Mycobacteria manipulation of host proteases and inflammatory pathways during infection within human macrophages and dendritic cells

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DISSERTAÇÃO

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2013
Mycobacteria manipulation of host proteases and inflammatory pathways during infection within human macrophages and dendritic cells

Dissertação orientada pela Prof. Doutora Elsa Anes (FFUL)
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Master Thesis
2013

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Communications in Scientific Meetings


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II-associated invariant chain peptide</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating factor</td>
</tr>
<tr>
<td>IFBS</td>
<td>Inactivated Fetal Bovine Serum</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>imDCs</td>
<td>Immature Dendritic Cells</td>
</tr>
<tr>
<td>mDCs</td>
<td>Mature Dendritic Cells</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>iI</td>
<td>Invariant chain</td>
</tr>
<tr>
<td>M0</td>
<td>Resting macrophages</td>
</tr>
<tr>
<td>M1</td>
<td>Classical activated macrophages</td>
</tr>
<tr>
<td>M2</td>
<td>Alternative activated macrophages</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose Receptor</td>
</tr>
<tr>
<td>OADC</td>
<td>Oleic acid, Albumin, Dextrose, Catalase</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNAs</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor- alpha</td>
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Abstract

Macrophages and dendritic cells (DCs) play an essential role during mycobacterial infection, acting as key effector cells in bacterial killing and antigen presentation. Cathepsins are host proteases involved in pathogen destruction and antigen presentation. This makes these proteases, as well as both cell types, perfect targets for mycobacterial manipulation.

In the first part of this thesis the role of mycobacteria during macrophage activation and DC maturation was addressed. By flow cytometry the surface expression of activation and maturation markers of mycobacteria infected human macrophages and DCs, was analyzed. It was demonstrated that: (1) *M. tuberculosis* H37Ra prevents the induction of the classical activated macrophage phenotype; (2) induces the maturation process of immature DCs; (3) the outcome of infection with *M. tuberculosis* H37Ra differs from that with *M. smegmatis*. These results indicate that *M. tuberculosis* infection has a differential role during macrophage activation and DC maturation and that *M. tuberculosis* H37Ra interferes with these processes differently of the non-pathogenic *M. smegmatis*.

In the second part, we aimed to decipher the role of cathepsins during mycobacteria infection of macrophages and DCs. Cathepsins protein levels were analyzed by western blot after infection with pathogenic or non-pathogenic mycobacteria. It was shown that cathepsin B and S levels during *M. tuberculosis* infection are distinct from those of host cells that internalized *M. smegmatis* and this is dependent on the host cell species tested. In addition, the role of these proteases in *M. tuberculosis* intracellular survival was evaluated by silencing cathepsins expression, using shRNA lentiviral vectors. It was observed, that cathepsin B and S knockdowns led to an increase in *M. tuberculosis* H37Ra intracellular survival. Altogether, these evidences point for a role of both cathepsins in the control of *M. tuberculosis* intracellular growth and demonstrate that *M. tuberculosis* modulates these cathepsins differently of the non-pathogenic *M. smegmatis*.

Keywords

Resumo

A tuberculose representa um dos mais graves problemas de saúde pública, causando anualmente cerca de dois milhões de mortes em todo o mundo. Estima-se que cerca de um terço da população mundial se encontra infectada com *Mycobacterium tuberculosis*, o microrganismo patogénico causador desta doença infecciosa. A emergência de estirpes de *M. tuberculosis* multirresistentes e extensivamente resistentes à terapêutica antibiótica utilizada, aliado ao facto de a única vacina disponível possuir baixa eficácia contra a tuberculose pulmonar, vem realçar a necessidade urgente de se encontrar novos alvos terapêuticos e novas estratégias para controlar a doença.

O sucesso deste patogénio reside, principalmente, na sua capacidade de sobreviver e de se dividir no interior de células imunitárias. Após ser internalizado pelos macrófagos, *M. tuberculosis* inibe a fusão do fagossoma com o lisossoma, escapando assim à acção das enzimas proteolíticas (catepsinas) e ao ambiente acídico do fagolisossoma. Em paralelo, os macrófagos infectados induzem uma resposta pró-inflamatória, que promove o recrutamento de células imunitárias da corrente sanguínea para o local da infeção. Forma-se o granuloma, a estrutura característica da tuberculose, que se pensa ser capaz de conter a infecção micobacteriana. Aqui, algumas micobactérias podem permanecer num estado de latência durante um longo período de tempo. Apenas 5 a 10% da população infectada desenvolve a doença activa, o que na maioria dos casos se deve a uma imunodepressão do hospedeiro.

Nos pulmões, *M. tuberculosis* é internalizado por macrófagos alveolares residentes, mas também por células dendríticas recrutadas da corrente sanguínea. Estes dois tipos de células apresentadoras de antígenos desempenham papéis distintos na imunidade contra a tuberculose. Os macrófagos ao induzirem os seus mecanismos bactericidas são os principais responsáveis pela destruição das micobactérias. Por outro lado, as células dendríticas são especializadas na apresentação de antígenos micobacterianos às células T naïve, que se encontram nos nódulos linfáticos. De modo a conseguirem desempenhar estas funções, é necessário que ocorra um processo de activação dos macrófagos assim como um processo de maturação das células dendríticas. Estudos anteriores mostram que as micobactérias têm a capacidade de interferir com estes processos.

As catepsinas são proteases que estão envolvidas num grande número de processos celulares em macrófagos e células dendríticas, tais como: destruição dos patogénios, processamento e apresentação de antígenos, processamento de várias enzimas celulares do hospedeiro e também apoptose. O seu vasto número de funções, e em particular o seu papel na destruição dos patogénios e na apresentação de antígenos, faz destas proteases alvos perfeitos para manipulação por micobactérias durante a infecção de macrófagos e células dendríticas.

Na primeira parte desta tese, é explorado o papel das micobactérias durante a activação dos macrófagos e a maturação das células dendríticas. Foram infectados macrófagos e células dendríticas, diferenciados a partir de monócitos isolados do sangue periférico de doadores humanos.
saudáveis, com micobactérias que expressavam a proteína verde fluorescente (GFP), nomeadamente a estirpe avirulenta M. tuberculosis H37Ra GFP e a espécie não patogénica M. smegmatis. Através de citometria de fluxo, foram selecionadas apenas as células infectadas com micobactérias fluorescentes e o efeito da infecção foi analisado pela medição da expressão de marcadores superficiais de activação e maturação. Ao infectar macrófagos não activados (M0) com M. tuberculosis H37Ra, verificou-se que a infecção impede a indução da clássica resposta pró-inflamatória M1, ao nível das moléculas apresentadoras de antígenos. Pelo contrário, a infecção de macrófagos M0 com M. smegmatis tem um efeito estimulatório, resultando num fenótipo idêntico ao induzido pela estimulação destas células com interferão-gama. Tendo em conta que o número de células infectadas com M. tuberculosis H37Ra GFP e com M. smegmatis GFP é semelhante, podemos concluir que as diferenças observadas ao nível dos marcadores de activação resultam, efectivamente, de diferenças de virulência entre ambas as espécies. Por outro lado, a infecção de macrófagos previamente estimulados com interferão-gama (M1), com M. tuberculosis H37Ra, não inibiu a activação destas células, pelo menos ao nível das moléculas apresentadoras de antígenos. Isto sugere que, com uma baixa multiplicidade de infecção, a activação induzida pelo interferão-gama consegue superar os mecanismos inibidores induzidos pelo M. tuberculosis H37Ra, ao nível das moléculas apresentadoras de antígenos.

Relativamente às células dendríticas, verificou-se que a infecção com M. tuberculosis H37Ra induz a maturação das células dendriticas imaturas, uma vez que se observou um aumento da expressão das moléculas apresentadoras de antígenos nas células infectadas. Foi também observado um aumento da expressão destes marcadores de maturação, após a infecção com M. smegmatis. No entanto, observou-se uma maior indução da maturação, pela estirpe avirulenta M. tuberculosis H37Ra, do que pela espécie saprófita M. smegmatis. As diferenças na virulência entre estas duas espécies poderão justificar o facto da infecção com M. tuberculosis H37Ra ser muito mais estimulatória do que com M. smegmatis. Estas evidências levam-nos a especular que a maturação das células dendriticas, observada após a infecção com M. tuberculosis H37Ra, poderá representar uma forma da micobactéria tirar partido das características migratórias das células dendriticas maturas e assim se disseminar pelo hospedeiro. Outra possível explicação seria que a indução da maturação pela infecção constitui um mecanismo de defesa do hospedeiro, na medida em que as células dendríticas infectadas representam fortes estimuladores das células T CD4+.

Concluindo, verifica-se que a infecção com a estirpe avirulenta M. tuberculosis H37Ra induz diferentes fenótipos, de acordo com o tipo de célula hospedeira infectada. Isto provavelmente está relacionado com as diferentes funções dos macrófagos e das células dendriticas na resposta imune contra a tuberculose. Em macrófagos humanos, esta estirpe avirulenta impede a expressão do fenótipo M1, ao nível das moléculas apresentadoras de antígenos, o que indica que provavelmente induz um fenótipo anti-inflamatório. Nas células dendríticas, a indução da maturação após a infecção com M. tuberculosis H37Ra pode reflectir um modo de disseminação utilizado pela micobactéria, ou um mecanismo de defesa do hospedeiro que lhe permita iniciar uma resposta imune celular específica. Para além disto, a infecção M. tuberculosis H37Ra induziu fenótipos distintos nas células imunes, comparativamente a M. smegmatis.
Na segunda parte desta tese, foi abordado o papel das catepsinas durante a infecção micobacteriana de macrófagos e células dendríticas. Focámos o nosso estudo nas catepsinas S e B, as quais estão envolvidas na apresentação de antigénios via moléculas MHC classe II. A expressão destas catepsinas foi avaliada ao nível das proteínas, em macrófagos e células dendríticas infectados com a estirpe virulenta *M. tuberculosis* H37Rv ou com a espécie não patogénica *M. smegmatis*, de forma a analisar se a infecção por micobactérias modula a expressão destas proteases. Demonstrou-se que, em macrófagos M0, a infecção com *M. tuberculosis* H37Rv diminui a expressão destas proteases, enquanto que com *M. smegmatis* não provoca qualquer alteração nestas proteases.

Por outro lado, a infecção micobacteriana afectou de forma diferencial a expressão das catepsinas S e B em células dendríticas e macrófagos humanos. Verificou-se que a infecção, tanto com *M. tuberculosis* H37Rv como com *M. smegmatis*, parece não interferir com os níveis de catepsina B nas células dendríticas. Relativamente à catepsina S, observou-se um aumento dos seus níveis após a infecção, tanto com *M. tuberculosis* H37Rv como com *M. smegmatis*. A infecção de células dendríticas imaturas com a estirpe virulenta resultou num aumento mínimo dos níveis de catepsina S, enquanto que a infecção de células dendríticas maduras resultou num grande aumento dos níveis desta protease. Apesar de não termos uma clara explicação para estes resultados, especulamos que, nas células dendríticas imaturas, *M. tuberculosis* H37Rv impede o aumento dos níveis de catepsina S, interferindo assim com apresentação de antigénios. Apesar de estas células após a infecção adquirirem um fenótipo de maduras, como anteriormente descrito, as moléculas apresentadoras de antigénios que estas expressam, encontram-se provavelmente num estado imaturo. Por outro lado, as células dendríticas maduras parecem ser capazes de superar este efeito inibitório e por isso se observa um grande aumento dos níveis desta protease.

Em paralelo, fomos também explorar o papel das catepsinas S e B na sobrevivência intracelular de *M. tuberculosis* em macrófagos. Para isso, monócitos humanos THP-1 foram silenciados para estas catepsinas, através do uso de vectores lentivirais a expressar *short hairpin RNAs*. Posteriormente, estas células foram diferenciadas em macrófagos e estes foram infectados com a estirpe virulenta *M. tuberculosis* H37Rv ou com a estirpe avirulenta *M. tuberculosis* H37Ra. Ao analisar os perfis de sobrevivência, verificou-se que o silenciamento da catepsina S aumenta significativamente a sobrevivência intracelular de *M. tuberculosis* H37Ra, enquanto que parece não interferir com a sobrevivência intracelular de *M. tuberculosis* H37Rv. Tendo em conta que a estirpe virulenta parece ter a capacidade de manipular a catepsina S em macrófagos humanos, é provável que o seu silenciamento mimetize esta modulação e, como tal, não se observem alterações na sua sobrevivência relativamente ao controlo sem silenciamento. A catepsina S parece ser relevante para o controlo do crescimento intracelular de *M. tuberculosis* H37Ra, mas enquanto a estirpe virulenta *M. tuberculosis* H37Rv parece desenvolver um mecanismo para evitar o efeito desta protease, a estirpe avirulenta *M. tuberculosis* H37Ra, parece não desenvolver o mesmo mecanismo de manipulação ou então desenvolver mas é menos efectivo. Foi também demonstrado que o silenciamento da catepsina B em macrófagos aumenta significativamente a sobrevivência intracelular de *M. tuberculosis* H37Ra e de *M. tuberculosis* H37Rv. Isto evidencia um papel desta protease no controlo da sobrevivência.
intracelular de ambas as estirpes em macrófagos.

Concluindo, verificou-se que os níveis de catepsina S e B, durante a infecção com *M. tuberculosis* H37Rv, são diferentes dos observados durante a infecção com *M. smegmatis*. Para além disso, também se observou que os níveis de catepsina S e B diferem consoante a espécie das células imunitárias infectadas, o que poderá estar relacionado com diferentes cinéticas de acidificação e de actividade proteolítica existentes entre elas. Para além disto, os resultados mostram um papel de ambas as catepsinas no controlo do crescimento intracelular de *M. tuberculosis* H37Ra e *M. tuberculosis* H37Rv em macrófagos. Porém, a estirpe virulenta *M. tuberculosis* H37Rv parece ser capaz de subverter os efeitos bactericidas da catepsina S, ao diminuir os seus níveis proteicos. No entanto, a estirpe avirulenta *M. tuberculosis* H37Ra parece ser mais susceptível aos efeitos de ambas as proteases.

**Palavras-chave**

*Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, macrófagos, células dendríticas, catepsinas, apresentação de antigénios.
1. Introduction

1.1. Tuberculosis

Tuberculosis, one of the oldest recorded human diseases, is considered one of the leading causes of infectious disease mortality worldwide (Daniel, 2006; WHO, 2012). During the first half of the XIX century, this disease was the leading cause of death in Europe, as a result of the population explosion and the growth of large urban centers. The only treatment then, which consisted of fresh air, a good healthy diet and rest, was available in the so called sanatoriums (Harries et al., 2006; Smith, 2003). The first microbiological advances in this disease were made when the German physician Robert Koch first identified the tubercle bacillus, in 1882 (Barry III and Cheung, 2009). During the XX century, the incidence of tuberculosis was greatly reduced in the developed countries, due to the improvement of public health practices, the widespread use of the live attenuated \textit{M. bovis} BCG (Bacillus Calmette-Guérin) vaccine, as well as the discovery of antibiotics, such as streptomycin, the first effective therapy for tuberculosis. By that time, many experts believed that tuberculosis had been nearly eradicated. However, in the mid-1980s the number of new cases increased with the deterioration of socio-economic conditions and the emergence of Human Immunodeficiency Virus (HIV), especially in southern and east Africa (Barry III and Cheung, 2009; Smith, 2003). This lead in 1993, to the World Health Organization to declare the disease as a global emergency (Harries et al., 2006).

Nowadays, tuberculosis still remains a major public-health problem with one-third of world’s population infected with \textit{Mycobacterium tuberculosis} and 8.7 million new cases of tuberculosis reported, in 2011 (World Health Organization, 2012). Although tuberculosis still causes almost two million deaths annually worldwide, the disease particularly affects developing regions such as Africa, which has the highest proportion of tuberculosis cases co-infected with HIV (Corbett et al., 2003; Smith, 2003; World Health Organization, 2012). The currently available BCG vaccine, which provides protection against the disease in childhood, has proven to be ineffective against the most frequent outcome of tuberculosis, the lung infection in adults (Soualhine et al., 2007). This situation became even more alarming with the emergence of multidrug and extremely drug-resistant strains of \textit{M. tuberculosis} worldwide (Nguyen and Pieters, 2009). Therefore, new strategies for the prevention and treatment of tuberculosis are urgently needed. A better understanding of the mechanisms of interaction between \textit{M. tuberculosis} and the host immune system is crucial to define novel therapeutic targets and strategies to control the disease.

1.2. Mycobacteria

The genus \textit{Mycobacterium} comprises rod-shape bacteria characterized by a very complex lipid-rich cell envelope that differs substantially from the typical cell wall structure of gram-positive and also gram-negative bacteria. The cell envelope contains, in addition to the cell membrane and
peptidoglycan layers, a large hydrophobic layer composed by mycolic acids associated to a vast array of other lipids and glycolipids (Forrellad et al., 2013; Glickman et al., 2001; Russell, 2001). This unique envelope confers intrinsic resistance to antibiotics and dehydration being also responsible for the acid-fast staining property used to identify mycobacteria, in Zhiel-Neelsen acid-fast stain (Forrellad et al., 2013; Scherr and Nguyen, 2009). The majority of the species that compose the genus are non-pathogenic environmental bacteria, such as *Mycobacterium smegmatis*, which presents similarities to soil bacteria from the genus *Streptomyces* (Cosma et al., 2003; Scherr and Nguyen, 2009). However, a few species are highly successful pathogens including *Mycobacterium leprae*, *Mycobacterium ulcerans* and those present in the *Mycobacterium tuberculosis* complex.

The *Mycobacterium tuberculosis* complex consists in a group of genetically closely related species namely *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium canetti*, *Mycobacterium caprae* and *Mycobacterium pinnipedii* (van Soolingen et al., 1997; Aranaz et al., 1999; Aranaz et al., 2003; Cosma et al., 2003; Cousins et al., 2003), which are the etiologic agents of tuberculosis in humans and other animals. Although, all the members of the complex are facultative intracellular pathogens that cause tuberculosis, they diverge in terms of their phenotypic properties, host tropisms and virulence (Ahmad, 2011). The most well known member is *Mycobacterium tuberculosis*, a slow-growing and obligate aerobe bacterium, which the only known natural host is the human species (Cosma et al., 2003; Pieters, 2001).

### 1.3. Pathogenesis of Tuberculosis

Tuberculosis is usually a lung infection but may affect almost any organ of the body. This infection is caused by inhalation of aerosols containing the infectious bacilli, generated from the cough of an infected individual which has developed the active respiratory disease (Mortellaro et al. 2009). In the lung, *M. tuberculosis* is internalized by resident alveolar macrophages but also by dendritic cells and monocytes recruited from the bloodstream (Bhatt and Salgame, 2007). Once inside macrophages, *M. tuberculosis* subverts the major killing mechanisms employed by these host cells (Hestvik et al., 2005; Smith, 2003). This pathogen prevents fusion of phagosomes with lysosomes (Armstrong and Hart, 1971) and restricts its phagosome acidification to a mild pH (Sturgill-Koszycki et al., 1994), escaping from the degrading forces of proteolytic enzymes and from an acidic environment inside the phagolysosome. Therefore, the bacilli have the capacity to avoid immediate destruction, which allows them to establish a niche inside the macrophage (Houben et al., 2006).

Simultaneously, the *M. tuberculosis* infected macrophages induce a localized pro-inflammatory response, through the release of cytokines and chemokines, which promotes the recruitment of additional macrophages and other immune cells from the neighboring blood vessels to the infection site. These cells are the building blocks for the granuloma, the hallmark structure of tuberculosis (Dietrich and Doherty, 2009; Russell, 2007). The granuloma is constituted by an organized cluster of immune cells composed by infected macrophages in the center, surrounded by additional macrophages and lymphocytes, which delineate the periphery of the structure in association with a fibrous extracellular matrix. Dendritic cells, neutrophils and natural killer cells may also populate
this structure (Cosma et al., 2003; Russell, 2007). Although the precise function of the granuloma is not completely understood, it is believed that the formation of this structure contributes to the physical containment of the mycobacterial infection, limiting the pathogen dissemination to the rest of the lung tissue (Ahmad, 2011; Forrellad et al., 2012). However, the pathogen is not completely eradicated in some individuals since some M. tuberculosis bacilli may remain dormant for a long period of time, during which there are no disease symptoms and the individual does not transmit the infection to others. In this stage the infection is referred to as latent tuberculosis (Ahmad, 2011; Forrellad et al., 2012; Smith, 2003). Thus, only 5-10% of the infected population will develop the active disease in their lifetime (Mortellaro et al., 2009). When the immune system of a latently infected individual becomes weakened as a consequence of malnutrition, old age or HIV co-infection, the dormant bacilli are able to reactivate and replicate into the lung and other tissues (Ahmad, 2011). Under this situation, the granuloma center becomes necrotic, undergoes caseation, resulting in the destruction of the surrounding host tissue (Houben et al., 2006). This leads to the rupture of the granuloma walls and to the release of infectious bacilli into the airways (Dietrich et al., 2009; Russell, 2007).

1.4. Interaction of mycobacteria with antigen presenting cells

The interaction of mycobacteria with antigen presenting cells (APCs) is a major feature in the pathogenesis of tuberculosis. Both macrophages and dendritic cells play a central role in inducing the immune response against M. tuberculosis infection. Macrophages act as key effector cells in mycobacterial killing and dendritic cells in mycobacterial antigen presentation and consequent stimulation of naïve T cells. Therefore, both cell types are perfect targets for mycobacterial induced manipulation (Hope et al., 2004; Mortellaro et al., 2009).

1.4.1. Macrophages as the main reservoir for mycobacteria

Macrophages constitute the first line of cellular defense against microbial invasion and are known to be the main reservoir of infection by M. tuberculosis. Upon reaching the local of infection, the major role of macrophages is the rapid elimination of the invading microorganisms (Hestvik et al., 2005; Russell, 2001). A variety of cell surface receptors promote M. tuberculosis phagocytosis, these include among others, complement receptors, the mannose receptor (MR) and Fc receptors (Pieters, 2008). Following uptake, M. tuberculosis and other pathogens are retained within a phagocytic vacuole called the phagosome. If the normal phagosome maturation cycle occurs, a series of sequential fusion events with vesicles from the endocytic pathway occur, the phagosome fuses with lysosome and the pathogen encounters a hostile environment that includes acidic pH levels, reactive oxygen (ROI) and nitrogen intermediates (RNI) and lysosomal proteases, such as cathepsins. Together, these mechanisms will promote the pathogen destruction as well as the processing and presentation of antigens to T cells, in the context of major histocompatibility complex (MHC) molecules (Hope et al., 2004; Poirier and Av-Gay, 2012).
Contrasting with this scenario, pathogenic mycobacteria have developed strategies to avoid these killing mechanisms (Hestvik et al., 2005; Smith, 2003). Armstrong and Hart (1971) showed that after being phagocytosed, *M. tuberculosis* arrests normal maturation of its phagosome and thus prevents its fusion with pre-formed lysosomes, avoiding direct exposure of the bacilli to the degrading force of lysosomal hydrolases. Moreover, phagosomes containing *M. tuberculosis* do not undergo further acidification, presenting a mild pH of 6.2-6.3, due to the exclusion of vacuolar proton-ATPase from the bacilli-containing phagosome membrane (Sturgill-Koszycki et al., 1994; Tailleux et al., 2003). These mechanisms not only allow the bacilli to evade proteolytic degradation but also contribute to keeping its antigens from being processed and loaded onto MHC class II molecules for antigen presentation (Baena and Porcelli, 2009). In addition to interfering with phagosome maturation, *M. tuberculosis* also manipulates intracellular trafficking in macrophages, retaining access to the recycling endosome system, in order to obtain the required nutrients for the bacilli intracellular growth (Clemens, 1996; Mortellaro et al., 2009).

**Macrophage activation and its consequences during *M. tuberculosis* infection**

In response to pathogen invasion and inflammation mediators, resting macrophages (M0) undergo different programs of activation (polarization). Activated macrophages have been generally classified into two groups: classical (M1) and alternative (M2) activated macrophages (Benoit et al., 2008). M1 macrophages result from the stimulation with type-1 cytokines (Interferon gamma - IFN-γ) and microbial products. These cells are inflammatory and their microbicidal activity is enhanced (Lugo-Villarino et al., 2011). In contrast, M2 macrophages are poorly microbicidal and play a critical role in the resolution of inflammation by producing anti-inflammatory mediators. M2 activation program is driven by the stimulation of resting macrophages with type-2 cytokines, interleukin 4 (IL-4) and interleukin 13 (IL-13). In general, polarization of M1 macrophages is part of the common host immune response against bacterial infection (Andrade et al., 2012; Benoit et al., 2008).

In resting macrophages, pathogenic mycobacteria in order to survive and replicate, inhibit phagosome-lysosome fusion. However, IFN-γ activated macrophages (M1) are able to overcome the blockage of phagosome maturation from infected cells. Furthermore, IFN-γ activated macrophage response is characterized by the secretion of large amounts of pro-inflammatory cytokines and chemokines, high production of ROI and RNI, and enhanced phagocytosis. It also enhances the expression of MHC class II and costimulatory molecules such as CD80 and CD86 for antigen presentation to T cells (Herbst et al., 2011; Lugo-Villarino et al., 2011; Poirier and Av-Gay, 2012).

**Antigen presentation pathway and their modulation by *M. tuberculosis***

Once activated by IFN-γ, macrophages have the capacity to present mycobacterial antigens to T cells. However, unlike dendritic cells which present mycobacterial antigens to naïve T cells in lymph nodes, macrophages present antigens to effector T cells at sites of infection (Baena and Porcelli, 2009).
Antigen presentation mediated by MHC molecules is a complex process that involves distinctive pathways. MHC class I molecules are expressed on all nucleated cells, presenting antigens to antigen-specific CD8+ T cells. This pathway is specialized to present endogenous antigens, which are processed in the cytosol via proteasome. In contrast, MHC class II molecules expression is restricted to the professional APCs, including macrophages, dendritic cells and B cells. This pathway allows the presentation of exogenous antigens, such as mycobacterial antigens, that are processed in the endosomal route and presented to CD4+ T cells (Crevel et al., 2002; Hsing and Rudensky, 2005). Moreover, there is another antigen presentation pathway involved. Nonclassical MHC-like molecules, such as CD1, present among others lipids, mycobacterial lipoproteins to CD1-restricted T cells, and are expressed on both macrophages and dendritic cells (Crevel et al., 2002). Besides this, dendritic cells and macrophages are also capable of performing cross-presentation. This pathway enables presentation of peptides derived from exogenous antigens on MHC class I molecules and consequent stimulation of CD8+ T-cells (Rock and Shen, 2005).

CD4+ T cell activation is essential for control of mycobacterial infection, therefore interfering with MHC class II pathway for antigen presentation in macrophages, might be a strategy employed by the bacterium to avoid destruction and to persist in host immune cells (Chapman, 2006; Koul et al., 2004). Indeed, several mechanisms have been described by which *M. tuberculosis* can affect antigen presentation, including downregulation of the expression of MHC class II and costimulatory molecules, and sequestering mycobacterial antigens from molecules required for CD4+ T-cell activation (Koul et al., 2004; Noss et al., 2000; Russell, 2001). Components of mycobacterial cell wall, such as the 19-kDa lipoprotein, down-regulate surface expression of MHC class II molecules, reducing the antigen presentation capacity of the infected macrophages (Noss et al., 2001). Furthermore, mycobacterial species were found to interfere with macrophage IFN-γ signaling in order to inhibit the synthesis of MHC class II molecules (Noss et al., 2000).

1.4.2. Dendritic cells as important targets during mycobacteria infection

Dendritic cells (DCs) are the most potent antigen presenting cells of immune system, given their unique ability to capture the pathogen at the infection site and to migrate to secondary lymphoid organs in order to present pathogen-derived antigens to naïve T-cells. Hence, they are likely to have a major role in initiation of the adaptive immunity against tuberculosis (Banchereau et al., 2000; Hermann and Lagrange, 2005; Savina, 2007).

Dendritic cells are monocytic bone marrow derived cells, present in most peripheral tissues such as the skin, intestine and the lungs, where they act as sentinel cells, monitoring the contact of the body surfaces with incoming pathogens (Demangel and Britton, 2000; Hope et al., 2004). In these sites, they are considered as immature dendritic cells. They express low to moderate levels of surface MHC class II and co-stimulatory molecules such as CD40, CD80, CD86, while having a high capability to phagocyte invading microorganisms. Therefore, immature dendritic cells are characterized by a high ability for antigen uptake, but low T cell stimulatory activity (Hope et al., 2004; Martino, 2008). In the lungs, immature dendritic cells underline alveolar spaces and upon mycobacterial encounter, these
cells effectively uptake the *M. tuberculosis* bacilli (Hanekom *et al*., 2003; Mortellaro *et al*., 2009). Upon interaction with pathogens, tissue injury or exposure to inflammatory mediators, these efficient antigen capturing cells undergo phenotypic and functional changes, a process termed maturation, that occur while they migrate into secondary lymphoid organs such as lymph nodes. During this maturation process, dendritic cells lose the capability to capture antigens, but acquire an increased ability to present antigens effectively, expressing high levels of antigen presenting molecules in their surface, such as MHC class II and CD1, and high levels of co-stimulatory molecules as CD40, CD80 and CD86 (Banchereau *et al*., 2000; Martino, 2008). In lymph nodes, mature dendritic cells present processed antigens in association with MHC class II molecules to naïve CD4+ T lymphocytes, inducing their activation and differentiation into effector T cells (Figure 1).

![Figure 1. Role of dendritic cells (DC) in the immune system.](image)

**Figure 1. Role of dendritic cells (DC) in the immune system.** In peripheral tissues, immature DCs, act as sentinel cells, monitoring these tissues against invading pathogens. Upon pathogen capture, immature DCs initiate their maturation and migration through the lymph into lymph nodes. Pathogen-derived antigens are presented by the mature DCs to naïve CD4+ T cells, inducing their activation and differentiation into effector T cells. This figure was adapted from Geijtenbeek *et al*. (2002).

Depending on the type of pathogen that is recognized by the dendritic cells and on the cytokine environment, CD4+ T cells differentiate into T helper 1 (Th1) cells which secrete IFN-γ, inducing the killing of intracellular pathogens or T helper 2 (Th2) cells characterized by the production of interleukin 4 (IL-4), effective against extracellular pathogens (Koul *et al*., 2004). During *M. tuberculosis* infection, the Type 1 cytokines IL-12, IL-1 and IL-18 are secreted, polarizing the T cell response towards Th1 phenotype (Mortellaro *et al*., 2009). Thus, the effector Th1 cells, activated following antigen presentation by dendritic cells, migrate to the site of infection and produce cytokines such as IFN-γ in order to activate *M. tuberculosis* infected macrophages and consequently eliminate the bacilli (Bodnar *et al*., 2001; Poirier and Av-Gay, 2012).

Because dendritic cells play a crucial role in induction and regulation of a protective immunity against pathogens, modulation of dendritic cell generation and/or maturation may be a significant mechanism by which these infectious agents evade immune surveillance and disseminate within the host (Martino *et al*., 2004). Considerable in *vitro* evidence exists that dendritic cells can internalize pathogenic mycobacteria by phagocytosis (Henderson *et al*., 1997), although the outcome of this
interaction is still not completely understood. In particular, the ability of these pathogens to interfere with dendritic cell maturation remains controversial. Henderson et al. (1997) found that human monocyte-derived dendritic cells uptake *M. tuberculosis* efficiently, promoting the up-regulation of cell surface maturation markers in infected cells, including surface molecules involved in antigen presentation and interaction with T cells. This phenotype was consistent with activation of the dendritic cells, suggesting that infected dendritic cells produced cytokines that lead to maturation (Henderson et al., 1997). Furthermore, Thurnher and colleagues (1997) described the effects of BCG on cultured human blood dendritic cells. Infection with BCG resulted in the down-modulation of endocytosis and the up-regulation of surface maturation markers. The maturation of infected dendritic cells was at least in part induced by the secretion of tumor necrosis factor-alpha (TNF-α) by these cells in response to BCG infection. In contrast to these findings, suggesting that dendritic cell maturation is induced by mycobacteria infection, other workers have reported that *M. tuberculosis* inhibits maturation of human monocyte-derived dendritic cells *in vitro*. Infected dendritic cells presented a minimal and reversible upregulation of cellular surface maturation markers and were compromised in their ability to activate naïve T cells (Hanekom et al., 2003). Altogether, these indicated that infection with mycobacteria might have diverse phenotypic effects on immature dendritic cells, *in vitro*.

1.5. **Mycobacterial manipulation of host cathepsins**

Cathepsins are a larger family of proteases composed mostly by cysteine proteases, with the exception of cathepsins D and E which are aspartic proteases and cathepsin A and G which are serine proteases (Beers et al., 2003; Zavasnik-Bergant and Turk, 2006). Their classification is based on their structure and catalytic type (Reiser et al., 2010). In general, the cysteine cathepsins are stable in acidic cellular compartments as lysosomes and endosomes (Reiser et al., 2010). All cathepsins are synthesized as inactive proenzymes that require activation by proteolytic cleavage (Zavasnik-Bergant and Turk, 2006). The major regulators of cathepsin activity are cystatins, endogenous protein inhibitors, which bind to the target enzyme, obstructing the active site and thereby preventing substrate hydrolysis (Hsing and Rudensky, 2005).

Cathepsins are involved in a number of important cellular processes in APCs such as pathogen destruction, processing of several host cell enzymes, antigen presentation and apoptosis. Considering antigen presentation, these proteases are implicated in the generation of mature MHC class II molecules and in antigen processing within endolysosomal compartments, where they degrade pathogens internalized by APCs into antigenic peptides presentable in MHC class II molecules (Baena and Porcelli, 2009; Conus and Simon, 2010; Zavasnik-Bergant and Turk, 2006).

Newly synthesized MHC class II αβ heterodimers assemble in the endoplasmic reticulum (ER) and bind to a chaperone, the invariant chain (Ii), before leaving ER towards endocytic compartments. The invariant chain not only function as a chaperone, assuring the proper folding and assembly of MHC class II molecules, but also directs trafficking of these molecules to the endosomal compartments, which contain the antigen peptides for loading onto MHC class II complex (Hsing and Rudensky, 2005; Riese et al., 1996). During the translocation through the endocytic pathway, the
invariant chain undergoes a stepwise proteolytic degradation (Figure 3) mediated by cathepsins that generate various cleavage intermediates, such as lip22 and lip10 intermediates. The initial cleavages can be executed by asparagine endopeptidase (AEP) although other proteases can perform this cleavage in its absence (Trombetta and Mellman, 2005). The processing of li culminates in the generation of the class II-associated invariant chain peptide (CLIP) (Beers et al., 2003; Nepal et al., 2006). The CLIP peptide remains in the MHC class II peptide binding groove and prevents premature peptide loading. A second chaperone, human leukocyte antigen (HLA)-DM, will then catalyze the removal of CLIP, in exchange for the antigen peptides. The resulting peptide-loaded MHC class II complexes are then transported to the cell surface and prepared for presentation to CD4+ T-cells in combination with co-stimulatory molecules (Chapman, 2006; Hsing and Rudensky, 2005; Torres et al., 2006).

![Diagram of invariant chain degradation events](image)

**Figure 2. Invariant chain degradation events.** The invariant chain (li chain) undergoes successive cleavages, during the translocation of li chain-MHC class II complex through the endocytic pathway of antigen presenting cells. This proteolytic degradation is initiated by asparagine endopeptidase (AEP) or other unidentified proteases, originating the li chain intermediate p10. Further cleavage (involving cathepsin S or L, depending on the cell types) results in the class II-associated invariant chain peptide (CLIP). Subsequently, a second chaperone, human leukocyte antigen (HLA)-DM, catalyzes the removal of CLIP in exchange for the antigen peptides. This figure was adapted from Trombetta and Mellman (2005).

Macrophages and dendritic cells are equipped with a diverse group of cathepsins, most of which are cysteine proteases. Cathepsin B, L and S are the major cysteine proteases expressed in these cells and have all been suggested to participate in the processing of internalized antigens for CD4+T cell presentation and/or processing of invariant chain (Hsieh et al., 2002; Nepal et al., 2006). Cathepsin S is the principal protease involved in the late steps of li degradation in macrophages, dendritic cells and B cells, specifically in the conversion of lip10 into CLIP, while in cortical thymic epithelial cells this process is mediated by cathepsin L (Nepal et al., 2006). Moreover, both cathepsin S and L have also been shown to be important in the generation of certain antigenic peptides (Hsing and Rudensky, 2005; Plüger et al., 2002), as well as cathepsin B (Matsunaga et al., 1993).

The infection with *M. tuberculosis* elicits a MHC class II-restricted CD4+T cell response that is essential to control the primary mycobacterial infection. Therefore, the integrity of the class II MHC
antigen presentation pathway is necessary to ensure that the bacilli are contained and that adaptive immunity is established. Since, cathepsins play an important role in MHC class II antigen processing and presentation, pathogenic mycobacteria modulate MHC class II pathway by interfering with these host proteases (Nepal et al., 2006). In this context, in vitro experiments showed that M. bovis BCG infection causes inhibition of IFN-γ induced cathepsin S expression, in infected human acute monocytic leukemia cell line (THP-1) cells (Sendide et al., 2005). This was associated with an intracellular accumulation of MHC class II molecules complexed with cathepsin S lip10 substrate and with the export of immature MHC class II molecules to the cell surface. This effect was reversed by the addition of neutralizing antibodies that suppress IL-10, restoring the expression of active cathepsin S and the export of mature MHC class II molecules to the surface of infected cells. Thus, M. bovis BCG inhibits cathepsin S expression through the induction of the inhibitory cytokine IL-10 (Sendide et al., 2005). In addition, the inhibition of expression or activity of other cathepsins, such as cathepsin L, in murine bone marrow-derived macrophages infected with either M. avium or M. tuberculosis, was also reported. In contrast, the cathepsin B and S activity has no obvious alteration upon mycobacterial infection. The inhibition of expression of this cathepsin may influence the types of T cell epitopes generated in antigen presenting cells (Nepal et al., 2006).

1.6. Thesis Goals

The major goal of this project is to characterize mycobacterial manipulation of host macrophage activation and dendritic cell maturation, in the context of host proteases such as lysosomal cathepsins. The study of these mycobacteria-host interactions may provide us the knowledge to develop new strategies for the prevention and treatment of tuberculosis.

In the first part of this thesis we intent to elucidate the role of mycobacteria during macrophage activation and dendritic cell maturation. For this purpose, human macrophages and DCs were infected with the avirulent M. tuberculosis H37Ra or the non-pathogenic M. smegmatis expressing green fluorescent protein (GFP) and their surface phenotype was analyzed using flow cytometry. We focused our analysis in the expression of surface activation or maturation markers, involved in antigen presentation and T cell stimulation. We also studied the influence that these mycobacteria species had on macrophage activation and DCs maturation, previously induced by IFN-γ and TNF-α, respectively.

The second part of this thesis, addresses the role of lysosomal cathepsins during mycobacteria infection of host macrophages and DCs. First, we evaluated whether mycobacteria infection modulates lysosomal cathepsins expression, by analyzing their protein levels in human macrophages and dendritic cells infected with the virulent M. tuberculosis H37Rv or the non-pathogenic M. smegmatis. Second, we investigated the importance of these cathepsins on M. tuberculosis intracellular survival within macrophages, by silencing cathepsins expression in THP-1 cells, using a shRNA lentiviral library, followed by infection with M. tuberculosis H37Rv or M. tuberculosis H37Ra strain and consequent quantification of intracellular survival through colony forming units counting.
2. Materials and Methods

2.1. Role of mycobacteria during macrophage activation and DC maturation

2.1.1. Bacterial cultures and growth conditions

*M. tuberculosis* H37Ra (25177, ATCC) expressing green fluorescent protein (GFP) (*M. tuberculosis* H37Ra GFP) was generated by electroporation with pMN437 plasmid (Addgene plasmid # 32362), which harbors a gene that encodes GFP protein (mycgfp2+) and an hygromycin resistance gene for selection (a kind gift from Michael Niederweis). This green fluorescent protein (GFP)-expressing strain was cultivated in complete growth medium containing Middlebrook’s 7H9 broth (Difco) supplemented with 0.2% (v/v) glycerol (Sigma), 10% (v/v) OADC (BD- Becton, Dickinson and Company) and 0.05% (v/v) Tyloxapol (Sigma). The medium was also supplemented with 50μg/ml of hygromycin (Invitrogen) to maintain the selective pressure.

*M. smegmatis* mc2155 (700084, ATCC) harbouring a p19-(long-lived) EGFP plasmid (*M. smegmatis* GFP) was grown in medium containing Middlebrook’s 7H9 broth (Difco) and Nutrient broth (Difco), supplemented with 0.5% glucose, 0.05% (v/v) Tyloxapol (Sigma) but also with 50μg/ml of hygromycin (Invitrogen) in order to stabilize GFP expression (Anes et al., 2006). Both cultures were incubated at 37°C with 100 rpm agitation.

Prior to infection, in order to obtain a single cell suspension, all mycobacterial cultures were centrifuged at 3000 x g for 10 minutes, washed with PBS (Phosphate buffer saline) and centrifuged a second time in the same conditions. Then, the bacterial pellet was resuspended in the desired cell culture medium without antibiotics. To remove the clumps, the mycobacterial suspension was passed through a 21G needle and then sonicated for 5 minutes. Residual aggregates were eliminated by a low speed centrifugation (350 x g) for 1 minute. Single cell suspension was verified by light microscopy. Finally, optical density at 600 nm (OD$_{600nm}$) was used to determine cellular density in the mycobacterial suspension, assuming that OD$_{600nm}$ = 0.1 is equivalent to 1x10$^7$ mycobacteria/ml.

Construction of *Mycobacterium tuberculosis* H37Ra GFP

- pMN437 plasmid purification – Transformation of *E. coli* with pMN437 plasmid

The chemically competent *E. coli* strain was placed on ice, in contact for 10 minutes with 100 ng of pMN437 DNA. The mixture was then incubated at 45 °C for 45 seconds (heat-shock transformation), followed by another 10 minutes on ice. The bacteria cells were inoculated into SOC medium (Super Optical Broth; Sigma) and incubated at 37 °C, under agitation for 1 hour. Following this, the cultures were plated in Luria-Bertani (LB) agar medium (Sigma) containing hygromycin and were incubated at 37 °C overnight (Hanahan and Harbor, 1983). As a confirmation of *E. coli* transformation with the pMN437 plasmid, colonies with GFP fluorescence were observed by fluorescence microscopy. The selected transformants were grown in LB broth with 50 μg/ml
hygromycin at 37 °C, under agitation, overnight. The pMN437 plasmid was purified using a PuroLink® Quick Plasmid Miniprep Kit (Invitrogen), according to the manufacturer's instructions, and was quantified using NanoDrop ND 100 (ThermoScientific).

- **Electroporation of *M. tuberculosis* H37Ra with pMN437 plasmid**

To prepare electrocompetent mycobacteria cells, *M. tuberculosis* H37Ra cultured as described above, was incubated on ice for 90 minutes and then centrifuged at 3000 x g for 10 minutes. Following this, mycobacteria cells were washed three times in ice-cold 10 % glycerol and before storage at -80 °C, bacterial cells were also resuspended in ice-cold 10 % glycerol.

The pMN437 plasmid (2 µg DNA) was electroporated into electrocompetent *M. tuberculosis* H37Ra, applying one single pulse of 1.8 kV, 25 µF, with the pulse-controller resistance set at 200 Ω resistance, in 0.2 cm cuvettes. Electroporated mycobacteria were incubated in Middlebrook’s 7H9 broth (described above) at 37 °C for 36 hours, to allow the expression of the antibiotic-resistant gene carried on the pMN437 plasmid. Subsequently, mycobacteria cells were plated on Middlebrook 7H10 agar medium (Difco) supplemented with 10 % (v/v) OADC (BD), 0.5 % (v/v) glycerol (Sigma) and 50 µg/ml of hygromycin, and incubated at 37 °C. After 4 weeks, transformant colonies were isolated and then inoculated in the Middlebrook’s 7H9 broth (described above) with 50 µg/ml of hygromycin. Adapted from (Goude and Parish, 2008).

### 2.1.2. Peripheral Blood Mononuclear Cells: Isolation Procedure

Peripheral Blood Mononuclear Cells (PBMCs) were obtained from buffy coat preparations kindly provided by Instituto Português do Sangue. The mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (GE Healthcare). Briefly, the buffy coat was diluted (1:1) in MACS Buffer, which was prepared by diluting MACS BSA (bovine serum albumin) Stock Solution 1:20 with autoMACS™ Rinsing Solution (both purchased from MACS, Miltenyi Biotec). Then, the diluted buffy coat was gently overlaid on Ficoll-Paque™ Plus (GE Healthcare) and an 800 x g centrifugation for 20 minutes at room temperature was performed without brakes and with minimal acceleration. The resulting interface, which contains the PBMCs, was collected and washed two times with cold MACS Buffer, through a 450 x g centrifugation for 10 minutes at 4 °C, followed by a second centrifugation at 350 x g for 5 minutes at 4º C. Subsequently, monocytes were positively selected from total PBMCs, using anti-CD14-labeled magnetic beads and MACS separation LS columns (MACS, Miltenyi Biotec), according to the manufacturer’s instructions. Depending on the following experiments, monocytes were plated at a desired density in the appropriate plates, in order to differentiate into macrophages and dendritic cells. Adapted from (Wang et al., 2010).
2.1.3. Human macrophage and dendritic cell differentiation

For macrophages differentiation, CD14 monocytes were resuspended in RPMI-1640 medium with GlutaMAX™ (Gibco) supplemented with 1 % HEPES (Gibco) at pH 7.4 and were allowed to attach to the plate for 2 hours at 37 ºC. Following this, cells were cultured with RPMI-1640 medium with GlutaMAX™ (Gibco) supplemented with 10 % (v/v) Heat-Inactivated Fetal Bovine Serum (iFBS) (Invitrogen), 1 % HEPES (Gibco) at pH 7.4, 1 % (v/v) sodium pyruvate (Gibco), 1 % (v/v) Penicillin-Streptomycin (Gibco), 0.1 % 2-Mercaptoethanol (Gibco) and 20 ng/ml M-CSF (macrophage-colony-stimulating factor) (Immunotools), for 7 days at 37 ºC in 5 % CO₂ atmosphere. On day 4, fresh medium with a full dose of M-CSF was added to the culture.

For dendritic cells differentiation, monocytes were resuspended in RPMI-1640 medium with GlutaMAX™ (Gibco) containing 10 % (v/v) iFBS (Invitrogen), 1 % HEPES (Gibco) at pH 7.4, 1 % (v/v) sodium pyruvate (Gibco), 1 % (v/v) Penicillin-Streptomycin (Gibco), 0.1 % 2-Mercaptoethanol (Gibco), 20 ng/ml IL-4 (Immunotools) and 10 ng/ml GM-CSF (granulocyte-macrophage colony-stimulating factor) (Immunotools), for 7 days at 37 ºC in 5 % CO₂ atmosphere. On day 2 and 4, new medium with a full dose of IL-4 and GM-CSF was added to the culture.

To analyze cell surface markers expression of human monocyte-derived macrophages and dendritic cells, monocytes were plated in 12-well plates, at a density of 1x10⁶ cells per well.

2.1.4. Treatment with IFN-γ and TNF-α

When required, human monocyte-derived macrophages were stimulated with human IFN-γ (Immunotools) at 100 UI/ml and human monocytes-derived dendritic cells were stimulated with TNF-α (Immunotools) at 50 ng/ml, overnight, and prior to infection.

2.1.5. Mycobacteria infection of human macrophages and dendritic cells

For analysis of cell surface markers expression of mycobacteria infected human monocyte-derived macrophages and dendritic cells, monocytes were plated in 24-well plates, at a density of 5x10⁵ cells per well and following 7 days of differentiation, human macrophages and dendritic cells were stimulated as described above or were left unstimulated. Then, these cells were infected with single-cell suspensions of M. smegmatis GFP or M. tuberculosis H37Ra GFP (prepared as described above), at a multiplicity of infection (MOI) of 1 and were incubated with the macrophage or dendritic cell differentiation medium without antibiotics (infection medium), for 3 hours at 37 ºC, to allow the uptake of mycobacteria. After 3 hours infection, in order to remove non-internalized extracellular mycobacteria, cells were washed with infection medium and cultivated for an additional 24 hours in infection medium supplemented with 10 μg/ml of gentamicin (Gibco). One day after infection, cells were analyzed by flow cytometry. Uninfected human macrophages and dendritic cells, non-stimulated and stimulated were used as controls.
2.1.6. Cell surface staining and flow cytometry

Uninfected or infected human macrophages and dendritic cells were washed with PBS, harvested by incubation with 5 mM EDTA (Ethylenediaminetetraacetic acid) (Gibco) in PBS for 10 minutes, followed by fixation with 4 % paraformaldehyde. Subsequently, cells were washed with MACS buffer (MACS, Miltenyi Biotec) and incubated for 30 minutes at room temperature with the appropriate fluorochrome-conjugated antibodies (Table 1). Cells were washed twice before cell surface markers expression was assessed, using Guava easyCyte™ HT flow cytometer (Millipore). Data analysis was performed using Guava InCyte software (Millipore) and results were expressed as the Mean Fluorescence Intensity (MFI) of each surface marker, percentage of positive cells for each surface marker or as the percentage of GFP-positive cells. A total of 5,000 events per sample were analyzed. Only viable cells were considered in the analysis.

2.2. Role of host cathepsins during mycobacteria infection in human macrophages and dendritic cells

2.2.1. Mycobacterial manipulation of cathepsins in human macrophages and DCs

2.2.1.1. Bacterial cultures and growth conditions

*M. smegmatis* was grown as described above but without hygromycin addition. *M. tuberculosis* H37Rv (25618, ATCC), was cultivated in complete growth medium containing Middlebrook’s 7H9 broth (Difco) supplemented with 0.2 % (v/v) glycerol (Sigma), 10 % (v/v) OADC (BD- Becton, Dickinson and Company) and 0.05 % (v/v) Tyloxapol (Sigma) and were incubated at 37 °C with 100 r.p.m agitation. Before any experiment, the individualization of mycobacterial culture was performed as previously described.

2.2.1.2. Mycobacteria infection of human macrophages and dendritic cells

CD14 monocytes isolated from the peripheral blood (as described above) were seeded at a density of 2x10⁶ cells per well, in a 6-well plate and differentiated into human macrophages and dendritic cells with the appropriate cytokines, for 7 days, as described above. The human monocyte-derived macrophages and dendritic cells (non-stimulated and stimulated) were infected with single-cell suspensions of *M. smegmatis* or *M. tuberculosis* H37Rv at a MOI of 1 for 3 hours. Following 3 hours infection, cells were washed and cultivated for an additional day, in infection medium supplemented with 10 μg/ml of gentamicin to destroy extracellular bacteria. To analyze the cathepsins expression, the infected cells were lysed and the proteins collected 24 hours post-infection.
2.2.1.3. Western Blot

The adherent cells were scraped with Laemmli buffer 2x (Sigma) diluted in PBS (1:2) and the cells in suspension were centrifuged and resuspended with the same buffer. The resulting cell lysates were denatured at 95 °C for 5 minutes. The same volume of protein extracts were loaded on a 12 % SDS-PAGE gel (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) and the protein separation was performed by electrophoresis at 200 V for 1 hour. Then proteins were transferred to a nitrocellulose membrane (0.45 µm pore size; Bio-Rad) at 30 V, 4 °C, overnight, in a wet transfer system, Mini Trans-Blot® module (Bio-Rad). The membrane was blocked for 1 hour in Blocking Buffer, prepared with 5 % Bovine Serum Albumin (BSA; Merck) in TBS-T (Tris-buffered saline 1x, 0.1 % Tween20), and then incubated for 2 hours at room temperature with the appropriate primary antibodies (Table 2(a)) diluted in TBS-T with 1 % BSA (Merck). After washing the nitrocellulose membrane with TBS-T, incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Table 2(b)) diluted in TBS-T with 5 % BSA (Merck) was performed, for 1 hour at room temperature. The membrane was washed again, before enhanced chemiluminescence detection. Cathepsin bands were visualized using ECL Prime Western Blotting Detection Reagents (Amersham). Quantification of blot bands was performed using ImageJ 1.45 software (National Institutes of Health, USA). Anti-tubulin antibody was used as a normalization control.

2.2.2. The effect of host cathepsins on mycobacteria intracellular survival

2.2.2.1. Bacterial cultures and growth conditions

*M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv were cultivated as described above but without hygromycin addition. Before any experiment, the individualization of mycobacterial culture was performed as previously described.

2.2.2.2. THP-1 cell line and growth conditions

Human acute monocytic leukemia cell line, THP-1 (TIB-202™, ATCC), was grown in RPMI-1640 medium with GlutaMAX™ (Gibco) supplemented with 10 % (v/v) iFBS (Invitrogen), 1 % (v/v) L-glutamine (Gibco), 1 % (v/v) HEPES (Gibco) at pH 7.4, 1 % (v/v) sodium pyruvate (Gibco), 1 % (v/v) MEM-non essential amino acids (Gibco) and 1 % (v/v) Penicillin-Streptomycin (Gibco), and incubated at 37 °C in 5 % CO₂ atmosphere.

2.2.2.3. HEK 293T cell line and growth conditions

Human embryonic kidney 293T cell line, HEK 293T (CRL-11268™, ATCC), was maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 10 % (v/v) iFBS (Invitrogen) and 1 % (v/v) Penicillin-Streptomycin (Gibco) and incubated at 37 °C in 5 % CO₂ atmosphere.
2.2.2.4. Stable knockdown of cathepsin genes in THP-1 cells using lentiviral vectors

To knockdown cathepsin gene expression in THP-1 cell line, we used a replication-incompetent lentiviral vector that infects the target cells, integrates into its genome, endogenously expressing short hairpin RNAs (shRNAs). The expressed shRNAs were subsequently processed by the RNA interference machinery of the cell, into small interfering RNAs (siRNAs), which will target the degradation of cathepsins mRNA (Silva and Schnierle, 2010; Stewart et al., 2003). Lentiviral particles were produced by co-transfecting a packing cell line with a three-plasmid expression system: a 2nd generation packaging plasmid pCMV-dR8.91 expressing viral enzymes and structural proteins, needed for infectious particle formation, an envelope plasmid pCMV-VSVG (Addgene plasmid #8454) containing the envelope glycoprotein of vesicular stomatitis virus (VSV-G), and the lentiviral vector, pLKO.1 puro plasmid (Addgene plasmid #8453) that expresses the shRNA and carries a puromycin resistance marker, for selection of the cells that stably express the shRNA of interest. Therefore, this method allows a long-term silencing of cathepsin genes within THP-1 cell line (Moffat et al., 2006; Pluta and Kacprzak, 2009).

Purification of shRNA-pLKO.1 plasmid, lentiviral production and lentiviral infection were performed according to TRC lentiviral protocols present at the RNAi Consortium website (https://www.broadinstitute.org/genome_bio/trc/publicProtocols.html).

- **Purification of cathepsin shRNA-pLKO.1 Plasmid**

  The cathepsins shRNA-pLKO.1 vectors were kindly provided as bacterial glycerol stocks by Prof. Luis Moita (Instituto de Medicina Molecular, Lisbon). In order to isolate shRNA-pLKO.1 plasmid, the *E. coli* transformants were grown in TB (terrific broth) medium containing 100 µg/ml carbenicillin (Sigma Aldrich®) for 16 hours at 37 ºC, 300 r.p.m agitation. Following 16 hours of incubation, the DNA plasmid was purified using a PuroLink® Quick Plasmid Miniprep Kit (Invitrogen), according to the manufacturer’s instructions, and was quantified using NanoDrop ND 100.

- **Lentiviral production in HEK 293T cell line**

  HEK 293T packaging cells were seeded at a density of 2.2x10⁴ cells per well in DMEM medium (Gibco) supplemented with 10 % (v/v) iFBS without Penicillin-Streptomycin (Gibco), in flat-bottom 96-well microplates. After 24 hours of incubation at 37 ºC in 5 % CO₂ atmosphere, packaging cells with ~ 70% confluence were co-transfected with 100 ng of the packaging plasmid pCMV-dR8.91, 10 ng of the envelope plasmid pCMV-VSV-G and the 100 ng of the shRNA-pLKO.1 puro plasmid, using FuGENE 6 transfection reagent (Roche) in Opti-MEM medium (Invitrogen). Following 18 hours of incubation at 37 ºC in 5 % CO₂ atmosphere, the medium containing the transfection reagent was replaced by DMEM medium supplemented with 30 % iFBS (Invitrogen) and 1 % Penicillin-Streptomycin (Gibco). Lentivirus were collected 24 hours later and then aliquoted and stored at -80 ºC.
o **Lentiviral Infection of THP-1 cell line**

Following production of shRNA lentivirus, transduction of THP-1 cell line was performed in order to knockdown cathepsin genes. THP-1 cells were seeded at a density of $5 \times 10^4$ cells per well in round bottom 96-well microplates. Cells were centrifuged and resuspended in 10 µl of lentivirus and 40 µl of fresh THP-1 culture medium containing 8 µg/ml of Polybrene B (Sigma Aldrich®) was added to each well. Microplates were spun at 900 x g for 90 minutes at 37 ºC (spinoculation). Following spinoculation, supernatants were replaced with fresh THP-1 culture medium and cells were incubated at 37 ºC in 5 % CO₂ atmosphere. After 48 hours, transduced cell selection was performed by replacing medium in each well with fresh THP-1 culture medium supplemented with 5 µg/ml of puromycin (Sigma). Cells were maintained under puromycin selection for 3 days and then each microplate was split into two replica plates and incubated at 37 ºC in 5 % CO₂ atmosphere, in order to expand transduced cells. The incubation period following split, was dependent on the subsequent assays performed. Previous to each assay, cells were collected and their number normalized.

o **Validation of cathepsin genes knockdown by western blotting**

Each cathepsin gene has five different shRNAs with different knockdown efficiencies. In order to access the level of knockdown of each cathepsin shRNAs, relative quantification of cathepsin genes expression was performed at protein level by western blotting.

THP-1 transduced cells were seeded in 6-well plate, at a density of $2 \times 10^6$ cells per well. In order to induce differentiation of THP-1 monocytes into macrophages, cells were incubated in THP-1 culture medium supplemented with 20 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 24 hours. Then, the THP-1 culture medium supplemented with PMA was replaced with new fresh PMA-free medium. Following 24 hours, in order to quantify at cathepsin protein expression, the cells were lysed, proteins collected and a western blot was performed, as previously described. The primary and secondary antibodies used in western blot were listed in Table 2 (a)(b).

2.2.2.5. **Quantification of mycobacteria intracellular survival in silenced macrophages**

Before infection, silenced THP-1 cells were seeded at a density of $7.5 \times 10^4$ cells per well in flat-bottom 96-well microplate, in THP-1 culture medium supplemented with PMA in order to differentiate into macrophages (as previously described). These cells were infected with single-cell suspensions of *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv at a MOI of 1 according with the previous protocol. After 3 hours infection, cells were washed three times with THP-1 culture medium without antibiotics and cultivated with 10 µg/ml of gentamicin for 24 hours. Culture medium was changed every two days during the course of the experiment.

To quantify mycobacteria intracellular survival, at day 5 post-infection cells were washed and lysed with 0.5 % Igepal (Sigma Aldrich®) solution in distilled water. Serial dilutions of the lysate were performed in distilled water before being plated on Middlebrook 7H10 agar medium (Difco).
supplemented with 10 % (v/v) OADC (BD) and 0.5 % (v/v) glycerol (Sigma). Colony forming units (CFU) were counted after 3 weeks of incubation at 37 °C.

2.2.2.6. Statistical analysis

Statistical treatment was performed using SigmaPlot 11.0. The analysis was made using One Way ANOVA followed by multiple comparisons versus a control group using Holm-Sidak test. The differences were considered statistically significant when p<0.05. The results are represented as the mean ± Standard deviation (SD).

2.3. Antibodies used to perform Flow Cytometry and Western Blot

Table 1: List of antibodies used to perform Flow Cytometry Assays

<table>
<thead>
<tr>
<th>Fluorochrome-conjugated antibodies</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-Mouse anti-human CD1a</td>
<td>BD Pharmingen™</td>
</tr>
<tr>
<td>Alexa Fluor® 488- anti-human CD14</td>
<td>Biolegend</td>
</tr>
<tr>
<td>PE-Mouse anti-human CD86</td>
<td>BD Pharmingen™</td>
</tr>
<tr>
<td>Alexa Fluor® 488- anti-human CD206(MMR)</td>
<td>Biolegend</td>
</tr>
<tr>
<td>PE-anti-human CD209 (DC-SIGN)</td>
<td>Biolegend</td>
</tr>
<tr>
<td>PerCP-anti-human HLA-DR</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

Table 2: List of antibodies used to perform Western Blot

<table>
<thead>
<tr>
<th>Primary Antibodies (a)</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal anti-human cathepsin B</td>
<td>1:2000</td>
<td>Abcam®</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-human cathepsin S</td>
<td>1:4000</td>
<td>Abcam®</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-human β-tubulin</td>
<td>1:5000</td>
<td>Abcam®</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibodies (b)</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse IgG (H+L)-HRP conjugated</td>
<td>1:2000</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Anti-rabbit IgG (H+L)-HRP conjugated</td>
<td>1:2000</td>
<td>Bio-Rad</td>
</tr>
</tbody>
</table>
3. Results

3.1. Role of mycobacteria during macrophage activation and DC maturation

In the first part of this project, we investigated the role of mycobacteria during macrophage activation and dendritic cell maturation. Initially, human peripheral blood monocytes were isolated from healthy human donors, differentiated towards human macrophages or human dendritic cells and their morphology and surface phenotype were characterized. Subsequently, we infected the human peripheral blood monocyte-derived macrophages and monocyte-derived dendritic cells with the *M. tuberculosis* H37Ra or *M. smegmatis* and examined surface expression of activation and maturation markers in these infected cells, using flow cytometry. We analyzed the outcomes of infection with both strains in these cell types and compared them to the effects of a positive control for macrophage activation and dendritic cell maturation. We also studied the outcomes of infection with these mycobacteria, in IFN-γ-induced macrophage activation and TNF-α-induced dendritic cell maturation.

To perform these experiments, we used green fluorescent protein (GFP)-expressing mycobacteria. We infected human macrophages and dendritic cells with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP and selected the infected host cells (GFP-positive cells) by flow cytometry. Using GFP-expressing mycobacteria enabled us to discriminate between mycobacteria infected and uninfected cells within the same well, which was fundamental since infection could be heterogeneous and the fraction of cells that remained uninfected (bystander cells) could have interfered in the interpretation of the results. Therefore, we guaranteed that the observed effects were simply from macrophages and dendritic cells that have internalized *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP.

3.1.1. Characterization of resting macrophages and immature dendritic cells

Macrophages were differentiated from CD14 peripheral blood monocytes cultured in presence of M-CSF for 7 days, as described above. It was evident from our observations, using optical microscopy, that human monocytes cultured in these conditions, acquired features of resting macrophages (M0). Some cells presented an elongated morphology but most of them remained with a round morphology after differentiation. They also exhibited an increase in size and adherence in comparison with human monocytes (results not shown). Moreover, surface phenotypic analysis, using flow cytometry, (Figure 3) also revealed that these cells expressed a macrophage-like phenotype. The cells expressed high levels of CD14, a surface marker for macrophages, and lacked expression of CD1a, which indicates together with the absence of DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), that these cells didn’t differentiate into DCs. Furthermore, they exhibited moderate levels of HLA-DR molecules and co-stimulatory molecules CD86. The cell surface expression of mannose receptor (CD206), which is a surface marker involved in the receptor-mediated uptake (Ernst, 1998), was also analyzed. As expected, these cells expressed intermediated levels of both surface markers. These results, together with the cellular morphology,
suggested that these cells acquired the morphologic and phenotypic characteristics of resting macrophages (Sallusto et al., 1995; Smith et al., 1998; Young et al., 1990).

Dendritic cells were generated from human peripheral blood monocytes cultured in the presence of GM-CSF and IL-4, as previously described. After 7 days of incubation, examination of the culture using optical microscopy revealed that cells attained the characteristic morphology of immature DC (imDCs), which included round cells with small cytoplasmic projections and tendency to aggregate, forming numerous floating clusters (results not shown). Analysis by flow cytometry of the cell surface markers (Figure 3) showed that DCs expressed high levels of CD1a but low levels of CD14 compared to resting macrophages. In addition, these cells constitutively express high levels of MHC class II molecules, and relatively moderate levels of the co-stimulatory molecules CD86. These cells also expressed elevated levels of mannose receptor and DC-SIGN, two important receptors present on imDCs for the efficient capture of a variety of antigens, including viable mycobacteria (Geijtenbeek et al., 2003). These results were in agreement with previous studies performed, that described the morphology and surface phenotype of imDCs, obtained using GM-CSF and IL-4 (Markowicz and Engleman, 1990; Sallusto et al., 1995). This suggests that these cells generated in our experiment were in an immature phenotypic state.

![Graph of surface markers expression](image)

Figure 3. Analysis of surface markers expression of human monocyte-derived macrophages and human monocyte-derived dendritic cells. CD14 monocytes were isolated from the peripheral blood as described in Materials and Methods and were cultured for 7 days, in the presence of M-CSF to develop into macrophages or in the presence of GM-CSF and IL-4 to generate DCs. Cells were then stained with specific antibodies (Abs) for the surface markers indicated and their surface phenotype was assessed by flow cytometry. A total of 5,000 events per sample were analyzed. Results are expressed as mean fluorescence intensities (MFI) from one single experiment.
3.1.2. Role of mycobacteria during macrophage activation

To investigate the influence of mycobacteria infection in macrophage activation, we initiated our study with the development of a positive control for classical macrophage activation (M1). In general, M1 macrophages are obtained by exposure of resting macrophages (M0) to two inflammatory stimuli, IFN-γ and LPS (Mosser, 2003). In preliminary experiments, we stimulated M0 with both cytokines and observed that IFN-γ induced a stronger up-regulation of the proinflammatory M1 phenotype, at the level of antigen presenting molecules, than LPS (data not shown). As a result, IFN-γ was selected to stimulate M0 macrophages.

M0 and M1 macrophages were then infected with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP and the number of GFP-positive cells was assessed by flow cytometry. The percentages of infected cells for each infection were determined (Figure 4): in M1 macrophages, the percentage of either *M. smegmatis* GFP or *M. tuberculosis* H37Ra GFP infected cells were lower than that observed in M0 macrophages. This may be attributed to the higher bactericidal activity of M1 activated macrophages in comparison with resting macrophages. The percentage of *M. smegmatis* GFP and *M. tuberculosis* H37Ra GFP infected cells was similar when M0 macrophages were used as host, whereas in M1 activated macrophages the percentage of *M. smegmatis* GFP infection was lower than the percentage of *M. tuberculosis* H37Ra GFP. This is probably a result of the higher bactericidal activity of M1 macrophages, in combination with the fact that the non-pathogenic mycobacteria *M. smegmatis* may be rapidly destroyed in the M1 environment, in contrast to *M. tuberculosis* H37Ra that developed survival mechanisms to persist inside host macrophages during a longer period of time.

![Figure 4. M. tuberculosis H37Ra GFP and M. smegmatis GFP infection of resting human monocyte-derived macrophages and IFN-γ-activated human monocyte-derived macrophages.](image)

(A) The results, obtained from 5,000 living cells, are expressed as the percentage of GFP-positive cells, which represent the percentage of cells infected with fluorescent mycobacteria. (B) Representative flow cytometry histograms show the GFP expression from resting macrophages (M0) and IFN-γ-activated macrophages (M1) infected with *M. tuberculosis* H37Ra or *M. smegmatis* GFP.
Next, the surface phenotype of M0 infected macrophages with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP at MOI 1, for 24 hours, was analyzed by flow cytometry (Figure 5). Since, HLA-DR and CD86 represent key molecules involved in antigen presentation and T cell stimulation, the cell surface expression of these markers was examined in infected macrophages and compared to that on both uninfected M0 and M1 polarized host cells.

**Figure 5.** Effect of mycobacteria infection during activation of human monocyte-derived macrophages. Resting macrophages (M0) differentiated as described in Materials and Methods were infected with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP at MOI 1 or were left uninfected. IFN-γ-stimulated macrophages (M1) were used as a macrophage activation control. Cells were then fixed and stained with specific Abs for the surface markers indicated, 24 hours after infection. Activation was determined by measuring through flow cytometry the cell surface expression of the costimulatory and antigen-presenting molecules, CD86 and HLA-DR, respectively. (A) Results are expressed as the mean fluorescence intensities (MFI) for each surface marker (shown on the right). (B) Representative flow cytometry histograms show the HLA-DR and CD86 expression from resting macrophages (M0), IFN-γ-activated macrophages (M1), M0 infected with *M. tuberculosis* H37Ra (H37Ra) and M0 infected *M. smegmatis* GFP (M. smeg).
As expected, IFN-γ-activated M1 macrophages showed up-regulation of HLA-DR and CD86 molecules, in comparison with the M0 phenotype. Infection of M0 macrophages with *M. smegmatis* GFP also induced further up-regulation of HLA-DR and CD86 molecules, evidenced by an increase in the percentage of positive cells and in the expression of both surface markers. In contrast to *M. smegmatis* GFP, the avirulent *M. tuberculosis* H37Ra GFP neither enhanced nor inhibited surface expression of CD86 and slightly down-regulated HLA-DR expression (~6% decrease) (Figure 5A and 5B). This lead us to speculate that the exposure of resting macrophages to avirulent *M. tuberculosis* H37Ra appears to prevent the induction of M1 pro-inflammatory phenotype at the level of the antigen-presenting molecules, while the non-pathogenic *M. smegmatis* induce this M1 pro-inflammatory phenotype, almost as effective as IFN-γ treatment.

In agreement with the previous results, we raised the question whether *M. tuberculosis* H37Ra and *M. smegmatis* would interfere with macrophage activation induced by the pro-inflammatory stimuli, IFN-γ. Therefore, M0 macrophages activated by treatment with IFN-γ were infected with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP at MOI 1. The surface expression of HLA-DR and the co-stimulatory molecules CD86 of infected and uninfected M1 (used as the control) was examined by flow cytometry. 24 hours post-infection. As shown in Figure 6, *M. tuberculosis* H37Ra GFP infected M1 macrophages displayed a slightly increase in surface expression of HLA-DR and CD86. When M1 cells were infected with *M. smegmatis* GFP, HLA-DR expression was slightly enhanced and the expression of CD86 in these cells did not change relative to the control. These results indicate that *M. tuberculosis* H37Ra did not impair IFN-γ-induced activation, at least at the level of antigen-presenting molecules, which might suggests that IFN-γ-induced activation was able to overcome the mechanisms of inhibition employed by these mycobacteria, observed in our previous results (Figure 5). *M. smegmatis* infected M1 cells present a similar phenotype to that of the control, which it was not surprising, since exposure of M0 macrophages to these environmental mycobacteria also induced a similar pro-inflammatory M1 phenotype to that induced by IFN-γ, as described in our previous results (Figure 5).

![Figure 6. Effect of mycobacteria infection on macrophage activation phenotype induced by IFN-γ.](image)

Resting macrophages (M0) generated as described in Materials and Methods were stimulated with IFN-γ for additional 24 hours, in order to activate these cells. IFN-γ-activated macrophages (M1) were either infected with *M. tuberculosis* H37Ra GFP (H37Ra) or *M. smegmatis* GFP (M. smeg) at MOI 1 or were left uninfected, as a macrophage activation control. Cells were then fixed, stained with the respective Abs and the cell surface expression of CD86 and HLA-DR was assessed by flow cytometry, 24 hours post-infection. Data are expressed as the mean fluorescence intensities (MFI) for each surface marker.
3.1.3. Role of mycobacteria during dendritic cell maturation

To decipher the role of mycobacteria infection during DC maturation process, a positive control for DC maturation was required. To accomplish this, imDCs were stimulated with TNF-α, for an additional day, in order to obtain mature DCs (mDCs). We exposed these cells to TNF-α, since the role of this cytokine, in the induction of DC maturation in vitro is well documented (Roake et al., 1995; Sallusto and Lanzavecchia, 1994).

imDCs and mDCs were then infected with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP and the number of GFP-positive cells was quantified by flow cytometry. Figure 7 displays the percentages of infected cells for each infection, as well as the phagocytic capacity of DCs generated in these experiments. One day after infection, the percentage of *M. tuberculosis* H37Ra GFP infected cells were slightly higher than the percentage of *M. smegmatis* GFP infected cells. Furthermore, a clear difference in the percentage of infected cells was observed, when comparing imDCs and mDCs (Figure 7A and 7B), which was 3.7-fold higher in imDCs relatively to mDCs in *M. smegmatis* GFP infection and 3-fold higher in imDCs compared with mDCs in *M. tuberculosis* H37Ra GFP infection. The fact that the number of infected cells in the mDCs population was lower than in imDCs confirms an effective maturation process, since mDCs are characterized by a lower ability to internalize bacteria (Martino, 2008).

![Figure 7.](image)

Figure 7. *M. tuberculosis* H37Ra GFP and *M. smegmatis* GFP infection of immature human monocyte-derived DCs and mature human monocyte-derived DCs. CD14 monocytes were isolated from peripheral blood and were cultured for 7 days with GM-CSF and IL-4 to develop into immature dendritic cells (imDCs). These cells were then cultured in the presence of GM-CSF and IL-4 either with or without TNF-α, to generate mature DCs (mDCs) or to maintain the immature phenotype. imDCs and mDCs were infected with *M. tuberculosis* H37Ra GFP (H37Ra) or *M. smegmatis* GFP (M. smeg) at MOI 1. Cells were then fixed and their GFP expression levels were measured using flow cytometry, 24 hours post-infection. (A) The results obtained from 5,000 living cells are presented as the percentage of DCs expressing GFP. (B) Representative flow cytometry histograms show the GFP expression from imDCs and mDCs, infected with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP.
Then, the surface phenotype of infected imDCs with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP at MOI 1, 24 hours post-infection, was examined by flow cytometry (Figure 8A and 8B). We focused our attention on surface maturation markers involved in antigen presentation and T cell interaction, such as CD1a, CD86 and HLA-DR. Infected imDCs phenotype was compared with that from both uninfected imDCs and mDCs. As shown in Figure 8, TNF-α-stimulation of imDCs resulted in a strong increase of HLA-DR and CD86 expression (~2-fold increase and 3-fold increase, respectively), but in a slightly down-regulation of CD1a molecules. An increase in HLA-DR and CD86 expression was also observed after infection of imDCs with both *M. tuberculosis* H37Ra GFP and *M. smegmatis* GFP. However, *M. tuberculosis* H37Ra induced somewhat higher up-regulation of HLA-DR and CD86 molecules than *M. smegmatis*, which indicates that both strains induce maturation of DCs, but the extent of induction by *M. tuberculosis* H37Ra is rather higher than by *M. smegmatis*. Relatively to the expression of CD1a, although TNF-α-stimulation of imDC resulted in a decrease in this surface marker expression, both *M. tuberculosis* H37Ra GFP and *M. smegmatis* GFP infection induced a similar up-regulation of this antigen-presenting molecule. This suggests that the increase in CD1a expression appears to be independent of the differences in virulence between both species.
Figure 8. **Effect of mycobacteria infection during phenotypic maturation of human monocyte-derived dendritic cells.** Immature human monocyte-derived dendritic cells (imDCs) generated as described in Materials and Methods were infected with *M. tuberculosis* H37Ra GFP (H37Ra) or *M. smegmatis* GFP (M. smeg) at MOI 1 or were left uninfected. In order to obtain mature DCs (mDCs) to use as a maturation control, some of the uninfected imDCs were stimulated with TNF-α. After one additional day in culture, cells were fixed, stained with the indicated Abs and surface maturation markers examined by flow cytometry. (A) Results are represented as the mean fluorescence intensities (MFI) for each surface marker. (B) Representative flow cytometry histograms show the HLA-DR, CD86 and CD1a expression from immature DCs, mature DCs, imDCs infected *M. smegmatis* GFP and imDCs infected with *M. tuberculosis* H37Ra.
Next, we decided to determine the effect of both *M. tuberculosis* H37Ra and *M. smegmatis* infection on DC maturation phenotype induced by TNF-α. To this, mature DCs were infected with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP at MOI 1 and the cell surface expression of CD1a, CD86 and HLA-DR was assessed by flow cytometry (Figure 9). Infected mDCs phenotype was compared with the surface phenotype of uninfected mDCs, used as the control. We observed that mDCs infected either with *M. tuberculosis* H37Ra GFP or *M. smegmatis* GFP displayed a maturation phenotype identical to the control, since almost no changes in surface expression of CD1a, CD86 and HLA-DR molecules were observed after infection. These results suggest that, neither *M. smegmatis* nor *M. tuberculosis* H37Ra appears to interfere with TNF-α-induced DC maturation. This absence of effect might be explained by the fact that mDCs lost their ability to internalize bacteria, as observed in our previous results by the lower percentage of mDCs infected with GFP-expressing mycobacteria (~15% infected cells) (Figure 7).

![Figure 9](image.png)

**Figure 9. Effect of mycobacteria infection on the DC maturation phenotype induced by TNF-α stimulation.** Immature human monocyte-derived dendritic cells (imDCs) generated as described in Materials and Methods were stimulated with TNF-α for 1 day to obtain mature DCs (mDCs). mDCs were either left uninfected, as a control, or were infected with *M. tuberculosis* H37Ra GFP (H37Ra) or *M. smegmatis* GFP (M. smeg) at MOI 1 and cultured for additional 24 hours. These cells were then fixed, stained with specific Abs for the surface markers indicated and analyzed by flow cytometry, 24 hours post-infection. Results shown are expressed as the mean fluorescence intensities (MFI) for each surface marker.
3.2. Role of cathepsins during mycobacteria infection of human macrophages and dendritic cells

In the second part of this project, we addressed the role of lysosomal proteases during mycobacteria infection of host cells. First, we examined the expression of different cathepsins, in primary human macrophages and dendritic cells infected with pathogenic and non-pathogenic mycobacteria, to decipher if mycobacteria infection modulates their expression. We focused our attention in cathepsin B and S, because they represent two major lysosomal cysteine proteases expressed in professional APCs, whose participation in the processing of internalized antigens and/or invariant chain degradation has been described (Nepal et al., 2008). Second, we investigated the importance of these same cathepsins on *M. tuberculosis* intracellular survival. For this purpose, we infected THP-1 macrophages silenced for each individual cathepsin, using shRNA lentiviral library, and monitor mycobacteria intracellular survival within these cells.

3.2.1. Mycobacterial manipulation of host cathepsins in human macrophages and dendritic cells

To evaluate if mycobacteria is able to modulate the expression of different cathepsins in human macrophages and DCs, we generated human resting macrophages (M0) and immature dendritic cells (imDCs) and stimulate them with IFN-γ (M1) and TNF-α (mDCs), respectively. Then, these cells (non-stimulated and stimulated) were infected with *M. tuberculosis* H37Rv at MOI 1. One day after infection, cathepsin S and B protein levels were assessed by western blot. These results were compared with those from uninfected cells but also from cells infected with a non-pathogenic *M. smegmatis* strain.

As observed in Figure 10A, while in uninfected macrophages non-stimulated (M0), cathepsin S was abundant, the exposure to *M. tuberculosis* H37Rv bacilli, lead to an almost 2-fold decrease in protein levels. In contrast, in M0 infected cells with the non-pathogenic *M. smegmatis*, cathepsin S levels remained practically unchanged. Next, we decided to determine if *M. tuberculosis* H37Rv infection also results in a decrease in cathepsin S induced by IFN-γ-stimulation, since it was reported that *M. tuberculosis* has the ability to interfere with IFN-γ-signaling pathways (Ting et al., 1999). As previously described (Chan, 2010), upon IFN-γ stimulation of M0 cells, an increase of cathepsin S was observed. Surprisingly, exposure of IFN-γ activated macrophages either to *M. tuberculosis* H37Rv or *M. smegmatis* bacilli resulted in a drastically decrease in this protease, to a level below the basal protein amounts observed in uninfected M0 cells (Figure 10A).

In DCs non-stimulated (imDCs), cathepsin S protein levels increased either after *M. tuberculosis* H37Rv or *M. smegmatis* infection (Figure 10B). However, the degree of up-regulation was different between both strains. *M. smegmatis* infected imDCs showed higher levels of this host cathepsin than *M. tuberculosis* H37Rv infected imDCs. Upon stimulation with TNF-α, we observed that DCs exhibited a slightly increase in cathepsin S, whereas *M. tuberculosis* H37Rv infection
strongly enhanced the amount of this protease in TNF-α-stimulated DCs. Infection with *M. smegmatis* also increased cathepsin S levels although to a lesser extent.

![Diagram](image)

**Figure 10. Manipulation of Cathepsin S expression by different species of mycobacteria.** Western Blot analysis of Cathepsin S (CathS), in non-stimulated (M0) and IFN-γ stimulated (M1) human monocyte-derived macrophages (A) and in non-stimulated (imDCs) and TNF-α stimulated (mDCs) human monocyte-derived dendritic cells (B), 24 hours after infection with *M. smegmatis* (M. smeg) or *M. tuberculosis* H37Rv (M. tb H37Rv) at MOI 1. β-Tubulin (β-Tub) was used as a normalization control. Quantification of western blots is shown on the right. Results are represented as the percentage of relative protein expression comparatively to cathepsin expression in uninfected non-stimulated macrophages or dendritic cells.

The results obtained from cathepsin B western blot analysis, in primary macrophages infected with pathogenic and non-pathogenic mycobacteria, were similar to those from cathepsin S, described above (Figure 10A). Figure 11A shows that infection of M0 cells with *M. smegmatis* resulted in levels of cathepsin B similar to those from uninfected M0 macrophages, whereas upon infection with *M. tuberculosis* H37Rv, the amount of this protease was specifically reduced (approximately 1.5-fold decrease). After infection of IFN-γ-activated macrophages with either *M. tuberculosis* H37Rv or *M. smegmatis* at MOI 1 (Figure 11A), a clear reduction in cathepsin B protein levels was observed similar to those observed for cathepsin S. Conversely, protein levels of cathepsin B, in infected human dendritic cells (Figure 11B) were slightly different from those of cathepsin S (Figure 10B). Upon *M.*
*smegmatis* or *M. tuberculosis* H37Rv infection of either imDCs or mDCs, the expression of cathepsin B remained unchanged relative to uninfected control.

![Graph A](image)

**A**

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Macrophages + IFN-γ</th>
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<tbody>
<tr>
<td>Uninf.</td>
<td>M. smeg M. tb</td>
</tr>
<tr>
<td>Uninf.</td>
<td>M. smeg M. tb</td>
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</table>

Relative Cathepsin B protein expression

![Graph B](image)

**B**

<table>
<thead>
<tr>
<th>Dendritic cells</th>
<th>Dendritic cells + TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninf.</td>
<td>M. smeg M. tb</td>
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<tr>
<td>Uninf.</td>
<td>M. smeg M. tb</td>
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Relative Cathepsin B protein expression

Figure 11. Manipulation of Cathepsin B expression by different species of mycobacteria. Western Blot analysis of Cathepsin B (Cath B), in non-stimulated (M0) and IFN-γ stimulated (M1) human monocyte-derived macrophages (A) and in non-stimulated (imDCs) and TNF-α stimulated (mDCs) human monocyte-derived dendritic cells (B), 24 hours after infection with *M. smegmatis* (M. smeg) or *M. tuberculosis* H37Rv (M. tb H37Rv) at MOI 1. β –Tubulin (β –Tub) was used as a normalization control. Quantification of western blots is shown on the right. Results are represented as the percentage of relative protein expression comparatively to cathepsin expression in uninfected non-stimulated macrophages or dendritic cells.

Altogether, these results indicate that *M. tuberculosis* H37Rv infection, contrary to that one of *M. smegmatis*, led to a reduction in basal cathepsin S and B protein levels in M0 macrophages, while in IFN-γ-activated macrophages, exposure to both *M. tuberculosis* H37Rv and *M. smegmatis* strains resulted in a dramatic decrease in protein amounts of these lysosomal proteases. Furthermore, in mature DCs, *M. tuberculosis* H37Rv infection led to a strong increase in cathepsin S contrary to cathepsin B. *M. smegmatis* seems to enhance cathepsin S expression both in imDCs and mDCs, while did not change cathepsin B protein levels in these cells.
3.2.2. Effect of cathepsins on *M. tuberculosis* intracellular survival within THP-1 macrophages

Next, we examined the importance of these host proteases on the pathogen intracellular survival within macrophages. To accomplish this, a shRNA lentiviral library was used, to express specific short hairpin RNAs (shRNAs), in order to knockdown cathepsin S and B in primary human macrophages and dendritic cells. However, in these cells, this silencing technique was not successful (data not shown), probably as a result of problems in spinoculation conditions. So, to proceed with this experiment, we decided to apply this same silencing method, to THP-1 monocytes instead of primary human cells, since it’s a well-studied human monocytic cell line, whose gene silencing using shRNA lentiviral vectors was already optimized in our laboratory.

After production of lentivirus expressing cathepsin B-shRNA oligomers, cathepsin S-shRNA oligomers or control shRNA oligomers, THP-1 monocytes were transduced with these vectors, in order to stably knockdown cathepsin S or B genes. Transduced cells were selected with 5 µg/ml puromycin and then, differentiated into macrophages by incubation with PMA overnight. THP-1 macrophages silenced for cathepsin S or B were then infected with *M. tuberculosis* H37Rv at MOI 1 (to avoid macrophages extensive cell death during the course of the experiment). To monitor the effect of knockdown of these genes on mycobacteria intracellular survival, CFU were determined at day 5 post-infection. Transduced cells were also infected with the avirulent *M. tuberculosis* H37Ra strain, at the same MOI and the results compared with those from virulent *M. tuberculosis* H37Rv.

To test cathepsin S silencing efficiency, in THP-1 macrophages transduced with different shRNA lentivirus, the levels this cathepsin were quantified by western blot. As shown in Figure 12B, expressed shRNA 2 and 3 considerably reduced cathepsin S levels in THP-1 macrophages while shRNA 1 did not significantly decrease cathepsin S levels. We observed that (Figure 12A) cathepsin S knockdown either of approximately 94% (shRNA 2) or 87% (shRNA 3) resulted in a significant increase in *M. tuberculosis* H37Ra CFUs. Strikingly, silencing of cathepsin S using the same shRNAs did not alter significantly *M. tuberculosis* H37Rv survival in comparison with the control. In macrophages transduced with the shRNA 1, either *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv survival was similar to the control. This result is consistent with the fact that shRNA 1 did not significantly knockdown cathepsin S, which means that the differences observed in the intracellular survival of *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv, when the other shRNAs were used, were in fact due to the absence of cathepsin S in macrophages.

Taken together, these data suggest that the knockdown of cathepsin S leads to increased survival of *M. tuberculosis* H37Ra in THP-1 macrophages, while does not seem to interfere with *M. tuberculosis* H37Rv survival. This absence of effect as a result of cathepsin S knockdown, might be explained by the fact that *M. tuberculosis* H37Rv already downregulates cathepsin S expression in human macrophages, as demonstrated by our previous results (Figure 10A). Therefore, cathepsin S silencing using lentiviral vectors, probably mimics the natural situation observed in infected control cells.
As shown in Figure 13B, transduction of THP-1 cells with shRNA 1 and 2, drastically decrease cathepsin B protein amounts (Figure 13B). shRNA 1 decreased cathepsin B levels around 89% and shRNA 2 reduced around 81%. Silencing of cathepsin B significantly increased the bacterial burden, in THP-1 macrophages infected either with *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv (Figure 13A). However, the cathepsin B knockdown appears to enhance the survival of *M. tuberculosis* H37Ra to a higher extent than that one of *M. tuberculosis* H37Rv.

These observations indicate that cathepsin B might be relevant to the control of both *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra intracellular growth, which is consistent with our previous results that showed that *M. tuberculosis* H37Rv down-regulates cathepsin B protein levels (Figure 11A).
Figure 13. Effect of Cathepsin B (Cath B) silencing on *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv intracellular survival within THP-1 macrophages. (A) THP-1 monocytes, transduced with lentiviral vectors that expressed short-hairpin RNAs (shRNAs) specific for cathepsin B (shRNA 1, 2) or control shRNA, were differentiated into macrophages by incubation with PMA overnight, followed by infection with *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv at MOI of 1. To measure mycobacteria intracellular survival, CFU were determined at day 5 post-infection. Data are expressed as the mean percentage of CFU relative to control ± SD of three replicates from one experiment. (B) Knockdown efficiency of cathepsin B. Levels of cathepsin B in THP-1 macrophages transduced with shRNA 1, 2 and control shRNA were quantified by western blot. β –Tubulin (β –Tub) was used as a normalization control. Results are represented as the mean percentage of relative protein expression comparatively to control ± SD from two independent experiments. *, statistically significant differences comparatively to control shRNA ( * P < 0,05) by One Way ANOVA followed by comparisons versus a control group using Holm-Sidak method.
4. Discussion

An effective immune response to *M. tuberculosis* involves APCs, such as alveolar macrophages and DCs, which represent the first lines of defense encountered by this pathogen, in the host. These two cell types have distinct roles in immunity against tuberculosis: while macrophages mainly induce microbicidal mechanisms for elimination of mycobacteria, DCs are specialized in the presentation of mycobacterial antigens to naïve T cells (Banchereau *et al*., 2000; Hestvik, Hmama, & Av-gay, 2005). Macrophages and DCs must undergo an activation and maturation process, respectively, so they can perform their specific functions. Therefore, in the first part of this thesis, we investigated whether mycobacteria infection interferes with macrophage activation and dendritic cell maturation. For this purpose, we used human macrophages and DCs, obtained by culturing PBMCs in vitro with the respective cytokines. These cells acquired morphologic features and a cell surface phenotype (Figure 3) that closely resemble resting macrophages (M0) (Smith *et al*., 1998; Young *et al*., 1990) and immature DCs (Chapuis *et al*., 1997; Sallusto and Lanzavecchia, 1994). Subsequently, we infected resting macrophages and imDCs with GFP-expressing mycobacteria strains. By using GFP expressing *M. tuberculosis* H37Ra and *M. smegmatis*, we were able to select by flow cytometry the cells that have internalized mycobacteria and thus, analyze the effect of infection on the expression of their surface activation and maturation markers.

Classically activated macrophages (M1) are developed in response to exposure to a microbial challenge or in response to type-1 cytokines, of which IFN-γ is probably one of the most relevant (Lugo-villarino *et al*., 2011; Pieters, 2008). It is widely described that IFN-γ has a critical role in immunity against intracellular pathogens, such as *M. tuberculosis* (Cooper *et al*., 1993; Flynn *et al*., 1993; Newport and Huxley, 1996). Considering the importance of IFN-γ-induced activation in the control of infection, it is not surprising that *M. tuberculosis* has evolved mechanisms to interfere with induction of this pro-inflammatory M1 phenotype (Benoit *et al*., 2008), in order to persist within host cell. Indeed, our results demonstrate that the infection of M0 macrophages with the avirulent *M. tuberculosis* H37Ra strain appears to prevent the induction of M1 pro-inflammatory phenotype at the level of the antigen presenting molecules, despite the low bacterial load used. In contrast, infection with the non-pathogenic *M. smegmatis* was clearly stimulatory of M1 phenotype, almost as effective as IFN-γ stimulation. Since the percentage of infected cells with *M. tuberculosis* H37Ra and with *M. smegmatis* was similar (Figure 4), the marked differences observed may actually be explained by virulence mechanisms of *M. tuberculosis* that does not exist in *M. smegmatis*. These findings are in line with a previous report, showing a slightly down-modulation of the expression of MHC class II molecules and no alterations in CD86 molecules, after *M. tuberculosis* H37Rv infection of human monocyte-derived macrophages (Giacomini *et al*., 2001). This suggests that prevention of induction of M1 phenotype at the level of antigen presenting molecules following infection with *M. tuberculosis* H37Ra, possibly impairs the capacity of macrophages to present mycobacterial antigens and thus to activate CD4+ T cells.

Interestingly, despite preventing the induction of the M1 phenotype, *M. tuberculosis* H37Ra infection did not impair IFN-γ-induced activation of macrophages, at least at the level of antigen
presenting molecules (Figure 6). This indicates that the IFN-γ-induced activation was able to overcome the inhibitory effects of *M. tuberculosis* H37Ra infection on antigen presenting molecules within M0 macrophages (Figure 5). In contrast, a previous study by Hmama et al. (1998) reported that a virulent *M. tuberculosis* strain decreased IFN-γ-induced cell surface expression of HLA-DR molecules in THP-1 cells (Hmama et al., 1998). Another report showed that in murine bone-marrow-derived macrophages, infection with viable *M. tuberculosis* H37Ra inhibited IFN-γ-induced expression of MHC class II molecules (Noss et al., 2000). We considered that, differences in the mycobacterial strain, the type of macrophages and the multiplicity of infection used, might explain this discrepancy between our results and those previously reported. The MOI used by Noss et al. (2000) was considerably higher than that used in our work, and resulted in inhibition of MHC class II surface expression in murine bone-marrow-derived macrophages. Hmama et al. (1998) observed attenuation in IFN-γ-induced expression of HLA-DR molecules, using a virulent *M. tuberculosis* strain while in our work we used the avirulent *M. tuberculosis* H37Ra strain.

Altogether, these results lead us to conclude that at a low MOI, *M. tuberculosis* H37Ra infection prevents induction of M1 pro-inflammatory phenotype at the level of the antigen presenting molecules but is not sufficient to inhibit the IFN-γ-induced macrophage activation. Taking in consideration the stronger bactericidal environment of M1 macrophages, also evidenced by our results (Figure 4), we speculate that with such low bacterial load, IFN-γ-induced activation overcomes the inhibitory effects employed by the avirulent *M. tuberculosis* H37Ra.

Dendritic cells play a central role in the initiation of a specific T-cell immune response to pathogens. Therefore, these most potent APCs are thought to be essential for the control of *M. tuberculosis* infections, by internalizing, processing and presenting mycobacterial antigens to naïve T cells in lymph nodes (Mortellaro et al., 2009; Steinman, 1991). The outcome of the interaction between pathogenic mycobacteria and DCs is still not completely understood and there are conflicting reports within the literature regarding the ability of these pathogens to interfere with DC maturation. In this study, we found that the infection of immature DCs with the avirulent *M. tuberculosis* H37Ra induces DC maturation, as evidenced by an up-regulation in surface expression of HLA-DR molecules and co-stimulatory molecules CD86 (Figure 8). Moreover, as expected (Martino et al., 2005), an enhanced expression of these maturation markers was also observed after infection with the non-pathogenic *M. smegmatis* (Figure 8). Despite this, *M. tuberculosis* H37Ra infection resulted in higher up-regulation of HLA-DR and CD86 molecules than that one with *M. smegmatis*, indicating that both species induce maturation of DCs, but the extent of induction is rather higher with *M. tuberculosis* H37Ra. This may be attributed to the fact that *M. tuberculosis* H37Ra is an avirulent mycobacterium strain highly related with the virulent *M. tuberculosis* H37Rv (Zheng et al., 2008), while *M. smegmatis* is environmental mycobacterium that can infect immune cells but does not persist in professional phagocytes (Jordao et al., 2008; Martino et al., 2005). Our results are in agreement with earlier in vitro studies showing that human DCs undergo maturation upon infection with different mycobacterial species, such as virulent *M. tuberculosis* (Giacomini et al., 2001) and the attenuated strain *M. bovis* BCG (Hashimoto et al., 2002; Kim et al., 1999). Contrasting with these studies and with our results, Hanekon et al. (2003) reported that exposure of DCs to *M. tuberculosis* H37Rv resulted in a minimal phenotypic maturation
of imDCs. In our opinion, the reason for the apparent discrepancy observed may lie in the positive control for maturation utilized. While we used a single inflammatory cytokine, TNF-α, whose role in induction of DC maturation in vitro is well documented (Roake et al., 1995; Sallusto and Lanzavecchia, 1994), Hanekon et al. (2003) stimulated imDCs with a potent maturation cocktail composed by three cytokines, which could have resulted in a higher expression of maturation markers than that from infected cells.

Taken together, this lead us to speculate that the *M. tuberculosis* H37Ra induced maturation of imDCs, observed in our experiments, perhaps reflects a strategy employed by the mycobacteria, to take advantageous of the migratory properties of mDCs, and thus disseminate within the host. On the other hand, from the perspective of the host, induction of DC maturation upon *M. tuberculosis* H37Ra infection could contribute to enhanced presentation of mycobacterial antigens and thus, this infected DCs could constitute potent stimulators of T cell response against this mycobacterium.

In addition to the classical MHC class II and I presentation pathway, DCs can also present lipid antigens to T cells, in the context of CD1 molecules (Crevel et al., 2002). Considering the lipid content of mycobacteria cell wall, we decided to analyze the effect of infection on the expression of CD1a molecules in imDCs. We observed that both *M. tuberculosis* H37Ra and *M. smegmatis* infections induced a similar up-regulation of these antigen presenting molecules. These results suggest that the increase in CD1a expression appears to be independent of the differences in virulence between both species. Previous studies (Stenger et al., 1998) revealed that the virulent *M. tuberculosis* H37Rv strain is able to down-regulate expression of CD1a molecules on the surface of monocytes co-cultured with GM-CSF and IL-4. It can be hypothesize that the down-regulation of CD1a reflects a further mechanism induced by pathogenic mycobacteria to circumvent immune recognition, that the avirulent *M. tuberculosis* strain H37Ra, might not be able to induce, even being highly related to *M. tuberculosis* H37Rv (Zheng et al., 2008).

We have also analyzed the effect of *M. tuberculosis* H37Ra and *M. smegmatis* infection on DC maturation phenotype induced by TNF-α treatment prior to infection. Our results showed that neither *M. smegmatis* nor *M. tuberculosis* H37Ra appears to interfere with TNF-α-induced DC maturation (Figure 9). This probably results from the fact that DCs stimulated with TNF-α, acquire a mature phenotype and thus, loss the ability to internalize bacteria (Sallusto and Lanzavecchia, 1994). Indeed, we observed that the percentage of TNF-α stimulated DCs infected with mycobacteria was extremely reduced in comparison imDCs (Figure 7), which confirms these previous results. Assuming that mDCs almost did not internalize either *M. smegmatis* or *M. tuberculosis* H37Ra species, it’s reasonable that no alterations in the phenotypic maturation of mDCs were observed.

Concluding this part of the thesis, it was demonstrated that the outcome of infection of macrophages and DCs differs with the non-pathogenic *M. smegmatis* and with the avirulent *M. tuberculosis* H37Ra. Moreover, it was interesting to observe that the infection with the avirulent *M. tuberculosis* strain induced distinct phenotypes according to the host cell type. This is probably connected with the different roles of these host cells in immunity against pathogens. In human macrophages that act as key effector cells in mycobacterial killing and antigen presentation at the site of infection, *M. tuberculosis* H37Ra prevents the induction of M1 pro-inflammatory phenotype at the
level of the antigen presenting molecules. This means that this mycobacterium probably manipulates these cells towards an anti-inflammatory phenotype. In future studies, the specific activation markers of M2 phenotype should be analyzed in infected cells, in order to support this theory. In human DCs, host cells responsible for mycobacterial antigen presentation to naïve T cells in the lymph nodes, the infection with *M. tuberculosis* H37Ra induces DC maturation. In this case we speculate that *M. tuberculosis* H37Ra-induced DC maturation might offer the possibility to the bacilli to use these cells as a vehicle to reach the lymph nodes and maybe other organs. On the other hand, the induction of this phenotype might also represent a defense mechanism of the host by which is initiated a specific T-cell immune response. Since maturation does not necessarily implicates that the cell is functionally capable of presenting antigen to T cells, it would be interesting, in a near future, to examine the capacity of *M. tuberculosis* H37Ra infected DCs to induce antigen specific T cell proliferation as well as to analyze the profile of secreted cytokines by infected cells.

Cathepsins are essential to the antigen presenting function of macrophages and DCs, and thus, to the effective CD4+ T cell immune response against pathogens, such as *M. tuberculosis*. It was previously reported that *M. tuberculosis* modulates MHC class II antigen presentation (Hmama et al., 1998; Noss et al., 2000; Pancholi et al., 1993) and that one of the mechanisms underlying this attenuation, is the modulation of host cathepsins that participate in this pathway (Nepal et al., 2006). Therefore, in the second part of this thesis, we aimed to decipher the role of cathepsins during mycobacteria infection of human monocyte-derived macrophages and DCs. We focused in cathepsin S and B, two major lysosomal cysteine proteases expressed in professional APCs, and involved in the MHC class II pathway for antigen presentation (Hsieh et al., 2012).

Cathepsin S plays an important role in MHC class II antigen presentation, being the main cysteine protease responsible for the late steps of invariant chain (Ii chain) degradation, in macrophages and DCs (Chapman, 2006; Riese et al., 1996).

Our results, concerning human macrophages, demonstrate that *M. tuberculosis* H37Rv infection, contrary to *M. smegmatis* infection, lead to a reduction of basal cathepsin S protein levels in these cells (Figure 10A). Since cathepsin S mediates the final steps of the li chain cleavage, this suggest that the decrease of this protease levels, probably results in a defect in li chain processing in infected macrophages. Indeed, it was previously described that infection of THP-1 macrophages with *M. bovis* BCG was associated with a reduction of li chain degradation and subsequent export of immature MHC class II molecules to the cell surface (Sendide et al., 2005). Therefore, we hypothesize that the virulent *M. tuberculosis* H37Rv strain by decreasing cathepsin S protein levels, probably attenuates the surface expression of mature MHC class II molecules. Surprisingly, infection either with *M. tuberculosis* H37Rv or *M. smegmatis* resulted in a drastic decrease in IFN-γ-induced cathepsin S protein levels in human macrophages (Figure 10A). We speculate that cathepsin S levels in IFN-γ-activated macrophages appear to be modulated by an intrinsic property of mycobacterial species. This property does not seem to be related with mycobacterial virulence, since both *M. tuberculosis* H37Rv and *M. smegmatis* down-regulate IFN-γ-induced cathepsin S expression, but the former is a virulent species and the latter a non-pathogenic one. A previous study, describing the ability of the attenuated
strain *M. bovis* BCG to inhibit IFN-γ-induced cathepsin S expression in human macrophages (Sendide *et al.*, 2005), represents an evidence that supports this hypothesis.

Interestingly, in dendritic cells when we analyzed the expression of cathepsin S upon mycobacteria infection (Figure 10B), clear differences were observed in comparison with human macrophages. In both imDCs and mDCs, cathepsin S protein levels increased upon *M. smegmatis* and *M. tuberculosis* H37Rv infection. However, infection with *M. tuberculosis* H37Rv, in immature DCs resulted in a minimal increase of cathepsin S levels, while in mature DCs strongly increased this protease levels. Although we have no clear explanation for these results, it can be hypothesize that in imDCs, the virulent *M. tuberculosis* H37Rv strain prevents the up-regulation of cathepsin S levels and thus, might impair the Ii chain processing, as previously observed in human macrophages (Sendide *et al.*, 2005). Although *M. tuberculosis* H37Rv infection of imDCs results in an increase in expression of antigen-presenting molecules, as a result of *M. tuberculosis* H37Rv-induced maturation (Henderson *et al.*, 1997), it might be an increase in immature MHC class II molecules instead of peptide-loaded MHC class II molecules. On the other hand, TNF-α-induced maturation appears to overcome this inhibitory effect employed by *M. tuberculosis* H37Rv, and thus, a strong increase in this protease levels is observed.

Soualhine and co-authors (2007) proposes that active cathepsin S have a direct role in abolishing mycobacterial control of phagosome-lysosome fusion, although its underlying mechanism is still unclear. This study demonstrated, that infection of macrophages with a BCG strain engineered to secrete active cathepsin S, accelerates fusion of its phagosomes with lysosomes which leads to intracellular killing and in turn, to an increase in antigen presentation by infected macrophages (Soualhine *et al.*, 2007). Rather than over-expressing cathepsin S in host cells by using an engineered mycobacterium, we decided to knockdown this protease in THP-1 macrophages, by using shRNA lentiviral vectors, in order to understand its influence on *M. tuberculosis* intracellular survival. We found that the silencing of cathepsin S in macrophages significantly increases intracellular survival of the *M. tuberculosis* H37Ra strain while does not interfere with intracellular survival of the *M. tuberculosis* H37Rv strain (Figure 12). These findings may reflect differences in virulence between both strains. The virulent *M. tuberculosis* H37Rv strain, as demonstrated in our results (Figure 10A), has the capacity to manipulate cathepsin S in human macrophages, by diminishing its protein levels. Therefore, no alterations on intracellular survival were observed, probably because we were mimicking this subversion mechanism employed by *M. tuberculosis* H37Rv, when we silenced cathepsin S in macrophages. On the other hand, the significantly increase on survival of the avirulent *M. tuberculosis* H37Ra strain reveals that this protease probably plays an important role in the control of *M. tuberculosis* H37Ra intracellular growth in human macrophages. Therefore, we conclude that cathepsin S might be involved in the control of *M. tuberculosis* intracellular growth, but while the virulent *M. tuberculosis* H37Rv developed a mechanism to avoid the effect of this protease, the avirulent *M. tuberculosis* H37Ra might not have the same manipulation mechanism or might have but is less effective than that of *M. tuberculosis* H37Rv.

Prior to CD4+T cell activation, exogenous antigens must be processed by lysosomal proteases to generate antigenic peptides for presentation via MHC class II molecules (Zhang *et al.*, 2005).
Cathepsin B which is mainly expressed in APCs has been proposed to be one of the lysosomal proteases involved in this process (Matsunaga et al., 1993; Mizuochi et al., 1994).

In the present work, concerning human macrophages, we found that infection of these cells with *M. tuberculosis* H37Rv decreases basal cathepsin B protein levels, while infection with the non-pathogenic *M. smegmatis* has no effect on this protease (Figure 11A). This suggests that the reduction in basal cathepsin B levels probably represents a specific subversion mechanism employed by pathogenic mycobacteria, since infection with the non-pathogenic *M. smegmatis* shows no alterations in this protease levels. Considering the key role of cathepsin B in antigen processing (Mort and Buttle, 1997), it can be speculate that the decrease in cathepsin B levels upon mycobacteria infection may interfere with the generation of protective T cell epitopes. Very little information exists on the role of cathepsin B during mycobacteria infection. One study by Rivera-Marrero et al. (2004) showed that the maturation of human THP-1 monocytes upon exposure to a virulent *M. tuberculosis* strain resulted in an increased expression of cathepsin B. It can be speculated that the discrepancy observed between this report and our work, might be explained by a differential modulation of cathepsin B levels by *M. tuberculosis* in human monocytes and M0 macrophages, probably according to the role of this protease in each host cell during infection. When we analyzed cathepsin B expression in infected macrophages previously activated with IFN-γ, we observed a strong decrease in the protease levels in either *M. tuberculosis* H37Rv or *M. smegmatis* infected cells. This drastic reduction of IFN-γ-induced cathepsin B levels may be caused by an inherent property common to several mycobacterial species and not necessarily related to virulence.

Concerning human DCs, we also analyzed the effect of mycobacteria infection on cathepsin B protein levels. Upon *M. smegmatis* or *M. tuberculosis* H37Rv infection of either imDCs or mDCs, the expression of cathepsin B remained unchanged relative to uninfected control (Figure 11B). Therefore, these results suggest that mycobacteria infection probably does not interfere with the protein expression of this lysosomal protease in dendritic cells.

In our work, we proposed to determine whether the down-regulation of cathepsin B would be relevant to *M. tuberculosis* intracellular survival in macrophages. Thus, we silenced this cathepsin in THP-1 macrophages, by using a shRNA lentiviral library. Our results demonstrate that the knockdown of cathepsin B significantly increases intracellular survival of *M. tuberculosis* H37Ra but also *M. tuberculosis* H37Rv. These findings suggest that cathepsin B might be important to the control of both strains intracellular growth in macrophages. The fact that the virulent *M. tuberculosis* H37Rv decreases the levels of cathepsin B (Figure 11A) also supports this hypothesis. We possibly observed an increased survival of this virulent strain when comparing to the control because cathepsin B down-regulation observed upon *M. tuberculosis* H37Rv infection might not be enough to overcome the effects of this protease in the control of intracellular growth.

In conclusion, it was demonstrated that *M. tuberculosis* modulates cathepsin S and B differentially of the non-pathogenic *M. smegmatis*. Furthermore, during mycobacteria infection cathepsin S and B levels appear to be specific of the host cell type being infected. This might be related with different kinetics of acidification and proteolytic activity existent between these cells (Trombetta and Mellman, 2005). At the same time, evidences point for a role of both cathepsins in the
control of intracellular growth of *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv strains within macrophages. However, the virulent *M. tuberculosis* H37Rv strain appears to be capable to subvert the negative effects of cathepsin S on the intracellular survival, by decreasing their protein levels. On the other hand, the avirulent *M. tuberculosis* H37Ra strain appears to be more susceptible to the killing effects of both cathepsins. Future studies evaluating cathepsin S and B activity in macrophages and dendritic cells after mycobacteria infection, may contribute to a better understanding of the mycobacterial manipulation mechanisms of these cathepsins.
5. References


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