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**Impact of methylthioxylose substituents on the biological activities of lipomannan and lipoarabinomannan in *Mycobacterium tuberculosis***

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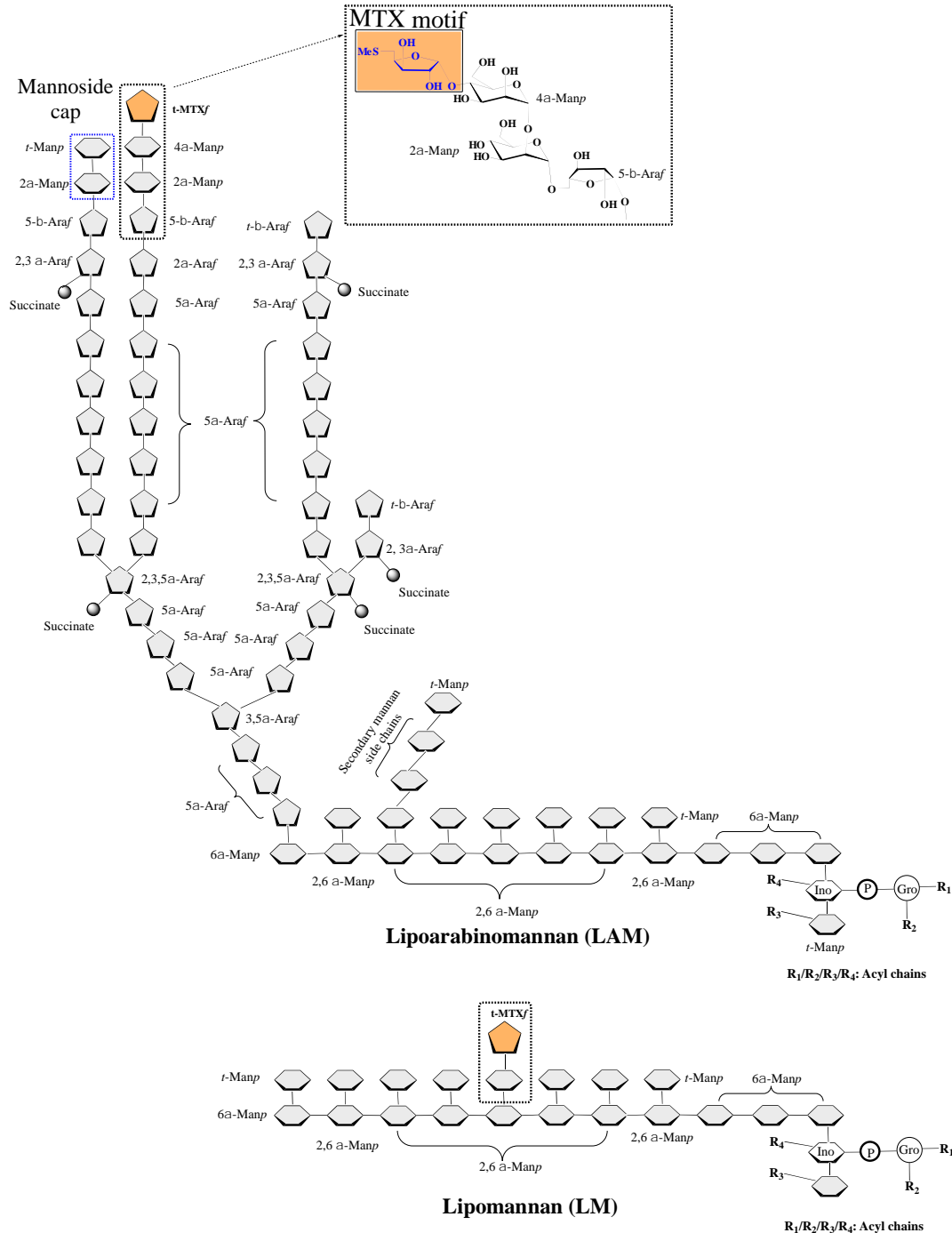
## Abstract

Two lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), play various albeit incompletely defined roles in the interactions of mycobacteria with the host. Growing evidence points to the modification of LM and LAM with discrete covalent substituents as a strategy used by these bacteria to modulate their biological activities. One such substituent, originally identified in *Mycobacterium tuberculosis* (*Mtb*), is a 5-methylthio-D-xylose (MTX) sugar which accounts for the antioxidative properties of LAM. The widespread distribution of this motif across *Mtb* isolates from several epidemiologically important lineages have stimulated interest in MTX-modified LAM as a biomarker of tuberculosis infection. Yet, several lines of evidence indicate that MTX may not be restricted to *Mtb* and that this motif may substitute more acceptors than originally thought. Using a highly specific monoclonal antibody to the MTX capping motif of *Mtb* LAM, we here show that MTX motifs not only substitute the mannoside caps of LAM but also the mannan core of LM in *Mtb*. MTX substituents were also found on the LM and LAM of pathogenic slow-growing nontuberculous mycobacteria. The presence of MTX substituents on the LM and LAM from *Mtb* enhances the pro-apoptotic properties of both lipoglycans on LPS-stimulated THP-1 macrophages. A comparison of the cytokines and chemokines produced by resting and LPS-activated THP-1 cells upon exposure to MTX-proficient versus MTX-deficient LM further indicates that MTX substituents confer anti-inflammatory properties upon LM. These findings add to our understanding of the glycan-based strategies employed by slow-growing pathogenic mycobacteria to alter the host immune response to infection.

Keywords: Lipoarabinomannan, lipomannan, *Mycobacterium*, methylthioxylose, apoptosis, inflammation.

Lipoarabinomannan (LAM) and lipomannan (LM) are abundant lipoglycans of the cell envelope of all mycobacteria [Fig. 1]. They are anchored in the inner and outer membranes where they play critical roles in cell integrity<sup>1,2</sup> and the interactions of mycobacterial pathogens with the host<sup>3-7</sup>. Like other prokaryotes, mycobacteria modify the core structure of their cell envelope (lipo)polysaccharides with discrete covalent substituents<sup>8, 9</sup>. Beyond altering the structure, biosynthesis and biological activities of these glycans, recent work from our laboratory indicates

that such modifications can have far-reaching consequences on the bacterium, altering their cell envelope permeability, susceptibility to antibiotics, surface properties, interactions with innate immune cells and virulence<sup>10-13</sup>.



**Figure 1: Schematic representation of the structures of *Mtb* LM and LAM.** Schematic representation of the 5-methylthio-D-xylose (MTX) motif (in orange) attached to the non-reducing arabinan termini of mannoside-capped lipoarabinomannan (LAM) and the mannan domain of lipomannan (LM) in *Mtb*.

Both LM and LAM are known to be modified with tailoring substituents many of which are species-specific. Among these, mannoside caps modifying the non-reducing arabinan termini of LAM in *Mycobacterium tuberculosis* (*Mtb*) and a number of other pathogenic slow-growing mycobacteria have been the focus of most studies to date for their role in promoting intracellular survival and immune evasion<sup>3, 5-7</sup>. Interestingly, the mannoside caps of *Mtb* LAM may further be substituted at the C4-position with 5-methylthio-D-xylose (MTX) residues [Fig. 1]. MTX motifs occur at the level of one unit per entire molecule of LAM, or one unit for every five to six mannoside caps<sup>14</sup>. We reported earlier on the biosynthetic origin of this uncommon sugar in prokaryotes and the nature of the glycosyltransferase involved in its transfer onto LAM<sup>15</sup>. Its finding on the LAM of *Mtb* clinical isolates from several epidemiologically important lineages suggests that it is highly conserved<sup>9</sup>. The fact that it was found to modify the LAM of *Mtb* cultured in laboratory medium or recovered from infected mouse lungs and clinical specimens from tuberculosis (TB) patients further indicates that it is expressed both *in vitro* and *in vivo*<sup>16-18</sup>. The widespread distribution and apparent constitutive production of MTX-modified LAM in *Mtb* isolates has stimulated interest in this motif as a TB biomarker<sup>16, 18</sup>. Yet, preliminary evidence indicates that MTX may not be specific to *Mtb*. Indeed, MTX motifs were reported to substitute an unspecified location of LAM from *Mycobacterium avium*<sup>19</sup> and the mannan domain of both LM and LAM in *Mycobacterium kansasii*<sup>20</sup>. Moreover, the five gene cluster dedicated to the biosynthesis of the MTX motif of LAM can be found in the genomes of a number of fast-growing and slow-growing *Mycobacterium* species as well as other Actinomycetes (e.g., *Mycobacterium smegmatis*, *M. vanbaalenii*, *M. marinum*, *M. ulcerans*, *Rhodococcus* *Streptomyces*, etc.)<sup>15</sup>, indicating that this motif may be more widespread than previously appreciated. Whether MTX substitutes the LM, the LAM or other acceptor molecules (e.g., glycolipids or glycoproteins) from these organisms is currently not known.

Another ill-defined aspect of the biology of the MTX motif concerns its role in the physiology of mycobacteria and their interaction with the host. The biosynthetic effort required to make this additional substituent of LAM would suggest that it confers upon *Mtb* and other MTX-producing *Mycobacterium* species a fitness advantage in the environment or during infection. Studies conducted on disaccharide mimetics of the MTX capping motif of *Mtb* LAM showed that the presence of MTX substituents on the mannoside caps prevented the latter from engaging the mannose receptor thereby potentially allowing *Mtb* to evade macrophage uptake through this C-

type lectin<sup>21</sup>. The MTX capping motif further accounts for the ability of purified LAM to scavenge oxygen radicals, possibly via a redox cycle of chemical oxidation of MTX followed by enzymatic reduction by host or bacterial methionine sulfoxide reductases<sup>22,23</sup>. Whether the MTX substituents of LAM and LM from *Mtb* and *M. kansasii* impact the inflammatory or apoptosis-inducing properties of these lipoglycans has not yet been investigated, despite evidence that LM from *M. kansasii* triggers THP-1 apoptosis<sup>20</sup> and the finding that disaccharide mimetics of the MTX capping motif of *Mtb* LAM slightly inhibit the production of TNF- $\alpha$  and IL-12p70 by activated THP-1 cells<sup>24</sup>.

Because knowledge of the fine structures of LM and LAM across *Mycobacterium spp.* is critical to the understanding of their structure-function relationships and to the assessment of their potential as biomarkers of mycobacterial infections, we here took advantage of the availability of a highly specific antibody to the MTX capping motif of *Mtb* LAM to revisit the distribution of the MTX motif in various *Mycobacterium* species, study the existence of potential alternative acceptor sites on LM and LAM, and determine its impact on the biological activities of these lipoglycans. Unexpectedly, our analyses revealed the presence of MTX substituents on the mannan domain of LM not only in *M. kansasii* but also in *Mtb* and *M. avium*. While MTX substituents had no apparent effects on the elongation, branching or mannoside capping of LM and LAM, they clearly impacted the pro-inflammatory and/or apoptosis-inducing properties of both lipoglycans. Collectively, the results reported herein shed new light on the nature of the cell envelope glycans serving as acceptors for MTX residues in tuberculous and nontuberculous mycobacteria and highlight previously unknown immunomodulatory properties associated with this discrete covalent substituent.

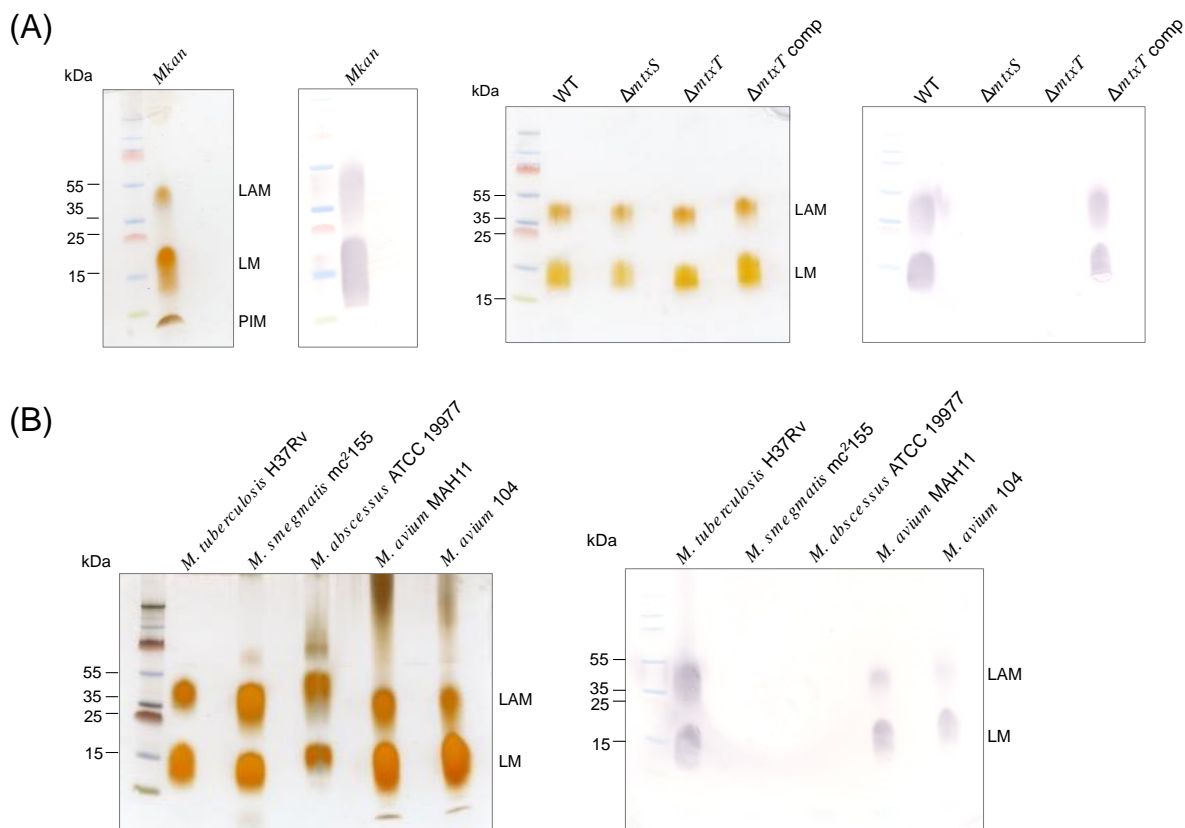
## RESULTS AND DISCUSSION

### ***Recognition of Mtb LM by a monoclonal antibody targeting the methyl-thio-D-xylose (MTX)-dependent epitope of LAM***

S4-20 is a recombinant monoclonal antibody isolated by Otsuka Pharmaceutical by phage display of scFV libraries generated from rabbits immunized with *M. bovis* BCG and screened for reactivity with mannoside-capped LAM<sup>25</sup>. This antibody is used in highly sensitive ELISA assays for LAM detection in sputum and urine from TB patients<sup>16,18</sup>. It was shown to recognize synthetic

mimetics of the MTX-substituted mannoside capping motif of *Mtb* LAM harboring two or three mannosyl residues<sup>18</sup>.

The ability of S4-20 to recognize *Mtb* LAM was first verified by immunoblot. Since MTX was reported to substitute the mannan domain of LM and LAM in *M. kansasii*<sup>20</sup>, we were curious to determine whether the antibody might also recognize LM from *Mtb*. Lipoglycan fractions from *Mtb* and *M. kansasii* containing a mixture of LM and LAM were run on SDS-PAGE and, upon transfer to nitrocellulose membranes, probed with the antibody. To our surprise, not only did S4-20 react with both LM and LAM from *M. kansasii*, it also revealed both lipoglycans in *Mtb* [Fig. 2A].



**Figure 2: Reactivity of the S4-20 antibody to the LM and LAM from *M. kansasii*, *Mtb*, *M. smegmatis*, *M. abscessus* and *M. avium*.**

(A) Lipoglycans from *M. kansasii* ATCC12478, WT *Mtb* H37Rv, *Mtb* $\Delta mtxS$ , *Mtb* $\Delta mtxT$  and *Mtb* $\Delta mtxT$ /pMV306H-*mtxT* were run on a 10-20% Tricine gel followed by periodic acid-silver staining (left panels) or revealed with S4-20 antibodies upon transfer to a nitrocellulose membrane (right panels). The results presented are representative of at least three independent SDS-PAGE runs using different lipoglycan preparations from each strain.

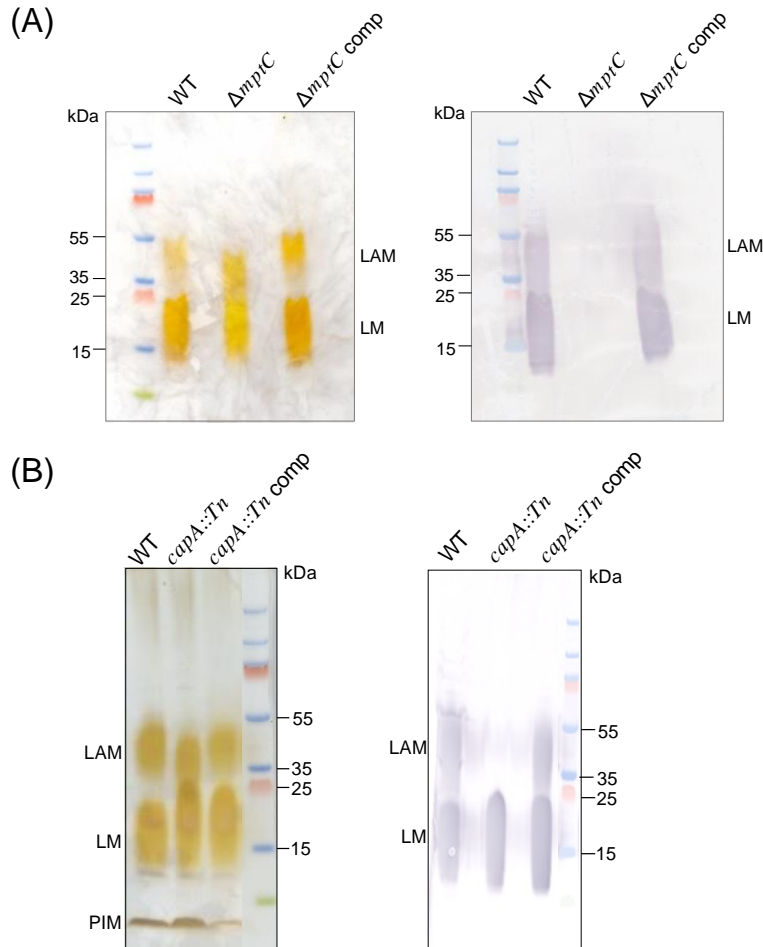
(B) Lipoglycans prepared from *M. smegmatis* mc<sup>2</sup>155, *M. abscessus* subsp. *abscessus* ATCC 19977 and *M. avium* subsp. *hominissuis* 104 and MAH11 were processed as in (A) and the Western blots revealed with the S4-20 antibody.

*MtbΔmtxS* and *MtbΔmtxT* are two *Mtb* knock-out mutants that produce a LAM devoid of MTX capping motif<sup>15</sup>. *MtbΔmtxS* is deficient in the synthesis of decaprenyl-phospho-MTX (DP-MTX), the dedicated MTX lipid donor in the synthesis of MTX-modified LAM, whereas *MtbΔmtxT* is proficient in DP-MTX synthesis but lacks the ability to transfer the MTX residue from DP-MTX to mannoside-capped LAM<sup>15</sup>. Since LM and LAM recognition by S4-20 was lost in *MtbΔmtxS*, we conclude that MTX-dependent epitopes are involved in the recognition of both *Mtb* lipoglycans by the antibody [Fig. 2A]. The fact that the LM and LAM prepared from *MtbΔmtxT* failed to react with S4-20 indicates that the same glycosyltransferase (MtxT; Rv0541c) is involved in the transfer of MTX from DP-MTX to both LAM and LM. LM and LAM reactivities were restored upon complementation of the *mtxT* mutant with a wild-type copy of the *mtxT* gene [Fig. 2A].

### ***The MTX motif substitutes the mannoside branches of the mannan domain of Mtb LM but not Mtb LAM***

The location of the MTX residues on the mannan domain of *Mtb* LM was first approached genetically. Reasoning that MtxT might specifically transfer MTX motifs to monomannoside or  $\alpha$ -1,2-linked di- or-tri-mannoside substituents such as the ones making the mannoside caps of LAM, we hypothesized that it likely transferred MTX to the structurally similar mono- and di-mannoside branches of the mannan domain of LM in *Mtb* and *M. kansasii*<sup>19</sup>. This hypothesis was tested by running an immunoblot on the lipoglycan fraction from *MtbΔmptC* (Rv2181), a knock-out mutant not only deficient in the branching of LM but also in the mannosylation of the mannoside caps of LAM past the first mannosyl residue added by the priming mannosyltransferase, CapA<sup>26,27</sup>. Neither the LM or LAM from *MtbΔmptC* were recognized by S4-20, thereby establishing the branching mannosyl residues of LM as the site at which MTX substitution occurs. LM and LAM reactivity was recovered in the *mptC* knock-out mutant upon complementation with a wild-type copy of the *mptC* gene [Fig. 3A]. The loss of reactivity of LAM in the *mptC* mutant validates the location of MTX on the mannoside caps of *Mtb* LAM and confirms that more than one mannosyl residue in the caps is required for the antibody to show

detectable binding by this approach. Consistent with these results, S4-20 recognized the LM but not the LAM of an *Mtb capA* transposon mutant (*Mtb capA::Tn*) which displays normal mannoside branching on LM but fails to add mannoside caps to LAM<sup>26</sup>. LAM reactivity was recovered upon complementation of *Mtb capA::Tn* with a wild-type copy of the *capA* gene [Fig. 3B].



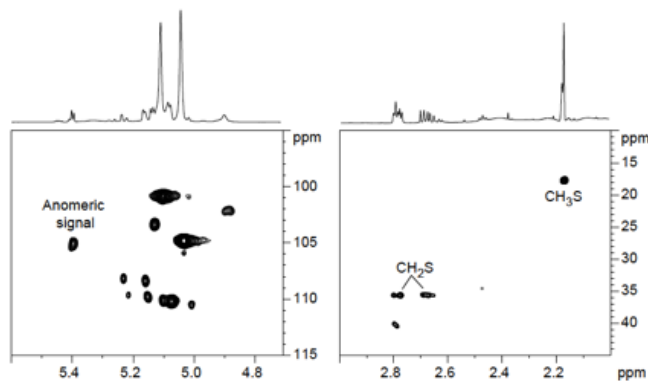
**Figure 3: Reactivity of the S4-20 antibody to the LM and LAM from *Mtb* mutants deficient in the mannoside caps of LAM and/or mannoside branching of LM.**

(A) Lipoglycans from WT *Mtb* H37Rv, *Mtb*Δ*mptC* and *Mtb*Δ*mptC*/pVV16-*mptC* were run on a 10-20% Tricine gel followed by periodic acid-silver staining (left panel) or revealed with S4-20 antibodies upon transfer to a nitrocellulose membrane (right panel). The results presented are representative of at least three independent SDS-PAGE runs using different lipoglycan preparations from each strain.

(B) Same as above using lipoglycan preparations from WT *Mtb* CDC1551, the *capA* transposon mutant (*capA::Tn*) and the complemented *capA* transposon mutant (*capA::Tn-comp*).

The presence and location of MTX on *Mtb* LM was independently confirmed by NMR and mass spectrometric analyses. While signals typifying MTX or MSX residues were not detectable

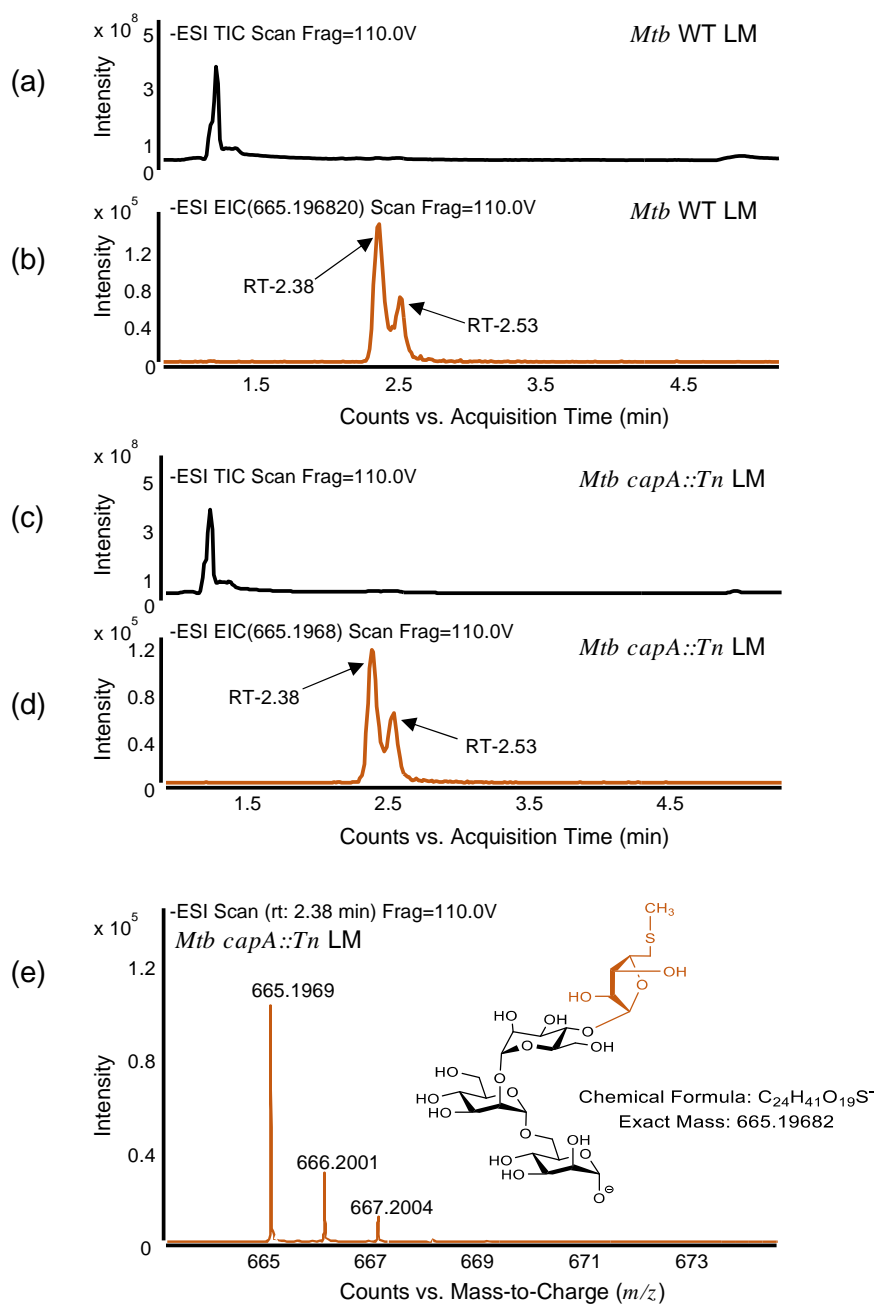
in the spectrum of LM derived from wild-type *Mtb* CDC1551 (data not shown), anomeric signals corresponding to 5'-methylthiopentose H1 at  $\delta$  5.40 and C1 at 105.1 were clearly observed in the 2D  $^1\text{H}$ - $^{13}\text{C}$  HMQC NMR spectrum of the LM purified from the *Mtb capA::Tn* mutant. The characteristic correlations at  $\delta$  2.68 and  $\delta$  2.79 /  $\delta$  35.6 corresponding to the methylene group linked to the sulfur atom, and at  $\delta$  2.17 /  $\delta$  17.7 ppm corresponding to the methyl group linked to the sulfur atom are indicated by arrows in Fig. 4.



**Figure 4:** NMR analysis showing the presence of MTX substituents on *Mtb* LM.

2D  $^1\text{H}$ - $^{13}\text{C}$  HMQC NMR spectrum of LM purified from *Mtb capA::Tn*. Two expanded areas are shown, one showing the anomeric signal of MTX, and the other one the methyl and methylene groups linked to the sulfur, typifying the MTX motif. Some contamination of LM by LAM is observed on the spectrum. However, as shown in Fig. S1, contaminating LAM from *Mtb capA::Tn* does not account for the MTX signals.

To further confirm the presence of MTX substituents on the mannan backbone of LM from *Mtb capA::Tn* and, potentially, the wild-type parent strain, *Mtb* CDC1551, purified deacylated LM from the two strains were treated with commercial Jack bean  $\alpha$ -mannosidase and endo- $\alpha$ -1,6 mannanase from *Bacillus circulans*<sup>28</sup>, and the digestion products analyzed by LC/MS. This analysis identified the primary MTX-containing enzymatic product as an MTX-Man<sub>3</sub> tetrasaccharide at  $m/z$  665.1968 as  $[\text{M}-\text{H}]^-$  [Fig. 5] whose likely structure is shown in panel (e). Smaller amounts of MTX-Man<sub>4</sub> and MTX-Man<sub>5</sub> were also found (data not shown). Thus, in agreement with the loss of reactivity of S4-20 against LM isolated from *Mtb*  $\Delta$ *mptC*, LC/MS results support the location of the MTX motif on the mannoside branches of the mannan domain. The fact that MTX substituents were not readily detectable by NMR on WT *Mtb* LM suggests that the degree of MTX substitution of LM in this strain is less than in *Mtb capA::Tn*. We tentatively attribute this result to the fact that, in the absence of mannoside caps on the mutant LAM, excess MTX residues are instead transferred to LM.



**Figure 5: LC/MS analysis showing the presence of MTX substituents on *Mtb* LM.** Negative mode LC/MS analysis was conducted on  $\alpha$ -mannosidase- and endo- $\alpha$ -1,6 mannanase-digested deacylated LM from *Mtb* CDC1551 WT and *Mtb capA::Tn*. The corresponding total ion chromatograms (TIC) are shown in panels (a) and (c), while panels (b) and (d) display the extracted ion chromatograms (EIC) at  $m/z$  665.1968. The EICs show the elution profiles of digested products with retention times (RT) of 2.38 and 2.53 min representing structural isomers of MTX-Man<sub>3</sub>. The MS spectrum of the peaks from *Mtb capA::Tn* indicates the presence of MTX-capped trimannoside with a  $m/z$  value of 665.1968 as  $[M-H]^-$  as shown in panel (e). The possible structure of the MTX-capped trimannoside based on the digestion pattern of the endo- $\alpha$ -1,6 mannanase<sup>28</sup> is illustrated in the inset. Note that the precise linkage of the MTX substituent remains to be established.

In line with the results of detailed mass spectrometric and NMR analyses of *Mtb* H37Ra LAM by Treumann *et al.*<sup>14</sup>, NMR analysis of LAM derived from the *capA* transposon mutant failed to indicate the presence of MTX substituents on the mannan domain of LAM [Fig. S1], a result further supported by the lack of reactivity of the S4-20 antibody against LAM from *Mtb capA::Tn* [Fig. 3B].

### ***Reactivity of S4-20 to LM and LAM from nontuberculous mycobacteria***

Immunoblots of lipoglycan fractions prepared from nontuberculous *Mycobacterium* (NTM) species that possess (*M. avium*, *M. smegmatis*, *M. kansasii*)<sup>15</sup> or, on the contrary, are devoid (*M. abscessus*) of the five gene cluster required for the biosynthesis of the MTX motif of LAM showed a clear reactivity of LM and LAM from *M. avium* subsp. *hominissuis* isolates 104 and MAH11 to the S4-20 antibody [Fig. 2B], as was observed with *Mtb* and *M. kansasii* [Fig. 2A]. Whether MTX motifs modify the exact same positions of LM and LAM in *M. kansasii* and *M. avium* as in *Mtb* remains to be determined. Based on the fact *M. kansasii* and *M. avium* produce mannoside-capped LAM<sup>29</sup>, and given the high degree of conservation of the five core enzymes of the MTX pathway (Pnp, GalE3, MtxS, MtxT and Rv0540)<sup>15</sup> across the three species (75 to 92% primary sequence identity), it is likely that the MTX motif substitutes the same positions of LM and LAM in *M. kansasii* and *M. avium* as in *Mtb*.

No signal was observed with *M. abscessus* lipoglycans consistent with the fact that this species lacks the genes required for MTX biosynthesis. The lack of reactivity of *M. smegmatis* LM, in contrast, may suggest that one or more of the genes involved in producing and transferring the MTX motif to LM is not active in this species. Alternatively, differences in the accessibility of the MTX-containing epitope of LM in *M. smegmatis* compared to slow-growing mycobacteria could explain this result. Finally, one cannot exclude that the MTX residues in this species are transferred to an as yet unknown acceptor such as a glycolipid or a glycoprotein instead of LM.

### **The presence of MTX substituents does not impact the core structure and mannoside capping of *Mtb* LM and LAM**

With reports in the literature of discrete covalent substituents controlling the biosynthesis of polysaccharides in Gram-negative and Gram-positive organisms<sup>30</sup>, including our recent finding

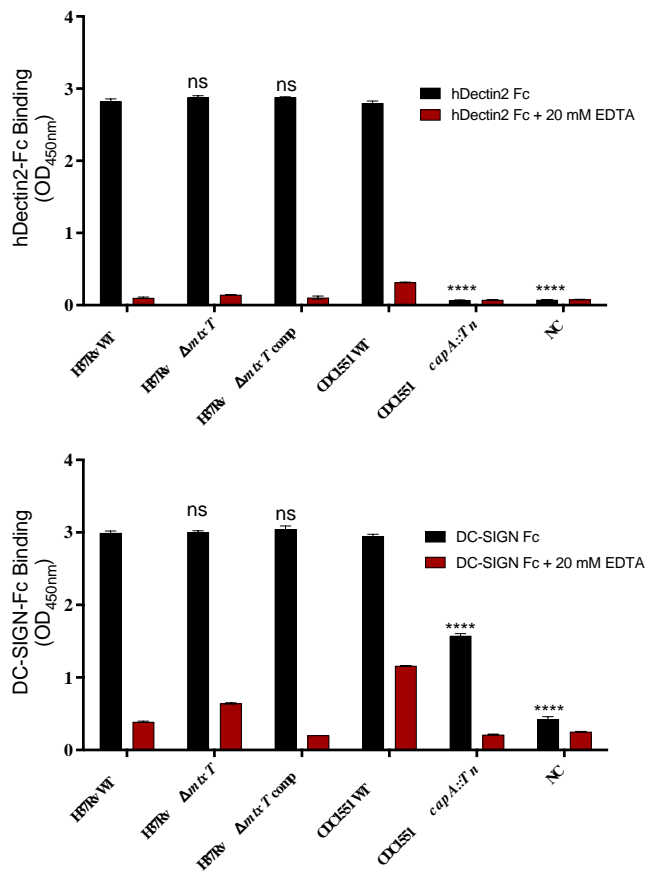
that succinyl substituents control the mannoside capping of LAM in *Mtb*<sup>12</sup>, we first wanted to determine whether the presence of MTX residues on the LM and LAM of *Mtb* impacted the elongation and branching of these lipoglycans. Quantitative analyses of the alditol acetate and per-*O*-methylated alditol acetate derivatives prepared from LM and LAM isolated from the wild-type strain, the *mtxT* mutant and the complemented *mtxT* mutant revealed near identical structures in all three strains [Tables S1-S4]. The degree of mannoside capping displayed by the LAMs purified from the wild-type strain and the *mtxT* mutant as determined by capillary electrophoresis was also comparable [Fig. S2], thereby excluding any major impact of the MTX residues on the elongation, branching and mannoside branching and capping of LM and LAM.

### ***Impact of the MTX motif on the biological activities of Mtb LM and LAM***

Our attention next turned to the potential impact that the addition of MTX substituents to LM and LAM might have on the interactions of both lipoglycans with innate immune cells. As stated earlier, studies using synthetic mimetics of the MTX capping motif of *Mtb* LAM showed that the presence of MTX substituents reduced the ability of mannoside caps to interact with the human mannose receptor<sup>21</sup> in addition to having a modest inhibitory effect on the production of TNF- $\alpha$  and IL-12p70 by activated THP-1 cells<sup>24</sup>. Moreover, while the type of substitution modifying the mannan domain of LM have been shown to affect the cytokine response of monocyte-derived THP-1 cells to LM<sup>31</sup>, the specific impact that MTX substituents might have on this activity has never been investigated. Neither has the role played by MTX substituents, if any, in the apoptosis-inducing activity of *M. kansasii* LM<sup>20</sup>. The question of the impact of MTX substituents on the biological activities of LM and LAM was addressed through a suite of assays detailed below.

Besides the mannose receptor, two other C-type lectins expressed at the surface of dendritic cells and macrophages, DC-SIGN and Dectin-2 (Dendritic-Associated C-type lectin-2), have been reported to bind *Mtb* LAM. This binding is dependent on the presence of  $\alpha$ 2-linked mannoside caps at the non-reducing end of the arabinan domain of LAM<sup>29, 32</sup>. To determine whether the presence of MTX substituents on the mannoside caps of LAM altered the ability of these lectins to recognize LAM, ELISA-based assays were used to compare the binding of purified LAM from *Mtb* H37Rv wild-type, the *mtxT* mutant and the complemented *mtxT* mutant to DC-SIGN and Dectin-2 Fc fusion proteins. LAM purified from wild-type *Mtb* CDC1551 and the corresponding

*capA::Tn* mutant were also included in the assays as controls. Consistent with the fact LAM bearing mannoside caps is the only known ligand of Dectin-2<sup>32</sup>, no significant binding of the Dectin-2 Fc fusion protein to LAM purified from *Mtb capA::Tn* was detected [Fig. 6]. Compared to LAM from wild-type *Mtb* CDC1551, the binding of the DC-SIGN Fc protein to *Mtb capA::Tn* LAM was reduced but not abrogated, as expected from the ability of DC-SIGN to bind more moieties of LAM than the only  $\alpha$ 2-linked mannoside caps<sup>29,33</sup>. In comparison, the binding of *mtxT* mutant LAM to either fusion protein did not significantly differ from that of wild-type *Mtb* H37Rv LAM, indicating that MTX substituents do not interfere with the recognition of *Mtb* LAM by these lectins [Fig. 6].

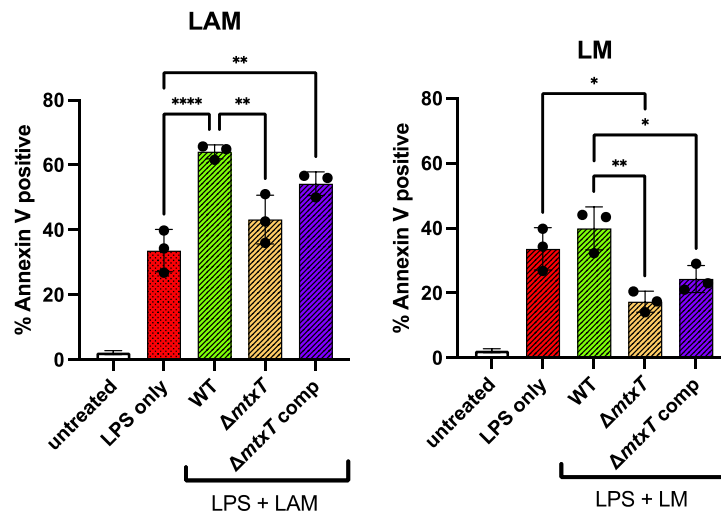


**Figure 6: Binding of Dectin-2-Fc and DC-SIGN-Fc proteins to LAM purified from wild-type *Mtb* H37Rv, wild-type *Mtb* CDC1551, the *Mtb* H37Rv *mtxT* mutant, the complemented *mtxT* mutant and the *Mtb* CDC1551 *capA::Tn* transposon mutant.**

LAMs purified from different *Mtb* strains (1  $\mu$ g per well in isopropanol) were coated in 96-well plates and compared for their ability to bind Dectin-2-Fc and DC-SIGN-Fc as described under Methods. EDTA was added to some wells from each treatment group to block the calcium-dependent binding of Dectin-2-Fc and DC-SIGN-Fc to their ligand. Shown are the means  $\pm$  SD

(error bars) of absorbances measured at 450 nm for two biological replicates. Asterisks denote statistically significant differences between wild-type (H37Rv or CDC1551) *Mtb* controls and their respective mutants (ns, not significant; \*\*\*\* $p < 0.0001$  using a one-way ANOVA with Fisher's LSD test for multiple comparisons). The results presented are representative of two independent experiments. NC, non-coated.

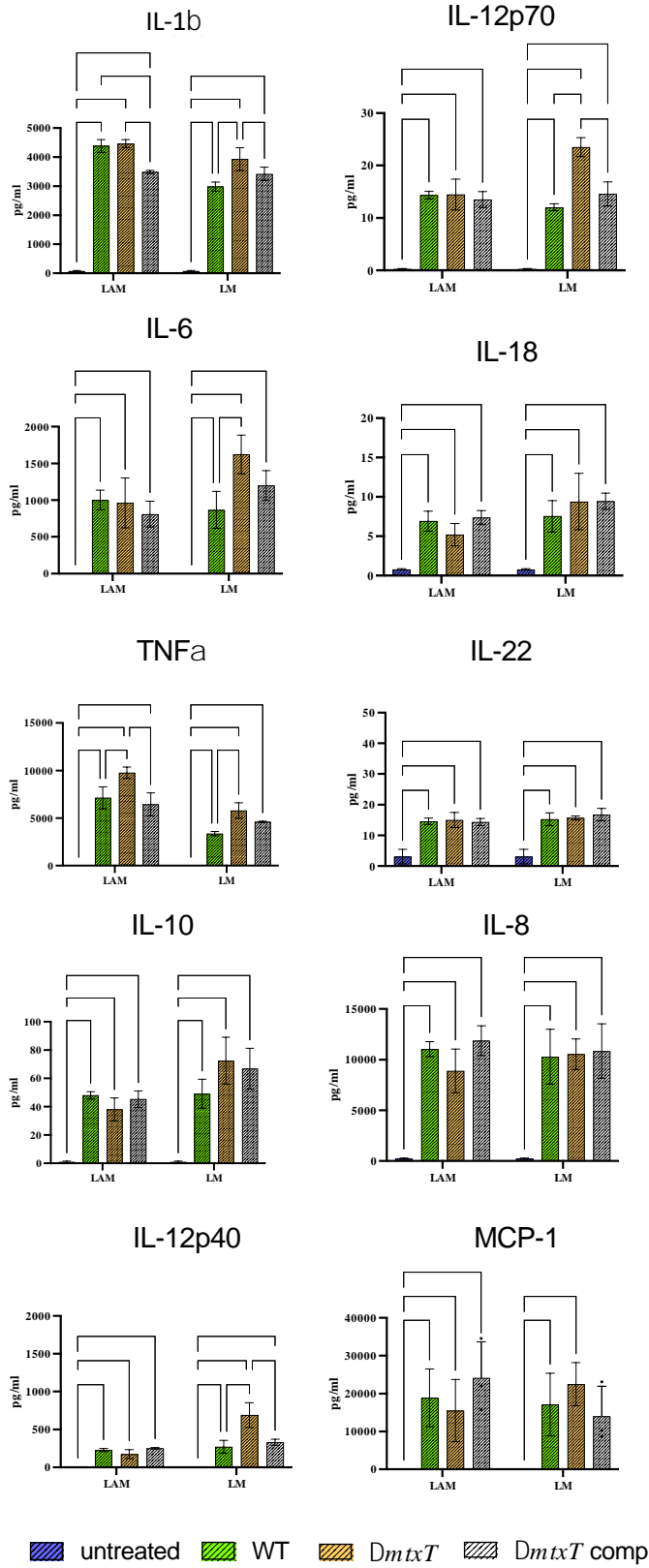
A report showing that LM from the MTX-bearing *M. kansasii* triggers THP-1 apoptosis<sup>20</sup> next prompted an examination of these cells when treated with purified LAM or LM from either wild-type *Mtb* H37Rv or the *mtxT* mutant to determine whether this motif may also contribute to increased programmed cell death. When cells were treated with the purified LAMs or LMs along with LPS for 48 h, both wild-type lipoglycans prompted increases in percentages of apoptotic THP-1 cells that was comparatively abrogated when treated with LAM or LM purified from the *mtxT* mutant. Increased LPS-mediated apoptosis was partially restored when cells were treated with lipoglycans purified from the complemented  $\Delta$ *mtxT* mutant strain, albeit not to statistically comparable levels to wild-type lipoglycans [Fig. 7].



**Figure 7: Apoptosis-inducing activity of purified LM and LAM from wild-type *Mtb* H37Rv, the *Mtb* H37Rv *mtxT* mutant and the complemented *mtxT* mutant.**

PMA-differentiated THP-1 cells were treated with  $150 \text{ ng mL}^{-1}$  LPS in the presence or absence of  $10 \text{ }\mu\text{g mL}^{-1}$  LAM or LM from WT *Mtb* H37Rv, *Mtb* $\Delta$ *mtxT* and *Mtb* $\Delta$ *mtxT*/pMV306H-*mtxT*. After 48 h of treatment, cells were harvested and stained with Annexin V-Alexa-Fluor-647 and propidium iodide. Annexin V positive cells were enumerated as percentage of the total cell number. Statistical differences between the different treatment groups (LPS with or without added purified LM or LAM from the different strains) were obtained using a two-way ANOVA with Tukey's multiple comparisons test with, \*\*\*\* $p \leq 0.0001$ ; \*\* $p \leq 0.005$  and \* $p \leq 0.05$ .

It was previously shown that treatment of THP-1 cells with purified *Mtb* LAM alone failed to induce or inhibit production of TNF $\alpha$  or IL-12p70, yet inhibition of these cytokines was observed when used together with a strong proinflammatory stimulus<sup>24,34</sup>. Accordingly, in the present study, the effect of the MTX motif of either LAM or LM on cytokine/chemokine production was determined in both the absence and presence of LPS. Figure 8 and Fig. S3 show secretion of eight cytokines (IL-1 $\beta$ , IL-6, TNF $\alpha$ , IL-10, IL-12p40, IL-12p70, IL-18 and IL-22) and two chemokines (IL-8 and MCP-1) from THP-1 cells treated with either LAM or LM from wild-type *Mtb* H37Rv, the *mtxT* mutant or the complemented mutant. Cells treated with LAM without added LPS [Fig. S3] secreted very little pro- or anti-inflammatory cytokines (exception made of chemokines IL-8 and MCP-1) and showed no significant differences between wild-type and mutant strains. Treatment with purified LAM followed by the addition of LPS showed significantly higher release of cytokines and chemokines, yet no differential secretion between LAM purified from the wild-type and mutant strains was observed, with the noted exception of TNF $\alpha$  whose levels were highest with the cells treated with MTX-deficient LAM [Fig. 8]. Interestingly, treatment of cells with wild-type LM (without addition of LPS) stimulated significantly less release of IL-1 $\beta$ , IL-6, TNF $\alpha$ , IL-10, IL-12p40 and IL-12p70 when compared to cells treated with the mutant LM [Fig. S3]. Treatment with the LM from the complemented mutant generally reverted responses for most of these cytokines to levels observed for wild-type treatment. Treatment of THP-1 macrophages with LM in the presence of LPS revealed differential levels of cytokine secretion between wild-type and mutant LM with, again, significantly less production of IL-1 $\beta$ , IL-6, TNF $\alpha$ , IL-12p40 and IL-12p70 observed in cells treated with wild-type LM compared to those treated with mutant LM [Fig. 8]. THP-1 treated with LPS and LM derived from the complemented mutant generally reverted to wild-type levels for these five cytokines with reduction significance achieved for IL-1 $\beta$ , IL-12p40 and IL-12p70. Together, these data support the perception that the MTX motif on the LM component of *Mtb* has a greater influence on host cell immunomodulation than MTX-modified LAM. By reduction of these powerful proinflammatory cytokines, this immune modulation by MTX serves to reduce host inflammatory responses that would likely alter immunity against the bacilli.



**Figure 8: Evaluation of chemokine and cytokine secretion by THP-1 cells treated with purified LM and LAM from wild-type *Mtb* H37Rv, the *Mtb* H37Rv *mtxT* mutant and the complemented *mtxT* mutant.**

THP-1 cells were treated with purified LAM or LM (10  $\mu\text{g mL}^{-1}$ ) from either WT *Mtb* H37Rv, *Mtb* $\Delta$ *mtxT* and *Mtb* $\Delta$ *mtxT*/pMV306H-*mtxT* for a total of 48 h in the presence of 30 ng mL<sup>-1</sup> LPS. LPS was added 24 h after LAM/LM treatment. Supernatants were analyzed for the indicated cytokines/chemokines using Luminex® multiplex assay. Statistical differences were obtained using a two-way ANOVA with Tukey's multiple comparisons test with, \*\*\*\* $p \leq 0.0001$ ; \*\*\* $p \leq 0.005$ ; \*\* $p \leq 0.01$  and \* $p \leq 0.05$ .

## CONCLUSION

The energetic cost associated with the biosynthesis of MTX substituents of LAM<sup>15</sup> has long suggested that this motif confers upon *Mtb* a fitness advantage in the environment or during infection. The findings reported herein provide support for this assumption by showing that MTX substituents not only modify LAM but also LM and, in doing so, significantly alter the biological activities of both lipoglycans. In addition to conferring upon LAM anti-oxidative properties<sup>23</sup> and a way for *Mtb* to potentially evade macrophage uptake through the mannose receptor<sup>21</sup>, the MTX motif enhances the pro-apoptotic activity of LAM on LPS-activated THP-1 cells. The MTX substituents of LM similarly enhances the pro-apoptotic activity of LM on LPS-stimulated THP-1 cells while dampening the pro-inflammatory properties of this lipoglycan on both resting and LPS-stimulated THP-1 cells. The significant increases in IL-1 $\beta$ , IL-6, TNF $\alpha$  and IL-12 observed upon treatment of cells with LM devoid of MTX suggest that this motif may serve to abrogate optimal macrophage inflammatory response, thereby facilitating the colonization of host niches by the bacilli.

The fact that MTX motifs also substitute the LM and the LAM of slow-growing NTM such as *M. avium* and *M. kansasii* strongly suggests that these MTX-driven mechanisms of immunomodulation involving LM and LAM are conserved across tuberculous and nontuberculous pathogens of the human lung. Future studies should now aim to investigate how these biological activities conferred by the MTX substituents of LM and LAM translate in the context of TB infection. From a diagnostic standpoint, the presence of MTX substituents on the LM and LAM of slow-growing NTM pathogens indicates that this motif may be a broader biomarker of mycobacterial infections than originally thought.

## METHODS

**Bacterial strains and growth conditions** – *Mtb* CDC1551, *Mtb* H37Rv ATCC 25618, *M. smegmatis* mc<sup>2</sup>155, *M. abscessus* subspecies *abscessus* ATCC 19977, *M. avium* *hominissuis* 104, *M. avium* *hominissuis* MAH11<sup>35</sup> and *M. kansasii* ATCC 12478 were grown under agitation at 37°C in Middlebrook 7H9 medium supplemented with 10 % albumin-dextrose-catalase (ADC) (BD Sciences) and 0.05 % Tween 80, or on Middlebrook 7H11 agar supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC) (BD Sciences). Hygromycin (50 µg mL<sup>-1</sup>) and kanamycin (25 µg mL<sup>-1</sup>) were added to the culture media as needed. *Mtb capA::Tn* was obtained from BEI Resources (NR-15700). The *mtxS* and *mtxT* knock-out mutants (*MtbΔmtxS* and *MtbΔmtxT*) and the *mptC* (*Rv2181*) knock-out mutant of *Mtb* H37Rv ATCC 25618 were reported previously<sup>15, 27</sup>. The pVV16-*Rv2181* plasmid used to complement the *mptC* knock-out mutant of *Mtb* H37Rv was described by Kaur *et al.*<sup>36</sup>. *Mtb capA::Tn* and *MtbΔmtxT* were complemented by expressing wild-type copies of the *capA* and *mtxT* genes, respectively, under control of their own promoters from the integrative plasmid pMV306H (a pMV306 derivative harboring a hygromycin-resistance gene). To this end, the *capA* gene and 297-bp of upstream sequence were PCR-amplified using primers pMV306H-CapA.fw (5'- GCTCTAGAGCGCCGGCGATCTGGGCAGC – 3') and pMV306H-CapA.rv (5' – GGATCGATTTACCGCGTTGACTTGACCACCT – 3') and cloned into the *Xba*I and *Cla*I restriction sites of pMV306H, yielding *MtbΔmtxT*/pMV306H-*capA* (*Mtb capA::Tn* comp). The *mtxT* gene and 300-bp of upstream sequence were PCR-amplified using primers pMV306H-MTX2.fw (5' – GCTCTAGAGCCGCAGCCAGTGGAGGCCG – 3') and pMV306H-MTX2.rv (5' – GGATCGATTTACCAGTTGGTGAAGATGAT – 3') and cloned into the *Xba*I and *Cla*I restriction sites of pMV306H, yielding *MtbΔmtxT*/pMV306H-*mtxT* (*MtbΔmtxT* comp).

**Preparation and analysis of lipoglycans** – Extraction of lipoglycans from mycobacteria followed earlier procedures<sup>10, 11</sup>. Briefly, total lipoglycans were extracted multiple times by ethanol reflux from delipidated cells prior to treatment with DNase, RNase, α-amylase and Proteinase K, and extensive dialysis against water. Purified LAM and LM used for structural analysis and biological activity studies were separated by gel exclusion chromatography. Lipoglycans were analyzed by

SDS-PAGE on commercial Novex 10-20% Tricine gels stained with periodic acid Schiff reagent. Alternatively, lipoglycans were transferred onto a nitrocellulose membrane (iBlot™ system, Invitrogen™) and membranes were blocked with 3% BSA in TBST buffer (Tris-buffered saline containing 0.1% Tween® 20) for 1 h at 25°C. S4-20 monoclonal antibodies (0.1 µg mL<sup>-1</sup> final concentration) (Otsuka Pharmaceutical) were used as primary antibodies. Alkaline phosphatase-conjugated polyclonal anti-rabbit IgG (whole molecule; Sigma A3687) diluted 1:30,000 was used as the secondary antibody. Labeled lipoglycans were revealed by short incubation with 1-Step™ NBT/BCIP substrate solution (Thermo Scientific).

For structural analyses, 50 µg of LM and 50 µg of LAM were used for permethylation and alditol acetates preparation and analyzed by GC/MS to determine monosaccharide composition and glycosyl linkage patterns as described<sup>10</sup>. The presence of mannoside capping motifs at the non-reducing arabinan termini of LAM from *Mtb* was analyzed by capillary electrophoresis<sup>37</sup>. The presence of MTX substituents on *Mtb* LM was determined by submitting purified, deacylated, LM to enzymatic digestions with commercial Jack bean α-mannosidase (Sigma) and endo-α-1,6 mannanase from *Bacillus circulans* as described<sup>28, 38</sup>, followed by LC/MS analysis of the enzymatic products. Digested oligosaccharides were analyzed on an Agilent 6546 LC/Q-TOF. LC separation was performed on an Atlantis T3 column (2.1 x 150 mm; 3 µm) at 40°C with a flow rate of 0.3 mL min<sup>-1</sup> using a gradient of solvent B (0.01 M ammonium acetate in acetonitrile) in solvent A (0.01 M ammonium acetate in water) as follows: 0–2.5 min (5% solvent B), 2.5-5 min (40% solvent B), 5-9 min (95% solvent B), 9-10 min (hold 95% solvent B), 10–11 min (5% solvent B). Data acquisition was performed in the negative electrospray ion mode with a mass-to-charge ratio (*m/z*) range of 100-3,200 at the rate of 1.25 spectra s<sup>-1</sup>. Dual AJS ESI source was used at the following settings: capillary voltage, 3,500 V; nebulizer gas pressure, 40 psig; drying gas flow, 9 L min<sup>-1</sup>; and drying temperature, 325°C. Data processing was carried out using Mass Hunter Workstation Software Qualitative Analysis (version B.07.00).

<sup>1</sup>H 1D spectra and <sup>1</sup>H-<sup>13</sup>C 2D HMQC NMR experiments were performed at 303K with a cryo-probed Bruker DRX600 spectrometer (Karlsruhe, Germany) using “zgpr” and “hsqcetgpsisp2.2” sequences. Native molecules were dissolved in D<sub>2</sub>O (Eurisotop, France) and analyzed in 200 x 5 mm UP5 NMR tubes (CortecNet). Proton and carbon chemical shifts are expressed in ppm downfield from the signal of external acetone (δH 2.22 and δC 30.89).

**Dectin-2 and DC-SIGN binding assays** - LAM (1  $\mu\text{g}$  per well in isopropanol) was coated on 96-well Maxisorp plates (Nunc). Dectin-2-Fc<sup>32</sup> and DC-SIGN-Fc<sup>39</sup>, soluble forms of the human Dectin-2 and DC-SIGN receptors, respectively, consisted of their C-terminal extracellular domains (amino acids 42 to 209 for Dectin-2, and amino acids 59 to 404 for DC-SIGN) fused at their N termini to the C terminus of the human IgG1-Fc1 fragment. Dectin-2-Fc or DC-SIGN-Fc proteins (1  $\mu\text{g mL}^{-1}$  in PBS buffer containing 1 mM  $\text{CaCl}_2$  and 1% BSA) were pre-incubated or not with 20 mM EDTA, and allowed to react with LAM for 2 h at room temperature in a total volume of 50  $\mu\text{L}$ . Wells were washed once with PBS and the bound Fc fusion proteins were detected using biotin-conjugated anti-human IgG Fc $\gamma$  specific antibodies (eBioscience) and avidin-horseradish peroxidase (eBioscience).

**Treatment of THP-1 cells with purified lipoglycans** – THP-1 cells were treated with 20  $\text{ng mL}^{-1}$  phorbol 12-myristate 13-acetate (PMA) in RPMI containing 10% fetal bovine serum, seeded at  $1.0 \times 10^5$  cells per well in 96-well flat bottom plates and allowed to differentiate for 24 h. After 24 h with PMA, the medium was removed from the adherent THP-1 cells and fresh medium was added. After an additional 24 h, purified *Mtb* LAM or LM were added to the wells at 10  $\mu\text{g mL}^{-1}$ . In some experiments 150  $\mu\text{g mL}^{-1}$  LPS was added to the wells at the same time as the lipoglycans. In other experiments, lipoglycans were added for 24 h followed by addition of 30  $\mu\text{g mL}^{-1}$  LPS. After 48 h of incubation with either lipoglycans and/or LPS, the supernatants were harvested and stored at  $-20^\circ\text{C}$  for multiplex Luminex<sup>®</sup> assays for cytokines and chemokines and for NO by the Griess Reagent assay. Cells were detached from the wells by treatment with ice-cold PBS and gentle dispersion by pipetting. Cells were then transferred to new 96-well round bottom plates for staining with Annexin V.

**Apoptosis and necrosis** - Detached cells were wash twice in 1x Annexin V buffer (10 mM HEPES pH=7.4; 140 mM NaCl; 2.5 mM  $\text{CaCl}_2$ ). Annexin V-Alexa-Fluor-647<sup>®</sup> (Invitrogen, Waltham, MA) was added according to the manufacturer's protocol. Following 20 min of staining, the cells were washed and resuspended in Annexin V buffer followed by addition of propidium iodide (PI) (Molecular Probes, Eugene OR) at 10  $\mu\text{g mL}^{-1}$ . Apoptotic vs. dead cells were resolved by flow cytometry with analysis of dot plots of FL3 (PI) vs. FL6 (Alexa-Fluor-647) and the percentage of apoptotic cell was those cells that were FL6<sup>+</sup> and FL3<sup>-</sup>. Necrotic cells were those that were FL6<sup>-</sup>

and FL3<sup>+</sup>. Flow cytometry was performed on a Beckman-Coulter Gallios multicolor flow cytometer. Flow cytometry data were analyzed using FlowJo<sup>®</sup> software (FlowJo, Ashland, OR).

***Multiplex immunoassay for cytokines and chemokines*** - Multiplex immunoassay using macrophage culture supernatants was performed using a Luminex bead-based multiplex ELISA kit (MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A, Millipore) according to the manufacturer's instructions. Sample data were acquired on a MAGPIX instrument running xPONENT 4.3 software (Luminex Corp.) and analyzed using a five-parameter logistic model with an 80-120% standard acceptance range. Data was graphed using Graphpad Prism version 4.00.

***Statistical analysis*** – Unless otherwise indicated in the figure legends, data are expressed as the mean ± SD values from triplicate assays. Statistical tests were performed as indicated in the figure legends. Calculations were performed using GraphPad PRISM version 9.5.1 for Windows or GraphPad PRISM 10.1.1 for MacOS (San Diego California USA).

## **Supporting Information**

The Supporting Information is available free of charge at: xxxxx

Details of the structural analysis of LM and LAM from the different *Mtb* strains: Alditol acetates and glycosyl linkage analysis; degree of mannoside capping of LAM determined by capillary electrophoresis; and NMR analysis of LAM purified from the *Mtb* CDC1551 WT *capA* transposon mutant (PDF).

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## **Notes**

The authors declare no competing financial interests.

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**GRAPHICAL ABSTRACT**

