Universitat Autònoma de Barcelona

In vitro effects of pollutants and novel therapeutic approaches for tuberculosis

PABLO RODRÍGUEZ FERNÁNDEZ

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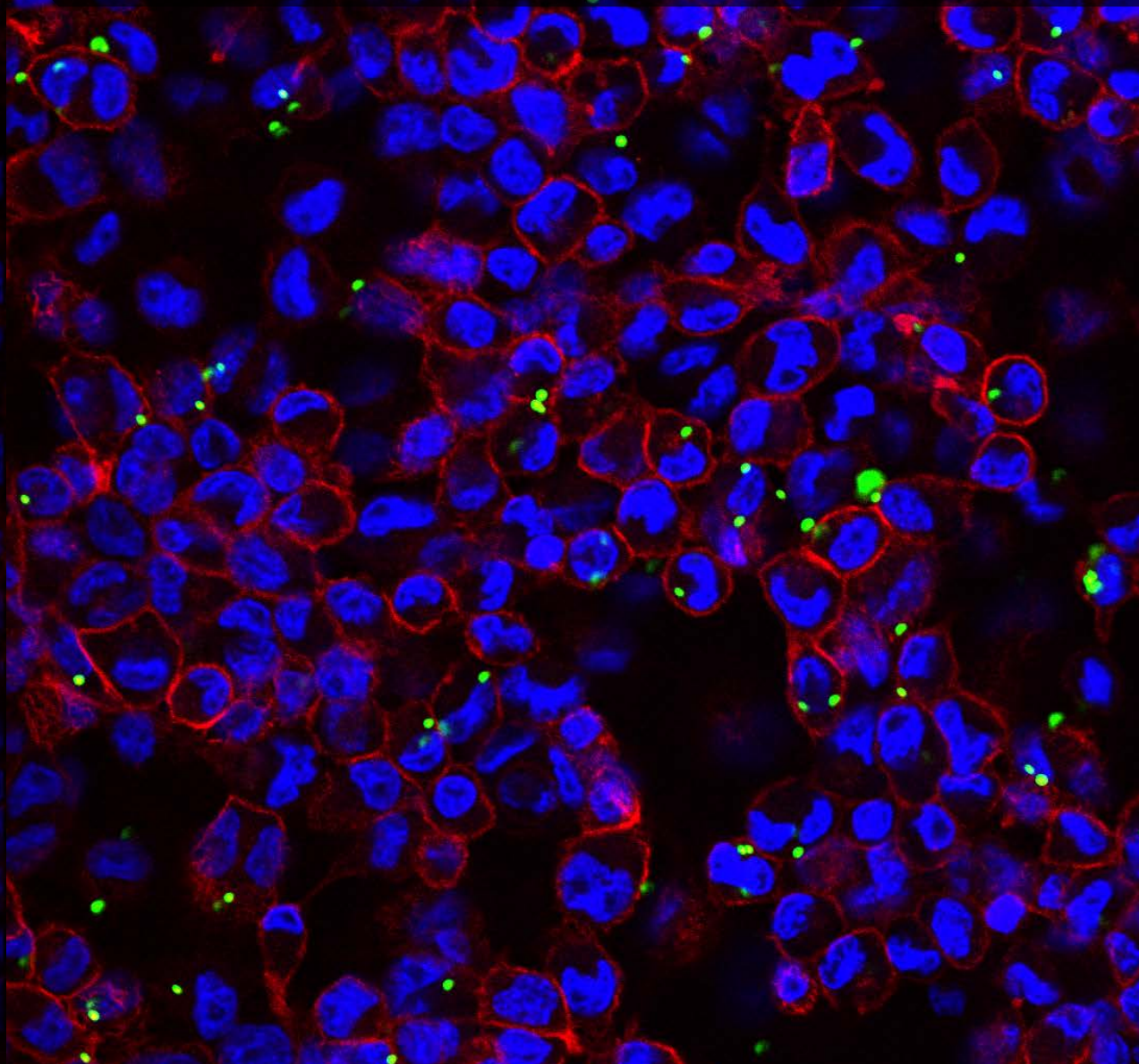

PABLO RODRÍGUEZ FERNÁNDEZ

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Doctoral Thesis

In vitro effects of pollutants and novel therapeutic approaches for tuberculosis

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To all people who deserved my time

and I could not give it because of the work on this thesis

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SUMMARY

Tuberculosis (TB) is the first cause of death caused by a single infectious agent, killing about 1.6 million people per year. TB is transmitted via inhalation, and when *Mycobacterium tuberculosis*, the causal agent of TB, reaches the alveolus, an inflammatory response is triggered. The immune response is conditioned by host factors, coexisting infections such as HIV, diversity of mycobacteria, but also by external factors like pollution, undernutrition, overcrowding or alcoholism. Air pollution is mainly due to particulate matter and some clinical studies have found associations with TB. Smoking is an established risk factor for TB infection, disease, relapse, fail in the treatment and drug-resistant TB and it is estimated that 15% of global TB deaths are attributed to smoking. Although hypothetically all deaths related to cigarette smoke (CS) are preventable, the alarming data is that more than one billion people are current smokers, and the number could be increased due to aggressive tobacco marketing in low and middle-income countries. Electronic cigarettes (e-cigs) are based on vaporizing a solution of glycerol that could contain flavours and nicotine. They are usually promoted as smoking cessation devices, giving the idea that they are harmless, although e-cigs could be the first step in the use of traditional cigarettes.

On the other side, treatment of TB takes at least six months and consists of two steps due to active and latent populations. Although effective, the emergence of drug-resistant TB and the limited pipeline make necessary to look for new therapeutic approaches. Encapsulation of current drugs could improve the treatment as it would be easier to reach the minimal inhibitory concentration at the site of infection and decrease general toxicity and side effects. Different materials for nanoparticles (NPs) synthesis and routes of administration are being explored. Host-directed therapies are based on modulating the response of the host by small molecules. It is a novel approach that will avoid the spread of resistance.

There are two main aims in this thesis. One of them is to evaluate the impact of diesel, electronic cigarette vapor (e-vapor) and CS on macrophages infected with *M. tuberculosis*, as an *in vitro* model of a TB patient exposed to these pollutants. To do this, we looked for the recovery of CFUs, percentage of infection, cytokines production and metabolomics profile (**Chapters I-IV**). The second one is to evaluate NPs resistant to acidic pH for oral treatment (**Chapter VII**) and combining the addition of drugs, NPs or molecules targeting virulence factors with exposure to CS (**Chapters V, VI & VIII**).

In **Chapter I** we summarized the interaction between environmental pollution and respiratory infections. Poor air quality of overcrowded and industrialized cities due to large quantities of pollutants, like diesel, has been related to asthma, lung cancer and respiratory infections, especially in children. Exposure to particulate matter increases the susceptibility to pneumonia and viral respiratory infections, causing inflammation, oxidative stress, alter cytokines production and affect T and NK cells. Children and elderly people are more susceptible. In **Chapter II** we explored the role of diesel, as a main contributor to urban pollution, in the susceptibility to mycobacterial infections, as pollution is rapidly increasing in areas where TB is endemic. In our experience, diesel particles were highly cytotoxic, although pollutants did not delay significantly the growth of the bacteria. We did not observe changes in intracellular bacteria burden nor impairment of internalization. However, extracellular recoveries of BCG were higher, which suggests that contention of BCG is impaired to some extent by diesel pollutants.

In **Chapter III** we studied the role of CS and e-vapor in *M. tuberculosis* phagocytosis and cytokine production. Intracellular *M. tuberculosis* burden was reduced when macrophages were exposed to e-vapor, although less than when exposed to CS. Percentage of infection was also reduced when evaluated by confocal microscopy infecting with *M. bovis* BCG and latex beads. The production of cytokines was evaluated by Luminex, observing that e-vapor generates a pro-inflammatory response, increasing the levels of TNF-α, IL-8 and IL-1β. In **Chapter IV** we used metabolomics to analyse the global profile of the metabolites in extracellular and intracellular fraction of cells exposed to CS or e-vapor and infected with *M. bovis* BCG. Main metabolomics differences in supernatants were due to maturation of the cultures, but e-vapor exposed samples also differed, mainly by the presence of lactic acid, nicotine and glycerol. Intracellular metabolites were mostly affected by time, but just after infection samples were differentiated by CS or e-vapor exposure. It was observed that tryptophan was not reduced in intracellular infected samples exposed to CS or e-vapor exposure, suggesting an impairment of the depletion of essential nutrients strategy against pathogens. Levels of kynurenine, an immune regulator, were also altered. All in all, e-vapor impairs the phagocytic function and the cytokine response to *M. tuberculosis*, as well as induces changes in the metabolome of THP-1 cells comparable to CS exposure. In our experience, e-cigs modify the metabolome in a comparable manner with CS.

As smoking has been associated with TB treatment default, we investigated in **Chapter V** the effects of CS and anti-TB drugs in macrophages infected with *M. tuberculosis*. Cells were exposed to CS, infected with *M. tuberculosis* and treated with anti-TB drugs. Intracellular recovery of CFUs was lower in cells exposed to CS extract due to an impairment in the phagocytosis. Macrophages treated with drugs showed a reduction in the intracellular burden compared with non-treated. The same pattern applied to the recovery of extracellular CFUs. We detected less concentration of rifampicin in smoked samples, due to a potential interaction with CS. We concluded that smoking and TB treatment is a harmful interaction that impairs the immunity of the host.

As treatment of TB is long and requires several drugs with severe side effects, leading to noncompliance and resistant TB, we explored the potential advantages of NPs on the next chapters. In **Chapter VI** we tested the activity of rifampicin and isoniazid encapsulated in silica NPs and explored their effect against human macrophages infected with *M. tuberculosis* exposed to CS. In our experience, silica NPs were highly toxic for THP-1 macrophages, although no effect on the growth of *M. tuberculosis* was detected. Silica NPs were administered to infected cultures exposed to CS, and no interaction NPs-CS was found. Poor drug load efficiency and high toxicity of the NPs made that administration of NPs had no advantage compared to free drugs. In **Chapter VII** we tested the activity of PLGA NPs loaded with rifampicin. NPs were emulsified to protect them against gastric conditions. NPs were able to cross an *in vitro* model of intestinal barrier and were protected from degradation under simulated gastric conditions. NPs were not cytotoxic to macrophages, and only slightly delayed the growth of *M. tuberculosis*. Time-kill kinetics at acidic pH experiments showed that unloaded NPs have some bactericidal action and loaded NPs have a sustained release of the drug. When macrophages were infected with *M. tuberculosis* and NPs or drugs were administered, loaded NPs showed a more effective elimination of *M. tuberculosis* than free drugs.

Finally, in **Chapter VIII** we explored a new approach targeting MptpB, a protein tyrosine that prevents phagosome maturation, allowing *M. tuberculosis* to survive inside macrophages. We studied the combined effect of MptpB inhibitor and CS in THP-1 macrophages infected with *M. bovis* BCG or *M. tuberculosis*. Our results showed that MptpB inhibitor had no cytotoxic effect. Growth of extracellular bacteria was not reduced by the MptpB inhibitor, only by rifampicin. Although the reduction of intracellular bacteria when the MptpB inhibitor was administered alone was very mild,

intracellular bacteria was significantly reduced by the combination of rifampicin and MptpB inhibitor. No major effects were observed by the combination of CS and the MptpB inhibitor. MptpB inhibitor is a promising candidate as a drug for TB treatment, especially in combination with other TB drugs like rifampicin, or as a vaccine.

This work highlights the importance of making global environmental policies and implementing tobacco cessation strategies as part of the TB treatment. Results show e-cigs cause a pro-inflammatory response that could favour TB disease. As drug resistance is inevitable, it is necessary to focus on new therapeutic approaches, as nanoparticles carriers or host-directed therapies. Our results support that the use of loaded PLGA NPs are an improvement of free drugs, and targeting virulence factors in addition to drugs administration potentiate the bactericidal effect against *M. tuberculosis*.

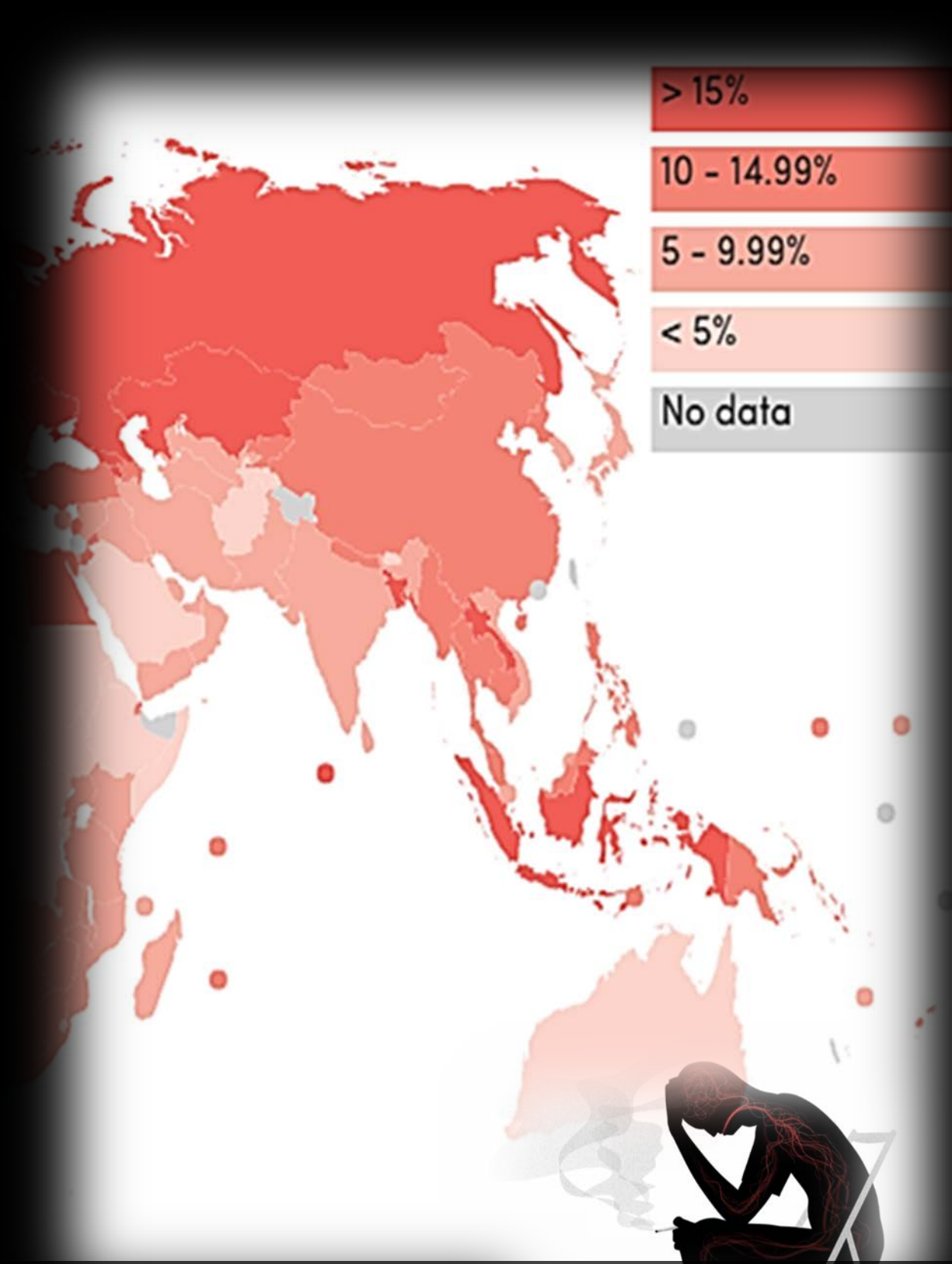
ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ADC	Albumin-dextrose-catalase
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CSE	Cigarette smoke extract
DEP	Diesel exhaust particles
DMSO	Dimethyl sulphoxide
DPBS	Dulbecco's phosphate-buffered saline
DR-TB	Drug-resistant tuberculosis
e-cig	Electronic cigarette
e-liquid	Liquid for electronic cigarettes
EMB	Ethambutol
ENDS	Electronic nicotine delivery systems
ERS	European Respiratory Society
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
e-vapor	Vapor generated from electronic cigarettes
FDA	Food and drug administration
GC	Gas chromatography
HAART	Highly active antiretroviral therapy
HDT	Host-directed therapy
HIV	Human immunodeficiency virus
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma release assay

IL	Interleukin
INH	Isoniazid
LTBI	Latent tuberculosis infection
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimal inhibitory concentration
MOI	Multiplicity of infection
Mptp	Mycobacterium protein tyrosine phosphatase
MS	Mass spectrometry
MSMD	Mendelian susceptibility to mycobacterial disease
MSNP	Mesoporous silica nanoparticle
MTBC	*Mycobacterium tuberculosis* complex
NPs	Nanoparticles
OADC	Oleic acid-albumin-dextrose-catalase
OPLS-DA	Orthogonal projection to latent structures discriminant analysis
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PG	Propylene glicol
PI	Phosphoinositide
PI3P	Phosphatidylinositol-3-phosphate
PLGA	Poly(lactic-co-glycolid) acid
PM	Particulate matter
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase

PZA	Pyrazinamide
RIF	Rifampicin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SNPs	Single nucleotide polymorphisms
TB	Tuberculosis
TNF-α	Tumour necrosis factor-alpha
TRAP	Traffic-related air pollution
Treg	Regulatory T cells
TST	Tuberculin skin test
VG	Vegetable glycerol
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

Percentage of tuberculosis-related deaths due to tobacco
The Tobacco Atlas©



1. INTRODUCTION

1.1 Tuberculosis disease: epidemiology and pathogenesis

In 2017, it was estimated that 10 million people had developed active tuberculosis (TB) disease, and 1.6 million people died **[1]**. Fifty-six per cent of the total TB cases were found in only five countries: India, Indonesia, China, the Philippines and Pakistan **[1]**. Furthermore, based on the interferon-gamma release assays (IGRAs) and tuberculin skin tests (TSTs), more than 1.5 billion people (1/4 worldwide population) might be latently infected by *Mycobacterium tuberculosis*, 5-10% of which will develop active TB during their lifetime **[1, 2]**. Because in the latent infection stage the pathogen is not transmitted, the efficiency of transmission should be extremely high to be able to maintain one-fourth of the global population infected. If the pathogen would cause a fatal disease in all individuals it would finally disappear; thus latency is an effective strategy to ensure its survival **[3]**. This stage enables to maintain a high incidence because most cases are due to reactivation of latent tuberculosis infection (LTBI) instead of primary infections **[4]**.

Infection begins when droplets containing *M. tuberculosis* enter the lungs via inhalation. Once a person is exposed, *M. tuberculosis* can either be eliminated by innate or adaptive immune responses of the host or could remain in the host in a latent form. Alveolar macrophages are fundamentally the first line of defence which will try to internalize the bacteria by phagocytosis. The differentiation from hematopoietic cells to monocyte-macrophages is highly dependent on the growth factors and cytokines of the local environment **[5]**. The interaction *M. tuberculosis* – professional phagocytes occurs through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) like toll-like receptors, C-type lectin receptors, Fc Receptors, Scavenger receptors or cytosolic DNA sensors and as a result, it has the phagocyte activation **[2]**. If phagocytosis takes place, *M. tuberculosis* is finally trapped in a vacuole named phagosome. Bacteria in the alveolar space of infected macrophages migrate to the lung parenchyma, where the recruitment of mononuclear cells, due to a local proinflammatory response, takes place (Figure 1a). Macrophages will try to destroy the internalized bacteria by fusion events of the phagosome with the lysosome. This process is regulated by the activity of Rab GTPases. On the contrary, *M. tuberculosis* will try to stop the maturation of the phagosome and will succeed it unless the macrophage is stimulated by inflammatory cytokines **[2]**. So, in this step, macrophages should be activated by interferon-gamma (IFN-γ) to activate the vacuolar proton ATPase, which generates many reactive oxygen intermediates that acidify the lumen (pH<5.2) in less than 15 minutes **[4]** in a process called phagosome maturation,

which involves the interaction with many intracellular organelles **[6, 7]**. This acidic condition could kill *M. tuberculosis*, so the pathogen tries to trigger an anti-inflammatory response, to block reactive oxygen intermediates and the acidification of the phagosome **[8]**.

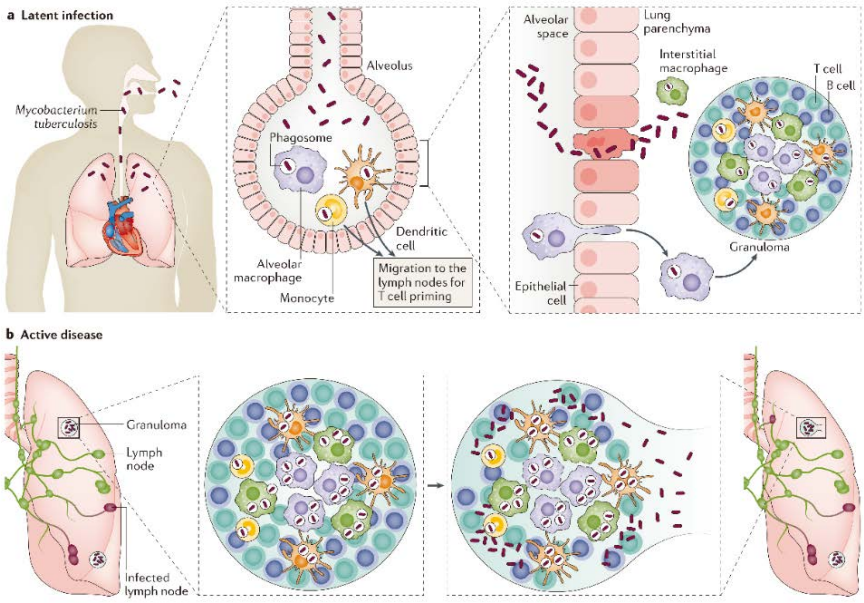


Figure 1. *Mycobacterium tuberculosis* infection. a. The starting of the infection and the latent state. b. Replication of the bacteria inside the granuloma conducts to active disease. Image from **[9]**.

On the other hand, monocytes transport *M. tuberculosis* to lymph nodes to allow the T cell priming, because although macrophages ingest the bacteria they cannot effectively kill them. T cell priming enhances the inflammatory response recruiting T and B cells for the formation of the tubercle or granuloma **[8, 9]**. The granuloma consists of a kernel of infected macrophages, surrounded by foamy giant cells and macrophages, with lymphocytes on the periphery ($CD4^+$ and $CD8^+$ T cells and B cells) and with collagen and other extracellular matrix components that delineate the structure. The granuloma's function is to limit the dissemination of infection, imprisoning the bacteria without killing them. This structure represents the containment phase (neither signs of disease nor transmission to others) which can endure from just some weeks to several years (Figure 1a) **[10]**. However, granuloma is a safe place for *M. tuberculosis* to establish latency **[8]**.

At this stage, the centre of the granuloma can be caseous or fibrotic. Fibrotic lesions are typical of latent infections and are mainly composed by fibroblast with a lower number of blood vessels entering into the structure, without hardly any macrophages **[11]**. Nevertheless, the granuloma could evolve to be caseous, indicating a necrotic state of death macrophages and other types of cells either due to latent infection or, more frequently, active disease (lung cavitation) **[11]**. Persistence and changes of the state of the granuloma depends on the existence of Th1 response of the T lymphocytes leading to the continual inflammation, antimicrobial activity and phagocytic activation **[5]**. In other words, during infection, different and contradictory immune responses often occur balancing or unbalancing the response *M. tuberculosis* - host **[2]**. It is supposed that the equilibrium dormant - persistent bacteria makes the difference from LTBI and active TB **[4]**. Although we use the terms LTBI and active TB, the reality is more complex and corresponds to a continuous spectrum of disease, with a diverse range of manifestations **[11]**.

Inside of the granuloma, *M. tuberculosis* can disrupt the membrane of the phagosome, either by ESX-1 system (transmembrane channels to secrete outside the bacteria involved in major virulent facts) and dimycocerosates or pthiocerol dimycocerosates (key virulent lipids) causing the mycobacterial DNA to be released into the cytosol, inducing type I interferon responses and promoting the growth of the pathogen **[6]**. This uncontrolled replication of *M. tuberculosis* will result in the destruction of the alveolar macrophage **[8]**, which in fact accelerates the growth of *M. tuberculosis*. This occurs because the growth inside the dead cells is even faster than in the extracellular environment **[12]**. The host mechanisms to prevent the bacteria from reaching the cytosol are the autophagy and programmed cell death **[4]**. The pathogen is trapped inside the host cell until the cell dies either by necrosis or apoptosis. Apoptosis of infected cells is regulated via tumour necrosis factor-alpha (TNF-α) and tries to prevent the uncontrolled replication of the pathogen and provides antigens to the adaptive immune response **[2]**. Necrosis consists in the release of both nuclear and cytoplasmic content. It is hypothesized that apoptosis could enhance the dissemination of *M. tuberculosis* and necrosis would trigger the replication **[2]**. If the granuloma's burden was so high (usually by a change in the immune status of the host), the wall of the granuloma could break down, releasing *M. tuberculosis* to the bloodstream (disseminated TB) or the respiratory tract (being the host infectious) **[9]** (Figure 1b). During pulmonary active TB, *M. tuberculosis* promotes the formation of cavities where it can proliferate and destruct the lung's extracellular matrix **[5]**.

In addition to this simplistic view of host-pathogen interaction, it is more and more evident that genetic and phenotypic characteristics of host and *M. tuberculosis* strains, modulated by environmental factors, play a vital role **[13]**. For example, about 27% of TB cases are attributable to undernutrition and 22% to indoor air pollution **[9]**. Moreover, although the incidence is declining annually by 1.5%, the appearance of MDR-TB strains is of particular concern. The average of MDR-TB is estimated at 5%, but it is as high as 20% in areas of the former Soviet Union. Countries like China and India arise a concern in the emergence of MDR-TB **[9]**.

1.2 Factors playing a key role in tuberculosis outcome

1.2.1 Host interindividual variability

Host genetic factors could explain why some people are susceptible to TB infection and disease while others are resistant. The hypothesis of the existence of a genetic component causing the hereditability susceptibility of TB was illustrated by the Lübeck disaster (Germany, 1927). A total of 251 newborns were accidentally vaccinated with virulent *M. tuberculosis* instead of BCG, resulting in 212 TB cases in children, from whom 77 died; while 39 did not develop any clinical symptoms **[14]**.

History resembles another interesting fact: Europeans have a greater resistance to TB than Africans. This is attributed to the time Europeans have been in contact with *M. tuberculosis*, which is longer than in Africans **[13]**. An old infectious disease which affects the fitness in or before reproductive age should sharp select the genetic variability of a population **[15]**; however, these polymorphisms which contribute to the resistance of TB probably also enhance autoimmune reactions **[13]**. This implies a potential lack of a high selective force in many genes, thus, difficulting the identification of specific genes affecting TB susceptibility.

There are two main approaches to detect DNA variations linked with TB: linkage analysis (studies of genome regions shared by affected relatives) and association studies (studies made to find genetic markers) with larger statistical power **[16]**. One of the most interesting polymorphism associated with TB is found on *Slc11a1* gen **[17]**.

Candidate genes by association studies are based on the putative function of the gene investigated, although coding genes represent less than 2% of the genome **[15]**. Associations with genes encoding SLC11A1, HLA, TLR or IFN-γ have been found **[16]**. Previous studies on Mendelian susceptibility to mycobacterial disease (MSMD) genes highlighted an association between IFN-γ receptor pathway and susceptibility to TB in children. MSMD, an inherited condition characterized by a predisposition to disease by weakly virulent mycobacteria **[18]**, involves germline mutations in seven autosomal genes (*Ifngr1*, *Ifngr2*, *Il12b*, *Il12rb1*, *Stat1*, *Irf8* and *Isg15*) and two X-linked genes (*Nemo* and *Cybb*) **[15, 19]**.

Genome-wide association studies analyse all the genome, coding and non-coding genes. The problems associated with this technique are the requirement of big sample sizes and controls **[15]**. Although many SNPs (single nucleotide polymorphisms) have been associated with susceptibility phenotypes, the susceptibility cannot be explained

only by the genetic variation (missing heritability), maybe because i) genes with minimal contributions to the susceptibility phenotype may not be found, ii) rare variants in genes or iii) epistasis (the effect of a genetic locus on a phenotype by the action of at least one other locus) **[15]**. Moreover, results remain inconsistent and are not reproducible in independent studies with different sample populations **[19, 20]**.

It is increasingly evident that gene-gene interactions (epistasis) take a crucial part in the missing heritability of complex diseases like TB; probably a reason for the lack of success in finding single polymorphisms **[21]**. Genetic functions are probably regulated by both complex mechanisms involving many genes and environmental factors **[22]**. A data-mining approach (the process of extracting hidden patterns and potentially useful information from large amounts of data) is a way to find epistasis efficiently **[22]**. However, functional epistasis is not always evident from statistical epistasis **[23]** and epistasis studies requires big samples sizes for their detection **[21]**.

It is also possible that epigenetic mechanisms, like mR146a **[13]**, and somatic mutations play an important role in the susceptibility to TB **[17]**. Not only genetic factors play a role in host variability, but also so does age. Children's immune system and pulmonary tissues are still under maturation process, while on the other hand, elderly people show immunosenescence, a decline in many immune parameters **[24, 25]**. For example, Spanish population over 64 years old in Catalonia have a greater risk of suffering TB disease **[26]**.

1.2.2 MTBC diversity

Mycobacterium sp. (lit. bacteria of fungal appearance) is an Actinomycete with 30% of their genes involved in the lipid synthesis, being mycolic acids responsible for the acid-fastness of the mycobacteria. Its thick cell wall makes *M. tuberculosis* able to resist desiccation, antimicrobials and host-immune mechanisms **[6, 27]**. Besides, 40% of their genes are annotated as having 'unknown' function, being no homologues in other microorganisms outside *Mycobacterium tuberculosis* complex (MTBC), which contributes to the special characteristics of this bacteria **[27]**.

During many years, it has been thought that in MTBC there was too little genetic diversity to consider. However, this 'clonal' idea does not explain why strains of MTBC differ in their content of SNPs, small and large insertion, duplication or deletion sequences **[28]**.

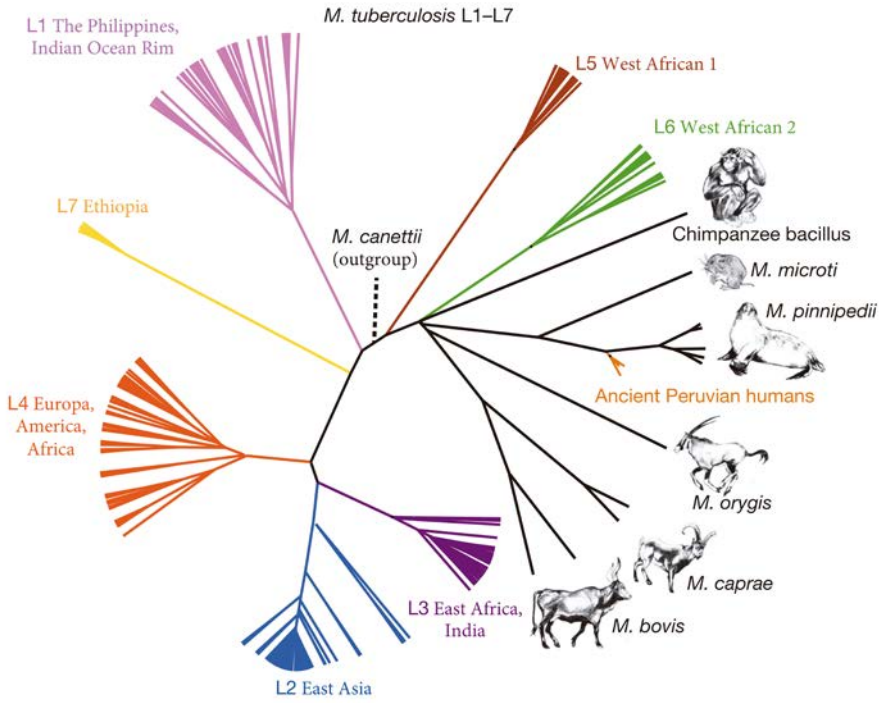


Figure 2. Phylogenetic analysis of MTBC. There are seven lineages (L1-L7) of MTBC. Modern MTBC (species with the region TbD1 deleted) is a monophyletic group that includes clades from L2 to L4. By contrast, ancestral MTBC are paraphyletic because they do not comprise a single phylogenetic group **[28]**. L5 and L6 clades includes *M. africanum*, where all animal MTBC originates. They occurred almost exclusively in West Africa for unknown reasons **[29]**. Image modified from **[30]**.

In contrast, MTBC is composed of seven phylogenetic lineages, including *M. tuberculosis* and *M. africanum* (obligate human pathogens) as well as other closely related species adapted to mammal species as *M. bovis* and other rarely isolated species (Figure 2) **[31]**.

MTBC is supposed to originate in Africa, travelling all over the world through human migrations, adapting to their local environments. This hypothesis is reinforced because Africa has the largest diversity of human MTBC (L1-L7), including L5 and L6 **[29]**. Dispersal of L4 could be traced by historical migrations out of Europe **[32]**. The genetic diversity of MTBC is probably due to stochastic variation in small populations or neutral variation with no fitness impact. Additionally, diversity could be selected by antibiotic pressure or independently due to heterogeneous immune responses of the host **[19]**.

Although MTBC diversity is not only the result of the environment, the role the immune system plays is not clear. Many SNPs have been identified which could explain the diversity of responses **[33]**. On the other side, surprisingly, human T cell epitopes are hyperconserved, implying that T cell responses may drive high selective forces and T cell epitopes are not under diversifying selection. In fact, 95% of the experimentally verified human T cell epitopes analysed are completely conserved in different representative strains of MTBC **[34]**. A high evolutionary conservation of the genomic region is a measure of the selective pressure; the importance of the sequence should be reflected in their conservation **[28]**.

The majority of pathogens evade the host immune response by antigenic variation, but it seems MTBC conserves the T cell responses ensuring a severe inflammatory response that causes tissue destruction and cavitation, which leads to a more efficient transmission. In fact, virulence and transmission are commonly associated with TB, contrary to other pathogens, although they do not always have a positive correlation **[35]**. Probably *M. tuberculosis* may benefit from going undetected by host mechanisms during certain parts of the infection cycle, while other parts take advantage from being detected and inducing high inflammatory responses **[36]**.

The diversity of MTBC could explain the differences in the secretion of cytokines, ability to inhibit apoptosis or trigger necrosis. Different strains unbalance the immune response differently, which is related to their level of virulence and transmissibility rates. Different MTBC strains and lineages differ in their growth rates or mycolic acid profiles. For example, a higher rate of MDR isolates and a higher virulence strains relates to L2 rather than others lineages, L2 and L4 have a higher progression rate to active TB disease than L6, and L4 is linked more with pulmonary TB while L2 and L3 are more associated with extrapulmonary TB **[19, 28]**. Modern lineages (L2-L4) are generally more virulent and more globally successful compared to other more geographically restricted lineages **[28]**. Treatment for TB also influences the diversity of MTBC: the more we treat and cure, the more we select MDR-TB.

1.2.3 External factors

1.2.3.1 Smoking

1.2.3.1.1 Tobacco products and electronic nicotine delivery systems

Tobacco is produced from the leaves of a plant, *Nicotiana tabacum*, which are dried and fermented. Tobacco products like cigarettes, cigars, bidis (India) or kreteks (Indo-

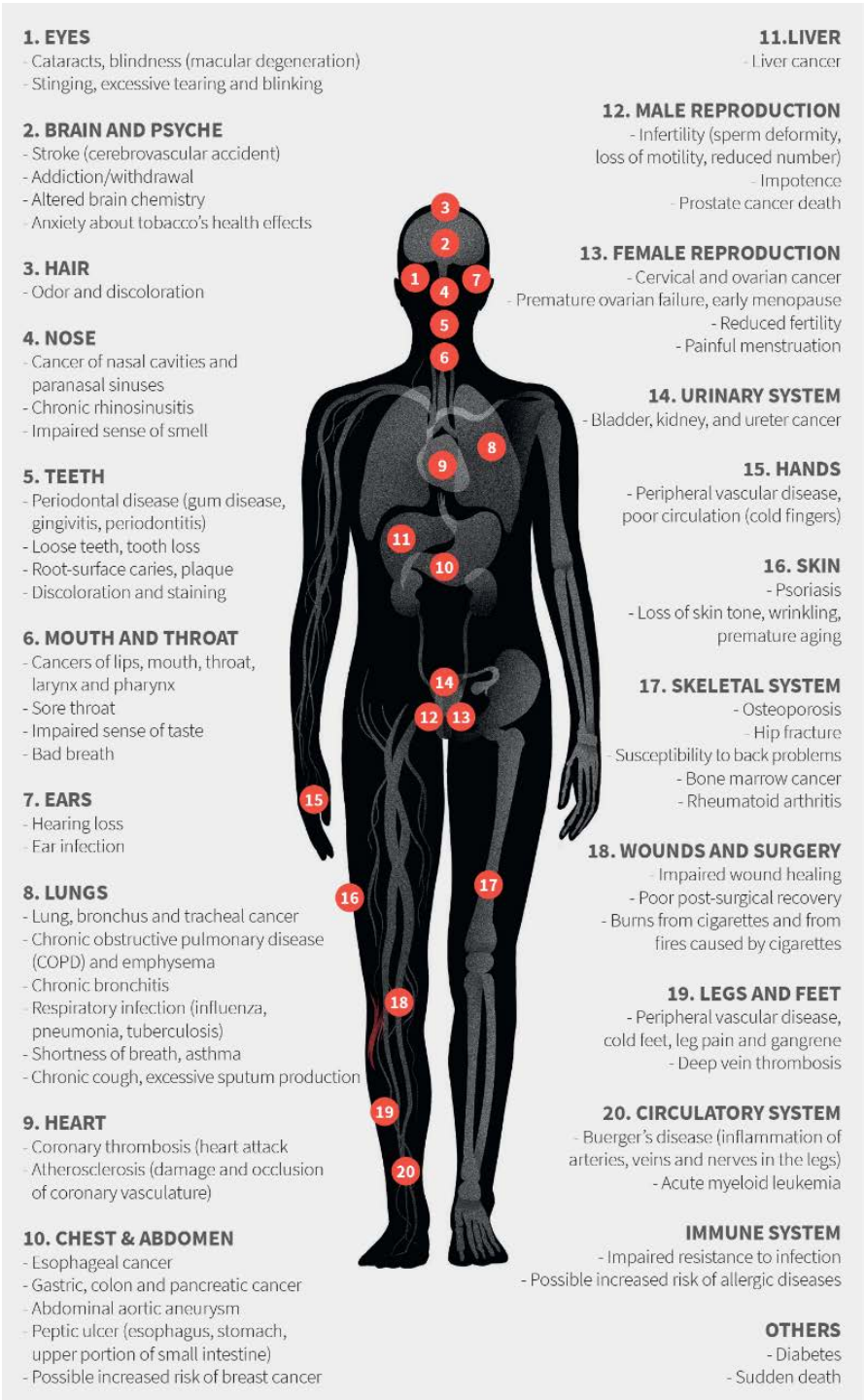


Figure 3. Health effects due to smoking cigarettes. Image from **[37]**.

nesia) are smoked, but others can be chewed or sniffed, like chewing tobacco, snus (Sweden), dip or snuff. The last ones are usually englobed as smokeless tobacco. Besides, there are special devices to smoke, as pipes or hookahs **[38]**. Tobacco products are known for containing nicotine, the compound that causes addiction, but cigarette smoke contains more than 7,300 compounds in the gas and tar (particulate) phases **[39]**. More than 5,200 compounds are described in an extensive book written by Rodgman and Perfetti **[40]**. Among them are human carcinogens and many toxic agents, including carbon monoxide, ammonia, acrolein, acetone, nicotine, benzopyrenes, hydroquinone or nitrogen oxides **[41]**.

Despite the devastating effects of cigarette smoke (CS) (Figure 3), globally more than 942 million men and 175 million women are current smokers **[37]**. People with mental disorders tend to smoke even four times more than the rate of the general population **[42]**. Male smoking prevalence increased in the last century while the peak of female smoking happened in the last decades, and only in very high incomes countries. Although prevalence in very high incomes countries has started to decline thanks to stronger tobacco control policies and taxes, prevalence in low and middle-income countries is arising **[37]**. Another concern is the increase of smoking in young people, especially females. Tobacco companies are making aggressive marketing in low and middle-income countries, where no strategies against tobacco have been implemented so far, despite the policies recommendations made by ERS (European Respiratory Society) and The Union in non-communicable diseases **[37, 43, 44]**. Figure 4 shows the increase in smoking prevalence in some African countries.

In the last years, Electronic Nicotine Delivery Systems (ENDS) have appeared on the market. Now they are classified as tobacco products, although they are not necessarily dependent on tobacco. Electronic cigarettes (e-cigs), which emerge in China in 2004, vaporize a solution with or without nicotine and with or without flavourings in a carrier medium (glycerol or propylene glycol). They are advertised as healthy products, badly delivers of nicotine and are marketing especially to young people with more than 7,000 flavours available in the market **[42]**. Among the compounds found in the aerosol, there are aldehydes, volatile organic compounds, phenolic compounds, polycyclic aromatic hydrocarbons, tobacco alkaloids, heavy metals and flavours **[41]**. Young people see them as easy to obtain, cool and a healthier option than traditional tobacco. Although the chemistry profile is much less harmful compared with tobacco smoke, they are not completely harmless **[45]**. Approximately 10% of e-cigs users are new

users, and two of the major concerns are the unclear risk e-cigs suppose in the repetitive use and that e-cigs could be the key to open the way to the traditional cigarettes [42]. It is not uncommon to users to be dual or polytobacco users.

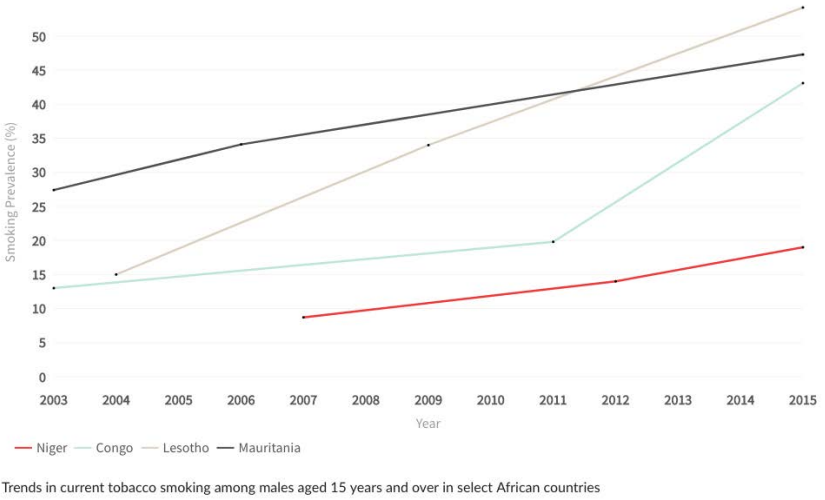


Figure 4. Smoking in Africa. Several sub-Saharan countries have seen a recent increase in smoking prevalence. Figure from [37].

The strategies for smoking cessation include both behavioural treatments and nicotine replacement therapy. In fact, a combination of both strategies has been proved to be the most effective strategy. Behavioural treatments include cognitive behavioural therapy, motivational interviewing, mindfulness, telephone support and social media support. Nicotine replacement therapy is based on stimulating the brain receptors targeted by nicotine, reducing cravings and withdrawal of symptoms [42]. Unfortunately, cessation support is not present in the majority of low-income countries [46]. Even more, a high proportion of smokers do not look for smoking-cessation strategies, and smokeless tobacco products have been suggested to be a harm-reduction approach to reduce the smoking prevalence [45]. In fact, e-cigs are promoted as smoking-cessation devices. However, there is an intense debate between those who believe e-cigs could help and those who believe e-cigs could ruin all the achievements made by tobacco control policies [45]. The same debate is related to adolescents' use: e-cigs could entrap them and be a gateway to smoking, but, at least theoretically, this could be protective against tobacco cigarettes, being a gateway from smoking. Long-term effects of e-cigs remain unknown.

1.2.3.1.2 Relation between tuberculosis and cigarette smoke

The association between smoking and TB was suggested for the first time in the literature in 1918 **[47]**. In 1956, adult patients with pulmonary TB in the UK were found to be more prone to be moderate and heavy smokers than non-smokers, both in males and females. Consequently, it was suggested that smoking and pulmonary TB could be related **[48]**. In 1961 and 1963 two studies from Australia and England associated TB with heavy drinking and not with smoking, suggesting that it was more probable to have TB if you were a smoker just because heavy drinkers were likely to be heavy smokers **[48, 49]**. The relationship between smoke and TB has remained unclear until the last twenty years when many studies in this area have been published. Smoking is recognized today as one of the driving forces in TB global pandemic **[50]**. In fact, the geographic overlap between areas of higher prevalence of cigarette smoking and regions with higher prevalence of latent and active TB is outstanding **[51, 52]**.

Despite all the studies, there were still some kind of risk of potential cofounders: smokers could be in areas of greater TB risk, higher transmission rates could be due to socializing rather than smoking *per se* and the fact that smokers are associated in general with a low socioeconomic status (i.e. crowding, malnutrition etc.) **[50]**. However, some comprehensive reviews and meta-analysis have evidenced the relationship between smoking and TB, independently of these other factors **[53-55]**. Alcohol and HIV are considered independent risk factors **[56, 57]**. Gender was not considered a risk factor for TB, and some studies found some measure of socio-economic class related with TB **[57]**, as we discuss later. In fact, smoking accounts for 15.8% of TB cases, being an important driving force of TB **[58]**. In Catalonia, smoking is the most important risk factor for Spanish people and immigrants **[26]**. The evidence of the effects of smoking on TB infection, disease, relapse, outcome of the treatment and probability of having drug resistant-TB are described below.

Smoking and risk of TB infection: Both primary and secondhand smoking has been associated with a positive TST in different countries and in different groups of ages **[59]**. Duration of smoking has also been found to be associated with TB infection. In a large cross-sectional study in China, similar results have been found with IGRAs (*in vitro* methods for infection diagnosis), being the annual risk of TB infection among ever-smokers 1.53 fold higher than non-smokers **[51]**. Globally, the annual risk of TB infection among ever-smokers has been found to be 1.4-1.6 fold higher as compared to never-smokers **[54]**.

Smoking and risk of TB disease: Many studies have investigated the relationship between smoking and developing active TB disease, distinguishing between current and former smokers, intensity (pack/years) and years of smoking **[50]**. The evidence for this association is very strong **[57]**. The association between years of smoking and TB has been demonstrated by case-control studies in Asia, North and South America, Europe and Africa **[49]**.

Smoking has been related with cavitary lesions, bacillary load, smear conversion delay, and related with false-negative IGRAs **[60]**.

Smoking and risk of TB relapse: Duration and intensity of smoking has been related to reactivation of TB **[56, 60]**.

Smoking and outcome of anti-TB treatment: A delayed response to the treatment has been described **[61]**. Some studies associate smoking with TB mortality **[50]**, and, in fact, many TB deaths are due to smoking (Figure 5).

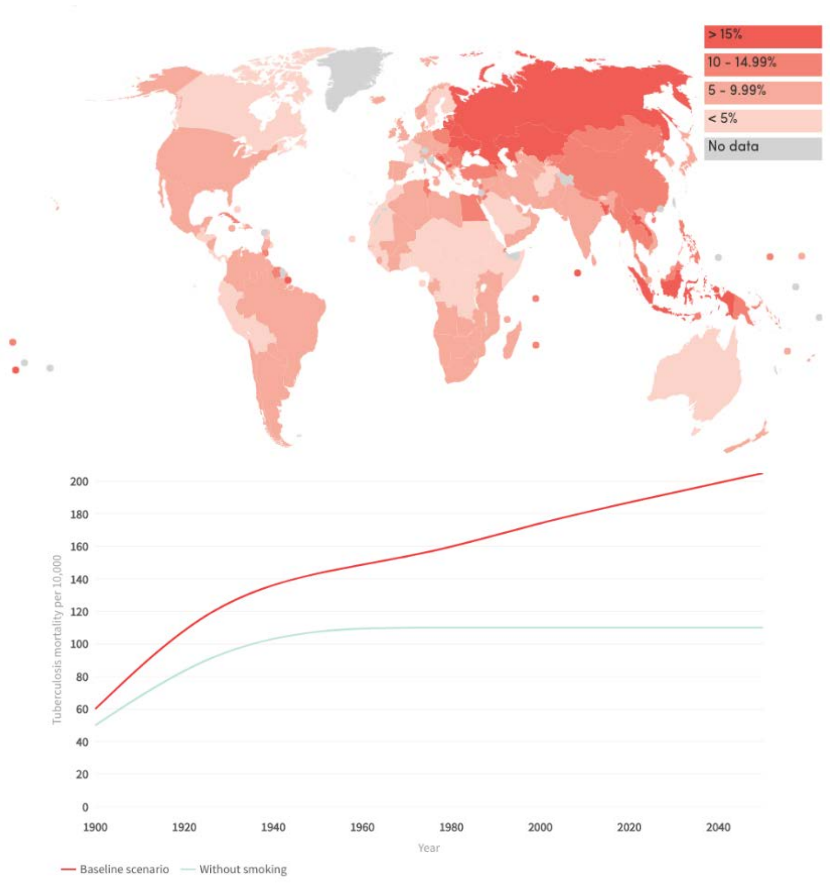


Figure 5. Smoking and tuberculosis mortality. A. Percentage of tuberculosis-related deaths due to tobacco by countries. B. Model graph predicting that smoking will greatly increment TB mortality in the forthcoming years. Image from **[37]**.

Smoking and risk of drug resistance: There is a significant association between smoking and drug resistant-TB, independently on the study design and smoking status **[62]**. In fact, smoking was associated with MDR-TB and even XDR-TB (extensively drug-resistant tuberculosis) **[62]**, being the risk of MDR-TB 1.45 times higher in smokers than in non-smokers. The great importance of these numbers is that about 25% of patients with MDR/rifampicin resistant-TB die because of TB **[1]**.

Similar results have been associated with secondhand or environmental smoke, an increased risk of LTBI and TB disease **[63, 64]**. Furthermore, there is a dose-response relationship between the number of cigarettes that family members smoke daily and the risk of developing TB in children **[49]**. It is plausible that in the future thirdhand smoke (tobacco residue deposited on surfaces) is found to impact TB **[65]**.
Although it is difficult to calculate the TB cases attributable to smoking the numbers range between 7-20% **[26, 58, 66]**; thus, it is advisable to combine smoking-cessation strategies with TB treatment **[67, 68]**. Furthermore, it is crucial in some countries like India, where 40% of TB cases and 50% of TB deaths are due to smoking **[69]**.

1.2.3.1.3 Effects of tobacco on the immune system

In general, smoke modifies lung architecture and interferes with the immune system **[70]**. At a DNA level, effects of CS include epigenetic changes (reversible changes) like histone modifications, DNA methylation and chromatin modifications, and DNA damage (irreversible changes) in form of mutations due to the oxidative stress **[71]**.

Although the inflammatory response is altered, the effect of CS is contradictory, causing both pro- and anti-inflammatory responses, mainly due to the high amount of reactive oxygen radicals present in the smoke. As a result, some metabolic pathways related to inflammation, oxidative stress or apoptosis are activated by smoke **[70]**. Macrophages exposed to smoke show (observing through the microscope) a vacuolated morphology with lysosomal inclusions of tobacco particles. These morphological changes in macrophages are suggested to be related with phagocytic activity, glycolysis rate and cytokine production impairment **[70]**. Exposure to smoke should upregulate the macrophage phenotype M2 (the anti-inflammatory type which repairs damaged tissues) and downregulate M1 (the pro-inflammatory type) as a compensatory response to inflammation, although the response differs depending on the areas of the smoker's lung **[72]**.

CS increases the number of macrophages in the smoker's lungs, increasing the lysosomal enzymes levels, which damage connective tissue and parenchymal cells of the lung. On the other hand, although there are more macrophages, they seem to be impaired in their function to phagocyte and kill bacteria **[41]**.

If innate and adaptive immune responses are modified, cytokine and antibody production are altered. Neutrophils, macrophages and CD8+ T cells modify their cytokine production, such as interleukin (IL)-8 or IL-1β. Aldehydes present in the smoke may play a vital role in the cytokine pattern changes **[73]**. Low levels in secretion of proinflammatory cytokines could be critical for the early responses to host-pathogen interaction and conditionate the susceptibility to TB infection **[41]**. As reviewed by López-Hernández *et al*. **[70]**, nicotine may conditionate the infection process by inhibition of IL-12, TNF-α and IFN-γ, inhibition of the growth of T cells, enhancing the immunosuppressive activity of Tregs, inhibition of autophagosome formation or modifying the migration rate of THP-1. In some studies, it has been observed that acrolein and nicotine impair *M. tuberculosis* control by macrophages. Studies show that macrophages are unable to contain *M. tuberculosis*, so colony-forming units tend to have greater numbers if they have been exposed to smoke **[70, 74, 75]**.

As smoking, e-cigs alter the innate immune response, but in a different way. E-cigs can provoke oxidative stress suppressing cellular defences and lead to DNA damage **[70]**. In general, damage caused by e-cigs seems to be lower than that caused by traditional smoke. However, they have also been shown to have a cytotoxic effect, cause pro-inflammatory response and inhibit phagocytosis **[76]**.

1.2.3.2 Air pollution

Ever since the observations in Paris, between 1858-1902, which concluded that the number of windows per household was related to TB mortality, it was suggested that TB and inadequate aeration of houses were related **[77]**. Association between cooking with biomass fuels, over-crowding or smoking with TB demonstrate that TB and indoor pollution are related **[55]**. Linking outdoor pollution with TB is much less studied. History supports that in the west, TB could be linked to industrialization. When industrialization takes place in a country there is a rise in TB incidence, while when the country switches to cleaner energy, the prevalence of TB decreases **[78]**. However, there could be many cofounders that took place with switching to cleaner energy, like drug administration, vaccines and socio-economic factors **[78]**.

Global environmental pollution may be an indicator that Earth is now on Anthropocene, a new geological era due to human impact [79]. Air pollution is caused mainly by particles in suspension called particulate matter (PM). Although the origin of PM could be natural sources, like volcanic eruptions or fires, PM originated by human activity causes more concern, especially those caused by factories and vehicles. PM size is usually smaller than 10 µm but could be smaller than 2.5 µm, which enables PM to reach easily the alveolus [80]. PM causes cytotoxic effects and generates a proinflammatory response due to the oxidative stress, with an impairment in cytokine production [81]. Furthermore, inflammatory responses could be indirectly due to pollution, by an industrialized microbiota, responsible for many non-communicable chronic diseases, like diabetes, cardiovascular diseases, cancer and chronic respiratory diseases [82]. Additionally, it is demonstrated that air pollution is linked with TB; and in a similar way, lower air pollution is linked with lower pulmonary TB disease rates [83]. Unfortunately, pollution is increasing in areas where TB prevalence is very high [81].

A relation between TB and traffic-related air pollution (TRAP) has been suggested as some of the compounds found in TRAP are also found in CS [84]. Diesel exhaust is one of the major components of TRAP in big cities. *In vitro* and *in vivo* experiments linked exposure to diesel exhaust particles (DEP) of engines with higher mycobacterial burden in infected human peripheral blood monocytes and mice [85, 86]. A clinical study related proximity to traffic with poor TB outcomes [84]. Diesel exhaust particles are probably also related to other epidemics like asthma [87]. Prevalence of asthma in industrialized countries is especially high, and according to WHO (World Health Organization) in 2050 it will affect one billion people [88]. Sulphur dioxides are also associated with an increased risk of TB [77].

1.2.3.3 Other relevant factors

HIV coinfection

Eleven per cent of TB is due to HIV (Human Immunodeficiency virus) and every year more than 300,000 TB deaths are HIV positive people, which correspond to about 18% TB deaths [89]. TB is the most common infection affecting HIV-positive people. It is not surprising that the prevalence of TB and HIV are related: about 72% of HIV infected - TB patients live in Africa [1, 90]. Extension of MDR, caused partially by HIV, is increasing dramatically in Southeast Asia, India, sub-Saharan Africa and South America [91]. HIV

accelerates between 12-20 times the progression from latent to active TB disease, but TB disease also accelerates the progression of HIV infection [92]. HIV and TB co-infected people have less symptoms of TB than negative HIV people with TB, or the signs and symptoms of TB like fevers, malaise or weight loss are supposed to be from HIV itself, which makes TB's diagnosis harder [92].

TB can occur in any moment during the course of HIV infection, and the risk is higher if CD4+ T cells number is low. When administering highly active antiretroviral therapy (HAART) TB's risk is reduced, but TB death continues to be higher than in HIV-negative people [93]. One important consideration is the drug-drug interactions between HIV treatment and rifamycins, key drugs in TB treatment. Rifampicin (RIF) induces cytochrome P450 enzymes, while many HAART inhibit them [91]. Because of public health issues, the priority is to treat TB for at least six months before starting the HAART [91]. When HAART is started, a worsening of TB symptoms (hectic fever, higher pre-existing TB lesions…) could appear. They are not due to failure to control the infection but are related to the restoration of the immune system [91]. In the last years, the percentage of TB patients co-infected with HIV in Europe has increased from 3 to 12% [94].

Diabetes mellitus

Diabetes mellitus triples the risk of developing TB and makes the risk of death, relapse or treatment failure, higher [95]. Seven per cent of TB cases are attributable to diabetes [89]. Even some studies suggest that there could be an association between diabetes and MDR-TB [96]. The association between TB and diabetes is not new; there is a complex interaction between nutrition and TB.

The prevalence of TB among diabetics is 4.1%. Diabetes enhances TB susceptibility via hyperglycemia and cellular insulinopenia and also affecting macrophages function [97]. Diabetes affects chemotaxis, phagocytosis, IFN-γ production and antigen presentation of *M. tuberculosis* [97]. On the other hand, infections, including TB, worsen the symptoms of diabetes. TB could induce hyperglycemia as well due to inflammation. Rifampicin also leads to hyperglycemia status, so the doses should be adjusted [95, 97].

Undernutrition, overcrowding and other related socioeconomic status

Undernutrition increases the risk of TB and TB can lead to malnutrition. Undernutrition negatively affects cell-mediated immunity, which is the first defence against *M. tuberculosis*. It is a risk factor for TB disease, TB death, TB relapse and increases the risk of drug toxicity **[98]**. Before anti-TB therapy, a rich diet in calories, proteins, fats and vitamins was considered of paramount importance in the treatment of TB **[99]**. High prevalence of HIV infection in developing countries further worsens the problem of undernutrition and TB **[99]**. Lack of education, low incomes or unemployment are responsible for an increased risk of TB due to limitations in the use of health care services even in healthy countries. TB rates in homeless people are even 20 times higher than in general population **[100]**. Injecting drug users have high rates of TB incidence due to poverty, unemployment, imprisonment or lack of access to health care centres **[100]**. Overcrowding and poor ventilation increase the risk of exposure **[101]**. Migrants, refugees, cross-border populations and itinerant people like gipsies are in higher risk of TB due to legal impediments to access to health care centres, traumatic experiences, awareness of rights and services provided, stigmatization and other social and economic difficulties **[100]**.

Alcohol consumption

About 10-17% of TB cases are related to alcohol abuse and, although it is difficult to quantify alcohol consumption, alcohol is related to various confounding factors as crowding, malnutrition, homelessness or imprisonment **[95, 101]**. Alcohol drinkers are considered immunocompromised because alcohol is considered to be the causal factor of more than 200 diseases, including TB **[95, 102]**. Alcohol consumption is an independent TB risk factor, at least for men **[102]**, making heavy drinkers more contagious and with more severe manifestations of the disease **[101]**. Furthermore, heavy drinking could act synergistically with smoking, increasing the risk of developing TB **[56]**.

Patients with biological response modifiers treatment

Biological therapy is based on boosting the host's immune system to fight against the pathogen, using interferons or interleukins. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) has recently revised the different types of drugs according to the targeted site or the impact on the risk factor for lung infections **[103]**. Recommendations include the performance of both TSTs and IGRAs,

and depending on the risks, screening for TB and prophylaxis. Drugs that are involved in these recommendations are: anti-TNF-α agents, interleukins (IL-1, IL-5, IL-17), intracellular signal pathways, lymphoid cells surface antigens (Cluster of Differentiation (CD)19, CD20, CD52), immune checkpoints inhibitors, cell adhesion inhibitors, sphingosine-1-phosphatase receptor modulators and proteasome inhibitors **[103]**. Patients with anti-TNF-α therapy (like infliximab, adalimumab, golimumab, certolizumab pegol or etanercept) have twice to four times the risk of developing active TB compared to healthy patients **[25, 104]**. Other drugs with additional risks are antimetabolites, anticalcineurin and corticoids **[105]**.

1.3 Treatment and innovative therapeutics formulations

1.3.1 Drugs against tuberculosis

Drugs against TB are classified in first- or second-line drugs. Based on current treatment guidelines, second-lines drugs could be classified in three additional groups: A, B or C **[106]**. The mechanism of action is summarized in Figure 6.

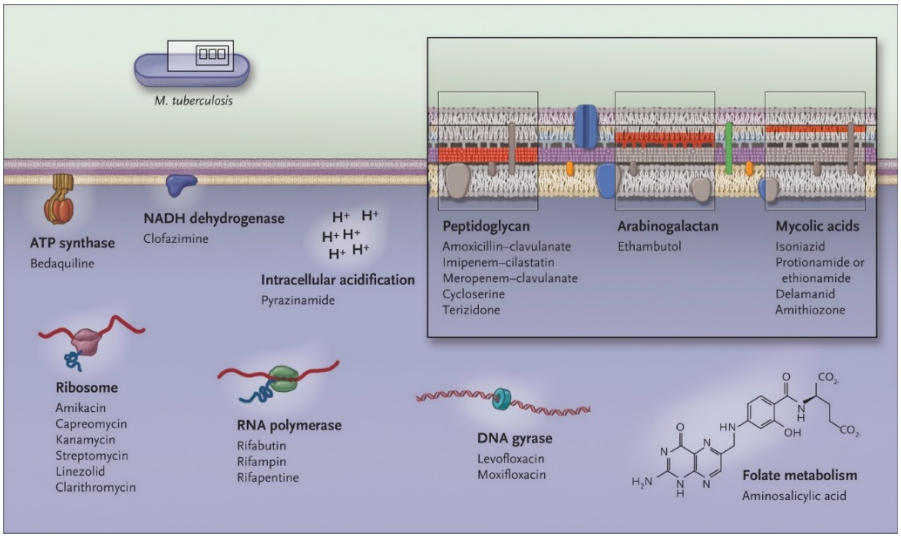


Figure 6. Mechanisms of action of antimycobacterial agents. Illustration from **[107]**.

First-line drugs:

Isoniazid

Isoniazid (INH, isonicotinylhydrazine) is a synthetic antimicrobial, first used clinically in 1952 with a highly specific spectrum, exclusively against *M. tuberculosis*, *M. bovis* and *Mycobacterium kansasii*. It is a pro-drug that needs to be activated by the mycobacterial enzyme KatG, a catalase-peroxidase hemoprotein **[108]**. The metabolites generated produce reactive oxygen species (ROS), such as isonicotinoyl radical. ROS cause damage to many cell components, and isonicotinoyl radical specifically inhibits InhA by covalent binding. InhA is a nicotinamide adenine dinucleotide (NADH)-specific enoyl-acyl carrier protein reductase involved in the mycolic acid biosynthesis pathway. The inhibition of InhA leads to the accumulation of long-chain fatty acids, inhibiting the cell wall synthesis and leading to cell death **[109]**.

It has both bactericidal and sterilizing effect against mycobacteria. In humans, it causes a rapid decrease in sputum bacilli in the first two weeks of treatment **[109]**. The metabolic pathway is primary by the liver. Resistance mutations occur in the target gene *inhA* and in the activating enzyme KatG **[110]**.

Rifampicin

Rifampicin (RIF) interferes with the RNA synthesis by its binding to the β subunit of the RNA polymerase activity, acting early in transcription. RIF does not inhibit the mammalian enzyme. Although it has a wide spectrum, its use is restricted to mycobacterial infections where it is used in combination with other drugs, due to the rapid emergence of resistant bacteria **[109]**. The majority of the mutations that occur on the *rpoB* gene confer resistance not only to RIF but also to many rifamycins **[110]**.

Its metabolism is mainly hepatic and affects some other drugs metabolism. Hepatitis and serious hypersensitivity reactions, shortness of breath, vomiting, diarrhoea or orange-red staining of all body fluids are on the main side effects (Table 1) **[111]**.

Ethambutol

Ethambutol (EMB) inhibits arabinosyl transferases involved in cell-wall biosynthesis, affecting mainly the synthesis of arabinogalactan and lipoarabinomannan **[109]**. Although there are multiple arabinosyl transferases, *embCAB* operon and particular *embB* gene seems to play an important role. Mutations in *embB* or *embA* result in much higher minimal inhibitory concentrations (MICs), although resistances can occur without *emb* mutations. Optic neuropathy and hepatoxicity are the main side effects of this drug **[109, 110]**.

Pyrazinamide

Pyrazinamide (PZA) is a pro-drug with sterilizing activity that only kills non-growing bacteria **[110]**. It is thought that pyrazinamide diffuses to *M. tuberculosis* where is converted by a pyrazinamidase into pyrazinoic acid, which is able to inhibit some functions in *M. tuberculosis* at acid pH, like interrupting fatty acid synthesis, the membrane transport and the membrane energy production **[109, 110]**. Resistance generally happens by mutation on pyrazinamidase gene *pnc*A, although mutations on *rsp*A or *pan*D could confer resistance **[112]**.

Table 1. Common adverse reactions to first-line TB drugs. Adapted from [111].

Caused by	Adverse Reaction	Signs and symptoms
Ethambutol	Eye damage	Blurred vision, changed colour vision
Isoniazid	Nervous system damage Peripheral neuropathy Hepatitis	Dizziness, tingling sensation, numbness, abdominal pain, fatigue, flu-like symptoms, nausea, vomiting, yellow skin
Pyrazinamide	Upset stomach Gout Hepatitis	Upset stomach, abnormal uric acid level, joint aches, abdominal pain, fatigue, flu-like symptoms, nausea, vomiting, yellow skin
Rifampicin	Bleeding problems Discolouration of body fluids Drug interactions Hepatitis	Easy bruising, slow blood clotting, orange urine, sweat or tears, interferences with certain medications, abdominal pain, fatigue, flu-like symptoms, nausea, vomiting, yellow skin

Second-line drugs:

Group A

Levofloxacin and moxifloxacin are quinolones that inhibit ATP-dependent enzymes DNA gyrase and topoisomerase IV. DNA gyrase is an essential enzyme involved in replication, transcription and repair of bacterial DNA. It is coded by *gyrA* and *gyrB*, where the mutations for quinolones resistance are mainly located [109].

Bedaquiline is an inhibitor of F_1F_0-mycobacterial ATP synthase, impeding the oxidative phosphorylation, and has a selective activity against many mycobacteria, including *M. tuberculosis* [113]. It has synergism with other anti-TB drugs. It has bactericidal activity, probably by the collapse of the pH gradient and the dissipation of the proton motive force. Although it is quite a new drug and the first TB drug approved in the last 40 years, resistance strains have already appeared [114].

Linezolid inhibits protein synthesis binding to 23S rRNA preventing the binding of formyl-methionine to tRNA to start the synthesis of peptides [109].

Group B

Clofazimine. The mode of action is not completely understood but it seems to be implicated in membrane perturbations, interfering with electron transport **[109]**. Cycloserine is an analogue of the D-alanine which inhibits alanine racemase and D-alanine ligase, both essential enzymes in the synthesis of peptidoglycan and cell-wall **[109]**. Terizidone is formed with two molecules of cycloserine and one of terephtalaldehyde, and its mode of action is similar to Cycloserine inhibiting the enzymes alanine racemase and D-alanine ligase. Although it is a broad-spectrum antibiotic, it is only recommended for TB treatment **[115]**.

Group C

Group C includes all other anti-TB drugs for the TB treatment when a regimen cannot be composed of drugs from group A and B. For a complete list see Table 2. From previous recommendations, kanamycin and capreomycin have been eliminated because of their association with less effective outcomes, other drugs are no longer available due to the withdrawals or concerns of dysglycemias. Clavulanic acid could be used, but only with carbapenems **[106]**.

1.3.2 Current tuberculosis treatment

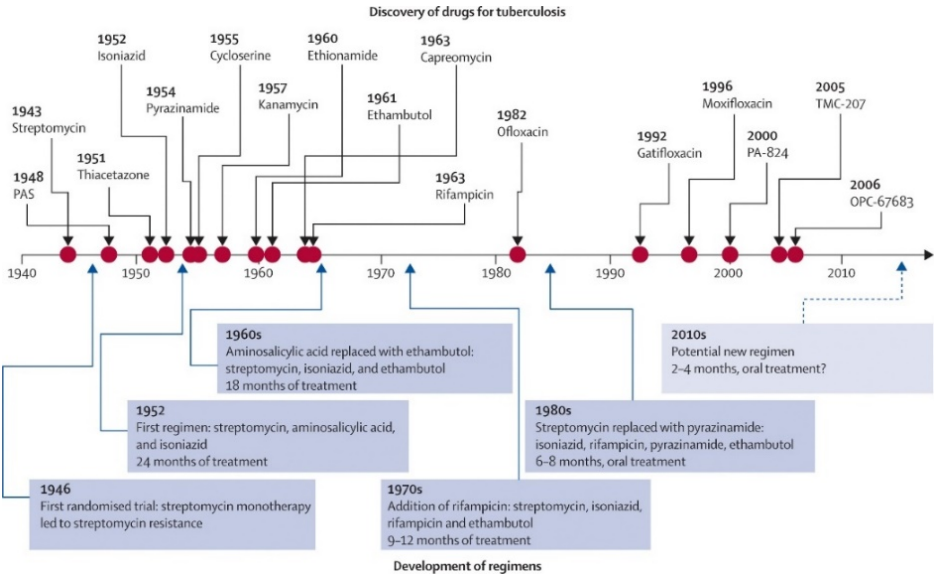


Figure 7. History of drug discovery and development of treatment regimens for TB. Figure from **[116]**.

Treatment of TB started with streptomycin, and it was later combined with *p*-aminosalicylic acid, but this initial treatment had many side-effects and the development of drug resistance was very high (Figure 7). In 1952 isoniazid (INH) was added to the treatment, thus improving the outcome and shortening the treatment to two years.

In the 1960s, ethambutol replaced *p*-aminosalicylic acid and in the 1970s rifampicin (RIF) was introduced, shortening the treatment to 9 months (Figure 7). Addition of pyrazinamide into first-line regimens let optimize the treatment to six months **[117]**. There are three main lines of treatment: for LTBI patients, drug sensible TB patients, and MDR-TB patients (Table 2). Current TB treatment consists on a 2 months initial phase of treatment to kill the active replicating bacilli followed by a 4 months continuation phase of two-drug regimen to eliminate the surviving bacilli (Table 2) **[1]**.

Table 2. Treatment guidelines summary for TB and RR/MDR-TB. Adapted from **[106, 118]**.

	Initial/Intensive phase		Continuation phase	
LTBI			INH	6-9 months
			RIF/rifapentine + INH	3-4 months
			RIF	3-4 months
TB	4 drugs combination: INH RIF PZA EMB	2 months	2 drugs combination: INH RIF/rifapentine	4 months
MDR-TB	At least 5 drugs: 3 Group A 2 Group B Group C: Add to complete when drugs from Group A or B cannot be used	Short regimen: 4 months* Longer regimen: 6-7 months	At least 5 drugs: 3 Group A 2 Group B Group C: Add to complete when drugs from Group A or B cannot be used	Short regimen: 5-8 months* Longer regimen: >12 months

INH: Isoniazid; **RIF**: rifampicin; **PZA**: pyrazinamide; **EMB**: ethambutol **Group A**: levofloxacin or moxifloxacin, bedaquiline, linezolid; **Group B**: clofazimine, cycloserine or terizidone; **Group C**: ethambutol, delamanid, pyrazinamide, imipenem-cilastatin or meropenem, amikacin or streptomycin, ethionamide or prothionamide, *p*-aminosalicylic acid. * Shorter regimen is not recommended to UE countries.

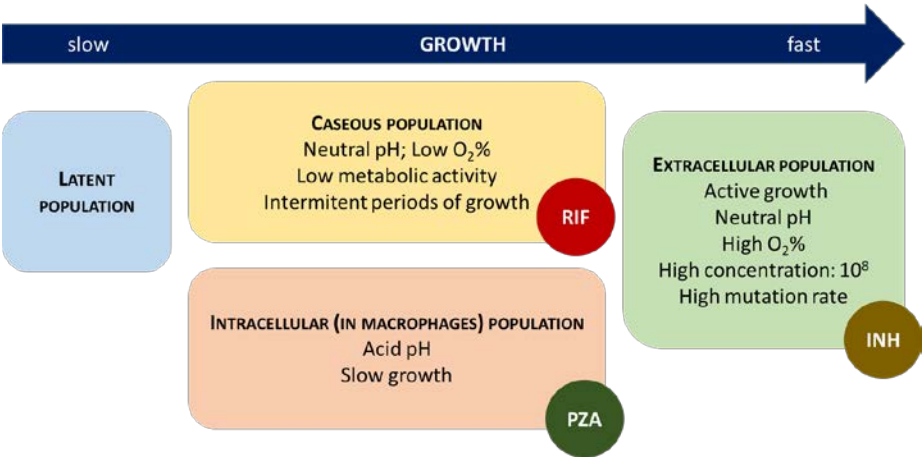


Figure 8. Different bacilli subpopulations and the main drug that interferes with their growth. Latent bacilli are mainly killed by the immune system of the host.

The two-step treatment is due to the active and dormant bacilli, which requires drugs with bactericidal and sterilizing effects. Bactericidal effects consist in killing the high replicating mycobacteria, producing the clinical recovery; sterilizing steps consist in the elimination of the semidormant bacteria **[119]**. Isoniazid and rifampicin are bactericidal, ethambutol inhibits monoresistant strains and reduces the burden and pyrazinamide sterilizes **[119]**. The mode of action is summarised in Figure 6. To prevent the emergence of resistant bacilli, multiple drugs are used simultaneously, thus eliminating the replicating bacilli extracellularly, intracellularly or in caseum lesions (Figure 8). Some factors that could increase the chance of developing drug-resistant TB are: not adhering to the treatment regimen, visiting areas where DR-TB is common or staying in contact with DR-TB patients, having malabsorption of drugs, not improving with the treatment or having a relapse **[111]**.

The therapy will take more than six months if the patient is HIV positive, is pregnant, has MDR/XDR-TB or in some extrapulmonary TB cases **[111]**. The treatment of MDR-TB is longer because it requires more and less effective drugs with a higher tendency to cause adverse reactions.

1.3.3 New approaches for tuberculosis treatment

1.3.3.1 Encapsulated drugs

When free drugs are orally administered, one of the main challenges is to maintain or even to reach, in prolonged treatments, the MIC at the site of infection. Due to

patients' noncompliance, malabsorption, drugs not arriving where the bacilli are, or because drugs are inactivated by pH or enzymes, this sub-therapeutic level could lead to the appearance of resistant strains. Two main kinds of efforts have been done to prevent it: avoiding the oral route to enhance the local concentration of drugs at the site of infection and encapsulating the drugs **[120]**.

Nebulization of TB-drugs, especially aminoglycosides, and administering them via inhalation, can inhibit bacillary growth better than free drugs. However, drug vehiculation seems to be necessary to ensure the macrophages uptake **[120]**.

Drug delivery in carriers seems to be an attractive solution to improve the uptake by macrophages. Some potential advantages are: large area volume ratio and improved bioavailability, minimal side effects, decreased toxicity to other organs, reduction of drug degradation in the gastrointestinal tract, preventing the disruption of commensal microbiota, reduction in the treatment's duration giving high local concentrations and reduction in the dose and frequency, which will improve patients' compliance **[120, 121]**. Besides, carriers could be targeted to deliver the drug specifically to macrophages or could release the drug only when in contact with specific secretions of bacteria **[121, 122]**.

Drug carriers can be classified, according to their size, in liposomes, microparticles and nanoparticles. Liposomes are carriers formed by a closed phospholipid bilayer of a size of about 200nm. Both hydrophilic and hydrophobic drugs can be encapsulated, and they have low toxicity and controllable kinetics **[123]**. The main advantage of this carrier is the high encapsulation efficiency **[120]**. Stabilization of the liposomes to prevent their fusion could be easily achieved by surface modifications with biocompatible polymers like polyethylene glycol (PEG) **[123]**. Despite their large size, liposomes are efficiently taken by macrophages and the efficiency of encapsulated drugs are in principle better than free antibiotics.

Microparticles have the advantage of protecting the drug against pulmonary metabolism and prolonging the drug release. Their size is higher than 100nm.

Nanoparticles (NPs) carriers are less than 100nm. One of the main drawbacks, when they are administered as an aerosol, is that NPs nebulization is very difficult to accomplish because the majority of the dose is exhaled. However, a strategy to solve this problem is to incorporate the NPs in a microparticle. The process is commonly called The Trojan Particle approach **[120]**.

The formulation of NPs could be very different: gold, silver, lipid-based, silica, titanium dioxide, magnetic, polymeric or biological, carbon nanotubes or quantum dots **[124]**. Toxic effects of NPs, especially for the potential to interfere with the immune system, are a major concern, and not only quantity and composition matters, but morphology, size, surface charge, agglomeration state, distribution and route of administration as well **[124, 125]**. For example, the smaller the size, the greater the toxic effect. Clearance of NPs is difficult because of the nano-size, which could result in pulmonary inflammation, fibrosis or lung cancer **[126]**.

The most common types of NPs are synthetic and natural polymers. Poly (lactic-co-glycolic) acid (PLGA) is a synthetic copolymer approved by the FDA (Food and Drug Administration) for therapeutic purposes, which has the advantages of having low systemic toxicity and being biodegradable. Besides, PLGA can easily cross the intestinal barrier **[122]**. Chitosan and alginate polymers are the most common natural polymers used for the synthesis of NPs. Besides the cited PLGA advantages, chitosan has a cationic nature which allows it to act as a permeation enhancer **[126]**. However, natural polymers could trigger inflammation by the immune system.

Another classification attending to the structure group NPs in dendrimers (repetitively branched molecules), quantum dots (semiconductor nanocrystals with a core-shell structure), micelles, polymeric and liposomal. Although, strictly, liposomal formulations are not NPs because the size is higher than 100nm (120-250nm) **[127]**.

More than 30 research articles about NPs for TB treatment were published in 2018 and the number has increased since then. Research is mainly done with first-line TB drugs: isoniazid, rifampicin and pyrazinamide.

1.3.3.2 Host-directed therapies

Host-directed therapy (HDT) is a new treatment concept in which the response of the host against TB is modulated by small molecules. This method exerts less selective pressure against *M. tuberculosis*, decreasing the emergence of antibiotic resistance. Furthermore, it is a promising strategy for MDR-TB and XDR-TB, where no effective antibiotics are available.

Following the classification suggested by Kolloli and Subbian **[128]**, HDT for *M. tuberculosis* could be arranged in five main groups depending on the target: granuloma formation, autophagy, inflammatory response, cell-mediated immune response and addition of anti-*M. tuberculosis* antibodies (Figure 9).

Targeting granuloma formation: Although granulomas can contain the bacilli, they difficult the access of antibiotics, playing a key role for bacterial survival and proliferation. Granuloma formation and its integrity is mainly controlled by TNF-α. Maintaining basal levels of TNF-α contributes to the disruption of granuloma and a higher rate of clearance of *M. tuberculosis* by antibiotics, but can also suppress host immunity **[129]**. There is a high angiogenesis rate in granulomas; so neutralizing Vascular Endothelial Growth Factor (VEGF) enhances the efficacy of antibiotics **[128]**.

Targeting autophagy: Vitamin D_3 upregulates the expression of autophagy-related proteins and induces the production of ROS and antimicrobial peptides. Autophagy-related proteins promote autophagolysosome formation, thus restricting *M. tuberculosis* growth. Some studies have shown different responses in the addition of

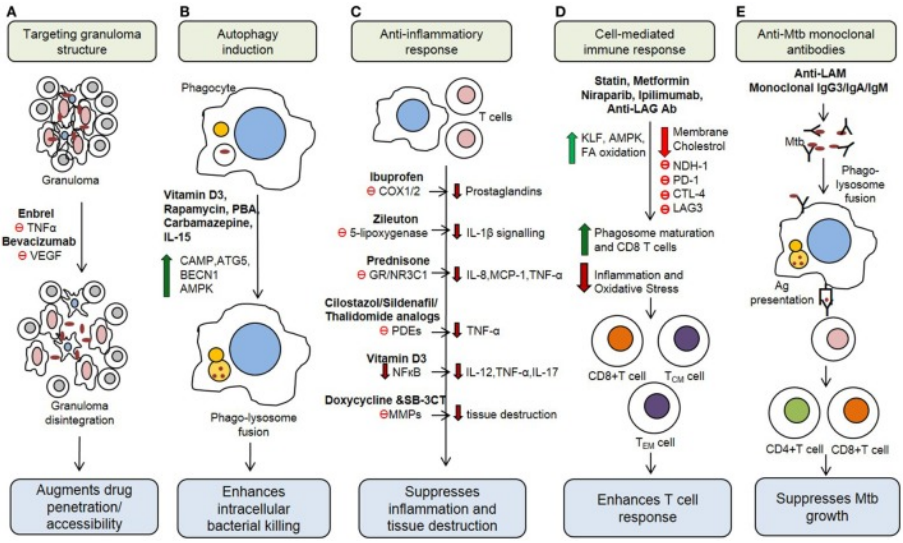


Figure 9. Potential host therapeutic targets against *Mycobacterium tuberculosis*. (A) Host-directed therapeutic (HDT) drugs change the integrity of granuloma and enhance drug accessibility. **(B)** Some HDT agents upregulate production of antimicrobial peptides, reactive oxygen and induce autophagy in infected cells. **(C)** HDT drugs suppress proinflammatory responses, which decrease inflammation and tissue damage during the active stage of the disease. **(D)** HDT agents regulate cell-mediated immune responses, including antigen-specific T cell responses. **(E)** Monoclonal antibody administration is other emerging HDT concept for TB treatment. VEGF, vascular endothelial growth factor; PBA, phenylbutyrate; CAMP, cathelicidin antimicrobial peptide; ATG5, autophagy-related protein 5; BECN1, beclin-1; AMPK, AMP-activated protein kinase; COX1/2, cyclooxygenase-1/2; GR, glucocorticoid receptor; PDE, phosphodiesterases; MMPs, matrix metalloproteinases; KLF, Kruppel-like factor; PD-1, programmed cell death 1 receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; LAG3, lymphocyte activation gene 3; LAM, Lipoarabinomannan. Figure and text from **[128]**.

D_3, suggesting that polymorphisms in genes associated with D_3 can influence the outcome of TB treatment **[128]**.

Anticonvulsant drugs, such as carbamazepine, or inhibitors of virulence factors like *Mycobacterium* protein tyrosine phosphatase (Mptp)B can stimulate autophagy through the depletion of PI3P (phosphatidylinositol-3-phosphate) and AMP-activated protein kinase activation **[128, 130]**.

Targeting the inflammatory response: An uncontrolled pro- or anti-inflammatory response could lead to a bad outcome against *M. tuberculosis*. Acetylsalicylic acid regulates the inflammatory pathology suppressing neutrophil migration and TNF-α production. Prostaglandin and thromboxane as vasoconstrictors facilitate platelet aggregation **[128]**. Ibuprofen in conjunction with antibiotics could reduce the inflammatory pathology and the bacterial burden **[131]**. Corticosteroids can control exacerbated inflammation. Inflammation can also be controlled inhibiting phosphodiesterases activity. Inhibition of the matrix metalloproteinases expressed by *M. tuberculosis* to degrade the pulmonary extracellular matrix and generate tissue damage and cavitation is another promising strategy **[128]**.

Targeting cell-mediated immunity: It is a crucial part against the TB fight inside the body, and many alternatives have arisen to modulate the adaptative immune response. One of the most promising HDT is statin. Statin, a drug known for reducing LDL cholesterol levels, inhibits inflammation and reduces tissue damage and the bacterial load. The reduction in viable *M. tuberculosis* is achieved by decreasing cholesterol levels in the membrane, which promotes the maturation of the phagolysosome and host autophagy **[132]**.

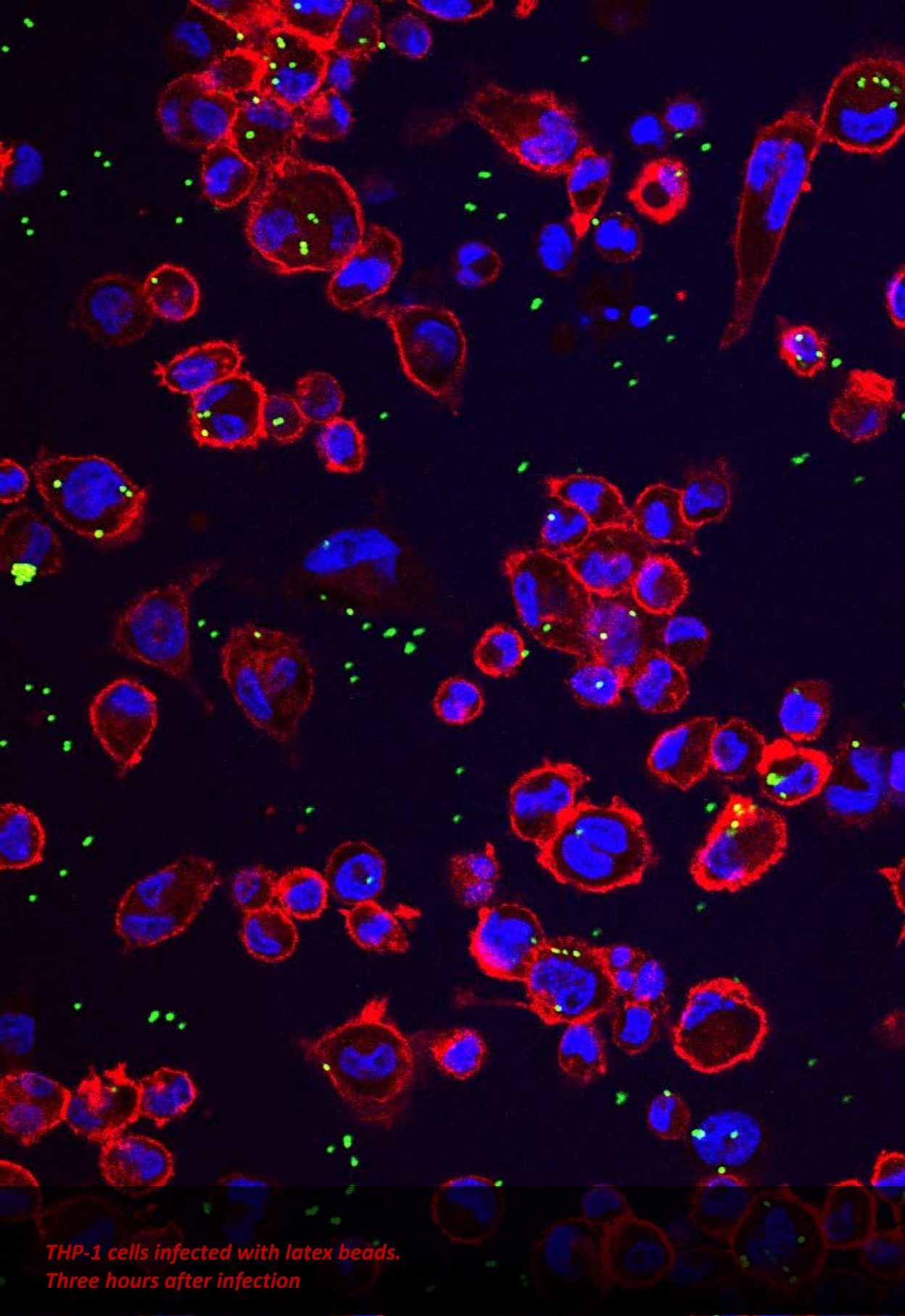
Another strategy is the supplementation with cytokines, like IFN-γ, a key cytokine activating the innate immune response. Supplementation of IFN-γ via subcutaneous injection or via aerosol in conjunction with anti-TB drugs has improved the results of the treatment in cavitary patients. Studies with IFN-α and IL-2 have not found clinical improvements, suggesting the complex roles of cytokines in regulating the immune functions **[128]**.

Antibodies against M. tuberculosis: Addition of FcR or anti-lipoarabinomannan antibodies enhance the maturation of the phagosome, improving the treatment and preventing the reactivation of TB. Antibodies can be used alone or in conjunction with current therapy, and they seem a good choice for patients in an immunocompromised

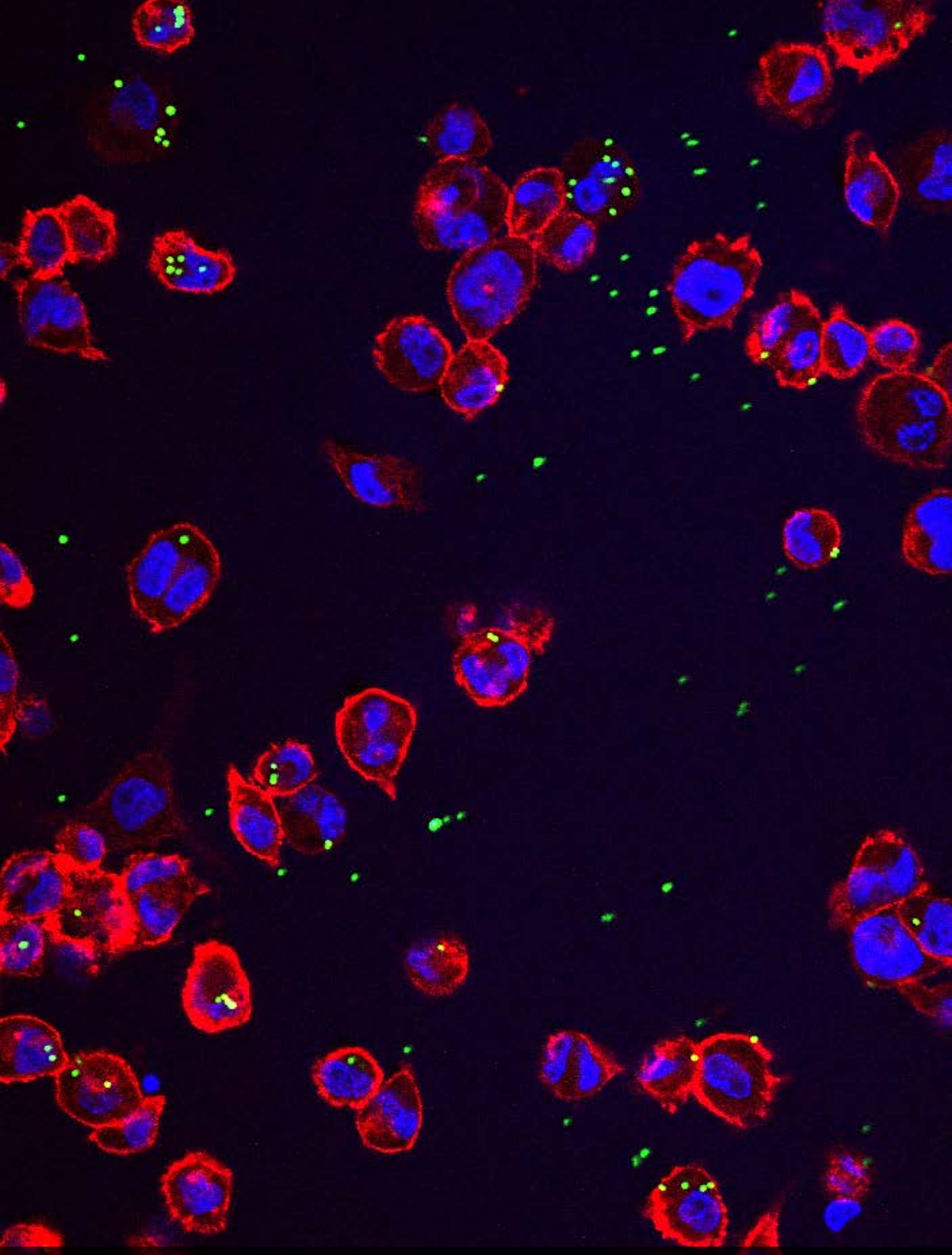
state **[128]**. Studies adding IFN-γ and Immunoglobulin A against *M. tuberculosis* showed promising results preventing *M. tuberculosis* infection **[133]**. However, one of the main drawbacks of using antibodies is the elevated cost.

There are many HDT promising candidates and further investigation is needed to use them for MDR-TB cases or to use in combination with current therapy to reduce the duration of the treatment, reduce drug toxicity, prevent the emergence of resistance and/or improve lung function **[128]**.

Recently, the relationship between microbiome and TB has been questioned. It has been observed that *M. tuberculosis* causes alterations in the microbiota, being variable among studies and usually of minor magnitude. However, anti-TB drugs alter the microbiota in long-lasting changes **[134]**. It is speculated that differences in the microbiome may identify individual resistance to initial infection or control LTBI, which could be used to prevent TB disease **[134]**. Further research is needed to answer these questions.



THP-1 cells infected with latex beads.
Three hours after infection



2. JUSTIFICATION

Despite all efforts in the fight against TB, it continues being a global pandemic with high prevalence and mortality. In fact, we are now very far from the End TB strategy of the WHO for 2030, as more than three people die every minute. In order to eradicate the disease, many efforts are being carried out to develop TB diagnosis methods with the hope to reduce the incidence. Furthermore, because not all patients have the same response, it is evident that the interaction host-pathogen plays a key role in the disease's outcome. Thus, many studies have evaluated the host's immune system role.

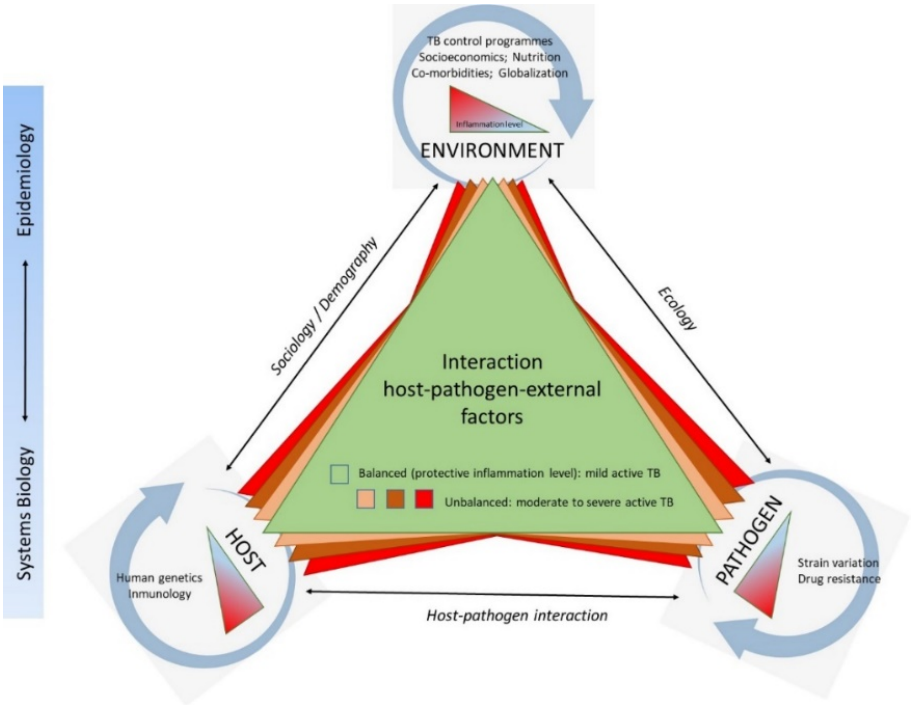
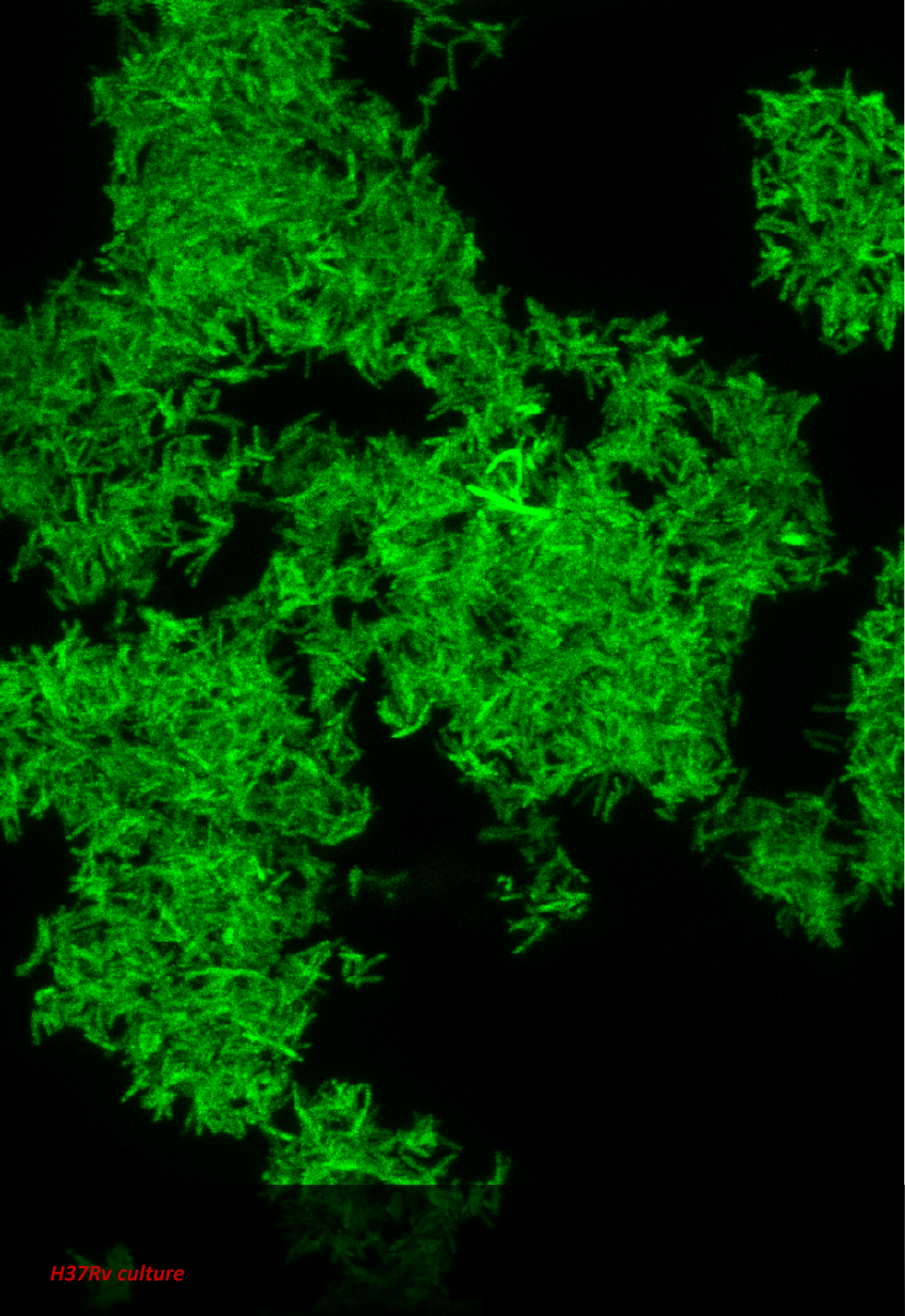


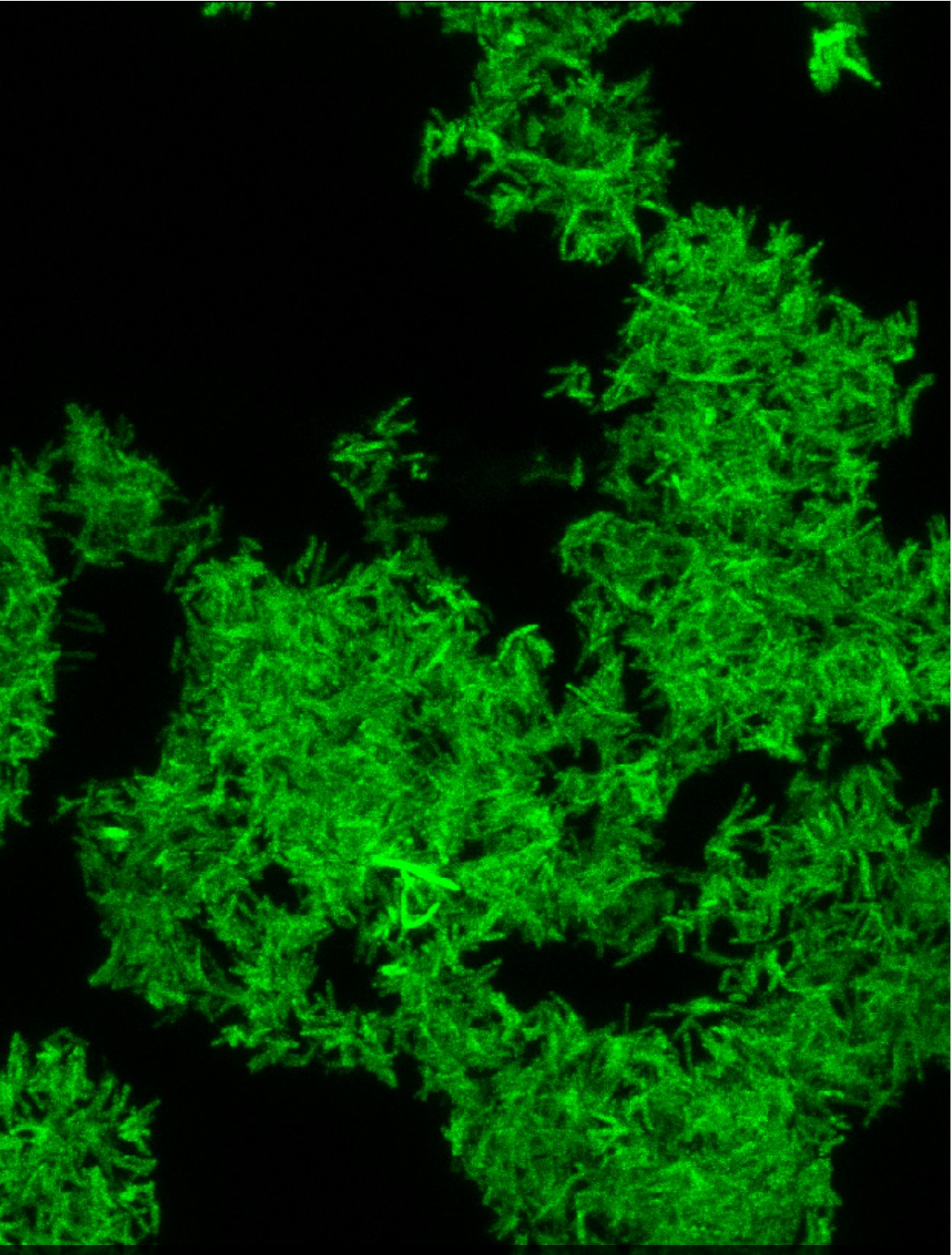
Figure 10. A systems epidemiology approach for TB research integrating the interaction between human host, *M. tuberculosis* pathogen and external factors. The balance between the three factors determines the inflammation level of the host and the final outcome of TB disease. Unbalanced immune responses leads to more severe active TB disease. Figure created from **[19, 31]**.

Recent evidence shows that, although the host's response is defined by the pathogen and the host itself, the environment and all the external factors, where host and pathogen are, play a crucial role in this interaction (Figure 10). The air quality is related to the health of the lung; but air pollution and diesel exhaust fumes are a great problem in big cities, especially in areas where TB incidence is high. Another ambient pollutant is smoke. Smoking is a leading factor for TB epidemic. Despite the vast clinical and epidemiological evidence, the reason why smoke affects TB outcome is not well

understood. E-cigs are marketed by Tobacco Companies as smoking cessation devices, and the sales of e-cig have dramatically increased in the last years. However, their long-term efficacy and safety have not been demonstrated. Evidence indicates that e-cigs are not only water vapour. Electronic vapour could also have an impact on TB, as cigarette smoking does.

On the other hand, another big challenge to control TB is the appearance and transmission of MDR- and XDR-TB. Patients' noncompliance, retreatment or subtherapeutic doses of drugs, among other factors, lead to multidrug resistance. The pipeline of drugs is very limited and it is mandatory to find new therapeutic options. One strategy is the vehiculization of the current drugs in nanocarriers. Nanoparticles have *a priori* many advantages in comparison with free antibiotics and many different compounds are available for the synthesis of these NPs, from silica to biopolymers. Nanoparticles gradually release the drug inside of macrophages, where bacilli are, potentially reducing the doses of drugs and side effects. Another promising strategy is to combine the drugs with HDT, trying to balance the immune response (Figure 10). This approach has the advantage of avoiding a high selective pressure in favour of drug resistance appearance.


H37Rv culture



3. OBJECTIVES

1. To study the effect of environmental pollutants and the outcome of TB disease.

    1.1 To summarize the interaction between air quality and the susceptibility to respiratory diseases, including TB **(Chapter I)**.

    1.2 To evaluate the impact of an environmental pollutant, diesel exhaust particles, on the persistence of *M. tuberculosis* within macrophages **(Chapter II)**.

2. To evaluate the effects of cigarette smoke and e-cig vapour in a cell culture infected with *M. tuberculosis*.

    2.1 To evaluate the ability of *M. tuberculosis* to penetrate and grow within macrophages in the presence of cigarette smoke or e-vapor and to measure the production of cytokines in these conditions **(Chapter III)**.

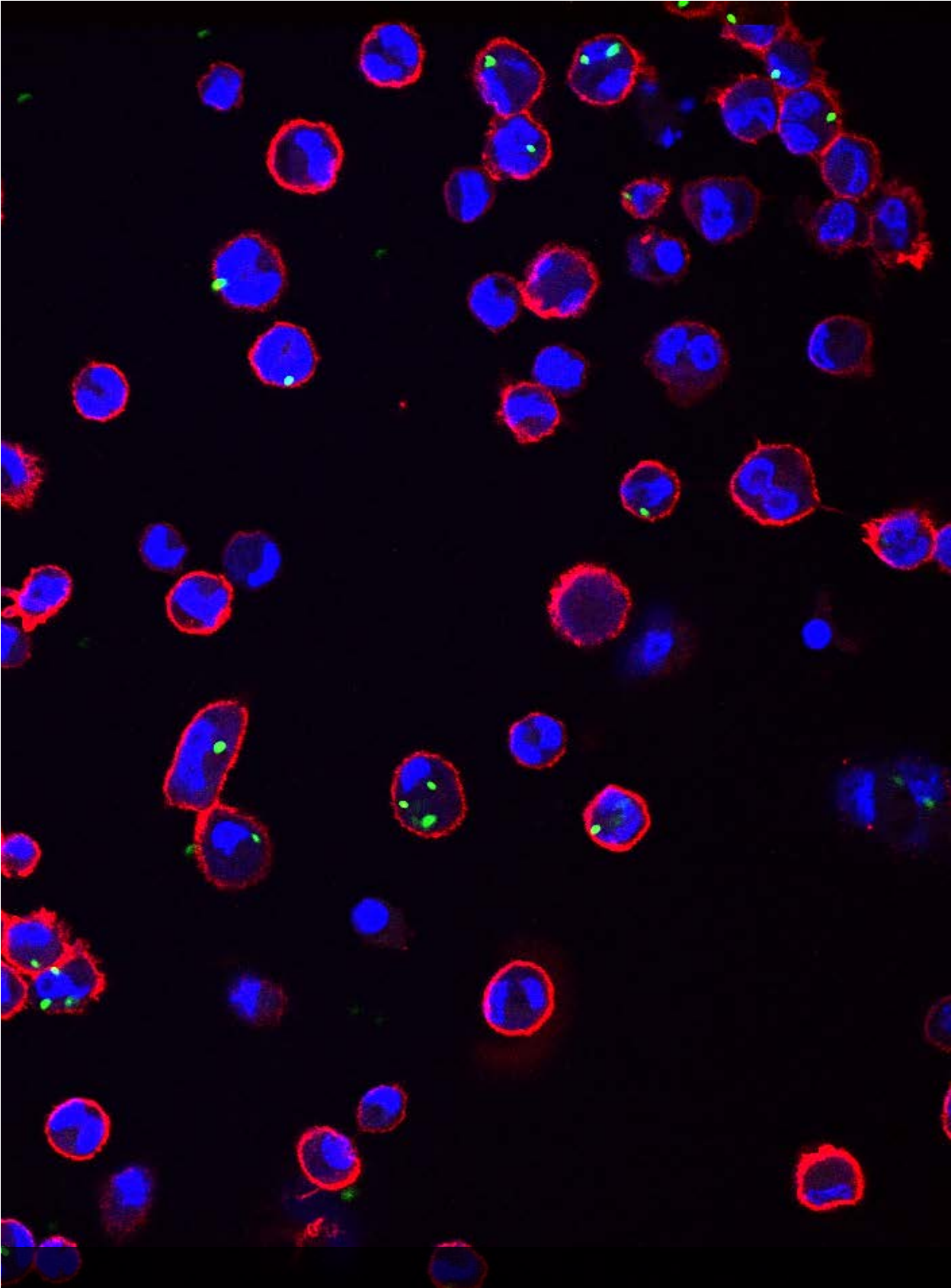
    2.3 To evaluate the effect of cigarette smoke and e-vapor on the metabolites of intra- and extracellular media of macrophages infected with *M. tuberculosis* **(Chapter IV)**.

    2.2 To evaluate the effect of cigarette smoke in macrophages infected with *M. tuberculosis* when anti-tuberculosis drugs are administered **(Chapter V)**.

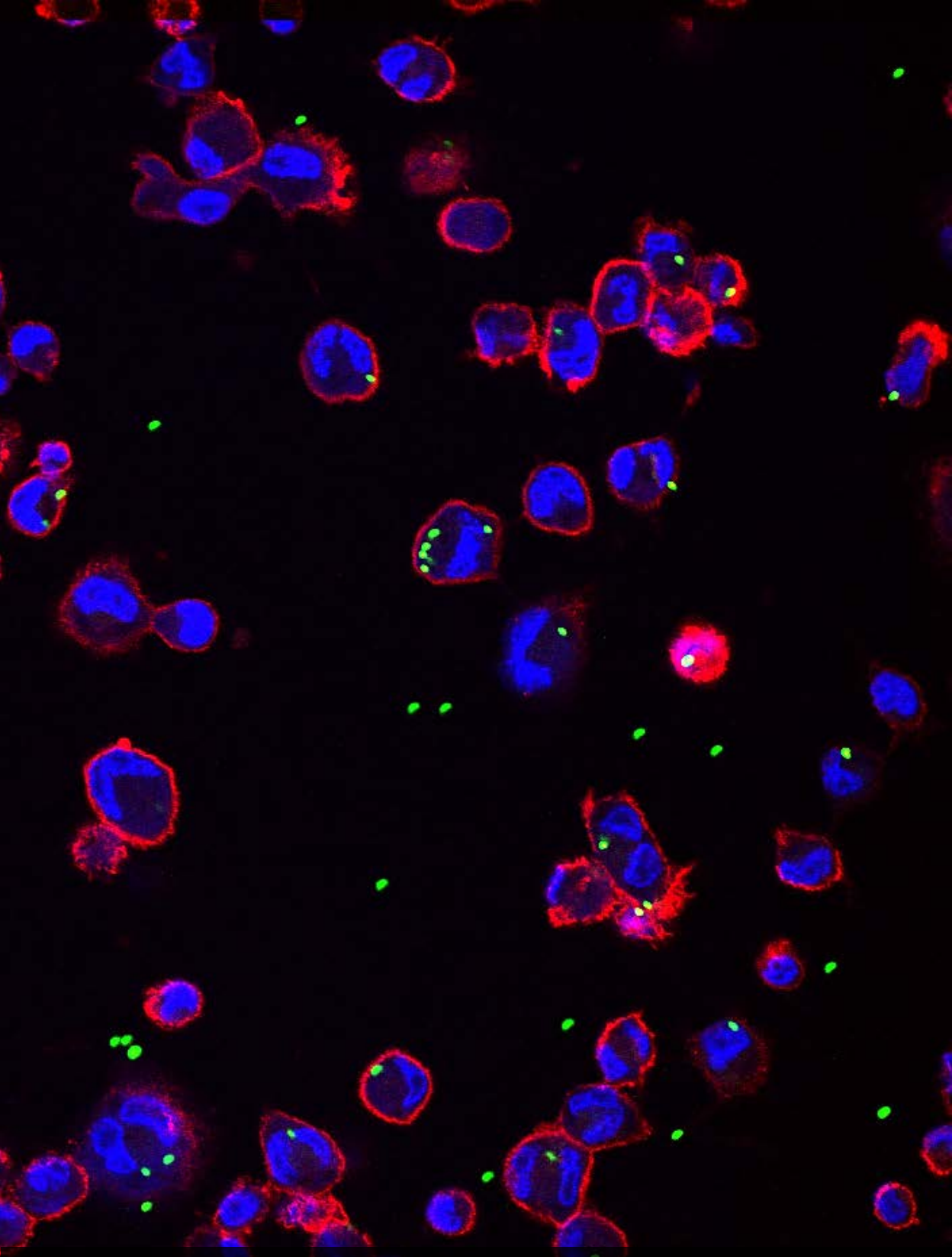
3. To evaluate novel therapeutic options against TB.

    3.1 To evaluate the effect of new drugs encapsulated in nanoparticles on infected *M. tuberculosis* macrophages **(Chapters VI & VII)**.

    3.2 To evaluate the effect of an inhibitor virulence factor of *M. tuberculosis* in infected macrophages treated with rifampicin and the possible interaction between cigarette smoke and the inhibitor **(Chapter VIII)**.



THP-1 cells exposed to cigarette smoke infected with latex beads.
Day 6 after infection



4. RESULTS

4.1 Chapter I: Interaction between Environmental Pollution and Respiratory Infections

The exposome is defined as the measure of all exposures of an individual during his or her lifetime and how these exposures affect health **[1]**. Humans are exposed to large quantities of compounds by inhalation, and the change from rural lifestyles to living in overcrowded, industrialized cities, together with the mass use of motor vehicles means that we are exposed to large amounts of contaminants via the respiratory tract. It is estimated that only 1 cubic centimetre of city air contains approximately 100 bacteria and around 10^7 small-diameter particles (less than 300 nm) **[2]**. In fact, we now know that the outcome of an infection depends not only on host- and pathogen-associated factors, but also on key external factors. For example, environmental changes influence the flyways of migratory birds, which are vectors of the virus influenza A, modifying the spread of new variants of the influenza virus **[3]**.

The major environmental pollutants are basically particles in suspension, known as particulate matter (PM), and include metals or silica, volatile organic compounds, and gaseous pollutants, such as ozone, sulfur dioxide, nitrogen monoxide and dioxide, and carbon monoxide. They are generally produced naturally by sand storms or volcanic eruptions, or by humans, in the form of biomass burning, traffic emissions, mining, and farming. In large cities, however, most pollution is caused by the combustion of diesel engines **[4]**.

It is estimated that 7 million people die every year as a result of environmental pollution **[5]**. Poor air quality is a risk factor for the development of numerous respiratory diseases, such as asthma, lung cancer, and respiratory infections, especially in children **[6]**. Respiratory infections cause the death of more than 4 million people annually. In Europe, almost half a million people die every year due to high concentrations of PM, 78,000 die from exposure to nitrogen dioxide, and more than 14,000 die from exposure to ozone **[7]**. Exposure to high levels of sulfur dioxide has been associated with an increase in sputum production, chronic cough, and bronchoconstriction, and more frequent bronchiectasis exacerbations **[8]**.

Environmental pollution particles cause inflammation, airflow changes, and altered defence mechanisms, both in the upper and lower respiratory tract **[9]**. Larger PMs can be eliminated by mucociliary transport, but the smaller ones can reach the bronchioles. In addition, $PM_{2.5}$ (with a diameter of less than 2.5 µm) that are not phagocytized by macrophages can spread systemically.

Exposure to PM increases susceptibility to bacterial pneumonia and viral respiratory infections [10]. Exposure to environmental pollution in children affects the proper functioning of the respiratory system, especially in the early years of life, when the respiratory and immune systems are not yet fully developed. There is an association between bronchitis and environmental pollution in children under five years of age [11]. In fact, prenatal exposure to $PM_{2.5}$ increases susceptibility to respiratory infections (bronchitis and pneumonia), as many environmental toxins can easily cross the placenta (especially $PM_{0.25}$ and smaller) and accumulate in the fetus in higher concentrations than in the mother [12]. In the elderly, immunosenescence might contribute to an increased propensity to respiratory infections, due, among other factors, to cytokine deregulation [12].

PM can cause oxidative stress by eliminating antioxidants and producing reactive oxygen species (ROS), which leads to an inflammatory response by producing cytokines IL-6, IL-8 and TNF-α [2]. Moreover, exposure to PM can alter the ability of macrophages to inactivate viruses, lyse bacteria, or inhibit the presentation of antigens [10].

Exposures to different nitrogen oxides can increase the risk of respiratory infections, affecting T cells and NK cells, which play important roles in the defence against viruses [4]. An accumulation of metals may also be a risk factor for infections since metal availability is generally a factor promoting the growth of microorganisms. In *Mycobacterium tuberculosis* or *Neisseria meningitidis*, an increase in the availability of iron in the host is related to more serious infections [10].

Tobacco smoke also causes exposure to high concentrations of PM, 15,000–40,000 µg PM per cigarette. In general, tobacco smoke exposure increases the risk of prolonging viral respiratory infections or developing bacterial infection by *Streptococcus*, *Legionella*, *Mycoplasma*, or *Haemophilus* [10]. It is also related to an increased susceptibility to infection by *M. tuberculosis*, and progression to active tuberculosis [10]. In cell cultures, we have observed that smoke negatively affects the phagocytic capacity of macrophages [13]. Exposure to tobacco smoke has also been linked with a delay in the negativization of mycobacterial cultures in treated patients [14]. In fact, exposure to smoke not only alters the immune response of the host but also has an impact on the microorganism, modifying its phenotype toward other more virulent variants [15].

Environmental pollution increases the rate of respiratory infections and is associated with increased costs and premature deaths in chronic patients and children. Some of

the causes can be acted upon, such as tobacco exposure and some aspects of environmental pollution. An understanding of how pollution affects the immune response to respiratory infections would help us establish effective epidemiological strategies, and improve clinical treatment and patients' quality of life.

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4.2 Chapter II: Impact of diesel exhaust particles on infected THP-1 macrophages with *Mycobacterium bovis* BCG

ABSTRACT

A link between pulmonary tuberculosis and the quality of the air has been suggested. Diesel is one of the main contributors to pollution and it is reported to be able to modify susceptibility to lung infections. In this study, we exposed THP-1 cells and *Mycobacterium bovis* BCG to diesel exhaust particles. High cytotoxicity was found on THP-1 cells at 3 and 6 days, but no effect was found on the growth of *M. bovis* BCG. Infection of THP-1 cells exposed to a non-cytotoxic diesel exhaust particles concentration showed no changes in intracellular bacteria burden but higher extracellular recoveries, suggesting no phagocytosis impairment but bad contention of *M. bovis* BCG. Unravelling the links between air pollution and impairment of human antimycobacterial immunity is vital, since pollution is rapidly increasing in areas where tuberculosis incidence is really high.

INTRODUCTION

Tuberculosis (TB) is a global infectious disease that continues being one of the main leading causes of death, causing more than 1.5 million deaths per year **[1]**. Although TB could be a disease in virtually any part of the body, the majority of patients have pulmonary TB. A link between pulmonary TB and the quality of the air has been suggested in many studies **[2-5]**. Air pollution is increasingly becoming a cause of concern. Ambient particulate matter, household air pollution and ozone are responsible for the death of more than six million people every year **[6]**. In Europe, 80% of the people live in places where particulate matter (PM) exceed levels of WHO air quality guidelines **[7]**. One main contributor for urban contamination are diesel exhaust fumes; so, operators of excavators, cranes, forklift trucks, workers of bus depots, car mechanics, railwaymen or miners, are especially exposed **[8]**. Occupational exposures are an important risk factor for chronic obstructive pulmonary disease (COPD); nevertheless, pollution is not restricted to them, since diesel exhaust is dispersed by transport vehicles like buses, cars, railway engines and especially trucks, resulting in air contamination of urban environments **[9]**. The conclusion of a study

conducted in California is that living near dense roads, as a measure of traffic-related air pollution, could be a risk factor for poor TB treatment outcome **[10]**.

One of the major traffic-related air pollutants is diesel exhaust. Although diesel engines produce much less carbon monoxide than petrol engines and diesel engines have a high efficiency in combustion (nearly 98%), the incomplete combustion generates lots of toxic compounds like carbon oxides, nitrogen, sulfur compounds and aromatic hydrocarbons **[9]**. Apart from the fumes (gas, vapors and semi-volatile organic and inorganic compounds), diesel engines emit small solid particles, especially when the motor is not warmed-up **[11]**. Furthermore, exhaust gas could condensate and form ultrafine particles, that could be grouped and be aggregated to 15-40 nm particles. Because the particles are of small size, they get very easily into the respiratory tract, where they could reach the alveoli **[9]**.

The PM of diesel fumes are called diesel exhaust particles (DEP) and include a variety of organic compounds, including quinones, polycyclic aromatic hydrocarbons (PAH) and nitro-PAH carcinogenic compounds **[12]**. DEP have a core of 10-30nm, but as the fumes, they can agglomerate and form aggregates of diameters of 60-200nm, with the black appearance **[13]**. The level of air pollution and increased adverse health effects are related, but DEP have a higher oxidative potential than biomass burning **[14]**. DEP are able to modify the susceptibility to lung infections, suppress the production of cytokines and inhibit macrophage function **[5, 15]**. Because DEP are ultrafine particles and are important compounds of the urban air pollution, they can accumulate in the lungs. Accumulation of DEP are associated with various respiratory diseases **[16]**. Since *M. tuberculosis* can survive in macrophages of the lungs, and the contention and elimination of the disease depends on the immune status of the host, it is of great importance to know whether DEP could influence the outcome of *M. tuberculosis* infections **[17]**. Levels of SO_2 are associated with a 7% increment risk in TB incidence **[18]**. TB and air pollution are related both in short- and long-term exposures **[2, 19]**. Countries with high diesel consumption like India or China have a high incidence of TB **[1, 20]**. DEP are associated with decreasing host defence against non-typeable *Haemophilus influenzae* and by modulating stress responses **[21]**. In this study, we evaluate the impact of DEP exposure on mycobacterial infections.

MATERIALS & METHODS

THP-1 cell line and mycobacteria growth conditions

The human monocytic cell line THP-1 (ATCC TIB-202™) was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMax (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, penicillin 10,000 units/ml, streptomycin 10 mg/ml and Fungizone® 25 μg/ml. The cells were passaged every 3 days. For the experiments, RPMI without antibiotics was used. THP-1 monocytes were stimulated to macrophages using 0.1 μM of Phorbol 12-Myristate 13-Acetate (PMA, Sigma, St Louis, USA) for 72 hours (37°C in 5% CO_2). RPMI was replaced by fresh medium 24 hours before the experiments.

Mycobacterium bovis BCG strain was grown in Middlebrook 7H9 supplemented with 0.05% tween 80, 0.08% of glycerol and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). Cultures were incubated at 37°C without agitation.

Preparation of diesel exhaust particles

DEP (Standard Reference Material (SRM) 2975) were purchased from the National Institute of Standards Technology (NIST) (Gaithersburg, MD, USA). The reported mean diameter of these particles was 11.2 ± 0.1 μm by area distribution, and the surface area, as determined by nitrogen gas adsorption, was 0.538 ± 0.006 m^2/cm^3. DEP were kindly provided by doctor Cruz M.J. of Pulmonology Service, Hospital Universitari Vall d'Hebron, Barcelona.

DEP (5 g/l) were prepared in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA). DEP were then exposed to ultrasonic irradiation for 20 minutes and agitated vigorously. Once prepared, they were stored frozen at -20ºC.

Cytotoxicity Assay

To determinate the cytotoxic effect of the DEP on THP-1 macrophages, viable cells were counted with trypan blue in an optic microscope. THP-1 monocytes were seeded at a concentration of 3×10^6 cells per well in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA) using RPMI containing PMA. The plates were incubated for 72 h, and then the medium was changed (37°C in 5% CO_2 atmosphere). Different concentrations of DEP (0-50 mg/l) were added to the wells and cells were incubated for 24 hours, 72 hours and 6 days before counting. Fresh medium was added

at 72h (with DEP) for the wells used at day 6. The results are expressed as viability percentage using cells untreated as a control.

M. bovis BCG growth curve

M. bovis BCG was adjusted to OD_{600nm} 0.01 in 7H9-tween medium supplemented with 0.05% tween 80, 0.08% of glycerol and 10% albumin-dextrose-catalase (ADC), with DEP (6 or 25 mg/l) or without. The absorbance was measured every 24 hours for two weeks.

Macrophage infection with _M. bovis_ BCG

THP-1 macrophages were infected following a protocol previously reported **[22]**, with some modifications. Briefly, 3×10^5 cells per well were PMA stimulated and seeded in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA)). Temperated DEP (6 mg/l) were added to the cells and incubated for 3 hours. For the infection, mid-log phase *M. bovis* BCG was washed twice with DPBS+0.05% tween and subsequently once with DPBS after which they stood for 5 min, before the supernatant was collected. The bacteria were then diluted in RPMI with DEP or not and added to the THP-1 macrophages at a multiplicity of infection (MOI) of 10. After 3 h of contact at 37°C in 5% CO_2, the macrophages were treated with 200 μg/mL of amikacin for 1 h and washed three times with DPBS to eliminate any extracellular bacteria. Lastly, 1 mL of RPMI with DEP was added to each well and incubated at 37°C in 5% CO_2. Fresh medium with DEP was added at day 3. Extracellular growth was assessed with serial dilution in PBS-tween plating onto Middlebrook 7H10 solid medium at days 0, 1, 2, 3 and 6. Intracellular growth was assessed by lysis of the monolayers by the addition of 500 μL of water followed by a 30 min incubation at room temperature and then plating on 7H10 medium. Colonies were counted after 3–4 weeks incubation at 37 °C and the average CFUs/mL determined.

Statistical analyses

Statistical analyses were performed using the GraphPad PRISM 6.0 software package (San Diego, California, USA). Differences between treatments were compared by the student's t-test. A *p*-value ≤0,05 was considered as a statistically significant.

RESULTS & DISCUSSION

We evaluated a range of DEP concentrations (6.25 to 50 mg/l) to determine the maximum non-cytotoxic dose in THP-1 macrophages, since 50 mg/l is the most frequent value found in the literature **[23, 24]**. DEP were dissolved in RPMI medium and macrophages were exposed for 6 days. Cell viability was assessed by trypan blue exclusion test at days 1, 3 and 6. After 24h of exposure, viability of cells exposed to 25 mg/l and 50 mg/l DEP decreased, but not the viability of cells exposed to concentrations of 12.5 mg/l or lower (Figure 1). However, at days 3 and 6, the viability of THP-1 was inversely correlated with the dose of DEP (Figure 1). Similar values showing high cytotoxicity were already reported **[25]**. We concluded that 6.25 mg/l DEP was a concentration that could be used in the experiments since THP-1 viability was in all days over 80% (Figure 1). Additionally, we observed that DEP tend to form aggregates and were phagocytized efficiently by macrophages (Figure 2). High internalization of DEP by macrophages was already described (engulfment over 99%), as well as rapid sedimentation of DEP **[16]**. DEP deposition on lung phagocytes has also been observed in mice **[11]**.

On the other hand, we evaluated the impact of DEP on *M. bovis* BCG growth. Bacterial cultures were exposed to DEP concentrations for two weeks and OD_{600nm} was measured every 24 hours. Growth of *M. bovis* BCG was not affected by the presence of DEP in the medium (Figure 3). *M. bovis* BCG was also exposed to a higher concentration of DEP in the medium (25 mg/l), and no significant change in the growth was observed (Figure 3).

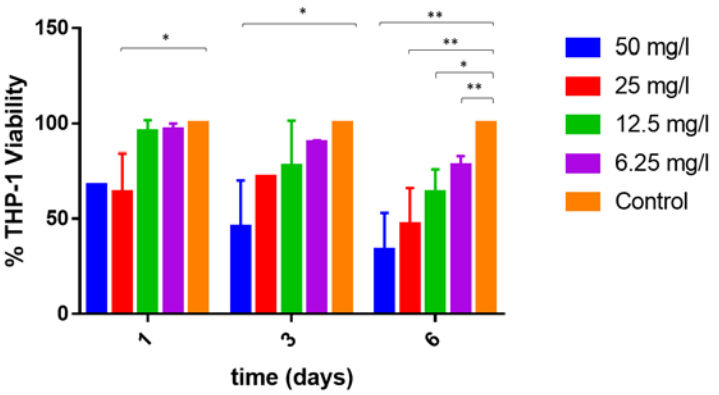

Figure 1. THP-1 viability after exposition to different DEP concentrations. Trypan Blue exclusion test was performed at 1, 3 and 6 days of exposure to DEP. The results are expressed as the average and standard deviation of two replicates of at least two independent experiments. *P<0.05 **P<0.01

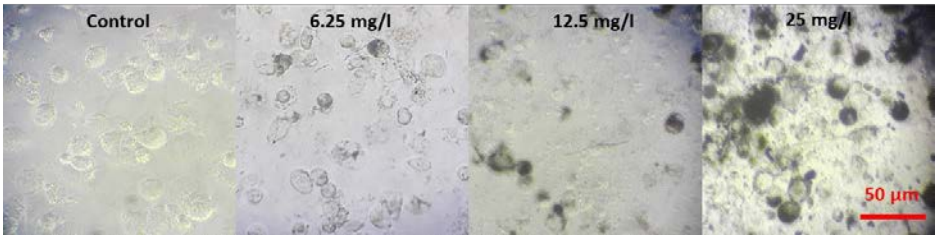

Figure 2. Optical microscope images of macrophages exposed to different DEP concentrations. THP-1 were visualized at the microscope 24 hours after being exposed to DEP (0-25 mg/l).

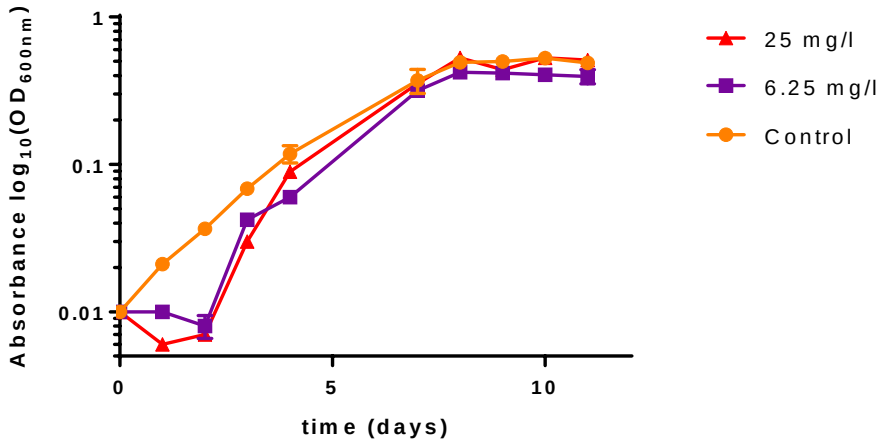

Figure 3. Growth of *M. bovis* BCG in 7H9 supplemented with 0.05% tween, 0.08% glycerol and 10% ADC and, exposed to DEP (6.25 and 25 mg/l). The results are expressed as the average and standard deviation of duplicates.

DEP exposure is related to susceptibility to bacterial infections in the lung **[12, 24, 26]**. It has been described that in mice DEP increases susceptibility to lung *Pseudomonas aeruginosa* infection, mice have higher inflammation and the clearance of the bacteria from the lungs is impaired in a DEP dependent manner **[11]**. We studied the effect of the DEP in the burden of *M. bovis* BCG in THP-1. Macrophages were exposed to DEP for three hours, as the mean time found in other studies **[21, 23, 27]**, and then infected with *M. bovis* BCG. Intracellular and extracellular CFUs were evaluated for six days. No significant changes were observed in the intracellular CFUs of macrophages exposed and unexposed to DEP (Figure 4.A).

However, extracellular CFUs were higher at days 3 and 6 when exposed to DEP (Figure 4.B). Intracellular CFUs were always higher than extracellular ones in at least one magnitude order (Figure 4). The differences observed in extracellular bacteria counts

between DEP-exposed and unexposed samples could be explained by a decrease in the killing rate on DEP exposed macrophages. Overburden of bacteria in macrophages could lead to their death, increasing the extracellular bacilli but not intracellular ones.

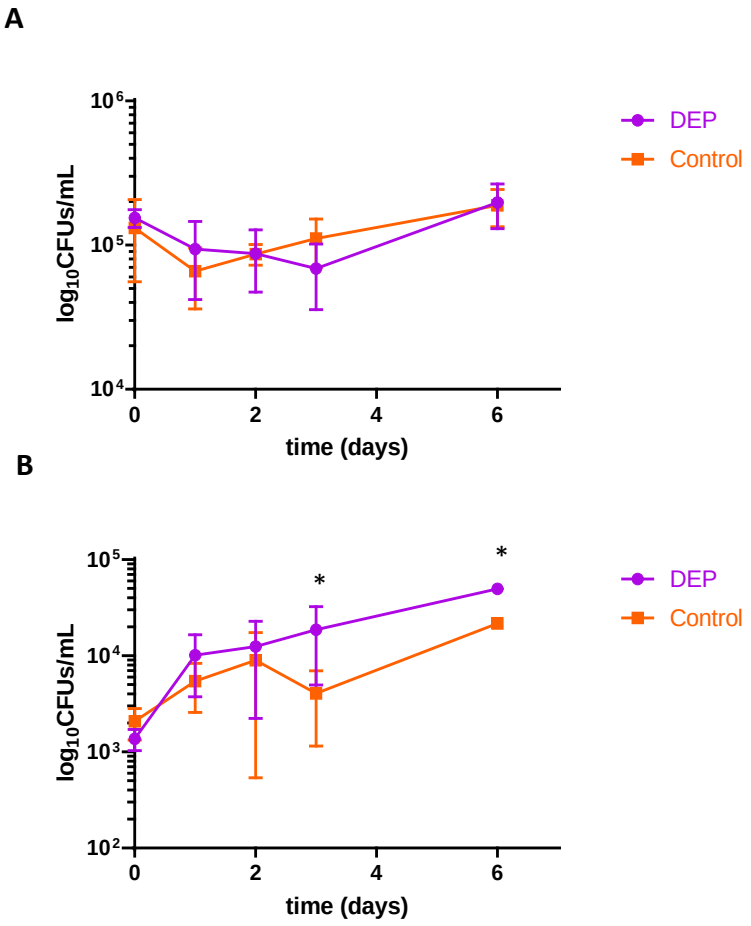

Figure 4. *M. bovis* BCG intra- and extracellular CFUs counts over 6 days. THP-1 macrophages were exposed to 6.25 mg/l DEP. A. Intracellular and B. extracellular growth of bacilli was assessed. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments. *P<0.05

Bacterial killing by alveolar macrophages is associated with the production of reactive oxygen and nitrogen species, cytokines, chemokines and interferon **[12, 24]**. It has been observed that DEP inhibit macrophage function and reduce cytokine production *in vitro* and *in vivo* **[5]**. Production of cytokines like TNF-α, IFN-γ and IL-12, which play important roles in killing mycobacterial infections, are altered in mice exposed to DEP **[5]**. TNF-α is reduced *in vitro* in murine alveolar macrophages infected with BCG and

non-infected when exposed to DEP and IL-12 in uninfected macrophages **[26]**. Moreover, the same study showed a reduction in the levels of mRNA of TNF-α in RAW264.7 macrophages **[26]**. IL-8 was reduced in alveolar macrophages exposed to high DEP concentrations and in A549 cells infected with *M. tuberculosis* when treated with PM **[23]**. All of these changes in cytokines resulted in a reduced macrophage bactericidal activity and prolonged bacterial survival **[12]**. In addition, levels of nitric oxide (NO) in response to IFN-γ are reduced in alveolar macrophages exposed to DEP. This response is a fundamental mediator of antibacterial action of activated macrophages **[5, 17]**. Reactive oxygen species are decreased as well in macrophages exposed to DEP **[24]**. In a study with Influenza virus in A549 cells, the oxidative stress generated by DEP was related to an increased susceptibility to viral infection due to a better attachment and entering into the respiratory cells **[27]**.

Additionally, studies with alveolar macrophages exposed to DEP showed impaired phagocytosis of heat-killed fluorescent yeast particles or inert latex particles **[28, 29]**. Other studies with *Streptococcus pneumoniae* showed that the rate of killing intracellular bacteria in alveolar macrophages and J774 cells are similar in the presence of concentrated ambient particles, although they detected an inhibition in internalization of bacteria. Consequently, they detect more bacteria surviving (extracellularly and intracellularly) on DEP exposed samples **[30]**. Yin *et al*. infected alveolar macrophages from rats and observed a decrease in the phagocytosis and killing of *Listeria monocytogenes* **[12]**. However, we neither observed any significant effect by DEP exposure on the uptake of bacteria at day 0, nor changes of intracellular burden at days 1-3 and 6.

Similar to our results, *in vitro* studies with mycobacteria, exposing to DEP for three days before infection with *M. bovis* BCG, showed no changes in survival of mycobacteria until day 10. Bonay *et al*. exposed human monocytes to DEP at the same time that infected, and they obtained similar results, suggesting that no functional changes such as activation of cells or impairing phagocytosis could happen **[16]**. They concluded that although DEP is a major pollutant with well adverse health effects, they do not seem to influence macrophages against mycobacteria **[16]**. Rivas-Santiago *et al*. exposed A549 cells to different PM sizes and concentrations for 18h followed by 18h of infection with *M. tuberculosis* at different MOI. CFU recovery was higher when exposed to 10 and 50 mg/l PM (but not in lower concentrations), and the impact was more visible when the MOI was higher and the PM smaller **[23]**.

Therefore, another question to consider, that may explain the discordance between the results of different experiments, is if a longer exposure time is needed to observe DEP effects. Long-term DEP exposure is related in animal studies with pulmonary inflammation, airway remodelling in the lungs as well as oxidative stress **[31]**. Experiments *in vivo* show that inhaling DEP for one month does not increase the mycobacterial CFUs in the lungs of mice, but two months increases slightly and 6 months increases significantly **[5]**. When *M. bovis* BCG and DEP are inoculated, BCG is present at higher quantities in lungs, liver and lymph nodes of mice after five weeks than in mice unexposed to DEP **[17]**.

In vitro models provide high standardization, acceptable reproducibility, low cost and absence of ethical concerns in the study. However, despite these advantages, they only provide a small part of what happens in the whole organism, since systemic reactions, buffering capacity or detoxify pathways can only be partly simulated **[13]**.

In summary, we have shown that exposure to DEP has high cytotoxic effects. DEP could lead to an impairment in the contention of the bacilli. A decrease in the killing rates and overburden of bacteria suggest that DEP are related with mycobacterial infections. Deciphering the links between air pollution and impairment of human antimycobacterial immunity is vital since pollution is rapidly increasing in areas where TB incidence is really high **[23]**.

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4.3 Chapter III: Exploring the effects in phagocytosis and cytokines production of e-cigarette vapor on THP-1 macrophages infected by *Mycobacterium tuberculosis*

ABSTRACT

Cigarette smoking and tuberculosis are a significant cause of death worldwide. Several epidemiological studies have demonstrated cigarette smoking is a risk factor for tuberculosis. Electronic cigarettes have recently appeared as a healthier alternative to conventional smoking, although their impact in tuberculosis is not well understood. The aim of this study was to explore the effect of electronic cigarettes in phagocytosis of *Mycobacterium tuberculosis* and cytokines production. To this end, *in vitro* infection was carried out by exposing THP-1 macrophages to four electronic vapor extracts and the intracellular burden of *M. tuberculosis* was determined. The percentage of infection was evaluated by confocal microscopy and the cytokine production by Luminex. A reduction of intracellular *M. tuberculosis* burden in THP-1 macrophages was found after its exposure to electronic vapor extract; the same trend was observed by confocal microscopy when *Mycobacterium bovis* BCG-GFP strain was used. Electronic cigarettes stimulate a pro-inflammatory cytokine response. We conclude that electronic cigarettes impair the phagocytic function and the cytokine response to *M. tuberculosis*.

INTRODUCTION

Both cigarette smoking and tuberculosis (TB) are a significant cause of death worldwide. The World Health Organization (WHO) estimates that 1.3 and 7 million people died in 2017 of TB and cigarette smoking respectively **[1, 2]**. It is noteworthy that countries with a high incidence of TB tend to also have an elevated proportion of smokers, suggesting a deadly interaction **[3]**. The link between TB and cigarette smoke has already been suggested since 1918 and it has been confirmed by several epidemiological studies **[4, 5]**. There is evidence that cigarette smoke is a risk factor for TB, aiding the infection, the progression to the active disease and the severity of the illness **[6, 7]**. In addition, it has been observed that cigarette smoke delays in the culture conversion during TB treatment, increasing the risk of transmission to the population **[8-10]**.

Many studies have been focused on understanding the cause-effect relationship between cigarette smoke and TB, however, many aspects currently remain unclear. Cigarette smoke effects include loss of phagocytic capacity, failure to contain the intracellular growth of mycobacteria in macrophages, attenuation of cytokine and chemokine response as well as attenuation of apoptosis **[11-15]**.

In the last years, Electronic Nicotine Delivery Systems (ENDS) and especially electronic cigarettes (e-cigs) have appeared as a cooler, cheaper and healthier alternative to conventional tobacco, proving more attractive to the younger population. However, in the medical community there is a growing concern due to: i) the health effects from ENDS, ii) the inexact labelling of the electronic cigarette liquid (e-liquid) components, iii) the lack of international laws for quality production, iv) non-smokers being converted by e-cigs into dual users **[16-18]**.

E-cigs deliver an aerosol (known as vapor) from e-liquids that can contain propylene glycol (PG), vegetable glycerol (VG), flavors and nicotine in different concentrations and ratios **[19]**. Although the percentage of toxic compounds found in e-cig vapor (e-vapor) are lower than cigarette smoke **[19]**, some studies have shown a toxic effect on cells like alveolar macrophages **[20]**, lung epithelial cells **[21-24]** and monocytic cells **[25]** as well as proteomic changes in the cells of the lower airways **[26]** and pro-inflammatory response **[21, 23, 25, 27]**. Moreover, function damage in macrophages has been observed and therefore an enhancement of infections caused by microorganisms such as *Haemophilus influenzae* **[28]**, *Escherichia coli* **[20]**, *Staphylococcus aureus* **[20, 23]** and *Streptococcus pneumoniae* **[29]**. In addition, it has been shown that e-cigs promote influenza A virus infection in mice **[29]**.

To obtain a better understanding of the relation between TB and e-vapor, we studied the influence of them on phagocytosis of *Mycobacterium tuberculosis* and cytokines production of infected THP-1 macrophages.

MATERIALS AND METHODS

THP-1 cell line and mycobacteria growth conditions

The human monocytic cell line THP-1 (ATCC TIB-202™) was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMax (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. The cells were passaged every 3 days. THP-1 monocytes were stimulated to macrophages using 0.1

µM of Phorbol 12-Myristate 13-Acetate (PMA, Sigma, St Louis, USA) for 72 hours (37°C in 5% CO_2). RPMI was replaced by fresh medium 24 hours before the experiments.

M. tuberculosis H37Rv and *Mycobacterium bovis* BCG expressing green fluorescent protein (BCG-GFP) strains were grown in Middlebrook 7H9 supplemented with 0.05% tween 80 and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). For *M. bovis* BCG-GFP, 0.08% of glycerol and 20 µg/mL of kanamycin were also added. The mycobacteria cultures were incubated at 37°C. *M. bovis* BCG-GFP was kindly provided by Carlos Martín from the University of Zaragoza, Spain.

Cigarette smoke and e-vapor extraction

Cigarette smoke extract (CS extract) was prepared from commercial cigarettes (Marlboro: 10 mg Tar, 0.8 mg Nicotine, Philip Morris Sàrl Neuchâtel, Switzerland) as was previously reported **[30]**. Briefly, one cigarette was combusted using a syringe-modified apparatus, which draws the smoke into a sterile glass containing 10 mL of RPMI medium or 7H9-tween. 60 mL of smoke was extracted for 10 sec following a 30 sec break; this process was repeated six times per cigarette and the CS extract was sterilized using a 0.22 µm filter. The resulting solution was considered as a 100% CS extract. The absorbance (320nm) was measured for each batch to assure reproducibility and adjusted to 1.2 ± 0.2 **[31, 32]**. The working solution was 10% CS extract, equivalent to the smoke of between half a pack and 2 packs of cigarettes per day **[33]**. For each experiment, fresh extract was used and added to the cultures within 30 min of preparation.

For e-vapor extract, a similar protocol was carried out. QHIT e-cigarette (Puff, Moncalieri, Italy) was used with a Cartomicer CE4 device, 3.7 V. The e-liquid base was a compound of 55% PG: 45% VG. The e-liquid base was used with nicotine (8 mg/g) and without, or with Coffee flavour (Irish Cloud) with and without nicotine (8 mg/g) (Puff It, Puff, Moncalieri, Italy). Coffee flavor was chosen because of the toxic effect observed in fibroblasts **[34]**. 60 mL of e-vapor was extracted for 3 sec, after which a 30 sec break followed; the process was repeated 40 times (average of puffs found in literature), in 10 mL of RPMI medium or 7H9-tween **[23, 28]**. The e-vapor extract was also sterilized using a 0.22 µm filter. Like for CS extract, fresh extract was used and added to cultures within 30 min of preparation at 100% concentration according to literature **[23, 28]**.

Cytotoxicity Assay

To determinate the cytotoxic effect of the cigarette smoke and e-vapor extracts on THP-1 macrophages, EZ4U (Biomedica, Vienna, Austria) assay was used following the manufacturer's instructions. Briefly, THP-1 monocytes were seeded at a concentration of 1×10^5 cells per well in 96-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA) using RPMI without phenol red containing PMA. The plates were incubated for 72 h (37°C in 5% CO_2 atmosphere). CS extract or e-vapor extract were diluted in 200 µL of RPMI without phenol red per well at different concentrations and incubated for 3 hours for CS extract and 24 hours for e-vapor extract. The monolayers were washed once with Dulbecco's PBS (DPBS) and then 200 µL of fresh medium without phenol red and 20 µL of EZ4U were added. The absorbance was read after 4 h of incubation (day 0), and also at days 3 and 6. For the plates of day 6, medium was changed on day 3. A microplate reader (Victor 3, Wallac, Waltham, USA) was used with a wavelength of 450 nm with 620 nm as reference. The results are expressed as viability percentage using cells untreated as a control.

***M. tuberculosis* Growth curve**

M. tuberculosis H37Rv was adjusted to OD_{600nm} 0.01 in 7H9-tween medium (ADC supplemented) with or without 100% e-vapor extract or 10% CS extract. The absorbance was measured every 24 hours for two weeks.

Macrophage infection with *M. tuberculosis*

THP-1 macrophages were infected following a protocol previously reported [35], with some modifications. Briefly, 3×10^5 cells per well were PMA stimulated and seeded in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA). Freshly prepared 10% CS extract or 100% e-vapor extract were added to the cells and incubated for 3 hours for CS extract and 24 hours for e-vapor extract at 37°C in 5% CO_2. For the infection, mid-log phase *M. tuberculosis* were washed twice with DPBS+0.05% tween and subsequently once with DPBS after which they stood for 5 min, before the supernatant was collected. The bacteria were then diluted in RPMI with CS extract or e-vapor extract and added to the THP-1 macrophages at a multiplicity of infection (MOI) of 0.1. After 3 h of contact at 37°C in 5% CO_2, the macrophages were treated with 200 µg/mL amikacin for 1 h and washed three times with DPBS to eliminate any extracellular bacteria. Lastly, 1 mL of RPMI with CS extract or e-vapor extract was added to each well and incubated at 37°C in 5% CO_2. Fresh medium with CS extract or e-vapor extract was added at day 3. Intracellular growth was assessed by lysis of the

monolayers by the addition of 500 µL of water followed by a 30 min incubation at room temperature and serial dilution in PBS-tween plating onto Middlebrook 7H10 solid medium at days 0, 1, 2, 3 and 6. Colonies were counted after 3–4 weeks incubation at 37 °C and the average CFUs/mL determined.

Confocal Microscopy

THP-1 macrophages were seeded on a 12mm circular coverslips in 24-well plate. The infection was performed as described above but using *M. bovis* BCG-GFP strain or 1-µm-diameter yellow-green latex beads (Sigma, St Louis, USA) with a MOI of 10. Latex beads were used in order to determine if phagocytosis was impaired in a general or specific mycobacteria pathway. After infection, cells were fixed overnight with 4% paraformaldehyde (PFA). Coverslips were washed twice with DPBS and incubated with DPBS-BSA 1% 10 min. For staining, coverslips were washed once with DPBS and then 200 uL of DPBS containing Hoechst 33342 (1:1000) and texas red-X-phalloidin (1:200) were added and incubated for 10 min. Hoechst and red-X-phalloidin were purchased in Invitrogen (Waltham, USA). Finally, the coverslips were washed once with DPBS, once with water and subsequently mounted with Prolong Gold Antifade Reagent (Invitrogen). For quantification, 900 cells from three independent experiments (300 cells/coverslip) were counted per day and treatment. The images were taken with an Olympus Fluoview 1000 microscope at days 0, 3 and 6 for the experiments with *M. bovis* BCG-GFP and at day 0 for the latex beads experiments. The images were analysed using ImageJ software **[36]**.

Cytokines detection

Cytokines production from infected and uninfected THP-1 macrophages, which were either exposed or non-exposed to e-vapor extract or CS extract, were assessed using the human magnetic Luminex assay kit (LXSAHM, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Analysis included detection of some of the most important cytokines involved in the immune response against *M. tuberculosis* infection as tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-10, IL-12, IL-18, IL-1β and interferon (IFN)-γ. Supernatants were recovered from infection assays (described above) with *M. tuberculosis* H37Rv strain and sterilized using a 0.22 µm filter. For the supernatants of day 6, 500 µL extra of fresh RPMI (with or without e-vapor extract or CS extract) were added on day 3 (this extra volume was taken in account in the calculation of cytokine production). Cytokine levels were calculated

using the Luminex® 200™ system and the xPONENT® 3.1 software (Luminex Technologies, Inc., Austin, USA).

Statistical analyses

Statistical analyses were performed using the GraphPad PRISM 6.0 software package (San Diego, California, USA). Differences between treatments were compared by the Mann-Whitney test. A *p*-value ≤0,05 was considered as a statistically significant.

RESULTS

e-vapor extract and cigarette smoke extract are not toxic for THP-1 and mycobacteria at the concentrations used

We evaluated if the concentrations found in literature: 10% CS extract and 100% e-vapor extract were nontoxic in THP-1 macrophages **[23, 28, 33]**. The macrophages were exposed to e-vapor or CS extracts for 6 days and the cell viability was assessed using the formazan-based cell proliferation assay EZ4U at days 0, 3 and 6. The assessed concentrations assessed of CS extract and e-vapor extract (both non-flavored and coffee flavored on a base with or without nicotine) did not affect the viability of THP-1 (Figure 1).

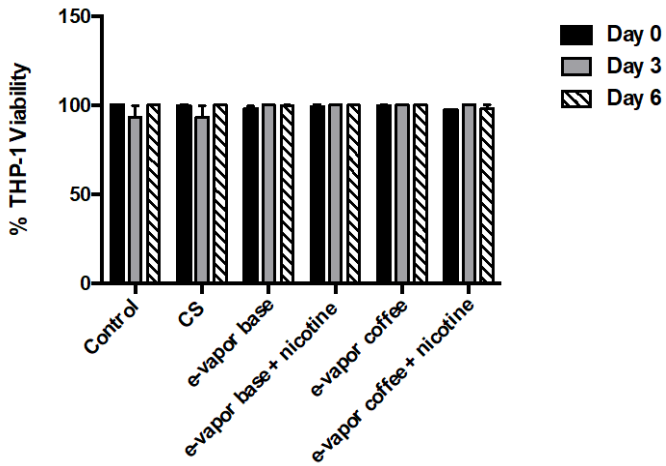

Figure 1. THP-1 viability after exposition to cigarette smoke (CS) extract and e-vapor extract. Formazan-based test EZ4U was performed at 4 hours (day 0), 3 and 6 days of exposition to 10% CS extract, 100% e-vapor base extract with and without nicotine and e-vapor coffee flavor extract with and without nicotine. The results are expressed as the average and standard deviation of six replicates of at least two independent experiments.

On the other hand, we studied the effect of e-vapor extract and CS extract on *M. tuberculosis* H37Rv growth. The cultures were exposed to e-vapor extract and CS extract for two weeks and the OD_{600nm} was measured every 24 hours. The results obtained showed no significant changes. A slight increase in the growth of *M. tuberculosis* was observed when it was exposed to e-vapor extract and a slight decrease when the bacteria were exposed to CS extract (Figure 2).

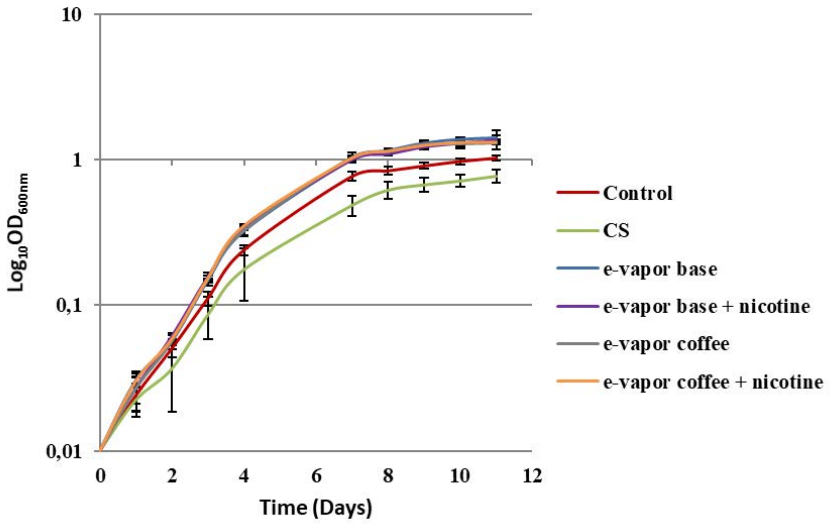

Figure 2. Growth of *M. tuberculosis* H37Rv in Middlebrook 7H9 supplemented with 0.05% tween 80 and 10% ADC, exposed to 10% CS extract, 100% e-vapor base extract with and without nicotine and e-vapor coffee flavor extract with and without nicotine. The results are expressed as the average and standard deviation of duplicates of at least two independent experiments.

e-vapor and cigarette smoke extracts reduce the phagocytosis of *M. tuberculosis* by THP-1 macrophages

As was previously reported in literature for other microorganisms, we studied if the extracts of e-vapor and cigarette smoke had any effect on the phagocytic function after infection with *M. tuberculosis*. Therefore, macrophages were exposed to e-vapor extract and CS extract for 24 hours and 3 hours respectively, and then infected with H37Rv strain. The intracellular CFUs were evaluated during 6 days. When the macrophages were exposed to e-vapor extract base and e-vapor extract base with nicotine, a reduction of the number of viable intracellular *M. tuberculosis* was observed compared with the control. Less mycobacteria were recovered when the cells were exposed to e-vapor extract base with nicotine compared with e-vapor extract base

without nicotine. A similar tendency was observed when the macrophages were exposed to e-vapor extract with coffee flavor, however the CFUs counts were higher than e-vapor extract base. The highest decrease in the CFUs counts were observed when the macrophages were exposed to CS extract compared with e-vapor extract and control. The highest decrease in the CFUs counts were observed when the macrophages were exposed to CS extract compared with e-vapor extract and control (Figure 3).

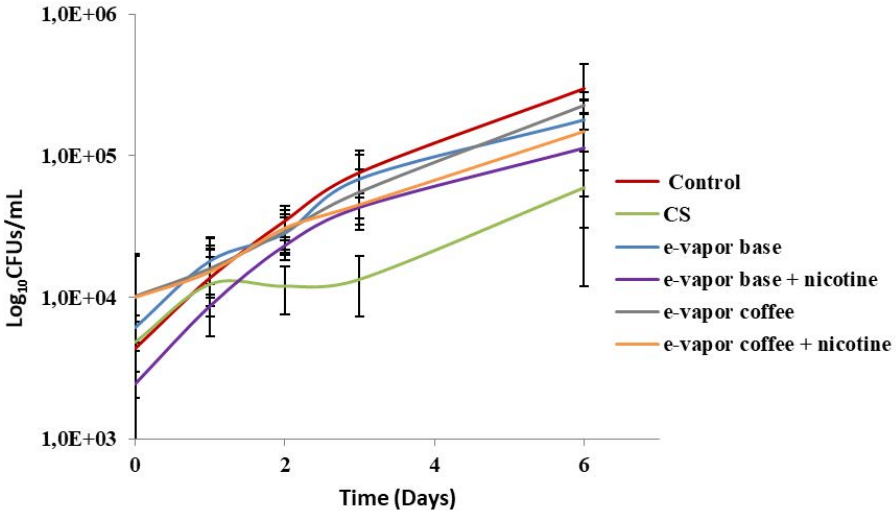

Figure 3. *M. tuberculosis* intracellular CFUs counts over 6 days. THP-1 macrophages exposed to 10% CS extract, 100% e-vapor base extract with and without nicotine and e-vapor coffee flavor extract with and without nicotine. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments.

In order to confirm the results obtained in CFUs counts, we determined the percentage of infected cells using confocal microscopy. The fluorescent strain *M. bovis* BCG-GFP was used, following the same infection protocol used with *M. tuberculosis*. As was observed in the CFUs counts, the results show that there is a significant reduction in the uptake of *M. bovis* BCG-GFP when the macrophages are exposed to CS extract compared with the control (Fig. 4). Moreover, there is a trend toward an increase in the uptake of mycobacteria after the exposition to e-vapor extract compared with CS extract, but to a lesser extent than the control sample (Figure 4). Subsequently, the results obtained by confocal microscopy confirm the results obtained by CFUs counts.

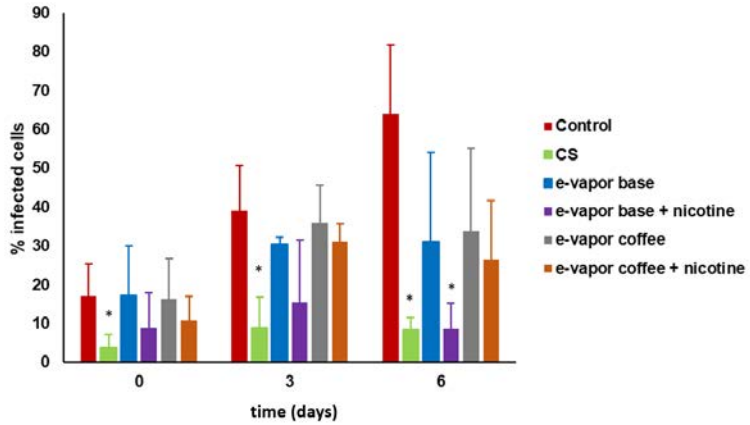

Figure 4. Phagocytosis of *M. bovis* BCG-GFP by THP-1 macrophages. Macrophages were exposed 10% CS extract, 100% e-vapor base extract with and without nicotine and e-vapor coffee flavor extract with and without nicotine. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments using confocal microscopy. Significance from control, $P<0,05$, Mann-Whitney *U* test.

To determinate if the reduction of mycobacteria uptake is due to a general or specific defect in the phagocytic function, we evaluated the effect of CS extract and e-vapor extract in the capacity to phagocyte inert material like latex beads. Macrophages were exposed to e-vapor or CS extracts and then the latex beads were in contact during 3 hours. The images were analyzed and the results show no major changes in phagocytosis, suggesting a specific defect in some specific pathway when the cells are exposed to CS extract (Figure 5). Less macrophages with latex beads inside were observed when the cells were exposed to e-vapor coffee extract with and without nicotine (Figure 5).

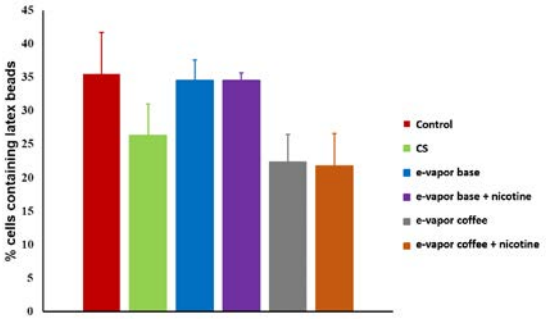

Figure 5. Phagocytosis of GFP-latex beads by THP-1 macrophages. Macrophages were exposed to 10% CS extract, 100% e-vapor base extract with and without nicotine and e-vapor coffee flavor extract with and without nicotine. 300 cells were counted. The results are expressed as the average and standard deviation of three independent experiments using confocal microscopy.

e-vapor extract and cigarette smoke extract alter cytokine production

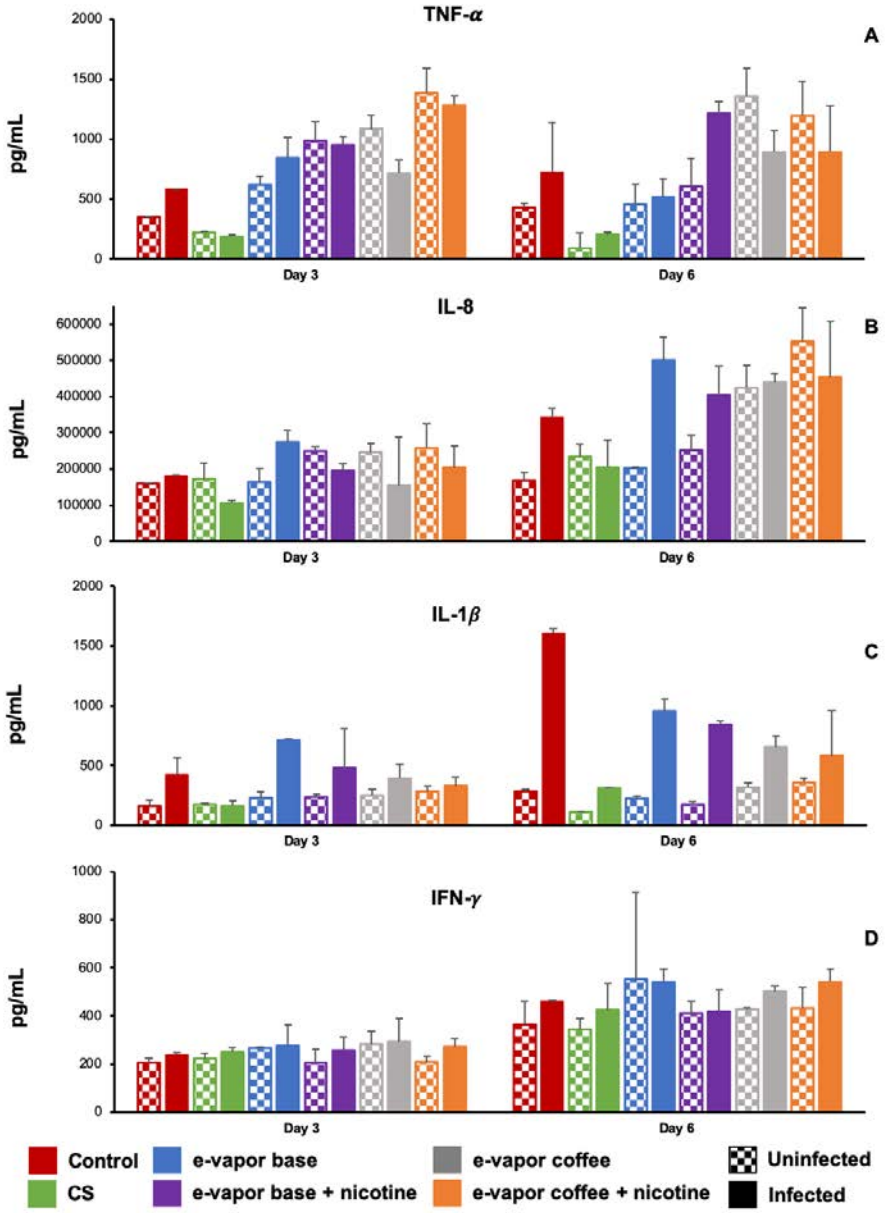

Figure 6. Cytokine production by THP-1 exposed and non-exposed to e-vapor extract and CS extract. Macrophages were exposed to 10% CS extract, 100% e-vapor base extract with and without nicotine and e-vapor coffee flavor extract with and without nicotine. Macrophages were uninfected or infected with *M. tuberculosis* H37Rv strain. **A**: tumor necrosis factor (TNF-α), **B**: interleukin 8 (IL-8), **C**: interleukin 1-beta (IL-1β), **D**: interferon gamma (IFN-γ). The results are expressed as the average and standard deviation of at least two independent experiments.

Given that previous studies have shown that e-vapor extract and CS extract alter the cytokine secretion and consequently the clearance of infections, we explored the cytokine response to e-vapor extract and CS extract in THP-1 macrophages. In general, the tendency observed was that the unexposed macrophages produced more cytokines when they were infected. It was also observed that macrophages exposed to CS produced less cytokine response than unexposed cells or cells exposed to e-cigs (Figure 6).

Regarding e-cigs vapor exposed cells, cytokine production was related to the cytokine and the e-cigs vapor extract used. As a general trend, the cytokine response was higher than the cells exposed to CS and even than unexposed cells. The production of TNF-α was higher in infected cells at day 6 exposed to e-vapor with coffee flavour (with and without nicotine) (Figure 6.A). IL-8 is was the cytokine produced in higher amount (Figure 6.B). The IL-1β was highly produced by cells exposed to *M. tuberculosis* infection (Figure 6.C). Finally, the production of IFN-γ was similar between uninfected and infected cells (Figure 6.D). IL-6, IL-10, IL-12 and IL-18 were all of them below the detection levels.

In Figure 7 is shown the ratio between the amount of cytokine produced by each exposed, infected and the non-infected cells. It is observed, that the cells exposed to CS and e- vapor extract with coffee flavor (with and without nicotine) have an impaired response to infection in comparison with unexposed cells and e-cigs base and without flavor or nicotine.

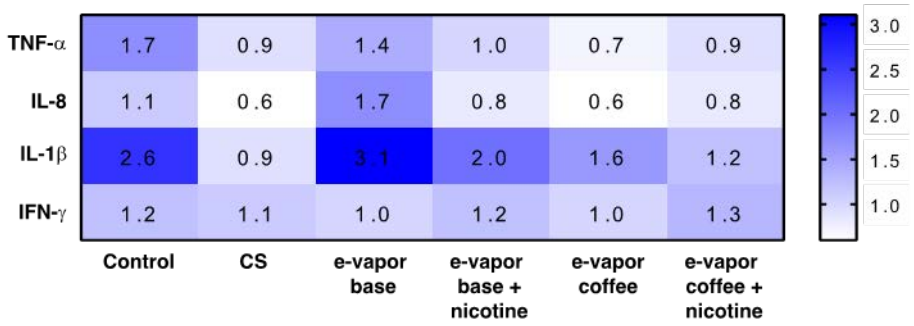

Figure 7. Increment of the cytokine production at day 3 in infected cells compared with the uninfected cells with the same exposure. The numbers in the figure are the result of dividing the amount of cytokine in infected cells between uninfected cells. Values higher than 1.0 mean production of cytokines in infected cells are higher than in uninfected. The higher the number, the higher the increment.

DISCUSSION

It has been widely proven that CS has an adverse impact on human health and despite the efforts oriented to reduce its use, it still remains as an issue worldwide. E-cigs have been marketed as a healthier option, however like CS, it has been related to cytotoxicity and abnormal immune response compromising the clearance of pathogens. In this study, we evaluated the impact of e-cigs and CS on the phagocytosis of *M. tuberculosis* and cytokines production of infected THP-1 macrophages. Our results show that e-cigs and CS have a deleterious impact on the immune response against TB.

We tested the effect of 100% e-vapor and 10% CS extracts on viability of THP-1 macrophages and we did not observe a cytotoxic effect, this result confirms that e-vapor is less toxic than CS, since it has been reported that higher than 10% concentrations of cigarette smoke are toxic in several cell lines **[32, 37, 38]**. This was also observed by us when higher concentrations than 10% were tested (data not shown). Free radicals are involved in the tobacco cytotoxicity and they are produced not only in the tobacco combustion but also when e-liquid is heated in the atomizer **[39]**. Scott *et al*. detected lower cytotoxicity in alveolar macrophages in the liquid form compared to the vaped **[40]**. The lower cytotoxicity observed in e-vapor extract can be related with the low concentration of toxicants due to the low temperature of evaporation of the e-liquid compared with the tobacco combustion when a cigarette is smoked **[34, 39, 41]**. On the other hand, our results are in line with the results obtained by Farsalinos *et al*. and Romagna *et al*. in which the presence of nicotine in e-vapor extract did not affect the cell viability **[34, 41]**. The results found in literature, regarding the cytotoxicity of e-vapor extract flavored are contradictory; some studies have shown a cytotoxic effect when tobacco, cherry, cinnamon and coffee flavors were tested **[34, 42-44]**. However, in agreement with our results, Ween *et al*. observed that cytotoxicity in THP-1 macrophages was not related with the e-cig flavor **[28]**. It has been suggested that cell line used, nicotine, flavors, PG/PV ratio, voltage and potency of the device can influence the toxicity effects of e-vapor **[45]**.

Innate immune response is crucial for the outcome of TB infection as well as for the adaptive immune response **[46]**. Macrophages are a pivotal piece of the innate immunity for TB and its response is affected by cigarette smoke and e-vapor. Our results showed a decrease of the intracellular *M. tuberculosis* in THP-1 macrophages under the exposition to e-vapor extract as well as CS extract, where even less viable *M.*

tuberculosis where found compared with the control. Similar trend was obtained by confocal microscopy using BCG-GFP strain. These results suggest that e-vapor extract and CS extract cause a phagocytosis impairment in THP-1. This kind of damage has been observed in monocytes from smokers TB patients **[14]**, in alveolar macrophages from smokers **[15]**, in lung-tissue and alveolar macrophages from smoking mice **[47, 48]** and in alveolar macrophages and THP-1 macrophages when cells exposed to CS extract were infected with *H. influenzae* **[28, 38]**. The reduction in the phagocytic ability has been related with the decrease of the expression of recognition molecules (CD44, CD91, CD31 and CD71) for apoptotic cells in alveolar macrophages **[15]**, the inhibition of Rac1 protein, involved in cytoskeletal rearrangements **[48]**, the blockage of PI3K signaling cascade **[38]** as well as the decrease of the expression of bacterial recognition receptors like Toll-like receptors 2 and 4 (TLR-2 and TLR-4, respectively) and scavenger receptor SR-A1 **[28, 49-51]**. However, it has been also observed opposite results. Shang *et al*. found an increase of viable intracellular *M. tuberculosis* in THP-1 macrophages after the exposition to CS extract (from 3R4F standard cigarette) compared with the macrophages non-treated. In the study of Shang *et al*., an increase in the intracellular burden was also found in alveolar macrophages obtained from autopsies of smokers **[11]**. Other studies showed similar results using the BCG and H37Ra strains in alveolar macrophages and monocyte-derived macrophages (MDM) **[12, 37]**. Moreover, Bai *et al*. observed that the nicotine component of CS extract reduces the number of autophagosomes leading an increase of *M. tuberculosis* burden in alveolar macrophages **[3]**. Although, it is important to highlight that the cell line, the strain, the type of cigarette/e-cig, the extraction method, the exposure dose and time all have an influence on the results obtained **[52]**.

On the other hand, in line with our results with cigarette smoke, other studies have shown similar results in e-cigs. In mice model, it has been observed that the exposure to e-vapor extract decreased also the phagocytic ability **[29]**. Phagocytosis of *H. influenzae* is also decreased in THP-1 macrophages by e-vapor flavored with or without nicotine but not with e-vapor base extract, suggesting that flavors could have a role in the phagocytosis impairment **[28]**. However, our results showed the opposite, as we did not observe that coffee flavor affect more the growth of H37Rv or BCG-GFP inside macrophages exposed to e-vapor extract flavored although in our case, the flavor tested is different from Ween *et al*. **[28]**. Additionally, our results showed that the macrophages exposed to e-vapor extract with nicotine had less intracellular mycobacteria than the cells exposed to e-vapor extract without nicotine, suggesting

that nicotine in e-vapor extract has an effect in the phagocytosis of mycobacteria. On the other hand, Ween *et al*. observed that the impairment of phagocytosis by e-cigs was also linked with the expression of scavenger receptor SR-A1 and TLR-2 **[28]**.

It has been observed that CS extract produce damage in specific phagocytosis pathways in alveolar macrophages **[38]**, and in our experience, the results obtained with *M. bovis* and with latex beads also suggest the same specific impairment to phagocyte alive particles with both, e-vapor with and without nicotine.

As important mediators of the immune system, macrophages release cytokines in order to regulate both innate and adaptive immunity **[53]**. Cytokines are produced by macrophages activated by *M. tuberculosis* infection **[54]**. This is in agreement with our results where the release of cytokines is stimulated for the infection in unexposed cells, compared with the uninfected control. It has been observed that another effect of e-cigs and CS on macrophages is the alteration of cytokines production, stimulating both inflammatory and anti-inflammatory responses **[47]**. TNF-α, IL-1β and IFN-γ are pro-inflammatory cytokines that are important in the host defence after *M. tuberculosis* infection **[54]**. TNF-α also initiates the activation of the phagocyte **[54]**. Similar to our results, several studies have found that CS extract decrease the production of TNF-α in uninfected alveolar macrophages, THP-1, Ana-1 macrophages, monocyte-derived macrophages and human peripheral blood mononuclear cells (PBMC) **[12, 28, 53, 55, 56]** and IL-1β also in uninfected THP-1, monocyte-derived macrophages and PBMC **[28, 55, 57]**. We observed the same tendency in infected cells exposed to CS extract, which produced less TNF-α and IL-1β compared with the infected cells non-exposed to CS extract. Despite some studies have shown that IFN-γ is decreased by CS **[9, 37, 58]**, we did not observe important changes in its release, compared to control in THP-1 macrophages. IL-8 is a neutrophil chemoattractant that mediates the inflammatory process **[54]** that is increased in uninfected THP-1 macrophages exposed to CS extract **[28]**. Our results show the same tendency.

The effect of e-vapor extract in the cytokines production is different from the observed in CS extract, with a tendency to produce a pro-inflammatory response. A pattern of elevated cytokine production after e-cig inhalation was observed in mice model **[23]**. Our results show a trend to increase TNF-α in infected and uninfected cells. Opposite results were obtained by Ween *et al*. where TNF-α was decreased in uninfected THP-1, however, the cytokine was measured at 24 hours when the production could be lower **[28]** and we speculate that e-vapor extract can require more time in order to see

the effect in the cytokines production. Additionally, an increase in the TNF-α production by e-vapor extract in HaCaT cells and by e-cig condensate in THP-1 was observed **[20, 24]**. On the other hand, the e-vapor extract effect observed on uninfected THP-1 as observed by Ween *et al*. showed a decrease of IL-1β **[28]** which we did not observe. However, we found this decrease in infected cells mainly on the last day of the experiment. Cervellati *et al*. showed that IFN-γ was increased in uninfected HaCaT cells exposed to e-vapor extract **[24]**, although we observed only a slight increase also the last day of the experiment. Contributing to the inflammation process, IL-8 is increased in infected and uninfected THP-1 macrophages; this finding is similar to several studies in uninfected cells including THP-1 **[20, 22, 24, 25, 28]**.

As it is observed in other studies where the e-cig are used, this study has some limitations: i) the use of only one type of e-cig of second-generation and only one flavour although there is a wide range of e-liquid brands and devices available on the market, ii) as an *in vitro* study, the lung environment is not completely represented, iii) not all experiments were done with virulent *M. tuberculosis* strain because of biosafety normative and cannot be assumed that response of nonvirulent mycobacteria would be the same, iv) it is difficult to estimate if the concentrations and duration of CS and e-vapor are representative of what happens in the smokers **[37]**. The topography between vaping and cigarette smoking is also different, and e-cigs users could vape more reaching a similar effect to tobacco.

In summary, e-cigs impair the phagocytic function of THP-1 macrophages as well as promote the pro-inflammatory response. Moreover, we confirm that CS affects also the phagocytic function and decreases the effector cytokines response (TNF-α, IL-1β and IL-8). Although e-cigs are advertised as a better option and its effect is not as strong as cigarette smoke, the dependency and long term consequences are not still well known. Our data contributes to explain some of the mechanisms behind the epidemiological link between TB and smoking, and to support the policy efforts to reduce the exposure to smoke and e-vapor as part of the TB control strategies.

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4.4 Chapter IV: Electronic and conventional cigarettes modify the metabolome of THP-1 macrophages infected with *M. bovis* BCG impairing the tryptophan starvation strategy

ABSTRACT

Electronic cigarettes are marketed as a harmless alternative to conventional cigarettes. Metabolomics, the study of the small molecule intermediates and products of the metabolism, is a powerful strategy to obtain a global profile of important metabolites in different conditions. As smoking is linked with tuberculosis, we wonder if electronic cigarettes could also be linked, altering the metabolome in a similar manner as cigarette smoke. THP-1 cells exposed to cigarette smoke or electronic vapor were infected with *M. bovis* BCG. Supernatants and cells were collected at 0h and 36h and then metabolites were extracted and analysed by gas chromatography coupled to mass spectrometry. Main metabolomics changes in supernatants were due to maturation of the cultures, but electronic vapor exposed samples were also differentiated, and mainly characterized by the presence of lactic acid, nicotine and glycerol. Intracellular metabolites were mostly influenced by maturation of the cells, but just after infection (0h) samples were differentiated by cigarette smoke or electronic vapor exposure. These different characteristics were lost at 36h. Levels of tryptophan are normally reduced in infected cells as a part of a protection strategy against pathogens, however, in this study, it was not reduced in intracellular infected samples exposed to cigarette or electronic vapor exposure. Levels of kynurenine, an immune regulator, were also altered. We found changes in the metabolome of THP-1 cells by electronic vapor exposure comparable to smoke exposure. Our results showed that electronic cigarettes have some impact on the metabolome.

INTRODUCTION

Sales of electronic nicotine delivery systems (ENDS), and especially electronic cigarettes (e-cigs), are rapidly increasing due to the popular belief that they are a harmless alternative to conventional cigarettes **[1]**. In an effort to undermine tobacco control policies, tobacco companies promote e-cigs as smoking cessation devices **[2]**. Nevertheless, there is a concern that e-cigs could reach the opposite aim, serve as a gateway to conventional cigarettes, especially young people **[3]**. Evidence in the

literature suggests that vapour from e-cigs, far from being just risk-free vapor, it causes oxidative stress, contains many toxic compounds and alters the cytokine production of the immune system **[4, 5]**.

Cigarette smoke (CS) is a leading factor for tuberculosis (TB) epidemics. It is estimated that more than 240,000 TB deaths/year are due to smoking **[6]**. Despite the conclusive clinical and epidemiologic evidence between CS and TB **[7]**, the hypothetical connection between e-cig and TB will not be available until clinical evidence arrives in the forthcoming years **[8]**.

Metabolomics is a powerful bioanalytical strategy that allows obtaining a global profile of the metabolites in a course of a biological process. It is based on the study of small molecules that are intermediates and products of the metabolism from cell reactions or foreign chemicals, like CS **[9]**. It is possible to compare the metabolites under normal conditions with an altered state, finding biomarkers that can support the molecular bases of complex processes like lung diseases **[10]**. Mass spectrometry (MS) is the most used analytical technique in metabolomics, given its high sensitivity for detecting low concentrated metabolites **[11]**. Before using MS, however, first, a separation technique such as gas or liquid chromatography is needed, to couple to the MS. Gas chromatography is based on separation of naturally volatile compounds (as ketones, aldehydes, alcohols, esters or some lipids) or compounds made volatile by derivatisation (as sugars, amino acids, lipids, peptides or organic acids) and thermally stable **[12]**. This is done to introduce to the MS the compounds one after another, to facilitate the metabolite identification **[11]**.

The analysis of the identification, quantification and characterization of these small molecules is carried out by chemometrics **[13]**. The simplest correlation is principal component analysis (PCA), a non-supervised method that allows detecting trends, groups and outliers **[11]**. Then, orthogonal projections to latent studies discriminant analysis (OPLS-DA) could be used for the classification and discrimination of metabolites into two or more possible classes **[14]**.

Although some changes in metabolites concentration have been found when cells are exposed to CS, to the best of our knowledge the metabolomics of CS and e-cig exposed samples infected with mycobacteria has not been tested **[15, 16]**. In this study, we tested whether e-cig vapour may affect the metabolome in a comparable manner to CS, which has been linked to TB. For that, we exposed THP-1 cells to e-cig vapour and CS extract, cells were infected and extracellular and intracellular metabolome were analysed.

MATERIALS & METHODS

Cell cultures and mycobacteria growth conditions

The human monocytic cell line THP-1 (ATCC TIB-202™) was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMax (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, penicillin 10,000 units/ml, streptomycin 10 mg/ml and Fungizone® 25 µg/ml (Gibco, Paisley, UK). The cells were passaged every 3 days. For the experiments, RPMI without antibiotics was used. THP-1 monocytes were stimulated to macrophages using 0.1 µM of Phorbol 12-Myristate 13-Acetate (PMA, Sigma, St Louis, USA) for 72 hours (37°C in 5% CO_2). RPMI was replaced by fresh medium 24 hours before the experiments.

Mycobacterium bovis BCG strain was grown in Middlebrook 7H9 supplemented with 0.05% tween 80, 0.08% glycerol and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). The mycobacteria cultures were incubated at 37°C without agitation.

Cigarette smoke extraction and e-vapor extraction

CS extract was prepared from commercial cigarettes (Marlboro: 10 mg Tar, 0.8 mg Nicotine, Philip Morris Sàrl Neuchâtel, Switzerland) as previously reported **[17]**. Briefly, one cigarette was combusted using a syringe-modified apparatus, which draws the smoke into a sterile glass containing 10 mL of RPMI. Sixty millilitres of smoke were extracted for 10 sec following a 30 sec break; this process was repeated six times per cigarette and the CS extract was sterilized using a 0.22 µm filter. The resulting solution was considered as 100% of CS extract. The absorbance at 320nm was measured for each batch to ensure reproducibility and adjusted to 1.2 ± 0.2 **[18, 19]**. The working solution was 10% CS extract, equivalent to the smoke of 0.5-2 packs of cigarettes per day **[20]**. For each experiment, fresh extract was used and added to the cultures within 30 min of preparation.

For e-vapor extract, a similar protocol was carried out. QHIT e-cigarette (Puff, Moncalieri, Italy) millilitres with a Cartomicer CE4 device, 3.7 V. The e-liquid base was a compound of 55% PG: 45% VG and used with nicotine (8 mg/g) (Puff It, Puff, Moncalieri, Italy). Sixty millilitres of e-vapor were extracted for 3 sec, after which a 30 sec break followed; the process was repeated 40 times (average of puffs found in literature), in 10 mL of RPMI medium or 7H9-tween **[4, 21]**. The e-vapor extract was also sterilized using a 0.22 µm filter. Like for CS extract, fresh extract was used and

added to cultures within 30 min of preparation at 100% concentration according to literature **[4, 21]**.

Macrophage infection with *M. bovis* BCG

THP-1 macrophages were infected following a protocol previously reported **[22]**, with some modifications. Briefly, $5{,}2\times10^5$ cells per millilitre were PMA stimulated and seeded in 75 cm^2 tissue culture flasks (Nunc®, Thermo Scientific, Denmark) in a final volume of 35 ml. Freshly RPMI containing 10% CS extract was added to the cells and incubated for 3 hours for CS extract at 37°C in 5% CO_2. For the infection, mid-log phase *M. bovis* BCG were washed twice with DPBS+0.05% tween and subsequently once with DPBS after which they stood for 5 min before the supernatants were collected. The bacteria were then diluted in RPMI with CS extract and added to the THP-1 macrophages at a multiplicity of infection (MOI) of 3. After 3 h of contact at 37°C in 5% CO_2, macrophages were washed three times with DPBS to eliminate any extracellular bacteria. Lastly, 18 mL of RPMI with CS extract was added to each flask and incubated at 37°C in 5% CO_2. Extracellular media were collected and cells were detached with scrapers (SPL Life Sciences, South Korea). Viability of cells was checked with trypan blue. Two washes with DPBS were made and samples were stored in 50 µl DPBS at a final quantity of 5×10^6 cells. Both extracellular media and cell suspension were frozen immediately at -80ºC. RPMI, CSE and bacteria inoculum were additionally frozen to act as controls.

Metabolic extraction and derivatization

Three independent metabolite extraction (5 million cells each) were performed for each condition. Samples were defrosted on ice and 450 µl extraction solution (MeOH/H_2O 9:1 v/v, with internal standard at 7 ng/µL) and one tungsten bead were added. Cell disruption and grinding was accomplished by using a vibration mill (30 Hz, 2 min). Then, beads were removed and samples were cooled on ice bath for 2 hours. Cells were centrifuged (14,000 rpm, 10 min, 4ºC) and supernatant was collected, and 100 µl metabolic extract were vacuum-dried and kept frozen until derivatization.

First, the dried extracts were dissolved in 15 µL of a solution of methoxyamine (15 µg/µL) in pyridine and shaken for 10 min. Then, samples were kept 1 h at 70ºC and at least 16h at RT. The solution was then mixed with 15µL of N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) (1% trimethylchlorosilane [TMCS]) for 1 h and then mixed with 15 µL heptane with methyl stearate 15 ng/µL. Finally, 50 µl heptane with methyl stearate 5 ng/µL was added to the samples.

GC-MS analysis

GC-MS and data collection were performed as described previously **[23, 24]**. Briefly, 1-µl aliquot of derivatized sample was injected (splitless mode) into an Agilent 6980 GC equipped with a 10 m or 30 m fused-silica capillary column chemically bonded with 0.18 mm DB5-MS stationary phase (J&W Scientific, Folsom, CA). The column effluent was introduced into the ion source of a Pegasus III TOFMS instrument (Leco Corp., St Joseph, MI). Masses were recorded from m/z 50 to 800 at a rate of 30 spectra s^{-1}, and files of acquired samples were exported to MATLAB 7.3 (R2006b) (Mathworks, Natick, MA) in NetCDF format for further data processing and analysis. All data from pre-treatment procedures, including baseline correction, chromatogram alignment, setting of time windows, and hierarchical multivariate curve resolution (H-MCR) were performed in MATLAB using custom scripts **[25]**. Alignment and smoothing using a moving average were carried out before H-MCR was used to resolve pure chromatographic and spectral profiles. Before multivariate modelling, all peak areas were normalized to the peak areas of 11 internal standards eluting over the entire chromatographic time range. Mass spectra of all detected compounds were compared with spectra in the NIST+ library, using the in-house mass spectrum library database established by Umeå Plant Science Center (UPSC). Although metabolites can be involved in several biological processes, for a functional interpretation of the results were grouped in metabolic pathways (carbohydrate, energy, nucleotide, lipid, amino acid and others).

Data mining

The H-MCR-processed GC-MS data were mean-centred and scaled to unit variance prior to multivariate data analysis. Principal component analysis (PCA) was used initially to study the main variation in the data. In a second step, orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed to model the systematic variation in the metabolomic data related and orthogonal to predefined sample classes among the samples, specifically infected vs. non-infected, cells vs. supernatants, 0h vs. 36h. Cross-validation was used to determine the predictive ability of the models. The OPLS-DA model loadings were used to highlight significant metabolites associated with detected class differences. All multivariate analyses were carried out with SIMCA-P software (version 15.0; Umetrics AB, Umeå, Sweden).

RESULTS & DISCUSSION

CS has a negative impact on human health and smoking cigarettes are related with a poor outcome on TB disease. Tobacco companies advert e-cigs as a healthier alternative, in part based on the lack of epidemiological data relating e-vapor and respiratory diseases. In this study, we evaluated the general impact of smoking and vaping on metabolomics in THP-1 macrophages infected by *M. bovis* BCG. Results showed that e-vapor altered metabolomics in our model even more than CS, and could impair some metabolic pathways in a similar way than CS.

We exposed THP-1 macrophages to e-vapor or CS and then infected with *M. bovis* BCG. Immediately after infection (time 0h) and 36 hours later, extracellular media of the culture flasks and cells detached were stored to study metabolomics on the supernatants and intracellularly. Cells were diluted or concentrated to obtain the same number of cells in all the samples, counting with trypan blue exclusion test, to make the samples comparable.

GC-MS metabolomics was used in a semi-quantitative mode (isotope standard was not provided for each metabolite). When PCA analysis for extracellular metabolites was carried out, we observed that samples were separated into two groups: 0h and 36h (Figure 1). The second component of the analysis, separated samples exposed to e-vapor from samples exposed to CS or unexposed (Figure 1). RPMI medium controls without cells were also separated from the samples (Figure 1).

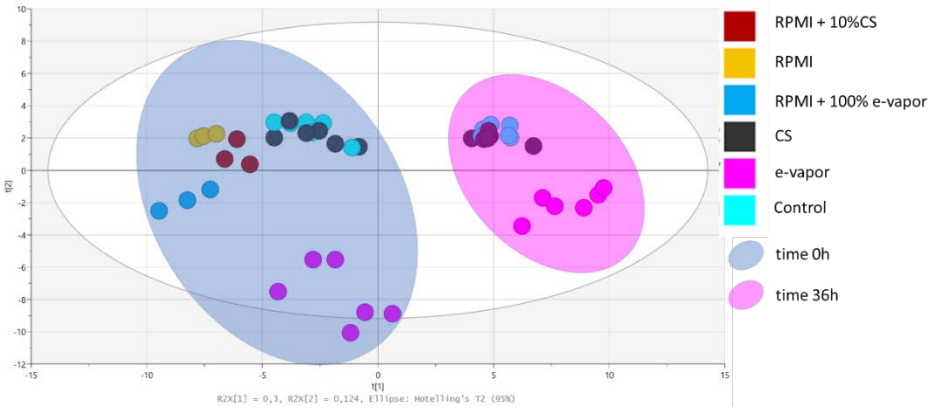

Figure 1. PCA analysis of extracellular metabolites. Samples were mainly separated by the time of the experiment, and a secondary separation was made by e-vapor exposure. RPMI medium without cells (RPMI + 10%CS; RPMI; RPMI +100% e-vapor) were all grouped together in the left of the plot.

We performed an OPLS-DA analysis of samples at time 36h. Three groups were separated: samples exposed to e-vapor, samples exposed to CS and samples unexposed (Figure 2).

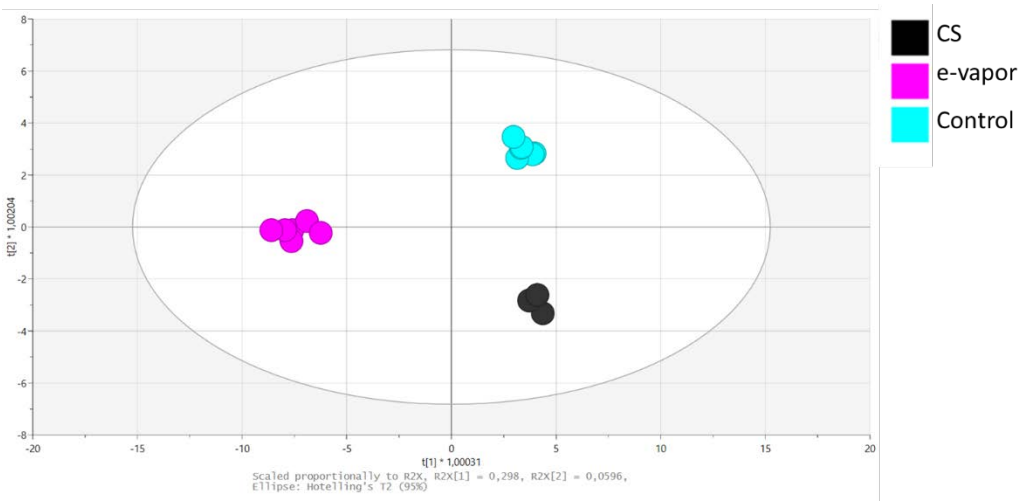

Figure 2. OPLS-DA analysis of extracellular metabolites of time 36h. Supernatants were separated by their metabolomic in three groups: samples exposed to e-vapor, CS or non-exposed. *P*-value 0.006.

The compounds that mostly contributed to e-vapor separation were: lactic acid, nicotine, benzoic acid and glycerol. Glycerol is the main compound of the e-liquid, and nicotine is present in the e-liquid at a concentration of 8mg/g. Benzene is reported to be formed from propylene glycol and glycerol, and sometimes benzoic is also part of the additives of the e-liquid **[26]**. Propylene glycol, when it is administrated intravenously in relatively high doses, can provoke lactic acidosis **[27]**. Furthermore, glycerol could be oxidized to dihydroxyacetone and lactic acid **[28]**. Nevertheless, the biggest differences between samples are not related with exposure to fumes or infection, but with the day of infection, suggesting that cell release of compounds and consumption of metabolites are the main changes we can observe (Figure 1 & 3). Our results suggest that e-vapor exposure makes media composition more different in a particular time point than any other condition we tested (exposure to CS, or the infection state) (Figure 1). A possible explanation that makes e-vapor supernatants more different than CS supernatants, compared to the control, is that CS contains many metabolites (more than 5,000) in small concentrations that probably are not on the library (that contains only 928 metabolites) or can not be detected.

E-vapor alteration of the metabolome was also observed in intracellular analysis. General analysis revealed 105 intracellular metabolites. The results were manually

cured: internal standards (14), metabolites with low-grade chromatographic peaks and suboptimal mass spectrum match score (under 700) were removed. The resulting list of 63 metabolites was used for further analysis. Uninfected samples exposed to e-vapor were excluded from the analysis due to a lack of metabolites detected.

PCA analysis of intracellular metabolites showed a clear separation between samples of 0h and 36h. Infected samples at time 0h non-exposed to smoke were the most similar samples to samples of 36h, although the second component of the analysis separated both groups (Figure 3.A). There were more metabolites detected at 36h. Samples were not sub-separated between infected -uninfected or between CS-e-vapor exposure (not shown).

If we considered only the infected samples, there is a clear separation on day 0 (immediately after the samples are infected) of samples exposed to e-vapor, CS and non-exposed (Figure 3.B).

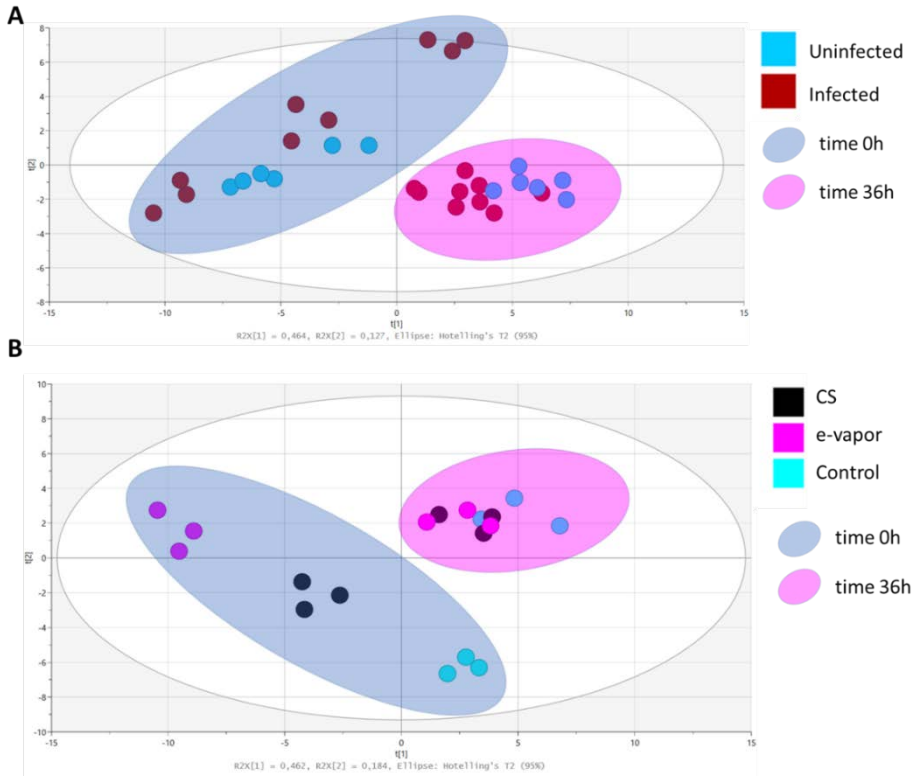

Figure 3. PCA analysis of intracellular metabolites. A. When PCA was carried out in all samples, a separation between samples of time 0h and 36h in the main component of the analysis was observed. B. When PCA was carried out only in infected samples, samples of time 0h were separated in different groups (e-vapor, CS, non-exposed) but not samples of time 36h.

Differences in metabolomics between samples exposed to CS or e-vapor and control at initial time are less evident at final time (36h from the last fume exposure), in which PCA analysis could not identify differences between the groups (Figure 3.B). Similar acute effects have also been observed in human bronchial epithelial cells exposed to CS or e-cig liquid and A549 cells exposed to CS, where the maximal deviation of the metabolome from untreated cells is produced at 1 or 2 hours after CS exposure **[8, 15, 29]**. Our results also suggest that CS or e-vapor provokes changes in metabolites immediately after exposure (Figure 3.B).

Regarding **carbohydrate metabolism**, we observed a reduction in glycerol-2-phosphate, glycerol-3-phosphate and ribose-5-phosphate in samples exposed to CS or e-vapor compared to control. Sorbitol and lactic acid were increased in non-infected samples exposed to CS at time 36h compared to the equivalent non-exposed. A similar decrease in metabolites of glycolysis and pentose phosphate pathway and increase in lactic acid and sorbitol were observed in A549 cells **[15]**. Similar to Vulimiri *et al*. **[15]**, we also observed levels of fructose and sorbitol changed in a consistent manner in samples uninfected exposed to CS from time 0h to 36h, sorbitol level is elevated at initial time probably as a response to CS stress, and then decreases, indicating that sorbitol likely generates fructose by sorbitol dehydrogenase. We did not observed the same tendency in infected samples. Aug *et al*. **[29]** observed that glucose was depleted and ATP and Krebs metabolites were increased in cells exposed to CS, reflecting the energy demand of the cellular recovery process. **Energy metabolism** was mostly unchanged in CS or e-vapor uninfected samples. Succinic acid, malic acid and isocitric acid were detected in similar concentrations than in THP-1 cells unexposed to CS or e-vapor, although citric acid was found higher in samples exposed to CS or e-vapor. Other studies found elevated several citric acid metabolites in samples exposed to CS **[15]**. However, these compounds were in lower concentrations in samples exposed to CS or e-vapor compared to the control when they were infected. We observed a reduction in **nucleotide metabolism** in CS/e-vapor samples, both in purines (hypoxanthine) and pyrimidines (uracil). Other studies found an increase in pyrimidines but a decrease in purines **[15]**. Regarding **amino acid metabolism**, glutamic acid was decreased in infected samples exposed to CS or e-vapor. Hsu *et al*. **[9]** observed that glutamate, a key nutrient in many metabolic pathways and regulating amino acid and carbohydrate metabolism, was significantly reduced in smokers. In a previous study, glutamate, glutamine, proline, histidine and arginine were found to be increased by the exposition to e-cig liquid in human bronchial epithelial cells **[8]**. Depletion of glutathione was also

found in previous studies in A549 and BEAS-2B cells lines in the first hours of contact with CS, highlighting the oxidative effects of smoke [8]. We did not detect glutathione in our analysis.

We also analysed the trend in same conditions between 0h and 36h. A difference in a level of certain metabolite between time points was considered valid when the average value of 3 (RSD <30) independent samples in a time point differed from another. Based on this algorithm, 3 groups of metabolites were observed: one increasing from 0h to 36h, and two decreasing from 0h to 36h (Table 1).

Table 1. Metabolites detected by GS-MS that increased or decreased in all samples or only in infected ones from day 0 to 1. Group 1 includes 13 metabolites (20.6% of all identified metabolites) that increase in all conditions. In Group 2 metabolites decrease on infected samples exposed to CS/e-vapor compared to the control, and the opposite in Group 3. Some metabolites could be included in more than one metabolic pathway.

	Group 1. Increase in all samples	Group 2. Decrease in infected samples exposed to CS/e-vapor	Group 3. Decrease in infected samples non-exposed to CS/e-vapor
Amino acid metabolism	β-Alanine, lysine	2-aminobutyric acid, maleic acid, S-methyl-cysteine, tyrosine	tryptophan, glutamic acid, sarcosine
Metabolism of other amino acids	hypotaurine, 1,5-diaminopentane		pyroglutamic acid
Carbohydrate metabolism	lactic acid, glycerol-3-phosphate, glycerol-2-phosphate, uridine 5-diphospho-N-acetylglucosamine	myo-inositol, scyllo-inositol, fructose,	Ribose
Energy metabolism	malic acid, citric acid	succinic acid	
Lipid metabolism	o-phosphoetanolamine	nonanoic acid	
Nucleotide metabolism	hypoxanthine		Uracil
Cofactor and vitamin metabolism	nicotinamide		
Others		phosphate-fragment	

Group 1 are metabolites that increase in all conditions, being putatively involved in the normal maturation of the cell culture, and includes lactic, malic and citric acids. Carbohydrate metabolism and energy metabolism increased during the time that the experiment lasted. Previous studies showed that on Chinese hamster ovary cells, intracellular levels of citric acid, lysine and alanine are increased, and lactate extracellular levels showed an increased in the first 24 hours [30]. The accumulation of

citric acid could be due to an altered Krebs cycle, as already described on activated macrophages **[31]**. Carbohydrate metabolism is upregulated on activated macrophages for the production of citrate. Citrate activates the lipid synthesis, important for the activation of macrophages, the production of inflammatory mediators and to allow membrane expansion needed for antigen presentation **[31, 32]**.

Groups 2 and 3 show a decrease or increase on metabolites of infected samples exposed to CS or e-vapor compared to non-exposed infected samples. Group 2 includes maleic, succinic acids, amino acids tyrosine and cysteine, *myo*- and *scyllo*-inositol while Group 3 includes tryptophan, glutamic and pyroglutamic acids, uracil and ribose (Table 1). A total of 50% of the metabolites found on these groups are part of the amino acids metabolism. Amino acids play a key role in the host-pathogen interaction, as pathogens depend on the host nutrients for their survival, and host try to keep away pathogens from their nutrients, including not only amino acids but transition metals **[33, 34]**.

An important amino acid described before in the host-pathogen interaction is tryptophan **[34, 35]**. We found that intracellular levels of tryptophan increased in uninfected THP-1 cells non-exposed to CS nor e-vapor, while decreased in uninfected THP-1 cells (Figure 4). Intracellular levels of tryptophan increased in THP-1 cells exposed to CS or e-vapor, regardless if they were infected or not. Metabolites in cells uninfected exposed to e-vapor could not be measured.

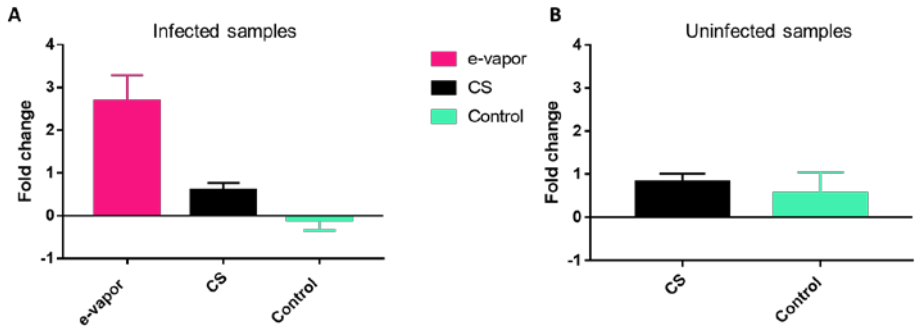

Figure 4. Fold change of intracellular tryptophan from 0h to 36h. A. Fold in infected samples and B. Fold in uninfected samples. Fold change was calculated as (B/A)-1, where B is the level of tryptophan at 36h and A at 0h. Control refers to THP-1 nonexposed to e-vapor or CS.

Levels of tryptophan increased during the day and a half of the experiment in uninfected samples probably due to active biosynthesis (Figure 4.B). In infected THP-1 cells control, level of tryptophan could be decreased as a protection strategy against the pathogen (Figure 4.A). Although *M. tuberculosis* constitutively synthesizes its own amino acids, these also play important functions regulating the immune system, as activating TLR signalling, Th1 or Th17 responses and secreting immunoglobulin A **[35]**. Amino acid starvation is also important to activate autophagy, a highly conserved cellular mechanism that triggers nutrient recycling but also enhances targeting and degradation of intracellular pathogens **[35]**. However, tryptophan level continued increasing in infected cells exposed to CS or e-vapor (Figure 4.A). Possibly the exposure to CS or e-vapor disrupts the protection strategy. A small change in an intracellular concentration of amino acid after infection, caused both by pathogen and host, has important effects on the outcome of the infection **[35]**.

We expected that indoleamine 2,3-dioxygenase (IDO), the enzyme that catalyses the reaction tryptophan to kynurenine, could be implicated in this process. The expression of IDO is regulated by TNF-α, IFN-γ or prostaglandins **[35]**. In **Chapter III** it was discussed that both CS and e-vapor dysregulate the expression of TNF-α. *Mycobacterium avium* subsp. *paratuberculosis* infection promotes IDO expression in human monocytes, decreasing tryptophan in those cells **[36]**. IDO has an antimicrobial activity by depleting tryptophan from the cells, but also balances the immunomodulatory response by its product kynurenine **[37]**. IDO activation restricts the growth of *M. avium* **[38]**. It has been observed in several studies that IDO activity is lower in smokers and, thus, the immunosuppression dependent on IDO **[39, 40]**.

As kynurenine was not detected in the first metabolomic screening, we decided to use a longer column (30m) for obtaining a better resolution of the metabolites. Tryptophan and kynurenine levels are shown in Figure 5.

As IDO is activated in infected macrophages, level of kynurenine increased during the experiment while the level of tryptophan was reduced (Figure 5). However, IDO activity was decreased on infected THP-1 cells exposed to CS, kynurenine was not increasing in the 36h of the experiment and levels of tryptophan were increased from 0h to 36h (Figure 5).

Kynurenine is a potent negative regulator of inflammation and T cell activity, enhancing the generation of regulatory T cells (Treg), promoting apoptosis and inhibiting ROS

production [34, 35]. Enhancement of IDO activity could reduce inflammation, typically associated with tobacco cigarette smoking [37].

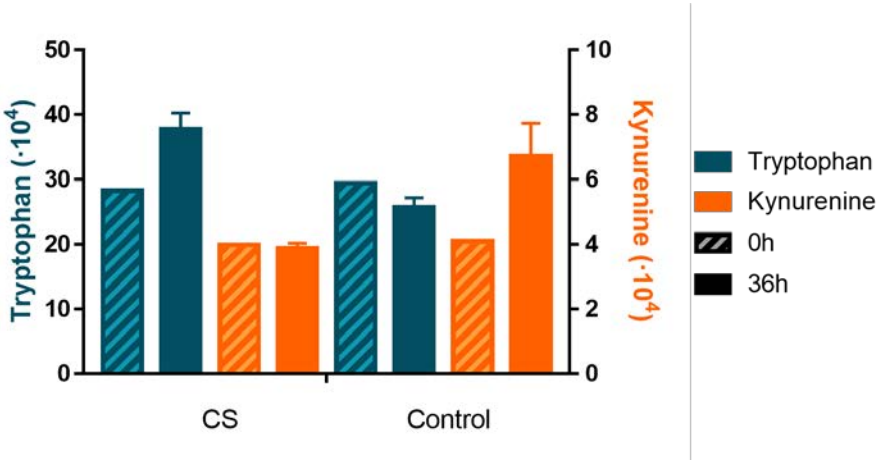

Figure 5. Levels of tryptophan and kynurenine in THP-1 cells infected with BCG. IDO catalyses the reaction tryptophan (substrate) to kynurenine (product). IDO activity was reduced on THP-1 cells exposed to CS. Stripped columns showed time 0h, solid columns showed time 36h, blue columns levels of tryptophan (left Y-axis) and orange columns levels of kynurenine (right Y-axis).

In conclusion, our results show the profound impact of e-vapor on metabolomics in supernatants of THP-1 cells, even more than CS or infection stress. E-vapor also altered the intracellular metabolomics profile, and some of their effects are comparable to CS. We observed that the protection strategy of tryptophan depletion by the host against pathogens is impaired by CS or e-vapor, and impairment in the immune system is suggested by abnormal kynurenine concentrations. Further studies focusing on chronicity of e-vapor use could reveal if the changes observed are maintained over time, supporting the hypothesis that e-cigs should be avoided.

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4.5 Chapter V: Effects of cigarette smoke on the administration of isoniazid and rifampicin to macrophages infected with *Mycobacterium tuberculosis*

ABSTRACT

Smoking and tuberculosis are both behind of more than eight million deaths every year. Smoking is a risk factor for tuberculosis infection and mortality, and it is associated with tuberculosis treatment default. Cytotoxicity of THP-1, J774A.1 and MH-S macrophages was evaluated with the reference and a commercial cigarette. THP-1 was the cell line most resistant to the toxic effects of smoke. THP-1 cells were exposed to cigarette smoke, infected with *Mycobacterium tuberculosis* and treated with anti-tuberculosis drugs. Intracellular recovery of CFUs was lower in cells exposed to cigarette smoke extract due to an impairment in the phagocytosis. Macrophages treated with drugs showed a reduction in the intracellular burden compared with non-treated. The same pattern applied to the recovery of extracellular CFUs except for rifampicin. We detected less concentration of rifampicin in smoked samples, due to a potential interaction with cigarette smoke. Smoking interferes with tuberculosis treatment impairing the immunity of the host.

INTRODUCTION

Smoking is the second global cause of death, being responsible for the death of over 20% of adult men and 5% of women **[1, 2]**. Smoking is generally associated with a lower incoming status, overcrowded places and malnutrition, characteristics that correlate as well with tuberculosis (TB) **[3]**. The majority of smokers (80%) live in countries with a high prevalence of TB, which probably makes smoking the largest risk factor for TB **[4]**. Studies reveal a fold increase in the risk of TB among current smokers, in a dose-response relationship with the number of cigarettes smoked per day and the number of years smoking **[5]**. In fact, there is an association between smoking and a positive tuberculin skin test (TST) **[6]** and it has been observed that smoking is a risk factor for TB infection, active TB disease and TB mortality **[7]**. The risk of transmission is also increased because smoking induces coughing, which could cause delays in the diagnosis of TB among smokers **[8]** and because there is a delayed time of sputum

conversion culture in smokers **[9, 10]**. Data suggests that aggressive tobacco control policies could avoid 27 million TB deaths by 2050 **[11]**.
Mainstream cigarette smoke (the smoke inhaled and exhaled by smokers) contains more than 6,000 compounds as well as 10^{15} free radicals per puff in the gas phase, 10^{17} free radicals per gram in the tar (tobacco particulate residues) phase and 15,000-40,000 µg of particulate matter per cigarette **[12, 13]**. *In vitro* and *in vivo* murine assays studying the effects of cigarette smoke on *Mycobacterium tuberculosis* control shows that the ability to the contention of the bacilli is compromised **[4, 5, 14-16]**.

Recently, smoking has been found as an additional risk factor not only for TB infection but for drug-resistant tuberculosis (DR-TB) infection or multidrug-resistant tuberculosis (MDR-TB) infection **[17]**. It is called MDR-TB when *M. tuberculosis* strain is resistant to at least isoniazid (INH) and rifampicin (RIF) **[18]**. Some studies correlate smoking with treatment default, suggesting the importance of quitting smoking for TB treatment. For example, the risk of a poor TB treatment outcome is 70% greater in smokers compared to non-smokers in Georgia **[8]**. The risk of treatment failure is 7.5 times greater in smokers in Malaysia **[19]**. Smear conversion rates were lower in smokers, followed by ex-smokers and non-smokers in Iran **[20]**. Similar results were found in Qatar, where smokers have 6 times more positive sputum **[21]**. Furthermore, it has been found, in a clinical study in Germany, that the impact of smoking or having MDR-TB had a similar effect in delaying culture conversion **[22]**. Despite the epidemiological evidence, why smokers have an increased risk of treatment default is not well understood. In the present study, we focus on the effects of cigarette smoke on an infected cell culture treated with anti-mycobacterial drugs.

MATERIALS AND METHODS

Cell cultures and mycobacteria growth conditions

The human monocytes THP-1 (ATCC TIB-202™) and the murine alveolar macrophages MH-S (ATCC CRL-2019™) were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMax (Gibco, Thermo Fisher Scientific, MA, USA) at 37°C in 5% CO_2. Murine macrophages J774A.1 (ATCC TIB-67™) were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose GlutaMax (Gibco, Thermo Fisher Scientific, MA, USA) at 37°C in 10% CO_2. All media were supplemented with 10% heat-inactivated fetal bovine serum. Cells were passaged every 3 days. THP-1 monocytes were stimulated to macrophages using 0.1 µM of Phorbol 12-Myristate 13-Acetate

(PMA, Sigma-Aldrich, St Louis, MO, USA) for 72 hours and RPMI medium was changed 24 hours before the experiments.

M. tuberculosis H37Rv, *M. bovis* and *M. bovis* BCG expressing green fluorescent protein (BCG-GFP) strains were grown in Middlebrook 7H9 supplemented with 0.05% tween 80 and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). For *M. bovis* BCG-GFP, 0.08% of glycerol and 20 µg/mL of kanamycin was also added. The mycobacteria cultures were incubated at 37°C. *M. bovis* BCG-GFP was kindly provided by Carlos Martin from University of Zaragoza, Spain.

Cigarette smoke extraction

Cigarette smoke extract (CSE) was prepared from commercial cigarettes (Marlboro: 10 mg Tar, 0.8 mg Nicotine, Philip Morris Sàrl Neuchâtel, Switzerland) or reference cigarettes (3R4F: 9.4 mg Tar, 0.73 mg Nicotine, University of Kentucky) as was previously reported **[23]**. Briefly, one cigarette was combusted using a syringe-modified apparatus, which draws the smoke into a sterile glass containing 10 mL of RPMI or DMEM. Sixty millilitres of smoke was extracted for 10 sec following a 30 sec break; this process was repeated six times per cigarette and the CS extract was sterilized using a 0.22 µm filter. The resulting solution was considered as 100% of CS extract. The absorbance at 320nm was measured for each batch to assure reproducibility and adjusted to 1.2 ± 0.2 **[24, 25]**. The working solution was 10% CS extract, equivalent to the smoke of 0.5-2 packs of cigarettes per day **[26]**. For each experiment, fresh extract was used and added to the cultures within 30 min of preparation.

Preparation of antimicrobials agents

Ethambutol, INH, RIF, levofloxacin, ethionamide and D-cycloserine were purchased to Sigma-Aldrich (St Louis, MO, USA). Amikacin and streptomycin were purchased to Apollo Chemicals Ltd (Tamworth, UK). Stock solutions of INH (4 mg/mL), amikacin (1.6 mg/mL), ethambutol (3.2 mg/mL), streptomycin (0.4 mg/mL) and D-Cycloserine (25.6 mg/mL) were prepared in distilled water. RIF (6.4 mg/mL) was prepared in methanol and then diluted in distilled water until 0.512 mg/mL. Levofloxacin (0.8 mg/mL) was prepared in 0.1 N NaOH and ethionamide (1.92 mg/mL) in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA). Stock solutions were sterilized using 0.22 µm filters and stored at -20ºC.

Cytotoxicity assay

To determine the cytotoxic effect of CSE on macrophages, PrestoBlue (Invitrogen, San Diego, USA) and EZ4U (Biomedica, Vienna, Austria) assays were used following the manufacturer's instructions. For PrestoBlue experiments, cells were seeded at a concentration of 5×10^4 cells in 96-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA). Plates were incubated for 24h, and then CSE were added and incubate for 3 hours. Absorbance was measured after 3h of contact with CSE and on days 1, 2, 3 and 6. Before measuring, monolayers were washed twice with Dulbecco's PBS (DPBS) and 100 µl of RPMI and 10 µl of PrestoBlue were added. Absorbance was read after 1 hour incubation at 570nm. EZ4U assay was conducted as reported previously **[27]**, making dilutions of CSE.

Mycobacteria growth curve

M. tuberculosis H37Rv was adjusted to OD_{600nm} 0.01 in RPMI supplemented with 10% FBS and 0.05% tween, with or without 10% CS extract. The absorbance was measured every 24 hours for two weeks.

MIC determination

Minimal inhibitory concentrations (MIC) of RIF (0.004-0.128 µg/mL), INH (0.03125-1 µg/mL), amikacin (0.125-4 µg/mL), ethambutol (0.25-8 µg/mL), streptomycin (0.03125-1 µg/mL), levofloxacin (0.0625-2 µg/mL), ethionamide (0.06-1.92 µg/mL) and D-cycloserine (2-64 µg/mL) were tested as previously described **[28]**. Briefly, mid-log phase *M. tuberculosis* cultures were diluted in 7H9 supplemented with ADC and 0,2% glycerol with or without 10% Marlboro CSE to a final OD_{600nm} of 0.01. Aliquots of 100 µl were added to each well of a 96-well plate containing 100 µl of 2-fold serial dilutions of each antibiotic in duplicate. Sterility (no bacteria) and growth (no antibiotic) controls were also prepared. Thirty microliters of resazurin (0.01%) were added after seven days of incubation at 37ºC. Plates were incubated for additional 24 h. The visual MIC was determined as the lowest antibiotic concentration which prevented colour change, from blue (oxidized state) to pink (reduced state).

Antibiotic activity on THP-1 infected and treated with CSE

Infected THP-1 cells were treated as previously reported **[27]** with the following modifications. Briefly, 3×10^5 cells per well were PMA stimulated and seeded in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA). Freshly prepared

10% CSE was added to the cells and incubated for 3 hours at 37°C in 5% CO_2. After 72 h of infection, plates were washed 3 times with DPBS and then it was added 1ml of RPMI containing rifampicin and isoniazid at 4 and 24 µg/ml for RIF and 5 and 7 µg/ml for INH in presence or absence of CSE. The drug concentrations used were chosen according to minimum and maximum concentrations in human serum **[27, 29]**. Intracellular growth of *M. tuberculosis* was assessed by lysis of the monolayers by the addition of 500 µL of water followed by a 30 min incubation at room temperature and serial dilution in PBS-tween plating onto Middlebrook 7H10 solid medium at days 0, 2 and 4. Colonies were counted after 3–4 weeks incubation at 37 °C and the average CFUs/mL determined.

Cell viability of infected cells via flow cytometry

Infection protocol was followed as described before, but infecting with BCG-GFP at a MOI of 10. At day 0 and 6 phagocytosis was analysed as follows: a wash was made with 1 ml DPBS before detaching cells by adding trypsin (Gibco, Thermo Fisher Scientific, MA, USA) for 10 min at 37ºC and neutralize with RPMI. Then, suspensions were centrifuged 1 min 2,000 rpm and the supernatant discarded. The staining was performed by adding 100 µl of Annexin Binding Buffer freshly prepared, 1.5 µL of Annexin V-PE and 3 µL of 7-Aminoactinomycin D (7-AAD) (PE Annexin V Apoptosis detection kit, BD Pharmigen, CA, USA). Cells were incubated at RT in darkness for 15 min and then centrifuged 5 min 2,000 rpm. The supernatants were eliminated and cells were fixed with 200 µL 4% paraformaldehyde for 30 min at RT. Finally, cells were centrifuged 5 min at 1,500 rpm and resuspended in 200 µL of DPBS. The samples were then read on a FACSCanto II (BD Biosciences). At least ten thousand events were collected and BD FACSDiva 7.0 software (Franklin Lakes, NJ, USA) was used to analyse the results.

RIF quantification by Mass spectrometry

Additionally, supernatants and intracellular of day 4 medium were recollected to perform assays by ultra-performance liquid chromatography (uPLC) (Agilent 1260 Infinity LC, California) and detection by triple quadrupole mass spectrometer (Agilent 6410BA). The analytical column used was Prontosil 120-5 C18 ACE 5µm 4,0x150mm (Bishchoff Chromatography, Germany). Briefly, calibrators (INH, RIF and omeprazole) and internal standard were spiked to 500 µl of culture cell medium to achieve the concentration ranges from 0 to 30 µg/mL in order to make the calibration curves. After adding the internal standard to the samples, these were pre- concentrated and purified

by using an SPE cartridge C_{18} BondElut (pH 4.5) (Agilent). The cartridge was preconditioned washing twice with 2 ml of methanol, 2 ml of water and 2 ml of 0.05M phosphate buffer pH 4.5. Then, the sample was added and after 5 min the cartridge was washed with 1 ml phosphate buffer. The elution phase was made with 1 ml of acetonitrile and 1 ml of methanol. After that, the sample was dried and reconstituted in 1 ml of 3% acetonitrile and 0.06% trifluoroacetic acid. uPLC elution phases were: phase A (3% acetonitrile, 0.3% trifluoroacetic acid) and phase B (80% acetonitrile and 0.1% trifluoroacetic acid).

Compounds were quantified with multiple reaction monitoring (MRM) by using the 823.4 units prec ion and 791.5 units prod ion for rifampicin. Results were analysed using the Mass Hunter software (Agilent, Atlanta, USA). Calibration curves and sample quantification was made by Quantitative software (Agilent, Atlanta, USA).

Statistical analyses

Statistical analyses were performed using the GraphPad PRISM 7.0 software package (San Diego, California, USA). Differences between conditions were compared by the Mann-Whitney test and Student's t-test. A *p*-value ≤ 0.05 was considered as statistically significant.

RESULTS

CSE 10% is not toxic in different cell lines

Macrophages THP-1, MH-S and J774A.1 were exposed to 10% CSE for 6 days. To check cytotoxicity in different cell lines, PrestoBlue assays were carried out at Marlboro 10% CSE. Results showed that MH-S and J774A.1 were less resistant to tobacco effects than THP-1 cells (Figure 1A), reason why all experiments were made with THP-1 cells. EZ4U assay was also used to test CSE cytotoxicity on THP-1 cells exposed to 3R4F and Marlboro 10% CSE. Results showed that 10% CSE is not toxic for the cell lines tested; only a mild decrease at day 6 was observed when 3R4F 10% CSE was used (Figure 1B).

Moreover, the results of cytotoxicity in THP-1 cells, dead and apoptotic cells were measured by flow cytometry, dying with Annexin V and 7-AAD, following the infection protocol. Apoptosis and cell death increased as the days passed (Figure 2). Uninfected cells tended to have lower rates of apoptosis than infected cells (Figure 2.A). Cells exposed to 10% CSE showed a reduction of apoptosis at days 3 and 6 compared with

unexposed (Figure 2.A). However, a higher percentage of cells were dead when they had been exposed to Marlboro 10% CSE for at least three days (Figure 2.B).

Cigarette smoke does not affect the growth of mycobacteria

M. tuberculosis H37Rv culture was exposed during two weeks to 10% of Marlboro or 3R4F CSE and absorbance was measured every 24 hours (OD_{600nm}). No significant changes were obtained, but a slight decrease in the growth of H37Rv strain exposed to 10% Marlboro CSE. No effect was seen on H37Rv 10% 3R4F CSE (Figure 3).

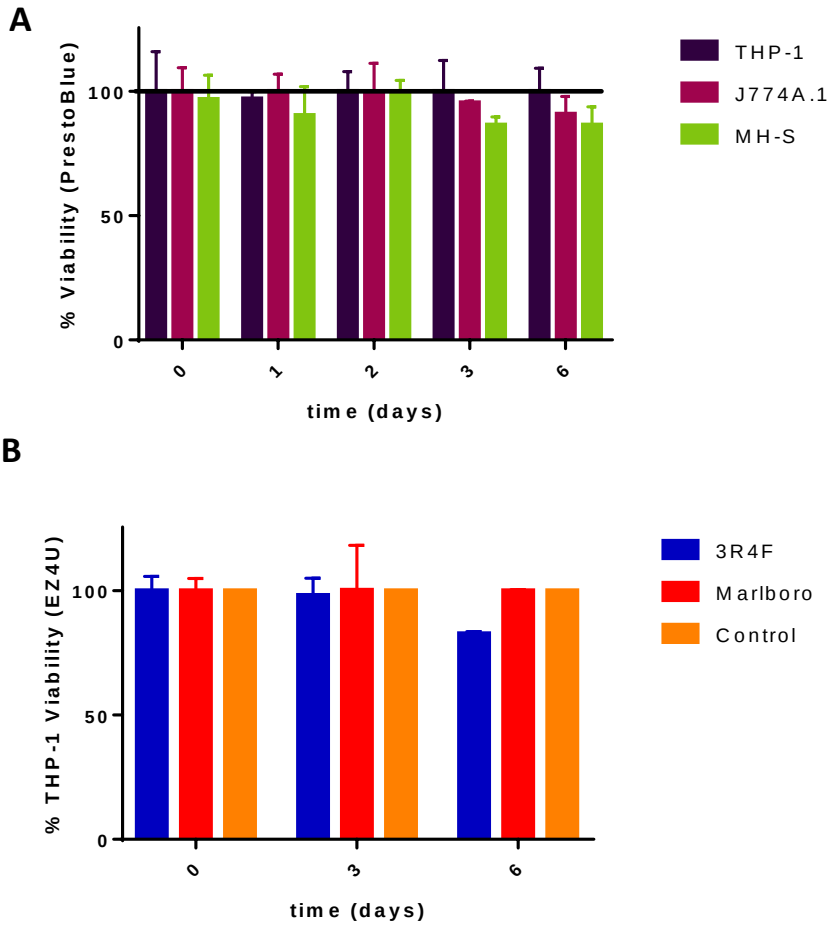

Figure 1. Cell viability after exposure to CSE. A. Viability of different cell lines after exposure to 10% Marlboro CSE. PrestoBlue test was performed at 3 hours (day 0), 1, 2, 3 and 6 days. Control is each cell line without exposure to 10% CSE and it is equivalent to 100%. B. THP-1 viability after exposure to 10% Marlboro or 10% 3R4F CSE. EZ4U test was performed at 4 hours (day 0), 3 and 6 days of exposure to 10% CSE. The results are expressed as the average and standard deviation of six replicates of at least three independent experiments.

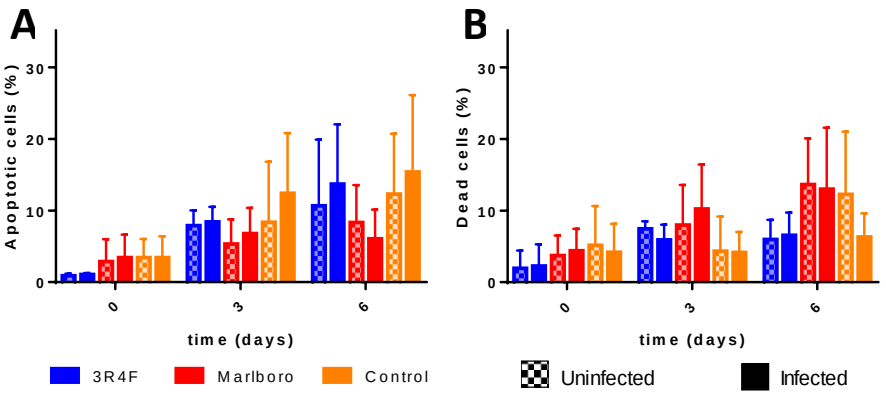

Figure 2. Viability of THP-1 cells after 10% CSE exposure analysed by flow cytometry. A. Percentage of apoptotic cells and B. Percentage of dead cells on days 0, 3 and 6 after exposure to 10% CSE and infected with BCG-GFP. The results are expressed as the average and standard deviation of at least two independent experiments.

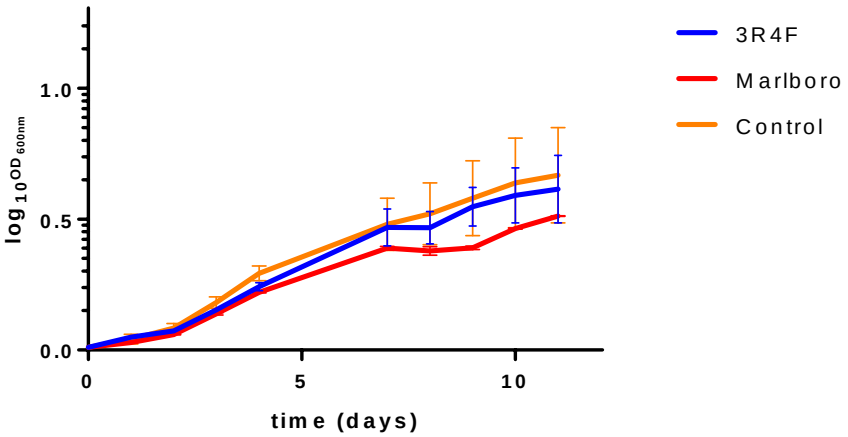

Figure 3. Growth of *M. tuberculosis* H37Rv in RPMI supplemented with 0.05% tween, exposed to 10% CSE extract. The results are expressed as the average and standard deviation of duplicates of at least two independent experiments.

Cigarette smoke affects the infection rates

THP-1 cells were infected with BCG-GFP and then analysed by flow cytometry at days 0 and 6. The percentage of infection was lower in cells exposed to 10% CSE compared to the control (Figure 4). As 3R4F CSE were more cytotoxic (Figure 1.B) and induced more apoptosis (Figure 2.A) than Marlboro CSE, while impairment of phagocytosis by

3R4F CSE was lower than by Marlboro CSE, we decided to conduct the next experiments only with Marlboro CSE.

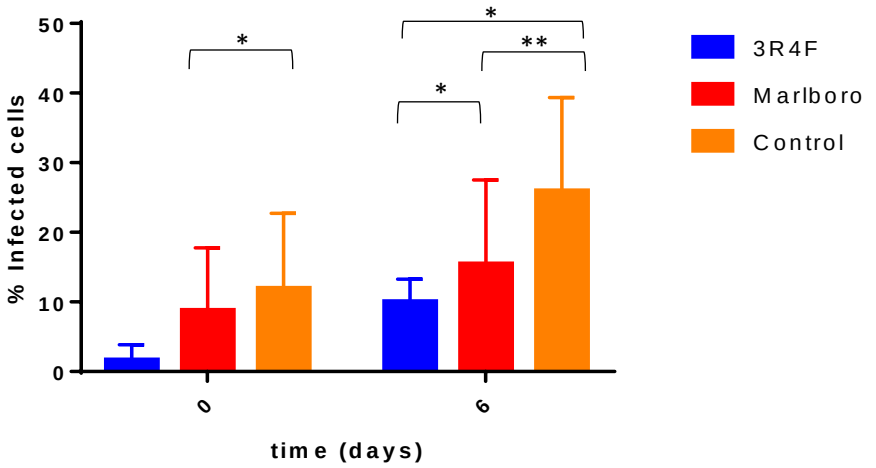

Figure 4. Infection rates after 10% CSE exposure analysed by flow cytometry. Percentage of infected cells on days 0 and 6 after exposure to 10% CSE and infected with BCG-GFP. The results are expressed as the average and standard deviation of at least two independent experiments. *P<0.05; **P<0.01.

Cigarette smoke does not affect the effect of antibiotics against mycobacteria

We determine the MIC of eight antituberculosis drugs against *M. tuberculosis* H37Rv exposed or not to 10% Marlboro CSE. No changes in the MICs were produced although for RIF the MIC was slightly higher in the case of bacteria exposed to 10% of CSE, going up from 0.064 (control) to 0.128 µg/ml (Table 1).

Table 1. Minimal inhibitory concentration (µg/ml) of eight antimicrobial drugs against *M. tuberculosis* H37Rv. *M. tuberculosis* was exposed to 10% Marlboro CSE and then to the drug. The results are expressed as the median and range (minimum-maximum) of duplicates of at least three independent experiments.

MIC	CSE	Control
Isoniazid	0.062 (0.062-0.125)	0.062 (0.062-0.125)
Rifampicin	0.128 (0.128)	0.064 (0.064)
Amikacin	0.5 (0.5-1)	0.5 (0.5-1)
Etambutol	1 (1)	1 (1)
Streptomycin	0.125 (0.125)	0.125 (0.125)
Levofloxacin	0.125 (0.125-0.25)	0.125 (0.125-0.25)
Ethionamide	0.48 (0.48-0.96)	0.48 (0.48-0.96)
D-cycloserine	8 (8)	8 (8)

Intracellular *M. tuberculosis* burden of THP-1 cells after administrate anti-TB drugs is modified by cigarette smoke exposure

We tested the efficacy of adding RIF and INH at two different concentrations in reducing *M. tuberculosis* H37Rv survival in THP-1 macrophages exposed to Marlboro CSE. When macrophages were treated with RIF, the recovery of CFUs was lower than in non-treated cells, whether they were exposed to CSE or not (Figure 5.A). If macrophages were exposed to CSE, the recovery of CFUs was lower than in non-CSE-exposed cells at days two and four (Figure 5.A). A similar trend was observed when macrophages were treated with INH. Recovery of CFUs were lower if macrophages were exposed to CSE or if INH was added to the infection assay, compared with the control non-exposed (Figure 5.B). The antimicrobial activity of the INH was higher than RIF at the concentrations tested since the survival of *M. tuberculosis* was lower in macrophages treated with INH than treated with RIF (Figure 5.A & B). On the other hand, when combinations of RIF and INH were added, the intracellular *M. tuberculosis* burden was lower than in non-treated cells (Figure 5.C). In a similar way, the recovery of CFUs in macrophages exposed to CSE was lower. However, the recovery of CFUs

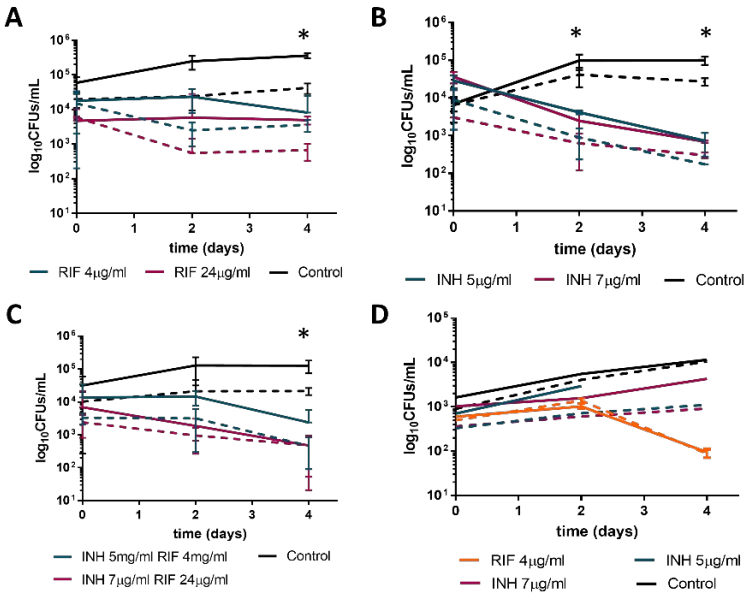

Figure 5. *M. tuberculosis* intracellular and extracellular CFUs counts over 4 days after drugs administration. Recovery of intracellular CFUs from THP-1 macrophages exposed to 10% Marlboro CSE and (A) RIF treatment, (B) INH treatment and (C) combinations of RIF and INH. (D) Extracellular *M. tuberculosis* recovery after exposure to CSE and RIF or INH. The continuous line corresponds with unexposed cells to CSE and the dashed line with exposed cells to CSE. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments. $P<0.05$

from macrophages treated with INH 7 or exposed to CSE tended to be the same at day four (Figure 5.C). Regarding extracellular bacteria, recovery of samples treated with RIF or INH was lower than in untreated samples (Figure 5.D). Samples treated with combinations of RIF and INH had no viable extracellular bacteria. Samples exposed to CSE tended to have lower recovery of CFUs (Figure 5.D).

The concentration of RIF in supernatants cultures does not change with cigarette smoke exposure

In order to measure the impact of CSE or infection on drugs, supernatants and cell lysates were collected and RIF was quantified by mass spectrometry. No significant differences were observed between infected cells and uninfected. RIF concentration was slightly higher in supernatants unexposed to CSE than exposed to CSE (Figure 6). The intracellular concentration of RIF could not be determined because it was below the detection limit.

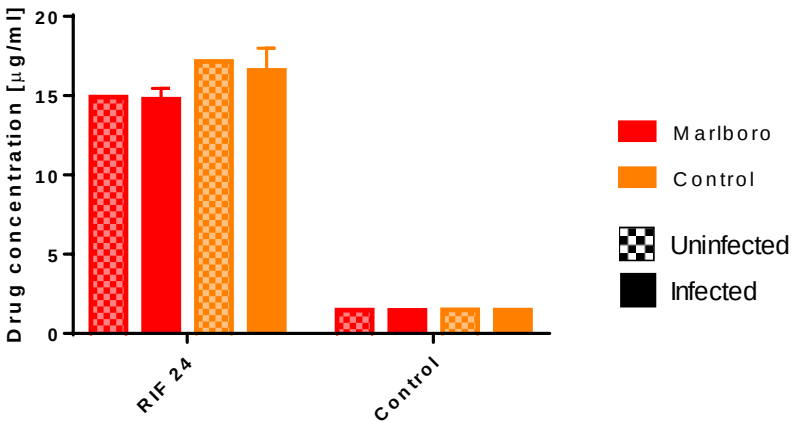

Figure 6. Extracellular RIF concentration of infected and non-infected THP-1 cultures. THP-1 macrophages were exposed to 24µg/ml RIF, 10% Marlboro extract and/or *M. tuberculosis*. Supernatants were collected four days after infection and analysed via mass spectrometry for quantification.

DISCUSSION

The relation between cigarette smoke and anti-TB therapy is not well understood. Smokers and secondhand smokers have an increased risk for LTBI and TB. The risk of treatment failure is also higher in smoking TB patients. In this study, we evaluated the impact of INH and RIF on THP-1 macrophages infected with *M. tuberculosis* treated

with tobacco smoke. Results showed that CSE had some toxic effects in only one cell line (MH-S), and no toxic effect on bacteria. We also observed that CSE modified intracellular *M. tuberculosis* burden even if anti-TB therapy was administered.

When we evaluated the effect of CSE on one human and two murine cell lines we observed different values for cytotoxicity, being THP-1 the most resistant. Some studies show the high cytotoxicity of CSE in cardiomyoblast cells or fibroblasts, suggesting that values higher than 12,5% would be toxics **[30-32]**. We tested the CSE effect in the viability of different cells lines before infection with *M. tuberculosis*, due to the different sensitivity previously described between cells lines. For example, Holownia *et al.* **[33]** did not detect cytotoxicity in THP-1 cells but found high cytotoxicity in human alveolar A549 cells at the same CSE concentration. Other studies have found the same kind of differences between cell lines **[34, 35]**. Additionally, we detected variability in the cytotoxicity between Marlboro brand cigarettes and the 3R4F Kentucky reference cigarettes. Moreover, other authors have reported different cytotoxicity and mutagenic potency between 3R4F and roll-your-own and factory-made commercial cigarettes **[36-38]**.

Apoptosis tended to be inhibited and cell death percentage was higher after three days of exposure to Marlboro CSE. This is in agreement with other publications, wherein alveolar epithelial cells and human umbilical vein endothelial cells (HUVEC) necrosis is induced and apoptosis inhibited **[39]**. Almost all mycobacteria can induce apoptosis, but virulent mycobacteria have mechanisms to evade apoptosis, since apoptosis is the host innate response against *M. tuberculosis* **[40]**. CSE would be acting in favour of the infection.

The effect of cigarette smoke seems less strong on bacteria than on cells, only finding a non-significant decrease in the mycobacteria growth ability compared to unexposed bacteria to CSE in RPMI medium. Although RPMI is not the optimal growth medium for *M. tuberculosis*, it is probably the most similar to *in vivo* experiments. We did not find an increase in the growth kinetics of *M. tuberculosis* exposed to CSE as a possible explanation of the poor treatment outcome of smoker TB patients although some of the compounds of CSE like nicotinic acid, nicotinamide or nicotinamide adenine dinucleotide have been reported to enhance the growth of *M. tuberculosis* **[41]**. The paradox that growth of the bacilli is not higher in the presence of CSE is in agreement with other publications where similar experiments have been done with *Staphylococcus aureus* **[42]**. However, in this study, authors supplemented

bronchoalveolar lavages (BAL) samples from non-smokers with CSE and found a higher *S. aureus* growth than in non-exposed samples at 30 min of exposure. They suggested that CSE impairs the BAL bacteriostatic properties because of a higher bioavailability of iron **[42]**.

One of the most important considerations between smokers and TB is the final outcome of the treatment. When we tested the most common TB drugs in infected cultures, RIF or INH, the intracellular *M. tuberculosis* burden tended to be lower in cultures exposed to CSE. An explanation for the paradox that CSE-drug treated cultures had lower CFUs than non-CSE ones is that smoke alters the phagocytic ability of the macrophage. An impairment of macrophage phagocytosis by CSE is described in different cell lines **[43-46]**. Furthermore, alveolar macrophages exposed to CSE show alveolar lysosomal accumulations of tobacco smoke and they lose the capacity to migrate to *M. tuberculosis* **[47]**.

Recovery of extracellular bacteria from macrophages was similar in samples exposed and unexposed to CSE. It would be expected that if CSE reduces phagocytosis, the extracellular bacteria would be higher in CSE samples. However, as we made intensive washings to remove extracellular bacteria, we eliminated the hypothetical differences between extracellular bacteria in samples exposed and non-exposed to CSE. This is in concordance with the fact that CSE affects the phagocytosis of macrophages but not the growth of the bacteria. The number of extracellular bacteria in media with antibiotic was lower than untreated samples.

On the other side, when we evaluated the MICs, we found that bacteria exposed to smoke and drugs had a slight increase in the MIC of RIF, which suggests a chemical interaction between smoke and RIF. Our results quantifying the RIF in the extracellular fraction of THP-1 macrophages showed higher amounts in cultures unexposed to CSE, suggesting less degradation. A great number of drug interactions have been associated with smoking because of pharmacokinetic interactions between nicotine and polycyclic aromatic compounds of tobacco, suggesting that smokers may need higher doses of drugs to reach similar effects **[48]**.

Although there are many epidemiologic studies which found smoking as an independent risk for DR-TB, many are observational studies that do not look for a correlation between CSE and the efficiency of the drugs, but with a poor treatment compliance in smoking TB patients, especially in males **[17, 49]**. A study focused on TB

patients in Russia found that smoking was associated with INH resistance, but no explanations were suggested for this association **[50]**.

Overall, cigarette smoke is a harmful combination of chemical compounds that interferes with TB treatment. The effect of smoke does not seem to interfere with the growth of the bacteria but impairs the immunity of the host, which makes that, despite applying the correct treatment, the outcome is worse than in non-smokers. This research is added to the clinical evidence that supports the relationship between smoking and MDR-TB, and especially to the evidence that smoking cessation should be considered as a part of the TB treatment.

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4.6 Chapter VI: Activity of mesoporous silica MCM-41 type nanoparticles on THP-1 macrophages infected with *M. tuberculosis versus* free rifampicin and isoniazid

ABSTRACT

Treatment against tuberculosis, a global epidemic with 10 million new cases every year, is long and requires many drugs with severe side effects, which lead to noncompliance and resistant tuberculosis. Many smokers are tuberculosis patients, and smoking is an additional risk factor for resistant tuberculosis and tuberculosis treatment default. Nanoparticles have emerged as a smart solution to reduce treatment doses, efficiently delivering the drug to the bacilli. In this study, we encapsulated rifampicin and isoniazid in MCM-41 mesoporous silica nanoparticles and studied their effect against THP-1 human macrophages infected with *Mycobacterium tuberculosis* exposed or not to cigarette smoke. The higher non-cytotoxic concentration of nanoparticles was determined as 75 µg/ml, and no effect on the growth of *M. tuberculosis* was detected at this concentration. When exposed to infected cultures, no lethal interaction nanoparticles – cigarette smoke was observed in this model. Moreover, administrating nanoparticles had no advantage compared to free drugs. Due to poor efficiency in drug loading and the high toxicity of the silica nanoparticles, made it impossible to increase the quantity of nanoparticles to the macrophage culture, arising as the major drawback of this study. Increasing the cargo of nanoparticles will probably lead to better results in future experiments.

INTRODUCTION

Every year about 10 million new tuberculosis (TB) cases are reported **[1]**. The aims of treating TB are to cure the patients and to minimize transmission; so, there is a global need for TB treatment success **[2]**. Although current treatment against TB is effective, its large duration is associated with severe side effects and noncompliance **[3]**. Nevertheless, not only adherence to the regimen matters, hydrophobic drugs like rifampicin (RIF) tend to have poor bioavailability or cannot reach the target by themselves **[4, 5]**. Furthermore, about 3.6% of new TB patients in the world have multidrug-resistant (MDR-)TB, and more alarming, around 20% of patients previously treated for TB are MDR-TB **[2]**. Resistance to the drugs is due to a reduced

permeability, enhanced efflux of drugs, enzymatic inactivation and/or modification of the drug target **[6]**. On the other hand, smoking is responsible for more than 15% of TB cases **[7]**. Cigarette smoke (CS) is an additional risk factor for drug-resistant and MDR-TB **[8]**, and some studies link smoking with TB treatment default **[9-11]**.

Nanoparticles (NPs) have recently arisen as a smart solution for maximizing the effect of drugs reducing to a minimum their side effects. The principle around NPs is based on the fact that macrophages efficiently internalize and deliver particles of very small size (50-200 nm) to acidified endosomes, therefore they access targets that otherwise would be very difficult to reach, and release the drug gradually **[3, 12, 13]**. These characteristics potentially enable reducing the doses and the frequency **[6]**. Encapsulation of drugs avoids systemic toxicity and protects the drugs against inactivation before reaching the target. NPs could be delivered via oral, parenteral or by inhalation. The oral route is the most convenient, cheap and has the greatest degree of compliance, although through this route NPs have to cross the thick barrier of the gastrointestinal tract, where they could be eliminated without being absorbed **[14]**, are potentially spread throughout the body maintaining many of the side-effects of free drugs, and could reach the target at sub-therapeutic levels **[3, 4]**. However, the gastrointestinal tract has an extensive surface area (300-400 m^2) for drug absorption and oral nanoparticles do not require sterility **[4]**.

Inorganic-based NPs, as mesoporous silica nanoparticles (MSNPs), are promising good candidates as nanocarriers. Size and pore structure are very customizable, they have high thermal stability and low-pH resistance, high pore volume and large surface area, enabling the accumulation of high doses of the drugs **[6, 14]**. Loading of both hydrophilic or hydrophobic drugs (like RIF) has been achieved **[3, 13]**. MSNPs provide protection for the drug against harsh environments in the body **[14]**. Furthermore, they can be functionalized with hydrophilic compounds like PEG (polyethylene glycol) to improve pharmacokinetic properties, such as drug targeting, a higher drug uptake, be more water-soluble or even enable a controlled drug release **[13, 14]**. Compared to other drug carriers, MSNPs are more blood stable than liposomes and polymeric NPs, and other biodegradable polymers such as poly(l-lactide-co-glycolide) (PLGA) **[13]**. As a proof of concept, MSNPs have been efficiently delivered to lysosomes and release the drugs intracellularly **[13, 15-18]**. In this study, we explore the effects of two different pore size MSNPs coated with PEG on human cells infected with *M. tuberculosis* exposed or unexposed to CS.

MATERIALS & METHODS

THP-1 cell line and mycobacteria growth conditions

The human monocytic cell line THP-1 (ATCC TIB-202™) was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMax (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, penicillin 10,000 units/ml, streptomycin 10 mg/ml and Fungizone® 25 µg/ml. The cells were passaged every 3 days. For assay conditions, RPMI without antibiotics was used. THP-1 monocytes were stimulated to macrophages using 0.1 µM of Phorbol 12-Myristate 13-Acetate (PMA, Sigma, St Louis, USA) for 72 hours (37°C in 5% CO_2). RPMI was replaced by fresh medium 24 hours before the experiments.

Mycobacterium tuberculosis H37Rv strain was grown in Middlebrook 7H9 supplemented with 0.05% tween 80 and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). Cultures were incubated at 37°C without agitation.

Preparation of nanoparticles

MSNPs (type MCM41) were synthesized in Universitat Politècnica de València and they were kindly provided by doctor Elena Aznar.

For isoniazid (INH)-NPs, 1 g of cetyltrimethylammonium bromide (CTAB) was dissolved in 480 mL of water at 50°C, 500 rpm. Then, 280 mg of sodium hydroxide were dissolved in 3.5 mL of water and mixed with the CTAB solution at 80°C. After that, 5 mL of tetraethyl orthosilicate (TEOS) were added with a syringe and needle dropwise (5 mL/min) stirring at 1200rpm for 2 h. Then, the suspension was cooled at room temperature, and the solid product was centrifuged and washed until reaching a neutral pH. After that, it was dried at 70 °C and grinded to obtain a white powder. To remove the surfactant (CTAB), nanoparticles were calcined at 550°C using an oxidant atmosphere for 5 h. One hundred milligrams of NPs and 60 mg of INH were resuspended in 5 ml of water for 24 h. Finally, they were vacuum filtered and dried.

For RIF-NPs, 0.96 g of cetyltrimethylammonium tosylate (CTAT) and 173.5 mg of triethanolamine (TEA) were resuspended in 50 ml of water at 80 °C for 1 hour, and then 7.8 ml of TEOS was quickly added into the surfactant solution. The mixture was stirred at 80 °C at 1000 rpm for another 2 hours. NPs were calcined at 550 °C using an oxidant atmosphere for 5 h to remove the CTAT. One hundred milligrams of NPs and

180 mg of RIF were resuspended in 5 ml of chloroform for 24 h. Finally, they were vacuum filtered and dried.

For the functionalization of NPs, 100 mg of NPs were resuspended in 5 ml of acetonitrile and 185 µL of 3-mercaptopropyl-trimethoxysilane. After stirring 5.5 h RT, 220.3 mg of 2,2'-dipyridyl disulfite was added and the mixture was stirred for an additional 12 h at room temperature (RT). The product was then filtered and dried under vacuum. Finally, 50 mg of this solid and 120 mg of PEG was suspended in 5 mL acetonitrile. After stirring for 12 h, the final material was isolated by centrifugation, washed with abundant water, dried under vacuum and grinded.

Efficiency load was 2.4 µg of RIF and 7.2 µg of INH per one mg of silica NPs.

Cigarette smoke extraction

CS extract was prepared from commercial cigarettes (Marlboro: 10 mg Tar, 0.8 mg Nicotine, Philip Morris Sàrl Neuchâtel, Switzerland) **[19]**. Briefly, one cigarette was combusted using a syringe-modified apparatus, which draws the smoke into a sterile glass containing 10 mL of RPMI. Sixty millilitres of smoke was extracted for 10 sec following a 30 sec break; this process was repeated six times per cigarette and the CS extract was sterilized using a 0.22 µm filter. The resulting solution was considered as 100% of CS extract. The absorbance (320nm) was measured for each batch to assure reproducibility and adjusted to 1.2 ± 0.2 **[20, 21]**. The working solution was 10% CS extract, equivalent to the smoke of 0.5-2 packs of cigarettes per day **[22]**. For each experiment, fresh extract was used and added to the cultures within 30 min of preparation.

Cytotoxicity Assay

To determinate the cytotoxic effect of the silica NPs on THP-1 macrophages, viable cells were counted with trypan blue in an optic microscope. THP-1 monocytes were activated with PMA and seeded at a concentration of 3×10^6 cells per well in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA) using RPMI. The plates were incubated for two days, the PMA was removed and macrophages were rested for one day. Different concentrations of NPs (0-600 µg/ml) were added to the wells and cells were incubated for four days. The results are expressed as viability percentage using cells untreated as a control (100% viability).

M. tuberculosis growth curve

M. tuberculosis H37Rv was adjusted to OD_{600nm} 0.01 in 7H9-tween medium (ADC supplemented) with NPs (75 µg/ml) or without. The absorbance was measured every 24 hours for two weeks.

MIC determination

Minimal inhibitory concentrations (MIC) of RIF-NPs and INH-NPs (18.75-600 µg/mL of NP) were tested as previously described **[23]**. Briefly, mid-log phase *M. tuberculosis* cultures were diluted in 7H9 supplemented with ADC and 0,2% glycerol with or without 10% CS to a final OD_{600nm} of 0.01. 100 µl of this culture were added to each well of a 96-well plate containing 100 µl of 2-fold serial dilutions of each antibiotic in duplicates. Sterility (no bacteria) and growth (no antibiotic) controls were also prepared. Thirty microliters of resazurin (0.01%) were added after seven days of incubation at 37ºC. Plates were incubated for additional 24 h. The visual MIC was determined as the lowest antibiotic concentration which prevented colour change, from blue (oxidized state) to pink (reduced state).

Macrophage infection with *M. tuberculosis* H37Rv

THP-1 macrophages were infected following a protocol previously reported **[24]**, with some modifications. Briefly, 3×10^5 cells per well were PMA stimulated and seeded in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA). CS extract was added to the cells and incubated for 3 hours. For the infection, mid-log phase *M. tuberculosis* H37Rv was washed twice with DPBS+0.05% tween and subsequently once with DPBS after which they stood for 5 min, before collecting the supernatant. Bacteria were then diluted in RPMI with or without CS and added to the THP-1 macrophages at a multiplicity of infection (MOI) of 0.1. After 3 h of contact at 37°C in 5% CO_2, the macrophages were treated with 200 µg/mL of amikacin for 1 h and washed three times with Dulbecco's PBS (DPBS) to eliminate any extracellular bacteria. Lastly, 1 mL of RPMI was added to each well and incubated at 37°C in 5% CO_2. After 72h, three additional intensive washings with DPBS were performed and NPs were added. Extracellular growth was assessed with serial dilution in PBS-tween plating onto Middlebrook 7H10 solid medium at day 4. Intracellular growth was assessed at days 0, 2 and 4 by lysis of the monolayers by the addition of 500 µL of water followed by a 30 min incubation at RT and then plating on 7H10 medium. Colonies were counted after 3–4 weeks incubation at 37 °C and the average CFUs/mL determined.

RESULTS & DISCUSSION

NPs are a promising strategy to deliver the cargo trapped in their pores, as they are avidly internalized by macrophages. We explored the effects of two different size-pore silica NPs in THP-1 macrophages infected with *M. tuberculosis*. We used as controls of the NPs efficiency cells exposed to free drug (INH or RIF) at the same concentration that the cargo of the NPs. Additionally, we performed the assays in the presence of CS to check if there were any negative effects.

Firstly, after the NPs were prepared they were visualized using Transmission Electronic Microscope (TEM) at Universitat Politècnica de València, as a control of the correct synthesis of the NPs (Figure 1).

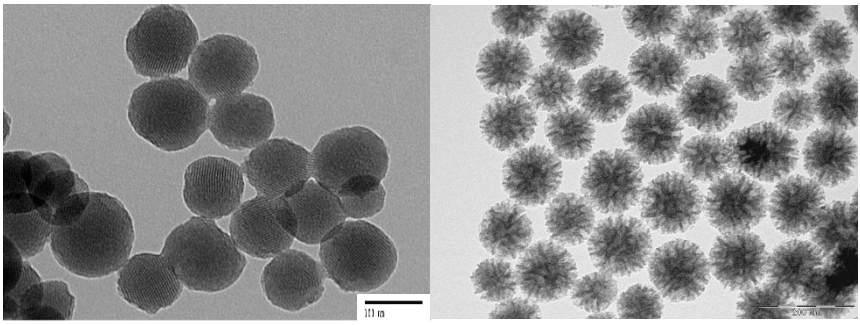

Figure 1. Transmission electronic microscopy of silica NPs at 40.000x. A. INH-NPs. The honeycomb-like structure of the NP is visualized with the parallel stripes and the hexagonally packed light dots. B. RIF-NPs. The images were kindly provided by Dr Elena Aznar.

THP-1 macrophages were exposed to 10% CS and INH or RIF-NPs (4.69-600 µg/ml of NP) during four days. To check viability, trypan blue exclusion assay was carried out. Results showed that viability decreased to less than 40% at 150 µg/ml or higher NPs doses (Figure 2). The highest non-toxic dose for all NPs types was 75 µg/ml, and the viability of THP-1 cells was over 80% in all cases (Figure 2). Toxicity of silica NPs seems a hot topic in the literature, however, many studies reflect that this toxicity concern may be overestimated: MSNPs neither reduce glutathione level, nor generate reactive oxygen species in mouse keratinocytes **[25]**, nor induce cytotoxicity on intestinal cells **[29]**. No significant signs of toxicity in chronically dosed animals have been seen **[26]**. On the contrary, MSNPs seems to have good biocompatibility, biodegradability and excretion properties **[6, 13]**. Independently of the impact in the long run of silica NPs in the body, our results showed that silica NPs are highly cytotoxic, at least compared

to poly(l-lactide-co-glycolide) (PLGA) NPs, were in similar conditions even 1,000 µg/ml results non-cytotoxic **[24]**.

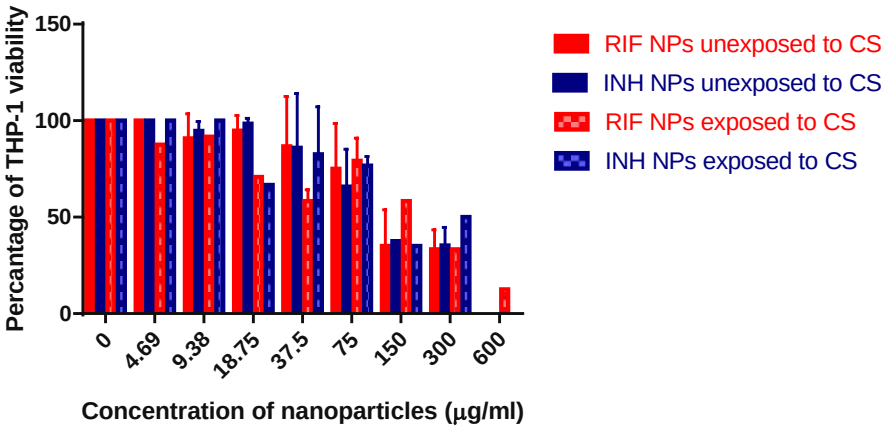

Figure 2. THP-1 viability after exposure to different NP concentrations. Trypan Blue exclusion test performed 4 days after exposure to RIF or INH-NPs.

Moreover, we studied the effect of a non-cytotoxic dose of NPs on *M. tuberculosis* H37Rv growth. The cultures were exposed to 75 µg/ml of loaded and unloaded NPs for two weeks and the OD_{600nm} was measured every 24 hours. The results obtained showed no significant changes compared with the control at 75 µg/ml (Figure 3); suggesting no bactericidal effects. It was not surprising that loaded NPs did not inhibit the growth of *M. tuberculosis*; the dose of the loaded antibiotic was small (0.18 µg/ml of RIF; 0.54 µg/ml of INH) and the efficiency ratio of loaded NP *versus* free drug is 1:1 **[13]**. Despite the absence of impairing the growth of *M. tuberculosis*, when drugs are delivered by silica nanocarriers, concentration of the antibiotic in macrophages could be even 10 times higher than in blood (extracellular medium) **[27]**.

We determined if the MIC of loaded and unloaded RIF or INH-NPs against *M. tuberculosis* H37Rv exposed or not to 10% CS was between 18.75-600 µg/ml and if it differed between them. *M. tuberculosis* was exposed to the NPs for a week and growth was assessed by reduction of the resazurin. MIC of unloaded NPs were higher than the concentrations tested (600 µg/ml). MIC was reduced when NPs were loaded, to 600 µg/ml in RIF-NPs and to 150 µg/ml in INH-NPs (Figure 4). Same results were obtained with and without CS exposure.

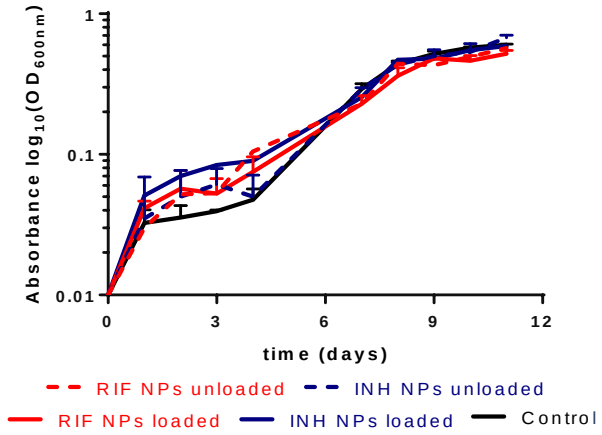

Figure 3. Growth of *M. tuberculosis* H37Rv in 7H9 medium, exposed to 75 µg/ml of MSNPs. The continuous line corresponds with loaded NPs and the dashed line with unloaded NPs. The results are expressed as the average and standard deviation of duplicates.

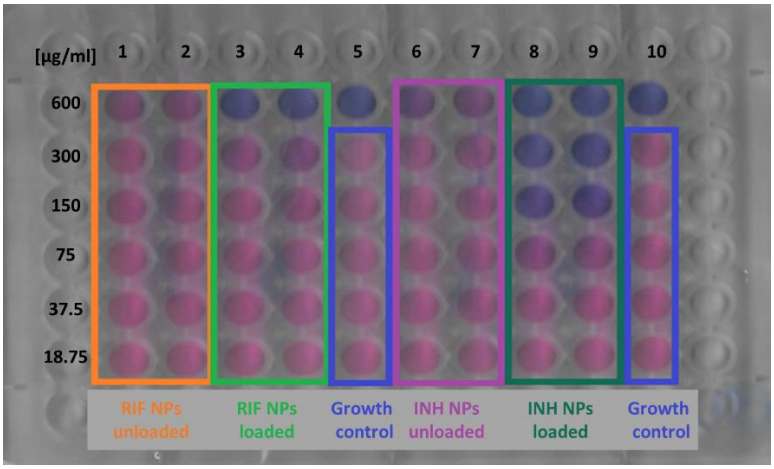

Figure 4. Minimal inhibitory concentration (µg/ml) of NPs against *M. tuberculosis* H37Rv. *M. tuberculosis* was exposed to 18.75-600 µg/ml of NPs and CS. Control lines (columns 5 and 10) were composed of one sterile well and five with *M. tuberculosis* inoculum as a growth control. The image shows duplicates of the conditions.

Finally, we studied the effect of the NPs on infected cultures exposed and not exposed to CS. Therefore, macrophages were exposed to CS for 3 hours and then infected with H37Rv strain. After 72h, NPs or free drugs were administrated to the cultures and the extracellular CFUs were evaluated at day 4 and intracellular CFUs were evaluated during the four days.

Addition of NPs to THP-1 cells did not reduce the extracellular CFUs in comparison with the untreated macrophages, no matter if they were exposed to CS or not or loaded with RIF or INH or unloaded (Figure 5.A & B). Free drugs reduced the extracellular CFUs (Figure 5.A & B). These results could be explained by the sustained release of the drug from the NP.

When the macrophages were exposed to CS, a reduction in the number of viable intracellular *M. tuberculosis* was observed compared with CS unexposed (Figure 5.C & D). This reduction is due to an impairment on the phagocytosis of THP-1 cells by CS (**Chapter III**). Less mycobacteria were recovered when the cells were exposed to free RIF or INH, comparing to the control (Figure 5.C & D). When loaded or unloaded NPs were added to the macrophages, no reduction of the intracellular burden was observed compared to the control (Figure 5.C & D).

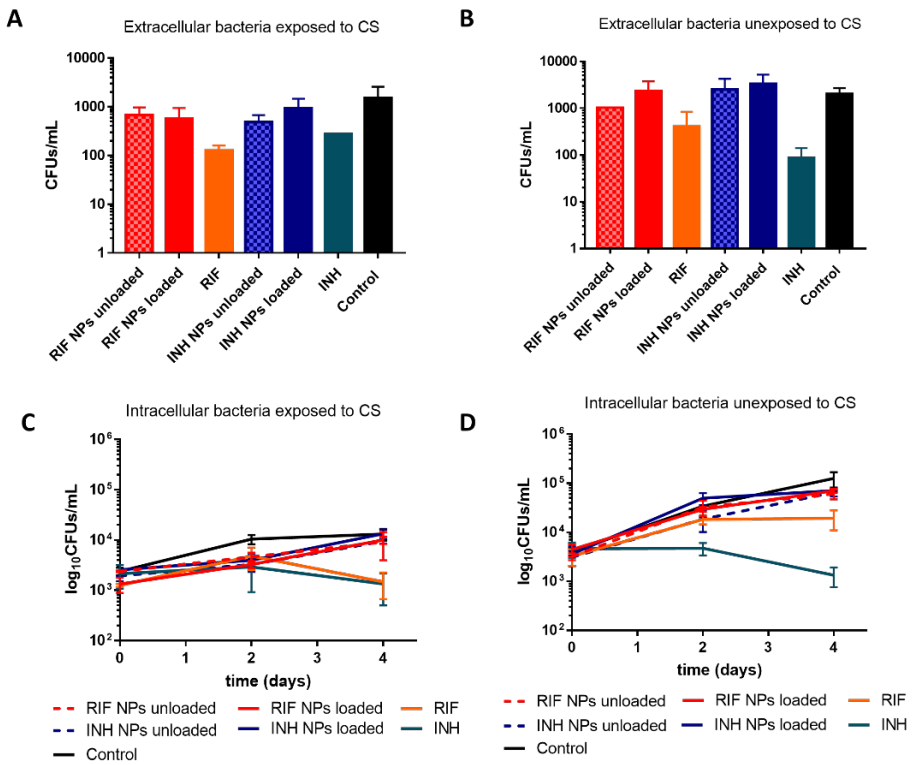

Figure 5. *M. tuberculosis* extracellular and intracellular CFUs counts over 4 days after NPs or drugs administration. Recovery of extracellular CFUs at day 4 after NPs administration from THP-1 macrophages (A) exposed to 10% CS extract and (B) not exposed. Recovery of intracellular CFUs during four days from THP-1 macrophages (C) exposed to CS extract and (D) not exposed. Free drug was at the same concentration that the drug inside loaded NPs. The results are expressed as the average and standard deviation of triplicates.

MSNPs are reported to be ingested avidly by human macrophages infected by *M. tuberculosis*, where they traffic to acidified endosomes and release the drug intracellularly **[5, 13]**. Furthermore, PEGylation give better results than uncoated MSNPs **[13]**. We suspect that the main drawback in the efficiency of the NPs here is the small quantity of cargo. However, loaded NPs were even less effective than free antibiotic at the same concentration than the drug in loaded NPs. This event could be explained by the fact that in NPs the release kinetics of the drug is not only dependent on dose and composition, but morphology, size, size distribution, surface charge, and agglomeration state **[28, 29]**. An additional consideration is that the release depends on the solubility of the drug. For example, RIF is relatively hydrophobic, which could make RIF undergo a phase transition that preferentially maintains the drug in the porous interior of the MSNP under aqueous conditions **[13]**. Although studies with similar NPs have shown that 95% drug release was achieved after 24 hours **[30]**, the NPs used in this study have a gate that is only opened in the presence of antioxidants, like in a glutathione reduced environment. It is also possible that not all the drug was unloaded from the NP in four days.

All in all, although NPs are emerging as a novel approach for a smarter use of the drugs, there is a need for testing if all types of NPs have an increased efficiency compared to free drugs. In this study, we compared the use of silica NPs with free antibiotics and they were less effective. This could be explained by the toxicity of the NPs, which does not allow adding more NPs to infected macrophages, the low-efficiency load, and we are uncertain that the gate is completely opened. Increasing the cargo of the NPs will probably lead to more promising results, since less side-effects and higher concentration of the drug inside of macrophages are theoretically achieved by NPs.

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4.7 Chapter VII: Matryoshka-type gastro-resistant microparticles for the oral treatment of *Mycobacterium tuberculosis*

ABSTRACT

Aim: Production of Matryoshka-type gastroresistant microparticles containing antibiotic-loaded PLGA nanoparticles against *Mycobacterium tuberculosis*. **Materials & methods:** The emulsification and evaporation methods were followed for the synthesis of PLGA nanoparticles and methacrylic acid-ethyl acrylate-based coatings to protect rifampicin from degradation under simulated gastric conditions. **Results & Conclusion**: The inner antibiotic-loaded nanoparticles here reported can be released under simulated intestinal conditions whereas their coating protects them from degradation under simulated gastric conditions. The encapsulation does not hinder the antituberculosis action of the encapsulated antibiotic rifampicin. A sustained antibiotic release could be obtained when using the drug-loaded encapsulated nanoparticles. Compare to the administration of the free drug, a more effective elimination of *M. tuberculosis* was observed when applying the nanoparticles against infected macrophages. The antibiotic-loaded PLGA nanoparticles were also able to cross an *in vitro* model of intestinal barrier.

INTRODUCTION

Mycobacterium tuberculosis is a well-known example of a highly spread pathogen causing tuberculosis (TB), a disease that greatly affects public health in most developing countries. *M. tuberculosis* is localized in the early endosomes of infected macrophages **[1]**. According to the last WHO report **[2]**, despite the moderate decline (1.5%) in the global incidence of TB observed during the last 15 years, being in 2015 a 18% lower than in 2000, TB still constitutes a major cause of morbidity and mortality worldwide. In 2016, there were 10.4 million new cases and 1.7 million people died from the disease worldwide ranking alongside HIV as a leading cause of death. In the treatment of drug-susceptible TB, in order to act on different bacterial subpopulations (extracellular and intracellular), current treatment is extended over long periods of time (6-9 months) with a combination of at least four of the five first-line drugs against the pathogen in order to prevent both relapse and development of antibiotic resistance. Rifampicin, isoniazid, pyrazinamide and ethambutol are given daily usually

during the first two months of treatment, leaving just rifampicin and isoniazid for the remaining period. Except for pyrazinamide, the other commonly used antibiotics require bacterial replication for their action **[3]**.

Being at least partially an intracellular pathogen, any treatment against TB should be selectively directed towards the intracellular reservoir of the bacterial load. The introduction of targeted drug delivery systems into the clinical practice for treating intracellular pathogens would allow applying drugs effectively, increasing patient compliance, extending the product life cycle, providing product differentiation and reducing healthcare costs. Over the last decades, significant progress has been made in the development of nanotechnological approaches to diagnose, screen, treat, and prevent disease, hence improving human health. As a consequence of this extensive research 43 nanomedicines have been approved for the clinical practice by the EMA and 71 by the US FDA mainly as drugs and medical devices and other 128 are currently undergoing clinical trials **[4]**. Their application is principally directed towards cancer treatment and antimicrobial therapy using drug delivery systems. In those systems, the nanoparticle (NP) acts as a drug depot either as suspensions or as coatings on medical devices. General applications of nanomaterials in the field of infectious diseases include their use as adjuvants or as antimicrobial and vaccine carriers, to achieve a sustained or controlled delivery depending on the need. In addition, several metallic nanomaterials (i.e., silver, copper, etc.) are antimicrobial by themselves releasing, after oxidation, metallic ions that show multiple mechanisms of biocidal action and consequently avoiding antimicrobial resistance. When multiple mechanisms of action take place against a pathogenic microorganism simultaneous gene mutations are difficult to occur and consequently, the development of resistance is reduced. In addition, nanoparticles can be used to achieve a targeted and selective delivery of the corresponding antimicrobial drug. Targeted strategies can be used by coupling the antimicrobial NP to a specific recognition moiety (i.e., antibody, peptide, oligonucleotides, etc.), or by electrostatic binding to the bacterial wall **[5]** or even the antimicrobial can be released just in response to a biomolecule secreted under the presence of bacteria **[6,7]**. Passive strategies take advantage of the nanoparticle-immune system recognition. Thus, macrophages detect, internalize and destroy not only pathogens and apoptotic cells, but also NPs due to the large number of non-specific surface receptors that those cells have[3]. NPs are internalized within those cells using the phagocytic route when proteins of the blood plasma (i.e., opsonins) are adsorbed on the NP surfaces. Also, other internalization non-phagocytic routes

(clathrin or caveolin-mediated endocytosis, macropinocytosis, etc.) can internalize different NPs within intracellular vesicles. Therefore, NPs can accumulate their antimicrobial cargos into intracellular compartments of infected cells. *In vitro* and *in vivo* experiments showed enhanced antimicrobial activity of antibiotic-containing NPs compared to the administration of the free drug; however, a specific co-localization of the NP and the pathogen in the same intracellular compartment is difficult to achieve. In this regard, Kalluru *et al.* **[8]** demonstrated that even though rifampicin loaded poly lactic-co-glycolic acid (PLGA) nanoparticles were able clear macrophages infected with *Mycobacterium bovis* (BCG) more efficiently compared to the administration of the free drug **[9]**, the drug-containing NPs remained inside phagolysosomes that were separated from the BCG-containing phagosomes. The authors postulated that the loaded hydrophobic drugs easily crossed by diffusion both, the phagolysosome membrane enclosing the NP (phagosome to cytoplasm) and the membrane of the phagosome enclosing the BCG. Therefore, a perfect subcellular co-localization inside the same vesicle is not necessary to show enhanced efficacy. In addition, the great benefit of using nanomaterials is that by selecting a specific size **[10]** or by using a simple surface modification, the same NP can be internalized following different routes **[11]**.

Oral administration of drugs is preferable over any other route of administration because it is not invasive and shows long term compliance and increased patient acceptance, allowing easily home therapy **[12]**. The main drawback of this route is the potential degradation of the active principle in contact with the acidic and enzymatic environment of the gastrointestinal tract and during the pre-systemic metabolism. Capsules have been designed to protect the drug from the degradation in the gastric fluid releasing its cargo in the intestine in a pH-responsive manner. For instance, Patel *et al.* **[13]** described the synthesis and sustained rifampicin release from pH-dependent chitosan-based nanoparticles intended for oral delivery. Also, pH-independent sustained-release formulations have been designed to increase the intestinal adsorption using mucoadhesive formulations and absorption enhancers.

The activity of rifampicin is based on the inhibition of the RNA synthesis, by means of van der Waals forces, of the hydrophobic side chains near the naphthol ring of the rifampicin with the bacterial DNA-dependent RNA polymerase **[14]**. Rifampicin is degraded (~26%) under gastric conditions, which can be responsible for its reduced bioavailability *in vivo*, but its nanoparticulated formulation can reduce the degradation

level to a 9% **[15]**. NPs have also been used to protect and transport active principles across the intestinal epithelium to the bloodstream by targeting them with specific proteins able to cross-polarized epithelial barriers **[16]** or by using appropriated sizes surface and charges opening the tight junctions between the epithelial cells **[17]**.

In this work, we describe a drug delivery system potentially able to reach infected alveolar macrophages using biodegradable and approved antibiotic-loaded carriers. The system is based on nanoparticulated formulations of PLGA (Resomer® RG504) loaded with rifampicin targeting *M. tuberculosis*. Those drug-loaded NPs are, in a second step, encapsulated inside (as Matryoshka dolls) microparticles (MPs) based on methacrylic acid-ethyl acrylate copolymer (1:1, Eudragit® L 100-55) to cause the drug-loaded NPs to first pass through the stomach without being dissolved (thanks to the shell made of Eudragit® L 100-55) to then dissolve in the intestinal fluid releasing the inner NPs made of PLGA.

MATERIALS & METHODS

Nano and microparticle synthesis

PLGA-rifampicin NPs and microcapsules based on Eudragit were synthesized in Universidad de Zaragoza and kindly provided by Dr Manuel Arruebo. For further details about the synthesis consult Materials and Methods of **Appendix II**.

Antimicrobial evaluation

For determining the antituberculosis activity of the samples, mycobacterial strains were grown at 37 °C in Middlebrook 7H9 broth supplemented with 10% ADC and 0.05% Tween 80 until reaching an OD_{600} of 0.8. Then, bacterial cultures were diluted in Middlebrook 7H9 broth supplemented with ADC and 0.5% glycerol in order to obtain a bacterial suspension of 10^5 CFU/mL. Aliquots of 100 µL were transferred to each well of a 96-wells plate that contained 100 µL of samples prepared from 2-fold serial dilutions in the same culture medium. The inoculated plates were incubated for 6 days at 37 °C and for an additional 2 days after the addition of the redox indicator (30 µL of a resazurin solution at 0.1 mg/mL). A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. Thus, the MIC (minimum inhibitory concentration) was defined as the lowest concentration of compound that prevented this colour change.

For estimating the release of rifampicin from NPs, 0.806 mg/mL of rifampicin-loaded PLGA NPs were incubated in 400 µL of culture media (Middlebrook 7H9 broth supplemented with ADC and 0.5% glycerol) adjusted at pH=7.0 or pH=4.5, at 37 °C. Samples were taken after 3, 7, 14, and 25 days, centrifuged for pelleting (10 min at 4500 rpm) and the NPs and the supernatants transferred to a new tube and kept frozen until use. Once all samples were ready, the supernatants were evaluated for the presence of rifampicin by using the protocol described above. As a negative and sterility control, 0.554 mg/mL of empty PLGA NPs were processed in the same way as rifampicin-loaded NPs were. In order to consider spontaneous degradation of rifampicin after being released from the NPs, tubes containing an amount of free rifampicin equal to that encapsulated in the PLGA NPs were incubated and processed in the same way. The experiments were performed in triplicate.

Bacterial growth and time-kill kinetics

The time-kill kinetics of *M. tuberculosis* H37Rv were determined by incubating cultures with NPs and free rifampicin and counting the number of viable bacteria at several time points **[18]**. Mycobacterial cells were cultured till exponential phase and then diluted to reach 10^7 CFU/mL in Middlebrook 7H9 broth. Four samples of 10 mL of this bacterial suspension were taken separately and added with: i) rifampicin, to reach an inhibitory concentration of 0.5 µg/mL, ii) rifampicin-loaded NPs, which contained rifampicin for final inhibitory concentrations of 0.06-0.07 µg/mL, and 4.9-6.0 µg/mL of PLGA (which are below inhibitory concentrations of PLGA), iii) empty PLGA NPs to reach the same final concentration of 4.9-6.0 µg/mL; the fourth sample did not receive any compound and was taken as control. All four samples were incubated at 37°C. On days 3, 7, 14, and 21, from each sample, an aliquot of 100 µL was taken for diluting and plating on Middlebrook 7H10 agar plates which were incubated further at 37°C until growth was observed in order to count the number of CFUs. The assay was performed in duplicate.

Loaded or empty nanoparticles at sub-inhibitory concentrations of PLGA and rifampicin (in the case of the loaded nanoparticles) were added to cultures of *M. tuberculosis* in order to estimate their impact on bacterial growth. Bacterial cells were cultured till exponential phase; they were diluted to reach 10^5 – 10^6 CFU/mL in fresh Middlebrook 7H9 broth, and separated into three samples of 10 mL. PLGA NPs containing rifampicin equalling one-fourth of its MIC (i.e., 0.004 µg/mL; MIC = 0.015 µg/mL) was added to the first culture; the second was added with the same amount of empty PLGA NPs (to reach 0.32 µg/mL of PLGA, well below its MIC, which is 7.8 µg/mL); the last sample was incubated without further additions, as control. Immediately, an aliquot was removed

for determining initial values of OD_{600} and CFU/mL. Cultures were incubated at 37 °C and new aliquots were taken and analyzed for OD_{600} and CFU/mL after 3, 5, 7, 10, 12, 14 and 21 days.

Cell culture

TC7 clone human epithelial colorectal adenocarcinoma (Caco2) cells, kindly donated by Dr MJ Rodriguez-Yoldi, were grown using Dulbecco's modified Eagle's medium (DMEM w/stable Glutamine; Biowest) in a humidified atmosphere containing 5% CO_2 at 37 °C. The growth medium was supplemented with fetal bovine serum (FBS, 10% (v/v); Thermo Fisher Scientific) and antibiotic-antimycotic (60 µg/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, Biowest).

MH-S murine alveolar macrophages were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with FBS 10% at 37 °C in a 5% CO_2 humidified incubator.

Cell viability assay

The *in vitro* cytotoxicity of rifampicin, unloaded-PLGA NPs and rifampicin-loaded PLGA NPs was evaluated using the resazurin based Alamar Blue™ assay (Invitrogen, US) according to the manufacturer's instructions. TC7 Caco2 (density of 8000 cells/well) cells were seeded in a 96-well plate 48 h prior to incubation with the different formulations and allowed to adhere. Non-loaded-PLGA and rifampicin-loaded PLGA NPs were added to the cells in complete growth medium at a concentration range of 0.0625-1 mg/mL and, then, cells were maintained in the standard conditions mentioned above for 24 h. Rifampicin was added to the cells at a concentration range of 0.5-8 µg/mL, which are equivalent drug concentrations to the ones contained in the rifampicin-loaded PLGA nanoparticles used. After incubation, cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, Biowest) and incubated with complete growth medium containing 10% (v/v) Alamar Blue dye for 4 h. Fluorescence of the medium was measured at 37 °C in a multi-mode Synergy HT Microplate Reader (excitation wavelength 530 nm, emission wavelength 590 nm; Biotek). Cell viability was expressed as a relative percentage to the untreated control cells value. The percentages obtained depict the average of four values.

To evaluate the cytotoxic effect of the PLGA NPs on the MH-S cell line, the EZ4U cell proliferation assay (Biomedica) was used following the manufacturer's instructions. Briefly, MH-S were seeded at a concentration of 1×10^5 cells per well in 96-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA) using RPMI without

phenol red. The plates were incubated overnight (37 °C in 5% CO_2 atmosphere). Non-loaded-PLGA NPs, rifampicin-loaded PLGA NPs or free rifampicin at minimum **[19,20]** and maximum **[21–23]** serum concentrations (4 and 24 µg/mL, respectively), were diluted in 200 µL of RPMI without phenol red per well and incubated for 24 h. The monolayers were washed once with DPBS and then 200 µL of fresh medium without phenol red and 20 µL of EZ4U were added. The absorbance was read after 4 h of incubation, and also at 48 h and 72 h. A microplate reader (Victor 3, Wallac) was used with a wavelength of 450 nm with 620 nm as reference. The results were expressed as a viability percentage using untreated cells as control.

Permeability experiments

For further details about the permeability experiments consult Materials and Methods of **Appendix II**.

***In vitro* infection of MH-S macrophages**

MH-S murine alveolar macrophages were plated at a concentration of 3×10^5 cells per well in 24-well tissue culture plates with clear bottoms (TTP®) and allowed to adhere overnight. For the infection, mid-log phase *M. tuberculosis* was washed twice with PBS with a 0.05% Tween, once with Dulbecco's PBS and then allowed to stand for 5 min, before the supernatant was collected. The bacteria were then diluted in RPMI and added to the MH-S cells at a concentration of $\sim 3 \times 10^4$ CFU/well. After 3 h of infection at 37 °C in 5% CO_2, macrophages were treated with 200 µg/mL amikacin for 1 h and washed three times with DPBS to eliminate any extracellular bacteria. Lastly, 1 mL of complete RPMI-1640 was added to each well and incubated for 72 h at 37 °C in a 5% CO_2 humidified incubator, in order to allow bacteria to grow inside the macrophages. After such incubation (day 0), fresh medium containing unloaded-PLGA NPs, rifampicin-loaded PLGA NPs or free rifampicin was added at 4 and 24 µg/mL (minimum **[19,20]** and maximum **[21–23]** serum concentrations). The quantity of rifampicin-loaded PLGA NPs added was calculated according to the rifampicin concentration inside the NPs. The quantity of empty PLGA NPs added was proportional to the quantity of loaded PLGA NPs. Intracellular survival and growth were assessed by lysing the monolayers (day 0, 2 and 4) by the addition of water followed by a 30 min incubation at 37 °C and enumeration of bacteria by serial dilutions in PBS-Tween plating onto Middlebrook 7H10 solid medium. Colonies were counted after 3–4 weeks incubation at 37 °C and the average CFU/mL determined.

RESULTS

Physico-chemical characterization of the micro- and nanoparticles produced

For physicochemical characterization consult Results of **Appendix II**.

Antimicrobial activity evaluation

The resazurin assay was used to evaluate the MIC of nanoparticles and drugs against actively growing cultures of *M. tuberculosis*. Unloaded PLGA NPs, rifampicin-loaded PLGA NPs and free rifampicin were evaluated. The evaluated MIC of rifampicin was 0.015 µg/mL, value in agreement with those described for strains susceptible to rifampicin **[24]**. The MIC of the empty PLGA NPs was 7.8 µg/mL, hence indicating a moderate antituberculosis effect for the polymer PLGA. This intrinsic antituberculosis activity was attributed to the potential accelerated hydrolysis of the ester bonds of the PLGA, hence producing lactic and glycolic acids that would further acidify the media being this process autocatalytic **[25]**. As a consequence, increases in media acidification would, in turn, result in an enhanced bactericidal action for *M. tuberculosis*.

Interestingly, the MIC of the rifampicin-loaded PLGA NPs was 1.95 µg/mL, which corresponded to 0.02 µg/mL of rifampicin (considering the DL), which is a value very close to the one obtained for the free rifampicin (0.015 µg/mL). These experiments suggested that rifampicin was being released from the nanoparticles, so we designed an experiment to monitor kinetics of drug release. Nanoparticles were incubated in culture media at pH 7.0 and the antituberculosis activity of nanoparticle-free supernatants was assayed against M. tuberculosis. We observed that in these conditions, free rifampicin was degraded upon time, rapidly after the first week, so we took this into consideration when analyzing the following results. After three days, the supernatants contained a 72.6% of the initial amount of rifampicin, and this value was 48.4% after 7 days (lower than the one obtained after three days due to rifampicin degradation). Longer incubation times resulted in much lower amounts of rifampicin, being 24.2% after 14 days and 6.0% after 25 days.

Sustained-release was also observed for 7 days in blood serum by Booysen *et al*. **[26]** when using rifampicin-loaded PLGA-PEG NPs administered orally in a rat model whereas only during the first 2 days those authors were able to detect the antibiotic in blood serum when administered as a free (non-encapsulated) drug. In addition, Pandey

and Khuller **[27]** also demonstrated the presence of systemic rifampicin 5 days after its oral administration in mice when encapsulated within PLGA NPs (~200-300 nm) whereas the free drug was present only during the first 12h. We also observed that the rifampicin released from NPs incubated at 37 °C during 25 days was still active and killed *M. tuberculosis*.

Under physiological conditions, mycobacteria reside in an acidic cellular compartment, so we investigated next the amount of rifampicin that would be released from the nanoparticles in such acidic conditions. For this, nanoparticles were incubated at pH 4.5 and the antituberculosis activity of supernatants at different time points was determined by the resazurin method; results are given in Table 1. A considerable amount of rifampicin could be detected in the nanoparticle supernatants (40.3%) after three days, indicating that the antibiotic was readily being released from the nanoparticles. We observed that the amount of rifampicin detected in the supernatants decreased after longer incubation times, which may be attributed to degradation of rifampicin in the culture media due to spontaneous reactions (as discussed above) **[15]**. In fact, after two weeks, <6.0% of rifampicin could be detected in the supernatants at pH 4.5, indicating that degradation of the antibiotic was faster under acidic conditions. In order to assess the latter, we performed a control assay from a solution of free rifampicin at pH 4.5 and detected that in fact, the amount of rifampicin that can be detected also decreases with time, although to a lesser extent than that obtained from the nanoparticle supernatants probably caused by a superior acidification of the medium due to the presence of lactic and glycolic acids as sub-products of the PLGA degradation (Table 1). Control experiments performed with the supernatants of empty nanoparticles did not show any antituberculosis activity even at the highest concentrations tested.

Table 1. Bioassay-based quantification of rifampicin released from PLGA NPs, in comparison with the amount detected from a free solution of rifampicin.

pH	Days	% Rif detected from Rif-loaded NPs	% Activity of a control Rif solution
4.5	3	40.3	98.1
	7	10.1	39.9
	14	<6.0	6.0
	25	<6.0	<6.0

We tested next the impact of empty and antibiotic-loaded PLGA nanoparticles in the growth of M. tuberculosis. We first detected that empty nanoparticles produced a slight delay in growth of the M. tuberculosis culture, which is consistent with moderate intrinsic antituberculosis properties of PLGA detected in both the MIC assays and the murine alveolar macrophages infection model, as reported in this work and elsewhere in the literature **[25]**. Interestingly, we observed that rifampicin-loaded nanoparticles containing subinhibitory concentrations (0.25 x MIC) of rifampicin caused a further slight delay in the growth of M. tuberculosis in comparison with the same amount of empty NPs. Figure 1 shows the outcome of a representative experiment. This delay could be detected in terms of both OD600 (Figure 1a) and also in the number of viable cells (Figure 1b). We attributed this effect to the release of the antibiotic from the nanoparticles. Given that rifampicin was used at a subinhibitory concentration, it only delayed but not arrested bacterial growth.

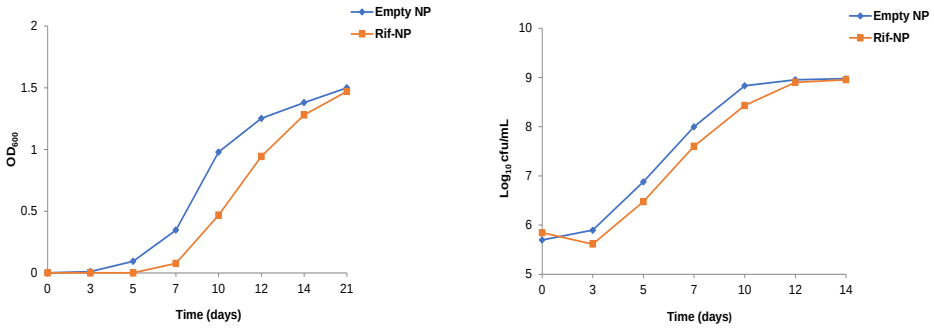

Figure 1. Impact of empty and rifampicin-loaded nanoparticles on the growth of M. tuberculosis on Middlebrook 7H9 broth at pH 7.0. The rifampicin-loaded nanoparticles (red lines) contained a sub-inhibitory concentration of rifampicin and caused a slight delay in the growth of M. tuberculosis in comparison with the same amount of empty nanoparticles (blue line) that was detected in terms of both OD600 (left panel) and also in the number of viable cells (right panel), as shown in this representative experiment.

In order to estimate the antimicrobial activity of the rifampicin-loaded PLGA nanoparticles in conditions similar to those encountered during antituberculosis treatment, we performed time-kill kinetics experiments at pH 4.5. The pH of the early endosome in which M. tuberculosis resides, ranges from pH 6.2 to 4.5 depending on the macrophage activation state **[28]**. We found that the free and the encapsulated rifampicin had similar levels of bacterial eradication, both causing a reduction larger than 8-log in the number of viable bacteria after 14 days of incubation (Figure 2). Empty PLGA NPs showed partial antimicrobial activity (2.5 log reduction compared to the non-treated bacterial cells, after 21 days of

incubation; Figure 2), which is consistent with the fact that the concentrations of empty NPs used in this experiment are below, but rather close to, the inhibitory concentrations of PLGA.

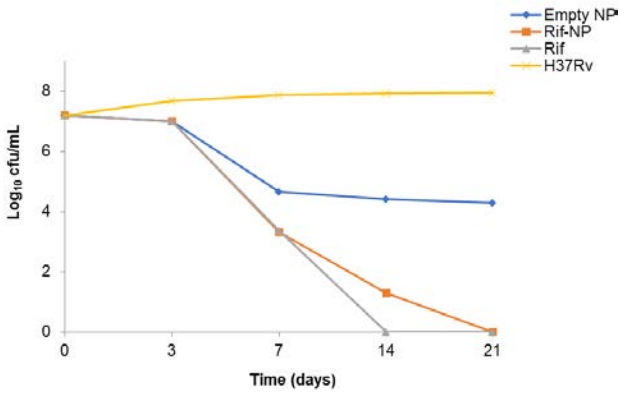

Figure 2. Time-kill kinetics at pH 4.5. High bacterial density (1.5 x 107 CFU/ml) cultures of *M. tuberculosis* were incubated at pH 4.5, conditions in which cells barely replicated (black line), and efficiency of several treatments for reducing the number of viable bacteria over time were compared. Treatment with 0.06-0.07 µg/mL of encapsulated rifampicin (between 4.5 and 6 times above the MIC) (red line) was as efficient as the free antibiotic at the same concentration (green line) to kill cells in a population, being capable of sterilizing the culture after two weeks. Intrinsic antimicrobial activity of PLGA was also detected (blue line), which was more than four orders of magnitude lower than that obtained for the antibiotic containing samples.

We investigated the impact of the treatment with free rifampicin or rifampicin-containing nanoparticles in the morphology of *M. tuberculosis* under both acidic and neutral conditions. As can be seen in Figure 3, at any condition tested the roughness of the surface of the bacteria wall under acidic conditions increased compared to the cells treated at physiological pH. Treatment with rifampicin either free or encapsulated enhanced the alterations observed in the cell shape and roughness.

We also tested whether the antituberculosis activity of the rifampicin-loaded PLGA nanoparticles was retained after being released from the Eudragit microparticles. For this, the complete Matryoshka MPs were immersed in PBS at pH=7.4 in order to release the nanoparticles that were tested for antituberculosis activity *in vitro*. Nanoparticles containing rifampicin were effective at doses above 0.035 mg/mL whereas empty (without antibiotic) PLGA nanoparticles released from MPs did not show any antituberculosis effect at the highest dose tested (1.12 mg/mL).

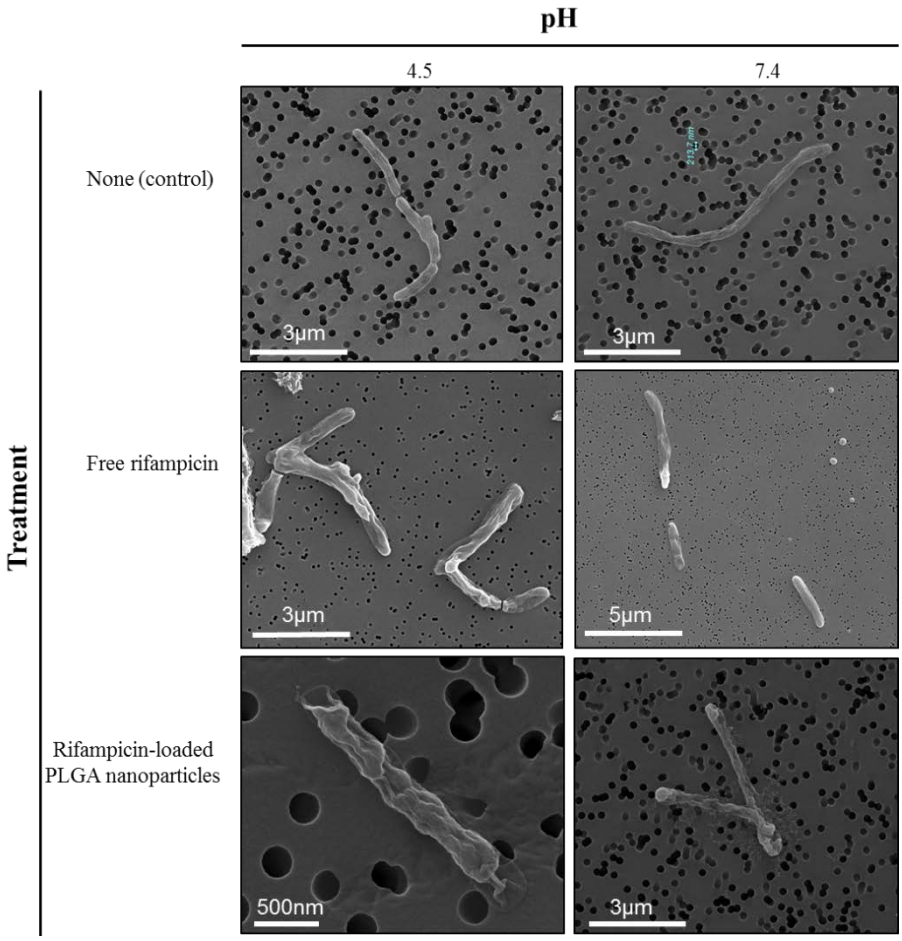

Figure 3. SEM photographs of the *M. tuberculosis* bacteria in contact with free rifampicin or with rifampicin-loaded nanoparticles under acidic (pH 4.5) and neutral (pH 7.4) conditions.

Cell viability assay

To study the influence of different PLGA formulations on cell viability, the potential cytotoxic effect of unloaded-PLGA nanoparticles, rifampicin-loaded PLGA nanoparticles and free rifampicin were assayed in two different cell lines, TC7 Caco2 and MH-S murine alveolar macrophages. Caco2 cell line was selected because it is one of the most used *in vitro* models to study the intestinal transport when drugs are orally administrated. The results showed that both, rifampicin-loaded PLGA nanoparticles and free rifampicin did not induce

cytotoxicity in the range of concentrations tested (0.0625-1 mg/mL PLGA nanoparticles and 0.5-8 µg/mL of rifampicin).

MH-S murine alveolar macrophages were chosen because infected macrophages are the target where the drug has to exert its action. We tested unloaded and rifampicin loaded PLGA NPs with rifampicin as well as free rifampicin at serum concentrations (4 µg/mL and 24 µg/mL), during 72 h.

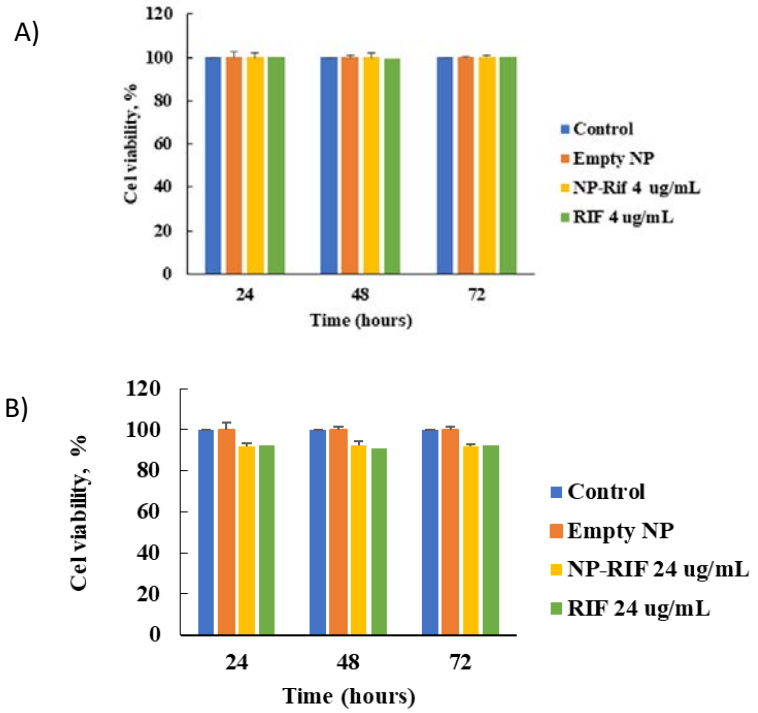

Figure 4. Cytotoxic effect of PLGA NPs on MH-S murine alveolar macrophages after 24, 48 and 72 hours of exposition to empty NPs, rifampicin-loaded NPs and free rifampicin at serum concentrations: A) 4 ug/mL and B) 24 ug/mL.

The results showed that the PLGA NPs empty or with rifampicin were not toxic for the macrophages at plasma concentrations. Moreover, it was not found any significant effect when free rifampicin was added to the macrophages (Figure 4).

Evaluation of Caco2 cell monolayer integrity and visualization of epithelial monolayers by transmission electron microscopy (TEM)

For evaluation of Caco2 cell monolayer integrity consult Results of **Appendix II**.

Permeability assay through the artificial membrane assay

For permeability assay consult Results of **Appendix II**.

Antimycobacterial effect of rifampicin-loaded PLGA nanoparticles in murine alveolar macrophages infection model

Finally, we investigated the proliferation of *M. tuberculosis* inside macrophages under the treatment with unloaded-PLGA NPs, rifampicin-loaded PLGA NPs and free rifampicin at serum concentrations (4 and 24 µg/mL). We found that at those two concentrations the exposure to PLGA NPs loaded with rifampicin caused a decrease in the growth of *M. tuberculosis* of 3.3 and 3.6 times respectively, compared with free rifampicin until day 4 (Figure 5a-b). It is possible that the effect can be stronger with a longer exposition, as it was observed when the bacteria were exposed to rifampicin-loaded PLGA NPs (Figure 2) and also as it was reported by others in experiments with BCG in murine macrophages [8,29].

The effect observed after the treatment with rifampicin-loaded PLGA NPs at maximum concentration (24 µg/mL) could be related with the synergy observed between the drug and PLGA, since the final concentration of PLGA added was higher (23.9 µg/mL) than the MIC value (7.8 µg/mL). However, in the minimum concentration (4 µg/mL), the effect observed can be related exclusively to the drug since the PLGA concentration present was lower (3.9 µg/mL) than the MIC value.

On the other hand, we also found that empty PLGA NPs had some antimicrobial activity when the same quantity of PLGA as the one contained in rifampicin-loaded PLGA NPs, was added (Figure 5a-b). Again, the concentration was higher (51.5-309 µg/mL) than the MIC value. The same tendency was observed in the time-kill kinetics experiments and also by Lawlor et *al*. [24] in THP-1 macrophages using PLGA microparticles, who suggested that the antimicrobial effect could be related with the size and morphology of the particles used. It is important to mention that the approach here reported could also be used to encapsulate the second-line injectable tuberculosis drugs utilized for the treatment of multidrug-resistant tuberculosis that generally present poor efficacy, are more toxic and require prolonged treatments [30].

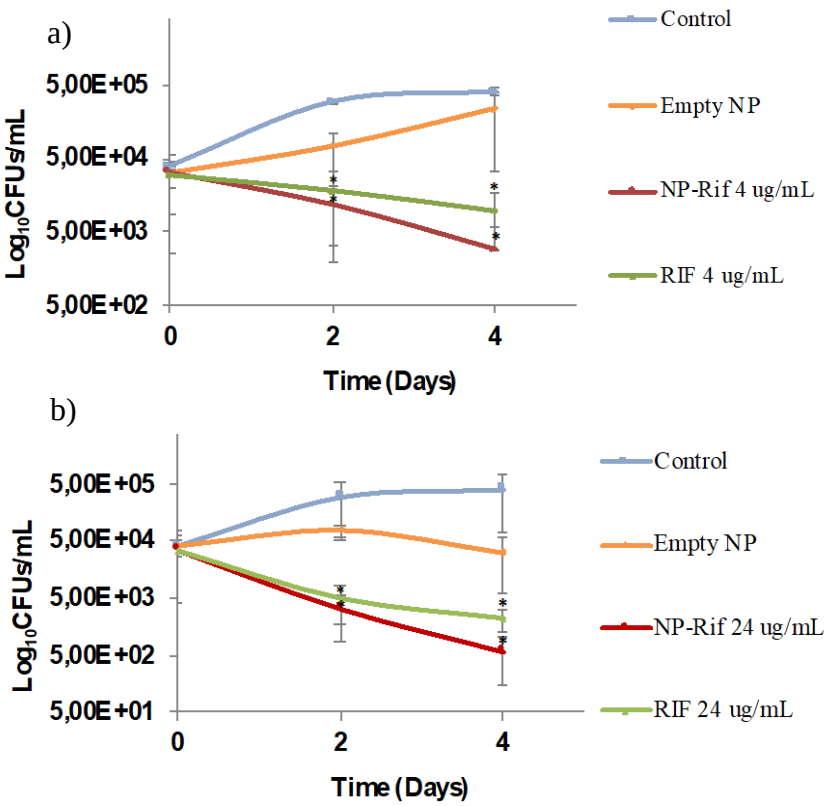

Figure 5. Growth of H37Rv strain inside MH-S macrophages after the treatment with empty PLGA NPs, rifampicin-loaded PLGA NPs and free rifampicin. (A) Minimum serum concentration (4 µg/mL). (B) Maximum serum concentration (24 µg/mL). Data are expressed as the average and standard deviation of triplicates of at least three independent experiments. *P<0,05 (Compared with MH-S control that was infected with H37Rv without NPs treatment, Mann-Whitney U test, GraphPad Software Inc.)

DISCUSSION

The targeted drug delivery to the intracellular compartments of infected alveolar macrophages where *M. tuberculosis* resides and multiplies could be a promising approach to improve the antibiotics therapeutic efficacy in the treatment of this disease. In passive targeting, organic and inorganic NPs are rapidly internalized within cells to be degraded as part of the innate immune response against any foreign body. Together with granulocytes, resident or circulating macrophages, as well as dendritic cells are all specialized in this clearance. Independently of the route of cellular entry (i.e., phagocytosis, macropinocytosis, clathrin or caveolin-mediated endocytosis, etc.), the internalized NPs remain inside intracellular vesicles where enzymatic and acidic

degradation take place. This NP uptake depends on the macrophage population considered and on its activation state [31]. As we mentioned before, Kalluru *et al*. [8] demonstrated that whereas rifampicin-loaded PLGA NPs are internalized within acidic phagolysosomes, *Mycobacterium bovis* BCG remained in early phagosomes using BCG-infected mouse bone macrophages and also RAW mouse macrophages. However, even though co-localization within the same vesicle was not achieved, drug diffusion into the cytosol cleared the infection. We postulated that intracellular accumulation and slow antibiotic release can be achieved using orally administered antibiotic-loaded PLGA NPs if they were able to cross the intestinal epithelium into the blood stream with the sufficient blood circulation half-life to reach *M. tuberculosis*-infected alveolar macrophages. Thus, the encapsulation of antituberculosis drugs in nanoparticle delivery systems could also increase the drug bioavailability and, therefore, reduce drug dosage frequency. This fact would also allow decreasing treatment-related side effects (i.e., hepatotoxicity, skin reactions, gastrointestinal disturbances and immunological reactions) and improve drug regime compliance by the patient, two of the main clinical problems in the current treatment of tuberculosis. Other factor that can contribute to improve the patient adherence to the treatment is the way in which therapy is administered. In that sense, oral drug administration is the preferable route for long-term therapy due to its noninvasive character, simplicity and patient compliance. However, the main disadvantage of this administration route is the variability in the drug absorption due to degradation by the stomach acidic pH and lytic enzymes. Our study demonstrates that free rifampicin degrades over time under culture conditions (Table 1) and even more under acidic conditions in agreement with the previous literature [15]. To overcome this drawback, rifampicin loaded NPs were encapsulated inside MPs based on methacrylic acid-ethyl acrylate copolymer, which remains insoluble in gastric conditions and dissolves in the intestinal fluid. In our case, we selected a polymer-based on an anionic methacrylic acid-ethyl acrylate copolymer to release its cargo (antibiotic-loaded nanoparticles) at the duodenum pH taking advantage of the thin mucous layer of this first section of the small intestine and its high vascularization [32]. In addition, at that level, it would be the nanoparticle carrier system that would act as a protector to minimize the rifampicin degradation induced by physiological conditions. Coatings targeting the intestine (based on hydroxypropyl methylcellulose phthalate) containing drug-loaded NPs have been used *in vitro* e *in vivo* in the systemic administration of insulin [17]. Those authors used a mixture of a non-acid dependent mucoadhesive polymer (Eudragit RS) with PLGA for the

formulation of the inner insulin-loaded NPs and they demonstrated a prolonged hypoglycemic effect in a diabetic rat model after oral administration.

Previous to the permeability study, we provide evidence of no cytotoxicity of developed PLGA NPs on TC7 Caco2 and MH-S murine alveolar macrophages. These results are in agreement with previous reports conducted with PLGA NPs. Thus, Tukulula *et al*. **[33]** reported no cytotoxic effect of PLGA NPs and curdlan-conjugated PLGA NPs in THP1 macrophages over a 72 h period (dose range from 0.13 to200 µg/mL). In primary murine macrophages, thioridazine and rifampicin loaded-PLGA NPs and unloaded PLGA NPs did not significantly reduce cell viability throughout a 9 days treatment **[34]**. Also, PLGA NPs were not toxic to Caco2 cells after 24 h exposition, as long as the concentration was lower than 500 µg/mL **[35]**. An *in vitro* study carried out by Zhang *et al*. **[36]** to evaluate the cytotoxicity of PLGA NPs, demonstrated that they were well tolerated by Caco2 cells at concentrations below 25 mg/mL.

We used ester-terminated PLGA NPs to avoid electrical charge to hinder mucus adsorption and we synthesized antibiotic NPs with reduced sizes (145 nm) to favour the potential transcytosis by the Microfold cells (M cells) present in the gut-associated lymphoid tissue of the Peyer's patches in a hypothetical *in vivo* application. Bhavsar and Amiji **[37]** demonstrated that a high number of intestinal cells expressing the gene for green fluorescent protein (GFP) were observed when 200 nm gelatin NPs containing a plasmid encoding the GFP and in turn encapsulated within capsules were administered orally in rats which is indicative of a successful NP intracellular internalization.

The reduced size of the rifampicin-loaded PLGA NPs (145 nm) would favour the permeation through the intestinal epithelium reaching the systemic circulation. In this regard, Desai *et al.* demonstrated that 100 nm PLGA NPs orally administered in mice were able to diffuse through the submucosa layers whereas larger nano- and microparticles (>500nm) remain trapped in the epithelial lining of the tissue **[38]**. Also, reduced NP sizes favour nanoparticle cellular internalization to achieve transcytosis considering that the paracellular transport constitutes only 1% of the total mucosal surface area **[39]**. Our *in vitro* permeability assay revealed that epithelial cells were able to uptake rifampicin-loaded PLGA NPs. Given that the paracellular transport is not viable due to the small size of interstitial space and that the intercellular junctions remain still tight during the permeability assay, the NPs internalization inside the cells may happen via endocytic uptake mechanisms. In that sense, previous works

demonstrated that the uptake of PLGA NPs was reduced at 4 ºC in Caco2 cells, indicating an endocytic uptake mechanism was involved in the NPs uptake for these cells **[31]**. The specific endocytosis pathways by which the particles may be translocated through the cells remain to be elucidated.

Also, we observed a sustained rifampicin release up to 25 days after incubation in culture media. Sustained-release was also observed for 7 days in blood serum by Booysen *et al.* **[26]** when using rifampicin-loaded PLGA-PEG NPs administered orally in a rat model whereas only during the first 2 days those authors were able to detect the antibiotic in blood serum when administered as a free (non-encapsulated) drug. In addition, Pandey and Khuller **[27]** also demonstrated the presence of systemic rifampicin 5 days after its oral administration in mice when encapsulated within PLGA NPs (~200-300 nm) whereas the free drug was present only during the first 12h. We also observed that the rifampicin released from NPs incubated at 37 ºC during 25 days was still active and killed *M. tuberculosis*. Also, empty PLGA NPs showed intrinsic antituberculosis activity that was attributed to the potential accelerated hydrolysis of the ester bonds of the PLGA, hence producing lactic and glycolic acids that would further acidify the media being this process autocatalytic **[25]**. As a consequence, increases in media acidification would, in turn, result in an enhanced bactericidal action for *M. tuberculosis*.

Finally, the assays in *M. tuberculosis* infected alveolar murine macrophages showed that the treatment with rifampicin-loaded PLGA NPs had an antimicrobial effect at both tested concentrations until day 4, significantly decreasing the intracellular bacterial load. It is possible that the effect can be stronger with a longer exposition, as it was observed when the bacteria were exposed to rifampicin-loaded PLGA NPs (Figure 2) and also as it was reported by others in experiments with BCG in murine macrophages **[8,29]**. It is important to mention that the approach here reported could also be used to encapsulate the second-line injectable tuberculosis drugs utilized for the treatment of multidrug-resistant tuberculosis that generally present poor efficacy, are more toxic and require prolonged treatments **[30]**.

Conclusions

By using the oil-in-water (O/W) emulsion solvent evaporation method it is possible to prepare monodisperse antibiotic-loaded PLGA-based NPs with high encapsulation efficiencies. Those antibiotic-loaded PLGA-based NPs can also be

encapsulated within (Eudragit-based) monodisperse microcapsules using the double W/O/W emulsification and evaporation method obtaining antibiotic-loaded PLGA NPs encased within gastroresistant microparticles based on methacrylic acid-ethyl acrylate copolymers. Under gastric simulated conditions, we demonstrated that the external coating remained unaltered and that the inner antibiotic-loaded NPs could be rapidly released under intestinal simulated conditions. We also demonstrated that those inner antibiotic-loaded NPs were able to cross an in vitro model of a simulated intestinal barrier composed of Caco2 cell monolayers. Therefore, those nanoparticles would be protected from gastric degradation and potentially reach systemic circulation. The antituberculosis action of the encapsulated rifampicin within the NPs was similar to that observed for the free drug indicating that the synthesis protocols did not alter the drug activity. This action was observed to be pH dependent considering both intracellular and extracellular milieus. We also demonstrated that those antibiotic-loaded NPs were non-cytotoxic on human cell lines but they were able to restrict the proliferation of M. tuberculosis inside infected macrophages. This novel delivery system shows a great potential for the successful targeted treatment of tuberculosis thanks to its demonstrated long-term release and its high cytocompatibility.

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4.8 Chapter VIII: Effect of a novel MptpB inhibitor and cigarette smoke on intracellular and extracellular persistence of *M. bovis* BCG and *M. tuberculosis* H37Rv on THP-1 cells

ABSTRACT

Tuberculosis treatment is long and complex and may lead to multidrug resistance if it is not properly administered. A new approach is to target virulence factors, like MptpB, a protein tyrosin involved in the dephosphorylation of host inositides, able to modify the immune response. Phosphoinositides regulate phagosome maturation and by their dephosphorylation *Mycobacterium tuberculosis* can survive inside macrophages. Inhibition of MptpB should lead to less intracellular survival. However, as many tuberculosis patients are smokers, which delays the time of sputum conversion, we studied the combined effect of MptpB inhibitor and cigarette smoke in THP-1 macrophages infected with *Mycobacterium bovis* BCG or *M. tuberculosis*. Our results showed that MptpB inhibitor had no cytotoxic effects. Growth of extracellular bacteria was not reduced by the MptpB inhibitor, only by rifampicin. Although the reduction of intracellular bacteria when the MptpB inhibitor was administrated alone was very mild, intracellular bacteria was significantly reduced by the combination of rifampicin and MptpB inhibitor. No major effects were observed by the combination of cigarette smoke and the MptpB inhibitor. As the MptpB inhibitor reduced dramatically the intracellular burden of mycobacteria, it is a promising candidate as a drug for TB treatment, especially in combination with other TB drugs like rifampicin, or as a vaccine.

INTRODUCTION

Treatment of tuberculosis (TB) requires at least six months and often leads to multidrug-resistant TB, a major worldwide threat. Rifampicin resistant *Mycobacterium tuberculosis* affects over 50 million people, and the incidence of multi or extensively drug-resistant TB is increasing **[1]**. Although traditional antibiotics, which inhibit cell synthesis or bacterial growth, are effective in killing the pathogen, there is a risk of drug resistance emergence **[1]**. New targets that generate less selective pressure could be virulence factors. The secretion of virulence factors by bacterial pathogens is required to modulate the host's responses. When *M. tuberculosis* is in contact with

macrophages, the first line of defence of the host, virulence factors are secreted to the media to interfere with host's signalling factors, stop phagosome maturation, modulate apoptosis, avoid the toxic effects of reactive nitrogen species, affect cell proliferation and migration, interfere with antigen presentation or suppress host's bactericidal actions **[2, 3]**.

Protein tyrosine kinases (PTKs) and phosphatases (PTPs) act as virulence factors modulating phosphorylation and dephosphorylation of host proteins: PTKs control the amplitude of the immune response and PTPs the rate and duration of the response **[4, 5]**. PTKs are conserved between species, but PTPs are structurally and mechanistically different **[4]**. Examples of well-studied bacterial PTPs are YopH in *Yersinia pestis* (responsible for the bubonic plague) and SptP of *Salmonella typhimurium* **[1]**. Three PTPs that supposedly inhibit phagosome maturation and target phosphatidylinositol-3-phosphate (PI3P) have been discovered in *M. tuberculosis*: SapM, MptpA and MptpB **[6]**. As mycobacterial genome lacks PTKs, PTPs should be involved in the dephosphorylation of host proteins **[2]**. SapM dephosphorylates PI3P from phagosomes and inhibits the fusion with late endosomes or lysosomes **[7]**. MptpA is present in both slow- and fast-growing mycobacteria, but MtptB is restricted to slow-growing species **[8]**. Both MptpA and MptpB, are secreted to the extracellular medium by *M. tuberculosis* growing in mid-log phase **[8]**. MptpB shares homology with human phosphatases like PI3Pase, dephosphorylating phosphoinositide (PI) compounds like PI3P or phosphatidylinositol 3,5-biphosphate (PI(3,5)P2), phosphotyrosine and phosphoserine-threonine **[6, 9, 10]**. MptpB has two conformations, open and closed, to resist oxidative inactivation, and might interfere with host signal transduction pathways facilitating the survival of *M. tuberculosis* in the phagosome **[3, 5]**.

As PI compounds are regulators of signal transduction cascades, cytoskeleton structures and membrane composition, *M. tuberculosis* targets these compounds to modulate the vesicle trafficking, impeding phagosome to acquire microbicide properties **[6]**. Calcium and PTKs are essential in phagosome maturation, but mycobacteria diminish calcium concentrations and interferes with PI signalling pathway because PTPs not only antagonize with PTKs but generate antagonistic signals **[2, 5]**. Immediately after infection, PI3P is associated with phagosomes containing *M. tuberculosis*, but PI3P is reduced rapidly after 2-4 minutes **[10]**. On the contrary, phagosomes with dead mycobacteria have high levels of PI3P **[7]**. PIs (PI3P and PI(3,5)P2) play a key role on recruitment of Early Endosomal Antigen 1 (EEA1) and Rab

GTPases proteins (Rab5 and Rab7) that directs phagosome maturation and clearance of infection **[10, 11]**. Normal maturation of the vacuoles containing *M. tuberculosis* should lose Rab5 and acquire Rab7, which is involved in vesicular fusion **[2]**. However, infected vacuoles do not acquire Rab7, which excludes lysosomal hydrolases and acidifying ATPases **[6, 7]**.

All this suggests that chemical inhibition of MptpB could lead to clearance of mycobacterial infections, and therefore prevent the persistence and prevalence of *M. tuberculosis* **[11]**. In fact, there are some studies identifying new inhibitors against MptpB with promising results in reducing mycobacterial survival in infected macrophages **[10, 11]**.

Another concern in the fight against TB is the high prevalence of smokers between TB patients. Cigarette smoke alters the immune response of the host facilitating the infection, the severity of the illness and delaying the time of sputum conversion **[12, 13]**. This delayed culture conversion is, in fact, similar in smoking TB patients that in non-smokers multidrug-resistant TB patients **[14]**. As MptpB inhibitors and cigarette smoke both affect the host's immune response, we investigated the effectiveness of rifampicin (RIF) and the MptpB inhibitor in cells exposed to cigarette smoke.

MATERIAL & METHODS

THP-1 cell line and mycobacteria growth conditions

The human monocytic cell line THP-1 (ATCC TIB-202™) was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 GlutaMax (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum, penicillin 10,000 units/ml, streptomycin 10 mg/ml and Fungizone® 25 µg/ml. The cells were passaged every 3 days. For the experiments, RPMI without antibiotics was used. THP-1 monocytes were stimulated to macrophages using 0.1 µM of Phorbol 12-Myristate 13-Acetate (PMA, Sigma, St Louis, USA) for 72 hours (37°C in 5% CO_2). RPMI was replaced by fresh medium 24 hours before the experiments.

M. tuberculosis H37Rv and *Mycobacterium bovis* BCG strains were grown in Middlebrook 7H9 supplemented with 0.05% tween 80 and 10% albumin-dextrose-catalase (ADC), or on 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). For *M. bovis* BCG, 0.08% of glycerol was also added. The mycobacteria cultures were incubated at 37°C.

Synthesis of the MptpB inhibitor

4-(3',5'-Dichloro-4'-hydroxy-3-biphenyl)-5-methylisoxazole-3-carboxylic acid was synthesized in University of Manchester as already described **[10]** and it was kindly provided by Professor Lydia Tabernero.

Briefly, trifluoromethane sulfonic anhydride was added to methyl 4-(3-hydroxyphenyl)-5-methylisoxazole-3-carboxylate and pyridine in dichloromethane at 0 °C, and the reaction mixture was stirred at room temperature (RT) for 6 h and then poured into water. The mixture was extracted with dichloromethane and the organic extracts were dried and concentrated under reduced pressure. Chromatography of the residue gave methyl 5-methyl-4-(3-trifluoromethanesulfonyloxyphenyl)-isoxazole-3-carboxylate as an off-white solid. This compound was resuspended in DMF with pinacolyl (3,5-dichloro-4-hydroxyphenyl)boronate and sodium carbonate in water at 0 °C. The reaction mixture was degassed by bubbling nitrogen. The catalyst $Pd(PPh_3)_4$ was added, and the mixture was heated to 90 °C for 3 h, cooled to RT, and poured into ice-water. The mixture was extracted with EtOAc, and the organic extracts were dried and concentrated under reduced pressure to afford the methyl 4-(3',5'-dichloro-4'-hydroxy-3-biphenyl)-5-methylisoxazole-3-carboxylate as an off-white solid used without purification.

Aqueous sodium hydroxide was added to the methyl ester in MeOH: THF at 0 °C and the mixture stirred for 1 h at 0 °C and then poured into ice–water. The mixture was acidified and concentrated to give an aqueous slurry that was extracted with EtOAc. The organic extracts were dried and concentrated to give an off-white solid that was crystallized to give the inhibitor as an off-white solid.

Preparation of antimicrobials agents

RIF was purchased to Sigma-Aldrich (St Louis, MO, USA). Amikacin was purchased to Apollo Chemicals Ltd (Tamworth, UK). Stock solutions of amikacin (10 mg/mL) was prepared in distilled water. RIF (6.4 mg/mL) was prepared in methanol and then diluted in distilled water until 0.512 mg/mL. Stock solutions were sterilized using 0.22 µm filters and stored at -20ºC.

MptpB inhibitor (80mM) was prepared in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA). Once resuspended, it was stored at -80ºC and used in the next two days.

Cigarette smoke extraction

Cigarette smoke extract (CSE) was prepared from commercial cigarettes (Marlboro: 10 mg Tar, 0.8 mg Nicotine, Philip Morris Sàrl Neuchâtel, Switzerland) **[15]**. Briefly, one cigarette was combusted using a syringe-modified apparatus, which draws the smoke into a sterile glass containing 10 mL of RPMI. Sixty millilitres of smoke was extracted for 10 sec following a 30 sec break; this process was repeated six times per cigarette and the CSE was sterilized using a 0.22 µm filter. The resulting solution was considered as 100% of CSE. The absorbance at 320nm was measured for each batch to assure reproducibility and adjusted to 1.2 ± 0.2 **[16, 17]**. The working solution was 10% CSE, equivalent to the smoke of 0.5-2 packs of cigarettes per day **[18]**. For each experiment, fresh extract was used and added to the cultures within 30 min of preparation.

Cytotoxicity Assay

To determine the cytotoxic effect of CSE on macrophages, PrestoBlue (Invitrogen, San Diego, USA) assay was used following the manufacturer's instructions. Cells were seeded at a concentration of 5×10^4 cells in 96-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA). Plates were incubated for 24h, and then CSE was added and incubate for 3 hours. Then, MptpB inhibitor (80 µM) and RIF (0.3 µg/ml). Absorbance was measured after 6h of contact with CSE and at 24 and 72 hours. Time of experiment and doses of RIF and MptpB inhibitor was used as suggested previously **[10]**. Before measuring, monolayers were washed twice with Dulbecco's PBS (DPBS) and 100 µl of RPMI and 10 µl of PrestoBlue were added. Absorbance was read after 1 hour incubation at 570nm.

Macrophage infection

THP-1 macrophages were infected following a protocol previously reported **[19]**, with some modifications. Briefly, 3×10^5 cells per well were PMA stimulated and seeded in 24-well tissue culture plates with clear bottoms (Falcon®, Tewksbury, USA). RPMI containing CSE was added to the cells and incubated for 3 hours. For the infection, mid-log phase *M. tuberculosis* H37Rv or *M. bovis* BCG was washed twice with DPBS+0.05% tween and subsequently once with DPBS after which they stood for 5 min, before the supernatant was collected. The bacteria were then diluted in RPMI with MptpB inhibitor (80µM), RIF (0.3 or 4 µg/ml) and/or CSE and added to the THP-1 macrophages at a multiplicity of infection (MOI) of 0.1 (for H37Rv) or 10 (for BCG). After 3 h of contact at 37°C in 5% CO_2, the macrophages were treated with 200 µg/mL amikacin for 1 h and

washed three times with DPBS to eliminate any extracellular bacteria. Lastly, 1 mL of RPMI with inhibitor, RIF and/or CSE was added to each well and incubated at 37°C in 5% CO_2. Fresh medium with MptpB inhibitor, RIF and/or CSE was changed at 24h post-infection. Extracellular growth was assessed with serial dilution in PBS-tween plated onto Middlebrook 7H10 solid medium at days 0, 1, 2 and 3. Intracellular growth was assessed by lysis of the monolayers by the addition of 500 µL of water followed by a 30 min incubation at RT and then plating on 7H10 medium. Colonies were counted after 3–4 weeks incubation at 37 °C and CFUs/mL determined.

Statistical analyses

Statistical analyses were performed using the GraphPad PRISM 6.0 software package (San Diego, California, USA). Differences between treatments were compared by the Student's t-test. A *p*-value ≤0,05 was considered as a statistically significant.

RESULTS & DISCUSSION

The pathogenicity of a bacteria depends on its ability to survive and replicate in the host **[8]**. Usually, intracellular pathogens require the modification of complex signalling events to survive in the host's cells, such as interfering in the phagosome-lysosome fusion and acidifying the phagosome.

We explored the effect of a novel inhibitor in avirulent and virulent mycobacteria, at different MOIs, and we studied the effect of cigarette smoke when the MptpB inhibitor was added. RIF was added to test if the MptpB inhibitor was more effective than RIF and to check if an improvement was achieved in combined therapy with the MptpB inhibitor.

Macrophages were exposed to MptpB inhibitor (80 µM), RIF (0.3 µg/ml) and CSE during three days and the cytotoxicity of the compounds was assessed using PrestoBlue assay at 6, 24 and 72 hours. Viability was over 80% in all conditions and days; suggesting that the compounds do not have major toxic effects on the cells at the concentrations assessed (Figure 1).

Toxicity of the MptpB inhibitor was not studied on the bacterial strains since MptpB inhibitors do not have bactericidal action by themselves but potentiate the impairment of mycobacterial growth within macrophages **[11]**. However, we studied the growth of the mycobacteria in the intracellular and extracellular fraction of infected cell cultures in the presence of the inhibitor, RIF and CSE.

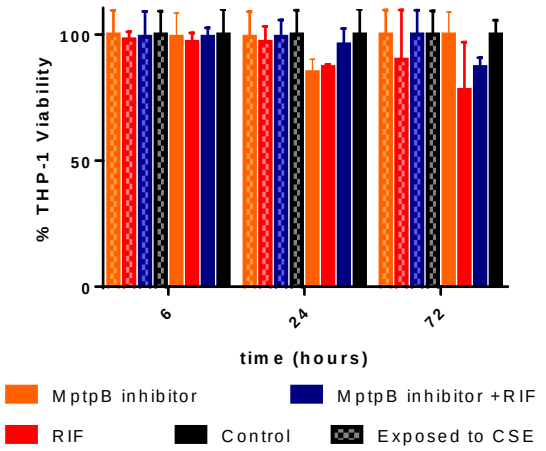

Figure 1. THP-1 viability after exposure to MptpB inhibitor, rifampicin and smoke. PrestoBlue assay was performed at 6, 24 and 72 hours after exposure to MptpB inhibitor, 0.3 µg/ml rifampicin and 10% CSE. The results are expressed as the average and standard deviation of three replicates.

For the extracellular fraction, macrophages were exposed to CSE for three hours and infected with *M. bovis* BCG. Extracellular CFUs tended to be higher in non-CSE exposed cultures compared to CSE exposed ones (Figure 2). Addition of MptpB inhibitor did not reduce the extracellular CFUs counts (Figure 2.A), while the addition of RIF 0.3 µg/ml, did (Figure 2.B). There were no major differences in the growth of BCG by adding RIF or the combination RIF and inhibitor (Figure 2.B & 2.C), suggesting that the inhibitor did not play a major role in the extracellular fraction. This is in agreement with previous reports where it was shown that this inhibitor does not affect the growth of extracellular mycobacteria, evidencing the lack of bactericidal activity **[10, 11]**. This also demonstrates that *mptpB* gene is not essential for the mycobacteria survival **[1]**. We performed the same experiments with *M. tuberculosis* H37Rv strain. There were no significant differences in the recovery of extracellular CFUs exposed to CSE compared to unexposed (Figure 3). No effect was observed by adding the inhibitor (Figure 3.A) neither RIF 0.3 µg/ml (Figure 3.B). As addition of RIF 0.3 µg/ml had no effect on the growth of H37Rv, we performed the experiments adding RIF 4 µg/ml, and the recovery of bacilli was null. Combination of RIF and inhibitor had no additional advantage to adding only RIF in the elimination of extracellular mycobacteria, as expected (Figure 3.C).

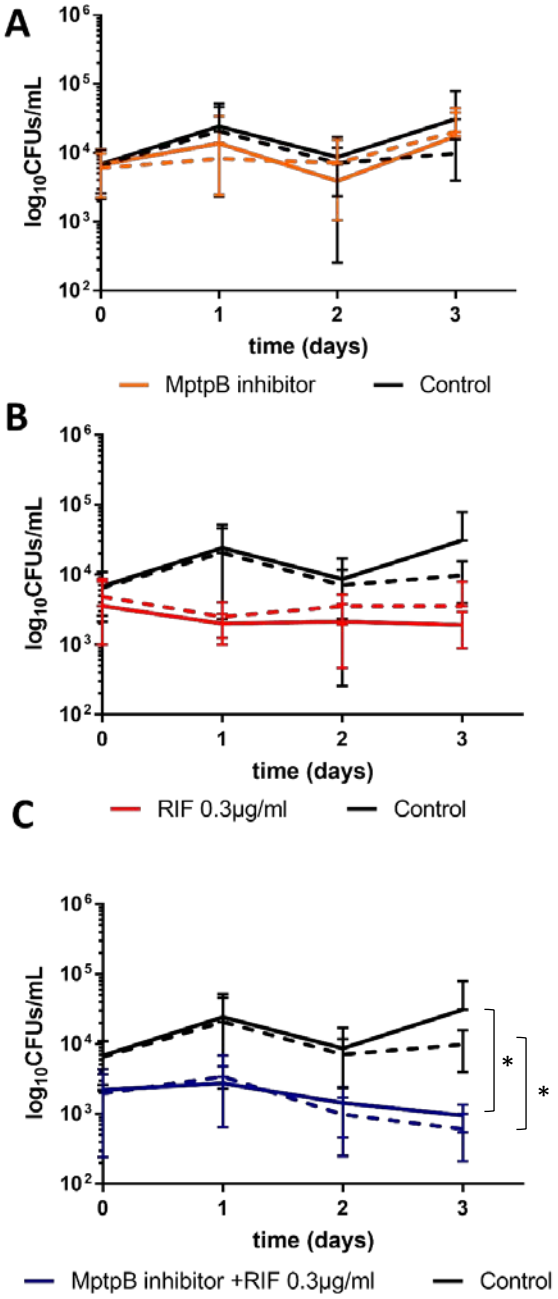

Figure 2. *M. bovis* extracellular CFUs counts over 3 days after drugs administration. Recovery of extracellular BCG from THP-1 macrophages exposed to 10% CSE and (A) MptpB inhibitor (80µM), (B) RIF 0.3 µg/ml and (C) combinations of MptpB inhibitor (80µM) and RIF 0.3 µg/ml administration. The continuous lines correspond with CSE unexposed cells and the dashed lines with CSE exposed cells. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments.*P<0.05 (day 3).

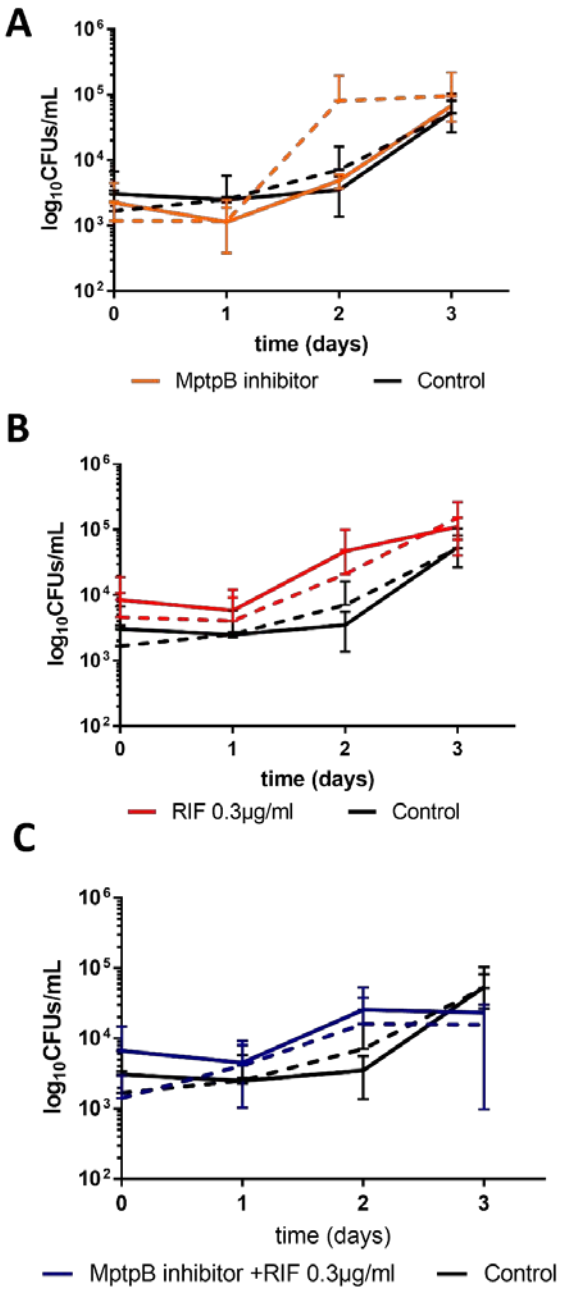

Figure 3. *M. tuberculosis* extracellular CFUs counts over 3 days after drugs administration. Recovery of extracellular CFUs from THP-1 macrophages exposed to 10% CSE and (A) MptpB inhibitor (80µM), (B) RIF 0.3 µg/ml and (C) combinations of MptpB inhibitor (80µM) and RIF 0.3 µg/ml administration. The continuous lines correspond with CSE unexposed cells and the dashed lines with CSE exposed cells. The results are expressed as the average and standard deviation of duplicates of at least two independent experiments.

Intracellular CFUs were assessed during three days, following the same protocol than for extracellular CFUs, but lysing the monolayers. In general, cells exposed to CSE and infected with BCG or *M. tuberculosis* had a lower intracellular burden (Figures 4 & 5). This is in agreement with previous investigations, where impairment of macrophage phagocytosis by CSE has been described in different cell lines **[20-23]**. Compare **Chapter III**.

A slight reduction in intracellular BCG was seen when MptpB inhibitor was added to the culture (Figure 4.A). Addition of RIF 0.3 µg/ml reduced significantly the intracellular burden (Figure 4.B). The addition of the combination RIF plus MptpB inhibitor reduced even more the internal burden, 67% more than the addition of RIF without MptpB inhibitor at 72 h post-infection (Figure 4.C). Similar results were obtained with H37Rv strain. Addition of the MptpB inhibitor reduced slightly the recovery of CFUs (Figure 5.A). Addition of RIF 0.3 µg/ml only caused a tiny reduction in the intracellular burden, but the addition of RIF 4 µg/ml reduced significantly the burden (Figure 5.B). Addition of both, MptpB inhibitor and RIF, reduced drastically the surviving bacilli, over 80% compared to RIF at day three (82.5% for RIF 0.3 µg/ml and 89.5% for RIF 4 µg/ml) (Figure 5.C). Similar to Vickers *et al.* **[10]**, we observed that inhibition of MptpB enhances mycobacterial killing by RIF.

Although the combination of RIF, even at very low doses, with MptpB inhibitor reduces dramatically the intracellular burden of mycobacteria in macrophages, it is not well understood why the effect of administrating the inhibitor alone is not so high. Probably a higher concentration of inhibitor or longer exposure times would decrease more the intracellular burden. It has been observed that this inhibitor shows dose-dependent efficacy in its ability to reduce BCG (up to 87% reduction in 72h) in J774 cells and H37Rv (up to 63%) in THP-1 cells and similar results were obtained with MDR-TB **[10]**.

Disruption of *M. tuberculosis mptpB* gene impairs the ability of the mutant strain to survive in macrophages J774A.1 and guinea pigs while the reintroduction of the gene into the mutant strain re-establishes the infection and survival at comparable levels as in the parental strain **[5]**. On the contrary, if MptpB is purified and added to the macrophages, the survival of H37Rv in macrophages is higher **[3]**. Overexpression of *mptpB* in RAW264.7 cells also increased the survival of intracellular H37Rv **[3]**. In the last years, several inhibitors have been developed but the efficiency in reducing *M. tuberculosis* survival has not been very high, at least comparing with MptpB deficiency **[24, 25]**.

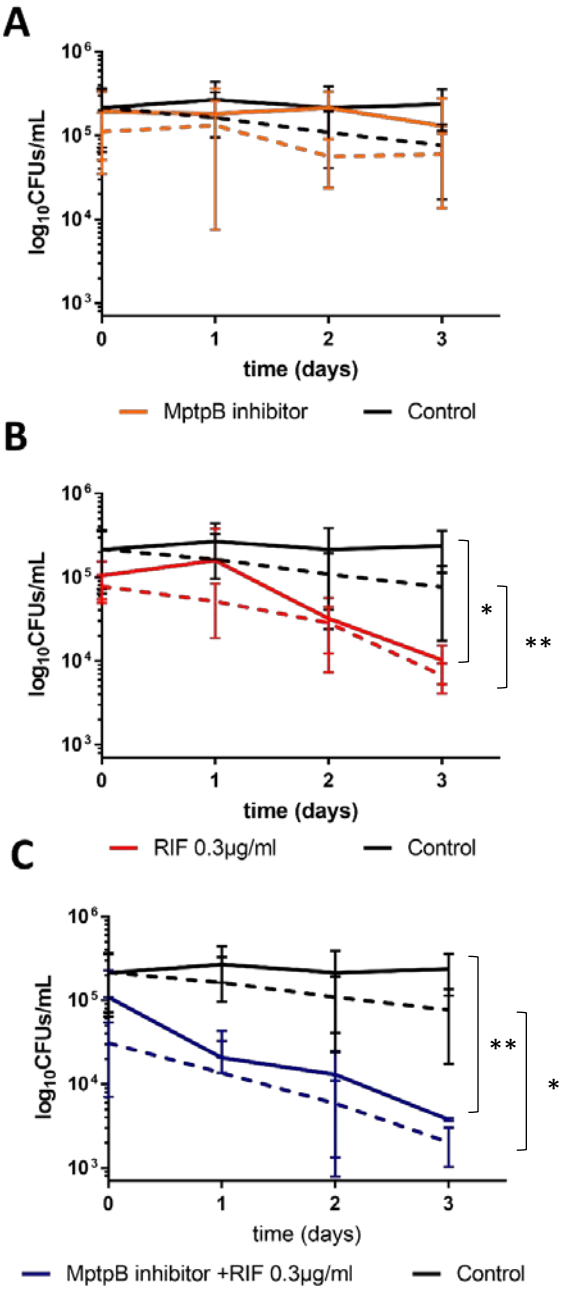

Figure 4. *M. bovis* intracellular CFUs counts over 3 days after drugs administration. Recovery of intracellular CFUs from THP-1 macrophages exposed to 10% Marlboro CSE and (A) MptpB inhibitor (80µM), (B) RIF 0.3 µg/ml and (C) combinations of MptpB inhibitor (80µM) and RIF 0.3 µg/ml administration. The continuous lines correspond with CSE unexposed cells and the dashed lines with CSE exposed cells. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments. **P*<0.05 ***P*<0.01 (day 3).

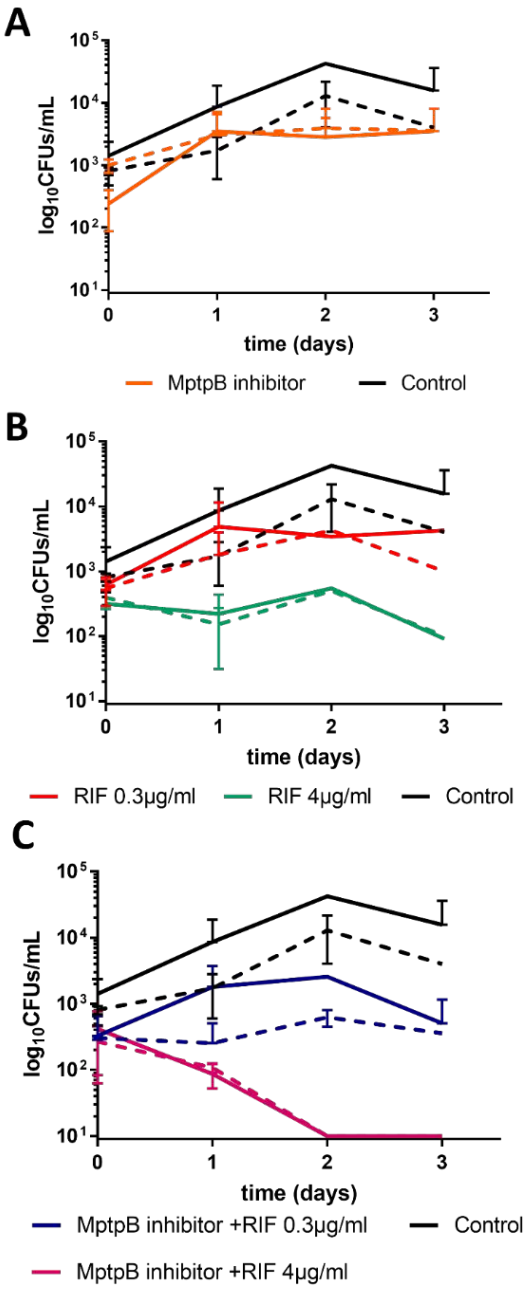

Figure 5. *M. tuberculosis* intracellular CFUs counts over 3 days after drugs administration. Recovery of intracellular CFUs from THP-1 macrophages exposed to 10% CSE and (A) MptpB inhibitor (80µM), (B) RIF 0.3 and 4 µg/ml and (C) combinations of MptpB inhibitor (80µM) and RIF 0.3 and 4 µg/ml administration. The continuous lines correspond with CSE unexposed cells and the dashed lines with CSE exposed cells. The results are expressed as the average and standard deviation of triplicates of at least three independent experiments.

The importance of MptpB in the interaction host-pathogen has been highlighted since disruption of *mptpB* had an effect reducing the intracellular burden on activated macrophages with IFN-γ, but not in resting macrophages **[2, 5]**. Nevertheless, other studies with other strain did not report the same difference **[11]**. Because some reports indicated that *M. tuberculosis* can survive better in IFN-γ activated macrophages with *mptpB* overexpressed, it was thought that MptpB could affect IFN-γ pathway **[3]**. In fact, MptpB suppresses IL-1β, IL-6 and apoptosis, hamper NF-kB and MAPK signal pathways and inhibit p53 and host inflammatory responses **[3]**. This effect is modulated by inhibition of MptpB **[1]**. Despite that IL-1β is an important proinflammatory cytokine in the host defence after *M. tuberculosis* infection and it is reported to be reduced in macrophages exposed to CSE **[22, 26, 27],** we did not find any antagonist effect between macrophages treated with inhibitor exposed and unexposed to CSE. No changes were observed between samples exposed and unexposed to CSE. Cells exposed to CSE tended to have a lower intracellular burden because of a phagocytosis impairment (**Chapter III**). The molecular factors responsible for controlling phosphoinositide levels in mycobacterium-infected macrophages remain to be unravelled, even when MptpB is playing an important role **[9]**. Further studies like administrating MptpB inhibitor only before or after infection would be necessary, as targeting virulence factors are promising candidates for drugs and vaccines **[8]**. One of the main reasons for the efficacy of the treatment with the inhibitor is probably that there is no need to cross the poorly permeable and thick mycobacterial cell wall **[10]**.

In summary, PI3P is a regulatory lipid essential for the phagosome-lysosomal fusion. In this study, we have shown that MptpB inhibitor did not kill the extracellular mycobacteria but reduced the intracellular burden, especially if administered with RIF. We found no interactions between MptpB inhibitor and CSE. Targeting virulence factors without bactericidal activity has the advantage of reducing selective pressure, and can be used in combination with the current treatment to reduce the establishment of an infection or even as a form of prevention **[1]**.

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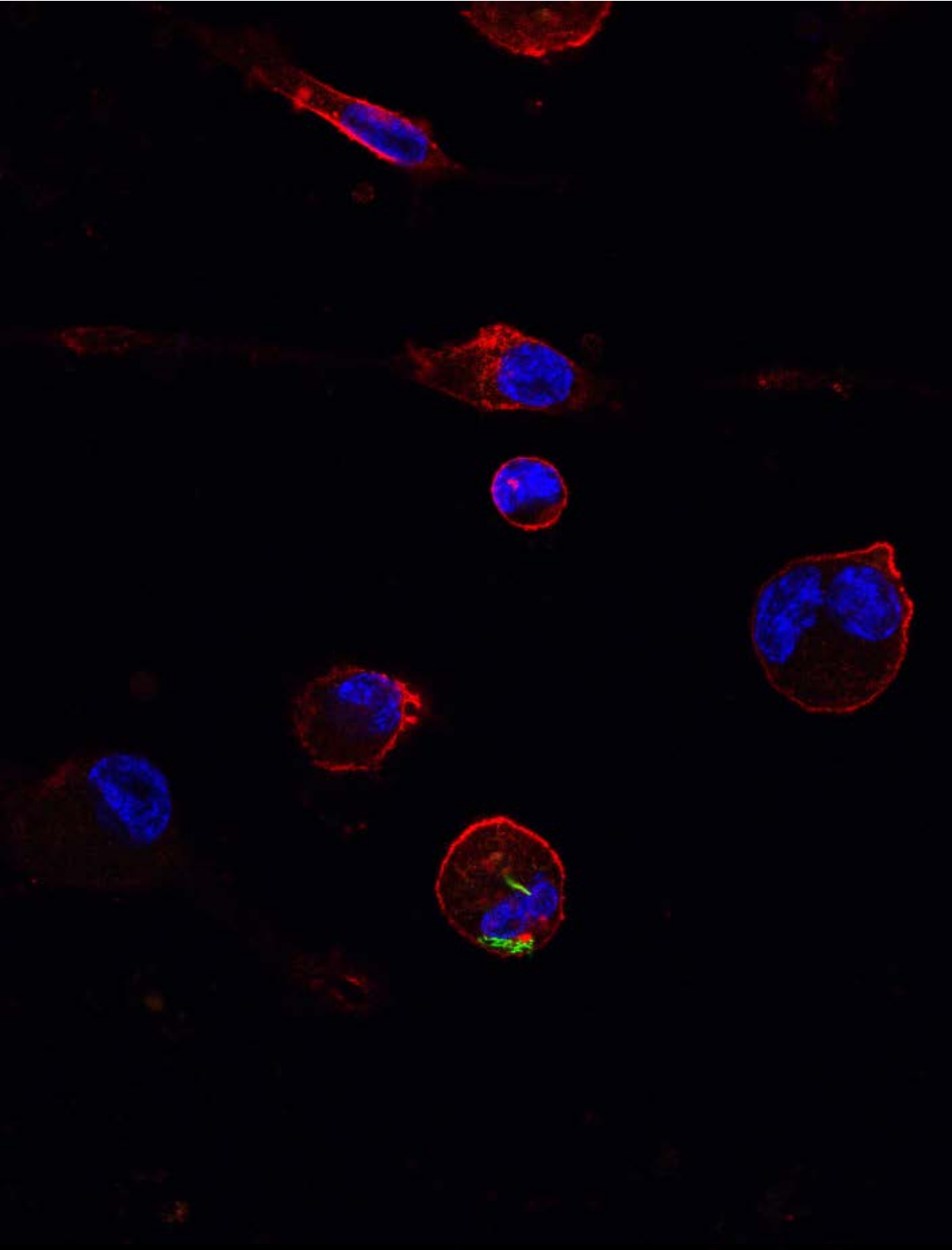
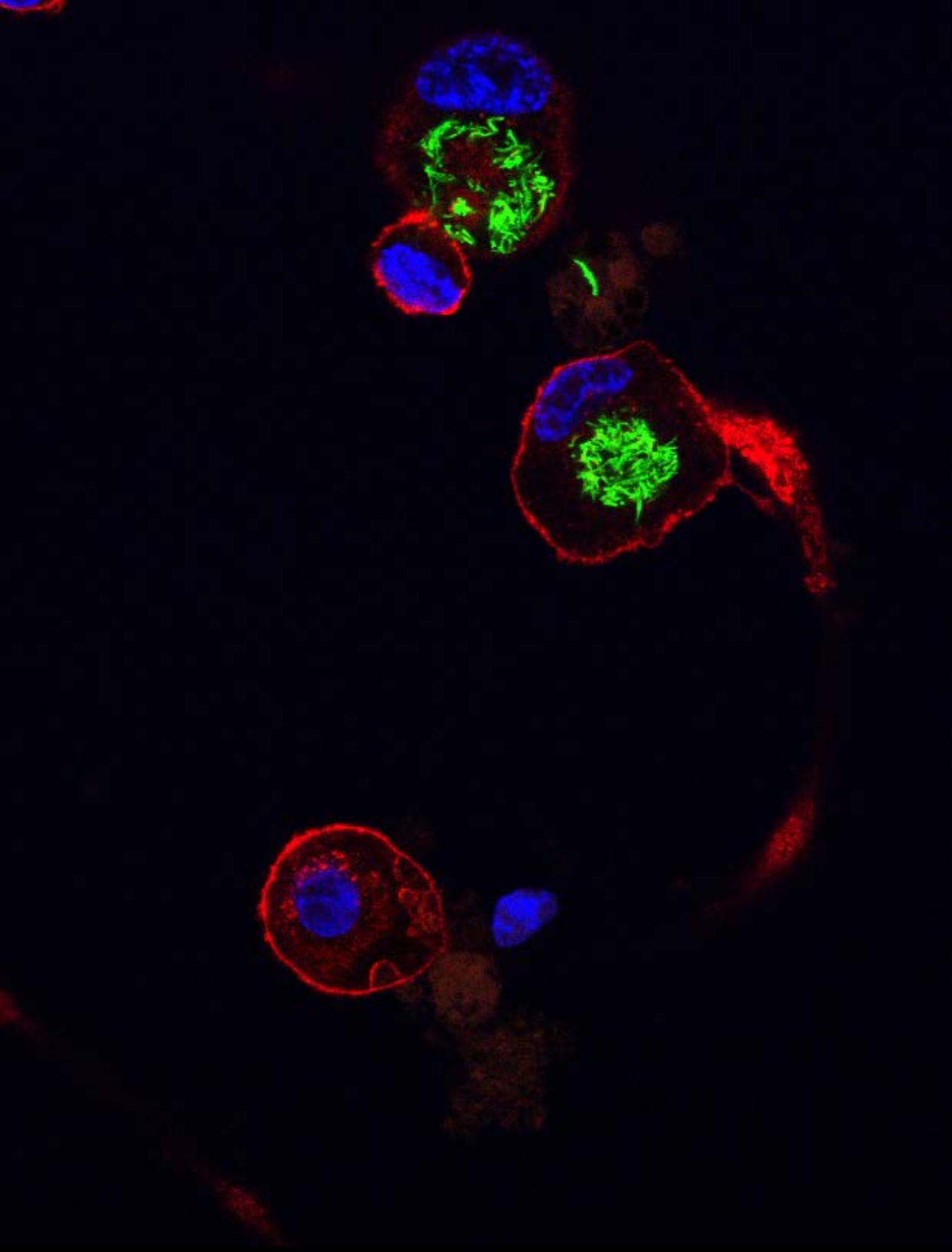


THP-1 cells infected with BCG-GFP.
Day 6 after infection



5. DISCUSSION

The outcome of an infection is determined by the host itself, the pathogen and external factors. TB remains one of the most widespread infectious diseases with a high mortality, partially because cigarette smoking is a risk factor that leads to TB epidemics. This thesis is focused on the impact of outdoor (diesel) and indoor (smoking and vaping) air pollution on TB. The quality of the air is linked with the health status of the lungs and the susceptibility to respiratory infections, and TB is not an exception. Moreover, the appearance of resistant TB makes it necessary to innovate on treatment, whether it is looking for more efficient delivery mechanisms or looking for new drugs to potentiate the host's immune status to kill the bacteria. Therefore, in this thesis, we discuss some novel therapeutic approaches: NPs and HDT.

This discussion is structured in two main topics: the impact of pollution on TB and the effectiveness of new therapeutic approaches.

5.1 Diesel, cigarette smoke and electronic vapor pollutants

We studied the toxicity of pollutants on the cell, the bacteria, the bacterial burden of infected cells, and the production of cytokines and metabolites.

Cytotoxicity of pollutants.

In **Chapter II**, macrophages were exposed to low concentrations of DEP because of the high cytotoxicity observed in THP-1 cells, as it was already reported **[135]**. Induction of pulmonary oxidative stress, pro-inflammatory responses and genotoxicity are behind ultrafine particles effects **[136]**. We observed a high internalization of the DEP on macrophages as well as rapid sedimentation. *In vivo* studies with mice have shown DEP deposition on lung phagocytes **[137]**. In **Chapters III & V**, we highlighted the high cytotoxicity of CS in comparison with e-vapor. Combustion generates many free radicals that could explain the different cytotoxicity between CS and e-vapor **[138-140]**. Although e-cigs do not produce combustion in the process to heat the liquid in the atomizer, many toxic compounds, reactive oxygen species and free radicals are generated, being some of the compounds generated carcinogens. In CS, we observed different cytotoxicity levels in different cell lines, as it was already published **[58, 141, 142]**, and that cytotoxicity differed between cigarette brands **[143-145]**. Apoptosis tended to be inhibited and cell death percentage was higher after three days **[146]**. Inhibition of apoptosis and the increased cell death due to CS would be acting in favour

of the pathogen, avoiding the killing and facilitating the dispersion. As happens with CS, toxicity of e-vapor depends on many variables: the cell line used, if they contain nicotine, type of flavors, the PG/PV proportion and the voltage and potency of the device **[147]**. Neither the presence of nicotine nor flavors affected the cytotoxicity of e-cigs, despite some studies with opposite results on the literature. Although flavors are considered safe for consumption, inhalation could have very different results. Comparison between refill liquids is difficult as more than 8,000 flavors exist on the market. Higher potency of e-cig battery and longer puffs are related with higher cytotoxicity **[138, 148-151]**. However, vaping sessions are very different from the topography of smoking, which makes it difficult to extrapolate the data from *in vitro* experiments of macrophages exposed to CS and e-vapor in similar ways.

Effects of pollutants on bacteria.

We studied the effects of pollutants on the bacteria in **Chapters II, III & V**. Growth of the bacteria was not impaired by DEP or e-vapor exposure. CS only delayed slightly the kinetics growth, both in 7H9 and RPMI, although some compounds of CS, like nicotinic acid, have been reported to enhance the growth of *M. tuberculosis* **[152]**. No effect has been described in other facultative intracellular pathogens such as *S. aureus* **[153]**. It is plausible that the higher bioavailability of iron in smokers enhances the growth of the mycobacteria in an infection *in vivo*, where iron is a limiting factor for its growth.

When we exposed bacteria to CS and drugs, we observed a slight increase in the MIC for RIF, suggesting an interaction. Quantifying the RIF in the extracellular fraction showed higher amounts of RIF in non-CS cultures, suggesting less degradation. Drug interactions have been associated with smoking because of pharmacokinetic interactions between nicotine and polycyclic aromatic compounds of tobacco, suggesting that smokers may need higher drug doses to reach similar effects **[154]**.

Effects of pollutants in the extracellular and intracellular burden.

In **Chapter II** we observed that exposure to DEP did not affect the intracellular BCG burden. We did not observe any impairment on phagocytosis as it is described in other microorganisms **[155-157]**. Similar to our results, *in vitro* assays with mycobacteria showed no impairment of phagocytosis before day 10 **[158]**. Intracellular *M. tuberculosis* burden in other cells showed differences in CFU recovery only when exposed to high doses of PM, and the impact was more visible when the MOI was

higher and the PM smaller **[81]**. Long-time exposure could be necessary to observe differences in intracellular burden on animals exposed to DEP **[85]**.

Extracellular BCG tended to be higher in samples exposed to DEP, which could be explained by a decrease in the killing rate on DEP exposed macrophages. Overburden of bacteria in macrophages could lead to their death, increasing the extracellular bacilli but not the intracellular ones.

On the other hand, as it was discussed in **Chapter III**, both CS and e-vapor reduced the intracellular burden of *M. tuberculosis*, BCG and latex beads, supporting the hypothesis that CS and e-vapor extract cause a phagocytosis impairment. Phagocytosis impairment has been observed in monocytes from smokers TB patients, in alveolar macrophages from smokers, in lung-tissue and alveolar macrophages from smoking mice, and in alveolar and THP-1 macrophages when cells exposed to CS extract were infected with *H. influenzae* **[141, 151, 159-163]**. The phagocytosis impairment could be explained by a decrease of the expression of recognition molecules (CD44, CD91, CD31 and CD71) for apoptotic cells in alveolar macrophages, the inhibition of Rac1 protein, involved in cytoskeletal rearrangements, the blockage of PI3K signaling cascade as well as the decrease of the expression of bacterial recognition receptors like Toll-like receptors 2 and 4 and scavenger receptor SR-A1 **[141, 151, 159-163]**. Furthermore, alveolar macrophages exposed to CS show alveolar lysosomal accumulations of tobacco smoke, losing their capacity to migrate to *M. tuberculosis* **[164]**. Opposite results have also been observed on virulent *M. tuberculosis*, BCG and H37Ra strains where intracellular burden is higher in cells exposed to CS **[58, 75, 165]**. Moreover, Bai *et al*. observed that nicotine reduces the number of autophagosomes leading to an increase of *M. tuberculosis* burden in alveolar macrophages **[74]**. Our studies with latex beads (inert particles) support that phagocytosis impairment by CS plays a vital role in the outcome of the experiments. Regarding e-vapor, we did not observe differences between flavors, unlike some previous research **[151]**. Macrophages exposed to e-vapor with nicotine had less intracellular mycobacteria, suggesting that nicotine in e-vapor extract has an effect on the phagocytosis of mycobacteria. Ween *et al*. observed that the impairment of phagocytosis by e-cigs was also linked with the expression of scavenger receptor SR-A1 and TLR-2 **[151]**. Contradictory results between experiments could be explained by the different size of DEP, time of exposure, multiplicity of infection, cell, line, strain, type of cigarette/e-cig and/or extraction method. Some studies showed opposite results after prolonged exposure times.

In **Chapter V** we discussed the effect of CS when RIF or INH was administered. Like macrophages unexposed to drugs, the intracellular *M. tuberculosis* burden tended to be lower in cultures exposed to CS. Extracellular *M. tuberculosis* was similar in samples exposed and non-exposed to CS, in agreement with the fact that CS affects the phagocytosis of macrophages but not the growth of the bacteria (see **Chapter III**). Although some studies link CS with MDR-TB, they do not look for a correlation between CS and the efficiency of the drugs, but with a poor treatment compliance in TB smoker patients, especially in males **[62, 166]**. Similar results were observed in **Chapters VI & VIII**, where samples exposed to CS had a reduced intracellular burden, and no interactions were found between treatment and CS. The exposure to CS does not generate greater differences in the extracellular or intracellular *M. tuberculosis* and BCG burden between the control-RIF, control-MptpB inhibitor and control-RIF-MptpB inhibitor. This indicates that there was no interaction between CS and MptpB inhibitor, although MptpB inhibitor favours the host while CS benefits the infection.

Effect of CS and e- vapor on cytokines production and metabolomics.

We described the effects of CS and e-vapor on the production of cytokines in **Chapter III**. Macrophages produced more pro-inflammatory cytokines like TNF-α, IL-1β and IFN-γ when they were infected with *M. tuberculosis*, as a natural response against the bacteria **[167]**. All of these are key cytokines in the host defence after *M. tuberculosis* infection, and TNF-α also initiates the activation of the phagocyte **[140, 167]**. CS and e-vapor stimulate both inflammatory and anti-inflammatory responses, although, in general, e-vapor showed a tendency to produce a pro-inflammatory response **[162, 168]**. Same pattern of elevated cytokine production after e-vapor inhalation was also observed in mice model **[168, 169]**. CS decreased the production of TNF-α and IL-1β in uninfected macrophages, as it was already described in THP-1 cells, macrophages and peripheral blood mononuclear cells (PBMCs) **[151, 170, 171]**. We observed the same tendency in infected cells exposed to CS extract. E-vapor increased TNF-α, as it was reported in other studies **[172, 173]**. We did not observed a decrease in IFN-γ release by CS, unlike some previous studies describe **[58, 174, 175]**. IL-8, a neutrophil chemoattractant that mediates the inflammatory process, was increased in uninfected macrophages exposed to CS, as it was already reported **[151, 167]**. E-vapor also stimulated the production of IL-8 **[151, 172, 173, 176, 177]**.

Unbalance in cytokine production caused by e-vapor and CS could be associated with a less effective immune response against pathogens, such as Th-1/Th-2 pattern

changes, which could explain the higher susceptibility of developing mycobacterial disease when smoking **[178, 179]**.

In **Chapter IV** we studied the metabolomics profile of THP-1 cells in samples exposed to CS and e-vapor. PCA analysis for extracellular metabolites revealed that maturation of the cultures was the most important variable altering the metabolomics profile, followed by e-vapor exposure. The compounds that most contributed to e-vapor separation (lactic acid, nicotine, benzoic acid and glycerol) were compounds of e-liquids or products of reactions between components of e-liquids **[180-182]**. A hypothesis explaining why e-vapor supernatants are more different than CS supernatants, compared to the control, is that CS contains many metabolites in small concentrations that probably are not in the UPSC library or could not be detected.

Alteration of the metabolomic profile due to e-vapor was also observed in the intracellular analysis. PCA analysis showed a clear separation between days. Our results also suggest that CS or e-vapor causes changes in metabolites immediately after exposure. This is in agreement with other studies, where maximal deviation of the metabolome is produced at 1 or 2 hours after CS exposure **[183-185]**. We observed a general reduction in carbohydrate metabolism and an accumulation of citric acid in uninfected samples as previously reported **[183, 184]**. Glutamic acid was reduced, as it was reported for smokers **[186]**. We also studied the trends in increasing or decreasing metabolites during time. Similar to previous studies, we observed an increase in lactic, malic and citric acids, indicating that these are products of maturing cell culture **[187]**. The accumulation of citric acid could be due to an altered Krebs cycle **[188]**. Citrate activates the lipid synthesis, important for the activation of macrophages, the production of inflammatory mediators and to allow membrane expansion needed to antigen presentation **[188, 189]**.

Between the metabolites that are decreased differentially in samples exposed to CS or e-vapor compared to control, we observed that 50% of the metabolites found in these groups are part of the amino acids metabolism. Amino acids play a key role in the host-pathogen interaction, as pathogens depend on the host nutrients for their survival, and the host tries to keep pathogens away from their nutrients **[190, 191]**. We found that tryptophan increased during the 36h of the experiment in uninfected samples, probably due to active biosynthesis. In infected cells unexposed to CS or e-vapor, levels of tryptophan could be decreased as a protection strategy against the pathogen. Although *M. tuberculosis* constitutively synthesizes its own amino acids, amino acids

also play important functions regulating the host's immune system, such as activating TLR signalling, Th1 or Th17 responses and enhancing targeting and degradation of intracellular pathogens indirectly by autophagy **[192]**. However, tryptophan levels continued to increase in infected cells exposed to CS or e-vapor. Possibly the exposure to CS or e-vapor disrupts the protection strategy.

We also observed that tryptophan and kynurenine levels were inversely correlated, indicating that indoleamine 2,3-dioxygenase (IDO), the enzyme that catalyses the reaction that transforms tryptophan into kynurenine, could be altered. IDO is regulated by TNF-α, IFN-γ or prostaglandins **[192]**. *Mycobacterium avium* infection promotes IDO expression, decreasing tryptophan and restricting the growth of the pathogen **[193, 194]**. In **Chapter III** we discussed that both CS and e-vapor dysregulate the expression of TNF-α. IDO also balances the immunomodulatory response by its product kynurenine **[195]**. Kynurenine is a potent negative regulator of inflammation and T cell activity, enhancing the generation of regulatory T cells (Treg), promoting apoptosis and inhibiting ROS production **[191, 192]**. It has been observed in several studies that IDO activity is lower in smokers and, thus, so is the immunosuppression dependent on IDO **[196, 197]**. Enhancement of IDO activity could reduce inflammation, typically associated with tobacco cigarette smoking **[195]**.

5.2 New therapeutic approaches

Cytotoxicity of MptpB inhibitor and encapsulated drugs.

Organic and inorganic NPs are rapidly internalized within cells to be degraded as part of the innate immune response against any foreign body. In **Chapters VI & VII** we discussed the cytotoxicity of NPs. Silica NPs were much more cytotoxic than PLGA NPs, although they were not highly toxic for the organism when exposed chronically. PLGA are biodegradable polymers that are not accumulated in the cells **[198-202]**. We did not find cytotoxicity of PLGA NPs on Caco2 and MH-S cells, similar to studies in other cells lines **[203-205]**. An *in vitro* study with Caco2 cells suggests that PLGA NPs are not cytotoxic even at 25 g/l **[206]**. Drug load did not influence the cytotoxicity of the NPs. In **Chapter VIII** we explained that MptpB inhibitor was not found cytotoxic at the dose used.

Effects of MptpB inhibitor and encapsulated drugs on the bacteria.

Effectivity of NPs against *M. tuberculosis* was discussed in **Chapters VI & VII**. No changes in the growth curve were observed using silica NPs, since NPs concentration was very low, as well as the load of the drug. PLGA NPs reduced the growth of the bacteria for the 21 days tested. The intrinsic antituberculosis activity of empty PLGA NPs could be explained due to the potential acidification caused by PLGA degradation **[207]**.

Bactericidal effects were also tested with both types of NPs. MIC of unloaded silica NPs was superior to the concentrations tested, but the MIC was reduced when NPs were RIF or INH loaded. Time-kill kinetics at acidic pH also demonstrated the capacity of RIF-PLGA NPs to sterilize the cultures after two weeks. However, unloaded PLGA NPs also presented some bactericidal activity. Effectivity of free RIF was higher than RIF-PLGA NPs, due to the sustained release of the drugs from NPs, as previously reported **[208, 209]**.

Toxicity of the MptpB inhibitor (**Chapter VIII**) was not studied on the bacterial strains since MptpB inhibitors do not have bactericidal action by themselves but rather potentiate the impairment of mycobacterial growth within macrophages **[210]**.

Effect of MptpB inhibitor and encapsulated drugs on infected macrophages.

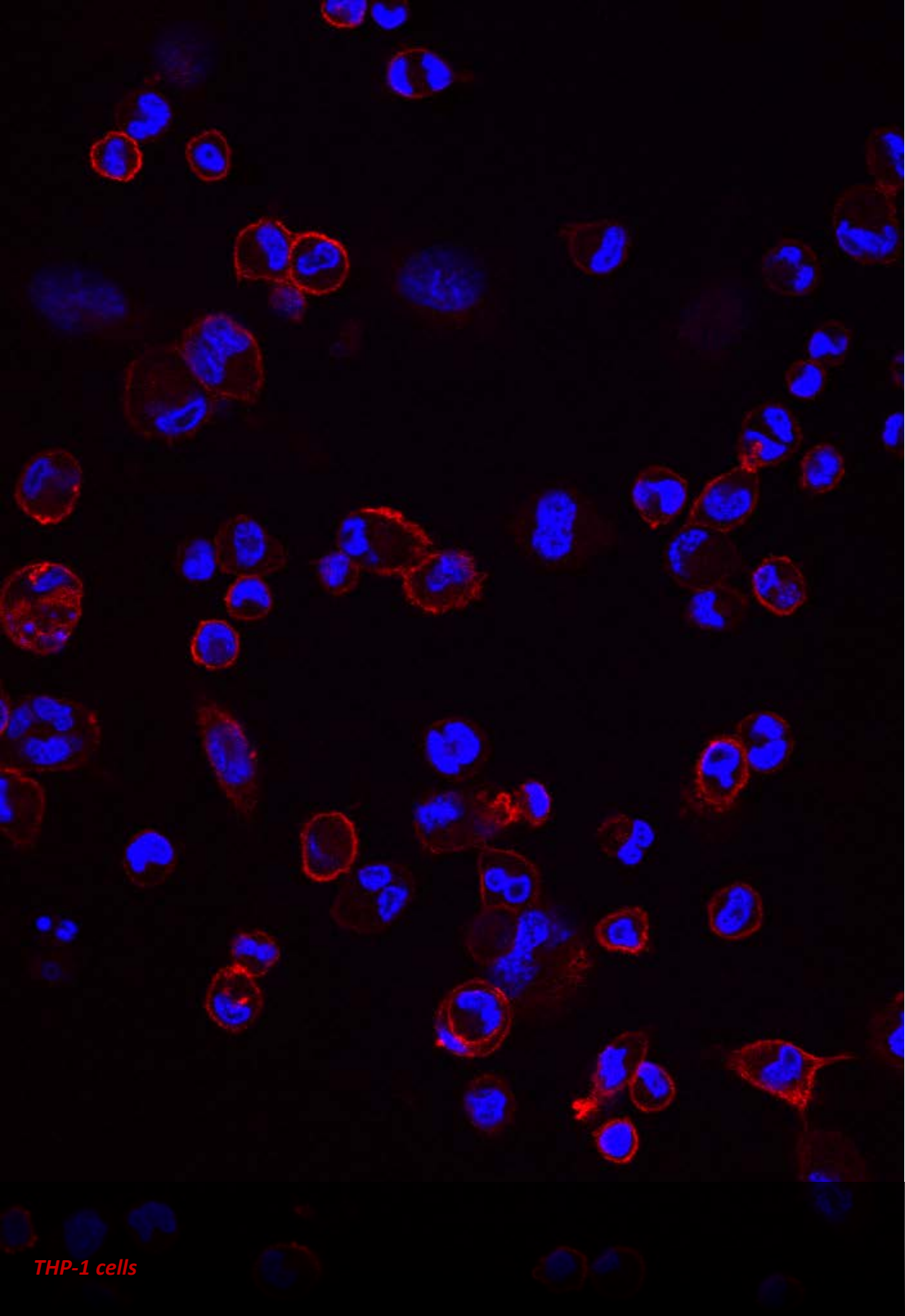
We evaluated the activity of silica NPs in **Chapter VI** on infected THP-1 macrophages. Silica NPs were less effective in both extracellular and intracellular burden than free antibiotics. The low impact could be explained by the small quantity of cargo. The fact that free drugs were more effective than loaded NPs could be explained by the release kinetics of the drug is not only dependent on dose and composition but also morphology, size, size distribution, surface charge, agglomeration state and solubility of the drug itself **[125, 201, 211, 212]**.

We observed in **Chapter VII** that intracellular bacterial load was significantly reduced by the addition of loaded PLGA NPs. Although NPs were not targeted to macrophages, they were rapidly internalized, even when internalization depends on the activation of macrophages **[213]**. Although colocalization does not occur because NPs are internalized in acidic phagolysosomes while *M. tuberculosis* remains in early phagosomes, drug diffuses to the cytosol and clears the infection. Our study was carried out in four days, but probably stronger effects could be observed with longer exposure time, as observed in similar studies **[202, 214]**. Encapsulating combinations of anti-TB drugs that generally present poor efficacy, are more toxic and require

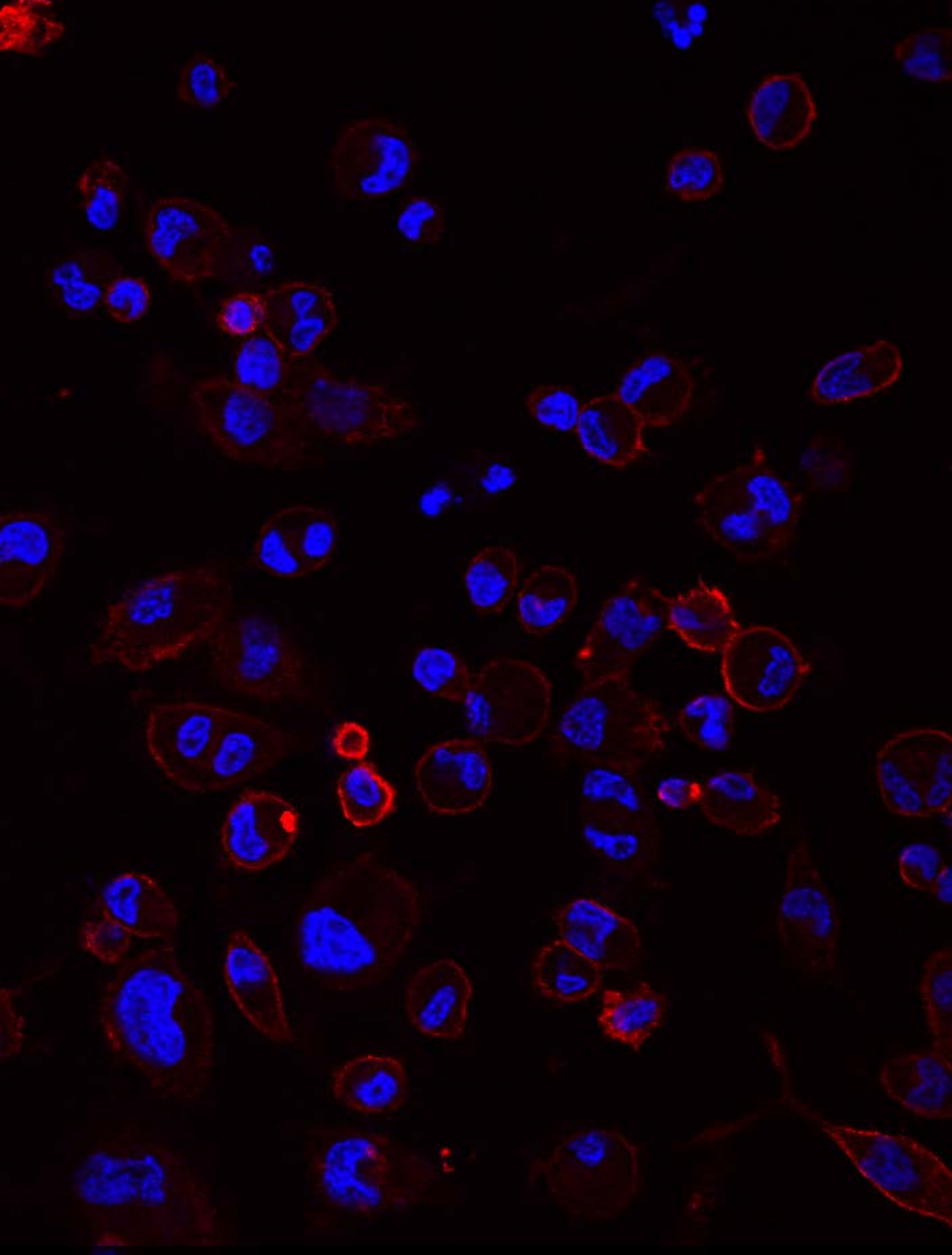
prolonged treatments could dramatically improve the treatment of MDR-TB patients **[215]**.

In **Chapter VIII** we discussed the effects of the addition of the inhibitor to macrophages infected. Extracellular CFUs counts in H37Rv and BCG were not affected by the inhibitor, although they were by RIF. Combination of RIF and the inhibitor had no additional advantage, being in agreement with the hypothesis that MptpB is not essential, does not affect the growth of extracellular mycobacteria and does not have bactericidal activity **[130, 210, 216]**.

Intracellular BCG and H37Rv burden was slightly reduced when the inhibitor was added to the culture in comparison with the addition of RIF. However, the addition of the combination RIF-inhibitor reduced dramatically the internal burden, 67% and 80% more than the addition of RIF alone in BCG and H37Rv respectively, similar to previous research **[130]**. Other studies have shown that overexpression of *mptpB* or addition of MptpB increased the survival of intracellular H37Rv, *mptpB* disruption impairs the ability of the mutant strain to survive in macrophages while the reintroduction of the gene into the mutant strain establishes the infection and survival **[217, 218]**. Some studies have shown that MptpB plays an important role on activated macrophages but not on resting macrophages, which suggests the importance of designing host-pathogen experiments when testing these compounds **[210, 218, 219]**.



THP-1 cells



6. CONCLUSIONS

6.1 Impact of outdoor and indoor air pollution on tuberculosis

1. Diesel exhaust particles are avidly phagocytosed by THP-1 cells and have high cytotoxic effects. Diesel exhaust particles could impair the contention of *M. bovis* by a decrease in the killing rates and overburden of bacteria, suggesting that diesel particles are related with mycobacterial infections.

2. Cigarette smoke is highly cytotoxic against different macrophage cell lines in comparison with electronic cigarettes vapor. Both e-vapor and cigarette smoke impair the phagocytic function of THP-1 cells against *M. tuberculosis*, *M. bovis* and latex beads. Nicotine but not flavor compounds of e-vapor seem to contribute to the phagocytosis impairment. E-vapor promotes pro-inflammatory responses while cigarette smoke decreases the cytokine response.

3. THP-1 cells exposed to e-vapor showed a profound impact on the metabolites present in supernatants, even more than cigarette smoke or infection stress. E-vapor also altered the intracellular metabolomic profile, with comparable effects to cigarette smoke. We observed that the protection strategy of tryptophan depletion by the host against pathogens is impaired by cigarette smoke or e-vapor, and that the immune system response is unbalanced by altered kynurenine concentrations.

4. Cigarette smoke does not interfere with the growth of the bacteria but reduces slightly the concentration of rifampicin in the extracellular fraction of macrophages cultures. Cigarette smoke also increases slightly the minimal inhibitory concentration of rifampicin. Intracellular burden of macrophages cultures exposed to cigarette smoke where rifampicin and/or isoniazid were administered do not differ from those unexposed to cigarette smoke, except for the impairment of phagocytic function.

6.2 Novel therapeutic approaches

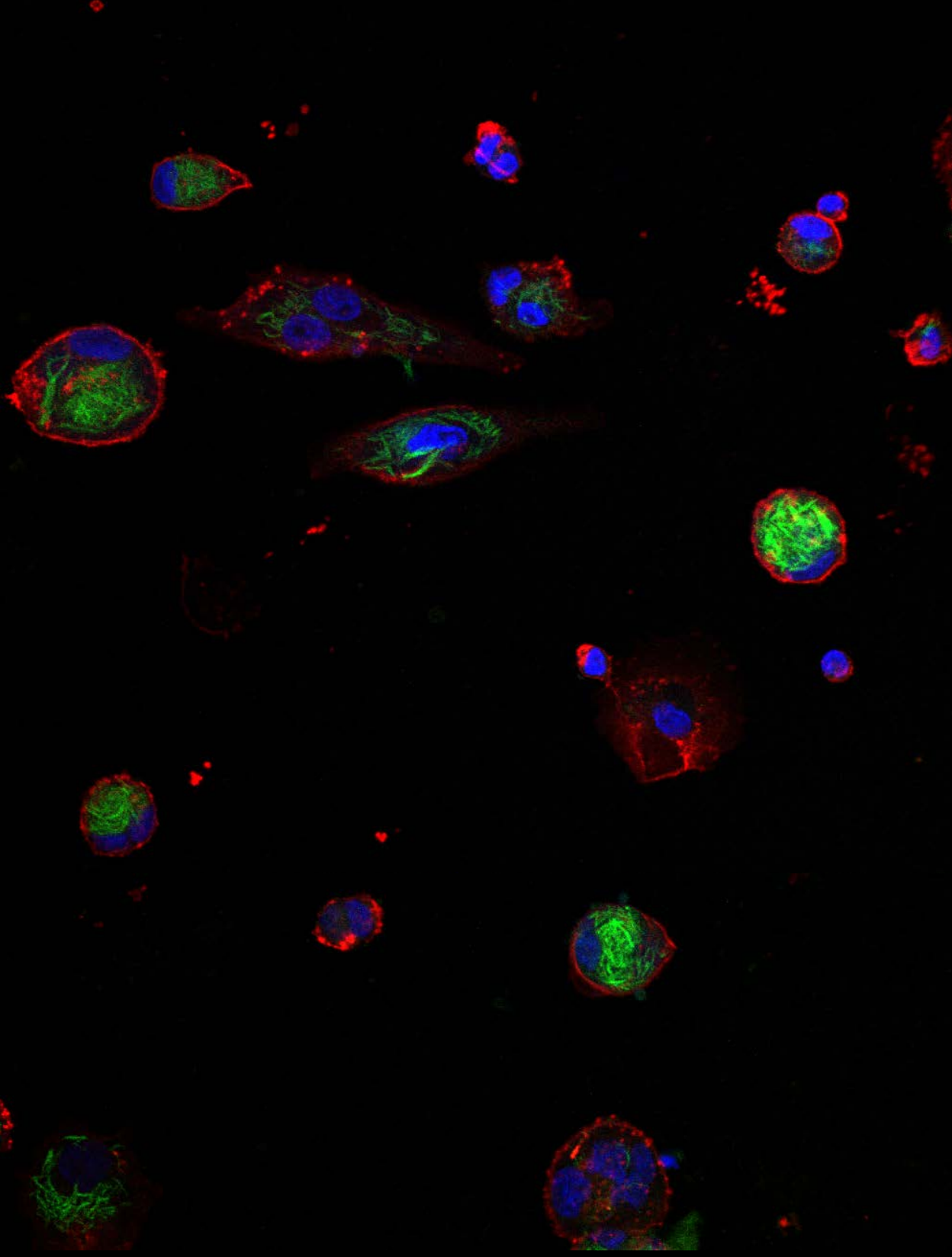
1. MCM41 silica nanoparticles loaded with rifampicin and isoniazid used in this study were less effective than free antibiotics. This is due to the sustained release of drug and toxicity of the nanoparticles themselves, that prevent concentration increase of nanoparticles added to macrophages. Nanoparticles had a very low load efficiency.

2. PLGA nanoparticles were less cytotoxic than silica nanoparticles and were resistant with Eudragit encapsulation to acidic pH, simulating gastric conditions. They were able

to cross an *in vitro* model of the intestinal barrier. The bactericidal action of nanoparticles loaded with rifampicin was similar to free rifampicin, indicating that rifampicin loaded in the nanoparticle is active. Unloaded PLGA nanoparticles have some bactericidal activity without being cytotoxic. Bactericidal activity of nanoparticles loaded with rifampicin against MH-S infected with *M. tuberculosis* was higher than the same concentration of free rifampicin.

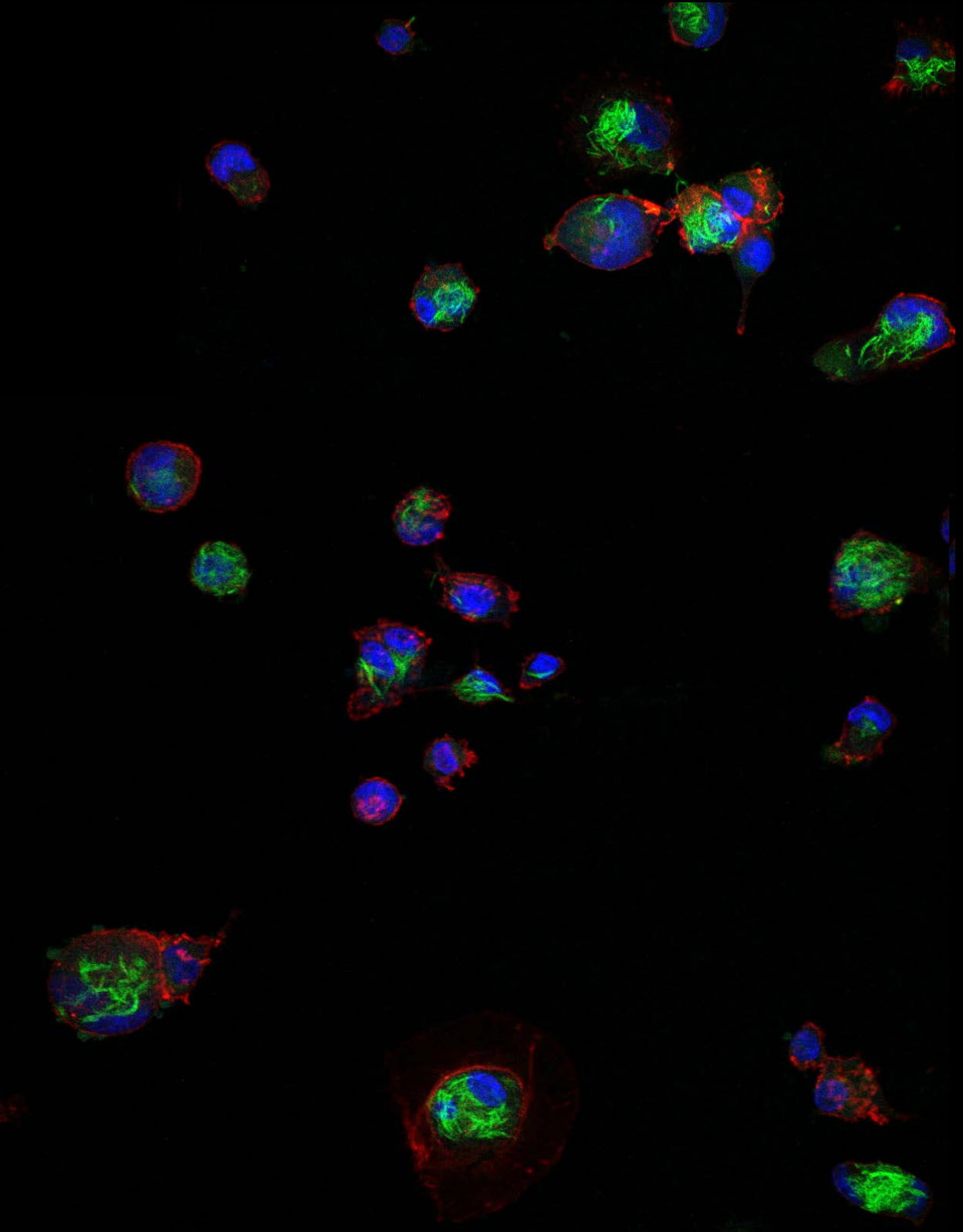
3. Inhibitor of MptpB was not cytotoxic on THP-1 cells. When it was administered to *M. tuberculosis* or BCG infected macrophages with a low dose of rifampicin, the intracellular burden was reduced significantly compared to treated only with a low dose of rifampicin. Extracellular burden was not affected by the administration of MptpB inhibitor, because it did not have bactericidal activity. MptpB inhibitor had the same effect on the intracellular burden compared to the control in the presence or absence of cigarette smoke, without interactions smoke-inhibitor.

It is summarized in the literature that environmental and indoor air pollution increase the rates of respiratory infections, causing oxidative stress, promoting inflammatory responses and altering defence mechanisms in the respiratory tract. Our in vitro *results confirm that pollutants cause disbalance on the immune response, phagocytosis impairment and metabolic changes, supporting a greater susceptibility to mycobacterial diseases. Infected macrophage models also support the use of loaded PLGA nanoparticles as an improvement of free drugs, and the use of host-directed therapies in addition to drugs to potentiate the response against mycobacteria.*



THP-1 cells infected with BCG-GFP.

Day 6 after infection



7. FUTURE PERSPECTIVES

Despite all efforts to reduce the environmental pollution and regulate policies against tobacco industries, there are still many steps to follow in the forthcoming years. Thus, it is necessary to jump to more green energies and avoid diesel exhaust particles and other pollutants that reduce the air quality of the cities, and the health status of the lungs. There is an urgent need to make global policies because fumes and smoke are risk factors for several diseases but particularly for TB global epidemics, and it is mandatory to implement them not only in European Union countries, but especially in countries where TB is endemic. Furthermore, it is necessary to incorporate smoking cessation as part of TB control strategies and as a part of TB treatment. Clinicians should be actively involved in helping their TB patients to stop smoking.

Scientific research will continue to focus on ENDS, e-cigs and IQOS tobacco heats systems, accumulating evidence of their impact on the long run. Genomics, transcriptomics, proteomics and metabolomics seem powerful bioanalytical strategies to give a global impact of these products, until clinical and epidemiological data appear. Improvement of big data management that allows a more comprehensive interpretation of -omics results is expected in the coming years.

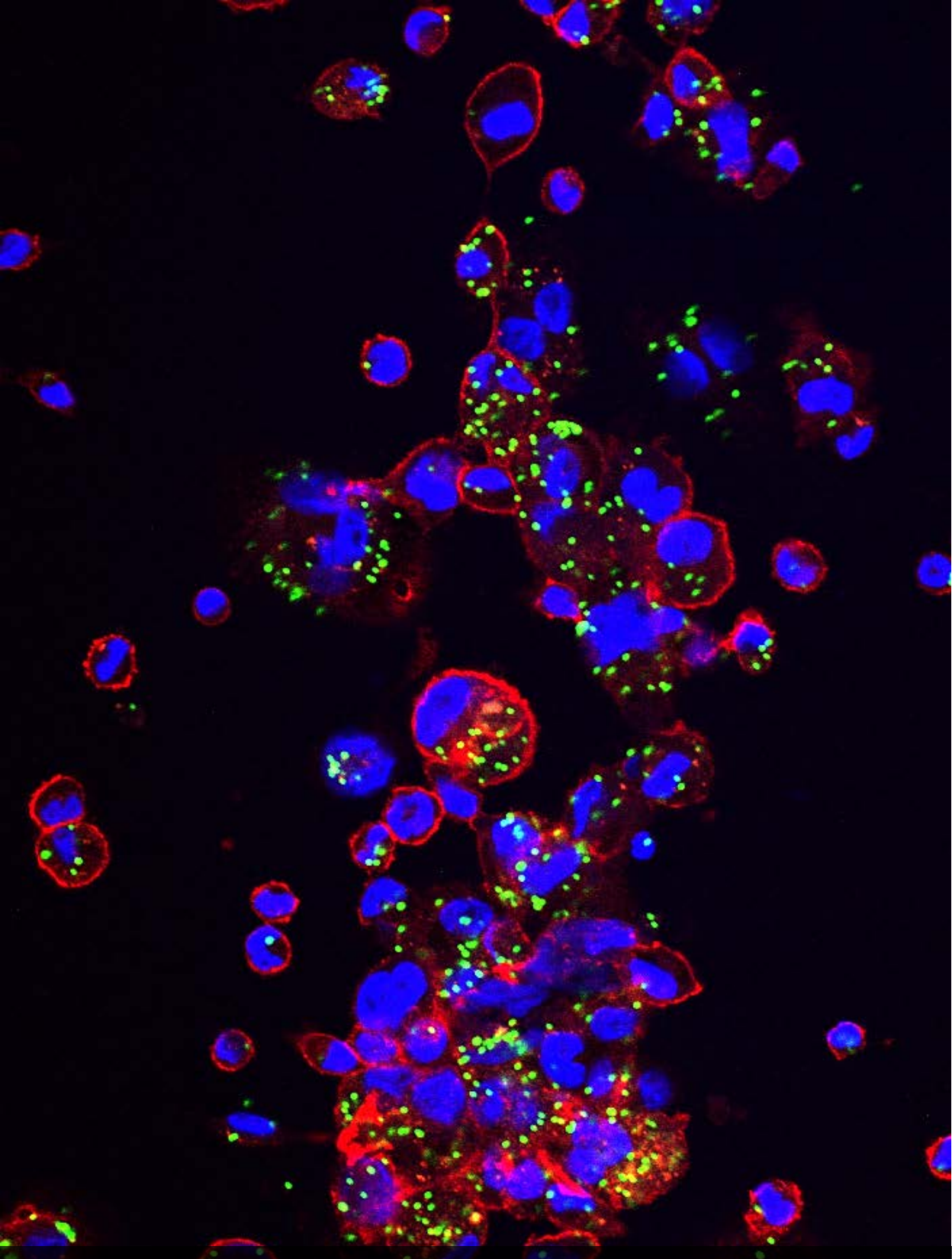
The alarming increase in multi- and extensively drug-resistant TB in combination with the scarce drugs available for TB treatment make necessary to focus on new strategies. Targeted delivery of encapsulated drugs could help to reduce systemic side effects, decrease the dosage needed and improve the compliance of the patients. Future research will probably be focused on host-directed therapies, boosting the host's immune response against the pathogens. As they are not directed towards the bacteria, they will not spread resistance. Publications on HDT have dramatically increased in the last years. Some of these compounds could be encapsulated and delivered with a specific target. Exploring new ways of administration, like aerosolized drugs or microneedles, seems a hot topic for the next years.

As the final outcome of a disease depends not only on the host and the pathogen, but external factors play important roles, there is a need to integrate in the laboratory models all three variables. External factors can modify the activity of drugs, the immunity of the host or pathogenicity of the bacteria, and, thus, the outcome of the disease.

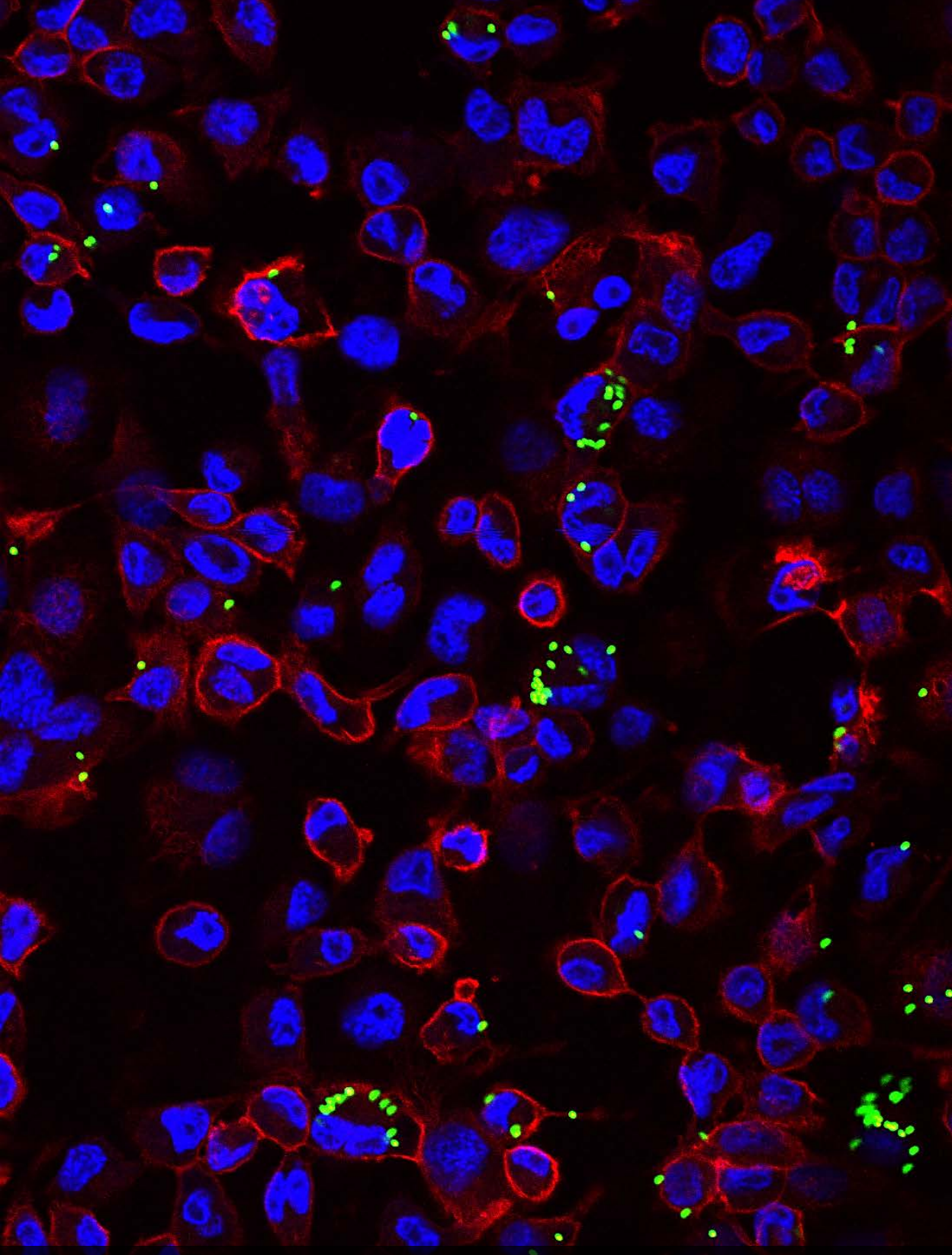
Moreover, although the *in vitro* models used in this thesis give some useful information, the reality is much more complex. Animal models are a more realistic

approach, but not without drawbacks. Mice are highly resistant to developing classic TB disease and their lungs are too small to form granulomas; in guinea pigs, it is difficult to evaluate the immune responses; rabbits are resistant to TB and there is a lack of immunological reagents; monkeys have a high cost and their handling is difficult. Even if the research continues in the *in vitro* model, some changes should be done in order to improve them. Three-dimensional cell culture is a proposal to give some 3D space to the cells. 3D printing of structures simulating lungs or granulomas could be a more realistic model. Air – liquid interface model seems a more physiologic approach than condensates of smoke. Probably *in silico* and mathematical modelling, in which computer programmes model a physiologic process, would be increased in the future, since they can combine some advantages of *in vivo* and *in vitro* experimentation.

In conclusion, it is mandatory to integrate political and clinical actions as well as basic and applied research to reach the stop TB aims.



THP-1 cells exposed to e-vapor infected with latex beads.
Three hours after infection



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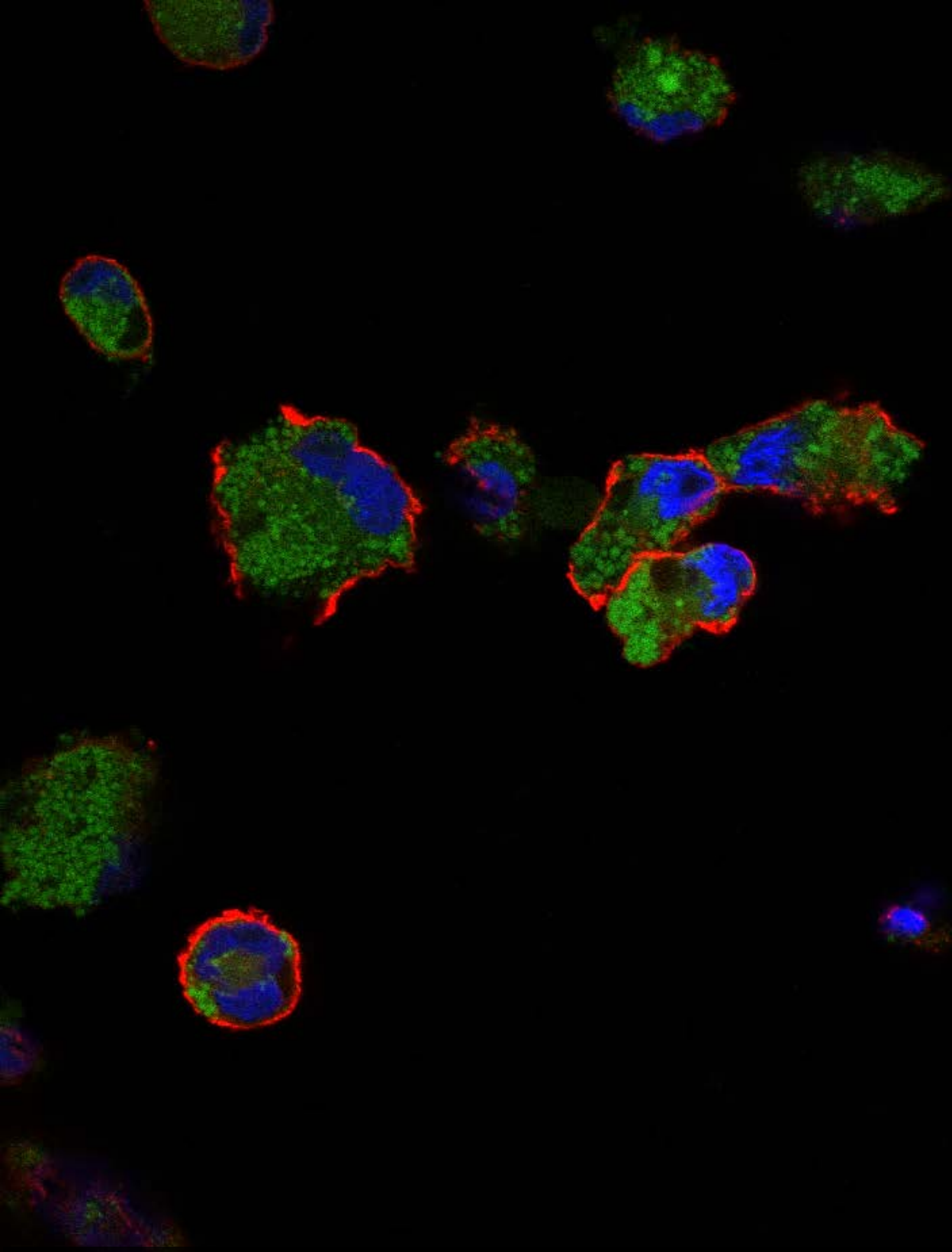
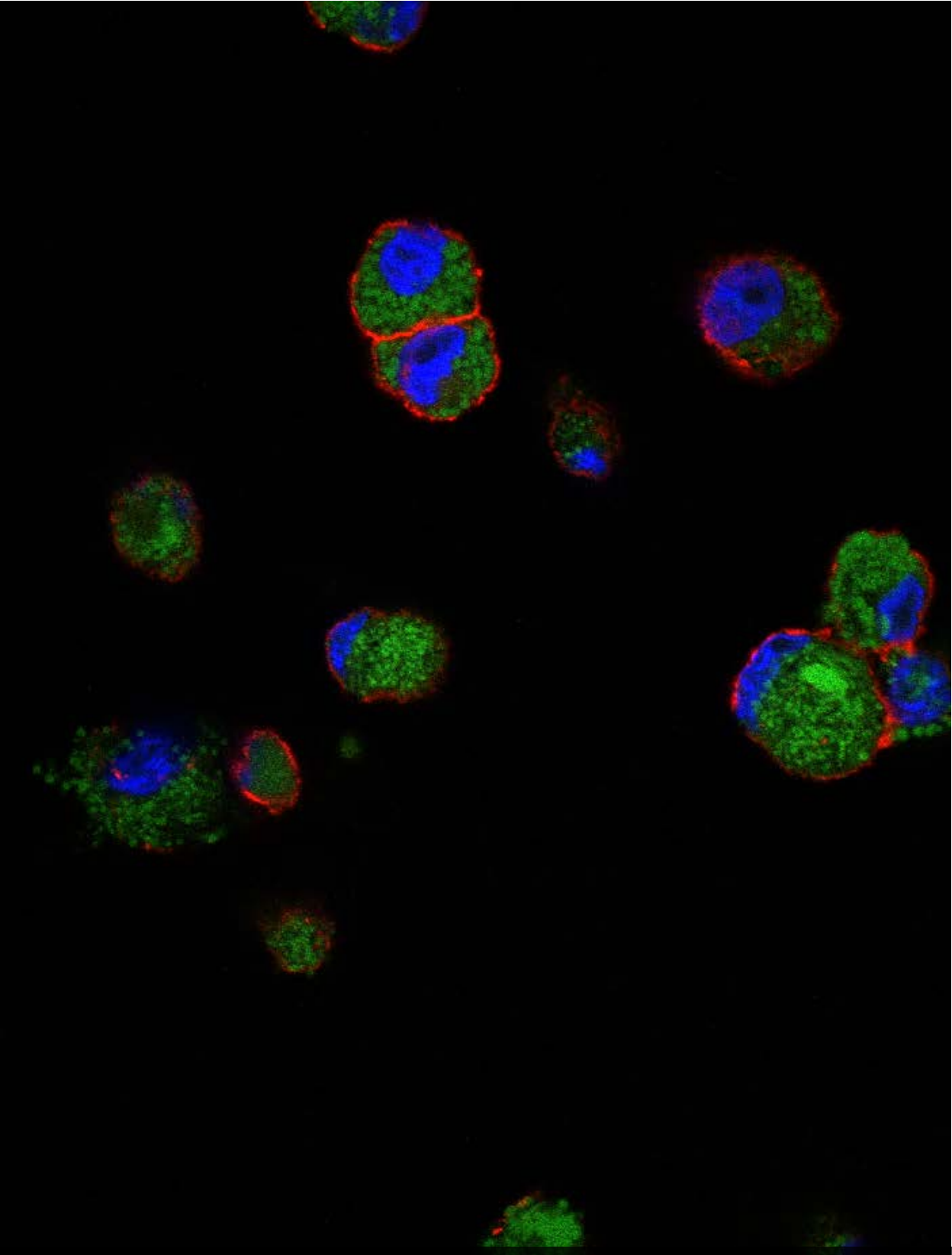


THP-1 cells exposed to cigarette smoke infected with BCG-GFP.
Day 6 after infection



9. APPENDIX

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Editorial

Interaction Between Environmental Pollution and Respiratory Infections☆

Interacción entre contaminación ambiental e infecciones respiratorias

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The exposome is defined as the measure of all exposures of an individual during his or her lifetime and how these exposures affect health.[1] Humans are exposed to large quantities of compounds by inhalation, and the change from rural lifestyles to living in overcrowded, industrialized cities, together with the mass use of motor vehicles means that we are exposed to large amounts of contaminants via the respiratory tract. It is estimated that only 1 cubic centimeter of city air contains approximately 100 bacteria and around 10^7 small-diameter particles (less than 300 nm).[2] In fact, we now know that the outcome of an infection depends not only on host- and pathogen-associated factors, but also on key external factors. For example, environmental changes influence the flyways of migratory birds, which are vectors of the virus influenza A, modifying the spread of new variants of the influenza virus.[3]

The major environmental pollutants are basically particles in suspension, known as particulate matter (PM), and include metals or silica, volatile organic compounds, and gaseous pollutants, such as ozone, sulfur dioxide, nitrogen monoxide and dioxide, and carbon monoxide. They are generally produced naturally by sand storms or volcanic eruptions, or by humans, in the form of biomass burning, traffic emissions, mining, and farming. In large cities, however, most pollution is caused by the combustion of diesel engines.[4]

It is estimated that 7 million people die every year as a result of environmental pollution.[5] Poor air quality is a risk factor for the development of numerous respiratory diseases, such as asthma, lung cancer, and respiratory infections, especially in children.[6] Respiratory infections cause the death of more than 4 million people annually. In Europe, almost half a million people die every year due to high concentrations of PM, 78,000 die from exposure to nitrogen dioxide, and more than 14,000 die from exposure to ozone.[7] Exposure to high levels of sulfur dioxide has been associated with an increase in sputum production, chronic cough, and bronchoconstriction, and more frequent bronchiectasis exacerbations.[8]

Environmental pollution particles cause inflammation, airflow changes, and altered defense mechanisms, both in the upper and lower respiratory tract.[9] Larger PMs can be eliminated by mucociliary transport, but the smaller ones can reach the bronchioles. In addition, $PM_{2.5}$ (with a diameter of less than 2.5 μm) that are not phagocytized by macrophages can spread systemically.

Exposure to PM increases susceptibility to bacterial pneumonias and viral respiratory infections.[10] Exposure to environmental pollution in children affects the proper functioning of the respiratory system, especially in the early years of life, when the respiratory and immune systems are not yet fully developed. There is an association between bronchitis and environmental pollution in children under five years of age.[11] In fact, prenatal exposure to $PM_{2.5}$ increases susceptibility to respiratory infections (bronchitis and pneumonia), as many environmental toxins can easily cross the placenta (especially $PM_{0.25}$ and smaller) and accumulate in the fetus in higher concentrations than in the mother.[12] In the elderly, immunosenescence might contribute to an increased propensity to respiratory infections, due, among other factors, to cytokine deregulation.[12]

PM can cause oxidative stress by eliminating antioxidants and producing reactive oxygen species (ROS), which leads to an inflammatory response by producing cytokines IL-6, IL-8 and TNF-α[2]. Moreover, exposure to PM can alter the ability of macrophages to inactivate viruses, lyse bacteria, or inhibit the presentation of antigens.[10]

Exposures to different nitrogen oxides can increase the risk of respiratory infections, affecting T cells and NK cells, which play important roles in the defense against viruses.[4] An accumulation of metals may also be a risk factor for infections, since metal availability is generally a factor promoting the growth of microorganisms. In *Mycobacterium tuberculosis* or *Neisseria meningitidis*, an increase in the availability of iron in the host is related to more serious infections.[10]

Tobacco smoke also causes exposure to high concentrations of PM, 15,000–40,000 μg PM per cigarette. In general, tobacco smoke exposure increases the risk of prolonging viral respiratory infections or developing bacterial infection by *Streptococcus*, *Legionella*,

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Mycoplasma, or *Haemophilus*.[10] It is also related to an increased susceptibility to infection by *M. tuberculosis*, and progression to active tuberculosis.[10] In cell cultures, we have observed that smoke negatively affects the phagocytic capacity of macrophages.[13] Exposure to tobacco smoke has also been linked with a delay in the negativization of mycobacterial cultures in treated patients.[14] In fact, exposure to smoke not only alters the immune response of the host, but also has an impact on the microorganism, modifying its phenotype toward other more virulent variants.[15]

Environmental pollution increases the rate of respiratory infections, and is associated with increased costs and premature deaths in chronic patients and children. Some of the causes can be acted upon, such as tobacco exposure and some aspects of environmental pollution. An understanding of how pollution affects the immune response to respiratory infections would help us establish effective epidemiological strategies, and improve clinical treatment and patients' quality of life.

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Research Article

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Nanomedicine

Matryoshka-type gastro-resistant microparticles for the oral treatment of *Mycobacterium tuberculosis*

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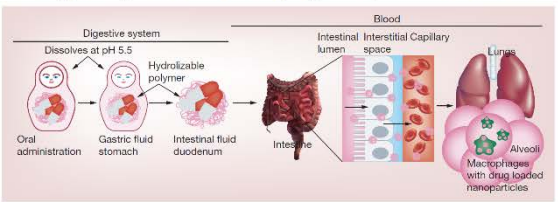
Aim: Production of Matryoshka-type gastroresistant microparticles containing antibiotic-loaded poly lactic-*co*-glycolic acid (PLGA) nanoparticles (NP) against *Mycobacterium tuberculosis*. **Materials & methods:** The emulsification and evaporation methods were followed for the synthesis of PLGA–NPs and methacrylic acid-ethyl acrylate-based coatings to protect rifampicin from degradation under simulated gastric conditions. **Results & conclusion:** The inner antibiotic-loaded NPs here reported can be released under simulated intestinal conditions whereas their coating protects them from degradation under simulated gastric conditions. The encapsulation does not hinder the antituberculosis action of the encapsulated antibiotic rifampicin. A sustained antibiotic release could be obtained when using the drug-loaded encapsulated NPs. Compared with the administration of the free drug, a more effective elimination of M. tuberculosis was observed when applying the NPs against infected macrophages. The antibiotic-loaded PLGA–NPs were also able to cross an *in vitro* model of intestinal barrier.

Matryoshka-type gastroresistant microparticles containing antibiotic-loaded poly lactic-*co*-glycolic acid nanoparticles against *M. tuberculosis* were produced to protect the antibiotic from degradation under simulated gastric conditions. The antibiotic-loaded poly lactic-*co*-glycolic acid nanoparticles were able to cross an *in vitro* model of intestinal barrier, being more effective in the elimination of *M. tuberculosis* when applied against infected macrophages compared with the use of the free drug.



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Future Medicine

Keywords: enteric coating • eudragit • gastroresistant • microparticles • *Mycobacterium tuberculosis* • oral delivery • PLGA • rifampicin • TEER • tuberculosis

Mycobacterium tuberculosis is a well-known example of a highly spread pathogen causing tuberculosis (TB), a disease that greatly affects public health in most developing countries. *M. tuberculosis* is localized in the early endosomes of infected macrophages [1]. According to the last WHO report [2], despite the moderate decline (1.5%) in the global incidence of TB observed during the last 15 years, being in 2015 a 18% lower than in 2000, TB still constitutes a major cause of morbidity and mortality worldwide. In 2016, there were 10.4 million new cases and 1.7 million people died from the disease worldwide ranking alongside HIV as a leading cause of death. In the treatment of drug susceptible TB, in order to act on different bacterial subpopulations (extracellular and intracellular), current treatment is extended over long periods of time (6–9 months) with a combination of at least four of the five first-line drugs against the pathogen in order to prevent both relapse and development of antibiotic resistance. Rifampicin, isoniazid, pyrazinamide and ethambutol are given daily usually during the first 2 months of treatment, leaving just rifampicin and isoniazid for the remaining period. Except for pyrazinamide, the other commonly used antibiotics require bacterial replication for their action [3].

Being at least partially an intracellular pathogen, any treatment against TB should be selectively directed towards the intracellular reservoir of the bacterial load. The introduction of targeted drug-delivery systems into the clinical practice for treating intracellular pathogens would allow applying drugs effectively, increasing patient compliance, extending the product life cycle, providing product differentiation and reducing healthcare costs. Over the last decades, significant progress has been made in the development of nanotechnological approaches to diagnose, screen, treat and prevent disease, hence improving human health. As a consequence of this extensive research 43 nanomedicines have been approved for the clinical practice by the European Medicines Agency (EMA) and 71 by the US Food and Drug Administration (FDA) mainly as drugs and medical devices and other 128 are currently undergoing clinical trials [4]. Their application is principally directed towards cancer treatment and antimicrobial therapy using drug-delivery systems. In those systems, the nanoparticle (NP) acts as a drug depot either as suspensions or as coatings on medical devices. General applications of nanomaterials in the field of infectious diseases include their use as adjuvants or as antimicrobial and vaccine carriers, to achieve a sustained or controlled delivery depending on the need. In addition, several metallic nanomaterials (i.e., silver, copper, etc.) are antimicrobial by themselves releasing, after oxidation, metallic ions that show multiple mechanisms of biocidal action and consequently avoiding antimicrobial resistance. When multiple mechanisms of action take place against a pathogenic microorganism, simultaneous gene mutations are difficult to occur and consequently the development of resistance is reduced. In addition, NPs can be used to achieve a targeted and selective delivery of the corresponding antimicrobial drug. Targeted strategies can be used by coupling the antimicrobial NP to a specific recognition moiety (i.e., antibody, peptide, oligonucleotides, etc.), or by electrostatic binding to the bacterial wall [5] or even the antimicrobial can be released just in response to a biomolecule secreted under the presence of bacteria [6,7]. Passive strategies take advantage of the NP-immune system recognition. Thus, macrophages detect, internalize and destroy not only pathogens and apoptotic cells, but also NPs due to the large number of nonspecific surface receptors that those cells have [3]. NPs are internalized within those cells using the phagocytic route when proteins of the blood plasma (i.e., opsonins) are adsorbed on the NP surfaces. Also, other internalization nonphagocytic routes (clathrin or caveolin-mediated endocytosis, macropinocytosis, etc.) can internalize different NPs within intracellular vesicles. Therefore, NPs can accumulate their antimicrobial cargos into intracellular compartments of infected cells. *In vitro* and *in vivo* experiments showed enhanced antimicrobial activity of antibiotic containing NPs compared with the administration of the free drug; however, a specific colocalization of the NP and the pathogen in the same intracellular compartment is difficult to achieve. In this regard, Kalluru *et al.* [8] demonstrated that even though rifampicin-loaded poly lactic-*co*-glycolic acid (PLGA) NPs were able clear macrophages infected with *Mycobacterium bovis* (BCG) more efficiently compared with the administration of the free drug [9], the drug-containing NPs remained inside phagolysosomes that were separated from the BCG-containing phagosomes. The authors postulated that the loaded hydrophobic drugs easily crossed by diffusion, both the phagolysosome membrane enclosing the NP (phagosome to cytoplasm) and the membrane of the phagosome enclosing the BCG. Therefore, a perfect subcellular colocalization inside the same vesicle is not necessary to show enhanced efficacy. In addition, the great benefit of using nanomaterials is that by selecting a specific size [10] or by using a simple surface modification, the same NP can be internalized following different routes [11].

Oral administration of drugs is preferable over any other route of administration because it is not invasive and shows long-term compliance and increased patient acceptance, allowing easy home therapy [12]. The main drawback of this route is the potential degradation of the active principle in contact with the acidic and enzymatic environment of the gastrointestinal tract and during the presystemic metabolism. Capsules have been designed to protect the drug from the degradation in the gastric fluid releasing their cargo in the intestine in a pH-responsive manner. For instance, Patel *et al.* [13] described the synthesis and sustained rifampicin release from pH-dependent chitosan-based NPs intended for oral delivery. Also, pH-independent sustained release formulations have been designed to increase the intestinal adsorption using mucoadhesive formulations and absorption enhancers.

The activity of rifampicin is based on the inhibition of the RNA synthesis, by means of van der Waals forces, of the hydrophobic side chains near the naftol ring of the rifampicin with the bacterial DNA-dependent RNA polymerase [14]. Rifampicin is degraded (~26%) under gastric conditions, which can be responsible of its reduced bioavailability *in vivo*, but its nanoparticulated formulation can reduce the degradation level to a 9% [15]. NPs have also been used to protect and transport active principles across the intestinal epithelium to the bloodstream by targeting them with specific proteins able to cross-polarized epithelial barriers [16] or by using appropriated sizes, surface functionalizations or charges opening the tight junctions between the epithelial cells [17].

In this work, we describe a drug-delivery system potentially able to reach infected alveolar macrophages using biodegradable and approved antibiotic-loaded carriers. The system is based on nanoparticulated formulations of PLGA (Resomer® RG504) loaded with rifampicin targeting *M. tuberculosis*. Those drug-loaded NPs are, in a second step, encapsulated inside (as Matryoshka dolls) microparticles (MPs) based on methacrylic acid-ethyl acrylate copolymer (1:1, Eudragit® L 100-55) to cause the drug-loaded NPs to first pass through the stomach without being dissolved (thanks to the shell made of Eudragit® L 100-55) to then dissolve in the intestinal fluid releasing the inner NPs made of PLGA. PLGA was selected as a polymer due to its well-known properties: predictable biodegradability and biocompatibility; FDA and EMA approvals for its application as building material in drug-delivery systems; well-described formulations and methods of production adapted to the encapsulation of various types of hydrophilic or hydrophobic active pharmaceutical ingredients; drug protection from biochemical degradation; possibility to target its based NPs to specific tissues or cells and the possibility of achieving a sustained release. Nanoparticulated formulations of PLGA can be produced by nanoprecipitation and emulsification with solvent evaporation, being the procedures that have been actively used during the last three decades to produce drug-loaded PLGA–NPs. Emulsification with solvent evaporation was selected against nanoprecipitation because by nanoprecipitation it was difficult to couple the rifampicin and the PLGA precipitation. On the contrary, by emulsification, rifampicin was selectively loaded inside the PLGA emulsion and afterwards it was entrapped during the precipitation of the polymer once the solvent was evaporated. Some other production techniques of drug-loaded nanoparticulated formulations, such as emulsion crosslinking or postdrug loading, were not considered due to the following concerns:

- PLGA is a polymer with rare possibilities to be crosslinked, unless it is functionalized. In this work, Resomer® RG504 was used and no further functionalization was feasible because it is an ester terminated polymer.
- Cross-linking of polymers is conducted via a controlled chemical reaction. It implies that not only the polymer but also the drug could react with the cross linker agent. If this would be the case, the drug could not have any therapeutic effect and even could be more cytotoxic that the unmodified drug.
- Post-drug loading implies that the drug molecules should permeate through the polymeric shell of the NP. Then, it would require a large drug concentration gradient to enable a sufficient drug loading. Considering that the drug release in PLGA is powered by the PLGA hydrolysis, the PLGA postdrug loading should require a larger processing time than the one selected by solvent evaporation, as well as a more expensive sorting procedure to separate the unloaded drug from the polymer and the surfactant molecules used.

Materials & methods

Nano & MP synthesis

The polymer, Eudragit® L 100-55 is an anionic methacrylic acid–ethyl acrylate copolymer (1:1) containing free carboxylic groups which are ionized in an aqueous media at pH $\geq$ 5.5 making it resistant to the acid environment of the gastric fluid, but slightly soluble at intestinal (duodenum) pH. Resomer® RG504 is an ester terminated poly(D,L-lactide-*co*-glycolide) 50:50 (Mw 38000–54000Da) widely used in controlled release systems. Both polymers were gifted by Evonik Industries AG (Darmstadt, Germany) and used as received. Drugs including

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rifampicin and dexamethasone (as internal standard); surfactants including sodium cholate hydrate and Pluronic® F-68 and solvents including ethyl acetate, ethanol absolute, acetonitrile and methanol (HPLC grade) were supplied by Sigma–Aldrich (MO, USA).

PLGA-rifampicin NPs were prepared by the oil-in-water (O/W) emulsion solvent evaporation method. In this method, 1% (w/v) of PLGA polymer (50:50), 0.2% (w/v) of rifampicin and 3% (w/v) of pluronic F68, used as surfactant, were dissolved in 5 ml of ethyl acetate. The organic phase was then emulsified with 10 ml of Milli Q water used as aqueous phase. This mixture was sonicated in an ice bath for 25 s with a sonicator (Digital Sonifier 450, MO, USA) using a probe of 0.13 inches in diameter and 40% of amplitude. After the formation of a stable emulsion, the organic solvent was evaporated under continuous stirring (600 rpm) for 3 h to allow polymer precipitation.

Microcapsules based on Eudragit were prepared by following the double emulsion solvent evaporation method (w/o/w) using antibiotic-loaded PLGA–NPs in the inner water phase. Briefly, 2% (w/v) of Eudragit L100-55 was dissolved in 2.5 ml of ethanol:ethyl acetate (1:4; organic phase) and emulsified with 1 ml of the antibiotic-loaded PLGA suspension (25 mg/ml; inner aqueous phase) by ultrasonication (Digital Sonifier 450) at a 40% of amplitude for 30 s in an ice bath. The formed w/o emulsion was then emulsified with 2 ml of a 1% (w/v) sodium cholate solution at 40% amplitude for 35 s to obtain the w/o/w emulsion. 10 ml of 0.3% (w/v) sodium cholate solution were also added before stirring to promote the stability of the final emulsion.

Antimicrobial evaluation

For determining the antituberculosis activity of the samples, mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% ADC (i.e., bovine albumin fraction V, glucose and catalase) and 0.05% Tween 80 until reaching an optical density measured at 600nm (OD_{600}) of 0.8. Then, bacterial cultures were diluted in Middlebrook 7H9 broth supplemented with ADC and 0.5% glycerol in order to obtain a bacterial suspension of 10^5 colony-forming units (CFU)/ml. Aliquots of 100 µl were transferred to each well of a 96-wells plate that contained 100 µl of samples prepared from twofold serial dilutions in the same culture medium. The inoculated plates were incubated for 6 days at 37°C and for an additional 2 days after the addition of the redox indicator (30 µl of a resazurin solution at 0.1 mg/ml). A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. Thus, the minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound that prevented this color change.

For estimating the release of rifampicin from NPs, 0.806 mg/ml of rifampicin-loaded PLGA–NPs were incubated in 400 µl of culture media (Middlebrook 7H9 broth supplemented with ADC and 0.5% glycerol) adjusted at pH = 7.0 or pH = 4.5, at 37°C. Samples were taken after 3, 7, 14 and 25 days, centrifuged for pelleting (10 min at 4500 rpm) and the NPs and the supernatants transferred to a new tube and kept frozen until use. Once all samples were ready, the supernatants were evaluated for the presence of rifampicin by using the protocol described above. As a negative and sterility control, 0.554 mg/ml of empty PLGA–NPs were processed in the same way as the rifampicin-loaded NPs were. In order to consider spontaneous degradation of rifampicin after being released from the NPs, tubes containing an amount of free rifampicin equal to that encapsulated in the PLGA–NPs were incubated and processed in the same way. The experiments were performed in triplicate.

Bacterial growth & time-kill kinetics

The time-kill kinetics of *M. tuberculosis* H37Rv were determined by incubating cultures with NPs and free rifampicin and counting the number of viable bacteria at several time points [18]. Mycobacterial cells were cultured till exponential phase and then diluted to reach 10^7 CFU/ml in Middlebrook 7H9 broth. Four samples of 10 ml of this bacterial suspension were taken separately and added with: rifampicin, to reach an inhibitory concentration of 0.5 µg/ml; rifampicin-loaded NPs, which contained rifampicin for final inhibitory concentrations of 0.06–0.07 and 4.9–6.0 µg/ml of PLGA (which are below inhibitory concentrations of PLGA); empty PLGA–NPs to reach the same final concentration of 4.9–6.0 µg/ml; the fourth sample did not receive any compound and was taken as control. All four samples were incubated at 37°C. On days 3, 7, 14 and 21, from each sample, an aliquot of 100 µl was taken for diluting and plating on Middlebrook 7H10 agar plates which were incubated further at 37°C until growth was observed in order to count the number of CFUs. The assay was performed in duplicate.

Loaded or empty NPs at subinhibitory concentrations of PLGA and rifampicin (in the case of the loaded NPs) were added to cultures of *M. tuberculosis* in order to estimate their impact on bacterial growth. Bacterial cells were cultured till exponential phase; they were diluted to reach 10^5–10^6 CFU/ml in fresh Middlebrook 7H9

broth, and separated into three samples of 10 ml. PLGA–NPs containing rifampicin equalling a fourth of its MIC (i.e., 0.004 μg/ml; MIC = 0.015 μg/ml) was added to the first culture; the second was added with the same amount of empty PLGA–NPs (to reach 0.32 μg/ml of PLGA, well below its MIC, which is 7.8 μg/ml); the last sample was incubated without further additions, as control. Immediately, an aliquot was removed for determining initial values of OD_{600} and CFU/ml. Cultures were incubated at 37°C and new aliquots were taken and analyzed for OD_{600} and CFU/ml after 3, 5, 7, 10, 12, 14 and 21 days.

Cell culture

TC7 clone human epithelial colorectal adenocarcinoma (Caco2) cells, kindly donated by MJ Rodriguez-Yoldi, were grown using Dulbecco's modified Eagle's medium (DMEM w/stable Glutamine; Biowest, Nuaille, France) in a humidified atmosphere containing 5% CO_2 at 37°C. The growth medium was supplemented with fetal bovine serum (FBS, 10% (v/v); Thermo Fisher Scientific, MA, USA) and antibiotic–antimycotic (60 μg/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, Biowest, Nuaille, France).

MH-S murine alveolar macrophages were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with fetal bovine serum 10% at 37°C in a 5% CO_2 humidified incubator.

Cell viability assay

The *in vitro* cytotoxicity of rifampicin, unloaded-PLGA–NPs and rifampicin-loaded PLGA–NPs was evaluated using the resazurin based Alamar Blue™ assay (Invitrogen, MA, USA) according to the manufacturer's instructions. TC7 Caco2 (density of 8000 cells/well) cells were seeded in a 96-well plate 48 h prior to incubation with the different formulations and allowed to adhere. Nonloaded-PLGA and rifampicin-loaded PLGA–NPs were added to the cells in complete growth medium at a concentration range of 0.0625–1 mg/ml, and then, cells were maintained in the standard conditions mentioned above for 24 h. Rifampicin was added to the cells at a concentration range of 0.5–8 μg/ml, which are equivalent drug concentrations to the ones contained in the rifampicin-loaded PLGA–NPs used. After incubation, cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS, Biowest) and incubated with complete growth medium containing 10% (v/v) Alamar Blue dye for 4 h. Fluorescence of the medium was measured at 37°C in a multimode Synergy HT Microplate Reader (excitation wavelength 530 nm, emission wavelength 590 nm; Biotek, VT, USA). Cell viability was expressed as a relative percentage to the untreated control cells value. The percentages obtained depict the average of four values.

To evaluate the cytotoxic effect of the PLGA–NPs on the MH-S cell line, the EZ4U cell proliferation assay (Biomedica, Vienna, Austria) was used following the manufacturer's instructions. Briefly, MH-S were seeded at a concentration of 1×10^5 cells per well in 96-well tissue culture plates with clear bottoms (Falcon®) using RPMI without phenol red. The plates were incubated overnight (37°C in 5% CO_2 atmosphere). Nonloaded-PLGA–NPs, rifampicin-loaded PLGA–NPs or free rifampicin at minimum [19,20] and maximum [21–23] serum concentrations (4 and 24 μg/ml, respectively), were diluted in 200 μl of RPMI without phenol red per well and incubated for 24 h. The monolayers were washed once with DPBS and then 200 μl of fresh medium without phenol red and 20 μl of EZ4U were added. The absorbance was read after 4 h of incubation and also at 48 and 72 h. A microplate reader (Victor 3, Wallac, Perkin Elmer, MA, USA) was used with a wavelength of 450 nm with 620 nm as reference. The results were expressed as a viability percentage using untreated cells as control.

Permeability experiments

For the permeability experiments, TC7 Caco2 cells were seeded on polycarbonate membrane inserts (0.47 cm^2; 0.4 μm pore size) into a 24-well plate at a density of 5×10^4 cells/cm^2. Cells were maintained in complete medium for 28 days, replacing it every other day, to obtain well-differentiated monolayers.

The integrity of the epithelial monolayers was evaluated by measuring the Transepithelial electrical resistance (TEER). TEER values were measured every 3–4 days using a Millicell ERS2 Voltohmmeter (Millipore, MA, USA) according to manufacturer's instructions throughout the experiment course. The final TEER values were obtained by multiplying the meter readings by the effective surface area of the filter membrane and were expressed as Ωcm^2. TEER values $\geq$400 Ωcm^2 over background were used as a reference point to indicate adequate monolayer integrity in order to carry out permeability assays with the TC7 Caco2 monolayers.

After verification according to TEER values, the integrity of the monolayer was measured by monitoring the Lucifer yellow (LY) rejection, a paracellular marker, across the cell monolayer. Prior to the experiment, the inserts were washed twice with calcium- and magnesium-free Hank's-balanced salt solution (HBSS; Biowest, Nuaille,

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France) and equilibrated for 60 min with prewarmed HBSS containing 10 mM HEPES. Immediately, 300 µl of 400 µM LY and 600 µl HBSS containing 10 mM HEPES were added into the apical and basolateral chambers, respectively. The plate was placed at 37°C in a 5% CO_2 humidified incubator for 1 h. After the finalization of permeability experiment, LY fluorescence in apical and basal compartments was measured at 485/535 nm excitation/emission wavelengths using a multimode Synergy HT microplate reader (Biotek) and the percentage rejection of LY was calculated.

Previous to the beginning of the transport experiments, the culture media in the donor and acceptor compartments were aspirated, cells were rinsed twice with prewarmed HBSS and, immediately, were incubated with HBSS supplemented with 10 mM HEPES and allowed to equilibrate in the transport medium at 37°C for 30 min. To evaluate transepithelial transport of rifampicin-loaded PLGA–NPs, 300 µl of NPs suspensions of different concentrations (2.5, 5 and 10 mg/ml) in transport buffer were loaded into the apical chamber and 600 µl HBSS containing 10 mM HEPES were added into the basolateral chamber. Then, cells were incubated at 37°C in a 5% CO_2 humidified incubator for 4 h. Then, 300 µl of the basolateral solution were collected and the rifampicin concentration was determined by HPLC following the analytical method described in the previous section. Briefly, samples containing rifampicin-loaded PLGA–NPs were mixed with 1 ml of acetonitrile and sonified in an ultrasonic bath for 30 min. Thereafter, 100 µl of an internal standard (dexamethasone, 300 ppm) was added and the mixture was shaken in a rotator mixer for 15 min at 4°C. After the addition of 100 µl of methanol, the solution was mixed in an ultrasonic bath during 15 min. Finally, the mixture was filtered using a 0.22 µm PTFE syringe filter and placed in a vial for UV–HPLC analysis.

In vitro infection of MH-S macrophages

MH-S murine alveolar macrophages were plated at a concentration of 3×10^5 cells per well in 24-well tissue culture plates with clear bottoms (TTP®) and allowed to adhere overnight. For the infection, midlog phase *M. tuberculosis* was washed twice with PBS with a 0.05% Tween, once with Dulbecco's PBS and then allowed to stand for 5 min, before the supernatant was collected. The bacteria were then diluted in RPMI and added to the MH-S cells at a concentration of approximately 3×10^4 CFU/well. After 3 h of infection at 37°C in 5% CO_2, macrophages were treated with 200 µg/ml amikacin for 1 h and washed three times with DPBS to eliminate any extracellular bacteria. Last, 1 ml of complete RPMI-1640 was added to each well and incubated for 72 h at 37°C in a 5% CO_2 humidified incubator, in order to allow bacteria to grow inside the macrophages. After such incubation (day 0), fresh medium containing unloaded-PLGA–NPs, rifampicin-loaded PLGA–NPs or free rifampicin was added at 4 and 24 µg/ml (minimum [19,20] and maximum [21–23] serum concentrations). The quantity of rifampicin-loaded PLGA–NPs added was calculated according to the rifampicin concentration inside the NPs. The quantity of empty PLGA–NPs added was proportional to the quantity of loaded PLGA–NPs. Intracellular survival and growth were assessed by lysing the monolayers (day 0, 2 and 4) by the addition of water followed by a 30 min incubation at 37°C and enumeration of bacteria by serial dilutions in PBS-Tween plating onto Middlebrook 7H10 solid medium. Colonies were counted after 3–4 weeks incubation at 37°C and the average CFU/ml determined.

Results

Physicochemical characterization of the MPs & NPs produced

Figure 1A shows schematically the experimental protocol used in the synthesis of the antibiotic-loaded PLGA–NPs here reported. Single emulsion was used to entrap and preserve the hydrophobic structure and activity of rifampicin. The colloidal hydrodynamic size measured in water was 145.2 ± 31.2 nm. The measured scanning electron microscopy (SEM) histograms revealed a particle size distribution centred at 149.1 ± 35.6 nm (Figures 1B–C) for the rifampicin-loaded PLGA–NPs. As can be seen in Supplementary Figure 1, the NP monodispersity was maintained in different independent batches, indicative of the robustness of the synthesis method.

HPLC analysis indicated that the encapsulation efficiency (EE) and drug loading (DL) achieved for the rifampicin-loaded PLGA–NPs were 5.4 ± 0.55 and 1.08 ± 0.11 w/w %, respectively. This DL is similar to those previously reported in the literature using PLGA [24] although higher values have been previously reported with other carriers [13]. To check the potential interaction between the antibiotic studied and the encapsulating PLGA–NPs, fourier-transform infrared spectroscopy analysis was performed by comparing the signals of the antibiotic-encapsulated NPs with a mixture of the corresponding free antibiotic and unloaded PLGA–NPs as control under the same proportional concentrations. Supplementary Figure 2 shows that the vibrations characteristic of the chemical bonds of the encapsulated antibiotic were similar to the ones retrieved from the simple physical mixture

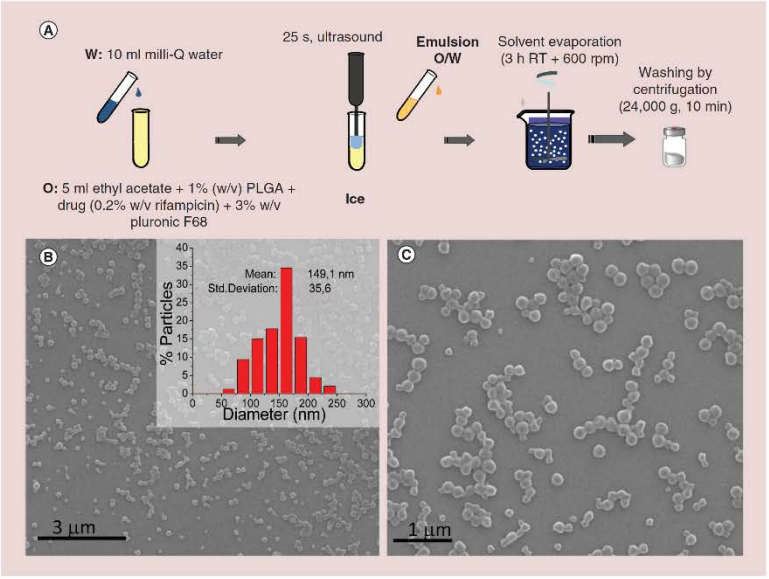


Figure 1. Synthesis and characterization of the prepared materials. (A) Synthesis procedure for the preparation of drug-loaded PLGA–NPs by single o/w emulsification and evaporation process. **(B–C)** Scanning electron microscopy images of rifampicin-loaded PLGA–NPs.
NP: Nanoparticles; o/w: Oil-in-water; PLGA: Poly lactic-*co*-glycolic acid; RT: Room temperature; SEM: Scanning electron microscopy; Std: Standard.

composed of the corresponding antibiotic and empty PLGA–NPs. This indicates that no new chemical bonds between the antibiotic and the PLGA encapsulating matrix were formed. This is a positive result as we preserved during the encapsulating process the chemical structure of the antibiotic in order to maintain its antituberculosis activity.

The schematic representation of the Matryoshka-type MPs reported is shown in Figure 2A where the potential application of the developed NPs for an efficient and controlled intracellular delivery of antibiotics against intracellular alveolar infected macrophages after oral administration is represented. In addition, the schematic synthesis based on the double w/o/w emulsification and evaporation method for the production of the gastroresistant microcapsules containing the inner drug loaded-PLGA–NPs is represented in Figure 3A. The SEM morphological analysis of the gastroresistant microcapsules made of Eudragit® L 100-55 with or without inner antibiotic-loaded PLGA–NPs (Figures 2B & C, respectively) revealed a bipyramidal structure of micrometric size (6.1 ± 1.9 µm). We observed that depending on the temperature and consequently on the viscosity of the precursor polymeric solution different morphologies were obtained; probably, thermal fluctuations on the formation of those polymeric shells might be responsible for this polyhedric morphology.

The schematic description of the evolution of the gastroresistant MPs with varied pH is represented in Figure 3B. Figures 3C and D show SEM images of the Eudragit-based microcapsules loaded with rifampicin–PLGA–NPs and Figure 3E shows an empty Eudragit-based microcapsule where no surface roughness is observed due to the lack of inner PLGA–NPs. After incubation during 2 h at 37°C in simulated gastric fluid (0.1N HCl; pH = 1.03), the morphology of the methacrylic acid–ethyl acrylate MPs remained unaltered (Figure 2C & 3H). Depending on the

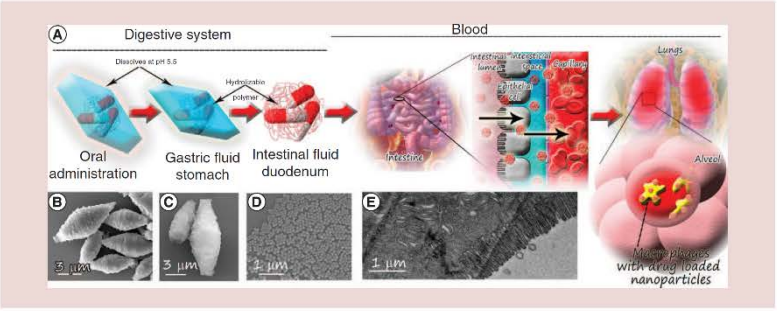


Figure 2. Schematic description of the concept here described and characterization fo the as prepared materials. (A) Schematic representation of the envisaged fate of the pH-responsive Matryoshka-like MPs and NPs for an efficient and controlled intracellular delivery of antibiotics against intracellular alveolar infected macrophages. **(B)** SEM of Eudragit microcapsules containing drug-loaded PLGA–NPs. **(C)** SEM of Eudragit microcapsules after 2 h in HCl 0.1N at 37°C (simulated gastric conditions). **(D)** SEM of the rifampicin-loaded PLGA–NPs-loaded inside the external microcapsules. **(E)** Transmission electron microscopy image of morphology of TC7 Caco2 epithelial cell barrier. MP: Microparticle; NP: Nanoparticle; PLGA: Poly lactic-*co*-glycolic acid; SEM: Scanning electron microscopy.

methacrylic copolymer formulation, the carboxyl/ester ratio can be adjusted to control the polymer dissolution pH.

The used gastroresistant polymer dissolves at pH above 5.5. To corroborate it, we encapsulated rifampicin-loaded PLGA–NPs (Figure 2D) within Eudragit L 100-55 microcapsules and then we immersed them in a simulated intestinal fluid based on PBS (pH 6.8) during 3 h at 37°C observing an immediate release of the inner PLGA–NPs. To corroborate that the NPs observed were actually the initial PLGA–NPs instead of a recrystallization or new particles composed of the polymer (Eudragit), we labeled the inner PLGA–NPs with gold–NPs following our previously published protocol [25]. As can be observed in Figure 3H, the MPs resisted the simulated gastric fluid but upon contact with the simulated intestinal fluid the inner PLGA–NPs were released almost immediately (Figures 3F & G). To observe the degradation process we immersed the MPs in the simulated intestinal fluid during a few seconds at a pH of 5.5 and we observed how partially degraded (Eudragit-based) microcapsules containing the interior PLGA–NPs started to be released (Figures 3 I & J). After 3 h the shell made of Eudragit was totally dissolved and the inner PLGA–NPs loaded with gold–NPs inside were released to the simulated intestinal fluid (PBS at pH 6.8 and at 37°C; Figure 3k). Hence, we can conclude that the inner PLGA–NPs were the ones we previously preloaded inside the MPs.

Antimicrobial activity evaluation

The resazurin assay was used to evaluate the MIC of NPs and drugs against actively growing cultures of *M. tuberculosis*. Unloaded PLGA–NPs, rifampicin-loaded PLGA–NPs and free rifampicin were evaluated. The evaluated MIC of rifampicin was 0.015 µg/ml, value in agreement with those described for strains susceptible to rifampicin [26]. The MIC of the empty PLGA–NPs was 7.8 µg/ml, hence indicating a moderate antituberculosis effect for the polymer PLGA. This intrinsic antituberculosis activity was attributed to the potential accelerated hydrolysis of the ester bonds of the PLGA, hence producing lactic and glycolic acids that would further acidify the media being this process autocatalytic [27]. As a consequence, increases in media acidification would in turn result in an enhanced bactericidal action for *M. tuberculosis*.

Interestingly, the MIC of the rifampicin-loaded PLGA–NPs was 1.95 µg/ml, which corresponded to 0.02 µg/ml of rifampicin (considering the DL), which is a value very close to the one obtained for the free rifampicin (0.015 µg/ml). These experiments suggested that rifampicin was being released from the NPs, so we designed an experiment to monitor kinetics of drug release. NPs were incubated in culture media at pH 7.0 and the antituberculosis activity of NP-free supernatants was assayed against *M. tuberculosis*. We observed that in these conditions, free rifampicin was degraded upon time, rapidly after the first week, so we took this into consideration

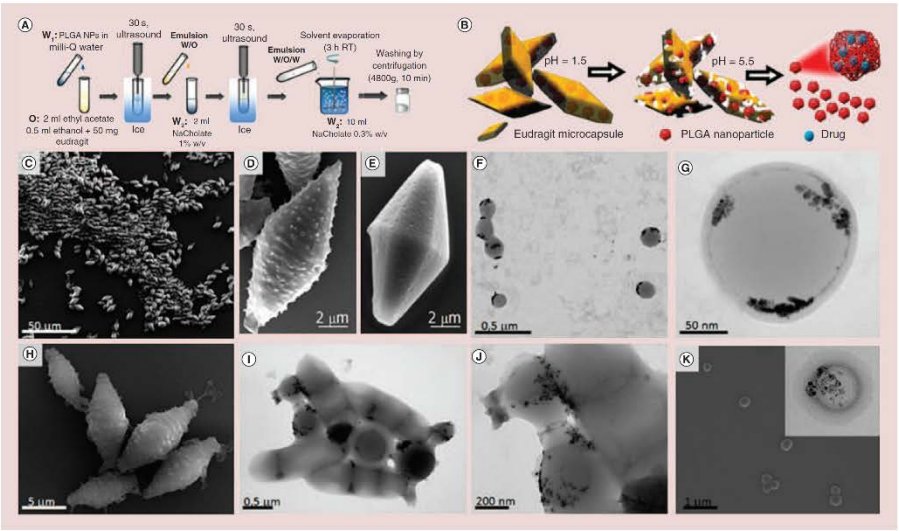


Figure 3. Characterization of the different materials under simulated conditions. (A) Schematic synthesis procedure for the synthesis of microparticles containing drug loaded-PLGA–NPs by a double w/o/w emulsification and evaporation method. **(B)** Schematic description of the evolution of the (Eudragit based) MPs with pH. **(C)** SEM image of Eudragit microcapsules loaded with rifampicin–PLGA–NPs, **(D)** SEM image, at a higher magnification, of a Eudragit microcapsule loaded with rifampicin–PLGA–NPs, **(E)** SEM image of a nonloaded Eudragit MP, **(F)** TEM image of Au-loaded PLGA–NPs used as beacons to demonstrate the successful PLGA–NPs encapsulation (Au–NPs appear as dark spots due to their high electronic density). **(G)** TEM image of a zoomed Au-loaded PLGA–NP; Au–NPs are trapped in the PLGA matrix. **(H)** SEM image of Eudragit microcapsules loaded with Au–PLGA–NPs after 2 h in HCl 0.1N at 37°C. **(I)** TEM image of the Eudragit microcapsules loaded with Au–PLGA–NPs at pH = 5.5. The microcapsule is partially degraded and Au–PLGA–NPs can be observed in the matrix. **(J)** TEM image with a high magnification of microcapsules loaded with Au–PLGA–NPs at pH = 5.5. **(K)** SEM image of Au–PLGA–NPs after the microcapsule dissolution at pH = 6.8 after 3 h at 37°C. Inset, TEM image of one of the inner Au–PLGA MPNPs. MP: Microparticle; NP: Nanoparticle; PLGA: Poly lactic-co-glycolic acid; RT: Room temperature; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy; W/O/W: Double emulsion solvent evaporation method.

when analyzing the following results. After 3 days, the supernatants contained a 72.6% of the initial amount of rifampicin and this value was 48.4% after 7 days (lower than the one obtained after 3 days due to rifampicin degradation). Longer incubation times resulted in much lower amounts of rifampicin, being 24.2% after 14 days and 6.0% after 25 days.

Sustained release was also observed for 7 days in blood serum by Booysen *et al.* [28] when using rifampicin-loaded PLGA–PEG–NPs administered orally in a rat model whereas only during the first 2 days those authors were able to detect the antibiotic in blood serum when administered as a free (nonencapsulated) drug. In addition, Pandey and Khuller [29] also demonstrated the presence of systemic rifampicin 5 days after its oral administration in mice when encapsulated within PLGA–NPs (~200–300 nm) whereas the free drug was present only during the first 12 h. We also observed that the rifampicin released from NPs incubated at 37°C during 25 days was still active and killed *M. tuberculosis*.

Under physiological conditions, mycobacteria reside in an acidic cellular compartment, so we investigated next the amount of rifampicin that would be released from the NPs in such acidic conditions. For this, NPs were incubated at pH 4.5 and the antituberculosis activity of supernatants at different time points was determined by the resazurin method; results are given in Table 1. A considerable amount of rifampicin could be detected in the NP supernatants (40.3%) after 3 days, indicating that the antibiotic was readily being released from the NPs. We

Table 1. Bioassay-based quantification of rifampicin released from poly lactic-*co*-glycolic acid nanoparticles, in comparison with the amount detected from a free solution of rifampicin.

pH	Days	% Rif detected from Rif-loaded NPs	% activity of a control Rif solution
4.5	3	40.3	98.1
	7	10.1	39.9
	14	<6.0	6.0
	25	<6.0	<6.0

NP: Nanoparticle; Rif: Rifampicin.

observed that the amount of rifampicin detected in the supernatants decreased after longer incubation times, which may be attributed to degradation of rifampicin in the culture media due to spontaneous reactions (as discussed above) [15]. In fact, after 2 weeks, <6.0% of rifampicin could be detected in the supernatants at pH 4.5, indicating that degradation of the antibiotic was faster under acidic conditions. In order to assess the latter, we performed a control assay from a solution of free rifampicin at pH 4.5 and detected that in fact, the amount of rifampicin that can be detected also decreases with time, although to a lesser extent than that obtained from the NP supernatants probably caused by a superior acidification of the medium due to the presence of lactic and glycolic acids as subproducts of the PLGA degradation (Table 1). Control experiments performed with the supernatants of empty NPs did not show any antituberculosis activity even at the highest concentrations tested.

We tested next the impact of empty and antibiotic-loaded PLGA–NPs in the growth of *M. tuberculosis*. We first detected that empty NPs produced a slight delay in growth of the *M. tuberculosis* culture, which is consistent with moderate intrinsic antituberculosis properties of PLGA detected in both the MIC assays and the murine alveolar macrophages infection model, as reported in this work and elsewhere in the literature [27]. Interestingly, we observed that rifampicin-loaded NPs containing subinhibitory concentrations (0.25 × MIC) of rifampicin caused a further slight delay in the growth of *M. tuberculosis* in comparison with the same amount of empty NPs. Figure 4 shows the outcome of a representative experiment. This delay could be detected in terms of both OD_{600} (Figure 4A) and also in the number of viable cells (Figure 4B). We attributed this effect to the release of the antibiotic from the NPs. Given that rifampicin was used at a subinhibitory concentration, it only delayed but not arrested bacterial growth.

In order to estimate the antimicrobial activity of the rifampicin-loaded PLGA–NPs in conditions similar to those encountered during antituberculosis treatment, we performed time-kill kinetics experiments at pH 4.5. The pH of the early endosome in which *M. tuberculosis* resides, ranges from pH 6.2 to 4.5 depending on the macrophage activation state [30]. We found that the free and the encapsulated rifampicin had similar levels of bacterial eradication, both causing a reduction larger than 8-log in the number of viable bacteria after 14 days of incubation (Figure 5). Empty PLGA–NPs showed partial antimicrobial activity (2.5 log reduction compared with the nontreated bacterial cells, after 21 days of incubation; Figure 5), which is consistent with the fact that the concentrations of empty NPs used in this experiment are below, but rather close to, the inhibitory concentrations of PLGA.

We investigated the impact of the treatment with free rifampicin or rifampicin-containing NPs in the morphology of *M. tuberculosis* under both acidic and neutral conditions. As can be seen in Figure 6, at any condition tested the roughness of the surface of the bacteria wall under acidic conditions increased compared with the cells treated at physiological pH. Treatment with rifampicin either free or encapsulated enhanced the alterations observed in the cell shape and roughness.

We also tested whether the antituberculosis activity of the rifampicin-loaded PLGA–NPs was retained after being released from the Eudragit MPs. For this, the complete Matryoshka MPs were immersed in PBS at pH = 7.4 in order to release the NPs that were tested for antituberculosis activity *in vitro*. NPs containing rifampicin were effective at doses above 0.035 mg/ml whereas empty (without antibiotic) PLGA–NPs released from MPs did not show any antituberculosis effect at the highest dose tested (1.12 mg/ml).

Cell viability assay

To study the influence of different PLGA formulations on cell viability, the potential cytotoxic effect of unloaded-PLGA–NPs, rifampicin-loaded PLGA–NPs and free rifampicin were assayed in two different cell lines, TC7 Caco2 and MH-S murine alveolar macrophages. The Caco2 cell line was selected because it is one of the most used *in vitro* models to study the intestinal transport when drugs are orally administrated. The results depicted in Supplementary

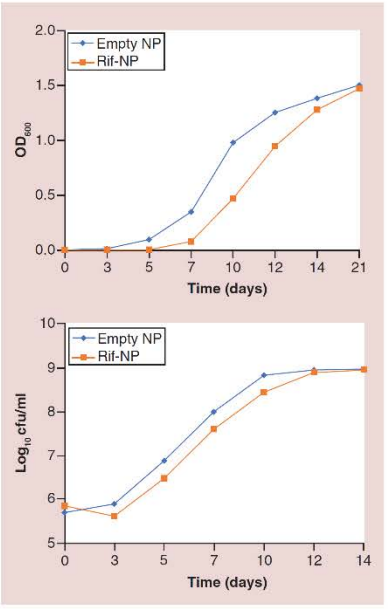


Figure 4. Impact of empty and rifampicin-loaded nanoparticles on the growth of *M. tuberculosis* on Middlebrook 7H9 broth at pH 7.0. The rifampicin-loaded NPs (red lines) contained a subinhibitory concentration of rifampicin and caused a slight delay in the growth of *M. tuberculosis* in comparison with the same amount of empty NPs (black line) that was detected in terms of both OD_{600} (upper panel) and also in the number of viable cells (bottom panel), as shown in this representative experiment.
NP: Nanoparticle; OD_{600}: Optical density measured at 600 nm; Rif: Rifampicin.

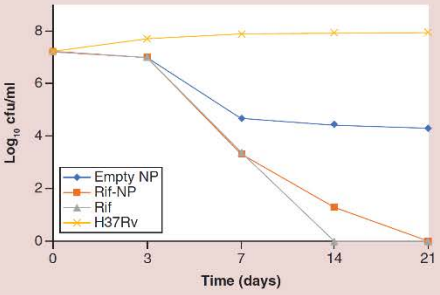


Figure 5. Time-kill kinetics at pH 4.5. High bacterial density (1.5 × 107 colony-forming unit/ml) cultures of *M. tuberculosis* were incubated at pH 4.5, conditions in which cells barely replicated (black line), and efficiency of several treatments for reducing the number of viable bacteria over time were compared. Treatment with 0.06–0.07 μg/ml of encapsulated rifampicin (between 4.5 and 6-times above the MIC; red line) was as efficient as the free antibiotic at the same concentration (green line) to kill cells in a population, being capable of sterilizing the culture after 2 weeks. Intrinsic antimicrobial activity of poly lactic-*co*-glycolic acid was also detected (blue line), which was more than four orders of magnitude lower than that obtained for the antibiotic containing samples.
NP: Nanoparticle; Rif: Rifampicin.

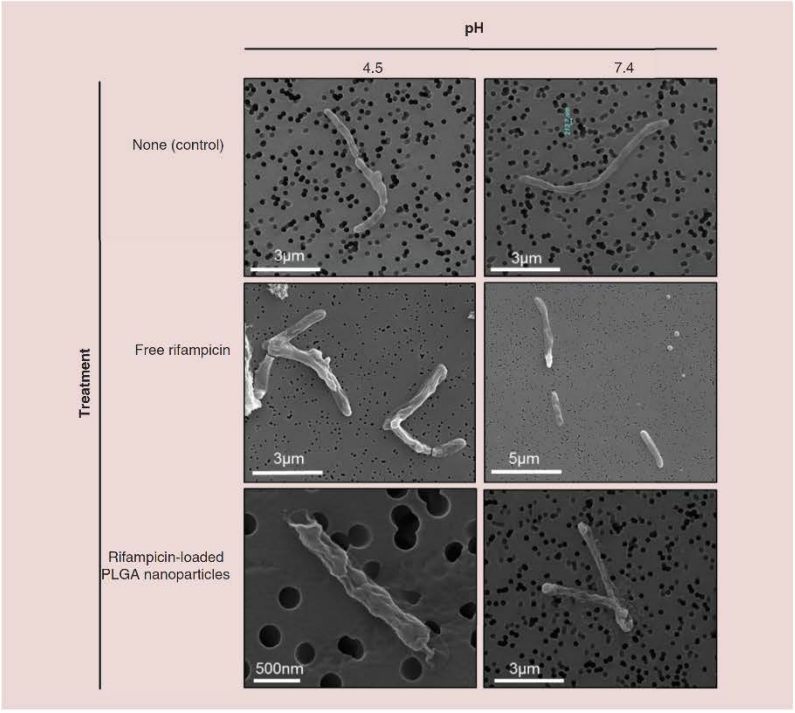


Figure 6. Scanning electron microscopy photographs of the *M. tuberculosis* bacteria in contact with free rifampicin or with rifampicin-loaded nanoparticles under acidic (pH 4.5) and neutral (pH 7.4) conditions.
PLGA: Poly lactic-*co*-glycolic acid.

Figure 3 showed that both, rifampicin-loaded PLGA–NPs and free rifampicin did not induce cytotoxicity in the range of concentrations tested (0.0625–1 mg/ml PLGA–NPs and 0.5–8 µg/ml of rifampicin).

MH-S murine alveolar macrophages were chosen because infected macrophages are the target where the drug has to exert its action. We tested unloaded and rifampicin-loaded PLGA–NPs with rifampicin as well as free rifampicin at serum concentrations (4 and 24 µg/ml), during 72 h. The results showed that the PLGA–NPs empty or with rifampicin were not toxic for the macrophages at plasma concentrations. Moreover, no significant effect was found when free rifampicin was added to the macrophages (Supplementary Figure 4).

Evaluation of Caco2 cell monolayer integrity & visualization of epithelial monolayers by transmission electron microscopy

As we mentioned in the experimental section, the integrity of Caco2 monolayers on membrane supports was assessed by TEER measurements and LY rejection assays. TEER values steadily increased during Caco2 cell differentiation, reaching a plateau value around 28 days after seeding, tight junctions were formed after that time and the monolayer integrity increased over time (data not shown). The medium TEER value achieved was 500 Ωcm^2. In general, a wide range of TEER values have been reported in the literature for these Caco2 cell clones. It has also been

described that this heterogeneity in TEER measurements can be due to diverse factors such as cell passage number, medium composition and cell culture period.

At that moment, the LY permeability, a fluorescent indicator of cell monolayer integrity, was evaluated in the apical to basolateral direction across Caco2 monolayers. In general, no fluorescence intensity was detected in the basal compartment, indicating the formation of intact epithelial monolayers with well-formed tight junctions. As selection criteria, only monolayers with % LY rejection values ≥99.9 were selected to assess the integrity of the monolayers within the course of the experiment. Immediately after, a second value of TEER was determined to control the status and the integrity of the cellular monolayer at the end of the permeability experiment. Along with the LY rejection values mentioned above, changes lower than 5% in TEER values compared with the initial readings were taken as an indicator of the maintenance of monolayer integrity.

Finally, the precise formation of a homogenous Caco2 epithelial barrier (Figure 2E) was monitored using a slightly modified transmission electron microscopy (TEM) protocol, previously reported [31]. In addition, TEM imaging allowed us to visualize the morphological organization of the epithelial barrier in detail. Thus, after 4 weeks in culture, TC7 Caco2 cells spontaneously formed a simple uniform epithelial layer with a structural organization similar to that of differentiated intestinal epithelial cells on the apical side of the transwell filters (Figure 7A3). This monolayer was constituted by polarized cuboidal-shaped cells that present a continuous brush border at their apical surface. The prominent brush border was composed of well-ordered and dense microvilli, presenting great homogeneity with regard to dimensions within and between cells. Different organelles such as mitochondria, lysosomes and the nucleus were observed in the intracellular compartments (Supplementary Figure 5). It is important to mention that tight junctions, the major subcellular structures in paracellular transport route, were visualized as intercellular contact areas between the membranes of two neighboring epithelial cells (Supplementary Figure 5B & C). Other types of cell–cell junctions, such as adherens junctions and desmosome were also visualized (Supplementary Figure 5B & C). Typical cell membrane folding, called interdigitations, were encountered between cells in the artificial epithelial monolayer (Figure 7A3 & Supplementary Figure 5D). Altogether, these results confirmed that artificial Caco2 cell monolayers closely mimic the characteristics of the *in vivo* barrier and; therefore, maintained the adequate morphological organization and integrity to carry out NP transport experiments.

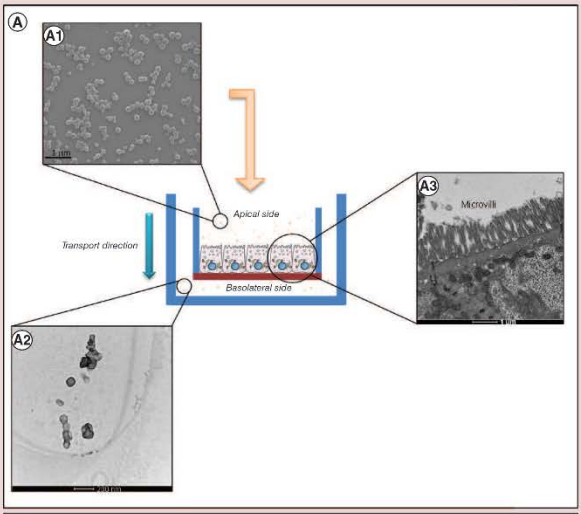
Permeability assay through the artificial membrane assay

The *in vitro* intestinal transport of rifampicin-loaded PLGA-based NPs was studied in TC7 Caco2 cells after the incubation with growing concentrations of NP suspensions for 4 h. TEM visualization of basolateral side samples (Figure 7A2) showed that, in all cases studied, a significant amount of rifampicin-loaded PLGA–NPs permeated through the artificial epithelial monolayer.

In addition, to complete the permeability study we quantified the transported rifampicin concentration presented in basolateral side samples by HPLC analysis. As shown in Figure 7B, the amount of transported drug that passed through Caco2 monolayers increased in a concentration-dependent manner, representing approximately 6% of the initial drug amount added to the apical chamber. These values further confirmed the results obtained by TEM imaging, indicating that the flux of rifampicin-loaded PLGA–NPs through TC7 Caco2 monolayer was 0.0046 μg/mm^{2}h (Figure 7B). It is important to mention that we cannot discard that a part of this rifampicin could be free drug that has been released from the loaded PLGA–NPs before or/and after their translocation through the artificial epithelial membrane.

On the other hand, the effect of NP suspensions on the monolayer integrity was investigated by monitoring TEER values after permeability experiments. Our data indicated that the modification of postexperiment TEER values was lower than 5% in relation to the initial TEER values (data not shown), suggesting that the opening of tight junctions did not happen in the cell monolayer after NP suspensions exposure.

Taken all together, these results suggest that epithelial cells were able to uptake rifampicin-loaded PLGA–NPs and those were able to translocate to the basolateral side. This means that in a potential *in vivo* application the NPs would be able to likely reach systemic circulation. We used ester-terminated PLGA–NPs to avoid electrical charge and hinder mucus adsorption. On the other hand, antibiotic NPs with reduced sizes (145 nm) were synthesized to favor the potential transcytosis by the Microfold cells (M cells) present in the gut-associated lymphoid tissue of the Peyer's patches in a hypothetical *in vivo* application.



B TC7 Caco2 epithelial monolayer

Rifampicin concentration, ppm		µg Rifampicin / mm² h	% transport
Apical side	*Basolateral side*		
65	4,20	0,0015	6.5
130	7,97	0,0028	6.1
260	12,94	0,0046	5.0

Figure 7. Description of the *in vitro* permeability assay and drug transport data. (A) Permeability assay through the artificial membrane assay. Scanning electron microscopy image of rifampicin-loaded PLGA–NPs before transport assay **(A1)**; transmission electron microscopy image of rifampicin loaded PLGA–NPs after transport assay **(A2)** and transmission electron microscopy image of morphology of TC7 Caco2 epithelial cell barrier **(A3)**. **(B)** Rifampicin concentration (ppm) presented in apical and basolateral side in TC7 Caco2 epithelial barrier. Rifampicin flux and transport data are also shown in both epithelial monolayers.
NP: Nanoparticle; PLGA: Poly lactic-*co*-glycolic acid.

Antimycobacterial effect of rifampicin-loaded PLGA–NPs in murine alveolar macrophages infection model

Finally, we investigated the proliferation of *M. tuberculosis* inside macrophages under the treatment with unloaded-PLGA–NPs, rifampicin-loaded PLGA–NPs and free rifampicin at serum concentrations (4 and 24 µg/ml). We found that at those two concentrations the exposure to PLGA–NPs loaded with rifampicin caused a decrease in the growth of *M. tuberculosis* of 3.3 and 3.6-times, respectively, compared with free rifampicin until day 4 (Figure 8A & B). It is possible that the effect can be stronger with a longer exposition, as it was observed when the bacteria was exposed to rifampicin-loaded PLGA–NPs (Figure 5) and also as it was reported by others in experiments with BCG in murine macrophages [8,32].

The effect observed after the treatment with rifampicin-loaded PLGA–NPs at maximum concentration (24 µg/ml) could be related with the synergy observed between the drug and PLGA, since the final concentration of PLGA added was higher (23.9 µg/ml) than the MIC value (7.8 µg/ml). However, in the minimum concentration (4 µg/ml), the effect observed can be related exclusively to the drug since the PLGA concentration present was lower (3.9 µg/ml) than the MIC value.

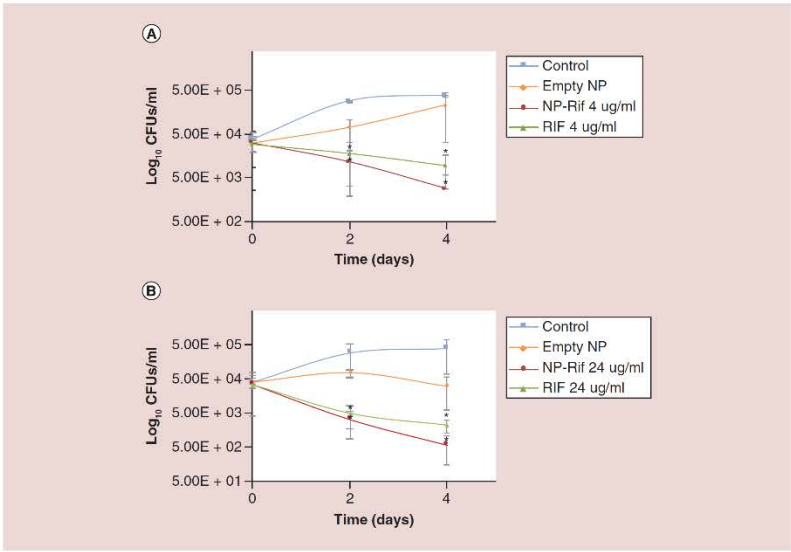


Figure 8. Growth of H37Rv strain inside MH-S macrophages after the treatment with empty poly lactic-*co*-glycolic acid nanoparticles, rifampicin-loaded poly lactic-*co*-glycolic acid nanoparticles and free rifampicin. (A) Minimum serum concentration (4 μg/ml). **(B)** Maximum serum concentration (24 μg/ml). Data are expressed as the average and standard deviation of triplicates of at least three independent experiments.
*$p < 0.05$ (compared with MH-S control that was infected with H37Rv without NPs treatment, Mann–Whitney U-test, GraphPad Software, Inc).
CFU: Colony-forming unit; MH-S: Murine alveolar macrophages; NP: Nanoparticle.

On the other hand, we also found that empty PLGA–NPs had some antimicrobial activity when the same quantity of PLGA as the one contained in rifampicin-loaded PLGA–NPs, was added (Figure 8A & B). Again, the concentration was higher (51.5–309 μg/ml) than the MIC value. The same tendency was observed in the time-kill kinetics experiments and also by Lawlor *et al.* [26] in THP-1 macrophages using PLGA–MPs, who suggested that the antimicrobial effect could be related with the size and morphology of the particles used. It is important to mention that the approach here reported could also be used to encapsulate the second line injectable TB drugs utilized for the treatment of multidrug-resistant TB that generally present poor efficacy are more toxic and require prolonged treatments [33].

Discussion

The targeted drug delivery to the intracellular compartments of infected alveolar macrophages where *M. tuberculosis* resides and multiplies could be a promising approach to improve the antibiotics therapeutic efficacy in the treatment of this disease. In passive targeting, organic and inorganic NPs are rapidly internalized within cells to be degraded as part of the innate immune response against any foreign body. Together with granulocytes, resident or circulating macrophages as well as dendritic cells are all specialized in this clearance. Independently of the route of cellular entry (i.e., phagocytosis, macropinocytosis, clathrin or caveolin-mediated endocytosis, etc.), the internalized NPs remain inside intracellular vesicles where enzymatic and acidic degradation take place. This NP uptake depends on the macrophage population considered and on its activation state [34]. As we mentioned before, Kalluru *et al.* [8] demonstrated that whereas rifampicin-loaded PLGA–NPs are internalized within acidic phagolysosomes, *Mycobacterium bovis* (BCG) remained in early phagosomes using BCG-infected mouse bone macrophages and also

RAW mouse macrophages. However, even though colocalization within the same vesicle was not achieved, drug diffusion into the cytosol cleared the infection. We postulated that intracellular accumulation and slow antibiotic release can be achieved using orally administered antibiotic-loaded PLGA–NPs if they were able to cross the intestinal epithelium into the blood stream with the sufficient blood circulation half-life to reach *M. tuberculosis*-infected alveolar macrophages. Thus, the encapsulation of antituberculosis drugs in NP-delivery systems could also increase the drug bioavailability and; therefore, reduce drug dosage frequency. This fact would also allow decreasing treatment-related side effects (i.e., hepatotoxicity, skin reactions, gastrointestinal disturbances and immunological reactions) and improve drug regime compliance by the patient, two of the main clinical problems in the current treatment of TB. Other factor that can contribute to improve the patient adherence to the treatment is the way in which therapy is administered. In that sense, oral drug administration is the preferable route for long-term therapy due to its noninvasive character, simplicity and patient compliance. However, the main disadvantage of this administration route is the variability in the drug absorption due to degradation by the stomach acidic pH and lytic enzymes. Our study demonstrates that free rifampicin degrades over time under culture conditions (Table 1) and even more under acidic conditions in agreement with the previous literature [15]. To overcome this drawback, rifampicin-loaded NPs were encapsulated inside MPs based on methacrylic acid–ethyl acrylate copolymer, which remains insoluble in gastric conditions and dissolves in the intestinal fluid. In our case, we selected a polymer based on an anionic methacrylic acid–ethyl acrylate copolymer to release its cargo (antibiotic-loaded NPs) at the duodenum pH taking advantage of the thin mucus layer of this first section of the small intestine and its high vascularization [35]. In addition, at that level, it would be the NP carrier system that would act as a protector to minimize the rifampicin degradation induced by physiological conditions. Coatings targeting the intestine (based on hydroxypropyl methylcellulose phthalate) containing drug-loaded NPs have been used *in vitro* and *in vivo* in the systemic administration of insulin [17]. Those authors used a mixture of a nonacid-dependent mucoadhesive polymer (Eudragit RS) with PLGA for the formulation of the inner insulin-loaded NPs and they demonstrated a prolonged hypoglycemic effect in a diabetic rat model after oral administration.

Previous to the permeability study, we provided evidence of no cytotoxicity of developed PLGA–NPs on TC7 Caco2 and MH-S murine alveolar macrophages. These results were in agreement with previous reports conducted with PLGA–NPs. Thus, Tukulula *et al.* [36] reported no cytotoxic effect of PLGA–NPs and curdlan-conjugated PLGA–NPs in THP1 macrophages over a 72 h period (dose range from 0.13 to 200 µg/ml). In primary murine macrophages, thioridazine and rifampicin loaded-PLGA–NPs and unloaded PLGA–NPs did not significantly reduce cell viability throughout a 9 day treatment [37]. Also, PLGA–NPs were not toxic to Caco2 cells after 24 h exposition, as long as the concentration was lower than 500 µg/ml [38]. An *in vitro* study carried out by Zhang *et al.* [39] to evaluate the cytotoxicity of PLGA–NPs, demonstrated that they were well tolerated by Caco2 cells at concentrations below 25 mg/ml.

We used ester-terminated PLGA–NPs to avoid electrical charge to hinder mucus adsorption and we synthesized antibiotic NPs with reduced sizes (145 nm) to favor the potential transcytosis by the Microfold cells (M cells) present in the gut-associated lymphoid tissue of the Peyer's patches in a hypothetical *in vivo* application. Bhavsar and Amiji [40] demonstrated that a high number of intestinal cells expressing the gene for green fluorescent protein were observed when 200 nm gelatin–NPs containing a plasmid encoding the green fluorescent protein and in turn encapsulated within capsules were orally administered in rats which is indicative of a successful NP intracellular internalization.

The reduced size of the rifampicin-loaded PLGA–NPs (145 nm) would favor the permeation through the intestinal epithelium reaching the systemic circulation. In this regard, Desai *et al.* demonstrated that 100 nm PLGA–NPs orally administered in mice were able to diffuse through the submucosa layers whereas larger NPs and MPs (>500 nm) remain trapped in the epithelial lining of the tissue [41]. Also, reduced NP sizes favor NP cellular internalization to achieve transcytosis considering that the paracellular transport constitutes only a 1% of the total mucosa surface area [42]. Our *in vitro* permeability assay revealed that epithelial cells were able to uptake rifampicin-loaded PLGA–NPs. Given that the paracellular transport is not viable due to the small size of interstitial space and that the intercellular junctions remain still tight during the permeability assay, the NPs internalization inside the cells may happen via endocytic uptake mechanisms. In that sense, previous works demonstrated that the uptake of PLGA–NPs was reduced at 4°C in Caco2 cells, indicating an endocytic uptake mechanism was involved in the NPs uptake for these cells [34]. The specific endocytosis pathways by which the particles may be translocated through the cells remain to be elucidated.

Also, we observed a sustained rifampicin release up to 25 days after incubation in culture media. Sustained release was also observed for 7 days in blood serum by Booysen *et al.* [28] when using rifampicin-loaded PLGA–PEG NPs administered orally in a rat model whereas only during the first 2 days those authors were able to detect the antibiotic in blood serum when administered as a free (nonencapsulated) drug. In addition, Pandey and Khuller [29] also demonstrated the presence of systemic rifampicin 5 days after its oral administration in mice when encapsulated within PLGA–NPs (~200–300 nm) whereas the free drug was present only during the first 12 h. We also observed that the rifampicin released from NPs incubated at 37°C during 25 days was still active and killed *M. tuberculosis*. Also, empty PLGA–NPs showed intrinsic antituberculosis activity that was attributed to the potential accelerated hydrolysis of the ester bonds of the PLGA, hence producing lactic and glycolic acids that would further acidify the media being this process autocatalytic [27]. As a consequence, increases in media acidification would in turn result in an enhanced bactericidal action for *M. tuberculosis*.

Finally, the assays in *M. tuberculosis*-infected alveolar murine macrophages showed that the treatment with rifampicin-loaded PLGA–NPs had an antimicrobial effect at both tested concentrations until day 4, significantly decreasing the intracellular bacterial load. It is possible that the effect can be stronger with a longer exposition, as it was observed when the bacteria was exposed to rifampicin-loaded PLGA–NPs (Figure 5) and also as it was reported by others in experiments with BCG in murine macrophages [8,32]. It is important to mention that the approach here reported could also be used to encapsulate the second line injectable TB drugs utilized for the treatment of multidrug-resistant TB that generally present poor efficacy, are more toxic and require prolonged treatments [33].

Conclusion

By using the oil-in-water (O/W) emulsion solvent evaporation method it is possible to prepare monodisperse antibiotic-loaded PLGA-based NPs with high encapsulation efficiencies. Those antibiotic-loaded PLGA-based NPs can also be encapsulated within (Eudragit-based) monodisperse microcapsules using the double W/O/W emulsification and evaporation method obtaining antibiotic-loaded PLGA–NPs encased within gastroresistant MPs based on methacrylic acid–ethyl acrylate copolymers. Under gastric simulated conditions we demonstrated that the external coating remained unaltered and that the inner antibiotic-loaded NPs could be rapidly released under intestinal simulated conditions. We also demonstrated that those inner antibiotic-loaded NPs were able to cross an *in vitro* model of a simulated intestinal barrier composed of Caco2 cell monolayers. Therefore, those NPs would be protected from gastric degradation and potentially reach systemic circulation. The antituberculosis action of the encapsulated rifampicin within the NPs was similar to that observed for the free drug indicating that the synthesis protocols did not alter the drug activity. This action was observed to be pH-dependent considering both intracellular and extracellular milieus. We also demonstrated that those antibiotic-loaded NPs were noncytotoxic on human cell lines but they were able to restrict the proliferation of *M. tuberculosis* inside infected macrophages. This novel delivery system shows a great potential for the successful targeted treatment of TB thanks to its demonstrated long-term release and its high cytocompatibility.

Future perspective

The increase of antibiotic resistant bacteria is a rising concern worldwide. The use of nanotechnological approaches can improve current antimicrobial treatments by providing a rational usage of antibiotics. Targeted therapies based on drug-loaded NPs with demonstrated benefits over the administration of the free drug will direct future research lines. Future research directions of the study here reported will be directed towards the *in vivo* evaluation of the antituberculosis effect of those antibiotic-loaded gastroresistant NPs compared with the administration of the free drug in mice infected with *M. tuberculosis* strain H37Rv.

Financial & competing interests disclosure

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Summary points

- Matryoshka-type gastroresistant microparticles containing antibiotic-loaded poly lactic-*co*-glycolic acid (PLGA) nanoparticles (NPs) against *M. tuberculosis* were produced by a multistage emulsification–evaporation method.
- The inner antibiotic-loaded NPs were released under simulated intestinal conditions whereas the coating protected them from degradation under simulated gastric conditions.
- The Matryoshka-type procedure of encapsulation did not hinder the antituberculosis action of the encapsulated antibiotic rifampicin.
- A sustained antibiotic release can be obtained when using the encapsulated NPs as well as a more effective elimination of *M. tuberculosis* when applied against infected macrophages compared with the use of the free drug.
- The potential colocalization of the drug-loaded NPs with the intracellular pathogen in the same intracellular vesicle represents a promising approach to improve the antibiotic therapeutic efficacy in the treatment of the disease.
- Antibiotic-loaded PLGA–NPs were able to cross an *in vitro* model of intestinal barrier and demonstrated their suitability as effective drug carriers in the oral treatment of tuberculosis.

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Supporting information

Matryoshka-Type Microparticles for the Oral Treatment of Intracellular Pathogens

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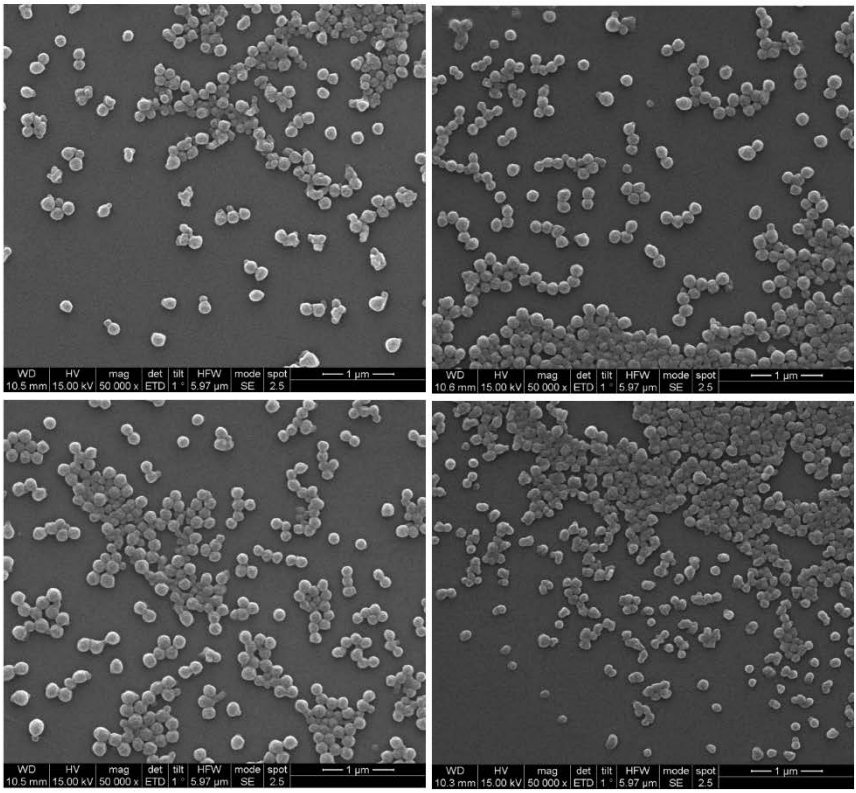


Figure S1. Inter-batch monodispersity is shown in those SEM images of 4 independent batches of rifampicin-loaded PLGA nanoparticles.

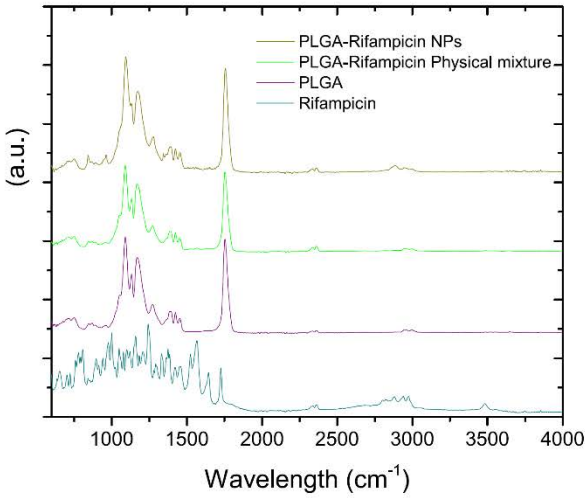


Figure S2. FTIR spectra of the corresponding antibiotic encapsulated PLGA nanoparticles and the physical mixture composed by the corresponding free antibiotic and the un-loaded PLGA nanoparticles. The free drugs and the PLGA are also added to facilitate the comparison

Figure S3. Cell viability of TC7 Caco2 cell lines upon exposure to free rifampicin and rifampicin-loaded PLGA NPs. Percentage values are expressed as mean ± standard deviation (n = 4).

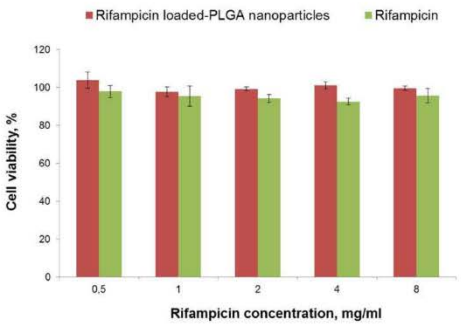
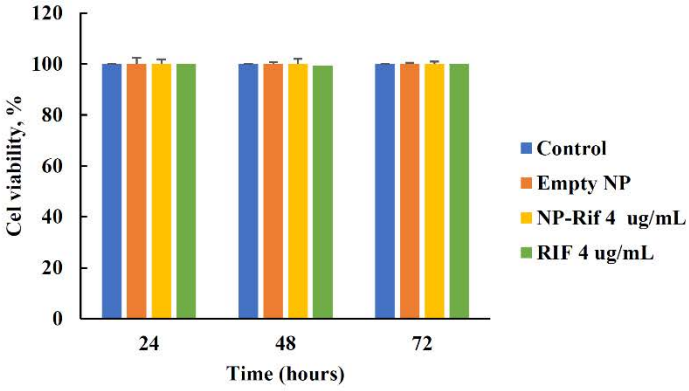


Figure S4. Cytotoxic effect of PLGA NPs on MH-S murine alveolar macrophages after 24, 48 and 72 hours of exposition to empty NPs, rifampicin-loaded NPs and free rifampicin at serum concentrations: A) 4 ug/mL and B) 24 ug/mL.

A)



B)

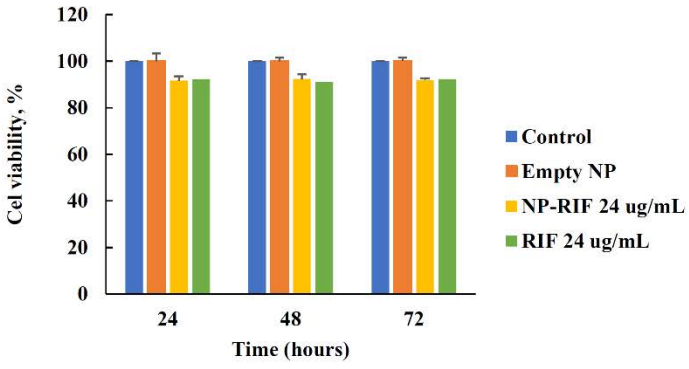


Figure S5. a-f) Morphology of TC7 Caco2 epithelial cell barrier. a-c, f) Transmission electron micrographs show well-differentiated cells with microvilli (Mv). b-d) TEM images showing cell-to-cell junctions (TJ: tight junction, AJ: Adherens junction and D: Desmosomes) and cellular interdigitations (I). c y e) Transmission electron micrographs show a relatively big nucleus and mitochondria (N and M, respectively, as indicated)