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Research article

**Liposomal Amikacin and *Mycobacterium abscessus*: Intimate interactions
inside eukaryotic cells**

Running Title: ALIS efficiency against intracellular *M. abscessus*

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21

22 **ABSTRACT (Words count: 246)**

23 **Background**

24 *Mycobacterium abscessus* (Mabs), a rapidly growing mycobacteria, is considered a multidrug
25 resistant organism. Among the standard antimicrobial multi-drug regimens against Mabs,
26 amikacin is considered as one of the most effective. Parenteral amikacin, as a consequence of
27 its inability to penetrate inside the cells, is only active against extracellular mycobacteria. The
28 use of inhaled liposomal amikacin may yield improved intracellular efficacy by targeting
29 Mabs inside the cells, while reducing its systemic toxicity.

30 **Objectives**

31 Our objectives were to evaluate the colocalization of an amikacin liposomal inhalation
32 suspension (ALIS) with intracellular Mabs, and then to measure its intracellular anti-Mabs
33 activity.

34 **Methods**

35 We evaluated the colocalization of ALIS with Mabs in eukaryotic cells such as macrophages
36 (THP-1 and J774.2) or pulmonary epithelial cells (BCi-NS1.1 and Mucilair), using a
37 fluorescent amikacin liposomal inhalation suspension and GFP-expressing Mabs, to test
38 whether ALIS reaches intracellular Mabs. We then evaluated the intracellular anti-Mabs
39 activity of ALIS inside macrophages using cfu and/or luminescence.

40 **Results**

41 Using confocal microscopy, we demonstrate fluorescent ALIS and GFP-Mabs colocalization
42 in macrophages and epithelial cells. We also showed that ALIS was active against
43 intracellular Mabs at a concentration of 32 to 64 mg/L, at 3 days and 5 days post-infection.
44 Finally, ALIS intracellular activity was confirmed when tested against 53 Mabs clinical
45 isolates, showing an intracellular growth reduction for nearly 80% of the isolates.

46 **Conclusions**

47 Our experiments demonstrate the intracellular localization and intracellular contact between
48 Mabs and ALIS, and the antibacterial activity against intracellular Mabs, showing promise for
49 its future use for Mabs pulmonary infections.

50

51

53 **INTRODUCTION**

54 *Mycobacterium abscessus* is a rapidly growing, multidrug resistant organism that accounts for
55 more than half of all nontuberculous mycobacterial (NTM) infections in patients with cystic
56 fibrosis (CF).¹ The standard treatment regimen against this mycobacterium comprises a
57 combination of at least three antibiotics, including cefoxitin or imipenem, a macrolide, and
58 amikacin which is the only effective aminoglycoside against *M. abscessus*.² The use of
59 parenteral aminoglycosides for NTM diseases, and *M. abscessus* in particular, for a long
60 duration of treatment is limited by severe side effects such as oto-, vestibular and nephro-
61 toxicity.^{3,4} These side effects occur at a lower prevalence if conventional water-soluble (free)
62 amikacin is inhaled rather than parenterally administered.⁵ So, the use of inhaled liposomal
63 drug formulas might allow improved efficacy and reduced systemic toxicity by delivering the
64 drug into closer proximity with the target.^{6,7}

65 Inhaled antibiotics packaged in a liposomal formula have been proposed as a treatment for
66 chronic lung infections for some time.^{8,9} They were first suggested as therapies against fungal
67 infections¹⁰ and, some years later, against bacterial infections¹¹⁻¹⁴ and notably in the context of
68 CF.^{6,15-18}

69 The treatment with amikacin liposomal inhalation suspension (ALIS) has already been tested
70 in CF patients to study responses against *Pseudomonas aeruginosa* infections^{6,18,19},
71 *Mycobacterium avium* complex²⁰⁻²² and recently in France, *M. abscessus*.²³

72 In the present study, we have assessed the ability of ALIS to reach a close proximity with
73 intracellular mycobacteria, by evaluating the colocalization of fluorescent ALIS and GFP-
74 expressing *M. abscessus* in macrophages and pulmonary epithelial cells. The purpose of using
75 human epithelial cells was to study interactions in cells typically infected by *M. abscessus*, in
76 the presence of mucus, and in the presence of a biofilm often formed by *M. abscessus* smooth

77 (S) morphotype after infection of epithelial cells. We also measured the intracellular
78 inhibitory activity of ALIS on *M. abscessus* in these cells. Finally, we tested the effect of
79 ALIS on numerous clinical strains.

80

81

82 **MATERIALS AND METHODS**

83

84 **Mycobacterial strains, cells and culture conditions.**

85 Mycobacterial strains were grown aerobically at 37 °C in Middlebrook 7H9 and on 7H11
86 (Sigma-Aldrich, Saint-Quentin-Fallavier, France) medium supplemented with 0.2% glycerol
87 and 1% glucose. When necessary, hygromycin (Invivogen, Toulouse, France), was added to
88 the growth medium at 1,000 mg/L. This was the case when we transformed the ATCC 19977
89 reference of *M. abscessus* strain with the plasmid pSMT3-GFP-LuxAB. This transformed
90 strain (Mabs-CIPS-GFP) allowed luminescence and fluorescence for measurement of
91 bacterial load and localization by confocal microscopy, respectively. The tested clinical
92 strains were provided from the strain collection of the Raymond Poincaré hospital-APHP.²⁴
93 For infection, mycobacteria were grown to mid-log phase in liquid medium and harvested by
94 centrifugation and suspended in a PBS solution. After 3 washes, the bacterial clumps were
95 disrupted by 20–30 passages through a 29G needle and the bacterial suspension was aliquoted
96 and stored at -80 °C before use.

97

98 Murine and human macrophage cell lines (J774.2 and THP-1 respectively), a bronchial
99 epithelial cell line with multipotent differentiation capacity (BCi-NS1.1),²⁵ primary bronchial
100 epithelial cultures (MucilAir, Epithelix, Switzerland) and dendritic cells were used.

101 Murine J774.2 macrophages (MΦ) (Sigma-Aldrich) were grown in DMEM-Glutamax
102 medium supplemented with 5% heat-inactivated FBS (Thermo Fisher Scientific, USA),
103 penicillin (100 IU/mL) and streptomycin (100 mg/L) at 37 °C in a humidified atmosphere of
104 5% CO₂. The human monocytic cell line THP-1 was maintained in RPMI 1640-Glutamax
105 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS at 37 °C with 5%
106 CO₂. For differentiation into macrophages-like cells, 2×10^5 THP-1 cells were seeded into
107 24-well plates and activated with 100 ng/mL of Phorbol 12-Myristate 13-Acetate (Sigma-
108 Aldrich) for 24 h.

109 The bronchial epithelial cell line with multipotent differentiation capacity BCI-NS1.1 was
110 grown on Falcon™ cell culture inserts with a 0.4 μm pore size (Fisher Scientific) in BEGM
111 medium (Lonza, Basel, Switzerland) till confluency. For differentiation the apical medium
112 was removed and the basolateral medium was replaced by DMEM/F12 with 2% Ultroser™ G
113 serum substitute (Sartorius Stedim France). After three weeks of culture, the BCI-NS1.1
114 cultures display several markers of differentiation (pseudo-stratification, polarization, tight
115 junctions, transepithelial potential difference and 10% ciliated cells).²⁵

116 The ready-to-use primary bronchial epithelial cultures MucilAir are provided on cell inserts,
117 displaying differentiated ciliated epithelium. The culture medium used was MucilAir medium
118 (Epithelix, Plan-les-Ouates, Switzerland).

119

120 Peripheral blood mononuclear cells were isolated from freshly collected blood samples
121 obtained from healthy voluntary blood donors (Ambroise Paré Hospital, France) by density
122 gradient centrifugation using a lymphocyte separation medium (Eurobio, Les Ulis, France) as
123 previously described.²⁶ Monocytes were purified by positive selection using anti-CD14-
124 coated magnetic micro beads (Miltenyi Biotech, Bergish Gladsbach, Germany). Monocytes
125 were differentiated into dendritic cells (DCs) for 7 days in DC-medium described as RPMI

126 10% FCS medium supplemented with 800 UI/mL GM-CSF and 1000 UI/mL IL-4 (R&D
127 Systems, Abingdon, UK). Fresh DC-medium is added to culture at days 2 and 6.

128 **Reagents**

129 Water-soluble amikacin sulfate and ALIS formulations were provided by INSMED. The
130 classical non-fluorescent labelled liposomal preparation is simply named ALIS in the
131 manuscript.

132 For ALIS dilution, the solution started at a concentration of 105 g/L was first diluted in 1.5%
133 NaCl solution to obtain a 50 × concentrated solution according to the final concentration
134 desired (*e.g.* 3.2 g/L for a final concentration of 64 mg/L). Then a second dilution was
135 established in a 300 mM lactose solution to produce a 5 × concentrated solution. To obtain the
136 final concentration, 1/5 of this solution was added to 4/5 of cell culture. For the blank controls
137 (only extracellular water-soluble amikacin treatment or liposomes alone), the same dilutions
138 were done with water plus NaCl and then lactose and added to the cell culture.

139 For experiments where fluorescent amikacin and liposomes were required, a proportion of the
140 amikacin sulfate solution (0.91%) was conjugated to tetramethylrhodamine (TAMRA), and
141 dipalmitoylphosphatidylethanolamine (DPPE) labeled with 0.01% AF647 was included in the
142 lipid component of the liposomes, as described in Zhang *et al.*²⁷ This preparation was called
143 ALIS-TAMRA in the manuscript.

144 Decanal (D7384) was purchased from Sigma-Aldrich (Saint-Louis, Missouri, USA). A
145 solution of 1% was prepared by dilution in distilled water. This solution was sonicated to
146 obtain an opaque white homogenous emulsion of decanal.

147

148 **MΦ infection and intracellular growth measurement**

149 MΦ were seeded in 24-wells plate at a concentration of 5×10^4 to 10^5 cells per mL of
150 medium. MΦ were infected at a MOI between 1 and 10 mycobacteria per MΦ in order to

151 avoid rapid cell lysis and incubated for 3 h at 37 °C.^{28,29} After infection, cells were washed
152 thoroughly with PBS (three times) to eliminate extracellular bacteria and re-fed with complete
153 medium containing amikacin (Mylan S.A.S., France) at 250 mg/L for a further 1 h incubation
154 at 37 °C. This step was essential to kill the remaining extracellular mycobacteria. The medium
155 containing amikacin was then discarded, and cells were washed again three times with PBS.
156 Infected cells were subsequently incubated in the presence of amikacin at 50 mg/L at 37 °C to
157 prevent any further extracellular growth of *M. abscessus*. Control dilution solutions (NaCl-
158 lactose or liposomes alone) or ALIS, at the final concentration of 32 mg/L or 64 mg/L, were
159 added until the time of analysis. For day 5 measurements, the culture medium was changed at
160 day 3.

161

162 **Cfu counts and luminescence measurement**

163 To assess the intracellular growth of mycobacteria, colony forming unit (cfu) counts were
164 performed at day 0 (*i.e.* 4 h post infection after the last wash), day 1, day 3 and day 5 by
165 lysing the cells with 1 mL cold distilled water, and plating 10-fold serial dilutions on
166 Columbia agar plates containing 5% sheep blood (Biomérieux, Marcy l'Etoile, France) as
167 described previously.³⁰ Colony enumeration was performed after 5–7 days of incubation at 37
168 °C.

169 For luminescence measurement, cells were lysed in the same way with cold water and two
170 aliquots of 200 µL for each experiment point were transferred to a white Nunc-96-well plate
171 (Thermo Fisher Scientific). A solution of 1% decanal corresponding to 1/5th of the culture
172 volume to analyze (*i.e.* 40 µL) was injected into each well in the Fluoroskan reader (Thermo
173 Fisher Scientific) and luminescence was immediately read.

174

175 **MIC determination**

176 MIC of the different *M. abscessus* strains for amikacin were determined using 96 well round
177 bottom plates. First, amikacin was diluted in Mueller-Hinton at 2 × concentration in a volume
178 of 100 μL and a control well without antibiotic was prepared. The inoculum was adjusted to 4 ×
179 10⁷ cfu/mL in Cation-adjusted Mueller-Hinton broth. After dilution, 100 μL of a 1 × 10⁵ cfu/mL
180 dilution of the different strains was added to each well and incubated at 30 °C for 4 days.
181 Amikacin concentrations tested were spread out from 2 to 128 mg/L. MIC values were
182 determined using the resazurin assay and fluorescence measurement.³¹

183

184 **Confocal Microscopy**

185 The cultures were fixed 5 min for BCI-NS1.1 and 10 min for MucilAir in 4%
186 paraformaldehyde without methanol, rinsed, permeabilized with triton X100 0.5% and stained
187 with Hoechst (Sigma) or with fluorescent phalloidin-Atto (Sigma).

188 The morphology of the cells and the localization of the liposomes in the infected cells were
189 examined with a WLL confocal Leica SP8 microscope. Image acquisition was performed
190 under ×20 or ×40 apochromatic lens. Image analysis was performed with the Image J
191 software.

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193

194

195 **RESULTS**

196

197 **Liposomes co-localized with *M. abscessus* in infected macrophages and epithelial cell**
198 **cultures**

199 Our first objective was to visualize within the cell the colocalization of ALIS with *M.*
200 *abscessus*. These experiments were prepared with red-fluorescent amikacin-containing
201 liposomes and a GFP-expressing *M. abscessus* strain (Mabs-CIPS-GFP). The location of
202 bacteria and liposomal amikacin inside macrophages or human epithelial cells was observed
203 with a confocal microscope.

204 Macrophages and epithelial cells were infected with a multiplicity of infection (MOI) of 10,
205 and 10 to 20, respectively. Labelled liposomes were then added at a concentration of 64 mg/L
206 to observe its colocalization with the fluorescent mycobacteria inside the cells.

207

208 After 3 hours of infection with Mabs-CIPS-GFP at a MOI of 10, the macrophages displayed
209 numerous infected cells as previously described.²⁸ The J774.2 and THP-1 macrophages were
210 then exposed to 64 mg/L ALIS-TAMRA during 1, 4 and 24 hours. After 1 hour, the ALIS-
211 TAMRA liposomes were already observed in a close vicinity to intracellular bacteria (Figure
212 S1, available as Supplementary data). They were still present in this location at 4 h (Figure 1a
213 and 1c) and 24 h (Figure 1b and 1d) after the introduction of liposomal amikacin.

214 Differentiated epithelial cell cultures were more difficult to infect, therefore the MOI was
215 increased to 20. Our previous observations on infected MucilAir cultures revealed that
216 cultures exposed to Mabs-CIPS during one or two weeks display heterogeneous morphology,
217 with normal areas characterized by apicobasal polarization and typical basal actin network,
218 and various abnormal areas with a disturbed actin network in the depth of the epithelium,

219 reduced intercellular cell contacts and eventual local epithelial detachment from the support.
220 We chose to infect the cultures for several days in order to obtain enough infected cells. After
221 two weeks of infection, the MucilAir cultures were exposed to liposome ALIS-TAMRA at 64
222 mg/L. After short term exposure (24 h), there were no liposomes in these cells (data not
223 shown). Colocalization of liposomes and mycobacteria inside the cells of the MucilAir
224 cultures was observed after 2 or 4 days of liposome exposure (Figure 1e and 1f).

225 The differentiated BCI-NS1.1 cell line cultures were infected with Mabs-CIPS-GFP for 6 days
226 and treated with 64 mg/L ALIS-TAMRA during 3 to 6 days, because for a very short time
227 exposure (1 h and 4 h), there were no liposomes in the cells (data not shown). The cultures
228 displayed numerous infected cells with a clear intracellular colocalization of liposomes and
229 mycobacteria as soon as 3 days of liposome treatment (Figure 1g) and the liposomes were still
230 present after 6 days of treatment (Figure 1h).

231
232 **Effect of liposomal amikacin on intra-macrophage *M. abscessus* complex reference**
233 **strains: ALIS impairs *M. abscessus* intracellular growth in macrophages and in human**
234 **primary cells.**

235 Our second objective was to evaluate the intracellular anti-*M. abscessus* activity of ALIS. For
236 this, we had an excellent comparator with the water-soluble amikacin that we use to prevent
237 extra-cellular growth of mycobacteria,²⁸⁻³⁰ which has an inability to reach and inhibit
238 mycobacteria once internalized by the cells.

239 Preliminary experiments were performed with ALIS or ALIS-TAMRA. A range of
240 concentrations of these formulations were tested, from 8 to 64 mg/L. The most significant
241 results were obtained with concentrations of 64 mg/L and 32 mg/L.

242 *M. abscessus*-infected macrophages treated with ALIS displayed a decrease in intracellular
243 bacteria as compared to the water-soluble amikacin (at 50 mg/L) control group and to empty

244 liposomes. This difference was observed at 3 days post-infection (dpi) and at 5 dpi (Figure 2a
245 to 2d). The results were confirmed by two different methods, luminescence (Figure 2a and 2c)
246 and cfu counts per well (Figure 2b and 2d).

247
248 Bactericidal activity of ALIS was also observed against the two other *M. abscessus* subspecies,
249 i.e. *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* (Figure 3a to 3d). Results
250 presented in Fig. 3 are those obtained with a concentration of 64 mg/L, also with the two
251 methods, i.e. luminescence (Figure 3a and 3c) and cfu counts per well (Figure 3b and 3d).

252 Dendritic cells derived from a blood donor were also used as a host for *M. abscessus* infection
253 with the purpose of testing ALIS efficiency (Figure 3e). Similar results were obtained,
254 confirming the intracellular activity of ALIS against internalized *M. abscessus*.

255

256 **ALIS intracellular activity against *M. abscessus* clinical isolates**

257 A total of 53 clinical strains were tested with ALIS. Amikacin MIC vis-à-vis *M. abscessus*
258 clinical isolates was assessed by broth microdilution susceptibility testing as described.³² We
259 did not identify clinical isolates with MIC > 64 mg/L within the clinical strains tested (Table S1
260 in Supplementary data).

261 According to their growth profiles in macrophages in the presence of ALIS, clinical isolates can
262 be classified into three categories : (i) strains with a significant decrease in macrophage
263 bacterial load (Figure 4a) (phenotype 1); (ii) strains with continuous intra-macrophage growth
264 and with an absence of any growth difference compared to the control (Figure 4b) (phenotype
265 2); (iii) strains that do not grow in the presence of ALIS, or with a slight decrease, but who's
266 decrease was not sufficient to demonstrate a significant difference compared to the control
267 (Figure 4c) (phenotype 3). Overall, 79% of clinical isolates showed an intracellular growth
268 reduction (phenotype 1 and 3), and 21% showed intracellular growth (phenotype 2), in the

269 presence of ALIS. What was important to note was that these intracellular growth or non-
270 growth results in the presence of ALIS is independent of the MIC measurement with respect to
271 water-soluble amikacin (Table S1 in Supplementary data). The mean MIC of each group was
272 not correlated with *ex-vivo* susceptibility since the mean MIC of grouped phenotype 1 and 3 is
273 18.3 mg/L (\pm 8.1) and those of phenotype 2 group is 19 mg/L (\pm 8.5).

274

275 **DISCUSSION**

276 In the treatment of NTM infections, and particularly for *M. abscessus*, aminoglycosides like
277 amikacin are recommended as part of the current guideline-based therapy (GBT).³³ This
278 mycobacterium is present in patients in two distinct morphotypes, a rough (R) and a smooth
279 (S). If the first one is mainly extracellular and cord-forming, the second one is mainly
280 intracellular,²⁸ rendering it difficult to access with antibiotics. Intracellular *M. abscessus* can
281 indeed evade antimicrobial therapy because water-soluble amikacin is unable to reach this
282 niche to inhibit mycobacteria once internalized by the cells.²⁸⁻³⁰ Thus, the NTM phagocytosed
283 by circulating macrophages are protected from antimicrobial compounds and furthermore
284 propagate in the lungs, rendering these infections difficult to treat.³⁴ In order to improve access
285 of antibiotics to the bacteria, the use of liposomes can be of great use. They also have an
286 advantage in optimizing the diffusion of antibiotics into the interstitial and intracellular
287 environment, specifically in the presence of extracellular biofilms.

288

289 Liposomes have a well proven track record as useful vehicles for the transport of drugs for
290 which crossing of cell membranes is essential, such as ciprofloxacin or amphotericin B.^{35,36}
291 The first experiments to develop liposome-encapsulated amikacin date back to the late
292 1980's,^{8,37-40} with *M. avium* as a model target. Therefore, liposome-encapsulated antibiotics
293 like ALIS may be beneficial agents in the fight against nontuberculous mycobacterial
294 pulmonary infections, since NTM can evade antimicrobial therapy by sequestration into
295 macrophages. The fact that liposome formulations can penetrate intracellular spaces including
296 macrophages can bring an improvement to therapeutic candidates. ALIS have already shown
297 promise as antibacterial candidates, for example in CF patients to study responses to
298 *Pseudomonas aeruginosa* infections, *Mycobacterium avium* complex or *M. abscessus*
299 recently.^{6,17-22,41} To our knowledge, no example of eukaryotic intracellular simultaneous

300 colocalization of bacteria with antibiotics (delivered by liposomes) is described in the
301 literature. In this study, using different cellular models, ALIS can be found inside those cells
302 and in contact with intracellular *M. abscessus*. In addition, we have shown its efficacy in the
303 same cell models against numerous clinical isolates.

304

305 Liposomal amikacin was found in close proximity with bacteria in J774.2 and THP-1
306 macrophages after a short exposure time to ALIS (1 h) and also 4 and 24 h. Our results are in
307 accordance with previous *in vitro* observations of the penetration of liposomal-encapsulated
308 amikacin in macrophages at 24 h.²⁷ In this study, Zhang *et al.* demonstrated by flow
309 cytometry that ALIS-TAMRA is in higher concentration in the macrophages than free
310 TAMRA-conjugated amikacin (ratio around 5:1 for the concentration we used here, 64
311 mg/L). But, in this study, colocalization of ALIS with bacteria in eukaryotic cells was not
312 evaluated. In addition, we have evaluated the entry of liposomal amikacin into infected-
313 epithelial cells in two differentiated bronchial epithelial cell culture models, namely, MucilAir
314 and BCI.NS1.1. The cultures of these cells retain several characteristics of native lung tissue,
315 among them apicobasal morphological polarization, tight junctions, cilia and coordinated
316 ciliary beating, with mucus production.^{25,42,43} To our knowledge this is the first time that the
317 internalization of liposomal amikacin and its colocalization with bacteria has been observed in
318 differentiated human airway epithelial cells.

319

320 We have shown in macrophages cell-models that this colocalization was correlated with a
321 reduction of the intracellular bacterial load, as measured by a decrease in mycobacterial cell
322 counts or the luminescence emitted by mycobacteria in infected cells at different time points
323 after infection. The efficacy of ALIS in reducing the bacterial load in infected cells was

324 observed in macrophage based cellular models (THP-1 and J774.2), but also in primary
325 dendritic cells derived from circulating human monocytes.

326

327 Finally, ALIS activity was shown both against the reference *M. abscessus sensu lato* strains,
328 and against a wide range of *M. abscessus* clinical isolates. All strains were *M. abscessus*
329 subsp. *abscessus*. If we amalgamate the results from the range of strains (group 1) for which
330 we observe a clear significant bacterial load reduction compared to untreated macrophages
331 and the third phenotype (group 3) for which ALIS was merely bacteriostatic (leading to no
332 significant difference with untreated macrophages). We therefore obtained a total of 42 strains
333 out of 53, for which ALIS had an effect on inhibiting intra-macrophagic growth.

334 We observe three phenotypes, and these three phenotypes are independent of the MICs
335 obtained for all isolates. We had a majority of smooth strains, with only 8 rough strains, but
336 which were in the two groups for which inhibitory activity is observed. Several studies have
337 shown an increase in the MIC of amikacin according to the S or R morphotype.^{44,45} Although
338 the number of R isolates is low, we cannot attribute these different phenotypes to the
339 morphology of the mycobacterium. All tested isolates belonged to the subspecies *abscessus*.
340 Here again we cannot imply a different subspecies which would be more or less sensitive to
341 the intracellular action of amikacin. A final aspect would be to sequence the complete genome
342 of the isolates to see if other genome-wide information, other than a mutation correlating with
343 a decrease in sensitivity to amikacin, not observed here by the comparison of MIC averages
344 by group, would indicate a different intracellular behavior between the 3 groups of isolates.
345 Several factors already described in our laboratory and by others, may favor the intracellular
346 growth of one isolate compared to another as recently described with the emergence of more
347 virulent clones, among all the clones isolated in the world.^{46,47} We believe that this reflects a
348 variety in the lifestyle of *M. abscessus* strains within an intra-macrophagic environment

349

350 In conclusion, we were able to observe colocalization of liposomal amikacin and *M.*
351 *abscessus* in various cellular strains belonging to different types of cells, macrophages and
352 epithelial cells. We have also shown that, contrary to water free-amikacin, liposomal amikacin
353 was active against intra-macrophagic *M. abscessus* at a conventionally used concentration and
354 that these inhibitory effects were also observed with numerous tested clinical isolates.

355

356

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363

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366

367 **Transparency declarations section**

368 None to declare.

369

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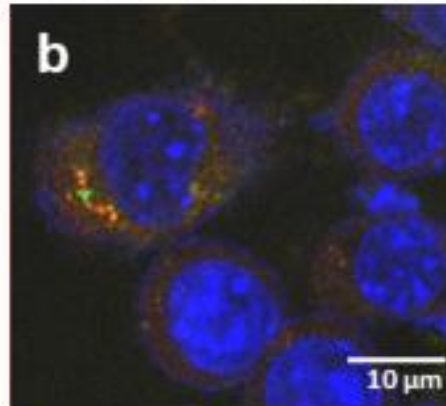
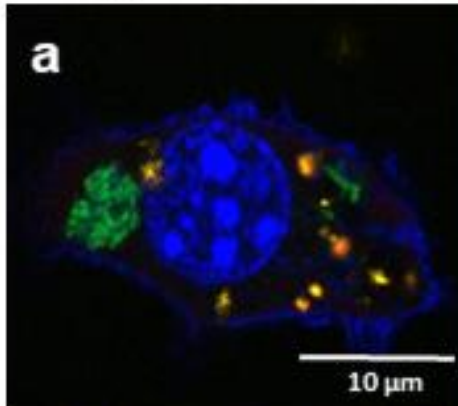
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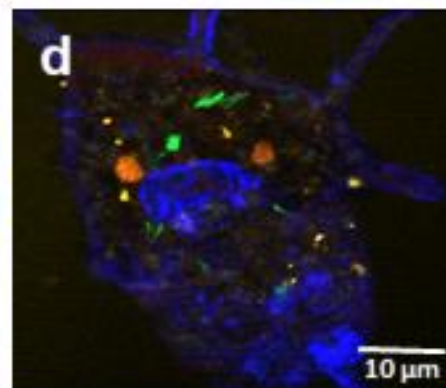
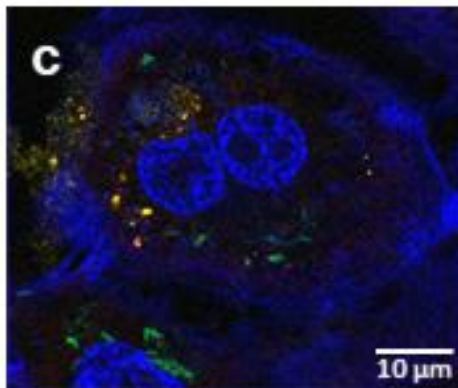
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Early observation **Late observation**

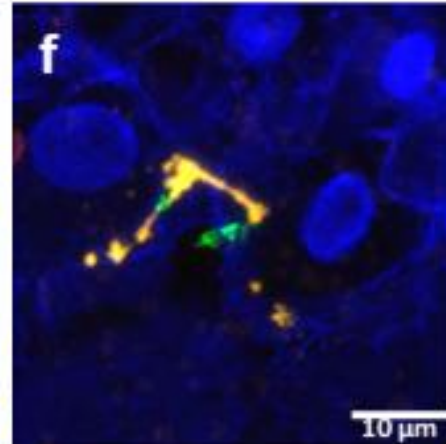
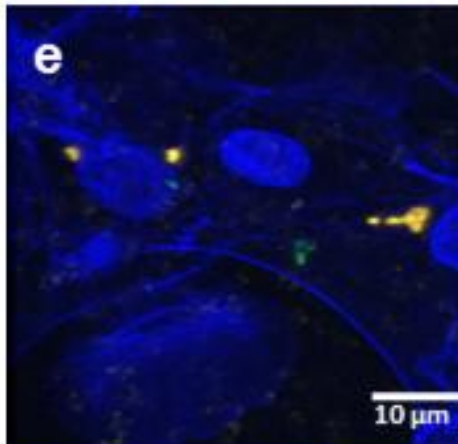
J774.2



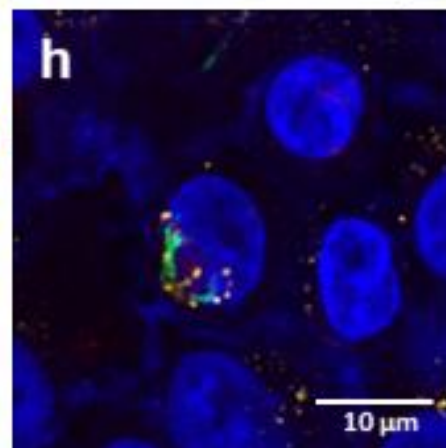
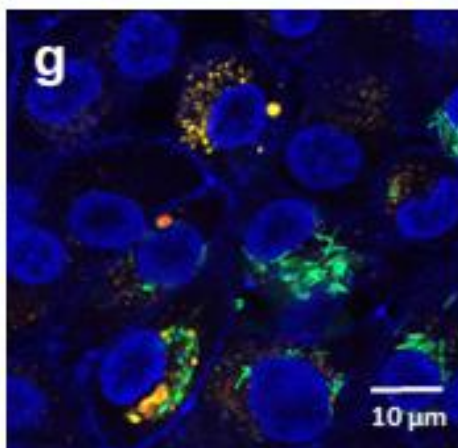
THP-1

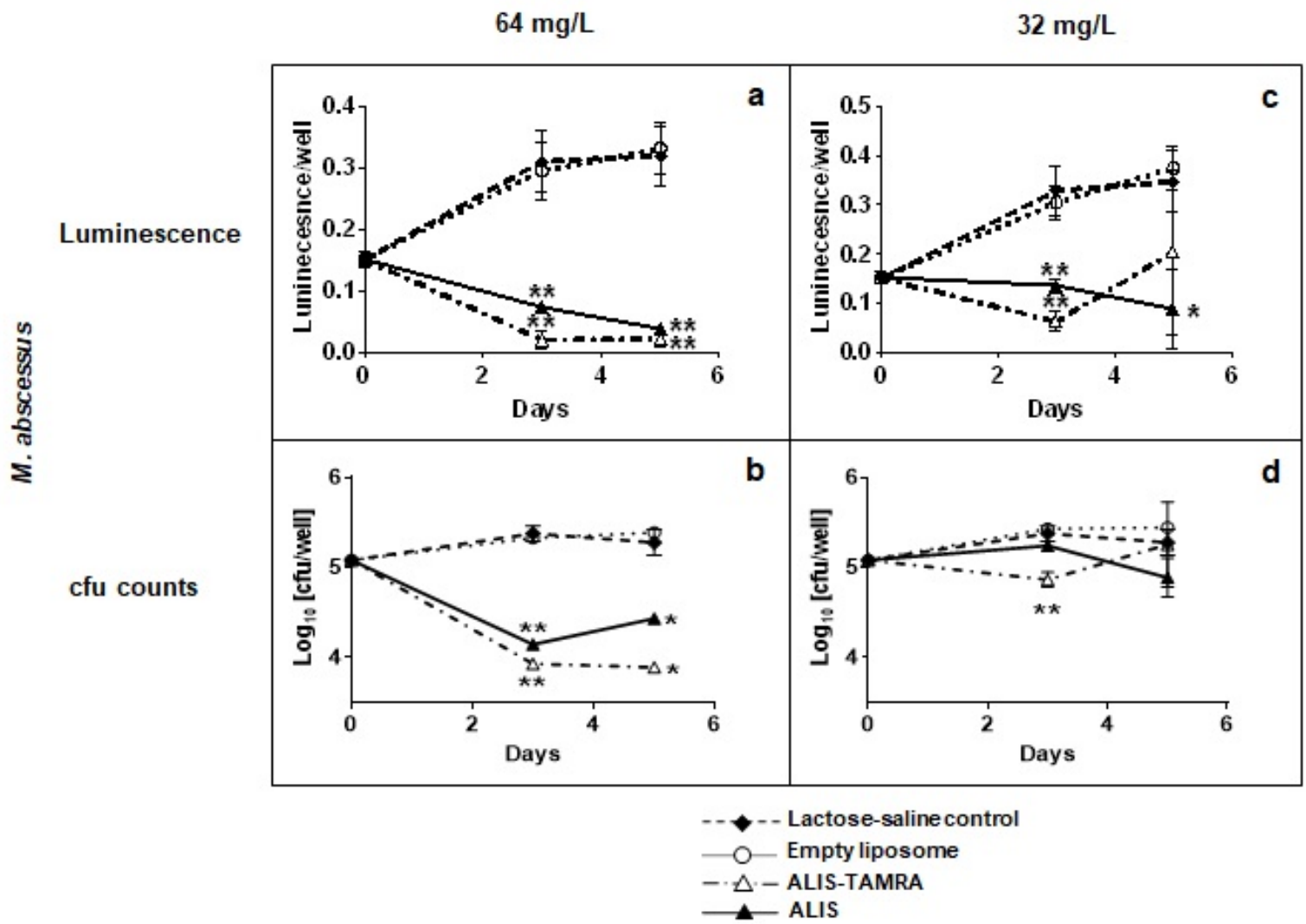


MucilAir
(Differentiated primary bronchial epithelium)



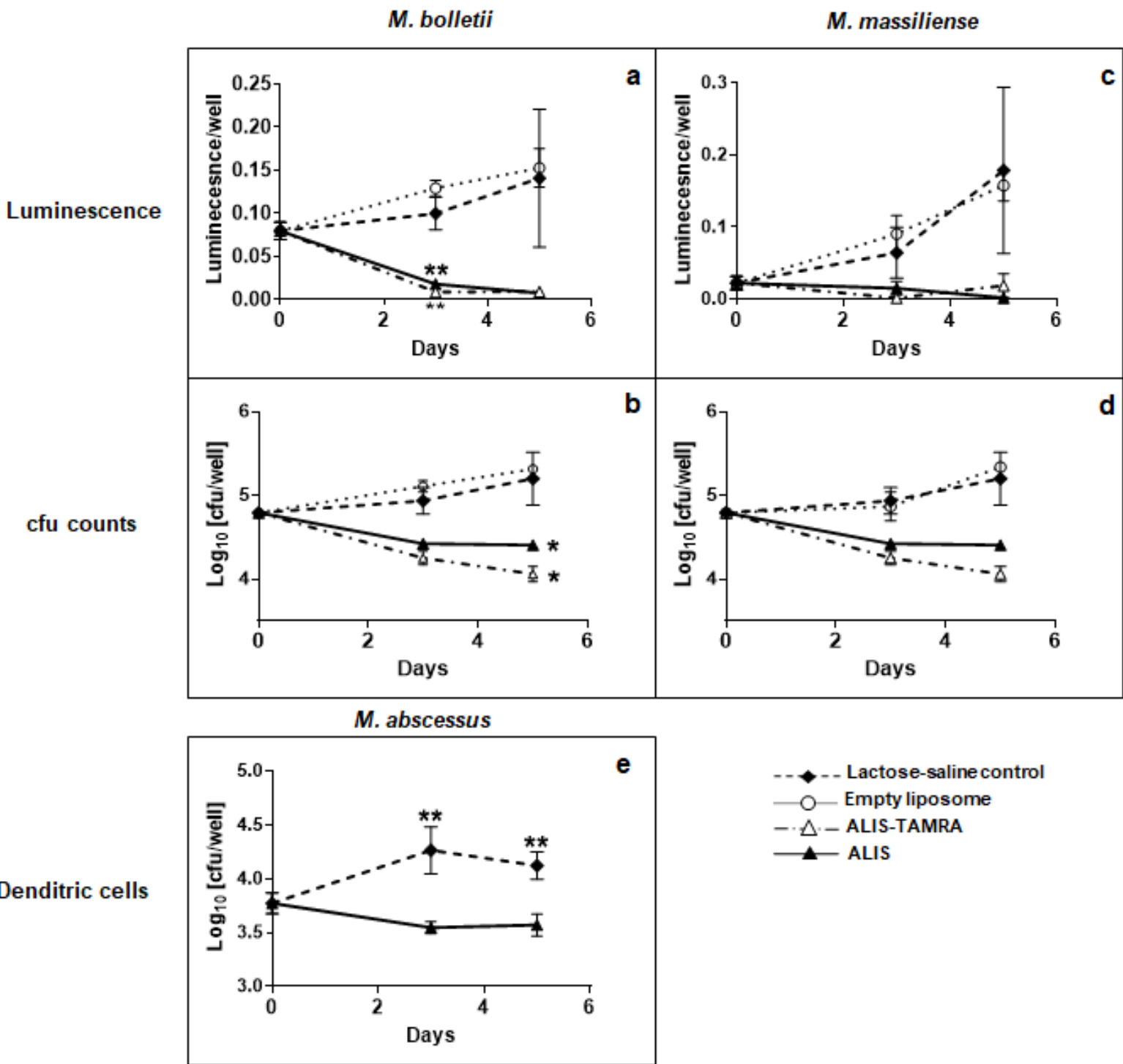
BCi-NS1.1
(Differentiated bronchial epithelial cells)



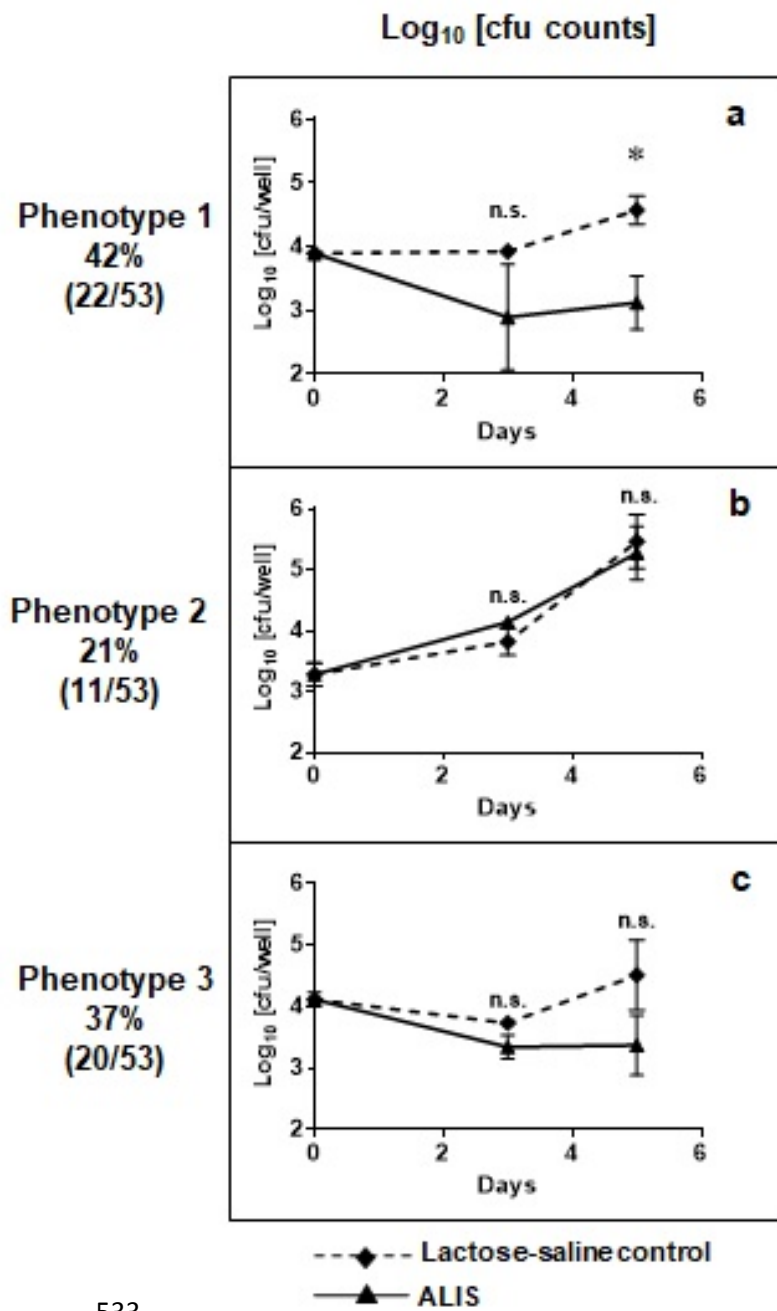


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532 **Figure 4**



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537 **Caption to Figures :**

538 **Figure 1 :** ALIS-*Mycobacterium abscessus* colocalization inside eukaryotic cells at different
539 time-points of ALIS treatment of Mabs-CIPS-GFP-infected cell cultures : J774.2 (a, b), THP-
540 1 (c, d), MucilAir (e, f) and BCI-NS1.1 cell cultures (g, h). Cell outline and nuclei were
541 highlighted by F-actin staining with 1/400 phalloidine-atto 390nm (Sigma Aldrich) and DNA
542 staining with 1/1000 Hoechst 33342 (Sigma Aldrich), respectively. For liposomal amikacin,
543 excitation and emission wavelengths were as follows: amikacin-TAMRA (ex 546 nm/em 579
544 nm), liposome Alexafluor (ex 650 nm/em 665 nm). Yellow: amikacin-TAMRA; Green:
545 Mabs-CIPS-GFP; Blue: DAPI-stained DNA.

546

547 **Figure 2 :** Quantification of intra-macrophagic *M. abscessus* presence by two different
548 methods, cfu counts (b and d) and luminescence (a and c), after liposomal amikacin treatment
549 or empty liposome (without amikacin) (open circles). Dilution liposome solution (lactose-
550 NaCl) was also used as control (filled diamond). Efficiency of ALIS (filled triangle) and
551 ALIS-TAMRA (open triangle) was tested at two different concentrations, 64 (a and b) and 32
552 mg/L (c and d), after 3 and 5 days of treatment in infected J774.2 macrophages. Differences
553 between means were analyzed by two-way ANOVA and the Tukey post-test, allowing
554 multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

555

556 **Figure 3 :** Quantification of intra-macrophagic *M. bolletii* (a and b) and *M. massiliense* (c and
557 d) presence by two different methods, cfu counts (b and d) and luminescence (a and c), after
558 liposomal amikacin treatment. Efficiency of liposomal amikacins (ALIS (filled triangle) and
559 ALIS-TAMRA (open triangle)) was tested at the concentrations of 64 mg/L, after 3 and 5
560 days of treatment in infected J774.2 macrophages. Dilution liposome solution (lactose-NaCl)

561 (filled diamond) and empty liposome (without amikacin) were used as control (open circles).
562 (e) cfu count of *M. abscessus* CIP S strain at 3 and 5 days in dendritic cells from a blood
563 donor with or without ALIS treatment at 64 mg/L. Differences between means were analyzed
564 by two-way ANOVA and the Tukey post-test, allowing multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

566

567 **Figure 4 : ALIS intracellular activity against *M. abscessus* clinical isolates in J774.2**
568 **macrophages.** Clinical isolates were tested against an ALIS concentration of 64 mg/L, after 3
569 and 5 days of treatment (filled triangle), in infected J774.2 macrophages or lactose-NaCl
570 control (filled diamond). Graphs presented are those of an individual strain representative of
571 each group, i.e., strains with a significant decrease in macrophages bacterial load (a), strains
572 with a continuous intra-macrophage growth and with an absence of growth difference with the
573 control (b) and strains that do not growth in presence of ALIS, or with a slight decrease, but
574 whose decrease is not sufficient to present a significant difference with the control (c).
575 Individual strains MIC and the mean MIC (\pm SEM) in mg/L of each population group is
576 indicated in Supplementary Table 1. Differences between means were analyzed by two-way
577 ANOVA and the Tukey post-test, allowing multiple comparisons. n.s. = non-significant; *, $p <$
578 0.05 ; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

579