Liposomal Amikacin and Mycobacterium abscessus: Intimate interactions inside eukaryotic cells

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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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ABSTRACT (Words count: 246)

Background

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

Objectives

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

Methods

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

Results

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

Conclusions

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

INTRODUCTION

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

Inhaled antibiotics packaged in a liposomal formula have been proposed as a treatment for chronic lung infections for some time.[8,9] They were first suggested as therapies against fungal infections[10] and, some years later, against bacterial infections[11-14] and notably in the context of CF. [6,15-18]

The treatment with amikacin liposomal inhalation suspension (ALIS) has already been tested in CF patients to study responses against *Pseudomonas aeruginosa* infections[6,18,19], *Mycobacterium avium* complex[20-22] and recently in France, *M. abscessus*.[23]

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

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

MATERIALS AND METHODS

Mycobacterial strains, cells and culture conditions.

Mycobacterial strains were grown aerobically at 37 °C in Middlebrook 7H9 and on 7H11 (Sigma-Aldrich, Saint-Quentin-Fallavier, France) medium supplemented with 0.2% glycerol and 1% glucose. When necessary, hygromycin (Invivogen, Toulouse, France), was added to the growth medium at 1,000 mg/L. This was the case when we transformed the ATCC 19977 reference of *M. abscessus* strain with the plasmid pSMT3-GFP-LuxAB. This transformed strain (Mabs-CIPS-GFP) allowed luminescence and fluorescence for measurement of bacterial load and localization by confocal microscopy, respectively. The tested clinical strains were provided from the strain collection of the Raymond Poincaré hospital-APHP.[24]

For infection, mycobacteria were grown to mid-log phase in liquid medium and harvested by centrifugation and suspended in a PBS solution. After 3 washes, the bacterial clumps were disrupted by 20–30 passages through a 29G needle and the bacterial suspension was aliquoted and stored at -80 °C before use.

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

Murine J774.2 macrophages (MΦ) (Sigma-Aldrich) were grown in DMEM-Glutamax medium supplemented with 5% heat-inactivated FBS (Thermo Fisher Scientific, USA), penicillin (100 IU/mL) and streptomycin (100 mg/L) at 37 °C in a humidified atmosphere of 5% CO_2. The human monocytic cell line THP-1 was maintained in RPMI 1640-Glutamax (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS at 37 °C with 5% CO_2. For differentiation into macrophages-like cells, 2×10^5 THP-1 cells were seeded into 24-well plates and activated with 100 ng/mL of Phorbol 12-Myristate 13-Acetate (Sigma-Aldrich) for 24 h.

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

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

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

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

Reagents

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

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

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

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

MΦ infection and intracellular growth measurement

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

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

Cfu counts and luminescence measurement

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

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

MIC determination

MIC of the different *M. abscessus* strains for amikacin were determined using 96 well round bottom plates. First, amikacin was diluted in Mueller-Hinton at 2 × concentration in a volume of 100 µL and a control well without antibiotic was prepared. The inoculum was adjusted to 4×10^7 cfu/mL in Cation-adjusted Mueller-Hinton broth. After dilution, 100 µL of a 1×10^5 cfu/mL dilution of the different strains was added to each well and incubated at 30 °C for 4 days. Amikacin concentrations tested were spread out from 2 to 128 mg/L. MIC values were determined using the resazurin assay and fluorescence measurement.[31]

Confocal Microscopy

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

Bactericidal activity of ALIS was also observed against the two other *M. abscessus* subspecies, i.e. *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* (Figure 3a to 3d). Results presented in Fig. 3 are those obtained with a concentration of 64 mg/L, also with the two methods, *i.e.* luminescence (Figure 3a and 3c) and cfu counts per well (Figure 3b and 3d).
Dendritic cells derived from a blood donor were also used as a host for *M. abscessus* infection with the purpose of testing ALIS efficiency (Figure 3e). Similar results were obtained, confirming the intracellular activity of ALIS against internalized *M. abscessus*.

ALIS intracellular activity against *M. abscessus* clinical isolates

A total of 53 clinical strains were tested with ALIS. Amikacin MIC vis-à-vis *M. abscessus* clinical isolates was assessed by broth microdilution susceptibility testing as described.[32] We did not identify clinical isolates with MIC > 64 mg/L within the clinical strains tested (Table S1 in Supplementary data).
According to their growth profiles in macrophages in the presence of ALIS, clinical isolates can be classified into three categories : (i) strains with a significant decrease in macrophage bacterial load (Figure 4a**)** (phenotype 1); (ii) strains with continuous intra-macrophage growth and with an absence of any growth difference compared to the control (Figure 4b**)** (phenotype 2); (iii) strains that do not grow in the presence of ALIS, or with a slight decrease, but who's decrease was not sufficient to demonstrate a significant difference compared to the control (Figure 4c**)** (phenotype 3). Overall, 79% of clinical isolates showed an intracellular growth reduction (phenotype 1 and 3), and 21% showed intracellular growth (phenotype 2), in the

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

DISCUSSION

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

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

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

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

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

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

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

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

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

AKNOWLEDGMENTS

We thank INSMED for providing us all the preparations of liposomal amikacin. We also warmly thank Dr Ben Marshall (University Hospitals of Southampton, UK) for a thorough review and correction of the manuscript.

These results were presented as a poster form at the 31st European Congress of Clinical Microbiology & Infectious Diseases (ECCMID), taking place from 9th to 12th July 2021

FUNDINGS

This study was supported by internal funding.

Transparency declarations section

None to declare.

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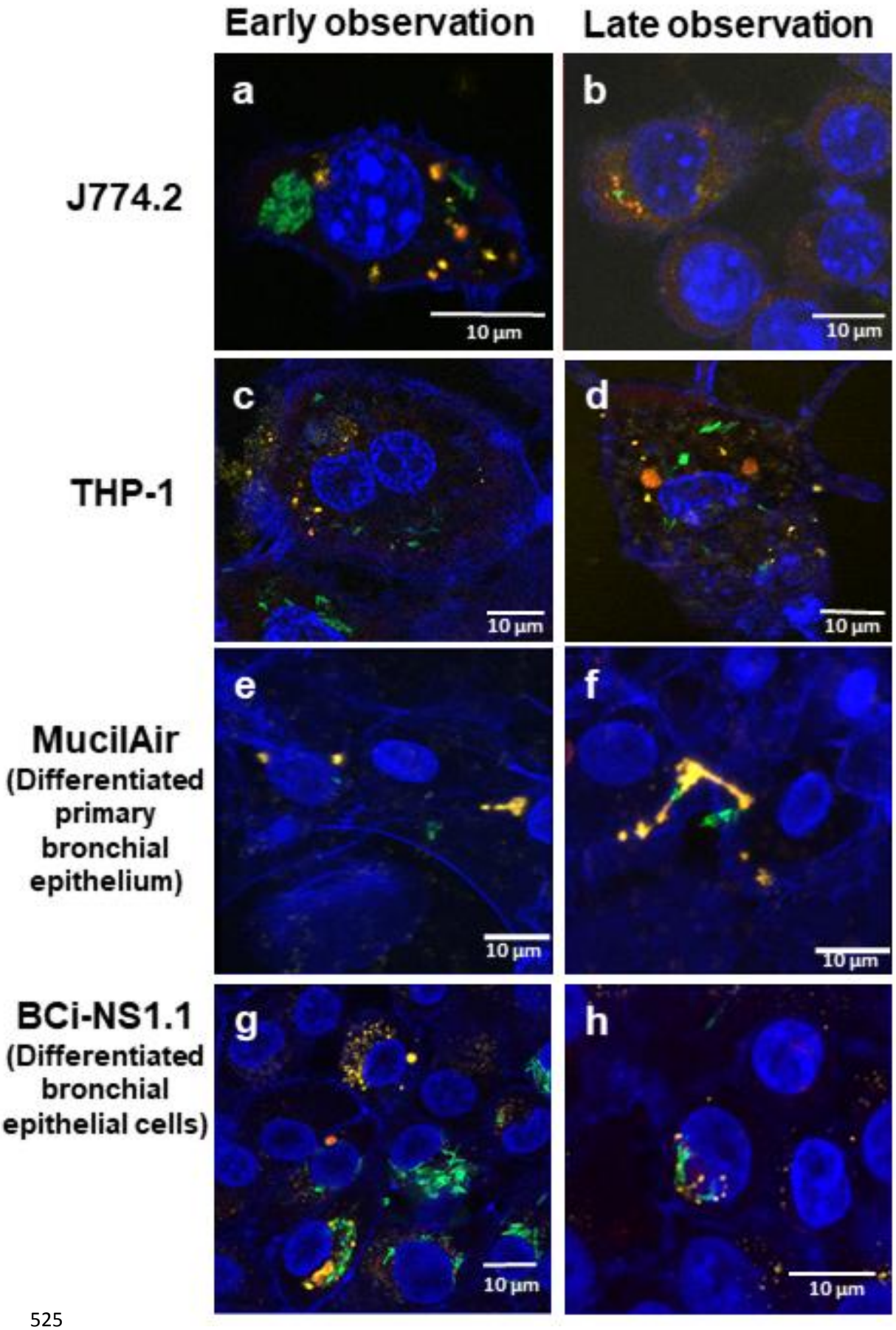
524 **Figure 1**



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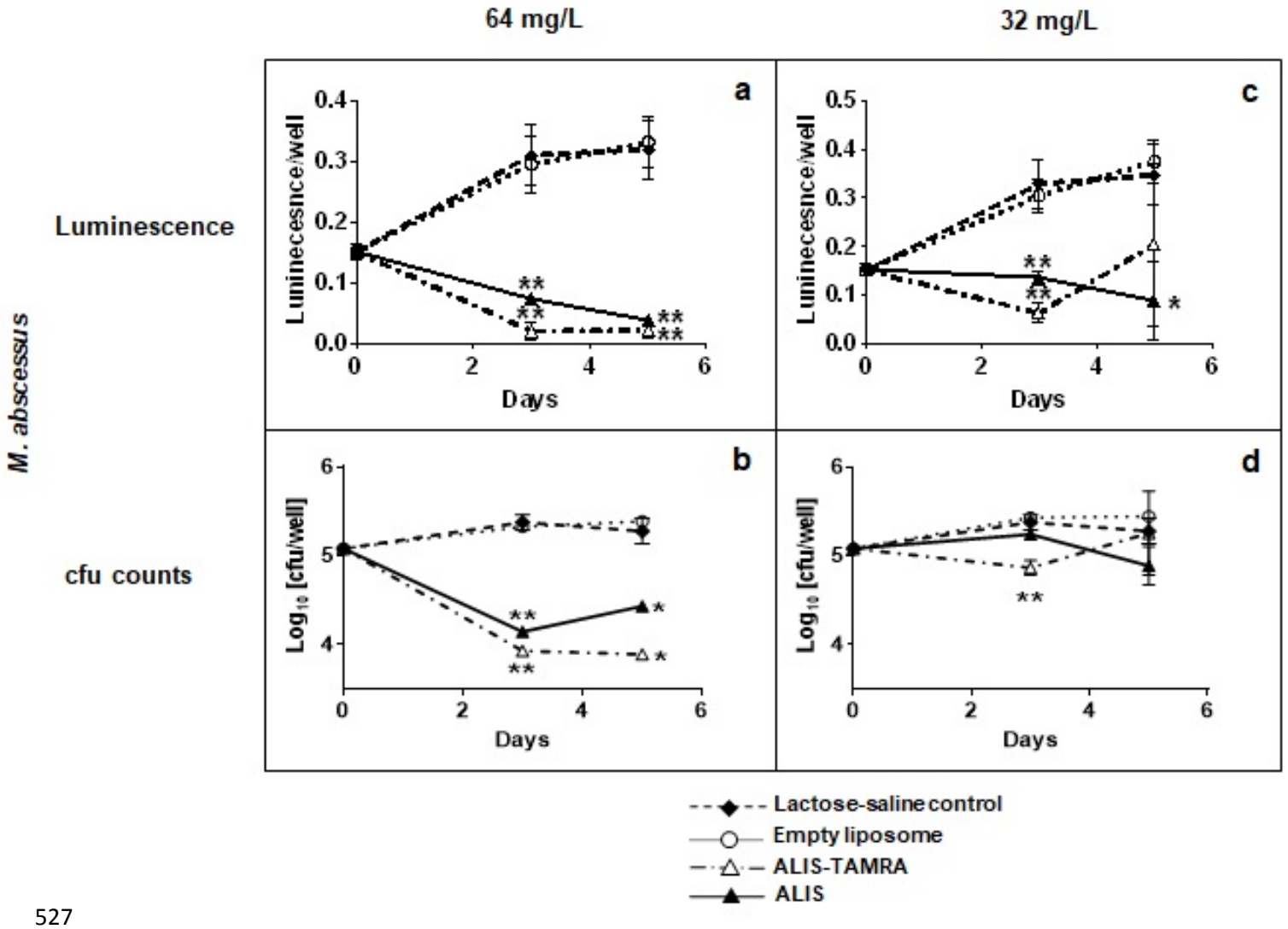
526 **Figure 2**



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529 **Figure 3**

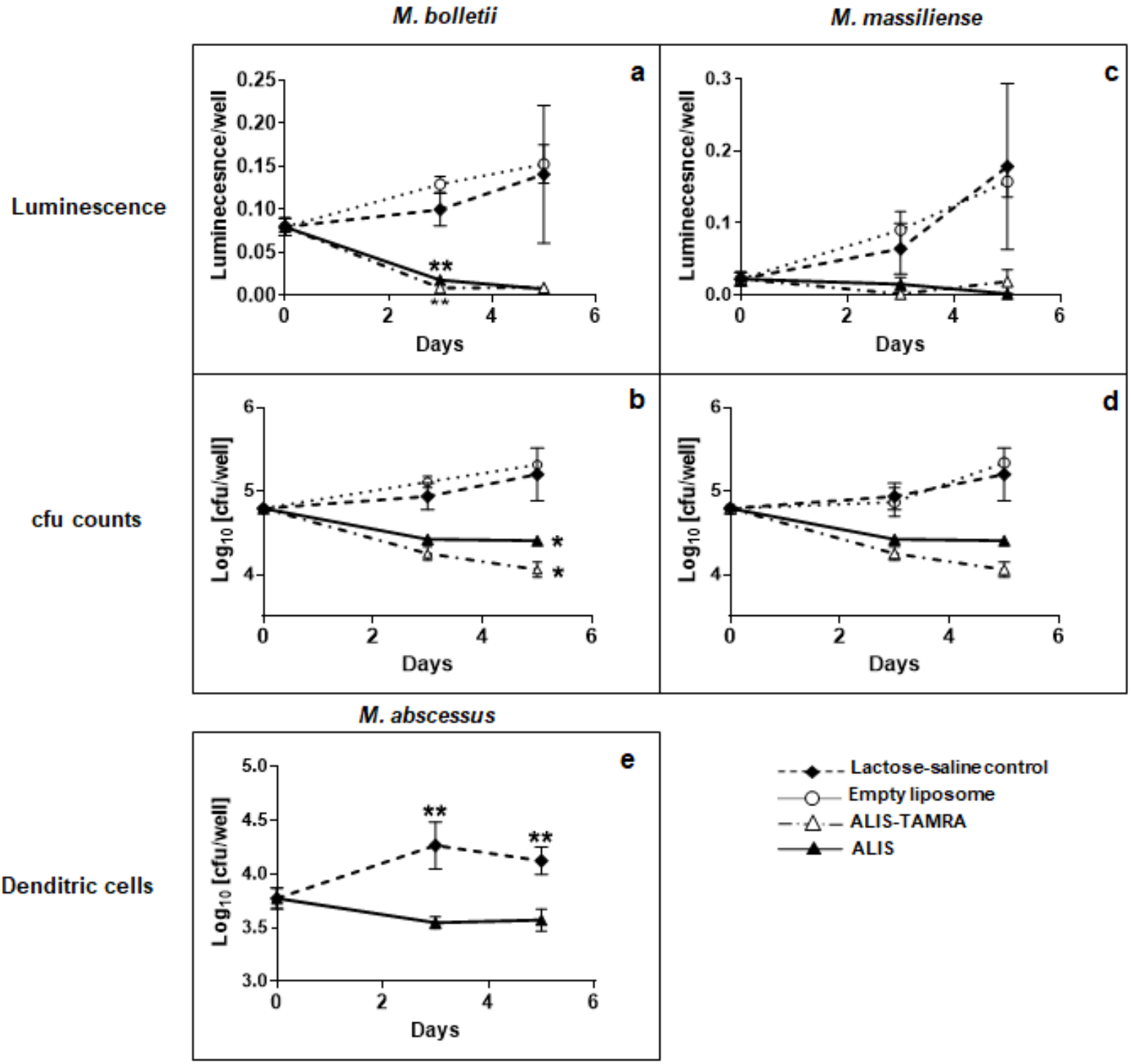

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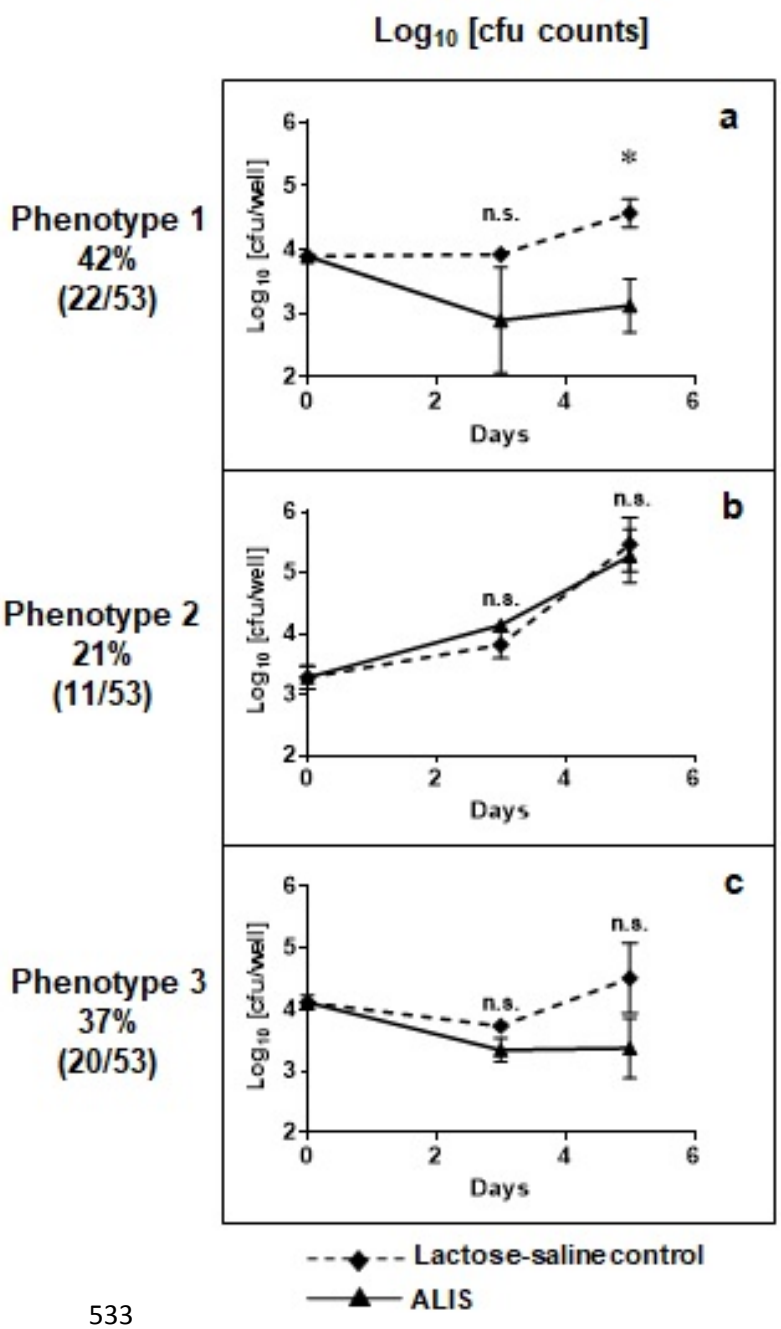
Figure 4


Log10 [cfu counts]
Phenotype 1
42%
(22/53)
Phenotype 2
21%
(11/53)
Phenotype 3
37%
(20/53)
a
b
c
n.s.
*
Log10 [cfu/well]
Days
Lactose-saline control
ALIS

Caption to Figures :

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

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

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

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

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