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Identification and Manipulation of Cystatins to Control *Mycobacterium tuberculosis* Infection of Human Macrophages

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Abstract

Tuberculosis (TB) is a disease caused by the facultative intracellular species *Mycobacterium tuberculosis* (Mtb), one of humankind's most ancient and successful pathogens. The eradication of Mtb infection has been a problem over the ages, due to pathogen's ability to survive in host cells and escape the immune defense mechanisms. One efficient mechanism to kill pathogens occurs within macrophages, innate immune cells that use, among others, phagocytosis, a mechanisms by which the macrophage engulfs the bacteria in a structure called the phagosome which matures and in which a series of killing events will the clear pathogens. Specifically, the content of the phagosome will acquire proteolytic enzymes, as is the case of cathepsins, that will take relevant role in pathogen destruction at the low pH environment of the phagolysosome and, not less relevant, processing pathogen antigens to be presented to lymphocytes and therefore to expand the immune response. Recent studies revealed that Mtb is able to regulate cathepsins at the level of the gene expression and activity, increasing its survival inside macrophages. Cathepsins are regulated by their inhibitors, cystatins, which bind their active site and down-regulate cathepsins activity. Since Mtb was shown to down-regulate cathepsin activity we hypothesize that the cystatin pathway might be explored to improve the processes dependent on cathepsin activity such as pathogen destruction and antigen processing by type II HLA complexes, to counter the ability of Mtb to survive during infection.

In the first part of the study we target cystatin C, since it is described to be a major inhibitor of cathepsin S, an important cathepsin enrolled in pathogen clearance and antigen presentation. By silencing the mRNA of cystatin C we do hypothesized to prevent it from inhibiting cathepsins activation during several models of mycobacteria infection including the vaccine species *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and the non-virulent species *Mycobacterium smegmatis* infection. Overall, our results after cystatin C silencing, indicate an increase in proinflammatory responses by infected macrophages, concomitant with an increase of mycobacteria killing, and an improvement of the expression of HLA-DR receptors on the cell surface.

In parallel, we focused on the influence of protease inhibitors (PIs) used in HIV therapy, as an alternative model to cystatin silencing, to control cathepsin activity within macrophages during mycobacterial infections. We assessed the effects of saquinavir and ritonavir on the antigen presentation HLA class II complexes expression at the cell surface and on the mycobacterial killing/survival effects. We found notable interactions between the infected cells and the protease inhibitors, particularly saquinavir was observed to increase the expression of HLA-DR on the cell surface and increased mycobacterial killing, while ritonavir decreased HLA-DR expression on the surface and contributed to an increased mycobacterial survival.

Altogether our results show that by manipulating cystatin C expression in macrophages and/or the treatment of infected cells with PIs, leads to macrophage interference of cathepsins and therefore with macrophages defense mechanisms. Thus suggests that cystatins and PIs manipulation are a potential tool to improve the host cellular immunity against Mtb and be used as an host-targeted therapeutic.

Key-words: tuberculosis, mycobacteria, macrophages, cathepsins, cystatins, saquinavir, ritonavir

Resumo

A tuberculose é uma doença infeciosa que se desenvolve, na maioria dos casos, nos pulmões, causada por um agente patogénico intracelular facultativo do complexo *Mycobacterium tuberculosis* spp que inclui a espécie *Mycobacterium tuberculosis* (Mtb) e o *Mycobacterium bovis.* Apesar do desenvolvimento de antibióticos para terapêutica e do melhoramento da qualidade sanitária ao longo dos anos, nos dias de hoje a tuberculose é considerada uma das 10 maiores causas de morte por doença infeciosa. Isto deve-se à capacidade adaptativa deste agente patogénico, que levou ao aparecimento de formas multirresistentes a antibióticos, tornando difícil a sua irradicação no mundo.

A capacidade adaptativa do *M. tuberculosis* levou à aquisição de mecanismo que contornam os mecanismos de defesa do hospedeiro. O sistema imunitário responde ao contacto pelos microrganismos, iniciando-se o recrutamento de células imunes inatas como, os macrófagos e células dendríticas, os quais são a primeira linha de defesa celular do hospedeiro. Os macrófagos têm a capacidade de destruir conteúdo extracelular através de um processo denominado fagocitose, este processo inicia-se quando os macrófagos reconhecem e internalizam a bactéria a vesícula chamada fagossoma, que passa por diferentes estádios de maturação através de uma série de fissões e fusões parciais do organelo com endossomas tardios. O estádio final de maturação após fusão com os lisossomas primários dá origem ao fagolisossoma. O conteúdo desta vesicula possuí enzimas hidrolíticas que no pH acídico desse lúmen ficam ativadas, proporcionando condições ambientais ótimas para a degradação dos microrganismos internalizados. Contudo, Mtb consegue escapar a este sistema, impedindo a sua maturação e bloqueando no seu nicho ao estado de vesicula fagossomal imatura. Deste modo persiste intracelularmente limitando a sua interação com estes mecanismos degradativos o que lhe permite sobreviver e inclusivamente replicar-se em macrófagos de granulomas nos pulmões. Assim, na maioria dos casos o balanço entre a sobrevivência, a replicação da bactéria e o recrutamento e ação das células do sistema imune leva a que a infeção fique confinada a uma estrutura denominada granuloma que permite o seu controlo. O indivíduo encontra-se infetado mas não demonstra ter sintomas de doença, existindo uma doença/infeção latente que pode ser ativada em qualquer momento que o organismo desenvolva imunodepressão.

Um grupo muito importante de enzimas proteolíticas que se encontram no lúmen das vesículas fagocitárias, são as catepsinas. Estas hidrolases lisossomais clivam as ligações peptídicas levando à degradação proteica e atuam no processamento de péptidos para apresentação de antígenos, contribuindo para a morte de agentes patogénicos. Foi observado em estudos que uma das catepsinas com maior nível de expressão durante a infeção, na fagocitose e nos macrófagos, é a catepsina S, cuja principal função é apresentar antigénios ao complexo principal de histocompatibilidade classe II (MHC II em ratinho ou HLA II em humanos). Apesar das catepsinas serem bastante expressas durante a infeção, elas são controladas pelos seus inibidores naturais as cistatinas, que se ligam ao sítio ativo das catepsinas inibindo a sua atividade proteolítica. Contudo, existem evidências que o bacilo da tuberculose interfere com a atividade proteolítica do macrófago para aumentar a sua sobrevivência durante a infeção, diminuindo a atividade das catepsinas. O mesmo foi demonstrado ao nível da expressão genética das cistatinas, mas não existem estudos que comprovem que esse mecanismo afeta também a atividade inibitória das cistatinas nem o efeito no controlo da infeção. Uma possível via para contornar a reação adversa da micobactéria, seria através da manipulação das cistatinas, tornando-as interessantes alvo de estudo.

A tuberculose é uma doença altamente associada à infeção pelo vírus da imunodeficiência humana (HIV) uma vez que a consequente imunodeficiência provocada pela ação do vírus leva à disrupção do granuloma que contém a infeção por Mtb e disseminação do bacilo pelos tecidos. Um conjunto de moléculas utilizadas para o tratamento do HIV, são os inibidores da protease viral (IP). Já foi demonstrado experimentalmente que os IP influenciam a atividade das catepsinas durante a infeção pelo HIV. Assim sendo, estes inibidores podem ser potencialmente úteis na manipulação das catepsinas durante a infeção por outros agentes patogénicos, incluindo o Mtb a fim de contribuir para controlar esses infeções.

O principal objetivo deste estudo foi investigar a interação entre cistatinas, Mtb e macrófagos, usando as cistatinas como ferramenta para manipular a proteólise por catepsinas durante a infeção e reforçar a resposta do hospedeiro na eliminação dos patogenos.

Neste estudo, usamos metodologias de RNA de interferência ('small interfering RNA; siRNA') específicos para silenciar o RNAm para a cistatina C durante a infeção por *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) e *Mycobacterium smegmatis*. Esta cistatina é expressa em todos os tecidos e é o maior inibidor natural da catepsina S.

Numa primeira fase, foi estudada a expressão génica da cistatina C durante a infeção por micobactérias através de PCR quantitativo (qPCR). Os nossos resultados mostram que a eficiência do silenciamento da cistatina C pela técnica usada em macrófagos, é cerca de 50% na redução dos níveis do RNA mensageiro comparativamente com os níveis obtidos no controlo, ou seja, em macrófagos nos quais foi usado um siRNA controlo inespecífico (scramble).

As consequências do silenciamento ao nível da resposta inflamatória, especificamente por análise de ELISA da secreção de interlucina-1β (IL-1β) em macrófagos não infetados, comparativamente com macrófagos infetados com *M. bovis* BCG e *M. smegmatis*, indicam que com silenciamento da cistatina C concentrações de IL-1β aumentaram ainda mais. Também foi possível verificar diferenças entre macrófagos infetados com *M. bovis* BCG e *M. smegmatis* com e sem silenciamento da cistatina C.

A análise da expressão dos marcadores apresentadores de antigénios à superfície dos macrófagos foi feita através da citometria de fluxo. O antigénio leucocitário humano tipo II (type II human leukocyte antigen, HLA-DR) foi usado nesta experiência pela sua capacidade de apresentar péptidos de bactérias fagocitadas aos linfócitos T. Os níveis de expressão foram analisados em macrófagos não infetados e macrófagos infetados com *M. bovis* BCG e *M. smegmatis*, com e sem silenciamento da cistatina C. Baixos níveis de expressão da HLA-DR foram observados em macrófagos infetados com *M. bovis* BCG, um efeito descrito em micobactérias patogénicas e que é comum quer ao Mtb quer ao BCG. No entanto, os macrófagos silenciados apresentaram uma restituição desses níveis da expressão da HLA-DR para níveis semelhantes a *M. smegmatis*, melhorando assim a resposta imune.

A sobrevivência das micobactérias durante a infeção foi estudada através da contagem de unidades formadoras de colónias (colony-forming unit, CFU) em macrófagos infetados com *M. bovis* BCG e *M. smegmatis*, com e sem silenciamento da cistatina C. A infeção por micobactérias em macrófagos leva a um aumento da sua sobrevivência, mas ao manipularmos a cistatina C, silenciando-a, observamos que existe uma redução da sobrevivência das micobactérias apontando possivelmente para um aumento da atividade proteolítica e capacidade de matar o patogeno.

Em paralelo, macrófagos infetados foram tratados com as inibidoras da protease viral do HIV (IP), especificamente saquinavir (SQV) e ritonavir (RTV), para estudar o seu efeito na modulação da resposta do hospedeiro após a infeção com *M. bovis* BCG. Foram analisados ao nível: da resposta inflamatória, através da análise da secreção da citoquina IL-1β; da expressão dos apresentadores de

antigénios à superfície, usando o HLA-DR como antigénio de análise, e na sobrevivência da micobactéria através da contagem de unidades formadores de colónias.

Quando as células infetadas foram tratadas com os IP, os resultados mostram que ao nível da resposta inflamatória, existe um aumento abruto da secreção da IL-1β quando infetadas com micobactéria comparativamente com as células não infetadas. No entanto, observaram-se diferenças significativas entre os tratamentos em células infetadas. No tratamento com RTV em macrófagos infetados, observou-se um maior aumento da secreção de IL-1β do que nos macrófagos infetados tratados com SQV.

Na análise da expressão dos marcadores apresentadores de antigénios, os tratamentos levam a uma variação significativa da expressão do HLA-DR. Contudo verificou-se que o saquinavir modula a resposta positivamente, aumentando os níveis de expressão da HLA-DR à superfície da célula, enquanto o ritonavir diminuí os efeitos do mesmo.

A contagem de unidades formadoras de colónias permitiu observar que ao longo do estudo, o tratamento com saquinavir diminuí a sobrevivência do *M. bovis* BCG, em contraste o tratamento com ritonavir aumenta significativamente a sobrevivência.

No conjunto, os resultados obtidos no silenciamento da expressão da cistatina C levaram a um aumento da resposta pró-inflamatória por macrófagos infetados, tal como um aumento da capacidade do macrófago de expressar moléculas apresentadoras de antigénios à superfície da célula e um aumento na morte intracelular das micobactérias. Isto sugere que a manipulação das cistatinas, que regulam negativamente as catepsinas, podem ser uma ferramenta viável para potenciar as células hospedeiras, no controlo da atividade proteolítica melhorando a resposta imune contra a infeção e eliminarem as micobactérias. Os resultados obtidos pelos IP mostram que células tratadas com ritonavir diminuem a capacidade de expressão de apresentadores de antigénios e aumentam a sobrevivência da micobactéria, enquanto o tratamento com saquinavir aumentou a capacidade de expressar moléculas apresentadoras de antigénios à superfície e um aumento da morte das micobactérias, tornando o saquinavir um potencial alvo de estudo para o desenvolvimento de uma nova terapia contra Mtb.

Palavras-chave: tuberculose, micobactéria, macrófagos, catepsinas, cistatinas, saquinavir, ritonavir

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Abbreviations

Mtb	*Mycobacterium tuberculosis*
TB	Tuberculosis
BCG	Bacille Calmette-Guérin
PIs	Protease inhibitors
GFP	Green Fluorescent Protein
MHC	Major Histocompatibility Complex
SQV	Saquinavir
RTV	Ritonavir
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
HIV	human immunodeficiency virus
siRNA	short interference RNA
RPMI	Roswell Park Memorial Institute
OADC	Oleic acid-albumin-dextrose-catalase
qRT- PCR	Quantitative reverse transcription polymerase chain reaction
MOI	Multiplicity of infection

1. Introduction

Tuberculosis (TB) is an infectious disease caused by the facultative intracellular bacteria of the *Mycobacterium tuberculosis* Complex (MTC) [1]. The disease occurs in the lungs by inhalation of airborne droplets containing the bacilli, settling in the respiratory system of the host and infecting other individuals using as propagation vehicle the cough of the host [2]. It caused 1.3 million deaths in 2017, becoming one of the top 10 leading causes of death and the main cause of death by a singular infectious agent worldwide [3]. Tuberculosis diagnosis had a swift development during the last 10 years, however even with TB therapy which intends to avoid complications and mortality, the number of new cases increased to 5.7 – 5.8 million per year [4]. These numbers reveal the importance of knowing and understanding the host defense mechanisms and the interactions between the pathogen and the cells of the immune system, in order to create new tools against *M. tuberculosis* and decrease the mortality of this disease.

1.1. MTb infection – transmission and the immune system

M. tuberculosis (Mtb) was presented for the first time to the Physiological Society of Berlin in 1882 by Robert Koch, who showed evidences for the isolation of a living rod-shaped organism causing the disease (TB) [5]. Mycobacteria are Gram-positive and acid-fast, they are aerobic and rod shaped and comprising a peptidoglycan-arabinogalactan polymer with covalently bound mycolic acids which are components of their walls [6]. This provides an extraordinary efficient permeability barrier to noxious compounds and many drugs. Treatments affecting mycolic acid biosynthesis and the production of extractable lipids resulted in an increase of cell wall permeability which drastically decrease the virulence of Mtb [7].

M. tuberculosis dynamics within the host begin by inhalation of bacilli expelled by an individual with active tuberculosis and installment of the bacteria in the respiratory tract, more specifically in the lungs. When in the lungs the immune system is activated, macrophages and dendritic-cells (DC) are recruited being the first line of defense of our immune system. After their activation they are able to kill intracellular bacteria and participate in a protective T helper cell type 1 (Th1) response [8].

Macrophages are recruited to the infection site, participating in the formation of the granuloma that arises in response to a persistent stimulus [9]. Granulomas are organized aggregates of immune cells in which macrophages and T cells play the central role. The structure of the granuloma develops with the fusion of macrophages resulting in multinucleated giant cells and lipid-filled foamy macrophages surrounded by a ring of lymphocytes encapsulated in a fibrotic cuff [10,11]. The structure main function is to contain the pathogen, providing an important niche during the infection. Granuloma differs between active and latent form of infection. In latent infection the center of granuloma is made of infected macrophages and other cells and fibrotic granulomas without bacilli also exist. In active disease is found granulomas composed of dead and alive macrophages at the center and few lymphocytes, necrotic neutrophilic cells, giant foam cells and epithelioid macrophages. The state of the granuloma is crucial for control of bacterial proliferation. In latent infection, Mtb resides in the hypoxic center, rich in lipids,

but in active disease, Mtb can replicate in peripheral oxygenated areas. Deficiency on some cytokines can lead to granuloma malformation leading to bacteria proliferation [12,13].

However, Mtb is able to resists inside the granuloma and persist in the lungs, escaping using its own strategies against the granuloma and proliferate to the lung tissue, leading to active pulmonary tuberculosis.

As pathogens have elaborated defense and survival strategies to persist during infection and replicate in a host organism, likewise the host organism developed strategies for their protection such as phagocytosis, strict control over the production of proinflammatory and anti-inflammatory cytokines, apoptosis, and others. The alveolar macrophages internalizing and exposing pathogens to the acidic and hydrolytically active environment of the phagosome. This environment takes an important role to process and present peptide antigens derived from bacterial degradation, to T lymphocytes to major histocompatibility complex class II (MHC II) molecules [14]. However, this pathogen survived by avoiding macrophages mechanisms and by replicating within these cells.

Macrophages are activated by $CD4^+$ and $CD8^+$ T cells, to enhance their antimicrobial effects. They recognize microbe-associated molecular patterns (MAMPs) of the bacteria via pattern recognition receptors (PRRs) being the responsible to initiate the innate and adaptive immune response to Mtb [15].

There are cytokines that potentiate the action of the cells of the immune system. Bacteria lipopolysaccharide (LPS) activated macrophages which release of proinflammatory cytokines like: (1) TNF-α, that contributes to inflammation and granuloma formation; (2) IL-1β, involved in the immune response such as TNF increased secretion, neutrophil chemotaxis, endothelial activation; (3) IL-6, and IL-12 working as synergistic activity on inflammatory pathway and to amplify this response leading to antigen presentation and IFN-γ secretion by $CD4^+$ and $CD8^+$ T-helper cells [16,17].

1.2. Phagocytosis

An important innate immune mechanism performed by macrophages to eliminate bacteria is phagocytosis. Phagocytosis begins when the pathogen is recognized by host cellular receptors that promote its internalization in a vacuole called the phagosome. Then the phagosomal membrane composition changes becoming accessible to early endosomes, and by fusion and fission events, its content matures and becomes able to fuse with late endosomes. After these events, it merge with lysosomes forming a phagolysosome. In the phagolysosome several aggressions are delivered to the bacteria: (1) the vacuolar H^+ - ATPase - whose activity leads to an acidification of the phagolysosomal lumen to a pH around 5; (2) release of reactive oxygen species (ROS) produced by NADPH-oxidase and reactive nitrogen species (NOS) produced by nitric oxide synthase; (3) increased activity of proteolytic enzymes such as cathepsins and also of lipolytic enzymes. This deadly combination promotes the bacteria degradation. However Mtb is capable to counter these macrophages mechanisms and survive[18–21].

There are many pathways that Mtb use to escape from the degradative aspects of the phagocytic process. The majority of them involve inhibition of the maturation of phagolysosomes. These may occur by: (1) inhibiting early endosome maturation to late endosome by acting above Rab GTPase network; (2) inhibiting the phagosomal acidification by secreting PtpA to inhibit H^+ ATPase complex; (3) blocking the fusion with lysosomes avoiding contact with degrative lysosomal enzymes through

recruitment of Coronin 1 to the phagosomal surface; (4) inhibiting autophagy using virulence components to overexpressing macrophages to regulate the apoptotic response [22,23].

Since Mtb is able to modify the phagosomal environment by interfering with several mechanisms of the macrophages, they are also interfering with antigen presentation by inhibiting this system. Toll-Like receptors (TLRs) are innate PRR receptors on the macrophages surface. They recognize Mtb, and produce proinflammatory cytokines required to control Mtb infection and the activation of $CD4^+$ T cell. During this process, proteases degrade bacteria into peptide antigens that are loaded to MHC class II and induce a $CD4^+$ T cell response and IFN- γ is expressed in control the infection. IFN- γ is important in this process not just by promoting effector mechanisms for containing pathogens but to induce MHC class II molecules.

Mtb infected macrophages decrease MHC class II expression and antigen presentation, and it's possibly related with mycobacterial lipoproteins which inhibit MHC class II molecule expression. These lipoproteins are agonist of TLR2 and MHC class II expression which antigen presentation are dependent of this receptor, prolonged TLR2 signaling may inhibit MHC class II antigen processing and presentation [24–26,27].

1.3. Cystatins and Cathepsins

During phagolysosome formation, the lysosome fuses with the phagosome, integrating components of its content such as degradative lysosomal enzymes. These enzymes contribute to lower pH and are involved in many processes as protein degradation, autophagy, direct pathogen killing, antigen presentation, among others. Thus, pathogens encased in macrophages phagosomes can be degraded by hydrolases such as cysteine proteases which are preponderant among lysosomal proteases.

Cysteine proteases, or cathepsins, irreversible cleave peptide bonds exhibiting broad specificity, cleaving their substrates preferentially after basic or hydrophobic residues and possessing a multitude of substrates consistent with their roles in intracellular protein degradation. There are three groups of cathepsins found in humans denominated according to the amino acid residue at the proteolytic site: aspartatic proteases, cathepsins D and E; serine proteases, cathepsins A and G and the cysteine proteases, cathepsins B, C, F, H, K, L, O, S, V, X and W. Some of these cathepsins interact with Mtb, more specifically B, D, G and S [28–32].

One particularity of almost all cathepsins is they have optimal activity at acidic pH and reduced or no activity at neutral and non-acidic pH. Cathepsins increase their activity in late endosomes and lysosomes which haves acidic pH content, important against intracellular bacteria. However, cathepsin S (Cat S) is an exception being capable to maintain its activity at neutral pH [33].

In the human lungs, were found high levels of Cat S expression. This particular protease maintains its activity and stability at neutral pH, and its main function is in antigen processing and presentation having high expression levels during phagocytosis and in antigen-presenting cells such as macrophages [31,32,34]. Cathepsin S is involved in the processing of maturation of MHC class II molecule. It was shown in *Mycobacterium bovis* spp. Bacille Calmette-Guérin (*M. bovis* BCG) infected cells that when Cat S expression was inhibited there was a decrease of MHC class II expression in macrophages, suggesting that by manipulating Cat S expression we are interfering with antigen presentation mechanism [31,32,35].

One important natural regulator of Cat S is cystatin C (CST C), a cysteine peptidase inhibitor, which binds reversibly to proteases at their active site, thus competing with the substrate, down-regulating its activity.

Cystatins come from a superfamily of evolutionary related proteins, each consisting in at least one domain of 100-120 amino acid residues with conserved sequence motifs. They are found in all living organisms (mammals, nematodes, arthropods etc.), and act in multiples biological processes as regulation of hormone processing, inflammation, antigen presentation and T-cell dependent immune response and modulators of proteolytic activity against bacterial and viral infection [36].

This superfamily is divided by its structure in three families: **Type I cystatins**, known as Stefins (Stefins A and B), are polypeptides of 98 amino acid residues without disulfide bonds nor carbohydrate side chains and with one cystatin domain. They are mainly detected intracellularly acting as inhibitors of cysteine proteases. **Type II cystatins**, cystatins C, D, E/M, F, S, SA, SN, CRES, testatin, cystatins 11, 12, 13 and 14, are bigger than type I cystatins with a single-chain polypeptides with 120 amino acid residues and have two conserved disulfide bridges. They can be secreted to extracellular space through the membrane cell by a signal peptide enhance their concentrations extracellularly. **Type III cystatins** are the most complex of all. They have approximately 350 amino acid residues, eight disulfide bonds and three type II cystatin domain. They are kininogens, intravascular proteins which are involved in the processes of protection against leaking lysosomal cysteine proteases [37–40].

The molecular constitution of cystatins makes them tight-reversible binding protein inhibitors, and it is their structure that allows the manipulation of cathepsins. They have a long five-turn α-helix and a five-stranded antiparallel β-pleated sheet, with an additional helix. These suggested a three conserved regions: the amino terminus and two hairpin loops, which together create a wedge-shaped edge cystatin structure highly complementary to the active-site of cathepsins, blocking them. [40,41]

The interaction between cystatins and their target proteases is determined by the differences in the structures of the interacting regions of the enzymes, which differ between every type of cystatins. In type I cystatins case, Stefin A is strongly related with the tissues in the first-line of defense, like the skin immune system, and it is highly expressed in follicular dendritic cells of germinal centers in secondary lymphoid organs, being able to prevent apoptosis [40,42]. On the other hand, Stefin B is expressed in all human cells but its expression is increased by lipopolysaccharide (LPS) stimulation in response to bacterial infections [40,43]. Type II cystatins act in different ways to help the immune system, but cystatin F and C have high expression in principal cells of the immune system. Cystatin F is found at dimeric and monomeric forms in dendritic cells, T cells and NK cells, regulating the activity of cathepsins by the endosomal/lysosomal pathway, but it is only activated when there is proteolytic cleavage at the N-terminus converting it from dimer to monodimer and thus becoming capable to inhibit its main targets, cathepsins F, H, K, L, S and V [44,45]. A feature of this cystatin is that it can be activated in its inactive dimeric form by inhibiting mannose-6-phosphate, which allows proteases to be trans-regulated in cells that do not express cystatin F [46]. However, the most abundant cystatin found in a variety human tissues, cells types and body fluids is **cystatin C.**

Cystatin C has a molecular mass of 13,343 Da and its encoding gene is a house-keeping gene, acting as an emergency inhibitor, neutralizing redundant proteolytic activity outside cells. Thus, it is considered the major inhibitor of cathepsins since its ability to bind tightly to the active-site of cathepsins, more specifically Cat B, H, L and S [47]. It was also shown interaction between CST C and Cat K, where CST C regulate Cat K which acts in the degradation of the bone matrix [48]. Many studies

suggest CST C strongly regulate cathepsin S activity it was shown in Sjögren's Syndrome patient tears where is a reduction of CST C activity and an increase of Cat S activity changing tear composition [49]. Beyond interfering in proteolytic activity by inhibit cathepsins, cystatin can also be involved in the production of reactive nitrogen species (NOS) in macrophages which is an important mechanism against microorganism, as previously mentioned. Some studies suggest that the combination of cystatin C and IFN-γ induced production of NOS promoting pathogen killing [38].

Cystatin C is implicated in several diseases: it's a marker of glomerular filtration rate and the kidney function [50]; it's involved in systemic lupus erythematosus inflammation process; cerebral amyloid angiopathy [51]; promotes atherosclerosis; it's involved in Alzheimer's disease; among others. This cystatin is also related with Mtb infection. One of the mechanisms of Mtb to inhibiting phagosome maturation is to avoid the contact with cathepsins. Previously studies shown the treatment with cystatin leads to an increase of Mtb survival rate in macrophages. That suggest a down-regulation of cathepsins in macrophages which consequently promotes Mtb survival [31]. Since CST C is a natural inhibitor of Cat S, this is a possible tool against Mtb infection.

1.4. Immunodepression, HIV and antiretroviral therapy

Tuberculosis and human immunodeficiency virus (HIV) are the main important infectious diseases with impact in resource-limited countries. In 2012, was estimated that 1.1 million of people were Mtb-HIV co-infected [52]. Tuberculosis and HIV act against the immune system. The co-infection is benefic to both diseases, HIV increases the risk of Mtb progression and Mtb increases the replication of the virus [53].

Some treatments against the HIV involve protease inhibition. It was already shown in some studies that HIV proteases+ inhibitors affect cathepsins during HIV infection [54]. In addition, it has been shown that Highly Antiretroviral Therapy (HAART), an HIV treatment, could decrease the impact on the immune system in the early stage of Mtb infection [55].

The human immunodeficiency virus (HIV) is a chronic infection leading to the acquired immunodeficiency syndrome (AIDS). It is estimated that almost 37 million people are living with this infection [56]. This infection has impact in the immune system leaving it compromised, and increases the probability of acquire other infections or diseases [57]. A customized combination of anti-retroviral drugs is a therapeutic strategy to delay the infection progress, strategy known by Highly Antiretroviral Therapy (HAART). This combination is made by nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTIs (nNRTIs), integrase inhibitors and protease inhibitors (PIs) [58]. Like MTb infection, HIV can infect macrophages in the lungs and interfere with the secretion of proinflammatory cytokines, such as type I interferons, IFN-γ and TNF- α which contribute to disease control in both cases [59]. In Mtb-HIV co-infection, was shown that cytokines production by Mtb-activated macrophages enhance replication of HIV-1 [60].

Recent studies showed a reduction of mortality in Mtb-HIV co-infected patients during HAART regimen. Since macrophages are common to both diseases, and one defense mechanism against pathogens is proteolytic activity, the PIs from HAART could be acting in both diseases changing the proteolytic activity reducing both infections [61].

Macrophages and DCs are both antigen presenting cells, they indeed **cross-present exogenous antigens**. One way to do cross-presentation is by endosomes or phagosomes, where proteins are partially

degraded by cathepsins and are presented to MHC-I [62,63]. Inhibited proteolytic activity breaks the role of cross-presented antigens with a consequent the increase of infection.

During HIV infection the **PIs** block the HIV aspartyl protease not allowing the cleavage of HIV Gag-Pol polyprotein and consequently the conversion of HIV particles into mature infectious virions [64]. PIs like ritonavir (RTV) and saquinavir (SQV) also inhibit the proteolytic activity of the proteasome and consequently intracellular accumulation of polyubiquitinated proteins [65,66].

Saquinavir was the first-generation of HIV protease inhibitors based on hydroxyethelene and hydroxyethelamine isosteres [64]. Another developed PI was Ritonavir, a potent inhibitor of cytochrome P450 3A and inhibitor of p-glycoprotein efflux pumps. Ritonavir in HAART, is used more frequently as a booster to increasing SQV absorption and decreasing the metabolism of SQV allowing for larger bioavailability of SQV [67,68]. Both anti-retrovirals are capable to bind the active site of proteases, inhibiting them, similarly to how they inhibit HIV proteases. Recent studies showed that SQV and RTV have a strong effect on cathepsins activities. RTV induced a reduction of cathepsin activities while SQV enhanced them [54]. These results suggest the hypothesis of using SQV and RTV during MTb infection to manipulate the proteolytic activity of human cathepsins to reduce the Mtb survival and improve other aspects of the immune response.

Objective

Macrophages use cathepsins to destroy the bacteria and process their antigens, but Mtb interferes with these mechanisms in favor of its survival. Understanding how these proteolytic mechanisms respond to infection will provide a valuable insight to modulate the immune response to tuberculosis. The main goal of this thesis is to modulate the phagosomal proteolytic activity in macrophages infected with *Mycobacterium bovis* BCG by manipulating the natural inhibitors of cathepsins, the cystatins, or by treatment with protease inhibitors used in HIV therapeutic.

2. Materials and Methods

2.1. Cell lines and culture conditions

a. Monocyte isolation

CD14 $^{+}$ monocytes were isolated from buffy coats from healthy donors provided by national blood institute (Instituto Português do Sangue e da Transplantação, Lisboa, Portugal).

Buffy coats were diluted ½ with MACS buffer containing EDTA 2 mM (Gibco), 0.5 % inactivated fetal bovine serum (FBS) (Corning), phosphate-buffered saline (PBS) pH 7.2 (Gibco) Leukocytes were separated by density gradient centrifugation (800 x *g*, 20 min) using Ficoll-Paque (GE Healthcare Life Science). This gradient leads to the formation of a top layer of plasma, a second layer where leukocytes are found, a third layer of Ficoll-Paque and at last the densest layer composed of erythrocytes. The leukocyte layer was recovered and the cells were resuspended in MACS buffer, and centrifuged at 500 x *g* for 10 min. The resulting supernatant was rejected. Cells were resuspended in MACS buffer, centrifuged (500 x *g*, 5 min) and the resulting cells pellet was resuspended in MACS buffer. $CD14^{+}$ were isolated by incubation of the resuspended pellet with Micromagnetic beads coated with $CD14^{+}$-specific antibodies (Miltenyi Biotec). Cells were then separated using with a magnetic column. Following separation, the cells were counted and their viability was assessed by trypan blue exclusion staining.

b. $CD14^{+}$ differentiation into Macrophages (Mø)

$CD14^{+}$ cells were cultured for 3 h in Roswell Park Memorial Institute medium (RPMI) (Gibco) complemented with 1% glutaMAX (Gibco) and 1% HEPES buffer (Hyclone) and allowed to adhere to the bottom of the wells. Following 3 h the medium was supplemented with 1:1 proportion of fresh medium in order to achieve a final concentration of 1% glutaMAX, 1 % HEPES buffer, 10 % FBS, 1 % sodium pyruvate (Hyclone), 1 % penicillin- streptomycin (Gibco), 0.1 % β-mercaptoethanol (Gibco) and macrophages colony-stimulating factor 20 ng/mL (M-CSF). Fresh complete medium, as above, was added again at the 4^{th} day of differentiation and left until the 7^{th} day following which were ready to be used.

c. Bacterial cultures

Mycobacterium bovis bacillus Calmette-Guérin (BCG) containing a GFP encoding plasmid (GFP), and *Mycobacterium smegmatis* part of the mycobacteria library of our research unit (Host-Pathogen Interaction Unit, iMED.ULisboa) were grown in Middlebrook 7H9 medium (Difco) supplemented with OADC enrichment (Difco) and 50 µg/ml hygromycin.

2.2. Infection of Mø

Bacteria cultures on exponential growth phase were centrifuged (3000 x *g*, 5 min), washed in phosphate-buffered saline (PBS) (Gibco) and centrifuged again (3000 x *g*, 5 min). Following that they

were resuspended in RPMI complete medium (without antibiotics) and submitted to an ultrasonic bath (5 min) to disrupt the bacterial clumps. Remaining clumps of bacteria were removed by a short spin (500 x *g*, 1 min). The resulting suspension was quantified measuring optical density at 600 nm (OD_{600}) to determine the concentration of bacterial cells in the suspension. Cells were washed with PBS and incubated with mycobacteria at MOI 2 for 3 h at 37° C in 5 % CO_2. Following incubation, cells were washed with PBS and fresh complete medium was added and cells were incubated at 37º C in 5% CO_2 for the rest of the experiments.

2.3. RNA extraction

Mø were seeded in 12-well plates at a density of 6 x 10^5 Cells/well and after infection RNA was extracted using the NZY Total RNA Isolation kit (NZYTech) and following manufacturer instructions. RNA bounded to the silica membrane column was eluted and its quantity and quality was determined by measuring the 260/230 and 260/280 nm absorbance ratios using NanoDrop™ 1000 spectrophotometer.

2.4. Quantitative Real-Time polymerase chain reaction (qRT-PCR)

The qRT-PCR was performed using NZY qPCR Green Master Mix (2x), ROX (NZYTech) following the manufacturer protocol. To synthesize cDNA from RNA, was used 2 µg of total RNA for cDNA synthesis performed according to NZY First-Strand cDNA synthesis Kit (NZYTech) protocol. qRT-PCR was performed using NZY qPCR Green Master Mix and the primers (Thermo Fisher Scientific) described in Table 1 at a final concentration of 0.5 µM. The qPCR reaction proceeded as follows: 1 cycle of 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s, 60 ºC for 30 sec and, 72 ºC for 30 sec. The mRNA expression profiles were normalized with respect to GAPDH (Glyceraldehyde 3phosphate dehydrogenase). The qRT-PCR was performed using an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System.

Table 2.1: Primers used in qRT-PCR

Target Gene	Primer	Sequence (5'-3')
Cystatin C	Forward	CAACAAAGCCAGCAACGACAT
	Reverse	AGAGCAGAATGCTTTCCTTTTCAGA
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAA
	Reverse	AATGAAGGGGTCATTGATGG

2.5. Transfection

The transfection with siRNA anti-cystatin C (Thermo Scientific Dharmacon ON-Target plus smart pool) was performed using the ScreenFect®A and following the manufacturer's instructions.

Cells were already adherent in a 96-well plate from the differentiation. In separate, 0.45 µl of ScreenFect®A were diluted in 10 µl of Dilution Buffer per well and 0.25 µl of siRNA anti-cystatin C were diluted in 10 µl of Dilution buffer per well. Diluted ScreenFect®A and siRNA anti-cystatin C were mixed and incubated for 20 min at room temperature to allow the complex formation. Following that were added 100 µl of freshly detached per well and resuspended the cells with the complexes. The plates were maintained at 37 ºC in a 5 % CO2 incubator overnight (16 – 18 h).

2.6. Flow cytometry

Mø were seeded in 48-well plates at density of 1.5 x 10^5 cells/well. Following infection with mycobacteria, HLA-DR expression were measured using HLA-DR specific antibody conjugated with PerCP (Biolegend). Briefly, cells were detached from the wells incubating with Accutase for 15 min at 4 °C and then centrifuged (500 x g, 5 min). The cell pellet was resuspended in 4 % paraformaldehyde for 30 min in the dark for cell fixation. Cells were washed with PBS containing 2 % of FBS and EDTA 2 mM and then incubated with the HLA-DR specific antibody for 30 min at 4 °C. The samples were analyzed in Guava easyCyte™5HT flow cytometer (Merck Millipore).

2.7. Quantification of mycobacteria intracellular survival

Mø were seeded in 96-well plates at a density of 5 x 10^4 cells/well and infected with mycobacteria, as previously described. After 3 h, 1, 3 and 5 days of infection Mø were incubated in 100 µl of 0.05 % Igepal aqueous solution (Sigma) at 37 °C for 10-15 min to lyse host cells and release bacteria. Serial dilutions of the resulting bacterial suspensions were plated in Middlebrook 7H10 supplemented with 10% OADC (Difco) and incubated for 2-3 weeks at 37° C before colonies were observable. When colonies became visible under the microscope, they were counted, and the formula N × Df⁄V was applied 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 (5 µl in this case).

2.8. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

The quantification of human IL-1β (Biolegend) in the supernatants of infected cells was performed by using Biolegend's ELISA MAX™ Deluxe Sets and reagents and by following the manufacturer's instructions. To stop the reaction, we used an acid solution 2N H_2SO_4 (Sigma). The absorbance was read by Tecan Infinite M200 Spectrophotometer at 450 nm and subtracted from the absorbance at 570 nm.

2.9. Treatment with SQV and RTV

Cells were pretreated 1 h with 5 μM of SQV and RTV kindly provided by Professor João Gonçalves (Instituto de Investigação do Medicamento, Faculdade de Farmácia da Universidade de Lisboa). The infection was performed as previously described, and the bacterial suspension was added over the treatment. After 1 h of infection the medium of infected cells were washed with PBS and fresh complete medium with 5 μM of SQV and RTV was added. The controls were treated in the same conditions but without addition of SQV and RTV.

2.10. Statistical Analysis

Data is presented as mean with the respective standard deviation or standard error (referred when used). Statistical analysis was performed with SigmapPlot 11. Multiple comparisons were made using one-way ANOVA followed by pairwise comparisons of the groups using Holm-Sidak test or Ranks. The considered nominal alpha criterion level was 0.05 below which differences between samples were considered significant.

3. Results

3.1. Manipulation of cystatin C by siRNA during mycobacteria infection in macrophages

The main objective of this study is to explore the potential of cystatin C manipulation to influence the immune response during mycobacteria infection. For this, we used strains of *Mycobacterium bovis* BCG and *Mycobacterium smegmatis* to perform our assays, as models of pathogenic and non-pathogenic bacteria related to *M. tuberculosis*. *M. bovis* BCG is an attenuated strain derived from *M. bovis*, the causative agent of bovine tuberculosis. However it activates the innate immune response and has similar properties to Mtb, making a suitable model to study the response to the virulent strains of mycobacteria [13,69]. *Mycobacterium smegmatis* is a nonpathogenic strain saprophytic bacteria that conserves significant proportion of genes and physiological mechanisms similar to other mycobacteria such as Mtb [70,71]. We selected *M. bovis* BCG because it is a non-virulent model of Mtb and *M. smegmatis* because it is a non-pathogenic mycobacteria, and both are safe to handle outside a Biosafety level-3 laboratory that otherwise would be required to manipulate Mtb.

3.1.1. Assessment of cystatin C mRNA silencing

We performed an assay to assess the extent of cystatin C silencing by quantitative real-time polymerase chain reaction (qRT-PCR) to quantify the amplification of cystatin C mRNA. We transfected macrophages with siRNA anti-cystatin C to compare the extent of mRNA amplification to the one obtained from control cells treated with a scramble siRNA.

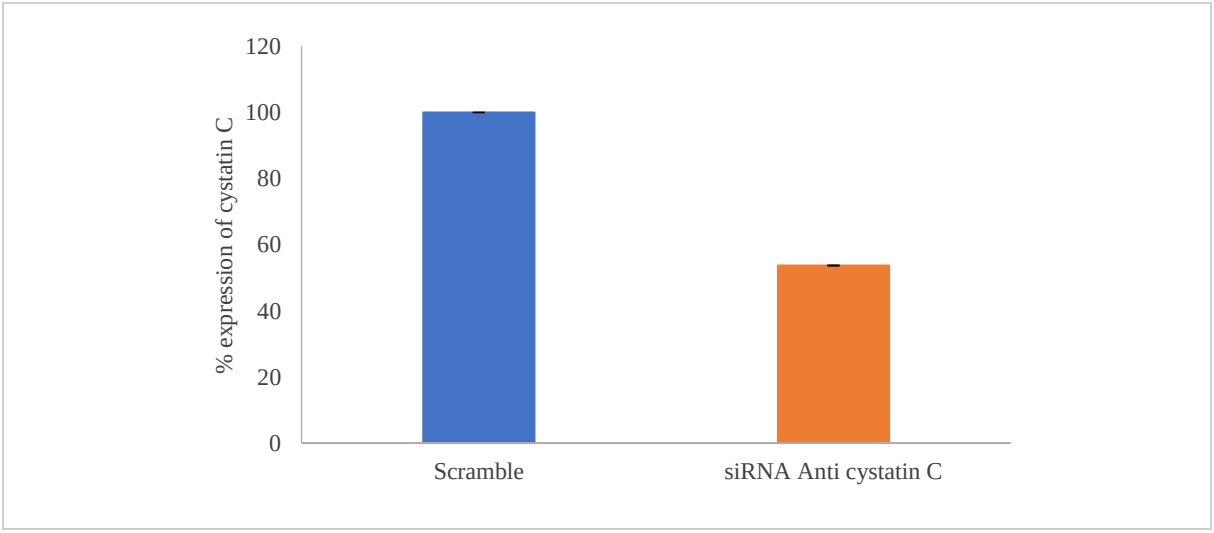


Figure 3.1: Cystatin C expression in macrophages. Cystatin C levels in macrophages were determine by real-time RT-PCR at 24 h postinfection after transfection with siRNA anti-cystatin C. Values are relative to non-infected cells transfected with scramble and are the average of biological duplicates.

As seen in Figure 3.1, the levels of cystatin C mRNA decrease approximately 50 % comparatively to the control. We hypothesize this achieved reduction in cystatin C expression will impact the cellular functions dependent on cathepsin activity.

3.1.2. Cystatin C and the pro-inflammatory response

The first step in *M. tuberculosis* infection is the encounter with innate immunity cells from the lung that recognize it by pattern recognition receptors. This first interaction leads to a production of chemokines and cytokines that recruit and activate other cells participating in the inflammatory response. The interleukin-1β (IL-1β) is one of such mediators of inflammatory response, activated by the inflammasome in macrophages upon recognition of Mtb molecules [72]. In order to evaluate the effects of cystatin C silencing in the proinflammatory response, we performed an Enzyme-Linked Immunosorbent Assay (ELISA) to measure the concentrations of IL-1β during *M. bovis* BCG and *M. smegmatis* infection. After 24 h of infection the supernatant was recovered for quantification of IL-1β.

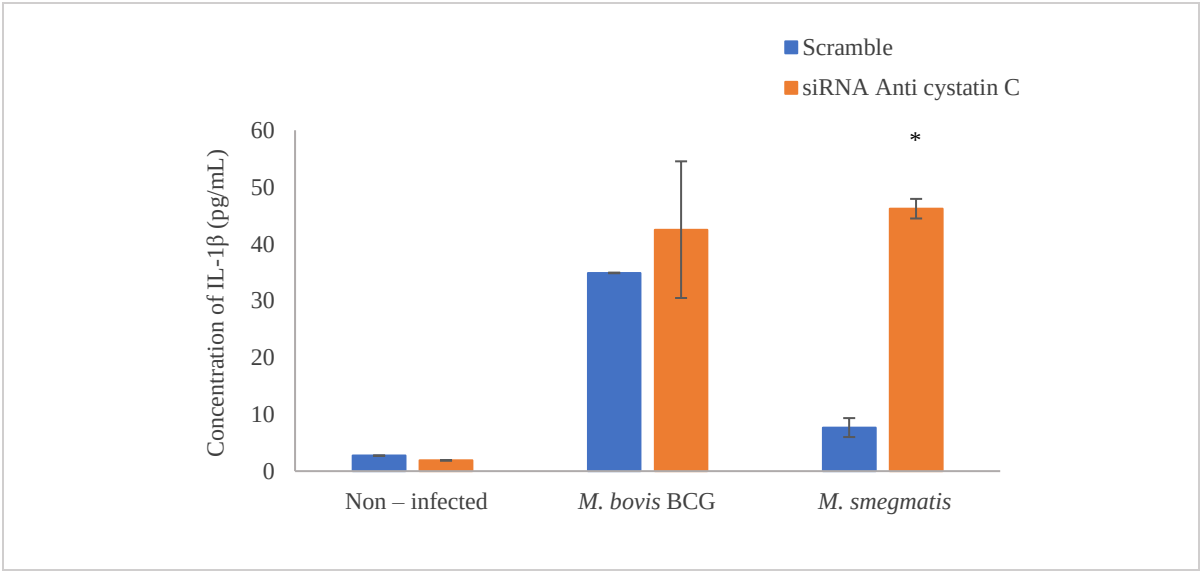


Figure 3.2: Silencing cystatin C increases the secretion of IL-1β in macrophages during infection. Primary human macrophages were transfected with anti-cystatin C siRNA and infected with *M. bovis* BCG and *M smegmatis*. Following 24 h of infection the supernatant was collected and the concentration of IL-1β was measured by ELISA. Values are relative to non-infected cells transfected with scramble control and represent the average of biological duplicates and the statistical significance is shown above the error bar (*$p<0.05$).

It is notable a difference between the conditions in Figure 3.2. As expected the resting non-infected cells show low secretion of IL-1β than the infected cells, confirming the ability of these cells to respond to infection. The *M. bovis* BCG infected cells show increased secretion of IL-1β comparatively to *M. smegmatis* infection which is consistent with previous reports demonstrated the need for specific Mtb molecules to induce activation of the inflammasome and production of IL-1β [72]. In both infections, silencing cystatin C resulted in an increase of IL-1β secretion although only statistically significant for *M. smegmatis* infection. Interestingly, the increased the levels of IL-1β achieved by cystatin C silencing during *M. smegmatis* infection lead to a similar response to that *M. bovis* BCG alone.

3.1.3. Cystatin C on the antigen presentation process during infection

Processing and presentation of antigens derived from phagocytized pathogens by the MHC class II complex is crucial to elicit an effective cellular and humoral immune response and control Mtb infection. The immune activation and expansion of activated T cells is identified by highly HLA-DR expression which are well-recognized markers of TB [73]. The expression of HLA-DR on the surface of macrophages indicates an ability to correctly assemble and express this type of antigen presentation machinery. Cathepsin S participates in antigen presentation by degrading the invariant chain that blocks HLA class II molecules from loading antigens. Pathogenic mycobacteria are reported to reduce the expression of HLA-DR class II molecules leading to lower activation of the adaptive immune response [24]. In order to analyze the result of cystatin C silencing on antigen presentation by macrophages we quantified the surface expression of a class II HLA molecule, HLA-DR, by flow cytometry after 24 h of infection with *M. bovis* BCG and *M. smegmatis*.

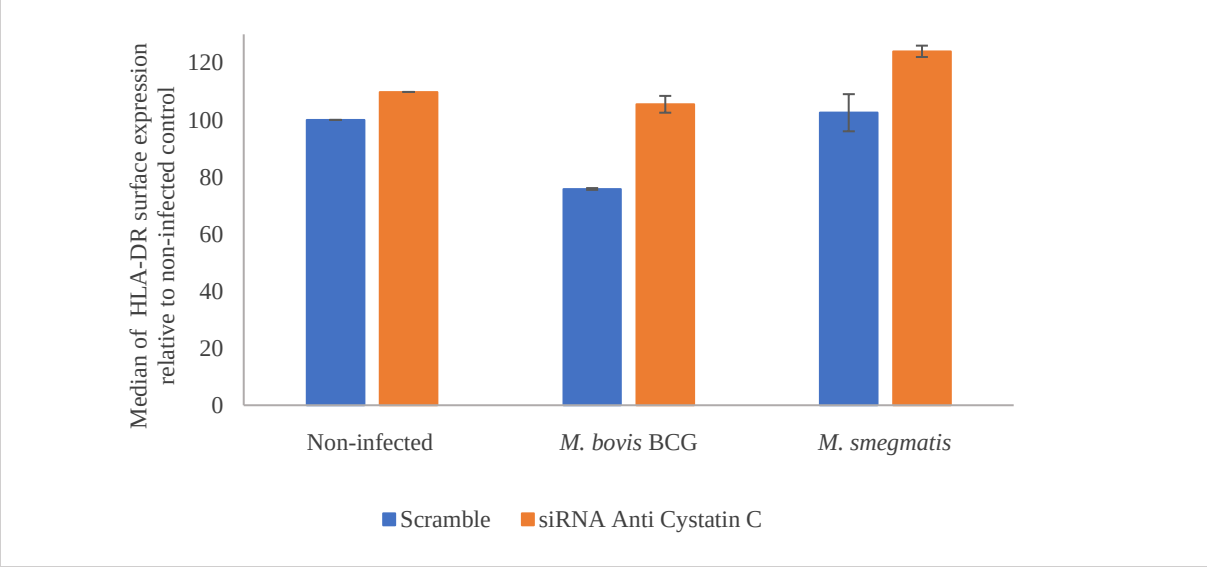


Figure 3.3: Silencing cystatin C increases HLA-DR surface expression in *M. bovis* BCG and *M. smegmatis* infected macrophages. HLA-DR surface expression was assessed by flow cytometry after 24 h of *M. bovis* BCG and *M. smegmatis* infection of primary human macrophages previously transfected with siRNA anti-cystatin C or scramble control. Control non-infected cells transfected with scramble are represented as 100% and the effect of cystatin C transfection and scramble was calculated as % of control.

Following 24 h of infection, the results featured a reduction of HLA-DR surface expression in macrophages infected with *M. bovis* BCG (Figure 3.3, yellow bars) in agreement with previous reports for pathogenic mycobacteria. However, by silencing cystatin C we could restore the normal levels of HLA-DR expression (Figure 3.3, green bars) during *M. bovis* BCG infection or for the case of *M. smegmatis* we could even improve them.

Cystatins inhibit cathepsins, and cathepsins are described to be involved in antigen presentation. In this assay, by silencing was observed an increase of HLA-DR expression on the cell surface indicating a correct assembly of the machinery of antigen presentation machinery. These results suggest that by

silencing cystatin C we are improving the proteolytic activity of cathepsins and consequently the processing of the antigen presentation machinery.

3.1.4. Role of Cystatin C on mycobacteria intracellular survival

Cystatin C is the main natural inhibitor of cathepsin S and other cathepsins and Mtb lowers cathepsin S expression during infection, which consequently, decreases the proteolytic activity and enhances Mtb survival [31]. In this assay we want to determinate the role of cystatin C in the intracellular survival of Mtb in macrophages. To observe this interaction, we transfected macrophages with siRNA against cystatin C and infected macrophages with *M. bovis* BCG and *M. smegmatis*. Intracellular mycobacteria were accessed by colony-forming units (CFUs) after 3 h and 24 h post-infection.

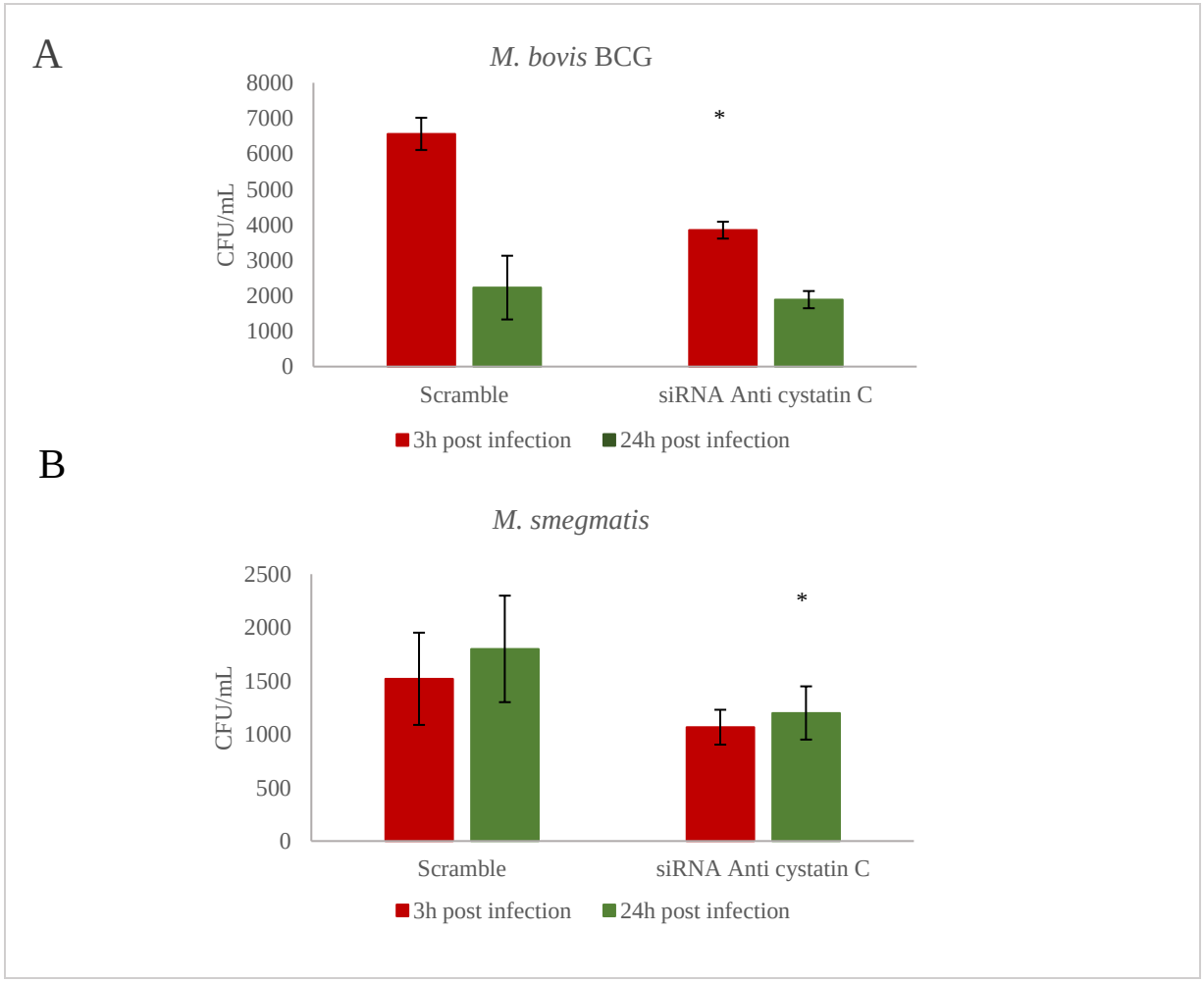


Figure 3.4: Cystatin C modulates *M. bovis* BCG and *M. smegmatis* survival in macrophages. Cells transfected with siRNA anti cystatin C were infected with *M. bovis* BCG **(A)**, or *M. smegmatis* **(B)** for 3 h or 24 h and then lysed and plated in 7H10 OADC medium. The intracellular mycobacteria were accessed by colony-forming units and the bar plots depict the average of biological triplicates. The standard error and the statistically significance comparatively to the control are shown for each time-point (* $p < 0.05$).

As is shown in Figure 3.4, there is an increase of mycobacteria death by down-regulating cystatin C expression. The burden of bacteria is higher for *M. bovis* BCG at all time points, as is expected from a model for pathogenic mycobacteria. Nevertheless, by silencing cystatin C we could obtain the highest increase of mycobacteria killing in *M. bovis* BCG infected macrophages comparative with *M. smegmatis* infected macrophages at 3 h post infection (Figure 3.4 A and B). After 24 h post infection, we obtained similar results yet more pronounced for *M. smegmatis* infection than with *M. bovis* BCG infected macrophages. These results are in agreement with our hypothesis that by down-regulating cystatins expression we are improving the proteolytic activity and consequently increasing the mycobacteria death.

3.2. Impact of Proteases Inhibitors in macrophages during mycobacteria infection

Tuberculosis and HIV infection have a significant overlap in various regions of the world. In this co-infection scenario, analyzing the interactions between the therapeutics and each infectious agent, gains particular interest. Moreover, previous studies revealed interactions between proteases inhibitors (PIs) used to treat HIV infection and cathepsins in HIV-infected macrophages [65]. These PIs can bind the active site of proteases, inhibiting their activity, in similar fashion to cystatins. Taking this in consideration, we hypothesize that these PIs might be used to manipulate the proteolytic activity during the infection of macrophages by mycobacteria.

3.2.1. PIs and the pro-inflammatory response

To assess if there is any interaction of PIs with proinflammatory response, we performed an ELISA to measure the concentrations of IL-1β during *M. bovis* BCG infection. The cells were treated with saquinavir or ritonavir 1 h prior infection at 5 μM concentration [54], and continued to be administered after infection. After 24 h of infection the supernatant was removed and IL-1β was quantified.

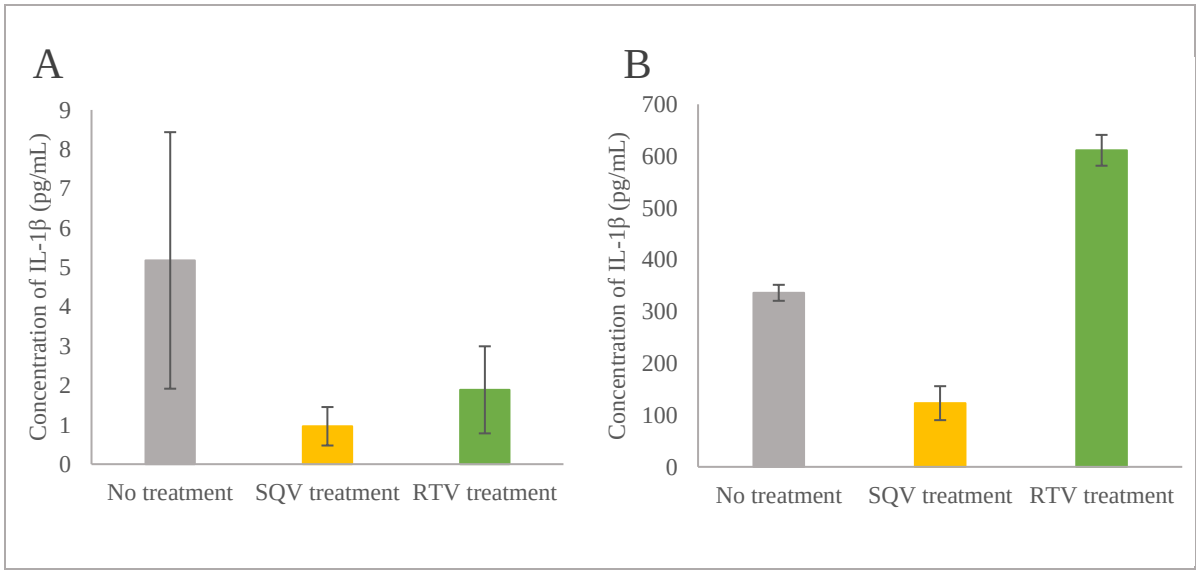


Figure 3.5. Saquinavir and ritonavir alters the secretion of IL-1β in macrophages during infection. Primary human macrophages were treated for 1h with 5 μM RTV, 5 μM SQV or left untreated (control) prior to infection with *M. bovis* BCG. **(A)** Represent non-infected macrophages and **(B)** represent *M. bovis* BCG infected macrophages Following 24 h of infection the supernatant was collected and the concentration of IL-1β was measured by ELISA. Values are relative to no treated cells and represent the average of biological duplicates ± standard deviation.

In non-infected macrophages the concentrations of IL-1β are extremely low (Figure 3.5 A) which is expected since there is no stimulus for proinflammatory cytokines productions. In *M. bovis* BCG infected macrophages as seen in Figure 3.5 B, infected macrophages treated with RTV increased the concentration of IL-1β while infected macrophages treated with SQV decreased its concentrations.

3.2.2. Influence of saquinavir and ritonavir on antigen presentation process during *M. bovis* BCG infection.

As previously mentioned, proteolysis is a crucial process for the processing and maturation of HLA class II antigen presentation complexes and the PIs are reported to interfere with host proteases. To evaluate the interference of PI with the antigen presentation process, we analyzed the surface expression of HLA-DR on *M. bovis* BCG infected cells. Infected macrophages were treated with saquinavir (SQV) and ritonavir (RTV) and the quantification of HLA-DR was accessed by flow cytometry 24 h post infection.

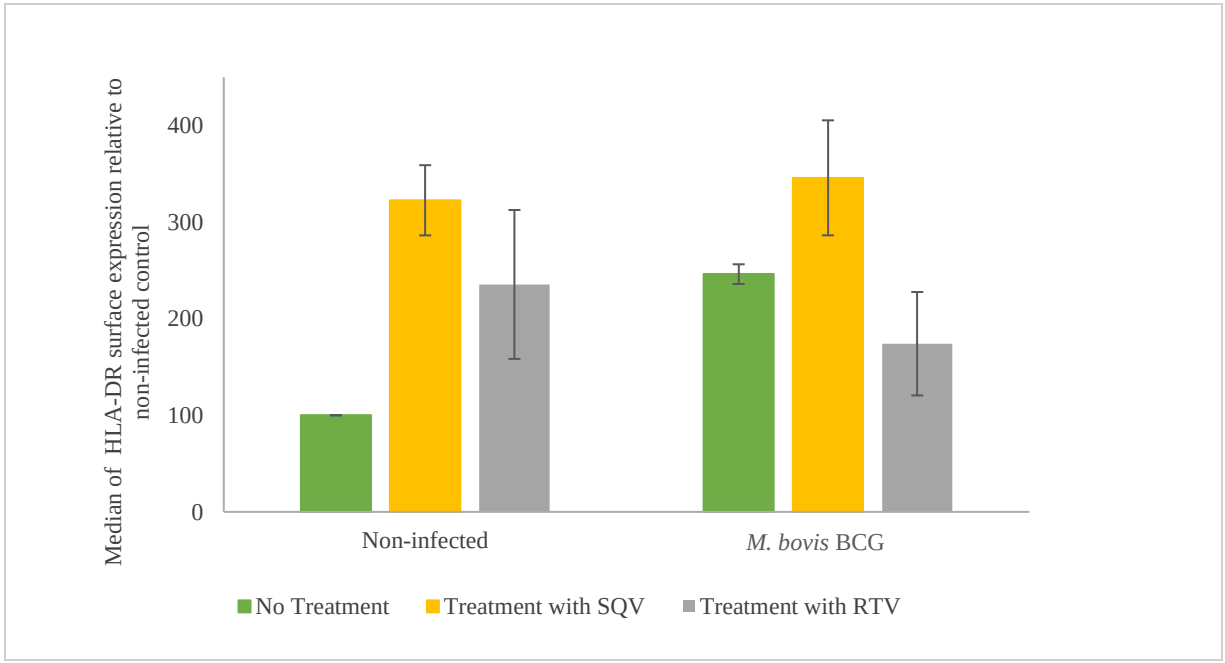


Figure 3.6: Treatment with saquinavir and ritonavir alter the expression of HLA-DR on macrophages surface. Cells were treated for 1h with 5 µM RTV, 5 µM SQV or left untreated (control) prior to infection with *M. bovis* BCG. The cells were treated until 24 h of infection. The HLA-DR surface expression was assessed by flow cytometry After 24 h of infection. Bar plots depict HLA-DR surface expression calculated as fluorescence intensity relative % to non-infected untreated control. This figure shows one representative experiment performed in duplicates ± standard deviation.

As seen in Figure 3.6, treatment with PIs interferes with the antigen presentation machinery during mycobacteria infections. In non-infected macrophages, both treatments enhance the HLA-DR expression comparing to the untreated control, however the expression of HLA-DR was higher in macrophages treated with SQV than with RTV. In *M. bovis* BCG infected macrophages after 24 h of infection there is an increase of HLA-DR expression during SQV treatment. The macrophages treated with RTV showed a decrease of HLA-DR expression compared with untreated macrophages. Treatment with SQV proved to be more consistent between treated and untreated macrophages, while treatment with RTV shown differences between the treated and untreated macrophages.

3.2.3. Quantification of mycobacteria intracellular survival during treatment with saquinavir and ritonavir

After PIs shown interfere with antigen presentation machinery, recent studies shown the interactions with proteolytic activity during infections. We used PIs as treatment to study their effects and quantify the intercellular survival of *M. bovis* BCG in macrophages. To this end, macrophages were pretreated with SQV and RTV followed by infection with *M. bovis* BCG. The intracellular mycobacteria were assessed by colony-forming units (CFUs) after 3 h, 24 h and 72 h of infection.

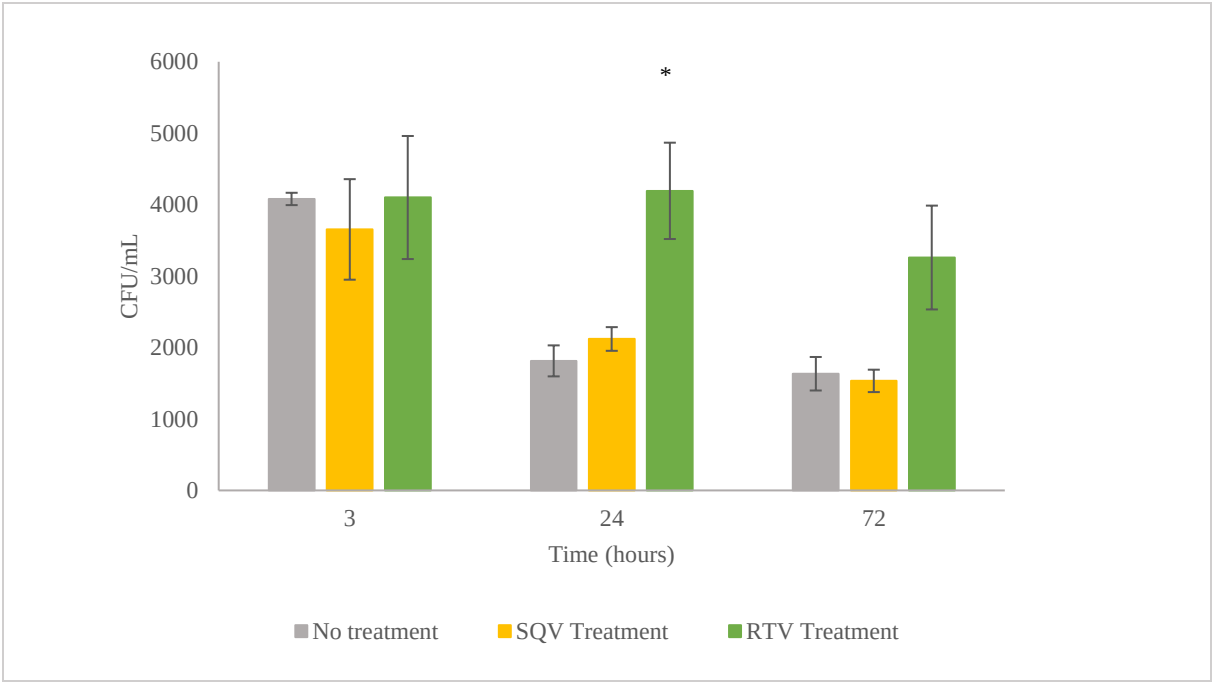


Figure 3.7: SQV and RTV alters *M. bovis* BCG survival in macrophages. Macrophages were pretreated for 1 h with 5 µM RTV, 5 µM SQV or medium (control) followed by infection with *M. bovis* BCG for 3 h, 24 h and 72 h. For each time point cells were lysed and plated in 7H10 OADC medium. The intracellular mycobacteria were assessed by counting colony-forming units and the bar plots depict the average of biological triplicates. The standard error and the statistically significance comparatively to the control are shown for each time-point (*$p<0.003$).

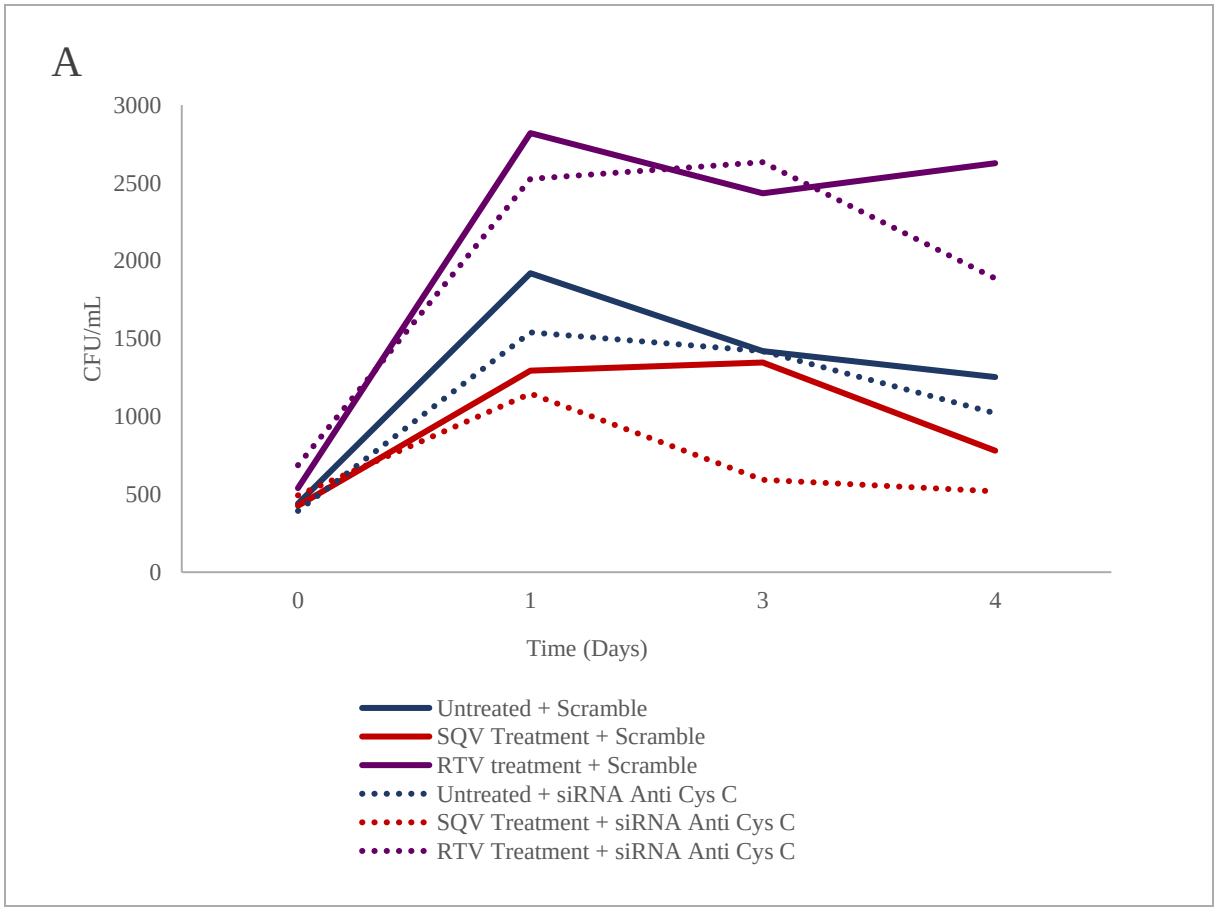
As seen in Figure 3.7, after 3 h of infection there were no changes in mycobacterial survival. Cells treated with SQV shown no significantly changes in the *M. bovis* BCG survival in any time-point, however cells treated with RTV increased survival after 24 h and 72 h of infection.

3.3. Silencing cystatin C during *M. bovis* BCG infection in macrophages treated with SQV and RTV

In the previous sections I've shown that cystatin C silencing or PIs treatment can be used to manipulate the infection. Following those positive results we decided to combine both strategies and analyze the outcome. To perform this assay, first we transfected macrophages with siRNA against cystatin C, followed treatment with SQV or RTV 1 hour before infection, and finally infected cells with *M. bovis* BCG.

3.3.1. Quantification of mycobacteria intracellular survival in siRNA anti cystatin C transfected macrophage, treated with SQV and RTV

As previously reported, proteolytic activity plays an important role against pathogens during infections [74]. PIs and cystatins interfere with proteolytic activity and our results proved they can influence mycobacteria death during infection. Following our promising results, we performed an assay where we transfected macrophages with siRNA anti-cystatin C and then treated them with saquinavir or ritonavir before infecting with *M. bovis* BCG. The results of mycobacteria intracellular survival were accessed by CFUs 3h, 1, 2 and 5 days post infection.



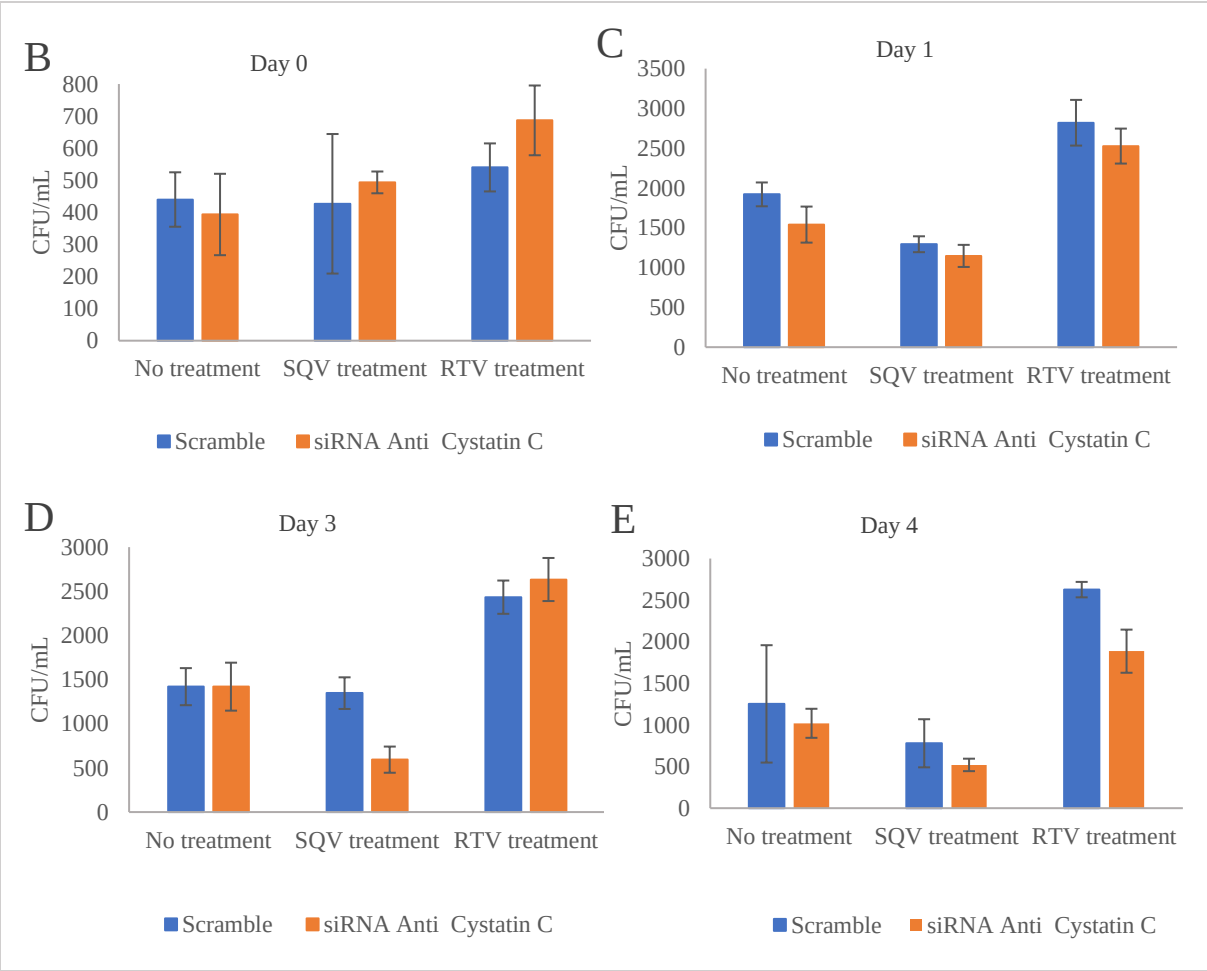


Figure 3.8: Silencing cystatin C and treatment of macrophages with SQV or RTV alters mycobacteria survival. Cells were transfected with siRNA anti cystatin C and in the next day were pretreated for 1 h with 5 µM RTV, 5 µM SQV or medium (control). After that, macrophages were infected with *M. bovis* BCG. The intracellular mycobacteria were accessed by colony-forming units at 3 h **(B)**, 1 **(C)**, 3 **(D)** and 4 **(E)** days postinfection by lysing the cells and plating the lysate in 7H10 OADC medium. The bar plots depict the average of biological triplicates.

As seen in Figure 3.8 A, the different treatments affect the mycobacterial survival. The SQV treatment enhanced mycobacterial killing while RTV treatment increased mycobacteria survival, with or without silencing cystatin C. Silencing cystatin C together with SQV produced the greatest increase of *M. bovis* BCG death.

The different behavior of macrophages starts after 1 day of infection (Figure 3.8 B). As previously seen in our results with singular treatments with PIs, in the presence of RTV, is shown an increase of mycobacterial survival in transfected macrophages with scramble and cystatin C, while in SQV presence, the mycobacterial survival is similar to the infected macrophages without treatment.

After 3 and 4 days of infection (Figure 3.8 D and E) we could observe an increase of *M. bovis* BCG death in macrophages transfected with cystatin C and treated with SQV. This impact of silencing cystatin C and treated them with SQV reinforce our previously results where we observed an increase of mycobacteria death by silencing cystatin C (Figure 3.4 A) and also suggesting SQV increase the proteolytic activity.

During the 4 days of infection there is a significant difference between macrophages treated with RTV than the others treatments. RTV and silenced cystatin C in macrophages increase the mycobacteria survival over time, suggesting that RTV can keep is action and favor the proliferation of infection.

4. Discussion

In the first part of this thesis the effects of silencing cystatin C on **the proinflammatory response** are analyzed. The proinflammatory cytokines are important against pathogens. Macrophages produce IL-1β, a proinflammatory cytokine mediator of inflammatory response, which is activated by a cysteine protease called caspase-1, whose activity is dependent on the activation of the inflammasome [75,76]. Its involved in cell proliferation, differentiation and apoptosis [77,78]. Was discovered that high levels of IL-1β leads to inhibition of Mtb growth by promoting additional antimicrobial effectors which restrict Mtb replication [79].

One other class of proteases is involved in an alternative-processing pathway to the maturation of IL-1β, the serine proteases [80]. Published reports used inhibitors of serine proteases during Mtb infection to evaluate the secretion of IL-1β. The use of inhibitors led to a decreased IL-1β secretion, and the authors demonstrated the manipulation of serine proteases was not correlated with cell death during Mtb infection [81]. Since inhibitors of serine proteases alter IL-1β secretion, we wanted to see if by manipulating the inhibitors of cysteine proteases, cystatins, we would produce such effect in IL-1β secretion. We down-regulated cystatin C expression using siRNA anti-cystatin C to quantify IL-1β secretion during *M. bovis* BCG and *M. smegmatis* infections and if is correlated with mycobacteria survival. While down-regulating the expression of cystatin C during infection there was an increase of IL-1β secretion for both infections (Figure 3.2) in contrast with the reaction of the proinflammatory response with the use of serine proteases inhibitors previous reported [81]. Curiously, the basal levels of IL-1β secretion were lower during *M. smegmatis* infection than during *M. bovis* BCG infection, yet this is consistent with previous reports that demonstrated the need for specific Mtb molecules to induce activation of the inflammasome and production of IL-1β [72]. Nevertheless, by silencing cystatin C we could restore the levels of IL-1β to those observed for *M. bovis* BCG infection. It is possible that serine proteases are also involved in the enhanced of IL-1β secretion, as was previously mentioned [81], and help in the restoration of proinflammatory response. Since *M. smegmatis* is a non-pathogenic saprophyte, it's possible other mechanisms are involved and enhance the secretion of IL-1β in macrophages. The increase of IL-1β is a strong indicator of inflammasome activation against the mycobacteria infection.

The observation that cystatin C silencing provoked an increased pro-inflammatory response prompted us to search for other crucial aspects of the immune response to infection that might also be modulated by cystatin regulation. One such aspect is the priming of adaptive immunity by an effective presentation of microbial antigens by phagocytes to lymphocytes. We proceeded to search for an interaction with the **antigen presentation** machinery by analyzing if the class II antigen presentation complex HLA-DR is being correctly expressed on the surface of infected macrophages.

Cystatin C is the major regulator of cathepsin S and cathepsin S takes an important role in antigen presentation since it degrade the invariant chain of the HLA class II complex [82]. The expression of antigen-loaded HLA class II complex leads to an activation of T cells. The presence of HLA-DR is an indicator of macrophage's ability to present antigens and is one way to confirm the antigen presentation machinery is being correctly assembled [83–86].

Previous evidence has suggested that cystatin C has no control on MHC class II (the murine equivalent of the human HLA class II) in dendritic cells [87]. However, since cathepsin S allows HLA class II molecules to load antigens, we wanted to know if the inhibition of cystatin C and consequent interference with the proteolytic activity of cathepsins alters antigen presentation.

Silencing cystatin C alters the expression of HLA-DR on the cell surface in both, *M. bovis* BCG and *M. smegmatis* infected macrophages, as seen in Figure 3.3. By inhibiting cystatin C we restored the normal levels of HLA-DR expression in *M. bovis* BCG infection, and in *M. smegmatis* was observed an improvement of HLA-DR levels.

Other studies support the degradation of the invariant chain of MHC class II is dependent on cathepsin L or S activity, as well as the peptides presented by MHC class II [88–90]. One possible explanation to the restoration of the HLA-DR levels during infection is that by silencing cystatin C we are increasing the activity cathepsin S and of other cathepsins and improving the antigen presentation machinery and possibly improve the activation of T-cells which is deficient during Mtb infection.

The results from **intracellular survival** complement our previous results from proinflammatory response and antigen presentation. Using siRNA anti cystatin C, in the initial phase of *M. bo*vis BCG infection was observed an increase of mycobacteria killing, but the effect decreased during infection, however still visible the mycobacteria death when cystatin C is silenced compared with the control (Figure 3.4 A). The same result was observed during *M. smegmatis* infection, being more pronounced the mycobacteria killing after 3 h of infection than 24 h of infection (Figure 3.4 B).

In the initial phase of infection, the macrophages already internalized the bacteria and the many steps of phagocytosis initiate. The most important step for bacteria killing is the phagosome fusion with late endosomes and lysosomes, forming a structure called phagolysosome which carries in its lumen a high content in cathepsins and activates them due to pH decrease [27,31,91].

Previous reports demonstrated interactions between cathepsins and Mtb: cathepsin L levels decrease in response to infection of IFN-γ-stimulated mouse bone marrow macrophages with Mtb [92], and down-regulation of cathepsin G was found to increase Mtb survival in THP1 cells [28].

Cathepsins such as cathepsin B, L and S, play an important role in pathogen killing and antigen presentation. Published reports shown a decrease of cathepsin B, L and S activity during Mtb and *M. smegmatis* infection in macrophages, leading to an increase of mycobacteria survival. These results showed the importance of the proteolytic activity of cathepsins against mycobacteria. The same study reports a down-regulation of cystatin C upon infection what was unexpected since cystatin C is a natural inhibitor of cathepsin B, L and S and was observed the down-regulation of these cathepsins during Mtb infection [31]. Our results shown an increase of mycobacteria killing by reduction of the expression of cystatin C during infection, what was expected since cystatins inhibits cathepsins. In fact, cystatin C is the major inhibitor of cathepsin S [49,93,94]. It is possible that silencing cystatin C leads to up-regulation of cathepsin S activity for its main functions such as: pathogen killing, that is observed in our results of increased mycobacteria killing; degradation of invariant (Ii) chain which allows HLA class II molecules to load antigens, and in our experiments resulted in a restoration of HLA-DR expression on infected macrophages. However, while I'm inferring that the direct responsible for these changes is cathepsin S, other intermediate or effectors might be at play. More assays are needed to quantify the expression and activity of cathepsin S in cystatin C silenced macrophages and to test if cathepsin S is in fact important for the phenotypes here described to occur.

As mentioned before, cystatins are the natural inhibitors of cathepsins by binding the active site inhibiting cathepsins activity. There are also other inhibitors of proteases in nature and synthesized in laboratory [95]. In the case of tuberculosis, there is a type of inhibitor that often co-exists with bacterial infection, such as those used in HIV therapy, whose co-infection is significantly prevalent.

Protease inhibitors (PIs) used in HIV therapy, act similarly to cystatins, they block the active site of proteases inhibiting their activity. PIs are used to block HIV aspartyl protease, thus inhibiting the

cleavage of the polyproteins Gag and Gag-Pol of HIV and preventing the maturation of non-infectious immature virions, to mature infectious virions [96,97]. In the second part of this thesis I analyze *M. bovis* BCG infected macrophages exposed to treatment with PIs, specifically saquinavir and ritonavir, since it was recently demonstrated that SQV and RTV modulate cathepsin S activity [54]. Following my presented evidences that cystatin manipulation can alter the macrophage response to mycobacterial infection, my second goal was to understand the influence in those immune processes of PIs used to treat HIV infection that have the potential to regulate proteolysis. Once again I focused on the ability of the macrophage to signal a proinflammatory response, engage the antigen presentation mechanisms and kill the bacterial pathogen.

The **proinflammatory** cytokines are important against pathogens. Previous reports demonstrated that SQV treatment in non-infected macrophages in vitro, leads to an increase of IL-1β production [98]. SQV interfere with proinflammatory cytokines therefore we wanted to observe if the same response occurs in infected macrophages. By analyzing our results from proinflammatory response, in non-infected macrophages the secretion of IL-1β is almost null compared with the infected macrophages. In the infected macrophages there is an increase of secretion of IL-1β in cells treated with RTV (Figure 3.5), that might be due to high number of bacteria stimulating the immune response, which later is confirmed in the results obtained by mycobacteria survival. However, the SQV treated macrophages has lower concentrations of IL-1β compare with RTV treated macrophages (Figure 3.5) and the concentrations of IL-1β from silenced cystatin C (Figure 3.2). These results suggested that SQV, RTV and silenced cystatin C could interfere with the proinflammatory response in different ways.

SQV and RTV can alter peptide degradation patterns modifying the epitope production and consequentially the MHC class I complex, and modify the antigen processing [66]. Since PIs influence the MHC class I complex, we verify if they influence the proteolytic activity of cathepsins and their interaction with the MHC class II complex and the **antigen presentation**. To find out, we analyzed the expression of HLA-DR on the macrophages surface.

SQV enhances the proteolytic activity of cathepsin S, as was recently described [54], which has an important role in processing antigen peptides and the MCH class II complex. Our results confirm that interaction by the increase of HLA-DR expression in cells treated with SQV (Figure 3.6) suggesting the proteolytic activity was enhanced, and that macrophages have correctly processed the antigen presentation machinery to prime T cells. In contrast, the expression of HLA-DR on macrophages surface was lower in RTV treatment. The decrease of the proteolytic activity and consequently the pool of antigen presentation complexes is a possible explanation to this contrast. However, the results from SQV treatment have similarities with the results of HLA-DR expression in infected macrophages silenced with cystatin C (Figure 3.3).

These results are correlated with the **intracellular survival** of *M. bovis* BCG where we observe a decrease of *M. bovis* BCG survival in cells treated with SQV (Figure 3.7), which explains the increase of HLA-DR expression. This immune response was very similar to what was observed in the previous results for silencing of cystatin C. In contrast, an increase of *M. bovis* BCG survival during RTV treatment (Figure 3.7), which correlated to lower surface expression of the antigen presentation. SQV and RTV are reported to differentially control the proteolytic activity by respectively increasing or decreasing its activity [54], and our results fit these evidences. These PIs also control cathepsin S activity which is involved in the invariant chain (iI) processing of MHC class II and in the bacteria degradation. The decrease of Cat S favors bacteria survival as was previously shown [31], this makes Cat S one possible responsible for the alteration in the expression of HLA-DR and the levels of *M. bovis* BCG survival that were observed during PI treatment.

Our previous results were very suggestive that by manipulating cystatin C expression or by using and PIs we can improve the immune response mediated by macrophages possibly by manipulating the proteolytic activity of cathepsins. Since both methods individually appear to produce similar results, we decided to perform an assay in which we employ both, by **silencing cystatin C while treating with SQV or RTV in infected macrophages**.

The previous results related with cystatin C suggested the silencing of cystatin C during infection may improve the killing of mycobacteria since the targets of cystatin C, namely cathepsins, would be free to perform their proteolytic activity. The treatments with PIs suggested that SQV, described for improving the proteolytic activity of cathepsin S enhances mycobacteria killing while in contrast, RTV described for decreasing the activity of cathepsin S increases mycobacteria survival[54].

In this last experiment, as single treatments, SQV and silencing cystatin produced the same results, increasing *M. bovis* BCG killing. When we combined both treatments we could see a synergistic improvement of bacterial killing mostly relevant for the latter stages of the infection (Figure 3.8 D and E). Since on one treatment we are lowering a cathepsin inhibitor and on the other treatment we are using an enhancer of cathepsin activity we might be further increasing the lysosomal proteolytic response leading to the destruction of the pathogen. In contrast, RTV treatment with silenced cystatin C showed the highest increase of *M. bovis* survival in all phases of infection (Figure 3.8). In our previous results macrophages treated with RTV increased the mycobacteria survival while silencing cystatin C increased their death. The RTV treatment together with silenced cystatin C, seems to nullify the effect of silencing cystatin C, increasing the mycobacterial survival. Possibly RTV could be interacting not only with proteolytic activity of cathepsins but also with other mechanisms that favor of mycobacteria survival. However, these results together demonstrate the capacity of PIs and silenced cystatin C in manipulating the immune response against the mycobacteria.

In short, silencing cystatin C led to: (I) an increase proinflammatory cytokines activated by the inflammasome against the mycobacteria, (II) a restore of HLA-DR expression on macrophages surface and (III) an improvement of mycobacteria killing. Altogether, these results consistently reinforce the idea that lowering the expression of the cathepsin inhibitor, cystatin C, improves the host cellular response to infection.

For PIs treatments was shown by conjugating ours results from proinflammatory response, antigen presentation and mycobacterial survival, that PIs from HIV therapy interact with the cathepsins activities during mycobacteria infection. They may blind directly to the catalytic site of cathepsins and inhibit the activity, or maybe they are involved in other pathways which might alter cathepsins activities and decrease or increase their activities against the bacteria.

We can also say silencing cystatin C as the same actions in the immune system as SQV, and both together could improve the immune system response against the bacteria.

5. Final considerations and future perspectives

M. tuberculosis is able to avoid the defense mechanism of macrophages, one of them the proteolytic activity of cathepsins. In this thesis we use two different strategies that act on proteolytic activity to see what changes could occur in host cells against mycobacteria: the first, through a host molecule itself, cystatin C, and the second, through molecules external to the system, proteases inhibitors from HIV treatment, saquinavir and ritonavir.

From the results obtained in the analysis of manipulating cystatin C, we may conclude that during mycobacteria infection in macrophages manipulating cystatin C may have had an impact on the proteolytic activity of cathepsins. Since cystatin C is the major inhibitor of cathepsin S activity, possibly, the silencing of cystatin C led to an increase of cathepsin S which intervening in the antigen presentation machinery and pathogens death.

The next step is to confirm the involvement of cathepsin S in these results, by analyze the activity and expression of its during silencing of cystatin C. It is also needed an analysis of other cathepsins and their involvement against mycobacteria infection during cystatin C silence. In future experiments we need try to understand the triggering of events when we silence cystatin C, from the moment the macrophage comes into contact with mycobacteria, until the end of the infection. However, we proved the manipulation of cystatin C led to changes in the immune response against the pathogen, opening new doors of study and possible development of new therapies against Mtb.

The use of PIs used in HIV therapy in mycobacteria infections suggested an interaction with the immune system response. They altered the secretion of proinflammatory cytokines, and also interfere with antigen presentation machinery and pathogen death. Treatments with SQV prove to increase the mycobacteria death while RTV decreases mycobacteria death, which may suggest an interaction with the proteolytic activity but the answer is still unknown. Studies demonstrated interactions with these PIs with cathepsin S [65] it is needed to performed more assays to verify the cathepsin S activity and if others cathepsins are also being influenced by the PIs. These treatments need to be further studied to see which other immune system pathways may be involved during mycobacteria infection. Yet, treatment with SQV proved to be viable pathway to improve the immune system against the pathogens.

This study supports the initial hypothesis that by interfering with cystatins we are capable to help the host macrophages against the pathogenic mycobacteria and thus improve the cellular response against the infection. In addition, treatments with SQV can also help the immune system recover their sets of weapons against pathogenic mycobacteria. These two systems could be used as a tool to developing new drugs or therapies that improves the immune system response through cathepsins activity which are important against Mtb infection.

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