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Efficacy of bedaquiline, alone or in combination with imipenem, against *Mycobacterium abscessus* in C3HeB/FeJ mice

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ABSTRACT (75 words)

Mycobacterium abscessus lung infections remain difficult to treat. Recent studies have recognized the power of new combinations of antibiotics such as bedaquiline and imipenem although *in vitro* data have questioned this combination. We report that the efficacy of the bedaquiline plus imipenem treatment relies essentially on the activity of bedaquiline in a C3HeB/FeJ mice model of infection with a rough variant of *M. abscessus*. The addition of imipenem contributed at clearing the infection in the spleen.

Note (1074 words)

Mycobacterium abscessus is a rapidly growing mycobacterial species, whose infections remain very difficult to treat, due to the limited panel of available antibiotics (1). Among them, the β-lactams, imipenem (IPM) and cefoxitin (FOX), are part of the *M. abscessus* multidrug therapy along with amikacin (AMK) and clarithromycin (CLR) (2-5). In addition, the development of specific β-lactamase inhibitors, enhancing the efficacy of IPM *in vitro* and *in vivo*, broadens the use of IPM in *M. abscessus* drug therapy (6-8). Other studies highlighted the potential of testing new drug combinations that include IPM and are associated with increased efficacy against *M. abscessus* (6, 9, 10), yet questioning the relevance of the bedaquiline (BDQ) plus IPM combination (11). BDQ targets the ATP synthase and exhibits activity against a wide panel of *M. abscessus* clinical isolates *in vitro* and in infected zebrafish, although its effect is bacteriostatic only (12). A recent study suggested that, by reducing the intracellular pool of ATP in *M. abscessus*, BDQ suppresses the effect of IPM and FOX, although the effect of the BDQ plus IPM combination was considered additive (11). This led the investigators to conclude that the addition of BDQ to a β-lactam-containing regimen may negatively affect the treatment outcome (11). In comparison, data from the hollow fiber model highlight that β-lactam is the most active and important part of the *M. abscessus* regimen (13). That these studies focused exclusively on the interaction of β-lactams and BDQ *in vitro*, confirmatory results in a pre-clinical animal model are warranted.

Herein, we explored the therapeutic efficacy of BDQ or IPM, alone or in combination, using the immunocompetent C3HeB/FeJ mouse model of *M. abscessus* infection. C3HeB/FeJ mice are highly susceptible to mycobacterial infections, particularly to *Mycobacterium tuberculosis* due to a deletion on the *Ipr1* (Intracellular pathogen resistance 1) gene located in the locus called *sst1* (14, 15). All animal experiments were performed according to ethical guidelines and with ethical committee (N°047 with agreement A783223) agreement APAFIS#11465.

First, we evaluated, the *in vitro* interaction between BDQ and several β-lactams or CLR against *M. abscessus* CIP104536 strain in cation-adjusted Mueller-Hinton broth (CaMHB) (Becton-Dickinson, Le Pont-de-Claix, France) using a 2-dimensional microdilution checkerboard method, as previously described (16-19). Our results confirm that the β-lactam plus BDQ combinations are indifferent, as it is the case with the CLR plus BDQ combination (Table 1).

Next, the performances of pulmonary and intravenous (IV) infection routes were compared in C3HeB/FeJ mice. Mice were infected intratracheally using agar bead-embedded bacteria to maintain a persistent infection, as reported previously for *Pseudomonas aeruginosa* (20). A significant increase in mortality was noticed when mice were infected intratracheally with a solution of agar beads containing 2.10^5 CFUs/mouse in 50 µl, leading only to 40% of mouse survival at 14 days post-infection (dpi) (see Fig. S1A in the supplemental material) correlated with an important increase in the CFU at 14 dpi suggesting accelerated bacterial growth in the lungs (Fig. S1B). In contrast, persistence occurs for up to 25 days after IV infection with 10^6 CFU/mouse as evidenced by CFU counting after plating of the organ homogenates (Fig. 1 and Fig. S2A and S2B) although as soon as the injected dose is less than 10^6 CFU, persistence in the organs is reduced (Fig. S2B). This represents an important asset over previously described murine models, characterized by a more rapid bacterial clearance (21-23).

The IV route of infection was subsequently used to evaluate and compare the activity of BDQ and AMK. Because AMK is bactericidal against *M. abscessus* while BDQ is bacteriostatic *in vitro*, we wondered whether BDQ would be more effective than AMK in an *in vivo* infection model. CFU were significantly reduced in mice receiving 30 mg/kg BDQ (oral administration) as compared to mice treated with 150 mg/kg AMK (subcutaneous administration) in the lungs and the spleen at 12 and 25 dpi (Fig. 2A and 2B). No significant differences were observed between the BDQ- or AMK-treated animals in the spleen at 12 dpi, but bacterial loads in these two groups were significantly lower compared to the control group (oral administration of DMSO) (Fig. 2C).

The efficacy of BDQ in this infection model prompted us to compare it with IPM (subcutaneous administration) either alone or as a companion drug, for 15 days of treatment. No significant differences were noticed between the animals treated with BDQ alone and the animals treated with BDQ plus IPM at 12 and 20 dpi, with the exception of the liver at 12 dpi (Fig. 3A to 3C), indicating that the overall activity of the BDQ plus IPM combination was mainly due to the intrinsic activity of BDQ. In general, BDQ alone or in combination with IPM exhibited an increased activity as compared to IPM in the liver and spleen but not in the lungs (Fig. 3). The spleens of treated and untreated mice were weighed as an additional marker of the effectiveness of the various treatments. These measures indicated that only treatments with BDQ plus IPM or IPM alone were associated with lower spleen weights, as compared to those of the untreated or BDQ-treated mice (Fig. 3D). Collectively, the reduced

bacterial burden, together with the lower spleen weights represent a marker for improved outcome of the infection.

BDQ is a diarylquinoline approved by the Food and Drug Administration and the European Medicines Agency for the treatment of multidrug-resistant tuberculosis. It is bacteriostatic against *M. abscessus in vitro,* displaying MIC_{50} of 0.125 µg/ml and a MIC_{90} > 16 µg/ml, and ECOFF values demonstrates that BDQ exhibits moderate activity (16, 24). Discordant results regarding the efficacy of BDQ were generated in various immunocompromised mouse models, raising the question of the influence of immunosuppression on antibiotic efficacy (25, 26). However, efficient responses to BDQ were observed in other animal models, such as zebrafish (12). Two studies reported poor or negative results for BDQ administration against NTM infected patients (27, 28). However, recent studies showed that the activity of BDQ can be potentiated with adjunctive therapy, by so improving BDQ-based treatments (16, 29). This study provides evidence that the BDQ plus IPM combination remains superior to IPM alone and equivalent to BDQ alone as judged by the comparable bacterial clearance in the spleens of the mice treated with BDQ plus IPM as compared to BDQ alone.

In summary, the IPM plus BDQ combination enhances the clearance of the infection. This supports also the importance of evaluating antibiotic activity in combination rather than separately against this highly drug-resistant mycobacterium.

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AUTHOR CONTRIBUTIONS STATEMENT

JLH, JN and ON designed the project and experiments; VLM, CR, FM and CD performed the experiments; VLM, CR, JN, ON, LK and JLH wrote and corrected the manuscript.

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Table 1. Interaction between bedaquiline and other drugs against *M. abscessus* CIP104536^T

Compound	MIC* (mg/l)	Interaction with BDQ		
		FICI$^{\$}$ (mean)	SD	Outcome
BDQ	0.125	-	-	-
IPM	16$^{\&}$	0.55	±0.06	Indifferent$^{£}$
FOX	32	0.52	±0.03	Indifferent
CLR	2	0.61	±0.09	Indifferent
AMP	>512	0.57	±0.02	Indifferent

(*) MIC were evaluated by REMA checkerboard assay in cation-adjusted Mueller-Hinton broth (CaMHB) (Becton-Dickinson, Le Pont-de-Claix, France). 10^5 bacteria were diluted in Mueller-Hinton media (Sigma-Aldrich). Plates were incubated for 3 days at 30°C then 20 µL (10% v/f) of Resazurin 0.025% were added to the wells and plates were incubated overnight at 30°C.

($) The fractional inhibitory concentration index (FICI) was calculated as follows: FICI = (MIC drug A in combination/MIC drug A alone) + (MIC drug B in combination/MIC drug B alone), where drug A was bedaquiline (BDQ) and drug B was clarithromycin (CLR, Sigma-Aldrich, France), imipenem (IPM, Mylan S.A.S, France), cefoxitin (FOX, Panpharma, France) or ampicillin (AMP, Euromedex, France).

(£) Interaction between the two compounds was defined as synergistic when FICI value was ≤0.5, indifferent when 0.5< FICI ≤4, and antagonistic when FICI was >4.

(&) Values showed in the table are the mean of four independent experiments ±SD.

Legend to figures

Figure 1. Bacterial persistence of *M. abscessus* CIP 104536^T (rough variant) in the lungs, spleen and liver of C3HeB/FeJ mice after infection in the tail vein with 10^6 CFU/mouse in a total volume of 200 µl of water containing 0.9% sodium chloride. The following day, three mice were euthanized and whole organs were harvested to determine baseline bacterial burden. Mouse lungs, spleens and livers were homogenized, serially diluted and plated onto VCAT (Vancomycin, Colistin sulfate, Amphotericin B, and Trimethoprim) chocolate agar plates (BioMérieux, France) and incubated for 5-6 days at 37°C prior to CFU count. Results are expressed as the $\log_{10}$ units of CFU at 1, 12 and 25 dpi.

Figure 2. *M. abscessus* R-infected C3HeB/FeJ mice (9.2×10^5 CFU/mouse) treated with Bedaquiline (BDQ) or Amikacin (AMK). Bacterial counts in the lungs (A), liver (B) and spleen (C) of C3HeB/FeJ mice infected IV, as described in Fig. 1. Antibiotic treatment began at 2 dpi. Mice were treated starting on day 2 for 7 days (D12) or 17 days (D25) by daily subcutaneous injections of 150 mg/kg AMK (Mylan laboratories) in saline solution or daily oral gavage of 30 mg/kg BDQ at in a total volume of 200 µl (BDQ solution in DMSO was diluted in 20 % 2-hydroxypropyl-β-cyclodextrin). A control group received a daily subcutaneous injection of saline and oral gavage of DMSO containing 20 % 2-hydroxypropyl-β-cyclodextrin. All solutions were administered five times weekly for latter time point. Mice were euthanized 3 days after antibiotic cessation to allow antibiotic clearance. Furthermore, given the long half-life and high protein binding capacity of BDQ, spleens, livers and lungs from drug-treated and control mice were homogenized in water supplemented with 10% bovine serum albumin (30) before dilution. Experimental groups of mice were evaluated for bacterial burden on day 1 (before treatment started), 12 and 25 as described in Fig. 1. n = 5 mice were used per experiment and bacterial load in each group are expressed as $\log_{10}$ units of CFU (± SD) cells. 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*. Experiment was realized once.

Figure 3. *M. abscessus* R-infected C3HeB/FeJ mice treated (2.7×10^5 CFU/mouse) by Bedaquiline (BDQ), Imipenem (IPM) or BDQ plus IPM. Bacterial loads in the lungs (A), liver (B) and spleen (C) were determined as reported in Fig. 1. Relative weight of spleen to mouse weight are shown in (D). Antibiotic treatment began 2 days after infection. Mice were treated starting on day 2 for 7 days (D12) or 13 days (D20) with twice daily (*i.e.* every 12 h) subcutaneous injection of IPM (MSD laboratories, France) in saline solution at 100 mg/kg or daily oral gavage of BDQ as described in Fig. 2 or both IPM plus BDQ. Experimental groups of mice were evaluated for bacterial burden on day 1 (before treatment started), 12 and 20 as described in Fig. 1. (D) Mouse spleens were weighed at 1, 12 and 20 dpi. The value represents the relative weight of each spleen relative to the weight of the mouse from which they were collected. n = 5 mice were used per experiment and bacterial load in each group are expressed as $\log_{10}$ units of CFU (± SD) cells. Differences between means were analyzed by two-way ANOVA and the Tukey post-test, allowing multiple comparisons. n.s. = non-

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Figure 1

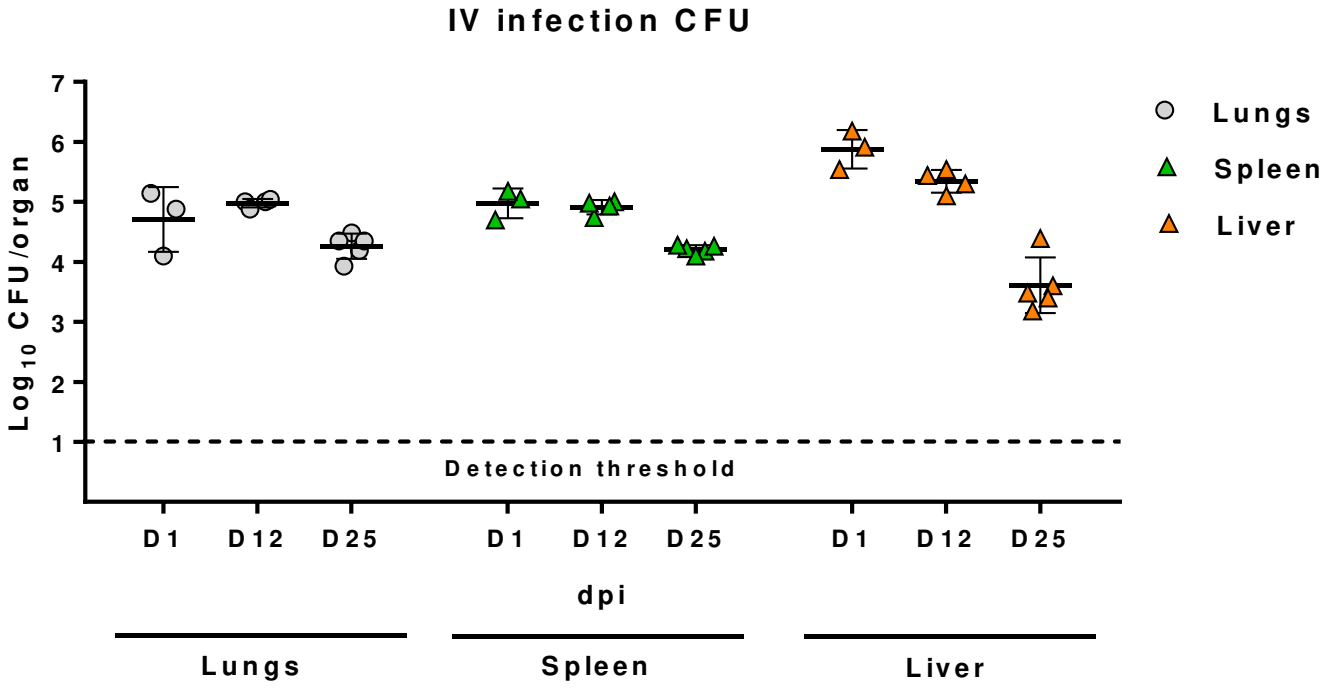

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Figure 2

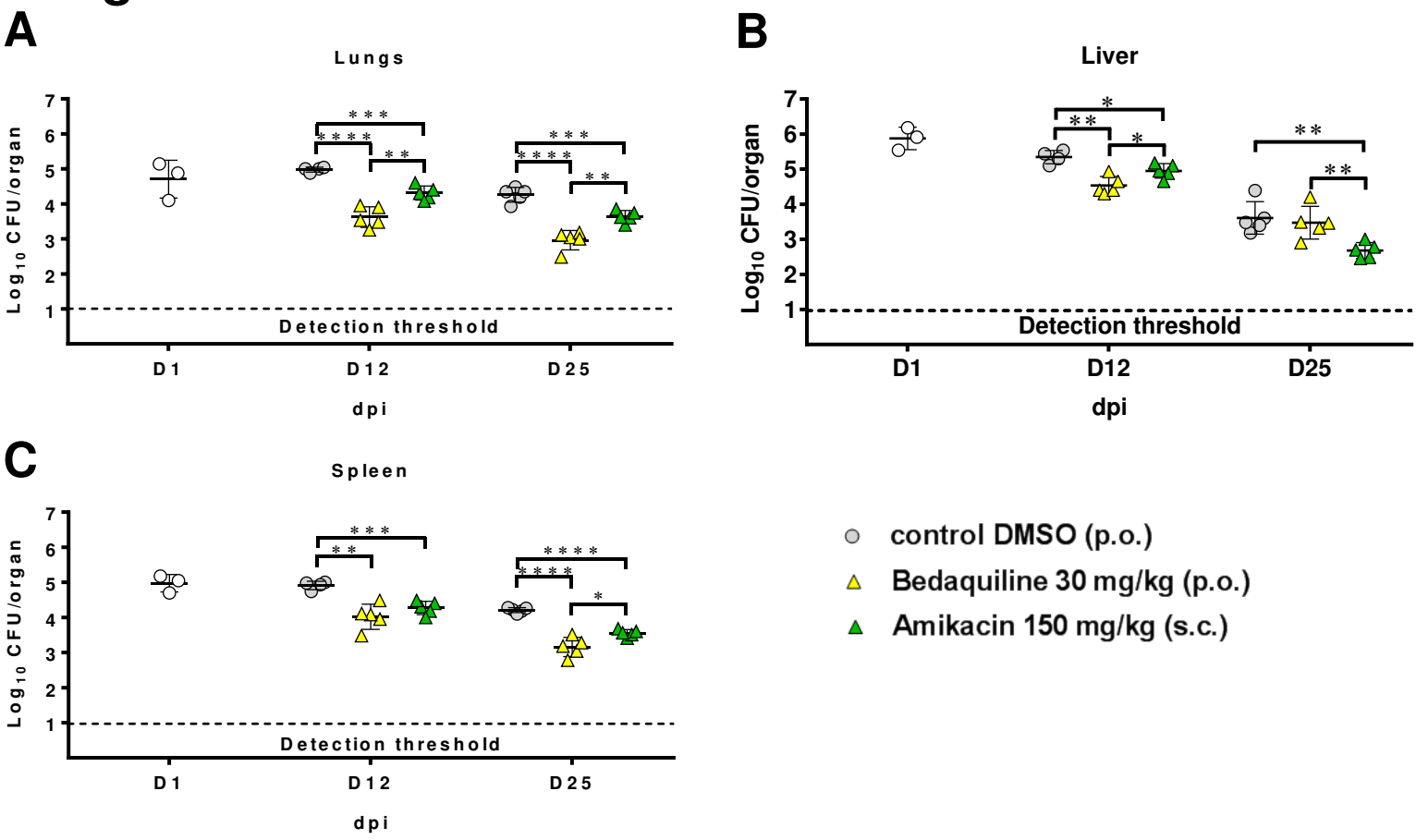

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Figure 3

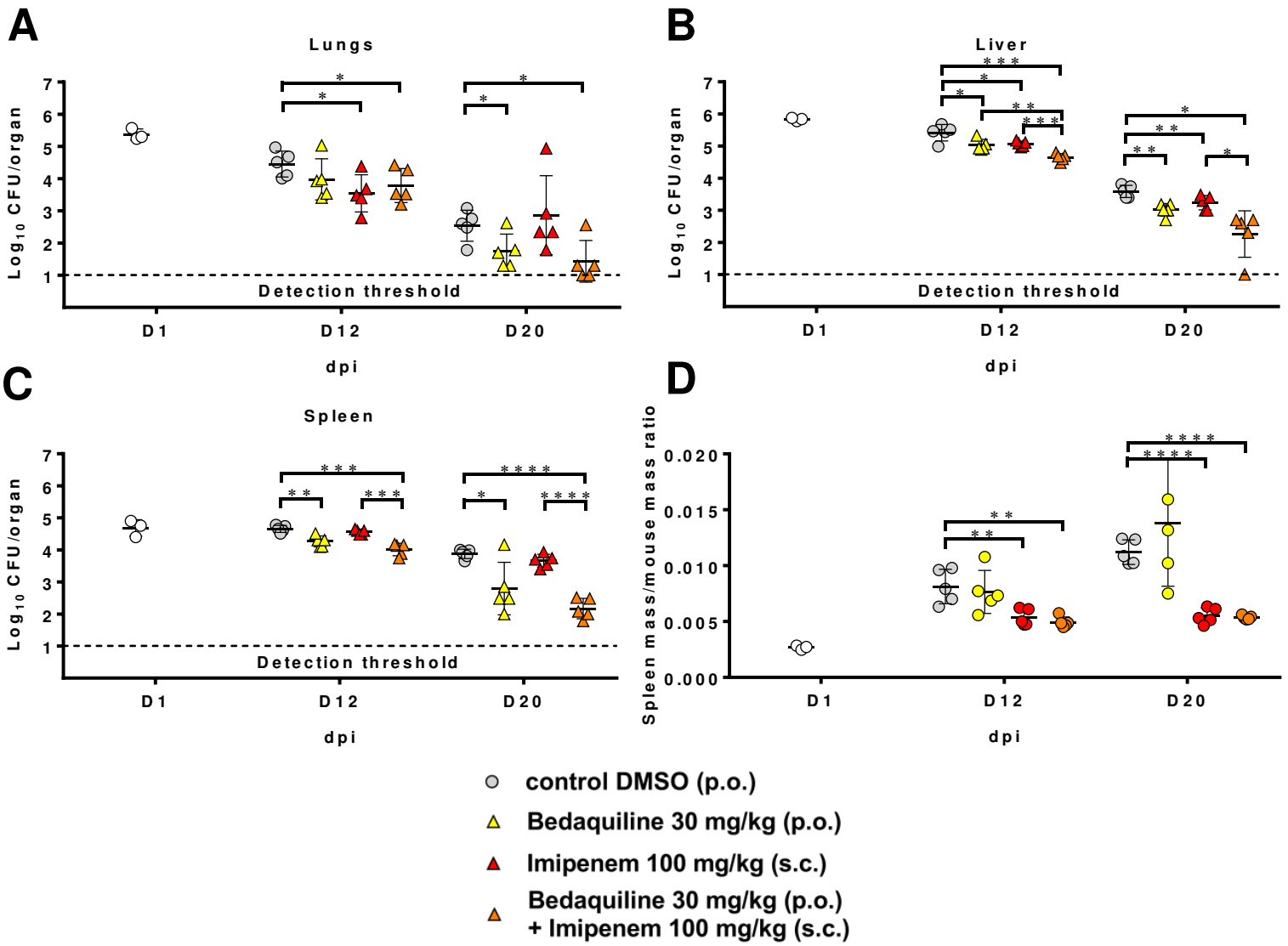


Figure 3. *M. abscessus* R-infected C3HeB/FeJ mice (2.7×10^5 CFU/mouse) treated by Bedaquiline (BDQ), Imipenem (IMP) or BDQ plus IPM. Bacterial loads in the lungs (A), liver (B) and spleen (C) were determined as reported in Fig. 1. Relative weight of spleen to mouse weight are shown in (D). Antibiotic treatment began 2 days after infection. Mice were treated starting on day 2 for 7 days (D12) or 13 days (D20) with twice daily (*i.e.* every 12 h) subcutaneous injection of IPM (MSD laboratories, France) in saline solution at 100 mg/kg or daily oral gavage of BDQ as described in Fig. 2 or both IMP+BDQ. Experimental groups of mice were evaluated for bacterial burden on day 1 (before treatment started), 12 and 20 as described in Fig. 1. (D) Mouse spleens were weighed at 1, 12 and 20 dpi. The value represents the relative weight of each spleen relative to the weight of the mouse from which they were collected. n = 5 mice were used per experiment and bacterial load in each group are expressed as $\log_{10}$ units of CFU (± SD) cells. 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*. Experiment was realized once.

Supplementary Figure 1

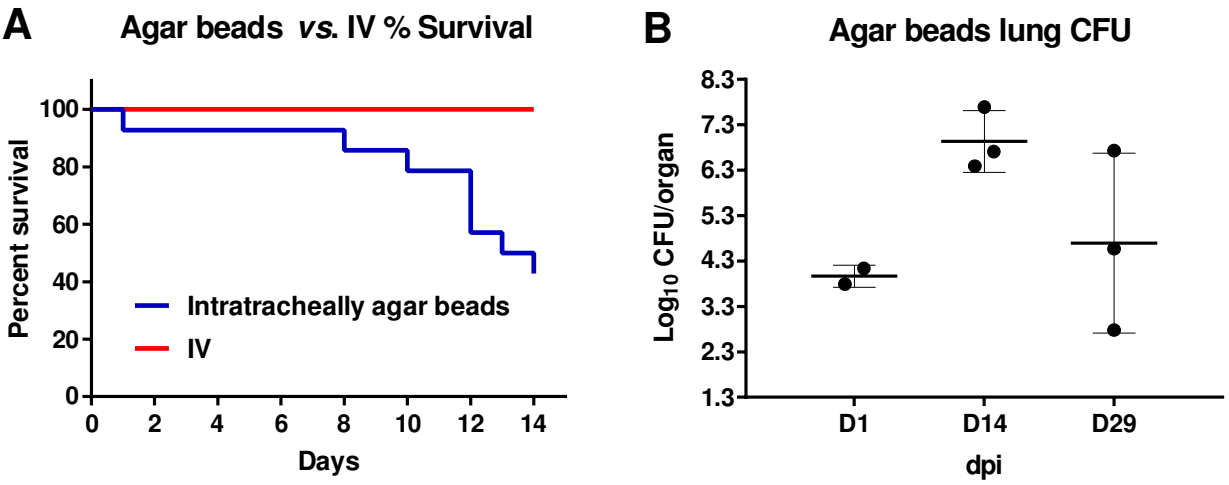
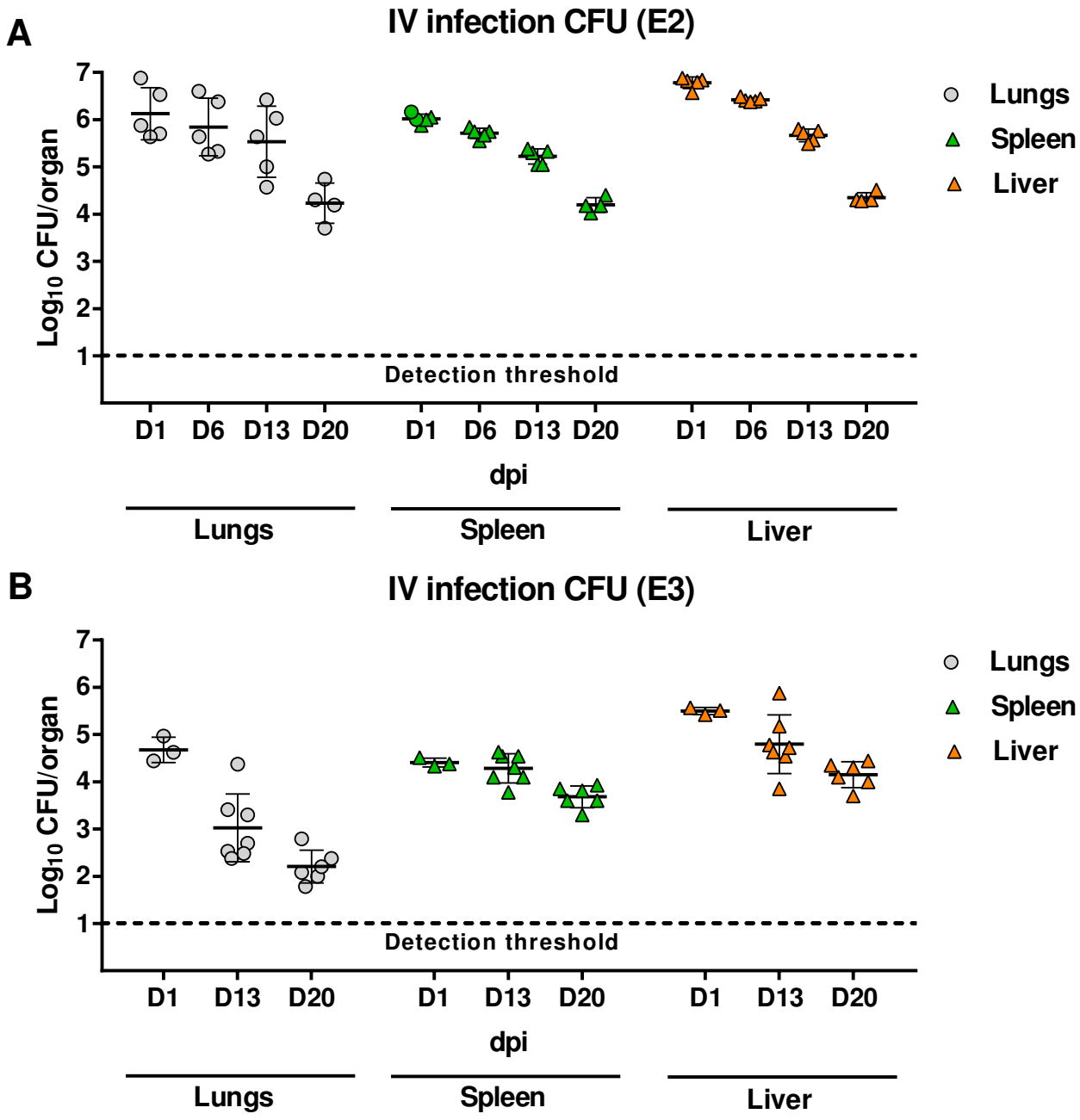


Figure 1. (A) Survival of C3HeB/FeJ mice infected intratracheally or intravenously (IV) with *M. abscessus* CIP 104536^T (smooth variant). (B) Persistence of *M. abscessus* in the lungs of intratracheally-infected C3HeB/FeJ mice. Agar beads where prepared as described previously (1). Mice were infected with a solution of agar beads containing 2.10^5 CFUs/mouse in 50 µl. Survival curves were generated over a 14 days post-infection experiment. Mouse lungs were collected and homogenized, serially diluted and plated onto VCAT (Vancomycin, Colistin sulfate, Amphotericin B, and Trimethoprim) chocolate agar plates (BioMérieux, France) and incubated for 5-6 days at 37°C prior to CFU count. Results are expressed as $\log_{10}$ units of CFU at 1, 14 and 29 dpi. Results are representative of one of two independent experiments (A and B) with similar results.

Supplementary Figure 2



Supplementary Figure 2. Bacterial persistence of *M. abscessus* CIP 104536^T (rough variant) in the lungs, spleen and liver of C3HeB/FeJ mice after infection in the tail vein with 4.8×10^6 (A) and 3.1×10^5 CFU/mouse (B) in a total volume of 200 µl of water containing 0.9% sodium chloride. The following day, three mice were euthanized and whole organs were harvested to determine baseline bacterial burden. CFU were determined as described in Fig.S1. Results are expressed as the $\log_{10}$ units of CFU at 1, (6), 13 and 20 dpi.

REFERENCES

1. Cigana C, Lorè NI, Riva C, De Fino I, Spagnuolo L, Sipione B, Rossi G, Nonis A, Cabrini G, Bragonzi A. 2016. Tracking the immunopathological response to *Pseudomonas aeruginosa* during respiratory infections. Sci Rep 6:21465.