

**UNIVERSIDADE DE LISBOA**  
**FACULDADE DE CIÊNCIAS**  
**DEPARTAMENTO DE BIOLOGIA VEGETAL**



**DECIPHERING HOST FACTORS FOR  
MYCOBACTERIA INTERNALIZATION DURING  
PHAGOCYTOSIS**

**DISSERTAÇÃO**

Pedro Manuel Dias Timóteo

**MESTRADO EM MICROBIOLOGIA APLICADA**

**2013**

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**Pedro Manuel Dias Timóteo**

**MASTER THESIS**

**2013**

This thesis was fully performed at Centro de Patogénese Molecular – Unidade de Retrovírus e Infecções Associadas (CPM-URIA) of the Faculty of Pharmacy of the University of Lisbon under the direct supervision of Prof. Dr. Elsa Anes.

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## Relevant Abbreviations

- **HIV / VIH** – Human Immunodeficiency Virus
- **BCG** – Bacillus Calmette-Guérin
- **MOI** – Multiplicity of Infection
- **HMDM** – Human Monocyte-derived Macrophages
- **GFP** – Green Fluorescent Protein
- **Rab GTPase** – Guanosine triphosphatase belonging to the Rab family of proteins
- **THP-1** – Human monocyte cell line THP-1 (ATCC® TIB-202™)
- **J774** – Mouse macrophage cell line J774A.1 (ATCC® TIB-67™)
- **shRNA** – Small hairpin RNA
- **siRNA** – Small interfering RNA
- **CD40 / CD17** – Cluster of differentiation receptor 40 / 17
- **MHC II** – Major Histocompatibility Complex class II
- **DMEM** – Dulbecco's Modified Eagle Medium
- **FBS** – Fetal Bovine Serum
- **RPMI** – Roswell Park Memorial Institute (where this culture medium was developed)
- **HEPES** - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **M-CSF** – Macrophage Colony Stimulating Factor
- **EDTA** – Ethylenediamine tetraacetic acid
- **HEK 293T** – Human Embryonic Kidney cell line ATCC® CRL-11268™
- **PMA** – Phorbol 12-myristate 13-acetate
- **PBS** - Phosphate Buffered Saline
- **OD** – Optical Density
- **FITC** - Fluorescein isothiocyanate
- **U-87MG** – Human glioblastoma cell line U-87 MG (ATCC® HTB-14™)
- **ESAT-6** – Early Secreted Antigenic Target Protein 6

## Resumo

A tuberculose é uma doença infecciosa que tem acompanhado historicamente a Humanidade. Caracteriza-se tipicamente por uma infecção pulmonar, manifestando-se através de tosse persistente, febre e especialmente por uma fraqueza generalizada. Apesar da sua ancestralidade, é ainda hoje a doença infecciosa responsável pelo maior número de mortes a nível mundial.

O agente etiológico da tuberculose é uma bactéria, *Mycobacterium tuberculosis*, descrita pela primeira vez por Robert Koch no século XIX. Pertence à ordem *Actinomycetales* e à família das *Mycobacteriaceae*; são bacilos, Gram-positivos, e apresentam por norma um crescimento lento e uma membrana com uma composição invulgar, rica em lípidos particulares como o lipoarabinomanano.

*M. tuberculosis* é um patógeno particularmente resistente por se encontrar extremamente adaptado ao hospedeiro humano, e o problema agrava-se com a actual epidemia do vírus de imunodeficiência humana (VIH). Indivíduos infectados com VIH não só têm uma probabilidade maior de desenvolver tuberculose, como apresentam morbidades mais elevadas, sendo a tuberculose a principal causa de morte entre doentes de VIH. Outro dos obstáculos mais significativos é a resistência a antibióticos, que nas últimas décadas se tem tornado mais proeminente em *M. tuberculosis*. Várias ocorrências de resistência aos antibióticos de primeira e segunda linha foram já documentados, e o surgimento de estirpes multirresistentes representa um sério problema de saúde pública. Continua a não existir uma vacina eficaz para substituir a BCG (Bacilo Calmette-Guérin, estirpe atenuada do agente da tuberculose bovina *Mycobacterium bovis*), que está em uso desde 1921, e cuja eficácia na protecção contra a tuberculose pulmonar em adultos é manifestamente insuficiente.

Muita da dificuldade em combater a tuberculose está também relacionada com a sua prevalência e persistência. Segundo a Organização Mundial de Saúde, um terço da população mundial pode ser portadora de *M. tuberculosis* no seu estado latente, ou seja, sem desenvolver doença activa. Ainda que a doença não seja contagiosa neste estado, a sua presença no hospedeiro pode vir a manifestar-se de forma oportunista em casos de enfraquecimento imunitário, como por VIH, drogas imunossupressivas, doença concomitante ou simplesmente pelo envelhecimento.

Por estas razões a investigação no combate à tuberculose está cada vez mais a dirigir-se no sentido de compreender os sistemas de imunidade do hospedeiro e a sua relação com este patogénio.

A pedra angular da interacção patogénio-hospedeiro é a fagocitose. É um processo de imunidade inata altamente conservado evolutivamente através do qual células eucarióticas são capazes de internalizar e digerir partículas externas ou microorganismos, eliminando-os. Certas células do sistema imunitário humano servem-se deste processo para neutralizar patógenos invasores, denominando-se células fagocitárias, ou fagócitos, entre os quais se destacam os macrófagos. *M. tuberculosis* é tipicamente inalado por aerossóis e nos pulmões entra em contacto com os macrófagos alveolares residentes, que oferecem a resposta fagocitária inicial.

Da fagocitose fazem parte várias etapas. Tem início pela internalização das partículas, que consiste na captura e envolvimento do alvo numa vesícula endocítica. Esta é depois transportada pelo citoplasma, via elementos do citoesqueleto, e vai fundindo com outras vesículas endocíticas. Quando o grau de maturação é compatível funde-se com lisossomas, cujo conteúdo enzimático assegura uma degradação proteolítica do alvo. A este processo dá-se o nome de biogénese do fagolisossoma, e nele inicia-se a acção de proteases denominadas catepsinas, bem como óxidos nítricos e superóxidos de efeito bactericida. Depois de digerido o conteúdo fagocitado, os antígenos resultantes serão utilizados para despoletar uma resposta imunitária adquirida, levada a cabo por células T.

Quando a partícula a fagocitar é o *M. tuberculosis*, este é capaz de subverter os mecanismos de defesa do fagócito, conseguindo sobreviver e multiplicar-se no interior da célula hospedeira. Sendo complexo o arsenal molecular de que o fagócito dispõe para combater invasores, também o é o modo como esta micobactéria o evita. O *M. tuberculosis* consegue impedir a maturação do fagossoma, controlando o hospedeiro através de processos que ainda não são totalmente compreendidos. Sabe-se, porém, que influencia a função de proteínas de tráfego vesicular e transporte membranar, bem como as referidas catepsinas. Sendo estes alguns dos alvos da sua virulência, tornam-se também importantes alvos de investigação biomédica, pois podem ajudar a combater a estratégia de infecção de *M. tuberculosis*, fortalecendo as defesas do hospedeiro.

A fase de internalização das bactérias por macrófagos é um ponto crítico na infecção, uma vez que representa um dos primeiros contactos entre o patogénio e o hospedeiro, e é através dela que têm início todos os restantes mecanismos. Interessa pois compreender como é regulada a internalização, e que influência terá *M. tuberculosis* nessa regulação.

Para estudar devidamente factores de internalização, é necessário em primeiro lugar definir o modelo de internalização de partículas inertes para estabelecer o que ocorre na fagocitose como fenómeno abrangente.

Criámos um desenho experimental que nos permitiu explorar um grande número de variáveis. Fizemos variar o tipo de micobactéria: destruída no fagócito (caso de *M. smegmatis*) versus sobrevivente intracelular (caso de *M. tuberculosis*), o tempo, a multiplicidade de infecção (MOI, representa o número de bactérias por cada macrófago) e o tipo de macrófago. Para este trabalho foram utilizados três tipos de macrófagos: macrófagos derivados de monócitos humanos (HMDM), macrófagos humanos da linha celular THP-1 e macrófagos de ratinho da linha celular J774. Utilizámos também três espécies dentro do género *Mycobacterium*: *M. tuberculosis*, *M. bovis* BCG e *M. smegmatis*, sendo as duas últimas consideradas não-patogénicas. Obtendo os valores de internalização ao longo de três pontos no tempo (30 minutos, 1 hora e 4 horas) tornou-se possível traçar perfis de internalização para cada combinação de variáveis.

As estirpes à nossa disposição expressavam proteína verde fluorescente (GFP) por meio de um plasmídeo replicativo, o que permitiu que os macrófagos que internalizaram micobactérias pudessem ser identificados em citometria de fluxo através da fluorescência no comprimento de onda do verde.

Armados desse desenho experimental e de um protocolo de infecção eficiente que permitiu testar várias variáveis em simultâneo, descrevemos curvas de internalização para as várias combinações de macrófago-micobactéria. Destes resultados foi possível chegar à conclusão esperada de que a internalização é fortemente influenciada pelo tempo e pela multiplicidade de infecção, ou seja, estes factores condicionam fortemente a resposta fagocitária dos macrófagos. Tornou-se também evidente que os HMDM são os agentes fagocitários mais eficientes de entre os macrófagos testados, tendo sido capazes de atingir elevados níveis de internalização mais rapidamente que os restantes. Uma observação que se nos revelou pertinente foi a de que ao fim de apenas 30 minutos, todos os tipos de macrófagos foram capazes de realizar uma considerável internalização das micobactérias na amostra. Põe-se assim em causa muito do *background* experimental em micobactérias, que prevê infecções de duração igual ou superior a 3 horas. Este nosso trabalho sugere que infecções mais breves são não apenas funcionais, mas até preferíveis para o estudo de reguladores das fases iniciais de fagocitose, como é o caso da internalização. Infecções demasiado prolongadas podem levar à perda desta informação e a uma alteração do estado fisiológico dos macrófagos, particularmente com cargas bacterianas elevadas.

Foi também importante observar que as diferentes espécies de *Mycobacterium* apresentam diferenças ao nível da internalização. *M. smegmatis* foi internalizada mais rapidamente e em maiores quantidades, seguida por *M. bovis* BCG e esta por *M. tuberculosis*. Ora, a esta ordem de internalização corresponde a ordem de inversa de patogenicidade, ou seja, com o aumento da patogenicidade diminui a internalização. Levanta-se imediatamente a sugestão de que a diminuição da internalização será um dos processos de virulência de *M. tuberculosis*. Parece haver uma contradição, pois sendo *M. tuberculosis* um patógeno intracelular esperar-se-ia que promovesse a sua internalização o mais possível. No entanto, uma carga microbiana demasiado elevada sobre as células do hospedeiro leva a processos de morte celular, privando assim *M. tuberculosis* do seu *habitat*. Força-se então um equilíbrio cuja regulação importa compreender.

Para investigar o papel de factores do hospedeiro que influenciem a internalização, realizámos uma triagem sobre 71 proteínas envolvidas em tráfego vesicular e na maturação do fagossoma, a maioria pertencente aos grupos das Rab guanosina trifosfatases (Rab GTPases), catepsinas e cistatinas. Pelo silenciamento de cada uma das proteínas individualmente torna-se possível avaliar a sua contribuição para o processo de internalização, ou seja, descobrir a sua função por redução ou ausência-de-função.

O silenciamento individual das proteínas foi feito por intermédio de bibliotecas lentivirais contendo plasmídeos que codificam para um pequeno RNA de interferência (*hairpin*), complementar ao RNA mensageiro de um gene específico. Graças a essa complementaridade o RNA mensageiro é destruído, impedindo-se assim a produção da proteína correspondente. Assim construímos uma linha *knockdown* de macrófagos THP-1, a qual submetemos a ensaios semelhantes aos que desenvolvemos na primeira parte dos trabalhos. Na abordagem experimental, as variáveis de tempo e MOI foram restringidas a apenas 1 hora e uma MOI de 10, pois estes valores representam um estímulo mais moderado e permitem assim realçar as eventuais diferenças provocadas pelos silenciamentos. Utilizámos nesta fase apenas *M. tuberculosis* e *M. smegmatis* na qualidade de controlo não-patogénico.

Nestas condições, a grande maioria dos silenciamentos provocou um decréscimo na internalização de micobactérias, o que levou a que as suas proteínas-alvo fossem classificadas como potenciais reguladores positivos da internalização. Aplicando limites para aquilo que deveria ser considerada uma diferença significativa face aos controlos não-silenciados, 26 proteínas emergiram como reguladores, sendo por isso denominados os *hits* da triagem. Este é um número surpreendentemente elevado, tendo em conta que as

bibliotecas lentivirais não foram desenhadas especificamente para ensaios de internalização.

Destes *hits* destacam-se especialmente aqueles que se manifestaram apenas em infecções com uma das micobactérias, pois permitem inferir uma relação directa com a patogenicidade. É o caso da catepsina L, identificada como *hit* apenas para *M. smegmatis*. Considerando que a ausência desta proteína levou a que *M. smegmatis* fosse internalizado em menor nível e, tendo presente a correlação demonstrada entre baixa internalização e patogenicidade, pode dizer-se que nesta situação *M. smegmatis* se comportou como uma micobactéria patogénica. Daqui se retira que o silenciamento da expressão de catepsina L pode ser uma das vias através das quais *M. tuberculosis* manifesta a sua patogenicidade.

Três outras proteínas demonstraram um papel regulatório na internalização apenas em infecções com *M. tuberculosis*: Rab37, Rab38 e catepsina W. Desta última são conhecidas funções na regulação da citotoxicidade de linfócitos, mas não é claro o seu mecanismo de acção. No caso de Rab37 e Rab38, este trabalho representa a primeira associação destas à infecção por *M. tuberculosis*. Tratando-se de uma triagem abrangente, só será possível definir o modo de funcionamento destas proteínas com novas experiências no futuro.

Os restantes 22 *hits* não são menos importantes; apesar de conhecida a sua associação à tuberculose, demonstraram pela primeira vez ser reguladores positivos da internalização.

Há limitações ao estudar infecções *in vitro*, pois as conclusões a retirar não são necessariamente igualmente válidas para o contexto real de infecções por *M. tuberculosis*. As diferenças acentuam-se se considerarmos que neste trabalho experimental se simulou um cenário de uma primo-infecção, ou seja, o primeiro contacto entre *M. tuberculosis* e células humanas, sendo este um cenário artificial que não corresponde à maioria dos casos de tuberculose em humanos. É uma concessão necessária para a investigação experimental e apesar de tudo não impede que estas descobertas tenham potencial terapêutico. As proteínas aqui identificadas poderão servir para controlar mais eficazmente a infecção, se aprofundado o conhecimento sobre a sua função.

Esta tese apresenta pois uma nova abordagem experimental, na forma de infecções breves e eficientes e de novos intervenientes nos primeiros passos do equilíbrio dinâmico entre patogénio e hospedeiro.

**Palavras-chave:** *Mycobacterium tuberculosis*, macrófago, fagocitose, internalização, triagem, citometria de fluxo, regulador positivo

## **Abstract**

*Mycobacterium tuberculosis* is a very successful pathogen that is able to survive and multiply inside the host's immune cells, thus triggering the active disease of tuberculosis or alternatively entering into an asymptomatic latent state. Its success lies on a powerful subversion of the fundamental innate immunity process of human-host cells: phagocytosis.

*M. tuberculosis* is known to resist destruction by phagocytes through a complex molecular sabotage of phagosome maturation, an integral part of the phagocytic process. On the other hand, internalization, the first and arguably most important step of phagocytosis, has not been so thoroughly investigated.

In this work we outline a method for quantifying internalization of green fluorescent mycobacteria through flow cytometry. With it we provide profiles of internalization-over-time for J774, THP-1 and human monocyte-derived macrophages (HMDM), which were infected with *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis*. From these experiments we gathered that HMDM are the most efficient in internalizing mycobacteria, that there is considerable internalization even after short time periods such as 30 minutes and also that *M. tuberculosis* is internalized to a lesser extent than its non-pathogenic counterparts. It is suggested that *M. tuberculosis* manipulates its own internalization. In order to decipher which are the host factors targeted by this subversive strategy, we performed an internalization-based screening on knockdown THP-1 macrophages. Protein knockdowns were achieved by shRNA interference from lentiviral libraries targeting a total of 71 proteins. Using the previously designed method, we identified 26 putative positive regulators of internalization including a large number of Rab GTPases and cathepsins, proteins associated with vesicular trafficking and proteolysis in the mature phagosome.

Through this novel approach we were able to quantify rates of internalization, which were previously only empirically estimated, and use them as a model to highlight new potential therapeutic targets in the ongoing battle against tuberculosis.

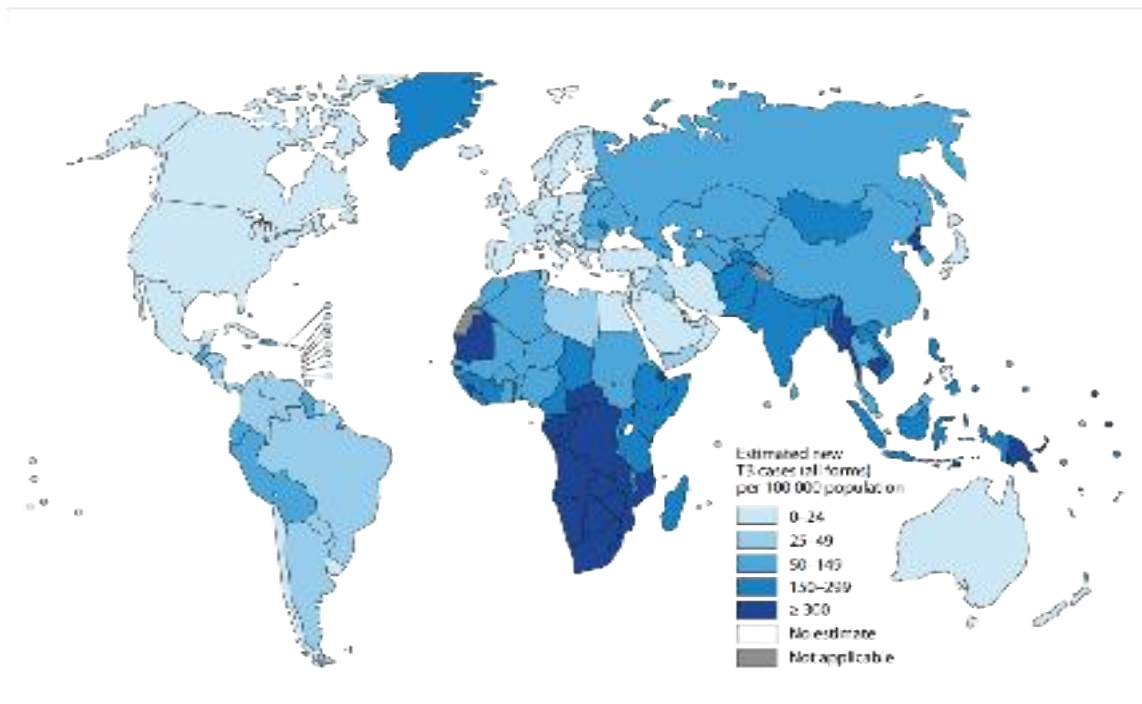
**Keywords:** *Mycobacterium tuberculosis*, macrophage, phagocytosis, internalization, screen, flow cytometry, positive regulator

# 1. Introduction

## 1.1 General aspects of tuberculosis

Tuberculosis has a record as one of the leading causes of death worldwide. It was known to be responsible for one in every four deaths in nineteenth century Europe, and is at present still a very widespread and burdensome disease, responsible for 1.4 million deaths and 8.7 million new infected individuals each year, as estimated by the World Health Organization <sup>1</sup>.

It is a disease that primarily affects the lungs, but can also cause systemic infections, and its symptoms include persistent coughs, often with blood or sputum, fever, weakness and weight loss. Infections on other organs yield different symptoms and are often difficult to diagnose.



**Figure 1.** Estimated tuberculosis incidence rates. Map and data from the *Global Tuberculosis Report 2012*, by the World Health Organization.

The etiological agent of tuberculosis is a bacterium, *Mycobacterium tuberculosis*, which was first identified by the microbiologist Robert Koch in the late nineteenth century. It has been widely studied since, and we now know of many particular molecular features that make it a very persistent threat.

Despite the complex cellular and molecular mechanisms underlying tuberculosis, it is to an extent a poverty disease, in that it is strongly correlated to low living standards. Poverty

and malnutrition can be considered the strongest risk factors for tuberculosis. Four out of every five cases of tuberculosis occur in sub-Saharan countries, and 80% of all cases can be attributed to just twenty-two low-income developing countries (Figure 1)<sup>1</sup>.

The sequencing of the *M. tuberculosis* genome in 1998 provided a valuable boost in tuberculosis research<sup>2</sup>, but current progress in combatting this age-old disease has been threatened in the past decades by the serious challenges of Human Immunodeficiency Virus (HIV) and resistance to antibiotics. HIV greatly increases the susceptibility to tuberculosis, such that the latter is responsible for almost 25% of deaths among people infected with HIV, which are also twenty times more likely to contract tuberculosis. The world is experiencing an HIV epidemic that is especially prevalent on sub-Saharan Africa, the region in which tuberculosis is more widespread, so this bacterium-virus synergy presents a difficult hurdle to overcome. Apart from HIV, there are other ways by which an individual's immune system can become compromised, such as immunosuppressive drugs, concomitant disease or quite simply old age, and so it becomes even clearer that poverty inescapably exacerbates the issue<sup>1</sup>.

As for antibiotics, multidrug-resistant strains are becoming increasingly common, appearing in around 10% of tuberculosis infections worldwide. In some particular eastern countries, like India and China, the incidence is much greater. The strongest first-line antibiotics rifampicin and isoniazid are growing ineffective<sup>3</sup> and as a consequence much of the research in the field is turning towards the search of new therapeutic targets.

Research on tuberculosis has been granted a large funding recently, in an effort to eliminate tuberculosis as a major health problem by the year 2050. Some hope lies on a novel vaccine. The current vaccine, Bacillus Calmette-Guérin (BCG) has been in use since 1921. It is the most used vaccine in the world, and is still administered to all newborns in Europe. Although it is capable of preventing tuberculous meningitis in children, its efficacy is severely low in protecting against pulmonary tuberculosis in adults. Moreover, there is a risk of disease for immunodeficient patients and children of HIV-positive mothers<sup>1</sup>.

Current research aims to develop treatments that rely on boosting the host's immune response in a way that is fast and cost-effective. Antibiotic-based therapies can take up to several months, which increases the risk of antibiotic resistance and also struggle with accessibility towards developing countries and isolated populations.

Hopefully, more natural and effective treatments will stem from the study of the host-pathogen relationship, aided by the recent advances in proteomics and systems biology.

## **1.2. Biology of Mycobacterium tuberculosis**

*M. tuberculosis* is a bacillus belonging to the family *Mycobacteriaceae*, within the phylum *Actinobacteria*. It is nonmotile, does not form spores and grows better on high-oxygen environments, hence its perceived preference towards the lungs. Mycobacteria have a particular type of cell wall that is rich in lipoarabinomannan and do not possess an outer cell membrane. Moreover, they are considered Gram-positive but recent electron microscopy associated with tomography showed an outer membrane similar to Gram-negative species. They are acid-fast bacilli, as they resist discoloration by acids. Its particular membrane structure is thought to play an important role in infection. Lipoarabinomannan is analogous to Lipopolysaccharides, a common bacterial antigen known to trigger an aggressive immune response, but many other lipids from mycobacteria were shown to be involved in virulence. Another consequence of their unique cell wall is a resistance to several antibiotics that target cell wall biosynthesis, such as penicillin<sup>4</sup>.

The genus *Mycobacteria* is divided into two groups based on growth rate. *M. tuberculosis* is a slowly growing mycobacterium, with a generation time of between 15 and 20 hours. This slowness is relevant to its virulence because it forces the mycobacteria to be internalized into macrophages, where they encounter the conditions to multiply freely: they are facultative intracellular pathogens.

## **1.3. Host-pathogen relationship**

*Mycobacterium tuberculosis* shows a marked adaptation to the human host, and its resistance to the natural human defense mechanisms suggests a very ancient relationship<sup>5</sup>. The association was thought to have begun upon the first domestications of cattle, in the Neolithic period, and *M. tuberculosis* was thought to have been a human variant of the bovine counterpart *Mycobacterium bovis*, the etiological agent of bovine tuberculosis. Recent studies instead suggest the pathogen's affinity towards humans extends further back in time, to the first African populations that subsequently migrated to Europe, Asia, and the rest of the world around 125 000 years before present<sup>6</sup>.

The known relationship between humans and *M. tuberculosis* actually challenges definition. The complex nature of their interaction is not a linear host-pathogen scenario, for in most cases of infection by *M. tuberculosis*, there is no active disease: the pathogen simply remains dormant inside the host's macrophages, and can evade immune surveillance for years or even decades without causing any symptoms: this is called a latent

infection<sup>7</sup>. The World Health Organization estimates that one in every three people is a carrier for *M. tuberculosis*, while the prevalence of active disease is largely inferior<sup>1</sup>. The bacterium's discreet dormancy state favors its continued existence<sup>8</sup>. It is only when the immune system is weakened that tuberculosis becomes active, and given that this is the only contagious stage of the disease, it can be seen simply as a way for the bacterium to seek a new host when the current one fails.

Only a small percentage (5-10%) of infected individuals develops the active and contagious stage of the disease throughout their lifetime. Another unknown number of people will have contracted and successfully eliminated the pathogen<sup>9</sup>. Given that the vast majority of the carriers of *M.tuberculosis* suffer no negative consequences from it, there are grounds to question the nature of this paradoxical host-pathogen relationship. At the very least, it can be one of commensalism (i.e. the bacterium benefits without harming the host) but it may be considered a case of mutualism, as the presence of the bacteria inside the host's immune cells is likely to stimulate them via production of interferon- $\gamma$  and other inflammatory cytokines, making them more effective in responses to other extraneous pathogens<sup>10</sup>.

The process that defines which path *M. tuberculosis* and indeed which path the disease will take is phagocytosis.

#### **1.4. Phagocytosis**

Phagocytosis is a highly-conserved process by which invasive particles are captured, internalized and neutralized by a host cell. These particles may be inert (for example, debris or latex beads) or living, such as bacteria or small eukaryotes. The term phagocytosis encompasses the steps of internalization, digestion, recycling and exocytosis of debris (Figure 2), although it may also be used to refer exclusively to the internalization step. Many human cells have the capacity to phagocytize, such as neutrophils, dendritic cells, monocytes and macrophages, all of which are considered professional phagocytes<sup>11</sup>.

*M. tuberculosis* is known to act upon phagocytosis: its success as a pathogen relies precisely on its sabotage of the otherwise very efficient phagocytic mechanisms, in a complex molecular tug-of-war.

### 1.4.1 Macrophages

In the context of an infection by *M. tuberculosis*, which commonly enters through the lungs in aerosols, the first host cells it encounters and invades are the alveolar resident macrophages<sup>12</sup>.

The alveolar macrophages in the lungs provide one of the first lines of defense against the invading pathogen. Peculiarly, macrophages are both the antagonist and the habitat of *M. tuberculosis*, thus it is crucial to understand the macrophages' physiology and the equilibrium between the two.

Macrophages can be in either a resting or an activated state. Activation may be triggered by bacterial antigens or inflammatory signals and induce significant morphological and molecular changes on the macrophage, the most relevant of which is the release of pro-inflammatory cytokines<sup>13</sup>. During a pulmonary infection, *M. tuberculosis* is phagocytized by resting alveolar macrophages, which then become activated and promote an inflammatory response<sup>14</sup>. Following activation, the macrophages become primed and generally more aggressive against *M. tuberculosis*, achieving higher levels of intracellular killing of bacteria<sup>15</sup>.

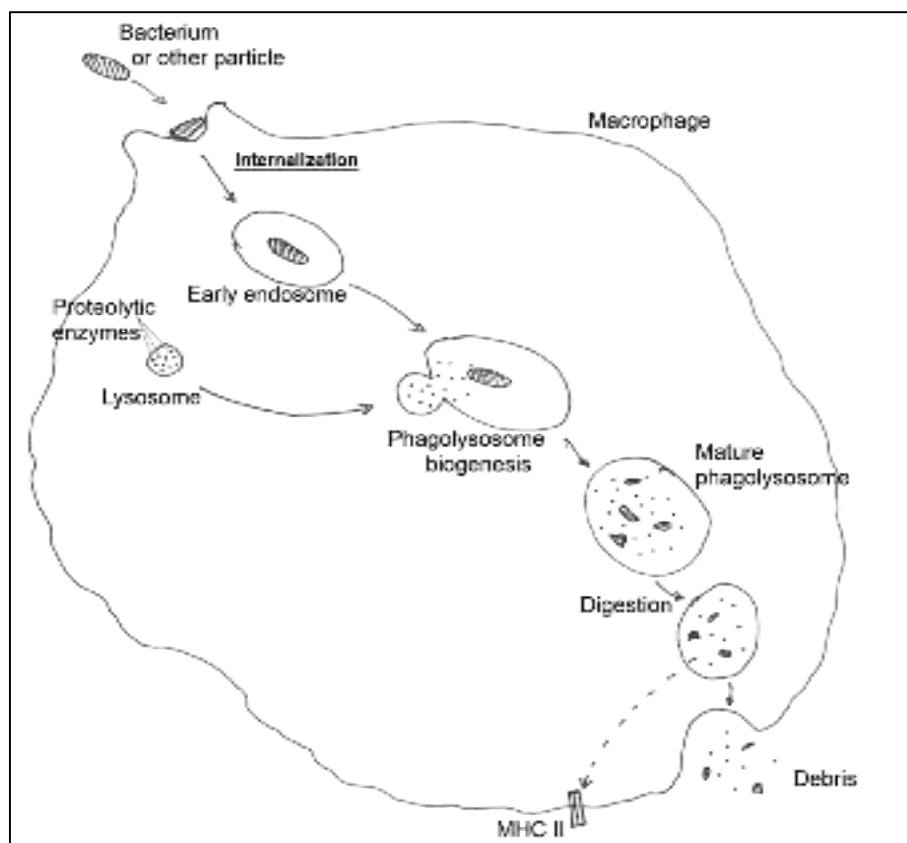
### 1.4.2 Internalization

The first step in phagocytosis is internalization, but many events must take place before the bacteria are truly engulfed by the macrophages. Internalization is mediated by cell surface receptors and ligands, with varying degrees of specificity, which bind to and promote the uptake of *M. tuberculosis*. Identified receptors include complement receptors, scavenger receptors, surfactant protein receptors, C-type lectins, Tumor Necrosis Factor receptors (e.g. CD40) and glycosylphosphatidylinositol-anchored receptors (e.g. CD14)<sup>16</sup>. Toll-like receptors are also relevant in the context of infection, bringing about the activation of the cytokine network and consequently the innate immune response<sup>17</sup>. A relevant and well-characterized ligand of *M. tuberculosis* is lipoarabinomannan, which often contains terminal mannose residues, thus becoming recognizable by the macrophage's CD14 receptors as well as the mannose receptors<sup>18</sup>.

Internalization is an important step in determining the outcome of the host response to the bacilli, as it represents the first physical contact between host and pathogen<sup>19</sup>. However, the results of this contact are not limited to internalization. The activation of receptors, and their type, will ultimately steer the response of the immune system. For example, both macrophages and dendritic cells express T-cell co-stimulants, triggering a

more thorough immune response<sup>17</sup>. If phagocytosis is completely effective, processed *M. tuberculosis* antigens are presented to T cells via the Major Histocompatibility Complex of the macrophages, thus bridging the innate and acquired immune systems and neutralizing the invading pathogen<sup>20</sup>.

The vesicles formed by internalization of bacteria or particles can vary in composition and size depending on what is being internalized, but all fall under the designation of phagosome – a phagocytic endosome. The uptake of bacteria is certainly a complex process. The cytoplasmic membrane at the phagocytic cup and the cytoskeleton require significant structural rearrangement in the early stages of phagocytosis in order to form the pseudopods, engulf the bacteria and encase them in the phagosome. Rather than a simple shift in position of membrane lipids, there is recruitment from the endocytic pathway<sup>21</sup>. As for the cytoskeleton, it has already been demonstrated that *M. tuberculosis* inhibits actin assembly<sup>22</sup>, suggesting that it is capable of interfering with its own internalization by macrophages and with the transport events leading to phagosome maturation.



**Figure 2.** Simplified diagram of phagocytosis.

### 1.4.3. Phagosome maturation

From early to late stages of phagocytosis, the phagosome containing the internalized bacterium suffers a series of maturation events, which consist in partial fusion and fission events with trafficking vesicles. This allows the exchange of luminal and membrane components that will turn the phagosome “older” or more mature, meaning more competent to fuse with lysosomes. This requires organized rearrangement of membrane and cytoskeletal components. After the fusion, the mature phagolysosome presents a very hostile environment for the internalized bacterium: it becomes rich in hydrolytic enzymes, nitric oxides and superoxides<sup>21</sup>.

The internal space of the phagosome, pH-neutral by default, is acidified, by way of strong adenosine triphosphatase activity in its membrane, and the lower pH promotes activation of proteases and their regulators. In standard cases as in phagocytosis of non-pathogenic bacteria or inert particles, this would be sufficient for the invading pathogen to be destroyed, its remains recycled and transported to the Major Histocompatibility Complexes II (MHC II) for antigen presentation.

However, it is upon these processes that *M. tuberculosis* is able to act, by inhibiting several of the host cell’s defenses: the acidification itself, the activation and action of proteases and the fusion of the early endosome with lysosomes. This leads to an incomplete and immature phagosome<sup>23</sup>, which has been referred to as the “four minute phagosome” precisely because it persists in a stage which would be commonly found at the four-minute mark in a standard phagocytic event<sup>24</sup>.

In this stage, *M. tuberculosis* may achieve its latent status, or it may remain active and even multiply inside the host cell. The entry into latency requires a major shift in the gene expression of the *Mycobacterium*, and that state may actually be enforced by the macrophage as a defense mechanism. In either case, the development is the result of biochemical dialogue between pathogen and host<sup>25</sup>.

There has been recent speculation on whether *M. tuberculosis* is capable of escaping the phagosome into the cytosol, in a way similar to *Salmonella sp.* or *Listeria sp.*<sup>26</sup>. While the cytosol would provide a rich environment for the mycobacterium, there is still no conclusive evidence of this escape, and electron microscopy studies have so far failed to replicate the initial results that originated this hypothesis.

#### 1.4.4. Vesicular traffic

Phagocytosis is, as a whole, a matter of intracellular traffic, with each step requiring biogenesis, transport or fusion of vesicles. These vesicles are not exclusively derived from the plasma membrane, as there is also transport to and from the endomembranes of the endoplasmatic reticulum, the trans-Golgi network and of course lysosomes<sup>27</sup>. For there to be an effective neutralization of the captured pathogen, the sequential processes of the endocytic route and phagosome maturation must be adequately coordinated. Rab guanosine triphosphatases (Rab GTPases) are a particularly important group of proteins that regulate membrane identity and direct the traffic and fusion events that play a major role in phagosome maturation<sup>28,29</sup>.

*M. tuberculosis* is able to interfere with these vesicular traffic events and as part of its survival strategy. Through down-regulation of several GTPases, *M. tuberculosis* blocks vesicles bound for the cytoplasmic membrane to form the phagocytic cup and also denies the fusion events leading to phagolysosome biogenesis<sup>30</sup>. Two examples of interest are Rab5 and Rab7. The first is involved in the early stages of phagocytosis, coordinating endocytosis from the plasma membrane and fusion between endosomes<sup>31</sup>. Rab7 acts in the later stages, regulating transport from the early to the late endosomes<sup>32</sup>. It has been shown that *M. tuberculosis* and even *M. bovis* BCG can affect these processes, limiting the expression and recruitment of those proteins even from within the endosome<sup>33</sup>.

Rab GTPases are potential targets for protecting against infection, as they are crucial for the fusion events leading to the mature phagolysosome. Not only that, but some Rab GTPases have been reported to also recruit cathepsins for the later stages of phagocytosis<sup>34</sup>.

#### 1.4.5. Cathepsins and Cystatins

Cathepsins are proteases that exist in all animals, and are more commonly found in lysosomes. There are several classes of cathepsins, based on the aminoacid residue in the enzyme's active center and their targets; most are cysteine proteases, such as cathepsins B, S or L.

The functions of cathepsins are not restricted to phagocytosis; quite the opposite, as their study and applications are very extensive and wide-reaching, including cancer research. They are, however, also studied as potential targets in regulating the host-pathogen interaction in *M. tuberculosis* infections, because they play an important role in the maturation and degradation events on the phagosome<sup>35</sup>. Specifically, they are important

in order to degrade the phagocytized bacteria into small peptides which can be delivered to the Major Histocompatibility Complexes, which in turn trigger the T-cell-mediated immune response<sup>36</sup>.

The majority of cathepsins require a low pH in order to function: many are self-regulated, by folding or autocatalysis that inactivates them while the environment is not sufficiently acidic<sup>37</sup>. If *M. tuberculosis* is able to prevent acidification, it also protects itself from the proteolytic action of cathepsins<sup>38</sup>.

Cystatins are known to be regulators of cathepsin activity<sup>39</sup>; therefore their role in the phagosome environment is also important, as is any interaction they may have with *M. tuberculosis*.

## **1.5. Objectives**

The study of *Mycobacterium tuberculosis* is, as we've shown, inseparable from the study of macrophages, and their interaction is the essential part of the disease of tuberculosis. The laboratorial standard to study this interaction is the infection experiment, which is typically performed as an "end-point experiment", whereby the infections with *M. tuberculosis* are usually carried over long periods of time (3 to 4 hours<sup>40</sup>).

The internalization process itself, via the cell membrane, is a complex process reliant on numerous signaling pathways. The molecular mechanisms behind them must be taking place very early on, certainly before the 3 to 4 hours of contact predicted in most protocols.

We therefore seek to resolve this incongruence between the theoretical and practical aspects of the internalization process on what constitutes an adequate infection time. We aimed to measure the internalization rates with several combinations of different macrophages and different mycobacteria, to obtain fundamental information on how the infection scenario is proceeding since its very early stages.

Using three time points with which to quantify the percentage of infected macrophages, we provide means to determine internalization rates by flow cytometry, and this can be done for several combinations of macrophages and mycobacteria.

We are then able to set a base model to use as a tool in order to screen for host factors influencing internalization. By measuring changes in internalization brought about by protein knockdowns, we are able to study the roles of those proteins by decrease or absence-of-function.

We targeted familiar proteins, with known functions in mycobacterial infections, including cathepsins, cystatins and Rab GTPases. The focus on internalization is an effort to complement the ongoing study of mycobacteria-host interactions in our laboratory, which in the past has experimented extensively with these same targets, measuring the survival of internalized mycobacteria via counting of colony forming units. We hope to uncover a clearer picture of the early regulatory processes happening in phagocytosis by using different techniques, such as flow cytometry and new experimental conditions, such as brief infections with a minimum of 30 minutes.

With these experiments, we also set the stage for *M. tuberculosis* to interfere so that we can study what its influence is on internalization and on the host factors that control it.

To conclude, the goals of this work were:

- ❖ To define the internalization rates of the several macrophages and mycobacteria that are used in tuberculosis research in order to compare their efficiency in the phagocytic process;
- ❖ To describe novel roles of certain proteins with previously unknown functions in the internalization process.
- ❖ To evaluate the influence of mycobacteria, particularly *M. tuberculosis*, on the internalization process.

## **2. Materials and Methods**

### **2.1. Mycobacteria cultures**

*Mycobacterium tuberculosis* strain H37Rv and *Mycobacterium bovis* BCG were cultured in 7H9 medium supplemented with 10% oleic acid albumin dextrose catalase and 0.05% tyloxapol. All manipulations of *M. tuberculosis* were performed in a Biosafety Level 3 environment.

*Mycobacterium smegmatis* (mc2 155) was grown in liquid culture medium containing 4,7g/l Middlebrook 7H9 broth and 5g/l Nutrient broth (*Difco*<sup>TM</sup>), supplemented with 0.05% tyloxapol and 0.5% glucose.

Each of these species of mycobacteria harbors a plasmid constitutively expressing Green Fluorescent Protein (GFP) and a hygromycin resistance as a selection marker. The culture medium contained 50 µg/ml hygromycin (*Sigma-Aldrich*®) in order to maintain selective pressure on the plasmid carriers.

All cultures were kept at 37°C and were subcultured until achieving exponential phase before being used on experiments: 7-10 days beforehand for *M. tuberculosis* and *M. bovis* BCG, but only overnight for the fast-growing *M. smegmatis*.

### **2.2. Cell cultures**

The murine macrophage cell line J774A.1 (ATCC® TIB-67<sup>TM</sup>), henceforth referred to as 'J774', was cultured in Dulbecco's Modified Eagle Medium (DMEM, *Gibco*®) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) glutamine and 1% antibiotic – penicillin-streptomycin (v/v).

The human monocyte cell line THP-1 (ATCC® TIB-202<sup>TM</sup>) was cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) glutamine, 1% (v/v) antibiotic – penicillin-streptomycin, 1% (v/v) HEPES buffer, 1% (v/v) sodium pyruvate and 1% (v/v) non-essential amino acids.

The formulations described above are also referred to as 'complete' DMEM or RPMI media. If antibiotic is not included, they are referred to as 'infection' media.

Primary human monocyte-derived macrophages were obtained from blood from donors. Monocytes were first isolated according to the protocol from *Miltenyi Biotec*, using the MidiMACS kit and LS columns. For the differentiation of monocytes into macrophages, 300,000 cells were seeded on a 24-well plate with 300 µl RPMI medium per well and

allowed to adhere to the plate bottom. After 2 hours, 300 µl of RPMI with 20% (v/v) FBS, 2% (v/v) penicillin-streptomycin, 2% (v/v) sodium pyruvate, 2% (v/v) β-mercaptoethanol and 0.6 µl of macrophage colony stimulating factor (M-CSF 1000x) for a final concentration of 20 ng/ml in each well. After approximately 72 hours 600 µl of complete RPMI containing 20 ng/ml M-CSF were added. At the seventh day the macrophages were collected via resuspension with a trypsin-EDTA solution and were ready to be plated for the infection experiments.

The HEK 293T cell line (ATCC® CRL-11268™) was used to amplify the viral constructs of the lentiviral knockdown libraries. They were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic – penicillin-streptomycin.

All of the above were cultivated at 37°C in an atmosphere containing 5% carbon dioxide.

## **2.3. Infection protocol**

### **2.3.1. Preparation of macrophages**

Cells were seeded onto 24 or 12-well plates (approximately 500,000 cells per well) with the appropriate culture medium (complete DMEM or RPMI).

THP-1 cells were plated for 24 hours in contact with 20 nanomolar phorbol 12-myristate 13-acetate (PMA, *Sigma*©), which differentiates the monocytes into macrophages, followed by 24 hours of *resting* in complete culture medium without PMA.

J774 macrophages were plated 24 hours in advance at half the desired concentration, so that they would multiply to the intended final concentration on the day of the experiment.

(See section 2.3.5 for alternate protocol)

### **2.3.2. Preparation of bacteria**

Disassembly of bacterial aggregates is necessary as large clumps may have a strong influence on phagocytosis. The steps involved are: washing with Phosphate Buffered Saline (PBS) at 1x concentration; resuspension in infection medium (DMEM or RPMI medium according to the cells to be infected); passaging several times with a 5ml syringe and a 21G needle; a 5-minute ultrasound bath followed by a short centrifugation at a low force: 1 minute, 300 G to pellet any remaining aggregates. After the last centrifugation, only the supernatant was collected.

By reading the optical density at 600 nanometers, bacterial suspensions were adjusted for concentrations corresponding to multiplicities of infection (MOI) of 1, 10 and 100 bacteria per macrophage. This calculation was based on the standard relationship between optical density (OD) and concentration of bacteria:

$$\{\text{OD } 0.1 \Leftrightarrow 1 \cdot 10^7 \text{ bacteria/ml}\}$$

### **2.3.3. Infection**

The contact between macrophages and bacteria was initiated by removing the cell culture medium and replacing it with the bacterial suspension in infection medium.

To achieve the three different lengths of infection, the samples were infected at different times, so that afterwards they could all be stopped and processed at the same time. Considering the stopping time of the infection as the “time point –zero”, samples were infected at –4 hours, –1 hour and –30 minutes. Between manipulations, samples were incubated at 37°C in an atmosphere containing 5% carbon dioxide.

(See section 2.3.5 for alternate protocol)

### **2.3.4. Processing samples after infection**

While remaining adhered to the plate, the cells were washed three times with warm PBS, thus removing the mycobacteria. Cells were then dislodged using 400µl trypsin with a 5-minute incubation at 37°C. Afterwards the cells were resuspended in 1 ml of infection (antibiotic-free) DMEM or RPMI medium to neutralize the proteolytic action of trypsin. This was followed by centrifugation for 10 minutes at 450 G. Fixation was then performed with 4% paraformaldehyde, for 30 minutes at room temperature, and the samples were then finally washed and resuspended twice in PBS with 1% fetal bovine serum and kept at 4°C until they were used for flow cytometry.

(See section 2.3.5 for alternate protocol)

### **2.3.5. Alternate protocol for screening of knockdown cells**

The experiments regarding protein knockdown were restricted to the time point of 1 hour and the MOI of 10 mycobacteria per macrophage. This was necessary to accommodate the large number of samples – 181. The values were chosen because they present a more moderate burden on the macrophages and at the same time allow for more expressive differences across all cell types.

The experimental handling itself differs from the previous sections in that it was performed on 96-well microplates in proportionally smaller volumes. The infection and post-infection protocols were otherwise identical to those described before.

### **2.3.6. Confirming the absence of extracellular bacteria**

Extracellular bacteria adherent to the macrophage, but not internalized, can compromise the readings of flow cytometry. To determine whether the post-infection protocol was effective in separating bacteria from the macrophages, a parallel experiment was conducted. Wild-type J774 macrophages were first plated in two different conditions: either onto glass coverslips placed on the plate wells (*group A*) or onto the plate wells' bottom (*group B*). Both samples were infected with *M. smegmatis* with a MOI of 100 for 1 hour. Group B was subjected to the entire post-infection protocol, while group A was not. All samples were stained with 25µg/ml of ethidium bromide at the end of the infection. Extracellular mycobacteria stained red, while internalized mycobacteria emitted only green fluorescence from GFP.

Samples were placed on microscope slides and observed under a Zeiss Axioskop 40 fluorescence microscope (Göttingen, Germany) using a FITC filter. Besides qualitative observations of the relative abundance of red mycobacteria around the macrophages, quantification was also made: from a random sampling, we determined the percentage of macrophages with neighboring red mycobacteria (Figure 12).

## **2.4. Protein knockdown**

### **2.4.1. Knockdown lentiviral libraries**

Two different lentivirus libraries were used for the protein knockdowns. The first, obtained from The RNAi Consortium, contained hits from a previous screen performed in our laboratory, whereby knockdowns of several membrane-associated proteins were shown to affect intracellular survival of mycobacteria in macrophages (Carmo, N. *et al*, unpublished data). The second library includes target siRNAs for several cathepsins and cystatins and was built in partnership with Professor Luís Moita from the Institute of Molecular Medicine (Lisbon, Portugal) and by using MEROPS, a protease database from the Sanger Institute (Cambridge, United Kingdom).

Both libraries were contained in 96-well microplates with pLKO.1 plasmids which coded for a different short specific RNA hairpin, lentiviral proteins and the resistance to puromycin as a selection marker. Each hairpin has a specific target on the host's messenger RNA.

#### **2.4.2. Building a stable THP-1 knockdown cell line**

Part of this work was performed on knockdown THP-1 cells. Applying the lentiviral library to wild-type THP-1 cells is a multi-step approach.

The plasmids contained in the library were transfected into *Escherichia.coli* via electroporation. After overnight growth at 37°C with mixing (220 revolutions per minute), the DNA was extracted, in plasmid form, from the bacteria, using a PureLink™ Quick Plasmid Miniprep Kit and its respective protocol (*Invitrogen*).

The DNA was then quantified using picoGreen, a fluorescent nucleic acid stain, and its corresponding protocol (*Life Technologies*). The plasmids were co-transfected onto HEK 293T cells together with additional lentiviral proteins. These provide the necessary materials for the assembly of mature viral particles. The HEK 293T cells then began producing these particles containing the desired DNA construct, whilst being selected for transfection via the addition of 5µg/ml puromycin to the growth medium.

After 24 hours, the virus particles were passaged into DMEM containing 30% fetal bovine serum and collected 24 hours later. These virus particles were used to infect wild-type THP-1 monocytes, on a 96-well microplate. The monocytes were maintained in culture medium with 5 µg/ml puromycin and passaged or split into new microplates to obtain larger numbers of cells with a stable knockout.

Before infection, THP-1 cells were counted, normalized and plated on a fresh 96-well microplate at a concentration of 375 000 cells/ml.

The negative controls for the protein knockdowns were infected with viruses that contained an RNA hairpin with no target in the human genome, meaning that it would not bind to any messenger RNA of the host cell nor silence protein production. These hairpins contain a scrambled sequence and are called shScramble.

#### **2.4.3. Confirming protein knockdown via Western Blot**

A stable line of knockdown THP-1 monocytes were denatured at 95°C for 5 minutes in the presence of Laemmli buffer (*Bio-rad laboratories*©) and loaded onto 12% sodium dodecyl sulphate polyacrilamide gels. Electrophoresis was set at 200 Volt for 1 hour. Proteins were transferred onto nitrocellulose membranes with an electrical intensity of 30

Volt overnight at 4°C. On the following day, membranes were blocked for 1 hour at 20°C with a blocking buffer containing TBS-T (Tris-buffered saline with 0.05% (v/v) Tween 20) plus 5% (w/v) bovine serum albumin. Membranes were then incubated with primary antibodies diluted in TBS-T plus 1% (w/v) bovine serum albumin for 2 hours at 20°C. After three washes with TBS-T, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies for 1 hour at 20°C. After three more washes with TBS-T, the membranes were stained for 5 minutes using the chemiluminiscent reagent Luminata Crescendo Western HRP Substrate (*Milipore*®). Chemiluminescence was acquired with ChemiDoc XRS+ system (*Bio-rad laboratories*®)

## **2.5. Data acquisition and analysis**

### **2.5.1. Quantification of internalization by flow cytometry**

Samples were read on a Milipore EasyCyte H6 Guava flow cytometer, using its proprietary InCyte software, as well as the freeware Flowing Software (Turku Centre for Biotechnology, Finland). Fluorescence was measured using the excitation and emission wavelengths of 488 and 525 nanometers with a 75 milliwatt blue laser. The macrophage population was gated based on forward and side scatter values, in such a way that extracellular bacteria and debris were excluded from the event count. 5000 events from inside that gate were acquired with an average rate of 260 events per second.

The mycobacteria constitutively expressed Green Fluorescent Protein, while the macrophages did not, and so internalization was measured by quantifying the percentage of total macrophages that emitted intense green fluorescence (Figure 3). Uninfected macrophage samples were used as negative controls to calibrate the readings of green fluorescence intensity for each experiment.

Using the values from different time points, the kinetics of internalization were determined and plotted using a custom function in R software. Data points were connected to create a growth-like profile for the internalization rates, which include the average values as well as the standard deviation for each data point (Figures 4, 5 and 6). All experiments were performed in triplicate.

### **2.5.2. Statistical treatment**

The software SigmaPlot 12.0 (*Systat Software Inc.*) was used for statistical analysis. Three-way ANOVAs were performed to evaluate the global differences in internalization

between different macrophages and different bacteria by taking into account the variables of time and MOI. Pairwise differences between types of macrophages and types of mycobacteria were evaluated using the Holm-Sidak method. Differences were considered significant for  $p < 0.05$ .

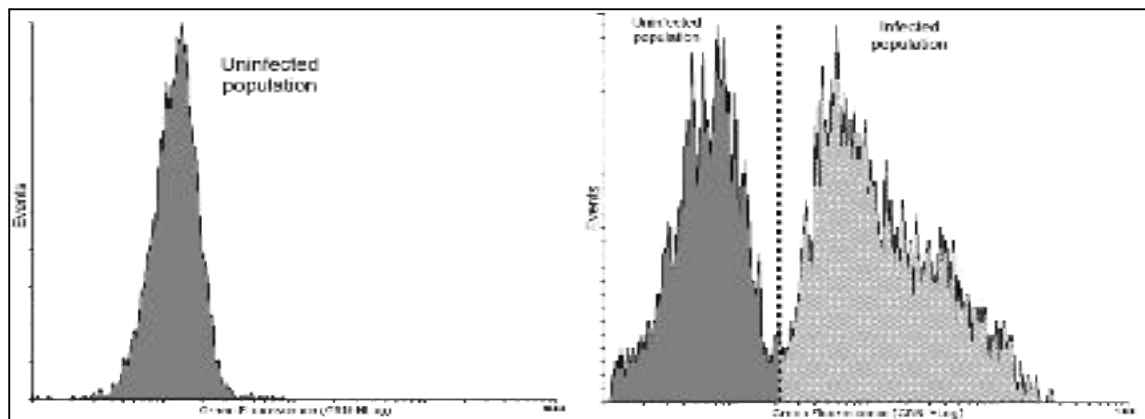
### **2.5.3. Labelling hits in the knockdown screen**

To evaluate whether the effect of knocking down the selected proteins was relevant for internalization, a criterion had to be fulfilled: two or more hairpins silencing each protein had to cause a difference in internalization of at least 2.5 times the standard deviation of the controls. This difference must necessarily have the same direction (positive or negative) among the considered hairpins.

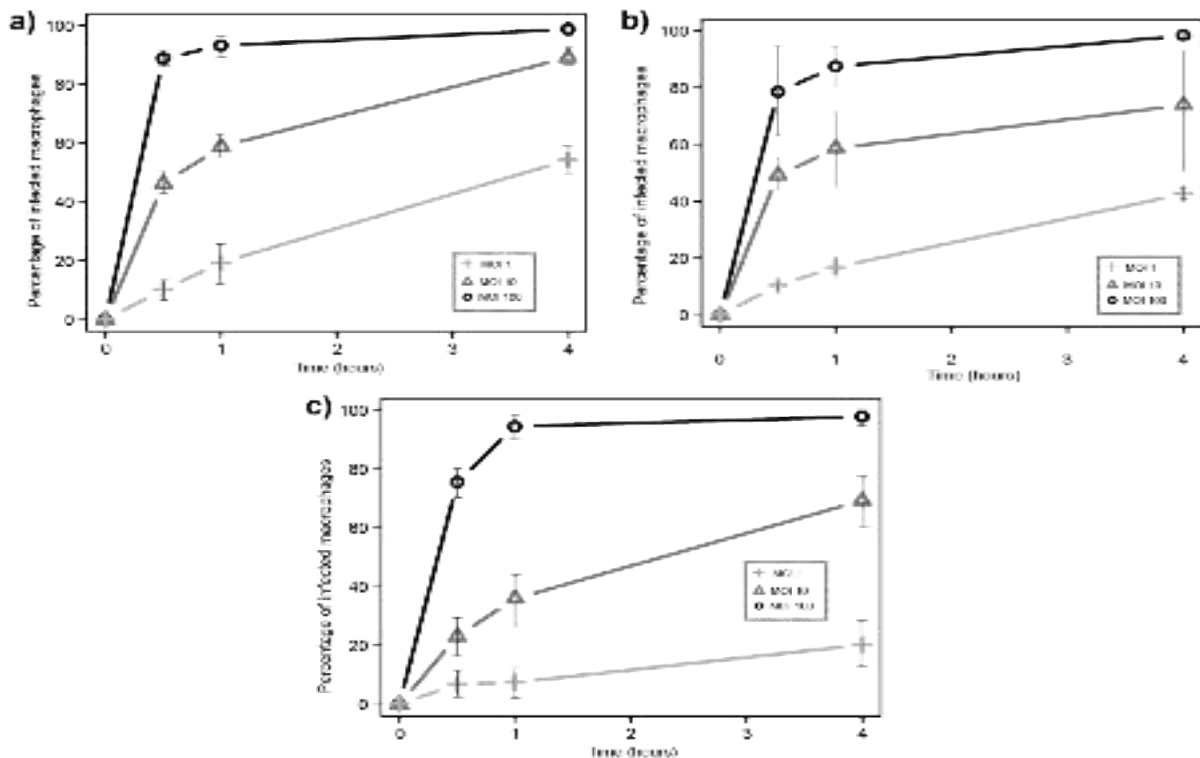
### 3. Results

#### 3.1. Rates of internalization

For this part of the experiment, the variables tested were: type of macrophage, type of mycobacteria, time and multiplicity of infection (MOI).

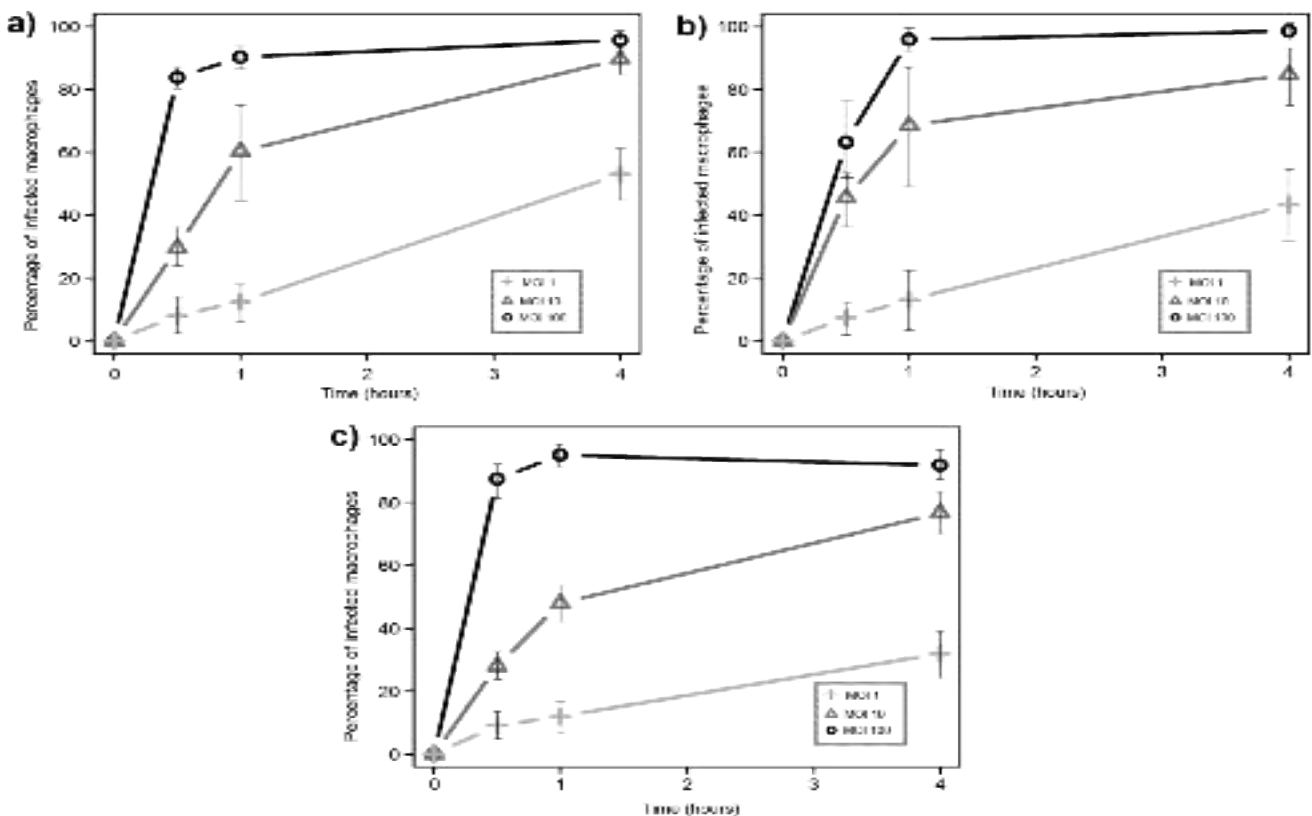


**Figure 3.** As described in section 2.5.1. Example populations of uninfected and infected macrophages, as obtained by flow cytometry and represented in histograms. **Left:** Uninfected control sample of THP-1 macrophages. **Right:** Sample of THP-1 macrophages infected with *Mycobacterium smegmatis* for 4 hours with a multiplicity of infection of 1. X axis represents intensity of green fluorescence in a logarithmic scale. Y axis represents number of events. The two samples are taken from the same experiment. With flow cytometry software we calculated the percentage of total events that are in each population.



**Figure 4.** Internalization by J774 mouse macrophages of different mycobacteria: **a)** *M. smegmatis*; **b)** *M. bovis* BCG; **c)** *M. tuberculosis*. The X-axis represents time, while the Y-axis represents percentage of infected macrophages. Each data point includes the average and standard deviation of three replicate experiments.

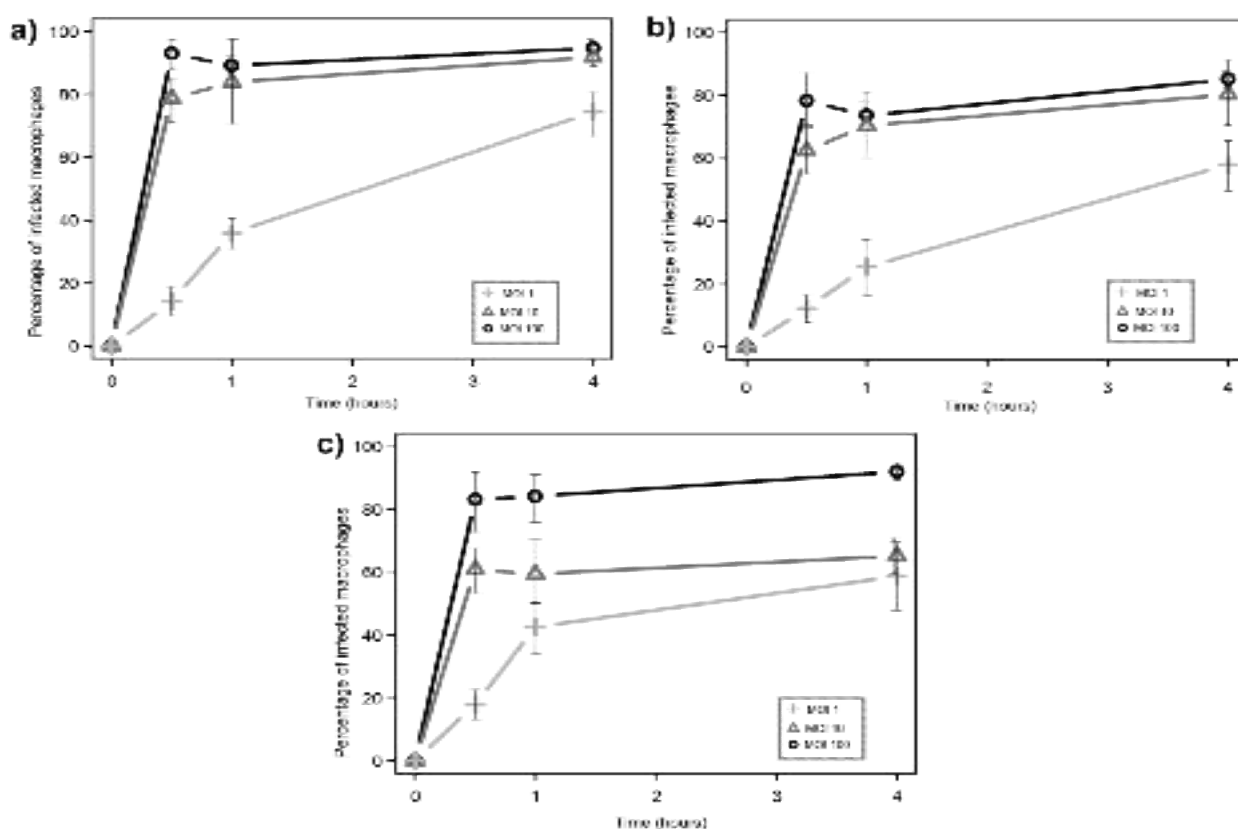
On Figure 4, detailing the internalization profiles of J774 mouse macrophages, we can see a marked difference between multiplicities of infection, as they maintain some graphical distance, with no overlap. With a MOI of 100 all of the mycobacteria achieved an almost ubiquitous infection of the macrophages, which is to be expected from such a heavy bacterial burden. The avirulent *M. smegmatis* is not only internalized faster, but also to a greater extent, reaching a higher percentage of infected macrophages than the other mycobacteria (although these differences were deemed non-significant by the Holm-Sidak pairwise comparison,  $p > 0.05$ ). *M. tuberculosis* appears to be internalized at a lower rate than the other mycobacteria during the first hour of contact with macrophages, which is particularly evident in the curve corresponding to a MOI of 1, which barely reaches 20% at the end point.



**Figure 5.** Internalization by THP-1 macrophages of different mycobacteria: **a)** *M. smegmatis*; **b)** *M. bovis* BCG; **c)** *M. tuberculosis*. The X-axis represents time, while the Y-axis represents percentage of infected macrophages. Each data point includes the average and standard deviation of three replicate experiments.

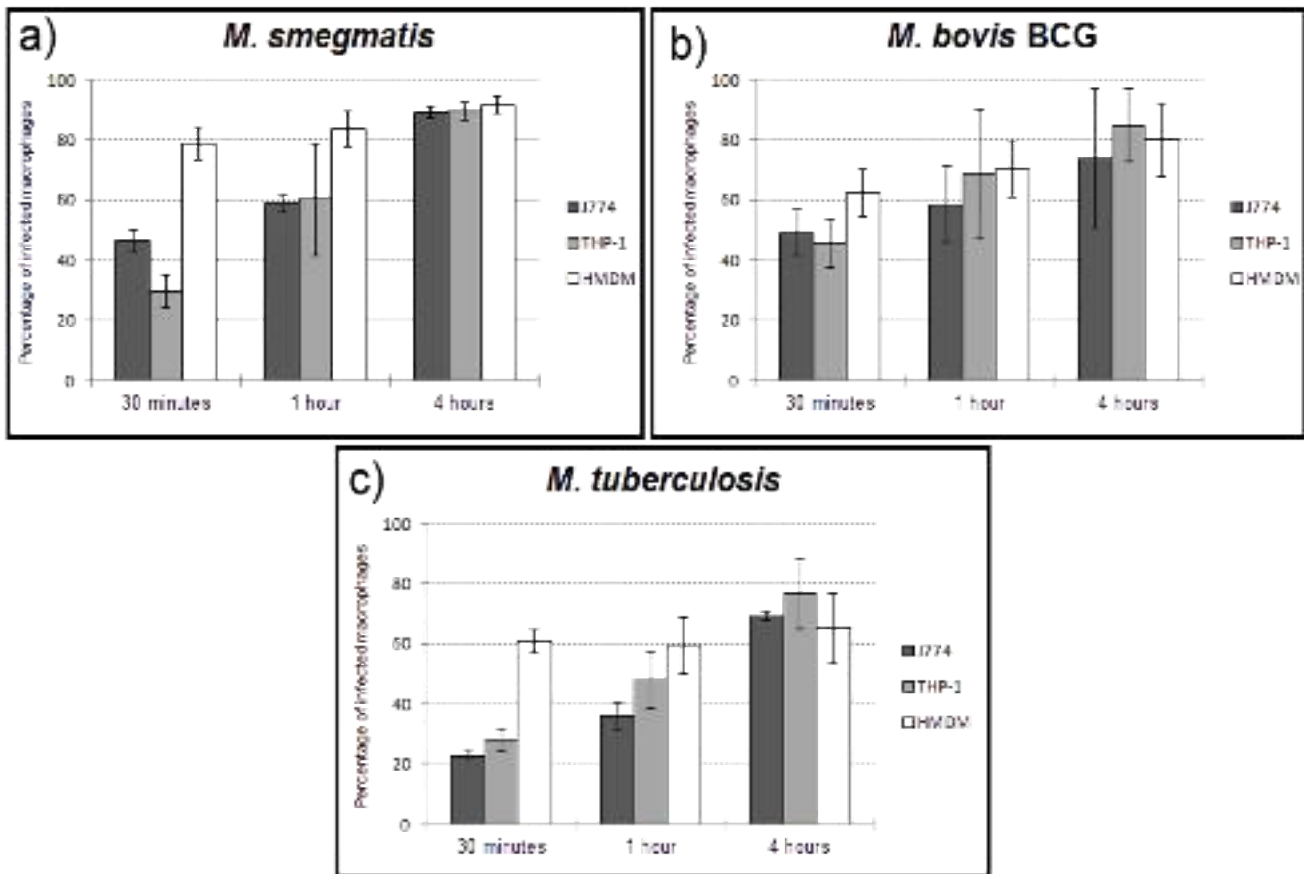
In Figure 5 we see no great difference between the species of mycobacteria, other than that *M. smegmatis* is slightly more internalized than its virulent counterparts. As will become evident in other experiments, the MOI of 100 tends to fall on extreme values, that is, extremely close to 100% of infected macrophages. *M. tuberculosis* shows a somehow

slower internalization when present in lower numbers (i.e. with a MOI of 1 or 10), indicated by a less steep curve, although it is able to recover the difference by the end of the experiment. Nonetheless, there is substantial difference in the kinetics of internalization of *M. tuberculosis* compared to the other mycobacteria at the early stages. Global differences were evaluated with the Holm-Sidak method, which determined the differences between *M. tuberculosis* and other mycobacteria were significant ( $p < 0.05$ ). The apparent decline on the 4 hour mark of MOI 100 in Figure 5.c is buffered by the standard deviation and is not likely to represent an actual decrease in internalization.



**Figure 6.** Internalization by human monocyte-derived macrophages of different mycobacteria: **a)** *M. smegmatis*; **b)** *M. bovis* BCG; **c)** *M. tuberculosis*. The X-axis represents time, while the Y-axis represents percentage of infected macrophages. Each data point includes the average and standard deviation of three replicate experiments.

Human monocyte-derived macrophages (HMDM), represented in Figure 6, appear to reach a plateau earlier on, as we can see a shorter difference between the time points of 1 hour and 4 hours, which is all the more evident in the case of *M. tuberculosis*. The short decrease observed between the first and second time points is odd, although the values increase again towards the 4 hour mark; this will be addressed in the *Discussion*.



**Figure 7.** Internalization efficiency of different macrophages, sorted by species of mycobacteria. Only the MOI of 10 is represented.

These profiles of internalization over time contain valuable information on the hardly-known kinetics of internalization, particularly during the first hour. However, comparison of the phagocytic efficiency of different macrophage types requires a different graphical approach.

The results in Figure 7 show that human monocyte-derived have great phagocytic efficiency, largely exceeding its counterparts in the first hour (significant for HMDM *versus* J774,  $p < 0.05$ ), although they seem to reach peak activity before the fourth hour. J774 and THP-1 cells cannot be said to differ significantly between them in these measurements (differences non-significant,  $p > 0.05$ ). A relevant observation is that *M. tuberculosis* is here shown to be internalized to a lesser extent, although it was present in the same concentrations as the other mycobacteria at the time of infection (significant,  $p < 0.05$ ). At the same time, there is an inverse correlation between virulence and percentage of internalization; we observe the latter is greater with *M. smegmatis*, lower with BCG and the lowest with *M. tuberculosis*, a pattern which is more easily observed in HMDM.

### **3.2. Effects of selective protein knockdown on internalization**

The samples were based on two different lentiviral libraries and the objective in this screen was to identify 'hits' from among the knockdowns: proteins that when silenced induced a significant change, either positive or negative, in the internalization capacity of macrophages. The controls, in this context, are the macrophages that were transfected with shScramble.

The overview of the results from the screens can be seen in Figures 9 and 10, which include the knockdown samples from each lentiviral library. The vast majority of knockdowns caused decreased internalization. Applying the criteria for what would count as a *hit* (described in section 2.5.3) we identified a total of 26 hits, which corresponded to 26 proteins with a presumed role in internalization. Only one of the hits was exclusive to infections with *M. smegmatis*, while three were exclusive to *M. tuberculosis* (Figure 13).

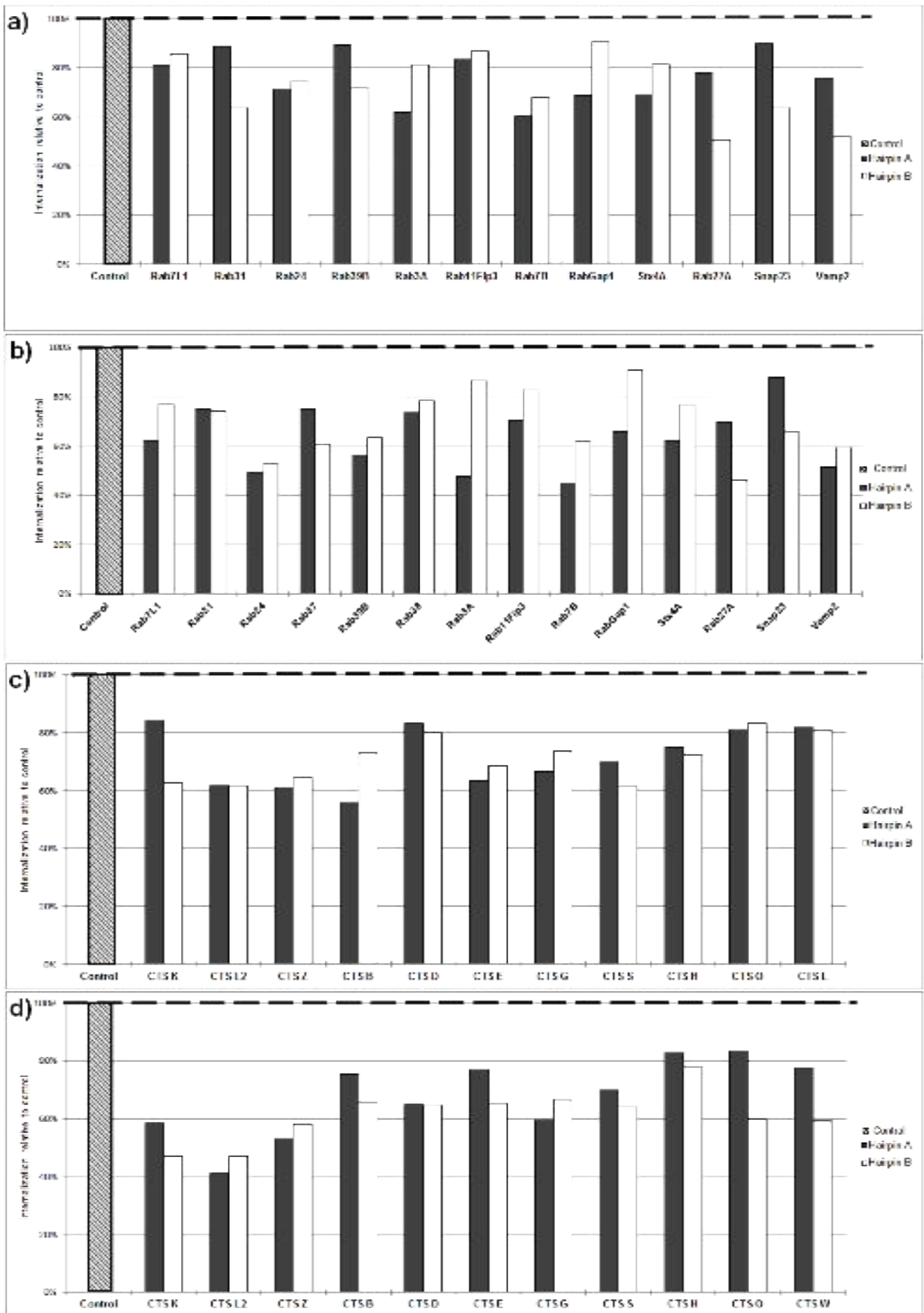
In this screen we identified mainly Rab GTPases and cathepsins, in addition to the individual cases of SNAP23, VAMP2 and STX4A. All of the identified hits had a negative influence on internalization, indicating that they are likely positive regulators of internalization. The overall pattern was rather similar between the two species of mycobacteria, despite the difference in baseline internalization rates between them. The average reduction, however, is higher in *M. tuberculosis* (33% reduction vs. 27.5% for *M. smegmatis*).

### **3.3. Evaluating protein knockdown of cathepsin L**

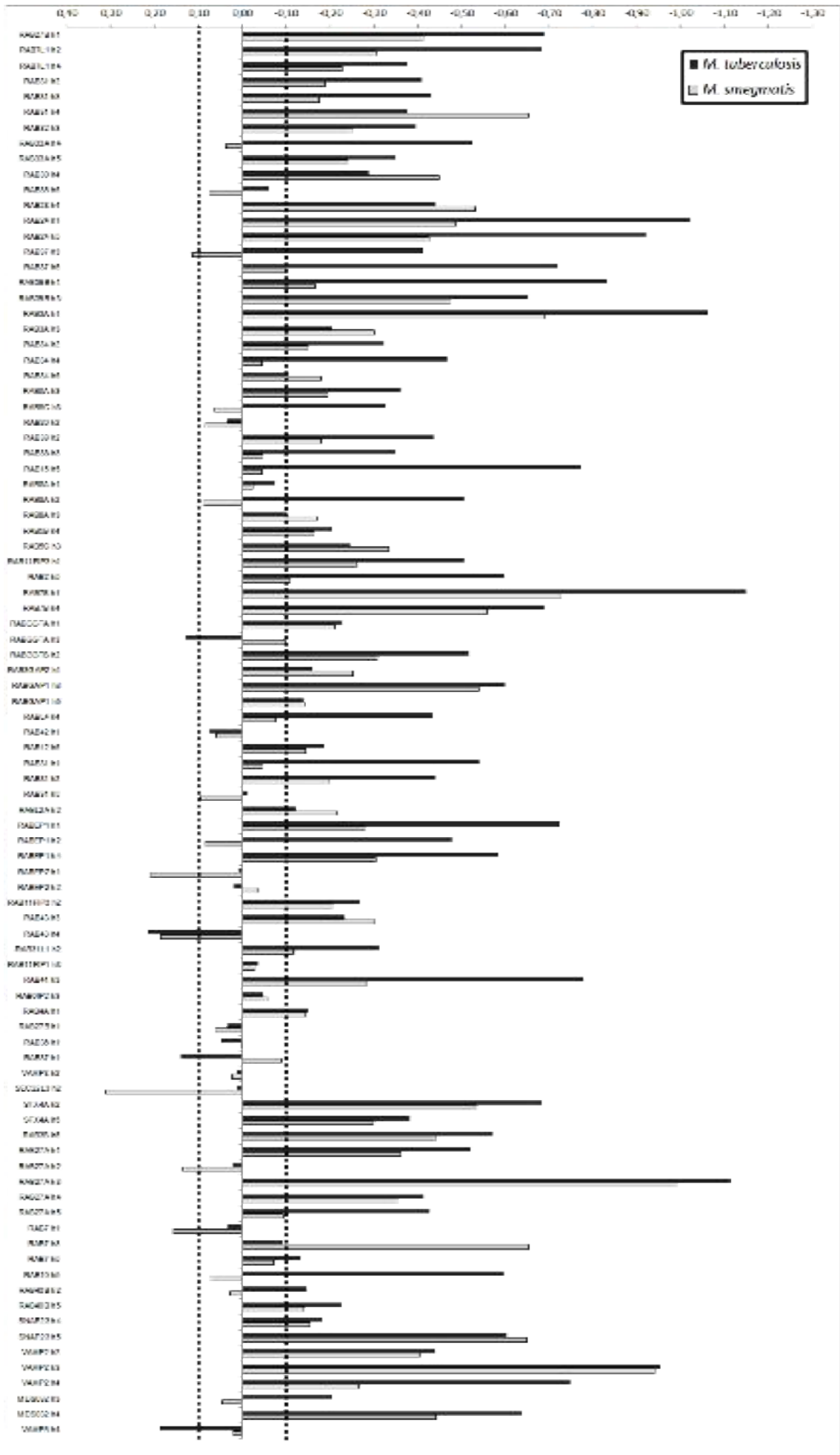
As an example of the effectiveness of the lentiviral library-based knockdown method, we measured cathepsin L production via Western Blot (Figure 11). Silencing of protein production was extremely effective which allows for some safety in discussing the knockdown-based screen.

### **3.3. Inside or outside the macrophage?**

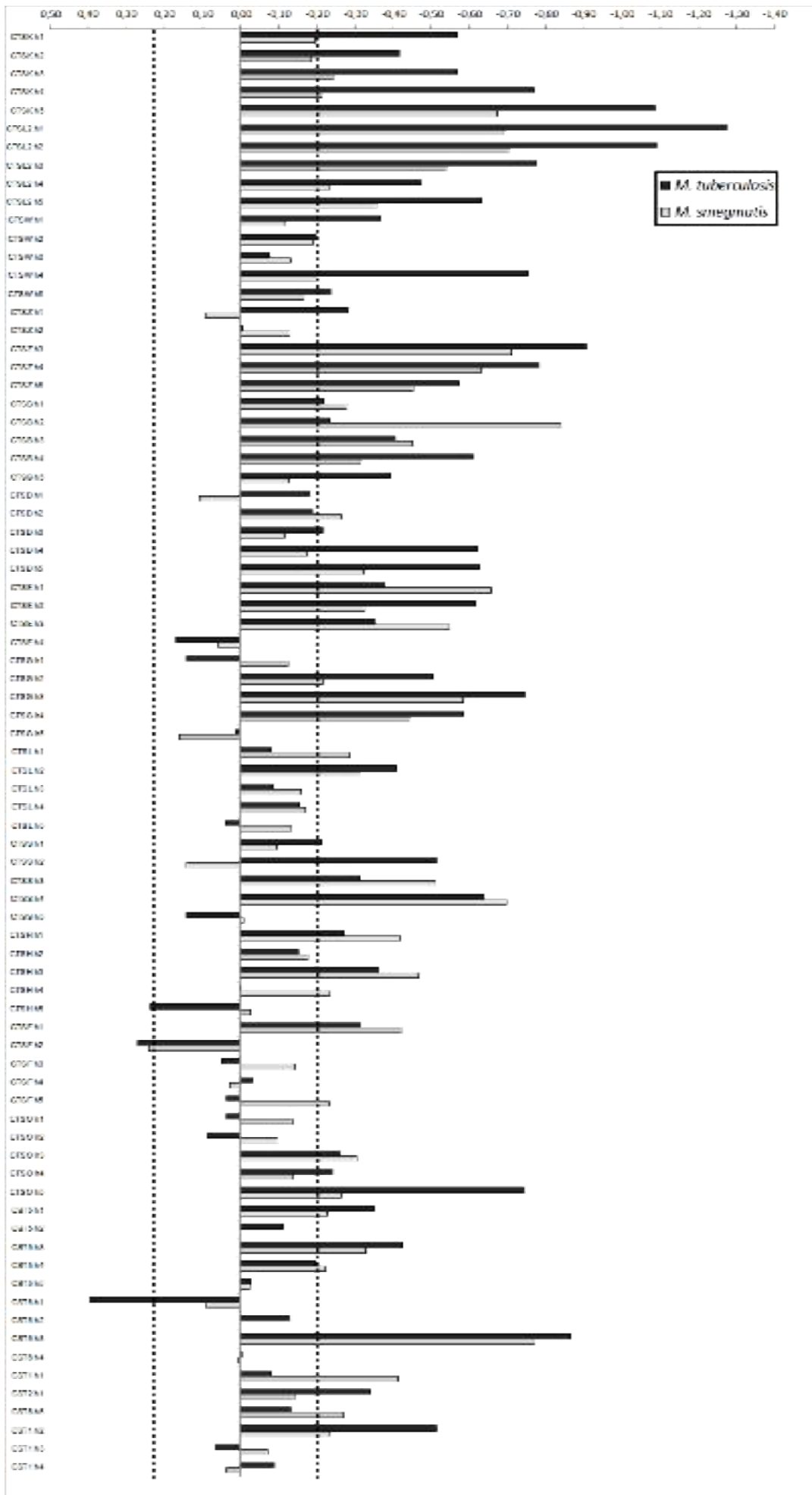
Applying the method described in section 2.3.6, some samples of infected macrophages were observed directly after the infection period (Figure 12.a), while others were subjected to every step of the post-infection protocol (Figure 12.b). From a random sampling, we calculated the percentage of macrophages with adjacent extracellular mycobacteria (Figure 12.c). The washed samples were completely clear of adherent mycobacteria, while 58% of the macrophages in the unwashed samples were not.



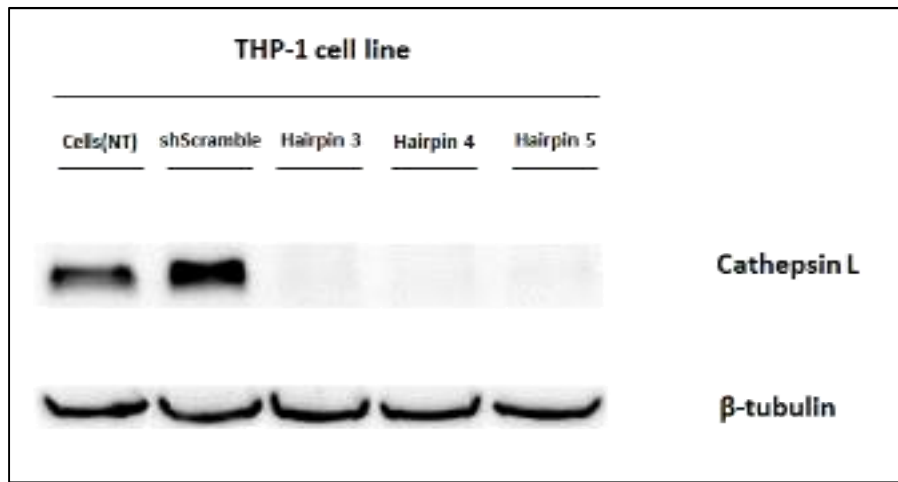
**Figure 8.** Efficiency of internalization for knockdown THP-1 macrophages infected with: **a)** and **c)** *M. smegmatis*; **b)** and **d)** *M. tuberculosis*. On the X-axis lay the labels of the proteins that were knocked down, on the Y-axis is the efficiency of internalization relative to the shScramble (no knockdown) control.



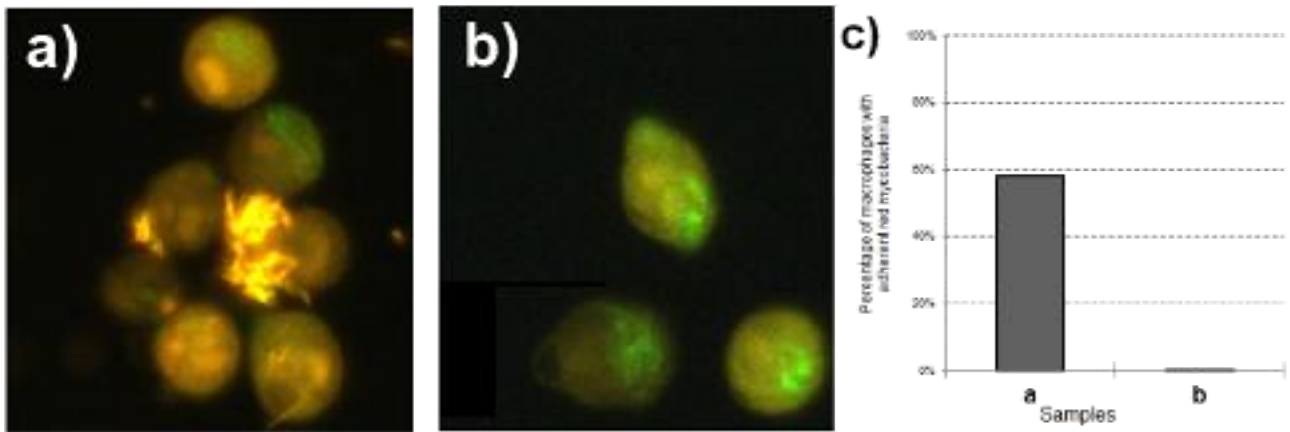
**Figure 9.** Overview of the screen performed on vesicular traffic proteins (the first lentiviral library). The different interference hairpins are sorted in the vertical axis. The horizontal axis expresses  $\log_2(\text{internalization} / \text{control})$ . Note that the horizontal axis is flipped so that negative values are on the right. The vertical lines represent the threshold of 2.5 times the standard deviation of controls, above which the knockdowns were deemed relevant.



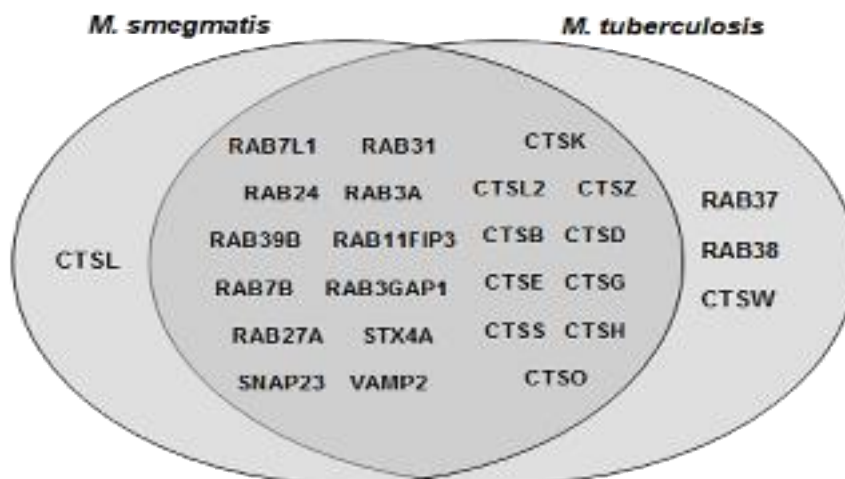
**Figure 10.** Overview of the screen performed on cathepsins and cystatins (the first lentiviral library). The different interference hairpins are sorted in the vertical axis. The horizontal axis expresses  $\log_2(\text{internalization} / \text{control})$ . Note that the horizontal axis is flipped so that negative values are on the right. The vertical lines represent the threshold of 2.5 times the standard deviation of controls, above which the knockdowns were deemed relevant.



**Figure 11.** Western Blot for cathepsin L quantification in knockdown THP-1 monocytes. The constitutively expressed  $\beta$ -tubulin is the positive control, and non-transfected cells (NT) are the negative control. All three of the represented hairpins caused a severe visible decrease in cathepsin L levels. Results generated by Joana Bugalhão, Nuno Carmo and David Pires in our laboratory.



**Figure 12.** Fluorescence microscopy images of macrophage samples infected with *Mycobacterium smegmatis*, MOI 100, 1 hour. In **a)** the sample was stained with ethidium bromide and immediately observed under a fluorescence microscope. In **b)** the sample was stained and subjected to the entirety of the post-infection protocol. **c)** Bar plot representing the quantification of adherent extracellular mycobacteria in the original samples from where images **a)** and **b)** were taken. Images captured with a Zeiss Axioskop 40 microscope and its software AxioVision 4 using a FITC filter.



**Figure 13.** Venn Diagram illustrating which hits in the screen were identified in infections with *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*

## **4. Discussion**

### **4.1. Experimental factors influencing internalization**

The greatest differences in internalization stemmed, not surprisingly, from the variables of time and multiplicity of infection (MOI). The maximum values of 4 hours and 100 bacteria per macrophage, respectively, represent a very heavy burden on phagocytic cells. The MOI of 100, in particular, is a far cry from the actual values in an actual infection by *M. tuberculosis* via aerosols. The goal of using such hyperbolic numbers was to test and calibrate the techniques and models for internalization, and not to mimic real-life conditions.

Our protocol and data treatment allow us to accurately distinguish between low and high MOIs, and between short and long infections, suggesting it can be robust enough to detect other factors influencing internalization.

However, the method was not without its weaknesses. The flow cytometer reads the fluorescence of each individual cell as a whole, and so cannot specifically pinpoint nor count the bacteria inside it. This limitation raised a serious issue, in that the observed fluorescence could also be emitted by non-internalized bacteria that were only clinging to the macrophage cell membrane. We resolved this issue by confirming, by fluorescence microscopy, that the post-infection protocol was quite efficient in clearing macrophages of adherent bacteria (Figure 12).

Our method has then shown to be adequate for the study we undertook, and assured us that by measuring the percentage of infected macrophages in the population through green fluorescence in flow cytometry, we achieve an accurate portrayal of the efficiency of internalization in the sample. With this reassurance we are then able to properly compare and discuss our results.

### **4.2. Internalization of mycobacteria occurs rapidly**

The uptake of *Mycobacterium tuberculosis* took place faster than expected. While the commonly used infection protocols recommend 3 to 4 hours of contact between macrophages and mycobacteria<sup>40</sup>, we were able to measure internalization in as much as 83% of the macrophages in only 30 minutes, a value obtained for human monocyte-derived macrophages infected with *M. tuberculosis* with a MOI of 100 mycobacteria per macrophage. Overall, considering a MOI of 10 – which is very frequently used – our results indicate that 1 hour would be sufficient to efficiently infect the macrophage culture.

The abbreviation of the infection procedures can save time and potentially allow for a more accurate study of the early steps of phagocytosis. For there to be effective internalization after only 30 minutes, clearly the interaction between macrophages and mycobacteria takes place very early on<sup>41</sup>. If we are to allow for 4 hours of infection, we will have the macrophages reach a more advanced stage of immune response, both regarding phagocytosis and activation. A more prolonged mycobacterial burden on the macrophages' cell biology and gene expression may muddle observations regarding, for example, membrane receptors or endocytic routes<sup>42</sup>. Thus we propose that keeping the infection experiments "short and sweet" will yield better results and potentially pave the way for discoveries on the critical initial moments of phagocytosis.

#### **4.3. Human monocyte-derived macrophages are efficient phagocytes *in vitro***

From the collected data, primary human monocyte-derived macrophages (HMDM) stand out as extremely competent in internalizing mycobacteria, particularly when analyzing the early time points in the infection experiments. In terms of numbers of infected macrophages, HMDM ranked consistently higher than THP-1 and J774 macrophages at the 30 minute mark. This remarkable quickness marks them as ideal for experiments shorter than 1 hour. This statement, however, is mellowed by the fact that global differences between macrophages were only significant for HMDM *versus* THP-1 and not J774 (Holm-Sidak method,  $p < 0.05$ ).

In some samples with high multiplicities of infection, HMDM showed a strange downward slope between the first and second time points (Figure 6). It is unlikely that this represents a real decrease in the internalization kinetics, as the internalization by macrophages is irreversible and thus should only increase with time<sup>18</sup>. A probable explanation is that of experimental artifact. Infections were asynchronous: in order to obtain different durations for infection, contact between macrophages and mycobacteria was initiated, rather than ended, at different times. This means the samples for the shortest duration were the last ones to be infected. The bacteria for all the samples were necessarily prepared from a single batch at the same time and while the waiting time would not be sufficient for them to multiply significantly, the fetal bovine serum in the infection medium in which they were suspended may have had some interference. Mycobacteria may have become tagged or opsonized by the serum's constituents and that would have facilitated binding and internalization. This holds true for all experiments, but the fact that such a slope

was only apparent for HMDM, it can be inferred that they are more sensitive to opsonization.

#### **4.4. Internalization is dependent on the species of mycobacteria**

The differences between different species of *Mycobacterium* are worthy of note, as they occur for every type of macrophage we tested. Figure 7 represents the percentage of internalization for each type of macrophage, so that the three *Mycobacterium* species are more easily comparable. At the 4 hour mark, representing the end of the infection period, we see a step-like profile across all cell types: *M. smegmatis* is internalized at a higher level, followed by *M. bovis* BCG and *M. tuberculosis*. This difference between mycobacterial species was already empirically expected but not yet confirmed, and the results are nonetheless interesting and open up new avenues of discussion.

The decreasing order of internalization among the tested mycobacterial species is coherent with the growth rate of each. Faster-growing mycobacteria were internalized at a higher rate and to a higher extent, and the reverse is also true (Figure 7). This leads us to question whether there may be bacterial growth during the experiment, tainting the results. This hypothesis can be dismissed for *M. bovis* BCG and *M. tuberculosis*, for which the doubling time greatly exceeds the 4-hour maximum duration of the experiment. *M. smegmatis*, on the other hand, has a doubling time of approximately 3 hours and could present a liability to the robustness of the method, given that the infection takes place at 37°C. However, when we consider that the bacteria were taken from a culture in exponential phase, thoroughly washed and individualized, significantly diluted and resuspended in a non-optimal medium, exponential bacterial growth is not expected to take place in these conditions.

The observed relationship between growth rate and internalization also happens when analyzing virulence. Indeed, *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis* are in a decreasing scale of virulence inversely proportional to the scale observed in internalization (Figure 7). It seems *M. tuberculosis* may be actively avoiding internalization.

#### **4.5. *Mycobacterium tuberculosis* may inhibit its internalization by macrophages**

Given that *M. tuberculosis* is a facultative intracellular pathogen, the observation that it limits its internalization seems counter-intuitive: *M. tuberculosis* should favor its

internalization in order to be captured and thus survive and multiply inside of the host's macrophages, but our evidence indicates this is not the case.

The reasons for this effect may be explained in an evolutionary context. *M. tuberculosis* requires a living host in order to survive and multiply. If the bacterial burden on the host cells is too high, it may trigger programmed cell death via necrosis or apoptosis and this is clearly not a favorable outcome for the bacteria<sup>43</sup>. It is then reasonable to deduce that *M. tuberculosis*, which has been coexisting and coevolving with humans for several thousand years<sup>5</sup>, may have developed ways by which to control its own internalization, balancing the invasion of the host's macrophages with a discreet immunogenic profile.

It was in order to understand which of the host factors are targeted by *M. tuberculosis* that we conducted a screen measuring the internalization efficiency of macrophages with select protein knockdowns.

#### **4.6. Host factors influence mycobacteria internalization**

We screened for proteins with a significant influence on internalization, measured via the percentage of infected macrophages after 1 hour of infection with a MOI of 10 mycobacteria per macrophage.

We selected some of the proteins featured in the screen from previous screens based on intracellular survival of mycobacteria performed in our laboratory (Carmo, N. *et al*, unpublished results), thus we already had the knowledge that they play a role in phagocytosis. We intended to complement these findings with an insight specifically into the internalization process via flow cytometry. From two lentiviral libraries and a total of 181 samples, we identified a total of 26 proteins belonging to five different groups (Table 1) which, when silenced by RNA interference, caused a significant change in the internalization efficiency of infected macrophages, and are thus labeled as hits.

It is then understood that these proteins have a contributing role to the efficiency of internalization of mycobacteria. The efficiency of internalization of the knockdowns towards the controls was, at minimum, 40% and, on average 65 to 70%. The overall pattern of reduction in internalization was similar between infections with *M. smegmatis* or *M. tuberculosis*, even when considering various hairpins for the same protein knockdown (Figures 9 and 10). However, the average drop in efficiency was greater for *M. tuberculosis*, which is remarkable when taking in consideration that *M. tuberculosis* is already internalized at lower levels in control conditions.

It is not surprising to see that proteins mostly associated with vesicular traffic and phagosome maturation exert influence on internalization, which is a vesicular and membrane process<sup>29</sup>. The fact that the silencing of these proteins significantly decreased the efficiency of internalization suggests that some cross-regulation exists between the chronologically separate systems of internalization and phagosome maturation. Particular attention should be given to the two major groups of proteins in this study: cathepsins and Rab GTPases.

**Table 1. Groups of hits identified in the internalization screen**

Group	Number of hits
Cathepsin (CTS)	12
Rab GTPase (Rab)	11
Syntaxin (STX)	1
Synaptosomal-associated protein (SNAP)	1
Vesicle-associated membrane protein (VAMP)	1

#### **4.7. Only positive regulators of internalization were found**

We tested a total of 181 hairpin RNA samples, spanning 71 proteins, and highlighting 26 hits among them. For every one of these hits, the knockdown of protein expression led to a decrease in internalization, which means that all of the identified hits are putative positive regulators of internalization. The absence of negative regulators is a relevant occurrence, for in the context of mycobacterial infections, we witnessed a clear reduction in internalization of *M. tuberculosis*, but for reasons that remain unclear. The picture that is being painted by these results is one where the mycobacteria negatively control the host's gene expression, hindering the production of relevant proteins and lowering the efficiency of phagocytosis. Thus it seems the negative regulation of internalization stems from the mycobacteria, whilst the positive functions lie in the host cell.

Granted, there were some hairpins that induced a positive change in internalization efficiency, which targeted the following proteins: Rab37, RabGGTA, RabEP2, Rab43, Rab27A, Rab7, SEC22L3, VAMP5 and CTS6. However, these were in some instances the only hairpin available for that particular protein and in others the single hairpin among several for a given protein to exhibit a positive effect. As a consequence, these listed proteins could not be considered hits in our screen because they did not fulfill the criteria we established (section 2.5.3) and thus had limited significance. The cases where only one hairpin was available were unfortunate products of the limitations in our lentiviral

knockdown libraries, and could present new possibilities if thoroughly investigated in the future. It is possible that they may yet be identified as positive regulators, which would be an interesting contrast to the landscape we observed in our present work.

#### **4.8. Rab Guanosine Triphosphatases are positive regulators of internalization**

Rab GTPases are regulators of membrane identity, which is to say they control the hierarchical traffic of endocytic and secretory pathways<sup>28</sup>. This definition clearly marks them as relevant for both internalization and phagosome maturation (also referred to as phagolysosome biogenesis). The sequential ordering of membrane trafficking is made possible by a network or, more accurately, a cascade of Rab GTPases, in which each one replaces another along the trafficking steps as they interact with other binding proteins, factors and activators<sup>28</sup>. Internalization of mycobacteria is undoubtedly an endocytic process and so it is safe to claim that Rab GTPases are involved in it.

The cascade of Rab GTPases plays a role in the events after recognition and binding, so the diminished efficiency of internalization of Rab GTPase knockdown is something to be expected<sup>34</sup>. In our experiments, knockdowns of eleven different Rab GTPases caused drops in internalization to between 40 and 80% of baseline levels, which is a large enough difference to highlight the contribution of the knocked-down proteins towards internalization.

However, no single one of the screened Rab GTPases caused a severe decrease in internalization efficiency (such as tenfold or higher), and so none could be said to be of critical importance to the internalization process. It is likely that there is some redundancy between Rab GTPases, their binding proteins and cofactors, and so the absent Rab GTPase's function would be replaced by another or even skipped altogether<sup>29</sup>, resulting in a slower, more imperfect but nonetheless active internalization of captured mycobacteria.

Rab37 and Rab38 knockdowns were only relevant in infections with *Mycobacterium tuberculosis*, and not the non-pathogenic *M. smegmatis*. There has been no previous mention of these proteins in regards to pathogenicity in the context of tuberculosis, but the exclusive association of both to *M. tuberculosis* was made clear through these results. Our experimental approach unfortunately does not allow for an insight into their function other than their apparent role as regulators that are sensitive to virulence factors.

#### **4.9. Some cathepsins have different roles in pathogenic and non-pathogenic mycobacteria**

One particularly interesting result from the screens regards cathepsin L. It was reported to be pertinent to control arsenic trioxide cytotoxicity, albeit in studies performed in U-87MG human glioblastoma cell lines<sup>44</sup>, but its role in mycobacterial infections has also been studied in the context of adaptive immunity: cathepsin L is a hydrolase responsible for cleaving bacterial peptides in the MHC II antigen-presenting routes, which are necessary to launch a T-cell immune response<sup>45</sup>. The same study concluded that cathepsin L was not essential for an effective antigen presentation and T-cell recruitment, but left the matter of innate immunity unattended, whereas here we show that cathepsin L may also play a role in internalization.

As a consequence of a knockdown of cathepsin L, our macrophages phagocytized significantly less mycobacteria than the controls (with significance here being derived from our established criteria), but this was the case only for *Mycobacterium smegmatis*. As shown before, an increase in pathogenicity is associated with a decrease in internalization, and so in this case of cathepsin L knockdown, *M. smegmatis* behaves more similarly to its virulent counterpart *M. tuberculosis*. Adding the fact that the same knockdown in infections with *M. tuberculosis* had little to no effect on internalization, we have an indicator that the suppression of cathepsin L may be a natural effect of *M. tuberculosis* on the host as a means to control its internalization, a hypothesis which is supported by literature<sup>38</sup>. It has already been established that *M. tuberculosis* can inflict changes to the host cell's gene expression as means of survival through subversion of defenses. It now appears that limiting the expression of cathepsin L is one form through which it achieves that goal.

Cathepsin W (also dubbed lymphopain) was a hit exclusive to *M. tuberculosis*. It has been associated to specific defense against mycobacteria regarding cytotoxicity in T-cells and lymphocytes<sup>46</sup>, although its concrete form of action is still unknown. Its role in internalization in exclusivity towards *M. tuberculosis* is difficult to explain without more data, but the first conjecture to explore would be regarding genes in the *Region of Difference 1*, a short DNA section containing ESAT-6 and other virulence factors, which is present in *M. tuberculosis* but absent in *M. smegmatis* and *M. bovis* BCG<sup>47,48</sup>.

While cystatins, known inhibitors of cathepsins, were also targeted by our investigation, none of them induced a change in internalization when absent via knockdown. From this observation, it seems their role may be restricted to the later stages of phagocytosis, bearing no influence on internalization.

#### **4.10. Membrane-associated proteins STX4A, VAMP2 and SNAP23 are involved in internalization**

While our work focused heavily on Rab GTPases and cathepsins, hits also included syntaxin 4A (STX4A), synaptosomal-associated protein 23 (SNAP23) and vesicle-associated membrane protein 2 (VAMP2) (Table 1).

These three proteins interact with each other, forming a complex that binds itself to the cell's membrane fusion machinery and thus they regulate specificity of vesicular transport<sup>49</sup>. Once again, it is not surprising to see vesicular transport proteins as regulators of phagocytosis.

STX4A in particular is described in literature as being involved in glucose-activated exocytosis of insulin, by interaction with actin in pancreatic beta cell line MIN6<sup>50</sup>. While a parallel with macrophage cell lines may not be straightforward to establish, it is notable that in our results STX4A stood as a positive regulator of internalization. Through interaction with actin it is entirely plausible for STX4A to be able to modulate endocytosis<sup>51</sup>, and in fact our results indicate that it does exert some influence.

SNAP23 has been described as a positive regulator involved in phagosome maturation as well as phagosome formation, which is a synonym for internalization. While our results provide no further insight into its yet unclear role, the fact that we were able to correctly identify SNAP23 as a positive regulator for internalization reinforces the validity of the methods we developed.

#### **4.11. Implications on in vivo infections**

This entire work was done on conditions intended to mimic a first infection by *M. tuberculosis*. The macrophages were in a resting phenotype and not in an activated state. The infection of macrophages by mycobacteria in the context of the experiments is to be regarded as a first contact between them, in controlled environmental conditions.

The protein targets that were screened mostly pertained to the later stages of phagocytosis rather than internalization. One could argue it would be wiser to focus on membrane receptors and ligands when analyzing internalization, but that work has already been done before, by members and colleagues of our laboratory<sup>19</sup>. The focus for this work fell on internalization precisely to expand our knowledge of the phagocytic process as a whole.

The fact that several hits were identified in our screen validates this initiative. It also shows that the various systems comprising phagocytosis are not closed, and the proteins acting upon one step may also be important in others.

The short time span of the experiment and of course the fact that it is *in vitro* doesn't allow for the whole array of responses we would see in a real *in vivo* infection scenario, particularly the processes of acquired immunity. Nonetheless, we are now aware of the role of several proteins on the first crucial moment of host-pathogen interaction: internalization.

#### **4.12. Drawbacks and future work**

As they stand, our results constitute only the first approach – a wide-ranging screen designed to highlight candidates for further detailed research, within the context of mycobacterial infection. Evidently, a screen does not aim to explain the functions of the tested proteins, only to label them as potential positive or negative regulators. Later studies can focus more thoroughly on individual hits, studying their function along with their putative inhibitors and activators.

We are left with two unanswered questions: what is the exact role of the highlighted proteins? And how does *M. tuberculosis* control their expression during infection?

It has been established that *M. tuberculosis* is capable of altering the host cell's gene expression, inhibiting several parts of the phagosome maturation process to ensure its own survival. We therefore propose that a similar sabotage affects internalization, either *a priori* or *a posteriori*. In order to clarify this, we could perform experiments whereby macrophages would be stimulated with supernatant from a growing *M. tuberculosis* culture and afterwards infected with mycobacteria to evaluate how the efficiency of internalization would change. This would indicate whether some compound secreted by *M. tuberculosis* inhibits internalization even before it occurs or if the presence of the bacterium is required.

We have presented a comparison of the internalization profiles of three species of mycobacteria, and studied the effects of protein knockdowns on two of them: *M. smegmatis* and *M. tuberculosis*. The former is a thoroughly used model for a non-pathogenic mycobacterium, as it expresses none of the virulence factors of *M. tuberculosis*, but necessarily presents very similar cell biology. In this work, *M. smegmatis* was extremely useful for us to be able to infer which protein knockdowns are potentially associated with *M. tuberculosis* pathogenicity. However, *M. smegmatis* cannot be said to represent a truly inert

subject of phagocytosis. Therefore, this work can be improved by the study of internalization of inert particles such as latex beads. The current protocols can easily accommodate fluorescent latex beads, whose internalization can also be measured by flow cytometry.

The protocols and analysis methods that were developed during the work for this dissertation have shown to be sound and productive. They can potentially be used for different knockdown libraries to uncover new regulators of internalization in phagocytosis, and even on studies on species other than mycobacteria: *Listeria sp.* and *Salmonella sp.*, for example. Both are intracellular parasites and are relevant public health issues, so any treatments based on the host-pathogen interaction rather than antibiotics would be of enormous benefit.

#### **4.13. Closing remarks**

With this work we have attempted to advance the understanding of the interaction between *Mycobacterium tuberculosis* and the human host by approaching the process of phagocytosis from a different perspective. We focused on the earliest of stages, internalization, to complement the already vast pool of knowledge pertaining to the late phagosome maturation events. With the methods we developed, we not only established standard rates of internalization for the most used macrophage models in tuberculosis research, but also uncovered interesting relationships between them, as well as between species of mycobacteria. By identifying regulators that were otherwise thought independent from internalization we hope to open new paths for the research into the internalization step of phagocytosis as a potential therapeutic target against tuberculosis.

Through our effort to bridge the gap between the early and late stages of phagocytosis we arrived at the conclusion that there seems to be no real gap to speak of. Indeed, the regulators we highlighted participate in other stages of phagocytosis, which reinforces the idea that we stand before a very complex process dependent on a wide network of agents.

We remain baffled by the cunning demonstrated by *M. tuberculosis* in avoiding and manipulating the defenses put up by the human host. We only hope to have slightly untangled this vast web of knowledge and contributed to rid mankind's lungs of their most persistent uninvited guest.

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Figure 1 was obtained from the World Health Organization website, under the URL:

<[http://gamapserver.who.int/mapLibrary/Files/Maps/Global\\_TB\\_incidence\\_2011.png](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_TB_incidence_2011.png)>

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