Introduction:

Tuberculosis, a threat:

The disease tuberculosis (TB) is constantly threatening the mankind since the prehistoric times. The evidence came from the description by Hippocrates as early as 400 B.C. and *Mycobacterium tuberculosis* DNA in Egyptian mummies from 2000 B.C. [Zink et al, 2003; Ducati et al, 2006]. Previously the disease was known as “consumption,” “wasting away,” “king’s evil,” “lupus vulgaris,” “the white plague” or “phthisis” based on its clinical manifestations [Ducati et al, 2006; Donoghue 2009].

*M. tuberculosis* generally infect a new person by its release from the lung of a infected person through the airways. During the 18th and 19th centuries in Europe, the Industrial Revolution led to congested living conditions in urbanized areas resulting in optimal conditions for spread of TB. This resulted in epidemic levels, with 20-30 % of all deaths being caused by the disease [Donoghue, 2009; Dye & Williams, 2010]. However, during the second half of the 19th century, mortality rates from TB radically decreased in Europe due to improvement in housing, diet, education, and sanitation, and with the launch of sanatoria where patients were exposed to fresh air and a healthy diet. The decrease in prevalence rate of TB in Europe occurred much before the discovery of antibiotics. This highlights the importance of living conditions and socio economic factors in controlling TB and also some of the difficulties that are still faced in low-income countries now [Snowden, 2008].

In 1943, the discovery of streptomycin made the disease treatable and the prevalence rate continued to drop in developed countries throughout the 20th century. However, TB continued to increase among the poor and susceptible populations in developing courtiers, as well as marginalized populations in developed countries [Snowden, 2008]. Last decade of 20th century is marked by the emergence of new tropical diseases, HIV epidemic, antibiotic resistance which made infectious disease into a recognized global terror. The development of multi drug-resistant TB (MDR-TB), the increased susceptibility of HIV-positive cases with poor immune systems to TB, increased mobilization of people from one part of the globe to another, and the relative
incompetence of the Bacillus Calmette-Guérin (BCG) vaccine have made the situation very critical, resulting in the disease as a global emergency [Navin et al, 2002; Dheda et al, 2010]. Although BCG vaccine has been used since the early 20th century, it has proven rather unsuccessful, especially in preventing adult pulmonary TB [Dheda et al, 2010]. More effective vaccines are extremely needed to control the deadly disease.

Urgency in public health and financial efforts together with better admittance to health care, enhanced control of spread, improved and more available diagnostics, increased treatment and cure rates are needed. To tackle the emergence of antibiotic resistance, new improved vaccine and new drugs should be developed. Also new scientific knowledge about the basic molecular and immunological mechanisms underlying TB is of the essence to overcome the disease [Snowden, 2008; Dheda et al. 2010].

**Global Scenario:**

According to World Health Organization (WHO) report, TB is a worldwide pandemic. Among the 22 countries with the highest estimated TB incidence rates, half are from six Asian countries, viz., Bangladesh, China, India, Indonesia, Pakistan and Philippines. A WHO fact sheet dated March 2010 [Geneva, WHO, 2010] on TB stated that overall one third of the world's population (over 2 billion) is currently infected with the TB bacillus.

In the global TB report 2013, 8.6 million new cases and 1.3 million cases of death was registered [Geneva,WHO, 2013]. The global community woke up to this disease when, in 1993, WHO declared TB as a global emergency. It was estimated that by 2004, the world as a whole would have achieved the Millennium Development Goal (MDG) of halting and reversing the incidence to half of its 1990's prevalence and mortality rate. Now the revised time limit to achieve that MDG is by 2015 [Geneva, WHO, 2006]. Directly observed treatment-short course (DOTS) is an internationally recognized strategy for delivering the basics of TB case-finding and cure. Worldwide, between 1995 and 2008, a cumulative total of 36 million TB patients were successfully treated in DOTS programs, and up to 6 million deaths were averted. The treatment
success rate (~86%) achieved in DOTS cohorts worldwide exceeded the global target of 85% for the first time in 2007 [Geneva, WHO, 2006].

The Southeast Asian region (SEAR) is critically important from the global perspective. It is home to 25% of the world human population; and with 30% of the world's poor living in this region, it suffers from high burdens of communicable and noncommunicable diseases [WHO, 2009; Mc Carthy, 2001]. SEAR accounted with an estimated 4.8 million prevalent cases and about 3.4 million incident cases and 450 000 deaths in 2012 resulting in 39.5% morbidity and 48% mortality of the global burden of TB (Figure 1A and B). Moreover, India accounts 26% of the world's incident cases. According to the WHO report on Tuberculosis Control in the South-East Asia Region 2014, young adults (age group 25–34 years), particularly male are mostly affected among the new cases. In spite of the decrease in the toll of the disease after the introduction of various measures like DOTS, the disease kills 450 000 lives each year in this SEAR.

**Estimated incidence of all forms of TB, classified by WHO region, 2012**

![Graph showing estimated incidence of all forms of TB by WHO region, 2012](image)

*Figure 1A. Estimated global TB incidence = 8 600 000 (8 300 000–9 000 000) cases (all forms of TB)*

Estimated mortality of all forms of tuberculosis, classified by WHO region, 2012

![Pie chart showing distribution of estimated global TB mortality by region: South East Asia (47.6%), Africa (24.3%), Western Pacific (11.6%), Eastern Mediterranean (10.6%), Europe (3.8%), and The Americas (2%).]

*Figure 1B. Estimated global TB mortality = 940 000 (790 000–1 100 000) cases (all forms of TB)*


**Indian Scenario:**

In India, TB has been mentioned in the Vedas and the old Ayurvedic scriptures. Historically speaking, fight against TB in India can be broadly classified into three periods: early period, before the discoveries of x-ray and chemotherapy; post-independence period, during which nationwide TB control programs were initiated and implemented; and the current period, during which the ongoing WHO-assisted TB control program is in place.

Even today in India, two deaths occur every three minutes from TB. Major challenges to control TB in India include poor primary health-care infrastructure in rural areas of many states; unregulated private health care leading to widespread irrational use of first-line and second-line anti-TB drugs; spreading HIV infection; poverty; lack of political will. A collaborative effort is in progress between NTCP and National Rural Health Mission (NRHM), which is a reform initiative of which the goal is to improve primary health care in rural areas. In addition to this,
NTCP has established several initiatives in coordination with the private sector and the Indian Medical Association (IMA) to improve TB care.

Since concurrent infection with HIV weakens the immune system, people with co-infection of HIV and TB are much more likely to develop TB; it is a leading cause of death among HIV-positive people. The belief that many individuals are able to clear the infection through an effective innate immune response indicates an important role for this part of the immune system in achieving sterilization of infection, and may provide a hint as to how we can boost the immune response to clear the pathogen.

![Estimated TB incidence rate, 2012](image)

**Figure 2. TB incidence around the globe. Source: Global Tuberculosis report 2013 by WHO.**

**The causative organism, *Mycobacterium tuberculosis***

TB is caused by a group of closely related bacterial species termed *M. tuberculosis* complex (MTC). Today the principal cause of human TB is *M. tuberculosis*. In 1881, the bacillus causing...
TB, *M. tuberculosis*, was discovered by Robert Koch by culturing crushed granulomas. *M. tuberculosis* is aerobic, intracellular, Gram positive, rod-shaped, generally non-sporulating [Ghosh *et al.*, 2009] with unique cell wall composition [Ducati *et al.* 2006]. The size measures around 1-4 μm in length and 0.5 μm in diameter. The complex, waxy cell wall confers the bacillus its acid-fast property. *M. tuberculosis* is generally very slow growing with a doubling time of about 24h [Ducati *et al.* 2006].

Other members of MTC include *M. bovis*, *M. microti* and *M. africanum*. *M. microti* is not known to cause TB in humans; infection with *M. africanum* is very rare, while *M. bovis* has a wider host range and is the main cause of TB in other animal species. Human TB is most often caused by an *M. tuberculosis* strain, but *M. africanum* and *M. bovis* infection can also lead to the development of TB [Ducati *et al.* 2006]. Humans become infected by *M. bovis*, usually via milk, milk products or meat from an infected animal [Prasad *et al.*, 2005; Srivastava *et al.* 2008]. Organisms are identified by their red color on acid-fast staining.

![A, M tuberculosis growth in Lowensten Jensen medium. B, Acid fast staining of M tuberculosis from pure culture. C, Acid fast bacilli in the sputum.](image)

**Figure 3.** A, *M tuberculosis* growth in Lowensten Jensen medium. B, Acid fast staining of *M tuberculosis* from pure culture. C, Acid fast bacilli in the sputum.

**Cell wall**

The cell wall of *M. tuberculosis* largely complex and composed with long-chain fatty acids termed mycolic acids linked to arabinogalactan, which is attached to the peptidoglycan. The cell wall also contains several lipoglycans including lipoarabinomannan (LAM), its precursors
lipomannan (LM), and phosphatidyl-myoinositol mannosides (PIM). These components are non-covalently attached to the plasma membrane through their GPI anchors, and they extend to the exterior of the cell wall [Ducati et al. 2006; Briken et al. 2004]. LAM acts as a virulence factor of *M. tuberculosis*, contributing to the inhibition of macrophage functions important for killing the pathogen by inhibiting phagosomal maturation and interfering with cell signaling and shifting the cytokine response from pro- to anti-inflammatory [Briken et al. 2004; Pathak et al. 2005; Nigou et al. 2001; Vregne et al. 2005]. LAM consists of a phosphatidyl-myoinositol anchor, a D-mannan polymer attached to the inositol ring, D-arabinose chains, and capping motifs at the end of the arabinose residues [Vregne et al., 2003]. The schematic representation of the mycobacterial cell wall is shown in figure no 5.

Virulent, slow-growing mycobacteria contain mannose-capped LAM (ManLAM) in their cell wall, while non-virulent species of mycobacteria such as *M. smegmatis* harbour non-capped AraLAM or phospho-myoinositol-capped LAM (PILAM), and the type of capping is important for virulence [Dao et al., 2004]. The cell wall of *M. tuberculosis* also contains a 19-kDa lipoprotein of unknown function which has been implicated in virulence through a role in host cell death and manipulation of bactericidal mechanisms [Ciaramella et al., 2000]. The 19-kDa lipoprotein of *M. tuberculosis*, as well as LM, and AraLAM from rapidly growing mycobacteria, provoke an inflammatory response in the host by binding to Toll-like receptors (TLR) on the host cell surface [Means et al., 1999, Ciaramella et al., 2000].
Figure 4. Schematic representation of the complex M.tuberculosis cell wall. [Park & Bendelac, 2000].

Mycobacterial strains

*M. tuberculosis* belongs to the genus *Mycobacterium*, which include approximately 100 species. The most familiar species are *M. tuberculosis* and *M. leprae* (leprosy) [Kassim & Ray, 2004]. *M. tuberculosis* are genetically diverse and significant phenotypic differences are present among the clinical isolates. *M. tuberculosis* exhibits a biogeographic population constitution and different strain lineages are related with different geographic regions. Microevolutionary variations have effect on the relative fitness and transmission dynamics of drug-resistant strains [Gagneux, 2009].

The Beijing strain is virulent, developing drug resistance and causing extra-pulmonary TB more often than other strains [Nocol & Wilkinson, 2008]. *M tuberculosis* H37 was isolated from a 19-year old pulmonary TB patient in 1905. H37 is a laboratory strain but later developed into a virulent (H37Rv) and an avirulent strain (H37Ra), depending on virulence in guinea pigs [Steenken et al, 1934]. Both the strains can be cultured in the laboratory. The H37Rv and H37Ra differ both genetically and phenotypically and only the H37Rv strain is able to replication inside human macrophages [Xhang et al, 1998]. The differences between the strains lies in the mutation in *phoP* gene, which is essential for adaptation in the intracellular environment [Lee et al, 2008; Ferre et al, 2009; Li et al, 2010]. The BCG, H37 Ra/Rv-strains and different clinical isolates are the common strains used to study the pathogenesis in *in vivo* and *in vitro* experiments. *M. marinum*, since it is less prone to cause disease in humans than *M. tuberculosis*, can be used to model tuberculous lesions by infection of mouse tails [Carlsson et al, 2010] and to infect zebrafish embryos and the amoeba *Dictyostelium* [Pozos & Ramakrishnan, 2004; Tobin & Ramakrishnan, 2008; Hagedorn et al, 2009]. However, the extrapolation of data obtained from the non-pathogenic mycobacteria should be done cautiously, as many mechanisms are specific for *M. tuberculosis*. The data presented in this thesis is based on work performed with live, heat killed BCG, H37 Ra/Rv-strains on human monocyte/macrophages system.
Genome

The genome of the H37Rv strain was published in 1998 [Sager Institute, 2007]. The genome size is 4 million base pairs, with 3959 genes; 40% of these genes have had their function characterized, with possible function postulated for another 44%.

The genome contains 250 genes involved in fatty acid metabolism, with 39 of these involved in the metabolism generating the waxy coat. Such large numbers of conserved genes show the evolutionary importance of the waxy coat to pathogen survival. Within the genome are also 6 pseudogenes.

About 10% of the coding capacity is taken up by two clustered gene families that encode acidic, glycine-rich proteins. These proteins have a conserved N-terminal motif, deletion of which impairs growth in macrophages and granulomas [Glickman & Jacobs, 2001]. Nine coding sRNAs have been characterised in M. tuberculosis, [Arnvig & Young, 2009] with a further 56 predicted in a bioinformatics screen [Livny et al, 2006].

Natural outcome of Mycobacterium tuberculosis infection

After the infection TB develops only among the 10% of the infected people. However, no particular symptom is seen in the latent phase of infection. The symptoms differ in the early and chronic phases of the disease.

After inhalation, mycobacteria are ingested by resident alveolar macrophages and dendritic cells (DC) through phagocytosis, by which the pathogens are killed. However, the mycobacteria escape the killing process and replicate inside the cell. After the initial interaction with the pathogen, these cells produce pro-inflammatory cytokines which leads to recruitment of more DC, monocytes and neutrophils from the blood stream and the infected cell become activated and migrate to the local lymph node leading to the activation of specific T cells. The cytokines (IL-12 and IL-18) released by the phagocytic cell induce NK cells which in turn produce IFN-γ. The released IFN-γ activates the macrophages to release various pro-inflammatory cytokines like
TNF-α and other microbicidals [Korbel et al, 2008, North & Jung, 2004]. By the above signaling pathway mediated by cytokine and chemokines, immune cells are recruited and the granuloma is formed which is the pathological hallmark of TB.

**Figure 5. Primary TB infection and its fate.** After inhalation of *M. tuberculosis* droplet nuclei, several scenarios may follow. Mycobacteria may be destroyed by alveolar macrophages, in which case no real infection will take place. Alternatively, *M. tuberculosis* may not be immediately killed, and so a primary complex consisting of a small infiltrate and a draining lymph node will develop. Small calcifications may be seen on radiographic examination and the tuberculin skin test (TST), as a marker of an *M. tuberculosis* specific T-cell response, becomes positive. Most often, infection is stabilized at this point. In a minority of cases active disease now develops (primary TB), either in the lungs or anywhere else after hematogenous dissemination of *M. tuberculosis*. Months or years afterwards, usually under conditions of failing immune surveillance, latent infection may reactivate (post-primary TB).

Macrophages differentiate into epitheloid cells or foamy macrophages, or fuse to form giant cells within the granuloma and become surrounded by lymphocytes and an outer cuff of fibroblasts and extracellular matrix proteins. The bacilli are contained within the granuloma till it fails owing to immunosuppression [Russell, 2007; Dechastellier, 2009]. Granuloma formation is
considered as favourable for the host as it coincided with the onset of adaptive immunity and reduction of bacterial growth in the lung. However, recent studies in zebrafish embryos infected with *M. marinum*, have indicated that mycobacteria too use the granuloma for their advantage upon initial infection by recruiting new macrophages to allow spread between host cells [Davis & Ramakrishnan, 2009]. In the active stage of TB, granuloma is caseous and contain necrotic Macrophages. However, in the advanced stage of TB, it forms cavities in lung. When this structure ruptures, the infecting bacilli come into the airways resulting in infection to a new person. [Russell, 2007; Dechastellier, 2009].

**Inflammatory responses by macrophage cells after activation with *M.tuberculosis***

![Diagram]

**Figure 6. Major inflammatory responses exerted by macrophages after *M. tuberculosis* interaction.**

Mycobacteria can infect any part of the body. The infection in the lung is considered as pulmonary TB (PTB) and the rest part of the body is extrapulmonary TB. The main concern of the thesis is PTB and also the extrapulmonary TB with the infection in chest (Pleural TB).
TB of the lungs, resulting in symptoms such as chronic bloody coughs, night sweats and weight loss, is the most common clinical manifestation of *M. tuberculosis* infection. However, any organ in the body can be affected by spread of bacteria through the lymphatics, causing disseminated or extra-pulmonary TB [Ducati *et al.*, 2006; Donoghue, 2009]. Extra-pulmonary TB can manifest itself as pericarditis, meningitis, or spinal TB, for example [Girling *et al.*, 1988]. In latent TB infection, on the other hand, the bacilli are thought to be contained in the granuloma or in other tissue, remaining in a dormant non- or slowly replicating state for decades, waiting for an opportunity to start replicating when the host is immunocompromised and unable to prevent growth [Ehlers, 2009].

**TB Pleural Effusion (TBPE):**

TBPE is the leading inflammatory pleural disease [Gopi *et al.*, 2006; Sinzobahanvya & Bhakta, 1989; Shah, 1992; Batungwanayo *et al.*, 1993; Elliott *et al.*, 1993; al Quorain *et al.*, 1994; Richter *et al.*, 1994; Valdis *et al.*, 1996]. TBPE is one of the most common extra pulmonary involvement in TB among the developing countries [Yoon *et al.*, 2004; Sharma & Mohan, 2004]. TB may affect the pleura at different stages of pulmonary or systemic disease by various mechanisms. TBPE may represent a manifestation of either primary infection or reactivation of latent disease, the latter being more common [Moudgil *et al.*, 1994]. Pleural effusion is believed to occur secondary to the rupture of a subpleural caseous focus in the lung in to the pleural space. In case of TB, the bacterial load in the patient is a useful parameter for determination of the protective immunity against the disease [Crofton, 1999]. Therefore, scanty bacterial load in the pleural TB patients reflects that the protective immunity is good in these patients compared to the patients with pulmonary TB, where bacterial load is very high. Others [Allen & Apicella, 1968] reported that the clinical syndrome, pleural effusion, is a reflection of an in situ delayed type hypersensitivity reaction. Additionally, high INF-γ in pleural fluid has also been documented [Jalapathy *et al.*, 2004].
Relapsed/recurrent TB (RTB):

According to the Revised national tuberculosis control programme (RNTCP based on DOTS), relapsed TB means a TB patient who was declared cured or treatment completed by a physician and who reports back to the health facility and is now found to be sputum smear positive is a relapse case” [RNTCP, 2011].

The term ‘Relapsed TB’ means ‘Recurrent TB’ which incorporates both the re-infection as well as the reactivation cases [American Thoracic Society, 2000]. The distinction between the endogenous reactivation and exogenous re-infection has important implications in the planning of clinical trials and national TB control programmes.

Moreover, relapse cases are one of the three subsets of retreatment cases of TB [WHO, 2010]. After completion of anti-tuberculous treatment not all the patients develop the disease again except few which might get relapsed. So we hypothesized lowered immune response might be the reason for the relapse.

Although no previous report has demonstrated immune responses of relapsed TB cases and compared it with new TB cases, a very recent study and our own study has shown lowered protective immune response among the retreatment than new TB cases [Wang et al, 2012].

![Figure 7. Chest X-ray finding of (A) Pulmonary TB, (B) Miliary TB, (C) Pleural TB](image)
**Miliary TB (MiTB):**

MiTB is a common manifestation of lymphohematogenous dissemination of tubercle bacilli throughout the lung and other viscera [Sharma *et al.*, 2005; Sahn & Neff, 1997; Sharma & Mohan, 2001; Baker & Glassroth, 2004; Divinagracia & Harris, 1999; Sharma & Mohan, 2006]. Miliary pattern of the chest radiograph is the hallmark of MiTB. Mortality from MiTB has remained high despite effective therapy being available. MiTB accounts for less than 2% of all cases of TB in various clinical studies in immunocompetent individuals. For a long time, miliary TB has been considered to be a childhood disease. However, during the last three decades, it is increasingly being recognized in adults as well. Several reasons are thought to be responsible for this changing epidemiological trend. These include: human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), ever increasing list of causes of immunosuppression, such as use of biologicals and immunosuppressive drugs for treatment of various medical disorders, increasing occurrence of organ transplantation, chronic haemodialysis programme, among others. Males seems to be more frequently affected by MiTB in childrens as well as adults [Sharma *et al.*, 2005; Sahn & Neff, 1997; Sharma & Mohan, 2001; Baker & Glassroth, 2004; Divinagracia & Harris, 1999; Sharma & Mohan, 2006].

**Diagnosis and chemotherapy:**

Diagnostic methods for the pulmonary TB cases include sputum smear positive by Ziehl-Neelsen (ZN) staining, chest X-rays consistent with TB [Leitch, 2002]. For miliary TB cases, presence of constitutional symptoms suggestive of TB, miliary mottling in the chest X-ray reports and favorable response to anti-TB regime by follow-up study for six months are the criteria. In case of pleural effusion patients, chest X-ray suggestive of effusion, positive tuberculin skin test (TST), adenosine deaminase (ADA) positivity (>40IU/L) with preponderance of lymphocyte count (lymphocyte/neutrophil > 0.75) in exudative pleural fluid [Perez-Rodriguez *et al.*, 2003] and favorable response to anti-TB regime in follow-up study for three months are very important diagnostic criteria. However, TB-PCR, immunological memory-based tests including the less specific tuberculin skin test and more specific IFN-γ release assays, phage amplification assays, solid culture and automated liquid culture, as well as several tests for antibiotic resistance are critical in diagnosing the disease. Rapid, reliable and economic diagnostic procedures are
extremely needed in the setting with poor resources for the confirmation and diagnosis of drug resistance pattern [Pai et al, 2010].

There is no gold standard for the diagnosis of latent TB infection (LTBI; asymptomatic state with no clinical or radiological evidence of active disease but with viable M. tuberculosis organisms within tissues). However, the discovery of new immunodiagnostic tools such as the IFN-γ release assays (IGRA) has improved our understanding about human TB [Dheda et al, 2009; Mack et al, 2009]. Although well known, it is not widely appreciated that a substantial proportion (perhaps up to 50%) of close contacts of microbiologically confirmed index cases, even in many (although not all) high burden settings, have no immunodiagnostic (positive TST) evidence of LTBI [Morrison et al, 2008].

After the diagnosis of TB, the treatment usually involves a combination of four first line antibiotics (rifampicin, isoniazid, pyrazinamide, and ethambutol) for two months, followed by two drugs for four months [Caminero et al, 2010]. These drugs act on the actively replicating bacilli and are ineffective against non- or slowly-replicating ones present in latent TB infection. Thus, long term treatment is required to eradicate both replicating and dormant bacilli [Wolf et al, 2007]. When the organism is resistant to multiple drugs, the treatment includes more than four drugs, selected from the five lines of available substances, for up to 18 months. [Caminero et al, 2010].

In spite of the progress made so far after TB was put back on the global health agenda in the early 1990’s, there is a vital need for better TB control as well as new vaccines and therapies as TB is the leading cause of death from infectious disease in the world [Ducati et al, 2006]. This requires understanding of various important facts including the initial interaction of host and pathogens and role of innate immune system in the disease process of TB and this thesis focuses on this issue, especially on the role of pro and anti-inflammatory cytokines in the pathogenesis. This type of knowledge is pivotal in developing effective immune responses by the host and could find new targets against the organism.
Immune response against *M. tuberculosis*:

**Innate immune response**

The innate immune response is the first line of defense against invading mycobacteria and thus plays a pivotal role in clearance of the infection and is the main concern of the present thesis. Surprisingly, mycobacteria have evolved strategies for intracellular survival and replication within macrophages indicating the importance of the innate immune cells in the pathogenesis. Anatomical barriers such as the skin as well as the complement system and several types of innate immune cells are the major components of innate immune system. The receptors of the innate immune cells include TLR, complement receptor (CR) 3, mannose receptor, scavenger receptors, and DC-specific intercellular-adhesion-molecule-3-grabbing nonintegrin (DC-SIGN). *M. tuberculosis* interacts with these receptors and leads to the induction of an inflammatory response that which determine the fate by either clearance of the bacilli or granuloma formation [Dheda *et al*, 2010; Davis & Ramakrishnan, 2009]. The alveolar macrophages and incoming monocytes from the bloodstream provide the bacteria with its niche, but may also be able to disarm the pathogen if stimulated correctly. The DC which is essential for antigen presentation to T cells in the draining lymph node also provides a replication niche for the *M. tuberculosis* [Dheda *et al*, 2010; Korbel *et al*, 2008]. On the other hand, *M. tuberculosis* disturbs the immune system by preventing both DC migration and antigen presentation [Wolf *et al*, 2007]. So the initial interaction between *M. tuberculosis* and the innate immune system is a complex process.

NK cells have also been found important in the immune response against *M. tuberculosis*. Mycobacterial infection is accompanied by massive influx of neutrophils which are among the first cells to respond to inflammatory stimuli by migrating to the infection site [Korbel *et al*, 2008] although the conflicting data exist.

After the activation by neutrophils kill the pathogen by various antimicrobial molecules present in their granules, including defensins, lactoferrin, cathelicidin, and lysozyme, which is transferred into the *M. tuberculosis*-containing phagosome upon fusion with granules [Korbel *et al*, 2008; Faurschou & Borregaard, 2003]. Neutrophils can also exert efficient killing of microbes by the assembly of the NADPH oxidase in the phagosomal membrane, which leads to the generation of superoxide followed by reactive oxygen species (ROS) in the phagosome
[Faurschou & Borregaard, 2003] and have been shown to be able to kill \textit{M. tuberculosis} in a Ca$^{2+}$ dependent manner [Majeed \textit{et al}, 1998]. Moreover, neutrophils activate macrophages through release of granule proteins [Tan \textit{et al}, 2006] and heat shock protein 72 (Hsp72). Hsp72 is released from apoptotic neutrophils, which have recently been found to induce macrophage activation in addition to having an inflammation resolving role [Persson \textit{et al}, 2008]. However, conflicting evidence comes from some \textit{in vivo}-studies as to whether neutrophils have a protective or tissue-damaging role in \textit{M. tuberculosis} infection [Dheda \textit{et al}, 2010; Korbel \textit{et al}, 2008; Martineu \textit{et al}, 2007]. NK cells are granular lymphocytes of the innate immune system that have cytotoxic functions exerted through perforin and granzyme or granulysin [Korbel \textit{et al}, 2008]. NK cells provide the stimulation signal to macrophage through IFN-$\gamma$ during \textit{M. tuberculosis} infection [Ducati \textit{et al}, 2006; Junqueira-Kipnis \textit{et al}, 2003]. NK cells activation involves the complex interactions between receptors and IL-12, IL-18, IFN-$\alpha$. In in-vitro models, NK cells can directly lyse the \textit{M. tuberculosis} -infected macrophages and exert a pro-inflammatory response, restricting \textit{M. tuberculosis} growth in an apoptosis-dependent manner [Korbel \textit{et al}, 2008]. Furthermore, NK cells can kill regulatory T cells that are at risk of dampening the immune response to \textit{M. tuberculosis} [Roy \textit{et al}, 2008]. The defect in the functionality of NK cells in TB patients was found to be an effect rather than cause of disease [Raja \textit{et al}, 2004]. So, the role of NK cells in human TB is not completely clear [Korbel \textit{et al}, 2008].

**Adaptive immune response**

The innate and adaptive immunity are closely connected. Macrophages and dendritic cells, the primary cell types involved in the innate immune response to mycobacteria, play a crucial role in the initiation of adaptive immunity. In principle, three processes contribute to the initiation of adaptive immunity: antigen presentation, costimulation, and cytokine production. Antigen presentation plays an important role in activating the adaptive immune response against \textit{M. tuberculosis}.

Patients with active TB may suffer from anergy or T-cell unresponsiveness [Hirsch \textit{et al}, 1999]. This may be caused by intrinsic defects or dynamic inhibition of one of these three processes. Infected DC and macrophages present \textit{M. tuberculosis} -antigens to T lymphocytes through MHC class I, to CD8+ cytotoxic T lymphocytes, and through MHC class II, to CD4+ T helper cells,
leading to the activation and proliferation of the lymphocytes. Additionally, CD1-restricted T cells can be activated through presentation of glycolipid antigens by DC, and γδ T cells through presentation of phospholipid antigens, and these contribute to protective immunity against TB by producing IFN-γ or exerting cytotoxic activity [Dheda et al, 2010; Raja et al, 2004]. Memory T cells also form upon *M. tuberculosis* infection. The infected macrophages and DC secrete cytokines including IL-12, IL-23, IL-7, IL-15 and TNF-α, leading to attraction of more leukocytes to the infection site [Dheda et al, 2010]. For a long time, *M. tuberculosis* was believed to always be contained inside an impermeable phagosome in the macrophage, giving rise to the question of how *M. tuberculosis* antigens can be presented via MHC class I as well as MHC class II [Clemens et al, 2002; Vergne et al, 2004]. However, recent evidence indicates that *M. tuberculosis* as well as *M. marinum* can escape its vacuole and also reside in the host cell cytosol, giving an explanation to this question [Weerdenburg et al, 2010; VanderWel et al, 2007]. This knowledge has been used to design a BCG vaccine strain expressing perfringolysin, which enables it to escape from the phagosome and trigger an enhanced immune response through MHC class I [Sun et al, 2009]. However, an alternative explanation for the presentation of *M. tuberculosis* antigens via MHC class I is an interaction between themycobacterial phagosome and the endoplasmic reticulum (ER) leading to proteasome degradation and MHC class I presentation of antigens [Guermonprez et al, 2003].

Depending on the cytokine environment, the CD4+ T cells can mount a Th1 response (IL-12, IL-18, IFN-γ), or a Th2 response (IL-4, IL-5, IL-13). A Th1 response leads to the release of pro-inflammatory cytokines including IFN-γ, which is thought to enhance killing of intramacrophage mycobacteria through NO and ROS production [Dheda et al, 2010; Russell, 2007; Flesch & Kaufmann, 1987; Rohde et al, 2007; Schaible et al, 1998]. This has been well characterized in mice, although the mechanism has not been fully elucidated in humans, and is thought to be different [Chan et al, 2001; Thoma-Uzsynski et al, 2001]. A Th2 response, on the other hand, leads to release of IL-4, IL-5, IL-10 and IL-13, promoting B lymphocyte activation leading to an antibody response, and promoting an anti-inflammatory macrophage response. Th17 cells, stimulated by IL-23, IL-6, IL-21, and low TGF-β levels, are involved in recruitment of cells of the innate immune system and Th1 cells, and secrete IL-17 [Dheda et al, 2010]. Regulatory T cells (Treg), stimulated by IL-2 and high TGF-β levels, can also be stimulated. Treg produce
anti-inflammatory cytokines such as IL-10 and can suppress microbicidal mechanisms in the macrophage, and the activity of these cells is elevated in TB patients [Dheda et al, 2010; Ribeiro-Rodrigues et al, 2006].

Specific activation of CD8+ cytotoxic T cells can lead to killing of \textit{M. tuberculosis} through a perforin and granulysin-mediated pathway by which the infected macrophage undergoes cell death, or by induction of apoptosis through the extrinsic pathway via Fas ligand [Dheda et al, 2010; Woodworth & Behar, 2006; Weerdenburg et al, 2010]. It is known that TB patients display a defect in the killing capacity of their cytotoxic T cells [Brighenti & Andersson, 2010]. Thus, a Th1/Th17 response, but also activation of cytotoxic T cells, is thought to be important aspects of the adaptive immune response to \textit{M. tuberculosis} infection [Dheda et al, 2010].

The role of B lymphocytes and a humoral response in protection against TB is unclear [Raja et al, 2004]. However, evidence from experimentally infected animals suggests that an antibody response can have an immunomodulating effect on cellular immunity through cytokine signaling, as well as a protective role against infection by inhibiting bacterial replication, neutralizing bacterial products, triggering of the complement system, and promoting antibody-dependent cellular cytotoxicity.

**Macrophage:**

**Function and activation**

The word macrophage means “big eater” in Greek. Macrophages, the large mononuclear cells of the innate immune system function as professional phagocytes and are capable of engulfing particles larger than 0.5 \( \mu m \), including microbes. The macrophages in the resting state internalize debris and apoptotic cells in non-inflammatory manner [Rohde et al, 2007]. During infection, these cells ingest and kill pathogens, recruit other cells of the immune system, and present antigens from the microbe to cells of the adaptive immune system. The monocytes are the precursor of macrophages and circulate in the blood stream. They are recruited at the site of infection after the stimulation. Monocytes differentiate into a macrophage, with increased phagocytic capacity and different morphology and adhesive properties. Resident macrophages are terminally differentiated and have a fixed location in the body, at strategic points where
infection can occur, *e.g.* alveolar macrophages are stationed in the lungs, Kupffer cells in the liver and microglia in the nervous system. Macrophages can become activated upon inflammatory or microbial stimulation. During the activation by the microbial components, such as LPS, the cell exerts antimicrobial properties necessary for clearance of the pathogen. However, the pathogen like *M. tuberculosis* has evolved the capacity to escape the antimicrobial action of the Macrophages. [Benoit *et al*, 2008; Flannagan *et al*, 2009].

**Macrophage polarization**

According to the type of stimulation, macrophage polarizes into either M1 or M2 types which are functionally different. M1, the classically activated macrophages polarization is induced by pro-inflammatory or Th1 cytokines (IFN-γ, TNF-α, GM-CSF) and microbial products. M1 macrophages have microbicidal and inflammatory properties (secreting IL-1, IL-12, TNF-α, IL-23, IL-6 and overexpressing the IL-1 receptor, MHC class II and TLR2 and -4). They express inducible nitric oxide synthase (iNOS) and produce ROS. On the other hand, M2, alternatively activated Macrophages polarization is induced by anti-inflammatory or Th2 cytokines (IL-4, IL-13, M-CSF, IL-10). M2 macrophages are not microbicidal and have regulatory properties and express arginase [Benoit *et al*, 2008; Mantovani *et al*, 2005].

Moreover, M2 macrophages have three subsets, all with different immunoregulatory properties, as well as roles in angiogenesis, tissue remodelling and repair. M2a macrophages are induced by IL-4 or IL-13, M2b by immune complexes and TLR agonists. M2c are induced by IL-10 and glucocorticoid hormones. M2a also express decoy IL-1 receptor and MHC class II. M2a and M2c macrophages secrete anti-inflammatory cytokines such as IL-10 or TGF-β, as well as different chemokines, and IL-1 receptor antagonist. M2b play important role in the immunoregulation and produce IL-10, IL-1, TNF-α, and IL-6 [Benoit *et al*, 2008; Mantovani *et al*, 2005; Gordon, 2003; Mantovani *et al*, 2004]. Additionally, chemokine receptors expressed differentially on the different classes of macrophages.
Macrophages polarization is reversible. Therefore, the same macrophages can contribute in both inductions as well as in resolution of inflammation [Porcheray et al, 2005]. Thus, dynamicity and the complexity of the macrophages highly influence the pathogenesis of TB.

**Pathogen recognition receptors**

Pathogen recognition receptors (PRR) are present in the innate immune cells and are located either on the cell surface or in the cytoplasm. The main function of PRR are to recognize a diverse range of bacterial products called pathogen-associated molecular patterns (PAMPs), or stress signals termed danger-associated molecular patterns [Korbel et al, 2008]. The interaction between PAMPs and PRR leads to either phagocytosis or to signaling events [Apostolopoulos and McKenzie, 2001; Boller & Felix, 2009]. The TLRs belong to the families of signaling PRRs which include 10 highly conserved transmembrane proteins termed TLR1-10. TLRs contain terminal leucine rich repeats (LRR) that recognize specific PAMPs, a transmembrane domain,
and finally a cytoplasmic signaling domain, homologous to the IL-1 receptor termed as Toll/IL-1 receptor (TIR) domain. The different members of the TLR family distinguish distinct microbial products, e.g. lipoproteins (TLR2), LPS (TLR4), single stranded (TLR7) or double stranded (TLR3) viral RNA, flagellin (TLR5) and unmethylated CpG sequences in DNA (TLR9). Binding of respective TLR to the PAMPs leads to signal transduction via TIR and signaling cascades leading to the activation of the transcription factor NF-κB [Dunne & O'Neil, 2003; Kawai & Akira, 2007]. NF-κB is a heterodimer of a p50 and p65 components which remains in the cytoplasm because of its interaction with inhibitory κB (IκB) proteins in the unstimulated cell. After the stimulation, a series of phosphorylation events follow TLR stimulation leading to polyubiquitination and degradation of IκB, and thus NF-κB localize to the nucleus. NF-κB binds to the DNA and leads to the transcription of pro-inflammatory cytokine genes. Thus, with interactions between the macrophage and other cells of the innate and adaptive immune systems through cytokine signaling and antigen presentation, an appropriate immune response can be mounted [Rohde et al, 2007, Kawai & Akira, 2007].

The NLR family consists of more than 20 different soluble proteins and serve as cytoplasmic PRRs inside macrophages to sense danger signals, such as microbial products as well as extracellular ATP and cell disruption (K+ efflux) [Martinon et al, 2009; Ting et al, 2008]. The mechanism as to how NLRs recognize these danger signals are various and evidence points to the association of a secondary messenger such as ROS [Martinon, 2010]. The NLRs are vital to a functional innate immune system. Among the various subfamilies of NLRs, the main ones are NACHT, LRR and PYD domains-containing protein (NLRP) family and the NACHT, LRR and CARD domains containing protein (NLRC) family. NLR proteins contain sensing LRR region, central oligomerization domain (NACHT) and effector domain [pyrin domain (PYD), caspase recruit domain (CARD), baculovirus inhibitor of apoptosis repeat domain or transactivation domain] which determines the type and function of the NLR [Martinon et al, 2009; Ting et al, 2008]. The activation of NLRs can bring about NF-κB activation and translocation to the nucleus resulting in transcription of proinflammatory cytokine genes. Additionally, NLRs including NLRP1, NLRP3, and ICE-protease activating factor (IPAF/NLRC4) can form molecular complexes termed inflammasomes together with CARD domain-containing proteases known as caspases. In the case of NLRPs, this occurs with the help of scaffolding proteins such as...
apoptosis-associated speck-like protein containing a CARD (ASC), which through homotypic interaction acts as a bridge between the NLRP and the CARD domain of the caspase. Subsequently, through inflammasome assembly, inflammatory caspases are activated and the pro-inflammatory cytokine precursors pro-IL-1β and pro-IL-18 are cleaved into their mature forms and released from the cell. The precursors are produced upon TLR engagement and NF-κB translocation to the nucleus [Martinon et al, 2009; Mariathasan & Monack, 2007]. Inflammasome activation is also dependent on upregulation of the NLR gene, which occurs upon receptor ligation [Thoma-Uszynski et al, 2001]. In addition to cytokine production, activation of the NLRP3 inflammasome can lead to induction of a type of cell death where the cell releases large amounts of pro-inflammatory cytokines, and displays signs of necrosis with permeabilization of the plasma membrane. This cell death pathway can be dependent on caspase-1, and is then termed pyroptosis, or independent of caspase-1 but dependent on NALP3 inflammasome assembly, and is then termed pyronecrosis. Inflammasome-related cell death can be beneficial for the host as it may kill the microbe and leads to recruitment of other immune cells, but can also be thought of as an “emergency exit” for a cell that is unable to handle an intracellular pathogen, as it inevitably leads to excessive inflammation [Ting et al, 2008].

Cytokine production

There are two major pathways for release of cytokines from the cell. Newly synthesized cytokine-precursor proteins in the ER of macrophages are folded, partially glycosylated, and then transported to the Golgi complex for further processing and glycosylation, ending up in the trans Golgi network (TGN) [Stow et al, 2009]. In the constitutive secretory pathway, recycling endosomes sort and carry proteins from TGN to the cell surface in continuous small amounts, and this pathway can be upregulated upon stimulation in macrophages to increase cytokine secretion. In the granule-mediated secretory pathway in professional secretory cells where proteins are stored into granules until degranulation is triggered, and the vesicle contents are released through fusion with the plasma membrane. The secretion pathway of IL-1β from the cytosol is not known [Stow et al, 2009]. The process of cytokine release from macrophages is tightly regulated and the response can be very quick. Upon activation with LPS, macrophages
release TNF-α, which can be detected both as a precursor in the TGN and as a cleaved mature cytokine at the cell surface as early as 20 min after stimulation [Shurety et al., 2000].

Thus, macrophages are the main players in innate immunity which can be stimulated in different ways to perform effector functions and also orchestrate other parts of the immune system.

**Antimicrobial properties of the macrophage**

The most important antimicrobial properties of macrophages are acidification of the phagosome, activation of the NADPH oxidase NOX2, activation of iNOS, as well as antimicrobial peptides and degradative proteins [Flannagan et al., 2009].

For the acidification of phagosome, a large number of vacuolar H^+-ATPases are needed in the phagosomal membrane. There are different theories as to where the vacuolar H^+-ATPase is recruited from, including the TGN [Fratti et al., 2003; Kinchen & Ravichandran, 2008], endosomes [Jordao et al., 2008], and tubular structures protruding from lysosomes [Sun-Wada et al., 2009]. The vacuolar H^+-ATPase enzyme complex is central for phagosomal maturation, both for acidification as a means to destroy pathogens as well as for activating hydrolytic enzymes that in turn degrade pathogens, and finally as a controller of membrane traffic [Flannagan et al., 2009; Huynh & Grinstein, 2007]. In addition to these functions, the vacuolar H^+-ATPase pumps H^+ in an electrogenic manner, which facilitates superoxide production as it counteracts the negative charges transported by the NADPH oxidase and the phagosomal H^+ can be combined with products of the oxidase, generating more complex ROS [Flannagan et al., 2009].
Macrophages use the NADPH oxidase NOX2 to generate ROS from O\textsubscript{2} for killing microorganisms, although this mechanism is more potent and in neutrophils. Upon activation, the enzyme complex subunits of the NADPH oxidase assemble in the phagosomal membrane in a Rac1- or Rac2-dependent manner. The active NADPH oxidase transfers cytosolic NADPH electrons to O\textsubscript{2} in the phagosome, producing superoxide (O\textsubscript{2} -) which forms hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) through dismutation in the phagosome. H\textsubscript{2}O\textsubscript{2} in turn further reacts with O\textsubscript{2}-, generating different ROS, which can kill the intraphagosomal pathogen [Roy \textit{et al}, 2008; Robinson, 2009].

\textbf{iNOS} (NOS2), also termed NOS2 in phagocytes, is synthesised \textit{de novo} upon microbial stimulation of macrophages. It functions to produce nitrogen radicals on the cytoplasmic side of the phagosome, which can then diffuse into the phagosome. iNOS has two subunits, which act in
concert to produce NO• and citrulline from L-arginine and O2. Upon reaction with oxygen radicals produced by the NADPH oxidase, NO• is converted to reactive nitrogen intermediates (RNI), which are very toxic to microbes in the phagosome, and can damage DNA, lipids and proteins [Roy et al, 2008]. The importance of RNI in protection against microbial infection in mouse macrophages has been well documented [MacMicking et al, 1997], and RNI are thought to play a role in human macrophage infection as well, although this is more controversial [Chan et al, 2001; Schon et al, 2004]. The mature phagosome contains many antimicrobial peptides and degradative proteins which help in the destruction of phagosomal pathogens. Antimicrobial agents can either deprive the microbe of nutrients or compromise its integrity by inducing membrane permeabilization. Iron scavengers, such as lactoferrin, and iron exporters, such as natural resistance-associated macrophage protein 1 (NRAMP1), remove iron thus depriving the microbe of an essential factor required for DNA synthesis and mitochondrial respiration [Flannagan et al, 2009; Nairz et al, 2010]. Antimicrobial peptides and proteins that more directly contribute to microbe killing by permeabilizing the bacterial cell membrane include defensins, cathelicidins, lysozymes, lipases, proteases, and hydrolases [Flannagan et al, 2009]. In neutrophils, these defence substances are packed in granules, while in macrophages they can be expressed upon activation in a manner dependent on vitamin D [Liu et al, 2006] or be acquired from other cell types [Tan et al, 2006, Ocho et al, 2001].

With their wide array of antimicrobial functions, it is evident that monocytes are key players in the innate immune response. In addition to the mentioned mechanisms in the phagosome, macrophages are capable of inducing programmed cell death through apoptosis when infected with an intracellular pathogen, as a type of “emergency exit.” This can function as a direct deprivation of the replication niche of the bacterium or virus, as well as a signal to the remaining immune system [Lamkanfi et al, 2010]. Another mechanism is autophagy, a process by which the cell degrades its own components through a pathway similar to that of phagosomal maturation described above, which has been implicated in enhanced killing of intracellular pathogens including *M. tuberculosis* [Deretic et al, 2009; Deretic, 2005].
Enhancing the antimicrobial properties of the macrophage

Inflammasome activation is an antimicrobial mechanism in macrophages, where microbial components activate caspase-1, which together with receptor signaling leading to transcription of the pro-IL-1β gene results in cleavage of pro-IL-1β and release of the mature cytokine from the cell. IL-1β signaling has in turn been implicated in improved phagosomal maturation [Master et al. 2008; Lazarevic & Martinon, 2008]. Other cytokines have also been implicated in enhancing (IFN-γ, TNF-α) or attenuating (IL-10) phagosomal maturation in human and mouse macrophages [Schaible et al, 1998; Via et al, 1998; Harris et al, 2008; O'Leary et al, 2011]. The link between macrophage activation and phagosomal maturation is not completely clear, but it is known that NF-κB translocation, achieved by TLR or pro-inflammatory cytokine receptor ligation, to the nucleus leads to transcription of genes encoding antimicrobial proteins as well as cytokines and co-stimulatory molecules, and it has been proposed that pro-inflammatory cytokine signaling has a direct effect on effector functions in the phagosome or an effect on fusion and fission events between the phagosome and the endocytic network [Thoma-Uszynski et al, 2001; Via et al 1998, Aliprantis et al, 1999; Underhill et al, 1999].

Conflicting evidence concerning the role of TLRs in phagosomal maturation exists. Blander and Medzhitov showed that absence of TLR signaling leads to a significant impairment in the acquisition of lysosomal markers and hypothesize that the accelerated phagosomal maturation achieved by TLR ligation or uptake through FcγR creates a hostile environment for microbes [Blander, 2007; Blander & Medzhitov, 2004; Blander & Medzhitov, 2006]. Conversely, Yates & Russell found that there was no difference in the maturation of phagosomes containing uncoated silica beads and silica beads coated with the TLR ligands LPS (a TLR4 ligand) and Pam3CSK4 (a TLR2 ligand) [Yates & Russell, 2005; Russell & Yates, 2007]. The authors postulated that although TLRs are enriched on the phagosome [Underhill et al, 1999] and their ligation may slightly enhance phagolysosomal fusion, ligation does not enhance the hydrolytic activity of the phagosome and TLR ligation is not key to enhancing phagosomal maturation [Yates & Russell, 2005; Russell & Yates, 2007].
Human and mouse macrophages

In mouse and probably human macrophages infected with *M. tuberculosis*, phagosomal maturation, RNI production and regulation of bacterial replication/ killing can be enhanced through stimulation with pro-inflammatory cytokines such as IFN-γ or TLR ligands [Flesch & Kaufmann, 1987; Rohde *et al*, 2007; Schaible *et al*, 1998; Thoma-Uszynski *et al*, 2001; Reljic *et al*, 2010]. However, the reports on human and mouse cells, and the failure to see a reduction in bacterial killing in human macrophages where iNOS activity has been blocked, indicate that different killing mechanisms are used by human and mouse Macrophages [Chan *et al*, 2001; Thoma-Uszynski *et al*, 2001]. On the other hand, induction of iNOS has been observed in human granulomas [Schon *et al*, 2004; Andersson *et al*, 2007]. The present study is based on human macrophages, to ensure a relevance of the results for human TB. To sum up, macrophages, their PRRs, microbicidal mechanisms and the cytokines they secrete play a central role in the innate immune response to infection, and the pro- or anti-inflammatory response elicited is essential in determining the outcome of infection.

*M. tuberculosis* interaction with macrophages

Recognition and ingestion

The macrophage is the main replication niche of *M. tuberculosis*, despite the bactericidal characteristics and functions that this cell type normally has. The bacillus has evolved several strategies for surviving in the otherwise hostile intracellular environment of the macrophages. *M. tuberculosis* interacts with the macrophage through several different receptors, including CR, MR, and FcγR, which leads to phagocytosis of the bacterium, and this is the route of entry employed by *M. tuberculosis* to gain access to its replication niche. The receptor engaged for uptake depends on whether the bacillus has been opsonized with complement or antibodies, or remains unopsonized, and can be important in determining the subsequent events in the host cell as well as the outcome of infection [Pieters, 2008], although this has been debated [Vergne *et al*, 2004]. Studies suggest that multiple receptors may be engaged simultaneously during phagocytosis of *M. tuberculosis* [Ernst, 1998].
**Toll Like Receptors (TLRs)**

Apart from engaging endocytic PRRs to mediate phagocytosis, PAMPs on the *M. tuberculosis* surface also bind signaling PRRs including TLRs. TLR2 forms a heterodimer with TLR1 or TLR6 on the macrophage cell surface and together with CD36 recognizes different bacterial structures, inducing a pro-inflammatory response [Berrington & Hawn, 2007]. TLR2 engagement by *M. tuberculosis* components leads to vitamin D-dependent production of cathelicidin, and may play a role in phagosomal maturation, as previously discussed [Korbel et al, 2008]. Several *M. tuberculosis* components are recognized by TLR2, including lipoproteins, the cell wall core structure, lipids, LM, PIM, and nonmannose- capped LAM. ManLAM from virulent *M. tuberculosis*, on the other hand, does not bind TLR2, circumventing the TLR2-mediated pro-inflammatory response [Quesniaux et al, 2004; Underhill et al, 1999]. The 19-kDa lipoprotein of *M. tuberculosis* is also recognized by TLR1, and an *M. tuberculosis* extract can be recognized by TLR6. TLR4, which is the receptor that together with CD14 binds LPS of Gram negative bacteria, recognizes a yet undefined heat-labile *M. tuberculosis* component, and the intracellular TLR9 recognizes *M. tuberculosis* DNA in the phagosome [Korbel et al, 2008; Quesniaux et al, 2004]. *In vivo* knock-out studies in mice have shown a redundancy of TLRs when it comes to combating *M. tuberculosis* infection, and have given contradicting results as to whether knock-out of TLR leads to increased susceptibility to *M. tuberculosis*.

However, it has been established that MyD88, an adaptor protein essential for NFκB activation, is required for an effective immune response against *M. tuberculosis* [Korbel et al 2008, Berrington & Hawn 2007]. It has been argued, however, that it is the vital role of MyD88 in IL-1R or IFN-γ signaling rather than in TLR signaling and pathogen recognition that leads to its central role in *M. tuberculosis* immunity [Reiling et al, 2008].

**Protein kinase Cs (PKCs)**

Protein kinase C (PKC) is a family of protein serine/threonine kinases centrally involved in intracellular signal transduction. The PKC isoforms are divided into 3 subfamilies based on their activation requirements: the conventional isoforms, PKC-α, -βI, -βII, and -γ, require calcium,
diacylglycerol, and phosphatidylserine; the novel isoforms, PKC-δ, -ε, -η, and -θ, require diacylglycerol and phosphatidylserine but are calcium independent; the atypical isoforms, PKC-ζ and λ/κ, require only phosphatidylserine [Newton, 2003]. Different isoforms of PKC are involved in such pivotal functions as cell growth, differentiation, apoptosis, motility, and secretion. Initial evidence for the involvement of PKC in TLR signaling came from observations that altering PKC activity in cells of the innate immune system affected cytokine secretion. Subsequently, LPS and other TLR ligands were shown to activate most of the PKC isoforms expressed in monocytes, Macrophages, dendritic cells, and neutrophils [Fronhofer et al, 2006; McGettrick et al, 2006; Kontny et al, 2000; Zhou et al, 2006; Asehnoune et al, 2005]. A large number of studies have shown that pharmacological inhibition of PKC or its depletion by long-term treatment with phorbol esters, decreases LPS-stimulated cytokine secretion [Fronhofer et al, 2006; West et al, 1997; Labeta et al, 1993; Cuschieri et al, 2006]. Accordingly, acute activation of PKC with phorbol esters increases cytokine secretion [McGettrick et al, 2006; West et al, 1997; Cuschieri et al, 2006; Knethen et al, 2007]. Given the fact that M. tuberculosis may respond to the intracellular milieu of the macrophages with the induction of environmentally regulated genes required for survival and growth of the bacteria it can be assumed that the protein kinases may also be the factors in Mycobacterium-Macrophages interaction.

Interestingly, one of the initial cellular events disrupted by M. tuberculosis is the transient elevation of intracellular Ca^{2+}. This calcium flux is required for the subsequent phagosome/lysosome fusion and induction of a Ca^{2+} flux upon an M. tuberculosis infection leads to phagosome maturation [Malik et al, 2001]. This calcium rise, which occurs upon infection with dead M. tuberculosis or live Staphylococcus aureus, is dependent on sphingosine kinase (SPK)3 activation. In contrast, live M. tuberculosis fails to activate SPK [Malik et al, 2003]. SPK is a key enzyme catalyzing the formation of sphingosine- 1-phosphate (S1P), a lipid messenger that is implicated in the regulation of a wide variety of important cellular events through both intracellular and extracellular mechanisms, which include Ca^{2+} mobilization, activation of MAPK, and vesicular trafficking [Monick et al, 2004; Koda et al, 2005; Meyer et al, 1998; Spiegel et al, 2002; Kluk et al, 2002]. Infection by mycobacteria leads to a signaling response by the host macrophage and subsequent production of proinflammatory mediators.
Cytokine response

Pro-inflammatory cytokine signaling is crucial for an effective immune response against intracellular pathogens, and mice lacking IL-1β, IFN-γ, IL-12, IL-6 or TNF-α quickly succumb to infections [Benoit et al, 2008]. The same cytokines play a great role in controlling human M. tuberculosis infection, and their downregulation through IL-10 or TGF-β can be detrimental to the host, despite the fact that these cytokines are crucial for limiting tissue damage [Flynn & Chan, 2001]. Early during M. tuberculosis infection of mice and humans, the macrophages are polarized towards an M1 phenotype, although some individuals have a more M2-like profile which can be reversed by antibiotic treatment [Benoit et al, 2008; Redente et al, 2010]. Macrophages from mouse bronchoalveolar lavage switch to a more M2- like profile as M. tuberculosis infection progresses, with high levels of IL-4 and a loss of iNOS but increase in arginase expression, while macrophages from inside mouse granulomae remain more M1-like [Redente et al 2010]. Th1 cytokines and an M1 macrophages response is thought to be essential for control of human M. tuberculosis. Concurrent helminth infection, which leads to a switch to a Th2 response, increases morbidity in TB patients [Elias et al, 2006]. In addition, reactivation of latent TB can be associated with a switch to a Th2 response [Howard & Zwilling, 1999]. Finally, Th2 cytokine treatment in M. tuberculosis infected mice deprives the macrophages of their killing mechanisms, leading to increased replication through increased availability of iron [Kahnert et al, 2006].

Although pro-inflammatory cytokines are necessary for the host response to M. tuberculosis, they are also responsible for tissue damage, again highlighting the importance of a tightly regulated immune response against M. tuberculosis infection [Carlsson et al, 2010; Howard & Zwilling, 1999]. In addition to the crucial cytokine response to M. tuberculosis infection, chemokines including RANTES, MIP1-α, MIP2, MCP-1, MCP-3, MCP-5 and IFN-γ-induced protein 10 kDa are also released upon macrophages infection with M. tuberculosis, and chemokine receptors including CCR5, RANTES receptor, MIP1-α receptor and MIP1-β receptor are expressed. Some of these have been found to be essential for protection against M. tuberculosis infection as well as for granuloma formation, although this response is less well characterized [Raja et al, 2004; Flynn & Chan, 2001].
**Immunity to *M. tuberculois** require the Th1 response**

![Diagram](image)

*Figure 10: Th1 and Th2 cytokine response by macrophages after *M. tuberculois* infection.*

**Inhibition of bactericidal mechanisms**

Pathogens have evolved different mechanisms to allow survival inside the macrophage. *Listeria* and *Shigella* spp. escape into the cytoplasm to avoid the degradative milieu of the lysosome and have evolved mechanisms of surviving extraphagosomally. *Coxiella* and *Leishmania* spp. can replicate inside phagolysosomes despite the hostile milieu. *Legionella, Brucella* and *Mycobacterium* spp., in contrast, inhibit phagosomal maturation [Rohde et al, 2007]. *M. tuberculois* owes its success as pathogen to its ability to interfere with the normally effective antimicrobial properties of the macrophages. Thereby, the bacterium creates a niche that supports its replication despite its localization inside the cell that was designed to kill microorganisms.

**Signal transduction mechanism in macrophages:**

Signal transduction mechanism involves the transmission of signals from outside of the cells to inside by various mechanisms including coupling of ligand-receptor interactions to many intracellular events. These events include phosphorylations by tyrosine kinases and/or serine/threonine kinases resulting in altered cellular activities and gene activation [Krebs & Beavo, 1989]. Ligand-stimulated cell signaling may be initiated at the cell surface by the direct activation of receptor tyrosine kinases (RTK) [Eagn & Weinberg, 1993; Sadowski et al, 1993].
Alternatively, signaling through the membrane receptor that lacks protein kinase domains may proceed indirectly via the activation of membrane associated, cytoplasmic, non-receptor PTK [Samelson & Klausner, 1992]. These initial rapid events may be linked to one of several downstream signaling elements such as PLC-γ. Activation of PLC-γ by tyrosine phosphorylation leads to the hydrolysis of phosphatidylinositol- (4, 5)-bisphosphate [ptdIns (4,5)P₂], generation of PKC activator diacylglycerol (DG) and the endogenous Ca²⁺ mobilizing agent inositol (1,4,5)-triphosphate [Ins(1,4,5)P₃] [Dekker & Parker, 1994]. These two second messengers activate Ca²⁺ and phospholipid dependent PKC, as well as other Ca²⁺-regulated protein kinases. The activation of the classical PKC (cPKC) essentially needs the binding of DG and phosphatidyl serine (PS) to the regulatory C1 region of PKC, the latter being constitutively present at the cell membrane. Ligand induced cell surface receptors may also initiate signaling through either classical heterotrimeric or small Ras-like GTP-binding proteins [Eagn & Weinberg, 1993]. In either instance, a cascade of signaling events ensues that involves multiple protein kinases. For example, certain receptors (such as those for angiotensin, bradykinin and histamine, and the phagocyte receptor for the chemotactic peptide fMLP) coupled to heterotrimeric G proteins activate PLC β, leading to the production of DG and Ins (1,4,5) P₃ [Simon et al, 1991]. Other receptors may recruit signaling pathways, which activate the mitogen activated protein kinases (MAPK) [Weinstein et al, 1992]. This may be regulated in a Ras-dependent manner involving a cascade of protein kinases, upstream and downstream of MAP kinase, including the protein kinase Raf-1, MAP-ERK kinase (MEK) and the ribosomal S6 protein kinase [Eagn & Weinberg, 1993; Weinstein et al, 1992; Lange-Carter et al, 1993]. Alternatively, activation of MAPK via MEKK may occur independently of Ras [Lange-Carter et al, 1993]. Treatment of macrophages with agonist e.g. LPS [Liu et al, 1994; Eagn & Weinberg, 1993], IFN-γ [Sadowski et al, 1993; Hunter, 1993] has also shown to induce rapid protein phosphorylation. For example, transcriptional activation of IFN-γ in macrophages involves tyrosine phosphorylation of a 91 kDa protein that is part of the IFN-γ activation factor transcriptional-regulatory complex and this may involve activated JAK-family PTK [Sadowski et al, 1993; Hunter, 1993]. Recently LPS induced cell activation leading to cytokine production has also been shown to involve activation of specific PTKs, including a CD14 associated Src-family PTK, p53/56 lyn, as well to other Src-family kinases, p58 hck and p59c-fgr [Stevanofa et al, 1993].
The PKC family of enzymes has been divided into three major groups based upon structural and regulatory properties: i) conventional PKC (cPKC) or type I isotypes (α, β-I, β-II and γ) that are regulated by Ca\(^{2+}\) and DG; ii) novel PKC (nPKC) or type II isotypes (δ,ε,η,μ) that are also activated by DG but are Ca\(^{2+}\)-independent; iii) atypical PKC (aPKC) or type III isotypes (ζ,τ,λ) independent of both Ca\(^{2+}\) and DG[Decker & Parker, 1994; Ways et al, 1994]. In quiescent cells, that majority of PKC is localized in the cytosol. Translocation of PKC to the membrane fraction is considered to reflect enzyme activation and in macrophage specifically, activation of the oxidative burst by phorbol esters with redistribution of PKC activity to the particulate fraction [Myers et al, 1985]. A role of PKC-α and PKC-β in LPS mediated cell signaling in macrophages has been suggested by the finding that treatment of human monocytes with LPS induces the quantitative translocation of these enzymatic activities to the membrane fraction [Bakouche et al, 1992]. PKC inhibitors abrogate both the activation and the translocation of PKC in these cells, and attenuate LPS induced secretion of IL-1.

Changes in the concentration of cytosolic intracellular free calcium are also a feature of macrophages activation. For example, the potent Macrophages peptide agonist fMLP induces the rapid increase of calcium from intracellular stores and this is associated with an oxidative burst and other functional responses including cytokine production [Richter et al, 1992]. Moreover, exposure to agents that either directly or indirectly inhibit Ca\(^{2+}\) regulated protein kinases has been shown to abrogate changes in cell function included by several activating ligands including LPS and IFN-γ [Kemmerich et al, 1986; Ohmori et al, 1992]. Mycobacteria subvert these signaling pathways by regulating the genes involved and ultimately control the pathogenesis.

**Immunotherapy in TB:**

Immunotherapy is one of the modern therapeutic approaches against TB. There may be three major potential uses for immunotherapy in TB patients. Firstly, patients with MDR-TB, current therapy is suboptimal, and adjunctive immunomodulation may facilitate initial bacillary clearance and increase cure rates. Immunotherapeutic strategies could include administration of Th1 cytokines such as IL-2 or IFN-γ, or of IL-12 and IL-18, which elicit IFN-γ production. Alternatively, natural inhibitors of TGF-β or anti–IL-10 antibodies could be used to
downregulate the Th2 response. MDR *M. tuberculosis* varies in drug susceptibility, and treatment regimens must be tailored for individual patients. Large randomized trials of immunotherapy in this setting would be a major logistical challenge, but this is the only way to confirm or refute the promising results of small studies using IL-2 and aerosolized IFN-γ [Johnson *et al*, 1997; Condos *et al*, 1997]. The second potential use for immunotherapy is to shorten the duration of treatment for drug-susceptible tuberculosis, reducing cost and increasing treatment completion rates. A distinctive role for immunotherapy could be to kill slowly replicating or “dormant” organisms more effectively than current antituberculous agents, perhaps by administering immunotherapy after the initial phase of treatment. However, a more sophisticated understanding of the biology of slowly replicating *M. tuberculosis* is necessary to develop such a strategy. The third use for immunotherapy is to downregulate the host inflammatory response, which can cause substantial morbidity during the early phase of antituberculosis therapy, usually in patients with severe disease. These problems are likely to be due to cytokine production, as the highest local and systemic levels of Th1 cytokines are found in patients with the most severe TB [Yamada *et al*, 2000; Tsao *et al*, 2002]. In these individuals, targeted strategies to reduce the inflammatory response, such as antibodies to IFN-γ or to TNF-α may have significant benefits, similar to those achieved with corticosteroids, but with reduced risk of immunosuppression. Administration of cytokines or cytokine antagonists alters only one aspect of a complex immune response. An alternative approach is to provide a stimulus that generates a multifaceted response favoring bacillary elimination. Administration of the environmental mycobacterium *M. vaccae* has been postulated to enhance the Th1 response and suppress the Th2 response, but adjunctive immunotherapy with *M. vaccae* in tuberculosis patients has not yielded conclusive evidence of clinical benefits in controlled trials [Mwinga *et al*, 2002]. Other strategies could include administering Toll-like receptor agonists that boost the innate immune response to infection, a cocktail of immunogenic peptides that stimulate protective T cell responses, or a DNA construct encoding these immunogenic peptides. During the past decade, we have seen explosive growth in our knowledge of the pathogenesis of disease due to *M. tuberculosis*, coupled with an improved understanding of the mechanisms that mediate protective immunity.
Identification of particular target will turn up an effective immunotherapy against mycobacteria. More studies on the detection of deficiencies in the host immune response will contribute in the successful immunotherapy in TB.