Universidade de Lisboa

Faculdade de Farmácia

Developing host-directed-therapies for *Mycobacterium tuberculosis* and HIV co-infection based on Cystatins manipulation

Manoj Kumar Mandal

Supervisors: Professora Doutora Elsa Maria Ribeiro dos Santos Anes

Professor Doutor David Alexandre Rodrigues Pires

Thesis specially prepared to obtain the degree of Doctor of Pharmacy
(speciality in Microbiology)

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Jury:

Luís Jaime Gomes Ferreira da Silva Mota, Associate Professor Faculdade de Ciências e Tecnologia - Universidade Nova de Lisboa
Paulo Jorge Gonçalves de Bettencourt, Assistant Professor Faculdade de Medicina da Universidade Católica de Lisboa
Irina Saraiva Franco, Assistant Professor Faculdade de Ciências e Tecnologia - Universidade Nova de Lisboa
Elsa Maria Ribeiro dos Santos Anes Full Professor, Faculdade de Farmácia, Universidade de Lisboa
Maria João Gracias Fernandes da Costa Catalão, Assistant Professor Faculdade de Farmácia, Universidade de Lisboa

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Azevedo-Pereira, J. M., Pires, D., Calado, M., Mandal, M., Santos-Costa, Q., & Anes, E. (2023). HIV/Mtb Co-Infection: From the Amplification of Disease Pathogenesis to an "Emerging Syndemic". *Microorganisms*, *11*(4), 853. https://doi.org/10.3390/microorganisms11040853.

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Mandal, M., Pires, D., Matos, A. I., Azevedo-Pereira, J. M., Florindo, H. F., & Anes, E. (2025). Chitosan-particle-based cystatin F RNA silencing as a therapeutic strategy to improve the control of Intracellular *Mycobacterium tuberculosis*. 45th Annual Congress of the European Society of Mycobacteriology, Lisbon (Portugal).

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Mandal, M., Pires, D., Calado, M., Azevedo-Pereira, J. M., & Anes, E. (2022). Manipulation of protease inhibitors for developing therapeutic strategies against *Mycobacterium tuberculosis* infection. 13th iMed.ULisboa Postgraduate Students Meeting, Faculty of Pharmacy, University of Lisbon, Lisbon (Portugal).

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Mandal, M., Pires, D., Matos, A. I., Azevedo-Pereira, J. M., Florindo, H. F., & Anes, E. (2025). Chitosan-particle-based cystatin F RNA silencing as a therapeutic strategy to improve the control of Intracellular *Mycobacterium tuberculosis*. 45th Annual Congress of the European Society of Mycobacteriology, Lisbon (Portugal).

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Abbreviations and acronyms

AIDS	Acquired Immunodeficiency Syndrome
AM	Alveolar macrophages
AMPs	Antimicrobial peptides
APCs	Antigen presenting cells
ART	Antiretroviral therapy
ATCC	American type culture collection
ATRA	All-trans retinoic acid
BPaL	Bedaquiline, pretomanid, and linezolid
cART	Combined antiretroviral therapy
CDC	Centers for Disease Control
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony-forming units
Clp	Caseinolytic proteases
CS-DS	Chitosan based delivery system
Cst	Cystatin
CstC	Cystatin C
CstF	Cystatin F
CTL	Cytotoxic T-lymphocyte
CTLs	Cytotoxic cells
DC	Dendritic cells
DCM	Dichloromethane
DMPC	Dimiristoyl phosphatidyl choline
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPC	Dioleoyl phosphatidyl choline
DOPE	Dioleoyl phosphatidyl ethanolamine
DSs	Delivery systems
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
ESAT-6	Early secreted antigenic targetof 6 kDa
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
HCV	Hepatitis C virus
HDT	Host-directed therapy

HIV	Human immunodeficiency virus
HLA-ll	Human leukocyte antigen class II
HMDM	Human monocyte-derived macrophages
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IFN-γ	Interferon γ
IL	Interleukin
INH	Isoniazid
IRIS	Immune reconstitution inflammatory syndrome
JAK	Janus kinase
Lip.	Lipid
LTBI	Latent tuberculosis infection
Ly	Lymphocyte
MDR	Multidrug-resistant
MHC	Major histocompatibility complex
MMPs	Metalloproteinases
Mø	Macrophage
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NK	Natural killer
NRTIs	Nucleoside reverse transcriptase inhibitors
OADC	Oleic albumin dextrose catalse
P.I.	Polydispersity index
PAMPs	Pathogen-associated molecular patterns
PAS	*para*-amino salicylic acid
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
PI	Protease inhibitor
PLA	Poly (L-lactic acid)
PLGA	Poly (lactic-co-glycolic acid
PLGA-man	Poly (lactic-co-glycolic acid (PLGA)-mannose
PZA	Pyrazinamide
RD	Regions of difference
RFU	Relative fluorescence units

RIF	Rifampicin
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RPMI	Roswell Park Memorial Institute
RR	Rifampicin-resistant
RSV	Respiratory syncytial virus
RTV	Ritonavir
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SQV	Saquinavir
TB	Tuberculosis
TLR9	Toll-like receptor 9
TNF-α	Tumor necrosis factor α
WHO	World Health Organization
XDR	Extensively drug-resistant

Abstract

Tuberculosis (TB), primarily caused by the bacterial species *Mycobacterium tuberculosis* (Mtb), remains a widespread fatal illness and a major public health problem despite the availability of antibiotics and preventive vaccines. TB re-emerged in the 1980s as the world's leading cause of death from a single infectious agent, affecting millions of people annually after decades of decline. The HIV pandemic was a major cause, as the infection led to immunodepression and to increased susceptibility to TB. Other contributing factors include the increased accessibility of travel from regions where TB is endemic, and the emergence of drug-resistant strains following decades of antibiotic therapy usage. The development of novel strategies is urgently required to improve treatment outcomes and alleviate the morbidity and mortality associated with both infections. The objective of this work is to identify alternative therapeutic strategies that may assist in the control of Mtb and HIV infections, particularly during coinfection were drug-drug interactions and a syndemic condition pose relevant challenges. Therefore, we focused on the development of host-directed therapies (HDTs) by targeting protease inhibitors (PIs), including saquinavir (SQV) repurposed here for TB, and cystatins which are natural inhibitors of lysosomal cathepsins.

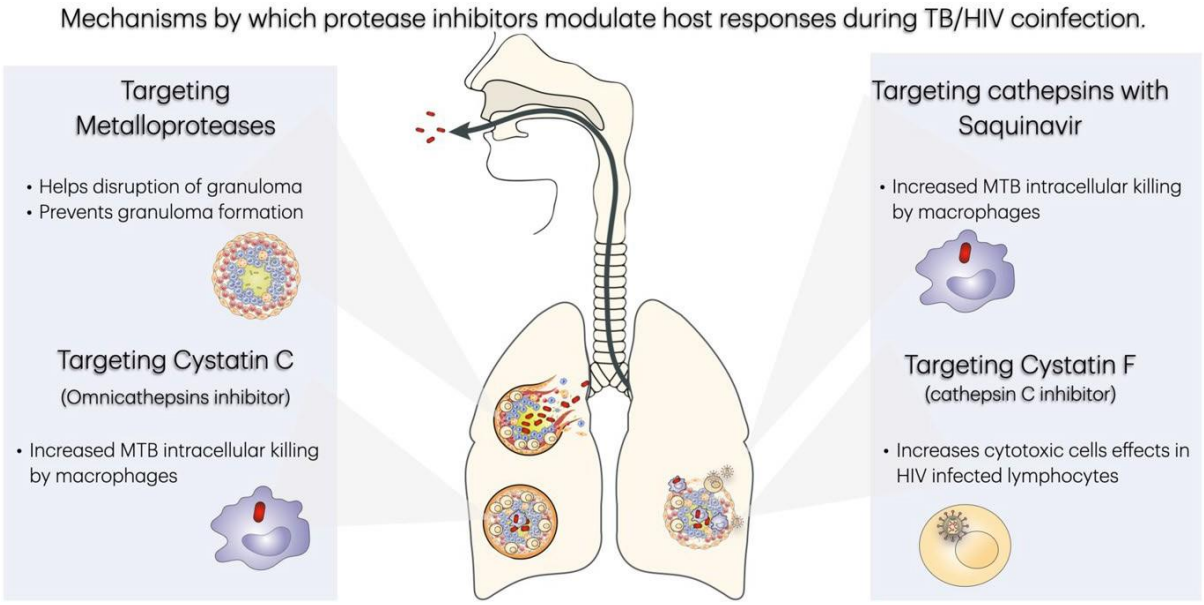
The findings of this study suggest that SQV, the first protease inhibitor used to control HIV infection, possesses the capacity to enhance the proteolytic activity of endolysosomal proteases overcoming the inhibition induced during infection by Mtb. Since treatment with SQV has some systemic toxicity, a liposomal drug delivery system was developed to target SQV to macrophages. This strategy had a significant impact on the intracellular concentration of SQV, achieving a substantial increase in Mtb killing, while concomitantly exhibiting reduced cytotoxicity.

The observation that Mtb infection leads to a decreased proteolytic activity of lysosomal cathepsins in macrophages, and simultaneously some cystatins were overexpressed during the early phase of infection, specifically between 24 h and 48 h post-infection, led to a subsequent investigation into the modulation of CstC and CstF, which were identified as the most differentially expressed. Likewise, their depletion by RNA silencing has demonstrated to enhance the intracellular killing of Mtb, including during infection with multidrug-resistant clinical strains. Furthermore, it was demonstrated that depletion of CstF in macrophages could also impact the level of control over HIV in infected lymphocytes infection during co-culture. The results indicate that a decrease in the CstF released by phagocytes leads to an increase in the major pro-granzyme convertase, cathepsin C, of cytotoxic immune cells derived from peripheral blood lymphocytes. Consequently, an observed augmentation of the granzyme B cytolytic activity leads to a significant reduction in viral replication in HIV-infected $CD4^+$ T-lymphocytes. Moreover, the development of drug delivery nanoparticle formulation based on

chitosan used for the targeting of siRNA to primary macrophages results in a significantly higher level of silencing when compared to the conventional *in vitro* transfection method. Altogether, the results of this thesis suggest that the development of new HDT based on protease inhibitors could improve the control of infections with Mtb or HIV, including during coinfection.

The graphical abstract summarizes the mechanisms by which protease inhibitors modulate host responses during TB/HIV coinfection. All except for the targeting of metalloproteases were investigated in this thesis.

Keywords: *Mycobacterium tuberculosis*, host-directed therapies, saquinavir, cystatins, HIV coinfection



Graphical Abstract. Figure from Mandal et al., 2025.

Resumo

A tuberculose (TB), é uma doença infeciosa causada nos humanos principalmente pela espécie bacteriana *Mycobacterium tuberculosis* (Mtb). A infeção continua a causar elevada mortalidade mundialmente e constitui um grande problema de saúde pública, apesar da existência de antibióticos e de vacinas preventivas. A TB ressurgiu na década de 1980 como a principal causa mundial de morte por um único agente infecioso, afetando milhões de pessoas anualmente após décadas de declínio. A pandemia do HIV foi uma das principais causas, pois esta infeção induz imunodepressão por depleção de linfócitos $CD4^+$ e, consequentemente, o aumento da suscetibilidade à TB. Outros fatores contribuintes incluem a maior facilidade de viajar de regiões onde a TB é endémica e o surgimento de estirpes resistentes aos antibióticos após décadas da implementação do seu emprego. É urgente o desenvolvimento de novas estratégias terapêuticas para contribuir para melhorar o tratamento e aliviar a morbilidade e a mortalidade associadas a ambas as infeções. O objetivo deste trabalho é o de identificar estratégias terapêuticas alternativas que possam auxiliar no controlo das infeções por Mtb e HIV, particularmente durante a coinfeção, contexto este onde as interações medicamentosas e uma condição sindémica representam desafios relevantes. Assim, este trabalho focou-se no desenvolvimento de terapias direcionadas ao hospedeiro (HDTs) tendo como alvo inibidores de protease (IPs), incluindo saquinavir (SQV) redirecionado aqui para controlo da TB, e cistatinas, que são inibidores naturais de catepsinas lisossomais.

Os resultados deste estudo sugerem que o SQV, o primeiro inibidor de protease utilizado para controlar a infeção pelo HIV, possui a capacidade de aumentar a atividade proteolítica das proteases endolisossomais, superando a inibição induzida durante a infeção por Mtb. Como o tratamento com SQV apresenta alguma toxicidade sistémica, foi desenvolvido um sistema de administração de fármacos lipossomais para direcionar o SQV aos macrófagos. Essa estratégia teve um impacto significativo na concentração intracelular do SQV, contribuindo substancialmente para a eliminação de Mtb, ao mesmo tempo em que levou a uma citotoxicidade reduzida.

As observações de que a infeção por Mtb leva a uma diminuição da atividade proteolítica das catepsinas lisossomais em macrófagos, e simultaneamente algumas cistatinas são subre-expressas durante a fase inicial da infeção, especificamente entre 24 h e 48 h pós-infeção, levou a uma investigação subsequente sobre a modulação de CstC e CstF, que foram identificadas como as mais diferencialmente expressas. Da mesma forma, sua depleção por silenciamento de RNA demonstrou aumentar a morte intracelular do Mtb, inclusivé durante a infeção por estirpes clínicas multirresistentes aos antibióticos. Além disso, foi demonstrado que a depleção de CstF em macrófagos

também pode aumentar o nível de controlo sobre a infeção por HIV em linfócitos infetados durante a cocultura. Os resultados indicam que uma diminuição na CstF libertada pelos fagócitos nestas condições leva a um aumento da atividade da principal pró-granzima convertase, a catepsina C, em células imunes citotóxicas derivadas de linfócitos do sangue periférico em cocultura. Consequentemente, um aumento observado da atividade citolítica da granzima B leva a uma redução significativa na replicação viral em linfócitos T $CD4^+$ infetados pelo HIV. Além disso, o desenvolvimento de uma formulação de nanopartículas para administração de fármacos baseados em quitosano, usada para direcionar siRNA a macrófagos, resulta num nível significativamente maior de silenciamento quando comparado ao método convencional de transfeção *in vitro*.

No seu conjunto, os resultados desta tese sugerem que o desenvolvimento de novas HDT baseadas em inibidores de protease pode contribuir para um melhor controlo de infeções por Mtb ou HIV, inclusive durante a coinfeção.

CHAPTER 1.

Introduction

This introductory chapter contains data published in:

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1.1. General Introduction

Tuberculosis (TB), primarily caused by the bacterial species *Mycobacterium tuberculosis* (Mtb), remains a widespread fatal illness and a major public health problem (World Health Organization, 2024). In 2023, TB has re-emerged as the world's leading cause of death from a single infectious agent, displacing coronavirus disease (COVID-19) and surpassing the deaths caused by acquired immunodeficiency syndrome (AIDS) due to human immunodeficiency virus (HIV) (World Health Organization, 2024). During that year, the total number of deaths due to TB was 1.25 million, accounting for 161,000 deaths among people living with HIV (World Health Organization, 2024). According to recent estimates, approximately one-quarter of the world's population has been infected with Mtb (Houben et al., 2016). However, not all infected individuals will develop TB. With an appropriate immune response, some people may clear the pathogen or contain the bacilli in a latent TB infection (LTBI). Coinfection with HIV is one of the causes that accelerates progression from LTBI to active disease (Kiazyk et al., 2017).

Since the advent of combined antiretroviral therapy (cART), HIV infection has become a chronic condition. Continuous cART treatment allows for the suppression and control of the viral load, thereby preventing the transmission of the virus. According to UNAIDS (UNAIDS Report, 2024), cART coverage reached approximately 76% of diagnosed individuals, with 40 million people living with HIV globally. In 2023, approximately 630,000 people around the world lost their lives to AIDS. It is estimated that at least 1.8 million people have AIDS, a condition usually associated with a late diagnosis and treatment of HIV infection. Recently, AIDS has become more prevalent among individuals who have discontinued cART (Kitenge et al., 2023). In addition to the health risks associated with this, the situation has been shown to increase the risk of HIV transmission and to add to the burden on health systems (Abdulrahman et al., 2019). TB remains the leading cause of death among people with HIV, responsible for approximately 30% of AIDS-related fatalities.

According to the 2024 WHO Global Tuberculosis Report (World Health Organization, 2024), Mtb is still responsible for an estimated 10.8 million new infections. Furthermore, Mtb has been linked with a significant degree of antimicrobial resistance. The proportion of individuals diagnosed with TB who have rifampicin-resistant TB (RR-TB) and multidrug-resistant TB (MDR-TB, defined as resistance to both rifampicin and isoniazid), collectively referred to as MDR/RR-TB, accounts for approximately 5–10% of the total TB caseload (400,000 cases globally) (World Health Organization, 2024).

A major challenge in the quest for an HIV cure is the emergence of drug resistance during cART, which is predominantly attributed to the high mutation rate of HIV, the prolonged duration of treatment, and inadequate adherence to therapy (World Health Organization, HIV Drug Resistance Report, 2024). The emergence of drug-resistant HIV variants has the consequence of compromising the effective inhibition of viral replication by antiretroviral drugs. In the present context, the investigation on host-directed therapies that enhance the host responses to control the infection and ameliorate immunopathological damage constitutes a promising strategy to improve disease outcome. This includes the search and repurposing of existing drugs already approved for other conditions that will improve the effectiveness of existing antimicrobials, minimize drug resistance, decrease treatment duration, and adverse effects.

1.1.1. Evolution and historical perspective

TB is the disease caused by members of the *Mycobacterium tuberculosis* (*Mtb*) complex (MTBC), a group comprising the closely related species *M. tuberculosis, M.bovis*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* and *M. canettii* (Cole et al., 1998; Coscolla et al., 2013; Wirth et al., 2008). In fact, the genus *Mycobacterium* is relatively recent, having appeared approximately 36 million years ago (Djelouadji et al., 2011; Cardona, 2025). It belongs to the phylum *Actinobacteria*, making it a Gram-positive bacterium that has undergone a transformation of its outer membrane. This transformation not only rebuilt the membrane but also enhanced it by incorporating exceptionally large fatty acids known as mycolic acids, which consist of about 90 carbon atoms (Cardona, 2025). On the other hand, the outer membranes of Gram-negative bacteria contain around 12 carbon atoms. The complexity of this structure requires significant energy investment, leading to a slow replication rate (Cardona, 2025).

The MTBC is thought to have evolved from *Mycobacterium kansasii* (Jankute et al., 2017), undergoing a loss of glycopeptide polarity in its outer membrane. About 3 million years ago, this evolutionary path gave rise to an organism resembling *M. canettii* that has been considered to be the most recent ancestor of the MTBC (Gutierrez et al., 2005). This gradual evolution has resulted in an enhanced capability for transmission via aerosolized droplets. It has been proposed that the control of fire one million years ago was pivotal in associating Mtb infection with early human ancestors. The inhalation of airborne particles and the spread of Mtb infection may have been facilitated by the use of enclosed shelters, campfires that were used as gathering spots, and poorly ventilated environments (Chisholm et al., 2016). Evidence supporting this includes the presence of tuberculous leptomeningitis in a fossilized frontal bone attributed to *Homo erectus*, dated to approximately 500,000 years ago, found in Kocabaş, Turkey (Kappelman et al., 2008). There are several studies suggesting an African origin for

MTBC (Hershberg et al., 2008; Wirth et al., 2008; Gutierrez et al., 2005; Comas et al., 2013). Molecular clock analysis suggests that the MTBC complex emerged around 70,000 years ago, aligning with the second out-of-Africa migration and the Neolithic expansion of modern humans. In the study by (Comas et al., 2005), they concluded that MTBC has been co-evolving with anatomically modern humans for tens of thousands of years. They justified it considering the common origin in Africa, the congruence in phylogeography, and the dating of major branching events. Moreover, the better health status among Paleolithic hunters-gatherers compared to Neolithic populations and the data obtained by (Ragonnet et al., 2021), which was from historical records of the pre-chemotherapy suggest TB as a well-tolerated, highly persistent disease and even in low-density populations, posing a negligible risk for human extinction (Cardona et al., 2022; Cardona, 2025).

These closely related bacterial sub-species have plagued both human and animal populations for thousands of years. TB has a complex history, and it has coexisted with humans for more than 40,000 years, since *Mycobacterium prototuberculosis*, the supposed ancestor of the MTBC, reached the Fertile Crescent. The results from the study (Wirth et al., 2008) has also supported the emergence of the MTBC clone from *M. prototuberculosis* progenitor pool and its co-migration with modern human populations out of Africa (Gutierrez et al., 2005). The study also suggested that approximately 20,000 to 30,000 years ago from the common MTBC ancestor, two main lineages arose and one of them spread exclusively among humans, subsequently migrating to Asia, Europe and continental Africa (Wirth et al., 2008). Although the evolutionary timing and spread of the MTBC remains largely unknown, it has evolved and differentiated into various lineages, following the main human migration routes to the present day (Wirth et al., 2008) (Fig. 1.1). In the context of virulence evolution among MTBC species, it has been revealed from a comparative genomic analysis that they share over 99.95 % of sequence homology (Brosch et al., 2002). However, the main differences between them when compared to Mtb are large genetic changes called sequence polymorphisms (de Jong et al., 2008). These differences are reflected by regions of difference (RD), which often appear as gene deletions (Brosch et al., 2002; Brosch et al., 2007; Cole et al., 1998; Marinova et al., 2017; Smith et al., 2009) or small insertions (Broset et al., 2015). These observations reinforce that Mtb has ancient roots and lost some genes during adaptation to animal hosts, gaining an advantage in its new environment but losing some resilience in humans (Anes et al., 2023) (Fig. 1.1). For *M. africanum*, lineage 5 is more closely related to Mtb-like groups, whereas lineages 6 and 9 have an RD more similar to animal-adapted species like *M. bovis* (Mostowy et al., 2004; Silva et al., 2022) (Fig. 1.1)

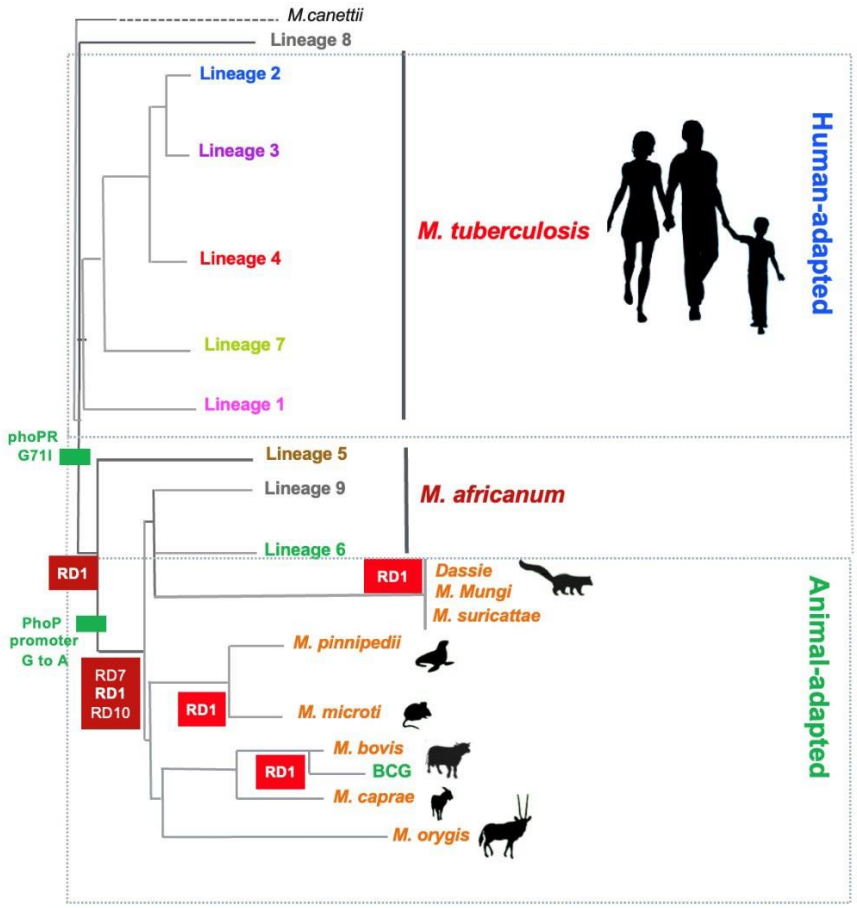

Figure 1.1. Virulence evolution among MTBC. Lineages of the MTBC with RD deletions and PhoPR mutations. Adapted from Anes et al., 2023 and MTBVAC, 2021.

The word "tuberculosis" derives from "tubercle", which was first described by Sylvius in 1650 as the histological lesion that appears in the organs affected by the infection. Then, J. Schoenlein christened the disease as "tuberculosis" in 1839, and later it was observed in the bone by Pott in the late eighteenth century (Cave, 1939). Tuberculous lesions and deformities compatible with the disease were found by molecular biology in human skeletons dating back to 5000 BC and in Egyptian mummies of 4000 BC (Nerlich et al., 1997).

The writings of ancient India and China also describe TB, respectively dated around 3300 and 2300 years ago (Morse, 1967; Brown et al.,1941). The clinical presentation of TB is clearly described as "schachepheth" in ancient Hebrew, which can be found in the books Deuteronomy and Leviticus of the Bible (Sharma, 1981). TB was a well-known disease as "phthisis" and "Corpus" in ancient Greece (5th century BC) and in the Hippocratic (III century BC) respectively, both of which represent the clinical onset of the disease precisely. Furthermore, writings were rediscovered, and they described tubercular lesions as the destruction of the lung tissue, subsequently giving rise to the chronic process accountable for the progressive consumption of affected patients (Daniel and Daniel, 1999).

At that time, the disease was not yet considered contagious, however, Aristotle was still able to recognize only the contagious nature of the pig's and ox's scrofula. In Roman times, Galen for the first time attempted to treat TB based on milk, fresh air and sea voyage (Meachen, 1936). Avicenna was the one who suggested that diabetes could be a risk factor for the development of TB and also speculated that isolation of patients could reduce the risk of TB transmission (Galien et al., 1821). In medieval times (1336), Guy de Chauliac was the first one to propose the surgical treatment of scrofula by the "myrtle leaf" incision (Barberis et al., 2017). In the Middle Ages, people used to believe that the kings of England and France could cure scrofula simply by touching those affected. A clear definition of TB as a contagious disease was not known until the 16th century, when Girolamo Fracastoro, who is also known as the father of the "doctrine of contagion", hypothesized its transmissibility (Riccardi et al., 2020). It was the year 1699 when the health council of the Republic of Lucca ordered the obligatory denunciation of TB, and in the year 1735, it laid the foundation of the "sanatorium" concept as a place of care and isolation for the affected people away from common hospitals (Martini et al., 2018). Due to the increasing incidence of the pathology during the industrial revolution between the 18th and 19th centuries in England, authors like Willis, Morton, and Marten spread the knowledge of TB (Keers and Richard, 1982). At that time, TB was also known as the great white plague because of the extreme anaemic pallor of those affected, and the common risk factors were malnutrition, unsanitary environment, and poor living conditions.

In 1810, Bayle was the first to describe the presence of tubercles in organs other than the lung and also recognized their possible dissemination to the whole organism, defining it as "miliary tuberculosis".
In 1865, Villemin informed the French Academy that "virus" is the causal agent for TB, supporting his assertion by inoculating tubercular material coming from a human lymph node into a rabbit and obtaining the typical tubercular lesion in the rabbit after some weeks (Daniel, 2006). On March 24, 1882, Robert Koch presented his work to the Physiological Society of Berlin. He announced the discovery of *Mycobacterium tuberculosis,* describing it as "thin, with length of half-a-quarter of the diameter of a red blood cell, very similar to the lepers' bacillus, but sharper". He was awarded the Nobel Prize for Physiology or Medicine in 1905 for his contributions (Koch, 1932).

The bacteriologist Franz Ziehl and the pathologist Friedrich Neelsen demonstrated the typical appearance of acid-fast bacilli by introducing the Ziehl-Neelsen stain. In the following years, mortality began to decline, possibly due to Koch's discovery of the causal agent of TB and Virchow's opinions that generated large public health campaigns, improving awareness of risk factors and correct behaviours among the general population (Riccardi et al., 2020). In 1908, Albert Calmette and Camille Guerin grew Koch's bacilli in several media in order to decrease their virulence and to increase the

capacity to produce immunity. In 1921, the BCG vaccine was first administered to humans, more specifically to a child, by Dr. Weil-Hale in Paris. The baby's mother and grandmother died due to tuberculosis just after the baby was born. However, the baby grew up in good health after being given 6 mg of BCG orally. The first active drug against TB "streptomycin" was discovered by Waksman in 1943, also for which he won the Nobel Prize for Physiology and Medicine in 1952 (Selman and Waksman, 2013). Unfortunately, the emergence of drug-resistant appeared rapidly after the first streptomycin trial carried out in London in 1950, constituting a contraindication to antibiotic monotherapy (Pyle, 1947; Mitchison, 1950; Canetti et al., 2020).

The addition of isoniazid (INH) to the two discovered antibiotics, *para*-amino salicylic acid (PAS) and streptomycin in the form of a "triple therapy" for 18-24 months was more successful (Murray et al., 2015). However, the side effects and drug resistance led to further drug development efforts. In 1961, ethambutol (EMB) was introduced, which eventually replaced PAS due to its greater efficacy and tolerability (Murray et al., 2015). In the 1970s, several clinical trials with rifampicin (RIF) and pyrazinamide (PZA) demonstrated these drugs as key components in TB therapy, enabling treatment reductions to 9 and 6 months, respectively (Short-course chemotherapy in pulmonary tuberculosis). A controlled trial by the British Thoracic and Tuberculosis Association, 1976; Singapore Tuberculosis Service/British Medical Research Council, 1979). Several other drugs were developed in the following decades, which include aminoglycosides, fluoroquinolones, bedaquiline or delamanid, and these drugs play important roles in the treatment of drug-resistant TB (Murray et al., 2015) (Figure 1.2)

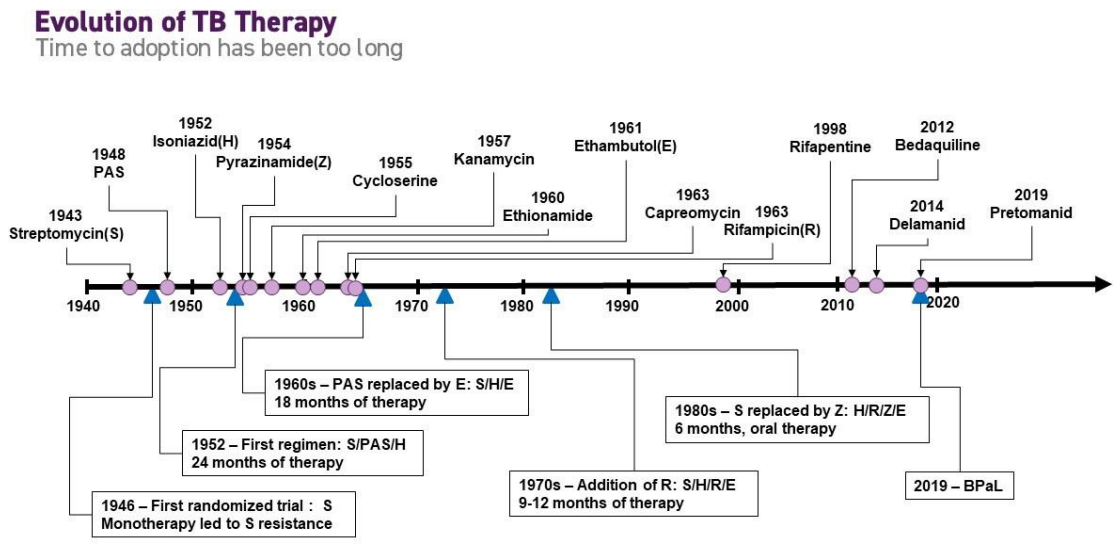

Figure 1.2. Timeline of the evolution of tuberculosis treatments during the 20th and 21st centuries. Retrieved from: https://www.tballiance.org/content/drugs-regimens-transforming-tb-drug-development. Reproduced with permission from TB Alliance.

In 1998, the sequencing of the genome of *Mtb* additionally improved the understanding and knowledge of the pathogen, which may facilitate diagnosis and drug resistance prediction, ultimately enabling its eradication (Cole et al., 1998; Cabibbe et al., 2018).

1.1.2. Epidemiology

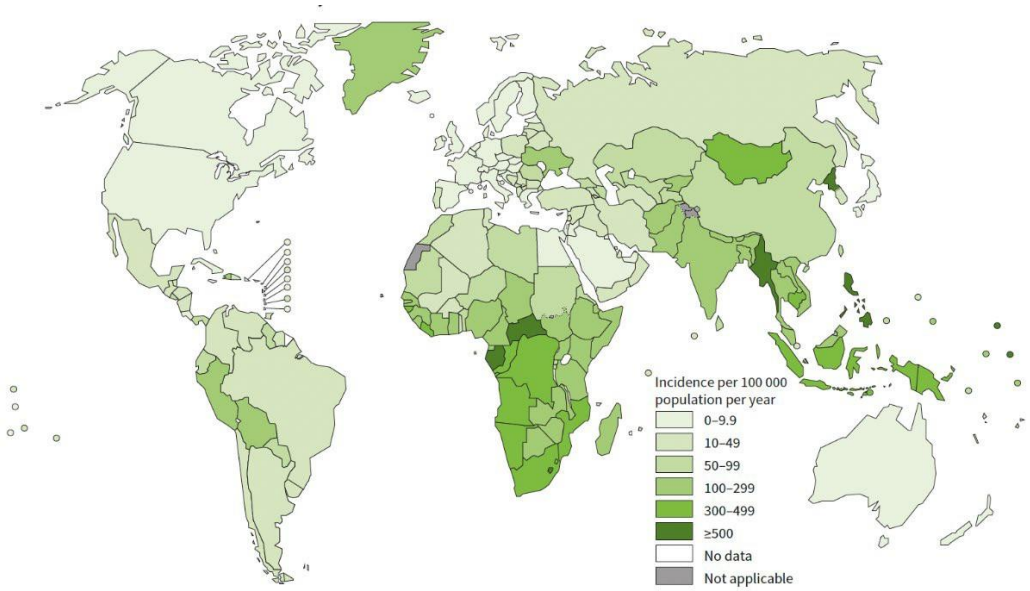

Figure 1.3. Worldwide estimated incidence rates of TB in 2023. Values per 100,000 individuals per year. Reproduced from Global Tuberculosis Report 2024 (WHO, 2023).

The total number of deaths caused by TB in 2023 was 1.25 million, including the estimated number of 161,000 among people with HIV. This number was down from estimates of 1.32 million, 1.42 million, and 1.42 million in 2022, 2021, and 2020, respectively (World Health Organization, 2024). In 2023, around 60 countries had a low incidence of TB, mostly in the WHO Region of the Americas and the WHO European Region, and in addition, a few countries in the WHO Eastern Mediterranean and Western Pacific regions. High TB burden countries included the Central African Republic, the Democratic People's Republic of Korea, Gabon, Lesotho, Myanmar, and the Philippines (World Health Organization, 2024).

The spread of TB and its impact on specific regions or populations are associated with several factors. Therefore, it is essential to distinguish between risk of exposure, risk of infection, and risk of developing the disease (Lienhardt, 2001). This includes the probability of contact between a susceptible host and an individual with active TB, depending on the prevalence of the disease in a specific region. Exogenous factors, such as the infectivity of the source case and the degree of contact between the susceptible

person and the infectious case may contribute to the risk of infection. However, it is also dependent on individuals' susceptibility to infection (Lienhardt, 2001). Risk factors for disease progression are mostly endogenous, including all the conditions that suppress the immune system and disturb the host-pathogen balance, such as HIV coinfection, diabetes, malnutrition, smoking, and alcohol consumption (Corbett et al., 2003; Cegielski and McMurray, 2004; Lin et al., 2007; Stevenson et al., 2007).

In 2023, among all incident cases of TB, 6.1 % were people living with HIV, with the highest proportion in the countries in the WHO African Region (World Health Organization, 2024). The WHO African and South-East Asia regions accounted for around 81% of the combined total number of deaths caused by TB among people with and without HIV. Amongst them, India accounted for 26 % of such deaths (World Health Organization, 2024). The total number of deaths caused by TB among individuals with HIV includes 48% of adult men, 36% of adult women, and 16% of young adolescents (World Health Organization, 2024). Although TB-HIV coinfection rates have been steadily declining over the years, a notable portion of the population is still affected.

Several strategies have been implemented over the years to control TB. Either the Short-course (DOTS) strategy of 1994 or the Stop TB Strategy of 2006 was implemented to improve overall treatment success and amplify the response against the emerging challenges of HIV-associated TB and drug-resistant TB (World Health Organization, 1994; World Health Organization, 2006a, 2006b). The new strategy, known as the End TB Strategy, developed in the context of the United Nations Sustainable Development Goals, aims to reduce the TB incidence and deaths by 90% and 95% respectively until 2035 (Floyd et al., 2018). However, COVID-related disruptions affect the progress, diverting healthcare resources and limiting access to diagnostics and treatments (World Health Organization, 2023a).

1.2. HIV

The Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). It is classified as a member of the *Lentivirus* genus of the *Retroviridae* family. There are two sub-types of virus, HIV-1 and HIV-2. HIV-1 is the most virulent and is responsible for AIDS pandemics. It was first isolated from a patient with lymphadenopathy in 1983 (Barré-Sinoussi et al., 1983). HIV-2, which is found primarily in West African countries was isolated in 1986 (Clavel et al., 1986), is less virulent with lower viral loads and decreased rates of disease progression.

HIV-1 and HIV-2 are enveloped RNA viruses which belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae,* and to the genus *Lentivirus*. They are characterized by a long gap between infection and symptomatic disease (Swanstrom & Coffin, 2012). HIV-1 is considered to be the main causative agent of AIDS. However, HIV-2, which differs from HIV-1 in terms of genomic structure and antigenicity, may also cause AIDS, although with slower disease progression (IARC 2012a; Januario et al., 2014). In the 1980s, HIV emerged as a new viral infection with reports of an acute infection in humans and followed by a chronic infection, resulting in massive depletion of $CD4^+$ T-cell and immunodeficiency (Centers for Disease Control (CDC), 1981; CDC, 1981; Siegal et al., 1981).

The natural history of HIV infection is characterized by three distinct stages: acute infection, chronic asymptomatic stage, and AIDS stage. The acute phase is characterized by a rapid increase in viral loads, which in turn results in a depletion of both mucosal and peripheral blood $CD4^+$ T-cells (Weiss et al., 1993). The duration of this phase is typically between four and eight weeks following the initial transmission. Subsequently, a robust HIV-specific cytotoxic T-lymphocyte (CTL) response is developed, which effectively suppresses viral loads in peripheral blood This marks the transition to the chronic phase, where there is a balance between viral replication, viral immune evasion, and the elimination of infected cells by the host immune response (Borrow et al., 1994; Avila-Rios et al., 2019; Huang et al., 2019). During the chronic phase of infection, even in the presence of a host immune response, HIV continues to replicate, and the number of T-CD4+ lymphocytes irreversibly declines. In the absence of antiretroviral therapy, this will result in severe immunodeficiency and the subsequent occurrence of opportunistic infections, which are characteristic of the AIDS stage of infection (Swanstrom & Coffin, 2012).

The number of new HIV infections annually exceeds 1.3 million, with approximately two million deaths per year resulting from AIDS (UNAIDS, 2024). Nevertheless, the introduction of combined antiretroviral therapy (cART) has enabled HIV infection to be regarded as a long-term chronic condition. However, while the treatment has been demonstrated to be effective in controlling viral replication, it has not been shown to be capable of eradicating the infection (Scandlyn, 2000; Pires et al., 2023). In 2022, it was estimated that approximately 9.2 million individuals infected with HIV were not receiving cART, while about 2.1 million individuals undergoing treatment were not virally suppressed (UNAIDS, 2024). In 2023, approximately 630,000 people lost their lives to AIDS (UNAIDS, 2024). Recent studies have indicated an increase in the prevalence of AIDS among individuals who have discontinued cART (Kitenge et al., 2023).

In the context of the HIV replication cycle, the virus undergoes a series of changes from the initial contact with the targeted cell to the production of new infectious viral particles, which are capable of

initiating the subsequent round of replication (Frank, 2013). The replication cycle of HIV is a complex process that can be divided into two phases: the early and the late phases of replication. The early phase initiates with the virion attachment to the cell surface, culminating in the integration of the proviral DNA into the host genome (Fig. 1.4). On the other hand, the late phase of replication begins with the initiation of proviral transcription and concludes with the release of fully infectious progeny virions (Frank, 2013; Fig. 1.4).

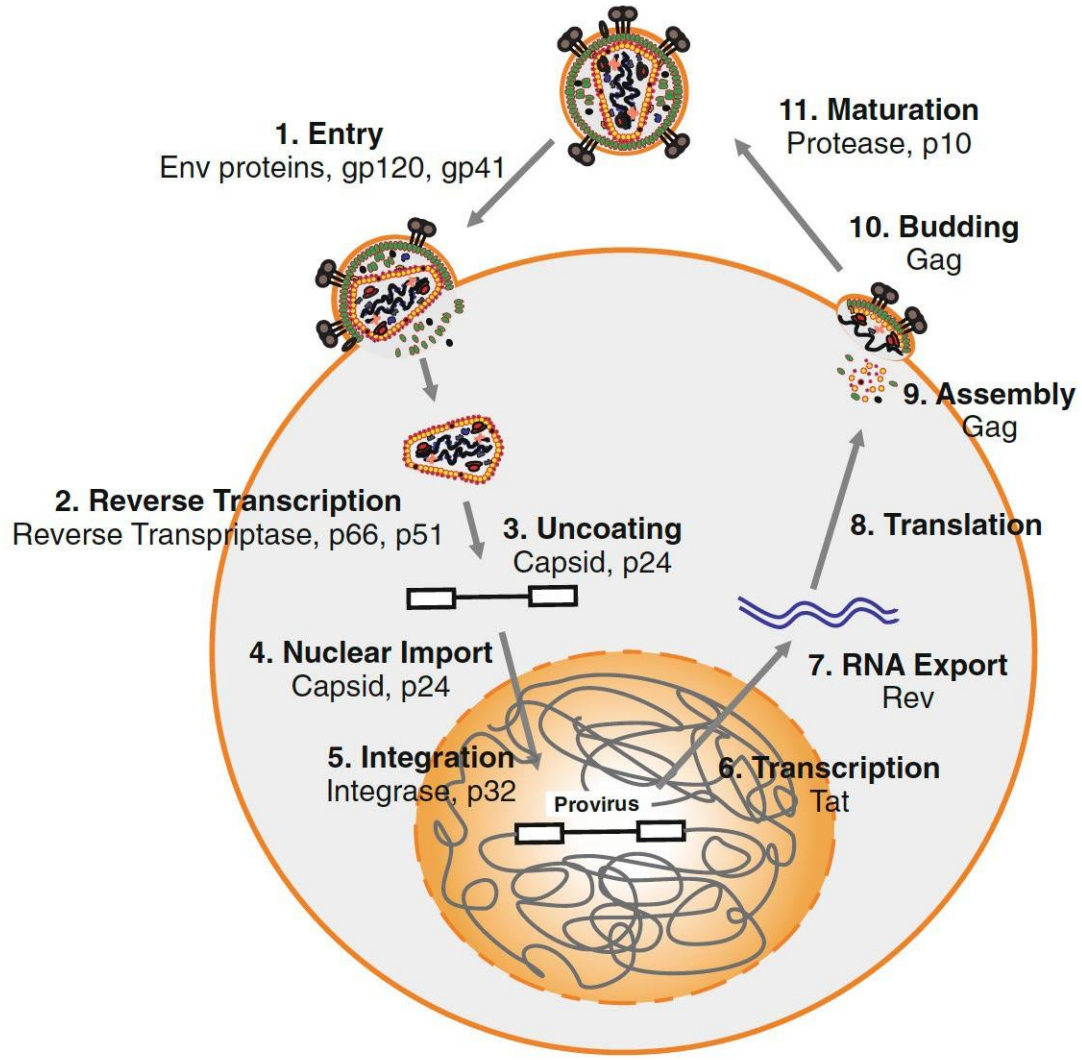

Figure 1.4. Overview of the HIV replication cycle. Retrieved from Kirchhoff, 2013. HIV Life Cycle: Overview. Encyclopedia of AIDS. doi: 10.1007/978-1-4614-9610-6_60-1. Reproduced with permission from author of the content in book: Encyclopedia of AIDS.© Springer Science+Business Media New York 2013.

In summary, the HIV replication cycle begins with the binding and fusion of HIV to the surface of the host cell. The primary receptor for HIV entry is the CD4 molecule. Additionally, viral envelope

glycoprotein gp120 must also bind to a coreceptor, namely the chemokine receptors CCR5 or CXCR4.. Following binding to the cell receptors, gp120 undergoes a series of conformational changes that ultimately result in the fusion of the viral envelope with the cell membrane. Following the completion of the fusion process, the viral capsid containing the viral genome and proteins is released into the cytoplasm of the cell and transported to the nuclear pores. During this transport, the genomic RNA is retrotranscribed into dsDNA by the viral reverse transcriptase. Within the nucleus, the capsid is disassembled and the dsDNA is integrated into the cell chromosomal DNA mediated by the viral integrase (Selyutina, et al., 2020; C. Li, et al., 2021). In the event of cell activation, the proviral DNA is then transcribed into multiple copies of mRNA by the host's transcription machinery. Among those copies of RNA, some become the genome of the new virus particles, whereas other copies of the RNA are used by the cells to produce new HIV proteins. The new viral RNA and HIV proteins are directed to the cell surface, where the new viral particles are assembled and released by a budding process that occurs at the cytoplasmic membrane. The newly formed virions are immature and noninfectious, characterized by a thick layer of radially arranged Gag and Gag-Pol precursors. During the process of budding or shortly after it, there is activation of the viral protease, which cleaves the Gag and Gag-Pol precursors into their mature final components, creating a mature infectious virus.

$CD4^+$ T cells are the primary target cells for viral replication. Despite the fact that HIV is capable of infecting and killing CD4+ helper T cells, there are other cell types that are also susceptible to infection with variation in the time frame of viral replication and the fate of the infected cells (Kirchhoff, 2013). For example, macrophages have the capacity to produce HIV over a period of several weeks and are capable of storing infectious virions intracellulary (Kirchhoff, 2013). A considerable amount of progress has been made; nevertheless, further research is required to elucidate the need for understanding the complex interactions between HIV and its host cell.

TB remains the leading cause of death among people with HIV, responsible for approximately 30% of AIDS-related fatalities. Thus, a closely associated opportunistic infection is with Mtb, producing a syndemic, a state in which HIV/Mtb coinfection exacerbates the morbidities of each pathogen (Azevedo-Pereira et al., 2023). Likewise, HIV remains an important risk factor for the development of latent TB infection to active TB disease. In 2022, it was estimated that 671,000 people were co-infected with both pathogens, accounting for 167,000 deaths (World Health Organization, 2023). Similarly, in 2023, around 662,000 people were estimated to have HIV-associated TB, leading to 161,000 deaths among this population (World Health Organization, 2024). Thus, TB and AIDS still remain as two of the most relevant human infectious diseases that cause high morbidity and mortality worldwide.

1.3. Immunopathogenesis of Mtb and during HIV coinfection

Mtb is transmitted to susceptible hosts via aerosolized secretions from the lower respiratory tract of TB patients. Upon reaching the alveoli, Mtb is phagocytosed by permissive, non-inflammatory alveolar macrophages, providing the appropriate intracellular environment for the establishment of latent infection (Anes et al., 2021; Cambier et al., 2014; Cohen et al., 2018). Mtb is a facultative intracellular pathogen that can reside and replicate within macrophages (Mandell et al.,2006). These cells are considered host professional phagocytes designed to destroy microorganisms within lysosomes. However, Mtb can subvert this pathway and survive in immature endosomal vesicles (Russell et al., 2001). In the initial phases of infection, the surface signatures of Mtb, known as pathogen-associated molecular patterns (PAMPs), are recognized by members of innate pattern recognition receptors (PRRs), expressed by immune cells such as alveolar macrophages and dendritic cells (Hosseinian et al., 2024). Upon interaction with PAMPs, there is activation of intracellular signaling pathways, which induce the production of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, and chemokines, all relevant in orchestrating the appropriate defense mechanisms (Hosseinian et al., 2024; Stamm et al., 2015).

The inhalation of respiratory droplets containing Mtb, subsequently triggers the activation of alveolar macrophages (AM). These phagocytic cells function as the primary line of defense, engulfing the bacteria and thereby containing their dissemination from the lungs to other organs (Woo et al., 2018). Pro-inflammatory cytokines released by these immune cells and newly arrived macrophages then initiate the formation of innate granulomas, which represent the earliest mechanism of defense against Mtb (Domingo-Gonzalez et al., 2016; Nisa et al., 2022) (Figure 1.6).

Following the innate phase, the adaptive immune responses are founded in $CD4^+$ and $CD8^+$ T-lymphocytes that react against Mtb-infected cells within the granuloma structure (Navasardyan et al., 2024; Azevedo-Pereira et al., 2023) (Figure 1.5). These lymphocytes are important for the activation of macrophages, to a more bactericidal M1 inflammatory state, and/or by exerting cytotoxic effects on Mtb-infected cells (Hosseinian et al., 2024) (Figure 1.6). Altogether, this leads to the formation of an adaptive granuloma, a structure that is much more effective in containing bacteria in the lungs compared to the initial innate granuloma (Navasardyan et al., 2024; de Martino et al., 2019). A caseating granuloma, which is defined by the presence of epithelioid macrophages surrounding a necrotic core consisting of foamy infected macrophages, is a hallmark of TB (Navasardyan et al., 2024; Cronan et al., 2022). Besides macrophages, a plethora of cell types have been identified within TB granulomas. These cells include neutrophils, dendritic cells (DCs), B- and T-cells, fibroblasts, natural

killer (NK) cells, and cells that secrete components of the extracellular matrix (Ramakrishnan et al., 2012). Uninterrupted secretion of chemokines by activated infected macrophages attracts neutrophils, monocytes, and lymphocytes, thereby continuously feeding the granuloma with new immune cells.

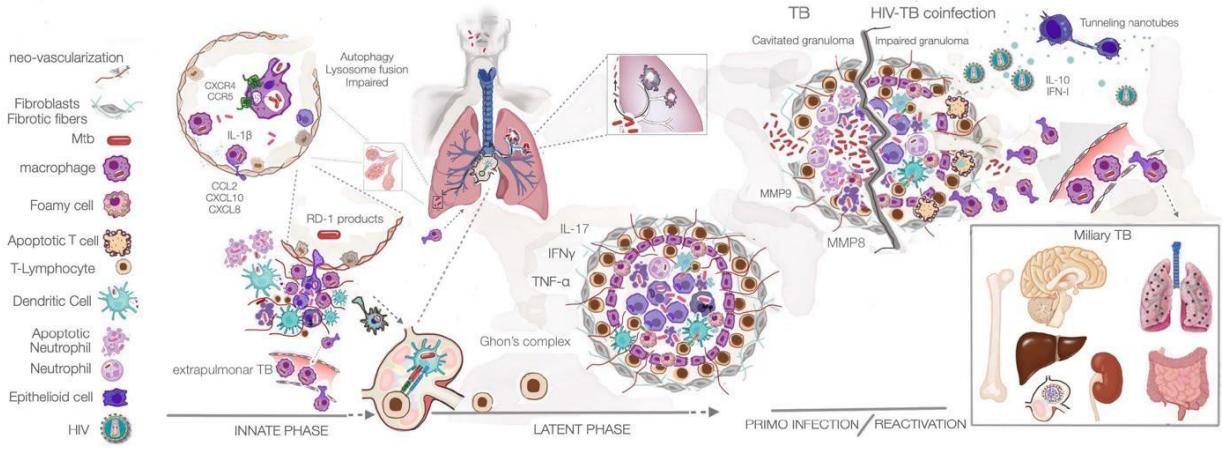

Figure 1.5. Pathogenesis of Mtb infection: implications in HIV coinfection. Inhaled aerosols containing up to three bacilli reach the alveoli in the lower part of the lung. Macrophages take up the pathogen, but Mtb impairs phagolysosome fusion and autophagy. This is enhanced during HIV coinfection, which helps Mtb to establish intracellular niches. In turn, Mtb increases the expression of the HIV coreceptors CXCR4 and CCR5 (helping HIV spread during coinfection). The secretion of IL-1β by infected macrophages and pneumocytes induces transmigration to the lung tissue. Mtb RD-1 products, together with chemokines secreted by infected macrophages, attract more innate cells, leading to the formation of the innate granuloma. If this response is strong, the infection is contained and, in 95% of people, it progresses to a latent phase. In about 5% of people, this phase leads to primary infection with pleural extrusion and eventual haematogenous dissemination. The latent granuloma is maintained by newly arrived T lymphocytes (after proper priming by dendritic cells in the mediastinal lymph nodes) and macrophages, providing a proper balance of cytokines (i.e., TNF-α, IFNγ, and IL-17). In a few granulomas, the pathogen may go into a low metabolic state of dormancy. Immunosuppressive conditions, such as malnutrition or ageing, may favour reactivation (usually in the upper part of the lung). The cytokine balance is disturbed towards increasing levels of TNF-α, leading to excessive neutrophil recruitment with the secretion of metalloproteinases (MMPs) and the caseation of the granuloma. Ultimately, after tissue cavitation, bacteria are released for transmission to new hosts. In the presence of HIV coinfection, extensive lymphocyte depletion, and low secretion of TNF-α, the granuloma dynamics and integrity are compromised. The environment of cytokines secreted by deactivated macrophages (e.g., IL-10 and IFN-I) will favour the formation of tunnelling nanotubes and the cell-to-cell spread of HIV throughout the body. We postulate that HIV-infected cells, or those under the influence of the viral Nef protein, will facilitate macrophage migration to other tissues, thereby contributing to the spread of Mtb and extrapulmonary TB. Figure from Azevedo-Pereira et al., 2023.

HIV is a master at suppressing the immune response of the host, with the potential to be the major cause of Mtb proliferation and reactivation of LTBI into active TB (Azevedo-Pereira et al., 2023). Individuals infected with HIV are more susceptible to infection with Mtb, partially attributable to the depletion of $CD4^+$ T-cells by apoptosis induced by the viral infection, which consequently alters their effective immune response (Bares et al., 2020). In the case of immunocompetent individuals, a concerted response from several immune cells is observed, working together to target Mtb-infected cells. As previously mentioned, $CD4^+$ T lymphocytes play a significant role in the control of the infection. $CD4^+$ T-cells function as both helper cells, producing interferon γ (IFN-γ) or IL-17 required to control Mtb infection, and as drivers of the formation of cytotoxic cells (CTLs) from $CD8^+$ lymphocytes, as well as in B-cells' cooperation in the production of antibodies (Bares et al., 2020) (Figure 1.6).

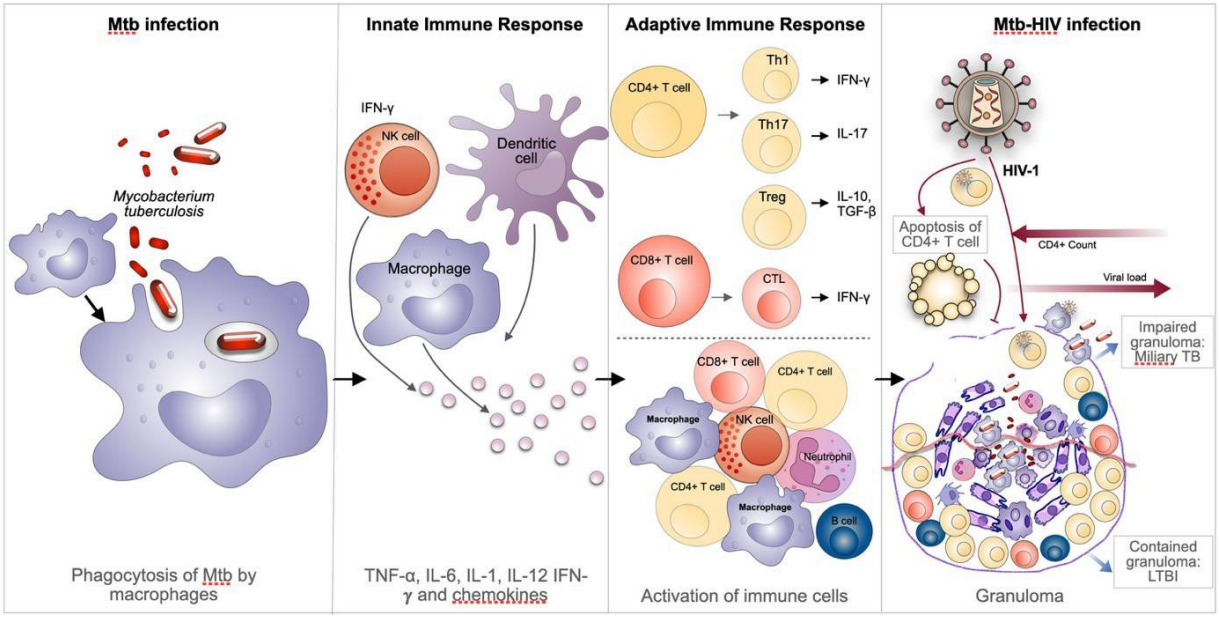

Figure 1.6. Immune response in TB and during HIV coinfection. Alveolar macrophages, as professional phagocytes, constitute the first innate immune cells to restrict the infection. However, Mtb can establish intracellular survival niches within endosomes. The innate immune response is executed by coordinating the activity of macrophages, NK cells, and DC, which secrete an array of effector cytokines (e.g., TNF-α, IFN-γ, IL-1) and various chemokines. This process recruits $CD4^+$ and $CD8^+$ T-cells to release their specific cytokines, such as IFN-γ, which activate infected macrophages to a more bactericidal state. This, in turn, triggers a concerted immune cascade that ultimately results in granuloma formation. Apoptosis of cells infected with Mtb is promoted by TNF-α following its activation by IFN-γ or by the cytotoxic activity of CTLs. This process contributes to the control of the infection in a latent form within the host. However, the coinfection with HIV results in a systemic immunodepression, which is characterized by a dramatic loss of functional $CD4^+$ T-cells by apoptosis. This

depletion of $CD4^+$ T-cells leads to a deficient supply of these lymphocytes to the granuloma, contributing to the disruption of that solid architecture. Figure from Mandal et al., 2025.

Consequently, the depletion of $CD4^+$ T-cells influences the number of activated macrophages, thereby contributing to the persistence and multiplication of Mtb. The overall reduction in $CD4^+$ T-lymphocytes, together with the entrance of HIV-infected $CD4^+$ T-lymphocytes in granulomas, constitutes a primary factor contributing to the disorganization of the granuloma (Diedrich et al., 2016). This impairment of the granuloma as a solid structure facilitates the easy dissemination of Mtb from the lungs to other organs, thus weakening the immune control over LTBI and enabling the progression to active infection (Figure 1.6).

Therefore, the coinfection of Mtb with HIV appears to be one of the most significant risk factors for the progression to active TB. As previously mentioned, there is an antibiotic therapy for treating TB and an established antiretroviral therapy (ART) to control chronic HIV infection. Still, the growing resistance to both treatments and the interactions between the various drug classes raise serious concerns about the effective control of these infections (Navasardyan et al., 2024; Bares et al., 2020; Jones et al., 2022; Sun et al., 2022; World Health Organization. HIV Drug Resistance Report 2021*;* Olivença et al.,2022; Pires et al., 2015; Pais et al., 2022). Accordingly, there is an imperative requirement for a more profound and detailed understanding of the pathways underlying Mtb-HIV interactions, with an urgent need to develop comprehensive strategies and new efficacious therapeutics to address the challenges posed by TB and TB-HIV coinfection.

1.4. Current therapeutic approaches in TB and during HIV coinfection

The first-line therapeutics for TB are rifampin, isoniazid, pyrazinamide, and ethambutol (RIPE). RIPE therapy is administered over a period of six to nine months, starting with an intensive phase of 2 months, followed by a continuation phase of four or seven months (Centers for Disease Control and Prevention, Available online: https://www.cdc.gov/tb/treatment/index.html). This well-planned approach effectively addresses TB, reducing the probability of relapses and the emergence of drug-resistant strains. However, in cases involving drug-resistant strains of Mtb, the employment of second-line agents, including injectable aminoglycosides, injectable polypeptides, oral and injectable fluoroquinolones, and bedaquiline, is imperative (Seung et al., 2015).

Rifampin can be administered either orally or intravenously. It can inhibit Mtb-encoded DNA-dependent RNA polymerase via interaction with the rpoB-encoded β subunit. This interaction prevents RNA synthesis by blocking the elongation of the RNA transcript (Beloor et al., 2025; Koch et al., 2014). Consequently, bacterial strains that possess chromosomal mutations in the rpoB gene exhibit

resistance to rifampin treatment (Koch et al., 2014). Isoniazid is a prodrug activated within the bacteria by the enzyme KatG. This results in the generation of radicals that disrupt the production of mycolic acid, an essential component of the Mtb cell wall (Timmins et al., 2006). In addition, isoniazid is activated by NADH and InhA, yielding isoniazid-NADH adducts that inhibit InhA, a pivotal enzyme involved in mycolic acid synthesis (Marrakchi et al., 2014). Mutations in KatG result in the emergence of resistance to isoniazid (Timmins et al., 2006). A similar mechanism is observed in the case of pyrazinamide, a prodrug that is activated to pyrazinoic acid by the action of the pyrazinamidase enzyme, encoded by the pncA gene in Mtb (Zhang et al., 2014). Strains of Mtb that lack pyrazinamidase activity are therefore resistant to pyrazinamide. In contrast, ethambutol exerts its effect by inhibiting arabinosyl transferase, an enzyme that is encoded by the bacterial gene embCAB (Palomino et al., 2014). Mutations in the embB gene have been associated with resistance to ethambutol, and it has also been demonstrated that ethambutol, when used in conjunction with beta-lactams, enhances their efficacy against Mtb by improving beta-lactams' access through the cell wall (Olivença et al., 2024). Strains of Mtb that are resistant to both isoniazid and rifampin (multidrug-resistant tuberculosis—MDR-TB) require treatment with second-line drugs (Ennassiri et al., 2018). Extensively drug-resistant tuberculosis (XDR-TB) strains are characterized by resistance to isoniazid, rifampin, a fluoroquinolone, and at least one of the following second-line agents: amikacin, capreomycin, or kanamycin, or otherwise bedaquiline or linezolid (Seung et al., 2015).

In the context of immunocompromised individuals, such as those infected with HIV, the emergence of drug-resistant strains of Mtb has become a particularly pertinent concern. The management of TB becomes significantly more complex in these cases due to the heightened risk of developing TB in HIV-positive individuals. This imposes regular testing for both LTBI and active TB infection. Another potential complication that should be considered is the occurrence of immune reconstitution inflammatory syndrome (IRIS) in individuals undergoing highly active antiretroviral therapy. This condition is characterized by an uncontrolled hyper-inflammatory response against opportunistic infections, which usually occurs within the first six months of treatment in people living with HIV (Lai et al., 2016; Sueki et al., 2018; Manzardo et al., 2015). The management of Mtb/HIV coinfection can be attained through a multifaceted approach, including the combination of ARTs and antibiotics, as well as the prevention of IRIS (Bruchfeld et al., 2015). However, it is crucial to acknowledge the sophistication of this treatment protocol and the potential for drug–drug interactions (DDIs). Furthermore, the duration of treatment should be taken into consideration, with shorter regimens being preferred. In addition, TB prophylactic treatment for individuals with HIV should be customized in accordance with their cART regimen to enhance the effectiveness of the treatment and to limit the adverse effects associated with the coinfection (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024).

The Centers for Disease Control (CDC) currently recommends a twelve-week, once-weekly regimen of isoniazid and rifapentine with cART in the absence of any DDIs (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024). Nonetheless, it is imperative to take special consideration when prescribing this combination due to its potential DDIs. This includes drugs like rifapentine and rifampin, which are contraindicated in HIV-positive individuals taking all protease inhibitors (doravirine, rilpivirine, bictegravir, cabotegravir, elvitegravir, temsavir, and lenacapavir) (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024; Niemi et al., 2003). Appropriate cART includes efavirenz once daily or raltegravir twice daily with either abacavir/lamivudine or tenofovir disoproxil fumarate/emtricitabine. However, rifampin and rifapentine significantly reduce the effective dose concentration of these drugs, thus necessitating adjustments to ART regimens, which in turn increase the likelihood of drug-related adverse effects (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024).

The adjustment in dosage of drugs is essential for the maintenance of adequate drug levels, which will provide efficient treatment for both TB and HIV without any risk of failure. For instance, the current guidelines recommend a dose of 600 mg of rifamycin to be given alone or co-administered with efavirenz (Schutz et al., 2010), but some sources suggested a higher dose of 800 mg for individuals with a body weight of more than 60 kg (Schutz et al., 2010). It is important to note that dolutegravir is not recommended for patients with integrase strand transfer inhibitor resistance, though it can also be used as a first-line ART (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024). Rifabutin, another drug belonging to the rifamycin family, has demonstrated its efficacy in the treatment of TB (Horne et al., 2011). An alternative option that has gained popularity is the utilization of shorter drug courses, which have been shown to exhibit higher efficiency and greater completion rates compared to traditional regimens (Ignatius and Swindells, 2020). The BRIEF TB/AF279 clinical trial demonstrated the efficacy of an ultra-short regimen comprising a one-month course of rifapentine and isoniazid, achieving a higher completion rate than the nine-month course of isoniazid in preventing TB infection in HIV-infected individuals (Swindells et al., 2019). This regimen is now recommended by the WHO (Swindells et al., 2019; World Health Organization, 2020). In addition, the development of several compounds has also been reported, including a family of N-alkyl nitrobenzamides that exhibit promising antitubercular activities and can even be classified as a structural simplification of previously known inhibitors of decaprenylphosphoryl-β-D-ribofuranose 2’-oxidase (DprE1), a critical enzyme of Mtb and a novel antitubercular target (Pais et al., 2024).

In HIV-positive patients with TB, $CD4^+$ T-cell counts are a crucial factor in determining the initiation of cART (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024). Based on the $CD4^+$ T-cell

counts, cART should be initiated within two weeks of TB treatment initiation if the $CD4^+$ T-cell count is less than 50 cells/mm^3, while it can be commenced within eight weeks of TB treatment initiation if the $CD4^+$ T-cell count is 50 cells/mm^3 or above (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2024; Niemi et al., 2003). Two main treatment options are currently available for individuals with TB and HIV infection. The first option involves a six-to-nine-month period of intensive treatment with a combination of drugs, specifically rifampin, isoniazid, pyrazinamide, and ethambutol for two months, followed by a four-month continuation phase of rifampin and isoniazid (Carr et al., 2022). The second option consists of a 4-month regimen of rifapentine-moxifloxacin, which can be administered to patients with a $CD4^+$ T-cell count of 100 cells/microliter, and an ART regimen including efavirenz (Centers for Disease Control and Prevention. TB Treatment for Persons with HIV. Available online: https://www.cdc.gov/tb/hcp/clinicalcare/hiv.html?CDC_AAref_Val=https://www.cdc.gov/tb/topic/treatment/tbhiv.htm). Additionally, a number of innovative therapeutic approaches for countering the coinfection have been proposed, including the use of dual-targeted anti-HIV/anti-TB heterodimers (Alexandrova et al., 2017) and the targeting of HIV's inhibition of NK cell-mediated immunity by HIV in response to Mtb infection (Yang et al., 2023).

Despite the advancements and developments in therapeutic approaches to treat and control TB and chronic HIV infection, the synergistic action of these pathogens, rising resistance to both treatments, and drug–drug interactions are leading to significant diagnostic and therapeutic challenges. Coinfection with Mtb increases the risk of death among HIV-positive patients, while late-stage HIV infection increases the risk of developing TB in latently infected individuals. After decades without signs of progress, the drug development pipeline for tuberculosis has finally seen the introduction of new antibiotics in clinical practice. The BPaL regimen, consisting of bedaquiline, pretomanid, and linezolid, has been recently recommended to treat drug-resistant TB (Migliori et al., 2022). The regimen lasts between 6 and 24 months, depending on resistance levels and response to treatment. This regimen has produced remarkable results in clinical trials, showing comparable results to the standard treatment when applied to drug-susceptible TB cases and high rates of success in treating XDR TB and unresponsive MDR TB, even in HIV-positive individuals (Conradie et al., 2020). Still, it suffers from the same drawbacks of the standard regimen, with several treated individuals showing adverse effects to the treatment, including 81% of the patients reporting peripheral neuropathy and approximately half the patients showing evidence of hematologic toxic effects.

The side effects associated with the standard RIPE regimen and the different drug-resistant TB regimens have been extensively documented. Severe side effects related to the termination of standard treatment can include hepatotoxicity, peripheral neuropathy, exanthema, gastrointestinal disorders, and arthralgia, among others (Schaberg et al., 1996; Awofeso et al., 2008; Nortey et al.,

2024; Sant' Anna et al., 2023). In MDR TB treatment, these are further complicated, with a study reporting gastrointestinal disturbance (18.4%), psychiatric disorder (5.5%), arthralgia (4.7%), hepatitis (3.9%), peripheral neuropathy (3.1%), hypothyroidism (2.3%), epileptic seizures (2%), dermatological effects (2%), ototoxicity (1.6%), and nephrotoxicity (1.2%) in the enrolled patients (Yang et al., 2017). These adverse effects pose additional ethical questions when considering preventive therapy offered to individuals presumptively infected with Mtb. The preventive regimens have seen substantial length reductions from six to nine months to one to four months (Walker et al., 2025). Yet, these are still long treatments in absolute terms, especially considering the low baseline risk of developing tuberculosis. In addition to these side effects, cases of bedaquiline resistance are rising in Africa following first treatment, providing evidence for a careful usage of this last available regimen for XDRTB, for which currently no good alternative exists (Derendinger et al., 2023).

Even accounting for the high success rates associated with the situation of full compliance to therapy, the concomitant costs, aggravated in cases of MDR and XDR TB, constitute a major challenge in high-TB-burden countries. According to the WHO, the percentage of TB patients and their households that experienced catastrophic total costs associated with TB and its treatment (defined as >20% of annual household expenditure or income) ranged from 13% to 92%, averaging at 49% (World Health Organization, 2023). All these factors highlight an ongoing and critical demand for complementary management approaches that can bypass the constraints of the existing antimicrobial treatment portfolio, addressing the problems of drug resistance, drug interaction, adverse drug effects, access to and cost of treatment, and patient compliance. Host-directed therapies, particularly those developed through drug repurposing, have the potential to fill this role as a complementary strategy to improve TB treatment.

1.5. Host-directed therapies: Current status and recent progress for the treatment of infections

From an evolutionary perspective, host-pathogen interactions can be regarded as states in which microbes exist without causing harm or manifesting overt disease (Zumla et al., 2016; Casadevall et al., 2000). The action of the host's innate and adaptive immune surveillance mechanisms determines whether the infection will be contained or whether it will progress to clinical disease, resulting in recovery or death (Zumla et al., 2016). It is imperative to acknowledge the crucial function of host-related events in the efficacy of antimicrobial treatment, particularly because disease progression subsequent to infection can result in tissue damage, which can lead to long-term functional impairment and increased mortality. The underlying mechanisms of this process are multifaceted and include, but are not limited to, immune dysregulation resulting from various etiologies, such as an excessive inflammatory response to infection, the use of immunosuppressive therapies,

disadvantageous socioeconomic conditions, HIV infection, or the presence of comorbidity, including non-communicable diseases such as diabetes, neoplasia, and chronic pulmonary disease (GBD 2013 Mortality and Causes of Death Collaborators, 2015).

In the context of infectious diseases, it is crucial to understand the complex interplay between the host and the pathogen to elucidate the mechanisms underlying pathogenesis. In the past few years, there has been a paradigm shift in scientific research, with a transition from a direct targeting of the pathogen to a focus on host factors. This paradigm shift has opened promising new avenues that hold the potential to enhance clinical outcomes, referred to as host-directed therapies. Host-directed therapies (HDTs) are defined as host-directed interventions that can modulate intracellular pathways involved in innate or adaptive immune responses to microbes to enhance immune response and reduce immunopathology (Wallis et al., 2023). About 90% of the tested HDTs have demonstrated comparable efficacy levels towards drug-resistant and drug-susceptible pathogens (Shapira et al., 2024). In addition to their efficacy in battling drug-resistant pathogens, HDTs have been hypothesized to reduce the likelihood of developing drug resistance by targeting multiple cellular and intracellular mechanisms that are critical for microbial replication and pathogenesis (Shapira et al., 2024).

HDTs can also enhance the efficacy of anti-microbial treatments. A recent study evaluated 183 host-directed drugs, of which 30% were found to have activity against more than one pathogen. The majority of these (52%) targeted close evolutionary relatives, while 48% were active against evolutionarily distinct groups at the levels of families, kingdoms, and domains (Shapira et al., 2024; Goletti et al., 2024). The HDTs comprise drugs with different mechanisms of action. Among these agents are those that enhance host immunity, such as CAR-T cells, which have been used in the therapeutic management of HIV-1-infected individuals, leading to a transient reduction in the HIV-1 viral reservoir (Liu et al., 2021). The administration of type I or III interferon (IFN) has also been used for the treatment of both SARS-CoV-2 infection and chronic hepatitis C (HCV) infection, respectively (Kamyshnyi et al., 2023; Muir et al., 2014; Tam et al., 2023) ; N-acetylcysteine, an antioxidant, has been utilized to protect cells from oxidative damage in TB (Mapamba et al., 2022) ; anti-pathogen antibodies have been used for the treatment of SARS-CoV-2 (Vita et al., 2022) and respiratory syncytial virus (RSV) infections (Raghuveer et al., 2024).

The immunopathology associated with the immune response to the pathogen can be mitigated by HDTs that attenuate inflammation. These HDTs comprise compounds that target cytokines, such as the interleukin (IL)-6 receptor blockade in COVID-19 (Ferraccioli et al., 2022), tumor necrosis factor (TNF) treatment in cases of TB meningitis (Goulenok et al., 2022), and the Janus kinase (JAK) inhibitors in COVID-19 (Ferraccioli et al., 2022). A number of other potential anti-inflammatory HDTs have been

identified, including the use of corticosteroids (Ferraccioli et al., 2022; Barnard et al., 1953), antioxidants such as N-acetylcysteine (Mapamba et al., 2022), vitamin D, statins, and cyclooxygenase 2 inhibitors (Chandra et al., 2024; Dutta et al., 2016; Mai et al., 2018).

HDTs are also expected to modulate the immune response to Mtb, thus emerging as a compelling antibiotic adjuvant therapeutic strategy. There is mounting evidence to suggest that several facets of the immune response, including reactive oxygen species production, antimicrobial peptide synthesis, cytokine production, autophagy induction, and cell-mediated immunity, can be modulated by HDT to hinder Mtb growth (Figure 1.7). One potential host therapeutic target against Mtb is the impairment of granuloma structure through TNF-α blockade by using etanercept (Enbrel, a soluble TNF-α receptor). This disruption enhances antibiotic access to the bacteria and curtails lung pathology (Bourigault et al., 2013) (Figure 1.7). Another recent clinical trial has demonstrated that etanercept-mediated TNF blockade increases $CD4^+$ cells amongst patients with HIV-associated TB in the early treatment phase of TB. Nevertheless, the administration of anti-TNF-α therapy has been demonstrated to exacerbate the severity of the disease by suppressing the host's immune response, a finding that has been established in animal models (Bourigault et al., 2013; Wallis et al., 2004).

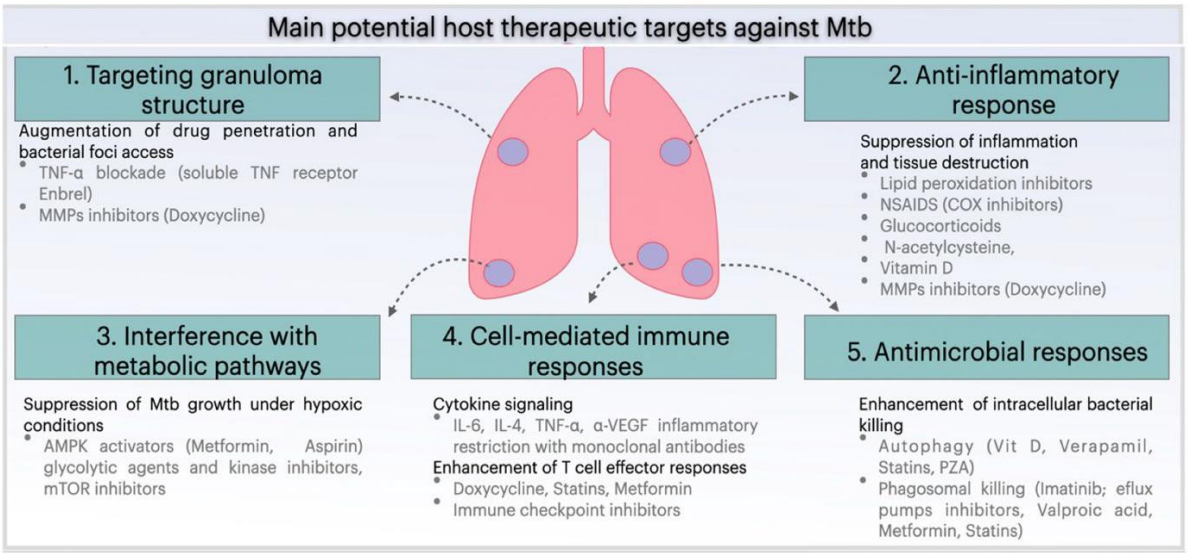

Figure 1.7. Host-directed therapies (HDT) against *Mycobacterium tuberculosis*. The main HDT used to improve the outcome of TB. (**1**) HDT can affect granuloma integrity to improve antibiotic penetration and access to bacterial foci. (**2**) HDT can act by suppressing the proinflammatory responses that contribute to tissue damage during TB. (**3**) The interference with metabolic pathways is a promising strategy for suppressing Mtb growth under hypoxic conditions. (**4**) HDT can target cell-mediated immune responses by enhancing T-cell responses

through doxycycline, statins and metformin. (**5**) Autophagy-inducing drugs such as vitamin D, verapamil, and statins contribute to the intracellular killing of Mtb in lysosomes. Figure from Mandal et al., 2025.

Furthermore, HDTs can modulate immune responses by targeting the vitamin D signaling pathway, which is critical for enhancing the Mtb-killing activity of macrophages. Vitamin D has been shown to induce autophagy by downregulating the mTOR protein (Wu and Sun, 2011). Additionally, CAMP gene expression can be activated by the co-administration of phenylbutyrate with vitamin D_3 and standard anti-TB drug therapy and has been shown to inhibit the intracellular growth of Mtb (Mily et al., 2013; Mily et al., 2015). A recent study revealed that the supplementation of vitamin D at a dose of 2.5 mg in combination with standard first-line anti-TB drugs effectively rectified the vitamin D deficiency. Still, no significant change in the Mtb culture conversion in pulmonary TB has been observed (Wejse et al., 2009; Martineau et al., 2011). A similar finding was reported for sputum conversion, which was enhanced with vitamin D supplementation in TB patients with the vitamin D receptor “tt” genotype (Martineau et al., 2011). These conflicting results may be attributable to variations in dose, treatment duration, disease stage, and other factors. Therefore, further studies with an adequate sample size and appropriate clinical conditions are required to understand the epidemiological effects of vitamin D supplementation in TB.

The potential of HDTs as an adjuvant in the treatment of TB also includes drugs that target the anti-inflammatory response, such as aspirin and ibuprofen (Cubillos-Angulo et al., 2022 (Figure 1.5). A study conducted in the C3H4B/Fej murine model demonstrated that aspirin exhibits a greater effect on bacillary load, resulting in enhanced survival in advanced disease (Marzo et al., 2014). In a different study, the administration of aspirin to BALB/c mice led to a potentiation of the action of pyrazinamide, resulting in enhanced clearance of viable mycobacteria in both the spleen and lungs. However, the combination of aspirin and isoniazid resulted in increased bacterial loads (Byrne et al., 2007). These findings collectively indicate that further clinical studies should be conducted to evaluate the therapeutic effect of concomitant aspirin administration when combined with other anti-TB drug combinations. In a mouse model mimicking active TB, the bacillary load was also reduced by ibuprofen treatment (Vilaplana et al., 2013).

Additionally, in a different study conducted using a murine model, ibuprofen was found to enhance the efficacy of pyrazinamide in TB treatment (Byrne et al., 2007). It is anticipated that HDTs should improve cell-mediated immunity. Such agents include cholesterol-reducing drugs such as statins. Several mechanisms against TB have been identified, which include the inhibition of foamy cell formation, the support of Mtb persistence via the reversal of cholesterol biosynthesis (Shim et al., 2020), and the induction of phagosomal maturation and autophagy (Parihar et al., 2014). For instance,

the autophagy inhibitor 3-methyladenine has been shown to reverse autophagy and the maturation of phagosomes. In contrast, the administration of the statin-simvastatin at a dose of 25 mg/kg has been shown to accelerate the clearance of bacteria in the lungs of mice when used in conjunction with standard therapy (Skerry et al., 2014; Wallis et al., 2015). However, an extensive retrospective study utilizing a national medical claims database revealed that statins did not offer protection against tuberculosis in patients with diabetes mellitus (Wallis et al., 2015; Kang et al., 2014), highlighting the need for further research to determine the most effective agent and dosing schedule for clinical trials. Other mechanisms involve an increase in the percentage of natural killer T (NKT) cells in the cultures and upregulation of co-stimulatory molecules on monocytes, along with higher IL-1β and IL-12p70 secretion (Pandey et al., 2008; Guerra-De-Blas et al., 2019) and inhibition of TGF-β (Rodrigues et al., 2010; Ma et al., 2013).

Various evidence and studies, as well as the number of ongoing interventional clinical trials, reveal HDTs as a promising solution by repurposing known effective drugs and targeting new candidates. The results from these trials will be relevant to determine their potential beyond pre-clinical evidence. Repurposed drugs for HDTs have the benefit of decades of clinical application. Still, evidence must be collected concerning their safety and bioavailability in TB patients who might show heightened susceptibility to adverse effects while also experiencing interactions with the antimicrobial therapy. As mentioned before, hepatic and kidney disorders are already described as significant adverse drug effects and reasons for patient non-compliance. The addition of further drugs might introduce an additional burden to the patient's liver and kidneys. One must also consider the diverse population that is affected by TB. Many repurposed drugs and candidate HDTs are studied in cohorts from high-income countries that may not reflect the populations affected by TB and HIV from high-burden countries. This is particularly relevant for severe manifestations of TB, such as meningitis and miliary TB, which are more often found in those regions.

1.5.1. Protease inhibitors as a strategy to control infectious diseases

Enzymes have long been recognized as ideal candidates for target-based drug development, as evidenced by decades of research on disease agent proteases. Screening for selective protease inhibitors has been a valuable strategy to deliver effective therapeutic solutions for treating some of the most important infectious diseases like malaria and COVID-19 (Sojka et al., 2021). A number of proteolytic targets for infectious diseases have been validated, including proteasomes of the malarial pathogen *Plasmodium falciparum*, *P. falciparum* aspartyl proteases, and SARS-CoV-2 viral proteases (Sojka et al., 2021). Furthermore, low-molecular-weight inhibitors of proteases have recently emerged

as promising therapeutic agents for treating numerous diseases, including hypertension, diabetes, and various cancers (Drag et al., 2010).

Indeed, proteases play crucial roles in nearly all biological processes, both within individual organisms and in extracellular events. This generally involves either the activation of other enzymes through their well-timed processing, resulting in the generation of active, accessible catalytic sites on substrates, or conversely, the inactivation of proteins through their proteolytic fragmentation (Bond et al., 2019). Proteases are classified according to the composition of their active site and catalytic reaction. To date, seven classes of proteases have been identified, which include metallo-, serine, aspartyl, cysteine, threonine, glutamate, and asparagine proteases (Rawlings et al., 2021). Among the five most abundant, serine proteolytic enzymes are found in the highest natural abundance, followed in decreasing order by metallo-, cysteine-, aspartate, and threonine proteases (Barrett et al., 2004). These are the five classes that have been identified in humans, accounting for approximately 3% of the genome (Puente et al., 2003) and participating in the regulation of a variety of biological processes, including ovulation, embryogenesis, organ formation, tissue remodeling, immune response, antigen presentation, pathogen infection, cell death, cell migration/invasion, cell signaling, and wound healing (Wang et al., 2021; Fortelny et al., 2014; Kappelhoff et al., 2017).

Proteolytic enzymes are regarded as a primary component of the major virulent factors of infectious agents, playing a pivotal role in their development, reproduction, and interactions with host/invertebrate vector tissues. Therefore, these enzymes are considered promising targets for designing new drug candidates for the treatment of infectious diseases (Renslo et al., 2006; McKerrow et al., 2006). In 1995, the discovery of HIV protease inhibitors (HIV-PIs), such as saquinavir, lopinavir, and ritonavir (Kitchen et al., 1995; Kosalaraksa et al., 2013), demonstrated the antiviral efficacy of enzyme-targeted drugs. These HIV-PIs function by selectively blocking the retroviral protease, thereby preventing the processing of the long polypeptide that the viral RNA genome encodes into its individual viral proteins (Ghosh et al., 2016).

The advent of novel inhibitors and their combinations has been fundamental to the development of effective and less toxic antiretroviral therapy, thereby transforming HIV infection from a fatal to a more manageable disease. The development of protease inhibition-based treatment has emerged as an attractive and potentially efficacious strategy against infections since the discovery and approval of HIV-PIs. For instance, the papain-like cysteine protease cruzain has been demonstrated to be essential for the life cycle and virulence of *Trypanosoma cruzi*, the causative agent of Chagas disease (McKerrow, 2018). Likewise, an irreversible cruzain inhibitor, vinyl-sulfone, has demonstrated efficacy against schistosomiasis, hookworm infections, and cryptosporidiosis (McKerrow, 2018). In the context of

treating multiple myeloma, selective proteasome inhibitors such as carfilzomib, bortezomib, and ixazomib have been developed as a therapeutic strategy (Richardson, 2014). In addition, the development of specific inhibitors is critical in the treatment of infectious diseases such as malaria, leishmaniasis, schistosomiasis, and Chagas disease (Bibo-Verdugo et al., 2017).

Another example that substantiates the efficacy of protease-inhibition-based drug development is evident in the repurposing of drugs designed to target SARS-CoV-2. These candidate compounds have been shown to inhibit the SARS-CoV-2 main protease (M^{pro}), which is known to play a crucial role in the viral replication process within infected cells (Hilgenfeld et al., 2014). The PL^{pro} protease of SARS-CoV-2 shares a high degree of sequence and structural similarity with PL^{pro} of the previously emerged coronavirus (SARS-CoV-1). It has been characterized as displaying analogous functions in virus replication and modulation of the host's immune responses (Rut et al., 2020). Thus, the inhibition of SARS-CoV-2 protease can block viral replication and enhance the innate immune responses in acute cases of SARS-CoV-2 infection. Several studies have demonstrated the efficacy of repurposing drugs developed for the treatment of SARS-CoV-1-PL^{pro} to inhibit SARS-CoV-2-PL^{pro} (Rut et al., 2020; Freitas et al., 2020; Shin et al., 2020).

Regarding tuberculosis, the Mtb proteasome has been the subject of recent studies probing protease inhibitors in compound libraries for potential interactions with mycobacterial proteases (Almeleebia et al., 2021; Tyagi et al., 2022; Mehra et al., 2015). Mtb caseinolytic proteases (Clp) have been explored as such potential targets. These serine proteases play important roles in proteostasis of eukaryotic and prokaryotic cells and are often associated with infectivity and virulence of several pathogens. The Clp system comprises the highly conserved caseinolytic protease P (ClpP) and AAA + (ATPases associated with diverse cellular activities) chaperone-unfoldases (Zhou et al., 2025). Mtb uniquely harbors two ClpP paralogs—ClpP1 and ClpP2—that function together as a heterotetradecameric complex with an essential role in maintaining protein homeostasis, degrading misfolded or damaged proteins, and modulating virulence factors (Benaroudj et al., 2011). These ClpP are essential for Mtb survival in vitro and during infection (Raju et al., 2012), making them attractive and validated drug targets (Yang et al., 2023; Moreira et al., 2017).

Several small-molecule inhibitors, such as boronic acid derivatives and cediranib-based compounds, have shown potent activity against MtbClpP1P2. Cediranib, originally developed as a human VEGFR-2 inhibitor for cancer treatment, was recently identified as a potent allosteric inhibitor of MtbClpP1P2, disrupting its activity and effectively suppressing Mtb growth (Yang et al., 2023). Bortezomib, a clinically used human proteasome inhibitor for multiple myeloma, has been identified as a potent inhibitor of mycobacterial ClpP1P2 using a target mechanism-based whole-cell screening approach

(Moreira et al., 2015). This compound showed growth inhibition and microbicidal activity in Mtb. However, bortezomib's lack of selectivity toward human proteasomes presents a major obstacle for its use as an anti-TB drug. Many ClpP inhibitors face challenges due to cross-reactivity with human mitochondrial ClpP, which can lead to mitochondrial dysfunction. Finding compounds designed to retain anti-Mtb activity while significantly reducing off-target effects on the human proteasome is paramount to improving their safety profile (Moreira et al., 2017, Towards Selective Mycobacterial). To address this, analogs with modified warheads, such as chloromethyl ketones, have been developed to retain anti-mycobacterial ClpP1P2 activity while significantly reducing off-target effects on human cells (Moreira et al., 2017 Bortezomib Warhead-Switch Confers Dual Activity). These analogs demonstrate dual inhibition of both the ClpP1P2 and the Mtb proteasome, a combination that appears particularly lethal to Mtb yet spares human proteasome function. Additionally, efforts to inhibit related components such as ClpC1, the AAA+ chaperone-unfoldase that cooperates with ClpP1P2, have also yielded promising quinoline-based inhibitors with potent antimycobacterial activity (Moreira et al., 2017, Towards Selective Mycobacterial).

1.6. Cathepsins and their natural inhibitors cystatins

Several proteolytic enzymes are involved in various physiological processes that are related to the maintenance of homeostasis in host cells (Turk et al., 2012). These enzymes have been shown to play an important role in the proteolytic killing of bacteria that have been taken up via the process of phagocytosis (Russell et al., 2009). It was the year 1901 when Metchnikoff, also known as the father of innate immunity, announced for the first time that mononuclear phagocytes aslo named as macrophages by him, take up exogenous material by phagocytosis into vacuoles conatining the proteolytic enzyme macrocytase (Metchnikoff 2021; Burster et al., 2010; Anes et al., 2021). Willstätter later suggested the term cathepsin, derived from the Greek word "kathepsein", describing the ability of a proteolytic enzyme to digest" in a slightly acidic environment (Willstätter, 1928). He also proposed that cathepsin originated in leukocytes was secreted after autolysis (Willstätter, 1928).

In fact, research on cathepsin is concomitant with the discovery of lysosomes which was found by de Duve in 1955 (Appelmans et al., 1955). Lysosomes play a significant role in cellular degradation being associated with the major degradative vacuoles in eukaryotic cells. They are packed with substantial amounts of cathepsins and provide a reducing and slightly acidic environment which is ideal for the optimal activity of these enzymes (Anes et al., 2021, Anes et al, 2022). In 1970, an enzyme was discovered from swine leukocytes, and it was cathepsin G because of its ability to digest gamma-globulin. Next, another enzyme called cathepsin F was isolated with the capacity to hydrolyze fibrin.

Currently, the term cathepsins describes proteases which on the basis of their catalytic mechanisms are divided into three different families. These include the primary lysosomal enzymes which consist of a set of 11 cysteine cathepsins. These cathepsins are identified as B, C, F, L, K, S, H, O, V (L2), W and X (P, Y, Z). Additionally, the aspartic proteases such as cathepsins D and E and the serine proteases A and G (Burster et al., 2010; Turk et al., 2012). Although they were originally thought to be lysosomal proteases responsible for bulk proteolysis of endogenous and exogenous proteins and for the direct digestion and destruction of engulfed pathogens, they are now understood to function in many other cellular contexts. These include (i) the cytoplasm, where they regulate cell death, autophagy (Repnik et al., 2012), and trafficking, processing, and turnover of cytokines (Orlowski et al., 2015), (ii) the nucleus, where they regulate cell cycle and transcription (Ferrer-Mayorga et al., 2015), and (iii) the plasma membrane, where they activate and shed surface receptors and mediate T-cell priming and signaling (Pires et al.,2017, Hsing et al., 2005).

Recent advances in the understanding of the spatial localization of cathepsins and their implications for immune activation and resolution during infections are summarized in our review (Anes et al., 2022) (Figure 1.8)

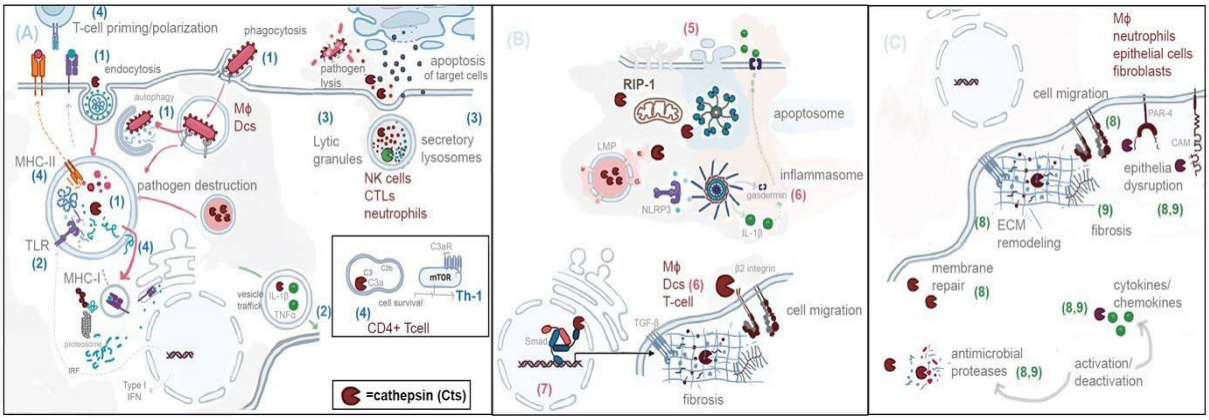

Figure 1.8. Schematic representation of the spatial localization of cathepsins and their roles in endocytic pathway **(A)** (blue numbers from 1 to 4), **(B)** cytosol and nucleus (pink numbers from 5 to 7), and **(C)** extracellular environment (green numbers 8 and 9). (1) Phagocytosis/endocytosis/autophagy. (2) Pattern recognition/cytokine activation. (3) Activation of proteases. (4) T-cell priming and polarization. (5) Programmed cell dead. (6) Inflammation. (7) Regulation of transcription. (8) ECM remodeling. (9) ECM inflammation during infection. Figure from Anes et al., 2022.

In host response to infections, cathepsins play crucial roles in innate and also in adaptive immune responses. Cathepsin B is shown to process and vesicle traffic immune modulators such as interleukin-1β (IL-1β) (Orlowski et al., 2015) and TNF-α (Ha et al., 2008), respectively; cathepsin K

activates toll-like receptor 9 (TLR9) (Wei et al., 2020), whereas cathepsin S is involved in major histocompatibility complex (MHC) class II-dependent CD4+ T-cell activation (Pires et al., 2017; Hsing et al., 2005). They also can be secreted outside the cell and act on extracellular matrix remodeling, mucosae host defense, angiogenesis, complement activation, and cell processes like migration and proliferation (Patel et al., 2018). Notably, protease activity must be properly regulated, and disruption of the firm interaction between proteases, substrates and inhibitors can result in the pathogenesis and advancement of a variety of diseases, including infectious diseases like TB and AIDS.

Mtb is a facultative intracellular pathogen with the potential to establish its primary niche within early endolysosomal vesicles. To this end, the bacilli hamper phagosomal maturation and subsequent intracellular macrophage efficacy (Russell et al., 2007; Welin et al., 2011). This dysregulation of host macrophages can affect the expression of proteolytic enzymes, such as lysosomal cathepsins, leading to an enhanced intracellular survival of Mtb (Pires et al., 2016). The abnormal activity of proteolytic enzymes must be tightly controlled, as it can result in severe dysfunction and pathology. Cystatins (Csts) are a well-known class of endogenous modulators of cathepsins, which generally control their excessive enzymatic activity by binding and sequestering them within cells, tissues, and body fluids (Turk et al., 2012). Pathogens, such as Mtb and HIV, could evolve strategies for manipulating these early events to prevent the activation of the microbicidal mechanisms, allowing their survival or replication within the cells. Therefore, Csts may emerge as potential targets that could be manipulated to restore cathepsin activity in the context of Mtb infection or coinfection with HIV.

Csts are well-characterized inhibitors that are further divided into four different families. Type I Csts (stefins) consist of CstA and B and exist within the cytosol and nucleus, while type II Csts include CstEM, S, SA, SN, and D, which are secreted and function as extracellular proteins (Magister et al., 2013; Ochieng et al., 2010). A subset of the secreted type II Csts (CstC and F) has been observed to be internalized by immune cells or to translocate from the secretory pathway, translocating into the cytosol of the immune cells, accumulating in endosomal/lysosomal vesicles (Lautwein et al., 2002; Colbert et al., 2011). Type III Csts include kininogens that circulate in the blood as precursors of the vasoactive peptide kinin (Cst families reviewed in (Magister et al., 2013; Ochieng et al., 2010)). Finally, type IV Csts comprise thyropines and are characterized as a Csts family originating from the observation that a fragment of the p41 invariant chain bound to MHC class II molecules inhibits cathepsin (Ogrinc et al., 1993; Anes et al., 2021). Cathepsins and their natural inhibitors are regarded as the key players in Mtb and HIV infection.

1.6.1. Cathepsins and inhibitors during TB infection and HIV-coinfection

Mtb and HIV are both intracellular pathogens, and during the course of coinfection, they must overcome mucosal barriers in order to establish intracellular niches in their host cells (Anes et al., 2021). Mtb transmission occurs via small droplets which have to overcome the strong innate immune antimicrobial milieu of the upper and lower respiratory tract before reaching the alveolar compartment where bacteria are phagocytized by macrophages and DCs (Figure 1.9) (Cambier et al., 2014).

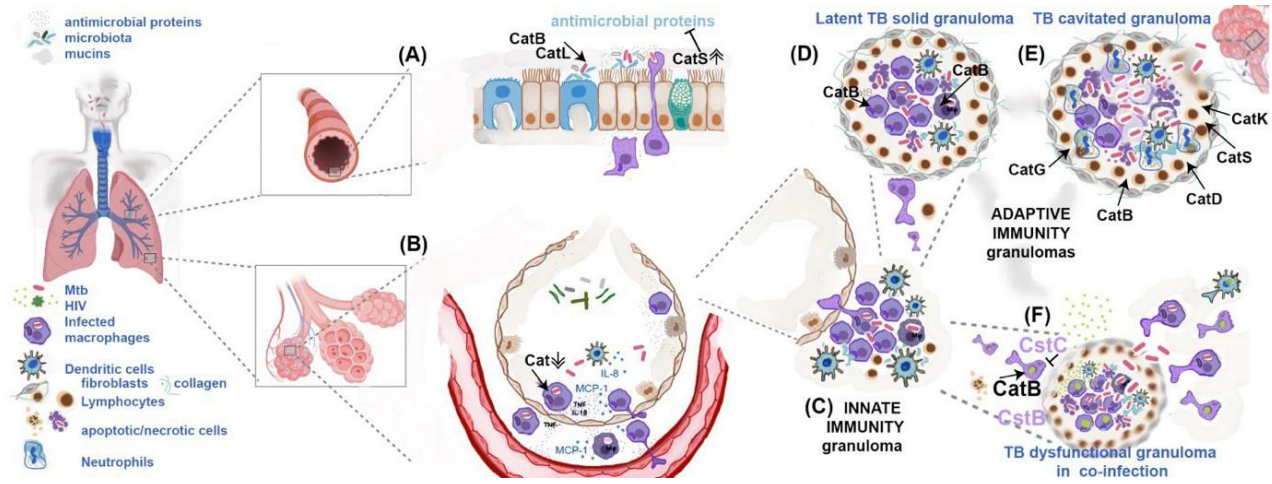

Figure 1.9. Cathepsins and inhibitors during TB infection and HIV-coinfection. **(A)** Mtb needs to overcome the strong mucosa inflammatory environment of the upper and lower respiratory tract, as cathepsins B, L, and S exert a direct killing effect. **(B)** After Internalization of Mtb by alveolar macrophages, there is downregulation of cathepsins that helps to establish intracellular niches. **(C)** Cells infected with Mtb will move to the deeper lung tissue, and there is the formation of an innate granuloma. **(D)** Next, there is formation of a dynamic structure i.e. adaptive immune granuloma upon apoptosis of infected macrophages induced by cathepsin B, releasing live bacteria in apoptotic bodies which are internalized by new arrival macrophages **(E)** During active TB, there is increased secretion of cathepsins G, K, D, S and B in the granuloma as well as from cells in the lung tissue being stimulated by Mtb products, and this leads to collagen destruction, cavitation and, bacilli spread to other individuals. **(F)** The arrival of HIV to the lung in latently infected macrophages which under an inflammatory environment of cytokines will initiate to replicate. Cathepsin B will associate in the formation of new viral particles via processing viral proteins. In contrary, Csts B and C will inhibit this mechanism. Coinfection with HIV causes depletion of lymphocyte, low secretion of TNF as well as death of monocytes at virus replicating site, compromising the structural dynamics and integrity of granuloma, resulting the spread of infected cells to other parts of the body. Retrieved from Anes et al., 2021. Reproduced with permission from Microbial Immunology, a section of the journal Frontiers in Immunology.

Mucosal barriers exert intrinsic microbicidal function primarily based on innate receptors, which include toll-like receptors (TLRs). These recognize microbial signatures from pathogens and microbiota (MAMPs), which stimulate the release of antimicrobial proteins and peptides. The secreted proteases include cathepsins, lysozyme, which is also known as the cell wall-degrading enzyme, the iron-chelating protein lactoferrin, the secretory leucoprotease inhibitor (SLPI), and cathelicidin, as well as antimicrobial peptides (AMPs) (Rogan et al., 2006; Anes et al., 2021). Other lungs specific secreted proteins include the surfactant proteins SP-A and SP-D from the complement system (Li Y et al., 2012). SPs A and D are involved in the early capturing of Mtb, which emphasizes their significance in surfactant homeostasis and the host's defence in the lung. The binding of these proteins to the terminal mannosyl oligosaccharides of the cell wall induces bacilli agglutination, improving phagocytosis by alveolar macrophages (Li Y et al., 2012; Anes et al., 2021).

The two most abundant endopeptidases in the lung environment are cathepsins B and L which are found predominantly in bronchial epithelial cells that constitute the first line of defence against pathogens. Cathepsin S is expressed prominently in macrophages and is also present on the cell surface of ciliated cells, and can facilitate the motility of cilia, preventing unspecific binding with plasma-derived proteins during circulation (Chapman et al., 1997). All mucosae consist of lactoferrin which is active against a number of viruses including HIV (Harmsen et al., 1995). It has been shown that cathepsins B, L, and S cleave and inactivate lactoferrin, SLPI and AMPs including defensins and cathelicidins (Figure 1.6) (Rogan et al., 2006; Taggart et al., 2003). The surfactant proteins A and D were shown as potent substrates of cathepsin B, L, and S, which may compromise their capacity to aggregate lipids as well as bind microorganisms (Lecaille et al., 2016; Anes et al., 2021). Although AMPs are resistant to proteolytic degradation, cathepsin S is the most potential cysteine cathepsin which can cleave all the AMPs, impairing their antimicrobial activity (Anes et al., 2021). In the context of chronic inflammatory lung diseases, overexpression of cathepsin S may lead to enhanced depletion of AMPs, promoting Mtb infection and colonization (Taggart et al., 2003, Anes et al., 2021). In contrary, certain AMPs might work as proteases inhibitors (Lecaille et al., 2016) with regulatory role in lung innate immunity such as cathelin domain which was proposed as cathepsin L inhibitor (Zaiou et al., 2003). Several pro-inflammatory cytokines, including IL-1b and TNF-α which respond to the detection of MAMPs through innate receptors, are associated with the regulate of cathepsin S release (Li Y et al., 2012). Extracellular cathepsins are associated with the direct modulation of immune environment at lower or upper female genital tract, affecting HIV transmission (Anes et al., 2021).

Cathepsin D, being regulated by hormones that are involved in the menstrual cycle (Bretschneider et al., 2008), together with the reported interference of cathepsins with a number of ant-HIV immune

factors (Harmsen et al., 1995; Taggart et al., 2003; McNeely et al., 1995; Hasan et al., 2006; Barrow et al., 1997) provide another degree of complexity to the regulation of HIV factors influencing HIV potential to infect cells at the genital mucosae. Accordingly, the cathepsin inhibitor Cst B was found to be over-abundant in the cervicovaginal fluid of women resistant to HIV, and this correlates with protection from HIV transmission via unprotected sexual intercourse (Burgener et al., 2011).

1.6.2. Cystatins in immune system

Cystatins consist of a large superfamily of homologous proteins with various biological activities. They were originally characterized as inhibitors of lysosomal cysteine proteases, but in recent years some additional roles of cystatins have been proposed (Magister et al., 2012). As mentioned earlier, Inhibitory cystatins comprise three families, family I (stefins), family II (cystatins) and family III (kininogens) (Magister et al., 2013; Ochieng et al., 2010; Magister et al., 2012). Stefin A is commonly implicated in the neoplastic transformation of epithelium, while another family I cystatin, stefin B, is proposed to have some role in neurodegenerative diseases. Cystatin C (Cst C), a typical type II cystatin, is present in numerous human tissues and cells (Magister et al., 2012). Expression of other type II cystatins is more specific.

Cystatin F (Cst F) is a protease inhibitor targeted to endo/lysosomes, specifically expressed in immune cells and hence assumed to act in immune-related processes (Lautwein et al., 2002; Colbert et al., 2011; Magister et al., 2012). Among type II cystatins, Cst C and Cst F are the most prominent ones in immune cells, the former being the most prevalent human cystatin. Cst C was originally found to be a ‘Post-y-globulin’ or ‘Y-trace’ and was the initial cystatin to be sequenced for amino acids (Grub et al., 1982). Later on, it was determined that its amino acid sequence was very similar to cystatin, present in chicken egg white (Turk et al., 1983). Cst C contains 120 amino acid residues and has a molecular mass of 13,343 Da. From the Cst C cDNA sequence, it was revealed that Cst C is synthesized as preprotein consisting of a 26-residue signal peptide (Abrahamson et al., 1987). Cst C coding gene is of the usual house-keeping type of gene, being expressed in a broad range of human tissues and cells. However, like most of the other type II cystatins, Cst C is secreted and with substantial amounts in body fluids, especially high levels have been found in seminal plasma and cerebrospinal fluid. It could be considered as a major human extracellular cysteine protease inhibitor, including strong inhibitor of all papain-like proteases (Abrahamson et al., 1986; Barrett et al., 1984) and asparaginyl endopeptidase/legumain (Alvarez-Fernandez et al., 1999). Cst C was also shown to be involved in the regulation of cathepsin S function and also in-variant chain processing in DCs (Pierre et al., 1998). However, several other studies ruled out involvement in the modulation of MHC ll-dependent antigen presentation in DCs (El-Sukkari et al., 2003). Moreover, there was reduction in levels of Cst C upon the

maturation of DCs and studies on colocalization do not support intracellular interactions between Cst C and its potential target enzymes, including cathepsins H, L and S in immature or mature DCs (Zavasnik et al., 2005).

On the other hand, Cst F was shown to be a better candidate to regulate the proteolytic activity of cysteine proteases in the DCs (Magister et al., 2012). Three independent groups discovered Cst F, identified by cDNA cloning and named it leukocystatin and Cst F by the first two groups, whereas the third group identified it as cystatin-like metastatic tumors (CMAP) upon finding overexpressed mRNA encoding Cst F in liver metastatic tumors (Halfon et al., 1999; Ni J et al., 1998; Morita et al., 2000). Human Cst F is synthesized as a pre-protein with 145 amino acids and a putative 19-residue signal peptide (Halfon et al., 1999; Ni J et al., 1998). Although it is composed with a signal sequence, only a minority proportion is secreted (Ni J et al., 1998) and interestingly, it is secreted as an inactive disulphide-linked dimer (Cappello et al., 2004) which is reduced to the active monomeric form (Langerholc et al., 2005). It is glycosylated (Halfon et al., 1999; Ni J et al., 1998) and upon modification of its *N*-linked saccharides, the endosomes and lysosomes are targeted (Colbert et al., 2009).

Cst F tightly inhibits several cathepsins, including F, K and V, however, cathepsins S and H are only inhibited with lower affinities (Ni J et al., 1998; Langerholc et al., 2005). The inhibitor is expressed selectively in immune cells which include cytotoxic T cells, NK cells, monocytes, DCs, and with level and localization controlled accordingly by the physiological state of the cells (Magister et al., 2012; Halfon et al.,1999; Ni J et al., 1998; Hashimoto et al., 2000; Obata-Onai et al., 2002). From the unique features of Cst F, it can be suggested that this immune-cell specific inhibitor has a significant role in immune response-related processes via inhibition of specific targeted enzymes. Among the protease targets of Cst F, cathepsin C is one of the cysteine proteases which is associated with the activation of granzymes in cytotoxic T cells, NK cells and also several of effector proteases of mast cells and neutrophils (Hamilton et al., 2008; Pham et al., 1999; Adkison et al., 2002). Cathepsin C in an N-terminally truncated form only can be strongly inhibited by Cst F (Hamilton et al., 2008). In the context of NK cells, the regulation of Cst F and consequently cathepsin C and granzymes by NK cell surface receptors plays a mechanistic role to turn them into regulatory NK cells (Magister et al., 2013). Cystatins are associated with numerous of normal and pathological conditions, exerting various immunomodulatory functions by controlling the activity of cysteine proteases or other mechanisms other than their inhibitory function. However, there are not numerous studies regarding implication of different types of cystatins in immune system.

1.7. Thesis scope and objectives

Despite continuous and extensive global efforts in the fight against TB, this infectious disease continues to exert a tremendous burden on public health concerns and deaths worldwide. TB is highly frequent in people living with HIV. The continuing epidemics of both chronic infections and the emergence of antimicrobial resistance, as well as the lack of effective diagnostic tools and drug–drug interactions, pose major challenges in the fight against these pathogens. There is an urgent need for the development of potential alternative therapeutic strategies to current antimicrobials that could boost the host immunity and effectively control Mtb infection or coinfection with HIV. In the present context, the investigation on host-directed therapies that enhance the host responses to control the infection and ameliorate immunopathological damage constitutes a promising strategy to improve disease outcome, helping alleviate the morbidity and mortality associated with both infections. This includes the search and repurposing of existing drugs already approved for other conditions that will improve the effectiveness of existing antimicrobials, minimize drug resistance, decrease treatment duration, and adverse effects. For example, a plethora of antiretroviral drugs are already available for the treatment of HIV infection. However, only a few of these (PIs) can interfere with virus production and release from macrophages during chronic infection. The proteostasis network, comprising chaperones, proteases, and the proteasome, plays a major role in the survival of Mtb under cognitive host immune stress (Lupoli et al., 2018). It has been demonstrated that HIV and Mtb interact synergistically, modifying the host immune environment, thereby promoting the replication and dissemination of both pathogens. Hence, it is plausible that during coinfection, HIV PI could interfere with the host proteases.

The potential and proven proteolytic targets against various infectious diseases provide compelling evidence for establishing protease inhibitors as a drug development platform and other therapeutic strategies. A more profound understanding of microbial dependence on cellular proteases and their inhibitors may establish a strong basis for designing novel host-directed strategies to control microbial infection. It has been demonstrated that HIV PIs, particularly SQV, may interfere with the proteases of the host during Mtb or Mtb/HIV infection. As previously mentioned, saquinavir (SQV) controls HIV infection by interfering with HIV protease activity, ultimately preventing virion infectivity and intercellular transfer. SQV could be repurposed to regulate a variety of protease-dependent infectious organisms. Within this scope, we hypothesize that repurposing SQV, a first-generation HIV PI, may improve intracellular killing of Mtb, emerging as a potential host-directed therapy for TB. Furthermore, the enhanced internalization of SQV in macrophages may significantly impact the proteolytic activity of cathepsins compared to that of free drug treatment, and possibly with less cytotoxicity. Advanced drug-delivery methods are of paramount importance for SQV, as its serum availability is low due to

first-pass metabolism by the liver (Cooper et al., 2003). This is further worsened by the use of SQV, and would require demonstration that its use in conjunction with targeted drug-delivery methods can overcome negative interactions with rifampicin and increase its bioavailability. Therefore, we also aim to develop a liposomal drug delivery system to overcome negative interactions with rifampicin and significantly increase the potential of SQV's role as an enhancer of macrophages' killing activity.

In other hand, several proteolytic enzymes are involved in various physiological processes that are related to the maintenance of homeostasis in host cells (Turk et al., 2012). These enzymes have been shown to play an important role in the proteolytic killing of bacteria that have been taken up via the process of phagocytosis (Russell et al., 2009). Mtb is a facultative intracellular pathogen with the potential to establish its primary niche within early endolysosomal vesicles. To this end, the bacilli hamper phagosomal maturation and subsequent intracellular macrophage efficacy (Russell, 2007; Welin et al., 2011). This dysregulation of host macrophages can affect the expression of proteolytic enzymes, such as lysosomal cathepsins, leading to an enhanced intracellular survival of Mtb (Pires et al., 2016). The abnormal activity of proteolytic enzymes must be tightly controlled, as it can result in severe dysfunction and pathology. Cystatins (Csts) are a well-known class of endogenous modulators of cathepsins, which generally control their excessive enzymatic activity by binding and sequestering them within cells, tissues, and body fluids (Turk et al., 2012). Pathogens, such as Mtb and HIV, could evolve strategies for manipulating these early events to prevent the activation of the microbicidal mechanisms, allowing their survival or replication within the cells. Similarly, HIV-infected patients frequently exhibit pleural effusion due to TB in the context of coinfection (Pires et al., 2021). Furthermore, higher levels of CstF have been detected in pleural effusion of TB patients compared to other inflammatory conditions (Cohen et al., 2015). Therefore, Csts such as Cst C and Cst F may emerge as potential targets that could be manipulated to restore cathepsin activity in the context of Mtb infection or coinfection with HIV.

Thus, we hypothesize that the depletion of these Csts expression could modulate the proteolytic activity of the macrophages' lysosomal cathepsins S, L, B as well as cathepsin C in the context of Cst F, which in turn improves the control of both Mtb and HIV pathogens. In order to knockdown the expression of Csts expression, invitro transfection method is applied. However, this invitro transfection methods are not suitable for the clinical translation of host-cell RNA silencing. Therefore, we also aim to develop different RNA delivery systems (DSs) i.e., nanomedicine based on chitosan to overcome this limitation and also to significant impact on the intracellular survival of TB bacilli.

The specific objectives of this thesis are as follows:

1. Repurposing Saquinavir as a host-directed strategy to Control *Mycobacterium Tuberculosis* Infection.
2. Delivery of Saquinavir loaded in liposome to Macrophages to overcome Cathepsin Blockade by *Mycobacterium tuberculosis*.
3. To define a specific pattern for cystatins expression and activity in macrophages obtained from healthy donors (buffy coats) in de novo infection from three different infections: Mtb, HIV, or both.
4. To characterize the involvement of cystatins by using high-content siRNA screening in the intracellular killing/survival of Mtb, in coinfection with HIV.
5. To assess the role of cystatins (specifically Cst C) in immune-activation, by using high-content siRNA screening focusing on programmed cell death (apoptosis vs necrosis), by cytokine secretion (TNF and IL1ß, IFN-γ) and by antigen presentation during de novo infection.
6. To investigate the impact of CstF depletion in macrophages during the coinfection of Mtb-infected phagocytes with lymphocytesinfected with HIV.
7. To develop nano-formulations with designed siRNAs to target host macrophages infected with Mtb and HIV as pre-therapeutic approach.

Overall, the present work underscores the pivotal role of HDTs in the mitigation of the substantial burden caused by infectious diseases, with particular emphasis on mycobacterial infection and Mtb/HIV coinfection. Moreover, it delves into innovative HDTs based on protease inhibitors and their clinical implications as adjunctive therapeutic regimens for the management of TB and HIV coinfection. These novel strategies include the repurposing of SQV (an HIV protease inhibitor) and the manipulation of Csts (the natural cathepsin inhibitors).

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CHAPTER 2.

Repurposing Saquinavir for Host-Directed Therapy to Control *Mycobacterium tuberculosis* Infection.

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I contributed to this work in experimental part of the study.

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I contributed to this work in experimental part by performing most of the experiments related with infection and validating the accuracy and reproducibility of the experimental results and data to ensure the reliability of the research.

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Abstract

Despite the available antibiotics, tuberculosis (TB) has made its return since the 90's of the last century as a global threat mostly due to coinfection with HIV, to the emergence of drug resistant strains and the lack of an effective vaccine. Host-directed strategies could be exploited to improve treatment efficacy, contain drug-resistant strains, improve immune responses and reduce disease severity. Macrophages in the lungs are often found infected with *Mycobacterium tuberculosis* (Mtb) and/or with HIV. The long-term survival of lung macrophages infected with Mtb or with HIV, together with their ability to produce viral particles, especially during TB, makes these niches major contributors to the pathogenicity of the infection. Among the available drugs to control HIV infection, protease inhibitors (PIs), acting at post-integrational stages of virus replication cycle, are the only drugs able to interfere with virus production and release from macrophages during chronic infection. For Mtb we recently found that the pathogen induces a general down-regulation of lysosomal proteases, helping bacteria to establish an intracellular niche in macrophages. Here we found that the PI saquinavir, contrary to ritonavir, is able to induce an increase of endolysosomal proteases activity especially of cathepsin S in Mtb infected macrophages and during coinfection with HIV. Our results indicate that saquinavir treatment of infected macrophages led not only to a significant intracellular killing of Mtb but also: (i) to an improved expression of the HLA class II antigen presentation machinery at the cell surface; (ii) to increased T-lymphocyte priming and proliferation; and (iii) to increased secretion of IFN-γ. All together the results indicate saquinavir as a potential host directed therapy for tuberculosis.

In this study, we demonstrate that incorporation in liposomes was able to increase the efficiency of saquinavir internalization in macrophages, reducing cytotoxicity at higher concentrations. Consequently, our results show a significant impact on the intracellular killing not only to reference and clinical strains susceptible to current antibiotic therapy but also to multidrug- and extensively drug-resistant (XDR) Mtb strains. Altogether, this indicates the manipulation of cathepsins as a fine-tuning strategy used by the pathogen to survive and replicate in host cells.

2.1. Introduction

Mycobacterium tuberculosis (Mtb) the causative agent of tuberculosis (TB) and the AIDS-associated human immunodeficiency virus (HIV), have in common macrophages (Mø) as immune cell reservoir. Both pathogens alter the Mø microbicidal and immune-activating functions and convert these cells into intracellular survival niches (Bell and Noursadeghi, 2018). In the case of Mtb, the WHO estimates that one quarter of the human population is latently infected and among these, 10% will develop the active disease. From the latently infected group about 600,000 people are estimated to be carriers of multidrug-resistant (MDR) and extensively drug-resistant Mtb strains (XDR) (World Health Organization, 2020). Main contributors to TB activation from latency are immunosuppressive conditions, especially HIV coinfection, malnutrition and aging.

While for HIV the infection became a chronic controlled situation with the available medicines, for TB and particularly during coinfection with HIV the scenario is a global threat for humankind. This includes, as aforementioned, (A) the increased MDR and XDR strains to current available antibiotics; (B) the condition that Mtb exacerbates HIV infection and vice versa leading to TB activation from latency; and (C) the fact that co-infected individuals contribute to viral spread and to MDR and XDR strains transmission (World Health Organization, 202; Souriant et al., 2019; Mancino et al., 1997). All together this led us to search for host targets that may be manipulated during infection to boost the immune responses blocked by the pathogens as an alternative therapeutic strategy to current antimicrobials. In this context, the repositioning of drugs represents a useful strategy in the search for new fast therapeutic approaches for TB control. Furthermore host-directed strategies could be exploited to improve treatment efficacy and outcome and reduce disease severity and mortality.

Combined antiretroviral therapies have been applied in HIV-infected patients for more than two decades and include a cocktail of nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTIs (nNRT1s), protease inhibitors (PIs), and integrase inhibitors (Kourjian et al., 2014). These were shown to efficiently suppress HIV replication, leading to partial immune restoration and turning AIDS into a chronic infection. A threat to this controlled situation arises from the fact that HIV in addition to infect $CD4^+$ T-lymphocytes also infects Mø. While the absolute number of infected Mø in the body is relatively low compared to $CD4^+$ T-cells this is not the case for HIV infected Mø in the lungs (Aquaro et al., 2002) particularly during coinfection with Mtb (Toossi et al., 2001). Furthermore, alveolar Mø simultaneously infected with HIV and Mtb, were isolated from a patient co-infected with both pathogens (Mwandumba et al., 2004). The long-term survival of lung Mø infected with these pathogens, together

with their ability to exacerbate the infection by each other, turns these viral reservoirs into a challenge to HIV eradication since they continue producing virus in this tissue despite antiretroviral therapy (Souriant et al., 2019; Mancino et al., 1997). Among the available drugs to control HIV infection, protease inhibitors (PIs), acting at post-integrational stages of virus replication cycle (Arts and Hazuda, 2012) are the only drugs able to interfere with virus production and release from Mø during chronic infection (Titanji et al., 2013). The anti-viral activity of PIs is based on inhibition of the HIV aspartic protease, responsible for the cleavage of the Gag/Pol polypeptide and the structural viral core proteins leading to the production of immature viral particles, the inhibition of viral replication and cell-to-cell spreading (Titanji et al., 2013; Roberts et al., 1990; Craig et al., 1991).

PI were recently shown to directly act as modulators of endolysosomal proteases activity, namely of cysteine cathepsins in human $CD4^+$ T-cells and in antigen presenting cells (APCs) as dendritic cells and Mø (Kourjian et al., 2016). Curiously, while saquinavir (SQV) activates omni-cathepsins enzymatic activity (omnicathepsins includes cathepsins B, L and S), ritonavir (RTV) displayed the opposite effect on cells obtained from non-infected individuals.

For Mtb we previously demonstrated that during infection of human Mø, a general down-regulation of cathepsins gene expression, concomitant with a decreased protease activity, occurs either in resting M0 or in IFN-γ M1 activated cells (Pires et al., 2016; Pires et al., 2017). This may be a strategy used by the pathogen to manipulate the host microbicidal responses in order to survive intracellularly in these immune cells and to prevent antigen presentation. Here we found that SQV, contrary to RTV, is able to enhance the omnicathepsins protease activity including a very significant increase in cathepsin S activity in Mtb infected Mø. The enhancement of the catalytic activity was able to overcome the enzymatic inhibition induced by the pathogen in a three-fold magnitude. The same was observed during HIV coinfection. Our results indicate that SQV treatment during Mtb infection led not only to an exacerbated intracellular killing of the bacteria but also to an improved expression of the HLA class II antigen presentation machinery at the cell surface, to $CD4^+$ T-lymphocyte priming and proliferation as well as to increased secretion of IFN-γ.

This work indeed demonstrates that saquinavir (SQV) incorporated in liposomes increases the efficiency of the HIV protease inhibitor internalization in macrophages, reducing their cytotoxicity at higher concentrations while impacting the proteolytic activity of cathepsins. By using this strategy, we aim to demonstrate that cathepsins are major players during Mtb infection and that overcoming the cathepsin activity blockade induced by the pathogen may contribute to controlling the infection. All together the results indicate SQV as a potential host directed therapy for tuberculosis.

2.2. Results

2.2.1. Treatment with saquinavir impacts cysteine cathepsins enzymatic activity in Mø infected with Mtb and HIV

Protease Inhibitors (PI) prescribed to HIV-infected patients were previously found to directly manipulate the proteolytic activity of endolysosomal cysteine cathepsins in APCs isolated from healthy non-infected donors (Kourjian et al., 2016). Here in the context of infected Mø with Mtb or during coinfection with HIV, we first aimed to assess the effect of HIV PIs saquinavir (SQV) and ritonavir (RTV) on omnicathepsin proteolytic activities (which measure the combined activities of cathepsin B, L, and S). Cathepsins B, L and S are all involved in intracellular killing of pathogens internalized by Mø through phagocytosis/endocytosis (Pires et al., 2016). Cathepsin S, in addition, is strongly expressed in APCs and also operates in the endocytic pathway with proteolytic activities required for antigen and MHC class II processing (Pires et al., 2017; Pierre et al., 1998; Lautwein et al., 2002).

The selected concentrations of SQV and RTV ranging from 5 to 20 µg/mL were based in previous studies (Kourjian et al., 2014; Kourjian et al., 2016) concerning the average levels found in the plasma of people treated with a single daily dose of 5 to 10 µg/mL (Kourjian et al., 2016; Pajonk et al., 2002; Acosta et al., 2000; van Heeswijk et al., 2000). Here, Mø were treated with SQV or RTV one hour before infection (as detailed in methods) and the drugs were left in contact with cells during the whole assay. The cleavage of a peptidase-specific fluorogenic peptide substrate was measured over 1.5 hours, 24 h post-infection. Cells treated with omnicathepsin inhibitor E-64d were used as control.

Figure 1A (upper panels) shows the effects of PIs on omnicathepsin proteolytic activities in Mø infected with Mtb or co-infected with HIV relatively to non-treated infected cells. Treatment with SQV led to a very significant increase of the proteolytic activity in a dose-dependent manner, while no effects were observed for RTV in all conditions tested. In non-infected cells **(Figure 1A** upper panels left and right) the effects of SQV on cathepsin kinetics was more exacerbated than in infected ones reinforcing our previous results that Mtb infection results in an overall decrease of cathepsins activity (Pires et al., 2016; Pires et al., 2017). The kinetics when using the concentration of 10 µg/mL was near the saturation level by the end of 60 min treatment (**Figure 1A** upper right panel).

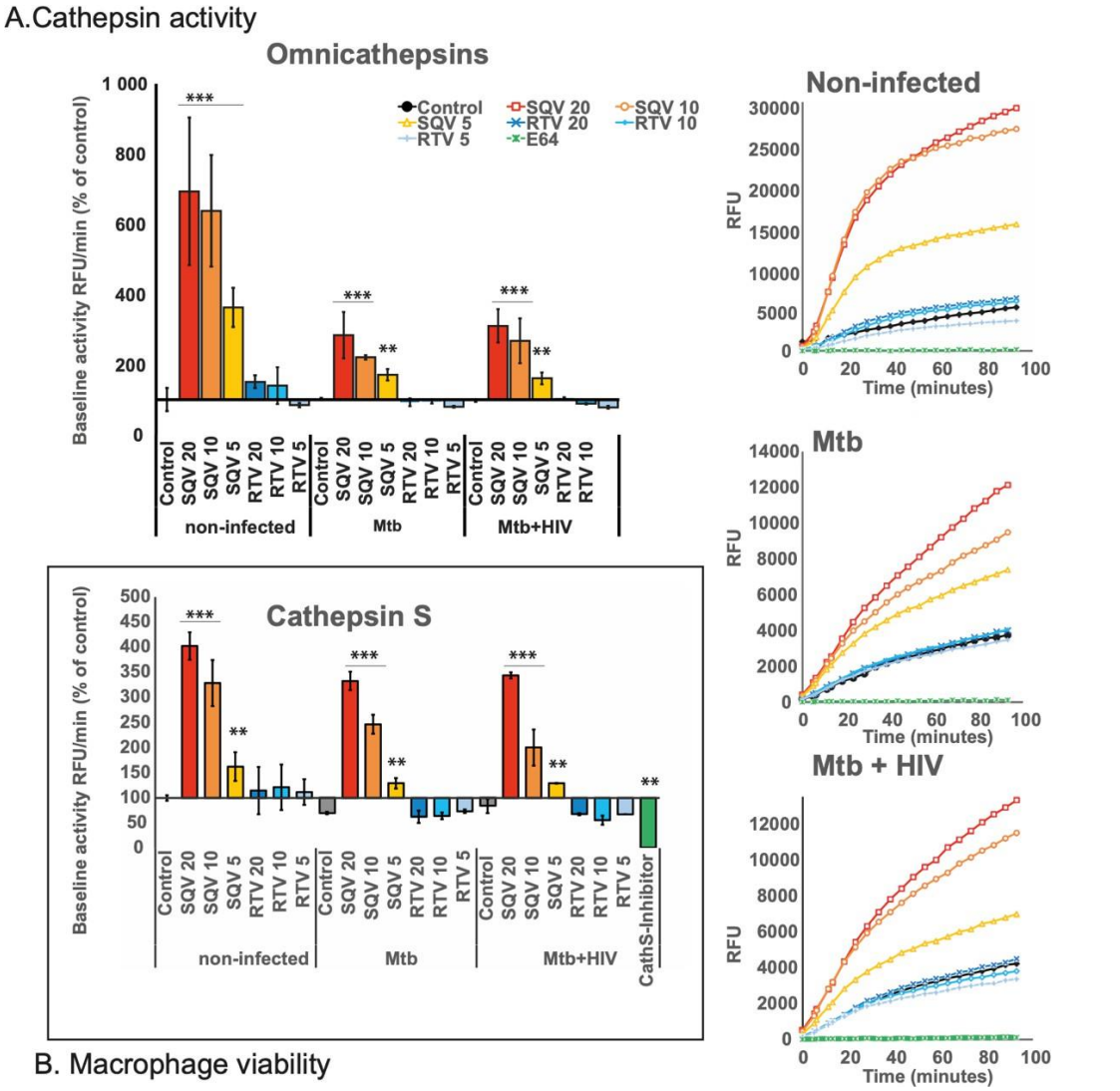

A.Cathepsin activity
Omnicathepsins
Baseline activity RFU/min (% of control)
Control
SQV 20
SQV 10
SQV 5
RTV 20
RTV 10
RTV 5
E64
non-infected
Mtb
Mtb+HIV
Cathepsin S
CathS-Inhibitor
Non-infected
Mtb
Mtb + HIV
RFU
Time (minutes)
B. Macrophage viability
SQV
RTV
DMSO
Viability(%)
Conc. (μg/mL)
Dilution(%)
Mtb
Percentage of cells
Necrotic
Late apoptotic
Early apoptotic
Live
RTV 100

Figure 1. HIV PIs alter cathepsins' activity in human macrophages infected with Mtb. **(A)** Omnicathepsin activity or cathepsin S activity alone were monitored with a specific fluorogenic substrate every 5 min in live cells pretreated with DMSO, RTV, SQV, or with specific inhibitors (E-64d or ZFL-COCHOO for cathepsin S). The slope of fluorescence emission in the presence of DMSO was represented as 100%, and the effect of each PI was calculated as a percentage of the DMSO control. Data are represented as average from three independent experiments and donors and data dispersion represented by the error bars as standard error (**P* < 0.05, ***P* < 0.01, ****P* < 0.001 relatively to control). **(B)** Cell viability (upper bar-plots) was measured in non-infected cells treated for 3 days with the PIs and using PrestoBlue resazurin-based solution by quantifying the emission of fluorescence in a plate reader. Cell death (lower bar-plot) was measured by flow cytometry after 24 h of infection using FITC-Annexin V and propidium iodide. Values show the average of three biological replicates from one representative experiment performed in triplicate while error bars depict standard deviation (**P* < 0.05, ***P* < 0.001 relatively to control).

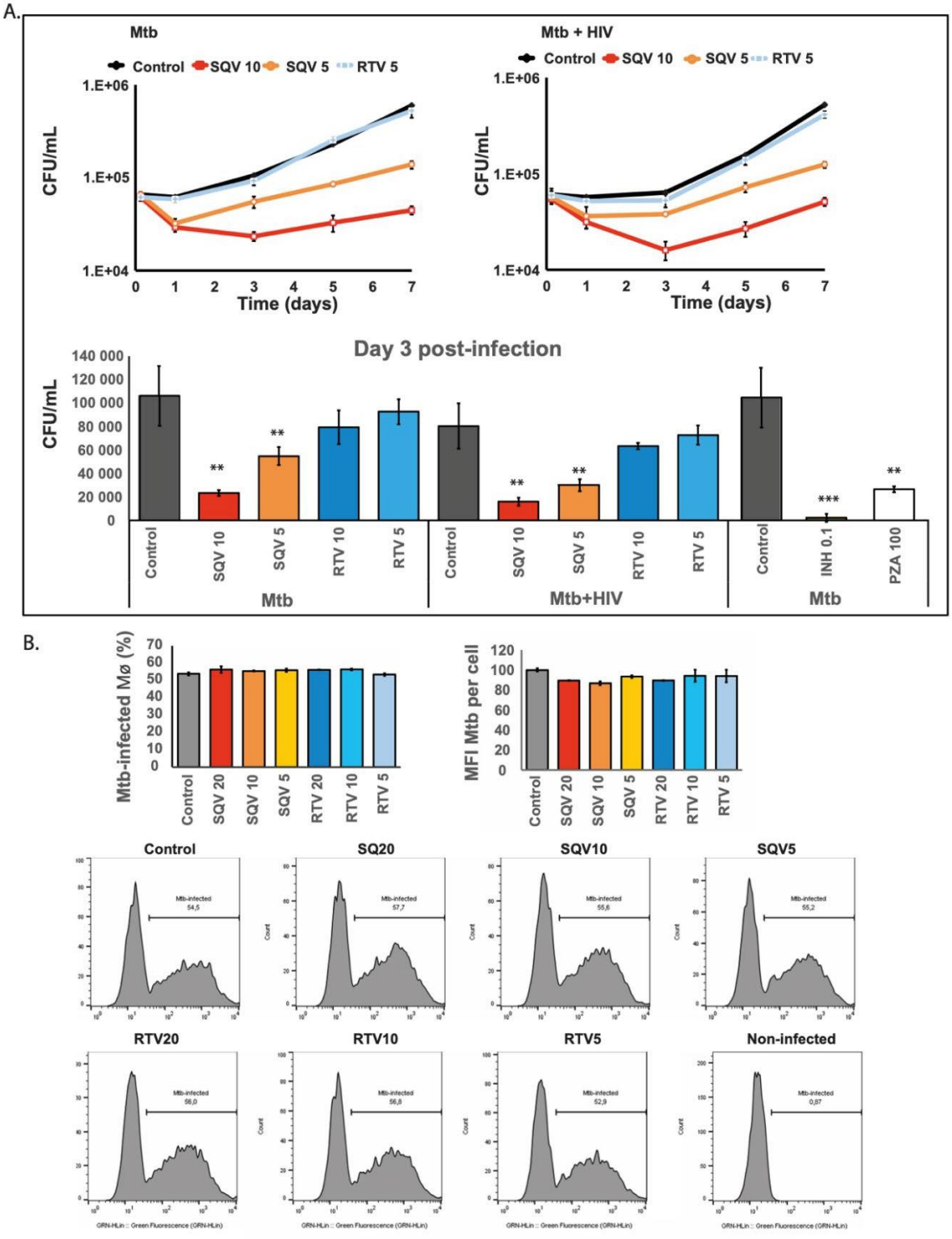
In parallel, we assessed the PIs effect on kinetics of cathepsin S activity alone using a cathepsin S cleavage-specific fluorogenic peptide substrate. As depicted in **Figure 1A** (lower panel) SQV strongly enhanced the proteolytic activity of cathepsin S in a dose dependent manner during Mtb infection and during coinfection with HIV contrary to RTV that presented kinetics similar to the control.

To confirm the effect of SQV and RTV in cell viability/cytotoxicity we performed the resazurin assay (**Figure 1B** upper panel) with results indicating cytotoxic effects on Mø viability at a concentration of 40 and 20 µg/mL for SQV and RTV, respectively, but without effects when using therapeutic concentrations of 5- 10 µg/mL. To further evaluate the impact on programmed cell death in infected Mø we used annexin-V and propidium iodide staining as markers for apoptosis and necrosis. We detected no increased toxicity on infected cells treated with concentrations of PIs ranging from 5 to 20 µg/mL, relatively to the control **(Figure 1B** lower panel; **Supplemental Figure 2)**.

2.2.2. Treatment with HIV PI saquinavir results in increased Mtb killing in primary human Mø during mono-infection and during HIV coinfection.

Once established that PI treatment of infected cells did not impact apoptosis neither necrosis in the experimental conditions used and, therefore, would not interfere with the amount of live bacteria recovered from infected Mø, we next tested the effects of SQV and RTV on Mtb intracellular killing. Our hypothesis is that SQV strongly increasing the proteolytic activity of omnicathepsins may reverse and largely compensate the induced inhibitory effect observed during Mtb infection (Pires et al., 2016). As shown in **Figure 2A** (upper and lower panel) pre-treatment with SQV in therapeutic concentrations significantly enhances the intracellular killing of Mtb during mono-infection or during HIV coinfection,

in a dose-dependent manner ($P < 0.001$). No effects were observed using therapeutic concentrations of RTV. Since cells were pre-treated before infection and the PIs were added again just once after bacteria internalization into Mø, the impact on bacteria killing was mainly observed during the first 3 days of infection with a concomitant recover of the intracellular replication afterwards. The effects on intracellular killing of Mtb were similar to those achieved using pyrazinamide (PZA) at a minimal inhibitory concentration estimated *in vitro* of 100 µg/mL (**Figure 2A** , lower panel).



C.

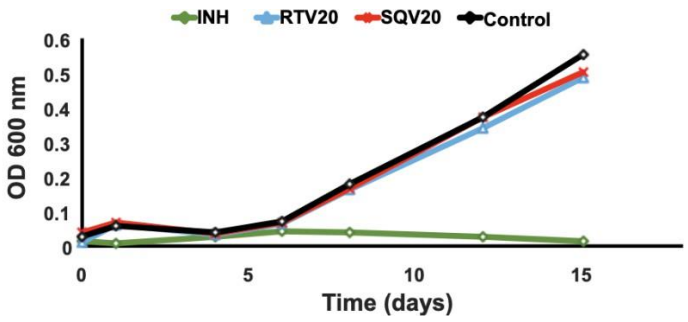


Figure 2. SQV decreases the intracellular survival of *Mycobacterium tuberculosis* (Mtb). **(A)** Intracellular survival of Mtb during mono- or coinfection with HIV along 7 days of infection. Data represents colony forming units (CFU) of intracellular bacteria recovered from Mø treated with the PI or DMSO control. Culture medium was changed on day 3 p.i. without addition of fresh PIs. Values depict mean CFU representative of three biological replicates from one representative experiment performed in triplicate. Error bars depict the standard deviation (*P*** < 0.01; ****P* < 0.001 relatively to control). **(B)** Percentage of Mø infected with Mtb and median fluorescence intensity of Mtb per Mø were measured by flow cytometry in Mø pre-treated with the PIs and after 3 h of infection with GFP-expressing Mtb. Bar-plots depict the average of three biological replicates and the error bars depict the standard error. Raw values from one representative replicate are presented in the fluorescence intensity histograms. **(C)** Mtb growth curves in broth medium treated with PIs and incubated for 15 days. Values represent the optical density measured at discrete time points from one representative experiment performed twice. Isoniazid (INH) was used as control for inhibition of growth.

To confirm that PI treatment did not impact on the ability of Mø to internalize bacteria, we assessed Mø containing GFP expressing bacteria by flow cytometry (**Figure 2B** , **Supplemental Figure 2**). The results show that approximately 50% of Mø were infected (left panel) and the infected population was loaded with similar amounts of bacilli (right panel), independent of the concentration of PIs used. Finally in order to disregard a microbicidal effect of the PIs directly in Mtb we evaluated the effect of higher concentrations of SQV and RTV than those used in *ex vivo* assays directly on bacteria replication in liquid media. Using turbidimetry assays at an OD of 600 nm, the *in vitro* growth of Mtb was similar to samples treated with 20 µg/mL either with SQV or RTV (**Figure 2C**). Isoniazid at a concentration of 0.1 µg/mL was used as control.

Altogether our results suggest that the Mtb intracellular killing effects of SQV are not attributed to a direct bactericidal effect of the drug but rather to an improved activity of omnicathepsins in the endocytic pathway. This is in accordance with our previous published results indicating that the limited non-toxic treatment with the omnicathepsin inhibitor E-64d helped Mtb survival in a 3 fold magnitude (Pires et al., 2016; Pires et al., 2017).

2.2.3. Treatment with HIV PI saquinavir results in increased surface expression of HLA class II antigen presentation machinery and $CD4^+$ T-Lymphocyte proliferation

Appropriate innate immune responses lead to destruction of pathogens during phagocytosis but also to adaptive immune responses that are crucial to control infections. SQV was demonstrated to enhance cathepsin S activity in non-infected cells (Kourjian et al.,2016) and here we show that SQV significantly enhances cathepsin S activity in infected cells. Since SQV regulates the activity of cathepsin S it may also be implicated in endosomal antigen and HLA class II processing required for appropriate antigen presentation (Pierre et al., 1998; Lautwein et al.,2002). Previously we hypothesized that the noticeable Mtb-induced decrease in cathepsin S expression during infection might be linked to poor antigen processing and presentation, compromising the adaptive immunity response to infection (Pires et al., 2017). Here we further analyzed if SQV or RTV interfere with HLA class II antigen presentation machinery during infection, thus helping to improve the adaptive immune responses.

For this, we first evaluated the effects of PIs during Mtb infection or during HIV coinfection and analyzed changes in the surface expression of HLA class II molecules compared to non PI-treated infected cells. For all concentrations tested, SQV treatment led to a significant increase of HLA class II expression at the cell surface as measured by flow cytometry (**Figure 3A** upper panel, **Supplemental Figure 3).** RTV used at the maximum therapeutic concentration found in plasma of treated patients achieved after a single dose administration (5 µg/mL), did not show any changes relatively to the control. In contrast, the lowest concentration of SQV (5 µg/mL) induced a significant increase of HLA class II presentation of endogenous antigens in non-infected cells, as observed in **Figure 3B** .

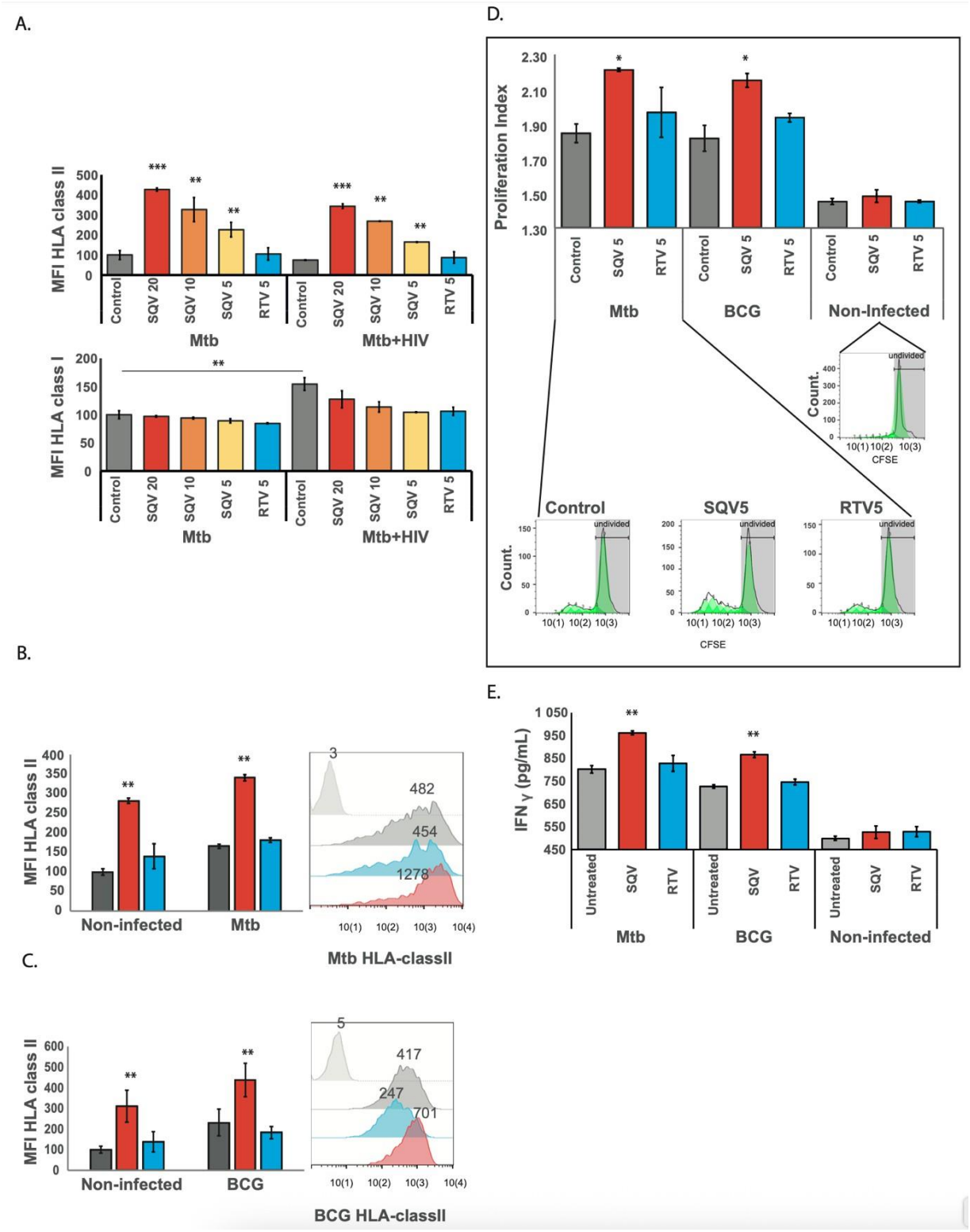


Figure 3. SQV results in increased expression of HLA class II antigen presentation machinery (A) Surface expression of human leukocyte antigen (HLA)-class II or class I on Mø infected with Mtb or co-infected with HIV. (B) Surface expression of human leukocyte antigen (HLA)-class II on Mø infected with Mtb or (C) BCG

compared to non-infected Mø. HLAs were measured by flow cytometry after 24 h of infection. Values in bar plots represent the average of median fluorescence intensity measured on three biological replicates from one representative experiment performed in triplicate relative to control. Error bars depict standard deviation (**P < 0.001, relatively to control). Raw values from one representative replicate are presented in the fluorescence intensity histograms. (D) $CD4^+$ T-cell proliferation after 5 days of coculture with Mtb or BCG-infected Mø. Following 24 h of the infection, CFSE stained $CD4^+$ T-cells were added to the infected Mø culture. After 5 days of coculture, $CD4^+$ T-cells CFSE fluorescence was measured by flow cytometry. Values in bar plots represent the proliferation index (average number of divisions per cell) of $CD4^+$ T-cell (*P < 0.01, relatively to control). Histograms from one representative replicate of the different treatments infected with Mtb are presented in the bottom. The green areas represent the $CD4^+$ T-cell populations after each division as modeled by the software. (E) IFN-γ was quantified in the supernatant after 5 days of cocultures of Mø with $CD4^+$T-lymphocytes by ELISA. Values depict mean concentration of three biological replicates from one representative experiment performed in duplicate. Error bars depict the standard deviation (**P < 0.01; ***P < 0.001, relatively to control).

Cathepsin S was demonstrated to be implicated in partial antigen processing for cross-presentation to $CD8^+$ T-lymphocytes (Shen et al., 2004) but without affecting the levels of HLA class I expression at the cell membrane (Kourjian et al., 2014; Wang et al., 2013). Therefore, as control we tested the expression of HLA class I at the cell surface by flow cytometry. The results indicate no changes in HLA class I expression in any treated samples when compared to control, during Mtb mono-infection. However, during coinfection with HIV, we observed a significant increase in the expression of HLA class I in control cells, relatively to control cells mono-infected with Mtb. This result confirms the overall induced effect of cytosolic viral peptides in increasing the expression level of HLA class I molecules (Figure 3A lower panel). The results also indicate that the treatment with SQV and RTV induced no differences on the expression of the antigen presentation machinery.

Since BCG has been used as TB vaccine for more than one century, and since it has been losing the efficacy to protect from infection, we next tested the effect of PIs in improving the HLA class II expression at the cell surface, required to improve antigen presentation. As observed in Figure 3C for BCG infected cells we noticed a highly significant (P < 0.01) increase in antigen presentation levels.
To further evaluate the consequences of PIs treatment on antigen presentation we performed cocultures of treated and infected Mø with autologous $CD4^+$ T-lymphocytes obtained from the same healthy PPD^+ donors and evaluated their ability to induce T-cell proliferation (Figure 3D). Following the same pattern of HLA surface expression, treatment with SQV in Mtb or BCG-infected cells induced a significant T-cell proliferation relatively to the control, after 5 days post-cocultures as evaluated by flow cytometry (Figure 3D). No changes were observed in cells treated with RTV.

We inferred that the induced T-cell proliferation would be concomitant with enhanced IFN-γ secretion and lately to indirect Mø activation and again the potentiation of the bactericidal effect. Therefore, we performed quantification of IFN-γ secretion from non-infected cells and compared to infected ones treated or not with SQV. We observed in agreement with T-cell proliferation increased secretion of IFN-γ in cocultures supernatants of Mtb or BCG infection exacerbated in conditions treated with SQV (Figure 3E); no significant alterations in IFN-γ secretion were detected in non-infected cocultures.

2.2.4. A negatively charged lipid composition results in high load of SQV incorporation in liposomes

As was mentioned before, HIV protease inhibitors, specifically SQV, were previously shown to impact human lysosomal proteases (Pires et al., 2021; Kourjian et al., 2016). To increase the effectiveness of SQV and to provide evidence of the crucial role of cathepsin manipulation for the success of tuberculosis bacilli in their intracellular survival, we decided to encapsulate SQV in a liposomal delivery system. The rationale for this approach is based on the fact that phagocytic cells efficiently internalize particles with sizes >0.1 μm, as is the case of liposomes, thus allowing loaded compounds delivery to infected cells at a high extent. In fact, the incorporation of antibiotics in negatively charged nanoformulations (Ahsan et al., 2002) has been effectively used both in tuberculous (Gaspar et al., 2008) and non-tuberculous infections (Gaspar et al., 2015). Consequently, we decided to evaluate the loading of SQV in different negatively charged or neutral lipid compositions. We tested three formulations: dimiristoyl phosphatidyl choline (DMPC) and dimiristoyl phosphatidyl glycerol (molar ratio 8:2); the lipid composition selected in the present work, composed of dioleoyl phosphatidyl choline (DOPC) (both negatively charged liposomal formulations) and dioleoyl phosphatidyl glycerol (DOPG); and dioleoyl phosphatidyl choline (DOPC) and dioleoyl phosphatidyl ethanolamine (DOPE) (molar ratio 8:2) (a neutral charged liposomal formulation). In all tested lipid compositions, the main choline phospholipid was always used at 80 mol%, namely DMPC, DOPC.

The preliminary results allowed us to conclude that a negatively charged liposomal formulation was better, particularly the one constituted by phospholipids with lower phase transition temperature (Tc), as in the case of DOPC (Tc = −17 °C), when compared to DMPC (Tc = +23 °C).

Table 1 shows the formulation composed of DOPC: DOPG that we selected because it displayed high SQV incorporation and because preliminary results indicated a high SQV internalization in macrophages. Indeed, SQV liposomes with a mean size ≈ 100 nm are highly homogenous, as demonstrated by the low polydispersion index (P.I.), presented E.E. superior to 90%.

Table 1.

Physicochemical characterization of liposomes unloaded or incorporating saquinavir.

Formulation	Lipid Composition (Molar Ratio)	(SQV /Lip)i (µg/µmol)	(SQV /Lip)f (µg/µmol)	E.E. (%)	Ø (nm) (P.I.)	ζ Pot (mV)
Loaded liposomes	DOPC: DOPG (8:2)	60.9 ± 2.7	53.4 ± 4.9	90.3 ± 6.7	116 ± 1 (<0.1)	−23 ± 2
Unloaded liposomes	DOPC: DOPG (8:2)	na	na	na	113 ± 1 (<0.1)	−23 ± 1
Liposomes labelled with rhodamine Rho-PE (0.1 mol%)						
Loaded liposomes	DOPC: DOPG (8:2)	42.7 ± 0.4	47.7 ± 0.7	109.1 ± 0.8	114 ± 1 (<0.1)	−24 ± 2
Unloaded liposomes	DOPC: DOPG (8:2)	na	na	na	111 ± 1 (<0.1)	−25 ± 2

DOPC—dioleoyl phosphatidyl choline; DOPG—dioleoyl phosphatidyl glycerol; SQV—saquinavir; lip—lipid; (SQV/Lip)i—initial saquinavir to lipid ratio; (SQV/Lip)f—final saquinavir to lipid ratio; E.E. (%) = (SQV/Lip)f /(SQV/Lip)i × 100; Ø—mean size; P.I.—polydispersity index; ζ Pot—zeta potential; na—not applicable.

2.2.5. SQV-loaded liposomes are effectively internalized by Mtb-infected macrophages

To test the efficacy of liposomal delivery of SQV to host macrophages, we analyzed the capacity of Mtb-infected cells to incorporate rhodamine-labelled SQV liposomes (Table 1). Macrophages infected with Mtb H37Rv were treated for 3 h with selected SQV liposomes (LipSQV) and then analyzed by flow cytometry and fluorescence microscopy. The SQV concentrations tested (ranging from 10 to 50 µg/mL) were selected based on prior studies by us and others (Pires et al., 2021; Kourjian et al., 2014; Pajonk et al., 2002) on the pharmacokinetics of the drug (Kourjian et al., 2016) and on the objective to increase the upper limit concentration tolerated without cytotoxic effects.

The results show that for all concentrations assayed, almost 100% of the population of macrophages incorporated LipSQV (Figure 4). Variations between treatments were mostly observable by flow cytometry analysis of the median fluorescence intensity (Figure 4a), indicating that on average LipSQV50 treatment resulted in macrophages with twice the fluorescence intensity of LipSQV20, with

the latter having 25% more intensity than LipSQV10. These results indicate that while LipSQV uptake is widespread even at lower concentrations, there is a relevant increase in the number of liposomes uptaken when higher concentrations are applied. Furthermore, fluorescence microscopy analysis (Figure 4b) shows higher liposome uptake by infected macrophages.

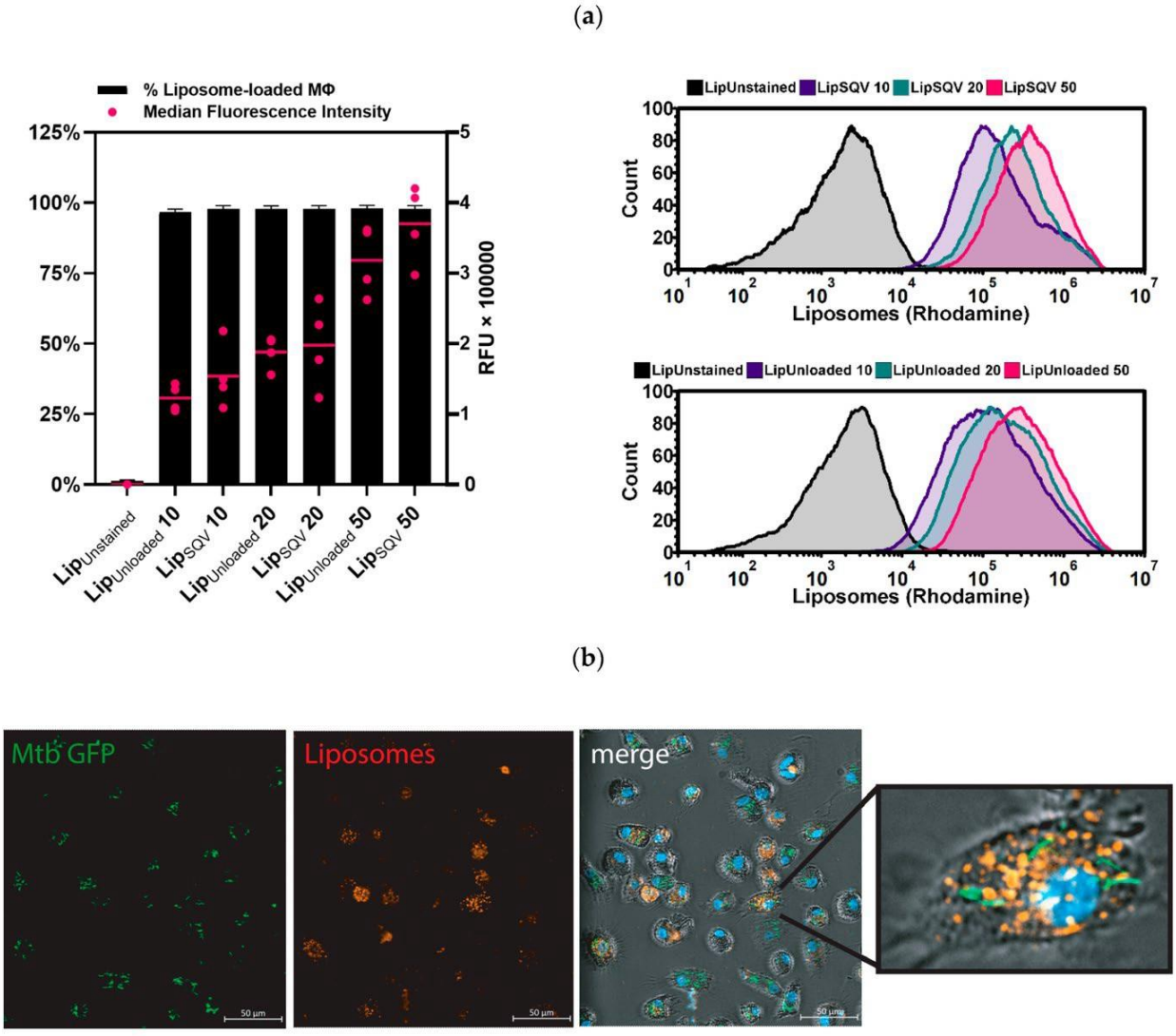


Figure 4. SQV-loaded liposomes are effectively internalized by Mtb-infected human macrophages. (a) Flow cytometry analysis of rhodamine-labeled liposomes incorporated by macrophages infected with Mtb H37Rv after 3 h of treatment. The bars represent the mean percentage of macrophages positive for liposomes (left *y*-axis). The dots represent the median fluorescence intensity per macrophage for each donor (right *y*-axis) and the horizontal lines represent the mean of all donors. The histogram displays one representative experiment. Error bars depict the standard error of the mean calculated from 4 independent experiments. RFU—relative fluorescence units. (b) Fluorescence microscopy analysis of the same conditions analyzed in (a). Mtb H37Rv is depicted in green and SQV-loaded liposomes are depicted in red; the nucleus is contrasted in blue

2.2.6. SQV-loaded liposomes present no cytotoxicity at therapeutic concentrations

SQV therapeutic application is highly influenced by its cytotoxicity in moderate concentrations (Pires et al., 2021; Pereira et al., 2022), difficulty in maintaining therapeutic levels in plasma (Van Heeswijk et al., 2000), and the known negative interactions with the current anti-TB drug rifampicin (McIlleron et al., 2007). Having established that the LipSQV formulations are effectively uptaken by infected macrophages, we assessed the cytotoxic effects of SQV using this nanoformulation. For this, we first analyzed non-infected macrophages treated with the selected concentrations of LipSQV and compared the results with cells treated with free SQV for 3 days (Figure 5a). This longer period of incubation served to confidently determine the effects of SQV exposure otherwise under-evaluated using a 3 h short treatment applied systematically to cytotoxicity assays. By using resazurin assays, the results show no cytotoxicity in all conditions tested, apart from an observed strong decreased viability of the cells during treatment with free SQV at 50 µg/mL. Remarkably, the treatment of cells using liposomal-loaded SQV at the same concentration of the drug resulted in no effect on macrophage viability.

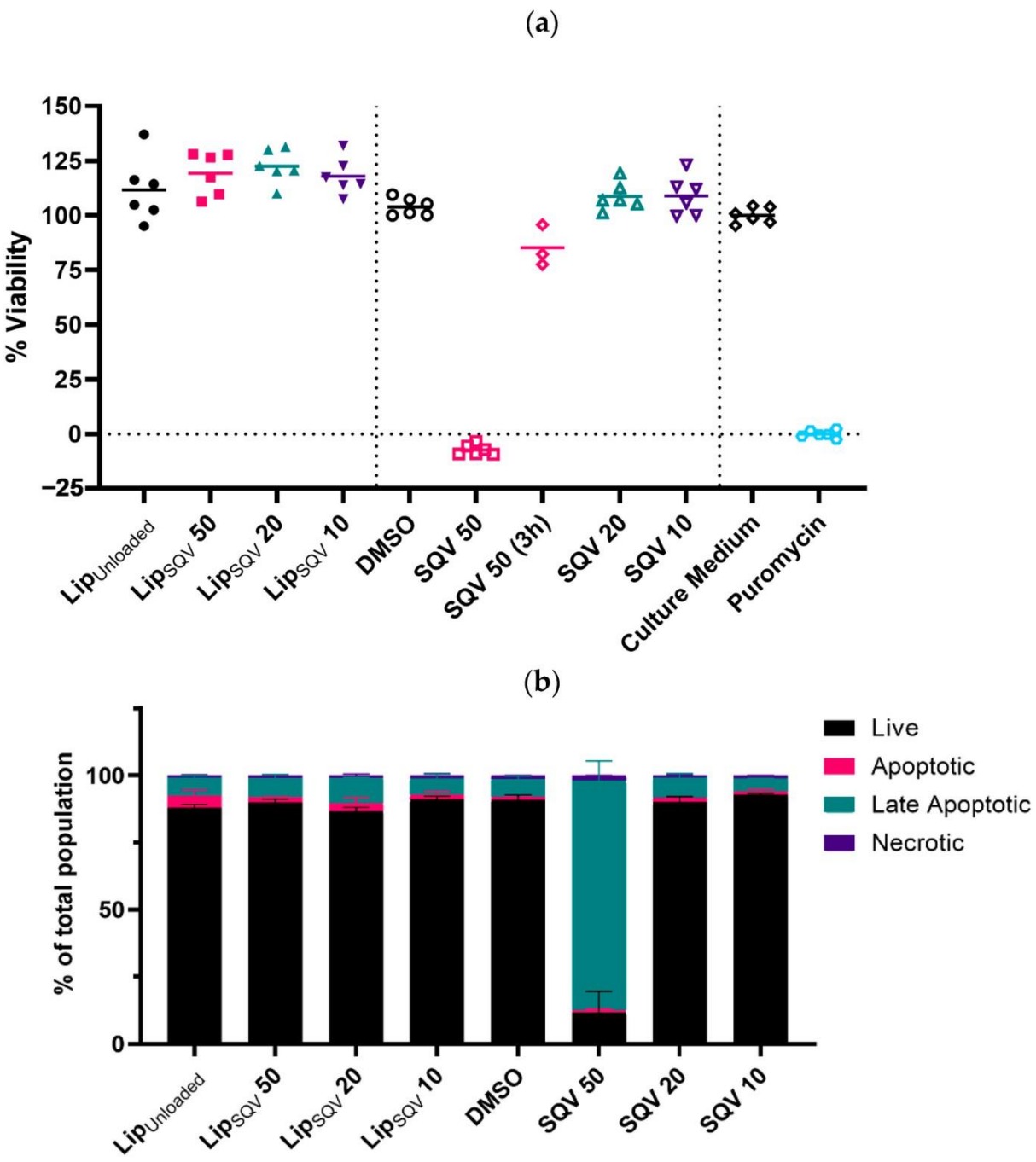


Figure 5. SQV-loaded liposomes present no cytotoxicity for human macrophages after 3 days of treatment. (a) Macrophages were treated with the selected concentrations of LipSQV or free SQV and incubated with the drugs for 3 days. Additionally, free SQV was tested at 50 µg/mL for only 3 h. Macrophage viability was measured using PrestoBlue (resazurin-based solution) by quantifying the fluorescence emission in a plate reader. LipUnloaded or DMSO were used as the negative controls and puromycin (5 µg/mL) as the positive control for cell death. The symbols depict the relative viability of cells from different donors compared to cells treated with culture medium alone. (b) Cell death was measured by flow cytometry analysis of Mtb-infected macrophages following 3 days of treatment, using Apotracker Green and Zombie Red viability dyes. Bars represent the average of two independent experiments and the error bars depict the standard error of the mean.

These results were confirmed by flow cytometry in Mtb-infected cells by quantifying the impact of the treatments on programmed cell death following 3 days of incubation (Figure 5b). Apotracker Green and Zombie Red were used as markers for apoptosis and necrosis, respectively. These dyes allow the fixation of the samples following staining and thus permit flow cytometry cell death analysis outside the BSL-3 laboratory. The results show no impact on cell death during all tested conditions, with the exception of free SQV at 50 µg/mL (Figure 5a,b).

To further establish the conditions for the following experiments, we decided to test whether a 3 h treatment using free SQV at 50 µg/mL could induce similar cytotoxicity. Following this treatment, cells were washed and incubated until day 3 without additional contact with the free drug. As shown in Figure 5a, the results indicate an average viability of 85%. We thus decided to establish a 3 h incubation for the following experiments when comparing the treatment of LipSQV with the free drug.

2.2.7. SQV-loaded liposomes significantly improve the free-SQV ability to increase cathepsin activity

Following the determination of the experimental conditions that produced a minimal effect on cell viability, we assessed the impact of the treatments in the proteolytic activity of cathepsins. General cathepsin activity was measured for 1.5 h using a fluorogenic peptide substrate specific for several cathepsins, including cathepsins B, L, and S. The inhibitor of most cathepsins, E-64d, was used as a negative control (Hook et al., 2015). For these experiments, we performed a 3 h treatment with the drugs, then washed the cells and incubated them for a further 24 h without additional treatments. Since we and others have already demonstrated SQV impact on human cathepsin activity (Pires et al., 2021; Bettencourt et al., 2016), we now sought to verify whether similar activity could be maintained 24 h after SQV or LipSQV removal from the culture medium. Indeed, the results show a marked increase in cathepsin proteolytic activity (Figure 6) in non-infected macrophages treated with SQV in liposomal form for 3 h, at 50, 20, and 10 µg/mL, in contrast to all conditions assayed with the free drug.

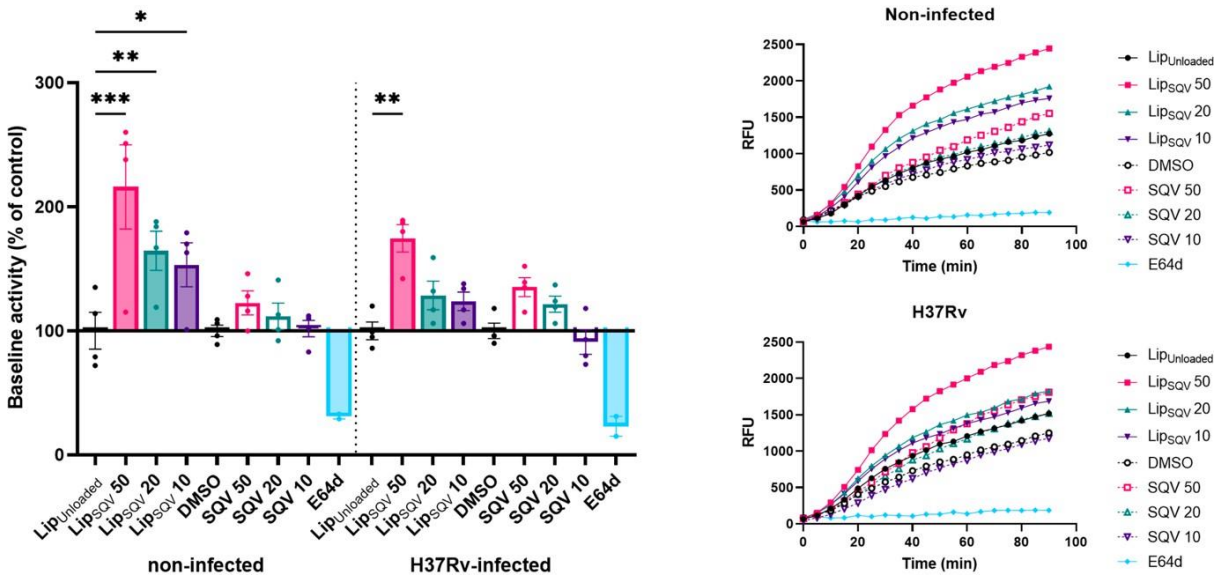


Figure 6. SQV-loaded liposomes increase the proteolytic activity of cathepsins in Mtb-infected human macrophages. Mtb-infected macrophages were treated for 3 h, then washed and incubated without drugs for an additional 24 h. Following that period, the general activity of cathepsins was monitored using a fluorogenic substrate and measured every 5 min for 90 min. General inhibitor of cathepsins, E-64d, was used as a negative control. Bars represent average baseline activity of four independent experiments. The symbols represent each donor tested and the error bars depict the standard error of the mean. Baseline activity was calculated as the maximum slope of fluorescence emission over 1 h. LipUnloaded and DMSO were used as references for LipSQV and free SQV treatments, respectively. Line plots display average fluorescence per time. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. RFU—relative fluorescence units.

When using Mtb-infected macrophages, only for the highest concentration (the LipSQV at 50 µg/mL), a statistically significant impact on cathepsin proteolytic activity was observed, confirming the strong inhibition of cathepsin activity induced by the pathogen. These results solidify the additional benefit of liposomal encapsulation of SQV in prolonging the effects on proteolytic activity in non-cytotoxic conditions and the impact during Mtb infection.

2.2.8. SQV-loaded liposomes improve the intracellular killing of Mtb reference laboratory and clinical strains with different drug resistance profiles

Saquinavir was already demonstrated to improve the intracellular killing activity of Mtb by human macrophages as a consequence of the increased proteolytic activity (Pires et al., 2021). In those studies, and in order to achieve the greatest effects, macrophages were incubated with SQV prior to

infection using 20 μg/mL, the highest concentration that was shown to provide no cytotoxic effects. Here, our hypothesis is that, by using liposomes as a vehicle, those results may be achieved without the requirement of a pre-incubation. Indeed, we do expect that by reducing the cytotoxic effects of SQV we can use higher concentrations previously unattainable. We therefore assessed the Mtb intracellular killing by macrophages using the conditions established in the previous sections and measured the colony-forming units (CFU) of bacteria recovered from infected cells over a period of 7 days (Figure 7). To account for strain-specific effects, and to test the effectiveness of the treatments in strains that accumulated mutations conferring drug resistance, we compared the effects of treatments using the H37Rv reference strain with clinical strains isolated from patients with tuberculosis. All were typified by the national reference institute for health (INSA) concerning antibiotic susceptibility. Here, we selected strains that were drug-susceptible, multidrug resistant (MDR), and extensively resistant (XDR) to antibiotics. The results indicate that LipSQV was able to significantly impact the macrophages' killing ability to all strains in a dose-dependent manner, as shown in Figure 7. However, no effects on Mtb killing were observed when using the free drug, except for the XDR strain for the SQV highest concentration of 50 μg/mL.

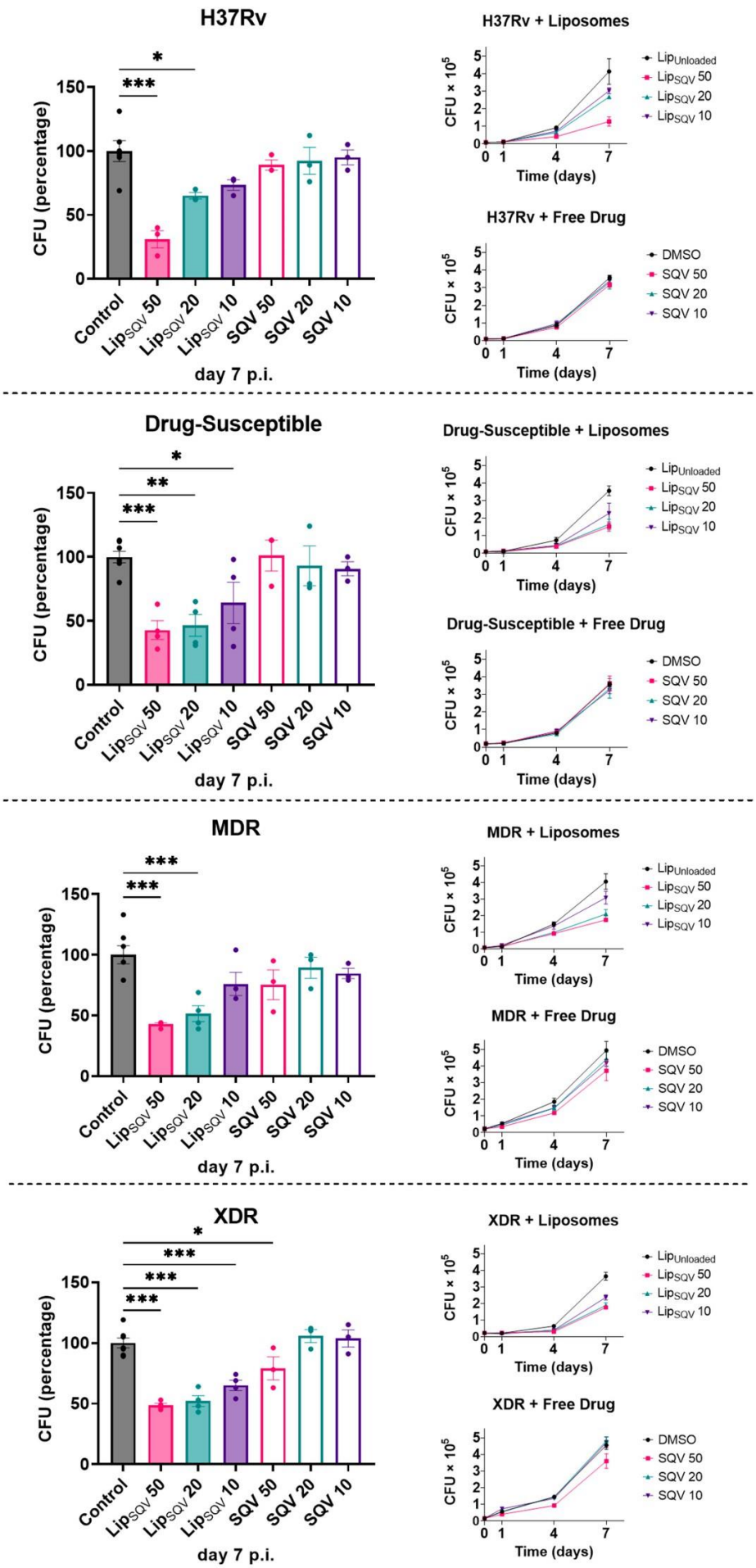

H37Rv
CFU (percentage)
day 7 p.i.
Control
Lip SQV 50
Lip SQV 20
Lip SQV 10
SQV 50
SQV 20
SQV 10
H37Rv + Liposomes
CFU × 10^5
Time (days)
Lip Unloaded
Lip SQV 50
Lip SQV 20
Lip SQV 10
H37Rv + Free Drug
DMSO
SQV 50
SQV 20
SQV 10
Drug-Susceptible
Drug-Susceptible + Liposomes
Drug-Susceptible + Free Drug
MDR
MDR + Liposomes
MDR + Free Drug
XDR
XDR + Liposomes
XDR + Free Drug

Figure 7. SQV-loaded liposomes improve the intracellular killing of Mtb laboratorial and clinical strains with different drug resistance phenotypes in human macrophages. Macrophages were infected with different Mtb strains for 3 h and then treated with selected concentrations of LipSQV and free SQV. LipUnloaded and DMSO were used as controls. To evaluate bacterial intracellular survival, at discrete time-points, macrophages were lysed, and serial dilutions of the bacterial suspension were plated on 7H10 agar plates. Colony-forming units were counted following 2–3 weeks. Lines depict average CFU per sample from at least 3 independent experiments. Bars represent average CFU relative to the respective controls at day 7 post-infection. Symbols represent each experiment with macrophages from a different donor. Error bars represent the standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

2.3. Materials and Methods

2.3.1. Cells and culture conditions

Human monocyte-derived Mø were obtained by isolating $CD14^+$ monocytes from buffy coats of healthy blood donors provided by the national blood institute (Instituto Português do Sangue e da Transplantação, Lisbon, Portugal) following a protocol established between Dr. Anes (Faculty of Pharmacy, University of Lisbon) and the Portuguese Institute for Blood, allowing access to buffy coats from healthy blood donors, for scientific research and academic purposes. The supplier provided no personal details from the donors. The cells were isolated using the MACS cell separation system (Miltenyi Biotec). Briefly, the mononuclear cell fraction was isolated using Ficoll-Paque PLUS (GE Healthcare) density gradient medium. This fraction was incubated with anti-CD14 magnetic beads and then passed across the MACS magnetic columns for positive monocyte selection. To induce differentiation to Mø, the isolated monocytes were allowed to adhere to 48- or 96-well plates at 1.5×10^5 or 5×10^4 cells per well, respectively for 2 h at 37°C, 5% CO_2, in RPMI-1640 medium (HyClone, GE Healthcare). Following adherence, the medium was supplemented to achieve a final concentration of 10% (v/v) FBS (HyClone, GE Healthcare), 1 mM sodium pyruvate (HyClone, GE Healthcare), 10 mM HEPES (HyClone, GE Healthcare), 0.1% β-mercaptoethanol (Gibco), and 20 ng/mL recombinant human M-CSF (Biolegend). Differentiation lasted for 7 days, and medium was renewed every three to four days until day 7. Purity of the isolated culture was verified by flow cytometry.

2.3.2. Bacterial cultures and HIV isolates

Mycobacterium tuberculosis H37Rv (ATCC 27294), H37Rv GFP-expressing strain and *Mycobacterium bovis* BCG Pasteur (ATCC 35734) were grown in Middlebrook's 7H9 medium supplemented with 10% OADC enrichment (Difco), 0.02% glycerol and 0.05% tyloxapol at 37°C (Pires et al., 2017). The clinical

strains were provided and characterized by the TB National Reference Laboratory from the Portuguese National Institute of Health's Dr. Ricardo Jorge (INSA). The clinical strain (INSA code 33427) is susceptible to streptomycin, isoniazid, rifampicin, and pyrazinamide (PZA); the MDR strain (INSA code 34192) is resistant to all those antibiotics plus ethionamide; the XDR strain (INSA code 163761) is resistant to all the previous antibiotics plus amikacin, kanamycin, capreomycin, moxifloxacin, and ofloxacin. Preceding the infections, bacterial cultures on exponential grown phase were centrifuged and washed in phosphate-buffered saline (PBS). Bacteria were resuspended in RPMI-1640 medium (Mø culture medium) without antibiotics. In order to dismantle bacterial clumps, the bacterial suspension was treated by ultrasonic bath for 5 min. Residual clumps were removed by 1-minute centrifugation at 500 × *g*. Single-cell suspension was verified by fluorescence microscopy and quantified by optical density at 600 nm.

Primary HIV-1 isolate UCFL1032 was obtained after cocultivation of infected patient's peripheral blood mononuclear cells (PBMCs) with PHA-stimulated PBMCs from uninfected individuals. Viral stocks were established in PBMCs from low-passaged supernatants of original cultures, aliquoted and maintained at -80° C until used. Viral concentration was measured by reverse transcriptase (RT) activity using an enzyme linked immunosorbent assay (Lenti-RT kit, Caviditech, Uppsala, Sweden). HIV-$1_{UCFL1032}$ was characterized both genetically and phenotypically: it belongs to subtype B and uses CXCR4 coreceptor to enter host cells. It has the ability to enter Mø that produce low amounts of viral progeny upon infection, a phenotype similar to what is described during the course of patients Mø infection (Honeycutt et al., 2017). This isolate is part of viral library created and maintained in our laboratory since the late Eighties, where a significant amount of HIV-1 and HIV-2 were characterized (Calado et al., 2010).

All experimental procedures using live Mtb and HIV were performed in the Biosafety Level 3 laboratory at the Faculty of Pharmacy of the University of Lisbon, respecting the national and European academic containment level 3, laboratory management and biosecurity standards, based on applicable EU Directives. All procedures have been approved by the faculty's biological safety committee.

2.3.3. Treatment and infection of Mø

Prior to infection, Mø were treated for 1 h with selected concentrations of saquinavir (SQV) (Merck Life Science) or ritonavir (RTV) (Merck Life Science) previously reconstituted in DMSO. Following pretreatment, the bacterial/viral suspension was added without removing the inhibitors. Mø were infected with a MOI of 1 of bacteria and inoculated with the equivalent of 1 ng of RT of HIV-$1_{UCFL1063}$.

After 3 h of infection at 37°C, 5% CO_2, the cells were washed with PBS to remove free bacteria/virus and cultivated in fresh complete medium supplemented with SQV or RTV. The controls were treated with the same concentration of DMSO as carried during treatments.

Phagocytosis of the bacteria was evaluated by flow cytometry using *M. tuberculosis* H37Rv GFP-expressing strain and following the procedures below. Monitorization of HIV infection was performed by fluorescence microscopy. Macrophages were fixed with 4% paraformaldehyde 4% sucrose solution in PBS for 1 h and quenched by incubating with 50 mM NH_4Cl in PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 1% BSA in PBS for 30 min. Cells were stained with anti-Gag antibody 1:100 (KC57, Beckman Coulter) in PBS BSA 1% for 1 hour, washed and then incubated with Alexa Fluor 555 Goat anti-Mouse IgG secondary antibody 1:1000 (Cell Signaling Technology) for 30 minutes. Coverslips were mounted using ProLong Gold Antifade Mountant (ThermoFisher Scientific) and visualized on a Zeiss Axioskop 40 fluorescence microscope. Analysis was performed on ImageJ software **(Supplemental Figure 1)**. To further confirm that the cell culture was infected with HIV, integration of the viral DNA into host genome was confirmed using nested polymerase chain reaction (PCR) as described (Kumar et al., 2002). Briefly, a first round of PCR amplification was done using an *Alu*-specific sense primer in combination with a *gag* antisense HIV-1 specific primer; the PCR products were then subjected to a second amplification reaction targeting the HIV-1 R/U5 region of LTR, leading to an amplicon with 391 bp **(Supplemental Figure 1)**.

2.3.4. Macrophage viability

Macrophages seeded in 96-well plates were treated with SQV, RTV or DMSO for 3 days. Next, samples were washed and incubated with PrestoBlue (Invitrogen) resazurin-based solution at 37°C, 5% CO_2, according to the manufacturer's instructions. After 3 h of incubation, fluorescence emission was analyzed in a Tecan M200 spectrofluorometer. Non-treated cells were used as reference and cells treated with RTV 100 µg/mL were used as control for cell death.

2.3.5. Enzymatic activity of cathepsins

Following 24 h of treatment and infection with *M. tuberculosis* H37Rv, or coinfection with HIV, Mø in a 96-well plate were washed with PBS and incubated in PBS with omnicathepsin (Z-FR-AMC, Z-Phe-Arg-AMC) (Enzo Life Sciences) or cathepsin S (Z-VVR-AFC) (BioVision) fluorogenic substrate for 1,5 h at 37°C in a Tecan M200 spectrofluorometer. Fluorescence readings were performed every 5 min. Essay specificity was verified by treating the cell lysates with general protease inhibitor E-64d or with specific cathepsin S inhibitor, provided in the kit.

2.3.6. Bacteria intracellular survival

When required, infected cells in 96-well plates were lysed in 0.05% Igepal solution for 15 min. Serial dilutions of the resulting bacterial suspension were plated in Middlebrook 7H10 with 10% OADC (Difco) and incubated for 2-3 weeks at 37 °C before colonies were observable and counted under the microscope.

2.3.7. Bacteria growth curves in broth medium

M. tuberculosis H37Rv in single-cell suspension were incubated in bacteria culture medium with selected concentrations of SQV, RTV and DMSO at 37°C, 5% CO_2 for 15 days. The optical density at 600 nm was measured at discrete time points. Isoniazid (INH) was used as control for inhibition of growth.

2.3.8. Flow cytometry

Following 24 h of treatment and infection with *M. tuberculosis* H37Rv, Mø in 48-well plates were recovered with HyQTase cell detachment solution (HyClone, GE Healthcare). For the identification of apoptotic and necrotic cells Annexin V Apoptosis Detection Kit with PI (Cat # 640914, Biolegend) was used following the manufacturer's instructions. Cells were incubated with annexin V and propidium iodide for 20 minutes, washed with the appropriate kit buffer and fixed in 4% paraformaldehyde for 1 h. Following fixation, cells were washed again in buffer and analyzed. For surface staining of HLA molecules, detached cells were promptly fixated for 1 h. Following fixation, cells were washed and incubated with Human TruStain FcX Fc receptor blocking solution (Biolegend) for 10 minutes and then stained for 20 min with antibodies specific for human HLA class I (Cat # 311422, Biolegend) and HLA class II (Cat # 361716, Biolegend) molecules. Samples were analyzed in Guava easyCyte™ 5HT flow cytometer.

2.3.9. $CD4^+$ T-Lymphocytes proliferation

Autologous $CD4^+$ T-lymphocytes were obtained from the same healthy PPD^+ donors according to the isolation protocol described above. Positive selection of the $CD4^+$ lymphocytes was performed using anti-CD4 magnetic beads (Miltenyi Biotec). Isolated lymphocytes were cultivated in 75 cm^2 flask at 2 × 10^6 cells per mL in RPMI-1640 medium (HyClone, GE Healthcare) supplemented with 15% (v/v) FBS (HyClone, GE Heaclthcare), 1 mM sodium pyruvate (HyClone, GE Heaclthcare), 10 mM HEPES (HyClone,

GE Healthcare) and 20 UI/ml of human recombinant Interleukin 2 (Biolegend) for 3 days prior to the experiment. Immediately before the experiment the lymphocytes were stained with Carboxyfluorescein diacetate succinimidyl ester (Cat # 423801, Biolegend) following the manufacturer's instructions. Macrophages infected with *M. tuberculosis* H37Rv or *M. bovis* BCG and treated for 24 h were washed and cocultivated with the lymphocytes at a ratio of 5 lymphocytes per macrophage for 5 days. $CD4^+$ lymphocytes were recovered after 5 days of coculture and analyzed using Guava easyCyte™ 5HT flow cytometer.

2.3.10. IFN-γ quantification

Supernatants from the previous assays were recovered following 24 h of infection and treatment and following an additional 5 days of coculture with $CD4^+$ lymphocytes and stored at -80 °C for posterior analysis of interferon-γ (IFN-γ) secretion. The quantification was performed by Sandwich Enzyme-Linked Immunosorbent Assay using ELISA Max Deluxe Set Human for IFN-γ (Cat # 430104, Biolegend) kits and following the manufacturer's instructions. Absorbance was measured by Tecan M200 spectrofluorometer at 570 nm.

2.3.11. Preparation and physicochemical characterization of saquinavir liposomes

The encapsulation of saquinavir (SQV) in liposomes was performed by an active loading method with an ammonium sulphate gradient as previously described (Gaspar et al., 2012; Aguiar et al., 2021). Briefly, the relevant phospholipids, dioleoyl phosphatidyl choline (DOPC), and dioleoyl phosphatidyl glycerol (DOPG), purchased from Avanti Polar Lipids, at a molar ratio of 8:2 and a lipid concentration of 30 µmol/mL, were dissolved in chloroform, and the organic solvent was removed by rotary evaporation. The formed homogeneous lipid film was hydrated with water and the so-formed suspension was frozen (−70 °C) and lyophilized in a freeze-dryer (Edwards, CO, USA) overnight. The rehydration of the lyophilized powder was performed with ammonium sulphate and trehalose (60 mM and 5%, respectively), pH 5.4 at room temperature for 30 min. To produce a homogeneous liposomal suspension, unloaded liposomes were filtered under nitrogen pressure (10–500 lb/in2) through polycarbonate membranes of proper pore size using a Lipex thermo-barrel extruder (Lipex: Biomembranes Inc., Vancouver, BC, Canada) until liposomes with a mean size of around 0.1 µm were attained. An ammonium sulphate gradient was created by replacing the extraliposomal medium with phosphate-buffered saline (PBS) buffer (pH 8.4), the pKa for SQV, using a Econo-pac 10 DG desalting column (Bio-Rad Laboratories, Hercules, CA, USA). SQV was incubated with unloaded liposomes at a final concentration of 50 µg / µmol of lipid for 60 min at room temperature. At this stage, a sample of the suspension was taken for further quantification in terms of SQV and lipid contents corresponding

to initial conditions. The non-encapsulated SQV was separated by ultracentrifugation at 250,000× *g* for 2 h at 15 °C in a Beckman LM-80 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA). The pellet was suspended in PBS (pH 8.4), thus corresponding to the final sample.

Fluorescent liposomes were prepared using the same methodology. In this case, rhodamine covalently linked to phosphatidyl ethanolamine (Rho-PE) obtained from Avanti Polar Lipids, USA was included in the lipid composition at 0.1 mol% related to total lipid. Liposomal formulations were characterized in terms of lipid and SQV contents and by the following incorporation parameters: initial and final SQV to lipid ratios ((SQV/Lip)i and (SQV/Lip)f, respectively. The Encapsulation Efficiency (E.E.) was defined as the percentage of:

$$[(SQV/Lip)f\,]/[(SQV/Lip)i] \times 100.$$

SQV incorporated in liposomes was determined by high-performance liquid chromatography (HPLC), as described in (Mudigonda et al., 2006). The HPLC system was an ELITE LaChrom Hitachi (Tokyo, Japan), comprising an L-2130 pump module, a Diode-Array L-2455 detector, and an autosampler L-2200 with a loop of 100 µL. The wavelength of the detector was set at 240 nm. The system was connected to a computer with specific software, Ez Chrom Elite, for integration and treatment of chromatograms. The analytical column was a LiChroCART® (250-4,6) Purospher® Star RP-8 (5 µm) (Merck, KGaA, Darmstadt, Germany) equipped with the respective guard column. The mobile phase, in an isocratic solvent system, was a mixture of 10 mM ammonium acetate buffer–acetonitrile (ACN) (35:65, *v*/*v*) pumped at a flowrate of 1.0 mL/min at 30 °C.

A calibration curve of SQV standards solubilized in acetonitrile, ranging from 80 to 1200 ng/mL, was used. Liposomal SQV samples were diluted in ACN in order to be within the range of the calibration curve. The Lipid content was determined using an enzyme-linked colorimetric method, Phospholipids Choline Oxidase-Peroxidase (Spinreact, St. Esteve de Bas, Spain).

The mean sizes of the liposomes were determined by dynamic light scattering, using a Zetasizer Nano S, Malvern Instruments, Malvern, UK, at a standard laser wavelength of 663 nm. The system also reports a polydispersity index, as a measure of particle size distribution, ranging from 0.0 for an entirely monodisperse sample up to 1.0 for a polydisperse suspension. Zeta potential of liposomal formulations was measured in a hydrodynamic sizing system, using a Zetasizer Nano Z (Malvern Instruments, Malvern, UK).

2.3.12. Fluorescence microscopy

Fluorescence microscopy was performed in live cells, counter-stained with Hoechst 33342 dye (Thermo Fisher Scientific, Waltham, MA, USA), directly on the culture plates. Images were captured using a Zeiss Axio Observer microscope and analyzed in Zen 3.6 Blue Edition software (Carl Zeiss Microscopy, Jena, Germany).

2.3.13. Statistical analysis

Data are presented as mean ± standard error except if stated otherwise. Statistical analysis was performed using SigmaPlot 12. Multiple group comparisons were made using ANOVA one parameter tests followed by pairwise comparisons of the groups using Holm-Sidak test. Two group comparisons were made using Student's t-test. All the prerequisites of the tests were verified. The considered nominal alpha criterion level was 0.05 below which differences between samples were deemed significant.

2.4. Discussion

The purpose of our study was to decipher whether the first-generation HIV protease inhibitor, SQV, could be repurposed as a host-directed therapy for tuberculosis especially during coinfection with HIV. Tuberculosis, the so call white plague disease until the beginning of the twentieth-century, remains a leading cause of mortality worldwide due to an infectious agent. While in the last hundred years the vaccine BCG and the introduction of antibiotics helped to control the disease, since 1980 with the emergence of HIV this scenario has completely changed. HIV coinfection exacerbates Mtb infection helping reactivation from latency (World Health Organization, 2020; Souriant et al., 2019; Mancino et al., 1997). Moreover, due to increased drug resistance strains, coinfection impacts the transmission of MDR. Accordingly, there is an urgent need to develop new medicines to control resistant bacteria and to redirect the immune responses of the host to effectively control the infection and the inflammatory responses. Within this context repurposing approved drugs will speed the process of improve therapy for the outcome of TB.

SQV was one of the first drugs developed to control HIV infection (Baker et al., 1995). It acts as an aspartic protease inhibitor interfering with HIV protease activity and therefore prevents the cleavage of Gag-Pol protein precursors. This inhibition ultimately blocks the infectivity of nascent virions and cell to cell spreading (Titanji et al., 2013; Roberts et al.,1990; Craig et al., 1991). It is likely that for other

pathogens dependent on proteases for their life cycle, SQV and other PIs could be repurposed to control the respective infections. In fact, they showed inhibitory effects against a wide spectrum of pathogens such as *Plasmodium falciparum* (Nsanzabana et al., 2011)*, Trypanosoma cruzei* (Castilho et al.,2018), the fungi *Fonsecaea pedrosoi* (Palmeira et al., 2018) and SARS-CoV and avian influenza viruses (Savarino et al., 2005).

It is expectable that HIV PI may also interfere with the host proteases. Among host proteases with relevance during immune responses to infections are cathepsins in the endocytic pathway and threonine proteases of the proteosome. Consistently, HIV PIs were shown to alter cathepsin activity of antigen presenting cells (Kourjian et al., 2016) and to interfere with proteosome peptide processing leading to accumulation of polyubiquitinated products (Kourjian et al., 2016; Pajonk et al., 2002). Accordingly, HIV PIs designed to inhibit the HIV aspartyl protease were described to alter the activity of aspartyl proteases like cathepsin D and E, as well as cysteine proteases, such as cathepsin S (Kourjian et al., 2016).

Our previous results indicated the ability of Mtb to down-regulate the activity of cathepsins in order to successful survive in human Mø (Pires et al., 2016). Mtb infection leads to a strong inhibition of cathepsins B, S and L (Pires et al., 2016; Pires et al., 2017) all of them involved in crucial activities during innate and adaptive immune responses. These results lead us to hypothesized that SQV, by inducing an increased activity of these cathepsins in non-infected immune cells, could be repurposed in the TB context to revert the blockade induced by the pathogen. Here, we demonstrated that SQV is able to increase omnicathepsins proteolytic activity during Mtb infection and during coinfection with HIV **(Figure 1A)**. In Mø, these endolysosomal enzymes are enrolled in pathogen killing as one of the first innate immune responses to infections. Likewise, we observed a significantly intracellular killing of Mtb in human Mø treated with SQV **(Figure 2A)**. Since either the infection with these pathogens and the treatment of host cells with SQV are inducers of apoptosis (Kourjian et al., 2016; Pajonk et al., 2002; Behar et al., 2010; Welin et al., 2011), we disregard this programmed cell death as inducer of pathogen killing by adjusting the experimental conditions **(Figure 1B)**. We may conclude that SQV induced pathogen killing was due to an increased activity of cathepsins along the endocytic pathway coincident with the same compartment of Mtb.

Cathepsin S contributes to the antigen presentation machinery by processing pathogen peptides and by generating of HLA-classII epitopes. Likewise, in infected cells treated with SQV a significant increase in HLA class II molecules were detected at the plasma membrane of infected cells leading to increased T-lymphocyte priming and proliferation **(Figures 3A, D)**. This was particularly relevant during BCG

infection indicating that SQV improves the capabilities of presenting vaccine antigens **(Figures 3C, D)**. Since the population in Portugal has been vaccinated for decades with BCG until 2017, most of the population are PPD+. Thus, we expect the blood from healthy donors to carry a significant population of memory/effector T cells that responded to the challenge of Mtb-infected macrophages (Farber et al., 2014; Nguipdop-Djomo et al., 2016).

Here, we found that SQV treatment induces higher levels of T-cell-secreted IFN-γ in a context that mimics bacteria replication during active TB **(Figure 3E).** This increased secretion of IFN-γ may have a dual effect: (1) activation of Mø to a more bactericidal state and, (2) indirectly contributing to a decreased IL-1β secretion. In fact, it was previous demonstrated that pretreatment of *M. tuberculosis*–infected Mø with IFN-γ specifically inhibited the release of IL-1β suggesting that during TB IFN-γ may suppress lung immunopathology induced by dysregulation of IL-1β (Mishra et al., 2013). These results suggest the cytokine environment might help achieve a better control of the immunopathology in the lungs, in accordance to published studies performed in murine models of Mtb mono-infection (Mishra et al., 2013). Moreover, SQV has been referred to possess anti-inflammatory effects especially in the lungs (Yu et al., 2017). This was attributed to the suppression of TLR4 signaling pathways of high-mobility group box 1 (HMGB1). The beneficial effects were linked to decreased levels of circulating and lung tissue inflammatory cytokines, such as IL-6, IL-1β, TNF-*α*, and iNOS.

Cathepsins S and L have been demonstrated to regulate autophagy (Chen et al., 2012). Mtb and HIV are known to inhibit autophagy: upon infection of Mø in the lungs, inhibition of the autophagic pathway by the first invader will likely benefit the second or induce a similar behavior in neighboring cells (Espert et al., 2015). It could be that an SQV-induced increase of the proteolytic activities of cathepsins S and L would improve autophagy. This would in turn help infected cells to eliminate not only of the pathogens but also of cytosolic aggregates and inflammatory signaling molecules, contributing to decreased tissue inflammation (Lim et al., 2019). Altogether our data and relevant literature indicates SQV as a potential host directed therapy for Tuberculosis.

One caveat of the study was the requirement to pre-treat macrophages with SQV to achieve the greatest effects and maintain the treatment throughout the infection. This would not be feasible to translate to the clinical setting. Moreover, constant exposure of macrophages to moderate doses of SQV as low as 20 μg/mL often induces cytotoxic effects.

In the present work, these limitations were overcome using SQV loaded in liposomes. Our results indicate a high internalization of the HIV protease inhibitor in macrophages while reducing its cytotoxicity at higher concentrations and effectively impacting the proteolytic activity of cathepsins

relative to free drug treatment. Moreover, we could achieve similar benefits of continuous treatment, restricting the exposure to the drug to only 3 h. This proved to be beneficial for the host macrophage microbicidal effects, enhancing Mtb intracellular proteolytic digestion.

Another potential limitation of the usage of SQV for tuberculosis treatment is the known interaction with rifampicin, resulting in a marked reduction in patients' serum levels (Centers for Disease Control and Prevention Updated Guidelines, 2004; Rae et al., 2001). Liposome-based delivery of SQV might constitute a solution to overcome these challenges by shielding SQV from rifampicin-induced drug metabolism. In fact, we observed the absence of cytotoxic effects when using liposomes to deliver concentrations of SQV much higher than those safely achieved in the serum of patients treated with the free drug. In comparison, the same concentrations of free SQV resulted in 85% apoptosis after 3 days of treatment. Notably in this context, tuberculosis is responsible for almost 30% of all deaths of HIV-infected patients (World Health Organization, 2022), and any improvement to the current ability to treat both infections with minimal adverse effects could have a significant impact on these individuals. The results indeed demonstrated the efficacy of SQV-loaded liposomes in contributing to control infections by Mtb clinical strains susceptible or resistant to the current antibiotic therapy. However, a more extensive liposomal characterization is needed to clarify the real drug concentration released into macrophages, allowing a possible application of liposomes in a clinical setting.

Data Availability Statement

The original contributions presented in the study are included in the article/ Supplementary Material .

2.5. References

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CHAPTER 3.

Modulation of Cystatin C in Human Macrophages Improves Anti-Mycobacterial Immune Responses to *Mycobacterium tuberculosis* Infection and Coinfection With HIV

This chapter contains data published in:

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I contributed to this work in experimental part by performing most of the experiments related with infection and verifying the accuracy and reproducibility of the experimental results and data to ensure the reliability of the research.

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Abstract

Tuberculosis owes its resurgence as a major global health threat mostly to the emergence of drug resistance and coinfection with HIV. The synergy between HIV and *Mycobacterium tuberculosis* (Mtb) modifies the host immune environment to enhance both viral and bacterial replication and spread. In the lung immune context, both pathogens infect macrophages, establishing favorable intracellular niches. Both manipulate the endocytic pathway in order to avoid destruction. Relevant players of the endocytic pathway to control pathogens include endolysosomal proteases, cathepsins, and their natural inhibitors, cystatins. Here, a mapping of the human macrophage transcriptome for type I and II cystatins during Mtb, HIV, or Mtb-HIV infection displayed different profiles of gene expression, revealing cystatin C as a potential target to control mycobacterial infection as well as HIV coinfection. We found that cystatin C silencing in macrophages significantly improves the intracellular killing of Mtb, which was concomitant with an increased general proteolytic activity of cathepsins. In addition, downmodulation of cystatin C led to an improved expression of the human leukocyte antigen (HLA) class II in macrophages and an increased $CD4^+$ T-lymphocyte proliferation along with enhanced IFN-γ secretion.

Available *in vitro* transfection methods are not suitable for the clinical translation of host-cell RNA silencing. To overcome this limitation, we developed different RNA delivery systems (DSs) that target human macrophages. In this work, a new potential nanomedicine based on chitosan (CS-DS) was efficiently developed to carry a siRNA-targeting cystatin C to the infected macrophage models. Consequently, an effective impact on the intracellular survival/replication of TB bacilli, including drug-resistant clinical strains, was observed. Altogether, these results suggest the potential use of CS-DS in adjunctive therapy for TB in combination or not with antibiotics. Overall, our results suggest that the targeting of cystatin C in human macrophages represents a promising approach to improve the control of mycobacterial infections including multidrug-resistant (MDR) TB.

3.1. Introduction

Tuberculosis (TB) is a transmittable disease caused by *Mycobacterium tuberculosis* (Mtb), a pathogen that latently infects about a quarter of the world's population. From the latently infected group, about 600,000 people are estimated to be carriers of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Mtb strains (World Health Organization, 2020). Coinfection with HIV is highly prevalent and constitutes simultaneously a major risk factor for TB activation from latency and an enormous public health threat by contributing to the spread of MDR–XDR strains (World Health Organization, 2020; Patel et al., 2009). Therefore, it is urgent to develop new therapeutic strategies to control the infection and overcome drug resistance.

Macrophages (Mø) are important immune cells in the pathobiology of TB. These cells play a dual role as the principal niche for Mtb persistence and as the main effector cell against the bacilli. Despite the fact that $CD4^+$ T cells are the main target for HIV, Mø also constitute relevant viral reservoirs in the context of the lung environment (Aquaro et al., 2002), particularly during coinfection with Mtb (Toossi et al., 2001). Both pathogens alter the Mø microbicidal functions converting these phagocytes into cellular reservoirs (Bell et al., 2018), and they modify the lung immune environment to one more favorable to pathogen replication. In fact, alveolar Mø can be simultaneously infected with HIV and Mtb as demonstrated in coinfected patients (Mwandumba et al., 2004). In addition, the long-term survival of infected lung Mø turns these reservoirs into a serious challenge for pathogen eradication. For instant, Mø were shown to continue producing HIV in the lung despite antiretroviral therapy, a situation that might be exacerbated in the context of TB-associated microenvironments (Souriant et al., 2019; Mancino et al., 1997).

Phagocytosis of Mtb by Mø in the lungs is an opportunity for destruction of the bacteria by phagosome fusion with lysosome, exposing the pathogen to lysosomal hydrolases. However, Mtb manipulates these events leading to its survival within vesicles of the endocytic/lysosomal pathway (Russell et al., 2007). Cathepsins are important acidic endolysosomal proteases involved at different levels during the processes of the innate and adaptive immune responses. In the endocytic pathway, they are major players in direct pathogen killing, processing of human leukocyte antigen (HLA) class II molecules, antigen processing and presentation, proinflammatory signalling molecular turnover, and secretion of proinflammatory cytokines (Ha et al., 2008; Hsing et al., 2005; Turk et al., 2012; Orlowski et al., 2015; Pires et al., 2016; Pires et al., 2017). While these cysteine proteases are optimally active in the acidic endolysosomal environment, they remain active in more neutral pH compartments (Claus et al., 1998;

Jordao et al., 2008). They also operate i) in the cytosol, regulating apoptosis, pyroptosis, and inflammasome activation (Bewley et al., 2011; Willingham et al., 2007; Amaral et al., 2018); ii) in the nucleus controlling gene expression (Ferrer-Mayorga et al., 2015); and iii) in the extracellular environment where they control extracellular matrix remodelling (Vidak et al., 2019). Extracellular secreted cathepsins were found relevant for HIV transmission through genital mucosae (cathepsin D) (Messaoudi et al., 1999) and for granuloma cavitation and lung parenchyma destruction during active TB (cathepsins K, G, and D) (Kubler et al., 2016; Walter et al.,2015; Rojas-Espinosa et al., 1974).

Not surprisingly, the abnormal activity of these proteases can lead to serious dysfunction and pathology and thus needs to be tightly controlled by endogenous protease inhibitors. Vital among endogenous protease inhibitors are cystatins (Csts), a group of evolutionarily related proteins. Under a normal physiological context, Csts control excessive cathepsin activity through trapping and blocking proteolytic activity in cells, extracellular milieu, organs, and body fluids. A slight imbalance in the equilibrium between Csts and cathepsins may result in unwanted inhibition of enzymatic activity (Turk et al., 2012). Type I Csts (also known as stefins) include CstA and B and are mainly found in the cytosol and the nucleus. In contrast, type II Csts are secreted and work as extracellular proteins, such as in the skin epithelia (Cst EM) and in saliva (Csts S, SA, SN, and D) [reviewed in (Magister et al., 2013; Ochieng et al., 2010)]. Some secreted type II Csts, such as CstC and F, can be internalized by immune cells or translocated from the secretory pathway, thus accumulating in endosomal/lysosomal vesicles (Lautwein et al., 2002; Colbert et al., 2011). Type III Csts family members include kininogens circulating in the blood as precursors of the vasoactive peptide kinin [Cst families reviewed in (Magister et al., 2013; Ochieng et al., 2010].

Upon the classical activation by lipopolysaccharide (LPS) or by interferon-γ (IFN-γ), Mø redirect gene expression to upregulate a variety of proteases involved in direct killing of intracellular pathogens or indirectly by having a critical role in antigen processing and presentation (Pires et al., 2016; Pires et al., 2017; Russell et al., 2009). We previously demonstrated that a general downregulation of cathepsins including cathepsin S occurs either in resting or in IFN-γ-activated human Mø infected with Mtb (Pires et al., 2016; Pires et al., 2017; Russell et al., 2009). This may be a strategy used by this pathogen to manipulate the host microbicidal responses in order to survive intracellularly. In contrast, the infection with the non-pathogenic species *Mycobacterium smegmatis* led to a strong upregulation of most cathepsins in resting Mø, but a slightly weaker response was noted in activated Mø. Furthermore, with the exception of cathepsin F, we provided evidence that most cathepsins are involved in Mtb killing (Pires et al., 2016). Additionally, manipulation of cathepsin S expression led to improved intracellular

killing of Mtb and increased MHC-II-antigen presentation and T-cell proliferation (Pires et al., 2017; Pires et al., 2021).
In line with this mechanism, during *de novo* infection, HIV is able to counteract lysosome-mediated total degradation by markedly decreasing the expression of lysosomal cathepsins B, C, S, and X (Harman et al., 2009).

Our previous findings pointed out potential roles of protease inhibitors during Mtb infection. Indeed, treatment of Mtb-infected Mø with synthetic cathepsin inhibitors, such as E-64d, helped the bacteria to survive. Accordingly, internalization of exogenous Cst C, the strongest inhibitor of cathepsins, led to a significant fivefold increase in Mtb survival rate 24 h after Mø infection (Pires et al., 2016). Since there have been no systematic studies in human primary Mø on the role of Csts during Mtb infection, especially during Mtb/HIV coinfection, we performed a transcriptomic analysis focusing on type I and II Csts. We found distinct gene expression profiles depending on Mø mono-infected with either Mtb or HIV or in Mø coinfected with both pathogens. In opposition to the profile found during infection with *M. smegmatis*, a species that is completely cleared in Mø in 24 to 48 h, we found Cst C with the most prominent increased gene expression along that time in all tested pathogenic conditions. Csts C and F are the inhibitors described to accumulate in endocytic pathway (Lautwein et al., 2002; Colbert et al., 2011). Our results revealed Cst F upregulated during *M. smegmatis* and Mtb infections but not during HIV or during Mtb/HIV coinfection. Altogether, the results suggest Cst C as a major target working in the endolysosomal pathway during infection with all pathogens.

In this work, we developed a delivery system (DS) for cystatin C siRNA, as the usual transfection methods are not suitable for clinical translation. The biomedical application of nanotechnology has opened up the way to combine drugs and corresponding carriers into a new class of pharmaceuticals with a particle size ranging from 1 to 1000 nm (Satalkar et al., 2016). This approach is expected to enhance drug targeting and reduce toxicity while increasing drug stability and improving drug absorption and efficacy (Martinelli et al., 2019; Wang et al., 2019; Kumari et al., 2019; Rani et al., 2018). Chitosan (Cs) is a natural non-toxic polymer with demonstrated properties such as biocompatibility and biodegradability, revealing good adhesion to mucosae and direct antibacterial properties for a variety of Gram-positive and Gram-negative species (Kucukoglu et al., 2019; Radwan-Praglowska et al., 2019; Rashki et al., 2021; Perinelli et al., 2018; Cunha et al., 2019), which makes it a suitable material for a DS. Here, we provide evidence substantiating a future clinical application of Cs-DS to treat drug-resistant TB by acting as a host-directed adjunctive therapy that improves the intracellular killing of Mtb. Overall, our results propose the targeted modulation of Cst C expression level in Mø as a potential therapeutic avenue to control Mtb infection including MDR-TB.

3.2. Results

3.2.1. Low multiplicity of infection of human macrophages with *Mycobacterium tuberculosis* combined with high HIV viral inoculum does not impact cell death

It is well established that HIV impairs the ability to control Mtb infection and vice versa (Souriant et al., 2019; Mancino et al., 1997; Diedrich et al., 2011). To standardize the infection of Mø in order to maintain similar numbers of viable cells during infection with either Mtb or HIV, or coinfection with both pathogens, we established different combinations of bacteria MOI and the viral infecting inoculum. Our previous results indicated that a MOI of up to 1 for Mtb in Mø derived from peripheral blood monocytes does not significantly impact cell death in our experimental conditions and time points analyzed (Bettencourt et al., 2013). Therefore, we investigated a standard bacteria MOI of 1 versus distinct viral inocula produced by serial dilution assays. The best ratio combination to observe a high percentage of infected cells with similar cell death was 1 ng of HIV-1 RT per 10^6 cells per ml. Cell death was evaluated by flow cytometry using annexin V to stain apoptotic cells and propidium iodide for necrotic cells at 24 and 48 h post infection (p.i.). As shown in Figure 1A, apoptosis was much more prominent than necrosis in all conditions tested, but with a similar percentage when comparing HIV and Mtb mono-infection with coinfection. Moreover, the total amount of Mø was similar between the distinct conditions, and no cell population changes in size and granularity that could interfere in the analysis were detected (Figure S1). Thus, we proceeded with MOI of 1 for Mtb and 1 ng of HIV for all subsequent infections. Ritonavir, a protease inhibitor formerly used for HIV therapeutics, was used in toxic concentrations as positive control (Pires et al., 2021).

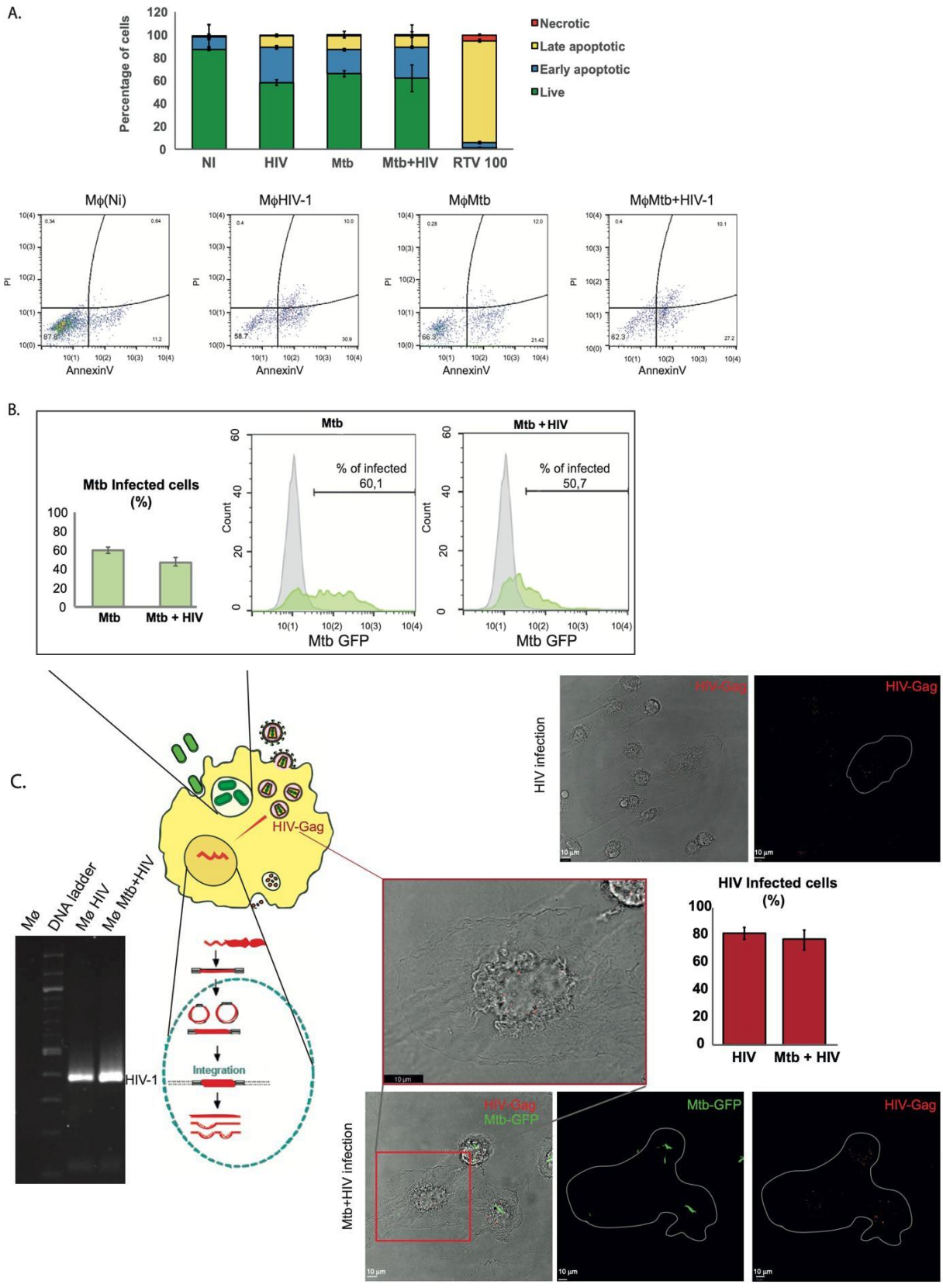


Figure 1. Infection with either Mtb or HIV, or coinfection with pathogens, in human Mø does not impact cell death. **(A)** Flow cytometry analysis of the percentage of infected cells stained for annexin V and/or with propidium iodide 48 h p.i. Results represent the mean of biological triplicates for each challenge. **(B)** Percentage of Mtb-infected Mø during mono-infection or during coinfection with HIV after 3 h p.i. determined by flow

cytometry. Bar plot depicts the mean ± SEM of three independent experiments. **(C)** HIV infection of Mø during mono- or coinfection with Mtb. Images represent z-axis maximum intensity projections of Gag/p24 protein depicted in red and Mtb depicted in green on a single plane of the bright-field channel, visualized by confocal microscopy. Bar plots represent the mean percentage of cells infected by HIV obtained from the microscopic analysis of 250 cells per treatment in ImageJ software. Error bars show the standard deviation. Gel electrophoresis shows the result of nested PCR amplification of HIV-1 (92US660) LTR with 391 base pairs (bp). Mtb, *Mycobacterium tuberculosis*; Mø, macrophages; p.i., post infection; LTR, long terminal repeat.

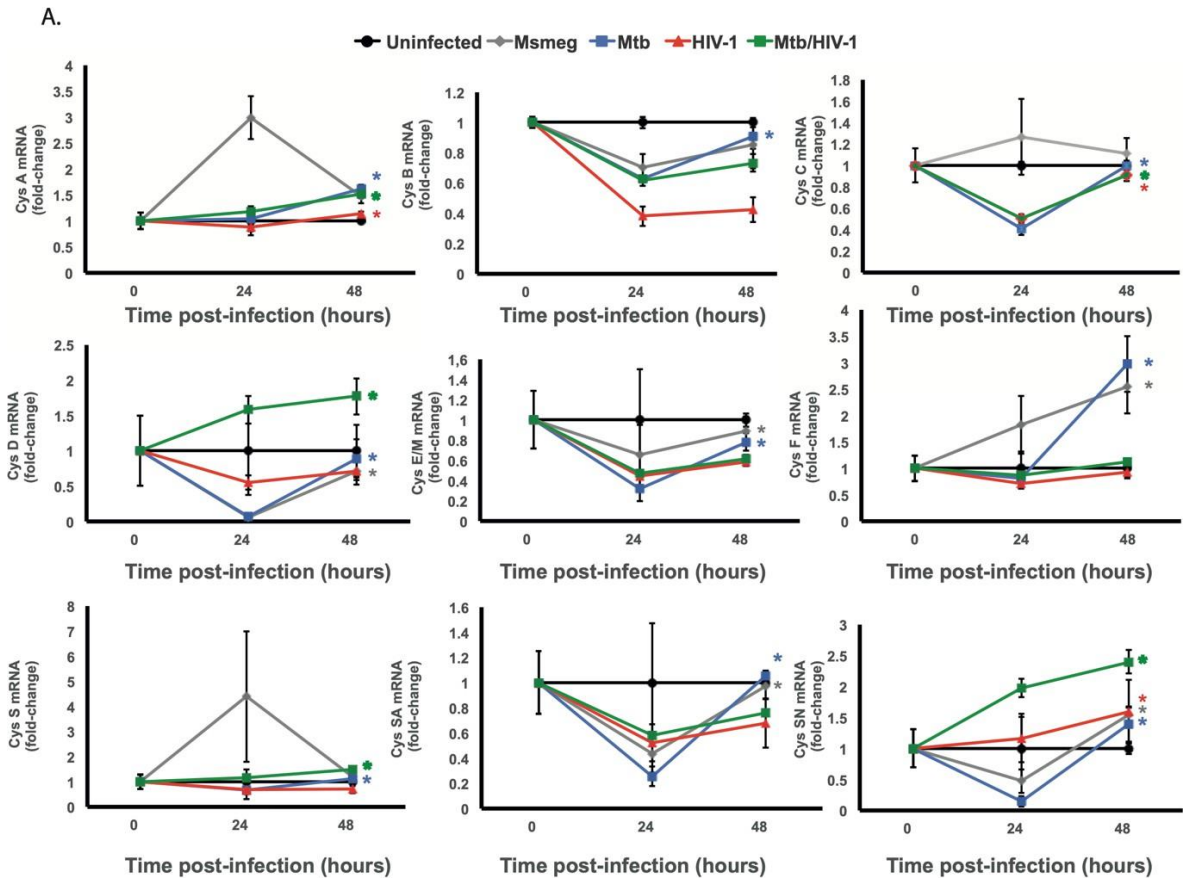
In an attempt to decipher if the simultaneous infection with both pathogens differentially interferes with internalization relative to mono-infections in our *in vitro* model, we quantified bacterial internalization by flow cytometry analysis of Mø infected with the Mtb-GFP strain. As expected, approximately 60% of all Mø were infected at 3 h p.i., with Mtb (**Figure 1B**). In experimental conditions, coinfection with HIV of up to 10% decrease in the internalization of Mtb was observed (**Figure 1B**). Viral internalization was evaluated by immunofluorescence labelling of the Gag protein in infected cells as shown in **Figure 1C**. Quantification of infected cells was done 3 h p.i. using a parallel culture of HIV-infected Mø exposed to the exact same conditions as the one used for coinfection with Mtb. No signal was detected in non-infected cells, confirming that the red fluorescence detected in the cytosol corresponds to virus-encoded Gag protein. ImageJ software analysis of the images showed that about 80% of cells expose to HIV particles internalized the virus. Most cells displayed small numbers of dots in the cytoplasm. A large number of cells tend to concentrate them at the nuclear region. This finding is in accordance with previous results showing viral capsid concentration in nuclear region before retrotranscription (Li et al., 2021) and with viral staining of Gag visualized by confocal microscopy 6 h p.i in cytosol and in perinuclear region of Mø (Hammonds et al., 2017). Since one of the steps of virus replication cycle is the integration of the retroviral DNA into the host genome, a nested PCR (**Figure 1C**) revealed an amplicon of 391 bp, thus further confirming HIV infection of Mø. Altogether, these results demonstrate an optimization of our mono- and coinfection models to study the modulation of the desired gene expression profile.

3.2.2. Cystatin expression is differentially regulated in macrophages during infection with either *Mycobacterium tuberculosis* or HIV, and coinfection with both pathogens

We next aimed to assess the pattern of type I and II Cst mRNA gene expression during our infection conditions in Mø. We performed a qRT-PCR analysis of type I and II Csts expressed in Mø at early stages of infection (24 and 48 h p.i.). This rationale was based on previous gene expression screens where it

was shown that the majority of the host's genes are modulated during the first 24–48 h p.i. with Mtb (Tailleux et al., 2008).

In addition, we infected Mø with *M. smegmatis* to assess gene expression differences in response to a non-pathogenic mycobacteria, which is usually cleared within Mø 24–48 h after challenge (Pires et al., 2016; Anes et al., 2006). As shown in **Figures 2A, B**, CstB, a type I Cst, was downregulated at 24 and 48 h p.i., independently of the type of microorganism challenge. By contrast, CstA, the other Cst of the same family, displayed an increasing expression tendency in response to pathogen challenge, including a prominent upregulation in response to *M. smegmatis* at 24 h p.i. (**Figures 2A, B**). For the other tested Csts belonging to the group II family, we observed a general downregulation tendency upon pathogen challenge with few exceptions. In the case of the *M. smegmatis* challenge, a species that is almost completely cleared in the first 24 h p.i (Anes et al., 2006)., we observed the upregulation of Cst C, Cst F, and Cst S. For Mtb, there was an upregulation of Cst F and Cst SN; for HIV, there was an upregulation of CstSN; and for Mtb and HIV coinfection, CstD, S, and SN were all strongly upregulated 48 h p.i. (**Figures 2A, B**).



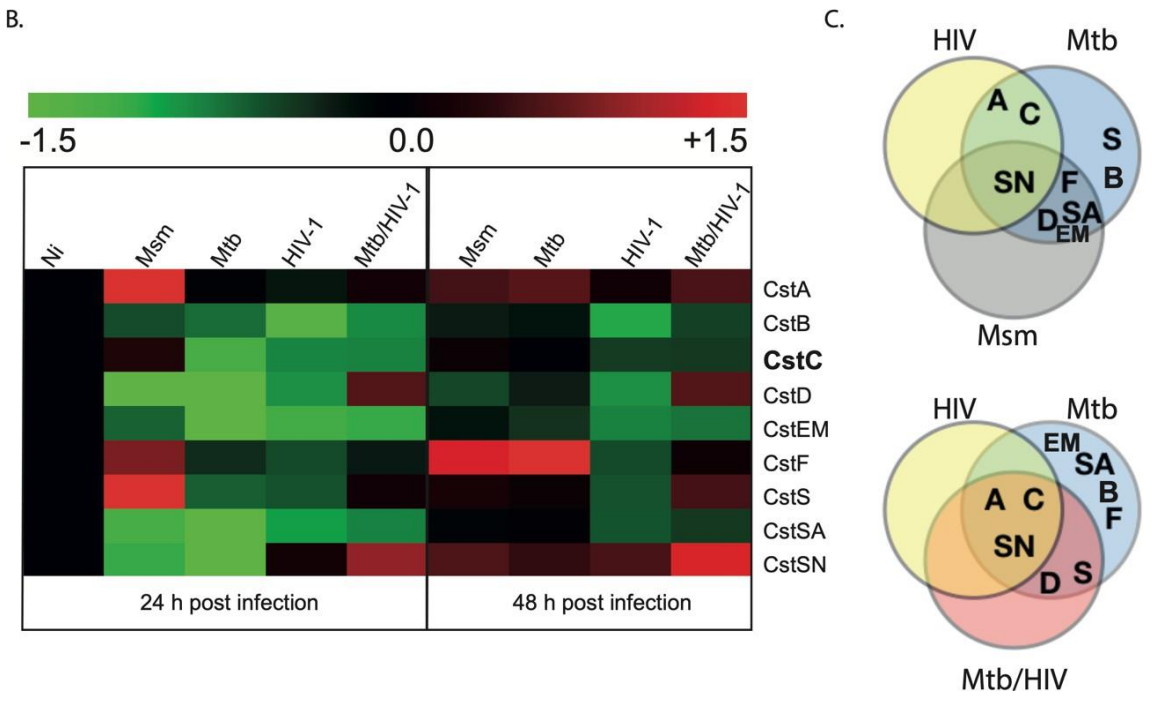


Figure 2. Cystatins are differentially regulated during mono-infection with Mtb or HIV, or coinfection with pathogens. **(A)** Gene expression kinetics of cystatins in Mø infected with Mtb or HIV, or coinfected with both pathogens, in comparison with the infection with *Mycobacterium smegmatis* for 24 and 48 h. Values are depicted relative to uninfected control and were previously normalized to GAPDH expression. Data are represented as the mean fold change per sample ± standard error. Statistical significance displayed refers to the values at 48 h relative to 24 h p.i. (*$p \leq 0.01$; n = 3). **(B)** Heatmap of qRT-PCR quantification of mRNA obtained from Mø after 24 and 48 h of infection. Values are depicted as log2 gene expression relative to uninfected Mø. **(C)** Venn diagram of the confirmed "hits" that exhibit significantly increased gene expression from 24 to 48 h p.i. Mtb, *Mycobacterium tuberculosis*: Mø, macrophages.

As stated before, a slight imbalance in the equilibrium between Csts and cathepsins may result in unwanted inhibition of enzymatic activity (Turk et al., 2012). This is particularly relevant for strong inhibitors of cathepsins such as CstC or F, contrary to weak inhibitors such as CstD, S, and SN, where a slight variance in protein concentration may induce a major switch from non-activity to strong enzymatic inhibition. We then compared the evolution of gene expression between the time points 24 and 48 h. In order to have an overview of this Cst differential gene expression, we performed a Venn diagram analysis (**Figure 2C**). In the Venn figure, CstSN (at the top) was unique in that it increases gene expression from 24 to 48 h p.i. with all microorganisms. Regarding the mono-infection data, we noticed that CstA and CstC are differently modulated during infection either with Mtb or HIV in comparison with *M. smegmatis* (**Figure 2C**, top panel). When we compared the mono-infections with Mtb or HIV

to the coinfection context, we confirmed that CstA and CstC are uniquely modulated by these pathogens (**Figure 2C**, bottom panel) and with statistically significant increased gene expression between those time points. Moreover, the analysis of Csts basal expression in non-infected cells (**Figure S2**) shows that Csts A, B, and C possess a high level of basal expression comparable with the housekeeping gene GAPDH, while the remaining Csts are 100- to 10,000-fold less expressed. Taking this into consideration, slight variations in gene expression for Csts A and C may result in more drastic effects on cathepsin activity. These findings indicate that CstA and CstC constitute obvious candidates for further evaluation in our infection model.

3.2.3. Inactivation of CstC expression in primary human macrophages results in increased *Mycobacterium tuberculosis* killing during mono- and coinfection with HIV

CstC is described as the most prominent Cst in immune cells and one of the few that accumulate in endolysosomal vesicles, with particular strong inhibitory effects on cathepsins such as B, L, and S (Lautwein et al., 2002; Colbert et al., 2011). In fact, our previous studies revealed that exogenous supplementation with CstC helped Mtb survival in human monocyte-derived Mø (Pires et al., 2016). Therefore, we decided to further investigate the downregulation of CstC expression during mono-infection with Mtb and coinfection with HIV, as potential effect by host Mø to counteract Mtb intracellular colonization. To this end, we performed siRNA-mediated gene silencing to decrease specifically the expression of CstC, as previously established in primary human Mø (Troegeler et al., 2014). As shown in **Figure 3A**, approximately 60% silencing of CstC mRNA was achieved, and that translated into a strong reduction of CstC protein level (**Figure 3A** and **Figure S3**). Importantly, no difference in cell death was observed between CstC-silenced Mø compared with scramble controls (cells transfected with a non-specific RNA; see *Materials and Methods*) (**Figures 3B** and **S4**). Next, we compared the effect of CstC silencing on the internalization and intracellular survival of Mtb in Mø during mono- and coinfection with HIV. Flow cytometry analysis showed that approximately 60% of Mø were infected with similar amounts of bacteria when comparing CstC-silenced Mø with scramble controls (**Figure 3C**). Next, bacterial survival was assessed by CFU counts of bacilli recovered from infected cells. As such, we observed a strong and significant reduction in CFU from bacteria recovered from CstC-silenced infected Mø relative to scramble controls ($p < 0.001$) (**Figure 3D**). At 24 h p.i., CstC silencing yielded around 70% increase in Mtb intracellular killing during mono-infection and 60% during coinfection with HIV. Interestingly, the impact CstC silencing on intracellular Mtb killing was similar to that obtained with PZA treatment (at a MIC of 100 μg/ml), a first-line antibiotic for TB. The effects on mycobacteria killing were maintained for up to 7 days p.i. We then proceeded to analyze the effects of CstC depletion on the intracellular survival of clinical strains isolated from TB patients

(**Figure 3E**). For the susceptible strain, the results were similar to those obtained with the reference laboratory strain H37RV. For the MDR strain, we found a significant killing effect induced by CstC depletion, a strain for which the first-line antibiotics isoniazid, rifampicin, and PZA, plus ethionamide, have lost their efficacy.

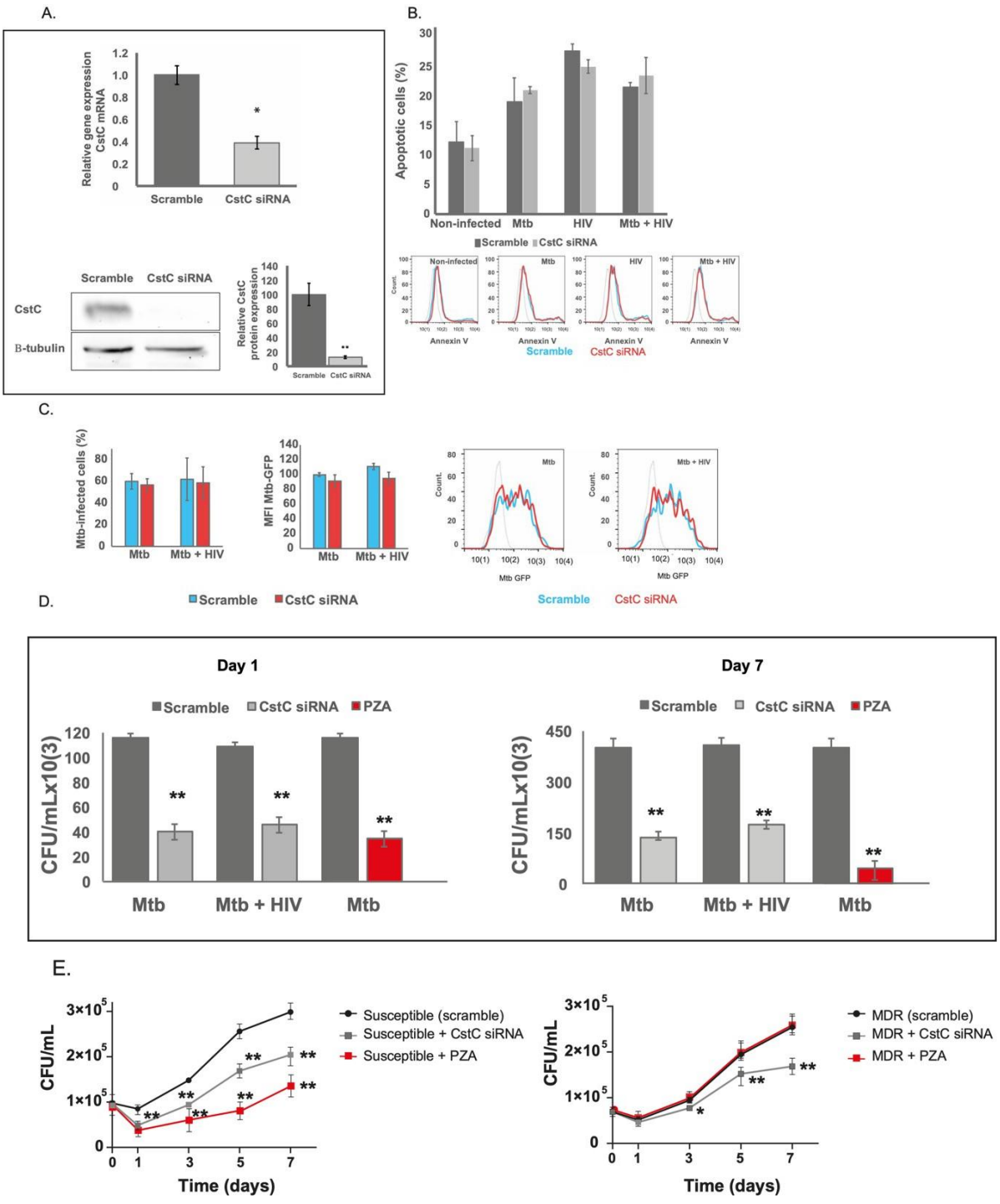


Figure 3. SiRNA-mediated gene silencing for CstC results in increased Mtb killing in primary human Mø during mono-infection and during HIV coinfection. **(A)** CstC was silenced by siRNA 3 days prior to infection in order to achieve maximum protein silencing. Relative gene expression of CstC mRNA in Mø was determined by RT-qPCR

and Western blotting at 24 h p.i. Values are relative to cells transfected with scramble and represent the average of biological triplicates (*$p < 0.01$; n = 3). **(B)** Effects of siRNA for CstC relative to scramble transfected cells on apoptosis in infected Mø. Apoptosis was measured by flow cytometry after 24 h of infection using fluorescent annexin V antibodies. Values show median fluorescence intensity (MFI) from one representative experiment performed in triplicate, while error bars depict the standard deviation. **(C)** Percentage of Mø infected with Mtb, and MFI of Mtb per Mø were measured by flow cytometry in scramble infected cells and in CstC-silenced infected cells, after 3 h of infection with a GFP-expressing Mtb strain. Bar plots depict the average of three biological replicates, and the error bars depict the standard error. Raw values from one representative replicate are presented in the fluorescence intensity histograms. **(D, E)** Intracellular survival of Mtb: reference laboratory strain H37Rv **(D)** and clinical strains **(E)**. Colony-forming units (CFU) of intracellular bacteria were recovered from Mø transfected with siRNA for CstC or with a scramble siRNA. Values depict mean CFU representative of three biological replicates measured in duplicate, while the error bars depict the SD. Asterisks indicate statistical significance between samples at the same time point relative to scramble control (*$p < 0.01$; **$p < 0.001$; n = 3). PZA was used as control for killing efficacy. Mtb, *Mycobacterium tuberculosis*; Mø, macrophages; PZA, pyrazinamide.

Overall, our results reveal that the modulation of CstC expression in Mø is important to control infection.

3.2.4. Inactivation of CstC expression impacts cysteine cathepsin enzymatic activity in macrophages infected with either *Mycobacterium tuberculosis* or HIV, or coinfected with both pathogens

To confirm if the impact of CstC silencing on the intracellular killing of Mtb was attributed to a direct effect on cathepsins, we assessed the OmniCathepsin proteolytic activities, which measures the combined activities of cathepsins B, L, and S. The cleavage of a peptidase-specific fluorogenic peptide substrate was measured over almost 2 h starting at 24 h p.i. The specificity of substrate cleavage was checked by preincubation of cells with E-64d, a cognate inhibitor of most cathepsins. As expected, Mtb infection induces a decrease in OmniCathepsin activity as shown by comparing the activity in scramble non-infected cells with Mtb scramble, or with scramble in coinfection. In all infection settings, CstC silencing leads to a significant increase in OmniCathepsin activity, with a twofold increase in the case of Mtb mono-infection (**Figure 4A**). In marked contrast, the silencing of CstC has no effect in cathepsin activities in non-infected cells, suggesting that the capacity of Mtb infection to lower cathepsin activity depends on CstC expression (**Figure 4A**). When compared with non-infected cells, mono-infection with HIV did not affect cathepsin activity (**Figure 4A**). However, the silencing of CstC led to a significant increase on OmniCathepsin activity of 0.2-fold during HIV infection (**Figure 4A**). Altogether, these results indicate that silencing of CstC impacts cathepsin B, L, and S activities in the endocytic pathway during Mtb and HIV mono-infections and during coinfection.

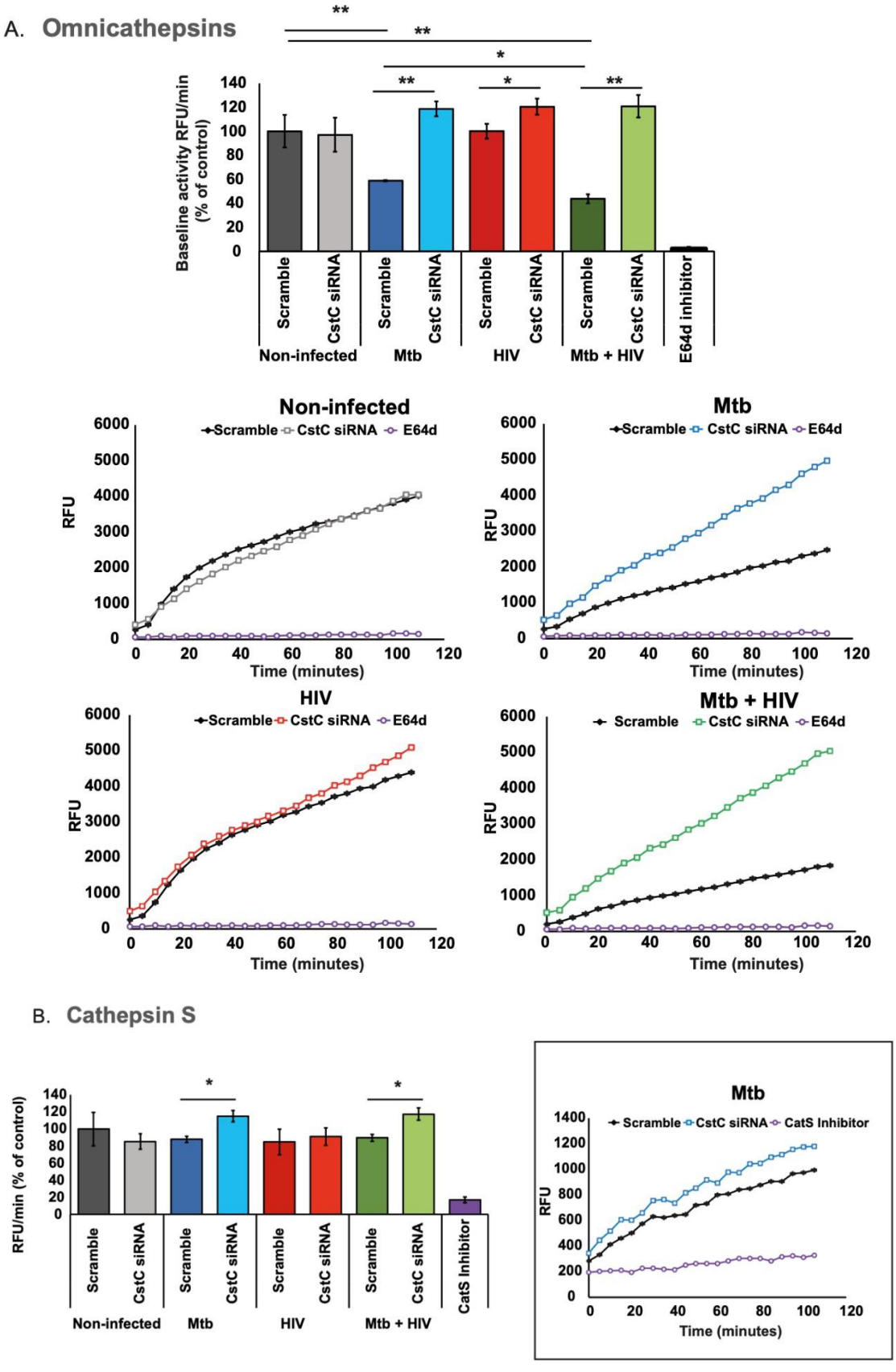

A. Omnicathepsins
Baseline activity RFU/min (% of control)
Scramble
CstC siRNA
E64d inhibitor
Non-infected
Mtb
HIV
Mtb + HIV
Non-infected
Mtb
HIV
Mtb + HIV
Scramble
CstC siRNA
E64d
RFU
Time (minutes)
B. Cathepsin S
RFU/min (% of control)
CatS Inhibitor
CatS Inhibitor
Mtb

Figure 4. SiRNA-mediated gene silencing for CstC alter cathepsin activity in human Mø infected with Mtb. **(A)** OmniCathepsin or **(B)** cathepsin S activity alone was monitored with a specific fluorogenic substrate every 5 min in live cells (scramble-control and CstC-silenced cells), or with specific inhibitors (E-64d or ZFL-COCHOO for cathepsin S). The slope of fluorescence emission in the scramble control of non-infected cells was represented as 100%, and the effect of each sample was calculated as a percentage relative to control. Data are represented as average from three independent experiments, and the error bars represent standard error (*p < 0.01, **p < 0.001; n = 3). Mtb, *Mycobacterium tuberculosis*; Mø, macrophages.

Due to the cathepsin S direct killing effect on intracellular pathogens, and its important role in adaptive immunity, we then followed the kinetics of cathepsin S activity. To do so, a cathepsin S-specific fluorogenic peptide substrate was employed along with a cathepsin S-specific inhibitor (see *Materials and Methods*) (Pires et al., 2017; Lautwein et al., 2002; Pierre et al., 1998). Accordingly, we show that by silencing CstC, a slight, but significant, increase in cathepsin S activity was observed during Mtb infection and coinfection with HIV (**Figure 4B**). CstC depletion did not impact cathepsin S activity in mono-infection with HIV (**Figure 4B**), suggesting that, in the case of HIV infection, cathepsin B and/or L activity should be modified.

3.2.5. CstC depletion increases the cell-surface expression of human leukocyte antigen class II and $CD4^+$ T-Lymphocyte proliferation along with IFN-γ secretion

CstC has been implicated in the impairment MHC class II processing and in endosomal antigen processing and presentation by regulating the activity of cathepsin S (Lautwein et al., 2002; Pierre et al., 1998). We hypothesized that the noticeable Mtb-induced increase in CstC expression might be linked to poor antigen processing and presentation, thereby compromising the adaptive immunity response to infection. To test this, we silenced CstC in non-infected cells, Mtb- or HIV-infected cells, or coinfected with both pathogens, and then we assessed the surface expression of HLA class II molecules by flow cytometry. In all conditions with Mtb infection, we found an increased cell surface of HLA class II expression (**Figure 5A**). Mono-infection with HIV failed to accomplish this effect, indicating that Mtb is responsible for this result in in coinfected cells (**Figure 5A**).

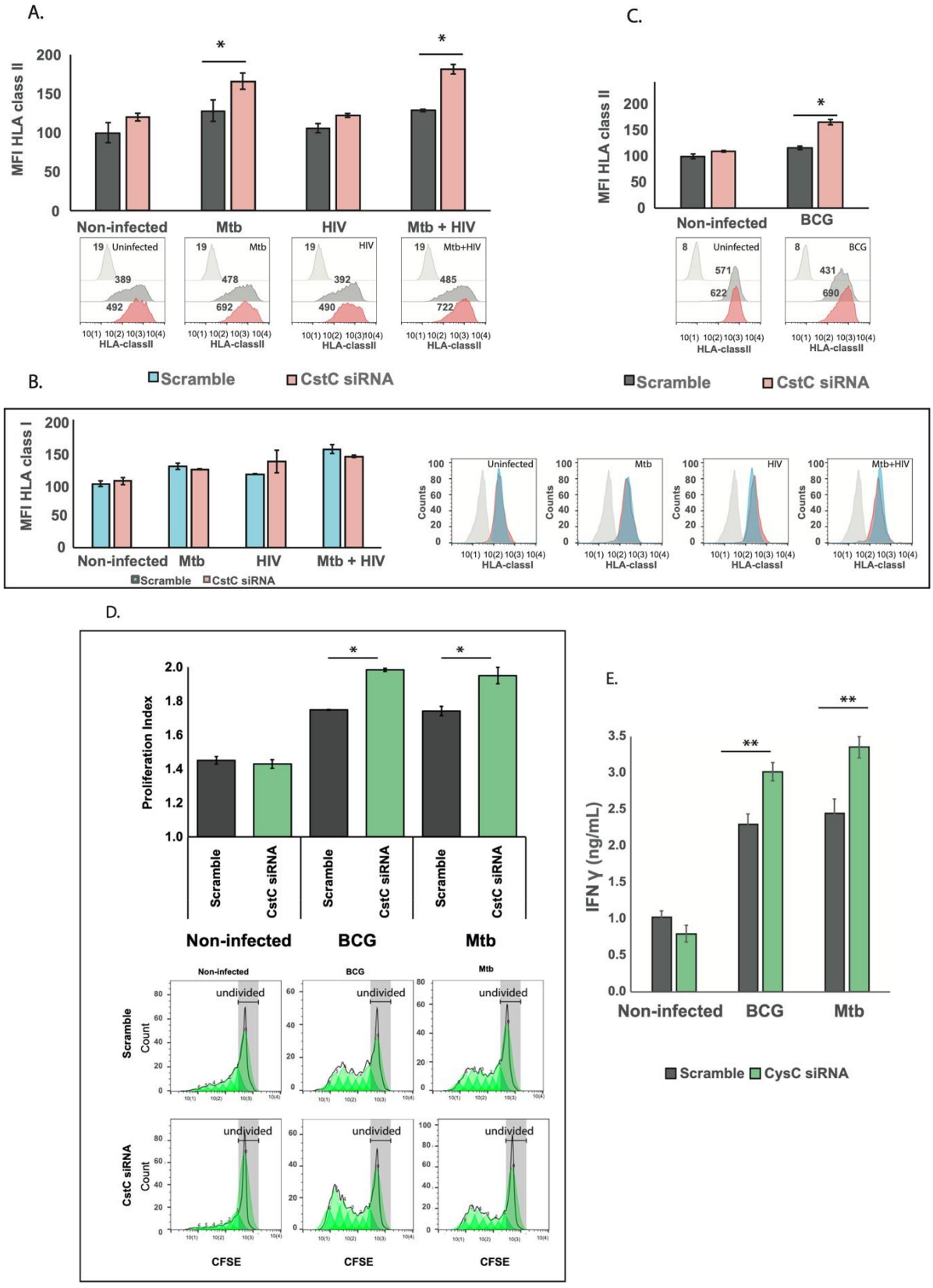


Figure 5. SiRNA-mediated gene silencing for CstC results in increased cell-surface expression of MHC class II and elevated T-cell proliferation and proinflammatory IFN-γ secretion. Cell-surface expression of human leukocyte antigen (HLA) class II **(A, B)** or class I **(C)** on Mø transfected with siRNA for CstC or with a scramble siRNA in the

different infection challenges compared with non-infected cells. HLA class II and I were measured by flow cytometry after 24 h of infection. Values in bar plots represent median fluorescence intensity (MFI) relative to the respective scramble controls from one representative experiment performed in triplicate, while error bars depict the standard deviation (*$p < 0.01$; n = 3). **(D)** $CD4^+$ T-cell proliferation after 5 days of coculture with Mtb- or BCG-infected Mø. Infected Mø were cocultivated with CFSE-stained $CD4^+$ T cells following 24 h of infection. After 5 days of coculture, $CD4^+$ T-cell CFSE fluorescence was measured by flow cytometry. Values in bar plots represent the proliferation index (average number of divisions per cell) of $CD4^+$ T cell (*$p < 0.01$, relative to scramble control; n = 3). Histograms from one representative replicate of the different treatments infected with Mtb are presented at the bottom. The green areas represent the $CD4^+$ T cell populations after each division, as modeled by the software. Each generation is identified by a 50% decrease in fluorescence caused by cell division. **(E)** IFN-γ was quantified in the supernatant after 5 days of cocultures of Mø with $CD4^+$ T cells by ELISA. Values depict mean concentration of three biological replicates from one representative experiment performed in duplicate. Error bars depict the standard deviation (**$p < 0.001$, relative to control; n = 3). Mtb, *Mycobacterium tuberculosis*; Mø, macrophages; CFSE, carboxyfluorescein succinimidyl ester.

Cathepsin S is also involved in partial antigen processing for cross-presentation to $CD8^+$ T lymphocytes (Shen et al., 2004), but with no effects on HLA class I expression at the cell membrane (Kourjian et al., 2014; Wang et al., 2013). We thus analyzed the expression of HLA class I at the cell surface by flow cytometry. No change was observed when comparing siRNA for CstC-treated cells relatively with scramble (**Figure 5B**).

We then focused on BCG infection. Since its first use in 1921, the BCG vaccine strain has lost its immunogenicity capacity. We decided to test if silencing of CstC results in improved surface expression of HLA class II molecules is required for antigen presentation, in the context of Mø infection with BCG. In a similar manner than for Mtb infection, the reduction of CstC gene expression significantly increases the cell surface of the MHC II class molecules in BCG-infected cells relative to scramble control infected cells (**Figure 5C**).

Finally, we questioned if the increase on cell-surface expression of MHC class II induced by CstC silencing would impact $CD4^+$ T-lymphocyte proliferation. To this end, we performed cocultures of infected Mø with autologous $CD4^+$ T lymphocytes obtained from the same healthy PPD^+ donors and evaluated their ability to induce T-cell proliferation (**Figure 5D**). Following the same pattern of HLA class II surface expression, CstC depletion in Mtb- or BCG-infected cells induced a significant increase of T-cell proliferation relative to scramble controls, after 5 days post-cocultivation as evaluated by flow cytometry (**Figure 5D**). As a consequence, we also observed an increased secretion of IFN-γ in coculture

supernatants of Mtb- or BCG-infected cells, which is further enhanced in CstC-silenced cells (**Figure 5E**); no significant alterations in IFN-γ secretion were detected in non-infected cocultures.

Collectively, our findings demonstrate that the modulation of CstC expression in human Mø has significant consequences to the innate immune control of Mtb intracellular growth, which is later amplified in the capacity of these cells to activate the adaptive immune response probably through a defective processing and presenting of antigen *via* MHC class II.

3.2.6. Physicochemical characterization of siRNA-incorporating particle formulations

Polymeric DSs were synthesized to deliver siRNA-targeting cystatin C (CstC). First, GlutCs/pARG–siRNA polyplexes were developed to allow the entrapment and subsequent release of the siRNA. The interaction between the positively charged chitosan (Katas et al., 2006; Mao et al., 2010) or arginine (Zhang et al., 2006; Zhao et al., 2012) and the negatively charged siRNA leads to the spontaneous formation of stable polyplexes in the aqueous milieu. Accordingly, GlutCs and pARG were used at two different ratios, 15:1 and 2.5:1 (*m*/*m*), for GlutCs/pARG:siRNA, respectively, to prepare the polyplexes through electrostatic-based interactions. To potentiate the interaction of DSs with macrophages, mannose-functionalized DSs were developed to improve their recognition by the mannose receptor (CD206) expressed at the macrophage surface by promoting receptor–ligand interaction and subsequently improving payload delivery (Uehara et al., 2022). siRNA-loaded DSs presented an average hydrodynamic diameter close to 200 nm, with a low polydispersity index, a near-neutral surface charge, and a spherical shape (Table 1 and Supplementary Figure S1). Macrophage-targeted NPs displayed high levels of entrapment efficiency and loading capacity for both siRNAs (Table 1).

Table 1. Physicochemical characterization of the particle formulations.

Formulation	Size (nm)	Polydispersity Index	ζ-Potential (mV)	Entrapment Efficiency (%)	Loading Capacity (μg mg^{-1})
CstC siRNA	194 ± 4	0.095 ± 0.008	−1.38 ± 0.42	96.44 ± 3.98	1.30 ± 0.05
Scramble	192 ± 0	0.112 ± 0.015	−1.37 ± 0.33	99.10 ± 0.77	1.33 ± 0.01

3.2.7. Particles loaded with anti-cystatin C siRNA have no cytotoxic effects on human primary macrophages

To define the optimal conditions for macrophage treatment with DSs, first, we assessed the highest concentration of DSs without cytotoxic effects using resazurin, and second, we evaluated the long-lasting incubation time with that concentration with no cell viability alterations. Figure 6 shows the results obtained in human monocyte-derived macrophages (HMDMs) treated for 72 h with different concentrations of DSs (Figure 6a) and for different periods of incubation using DSs (Figure 6b) with a fixed concentration of 1 mg/mL. For both formulations of DSs, the highest concentration with no significant cytotoxic effects corresponds to 1 mg/mL. In addition, the exposure to DSs for as long as 72 h did not show any effects on cell viability. A common protocol using a commercial transfection reagent served as a control reference and revealed that DSs produce comparable results to the transfection reagent for 6 h and 24 h, while for 72 h, only the treatment with the transfection reagent produced statistically significant cytotoxic effects.

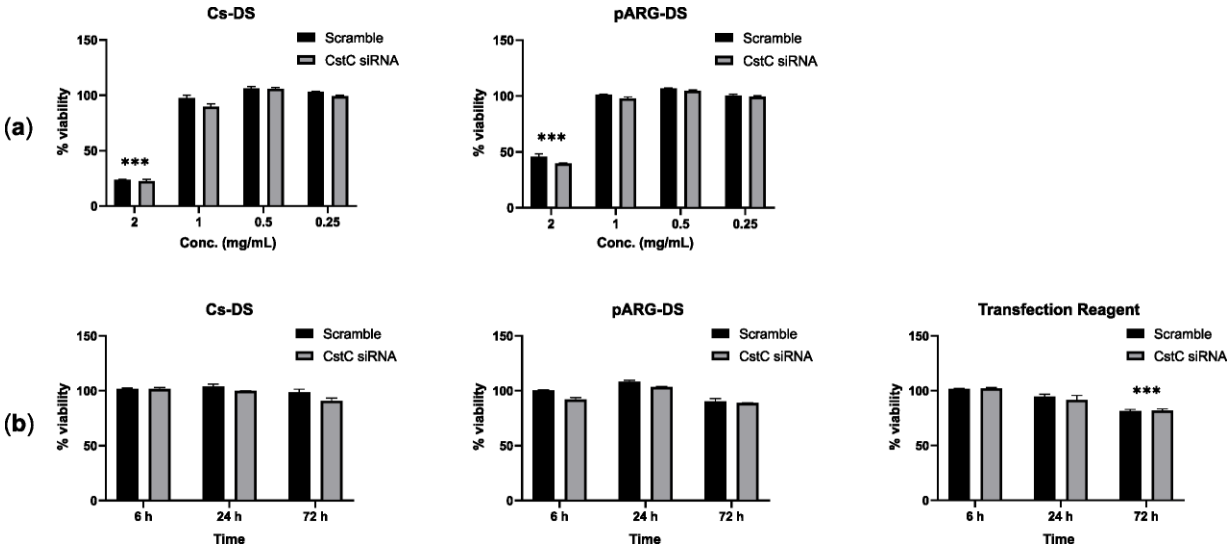


Figure 6. Effect of Cs and pARG particles on the viability of human monocyte-derived macrophages: (**a**) Macrophages were treated with different concentrations of DSs for 72 h or (**b**) treated with 1 mg/mL of DSs for different periods. The tested DSs were loaded with anti-CstC siRNA or scramble control siRNA. A transfection reagent (ScreenfectA) was used for comparison. Macrophage viability was measured using PrestoBlue (resazurin-based solution) by quantifying the fluorescence emission in a plate reader. Results were calculated relative to untreated macrophages (100% viability) and 0.05 % Igepal-treated macrophages (0% viability). Bars represent the average of three independent experiments, and the error bars depict the standard error of the mean. *** $p \leq$ 0.001 is relative to the control and all other concentrations tested (**a**) or to all other treatment times (**b**).

3.2.8. Chitosan particles loaded with anti-cystatin C siRNA effectively induce silencing in primary human macrophages and in THP-1 cells

Having defined the conditions without statistically significant cytotoxic effects, next, the efficacy of these DSs in delivering CstC siRNA and silencing CstC mRNA was evaluated. HMDMs were treated with 1 mg/mL of DSs for distinct periods of time, after which the cells were washed (to renew the culture medium and remove eventual remaining extracellular DSs). Incubation further proceeded until 72 h post-exposure to DSs. The quantification of CstC mRNA using qPCR indicated that Cs-DS-silencing effects were obtained 6 h after exposure, while maximum silencing was achieved by 72 h (Figure 7a). By contrast, for pARG-DS, no silencing was detected, and the treatment produced more erratic effects that, in some cases, would even induce CstC expression rather than silencing.

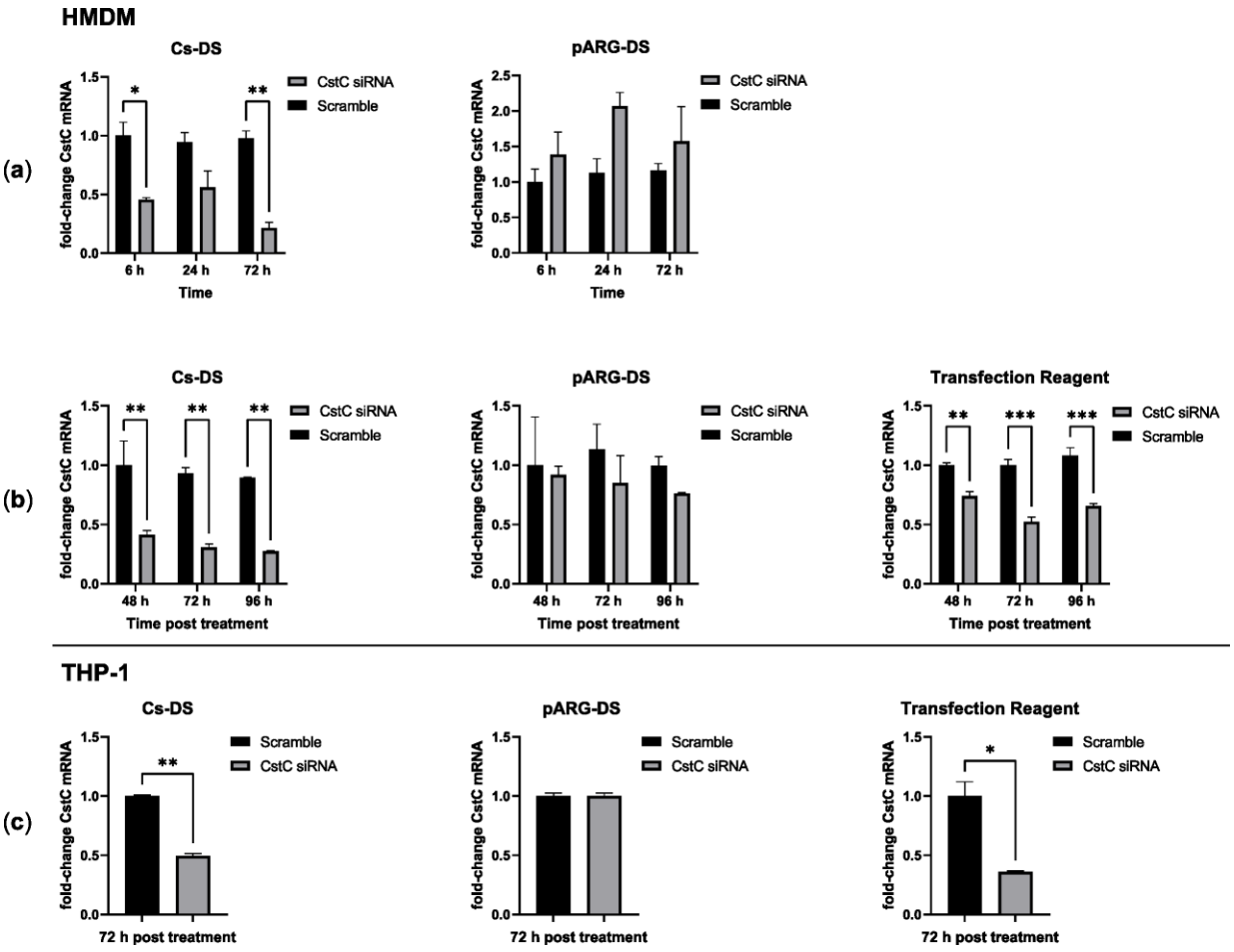


Figure 7. Cystatin C mRNA-silencing efficacy of Cs and pARG particles loaded with anti-CstC siRNA: (**a**) CstC mRNA levels were measured using qPCR in HMDMs treated with 1 mg/mL of DSs for different periods, after which the cells were washed and incubated until 72 h post-treatment; (**b**) alternatively, cells were left in contact with DSs

for the selected periods; (**c**) THP-1 macrophages were also treated under the same conditions but only for 72 h. A transfection reagent (ScreenfectA) was used for comparison. Bars represent the average of three independent experiments, and the error bars depict the standard error of the mean. For each plot, the values are presented relative to the scramble control from the earliest time point. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Since longer periods of exposure produced increased silencing of CstC, we further tested extending the time of contact of the cells to DSs (without the washing step). The results show that Cs-DS effectively silence CstC mRNA (Figure 7b) for 72 h with no benefit in increasing exposure time. Again, the results for pARG-DS were less consistent, and no statistically significant silencing of CstC mRNA was detected. As before, a commercial transfection reagent was used as a control reference, showing the effective silencing of CstC mRNA but with a comparatively less extent of silencing than with Cs-DS. Besides HMDMs, it was decided to test DSs in macrophages derived from the monocytic cell line THP-1 using the most optimal previously defined treatment conditions of 1 mg/mL for 72 h. This cell line is usually regarded as difficult to transfect. Our results were similar to those achieved for HMDMs with Cs-DS or the transfection reagent, leading to an effective reduction in CstC mRNA levels, while pARG-DS had no effect.

3.2.9. Chitosan particles are efficiently internalized by primary human macrophages and THP-1 cells

To control the distribution of DSs in the population of macrophages, we proceeded to analyze the capacity of HMDMs and THP-1 cells to internalize DSs. For this, we labelled Cs-DS with the fluorophore Cy5 to track them via fluorescence. Macrophages were treated with 1 mg/mL of Cy5-labeled Cs-DS for 6 h and then analyzed via flow cytometry and confocal microscopy. Figure 8 shows that for both types of macrophages, almost all cells were positive for DSs, and the results were very consistent, resulting in imperceptible values for standard error (Figure 8c). Concerning the number of DSs internalized, the mean fluorescence levels were one log higher for HMDMs than for THP-1 cells (Figure 8c), indicating an increased capacity of Cs-DS to target these cells. Furthermore, no differences were detectable when cells were treated with empty DSs or with DSs carrying siRNA.

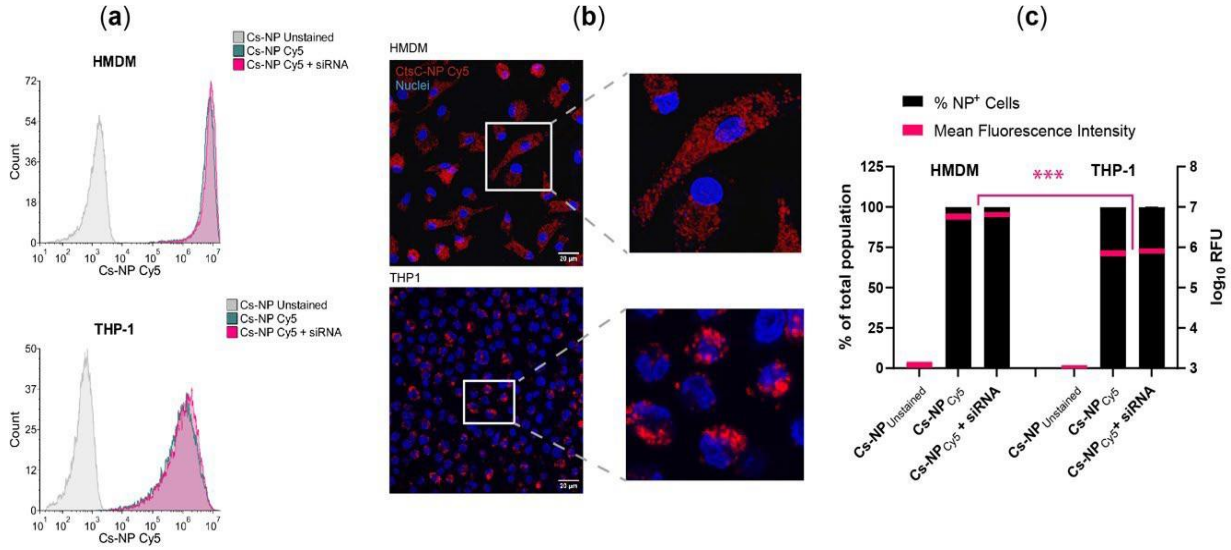


Figure 8. Cs-DS is effectively internalized by human monocyte-derived macrophages and THP-1 macrophages. Macrophages were treated for 6 h with Cy5-labeled empty Cs-DS or siRNA-loaded DSs, and their NP content was analyzed using (**a**) flow cytometry and (**b**) confocal microscopy: (**a**) flow cytometry histograms were obtained from one representative experiment; (**b**) confocal images from one representative experiment. DSs labeled with Cy5 are shown in red, and the cell nuclei labeled with DAPI are in blue; (**c**) the bar plot represents the percentage of macrophages loaded with DSs (left y-axis), and the red lines within the bars represent the mean fluorescence intensity of DSs per macrophage (right y-axis) calculated from three independent samples via flow cytometry. The thickness of the red lines represents min/max data dispersion. *** $p \leq 0.001$.

3.2.10. Chitosan particles loaded with anti-cystatin C siRNA impact the intracellular killing of Mtb strains either with susceptibility or with distinct drug-resistance profiles

As mentioned, we have previously shown that the targeting of CstC by siRNA improves the macrophages' response to Mtb infection (Pires et al., 2021). Likewise, here, we proceeded to evaluate the effect using Cs-DS as a delivery system of siRNA. For this, HMDMs were treated with 1 mg/mL of Cs-DS carrying CstC siRNA for 72 h prior to infection with Mtb. In these experiments, we compared different strains of Mtb, including the reference laboratory strain H37Rv and three clinical strains: one susceptible to drug therapy, one multi-drug-resistant (MDR), and one extensively drug-resistant (XDR) strain. The characterization of these strains was performed by the National Institute of Health (INSA, Portugal). This enabled us to account for strain variability and the potential outcomes related to mutations conferring drug resistance. Intracellular Mtb was quantified by measuring the colony-forming units recovered from infected macrophages over a 7-day period. The results show that for all the strains tested, treatment with Cs-DS loaded with CstC siRNA results in a significant impact on the

intracellular burden of Mtb compared with Cs-DS carrying the scramble siRNA control (Figure 9a). This reduction in the intracellular survival of Mtb was statistically significant from day 3 of infection onwards and had further impacts over time. Furthermore, it was found that even though the levels of CstC mRNA decreased over time after infection in the scramble control (as expected), Cs-NP CstC siRNA treatment was able to further impact the extent of the decrease in CstC mRNA for 72 h after all DSs were extracellularly washed out (Figure 9b). Moreover, to verify that mRNA silencing translates to lower CstC protein levels, we quantified the levels of CstC protein at 72 h post-treatment with DSs. The results in Figure 9b, right panel, indicate a 60% reduction in CstC protein expression, confirming the efficacy of NP-induced silencing.

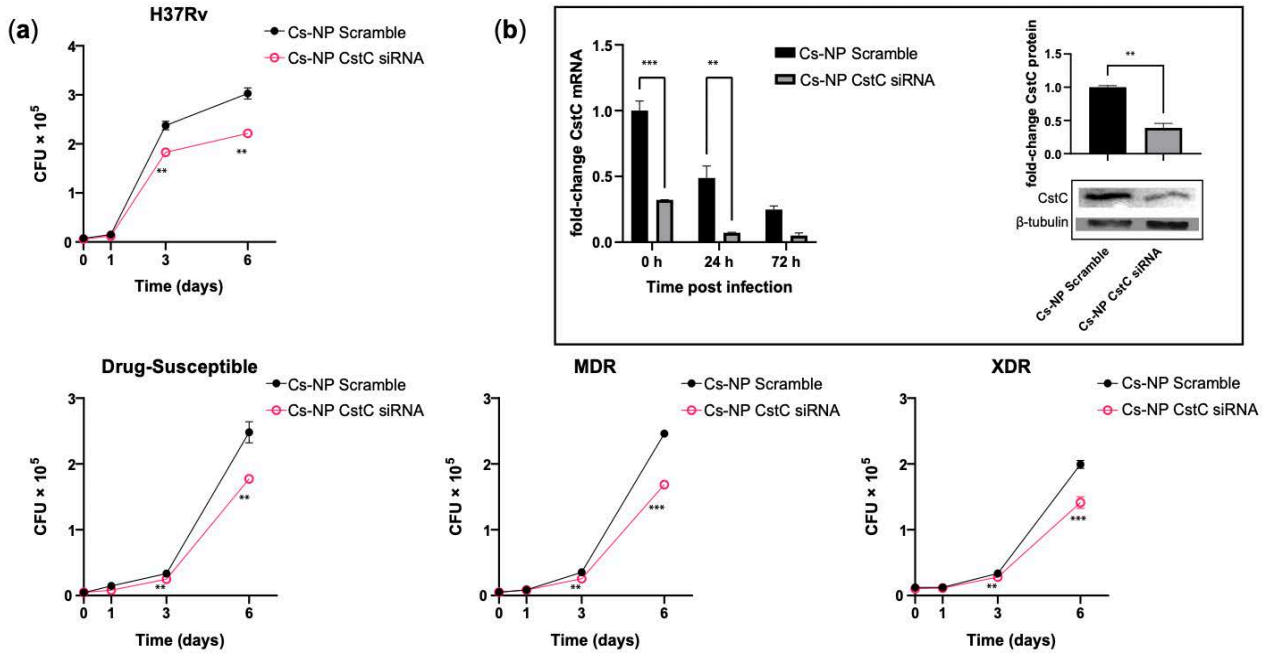


Figure 9. Cs-DS loaded with anti-cystatin C siRNA improves the intracellular killing of laboratory and clinical strains of Mtb with different drug-resistance phenotypes in human macrophages. Human monocyte-derived macrophages were treated with Cs-DS loaded with anti-CstC siRNA or scramble control siRNA 72 h before infection with (**a**) the laboratory strain H37Rv and three clinical strains of Mtb with different levels of drug resistance. Bacterial intracellular survival was evaluated at discrete time points. Line plots depict the average CFU per sample; (**b**) the bar plot of CstC mRNA levels demonstrates the silencing efficacy of Cs-DS during infection with H37Rv. Western blot image demonstrates the silencing of CstC protein by Cs-DS at the moment of infection (t = 0 h). The respective bar plot was calculated from band intensity using β-tubulins as a calibrator. The values depicted are the average of three independent experiments. Error bars represent the standard error of the mean. ** $p \leq 0.01$, and *** $p \leq 0.001$.

3.3. Materials and Methods

3.3.1. Cells and culture conditions

Primary human monocyte-derived Mø were obtained from buffy coats of healthy donors provided by the National Blood and Transplantation Institute (Instituto Português do Sangue e da Transplantação, Lisbon, Portugal) following a protocol established between Dr. Anes (Faculty of Pharmacy, University of Lisbon) and the blood institute. The personal details of the donors were not provided by the supplier. Briefly, peripheral blood mononuclear cells (PBMCs) were first isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). The PBMC fractions were incubated with anti-CD14 magnetic beads (Miltenyi Biotec), and the $CD14^+$ monocytes were isolated using magnetic-activated cell separation (MACS) cell separation magnetic columns. Monocyte differentiation to Mø was induced by allowing them to adhere to 12-, 48-, or 96-well plates at 1×10^6, 1.5×10^5, or 5×10^4 cells per well, respectively, for 2 h at 37°C, 5% CO_2, in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, GE Healthcare). Following adherence, the medium was supplemented to achieve a final concentration of 10% (v/v) fetal bovine serum (FBS) (HyClone, GE Healthcare), 1 mM of sodium pyruvate (HyClone, GE Healthcare), 10 mM of HEPES (HyClone, GE Healthcare), 0.1% β-mercaptoethanol (Gibco), and 20 ng/ml of the recombinant human M-CSF (BioLegend). The cell culture medium was renewed every 3 to 4 days until day 7 of differentiation. Purity (>99%) of the isolated culture was verified by flow cytometry (data not shown). The human monocytic cell line THP-1 (ATCC TIB202) (American Type Culture Collection, VA, USA) was cultivated as previously described (Jordao et al., 2008) and was differentiated to macrophages via incubation with 20 nM phorbol 12-myristate 13-acetate (PMA) overnight.

3.3.2. Bacterial cultures and HIV isolates

M. tuberculosis H37Rv [American Type Culture Collection (ATCC) 27294], H37Rv GFP-expressing strain, *Mycobacterium bovis* BCG Pasteur (ATCC 35734), and the clinical strains isolated from patients with active TB were grown in Middlebrook's 7H9 medium supplemented with 10% OADC enrichment (Difco), 0.02% glycerol, and 0.05% tyloxapol at 37°C (Pires et al., 2017). The strain *M. smegmatis* mc^2155, containing a p19 (long lived) EGFP plasmid, was kindly provided by Dr. Douglas Young (The Francis Crick Institute, London, UK), and it was grown in medium containing Middlebrook's 7H9 Medium (Difco) supplemented with 0.5% glucose and 0.05% tyloxapol at 37°C on a shaker at 200 rpm (Anes et al., 2003). The clinical strains were provided and characterized by the TB National

Reference Laboratory from the Portuguese National Institute of Health Dr. Ricardo Jorge (INSA). The clinical strain (INSA code 33427) is susceptible to streptomycin, isoniazid, rifampicin, and pyrazinamide (PZA); and the MDR strain (INSA code 34192) is resistant to all those antibiotics plus ethionamide.
The primary isolate HIV-$1_{UCFL1032}$ is part of our viral library stablished and maintained during the last three decades. This viral library contains a significant amount of HIV-1 and HIV-2 isolates characterized both genetically and phenotypically (Calado et al., 2010). HIV-$1_{UCFL1032}$ was isolated from a seropositive individual after cocultivation of infected patient's PBMCs with phytohemagglutinin (PHA)-stimulated PBMCs from uninfected individuals. Viral stocks were established in PBMCs from low-passaged supernatants of original cultures, aliquoted, and maintained at −80°C until used. Viral concentration was measured by reverse transcriptase (RT) activity using an ELISA (Lenti-RT kit, CavidiTech, Uppsala, Sweden). Phenotypic characterization made as described (Calado et al., 2010) on GHOST CD4+ cells individually expressing different coreceptors revealed that it uses CXCR4 coreceptor to enter host cells and has the ability to infect Mø producing low amounts of viral progeny upon inoculation, a phenotype similar to what is described during the course of Mø infection in patients (Honeycutt et al., 2017). Phylogenetically, it belongs to subtype B. The usage of CXCR4 was confirmed by preincubating PBMC-derived Mø with AMD3100, an antagonist of CXCR4 as described previously (Espirito-Santo et al., 2012; Simmons et al., 1998; Borrajo et al., 2019). This was also confirmed by the absence of proviral DNA integration by nested PCR. The usage of CXCR4 has been referred as a possible viral entry route for Mø tropic HIV; and in HIV-1-infected individuals, it was shown that this coreceptor usage broadens as the disease progresses (Simmons et al., 1998; Borrajo et al., 2019; Gorry et al., 2001; Verani et al., 1998).

All experimental procedures using live Mtb and HIV were performed in the Biosafety Level 3 laboratory at the Faculty of Pharmacy of the University of Lisbon, respecting the national and European containment level 3 laboratory management and biosecurity standards, based on applicable EU Directives. All procedures have been approved by the faculty's biological safety committee.

3.3.3. Macrophage infection

Prior to infection, bacterial cultures on exponential grown phase were centrifuged and washed in phosphate-buffered saline (PBS) and then resuspended in Mø culture medium without antibiotics. Bacterial clumps in the suspension were dismantled by ultrasonic bath treatment for 5 min. The suspension was further centrifuged for 1 min at 500 × *g* to remove residual clumps. Single-cell suspension was verified by fluorescence microscopy and quantified by optical density at 600 nm.

The infection was performed with a multiplicity of infection (MOI) of 1 bacterium per Mø and with the equivalent of 1 ng of RT of HIV-$1_{UCFL1063}$ per ml. After 3 h of infection at 37°C, 5% CO_2, the cells were washed with PBS to remove free bacteria/virus and cultivated in fresh complete medium.

Phagocytosis of the bacteria was evaluated by flow cytometry using *M. tuberculosis* H37Rv GFP-expressing strain and following the procedures described below. Monitoring of HIV infection was performed by fluorescence microscopy. Infected Mø were fixed with 4% paraformaldehyde–4% sucrose solution in PBS for 1 h and quenched with 50 mM of NH_4Cl in PBS for 15 min. Cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. Cells were stained with anti-Gag antibody diluted 1:100 (KC57, Beckman Coulter) in 1% BSA for 1 h, washed, and then incubated with Alexa Fluor 555 Goat anti-Mouse IgG secondary antibody (dilution of 1:1,000; Cell Signaling Technology) for 30 min. Coverslips were mounted using ProLong Gold Antifade Mountant (Thermo Fisher Scientific) and visualized on a Leica TCS SP8 confocal microscope. Non-infected cells were used in parallel as a negative control for the specificity of anti-Gag antibody labelling. Analysis was performed using Leica Application Suite X and ImageJ software. To further confirm the integration of the viral DNA into the host genome, a nested PCR was performed as previously described (Kumar et al., 2002). Briefly, the first round of PCR amplification was performed using an *Alu*-specific sense primer in combination with a *gag* antisense HIV-1 specific primer; the PCR products were then subjected to a second amplification reaction targeting the HIV-1 R/U5 region of long terminal repeat (LTR).

Macrophages were treated with GlutCs particles (Cs-DS) or pARG particles (pARG-DS) loaded with anti-cystatin C (CstC) siRNA SMARTpool ON-TARGETplus human CST3 siRNA (Agilent Technologies, Inc, Santa Clara, CA, USA) with target sequences (from 5’ to 3’) CAAUGACCUUGUCGAAAUC, CGUCGGCGAGUACAACAAA, GAACCACGUGUACCAAGAC, and UAGCUAGGGGUGAACUACUU or the respective siRNA non-targeting (scramble) control (Agilent Technologies, Inc, Santa Clara, CA, USA), with target sequences (from 5’ to 3’) UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, and UGGUUUACAUGUUUUCCUA. For comparison, macrophages were transfected with the same siRNAs using a ScreenFectA (ScreenFect GmbH, Eggenstein-Leopoldshafen, Germany) transfection reagent and following the manufacturer’s protocol. Macrophages were incubated with the transfection reagent and 100 nM of siRNA, which was calculated to be the same concentration of siRNA present in 1 mg/mL of DS.

3.3.4. Macrophage viability

Macrophages treated with DSs were incubated with a 10% (*v*/*v*) of PrestoBlue (Invitrogen, Carlsbad, CA, USA) resazurin-based solution at 37 °C and 5% CO_2, for 3 h. Fluorescence was quantified according to the manufacturer's instructions using a Tecan M200 Pro spectrofluorometer (Tecan, Männedorf, Switzerland). Non-treated macrophages served as references for 100% viability, and Igepal-treated macrophages (0.05%) were used as references for 0% viability.

3.3.5. Flow cytometry

Following 24 h of infection, Mø in 48-well plates were recovered with HyQTase cell detachment solution (HyClone, GE Healthcare). For the identification of apoptotic and necrotic cells, Annexin V-FITC Kit (Miltenyi Biotec) was used following the manufacturer's instructions. Cells were incubated with annexin V and propidium iodide for 20 min, washed with the appropriate kit buffer, fixed in 4% paraformaldehyde solution, and prepared using the same buffer, for 1 h. Following fixation, cells were washed again in buffer and analyzed. For surface staining of HLA molecules, detached cells were promptly fixated for 1 h. Following fixation, cells were washed and incubated with Human TruStain FcX Fc receptor blocking solution (BioLegend) for 10 min and then stained for 20 min with antibodies specific for human HLA class I (Cat # 311422, BioLegend) and HLA class II (Cat # 361716, BioLegend) molecules. Samples were analyzed in Guava easyCyte™ 5HT flow cytometer.

3.3.6. Reverse transcriptase–qPCR

Immediately following a 24- or 48-h infection, RNA was isolated from Mø in 12-well plates. RNA isolation was performed using NZY Total RNA Isolation kit (NZYTech), following the manufacturer's instructions. Total RNA measuring 200 ng was used for cDNA synthesis with NZY First-Strand cDNA Synthesis Kit (NZYTech), according to the manufacturer's instructions. qPCR was performed using NZY qPCR Green Master Mix (NZYTech) with the different sets of primers (Table 1) (Eurofins Genomics) at a final concentration of 0.5 μM.

TABLE 1 | List of qPCR primers.

Target gene		Target sequence (5′–3′)
Cystatin A	Forward	AAACCCGCCACTCCAGAAAT
	Reverse	TTATCACCTGCTCGTACCTTAAT
Cystatin B	Forward	TGTCATTCAAGAGCCAGGTG
	Reverse	AGCTCATCATGCTTGGCTTT
Cystatin C	Forward	CAACAAAGCCAGCAACGACAT
	Reverse	AGAGCAGAATGCTTTCCTTTTCAGA
Cystatin D	Forward	GATGAGTACTACAGCCGCCC
	Reverse	AGCAGAACTCTTCCTCTTTCAGT
Cystatin E/M	Forward	TCCGAGACACGCACATCATC
	Reverse	TCACAGCGCAGCTTCTCCT
Cystatin F	Forward	TCCCCAGATACTTGTTCCCAGG
	Reverse	TTCTGCCAATTTCCACCTCCA
Cystatin S	Forward	GCTCCAGCTTTGTGCTCTGCCT
	Reverse	GTCTGCTCCCTGGCTCGCAG
Cystatin SA	Forward	CTGCGGGTGCTACGAGCCAG
	Reverse	GGAGGGAGGGCAGAGTCCCC
Cystatin SN	Forward	TCCCTGCCTCGGGCTCTCAC
	Reverse	ACCCGCAGCGGACGTCTGTA
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAA
	Reverse	AATGAAGGGGTCATTGATGG

The PCR proceeded as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The qPCR was performed using a QuantStudio™ 7 Flex System (Thermo Fisher). The data were analyzed using the ΔΔCt method in the Applied Biosystems™ Analysis Software. The mRNA expression profiles were normalized with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene and finally calculated relative to non-infected samples. For each condition, three biological replicates were tested; and for every biological replicate, two technical replicates were performed. Statistical analysis was performed by ANOVA two-parameter. Gene expression heatmaps were generated using TM4 MultiExperiment Viewer software.

3.3.7. Transfection

Transfection with anti-Cst C siRNA or with scramble control siRNA was performed with ScreenFect A (ScreenFect) transfection reagent and following the manufacturer's protocol. Mø were incubated for 24 h with the transfection reagent and 50 nM of SMARTpool ON-TARGETplus human CST3 siRNA (Dharmacon, USA; target sequences: CAAUGACCUUGUCGAAAUC, CGUCGGCGAGUACAACAAA, GAACCACGUGUACCAAGAC, and UAGCUAGGGGUGAACUACUU) or the respective siRNA non-targeting control (Dharmacon, USA; target sequences: UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, and UGGUUUACAUGUUUUCCUA) in antibiotic-free medium. Following this incubation, fresh medium was added, and the cells were

incubated for an additional 2 days prior to any experiment in order to achieve maximum silencing. Silencing efficacy was measured by qPCR and Western blotting.

3.3.8. Western blotting

Total proteins were harvested using Laemmli buffer (Sigma-Aldrich) and heated at 95°C for 5 min. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 15% polyacrylamide gel and transferred to 0.2-µm pore nitrocellulose membrane (Amersham Protran, Cytiva). Membrane was blocked in 5% low-fat milk PBS with 0.1% Tween 20. Following blocking, the membrane was incubated in 1:2,000 dilution of primary antibodies specific for CstC (Cat # ABC20, Sigma-Aldrich) and β-tubulin (Cat # ab6046, Abcam) overnight. Membranes were washed in PBS-Tween and incubated with secondary horseradish peroxidase (HRP)-conjugated antibody (Cat # 1706515, Bio-Rad) for 1 h. Bands were visualized by chemiluminescence using NZY Supreme ECL HRP substrate (Cat # MB19301, NZYTech) in a ChemiDoc XRS (Bio-Rad). Quantification of band intensity was performed on ImageJ software.

3.3.9. Colony-forming unit assay

When required, infected Mø in 96-well plates were lysed in 0.05% Igepal solution. Serial dilutions of the resulting bacterial suspension were plated in Middlebrook's 7H10 with 10% OADC (Difco) and incubated for 2–3 weeks at 37°C before colonies were observable.

3.3.10. Enzymatic activity of cathepsins

Following 24 h of infection, Mø in a 96-well plate were washed with PBS and incubated in PBS with OmniCathepsin (Z-FR-AMC, Z-Phe-Arg-AMC) (Enzo Life Sciences) or cathepsin S (Z-VVR-AFC) (BioVision) fluorogenic substrate at 37°C in a Tecan M200 spectrofluorometer. Fluorescence readings were performed every 5 min. Assay specificity was verified by treating the cell lysates with general protease inhibitor E-64d or with specific cathepsin S inhibitor provided in the kit.

3.3.11. $CD4^+$ Lymphocyte proliferation

Autologous $CD4^+$ lymphocytes were obtained from healthy PPD^+ donors according to the isolation protocol described above. Positive selection of the $CD4^+$ lymphocytes was performed using anti-CD4 magnetic beads (Miltenyi Biotec). Isolated lymphocytes were cultivated in 75-cm^2 flask at 2×10^6 cells

per ml in RPMI-1640 medium (HyClone, GE Healthcare) supplemented with 15% (v/v) FBS (HyClone, GE Healthcare), 1 mM of sodium pyruvate (HyClone, GE Healthcare), 10 mM of HEPES (HyClone, GE Healthcare), and 20 UI/ml of human recombinant interleukin-2 (BioLegend) for 3 days prior to the experiment. Immediately before the experiment, the lymphocytes were stained with carboxyfluorescein diacetate succinimidyl ester (Cat # 423801, BioLegend) following the manufacturer's instructions. Mø infected with *M. tuberculosis* H37Rv or *M. bovis* BCG for 24 h were washed and cocultivated with the lymphocytes at a ratio of five lymphocytes per Mø for 5 days. $CD4^+$ lymphocytes were recovered after 5 days of coculture and analyzed using Guava easyCyte™ 5HT flow cytometer.

3.3.12. Sandwich ELISA for IFN-γ quantification

Supernatants from the previous assay were recovered following 5 days of coculture with $CD4^+$ lymphocytes and stored at −80°C for further analysis of IFN-γ secretion. The quantification was performed by sandwich ELISA using ELISA Max Deluxe Set Human for IFN-γ (Cat # 430104, BioLegend) kit and following the manufacturer's instructions. Absorbance was measured by Tecan M200 spectrofluorometer at 450 and 570 nm.

3.3.13. Preparation and physicochemical characterization of the particle formulations

Materials and reagents

Poly (L-lactic acid) (PLA) (2,000 Da) with a weight-averaged molecular mass (Mw) of 2000 was purchased from PolySciences Europe GmbH. Poly (lactic-co-glycolic acid (PLGA)-mannose (PLGA-man) was synthesized and characterized based on Conniot et al. (Conniot et al., 2019). PLGA (Resomer 503H, Mw 24,000–38,000), *D*-mannosamine hydrochloride (mannosamine·HCl), dimethylformamide, 4-dimethylaminopyridine, *N,N'*-dicyclohexylcarbodiimide, methanol, anhydrous sodium sulfate, poly(vinyl alcohol) (PVA, M_w 13,000–23,000 Da, 99% hydrolyzed), D-alpha-tocopherol polyethylene glycol 1000 succinate (TPGS), Pluronic® F-127 (PF-127), dichloromethane (DCM), and deuterated chloroform ($CDCl_3$) were purchased from Sigma-Aldrich. *N*-butyl poly-L-arginine hydrochloride (pARG, M_w range 3000–3400) was purchased from Polypeptide Therapeutic Solutions (Valencia, Spain). Glutamate chitosan (GlutCs; Protasan UP G113) was purchased from NovaMatrix (Sandvika, Norway). Quant-iT™ RNA Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Agarose, tris-acetate-EDTA (TAE) 50× buffer, and the loading buffer were purchased from VWR Scientific (Radnor, PA, USA).

3.3.14. Preparation of chitosan/arginine–siRNA polyplexes

GlutCs, pARG, and siRNA were dissolved in RNase-free water at 20.13, 3.355, and 1.342 mg mL^{-1} (100 mM), respectively. GlutCs/pARG–siRNA polyplexes were formed by quickly mixing the siRNA solution with an equal volume of GlutCs (15:1 (*m*/*m*) GluCs:siRNA ratio) or pARG (2.5:1 (*m*/*m*) pARG:siRNA ratio) solution dropwise. This mixture was further incubated under slow stirring, for 1 h at room temperature.

3.3.15. Synthesis of polymeric multifunctional DSs

PLGA-man/PLA DSs were prepared via a double-emulsion (water-in-oil-in-water (*w/o/w*)) solvent-evaporation method (Conniot et al., 2019). A PLGA-man/PLA (2:8) blend was dissolved in DCM at 50 mg mL^{-1}. A 10% (*m*/*v*) PVA aqueous solution that contained 100 µM of siRNA previously complexed with GlutCs or pARG (100 µL) was added to the organic phase containing the polymer blends dissolved in DCM. The internal aqueous phase used for the synthesis of empty NPs contained the GlutCs or pARG dissolved in the 10% (*m*/*v*) PVA. The mixture was emulsified under continuous sonication at 20% of amplitude for 15 s, using a microprobe ultrasonic processor. A second emulsion was performed by adding a 2.5% (*m*/*v*) TPGS aqueous solution (400 µL) to that w/o emulsion under the same conditions. The resultant w/o/w double emulsion was subsequently added dropwise into a 0.125% (*m*/*v*) PF-127 aqueous solution and stirred for 1 h at room temperature. NPs were separated via centrifugation at 20,000× *g* for 45 min at 4 °C (Beckman Coulter Allegra 64R High-Speed Centrifuge, Brea, CA, USA), washed with ultrapure water, and resuspended in PBS. Cy5-labeled NPs were prepared by adding 2.5 mg mL^{-1} of Cy5-grafted PLGA to the polymer blend.

3.3.16. Size distribution and ζ potential measurements

A Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) was used to determine the NP hydrodynamic mean diameter and polydispersity index (PdI) via dynamic light scattering (Conniot et al., 2019). The same equipment allowed for the determination of ζ potential of NPs measured via laser Doppler velocimetry in combination with phase analysis light scattering (Conniot et al., 2019). NPs were diluted in PBS, and their Brownian motion based on laser light scattering (NP size) and electrophoretic mobility using the Helmholtz–von Smoluchowski model (ζ potential) were determined at 25 °C via cumulative analysis (Conniot et al., 2019).

3.3.17. Entrapment efficiency and loading capacity of siRNA

The amount of siRNA anti-cystatin C (CstC siRNA) and negative control (scramble) entrapped in NPs was indirectly quantified in the supernatants collected from centrifugation following NP preparation. Entrapment efficiency (EE, Equation (1)) and loading capacity (LC, Equation (2)) were quantified using a Quant-iT™ RNA Assay Kit (broad range), following the manufacturer's instructions. The relative fluorescence for the RNA assay kit was measured using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 644 nm excitation and 673 nm emission wavelengths.

$$\text{EE}\ (\%) = \frac{\text{initial amount of siRNA} - \text{amount of siRNA in the supernatant}}{\text{initial amount of siRNA}} \times 100 \quad (1)$$

$$\text{LC}\ \left(\mu\text{g mg}^{-1}\right) = \frac{\text{initial amount of siRNA} - \text{amount of siRNA in the supernatant}}{\text{total amount of polymer}} \quad (2)$$

3.3.18. Confocal microscopy

Macrophages treated with cyanine-5-labeled DSs were fixed with 4% paraformaldehyde for 15 min and quenched with 50 mM of NH_4Cl in PBS for 15 min. Cells were permeabilized with 0.1% Triton X-100 for 5 min and counter-stained with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Coverslips were mounted using ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) and visualized on a Leica TCS SP8 confocal microscope (Leica Camera AG, Wetzlar, Germany). Analysis was performed using the Leica Application Suite X (Leica Camera AG, Wetzlar, Germany) and Fiji software (Schindelin et al., 2012).

3.3.19. Statistical analysis

Statistical analysis was performed using SigmaPlot 12. Multiple group comparisons at different time points of qPCR data were performed using ANOVA two-parameter test followed by pairwise comparisons of the groups using the Holm–Sidak test. Multiple group comparisons of the rate of cathepsins' proteolytic activity were made using one-parameter ANOVA followed by pairwise comparisons of the groups using the Holm–Sidak test. Two group comparisons of gene silencing

efficacy, bacteria colony-forming unit (CFU), Mø HLA expression, lymphocyte proliferation, and IFNγ secretion between scramble control and CstC siRNA were made using Student's t-test. All the prerequisites of the tests were verified. The considered nominal alpha criterion level was 0.05, below which differences between samples were deemed significant.

Statistical analysis was also conducted in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed using one-way ANOVA followed by a Holm–Sidak post hoc test. Two group comparisons were performed using Student's *t*-test. Differences were considered significant when the calculated adjusted-*p* value was equal to or below the alpha level of 0.05.

3.4. Discussion

Cathepsins were first described as endolysosomal proteases involved in the elimination of microorganisms or cell debris especially by professional phagocytic cells such as Mø (Magister et al., 2013; Kopitar-Jerala et al., 2006). After particle internalization and entrapment into a phagocytic vesicle, its content progressively acidifies. The phagosome will ultimately fuse with lysosomes, with subsequent acquisition and activation of cathepsins, culminating in the total digestion of the phagolysosomal content (Russell et al., 2009). In addition, cathepsins were associated with processing of microbial antigens as well as antigen presentation machinery to generate effective MHC–antigenic peptide complexes priming the adaptive immune response (Hsing et al., 2005; Pierre et al., 1998). It is conceivable that pathogens evolved strategies to manipulate these early events in order to avoid the activation of the microbicidal mechanisms and survive within these cells that otherwise would destroy them. This is the case for Mtb and HIV. Both pathogens manipulate the microbicidal mechanisms of Mø to establish chronic intracellular niches. In the case of HIV infection, the cleavage and processing of viral proteins for the assembly of new virus particles are performed by the host cathepsin B (Ha et al., 2012). An inhibition of this process impairs the infectivity of nascent virions and cell-to-cell spreading, keeping virus infection undetected by the immune system (Roberts et al., 1990; Craig et al., 1991; Titanji et al., 2013). This may account for the chronic infection in Mø limiting the spread of new viruses in normal conditions. For Mtb, we found out early that during establishment of the infection, the pathogen downregulates most cathepsins and that, with the exception of cathepsin F, most of these proteases were implicated in pathogen killing (Pires et al., 2016).

Since cathepsin proteolytic activity is regulated by Csts (Turk et al., 2012), we investigated here the role of these protease inhibitors during infection of Mø with Mtb or HIV or during coinfection. Initially,

we analyzed the gene expression of type I and II Csts during early events of infection. For *M. smegmatis*, a non-pathogenic species that is destroyed by Mø within 48 h, we found an early upregulation at 24 h of Csts A, C, F, and S. With the exception of CstSN, a Cst that is usually secreted out of the cell, all Csts showed a decreased gene expression relative to the first 24 h p.i. concomitant with the bulk destruction of bacteria in phagolysosomes (Anes et al., 2006). Thus, this may provide evidence that Csts, in physiological conditions, are operating to bring protease activity back to basal levels, following clearance of the bacteria. For Mtb at 24 h p.i., all Csts were downregulated except for CstA, which was similar to that observed for non-infected cells. Along the infection, Csts A, C, F, and SN had the most significant increases of gene expression, coincident with intracellular bacillus multiplication. Likewise, we noticed that the highest gene expression increases after 24 h p.i. for Csts A, C, and SN in mono-infection with Mtb or HIV, and coinfection with both pathogens. Since CstSN displayed an increased gene expression upon challenge with the non-pathogen *M. smegmatis*, it became less interesting to us for further investigation compared with Csts A and C, the most prominent hits in the context of pathogenic infection. Among the latter two candidates, while CstA accumulates more in the cytosol and in the nucleus (Magister et al., 2013; Ochieng et al., 2010), CtsC tends to be trafficked to the endocytic pathway (Lautwein et al., 2002; Colbert et al., 2011). This indicates that CstC could be an important target for pathogens that co-localize within the same compartments.

To further examine the impact of increased CstC expression along infection, we performed siRNA silencing of CstC in Mø prior to infection. We found that siRNA silencing of CstC had a significant antimicrobial effect against Mtb either during mono-infection or during coinfection with HIV, leading to significant reduction of CFU similar to that obtained with PZA treatment, a first-line antibiotic against Mtb. An improved killing effect following CstC depletion was also observed during infection with clinical Mtb strains including a MDR-TB. As the silencing did not affect cell death, nor alter the internalization of bacilli, we infer that the impact on bacterial killing was attributed to a direct decreased inhibition of lysosomal enzymes. CstC is a potent inhibitor of most cathepsins including cathepsins B, L, and S; and it has been shown to accumulate in endolysosomal compartments (Lautwein et al., 2002; Colbert et al., 2011). Our results indicate that the silencing of this inhibitor strongly impacts cathepsin proteolytic activity, suggesting a direct effect in the very same compartment that usually contains Mtb and, therefore, contributing to increased pathogen killing. This is particularly relevant for cathepsin S, which is active across a broad pH range (Claus et al., 1998) and strongly contributes, through the phagolysosomal system, to kill intracellular bacilli (Pires et al., 2016; Pires et al., 2017).

Previous studies have demonstrated CstC antiviral role *via* an inhibitory effect on viral proteases. In line with these evidences, CstC has been found to interfere with coronavirus replication in human lung cells (Collins et al., 1991), and in herpes simplex virus in human submandibular–sublingual and parotid cells (Björck et al., 1990), as well as with HIV in *in vitro* assays (Vernekar et al., 2015). An abnormal activity of CstC was found to target IdeS, the IgG cleaving protease of *Streptococcus pyogenes*; rather than acting as inhibitor, it enhanced IdeS activity (Vincents et al., 2008). During infection with parasites, in a murine model of *leishmaniasis*, CstC was associated with T-cell conversion from Th1 into Th2, skewing the host immune system to favor parasite propagation by inducing the secretion of the immunosuppressive IL-10 (Zi et al., 2018). Our results in HIV-infected cells lead us to propose that the increased CstC expression observed during HIV infection will affect viral spread through an inhibitory effect on viral proteases, or by affecting cathepsin activity required to process virus particles. This will contribute to maintain virus infection silenced from immune surveillance, while maintaining provirus integrated in the host genome. In fact, for HIV, CstC silencing was translated into a significant impact on OmniCathepsin enzymatic activity (albeit not as prominent as for Mtb) with a higher magnitude than measured for cathepsin S alone during Mtb infection. Altogether, our results suggest these pathogens have evolved an interesting strategy to inhibit protease activity and enhance their intracellular survival and spread, or perhaps to remain undetected within infected cells against immunosurveillance.

CstC, as a cathepsin S inhibitor and regulator, plays a pivotal role in the control of cleavage and removal of the MHC class II invariant chain (Ii) (Lautwein et al., 2002; Pierre et al., 1998). It also downregulates the MHC-II chaperon H2-DM, resulting in diminished MHC-II–peptide presentation and reduced T-cell proliferation (Zhang et al., 2019). CstC and cathepsin S have been shown to contribute to MHC class II antigen processing and presentation (Hsing et al., 2005; Zhang et al., 2019). Here, we demonstrated that the silencing of CstC induces a significant increased expression of HLA class II at the cell surface during Mtb infection and coinfection with HIV, but not during HIV mono-infection. This is in accordance with the increased cathepsin S activity during bacterial infection. This translated into a better priming of $CD4^+$ T lymphocytes in terms of high proliferation and increased IFN-γ secretion. All these results support our previous findings showing that by enhancing cathepsin S activity, a better priming of T cells by infected Mø can be achieved (Pires et al., 2017). The observed increase of IFN-γ secretion will certainly lead to proinflammatory activation of Mø with enhanced microbicidal activity against Mtb (Russell et al., 2009). IFN-γ may also contribute to control inflammation during active TB in accordance with previous studies showing that it inhibits the release of IL-1β and probably reduces lung immunopathology (Mishra et al., 2013).

Finally, we also show that silencing CstC could significantly impact the adaptive response induced by infection with the BCG, indicating that modulation of its expression may improve vaccination approaches. Previously, it was shown that the IL-10-dependent inhibition of cathepsin S observed in BCG led to decreased vaccine capacity (Sendide et al., 2005). Moreover, a recombinant BCG strain expressing active cathepsin S was able to overcome the inhibitory effects induced by IL-10 (Soualhine et al., 2007). Altogether, this suggests the potentiality of modulating cathepsin S activity by overexpression of the protein or by CstC depletion to strengthen the adaptive immune responses to infection.

Overall, the results indicate CstC as a potential therapeutic target in the Mø control of Mtb infection, which may also be proposed as a target in the context of Mtb/HIV coinfection. Here, we open new avenues for the development of future drug delivery systems for siRNA-based depletion of CstC in infected cells. Their inclusion in nanoparticles or liposomes targeting Mø receptors would allow their specific delivery to these immune cells and to concentrate them in the intracellular milieu. The resulting interference with CstC will improve cathepsin intracellular activity, overcoming the pathogen-induced blockade. Thus, CstC by restoring protease activity/inhibition balance emerges as an important new target to control infection. Also, microorganisms that depend on cellular proteases and their inhibitors might provide a solid frame for future research not only to better understand cathepsins/Cst function on pathogen replication and survival but also particularly to establish new therapeutic interventions where conventional antimicrobials have lost their efficacy.

Cs-DS was tested in the context of Mtb infection by the reference strain H37Rv and by three clinical strains with different phenotypes of drug resistance. The results show an effective capacity to silence CstC for at least 72 h during infection and following six days from the single-dose administration of Cs-DS. This treatment resulted in improved intracellular killing for all strains, despite their different drug-resistance profiles and growth kinetics. This result is in line with our previous evidence using common commercial transfection reagents (Bettencourt et al., 2013; Pires et al., 2021). In fact, taking into consideration (1) that common in vitro cell transfection methods are not translatable for the clinic; (2) that the present DS was demonstrated to be more efficient in silencing cystatin C mRNA over 96 h; and finally, (3) that it targets the siRNA to macrophages via mannose receptor where it overcomes endolysosomal degradation, it is evident they have the potential for application in complex host organisms.

For Cs-DS, the results for CstC silencing efficacy, from flow cytometry and confocal microscopy, demonstrate the success of this approach in targeting primary macrophages and in macrophages

derived from the monocytic cell line THP-1, both regarded as difficult to transfect. Altogether, these results demonstrate the potential for a chitosan-particle-based solution to modulate cystatin C expression, overcome the Mtb-induced blockade of the proteolytic function of macrophages, and improve the control of the infection. Further studies will be necessary to determine if the results obtained in this study can be replicated in vivo and to evaluate the pharmacokinetics of the treatment. It would also be of significant interest to the TB field to evaluate the efficacy of this treatment during latent Mtb infection. Here, we propose this delivery system for silencing cystatin C as a potential host-directed therapy that can be applied to complement the current antibiotic therapy and overall contribute to overcoming drug resistance.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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CHAPTER 4.

Modulation of Cystatin F in Human Macrophages Impacts Cathepsin-Driven Killing of Multidrug-Resistant *Mycobacterium tuberculosis*

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In this work I contributed to methodology and formal analysis as well as to experimental part by performing most of the experiments and validating the accuracy and reproducibility of the experimental results and data to ensure the reliability of the research. I also contributed to writing original draft preparation, review and editing.

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Abstract

Tuberculosis (TB) treatment relies primarily on 70-year-old drugs, and prophylaxis suffers from the lack of an effective vaccine. Among the 10 million people exhibiting disease symptoms yearly, 450,000 have multidrug or extensively drug-resistant (MDR or XDR) TB. A greater understanding of host and pathogen interactions will lead to new therapeutic interventions for TB eradication. One of the strategies will be to target the host for better immune bactericidal responses against the TB causative agent *Mycobacterium tuberculosis* (Mtb). Cathepsins are promising targets due to their manipulation of Mtb with consequences such as decreased proteolytic activity and improved pathogen survival in macrophages. We recently demonstrated that we could overcome this enzymatic blockade by manipulating protease inhibitors such as cystatins. Here, we investigate the role of cystatin F, an inhibitor that we showed previously to be strongly upregulated during Mtb infection. Our results indicate that the silencing of cystatin F using siRNA increase the proteolytic activity of cathepsins S, L, and B, significantly impacting pathogen intracellular killing in macrophages. Taken together, these indicate the targeting of cystatin F as a potential adjuvant therapy for TB, including MDR and XDR-TB.

4.1. Introduction

Human tuberculosis (TB) caused by the species *Mycobacterium tuberculosis* (Mtb) represents one of the deadliest infections in the world (World Health Organization, 2022). Mtb is a highly successful pathogen that primarily affects the lungs, leading to pulmonary TB, a condition required for transmission (World Health Organization, 2022; Cambier et al., 2014; Anes et al., 2021; Anes et al., 2023; Perrin et al., 2015; Wilson et al., 2005; Dheda et al., 2016; Dheda et al., 2017). The infection of other organs and tissues may also occur during extrapulmonary TB but constitutes a dead end for the pathogen (Anes et al., 2021; Anes et al., 2023; Azevedo-Pereira et al., 2023). Approximately 25% of the world's population has an immunologic activation status indicative of prior infection with Mtb while not presenting disease symptoms (World Health Organization, 2022; Anes et al., 2021; Kauffman et al., 2018; Ulrichs et al., 2006). In this latent infection, Mtb remain quiescent for decades until the reactivation of the infection, a phenomenon that will result in disease (active TB) in 5 to 10% of latent cases (World Health Organization, 2022). The most recent WHO report indicates that in 2021 ten million people developed active TB, with 1.6 million deaths. Among the active TB cases, 450,000 were caused by Mtb strains with multidrug (MDR) or extensive (XDR) drug resistance to available antibiotics. First-line chemotherapy includes the four first-line antibiotics rifampicin, isoniazid, pyrazinamide, and ethambutol for the first two months and an extension for four months with rifampicin and isoniazid (Peloquin et al., 2021). With the emergence of MDR (resistant to isoniazid and rifampicin) and XDR (MDR with resistance to any fluoroquinolone and one of three injectable second-line drugs) TB, treatment regimens are more complicated and lengthier (lasting for up to two years) with the inclusion of alternative less efficient second-line agents and high rates of failure (World Health Organization, 2022; Ramachandran et al., 2015; Quenard et al., 2017). The accelerated approval of clinical trials that are still in phase three for delamanid, bedaquiline, and pretomanid for complicated cases of MDR/XDR alone allows a limited treatment time but toxic issues and newly revealed drug resistance are still a concern (Dheda et al., 2019; Shean et al., 2013; Khan et al., 2019).

The public health threats of MDR and XDR-TB call for innovative therapeutic approaches, including host-directed therapies that can also be applied as adjunctive anti-microbial treatment. Professional phagocytes such as macrophages are archetype cells for pathogen destruction yet paradoxically contribute to a successful Mtb infection (Srivastava et al., 2014). They contribute to the establishment of Mtb intracellular niches in early phagosomes (Cambier et al., 2014; Russell et al., 2007) and to the expansion of infected cells in the lung parenchyma in structures typical of TB: the granuloma (Cambier et al., 2014; Cambier et al., 2014; Sia et al., 2019; Refai et al., 2018). Finally, macrophages provide

means for the induction of an exacerbated inflammatory response required for necrosis, a condition concomitant with uncontrolled Mtb replication (Dallenga et al., 2017; Welin et al., 2011; Lerner et al., 2017). The consequent lung cavitation will lead to pathogen transmission.

Endolysosomal cathepsins in macrophages play relevant innate and adaptive immune responses to control pathogens, including Mtb (Anes et al.,2021; Anes et al., 2022). They are directly involved in phagolysosomal pathogen proteolytic digestion and killing while making bridges for adaptive T-lymphocyte activation (Russell et al., 2007; Jordao et al., 2008; Pires et al., (Russell et al., 2007; Jordao et al., 2008; Pires et al., 2016; Pires et al., 2017).

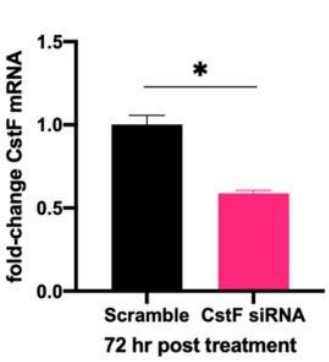
Our group provided evidence of Mtb's ability to manipulate lysosomal cathepsins and their natural inhibitors, cystatins (Pires et al., 2016; Pires et al., 2017; Pires et al., 2021), leading to their intracellular survival and poor T cell activation. Furthermore, particularly for cystatins, we found a significantly increased gene expression in the early stages of infection for cystatins C, A, and SN common to infection with Mtb and HIV (Pires et al., 2021). We overcame the Mtb-induced inhibition of cathepsins' proteolytic activity using different approaches such as targeting the regulation of gene expression using microRNAs or repurposing the HIV protease inhibitor saquinavir (Pires et al., 2017; Bettencourt et al., 2016; Pires et al., 2021; Pires et al., 2023) that we demonstrate to activate cathepsins B, L, and S. One very successful attempt to reverse the cathepsins' manipulation using Mtb was achieved by targeting cystatin C using RNA silencing (Pires et al., 2021; Pires et al., 2023).

During those gene expression studies, cystatin F (CstF) gene (CST7) emerged as the most upregulated gene during mycobacteria infection of human macrophages, including Mtb and the non-pathogen saprophyte species *Mycobacterium smegmatis* (Pires et al., 2021). Indeed, cystatin F is among the most upregulated transcripts in dendritic cells activated by bacterial lipopolysaccharide (Hashimoto et al., 2000) and was described to be downregulated in all-trans retinoic acid (ATRA)-stimulated U937 cells (causing monocytic differentiation towards macrophages) (Nathanson et al., 2002). However, because there are almost no reports on this cathepsin inhibitor during Mtb infection of human macrophages, in this work, we aim to decipher their role in Mtb intracellular killing, including during infection with MDR and XDR clinical Mtb strains.

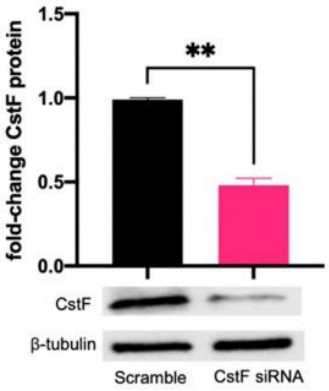
4.2. Results

4.2.1. siRNA-Mediated gene silencing effectively lowers cystatin F expression in human primary macrophages without cytotoxic effects

The manipulation of cystatin F (CstF) using siRNA-mediated gene silencing was performed, and the extension of gene expression silencing efficiency and cytotoxic effects was evaluated in macrophages. The transfection with anti-CstF was compared to a control of scramble siRNA, as previously described in the Materials and Methods section. Results indicate approximately 50% silencing of CstF mRNA (Figure 1a) and 60% of the protein amounts (Figure 1b). Next, the extent of silenced macrophages during infection with Mtb was investigated. From the qPCR data analysis, it was found that 3 h and 24 h post-infection with Mtb, CstF was silenced by approximately 60%. Moreover, after 72 h of infection, CstF mRNA remained at low levels of gene expression (Figure 1c). The results justify the efficacy of siRNA-mediated silencing in human macrophages pre- and post-infection with Mtb. Notably, there was no difference in cell viability when comparing CstF-silenced macrophages to cells transfected with a non-specific RNA (scramble) (Figure 1d).



(a)



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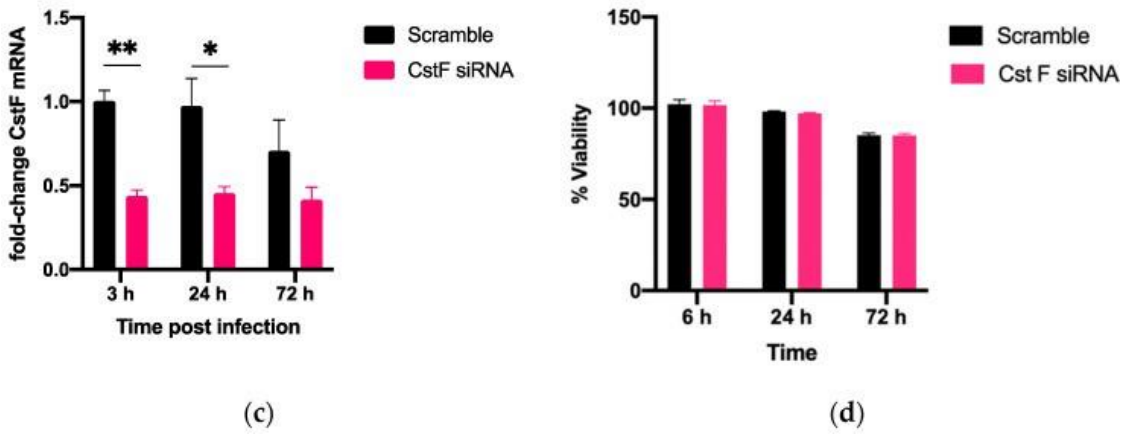
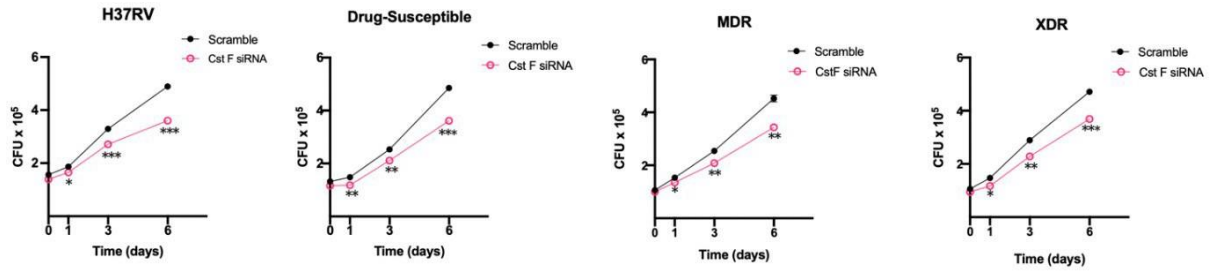


Figure 1. siRNA-mediated gene silencing effectively decreases CstF expression in human macrophages. To achieve maximum silencing, CstF was silenced by siRNA 3 days prior to infection with Mtb. (**a**) Relative gene expression of CstF mRNA in macrophages was obtained using RT-qPCR after 72 h of transfection. (**b**) The Western blot image demonstrates the silencing of the CstF protein by siRNA at the moment of infection. The respective bar plot was calculated from two independent experiments measuring band intensity using β-tubulin as a calibrator. The error bars represent the standard error of the mean. (**c**) RT-qPCR measured the relative gene expression of CstF mRNA in transfected cells 3 h, 24 h, and 72 h post-infection. The bar plots represent the average of three independent experiments, and the error bars demonstrate the standard error of the mean. (**d**) Effects of silencing on the viability of cells transfected with siRNA for CstF relative to scramble transfected cells. This was measured using PrestoBlue (resazurin-based solution) and quantifying the fluorescence emission in a plate reader. Untreated macrophages with 100% viability and 0.05% Igepal-treated macrophages with 0% viability were used as controls. * $p < 0.05$, ** $p < 0.01$.

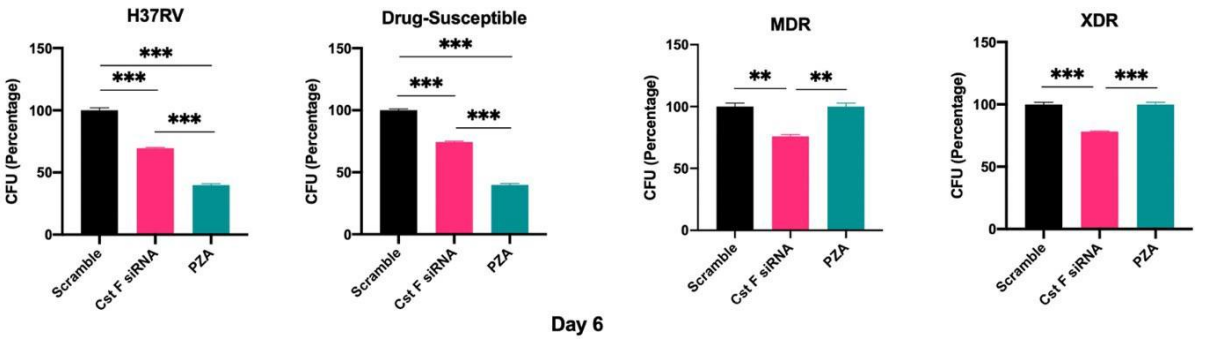
4.2.2. Silencing of CstF expression improves the intracellular killing of Mtb including clinical strains with distinct drug resistance profiles

CstF is primarily expressed in the immune cells, most prominently in dendritic cells, T cells, and NK cells (Ni et al., 1998). We decided to investigate the expression of this endogenous protein inhibitor in primary human macrophages, specifically during Mtb infection. Therefore, we targeted CstF through siRNA-mediated gene silencing as previously established in primary human macrophages (Troegeler et al., 2014). To evaluate the impact of CstF silencing on macrophages' response to Mtb infection, the quantification of the intracellular survival of Mtb was performed through colony forming units (CFU) counts of bacteria recovered from infected cells over six days (Figure 2a). The results indicate a significant impact on the macrophages' killing ability towards all Mtb strains, whether the Mtb reference laboratory or clinical strains with different drug resistance profiles. Figure 2a,b show a

significant reduction in CFU from bacteria recovered from CstF-silenced infected macrophages compared to scramble controls ($p < 0.001$). To assess if the effects on CFU counts were not affected by differences on the ability of macrophages to internalize bacteria, the amounts of Mtb in CstF-silenced macrophages was compared to those in the scramble control using flow cytometry. The results indicate similar amounts of bacteria in both conditions (Figure 2c). Moreover, the results, indeed, show no interference of cell death when comparing clinical and reference strains that could impact differences on CFU counts (Figure 2d). Overall, these results together with previous results (Figure 1d) showing no cytotoxic effects due to CystF silencing, indicate that the modulation of CstF expression in macrophages significantly impacts the intracellular killing of Mtb infection.



(a)



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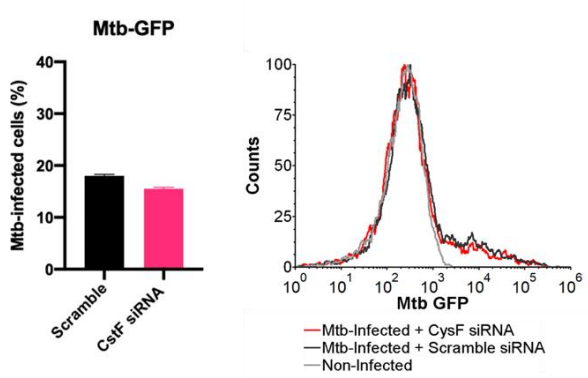


(c)

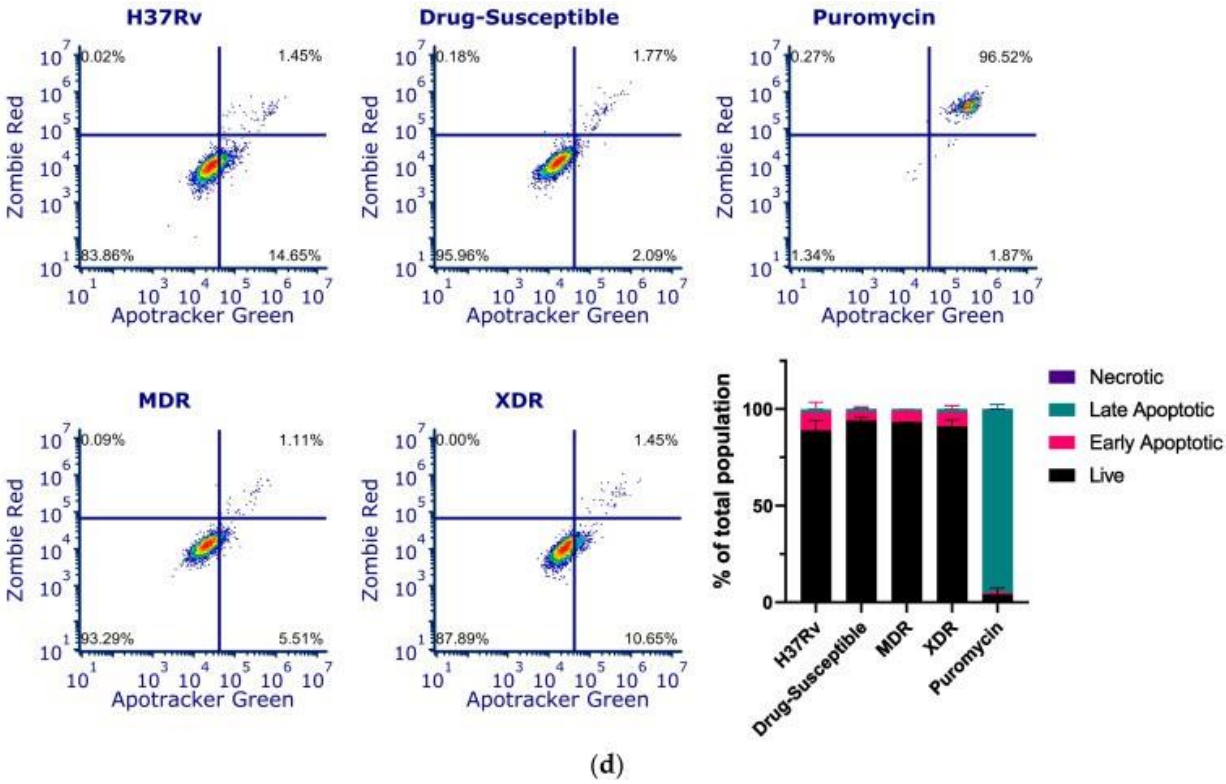
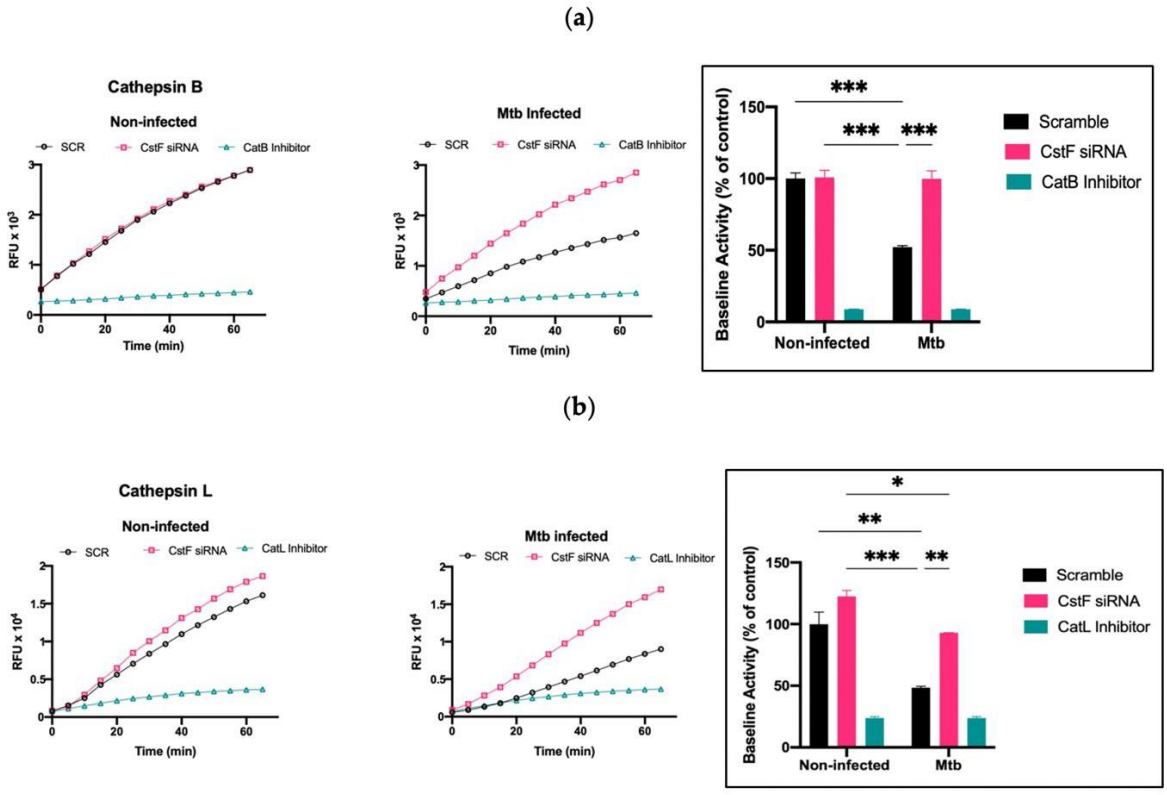


Figure 2. Silencing of CstF expression results in increased intracellular killing of reference laboratory and clinical strains of Mtb with distinct drug resistance profiles in primary human macrophages. Colony-forming units (CFU) of intracellular bacteria were recovered from human monocyte-derived macrophages transfected with anti-CstF siRNA or scramble control siRNA 72 h before infection with (**a**) Mtb reference laboratory strain H37RV and clinical strains. The intracellular survival of bacteria was evaluated at different time points. The line plots depict mean CFU per sample. (**b**) The bar plots demonstrate average CFU compared to the respective controls at Day 6 post-infection. The error bars represent the standard error of the mean. Pyrazinamide (PZA) was used as a reference to determine the killing efficacy. (**c**) The percentage of Mtb-infected macrophages was determined using flow cytometry in the scramble and CstF-silenced infected cells using a GFP-expressing Mtb strain (H37Rv). The bar plots represent the average of three biological replicates, while the error bars depict the standard error. The fluorescence intensity histograms present raw values from one of the replicates. (**d**) Cell death quantified using flow cytometry by staining macrophages with Apotracker Green (apoptosis) and Zombie Red (dead cells) dyes following 3 days of infection with laboratory and clinical strains. Live apoptotic cells were considered "early apoptotic", dead apoptotic cells were considered "late apoptotic", while non-apoptotic dead cells were considered "necrotic". The bar plot represents the mean of two independent experiments and the error bars represent the standard error of the mean. The dot plots depict representative results from one experimental replicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.3. Silencing of CstF expression significantly impacts cysteine cathepsin enzymatic activity in macrophages infected with Mtb

The modulation of CstF expression impacts the intracellular survival of Mtb in human macrophages during infection. To investigate whether the intracellular killing of Mtb was attributed to an effect on cathepsins, the proteolytic activity of cathepsins was evaluated. The activity of several cathepsins, including cathepsins B, L, and S, was measured over one hour starting at 24 h post-infection using fluorogenic peptide substrates specific for those cathepsins. Cathepsin-specific inhibitors were used as negative controls. As expected, there was a decrease in the proteolytic activity of cathepsins after Mtb infection, as shown when comparing the activity in scramble non-infected cells with Mtb scramble (Figure 3a–c). In all infected conditions, the depletion of CstF leads to an increased proteolytic activity of cathepsins (Figure 3a–c). Comparatively, CstF silencing does not affect cathepsin activities in non-infected cells.



(**c**)

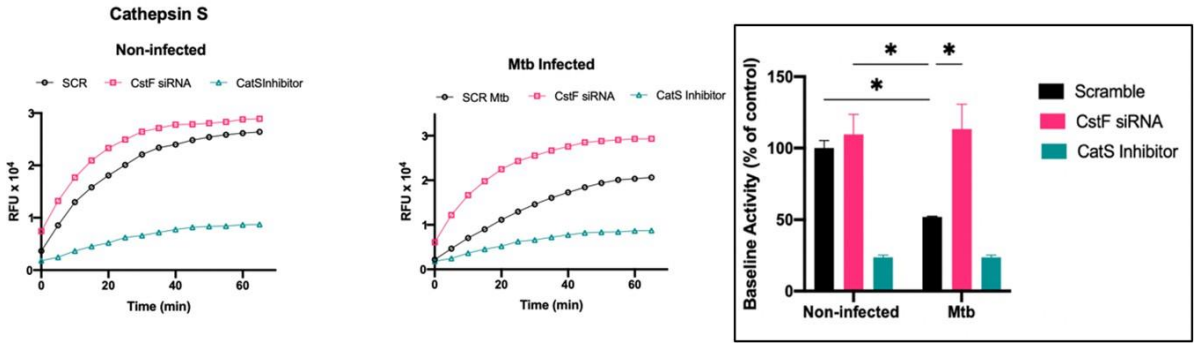


Figure 3. Silencing of CstF expression alters the proteolytic activity of cathepsins in human macrophages infected with Mtb. (**a**) Cathepsin B, (**b**) cathepsin L, and (**c**) cathepsin S activity was measured in scramble control and CstF-silenced cells using cathepsin-specific fluorogenic substrate every 5 min for 60 min. Specific inhibitors were used as negative controls. The bar plots represent average baseline activity calculated as the largest slope of fluorescence emission over 1 h. The slope of fluorescence emission in the control (scramble) for non-infected cells was represented as 100%, and each sample's effect was shown in a percentage relative to the control. The error bars represent the standard error of the mean. The line plots demonstrate average fluorescence per time. All cathepsin-specific inhibitor controls produced statistically significant inhibition of proteolytic activity ($p <$ 0.001). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Mtb, *Mycobacterium tuberculosis*; RFU, relative fluorescence units.

4.3. Materials and Methods

4.3.1. Cell isolation and culture conditions

Primary human monocyte-derived macrophages were isolated and then differentiated from buffy coats of healthy human donors, which were provided by the National Blood Institute (Instituto Português do Sangue e da Transplantação, I.P., Lisbon, Portugal) following a previously described protocol (Pires et al., 2021).

4.3.2. Bacterial cultures

M. tuberculosis H37Rv (ATCC 27294) (American Type Culture Collection, Manassas, VA, USA) and clinical strains provided and characterized by the TB National Reference Laboratory from the Portuguese National Institute of Health Dr. Ricardo Jorge (INSA) were grown in Middlebrook's 7H9 medium supplemented with 10% OADC enrichment (BD Difco, Franklin Lakes, NJ, USA), 0.02% glycerol, and 0.05% tyloxapol (Merck, KGaA, Darmstadt, Germany) at 37 °C. The clinical strain (INSA code 33427) is susceptible to streptomycin, isoniazid, rifampicin, and pyrazinamide; the MDR strain (INSA code 34192) is resistant to all those antibiotics in addition to ethionamide. The XDR strain (INSA code

163761) is resistant to all of the previous named antibiotics, and, additionally, to amikacin, kanamycin, capreomycin, moxifloxacin, and ofloxacin. All experimental procedures using live Mtb strains were performed in the biosafety level 3 laboratory at the Faculty of Pharmacy of the University of Lisbon, maintaining the national and European containment level 3 laboratory management and biosecurity standards based on applicable EU directives. The faculty's biological safety committee has also approved all those procedures.

4.3.3. Macrophage infection

Before infection, all Mtb strains were cultivated for approximately seven days at 37 °C and 5% CO_2 until the exponential growth phase was reached. On the day of infection, the bacterial suspensions were centrifuged and washed in phosphate-buffered saline (PBS) and resuspended in macrophage culture medium without antibiotics. Clumps of bacteria in the suspension were disrupted using an ultrasonic bath treatment for 5 min and removed using centrifugation at a low speed of 500× *g* for 1 min. The obtained single-cell suspension was verified through fluorescence microscopy and quantified by measuring the optical density at 600 nm. Then, the infection was performed with a multiplicity of infection (MOI) of 1 bacterium per macrophage for 3 h at 37 °C and 5% CO_2. Following this incubation period, the cells were washed with PBS to remove free bacteria and with an added fresh, complete medium.

4.3.4. Transfection

Macrophages were transfected 72 h before infection to achieve maximum silencing. Transfection with anti-CstF siRNA or with scramble control siRNA was performed using ScreenFect A (ScreenFect GmbH, Eggenstein-Leopoldshafen, Germany) transfection reagent according to the manufacturer's protocol. Macrophages were incubated for 72 h with the transfection reagent and SMARTpool ON-TARGETplus Human CST7 siRNA (Agilent Technologies, Inc., Santa Clara, CA, USA); target sequences: AGUGAAAGGCCUGAAAUAU, GAAAUUGGCAGAACUACCU, GGAUGACUGUGACUUCCAA, and CAAGGGCCCUAGUUCAGAU) or the respective siRNA non-targeting (scramble) control (Agilent Technologies, Inc., Santa Clara, CA, USA); target sequences: UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, and UGGUUUACAUGUUUUCCUA) in the medium without an antibiotic. Silencing efficacy was evaluated using qPCR data analysis.

4.3.5. Macrophage viability

Macrophages after transfection with anti-CstF siRNA or with scramble control siRNA were incubated with 10% (V/C) PrestoBlue (Invitrogen, Carlsbad, CA, USA) resazurin-based solution at 37 °C and 5% CO_2. After 2–3 h of incubation, fluorescence emission was analyzed according to the manufacturer's instructions using a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). Non-transfected macrophages were used as a reference for 100% viability, whereas macrophages treated with 0.05% Igepal were used as a reference for 0% viability.

4.3.6. Reverse transcriptase-qPCR

RNA was isolated from macrophages using NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) following the manufacturer's instruction. Total RNA measuring 100 ng was used for cDNA synthesis using an NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) according to the instructions provided by the manufacturer. To perform qPCR, an NZY qPCR Green Master Mix (NZYTech, Lisbon, Portugal) with a primer set specifically for CST7 mRNA (Forward-TCCCCAGATACTTGTTCCCAGG; Reverse-TTCTGCCAATTTCCACCTCCA) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (Forward-AAGGTGAAGGTCGGAGTCAA; Reverse-AATGAAGGGGTCATTGATGG) was used proceeding according to the previously described conditions (Pires et al., 2021). The qPCR was performed using a QuantStudio™ 7 Flex System (De Novo Software, Pasadena, CA, USA) (Thermo Fisher Scientific, Waltham, MA, USA), and data analysis was carried out using the ΔΔCt method. The mRNA expression profiles were normalized to the GAPDH housekeeping gene and calculated relative to the samples treated with the scramble control siRNA.

4.3.7. Western blotting

Proteins were harvested using a RIPA buffer (Merck, KGaA, Darmstadt, Germany). All the protein samples were diluted 1:1 in Laemmli buffer (Merck, KGaA, Darmstadt, Germany) and heated at 95 °C for 5 min before running the gel. Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA). They were transferred to the nitrocellulose membrane through the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). Next, the membrane was processed and stained using the iBind Western system (Thermo Fisher Scientific, Waltham, MA, USA) and primary antibodies specific for CstF (1:2000 dilution of #SAB2700222, Sigma-Aldrich), ß-tubulin (1:4000 dilution of #ab6046, Abcam, Cambridge, UK), and horseradish peroxidase

(HRP)-conjugated secondary antibody (1:4000 dilution of #1706515, Bio-Rad Laboratories, Hercules, CA, USA). The visualization of bands was performed through chemiluminescence using an NZY Supreme ECL HRP Substrate (NZYTech, Lisbon, Portugal) in a ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA). The band intensity was quantified using ImageLab software on USB drive #12012931 (Bio-Rad Laboratories, Hercules, CA, USA).

4.3.8. Flow cytometry

Macrophages were infected for three hours with a GFP-expressing Mtb strain to quantify bacterial internalization or with non-fluorescent H37Rv and clinical strains to quantify cell death. Macrophages were washed with PBS following three hours of infection and immediately detached (internalization experiments) or incubated for an additional three days (cell death experiments). Infected macrophages were detached using accutase. For cell death staining, Apotracker Green and Zombie Red (Biolegend, San Diego, CA, USA) dyes were used to stain apoptotic and dead cells, respectively. Furthermore, they were fixed in 4% paraformaldehyde for an hour and then analyzed in a Cytek® Aurora flow cytometer (Cytek® Biosciences, Fremont, CA, USA). Data analysis was performed in FCS Express 7 (De Novo Software, Pasadena, CA, USA).

4.3.9. Bacterial intracellular survival: Colony-forming unit assay

Infected macrophages at the selected time points (T_0, T_1, T_3, and T_6) of infection were lysed to recover intracellular bacteria using 0.05%of Igepal solution for 15 min. The resulting bacterial suspensions were serially diluted and plated in Middlebrook's 7H10 solid medium with 10% OADC (BD Difco, Franklin Lakes, NJ, USA), and incubated for 2–3 weeks at 37 °C and 5% CO_2 before colonies were observable and able to be counted under a microscope.

4.3.10. Enzymatic activities of cathepsins

After 24 h of infection, macrophages in 96-well plates were lysed with chilled cathepsin-specific lysis buffer and incubated on ice for 10 min. Cells were centrifuged at maximum speed for 5 min to recover the supernatant and a reaction buffer and substrate provided in the kit for cathepsin B or for cathepsin L (Abnova™; Thermo Fisher Scientific, Waltham, MA, USA), or cathepsin S (Biovision/Abcam, Cambridge, UK) were also added. Cathepsin-specific inhibitor supplied in the kit was used as a control to verify the assay specificity. The mixture was incubated at 37 °C for 1–2 h in a Tecan M200 spectrofluorometer (Tecan Group, Männedorf, Switzerland), taking fluorescence readings every 5 min.

4.3.11. Statistical analysis

Statistical analysis was performed in GraphPad Prism 9. Multiple group comparisons were conducted using one-way ANOVA followed by a Holm–Sidak post hoc test. Two group comparisons were made using Student's *t*-test. Differences were considered significant when the calculated adjusted *p* value was equal to or below the alpha level of 0.05.

4.4. Discussion

Proteolytic enzymes participate in several important physiological processes that maintain host cell homeostasis (Turk et al., 2012), one of which is the digestion of bacteria uptaken by phagocytosis (Russell et al., 2009). Mtb are intracellular pathogens that have their main niche in the phagosomes of host phagocytes such as macrophages. To establish themselves in this niche, the bacilli impair phagosomal maturation and the ensuing intracellular bactericidal mechanisms deployed by the macrophage (Russell et al., 2007; Welin et al., 2011). Our previous works have established how this Mtb-specific manipulation of the host macrophages interferes with their ability to express proteolytic enzymes such as lysosomal cathepsins and how the consequent defect in their activity leads to an improved Mtb intracellular survival (Pires et al., 2016). We have thus searched for different strategies to restore cellular proteolytic activity and increase the macrophages' ability to control the infection.

Cystatins are endogenous regulators of cathepsins and thus represent potential targets to be manipulated to restore cathepsin activity during infection. To this end, we have recently explored the manipulation of cystatin C and successfully developed a macrophage-directed solution that restores cathepsin activity and increases the intracellular killing of Mtb by targeting cystatin C expression (Pires et al., 2021; Pires et al., 2023). Our previous choice for cystatin C was related to its abundant expression and strong inhibitory effect on the most relevant lysosomal cathepsins, such as B, L, and S. In the present work, we have analyzed the potential of a different cystatin, cystatin F, to manipulate the macrophages' proteolytic activity and improve Mtb killing. Our previous evidence had shown cystatin F to be highly expressed during Mtb infection of primary human macrophages (Pires et al., 2021). Yet, contrary to cystatin C, which is ubiquitously expressed and mainly functional in the extracellular milieu (Nathanson et al., 2002; Turk et al., 2012; Russell et al., 2009), cystatin F is expressed uniquely in immune cells (Nathanson et al., 2002; Hamilton et al., 2008; Halfon et al., 1998). Moreover, while potentially secreted out of cells, cystatin F is mainly concentrated in the endolysosomal pathway on the producer cell or nearby immune cells that internalize this cystatin via the Man-6-phosphate

receptor (Nathanson et al., 2002; Hamilton et al., 2008; Halfon et al., 1998; Magister et al., 2013). This makes cystatin F a more promising target for the specific regulation of Mtb-infected immune cells with potentially fewer side effects.

Accumulation of cystatin F in endosomal/lysosomal vesicles directly regulates the activity of intracellular cysteine cathepsins (Lautwein et al., 2002). The monomeric form of cystatin F was shown to highly inhibit cathepsins L, V, K, and F, with a lesser extent of inhibition for cathepsins S and H and no effect on cathepsins B and C (Ni. Et al., 1998; Langerholc et al., 2005). However, a post-transcriptionally processed form of cystatin F was shown to efficiently inhibit cathepsin C with consequences to the proteolytic activation of serine proteases in T cells, NK cells, neutrophils, and mast cells (Hamilton et al., 2008). The consequent non-activated proteases include granzymes A and B, cathepsin G, elastase, proteinase 3, and mast cell chymase (Salvesen et al., 1990).

As mentioned before, cystatin F is highly expressed in dendritic cells, particularly in those undergoing LPS-induced maturation (Hashimoto et al., 20009. On the other hand, in the monocytic cell line U937, cystatin F downregulation has been described in response to phorbol ester stimuli inducing differentiation towards macrophages (Nathanson et al., 2002). Here, for the first time, we showed that interference in cystatin F expression in primary human macrophages impacts the expression of cathepsins B, L, and S during Mtb infection, demonstrating a role for this cystatin in regulating these lysosomal cathepsins in macrophages. Per our previous results (Pires et al., 2016; Pires et al., 2021 (Modulation Cst C); Pires et al., 2021 (Repurposing SQV)), when macrophages were infected with Mtb, a concomitant decrease in cathepsin activity was observed for the three cathepsins analyzed. By targeting cystatin F expression, the levels of proteolytic activity could be restored to the basal levels detected before infection. For cathepsins L and S, these results agree with previous reports showing direct regulation using cystatin F (Ni et al., 1998; Langerholc et al., 2005), whereas no evidence of direct inhibition was found for cathepsin B. Our results do not distinguish between a direct or indirect role for cystatin F, which might explain the contradictory results.

As previously mentioned, reports on cystatin F's role in immune system cells have been primarily focused on its negative regulation of cathepsin-mediated activation of granzymes and the cytotoxic activity of cytotoxic T cells, NK cells, and granulocytes (Kos et al., 2018). Here, we demonstrated the impact of cystatin F on primary macrophages' ability to control Mtb's intracellular burden. Silencing cystatin F resulted in a lower intracellular survival of the bacteria. Furthermore, we could reproduce those results in several clinical strains with different drug resistance phenotypes. This is the first report connecting cystatin F and a bacterial infection in macrophages. It agrees with the hypothesis that Mtb

interferes with macrophages' cathepsins and that we can restore their function by silencing cathepsin inhibitors, thus improving Mtb killing. This is in line with our previous finding for a different cathepsin inhibitor, cystatin C, also producing similar results in macrophages (Pires et al., 2021).

Altogether, our results reveal a promising new host molecule that can be targeted to improve the control of Mtb infection, even in cases of multiple and extensive drug resistance. Being a more specific cystatin of immune cells, future studies should explore if other aspects of the immunopathology induced by Mtb can be interfered with by targeting this cystatin.

4.5. References

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CHAPTER 5.

Cystatin F Depletion in *Mycobacterium tuberculosis*-Infected Macrophages Improves Cathepsin C/Granzyme B-Driven Cytotoxic Effects on HIV-Infected Cells during Coinfection

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In this work I contributed to methodology and formal analysis as well as to experimental part by performing most of the experiments and validating the accuracy and reproducibility of the experimental results and data to ensure the reliability of the research. I also contributed to writing original draft preparation, review and editing.

5. CstF depletion from Mtb-infected macrophages increases the cytotoxic activity to HIV infected cells

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5. CstF depletion from Mtb-infected macrophages increases the cytotoxic activity to HIV infected cells

Abstract

Cystatin F (CstF) is a protease inhibitor of cysteine cathepsins, including those involved in activating the perforin/granzyme cytotoxic pathways. It is targeted at the endolysosomal pathway but can also be secreted to the extracellular milieu or endocytosed by bystander cells. CstF was shown to be significantly increased in tuberculous pleurisy, and during HIV coinfection, pleural fluids display high viral loads. In human macrophages, our previous results revealed a strong upregulation of CstF in phagocytes activated by interferon γ or after infection with *Mycobacterium tuberculosis* (Mtb). CstF manipulation using RNA silencing led to increased proteolytic activity of lysosomal cathepsins, improving Mtb intracellular killing. In the present work, we investigate the impact of CstF depletion in macrophages during the coinfection of Mtb-infected phagocytes with lymphocytes infected with HIV. The results indicate that decreasing the CstF released by phagocytes increases the major pro-granzyme convertase cathepsin C of cytotoxic immune cells from peripheral blood-derived lymphocytes. Consequently, an observed augmentation of the granzyme B cytolytic activity leads to a significant reduction in viral replication in HIV-infected $CD4^+$ T-lymphocytes. Ultimately, this knowledge can be crucial for developing new therapeutic approaches to control both pathogens based on manipulating CstF.

5.1. Introduction

Mycobacterium tuberculosis (Mtb) and the human immunodeficiency virus (HIV) are syndemic interaction pathogens (Waters et al., 2020; Bell et al., 2018; Azevedo-Pereira et al., 2023). They synergize an accelerated progression to tuberculosis (TB) and to acquired immune deficiency syndrome (AIDS) during coinfection (Bell et al., 2018; Montales et al., 2015; World Health Organization, 2023). Both are responsible for a paradoxical effect observed in coinfected patients after the initiation of antiretroviral therapy (ART), referred to as immune reconstitution inflammatory syndrome (IRIS), a severe local and systemic inflammatory response (Lai et al., 2016). Approximately 13 million people are estimated to be coinfected with both pathogens, accounting for 250,000 deaths in 2022, with about 1.3 million new infections by HIV (UNAIDS, Available online: https://www.unaids.org/en) and 1.4 million with Mtb (World Health Organization, 2023). While antibiotic therapy to treat TB exists, as well as an established ART for controlling chronic HIV infection, the rising resistance to both treatments and drug–drug interactions are posing serious concerns for the effective control of pathogens and instructing an urgent need for new therapies (Bares et al., 2020; Jones et al., 2022; Sun et al., 2022; Navasardyan et al., 2024; World Health organization. HIV Drug Resistance Report 2021; Olivença et al., 2022; Pires et al., 2015; Pais et al., 2022).

TB is a leading cause of death among HIV-infected people (UNAIDS Topic: Tuberculosis, 2024). The interactions between HIV and Mtb that contribute to tuberculosis progression from latency, as a result of the immunosuppressive environment of type I interferons and IL-10, have been the subject of more extensive studies than those that Mtb employs to enhance virus replication and persistence (Azevedo-Pereira et al., 2023; Bares et al., 2020; Toor et al., 2014; Dupont et al., 2022; Dupont et al., 2020; Souriant et al., 2019). A deeper understanding of the pathways underlying these interactions may contribute to the control of both pathogens and the identification of new targets for the development of new efficacious therapeutics, particularly in the context of coinfection.

Our group has investigated the role of lysosomal cathepsins and their inhibitors, cystatins, during Mtb infection (Pires et al., 2016; Anes et al., 2022; Pires et al., 2023) and during HIV coinfection (Pires et al., 2021; Anes et al., 2021). The results revealed that Mtb can block cathepsin proteolytic activity, which contributes to its intracellular survival in macrophages (Mϕs) and poor activation of T lymphocytes. Concerning the natural inhibitors, there was a significant increase in gene expression for cystatins C, A, and SN during the early stages of infection, which was evident and common in both Mϕ monoinfection and coinfection with Mtb and HIV (Pires et al., 2021). Cystatin F (CstF) showed the

highest upregulation among the inhibitors, during Mtb monoinfection of Mϕs or in cells activated by interferon γ (IFN-γ) (Pires et al., 2021 Cst C Modulation). Indeed, we have developed various strategies to overcome the blockade induced by Mtb, including the regulation of gene expression with microRNAs (Pires et al., 2017) or using RNA silencing for cystatins (Pires et al., 2023; Pires et al., 2021; Mandal et al., 2023). In addition, we have demonstrated that saquinavir, an HIV protease inhibitor used in ART, can impact cathepsin enzymatic activity and overcome the Mtb-induced blockade (Pires et al., 2021; Pires et al., 2023). These results collectively demonstrate the potential of target protease inhibitors to control Mtb infection.

Mtb infects Mϕs, where it establishes intracellular niches (Mwandumba et al., 2004; Anes et al., 2003; Jordao et al., 2008). Appropriate immune responses for their intracellular clearance require $CD4^+$ T lymphocytes, particularly Th1, as well as other lymphocytes, including $CD8^+$ T cells (CTLs) and natural killer cells (NK) (Kaufmann et al., 2023; Portevin et al., 2012). Most of the effects on Mtb-infected cells are based on IFN-γ released by those lymphocytes that mediate Mϕ activation, leading to a more microbicidal state (Russell et al., 2009). The cytotoxic effector cells that result from this cytokine-activated infection environment will contribute to the control of the infection via the release of granzyme–perforin-mediated macrophage apoptosis. Infected and newly arrived noninfected Mϕs and lymphocytes come into close contact in one structure, the granuloma, which is a hallmark of TB, or in nearby tissues during the establishment of the infection. HIV-1 has a cell tropism for $CD4^+$ T cells and establishes intracellular reservoir sanctuaries in Mϕs (Aquaro et al., 2002). Although the simultaneous coinfection of Mϕs with both pathogens is possible in vitro, it has not been demonstrated in vivo (Bell et al., 2018; Pires et al., 2021). Cytotoxic immune cells, including Th1 cells but mainly NK cells and CTLs, induce the cell death of infected cells mediated by granzyme and perforin, constituting a pivotal response to control viral infections. However, an ineffective viral clearance occurs during HIV infection (Alter et al., 2007; Borrow et al., 2008; Asquith et al., 2006; McMichael et al., 2010).

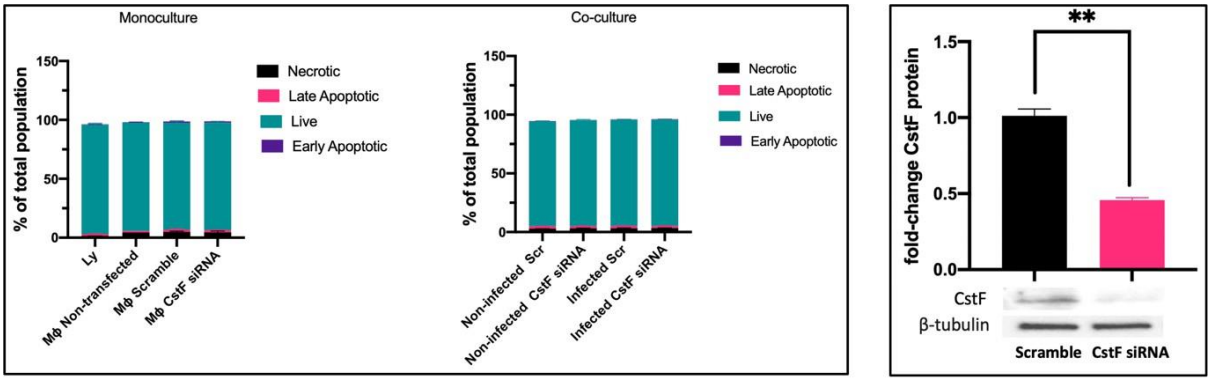
One of the most frequent manifestations of TB during HIV coinfection is pleurisy (Cohen et al., 2015). Both the pleural milieu and the granuloma structure provide the appropriate contact between Mtb-infected cells and noninfected phagocytes with HIV-infected lymphocytes in a particular environment of cytokines and other factors that, all together, help viral replication and spread (Toosi et al., 2001; Ferrer et al., 1997). Surprisingly, CstF levels were found to be significantly increased in the pleural fluids of TB patients compared to other inflammatory conditions (Werle et al., 2003).

In addition to the previously demonstrated Mtb microbicidal effects, this study aims to assess the potential of cystatin F manipulation to control HIV infection at the coinfection interface. To achieve this, we depleted cystatin F by siRNA in Mϕs prior to infection with Mtb and evaluated the trans effects on cultures of peripheral blood-derived lymphocytes infected with HIV. In conclusion, the results of this study indicate potential future directions for controlling both syndemic pathogens through CstF manipulation.

5.2. Results

5.2.1. Experimental Conditions for Transfection and Infection Produce Comparable Low Levels of Cell Death

We established the experimental conditions that produce comparable and low levels of cell death between infected and noninfected cells. Mtb-infected Mϕs and HIV-infected lymphocytes were analyzed for their effect on cell viability after transfection and 12 h of infection. Infected cells in monocultures were also compared to those in cocultures. Cell death was evaluated by flow cytometry using markers for apoptosis and necrosis, namely Apotracker Green and Zombie Red, respectively. Lymphocytes were distinguished from monocytes using CD3-specific Alexa Fluor® 700 antibodies. Figure 1a shows that there were no differences observed in Mϕ monoculture conditions when comparing CstF-silenced phagocytes to cells transfected with scramble or non-transfected. Notably, during coculture, noninfected (scramble) or CstF siRNA-treated cells displayed similar viability to the respective infected conditions (Figure 1a, coculture). Indeed, monocultures of HIV-infected lymphocytes displayed high viability by the end of 12 h p.i. Therefore, it can be concluded that the experimental conditions established here and previously (Calado et al., 2010) produce cells with similar viability and low impact at the early time points of the coinfection, resulting in low interference for the next coculture assays. CstF was depleted by siRNA prior to the infection of macrophage cultures with Mtb. As shown in Figure 1b, approximately 60% of protein depletion was achieved. The CstF siRNA-treated cells were used in all subsequent experiments, ensuring that the silencing level remained consistent across all conditions assayed.



(**a**) (**b**)

Figure 1. (**a**) Flow cytometry analysis of the percentage of live cells relative to those in programmed cell death (**a**). Apotracker Green (apoptosis), Zombie Red (necrotic cells), and CD3 Alexa Fluor 700 (lymphocytes) dyes were used to stain monocultures for each pathogen or cocultures of Mtb-infected Mϕs and HIV-infected lymphocytes. No interference in cell viability was observed on either transfected cells (3 days post-treatment) or infected cells (12 h after infection with pathogens). There were no differences observed in programmed cell death when CstF expression was silenced in Mtb-infected Mϕs cocultured with HIV-infected lymphocytes (right panel; coculture conditions). (**b**) The Western blot image demonstrates the silencing of the CstF protein by siRNA at the moment of infection. The respective bar plot was calculated from three independent experiments measuring band intensity using β-tubulin as a calibrator. The error bars represent the standard error of the mean (** $p \leq 0.01$).

5.2.2. Decreased CstF Levels from Mtb-Infected Macrophages Are Correlated with Increased Enzymatic Activity of Cathepsin C in Lymphocytes during Coinfection

CstF can be internalized from the extracellular milieu into the endocytic pathway of cytotoxic immune cells, where the inhibition of the pro-granzyme convertase cathepsin C occurs (Nanut et al., 2017; Prunk et al., 2020). As we previously observed the high expression of CstF during Mϕ infection with Mtb and in noninfected phagocytes, activated by IFN-γ (Pires et al., 2021), here, we depleted CstF using siRNA on infected phagocyte cultures and evaluated its effects on cathepsin C during coculture with lymphocytes. The enzymatic activity was measured by continuously monitoring the formation of fluorescent degradation products using a specific fluorogenic substrate for cathepsin C. A cathepsin-specific inhibitor was used as negative control. Figure 2 shows the significantly higher enzymatic activity of cathepsin C when co-cultivated Mϕs were treated with CstF siRNA compared to the respective scramble control during HIV coinfection conditions. No detectable effects on cathepsin C activity in response to CstF silencing were observed for Mϕ cocultures infected with Mtb with noninfected lymphocytes (Figure 2). Overall, the results indicate that countering CstF overexpression by depleting CstF in Mtb-infected Mϕs impacts the enzymatic activity of cathepsin C in lymphocytes during coinfection with HIV (Liu et al., 2019; Alam et al., 2019).

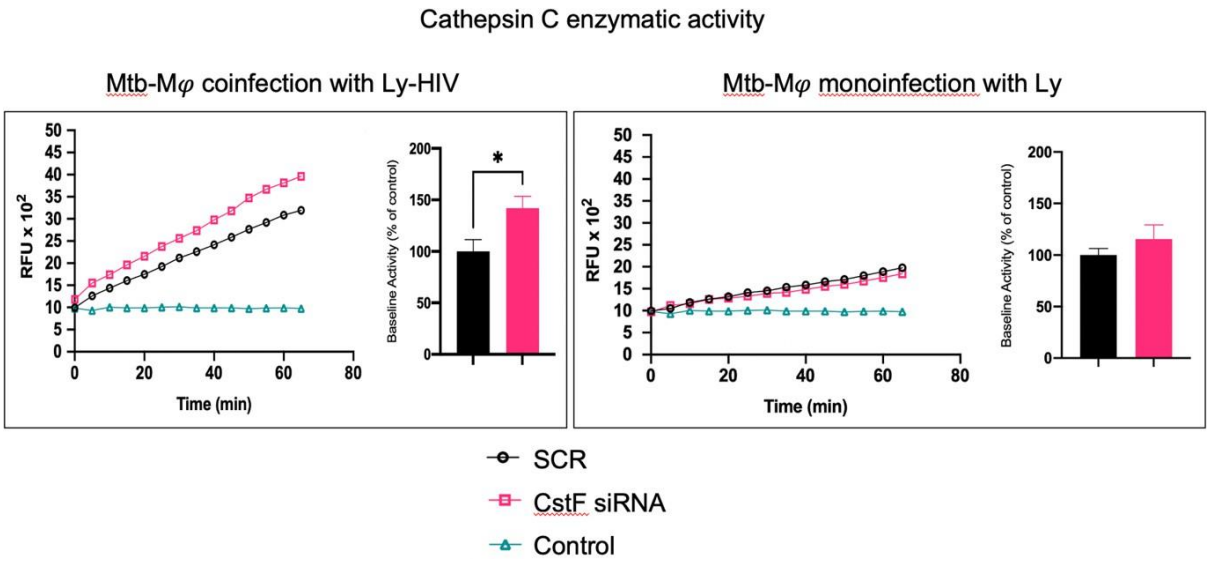


Figure 2. Silencing of CstF expression affects the proteolytic activity of cathepsin C. The enzymatic activity was measured in both scramble control and CstF-silenced cells during cocultures of macrophages with lymphocytes during monoinfection with Mtb or during coinfection with lymphocytes infected with HIV. A cathepsin C-specific fluorogenic substrate was used every 5 min for 60 min. A specific inhibitor was used as a negative control. The bar plots represent the average baseline activity calculated as the largest slope of fluorescence emission over 1 h from three biological replicates. The slope of fluorescence emission in the scramble control was represented as 100%, and each sample's effect was shown in a percentage relative to the control. The error bars represent the standard error of the mean. The line plots represent fluorescence over time from one representative experiment. (* $p \leq 0.01$). Mϕ, macrophages; Mtb, *Mycobacterium tuberculosis*; RFU, relative fluorescence units.

To further elucidate the contribution of noninfected Mϕs and Mtb-infected cells as sources of cathepsin C and a potential activation of cytotoxic activity from phagocytes to lymphocytes, we assessed the effects of IFN-γ in substitution of the activation provided by lymphocytes. In this manner, the possibility of lymphocytes serving as a potential source of cathepsin C, as expected in coculture, is eliminated.

As shown in Figure 3a, the treatment of noninfected monocultures with IFN-γ resulted in an increase in the enzymatic activity of cathepsin C when compared to the scramble control. However, this activity is not observed in Mtb-infected cells, where IFN-γ treatment results in a further reduction in cathepsin C activity in comparison to the scramble (Figure 3a). This finding corroborates our previous results on gene expression for cathepsin C, which indicated a downregulation of the irrespective gene [21]. Moreover, no differences in cathepsin C activity were observed in monoculture conditions with Mtb infection between resting Mϕs, Mϕs activated by IFN-γ (Figure 3a), or phagocytes in coculture with

noninfected lymphocytes (Figure 2). Furthermore, the depletion of CstF had no impact on the enzymatic activity of cathepsin C in IFN-γ-activated Mϕs with Mtb in comparison to the scramble control (Figure 3b).

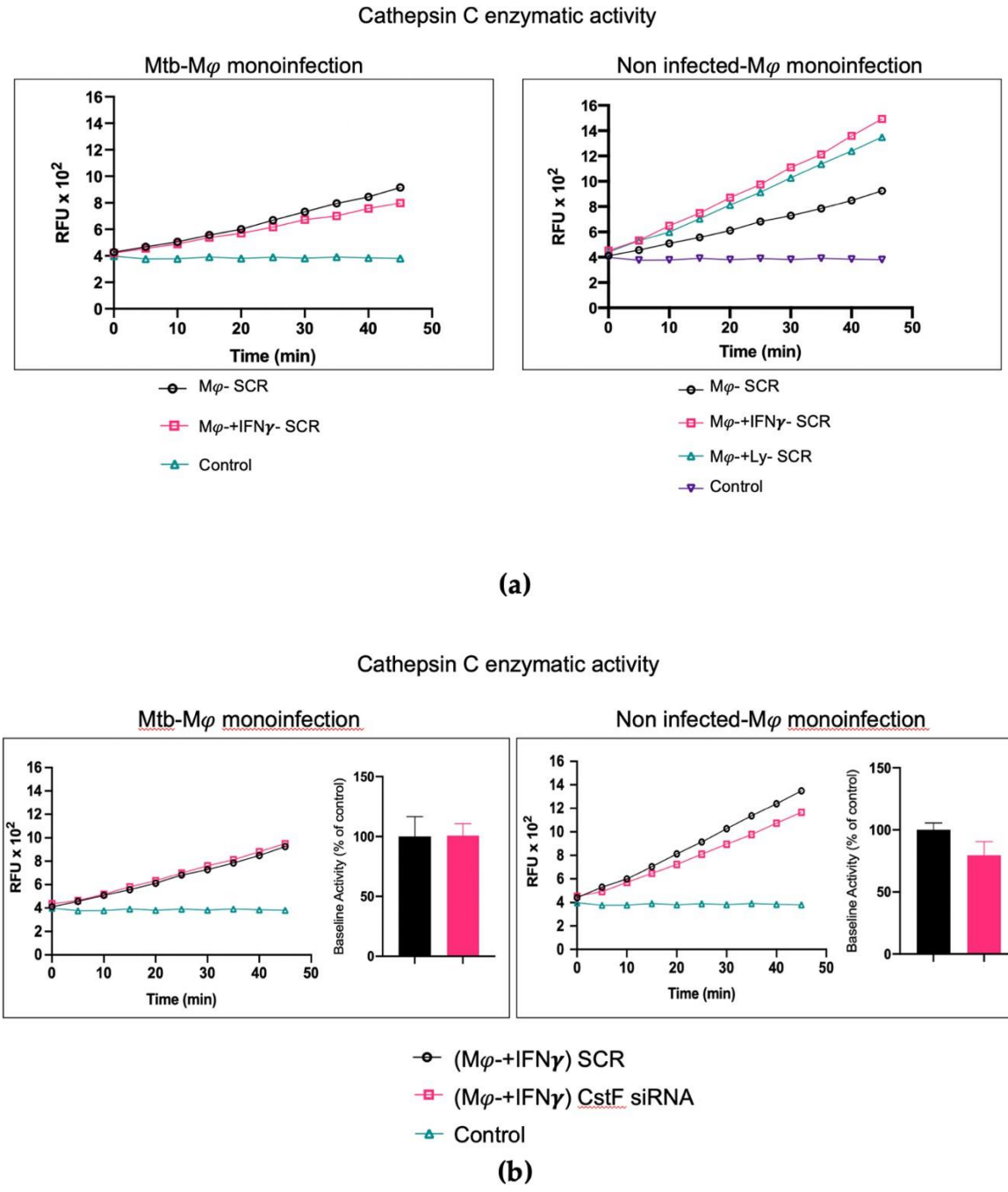


Figure 3. In contrast to noninfected cells, IFN-γ-activated Mϕs infected with Mtb do not serve as a source of cathepsin C. (**a**) The enzymatic activity of cathepsin C was compared between resting macrophages and macrophages treated with IFN-γ or in coculture with lymphocytes relative to the control. (**b**) The silencing of CstF expression does not affect the proteolytic activity of cathepsin C in noninfected cells or in cultures infected with Mtb. The enzymatic activity was quantified in both the scramble control and CstF-silenced cells during

monocultures of macrophages, which were either activated or not by IFN-γ. A cathepsin C-specific fluorogenic substrate was used at 5 min intervals over a 60 min period. A specific inhibitor was used as a negative control. The bar plots represent the mean baseline activity, calculated as the greatest slope of fluorescence emission over one hour from three biological replicates. The fluorescence emission slope in the scramble control was set at 100%, and the effect of each sample was expressed as a percentage relative to this control. The error bars represent the standard error of the mean. The line plots represent the temporal evolution of fluorescence in a single representative experiment. Mϕ, macrophages; Mtb, *Mycobacterium tuberculosis*; RFU, relative fluorescence units.

In contrast, in noninfected cells, a significant effect on cathepsin C activity is observed in IFN-γ-treated cells or in coculture with lymphocytes in comparison to resting Mϕs (Figure 3a), with non-significant effects in CstF-depleted cells compared to scramble control (Figure 3b). It can be concluded that the effect of CstF depletion on cathepsin C activity was only observed in conditions of the coculture of Mtb-infected Mϕs with HIV-infected lymphocytes.

5.2.3. CstF Depletion Is Correlated with Increased Cathepsin C–Granzyme B-Driven Cytotoxic Effects

It was next investigated whether higher levels of cathepsin C-induced proteolysis led to increased granzyme B activity in cytotoxic lymphocytes. As expected, granzyme B activity was significantly higher in CstF-silenced conditions and coculture conditions during coinfection with HIV compared to the scramble control (Figure 4a). However, no effects were observed during the coculture of Mtb-infected Mϕs with noninfected lymphocytes since the depletion of CstF did not impact the granzyme B activity of those cells compared to the scramble control (Figure 4) (Kim et al., 2007; Velotti et al., 2020; Boivin et al., 2009). To further confirm that the increased activity of granzyme originates from lymphocytes rather than from Mϕs, the effects were evaluated in Mϕs activated by IFN-γ in accordance with the results on cathepsin C described above. As shown in Figure 4b, no differences in granzyme activity were observed when comparing IFN-γ-activated Mϕs with non-treated controls. Moreover, a significant increase in cathepsin C activity was observed in noninfected phagocytes, which did not result in any discernible impact on granzyme proteolytic activation. Indeed, the depletion of CstF did not affect granzyme activity when it was compared with the scramble conditions. Upon the evaluation of the cocultures of noninfected macrophages with lymphocytes (Figure 4b), no effects on the depletion of CstF were detected. Conversely, a general increase in granzyme activity was observed when lymphocytes were infected with HIV. This is analogous to the increase in granzyme activity observed during Mtb and HIV coinfection (Figure 4a).

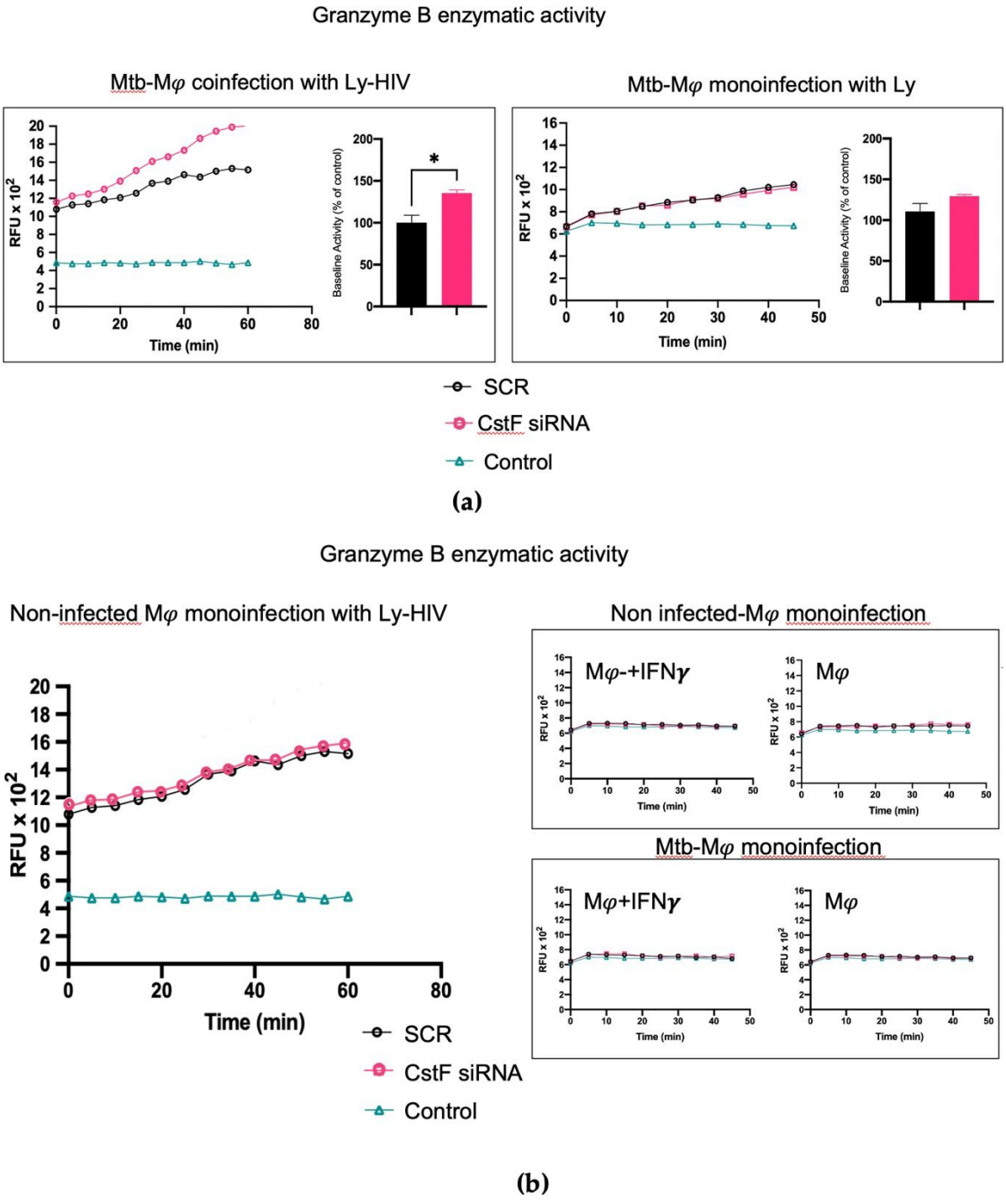


Figure 4. The silencing of CstF expression impacts the cytotoxic activity of granzyme B during Mtb coinfection with HIV. (**a**) The coculture of Mtb-infected macrophages with lymphocytes during coinfection with HIV or during monoinfection with Mtb. (**b**) Effects on monocultures of macrophages, either activated by IFN-γ or not, and on noninfected macrophages in a coculture with lymphocytes infected with HIV. The enzymatic activity of granzyme B was quantified in the cells. This was carried out in both a scramble control and in CstF-silenced cells. A granzyme B-specific fluorogenic substrate was used at 5 min intervals over a 60 min period. A specific inhibitor was used as a negative control. The bar plots represent the mean baseline activity, calculated as the greatest slope of fluorescence emission over one hour from three biological replicates. The fluorescence emission slope of the

scramble control was set at 100%, and the effect of each sample was expressed as a percentage relative to this control. The error bars represent the standard error of the mean. The line plots illustrate the fluorescence per time from a single representative experiment. (* $p \leq 0.01$). Mϕ, macrophages; Mtb, *Mycobacterium tuberculosis*; RFU, relative fluorescence units.

We conclude that the depletion of CstF interferes with the granzyme enzymatic activity of lymphocytes exclusively in the context of Mtb–HIV coinfection. The results indicate that the cytotoxic activity is provided through a decreased secretion of CstF from Mϕs rather than through granzyme activation in these phagocytes. Indeed, from the results obtained, it may be inferred that the increase in granzyme B is not achieved through a potential effect of CstF depletion on noninfected macrophages, which would potentially lead to an increased secretion of cathepsin C from macrophages to lymphocytes.

5.2.4. CstF Depletion Improves Cathepsin C/Granzyme B-Driven Reduction in Viral Replication During Mtb–HIV Coinfection

We next aimed to determine whether the increased cytotoxic effects of granzyme B could decrease HIV replication in Mtb–HIV cocultures. As shown in Figure 5, there was a significant decrease in viral particle production at the end of 72 h p.i., as assessed by reverse transcriptase analysis of culture supernatants. Noninfected lymphocytes were used as a negative control.

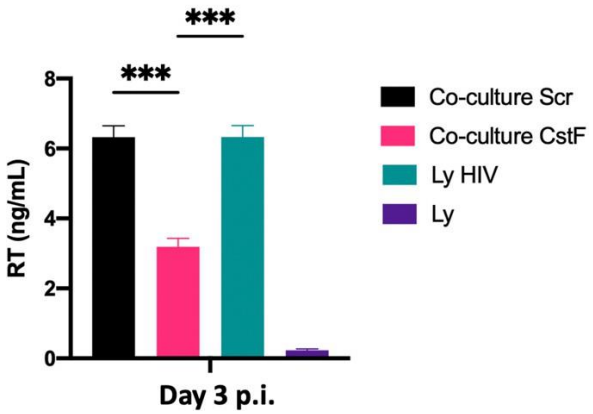


Figure 5. Depletion of CstF in Mtb-infected Mϕs enhances cathepsin C/granzyme B-mediated cytotoxic effects on HIV-infected cells during coinfection. Mtb-infected cells, whether CstF-depleted or scrambled, were cocultured with HIV-infected lymphocytes. A significant decrease in viral replication, as determined by RT activity in culture supernatants, was observed in Mtb-infected cells with CstF depletion. HIV-infected lymphocytes were used as a positive control and, as a negative control, we used cells that were not inoculated with HIV. Bar plots represent the average of three biological replicates from one representative experiment performed in duplicate. The error bars represent the standard error of the mean. Scr, scramble (*** $p \leq 0.001$).

The results indicate that the manipulation of CstF during coinfection conditions may represent a potential strategy for controlling HIV infection at the interface of the infection with Mtb and HIV. Finally, to provide further evidence on the effects of CstF manipulation on HIV depletion, the impact of granzyme B-driven apoptosis was evaluated. Figure 6 shows that apoptosis was more pronounced in CstF-silenced cocultures of Mtb-infected Mϕs and HIV-infected lymphocytes when compared to the scramble control by the end of three days post-infection. This suggests that the depletion of CstF from Mtb-infected Mϕs has a significant impact on cathepsin C/granzyme B-driven apoptosis of lymphocytes.

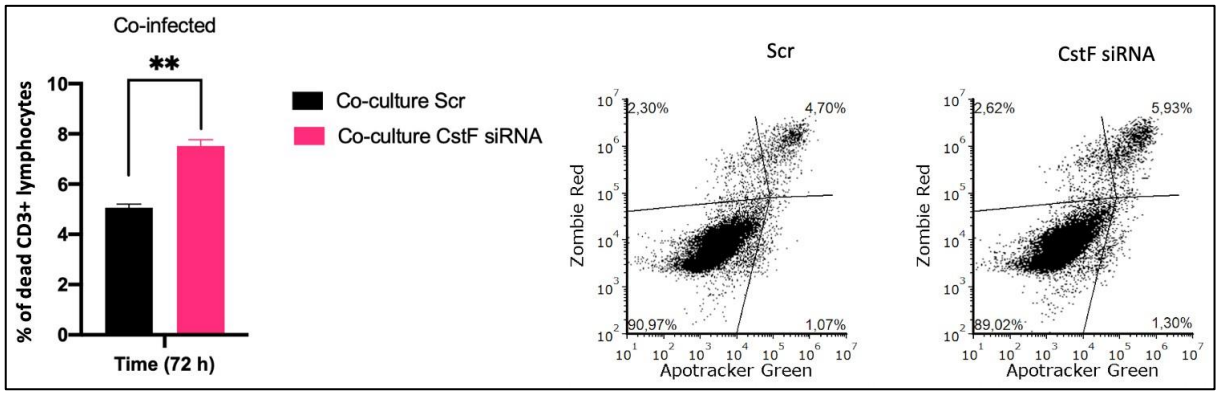


Figure 6. Manipulation of CstF expression during the coinfection of Mtb-infected Mϕs with HIV-infected lymphocytes induces granzyme B apoptotic effects. Apotracker Green (apoptosis), Zombie Red (dead cells), and CD3 Alexa Fluor 700 (lymphocytes) dyes were used for staining, and analysis was performed using flow cytometry 72 h p.i. Results represent the mean of three biological replicates (** $p \leq 0.01$). The error bars represent the standard error of the mean. The dot plots depict representative results from one experimental replicate.

Overall, the results demonstrate that the depletion of CstF in Mtb-infected cells correlates with increased activity of cathepsin C and granzyme B on lymphocytes, resulting in higher cytotoxicity of lymphocytes towards HIV-infected cells and leading to a reduction in viral replication.

5.3. Materials and Methods

5.3.1. Cell Isolation and Culture Conditions

Primary human monocyte-derived Mϕs were isolated and then differentiated from anonymous buffy coats of healthy human donors, which were provided by the National Blood Institute (Instituto Português do Sangue e da Transplantação, I.P., Lisbon, Portugal) following a previously described protocol (Pires et al., 2021). Autologous lymphocytes were obtained from the peripheral blood mononuclear cell (PBMC) fractions by lysing red blood cells. The lymphocytes were then stimulated with 3 µg/mL of Phytohemagglutinin-L (PHA-L) (ThermoFisher, Waltham, MA, USA) for three days prior to infection with HIV. They were further cultured in a 75 cm^2 flask at 2×10^6 cells per mL in a Roswell Park Memorial Institute (RPMI) medium (RPMI-1640) (Hyclone, GE Healthcare, Hertfordshire, UK) supplemented with 15% (*v*/*v*) Fetal Bovine Serum (FBS) (Hyclone, GE Healthcare), 2 mM L-glutamine (Gibco), and 20 UI/mL of human recombinant interleukin-2 (BioLegend, San Diego, CA, USA). IL-2 or M-CSF were not further added during coculture procedures. IFN-γ was used at a concentration of 100 IU/mL for the activation of Mϕs when required.

5.3.2. Bacterial Cultures and HIV Isolates

M. tuberculosis H37Rv (ATCC 27294) (American Type Culture Collection, Manassas, VA, USA) (Mtb) was grown in Middlebrook's 7H9 medium supplemented with 10% Oleic acid–Albumin–Dextrose–Catalase enrichment (OADC) (Difco, Omagh, UK), 0.02% glycerol, and 0.05% tyloxapol at 37 °C. The primary HIV-$1_{UCFL1032}$ isolate was obtained by coculturing PBMCs isolated from the infected patient with PBMCs from uninfected individuals, as described (Calado et al., 2010). After isolation, viral stocks were established in PBMCs from low-passaged supernatants of original cultures and stored at −80 °C until further use. All experimental procedures using Mtb and HIV were performed in the biosafety level 3 laboratory at the Faculty of Pharmacy of the University of Lisbon, maintaining the national and European containment level 3 laboratory management and biosecurity standards based on applicable EU directives.

5.3.3. Macrophage Infection

Before infection, Mtb was cultivated at 37 °C, 5% CO_2, until the exponential growth phase was reached. On the day of infection, the bacterial suspensions were treated for clump removal and a single cell was

obtained, as described before (Pires et al., 2023; Pires et al., 2021; Mandal et al., 2023), centrifuged, washed in phosphate-buffered saline (PBS), and resuspended in RPMI culture medium without antibiotics. Clumps of bacteria in the suspension were disrupted by ultrasonic bath treatment for 5 min and removed by centrifugation at a low speed of 500× *g* for 1 min. The obtained single-cell suspension was verified by fluorescence microscopy and quantified by measuring optical density at 600 nm. The infection was performed with a multiplicity of infection (MOI) of 1 bacterium per Mϕ for 3 h at 37 °C, 5% CO_2. Following this incubation period, cells were washed with PBS to remove free bacteria and added to a fresh complete medium.

5.3.4. Transfection

Mϕs were transfected 72 h before infection to achieve maximum RNA silencing. Transfection with anti-CstF siRNA or with scramble control siRNA was performed with ScreenFect A (ScreenFect GmbH, Eggenstein-Leopoldshafen, Germany) transfection reagent according to the manufacturer's protocol, as previously described (Mandal et al., 2023). Mϕs were incubated for 72 h with the transfection reagent and SMARTpool ON-TARGETplus Human CST7 siRNA (Dharmacon, Lafayette, CO, USA; target sequences: AGUGAAAGGCCUGAAAUAU, GAAAUUGGCAGAACUACCU, GGAUGACUGUGACUUCCAA, and CAAGGGCCCUAGUUCAGAU) or the respective siRNA non-targeting (scramble) control (Dharmacon, USA; target sequences: UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, and UGGUUUACAUGUUUUCCUA) in the medium without antibiotic. The same transfected or scrambled cells were split and used for the whole required further experiments.

5.3.5. Enzymatic Activities of Cathepsin C and Granzyme B

After 48 h of infection, Mϕ cultures in 96-well plates were lysed with chilled lysis buffer, 25 mM 2-(N-morpholino)ethanesufonic acid (MES) (MP Biomedicals, Singapore), 100 mM NaCl, and 5 mM cysteine, pH 6 for cathepsin C, and 50 mM Tris-HCl and 100 mM NaCl, pH 7.4 for granzyme B. Cells were centrifuged at 16,000× *g* for 20 min at 4 °C to recover the supernatant and further added to reaction buffer for 15 min at room temperature for cathepsin C or for 30 min at 37 °C for granzyme B. The specific fluorogenic substrates, 70 µM H-Gly-Phe-7-amino- 4-methylcoumarin (AMC) (Bachem, Bubendorf, Switzerland) for cathepsin C and 50 µM acetyl-Ile-Glu-Pro-Asp-AMC for granzyme B (Bachem), were then added, and the formation of fluorescent degradation products was measured continuously with excitation at 370 nm and emission at 460 nm in a Tecan M200. The activity of the control sample was set to 100% and activities for the other samples were adjusted accordingly. Noninfected Mϕs were subjected to the same treatment for enzymatic activity assessment.

5.3.6. Western Blotting

Total proteins were harvested using a RIPA buffer (Merck, KGaA, Darmstadt, Germany). The protein samples were diluted 1:1 in Laemmli buffer (Merck, KGaA, Darmstadt, Germany) and heated at 95 °C for 5 min before running the gel. The protocol followed the same as described in [27]. Proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to the nitrocellulose membrane through the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). Furthermore, the membrane was processed and stained using the iBind Western system (Thermo Fisher Scientific, Waltham, MA, USA) and primary antibodies specific for CstF (1:2000 dilution of #SAB2700222, Sigma-Aldrich), ß-tubulin (1:4000 dilution of #ab6046, Abcam, Cambridge, UK), and horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000 dilution of #1706515, Bio-Rad Laboratories, Hercules, CA, USA). The visualization of bands was performed through chemiluminescence using an NZY Supreme ECL HRP Substrate (NZYTech, Lisbon, Portugal) in a ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA). The quantification of band intensity was performed using ImageLab software version 6.1, on USB drive #12012931 (Bio-Rad Laboratories, Hercules, CA, USA).

5.3.7. Lymphocyte Infection with HIV and Coculture with Macrophages Infected with Mtb

Autologous lymphocytes were obtained from the PBMC fractions, stimulated, and further cultured according to the protocol described above. On the day of infection, lymphocytes were infected with 1000 TCID50/mL of HIV-$1_{UCFL1032}$ or left uninfected as controls. Briefly, viruses were added and incubated for 3 h in the presence of 3 µg/mL of polybrene (Sigma-Aldrich, St. Louis, MO, USA). Cells were then washed with PBS to remove any unadsorbed virus particles and cultured in an appropriate medium (500 µL/well). Mϕs were allowed to internalize Mtb for 3 h. After this chase period, they were washed with PBS to remove extracellular bacteria and cocultivated with the HIV-infected lymphocytes at a ratio of 1:2. Culture supernatants were collected at days 3 and 9 to recover virus particles and quantified by reverse transcriptase activity.

5.3.8. HIV Quantification

Supernatants collected from cocultures with lymphocytes infected with HIV, as described above, were used for viral replication quantification. This was assessed by using a colorimetric enzyme

immunoassay (Roche, Merck KGaA, Darmstadt, Germany) for the quantitative determination of retroviral reverse transcriptase activity by the incorporation of digoxigenin- and biotin-labeled dUTP into DNA. Absorbance was measured by Tecan M200 spectrofluorometer at 405 and 490 nm.

5.3.9. Cell Death and Viability Assays Using Flow Cytometry

For the assessment of apoptotic or necrotic cells, the Apotracker Green and Zombie Red (Biolegend, San Diego, CA, USA) dyes were used, respectively. Mϕs were allowed to internalize Mtb and lymphocyte HIV particles for 3 h. After the internalization step, extracellular bacteria were removed by washing Mϕ cultures with PBS, and extracellular viruses were eliminated as described previously. Monocultures or cocultures were further incubated for additional timing with the recommended cell death kit experiments reagents and evaluated at the end of 12 h or after 3 days of treatment. The corresponding noninfected cells treated or not with transfection reagents and siRNAs were evaluated in parallel using the same kit. After those timings, cultured cells were detached using 5 mM EDTA. Human peripheral blood lymphocytes were stained with Alexa Fluor® 700 anti-human CD3 antibody (Biolegend, San Diego, CA, USA). Cells were fixed in 4% paraformaldehyde for one hour and then analyzed in a Cytek® Aurora flow cytometer (Cytek® Biosciences, Fremont, CA, USA). Data analysis was performed in FCS Express 7 (De Novo Software, Pasadena, CA, USA).

5.3.10. Statistical Analysis

Statistical analysis was performed in GraphPad Prism 9. Multiple group comparisons were conducted using one-way ANOVA followed by a Holm–Sidak post hoc test. Two group comparisons were made using Student's *t*-test. Differences were considered statistically significant when the calculated adjusted *p*-value was equal to or below the alpha level of 0.05 ($p \leq 0.05$).

5.4. Discussion

Previous work from the group revealed that CstF, a protease inhibitor of lysosomal cathepsins, plays a significant role in Mtb infection, contributing to the intracellular survival of Mtb in human Mϕs (Mandal et al., 2023). The depletion of CstF resulted in the control of the infection, even in clinical strains of Mtb that are resistant to first-line antibiotics used to treat TB (Mandal et al., 2023). Moreover, a significant increase in the expression of the protease inhibitor during Mtb infection was previously demonstrated, which impacts the proteolytic activity of lysosomal proteases (Pires et al.,

2021). In contrast, HIV infection did not contribute to an increase in CstF gene expression. These results are consistent with previous studies showing a general decrease in CstF gene expression in $CD4^+$ T lymphocytes infected with HIV, as well as in genes related to cytotoxicity (Zhang et al., 2023).

TB remains a significant public health concern, with one of the contributing factors being the synergistic effect of the coinfection with HIV. Although HIV can also infect Mϕs, $CD4^+$ T lymphocytes are the primary target cells. While our recent work has demonstrated in vitro Mϕ infection with both pathogens (Pires et al., 2021), this has not yet been observed in vivo (Bell et al., 2018). The objective of our experiments was to replicate the conditions observed in vivo in the lungs of patients simultaneously infected with Mtb and HIV. Therefore, Mϕs were infected with Mtb and cocultured with autologous peripheral blood-derived lymphocytes infected with HIV. To achieve this, lymphocytes were isolated from the blood of healthy donors, including $CD4^+$ and $CD8^+$ naive T lymphocytes and conventional and unconventional NK cells. Since the Portuguese population has been vaccinated for BCG until the last 5 years, it is expected that PBMCs from healthy donors also contain effector and memory T lymphocytes that recognize autologous Mϕs infected with Mtb.

CstF was shown to be secreted from immune cell producers into the extracellular milieu and internalized by bystander cells (Nanut et al., 2017; Kos et al., 2018; Sawyer et al., 2021; Colbert et al., 2011). A key target of the protease inhibitor is cathepsin C, a major progranzyme convertase Magister et al., 2015). Likewise, the internalization of CstF was observed to have an inhibitory effect on cytotoxic cells, both in NK (Nanut et al., 2017) and in $CD8^+$ cytotoxic T lymphocytes (CTL) (Prunk et al., 2020), leading to anergy split, a condition where these cells lose the ability to secrete granzyme and perforin (Magister et al., 2015). Additionally, human NK cells displayed a 30-fold increase in CstF compared to CTL (Obata-Onai et al., 2002). However, it was not demonstrated whether this difference resulted from the accelerated synthesis and/or increased internalization of secreted CstF by closely interacting immune cells (Magister et al., 2015).

In this study, we depleted CstF from Mϕs prior to Mtb infection by using siRNA, based on our previous results, indicating a high gene expression of CstF from Mϕs activated with IFN-γ and/or in Mtb-infected phagocytes (Pires et al., 2016). We designed an in vitro experiment to assess whether the manipulation of CstF could contribute to the control of HIV replication during coinfection with Mtb.

As in previous work, here we provided evidence of successful CstF silencing at the protein synthesis level (Mandal et al., 2023). The current results indicate that the depletion of CstF from Mtb-infected Mϕs enhances cathepsin C activity in cocultured lymphocytes, thereby augmenting their granzyme

cytototoxic effects. However, no effects of CstF depletion were observed in an autocrine manner in activated Mϕs with regard to cathepsin C or granzyme B. Such effects are observed in monocultures of IFN-γ-stimulated noninfected Mϕs or during Mtb infection. Indeed, while Mϕs potentially can be activated by IFN-γ to a cytotoxic phenotype under certain conditions by activated effector Th1 lymphocytes (Liu et al., 2019; Alam et al., 2019; Kim et al., 2007; Velotti et al., 2020; Boivin et al., 2009), here, no higher granzyme activities were observed following CstF depletion or after IFN-γ activation in Mϕs. The results suggest that the observed increase in granzyme activity is driven by cytotoxic lymphocytes in coculture, rather than by activated Mϕs. Since an increase in cathepsin C was observed in noninfected Mϕs following IFN-γ stimulation, but not in Mtb-infected Mϕs, it can be hypothesized that noninfected Mϕs in culture conditions may contribute via cathepsin C secretion to lymphocytes, thereby driving granzyme activation and subsequent internalization in bystander cells. Nevertheless, no effects on CstF depletion were observed in either monocultures of Mϕs or cocultures of noninfected Mϕs with lymphocytes infected with HIV.

We conclude that the observed increase in cathepsin C enzymatic activity during coculture assays in Mtb–HIV coinfection is a result of the activation of this enzyme from cytotoxic lymphocytes following the depletion of CstF from Mtb-infected Mϕs. This activation does not occur in noninfected phagocytes. Ultimately, the depletion of CstF in noninfected Mϕs may result in an increased activity of cathepsin C in phagocytes, and their secretion could potentially lead to granzyme activation in lymphocytes. However, no effect of CstF depletion was observed in monocultures or in cocultures with lymphocytes from noninfected cells.

It can be inferred that a decrease in cathepsin C and an increase in CstF secretion, induced by Mtb, will result in the evasion of cytolytic activity by lymphocytes directed to Mtb-infected cells. This scenario will not be observed in noninfected phagocytes.

HIV infection can evade the early immune response, resulting in ineffective viral clearance. Cytotoxic lymphocytes including NK cells and HIV-specific adaptive cells such as CTLs or even Th1 are crucial for the outcome of infection and arise shortly after infection (Waters et al., 2020; McMichael et al., 2010). Additionally, high levels of NK recruitment have been observed in tuberculous pleural effusions and in early innate granulomas (Ota et al., 1990). Pleurisy is a common manifestation of TB, often observed during the primo infection (Cohen et al., 2015). It is also frequently observed in HIV-coinfected patients, where high levels of virus particles are present at the sites of Mtb infection (Toossi et al., 2001; Ferrer et al., 1997). Coincidently, higher levels of CstF were found in pleural effusion of TB patients than in other inflammatory conditions (Werle et al., 2003). Here, our results indicate that in

vitro, when this environment of cells and cytokines is reproduced, a clear impact of CstF depletion from Mtb-infected macrophages increases the cytotoxic activity of lymphocytes, with consequences on HIV replication and viral loads (Figure 7).

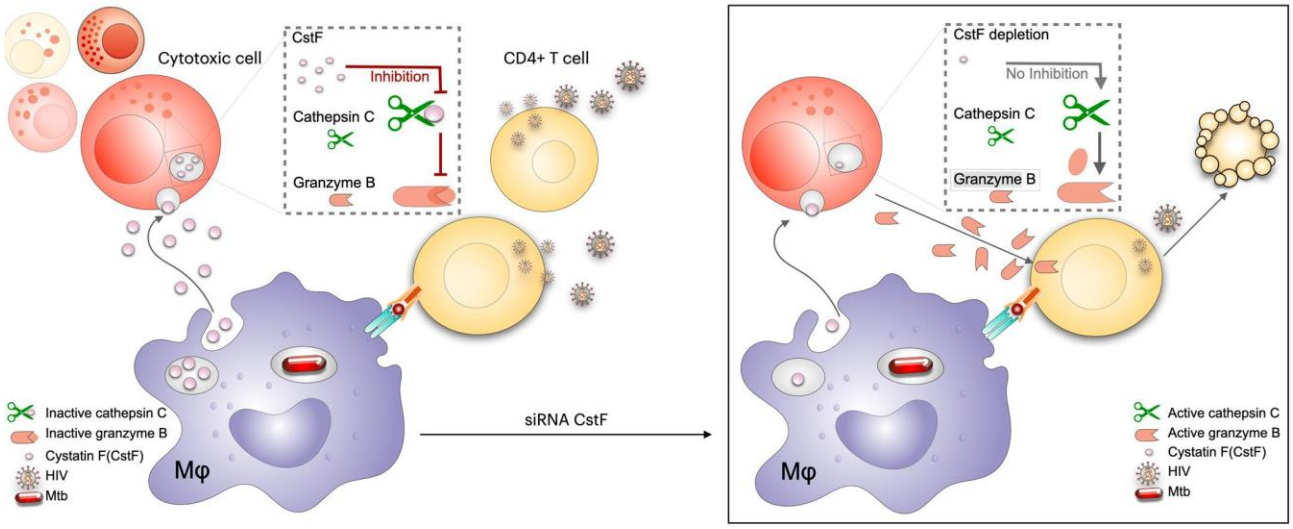


Figure 7. Impact of CstF depletion from Mtb-infected macrophages on the cytotoxic activity of lymphocytes. During the coinfection of Mtb-infected macrophages with lymphocytes infected with HIV, CstF depletion enhances cathepsin C proteolytic activity, which in turn activates granzyme B. This process results in a notable decrease in the viral load.

The results also suggest the existence of an evasion mechanism that enables early HIV replication during coinfection with Mtb at the interface environment through the CstF/cathepsin C/granzyme B axis. Ultimately, this knowledge can be crucial for developing new therapeutic approaches to control both pathogens based on the manipulation of CstF.

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CHAPTER 6.

CONCLUSIONS

CONCLUSIONS

Despite the available antibiotics, TB remains a major global threat. HIV infection is the most potent known risk factor for Mtb infection progress to active disease, with a 20-fold risk of reactivation of latent TB. On the other hand, TB is also a major cause of death in people living with HIV. Mtb and HIV thus act synergistically, which in turn accelerates the decline of immunological functions, leading to subsequent death if not treated. This thesis addresses the challenges to fight these pathogens through identification and development of new host-directed strategies based on protease inhibitors. In this study, we proposed novel strategies which include the repurposing of SQV for TB control and the modulation of Csts C and F to control both pathogens either during mono-infection or during coinfection.

The observation that the HIV protease inhibitor SQV increases cathepsin activity in non-infected cells (Kourjian et al., 2016) together with our results that Mtb infection downregulates cathepsin activity (Pires et al., 2017) lead us to hypothesise that it could be repurposed for Mtb therapy. The results indicate that SQV indeed increases the activity of cathepsins including in macrophages infected with Mtb overcoming the pathogen induced blockade with concomitant increase of the pathogen intracellular killing (Pires et al., 2021). Thus, in the next approach and to deliver SQV directly to macrophages, increase the efficiency of their internalization at higher concentrations while decreasing the systemic concentration we use liposomes as strategy. Using liposomal drug delivery system, we were able not only to increase the intracellular concentration, with less cytotoxicity than the free drug but also to induce a significant impact on the intracellular killing of Mtb, not only in reference strain but also MDR and XDR strains (Mandal et al., 2023). In addition, SQV treatment also showed to improve in the expression of HLA II antigen presentation machinery at the cell surface, increment in T-lymphocyte priming and proliferation as well as high secretion of IFN-γ. We conclude that altogether, these results reveal SQV as a potential HDT for TB.

We demonstrated that pathogens like Mtb and HIV can decrease cathepsins activity by increasing the activity of their natural inhibitors cystatins (Pires et al., 2021). Among Csts, CstC and CstF were selected as potential targets as we previously observed to be the most upregulated early between the first 24 h and 48 h of macrophage Mtb infection (Pires et al., 2021). Therefore, their manipulation through RNA silencing could restore cathepsin activity during Mtb infection or Mtb/HIV coinfection. Furthermore, we realised that modulation of Csts using *in vitro* transfection methods is not suitable for the clinical translation of host-cell RNA. Therefore, we overcome this limitation with a nanomedicine approach utilizing chitosan particles to deliver CstC-targeting siRNA (Mandal et al., 2023).

The results from these studies demonstrated that knocking down CstC and CstF mRNA modulates the proteolytic activity of the macrophages' lysosomal cathepsins, which consequently improves the intracellular killing of Mtb. Moreover, chitosan-based nanoparticles used for siRNA delivery demonstrated the success of this approach in targeting primary macrophages, showing efficient silencing compared to the *in vitro* transfection method with very low toxicity. The results also show an improved intracellular killing of Mtb with different drug-resistance profiles. We conclude that these two Csts (CstC and CstF) could be a promising strategy to improve the control of Mtb infection.

Finally, we focused our attention on CstF as a potential target in the context of Mtb/HIV coinfection. Since previous results from the group indicated the higher gene expression of CstF among all cystatins during macrophage infection with Mtb we hypothesize that the depletion of CstF could improve control over HIV during coinfection infection. Macrophages infected with Mtb and lymphocytes infected with HIV are found in close contact within TB granulomas (Azevedo Pereira et al., 2023). In this approach, we investigated the impact of silencing CstF in macrophages infected with Mtb which were further cocultured with lymphocytes infected with HIV. Overall, the results obtained indicate that decrement in CstF release by phagocytes increases the major pro-granzyme convertase cathepsin C of cytotoxic immune cells from peripheral blood-derived lymphocytes. The enhancement in the cytolytic activity of granzyme B induces the reduction of viral replication in HIV-infected $CD4^+$ T-lymphocytes. We conclude, that targeting CstF could be a potential therapeutic strategy against both Mtb and HIV pathogens, providing a promise future for co-infected individuals.

Overall, the results presented in this thesis indicates for the pivotal role of host-directed based on protease inhibitors as strategies to be included in coordination with antibiotics or vaccinal in future adjunctive therapeutic regimens.

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Supplementary information

Repurposing Saquinavir for Host-Directed Therapy to Control *Mycobacterium tuberculosis* Infection

David Pires [1], Sofia Valente [1], Marta Calado [1], Manoj Mandal [1], José Miguel Azevedo-Pereira [1], Elsa Anes [1,*]

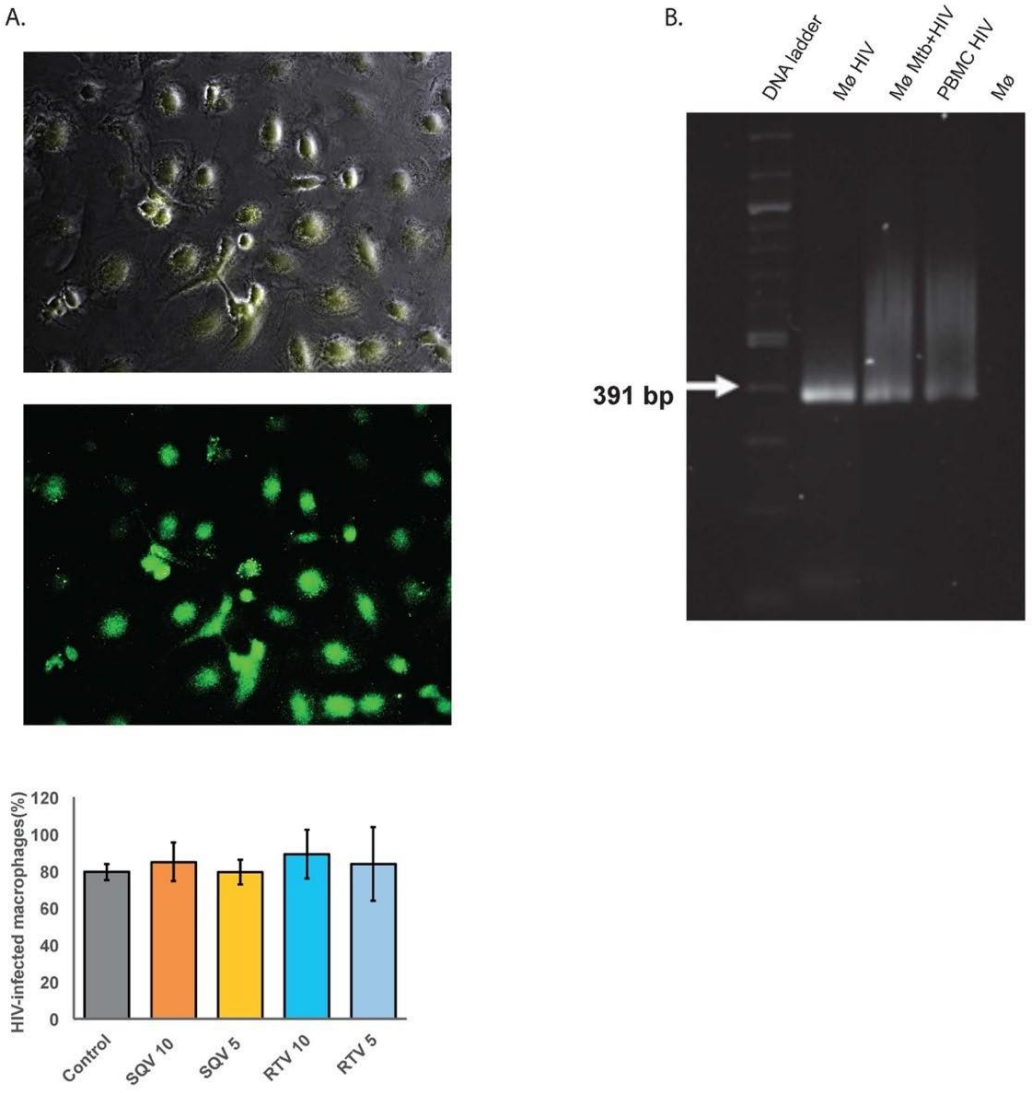


Figure 1 supplementary: Macrophage infection with HIV in treated cells.
(A) Virus particles visualized in infected cells by fluorescence microscopy and phase contrast. Bar-plots represent the mean percentage of cells infected by HIV obtained from the microscopic analysis of 250 cells per treatment in ImageJ software. Error bars show the standard deviation.
(B) Proviral DNA integration: gel electrophoresis of a PCR amplicon with 391bp.

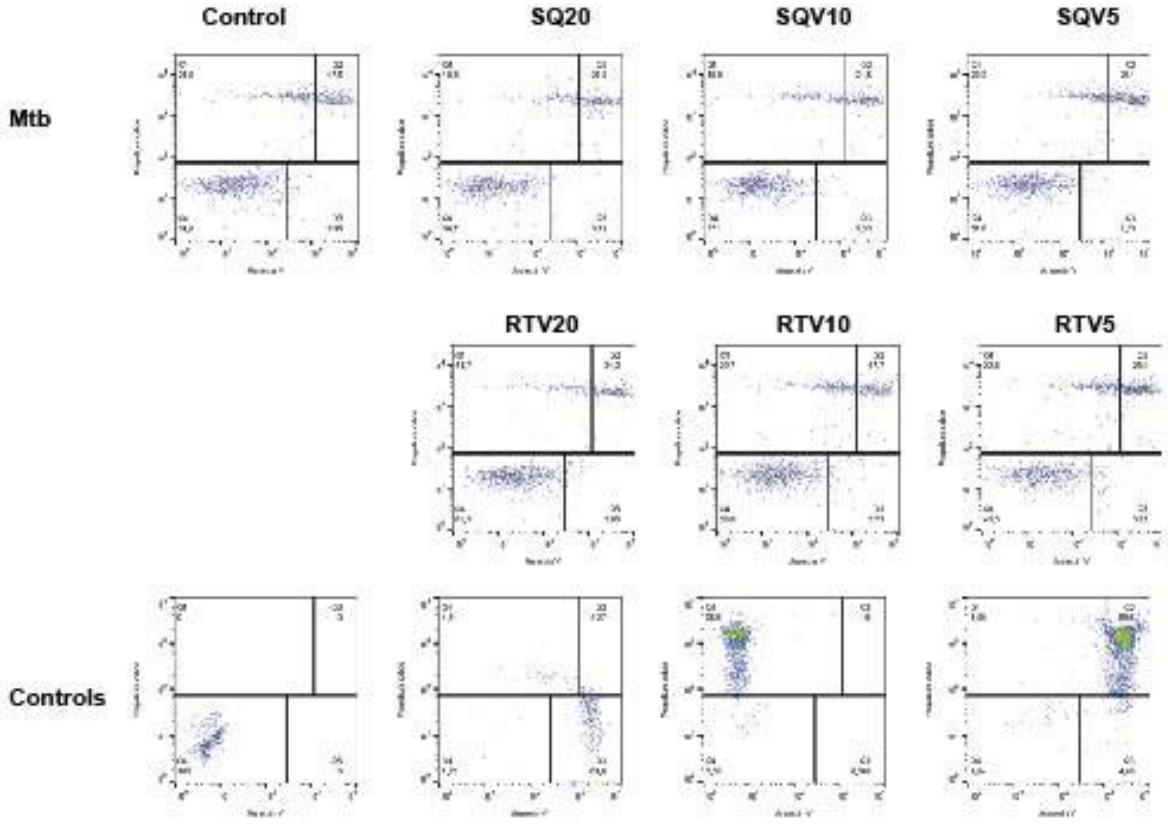


Figure 2 supplementary: Macrophage Cell death in Mtb-infected and treated cells.
Dot-plots representative of the data shown in Figure 1B (lower bar-plot).

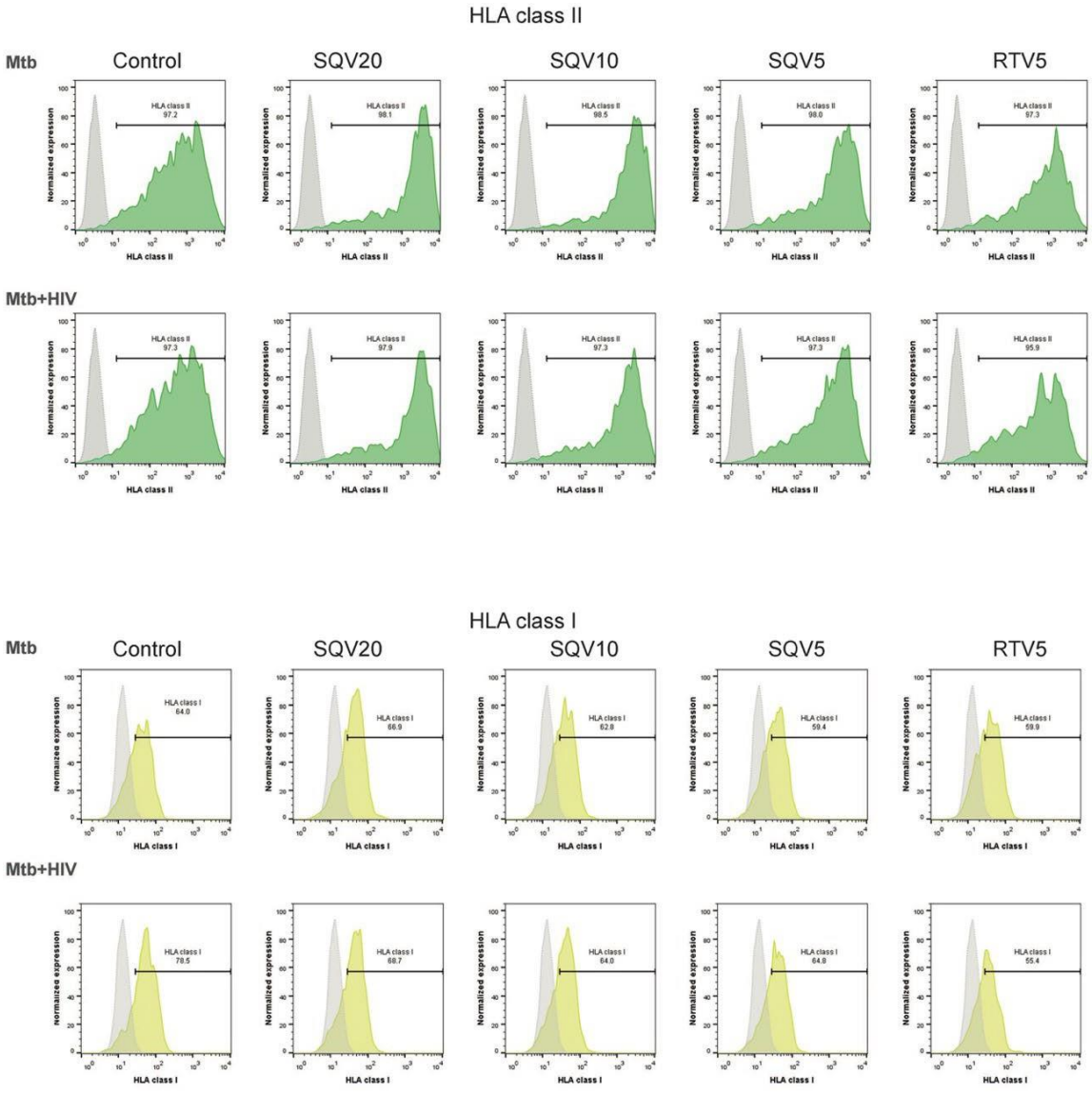


Figure 3 supplementary: HLA class II and I surface expression on macrophages infected and treated. Histograms representative of the data shown in Figure 3A.

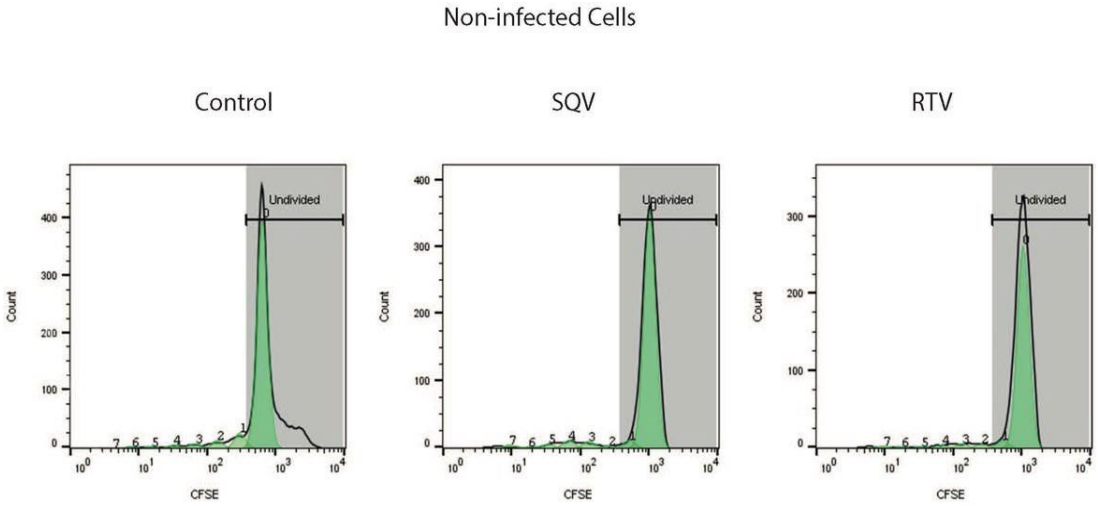
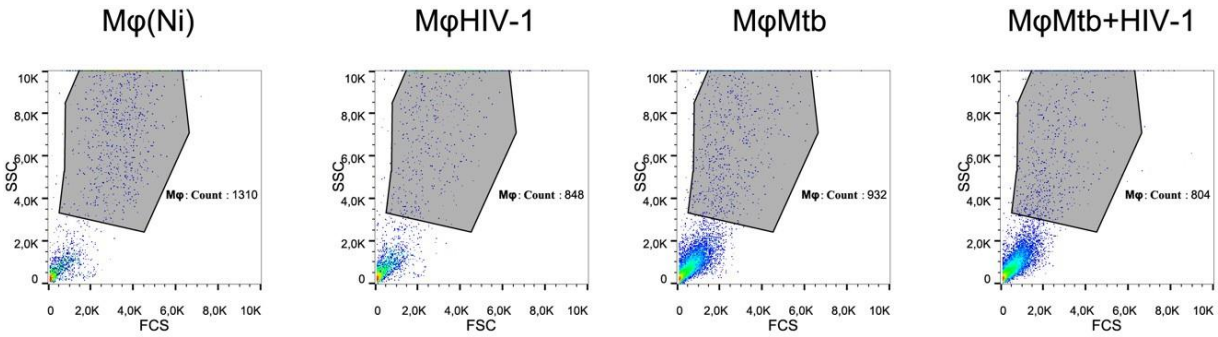


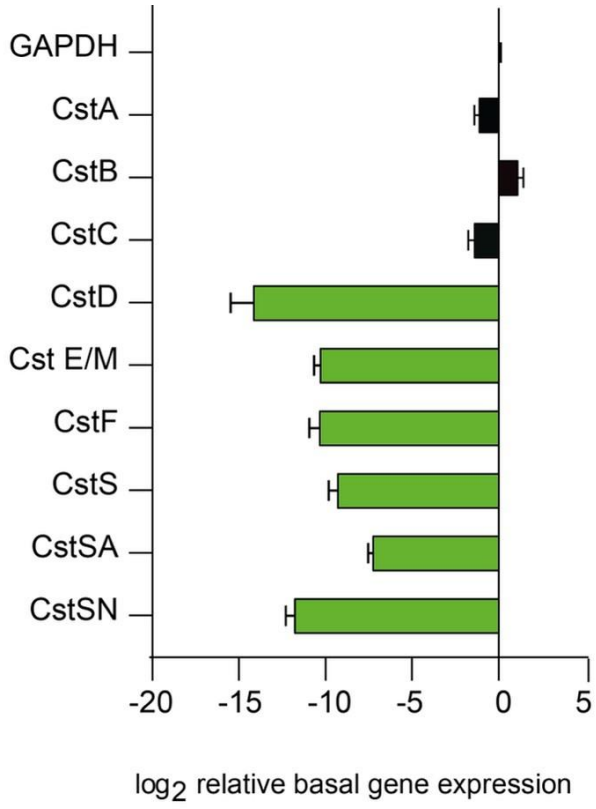
Figure 4 supplementary: T-cell proliferation in non-infected cocultures treated or not with PIs
Histograms representative of the data shown in Figure 3D.

Modulation of Cystatin C in Human Macrophages Improves Anti-Mycobacterial Immune Responses to *Mycobacterium tuberculosis* Infection and Coinfection With HIV

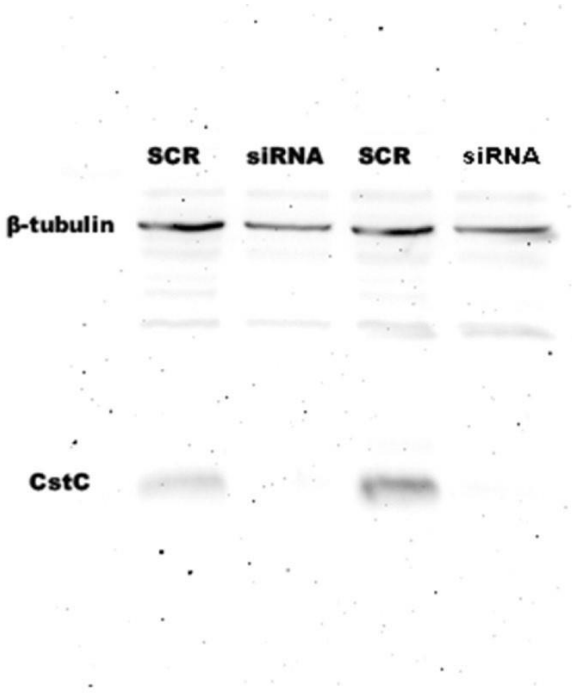
David Pires [1], Marta Calado [1], Tomás Velez [1], Manoj Mandal [1], Maria João Catalão [1], Olivier Neyrolles [2], Geanncarlo Lugo-Villarino [2], Christel Vérollet [2], José Miguel Azevedo-Pereira [1], Elsa Anes [1,*]



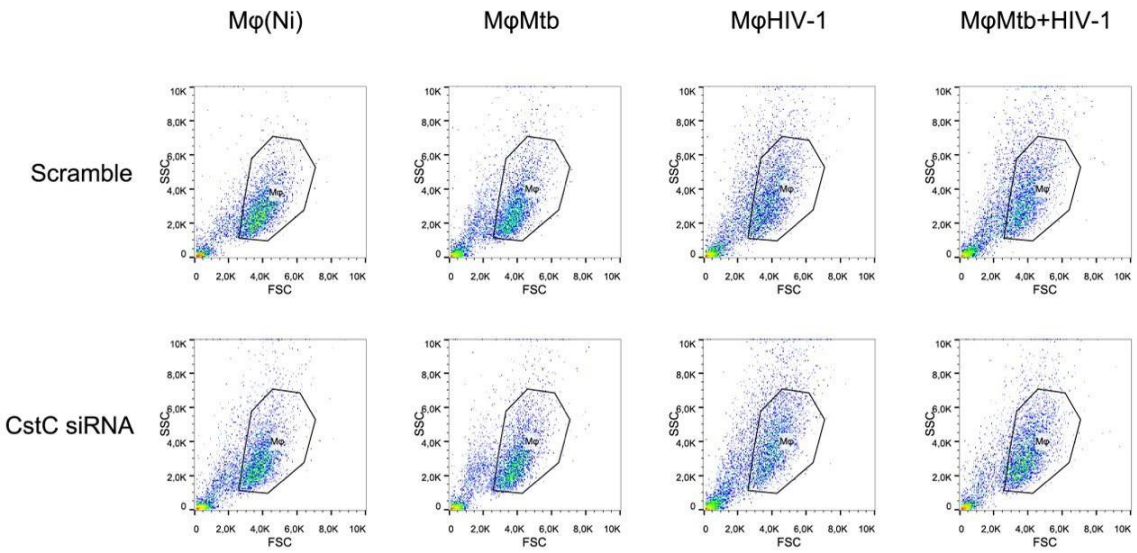
S1 Fig. Macrophage population gated for cell death analysis in Figure 1A.



S2 Fig. Basal gene expression of cystatins in non-infected cells relatively to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).



S3 Fig. siRNA silencing of cystatin C protein expression from two independent experiments.



S4 Fig. Macrophage population gated for apoptosis analysis in Figure 3B.

Development of chitosan particles loaded with siRNA for cystatin C to control intracellular drug-resistant *Mycobacterium tuberculosis*

David Pires [1,2,†], Manoj Mandal [1,†], Ana I. Matos [3], Carina Peres [3], Maria João Catalão [1], José Miguel Azevedo-Pereira [1], Ronit Satchi-Fainaro, Helena F. Florindo [3] and Elsa Anes [1,*]

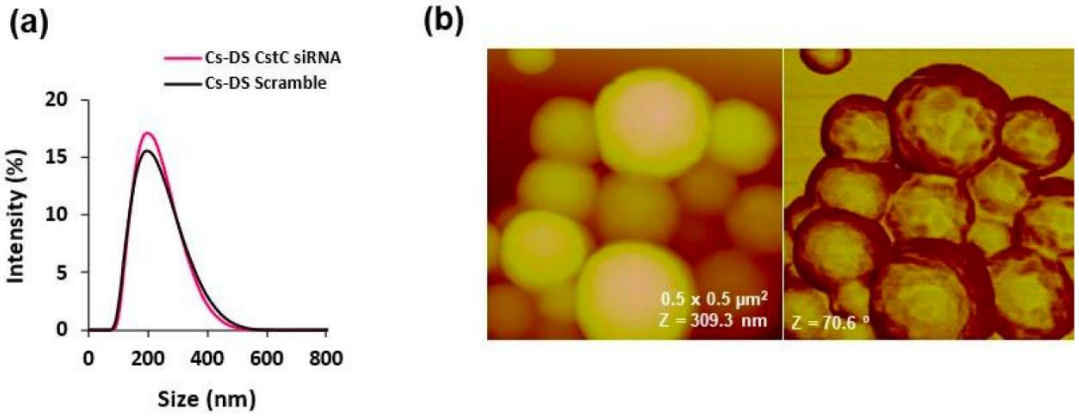


Figure S1. Physicochemical and morphological properties of Cs-DS. Dynamic light scattering analysis (a) and atomic force microscopy images (topography – left; phase – right) (b) showed a uniform-size polydispersity for slightly rough spherical particles.