0.396	−0.402	0.0063	IMR	Aldehyde dehydrogenase
MmpL7	11.117	1.974	4.532	2.141	0.408	−0.390	0.0173	CWCP	Involved in translocation of DIM
Rv1489	11.853	1.955	5.128	2.348	0.433	−0.364	0.0189	CH	Unknown
Rv2672	6.068	1.695	2.703	0.196	0.445	−0.351	0.0269	IMR	Secreted protease
FadE15	5.110	0.184	2.427	1.479	0.475	−0.323	0.0356	LM	Possibly involved in lipid degradation
Rv2073c	4.577	1.073	2.231	0.883	0.488	−0.312	0.0431	IMR	Probable short chain dehydrogenase
Mpt64	19.796	3.437	9.994	1.138	0.505	−0.297	0.0094	CWCP	Immunogenic protein
Efp	6.446	1.053	3.268	0.832	0.507	−0.295	0.0149	IP	Probable elongation factor
ProS	29.869	3.616	17.984	6.393	0.602	−0.220	0.0487	IP	Probable prolyl-tRNA synthetase
Zwf2	15.238	1.367	9.559	2.781	0.627	−0.203	0.0337	IMR	Probable glucose-6-phosphate 1-dehydrogenase
Rv2302	9.130	1.060	6.231	1.414	0.683	−0.166	0.0468	CH	Unknown
Rv1006	46.865	6.275	32.170	0.988	0.686	−0.163	0.0160	CH	Outer membrane protein
HtdY	35.011	1.925	24.052	3.154	0.687	−0.163	0.0068	IMR	Probable 3-hydroxyacyl-thioester dehydratase
Rv3676	41.621	2.196	28.753	2.218	0.691	−0.161	0.0020	RP	Crp transcriptional regulatory protein
Qor	37.858	3.530	26.392	2.408	0.697	−0.157	0.0097	IMR	Quinone reductase
RphA	19.035	1.804	13.338	2.797	0.701	−0.154	0.0414	IP	Ribonuclease
PknG	14.245	0.078	9.999	2.380	0.702	−0.154	0.0366	RP	Serine/threonine-protein kinase
GrcC1	19.893	0.962	14.005	1.060	0.704	−0.152	0.0021	IMR	Polyprenyl-diphosphate synthase
Rv3224	74.120	3.524	57.842	1.764	0.780	−0.108	0.0020	IMR	Iron regulated short chain dehydrogenase
RpoB	98.623	2.842	113.250	8.381	1.148	0.060	0.0458	IP	RNA polymerase subunit B
Rho	27.969	3.708	35.071	1.789	1.254	0.098	0.0404	IP	Transcription terminator factor
TpX	33.950	3.107	44.688	4.247	1.316	0.119	0.0241	VDA	Thiol peroxidase
AceE	54.181	6.877	72.160	8.478	1.332	0.124	0.0463	IMR	Pyruvate dehydrogenase
Ppa	30.964	3.321	42.103	4.046	1.360	0.133	0.0211	IMR	Inorganic pyrophosphatase
Rv1099c	14.796	0.972	20.195	2.280	1.365	0.135	0.0195	IMR	GlpX, involved in gluconeogenesis
Rv2258c	12.910	1.080	18.011	2.831	1.395	0.145	0.0434	RP	Possible transcriptional regulatory protein
GroEl2	455.010	99.795	679.497	62.115	1.493	0.174	0.0297	VDA	Chaperone
AccD5	34.809	3.209	59.571	8.499	1.711	0.233	0.0092	LM	Propionyl-CoA carboxylase
TrpB	13.317	3.400	23.886	2.180	1.794	0.254	0.0106	IMR	Tryptophan biosynthesis
SdhA	5.184	1.201	9.537	2.411	1.840	0.265	0.0488	IMR	Involved in TCA, fumarate reductase
SigH	9.950	4.299	21.134	3.325	2.124	0.327	0.0235	IP	Sigma factor H
SigA	25.694	15.880	56.868	5.162	2.213	0.345	0.0319	IP	Sigma factor A
BfrB	84.641	12.498	193.920	63.413	2.291	0.360	0.0429	IMR	Bacterioferritin, involved in iron storage
GgtA	2.823	1.695	6.910	1.812	2.447	0.389	0.0463	IMR	Probable bifunctional acylase
Rv1159A	3.396	3.216	10.574	1.500	3.114	0.493	0.0248	CH	Unknown
CysK1	3.946	2.762	14.514	3.306	3.678	0.566	0.0132	IMR	Cysteine synthase
Rv3127	14.340	13.644	55.636	18.609	3.880	0.589	0.0362	CH	Conserved protein
Rv0670	2.527	2.310	9.970	3.103	3.945	0.596	0.0290	IP	Probable endonuclease IV End
FtsZ	0.527	0.913	3.960	0.524	7.512	0.876	0.0048	CWCP	Essential for cell division
Rv2627c	1.403	0.172	12.361	0.986	8.809	0.945	0.0000	CH	Conserved protein
AhpD	0.256	0.443	5.499	0.397	21.491	1.332	0.0001	VDA	Alkyl hydroperoxide reductase D protein

[a] Average of the Normalized Total Spectra of three biological replicates.
[b] Standard Deviation of the Normalized Total Spectra of three biological replicates.
[c] Significant statistical differences between strains for each protein were calculated using a Student's t-test of the average Normalized Total Spectra of each protein per strain. Values with p-value < 0.05 were considered significant.
[d] Functional categories are as follows: Conserved Hypothetical (CH), Cell Wall and Cell Processes (CWCP), Intermediary Metabolism and Respiration (IMR), Regulatory Proteins (RP), Information Pathways (IP), Virulence, Detoxification and Adaptation (VDA), Unknown (UK), PE/PPE (PE (Proline-Glutamic) family of

proteins).

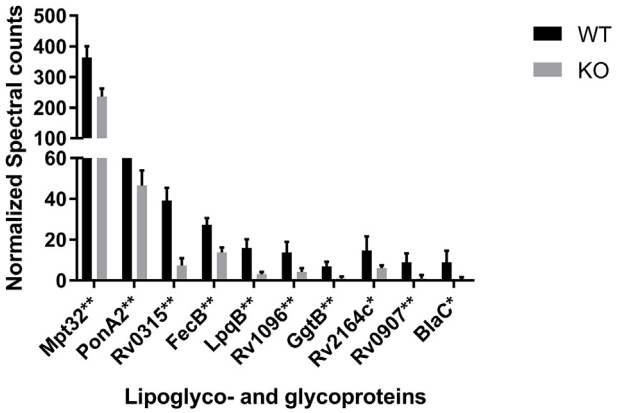

Fig. 1. Abundance of lipoglyco- and glycoproteins in the secreted fraction of mc2 6206 H37Rv ΔleuCDΔpanCD ΔRv1002c vs. mc2 6206 H37Rv ΔleuCDΔpanCD (Rv1002c WT). Black bars represent proteins levels in the Rv1002 WT strain. Grey bars represent proteins levels in ΔRv1002 strain. **Significance at p-value < 0.05, *Significance at p-value < 0.1. Lipoglycosylated proteins include FecB, LpqB, GgtB and BlaC. All others are glycosylated proteins.

inefficiencies in the acylation pathway, and resulting in an excess of unmodified protein that is subsequently shed into the extracellular milieu. Our proteomic findings show a general trend of lower abundance of lipoglyco- and glycosylated proteins in the secreted fraction of the ΔRv1002c mutant (Fig. 1) which may indicate an increased susceptibility to proteolysis, in support of the studies of Hermann and Belisle. Alternatively, this lower abundance may be due to inefficiency of the secretion mechanism in the absence of protein glycosylation, in support of the alternate hypothesis introduced above. It is also possible that the decreased abundance of these proteins in the secreted fraction may be directly related to an overall decreased in expression of these genes in the ΔRv1002c mutant. Additional studies are needed to corroborate any of these hypothesis.

Taken all together, it is likely possible that Rv1002c may be required for correct processing of the pro-glyco- and lipoglycoproteins.

3.3. Protein acylation of glycosylated proteins

Near 50% of all known glycosylated proteins are acylated. These are lipoglycoproteins that are often found in *Mtb*'s envelope. Given this co-occurrence, we will briefly discuss the mechanisms of protein acylation in mycobacteria.

Protein acylation is the covalent linkage of a fatty acid chain to selected amino acid residues in a protein. In Mycobacteria the only recognized type of protein acylation is that of the *N*-terminal cysteine of the mature protein. Such acylation effectively anchors the lipoprotein into the cytoplasmic membrane. In other organisms, acylation of serine and threonine such as mycoloylation in *Corynebacterium glutamicum* (i.e.modification of outer membrane porines by mycolic acids) at key serine residues has also been observed. *C. glutamicum* belongs to the bacterial order of Corynebacteriales which includes the *Mycobacterium* genus, and thus opens the possibility that *Mtb* or other mycobacteria could also include mycoloylation as part of their PTMs, however, this hypothesis remains to be tested [52–54].

Acylation of lipoproteins is dependent upon successful translocation of the pre-lipoprotein into the membrane. Translocation is performed primarily via the Sec pathway, but in a handful of proteins, it is performed via the Twin arginine translocation (Tat) system [55,56]. Currently, there is no indication that the acylation mechanism differs between Sec- and Tat-secreted proteins. Before cleavage of the signal sequence by the signal peptidase (LspA), Lgt, a phosphatidylglycerol:preprolipoprotein diacylglyceryl transferase attaches a diacylglyceryl molecule via a thioether bond to the cysteine located in the lipobox at the signal sequence cleavage site [57,58]. After cleavage of the signal sequence, the resulting diacylated *N*-terminal cysteine is then *N*-acylated at its free amino group by the apolipoprotein *N*-acyltransferase (Lnt) [57,59–61] (Fig. 2).

The first enzyme in the acylation pathway, Lgt, has been shown to be essential in *Mtb* H37Rv [62–64]. The essentiality of this enzyme hinders the ability to create Lgt KO mutants, making functional studies on *Mtb* protein acylation difficult. Thus, such studies have been carried out in the model organism *M. smegmatis* where Lgt appears to have two homologues (MSMEG_5408 and MSMEG_3222). Tschumi and colleagues were able to delete the closest *Mtb* Lgt homologue (MSMEG_3222) and demonstrated the lack of acylation in *M. smegmatis* lipoproteins as well as an increased release of these proteins into the extracellular milleu. This provided evidence that Lgt's activity results in the first acylation step required for anchoring of the protein into the cell membrane [64]. In the same study, the authors found that the majority of lipoproteins identified in the secreted material of the *M. smegmatis* Δlgt mutant appeared to be lacking their signal peptide. The authors suggested that this may be due to Lgt independent cleavage by either

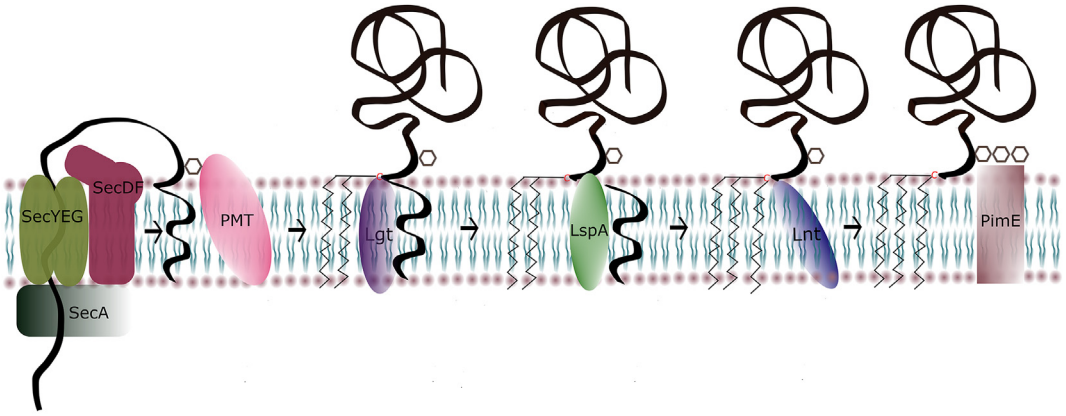

Fig. 2. Current understanding of the Protein Glycosylation and Acylation Process in *Mtb*. From left to right: PMT (Rv1002c) decorates the protein with a single mannosyl residue before or during export by the Sec Pathway (Multiple glycosylation sites may be present. Only one shown in figure). Lgt catalyzes the formation of a thioether linkage between a diacylglycerol and the *N*-terminal cysteine located immediately downstream of the signal sequence. After di-acylation, LspA cleaves the signal sequence. Lnt catalyzes the *N*-acylation of the *N*-terminal Cysteine of the mature lipoglycoprotein. Finally, PimE elongates the mannosyl chain.

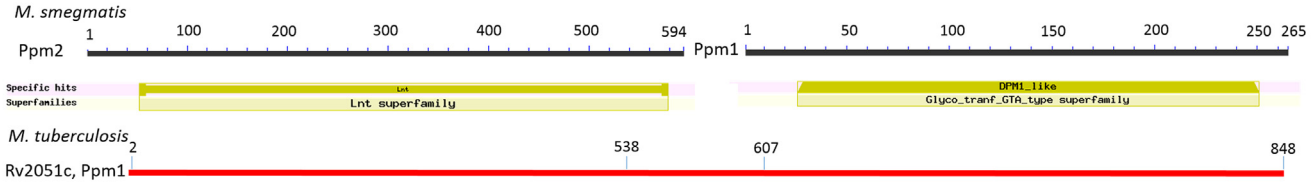

Fig. 3. Ppm1/2 gene in *M. smegmatis* and *M. tuberculosis*. In *M. smegmatis*, Ppm2 corresponds to the Lnt domain with *N*-acyl transferase activity, while Ppm1 corresponds to the domain with glycosyl transferase activity. In *Mtb*, both domains are present in Ppm1 (Rv2051c).

LepB (SpaseI), LspA or other proteases, also contributing to the release of the proteins into the media as has been observed in other bacteria [64–66]. Yet, other lipoproteins such as LppX and LpqH seemed to be released with their signal sequence intact, pointing out differential requirements of LspA in the absence of the diacylglycerol modified residues [64].

The majority of information on mycobacterial lipoproteins has stemmed from studies in fast growing saprophytic mycobacteria such as *M. smegmatis* [57,64,67]. However, recent analysis of lipoproteins expressed in *M. bovis* BCG confirmed that slow growing mycobacterial lipoproteins are also subjected to acylation resulting in triacylated proteins with different combinations of saturated and unsaturated C16, C18 and C19 fatty acids. In *M. bovis* BCG, the apolipoprotein *N*-acyltransferase (Lnt) is encoded by BCG_2070c, a homologue of *Mtb* Rv2051c (annotated as polyprenol-monophosphomannose synthase, Ppm 1 [57,68]. In *M. smegmatis*, *M. avium* and *M. leprae* the homologue of Ppm1 (Rv2051c) is split into two genes. *In M. smegmatis*, these genes are MSMEG_3859 (Ms-Ppm1) and MSMEG_3869 (Ms-Ppm2) (Fig. 3). While Ms-Ppm1 appears to be essential for survival of the mycobacteria, Ms-Ppm2 is not. Ms-Ppm1 is essential for synthesis of PIMs and lipomannan [69], while Ms-Ppm2 is expected to function as the *N*-acyl transferase in *M. smegmatis* [67]. In pathogenic mycobacteria, the essentiality of the gene is controversial. While BCG_2070c appears to be not essential in *M. bovis* BCG, Ppm1 in *Mtb* was found to be essential in one study [62] and non-essential in another [63] even though both were based in Himar1-based transposon mutagenesis. Zhang and colleagues suggested that these contradictory findings may be directly related to the gene domain where the transposon insertion occurred. They found that Ppm1 could sustain many insertions in the region encoding the carbon-nitrogen hydrolase domain, while the region encoding the *C*-terminal glycosyl transferase domain was required for optimal growth. This suggests that *N*-acylation function, although not essential, is of significant importance in pathogenic mycobacteria [70].

LspA, the signal peptidase involved in cleavage of lipoprotein signal sequence is not essential in *Mtb*. However, its deletion results in marked reduction of virulence in the mouse model and during *in vitro* infection of macrophages [71,72]. At least 73 Mtb lipoproteins have been identified as potential LspA substrates [73], many of which are involved in host-pathogen interactions or have a direct role in the physiology of the organisms (See other sections of this review for additional details). Lack of signal peptide cleavage probably results in a deficiency in *N*-acylation and thus impaired function or recognition by the immune system of some of these important lipoproteins.

3.4. Lipid and protein glycosylation cross pathways

The *Mtb* cell wall is unique in its glycolipid composition with lipids often carrying oligomannosyl and oligoarabinosyl chains. Lipoproteins are also often modified by glycosylation [74] (Table 3). It is thus expected that protein and lipid glycosylation pathways may share some common enzymes and substrates.

In *C. glutamicum*, the *N*-acyl transferase, Ppm2 affects both *N*-acylation and glycosylation of *Mtb* LppX suggesting that protein glycosylation in this bacteria occurs after full triacylation of the lipoprotein [29]. Triacylation and glycosylation of LppX were recovered when the Δppm2 mutant was complemented with either *C. glutamicum* ppm2 or ppm1, as well as with *Mtb* Rv2051c (ppm1). The authors then hypothesized that the absence of Ppm2 may affect the stability or activity of *C. glutamicum* glycosyl transferase. This activity can be complemented by either Ppm2 or Ppm1. It is unclear if the same mechanism occurs in *Mtb* where Rv2051c functions as both Ppm1 and Ppm2 (Fig. 3). Mass spectrometry evidence of different proteoforms of *Mtb* LpqH expressed in *M. smegmatis*, suggests that the first mannosylation step by the protein mannosyl transferase (PMT) occurs concomitantly with Sec translocation, before any acylation by Lgt and Lnt occurs (Fig. 2). However, in addition to non-acylated glycosylated forms of LpqH, Parra and colleagues also identified low abundant diacylated isoforms of non-glycosylated LpqH. This suggests that glycosylation may also occur after the protein has been diacylated by LgtA, but before *N*-acylation by Lnt [75]. This would indicate that protein glycosylation in *Mtb* lipoglycoproteins may be independent of *N*-acylation, but in some instances could be dependent on Lgt activity. Additional studies are needed to confirm any cross-dependency of the glycosylation and acylation pathways in *Mtb*.

Gurcha and colleagues first described the function of Rv2051c (ppm1) in the biosynthesis of polyprenol phosphomannose (PPM) [68]. Rv2051 has since been noted as essential for lipoglycan synthesis [69]. So far it is unclear if the lack of PPM biosynthesis activity (i.e. Ppm2 in *M. smegmatis*, Ppm1 (Rv2051c) in *Mtb*) results in lower levels of glycosylation (even in non-acylated proteins), an effect that may be expected due to lower levels of the mannose donor PPM. If that is the case, it would indicate that the lower levels of lipoglycoproteins observed in *C. glutamicum* ppm2 mutants may be a compounding effect due to a deficiency of mannosyl residues available for transfer from the lipid donor into the peptidyl acceptor by Rv1002c. This effect would be expected to occur in addition to (or independently of) the *N*-acylation deficiency.

Beyond the link between Ppm enzymes and glycosylation, other enzymes involved in lipid glycosylation are also expected to have a role in protein glycosylation. After glycosylation with the first mannosyl residue, subsequent elongation of the mannosyl chains is expected to happen in a stepwise process, similar to that of *O*-mannosylation in eukaryotes [26]. Given the α 1–2 interglycosidic linkage observed in mannosylated proteins, it is expected that elongation will occur through an α 1–2 mannosyl transferase, similar to the elongation of oligomannosyl chains in phosphatidyl inositol mannosides (PIM6), lipomanan (LM) and lipoarabinomannan (LAM) [30,76–78]. Until recently, the identity of this enzyme was unknown, but analyses of *M. smegmatis* strains constructed with deletions of two expected α 1–2 manosyl transferases (MSMEG_5149 (PimE) and MSMEG_4247), demonstrated that stepwise elongation of the oligomannoside chain in the *M. smegmatis* glycosylated protein FasC was due to the activity of PimE, an

Table 3
Known glycosylated proteins in Mycobacteria.

Name	Acylation	Reference	Function
Apa/Mpt32/45 KDa (Rv1860)	No	[16,17,89]	Adhesin [114]; immunomodulatory antigen [33,119]
LpqH (19KDa/Rv3763)	Yes	[19,22,30,89]	TLR-2 agonist [31,97,98]
LprA (Rv1270c)	Yes	[74,89]	TLR-2 ligand [87]
LprG (Rv1411c)	Yes	[39,74]	TLR-2 ligand (21–25); LAM transport [39,85,121]; regulation of triglyceride levels [84,139]
LprI (Rv1541c)	Yes	[22]	Lysozyme inhibitor [140]
LppX (Rv2945c)	Yes	*C. glutamicun* [29], *M. abscessus* [138]	PDIM transport [123]
LppO (Rv2290)	Yes	[89]	Lipoprotein, function unknown
LppQ (Rv2341)	Yes	[22]	Unknown
LpqR (Rv0838)	Yes	[39]	Unknown
LppN (Rv2270)	Yes	[22]	Unknown
LpqW (Rv1166)	Yes	[74]	Regulation of LAM biosynthesis [124,129]
LpqB (Rv3244c)	Yes	[74]	Regulation of the Two-Component System MtrA-MtrB involved in cell division [130]
LpqF (Rv3593)	Yes	[74]	Unknown
LppZ (Rv3006)	Yes	[74]	Immunogenic protein [21,141]
LpqN (Rv0583c)	Yes	[74]	Unknown
LprF (Rv1368)	Yes	[74,127]	Interacts with KdpD and may be involved in sensing of osmotic stress [128]
LpqT (Rv1016c)	Yes	[74]	TLR-2 ligand [142]
LppC (Rv1911c)	Yes	[74]	Unknown
GgtB (Rv2394)	Yes	[74]	Gamma-glutamyltransferase
GlnH (Rv0411c)	Yes	[89]	Active import of glutamine
SodC (Rv0432)	Yes	[20]	Immunogenic protein [105]
PstS1/38Kda Ag (Rv0934)	Yes	[74]	Phosphate binging protein/Phosphate uptake
PstS3 (Rv0928)	Yes	[74]	Phosphate binging protein/Phosphate uptake
PonA2 (Rv3682)	Yes	[74]	Penicillin binding protein involved in peptidoglycan biosynthesis [126]
Mpt83 (Rv2873)	Yes	[22,74]	Adhesin factor [108], TLR-2 agonist [109]
BlaC (Rv2068c)	Yes	[89]	Beta-lactamase
OppA (Rv1280c)	Yes	[74]	Glutathione transporter [112]
Rv0907	Yes	[74]	Unknown, possibly involved in cell wall biosynthesis
Rv0175	No	[39,89]	Mce associated, function unknown
Rv0281	No	[74]	Possible S-adenosylmethionine-dependent methyltransferase
Rv0315	No	[39]	Possible beta 1,3-glucanase precursor
Rv0988	No	[74]	Unknown
Rv1084	No	[74]	Unknown
Rv1096	No	[89]	Peptidoglycan deacetylase [143]
Rv1324	No	[74]	Possible thioredoxin
Rv1887	No	[89]	Unknown
Rv2164c	No	[89]	Unknown
Rv2672	No	[74]	Mycobacterial secreted hydrolase, hydrolysis host lipids [118]
Rv2799	No	[89]	Probable membrane protein
35 KDa Ag/Rv2744c	No	[89]	Phage shock protein (PspA ortholog) (Armstrong et al., 2016)
Rv3835	No	[89]	Membrane protein
Rv3491	No	[39,89]	Unknown
BfrB (Rv3841)	No	[74]	Bacterioferritin, involved in iron storage
FhaA (Rv0020c)	No	[74]	Involved in signal transduction
OmpA (Rv0899)	No	[74]	Porin, peptidoglycan binding protein [144]
PpiB (Rv2582)	No	[74]	Cyclophilin B (Ppetidyl-prolyl *cis*-trans isomerase. Modulates the immune response *in vitro* [145]

enzyme which is also responsible for the mannosylation of PIMs [79]. Interestingly, the deletion of MSMEG_5149 (PimE) and subsequent defect in elongation of oligomannoyl chain in FasC as well as loss of polar PIMs was successfully complemented with *Mtb* Rv1159 (pimE) gene indicating an expected similar function of PimE in *Mtb* [30]. Additional support for the stepwise elongation of mannosylated residues was later provided by analysis of recombinant LpqH expressed in *M. smegmatis* and high resolution mass spectrometry of different proteoforms of this lipoglycoprotein [75].

4. Glycosylated proteins and their role in *Mtb* physiology and host-pathogen interactions

Recognition of glycosylated proteins in *Mtb* led to an increased interest in the field. Proteomic and bioinformatic tools were applied to the identification of proteins with glycosyl residues at a global level. However, one of the main impediments for identification of *O*-glycosylation, which is in contrast to *N*-glycosylation, is the lack of a well-defined motif. Nevertheless, studies of *O*-glycosylation in other organisms have shown certain characteristics found in known *O*-glycosylated motifs such as the presence of alanine and proline residues around glycosylated serine and threonine. This information has been used to build an algorithm (i.e. NetOGlyc) for the 'in silico' identification of potential *O*-glycosylated proteins [80–83]. Using this algorithm, Herrmann and colleagues identified potential glycosylated proteins in *Mtb* and confirmed their glycosylation status using a mycobacterial expression system followed by ConA reactivity. The proteins identified in this study included LprI, GlnH, PstS1 (38 KDa Ag), SodC, Mpt83, LppQ, LppN and LpqH, while 3 additional products were negative for ConA binding [22]. Their approach was confined to potential lipoglycoproteins and thus, many other non-acylated glycoproteins were not identified.

The first glycoproteomics approach used 2D gel analyses of *Mtb* secreted proteins purified by ConA chromatography followed by selection of ConA reactive spots and further identification by LC-MS/MS. This approach resulted in the identification of more than 40 putative mannosylated proteins. Similar to previous findings, the majority of glycosylated proteins were lipoproteins, including well known TLR-2 ligands such as LprG and LprA [84–88], with the 19 KDa (LpqH) and 38 KDa (PstS1) antigens being among the most prominent ConA reactors [74]. Nevertheless, proteins across a range of functional categories were also identified, indicating protein glycosylation may be a

more common phenomena than previously thought.

In the most recent glycoproteomics approach to identify glycosylated proteins in *Mtb*, Smith and colleagues used ConA enrichment techniques and LC-MS/MS with a combination of different collision energy dissociation types which allowed for the successful fragmentation and further identification of glycosylated peptides. A total of 13 glycoproteins and their glycosylated peptides were identified. The majority of them were previously known glyco- and lipoglycoproteins such as Rv1096, BlaC, Rv2799, LprA, Apa (45 KDa Ag) and Rv2394. The newly identified glycosylated proteins were Rv2164c (conserved proline rich membrane protein), Rv0175 (mce associated membrane protein), Rv1887, 35 KDa protein (Rv2744c), Rv3835, and LppO [89].

4.1. Lipoglycoproteins modulate the host immune response

Lipoproteins, including lipoglycoproteins are well known TLR-2 agonists [90]. Lipoglycoproteins, such as LpqH and SodC have been found to be enriched in membrane vesicles from *Mtb*, which may explain the vesicles immunogenicity and potency as TLR-2 agonists [91–93]. LpqH, the best characterized lipoglycoprotein in terms of the extent of its lipidic and glycosyl moieties [75], is essential for full virulence in mice [94], and induces Th1 immune response, macrophage apopotosis and antibacterial autophagy in a TLR-2/1 dependent manner. The latter of these involves specific Vitamin D receptor signaling [95–102]. LpqH mediated apoptosis and autophagy are also reviewed by Lieu et al., 2015 [103]. SodC, an active superoxide dismutase, is also a lipoglycoprotein [104] that elicits an antibody response during natural infection [21] and contributes to survival of the bacteria in activated macrophages by protecting against reactive oxygen species [105]. SodC from *M. leprae* expressed in *M. smegmatis* appears to have high reactivity to langerin, a *C*-type lectin of Langerhans cells and thus, may facilitate infection of *M. leprae* in these cells [106]. Mpt83 is a secreted lipoglycoprotein that induces a CD8 T cell dependent protective responses in mice [107], act as an adhesin [108] and is expected to activate TLR-2, inducing apoptosis in infected macrophages [109]. PstS1 and PstS3 are virulence determinants that act as phosphate binding proteins involved in phosphate uptake [110] and can induce Th1 and Th17 responses [111]. OppA is a periplasmic lipoglycoprotein involved in transport of glutathione regulating the immune response in infected macrophages [112]. This protein has been shown to be important for virulence in *M. avium* [113].

Information about host-pathogen interactions is scarce for the majority of known glycosylated proteins that are not acylated. However, it is believed that mannosylated proteins can act as potential adhesins. For instance, Mpt32 (Apa, 45 Kd antigen) binds lung surfactant protein A (SP-A) [114] as well as the *C*-type lectin DC-SIGN [115]. Rv0315, an immunostimulatory antigen [116] is also annotated as a beta-1-3 gluconase, although its enzymatic activity is predicted to be non-functional [117]. Other functions, include that of Rv2672 which was recently annotated as a mycobacterial secreted hydrolase (Msh1) appears to catalyze hydrolysis of host lipids and is needed for replication inside foamy macrophages [118].

The specific role (if any) of glycosylation in immune recognition is not well known. Evidence from Mpt32 mannosylation indicates that glycosylation motifs may be key for T cell antigenicity [119], and may also be involved in the presentation to CD8 T cells by HLA-E [33]. It is not known if glycosylation motifs in other proteins serve the same role.

4.2. Lipoglycoproteins are essential for lipid localization and cell wall biosynthesis

In addition to their well-documented activity in host-pathogen interactions, lipoglycoproteins have a wide array of functions in the mycobacterial physiology. For instance, LprG (Rv1411c) is required for optimal growth *in vitro* [39], as well as virulence in mice [120]. This requirement is possibly due to LprG's central role in LAM translocation to the cell wall [39,85,121]. LppX is also important for virulence [122], being involved in translocation of PDIM to the outer membrane [123]. LpqW is an essential protein required for the conversion of PIM into LAM. Its precise function appears to be in regulating the flux of early PIM precursors (such as AcPIM4) resulting in overall regulation of polar PIM and LAM biosynthesis [124]. In Corynebacteria LpqW has also been shown to be essential for the mannosylation of periplasmic glycolipids [125]. Lipids are not the only target of lipoglycoproteins. PonA2, a penicillin binding protein, is involved in synthesis of peptidoglycan, a function that is essential for mycobacteria [126]. LprF on the other hand, appears to be involved in response to osmotic stress by interacting with the histidine kinase KdpD [127–129]. LpqB interacts with the Two component system MtrA-MtrB in a coordinated effort to regulate cell division [130]. Of note, the lipoglycoprotein BlaC is a beta lactamase responsible for resistance to some β-lactam antibiotics [131,132]. BlaC is also the only known glycoprotein exported by the twin and arginine transport system [55,133], suggesting that the glycosylation activity of the PMT (Rv1002c) may also act on fully folded substrates. This hypothesis is one of the questions that remain to be answered.

5. Conclusions and knowledge gaps

Post-translational modifications are key to the function of many proteins or enzymes. In *Mtb*, acylation and glycosylation are two important PTMs that are often found alongside each other. Acylation is important for the localization of proteins in the mycobacterial cell wall, as well as a major contributor to host-pathogen interactions, being recognized by the TLR-2/1 receptors in mammal cells.

The mechanism and function of protein glycosylation are only starting to be unearthed. Many glycosylated proteins are annotated with unknown function and others are only recognized by their role in host-pathogen interactions. Other glycosylated proteins appear to play important functions in mycobacteria. Table 3 provides a full list of known glycoproteins and their functions if known. Glycosylated proteins are often immune-dominant antigens eliciting both cellular and humoral responses. Some lipoglycosylated proteins have been highlighted because of their role in lipid transport, including virulence factors such as PDIM and LAM. Despite these findings, the full impact of protein glycosylation on the physiology of mycobacteria and immunity to *Mtb* are areas for future research efforts. Additionally, the impact of glycosylation of the function of individual proteins needs to be addressed.

The knowledge gaps that represent new opportunities for research are:

1. Does glycosylation truly protect against proteolysis and shedding into the secreted milieu? If so, which proteases are responsible for this shedding?
2. What is the broader role of protein glycosylation in the physiology and cell biology of mycobacteria?
3. What is the relationship between glycosylation and the function of lipoglycosylated proteins involved in lipid transport? Which of the glycolipids are affected (localization and levels) when glycosylation is inhibited?
4. Does glycosylation regulate the enzymatic activity of post-translationally modified proteins?
5. How exactly are the Sec and Tat Pathways associated with the Protein *O*-Mannosyl Transferase? Are they distinctly co-localized in the cell membrane?
6. Does inhibition of enzymes involved in protein acylation, resulting in altered levels of PPM, also result in altered levels of non-acylated glycoproteins (i.e. decrease mannose units available for modification)?
7. Are other types of glycosylation such as GalNac *N*-glycosylation in *C. jejuni* [134] or pseudaminic acid –like monosacharide *O*-

glycosylation in a variety of organisms [135–137] also present in mycobacteria?

From a broader view, the significance of glycosylation in *Mtb* (aside from a host-pathogen interaction stand point) still remains to be understood. Recent reports indicate that glycosylation of lipoprotein affects cell permeability and drug resistance in *M. abscessus*. Specifically, absence of the PMT in *M. abscessus* resulted in increased susceptibility of the organism to β-lactams and large antibiotics such as bacitracin, vancomycin and rifampicin [138]. Is this effect similar in *Mtb*? Do non-acylated glycoproteins have broader functions in the physiology of the mycobacteria?

Finally, while identification of glycoproteins and their specific glycosylated motifs has been advanced due to the power mass spectrometry coupled with enrichment methods, we may still be just looking at the tip of the iceberg. Additional systems biology studies are needed to not only identify and characterize novel glycoproteins, but also to better understand the impact of glycosylation on mycobacterial physiology and host-pathogen interactions.

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Appendix A. Supplementary data

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