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Macrophage Infection by Mycobacteria

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Abstract

Mycobacterium tuberculosis is the etiological agent of tuberculosis. It is a pathogen that continues to draw international concerns particularly due to the emergence of multi-drug resistance and to the difficulties associated with the diagnosis and treatment of latent tuberculosis. A key process in the pathogenesis of this disease is the interaction between this pathogen and host macrophages. Invasion of the macrophages protects the pathogen from attack by the immune system, allows it to multiply while protected within the macrophages, and alters the immune response as it influences the profiles of the cytokine and chemokine responses. Key to the intracellular survival of the pathogen within the macrophages is the specific interaction between the pathogen and its virulence factors with the host. This review provides an a summary of pertinent literature on the topic of macrophage receptors utilized by the pathogen, its survival strategies within the macrophage, and the general profile of immune signalling upon exposure to the pathogen. The importance of specific macrophage receptors and certain components of the pathogen to the direction of the immune response are also discussed.

Keywords: Mycobacteria; Macrophage; Phagosome; Tuberculosis

Introduction

Certain members of the Mycobacteria, particularly the slow growers, are facultative intracellular aerobic pathogens. Phylogenetically members of this family are classified as Gram-positive bacteria. Members of the Mycobacteria share a characteristic cell wall that is hydrophobic, waxy and rich in mycolic acid. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by arabinogalactan (AG), a biopolymer consisting of galactose and arabinose [1-3]. Other molecules and biopolymers found within the cell envelop includes lipomannans (LM), lipoarabinomannans (LAM), glycolipids, glycoproteins [1-3].

Pathogenic mycobacteria uses these surface-exposed molecules attach, enter, and infect host cells. These surface molecules specifically bind certain surface receptors on the host cell; most relevant in the study of pathogenesis of tuberculosis (TB) are the alveolar macrophages [4]. The process of phagocytosis is the first critical step for the infection of macrophages by mycobacteria and it involves microbial ligands and macrophage surface receptors that are now well characterized. Following phagocytosis a complex network of intracellular signaling changes take place and result in the survival of the internalized pathogen and causes attenuation in the secretion of and response to cytokines by the macrophage; events that eventually dictate the outcome of the disease.

Mycobacterium tuberculosis is the etiological agent of TB and it is endowed with many virulence factors that confer resistance to survive and multiply within the host. This pathogen infects large numbers of people every year around the globe and, as the World Health Organization reports [5], this disease remains to be one of the main causes of death. The initial infection can be active or latent, where the symptoms of the disease may be absent for years or decades. The latent form of the disease may at any time become active; a case referred to as reactivation TB. It is possible that about one third of the human population is latently infected with *M. tuberculosis* [5]. Although the initial and primary target of the pathogen is the human alveolar macrophage the infection can spread over to other organs from the lungs. The outcome of the infection depends on the first interactions between *Mycobacterium tuberculosis* and human cells and on the surface features of each and the virulence factors that the pathogen elaborates during the infection. The intracellular fate of this pathogen in the host depends on which receptor is involved and the interaction with phagosomal membrane. At the cellular level, the response of the host macrophage is regulated by intracellular signalling networks activated by the interaction with the pathogen and its virulence factors. Macrophages are important cells of the innate immune response and have a key role in inactivating pathogens and stimulating the first steps of immune responses. After its phagocytosis within the macrophages, *Mycobacterium tuberculosis* uses certain mechanisms to survive within the host and highly interfere with the signalling pathways of the cells; thus modulating the host immune response. This interference occurs at many levels: inhibition of phagosome maturation, down regulation of signalling pathways that lead to activation of the macrophage and to the immune response, inhibition of antigen presentation and inhibition of reactive oxygen/nitrogen intermediates.

Importance of cellular immunity in tuberculosis

The type of cells first recruited to the site of the infection is typically the neutrophils. They are attracted to the site of the infection by the interleukins secreted by infected alveolar macrophages and, once arrived in the lungs, they differentiate to form other mature macrophages [6]. These cells are very important in phagocytosis and intracellular killing action of bacteria and they can also help induce the adaptive immune response through cytokines signalling. In most of the cases, innate immune system is not effective to destroy completely the pathogens so the adaptive immunity is activated. The adaptive immune cells have a central role in response against tuberculosis. Among all these cells, CD4 and CD8 T cells are important in producing INF-γ, one of the main cytokines involved in the immune response. Its importance has been shown in some studies [7-10] where INF-γ gene knockout or the lack of its receptor led to higher susceptibility to *Mycobacterium tuberculosis*.

CD4 T cells have a central role in the protective response and also in the latent disease. Some experiments [11] have demonstrated CD4 T cells role and functions. In a model of latent tuberculosis, the infection was reactivated after the depletion of this kind of cells and the amount of CD8 T cells and INF-γ production by them was increased to

compensate the lack of CD4 T cells. However, this stronger response by CD8 T cells was not enough to fight the infection, suggesting the importance of CD4 T cells. Other functions of these immune cells have been found: they can induce the apoptosis of infected macrophages [12], they produce a wide number of cytokines such as IL-2 and TNF-α [13], they stimulate the activation of macrophages through direct contact and their production of relevant cytokines as IL-15, IL-10 and IL-10 [14,15]. NK and CD8 T cells contribute as well. CD8 T cells help by producing cytokines and lysing the infected cells [16,17]; NK cells recognize and destroy infected host cells, produce INF-γ to enhance the immune response and induce the expansion of T cells by stimulating macrophages to produce cytokines such as IL-12, IL-15 and IL-18 [18,19].

Autophagy

Autophagy is one of the main innate immune defense mechanisms used by infected macrophages to destroy *Mycobacterium tuberculosis*. With autophagy, the cells can degrade their own intracellular environment to control the infection [20]. When the macrophages are stimulated and the pathogens are engulfed, their activation leads to the production of IRGM1, a GTPase involved in stimulation of INF-γ that is the key cytokine in inducing autophagy [21]. Singh and co-workers [22] have shown in their work that IRGM1 probably translocate in the mitochondrial to regulate the autophagy in association with mitochondrial fission. Mycobacteria have evolved a strategy to survive also to autophagy. It has been found by Shin and colleagues a gene called eis ("enhances intracellular bacterial survival") that is present in *Mycobacterium tuberculosis* genome and can inhibit autophagy increasing the probabilities of mycobacterial survival.

Pathogenesis of tuberculosis

Lurie [23] has found that the pulmonary tuberculosis can be divided into four main stages. First, the bacilli have to be inhaled and, consequently, engulfed by the alveolar macrophages; during this stage, most bacteria are destroyed by microbicidal skills of the host cells. Mycobacteria that survive to this first intracellular destruction can start multiplying and, thereby, cause the macrophages disruption leading, in most of the cases, to an active disease. Because of that and because of the pro-inflammatory chemokines and cytokines released by the macrophages, other macrophages and monocyte-derived dendritic cells are attracted to the lungs to limit the infection. These immune cells can ingest the bacteria but not neutralize them; for this reason, at this second stage, mycobacterial grown and multiplication can be evident in the patient with little tissue damage being produced. Two or three weeks after the infection, T cells-mediated immunity develops with antigen-specific T lymphocytes and starts activating macrophages to kill the intracellular pathogens. At this stage, the bacilli growth stops and the disease can become stationary. The outcome of the infection depends on the successful interaction between T cells and infected macrophages and on the action of many factors such as INF-γ and TNF-α that can regulate mycobacterial growth, granuloma formation and initiation of adaptive immune response. The innate and adaptive immune systems are very important to determinate the outcome of the exposure to mycobacteria. A small percentage of individuals can remain uninfected because of a very effective immune system; however, in the majority of them, the innate immunity cannot protect them from the infection and the adaptive immunity is activated. The bacteria have evolved the skill to live in balance with human adaptive immune system cells: in this case, the result of infection is a latent disease and it can be maintained for the lifetime of the patient. If the balance is perturbed, the bacteria start reactivating and replicating and a reactivation of the disease occurs, leading to active and contagious tuberculosis. The reactivation of latent disease can depend on many factors like features of the pathogens and the host, but it always occurs if the immunity is suppressed or compromised.

Granuloma

After the initial infection of macrophages, the immune response is activated: the macrophages begin to produce inflammatory cytokines and chemokines that induce the immunity cells migration to the site of infection. Once macrophages and dendritic cells engulfed the bacteria, they migrate to the lymph nodes to present the mycobacterial antigen to CD4 and CD8 T cells and activate them.

These lymphocytes migrate back to the lungs following the chemokines as signal [24-26]. If the innate and adaptive immunity are not able to rapidly kill the pathogens, their migration to the site of infection can lead to the formation of granuloma. Granuloma is the characteristic histopathological feature of human pulmonary tuberculosis in which the bacteria can persist for many years. Granulomas are mainly composed of macrophages, neutrophils, dendritic cells and lymphocytes that are located around the bacteria to limit the spread of infection. When the granuloma is mature, fibroblasts are also present to surround the whole structure [27]. In the middle of granuloma, there are mature mycobacteria that develop a necrotic and caseous center, probably formed by the lipids released by the macrophages that, within the granulomas, are lipid-rich cells [27]. Besides limiting the spread of infection, this structure physically contains the bacteria and hinders their growth by stressing them with starvation, exposition to reactive oxygen and nitrogen intermediates and hypoxia. From some recent studies [27,28], it has been shown that mycobacteria can survive to those stresses by using host cholesterol as nutrition and using their proteasomes to fight oxidative stress (Figure 1). In most of the cases, granuloma is able to contain the infection but if some bacteria survive, it leads to a latent disease.

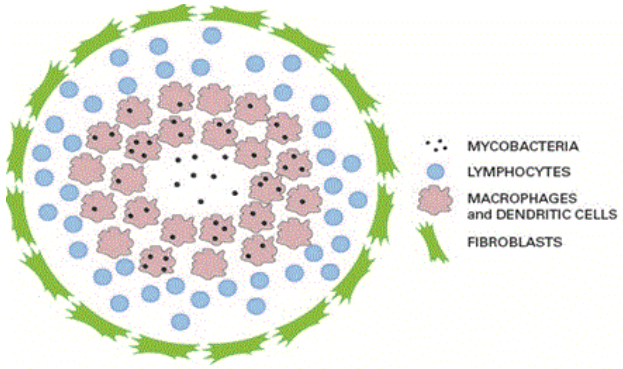

Figure 1: A schematic section through a granuloma highlighting the arrangement of different host cell types. The interior becomes liquefied mostly over time but live bacilli persist within.

Mechanisms used by *mycobacteria* to evade immune system and survive in the host cells

Phagosome maturation: The formation of a new phagosome is the result of the uptake of mycobacteria by phagocytes, a process that is meant to neutralize the pathogen; this vesicle is then manipulated by the pathogen to ensure its intracellular survival. During the normal process, the nascent phagosome follows a progressive maturation process through a sequential fusion with early endosomes, late endosomes and then lysosomes. Mycobacteria-containing phagosomes are not secluded from the whole cell vesicular network as it fuses with early endosomes and thus allow the pathogen to access nutrients. Beyond this step the phagosome maturation process is blocked. The molecular bases of this blockage have been somewhat characterized.

Manipulation of intraphagosomal pH: Mycobacteria can modulate and inhibit the acidification of the phagosomal vesicle by expressing a urease and a glutamine synthetase that increase the intraphagosomal pH producing ammonia. This alkaline pH hinders the transport of material from early endosomes to late endosomes arresting the phagosome maturation [29]. In human macrophages Jung and Robinson [30] have shown that infected macrophages express the P28 and the Epstein-Barr virus induced gene s (EBI3) proteins and the cell secretes the active IL-27. The IL-27 then engages its receptor on the same cell and down regulates the expression of the v-ATPase. This also contributes to reduced acidification of the phagosome. IL-27 signalling also negatively regulates Cathepsin D and CD63 (lysosomal integral membrane protein-1; LIMP-1). Both of the latter events are important for the maturation of the phagosome as well.

Manipulation of phagosomal membrane: For its maturation, the phagosome needs the assembly of appropriate fusion machinery on the phagosomal membrane for the interaction with early and late endosomes and then with the lysosomes. The vesicle fusion is, in part, regulated by Rab proteins which belong to the GTPase family. In particular, mycobacteria-containing phagosomes block the step where Rab7 is involved. Rab7 is a small GTPase that is involved in the late endocytic compartments and it controls the maturation of early endosomes to late endosomes. Mycobacteria blocks the Rab7 acquisition or interfere with the factors involved in its recruitment to the phagosomal membrane; thereby preventing phagosome maturation [31,32].

Another Rab protein important for the maturation process is Rab14 (Figure 2). This protein transiently associates with the phagosomal membrane and is normally absent in the late stages of the process. It has been shown that mycobacteria-containing phagosomes block the release of Rab14 from the phagosomal membrane [33]. Using overexpression of dominant-negative mutants and knockdown of endogenous Rab14 using si-RNA for Rab14 reverses the maturation block [33]. Phosphatidylinsoitol-3-phosphate (PIP3) is another phagosomal membrane marker important for the phagosomal maturation process (Figure 2).

Phagosomes undergoing normal maturation have PIP3 in the phagosomal membrane. Phagosomes containing *M. tuberculosis* have been shown to release PIP3 [34]. This pathogen secretes an acid phosphatase called SapM [35]. This enzyme has been shown to hydrolyse PIP3 during infection of macrophages with *M. tuberculosis* [34]. This activity results in the exclusion of PIP3 from the phagosomal membrane and further contributes to blocking the maturation process. A glycolipid of *M. tuberculosis* known as ManLAM (mannose-capped Lipoarabinomannan) is another surface component that is involved in blocking phagosome maturation. This glycolipid is essentially a glycosylated phosphatidylinositol and has been shown to interfere with the phagosomal acquisition of lysosomal content and syntaxin 6. Syntaxin 6 is required to recruit the early endosome auto antigen 1 (EEA1) [36], events dependent on the activity of the phosphatidylinositol 3-kinase and is required for proper maturation of the phagosome [37].

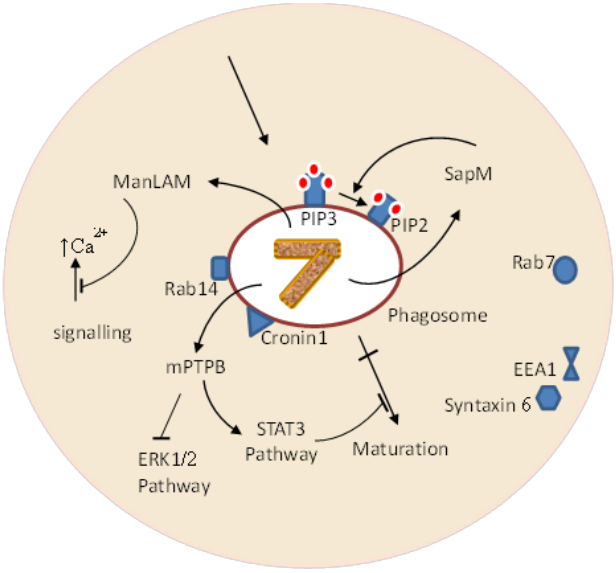

Figure 2: Proposed role of the various virulence factors of *Mycobacterium tuberculosis* in blocking the maturation of the pathogen-containing phagosome. PIP3 levels are affected by the phosphatase activity of SapM. PIP3 and Ca^{2+} levels are affected by ManLAM. These effects have consequences on recruitment of vesicle fusion effectors such as Rab 7, Syntaxin 6, and EEA1. The mPTPB has a role in down regulating ERK 1/2 signalling and up regulating STAT 3 signalling; effectively contributing to the maturation block.

Manipulation of host cytoskeletal system: Mycobacteria can also affect the interaction of the phagosome with the host cell actin cytoskeleton. When the pathogen is engulfed by the macrophage, the coronin1 accumulates around the bacteria-containing phagosome. Coronin1 is a cell cortex protein with tryptophan and aspartate residues and with actin binding sites important to promote the interaction between the phagosome and the cell cortical actin network. Some studies [38-40] have shown that the accumulation of this coat protein could be critical to prevent the function of the factors that promote the phagosome maturation.

Modulation of antigen presentation

After the infection, the pathogens move from the lungs to the lymph nodes within the dendritic cells that are the antigen-presenting cells that can present the antigen to T cells and activate the adaptive immune response. The pathway for processing and presentation of the antigens is a key process for the activation and the function of immune system. There are three different pathway of antigen presentation:

1) Mycobacterial peptide antigens can be processed and presented on MHC class II molecules by antigen-presenting cells (APCs) to CD4

T cells. Once these lymphocytes are activated, they can start killing the intracellular bacteria and the infected host cells by producing INF-γ and TNF-α.

2) Mycobacterial peptide antigens can be processed and presented on MHC class I molecules by APCs to CD8 T cells that can kill intracellular bacteria and infected cells by producing toxic granules.

3) Mycobacterial lipid or glycolipid antigens such as ManLAM and mycolic acids can be recognized and neutralized by natural killer cells (NK) or CD8 T cells. Mycobacteria have evolved some strategies to modulate and interfere with antigen presentation. While the processing of MHC class I molecules does not appear to be affected, mycobacterial surface ManLAM can attenuate the expression of MHC class II molecules induced by INF-γ on the APCs. This mechanism is not clearly understood yet, but it could involve an intracellular sequestration of non-mature MHC class II heterodimers, an inhibition of INF-γ gene expression, and/or a down regulation of class II molecules trans activator expression [41,42]. The MHC class II main trans activator is CIITA. In the normal pathway, MHC class II expression is induced by INF-γ that is produced after infection. INF-γ binding to its receptor leads to Janus kinase-signal transducer and activator of transcription (JAK-STAT) activation, resulting in STAT1α phosphorylation and dimerization. In this way, STAT1α can translocate into the nucleus and bind to the promoter GAS leading to the induction of CIITA that can activate MHC class II genes expression (Figure 3). Mycobacteria can interfere with this pathway through Toll-like receptors (TLR), leading to decreased expression of MHC class II molecules. TLR signalling occurs through MYD88 and downstream activation of nuclear factor kB (NF-kB) and mitogen-activated protein kinase (MAPK). In the nucleus, this pathway can induce the expression of many genes such as C/EBP that, by binding to CIITA promoter, can inhibit its expression and, thereby, MHC class II molecules expression and antigen presentation.

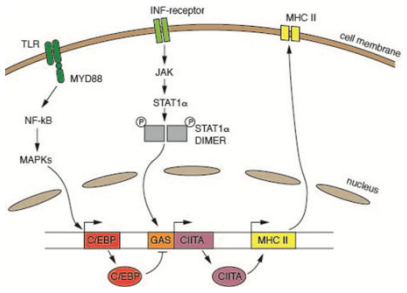

Figure 3: Major intracellular signalling pathways activated by pathogenic *mycobacteria* or their components.

Another mycobacterial molecule can further down regulate the production of MHC class II molecules: the 19-kDa lipoprotein. From some studies [43], it has been found that this component can hinder the antigen processing and presentation and its inhibitory effect depends on Toll-like receptor 2 (TLR2) that is very important in initiating the immune response after mycobacteria infection. The TLR2-dependent interference with the antigen presentation occurs only after the treatment with the 19-kDa lipoprotein and it is a strategy for the bacteria to evade the immune response. Ramachandra and colleagues [44] have showed evidence that mycobacteria can inhibit the antigen processing and presentation. Phagosomes containing *M. tuberculosis* are competent antigen-presenting organelles in which the MHC class II antigens are processed with mycobacterial antigen. The phagosomes with live pathogens show a smaller amount of this complex, compared to the phagosomes with dead bacilli. This suggests that live mycobacteria can attenuate antigen processing and presentation, besides phagosome maturation.

Reactive nitrogen intermediates and nitric oxides

After the infection with mycobacteria and the early activation of innate immunity, T cells are stimulated to produce INF-γ. This molecule can further stimulate the macrophage and up regulate the activity of nitric oxide synthase 2 (NOS2) and, thereby, the production of reactive nitrogen intermediates (RNIs) by macrophage. NOS2 catalyses the nitric oxide production (NO) from arginine; NO can react with some molecules to form other RNIs that can damage bacterial DNA, proteins and lipids [18,45]. RNIs are essential for the containment of the infection and for killing mycobacteria because these molecules are toxic for this kind of pathogen [18,46]. Moreover, nitric oxide also reacts with glutathione to form another molecule that is toxic for the bacteria: S-nitro glutathione [47]. These strategies that occur into the macrophages have been demonstrated in some studies: Rosen Zweig et al [48] have found that mutation of INF-γ receptor leads the individuals to have a strong susceptibility to *mycobacteria* infection; their experiment suggests that INF-γ and its pathway in the macrophage is very important to mediate the anti-mycobacterial action.

From other studies [49], we know that in the infected alveolar macrophages a high level of NOS2 is expressed and an inhibition of this enzyme can lead to have an increase of pathogens growth and repression of NOS2 anti-mycobacterial activity. Moreover, it has been shown, from other experiments [50-53], that if we inhibit NOS2 pathway and RNIs production, the susceptibility to *M. tuberculosis* is strongly enhanced and the persistent infection can be reactivated. Like for the other mechanisms of defense that occur in the host cells, *M. tuberculosis* has evolved some strategies to survive even in presence of RNIs. By screening a DNA library derived from a kind of *M. tuberculosis* highly resistant to RNIs, Ehrt and colleagues [54] have discovered noxR1 as a gene that confers resistance to reactive oxygen intermediates (ROI) and to RNIs in *E. coli* and *M. smegmatis*, and noxR3 in *Salmonella typhimurium* [54,55].

Macrophage receptors and signalling pathways involved in infection by *mycobacteria*

Signalling in the macrophage is at the cell surface by the interaction between its receptors and pathogen. Once phagocytized, it continues to create new surface and secreted molecules within the host cell to interact with the macrophage-signalling pathway. *M. tuberculosis* interacts with many cellular receptors including the complement receptor types 1, 3 and 4 (CR1, CR3 and CR4) [56], the mannose receptor [57,58], the surfactant protein A receptor (SPA-R) [58,59], Toll-like receptor (TLR) [60], scavenger receptor [61], CD14 [62] and dendritic cell-specific intercellular adhesion molecule-3- grabbing non integrin (DC-SIGN) [63,64]. The interaction of bacteria with macrophages can be direct or mediated by the host components. For the direct communication, are implicated the mannose receptor or the complement receptor type 3 and the surface polysaccharides and glycoproteins. There is a direct interaction also between lipoproteins and lipopolysaccharides (LPS) and the Toll-like receptor.

The mediated interaction involves some host molecules as antibody's Fc receptor, surfactant protein A receptor (SPA-R), complement receptor types 1, 3 and 4, and CD14. All these macrophage receptors are engaged in specific signalling pathways that lead to phagocytic uptake of the pathogen, phagosome maturation, and/or cytokine release. The *M. tuberculosis* infection does not involve only one receptor and its pathway, but multiple receptors play different roles during the infection.

Especially, it has been shown that the binding between mannose receptor and ManLAM can interfere, block the phagosome maturation and mediate phagocytosis of mycobacteria. This has been demonstrated by Kang and co-workers [65] who have found that the phagosome-lysosome fusion, during the uptake of ManLAM microspheres, was highly reduced in normal human macrophages but not in the cells lacking the mannose receptors [65]. The binding of ManLAM inhibits the increase of the cytosolic calcium level in the macrophages and, in this way, also the interaction between calmodulin and phosphatidylinositol 3 kinase(PI3K). Thereby, the recruitment of Rab proteins and antigen 1 is arrested. Antigen 1 is necessary for the delivery of lysosomal components from Golgi network to the phagosome and for their fusion. This process of phagosome-lysosome fusion inhibition is specific for ManLAM and mannose receptor and it does not occur if Man LAM binds to another receptor. When mycobacteria are internalized in the phagosomes, their surface molecules, including lipids, are released into the endocytic network and contribute to macrophage infection. Phagosome maturation depends on membrane-associated proteins and on the properties of the lipid bilayer. The mycobacterial lipids are hydrophobic molecules that confer solubility on the biological membranes; they can intercalate into the host cell membranes altering their physical properties and lipid composition or interact with membrane-associated receptors if they are lipids bearing peptides or oligosaccharides. The insertion of mycobacterial lipids into the host cell membranes can lead to the block of phagosome maturation through some different processes as alteration of the lipid bilayer, formation of phospholipid domains, constrictions of membrane movements and alteration in membrane fluidity and permeability.

Toll-like receptors and its pathway

One of the most important key receptors in macrophages is the Toll-like receptor (TLR). They are called pattern-recognition receptors (PRRs) because they recognize and interact with pathogen-associated molecular patterns (PAMPs) that are molecules associated with the pathogens that are recognized by cells of the innate immune system through Toll-like receptors and the other pattern recognition receptors. TLRs are trans membrane proteins with an extracellular domain with motifs rich in leucine to recognize microbial products. The result of TLR activation is the production of pro-inflammatory cytokines to have a protective response. The production of this type of cytokines by TLR pathway is mediated by NK-Kb, while the production of the last factors is mediated by extracellular signal-regulated kinase (ERK1/2) and p38 through the activities of GTPase, atypical protein kinase C (PKCζ) and phosphatidylinositol 3-kinase (PI3K) [66]. The bacteria or their components phagocytosis by macrophages also activates sphingosine kinase (SPK) and increases the intracellular calcium levels leading to the activation of PKCα/β and Ca^{2+}/calmodulin-dependent kinase (CamK), respectively. Both phagocytosis signalling and TLR signalling converge downstream on ERK1/2 [67]. ERK1 and ERK2 belong to the mitogen activated protein kinase (MAPK) family. Some other MAP kinases are JNK and p38 that, with ERK1/2, are the most important MAPK involved in *mycobacteria*-infected macrophages. When the levels of ERK1/2 and the production of TNF-α increase, the result is the activation of the macrophages. Conversely, when p38 activity is elevated, the production of mycobacterium-induced IL-10 increases. The balance between these signals is an important factor that influences the macrophage capability to kill the pathogen. The results of macrophage infection by non-pathogenic *mycobacteria* are high levels of TNF-α and low levels of IL-10, and this leads to the activation of the macrophage and suppression of the bacterium.

Infection of macrophages with pathogenic *mycobacteria*, conversely, leads to opposite result: high levels of IL-10 and low levels of TNF-α leading to the inhibition of macrophage activation and survival of the pathogen.

Stat pathway

Although seven members of the STAT family have been identified thus far in mammalian systems it appears that STAT1, STAT4, and STAT3 are the most involved in mycobacterial infections. STAT 1 and STAT 4 are homologous and produce similar effects upon activation; that is pro-inflammatory and anti-proliferative effects. STAT3 activation has the opposite effects and is associated with anti-inflammatory, anti-apoptotic, and pro-proliferative effects [68]. Activation of STAT1 and STAT4 pathways is the result of the stimulation of macrophages by INF-γ and IL-12, respectively. INF-γ is a cytokine produced by T cells and natural killer cells. When the macrophages are stimulated and activated by pathogens, their gene expression is altered and these cells produce cytokines and chemokines that stimulate T cells and natural killer cells. In this way, INF-γ is produced and further activates macrophages and enhances their bactericidal activities [69]. This pathway is not responsive or suppressed during macrophage infection with *M. tuberculosis* [70]. STAT3 pathway on the other hand appears to be more active or up regulated during such infections particularly as a result of increased secretion of IL-27 [70]. In both cases however, suppressor of cytokine signal negative regulator-1 and -3 (SOC-1 and SOC-3) are up regulated and the STATs pathway is down regulated as a consequence [71]. It can therefore be concluded that the final response of the macrophage to the infection is determined by how these two pathways with opposing effects are balanced by the SOCs (Figure 4).

C-type lectin receptor

The C-type lectin receptors (CLRs) are calcium-dependent glycan-binding proteins with a carbohydrate-binding C-type lectin domain [72]; these receptors are very important in mycobacterial binding and in inducing inflammatory responses because they detect the presence of carbohydrate-rich surface molecules of mycobacteria. This family of receptors includes a wide number of proteins with one or more C-type lectin-like domains, as selectins, collectins, proteoglycans and endocytic and phagocytic receptors like mannose receptors and complement receptors [72].

CLRs have a cytoplasmatic domain that mediates some downstream events as endocytosis and signal transduction, activated after its binding to the ligand. Some of the biological mechanisms activated by the interaction with mycobacteria are the pro-inflammatory response and the anti-inflammatory response.

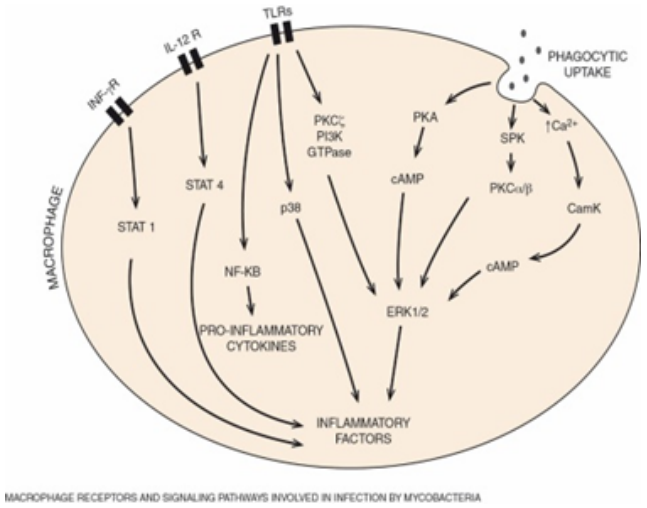

Figure 4: Major intracellular pathways activated in macrophages following exposure to pathogenic *mycobacteria* or their components and leading to proinflammatory cytokine secretion.

Pro-inflammatory response: The recognition of mycobacterial molecules by CLRs leads to the induction of expression of pro-inflammatory cytokines as IL-6, IL-2 and TNF and their production by the macrophages. These cytokines are essential for the immune response against mycobacterial infection [73,74].

Anti-inflammatory response: The binding between CLRs and mycobacteria also induces the production of anti-inflammatory and immunosuppressive factors as IL-10 and TGF-β [75,76].

The respiratory system and its mucosal sites continually touch the outer environment therefore it needs a quick and efficient immune system. The respiratory ways are exposed not only to pathogens but also to many foreign particles and allergens, which can cause an unnecessary and dangerous reaction like chronic inflammation responses and damages in local tissues. For this reason, an additional mechanism to control and to shut down the pro-inflammatory response is necessary. CLRs have this role and help to protect the tissue integrity: they stimulate the production of anti-inflammatory cytokines as IL-10 and TGF-β. Once IL-10 is produced, it activates a signalling pathway which is involved in the control of immune system, especially in mucosal sites where the exposure to external environment and foreign pathogens is continuous [77-80]. If these factors are excessively produced, the result is a change in macrophages killing ability, a decrease of production and action of pro-inflammatory cytokines and a prevention of immune system cells recruitment to the site of infection. In the case of mycobacteria infection, the anti-inflammatory factor IL-10 production can be further stimulated by the pathogen that has evolved this mechanism to survive into the host cells. Mycobacteria can bind to DC-SIGN to manipulate the immune response and increase the IL-10 concentration. Interacting with DC-SIGN, mycobacteria can induce the IL-10 production because ManLAM is able to activate the kinase Raf1 pathway: this leads to the transcription factor NF-kB phosphorylation and, in this manner, it can enter the nucleus and enhance the IL-10 transcription [63,75]. In some studies, it has been shown the inhibiting activity of mycobacteria-induced IL-10 on the maturation of dendritic cells and on their recruitment to the site of the infection [81]. It also hinders the activity of CD4 and CD8 T cells by down regulating the costimulatory factors on macrophages and inhibits the T cells proliferation [82].

Complement receptor types 1, 3, & 4

There are two distinct structural forms of the phagocyte complement receptors (CR). Complement receptor type 1 (CR1) is a monomeric trans membrane protein able to bind to complement-opsonized particle C3b and C4b but not C3bi. Complement receptors type 3 and 4 (CR3, CR4) are heterodimeric proteins from the integrin family [83,84]. They contain identical β subunits and distinct α subunits and they bind to C3bi. CR3 also contains a glucan binding site that has high specificity for polysaccharides. CR3 is a principal phagocytic receptor expressed on neutrophils, natural killer cells, monocytes and macrophages and, because it is part of the class of integrin adhesion receptors, interacts with the actin cytoskeleton and intracellular signalling pathways [85,86]. CR1, CR3 and CR4 are involved in phagocytosis and are very important to promote and enhance mycobacteria ingestion by the host cells. Among the complement receptors, CR3 is the major one involved in mediating mycobacteria ingestion: it mediates about 80% of complement-opsonized *M. tuberculosis* phagocytosis. In some works, it has been studied its lack or block and the result is a reduced or inhibited phagocytosis of the pathogens [57,85]. Like in other bacteria, in *M. tuberculosis*, an alternative pathway of complement activation can be activated, leading to the opsonisation with C3b and C3bi. Bacteria that are coated with these molecules can bind to CR1, CR3 and CR4 and be phagocytized in phagosomes [56,57,86]. Pathogenic mycobacteria have also developed one more mechanism to acquire opsonic C3 peptides: they can obtain the complement fragment C2a to form a C3 convertase on their surface generating opsonically active C3b and, thereby circuiting the complement cascade [87]. *M. tuberculosis* binds to the CR3 lectin site probably through the envelope polysaccharides, including D-glucan. This binding with CR3 mediated by polysaccharides provides a mechanism for complement-independent binding and a mode of binding that promoted CR3-directed phagocytosis.

Mannose receptor and lipoarabinomannans

The macrophage mannose receptor (MR), also called CD207, is another receptor belonging to the pattern recognition receptors (PRRs) family and it is a monomeric trans membrane glycoprotein, consist of a large extracellular domain with eight carbohydrate-recognition domains dependent and a little cytoplasmic domain containing a tyrosine-based motif involved in phagocytosis, endocytosis and endosomal sorting [88]. MRs are expressed only on the adult macrophages but not on fresh monocytes and they interact with mannose residues of glycoconiugates, expressed on the surface of virulent strains of mycobacteria, as lipoarabinomannan (LAM) and mannose-capped lipoarabinomannan (ManLAM), even if LAM can bind also to the complement receptor type 3. The binding between these molecules and mannose receptor is the first interaction occurring during bacteria phagocytosis by macrophages; however, this association can depend on the amount of bacterial surface ManLAM. After the initial step of phagocytosis and macrophages activation by mycobacterial antigens, the MR expression is strongly down regulated and the binding of *M. tuberculosis* is reduced; hence, the role of MR after the early stages of infection is likely to be minor. LAM is an amphipathic lipoglycan consisting of a glycerolphosphatidyl anchor, a

D-mannan core, a D-arabinan domain and various carbohydrate capping motifs. Lipoarabinomannan is synthesized through addition of mannose residues to phosphoinositol by a series of mannosyltransferases to produce phosphatidylmyo-inositol mannosides (PIMs) and lipomannan (LM). PIM and LM are then glycosylated with arabinan to form LAM. LAM, besides to be an important cell wall component, is one of the most important virulence factors of mycobacteria because is involved in the bacterium uptake by macrophages and in the modulation of macrophage response activating anti-inflammatory effects. These mechanisms include the inhibition of the activity of macrophage PKC and MAPK leading to the arrest of INF-γ transcription and the block of respiratory burst leading to neutralization of cytotoxic oxygen free radicals produced by macrophages [43,89]. One type of LAM is mannose-capped lipoarabinomannan (ManLAM): they are characterized by the presence of mannosyl caps on the terminal D-arabinan. This modification is present only in some species of mycobacteria like *Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium leprae* and *Mycobacterium avium*: the other species can carry some variations of it or no ManLAM. ManLAM has been shown to have immunosuppressive effects by stimulating IL-10 production: it inhibits the production of IL-12 interfering with TLRs, and TNF production. It also modulates *M. tuberculosis*-induced macrophage apoptosis through the binding with host mannose receptors; this is particularly important in deactivating host macrophages to allow the bacteria to survive and multiply within them.

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a C-type lectin receptors expressed mainly on the surface of dendritic cells but also on the macrophages [90]. It is a carbohydrates recognition receptor that can bind to the bacterial surface mannose type carbohydrates such as Man-LAM and lipoarabinomannans: through this binding, it can induce bacteria phagocytosis and IL-10 production to promote an anti-inflammatory response, as already mentioned [63,75].

Collectin family

Collectins (collagen-containing C-type lectins) belong to the innate immune system and they are pattern recognition receptors (PRRs) forming a family of collagenous Ca2+-dependent defense lectins [64]. Their function is to bind to lipids or oligosaccharide structures located on the surface of microorganisms. Binding of collectins to microorganisms enhances their elimination through mechanisms as **complement** activation, opsonisation and **phagocytosis**. Surfactant protein A (SP-A) and surfactant protein D (SP-D) are proteins belonging to the collectin family and they are produced by the respiratory epithelium. As surfactant proteins, they are very important to maintain lung physiology and integrity because they are also pattern recognition receptors (PRRs) involved in the innate immunity system [91,92]. Their calcium-dependent binding to the surface glycoconjugates of the pathogens enhances the uptake of the pathogen into the macrophages [93,94]. SP-A and SP-D are important to modulate the early stages of interaction between the host and mycobacteria upon the lung [95-99]. Both of them can bind to mycobacterial lipoarabinomannans but only SP-A can interact additionally with exposed glycoproteins in the cell wall of the pathogens [100-102]. It also can stimulate the expression of the other receptors involved in immunity response like complement receptor 3 and scavenger receptor [103,104]. Moreover, they can modulate the inflammatory response by regulating the cytokine production, the oxygen and nitrogen reactive species formation and functioning as chemo attractants for alveolar **neutrophils** and **monocytes** [105].

Scavenger receptors

Scavenger receptors belong to a wide family of cell surface trans membrane glycoproteins and they recognize macromolecules having a negative charge including lipopolysaccharides (LPS) of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria [106]. It is not yet known if scavenger receptors can activate the cytoskeleton to internalize bacteria but we know they play an important role in innate immunity and macrophage regulation. CD163 (cluster of differentiation 163) is a cell-surface glycoprotein receptor cysteine-rich belonging to superfamily class B; it is a receptor for haemoglobin-haptoglobin complex but it is also located on monocytes and macrophages to recognize bacteria. It can bind to both Gram-positive and Gram-negative bacteria and act as innate immune sensor. Its interaction with pathogens promotes the pro-inflammatory cytokines and other inflammatory mediators' production as TNF-α, IL-1β, IL-6 and IL-10, proving the CD163 role in immune response [107-112].

CD14

CD14 is a cell surface receptor without a cytoplasmatic and trans membrane domain, anchored to the cell through a lipid structure and modified by a glycan motif. It especially binds to Gram-negative bacteria LPS but it can also bind to Gram-positive lipoteichoic acids and peptidoglycan. CD14 can also interact with mycobacterial LAM and, thereby, stimulates the secretion of IL-8 by macrophages [113,114]. It has been found that CD14 is not so important in mediating phagocytosis but, after mycobacterial infection, its expression on the surface is up regulate, demonstrating that this pathogen can modulate the host immune response [115,116].

Cytokines and chemokine production driven by *Mycobacterium tuberculosis*

Pro-inflammatory cytokines: The recognition and the phagocytosis of mycobacteria lead the macrophages and dendritic cells to produce pro-inflammatory cytokines and chemokines that have an important role in inflammatory response and in the outcome of the infection.

TNF-α: This cytokine has a key role in regulating the pathology of tuberculosis and the immune response. It can act through the binding with its receptor, TNF-αR, and induce further macrophages activation, apoptosis of infected macrophages and production of reactive nitrogen intermediates and the formation of granuloma.

Mycobacteria can hinder the action of TNF-α and increase the probability of survival. The importance of this strategy has been demonstrated [12,117]: mice treated with antibodies against TNF-α or its receptor had increased susceptibility to the infection and showed malformed granulomas.

IL-6: this cytokine is produced early at the site of infection and it seems to have both pro- and anti-inflammatory features but its functions are not fully understood yet. In some experiments [118,119] it has been demonstrated IL-6 anti-inflammatory role. Indeed, it inhibited the production of TNF-α and other pro-inflammatory cytokines and, *in vitro*, it enhanced mycobacteria growth. Another

work [120] supports its pro-inflammatory function since the lack of IL-6 increased the susceptibility to the disease.

IL-12: it is produced mainly by phagocytic cells after the phagocytosis of the pathogens. IL-12 has an important role in inducing INF-γ production. IL-12 knockout mice and patients with genetic mutation in the genes encoding IL-12 have a reduced capability to produce INF-γ and an enhanced susceptibility to be infected [121-124].

INF-γ: this cytokine is particularly important in response to tuberculosis and is synthetized by CD4 and CD8 T cells and natural killer cells after the infection in response to mycobacterial infection. INF-γ has an essential role in activating macrophages and stimulating the antigen processing and presentation. Its lack can be harmful: it enhances extremely the susceptibility to tuberculosis because it wreaks defective macrophage activation and a decreased production of NOS2 and thereby of reactive nitrogen intermediates.

IL-18/IL-15: these molecules are involved in induction of INF-γ production as IL-12. They also have a role in stimulating the production of other cytokines, chemokines and transcription factors essential for immune response. Moreover, IL-15 is important in stimulating T cells and natural killer cells proliferation and activation [125].

Chemokines

Chemokines are chemotactic cytokines responsible for recruitment of immune system cells to the site of the infection. A wide number of chemokines has been found but some of them have a major importance such as IL-8 and MCP-1. IL-8 is synthetized by macrophages and by pulmonary epithelial cells after pathogens phagocytosis or stimulation with mycobacterial antigens and it attracts immune cells like T lymphocytes, neutrophils and monocytes. Like for the other chemokines, IL-8 production depends on the presence of TNF-α and its pathway because, if this cytokine is missing, IL-8 production is blocked [126]. Another important chemokine is monocyte chemo attractant protein 1 (MCP-1) that is produced by monocytes and macrophages to let it act on themselves. A lack or deficiency of this protein or its receptor leads to have problems in the granuloma formation and a low and not effective immune response [127,128].

Anti-Iflammatory cytokines

After mycobacteria infection, the host starts producing also anti-inflammatory cytokines to hinder the pro-inflammatory cytokines effects due to the pathogens.

IL-10: this cytokine is formed by infected macrophages after bacteria phagocytosis or after stimulation with LAM. Its effect is down regulation of production of INF-γ, TNF-α and IL-12 [129].

TGF-β: it is produced by dendritic cells and monocytes to inhibit cell-mediated immunity. In macrophages, it hinders the antigen processing and presentation, the pro-inflammatory cytokines production and the cell activation. In T cells, it opposes to cells proliferation and INF-γ production [130].

IL-4: like TGF-β, it can suppress INF-γ production and macrophages activation.

High levels of IL-4 lead to a further progression of the infection and with a reactivation of a latent disease [131,132].

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