

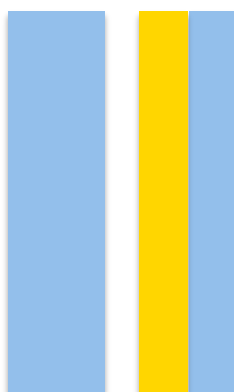
PROGRAMA DOUTORAL EM BIOLOGIA MOLECULAR E CELULAR

Advancing drug discovery with improved mycobacterial infection models

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**ADVANCING DRUG DISCOVERY WITH IMPROVED
MYCOBACTERIAL INFECTION MODELS**

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Publishing statement

According to the relevant national legislation, the author declares that she participated in the conception and execution of the experimental work, as well as in the interpretation of the results and in the writing of the publications referenced below, including those already published or in the process of being published and which form part of the thesis, presented here:

1. **Bento CM**, Gomes MS, Silva T (2021) Evolution of Antibacterial Drug Screening Methods: Current Prospects for Mycobacteria. *Microorganisms*, 9(12), 2562. <https://doi.org/10.3390/microorganisms9122562>

Authorship credit¹: Conceptualization, Visualization, Writing.

2. **Bento CM**, Silva AT, Mansano B, Aguiar L, Teixeira C, Gomes MS, Gomes P, Silva T, Ferraz R (2023) Improving the Antimycobacterial Drug Clofazimine through Formation of Organic Salts by Combination with Fluoroquinolones. *Int J Mol Sci*, 24, 1402 <https://doi.org/10.3390/ijms24021402>

Authorship credit¹: Methodology, Investigation, Formal analysis, Visualization, Writing. The author performed the experiments to assess antimicrobial activity and toxicity toward host cells, which are included in **Chapter 1** of the **Findings** (p. 57).

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3. **Bento CM**, van Calster K, Piller T, Oliveira GS, de Vooght L, Cappoen D, Cos P, Gomes MS, Silva T (2024) Characterization of novel double-reporter strains of *Mycobacterium abscessus* for drug discovery: a study in mScarlet. *Microbiol Spectr*, 12(10) e00362-24 <https://doi.org/10.1128/spectrum.00362-24>

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List of communications

The work showcased in this thesis was presented by the author at national and international conferences and workshops, as referenced below:

Oral communications

1. **Bento CM**, Geerts L, Oliveira GS, Gomes MS, Silva T. Optimizing macrophage infection with mycobacteria in different replicative stages for drug development. 11th i3S Annual Meeting, Póvoa de Varzim, Portugal. November 2024.
2. **Bento CM**, Geerts L, Oliveira GS, Gonçalves R, Calster KV, Cos P, Gomes MS, Silva T. Development of complex in vitro models of mycobacterial infection for drug screening. 10th Molecular and Cell Biology Symposium - A decade building science, Instituto de Investigação e Inovação em Saúde (i3S), Porto, Portugal. April 2024.
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5. **Bento CM**, Silva M, Olsson A, Gomes MS, Silva T. Lung organoids for the development of drugs against mycobacterial infections. REMODEL Summer School: Next-Generation Organoids and organ-on-chip at Instituto de Investigação e Inovação em Saúde (i3S), Porto, Portugal. September 2022.
6. **Bento CM**, Silva T, Aguiar L, Teixeira C, Gomes P, Ferraz R, Gomes MS. Use of Ionic Liquids to Fight Mycobacteria. Colóquio "A Small Couple Against the Big Three", Escola Superior de Saúde do Instituto Politécnico do Porto, Porto, Portugal. December 2021.
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Poster communications

1. **Bento CM**, Calster KV, Piller T, Oliveira GS, Voogh LD, Cappoen D, Cos P, Gomes MS, Silva T. *Mycobacterium abscessus* double-reporter strains: new tools for drug discovery. MicroSummit 2024, Instituto Superior Técnico, Lisboa, Portugal. November 2024.
2. **Bento CM**, Geerts L, Oliveira GS, Gomes MS, Silva T. The response of macrophages to infection is dependent on mycobacterial growth phase. 48th Congress of the Federation of European Biochemical Societies – Mining biochemistry for human health and well-being, Allianz MiCo – Milano Convention Centre, Milano, Italy. June 2024. Proceedings: FEBS Open Bio 14 (2) (2024) p. 159, doi: 10.1002/2211-5463.13837.
3. **Bento CM**, Calster KV, Piller T, Oliveira GS, Vooght LD, Cappoen D, Cos P, Gomes MS, Silva T. New tools for drug susceptibility testing against non-tuberculous mycobacteria. Brilliant PhDs, brighter future: Shaping Science together, Instituto de Investigação e Inovação em Saúde (i3S), Porto, Portugal. October 2023.
4. **Bento CM**, Calster KV, Piller T, Oliveira GS, Vooght LD, Cos P, Gomes MS, Silva T. Characterization of novel double-reporter strains of *Mycobacterium abscessus* for drug discovery: A study in mScarlet. 43rd Annual Meeting of the European Society of Mycobacteriology, Tirana, Albania. June 2023.
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6. **Bento CM**, Silva T, Aguiar L, Teixeira C, Gomes P, Ferraz R, Gomes MS. New ionic liquids to treat mycobacterial infections. EMBO Workshop on Tuberculosis 2022, Institut Pasteur, Paris, France. September 2022.
7. **Bento CM**, Gomes MS, Silva T. New infection models to test drugs against nontuberculous mycobacteria. MICROBIOTEC'21 – Congress of Microbiology and Biotechnology 2021, Sociedade Portuguesa de Microbiologia and Sociedade Portuguesa de Biotecnologia, Lisboa, Portugal. November 2021.
8. **Bento CM**, Silva T, Aguiar L, Teixeira C, Gomes P, Ferraz R, Gomes MS. Repurposing conventional antimycobacterial drugs using ionic liquids. ECMC 2021 – 7th International Electronic Conference on Medicinal Chemistry, MDPI, Basel, Switzerland. November 2021.

Abstract

Non-tuberculous mycobacteria (NTM) include all *Mycobacterium* species that do not cause leprosy or tuberculosis. Most NTM species are found in soil and water environments, while a few can be isolated from humans and animals, particularly those from the *Mycobacterium avium* and *Mycobacterium abscessus* complexes. Since the beginning of the century, there has been an increase in NTM cases, linked not only to a rise in susceptible individuals but also to growing awareness of these infections and improved diagnostic methods.

NTM primarily affect immunocompromised patients, such as those with AIDS or cancer, as well as individuals with underlying pulmonary diseases like cystic fibrosis. NTM disease requires long, multi-drug treatments that are often associated with poor patient compliance and low success rates. This situation is largely due to the ineffectiveness of current antibiotics, highlighting the urgent need for new and more effective treatments. Ionic liquids present promising alternatives to traditional antibiotic therapy by combining two molecules into a single, more stable, and bioavailable formulation. However, conventional methods for evaluating a compound's in vitro activity inadequately mimic the complexity of the infection and fail to predict clinical effectiveness. These challenges stem from intrinsic factors of mycobacteria, such as mostly being intracellular organisms, their capacity to enter a dormant state, and their tendency to aggregate and adhere to surfaces. All of these characteristics provide protection against antibiotics, complicating susceptibility assessments through gold-standard methods.

Our primary objective is to develop in vitro infection models with varying levels of complexity to test new compounds and closely predict their in vivo activity. We have created fluorescent and luminescent reporter *M. abscessus* strains to serve as tools for high-throughput screening of thousands of compounds rapidly and reliably, which can also be applied to monitor infection in animals.

We optimized intracellular infection models using macrophages, finding that the replicative state of the mycobacteria upon infection affects their antibiotic susceptibility; we also utilized organoid-derived alveolar epithelial cells to closely mimic the environment mycobacteria encounter after inhalation. Additionally, we implemented a model to test compounds on a large scale against *M. abscessus* biofilms.

Overall, we are contributing to speeding up the drug discovery pipeline by establishing practical and reliable in vitro tools to select clinically promising drug candidates.

Keywords: Non-tuberculous mycobacteria; *Mycobacterium abscessus*; *Mycobacterium avium*; in vitro drug susceptibility testing; reporter mycobacterial strains; macrophages; biofilms; alveolar organoids.

Resumo

As micobactérias não tuberculosas (NTM) incluem todas as espécies do género *Mycobacterium* que não causam lepra nem tuberculose. A maioria das espécies de NTM são encontradas em ambientes terrestres e aquáticos, enquanto algumas podem ser isoladas de humanos e animais, particularmente as que pertencem aos complexos de *Mycobacterium avium* e de *Mycobacterium abscessus*. Desde o início do século que se tem assistido a um aumento de casos de infeção por NTM, ligado não só ao aumento de indivíduos suscetíveis, mas também à crescente consciencialização acerca destas infeções e à melhoria dos métodos de diagnóstico.

As NTM afetam principalmente doentes imunocomprometidos, tais como aqueles com SIDA ou cancro, bem como indivíduos com doenças pulmonares subjacentes, como a fibrose quística. As doenças provocadas por NTM requerem tratamentos longos e com vários antibióticos, que estão frequentemente associados a uma baixa adesão por parte do doente e a baixas taxas de sucesso. Esta situação deve-se em grande parte à ineficácia dos antibióticos atuais, realçando a necessidade urgente de tratamentos novos e mais eficazes. Os líquidos iónicos são alternativas promissoras à terapia tradicional à base de antibióticos, combinando duas moléculas numa única formulação, mais estável e mais biodisponível. No entanto, os métodos convencionais para avaliar a atividade in vitro de um composto não mimetizam adequadamente a complexidade da infeção e não conseguem prever eficazmente a eficácia clínica. Estes desafios decorrem de fatores intrínsecos das micobactérias, tais como serem, na sua maioria, organismos intracelulares, a sua capacidade de entrar num estado dormente e a sua tendência para se agregarem e aderirem a superfícies. Todas estas características conferem proteção contra os antibióticos, complicando as avaliações de suscetibilidade através de métodos convencionais.

O nosso objetivo principal é desenvolver modelos de infeção in vitro com níveis de complexidade variados para testar novos compostos e prever a sua atividade in vivo. Foram criadas novas estirpes repórteres fluorescentes e luminescentes de *M. abscessus* para servir como ferramentas para o rastreio de milhares de compostos de forma rápida e fiável, podendo também ser aplicadas para monitorizar infeções em animais.

Otimizámos a infeção de macrófagos, e descobrimos que o estado replicativo das micobactérias no momento da infeção afeta a sua suscetibilidade aos antibióticos, uma vez dentro dos macrófagos; utilizamos também células epiteliais alveolares derivadas de organoides para replicar o ambiente que as micobactérias encontram após serem inaladas. Além disso, implementámos um modelo para testar compostos em larga escala contra biofilmes de *M. abscessus*.

Com este estudo, estamos a contribuir para acelerar o processo de descoberta de novos fármacos, estabelecendo ferramentas in vitro práticas e fiáveis para selecionar candidatos clinicamente promissores.

Palavras-chave: Micobactérias não tuberculosas; *Mycobacterium abscessus*; *Mycobacterium avium*; testes in vitro de susceptibilidade a fármacos; estirpes repórteres de micobactérias; macrófagos; biofilmes; organoides alveolares.

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INTRODUCTION

1. Non-tuberculous mycobacteria

1.1. Epidemiology of mycobacterial infections

Species from the *Mycobacterium* genus are members of the Actinobacteria phylum, which comprises filamentous bacteria characterized by a high guanine-cytosine content. These are mostly recovered from an extensive range of aquatic and terrestrial habitats [1,2]. The classification of mycobacterial species is constantly evolving due to the recurrent identification of new species by advanced sequencing technologies [3,4]. Still, the almost 200 species of mycobacteria known by now can be separated into tuberculosis-causing mycobacteria, nontuberculous mycobacteria (NTM), and *Mycobacterium leprae* (**Figure 1**) [5]. In the first group are the strictly pathogenic species belonging to the *Mycobacterium tuberculosis* complex. The latter is the causative agent of leprosy, a neurological and dermatological disease that still has over 200.000 cases reported every year, 16 million in the past 20 years [6]. The numbers for *M. tuberculosis*, the world's second-leading infectious cause of death only after SARS-CoV-2, are stark. Global efforts are underway to reduce tuberculosis' impact, resulting in fewer deaths and higher treatment success rates, yet resistance to available antimycobacterial agents remains a significant concern across all mycobacterial species.

NTM include all *Mycobacterium* species that do not cause leprosy or tuberculosis (TB). Most can be found in soil and water environments, and a few can be isolated from humans and animals, especially the species from the *Mycobacterium avium* complex – MAC (*M. avium*, *M. intracellulare* and *M. chimaera*) and the *Mycobacterium abscessus* complex (*M. chelonae* and *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletti* and *M. abscessus* subsp. *massiliense*) (**Figure 1**) [2]. A recent systematic review by Dahl et al. [7] presented an overview of the global NTM infection and disease trends. According to 47 studies from 18 countries, there has been an increase in NTM infection cases since the beginning of the century (**Figure 2**), particularly the ones caused by MAC and *M. abscessus* complex, the first and second most frequently isolated, respectively. The rise in NTM cases is linked to an increase in individuals vulnerable to these infections. This vulnerability stems from longer life expectancies, a greater prevalence of chronic diseases, and intensified immunosuppressive treatments. The broad use of long-term antibiotic regimens and better culture and detection systems also explain the increase in NTM cases [7,8]. Brode et al. [9] reported in 2014 that in many industrialized regions of the world, a local decline in TB incidence is coincidental with an increase in NTM infections. Among other associations that have not yet been proven, such as the possibility of cross-immunity between *M. tuberculosis* and NTM, a plausible one is related to public health conditions. While improving ventilation and plumbing in these regions was essential to reduce the incidence of TB, the

implementation of centralized water supplies, the disinfection of drinking water and the habit of showering are associated with NTM colonization by facilitating exposure to these environmental mycobacteria through aerosolization and by selection of bacteria naturally resistant to chloride, as is the case of NTM [10]. Indeed, several studies highlight environmental factors like water exposure and soil composition as being associated with an increased risk of NTM infections [7,11]. However, the most consensual explanation for the increasing trend of NTM is the growing awareness of these infections and better diagnosis methods to correctly identify the isolated mycobacterial species.

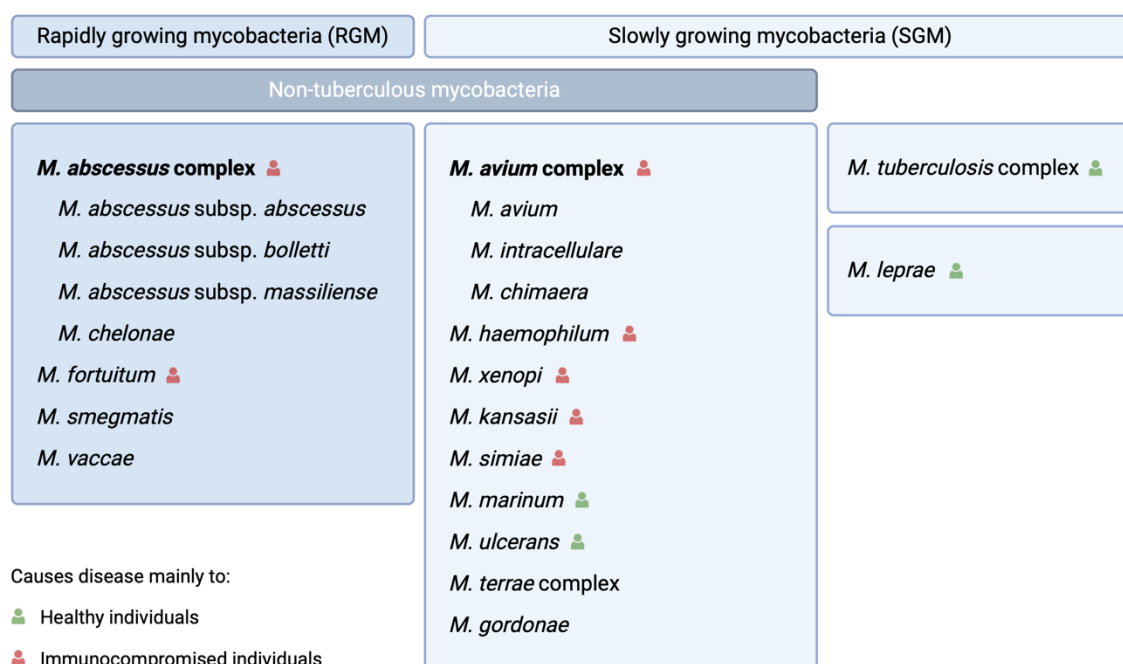


Figure 1. The more than 200 species of the *Mycobacterium* genus can be divided into rapidly and slowly growing mycobacteria. They can then be separated into the species that cause tuberculosis (*M. tuberculosis* complex), *M. leprae*, and the non-tuberculous mycobacteria (NTM), the latter including the majority of the species. Among NTM, there are strictly pathogenic species that cause disease in otherwise healthy individuals, opportunistic pathogens that only cause disease in immunocompromised patients and to those with underlying conditions, and non-pathogenic saprophytes. The image was created with BioRender, adapted from [2].

Still, the clinical awareness of NTM disease is far from that of TB. Additionally, the lack of resources allocated to monitoring and reporting NTM infections is probably associated with an underestimation of the number of NTM cases worldwide. The resemblance in symptoms between NTM disease and TB often results in misdiagnosis, mainly in TB endemic regions with low resources, like many African countries, where all patients with TB-like symptoms are treated for it without an identification of the causative microorganism [12].

As an example, a study conducted in Mali [12] showed that 18% of diagnosed chronic TB patients receiving treatment for multi-drug resistant TB (because first-line treatment failed) were indeed infected with NTM, mainly *M. avium*. This misdiagnosis leads to unnecessary expenses on ineffective treatments, worsens the patient's clinical condition and potentiates resistance development. A study from Ecuador [13] reported three patients with NTM lung disease who were initially diagnosed with TB by sputum smear microscopy and treated with anti-TB drugs. Two were immunocompetent, one had HIV/AIDS, and the MAC diagnosis was only determined after two of them died and the third was lost to follow-up. Curiously, these were the first NTM documented cases in Ecuador, a country without official reports on the incidence of NTM cases. It is, therefore, urgent to increase awareness of NTM infections in developing countries, increase funding for diagnosis centers and establish appropriate treatment guidelines to avoid misdiagnoses.

1.2. NTM epidemiology in Portugal

In Portugal, a study by Santos et al. [14] analyzed the trends in NTM circulation between 2014 and 2020 and concluded that the number of NTM isolations remained stable throughout time. The most common species isolated belonged to MAC (40.8%), followed by *M. abscessus* complex (9.6%) and *M. fortuitum* (6.3%) (**Figure 2**). Although considered not pathogenic, *M. gordonae* is also considerably represented (17.8%). This study reports that most patients were over 65 years old, and the number of NTM cases is not dependent on population density. As it happens in the rest of the world, the lack of diagnosis guidelines and patient follow-up, and being NTM a non-notifiable disease, results in faulty clinical data and an underestimation of the number of cases. Having that information would help understand the incidence and prevalence of NTM disease and its association with other pathologies. A previous study by Santos-Silva et al. [15] analyzed all reported NTM infection cases in Portugal between 2003 and 2016 and correlated microbiological data with clinical and social data. Of the 938 clinically relevant cases, 54.5% were MAC isolates. Among the rest were *M. chelonae*, *M. kansasii* or *M. xenopi*. These infections were commonly associated with co-infection with HIV and precarious social living conditions. A study by Rocha et al. [16] correlated NTM geographical incidence in Portugal between 2009 and 2013 with environmental data from public databases, such as population density, age, sex and HIV/AIDS incidence (from the 2011 nationwide census) and factors like temperature, humidity, precipitation or soil pH. Population density positively influenced the incidence of NTM. Age over 45 years old, HIV/AIDS incidence, humidity and precipitation also had a positive effect, while temperature and pH had a negative effect on NTM geographical incidence. Of course, the conclusions taken from this study are limited to a model that predicts NTM incidence by geographical distribution and overlaps it with environmental and

demographical data. As mentioned before, obtaining real data for each NTM isolation is crucial to help identify risk factors, eventual NTM environmental reservoirs, and healthcare access issues. However, this can only be achieved if adequate resources are allocated to report, monitor and follow up on NTM patients, just as it is done for TB patients.

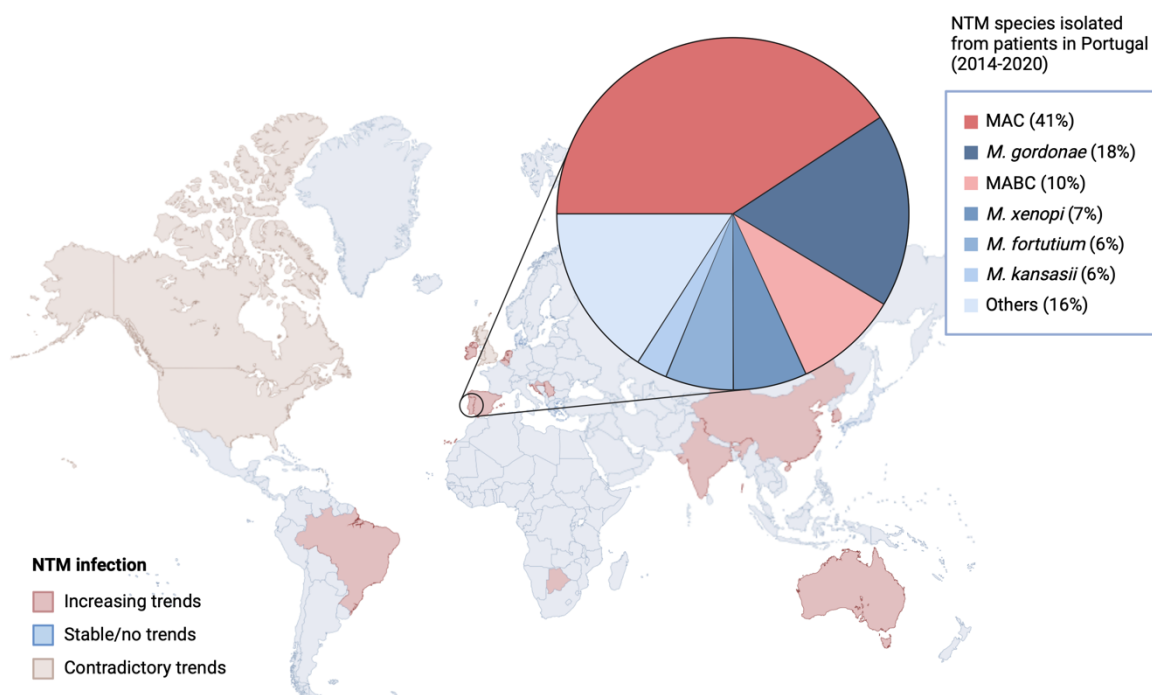


Figure 2. Worldwide, there has been a rise in NTM infections since the start of the century. In line with the rest of the world, the most frequently isolated pathogenic species in Portugal belong to the *M. avium* complex (MAC) and *M. abscessus* complex (MABC). The image was created with BioRender, adapted from [7,14].

1.3. Main biological traits of mycobacteria

Mycobacteria are aerobic bacilli with a uniquely thick cell wall. Although they share characteristics with Gram-positive and Gram-negative bacteria, mycobacteria's high content of lipids in the cell wall makes them acid-fast, as they resist a decolorization step with acidic alcohol. Therefore, the standard method for staining mycobacteria is the Ziehl-Neelsen staining [17]. Mycobacteria's cell wall (**Figure 3**) comprises a thick layer of peptidoglycan just outside the cytoplasmic membrane, covalently linked to arabinogalactan, a polymer of sugars. Esterified to arabinogalactan are long-chain high molecular weight fatty acids, the mycolic acids. This outer layer of mycolic acids represents up to 60% of the mycobacterial cell wall's weight, conferring strong hydrophobicity and impermeability to several molecules. Interacting with the mycolic acids is a layer of glycolipids, lipoglycans and loose proteins, including porins, that allow the passage of hydrophilic molecules, lipids

and glycans [17-20]. The high-lipidic content of the mycobacterial cell wall facilitates aerosolization, potentiating the infection of human and animal hosts with these environmental bacteria [10].

Given the complexity of its cell wall, mycobacteria require specialized mechanisms for cell division that allow them to synthesize several layers and, at the same time, maintain structure and shape [17]. Mycobacteria can be divided according to the time they take to grow on solid medium: slowly growing mycobacteria (SGM) take more than 7 days to form visible colonies on agar; rapidly growing mycobacteria (RGM) form colonies on agar in less than 7 days [2].

When growing on agar medium, mycobacteria present different morphotypes largely depending on the species and cell wall composition [2]. Mycobacterial colonies can be smooth, with a polished and well-defined surface and borders, or rough, with a wrinkled surface and irregular borders. *M. tuberculosis* colonies are usually rough, whereas *M. avium* colonies can be rough or smooth and even differentiated into smooth transparent or smooth opaque [21]. For most NTM, the colony morphotype depends on the presence or absence of glycopeptidolipids (GPL) and its degree of glycosylation on the surface of the cell wall. In *M. abscessus*, transitioning from smooth to rough morphotype highly influences the interaction with the host and the environment [2,22,23]. In *M. abscessus* smooth morphotypes, GLP is produced in the cytoplasm and is translocated by two proteins – MmpL4a and MmpL4b – to the outer layer of the cell wall (**Figure 3**) [24]. Smooth variants have increased motility on agar and form pellicle-like structures at air-liquid interfaces [24,25]. The production of GPL by these variants protects the bacteria from the host's immune system by masking the bacterial surface [26]. When the expression of MmpL4a and MmpL4b is hampered, GPL is not translocated to the surface and the colonies display a rough morphotype (**Figure 3**). The transition from smooth to rough morphotype is irreversible [27]. Rough *M. abscessus* easily aggregates and grows as serpentine cords, facilitating the formation of biofilms. These cording structures become bigger than the host immune cells, escaping their phagocytosis [28]. Using animal models helped understand that NTM's morphotype is associated with different levels of virulence. Rough *M. abscessus* is more virulent than smooth *M. abscessus*, as it is observed in mice [29] and zebrafish [28]. In MAC, rough variants replicate faster and are responsible for higher mortality in mice than smooth variants [30]. Among smooth MAC variants, the origin of the isolate highly influences its virulence, with bacteria isolated from animals (birds, pigs, and other species) being more virulent in mice than human or environmental isolates [31].

Mycobacteria are also characterized by their ability to enter a dormancy state when confronted with stressful conditions, such as nutrient starvation [32] or reduced oxygen levels [33]. They can remain dormant within host cells, extracellularly in the center of cell

aggregates, or within biofilm structures. This non-replicative state is associated with antibiotic resistance, as the targets of most drugs are not expressed under these conditions.

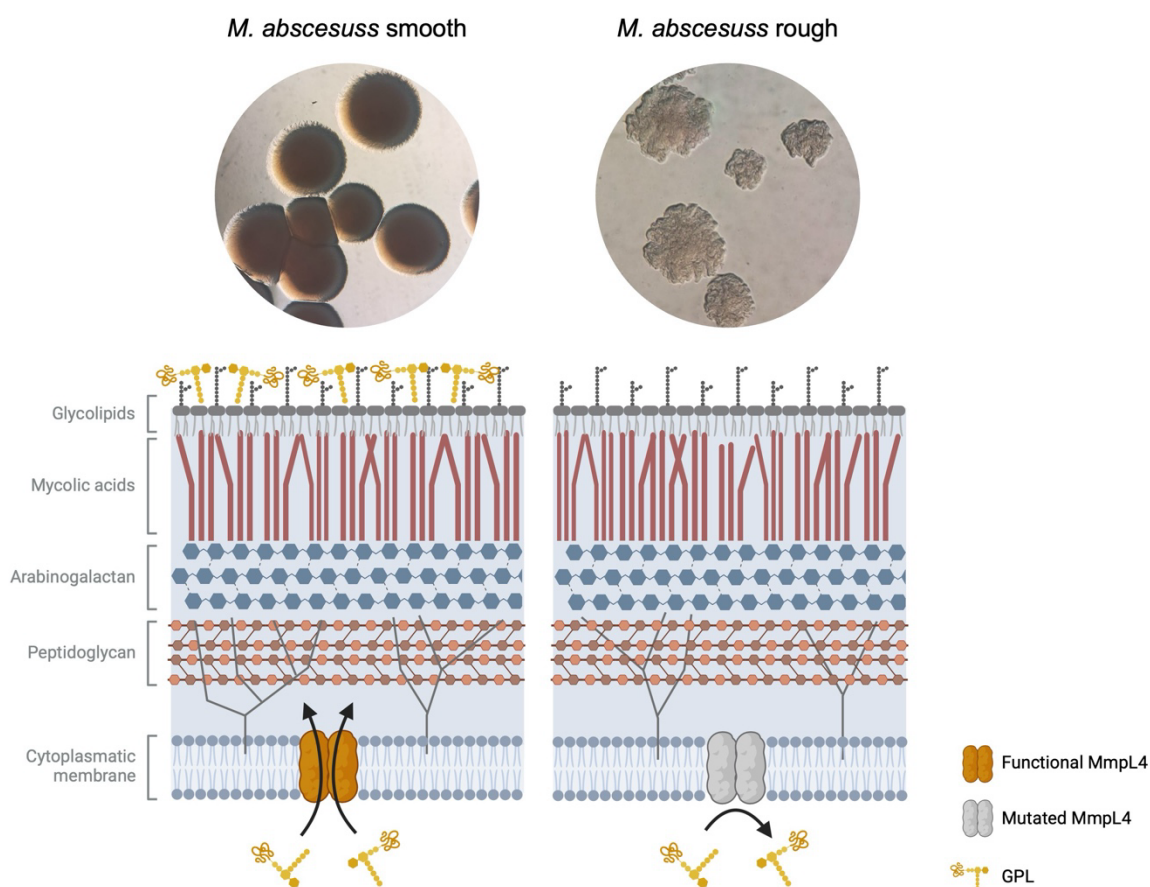


Figure 3. *M. abscessus* growing on agar medium presents two morphotypes: smooth and rough. Left – in smooth morphotypes, glycopeptidolipids (GPL) are produced in the cytoplasm and translocated by MmpL4 proteins to the outer layer of the cell wall. Smooth colonies have polished and well-defined surfaces and borders. Right – in rough morphotypes, the expression of MmpL4 is hampered and GPL is not translocated to the surface. Rough colonies display a wrinkled surface and irregular borders. The image was created with BioRender, including colony pictures from our *M. abscessus* laboratory strains.

1.4. NTM disease pathology

The most common manifestation of NTM infection is lung disease (**Figure 4**). The symptoms are very similar to those caused by TB or other pulmonary infections, including cough, sputum production, hemoptysis, fever, and weight loss, among others [34]. Three forms of chronic pulmonary NTM disease were described: i) fibro-cavitary disease, which occurs more often in older male patients with pre-existing lung disease like cystic fibrosis (CF), chronic obstructive pulmonary disease, bronchiectasis or smoking history; ii) nodular bronchiectasis disease, which occurs in non-smoking, post-menopausal women with

bronchiectasis and a few lung nodules; and iii) hypersensitivity pneumonitis, also known as “hot tub lung”, that occurs due to inhalation of aerosols in indoor hot tubs or household showers [8,34,35]. As the most commonly isolated NTM from respiratory samples, MAC species were associated with the three forms of NTM pulmonary disease [34,36]. However, *M. avium* can also disseminate to the central nervous system, causing meningitis and brain abscesses, or to lymph nodes, causing lymphadenitis [36]. *M. avium* is the NTM most involved in disseminated infections in severely immunocompromised people, mainly AIDS patients [37,38]. A study by Ricotta et al. [38] analyzed a database of all US patients hospitalized with extrapulmonary NTM disease from 2009 to 2014. It concluded that MAC was the most common isolate in patients with skin and soft tissue, blood, bone and joint, central nervous system, and abdominal and urinary infections, followed by *M. fortuitum* and *M. abscessus*. While MAC infections were the most common in HIV/AIDS patients, people with cancer were more likely to be infected with RGM [38]. Infection by *M. abscessus* is also responsible for pulmonary disease, mainly in CF patients. Indeed, *M. abscessus* has become the most prominent and worrisome NTM in cohorts of CF patients [39,40]. It can also cause infections in deep tissue and localized skin infections not only by dissemination from the lung but directly by exposure to environmental sources, through surgical wounds, cosmetic procedures, tattooing and acupuncture [40,41]. In HIV-negative patients, *M. abscessus* is the main responsible for meningitis and brain abscesses [42], but it can also cause ocular infections and bacteremia associated with catheter use [41]. Although it mainly affects immunocompromised patients, *M. abscessus* was isolated from immunocompetent people with symptoms of pulmonary or disseminated disease without a history of chronic lung disease [43,44].

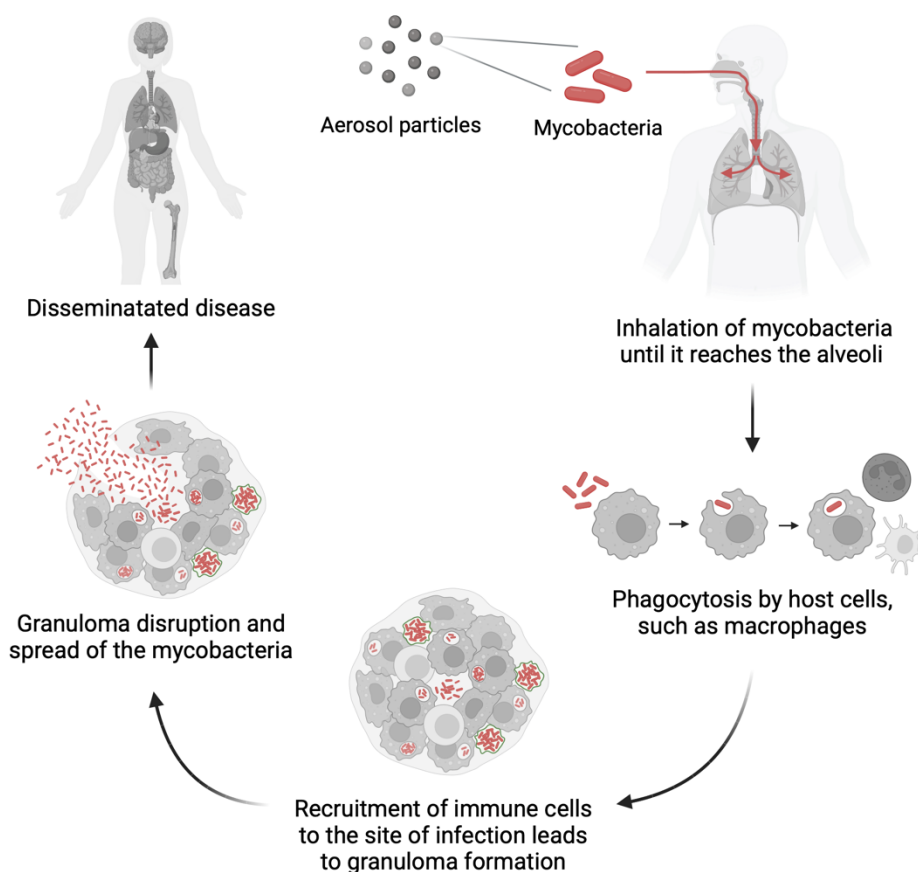


Figure 4. Mycobacteria are inhaled through aerosol particles and reach the alveoli, where they can cause pulmonary disease. The host's immune system attempts to control the infection by containing the mycobacteria in aggregates of immune cells known as granulomas. When these structures are disrupted, mycobacteria can spread and cause disseminated disease. The image was created with BioRender.

2. Treatment of NTM disease

2.1. Challenges

The similarities in the clinical presentation of pulmonary NTM and TB infections mean that, initially, anti-TB drugs were used to treat NTM infections. However, their effectiveness was limited [45]. The emergence of AIDS and infections caused by MAC led to the discovery of antibiotics with better efficacy against NTM infections, such as macrolides [46]. However, challenges persisted due to the high number of NTM species that cause similar clinical features but have differences in antibiotic susceptibility. Furthermore, the lack of correlation between in vitro susceptibility patterns and clinical response has led to delays in implementing treatment guidelines, resulting in incorrect administration of antibiotics and resistance development [47]. Treatment recommendations for most NTM species, such as

M. abscessus, are not evidence-based, leaving clinicians to make case-by-case decisions. However, even in cases of MAC infection, for which guidelines specify evidence-based treatment regimens, physicians frequently do not follow those recommendations [48], contributing to low treatment success rates. Independent of the mycobacterial species, NTM disease always involves using several oral and parenteral drugs for at least one year. Indeed, in most cases, treatment should last for at least 12 more months after the sputum is culture-negative [49]. Those drugs are associated with many side effects, especially because the patients have concomitant pulmonary disorders. Thus, the therapy risks and benefits should be discussed with the patient, and their willingness to receive treatment should be assessed. In some cases, antibiotic treatment is not administered, and instead, the disease's progression is monitored. Surgical resection of the diseased lung is also an option in more severe cases [49].

2.2. Treatment of MAC infections

The antibiotic regimen for treating MAC disease is the most well-established among NTM infections, and has evidence of efficacy (**Figure 5**) [50]. The basis is a macrolide, with clarithromycin or azithromycin being the usual option. Although clarithromycin has been the preferred choice and is still recommended for drug susceptibility testing in vitro, the current guidelines recommend an azithromycin-based regimen due to its better tolerance, fewer drug interactions and easiest administration [49]. Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit. However, in some NTM species, exposure to macrolides induces the expression of the gene *erm41* and consequent adenine methylation in 23S rRNA, modifying the drugs' target and inducing resistance [2,51]. Therefore, to prevent the development of macrolide resistance, MAC patients are treated with at least three drugs, including a macrolide, ethambutol and a rifamycin. Ethambutol inhibits the protein arabinosyltransferase, interfering with mycobacterial cell wall synthesis. It increases cell wall permeability, facilitating the passage of other antimycobacterial drugs [36]. Rifamycins such as rifampicin and rifabutin act by binding to and inhibiting DNA-dependent RNA polymerases, thus disrupting RNA synthesis early in the transcription process, which results in bactericidal activity also against intramacrophagic and nonreplicating bacteria [52]. Rifabutin, although less tolerated by patients with underlying chronic pulmonary disease [46], is not as affected by ADP-ribosylation as rifampicin and has synergistic activity with other drugs in vitro [2], therefore being preferred against disseminated disease.

In more severe cases of disease and with the emergence of antibiotic resistance, other drugs are often added to the regimen. Aminoglycosides like amikacin and streptomycin are recommended for patients with cavitary, advanced/severe bronchiectatic or macrolide-

resistant MAC pulmonary disease, in addition to the baseline regimen [49]. Aminoglycosides are inhibitors of protein synthesis by hampering tRNA binding and interfering with mRNA decoding [36]. Amikacin and macrolides are the only drugs in which in vitro susceptibilities predict treatment responses for MAC disease, allowing the establishment of minimum inhibitory concentration (MIC) break points of “susceptibility” and “resistance” [50].

Fluoroquinolones, like moxifloxacin, ofloxacin or norfloxacin, inhibit the mycobacterial DNA gyrase, impairing nucleic acid synthesis. They exhibit activity against *M. avium* in vitro and in infected mice [53]. However, including a fluoroquinolone in substitution for ethambutol or a rifamycin in the recommended three-drug therapy of MAC patients has no advantage, as it yields similar outcomes but with increased side effects [54]. They are, however, first-line drugs against *M. xenopi* pulmonary disease and are recommended against rifampicin-resistant *M. kansasii*, both SGM [49].

Clofazimine is part of the standard treatment against leprosy. It appears to be well-tolerated and effective against *M. avium* infections [50], being currently in a Phase II clinical trial to assess its clinical efficacy and safety for treating MAC pulmonary disease [55]. Clofazimine's mechanism of action is still unclear but multifactorial. It acts on the mycobacterial respiratory chain and ion transporters, inhibiting them. Its oxidation leads to the production of reactive oxygen species (ROS), increasing its antimycobacterial activity. It can also bind to the DNA and enhance the effect of bacterial phospholipase A2 [56]. While clofazimine influences the host's innate response by inducing macrophage apoptosis [57], its capacity to improve the humoral immune response benefits immunocompromised NTM patients [58].

2.3. Treatment of *M. abscessus* infections

The recent emergence of *M. abscessus* as a pathogenic agent, coupled with the reduced number of patients enrolled in treatment regimens, means no specific evidence-based guidelines have been established for treating these infections. Additionally, this species is characterized by a high antibiotic resistance [59,60]. Therefore, testing in vitro antibiotic susceptibility of *M. abscessus* isolates is required to determine which agents should be included in the regimen besides a macrolide (**Figure 5**), as this class of antibiotics is very active against susceptible *M. abscessus* strains [59]. Indeed, even if the isolate is macrolide-resistant due to a functional *erm41* gene, a macrolide is used for its immunomodulatory properties [49]. *M. abscessus* is intrinsically resistant to ethambutol and fluoroquinolones due to polymorphisms in *embB*, which encodes the arabinosyltransferase targeted by ethambutol, and in *gyrA*, which encodes the mycobacterial DNA gyrase targeted by fluoroquinolones [60].

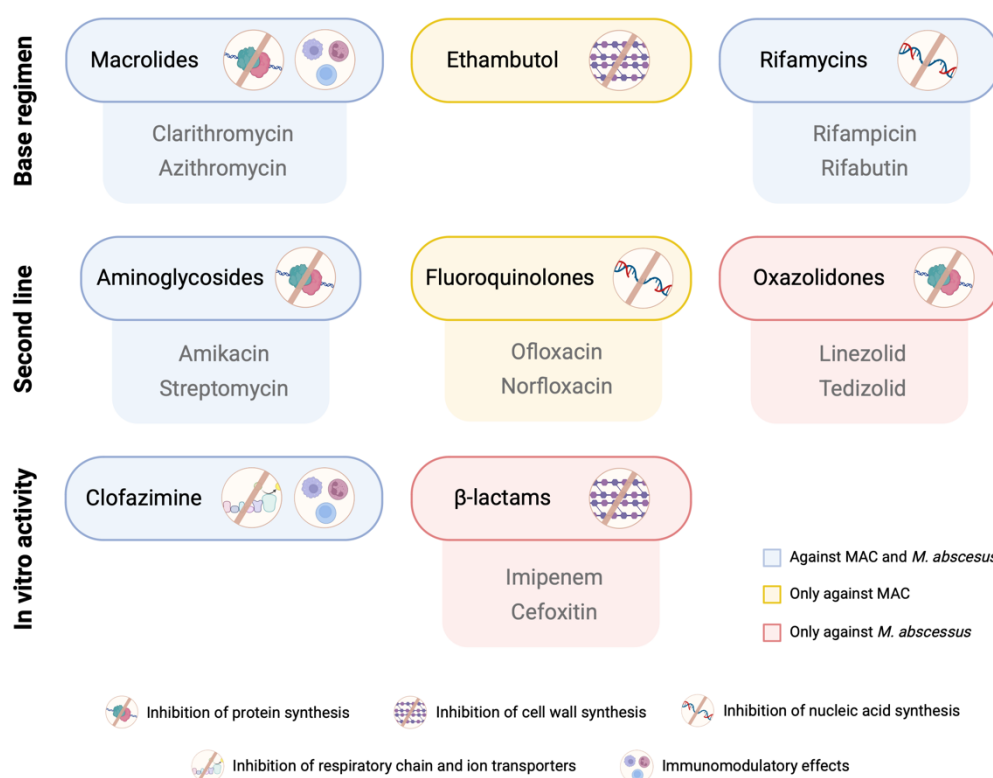


Figure 5. Treatment for MAC disease is based on a multi-antibiotic regimen that includes a macrolide, ethambutol, and a rifamycin. In more severe cases, second-line antibiotics are added to the regimen. Antibiotics with in vitro activity are currently being evaluated for their clinical safety and efficacy. Due to its high antibiotic resistance, *M. abscessus* disease is approached on a case-by-case basis. However, there are recommended treatment regimens based on other mycobacteria. The image was created with BioRender and is based on [49,50,59].

Although the in vitro MIC breakpoints for susceptibility and resistance against RGM only have clinical relevance for macrolides [59], amikacin is also recommended for *M. abscessus* pulmonary disease based on in vitro susceptibility results as they are often associated with treatment success [61]. However, the inclusion of other drugs that, although performing unpredictably, have low MICs against *M. abscessus*, must be considered to avoid acquired mutational resistance to both the macrolide and amikacin. Those include the β -lactams imipenem and cefoxitin, which inhibit the cross-linking of peptidoglycan during cell wall synthesis by inactivating penicillin-binding proteins. Although being resistant to most β -lactams due to the high impermeability of the cell wall and the production of β -lactamases, *M. abscessus* produces a class A β -lactamase, Bla_{Mab}, that hydrolyses imipenem and cefoxitin at a slower rate than other β -lactams [62], which may explain their higher activity.

Linezolid, an oxazolidinone, impairs protein synthesis by binding the ribosome and interfering with the positioning of tRNA. Although not very well tolerated by patients due to

associated adverse reactions [49], linezolid has demonstrated high activity against *M. abscessus* [63]. Tedizolid and delapazolid, the latter still in phase II clinical trials, are two more recent and promising oxazolidinones that overcome linezolid's toxicity and resistance issues [64,65].

Treating *M. abscessus* infections, especially in antibiotic-resistant cases, is far from dogmatic. It is approached on a case-by-case basis, relying mainly on each physician's expertise and data obtained for other NTM. The fact that it strongly depends on in vitro susceptibility testing, yet those results rarely translate to clinical efficacy, urges the development of new in vitro and in vivo models to test existing and new compounds to achieve a successful antimicrobial regimen.

3. NTM drug development

New antibiotics are being studied to treat specifically NTM infections, as was reviewed recently by Cantelli et al. [45]. Some of these new molecules follow classical mechanisms of action, like inhibition of the cell wall, DNA or protein synthesis. To avoid increasing drug resistance, others have new targets, like the MmpL3 protein, a transporter of trehalose monomycolate across the mycobacteria's inner membrane essential for the cell wall synthesis [66]; or the GyrB subunit of DNA gyrase, instead of the GyrA subunit, the target of fluoroquinolones [67]. SPR720, a benzimidazole that inhibits the ATPase activity of GyrB, completed a phase 2 clinical trial for its use against MAC pulmonary disease [68]. The discovery of these new molecules still occurs within the anti-TB drug development pipeline rather than as an investment in anti-NTM drug discovery. Consequently, the number of new drug candidates is lower than it could be, which can become a serious health problem in the near future, especially due to the multi-drug-resistant *M. abscessus*.

3.1. Ionic liquids as a new approach for drug development

As described, the treatment of NTM infections is based on the administration of different drugs at the same time for several months. The required dosages are associated with toxicity and side effects that can end the administration of one or more of them, hampering the treatment. Monotherapy against NTM infections should never be an option because of the risk of developing resistance. Furthermore, the fact that most patients are also being treated for other concomitant diseases increases the antibiotic burden. Therefore, combination therapy, i.e., combining antibiotics with other active molecules, emerges as an important approach to expand the antibiotic's spectrum of action, reduce antibiotic resistance and dosages and eventually create synergistic effects that enhance the activity of conventional drugs [69]. A straightforward example is the combination of β -lactam antibiotics with β -lactamase inhibitors. This approach is particularly interesting against *M.*

abscessus and other β -lactamase-producing pathogens as a way to increase the ability of the β -lactams to reach their targets [70]. Another approach has been the combination of agents that act on components of the mycobacterial biofilm's extracellular matrix, such as Tween-80 and N-acetylcysteine, with antimycobacterial drugs. These combinations have a synergistic effect against RGM biofilms, reducing the viability of mycobacteria and the biofilm's thickness and coverage area [71]. Antimicrobial peptides (AMPs), a part of the host's innate immune response, can be used as a monotherapy or in combination with antibiotics, having been described as active against several pathogens, including mycobacteria [72]. For example, LFcin17-30, a short version of the bovine antimicrobial peptide lactoferricin, significantly inhibits the axenic growth of *M. avium* [73] and increases the antimicrobial action of ethambutol inside macrophages by increasing the formation of lysosomes and autophagosome-like vesicles [74].

The combination of active pharmaceutical ingredients (APIs) formulated into a single ionic liquid (IL) has been used as a strategy for drug repurposing [75-77]. Indeed, when rescuing old and less-used drugs, common problems include unfavorable pharmacological properties such as low solubility, crystallization, and high toxic dosages [76]. These can be overcome by creating non-crystalline forms of the drugs called ILs. ILs are organic salts (OSs) with a melting point below 100°C [76,77] made by combining two molecules with opposite polarities. These two molecules establish weaker Coulombic interactions and lower cohesive energies than in a solid phase due to a lack of ion symmetry and low charge density. This contributes to lower melting points than conventional salts and higher solubility than their parent organic molecules [76]. The exact mechanism of action of ILs has yet to be entirely understood. Nonetheless, specific structural features, such as the length of the cation side chain and the inclusion of polar functional groups, can modify properties like lipophilicity and surface tension, which are known to affect the activity of these compounds [78]. The right combination of APIs into ILs can cost-effectively create innovative compounds with antimicrobial activity [79].

3.2. Challenges associated with NTM drug development

Falkinham highlighted the challenges associated with anti-NTM antibiotic development in a review paper in 2018 [80]. They mainly arise from mycobacteria's intrinsic factors, which are reflected in the methodological challenges of determining susceptibility in the laboratory. The challenges include i) the highly hydrophobic and impermeable lipid-rich mycobacterial cell wall that prevents antibiotics from penetrating effectively; ii) the fact that most NTM are intracellular organisms, which provides an additional barrier against antibiotics; and iii) the ability of NTM to enter a dormant state, making them less susceptible to antibiotics that target actively dividing cells; iv) their ability to aggregate, adhere to surfaces and form

biofilms, which protects them from antibiotics and complicates susceptibility assessment by conventional methods. It is also important to consider that among the same mycobacterial species, not all strains exhibit the same level of resistance or growth characteristics. Therefore, the drug screening results for one strain, the only available option most of the time, should not be blindly extrapolated to the whole species or genus.

Developing new antimicrobial agents heavily relies on determining whether their effectiveness in vitro translates to similar effectiveness in the host. Lin et al. [81] showed the effect of medium type and composition on the assessment of antimicrobial activity. Resistance of several pathogens to azithromycin was overcome by switching from bacterial culture medium to tissue culture media [81]. It was also observed that macrolides' MIC against *P. aeruginosa* in standard bacterial culture media decreased to values close to those measured in tissue culture media by supplementing the bacterial media with serum or bronchoalveolar lavage [82]. The authors explain the differences in susceptibility with increased permeability of the bacteria's outer membrane in tissue culture media, which leads to accumulation of the antibiotics inside the bacteria and concomitant reduction of the activity of efflux systems [82]. This shows that the context in which a drug is tested highly influences its antimicrobial activity. Therefore, testing new compounds in settings that accurately simulate the complexity of real infections is crucial.

4. Drug susceptibility testing against mycobacteria

4.1. Gold-Standard Methods

Dilution methods are the reference methods for drug susceptibility testing (DST) in bacteria. Various organizations publish international standards and guidelines for DST, including the Clinical and Laboratory Standards Institute (CLSI) [83], the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [84], and the European Centre for Disease Prevention and Control (ECDC) [85].

The broth and agar dilution methods are used to find the minimum inhibitory concentration (MIC) of a drug, which is the lowest amount of a compound that stops the growth of a microorganism [86]. Broth dilution can be done in tubes or microtiter plates. In this process, the test compound is diluted and mixed with a standardized concentration of bacterial suspension. The MIC is determined by the first tube or well that shows no visible growth, evaluated by turbidity [87-89]. Detecting turbidity can be difficult as it can be interpreted differently by different people. Besides, the dilution range may not give enough drug concentrations to determine the MIC reliably. Some improvements can be applied to broth dilution methods, like adding tetrazolium salts (MTT or XTT) or resazurin to the bacterial suspension [87]. The colorimetric results can be used to plot a dose-response

curve and calculate the drug concentration that inhibits 50% (IC_{50}), 90% (IC_{90}), or other percentages of bacterial growth using mathematical models that fit the experimental results.

When recurring to an agar dilution method, the bacterial load after exposure to different antibiotic concentrations is determined by counting colonies and calculating colony-forming units (CFUs) [88]. This method overcomes issues presented by broth dilution, like allowing for prompt identification of contaminant colonies [87,90]. However, it takes longer to have a read-out compared to broth dilution methods, especially in the case of mycobacteria. For slowly growing NTM, the activity of a compound takes at least one week to assess, while a minimum of three weeks is needed for the *M. tuberculosis* complex.

Other methods traditionally used for fast-growing microorganisms should not be applied to mycobacteria. Agar diffusion methods, for example, are based on the concept that an inhibition zone is created when a compound in a disk or a strip comes into contact with microorganisms on an agar plate. However, slow-growing microbes may not reach a sufficient bacterial density when the antibiotic concentration around the disk falls below the expected level. This can lead to smaller inhibition zones, making it difficult to interpret the results accurately [90]. That is why the CLSI guidelines do not recommend performing DST with mycobacteria, especially SGM, using agar diffusion methods [91]. DST with RGM using disk diffusion assays shows a poor correlation with broth dilution methods [92,93]. Furthermore, the disk diffusion method has the major limitation of not providing the MIC of the antibiotic [87,88].

4.2. Light-based tools

Traditional methods for assessing antibiotic efficiency are simple and reliable, but they are typically low-throughput and qualitative, failing to capture the drug's dynamics in an infected individual. Due to these limitations, new tools that can be applied to DST were developed based on novel technological advancements.

Bacteria's adenosine triphosphate (ATP) production is used to assess cell viability for DST using the ATP bioluminescence assay [94,95]. This assay is based on converting D-luciferin into oxyluciferin by luciferase in the presence of ATP and Mg^{2+} , generating light [87]. The luminescent signal is captured and translated into relative light units (RLU), which are proportional to the intracellular ATP concentration and, therefore, to bacterial viability. It can be performed using commercial kits, like the BacTiter-Glo microbial viability assay (Promega, Madison, WI), which is particularly suitable for high-throughput assays. Kapoor et al. [96] adapted the assay to three RGM – *M. immunogenum*, *M. chelonae*, and *M. abscessus* – and reported a linear relationship between intracellular ATP (RLU) and bacterial load (CFU/mL) in a considerably smaller amount of time: 1.5 h, compared to the 3 to 5 days required by CFU assay. Using a similar luciferin-luciferase reagent, Beckers et al.

[97] showed results for *M. tuberculosis* after 5 days of incubation instead of the 3 to 4 weeks required by a standard dilution method. This type of bioluminescence assay is highly specific and allows to get results quickly. However, it implies the lysis of the bacteria and the addition of luciferase and D-luciferin to measure the production of ATP. This can be overcome by using bioluminescent reporter bacteria.

ATP production can be measured without adding any reagent by genetically modifying bacterial strains to incorporate the luciferase gene. There are two types of bioluminescent reporter strains: one expresses the luciferase enzyme and requires the addition of D-luciferin to produce light, while the other can produce light without any additional substances by expressing the whole luciferase operon (lux operon). Andreu et al. [98] transformed *M. tuberculosis* to express the firefly luciferase (FFluc) or the lux operon and obtained antibiotic susceptibility results for extracellular and intracellular assays aligned with those obtained by resazurin assay and CFU counting in less time. Larsson et al. [99] performed a similar assay with *M. tuberculosis* expressing *Vibrio harveyi* luciferase. Both studies show that bioluminescent mycobacteria can be used to assess drug susceptibility rapidly and in a high-throughput way. However, their intracellular method requires lysis of the host cell before measurement of the bacteria's luminescent signal, and it is not microscopy-based, which does not allow, for example, to monitor bacterial viability over time and assess the drug's effect on the host cells.

Bioluminescent reporter strains can be used to follow the infection live and assess drug activity in animals using in vivo imaging systems [100,101], as the luminescent signal has low background and good penetration through living tissue [102]. Bioimaging reduces the number of mice per experiment, as the same animals are repeatedly imaged instead of euthanized at each time-point, thus allowing us to monitor the infection's progression over time and obtain more reliable results in drug efficacy studies [103].

Bacteria can also be fluorescent by expressing a varied range of fluorescent proteins. Fluorescent reporter strains are a valuable tool for high-throughput DST as the bacterial load can be assessed by different techniques, like microscopy, flow cytometry, or simply using a plate reader [102]. Compared to bioluminescence, fluorescent bacteria can be imaged inside fixed or living host cells and tissues. Moreover, this intrinsic fluorescence allows us to visualize bacteria non-terminally without adding extra reagents, antibodies or kits. Several studies show the use of fluorescent reporter mycobacteria in high-throughput screenings of large compound libraries against extracellular and intracellular bacteria, with the results being in line with the ones obtained using conventional readouts [104-107].

Ultimately, it is beneficial to engineer bacteria to produce both fluorescence and bioluminescence. Recombinant bacteria expressing both types of genes can serve as

markers for bacterial load, viability and infection progress, both in vitro and live on animals, in a cost-effective and non-invasive manner.

4.3. Complex in vitro models of infection

The current standardized methods for DST fail to mimic the environment that the bacteria experience in the host as they analyze the antibiotic's effect against free-floating bacteria, not accounting for the barriers that the drug must overcome in vivo. It's essential to consider the ability of antibiotics to penetrate biofilms, host cells, or granulomas, where bacteria may exist in non-replicative states. There have been efforts toward creating in vitro models of mycobacterial infection that incorporate most of those conditions. In most cases, these models use reporter strains and are adapted to high-throughput settings to rapidly screen a large set of new potential compounds. More complex models, like organoids and organ-on-a-chip, are hardly adapted to high-throughput screening but can be valuable tools for characterizing potential drug candidates in later phases of the drug development pipeline before reaching animal and human trials.

4.3.1. Mycobacterial biofilms

Biofilms are associations of microorganisms enclosed by an extracellular matrix (ECM) of polysaccharides, DNA, and other molecules [108]. Bacteria establish biofilms in the most diverse environments, like medical devices or aquatic surfaces, to protect themselves from external aggressions [109]. The establishment of biofilms in living tissue is also described, for example, for *M. abscessus*, which aggregates in the lung interstitial space [110].

The characteristic presence of hydrophilic GPL in the mycobacterial membrane of smooth variants is associated with pellicle formation in air-liquid interfaces, whereas the lack of GPL in rough variants facilitates extracellular growth and cording [2]. Dokic et al. [111] characterized *M. abscessus* pellicular biofilm and concluded that it comprises an ECM abundant in lipids and extracellular DNA, proteins and carbohydrates. Chakraborty et al. [112] showed that cellulose is present in the ECM of pellicle biofilms and microcolonies of *M. avium*, *M. abscessus* and *M. fortuitum*, and it has a structural role in the biofilm, as the addition of cellulase disintegrates it. As cellulose is also present in *M. tuberculosis* biofilms [113], the authors suggest that this polysaccharide can be used as a biomarker of mycobacterial biofilms [112].

Mycobacterial biofilms are easily sampled from showerheads or water taps, especially if the area was disinfected before, as NTM's tolerance to disinfectants selects for these microorganisms [80]. Mycobacteria in biofilm structures are usually more resistant to antibiotics than planktonic bacteria. One explanation must be the complexity of the ECM, which hampers the penetration of antibiotics. Moreover, it creates anaerobic areas where

some compounds are inactive, mainly because the bacteria are dormant [109]. Yam et al. [114] showed that *M. abscessus* is viable in the absence of nutrients and oxygen for long periods by entering a non-replicative persistence stage. These persistent mycobacteria are extremely resistant to antibiotics, a pattern also observed in biofilm models.

Therefore, when performing DST, it is essential to consider the ability of the drug to surpass the barriers conferred by the biofilm as well as the bacteria's replicative stage in these structures. Traditional bacterial culture tools such as microtiter plates can be adapted to study in vitro biofilm formation by allowing the bacteria to grow and adhere to the surface of the wells or on pegs in the microplate's lid [115,116], and then exposing them to different antibiotic concentrations. The most common method to quantify biofilm mass is by staining it with crystal violet (CV) and measuring the optical density after dissolution with an organic solvent [117]. However, the CV staining does not give information about bacterial viability, as it stains the peptidoglycan of viable, dormant and dead cells and other polysaccharides in the ECM. In this case, the measurement of bioluminescence can be useful. Sanchez et al. [118] demonstrated this using the BacTiter-Glo viability assay. Their results indicated a correlation between bacterial viability measured by RLU and colony counting. More interestingly, Wang et al. [119] used a *Pseudomonas aeruginosa* bioluminescent reporter strain to assess biofilm adhesion by, in parallel, directly observing the biofilm using a chemiluminescent imager, measuring the bioluminescent signal with a plate reader and performing CFU counting to corroborate the results. Although bioluminescence has not been described as a readout to study mycobacterial biofilms, it has the potential to be an important tool for assessing drug efficacy in these settings.

Contrary to the static models mentioned above, parameters like cell density, ECM, nutrients, gas, or metabolic products can be modulated in dynamic biofilm systems to reflect better the conditions experienced by natural biofilms [120]. In the flow cell biofilm model, fluorescent bacteria form biofilms on microscope coverslips. The coverslips are connected to vessels supplying broth culture and antibiotics through a peristaltic pump. The biofilm's viability can be monitored in real time using confocal laser scanning microscopy [117]. Another example of an open and dynamic system is the CDC biofilm reactor. This system rotates to allow bacteria to form biofilms on coupons placed on polypropylene holders, and the flow speed and temperature can be controlled. Coupons can be removed for analysis at any time [117]. Armbruster et al. [121] used a CDC biofilm reactor to grow biofilms of four opportunistic bacteria found in potable water distribution systems, including *M. mucogenicum*, and tested the biofilm susceptibility to a water disinfectant. These dynamic biofilm models lack the simplicity and lower cost of static models, being harder to adapt to high-throughput conditions for DST. Of course, both models are also limited by not

incorporating a host-pathogen interaction dimension, which can more easily be overcome by in vivo biofilm models [120].

4.3.2. *Mycobacteria-host interaction*

Mycobacteria usually infect a host by inhalation of aerosols. The bacilli travel through the airways until they reach the alveoli. There, they are phagocytized by alveolar macrophages, which migrate into the underlying tissue, or cross the epithelium by infecting alveolar epithelial cells (**Figure 6**) [122]. The infected macrophages trigger innate and adaptive immune responses, leading to the recruitment of immune cells to the site of infection and the formation of cell aggregates that contain the bacteria, called granulomas [123].

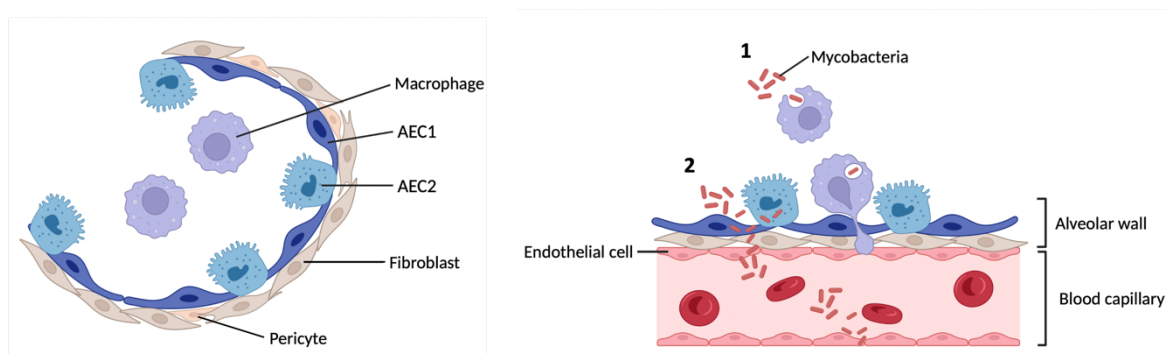


Figure 6. After reaching the alveoli, (1) mycobacteria are phagocytized by alveolar macrophages that transverse the alveolar barrier by diapedesis into the lung interstitium. (2) Some bacilli invade and replicate inside alveolar epithelial cells to reach the circulation and disseminate systemically. The image was created with BioRender.

4.3.2.1. *Macrophage infection*

After phagocytosis, macrophages try to kill the invading pathogen by fusing the phagosomes, in which the bacilli are enclosed, with lysosomes, exposing the bacteria to the lysosome's acidic and proteolytic content. *M. avium* can arrest that fusion by interfering with intracellular trafficking, avoiding the lysosomal antimicrobial mechanisms and, at the same time, having access to nutrients through the endosomal pathway, which is critical for the bacilli replication inside the host cells [124,125]. Through toll-like receptor (TLR) signaling, MAC-infected macrophages produce pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-12 [126]. Cytokine production promotes antimycobacterial responses, including apoptosis, as the macrophage is unable to kill the mycobacteria by common bactericidal mechanisms [127,128]. Although it was described that MAC-infected macrophages are less apoptotic and have reduced caspase activity than non-infected cells [129], if it eventually happens, some bacilli can survive in apoptotic

macrophages, escape to the extracellular media and infect surrounding macrophages [130,131].

In the case of *M. abscessus*, the presence or absence of a GPL layer at the bacterial surface highly influences how the macrophage is infected [2]. Smooth (S) variants, rich in GPL, seem to behave like SGM, as the latter also have a phenolic glycolipid (PGL) in the membrane that masks mycobacteria's TLR2 ligands [132]. *M. abscessus* S variants are taken up by the macrophage in phagosomes as individual bacilli, where the bacterial surface is kept in close apposition with the phagosome membrane, blocking its maturation into phagolysosomes. S bacilli can remain alone and "hidden" in these non-mature phagosomes for a long time, as they do not activate strong apoptosis and autophagic pathways. It was reported that the membrane of S variant-containing phagosomes shows signs of damage that indicate communication with the cytosol [133]. On the other hand, rough (R) variants are deprived of GPL and form aggregates of bacilli that are not easily phagocytized by macrophages and grow extracellularly, forming cords near the host cell's surface. Macrophages can, although, take up smaller aggregates that are enclosed in the same social phagosome. These social phagosomes are quickly fused with lysosomes as a way to acidify the compartment. However, mycobacteria can resist degradation in the first hours post-infection [133] and replicate abruptly until the phagosome reaches its capacity and is disrupted. The bacteria escape to the cytosol, a strong autophagic response is activated, and the macrophages become apoptotic, eventually releasing the bacteria into the extracellular environment [134]. Bacterial DNA in the cytosol was reported to activate the Type I IFN signaling pathways, allowing cell-to-cell spreading of the mycobacteria [134]. All that seems to indicate that an S morphotype, resistant to macrophage action, is more prevalent in the initial stages of infection and that it transits to an R morphotype to gain virulence and actively replicate intracellularly and extracellularly.

As the primary host cell of mycobacteria, macrophages are the most used cells to study mycobacterial intracellular infection and perform DST. Macrophages are usually obtained from primary cultures of murine – bone marrow-derived macrophages (BMDMs) – or human precursors – peripheral blood mononuclear cells (PBMCs) – that can be stimulated to differentiate into macrophages [74,135]. There are also macrophage cell lines, such as RAW 264.7 murine macrophage cells [136] or the THP-1 human monocyte cell line [137]. Recent studies use fluorescent reporter strains and large-scale formats to obtain relevant information quickly, avoiding CFU counting. For example, Brzostek et al. [138] infected the murine macrophage cell line J774 and THP-1 cells with *M. abscessus* mCherry and used flow cytometry-based methods to study how the macrophage's morphological features relate to bacterial burden. Sun et al. [139] used human pluripotent stem cell-derived macrophages to study *M. abscessus* GFP infection and perform in vitro drug screening.

Infection of macrophages with mycobacteria is a well-established model that gives insightful information before testing in animal models, such as the toxicity of new drugs to the host cells. However, it has limitations, as it only represents one type of host cells, while in vivo mycobacteria also interact with other cell types.

4.3.2.2. Lung epithelial cell infection

The alveolar epithelium comprises two types of cells: alveolar epithelial type I cells (AEC1) and alveolar epithelial type II cells (AEC2). AEC1 provide an extensive surface area for gas exchange with the capillaries, and AEC2 are responsible for secreting surfactants and other proteins [140]. Furthermore, AEC2 express pattern recognition receptors that sense the bacteria and start secreting cytokines, chemokines and antimicrobial peptides that attract alveolar macrophages to the mycobacteria. However, it is described that the epithelial cells are also infected by mycobacteria and contribute to the bacilli's passage across the epithelium [122]. This was shown using a human ex vivo tissue culture model of mycobacterial infection [141]. AEC2 cells were infected with *M. tuberculosis*, *M. abscessus* or *M. avium*, although to a lesser extent than macrophages (around 13% and 35%, respectively). The infection was dependent on the mycobacterial species – AEC2 were more infected with *M. tuberculosis* (22%) than *M. abscessus* (9%) and *M. avium* (7%) – and was associated with cell injury.

Most in vitro studies on infection of alveolar epithelial cells were performed using cell lines, particularly the alveolar basal epithelial cell line A549. They show that *M. tuberculosis* enters A549 cells with low efficiency and utilizes adhesins and toxins for a macrophage-independent crossing of the alveolar barrier, contributing to its disintegration [142-144]. Studies with *M. avium* show it can invade and replicate inside A549 cells. Its antibiotic susceptibility profile differs from that of intramacrophagic *M. avium*, as it is more resistant to conventional drugs inside the epithelial cells [145-147]. Studies with *M. abscessus* are more recent. Leestemaker-Palmer et al. [148] showed that *M. abscessus* can infect and replicate inside A549 cells, activating similar metabolic pathways to macrophage infection and causing loss of the epithelial membrane integrity. Ribeiro et al. [149] compared *M. abscessus* subsp. *abscessus* with *M. abscessus* subsp. *massiliense* and concluded both have an internalization rate of approximately 30% in A549 cells. However, *M. abscessus* subsp. *massiliense* survived better inside the cells than *M. abscessus* subsp. *abscessus* by reducing the acidification of the phagosomes. Moreover, *M. abscessus* subsp. *massiliense* recovered from infected RAW macrophages grew more inside A549 cells than *M. abscessus* subsp. *abscessus* in the same conditions. There is, thus, diversity at the subspecies level when it comes to *M. abscessus*, reinforcing how unpredictable this species is.

The infection of alveolar epithelial cells with mycobacteria is physiologically relevant to test drugs in an early phase of colonization before the infection is established in sites of more difficult access, like granulomas deep in the lung tissue. There is a lack of studies with primary alveolar epithelial cells. However, validating the studies with cell lines and developing more robust models of in vitro infection would be important. Complementing cultures of primary alveolar epithelial cells with macrophages would mimic even better the environment experienced by mycobacteria after inhalation.

4.3.2.3. *In vitro Granuloma Models*

The formation of granulomas is one of the hallmarks of mycobacterial infections. The cascade of innate and adaptive immune responses starts with infected macrophages in the lung interstitium recruiting peripheral monocytes and T-lymphocytes to the site of infection. Those cells surround a core of differentiated macrophages – foamy and epithelioid macrophages and multinucleated giant cells – monocytes, neutrophils, dendritic cells, and intracellular or free mycobacteria. This multicellular aggregate is coated with fibroblasts and collagen, forming a granuloma [123]. The mycobacteria inside the hypoxic environment of the granuloma develop into a dormant state, characterized by the accumulation of intracytoplasmic lipid inclusions, loss of acid fastness, and resistance to antibiotics. The containment of mycobacteria in a granuloma is essential to control this infection. The presence of CD4⁺ T cells that produce IFN- γ is crucial, as shown by the high propensity of AIDS patients, who have low counts of CD4⁺ T cells, in developing mycobacterial infections, especially by MAC [150]. Indeed, the depletion of IFN- γ and IL-12 is associated with an increased risk of infection by *M. avium* [151]. Besides, the production of TNF is important to maintain the structure of the granuloma, as its depletion is associated with the apoptosis of granuloma cells [152,153]. Disruption of the granuloma is followed by the release of extracellular mycobacteria and dissemination throughout most organs. In *M. abscessus* infections, this happens upon transition from a smooth to rough phenotype, which leads to granuloma disruption and extracellular cord formation [28].

Developing in vitro granuloma models is important to assess whether the drug can pass through the several layers of host cells and have activity against replicative or non-replicative bacteria. A three-dimensional in vitro granuloma can be achieved by infecting PBMCs and culturing them in conditions that inhibit surface contact [154]. Human cells are essential since the formation of granulomas is not easily replicated in other animals [155]. Several approaches are described to assemble an in vitro granuloma using different supports/holders [156-158], extracellular matrixes to accommodate the granuloma [159,160], levels of incorporation of immune cells [161,162], and mycobacterial species [154,163,164]. Guirado et al. [165] isolated PBMCs from TB-infected patients, showing

there is a significant influence of immune memory on granuloma formation: PBMCs from latent TB-infected patients incubated with autologous serum and virulent *M. tuberculosis* develop into a granuloma-like structure, with the bacteria showing signs of latency earlier than when using PBMCs from TB-naïve individuals.

The culture of granulomas can be done on a large scale in order to perform high-throughput drug screening. For example, Silva-Miranda et al. [166] used 384-well plates to assess the activity of antimycobacterial drugs against human PBMCs infected with a fluorescent reporter strain of *M. tuberculosis*. They noticed differences in the MICs obtained from the granuloma model compared to those from extracellular bacterial assays. This demonstrates the inadequacies of traditional methods in replicating the diverse barriers that a drug must surpass to reach bacteria in a living organism. Bielecka and Tezera et al. [167,168] developed alginate-collagen microspheres incorporating human PBMCs, *M. tuberculosis* fluorescent and bioluminescent reporters and extracellular matrix as a way to mimic the stress encountered by bacteria in vivo. Indeed, *M. tuberculosis* in this model showed susceptibility to pyrazinamide, which is only observed in vivo. This model can be refined by adjusting the microsphere composition to enable immune cell recruitment and create oxygen gradients [169].

There are few reports of in vitro granuloma models with NTM, particularly RGM. Their shorter replication time and tendency to grow extracellularly hamper the maturation of the granulomas, contrary to what is observed for *M. tuberculosis*. Je et al. [170] infected PBMCs with a very low dose of *M. abscessus* subsp. *massiliense* and observed cellular aggregates at 7 days post-infection attached by large amounts of extracellular mycobacteria that appeared to have escaped infected macrophages after necrosis. They also observed lower levels of inflammatory cytokine production than in *M. tuberculosis* infections, which can also be associated with the impairment of the granuloma's maturation and facilitation of the mycobacteria's dissemination. Therefore, in vitro granulomas may not be the best model for DST against RGM like *M. abscessus*. However, it is described that SGM NTM, like *M. avium*, can infect PBMCs and form granuloma-like structures that grow over time without extracellular growth and produce pro-inflammatory cytokines [154,171].

Overall, in vitro granuloma models get very close to in vivo conditions at a reduced cost and increased control. However, the high heterogeneity among donors and the difficulty of accessing large amounts of cells are limitations of using PBMCs as a laboratory model. Using reporter mycobacterial strains is essential to follow the infection and assess the bacterial viability in a situation where bacilli are expected to be in very different metabolic states and present distinct susceptibility to drugs.

4.3.2.4. Lung organoids

Another way to have an infection model with host cells organized in a three-dimensional structure is by using organoids. This approach starts from a less limited source of material, allows for a greater variety of cell types, and overcomes challenges posed by animal models, which often fail to accurately replicate the complexity of human diseases. Organoids comprise a variety of cells that self-organize into a three-dimensional structure that exhibits tissue organization, immune response, and physiological characteristics similar to an in vivo organ [172]. The starting point is a stem cell – a pluripotent stem cell (iPSC) or an organ-specific adult stem cell (ASC) – that is programmed to differentiate and multiply into the diversity of cell types that compose a specific tissue, from retinal, cerebral, or hippocampal to liver, intestinal, or lung [172]. iPSCs can differentiate into all specialized body cell types and are not subjected to donor-to-donor variability but require high expertise and lengthy and expensive protocols. ASCs are specific to a particular organ and their potential for self-renewal means that the organoids are genetically stable throughout several passages and can be preserved in biobanks [172].

Organoids can be used for several purposes, including host-pathogen interactions. Experimental studies have been published on infections by *Shigella*, *Escherichia coli*, *Clostridium difficile*, or *Salmonella* Typhi in intestinal organoids or *Helicobacter pylori* in gastric organoids [173,174]. A veterinary study of *M. avium* subsp. *paratuberculosis* infection of bovine-derived intestinal organoids has been published, as this species infects intestinal barrier cells after being ingested by calves [175].

Several approaches have been described to develop lung organoids [176], including their application to high-throughput screening of compounds. Danahay et al. [177] grew bronchospheres, which are lung organoids derived from primary human airway basal cells, in 384-well plates and screened 5000 different secreted proteins to identify which could influence basal cell differentiation into goblet cells, the lung cells responsible for producing mucus. During the COVID-19 pandemic, Han et al. [178] used lung organoids derived from human pluripotent stem cells infected with SARS-CoV-2 to identify antiviral compounds among a library of FDA-approved drugs. Indeed, due to the pandemic, there was an investment in developing organoids that represent the human alveoli, that is, that incorporate alveolar epithelial cells [179,180] and even engraftment with tissue-resident macrophages [181].

Studies of lung organoid infection with mycobacteria are still scarce and restricted to organoids from the upper part of the airways. Iakobachvili et al. [182] developed human airway organoids (AirOs) composed of lung basal, ciliated and goblet cells with a functional mucociliary system, following the protocol described by Sachs et al. [183]. Then, they microinjected fluorescent reporter *M. tuberculosis* or *M. abscessus* into the lumen of the

AirO and observed the bacteria's interaction with the epithelial cells by confocal microscopy. CFU counting showed decreased *M. tuberculosis* bacterial load after a week of infection but recovery by week 3 post-infection, whereas *M. abscessus* bacterial load increases over 6 days of infection. RT-qPCR analysis showed induced cytokine and antimicrobial peptide gene expression upon *M. tuberculosis* infection but none by *M. abscessus*. The *M. abscessus* strain used in this study was a smooth morphotype, so the authors suggest that the presence of GPL in the bacteria's membrane masked them from the host cells. Both mycobacterial species downregulated the expression of mucins, which are part of the airways' clearance system enhanced by inflammatory cues, such as IL-1 β , which was also downregulated in both cases. In the same study, the authors added PBMC-derived macrophages to the collagen matrix containing *M. bovis*-infected AirOs. They observed that the macrophages migrated towards the basal side of the AirOs, where they interacted and ingested mycobacteria but did not transverse to the AirO lumen. It is relevant to highlight that this model can mimic the epithelial wall of the upper airways but does not include alveolar epithelial cells. Besides, the lack of immune cells in the lumen of the AirO is noteworthy. In fact, the majority of the mycobacteria do not enter the AirO cells and grow extracellularly – only 2% of the AirO cells were infected with *M. tuberculosis* after 7 days [182]. However, that percentage increases to 15% in only 24 hours if the AirO cells are infected only after being seeded into a 2D monolayer. The authors suggest that the active mucociliary system in the 3D AirO hampers mycobacterial infection, and losing this mechanism in 2D enhances bacteria's internalization by the epithelial cells. This shows that the AirO model is a good reflection of the airway environment, but an alveolar organoid model may be a better option to study mycobacterial infections.

More recently, using the same AirO model – with basal, ciliated and goblet cells but no alveolar cells – Leon-Icaza et al. [184] studied the difference between *M. abscessus* smooth (S) and rough (R) organoid infection. Both variants grew over 12 days of infection in the lumen of the AirO, with no significant internalization by the AirO cells. Interestingly, *M. abscessus* S formed aggregates in contact with the apical side of the epithelial cells surrounded by an extracellular polymeric substance, which suggests biofilm formation. On the contrary, *M. abscessus* R formed serpentine cords of bacteria aligned parallelly. The R variant was associated with more cell death and production of mitochondrial ROS and H₂O₂ than the S variant, reflecting its higher virulence. Like Iakobachvili et al. [182], this study reports a decrease in the expression of several immune components, such as pro-inflammatory cytokines, the AMP lactoferricin and mucins. Additionally, the authors derived AirOs from the lung tissue of cystic fibrosis patients (CF-AirOs), obtaining organoids with mutations in the CFTR channel that accumulate mucus in the lumen and have enhanced oxidative stress, just as is in vivo. Both *M. abscessus* variants replicated more in the CF-

AirOs than in healthy AirOs, with enhanced formation of cords by *M. abscessus* R. Also, there was more oxidative stress and cell death caused by the infection than in healthy AirOs, suggesting higher susceptibility of CF-AirOs to *M. abscessus*. Treatment with the antibiotic cefoxitin, a β -lactam used in the clinic against *M. abscessus*, alone or combined with the antioxidant sulforaphane, reduced bacterial load from 92% to 99% of inhibition, respectively. Therefore, AirOs infected with mycobacteria are susceptible to antibiotics, potentiating this model as a complex tool for in vitro DST.

One of the major limitations of lung organoids is the lack of air exposure on the cells' apical side, as in vivo. This can only be achieved by enzymatically and/or mechanically dissociating the organoid 3D structure and seeding the cells at the bottom of a plastic well or in a transwell [174]. Coating with collagen, fibronectin or gelatin matrix is often required to promote cell adhesion to the surface. Having organoid monolayers facilitates access to the cell's apical side, easing the renewal of culture media and, most importantly, adding pathogens without needing a microinjector, which is very expensive and requires technical expertise and space. Transwell inserts also allow access to the cell's basal side, mimicking the epithelial barrier better by co-culturing epithelial cells with other cells, such as endothelial cells and immune cells that can even migrate to the upper and lower compartments [174]. Exposing the upper compartment to air while maintaining the cells in contact with a nutrient- and growth factor-rich medium on their basal side creates an air-liquid interface (ALI) [185], allowing, for example, ciliary movement and mucus production by airway epithelial cells [186]. Furthermore, 2D monolayers are more easily adapted to high-throughput settings than 3D organoids.

The association of organoid models with microfluidic systems resulted in the organ-on-a-chip technology, which can be adapted to several organs, including the lung [187]. Thacker et al. [188] established a murine ALI lung-on-a-chip model with *M. tuberculosis* to assess infection dynamics. The chip comprises two compartments separated by a porous membrane: the upper one is in contact with the air and populated by alveolar epithelial cells; the lower has endothelial cells in contact with a flowing liquid medium. They added fluorescent-tagged macrophages and observed their migration between the two compartments in response to the infection by time-lapse microscopy.

Investing in organoid science as a new approach to finding new treatments for infectious diseases is of extreme value. By now, it is the closest we get to simulating human disease without depending on animal testing, which is laborious and expensive, requires a high level of expertise, inevitably raises ethical issues, and often correlates poorly with clinical outcomes [189].

However, some barriers remain, particularly with lung organoid models, such as introducing immune cells and vasculature. Vascularizing the hydrogels that sustain the 3D structure of the organoid culture [190] can answer the latter.

Different in vitro infection models answer different scientific questions (**Figure 7**). A simpler model can be ideal for an initial screen with thousands of potentially active molecules. A more complex model can be applied on a smaller scale to select the most active compounds before the subsequent phases of the drug development pipeline. Nonetheless, the models complement each other. Mycobacteria are diverse at the species and strain levels regarding replication rate, metabolic state, preferable environment, and interaction with the host. The more available drug susceptibility models there are, the closer we will get to finding new solutions to antibiotic resistance and improving patient outcomes.

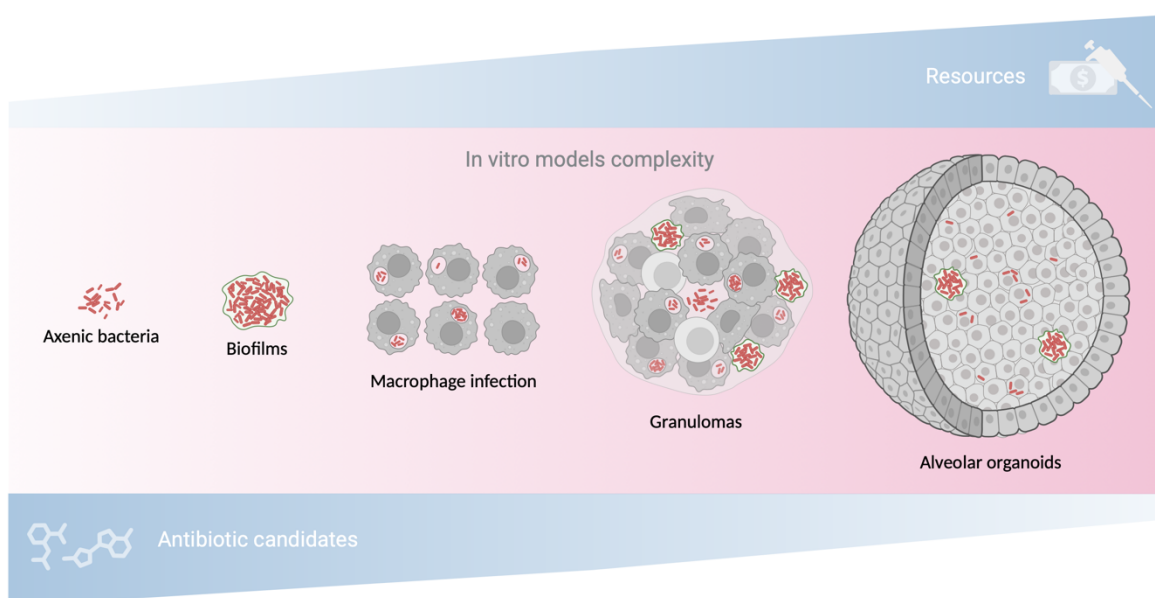


Figure 7. Mycobacterial in vitro infection models. As the complexity of the models increases, we come closer to mimicking in vivo conditions. Consequently, the resources allocated to each model increase while the throughput decreases. The image was created with BioRender.

OBJECTIVES

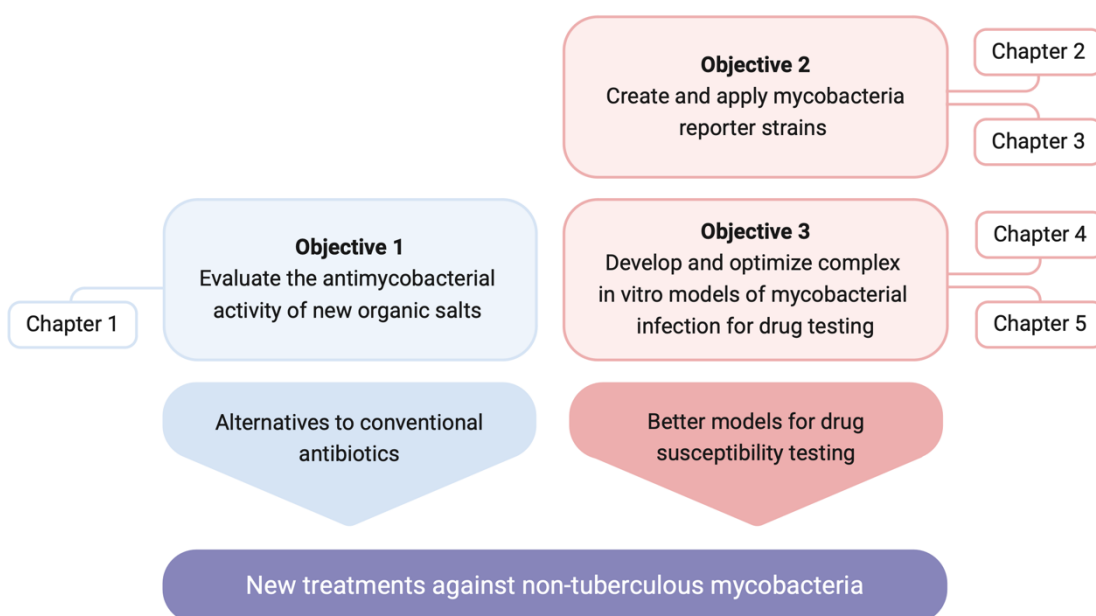
The overarching purpose of this work is to contribute to the discovery of new treatments against non-tuberculous mycobacteria. It includes two approaches: the first seeks alternatives to conventional antibiotics, while the second focuses on optimizing the models for drug susceptibility testing. These approaches complement each other, as testing new compounds using more complex and informative infection models leads to a more reliable selection of new drug candidates.

The first objective of this thesis aligns with the first approach, while the other two correspond to the second approach. They are as follows:

Objective 1. Evaluate the antimycobacterial activity of new organic salts

Objective 2. Create and apply mycobacteria reporter strains

Objective 3. Develop and optimize complex in vitro models of mycobacterial infection for drug testing



FINDINGS

Ethics statement

Human cells and tissues

This study uses primary human cells, namely peripheral blood mononuclear cells (PBMC), in Chapter 2. Blood samples from healthy individuals were collected at i3S medical facilities, with informed consent, privacy and confidentiality.

Fragments of excess lung tissue for the alveolar organoid experiments (Chapter 5) were obtained from resected pieces of patients who underwent lung resection. The protocol was approved by the ethics committee of the Centro Hospitalar Universitário de São João (Porto, Portugal), number CES 72/19. Samples were collected with informed consent, privacy and confidentiality.

All procedures comply with international guidelines and current national legislation, and agree with the World Medical Association Helsinki Declaration on Ethical Principles for Medical Research Involving Human Subjects.

Animals

This study uses the mouse to obtain bone marrow-derived macrophages (Chapters 1, 2, 3 and 4), mouse alveolar organoids (Chapter 5) and in vivo infections (Chapter 3). Mice were obtained from the i3S internal production. The procedures were done under the 3Rs principles, following the European Council Directive (2010/63/EU) and the Portuguese law (DL 113/2013) for animal welfare on experimentation. i3S Animal Facility is licensed by the national competent authority Direção Geral de Alimentação e Veterinária (DGAV) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Authorization for mouse experiments was submitted to the i3S Animal Ethics Committee and DGAV. The author is trained and certified at the FELASA C category (functions A+B+D), and by DGAV for research with animals.

Environment & Health Safety

This study involves the manipulation of mycobacterial species and mycobacteria-infected animals. Thus, all experiments were performed under biosafety levels (BSL) 2 conditions, according to Portuguese and EU law. The author has specific BSL2 training implemented at i3S. All procedures were performed under strict and periodically reviewed Standard Operation Procedures. i3S has a Biosafety Committee responsible for the revision, approval and supervision of all procedures involving pathogens.

CHAPTER 1

Improving antimycobacterial drugs through the formation of organic salts

Part of **Chapter 1** has been published in:

Bento CM, Silva AT, Mansano B, Aguiar L, Teixeira C, Gomes MS, Gomes P, Silva T, Ferraz R (2023) Improving the Antimycobacterial Drug Clofazimine through Formation of Organic Salts by Combination with Fluoroquinolones. *International Journal of Molecular Sciences*, 24, 1402. <https://doi.org/10.3390/ijms24021402>

This work was conducted in collaboration with LAQV-REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade do Porto, Portugal.

CHAPTER 1. Improving antimycobacterial drugs through the formation of organic salts

1.1. Introduction

Combining more than one drug in a single formulation is an appealing alternative to a conventional multidrug antibiotic regimen, as is the case with antimycobacterial therapies. Doing so by creating an ionic liquid (IL) can overcome physicochemical issues presented by the isolated parental molecules. Together with Paula Gomes' group at LAQV-REQUIMTE, University of Porto, we developed two new compounds that establish the same type of unique interactions as ILs: weaker Coulombic interactions and lower cohesive energies than in the solid phase, attributed to a lack of ion symmetry and low charge density. However, their melting point is above 100°C, classifying them as organic salts (OSs) instead of ILs. Nevertheless, OSs have melting points lower than their respective parent conventional salts, improving their thermostability, and are also more soluble [76].

The OSs were designed to ally the potent antimycobacterial activity of clofazimine, a first-line drug against *M. leprae* infections and currently undergoing clinical trials for the treatment of MAC pulmonary disease [55], with one of two fluoroquinolones, ofloxacin or norfloxacin, molecules with clinical effectiveness against MAC disease [53]. Clofazimine and the fluoroquinolones present opposite polarities when ionized, making them suitable for conjugation as an OS.

The objective of this study is to assess whether combining clofazimine with fluoroquinolones in an OS form can be an alternative to using the parent drugs to treat *M. avium* infections.

1.2. Materials and methods

1.2.1. Bacteria

Mycobacterium avium 2447 smooth transparent (SmT) strain, kindly provided by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium, was grown to exponential phase in Middlebrook 7H9 broth (BD Bioscience) containing 0.2% glycerol (VWR International) and 10% Albumin-Dextrose-Catalase (ADC) supplement at 37°C.

1.2.2. Direct effect of test compounds against mycobacteria

The antimicrobial activity of the different compounds was assessed by broth microdilution, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [83], using stock solutions of the compounds in DMSO due to the negligible solubility of clofazimine in water [191]. Briefly, $1 - 5 \times 10^5$ CFU/mL of *M. avium* was seeded in 96-well plates with increasing

concentrations of the compounds. Each condition was tested in triplicate. The plates were incubated at 37°C in a humid atmosphere. After six days of incubation, bacterial viability was assessed by resazurin reduction. 10% (v/v) of resazurin (Sigma-Aldrich) at 2.5 mM in phosphate-buffered saline (PBS) was added to each well. After 24 hours of incubation at 37°C, the fluorescence of resorufin, resulting from the conversion of resazurin by metabolically active cells, was measured at $\lambda_{\text{ex}} = 530 \text{ nm}$ and $\lambda_{\text{em}} = 590 \text{ nm}$ in a Synergy™ Mx microplate reader (BioTek, Agilent Technologies) using the Gen5 software. The results are expressed as the percentage of fluorescence obtained in treated wells relative to nontreated wells.

1.2.3. Infection of bone marrow-derived macrophages (BMM)

Macrophages were derived from the bone marrow of male C57BL/6 mice bred at the i3S animal facility. Briefly, the animal was euthanized by CO₂ inhalation, and its femurs and tibias were removed. Each bone was flushed with Hank's Balanced Salt Solution (HBSS, Gibco). The resulting cell suspension was centrifuged for 10 minutes at 259 g, 4°C, and re-suspended with Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10% Fetal Bovine Serum (FBS, Biowest), and 10% of L929 cell conditioned medium (LCCM) as a source of Macrophage Colony Stimulating Factor (M-CSF). Cells were cultured for at least 4 hours at 37°C in a 7% CO₂ atmosphere to remove fibroblasts. Non-adherent cells were collected, and the plate was washed three times with cold HBSS. The cellular suspension was centrifuged at 259 g, 4°C for 10 minutes, re-suspended with DMEM and the number of cells counted using a Neubauer chamber. The cell concentration was adjusted to 4×10^5 cells/mL with supplemented DMEM/10% LCCM, and the cell suspension was plated on 24-well plates (1 mL per well). The plates were incubated at 37°C in a 7% CO₂ atmosphere, and four days after seeding, 10% of LCCM was added to the culture medium. On the seventh day, the culture medium was renewed. On day 10 of culture, the cells are considered fully differentiated into macrophages.

On the 10th day, the culture medium was removed, and the adherent cells were incubated with a bacterial suspension of *M. avium* 2447 SmT (1:2.5, bacteria:cells) in supplemented DMEM for 4 hours at 37°C in a 7% CO₂ atmosphere. After incubation, cells were washed four times with warm HBSS to remove non-internalized bacteria and re-incubated with new supplemented DMEM/10% LCCM with each compound at 0.1, 0.3 and 5 μM . Each condition was tested in triplicate. The intracellular growth of *M. avium* 2447 SmT was evaluated five days after infection by colony-forming units (CFUs) assay, plating the bacteria in Middlebrook 7H10 agar (BD Difco) supplemented with 0.5% glycerol and 10% Oleic Acid-

Albumin-Dextrose-Catalase (OADC) for at least seven days at 37°C. The viability of macrophages was determined by resazurin reduction, as detailed above for mycobacteria.

1.2.4. IC_{50} and LD_{50} determination

The viability of both mycobacteria and host macrophages was determined by resazurin reduction, as detailed above. The IC_{50} (concentration that inhibits 50% of mycobacterial growth after seven days of incubation) and LD_{50} (concentration that inhibits 50% of macrophage viability) values were interpolated by fitting the experimental data through a 4PL nonlinear sigmoidal curve using the GraphPad Prism software version 9 (GraphPad Software Inc.). The respective 95% confidence interval of the interpolations was also retrieved.

1.3. Results

1.3.1. *In vitro* activity of the clofazimine-fluoroquinolone OSs against *M. avium*

The antimycobacterial activity and toxicity to host cells of both clofazimine-fluoroquinolone OSs, NfC and OfC (**Figure 1**), were tested *in vitro* and are summarized in **Table 1**.

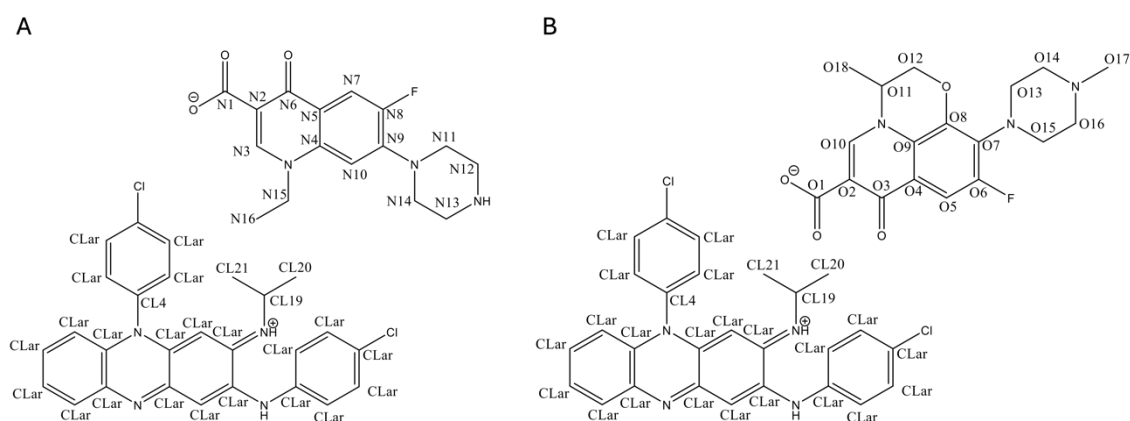


Figure 1. Chemical structure of NfC, the OS that combines norfloxacin (Nf) with clofazimine (C) (**A**), and of OfC, the OS that combines ofloxacin (Of) with clofazimine (**B**). The images were obtained using the ChemDraw software (Revvity Signals).

The susceptibility of *M. avium* to the clofazimine-fluoroquinolone OSs and their parent drugs was first assessed in axenic culture to determine the IC_{50} of each test compound. To this end, the bacteria were incubated with increasing concentrations of the compounds for seven days at 37 °C, and their viability was assessed by the resazurin assay. Both OSs were found to be equipotent to clofazimine and more potent than each of the individual fluoroquinolones (**Figure 2, Table 1**).

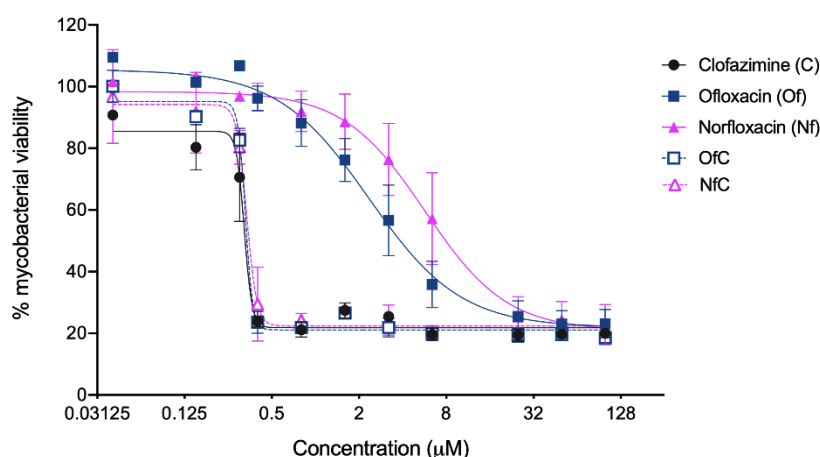


Figure 2. Antimycobacterial activity of the clofazimine-fluoroquinolone OSs and their parent drugs. *M. avium* 2447 SmT in liquid culture was incubated with increasing concentrations of OfC, NfC, clofazimine (C), ofloxacin (Of) and norfloxacin (Nf) for 7 days at 37°C. Mycobacterial viability was measured by resazurin reduction to resofurin at 530/590 nm. The symbols represent the average $\pm$ standard deviation of two to three independent experiments, presented as percentages of viable mycobacteria relative to non-treated mycobacteria. The lines represent the non-linear 4PL regression obtained in the GraphPad software for each experimental condition.

Considering that, after invading its mammalian host, *M. avium* replicates mainly inside macrophages, the toxicity of the OSs to infected murine bone marrow-derived macrophages (BMM) was also assessed. This was done by treating BMM infected with *M. avium* 2447 SmT with increasing concentrations of the test compounds for five days at 37 °C and 7% CO₂. Macrophage viability was assessed through a resazurin reduction assay. As shown in **Figure 3** and **Table 1**, though being more toxic to mammalian cells than their parent fluoroquinolones, the clofazimine-derived OSs seem less toxic to BMM than clofazimine itself. We are aware that the confidence intervals for LD₅₀ values shown in **Table 1** for clofazimine and its derived OSs have some degree of overlap, which, alongside the large standard deviations depicted on the macrophage viability curves shown in **Figure 3**, result in a non-statistically difference. Still, visual inspection of the viability curves in **Figure 3** suggests a decrease in toxicity compared to the parent clofazimine, and the same is hinted at by the LC₅₀ confidence intervals in **Table 1**.

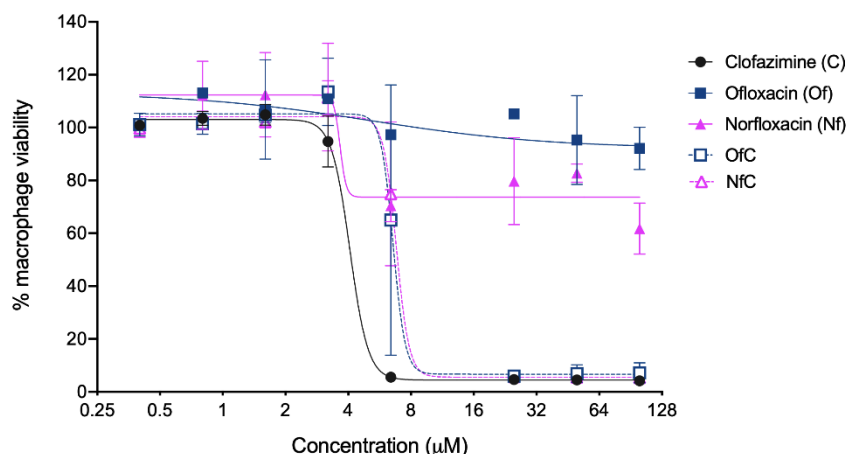


Figure 3. Toxicity of the clofazimine-fluoroquinolone OSs and their parent drugs to host mammalian cells. *M. avium* 2447 SmT in liquid culture was incubated with increasing concentrations of OfC, NfC, clofazimine (C), ofloxacin (Of) and norfloxacin (Nf) for 5 days at 37°C, 7% CO₂. Macrophage viability was measured by resazurin reduction to resofurin at 530/590 nm. The symbols represent the average $\pm$ standard deviation of two to three independent experiments, presented as percentages of viable macrophages relative to non-treated macrophages. The lines represent the non-linear 4PL regression obtained in the GraphPad software for each experimental condition.

Then, to test the activity of the OSs against intracellular mycobacteria, BMM infected with *M. avium* 2447 SmT were treated with three concentrations of the compounds that were not toxic to the macrophages (0.1, 0.3 and 0.5 μ M) for 5 days at 37 °C and 7% CO₂. The intracellular bacterial loads were quantified by the colony-forming units (CFUs) assay (**Figure 4**). Relevantly, even though the clofazimine-fluoroquinolone OSs were less active than the parental clofazimine, they still reduced the bacterial load up to 80% at the tested concentrations, being considerably more active against intracellular *M. avium* than their parent fluoroquinolones.

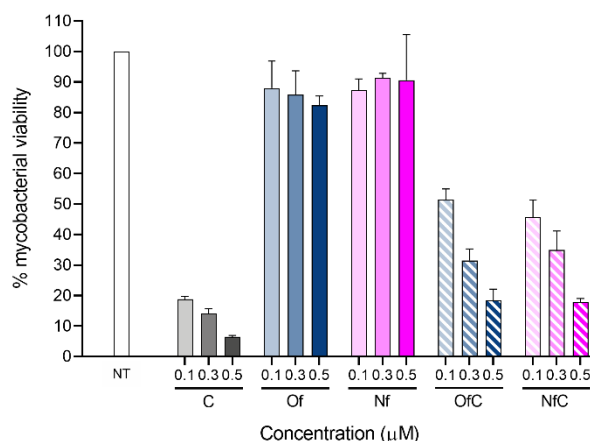


Figure 4. Activity of the clofazimine-fluoroquinolone OSs and respective parent drugs against intracellular mycobacteria. *M. avium* 2447 SmT in liquid culture was incubated with increasing concentrations of OfC, NfC, clofazimine (C), ofloxacin (Of) and norfloxacin (Nf) for 5 days at 37°C, 7% CO₂. Intracellular bacterial load was assessed by CFU counting. The graph shows the average + standard deviation of three replicates of one representative experiment, presented as the percentage of bacterial load in treated macrophages relative to nontreated macrophages. NT – non-treated.

Table 1. Physicochemical properties and in vitro effects of the clofazimine-fluoroquinolone OSs and their parent drugs. The compounds were tested against both axenic cultures of *M. avium* 2447 SmT (IC₅₀) and mammalian host cells (bone marrow-derived macrophages, BMM, LC₅₀) infected with *M. avium* 2447 SmT¹.

Compound	Molecular weight/g·mol ⁻¹	Net Charge ²	Melting Point [Decomposition Temperature]/°C	Solubility / mg·mL ⁻¹ (mM)	IC ₅₀ /μM ³	LC ₅₀ /μM ⁴
C	472.12	+1	221.9 [258.0]	4.8 (10.2)	0.33 (0.38 to 0.31) ⁵	4.1 (4.9 to 3.7) ⁵
Of	361.37	-1	274.8 [318.8]	5.7 (15.7)	3.85 (4.78 to 3.20) ⁵	>100
Nf	319.33	-1	206.1 [222.0]	11.0 (34.4)	7.93 (10.94 to 6.25) ⁵	>100
OfC	833.26	0	212.0 [274.3]	13.9 (15.8)	0.34 (0.36 to 0.32) ⁵	6.7 (13.04 to 3.42) ⁵
NfC	791.25	0	202.0 [354.6]	13.0 (17.3)	0.35 (0.38 to 0.33) ⁵	6.9 (13.44 to 3.57) ⁵

¹Due to the negligible solubility of clofazimine in water, stock solutions of all compounds were prepared in dimethylsulfoxide, so solubility data refer to this solvent (see text); ²at physiological pH 7.4; ³IC₅₀ is the concentration of the compound that inhibits by 50% the mycobacterial viability in axenic culture, obtained by interpolation of nonlinear regression (4PL) of the experimental data; ⁴LC₅₀ is the concentration of the compound that inhibits by 50% the viability of the host cells, obtained by interpolation of nonlinear regression (4PL) of the experimental data; ⁵the values within the parenthesis represent the % confidence interval of the interpolation. C – clofazimine; Of – ofloxacin; Nf – norfloxacin.

1.4. Discussion

This study aimed to evaluate the antimycobacterial activity of the new clofazimine-fluoroquinolone OSs. We tested the in vitro activity of these OSs against planktonic and intracellular *M. avium* and assessed their toxicity to host cells. Our results led us to conclude that the OSs maintained the same direct activity as clofazimine, significantly decreasing the IC₅₀ compared to the isolated fluoroquinolones (**Figure 2, Table 1**). In a more physiological environment, against infected macrophages, the OSs' activity at low concentrations is less potent than clofazimine's but significantly higher than the fluoroquinolones (**Figure 4**). However, the OSs' concentration can still be increased tenfold without being toxic to the host cells (**Figure 3**). A slight decrease in the OSs' toxicity compared to clofazimine consolidates the potential of these new formulations as alternatives to a regimen where clofazimine is administered independently. Besides, the new OSs can rescue the inclusion of fluoroquinolones in MAC disease treatment, as fluoroquinolones alone did not bring advantages in terms of activity when substituting ethambutol or rifamycins [54]. However, together with clofazimine in OS form, they are more active and can be good alternatives to help avoid antibiotic resistance.

Pairing ionically clofazimine with either ofloxacin or norfloxacin significantly improved their solubility and thermostability (results not shown). That is relevant given clofazimine's poor solubility in aqueous/polar environments, including gastro-intestinal luminal fluids [192,193], which limits its oral bioavailability. This issue was approached in other studies by encapsulation in different types of nanoparticles [194,195], nanosuspensions [196] or emulsions [197]. However, our OSs were obtained by mixing two existing molecules, which is undeniably a much simpler and more cost-effective way to ameliorate clofazimine than any nanoformulation strategy. Furthermore, clofazimine's activity against other pathogens, including *Staphylococcus* sp., *Streptomyces* sp., *Listeria* sp. and, of course, *M. leprae* [198], broadens the use of these OSs in other clinical presentations and co-infection conditions, as often happens in leprous lesions [199].

These results also open the path to the design of new formulations that can be interesting alternatives to multi-drug regimens associated with toxicity and low bioavailability. A number of combinations can be made with molecules with opposite polarities that can enhance the activity of the parent drugs by creating synergistic effects or allowing for decreased therapeutic dosages. Nevertheless, a new formulation, even if based on already clinically approved drugs, is required to enter the drug discovery pipeline to, among other factors, find the best administration route, dosage and metabolization, which can be particularly tricky for OSs or ILs, as they are composed of two molecules that must remain together until they reach the target. It is, therefore, essential to prove that they present advantages compared to existing drugs, and that can only be done by validating their activity in more

physiological settings. Indeed, in this study, we employed gold-standard methods to evaluate the antimycobacterial activity of the new compounds. Although informative, the following steps should include testing in more complex in vitro infection models to enhance their chances of progressing through the animal testing phase. The activity of the OSs can even be improved in different settings, with other players involved, such as different culture media, cell types and organizations.

This study, combined with our group's previous experience, highlighted the need to improve the models used in in vitro drug susceptibility testing.

CHAPTER 2

Construction and characterization of *Mycobacterium abscessus* fluorescent reporter strains

The data presented in **Chapter 2** have not yet been published.

CHAPTER 2. Construction and characterization of *Mycobacterium abscessus* fluorescent reporter strains

2.1. Introduction

Bacterial reporter strains are essential tools for adapting infection models to high-throughput drug screening (HTS) and reliably and affordably identifying hits. Relying on CFU counting or colorimetric methods like resazurin reduction for HTS is non-feasible. Therefore, reporter-based assays emerge as a simple, sensitive and robust solution.

We were provided with two distinct plasmids containing either the gene of a green fluorescent protein, mClover3, or a red fluorescent protein, mCherry, both designed in Paul Cos' research group, University of Antwerp. Both proteins have been well-described, are photostable and are widely used in mycobacterial reporter strains. Having both strains of mycobacteria, one expressing green fluorescence and the other red, expands their range of applications, from planktonic to intracellular assays. The bacteria's intrinsic fluorescence enables visualization under a microscope in live or fixed host cells without the need for dyes or antibodies. This is crucial for HTS as it saves time and enhances the reliability of results by requiring less manipulation.

Therefore, the goal of this work was to develop new fluorescent reporter *M. abscessus* and characterize them for drug susceptibility testing (DST).

2.1. Materials and Methods

2.1.1. Bacteria

DH5 α chemo-competent *Escherichia coli* (Invitrogen) was grown in Luria-Bertani (LB) (Merck Life Science) broth at 37°C. *M. abscessus* ATCC 19977, kindly provided by Paul Cos, University of Antwerp, Belgium, was grown in Middlebrook 7H9 broth (BD Bioscience) containing 0.2% glycerol (VWR International), 10% Albumin-Dextrose-Catalase (ADC) supplement and 0.05% Tween-80 (Merck Life Science) at 37°C, with agitation.

2.1.2. Plasmids

The plasmids pMV306G13+mclover and pMV306G13+mcherry (**Figure 1**), kindly provided by Paul Cos, University of Antwerp, Belgium, express the green fluorescent protein mClover3 and the red fluorescent protein mCherry, respectively. They also contain the antibiotic selection gene *KanR*, which provides resistance to kanamycin.

2.1.3. Transformation of *E. coli* with *mClover* and *mCherry* plasmids

pMV306G13+mclover or pMV306G13+mcherry were recovered and added to DH5 α chemo-competent *Escherichia coli* cells. The transformation occurred by heat shock technique, incubating at 42°C for 90 seconds. The resulting bacteria were incubated in LB at 37°C for 1 hour and then in LB agar with 50 μ g/mL of kanamycin (Gibco) overnight at 37 °C. Colonies were picked and incubated in LB broth with 50 μ g/mL of kanamycin at 37 °C for 16 hours. The plasmid was extracted from the cultures using the Miniprep technique (NZYMiniprep MB01001, NZYtech) and linearized using restriction enzymes EcoRI (EC0271, Thermo Scientific) and Sall (EC0641, Thermo Scientific). To verify the restriction digest, gel electrophoresis (1.2% agarose gel) of the plasmid cut with EcoRI alone and cut by both EcoRI and Sall was carried out after adding NZYDNA Loading dye 6x (MB13101, NZYtech) to the DNA. The mClover and mCherry genes were amplified using the polymerase chain reaction (PCR) technique. The PCR used the primers forward: 5'-GGTCGCAAAAGCGCCGAG-3' and reverse: 5'-GCCTGGCAGTCGATCGTAC-3', which were designed using SnapGene® software (Dotmatics) and synthesized by STAB VIDA and the NZYTaq II 2x Green Master Mix (MB35801, NZYtech). The amplified DNA was verified by gel electrophoresis (1.2% agarose gel) and sequenced with a Sanger sequencing reaction after purification with ExoSAP-IT® Express (Affymetrix) at the i3S Genomics platform.

2.1.4. Making *M. abscessus* electrocompetent for electroporation

An exponential culture of *M. abscessus* was centrifuged at 2879 g, 4°C for 10 minutes, and the pellet was resuspended in washing solution (ultrapure H₂O with 10% glycerol, 0.5 M sucrose and 0.025% Tween-80) on ice, followed by a series of centrifugations and resuspensions in 10 mL, 5 mL and 0.4 mL of washing solution.

2.1.5. Transformation of *M. abscessus* with *mClover* and *mCherry* plasmids

Electrocompetent *M. abscessus* was transformed with the plasmid pMV306G13+mclover or pMV306G13+mcherry by electroporation (2500 V, 25 μ F, 1000 Ω) using 2 mm gap electroporation cuvettes (5520, Molecular BioProducts) and a MicroPulser Electroporator (Bio-Rad). The bacteria were incubated in complete 7H9 broth overnight at 37°C with agitation and then centrifuged and resuspended in 0.4 mL complete 7H9 broth. The bacterial suspension was plated in Middlebrook 7H10 containing 0.5% glycerol, 10% OADC and 50 μ g/mL of kanamycin at 37°C.

2.1.6. Confirmation of the *M. abscessus* transformation with mClover and mCherry

Transformed *M. abscessus* was grown to exponential phase in complete 7H9 broth and centrifuged at 2879 g, 4°C for 6 minutes. The pellet was resuspended in H₂O RNase-free water and transferred to a 2 mL cryotube with 0.1 mm glass beads (11079101, BioSpec Products) and TEN buffer (0.1 M Tris-HCl pH=8.0 + 0.01 M EDTA pH=8.0 + 1 M NaCl). The bacteria were lysed using FastPrep-24 (MP Biomedicals) in 2 cycles of 30 seconds at 5.0 m/s. The lysate was incubated in chloroform (Fisher Chemical) for 2 minutes, centrifuged at 13800 g, 4°C for 5 minutes, and the aqueous phase was removed, incubated in chloroform and centrifuged. The aqueous phase was incubated with 3 M sodium acetate (Merck Life Science) and ice-cold absolute ethanol (Merck Life Science) for 2 minutes. The DNA precipitated overnight at -20°C and then was centrifuged at 16200 g, 4°C for 5 minutes. The pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer (0.01 M Tris-HCl pH=8.0 + 0.001 M EDTA pH=8.0).

The genomic DNA extracted from *M. abscessus* transformed with mClover or mCherry was amplified by PCR as described before for the *E. coli* transformation and verified using gel electrophoresis (1.2% agarose).

2.1.7. Growth curves

M. abscessus mClover and *M. abscessus* mCherry were grown to exponential phase in complete 7H9 broth + 50 µg/mL kanamycin, diluted to OD₆₀₀ = 0.05, and incubated at 37°C, 90 rpm for 96 hours. At each time point, OD₆₀₀ of a sample diluted 1:10 in culture medium was measured, and CFUs were determined by plating the bacteria in 7H10 agar + 10% OADC. Optical density was plotted against CFU counting, and Pearson correlation was performed using the software Prism9 (GraphPad Software Inc.). The analysis provides the Pearson correlation coefficient (r) value, where r = 1 means a perfect correlation.

2.1.8. Reporter strains susceptibility to conventional antibiotics

M. abscessus mClover and *M. abscessus* mCherry were grown to exponential phase in complete 7H9 broth and 1 – 5 × 10⁵ CFU/mL of bacteria were seeded in 96-well plates in the presence of 50 µg/mL of kanamycin, azithromycin, a combination of both, or increasing clarithromycin concentrations (0.391 – 100 µg/mL). The antibiotics were obtained from Merck Life Science. Each condition was tested in triplicate. The plates were incubated at 37°C in a humid atmosphere. *M. abscessus* mClover and *M. abscessus* mCherry viability were assessed by measuring their intrinsic fluorescence at $\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 519 \text{ nm}$ or $\lambda_{\text{ex}} = 578 \text{ nm}$, $\lambda_{\text{em}} = 603 \text{ nm}$, respectively, in a Synergy™ Mx plate reader and Gen5 software (BioTek). Results are expressed as Relative Fluorescence Units (RFU), corresponding to

the raw values provided by the equipment (kanamycin-azithromycin experiment) or as the percentage of fluorescent intensity obtained in experimental wells relative to nontreated wells (clarithromycin experiment).

2.1.9. Infection of bone marrow-derived macrophages and intracellular antibiotic activity

Macrophages were derived from the bone marrow of male C57BL/6 mice bred at the i3S animal facility, as described in **Chapter 1 (Section 1.2.3)**. On day 10 of culture, the culture medium was removed, and the adherent cells were incubated with a bacterial suspension of *M. abscessus* mCherry (1:2.5, bacteria:cells) in supplemented DMEM for 4 hours at 37°C in a 7% CO₂ atmosphere. After incubation, cells were washed four times with warm HBSS to remove non-internalized bacteria and re-incubated with new supplemented DMEM/10% LCCM with clarithromycin at 0.1, 1 and 5 µg/mL. Each condition was tested in triplicates. The intracellular growth of *M. abscessus* mCherry was evaluated two days after infection by CFU assay. The macrophages were lysed with 0.1% saponin, and the bacterial suspension was serially diluted in water containing 0.05% Tween-80 and plated in 7H10 agar + 10% OADC.

2.1.10. Infection of PBMC spheroids in a collagen-fibronectin matrix

Whole blood from a healthy donor was collected and diluted in PBS + 2% FBS to a final volume of 30 mL. The blood was slowly added to 15 mL of Histopaque (10771, Sigma) on a SepMate-50 tube (85450, StemCell Technologies) and centrifuged for 10 minutes at 1200 g, 4°C. The top layer was separated from the erythrocytes and diluted in PBS + 2% FBS to a final volume of 50 mL. Following a centrifugation of 10 minutes at 400 g, 4°C, the pellet was resuspended in 30 mL of PBS + 2% FBS and centrifuged again. The pellet was resuspended in 4 mL of RPMI Medium 1640 (Gibco) supplemented with GlutaMAX-I (61870-010, Gibco) + 20% FBS and the number of cells counted using a Neubauer chamber. The cell concentration was adjusted to 5×10^6 cells/mL, the cells were mixed with a suspension of *M. abscessus* mClover (1:500, bacteria:cells), and 50 µL was added to an 8-well IBIDI plate. The PBMCs + *M. abscessus* mClover suspension was carefully mixed with a matrix constituted of 950 µL of 3 mg/mL PureCol (5005, Advanced BioMatrix), 4 µL of 1 mg/mL human fibronectin (354008, Corning), 50 µL of PBS 10x and 10 µL of 1M NaOH. The plate was incubated for 45 minutes at 37°C to promote the solidification of the matrix, and 100 µL of supplemented RPMI + 20% FBS was added on top. The plate was incubated at 37°C, 7% CO₂.

2.1.11. Fluorescence microscopy

M. abscessus mClover and *M. abscessus* mCherry were grown to exponential phase in complete 7H9 broth. A sample of each culture was collected, placed on a glass slide, and air-dried. The bacteria were visualized in a Zeiss Axio Imager Z1 microscope equipped with a Plan-Apochromat 63x/1.40 Oil DIC objective and an Axiocam MR ver3.0 camera (Carl Zeiss). The samples were excited using filter sets at 470 nm and 550 nm, and the emitted fluorescence was detected at 525 nm and 605 nm, for *M. abscessus* mClover and *M. abscessus* mCherry, respectively.

Bone marrow-derived macrophages infected with *M. abscessus* mClover or *M. abscessus* mCherry in 8-well IBIDI plates were imaged live (37°C, 5% CO₂) in a Leica DMI6000 Timelapse microscope with a HCX PL FLUOTAR 40x/0.75 DIC objective and Hamamatsu FLASH4.0 camera, one day after infection. After three days of infection, the same infected cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes, permeabilized with PBS + 0.1% Triton for 15 minutes, stained with DAPI (nuclei) for 30 minutes and imaged in the same microscope. Macrophages were visualized with phase contrast (one day post-infection) and nuclei excited at 340-380 nm with emission at 425 nm (three days post-infection). The mycobacteria were excited using filter sets at 460 nm and 560 nm, and emission was detected at 527 nm and 645 nm, for *M. abscessus* mClover and *M. abscessus* mCherry, respectively.

PBMC spheroids infected for three days with *M. abscessus* mClover in a collagen-fibronectin matrix were incubated with 0.5 µM CellTracker deep red (C34565, ThermoFisher Scientific) and imaged live with a Laser Scanning Confocal Microscope Leica TCS SP5 II using the 496 nm (*M. abscessus* mClover) and 633 nm (CellTracker deep red) lasers and an HC PL APO Lbl. Blue 63x/1.40 Oil objective.

2.2. Results

2.2.1. Transformation of *E. coli* with the mClover and mCherry plasmids

To obtain the quantity of DNA necessary to transform mycobacteria, DH5α chemo-competent *E. coli* was transformed by heat shock with a plasmid containing either a green fluorescent protein gene, mClover, or a red fluorescent protein gene, mCherry (**Figure 1**).

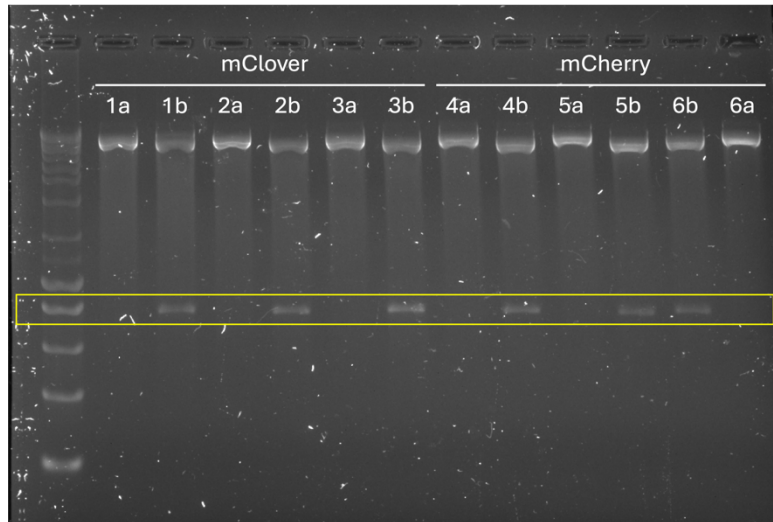


Figure 2. Gel electrophoresis on 1.2% agarose gel of the plasmids after linearization with the restriction enzyme EcoRI (a) or with the restrictions enzymes EcoRI and Sall (b) from three colonies of *E. coli* transformed with the mClover (1-3) or mCherry (4-6) plasmid. The yellow box indicates the bands with ca. 800 bp, given by the Ladder NzyDNA III (NZYtech). The image was obtained with a Gel Doc XR+ System (Bio-Rad).

The size of the bands after digestion by EcoRI and Sall was around 800 bp, which is the size of the mClover (835 bp) and mCherry (844 bp) genes, pointing to a successful transformation of *E. coli* with the mClover and mCherry plasmids. To further confirm this, the mClover and mCherry genes were amplified by PCR and analyzed by gel electrophoresis and DNA sequencing. Each amplified DNA sequence matched the respective gene sequence, confirming that *E. coli* was correctly transformed with the plasmids containing the gene with the gene to each fluorescent protein.

2.2.2. Transformation of *M. abscessus* with the mClover and mCherry plasmids

To obtain mClover and mCherry expressing mycobacteria, the plasmids were isolated from *E. coli* and inserted into electrocompetent *M. abscessus* ATCC 19977 by electroporation. Transformed colonies were selected after incubation in kanamycin-containing medium. The colonies' color, in a green or red tone, indicated that mClover or mCherry were being expressed by the bacteria, respectively. Genomic DNA was extracted to confirm the integration of the fluorescent genes in the *M. abscessus* genome. The fluorescent gene's sequence was amplified by PCR and analyzed by agarose gel electrophoresis (**Figure 3**). The bands on the agarose gel were about 800 bp, confirming the correct transformation of *M. abscessus* with mClover or mCherry.

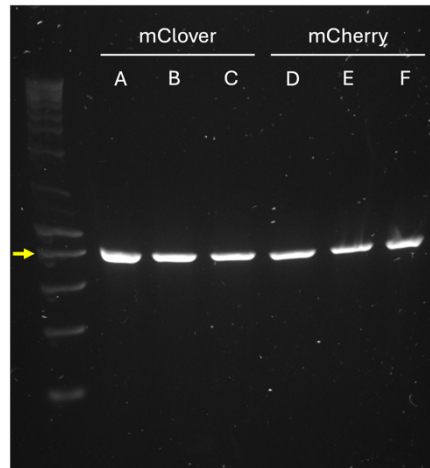


Figure 3. Gel electrophoresis on 1.2% agarose gel of the PCR products from three colonies of *M. abscessus* transformed with mClover (A-C) or mCherry (D-F). The yellow arrow indicates the bands with ca. 800 bp, given by the Ladder NzyDNA III (NZYtech). The image was obtained with a Gel Doc XR+ System (Bio-Rad).

2.2.3. Characterization of the *M. abscessus* mClover and mCherry strains

To evaluate how the *M. abscessus* fluorescent reporter strains grow in liquid media, the optical density (OD) was measured over 96 hours (**Figure 4A**). Both strains grow similarly, at a lower rate in the first 30 hours and then enter the exponential phase. Furthermore, by plotting OD against CFU counting at the same time points, it is possible to conclude that both parameters have a good correlation.

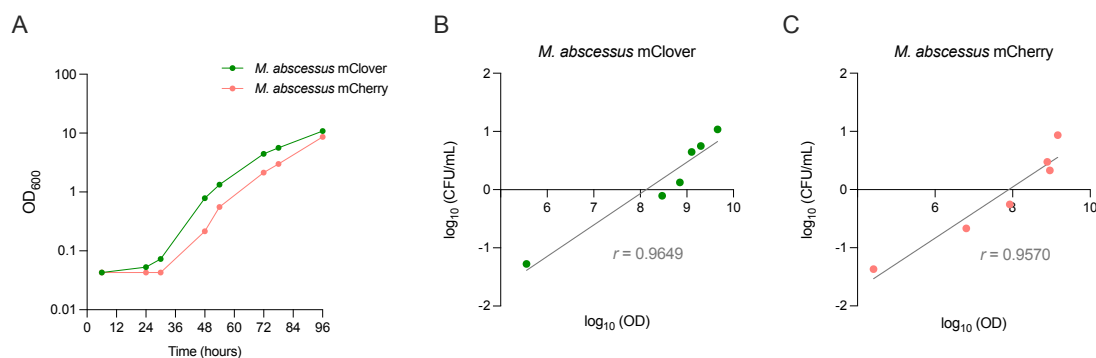


Figure 4. Growth profile of the *M. abscessus* mClover and mCherry strains. **(A)** Liquid cultures of each strain were incubated in the presence of 50 $\mu\text{g/mL}$ of kanamycin at 37°C, 90 rpm, for 96 hours, and the OD₆₀₀ was measured at each time point. **(B-C)** Pearson correlation between OD₆₀₀ and CFU counting from the same liquid cultures of *M. abscessus* mClover **(B)** and mCherry **(C)**. The lines represent a nonlinear fit, and r is the Pearson correlation coefficient calculated with GraphPad Prism 9. The graphs represent data from one culture for each *M. abscessus* strain.

To assess if the transformed strains are stable without the antibiotic selection pressure, the fluorescent signal of the reporter strains was measured in the presence or absence of the selection antibiotic kanamycin and/or the antimycobacterial agent azithromycin (**Figure 5**). Both strains grow equally in the presence or absence of kanamycin. The presence of kanamycin in the culture medium did not affect the growth inhibition activity of azithromycin. These results corroborate the stability of the transformation up to 96 hours and show that fluorescence can be used as a measure of bacterial load in liquid culture.

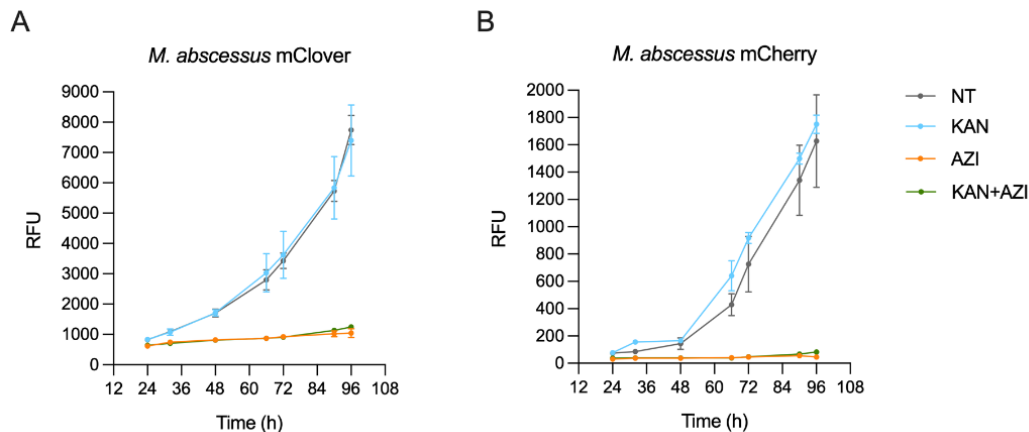


Figure 5. *M. abscessus* fluorescent reporter strains' growth is independent of the selection antibiotic kanamycin. Liquid cultures of *M. abscessus* mClover (**A**) and *M. abscessus* mCherry (**B**) were incubated in the presence of 50 $\mu\text{g/mL}$ of kanamycin (KAN), azithromycin (AZI), a combination of both (KAN+AZI) or neither (non-treated, NT) at 37°C, 90 rpm, for 96 hours, and the fluorescence (in RFU) was measured at each time point at 495/519 nm (mClover) or 578/603 nm (mCherry), respectively, using a Synergy™ Mx (BioTek). The symbols represent the average $\pm$ standard deviation of three replicates of one experiment.

To address if the transformation, and specifically the insertion of a resistance gene, altered the fluorescent strains' susceptibility to conventional antibiotics used in the clinic, *M. abscessus* mClover and mCherry strains were incubated with increasing concentrations of clarithromycin for 3 days at 37°C. The mycobacterial viability was evaluated by measuring the fluorescent signal of the reporter strains (**Figure 6A**). Both strains present a similar dose-response curve to clarithromycin and similar IC_{50} values: 4.28 $\mu\text{g/mL}$ for the mClover strain and 5.92 $\mu\text{g/mL}$ for the mCherry strain. The fluorescent signal of each strain was also detected by fluorescence microscopy, allowing the visualization of the individual bacilli expressing either the green fluorescent protein mClover (**Figure 6B**) or the red fluorescent protein mCherry (**Figure 6C**).

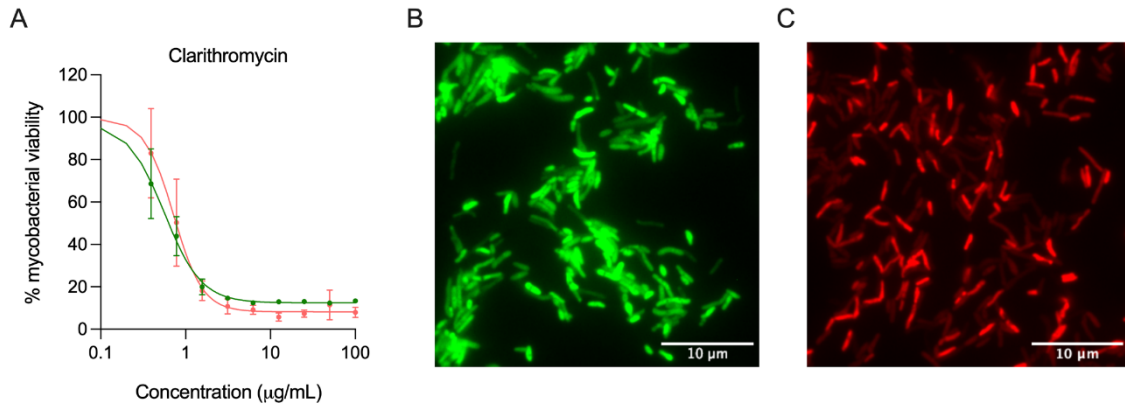


Figure 6. The fluorescent *M. abscessus* strains are susceptible to clarithromycin. **(A)** Clarithromycin susceptibility profile of *M. abscessus* mClover (green) and *M. abscessus* mCherry (pink). Each strain was incubated with increasing clarithromycin concentrations for 3 days. Mycobacterial viability was assessed by measuring each strain intrinsic fluorescence at 495/519 nm (mClover) or 578/603 nm (mCherry). The symbols represent the average $\pm$ standard deviation of three replicates of one experiment, presented as percentages of viable mycobacteria relative to non-treated mycobacteria. The lines represent the nonlinear regression (4PL) obtained in the GraphPad Prism 9. Fluorescence images of planktonic *M. abscessus* mClover **(B)** and *M. abscessus* mCherry **(C)** obtained with a Zeiss Axio Imager Z1 microscope.

Mycobacteria are intracellular pathogens that have the macrophage as one of the main host cells. To evaluate whether the fluorescent reporter strains can be used to perform intramacrophagic drug sustainability testing, murine bone marrow-derived macrophages were infected with *M. abscessus* mCherry. It was possible to observe that the intracellular mycobacterial load increased by approximately 2.5 logs in two days (**Figure 7A**). Besides, the intracellular mycobacteria are susceptible to increasing concentrations of clarithromycin. By fluorescence microscopy (**Figure 7B-E**), the mClover and mCherry transformed bacilli are visible inside macrophages.

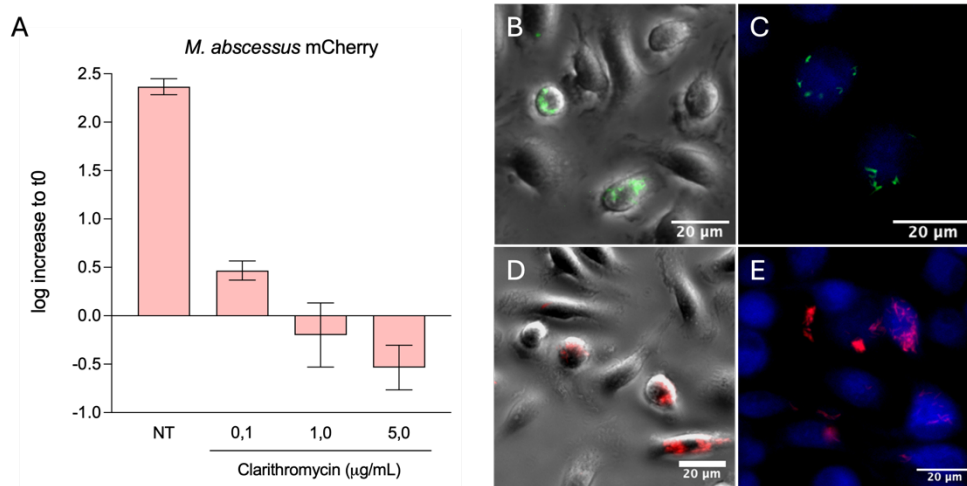


Figure 7. Infection of macrophages with *M. abscessus* fluorescent reporter strains. **(A)** BMM were infected with *M. abscessus* mClover for 2 days and treated with clarithromycin (CLA) at 0.1, 1 and 5 μg/mL. Log increase is the difference in terms of log₁₀ CFU/mL between day 2 and immediately after the infection (t0). NT – non-treated. The graph shows the average ± standard deviation of three replicates of one experiment. Representative fluorescence microscopy images of macrophages one **(B,D)** or three **(C,E)** days after being infected with *M. abscessus* mClover **(B,C)**, in green, or *M. abscessus* mCherry **(D,E)**, in red; in blue, nuclei (DAPI); macrophages in phase contrast in **B** and **D**. The images were obtained with a Leica DMI6000 Timelapse microscope.

Moving to a more complex in vitro infection model, human peripheral blood mononuclear cells (PBMCs) spheroids in a collagen-fibronectin matrix were infected with a low dose (1:500, bacteria:cells) of *M. abscessus* mClover. Although no quantification was made to assess intracellular bacterial load, the images obtained by confocal fluorescence microscopy show the bacilli inside individual host cells organized in a three-dimensional structure after three days of infection (**Figure 8**).

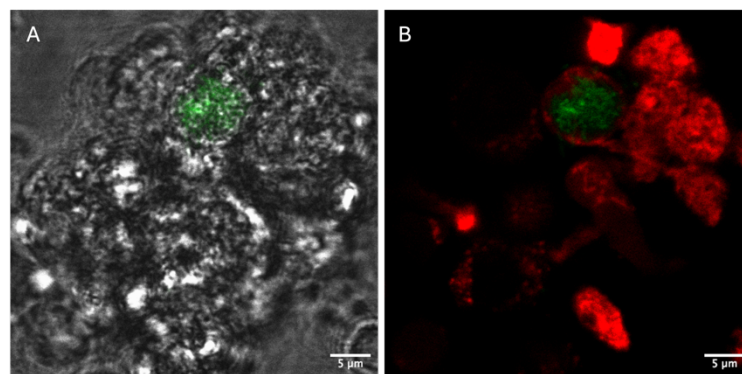


Figure 8. *M. abscessus* mClover infects three-dimensional aggregates of human host cells. PBMCs were incubated with *M. abscessus* mClover (1:500, bacteria:cells) in a collagen-fibronectin matrix for three days. Macrophages visualized in phase contrast **(A)** or stained with Cell Tracker deep red (red)

(B). In green, *M. abscessus* mClover. The images were obtained with a Leica SP5 II confocal microscope.

2.3. Discussion

We aimed to obtain a fluorescent *M. abscessus* strain. To achieve that, we transformed a laboratory strain of *M. abscessus* (ATCC 19977) to integrate the gene for a green fluorescent protein, mClover3, or a red fluorescent protein, mCherry, into the bacterial genome. Due to the thickness and high lipid content of the mycobacterial cell wall, we inserted the plasmids into the mycobacteria by electroporation, in which an electrical field is applied to increase the permeability of the cell membrane momentarily. To facilitate electroporation, the bacteria were previously treated with a detergent and sugar solution to improve the cell wall permeability [200], making them electrocompetent. The reduced number of electroporation products compared to the number of transformed *E. coli* after a heat shock, which involves simply exposing competent bacteria to a sudden temperature increase, highlights the challenges associated with mycobacterial transformation.

In addition to the fluorescent protein gene, the plasmids contain a gene that confers resistance to the antibiotic kanamycin, an aminoglycoside used to treat multi-drug-resistant *M. tuberculosis* infections [201]. Therefore, the *M. abscessus* that grew on kanamycin-containing agar carries the plasmid and, thus, the fluorescent protein gene. Visual inspection of the colonies showed that they presented a green or red coloration due to the expression of the respective fluorescent protein. That factor continually reassured us during the subsequent assays that our mycobacteria preserved their genetic modification. We further confirmed the presence of the fluorescent genes by amplifying the bacterial genomic DNA and performing gel electrophoresis (**Figure 3**). Moreover, fluorescence microscopy allowed us to observe the individual bacilli expressing either green or red fluorescence (**Figure 6B,C**).

We proceeded to characterize the new fluorescent reporter strains to determine whether the transformation altered any relevant factors for using them as DST tools. We observed that *M. abscessus* mClover and mCherry grow with time in liquid culture and that there is a good correlation between OD and CFU counting (**Figure 4**). That allows us to correctly and quickly determine the bacterial load of a culture at a particular time point by measuring the OD. We also confirmed that the fluorescent signal increases with time and that mClover emits a stronger signal than mCherry, although with higher background interference (**Figure 5**). We observed that the transformation was stable in the absence of the selection antibiotic, as the fluorescence emission was the same in the presence and absence of kanamycin for both strains. Importantly, the acquired resistance to kanamycin did not lead to cross-resistance with azithromycin, a macrolide, as the fluorescent signal remained at

basal levels in the presence of azithromycin (**Figure 5**). Both strains also show susceptibility to clarithromycin, presenting a similar dose-response curve to this macrolide, using fluorescence as a read-out (**Figure 6A**). Interference with other antimycobacterial agents should be studied to confirm the suitability of these strains for DST. We acknowledge that using a strain resistant to an antibiotic from the aminoglycoside class, which is crucial for treating NTM disease, poses a limitation in this transformation process. It could lead to false-negative results when evaluating the activity of compounds with a similar chemical structure to kanamycin.

To further characterize the fluorescent *M. abscessus* strains in a more complex and physiological setting, we infected macrophages, one of mycobacteria's primary host cells. The bacilli were visible inside the macrophages, which was corroborated by CFU counting (**Figure 7**).

Our results show that the *M. abscessus* mCherry intracellular load decreases when the infected macrophages are incubated with clarithromycin in a dose-dependent way (**Figure 7A**). Further fluorescence quantification and comparison with the CFU counting results would allow us to understand if fluorescence can be used to assess intramacrophagic bacterial load in a fast and reliable way.

We conducted a proof-of-concept assay to evaluate whether reporter strains could be utilized to monitor infection in a three-dimensional in vitro model using human host cells within a collagen-fibronectin matrix, aiming to replicate a granuloma in vitro model. Our results show that the fluorescent *M. abscessus* was internalized by the PBMCs (**Figure 8**). However, only a stacked analysis permits the observation of all infected cells in the aggregate, which imposes limitations on time and data generation for HTS assays. Other limitations are related to the low yield of PBMCs obtained from a donor's blood sample, which limits the number of conditions to be tested in each experiment, and the difficulty of staining the aggregated host cells embedded in a matrix for fluorescence microscopy.

Overall, the results presented in this chapter show the high potential of mycobacterial reporter strains to improve in vitro DST models by reducing time and resources and requiring less user interference as with gold-standard methods. Still, the robustness of these data should be enhanced by conducting side-by-side comparisons with the non-transformed *M. abscessus* strains, expanding the number of antibiotics tested to confirm the suitability of the reporter strains for susceptibility testing, and overall increasing the number of experiments in each case. Nonetheless, this study enabled us to develop expertise in molecular biology and bacterial transformation techniques and to grasp the essential aspects that need to be addressed to thoroughly characterize novel reporter strains for DST, which could be put into action in the study presented in **Chapter 3**.

CHAPTER 3

Application of double-reporter

***Mycobacterium abscessus* strains to drug testing**

Part of **Chapter 3** has been published in:

Bento CM, van Calster K, Piller T, Oliveira GS, de Vooght L, Cappoen D, Cos P, Gomes MS, Silva T (2024) Characterization of novel double-reporter strains of *Mycobacterium abscessus* for drug discovery: a study in mScarlet. Microbiology Spectrum, 12 (10) e00362-24. <https://doi.org/10.1128/spectrum.00362-24>

This work was conducted in collaboration with the Paul Cos' research group at the Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Belgium.

CHAPTER 3. Application of double-reporter *Mycobacterium abscessus* strains to drug testing

3.1. Introduction

The advantages of fluorescent reporter strains can be enhanced by having the mycobacteria also express luminescence. Since light emission occurs only in the presence of ATP, the signal serves as a measure of bacterial viability. With double-reporter strains, we can simultaneously assess the bacterial load, given by fluorescence, and the viability of the mycobacteria, given by luminescence. Furthermore, luminescence enables real-time visualization of bacteria during in vivo experiments.

M. abscessus double-reporter strains, Mab operon_mScarlet and Mab FF_mScarlet, were engineered to improve reporter gene expression levels and signal intensity compared to previously used systems. The recently developed red fluorescent protein mScarlet, recognized for its exceptional brightness, is expressed from an innovative synthetic promoter element (Pleft*), outperforming other fluorescent proteins like mCherry [202]. Mab operon_mScarlet is an autoluminescent strain, as it expresses the entire luciferase operon (lux operon), including its substrate. Mab FF_mScarlet expresses a codon-optimized, red-shifted thermostable variant of the firefly (FF) luciferase. These characteristics have shown the highest bioluminescent signal when compared to several other combinations [101,103]. Their application in mycobacterial infection models, ranging from broth cultures to macrophage and in vivo infection, can enhance their potential for HTS of thousands of compounds in a fast and reliable way.

The objective of this work was to fully validate Mab operon_mScarlet and Mab FF_mScarlet for use in DST.

3.2. Materials and methods

3.2.1. Bacteria

M. abscessus ATCC 19977 (from now on designated as Mab WT) was transformed by Paul Cos's group at the University of Antwerp, Belgium, using plasmids pMV306DIhsp+LuxG13+mScarlet or pMV306DIG13+FFrtCO+mScarlet (**Figure 1**). The transformation resulted in two new *M. abscessus* strains: Mab operon_mScarlet and Mab FF_mScarlet, which express the fluorescent protein mScarlet and emit luminescence either through the expression of the *LuxABCDE* operon or the enzyme firefly (FF) luciferase, respectively. They were grown in Middlebrook 7H9 broth (BD Bioscience) containing 0.2% glycerol (VWR International), 10% Albumin-Dextrose-Catalase (ADC) supplement and 0.05% Tween-80 (Merck Life Science) at 37°C, with agitation.

3.2.2. Transformation stability by flow cytometry

Mab operon_mScarlet, Mab FF_mScarlet and Mab WT cultures in exponential to stationary phase were passaged five times. Each sub-culture was sampled twice for cytometry. Briefly, 0.5 mL of bacterial suspension was collected, centrifuged at maximum speed for 5 minutes, and fixed with 4% paraformaldehyde (PFA) for 15 minutes. The bacteria were then resuspended in PBS with 2% FBS, 1 mM EDTA, and 0.1% sodium azide and filtered through a 35 μ m nylon mesh (Corning). The acquisition was performed in a BD LSRFortessa™ Cell Analyser (BD Biosciences) using the 610/20 nm filter, and data analysis was conducted in the FlowJo™ software (BD Biosciences).

3.2.3. Luminescence stability in liquid culture

Exponential phase cultures of Mab operon_mScarlet and Mab FF_mScarlet were plated in 96-well white plates (Thermo Scientific) and the luminescent signal was acquired in 3-minute intervals (integration time = 0.1 s) over 30 minutes using a Synergy™ Mx microplate reader and the Gen5 software (BioTek, Agilent Technologies). In the case of Mab FF_mScarlet, IVISbrite™ D-luciferin potassium salt (Revvity) at different concentrations in H₂O was added to the wells (10% v/v). Results are expressed as Relative Luminescence Units (RLU), corresponding to the raw values provided by the equipment.

3.2.4. Growth curves

Mab operon_mScarlet, Mab FF_mScarlet and Mab WT cultures in exponential phase were diluted to OD₆₀₀ = 0.05 and incubated at 37°C, 90 rpm for ten days. Every 24 hours, a sample of each bacterial culture was transferred to a 96-well black plate (Thermo Scientific) for fluorescence measurement (λ_{ex} = 569 nm; λ_{em} = 594 nm) or to a 96-well white plate (Thermo Scientific) for luminescence measurement (integration time = 0.1 s) using a Synergy™ Mx plate reader and Gen5 software (BioTek). For Mab FF_mScarlet, IVISbrite™ D-luciferin potassium salt at 0.5 mg/mL was added to the wells (10% v/v) before measuring the luminescence. At the same time, OD₆₀₀ of a sample diluted 1:10 in culture medium was measured, and CFU were determined by plating the bacteria in 7H10 agar + 10% OADC. Results are expressed either as Relative Fluorescence Units (RFU) or RLU, corresponding to the raw values provided by the equipment. Optical density, fluorescence, and luminescence were plotted against CFU counting, and Pearson correlation was performed using the software Prism9® (GraphPad Software Inc.). The analysis provides the Pearson correlation coefficient (r) value, where r=1 means a perfect correlation.

3.2.5. *Mycobacterial susceptibility against conventional antibiotics*

The following antibiotics were used: clarithromycin (0.0005–4 µg/mL), amikacin (0.016–32 µg/mL), linezolid (0.016–128 µg/mL), and moxifloxacin (0.016–32 µg/mL). The antimicrobial activity was assessed by broth microdilution, following the CLSI guidelines [83]. Briefly, $1 - 5 \times 10^5$ CFU/mL of Mab operon_mScarlet, Mab FF_mScarlet and Mab WT in exponential phase were seeded in 96-well white (Mab operon_mScarlet and Mab FF_mScarlet) or black (Mab WT) plates with increasing antibiotic concentrations. Each condition was tested in duplicate. The plates were incubated at 37°C in a humid atmosphere. After 3 days of incubation, Mab operon_mScarlet and Mab FF_mScarlet viability were assessed by luminescence measurement, as described above. Mab WT viability was assessed by resazurin reduction. Briefly, resazurin at 2.5 mM in PBS was added to the wells (10% v/v) and incubated for 8 hours at 37°C. The fluorescence of resorufin, resulting from the conversion of resazurin by metabolically active cells, was measured at $\lambda_{\text{ex}} = 530$ nm and $\lambda_{\text{em}} = 590$ nm in a Synergy™ Mx plate reader and Gen5 software (BioTek). The results are expressed as the percentage of luminescent or fluorescent intensity obtained in experimental wells relative to nontreated wells.

3.2.6. *MIC and IC₉₉ determination*

Each antibiotic's minimal inhibitory concentration (MIC) was obtained by observing the first well without visible turbidity in each experiment. The results represent the minimum and maximum values (interval), in µg/mL, observed in the different independent experiments. The IC₉₉ (antibiotic concentration that inhibits by 99% the mycobacterial viability) values were interpolated by fitting the luminescent (for Mab operon_mScarlet and Mab FF_mScarlet) or fluorescent (for Mab WT) experimental data through a 4PL nonlinear sigmoidal curve using the software Prism9® (GraphPad Software Inc.). The respective 95% confidence intervals of the interpolations were also retrieved.

3.2.7. *Strains suitability for high-throughput drug screening assays by Z'-factor determination*

One to 5×10^5 CFU/mL of Mab operon_mScarlet, Mab FF_mScarlet and Mab WT in exponential phase were seeded in a 96-well white (Mab operon_mScarlet and Mab FF_mScarlet) or black (Mab WT) plate. Thirty wells were incubated with clarithromycin at 2 µg/mL (CLA 2), and the other 30 wells were left untreated (CLA 0). After 3 days of incubation, the luminescent (for Mab operon_mScarlet and Mab FF_mScarlet) or resorufin fluorescent (for Mab WT) signal was measured as described above. The Z'-factor was calculated using the formula below:

$$Z'\text{-factor} = 1 - \frac{3(\sigma_{CLA2} + \sigma_{CLA0})}{|\mu_{CLA2} - \mu_{CLA0}|},$$

in which σ is the standard deviation; μ is the luminescence/fluorescence intensity average.

$Z'=1$, ideal assay, allowing for complete discrimination between negative and positive results; $1 > Z' \geq 0.5$ excellent; $0.5 > Z' > 0$, marginal; $Z'=0$, nominal; $Z' < 0$, unacceptable [203].

3.2.8. Infection of bone marrow-derived macrophages and intracellular antibiotic activity

Macrophages were derived from the bone marrow of male C57BL/6 mice bred at the i3S animal facility as described in **Chapter 1 (Section 1.2.3)**. Macrophages in 96-well PhenoPlate™ microplates (Revvity) (4×10^4 cells per well) were infected with Mab operon_mScarlet, Mab FF_mScarlet or Mab WT (MOI = 1) and treated with clarithromycin at 2 and 10 $\mu\text{g/mL}$, moxifloxacin at 2 and 8 $\mu\text{g/mL}$ or rifampicin at 8 and 16 $\mu\text{g/mL}$. The antibiotics were obtained from Merck Life Science. Each condition was tested in triplicate. The intracellular growth was assessed by CFU assay or fluorescence microscopy (for the transformed strains). Briefly, two days after infection, the infected cells were fixed with PFA 4% for 10 minutes, permeabilized with PBS + Triton 0.1% for 15 minutes, and stained with DAPI and HCS CellMask Deep Red (H32721, Invitrogen) for 30 minutes. The plates were screened in the automated high-content confocal microscope Opera Phenix™ Plus (Revvity), and the data was analyzed using Harmony High-Content Imaging and Analysis Software (Revvity) with mScarlet fluorescent signal being used as a measure of bacterial load. The images were acquired with 405 nm laser for DAPI (nuclei), 561 nm laser for Mab FF_mScarlet and 640 nm laser for HCS Cell Mask Deep Red (cytoplasm) and a 20x Air/0.4 objective. The fluorescence microscopy images were analyzed with ImageJ/Fiji software. Statistical analysis was performed using the software Prism9® (GraphPad Software Inc.), using a two-way ANOVA with Šidák's multiple comparisons test.

3.2.9. *M. abscessus* biofilm growth in 96-well plates and treatment with antibiotics

A Mab FF_mScarlet culture in exponential phase was centrifuged for 10 minutes at maximum speed, 4°C and the pellet was resuspended in culture medium without Tween-80 (biofilm medium) to a final concentration of 10^7 CFU/mL. 0.1 mL of bacterial suspension was added to the wells of a U-bottom, transparent polystyrene 96-well plate. The plate was sealed with parafilm and incubated for 7 days at 37°C in a humidified environment. After that time, the biofilm is fully formed inside the wall of each well, in contact with the air, above the liquid medium, with the appearance of a semi-opaque pellicle. Without touching the inside walls, the wells were washed twice with 0.2 mL of PBS. The biofilms were incubated with antibiotics amikacin, clarithromycin, rifabutin, linezolid (in 1% DMSO at the highest

concentration), moxifloxacin or imipenem (in H₂O) (Merck Life Science) for 2 days at 37°C in a humidified environment.

3.2.10. Quantification of biofilm luminescence and fluorescence

The wells were washed twice with PBS, and a solution of 0.5 mg/mL IVISbrite™ D-luciferin potassium salt (Revvity) in purified H₂O was added. Within 30 minutes, the luminescent signal emitted by Mab FF_mScarlet was captured with a ChemiDoc System (Bio-Rad) and a Newton 7.0 Imaging System (Vilber). The fluorescent signal was captured with Newton after exposure at 580 nm. Pixel intensity in each well was quantified and expressed using intensity mean values with the Image Lab™ software (Bio-Rad) for the luminescence obtained with ChemiDoc or using Kuant software for the luminescence and fluorescence obtained with Newton. The mean value obtained for the Triton X-100-treated wells (background) was subtracted to all wells.

3.2.11. Quantification of biofilm biomass

The D-luciferin solution was removed, and 0.2 mL of methanol (Merck Life Science) was added to each well. The plate was incubated for 15 minutes at room temperature. The methanol was removed, and the wells were air-dried. 0.2 mL of 0.1% crystal violet (CV) solution (Merck Life Science) in purified H₂O was added to each well, and the plate was incubated for 10 minutes at room temperature. The CV solution was removed, and the wells were washed twice with 0.2 mL of PBS. The CV stain was dissolved by adding 0.2 mL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm in a Synergy™ Mx plate reader and Gen5 software (BioTek).

3.2.12. Infection of nude mice with *M. abscessus*

Eight adult nude CBA mice bred at the i3S Animal Facility were kept in Biosafety level 2 (BSL2) conditions inside individually ventilated cages with HEPA filters and fed with sterilized food and water ad libitum. The mice were infected with 1.4x10⁸ CFU of Mab FF_mScarlet intravenously into one of the lateral tail veins with a 26G needle using a Hamilton syringe. At days 3, 7, 14 and 17 post-infection, after anesthesia and bioluminescence imaging, the two animals were euthanized by cervical dislocation. The spleen, liver and lung were collected and homogenized in circa 2.5 mL (spleen and lung) or 5 mL (liver) of purified H₂O with 0.5% Tween-80, and the bacterial load in the whole organ was assessed by CFU assay.

3.2.13. Bioluminescence imaging

Luminescence was measured using the IVIS® Lumina III *In vivo* Imaging System (Revvity) and the signal was analyzed with the Living Image v4.3.1 software and quantified in radiance.

Mab operon_mScarlet, Mab FF_mScarlet and Mab WT cultures in stationary phase were serially diluted 1:2 in a 96-well white plate. In the case of Mab FF_mScarlet, 0.5 mg/mL IVISbrite™ D-luciferin was added to the wells (10% v/v), immediately before visualization.

Imaging of the mice infected with Mab FF_mScarlet was performed immediately after (day 0) or 3, 7, 14, or 17 days post-infection. Two mice were injected subcutaneously with 30 mg/mL of IVISbrite D-luciferin Ultra RediJect (Perkin Elmer) and anesthetized with isoflurane using an XGI-8 Anesthesia System.

3.3. Results

3.3.1. Double-reporter plasmids

The two double-reporter *M. abscessus* strains – Mab operon_mScarlet and Mab FF_mScarlet – result from transforming the laboratory strain *M. abscessus* ATCC 19977 with one of two plasmid systems (**Figure 1**). Therefore, the double-reporter strains emit luminescence (**Figure 1D**) through either a bacterial luciferase encoded by the *LuxABCDE* operon (Mab operon_mScarlet, **Figure 1A**) or the red-shifted derivative of the firefly luciferase (FFrtCO) (Mab FF_mScarlet, **Figure 1B**) in addition to expressing the fluorescent protein mScarlet (**Figure 1C**). The resulting strains were validated through whole-genome sequencing.

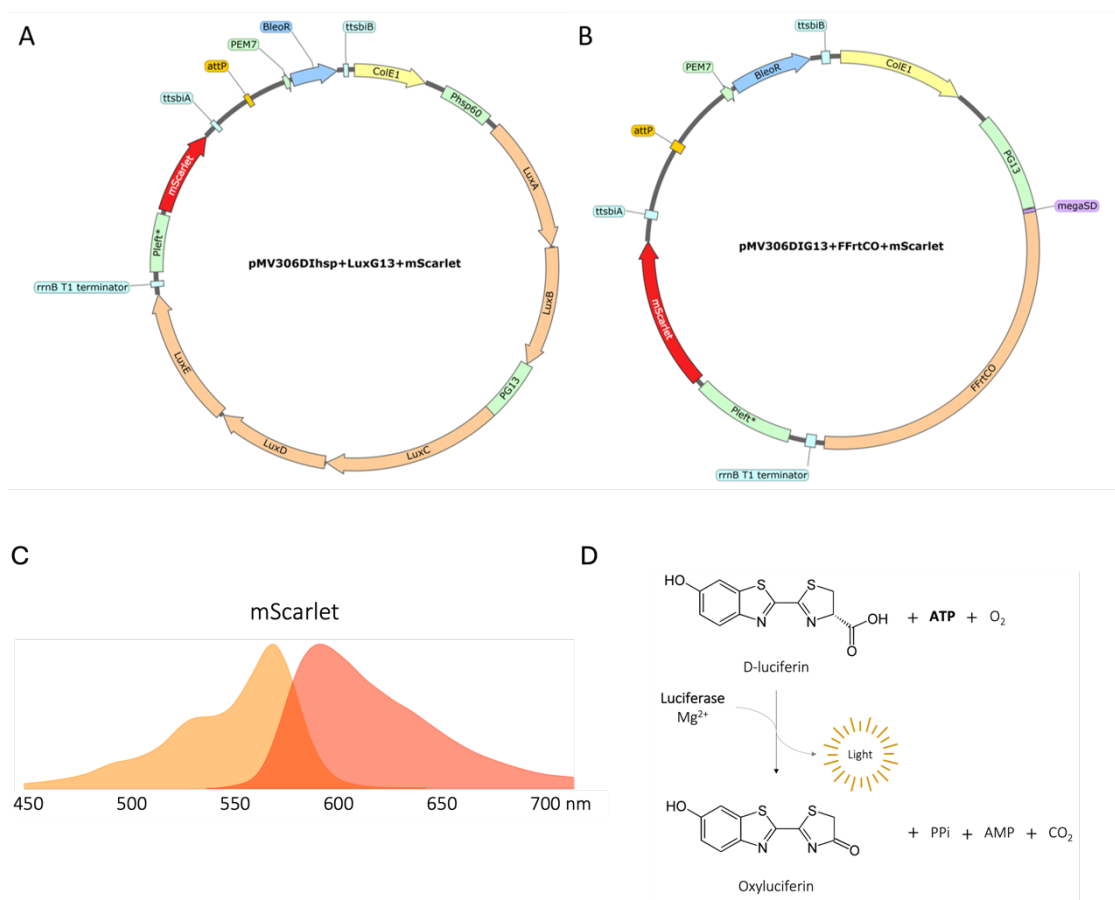


Figure 1. Visualization of the plasmids pMV306DIhsp+LuxG13+mScarlet **(A)** and pMV306DIG13+FFrtCO+mScarlet **(B)** used to transform Mab WT and generate Mab FF_mScarlet and Mab operon_mScarlet, respectively. The images were obtained using SnapGene® software (from Dotmatics; available at [snapgene.com](https://www.snapgene.com)). BleoR – zeocin resistance cassette; tetsiA – bi-directional intrinsic terminator; tetsiB – bi-directional intrinsic terminator B; Plef* – synthetic promoter element; megaSD – optimized mega Shine Dalgarno sequence; FFrtCO – red-shifted codon optimized firefly luciferase; PEM7 – EM7 promoter; Phsp60 – hsp60 promoter; PG13 – G13 promoter; attP – phage L5 attachment site; ColE1 – *E. coli* origin of replication. **(C)** Fluorescence spectra of mScarlet. The image was adapted from fpbase.org/protein/mScarlet (last accessed in January 2025). **(D)** Bioluminescence reaction catalyzed by luciferase. Imaged from [204].

3.3.2. Fluorescence and luminescence are stable

After the generation of the double-reporter strains, the next step was to evaluate if the transformation was stable. Specifically, if the double-reporter *M. abscessus* strains maintain their fluorescence throughout time when sub-culturing in a medium without the selection antibiotic (zeocin). To achieve this, mScarlet expression was evaluated by flow cytometry (**Figure 2**). The histograms show a clear difference between the fluorescence signal emitted by samples of Mab operon_mScarlet (**Figure 2A**) or Mab FF_mScarlet (**Figure 2B**)

compared to the non-transformed strain, here designated Mab WT. These results show that even after five passages without selective pressure from zeocin, the expression of mScarlet is maintained. We can thus presume that the expression of the luminescence genes is also maintained over time.

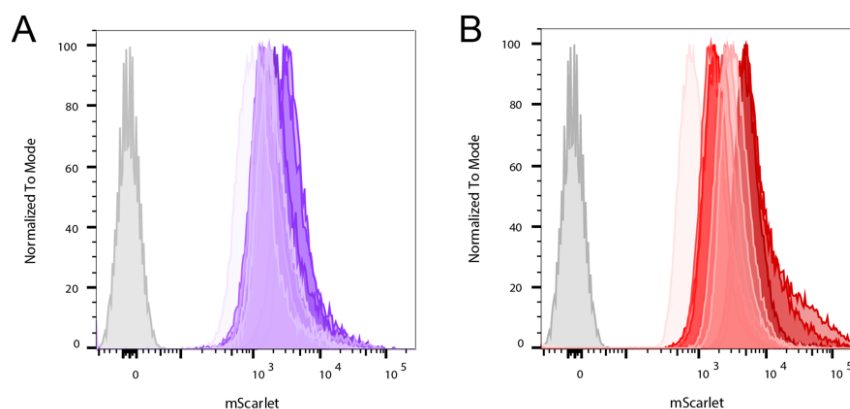


Figure 2. Expression of the mScarlet protein in liquid cultures, as evaluated by flow cytometry, of **(A)** Mab operon_mScarlet or Mab FF_mScarlet **(B)**. The three strains were cultured in 7H9 broth, passaged five times, and sampled twice for cytometry in each sub-culture. Each subculture is depicted in a different color, going from a lighter to a darker tone as the number of passages increases. The samples were analyzed in a BD LSRFortessa™ Cell Analyser (BD Biosciences) using the 610/20 nm filter, and the histograms were obtained with FlowJo™ software. The grey peak corresponds to Mab WT.

Regarding luminescence, the strain Mab FF_mScarlet only expresses the enzyme luciferase. For luminescence to occur, the enzyme's substrate, D-luciferin, must be added at the desired time point. Thus, the concentration of D-luciferin and the incubation time needed to obtain the maximum value were assessed in two Mab FF_mScarlet cultures differing in the number of cells.

We observed that 0.5 mg/mL of D-luciferin produces an intense and stable luminescent signal for up to 30 minutes, and this stability was independent of the number of bacteria in the culture (**Figure 3B**). Therefore, this was the concentration selected to proceed with the antibiotic susceptibility assays, and 10 minutes was the period chosen to measure the luminescent signal after adding D-luciferin. Similarly, the autoluminescence of two liquid cultures of Mab operon_mScarlet at different optical densities was also measured for 30 minutes (**Figure 3A**), confirming the stability of the luminescent signal.

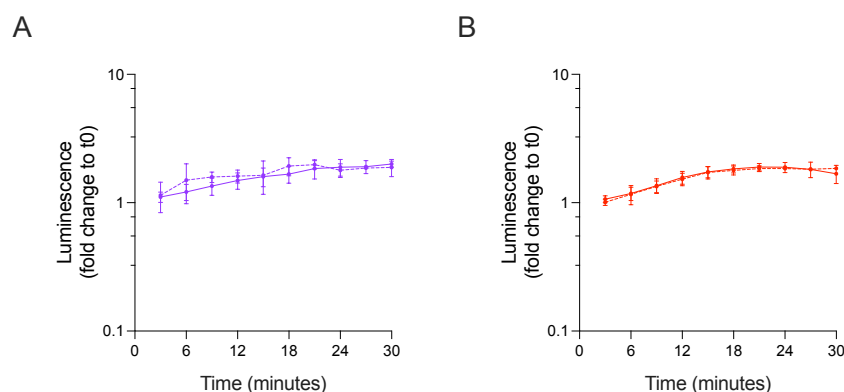


Figure 3. The luminescent signal was measured in 3-minute intervals of **(A)** Mab operon_mScarlet cultures after the addition of D-luciferin at 0.5 mg/mL (10% v/v) ($t=0$) and **(B)** Mab FF_mScarlet cultures. The dashed line represents a bacterial suspension ten times more diluted than the one represented by the full line. The results are expressed as fold change to RLU at $t=0$. The symbols represent the average $\pm$ standard deviation of two independent experiments.

3.3.3. The double-reporter *M. abscessus* strains grow similarly to the non-transformed strain, and their fluorescence and luminescence correlate with CFU counting

The growth profiles of the double-reporter *M. abscessus* strains were compared with Mab WT by simultaneously measuring OD₆₀₀, fluorescence, luminescence, and CFU. The OD and CFU results show that the double-reporter strains grew similarly to Mab WT (**Figure 4A, B**). The fluorescent and luminescent signals were similar between the two transformed strains, except that Mab operon_mScarlet had a lower luminescent signal when compared to Mab FF_mScarlet; after day 4, there was a slight decline in luminescence intensity (**Figure 4D**).

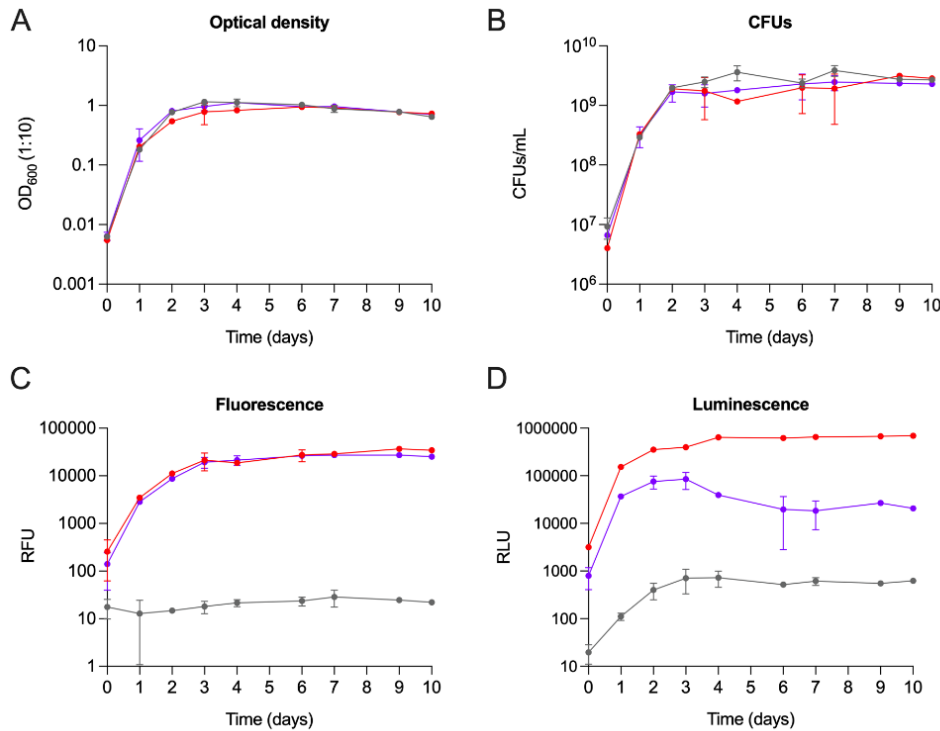


Figure 4. Growth curves of the double-reporter *M. abscessus* strains and the non-transformed *M. abscessus* strain. Liquid cultures of Mab WT (grey), Mab operon_mScarlet (purple), and Mab FF_mScarlet (red) were incubated at 37°C, 90 rpm, for ten days. In each time-point, samples were taken for **(A)** OD₆₀₀ (1:10), **(B)** CFU counting, **(C)** fluorescence at 569/594 nm, and **(D)** luminescence. Fluorescence and luminescence were read using a Synergy™ Mx (BioTek). The symbols represent the average $\pm$ standard deviations of two to three independent experiments. RFU – relative fluorescence units; RLU – relative luminescence units.

Moreover, by plotting OD, fluorescence, and luminescence parameters against CFU, it is possible to conclude that the fluorescent and luminescent signals emitted by the double-reporter *M. abscessus* strains, as well as the OD₆₀₀, have an excellent correlation with the CFU counting (**Figure 5**). In terms of luminescence, the *r* value for Mab operon_mScarlet indicates a good correlation, although it is not as good as the Mab FF_mScarlet results. This lower correlation is associated with decreased luminescent intensity after day 4 (**Figure 4D**), which is not in line with the CFU counting in that period (**Figure 4A**). Notably, the fluorescent and luminescent signals were similar in the presence of zeocin (data not shown) or in the absence of zeocin (**Figure 4**). Overall, the data above show the suitability of these strains for drug screening.

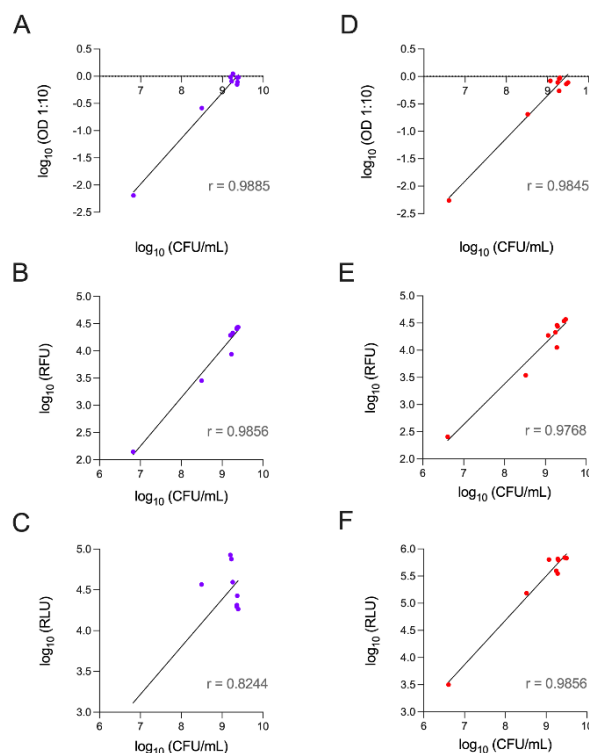


Figure 5. Pearson correlation between (A, D) OD₆₀₀ (1:10), (B, E) fluorescence, and (C, F) luminescence of the double-reporter *M. abscessus* strains and the CFU counting. (A-C) Mab operon_mScarlet; (D-F) Mab FF_mScarlet. The symbols represent the average of the experiments from **Figure 3**. RFU – relative fluorescence units; RLU – relative luminescence units. The lines represent a nonlinear fit, and r is the Pearson correlation coefficient calculated with GraphPad Prism 9.

3.3.4. The double-reporter *M. abscessus* strains have similar susceptibility to antibiotics as the non-transformed strain

To address if the transformation, and specifically the insertion of a resistance gene, altered the *M. abscessus* susceptibility to conventional antibiotics used in the clinic, the three *M. abscessus* strains were incubated with increasing concentrations of amikacin, linezolid, moxifloxacin, and clarithromycin for 3 days at 37°C. The mycobacterial viability was evaluated by measuring the luminescent signal of the double-reporter strains or by resazurin assay for Mab WT (**Figure 6**).

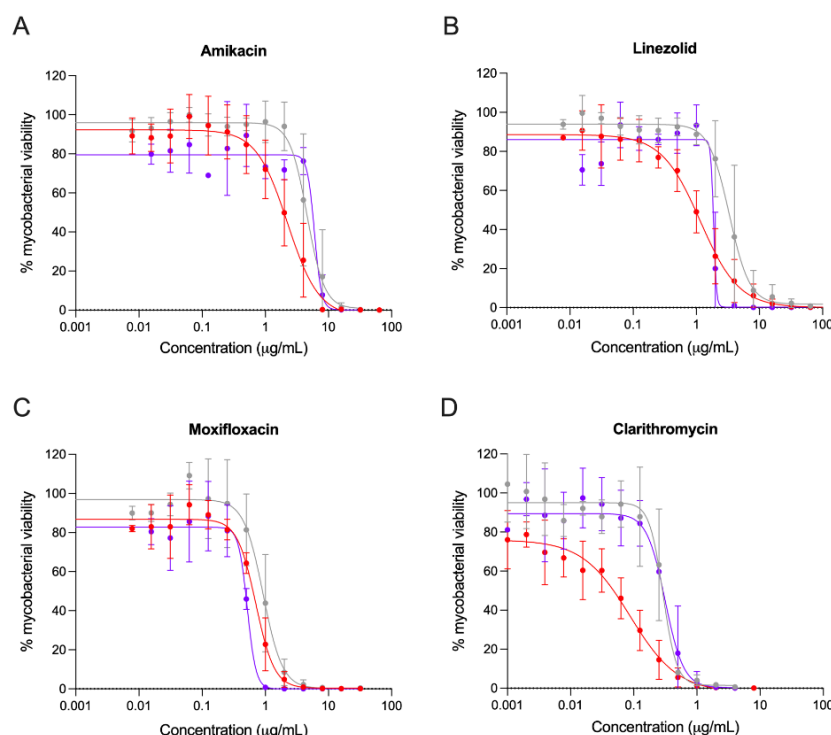


Figure 6. Antibiotic susceptibility profile of Mab operon_mScarlet (purple), Mab FF_mScarlet (red), and Mab WT (grey). Each strain was incubated with increasing concentrations of **(A)** amikacin, **(B)** linezolid, **(C)** moxifloxacin, and **(D)** clarithromycin for 3 days. The symbols represent the average $\pm$ standard deviations of three to seven independent experiments, presented as percentages of viable mycobacteria relative to the non-treated mycobacteria, measured by luminescence for Mab operon_mScarlet and Mab FF_mScarlet and by resorufin fluorescence for Mab WT. The lines represent the nonlinear regression (4PL) obtained in the GraphPad Prism 9 for each condition.

Overall, the antibiotic susceptibility profile of the double-reporter *M. abscessus* strains is similar to Mab WT, as reflected in the dose-response curves depicted in **Figure 6**. That is corroborated by comparing the values of the minimum inhibitory concentration (MIC) obtained by visual observation and the concentration of the antibiotic that inhibits by 99% the mycobacterial viability (IC_{99}) calculated with the luminescence or fluorescence data (**Table 1**).

Conventionally, the potency of an antibiotic is measured by the determination of the MIC value – the minimum antibiotic concentration for which no growth of bacteria is detected – through visual inspection of a liquid culture. This evaluation of antibiotic activity is, however, prone to error due to the subjectivity of the reading. Using fluorescence or luminescence as measures of bacteria viability allows the calculation of IC_{99} (as a *proxi* to complete growth inhibition) from dose-response curves. **Table 1** shows that the IC_{99} calculated from luminescence (double-reporter strains) correlates better with the MIC values for each antibiotic than the IC_{99} calculated from resazurin/resorufin fluorescence (Mab WT).

Therefore, luminescence is more sensitive than the resazurin assay to evaluate antibiotics' activity, especially for detecting low quantities of bacteria. The MIC and IC₉₉ values found for the double reporter strains did not significantly differ from those of Mab WT, except for an apparent discrete increase in susceptibility to linezolid by Mab operon_mScarlet.

Table 1. Activity of amikacin, linezolid, moxifloxacin, and clarithromycin against liquid cultures of the double-reporter *M. abscessus* strains and Mab WT.

Antibiotic	Mab operon_mScarlet		Mab FF_mScarlet		Mab WT	
	MIC interval ^a (µg/mL)	IC ₉₉ (µg/mL) ^b	MIC interval ^a (µg/mL)	IC ₉₉ (µg/mL) ^b	MIC interval ^a (µg/mL)	IC ₉₉ (µg/mL) ^b
Amikacin	[8, 32]	10.71 (5.60-n.c.)	[8, 32]	12.96 (7.99-n.c.)	[8, 16]	31.42 (10.03-n.c.)
Linezolid	[2, 8]	2.41 (n.c.)	[4, 32]	28.67 (10.15-n.c.)	[4, 32]	n.c.
Moxifloxacin	[2, 4]	1.04 (0.56-n.c.)	[2, 8]	3.64 (1.98-n.c.)	[2, 8]	4.91 (2.17-n.c.)
Clarithromycin	[0.5, 1]	1.58 (0.57-n.c.)	[0.5, 1]	1.36 (0.63-n.c.)	[0.5, 1]	n.c.

^a MIC: minimum inhibitory concentration, the first well without visible turbidity, is represented as an interval between the lowest and highest concentration detected in three to seven independent experiments. ^b IC₉₉ (antibiotic concentration that inhibits by 99% the mycobacterial viability) was measured by luminescence for Mab operon_mScarlet and Mab FF_mScarlet and by resorufin fluorescence for Mab WT. The IC₉₉ values were obtained by interpolation of nonlinear regression (4PL) of the experimental data obtained in the GraphPad Prism 9. The parenthesis values represent the interpolation's 95% confidence interval. n.c. – not possible to calculate.

In high-throughput drug screening, one way to evaluate the quality of the assay is by calculating the Z'-factor. The Z'-factor describes how well separated the positive and negative controls are and indicates the likelihood of false positives or negatives. Thus, we have determined the Z'-factor for all *M. abscessus* strains using 2 µg/mL of clarithromycin as the positive control (low quantity of bacteria) and non-treated bacteria as the negative control (**Table 2**).

Table 2. Z'-factor of a clarithromycin susceptibility assay against the double-reporter *M. abscessus* strains and the non-transformed *M. abscessus* strain.

Strain	Clarithromycin at 2 µg/mL		Clarithromycin at 0 µg/mL		Z'-factor ^b
	Luminescent intensity average (RLU) ^a	Standard deviation	Luminescent intensity average (RLU) ^a	Standard deviation	
Mab operon_mScarlet	712.73	252.76	29178.07	1627.39	0.80
Mab FF_mScarlet	3725.20	299.56	63009.43	3808.39	0.79
Strain	Resorufin intensity average (RFU) ^a		Resorufin intensity average (RFU) ^a		Z'-factor ^b
	Standard deviation		Standard deviation		
Mab WT	9753.27	1055.66	56584.93	4800.34	0.62

^a Average of luminescence (for the transformed strains) or resorufin fluorescence (for the non-transformed strain) intensity values of 30 wells treated or not with clarithromycin. ^b Z'-factor can be interpreted as follows: 1>Z'≥0.5 excellent.

As the calculated Z'-factors for all *M. abscessus* strains are between 0.5 and 1, the assay is considered excellent and can be used in a high-throughput context. Besides, the Z'-factor value is higher for the double-reporter *M. abscessus* strains than for Mab WT, which supports using these new strains' luminescence as a drug screening tool instead of conventional methods like the resazurin assay.

3.3.5. The double-reporter *M. abscessus* strains can infect mammalian host cells, and the intracellular bacterial load can be assessed by fluorescence

To evaluate the suitability of the double-reporter strains to infect host cells, murine bone marrow-derived macrophages were infected with the three *M. abscessus* strains. **Figure 7A** indicates that all strains grow similarly inside macrophages, having similar intracellular bacterial load after two days of infection. Moreover, the expression of mScarlet by the double-reporter strains allows us to monitor the infection by fluorescence microscopy, as seen in **Figure 7B**. The images were acquired using an automated high-content confocal microscope (Opera Phenix™ Plus, Revvity) that also quantifies the mScarlet fluorescent signal inside the host cells.

This quantification was used to evaluate the effect of different antimycobacterial agents (**Figure 7C-D**) at non-cytotoxic concentrations to the macrophages. mScarlet fluorescence showed to be a reliable measure of intramacrophagic bacterial load, allowing the detection of active antibiotics. The magnitude of the antibiotic effect evaluated by the fluorescent signal of the double-reporter strains was overall similar to that assessed by CFU. Interestingly, especially in the case of Mab FF_mScarlet, the apparent effect of the antibiotic was lower when measured by fluorescence. This can be related to the fact that mScarlet is

still present in bacteria that are not viable and cannot form colonies. Nonetheless, the transformed strains maintain the same susceptibility profile to the antibiotics as Mab WT in the intra-macrophagic assay.

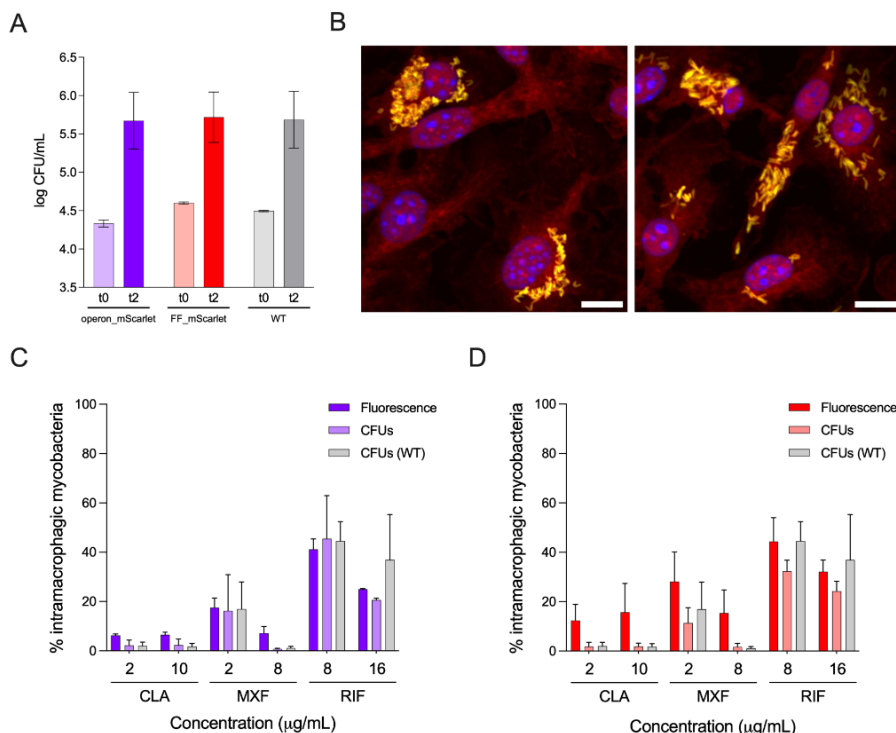


Figure 7. Infection of macrophages by the double-reporter *M. abscessus* strains. **(A)** Intracellular bacterial load immediately after infection (t0) and two days after infection (t2) with Mab operon_mScarlet (purple), Mab FF_mScarlet (red) or Mab WT (grey) by CFU counting. The graph shows the average $\pm$ standard deviations of two to eight independent experiments. **(B)** Representative fluorescence microscopy images of macrophages two days after being infected with Mab operon_mScarlet (left) or Mab FF_mScarlet (right), in yellow; in blue, nuclei (DAPI); in red, cytoplasm (HCS CellMask); magnification: 63x; scale bar: 10 μ m. Intracellular susceptibility of **(C)** Mab operon_mScarlet and **(D)** Mab FF_mScarlet to clarithromycin (CLA), moxifloxacin (MXF), and rifampicin (RIF), determined either by the mScarlet fluorescent signal or by CFU assay, in comparison to Mab WT by CFU. The graphs show the percentage of bacterial load in treated macrophages compared to nontreated macrophages two days post-infection and treatment, expressed as the average + standard deviations of two to three independent experiments.

3.3.6. *M. abscessus* biofilms' luminescence and fluorescence can be used for drug susceptibility testing

Mab FF_mScarlet biofilms were formed by culturing the bacteria in U-bottom 96-well plates for seven days. After the incubation, the biofilm is formed inside the wall of each well, partially above the liquid medium, in contact with the air. The semi-opaque pellicle that

constitutes the biofilms shows a slightly red color from the expression of the mScarlet fluorescent protein. Planktonic mycobacteria suspended in the liquid medium and aggregated at the bottom of the well are washed away before drug susceptibility testing.

To evaluate whether *M. abscessus* luminescence and fluorescence can be used for high-throughput drug screening, Mab FF_mScarlet biofilms were incubated for two days with increasing concentrations of six antibiotics conventionally used in the clinic to treat NTM disease: amikacin, clarithromycin, imipenem, linezolid, moxifloxacin and rifabutin. Additionally, the surfactant Triton X-100 was used as a positive control of biofilm disruption.

After two days of treatment, planktonic bacteria were washed, and D-luciferin was added. The plates were imaged using chemiluminescence detection equipment (ChemiDoc System, Bio-Rad) (**Figure 8A**). The intensity of the luminescent signal is represented by black pixels in the image, clearly limited to the walls of each well. The non-treated biofilms show an intense luminescence signal, contrary to Triton X-100-treated wells. It is also possible to observe that an increase in the antibiotic concentration results in a decrease in luminescence.

The fluorescent signal emitted by the biofilm was imaged using a Newton 7.0 Imaging System (Vilber) (**Figure 8B**). The intensity of the fluorescent signal follows a similar pattern as that obtained for the luminescent signal, with almost no signal in the Triton X-100-treated wells and a visible decrease in intensity accompanying the antibiotics' concentration increase. This equipment can also detect luminescence. Therefore, the luminescence signal after incubation with D-luciferin was also assessed (**Figure 8C**), corroborating the ChemiDoc results.

Crystal violet (CV) staining was performed on the same plate, following luminescence and fluorescence readings. CV is conventionally used to assess biofilm biomass, as this dye binds to negatively charged molecules, such as proteins and DNA, in the biofilm's extracellular matrix. With this technique, the biofilm is clearly visible to the naked eye, and the results align with the luminescence and fluorescence ones (**Figure 8D**).

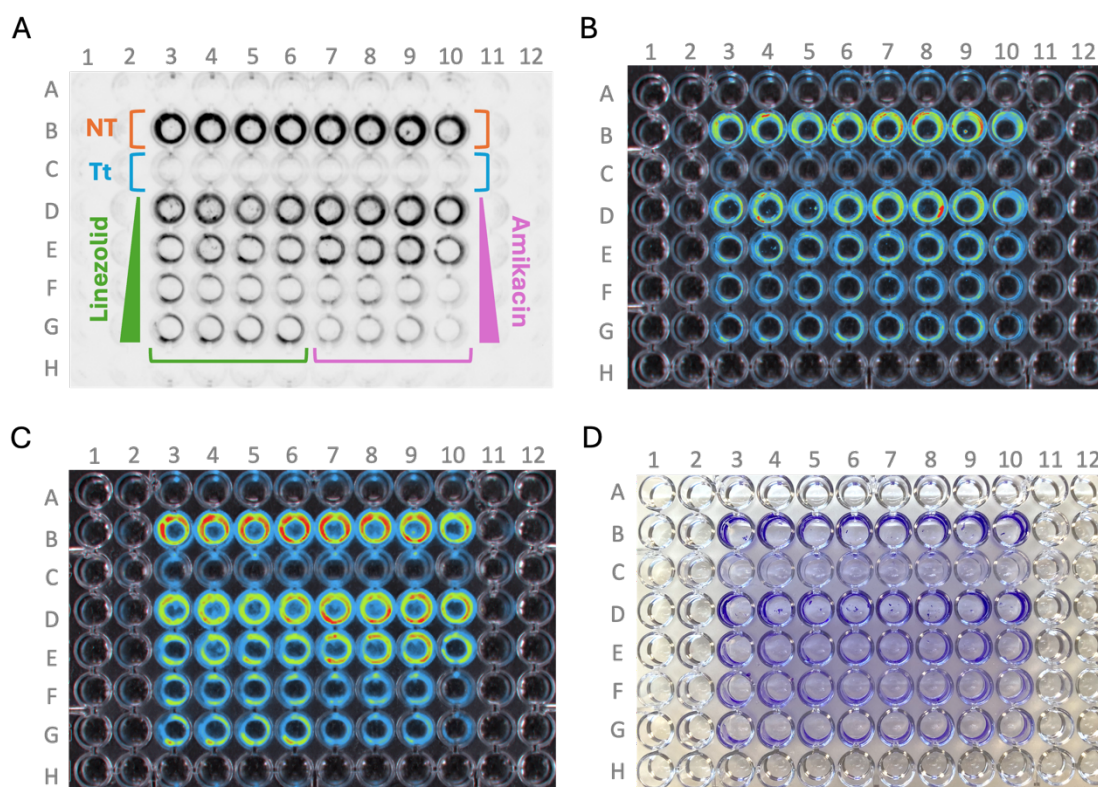


Figure 8. Luminescence and fluorescence measurement allow to visualize Mab FF_mScarlet biofilms using the same plate. Mab FF_mScarlet biofilms were treated with 1, 10, 50 and 100 $\mu\text{g/mL}$ of linezolid and amikacin, and 0.1% of Triton X-100 (Tt) for 2 days. Non-treated (NT) wells were incubated only with biofilm medium. The bacterial luminescent signal was captured in a ChemiDoc (Bio-Rad) **(A)** and in Newton 7.0 (Vilber) **(C)**. The bacterial fluorescent signal was captured in Newton 7.0 **(B)**. The biofilm biomass was stained using crystal violet assay **(D)**.

Figure 9 shows the dose-response curves of each antibiotic against Mab FF_mScarlet biofilms obtained by quantifying the bacteria's luminescent and fluorescent signal, and by performing CV staining. The susceptibility profiles obtained with the three parameters are similar for most antibiotics, even using different equipment. That is particularly evident with the two luminescence profiles obtained with ChemiDoc – luminescence (C) – and Newton – luminescence (N). The luminescence and fluorescence profiles of moxifloxacin vary the most from each other. With amikacin, imipenem and linezolid, the luminescence profile describes the activity of the intermediate concentrations more adequately than the fluorescence and CV profiles.

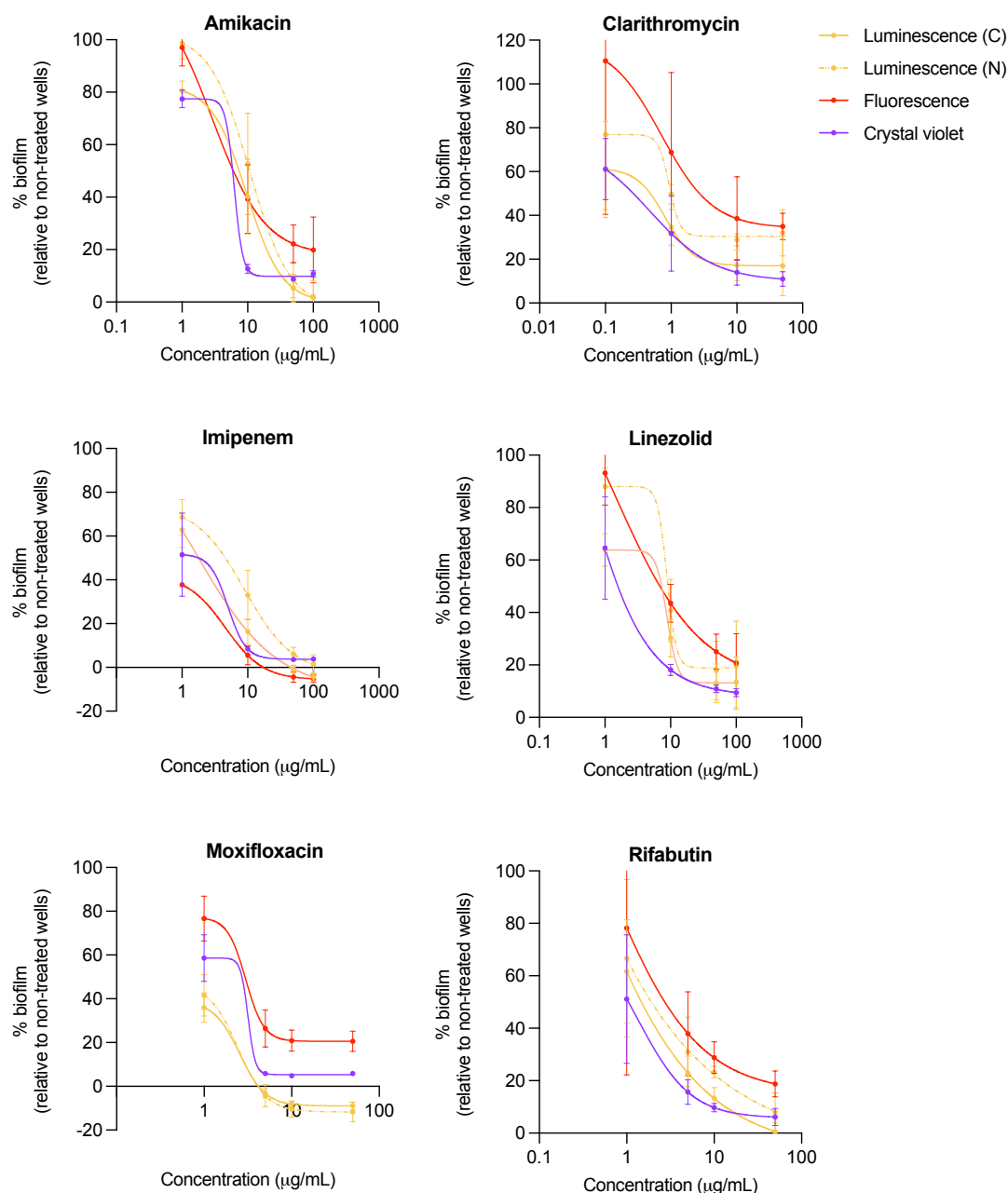


Figure 9. Antibiotic susceptibility profile of *M. abscessus* biofilms. Mab FF_mScarlet biofilms were incubated with increasing concentrations of amikacin, clarithromycin, imipenem, linezolid, moxifloxacin and rifabutin, for 2 days. The anti-biofilm activity was obtained by luminescence using ChemiDoc (Bio-Rad) (full orange line) and Newton 7.0 (Vilber) (dashed orange line), by fluorescence using Newton 7.0 (red) or by CV staining using a Synergy™ Mx (BioTek) (purple). The symbols represent the average $\pm$ standard deviations of two to three independent experiments, presented as the percentage of biofilm in treated wells relative to non-treated biofilms after subtracting the mean value of Triton X-100-treated wells. The lines represent the nonlinear regressions (4PL) obtained in GraphPad Prism 9 for each condition.

3.3.7. Monitoring a systemic *M. abscessus* infection in a mouse model using bioimaging

To understand if the new double-reporter *M. abscessus* strains can be used to monitor infection in vivo, we first measured the luminescent signal emitted by the three *M. abscessus* strains using an in vivo imaging system. Only Mab FF_mScarlet emitted a quantifiable luminescent signal in the IVIS system, and the signal correlated with increased bacterial density (**Figure 10**). In agreement with what was observed in vitro (**Figure 4D**), the luminescence of Mab operon_mScarlet in these settings was very low, and it did not correlate with bacterial density, being similar to Mab WT (**Figure 10B**). Considering this, we only proceeded with Mab FF_mScarlet for in vivo studies.

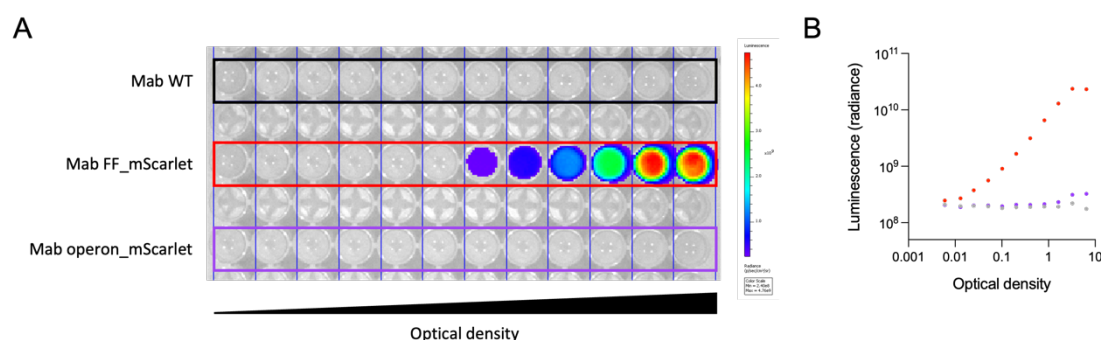


Figure 10. The luminescent signal of Mab FF_mScarlet is detected by bioimaging equipment. **(A)** Bacterial suspensions of the three *M. abscessus* strains were successively diluted 1:2 in a 96-well plate, and the luminescent signal was measured after exposing for 0.5 seconds using an IVIS® Spectrum In Vivo Imaging system (Revvity). The luminescence expressed in radiance by Mab WT (grey), Mab FF_mScarlet (red), and Mab operon_mScarlet (purple) are plotted in **(B)** and were analyzed with the Living Image v4.3.1 software.

To further characterize the Mab FF_mScarlet strain and its ability to infect organisms while maintaining a proper luminescent signal, Mab FF_mScarlet was used to infect eight nude mice intravenously. Nude mice have a genetic mutation that causes a diminished or absent thymus, resulting in an inhibited immune system, which facilitates the establishment of a chronic *M. abscessus* infection. At each time point post-infection, two mice were imaged after being subcutaneously injected with D-luciferin. Subsequently, the same mice were euthanized, and the bacterial load of the whole spleen, liver and lung was assessed by CFU counting. Immediately after the infection (day 0), the luminescent signal indicates that Mab FF_mScarlet is systemically spreading through the entire animal (**Figure 11A**). In the following days, the mycobacteria are localized in organs usually associated with intravenous mycobacterial infection, mainly the liver (days 3 and 7) and, later, with small blisters in the mouse's skin, mainly in the paws, that were visible to the naked eye (days 14 and 17). The CFU counting shows a decline in the bacterial load in the organs usually

associated with systemic mycobacterial infections (**Figure 11B**). The fact that bacteria are quantified in those organs at days 14 and 17 but no luminescent signal shows in the IVIS indicates that this equipment is not sensitive enough to detect bacterial loads below $\sim 10^5$ CFUs/mL. That is also noticeable by the lower bacterial amounts in the lung, which the IVIS does not detect. The formation of lesions in the skin seem to be associated with *M. abscessus* infection and can be a sign of dissemination of the bacilli from the other organs.

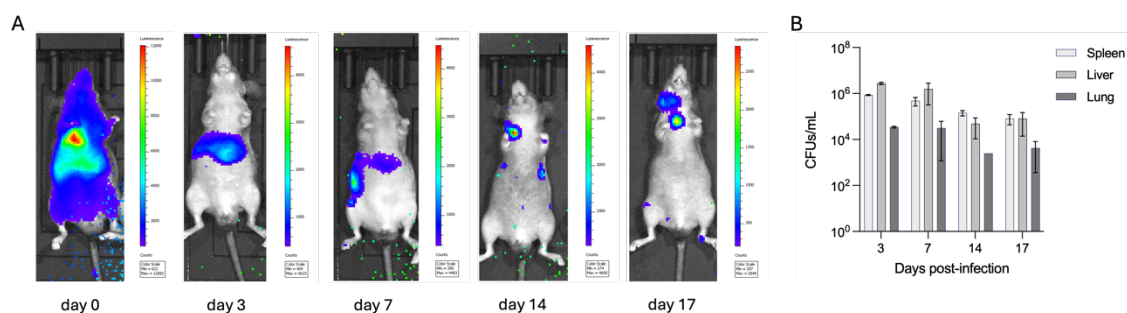


Figure 11. The luminescence emitted by Mab FF_mScarlet allows to follow the infection live by bioimaging. **(A)** Imaging of a representative mouse immediately after (day 0) intravenous infection with 1.4×10^8 CFU of Mab FF_mScarlet and 3-, 7-, 14- and 17-days post-infection. Before imaging, the mice were injected subcutaneously with 30 mg/mL of IVISbrite D-luciferin Ultra RediJect (Perkin Elmer). The images were acquired using an IVIS Lumina series III (Revvity). Exposition time: 300 seconds (days 0 to 14) or 120 seconds (day 17). The scales are presented in counts of luminescence. **(B)** Bacterial load in the whole spleen, liver or lung at days 3, 7, 14 and 17 post-infection. The graph shows the average $\pm$ standard deviations of two animals per time point.

3.4. Discussion

The main objective of this study was to develop a new, efficient, and reliable tool for drug screening against *M. abscessus* that can be used in vitro for high throughput hit identification and later in animal models to select drug candidates for future clinical trials.

In this study, the kanamycin selection gene used in the mClover and mCherry plasmids (**Chapter 2**) was replaced with a more efficient and potent zeocin resistance marker (BleoR) [205]. Zeocin is a formulation of phleomycin D1, a glycopeptide antibiotic that belongs to the bleomycin family, stabilized with a copper ion that, once reduced inside the cell, allows phleomycin D1 to intercalate with DNA, causing damage and consequent cell death [206]. This is a more suitable selection antibiotic as it is not usually used to treat mycobacterial infections, unlike the aminoglycoside kanamycin, reducing the chances of acquired resistance that would impair the selection. Our results show that the transformation was stable in the absence of zeocin as it did not affect fluorescence and luminescence emission (**Figures 2,3**) or change the growth profile (**Figure 4**) or macrophage infection capability

(**Figure 7**) of the non-transformed bacteria. Importantly, we observed that the expression of the selection antibiotic gene did not lead to cross-resistance to conventional antibiotics (**Figure 6**). Nevertheless, when screening new compounds using these strains, we must be aware of molecules with a similar structure to phleomycin that may appear inactive.

First, we confirmed that the fluorescent and luminescent signals were stable. Both strains keep the expression of mScarlet in liquid culture for several passages (**Figure 2**), indicating that the inserted genetic information, which includes the luminescent genes, is well integrated into the bacterial genome and is maintained from generation to generation. The luminescent signal is also stable for a long period, which is particularly important for the Mab FF_mScarlet strain, where D-luciferin needs to be added prior to measurement.

Our results also show that introducing new genetic information in the bacterial genome did not affect the growth profile of the strains (**Figure 4**). Most importantly, we show an excellent correlation between CFU counting, the reference and most reliable conventional method, and fluorescence and luminescence (**Figure 5**). That correlation is less strong for the luminescence of Mab operon_mScarlet, which coincides with a decrease in signal after day 3 of the growth curve. As the luminescence of this strain is independent of the addition of a substrate, we can hypothesize that after three days in batch culture, the bacteria reduce their production of the bacterial luciferase, encoded by the genes *luxAB*, and/or of its long-chain aldehyde substrate, encoded by *luxCDE*. This hypothesis requires future experimental confirmation. That is different for Mab FF_mScarlet, as we add the substrate, D-luciferin, to the bacterial culture right before measuring the luminescent signal, which boosts light emission in that period.

We found that luminescence is an accurate and sensitive readout of bacterial viability and a great alternative to conventional methods to perform DST. As was observed with the fluorescent reporter strains, the luminescent or fluorescent values can be transformed into a drug-response curve (**Figure 6**), allowing the calculation of the IC₉₉ instead of obtaining the MIC by turbidity assessment, which is subjected to person-to-person and assay-to-assay variability. Indeed, that variability resulting from visual observation of the wells is reflected in the MIC interval obtained from the independent experiments (**Table 1**). Furthermore, for Mab WT, it was not possible to calculate the IC₉₉ for linezolid and clarithromycin using the resazurin/resorufin fluorescence. On the other hand, the IC₉₉ values calculated by luminescence (double-reporter strains) correlated better with the respective MIC values and with published values for *M. abscessus* isolates [67,207].

To support the use of the double-reporter strains' luminescence as a tool for drug screening in an HTS context, we evaluated how different the luminescent signals emitted by a high and a low number of bacteria are, which would allow us to distinguish non-active from active molecules with high precision. Thus, we calculated the Z'-factor as a measure

of statistical effect size by comparing the luminescent signal of non-treated *M. abscessus* and *M. abscessus* exposed to a lethal dose of clarithromycin (**Table 2**). The calculated Z'-factor values for the double-reporter strains indicate that the assay is excellent and suitable for a full-scale high-throughput screening [203]. Additionally, these values are higher than the Z'-factor obtained for Mab WT using a resazurin assay, reinforcing luminescence as a drug susceptibility screening readout.

We then wanted to assess the applicability of the double-reporter strains for high-throughput DST inside host cells. We used an automated high-content screening microscope to quantify the fluorescence signal emitted by the bacteria inside murine macrophages. This equipment acquires images with a confocal mode of the whole well of a 96-well plate in minutes. Then, an integrated user-friendly analysis software identifies the macrophages and the intracellular bacteria by their fluorescent signal (**Figure 7B**). We observed no statistically significant differences between the bacterial load quantified by fluorescence and CFU for both double-reporter strains (**Figure 7C,D**). Besides, there were no differences between the CFU quantification for the double-reporter and the WT strains (**Figure 7A**). These results indicate that the transformation did not alter the intracellular antibiotic susceptibility of the bacteria and that the double-reporter's fluorescence can be used to quantify the bacterial load inside host cells. A limitation of using fluorescence to assess antibiotic activity is that some antibiotics, like clarithromycin [208,209], can have bacteriostatic activity, stalling the bacteria's growth without eliminating them. Therefore, a non-viable bacillus can still emit fluorescence through the presence of mScarlet but would not emit luminescence. However, an automated fluorescent microscope cannot detect luminescence, and the number of bacteria in intracellular assays is usually not high enough to be detected in a microplate reader. Therefore, in the experimental settings we have developed, fluorescence microscopy is the better strategy for monitoring intracellular infection in both fixed and live cells. This approach reduces time and variability between assays, which is a significant advantage when considering the purpose of HTS assays.

To replicate the conditions encountered by mycobacteria in aquatic environments, as well as on medical devices, showerheads, or water taps, we aimed to create a mycobacterial biofilm model using our double-reporter strain Mab FF_mScarlet and adapted it for high-throughput DST. By allowing *M. abscessus* to grow steadily on a plate for a week at 37°C in a humid environment and in non-renovating, detergent-free culture medium, we observed that it forms a semi-opaque pellicle at the air-liquid interface that extends to cover the inside wall of each well, just above the liquid medium. The biofilm pellicle exhibits a faint red color due to the expression of the mScarlet fluorescent protein, which makes it easier to observe with the naked eye. Bacterial aggregates are deposited at the bottom of each U-shaped well but are easily washed away when the culture medium is

removed. Therefore, we considered the pellicle ring that remained on the wall of each well even after multiple washes as the *M. abscessus* biofilm.

By utilizing three methods and two pieces of equipment, we could observe, quantify, and extract diverse information regarding the anti-biofilm activity of conventional antibiotics. The luminescent signal captured by ChemiDoc provides a clear image of the biofilm ring (**Figure 8A**). Indeed, the common purpose of a ChemiDoc system is to image nucleic acid and protein gels and blots, but it also works very well with luminescent bacteria. It allows us to perfectly distinguish wells containing a biofilm from those without one or where the biofilm viability has decreased, being sensitive enough to detect slight changes in luminescent intensity. The background signal is very low using this method. However, because a transparent plate was used, some signal transference was observed across wells. That is more visible in the Triton X-100 treated wells, where this detergent destroyed the biofilm, as proved by CV staining (**Figure 8D**), but some pixels are still quantified as positive signals. For that reason, we decided to subtract the mean value obtained for the Triton X-100 wells, which also includes the signal emitted by the plate itself, from each non-treated and antibiotic-treated value. The same was done for the other techniques. Indeed, the signal transference across wells is the most evident in the luminescent signal obtained with Newton (**Figure 8C**), given its high intensity even with a low exposition time (11.8 seconds, comparing to 49.8 seconds with ChemiDoc). We used Newton, an in vivo imaging system, because the ChemiDoc system we have access to does not measure red fluorescence. Newton is an instrument adapted to image animals, so we noticed some limitations regarding camera positioning and software analysis that disfavor its use. Nevertheless, Newton's fluorescent (**Figure 8B**) and luminescent (**Figure 8C**) signals follow the same pattern as the ChemiDoc and CV staining. Of note, we acquired the plate's fluorescence before washing, that is, before adding the luciferin solution for luminescence reading (results not shown). We conclude that although there were some visual and quantitative differences between the antibiotic concentrations, the difference compared to the non-treated wells was low, even at the higher antibiotic concentrations. Furthermore, Triton X-100-treated wells emitted a signal comparable to the non-treated wells. This may indicate that the integrity of the biofilm was compromised, but the bacteria were not eliminated, remaining in the liquid medium or accumulating at the bottom of the well.

Our antibiofilm assays show that the profiles obtained by luminescence, fluorescence and CV staining are similar for the six antibiotics (**Figure 9**). Most importantly, it is possible to distinguish an active compound by luminescence or fluorescence from a non-active one, which is essential for identifying hits by HTS. Additionally, fluorescence measures the bacterial content in the biofilm, while luminescence is intrinsically linked to the biofilm's metabolic state. Both methods are advantageous over conventional CV staining, which only

offers insights into the integrity of the biofilm's ECM. Moreover, measuring fluorescence and luminescence requires less time and manipulation of the biofilm than the CV protocol. It is also a non-terminal procedure, as the biofilm may be used for subsequent assays.

Finding compounds active against bacterial biofilms is urgent but challenging, and in vitro models like this can help reduce the gap between in vitro DST and clinical effectiveness. We can find evidence of that by comparing the activity of the antibiotics tested in this setting with their activity against planktonic Mab FF_mScarlet (**Figure 6**). All of them are less effective against biofilms than against isolated bacteria.

Once the double-reporter strains were validated for in vitro drug screening, it was important to investigate whether we could use their reporter characteristics in vivo. We infected nude mice with Mab FF_mScarlet, as we observed that the luminescent signal emitted by Mab operon_mScarlet was not intense enough to be detected with an IVIS system (**Figure 10**). Indeed, expressing an improved firefly luciferase, Mab FF_mScarlet has the advantage of having an emission spectrum shift towards the red (maximum at 620 nm) compared to reporters expressing other luciferases, which emit at lower wavelengths [101]. Mab operon_mScarlet expresses the whole lux operon that produces light in the blue range (490 nm), which is more susceptible to tissue absorption and scattering [103,210]. We proceeded only with Mab FF_mScarlet, although a D-luciferin injection is required before imaging. Fluorescence could not be used to monitor the infection as the animal's skin presents autofluorescence precisely in the same wavelengths as mScarlet. However, fluorescence can be used ex vivo to visualize the individual bacilli in slices of infected organs or by flow cytometry [202].

Our preliminary experiment results showed that the luminescent signal is detected inside the mice (**Figure 11A**). The luminescent signal indicates an initial colonization of the liver, which is controlled with time. CFU counting shows that at least the spleen and the lungs are also infected (**Figure 11B**), but the mycobacterial load is not high enough to be detected by luminescence. In a later phase of the infection, a luminescent signal is detected in the animal's skin, mainly in their paws, associated with blisters that appeared in those locations at that time. *M. abscessus* infection is associated with skin lesions that can emerge from dissemination from internal organs [40]. More experiments should be performed to confirm that association.

With the appropriate mouse model, we are confident that Mab FF_mScarlet can be a valuable tool for in vivo experiments. In fact, together with our collaborators, we have already observed that this strain can be utilized to monitor the infection in a *Galleria mellonella* model [211]. Bioimaging is the way forward with animal experimentation, as it enables monitoring the infection over time, producing more reliable results, and simultaneously reducing the number of animals used in each experiment.

Overall, these findings indicate that the double-reporter *M. abscessus* strains reliably and accurately reflect the activity of antibiotics, maintaining the same fundamental characteristics as the non-transformed strain while enhancing its potential for high-throughput screening of thousands of compounds swiftly, even in intracellular assays. Additionally, we observed that Mab FF_mScarlet can be employed to assess biofilm viability in a high-throughput manner and to monitor infections in vivo, making it also a valuable asset in a later phase of the drug discovery pipeline.

CHAPTER 4

Optimization of macrophage infection with mycobacteria for drug development

The data presented in **Chapter 4** have not yet been published.

Part of the work was conducted while mentoring Lisa Geerts, a Pharmacy Master's student from the University of Antwerp, Belgium, on an ERASMUS+ mobility internship.

CHAPTER 4. Optimization of macrophage infection with mycobacteria for drug development

4.1. Introduction

Infection models have limitations that arise not only from the host elements but also from the pathogen. The host cell types, their organization, culture media, and incubation conditions all influence the host-pathogen interaction and, consequently, the growth of mycobacteria. However, the state of the bacteria at the time of infection can also impact their growth.

Most studies involving experimental infection with mycobacteria start from a stock frozen inoculum. This approach has significant practical advantages, such as guaranteeing reproducibility in terms of bacterial concentration and being independent of the growth performance of pre-cultures and the time they take to prepare. However, it does not reproduce a physiological situation. Of note, it is well known that mycobacteria can exist in various replication stages within the host. They may be actively replicating inside immune cells, exist as dormant bacteria at the core of the granuloma with limited access to oxygen and nutrients, or transition from one state to another.

In vitro macrophage infection is a valuable model in the antimycobacterial drug development pipeline. It allows the evaluation of potential drugs' capacity to reach the bacteria inside the host cell and simultaneously allows the assessment of their toxicity.

In this study, we investigated whether the growth phase of the mycobacteria at the time of macrophage infection influenced their intracellular growth and susceptibility to antibiotics. This way, we intend to optimize in vitro susceptibility testing methods to make them more informative relative to in vivo efficacy.

4.2. Materials and methods

4.2.1. Bacteria

The *M. abscessus* strain Mab FF_mScarlet (**Chapter 3**) and the *M. avium* FF_mScarlet strain, kindly provided by Paul Cos, University of Antwerp, Belgium, were grown in Middlebrook 7H9 broth (BD Bioscience) containing 0.2% glycerol (VWR International) and 10% Albumin-Dextrose-Catalase (ADC) supplement at 37°C, with agitation. For Mab FF_mScarlet, the media also contained 0.05% Tween-80 (Merck Life Science).

4.2.2. *M. abscessus* and *M. avium* growth curves

The Mab FF_mScarlet and *M. avium* FF_mScarlet cultures were diluted to $OD_{600} = 0.05$ and incubated at 37°C, 90 rpm for seven days. At each time point, OD_{600} of a direct sample

(*M. avium*) or a sample diluted 1:10 in culture media (*M. abscessus*) was measured, and CFUs were determined by plating the mycobacteria in 7H10 agar + 10% OADC.

4.2.3. Macrophage infection, intracellular bacterial load quantification and antibiotic activity

Macrophages were derived from the bone marrow of male BALB/c mice bred at the i3S animal facility, as described in **Chapter 1 (Section 1.2.3)**. Macrophages in 96-well microplates (4×10^4 cells per well) were infected with Mab FF_mScarlet or *M. avium* FF_mScarlet. The infections were performed using frozen inoculum or bacterial cultures at different growth phases (i.e., lag, exponential, and stationary). Moreover, macrophages were infected with various multiplicities of infection (MOI), 0.2:1, 1:1, 5:1 or 10:1 (bacteria:cells). Prior to infection, each bacterial growth phase culture was collected and centrifuged for 5 minutes at 20000 g, washed with PBS, centrifuged again and resuspended in complete DMEM. After four hours, the cells were washed three times with HBSS using a microplate washer (BioTek) and incubated with complete DMEM + 10% LCCM, with or without antibiotic, for two (*M. abscessus*) or five (*M. avium*) days. The antibiotics (all obtained from Merck Life Science) and concentrations used were the following: clarithromycin (0.03 – 1 $\mu\text{g/mL}$), rifabutin (0.12 – 4 $\mu\text{g/mL}$), linezolid (1 – 32 $\mu\text{g/mL}$), imipenem (0.5 – 16 $\mu\text{g/mL}$) and amikacin (1 – 16 $\mu\text{g/mL}$).

For CFU counting, the macrophages were washed three times with PBS, lysed with 0.1% saponin, and the bacterial suspension was serially diluted in water containing 0.05% Tween-80 and plated in 7H10 agar + 10% OADC. Statistical analysis was performed using the software Prism9® (GraphPad Software Inc.) using a two-way ANOVA with Tukey's multiple comparisons test.

For fluorescence quantification, the macrophages were fixed with 4% PFA for 10 minutes at room temperature and washed three times with PBS. The cells were permeabilized with PBS + 0.1% Triton, washed three times and stained with DAPI and HCS CellMask Deep Red (H32721, Invitrogen) for 30 minutes. The plates were screened in the automated high-content confocal microscope Opera Phenix™ Plus (Revvity), and the data was analyzed using Harmony High-Content Imaging and Analysis Software (Revvity) with mScarlet fluorescent signal being used as a measure of bacterial load.

4.2.4. Cytokine quantification in extracellular media

Cytokine production by *M. avium* FF_mScarlet-infected BMM was quantified in the extracellular media on days 1 and 5 after infection.

TNF- α was quantified by ELISA, using the LEGEND MAX™ Mouse TNF- α ELISA Kit (430907, BioLegend), following the manufacturer's instructions. Briefly, the samples and TNF- α standards were added to a 96-well plate pre-coated with hamster monoclonal anti-

mouse TNF- α antibodies for 2 hours. The plate was washed and incubated with biotinylated rabbit polyclonal anti-mouse TNF- α antibodies for 1 hour. The plate was washed and incubated with Avidin-Horse Radish Peroxidase (HRP) B solution for 30 minutes. The plate was washed, Substrate Solution D was added to each well for 15 minutes, and the reaction was stopped. The absorbance was read at 450 nm and 570 nm in a μ Quant spectrophotometer (BioTek) using the Gen 5 software. The TNF- α concentration was determined by interpolating the standard curve using Microsoft Excel.

IL-6 and IL-1 β were quantified using the Bio-Plex Pro™ Mouse Cytokine Th17 Panel A 6-Plex (M6000007NY, Bio-Rad), following the manufacturer's instructions. This 96-well plate immunoassay kit includes standards, detection antibodies, streptavidin-PE and all the necessary buffers. It uses 6.5 μ m color-coded magnetic beads (xMAP technology) for magnetic washing and detection in the same well of IFN- γ , IL-1 β , IL-6, IL-10, IL-17A and TNF- α using a Bio-Plex 200 system. The cytokine concentration was obtained with the Bio-Plex Manager software (Bio-Rad).

4.2.5. Detection of macrophage autophagy and acidic organelles

Two or five days after macrophage infection with Mab FF_mScarlet or *M. avium* FF_mScarlet, respectively, the cells were stained for autophagy detection with the CYTO-ID® Autophagy Detection Kit 2.0 (ENZ-KIT175, Enzo Life Sciences) or for acidic organelles detection with the LYSO-ID® Green Detection Kit (ENZ-51034, Enzo Life Sciences), following the manufacturer's instructions. Briefly, the cells were washed and incubated with a green fluorescent probe and Hoechst stain for 30 minutes at 37°C. After washing, the stained infected macrophages in complete DMEM were imaged live at 37°C, 5% CO₂, in an automated high-content confocal microscope Opera Phenix™ Plus (Revvity). The fluorescent probes' intensity divided by the number of cells in each well was calculated with the Harmony High-Content Imaging and Analysis Software (Revvity).

4.3. Results

4.3.1. Mycobacteria's growth phase affects the intracellular growth

To understand how the mycobacteria growth phase implicates macrophage infection, bone-marrow-derived macrophages were infected with *M. abscessus* or *M. avium* that are either in the lag, exponential or stationary phase of its growth curve (**Figure 1**) and compared with infection with frozen mycobacterial inoculum. The frozen inoculum is prepared by growing mycobacteria to exponential phase, desegregating them into single cells and freezing them at -80°C until the time of infection. The mycobacteria growth phases were divided and

classified into lag, exponential and stationary by using the OD values and CFU counts of various independent liquid cultures (**Figure 1**).

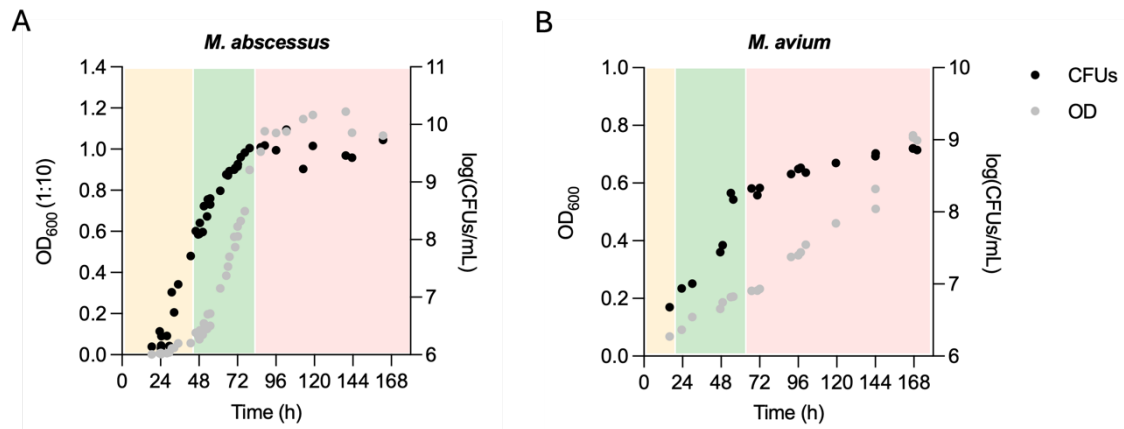


Figure 1. *M. abscessus* and *M. avium* growth curves. Mab FF_mScarlet (**A**) or *M. avium* FF_mScarlet (**B**) was grown in liquid culture for seven days at 37°C, with agitation. At each time point, samples were taken for OD₆₀₀ (grey) and CFU counting (black). The graphs represent data from eight to nine independent cultures. The colors limit the regions attributed to each mycobacterial growth phase: lag (yellow), exponential (green) and stationary (red).

The results show that *M. abscessus* in exponential phase at the time of infection grows more inside the macrophages than when in the other phases (**Figure 2C**). The same effect is also observed for *M. avium* infection (**Figure 2D**). Moreover, the infection with *M. avium* in stationary phase results in significantly lower bacterial loads than the other phases, a tendency that is not so evident for *M. abscessus* infection. Notably, the infection with frozen *M. abscessus* results in more dispersed bacterial loads among replicates than with the three growth phases. That does not apply to *M. avium* infection, where the results are more precise. By infecting the macrophages with *M. abscessus* (**Figure 2E**) or *M. avium* (**Figure 2F**) at different MOI, it is possible to conclude that independently of the number of mycobacteria given to the macrophages, *M. abscessus* and *M. avium* in exponential phase at the time of infection always grow better intracellularly than the other phases. *M. abscessus* in stationary phase grows better independently of the MOI than the frozen *M. abscessus*, contrary to what happens with *M. avium* infection. For MOIs higher than 1, *M. abscessus* in lag phase at the time of infection grows to the same extent, independently of the number of bacteria given to the macrophages (**Figure 2E**).

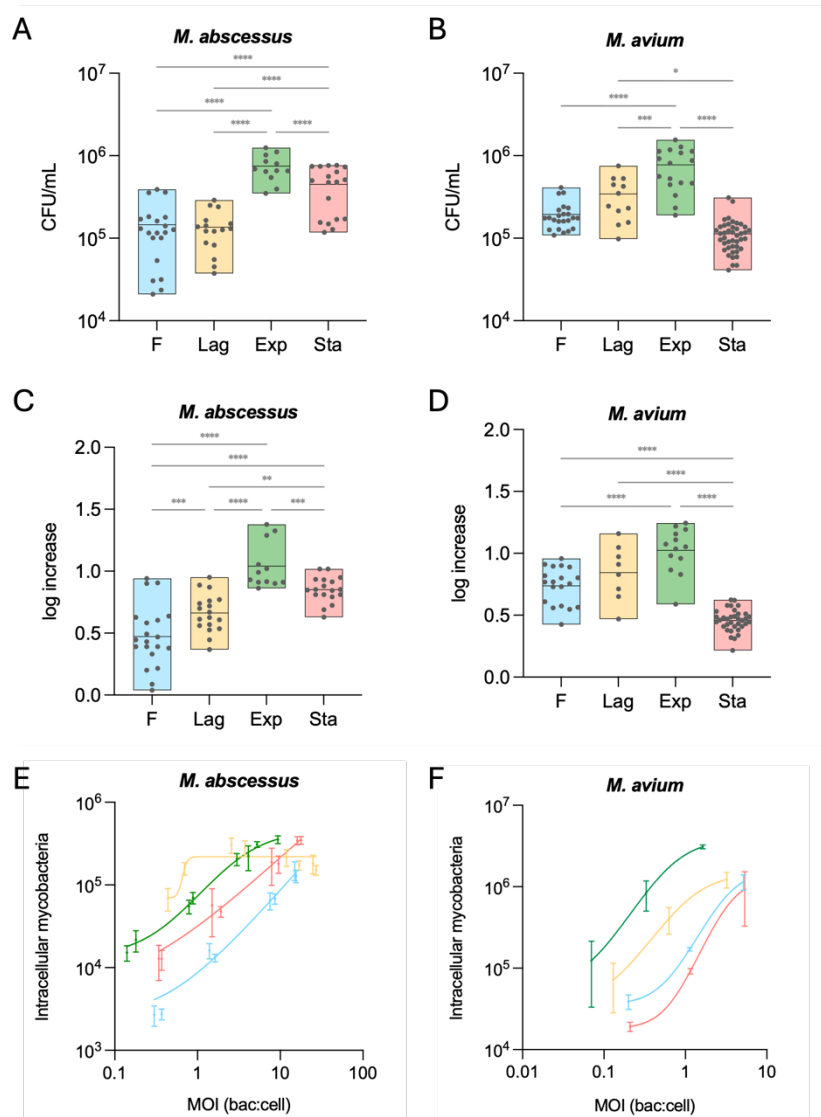


Figure 2. The intracellular growth depends on the mycobacteria's growth phase. BMM were infected with frozen inoculum (blue), lag (yellow), exponential (green) or stationary (red) growth phases of Mab FF_mScarlet (**A, C, E**) or *M. avium* FF_mScarlet (**B, D, F**), for 2 or 5 days, respectively. (**A-D**) The infection was done at MOI=1. (**A, B**) The intracellular bacterial load was assessed by CFU counting. (**C, D**) Log increase is the difference in terms of log₁₀ CFU/mL, between the last and the first day of infection. The graphs show the dispersion (floating bars) and the mean of all replicates of four to five independent experiments. Statistical analysis was performed with GraphPad Prism using a two-way ANOVA with Tukey's multiple comparisons test. * $p = 0.0421$, ** $p = 0.0027$, *** $p = 0.0006$ (**C**) or $p = 0.0002$ (**D**), **** $p < 0.0001$. (**E-F**) BMM were infected with increasing MOIs. (**E**) Mab FF_mScarlet intracellular load was assessed by fluorescent microscopy, using the mScarlet signal. (**F**) *M. avium* FF_mScarlet intracellular load was assessed by CFU counting. The graphs show the average + standard deviation of twelve replicates of two independent experiments (**E**) or of three replicates of one experiment (**F**). The lines represent the nonlinear regression (4PL) obtained in the GraphPad Prism.

4.3.2. *Mycobacteria's growth phase alters the macrophage cellular response*

The interaction between the macrophage and the mycobacteria in different growth phases may affect how the macrophages respond to infection. Thus, we set to evaluate if the mycobacterial growth phase influences the production of pro-inflammatory cytokines normally secreted by infected macrophages (**Figure 3**). The heterogeneity of the results, especially the ones obtained using a multiplex system (**Figure 3B,C**), makes it hard to draw definitive conclusions. However, it is possible to observe an accumulation of cytokines throughout the infection for all phases except the stationary phase. Indeed, it is notable that the production of TNF- α , IL-6 and IL-1 β by macrophages infected with *M. avium* in stationary phase is almost residual compared to the other phases.

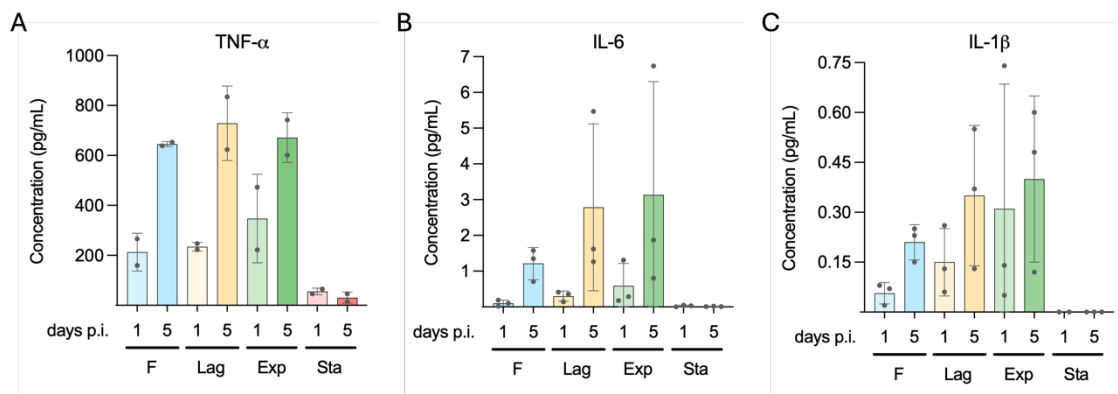


Figure 3. Pro-inflammatory cytokine production depends on the *M. avium* growth phase at the time of infection. The production of TNF- α (**A**), IL-6 (**B**) and IL-1 β (**C**) by macrophages infected with *M. avium* FF_mScarlet in different growth phases after 1 or 5 days was quantified using the LEGEND MAX™ Mouse TNF- α ELISA Kit (BioLegend) (**A**) or Bio-Plex Pro™ Mouse Cytokine Th17 Panel A 6-Plex (Bio-Rad) (**B,C**). The bars represent the average $\pm$ standard deviation of three independent experiments.

Mycobacterial infection can also have an impact on the macrophage's vesicular trafficking. Thus, cellular response kits (CYTO-ID® and LYSO-ID®, Enzo Life Sciences) containing fluorescent probes design for specific organelles or cellular compartments were used (**Figure 4A,B**). The probes signal was quantified by live high-content screening fluorescence microscopy after two (*M. abscessus*) or five (*M. avium*) days of infection. The results show that the autophagic flux is decreased when macrophages are infected with *M. abscessus* in stationary phase (**Figure 4C**). On the other hand, the amount of acidic compartments, such as lysosomes, is not affected by the *M. abscessus* growth phase, with the values being in line with the ones obtained for non-infected (NI) macrophages (**Figure 5D**). The influence of *M. avium* infection on these two processes (**Figure 4E,F**) was only

evaluated in one experiment, so no definite conclusions should be drawn. However, there seems to also be a decrease in the autophagic flux due to infection with *M. avium* in stationary phase. The increase in autophagy vesicles and acidic compartments after infection with *M. avium* in lag phase should be further explored.

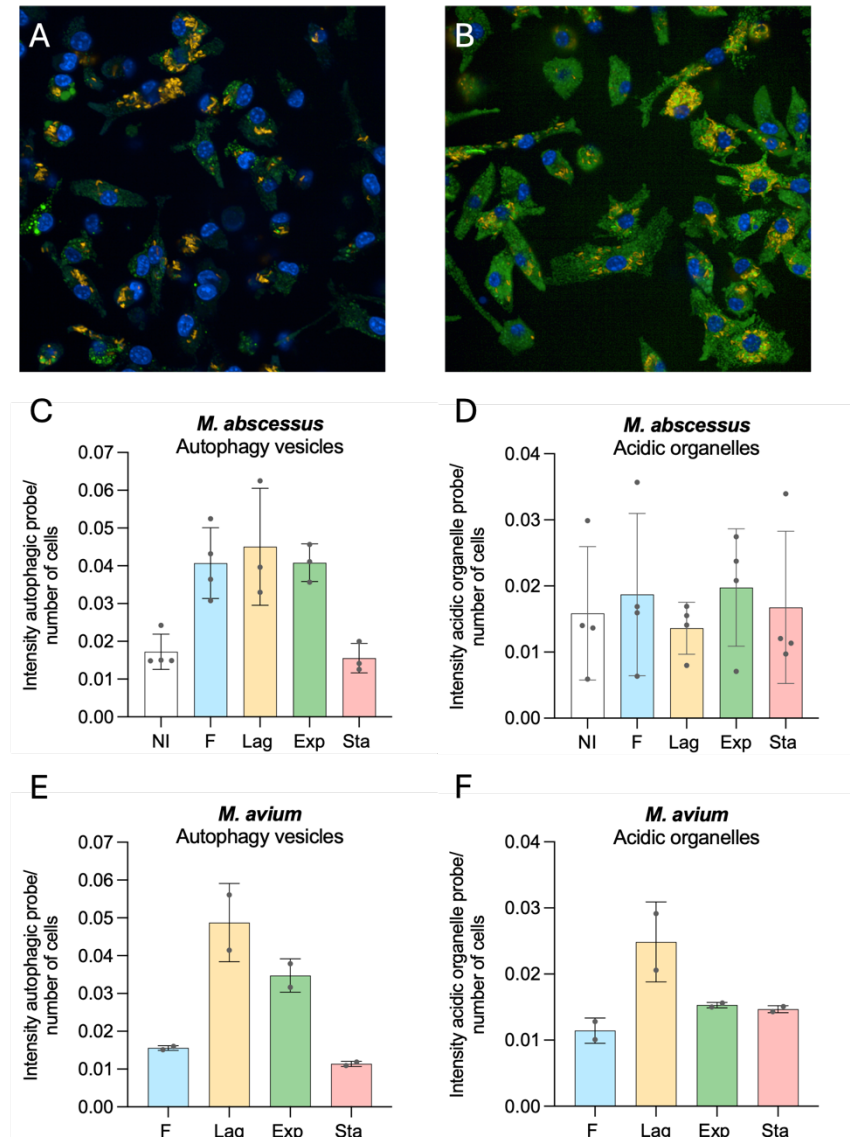


Figure 4. Vesicular trafficking processes are altered upon infection with mycobacteria in different growth phases. Macrophages were stained with the CYTO-ID® Autophagy Detection Kit 2.0 (**A,C,E**) or the LYSO-ID® Green Detection Kit (**B,D,F**) (Enzo Life Sciences) two (Mab FF_mScarlet) or five (*M. avium* FF_mScarlet) days after infection at a 1:1 (bacteria:cells) proportion. (**A,B**) Representative fluorescence microscopy images of *M. avium* FF_mScarlet (orange) -infected macrophages (nuclei in blue) with autophagic vesicles (**A**) or acidic compartments (**B**) in green. The cells were imaged live at 37°C, 5% CO₂ using Opera Phenix™ Plus. (**C-F**) The fluorescent probes' intensity divided by the number of cells in each well was calculated with the Harmony High-Content Imaging and Analysis

Software (Revvity). The bars represent the average $\pm$ standard deviation of three to four independent experiments (**C,D**) or of two replicates of one experiment (**E,F**).

4.3.3. Antibiotic susceptibility differs depending on the mycobacteria's growth phase

Intracellular antibiotic susceptibility testing is typically conducted after infecting host cells with mycobacteria that come from a frozen inoculum. Considering the differences in intracellular growth and macrophage response between frozen mycobacteria and those in different growth phases, it is important to determine whether antibiotic susceptibility is also affected, as this is crucial for performing in vitro DST reliably. Increasing concentrations of four conventional antimycobacterial agents with different mechanisms of action were added to *M. abscessus*-infected macrophages right after infection, and their activity was assessed by fluorescence measurement after two days. For rifabutin (**Figure 5B**), the profile is similar for all the growth phases and the frozen mycobacteria. In most cases, and more evidently for clarithromycin (**Figure 5A**), when the macrophages are infected with frozen mycobacteria, there seems to be no dose-dependent response to the antibiotic. Also, at higher concentrations, the antibiotics only inhibit up to 70 to 80% of the intracellular *M. abscessus* that came from a frozen inoculum, which does not happen when the mycobacteria are in the different growth phases. That can lead to misinterpretations of the compounds' activity when testing large screening setups.

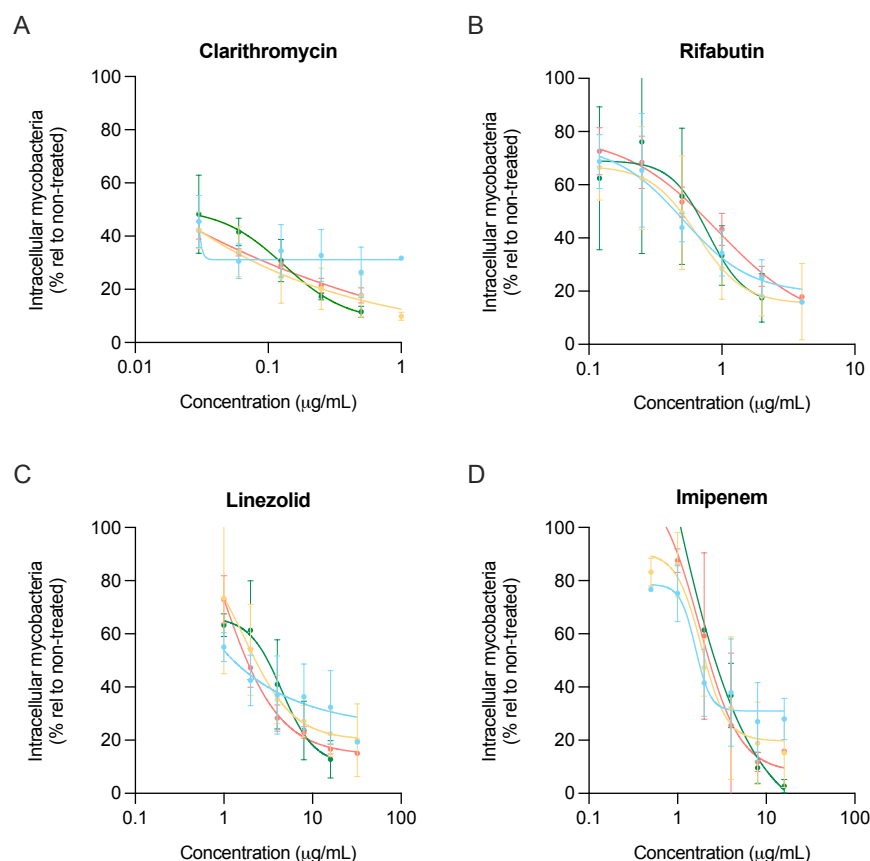


Figure 5. The intracellular antibiotic susceptibility is dependent on the *M. abscessus* growth state upon infection. BMM were infected with Mab FF_mScarlet frozen (blue), or in lag (yellow), exponential (green) or stationary (red) phase (MOI=1), and then treated with increasing concentrations of clarithromycin (**A**), rifabutin (**B**), linezolid (**C**) or imipenem (**D**) for two days. The intracellular mycobacterial load was assessed by the mScarlet fluorescent signal. The graphs show the average $\pm$ standard deviation of three to four independent experiments. The lines represent the nonlinear regression (4PL) obtained in the GraphPad Prism 9.

The same conditions were tested against *M. avium*-infected macrophages. A lot of variability is observed across experiments, leading to somewhat erratic susceptibility profiles, especially with linezolid and imipenem (**Figure 6C,D**). The profiles of clarithromycin and rifabutin (**Figure 6A,B**) are more explicit and indicate a lack of dose-dependent response when macrophages are infected with frozen *M. avium*. This lack is not entirely overcome by testing against the growth phases, though the range of inhibition is at least broader.

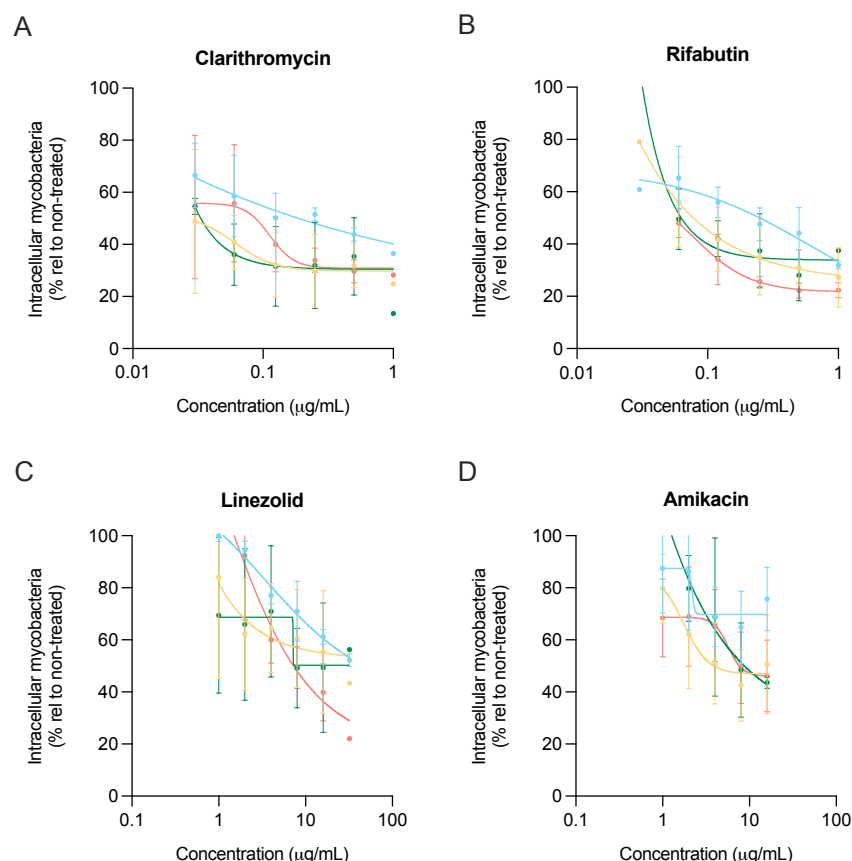


Figure 6. The antibiotic susceptibility against intracellular *M. avium* varies between growth phases. Macrophages were infected with *M. avium* FF_mScarlet frozen (blue), or in lag (yellow), exponential (green) or stationary (red) phase (MOI=1), and then treated with increasing concentrations of clarithromycin (A), rifabutin (B), linezolid (C) or imipenem (D) for two days. The intracellular mycobacterial load was assessed by the mScarlet fluorescent signal. The graphs show the average $\pm$ standard deviation of three to four independent experiments. The lines represent the nonlinear regression (4PL) obtained in the GraphPad Prism 9.

4.4. Discussion

We aimed to determine whether the growth phase of the mycobacteria at the time of infection influenced intracellular growth, cellular response, and bacterial susceptibility to antibiotics.

The most relevant information from this study is that mycobacteria in exponential phase at the time of infection can replicate more than the other phases inside the macrophage, independently of the bacteria-to-cell ratio (the multiplicity of infection – MOI) (Figure 2). This is likely due to the high metabolic activity of mycobacteria in exponential phase, which is sustained within the host cell. That high replicative state is also maintained by some bacilli that were neither internalized nor washed out four hours after being incubated with the macrophages. In fact, a visual inspection of the wells revealed that *M. abscessus* in

exponential phase showed a greater tendency to accumulate extracellularly, particularly at the periphery of the wells.

Our results also allow to conclude that mycobacteria in stationary phase, especially *M. avium*, show a significantly different behavior from the other phases. They grow less inside the macrophages (**Figure 2**), induce almost no immune response (**Figure 3**), and stimulate less accumulation of autophagic vesicles in the macrophage (**Figure 4**). This lack of response by the macrophage may indicate that the mycobacteria are “hidden” from the host cell, maybe in a latent form with less expression of antigen and recognition factors, leading to less pro-inflammatory cytokine production and autophagy induction by the macrophage [212]. These events would eventually lead to hampered activation of the adaptive immune response, allowing latent mycobacteria to persist inside the macrophage. A whole vision of the bacteria's transcriptome would help identify differences in gene expression between the mycobacterial growth phases and explain the distinct responses of the macrophage.

The main conclusion regarding *M. abscessus* antibiotic susceptibility is that relying solely on frozen mycobacteria can lead to misinterpretations of compound activity, especially when screening large compound libraries in which only one concentration is tested. The difference between an active and a non-active concentration, visible in the growth phase profiles (**Figure 5**), is not so evident in the frozen dose-response curves. Therefore, when testing new compounds against mycobacteria, it is essential to consider the bacteria's replicative stage to draw more confident conclusions. Depending on the mycobacterial phase, the antibiotic target's expression may be up or downregulated, and bacteria's defense mechanisms may also vary. Again, analysis of the bacteria's transcriptome can answer some of those questions.

The *M. avium* antibiotic susceptibility results are highly erratic (**Figure 6**), making it impossible to draw definitive conclusions. The instability of the *M. avium* FF_mScarlet transformation may explain the observed variability. This strain was designed and transformed by our collaborators at the University of Antwerp, but its fluorescence did not demonstrate the same stability as the *M. abscessus* double-reporter strains. Flow cytometry, fluorescence microscopy, and CFU counting revealed that approximately 15% of the bacilli/CFU in a liquid culture did not express mScarlet (results not shown). Since the results we obtained for antibiotic susceptibility (**Figure 6**) were derived from fluorescence imaging, we hypothesize that the pressure placed on the mycobacteria during macrophage infection could result in even greater instability and a loss of fluorescence compared to liquid culture.

The advantage of infecting the macrophages with quantified frozen inoculum is that the amount of inoculum needed to achieve a certain MOI is known. That was one of the most significant difficulties when infecting with the different mycobacterial growth phases, leading

to differences between the expected and actual MOI. Curiously, those differences were more accentuated with lag and exponential mycobacteria than with stationary mycobacteria, for which the calculated and actual MOI mostly aligned, even between independent experiments. However, that variability was not reflected in the results. For *M. abscessus* intracellular growth, the replicates were, in fact, more dispersed with frozen inoculum (**Figure 2**). In the macrophage response (**Figure 3,4**) and antibiotic susceptibility assays (**Figure 5,6**), the growth phase results are not, in general, more heterogeneous than the frozen ones. Therefore, although the experimental setup becomes more nuanced, infecting macrophages with mycobacteria at different stages of the growth curve provides valuable information that should be considered when evaluating the activity of new compounds against mycobacteria.

CHAPTER 5

Alveolar organoids as a model of mycobacterial infection

The data presented in **Chapter 5** have not yet been published.

This work was conducted within the framework of the “REMODEL – Research models in infection, cancer and regeneration: replacement and translation” project, funded by the European Union’s Horizon 2020 Research and Innovation Program.

Part of the work was conducted in collaboration with Rute Gonçalves, Joana Couto and Margarida Saraiva from the Immune Regulation group at i3S, Porto, Portugal.

CHAPTER 5. Alveolar organoids as a model of mycobacterial infection

5.1. Introduction

Organoids are among the most complex in vitro models. They aim to closely mimic the environment of specific organs by being made of cells from a donor, whether animal or human. The appropriate differentiation cocktail activates and inhibits specific signaling pathways, ensuring that the isolated stem cells differentiate into cells of the intended adult organ, replicating the tissue organization and physiological characteristics observed in vivo.

Mycobacterial infections most commonly affect the lungs. Therefore, lung organoids are of great interest for studying mycobacterial infections and testing potential new drugs. Various types of lung organoids have been developed. Alveolar organoids (AOs) mainly consist of alveolar epithelial type I cells (AEC1), which facilitate gas exchange between outside air and the underlying vasculature, and alveolar epithelial type II cells (AEC2), which are responsible for secreting surfactant that reduces surface tension, allowing for alveolar expansion without collapse [140]. AOs can be derived from mouse or human lung cells. Studies indicate that alveolar epithelial cells can be infected by mycobacteria and play a role in the bacilli's passage across the epithelium [122]. Thus, AOs appear particularly relevant for mycobacterial infections.

The objective of this work was to adapt AO models for mycobacterial infection and add a complex, highly predictive step to the evaluation pipeline of potential new drugs before they reach animal testing.

5.2. Materials and methods

5.2.1. Generation of mouse and human alveolar organoids

Alveolar organoids (AOs) were obtained by adapting the protocol from Sachs et al. [183]. Briefly, to obtain mouse alveolar organoids (mAOs), a C57BL/6 mouse was euthanized, and its lungs were exposed by cutting the rib cage. Blood was flushed out of the lungs by perfusing with cold PBS through the right ventricle. The lungs were transferred to a tube containing cold Adv⁺⁺⁺ DMEM/F-12 (advanced DMEM/F-12 media supplemented with 1x GlutaMax, 10 mM HEPES and 100 mg/mL of penicillin/streptomycin).

For human alveolar organoids (hAOs), a portion of the healthy parenchyma of a lobectomy from lung cancer patients at Centro Hospitalar Universitário São João (CHUSJ) was collected in a tube with Adv⁺⁺⁺ DMEM/F-12. The lung portion was then perfused with cold PBS to remove excess blood.

In a Petri dish, the whole lung (mouse) or lung portion (human) was minced into small pieces using a scalpel. The tissue was added to 10 mL of Adv⁺⁺⁺ DMEM/F-12 with 1 mg/mL of collagenase type II (17101015, Gibco) and incubated at 37°C with shaking for 1 hour.

The digested tissue suspension was sheared using 5 mL pipettes, P1000 and P200 tips, and passed through a 100 μm and a 70 μm cell strainer. 2% FBS was added, and the suspension was centrifuged for 5 minutes at 400 g, 4°C. To remove erythrocytes, the pellet was resuspended with red blood cells lysis buffer (00-4333-57, Invitrogen) for 5 minutes at room temperature. 10 mL of Adv⁺⁺⁺ DMEM/F-12 was added, and the cell suspension was centrifuged for 5 minutes at 400 g, 4°C.

For the mAOs, the cell pellet was resuspended in cold Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free (356231, Corning). 50 μL cell-containing Matrigel domes were plated in the center of a pre-warmed 24-well plate. After 30 minutes at 37°C, 0.5 mL of warm mouse alveolar organoid (mAO) media (composition in **Table 1**) media was added above each dome.

For the hAOs, the number of cells was assessed with a Neubauer chamber, and each Matrigel dome plated contained 2×10^5 cells. The domes were covered with 0.5 mL of warm PneumaCult™ Alveolar Organoid Expansion (AvOE) Medium (composition in **Table 2**). When intended, after 10 days, the media was exchanged for PneumaCult™ Alveolar Organoid Differentiation (AvOD) Medium (composition in **Table 2**).

The plates were maintained in a humidified incubator at 37°C, 5% CO₂ and the AO's development was monitored with a ZOE Fluorescent Cell Imager (Bio-Rad). The culture media above the Matrigel domes was renewed every 2-3 days.

Table 1. Composition of the mAO media and final concentrations of the components. The signaling pathways activated or blocked by each component, as well as the respective supplier and catalog number, are also presented. FGF – Fibroblast growth factor.

Media component	Signaling pathway		Supplier	Catalog number	Final concentration
	Activation	Block			
R-Spondin 1	Wnt/β-catenin		Peprotech	120-38	500 ng/mL
FGF-7	FGFR2b		Peprotech	100-19	25 ng/mL
FGF-10	FGFR2b		Peprotech	100-26	100 ng/mL
Noggin	TGF-β		Peprotech	120-10C	100 ng/mL
A83-01	TGF-β		Tocris	2939	500 nM
Y-27632	ROCK		Abmole	Y-27632	5 μM
SB202190	p38 MAPK		Merck Life Science	S7067	500 nM
B27 supplement	Insulin (among others)		Gibco	17504-44	1x
CHIR99021	Canonical Wnt		Merck Life Science	SML1046	3 μM
	GSK3-β				
N-Acetylcysteine	Antioxidant		Merck Life Science	A9165-5g	1.25 mM

Nicotinamide	Co-enzyme precursor	Merck Life Science	N0636	5 mM
Primocin	Antibiotic/antimycotic	Invitrogen	12634-034	50 mg/mL
HEPES	Buffer	Invitrogen	15630-056	10 mM
Penicilin/Streptomycin	Antibiotics	Invitrogen	15140-122	100 U/mL / 100 mg/mL
GlutaMax 100x	Nutrient	Invitrogen	Ant-pm-1	1x
Advanced DMEM/F12	Base medium	Invitrogen	12634-034	1x

Table 2. Composition of the hAO media. All components were acquired from Stemcell Technologies.

Culture media	Media component	Catalog number	% in final media
PneumaCult™ Alveolar Organoid Expansion (AvOE) Medium	PneumaCult™ AvOE Basal Medium	100-0848	88.75 – 89.75 *
	PneumaCult™ AvOE Supplement	100-0849	10
	Heparin solution	07980	0.25
	PneumaCult™ AvOE Passage Supplement *	100-0860	1
PneumaCult™ Alveolar Organoid Differentiation (AvOD) Medium	PneumaCult™ AvOD Basal Medium	100-0862	90
	PneumaCult™ AvOD Supplement	100-0863	10

* PneumaCult™ AvOE Passage Supplement is included only during the first 2-3 days after plating the hAOs and consistently used with hAO-derived monolayers.

5.2.2. AO passage

The culture media was removed, and the AO-containing Matrigel domes were sheared by adding ice-cold PBS and thoroughly pipetting the well's content. The AO fragments were centrifuged for 5 minutes at 400 g, 4°C, resuspended in fresh Matrigel and plated as described before.

5.2.3. Infection of mAOs by shredding

The mAO media was removed, and the AO-containing Matrigel domes were sheared with ice-cold PBS. The AO fragments were centrifuged for 5 minutes at 400 g, 4°C and resuspended in mAO media without antibiotics. The number of cells was assessed by enzymatically digesting a portion of the cell suspension with TrypLE™ Express (12604013, Gibco) for 10 minutes at 37°C, centrifuging for 5 minutes at 400 g, 4°C, resuspending in mAO media and counting with a Neubauer chamber. *M. avium* FF_mScarlet was added to the AO fragments at a 1:10 (bacteria:cells) proportion and incubated for 4 hours at 37°C.

The suspension was centrifuged and washed three times with DMEM/F-12 to remove extracellular mycobacteria. The pellet was resuspended in fresh Matrigel and plated as described before. The AO media above the domes was antibiotic-free.

5.2.4. Flow cytometry of mAOs

The mAOs were disrupted into single cells by enzymatic digestion with TrypLE Express for 20 minutes at 37°C with agitation. The cells were centrifuged for 5 minutes at 400 g, 4°C and resuspended in PFA 4% for 10 minutes at room temperature. The fixed cells were centrifuged and washed twice with PBS. The cells were permeabilized with PBS + 2% FBS + Triton 0,1% for 5 minutes, washed twice with PBS and resuspended in the primary antibody (**Table 2**) in FACS buffer (PBS + 2% FBS + 1 mM EDTA + 0,1% sodium azide) for 30 minutes, 4°C, in the dark. The cells were centrifuged and resuspended in the secondary antibody (**Table 2**) in FACS buffer for 30 minutes, 4°C, in the dark. Each sample was filtered through a 35-µm nylon mesh (Corning) before acquisition in a BD FACS ARIA II (BD Biosciences) using the 530/30 (FITC) filter. The data analysis was performed using the FlowJo software (BD Biosciences).

5.2.5. Confocal microscopy of mAOs

M. avium FF_mScarlet infected mAOs were recovered from the Matrigel domes by adding Cell Recovery Solution (354253, Corning) and transferring the mAO suspension to a tube using an opened tip to avoid damaging the 3D structure. After incubation on ice for 45 minutes, the tube was centrifuged for 3 minutes at 100 g, 4°C. The mAOs were fixed by carefully resuspending in PFA 4% for 20 minutes at room temperature. After centrifuging, the mAOs were washed with PBS + 1% BSA and permeabilized with PBS + 1% BSA + 1% Triton X-100 for 1 hour at room temperature. The mAO suspension was washed three times and incubated with DAPI (nuclei) in PBS + 1% BSA + 1% Triton X-100 + 5% FBS for 15 minutes at room temperature in the dark. After three washes, the mAOs were transferred to an 8-well IBIDI® plate and imaged with an HC PLAPO Lbl. Blue 63x/1.40 Oil objective using a Laser Scanning Confocal Microscope Leica TCS SP5 II. The samples were excited using the 405 nm (for nuclei) and 561 nm (for bacteria) lasers. The images were analyzed using ImageJ/Fiji software.

5.2.6. Histology of mAOs

The *M. avium* FF_mScarlet infected mAO samples were prepared for histology by recovering the mAOs from the Matrigel domes with Cell Recovery Solution and resuspending in formaldehyde. Paraffin inclusion, hematoxylin and eosin (H&E) and Ziehl-

Neelsen staining were performed by the Histology and Electron Microscopy platform at i3S. The images were acquired with a Leica DM2000 LED brightfield microscope.

5.2.7. *mAOs monolayers and M. avium infection*

The mAO media was removed, and the AO-containing Matrigel domes were sheared with ice-cold PBS. The AO fragments were centrifuged for 5 minutes at 400 g, 4°C and resuspended in TrypLE Express for 10 minutes at 37°C. The suspension was diluted in Adv⁺⁺⁺ DMEM/F-12 + 10% FBS to stop the trypsinization, centrifuged for 5 minutes at 400 g, 4°C and resuspended in Adv⁺⁺⁺ DMEM/F-12 + 10% FBS. The number of cells was assessed with a Neubauer chamber, and 5-10 x 10⁴ cells/mL in mAO media without antibiotics and CHIR99021 (mAO monolayer media) were seeded in each well of a 24-well plates (0.4 mL per well). The cells were incubated at 37°C, 5% CO₂ for 8 days, after which they were infected with *M. avium* FF_mScarlet at a 2.5:1 (bacteria:cells) proportion and incubated for 4 hours at 37°C. The cells were washed three times with warm HBSS and incubated in mAO monolayer media at 37°C, 5% CO₂. *M. avium*'s intracellular growth was evaluated on the day of infection (t0) and 4 and 7 days after infection by CFU assay.

5.2.8. *hAOs monolayers and M. abscessus infection*

To promote adhesion, the wells of a 96-well PhenoPlate microplate (6055302, Revvity) were coated with 50 µg/mL of collagen type I (PureCol, 5005, Advanced Biomatrix) for 1 hour at 37°C, washed twice with HBSS and air-dried. The hAOs were shredded into single cells, as described in **Section 5.2.7**, and 1 x 10⁴ cells were added to each well in PneumaCult™ AvOE Medium. The cells were incubated at 37°C, 5% CO₂ for 7 days, after which they were infected with Mab FF_mScarlet at a 100:1 (bacteria:cells) proportion and incubated for 6 hours at 37°C. The extracellular media was removed, and the infected cells were incubated with 200 µg/mL of amikacin (PHR1860, Merck Life Science) for 2 hours to kill non-internalized mycobacteria. The cells were washed with HBSS and incubated with PneumaCult™ AvOE Medium for 2 (immunofluorescence – **Section 5.2.9**) or 3 days (live imaging). Live imaging of the monolayers was performed at 24, 48 and 72 hours after infection using an Operetta CLS™ high-content analysis fluorescent microscope (Revvity) with temperature and CO₂ level control. The images were acquired with phase contrast for the cell monolayer and 561 nm laser for Mab FF_mScarlet. The bacterial aggregates' height was calculated using Harmony High-Content Imaging and Analysis Software (Revvity).

5.2.9. Immunofluorescence staining of mAO monolayers

The mAO monolayer was washed once with PBS and incubated with 4% PFA for 20 minutes at room temperature. The cells were washed three times and permeabilized with PBS + 0.3% Triton X-100 for 7 minutes at room temperature. After washing three times with PBS + 0.05% Tween-20 with 5 minutes of soaking between washes, the cells were blocked with PBS + 0.05% Tween-20 + 10% FBS for 1 hour at room temperature. The monolayer was incubated with the primary antibody (**Table 3**) in PBS + 0.05% Tween-20 + 5% FBS for 24 hours at 4°C, washed three times and incubated with the secondary antibody (**Table 3**) in PBS + 0.05% Tween-20 + 5% FBS for 45 minutes at room temperature. The cells were washed twice, stained with DAPI in PBS for 10 minutes at room temperature, washed one final time with PBS and imaged with an Axio Observer Z1 fluorescence microscope using the 365 nm (for nuclei), 470 nm (for targets in **Table 3**) and 500 nm (for bacteria) lasers and a 63x oil objective. The images were analyzed using ImageJ/Fiji software.

Table 3. List of antibodies used for flow cytometry and fluorescence microscopy of mAO cells.

Primary antibody	Target	Supplier	Catalog number	Dilution ratio
Aquaporin-5	AEC1	Invitrogen	PA597290	1:100
SFTPC	AEC2	Invitrogen	PA5102493	1:300
Phalloidin	Actin	Invitrogen	A22287	1:1000
Secondary antibody	Supplier		Catalog number	Dilution ratio
Donkey anti-Rabbit IgG, Alexa Fluor 488	Invitrogen		A21206	1:500

5.2.10. Immunofluorescence staining of *M. abscessus*-infected hAO monolayers

The *M. abscessus*-infected hAO monolayer was washed three times with HBSS, incubated with 4% PFA for 15 minutes at room temperature and washed three times with HBSS. The cells were permeabilized with PBS + 0.1% Triton X-100 for 15 minutes at room temperature, washed with PBS and blocked with PBS + 1% BSA for 30 minutes at room temperature. The monolayer was incubated with the primary antibody in PBS with or without BSA (see **Table 3**) overnight at 4°C. After 30 minutes at room temperature, the cells were washed three times with PBS with 5 minutes of soaking between washes and incubated with the secondary antibody in PBS with or without BSA (see **Table 4**) for 1 hour at 4°C. After washing twice, the cells were incubated with HCS Cell Mask Deep Red stain (H32721, Invitrogen) and DAPI for 15 minutes at room temperature, and washed one final time with PBS. The plate was imaged with an Operetta CLS™ high-content analysis fluorescent microscope (Revvity), and the data were analyzed using Harmony High-Content Imaging

and Analysis Software (Revvity) with mScarlet fluorescent signal being used as a measure of bacterial load. The images were acquired with 405 nm laser for DAPI (nuclei), 488 nm laser (for targets in **Table 4**), 561 nm laser for Mab FF_mScarlet and 640 nm laser for HCS Cell Mask Deep Red (cytoplasm) and a 20x Air/0.4 objective. The images were analyzed using ImageJ/Fiji software.

Table 4. List of antibodies used for fluorescence microscopy of hAO cells.

Primary antibody	Target	Supplier	Catalog number	Dilution ratio	Solvent
GPRC5a	AEC1	Merck Life Science	HPA007928	1:250	PBS+1% BSA
Pro-SPC	AEC2	Merck Life Science	AB3786	1:150	PBS+1% BSA
CLH2	Mucin-5AC	Merck Life Science	MAB2011	1:1000	PBS+10% BSA
SNA-FITC conjugate	SNA lectin	Invitrogen	L32479	1:250	PBS
Secondary antibody	Supplier		Catalog number	Dilution ratio	
Donkey anti-Rabbit IgG, Alexa Fluor 488	Invitrogen		A21206	1:500	

5.3. Results

5.3.1. Development and characterization of mouse alveolar organoids

Mouse alveolar organoids (mAOs) were obtained by isolating lung-resident adult stem cells from C57BL/6 mice. The stem cells were resuspended in a matrix, Matrigel® (Corning), supplemented with a cocktail of growth factors. It takes around 15 days to observe mAOs in the microscope. They typically present a cystic structure with apical-basal polarization, but mAOs with a denser and not-so-well-defined structure are also visible (**Figure 1A**). After that time, each mAO expands and loses its cystic shape, becoming large enough to be visible to the naked eye. Before that shape transformation starts, they should be passaged to ensure space in the matrix for all the individual mAOs. In this process, the organoid is disrupted into single cells and plated in new matrix, with fresh supplementation. The cells with stem potential will start the differentiation process and give origin to new organoids in less than 7 days (**Figure 1B,C**). This passaging process allows to work with organoids that derive from a lung sample from a single mouse for 10 to 12 passages.

By flow cytometry, after the three-dimensional structure was disrupted into single cells, it was possible to conclude that around 60% of the cells in mAOs 14 days after the tenth passage express a marker characteristic of AEC1 (**Figure 1D**), and around 20% express a marker characteristic of AEC2 (**Figure 1E**).

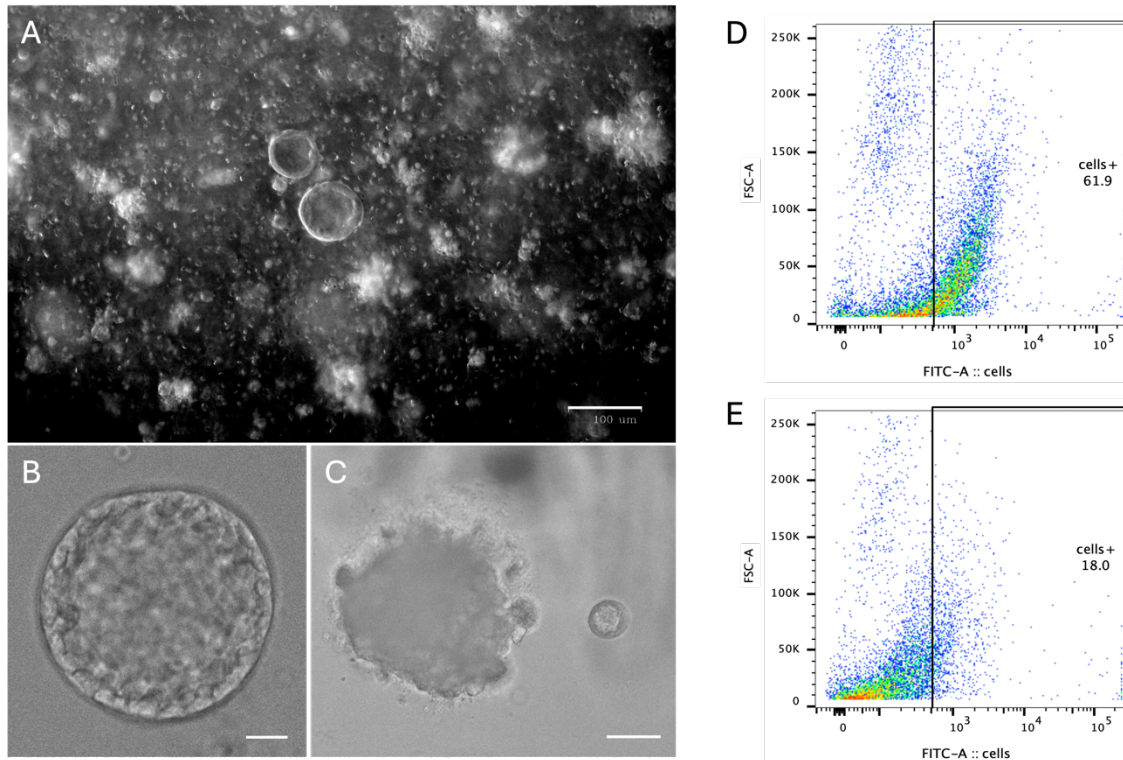


Figure 1. Appearance and cellular composition of mouse alveolar organoids (mAOs). **(A)** Phase contrast image of mAOs before the first passage. Scale: 100 μm . **(B,C)** Brightfield images of mAOs five days after the third passage. Scale: 25 μm **(B)** and 81 μm **(C)**. Images acquired with a ZOE Fluorescent Cell Imager (Bio-Rad). **(D,E)** Total number of single cells obtained after disruption of mAOs at 14 days after their tenth passage. The “cells+” subpopulation is positive for the expression of the AEC1 marker Aquaporin-5 **(D)** or the AEC2 marker SFTPC **(E)**. The samples were analysed in a BD FACS ARIA II (BD Biosciences) using the 530/30 (FITC) filter, and the plots were obtained with FlowJo software.

5.3.2. Infection of mAOs with *M. avium*

mAOs were infected with red fluorescent reporter *M. avium* by shredding the organoids and incubating the cellular aggregates with the mycobacteria (1:10, bacteria:cells) for four hours. The infected mAOs were reassembled in Matrigel and imaged by confocal microscopy. This technique allowed to observe the complex structure of a cystic mAO in detail (**Figure 2A**), but few mycobacteria were detectable. However, some bacilli were visibly interacting with the mAO cells (**Figure 2B**). *M. avium* infected mAOs were also processed for histological analysis. Hematoxylin and eosin (H&E) staining shows that the mAOs are predominantly cystic and have different sizes (**Figure 2C**), which could indicate different growth rates in the same sample. By Ziehl-Neelsen staining, which stains acid-fast bacteria like mycobacteria, it was possible to observe bacilli inside the mAO cells (**Figure 2D**).

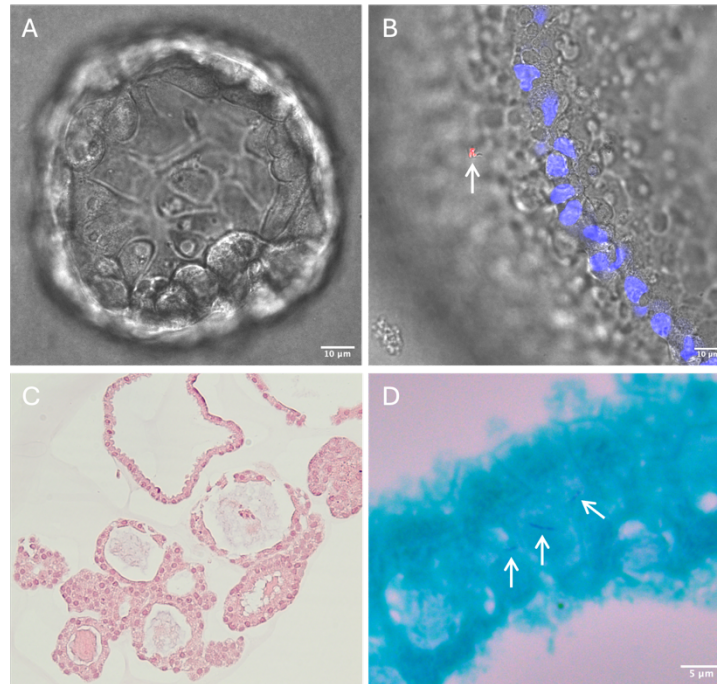


Figure 2. mAOs infected with *M. avium*. mAOs were shredded, incubated with *M. avium* FF_mScarlet for four hours and reassembled in Matrigel. **(A)** Phase contrast image of a mAO fifteen days after the fifth passage. **(B)** mAO in phase contrast merged with the nuclei in blue (DAPI) and *M. avium* FF_mScarlet in red (arrow). The images were acquired with a Leica SP5 II confocal microscope. Scale: 10 μ m. **(C,D)** Histological slices of mAOs fifteen days after the sixth passage stained with H&E **(C)** and Ziehl-Neelsen **(D)** show mAOs infected with *M. avium* FF_mScarlet (arrows). Scale: 5 μ m. The images were acquired with a Leica DM2000 LED.

Flow cytometry of the infected mAOs was performed to understand the efficiency and distribution of the infection among the cell types that constitute the mAOs. It was possible to conclude that among the total number of AEC1-positive or AEC2-positive cells, around 20% were infected with red fluorescent *M. avium* **(Figure 3)**. Importantly, these percentages were only achieved by increasing the multiplicity of infection to 10:1 (bacteria:cells).

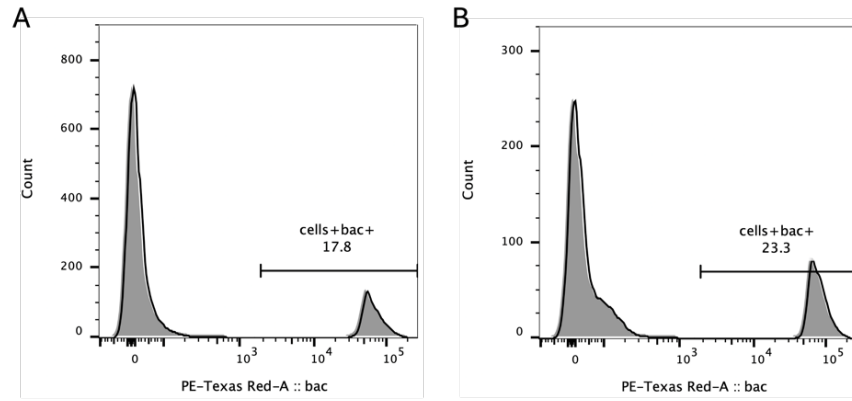


Figure 3. Percentage of mAOs cells infected with *M. avium* FF_mScarlet. Histogram of Aquaporin-5 (AEC1) positive cells (**A**) and SFTPC (AEC2) positive cells (**B**) that also are positive for mScarlet (“cells+bac+” subpopulation). The mAOs used were disrupted into single cells 14 days after their tenth passage. The samples were analysed in a BD FACS ARIA II (BD Biosciences) using the 530/30 (FITC) and 610/20 (PE-Texas Red) filters, and the plots were obtained with FlowJo software.

5.3.3. Infection of mAO cell-derived monolayers with *M. avium*

As the infection of the three-dimensional structure presented several limitations, the mAOs were disrupted, and their cells were seeded into a monolayer to facilitate the mycobacteria's access to the apical side of the alveolar cells. The way the cells are organized differs in this model from that of the organoid, but the constitution of the alveolar wall is still mimicked using differentiated primary alveolar cells, as confirmed by immunofluorescence staining (**Figure 4A,B**). The cells were infected with *M. avium* at 2.5:1 (bacteria:cells), and the intracellular bacterial load was assessed at 4 and 7 days post-infection. The results confirm the internalization and multiplication of the mycobacteria inside the mAO cells. However, a significant amount of *M. avium* also grows extracellularly (**Figure 4C**).

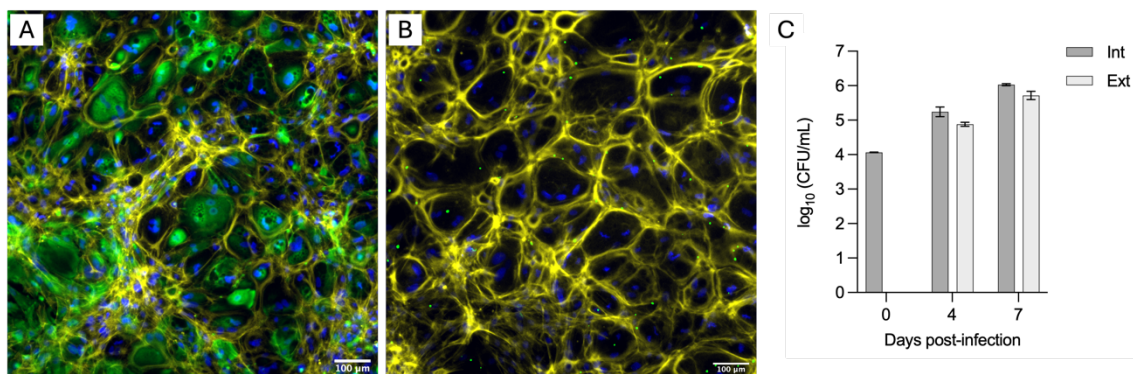


Figure 4. mAO cell-derived monolayer infected with *M. avium*. mAOs were mechanically and enzymatically disrupted and seeded at 5-10 x 10⁴ cells/mL in a 24-well plate. (**A,B**) Representative fluorescence microscopy images of mAO cell monolayers with actin in yellow (phalloidin), nuclei in

blue (DAPI) and AEC1 (Aquaporin-5) **(A)** or AEC2 (SFTPC) **(B)** in green. The images were acquired with an Axio Observer Z1 (Zeiss). Scale: 100 μ m. **(C)** Intracellular (Int) and extracellular (Ext) bacterial load in mAO cell-derived monolayers immediately after (0), 4- and 7-days post-infection with *M. avium* FF_mScarlet. The graph shows the average $\pm$ standard deviation of two replicates of one experiment.

5.3.4. Development and characterization of human alveolar organoids

Like what was done with mAOs, the human alveolar organoids (hAOs) developed in a Matrigel® matrix, but in this case, a commercial culture media (PneumaCult™ Medium, Stem Cell Technologies) was the source of differentiation factors to alveolar cells. The hAOs were maintained in an “expansion” media (PneumaCult™ AvOE Medium) for 10 days, which favoured the differentiation of most stem cells into AEC2, and then exchanged to a “differentiation” media (PneumaCult™ AvOD Medium), that promotes the differentiation of AEC2 into AEC1. Immunofluorescence confirmed the predominant expression of AEC2 or AEC1-specific cellular markers when the hAOs were in expansion or differentiation media, respectively (results not shown). Histological analysis of hAOs in expansion media shows the organoid is constituted of cuboidal-like cells, the characteristic morphology of AEC2, and hAOs in differentiation media are comprised mostly of flat cells, typically associated with AEC1 (results not shown). As a model for mycobacterial infection, it is more interesting to have AOs constituted by AEC2 than by AEC1, as the literature describes that mycobacteria infect the former. Therefore, we proceeded with hAOs in expansion media.

Compared with mAOs, hAOs in expansion media vary more in structures and size from one another in the same Matrigel® dome, and their development progresses differently from patient to patient. Still, like the mAOs, they are mostly cystic (**Figure 5**).

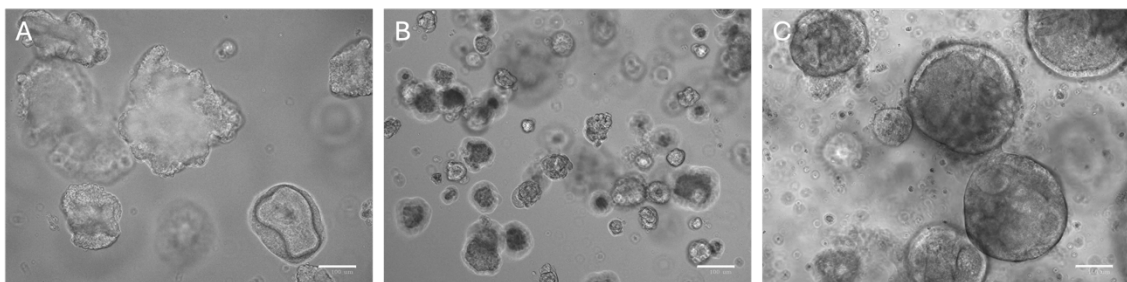


Figure 5. Appearance of human alveolar organoids (hAOs) in expansion media. Brightfield images of patient 2's hAOs in their second passage **(A)** and in their third passage **(B)**, and patient 12's hAOs in their sixth passage **(C)**. Scale: 100 μ m. The images were acquired with a ZOE Fluorescent Cell Imager (Bio-Rad).

5.3.5. Infection of hAO cell-derived monolayers with *M. abscessus*

hAOs were disrupted and seeded on top of a type-I collagen coating in 96-well plates. The cell monolayer is established as the cells adhere and multiply in the well for around 7 days, mimicking the human alveolar epithelial barrier. The cells were infected with 1×10^6 CFU of Mab FF_mScarlet for 6 hours, washed, and incubated for 2 days in expansion media. To assess the cellular constitution of the hAO-derived monolayer, the cells were fixed and immunofluorescence staining was performed. The cells in the monolayer express GPRC5a, a membrane marker of AEC1 (**Figure 6A**), and pro-SPC, a prosurfactant protein produced by AEC2 (**Figure 6B**). The cells also express the SNA lectin, typical of AEC2 (**Figure 6C**), and some of them produce MUC5AC, a mucin typically secreted by airway cells (**Figure 6D**). The percentage of *M. abscessus*-infected cells after 2 days, obtained by measuring the fluorescence intensity of the bacteria, is around 20%.

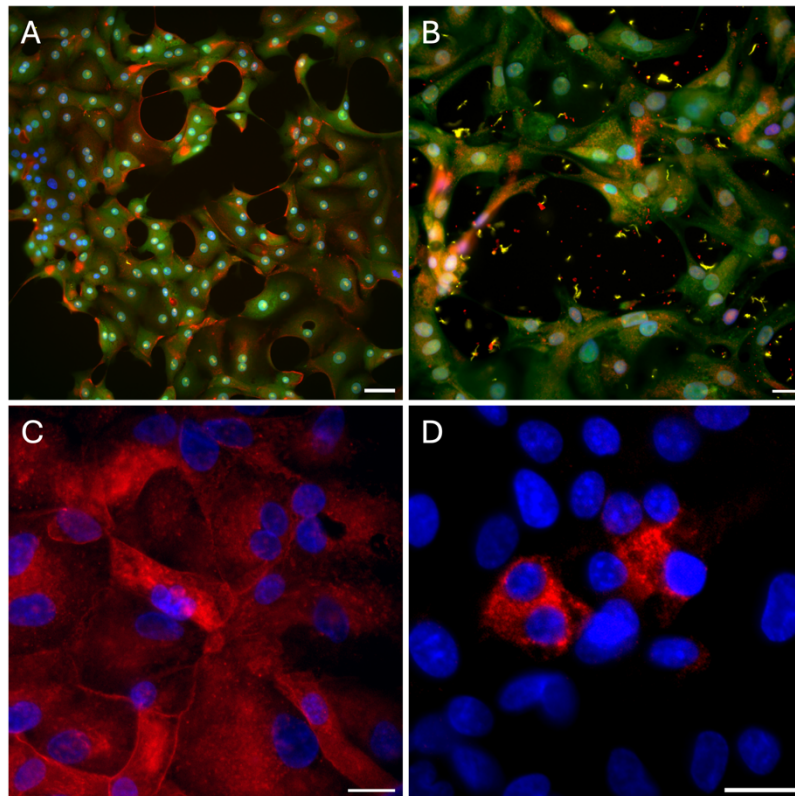


Figure 6. Confocal fluorescence microscopy images of hAO-derived alveolar monolayers infected with Mab FF_mScarlet. **(A)** Mab (yellow), GPRC5a (AEC1 marker, in red), CellMask™ plasma membrane (green), nuclei (blue). **(B)** Mab (yellow), proSP-C (AEC2 marker, in red), CellMask™ plasma membrane (green), nuclei (blue). **(C)** SNA lectin (red), nuclei (blue). **(D)** CLH2 (Mucin-5AC marker, red), nuclei (blue). Scale: 20 μ m. The images obtained with Operetta CLS™.

Although internalization and intracellular growth seem to occur to some extent, fluorescence quantification and CFU counting indicate significant extracellular growth. Indeed, live imaging of the wells over 72 hours revealed that *M. abscessus* replicates on top of the alveolar cells instead of growing intracellularly (**Figure 7**). These bacterial aggregates, around 12 μm in height, resemble biofilms, suggesting that these structures are formed not only on plastic but also on living tissue.

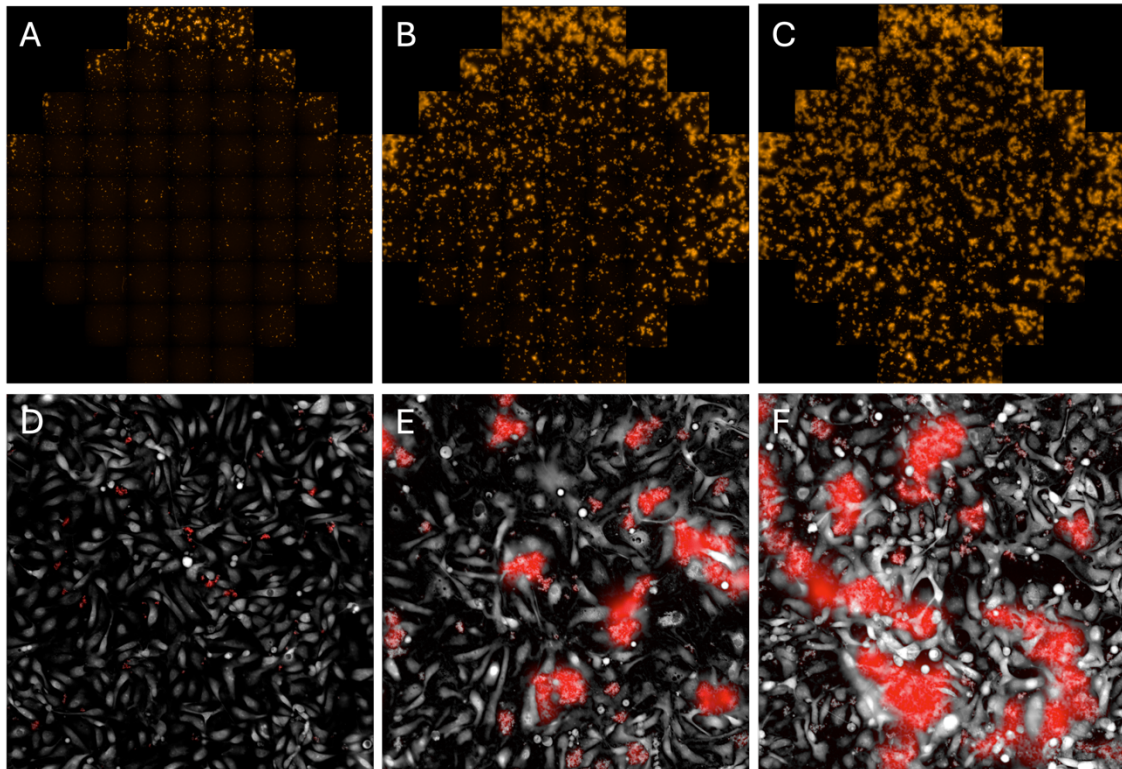


Figure 7. Infection of organoid-derived alveolar cells with *M. abscessus*. The images show the Mab FF_mScarlet (orange) in a whole well of a 96-well plate after 24 (**A**), 48 (**B**) and 72 (**C**) hours of infection. The close-up of one field of each well (**D-F**, in respect to **A-C**) shows the aggregates of Mab FF_mScarlet (red) on top of the hAO-derived alveolar cell monolayer (phase contrast). The images were obtained by live imaging with Operetta CLS™ (Revvity).

5.4. Discussion

This work aimed to establish an alveolar organoid model infected with mycobacteria for drug susceptibility assessment.

Starting with the whole lung of a C57BL/6 mouse, we obtained mAOs that lasted approximately seven months, demonstrating that this model significantly reduces the use of animals as a source of primary cells. Our flow cytometry results (**Figure 1D,E**) show that the mAOs are mainly constituted by AEC1 and AEC2, as expected, but cell types from the upper lung may also be present. These results suggest the cellular composition of the mAO

at the day and passage number they were analyzed and may not translate to other time points. However, they allow to conclude that most of the cells in the mAO are epithelial alveolar cells, validating the differentiation process even after ten passages.

To infect the mAOs with *M. avium* we opted for the shredding strategy. This imposes limitations, mainly because the newly formed organoids, after the shredding process, arise from stem cells. If those cells were not infected, then it is possible that the new AOs do not contain infected cells [174]. AEC2 have, however, stem potential, as they can self-renew and differentiate into AEC1 [185]. Our histology results (**Figure 2D**) indicate that *M. avium* was internalized by the mAO cells, suggesting that some AEC2 were infected during shredding, while others, including AEC1, may have been infected by bacilli that were trapped in the mAO's lumen after reassembling. Our flow cytometry results (**Figure 3**) indicate around 20% of infected cells of each epithelial type, which is a higher percentage than described for AEC2 *M. avium* infection at a higher MOI [141]. However, the number of experiments should be increased to confirm these results. Opting for an infection strategy that preserves the organoid's 3D structure, like microinjection, would be more physiological and susceptible to fewer errors.

Given the limitations encountered with the shredding approach, we next decided to seed the AO cells into a monolayer to facilitate access to the cell's apical side and to monitor their development and infection more easily through microscopy. We confirmed that our mAO-derived monolayers maintained an alveolar phenotype, as most cells expressed AEC1 or AEC2 markers and showed high confluency (**Figure 4A,B**). These cells were infected with *M. avium*. CFU counting results confirmed internalization and the intracellular bacterial load increased by one log every three to four days until day 7 post-infection (**Figure 4C**). The quantity of bacteria in the extracellular medium was curiously similar to the amount inside the cells, which suggests that these cells are infected less efficiently than macrophages. Further assays to quantify intracellular bacterial load and assess antibiotic susceptibility, for instance, using reporter mycobacteria and high-content microscopy analysis, would provide insights into the adaptability of AO-derived monolayers for HTS of antimycobacterial agents.

Since we had access to human lung samples, we transitioned to this more relevant organoid model. The protocol for obtaining the hAOs was the same as that for the mAOs. However, preparing the mAO media involved adding each component individually, which increased the chances of errors and variability between experiments. Therefore, for hAOs, we used a commercial alveolar organoid culture medium to avoid variations. This was especially important for hAOs, as the lung material would be sourced from different donors, which naturally implies inter-assay variability. We initiated each organoid culture with a small piece of healthy lung tissue from lung cancer patients undergoing surgery. Working with this type of cell source presented two types of constraints. The first constraint is quantitative:

each sample yielded a lower number of cells and, consequently, fewer cells with stem potential than we obtained using the mouse whole lung. This resulted in fewer hAOs before the first passage. Nonetheless, after a few passages, it became possible to obtain a number of hAOs comparable to mAOs (**Figure 5**). The second constraint when working with human samples is the variability of the differentiation process, which primarily relates to the variations in the lung location from which the sample was extracted. Some samples were taken from the upper lobe, some from the middle lobe, and others from the lower lobe. As the airway epithelium comprises different cell types, the cells isolated from each donor would inherently have different stem potentials. For example, basal cells are only present in the upper airway epithelium and have the stem potential to differentiate into any cell type, including alveolar cells [185]. Club cells, also present in the upper airways, possess the stem potential to differentiate into basal cells, as well as AEC2 and AEC1, resulting in alveolospheres or alveolar organoids [213]. Since AEC2 have the stem potential to self-renew and differentiate into AEC1 [185], a sample from the lower lobe rich in alveoli would primarily give rise to alveolar organoids. However, a study conducted by i3S colleagues concluded that the expression of AEC2 was enhanced by using the entire mouse lung rather than just the bottom half [214]. Using culture media with specific alveolar differentiation factors ensures that every cell with the stem potential to become an alveolar cell will do so. However, if no cells with that potential were isolated from the lung sample of a particular donor, it may explain why some organoid cultures were completely unsuccessful. Additionally, we are uncertain whether cells like basal cells, also isolated from the sample, cannot differentiate into other cell types under those conditions. We believe their presence or absence, depending on the location of the sample, can influence the composition of the organoids and result in variations depending on the donor.

Regarding the infection strategy, the limitations observed for the mAOs led us to move directly to the infection of hAO-derived monolayers. Using 96-well plates, we could optimize the model for HTS. Immunostaining of the monolayers confirmed the presence of AEC1 cells, given the expression of GPRC5a at the cell membrane (**Figure 6A**), which encodes a 3 G protein-coupling receptor [215]. We can also observe pro-SPC expression inside and outside the cells (**Figure 6B**), a marker of the surfactant protein C produced exclusively by AEC2 [216]. The presence of AEC2 is reinforced by the binding of the *Sambucus nigra* (elderberry bark) lectin (SNA) (**Figure 6D**) to the AEC2 membrane [217]. Detection of mucin-5AC (**Figure 6D**), a gel-forming glycoprotein secreted by goblet cells and club cells at the upper airway epithelium [218,219], indicates those cells are present to some extent in the hAOs. Staining with specific goblet and club cell markers or evaluating gene expression would confirm the composition and abundance of the different lung cell types in the hAO-derived monolayers.

Donor-to-donor variability was observed in the ability of hAOs to form monolayers. Although the majority would adhere to the plate, some hAO cells, after organoid dissociation, would not adhere to the plate and instead aggregated to each other as if they favored a 3D structure, even without a matrix. Coating the wells with type-I collagen improved cell adherence and was essential to avoid cell loss in the washing steps of infection and immunofluorescence protocols. Nevertheless, the alveolar epithelial cells adhered poorly to the plates compared to macrophages. Additionally, the fact that the hAOs consisted of a mix of cell types with distinct morphologies led to a non-confluent cell arrangement, as shown by the fluorescence microscopy images (**Figure 6**). We believe this lack of full confluency influenced the infection with *M. abscessus*, as some of the bacilli would adhere to the bottom of the well rather than interacting with the cells, which is unsurprising due to this species' ability to adhere to surfaces.

Quantification of intracellular *M. abscessus* indicated that around 20% of the hAO-derived alveolar epithelial cells were infected after two days, which is in line with studies with the AEC2 cell line A549 [148,149], even though our hAOs also have other cell types. But most interesting was the high level of *M. abscessus* extracellular growth. Live fluorescence microscopy close-up images (**Figure 7D-F**) show an intact hAO-derived monolayer covered on top with mycobacteria, forming huge and thick aggregates. The extracellular growth probably arises from bacilli that were never internalized by the cells, as the ones that adhered to the well on the spaces between cells. This can suggest the formation of *M. abscessus* biofilms on top of the hAO-derived alveolar monolayer, which resembles the observation made by Leon-Icaza et al. [184] of *M. abscessus* S variant forming aggregates surrounded by a polymeric substance on the apical side of AirO cells. A closer inspection of our *M. abscessus* aggregates, such as through electron microscopy, could support this hypothesis.

Although the AO were useful to identify important features of the growth of NTM and their interaction with different types of host cells, we did not reach our initial objective of setting hAO as a high-throughput setting for drug screening. Indeed, the high variability we observed with the hAOs, from their capacity or inability to form organoids and monolayers to their significant susceptibility to washing protocols, was not a good indicator for establishing a model that needs to be consistent and reproducible. Future studies may allow further optimization of this type of model to study mycobacterial infections.

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

This work ultimately aimed to contribute to the fight against NTM disease. We approached this issue in two ways. First, we evaluated the effectiveness of two new compounds with an alternative formulation to traditional antibiotics. The enhanced physicochemical characteristics of the clofazimine-fluoroquinolone OSs, without losing antimycobacterial activity, demonstrate the potential of investing in the refinement of conventional drugs. Second-line drugs like clofazimine and fluoroquinolones in OS form can ultimately become part of the therapeutic regimen against mycobacterial infections by substituting or reducing the dosages of more toxic antibiotics or those with mechanisms of action more susceptible to resistance. However, to achieve that ideal scenario, and even before proceeding to animal testing, potential compounds like these should be evaluated in more complex settings. Drug discovery research should always have in mind the 3 Rs of animal experimentation: Replacement, Reduction, and Refinement. The number of animals can be reduced if fewer compounds reach this phase, which can be accomplished by replacing animals with models that closely mimic host conditions. That is why we also approached the fight against NTM infections by enhancing the complexity of *in vitro* models while considering the plastic characteristics of mycobacteria.

In the first phase of DST, we need to balance the complexity of the *in vitro* model with the speed of achieving results. Often, a pool of compounds large enough to fill dozens of microtiter plates must be screened without being too time- or resource-consuming. That is where reporter bacteria come in handy. Our fluorescent reporter strains brought several advantages to planktonic and intracellular assays compared to CFU counting or the resazurin assays used to test the OSs. The most important aspect is the ability to use fluorescence microscopy to monitor the infection. Regardless of the setup, whether axenic, macrophages, or spheroids, we can always track the bacilli and observe how they interact with the host cells. This provides us with a very different type of information than counting colonies on agar. It allows us to see the conditions the bacteria are in, whether they are growing intracellularly or extracellularly, while also monitoring the host cells themselves. Assessing the bacterial load becomes pointless if the host cells are not viable. Quantifying an intrinsic property of the mycobacteria, such as fluorescence, is also more direct than acquiring the intensity of an externally added reagent. Fluorescence gives us a measure of bacterial load that only depends on the bacteria's presence or absence. This is very valuable for identifying hits in an extensive screening, where we must quickly distinguish an active from an inactive compound. However, relying solely on fluorescence can be a limitation. For example, if we want to step into *in vivo* models using the same reporter strain, which is always advantageous, the high fluorescent background of living tissues does not allow us to distinguish the bacteria's signal. Autofluorescence is also a limitation in culture

media supplemented with molecules with an aromatic side chain, like the ones in serum or the pH indicator phenol red. In these conditions, the bacterial fluorescent signal can be detected, but the high background limits the technique's sensitivity. Additionally, fluorescence is not a measure of viability, but only depends on the presence or absence of the fluorescent protein. The emission of fluorescence by dormant or non-replicating mycobacteria is something we need to further explore.

Assessing mycobacterial viability is essential for drug screening purposes and can be determined by luminescence. Luminescence is dependent on the availability of ATP. Therefore, light is only emitted when the bacteria are metabolically active.

Developing double-reporter *M. abscessus* strains enabled us to assess fluorescence and luminescence simultaneously. Besides direct antibiotic susceptibility testing, we applied the double-reporters luminescence to measure biofilm viability and follow the infection in vivo. For the former, discovering we could use accessible and user-friendly equipment like the ChemiDoc to capture the luminescent signal was a highlight. The Image Lab™ software was also easily adjusted to measure the pixels in the biofilm ring area. Additionally, bacterial fluorescence can be measured, adding another layer of information on the biofilm status. This is particularly pertinent in a biofilm as bacteria at its core are generally deprived of nutrients and oxygen, being in a latent or non-replicative state, not emitting luminescence but potentially expressing mScarlet. There are various advantages of analyzing a biofilm by fluorescence or luminescence instead of recurring to conventional techniques. CV staining only measures the extracellular matrix integrity, and its protocol requires much more manipulation, which can lead to material loss. CFU counting of a biofilm can be performed to quantify bacterial load. However, it can lead to an underestimation of the total number of bacteria due to bacteria that cannot grow on agar after being in stressful conditions, like the ones experienced on the biofilm. Additionally, to perform CFU counting, it is mandatory to obtain a single-cell suspension, which is difficult in the case of a biofilm. The biofilm must be removed from the plastic using manual scraping or sonication, and the aggregates should be disrupted with a needle. Besides being fallible, all these procedures are highly hazardous, as *M. abscessus* is a BSL2 microorganism that easily aerosolizes.

Thus, the objective of establishing a mycobacterial biofilm model for high-throughput drug screening was successfully achieved.

For the in vivo assays, luminescence was essential to overcome the issue of tissue autofluorescence. Ideally, we would use our autoluminescent *M. abscessus* strain to eliminate the need to inject the mice with D-luciferin. However, the signal intensity was not sufficient for detection. Even with a boost in light emission from the addition of D-luciferin, the equipment lacked the sensitivity to detect low quantities of bacteria. This is likely because mycobacteria are very small, and thus, it is necessary to have a high number of

bacteria in the same place to achieve a detectable signal. As a comparison, bioimaging in mice infected with parasites, which are significantly larger organisms, is very effective. With smaller animal models, such as the larvae *Galleria mellonella*, our collaborators could efficiently detect Mab FF_mScarlet, as the bacteria are less dispersed throughout the larval body. Therefore, with the appropriate in vivo model, the double-reporter strains are undoubtedly useful.

For setups like biofilms or animal models, where a strong luminescent signal is needed, only Mab FF_mScarlet can be used. However, for rapidly assessing bacterial viability at a large scale, Mab operon_mScarlet has proven to be essential. Colleagues in our group optimized a protocol for screening thousands of compounds against Mab operon_mScarlet growing in 384-well plates [220]. The use of liquid-handling robotics, combined with not needing to add D-luciferin, enhances the efficiency of the process. Even with small working volumes (30 μ L), meaning a low number of mycobacteria per well, the autoluminescent signal was sufficiently strong to be detected, and the measurement of the statistical effect size (Z' factor) confirmed the assay could be employed for high-throughput drug screening, leading to the identification of hits to be further explored in more complex infection models.

Importantly, the constructs we used to transform *M. abscessus* can also be applied to transform any other mycobacteria species. From a host-directed therapy perspective, it would be interesting to transform clinical isolates to express fluorescence and luminescence. However, this process is not as straightforward as transforming laboratory strains, as researchers have selected those for being easy to transform. Different clinical isolates will exhibit varying amenabilities to transformation and possess natural adaptations for survival in the host, the most evident being carrying antibiotic resistance genes, which may interfere with the selection of transformants. Therefore, customized transformation protocols and further optimizations would be necessary to obtain reporter isolates, which may not provide benefits for quickly determining treatment for a patient.

In the pursuit of finding complex infection models to fill the gap between in vitro and animal testing, we concluded that the way we used to infect macrophages with frozen mycobacteria was probably not the most appropriate. We could clearly see that for most antibiotics, no dose-response was observed when macrophages were infected in this way. When mycobacteria encounter a macrophage within the host some bacilli may be highly replicative while others are not. Although there may not be an in vitro growth phase that fully represents mycobacteria in the host, we can confidently state they are not frozen before encountering a host cell. Thus, it is useful to perform drug screening against different replicative states to understand the full potential of a new drug.

Two-dimensional models have a lot of advantages but also limitations. In the host, cells do not lie on a flat surface but interact with the neighboring cells and the environment in all

three dimensions. Embedding the cells in a matrix provides mechanical and biochemical cues similar to those found *in vivo*, allowing them to form a more natural tridimensional arrangement. Therefore, we tried to establish 3D models of mycobacterial infection. We created a spheroid of PBMCs, consisting of a mixture of immune cells like monocytes, lymphocytes, and dendritic cells, within a collagen-fibronectin matrix to simulate a granuloma. Due to limitations of the microscopy technique, we could not see if mycobacteria were contained in the core of the immune cell aggregate. This technical impediment did not allow us to quantify a drug's ability to eliminate those bacteria. Additionally, we did not proceed with this model because of the challenges in obtaining blood donors to isolate PBMCs and achieving a large enough cell yield to test multiple conditions per experiment. We are aware that developments have been made to address issues with visualizing 3D structures using microscopy. These include protocols for clearing the matrix of auto-fluorescent proteins and lipids, as well as the use of equipment like light sheet microscopes. These microscopes enable the acquisition of images from very thin slices of the spheroid, allowing for the reconstruction of the entire 3D structure. Also, high-content microscopes like Opera™ are equipped with technology that allows for screening an entire microplate with multiple spheroids per well and analyzing each one individually. Therefore, we still have a lot to explore regarding mycobacterial infection of granuloma *in vitro* models and how they can be applied for high-throughput drug screening.

Finally, we stepped into another 3D model, in this case, one that incorporates the cell types that constitute an entire organ or part of it. Our alveolar organoids were very promising, firstly because, contrarily to the PBMC spheroids, we could obtain a lot of material from a reduced source. This is certainly an advantage of organoid models. Even when starting from a mouse sample, if we can use organoids to test drugs instead of using animals, we can significantly reduce the number of animals further in the pipeline.

The organoids' main disadvantage was the heterogeneous behavior of the cells obtained from each donor. This variability hindered our ability to establish a stable and reliable high-throughput setting for drug screening. Additionally, the absence of immune cells remains a significant drawback. However, we acknowledge that organoids are undeniably useful for studying aspects of bacterial biology in an environment closely resembling the host. For our purposes, lung organoids can be crucial for evaluating the activity of drug candidates in later stages of the drug discovery process, prior to animal testing, although they are not suitable for the initial screening of thousands of compounds. On the other hand, having access to samples from human donors and being able to create a biobank of human primary cells that can be cross-referenced with patients' medical histories are valuable assets for host-directed therapies.

Future Perspectives

The space for improving in vitro models of infection is unlimited. In the future, we aim to continue investing in different ways of adapting existing models, drawing inspiration from what is being done with other pathogens, taking ideas from completely different scientific areas, or inventing new ones.

Our immediate ideas consist of continue exploring lung organoid-derived monolayers, maybe going back to mouse organoids as a source of cells, as we do not depend so much on donor variability. We pretend to establish monolayers on transwells, allowing the cells to be exposed to air on their apical side, which more closely mimics the alveolar epithelium. We started doing it with hAO-derived monolayers, however we were unable to achieve a level of confluency that would permit air exposure without leading to cell death. We believe that by optimizing cell density, culture conditions and finding helpful monitoring strategies and equipment we could develop a very interesting in vitro model.

We also want to invest in establishing a suitable mouse model for *M. abscessus* infection. Several factors need optimization, starting with the mouse strain. Immunocompetent mice like C57BL/6 [221] or C3HeB/FeJ [222], which closely mimic *M. tuberculosis* pulmonary infections, can clear *M. abscessus* infections within a few days. Some immunocompromised mouse strains, like nude mice [222], can maintain bacterial burden but lack a complete immune response and are very expensive. This affects factors related to host-pathogen interaction and pathology development, not being the ideal models for drug susceptibility testing. To overcome those issues, one of the most recent *M. abscessus* mouse models administers corticosteroids to immunocompetent mice to establish a chronic *M. abscessus* infection and assess antibiotic susceptibility [223,224]. That model uses aerosol infection, which is more physiological than an intravenous one. By establishing a model of chronic infection that develops structures such as granulomas, we could have enough mycobacteria concentrated in one region to enable the detection of luminescent signals and accurately quantify the effectiveness of compounds in reducing the bacterial burden.

Related to a different scientific question than the assessment of drug susceptibility, we want to contribute to a better understanding of the behavior of *M. abscessus* in the host. From various experiments, we observe that *M. abscessus* is more of an extracellular grower than an intracellular pathogen. The fact that we often observed extracellular bacteria during the course of macrophage or alveolar epithelial cell infection, even in the presence of antibiotics in the extracellular media, can stem from three scenarios. The most likely explanation is that the macrophage never took in those mycobacteria, nor were they washed away from the culture media right after infection, allowing them to continue replicating extracellularly. Another explanation may arise from a transition of *M. abscessus* morphotypes from smooth (S) to rough (R). It is described that S variants are phagocytosed

individually by macrophages and remain masked within the phagolysosomes, whereas R variants tend to aggregate and grow extracellularly. Our *M. abscessus* strains were S variants. However, the transition from S to R occurs during infection, modulated by the bacteria in response to still unidentified environmental cues. The third scenario is that the macrophage is taking up small bacterial aggregates that replicate inside a social phagosome until it reaches its capacity and disrupts, leading to escape into the cytosol and induction of autophagic and apoptotic responses that result in the release of bacteria into the extracellular medium.

We can thoroughly explore these questions and understand if there is a possible S-to-R transition occurring during infection by several approaches. The simplest consists of comparing the morphotype of the *M. abscessus* colonies before and after infecting the host cells. Another is conducting live tracking microscopy of the infection over an extended period and observing the entire process instead of time-point by time-point. Many questions can also be clarified by performing RNA sequencing and having a whole vision of the bacteria's transcriptome before the infection, intracellularly or after eventually escaping the host cells. We believe there will be differences depending on the mycobacterial growth phase. Identifying genes associated with metabolism, virulence, and phenotypic differences like growth rate or biofilm formation can also shed light on the most effective antibiotic targets for each replicative stage, expediting the development of new antimycobacterial drugs.

There is still much to be done before preclinical models can accurately predict the activity of a potential new drug on the host. We envision that the disadvantages of one model are counterbalanced by the advantages of another, and that many questions can be addressed by each of them. A compound never succeeds in the drug discovery pipeline if it does not pass through various in vitro and in vivo models. Utilizing high-throughput tools and automated processes allows this to be achieved in a shorter timeframe. Having different layers of complexity ensures that we gain a comprehensive understanding of a compound's efficacy in various scenarios, increasing the chances of finding new solutions to combat infectious diseases.

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