Host- and age-dependent transcriptional changes in *Mycobacterium tuberculosis* cell envelope biosynthesis genes after exposure to human alveolar lining fluid

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Running Title: Human lung mucosa and *M. tuberculosis* infection

Keywords: *Mycobacterium tuberculosis*, alveolar lining fluid (ALF), lung mucosa, cell envelope biosynthesis, gene expression

Abstract

Tuberculosis (TB) infection, caused by the airborne pathogen *Mycobacterium tuberculosis* (*M.tb*), resulted in almost 1.4 million deaths in 2019 and the number of deaths is predicted to increase by 20% over the next 5 years due to the COVID-19 pandemic. Upon reaching the alveolar space, *M.tb* comes in close contact with the lung mucosa before and after its encounter with host alveolar compartment cells. Our previous studies show that homeostatic innate soluble components of the alveolar lining fluid (ALF) can quickly alter the cell envelope surface of *M.tb* upon contact, defining subsequent *M.tb*-host cell interactions and infection outcomes *in vitro* and *in vivo*. We also demonstrated that ALF from 60+ year old elders (E-ALF) *vs.* healthy 18- to 45-year-old adults (A-ALF) is dysfunctional with loss of homeostatic capacity and impaired innate soluble responses linked to high local oxidative stress. In this study, a targeted transcriptional assay demonstrates that *M.tb* exposure to human ALF alters the expression of its cell envelope genes. Further, our results indicate that A-ALF-exposed *M.tb* upregulates cell envelope genes associated with lipid, carbohydrate, and amino acid metabolism, as well as genes associated with redox homeostasis and transcriptional regulators. Conversely, *M.tb* exposure to E-ALF shows lesser transcriptional response, with most of the *M.tb* genes unchanged or downregulated. Overall, this study indicates that *M.tb* responds and adapts to the lung alveolar environment upon contact, and that the host ALF status determined by factors such as age might play an important role in determining infection outcome.

INTRODUCTION

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis (TB), is one of the top leading causes of mortality worldwide due to a single infectious agent, with ~1.4 million attributed deaths in 2019 [1]. However, global estimates indicate that worldwide disruptions in the healthcare system during the current COVID-19 pandemic could lead to an additional 6.3 million new TB cases between 2020 and 2025 and an added 1.4 million more TB deaths [2, 3]. Strict lockdowns have prevented patients from having access to TB medications and clinical evaluations, and have led to decreased TB diagnosis rates, since available resources have been redirected to prevent the spread of COVID-19 [4, 5]. These factors are predicted to cause not only an increase in the number of TB cases, but also to promote the development of drug-resistant TB, stressing the need for the development of new anti-TB therapies [6].

Most current drugs target the *M.tb* cell envelope, a highly complex and dynamic structure comprised mainly of carbohydrates and lipids, which provide structural support and resistance to osmotic changes, as well as a critical immunoregulatory role during *M.tb* infection [7-10]. It consists of four main layers: 1] an inner plasma membrane with periplasmic space; 2] a peptidoglycan (PG) core covalently linked to arabinogalactan (AG) and mycolic acids (MAcs); 3] a peripheral layer of non-covalently linked lipids, glycolipids, and lipoglycans [*e.g.* phthiocerol dimycocerosates (PDIMs), trehalose dimycolate (TDM) and monomycolate (TMM), sulfolipids (SLs), phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM), and mannose-capped lipoarabinomannan (ManLAM), among others], and; 4) the outermost layer or capsule [11, 12]. The pathogenesis of *M.tb* is inherently linked to its heterogeneous and dynamic cell envelope surface, and cell envelope remodeling has been observed during infection and in response to

70 environmental stresses [10]. Thus, the particular cell envelope composition of a mycobacterial cell
71 at a given moment will define *M.tb*-host cell interactions and determine the infection outcome.
72 However, it remains poorly understood how the *M.tb* cell envelope changes and adapts to the host
73 lung environment during the natural course of pulmonary infection, a critical gap in our knowledge
74 for defining new drug targets against *M.tb* relevant to bacteria in the lung environment.
75
76 After the host inhales droplets containing *M.tb*, the airborne pathogen is deposited in the lung
77 alveolar space. Here it first comes in contact with soluble components of the alveolar lining fluid
78 (ALF) for an undefined period of time (from minutes to hours/days) [13-16], before and after its
79 encounter with host alveolar compartment cells such as alveolar macrophages (AMs) or alveolar
80 epithelial cells (ATs), and immune cells such as neutrophils [17, 18]. Our previous studies have
81 demonstrated that homeostatic ALF hydrolytic enzymes, whose function is to promote lung health,
82 can modify the *M.tb* cell envelope without reducing *M.tb* viability [19, 20], including a reduction
83 in major *M.tb* virulent factors mannose-capped lipoarabinomannan (ManLAM) and trehalose
84 dimycolate (TDM), from the *M.tb* cell surface. These ALF-derived *M.tb* cell envelope
85 modifications have an impact on *M.tb* infection outcomes *in vitro* and *in vivo*, since they allow for
86 a better recognition by cells of the immune system and improved control of the infection [19-23].
87 Indeed, exposure to ALF results in decreased *M.tb* association and intracellular growth within
88 human macrophages, as well as altered intracellular trafficking and increased pro-inflammatory
89 responses [19]. Neutrophils also possess an enhanced innate ability to recognize and kill
90 intracellular ALF-exposed *M.tb*, while limiting excessive inflammatory responses [21]. *In vivo*
91 infections using ALF-exposed *M.tb* demonstrate better control of infection in the mouse model
92 [22]. Further, *M.tb* fragments released after ALF exposure by the action of the ALF hydrolases,

93 are capable of priming neutrophils and modulating macrophages in an IL-10 dependent manner to
94 contribute further to the control of *M.tb* [20, 23].
95
96 Importantly, the levels and functionality of ALF soluble components are altered in certain human
97 populations such as the elderly, with increased pro-oxidation and pro-inflammatory pathways,
98 altered complement and surfactant levels, and decreased binding capability of surfactant protein
99 A (SP-A) and D (SP-D) in the aging lung, defining what we call 'dysfunctional ALF'.
100 Consequently, *M.tb* exposed to elderly dysfunctional ALF, while maintaining its viability, show
101 increased intracellular growth in macrophages and ATs, as well as increased bacterial burden in
102 mice with increased lung tissue damage [22, 24, 25]. Altogether, these results indicate that the host
103 ALF functional status plays a key role in shaping the *M.tb* cell envelope during the initial stages
104 of infection. However, the overall impact of these different ALF microenvironments on *M.tb*
105 adaptation to the human host and subsequent infection progression is still largely unknown.
106
107 Since hydrolytic enzymes present in functional human ALF (from healthy adult individuals)
108 modify the *M.tb* cell envelope [19], we hypothesize that *M.tb* will compensate for these ALF-
109 driven modifications by altering the expression of genes related to cell envelope biogenesis.
110 Conversely, *M.tb* exposed to ALF with decreased functionality (such as elderly ALF) will show
111 little to no changes. In addition, our published data [20, 26] demonstrate that 15-minute exposure
112 to human ALF is enough to alter the *M.tb* cell envelope and its interactions with host cells and that
113 these modifications are maintained for up to 24 h [20, 26]. In this study, we first aim to determine
114 if short (15 min) or long (12 h) exposure to human ALF has any effects on the expression of
115 targeted *M.tb* cell envelope genes, indicating that gene expression is altered at 15 min and that

116 these changes are maintained up to 12 h. Then, we use a multiplex qPCR assay to compare an
117 extensive transcriptional profile of *M.tb* cell envelope genes associated with lipid, carbohydrate
118 and amino acid metabolism, among others, after exposure to functional healthy adult (A)- *vs.*
119 dysfunctional healthy elderly (E)-ALF. Our results show significant differences in gene
120 expression, where A-ALF exposed *M.tb* upregulated genes involved in cell envelope remodeling,
121 thus implicating this remodeling in subsequent *M.tb*-host interactions during the infection process.
122
123
124 **MATERIALS AND METHODS**
125 **Human subjects and ethics statement** - Human ALFs used in this study were previously isolated
126 from collected bronchoalveolar lavage fluid (BALF) from healthy adult and elderly volunteers, in
127 strict accordance with the US Code of Federal and approved Local Regulations (The Ohio State
128 University Human Subjects IRB numbers 2012H0135 & 2008H0119 and Texas Biomedical
129 Research Institute/UT-Health San Antonio/South Texas Veterans Health Care System Human
130 Subjects IRB numbers HSC20170667H & HSC20170673H), and Good Clinical Practice as
131 approved by the National Institutes of Health (NIAID/DMID branch), with written informed
132 consent from all human subjects. Healthy adults (18-45 years old) and elderly (+60 years) were
133 recruited from both sexes and no discrimination of race or ethnicity.
134
135 **Collection of BALF and ALF** - BALF was collected from healthy adult or elderly donors in
136 sterile endotoxin free 0.9% of NaCl, filtered through 0.2 µm filters, and further concentrated 20-
137 fold using Amicon Ultra Centrifugal Filter Units with a 10-kDa molecular mass cut-off membrane
138 (Millipore Sigma) at 4°C to obtain ALF with a physiological concentration reported within the

139 human lung (1 mg/mL of phospholipid), as we previously described [19-24, 27]. ALF was
140 aliquoted in low-protein binding tubes and stored at -80°C until further use.
141
142 **Bacterial cultures and ALF exposure** - *M.tb* strains GFP-Erdman (kindly provided by Dr.
143 Marcus Horwitz, UCLA) and $H_{37}R_v$ (ATCC# 25618) were cultured in 7H11 agar plates (BD BBL),
144 supplemented with oleic acid, albumin, dextrose and catalase (OADC) at 37°C for 14 days. Single
145 bacterial suspensions (~$1x10^9$ bacteria/ml) were obtained as previously described [19-23].
146 Bacterial pellets were exposed to individual ALFs (from different donors) for 15 min or ~12 h at
147 37°C. After exposure, ALF was removed and bacterial pellets were directly incubated in
148 RNAProtect (Qiagen) for 10 min at room temperature (RT), centrifuged at 13,000 x *g* and stored
149 at -80°C until further use. For each of the ALFs, corresponding heat-inactivated ALFs (2 h at 80°C)
150 [21] were used as controls in parallel.
151
152 **RNA extraction and cDNA synthesis** - RNA from ALF- exposed bacterial pellets was extracted
153 using the Quick-RNA Fungal/Bacterial Miniprep kit (Zymo Research), following the
154 manufacturer's protocol. Briefly, bacterial pellets were resuspended in Lysis buffer and transferred
155 to a ZR BashingBead Lysis tube containing 0.1 mm and 0.5 mm ceramic beads. Bead beating
156 procedure was performed to break the tough-to-lyse mycobacterial cell envelope in a Disruptor
157 Genie [10 cycles of 1 min at maximum speed with 1 min intervals on ice]. RNA was isolated from
158 the supernatant using Zymo-spin columns, including an in-column DNAse I treatment, and eluted
159 in nuclease-free water. To completely remove the genomic DNA, a second DNAse treatment was
160 performed on the isolated RNA using TURBO DNAse reagent (Thermo Fisher Scientific) for 30
161 min at 37°C. Final RNA concentration and quality were measured with a Qubit 4 Fluorometer

162 using the HS RNA kit (Thermo Fisher Scientific), and a Nanodrop One^{C}, respectively. RNA (500
163 ng) was used for the synthesis of cDNA using the RevertAid H Minus First Strand cDNA Synthesis
164 kit (Thermo Fisher Scientific) with random hexamer primers, following the manufacturer's
165 guidelines.
166
167 **qPCR analysis of targeted genes** - Real-time quantitative PCR (qPCR) was performed to measure
168 the expression of ten genes associated with the *M.tb* cell wall biosynthesis pathways (*pmmA,*
169 *manB, whiB2, pimA, pimB, pimF, embC, pmmB, pmm1, manA*) after exposure of *M.tb* $H_{37}R_v$ to
170 healthy human ALF (n=2 biological replicates, from two different donors). cDNA and primers
171 were used in a 20 µl qPCR reaction with PowerUp SYBR Green Master Mix (Applied
172 Biosystems), following manufacturer's instructions. Reactions were run in an Applied Biosystems
173 7500 Real-Time PCR instrument with the following settings: reporter SYBR Green, no quencher,
174 passive reference dye ROX, standard ramp speed, and continuous melt curve ramp increment.
175 Expression was calculated relative to housekeeping genes *rpoB* or *sigA* using the $2^{-\Delta\Delta CT}$ method
176 [28].
177
178 **High-throughput multiplex qPCR** - Primer pairs for multiplex qPCR assay were designed using
179 BatchPrimer3 (https://wheat.pw.usda.gov/demos/BatchPrimer3/) [29] and PrimerQuest (IDT,
180 https://www.idtdna.com/pages/tools/primerquest), with the following settings: primer size 18-21
181 ntds, T_m of ~ 54-60°C, and maximum 3' self-complementarity of 3 ntd. Best primers within these
182 parameters were selected and aligned to the *M.tb* $H_{37}R_v$ reference genome (Genbank accession
183 number: NC_000962.3) [30-32] to confirm that they uniquely aligned to the targeted gene regions.
184 A high-throughput multiplex qPCR targeting more than 80 genes associated with *M.tb* metabolism

185 and cell wall biogenesis was performed in *M.tb* Erdman exposed to ALF using the Biomark 96.96
186 Dynamic Array IFC for Gene Expression in a Biomark HD instrument (Fluidigm). Briefly, 1.25
187 µl of cDNA was pre-amplified in a 5 µl reaction with our pool of specific primers (500nM) using
188 the Fluidigm Preamp Master Mix for a total of 10 cycles. Then, a 1/10 dilution of the pre-amplified
189 cDNA and 100 µM of combined forward and reverse primers were used to prepare the sample pre-
190 mix and assay mix, respectively. The 96.96 IFC chip was loaded and the assay run in a Biomark
191 HD following the manufacturer's instructions for Gene expression using Delta Gene Assays.
192 Relative expression for each of the genes was calculated in the Fluidigm Real-Time PCR Analysis
193 software using the $2^{-\Delta\Delta CT}$ method with *rpoB* as the reference gene, and reported as the $\log_2$ fold
194 change of A-ALF (n=3 biological replicates using ALFs from different donors) or E-ALF (n=3
195 biological replicates using ALFs from different donors) exposed *M.tb* Erdman compared to control
196 samples (*M.tb* Erdman exposed to corresponding heat-inactivated ALFs). As a control to compare
197 both methods (Biomark *vs.* targeted qPCR), we included *M.tb* $H_{37}R_v$ exposed to adult ALF in the
198 Biomark multiplex qPCR assay.
199
200 **Statistical analysis** - Statistical significance between the two qPCR methods used in this study
201 was calculated in GraphPad Prism v9.0.1 for each of the genes with a two-way ANOVA using the
202 Sidak's correction for multiple comparisons test with a 95% confidence interval.
203
204
205 **RESULTS**
206 **Exposure to human ALF alters the expression of cell envelope PIMs/LM/ManLAM**
207 **biosynthesis genes in *M.tb***

208 Our laboratory has previously demonstrated that ALF hydrolases modify the *M.tb* cell envelope,
209 and that these cell envelope modifications occur in as little as 15 min of *M.tb* being in contact with
210 ALF and are maintained up to 24 h, without compromising *M.tb* viability [19]. Importantly, these
211 ALF-driven alterations of the *M.tb* cell envelope had an effect on *M.tb* infection outcomes *in vitro*
212 and *in vivo* [19-23]. Based on these results, we sought to determine if *M.tb* alters its cell wall
213 biosynthetic pathways as a direct consequence of ALF exposure. Because one of the main *M.tb*
214 cell envelope components, ManLAM, is decreased by the action of ALF hydrolases [19] and PIMs,
215 LM and ManLAM are thought to be part of the same biosynthetic pathway, we first selected a few
216 key *M.tb* cell envelope genes associated with biosynthesis of initial mannose donors GDP-Man
217 [*manA/Rv3255c, manB/Rv3264c* (previously annotated as *manC*), *pmmA/ Rv3257c* (previously
218 annotated as *manB*), *pmmB/ Rv3308*] and polyprenylphosphate-based mannose or PPM
219 (*pmm1/Rv2051c*), the transcriptional regulator *whiB2/Rv3260c* [33], and mannosyl-
220 (*pimA/Rv2610c, pimB/Rv2188c, pimF/ Rv1500*) and arabynosyl- (*embC/Rv3793*) transferases (**Fig**
221 **1** and **Table 1**) [34, 35], all involved in this pathway. Expression was determined by RT-qPCR
222 from *M.tb* previously exposed to human ALF for 15 min and 12 h. Relative expression was
223 normalized to housekeeping genes *rpoB* (**Fig 2A**) or *sigA* (**Fig 2B**). *M.tb* preparations exposed to
224 the same heat-inactivated ALFs were used as reference samples.
225
226 The majority of targeted *M.tb* cell envelope genes were moderately upregulated after 15 min of
227 exposure to ALF when compared to *M.tb* exposed to heat-inactivated ALF (depicted as $\log_2$ fold
228 changes), except for *manA* that was slightly downregulated and *manB* and *pimB* that did not show
229 any changes **(Fig 2)**. Indeed, *ppm1* (polyprenol-monophosphomannose synthase), which transfers
230 mannose from GDP-mannose to endogenous PPM [36, 37] in the PIMs/LM/ManLAM

231 biosynthesis pathway, showed the highest $\log_2$ fold change (1.84 and 1.89 when using *rpoB* or
232 *sigA* as reference genes, respectively) after 15 min of exposure compared to heat-inactivated ALF,
233 although its expression was too low to be detected in our qPCR assay after 12 h (depicted as ND,
234 **Fig 2**). Similarly, *manA* was below the detection limit at 12 h and expression differences could not
235 be quantified (ND, **Fig 2**). A few of the tested genes increased their expression at 12 h post-ALF
236 exposure (*manB*, *pimB*, and *embC*) compared to heat-inactivated ALF, while others showed no
237 major transcriptional changes or were slightly downregulated.
238
239 When comparing 15 min *vs.* 12 h post-ALF exposure, some PIMs/LM/ManLAM biosynthesis-
240 associated genes decreased their expression (*pmmA, whiB2, pimF, pimA*) while others increased
241 (*manB, pimB, embC*) (**Fig 2** and **Table 2**). This suggests a temporal and dynamic adaptation of
242 *M.tb*'s cell envelope components in response to ALF, in agreement with previous reports showing
243 cell envelope remodeling of *M.tb* during infection and in response to different environmental
244 conditions [33, 34, 38-43]. *pmmB* expression was too low to be detected by our qPCR assay at
245 either timepoints tested, thus no $\log_2$ fold changes are shown.
246
247 **Effects of A- *vs.* E-ALF exposure on the expression of cell envelope biosynthesis genes in *M.tb***
248 Since we observed changes in the expression of key *M.tb* cell envelope genes in response to ALF,
249 and based on our previous publications showing that ALF status influences *M.tb*-host interactions
250 via multiple factors, including age [22, 24], we next sought to determine if contact with different
251 host ALFs would result in different transcriptional profiles of the *M.tb* cell envelope. We exposed
252 *M.tb* Erdman to individual A-ALFs or E-ALFs for 12 h as described in our previous work [19],
253 and calculated the relative expression of 83 genes related to cell envelope biosynthesis using a

254 multiplex qPCR assay in a Biomark HD platform [44]. Results from some of the genes were
255 compared to the previous targeted qPCR assay, showing concordance in gene expression between
256 the two qPCR methods used in this study (**Supplemental Figure 1**). Small differences observed
257 could be attributed to inherent variability within human ALF, since we used different human
258 donors.
259
260 Genes selected for the multiplex qPCR are related to the biosynthesis of key *M.tb* cell envelope
261 lipid and carbohydrate components (**Supplemental Table 1**). For lipid metabolism, we targeted
262 genes from the following pathways: fatty acid metabolism, glycerolipid and glycerophospholipid
263 metabolism, phosphatidylinositol (PI, precursor for more complex glycolipids such as PIM and
264 LAM), PIMs/LM/ManLAM biosynthesis, mycolic acid biosynthesis, and linoleic acid metabolism
265 [9]. For carbohydrate metabolism, we included genes related to: carbohydrate biosynthesis,
266 glycolysis and gluconeogenesis, mannose and fructose metabolism, galactose metabolism, citrate
267 cycle or TCA, glyoxylate and dicarboxylate metabolism, and inositol phosphate metabolism [45].
268 Gene names, with corresponding function, pathway and functional categories, as well as primer
269 sequences are listed in **Supplemental Table 1**.
270
271 As shown in **Fig 3A**, most of the cell envelope genes associated with lipid metabolism from the
272 different *M.tb*-targeted pathways were significantly upregulated in *M.tb* exposed to A-ALFs (A_1
273 to A_3, three different adult donors) when compared to the same *M.tb* strain exposed to E-ALFs (E_1
274 to E_3, three different elderly donors), with A_1-ALF having the highest $\log_2$ fold changes (**Fig 3A**
275 and **Supplemental Table 2**). Only a few genes were downregulated in *M.tb* exposed to A-ALFs,
276 including *fabH* and *fadD25* (FA metabolism, the latter involved in lipid degradation), and *adhE1*,

277 *glpK*, and *cdsA* (glycerolipid and glycerophospholipid synthesis). ATP-binding cassette
278 transporter Rv1747, thought to be involved in the export of lipooligosaccharides (LOS) through
279 the mycobacterial membrane, was also downregulated in A-ALF-exposed *M.tb*, whereas negative
280 regulator of Rv1747, named Rv2623 [46], showed increased expression (**Fig. 3A**). Interestingly,
281 *M.tb* exposed to two of the E-ALFs showed upregulation of Rv1747 (**Fig. 3A**), contrary to A-ALF
282 exposure, indicating a potential increase in *M.tb* PIM export after exposure to elderly ALF. All
283 genes involved in mycolic acid synthesis tested in this study were upregulated in *M.tb* exposed to
284 A-ALF. Finally, exposure to E-ALFs showed lesser effects on the overall expression of *M.tb* lipid
285 metabolism when compared to A-ALFs, with several genes showing moderate upregulation
286 (especially in E_1-ALF, which showed the highest upregulation among the elders for most of the
287 genes), and others with no effects or even decreased expression (**Fig. 3A**).
288
289 A similar trend was observed for carbohydrate metabolism genes (**Fig. 3B**), where most of the
290 genes from the different pathways studied showed significant increased expression in *M.tb*
291 exposed to A-ALFs when compared to E-ALFs. Particularly, 8 out of 12 genes associated with
292 fructose metabolism and the mannose donor (GDP-Man/PPM) biosynthesis pathways were highly
293 expressed in A-ALF-exposed *M.tb*, with only 2 genes downregulated (*pmmA*, which converts D-
294 mannose 1-phosphate in D-mannose 6-phosphate, and *pfkA*, also a key enzyme involved in
295 glycolysis) [47], and 2 other genes with variable expression across different A-ALF-exposed *M.tb*
296 (*ppm1* and *fbA*) (**Fig 3B**). Galactose metabolism genes (*galK, galT, galU*, and *aglA*) essential for
297 the biosynthesis of the cell envelope galactan core [48], were also upregulated after exposure to
298 A-ALFs, while no changes or even decreased expression were observed in *M.tb* exposed to E-
299 ALFs. Interestingly, predicted alpha-glucosidase *aglA* was significantly increased after exposure

300 to E_3-ALF (**Fig 3B**). Further, all tricarboxylic acid cycle (TCA)-associated genes were upregulated
301 by A-ALF exposure, with the exception of *sdhC* (membrane-anchored subunit of Sdh2, implicated
302 in *M.tb* growth linked to the TCA cycle under hypoxia conditions) [49] that was downregulated
303 upon both A- and E-ALF *M.tb* contact. Glyoxylate and dicarboxylate genes were decreased in E-
304 ALFs when compared to A-ALFs-exposed *M.tb*, whereas the only inositol phosphate gene tested
305 *ino1*, catalyzing the first step in inositol synthesis for the production of major thiols and cell wall
306 lipoglycans [50], had decreased expression after *M.tb* exposure to all ALFs tested, except for E_2-
307 ALF (**Fig 3B**).
308
309 In addition to lipid and carbohydrate pathways, we studied genes belonging to other categories
310 such as amino acid metabolism, redox homeostasis, and transcriptional regulators (**Fig. 3C** and
311 **Supplemental Table 1**). Results indicate that *M.tb* exposed to A-ALFs have major transcriptional
312 changes compared to *M.tb* exposed to E-ALFs. Indeed, all genes associated with amino acid
313 metabolism were highly upregulated in *M.tb* exposed to A-ALFs, except for *serC* (serine
314 metabolism, **Fig 3C**) [51]. Other cell wall-associated genes were upregulated in *M.tb* exposed to
315 A-ALFs including the putative membrane protein EccB3, part of the ESX-3 secretion system,
316 important for zinc and iron uptake and homeostasis [52], and the cutinase precursor Cfp21, a
317 lipolytic enzyme with immunogenic properties shown to elicit T and B cell responses [53, 54].
318 Similarly, genes *ahpC* and *sodC*, involved in the oxidative stress response [55, 56], and
319 transcriptional regulators *dosR* and *phoP* had significantly increased expression in *M.tb* exposed
320 to A-ALFs, whereas *virS* [57] was not significantly increased. Overall, our results demonstrate that
321 exposure to functional A-ALF results in broad changes in *M.tb* cell envelope biosynthesis,
322 suggesting highly dynamic cell envelope changes with constant remodeling within the lung

323 alveolar environment, whereas *M.tb* exposed to dysfunctional E-ALF [22, 24, 25] showed more
324 limited effects.
325
326
327 **DISCUSSION**
328 The cell envelope of *M.tb* is mainly composed of lipids and carbohydrates, and constitutes a
329 dynamic structure known to adapt to the changing local host environment, especially during
330 different stages of infection [8, 10]. Human ALF contains hydrolases whose homeostatic function
331 is to maintain lung health. We have demonstrated that healthy adult individuals have up to 17
332 hydrolase activities capable of altering the *M.tb* cell wall. Indeed, exposure of *M.tb* to these adult
333 ALFs significantly alters the *M.tb* cell wall reducing the content of two major cell envelope
334 components, ManLAM and TDM, without compromising *M.tb* viability [19]. Importantly, these
335 hydrolase activities are decreased in ALFs from healthy elders [24]. Thus, here, we demonstrate
336 for the first time that exposure to human ALF, the first environment encountered by *M.tb* upon
337 infection, results in transcriptional changes in key *M.tb* cell envelope biogenesis genes. Further,
338 exposure to healthy A-ALFs resulted in increased expression of most of the *M.tb* cell envelope-
339 associated genes from lipid, carbohydrate and amino acid metabolic pathways, among others. In
340 contrast, *M.tb* exposed to E-ALFs from healthy elderly donors, which we demonstrated constitutes
341 a more oxidized, pro-inflammatory and dysfunctional environment [22, 24], did not show many
342 significant changes in gene expression.
343
344 We first assessed essential genes from the PIMs/LM/ManLAM synthesis and the mannose
345 metabolism pathway (carbohydrate metabolism) (**Figs 1** and **2**), since our previous studies showed

346 a decrease of ManLAM in the cell envelope of *M.tb* after ALF exposure [19]. PIM/LM/ManLAM
347 molecules are essential to regulate *M.tb* recognition, uptake, survival and modulate the host
348 immune response [58, 59]. Indeed, ManLAM has been shown to block phagosome-lysosome (P-
349 L) fusion by inhibiting the Ca^{2+}/Calmodulin phosphatidyl-inositol-3-kinase (PI3K) hvps34
350 pathway, promoting *M.tb* intracellular survival [60-62]. The biosynthesis of these molecules
351 depends on mannose donors such as GPD-Man and PPM. Previous studies suggest that mannose
352 donor levels are altered during the course of the infection. Indeed, *M.tb* mannose donor
353 biosynthesis genes had increased expression levels 2 h after macrophage infection and then
354 gradually decreased [33]. These were also found upregulated in an *in vitro* granuloma model [34].
355
356 In our study, most of the genes involved in the PIM/LM/ManLAM biosynthesis pathway were
357 upregulated after exposure to A-ALFs (**Figs 3A** and **3B)**. We speculate that *M.tb* is trying to
358 compensate for the loss of ManLAM and other mannose-containing cell envelope surface
359 components due to the action of A-ALF hydrolases [19], with major implications for disease
360 progression. In this regard, bacilli exposed to functional A-ALF might get taken up by antigen-
361 presenting cells (APCs) before they can reconstitute ManLAM and will be cleared, while bacilli
362 that rapidly upregulate and replenish ManLAM on the cell surface before its encounter with APCs
363 will be able to block P-L fusion and survive withing the host cells. Conversely, exposure to E-
364 ALFs did not have major transcriptional effects in genes from both mannose donors and
365 PIM/LM/ManLAM biosynthesis pathways (**Figs 3A** and **3B)**, likely because E-ALF have less
366 hydrolase activities [24]. This E-ALF deficiency in hydrolase activities could be directly linked to
367 E-ALF being a highly oxidative environment [22, 24], therefore impacting the *M.tb* cell envelope
368 and subsequent remodeling to a much lesser degree during infection. Since removal of surface

369 lipids in *M.tb* enhances trafficking to acidic compartments [61], fewer ALF-driven alterations after
370 E-ALF exposure might partially explain why *M.tb* replicates faster in the elderly lung environment
371 by residing in its protective intracellular niche [22, 25]. Further studies will be necessary to
372 determine the specific impact of elderly ALF hydrolases on the *M.tb* cell envelope.
373
374 Most lipid metabolism genes associated with mycolic acid synthesis, fatty acid metabolism,
375 glycerolipid and glycerophospholipid synthesis, and linoleic acid metabolism were also
376 significantly upregulated in A-ALF compared to E-ALF-exposed *M.tb* (**Fig 3A**). A similar trend
377 was observed for carbohydrate metabolism, amino acid metabolism, and genes involved in redox
378 homeostasis, stress response, and transcriptional regulation (**Figs 3B** and **3C**). Nitrogen and amino
379 acid metabolism are important for *M.tb* pathogenesis and host colonization during infection, where
380 intracellular bacteria exploit host nitrogen sources for growth and replication [63, 64]. Importantly,
381 amino acids acquired from the host such as Ala and Gly might be directly assimilated for the
382 synthesis of cell wall biomass and incorporated into the PG layer [65-67]. Transcriptional
383 regulators DosR and PhoP also showed increased expression upon contact with functional A-ALF.
384 These proteins are part of two-component systems implicated in a large number of *M.tb* adaptive
385 responses, such as low oxygen levels during dormancy and persistence in the granuloma
386 environment [68, 69], but they also play key roles as regulators of *M.tb* virulence [70, 71].
387 Interestingly, DosR has been shown to play a role in lipid accumulation during oxidative stress
388 and iron starvation in certain *M.tb* clinical strains [72, 73].
389
390 These results indicate that the *M.tb* response to the ALF environment is not only limited to *M.tb*
391 cell envelope remodeling, but also potentially affects overall *M.tb* metabolism and virulence [19-

392 25, 27], with major implications in infection progression and TB disease outcome. Further global
393 transcriptomic studies will be able to provide clues as to which other metabolic pathways are
394 altered by the human ALF environment, with the potential to decipher key upregulated bacterial
395 determinants during early stages of infection that can be targeted for the development of new
396 preventative and therapeutic strategies [74]. Indeed, *M.tb* is exposed to ALF during the first
397 infection stages upon deposition to the alveolar space, but also when escaping from necrotic cells
398 or in cavities during active TB episodes leading to transmission [17]. Thus, it is plausible that *M.tb*
399 adapts its cell envelope to the alveolar environment, upregulating the expression of specific genes
400 to compensate the changes generated upon contact with ALF hydrolases and thus, determining
401 interactions with host cells. In this regard, timing is expected to be important, as *M.tb* bacilli not
402 able to restore its cell wall constitution before encountering antigen presenting cells such as
403 alveolar macrophages might be cleared [19-21].
404
405 Key genes such as Rv1747, thought to participate in the export of PIMs to the cell envelope through
406 negative regulation by stress protein Rv2623 [46], were highly downregulated in *M.tb* exposed to
407 A-ALFs. Indeed, an ΔRv1747 mutant showed decreased levels of PIMs and was growth-
408 attenuated, while ΔRv2623 had enhanced PIM expression and was hypervirulent in mice [75, 76].
409 Our data suggest that contact with functional A-ALF reduces the expression of PIM transporter
410 Rv1747 through increased expression of Rv2623, modulating the export of immunomodulatory
411 PIMs and potentially influencing bacterial growth and virulence.
412
413 Taken together, these results suggest that *M.tb* compensates for the loss of cell surface components
414 (due to the action of ALF hydrolases) by upregulating and activating different cell envelope

415 biosynthesis pathways to rebuild its cell wall, at the detriment of downregulating some key genes
416 (*e.g*. Rv1747) involved in the transport of cell envelope components to the surface [19-21, 23, 46].
417 This shift between ALF innate homeostatic mechanisms and *M.tb* countermeasures in the ALF
418 microenvironment, dependent on the host ALF status (A-AF *vs.* E-ALF), will likely determine
419 subsequent interactions between *M.tb* and host cells, as well as intracellular trafficking and
420 infection outcomes [19-21]. Further studies are needed to provide better insight to why E-ALF-
421 exposed *M.tb* replicates faster than A-ALF-exposed *M.tb* in both professional and non-professional
422 phagocytes, and to explain why E-ALF status in old age enhances *M.tb* infection *in vitro* and *in*
423 *vivo* [22, 24, 25] and contributes to elders being more susceptible to respiratory infections in
424 general. Finally, it is important to consider that the cell envelope composition of *M.tb* is strain-
425 specific, with differences observed in *M.tb* strains from different lineages, and thus, different ALF-
426 driven alterations in *M.tb* metabolism may drive different infection progression [77].
427
428 In summary, our study provides evidence that *M.tb* contact within the ALF shapes the composition
429 of its cell envelope which, depending on the ALF status ('functional A-ALF' *vs.* 'dysfunctional E-
430 ALF') [22, 24, 25], is likely to define subsequent *M.tb*-host cell interactions. Indeed, E-ALF-
431 exposed *M.tb* presented minimal transcriptional changes when compared to A-ALF-exposed *M.tb,*
432 which we speculate provides a fitness advantage to *M.tb* as its cell wall remains intact, and thus, it
433 has the energy reserves required to efficiently infect and replicate faster within host cells of elderly
434 individuals. In contrast, *M.tb* undergoes significant alterations on its cell wall (significant loss of
435 virulent factors ManLAM and TDM, among others) upon exposure to A-ALF [19]. This triggers
436 a greater transcriptional change that we interpret as efforts of A-ALF-exposed *M.tb* to reprogram
437 its metabolism to quickly rebuild its cell wall, specifically upregulating genes involved in the

438 biosynthesis of *M.tb* virulent factors such as ManLAM, with the ensuing energy requirements.
439 This can be detrimental for A-ALF-exposed *M.tb* and favor host cells to control infection better in
440 adult individuals. Future studies will investigate the metabolic status of E-ALF-exposed *M.tb* after
441 infection of professional and non-professional phagocytes *in vitro* and *in vivo*, and will correlate
442 bacterial and host determinants associated with increased susceptibility to infection in old age.
443
444
445 **CONFLICT OF INTEREST**
446 The authors declare no conflict of interest.
447
448 **AUTHORS CONTRIBUTIONS**
449 AAG performed experiments and analyzed data. AGV analyzed data. AOF processed BAL
450 samples. JP and DJM performed the bronchoalveolar lavage procedures in humans. AAG and JBT
451 conceptually developed the study and wrote the manuscript. JT and LSS provided critical analysis
452 of the data and editing of the manuscript. JT, LSS, and JBT provided funding. All authors read and
453 approved the final version of this manuscript.
454
455 **FUNDING**
456 This study was supported by the National Institute on Aging (NIA) of the National Institutes of
457 Health (NIH) under Award Number P01AG051428 to JT, LSS, and JBT. JBT was partially
458 supported by the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation. The content is solely
459 the responsibility of the authors and does not necessarily represent the official views of the
460 National Institutes of Health.

461

462 **ACKNOWLEDGEMENTS**

463 We want to thank Sean Vargas from the University of Texas San Antonio (UTSA) Genomics Core

464 for his assistance with the multiplex qPCR assay (Biomark HD).

465

466 **TABLES**

467 **Table 1. List of primers targeting the PIMs/LM/ManLAM biosynthesis pathways in *M.tb*.**

Locus tag $H_{37}R_v$	Gene name	Product and Function	Primer sequence (5′ to 3′)	Tm (°C)	Ref.
Rv0667	*rpoB*	Reference gene	rpoB-F: CCTGGAAGAGGTGCTCTACG	60	[33]
			rpoB-R: GGGAAGTCACCCATGAACAC	60	[33]
Rv2703	*sigA*	Reference gene	sigA-F: CTCGGTTCGCGCCTACCTCA	68	[35]
			sigA-R: GCGCTCGCTAAGCTCGGTCA	68	[35]
Rv3255c	*manA*	mannose-6-phosphate isomerase / GDP-Man biosynthesis: formation of Man-6-P	manA-F: GTTCACCACCTGGATTACCG	60	[33]
			manA-R: AACCCTCGGTGCATAACAAG	60	[34]
Rv3264c	*manB*[a]	D-alpha-D-mannose-1-phosphate guanylyltransferase / GDP-Man biosynthesis: formation of GDP-Man	manB-F: ACATCGCCGTTAAACACCAT	60	[34]
			manB-R: GTTCCTCACCCATCTGCTGT	60	[34]
Rv3257c	*pmmA*[b]	phosphomannose mutase / GDP-Man biosynthesis: formation of Man-1-P	pmmA-F: GATCACGTTGTGGATGATGG	60	[33]
			pmmA-R: GTGGATCTGCAGGCCTATGT	60	[34]
Rv3308	*pmmB*	phosphomannose mutase / GDP-Man biosynthesis: formation of Man-1-P	pmmB-F: ATACAGATCACGGCGTCACA	60	[34]
			pmmB-R: CGCTGGATATAACGGTCGAT	60	[34]
Rv2051c	*ppm1*	Polyprenol-monophosphomannose synthase / PPM biosynthesis	pmm1-F: TGGTTGAAGTCGATCCTTCC	60	[33]
			pmm1-R: GCGAACAAGACCAGGCATATG	63	[33]
Rv3260c	*whiB2*	Transcript. regulatory protein	whiB2-F: CCATTCGAGGAACCTCTGC	61	[33]
			whiB2-R: CAGGGCGTACTCCAGACACT	60	[33]
Rv2610c	*pimA*	alpha-(1-2)-phosphatidylinositol mannosyl-transferase / PIM biosynthesis (1st step)	pimA-F: CCGCACTGCCTGATTACTTT	60	[34]
			pimA-R: CGGCTCGTGTAGATGCAGTA	60	[34]

Rv2188c	*pimB*	alpha-(1-6)-phosphatidylinositol mannosyl- transferase / PIM biosynthesis (2nd step)	pimB-F: CTCGGTGGTCAAGGTACTCG	61	[34]
			pimB-R: GTGGTCACCTTTGGGAACAT	60	[34]
Rv1500	*pimF*	glycosyltransferase / LM/ManLAM biosynthesis	pimF-F: CGCCGACGTAGTATTTGGTT	60	[34]
			pimF-R: TGCGTACATAGTCGGCTGTC	60	[34]
Rv3793	*embC*	Arabynosyl-tranferase / ManLAM biosynthesis	embC-F: ATCACCGAGCTGCTGATG	58	[35]
			embC-R: TGCGAGTCACCGTTCCTA	59	[35]

468 [a] Previously annotated as *manC*.
469 [b] Previously annotated as *manB*.

470

471 **Table 2. Comparison of $\log_2$ FC between 15 min and 12 h of ALF exposure for targeted qPCR**
472 **genes.** Differences in the expression of targeted *M.tb* cell envelope genes between 15 min and 12
473 h after ALF exposure were calculated ($\log_2$ FC at 12 h – $\log_2$ FC at 15 min) for each reference
474 gene. Average differences for *rpoB* and *sigA* are also shown.

Genes	15 min *vs.* 12 h (*rpoB*)	15 min *vs.* 12 h (*sigA*)	Mean *rpoB* and *sigA*
manB	0.107	0.425	0.266
pmmA	-0.580	-0.421	-0.500
whiB2	-0.461	-0.276	-0.368
pimA	-0.418	-0.224	-0.321
pimB	-0.019	0.252	0.116
pimF	-0.552	-0.406	-0.479
embC	-0.024	0.222	0.099

475

476

477 **FIGURE LEGENDS**

478 **Fig 1. *M.tb* GDP-Man, PPM and PIM/LM/ManLAM biosynthetic pathways.** GDP-Man can
479 be biosynthesized directly from gluconeogenesis, through formation of Fruc-6-P from Glc-6-P (by
480 the action of Glc-6-P isomerase), formation of Man-6-P (by the action of Man-6-P isomerase or
481 ManA/Rv3255c), formation of Man-1-P (by the action of phosphomutases PmmA/Rv3257c,
482 previously annotated as ManB, and PmmB/Rv3308), and finally formation of GDP-Man (by the
483 action of Man-1-P guanylyn-transferase or ManB/Rv3264c, previously annotated as ManC).

484 Further, Man-6-P can also be directly formed from Man by the action of a hexokinase (HK). PPM
485 is formed from GDP-Man by the action of polyprenyl monophosphomannose synthase or
486 Ppm1/Ppm2). Further, CDP-DAG together with inositol by the action of PI synthase (Rv2612c)
487 forms PI, which is further mannosylated by several mannosyltransferases (PimA to PimF) to
488 generate higher orders PIMs using GDP-Man and PPM as mannose donors. At one point, from
489 PIM_4 and using PPM as the major mannose donor, PIM_4 is heavily mannosylated by an undisclosed
490 number of mannosyl-transferases generating LM, and further arabinosylated with arabinosyl-
491 transferases generating LAM, which can be further mannose-capped by the mannosyl-transferases
492 action. LAM can also contain methylthio-$_D$-xylose (MTX) capping motifs [78, 79], where MtxT
493 (Rv0541c) transfers MTX to the mannoside caps of LAM [80]. Note: For simplicity,
494 acyltransferases (*e.g.* Rv2610c) are not depicted, and neither is the formation of MTX-P-C50 by
495 MtxS (Rv0539).
496
497 **Fig 2. Relative expression of selected PIM/LM/ManLAM biosynthesis genes in *M.tb* $H_{37}R_v$**
498 **after exposure to ALF.** *M.tb* was exposed for 15 min and 12 h to healthy human ALF (n=2
499 biological replicates, from two independent ALF donors), using *rpoB* (**A**) or *sigA* (**B**) as reference
500 genes. Expression values are shown as $\log_2$ fold changes, and were calculated using the $2^{-\Delta\Delta CT}$
501 method (ALF-exposed *M.tb vs.* heat-inactivated ALF-exposed *M.tb*) and plotted as the mean ±
502 SEM using Prism v9. ND: not detected (below limit of detection).
503
504 **Fig 3. Relative expression of cell envelope biogenesis and metabolism genes in *M.tb* Erdman**
505 **exposed to A-ALF and E-ALF.** Heatmap showing relative expression of cell wall genes
506 associated to (**A**) lipid metabolism; (**B**) carbohydrate metabolism; and (**C**) other pathways, in *M.tb*

507 after being exposed to A-ALFs (n=3 biological replicates, A_1- to A_3-ALFs), or to E-ALFs (n=3
508 biological replicates, E_1- to E_3-ALFs). Expression was normalized using *rpoB* as reference gene
509 and calculated using the $2^{-\Delta\Delta CT}$ method (ALF-exposed *M.tb vs.* heat-inactivated ALF-exposed
510 *M.tb*). Heatmap was constructed using Prism v9, with downregulated genes in blue (0 to 1 $\log_2$
511 fold changes) and upregulated genes in yellow (1 to 5 or more $\log_2$ fold changes). Genes are
512 grouped based on their assigned pathways (see **Supplemental Table 1**). Note that genes *pimB*,
513 *accD3*, *adhC* (lipid metabolism), *pmmB*, Rv0794c (carbohydrate metabolism), and *metZ* (others)
514 were below the limit of detection in one or more of the samples, and have not been included in the
515 heatmaps.
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Fig 1

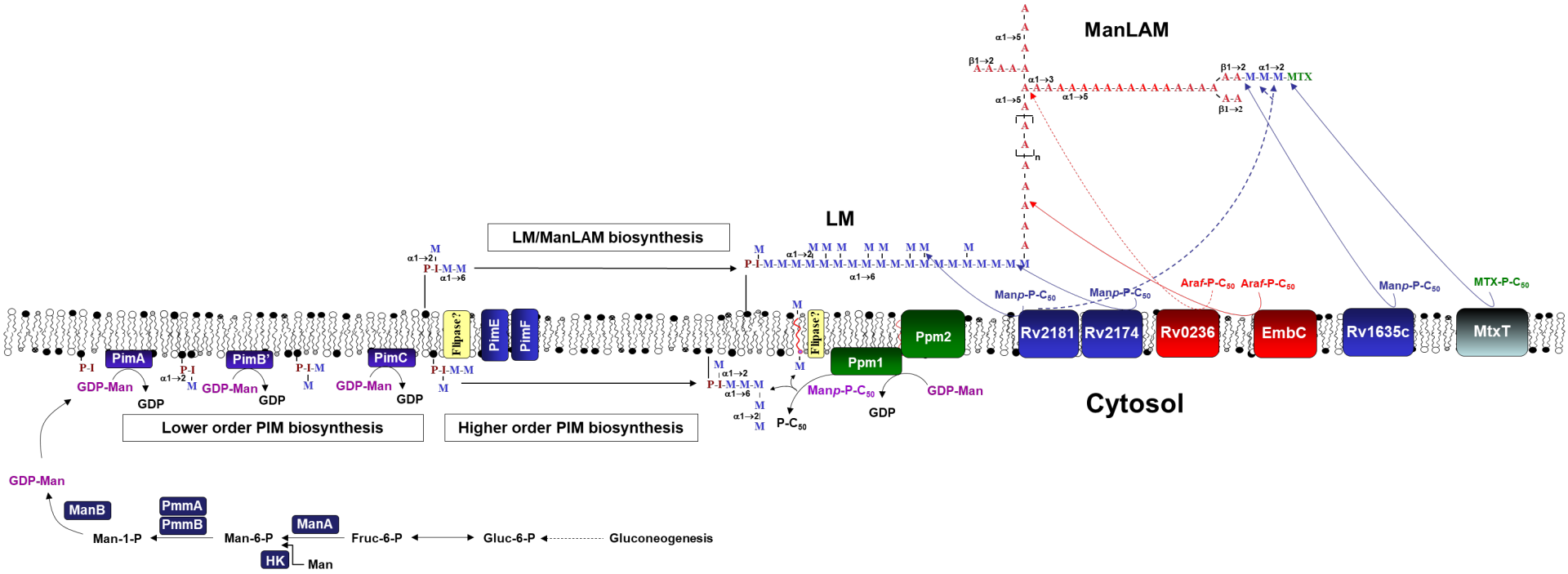

ManLAM
LM
LM/ManLAM biosynthesis
Lower order PIM biosynthesis
Higher order PIM biosynthesis
Cytosol
PimA
PimB'
PimC
PimE
PimF
Flipase?
Ppm1
Ppm2
Rv2181
Rv2174
Rv0236
EmbC
Rv1635c
MtxT
GDP-Man
GDP
Manp-P-C50
Araf-P-C50
MTX-P-C50
P-C50
ManB
PmmA
PmmB
ManA
HK
Man
Man-1-P
Man-6-P
Fruc-6-P
Gluc-6-P
Gluconeogenesis

Fig 2

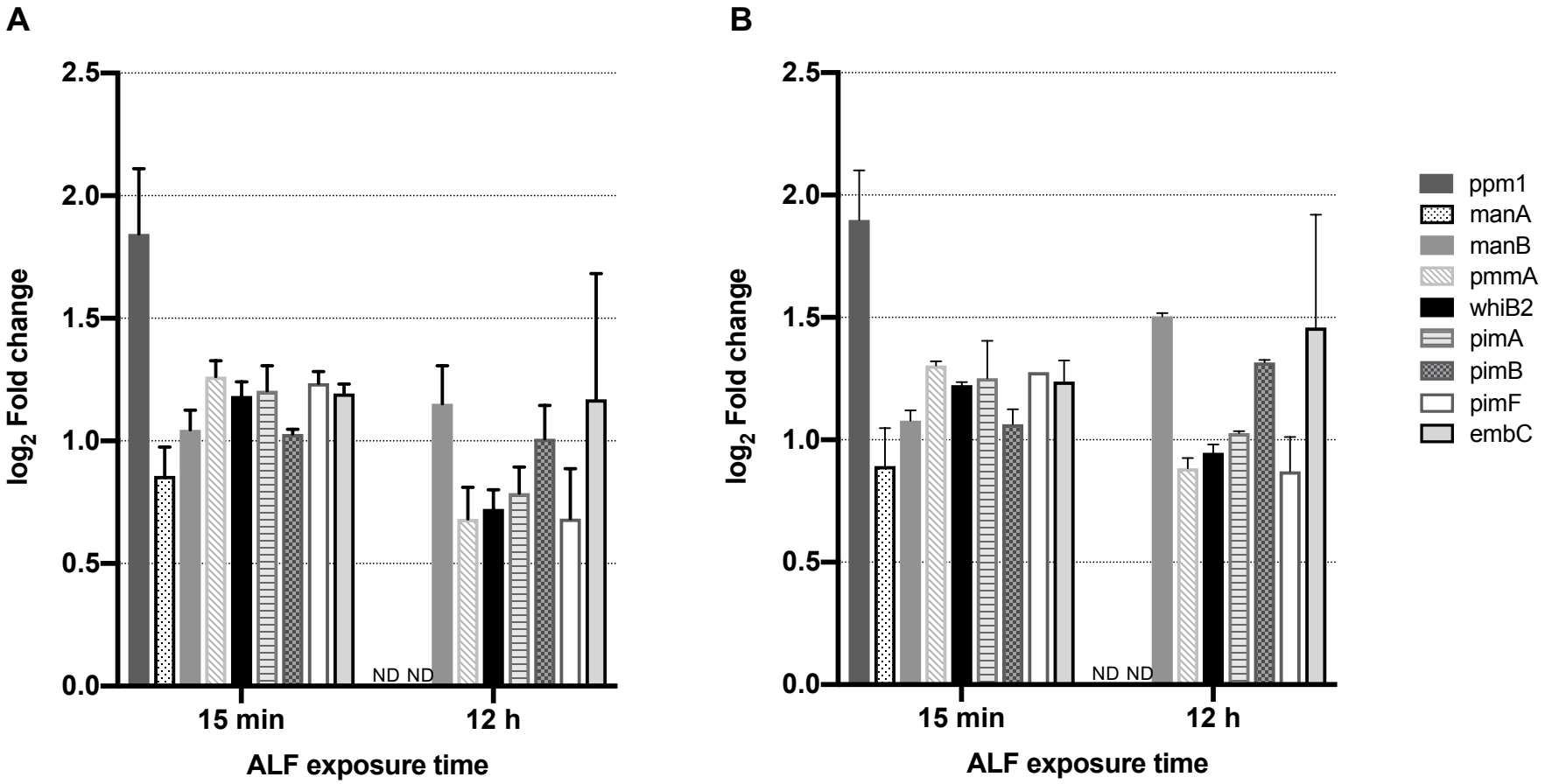
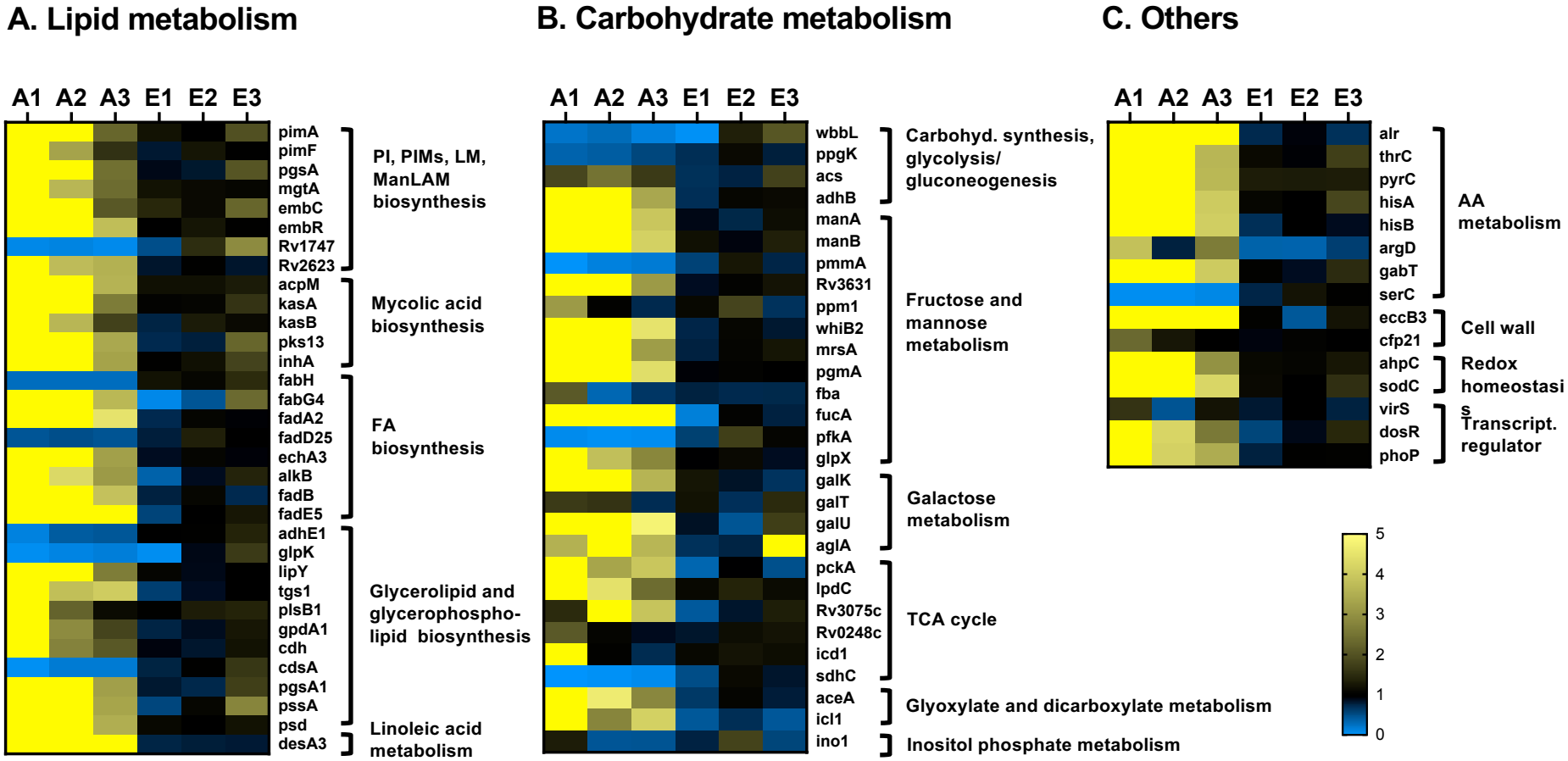

A
B
2.5
2.0
1.5
1.0
0.5
0.0
log2 Fold change
ND ND
15 min
12 h
ALF exposure time
ppm1
manA
manB
pmmA
whiB2
pimA
pimB
pimF
embC

Fig 3


A. Lipid metabolism
B. Carbohydrate metabolism
C. Others
A1 A2 A3 E1 E2 E3
pimA
pimF
pgsA
mgtA
embC
embR
Rv1747
Rv2623
PI, PIMs, LM, ManLAM biosynthesis
acpM
kasA
kasB
pks13
inhA
Mycolic acid biosynthesis
fabH
fabG4
fadA2
fadD25
echA3
alkB
fadB
fadE5
FA biosynthesis
adhE1
glpK
lipY
tgs1
plsB1
gpdA1
cdh
cdsA
pgsA1
pssA
psd
desA3
Glycerolipid and glycerophospho-lipid biosynthesis
Linoleic acid metabolism
wbbL
ppgK
acs
adhB
Carbohyd. synthesis, glycolysis/ gluconeogenesis
manA
manB
pmmA
Rv3631
ppm1
whiB2
mrsA
pgmA
fba
fucA
pfkA
glpX
Fructose and mannose metabolism
galK
galT
galU
aglA
Galactose metabolism
pckA
lpdC
Rv3075c
Rv0248c
icd1
sdhC
TCA cycle
aceA
icl1
Glyoxylate and dicarboxylate metabolism
ino1
Inositol phosphate metabolism
alr
thrC
pyrC
hisA
hisB
argD
gabT
serC
AA metabolism
eccB3
cfp21
Cell wall
ahpC
sodC
Redox homeostasis
virS
dosR
phoP
Transcript. regulator
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