Mycobacterium tuberculosis subversion of host vesicular traffic pathways

Joana Margarida Nunes Bugalhão

Dissertação
Mestrado em Microbiologia Aplicada
2013
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Dissertação orientada por Prof.ª Dr.ª Elsa Anes (FFUL)
e Prof.ª Dr.ª Margarida Gama Carvalho (FCUL)

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This thesis was fully performed at CPM-URIA of Faculty of Pharmacy of University of Lisbon under the supervision of Prof.ª Dr.ª Elsa Anes.

Prof.ª Dr.ª Margarida Gama Carvalho was the internal designated supervisor in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisbon.
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Acknowledgements

I want to acknowledge my supervisor, Prof. Elsa Anes, for the great opportunity to integrate her research team and for her availability and constant motivating words during the critical review of this thesis, which encouraged me to do my best. I’m grateful to CPM-URIA of the Faculty of Pharmacy, University of Lisbon, for the use of their facilities to perform this work. I’m also grateful to Prof. Margarida Gama Carvalho, my internal supervisor, for the final review of this thesis and for her helpful suggestions.

I want to acknowledge my office and lab colleagues, Nuno Carmo, David Pires, Joana Marques, Paulo Bettencourt, Pedro Timóteo e João Pombo, for turning the lonely moments of scientific research a little more pleasant.

Thank you, Nuno Carmo, for allowing me to contribute to this exciting project, for your ideas and advices and specially for giving me the lab training to perform this work. I'm grateful for everything that I've learned and for the opportunity to think and work more autonomously, thus evolving as an aspiring scientist.

Thank you, David Pires, for your ideas to improve my work and also for your help with several techniques, mainly western blot and qRT-PCR.

Unfortunately, the following words are insufficient to express my gratitude to you, Joana Marques. I thank you for scrolling through this journey with me and for becoming such a good friend. I will always remember that we stayed together and supported each other. A special acknowledgment for all the days you chose to mark 96 different volumes on pipettes to make my high-throughput work easier, when everything else was more exciting.

I also thank my colleagues from the neighboring labs for allowing me to use their equipments and especially for their advices concerning the P3 facility.

I want to demonstrate my sincere gratitude to all my friends and family, especially to those who were particularly patient with me during my master thesis.

Thank you, “amores” (Mafalda and Margarida), for always having the right words to encourage me. Your unconditional friendship was very important to me. I also thank you, Margarida, for reading this thesis, giving me an external point of view of my work.

Thank you, “Ouriense friends”, for the constant reminding that there is more in life beyond the scientific world. Thank you, boys, for the moments of relaxation and also for letting me be “one of the guys”. Perhaps one day I leave microorganisms alone and make you proud of me for creating the so desired “pink midget elephant”.

A special acknowledgment to my parents, who try their best to understand what I do and always support my decisions. Thank you for being more than standard parents, being my best friends instead. My strength and courage to pursue my goals are greatly due to your constant motivation.
Thank you so much, Vitor, for being with me as my friend and my love, giving sense to everything that I do. Thank you for being so understanding when I was absorbed by my work and for trying to cheer me up every day. Thank you for your unconditional belief in my abilities. Once again I realized that I’m capable of everything since I have you by my side.

Finally, the support from the Portuguese Funding Agency, Fundação para a Ciência e Tecnologia (FCT), Projects PTDC/BIA-BCM/102123/2008;PTDC/SAU-MII/098024/2008 and PIC/82859/2007, is gratefully acknowledged.
Communications in Scientific Meetings


Abbreviations

BCG - Bacillus Calmette-Guérin
CFU - Colony-forming units
GTPase - Guanine triphosphatase
HIV - Human immunodeficiency virus
MOI - Multiplicity of infection
OADC - Oleic acid, albumin, dextrose, catalase
OD - Optical density
PBS - Phosphate buffer saline
PMA - phorbol 12-myristate 13-acetate
qRT-PCR - quantitative real time polymerase chain reaction
Rab - Rat brain protein ras-related
RFU - Relative fluorescence units
SNARE - Soluble N-ethylmaleimide-sensitive-factor attachment receptor
tdTomato - tandem dimmer tomato
V-ATPase - Vacuolar adenosine triphosphatase
Resumo

A tuberculose é uma doença infecciosa que afecta principalmente o sistema respiratório e está documentada em toda a história da humanidade. Acredita-se que o seu agente etiológico, *M. tuberculosis*, tenha provocado mais mortes que qualquer outro patogénio humano.

Apesar da melhoria nas condições de saúde pública e da evolução a nível de prevenção, diagnóstico e terapêutica, são registados anualmente cerca de 9 milhões de novos casos de tuberculose e aproximadamente 2 milhões de mortes, sendo considerada a principal causa de morte a nível mundial provocada por um agente bacteriano infeccioso. O crescente aumento de co-infeccões com o vírus da imunodeficiência humana (VIH) e a emergência de estirpes resistentes a antimicobacterianos têm tornado a infecção ainda mais alarmante. Isto porque a vacina disponível é ineficaz em adultos e a única terapia se baseia na administração prolongada de antibióticos. Facilmente se depreende a necessidade de desenvolvimento de novas estratégias de combate à tuberculose, sendo imperativo o estudo científico da interacção de micobactérias com o seu hospedeiro humano para melhor compreender a sua patogenicidade e descobrir novos alvos terapêuticos.

*M. tuberculosis* é um microrganismo intracelular facultativo de crescimento lento, que possui factores de virulência únicos, incluindo componentes do envelope celular, proteínas necessárias à aquisição de nutrientes e proteínas envolvidas na subversão das respostas imunitárias inatas e adaptativas do hospedeiro. Uma das características da infecção com *M. tuberculosis* é a sua capacidade de sobreviver no interior de macrófagos alveolares humanos. De entre os vários factores de virulência, considera-se que a inibição da fusão do fagossoma com o lisossoma é a chave para o seu sucesso como patogénio intracelular. O bloqueio da maturação fagossomal parece ser alcançado através da interferência com factores de tráfego vesicular do hospedeiro.

Nas células eucarióticas, o tráfego vesicular é regulado por proteínas SNARE (acrónimo de Soluble NSF (N-ethylmaleimide sensitive factor) Attachment Protein Receptor) pertencentes à maquinaria de fusão e por Rab GTPases que controlam o tráfego de vesículas e tornam específicos os eventos de fusão. Estas proteínas membranares asseguram a correcta entrega de cargas entre vesículas e organelos, sendo responsáveis pela coordenação espacial e temporal da fusão de fagossomas recém-formados com diversas vesículas. A via de transporte normal para os lisossomas inclui proteínas Rab e SNARE, na qual o organismo fagocitado é transportado de um fagossoma nascente para um compartimento endossomal precoce contendo Rab5. A reciclagem de proteínas membranares leva à perda de Rab5 e aquisição de Rab7, o que permite a maturação para fagossoma tardio antes de ser entregue ao destino final, o compartimento lisossomal. Contrariamente aos estádios de maturação anteriores, o fagolisossoma possui um pH ácido e proteases activas, que permitem a destruição e degradação de bactérias.

Considerando o papel das Rabs e SNAREs na biogénese do fagolisossoma, é inequívoca a sua potencialidade como alvos de manipulação por microrganismos patogénicos intracelulares. Efectivamente, vários estudos indicam que fagossomas contendo *M. tuberculosis* permanecem
imaturos devido à exclusão ou aquisição transitória de proteínas características de endossomas tardios, enquanto retêm proteínas que permitem manter a sua identidade como fagossomas precoces. Isto é exemplificado pelo bloqueio da transição Rab5-Rab7 em fagossomas contendo micobactérias patogénicas. Apesar dos factores de tráfego vesicular serem indicados como alvos de manipulação micobacteriana, a maioria dos estudos não esclarece o seu envolvimento na sobrevivência/morte intracelular de *M. tuberculosis* em macrófagos humanos.

O presente trabalho teve como objectivo identificar Rab GTPases e SNAREs envolvidas na sobrevivência/morte de *M. tuberculosis* em macrófagos humanos THP-1, recorrendo a metodologias adequadas a ensaios em larga escala. Foi desenvolvido um método fluorimétrico com dois repórteres fluorescentes para quantificação das micobactérias intracelulares e dos macrófagos hospedeiros, uma vez que o método convencional de contagem de unidades formadoras de colónias (UFC) é não só dispendioso, mas também laborioso e inapropriado para rastreios em larga escala. Paralelamente foi produzida uma biblioteca de lentivirus a expressar *short hairpin* RNAs (shRNAs) específicos para silenciar os RNAs mensageiros (mRNAs) de Rab GTPases e SNAREs nas células hospedeiras. Deste modo foi possível avaliar o impacto do silenciamento de factores do hospedeiro na sobrevivência/morte intracelular de *M. tuberculosis*.

A proteína vermelha fluorescente tdTomato foi seleccionada como repórter da sobrevivência intracelular de *M. tuberculosis* H37Ra. A estirpe H37Ra foi transformada com o plasmídeo pASTA3, que contém o gene para tdTomato. A transformação foi eficiente, detectando-se a presença de micobactérias fluorescentes após vários dias de crescimento em meio líquido. Os nossos resultados revelaram uma boa correlação entre fluorescência e densidade óptica, demonstrando que a fluorescência de tdTomato permite discriminar diferentes concentrações micobacterianas *in vitro*.

Verificámos também que o método fluorimétrico permite avaliar a sobrevivência intracelular de *M. tuberculosis* H37Ra (tdTomato) em macrófagos THP-1 até ao terceiro dia após a infecção. De forma semelhante ao método de contagem de UFC, o método fluorimétrico permitiu distinguir entre multiplicidade de infecção (MOI) 1 e MOI 5 e apresentou o mesmo perfil de sobrevivência micobacteriana. No entanto, tendo em conta que a estirpe H37Ra só começa a multiplicar-se intracelularmente ao terceiro dia após a infecção e que a morte dos macrófagos aumenta ao sétimo dia, considerámos que o dia 5 seria o tempo de medição ideal para avaliação da sobrevivência intracelular. O método fluorimétrico não permitiu detectar a presença de micobactérias fluorescentes em células infectadas com MOI 1 ao quinto dia após a infecção, os quais correspondem à MOI e tempo de infecção pretendidos para avaliar a sobrevivência intracelular na triagem dos factores de tráfego vesicular. A quantificação micobacteriana nestes parâmetros foi possível apenas pelo método convencional de contagem de UFC, o qual optámos por manter nos nossos ensaios. Não obstante, *M. tuberculosis* H37Ra fluorescente pode ser útil em estudos que utilizem aparelhos mais sensíveis, destacando-se a citometria de fluxo e a microscopia de fluorescência.

Nos ensaios de sobrevivência intracelular de micobactérias, geralmente desconhece-se a viabilidade dos macrófagos. Este parâmetro é de extrema importância, permitindo revelar se há, efectivamente, um decréscimo de bactérias intracelulares ou se a diminuição se deve à morte dos hospedeiros. Os nossos resultados confirmaram que a intensidade de fluorescência resultante da
metabolização de alamarBlue está positivamente correlacionada com o número de macrófagos THP-1. Este método é, portanto, adequado para avaliar a viabilidade de macrófagos durante ensaios de infecção.

Para compreender o papel dos factores de tráfego vesicular na infecção micobacteriana, procedemos a uma triagem preliminar usando a estirpe avirulenta *M. tuberculosis* H37Ra. Após o silenciamento com lentivírus e selecção com puromicina, os monócitos THP-1 foram diferenciados em macrófagos e infectados com MOI 1. O número de micobactérias intracelulares foi determinado ao dia 5 após a infecção por contagem de UFC e a viabilidade dos macrófagos foi avaliada por fluorimetria usando alamarBlue. A carga micobacteriana no interior de macrófagos THP-1 silenciados para Rab GTPases e SNAREs foi comparada com o controlo scramble (sem alvo no genoma humano). Atribuímos a designação de “hot targets” aos factores cujo silenciamento com pelo menos um shRNA resultou numa carga micobacteriana com diferenças estatisticamente significativas relativamente ao controlo. Os resultados emergentes da triagem preliminar são o produto de 3 experiências independentes, onde 13 mRNAs alvo foram individualmente silenciados nas células THP-1 com diferentes shRNAs. Apresentamos nesta tese os resultados para 5 factores, cujos níveis de silenciamento foram determinados usando a reação de polimerase em cadeia em tempo real (qRT-PCR). Usando esta estratégia foi possível identificar Rab7, Rab14 e Rab34 como potenciais alvos de manipulação micobacteriana, uma vez que a redução destas proteínas alterou a sobrevivência intracelular da estirpe H37Ra sem afetar a viabilidade dos macrófagos transduzidos.

De entre as várias características de virulência que distinguem H37Ra de H37Rv, destaca-se a capacidade inferior da estirpe avirulenta para crescer intracelularmente. Além disso, grau de associação de Rabs com fagossomas micobacterianos difere entre as duas estirpes. Considerando estes aspectos, os fenótipos obtidos para os potenciais alvos Rab14 e Rab34 não foram apenas confirmados, mas também comparados com a estirpe virulenta, *M. tuberculosis* H37Rv. Com base nos resultados de sobrevivência intracelular, demonstrámos que as duas estirpes têm diferentes capacidades de manipulação das vias de tráfego vesicular do hospedeiro.

Com o intuito de revelar as implicações do silenciamento de mRNA, os níveis de Rab14 e Rab34 foram analisados ao nível da tradução. Os resultados de western blot mostraram que a transdução lentiviral diminuiu, de facto, a expressão das proteínas Rab14 e Rab34.

Com o presente trabalho foi possível demonstrar que Rab7 e Rab34 são importantes para a capacidade dos macrófagos destruírem micobactérias. A diminuição destas proteínas aumentou de forma significativa a capacidade de sobrevivência intracelular de *M. tuberculosis* H37Ra, mas não afectou a estirpe virulenta H37Rv. Estas proteínas Rab são importantes para a função e posicionamento de lisossomas, tendo sido anteriormente indicadas como reguladores da maturação fagossomal. Efectivamente, estudos anteriores desenvolvidos em macrófagos de ratinho mostraram que Rab7 e Rab34 são recrutadas apenas transientemente para fagossomas contendo *M. tuberculosis* H37Rv. A sua acumulação é superior em fagossomas contendo a estirpe H37Ra, sendo ainda mais elevada quando se trata de *Staphylococcus aureus*. Isto significa que as duas estirpes micobacterianas interferem com a associação fagossomal de Rab7 e Rab34, embora a manipulação exercida pela estirpe H37Rv seja mais acentuada. Em concordância com a literatura, os nossos
resultados indicam que H37Rv exclui Rab34 do seu fagossoma e, deste modo, o silenciamento de Rab34 não interfere com a sobrevivência intracelular. Apoiando os nossos resultados para a estirpe avirulenta, num estudo recente usando macrófagos de ratinho observou-se um aumento da sobrevivência intracelular da estirpe atenuada M. bovis BCG, face ao silenciamento de Rab34.

Rab14 está envolvida no tráfego entre o complexo de Golgi e endossomas precoces, mas o seu papel na maturação fagossomal permanece controverso. Está descrito na literatura a acumulação de Rab14 em fagossomas contendo micobactérias e que o seu silenciamento promove a biogênese do fagolisossoma. Contrariamente, nos estudos de Seto et al. (2011) a expressão de formas constitutivamente activas ou inactivas de Rab14 não interferiu com a capacidade de fusão entre fagossoma e lisossoma. Usando a nossa estratégia foi possível revelar o verdadeiro impacto da redução de Rab14 na sobrevivência de M. tuberculosis. A carga bacteriana de H37Ra diminuiu com o silenciamento de Rab14, enquanto o número de bactérias virulentas foi semelhante ao controlo. Estes resultados sugerem que Rab14 é essencial para sustentar a sobrevivência de M. tuberculosis H37Ra, contribuindo para o estabelecimento de um nicho replicativo. Contrariamente, a grande capacidade de H37Rv para sobreviver e proliferar parece ser independente da sua presença, provavelmente devido à superior habilidade para subverter os mecanismos do hospedeiro.

Rab10 está envolvida na reciclagem membranar e na maturação fagossomal, regulando a transição de um fagossoma nascente para um fagossoma precoce. Verificámos que uma diminuição de aproximadamente 50% nos níveis de mRNA de Rab10 não interferiu com a sobrevivência de H37Ra em macrófagos humanos. Segundo estudos anteriores em macrófagos de ratinho, o recrutamento de Rab10 para fagossomas contendo bactérias vivas da estirpe atenuada M. bovis BCG é inferior comparando com M. bovis BCG mortas por calor. Os efeitos provocados pelo silenciamento de Rab10 parecem ser similares à exclusão de Rab10 de fagossomas contendo micobactérias vivas, o que está de acordo com os nossos resultados. À semelhança de M. bovis BCG, H37Ra parece ser também capaz de diminuir o recrutamento de Rab10 para a membrana do fagossoma, o que explica a sobrevivência inalterada face ao silenciamento de Rab10.

A sintaxina 4 é uma t-SNARE (do inglês target SNARE) que se localiza nas membranas citoplasmáticas e endossomais. Um lípido micobacteriano, fosfatidil inositol manosídeo, é referido na literatura como responsável pela retenção de sintaxina 4 em membranas fagossomais. Esperávamos, portanto, que uma diminuição na expressão de sintaxina 4 limitasse a fusão entre fagossomas e endossomas, com uma consequente redução na carga micobacteriana. No entanto, um decréscimo de 30% e 50% nos níveis de mRNA de sintaxina 4 não alteraram o número de bactérias intracelulares da estirpe H37Ra. Por um lado, esta sintaxina pode ser um componente passivo durante a maturação fagossomal. Em alternativa, a retenção de outras SNAREs na membrana de fagossoma pode ser suficiente para assegurar a fusão com endossomas. Não excluindo nenhuma das hipóteses anteriormente sugeridas, é também possível que os níveis de silenciamento nas nossas experiências possam ter sido insuficientes para ter algum impacto, uma vez que não foram significativamente diferentes do controlo.

Os resultados apresentados nesta tese estão em concordância com a maioria das evidências postuladas na literatura, reafirmando que M. tuberculosis exclui factores que permitem a progressão
do fagossoma para fagolisossoma, tais como Rab10, Rab7 e Rab34, evitando assim a sua destruição e reconhecimento imunitário. Contrariamente, *M. tuberculosis* tem a capacidade de recrutar e reter nos seus fagossomas proteínas que regulam a fusão com endossomas precoces, tal como Rab14, tendo assim acesso a nutrientes e precursores biossintéticos para a sua sobrevivência.

**Palavras-chave:** *Mycobacterium tuberculosis*, macrófago, tráfego vesicular, maturação do fagossoma, Rab GTPase, SNARE, proteína fluorescente tdTomato.
Abstract

The success of *Mycobacterium tuberculosis* as an intracellular bacterial pathogen greatly relies in its ability to subvert the host vesicular trafficking pathways within macrophages. *M. tuberculosis*-containing phagosomes fail to fuse with late endosomes and lysosomes. Rab GTPases are responsible for fusion specificity and SNAREs are components of the vesicle fusion machinery. These host proteins are indicated as potential targets for mycobacterial manipulation, but their role in *M. tuberculosis* survival/killing in human macrophages remains unclear.

In this thesis work we developed a dual fluorescent reporter method for mycobacteria and macrophage quantification. We found that intracellular *M. tuberculosis* expressing tdTomato, a red fluorescent protein, can be quantified in human THP-1 macrophages infected with MOI 1 by fluorescence measurement. Although the method was reliable until day 3 post-infection, did not showed to be sensitive at later times for mycobacterial quantification, in opposition to the conventional method of colony forming units counting. Macrophage viability was successfully assessed by fluorimetry with alamarBlue reagent.

Using a high-throughput shRNA-based screen we identified Rab7, Rab14 and Rab34 as “hot targets” for mycobacterial manipulation and the levels of silencing were evaluated by qRT-PCR. A reduction of Rab7 and Rab34 significantly increased *M. tuberculosis* H37Ra survival in THP-1 macrophages, while Rab14 knockdown decreased mycobacterial burden. In contrast, no effects were observed after lentiviral silencing of Rab10 and syntaxin 4.

The phenotypes obtained in the preliminary screen for Rab14 and Rab34 knockdown were successfully confirmed and compared with the virulent strain H37Rv. The levels of silencing were determined by western blot. In opposition to H37Ra, Rab14 and Rab34 depletion did not alter intracellular bacterial burden of H37Rv, indicating that differences in virulence interfere with mycobacterial competence to manipulate host vesicular trafficking.

**Keywords:** *Mycobacterium tuberculosis*, macrophage, vesicular trafficking, phagosome maturation, Rab GTPase, SNARE, tdTomato fluorescent protein.
1. Introduction

1.1. Tuberculosis

Tuberculosis is one of the oldest infectious diseases affecting mankind and the causal agent is *Mycobacterium tuberculosis* (*M. tuberculosis*). It is believed that the disease has been present in all human history and pre-history, based on several records with more than 5000 years (Daniel, 2006).

Tuberculosis is highly contagious and is characterized by severe pulmonary infections (pulmonary tuberculosis), but may also extend to other organs (extrapulmonary tuberculosis) (World Health Organization, 2011). In the 18th century, tuberculosis reached epidemic proportions in North America and Europe and was designated as the “great white plague”. By that time, the concept of sanatoriums emerged, where people were treated only by a healthy diet, rest and fresh air (Harries and Dye, 2006). The understanding of tuberculosis pathogenesis was only achieved in the 19th century after fundamental microbiological findings. For instance, Jean-Antoine Villemin discovered that *M. tuberculosis* infection is transmissible and Robert Koch indentified and described the tubercle bacilli (Daniel, 2006; Harries and Dye, 2006).

Since these periods, the major contributions to control the spread of tuberculosis in developed countries were the implementation of public health measures, the advent of antibiotics and the massive vaccination with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine (Daniel, 2006). Altogether, these advances decreased the incidence and prevalence of the illness, but the down-trend was inverted in the mid-1980. The main reasons were an augment in poverty in developing countries and the emergence of co-infections with human immunodeficiency virus (HIV), the agent of acquired immunodeficiency syndrome (Mortellaro et al., 2009). Since active HIV co-infection compromises host defense, the risk of developing active tuberculosis disease is increased (Harries and Dye, 2006).

At the present time, tuberculosis is still the leading cause of death from a bacterial infectious disease, with around 9 million new cases and almost 2 million deaths each year (Harries and Dye, 2006; World Health Organization, 2011). The therapy for tuberculosis requires treatment with multiple antibiotics for a long period of time, which difficult the compliance of the regimes in regions with less resources (Russell, 2011). Moreover, nowadays emerging multidrug resistant and extensively drug resistant mycobacterial strains, contribute to the increment in tuberculosis concerns (Van Rie and Enarson, 2006).

The battle against tuberculosis requires the improvement of diagnosis methods, allied to the development and implementation of effective drugs for resistance bacilli and with short time regime therapies.
1.2. Mycobacteria

Mycobacteria are immobile, aerobic and rod-shaped bacteria of the phylum Actinobacteria and belong to the family Mycobacteriaceae (Scherr and Nguyen, 2009). According to gram staining mycobacteria are classified as gram positive, however its multi-layered cell wall structure is more similar to that of gram negative bacteria. In fact, mycobacteria have a thick outer-membrane that causes acid-fastness, allowing the identification of *Mycobacterium* spp. by Zhiel-Neelsen stain (Forrellad *et al.*, 2013; Scherr and Nguyen, 2009).

The genus *Mycobacterium* comprises more than 50 species, usually grouped according with the growth rate. The majority are fast-growing non-pathogenic environmental bacteria, such as *M. smegmatis* and *M. terrae*, but a few slow-growing pathogens are responsible for severe diseases. For instance, *M. tuberculosis*, *M. bovis* and *M. leprae* cause human tuberculosis, bovine tuberculosis and leprosy, respectively (Cosma *et al.*, 2003). Mycobacterial species that cause tuberculosis in humans and animals are included in the *M. tuberculosis* species complex. The complex comprises the genetically identical species *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. bovis*, *M. microti*, *M. caprae* and *M. pinnipeddi*, which have different phenotypes, host ranges and pathogenicity (Cosma *et al.*, 2003; Forrellad *et al.*, 2013).

*M. tuberculosis* is a slow-growing facultative intracellular pathogen, with bacterial virulence factors that allow its survival and replication inside human macrophages (Forrellad *et al.*, 2013). The characteristic mycobacterial cell wall envelope impairs *M. tuberculosis* destruction during host infection. In fact, the high content in complex lipids and lipoglycans in mycobacterial membranes difficult the entry of nutrients leading to slow growth, but also confer resistance to dehydration, chemical damage, antibiotics and degradation by host lysosomal enzymes (Scherr and Nguyen, 2009). In addition to the components of the cell wall envelope and the genes involved in their metabolism, other virulent factors contributing to *M. tuberculosis* pathogenesis were identified. These factors include proteins required for the uptake of nutrients and ions and proteins related to mycobacterial subversion of host immune responses (Forrellad *et al.*, 2013).

1.3. The highlights of *M. tuberculosis* infection

*M. tuberculosis* transmission occurs from person to person, through the inhalation of viable bacilli in microscopic droplets from atmosphere (World Health Organization, 2011). When bacteria reach the lungs, host immunity is triggered by alveolar macrophages and dendritic cells (Hope *et al.*, 2004). These phagocytes internalize bacteria in vacuoles called phagosomes and initiate innate immune responses, including destruction by lysosomal acidic enzymes following phagosome-lysosome fusion and the release of toxic reactive oxygen and nitrogen intermediates (Deretic *et al.*, 2006; Hope *et al.*, 2004). However, *M. tuberculosis* developed mechanisms to avoid intracellular killing, such as detoxification of free radicals and blockage of phagosome maturation. (Deretic *et al.*, 2006; Via *et al.*, 1997).

Infected macrophages expressing surface chemo-attractants and chemokines attract monocyte derived macrophages and dendritic cells from the blood vessels to contain the infection in the lung (Hope *et al.*, 2004; Jordao and Vieira, 2011). However, besides escaping innate immunity, it is thought
that *M. tuberculosis* is also able to retard the migration of infected dendritic cells from the infection focus to lymph nodes, where T-lymphocytes are primed against mycobacterial antigens. Therefore, the initiation of adaptive immunity is delayed for two-three weeks (Gallegos *et al.*, 2008; Ottenhoff, 2012; Wolf *et al.*, 2008). When T-helper lymphocytes are finally differentiated they release important proinflammatory cytokines, such as interferon-gamma (IFN-γ), which in turn activate macrophages to produce tumor necrosis factor alpha (TNF-α) and other microbicidal effectors for mycobacteria (Korbel *et al.*, 2008).

The recruitment and accumulation of immune cells to the site of infection origins the granuloma, the hallmark of tuberculosis, which limits bacterial dissemination (Russell, 2007). At this stage, the progression of the infection greatly varies according to the condition of the patients’ immune system. If granuloma formation is insufficient to eliminate the bacilli, latency may occur allowing dormant bacilli to survive in a non-replicating and resistant form for decades (Ramakrishnan, 2012; Russell, 2007). Nowadays, it is estimated that only 10% of the infected individuals will develop active disease in their lifetime and this is usually caused by immune depression (Harries and Dye, 2006).

1.4.  *M. tuberculosis* subversion of host vesicular traffic pathways

As referred above, *M. tuberculosis* possesses virulent factors and elicits microbial mechanisms to manipulate host defense in order to avoid degradation and escape immune detection. As other facultative intracellular bacterial pathogens, such as *Listeria monocytogenes* and *Salmonella enterica*, *M. tuberculosis* survives within human macrophages, the cells programmed to kill invading organisms (Brumell and Scidmore, 2007). One of the main mechanisms triggered by *M. tuberculosis* for intracellular survival is the inhibition of phagolysosome biogenesis (Deretic *et al.*, 2006). The key regulators of vesicular traffic and fusion in eukaryotic cells are Rab GTPases (Rat Brain Protein Ras-related) and SNAREs (soluble N-ethylmaleimide-sensitive-factor attachment receptors) (Somsel Rodman and Wandinger-Ness, 2000). The arising data are indicating that *M. tuberculosis* manipulates host vesicular traffic pathways through the selective exclusion or retention of Rabs and SNAREs at the membranes of their phagosomes (Brumell and Scidmore, 2007; Stein *et al.*, 2012).

1.4.1. Rabs and SNAREs functions

Rab proteins are a large family of monomeric GTPases and were already identified more than 70 Rabs encoded by the human genome. Since different Rab family members associate preferentiality with different populations of vesicles and organelles, they have a fundamental role in specifying their identity and routing (Schwartz *et al.*, 2007).

Rabs are localized at the cytoplasmic side of organelles and vesicles membranes, where they initiate the formation of a complex that allows specific membrane traffic and fusion (Figure 1) (Somsel Rodman and Wandinger-Ness, 2000). Several components of the fusion machinery participate in this process, including N-ethylmaleimide-sensitive factors (NSFs), NSF attachment proteins (SNAPs) and SNAREs (Weber *et al.*, 1998). The pairing of an organelle target SNARE (t-SNARE) with the vesicle SNARE (v-SNARE) originates a four-helix trans-SNARE bundle that pulls membranes together, allowing the fusion of lipid bilayers. Posterior to membrane fusion, the pair of SNAREs bound to the
membrane is designated as cis-SNARE and is separated into t-SNARE and v-SNARE through the recognition of the cis-SNARE complex by α-SNAP and the action of the NSF ATPase that interacts with α-SNAP (Band et al., 2002; Vieira et al., 2002; Weber et al., 1998).

Since SNAREs have some degree of promiscuity, Rab GTPases and their effectors also regulate docking and fusion specificity. Rabs act as molecular switches, being active when bound to GTP and inactive when bound to GDP. The spatial and temporal recruitment of GDP-GTP exchange factors (GEFs) and GTPase activating proteins (GAPs) is responsible for the correct transitions between Rabs. Briefly, the soluble inactive GDP-bound Rab is a substrate for GDP dissociation inhibitor (GDI). At the acceptor membrane, the complex interacts with GDI dissociation factor (GDF), which removes GDI from the complex, allowing the insertion of the Rab in the membrane. At this stage, GEF acts on the membrane-inserted Rab to convert it to a GTP-bound active state, which in turn interacts with specific effectors, such as phosphatidylinositol kinases and phosphatases (Hutagalung and Novick, 2011; Ng et al., 2012; Somsel Rodman and Wandinger-Ness, 2000). Through their effectors, Rabs control organelles maturation, vesicle movement along microtubule tracks, vesicle tethering, docking and fusion, cargo sorting and recruitment of signaling molecules (Castaño and Rojas, 2010).

The endosomal system involves a highly coordinated network of Rabs and SNAREs that control cargo transport from the cell surface and their delivery to acceptor vesicles such as lysosomes. Therefore, the identification of Rabs and SNAREs involved in phagosomal maturation might contribute to the understanding of the regulatory mechanisms involved in this process as well to serve as potential targets to be manipulated by intracellular pathogens.

**Figure 1 – Vesicle fusion.** (1) Tethering. The donor vesicle moves to the acceptor compartment and becomes tethered to the acceptor compartment by the combination of a GTP bound Rab and a tethering factor. (2) Docking. The v- and t-SNAREs assemble into a four-helix bundle. (3) Fusion. This trans-SNARE complex promotes fusion of the vesicle and acceptor lipid bilayers. Cargo is transferred to the acceptor compartment, and the SNAREs are recycled (adapted from Bonifacino and Glick, 2004).
1.4.2. The association of Rabs with phagosomes during phagolysosome biogenesis

Rab GTPases have been identified as regulators of the processes occurring during bacterial infections, such as bacterial phagocytosis and the transport of internalized pathogens to lysosomes (Castaño and Rojas, 2010).

After internalization of bacteria by macrophages, the nascent phagosome matures through stepwise sequential fusion and fission events with early, late endosomal and lysosomal vesicles. This process culminates with the formation of the phagolysosome, a compartment highly microbicidal able to eliminate the majority of internalized bacteria (Figure 2) (Seto et al., 2011).

Phagosomal maturation refers to a “kiss and run” model, with a series of fusion and fission events, where phagosomes acquire new molecules and recycle others (Desjardins et al., 1994). The recently formed phagosome binds to microtubules and in a few minutes loses components from the cytoplasmic membrane while others are acquired from “older vesicles”. Subsequently, a Rab5 dependent fusion with early endosomes occurs and the phagosome rapidly acquires the properties of this endocytic organelle. These include the acquisition of Rab5 and the transferrin receptor on their membranes. Indeed they are characterized by a moderate pH around 6 and inactive hydrolytic enzymes, thus being unable to kill microorganisms (Desjardins et al., 1994; Pitt et al., 1992). As the maturation proceeds, the early endosomal markers are recycled and the phagosome acquires molecules associated with late endosomes. The acquisition of Rab7 and the loss of Rab5 are crucial for the fusion of the early phagosome with late endosomes and lysosomes. A phagosome enriched in late endosomal components, such as Rab7, lysosome associated membrane protein 1 (LAMP 1) and proton pump vacuolar adenosine triphosphatases (V-ATPases), is able to mature into the phagolysosome (Fratti et al., 2001; Via et al., 1997). The phagolysosome has an acidic luminal pH around 4.5-5 due to the acquisition of V-ATPases and the fusion with acidic organelles. The acidic pH has a direct microbicidal effect in bacteria and also enhances the activity of lysosomal enzymes and the production of reactive oxygen and nitrogen species (Vieira et al., 2002).

In addition to Rab5 and Rab7, several other Rabs were shown to associate to phagosomes during the maturation process (Brumell and Scidmore, 2007).

1.4.3. M. tuberculosis manipulation of Rabs and SNAREs during host infection

In the 1970’s, Armstrong and Hart demonstrated for the first time that the viability of intracellular M. tuberculosis in infected macrophages was correlated with their presence in organelles that did not fuse with lysosomes (Armstrong and Hart, 1971; Armstrong and Hart, 1975). Several posterior studies have shown that mycobacteria-containing phagosomes are mild acidified (pH around 6) due to a deficient recruitment of V-ATPases (Crowle et al., 1991; Sturgill-koszycki et al., 1996). M. tuberculosis-containing phagosomes has the transferrin receptor at the phagosomal membrane meaning that is an immature early phagosome. Therefore it is competent to fuse with early endosomes, which contributes to the access of nutrients for bacterial survival (Sturgill-koszycki et al., 1996).

It is still unclear the precise point of maturation blockage, but the observation of Rab5 accumulation and absence of Rab7 in mycobacterial phagosomes, firstly indicated that the process was arrested during Rab5-Rab7 conversion (Figure 2) (Via et al., 1997). Posterior experimental
studies detected the presence of Rab7 in mycobacterial phagosomes, demonstrating that *M. tuberculosis*-containing phagosome has receptor machinery for Rab7 and therefore the maturation block could occur subsequently to Rab7 acquisition or were influenced by other components (Clemens *et al.*, 2000). A more recent study from Seto and colleagues helped to clarify the previous results, by demonstrating that Rab7 is transiently recruited to and subsequently released from mycobacterial phagosomes. This phenomenon contributes to maturation block, by limiting the recruitment of the cathepsin D protease and the Rab-interacting lysosomal protein (RILP) (Rab 7 effector) to phagosomes (Seto *et al.*, 2011).

The crucial functions of Rabs and SNAREs in vesicular traffic pathways led to the search of other proteins that could be manipulated by mycobacteria to inhibit phagosome maturation.

Studies in mice macrophages performed by Cardoso and colleagues showed that the recruitment of Rab10 to phagosomes containing live *M. bovis* BCG is lower comparatively to heat-killed BCG. Moreover, Rab10 knockdown delayed phagolysosome formation. Since Rab10 acts upstream to Rab5, these results suggest that Rab10 may be manipulated by mycobacteria to arrest phagosome maturation (Cardoso *et al.*, 2010).

It is known that Rab14 controls the traffic between Golgi and early endosomes (Junutula *et al.*, 2004; Kyei *et al.*, 2006). Kyei and colleagues observed the accumulation of Rab14 on *M. tuberculosis*-containing phagosomes and the reduction of Rab14 expression by small interfering RNA promoted the fusion with lysosomes. On the other hand, a study performed by Seto *et al.* in mice macrophages showed that the expression of constitutively active or dominant negative forms of Rab14 do not alter the fusion of phagosomes with lysosomes (Seto *et al.*, 2011). Taking into account the controversial results, the role of Rab14 during *M. tuberculosis* infection of human macrophages remains uncertain.

In the same study performed by Seto *et al.*, other Rabs were highlighted as regulators of *M. tuberculosis*-containing phagosome maturation. For instance, they demonstrated that Rab34 are only transiently recruited to mycobacterial phagosomes. Similarly to Rab7, Rab34 is a late endosomal Rab involved in the recruitment of cathepsin D to phagosomes (Seto *et al.*, 2011).

In addition to Rab GTPases, proteins of the fusion machinery were also suggested to be targets for mycobacterial manipulation. The v-SNARE cellubrevin, also known as VAMP3, is involved in endosomal recycling and endosomal interactions with post-Golgi compartments, and is usually acquired in phagosomes. Fratti *et al.* appointed cellubrevin as a mycobacterial target due to the observation of a discrete degradation of this SNARE during mycobacterial infection (Fratti *et al.*, 2002). Other SNARE that appears to be a target for *M. tuberculosis* is syntaxin 4, which is a recycling endosomal and plasma membrane t-SNARE. It was observed that syntaxin 4 is retained in phagosomes containing phosphatidylinositol mannoside (PIM)-coated latex beads, whereas the accumulation of this SNARE is lower in phagosomes containing uncoated beads. Since PIM is a mycobacterial glycolipid that impregnates host cell endomembranes, these findings indicate that PIM contributes to the fusion of *M. tuberculosis*-containing phagosomes with endosomal compartments, by retention of syntaxin 4 in the phagosomal membrane (Vergne *et al.*, 2004).

Despite the recent discoveries, little is known about how these proteins are manipulated during infection or if they contribute to *M. tuberculosis* survival/killing inside phagosomes.
Figure 2. The process of phagosome maturation. Microorganisms are internalized by macrophages and contained in phagosomes. Phagocytosis of inert particles or dead bacteria (brown rectangles) leads to acquisition of early endosomal proteins, such as Rab5. The maturation proceeds through the recycling of these markers and replacement with late endosomal proteins, such as Rab7. The process culminates with phagolysosome biogenesis and degradation of internalized particles. M. tuberculosis (purple rectangles) arrests phagosomes through retention of early endosomal proteins, such as Rab5 and exclusion or only transient acquisition of late endosomal proteins, such as Rab7. Therefore, M. tuberculosis remains within a phagosome suitable for its survival and replication. The broken arrow represents M. tuberculosis blockage of phagosome maturation into the phagolysosome (adapted from Seabra et al., 2002).

1.5. Fluorimetric methods to quantify intracellular M. tuberculosis survival

The standard methods to quantify intracellular mycobacterial survival are based on colony-forming units (CFU) counting. This technique is extremely laborious, expensive and CFU counting of mycobacterial species with fastidious growth rates is only possible after several weeks of incubation. This approach is not suitable for high-throughput screens and therefore is essential the development of faster and more suitable methods (Meyers et al., 1998).

Fluorescent proteins allow the monitoring of the cellular growth with high spatial and temporal resolution by fluorimetric detection using fluorimeters. Several fluorescent reporters are non-toxic cytoplasmic proteins and are continuously synthesized, which minimizes the effect of fluorescence signal dilution during bacterial replication (Müller-Taubenberger and Anderson, 2007). These reporters don’t require reagent addition and can be used in experiments using a microplate format suitable for high-throughput screens (Kremer et al., 1995; Meyers et al., 1998).

Fluorescent proteins are genetically encoded and have been used as reporters in transcriptomic and proteomic studies. In 1960, the Green fluorescent protein (GFP) was isolated from Aequorea Victoria and was used in 1994 for the first time to report gene expression in prokaryotes and eukaryotes (Chalfie et al., 1994). DsRed protein cloned from the coral Discosoma striata is also commonly used as a fluorescent reporter. However, DsRed has the disadvantages of tetramers.
formation that can originate toxic aggregates, and has a slow maturation time via a green intermediate (Müller-Taubenberger and Anderson, 2007). To overcome these problems, Shaner and colleagues generated an extremely bright and non-toxic derivate of DsRed, the tandem dimmer tomato (tdTomato) protein (Shaner et al., 2004). tdTomato is a useful red fluorescent protein that can be detected when expressed from mycobacterial promoters. No adverse effects on fitness were seen in *M. tuberculosis* H37Rv (Carroll et al., 2010).

Fluorimetric methods have potential to overcome the constraints of CFU counting techniques. These tools can improve the study of mycobacteria-host interactions and contribute as efficient tools for a faster screen of new antimycobacterial agents.

### 1.6. Thesis goals and strategies

The main goal of this thesis is the identification of human vesicular traffic factors involved in *M. tuberculosis* survival or killing within human macrophages. This work focuses in the roles of Rab GTPase and SNARE proteins during mycobacterial infection and the development of a dual fluorescent method for rapid quantification of their effects on mycobacterial survival.

THP-1 monocytes were silenced for Rab GTPases and SNAREs using a lentiviral library and infected with *M. tuberculosis* H37Ra after monocytes differentiation into macrophages. We hypothesized that some of the shRNA targeting genes have a role in the maturation process of *M. tuberculosis*-containing phagosome. Therefore, we expected higher or lower values of intracellular mycobacteria compared with the control.

The final step of this work consists on the selection and validation of genes, whose lentiviral transduction of host cells seemed to affect intracellular *M. tuberculosis* survival. To validate the phenotypes obtained in the preliminary screen, THP-1 cells were transduced with new batches of lentivirus targeting the genes of interest and infected with both avirulent and virulent *M. tuberculosis* strains. We hypothesized that differences in virulence between both strains could result in different phenotypes.

The study of Rabs and SNAREs in human macrophages infected with *M. tuberculosis* is essential to understand how this pathogen manipulates host cell function to avoid destruction and cause disease and also to identify other therapeutic targets to fight tuberculosis.
2. Material and Methods

2.1. Mycobacterial cultures

The avirulent *M. tuberculosis* strain H37Ra (25177™, ATCC) and the virulent *M. tuberculosis* strain H37Rv were maintained in Middlebrook’s 7H9 broth medium (Difco) supplemented with 10% (v/v) OADC (Becton, Dickinson and Company), 0.5% (v/v) glycerol (Sigma) and 0.05% (v/v) Tyloxapol (Sigma) at 37°C with stirring until exponential growth phase. *M. tuberculosis* H37Ra harboring pASTA3 plasmid (Add gene ID: 24657), which carries the genes for tdTomato protein and for hygromycin resistance were cultured in the same medium supplemented with 50 μg/ml hygromycin (Sigma-Aldrich) for selection.

Prior to any experiment, mycobacterial cultures at exponential growth phase were centrifuged at 3000 g, 37°C for 10 minutes, washed in phosphate buffer saline (PBS) pH 7.4, centrifuged again in the same conditions and resuspended in the appropriate medium. An individualization procedure was also performed in order to disaggregate bacterial clumps. A single-bacillus suspension was obtained by passing 20 times through a sterile syringe equipped with a 21-gauge needle, followed by ultrasonic treatment of bacterial suspensions in an ultrasonic water bath for 5 minutes. The remaining bacterial clumps were eliminated by centrifugation at 350 g for 1 minute and single-cell suspension was verified by light microscopy.

2.2. THP-1 cell line

Human acute monocytic leukemia cell line, THP-1 (TIB-202, ATCC), were maintained in RPMI-1640 Glutamax medium (Gibco), containing 10% (v/v) FBS (Gibco), 1% (v/v) L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco) at pH 7.4, 1x MEM-non essential amino acids (Gibco), 100 IU/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) and incubated in all the experiments at 37°C in 5% CO₂ atmosphere.

The concentration of THP-1 monocytes was determined by flow cytometer 48 hours prior to infection and cells were seeded at 75000 cells/well in infection medium (cell culture medium without penicillin and streptomycin) supplemented with 20 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) to induce differentiation of monocytes into macrophages. Cells were seeded onto 96-well microplates (Corning) with flat bottom transparent or flat bottom black when mycobacterial fluorescence measurement was required in infection assays. After 24 hours post-seeding, the medium was replaced with medium without PMA for more 24 hours, allowing macrophages to restore a resting phenotype.

2.3. HEK 293T cell line

Human embryonic kidney 293T cells, HEK 293T (CRL-11268, ATCC), were maintained in Dulbecco’s Modified Eagle medium (DMEM) (Gibco), supplemented with 10% (v/v) FBS, 100 IU/ml
penicillin and 100 μg/ml streptomycin and incubated in all the experiments at 37°C in 5% CO₂ atmosphere.

Cells were seeded, 24 hours before the experiments, in DMEM medium without penicillin and streptomycin at 2x10⁴ cells/well onto 96-well microplates (Corning).

2.4. Transformation of *M. tuberculosis* H37Ra with pASTA3 plasmid

2.4.1. Propagation of pASTA3 plasmid in *Escherichia coli* (E. coli)

The plasmid pASTA3 (Add gene ID: 24657) (Figure 3) is suitable for tdTomato expression in different bacterial species and contains a hygromycin resistance gene for selection (Carroll et al., 2010).

After 10 minutes of incubation on ice, a mixture containing chemically competent *E. coli* and 100 ng pASTA3 DNA was placed at 42°C for 45 seconds (heat-shock), followed by 10 minutes on ice to restore membrane integrity. Transformed cells were inoculated into SOC medium (Sigma) and incubated at 37°C with stirring for 1 hour. Bacterial cultures were plated in Luria-Bertani (LB) (Sigma) agar medium containing hygromycin and incubated overnight at 37°C (adapted from Hanahan, 1983). At the following day, fluorescence microscopy confirmed that the bacterial cells had red fluorescence, as a result of tdTomato fluorescent protein expression. Transformants were grown in LB broth plus 50 μg/ml hygromycin and incubated at 37°C with stirring overnight. The plasmid pASTA3 was isolated using PureLink™ Quick Plasmid Miniprep Kit, according to suppliers instructions (Invitrogen).

2.4.2. Electroporation of *M. tuberculosis* H37Ra with pASTA3 plasmid

*M. tuberculosis* H37Ra was grown in mycobacterial medium at 37°C with stirring until OD₆₀₀nm = 0.5–1. In order to obtain electrocompetent cells, bacterial suspension was incubated on ice for 90 minutes to improve the transformation efficiency and centrifuged at 3000 g for 10 minutes. The pellet was washed 3 times in ice-cold 10% (v/v) glycerol, reducing the volume each time (10 ml, 5 ml and 2.5 ml).

A mixture containing electrocompetent mycobacteria and 1μg pASTA3 plasmid DNA was left on ice for 10 minutes and transferred to a 0.2 cm electrode-gap electroporation cuvette, previously chilled on ice. The cuvette was placed in an electroporation chamber and subjected to one single pulse of 1.8 kV, 25 μF, with the pulse-controller resistance set at 200 Ω and then placed on ice for 10 minutes. The transformants were inoculated into mycobacterial medium and incubated with stirring at 37°C for 36 hours to express the hygromycin resistance gene carried on the DNA. Bacterial cells were harvested by centrifugation at 3000 g for 10 minutes and plated out suitable dilutions on mycobacterial solid medium containing Middlebrook’s 7H10 agar (Becton, Dickinson and Company), 0.5% (v/v) glycerol and 10% (v/v) OADC. Mycobacterial red fluorescence was confirmed by fluorescence microscopy after 2 weeks of incubation at 37°C. Red fluorescent colonies were inoculated in mycobacterial broth medium plus 50 μg/ml hygromycin and incubated with stirring at 37°C. The transformants were frozen in 20% glycerol at -80°C (Carroll et al., 2010).
Figure 3 – pASTA3 plasmid. Description and features: pASTA3 plasmid is a vector suitable for bacterial expression, and results from a codon-optimised tdTomato in pSMT3-S. Psmyc – strong mycobacterial promoter; tdTomato – inserted gene that codifies tandem dimmer tomato, a red fluorescent protein; pAL5000, mycobacterial replicon; Hygromycin B – Hygromycin resistance gene for bacterial selection; Ecoli OriE: E. coli origin of replication (Carroll et al., 2010).

2.5. Optical density and fluorescence measurements

Optical density and fluorescence measurements were performed in Tecan’s M200 spectrophotometer. Optical density at 600nm (OD$_{600nm}$) was measured to determine mycobacterial concentration (OD$_{600nm}$=0.1 is equivalent to 1x10$^7$ bacteria/ml). Relative fluorescence units (RFU) of *M. tuberculosis* H37Ra (tdTomato) were measured at an excitation of 554 nm, an emission of 586 nm and gain 200. RFU of cells incubated with alamarBlue reagent (Invitrogen) were measured at an excitation of 570 nm, an emission of 595 nm and gain 100.

2.6. Quantification of *M. tuberculosis* H37Ra in vitro

*M. tuberculosis* H37Ra (tdTomato) cultures were resuspended in infection medium and individualized as described above. After OD$_{600nm}$ determination, serial two-fold dilutions were performed in a 96-well microplate with flat bottom black and fluorescence was measured in Tecan’s M200 spectrophotometer. Samples were analyzed in triplicate and wells with infection medium were used as blanks.

2.7. Quantification of intracellular mycobacteria

THP-1 macrophages were infected with a multiplicity of infection (MOI) of 1, 2.5 or 5 mycobacteria/macrophage and incubated for 3 hours to allow the uptake of bacteria. Macrophages
were washed 3 times with infection medium to remove non-internalized bacteria and incubated for 21 hours in infection medium plus 10 µg/ml gentamicin (Gibco) to kill the remaining extracellular bacteria. Infected cells were incubated for five days, changing medium every other day.

Mycobacterial growth was determined by fluorescence measurement and/or CFU counting. At day 1, 3 and/or 5 post-infection, bacterial fluorescence was measured in Tecan’s M200 spectrophotometer, followed by cell lysis in distilled water containing 0.5% (v/v) IGEPAL (Sigma-Aldrich). Serial dilutions were performed in distilled water and plated on Middlebrook’s 7H10 agar (Difco) supplemented with 10% (v/v) OADC and 0.5% (v/v) glycerol. CFUs were counted after 3 weeks of growth at 37°C.

2.8. Fluorimetric quantification of THP-1 macrophages

The active ingredient of alamarBlue reagent (Invitrogen) is resazurin, a non-fluorescent dye, which is converted to the bright red–fluorescent resorufin via the reduction reactions of metabolically active cells. The amounts of fluorescence produced are proportional to the number of living cells, generating a quantitative measurement of cell viability (Invitrogen).

Serial two-fold dilutions of THP-1 monocytes were seeded onto a 96-microplate with flat bottom transparent (Corning) and differentiated into macrophages as described above. 48 hours post-seeding macrophages were incubated in infection medium containing 10% (v/v) alamarBlue and fluorescence was measured after 3 hours. Wells containing only infection medium plus 10% (v/v) alamarBlue were used as blanks.

2.9. Determination of minimum puromycin concentration to kill THP-1 cells

THP-1 cells were seeded onto 96-well microplates (Corning) at 5x10^4 cells/well in cell culture medium supplemented with different puromycin concentrations (1.25, 2.5, 5 and 10 µg/ml). Cells seeded in puromycin-free medium were used as controls. After 4 days of incubation, cells were incubated in cell culture medium containing 10% (v/v) alamarBlue reagent and fluorescence was measured after 3 hours.

Wells containing only infection medium plus 10% (v/v) alamarBlue were used as blanks.

2.10. RNA interference (RNAi) method to knockdown vesicular traffic genes

2.10.1. The shRNA lentiviral library

The RNAi Consortium (TRC) is a public-private effort whose mission is to create a short hairpin RNA (shRNA) library as well to validate tools and methods for functional genomics research and make them available to scientific community. The TRC library was designed to cover entire human and mouse genes and includes 3-5 shRNA constructs to target each messenger RNA (mRNA) transcript in order to produce different levels of gene knockdown. This library allows high-efficiency delivery of the shRNA and enables stable long-term gene suppression in a broad range of cell types (Moffat et al., 2006; Root et al., 2006).
The library was cloned into a lentiviral vector, pLKO.1 (Figure 4), which drives shRNA expression from a human U6 promoter and carries a puromycin-resistance gene to select the transduced cells. Self-inactivated viruses are generated using a three-plasmid packaging system: the shRNA-containing hairpin-pLKO.1 plasmid, the packaging plasmid containing the HIV gag, pol and rev genes (pCMV-dR8.91) and an envelope plasmid with the gene encoding the Vesicular stomatitis virus (VSV)-G coat. This reduces the possibility of recombination to create replication competent virus (Moffat et al., 2006; Root et al., 2006). When cells are infected with lentivirus, the viral genome is integrated into the host cell genome and the cassette produces a shRNA molecule that will be processed by the RNAi machinery and target the degradation of the complimentary mRNA.

Experimental procedures related to plasmid preparation, lentivirus production and lentivirus infection were performed as described at the TRC website: (https://www.broadinstitute.org/genome_bio/trc/publicProtocols.html). The plasmids used in this thesis were a kind gift of Dr. Luis Moita from Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa.

2.10.2. Hairpin-pLKO.1 plasmid preparation

Well sterile growth plates, 96 deep (Corning) were pre-filled with 1.2 ml of terrific broth (TB) medium (Invitrogen) containing 100 µg/ml carbenicillin (Sigma-Aldrich). The hairpin-pLKO.1-containing bacterial glycerol stocks were inoculated at 5 µl/well into the TB pre-filled 96 deep well plates and grown for 16 hours at 37°C with stirring (300 rpm). After incubation, plates were centrifuged at 1500 g for 8 minutes at 4°C. Bacterial pellets were resuspended in 200 µL of Resuspension buffer with RNase A (50 mM Tris-HCl, 10 mM EDTA pH 8.0 and 0.1 mg/ml RNase A) and further lysed in 210 µl lysis buffer containing Alkaline Protease (200 mM NaOH, 1% (w/v) SDS and 25 units/ml of Alkaline Protease) for 4 minutes at room temperature. Bacterial cell lysates were neutralized by adding 300 µl Neutralization buffer (3.75 M Guanidinium Hydrochloride, 0.9 M KOAc and 1.4 M HOAc, pH 4.35). After centrifugation at 3000 g for 30 minutes at 4°C, the clear liquid lysate from each well was transferred to a clarification filter plate (Whatman) and centrifuged again at 3000 g for 5 minutes at 4°C. Lysates were transferred to a pDNA binding plate (Whatman), centrifuged at 1800 g for 2 minutes at 4°C and supernatants discarded. Two washes were performed by centrifuging the pDNA binding plates at 1800 g for 2 minutes at 4°C in the presence of 600 µl of Wash buffer (480 ml of 100% ethanol added to a 120 ml of a solution containing 15 mM NaCl, 40 mM Tris-HCl and 25 mM Tris, pH 6.65). The plasmid DNA was eluted by adding 140 µl of Elution buffer (10 mM Tris-HCl, pH 8.0), followed by 10 minutes of incubation at room temperature and centrifugation at 1800 g for 5 minutes at 4°C.

Plasmid DNA concentration was assayed by spectrophotometry in Tecan’s M200 spectrophotometer using Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) following manufacturer’s protocol, and further stored at -80°C. When higher concentrations of specific plasmids were required, higher volumes of cultures of the corresponding bacterial glycerol stocks were grown as described above and plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen) and plasmid DNA concentration was determined with Nanodrop ND 1000 (Thermo Scientific).
2.10.3. Lentivirus production

There was produced shRNA lentivirus targeting human intracellular traffic genes already reported to be involved in \textit{M. tuberculosis} infection.

HEK 293T cells were seeded at \(2 \times 10^4\) cells/well onto 96-well microplates (Corning) in DMEM medium without penicillin and streptomycin. After 24 hours of incubation, cells were co-transfected with the packaging plasmid (10 ng of pCMV-dR8.91), the envelop plasmid (100 ng of VSV-G/pMD2.G) and 10 ng Harpin-pLKO.1 plasmid, using FuGENE HD transfection reagent (Roche) in Optimem medium (Gibco) in a final volume of 35 µl/well. After 30 minutes, the mixture was added to the 60\%-80\% confluent cells. After 24 hours of incubation, the medium was replaced by 180 µl of viral harvest medium (DMEM medium containing 30\% FBS). The supernatants with lentiviral particles (150 µl) were collected after 24 hours and stored at -80°C.

Figure 4 – Vector map for the pLKO.1 lentiviral vector. The self-inactivating lentiviral vector backbone contains elements for efficient viral packaging and shRNA expression. These include \(\Psi\), the lentiviral packaging site; RRE, the Rev-responsive element; cPPT, central polypurine tract. RSV 5' LTR is a hybrid of the Rous Sarcoma virus promoter and the HIV 5' LTR. SIN 3' LTR is the HIV 3' LTR with a self-inactivating U3 deletion. Expression of the shRNA is driven by the human U6 promoter (hU6). The lentiviral vector also contains the mammalian selection marker puromycin resistance gene (PAC) under the control of the PGK promoter as a mammalian selection marker. pUCori is the bacterial origin of replication of the plasmid, F1 ori is the single-stranded phage F1 origin of replication, and AmpR is the ampicillin resistance gene (Root et al., 2006).
2.10.4. Silencing of vesicular traffic genes in THP-1 monocytes

For THP-1 monocytes transduction, 5x10^4 cells/well were seeded in cell culture medium onto round-bottom 96-well microplates (Thermo scientific nunc) and centrifuged at 450 g, 37°C for 5 minutes. After supernatant removal, cells were infected with 10 µl lentivirus and resuspended 3 times. Besides having lentivirus for target genes, the screen also included a GFP expressing lentivirus for transduction control and a scrambled shRNA as a negative control, which has no target in the human genome. Cell culture medium (40 µl) supplemented with 8 µg/ml Polybrene (Hexadimethrine bromide; Sigma) was added to each well. To improve viral infection and decrease the length of exposure of cells to Polybrene, cells were centrifuged at 900 g, 37°C for 90 minutes. After centrifugation, the medium was replaced for cell culture medium and cells were incubated for 48 hours. Transduced cells were selected with 5 µg/ml Puromycin (Sigma) for 48 hours and then split and expanded to another microplates to be used in further assays, namely infection with mycobacteria, western blot and quantitative real time polymerase chain reaction (qRT-PCR).

2.11. RNA extraction, Reverse transcription and Quantitative Real-time PCR (qRT-PCR)

THP-1 transduced cells were lysed with TRIzol (Invitrogen) and total RNA was isolated and purified according to manufacturer’s protocol. RNA concentration was determined in Nanodrop ND 100 (Thermo Scientific) and RNA was reverse transcribed to complementary DNA (cDNA) with Superscript II reverse transcriptase following the supplier instructions (Invitrogen). The reaction volume (20 µl) for cDNA synthesis contained 200 ng of Random Hexamer Primers (Fermentas), 1 mM deoxyribonucleotide triphosphates (dNTPs) Mix, First Strand Buffer 1x (50 mM Tris-HCl, 75 mM KCl, 3mM MgCl2), 10 mM dithiothreitol (DTT), 40 U RNaseOUT, 200 U SuperScript II reverse transcriptase and 1µg of total RNA. The mixture was incubated at 42°C for 50 minutes, and then the reaction was inactivated by heating at 70°C for 15 min.

For qRT-PCR, cDNA (diluted 1:5 in water) was amplified in the presence of SYBR Green PCR master mix (Applied Biosystems), which contains SYBR Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, passive reference and the manufacturer’s optimized buffer components. Each reaction was made using 10 µl of SYBR green PCR master mix, 1 µl of each forward and reverse primer (10µM), 3 µl of water and 5 µl of cDNA. Primers (Eurofins MWG Operon) (Table 1) for qRT-PCR were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) and their specificity was confirmed using Basic Local Alignment Search Tool (BLAST) available from the “National Center for Biotechnology Information”. Reactions were performed in ABI 7000 Sequence Detection System with the ABI Prism SDS 7000 software (Applied Biosystems) with the following conditions: 10 minutes at 95°C to activate the AmpliTaq Gold® DNA polymerase followed by 40 cycles of denaturing at 95°C for 15 minutes and annealing/extendning at 60°C for 1 minute. A dissociation step was also performed to verify that the primers were binding only to their specific products. All samples were analyzed in duplicate and normalized against the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control reactions were made using water or total RNA instead of cDNA. The relative expression of each gene was calculated using Pfaffl’s method (Pfaffl, 2001).
Table 1 – List of primers used in qRT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>AAGGTGAAGGTCGGAGTCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AATGAAGGGGTCAATTGATGG</td>
</tr>
<tr>
<td>Rab14</td>
<td>Forward</td>
<td>ATGGCAACTGCAACCATAACAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCTCCGTTGTAACGCCCTA</td>
</tr>
<tr>
<td>Rab34</td>
<td>Forward</td>
<td>TGAGGGCTGAGGAGACTGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCTACCCTCTGTGGGGAAT</td>
</tr>
<tr>
<td>Syntaxin 4</td>
<td>Forward</td>
<td>TGGAGCTCATCAACAAGTGCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TAAGGCCTGTGAGTCACCT</td>
</tr>
<tr>
<td>Rab10</td>
<td>Forward</td>
<td>CACTTCTTCCCTTGCTGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAAGGCATCTGGGACACATT</td>
</tr>
<tr>
<td>Rab7</td>
<td>Forward</td>
<td>CGCTTCTGTCCCTCGGTTTAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACCATCACCTCCTTGGTCAG</td>
</tr>
</tbody>
</table>

2.12. Western-blot

Transduced cells were denatured at 95°C for 5 minutes in the presence of Laemmli buffer (BioRad), before loaded 20 µl on 12% Sodium Dodecyl Sulphate polyacrilamide gels. Electrophoresis was performed at 200 V for 1 hour. Proteins were transferred onto nitrocellulose membranes with an electrical intensity of 30 V overnight in a cold room. At the following day, membranes were blocked for 1 hour at room temperature. The blocking buffer contains Tris-buffered saline plus 0.05% (v/v) Tween 20 (TBS-T) and 5% (w/v) Bovine Serum Albumin (BSA) in order to avoid unspecific binding of the detection antibody. Following that, membranes were incubated with primary antibodies (Table 2) diluted in TBS-T plus 1% (w/v) BSA for 2 hours at room temperature. After 3 washes in TBS-T, membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2) for 1 hour at room temperature. After 3 washes in TBS-T, membranes were stained for 5 minutes with the chemiluminescent reagent, Luminata Crescendo Western HRP Substrate (Millipore). Chemiluminescence was detected and captured with ChemiDoc XRS+ system (Bio-Rad).

Table 2 – List of antibodies used to perform Western Blot, dilutions and incubation conditions

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Reference</th>
<th>Company</th>
<th>Dilution</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab14</td>
<td>sc-271401</td>
<td>Santa Cruz Biotecnology</td>
<td>1:300</td>
<td>2 hours, RT</td>
</tr>
<tr>
<td>Rab 34</td>
<td>ab73383</td>
<td>Abcam</td>
<td>1:1000</td>
<td>2 hours, RT</td>
</tr>
<tr>
<td>Beta-tubulin</td>
<td>ab20775</td>
<td>Abcam</td>
<td>1:4000</td>
<td>2 hours, RT</td>
</tr>
<tr>
<td>anti-mouse</td>
<td>172-1011</td>
<td>Bio-Rad</td>
<td>1:4000</td>
<td>1 hours, RT</td>
</tr>
<tr>
<td>anti-rabbit</td>
<td>170-6515</td>
<td>Bio-Rad</td>
<td>1:4000</td>
<td>1 hours, RT</td>
</tr>
</tbody>
</table>
2.13. Statistical analysis

Statistical analysis was made using SigmaPlot 11.0. Analyses were performed using the unpaired Student’s *t* parametric test or analysis of variance (ANOVA) parametric tests. Normal distributions were confirmed using the Kolmogorov-Smirnov test. To make multiple comparisons versus control group was used the Holm-Sidak method. The differences were considered statistically significant when *p*<0.05. To determine the correlation between two variables a linear regression was used as well as a Pearson Product Moment Correlation.
3. Results

3.1. Fluorimetric methods as alternative tools to study *M. tuberculosis* infection

3.1.1. Fluorescence intensity of tdTomato allows *M. tuberculosis* quantification in vitro

Tandem dimmer tomato (tdTomato) is a bright fluorescent protein. Carroll and colleagues demonstrated that tdTomato fluorescence is detectable in the virulent strain *M. tuberculosis* H37Rv (Carroll et al., 2010). To investigate if this fluorescent reporter is functional in the avirulent strain, *M. tuberculosis* H37Ra, the strain was transformed with pASTA3 plasmid, which carries the gene for tdTomato protein. To test if fluorescence intensity of tdTomato could be used to quantify *M. tuberculosis* H37Ra *in vitro*, several concentrations of bacteria were measured by fluorescence and the results were compared with the optical density at 600nm (OD<sub>600nm</sub>) method commonly used to quantify mycobacteria.

Our results show statistical strong correlation between the two measurements (r<sup>2</sup>=0.9986; p<0.05), which suggests the great potential of this fluorimetric method to accurately quantify *M. tuberculosis* H37Ra *in vitro* (Figure 5).

![Figure 5](image_url)

**Figure 5 - Quantification of *M. tuberculosis* H37Ra tdTomato by fluorescence (RFU) and optical density (OD<sub>600nm</sub>).** Data is represented as the mean of RFU (excitation 554, emission 586, gain 200) ± standard deviation of three replicates for each OD<sub>600nm</sub> value. Wells with infection medium were used as blanks for RFU. The line represents a linear regression between RFU and OD<sub>600nm</sub> (r<sup>2</sup>=0.9986; p<0.05 by Pearson Correlation).
3.1.2. Intracellular survival of *M. tuberculosis* H37Ra might be assessed by tdTomato fluorescence intensity

Since we obtained a positive correlation between the two methods - optical density and tdTomato fluorescence – *in vitro*, we then investigated if fluorimetry could be used *ex vivo* to quantify *M. tuberculosis* H37Ra (tdTomato) survival within THP-1 macrophages.

THP-1 macrophages were infected with *M. tuberculosis* H37Ra (tdTomato) at MOI 1 and 5. Mycobacterial burden was determined after 3 hours, 1 day and 3 days post-infection by fluorescence measurement. To validate our data, the results of intracellular mycobacterial kinetics obtained by fluorescence were compared with those obtained using the traditional method of CFU counting (Figure 6). We observed that an increase in MOI corresponds to an increase in CFU (Figure 6a) as well as an increase in RFU (Figure 6b). The results obtained by CFU determination validate indeed *ex vivo* those obtained by fluorimetry. The number of intracellular bacteria decreased at day 1 and increased at day 3 post-infection (Figure 6). However, the signal to background ratio was very low, especially when a MOI 1 was used. The ratio was never higher than 1.2 for a MOI 1 and was at most 1.7 for a MOI 5.

Concerning the kinetic of intracellular bacterial growth, the results obtained by fluorescence show a linear dependence relatively to those obtained by CFU counting and the statistical correlation between the two measurements was 0.8002 (p<0.05) (Figure 6c). These results indicate that fluorimetric quantification of fluorescent intracellular mycobacteria as the potential to overcome the constraints of the CFU counting, being a faster and less laborious method.
THP-1 macrophages were infected with *M. tuberculosis* H37Ra (tdTomato) at MOI 1 and 5. Intracellular bacteria burden was assessed by CFU counting and fluorescence measurement at 0, 1 and 3 days post-infection.  

**a)** Quantification of bacteria by CFU counting represented as CFU/Sample;  
**b)** Quantification of bacteria by fluorescence measurement represented as RFU after background removal (excitation 554, emission 586, gain 200). Non-infected cells were used as blanks for RFU normalization. Data are represented as the mean ± standard deviation of three replicates.  
**c)** Correlation between RFU and CFU/Sample. The line represents a linear regression between CFU/Sample and RFU from data of a) and b), respectively ($r^2 = 0.8002$; p<0.05 by Pearson Correlation).

---

**Figure 6 - Quantification of intracellular mycobacteria by fluorimetry and CFU counting in THP-1 cells.**

THP-1 macrophages were infected with *M. tuberculosis* H37Ra (tdTomato) at MOI 1 and 5. Intracellular bacteria burden was assessed by CFU counting and fluorescence measurement at 0, 1 and 3 days post-infection. **a)** Quantification of bacteria by CFU counting represented as CFU/Sample; **b)** Quantification of bacteria by fluorescence measurement represented as RFU after background removal (excitation 554, emission 586, gain 200). Non-infected cells were used as blanks for RFU normalization. Data are represented as the mean ± standard deviation of three replicates. **c)** Correlation between RFU and CFU/Sample. The line represents a linear regression between CFU/Sample and RFU from data of a) and b), respectively ($r^2 = 0.8002$; p<0.05 by Pearson Correlation).
3.1.3. Low sensitivity of the fluorimetric method to detect low burdens of intracellular mycobacteria

Assays performed in our laboratory demonstrated that the avirulent strain *M. tuberculosis* H37Ra does not grow in THP-1 macrophages until 3 days post infection and the number of CFU slightly decreases and stabilizes for the following days. It was also observed, with a light microscope, macrophage death at day 7 post-infection (data not shown). For these reasons we selected day 5 post-infection as a reference time-point for our studies. Therefore the intracellular *M. tuberculosis* H37Ra (tdTomato) in THP-1 were quantified by fluorescence measurement (Figure 7a) and the results compared with those obtained by CFU counting (Figure 7b). As in the previous experiment, the fluorescent signal to background ratio was very low at a MOI 1 here, and MOI 2.5 was used.

As depicted in Figure 7a, there were only statistically significant differences between the fluorescence from mycobacteria and blank (non-infected cells) for samples where MOIs were 2.5 and 5. The new set-up fluorimetric method was unable to quantify intracellular bacteria in samples infected at lower MOI such as 1, while this quantification was possible by CFU counting (Figure 7b). In addition, the signal to background ratio was around 2.7 for MOI 5, but at most 1.3 for MOI 2.5 (Figure 7a).

We chose a MOI 1 (low infectivity) for the subsequent experiments to screen vesicular traffic factors involved in mycobacterial infection. Despite the good correlations between fluorescence measurement and conventional methods of mycobacterial quantification, we considered that the fluorimetric method based on tdTomato fluorescence is not sensitive enough to accomplish our purpose. It was not possible to discriminate background from fluorescent mycobacteria using MOI 1. Moreover, if the screen includes genes manipulated by mycobacteria to survive and replicate, its silencing could increase bacterial killing. Therefore, bacterial burden at day 5 post-infection would be even lower than the obtained in this experiment and would be detected as CFU, but not by fluorimetry.

**Figure 7 - Comparison between fluorimetry and CFU counting to quantify *M. tuberculosis* H37Ra in THP-1 cells.** THP-1 macrophages were infected with *M. tuberculosis* H37Ra (tdTomato) at MOI 1, 2.5 and 5. **a)** RFU (excitation 554, emission 586, gain 200) at day 5 post-infection. **b)** CFU/Sample at day 5 post-infection. Data are represented as the mean ± standard deviation of three replicates (ANOVA; Holm-Sidak; *p*<0.05). *p*<0.05 and ** *p*<0.001 comparatively to blank (non-infected cells).
3.1.4. Fluorimetric quantification of THP-1 cells using alamarBlue

In most published data quantification of intracellular mycobacteria is usually performed by CFU without viable macrophage ratio correlation. This could lead to inaccurate quantification of mycobacterial infection.

The alamarBlue is a reagent used to determine cell viability. The active component of alamarBlue is converted into a fluorescent dye when metabolized by viable cells. Different cell numbers of THP-1 cells were seeded with PMA to differentiate monocytes into macrophages. After 48 hours macrophages were incubated with alamarBlue for 3 hours and fluorescence was measured with a fluorimeter. As seen in Figure 8, fluorescence intensity of alamarBlue is positively correlated with the number of macrophages ($r^2 = 0.9936; p<0.05$), supporting that alamarBlue method is a valuable tool to accurately quantify viable THP-1 macrophages during infection.

![Figure 8](image)

**Figure 8** - Fluorimetric quantification of THP-1 macrophages using alamarBlue reagent. Data is represented as the mean RFU (excitation 570, emission 595, gain 100) ± standard deviation of three replicates for each cell number. The line represents a linear regression between RFU and cell number ($p<0.05$ by Pearson Correlation).

3.2. The role of host vesicular traffic factors in mycobacterial infection

3.2.1. Determination of minimum puromycin concentration to select transduced cells

Our goal was to find Rab GTPases and SNAREs involved in host susceptibility or resistance to *M. tuberculosis* infection. Therefore, we first produced a lentiviral vector library that was used to silence our targets in THP-1 monocytes. This method allows persistent expression of siRNAs targeting the mRNAs of interest, which promotes target knockdown and also confers puromycin resistance to transduced cells. In order to determine the minimum puromycin concentration to further select lentiviral transduced cells, THP-1 cells were seeded with different puromycin concentrations and
alarBlue method was performed at day 4 post-seeding to evaluate cell viability. Cells incubated with puromycin-free medium were used as controls.

The results demonstrate that cell death increases with higher puromycin concentrations (Figure 9). The fluorescence intensity of cells incubated with 1.25 µg/ml puromycin was similar to control, which indicates that this concentration is insufficient to affect cell survival. On the other hand, fluorescence data indicate that the number of cells incubated with 10, 5 and 2.5 µg/ml puromycin was significantly different relatively to control (cells without puromycin). Comparing the fluorescence of samples incubated with 10 µg/ml and 5 µg/ml puromycin with the blank (alarBlue), no significant differences were achieved. Given these results, we determined that 5 µg/ml is the minimum puromycin concentration that causes total cell death.

![Figure 9 - Fluorimetric determination of puromycin concentration that kills THP-1 cells.](image)

**Figure 9 - Fluorimetric determination of puromycin concentration that kills THP-1 cells.** Data are represented as the mean RFU (excitation 570, emission 595 and gain 100) ± standard deviation of three replicates for each puromycin concentration. Cells incubated with 0 µg/ml puromycin were used as controls and alamarBlue diluted in infection medium was used as a blank for RFU. (ANOVA; Holm-Sidak; p<0.05 ) * p<0.05 comparatively to the control).

### 3.2.2. The silencing of Rab GTPases and SNAREs affects *M. tuberculosis* H37Ra survival

After lentiviral transduction and selection with puromycin, THP-1 monocytes were differentiated into macrophages and infected with *M. tuberculosis* H37Ra at a MOI 1. The number of intracellular mycobacteria was determined at day 5 post-infection by CFU counting (Figure 10a) and the viability of THP-1 macrophages was assessed using alamarBlue reagent (Figure 10b). Mycobacterial burden inside THP-1 macrophages knocked-down for vesicular traffic genes was compared with the scramble control (not targeting any human gene), and differences were termed “hot genes”. We designated as “hot genes”, genes whose knockdown with at least one shRNA resulted in intracellular mycobacterial burden with statistically significant differences relatively to scramble control (ANOVA; Holm-Sidak; p<0.05). The results of this screen are a product of 3 independent experiments where 13 mRNAs were individually knocked-down in THP-1 cells with several shRNAs targeting each one. From the 13
targets analyzed we present here the results for 5, whose levels of knockdown were confirmed by qRT-PCR (Figure 11).

As determined using alamarBlue, lentiviral silencing of the targets mRNA did not significantly affect macrophage viability comparatively to control with all the shRNAs tested (Figure 10b). Therefore, it was possible to correlate the effects in mycobacterial survival with the levels of mRNA knockdown.

As shown in figure 10a, the number of intracellular mycobacteria was significantly higher in cells silenced for Rab7 and for Rab34 comparatively to control. The 90% knockdown of Rab7 mRNA (Figure 11) increased 50% the number of intracellular bacteria (Figure 10a). The lentiviral silencing targeting Rab34 mRNA decreased mRNA levels around 60% with shRNA1 and 70% with shRNA2 (Figure 11), which led to an augment of 30% in mycobacterial survival (Figure 10a). The enhanced capacity of *M. tuberculosis* H37Ra to survive within THP-1 macrophages silenced for Rab7 and Rab34 indicates that these proteins regulate mechanisms employed by macrophages to kill mycobacteria.

The expression of Rab14 mRNA after lentiviral silencing was approximately 50% using shRNA1 and 70% with shRNA2 (Figure 11). These levels of mRNA were not statistically different to scramble control. Nonetheless, the reduction in Rab14 mRNA was sufficient to significantly decrease the number of intracellular mycobacteria, approximately 20% with shRNA1 and 40% with shRNA2. Our data indicate that Rab14 is important in maintaining intracellular *M. tuberculosis* H37Ra survival in THP-1 macrophages.

In contrast to the previous results, the number of intracellular mycobacteria after lentiviral silencing of Rab10 and Syntaxin 4 was similar to control (Figure 10a). The number of viable macrophages decreased around 30% in cells transduced for Rab10 and 20% for Syntaxin 4, however these differences were also not statistically significant (Figure 10b). In order to verify whether the lack of effect were due to inefficient RNA silencing, the validation of the knockdown was assessed by qRT-PCR. In fact, the levels of Rab10 mRNA decreased 50% and Syntaxin 4 mRNA levels decreased 50% in cells transduced with shRNA1 and 30% in cells transduced with shRNA2 (Figure 11). Despite the reduced levels of Rab10 and Syntaxin 4 mRNAs after lentiviral transduction, intracellular *M. tuberculosis* H37Ra survival was not affected. The knowledge of the biological functions of these proteins could explain if they are passive markers during phagosomal maturation or are naturally manipulated by mycobacteria and already excluded from *M. tuberculosis* H37Ra-containing phagosomes.
Figure 10 - Screen of vesicular traffic genes with potential role in *M. tuberculosis* H37Ra infection. THP-1 monocytes were transduced with short-hairpin RNAs specific for the genes of interest or with scramble control using lentiviral vectors. After selection with puromycin and differentiation with PMA, macrophages were infected with *M. tuberculosis* H37Ra at a MOI 1 for 3h. At day 5 post-infection cells were incubated with alamarBlue and fluorescence was measured after 3 hours. Quantification of bacteria at day 5 post-infection was made by CFU counting. 

**a)** Percentage intracellular *M. tuberculosis* H37Ra in THP-1 macrophages knocked-down for Rab GTPases and SNAREs expression. CFU values are relative to scramble control shRNA and result from the mean ± standard deviation of 3 independent experiments (ANOVA; Holm-Sidak; p≤0.05), (t-test; p≤0.05). * p<0.05 comparatively to control. 

**b)** Fluorimetric quantification of THP-1 macrophages using alamarBlue represented as RFU (excitation 570, emission 595, gain 100).
3.2.3. Validation of the host factors involved in *M. tuberculosis* H37Ra infection and comparison with the virulent strain H37Rv

Our results point for a validation as “hot targets” for Rab7, Rab14 and Rab34, since the knockdown of the corresponding mRNA affects mycobacterial survival at day 5 post-infection. In order to confirm the phenotypes observed in cells silenced for Rab14 and Rab34, a second round of silencing experiments were performed with new batches of lentivirus. To reveal if both avirulent and virulent mycobacterial strains were similarly affected, lentiviral transduced cells were infected with *M. tuberculosis* H37Ra and were compared to the strain H37Rv. Mycobacterial survival and macrophage’ viability was determined at day 5 post-infection by CFU counting and alamarBlue method, respectively (Figure 12). To confirm the implications of mRNA silencing, the levels of the target protein expression were assessed by western blot (Figure 13).

3.2.3.1. Rab14 knockdown has a different impact in intracellular survival of avirulent and virulent mycobacteria

As shown in Figure 12a, the knockdown of Rab14 reduced the number of intracellular *M. tuberculosis* H37Ra, which is in agreement with the preliminary assays (Figure 10a). For the two shRNAs tested, the number of intracellular mycobacteria was around 25% lower relatively to scramble control (Figure 12a) for a number of viable macrophages identical in both experimental conditions (Figure 12b). As assessed by western blot, Rab14 expression in THP-1 cells was about 93% reduced when shRNA1 was used for silencing (Figure 13). This level of knockdown was almost 55% higher...
than determined by qRT-PCR (Figure 11), suggesting that silencing efficiency was higher in this experiment. It was not possible to verify whether the discrepancies between qRT-PCR and the results of western blot were effectively due to different sensibilities of the techniques or rather by real differences between mRNA and protein levels. In fact, a silencing may occur via RNA degradation or reduced association with ribosomes.

In cells silenced with shRNA2, the level of knockdown quantified by western blot was 30% (Figure 13), which was in accordance with the results obtained by qRT-PCR (Figure 11).

Infection of transduced cells with the virulent mycobacterial strain, *M. tuberculosis* H37Rv, produced different results. The viability of macrophages was similar to control (Figure 12b), although intracellular mycobacterial survival was not affected by Rab14 silencing (Figure 12a).

In contrast to *M. tuberculosis* H37Ra, the capacity of virulent mycobacteria to survive within macrophages seems to be independent of Rab14.

3.2.3.2. Rab34 is only crucial to kill avirulent mycobacteria

Figure 12a shows that Rab34 knockdown in THP-1 cells enhances *M. tuberculosis* H37Ra survival, which is in agreement with our previous results (Figure 10a). The number of intracellular bacteria increased 30% in cells transduced with shRNA1, 26% with shRNA2 (Figure 12a) and no effect on the viability of macrophages were observed in both experiments (Figure 12b). Western blot results show that the levels of Rab34 decreased almost 40% in cells transduced with shRNA1 and around 60% with shRNA2 (Figure 13), while with qRT-PCR performed in the preliminary assays the corresponding results were 41% and 24% (Figure 11).

In cells silenced for Rab34 and infected with *M. tuberculosis* H37Rv, intracellular mycobacterial survival was not affected (Figure 12a). CFU results were not caused by differences in macrophage' viability, since the number of THP-1 macrophages was similar to control (Figure 12b).

Our data demonstrate that Rab34 silencing decrease the capacity of THP-1 macrophages to kill the strain H37Ra, but not the virulent strain.
Figure 12 - The effect of Rab14 and Rab34 knockdown in intracellular survival of avirulent and virulent mycobacteria. THP-1 monocytes were transduced with short-hairpin RNAs specific for Rab14, Rab34 or with scramble control using lentiviral vectors. After selection with puromycin and differentiation with PMA, macrophages were infected with mycobacteria at a MOI 1 for 3h. Mycobacterial survival and macrophage viability was determined at day 5 post-infection. a) Percentage intracellular M. tuberculosis H37Ra in THP-1 macrophages transduced with Rab14, Rab34 or with scramble control. CFU values are relative to control and result from the mean ± standard deviation of 3 independent experiments (ANOVA; Holm-Sidak; p<0.05). * p<0.05 comparatively to control. b) Fluorimetric quantification of THP-1 macrophages using alamarBlue. RFU (excitation 570, emission 595, gain 100) data is relative to control and are represented as the mean ± standard deviation of 3 independent experiments.
Figure 13 - Validation of Rab14 and Rab34 knockdown in transduced THP-1 cells by western-blot. THP-1 monocytes were transduced with short-hairpin RNAs specific for the genes of interest or with scramble control using lentiviral vectors. a) Percentage Rab14 expression relative to scramble control and western blot showing levels Rab14 and β-tubulin. b) Percentage Rab34 relative to scramble control and western blot showing levels of Rab34 and β-tubulin. β-tubulin was used as a protein loading control.
4. Discussion

Vesicular trafficking pathways in eukaryotic cells are regulated by Rab GTPases and SNAREs, which ensure the correct delivery of cargo between vesicles and organelles. These proteins have raised interest in scientific research, since their dysfunction has been associated with several diseases, from cancers and neurological disorders to infections (Hutagalung and Novick, 2011). In particular, the study of these factors during intracellular infections is revealing the mechanisms employed by bacterial pathogens to survive in their hosts and cause diseases (Brumell and Scidmore, 2007; Stein et al., 2012).

Many reports on the literature refer to the interference at the level of these proteins a maturation blockage of *M. tuberculosis*-containing phagosomes, which is the main mechanism manipulated by this pathogen to alter its intracellular localization and avoid destruction (Brumell and Scidmore, 2007; Seto et al., 2011). However, the majority of studies were performed in mice macrophages and do not reveal if these factors are directly implicated in mycobacterial ability to survive intracellularly in a more realistic model of tuberculosis: the human macrophages.

With this work we took advantage of several techniques suitable for large scale studies in order to develop a high-throughput methodology to identify host vesicular traffic factors involved in *M. tuberculosis* infection within human macrophages. We set up a dual fluorescent reporter assay for rapid quantification of intracellular mycobacteria and host viable macrophages. In parallel we performed a lentiviral shRNA based screen of vesicular traffic factors, in order to knockdown Rab GTPases and SNARES of host cells and assess their role on the intracellular mycobacterial survival.

*M. tuberculosis* H37Ra, the counterpart of the virulent strain H37Rv, was selected for our preliminary screen for having less safety constraints. Both mycobacterial strains are derived from their virulent parent strain H37, which was isolated from a patient with pulmonary tuberculosis (Zheng et al., 2008). The cell line THP-1 is a laboratorial model of human macrophages and was suitable for our studies, because of its high efficiency of transduction with lentivirus and its subsequent viability after infection with *M. tuberculosis* (Mishra et al., 2010).

The standard method to determine intracellular mycobacterial survival is based on CFU counting. Since the growth rate of *M. tuberculosis* is about 24 hours, the quantification of bacterial numbers by CFU become not only expensive, but also very time-consuming, especially when working with large numbers of samples (Collins et al., 1998; Meyers et al., 1998). Several recent studies are using fluorescent proteins as alternative tools to monitor cellular growth with high spatial and temporal resolution (Müller-Taubenberger and Anderson, 2007). A great advantage is that the fluorescent signal is not diluted during bacterial replication and can also be read *in vivo* without lysis or permeabilization of the cells. This is appropriate to monitor intracellular bacterial survival throughout several days after macrophage infection, allowing the evaluation using the same samples (Kremer et al., 1995; Meyers et al., 1998; Müller-Taubenberger and Anderson, 2007). Moreover, and in opposition to other’s gene-
reporter methods, such as luciferase, fluorescent proteins do not require the addition of reagents or co-factors (Collins et al., 1998).

As a reporter to monitor \textit{M. tuberculosis} H37Ra growth in THP-1 macrophages we selected tdTomato, since Carroll and colleagues demonstrated that its fluorescence is detectable in \textit{M. tuberculosis} H37Rv when expressed from mycobacterial promoters and without affecting its fitness (Carroll et al., 2010). To achieve our purpose, we transformed \textit{M. tuberculosis} H37Ra with pASTA3 plasmid carrying the gene for tdTomato. The transformation was efficient and the fluorescence of tdTomato was detected after several days of growth in mycobacteria liquid medium. Indeed, fluorescence had a significant positive correlation with optical density, indicating that tdTomato fluorescence permit to discriminate between different bacterial culture concentrations \textit{in vitro} (Figure 5).

We also found that tdTomato fluorescence allows the detection of intracellular \textit{M. tuberculosis} H37Ra until 3 days post-infection by reading fluorescence directly from microplates (Figure 6b). These made possible to obtain the results immediately without spending material, reagents and time, which are drawbacks of the CFU counting method.

Similarly to CFU (Figure 6a), tdTomato fluorescence allowed the discrimination between MOI 1 and MOI 5 throughout the infection (Figure 6b). The growth kinetic was also similar with both methods and strongly correlated (Figure 6c). Through fluorimetric and CFU quantification, we observed that the avirulent mycobacterial strain does not growth in THP-1 macrophages until day 3 post-infection. We previously observed that the number of intracellular bacteria slightly decreases after day 3 post-infection and stabilizes for the following days, with an increase in macrophage death at day 7 post-infection (data not shown). Therefore, we selected day 5 as a reference time-point however, we realized that fluorescent mycobacteria were not detected by fluorimetry when MOI 1 was used (Figure 7a). Nevertheless the presence of viable bacteria in those samples was detected by CFU counting (Figure 7b).

Our goal was to use fluorimetry to quantify \textit{M. tuberculosis} H37Ra during our set up screen of vesicular traffic genes. We selected a MOI 1 to minimize the damage of macrophages and thus be able to quantify intracellular mycobacteria at longest times post infection. On one hand, we were aware that avirulent strains cause macrophage apoptosis more than virulent ones and in a dose-dependent manner with increasing MOIs (Riendeau and Kornfeld, 2003). On the other hand, virulent strains grew significantly more rapidly inside macrophages and consequently higher MOIs can also lead to macrophage disruption due to necrosis (Zhang et al., 1998). Furthermore, a low infectivity reduces the formation of typical mycobacterial clumps during initial infection and is more approximate to what occurs in nature (Riendeau and Kornfeld, 2003; Zhang et al., 1998).

Using the fluorimetric method we were not able to discriminate the background from fluorescent mycobacteria at day 5 post-infection using MOI 1. We achieved statistically significant differences between MOI 2.5 and 5 and background, but the signal to background ratio was very low for MOI 2.5 (Figure 7a). Overall, our data establishes that fluorimetric method based on tdTomato fluorescence is not sensitive enough to accomplish our purposes. We expected that the silencing of genes in THP-1 macrophages could lead to intracellular bacterial burdens inferior to those obtained in this experiment,
which would not be detected by fluorimetry, and consequently will lead to incorrect interpretations of the obtained results. Nonetheless, mycobacteria expressing tdTomato have potential applications for other purposes. Fluorescent mycobacteria are not only detected by fluorimeters but can also be useful in studies using more sensitive devices, such as flow cytometer and fluorescence microscopes.

We adopted the accurate CFU counting technique for our experiments, with adjustments suitable for our high-throughput screen. In fact, we used square dishes containing mycobacterial solid medium, with the capacity for 96 samples, which effectively facilitated the handling of large numbers of samples.

The viability of macrophages is a parameter commonly unknown when determining intracellular mycobacterial survival by CFU counting. This parameter reveals if the number of intracellular bacteria is effectively decreasing or if it is caused by macrophage death. For instance, macrophage death could be enhanced by mechanisms employed by intracellular pathogens or due to the silencing of genes important for host cell survival (Riendeau and Kornfeld, 2003; Zhang et al., 1998). To overcome these limitations we used alamarBlue reagent, a proven indicator of cell viability (Invitrogen). The fluorescence intensity of alamarBlue is strongly correlated with the number of viable THP-1 macrophages (Figure 8). These results support the applicability of this fluorimetric method to monitor macrophage viability during infection assays.

Since alamarBlue method is highly sensible, with the capacity to detect as few as 50 viable cells (Invitrogen), we determined by fluorimetry that 5 µg/ml is the minimum puromycin concentration that kills THP-1 cells (Figure 9). This concentration was the lowest affecting significantly cell survival, with RFU values similar to the blank (amarBlue) and therefore was used to further select lentiviral transduced cells.

Following the establishment of CFU counting method to quantify intracellular mycobacteria and alamarBlue to evaluate infected macrophage viability, we produced a transducing shRNA lentiviral library targeting human intracellular traffic genes. The commonly used method of gene silencing is transfection with synthetic siRNAs. This method is not suitable for all cell types and gene silencing activity has a peak around 24 hours and then decreases within 48 hours (Rao et al., 2009). In contrast, shRNA carried on a lentiviral vector can be introduced to a wide range of cells, allowing persisting expression of siRNAs and consequently promotes effective and long-term gene silencing (Moffat et al., 2006; Rao et al., 2009).

In the preliminary screen the strain H37Ra was used and our results were a product of 3 independent experiments where target mRNAs were individually knocked-down in THP-1 cells by silencing with several shRNAs for each one. The levels of silenced targets were assessed at transcriptional level by qRT-PCR (Figure 11). Here, we identified Rab7, Rab14 and Rab34 as “hot targets” for mycobacterial manipulation, since a reduction of these proteins altered M. tuberculosis H37Ra survival in THP-1 macrophages at day 5 post-infection (Figure 10a), without affecting the viability of transduced macrophages (Figure 10b).

As mentioned before the strain H37Ra is related with H37Rv, but with attenuated virulence phenotype characteristics. For instance, the avirulent strain displays a decreased survival ability inside macrophages, has no skills to disrupt phagosomal membranes and was demonstrated to have loss of
virulence in guinea pigs and mice (Zheng et al., 2008). Moreover, Seto et al. reported that the degree of association of Rab GTPases with mycobacterial phagosomes differs when H37Ra or H37Rv is used (Seto et al., 2011). With these facts in mind, we were indeed able to confirm the phenotypes obtained in the preliminary screen for Rab14 and Rab34 knockdown. THP-1 cells were transduced with new batches of lentivirus and infected with both avirulent and virulent mycobacterial strains. We confirmed that differences in virulence interfered with their competence to manipulate host vesicular traffic pathways. In order to reveal the implications of mRNA silencing we analyzed the levels of Rab14 and Rab34 knockdown at protein levels. Western blot results show that lentiviral transduction effectively decreased the levels of Rab14 and Rab34 expression (Figure 13).

We found that Rab7 and Rab34 are effectively important for macrophage capacity to kill mycobacteria. The decrease of these proteins in THP-1 macrophages significantly increased the ability of M. tuberculosis H37Ra to survive intracellularly (Figure 10a). However, when M. tuberculosis H37Rv was used, Rab34 knockdown did not significantly altered the number of intracellular bacteria (Figure 12a).

Rab7 is important for lysosome function and positioning in cells and is a well known regulator of phagosome maturation, since the fusion of an early phagosome with late endosomes and lysosomes requires the acquisition of Rab7 and loss of Rab5 (Seto et al., 2009). Rab34 localizes at the trans-Golgi network and is also important for lysosome positioning (Wang and Hong, 2002). Evidence from Seto and colleagues suggests that both Rab7 and Rab34 are involved in phagosomal acidification and recruitment of cathepsin D and RILP to phagosomes. In mice macrophages, these Rabs were only transiently recruited to M. tuberculosis-containing phagosomes and their accumulation was higher in phagosomes containing the avirulent mycobacterial strain and even superior in those containing Staphylococcus aureus (Seto et al., 2011). This means that both mycobacterial strains interfere with the association of Rab7 and Rab34 with phagosomes, although the manipulation by H37Rv is more severe. Altogether, our results are in agreement with the previous indicating that M. tuberculosis H37Rv markedly excludes Rab34 from its phagosome and therefore Rab34 knockdown had less impact in its capacity to survive and proliferate, comparatively to H37Ra. A very recent study identified Rab34 as a crucial factor regulating the delivery of cargo and membrane proteins from lysosomes to phagosomes, independently of Rab7. The knockout of Rab34 in mice macrophages increased the survival of the attenuated M. bovis BCG, which is in conformity with our results for the avirulent strain of M. tuberculosis H37Ra (Kasmapour et al., 2012).

It is known that Rab14 controls the trafficking between Golgi complex and early endosomes (Junutula et al., 2004; Kyei et al., 2006), but its role in phagosome maturation is still controversial. Kyei and colleagues observed the accumulation of Rab14 on mycobacteria-containing phagosomes and that the reduction of Rab14 expression by small interfering RNA promoted the fusion with lysosomes (Kyei et al., 2006). On the other hand, Seto et al. experiments in mice macrophages showed that the expression of constitutively active or dominant negative forms of Rab14 do not alter the fusion of M. tuberculosis H37Rv-containing phagosomes with lysosomes (Seto et al., 2011).

Our results show the impact of Rab14 reduction in M. tuberculosis survival. While the mycobacterial burden of H37Ra decreased with Rab14 knockdown (Figure 10a and Figure 12a), the
number of intracellular virulent bacteria was similar to control (Figure 12a). These results suggest that Rab14 is essential to sustain *M. tuberculosis* H37Ra survival and that the great capacity of the H37Rv strain to survive and proliferate is independent of the presence of Rab14. We believe that Rab14 is important to the delivery of nutrients to *M. tuberculosis*-containing phagosome, allowing fusion with early endosomes. Therefore, this protein may contribute to the establishment of a replicative niche, but is not essential for *M. tuberculosis* H37Rv, since this virulent strain has a superior ability to subvert host mechanisms to sustain its survival (Junutula et al., 2004; Kyei et al., 2006; Stein et al., 2012).

Rab10 participates in plasma membrane recycling and is involved in phagosome maturation (Stein et al., 2012). Indeed, Rab10 acts upstream to Rab5 acquisition, regulating the transition from a nascent phagosome to an early phagosome (Cardoso et al., 2010). We found that a depletion of approximately 50% in Rab10 mRNA levels (Figure 11) did not affect intracellular *M. tuberculosis* H37Ra survival (Figure 10a) in human macrophages. According to Cardoso et al., the recruitment of Rab10 to phagosomes containing live *M. bovis* BCG is lower comparatively to heat-killed *M. bovis* BCG and Rab10 knockdown delays phagolysosome formation in mice macrophages containing heat-killed mycobacteria (Cardoso et al., 2010). This indicates that the effects of Rab10 knockdown may be similar to the exclusion of Rab10 in phagosomes containing live *M. bovis* BCG, which concur with our results for *M. tuberculosis* H37Ra. Similarly to *M. bovis* BCG, we believe that H37Ra is also able to impair the recruitment of Rab10 to its phagosomal membrane, which explains why Rab10 knockdown did not alter its capacity to survive intracellularly. The exclusion of Rab10 perhaps delays the process of membrane recycling to acquire late endosomal markers, which allows phagosomal access to transferrin and iron for mycobacteria (Cardoso et al., 2010).

The lack of effect in mycobacterial survival after lentiviral silencing was also observed for syntaxin 4, a recycling endosomal and plasma membrane t-SNARE required for membrane fusion. Vergne and colleagues observed that syntaxin 4 is retained in phagosomes containing PIM (phosphatidylinositol mannoside)-coated latex beads, whereas the accumulation of this SNARE is lower in phagosomes containing uncoated beads (Vergne et al., 2004). We expected that the absence of this SNARE protein could limit phagosome-endosome fusion, restricting the delivery of nutrients to mycobacterial phagosomes and consequently reduce the number of intracellular mycobacteria. However, the decline of 30% and 50% in syntaxin 4 mRNA levels (Figure 11) did not alter intracellular mycobacterial survival (Figure 10a). Syntaxin 4 might be a passive membrane component during phagosome maturation. Alternatively, PIM and other mycobacterial lipids may contribute to the retention of other SNAREs in phagosomal membranes, which in turn may be sufficient to ensure phagosomal fusion with early and recycling endosomes. However, we did not discard the hypothesis that the levels of knockdown in our experiment may have been insufficient to have an impact in *M. tuberculosis* H37Ra-containing phagosome maturation, since the levels were not significantly different to scramble control (Figure 11).

Intracellular *M. tuberculosis* secretes virulent factors to subvert host vesicular traffic and remain inside early phagosomes (Forrellad et al., 2013). Our study is in agreement with most published data. On one hand, *M. tuberculosis* needs to modulate vesicular trafficking very soon after internalization by macrophages to avoid destruction and immune detection. The inhibition of phagolysosome biogenesis
seems to be achieved by exclusion of factors regulating phagosome maturation, such as Rab10, Rab7 and Rab34. Conversely, *M. tuberculosis* seems to hijack in its phagosomal membrane proteins regulating the fusion with early endosomes, such as Rab14, thus gaining access to nutrients and biosynthetic precursors for bacterial survival.

In order to decipher the roles of the Rabs and SNAREs studied in this work, more experiments are required in the future to define the biological nature of the phenotypes obtained. The factors with potential role in intracellular trafficking need to be tested for a role in phagosome maturation. This may be achieved by characterizing the phagosomal maturation level of THP-1 cells transduced with the shRNA constructs that, from all 5, lead to the highest knockdown when compared with the scramble control. For instance, phagosomal maturation may be assessed by measuring the luminal phagosomal pH and/or the co-localization with enzymes required for production of reactive oxygen species. Since the knockdown of Rab14 and Rab34 affected the survival of H37Ra, but not H37Rv, it will be interesting to compare maturation of latex bead, heat killed mycobacteria and live mycobacteria-containing phagosomes in transduced macrophages, to discern accurately if the absence of effect is due to mycobacterial manipulation of phagosome maturation.

Our study supports the utility and efficiency of lentivirus-mediated gene silencing to decipher the function of specific proteins. The results presented in this thesis allied to the characterization of phagosome maturation will contribute to accurately select vesicular traffic factors involved in mycobacterial infection and will highlight new targets to develop antimycobacterial drugs.


Scherr, N., and Nguyen, L. (2009). *Mycobacterium* versus *Streptomyces*--we are different, we are the same. *Current Opinion in Microbiology*, 12(6), 699–707.


