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# How *Mycobacterium tuberculosis* Manipulates Innate and Adaptive Immunity – New Views of an Old Topic

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## 1. Introduction

Tuberculosis (TB), once regarded an historical disease due to the discovery of antibiotics, is one of the most wide-spread human infections today, and a major cause of death from bacterial infections. The causative agent, *Mycobacterium tuberculosis*, has evolved over ages along with the human species, the oldest human finding being 9 000 year-old skeletons with tuberculosis lesions (Hershkovitz, Donoghue et al. 2008). The most widely known form of tuberculosis, pulmonary TB, affects the lungs and is characterized by cough with bloody expectorations associated with fever, night sweats and weight loss. Extrapulmonary TB can be found almost anywhere in the body, either localized in organs such as the lymph nodes, pleura, abdomen, bones, joints and the central nervous system or appearing in a more disseminated form known as miliary TB. Active TB disease develops in 5-10% of infected individuals, whereas most exposed individuals contain the infection in the form of latent disease or more rarely eradicate the bacteria. To prevent progression of latent TB to active disease, an equilibrium between the host and the microbe has to be maintained. Today, about one third of the world population are carriers of TB infection, which constitutes an enormous reservoir for potential spread of disease.

### 1.1 Preface

Despite decades of research on TB, studies of the cellular mechanisms involved in TB pathogenesis have lagged behind, particularly in human systems. In addition, TB immunology is a competitive area of research and available data is still controversial and often based on interpretations of results obtained from artificial experimental systems. The events associated with the induction and maintenance of *M. tuberculosis*-specific immune responses are complex and there are still many questions to be answered. This chapter describes the mechanisms by which *M. tuberculosis* manipulates host cell functions and the specific immune responses that are induced during mycobacterial infection. The balance between the bacteria and the host is delicate and a large number of research studies have

been devoted to better understanding of the critical factors involved in the cellular and molecular regulation of this balance. As will be discussed, the interplay between innate and adaptive immune responses as well as different cells of the immune system is closely interconnected. A complex network of cells at different locations and stages of differentiation contribute to the induction of the *M. tuberculosis*-specific immune responses and the regulation of TB disease. In order to understand the pathogenesis of TB, it is therefore necessary to study and relate host-specific immune responses to the microbiological and biochemical events initiated by the bacteria that result in the establishment and progression of *M. tuberculosis* infection. This chapter describes experiments ranging from macrophage infections to immunological analysis of pathogenesis in clinical tissue and cell samples obtained from the site of infection of TB infected patients. The presented methods include molecular analysis of mycobacterial cell wall components, a method for rapid determination of bacterial numbers and *in situ* quantitative image analysis. The chapter is based on current literature where animal models and patient materials is integrated with comprehensive cellular and tissue model systems, which are used to explore *M. tuberculosis*-specific host-pathogen interactions in human TB. Basic knowledge and the recent advances in the field of host-mycobacterial interactions and how pathogenic mycobacteria can manipulate and escape the immune system will be discussed with a special focus on the knowledge obtained with experimental models in relation to human TB.

## 1.2 Mycobacterial structure

*M. tuberculosis* is a rod-shaped bacterium belonging to the family of Mycobacteria (*Mycobacteriaceae*), which are gram-positive bacteria. The mycobacterial cell wall is rich in waxes and lipids, which contribute to the virulence of the bacteria in different ways (Shui, Petzold et al. 2011). Mycobacterial waxes are composed of diverse mycolic acids, which form a layer reminiscent of the outer membrane of Gram-negative bacteria (Bhamidi, Scherman et al.; Sani, Houben et al.). The waxy cell wall together with the recently described capsular layer, the major component of which is  $\alpha$ -glucan, contribute to the extraordinary resistance of mycobacteria to stresses such as drought, low pH and antibiotics (Sani, Houben et al.; Liu, Barry et al. 1996). Pathogenic mycobacteria can regulate their cell wall thickness in response to stresses posed by host immunity (Cunningham and Spreadbury 1998) and there is recent evidence that the altered cell wall in stressed bacteria is refractory to acid-fast staining (Seiler, Ulrichs et al. 2003; Deb, Lee et al. 2009), a finding which may have important implications for diagnosis (Garton, Waddell et al. 2008).

## 1.3 The immunological checkpoints *M. tuberculosis* needs to pass

*M. tuberculosis* is a highly successful intracellular pathogen that has developed strategies to survive even in the presence of high immune pressure. The usual site of entry into the human body is through the airways, beginning with the inhalation of infected droplets expelled from another infected individual through coughing. The bacilli are transported into the respiratory tract to be engulfed by alveolar macrophages, cells that are designed to kill bacteria. Although being caused by a quite simple microorganism, TB is a multifaceted disease with a spectrum of antimicrobial effector pathways at play during different stages of infection, ranging from early innate to late adaptive immune responses during acute and

chronic infection. The infectious dose (the number of microorganisms required to cause infection) is very low, but nevertheless, most exposed individuals maintain the infection in a latent state. As the ability to control *M. tuberculosis* infection is strongly correlated with intact immune functions of the infected human host, individual differences in the ability to mount a proper immune response delivers an explanation for the low percentage of disease progressors. In order to cause active TB, *M. tuberculosis* has to pass several host immunity checkpoints (Barry, Boshoff et al. 2009). These checkpoints, illustrated in figure 1, include the initial attacks posed by innate immune mechanisms and the following adaptive immune response.

### **1.3.1 Checkpoint one: Avoiding being killed by the macrophage**

The first checkpoint that *M. tuberculosis* has to overcome is to prevent itself from being killed by the antimicrobial effector mechanisms harboured by macrophages. Macrophage effector mechanisms include acidification of the phagosome, exposure to proteases and antimicrobial peptides and the generation of reactive oxygen and nitrogen species. The pathogen has evolved strategies to evade and/or tolerate these stresses, and manages to survive in cells that are otherwise effective killers of most microorganisms. However, there may be circumstances under which the bacteria are actually eradicated inside the human host. For example, it is well established that many subjects, who are continuously exposed to *M. tuberculosis* (e.g. household contacts of TB patients), do not display any immunological memory of the pathogen, as evidenced by the lack of reaction to interferon- $\gamma$  release assays (IGRA) and tuberculin skin test (TST). These individuals possibly exert a massive innate immune pressure on the inhaled pathogens, making any adaptive immune reaction unnecessary. A clue to the mechanism behind this phenomenon comes from the observation that healthy household contacts of TB-patients produce high amounts of bactericidal compound nitric oxide (NO) (Idh, Westman et al. 2008). Although this observation needs further confirmation in human *in vitro* systems, it is possible that the ability of macrophages to produce sufficient amounts of NO actually allows the innate immune system to eradicate *M. tuberculosis* infection.

### **1.3.2 Checkpoint two: Defeating innate immunity**

Failure of innate immune mechanisms to control the growth of the bacteria, possibly related to insufficient production of NO and other immune mediators, admits *M. tuberculosis* through the second checkpoint, after which adaptive immunity becomes important. The increasing immune pressure mounted by the adaptive immunity restores the immunological control. Latent TB is characterized by immune reactivity towards TB antigens (e.g. IGRA or TST) along with absence of any clinical symptoms. In the lungs tissues, healed or active granulomatous lesions at different stages may be present. Individuals with latent TB include non-progressors, in whom the infection is kept at equilibrium through host immune mechanisms and progressors, in whom the infection progresses towards active disease.

### **1.3.3 Checkpoint three: Defeating adaptive immunity**

As long as immune control is maintained, *M. tuberculosis* infection is kept latent; however, sooner or later, the bacterium may take advantage of a declining immunocompetence of the

host, e.g. due to ageing, malnutrition, drug abuse, HIV infection or other immunosuppressive diseases or drug treatments. Thus, by passing the last checkpoint of adaptive immunity *M. tuberculosis* will inevitably cause its host to transmit the infection and eventually succumb, if left untreated. On the following pages, we will describe the protective immune defence mechanisms and the strategy that *M. tuberculosis* employs to get beyond these checkpoints.

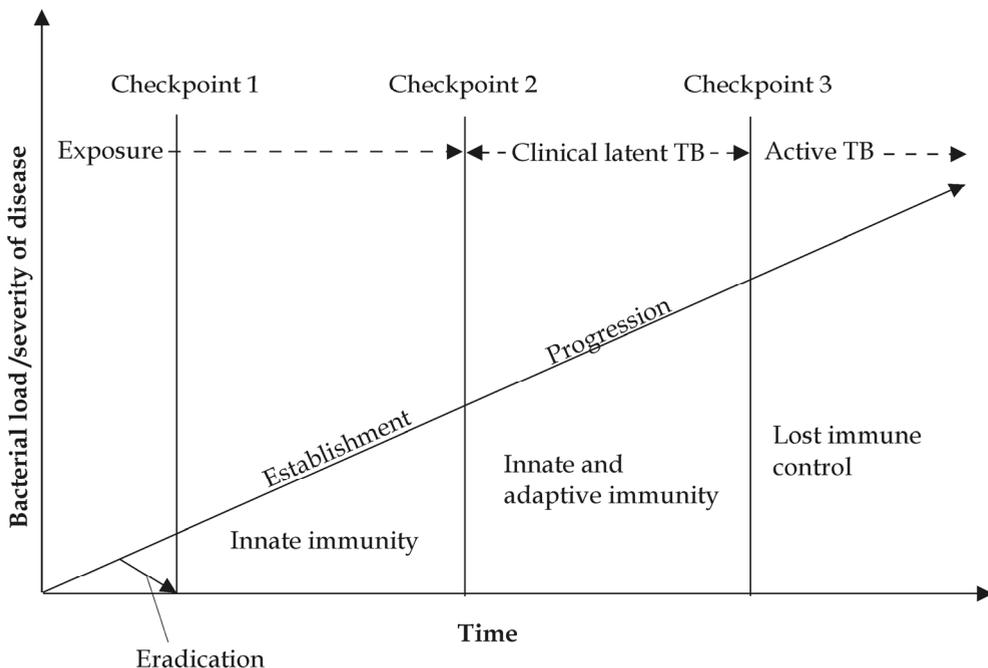


Fig. 1. The checkpoints that *M. tuberculosis* has to pass in order to cause TB. Checkpoint 1 is to avoid being killed by the very early immune mechanisms. Checkpoint 2 is to overcome innate immune control and thereby passing on to presentation to adaptive immunity. Checkpoint 3 is to defeat the effector mechanisms of adaptive immunity. The lost immune control admits effective replication of bacteria, which leads to necrosis and spread to other individuals.

## 2. Beyond checkpoint one: Establishment of *M. tuberculosis* infection

As mentioned above, the macrophage is the major host cell for *M. tuberculosis* infection, and the alveolar macrophage is described as the first cell that encounters *M. tuberculosis* on its journey within the host. As it is difficult to investigate the very initial events of mycobacterial infection in a living host, a major part of the studies on initial events is performed with macrophages monocultures, most often cell lines of human or murine origin or with macrophages derived from human or murine monocytes. It is well established that production of NO is strongly induced in murine macrophages during mycobacterial infection, but that isolated human macrophages fail to do the same upon *in vitro* infection.

Thus, there are important differences between mouse and human macrophages, which suggests that different macrophage immune mechanisms may exist in the two species. It is also important to realise that macrophages are heterogeneous even if obtained from the same individual and thus can have substantially different phenotypes depending on the protocol used for differentiation. In the body, the circulating precursor cells, the monocytes, are already heterogeneous when they enter the blood stream from the bone marrow. Subsequent differentiation in the various tissues further adds to the complexity. Nevertheless, experiments performed with pure macrophage cultures have substantially contributed to the understanding of *M. tuberculosis*.

## 2.1 Manipulation of phagosomal maturation

The alveolar macrophage is able to take up microorganisms into a vacuole that subsequently undergoes a process known as phagosomal maturation, where a series of fusion and fission events takes place to localize microbicidal activity to the vacuole. *M. tuberculosis* has evolved mechanisms to inhibit this process, and these mechanisms have been studied in detail (for review, see Vergne, Chua et al. 2004). The process of phagosomal maturation is traditionally viewed as a gradual process of complete fusion of lysosomes, which contain microbicidal effector molecules, with phagosomes. However, it turns out that this process is much more complex and selective than previously thought. Desjardins suggested that phagosomal maturation is a continuous process of fusion and fission of cellular organelles, the transfer of membranes and materials from these being a rather transient and highly specific event (Desjardins, Houde et al. 2005). There is evidence in the literature for separate sources of lysosomal proteins such as LAMPs and the proton pumps that are required for lowering the phagolysosomal pH. Whereas LAMPs can be found on both lysosomes and endosomes, the proton pumps can be recruited to the phagosome from the ER. Therefore, studies solely based on traditional markers for phagolysosomal fusion such as LAMPs may not reflect the actual milieu inside the phagolysosome. Instead, assays addressing more functional aspects of the phagosome must be employed. Fully matured phagolysosomes are known to have a pH below 5.0 (Russell, Vandervan et al. 2009), and the low pH is a prerequisite for functionality of the proteolytic, degradative enzymes delivered by lysosomes. Thus, phagosomal acidification, which can be studied using pH-reactive, fluorescent dyes, is a correlate of phagosomal activity.

### 2.1.1 Inhibition of phagosomal maturation through lipoarabinomannan

Mycobacterial lipids are known to be important for the bacterium to resist the host immune functions, the waxes being more like passive protectors, whereas other lipids exert more specific effects. The most studied glycolipid, lipoarabinomannan (LAM), is composed of glycosylphosphatidylinositol (GPI), which functions as a lipid anchor, and a branched arabinomannan residue that extends through the mycobacterial cell wall to be exposed on the outside of the bacterium (Shui, Petzold et al. 2011). LAM differs between virulent, slow growing mycobacteria and their non-virulent, fast-growing relatives in that the virulent species carry mannosylated LAM (ManLAM) (Dao, Kremer et al. 2004). ManLAM has been shown to inhibit phagosomal maturation in both murine and human macrophages (Fratti, Chua et al. 2003; Hmama, Sendide et al. 2004; Kang, Azad et al. 2005), thus playing a central role in manipulation of an important host cell function. LAM can interact with different

receptors on the host cell surface, including the mannose receptor and the complement receptor (Le Cabec, Carreno et al. 2002; Kang, Azad et al. 2005) and during prolonged infection of cell cultures, the glycolipid is trafficked throughout the membrane compartments of the host cell (Xu, Cooper et al. 1994). In fact, LAM can be detected in the urine of TB-patients, illustrating the extensive distribution of this molecule in *M. tuberculosis*-infected hosts (Hamasur, Bruchfeld et al. 2001). Although the ability of LAM to inhibit phagosomal maturation is well established, the mechanism by which it causes this effect has not been addressed by many groups. To date, there is no mycobacterial strain, which is specifically deficient in the production of ManLAM, making mechanistic studies difficult. A frequently used model to study LAM function is based on LAM-coated latex beads (Hmama, Sendide et al. 2004; Kang, Azad et al. 2005), and these studies have provided evidence for the inhibitory effect of ManLAM on phagosomal maturation but failed to provide a mechanism by which this occurs. In order to allow mechanistic studies of LAM functions, we created a unique model based on the previously established fact that LAM can be intercalated into the host cell membrane via its GPI anchor (Ilangumaran, Arni et al. 1995). Assuming that at least initially, the membrane surrounding the phagosome is more or less derived from the plasma membrane, we allowed cells that had incorporated LAM in their plasma membrane to phagocytose opsonised zymosan particles. Resulting phagosomes displayed a reduced ability to attract markers of phagosomal maturation in comparison to control conditions (Welin, Winberg et al. 2008), a phenomenon which was dependent on the ability of LAM to incorporate into the lipid rafts. There are ongoing studies investigating this phenomenon in more detail (Torrelles and Schlesinger 2011). Albeit a totally unrelated pathogen, the protozoan parasite *Leishmania donovani* carries a glycolipid lipophosphoglycan (LPG), which is structurally related to LAM, in its cell membrane. In analogy with LAM, LPG is shed into the lipid rafts of host macrophages during infection with *L. donovani*, also causing inhibition of phagosomal maturation, thereby providing the parasite with a suitable niche inside the host cell (Winberg, Holm et al. 2009).

### 2.1.2 The abilities of non-virulent vs. virulent strains to manipulate host cell functions

Many studies on the ability of *M. tuberculosis* to inhibit phagosomal maturation are based on the attenuated vaccine strain of *M. bovis*, better known as Bacillus Calmette-Guérin (BCG). Although this strain is not able to grow inside macrophages or to cause disease (at least not in immunocompetent individuals), it seems to be as capable as virulent strains of its relative, *M. tuberculosis* to manipulate phagosome functions (Jayachandran, Gatfield et al. 2008; Sun, Wang et al. 2010). Indeed, LAM from the BCG strain is of the ManLAM type, showing no major structural difference to ManLAM from virulent strains (Prinzis, Chatterjee et al. 1993). Thus, the BCG strain and other attenuated strains of *M. tuberculosis* can be exploited as a relevant model for initial events during mycobacterial infection (Rhoades, Hsu et al. 2003; Jayachandran, Sundaramurthy et al. 2007). The reason for the lacking virulence of BCG is attributed to the deletion of the Region of Difference-1 (RD1) in its genome (Lewis, Liao et al. 2003). This region encodes a Type VII secretion system and proteins that are secreted via this system. These proteins, termed Early Secreted Antigenic Target-6 (ESAT-6) and Culture Filtrate Protein-10 (CFP-10) have both been identified as important for virulence (Smith, Manoranjan et al. 2008). Interestingly, they seem to be secreted as a one-to-one complex, the CFP-10 protein being a putative chaperone to assist ESAT-6 folding (de Jonge, Pehau-Arnaudet et al. 2007). There is no evidence of a role for ESAT-6 for the inhibition of

phagosomal maturation, but instead it is involved in the events that takes place after the initial uptake. Therefore, extended infection experiments are required to obtain a more holistic picture of *M. tuberculosis* infection.

## **2.2 The interaction between *M. tuberculosis* and the macrophage during prolonged infection**

Most of the earlier studies on the interaction of *M. tuberculosis* with the host macrophage are based on short-term experiments of a few hours up to one day. Until recent years, researchers have underestimated the importance of extended infection experiments to study a chronic infection with a slow-growing bacterium such as *M. tuberculosis*. Thus, later studies performed with human macrophages and virulent bacteria have provided insight into the broader strategy of *M. tuberculosis*, which is certainly not limited to a life within a vacuole. Instead, it seems that the pathogen, after adapting to the intracellular environment by interfering with phagosomal maturation, will disrupt the phagosome, enhance replication and eventually kill the host cell.

### **2.2.1 New experimental models for studies of *M. tuberculosis* interaction with the host macrophage**

As outlined above, the fate of mycobacteria inside macrophages is more difficult to predict than previously assumed and it becomes crucial to study mycobacterial replication inside macrophages under varying conditions. The available methods for assessment of intracellular bacterial growth have long been limited to viable counts or incorporation of radioactive markers into growing bacteria. Therefore, alternative methods for these studies of *M. tuberculosis* that could be performed more effectively are convenient. Such methods, run on a medium-throughput scale, allow more thorough analysis of intracellular fate of *M.tuberculosis* in terms of extended time of infection, bacterial factors contributing to the ability of the bacterium to establish infection and varying conditions that support the host cell to control the infection. To this end, we validated the use of *M. tuberculosis* bacteria, engineered to express luciferase, against the gold standard determination of colony forming units (CFU). We found that luciferase-based determination of bacterial numbers was actually superior to the CFU-method in accuracy as determined by intraassay variation (Eklund, Welin et al. 2010) when run in a 96-well plate format. The use of luciferase, which requires a co-factor, FMNH<sub>2</sub> that is present only in living bacteria, allows inclusion of viable bacteria only. Fluorescent markers such as GFP are also used to enumerate bacteria; however, a loss of viability does not necessarily correlate with loss of fluorescence. Many studies that measure replication of mycobacteria inside cells do not include parallel measurements of extracellular and intracellular bacteria. The easy handling of the 96-well plates and the efficient readings with the plate reader with luminometry function admit measurements of both extracellular and intracellular growth, without the need for additional manipulation of the system (e.g. addition of extracellular antibiotics). Another shortcoming of many studies on intracellular growth of mycobacteria is that cell viability is not addressed. If the number of bacteria is determined in a lysate of adherent cells, it is very important to know how many cells were left, as loss of cells would result in loss of measurable bacteria. Thus, many studies that conclude that mycobacteria were killed by the macrophages cannot rule

out cell loss as cause of reduced numbers of CFUs. The use of luminometry for determination of bacterial numbers as in our system allows fluorescence-based measurement of cell viability in the same wells, which gives reliable data on the host-pathogen balance in the individual experiments.

### 2.2.2 Phagosomal acidification and control of mycobacterial growth

Using the described method, we have established that depending on the initial bacterial load, non-activated human macrophage cultures will experience different fates when infected with virulent *M. tuberculosis*. Whereas a higher bacterial load results in effective bacterial replication and rapid cell death (further discussed below); a lower bacterial load results in a state where the macrophage controls the pathogen. The latter situation provides us with a tool to study the immune mechanisms that the macrophage employs to control the infection (“balanced infection”).

By assessing the macrophage functions that were at play during the balanced infection, we narrowed down acidic pH of the phagosome and functional cathepsin D as essential for controlled infection, whereas the generation of reactive oxygen species did not appear to play a role in this context (Welin, Raffetseder et al. 2011). We found that the restricted intracellular growth of *M. tuberculosis* the ability of the macrophage to acidify phagosomes, but not to translocate LAMPs. This finding is supported by earlier studies, where the proton pumps (v-ATPase) but not LAMP-1, were excluded from mycobacterial phagosomes (Sturgill-Koszycki, Schlesinger et al. 1994). We were not able to delineate whether the acidic pH itself was harmful to the bacteria, but given the relative resistance of mycobacteria to low pH, it was more likely the activation of hydrolytic enzymes (such as cathepsin D) inside the acidic compartments that conveyed the killing activity.

### 2.2.3 Killing of *M. tuberculosis* or just growth restriction

The reduction of the net growth of the bacteria observed with the balanced infection could be explained by either absence of growth or by a steady-state between growth and killing. Importantly, we have never been able to see a reduction in bacterial numbers using the human macrophages, which would point to a net killing of bacteria. Other groups have reported killing of mycobacteria by macrophages (Thoma-Uszynski, Stenger et al. 2001; Fortune, Solache et al. 2004), however, many reports interpret reduced growth of mycobacteria as killing. Ehrt and colleagues showed that even in IFN- $\gamma$ -activated mouse macrophages, virulent *M. tuberculosis* were not killed but rather subject to growth restriction (Vandal, Pierini et al. 2008). Only deletion of a specific membrane protein of *M. tuberculosis*, Rv3671c, resulted in loss of bacterial viability. The authors further showed that this virulence factor is crucial for the maintenance of intrabacterial pH, as assessed by a pH sensitive variant of GFP. Together, these studies suggest that replication and induction of host cell death is only one strategy employed by *M. tuberculosis* when it enters the cell. The other strategy, which occurs if the macrophage is able to mount a significant level of phagolysosomal activity, is to switch the phenotype to a slower growth rate associated with enhanced ability to resist the harsh environment inside the host cell, allowing it to persist for extended time. Thus, the inhibition of phagosomal maturation may be rather a means to buy time to induce the optimal phenotype. Ageing of the

macrophage eventually unleashes the growth, but as long as immunosurveillance is strong, uptake by neighbouring cells will not result in significant net growth. This hypothesis is summarized in figure 2.

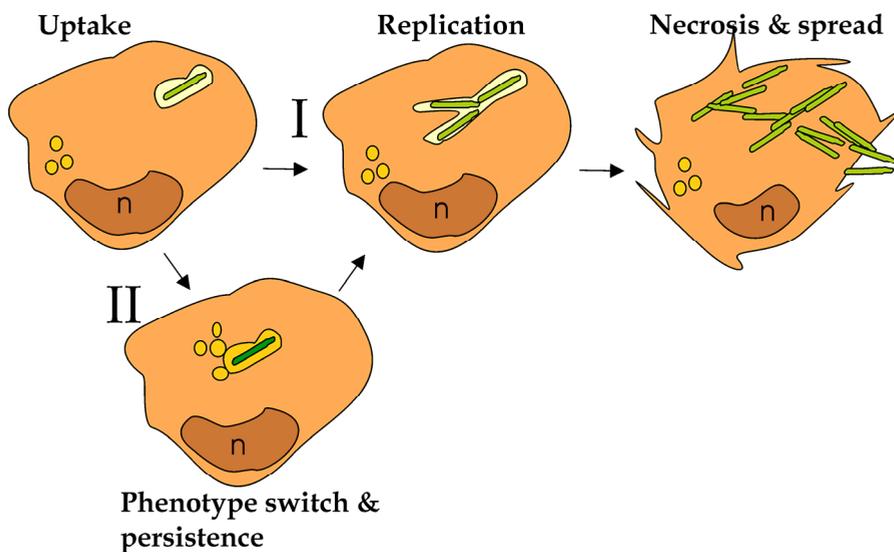


Fig. 2. Schematic view of two strategies that *M. tuberculosis* employs during infection of the macrophage. The first one (I) is to replicate after successfully inhibiting phagosomal maturation. The second (II), which can be used if the macrophage manages to acidify the phagosome, is to switch to a phenotype that tolerates the stresses posed by the macrophage. Ageing of the macrophage eventually allows the bacterium to resume growth.

#### 2.2.4 Host-protective apoptosis vs. detrimental necrosis

In 2007, Peter Peters and colleagues demonstrated that *M. tuberculosis* can, like the close relative *M. marinum* (Stamm, Morisaki et al. 2003), escape from the phagosome and replicate inside the macrophage cytoplasm (van der Wel, Hava et al. 2007). The issue has been a matter of contradiction during the past few years and many studies have emphasized the absence of bacilli void of phagosomal membranes in many experimental settings and cells obtained from infected humans. Given the facts that host cell death follows the mycobacterial escape from the phagosomes and that remnants of dead cells are rapidly removed in tissues by residual macrophages, it is logical to assume that any such event is difficult to capture in alveolar macrophages and tissues. Regardless, the protocols used for preparation of and the origin of macrophages (mouse *vs.* man) may be crucial determinants of the outcome for *M. tuberculosis* during experimental infection, and this may cause different results in the hands of different research groups. The mechanisms by which the phagosomal escape occurs are currently being investigated. A favoured candidate is ESAT-6, which plays an important role for the phagosomal escape in *M. marinum*, probably through its membrane-damaging properties (Smith, Manoranjan et al. 2008). In any case, phagosomal escape is likely to precede host cell death. There is conflicting evidence for the

mode of cell death induced by *M. tuberculosis*. In fact, there is convincing evidence that *M. tuberculosis* actively prevents apoptosis by the action of the protein nuoG (Velmurugan, Chen et al. 2007). The mechanism, by which this occurs, remains elusive, but a microbial rationale for apoptosis prevention is that this mode of cell death seems to be lethal to intracellular pathogens (Keane, Remold et al. 2000). In contrast to apoptotic cell death, which proceeds in a programmed, quiescent manner to avoid excessive inflammation, necrosis is an uncontrolled loss of cell integrity caused by different kinds of stresses such as mechanical forces, heat or infections. Typically, cellular contents, such as ATP, High Mobility Group Box (HMGB)-1 protein and DNA are released in the extracellular matrix. These endogenous danger signals attract the attention of innate immunity by interacting with purinergic and pattern recognition receptors, causing release of inflammatory cytokines and chemokines. Active tuberculosis lesions are associated with excessive inflammation and necrosis. It is a matter of debate whether inflammation is good or bad for *M. tuberculosis*. An illustrative example comes from the observations with IL-1 $\beta$ . This pro-inflammatory cytokine is crucial for the control of mycobacterial infection in mice as deficiencies in the production of the cytokine or its receptor causes more severe symptoms (Sugawara, Yamada et al. 2001; Mayer-Barber, Barber et al. 2010). On the other hand, *M. tuberculosis* has been shown to activate the NLRP3 inflammasome, which is a protein complex responsible for IL-1 $\beta$  production in macrophages (Petrilli, Dostert et al. 2007). Mycobacteria-induced production of IL-1 $\beta$  causes destructive inflammation in the mouse, the conclusion being that IL-1 $\beta$  is deleterious for the host (Carlsson, Kim et al.). Thus, IL-1 $\beta$  can act like a double-edged sword, in analogy to the pro-inflammatory cytokine TNF- $\alpha$ , which is required for TB control, but is also a component of accelerated inflammation during disease progression (Mootoo, Stylianou et al. 2009).

The recently described modes of cell death termed pyroptosis and pyronecrosis due to the association with the pyrogenic (fever-causing) cytokine IL-1 $\beta$ , has been described to occur during *Salmonella* and *Neisseria* infection, respectively (Fink and Cookson 2007; Duncan, Gao et al. 2009). As we found that a higher bacterial load resulted in cell death and a massive release of IL-1 $\beta$ , we sought to determine whether either of these novel modes of cell death was at play. Thorough analysis of the pattern of cell permeability, nuclear fragmentation and mitochondrial damage pointed to a more disorganized form of cell death of human macrophages infected with virulent *M. tuberculosis*. Any attempt to inhibit apoptosis (Caspase-dependent), pyroptosis (Caspase-1-dependent) and pyronecrosis (Cathepsin-B-dependent) failed, suggesting that the fate of the cells was necrosis. We turned to investigate the microbial factors that were causing necrosis, selecting ESAT-6 as a possible candidate. Deletion of this well-known but poorly understood mycobacterial virulence factor showed a clear relationship with the necrosis-causing ability of *M. tuberculosis*. Another report arrived at the same conclusion that macrophages are killed by *M. tuberculosis* in a necrosis-like fashion. However, the authors correlated the ability of the bacterium to induce cell death to functional PhoP two-component signalling instead of an intact RD-1 locus, in which ESAT-6 and the ESX-1 machinery is localized. The reason for this discrepancy remains elusive, but again, the different cell types used (human monocyte-derived macrophages in our study *vs.* bone-marrow-derived mouse macrophages in the other study) are possible reasons for the differing results.

### **3. Beyond checkpoint two: Interplay between innate and adaptive immunity and *M. tuberculosis***

As described above, *M. tuberculosis* is able to survive within the host even during a strong inflammatory response. The ability of the bacterium to enter and persist in the phagosomal system (Clemens and Horwitz 1995) is essential for establishment of infection, but also to avoid and/or manipulate protective host immune responses (Flynn and Chan 2003). Elucidation of the immune mechanisms that control the initial infection and prevent reactivation of latent TB is ongoing. Certainly genetic and environmental variations exist within the human population that may significantly affect an individual's susceptibility to develop active TB disease. However, pathogenic mycobacteria and their virulence factors can also modulate specific host immune responses, delay the onset of crucial anti-TB immunity and evade antimicrobial effector functions. Pathogenic mycobacteria are well adapted to the human host and have a range of complementary evasion mechanisms that contribute to the ability to avoid elimination by the immune system and establish a persistent infection (Tufariello, Chan et al. 2003; Gupta, Sharma et al. 2010). A close interplay between both innate (Jordao and Vieira 2011) and adaptive (Urdahl, Shafiani et al. 2011) antimicrobial effector pathways are required to control the progression of *M. tuberculosis* infection. Since the induction of the adaptive immune response including clonal expansion of antigen-specific T cells takes time, the innate immune system provides a first line of defence upon infection. In this section, we describe and discuss different antimicrobial effector pathways that are vital to maintain a balance between the mycobacteria and its host.

#### **3.1 Induction and regulation of innate immune responses in *M. tuberculosis* infection**

While macrophages are the primary host cell to be infected with *M. tuberculosis*, providing a shelter for growing bacteria, macrophages are also key effector cells with the ability to eliminate intracellular bacteria. Another innate immune cell with important antigen presenting and T cell activating functions is the dendritic cell (DC). Both macrophages and DCs are major players in the induction of proinflammatory responses in the early stages of TB infection and later on bridge innate immunity to the adaptive immunity.

##### **3.1.1 Nitric oxide**

In the initial phase of *M. tuberculosis* infection, infected host macrophages can be activated to become bactericidal by producing compounds such as oxygen and nitrogen radicals, primarily NO, which is important in order to restrict intracellular growth of bacteria. NO has been shown to be critical to control *M. tuberculosis* infection in mice (Chan, Xing et al. 1992; MacMicking, North et al. 1997; Scanga, Mohan et al. 2001; Reece, Loddenkemper et al. 2011) and it was recently shown that NO-mediated apoptosis of *M. tuberculosis*-infected murine macrophages was required for efficient mycobacterial killing (Herbst, Schaible et al. 2011). Moreover, inhibition of phagolysosomal maturation mediated by the *M. tuberculosis*-specific lipid trehalose dimycolate (TDM) is fully abrogated in IFN- $\gamma$ /LPS-activated murine macrophages and involves NO synthase (NOS2) and reactive nitrogen intermediates (RNI), which suggests that macrophage-specific NO can promote inactivation of a mycobacterial molecule with an essential virulence function (Axelrod, Oschkinat et al. 2008). These results also support the assumption that NO-mediated mycobacterial clearance is dependent on acidification of *M. tuberculosis*-containing phagosomes (MacMicking, Taylor et al. 2003).

Despite substantial evidence of a protective role of NO in the mouse, the importance of NO-mediated elimination of *M. tuberculosis* in humans is controversial. Our group as well as others have used immunohistochemistry or mRNA analysis to show that NO expression can be detected in human *M. tuberculosis*-infected macrophages *in vivo* (Nicholson, Bonacini-Almeida Mda et al. 1996; Nathan 2002; Schon, Elmberger et al. 2004; Andersson, Samarina et al. 2007). There is also a synergistic effect of vitamin D and IFN- $\gamma$  treatment to enhance *M. tuberculosis*-induced NO synthesis and bacterial killing in human monocyte-derived macrophages *in vitro* (Lee, Yang et al. 2009). Inhibition of NO-production *in vitro* using compounds such as L-NMMA or NMA promote intracellular *M. tuberculosis* growth, which suggests that NO indeed has a significant function in the human defence against *M. tuberculosis* (Jagannath, Actor et al. 1998). However, some strains of *M. tuberculosis* have developed strategies to neutralize the actions of NO and RNI by expressing a detoxifying protein encoded by the AhpC gene (Chen, Xie et al. 1998; Master, Springer et al. 2002). Interestingly, apart from its bactericidal activity, NO may possess regulatory effects on T cell activation leading to impaired effector functions (Bingisser, Tilbrook et al. 1998; Angulo, de las Heras et al. 2000; Hoffman, Mahidhara et al. 2002). As local production of oxidative stress and NO may severely impair the immune system, these effectors can mediate both beneficial and detrimental effects during mycobacterial infection.

Whereas NOS2 gene-disrupted mice have been used to show that NO and RNI are required to control murine TB (Scanga, Mohan et al. 2001; Reece, Loddenkemper et al. 2011), most methods to evaluate the relevance of NO in humans are indirect and dependent on detection of different NO synthases or specific NO-metabolites such as nitrate. Thus, reliable methods for direct assessment of NO are missing. The detection of NO radicals in tissues is particularly difficult due to the short lifetime and relatively low concentration of these compounds. Hence, better techniques are required to be able to test if the expression of NO synthases corresponds to the induction of an active enzyme. Simultaneous assessment of the NO synthase and metabolites can be used to overcome part of this problem. Furthermore, an important difference between mouse and human macrophages is their ability to induce either NO or the NO synthase *in vitro*. It is widely accepted that LPS and IFN- $\gamma$  can be used to activate robust NO production in murine macrophages, while very few publications show a similar induction of NO in human macrophages *in vitro*. The failure to induce NO in human macrophages under the same conditions that works for mouse macrophages (Arias, Zabaleta et al. 1997), support the hypothesis that NO possibly plays a more profound role in murine TB compared to human TB. It will be a future challenge to develop better model systems to continue to study and evaluate the bactericidal function of NO in human TB and also in relation to the potential suppressive effects on T cell responses at the site of TB infection. In this regard, we are currently establishing TB infection in a lung tissue model that we use to mimic human TB infection in the context of a more relevant physiological environment. The model consists of 3-dimensional organotypic cultures of stromal cells as well as primary human macrophages infected with virulent *M. tuberculosis*, which provides us with an experimental tool that can be used for mechanistic studies on human TB.

### 3.1.2 Antimicrobial peptides

Innate immune effector mechanisms in TB also include the induction and action of antimicrobial peptides (AMPs) such as human cathelicidin, LL-37 (Liu, Stenger et al. 2007;

Martineau, Newton et al. 2007). These are small cationic molecules, with the potential to kill microbes at mucosal surfaces. In addition to its antimicrobial properties, LL-37 is pro-inflammatory and could stimulate migration of various cell types (Coffelt, Marini et al. 2009) and also affect the inflammatory functions of neutrophils. Importantly, AMPs may be crucial to prevent initial uptake of mycobacteria in the respiratory tract. It has also been shown that LL-37 confers protection against *M. tuberculosis* infection by the induction of autophagy in human monocytes (Yuk, Shin et al. 2009). Autophagy is an important physiological process that involves degradation of intracellular components after fusion of autophagosomes and lytic lysosomes. Among other functions, autophagy is an important defence mechanism to inhibit intracellular survival of mycobacteria since it can overcome the phagosomal maturation block induced by *M. tuberculosis* (Gutierrez, Master et al. 2004). Although activated by different signalling pathways, the induction of both LL-37 and autophagy in human macrophages is dependent on the presence of active vitamin D. It is well known that individuals with vitamin D deficiencies have an increased susceptibility to TB, which may be associated with reduced production of LL-37 as well as reduced autophagy (Liu, Stenger et al. 2006; Liu, Stenger et al. 2007). Our unpublished observations suggest that vitamin D deficiency in a cohort of Russian TB patients with chronic pulmonary TB correlates with reduced levels of LL-37 in pulmonary TB lesions. More studies are needed to investigate the regulation of LL-37 expression *in vivo* and *in vitro* and whether mycobacteria have developed strategies to circumvent the function of this antimicrobial peptide.

### 3.1.3 Toll-like receptors

The capacity of the innate immune system to recognize foreign antigens is restricted to a set of conserved molecular patterns called pathogen-associated molecular patterns (PAMPs). The receptors for PAMPs are called pattern-recognition receptors (PRR), to which the Toll-like receptors (TLRs) belong. TLRs are PRRs that specifically recognize certain microbial antigens, including mycobacterial proteins and lipids. Antigen presenting cells (APCs), such as macrophages and DCs, express several different TLRs, which control the activation and induction of antimicrobial functions in the cells. Furthermore, TLR-stimulated APCs will interact with *M. tuberculosis*-specific T cells and induce clonal differentiation and expansion of these T cells. During mycobacterial infection, recognition by TLR 1, 2, 4, 8 and 9 are important for maturation of APCs including up-regulation of antigen-presenting molecules, production of inflammatory cytokines and chemokines and also the induction of antimicrobial effector functions (Means, Wang et al. 1999). TLRs mainly recognize lipid-rich mycobacterial molecules such as LAM (van Crevel, Ottenhoff et al. 2002) and TDM (Bowdish, Sakamoto et al. 2009). TLR2/1 activation, which triggers LL-37 production in a Vitamin D-dependent manner, has been shown to enhance the killing of *M. tuberculosis* bacilli in human macrophages (Liu, Stenger et al. 2006). In addition, mycobacterial cell wall products can regulate CD1 antigen presentation through TLR2 signalling (Roura-Mir, Wang et al. 2005). On the other hand, pathogenic mycobacteria have developed mechanisms to manipulate TLR-induced signalling pathways and may thus suppress important antimycobacterial immune responses (van Crevel, Ottenhoff et al. 2002; Bowdish, Sakamoto et al. 2009). In this regard, *M. tuberculosis* ManLAM can bind to the C-type lectin DC-SIGN on the surface of DCs and actively interfere with TLR-mediated DC maturation (Geijtenbeek, Van Vliet et al. 2003). Prolonged exposure to *M. tuberculosis* 19kD lipoprotein

inhibits MHCII expression as well as alternative MHC-1 antigen processing and presentation by IFN- $\gamma$  activated macrophages via TLR2, which may allow the bacteria to decrease recognition by CD4+ and CD8+ T cells and maintain chronic infection (Noss, Pai et al. 2001; Tobian, Potter et al. 2003; Pecora, Gehring et al. 2006). The ESAT-6 protein of *M. tuberculosis* can also interact with TLR2 and inhibit TLR signalling in macrophages in order to restrict innate immune responses (Pathak, Basu et al. 2007). Moreover, an elegant study recently provided strong evidence that active release of mycobacterial vesicles contain TLR2 lipoprotein agonists, which contribute to an enhanced pulmonary inflammation and progression of TB in infected mice (Prados-Rosales, Baena et al.). Cytokine analysis indicated the induction of atypical proinflammation since large amounts of immunosuppressive IL-10 was also produced by macrophages treated with mycobacterial vesicles. Altogether, these studies show that TLR interactions with *M. tuberculosis* ligands can activate antimicrobial and antigen presenting functions of macrophages and DCs but can also be exploited by the bacteria to escape the host immune response.

### **3.2 Induction and regulation of adaptive immune response in *M. tuberculosis* infection**

Induction of cell-mediated immunity and the formation of a granulomatous response are mandatory in human TB. Many immune cells are involved in this process that is guided by CD4+ helper T cells and CD8+ cytolytic T cells (CTLs). These T cell subsets possess complementary and interacting functions, which are therefore hot targets for mycobacterial manipulation, as improper activation of this arm of immunity facilitates bacterial invasion of the host.

#### **3.2.1 Th1 effector cells**

CD4+ T cells are so-called helper T cells (Th cells) with a specialized function in cytokine production in order to activate CTLs as well as B cells and other professional APCs. However, CD4+ T cells are also important for the activation of immune cells through specific receptor-ligand interactions. Differently polarized subsets of CD4+ T cells including Th1, Th2 and Th17 cells secrete distinct patterns of cytokines, which will control the fate of the CD4+ mediated immune response in TB. Therefore CD4+ T cells play a central role in the induction and maintenance of immune regulation during TB infection. Th1 effector cells mainly produce IL-12, IFN- $\gamma$ , TNF- $\alpha$  and IL-2 and induce cell-mediated immunity which is fundamental for immune protection in TB infection. Accordingly, patients with mutations in the IL-12 or IFN- $\gamma$  receptor genes have a strongly enhanced susceptibility to develop active TB (Newport, Huxley et al. 1996; de Jong, Altare et al. 1998). Particularly, IFN- $\gamma$  activation renders the macrophages capable of killing intracellular mycobacteria by overcoming the block in phagosomal maturation and also stimulates production of microbicidal effectors including NO. In addition, patients with latent TB who are treated with anti-TNF- $\alpha$  antibodies rapidly develop active TB (Keane, Gershon et al. 2001; Bruns, Meinken et al. 2009). A newly discovered subset called Th17 cells produce IL-17, IL-22 and IL-23, which has been shown to regulate the production of antimicrobial peptides (Liang, Tan et al. 2006) as well as the activation and recruitment of IFN- $\gamma$  expressing T cells at mucosal sites including the lung (Khader, Bell et al. 2007). Th1 and Th17 effector cells cooperate to induce protective immunity in TB.

### 3.2.2 Th2 effector cells

In contrast, Th2 effector cells producing IL-4, IL-5, IL-9 and IL-13, regulate the differentiation of antibody secreting plasma cells that has been shown to enhance intracellular persistence of *M. tuberculosis* (Potian, Rafi et al.). Thus, a dominant Th2 response can undermine Th1-mediated immunity and drive inappropriate alternative activation of macrophages (Rook 2007). The traditional view is that IFN- $\gamma$  activated or classically activated (M1) *M. tuberculosis*-infected macrophages, produce high amounts of proinflammatory cytokines and NO and become highly bactericidal. However, recent evidence suggests that the initial M1 activation is followed by alternative activation (M2) of *M. tuberculosis*-infected macrophages in the lung, which would support a switch in macrophage polarization upon progression of TB disease (Redente, Higgins et al. 2011). IL-4 and IL-13 could promote alternative macrophage activation characterized by collagen deposition and formation of fibrosis in the inflamed tissue, which are typical traits of advanced TB disease. IL-4 and IL-13 have also been shown to block autophagy-mediated killing of mycobacteria in both murine and human macrophages (Harris, De Haro et al. 2007). Thus, *M. tuberculosis* virulence factors may interfere with M1 polarization and instead promote polarization of alternatively activated M2 macrophages or deactivated macrophages that are immunosuppressive and poorly microbicidal (Benoit, Desnues et al. 2008).

### 3.2.3 Regulatory T cells

CD4<sup>+</sup> T cells may also be induced to acquire regulatory functions and to secrete immunosuppressive cytokines like TGF- $\beta$  and IL-10 rather than the classical Th1 or Th2 cytokines. There is plenty of evidence that *M. tuberculosis* can induce specific regulatory T (Treg) cells that potentially restrict protective immune responses during human TB. Increased levels of FoxP3<sup>+</sup> Treg cells have been discovered in blood (Ribeiro-Rodrigues, Resende Co et al. 2006), lung (Guyot-Revol, Innes et al. 2006), lymph nodes (Rahman, Gudetta et al. 2009) and pleura (Chen, Zhou et al. 2007) of patients with active progressive TB. Induction of FoxP3<sup>+</sup> Treg cells is also evident in murine TB (Shafiani, Tucker-Heard et al.). *In vitro* experiments demonstrate that ManLAM induces expansion of human Treg cells through mechanisms that depend on and prostaglandin E2 (PGE2) (Garg, Barnes et al. 2008) or PD1 (Periasamy, Dhiman et al.). These Treg cells produced significant amounts of TGF- $\beta$  and IL-10 and inhibited IFN- $\gamma$  expression by autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Several studies provide evidence that Treg cells could suppress antigen-specific IFN- $\gamma$  production by human T cells, by which mechanism they would limit immunopathology but also down-regulate cellular immunity in TB (Hougardy, Place et al. 2007; Li, Lao et al. 2007; Li and Wu 2008). Thus, local CD4<sup>+</sup> T cell responses could be inhibited, which may result in a failure to recruit CD8<sup>+</sup> effector T cells to the granulomatous lesions in TB. Premature induction of an immunosuppressive Treg response may blunt important CTL activity and instead enhance pathological alterations in TB infected tissue.

### 3.2.4 Polyfunctional T cells

A popular concept that has been intensively studied is the induction of polyfunctional T cells that has been demonstrated to correlate with immune protection in several chronic

intracellular infections including TB (Darrah, Patel et al. 2007; Forbes, Sander et al. 2008). Polyfunctional T cells are characterized by the simultaneous production of effector molecules such as several Th1 cytokine co-expressed with inflammatory chemokines and markers for CTL degranulation. Even if several studies show that polyfunctional T cells have an impact on TB immunity, we still lack specific immune signatures or correlates of immune protection that could be used as specific biomarkers in novel vaccine- and drug development. Expansion and activation of Treg cells and Th2 (IL-4, IL-13) cells in chronic TB infection may prevent important polyfunctional Th1 responses and the development of fully functional CTLs. Our research group applies multicolor flowcytometry, multiplex mRNA and luminex analysis to characterize polyfunctionality and unravel the complex network and functional relationship between cytokine and chemokine profiles in lung vs systemic circulation of patients with active TB compared to uninfected controls. We also plan to use a systems biology approach to address immunological defects induced by mycobacteria and associated with progression of clinical disease.

### 3.2.5 Cytolytic T cells

CD4<sup>+</sup> Th1 cells activate CD8<sup>+</sup> CTLs which act in concert with natural killer (NK) cells as professional killers of *M. tuberculosis*-infected target cells. Activation mediated by CD4<sup>+</sup> T cells or provision of other co-stimulatory signals provided by professional APCs are necessary to trigger the differentiation and maturation of CTLs. CTL-induced destruction of *M. tuberculosis*-infected cells is characterized by lysis of the cell membrane as well as disruption of the cell nucleus and characteristic fragmentation of target cell DNA. CTL-mediated lysis is primarily executed by one of two different mechanisms, involving either perforin- or Fas/Fas-ligand(L)-based killing. However, whereas perforin-mediated killing seems to be crucial for the elimination of infected target cells (Canaday, Wilkinson et al. 2001), Fas-mediated killing seems to play a major role in lymphocyte homeostasis (Kremer, Estaquier et al. 2000; Watson, Hill et al. 2000). Interestingly, *M. tuberculosis* can induce an increased expression of FasL on infected macrophages which may induce apoptosis among Fas expressing cells like CTLs and Th1 cells (Mustafa, Phyu et al. 1999). Death receptor ligand-mediated apoptosis could also lyse *M. tuberculosis*-infected cells in the absence of bacterial killing and thus contribute to activation-induced cell death of CTLs. Here, it has been suggested that disease progression is associated with Fas/FasL mediated apoptosis of *M. tuberculosis*-reactive T cells (Li, Bassiri et al. 1998; Rios-Barrera, Campos-Pena et al. 2006).

Hence, only CTLs using the perforin-dependent, granule-mediated pathway of target cell killing are effective in decreasing the viability of intracellular *M. tuberculosis* and properly control the infection (Stenger, Mazzaccaro et al. 1997; Lewinsohn, Bement et al. 1998; Silva and Lowrie 2000; Samten, Wizel et al. 2003). Perforin is a pore-forming protein that is stored in the cytolytic cell within cytoplasmic granules together with other lytic and antimicrobial molecules called granzymes and granulysin (Stenger, Hanson et al. 1998; Okada, Li et al. 2003). Similar to the actions of human cathelicidin, the antimicrobial peptide granulysin can interact with the negative cell surface of the bacteria and induce osmotic lysis (Ernst, Thoma-Uszynski et al. 2000). Importantly, mice lack a known homologue of granulysin, which again indicates that mouse TB is regulated differently from human TB. The capacity

of granzysin to kill *M. tuberculosis* bacilli located inside infected cells is dependent on the pore-forming properties of perforin. Upon binding to a specific target cell, the CTL reorients these lytic granules to the site of cell-cell contact and releases its contents in the intercellular space between itself and the infected target cell. Perforin will integrate into the target cell membrane in a  $\text{Ca}^{2+}$ -dependent manner and create polyperforin pores, which result in permeabilization and osmotic failure of the target cell. It is also believed that perforin forms pores in cellular membranes to facilitate entry and endosome-mediated transportation of granzymes and/or granzysin to the intracellular compartments via a newly identified membrane-repair mechanism (Keefe, Shi et al. 2005). Interestingly, we have found that short peptides of granzysin are particularly effective in killing of slow-growing multidrug resistant TB (MDR-TB) strains with a reduced fitness phenotype (Toro, Hoffner et al. 2006). These results suggest that a cost of resistance, measured as reduced growth among MDR-TB strains, could be associated with increased susceptibility to natural immune defence mechanisms, such as antimicrobial peptides of granzysin. However, a robust cell wall as well as the membrane of host cells still provides physical shelter for the bacteria that may spare them from being killed.

Individuals with active, progressive TB disease most likely have an inadequate up-regulation of *M. tuberculosis*-specific CD8+ CTLs and cytolytic effector molecules, resulting in insufficient killing of infected cells and bacilli at local sites of TB-infection. Importantly, impaired CTL function has been associated with the progression of clinical TB disease, particularly in anti-TNF treated patients with autoimmune diseases, who demonstrate selective depletion of perforin and granzysin expressing CD8+ CTLs (Bruns, Meinken et al. 2009). We have previously discovered that an impaired expression of both perforin and granzysin at the site of TB infection in pulmonary (Andersson, Samarina et al. 2007) and lymph node (Rahman, Gudetta et al. 2009) lesions from TB patients correlated with the progression of active TB disease. The cause and mechanism behind this impaired expression of antimicrobial effector molecules is unknown, but an important subject for future studies on human TB.

### 3.2.6 Mycobacterial manipulation of T cell effector functions

Bacterial factors are critical determinants in the decision between Th1 or Th2 polarization, and although *M. tuberculosis* does induce a strong inflammatory host response, Th1 responses may be delayed and insufficient to eradicate the infection. Mycobacteria may skew immune activation toward an improper regulatory or Th2 profile and simultaneously inhibit a Th1/Th17 response and a subsequent CTL response. Recent results demonstrate that the ESAT-6 protein from *M. tuberculosis* directly inhibits human T cell proliferation and  $\text{IFN-}\gamma$  production in a p38 MAPK-dependent manner (Peng, Wang et al. 2011). It has also been shown that apart from  $\text{IFN-}\gamma$ , ESAT-6 can inhibit IL-17 and  $\text{TNF-}\alpha$  production as well as expression of early activation markers on human T cells (Wang, Barnes et al. 2009). Similarly, the Th1 cell surface molecule Tim3 can stimulate antimicrobial immunity in *M. tuberculosis*-infected macrophages but simultaneously inhibit the expansion of Th1 cells to prevent excess tissue inflammation (Jayaraman, Sada-Ovalle et al. 2011). It has also been shown that PD1 and its ligands, can inhibit CTL function in human TB (Jurado, Alvarez et al. 2008), suggesting that PD1 interferes with T cell effector functions against *M. tuberculosis*.

Thus, the pathogen may have evolved mechanisms to modulate the expression of negative regulators including Tim-3 and PD1 to favour bacterial persistence. On the other hand, PD1-deficient mice are highly susceptible to progressive TB infection and show abnormal immune activation including a dramatic increase in proinflammatory cytokines but low numbers of lymphocytes infiltrating the lung (Lazar-Molnar, Chen et al. 2011). Importantly, a shift of the immune response towards excessive inflammation characterized by extensive production of proinflammatory cytokines such as IL-1, TNF- $\alpha$ , IL-6 and IL-17 may promote pathology and result in severe tissue damage instead of immune control (Lazar-Molnar, Chen et al. 2011; Torrado and Cooper 2011). Hence, a balance between Th1 and Th17 responses needs to be achieved to control bacterial growth and limit immunopathology in the chronic phase of TB infection.

### 3.3 Passing checkpoint three – loss of immune control and progression to TB

Intracellular replication of *M. tuberculosis* is followed by the release of inflammatory mediators and the recruitment of additional immune cells including monocytes, macrophages, neutrophils and lymphocyte to the site of infection. The resulting clusters of immune cells are known as granuloma, which are immunopathological hallmarks of tuberculosis. The formation and organization of the granulomatous inflammatory response is believed to contain the infection and to generate an immunological balance between the pathogen and the host. Thus, the function of the granuloma is generally described to be host-protective in its nature; however, recent research has also provided evidence that *M. tuberculosis* bacteria may use the granuloma as a vehicle to seed the infection in the local environment of the lung. It is noteworthy that the major route of spread of the infection to other individuals is through the rupture of fully matured caseating granuloma, which results in drainage of viable bacteria into the airways. This implies that *M. tuberculosis* would not be a successful pathogen if it would not be able to cause necrotic granulomas.

#### 3.3.1 The granuloma –good or bad for the *M. tuberculosis*-infected host?

Formation of *M.tuberculosis* granulomas typically requires the initiation of a delayed-type hypersensitivity reaction and chronic inflammation. Granulomas are composed of clusters of infected macrophages surrounded by sheets of lymphocytes and fibroblasts that are recruited to the site of infection with the aim to contain the infection (Flynn, Chan et al. 2011). The specific host and bacterial factors that orchestrate granuloma development are still poorly defined. Whereas it is well established that the granuloma is formed as part of the protective immune response to *M. tuberculosis*, there is evidence that mycobacteria can also utilize early granuloma formation to seed the infection to uninfected macrophages that are recruited to the site of granuloma (Davis and Ramakrishnan 2009). This ability of pathogenic mycobacteria to exploit granuloma function is dependent on the ESX-1 secretion system that promotes cell death of infected macrophages, which further enhances uptake and expansion of bacteria in the local environment. Granulomas are highly dynamic structures with multiple appearances in infected organs during active TB disease, including solid non-necrotizing and caseous necrotic granulomas (Kaplan, Post et al. 2003; Rahman, Gudetta et al. 2009). Rupture of mature granulomas drains the caseous necrotic fluid, which is full of

viable bacteria, into the airways of patients with TB, followed by expectoration in the form of infectious aerosols. This is the only known mechanism for *M. tuberculosis* to spread to other individuals. Therefore, although facing the risk of being restricted or killed by host immunity, *M. tuberculosis* is dependent on mature granulomas and host immunity. Interestingly, a recent analysis showed that many T cell antigens are actually conserved in virulent *M. tuberculosis* strains, implying that the bacterium actually wants to be presented to the host immune system (Comas, Chakravartti et al. 2011). Taken together, at least in the initial and late stages of *M. tuberculosis* infection, the bacterium takes advantage of the host immune response, and host immunity is therefore both protective and detrimental for the host during mycobacterial infection, which adds a level of complexity to the understanding of TB.

### 3.3.2 Manipulation of T cell functions in the granuloma

Our research group has built a technological platform based on quantitative cell- and tissue analysis of clinical materials from TB patients and controls. We have obtained patient samples from the site of TB infection and used immunohistology and *in situ* computerized image analysis to assess expression and distribution of CD4<sup>+</sup> T cells, CD8<sup>+</sup> CTLs and antimicrobial effector molecules in *M. tuberculosis*-infected tissue. Importantly, immune cells are recruited and collected at sites where the bacilli reside, *i.e.* in infected macrophages in the lung, lymph nodes, pleura or other organs (Barnes, Mistry et al. 1989; Schwander, Torres et al. 1998; Wilkinson, Wilkinson et al. 2005; Jafari, Ernst et al. 2008; Nemeth, Winkler et al. 2009). Hence, organ-specific cell-cell interactions and the functional expression of different proteins can be studied in a physiological environment. Protein expression can be quantified at the single-cell level (Bjork, Andersson et al. 1994) using microscopy and a highly sensitive digital image analysis system with the ability to detect and separate many million different colours. In our studies, we have found that the abundance of CD8<sup>+</sup> CTLs expressing the important anti-TB effectors perforin and granulysin, are very low in the granulomatous lesions (Andersson, Samarina et al. 2007; Rahman, Gudetta et al. 2009), indicating bacterial manipulation of host immune functions (Fig. 3). Quantitative real-time mRNA analysis of tissue from *M.tuberculosis* infected lymph nodes revealed that IFN- $\gamma$ , TNF- $\alpha$  and IL-17 were not up-regulated while there was a significant induction of TGF- $\beta$  and IL-13. Whereas CD8<sup>+</sup> CTLs were absent from the TB lesions, NO-producing macrophages were abundant and the expression of the *M. tuberculosis*-specific antigen MPT64 was high (Fig. 3). These results suggest that CTL activation is impaired and that the few effector CTLs that are present in the tissue cannot make contact with and kill *M. tuberculosis*-infected cells, which accumulate in the granuloma. Instead, these lesions may provide a protective niche for the bacteria, especially at later stages of *M. tuberculosis* infection after induction of adaptive immunity. Whereas compartmentalization of immune responses involved few CD8<sup>+</sup> CTLs, we found that the levels of FoxP3<sup>+</sup> Treg cells and also TGF- $\beta$  were significantly elevated in the tuberculous granulomas, suggesting that active immunosuppression takes place at this site (Rahman, Gudetta et al. 2009). This methodology provides important information about the regulation of immune responses in the microenvironment of the human TB granuloma and generates a valuable contribution and complement to the knowledge gained from animal and *in vitro* experimental systems.

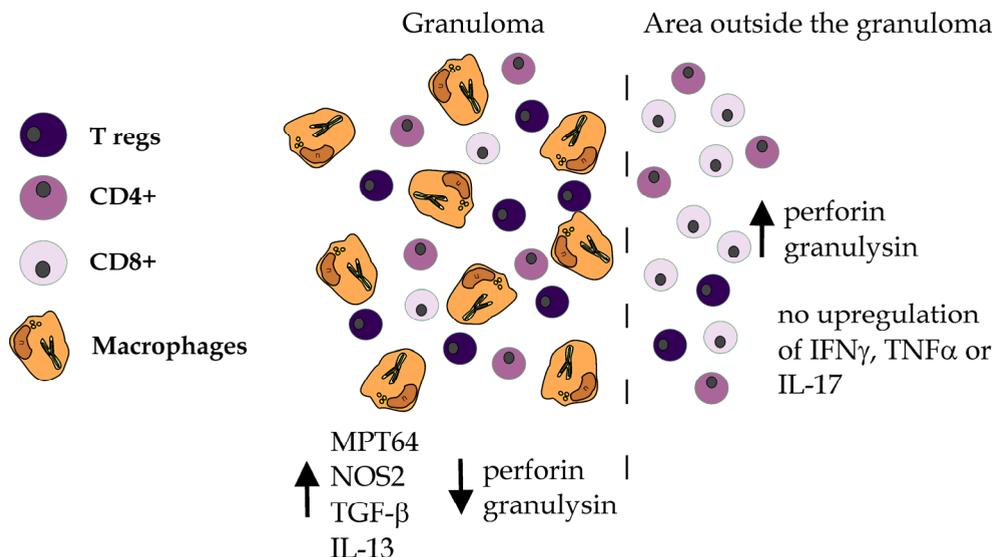


Fig. 3. Schematic illustration of the expression and distribution of *M. tuberculosis* infected macrophages, T cell subsets and cytolytic and antimicrobial effector molecules inside and outside the human granuloma. While macrophages expressing high amounts of the *M. tuberculosis*-specific antigen MPT64 as well as NO synthase are accumulated at the site of infection in the granuloma, the ratio of CD8+ CTLs and Treg cells are significantly higher outside the lesions. Expression of the important anti-TB effectors perforin and granulyisin, is low in the infected tissue, and almost absent in the *M. tuberculosis* granuloma. Impaired CTL activation correlates with a low induction of Th1 cytokines, IFN- $\gamma$ , TNF- $\alpha$  and IL-17, but an increased expression of the immunosuppressive cytokines TGF- $\beta$  and IL-13. This type of immune response will provide a protective niche for the bacteria inside the granuloma and favour bacterial persistence in the host.

#### 4. Conclusion

The complex mechanisms that are at play during the development of chronic and acute TB involve host immunity and mycobacterial manipulation of both innate and adaptive immunity. The tug-of-war between the bacteria and host immune cells has been illustrated in this chapter as a spectrum between early infection and active disease. The described checkpoints, which *M. tuberculosis* has to pass in order to cause acute infection, are represented by immune mechanisms of increasing complexity, ranging from early innate defence to the multifaceted antibacterial effects posed by adaptive immunity. If immunological control fails, the pathogen takes advantage of immune activities such as excessive release of inflammatory mediators, thus creating the necrotic tissue through which it conveys its spread to other individuals. Innovative thinking and new models that can be used to address questions on the interaction between host immunity and *M. tuberculosis* are required to move the research field forward towards new knowledge. Thus, by questioning old dogmas, many research groups are now approaching the pathogenicity mechanisms of *M. tuberculosis* from new angles, and this is already paying off as improved understanding of the disease it causes, which is a promising step towards new therapies.

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## 6. References

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