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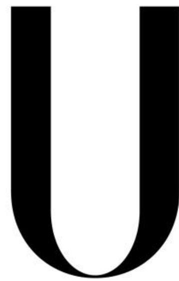

MANIPULATION OF CYSTEINE CATHEPSINS DURING *MYCOBACTERIUM TUBERCULOSIS* INFECTION OF HUMAN MACROPHAGES

Dissertação

João Palma Neves Pombo

MESTRADO EM MICROBIOLOGIA APLICADA

2013

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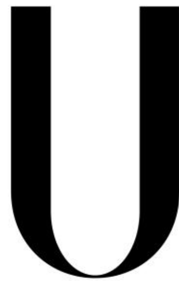

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Dissertação orientada pelas Professoras Doutoras Elsa Anes (Faculdade de Farmácia da Universidade de Lisboa) e Margarida Gama Carvalho (Faculdade de Ciências da Universidade de Lisboa)

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João Palma Neves Pombo

MASTER THESIS

2013

This thesis was fully performed at the Mycobacteria-Host Interactions Unit of the Centre for Molecular Pathogenesis – Retrovirus and Associated Infections Unit (CPM-URIA), in the Faculty of Pharmacy of the University of Lisbon, under the direct supervision of Prof. Dr. Elsa Anes.

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

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Communications in scientific meetings

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Abbreviations

HIV-AIDS – human immunodeficiency virus-acquired immunodeficiency syndrome

DNA – deoxyribonucleic acid

RNA – ribonucleic acid

miRNA – microRNA

mRNA – messenger RNA

siRNA – short interference RNA

shRNA – short hairpin RNA

PBMC – peripheral blood monocytic cell

HMDM – human monocyte derived macrophages

RPMI – Roswell Park Memorial Institute

DMEM – Dulbecco's modified Eagle medium

HI-FBS – heat-inactivated fetal bovine serum

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

OADC – oleic acid-albumin-dextrose-catalase

PBS – phosphate buffered saline

PMA – phorbol 12-myristate 13-acetate

MCSF – macrophage colony stimulation factor

LB – lysogeny broth

rpm – revolutions per minute

h – hour or hours

min – minute or minutes

OD – optical density

MOI – multiplicity of infection

CFU – colony forming unit

PCR – polymerase chain reaction

qRT-PCR – quantitative real-time PCR

BSA – bovine serum albumin

SDS – sodium dodecyl sulfate

SDS-PAGE – SDS-polyacrilamide gel electrophoresis

dH_2O – deionized water

TBS-T – tris buffered saline-Tween 20

HRP – horseradish peroxidase

EDTA – ethylenediaminetetraacetic acid

dNTP – deoxyribonucleotide-triphosphate

Abstract

Mycobacterium tuberculosis (*Mtb*), the main causative agent of tuberculosis, is among the world's most ancient and most successful pathogens. Its success can be attributed to its ability to avoid phagosomal destruction, survive and proliferate inside its host cells, of which the most important are the human macrophages. Besides being able to block phagosome-lysosome fusion, *Mtb* is able to inhibit the expression of lysosomal hydrolases, such as cathepsins. Cathepsins are a major class of lysosomal enzymes which have various functions. One in particular, cathepsin S, has been deeply implicated in peptide processing for antigen presentation, assuming in this way an important role in mediating the innate and adaptive immune responses to external invaders in the human body. In this work we analyzed a putative cathepsin gene regulation mechanism employed by *Mtb*, making use of miRNAs, small RNAs which post-transcriptionally regulate gene expression.

The first part of this work was dedicated to unraveling the effect of *Mtb* in the expression of cathepsin S and the candidate regulator miRNA-106b-5p (miR-106b-5p) in the context of macrophage infection. We have determined that miR-106b-5p is up-regulated, while cathepsin S is down-regulated during *Mtb* infection of macrophages, in contrast with the non-pathogenic *M. smegmatis* which seems to have no effect in either.

Bioinformatics data point out that the cathepsin S gene (CtsS) is a target for miR-106b-5p. As such, in the second part of this thesis we experimentally tested this hypothesis. First, the transfection of THP-1 cell line with miR-106b-5p mimics resulted in a decrease of cathepsin S, but not as exacerbated as we expected. The same effect has been reported for proteins that were shown to be very stable along time. Secondly, by using luciferase expression vectors and subsequent luciferase activity assays, we showed that the mRNA of CtsS is indeed a target for miR-106b-5p. Altogether, our results indicate that *Mtb* inhibits cathepsin S expression via miR-106b-5p up-regulation, during macrophage infection.

Keywords:

Mycobacterium tuberculosis, miRNAs, miR-106b-5p, cathepsin S, RNA interference

Resumo

A tuberculose é uma doença infecciosa que atormenta a humanidade desde há cerca de 9000 anos. A partir do século XVII, com a industrialização da sociedade e a concentração das pessoas em áreas urbanas, a tuberculose atingiu proporções catastróficas e transformou-se num verdadeiro flagelo, tornando-se numa das principais causas de morte na Europa. Ao longo dos tempos foi ganhando diferentes nomes, tais como tísica pulmonar, ou peste branca. Por ser uma doença tão devastadora e não ter ainda uma cura nessa época, a tuberculose era frequentemente abrangida e descrita em obras de arte e literatura como uma força inescapável do destino, semelhante à própria Morte. Com a descoberta do agente etiológico da doença, bem como o aparecimento dos antibióticos e o melhoramento da higiene e condições sanitárias, a incidência e mortalidade atribuídas à tuberculose começaram a declinar, e a doença foi sendo mantida sob controlo durante o século XX. No entanto, em 1992, a Organização Mundial de Saúde (OMS) declarou o ressurgimento da tuberculose como uma das doenças mais alarmantes a nível mundial: o surgimento e prevalência de estirpes com diversas formas de multi-resistência a antibióticos, e o consórcio oportunista entre o *Mycobacterium tuberculosis* e o vírus da imunodeficiência humana (VIH), foram as principais causas apontadas para este ressurgimento. Actualmente, a tuberculose é uma das doenças, associada a um agente patológico, que mais mortes causa no mundo; apenas em 2011, 8.7 milhões de pessoas contraíram a doença e 1.4 milhões morreram da doença.

O primeiro agente etiológico da tuberculose a ser descoberto, no século XIX, foi o *Mycobacterium tuberculosis*, um ser procariota pertencente ao filo *Actinobacteria*. Mais tarde, 6 outras espécies do género *Mycobacterium* foram identificadas como igualmente capazes de causar a doença em humanos, e hoje integram um grupo designado por complexo *Mycobacterium tuberculosis* (CMTB); ainda assim, *M. tuberculosis* é o mais comum agente da doença a nível mundial. O género *Mycobacterium*, compreende maioritariamente espécies inofensivas e saprófitas, contendo apenas um pequeno número de espécies patogénicas, de entre as quais se pode listar *M. leprae* e *M. ulcerans*, agentes causadores da lepra e da úlcera de Buruli respectivamente, para além de *M. tuberculosis*.

As micobactérias do CMTB estão entre os patogénios mais bem sucedidos do mundo. Grande parte deste sucesso pode atribuír-se à sua capacidade de conseguirem sobreviver dentro dos macrófagos humanos após terem sido fagocitadas. No processo geral de fagocitose em que o conteúdo internalizado é digerido, a maturação do fagossoma consiste numa série de fissões e fusões parciais do organelo com outros, em diferentes estádios de maturação com trocas parciais de componentes membranares e do lúmen. Estes fenómenos ocorrem durante o tráfego celular da periferia da célula até à região perinuclear onde existem

os lisossomas funcionais e ao longo de elementos do citoesqueleto. Durante o transporte o fagossoma vai maturando, isto é, torna-se competente para fundir com o lisossoma, fusão essa da qual resulta o fagolisossoma, vesícula que contém enzimas hidrolíticas num lúmen acidificado a pH aproximadamente igual a 5, ambiente que proporciona uma forte digestão dos seus conteúdos. O *M. tuberculosis* e espécies aparentadas conseguem bloquear a maturação do fagossoma e desse modo controlar o seu próprio destino intracelular; estão descritos diversos mecanismos através dos quais estas micobactérias conseguem subverter as etapas da fagocitose em seu favor, e também impedir a sua morte intracelular por apoptose do macrófago infectado, originando uma resposta imunitária exacerbada e desregulada que, em última análise, origina os próprios sintomas da doença. Porém, na maior parte dos casos, e tratando-se de indivíduos à partida saudáveis e imunocompetentes, o sistema imunitário é capaz de controlar a infecção, confinando as bactérias em estruturas histológicas pulmonares denominadas granulomas; deste modo, diz-se que há uma infecção latente, em que a bactéria está viva e dentro do organismo, mas não causa sintomas de doença. Vários factores, tanto fisiológicos como imunitários, podem então desencadear o desenvolvimento de tuberculose activa a partir de uma infecção latente, ou então fazer com que a infecção primária origine imediatamente uma situação de doença activa.

A nível sub-celular, o lisossoma contém vários tipos de enzimas hidrolíticas, de entre as quais se destaca um grupo de proteases denominadas catepsinas. Estas hidrolases lisossomais não só actuam na degradação de proteínas e processamento de péptidos para apresentação de antigénios como contribuem para a remodelação da matriz extracelular e para a reabsorção óssea. A maior parte das catepsinas são proteases de cisteína (catepsinas B, C, F, H, K, L, O, S, V, X e W), sendo duas delas proteases de serina (catepsinas A e G) e outras duas proteases de aspartato (catepsinas D e E).

Os microRNAs (miRNAs) são pequenas moléculas de RNA em cadeia simples, compostas por cerca de 22 nucleótidos, que possuem funções de regulação da expressão genética a nível pós-transcricional. Até há cerca de três décadas atrás, o Dogma Central da Biologia estava bem estabelecido: segundo este, DNA origina RNA, e RNA origina proteínas, atribuindo ao RNA um papel simples, intermediário e passivo. A descoberta dos primeiros miRNAs veio revolucionar esta visão e modernizar o pensamento científico em genética, introduzindo pela primeira vez o conceito de um tipo de RNA com funções regulatórias. A biogénese e maturação dos miRNAs é complexa, envolvendo várias etapas de processamento e modificação, desde que são transcritos do genoma até se tornarem maduros e funcionais. Os miRNAs maduros encontram-se associados ao complexo silenciador induzido por miRNA (miRISC), e é nesta forma que exercem a sua função: os miRNAs possuem afinidade para uma sequência de 7-8 nucleótidos contida na região não traduzida a 3’ (3’-UTR) do mRNA dos seus genes alvo, e ao se ligarem ao mRNA através desta sequência, por complementaridade

total ou parcial de bases, impedem que ele seja traduzido pelos ribossomas, provocando a sua degradação, ou destabilizando a sua estrutura molecular.

O objectivo deste trabalho é investigar uma relação causal entre a infecção de macrófagos humanos por *M. tuberculosis*, miRNAs, e catepsinas. A catepsina S, uma protease de cisteína, tem sido fortemente implicada no processamento peptídico para apresentação de antigénios através do complexo principal de histocompatibilidade classe II (MHC-II); estudos anteriores já constataram a manipulação desta catepsina por *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) em macrófagos, porém ainda nenhum estudo relacionado com a catepsina S foi feito em *M. tuberculosis*. Também, experiências preliminares evidenciaram que alguns miRNAs são diferencialmente expressos em macrófagos infectados com *M. tuberculosis*, entre os quais o miRNA-106b-5p (miR-106b-5p). Por análise bioinformática foi possível prever que o gene da catepsina S humana (CtsS) é um potencial alvo para o miR-106b-5p; deste modo, a hipótese de um modelo de regulação génica, durante a infecção, baseado na manipulação do miR-106b-5p e consequente alteração da expressão de CtsS, pode ser explorada.

Na primeira parte do trabalho explorámos a existência ou não de associações entre *M. tuberculosis* e CtsS, e entre *M. tuberculosis* e o miR-106b-5p, no contexto da infecção. Numa triagem preliminar, usámos uma biblioteca de lentivírus para silenciar macrófagos, diferenciados a partir de células monocíticas da linha celular THP-1, para as diferentes catepsinas. As linhas celulares slenciadas para cada catepsina foram então infectadas com a espécie não patogénica *M. smegmatis*, e a sobrevivência intracelular das bactérias foi analisada através do método das unidades formadoras de colónias (CFUs). Os resultados mostram que várias catepsinas, incluindo a catepsina S, possuem um efeito bactericida induzindo a morte intracelular de *M. smegmatis*. De todas as catepsinas testadas procedemos a um estudo dirigido à função da catepsina S no contexto da infecção por um patogénio, o *M. tuberculosis*, e, no seguimento dos dados bioinformáticos que indicavam uma potencial regulação da catepsina S pelo miR-106b-5p, observámos que a expressão de catepsina S em macrófagos infectados com *M. tuberculosis* é marcadamente reduzida em comparação com macrófagos não infectados ou infectados com *M. smegmatis*. Estabelecemos ainda a relação com a expressão genética do miR-106b-5p que em macrófagos infectados com *M. tuberculosis* está claramente sobre-expresso, comparativamente com os infectados com *M. smegmatis*. Concluímos que os mecanismos de virulência do *M. tuberculosis* controlam o aumento na expressão genética do miR-106b-5p, acompanhado de uma redução da quantidade de proteína da catepsina S, durante a infecção em macrófagos humanos.

Em seguida procurámos averiguar experimentalmente os dados bioinformáticos preditivos que obtivemos para o miR-106b-5p, ou seja, se o mRNA CtsS é realmente um alvo para este miRNA. Transfectámos macrófagos humanos com mimetizadores do miR-106b-5p para

exacerbar o efeito do miRNA e avaliar os efeitos na quantidade de catepsina S. Os resultados mostram uma redução na quantidade de proteína catepsina S em macrófagos transfectados com os mimetizadores que não é muito exacerbada, mas que existe; o mesmo tipo de efeito tem sido reportado quando as proteínas celulares são estáveis, não se esperando valores diferenciais maiores que 20%. De seguida demonstrámos o efeito do miR-106b-5p sobre o seu alvo putativo, o mRNA do gene CtsS. Para estas experiências foram usados vectores plasmídicos produtores de luciferase, dentro dos quais foi clonado um fragmento da 3'-UTR do gene CtsS contendo uma cópia da sequência alvo do miR-106b-5p; este fragmento foi inserido a jusante do gene da luciferase no vector, e desse modo, ao ser transcrito, o mRNA do gene da luciferase irá também conter o fragmento clonado. Os vectores recombinantes obtidos desta forma foram então transfectados para células da linha HEK 293T juntamente com mimetizadores do miR-106b-5p; posteriormente, estas células foram lisadas para ser realizado um ensaio de leitura da actividade da luciferase nestas condições. Os resultados que obtivemos indicam uma redução da actividade da luciferase na presença do mimetizador do miR-106b-5p, em comparação com o controlo em que se transfectou com o vector mas sem o mimetizador. Uma redução na actividade da luciferase nestas condições significa que o mimetizador bloqueou a tradução do mRNA do gene da luciferase, fazendo com que este não fosse traduzido para proteína; sendo assim, com esta experiência foi possível concluir que o gene CtsS humano é um alvo para a regulação genética mediada pelo miR-106b-5p.

O trabalho reflectido nesta tese permitiu-nos esclarecer os alicerces principais do modelo inicialmente proposto: determinámos que, durante a infecção em macrófagos, *M. tuberculosis* provoca um aumento na expressão do miR-106b-5p e uma diminuição na expressão da catepsina S. Demonstrámos que o mRNA CtsS é um alvo para o miR-106b-5p, e que a redução da catepsina S durante a infecção se deve, pelo menos em parte, à acção regulatória do miR-106b-5p, que se encontra sobre-expresso. Deste modo contribuímos para decifrar parte da complexidade de mecanismos que as micobactérias do CMTB realizam para sobreviverem dentro do seu hospedeiro humano.

Palavras chave

Mycobacterium tuberculosis, miRNAs, miR-106b-5p, catepsina S, interferência de RNA

1. Introduction

1.1. Tuberculosis, a past and present day health menace

Tuberculosis, also known in the past as phthiasis, consumption, and the “white plague”, has tormented humanity for thousands of years; the oldest record of tuberculosis infecting human beings dates back to approximately 9000 years ago (Hershkovitz et al., 2008). From the 17th century up to modern age, tuberculosis was one of the main causes of death in Europe, and still had no cure. It came to be seen as a “romantic” disease, taking the role of a tragic force of fate in important works of literature such as Victor Hugo’s *Les Miserábles* (1862) and *La Dame aux Camélias* (1848), by Alexandre Dumas. During the 19th century, the tuberculosis epidemic in Europe was at its peak, holding responsibility for 25% of human deaths in all of Europe (Lawn and Zumla, 2011). *Mycobacterium tuberculosis*, the first identified causative agent of the disease, was discovered by Robert Koch (Koch, 1882), thus becoming one the first microorganisms in history to have been scientifically recognized as a cause of illness. Prevention against tuberculosis became increasingly effective as health and sanitary habits improved during the 20th century; at the same time, the death toll due to the disease declined during this period as new antituberculosis drugs were discovered (Lawn and Zumla, 2011).

Nowadays, active tuberculosis is curable and treatable in most cases, with a 6-month course of antibiotics; however, despite all the advances in medicine and health management, tuberculosis still represents a heavy burden throughout the world, particularly in developing countries where access to health services is still difficult (WHO, 2013). Since 1992, the scientific and health-care community acknowledged the re-emergence of tuberculosis as a global health menace, identifying the insurgences of multidrug-resistant *M. tuberculosis* strains (MDR-TB), together with the opportunistic consortium of mycobacteria infection with HIV-AIDS, as the main causes of this event (1992). Currently, tuberculosis is considered by the WHO to be the second infectious disease, associated with a single biological agent, which causes the highest number of human deaths in the world, surpassed only by AIDS: in 2011, 8.7 million people fell ill with tuberculosis and 1.4 million died from the disease (WHO, 2012).

1.2. Mycobacteria and pathogenesis

Mycobacterium is a genus belonging to the phylum *Actinobacteria*, which comprises both high and low G+C containing (Ghai et al., 2012), mostly Gram-positive species. Like the

majority of the members of this phylum, most species of *Mycobacterium* are non-pathogenic, environmental commensals, and only small groups have evolved to exhibit pathogenicity to human beings and other animals (Madigan et al., 2009); even so, besides tuberculosis, other prestigious maladies such as leprosy and Buruli ulcer are caused by mycobacteria as well (Einarsdottir and Huygen, 2011; Irgens, 2002). Human tuberculosis can result from infection by any member of the *Mycobacterium tuberculosis* complex (MTC), a group of several closely related species, which share a common ancestor: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. pinnipedii* and *M. microti* (Aranaz et al., 2003; Ernst et al., 2007). Even so, approximately 95% of cases are caused by *M. tuberculosis* alone (Parsons et al., 2002).

The success of MTC mycobacteria can be attributed to their ability to survive and proliferate inside phagocytic immune cells: they are able to avoid intracellular destruction by manipulating the host's biochemical and trafficking mechanisms in a number of different ways (Deretic and Fratti, 1999; Wong et al., 2011). In the case of healthy, immunocompetent individuals, the immune response generated by the infection is generally capable of stopping the spread of the bacilli, containing them in histological structures called granulomas (Russell, 2007). The presence of granulomas indicates latent infection, a situation where the person is infected however is healthy, and does not spread the pathogen to other people; however, several immune, genetic and physiological factors can trigger the progression from latent infection to active disease (Guzzetta and Kirschner, 2013; Leistikow et al., 2010; Tufariello et al., 2003). The most common and easiest form of transmission is the inhalation of airborne droplets containing bacilli, expelled orally by individuals whose infection has already progressed to active, acute pulmonary tuberculosis (Erkens et al., 2010). As such, pulmonary tuberculosis is the most frequent form of the disease. However, depending on the severity of the pulmonary infection and/or the occurrence of other less common entry points for the bacilli into the body, tuberculosis can be developed in other organs and body regions such as the central nervous system, bones and joints, lymph nodes, or even the eyes (Carrol et al., 2001).

1.3. Phagocytosis and host cell factors — cathepsins

The main mechanism by which professional phagocytes, such as macrophages, eliminate bacteria and other external particles is phagocytosis, that is, the internalization of those particles by enveloping them in an intracellular plasma membrane-derived compartment called phagosome; the phagosome then undergoes several alterations until it fuses with the lysosome, in a phenomenon referred to as phagosome maturation or phagolysosome biogenesis (Kinchen and Ravichandran, 2008). Fusion with the lysosome allows the several types of hydrolytic enzymes contained in the lysosome to reach the contents of the phagosome, thereby promoting the degradation of those contents (Coutinho et al., 2012).

Cathepsins are a large group of lysosomal proteases, mostly cysteine proteases, each with different intracellular and/or extracellular functions, such as antigen processing and presentation, protein degradation, bone resorption, and hormone processing (Chapman, 1998; Dunn et al., 1991; Kakegawa et al., 1993; Turk et al., 2000). Cysteine cathepsins, in particular, are among the most studied enzymes; likewise, 11 cysteine cathepsins (cathepsins B, C, F, H, K, L, O, S, V, X and W) have been identified in humans (Rossi et al., 2004). Moreover, recent studies have already demonstrated that cathepsins are manipulated by *M. tuberculosis* during macrophage infection, effectively preventing phagosome maturation, apoptosis, and antigen presentation (Danelishvili et al., 2011; Nepal et al., 2006; Welin et al., 2011).

One in particular, cathepsin S, has been shown to have a critical role in antigen degradation and presentation in antigen-presenting cells (APCs) (Bania et al., 2003; Hsieh et al., 2002; Liu and Spero, 2004). *Mycobacterium bovis spp.* Bacille Calmette-Guérin (*M. bovis* BCG), an attenuated strain of the pathogenic *M. bovis*, has been shown to inhibit the expression of major histocompatibility complex class II (MHC class II) molecules in macrophages, by manipulating cathepsin S expression (Sendide et al., 2005; Soualhine et al., 2007), suggesting that other, more pathogenic mycobacteria might employ similar strategies.

1.4. MicroRNAs

MicroRNAs (miRNAs) are a class of very small, non-coding, regulatory RNAs that exist in the cells of higher eukaryotes. The Central Dogma of Biology, which had been established since five decades ago, stated that DNA codes for RNA and RNA is translated into protein, thus depicting RNAs as simple intermediate molecules; as such, the discovery and characterization of the first miRNAs in *Caenorhabditis elegans* revolutionized modern thinking in genetics, unveiling the much more diverse and complex roles that RNA actually has (Ke et al., 2003; Lee et al., 1993; Reinhart et al., 2000).

miRNAs are single-strand RNAs made of about 22 nucleotides. Newly transcribed miRNA precursors go through several processing steps from the moment they are transcribed to the moment they are mature and functional (Figure 1). Mature miRNAs, incorporated in the miRISC protein complex, then bind, through their “seed” sequence which comprises 7-8 nucleotides, to a specific complementary short sequence in the 3’-UTR region of their target mRNA, and effectively block translation of their target gene (Ul Hussain, 2012). Research on miRNAs exponentially grew since about ten years ago, and is still very active and productive today; miRNAs are currently known to be implicated in an array of diseases, such as viral diseases, heart disease and, above all, cancer (Kincaid and Sullivan, 2012; Lee and Dutta, 2009; Sun and Tsao, 2008).

It has also been recently reported that during mycobacteria infection in macrophages, some miRNAs are differentially expressed (Bettencourt et al., 2013; Singh et al., 2013; Wang et al., 2013).

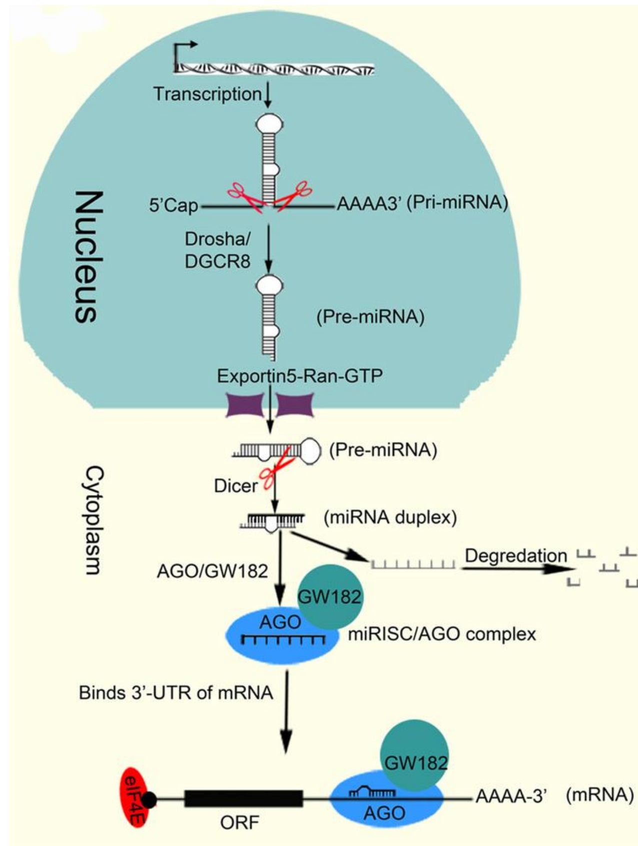

Figure 1. Currently accepted model for miRNA biogenesis and maturation. The miRNA precursor (pri-miRNA) is transcribed from the genome and is then processed by the Drosha/DGCR8 complex, originating a pre-miRNA with a 2 nucleotide 3' overhang. The pre-miRNA is then exported out of the nucleus via an exportin-5/Ran-GTP transmembrane complex; once in the cytoplasm, the pre-miRNA is further processed by the Dicer enzyme, originating a double-stranded RNA structure (miRNA duplex) with 2-nucleotide 3' overhangs. One strand of the miRNA duplex is degraded and the other strand is assimilated into the miRNA-induced silencing complex (miRISC); miRISC is a molecular machinery primarily composed of proteins from the Argonaute (AGO) family and the glycine-tryptophan (GW182) protein class. The miRISC complex then binds to the 3'-UTR of the target mRNA with perfect or nearly perfect base complementarity, and effectively blocks translation. This scheme was adapted from Hussain (2012).

1.5. Objectives

The work presented in this thesis contributed to clarify a novel putative control pathway employed by *M. tuberculosis* to survive the intracellular killing mechanisms of the human macrophage.

The first part of the thesis shall be dedicated to unraveling a connection between both the CtsS and miR-106b-5p genes and *M. tuberculosis*, in the context of macrophage infection. To perform this study, we explored two paths: the effect of the CtsS gene in the intracellular survival of mycobacteria during macrophage infection; and the effect of mycobacteria in the natural expression of these genes during infection. To work on the first path, macrophages with cathepsin gene knockdowns were infected with mycobacteria, and intracellular survival was assessed in each condition via CFU retrospective count. To explore the second path, RNA was extracted from macrophages infected with *M. tuberculosis* and a qRT-PCR was performed in order to compare miR-106b-5p gene expression in different time points post-infection; also, the whole cell proteins were extracted at different time points post-infection, and Western blots were performed to quantify cathepsin S protein expression during infection. Our findings provided evidence that cathepsin S negatively affects intracellular survival of mycobacteria during infection; also, we found that *M. tuberculosis* clearly up-regulates miR-106b-5p expression in human macrophages during infection, as opposed to the non-pathogenic *M. smegmatis* which seems to have no effect; finally, Western blot analysis revealed that the amount of cathepsin S is diminished during macrophage infection by *M. tuberculosis*.

In the second part of this thesis, we will focus on the relationship between the miRNA 106b-5p (miR-106b-5p) and the human cathepsin S (CtsS) gene. Based on bioinformatics data, we theorize that the 3´-UTR of the human CtsS gene transcript is a target for miR-106b-5p, and as such it can suppress CtsS gene expression by blocking translation of this transcript. To assess these hypotheses, we constructed recombinant luciferase-producing plasmid expression vectors harboring a fragment of the 3'-UTR of the human CtsS gene, containing the seed sequence of miR-106b-5p. Luciferase activity was then analyzed between recombinant and non-recombinant plasmids in the presence of miR-106b-5p mimics, to draw conclusions about the effect of miR-106b-5p on the 3'-UTR of the human CtsS transcript. It was demonstrated here that miR-106b-5p has a significant affinity for the transcript; together with the bioinformatics analysis, this strongly enforces the hypothesis that miR-106b-5p down-regulates post-transcriptional expression of the human CtsS gene. Altogether, these findings shed more light into the complex and still largely unknown mechanisms of intracellular survival of MTC mycobacteria.

2. Materials and methods

2.1. Strains and media

In this work, the mc[2] 155 strain of the non-pathogenic *Mycobacterium smegmatis* (ATCC® #700084™) and the H37Rv virulent strain of *M. tuberculosis* (ATCC® #25618™) were used to perform *in vitro* infections. As host cells in these infections, human monocyte derived macrophages (HMDMs) were used, as well as cells from the human acute monocytic leukemia cell line THP-1 (ATCC® #TIB-202™). Human peripheral blood monocytic cells (PBMCs) were extracted from the blood of healthy donors (courtesy of Instituto Português do Sangue, Lisbon).

For plasmid propagation and cloning assays, the JM109 strain of *Escherichia coli* (ATCC® #53323™) was used, and all plasmid and miRNA cotransfection assays were performed on human cells from the HEK 293T cell line (ATCC® #CRL-3216™).

THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% HI-FBS (Gibco®), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM GlutaMAX™ (Gibco®), 1 mM sodium pyruvate (Gibco®), 1X MEM Non-Essential Amino Acids (Gibco®), and 10 mM HEPES (Gibco®). THP-1 cells were grown in a humid incubator at 37º C with 5% CO_2, and kept at a concentration between 0.2×10^6 and 1.0×10^6 cells/ml. During infection assays, THP-1 cells were maintained in RPMI-1640 medium supplemented as above but without Penicillin or Streptomycin.

PBMCs were grown in 96-well culture plates, and maintained in RPMI-1640 medium supplemented with 10% HI-FBS, 2 mM GlutaMAX™, 1 mM sodium pyruvate, 50 µM of 2-mercaptoethanol (Gibco®) and 10 mM HEPES, and grown in a humid incubator at 37% with 5% CO_2. During the differentiation process before infections, the medium was supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin.

HEK 293T cells were maintained in 10 ml of DMEM supplemented with 10% HI-FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM GlutaMAX™. HEK 293T cells were grown in a humid incubator at 37º C with 5% CO_2, and were routinely passed upon reaching 90-95% confluence. The protocol of cell passage for HEK 293T cells consisted of detaching the cells from the culture flask matrix by trypsinization, and diluting at 1:5 volumes in fresh supplemented DMEM; the trypsinization protocol used in this work consisted of removing the culture medium from the flask, then washing the cells by gently adding 10 ml of 1X PBS (Gibco®), removing the PBS, and adding 1 ml of trypsin at 500 µg/ml to the cells, tilting the flask to ensure that the trypsin covers the whole cell covered surface; the cells are then incubated at 37ºC, 5% CO_2, for 5 min, to allow trypsin activity, and finally 9 ml of fresh supplemented DMEM are added to the cells, inactivating the trypsin. For plasmid and miRNA

transfection assays, HEK 293T cells were grown in DMEM supplemented as described, but without penicillin or streptomycin.

E. coli cells were maintained in LB (10 mg/ml NaCl, 5 mg/ml Bacto™ Yeast Extract, 10 mg/ml Bacto™ Tryptone for liquid LB; for solid LB, 15 mg/ml Bacto™ Agar were added to the liquid formula). For transformation assays, *E. coli* were plated in solid LB supplemented with 100 µg/ml ampicillin and grown in a humid incubator at 37ºC. For miniprep assays, *E. coli* were maintained in liquid LB with 100 µg/ml ampicillin, and grown in an orbital shaker at 37ºC, at 140 rpm.

M. smegmatis cells were maintained in 7H9 broth supplemented with nutrient broth and glucose, each at 5 mg/ml, and *M. tuberculosis* cells were maintained in 7H9 broth supplemented with 10% BBL™ OADC Enrichment; both strains were grown in an orbital shaker at 37ºC and 140 rpm. For intracellular survival assays, both *M. smegmatis* and *M. tuberculosis* were plated in 7H10 solid broth with 10% BBL™ OADC Enrichment.

2.2. Extraction of PBMCs from human blood

PBMCs were extracted from buffy coats derived from the blood of healthy human donors through gradient centrifugation. Buffy coats were diluted 1:1 in autoMACS® Running Buffer, and then carefully overlaid on Ficoll-Paque™ PLUS reagent (GE Healthcare) on a 2:1 fraction, followed by centrifugation at 800 × g for 20 min, without acceleration or breakdown. The interface between the resulting two phases was recovered to a 50 ml Falcon tube, and then autoMACS® Running Buffer was added to make 50 ml total volume. Then it was centrifuged at 350 × g for 5 min, the supernatant was discarded, and the pellet was resuspended in 5 ml ice-cold autoMACS® Running Buffer; at this point, 300 µl of human CD14 MicroBeads (MACS) were added to the cell suspension, and the cells were incubated like this for 30 min at room temperature, with agitation. After, 12 ml of cold autoMACS® Running Buffer was added to the cell suspension, and the cells were centrifuged at 350 × g for 10 min, at 4º C. The resulting pellet was resuspended in 5 ml cold autoMACS® Running Buffer, and was then slowly added to an LS column (MACS); after all the suspension had gone through, the column was washed by adding 3 ml autoMACS® Running Buffer and letting it flow through, by gravity force, into the collector tube, two to three more times. Finally, to elute the cells bound to the column, the column was removed from its magnetic stand and placed on top of a new collector tube; 5 ml of autoMACS® Running Buffer were added to the column, and after 2 ml had gone through by gravity force, the remaining 3 ml were pressed through using the column plunger, eluting the remaining cells. Cells were then diluted 1:1 in Trypan Blue (Sigma-Aldrich) and counted in a hemocytometer, to determine cell concentration and viability.

2.3. *In vitro* macrophage infection assays

In vitro infections were performed according to the same protocols for all cell types, and all bacterial species and strains. The infections comprised three stages: the earlier seeding of human cells in multiwell plates, followed by chemical activation of these cells, to induce differentiation of monocytic cells into macrophages; the preparation of the bacterial suspension to be used in the infection; and the mixing of the seeded human macrophages with the prepared bacteria.

2.3.1. Seeding human cells

For this work, THP-1 cells were seeded in 96- or 24-well culture plates, and PBMCs were seeded in 24-well culture plates. The seeding/differentiation protocol lasts for 3 days and comprises the inoculation of a cell suspension in the wells, followed by the differentiation of these cells into macrophages. Cells were inoculated at 5×10^4 cells per well, for 96-well culture plates, or 3.0×10^5 cells per well, for 24-well culture plates.

If working with THP-1 cells, cells are then incubated at 37ºC, 5% CO_2, for 24h, with 1 ml or 100 µl of medium (if working in 24- or 96-well plates respectively) to allow adherence to the bottom of the wells. After incubating for 24h, the medium was removed from the wells and replaced with the same volume of medium plus 20 nM PMA (ImmunoTools); subsequently, the cells were again incubated as before for 24h, to allow differentiation overnight. After this second incubation, the cells are differentiated into macrophages and ready for infection.

If working with PBMCs, cells are incubated at 37ºC, 5% CO_2, in 400 µl of simple RPMI-1940 medium plus 2 mM GlutaMAX™ and 10 mM HEPES, for 2 to 3 h, to starve the cells and allow them to better adhere to the bottom of the wells; after, 400 µl of RPMI-1640 medium plus 20% HI-FBS, 2 mM GlutaMAX™, 200 U/ml penicillin, 200 µg/ml streptomycin, 2 mM sodium pyruvate, 100 µM 2-mercaptoethanol, 10 mM HEPES and 40 ng/ml MCSF (ImmunoTools) are gently added to each well, and the cells are incubated for 3 days to allow differentiation. Then, the medium is removed from the wells and replaced with 1 ml of supplemented RPMI-1640 medium (as described in section 2.1), and the cells are again incubated for 48 h. After this incubation, the cells are ready for infection.

2.3.2. Preparing the bacterial suspension

Prior to performing the infection, on the same day, the bacteria were collected from an exponential growth phase liquid culture and submitted to a protocol in order to remove traces

of bacterial culture medium and, above all, disperse and discard bacterial clumps. This procedure is the same for each bacterial strain used in infections.

First, 10 ml of bacterial exponential growth culture are centrifuged at 3000 × g for 7 min, the supernatant is removed, and the pellet is resuspended in 10 ml of PBS; the suspension is again centrifuged as before, the supernatant is removed, and the pellet is resuspended in 5 ml of infection medium (specified for THP-1 cells and PBMCs in section 1.1). Following this, procedures are made to rid the suspension of bacterial clumps: the 5 ml of bacterial suspension are flushed up and down, around 20 times, with a 5 ml syringe, and the resulting suspension is then placed in an ultrasound bath for 5 min; finally, the suspension is centrifuged at low speed (350 × g) for 1 min to pellet the remaining clumps, the supernatant is transferred to a new recipient and the pellet is discarded; a 5 µl aliquot of the suspension is then observed under the microscope to confirm the absence of clumps, and after this, the bacteria are ready for infection.

To determine the concentration of bacterial cells in the suspension, the OD_{600} of the suspension was read, and the suspension was subsequently diluted to achieve an OD_{600} of 0.1, corresponding to 10^7 cells/ml, (Anes et al., 2006); after this, necessary calculations were made in order to achieve the desired number of bacterial cells in the desired volume of culture medium.

2.3.3. Infection of human macrophages in multiwell plates

The infection procedure is the same for THP-1 macrophages and for HMDMs, save for the different culture media used in each case.

A MOI of 10 — 10 bacteria per 1 host cell — was used in *M. smegmatis* infections, and a MOI of 1 was used in *M. tuberculosis* infections. When the cells were ready for infection, the culture medium was removed from the wells, and each well was washed once with 50 µl of PBS (if working with 96-well plates) or 500 µl of PBS (if working with 24-well plates); this washing step consists of gently adding the PBS to the wells, and removing the PBS immediately after, being careful not to let the cells dry or detach from the bottom of the wells. After washing, 50 µl or 500 µl (depending on the type of plate) of bacterial suspension were added to each well. Finally, after adding the bacterial suspension, the plates were incubated in a humid incubator at 37ºC, 5% CO_2, for 1 h (for *M. smegmatis* infections) or 3 h (for *M. tuberculosis* infections), to allow intracellular infection. An exception to this was when the infected cells were to be used in downstream protein extraction and Western Blot assays; in these cases, because infection conditions needed to be normalized between all bacterial strains used to collect data from, *M. smegmatis* infection plates were incubated for 3 h, in the same way as *M. tuberculosis* plates.

After incubating, the infection medium is removed from the wells, and each well is washed with PBS in the same manner as above. After this, 50 µl or 500 µl of infection medium plus 10 µg/ml gentamycin are added to each well, except for the wells that will be used in the present time point for downstream assays.

2.4. Quantification of intracellular survival of mycobacteria after macrophage infection

For this work, we used the method of CFUs determination to assess the intracellular survival of *M. smegmatis* and *M. tuberculosis* after macrophage infection. These assays were executed in different time points after *in vitro* infections, according to the bacterial strain or strains involved and their respective growth rates. CFU assays of infections with *M. smegmatis* were done 1 h and 24 h post-infection, whereas CFU assays of infections with *M. tuberculosis* were done 3 h, 24 h, 3 days, 5 days, and 7 days post-infection.

First, the culture medium is gently and thoroughly from the wells, and then 100 µl (if working with 96-well plates) or 500 µl (if working with 24-well plates) of 0.05% (if performing an *M. smegmatis* infection) or 0.5% (if performing an *M. tuberculosis* infection) IGEPAL aqueous solution are added to each well, to lyse host cells and release the bacteria. The wells are then incubated at 37º C for 10-15 min. After this, the well contents are vigorously resuspended with the pipette, and serial dilutions are made from these initial suspensions.

Finally, a 5 µl drop from each of the selected dilutions is placed on the dry surface of a solid agar plate (medium specifications in section 1.1), without spreading; the drops are allowed to dry, and are then incubated at 37º C.

Fast growth mycobacteria such as *M. smegmatis* take 24-48 h to form visible colonies, whereas slow growth mycobacteria such as *M. tuberculosis* take 2 to 3 weeks. When colonies become visible under the microscope, they are counted, and the formula $N \times Df / V$ can be used to calculate the number of CFU/ml, where N = number of colonies counted, Df = dilution factor regarding the initial suspension, and V = volume (ml) of inoculum used (0.005 ml in this case).

2.5. Quantification of miRNA gene expression

qRT-PCRs were performed to assess gene expression during mycobacteria infection in macrophages. RNA was extracted from infected cells with TRIzol® Reagent (Invitrogen), following the reagent manufacturer's instructions. In this work, qRT-PCRs were performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon), which contained a cDNA synthesis kit and an RT-PCR kit. All steps were performed according to the manufacturer's instructions.

Complementary DNA (cDNA) was synthesized using the Universal cDNA synthesis kit II: volumes of reaction components (5X Reaction buffer, nuclease-free water, enzyme mix and 5 ng/µl template RNA) were set according to the kit's protocol; the reaction was incubated at 42º C for 60 min to allow cDNA synthesis, followed by 5 min at 95º C to inactivate the reverse transcriptase, and immediately cooled down and stored at 4º C.

RT-PCRs were performed with the ExiLENT SYBR® Green master mix. The hsa-miR-106b-5p LNA™ PCR primer set, UniRT (Exiqon, no.: 205884), which contained the forward and reverse primers, was used in this assay. Volumes of reaction components (PCR Master mix, PCR primer mix, and 80X diluted cDNA template) were again set according to the kit's protocol. The reaction was then incubated at 95º C for 10 min to activate the polymerase and denature the DNA strands; this was followed by 40 amplification cycles of [DNA strand denaturation at 95º C, 10 s; primer annealing and amplification at 60ºC, 1min]. In the end, a melting curve analysis was performed to assess the specificity of the PCR primers to their designated targets.

2.6. Analysis of the effect of cathepsin and miRNA expression during mycobacteria infection of macrophages

2.6.1. Lentiviral infection

To determine the effect of cathepsins in the intracellular survival of mycobacteria during macrophage infections, we used the RNAi Consortium (TRC) technology, developed by the Broad Institute (Cambridge, US). shRNA-producing lentiviral vectors (kindly provided by Dr. Luís Moita, Instituto de Medicina Molecular) were used to infect THP-1 cells and thus produce stable cathepsin gene knockouts in their transcriptomes. Lentiviral infection was done accordingly with the Broad Institute's suggested protocol. Briefly, before infection, THP-1 cells were seeded in 96-well plates and differentiated into macrophages; the next day, the medium was removed from the wells and replaced with medium containing polybrene, and then lentivirus were added to the cells; the plates were then centrifuged at 800 × g, 37º C, for 90 min, to enhance infection. The medium was then removed and replaced with fresh medium without antibiotics, and the plates were incubated overnight at 37º C, 5% CO_2. Finally, the next day, puromycin was added to the wells at 5 µg/µl, to eliminate uninfected cells by drug selection. Infected cells were grown in the appropriate culture medium (section 2.1) plus 5 µg/µl puromycin from this point on.

2.6.2. Transient transfection

To assess the effect of cathepsin expression and miRNA intracellular presence/absence during mycobacteria infection in HMDMs, cells were transiently transfected with miRNA mimics and hairpin inhibitors, and with siRNA against cathepsin genes, using the HiPerFect® Transfection Reagent (QIAGEN). miRNA mimics, miRNA hairpin inhibitors, siRNA against cathepsin genes and all respective scramble controls were purchased from Thermo Scientific Dharmacon. The protocol is identic for transfection of cathepsin gene siRNAs, miRNA mimics, hairpin inhibitors, and scramble controls. Cells were seeded at 3×10^5 per well in 24-well plates, and were differentiated into macrophages in the day before transfection. The next day, the culture medium was gently removed, cells were gently washed with 250 µl of culture medium without antibiotics to remove traces of antibiotic and non-attached cells; the medium was again removed and 200 µl of the same culture medium were added to each well. Then, for each well, 81 µl of simple, non-supplemented RPMI medium (RPMI (-)), 9 µl HiPerFect® Reagent and 10 µl of 20 µM siRNA (final siRNA concentration of 166 pM) were mixed, and the mixture was incubated at room temperature for 20 minutes to allow formation of transfection complexes. After this, 100 µl of the mixture were added to each well, dropwise and slowly, tilting the plate between drops to accelerate dilution and reduce the cytotoxicity of the transfection reagent. Cells were then incubated at 37º C, 5% CO_2, for 4 to 6 h, to allow transfection. Finally, after incubation, 300 µl of PBMC culture medium plus 200 U/ml penicillin, 200 µg/ml streptomycin and 40 ng/ml MCSF were added to each well, and the cells were incubated ta 37º C, 5% CO_2, for 48 h before downstream assays.

2.7. Plasmid cloning and propagation

Plasmid cloning assays were performed in order to produce recombinant plasmid DNA to use in downstream luminescence assays. The pmirGLO Dual Luciferase miRNA Target Expression Vector (Promega Corporation) was used in these assays. The first step was to amplify the insert fragment, which will later be ligated to the plasmid vector; then both the insert and the vector are digested by restriction enzymes in order to create cohesive extremities, and finally the insert is ligated to the vector by means of a ligation reaction.

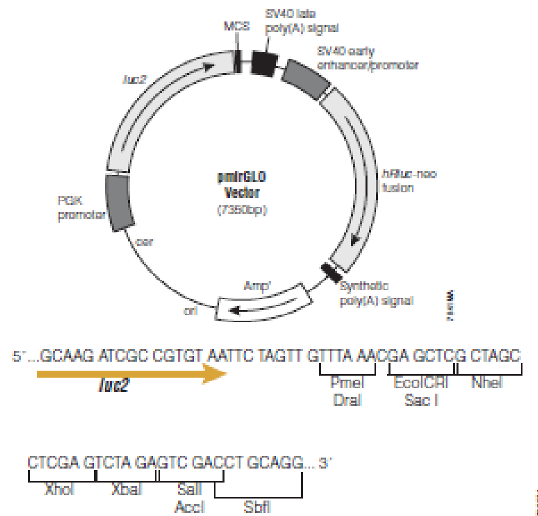

Figure 2. pmirGLO vector map, and multiple cloning site (MCS).

2.7.1. DNA extraction

Human DNA was extracted from THP-1 cells in exponential growth phase. 10 ml of THP-1 culture were centrifuged at 450 × g for 5 min, the culture medium was removed, and the pellet was resuspended in 780 µl of lysis buffer (0.1 M NaCl, 50 mM Trizma® HCl, 0.1 M EDTA, 1% SDS) with 0.77 mg/ml proteinase K and transferred to a 1.5 ml microtube; the tube was then incubated overnight at 55º C. After incubating, the lysate was mixed by inversion for 5 min, and then 300 µl of 5 M NaCl were added; the lysate was again mixed by inversion for 10 min, and then centrifuged at 16000 × g for 5 to 10 min. After, 850 µl of clean supernatant were transferred to a new tube, 750 µl of isopropanol were added, and the mixture was mixed by inversion for 2 min. The mixture was centrifuged at 16000 × g for 5 min, the supernatant was discarded, and the pellet was washed with ethanol at 70% (v/v); the mixture was centrifuged as above for 2 min, and the supernatant was carefully discarded, so as not to disturb the pellet. The tube was left open until the inside was completely dry, and then the pellet was detached and resuspended in 200 µl dH_2O. Finally, making sure that the pellet has been completely resuspended and the DNA solution is not viscous (if so, the tube would be incubated at 55º C overnight, before proceeding), the $OD_{260\ nm}$ of the solution was read to determine DNA concentration, and the sample was stored at -20º C.

2.7.2. Insert fragment amplification

A 413 bp fragment of the 3’-UTR region of the human cathepsin S (CtsS) gene, containing one copy of the seed sequence of miR-106b-5p (GCACTTT), was amplified by standard PCR (forward primer: 5’-GCGAGCTCCAAGAAATATGAAGCACTTTCTC-3’; reverse primer: 5’-

CCCTCGAGTTTTTTGAAACAGAGTCTCCACT-3') using the Phusion® Hot Start II DNA Polymerase (New England BioLabs®). Volumes and concentrations of PCR components (dH_2O, dNTPs, 5X Phusion® HF Buffer, forward and reverse primers, DNA polymerase and DNA template) were set according to the enzyme manufacturer's instructions. The PCR program was executed as follows:

1. 1 initial polymerase activation step of:
   - 98º C — 30 s
2. 35 denaturation/annealing/elongation cycles of:
   - 98º C — 10 s
   - 61º C — 20 s
   - 72º C — 12 s
3. 1 last elongation step of:
   - 72º C — 5 min

5 µl of PCR product mixed with 1 µl of 6X Orange DNA Loading Dye (Fermentas) were run in a 1.2% (m/v) agarose gel (plus 0.5 µg/ml ethidium bromide) electrophoresis (100 V, 2 h), to confirm the molecular mass, alongside 5 µl of O'GeneRuler 50 bp DNA Ladder (Fermentas). PCR products were then purified using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

2.7.3. Enzyme restriction

Restriction reactions were performed in order to generate compatible ends between the insert fragments and the plasmid vector. The vector contains a multiple cloning region with various restriction sites (Figure 1) and, for this experiment, restriction enzymes *Sac*I (5'-GAGCT🡇C-3') and *Xho*I (5'-C🡇TCGAG-3'), both from New England BioLabs®, were used. Both enzymes were used simultaneously in one double restriction reaction. The final reaction volume was 50 µl, and the quantities of the reaction components were set as follows:

- 0.4-1 µg purified DNA
- 20 U *Sac*I per 1 µg DNA
- 20 U *Xho*I per 1 µg DNA
- 5 µl restriction enzyme buffer (10X concentrated)
- 100 µg/ml BSA
- Deionized, nuclease-free H_2O, up to 50 µl final reaction volume

After adding all components, they were incubated at 37º C for 1,5 h to allow restriction enzyme activity, and after this the reaction was stopped by incubating at 65º C for 20 min. Restriction products were then purified with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

2.7.4. Ligation

Ligation reactions were performed in order to ligate the DNA insert fragment to the plasmid vector, and thus generate recombinant plasmid DNA, necessary for downstream luminescence assays. T4 DNA Ligase (New England BioLabs®) was used in these reactions. The final reaction volume was set to 30 µl. Reaction components were set as follows:

- 110 ng plasmid vector DNA
- 180 ng insert DNA
- 3 µl T4 DNA Ligase Reaction Buffer (New England BioLabs®)
- 1 µl T4 DNA Ligase
- Deionized, nuclease-free dH_2O, up to 30 µl final reaction volume

After adding all components, the mixture was incubated at room temperature for 30 min, and after that the reaction was finished.

2.7.5. Plasmid propagation

Vector plasmids and recombinant plasmids were transformed into and propagated in JM109 *E. coli* cells.

JM109 cells were made chemically competent by treatment with Ca^{2+}. 100 ml of JM109 cell culture in exponential growth phase were chilled on ice for 15 min, and then centrifuged at 3220 × g, 4º C, for 10 min; the pellets were resuspended in 40 ml of ice-cold 0.1 M $CaCl_2$, and incubated on ice for 30 min; cells were then centrifuged as above, and each pellet was resuspended in 6 ml of ice-cold 0.1 M $CaCl_2$ plus 15% UltraPure™ Glycerol (Invitrogen™). Cells were then stored at -80º C.

Chemically competent cells were then transformed by heat shock treatment. Competent cells were thawed on ice for 10 min, and then 50 ng of plasmid DNA were added to 100 µl of competent cells suspension. The mixture of cells and DNA was placed in a heating block at 42º C for 1 min, and then immediately placed back on ice for 2 min. Then, 1 ml of LB (with no antibiotic) was added to the cells, and they were incubated at 37º C with agitation (140 rpm) for 1 h; then, 100 µl of the resulting culture were spread on an LB plate with 100 µg/ml ampicillin,

and incubated at 37º C. Colonies were picked after 12 to 16 h of incubation, each colony was inoculated in 5 ml of liquid LB with 100 µg/ml ampicillin, and incubated overnight at 37º C with agitation; finally, each 5 ml culture was aliquoted, supplemented with 20% UltraPure™ Glycerol, and stored at -80º C.

Plasmid DNA was extracted from host cells and purified for downstream assays using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen™), following the manufacturer's provided protocol. Briefly, 1 to 5 ml of liquid cell culture in exponential growth phase were centrifuged and resuspended in Tris-HCl (pH = 8.0) buffer with RNase A; then the kit's lysis buffer was added, to allow cell lysis, and then the kit's precipitation buffer was added to precipitate chromosomal DNA. This was followed by a 10 min centrifugation at 16000 × g. The supernatant was transferred to a spin column, briefly centrifuged at 16000 × g to bind DNA to the filter, the flowthrough was discarded and the DNA was washed by adding the kit's wash buffer to the column and centrifuging shortly as above; finally, the DNA in the filter was eluted by adding dH_2O to the filter and centrifuging again as above. The spin column was discarded and the purified plasmid DNA was stored at -20º C.

2.8. Determination of miR-106b-5p effect on the 3'-UTR of the human cathepsin S gene transcript

The effect of the human miR-106b-5p on the 3'-UTR of the human cathepsin S (CtsS) gene was assessed through luminescence assays, using a recombinant pmirGLO vector containing the 3'-UTR of the CtsS gene (further referred to as pCTSS) to produce and measure luminescence differences.

Plasmids (pmirGLO non-recombinant vector and pCTSS) and miRNA mimics (miR-106b-5p mimic and miRNA mimic scramble) were first transfected into HEK 293T cells using the HiPerFect® Transfection Reagent (QIAGEN). HEK 293T cells were seeded in 12-well plates, each well containing 2.5×10^5 cells in 1 ml culture medium, so that after 24h each well was approximately 80% confluent. 24 h after seeding, the transfection protocol started: 300 ng of plasmid DNA and 10 ng of miRNA mimic (or only plasmid DNA) were diluted in non-supplemented DMEM to a final volume of 200 µl, for each well; 6 µl of HiPerFect® reagent were added to each plasmid/mimic dilution, which was then incubated at room temperature for 10 min; then, 200 µl of culture medium were removed from each well, and 200 µl of each plasmid-mimic-HiPerFect® dilution was slowly added to its respective well, dropwise. The cells were then incubated at 37º C, 5% CO_2, for 24 to 48 h, to allow transfection.

The Dual-Luciferase® Reporter (DLR™) Assay System (Promega Corporation) was used to measure luciferase activity, and the assay was performed according to the manufacturer's instructions. In brief, the transfected HEK 293T cells were lysed using the kit's Passive Lysis

Buffer, the lysate was added to the kit's firefly luciferase substrate, and luminescence from firefly luciferase activity was read immediately after, in the Tecan Infinite® M200 microplate reader; finally, the *Renilla* luciferase substrate was added, and *Renilla* luciferase activity was measured.

2.9. Quantification of cathepsin S by Western blot

Cathepsin amount in human cells was assessed by Western blot. Protein samples were extracted from adherent cells in multiwell plates using Laemmli Sample Buffer 2X Concentrate (Sigma-Aldrich), diluted 1:1 in PBS. After removing the culture medium, 250 µl of Laemmli buffer were added to each well, resuspended with a 200 µl pipette tip to reduce viscosity, and then transferred to a 1.5 ml microtube; the tubes were then incubated in a heating block at 95º C for 5 min, and then stored at -20º C.

20 µl of protein extract were run alongside 5 µl of NYZColour Protein Marker II (NYZTech) in a 12% SDS-PAGE (150 V, 400 mA) for 1 h or until the bands of the protein marker were widely separated from each other (running buffer: 25 mM Trizma® Base, 192 mM Glycine, 0.1% SDS). Proteins were then transferred from the gel to a 0.45 µm nitrocellulose membrane (Bio-Rad) by electrophoresis (150 V, 400 mA) for 2 h (transfer buffer: 25 mM Trizma® Base, 192 mM Glycine). The membrane was then immersed in TBS-T (15.4 mM Trizma® HCl, 137 mM NaCl, 0.1% (v/v) Tween 20) plus 5% (m/v) of BSA and incubated at room temperature for 15 min with angular rocking; afterwards, the membrane was incubated with Rabbit Anti-Cathepsin S antibody (Abcam®, ref.: ab92780, diluted 1:1500) and Rabbit Anti-beta-Tubulin antibody (Abcam®, ref.: ab6046, diluted 1:2000), in TBS-T plus 1% (m/v) BSA, overnight, with angular rocking, in a 4º C refrigerated chamber. Afterwards, the membrane was washed three times in TBS-T with angular rocking (10 min the first time, 5 min the other two times), and was then incubated with Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (Bio-Rad, diluted 1:5000) for 2 h, with angular rocking. After, the membrane was washed three times, the same way as before, and finally 5 ml of Luminata™ Crescendo Western HRP Substrate (Millipore) were added to the membrane; after reacting for 5 min, luminescence was read in the ChemiDoc™ MP System (Bio-Rad), and later quantified using ImageJ free software.

2.10. Statistical analysis

Multiple group comparisons were made using one-way ANOVA tests, with SigmaPlot 11.0 software. All prerequisites of this test were met. The adopted confidence level alpha was 0.001, below which differences were considered significant. All analyzed experiments were conducted in triplicates (n = 3).

3. Results

3.1. Manipulation of cathepsin S and miR-106b-5p expression by *M. tuberculosis* during macrophage infection

3.1.1. Several cathepsins are relevant for the intracellular survival of mycobacteria

Previous studies have stated that lysosomal cathepsins have a role in the intracellular killing of mycobacteria by macrophages (Welin et al., 2011). As such, we devised an experimental plan to assess intracellular survival of the fast-growth, non-pathogenic *M. smegmatis* inside THP-1 macrophages with knockdowns for several different cathepsins as well as their respective inhibitors: THP-1 macrophages with cathepsin gene knockdowns were infected with *M. smegmatis*, and the intracellular survival of the bacteria was assessed 24 h post-infection. *M. smegmatis* is easier to manipulate than *M. tuberculosis*, since it has a fast growth rate and does not require a biosafety level 3 laboratory; other studies have also demonstrated *M. smegmatis* to be genetically quite similar to *M. tuberculosis,* even regarding some virulence genes (Altaf et al., 2010; Reyrat and Kahn, 2001)*,* which is why we chose this bacterium to perform this preliminary experiment. The results show that THP-1 knockdowns for some cathepsins had significantly higher bacterial intracellular survival rates, indicating that those cathepsins have a role in the killing of mycobacteria during infection (Figure 3).

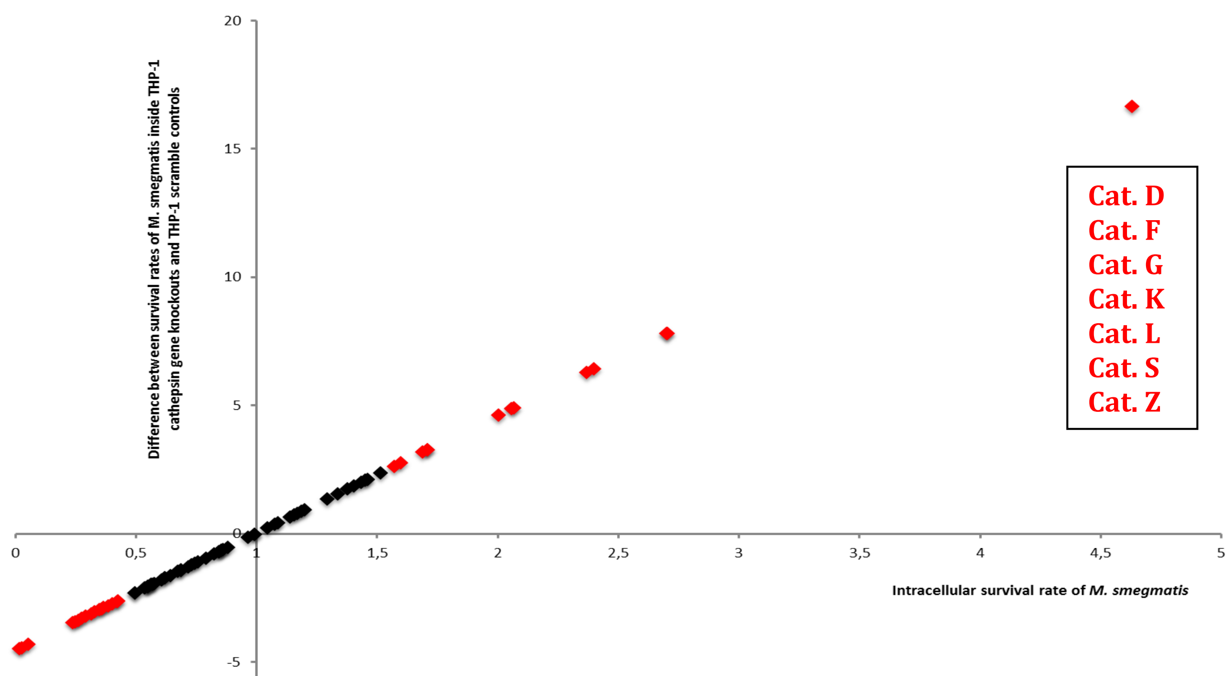

Figure 3. Relative intracellular survival rates of *M. smegmatis* 24 h post-infection in THP-1 macrophages harboring cathepsin gene knockouts. Survival rate values were calculated as (CFUs after the experiment ÷ number of bacteria before the experiment) for each condition. Y-axis values were calculated as ((survival rate – mean survival rate of the scramble controls) ÷ standard deviation of the scramble controls). All values were divided by the mean of survival rates in the scramble controls as normalization. Values of Y greater than 2.5 or less than -2.5 were considered hits and colored red as opposed to black. Positive hits are labeled in the box on the right with their respective cathepsin gene knockdowns. This hit identification method was adapted from Bard et al. (2006).

3.1.2. miR-106b-5p expression is augmented during *M. tuberculosis* infection, while cathepsin S expression in reduced

It was demonstrated in our recent studies that some miRNAs are diferentially expressed during mycobacteria infection (Bettencourt et al., 2013). The miRDB online bioinformatics database at http://mirdb.org (Wang, 2008; Wang and El Naqa, 2008) predicts that the CtsS gene transcript is a target for miR-106b-5p, which correlates to the fact that cathepsin S was one of the genes identified in figure 3 that significantly reduced intracellular survival of mycobacteria in macrophages. As such, gene expression of miR-106b-5p was analyzed in human macrophages, after 24 h of infection with both *M. smegmatis* and *M. tuberculosis* via qRT-PCR. The results show that miR-106b-5p is significantly more expressed in cells infected with *M. tuberculosis*, comparatively with non-infected cells and cells infected with *M. smegmatis* (Figure 4).

Following this, we assessed cathepsin quantification during macrophage infection by *M. smegmatis* or *M. tuberculosis* via Western blot. The results were in accordance with our hypothesis, as cathepsin S expression was significantly reduced 24 h post-infection with *M. tuberculosis* in human monocyte derived macrophages (HMDMs): infection with *M. tuberculosis* reduced cathepsin S expression by approximately 40%, in contrast with *M. smegmatis* which reduced cathepsin S expression by less than 10% in comparison with the non-infected control (Figure 5).

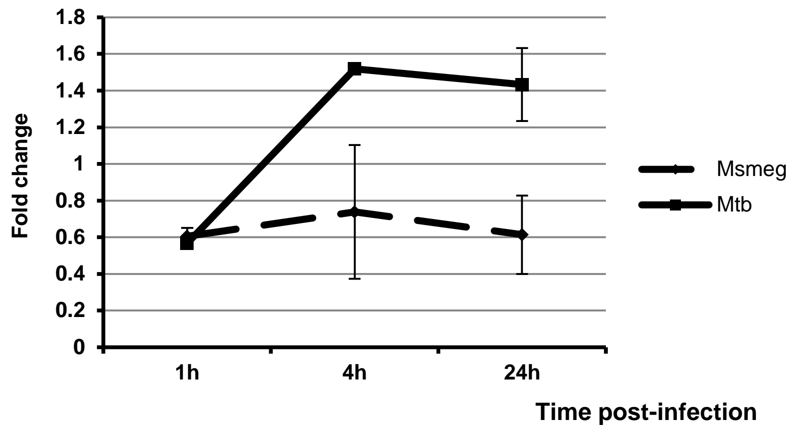

Figure 4. miR-106b-5p gene expression in HMDMs infected with the non-pathogenic *M. smegmatis* (Msmeg) and *M. tuberculosis* (Mtb). Y-values are shown as (mean fold change fluorescence intensity for each bacterium – mean fold change fluorescence intensity for the uninfected control) ± standard deviation (STD).

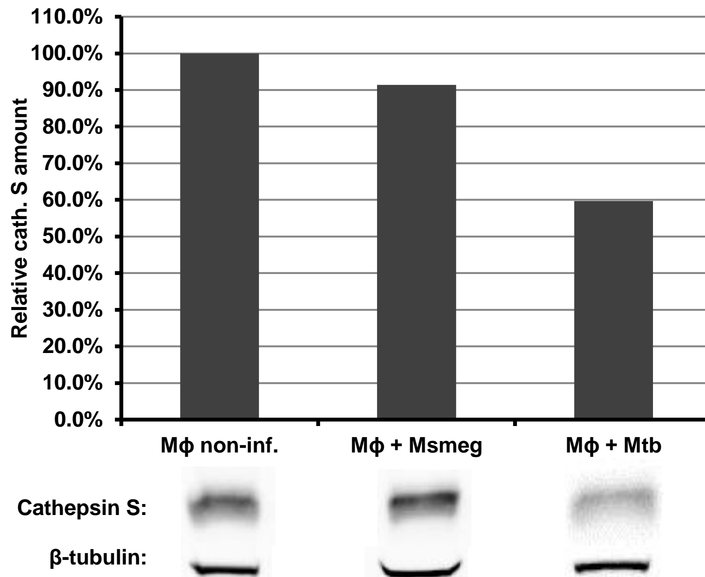

Figure 5. Relative cathepsin S expression 24 h post-infection of *M. smegmatis* and *M. tuberculosis* in HMDMs (Mϕs), shown by densitometry analysis. Presence of cathepsin S and β-tubulin was analyzed via Western blot. Band chemiluminescence was captured with the ChemiDoc™ MP System (Bio-Rad) and then quantified with ImageJ free software as a direct measure of band intensity. Graphic values are presented as percentage of (cathepsin band intensity ÷ β-tubulin band intensity), in comparison with the non-infected control. Below the chart, cathepsin S and β-tubulin bands are presented for each respective condition.

3.2. Influence of miR-106b-5p on cathepsin S gene translation

3.2.1. miR-106b-5p reduces cathepsin S in HMDMs

M. tuberculosis seems to influence miR-106b-5p expression and therefore cathepsin S during infection in macrophages: we verified that, during macrophage infection, *M. tuberculosis* controls cathepsin S, and also increases miR-106b-5p expression in the cell. In this way, a model of cathepsin S regulation via modulation of miR-106b-5p expression by *M. tuberculosis* during infection could be devised. To analyze the direct effect of miR-106b-5p on the amount of cathepsin S, we transfected HMDMs with miR-106b-5p mimics and assessed cathepsin S expression via Western blot, 24, 48 and 72 h post-transfection (Figure 6). The results indeed show that miR-106b-5p reduces the amount of cathepsin S, since it lowered about 40% from 24 h to 48 h post-infection in presence of the miRNA; nonetheless, they are not very exacerbated, since cathepsin S expression in cells transfected with miRNA scrambles lowered as well.

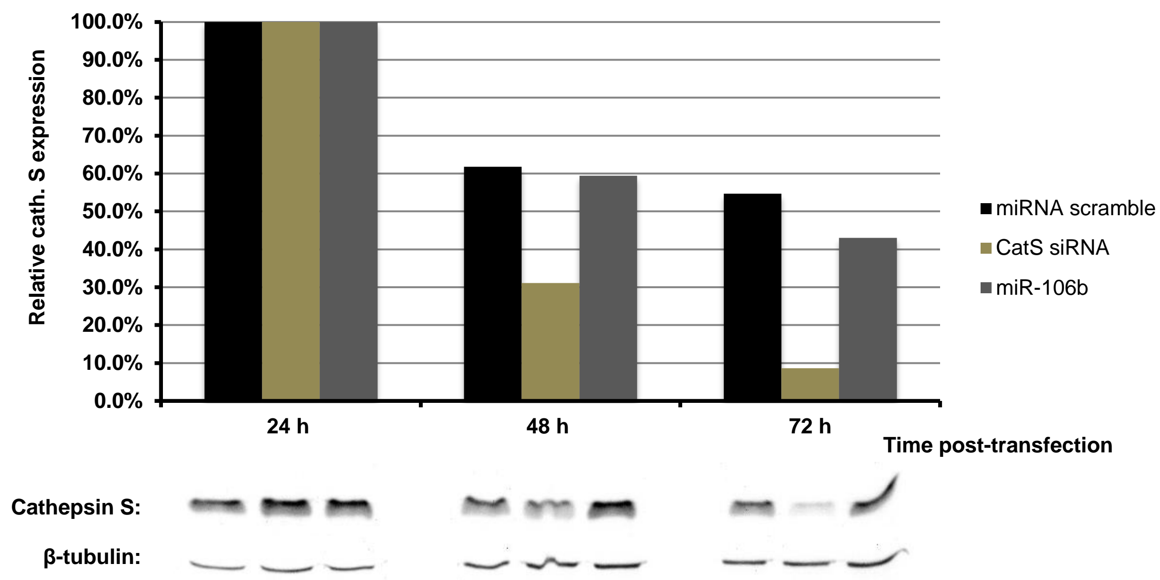

Figure 6. Relative cathepsin S expression in HMDMs 24, 48 and 72 h post-transfection with miR-106b-5p mimics, miRNA scramble (negative control) and anti-cathepsin S siRNA (positive control), shown by densitometry analysis. Presence of cathepsin S and β-tubulin was analyzed with Western blot. Band chemiluminescence was captured with the ChemiDoc™ MP System (Bio-Rad) and then quantified with ImageJ free software as a direct measure of band intensity. Graphic values are presented as percentage of (cathepsin band intensity ÷ β-tubulin band intensity), in comparison with the 24 h time point. Below the chart, cathepsin S and β-tubulin bands are presented for each respective condition.

3.2.2. The human cathepsin S transcript is a target for miR-106b-5p

To further consolidate the hypothesis of cathepsin S regulation through miR-106b-5p activity, we investigated whether miR-106b-5p is able to block translation of the human CtsS gene transcript. To determine this, a fraction of the 3'-UTR of the CtsS gene containing the seed sequence of miR-106b-5p was cloned into the pmirGLO Dual Luciferase miRNA Target Expression Vector; afterwards, recombinant plasmids were transfected into HEK 293T cells, and luciferase activity was read, as a direct proportion of the miRNA-transcript association rate (Figure 7). The results show that there is a significant decrease in luciferase activity associated with the recombinant plasmid, in comparison with the non-recombinant vector, in the presence of miR-106b-5p mimics; hence, this represents more strong evidence in favor of our initial hypothesis (Figure 8).

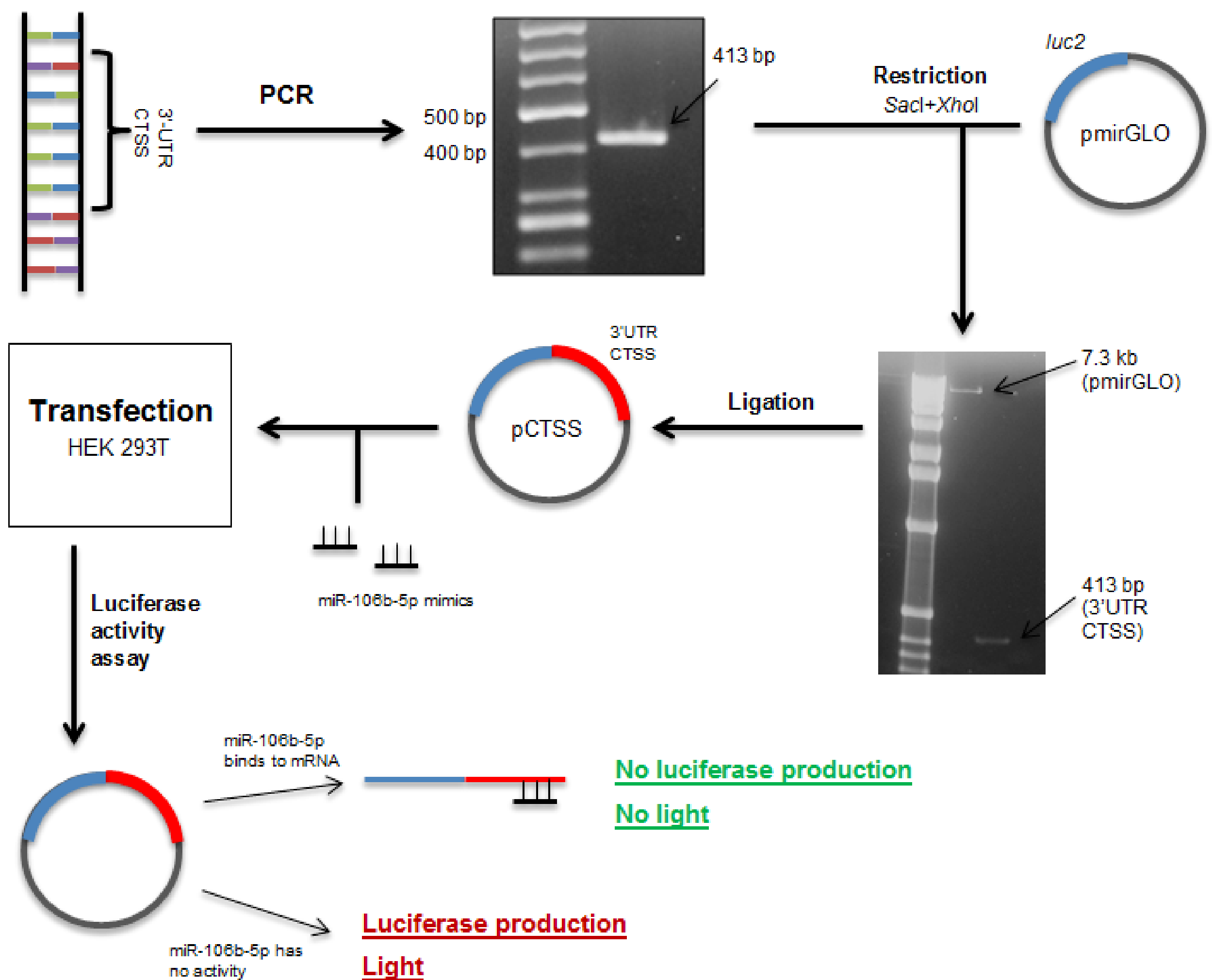

Figure 7. Flowchart scheme of the experimental plan for assessing miR-106b-5p interaction with the 3'UTR of the human CtsS gene transcript. A 413 bp fragment of the CtsS 3'UTR was amplified by standard PCR from human genomic DNA, and then digested with *Sac*I and *Xho*I, along with the pmirGLO vector. The fragment was then ligated to the vector to originate a recombinant plasmid (pCTSS-3'UTR), with the CtsS 3'-UTR fragment inserted immediately downstream of the open reading frame (ORF) of the firefly luciferase gene (*luc2*). The recombinant plasmid was transfected into HEK 293T cells, along with miR-106b-5p mimics, and incubated for 24 h at 37º C, 5% CO_2. After this, the cells were lysed, and luciferase activity was analyzed in the lysates: if there is activity, miR-106b-5p will bind to the *luc2* transcript, block translation of firefly luciferase, and ultimately prevent emission of light from luciferase activity; on the other hand, if there is no miRNA activity, the firefly luciferase transcript is translated, and emission of light can be observed.

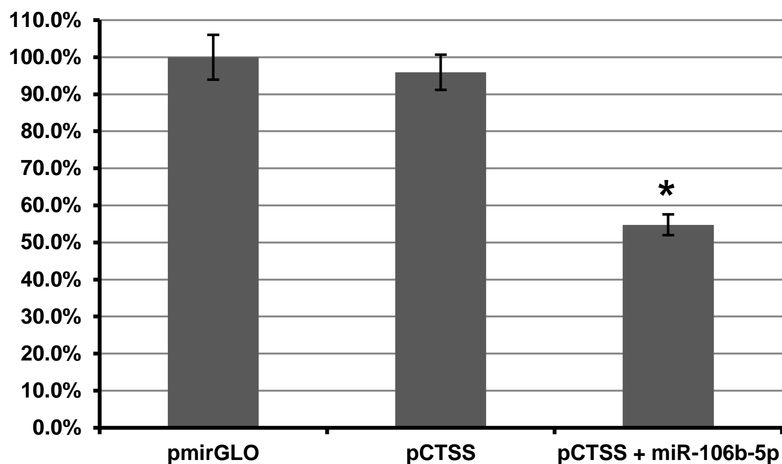

Figure 8. Quantification of firefly luciferase activity in transfected HEK 293T cell lysates. Cells were transfected with the non-recombinant vector (pmirGLO), with the recombinant vector (pCTSS) and with the recombinant vector plus miR-106b-5p mimics. Values are presented as percentage of the mean of (luminescence intensity from firefly luciferase ÷ luminescence intensity from *Renilla* luciferase) ± STD, for each condition, in comparison with the pmirGLO transfected control (*$p<0.001$, comparatively with both controls).

4. Discussion

Tuberculosis can be seen and understood as an immunopathology: the bacterium infects phagocytes, and subverts their mechanisms of intracellular killing, disturbing the immune regulation and ultimately rendering the body susceptible to the exacerbated immunological response triggered by the bacterium, which in turn become the disease itself (Divangahi et al., 2013; Rook et al., 1991). Knowledge of these matters has driven the scientific community into exploring possibilities of treating tuberculosis through modulation of immune system components, rather than using antibiotics, which are becoming increasingly less effective due to the various multidrug-resistant strains that arose and are currently spreading throughout the world.

In the first part of this thesis, we obtained encouraging results. In the triage represented in Figure 3, several cathepsins were identified as influential in the intracellular killing of *M. smegmatis*, among which cathepsins D and G, which have already been shown to be relevant to the intracellular killing of mycobacteria (Rivera-Marrero et al., 2004; Welin et al., 2011); after these observations, we will need to perform the same experiment with *M. tuberculosis* and HMDMs to better establish a connection between cathepsins and mycobacterial pathogenesis during infection, that is, between cathepsins and the disease. Focusing on cathepsin S and miR-106b-5p, we also had interesting results. *M. tuberculosis* indeed seems to reduce cathepsin S expression during infection, as opposed to *M. smegmatis*, suggesting that manipulation of cathepsin S is associated with virulence in mycobacteria. Results obtained with miR-106b-5p expression have also been significant: *M. tuberculosis* up-regulates expression of this miRNA, again in contrast with *M. smegmatis*, hinting that, again, manipulation of the miRNA is related to mycobacterial virulence in humans. After having somewhat clarified the influence of *M. tuberculosis* in cathepsin S expression, it is necessary to explore the influence of the bacterium in cathepsin S function. As was stated before, recent studies have deeply implicated cathepsin S in antigen presentation via the major histocompatibility class II (MHC-II) molecules in humans (Liu and Spero, 2004; Nakagawa and Rudensky, 1999); also, it has been demonstrated in earlier studies that infection with *M. tuberculosis* causes a reduction in MHC-II expression in macrophages (Hmama et al., 1998). As such, the next goal for this work shall be to explore the correlation between *M. tuberculosis* infection, cathepsin S down-regulation, and subsequent decrease in MHC-II expression in macrophages.

Regarding the second part of this thesis, we obtained some ambiguous results, along with some expressive results. Transfection assays will need to be repeated in order to confirm cathepsin S down-regulation in macrophages transfected with miR-106-5p mimics (Figure 6);

in the eventuality that these results remain the same after repeated assays, new approaches should be taken, such as exploring methods of increasing miR-106-5p concentration in the cells other than transfecting them with mimics (Jadhav et al., 2012). As for the assessment of miR-106b-5p effect on the human CtsS transcript, the evidence points out that indeed the 3'UTR of CtsS is a target for miR-106b-5p regulation. However, to further consolidate this data, we will need to perform an affinity/specificity analysis, in addition to the affinity analysis that was already carried out: creating a new recombinant vector, with a 1-nucleotide mismatch in the seed sequence of miR-106b-5p, would allow us to understand how specific miR-106b-5p is to the human CtsS 3'UTR, as was described by Bettencourt et al. (2013).

Overall, considering our initially proposed model, I can say that it was clarified and consolidated to a considerable extent. Still, with every new thing discovered, more questions arise: if *M. tuberculosis* really does manipulate miR-106b-5p expression during infection, what is the molecular/physiological mechanism behind this? Does *M. tuberculosis* modulate cathepsin S expression in any other way? Does regulation of other target genes of miR-106b-5p interfere with the intracellular survival of *M. tuberculosis*? Answers to these questions would support and complete our hypothetic model further still (Figure 9).

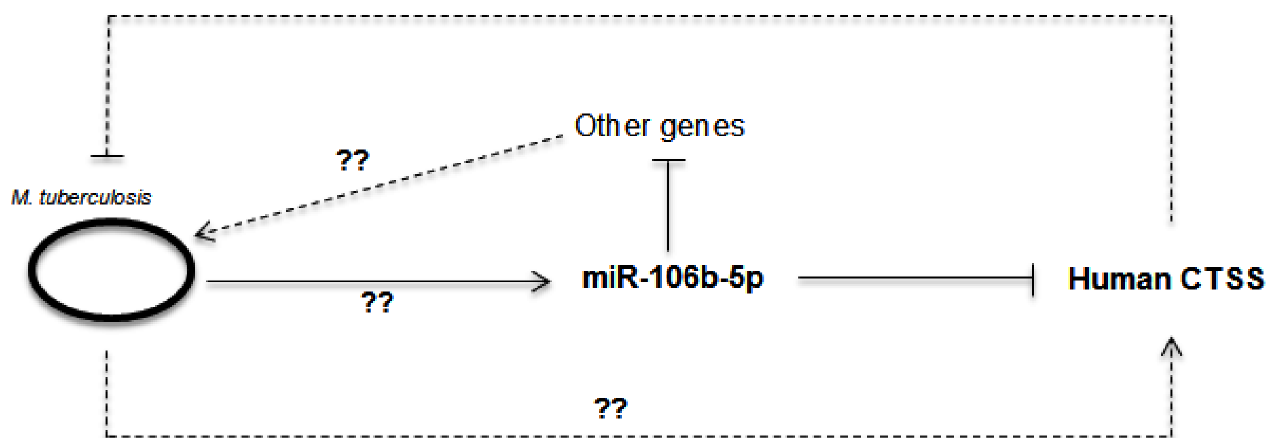

Figure 9. Our hypothesized model for cathepsin S gene (CtsS) manipulation, through modulation of miR-106b-5p, by *M. tuberculosis* during macrophage infection. The up-regulation of miR-106b-5p by *M. tuberculosis* during infection has been put to light, however the way this is processed remains unknown. Up-regulation of miR-106b seems to cause down-regulation of CtsS, which in turn reduces intracellular survival in *M. smegmatis*, in normal levels; however, since *M. smegmatis* is not pathogenic and does not reduce CtsS expression during infection, the fact that *M. tuberculosis* reduces CtsS expression during infection suggests that cathepsin S might inhibit intracellular survival of *M. tuberculosis* as well. CtsS is the first miR-106b-5p target gene to have been associated with *M. tuberculosis* infection in macrophages; however, other target genes for this miRNA might play a role in this scenario as well. Finally, we clarified that *M. tuberculosis* reduces CtsS expression during infection and that modulating miR-106b-5p levels seems to be a way to achieve this; still, reduction of CtsS expression during infection might be achieved through other different pathways as well.

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