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A truncated lipoglycan from mycobacteria with altered immunological properties

Helen L. Birch[a,1], Luke J. Alderwick[a,1], Ben J. Appelmelk[b], Janneke Maaskant[b], Apoorva Bhatt[a], Albel Singh[a], Jerome Nigou[c], Lothar Eggeling[d], Jeroen Geurtsen[b], and Gurdyal S. Besra[a,2]

[a]School of Biosciences, University of Birmingham, Birmingham B15 2TT, United Kingdom; [b]Department of Medical Microbiology and Infection Control, VU University Medical Center, 1081 BT Amsterdam, The Netherlands; [c]Département Mécanismes Moléculaires des Infections Mycobactériennes, Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5089, 31077 Toulouse cedex 4, France; and [d]Institute for Biotechnology 1, Research Centre Juelich, D-52425 Juelich, Germany

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Maintenance of cell-wall integrity in *Mycobacterium tuberculosis* is essential and is the target of several antitubercular drugs. For example, ethambutol targets arabinogalactan and lipoarabinomannan (LAM) biosynthesis through the inhibition of several arabinofuranosyltransferases. Apart from their role in cell-wall integrity, mycobacterial LAMs also exhibit important immunomodulatory activities. Here we report the isolation and detailed structural characterization of a unique LAM molecule derived from *Mycobacterium smegmatis* deficient in the arabinofuranosyltransferase AftC (AftC-LAM). This mutant LAM expresses a severely truncated arabinan domain completely devoid of 3,5-Ara*f*–branching residues, revealing an intrinsic involvement of AftC in the biosynthesis of LAM. Furthermore, we found that ethambutol efficiently inhibits biosynthesis of the AftC-LAM arabinan core, unambiguously demonstrating the involvement of the arabinofuranosyltransferase EmbC in early stages of LAM-arabinan biosynthesis. Finally, we demonstrate that AftC-LAM exhibits an enhanced proinflammatory activity, which is due to its ability to activate Toll-like receptor 2 (TLR2). Overall, our efforts further describe the mechanism of action of an important antitubercular drug, ethambutol, and demonstrate a role for specific arabinofuranosyltransferases in LAM biosynthesis. In addition, the availability of sufficient amounts of chemically defined wild-type and isogenic truncated LAMs paves the way for further investigations of the structure–function relationship of TLR2 activation by mycobacterial lipoglycans.

arabinofuranosyltransferase | lipoarabinomannan | *Mycobacterium tuberculosis* | cell wall | ethambutol

Tuberculosis (TB) affects a third of humankind and causes 1.7 million deaths annually (1). The spread of TB has been facilitated in recent decades due to the susceptibility of HIV-infected individuals to *Mycobacterium tuberculosis*, the etiological agent of TB (2). The problem has been compounded by the emergence of multi- and extensively drug-resistant *M. tuberculosis* strains (3). *M. tuberculosis* resides within the family of *Corynebacteriaceae*. A common feature of this family is that they possess an unusual cell-wall architecture dominated by an essential heteropolysaccharide termed arabinogalactan (AG), which is linked to both mycolic acids and peptidoglycan, forming the mycolyl–arabinogalactan–peptidoglycan complex (4–6). The formation of the arabinan domain of AG results from the subsequent addition of arabinofuranose (Ara*f*) residues by a set of unique arabinofuranosyltransferases (AraTs). The front-line drug ethambutol (EMB) has been shown to target at least three AraTs (EmbA, EmbB, and EmbC) (7, 8), but shows no inhibitory effects against the other recently identified cell-wall AraTs, such as AftA (9), AftB (10), and AftC (11). In a previous study, we successfully deleted *MSMEG2785* (Ms-*aftC*) (11) and showed that this leads to expression of a severely truncated AG structure with branching defects in its arabinan domain. Apart from AG, mycobacteria contain several other glycoconjugates. Lipoarabinomannan (LAM), which contains an arabinan domain that is structurally similar to that of AG, is a major component of the cell wall. It consists of a core mannan domain covalently linked to a mannosyl–phosphatidyl–*myo*–inositol (MPI) anchor, which makes it structurally similar to its biochemical precursor lipomannan (LM) (12–14). Both LAM and LM exhibit immunomodulatory functions that may influence the host immune response (reviewed in ref. 15). Species-specific differences in the "capping-motifs" of the nonreducing termini of the arabinan domain—for which three variants exist, i.e., AraLAM, PI-LAM, and ManLAM (16–20)—have been shown to be important for this function.

The arabinan domain of LAM is attached to an as-yet-unidentified region of the mannan backbone and is thought to be synthesized in a similar manner to that of arabinan found in AG (12–14). To date, only one AraT has been implicated in the biosynthesis of LAM. This enzyme, EmbC, has also been shown to be targeted by EMB, but to a lesser extent than the cell-wall core AraTs, EmbA and EmbB (21, 22). The formation of the arabinan domain of LAM requires an α(1→3) AraT in a similar manner to AG, thus resulting in the branched motif of LAM. Here we investigated the potential role of AftC in LAM biosynthesis. By analyzing the structure of LAM from a *M. smegmatis* Δ*aftC* mutant, we demonstrate that AftC carries dual functionality and is responsible for introducing 3,5-Ara*f* branches into LAM in addition to AG. Furthermore, we show, by treating an *M. smegmatis* Δ*aftC* mutant with EMB, that EmbC is involved in the very early steps of the LAM arabinan core synthesis and that truncation of this domain modulated the immunological properties of the molecule.

Results

Effects of *aftC* Inactivation on LM/LAM Biosynthesis. *M.* smegmatis wild type (WT) (Fig. 1, lane 1) and *M. smegmatis* Δ*aftC* lipoglycans (Fig. 1, lanes 2–4) were purified using conventional methods (20) resulting in the recovery of a highly purified lipoglycan with an intermediate size between *M. smegmatis* LAM and LM, now termed AftC-LAM (Fig. 1, lane 3). Complementation of *M. smegmatis* Δ*aftC* with Ms-*aftC* restored the lipoglycan profile to WT *M. smegmatis* (Fig. 1, lane 5). Plasmid-borne Mt-*aftC* also resulted in complementation of the mutant (Fig. 1, lane 6). The molecular weight of AftC-LAM was investigated by negative-ion matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MS). The mass of WT-LAM and AftC-LAM exhibited broad unresolved peaks centered at

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[1]H.L.B. and L.J.A. contributed equally to this article.

[2]To whom correspondence should be addressed. E-mail: g.besra@bham.ac.uk.

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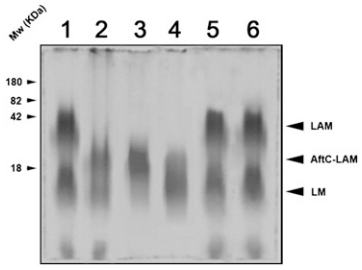

Fig. 1. SDS–PAGE analysis of lipoglycans extracted from *M. smegmatis* and *M. smegmatis* Δ*aftC*. Lane 1, lipoglycans extracted from *M. smegmatis*; lane 2, lipoglycans extracted from *M. smegmatis* Δ*aftC*; lane 3, purified AftC-LAM from *M. smegmatis* Δ*aftC*; lane 4, purified LM from *M. smegmatis* Δ*aftC*; lane 5, lipoglycans extracted from *M. smegmatis* Δ*aftC* pMV261-Ms-*aftC*; and lane 6, lipoglycans extracted from *M. smegmatis* Δ*aftC* pMV261-Mt-*aftC*.

m/z 15,000 and 8,000, respectively, indicating a weight decrease of ~7 kDa for the mutant LAM (Fig. S1 *A* and *B*).

Structural Characterization of AftC-LAM. The ratio of Ara to Man in WT-LAM and AftC-LAM was determined using gas chromatography (GC) of alditol acetate derivatives (11) (Fig. S2 *A* and *C*). WT-LAM had a molar ratio of Ara:Man of 2.7:1, which is consistent with previously reported data (22), whereas, for AftC-LAM, the Ara:Man ratio was 0.59:1 (Fig. S2 *A* and *C*). Complementation of *M. smegmatis* Δ*aftC* with either Ms-*aftC* or Mt-*aftC* restored the Ara:Man ratio to that of the wild-type LAM (Fig. S2 *E* and *G*). In *M. smegmatis*, LAM consists of approximately 71 Ara, 27 Man, and 1 Ins units (22, 23). The loss of 7 kDa for AftC-LAM equates to 45 Ara residues, suggesting that AftC-LAM contains one or more short arabinan domains of up to ~16 Ara residues. The ^{1}H-NMR spectrum of WT-LAM (Fig. 2*A*) was much more complex than the anomeric region of AftC-LAM (Fig. 2*B*). Indeed, the AftC-LAM 1D ^{1}H spectrum exhibits three major, well-defined resonances characterized by several overlapping resonances arising from six different classes of glycosidic residues. On the basis of our data for WT-LAM and previously published work (19), the ^{13}C resonance at δ101 ppm that correlated to an anomeric proton at δ5.15 ppm with a $^{1}J_{H1,C1}$ coupling constant of ≈170 Hz was assigned as 2,6-Man*p*. The resonances at δ105 and δ102.3, correlating to protons at δ5.07 and δ4.90, were assigned as *t*-Man*p* and 6-Man*p*, respectively. The *t*-β-Ara*f* residues corresponded to δ103.4 with ^{1}H at δ5.16. The well-separated spin systems for 2-α-Ara*f* attached to the 3-position (2-α-Ara*f*→3, δ108.2, δ5.27 ppm) and the 5-position (2-α-Ara*f*→5, δ108.5, δ5.20) of the 3,5-Ara*f* were also visible in the spectra of WT-LAM. Several spin systems were observed (δ110.3 and δ5.19 ppm, δ110.3 and δ5.14 ppm, and δ110.3 and δ5.11 ppm) assigned to 5-α-Ara*f* in different chemical environments with one overlapping set of 3,5-α-Ara*f* (δ110.2-δ5.12) for WT-LAM. As reported, two distinct chemical shifts of *t*-α-Ara*f* could occur for the respective arms of a branched 3,5-α-Ara*f*, as observed for 2-α-Ara*f*→3 and 2-α-Ara*f*→5 (19). In AftC-LAM (Fig. 2*B*), the resonances associated with the mannan core (*t*-Man*p*, 6-Man*p*, and 2,6-Man*p*) are conserved, but resonances associated with Ara*f* residues are notably less abundant. In particular, 2-α-Ara*f*→3 is absent and the complex set of signals for the different 5-α-Ara*f* are much more simplified due to the loss of 3,5-α-Ara*f* (Fig. 2*B*). GC/MS of per-*O*-methylated alditol acetate derivatives confirmed this result in that it showed an unaltered glycosidic linkage profile for the mannan backbone of both WT- and AftC-LAM and a complete loss of 3,5-Ara*f* and a significant reduction in *t*-Ara*f*, 2-Ara*f*, and 5-Ara*f*-linkages for the latter LAM (Fig. S2 *B* and *D*). Complementation of *M. smegmatis* Δ*aftC* with either Ms-*aftC* or Mt-*aftC* restored the glycosyl linkage profile to that of WT (Fig. S2 *F* and *H*), demonstrating that MSMEG2785 (Ms-*aftC*) and Rv2673 (Mt-*aftC*) are functional orthologs. Overall, the compositional analysis suggests that, as compared to WT-LAM, AftC-LAM has an unaltered mannan domain composed of an α(1→6)-Man*p* backbone substituted by *t*-Man*p* units at *O*-2 positions, to which is attached one or more short α(1→5)-linked linear arabinan chains (in total representing ≈12–15 Ara*f* units) terminating in a single β(1→2) Ara residue (Fig. S3).

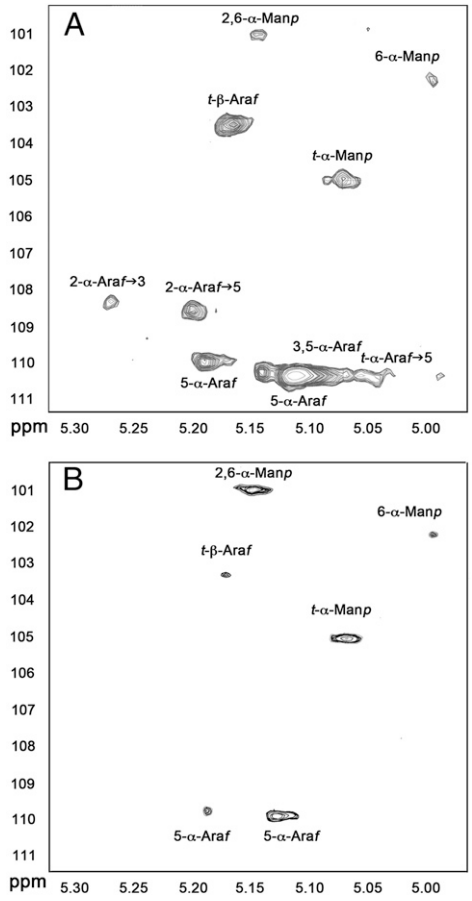

Fig. 2. Two-dimensional NMR spectra of WT-LAM and AftC-LAM purified from *M. smegmatis* and *M. smegmatis* Δ*aftC*. Structural characterization of WT-LAM (*A*) and AftC-LAM (*B*). ^{1}H, ^{13}C HSQC NMR spectra were acquired in D_2O at 313K. Expanded regions (δ ^{1}H: 5.0–5.30; δ ^{13}C: 101–111) are shown.

Effect of Ethambutol on AftC-LAM Formation. EMB blocks cell-wall arabinan biosynthesis by targeting the Emb proteins (7, 8). Although EmbC is known to be involved in LAM arabinan biosynthesis (22), its precise role is as yet inconclusive. We exploited the phenotype of *M. smegmatis* Δ*aftC* and the structural properties of AftC-LAM to investigate this role. For this, we treated [^{14}C]-glucose-labeled cultures of *M. smegmatis* with subinhibitory concentrations of EMB, extracted the lipoglycans, and performed a radiochemical quantification of [^{14}C]-incorporated sugars. SDS–PAGE analysis of the [^{14}C]-LM/LAM pool of WT *M. smegmatis* revealed two broad bands corresponding to LAM and LM migrating to their expected sizes (Fig. 3*A*, lane 1) and an Ara:Man ratio of 1.2:1 (Fig. 3*B*, lane 1). Subsequent analysis of the [^{14}C]-LM/LAM lipoglycan pool from *M. smegmatis* Δ*aftC* resulted in labeling of AftC-LAM and LM (Fig. 3*A*, lane 2) and an Ara:Man ratio of 0.5:1 (Fig. 3*B*, lane 2). Addition of EMB at 0.5 μg·ml^{-1} resulted in a decrease in the size of the AftC-[^{14}C]-LAM, an accumulation of [^{14}C]-LM (Fig. 3*A*, lane 3), and a corresponding change in the Ara:Man ratio of 0.3:1 (Fig. 3*B*, lane 3). Because

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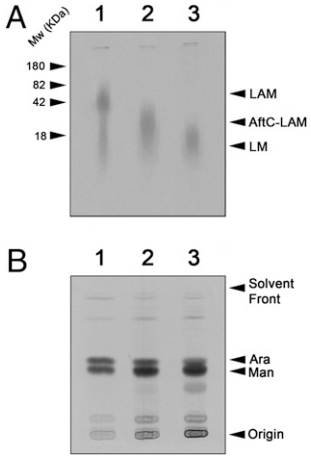

Fig. 3. SDS–PAGE (*A*) and total sugar analysis (*B*) of [^{14}C]-labeled lipoglycans extracted from *M. smegmatis* and *M. smegmatis* Δ*aftC* treated with EMB. Growing cultures of *M. smegmatis* and *M. smegmatis* Δ*aftC* were labeled with [^{14}C]-glucose, and their lipoglycans were extracted and analyzed by SDS–PAGE (*A*). Lane 1, *M. smegmatis*; lane 2, *M. smegmatis* Δ*aftC*; and lane 3, *M. smegmatis* Δ*aftC* + 0.5 μg·ml^{-1} EMB. The above lipoglycans were hydrolyzed in 2 M TFA and analyzed by TLC to determine the total sugar composition (*B*). Lane 1, *M. smegmatis*; lane 2, *M. smegmatis* Δ*aftC*; and lane 3, *M. smegmatis* Δ*aftC* + 0.5 μg·ml^{-1} EMB.

AftC-LAM contains an intact mannan core, the only possible effect of EMB is the direct inhibition of EmbC activity [addition of the terminal β(1→2) Ara*f* residues by AftB is insensitive to EMB (9)], thus showing its involvement in the very early stages of LAM arabinan biosynthesis and inhibition of an α(1→5) AraT. Notably, we observed that residual [^{14}C]-Ara labeling always remained upon EMB treatment (Fig. 3*B*, lane 3), suggesting that another AraT possibly adds the first Ara*f* residue to LM, akin to AftA in AG biosynthesis (9).

AftC-LAM Displays Proinflammatory Properties. LMs, in contrast to LAMs, are potent proinflammatory lipoglycans (24–27). This difference has been attributed to the presence of the arabinan domain in LAM, which masks the "bioactive" mannan core (24, 28). To investigate the consequence of the *aftC* mutation on the proinflammatory activity of LAM, we compared WT- and AftC-LAM for release of TNF-α by human THP1 cells. Consistent with earlier studies (24), truncated AftC-LAM, as compared to WT-LAM, exhibited an increased proinflammatory activity (Fig. 4*A*). LM is a known agonist for TLR2 (29). Therefore, we investigated the ability of WT- and AftC-LAM to activate this receptor. HEK293 cells expressing TLR2 were stimulated with increasing amounts of LAM after which TLR2-dependent IL-8 production was determined. As shown in Fig. 4*B*, AftC-LAM induced a much stronger TLR2 activation than did WT-LAM (~10-fold). This result demonstrates that the presence of the full-length arabinan domain somehow hampered TLR2 activation. Previously, it has been reported that immune stimulatory activity of some *M. smegmatis* LAM preparations may have been caused by lipopeptide contamination (26). To investigate this issue, both WT and AftC-LAM were pretreated with H_2O_2 [a procedure that inactivates lipopeptides (30, 31)], after which they were retested for their activity on HEK293 TLR2 cells. As shown in Fig. 4*C*, pretreatment with H_2O_2 substantially reduced the activity of both WT- and AftC-LAM. However, in contrast to WT-LAM, which lost all of its activity, a substantial part of the activity arising from AftC-LAM was sustained for up to 168 h of treatment (Fig. 4*C*). This was not due to the inactivation of the H_2O_2 itself because addition of fresh H_2O_2 after 96 h did not further reduce the activity (Fig. 4*C*). Therefore, we conclude that both LAM preparations were probably contaminated with lipopeptides to a certain extent. Nevertheless, inactivation of these molecules with H_2O_2 clearly demonstrated that AftC-LAM was able to activate TLR2, whereas WT-LAM became completely inactive for concentrations up to 100 μg·ml^{-1} (Fig. 4*D*).

To determine whether the increased proinflammatory activity of AftC-LAM indeed coincided with a more exposed mannan domain, the ability of WT- and AftC-LAM to interact with a human immunoglobulin G Fc construct harboring the extracellular domain of the C-type lectin dendritic-cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) was investigated. DC-SIGN is highly expressed on dendritic cells and recognizes high-mannose structures (32, 33) including LM (34) but not PILAM (35). As shown in Fig. 4*E*, the reactivity of DC-SIGN–Fc toward AftC-LAM was stronger as compared to the reactivity against WT-LAM, demonstrating that the mannan core of AftC-LAM was more accessible.

Discussion

The data described in this article unequivocally demonstrate that AftC is responsible for introducing 3,5-Ara*f* branching in the LAM arabinan domain of *M. smegmatis* and proposes a previously undescribed model for LAM biosynthesis (Fig. 5). Furthermore, we show that EmbC is involved in the very early stages of LAM arabinan biosynthesis and that, with the truncation of the arabinan domain, LAM gains the ability to activate TLR2. Although the structure of the arabinan domain of LAM is well understood, less is known of the enzymes involved in its biosynthesis. One important reason is that *Corynebacterium glutamicum*, a preferred model organism to study mycobacterial AG and LM biosynthesis (9–11), does not produce a convoluted LAM, as present in mycobacteria. During our investigation of various putative GT-C glycosyltransferases, we deleted *msmeg2785* (AftC) from *M. smegmatis*, which resulted in a phenotype that displayed a severely truncated AG (11). In the present study, we revisited this mutant in an attempt to investigate the potential role of AftC in LAM biosynthesis. Chemical analysis revealed that LAM isolated from the *aftC* mutant contained an unaltered mannan core, but with one or more simple arabinan moieties of ≈12–16 Ara*f* units composed of α(1→5) linkages terminating in a single β(1→2) fashion. It is reasonable to conclude from our data that AftC is not involved in early arabinan LAM biosynthesis because a more pronounced truncation of LAM would then be expected. AftC has dual functionality in terms of its involvement in the biosynthesis of both AG and LAM. Attempts to generate an *aftC*-deficient strain of *M. tuberculosis* proved unsuccessful (36), highlighting the essentiality of *aftC* in *M. tuberculosis* and the species' intolerance to cell-wall changes. Hence, AftC represents an excellent drug target.

EmbC has long been implicated in the biosynthesis of mycobacterial LAM and has been shown to be a target of the front-line drug EMB (21, 22). However, speculation around its precise enzymatic function has remained controversial. By using the unique phenotype of *M. smegmatis* Δ*aftC*, we provide unequivocal evidence that EmbC is an α(1→5) AraT, which is inhibited by EMB (Fig. 3). We now propose a working model, whereby an as-yet-unidentified GT-C AraT primes the mannan backbone with singular Ara*f* residues in much the same way as AftA primes the galactan backbone of AG (9). After this priming, EmbC extends the arabinan chain in an α(1→5) fashion before AftC branches the polysaccharide with an α(1→3) Ara*f* residue, and the domain is further matured by a combination of AraTs, including AftC, AftB, and perhaps other as-yet-unidentified enzymes (Fig. 5).

Several mycobacterial cell-wall lipoglycans are proinflammatory stimuli of the host immune response. Both LM and LAM are members of a family of mycobacterial lipoglycans that all contain a conserved MPI anchor (29). Interestingly, LM, in addition to being a potent inducer of proinflammatory cytokines, also displays strong anti-inflammatory properties and represses lipopolysaccharide (LPS)-induced cytokine responses in macro-

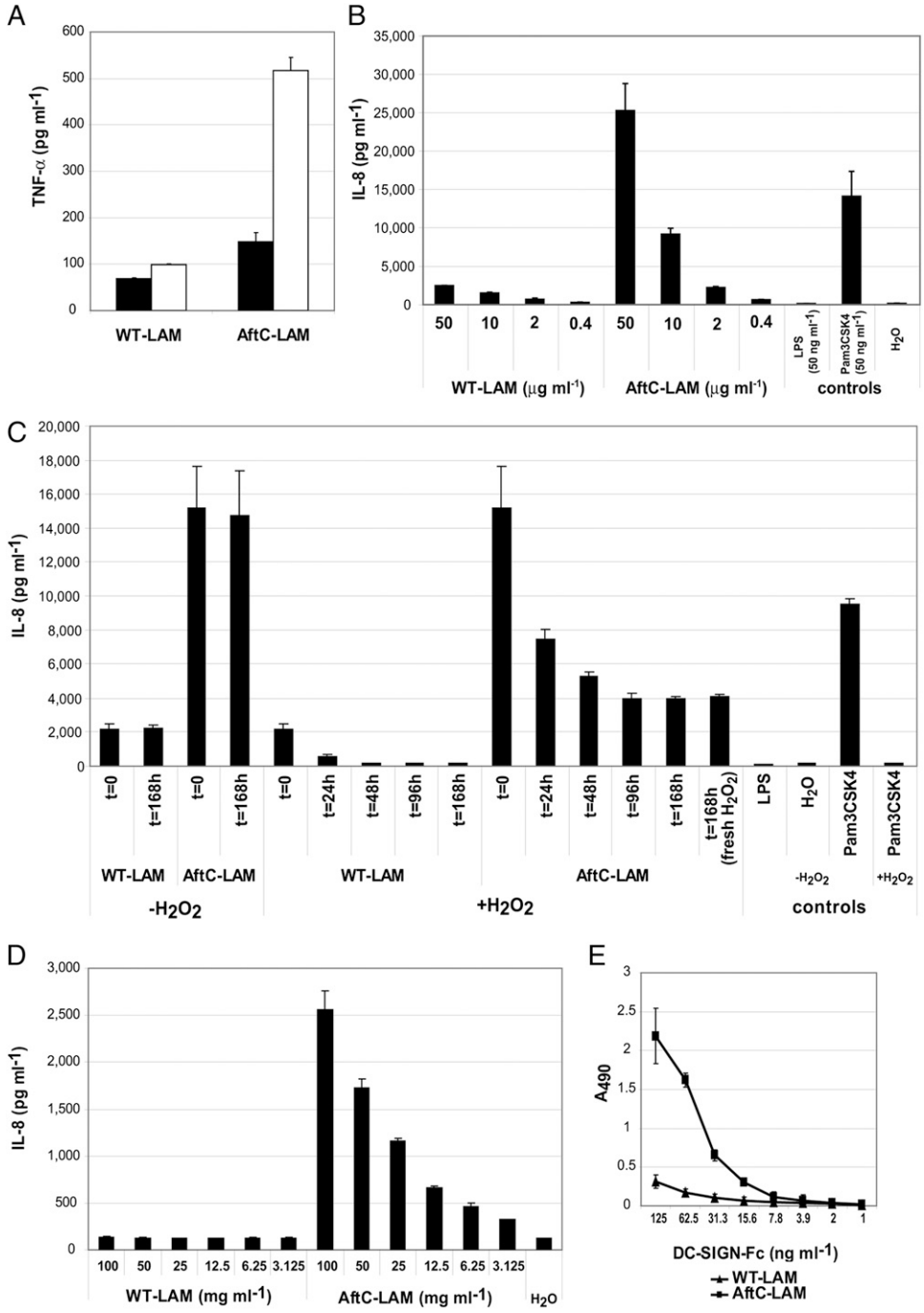

Fig. 4. TNF-α production by human THP-1 cells and IL-8 production by HEK293 TLR-2 cells in response to WT-LAM and AftC-LAM. (*A*) Human THP-1 cells were incubated with 1 $\mu g \cdot ml^{-1}$ (solid bars) or 10 $\mu g \cdot ml^{-1}$ (open bars) WT- or AftC-LAM. TNF-α was quantified after 24 h by ELISA. (*B–D*) HEK293 cells transfected with TLR2 were stimulated with (*B*) increasing amounts of WT- or AftC-LAM with LPS (50 $ng \cdot ml^{-1}$), with Pam_3CSK_4 (50 $ng \cdot ml^{-1}$), or with H_2O as a negative control; (*C*) 50 $\mu g \cdot ml^{-1}$ of WT- or AftC-LAM [with and without 1% hydrogen peroxide ($\pm H_2O_2$) for the indicated time period] with LPS (50 $ng \cdot ml^{-1}$), with Pam_3CSK_4 (50 $ng \cdot ml^{-1}$) ($\pm H_2O_2$ for 48 h), or with H_2O as a negative control; and (*D*) with increasing amounts of WT- or AftC-LAM (treated with H_2O_2 for 168 h). In all cases, cells were stimulated for 24 h at 37 °C, after which the supernatants were harvested and analyzed for IL-8 by ELISA. (*E*) Binding of DC-SIGN–Fc to WT- (▲) or AftC-LAM (■) as determined by ELISA. Absorption was measured at 490 nm. In all panels, data are expressed as the mean ± SD from one representative of three independent experiments.

phages (25). Recently, by making use of LM preparations, separated by their degree of acylation, it was demonstrated that these pro- and anti-inflammatory properties are, at least in part, dependent on the degree of acylation (27, 37). Whereas, tri- and tetra-acylated LM forms displayed strong proinflammatory properties, mono- and di-acylated molecules did not (37). In contrast, di-acylated LM was found to be the most potent inhibitor of LPS-induced cytokine responses, a feature that was independent of TLR2, mannose receptor, or SIGNR1 (27). In addition to the degree of acylation, additional structural features of MPI-anchored lipoglycans determine their biological activity. Recently, it was shown that the chain length of the mannan core

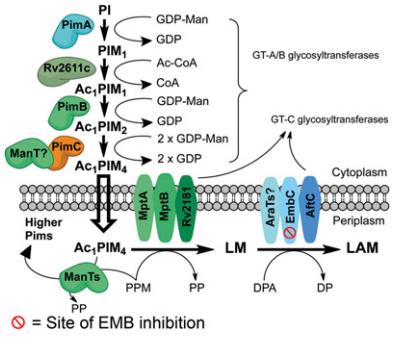

Fig. 5. Mycobacterial LAM biosynthesis and the role of AftC. The GT-A/B family of glycosyltransferases performs sequential glycosidic transfer of mannose residues using the high-energy nucleotide GDP-Man to a PI-based anchor in the cytoplasm. The GT-C family of glycosyltransferases then continue LM and LAM biosynthesis by elaborating Ac_1PIM_4 with mannose and arabinose residues. MptA, MptB, and Rv2181 are the only known ManTs involved in core mannan biosynthesis, for which they use the lipid-linked substrate polyprenylphosphomannose (PPM) as the sugar donor. Decaprenyl-1-monophosphoarabinose (DPA) is used by an unknown AraT to prime LM at an as-yet-unidentified position. EmbC and AftC are the other two identified AraTs, which mature the final LAM molecule before species-specific capping occurs.

of MPI-anchored lipoglycans directly correlated with their ability to activate TLR2 (26). Whereas lipoglycans with short oligomannopyranosyl backbones, such as the PIMs, were marginally active, the TLR2-activating potency of lipoglycans with longer backbones, i.e., LM, was strongly enhanced (26). In addition to mannan chain length, the type of substitution of the mannan core was found to be critical, with activity being retained in the case of Man*p* substitutions but absent when the core was substituted with Ara*f* residues (26). This was evidently clear for mycobacterial LAMs, which carry a bulky arabinan domain. Although their LM core would in principle allow for TLR2 activation, the LAMs were found to be inactive (26). The assumption that the arabinan domain is directly involved in "silencing activity" is further sustained by the observation that chemical degradation of the arabinan domain restores the proinflammatory properties of LAM (24). Our results are in agreement with these findings as AftC-LAM, which expresses a severely truncated arabinan domain, exhibited an increased proinflammatory activity (Fig. 4). Because WT and AftC-LAM did not differ in their pattern of acylation or core mannan composition, the increased proinflammatory activity must be directly related to the shortening of the arabinan domain. Exactly how the arabinan domain prevents TLR activation is currently not fully understood. One thought is that the arabinan domain exerts its inhibitory effect by steric hindrance (24). Although several lines of evidence point to this direction, the exact mechanism and the level at which this occurs remains unclear. Furthermore, Nigou and coworkers (26) demonstrated that *M. bovis* bacillus Calmette–Guérin LAM complexed with soluble CD14 efficiently competes with Pam_3CSK_4 for binding to TLR2, suggesting that binding to, and signaling through TLR2, may not be equal. In several mycobacterial species, the nonreducing termini of the branched arabinan domain are modified by capping motifs consisting of either oligomannosyl, i.e., ManLAMs, or PILAM units such as in *M. smegmatis* (16). Whereas reports on the immune stimulatory properties of ManLAMs are consistent and unambiguously demonstrate that these types of LAMs are inactive (24, 26), some controversy exists on the activity of PILAMs. Early reports demonstrate that PILAM is proinflammatory and signals via TLR2 (17, 24, 38–40). However, the mechanism by which a low degree of inositol–phosphate capping would confer this proinflammatory activity has never been explained. One hypothesis is that the *myo*–inositol–phosphate motif in the MPI-anchored lipoglycans may mimic the inositol–phosphate caps on PILAMs, thereby conferring activity. However, the notion that the unsubstituted phosphoinositol (PI) caps are unlikely to mimic the acylated MPI anchor, plus the observation that PIMs are only marginally active, clearly demonstrates that the PI motif alone does not confer activity (24). Interestingly, in a more recent study, Nigou et al. (26) demonstrated that highly purified PILAMs from *M. smegmatis* and *M. fortuitum* were equivalent in activity to ManLAMs. Therefore, in contrast to what was previously suggested, the authors concluded that the presence of PI caps did not make LAM proinflammatory, and they suggested that the activity in earlier experiments was probably due to lipopeptide contamination. Even though contaminant lipopeptides may not be detected by the current analytical methods, trace amounts of these molecules (<0.1%) are enough to influence the outcome of much more sensitive biological assays. We therefore determined the effect of H_2O_2 treatment on the TLR2-stimulating activity of both WT and AftC-LAM. As shown in Fig. 4*C*, treatment with H_2O_2 severely reduced the activity of WT-LAM (PILAM), suggesting that the earlier observed activity was indeed due to lipopeptide contamination. In contrast, although the activity of AftC-LAM was also substantially reduced, a stable part of the activity was insensitive to H_2O_2 treatment (Fig. 4*C*). Although we cannot exclude that H_2O_2 also acts on LAM and may thereby abrogate its activity, the observation that the residual activity of AftC-LAM stabilized after 4 days of treatment, instead of gradually declining further, argues against this notion. Furthermore, H_2O_2 treatment fully inactivated the lipopeptide Pam_3CSK_4, demonstrating that inactivated lipopeptides do not display any residual activity (Fig. 4*C*). Overall, these data strongly suggest that the proinflammatory activity observed in some PILAM preparations was indeed the result of contaminating lipopeptides. Nevertheless, on the basis of our experiments, we conclude that truncation of the arabinan domain by the *aftC* mutation resulted in an increased proinflammatory activity of AftC-LAM.

Materials and Methods

Bacterial Strains and Growth Conditions. *M. smegmatis* was grown in tryptic soy broth (Difco) containing 0.005% Tween 80 (TSBT); solid media included 1.5% agar. Selective media contained 100 $\mu g \cdot mL^{-1}$ hygromycin or 20 $\mu g \cdot mL^{-1}$ kanamycin. Generation of *M. smegmatis* Δ*aftC* mutants and complementants was previously reported (11). Minimal inhibitory concentrations were determined on solid media with various concentrations of EMB and defined as the lowest EMB concentration inhibiting 100% of growth after 5 days (8). *M. tuberculosis* H37Rv DNA was obtained from the National Institutes of Health Tuberculosis Research Materials and Vaccine Testing Contract at Colorado State University. All chemicals (unless specified) were from Sigma-Aldrich.

Extraction and Purification of Lipoglycans. Lipoglycans were extracted as described previously (20, 41). Briefly, dried cells were resuspended in water and disrupted by sonication; an equal volume of ethanol was added and the mixture refluxed, followed by centrifugation and recovery of the supernatant. This extraction process was repeated five times and the combined supernatants were dried, subjected to hot-phenol treatment, and dialyzed against water. The retentate was dried, resuspended in water, and digested with α-amylase, DNase, RNase, chymotrypsin, and trypsin. The retentate was dialyzed and subjected to hydrophobic and gel exclusion chromatography (20, 41). Eluates were collected, dialyzed, concentrated, and analyzed by SDS–PAGE using Pro-Q emerald glycoprotein stain (Invitrogen).

Glycosyl Composition and Linkage Analysis of WT-LAM and AftC-LAM by NMR Spectroscopic Analysis and GC/MS. The glycosidic linkage profile of WT-LAM and AftC-LAM was determined as described (11, 41). Full details of WT-LAM and AftC-LAM NMR and GC/MS analysis are provided in *SI Materials and Methods*.

Treatment of WT-LAM, AftC-LAM, and Pam_3CSK_4 with H_2O_2. WT-LAM, AftC-LAM, and Pam_3CSK_4 (Invitrogen) were treated with H_2O_2 as described (30, 31). In short, WT-LAM and AftC-LAM (0.2 $mg \cdot mL^{-1}$) and Pam_3CysK_4 (1 $\mu g \cdot mL^{-1}$) were incubated in the dark at 4 °C in the absence or presence of 1% H_2O_2. After incubation for various periods of time, the samples were snap-frozen using liquid nitrogen and lyophilized.

Cell Culture. THP-1 cells were grown (37 °C, 5% CO_2) in RPMI 1640 (Lonza) with 10% FCS (Lonza). HEK293 cells transfected with TLR2 (42) were kept in DMEM (Invitrogen) containing 10% FCS, 100 units·ml^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 0.5 mg·mL^{-1} G418, 2 mM L-glutamine, and 110 mg·L^{-1} pyruvate.

Cell Stimulation Assays. Cells (HEK293 cells were first released by trypsinization) were washed with and resuspended in culture medium at 1.25×10^6 cells·mL^{-1}. A total of 80 μL (1×10^5 cells) was transferred to a 96-well U-bottom plate (Greiner) and left for 2 h, followed by incubations in triplicate with H_2O_2-treated WT-LAM, AftC-LAM, and (Pam_3CSK_4 or with LPS [from *Salmonella enterica* serovar Abortusequi (Sigma-Aldrich L5886)] in a final stimulation volume of 100 μL. Unstimulated cells served as controls. Culture supernatants were harvested after 24 h by centrifugation and stored at −80 °C for cytokine measurements using ELISA.

Cytokine ELISA. Human IL-8 and TNF-α concentrations were determined in ELISA according to the manufacturer's instructions (Invitrogen and R&D Systems, respectively).

DC-SIGN–Fc ELISA. WT-LAM and AftC-LAM [1 μg·mL^{-1} in saline (100 μL)] were coated on NUNC Maxisorp plates overnight at 4 °C. Plates were blocked with 1% bovine serum albumin, and serially diluted DC-SIGN–Fc (43) was added for 2 h at room temperature. Then the plates were washed four times with Tris-buffered saline with 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.05% Tween-80 (TSMT) and incubated with goat-anti-human IgG antibody conjugated with peroxidase (Jackson Immunoresearch). Plates were washed eight times with TSMT and developed using *o*-phenylenediamine dihydrochloride and absorption measured at 490 nm.

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