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1 **Effect of glycosylation on the affinity of the MTB protein Ag85B for specific antibodies:**
2 **Towards the design of a dual-acting vaccine against tuberculosis**

3

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16

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25 **Keywords:** lipoarabinomannan, tuberculosis, *Mycobacterium*, glycoconjugate, antibodies, vaccine,
26 immune response

27

28 **Abstract (max 350 words)**

29 To create a dual-acting vaccine that can fight against tuberculosis, we combined antigenic arabino-
30 mannan analogues with the Ag85B protein. To start the process, we studied the impact of
31 modifying different parts of the Ag85B protein on its ability to be recognized by antibodies.
32 Through our research, we discovered that three modified versions of the protein, rAg85B-K30R,
33 rAg85B-K282R, and rAg85B-K30R/K282R, retained their antibody reactivity in healthy
34 individuals and those with tuberculosis. To further test the specificity of the sugar AraMan for
35 AraMan antibodies, we used Human Serum Albumin glycosylated with AraMan-IME and
36 Ara3Man-IME. Our findings showed that this specific sugar was fully and specifically modified.

37 Bio-panning experiments revealed that patients with active tuberculosis exhibited a higher antibody
38 response to Ara3Man, a sugar found in lipoarabinomannan (LAM), which is a major component of
39 the mycobacterial cell wall. Bio-panning with anti-LAM plates could eliminate this increased
40 response, suggesting that the enhanced Ara3Man response was primarily driven by antibodies
41 targeting LAM. These findings highlight the importance of Ara3Man as an immunodominant
42 epitope in LAM and support its role in eliciting protective immunity against tuberculosis.

43 Further studies evaluated the effects of glycosylation on the antibody affinity of recombinant
44 Ag85B and its variants. The results indicated that rAg85B-K30R/K282R, when conjugated with
45 Ara3Man-IME, demonstrated enhanced antibody recognition compared to unconjugated or non-
46 glycosylated versions. This suggests that coupling Ara3Man to rAg85B-K30R/K282R could lead to
47 the development of effective dual-acting vaccines against tuberculosis, stimulating protective
48 antibodies against both AraMan and Ag85B, two key tuberculosis antigens.

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52

53 **Introduction**

54 Tuberculosis (TB) is a significant global health challenge, with over 10 million new cases and 1.5
55 million deaths reported annually (1). Drug resistance is a major obstacle to TB control. Multi-drug-
56 resistant TB (MDR-TB) is TB that is resistant to both isoniazid and rifampicin, the two most potent
57 first-line anti-TB drugs. Extensively drug-resistant TB (XDR-TB) is even more resistant, with
58 resistance to at least three of the six most effective anti-TB drugs. Host-directed therapies (HDTs)
59 are a new approach to TB treatment that targets the host's immune system to help it fight the
60 infection. HDTs have shown promise in early clinical trials, but they are still in development. (2,
61 3). Vaccines are the most effective way to prevent infectious diseases, including TB. However, the
62 only licensed TB vaccine, Bacille Calmette-Guérin (BCG), is not effective enough in preventing TB
63 disease in adults. BCG is more effective in preventing TB meningitis in children, but it does not
64 provide long-term protection against pulmonary TB (4, 5).

65 Extensive research has been carried out on non-natural glycoproteins and glycolipids to develop
66 new therapeutic approaches for infectious diseases and cancer (6, 7). Carbohydrate-based vaccines
67 have been investigated as a potential treatment option (8-11). To create anti-infective vaccines,
68 antigenic oligosaccharides naturally present on the pathogen's membrane are chemically conjugated
69 with specific immunogenic carrier proteins like diphtheria toxoid, tetanus toxoid, and cross-reactive
70 material 197 (a mutant form of diphtheria toxoid) (12, 13). The use of natural oligosaccharides
71 obtained from pathogens allowed the development of efficient vaccines against different serotypes
72 of *Haemophilus influenzae* type b (Hib) virus and *Streptococcus pneumoniae*, or *Neisseria*
73 *meningitidis* (14) and more recently Varicella-zoster virus (15). Synthetic oligosaccharides have
74 been used as an alternative for the preparation of a vaccine against Hib (16) to avoid the various
75 problems related to the production of pure oligosaccharides from natural sources.

76 Arabinomannan (AM) is the major carbohydrate antigen of MTB and is part of the
77 lipoarabinomannan (LAM) membrane glycolipids. This oligosaccharide has been considered for the
78 development of new glycovaccines against TB. However, it is very complex and difficult to be
79 prepared by total synthesis. For this reason, synthetic analogues have been recently investigated as
80 antigens for the development of carbohydrate-based TB vaccines (17-20).

81 New synthetic strategies are continuously proposed to reduce the drawbacks of the chemical
82 synthesis of oligosaccharides. In this context, biocatalysis is considered an important tool to reduce
83 the synthetic steps (21). In past years, we have developed enzymatic strategies for the synthesis of
84 acetylated sugar building blocks with free hydroxyl groups in the desired position (22, 23) that can
85 be then chemically assembled to obtain complex oligosaccharides using the acetyl as the only
86 protecting group (24). This approach has been recently employed for the chemoenzymatic synthesis
87 of antigenic AM analogues composed by the combination of different units of mannose and
88 arabinose (17).

89 On the other hand, subunit vaccines obtained by gene fusion of different antigenic TB proteins are
90 under investigation. Ag85B, the major protein antigen of MTB, has been considered for the
91 development of several subunit vaccines that have been submitted to clinical investigation, aiming
92 to develop efficient vaccines alternative to the BCG (25).

93 Ag85B was also conjugated to natural AM isolated from MTB, to obtain vaccines with high
94 antigenic properties prepared by combining two different MTB antigens (10, 26, 27). However, the
95 biological activity of neo-glycoproteins based on antigenic proteins could be negatively affected by
96 the glycosylation of the protein epitopes (28, 29). In fact, the T-cell activity of Ag85B was strongly
97 depressed by chemical glycosylation targeting lysines (the most used approach for the preparation
98 of neo-glycoproteins) regardless the kind of sugar used (28). Several works dealing with the
99 characterisation of the T-cell epitopes of this protein have been published (28, 30). Still, only a few
100 of them contained data on the characterization of B-cell epitopes recognized by human antibodies
101 (28, 29). A detrimental effect induced by chemical glycosylation of recombinant Ag85B (rAg85B)

102 on T-cell activity was observed and ascribed to the modification of two lysines involved in relevant
103 epitopes (31). Accordingly, rAg85B variants obtained by replacement of these lysines with
104 arginines have been designed to avoid their glycosylation. Thus, rAg85B, which bears seven
105 additional amino acid residues at the N-terminal region with respect of the sequence of the wild-
106 type (wt) Ag85B (32), was compared with its variants obtained by Lys (K) to Arg (R) substitution
107 in position 30 and 282 (corresponding in the wild-type protein to K23 and K275, respectively).
108 These rAg85B variants maintained the T-cell activity after glycosylation, unlike non-mutated
109 rAg85B (31).

110 Little is known about the effect of glycosylation on the recognition of antigenic protein by human
111 antibodies. Therefore, in order to develop a dual-acting vaccine obtained by conjugation of
112 antigenic AM analogues with Ag85B, we evaluated the effect induced by glycosylation of rAg85B
113 on the affinity for antibodies present in the serum of TB patients. This work reports the preparation
114 of different neo-glycoproteins using sugars activated with an iminomethoxyethyl (IME) reactive
115 linker and different proteins. The different products were evaluated in the binding of antibodies
116 obtained from patients with active TB and from healthy controls.

117

118 **Materials and methods**

119 **Production of proteins and neo-glycoproteins**

120 Recombinant Ag85B (rAg85B) and its variants (obtained by substitution of a Lys residue with Arg
121 in position 30 and/or 282) were prepared according to the previously reported methods (31, 32).
122 This approach yields proteins with seven additional amino acid residues at the N-terminal compared
123 to the sequence of the wild-type (wt) Ag85B. Thus, positions 30 and 282 in rAg85B correspond to
124 positions 23 and 275 in the sequence of the wt protein.

125 The synthesis of the different glycans activated by an IME linker (17, 33), the chemical
126 glycosylation of proteins, and the characterization of the neo-glycoproteins obtained were carried
127 out according to the procedures previously reported for the different supports: commercial

128 recombinant human serum albumin (rHSA, Oryzogen, Wuhan, China) (17), rAg85B (28) and
129 variants (31).

130

131 **Studied population**

132 The study enrolled 47 individuals with newly diagnosed and untreated active pulmonary
133 tuberculosis, along with 40 healthy individuals with no history of tuberculosis exposure (referred to
134 as TB unexposed controls). In all cases, the diagnosis of active TB was confirmed through *M.*
135 *tuberculosis* culture isolation.

136

137 **ELISA assay**

138 Antibodies against Ag85B proteins and their glycoderivatives were detected using an ELISA assay
139 with minor modifications based on a previously described protocol (34).

140 The ELISA assay was performed in 96-well microplates (Nunc Maxisorb, Becton Dickinson). The
141 antigens (recombinant proteins: rAg85B, rAg85B-K30R, rAg85B-K282R, rAg85B-K30R/K282R,
142 synthesized oligosaccharides: AraMan, Ara₃Man, Man₂ and the conjugated glycoproteins: rAg85B
143 + Man₂, rAg85B-K30R/K282R + Man₂, rAg85B-K30R/K282R + Ara₃Man)

144 were diluted to a final concentration of 10 µg/mL in 100 µL of carbonate buffer (pH 9.6) and
145 incubated for 2 hours at room temperature followed by overnight incubation at 4°C. The wells were
146 then washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) to
147 remove any unbound proteins.

148 To block the non-specific binding sites, the wells were incubated (37 °C, 1 h) with PBS-T
149 containing 3% bovine serum albumin (BSA). Subsequently, the plates were incubated for 1 hour at
150 room temperature with 100 µL of serum diluted 1:100.

151 For the ELISA panning assay. plates were coated with lipoarabinomannan (LAM BEI Resources,
152 NIAID, USA) diluted (final concentration 2 µg/mL) in 100 µL of carbonate buffer (pH 9.6), then
153 incubated, washed and blocked using the same procedure described before. The sera were incubated

154 (RT, 1 h) with 100 μ L of serum (1:100), and the procedure was repeated three times (in three
155 different plates subsequently), keeping all other conditions identical as before. After the incubation
156 with sera, all the plates (those analyzed with the antigens and those analyzed with LAM for the
157 panning) were incubated (RT, 1h) with anti-human IgG Secondary Antibody HRP conjugated
158 (Invitrogen, Italy). The absorbance (OD) at 492 nm of antigen- and buffer-coated wells was
159 measured, and the difference in mean OD values was calculated. All samples were assayed in
160 duplicate to increase precision..

161

162 **Statistical analysis**

163 Data were reported as the mean values \pm standard error of the mean (SEM) or standard deviation
164 (SD). Statistical comparisons between two groups were performed by Student's t-test. *p* values < 0.05
165 were considered to be statistically significant. GraphPad Prism version 5.0 (GraphPad Software,
166 Inc., La Jolla, CA) was used for all statistical analyses and graphs.

167

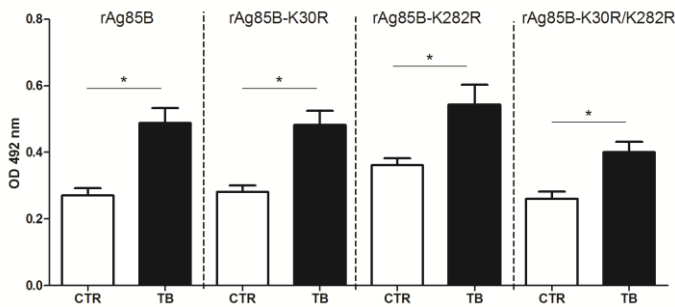
168

169 **Results**

170

171 **Ag85B variants preserved the antibody reactivity in healthy and TB human samples.** To
172 evaluate the impact of substitutions of Lys30 and/or Lys282 of Ag85B with arginine (rAg85B-
173 K30R, rAg85B-K282R and rAg85B-K30R/K282R, respectively), sera from control and TB subjects
174 were investigated. Control sera indicated comparable antibody levels against rAg85B and its
175 variants (Fig. 1), except a slightly higher value for the K282R variant. In TB patients, significantly
176 increased antibody levels were detected for rAg85B and its variants, compared to controls. This
177 enhancement in TB patients was uniform for each antibody (+50-80%) relative to each control; no
178 significant difference was observed between rAg85B and its variants in TB patients. This analysis
179 demonstrates that, while antibody levels against the different rAg85B variants are increased in TB vs

180 controls, no significant differences can be detected when normal subjects were compared.
181 Therefore, the introduced substitutions do not alter the antibody response.



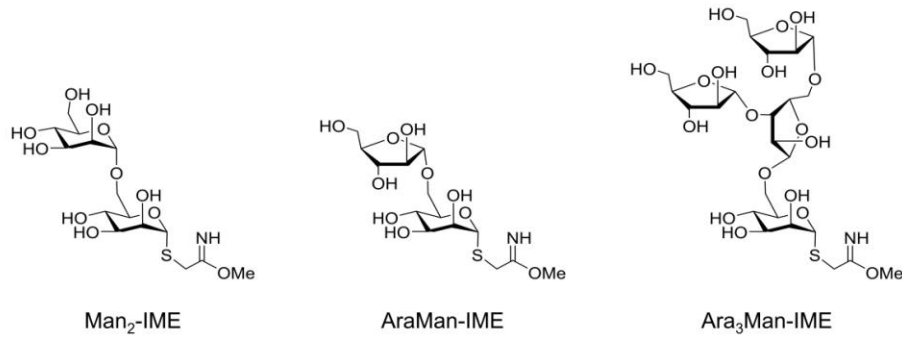
182

183 Fig. 1: Levels of antibodies (IgG) reactive against the rAg85B and its variants in TB patients (TB:
184 47 samples) and healthy subjects (CTR: 40 samples). All samples were measured in duplicate and
185 repeated three times. The data are presented as mean values \pm standard error of the mean (SEM).
186 OD = optical density; $*=p \leq 0.05$ by Student's t-test.

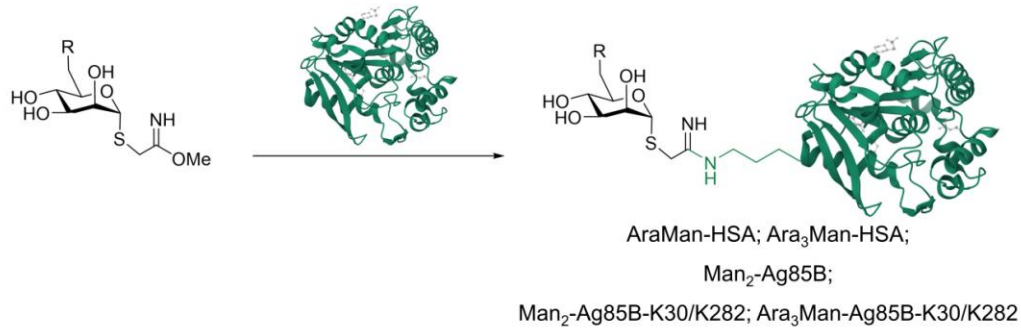
187

188 **Preparation and characterization of neo-glycoproteins.** The Man₂, AraMan and Ara₃Man
189 glycans, activated in an anomeric position with the IME reactive linker (Fig. 2a), which selectively
190 targets the amino group of lysine residues (33), were used to prepare the different neo-glycoproteins
191 (Fig. 2b) considered in the evaluation of antibody affinity.

a



b



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201 Fig. 2. a) Chemical structure of the different glycans activated with IME linker. b) Chemical
202 glycosylation of proteins. Reactions were performed in 100 mM sodium tetraborate pH 9.5 at 25 °C
203 for 24 h; glycosidic reagent/protein molar ratio: 250:1 for Ara₃Man-rAg85B-K30R/K282R, and
204 200:1 for the others; protein concentration: 2 mg/mL. In rAg85B and rAg85-K30R/K282R

205 glycosylation, 1mM benzamide hydrochloride was added in the reaction mixture to avoid potential
206 proteolysis (31). Protein graphical representation depicts Ag85B protein and was taken from the
207 Protein data Bank (PDB DOI: 10.2210/pdb1f0p/pdb).

208

209 After conjugation, , the degree of glycosylation of the resulting neo-glycoproteins was determined
210 by analyzing the residual presence of unmodified protein and the average glycan loading..

211 For rHSA derivatives, conjugation was assessed by hydrophilic interaction liquid chromatography
212 (HILIC) and high resolution mass spectrometry (HRMS), as previously described (17). Both
213 samples, AraMan and Ara₃Man, showed the complete modification of rHSA and an average loading
214 of 9.3 and 14.5 (17) mol/mol, respectively.

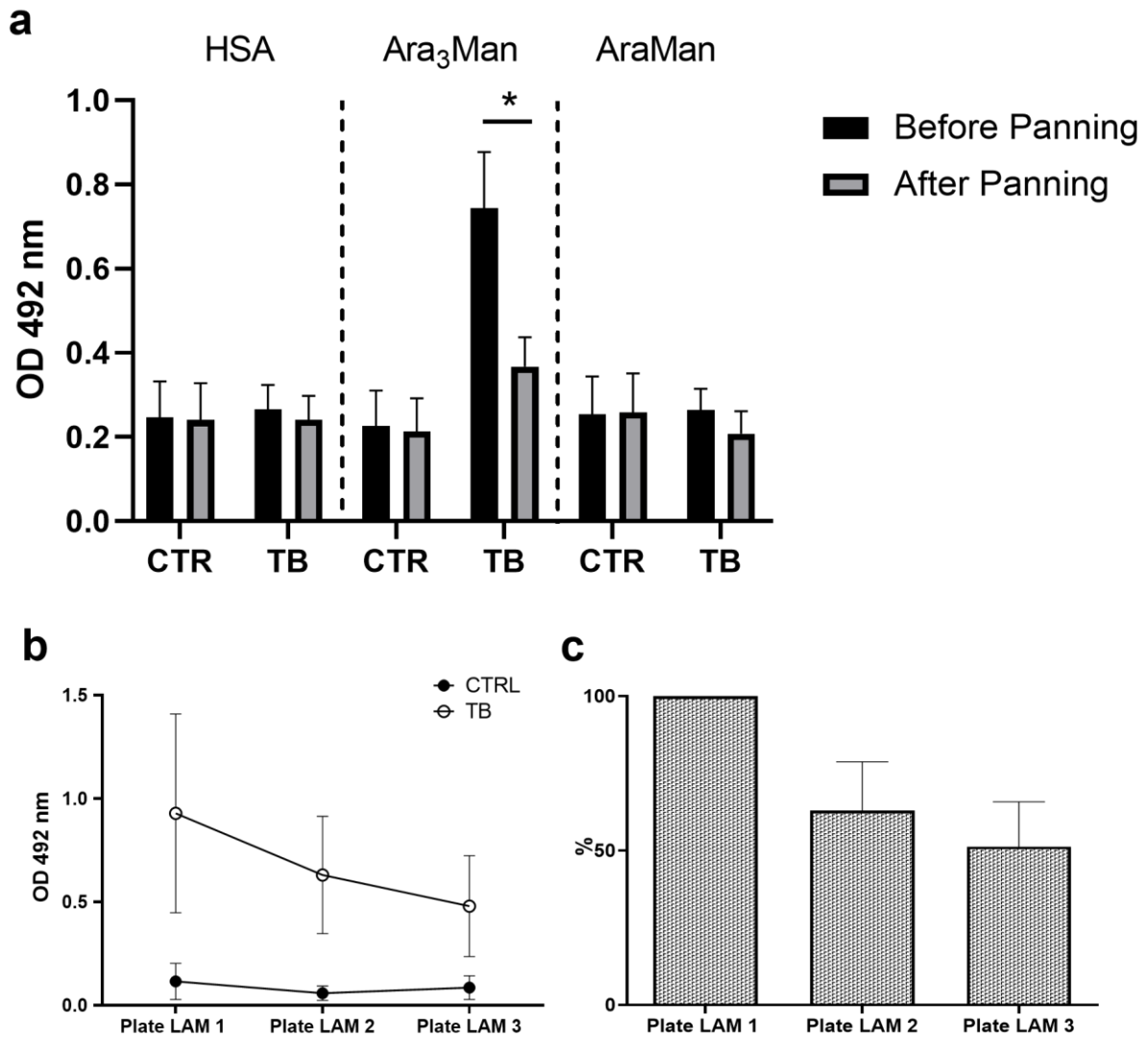
215 For Man₂-rAg85B the conjugation was quantitative, with no residual presence of unmodified
216 protein and an average loading of 4.8 disaccharide units *per* protein. The substitution of lysine 30
217 and 282 significantly reduced protein reactivity, as previously described (31), yielding to a 98%
218 glycosylation degree and an average loading of 2.5 mol/mol. To compensate the further reduction of
219 reactivity due to the increased saccharide size, the glycan/protein molar ratio was increased to 250:1
220 in the preparation of Ara₃Man-Ag85B-K30R/K282R. This resulted in 100% glycosylation yield and
221 an average loading of 2.8 mol/mol.

222

223 **The LAM-specific antibodies were subtracted by three rounds of bio-panning.** To characterize
224 the antibody reactivity tot the AM mimetic sugar antigen, the AraMan and Ara₃Man motifs were
225 examined after conjugation with HSA. Control and TB sera were placed into wells containing a
226 non-immunogenic protein (HSA) or the same protein glycosylated with the disaccharide (AraMan-
227 rHSA) or the tetrasaccharide (Ara₃Man-rHSA). To determine whether antibodies against these
228 motifs were specific for LAM, negative affinity purification of antibodies was also performed. In

229 detail, samples of sera were placed into wells containing LAM for three rounds of subtractive
230 (negative) panning *in vitro*. Sera negativity to rHSA was tested by ELISA in the same experiment.
231 Before the panning, the ELISA test showed a higher Ara₃Man antibodies level in TB subjects
232 compared to experiments performed with the non-glycosylated protein and with the disaccharide
233 AraMan (p<0.0037 and p<0.0031 respectively) (Fig. 3). However, this difference disappeared after
234 three rounds of bio-panning; the reduction in the response against Ara₃Man was statistically
235 significant (p<0.0215) compared to the levels obtained before the bio-panning. These results
236 indicate that the increase in Ara₃Man response level was mainly caused by LAM-specific
237 antibodies.

238 To highlight the anti-LAM antibody level present in the sera, LAM-coated plates were set up for
239 bio-panning. Figure 3 shows the levels of anti-LAM antibodies expressed as OD (panel b) or
240 percentage relative to Plate LAM 1 (panel c) in the three different plates used to purify the sera.
241 There is a decrease in anti-LAM antibodies in each bio-panning step for TB patients. This indicates
242 that some of the LAM-specific antibodies are removed from the sera of TB patients using LAM
243 purified from *M. tuberculosis*. The experiments also show that Ara₃Man is a motif contained in the
244 LAM.



245

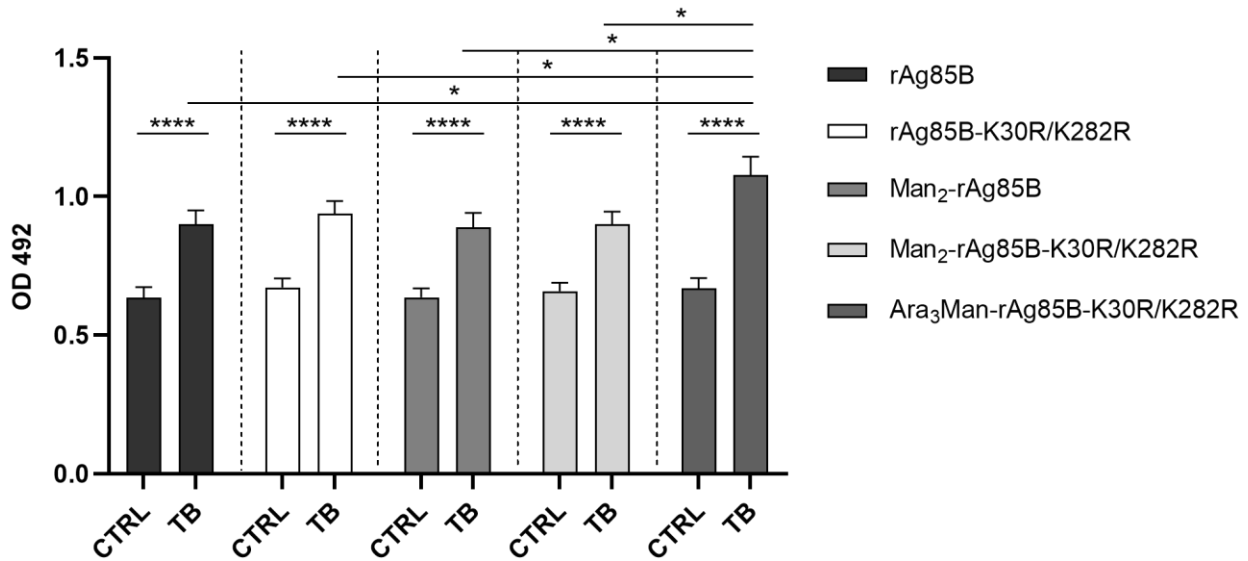
246 Fig. 3. ELISA analysis. (a) ELISA analysis for the determination of antibody (IgG) levels against
 247 HSA, Ara₃Man-rHSA and AraMan-rHSA was carried out using samples obtained before and after
 248 three rounds of selection against LAM in healthy subjects (CTR) and in TB patients. Absorbance
 249 values are the mean of 9 control samples and 11 TB samples in each group. All samples were
 250 measured in duplicate and repeated three times. Error bars show standard error of the mean (SEM)
 251 for each set of data. $*=p \leq 0.05$ by Student's t-test. The level of antibodies against Ara₃Man is very
 252 high in TB patients, and is reduced by about 50% in TB patients when LAM post-adsorption serum
 253 was tested. (b, c) Detection of anti-LAM antibody level upon 3 bio-panning procedures. The level
 254 of specific antibodies against LAM was measured after each bio-panning step. The same sera were

255 tested on three adsorbed LAM plates consecutively (from Plate LAM 1 to Plate LAM 3); the
256 reduction is expressed in OD mean values \pm standard error of the mean (SEM) (b) and in percentage
257 (c) of healthy control and TB subjects (b) or only TB (c), respectively. High levels of anti-LAM
258 antibodies are detected in TB patients on bio-panning steps (reduced by about 50% from step 1 to
259 step 3), while no anti-LAM antibodies could be found in healthy controls.

260

261 **Effects of glycosylation on antibody reactivity.** rAg85B and the rAg85B-K30R/K282R variant
262 proteins were glycosylated to evaluate the effect of the conjugation with the di- or tetrasaccharide
263 on the antibody affinity of the different glycoproteins. The experiments were performed by ELISA
264 test on sera from healthy controls and TB subjects. The results showed that there was a statistically
265 significant difference between TB samples and their controls for rAg85B and its double variant.
266 However, healthy subjects did not show increased antibody reactivity to all glycosylated proteins.
267 Notably, TB subjects show a statistically significant increase in affinity for Ara₃Man-rAg85B-
268 K30R/K282R when compared with all TB groups ($p \leq 0,0217$ vs rAg85B; $p \leq 0,0513$ vs rAg85B-
269 K30R/K282R; $p \leq 0,0173$ vs Man₂-rAg85B; $p \leq 0,0178$ vs Man₂- rAg85B-K30R/K282R) (Fig. 4).
270 Therefore, TB subjects have the highest levels of antibodies with reactivity towards the rAg85B-
271 K30R/K282R variant conjugated with Ara₃Man, due to the synergistic effect of the two antigens
272 (protein and sugar moiety), which may provide the basis for a dual-acting vaccine.

273



274

275 Fig. 4: Distribution of antibodies (IgG) levels reactive with rAg85B, its double variant and the
 276 corresponding glycoderivatives, in TB patients and healthy subjects (14 TB samples and 17
 277 control). All samples were measured in duplicate and repeated three times. The data are presented
 278 as mean values \pm standard error of the mean (SEM). OD = optical density; CTRL: controls; TB =
 279 tuberculosis. $p \leq 0.05 = *$, $p \leq 0.0001 = ****$ were calculated by Student's t-test.

280

281 Discussion

282 In this study, we first analyzed the effect of selected Ag85B modification on antibody recognition.
 283 Then, Human Serum Albumin (HSA) glycosylated with AraMan-IME and Ara₃Man-IME was
 284 considered to assess the specificity of the last sugar for AM antibodies.

285 In addition, rAg85B and its variant obtained by K/R substitution in positions 30 and 282
 286 (rAg85BK30R/K282R) and glycosylated with Man₂-IME was studied to assess the effect of the
 287 glycosylation on the antibody affinity of these antigenic proteins. Finally, conjugation of rAg85B-
 288 K30R/K282R with Ara₃Man-IME has been investigated as a proof of concept about the potential
 289 developing dual-acting vaccines against TB targeting antibodies specific for two different types of
 290 MTB antigens (the AM sugar and the Ag85B protein).

291 Given the success of several carbohydrate-based anti-bacterial vaccines, including those against
292 facultative intracellular organisms (35-37), we focused our efforts on constructing a carbohydrate-
293 protein conjugate vaccine against TB using an oligosaccharide that mimics the LAM antigen of
294 MTB. For this purpose, Ag85B was selected as the carrier protein in order to design a double-acting
295 vaccine, since it is one of the most potent antigenic species expressed by MTB.

296 Therefore, a recombinant form of Ag85B (rAg85B) (32) and the K30R and/or K282R variants were
297 prepared. The substitution of these amino acids, which are involved in the main T-cell epitopes of
298 Ag85B, proved to be conservative for protein conformation: *ex vivo* ELISPOT experiments
299 demonstrated that all protein variants maintained the original T-cell immunogenic activity exhibited
300 by rAg85B (31). The activity of the Ag85B variants was also maintained after glycosylation, unlike
301 the wild-type protein, since the introduced substitutions avoid glycosylation of the main T-cell
302 epitopes (31). In the present work, to complete the evaluation of the immunological response to
303 these recombinant proteins, the antibody interaction of wt rAg85B and of its variants was
304 investigated. In an ELISA test, all recombinant proteins showed similar efficiency of recognition of
305 antibodies present in the serum of MTB-infected patients (Fig. 1). In addition, glycosylation with
306 non-antigenic disaccharide (Man₂ and AraMan) had little effect on the immuno-reactivity of these
307 proteins, confirming them as putative antigenic carriers functional for the design of effective
308 glycoconjugated dual-acting vaccines against MTB (Fig. 3a and 4) using a double hit approach
309 (combining sugar and protein antigens).

310 LAM has been extensively studied for its immunomodulatory properties and as a structurally
311 unique glycolipid component of the envelope of all mycobacterial species (38) and is therefore
312 considered an attractive vaccine candidate (39, 40) to evoke immune responses against MTB. Anti-
313 LAM antibodies are induced during MTB infection (41-43) and have been associated with bacterial
314 opsonization and restriction of intracellular growth (44, 45).

315 Pure oligosaccharides are poor immunogens as they fail to recruit CD4⁺ T cell help. They are
316 therefore limited to T cell-independent B cell immune responses. However, conjugating a bacterial

317 polysaccharide to an immunogenic carrier protein that provides T cell epitopes creates a T cell-
318 dependent antigen that can induce protective immunity. Indeed, AM isolated from LAM derived
319 from the MTB cell wall and conjugated to various immunogenic carrier proteins has been used to
320 generate new glyco-conjugate TB vaccine candidates (46). However, AM-conjugated products
321 using different vaccination protocols showed modest protection, never exceeding the effect of BCG
322 vaccination (26, 27, 47-49). In addition, natural AM is too complex for chemical synthesis to
323 develop semi-synthetic glyco-vaccines. For this reason, we studied the synthesis of AM analogues
324 structurally related to the natural MTB antigen, which allowed the synthesis of the Ara₃Man that
325 showed affinity to LAM antibodies of infected patients (17).

326 In the present work, we have demonstrated the specificity of this oligosaccharide for the LAM
327 antibodies of TB patients and its synergic activity towards human TB-antibodies after conjugation
328 with a variant of rAg85B. Actually, ELISA experiments showed that the increased affinity induced
329 by Ara₃Man after conjugation with HSA (Fig. 3) was caused by LAM-specific antibodies. To
330 further validate this specificity, LAM-specific antibodies were selectively removed from TB patient
331 sera using purified LAM from *M. tuberculosis* (Fig. 3). This depletion of LAM-specific antibodies
332 led to a significant decrease in immune recognition of TB samples, confirming the strong
333 correlation between LAM and the Ara₃Man motif.

334 In addition, the immunogenic activity of rAg85B-K30R/K282R variant conjugated with different
335 synthetic oligosaccharides was investigated. Glycosylation with non-antigenic disaccharides
336 maintained the antibody affinity of the Ag85B mutant, while conjugation with Ara₃Man
337 tetrasaccharide enhanced it. This improvement was likely due to an additional interaction with
338 LAM-specific antibodies (Fig. 4).

339 ELISA experiments revealed that TB patients had significantly higher levels of antibodies against
340 the rAg85B-K30R/K282R variant conjugated with Ara₃Man compared to the non-conjugated
341 protein or the one conjugated with Man₂, a non-antigenic disaccharide. These findings suggest that
342 Ara₃Man could serve as a promising glycan component for developing more effective

343 glycoconjugate TB vaccines. By combining Ara3Man with rAg85B or other relevant antigens, a
344 synergistic effect could be achieved, potentially leading to enhanced immune responses and
345 improved protection against TB. For this reason, the results provide a proof-of-concept for the
346 development of a dual-acting vaccine that targets two different MTB antigens.

347 The next steps will involve evaluating the immunogenicity of this conjugate in animal models and
348 assessing its protective efficacy after the challenge. Additionally, further investigations will explore
349 alternative AM-mimetic oligosaccharides to optimize the immune response mediated by the sugar
350 antigen.

351

352

353 **Declarations**

354 **Ethics approval and consent to participate**

355 The Ethics Committee of the University of Rome “Tor Vergata” (Rome, Italy) approved the study
356 protocol (protocol number 173/19) “Valutazione biologica Ex Vivo di nuovi glicoconiugati semi-
357 sintetici derivati da proteine antigenetiche ricombinanti di *Mycobacterium tuberculosis* - Ex Vivo
358 biological evaluation of new semi-synthetic glycoconjugates derived from recombinant antigenic
359 proteins of *Mycobacterium tuberculosis*”.

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361 **Consent for publication**

362 Not applicable.

363

364 **Availability of data and materials**

365 The datasets used and/or analysed during the current study are available from the corresponding
366 author on reasonable request.

367

368 **Competing interests**

369 The authors declare that they have no competing interests.

370

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374

375 **Authors' contributions**

376 Conceptualization, experimental design, RB, ST; writing original draft preparation, RB, ST and
377 MT; design of *ex-vivo* experiments, RB, MM and SM; *ex-vivo* experiments, RB and BAM;
378 synthesis of LAM mimetic compounds, ZL; enzymatic synthesis of sugar building blocks, PC;
379 protein conjugation, TB; protein and glycoprotein analysis, ST; preparation of recombinant Ag85B
380 proteins, LPi and LPo; responsible for protein and glycoprotein analysis, CT; responsible for LAM
381 mimetic synthesis, YZ; responsible for financial support, MT; all authors read and approved the final
382 manuscript.

383

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