Influence of virulence-associated genes of *M. bovis* BCG on the interaction with host cells

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1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is an aerobic, obligate intracellular, non-motile, non-capsulated, non-spore forming pathogen which causes tuberculosis in humans and several animal species.

The genus *Mycobacterium* consists of more than 100 species which belong to the family of *Mycobacteriaceae.* Strains causing tuberculosis are summarised in the *Mycobacterium tuberculosis* complex (MTBC) namely *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, *M. pinnipedii* and *M. caprae.* All other species which cause pulmonary disease or lymphadenitis, skin disease, soft tissue disease or disseminating diseases are classified as Nontuberculous mycobacteria (NTM), also known as environmental or atypical mycobacteria. Although members of MTBC are distinguished by phenotypic characteristics and mammalian host ranges they all share a remarkable genetic homogeneity, with about 0.01% to 0.03% synonymous nucleotide variation [1-4] without showing significant traces of genetic exchange among them [2, 5]. It is therefore believed, that all members of the MTBC are descendants of a precursor strain that occurred 20,000 to 35,000 years ago [4, 6]. Findings dating back to early history support the suggestion that bovine and human tuberculosis evolved in a kind of co-evolution with humankind rather than a direct transmission from bovines to human beings. Using archaeological specimens from human beings, Spigelman and colleagues [7] first discovered ancient DNA from *M. tuberculosis* and other members of the MTBC by amplifying the highly conserved insertion sequence IS*6110* by polymerase chain reaction. This method helped to detect tuberculosis DNA [8] from bones of a long extinct bison from North America (17,000 years before present) whereas the first domestication of cattle occurred around 7000 years BC. Evidence of *M. tuberculosis* was also found in up to 1400 years old human bones from all over the world (Europe, Turkey and Borneo) [9].

Medieval Europe suffered heavily under epidemic plagues like pestilence and cholera, whereas tuberculosis played only a minor role because of the sparsely populated cities. This changed in modern times, where the rapidly increasing urbanisation led to rising incidence rates. In the second half of the 19th century more than 100,000 people died of tuberculosis in the German Reich.

In 1882 Robert Koch discovered *M. tuberculosis* as the causative agent of tuberculosis. For this discovery he was awarded the 1905 Nobel Prize in Physiology or Medicine.

At this time members of the MTBC complex were responsible for about 6% of deaths in Europe. The introduction of milk pasteurisation and the development of the vaccine strain Bacille Calmette-Guérin (BCG) helped reducing the incidence rates. In its time tuberculosis had a strong influence on the socioeconomic change. In the 18th and 19th century tuberculosis was known as “the romantic affliction”, at the end of the 19th and early 20th century as “ailment of the proletariat”, in National Socialism time as “antisocial ailment” and in the 1970s as “defeated ailment”. Nevertheless tuberculosis is on the march.

In 1993, due to the worldwide high death rate and morbidity, tuberculosis was declared as a global emergency by World Health Organisation (WHO). Despite all the efforts the incident cases of tuberculosis worldwide rose to 9.4 million in 2009 – more than ever before in history [10]. Nearly one third of the human population is latently infected with *M. tuberculosis*. About 10% of latently infected individuals are at risk of developing an active disease during their lifetime. In the case of a co-infection with Human immunodeficiency virus (HIV) the risk increases whereas the manifestations are much more severe, characterised by frequent extrapulmonary and miliary disease, and high mortality rates [11]. About 80% of the infected individuals are living in only 22 countries; mostly in China, South Africa, Nigeria, and Indonesia (Figure 1). Due to the rapidly increasing world population it can be assumed that the incident cases of tuberculosis will rise in the same extent. Another growing problem is the co-infection with HIV. In 2010 about 12% (1.1 million cases) of the worldwide tuberculosis cases were HIV-associated [10]. Countries from the sub-Saharan Africa region and Southeast Asia are particularly affected (Figure 2). In countries with high HIV prevalence the incident cases of tuberculosis have more than tripled [12].

Today there are seven vaccine strains, all of them derivates of the BCG strain, which was originally isolated from a cow infected with tuberculosis. This *M. bovis* strain was cultivated over a period of 13 years where it lost most of its virulence but retained strong enough immunogenicity to be used as a vaccine. Since 1921 an estimated one billion children were vaccinated with this live vaccine. In young children the estimated protective efficacy rates are very high when it comes to prevention of tuberculous meningitis or miliary tuberculosis [13].

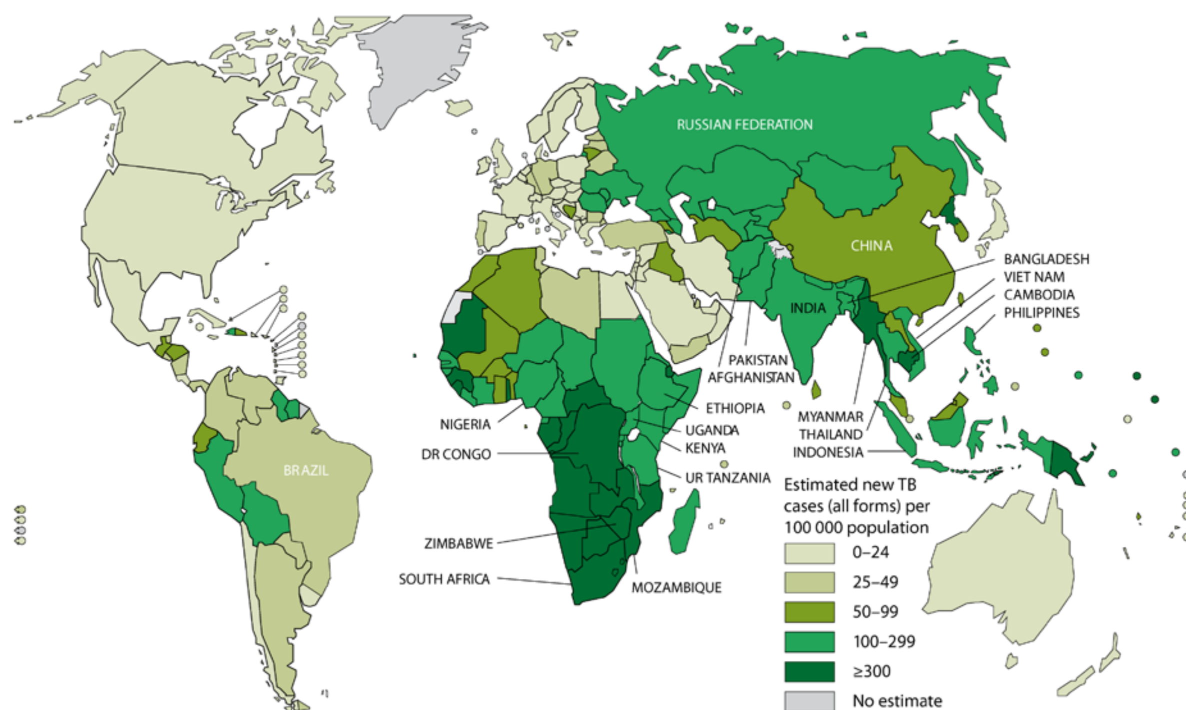


Figure 1. Estimated tuberculosis incidence rates, 2010

[Source: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_TB_incidence_2010]

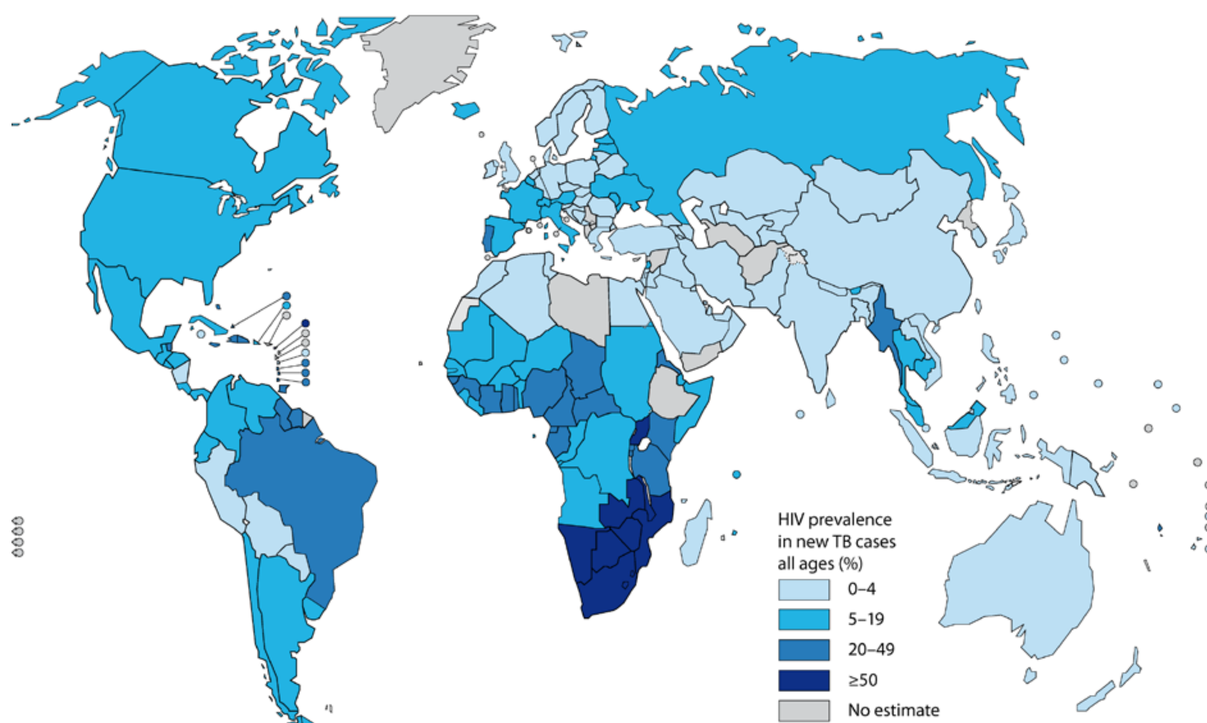


Figure 2. Estimated HIV prevalence in new tuberculosis cases, 2010

[Source: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_HIVprevalence_TBcases_2010]

A BCG vaccination grants 50% protection against tuberculosis [14]. Sometimes older children and adults develop no protective immune response from vaccination as shown in India from 1968 to 1971 [15].
Albeit tuberculosis can be cured by chemotherapy, the treatment lasts between six to nine months it is very expensive and requires the use of four different antibiotics. Especially in the world's poorest countries non-compliance of medication remains one of the major obstacles for an efficient treatment and is held responsible for the emergence of multidrug resistant (MDR) and extensively drug-resistant tuberculosis (XDR) [16]. The lack of cheap diagnostic tests, lack of effective vaccine and badly equipped health systems in developing countries make it difficult to control tuberculosis effectively. It is therefore not surprising tuberculosis remains the most dangerous bacterial pathogen worldwide despite all efforts to eradicate it.

1.1 Pathogenesis

Tuberculosis is a notifiable contagious disease which is normally transmitted by droplet infection and very rarely by smear infection or ingestion. Coughing patients, infected with *M. tuberculosis*, exhale droplets of respiratory secretions containing three to ten tubercle bacilli [17]. These bacteria remain viable over a longer period of time even though their surrounding confronts them with different stress situations like a drop in temperature, nutrient deficiency and a changed oxygen tension. After inhaling these droplets the bacilli reach the lung. Upon contact with mannose receptor [18], DC-specific C-type lectin DC-SIGN [19], and complement receptors [20] on the cell surface of alveolar macrophages and dendritic cells the bacilli are phagocytosed and trapped within phagosomes. Internalization is followed by phagolysosome fusion and maturation whereupon the bacteria come into contact with lytic enzymes, oxygenated lipids, fatty acids, and reactive oxygen and nitrogen intermediates [21].
The binding to Toll-like receptors also triggers a cascade of down-stream signalling pathways which are responsible for the release of immune modulatory cytokines (see section 1.2.1). These cytokines activate the cells and mechanisms of the non-specific immune system which is responsible for recognising and responding to pathogens in a general way without conferring a long lasting protective immunity to the host.

In the course of evolution *M. tuberculosis* developed a number of strategies which enables them to survive in phagosomes. Preventing the phagolysosome fusion [22-24] is only one strategy.

Other strategies are the triggering of anti-inflammatory responses, blocking reactive oxygen intermediates, reactive nitrogen intermediate production and reducing the acidification of the *M. tuberculosis*-containing phagosome [25]. These serious interventions on key functions of alveolar macrophages enable *M. tuberculosis* to persist and multiply in the early phagosome. Sooner or later infected macrophages disrupt and attract blood monocytes and dendritic cells to the infection site. Arriving monocytes differentiate into macrophages which again ingest mycobacteria without destroying them completely. By presenting mycobacterial antigens and expression of costimulatory signals and cytokines more and more blood-derived macrophages accumulate around the infection site without causing severe tissue damage. Despite the influx of macrophages the bacteria continue to multiply. Activated dendritic cells migrate to the draining lymph nodes where they stimulate naive T cells. Two to three weeks after the infection took place T cell immunity develops. Arriving antigen-specific T lymphocytes proliferate within the early lesions and start secreting IFN-γ. Macrophages, activated by IFN-γ, continue with their phagosomal maturation [26] and start killing intracellular bacteria [27].

In the meantime, infected macrophages surrounded by recruited mononuclear cells have formed a histological structure known as granuloma, consisting mainly of monocytes, lymphocytes and fibroblasts [28, 29]. When the early lesions calcify the bacteria are trapped inside the granulomas. With the loss of their ability to invade surrounding tissue the bacteria become dormant. Approximately 90% of infected individuals remain asymptomatic for years or decades.

Latently infected hosts have a lifetime chance of 5% to 10% that the bacteria get reactivated, causing a progressive tuberculosis. Reasons are manifold and may have something to do with changes in host cytokine/chemokine networks, genetic or environmental causes or failure to develop and maintain immune signals. Even stress or old age seems to be associated with reactivation [30]. In opposition to the primary infection the reactivation features granuloma failure, lung caviation and pulmonary disease [31-33].

1.2 The central role of alveolar macrophages

Alveolar macrophages belong to the unspecific, innate immune system. Their primary role is to detect and destroy pathogens like *M. tuberculosis* that have penetrated the alveoli deep in the lung. Identification of pathogens like *M. tuberculosis* takes place via Toll-like receptors (TLR). Eleven members of the Toll-like receptor family have been identified in the human genome. Mainly TLR2 in combination with TLR1/TLR6 or TLR4 [34, 35] seem responsibly for recognising mycobacterial components like the soluble tuberculosis factor (STF) [36] or the 19-kDa lipoprotein from *M. tuberculosis* [37].

As soon as there is direct contact between TLR on the cell surface of macrophages and mycobacterial components like lipopolysaccharide (LPS), the pathogens are first phagocytosed and then killed or at least limited in their dissemination. To this end macrophages have several effective mechanisms at their disposal. A) Following phagocytosis macrophages kill the bacteria by phagolysosome fusion whereupon the bacteria come into contact with lytic enzymes, acid and reactive oxygen and nitrogen intermediates [21]. B) The production of nitric oxide triggers apoptosis [38]. C) Secreted cytokines stimulate other cells of the innate immune system, triggering an inflammatory response and activating cells of the adaptive immune system, which confers a long lasting protective immunity, and D) by granuloma formation [39]. For this reason alveolar macrophages play a pivotal role when it comes to combating, controlling and eliminating *M. tuberculosis*. Following is a short overview of some of the induced immunologic relevant factors which are important for elimination and containment of *M. tuberculosis*.

1.2.1 Cytokines, mediators of the innate and adaptive immune system

Cytokines are messengers of the immune system and as such an important component of both the unspecific and the adaptive immune system. Immediately after contact with foreign pathogens monocytes, macrophages, granulocytes and natural killer cells induce the production of cytokines, long before cytotoxic T cells or antibodies are in place. Acting as a link between the innate and adaptive immune system, cytokines control the interaction of the immunological effector systems with the objective of promoting a powerful immune response against pathogens.

Cytokines unfold their biological activity after binding to specific receptors on the cell surface of their target cells. Here they induce a signal cascade which at the end results in a specific effect. Without these chemical mediators the innate and adaptive immune response would not be able to control or eliminate pathogens.

Cytokines are small, non-structural proteins with molecular weights of up to 40-kDa without a consistent amino sequence motif or three-dimensional structure. Due to their biological activities they can be divided by their role in infection and/or inflammation. Cytokines promoting inflammation are called pro-inflammatory cytokines (e.g. interleukin-1β, interferon-γ, tumor necrosis factor-α), whereas cytokines which block the signal cascade are called anti-inflammatory cytokines (e.g. interleukin-10). Chemokines like interleukin-8 are differrent from the above mentioned cytokines because they attract other immune cells by chemotaxis so that they accumulate around the source of chemokine production. In this manner they promote the passage of circulating leucocytes into the tissues.

The outcome of an infection depends on the sum of various factors influencing each other. Disturbances of the balance of this highly complex and intricate network of cytokines and effector cells by genetic, environmental, or microbial elements might have harmful consequences [40, 41]. Natural selection has generated pathogens with the ability to alter the cytokine synthesis, degrade pro-inflammatory cytokines or use cytokine receptors as portals of entry for cellular invasion [42].

In the case of *M. tuberculosis* it is known that phagocytosed bacteria reside in the phagosomal compartment of alveolar macrophages where the proteins have ready access to the major histocompatibility complex (MHC) class II processing and presentation machinery [43]. The macrophages get activated and start secreting cytokines further stimulating the complex interacting machinery of the host immune system. It is assumed that the inflammatory response is essential for granuloma formation and long term survival of *M. tuberculosis* [44]. Factors like TNF, IFN-γ, IL-12, reactive oxygen (ROIs) and nitrogen intermediates (RNIs) are important for the maintenance of the granuloma structure [45].

1.2.1.1 Interleukin-1 beta (IL-1β)

IL-1β is mainly produced by monocytes/macrophages [46], and dendritic cells [47] after the recognition of mycobacterial lipoproteins which are potent activators of the TLR2 [48, 49]. TLR activation leads to an increased production of the precursor molecule pro-IL-1β which co-localizes in the cytosol with pro-caspase-1 [50]. A protein complex termed "IL-1β inflammasome" [51] converts the inactive procaspase-1 in active caspase-1, which in turn is responsible for the activation of IL-1β. The active form of IL-1β is secreted into the extracellular environment and known to be an important first line defence against microbes [52] since it promotes phagosome maturation.

1.2.1.2 Interferon-gamma (IFN-γ)

IFN-γ belongs to the pro-inflammatory cytokines. It is synthesised by T lymphocytes and natural killer (NK) cells [53, 54] and in this regard responsible for the activation of macrophages which in turn produce inflammatory cytokines. Furthermore activated macrophages show a higher expression of cytokine/chemokine receptors and MHC-I and II molecules on their cell surface, enhancing macrophage antigen presentation to T cells [53]. IFN-γ also promotes the monocyte fusion and plays an important role in the multinucleated giant cell formation [55-57]. Patients with defects in IFN-γ receptors are highly susceptible towards mycobacterial infections [58, 59] and have a higher risk receiving recurrence of those infections [60, 61]. IFN-γ knockout mice are particularly susceptible to *M. tuberculosis* [62], furthermore it was shown, that the macrophage activating cytokines IFN-γ and TNF-α are important for the initial control of tuberculosis [60, 62].

1.2.1.3 Tumor necrosis factor-alpha (TNF-α)

TNF-α is regarded one of the most important pro-inflammatory cytokines when it comes to battling *M. tuberculosis*. It is released by monocytes, macrophages [63] and dendritic cells [64] after stimulation with the cell-wall component lipomannan (LM) or other mycobacterial antigens in a TLR2-dependent manner [65]. Its macrophage-activating functions are based upon its ability to promote phagocytosis, intracellular killing, MHC molecule expression, stimulating T lymphocyte activation and granuloma formation [66-68].

TNF-α activated macrophages release chemokines which attract additional immune cells to the infection site [69]. The ability of TNF-α to induce apoptosis underlines its immunoregulatory properties [70] making it important for confining intracellular pathogens and clearing some infections. On the other hand it is important that TNF-α is balanced by the anti-inflammatory cytokine IL-10. Without the regulatory effect of IL-10, TNF-α might induce tissue damage and necrosis [71].
Similar effects are seen in TNF-α knockout mice. When these mice have lost their ability to induce granuloma formation they are unable to limit bacterial dissemination whereupon they die from the consequences of tuberculosis [39]. Otherwise mice with latent tuberculosis see reactivation when TNF-α is subsequently blocked [72].

1.2.1.4 Interleukin-10 (IL-10)

The anti-inflammatory cytokine IL-10 is released by macrophages after phagocytosis of *M. tuberculosis*, but also by dendritic cells [73, 74], T cells [75, 76], B cells, neutrophils, eosinophils, and mast cells [77]. Several cytokines are known to promote the IL-10 secretion including IL-12 [78] and IL-6 [79]. The exact pathway responsible for the secretion of IL-10 is not fully understood, but there is some evidence, that antigens from infectious agents are recognized by TLR2 [80].
As an antagonist to pro-inflammatory cytokines IL-10 limits production of ROIs and RNIs in macrophages [81]. IL-10 furthermore alleviates the process of inflammation in monocytes by limiting the IL-8, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and (G-CSF) [82] production. By this means IL-10 interferes with all of the above mentioned functions of IFN-γ and TNF-α. In its immunosuppressive role IL-10 prevents an over-reaction of the inflammation promoting factors, counteracts cell damage, leads to a balance between the pro- and anti-inflammatory factors and is therefore essential for the outcome of disease. Cyktor and colleagues [80] have shown that the disruption of the IL-10 gene in mice or the blockage of the IL-10 receptor promotes the elimination of a majority of intracellular pathogens, however many of the laboratory animals died an inflammation-associated death.

1.2.1.5 Interleukin-8 (IL-8)

This chemokine has a pro-inflammatory character. Its primary function is to attract circulating leukocytes to invade inflammated tissues [83]. IL-8 is mainly secreted by granulomas infiltrated by neutrophils [84], macrophages infected with *M. tuberculosis* [85] and other cells with TLR involved in the unspecific immune response. Increasing number of lymphocytes and neutrophils reach the site of infection, latter-mentioned degranulate causing tissue damage [86] or boost the inflammatory response. The IL-8 production is negatively regulated by IL-1β and TNF-α [85]. The importance of IL-8 for bacterial containment was seen when it was totally blocked in rabbits whereupon the granuloma formation was inhibited [87].

1.2.2 Nitric oxide (NO)

Nitric oxide synthase (NOS), first described in 1989, is a catalytic enzyme with three known isoforms with only one of them showing antimycobacterial properties [88, 89]. The inducible nitric oxide synthase (iNOS; also known as NOS2) is expressed in activated innate immune cells like macrophages and neutrophils. Here it catalyses the production of (NO) and related RNIs which are collectively responsible for damaging bacterial DNA, proteins and lipids [90]. RNIs induce the synthesis and release of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-8 [91, 92]. The beneficial effect of RNIs on mycobacterial infections might be mediated by direct killing of mycobacteria or by prevention of dissemination.

In murine and human macrophages pro-inflammatory cytokines like IFN-γ, TNF-α, and IL-1β [93, 94] as well as the mycobacterial cell wall component lipoarabinomannan [95] are directly involved in the induction of iNOS expression and NO production. Whereas the antimycobacterial effects of NO have been demonstrated in mouse models, similar observations in humans were contradictory [96], although high amounts of NO were found in alveolar macrophages from patients with active pulmonary tuberculosis [97]. NO is capable of blocking the aerobic respiration in *M. tuberculosis* whereupon the bacteria activate a set of genes which are very similar to the dormancy survival regulon (see section 1.5). These genes are up-regulated under hypoxic conditions [98, 99] and other stress factors and are presumably responsible for long-term survival in human granulomas.

This supports the conclusion, that either different or possible overlapping pathways are responsible for the adaptive capabilities of *M. tuberculosis*.

1.3 The mycobacterial cell wall

The extremely complex cell wall of mycobacteria is unique and distinguishes them from other bacteria. The cytoplasmic membrane is overlaid by layers of peptidoglycan (PG), arabinogalactan (AG), and unusually long branched mycolic acids (MA), containing 70-90 carbons, which are covalently linked among each other forming the MA-AG-PG complex [100] better known as the cell wall core. This cell wall core is intercalated by phosphatidyl-myo-inositol mannosides (PIMs) and lipomannan (LM), the biosynthetic precursors of lipoarabinomannan (LAM) [101], also responsible for the induction of important immunomodulatory functions. The cell wall core is connected to the outer layer by extractable glycolipids consisting of mycolic acids, such as trehalose-6,6´-dimycolate (TDM) and trehalose-6-monomycolate (TMM) [102], important signalling and effector molecules of mycobacteria interacting with the immune system. The importance of these lipids for the outcome of tuberculosis was obvious as delipidation led to a decreased capability of *M. tuberculosis* to induce granuloma formation [103]. The cell wall glycolipids TDM and PIMs are responsible for granuloma formation in mice [104, 105], whereas LM and PIMs induce a strong pro-inflammatory response by TNF-α, IL-8, and IL-12 secretion [101, 106]. The structure of the cell wall constitutes many morphological and physiological characteristics of mycobacteria important for their virulence associated capabilities, many of them induced under microaerobic and anaerobic conditions. The mycobacterial cell wall also constitutes an efficient permeability barrier, thought to be largely responsible for their slow growth rates and their natural resistance against antibiotics and long-term survival in the hostile environment [107].
The high lipid content in the cell wall accounts for about 60% of the dry weight of mycobacteria. These lipids are responsible for the resistance of the bacteria to Gram staining. *M. tuberculosis* therefore is neither gram-positive nor gram-negative but instead described as acid-fast. For diagnostic methods the Ziehl–Neelsen stain is used. The carbolfuchsin dye penetrates the cell wall and then resists decolourisation by acid/alcohol leaving behind red coloured rod-shaped bacilli.

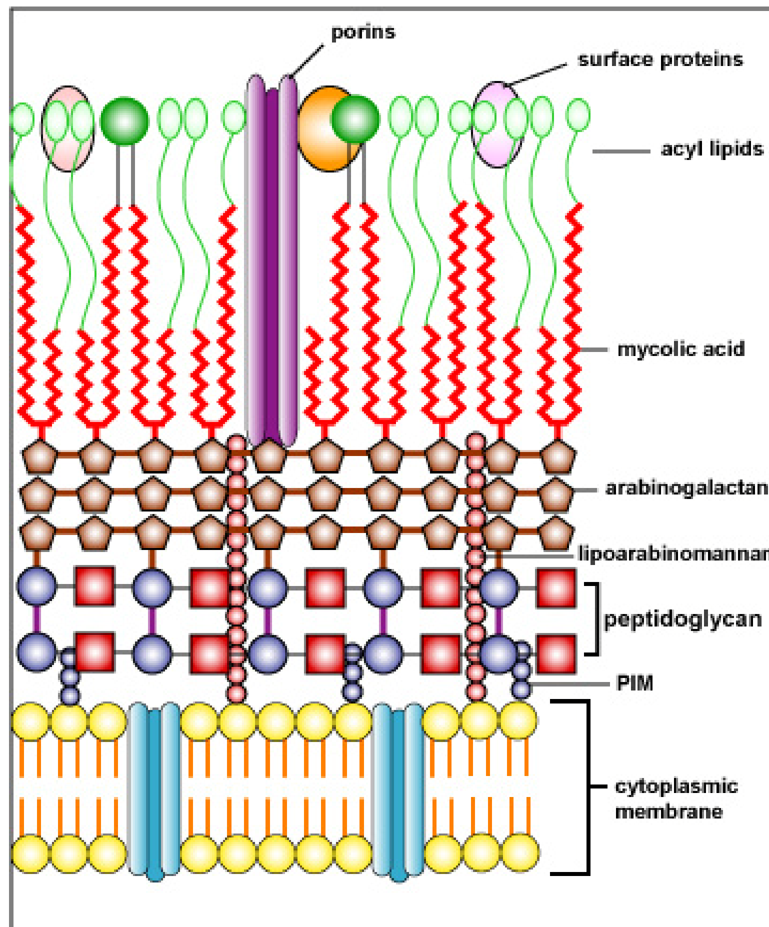


Figure 3. Schematic representation of the mycobacterial cell wall
[Source: http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit4/innate/u1fig11.html]

1.4 Multinucleated (giant) cells and granulomas

The granuloma formation constitutes the preliminary endpoint of a failed attempt from alveolar macrophages to eliminate phagocytosed tubercle bacteria. Granuloma formation is the result of a well-orchestrated interaction of host cytokines and chemokines secreted by infected alveolar macrophages triggering an inflammatory response, which coordinates the recruitment of immature blood-derived macrophages and T lymphocytes. The macrophages form aggregates which are first surrounded by T lymphocytes and then, at later stages, by fibroblasts forming a sealed envelope which encloses the structure [39]. Macrophages inside these granulomas fuse with each other forming multinucleated cells (MC) and continue to do so until they differentiate to multinucleated giant cells (MGC).

MGCs were first described by Langhans in 1868 [108] (also called Langhans cells) in conjunction with tuberculosis granulomas, which are a typical histopathologic feature of various granulomatous diseases (including leprosy, schistosomiasis, and sarcoidosis). The development of MGCs depends on the infecting mycobacterial species. Representatives from the MTBC induce large MGCs with more than 15 nuclei whereas the fusion process of opportunistic and avirulent strains like *M. avium* and *M. smegmatis* ceases at the point of MCs (less than 15 nuclei) [109].
MGCs show strong antigen presenting capabilities but have lost their ability to phagocytose further bacteria [109]. Even though the composition of MGCs and granulomas is known, the specific functions and complex interactions between the different cell types are not fully understood.
It is assumed that both sides, the bacteria and the host, profit from the granuloma formation. Whereas the bacteria are “walled off” from the host immune system, especially from the T cells releasing macrophage-activating cytokines, the granuloma also prevents the bacteria from spreading into healthy tissues and organs [110] but cannot eliminate the infection [33]. The influence of macrophage-activating cytokines like IFN-γ on granuloma formation was shown in immune-deficient individuals who were hampered in developing granulomatous structures and died of mycobacterial infection [59].
The direct contact between monocytes and bacteria is essential for multinucleated (giant) cell formation but phagocytosis alone is apparently not sufficient since many pathogens are phagocytosed without initiating cell fusion. This leads to the assumption that beside intercellular contact, surface molecules and adhesion molecules [111] or other distinctive features of mycobacteria, like the cell wall component TDM [112] are responsible for triggering the fusion process. Inside the granuloma there is a special micro-environment, thought to be predominated by nutrient limitation, low pH, hydrolytic enzymes, reactive nitrogen and reduced oxygen tension [113]. The bacilli adapt their metabolism to anaerobiosis by switching to nitrate respiration [114] and reductive amination of glyoxylate [115]. These metabolic changes are accompanied by chromosome duplication [116], cell wall modification [117] and the bacteria eventually attain a non-replicating and non-respiring state called dormancy [118, 119].

During this period infected individuals do not develop clinical disease. When infected cells die, macrophages from the periphery of the granuloma are ready to phagocytose released mycobacteria and cellular components in an ever repeating process [120].

When the immune system of the host fails to maintain immune signals because of unknown reasons, the granuloma structure disrupts resulting in lung cavitation and pulmonary disease [27, 31, 33]. When the lesion gets access to the bronchus, the bacteria might leave the body posing a risk for others to get infected with *M. tuberculosis*.

1.5 The dormancy survival regulon (DosR)

Spore forming bacteria are known to cease growing under conditions of nutrient depletion. Spores survive long periods of time until their surroundings change to more favourable conditions. To do so, they have to adapt their metabolism to a less profligate way [121]. Even non-sporulating pathogens like *M. tuberculosis* have proven that they are able to survive for more than ten years in sealed liquid cultures [122] or inside granulomas of latently infected hosts without losing viability. For developing new therapies against tuberculosis or preventing the reactivation from latent state bacilli, it is important to understand the genetic mechanisms behind their survival strategy.

M. tuberculosis and other intracellular mycobacteria trapped in a granuloma, are confronted with dramatic changes in their environment. Toxic necrotic products and hypoxia have the greatest influence on the intracellular survival [123, 124]. First experiments to find key genes which are influenced by declining oxygen concentrations showed that liquid cultures of *M. tuberculosis* were not able to survive sudden changes from aerated cultures to anaerobic conditions. When this change was precipitated in liquid medium without agitation the oxygen level in the sediment reached micro-aerophilic conditions. The bacteria not only adapted to the micro-aerophilic conditions [125] but exhibit synchronised replication when resuspended and diluted into oxygen-rich medium [126]. This experiment showed a connection between oxygen limitation and adaption to anaerobiosis.

It was inappropriate to examine the gradual change of the bacterial metabolism and the underlying genetic mechanisms which are responsible for the achievement of the dormant state.

Using stirring cultures the oxygen level declined gradually and the tubercle bacilli reached a homogeneous metabolic state. An oxygen saturation of 1% to 0.06% matches a micro-aerobic state called non-replicating persistent (NRP) stage 1.

This state was characterised by a slow increase in turbidity whereas the numbers of colony forming units (CFU), DNA synthesis, and ATP concentration did not change although a sudden increase in glycine dehydrogenase was initiated and sustained. Once the oxygen concentration dropped below 0.06% the bacilli shifted abruptly to an anaerobic state, designated NRP stage 2. At this stage, the turbidity remained constant whereas the concentration of glycine dehydrogenase declined notably. RNA and protein synthesis almost came to a standstill, which is why the bacteria were resistant to many antibiotics.

Experience has shown that mycobacteria must spend some time in the NRP stage 1 to survive anaerobic conditions [116]. This model was later called "*NRP oxygen depletion*" or "*Wayne-Dormancy*" model.

This Wayne-Dormancy model provided the basis for several other models most notably the "*Long-term nutrient depletion model*" [127], the "*Reduced oxygen in a chemostat controlled steady-state culture model*" [128], "*Standard stationary phase culture model*" [129], and the "*Static culture oxygen depletion model*" [130]. The transcriptional adaptability of *M. tuberculosis* was also tested in IFN-γ activated murine macrophages [1], under acidic conditions in phagosomes [131], in Balb/c and SCID mice [132], and guinea pigs [133]. Techniques like whole-genome microarrays [131, 134] and two-dimensional (2D) gel electrophoresis [1] gave a deeper insight in the transcriptional adaptations to some of the above mentioned experimental designs.

A complex regulated gene cluster consisting of three genes (Rv3134c/3133c/3132c) was significantly involved in the process of the above mentioned adaptations. This process is induced whenever the aerobic respiration is blocked by hypoxia, nitric oxide and carbon monoxide. Two of these genes encode the sensor histidine kinases DosS (Rv3132c) and DosT (Rv2027c), which both respond to NO. The third gene functions as a response regulator, DosR (Rv3133c) [99, 129, 130, 135].

Whereas DosT is more dominant in the early stage of hypoxia, DosS alone maintains induction of the DosR regulon when oxygen becomes limited [136, 137]. As with many other two-component systems, the dosRS operon is autoregulated [138]. Investigating the DosR regulon under many different stress conditions [139], it became apparent that it is the most highly induced, co-regulated gene cluster, although not all genes of this cluster were equally expressed under all these conditions [99, 129, 130]. This observation supported the conclusion that subsets of the dormancy genes were differently regulated.

To find only those genes which are really under control of the DosR regulon, it was decided that these genes had to fulfil some criteria. Most important was a strong up-regulation during (A) oxygen limitation, (B) nitric oxide exposure, (C) induction during the NRP model and (D) expression must be blocked by cyanide and by *dosR* mutation [99]. All in all 48 genes fulfilled these criteria and are therefore referred as "dormancy-associated genes", among them *rv0079*, *rv1130*, *rv1738*, *rv2626c*, *rv3127* and *rv3154c*.

Protein names and Rv numbers are according to the *M. tuberculosis* H37Rv genome annotation [140]. Several studies have shown that these genes play an important role in long-term adaptation of *M. tuberculosis* and *M. bovis* to the immune system of the host and to survive long periods of anaerobiosis [141, 142].

For many years it was thought that granulomas are anything but dynamic. Recent investigations in mice have shown that cellular accumulations differ over time [143] and are more dynamic with respect to oxygen tension [144]. It is therefore very likely that *M. tuberculosis* repeatedly experience changing levels of oxygen concentrations and it should be able to quickly switch their metabolism between aerobic respiration and nitrate respiration.

Investigations of a *DosR* mutant in *M. bovis* BCG have shown that this regulon is not only important for long-term survival under anaerobic conditions but also for recovering from anaerobiosis [145]. Without ability to switch quickly between aerobic and nitrate respiration, the bacteria are unable to maintain energy levels, which are pivotal for metabolic processes. Since the DosR regulon plays an important role for rapid adaptations to metabolic changes based on hypoxia [145] it is therefore essential for survival. A high number of DosR regulated genes have a strong T cell and IFN-γ inducing capacity [146]. Apart from that little is known about the functions and interactions of the dormancy regulated genes.

1.6 The conserved hypothetical protein Rv2626c

Rv2626c is one of the most strongly up-regulated genes in the DosR regulon [129, 147, 148]. It is predicted to consist of two cystathionine beta synthase (CBS) domains (accession number PF00571), a recently discovered domain named after the enzyme where it was originally identified [149] and which is found in all kingdoms of life. As opposed to other CBS domains this one does not appear to bind adenosinmonophosphat (AMP). The original enzyme catalyses the first step of the trans-sulfuration pathway which is important for the amino acid metabolism and instrumental for the conversion of homocysteine to cystathionine.
Although not much is known about the function of the CBS domain in Rv2626c, it is thought to be involved in protein interaction and protein regulation.

Rv2626c was originally found to be up-regulated under different *in vitro* and *in vivo* induced stress situations like hypoxia [134], treatment with nitric oxide [99] and in IFN-γ activated murine macrophages [1]. Rv2626c was not or only weakly expressed in exponentially growing liquid cultures [150]. In 2005 Sharpe *et al.* [151] found “another” gene which was strongly up-regulated under hypoxic conditions and named it hypoxic response protein 1 (HRP1) without knowing that it is identical to Rv2626c. Further sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) studies regarding the structure of this protein demonstrated the presence of a 32-kDA dimer, a 16-kDa monomer and small amounts of degradation product below [151]. Since Rv2626c was detected in culture filtrates by peptide mass fingerprinting [152], the immunogenicity of this and seven other DosR regulated proteins were analysed in Balb/c and C57BL/6 mice by plasmid DNA vaccination. It turned out that seven out of eight proteins induced strong humoral and/or cellular Th1-type (IL-2 and gamma interferon) immune responses. Rv2626c induced very strong T and B cell responses [153]. This observation was later confirmed by Bashir *et al.* [154] using recombinant Rv2626c. They revealed that this protein binds to murine macrophages, where it induces iNOS expression and NO production with simultaneous expression of nuclear factor (NF)-κB. This led to the speculation that the (NF)-κB-dependent pathway was involved.

After contact to the cell surface receptors Rv2626c stimulated pro-inflammatory and Th-1 type cytokines such as TNF-α, IL-12 and IFN-γ together with various costimulatory molecules such as B7-1, B7-2 and CD40. Similar results were seen in peripheral blood mononuclear cultures of tuberculosis patients [146].

Further studies with immunogold-labeled *M. tuberculosis* cultures showed a uniform distribution of protein around the bacilli and within the bacteria [147]. For now it is not known how the protein is secreted. Missing secretion signals led to the conclusion that another pathway than the well characterised Sec and Tat secretions systems are responsible for the secretion [147]. It is suggested that a recently “type VII” secretion system might be involved [155].

Since its immunogenic and antibody stimulating properties, Rv2626c is seen as a promising candidate for immuno-profiling of disease states [156] and vaccine development.

1.7 The mycobacterial DNA-binding protein 1 (MDP1)

This protein was first mentioned in 1998 as histone-like protein of *M. tuberculosis* (HLPMt), also designated HupB or Rv2986c [157], MDP1 in *M. bovis* BCG [158] or laminin binding protein of *M. leprae* [Lbp; [159]. Years later a 28-kDa cell wall associated homologue was found in *M. smegmatis* together with the siderophores mycobactin and carboxymycobactin. Since this protein showed an iron regulated expression it was called irep-28 [160]. A recent study about irep-28 proofed its ability to directly bind Fe^{3+} preventing the Fenton reaction and protecting DNA by ferroxidase activity [161]. Over the years other homologues of this gene have been found in various mycobacterial strains like *M. kansasii*, *M. avium*, *M. fortuitum*, *M. marinum* and *M. ulcerans*. By now there is no agreement for a uniform nomenclature so it was decided to use the designation *mdp1*-gene.

MDP1 is composed of 205 amino acids, predominantly alanine, arginine, lysine, proline and threonine with a calculated molecular weight of 21-kDa and an isoelectric point of 12.4. It constitutes approximately 7 to 10% of total proteins in *M. bovis* bacillus Calmette-Guérin (BCG) [158]. Genome sequencing of BCG showed that *mdp1* is a single copy gene.

MDP1 is furthermore important for growth regulation [162], stress response [163], accumulation in stationary phase and under anaerobic conditions [163, 164]. The gene is not under control of the DosR regulon. In slow growing mycobacteria, like *M. tuberculosis* or *M. bovis* BCG, *mdp1* proved to be essential [165]. It has been shown to control the transfer of mycolic acids to sugars by Ag85 complex proteins modifying the cell wall composition in advanced cultures of *M. smegmatis* and *M. bovis* BCG [146]. Furthermore antibodies against MDP1 were found in the sera of tuberculosis patients [160] making it an interesting candidate for vaccine design.

1.8 *M. bovis* BCG contra *M. tuberculosis*

To find out to which extent the genes *rv2626c* and *mdp1* influence virulence associated capabilities, *M. bovis* BCG was used in this study since *M. tuberculosis* is a biosafety level 3 organism, hence experiments are subject to experimental limitations. The attenuated strain *M. bovis* BCG features many properties of *M. tuberculosis* and *M. bovis* although according to Mahairas and colleagues [166] this strain has lost three distinct genomic regions of difference (designated RD1 to RD3), encompassing altogether 29.5 kb DNA. Others found that more than 100 genes were deleted in *M. bovis* BCG [167, 168].

In recent years enough data about BCG was published so it can be assumed, that investigations linked to the topic of this work are comparable to *M. tuberculosis*. Crucial for this decision was the fact that BCG reacts quite similar in the oxygen-limited “Wayne dormancy” culture system [169, 170] and that a DosR mutant in BCG lost its ability to survive hypoxia [142]. Two recent studies with a *DosR* mutant in *M. tuberculosis* showed a lesser impact on the survivability towards hypoxia [99, 171]. Although MDP1 is not DosR regulated, it nevertheless was seen to accumulate in stationary phase and anaerobic conditions, which implies that it is important for the growth-state dependent adaption of *M. bovis* BCG [172].

1.9 Objectives

The aim of this thesis was to investigate possible virulence associated capabilities of genes from *M. bovis* BCG which might have an impact on the viability in host cells. In the recent past, genome wide microarray and 2D gel electrophoresis studies investigated genes under certain stress conditions like hypoxia, nutrient depletion and acidic conditions which help *M. tuberculosis* to survive in a hostile environment. A new approach was used by single gene mutagenesis by up-regulation, down-regulation and deletion of the specific genes in *M. bovis* BCG whereas all other studies used wild-type strains of *M. tuberculosis* and *M. bovis*.

Genes chosen for this work had to fulfil the following criteria: they had to be up-regulated under three or more stress conditions and the up-regulation should be more than five times higher than in controls without stress. From all candidates, fulfilling these criteria, six hypothetical genes were selected: *rv0079*, *rv1130*, *rv1738*, *rv2626c*, *rv3127* and *rv3130c*.

Due to the manifold properties in pathological important processes and its up-regulation during dormancy and anaerobic conditions the mycobacterial DNA-binding protein 1 (MDP1) was also chosen as a promising candidate to study the issue of this work.

Genetic modifications performed in this study resulted in recombinant BCG strains in which the corresponding genes were over-expressed, down-regulated or deleted. For the first time comparative studies were applied to investigate the impact of these differentially regulated genes under *in vitro* and *in vivo* conditions simulating the natural course of infection. Primary human derived cells, a mouse cell line and Balb/c mice were used to investigate the ability of these genes to influence the reaction of the bacteria towards conditions met during infection. The focus was on investigations regarding the survival under pH stress condition, the intracellular survival in human monocytes/macrophages, cytokine expression in human peripheral blood mononuclear cells, macrophage fusion and intracellular survival in mice. This thesis summarises the results obtained for the genes *rv2626c* and *mdp1*.

2. Material & Methods

The following strains and plasmids were used in this study (Table 1).

Table 1. Strains and plasmids used in this study

Strains	Characteristics	Reference/Source
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Stratagene
M. bovis BCG Copenhagen	Attenuated strain of *M. bovis* used as vaccine	Statens Serum Copenhagen
M. bovis (pMV261)	BCG control strain with plasmid pMV261	[165]
M. bovis (pRv2626c)	BCG strain over-expressing the *rv2626c*-gene	This study
M. bovis ΔRv2626c::hyg	BCG mutant lacking the *rv2626c*-gene	This study
M. bovis (pMDP1)	BCG strain over-expressing the *mdp1*-gene	This study
M. bovis (pAs-MDP1)	BCG strain down-regulating the expression of the *mdp1*-gene	[165]
Plasmids		
pMV261	*Mycobacterium*/*E. coli* shuttle vector with the kanamycin resistance *aph*-gene from transposon Tn903 and the promoter from the *hsp*60 gene from *M. tuberculosis,* empty multiple cloning site; (see Attachment 8.2.1)	[173]
pRv2626c	Over-expression plasmid; pMV261 with the *rv2626c*-gene from *M. bovis* BCG Copenhagen downstream of the promoter *hsp*60 from *M. tuberculosis*	This study
pAs-MDP1	Antisense plasmid; pMV261 with 102 bp coding sequence plus 11 bp of the untranslated upstream region containing the Shine-Dalgarno Sequence of the mycobacterial *mdp1*-gene from *M. bovis* BCG Copenhagen cloned behind the promoter *hsp*60 from *M. tuberculosis*	[165]
pMDP1	Over-expression plasmid; pMV261 with the *mdp1*-gene from *M. bovis* BCG Copenhagen cloned downstream of the promoter *hsp*60 from *M. tuberculosis*	This study
pYUB854	*E. coli* cosmid with two multiple cloning sites flanking a hygromycin resistance gene (see Attachment 8.2.2)	[174]

2.1 Bacterial strains and culture conditions

E. coli DH5α was used for cloning experiments as recipient for plasmids, which were designed for the generation of over-expression strains, down-regulated strains and deletion mutants of *M. bovis* BCG (Bacillus Calmette-Guérin). *E. coli* DH5α was grown in Luria-Bertani- (LB) medium at 37°C [175] and later on LB agar plates, supplemented with 100 µg ml^{-1} kanamycin or 100 µg ml^{-1} hygromycin for selection of transformed bacteria. *M. bovis* BCG was grown in Middlebrook 7H9 broth (BD Biosciences, Heidelberg, Germany) supplemented with 10% ADC (2 g glucose, 5 g BSA, 0.85 g NaCl in 100 ml dH_2O) and 0.05% Tween80 (Roth GmbH, Karlsruhe, Germany). After electroporation *M. bovis* BCG was plated on Middlebrook 7H11 agar (BD Biosciences, Heidelberg, Germany), supplemented with 10% OADC (BD Biosciences, Heidelberg, Germany) and 0.5% glycerol (Roth GmbH, Karlsruhe, Germany). For selecting over-expressing and down-regulating strains or deletion mutants the Middlebrook agar was supplemented with 50 µg ml^{-1} hygromycin or 100 µg ml^{-1} kanamycin. Under experimental conditions the strains *M. bovis* BCG and *M. bovis* ∆Rv2626c::hyg were grown in Middlebrook broth without antibiotics, whereas the strains *M. bovis* (pMV261), *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1) were supplemented with 25 µg ml^{-1} kanamycin. All strains were incubated at 37°C without shaking. Strains and plasmids used in this study are described in Table 1.

2.1.1 Growth experiments under neutral and low pH conditions

All strains were grown in Middlebrook broth at 37°C and when necessary supplemented with 25 µg ml^{-1} kanamycin, until they reached a late growth phase with an optical density $(OD)_{(600\ nm)}$ of 3. At this state they were inoculated in 25 ml Middlebrook broth (pH 7) until an $OD_{(600\ nm)}$ of 0.02 to 0.04 was achieved. To measure the susceptibility of the new generated strains to low pH, parallel cultures were inoculated in 25 ml Middlebrook broth, adjusted to pH 5.3, until an $OD_{(600\ nm)}$ of 0.02 to 0.04 was achieved. Cultures of *M. bovis* BCG carrying the shuttle vector pMV261 or a plasmid, which was derived from pMV261, were furthermore supplemented with 25 µg ml^{-1} kanamycin. Once per week the OD was measured, 100 µl samples were taken and frozen at -20°C to determine the number of viable bacteria by adenosinetriphosphate (ATP) measurement.

Before starting the ATP measurement the frozen cultures were thawed, vortexed and then diluted 1 to 10 in dH_2O. Thereof 50 µl of the diluted samples, together with 50 µl of the luciferase reagent from the BacTiter-Glo™ Microbial Cell Viability Assay, were pipetted into 96 well plates (Nunc F96 MicroWell Plates, GmbH, Langenselbold, Germany). Bacteria and reagent were incubated for 5 minutes, shaken for 1 minute and then incubated for another 10 minutes. The luminescence as a measure of the ATP concentration was read in a Berthold-Luminometer LB 96 P (Berthold technologies, Bad Wildbad, Germany). For more information see manufacturer's instructions.

2.1.2 Cultivation of bacteria under pH stress for protein isolation

For protein isolation under pH stress conditions bacterial cultures were grown in 50 ml Middlebrook broth at 37°C and when necessary with 25 µg ml^{-1} kanamycin, until they reached an $OD_{(600\ nm)}$ of 3. At this point, 10 ml of each culture was centrifuged at 6000 x g for 20 minutes and 4°C. The supernatants were discarded and the pellets stored at -80°C. The rest of the cultures were again centrifuged for 20 minutes at 3500 x g, the pellets resuspended in Middlebrook broth adjusted to pH 5.3 and supplemented with 10% OADC. The cultures were returned to the incubator and incubated at 37°C for another 24 and 48 hours, respectively. Further samples were taken at these time points for adjoining protein extraction (see section 2.3.1).

2.2 Cloning techniques

2.2.1 Molecular biology techniques

Molecular biology techniques were accomplished according to standard protocols described by Sambrook *et al.* [175] or according to manufacturer's instructions of kits and enzymes (see Table 2). Transformation of *E. coli* DH5α was performed according to the method of Hanahan *et al.* [176], whereas the electroporation of *M. bovis* BCG was accomplished as described by Sharbati-Tehrani et al. [177]. Primers were purchased from Metabion (Martinsried, Germany). All sequencing reactions were performed by using the Prism Big Dye Terminator 3.1 FS Terminator Cycle Sequencing Ready Reaction Kit from PE Applied Biosystems 2500 (Darmstadt, Germany) in combination with an Applied Biosystems 3500xl Dx Genetic Analyzer.

Table 2. Kits used in this study

Method	Kit*
ATP measurement	BacTiter-Glo™ Microbial Cell Viability Assay, Promega, Mannheim, Germany
Cell staining	Diff-Quick Fix Medion Diagnostics, Langen, Germany
Cytokine measurement	ELISA Kits Ready-SET-Go for IL-1β, IL-8, IL-10, IFN-γ, TNF-α, eBioscience, delivered by Natutec, Frankfurt, Germany
Plasmid isolation	Qiagen Plasmid Maxi Kit, Qiagen, Hilden, Germany
DNA digestion	FastDigest Restriction Enzymes, Fermentas, St. Leon-Rot, Germany
DNA gel extraction	QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany
DNA ligation	T4 DNA Ligase, Fermentas, St. Leon-Rot, Germany
PCR purification	QIAquick PCR Purification Kit, Qiagen, Hilden, Germany
PCR amplification	DreamTaq™ DNA Polymerase Kit and dNTPs, Fermentas, St. Leon-Rot, Germany
Protein measurement	BCA™ Protein Assay Kit, PIERCE, Rockford, USA
Southern Blot	Digoxigenin Labeling Kit, Roche, Mannheim, Germany
Staining of mycobacteria	Tb-color modified (Ziehl-Neelsen), Merck, Darmstadt, Germany
Western blot	BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit), Roche, Mannheim, Germany

*All kits were used according to the manufacturer's specifications.

2.2.2 Mutagenesis of the *rv2626c*-gene in *M. bovis* BCG

2.2.2.1 Construction of the rv2626c over-expression plasmid

To accomplish the over-expression of the gene *rv2626c* was amplified from *M. bovis* BCG by using the primer combination Rv2626c_FW_BamHI and Rv2626c_BW_EcoRI. The amplified product was purified by using a polymerase chain reaction (PCR) purification kit from Qiagen. Afterwards the PCR product and the shuttle vector pMV261 were double digested with BamHI and EcoRI (Fermentas). Troublesome small DNA molecules were removed and the gene sequence ligated in the multiple cloning site (MCS) of the shuttle vector by using a T4 DNA Ligase from Fermentas. The plasmid pRv2626c was then transformed into *E. coli* DH5α.

Plasmids were isolated from densely grown cultures by using a Qiagen Plasmid Maxi Kit, then the inserts were sequenced to confirm the absence of mismatches and a plasmid without mismatches was used for electroporation of *M. bovis* BCG, as described by Sharbati-Tehrani *et al.* [177].

2.2.2.2 Site-directed mutagenesis of rv2626c

Site-directed mutagenesis was chosen for the deletion of the mycobacterial gene *rv2626c* in *M. bovis* BCG (Figure 4) and care was taken to ensure that the flanking genes are not disturbed. The cosmid pYUB854 (3893 bp) was used to generate the deletion plasmid which was used for mutagenesis. The deletion plasmid was constructed in two consecutive steps. First the downstream region of *rv2626c* was amplified by PCR using the primer combination Rv2626_LF_FW_StuI and Rv2626_LF_BW_XbaI (Figure 4A). Subsequently pYUB854 and the resulting 1209 bp long PCR product were digested with the restriction enzymes StuI and XbaI. Interfering small DNA molecules were removed by using the QIAquick PCR Purification Kit. Both products were ligated with each other (Figure 4B) using a T4 DNA Ligase. Afterwards the plasmid was transformed into *E. coli* DH5α. The plasmid was isolated from a densely grown culture by using the Qiagen Plasmid Maxi Kit and then used for a second cloning step. For this purpose the upstream region of *rv2626c* was amplified by PCR using the primer pair Rv2626_RF_FW_XhoI and Rv2626_RF_BW_SpeI (Figure 4C). The amplified 1090 bp long PCR product and pYUB854 with the downstream region of *rv2626c* were double digested using the restriction enzymes SpeI and XhoI. Interfering small DNA molecules were removed by using the QIAquick PCR Purification Kit. Afterwards the upstream region of *rv2626c* was ligated into the second MCS of pYUB854 (Figure 4D). Again *E. coli* DH5α was used as recipient of the plasmid. To generate the recombination substrate consisting of the hygromycin resistance gene framed in the DNA sequences flanking *rv2626c*. The plasmid was again isolated from a densely grown culture and then double digested with the restriction enzymes StuI and SpeI (Figure 4E). By running a 0.8% agarose gel the recombination substrate (about 4400 bp long) was separated and eluted by using a Gel Extraction Kit from Qiagen. For site-directed mutagenesis 2 µg of the recombination substrate were electroporated [177] into the wild-type strain of *M. bovis* BCG (Figure 4F).

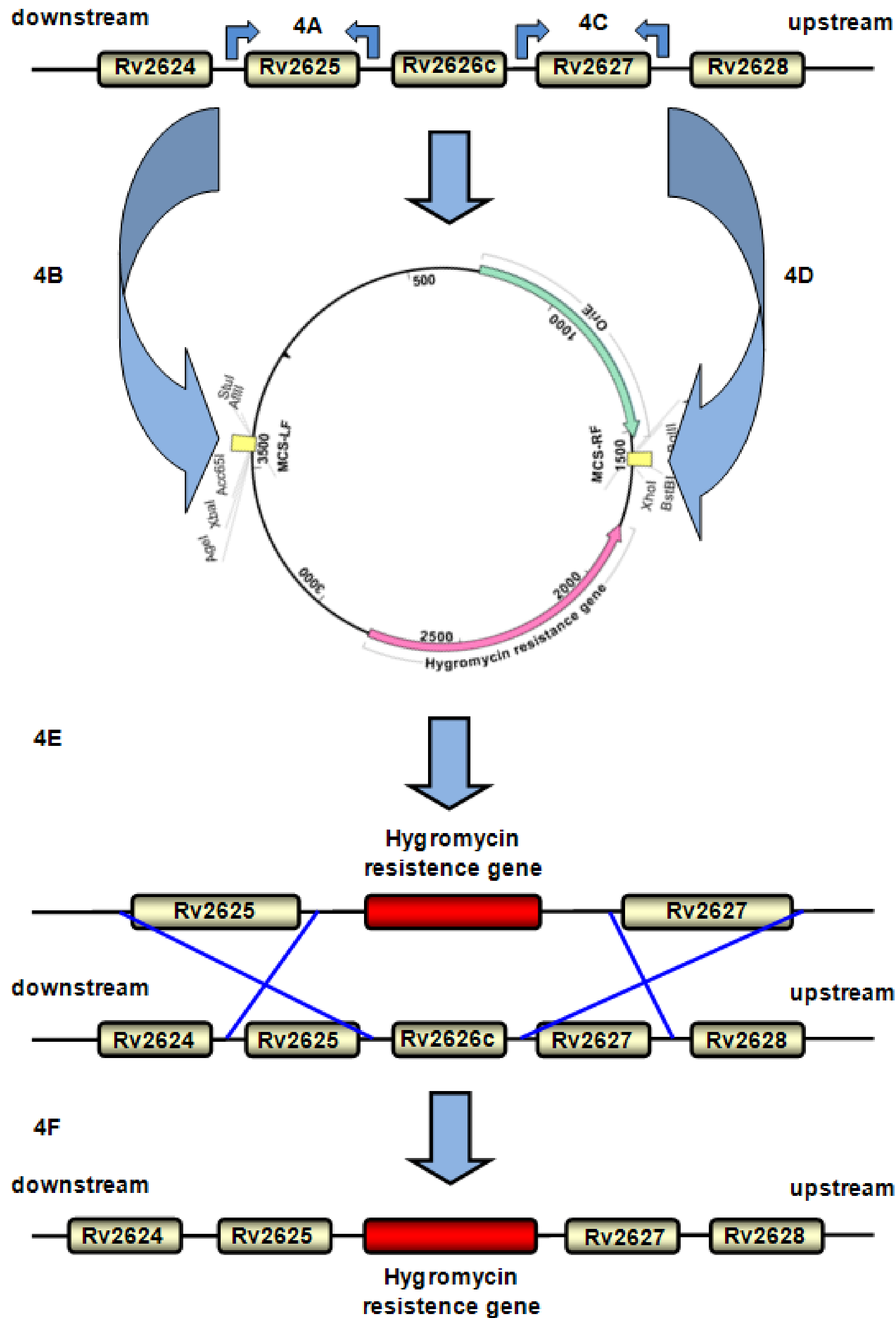


Figure 4. Generation of the deletion mutant M. bovis ΔRv2626c::hyg

A) Amplification of the downstream region of *M. bovis* BCG by PCR using the primer combination Rv2626_LF_FW_StuI and Rv2626_LF_BW_XbaI; **B)** ligation of the PCR product and pYUB854; **C)** amplification of the upstream region of *M. bovis* BCG by PCR using the primer combination Rv2626_RF_FW_XhoI and Rv2626_RF_BW_SpeI; **D)** ligation of the second PCR product and pYUB854; **E)** digestion of the deletion plasmid by StuI and SpeI followed by **F)** electroporation and site-directed mutagenesis in *M. bovis* BCG.

The electroporated bacteria were plated on Middlebrook agar supplemented with 10% OADC and 25 µg ml^{-1} hygromycin. After four weeks the colonies were screened by PCR (see Table 3). The successful deletion was ascertained by using the primer combination KoRv2658_FW/KoRv2660_BW and Rv2626c_FW/Rv2626c_BW. To examine the complete deletion of *rv2626c* in *M. bovis* BCG a Southern Blot was performed. For this purpose 1 µg genomic DNA from *M. bovis* BCG and from the deletion mutant *M. bovis* ∆Rv2626c::hyg were digested with ApaI and SmaI, respectively, separated on a 0.8% agarose gel and capillary transferred to positively charged nylon membrane (GE Healthcare, Buckinghamshire, UK) by following a standard protocol [175].
The hygromycin resistance gene (1818 bp) from pYUB854 was amplified using the primer pair Hyg2K FW and Hyg2K BW whereas the primer pair Rv2626c_FW_BamHI and Rv2626c_BW_EcoRI was used to amplify the gene sequence of *rv2626c* from the genomic DNA of *M. bovis* BCG. Both PCR products were labelled using a Digoxigenin Labelling Kit from Roche and then applied as probes in hybridisation using nylon membrane with the digested DNA from *M. bovis* BCG and the mutant. Probe detection was carried out by using anti-digoxigenin-AP conjugate and CDP-star from Roche according to the manufacturer's instructions.

Table 3. Primers used for mutagenesis of Rv2626c

Primers	Sequence*	Ann. Temp.	Product size
Rv2626c_FW_BamHI Rv2626c_BW_EcoRI	5´-CC *GGATCC* ATG ACC ACC GCA CGC GAC AT-3´ 5´-CC *GAATTC* CTA GCT GGC GAG GGC CAT GG-3´	61.8°C	432 bp
KoRv2658_FW KoRv2660_BW	5´-TCT TGA CGA GCT TGC CCG CGG CCT-3´ 5´-TGT CAT TGC GGC ACC CAG AGC CGC-3´	66.0°C	3200 bp
Hyg2K_FW Hyg2K_BW	5´-CAC CGT ACG TCT CGA GGA ATT CCT G-3´ 5´-GCG TCG TGA AGA AGG TGT TGC TGA-3´	64.3°C	1818 bp
Rv2659_RF_FW_XhoI Rv2659_RF_BW_SpeI	5´-TTT ATC CGC CAG CAT CGG AGG TAC C-3´ 5´-ATC ACC CCG CAT CGG AAA ACC TG-3´	64.9°C	1210 bp
Rv2659_LF_FW_StuI Rv2659_LF_BW_XbaI	5´-TGC ATG ACG AAC TCG GCC TGA ACA-3´ 5´-GCG AAC ACT AGG GTC GTT TGA CCC G-3´	64.3°C	1091 bp

*Restriction sites added to the primers for cloning purposes are italicised and underlined.

2.2.3 Mutagenesis of the *mdp1*-gene in *M. bovis* BCG

2.2.3.1 Construction of the mdp1 over-expression plasmid

The approach for constructing a MDP1 over-expression plasmid is similar to the above mentioned procedure for over-expressing *rv2626c* (see section 2.2.2.1) in *M. bovis* BCG and therefore described in an abbreviated form. The *mdp1*-gene (=*rv2986c*) was amplified by using the primer combination Rv2986c_FW_Pst and Rv2986c_BW_HindIII (Table 4). The amplicon was gel purified, digested with the restriction enzymes PstI and HindIII, ligated into the multiple cloning site of the shuttle vector pMV261 and then transformed into *E. coli* DH5α. The plasmid was then isolated from a densely grown culture and sequenced to confirm the absence of mismatches. After electroporating the shuttle vector into *M. bovis* BCG [177] the plasmid was again isolated and again sequenced.

2.2.3.2 Site-directed mutagenesis of mdp1

The procedure chosen for the deletion of the *mdp1*-gene in *M. bovis* BCG was the same as for *rv2626c* (see section 2.2.2.2), with the following modifications. The 915 bp long downstream region of *mdp1*-gene from *M. bovis* BCG was amplified by PCR using the primer combination Mb3010c_LF_FW_SpeI and Mb3010_LF_BW_BstBI. For the 704 bp long upstream region of *mdp1* the primer combination Mb3010c_RF_FW_Xba and Mb3010c_RF_BW_AflII was used. The recombination substrate was amplified by PCR using the primer combination Mb3010c_LF_FW_SpeI and Mb3010c_RF_BW_AflII. For site-directed mutagenesis 2 µg of the recombination substrate were electroporated into *M. bovis* BCG as described by Sharbati-Tehrani *et al.* [177]. See also Figure 4 for further details.

2.2.2.3 Construction of the mdp1 antisense plasmid

The construction of the antisense-plasmid pAs-MDP1 was recently described in Lewin *et al.* [165]. The plasmid contains a 113 bp fragment of BCG-DNA covering the first 102 bp of the coding sequence from the MDP1 gene and 11 bp of the untranslated upstream region covering the Shine-Dalgarno Sequence.

The fragment was inserted into the shuttle vector pMV261 downstream of the *hsp*60 promoter in antisense-orientation. The expression of MDP1 is reduced by about 50% in *M. bovis* BCG (pAs-MDP1) grown in broth culture if compared to *M. bovis* BCG containing the empty vector pMV261 [165].

Table 4. Primers used for mutagenesis of *mdp1*

Primers	Sequence of primers*	Ann. Temp.	Product size
Rv2986c_FW_PstI Rv2986c_BW_HindIII	5`-AA *CTGCAG* ATG AAC AAA GCA GAG CTC ATT GAC-3` 5`-CC *AAGCTT* CTA TTT GCG ACC CCG CC-3`	61.0°C	645 bp
Mb3010_LF_BW_BstBI Mb3010c_LF_FW_SpeI	5´- GGG *TTCGAA* CTT TG C GAT TGC GGG ACT TGT CG-3´ 5´-GGG *ACTAGT* CAC ATC CAC GAC CGC GAG CAC-3´	64.5°C	915 bp
Mb3010c_RF_FW_Xba Mb3010c_RF_BW_AflII	5´-GGG *TCTAGA* ATT TCA CTG AGC CAA CGA CCG-3´ 5´-GGG *CTTAAG* ACC CAC TCT GGT ATT GGC GTG-3´	60.6°C	704 bp
KoMb3009_FW KoMb3011c_BW	5´-ATT GTT CGA ATC CGC ACT GGA CCC-3´ 5´-GCG GCC GCA CAC ATT TGG TG-3´	64,2°C	2796 bp
pMV261_FW6 Mb3010c_LF_BW_Xba	GAC GAG ACG GGG TTC TAC GAA TCT TG GGG *TCTAGA* GGT CCT TCT GCC GGG AGA CGC	63.5°C	1017 bp
Rv2986c_ko_FW_Pst Rv2986c_ko_BW_Sal	5`-GG-*CTGCAG*-C ACG GAT ACC CTT CGT CAC A-3` 5`-AA *GTCGAC* GGC GAA ATT TCA GCG TGA C-3`	60.5°C	900 bp
pMV261_FW pMV261_BW	5´-GAG GAA TCA CTT CGC AAT GGC-3´ 5´-TAA CAT CAG AGA TTT TGA GAC ACA ACG-3´	60°C	231 bp

*Restriction sites added to the primers for cloning purposes are italicised and underlined.

2.3 Protein analysis

2.3.1 Protein extraction

Frozen bacterial pellets from the pH stress experiment were washed two times in 1 ml icecold PBS (10 mM sodium phosphate, 126 mM sodium chloride, pH 7.2) plus 0.2% EDTA. After the last washing step 1 µl proteinase inhibitor cocktail (SIGMA-ALDRICH, Taufkirchen, Germany) was added, the bacteria heated for 30 minutes at 80°C and then centrifuged for ten minutes at 13,000 x g and 4°C. The pellet was resuspended in 250 µl Tris-HCl, pH 8.0. Disintegration of the cells was achieved by using the cell disruptor PreCellys (PeqLab, Erlangen, Germany) (program: two times 26 seconds at 3,300 x g with a 30 seconds pause in between) followed by sonification (Branson Ultrasonics Corporation, Danbury, USA; duty cycle 50%, output control 3.5) for 10 minutes. Protein precipitation occurred over night in 500 µl icecold aceton with 20 mM Dithiothreit (Roth, Germany) at -20°C. After centrifugation (10 minutes at 12,500 x g) the proteins were resolved in 500 µl 50 mM Tris-HCl plus 0.3% SDS, pH 8.0. The protein concentration was measured with the BCA Protein Assay Kit from Pierce.

2.3.2 Western blot & immunodetection

For the Western blot 30 to 50 µg of proteins were separated in 10% SDS gels using the Maxigel System (Biometra, Göttingen, Germany). The gels were equilibrated for 15 minutes in Towbin buffer (25 mM Tris, 192 mM glycin and 20% methanol, pH 8.3). Simultaneously a PVDF-membrane (Roti-PVDF transfer membrane T830.1, pore size 45 µm, Carl Roth GmbH, Germany) was shortly equilibrated in methanol, washed for 2 minutes in dH_2O and than incubated for 5 minutes in Towbin buffer. The blotting was done in a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Munich, Germany) for 30 minutes at 1.2 mA per cm^2 constant, washed two times with TBS (50 mM Tris, 150 NaCl, pH 7.5) and then blocked over night in 1% casein/TBS at 4°C. A polyclonal rabbit antiserum against the peptide CLAAGLDPNTATAGE-KLH (BioGenes, Berlin, Germany) from Rv2626c was used for immunoblot experiments.
The membrane was incubated for 1 hour with a 1:3500 dilution of this primary antibody, followed by four successive 10 minutes lasting washing steps, two times in 50 ml TBST (50 mM Tris, 150 NaCl, 0.1% [v/v], Tween20, pH 7.5) and then two times in 0.5% casein blocking solution.

The secondary antibody (Peroxidase-conjugated AffiniPure F[ab`]$_2$ Fragment Goat Anti-Rabbit IgG [H+L], Jackson Immuno Research, Soham, UK) POD marked was used at a concentration of 1:7500 in 0.5% blocking solution over a time period of 30 minutes. Afterwards the membrane was washed four times in 100 ml TBST, drained from excess buffer and then incubated for 1 minute with detection reagent using the BM Chemiluminescence Western Blotting Kit [Mouse/Rabbit] from Roche. Drained of excess detection reagent the membrane was quickly transferred to an Amersham Hyperfilm ECL (GE Healthcare, Buckinhamshire, UK) in a dark room exposed for 2 to 15 minutes and then developed using an AGFA Curix 60 (Siemens, Germany, Erlangen). For more details see manufacturer's instructions.

2.4 Cell culture experiments

For cell culture experiments Buffy coats from anonymous healthy volunteers were obtained from the German Red Cross. For infection experiments only bacterial strains were used which had reached a late growth phase of $OD_{(600\ nm)}$ 3. Aliquots from corresponding cultures were frozen at -80°C and thawed shortly before the trial.

2.4.1 Methods of cell isolation from human-derived blood cells

Depending on the kind of experiment peripheral blood mononuclear cells (PBMCs) or monocytes were isolated from human-derived blood cells. Before using isolated PBMCs or monocytes for experimental purposes the cells were counted in a Neubauer counting chamber (Marienfeld, Lauda-Königshofen, Germany) and then used for infection experiments.

2.4.1.1 PBMCs Isolation

PBMCs were isolated by Ficoll-Paque Plus density centrifugation (GE Healthcare, Munich, Germany). For more details see manufacturer's instructions. Isolated PBMCs were used to measure the secretion of cytokines by infected cells (see section 2.4.2.1).

2.4.1.2 Monocytes isolation

Blood monocytes were obtained from human-derived PBMCs (see section 2.4.1.1) by Percoll density gradient centrifugation (see manufacturer's instructions, GE Healthcare, Munich, Germany). To remove remaining erythrocytes from the cell pellets the cells were resuspended in 10 ml ACK-lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.3) and then incubated for 5 minutes on ice. Afterwards 10 ml PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.4) were added to the lysis buffer, the cells were washed with PBS twice and then resuspended in 40 ml IMDM medium with L-Glutamine (PAA, Paching, Austria) plus 3% human AB serum (PAA, Paching, Austria). Monocytes were used to measure the intracellular survival of mycobacteria and the fusion rates of infected monocytes.

2.4.2 Infection experiments

Following isolation PBMCs were used for measuring the cytokine concentration (see section 2.4.2.1) in the supernatants of infected cells whereas monocytes were applied for intracellular survival (see section 2.4.2.2) and fusion experiments (see section 2.4.2.3).

2.4.2.1 PBMC infection and Cytokine measurement

One million isolated PBMCs per strain and per sampling point were seeded in 24 well Tissue Culture Plates (TPP, Trasadingen, Germany) and immediately infected by a multiplicity of infection (MOI) of 1. Five wells per strain were infected. Cells used as positive control were pre-activated with 10 ng ml^{-1} human IFN-γ (Invitrogen, Darmstadt, Germany) and 10 ng ml^{-1} LPS or remained untreated in the case of the negative control. The infection took place over night at 37°C and 5% CO_2. After 24 hours supernatants were taken and stored at -20°C. Later on the supernatants were thawed and the cytokine levels for IL-8, IL-10, IL-1β, IFN-γ and TNF-α were measured with ELISA Kits Ready-SET-Go from eBioscience by following the manufacturer's instructions.

2.4.2.2 Monocyte infection and intracellular survival

Following the isolation of monocytes (see section 2.4.1.2) five wells per strain and sampling point were seeded in 24 well Tissue Culture Plates with 1 million cells per well. The monocytes were allowed to adhere over night at 37°C and 5% CO_2, supplemented with 10 ng ml^{-1} human IFN-γ and 10 ng ml^{-1} LPS for pre-activation or remained unstimulated. The intracellular survival experiment of *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1) was exclusively performed with unstimulated monocytes.
Prior to infection non-adherent cells were removed by washing the wells twice with 1 ml RPMI 1640 medium (Biochrom, Berlin, Germany). Afterwards the monocytes were infected with a MOI of 1 and bacteria and monocytes were brought into closer contact by centrifuging (250 x g) the plates for 10 minutes at room temperature. After four hours of incubation at 37°C and 5% CO_2 the wells were again washed twice with RPMI 1640 medium to remove extracellular and non-adhered bacteria. The cells were then incubated for another two hours with 200 ng ml^{-1} amikacin disulfate salt (SIGMA, Taufkirchen, Germany) to kill remaining extracellular bacteria. After another washing step the cells were incubated in IMDM medium with L-Glutamine plus 3% human AB serum (PAA, Pasching, Austria) and 2 ng ml^{-1} amikacin disulfate salt. First samples were taken 4 hours after the onset of the infection by lysing the infected monocytes in 500 µl dH_2O for 15 minutes at 37°C to release BCG. Further samples were taken after 24, 48, 72 and 120 hours. All samples were stored at -20°C.

2.4.2.3 Monocyte infection and fusion of monocyte-derived macrophages

The infection was performed under the same conditions as for the intracellular survival experiment (see section 2.4.2.2) with two notable exceptions: sterile glass slides were prepared incubating them over night at room temperature in 250 ml dH_2O and 0.5 ml concentrated HCl. Afterwards they were consecutively washed twice with an excess of dH_2O and once with 95% methanol. The glass slides were air-dried over night at 37°C and than sterilized. These sterilized glass slides were placed into wells of 24 Tissue Culture Plates for cell adherence, to allow later staining and microscopy.

Contrary to the intracellular infection experiment monocytes were not only infected but also stimulated for 4 hours with 10 ng ml^{-1} human IFN-γ (Invitrogen, Darmstadt, Germany) and 10 ng ml^{-1} LPS or remained untreated. Stimulated and untreated monocytes were used as positive and negative controls respectively. After seven days, the glass slides were removed from the wells and adherent cells stained with Ziehl-Neelsen (Tb-color modified and malachite green, Merck, Darmstadt, Germany; see manufacturer´s instructions) to examine the uptake of mycobacteria or stained by Diff-Quick Fix (Medion Diagnostics, Langen, Germany; see manufacturer´s instructions) to determine if the mutagenised strains had influenced the capability of monocytes/macrophages to fuse with each other. The fusion index was determined by multiplying the numbers of nuclei within multinucleated cells (minimum 3 nuclei per cell) divided by the total number of nuclei multiplied by 100. All in all 6000 nuclei were counted per strain.

$$FI\,[\%] = \frac{\text{number of nuclei in multinucleated cells}}{\text{total number of nuclei}} \times 100$$

2.4.2.4 J774.A1 mouse macrophages infection and Griess assay

J774.A1 mouse macrophages (DSMZ no. ACC170) were grown in RPMI 1640 medium with 5% Foetal Calve Serum (FCS; Biochrom, Berlin, Germany) at 37°C and 5% CO_2 until the cells formed a monolayer at the bottom of the Tissue Culture Flasks (TPP, Trasadingen, Germany). For the purposes of the experiment 3.5 x 10^5 cells per well were seeded in 96 well Tissue Cultures-treated Plates (TPP, Trasadingen, Germany) and, with the exception of the positive and negative control, pre-activated over night with 10 ng ml^{-1} mouse IFN-γ. The next day the cells were washed twice with 200 µl RPMI 1640 medium to remove non-adherent cells and then infected using a MOI of 50. For the positive control the macrophages were activated with 10 ng ml^{-1} mouse IFN-γ and 10 ng ml^{-1} LPS whereas the negative control remained untreated.

For the standard, a 1 mM sodium nitrite ($NaNO_2$) solution was prepared in dH_2O and diluted (end concentration 100 µM, 80 µM, 60 µM, 50 µM, 40 µM, 30 µM, 20 µM, 10 µM, 6 µM, 2 µM and 0 µM). After 24 hours 100 µl of the sample supernatants and the standard were transferred in duplicate in fresh wells.

Both substances were then mixed with 100 µl of the freshly prepared Griess Reagent consisting of 1% sulfanilamide (resolved in 5% o-phosphoric acid) and 0,1% [w/v] N-(1-napthyl)-ethylenediamine dihydrochloride (mixing ratio 1:1).
After shaking for 10 minutes the absorbance of the samples at 570 nm was measured by an ELISA reader (TECAN Infinite M 200 Pro, Crailsheim, Germany). This assay relies on a diazotization reaction that was originally described by Griess in 1879 [178]. Modifications have been made to the original reaction through the years. The concentration of nitrite was interpolated from the $NaNO_2$ standard curve.

2.4.3 DNA extraction methods

Two different methods were used for extraction of DNA from human-derived blood monocytes (see section 2.4.3.1) and mouse tissues (see section 2.4.3.2).

2.4.3.1 DNA extraction from human-derived blood cells

Prior to DNA extraction the frozen cell lysates were thawed and 200 µl mixed with 100 µl 2 x TE9 buffer [500 mM Tris, 20 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaCl, 1% SDS and 6 µl Proteinase K (50mg/ml), pH 9 [179]. The suspension was first incubated for 60 minutes at 58°C and then for another 30 minutes at 97°C.
DNA extraction was done in two steps. First 300 µl phenol/chloroform/isoamyl alcohol were added, well mixed and the suspension was centrifuged for 10 minutes at 16,100 x g. In the second step 200 µl of the supernatants were mixed with 200 µl chloroform/isoamyl alcohol and again centrifuged for 10 minutes at 16,100 x g. DNA precipitation took place over night at -20°C after mixing 125 µl of the supernatants with 100% ethanol and 12.5 µl 5 M Na-acetate, pH 5.0. The DNA was pelleted by centrifugation for 20 minutes at 16,100 x g and then washed with 70% ethanol. The pellet was dried and resuspended in 50 µl sterile dH_2O. DNA quantification was done by using 10 µl for real-time-PCR.

2.4.3.2 DNA extraction from mouse tissues

Organs obtained from killed Balb/c mice were homogenised, 50 µl resuspended in 310 µl dH_2O and 40 µl 10 x TE8 buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH8). The suspension was heated for 30 minutes at 80°C, supplemented with 5 µl lysozyme (150 mg ml^{-1}) and then shaken over night at 37°C. Furthermore 70 µl of 10% SDS and 2 µl Proteinase K (50 mg ml^{-1}) [179] were added and the suspension again shaken for 2 hours at 65°C. After adding 100 µl of 5 M NaCl and 100 µl CTAB (10% CTAB in 0.7 M NaCl) the suspension was mixed and then heated for 10 minutes at 65°C. DNA extraction was done in three steps first by adding 700 µl chloroform/isoamyl alcohol and mixing followed by 30 minutes centrifugation at 16,100 x g. About 500 µl of the supernatants were afterwards mixed with phenol/chloroform/isoamyl alcohol and then centrifuged for 10 minutes at 16,100 x g. For the last step 400 µl of the supernatants were mixed with 400 µl chloroform/isoamyl alcohol and centrifuged for 10 minutes at 16,100 x g. DNA precipitation took place over night at -20°C by mixing of the supernatants with 300 µl isopropanol. The DNA was pelleted by centrifugation (30 minutes at 16,100 x g), washed with 70% ethanol, dried and then resuspended in 50 µl sterile dH_2O. DNA quantification was done by using 10 µl for real-time-PCR.

2.4.3.3 DNA quantification by real-time-PCR

Following DNA extraction from infected blood monocytes or mouse tissues the DNA quantification of intracellularly grown mycobacteria was done by real-time PCR [180]. For this purpose a region of the 85B antigen gene was amplified using the primers MY85FW and MY85BW [181] (Table 5) together with the FAM/TAMRA-labelled My85B probe [182] (Table 5). The amount of DNA was determined by using a standard with an established amount of genomic DNA from *M. bovis* BCG.

Table 5. Primers used for detection of intracellular grown mycobacteria

Primers	Primer sequences	Ann. Temp.	Product size
My85B_FW	5´-TCA GGG GAT GGG GCC TAG-3´		
My85B_BW	5´-GCT TGG GGA TCT GCT GCG TA-3	60°C	130 bp
MyQuant	(FAM)-TCG AGT GAC CCG GCA TGG GAG CGT-(TAMRA)		

2.5 Animal Experiment

Six female, six-weeks old Balb/c-mice (from Charles Rivers, Rockland, Mass., USA), per mycobacterial strain and time point, were infected. About 5.0 x 10^5 bacteria of *M. bovis* BCG or *M. bovis* BCG ∆Rv2626c::hyg were intravenously injected in the tail vein. The infected and three non-infected control animals were killed after 17 and 25 days. Liver, lung, lymph nodes and spleens were obtained, fixed in 10% formalin and histological analysed for tissue changes by members of the Veterinary Medicine Institute (Freie Universität, Berlin). Furthermore parts of these organs were homogenised to calculate the bacterial number by real-time PCR (see section 2.4.3.3).

3. Results

Recent investigations indicated that the mycobacterial genes *rv2626c* and *mdp1* from *M. tuberculosis* and *M. bovis* BCG possess virulence associates capabilities which might be important in the course of pathogenesis.

In order to investigate this matter *M. bovis* BCG was: A) mutagenised to construct new strains in which these genes were over-expressed, down-regulated or deleted and then B) examined if these genetic modifications had some influence on the viability of the strains and to what extend cells of the innate immune system reacted on the genetic modifications.

Wherever possible primary cells from human volunteers (as in the case of the intracellular survival, cytokine and fusion experiments) were used. In cases, where it was not possible, macrophages from a mouse cell line (for measuring the NO concentration), Balb/c mice (for intracellular survival) or artificial conditions for the pH experiment in liquid cultures, which correlates to the internal pH conditions in human macrophages, were applied.

The experimental setup was chosen to reflect the course of a natural infection beginning with the phagocytosis of the bacteria by macrophages (pH stress and intracellular survival experiment), the response of the innate immune system in particular PBMCs (by measuring the cytokine production) and macrophages (especially their ability produce nitric oxide compounds, to kill phagocytosed mycobacteria and to form multinucleated cells). Furthermore the viability of the deletion mutant was investigated in a mouse model.

The following strains were included in this investigation: *M. bovis* BCG, *M. bovis* ∆Rv2626c::hyg, *M. bovis* (pMV261), *M. bovis* (pRv2626c), *M. bovis* (pMDP1) and *M. bovis* (pAs-MDP1). In this context *M. bovis* BCG was used as a reference strain for *M. bovis* ∆Rv2626c::hyg whereas *M. bovis* (pMV261) was the reference strain for the remaining strains.

This chapter is sub-divided into two sections, one dealing with the mutagenesis and verification of the genetic modifications of recently generated strains (see section 3.1) the other with experiments designed to investigate the aforementioned issues (see section 3.2).

3.1 Mutagenesis of *rv2626c* and *mdp1*

3.1.1 Mutagenesis of the *rv2626c*-gene

3.1.1.1 Confirmation of the deletion mutant M. bovis ΔRv2626c::hyg

The gene *rv2626c* from *M. bovis* BCG was replaced by the hygromycin resistance gene from the plasmid pYUB854 by means of site-directed mutagenesis. In order to verify the genetic modification about 1 µg DNA from *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg were digested with the restriction enzymes ApaI and SmaI. The DNA was first transferred onto a membrane and then hybridized with the digoxigenin labelled sequence of *rv2626c* (amplified from *M. bovis* BCG) or the hygromycin resistance gene (amplified from the plasmid pYUB854). The hybridisation of the *rv2626c* probe confirmed that the gene is present in *M. bovis* BCG (Figure 5A, lane 1&2) while no hybridisation was seen (Figure 5A, lane 3&4) to confirm that the gene was absent in *M. bovis* ΔRv2626c::hyg. The probe for the hygromycin resistance gene hybridised to pYUB854 (Figure 5B, lane 5) and *M. bovis* ΔRv2626c::hyg (Figure 5B, lane 8&9) verifying that the hygromycin resistance gene had replaced *rv2626c*. At the same time no hybridisation was seen in *M. bovis* BCG (Figure 5B, lane 6&7) because wild-type strain does not contain the hygromycin gene. Therefore the Southern Blot confirmed the deletion of *rv2626c* which was also confirmed by sequencing and PCR (data not shown).

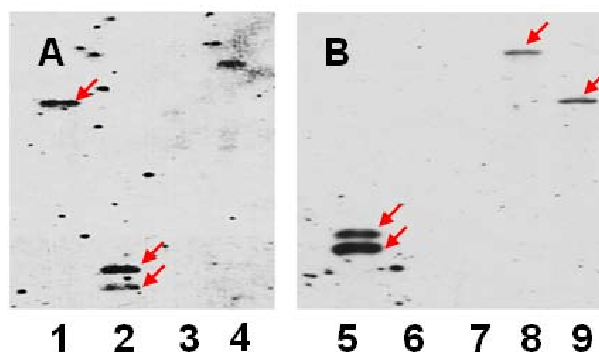


Figure 5. Confirmation of the deletion of *rv2626c* in *M. bovis* ΔRv2626::hyg by means of Southern Blot

A) Hybridisation with a probe covering the gene *rv2626c*, **B)** hybridisation with a probe covering a part of the hygromycin resistance gene; **Lane 1, 6)** *M. bovis* BCG digested with ApaI; **Lane 2, 7)** *M. bovis* BCG digested with SmaI; **Lane 3, 8)** *M. bovis* ΔRv2626c::hyg digested with ApaI, **Lane 4, 9)** *M. bovis* ΔRv2626c::hyg digested with SmaI; **Lane 5)** undigested pYUB854.

3.1.1.2 Confirmation of the over-expressing strain M. bovis (pRv2626c)

The gene sequence of *rv2626c* was amplified from *M. bovis* BCG, ligated into the MCS of the shuttle vector pMV261 and then electroporated into *M. bovis* BCG. The resulting strain was named *M. bovis* (pRv2626c). The success of the cloning procedure was reviewed by PCR, sequencing (data not shown) and Western blot (see Figure 8 and 15).

3.2.1 Mutagenesis of the *mdp1*-gene

3.2.1.1 Deleting the mdp1-gene has lethal consequences for M. bovis BCG

Once the gene sequences flanking the *mdp1*-gene were ligated into pYUB854 the deletion substrate was isolated and purified by gel extraction and then electroporated into competent *M. bovis* BCG bacteria. Although the experiment was repeated several times, all attempts failed since no colonies had grown on MB-agar plates. It was therefore concluded that the deletion of the *mdp1*-gene has lethal consequences for *M. bovis* BCG.

3.2.1.2 An over-expression of the mdp1-gene in M. bovis BCG is impossible

In order to generate the *mdp1* over-expressing strain the constructed plasmid pMDP1 was electroporated into *M. bovis* BCG. After several electroporations, only four colonies were grown on selective medium. The plasmid pMDP1 was isolated from these colonies and the heat shock promoter and the *mdp1*-gene were amplified by PCR and further analysed.

PCR products were separated on a 1.0% agarose gel (Figure 6). The bands for the *mdp1*-gene using BCG DNA as template (lane 2) and for the PCR product of the cloned *mdp1*-gene from pMDP1 (lane 4) were running at the expected heights (900 bp and 800 bp). Apart from this, the band for the PCR product representing the promoter region from pMDP1 (lane 3) was running significantly lower than expected (about 900 bp instead of 1017 bp). Irregularities like these were found in all four clones.

For this reason the *hsp*60 heat shock promoter and the *mdp1*-sequence from pMDP1 were sequenced, and the results then compared to the respective genes in the genomic DNA, to acknowledge if these irregularities are the result of mutations.

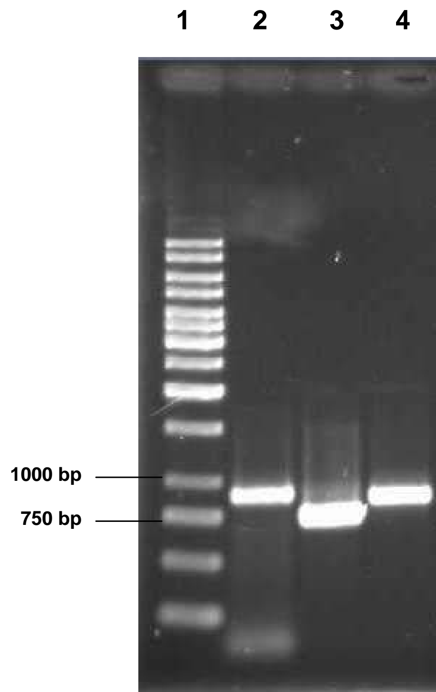


Figure 6. PCR amplificates for validation of the MDP1 over-expression plasmids
Lane 1) 1 kb marker; **Lane 2)** PCR product of the genomic sequence of *mdp1*; **Lane 3)** PCR product of the promoter region from pMDP1; **Lane 4)** PCR product of the cloned *mdp1*-gene from pMDP1.

The amplification of the heat shock promoter *hsp*60 from the over-expressing plasmid pMDP1 was done by using the primers pMV261_FW6 and Mb3010c_LF_BW_Xba (product size: 1017 bp) whereas the sequence of the *mdp1*-gene on the same plasmid was amplified by the primers pMV261_FW and pMV261_BW (product size: 800 bp). As control the genomic sequence of the *mdp1*-gene from *M. bovis* BCG was amplified with the primers Rv2986c_ko_FW_Pst and Rv2986c_ko_BW_Sal. See Figure 7 for a schematic illustration of the amplificated products and the primers used. All primers used can be found in Table 4.

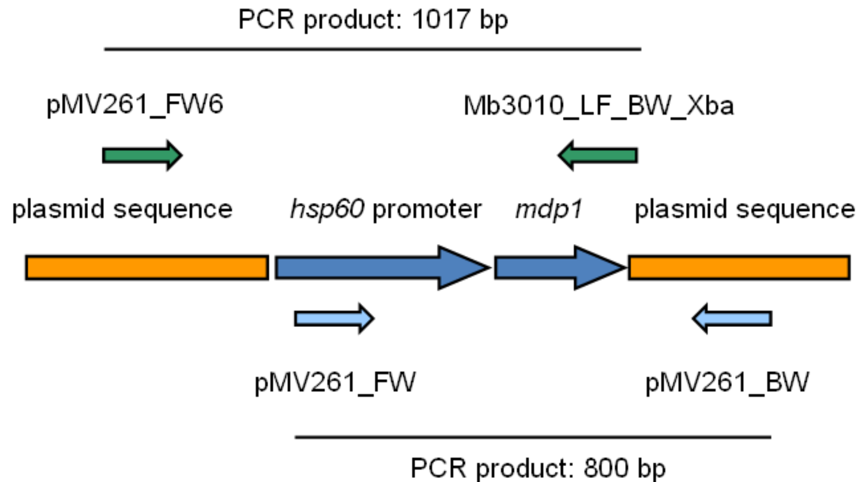


Figure 7. Graphic display of the *mdp1* over-expressing plasmid pMDP1
The graphic depicts primers which were used to examine the sequences of the heat shock promoter *hsp*60 (pMV261_FW6 and Mb3010c_LF_BW_Xba) and the *mdp1*-gene (pMV261_FW and pMV261_BW) ligated into the MCS of the shuttle vector pMV261.

The PCR products were sequenced and then compared to the existing sequences of the original plasmid pMV261 and the *mdp1*-gene using the Blast-Tool from NCBI and the program Megalign (DNA Star). From these comparisons it was determined that all four clones displayed deletions in the heat shock promoter *hsp*60 of pMV261, and point mutations in the start codon of the *mdp1* sequence integrated in the multiple cloning site behind the heat shock promoter (data not shown).
It was therefore assumed that neither of the clones was suitable to over-express MDP1. This assumption was supported by a protein ELISA which showed identical protein concentrations for *M. bovis* (pMDP1) and *M. bovis* (pMV261) (data not shown). Since all efforts to generate an over-expression strain or a deletion mutant failed it was believed that both approaches were impossible and therefore, this part of the cloning project was no longer pursued.

3.2.1.3 Antisense technique as means of choice to down-regulate MDP1

A recently generated *mdp1* antisense strain has demonstrably shown to down-regulate the MDP1 concentration in *M. bovis* BCG by about 50%. This strain regulated the growth of *M. bovis* BCG in broth culture, influenced the aggregation and increased the persistence in MM6 and J774.A1 macrophages [165].

3.2 Determination of the virulence associated capabilities of *rv2626c* and *mdp1*

3.2.1 Expression of *rv2626c* in exponential and stationary phase cultures

Standing cultures with a gradually declining oxygen concentration were expected to induce the DosR regulon which in turn activates the dormancy associated genes including *rv2626c* [142]. To confirm the growth conditions were sufficient to activate the DosR regulon, as shown in the NRP oxygen depletion model defined by Dr. Wayne [116], *M. bovis* BCG, *M. bovis* (pMV261), *M. bovis* ∆Rv2626c::hyg and *M. bovis* (pRv2626c) were incubated in Middlebrook 7H9 broth. When the cultures reached the exponential growth phase (OD 2) aliquots were taken and pellets were frozen at -80°C. The remaining cultures were incubated until they entered the stationary phase (OD 3). Proteins were isolated from the bacterial pellets and the expression of Rv2626c detected by Western blot analysis.

No band of Rv2626c was seen in *M. bovis* BCG, *M. bovis* (pMV261) and *M. bovis* ∆Rv2626c::hyg under the conditions of the exponential growth phase (Figure 8, lane 1-3). This means that the DosR regulon on one hand is not active and on the other hand *rv2626c* is not regulated otherwise. In the case of *M. bovis* (pRv2626c) the *rv2626c*-gene was cloned behind the *hsp*60 heat shock promoter, which is constitutively active, that is why Rv2626c was expressed in this strain even in exponentially grown cultures (Figure 8, lane 4).

Proteins which were isolated from stationary phase cultures and used for Western blotting showed bands in all strains (Figure 8, lane 5, 6 & 8) except *M. bovis* ∆Rv2626c::hyg (Figure 8, lane 7) where the gene was deleted. It was therefore not only proven that the growth conditions under stationary phase induced the expression of *rv2626c*, it was also demonstrated that the deletion of this gene in *M. bovis* ∆Rv2626c::hyg was successful. The culture conditions were therefore maintained to cultivate bacteria which were later used for infection experiments.

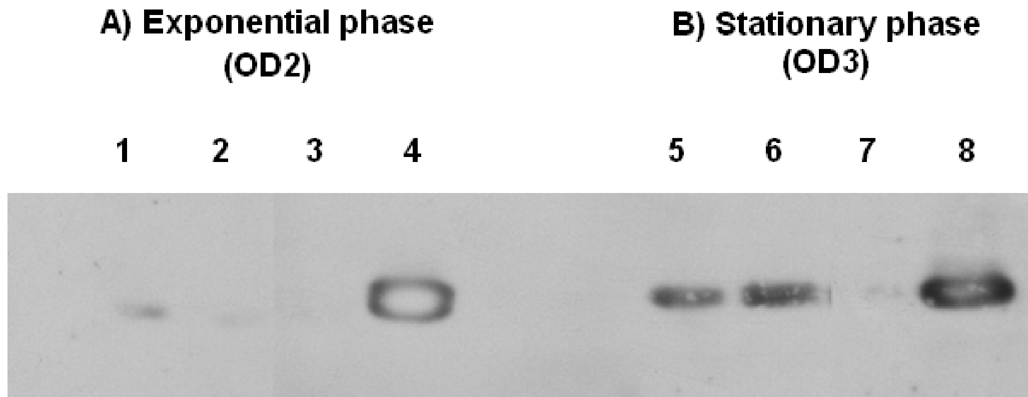


Figure 8. Expression of Rv2626c during A) exponential (OD2) and B) stationary growth phase (OD3) by means of Western blotting

All strains were grown in Middlebrook broth until they had reached the desired growth phase. 50 µg of total protein from *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg, *M. bovis* (pMV261) and *M. bovis* (pRv2626c) were separated in a 10% SDS-PAGE, transferred onto a PVDF membrane and incubated with a polyclonal rabbit antiserum against a peptide from Rv2626c. **Lane 1, 5)** shows *M. bovis* BCG, **Lane 2, 6)** *M. bovis* (pMV261), **Lane 3, 7)** *M. bovis* ΔRv2626c::hyg and **Lane 4,8)** *M. bovis* (pRv2626c).

3.2.2 Adaption of mutagenised *M. bovis* BCG strains to low pH

To see whether the genes *rv2626c* and *mdp1* are able to manipulate the adaption to low pH and to simulate the conditions inside the phagosomes *M. bovis* BCG, *M. bovis* (pMV261), *M. bovis* ΔRv2626c::hyg, *M. bovis* (pRv2626c), and *M. bovis* (pAs-MDP1) were first inoculated into Middlebrook 7H9/ADC broth until they reached stationary phase (OD 3). In this growth phase both proteins were expressed (Figure 8) [172]. The bacteria were then transferred in fresh neutral (pH 7) and acidified Middlebrook 7H9/OADC broth (pH 5.3). Once a week samples for OD and ATP measurement were taken over a period of 42 days to measure the growth of the strains.

3.2.2.1 Rv2626c influences the adaption of BCG to low pH

By comparing the growth curves of *M. bovis* (pMV261) and *M. bovis* (pRv2626c) in medium with neutral pH (7) and low pH (5.3) it became evident that the over-expression of Rv2626c had a negative effect on the growth of *M. bovis* (pRv2626c). Both strains showed an identical growth over the first seven days when inoculated in neutral medium. From then on *M. bovis* (pMV261) showed an accelerated growth compared to *M. bovis* (pRv2626c). With increasing age of the cultures the adverse effect of the over-expression of Rv2626c lessened and after 42 days both strains reached nearly an identical cell density (Figure 9A).

In medium with low pH (Figure 9B) the over-expression of Rv2626c hampered the growth of *M. bovis* (pRv2626c). The turbidity of the culture did not change much over a period of 28 days. From then on it increased until day 42 but the cell density reached only one third of *M. bovis* (pMV261).

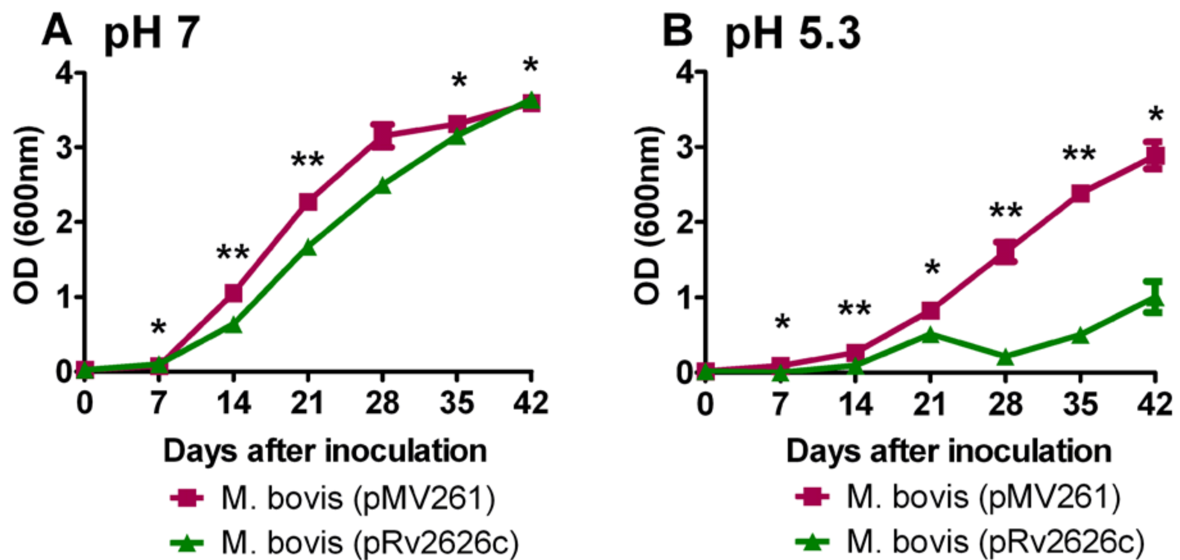


Figure 9. Adaptation of *M. bovis* (pMV261) and *M. bovis* (pRv2626c) to pH stress measured by OD

In Middlebrook broth grown cultures of *M. bovis* (pMV261) and *M. bovis* (pRv2626c)**,** having reached stationary growth phase were inoculated into **A)** neutral media (pH 7) and **B)** media with pH 5.3. Statistical analyses were done using a two-tailed, paired student's t-test. Results are expressed as mean with standard deviations, each value representing three cultures. This experiment was repeated three times with similar results. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

To get a better overview, an ATP quantification method was additionally used to measure the growth rate of metabolically active bacteria. The results obtained for neutral pH conditions were less obvious than the ones measured by OD but showed the same tendency.

Under both conditions *M. bovis* (pRv2626c) reached lower Relative Light Units (RLU) values than *M. bovis* BCG (Figure 12 A&B). In the case of the pH 5.3 the RLU values for *M. bovis* (pMV261) were two-fold higher than for *M. bovis* (pRv2626c). This result impressively underscores the problems of *M. bovis* (pRv2626c) to adapt to pH stress (Figure 10B).

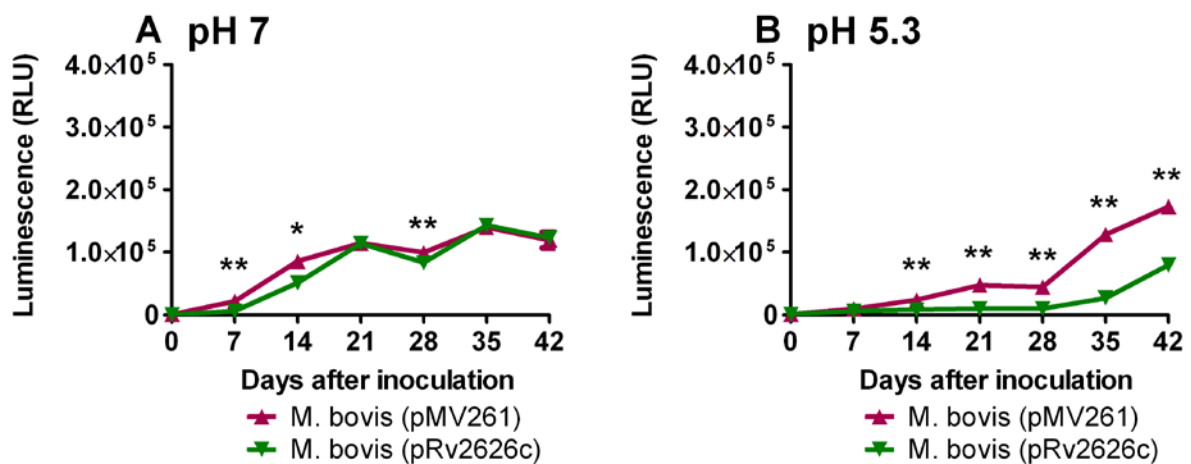


Figure 10. Adaptation of *M. bovis* (pMV261) and *M. bovis* (pRv2626c) to pH stress measured by ATP quantification

Middlebrook broth cultures of *M. bovis* (pMV261) and *M. bovis* (pRv2626c), having reached stationary growth phase were inoculated into **A)** neutral media (pH 7) and **B)** media with pH 5.3. Statistical analyses were done using a two-tailed, paired student's t-test. Results are expressed as mean with standard deviations, each value representing three cultures. This experiment was repeated three times with similar results. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

When *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg were inoculated under the same conditions in media with neutral pH both strains showed identical growth rates (Figure 11A). This changed when both strains were inoculated in media with pH 5.3. Here *M. bovis* ΔRv2626c::hyg showed a significant growth advantage. Whereas both strains needed more time to reach higher cell densities, *M. bovis* ΔRv2626c::hyg recovered faster than *M. bovis* BCG, but at the end both strains had reached almost identical cell densities (Figure 11B).

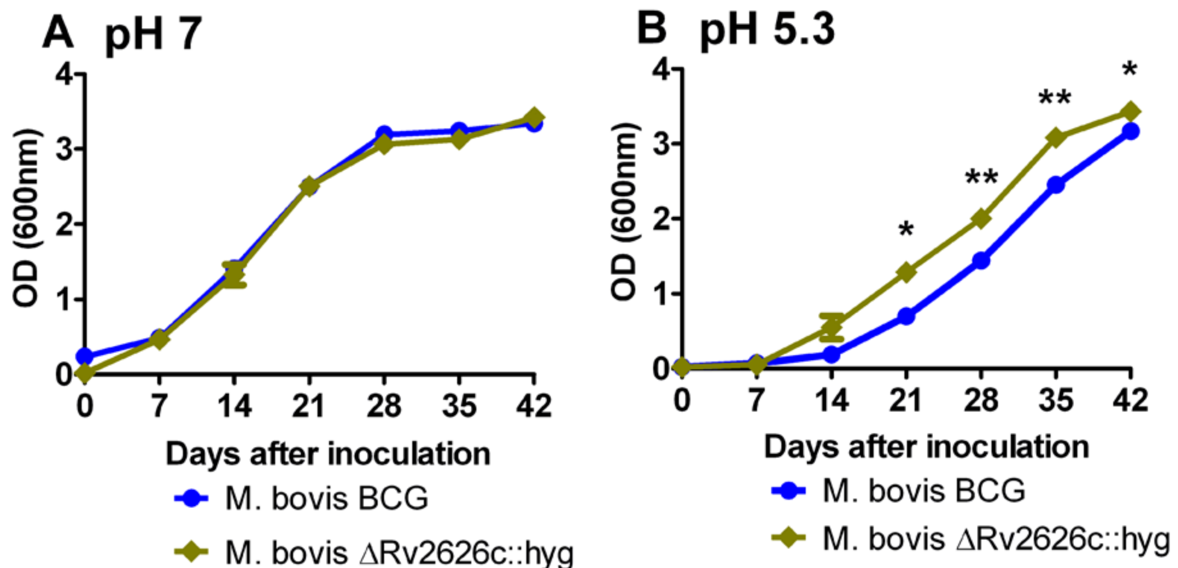


Figure 11. Adaptation of *M. bovis* BCG and *M. bovis M. bovis* ΔRv2626c::hyg to pH stress measured by OD

In Middlebrook broth grown cultures of *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg, having reached stationary growth phase, were inoculated into **A)** neutral media (pH 7) and **B)** media with pH 5.3. Statistical analyses were done using a two-tailed, paired student's t-test. Results are expressed as mean with standard deviations, each value representing three cultures. This experiment was repeated three times with similar results. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

When analysing the RLU values, it was noticed that the ATP content of *M. bovis* ΔRv2626c::hyg grown at neutral pH was significantly lower than for *M. bovis* BCG on days 7, 14 and 28 (Figure 12A), although the OD measurement showed identical growth rates (Figure 11A). The same phenomenon was observed under pH stress conditions where the growth advantage of *M. bovis* ΔRv2626c::hyg was less evident according to the ATP measurement (Figure 12B) compared to the OD measurement (Figure 11B). In summary, the loss of *rv2626c* from *M. bovis* BCG has a positive effect on the adaption to pH stress while the over-expression of *rv2626c* hinders pH stress adaption.

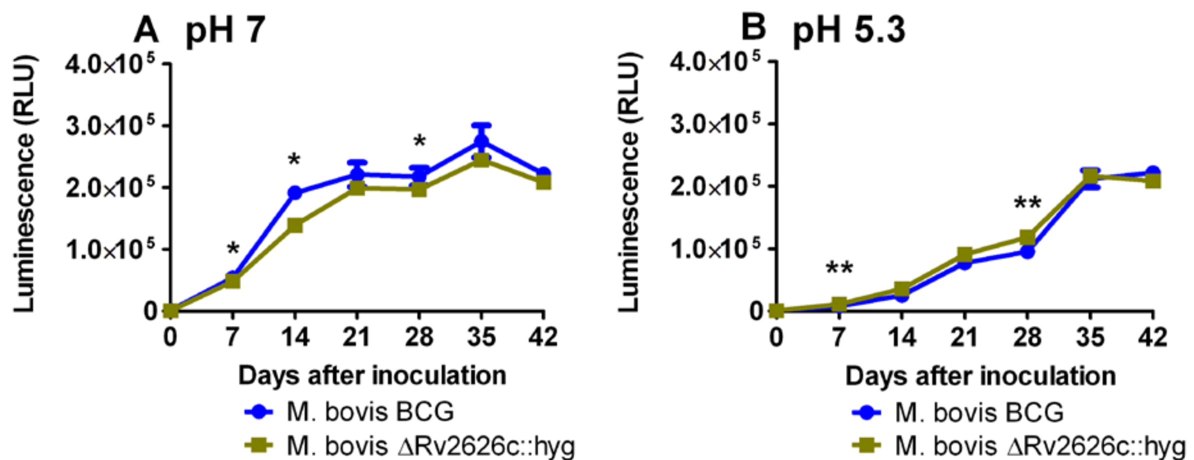


Figure 12. Adaptation of *M. bovis BCG and M. bovis ΔRv2626c::hyg* to pH stress measured by ATP quantification

In Middlebrook broth grown cultures of *M. bovis* BCG *and M. bovis* ΔRv2626c::hyg having reached stationary growth phase were inoculated into **A)** neutral media (pH 7) and **B)** media with pH 5.3. Statistical analyses were done using a two-tailed, paired student's t-test. Results are expressed as mean with standard deviations, each value representing three cultures. This experiment was repeated three times with similar results. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.2.2.2 MDP1 influences the adaption of BCG to low pH

In medium with neutral pH (7) *M. bovis* (pAs-MDP1) showed an accelerated growth and reached a higher cell density than *M. bovis* (pMV261) (Figure 13A). This advantage was reversed under low pH conditions where the antisense strain proved incapable to grow whereas it took *M. bovis* (pMV261) 14 days to adapt to the low pH conditions and start growing (Figure 13B). After 42 days, *M. bovis* (pMV261) almost reached the same cell density (OD 2.88) as under neutral pH conditions (OD 3.59) (Figure 13A). This corresponds to a cell density which is about 20% less than under normal pH conditions.

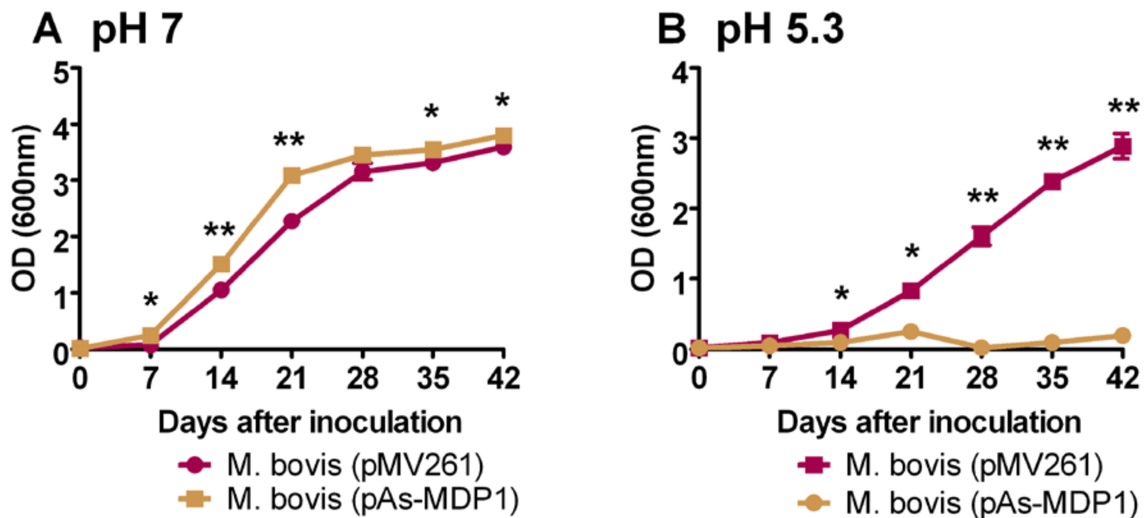


Figure 13. Adaptation of *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1) to pH stress measured by OD

In Middlebrook broth grown cultures of *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1) having reached stationary growth phase were inoculated into **A)** neutral media (pH 7) and **B)** media with pH 5.3. Statistical analyses were done using a two-tailed, paired student's t-test. Results are expressed as mean with standard deviations, each value representing three cultures. This experiment was repeated three times with similar results. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

The ATP measurement showed similar results for the neutral pH conditions (Figure 14A). Under low pH conditions the curve progression reflected the OD measurement (Figure 13B). The RLU values for *M. bovis* (pAs-MDP1) were 400 fold less than *M. bovis* (pMV261), which underlines the fact that this strain is totally unable to adapt to low pH conditions (Figure 14B).

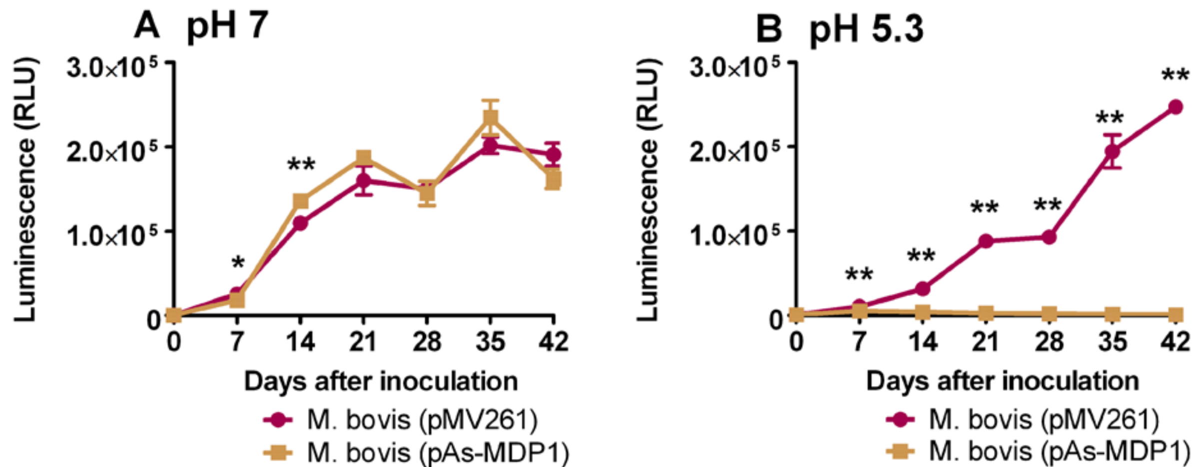


Figure 14. Adaptation of *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1) to pH stress measured by ATP quantification

In Middlebrook broth grown cultures of *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1), having reached stationary growth phase, were inoculated into **A)** neutral media (pH 7) and **B)** media with pH 5.3. Statistical analyses were done using a two-tailed, paired student's t-test. Results are expressed as mean with standard deviations, each value representing three cultures. This experiment was repeated three times with similar results. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.2.3 Rv2626c is expressed under pH stress conditions

Fisher and colleagues [131] recently mimicked the phagosomal acidification by incubating *M. tuberculosis* H37Rv for up to 30 minutes in low pH (5.5) conditions. During a subsequent genome wide microarray research *rv2626c* was not up-regulated. Since the pH stress experiment indicated a significant influence of *rv2626c* on the adaption/survival of *M. bovis* BCG under pH stress, it was decided to investigate this phenomenon by Western blotting. *M. bovis* BCG, *M. bovis* (pMV261), *M. bovis* ΔRv2626c::hyg and *M. bovis* (pRv2626c) were therefore cultivated in medium with neutral pH until all strains reached an $OD_{(600\ nm)}$ of 2. Samples were taken for protein isolation and the rest of the cultures were resuspended in liquid media with pH 5.3 for 24 and 48 hours respectively.

Proteins were also isolated from these samples and Rv2626c then detected by Western blot analysis.

In neutral media the autoradiogram of *M. bovis* BCG showed low amounts of Rv2626c (Figure 15A, lane 1) and high amounts for the over-expressing strain *M. bovis* (pRv2626c) (Figure 15A, lane 4). Under the same growth conditions no protein bands were detected for *M. bovis* ΔRv2626c::hyg and *M. bovis* (pMV261) (Figure 15A, lane 2&3).

When the same cultures were transferred into media with pH 5.3 the autoradiogram confirmed more prominent but consistent strong bands for Rv2626c in *M. bovis* BCG regardless whether the culture was grown for 24 or 48 hours under pH stress (Figure 15B &C, lane 5 & 9). Under the same conditions similar amounts of Rv2626c were detected in *M. bovis* (pMV261) (Figure 15B & C, lane 7 & 11). In the case of *M. bovis* ΔRv2626c::hyg the autoradiogram showed no protein bands (Figure 15, lane 6 & 10). When *M. bovis* (pRv2626c) was inoculated into low pH, the autoradiogram showed two bands indicating that Rv2626c exists in two different forms as predicted by Sharpe and colleagues [151] (Figure 15B & C, lane 8 and 12).

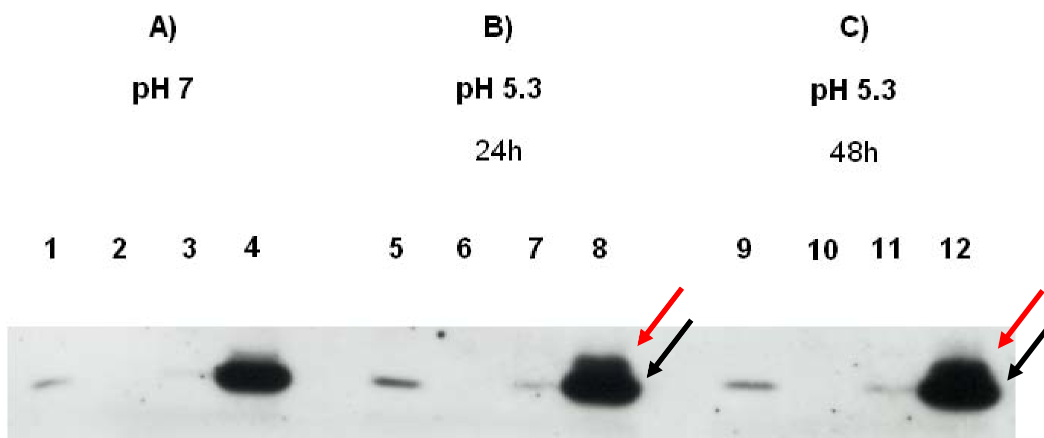


Figure 15. Confirmation of the over-expression of Rv2626c in *M. bovis* (pRv2626c) and the expression of Rv2626c under pH stress condition by means of Western blotting

The strains were grown in neutral Middlebrook broth until they had reached an $OD_{(600\ nm)}$ of 2 and were then transferred in medium with pH 5.3 for 24 and 48 hours. About 30 µg of total protein from *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg, *M. bovis* (pMV261) and *M. bovis* (pRv2626c) were separated in a 10% SDS-PAGE, transferred onto a PVDF membrane and incubated with a polyclonal rabbit antiserum against a peptide from Rv2626c. **Lane 1, 5, 9)** shows *M. bovis* BCG; **Lane 2, 6, 10)** *M. bovis* ΔRv2626c::hyg; **Lane 3, 7, 11)** *M. bovis* (pMV261) and **Lane 4, 8, 12)** shows *M. bovis* (pRv2626c). **Black arrow)** Rv262626c (16 kDa); **Red arrow)** Rv2626c probably 32 kDa.

3.2.4 Influence on the intracellular survival in human monocyte-derived macrophages

Highly pathogenic mycobacteria are known to survive inside acidified phagosomes of macrophages. Once it was shown that both genes interfered in the adaption/survival in liquid media at low pH, human monocyte-derived macrophages were infected with these strains using a MOI of 1. Samples were taken over a period of 5 days, beginning with 4 hours post infection. To calculate bacterial numbers in lysed monocyte-derived macrophages their DNA was isolated and then quantified by real-time PCR.

Analysing the data from three independent experiments with unstimulated monocytes-derived macrophages infected with *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg, *M. bovis* (pMV261) and *M. bovis* (pRv2626c) showed no significant growth advantage between the strains (data not shown). The experiment was then repeated with pre-activated monocyte-derived macrophages, but the result remained the same (Figure 16A & B).

In the case of *M. bovis* (pAs-MDP1) non-stimulated monocyte-derived macrophages were used to investigate the influence of MDP1 on the intracellular survival. The evaluated data indicate that the down-regulation of MDP1 hampers the growth of *M. bovis* (pAs-MDP1). The amount of phagocytosed bacteria was identical for both strains (after 4 hours of infection) but from thereon the bacterial numbers of *M. bovis* (pMV261) were increasing, whereas the numbers of *M. bovis* (pAs-MDP1) remained stagnant for the test period. At the end bacterial numbers of *M. bovis* (pMV261) were fourfold higher than for *M. bovis* (pAs-MDP1) whose bacterial numbers compared to the test start were slightly decreased (Figure 16C).

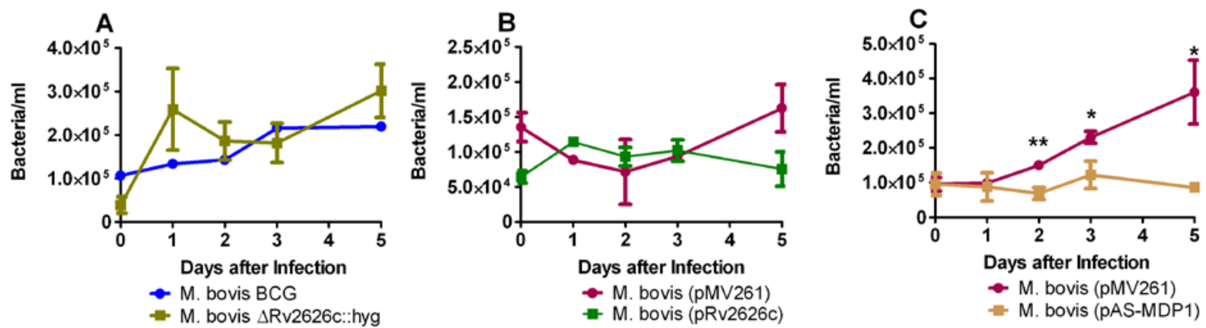


Figure 16. Intracellular survival of different *M. bovis* BCG derivates in human macrophages
Monocytes (1,0x10^{6}) from healthy volunteers were pre-activated and infected (MOI 1) with **A)** *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg or **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c). Unstimulated monocytes were infected with **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Infected monocytes were lysed at day 0 (after 4 hours), 1, 2, 3 and 5, the DNA isolated and then the amount of bacteria quantified by real-time PCR. The figures shown depict one representative result from three independent experiments. The values represent the mean of three infections with the standard deviation. Statistical analysis was done using a two-tailed, paired student's t-test. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.2.5 The influence on the secretion of selected cytokines by human PBMCs

PBMCs from human volunteers were infected with bacteria using a MOI of 1. The supernatants were taken 24 hours later, frozen at -20°C and measured at a later time point. Non-infected and non-stimulated PBMCs were used as negative control, whereas positive controls consisted of LPS and IFN-γ stimulated PBMCs. All chosen cytokines were induced after stimulation with LPS and IFN-γ (data not shown).

3.2.5.1 IL-1β secretion

PBMCs from human donors showed high fluctuations in the IL-1β concentration between different donors and strains. The experiments concerning the gene *rv2626c* were repeated eight times. The measured IL-1β concentration in the supernatants from cultures infected with *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg showed only minor distinctions between both strains (Figure 17A). Contrary to this result PBMCs showed a strong significant up-regulation of IL-1β (P=0.0061) when they were infected with *M. bovis* (pRv2626c) (Figure 17B). Obviously the over-expressing strain induced such a strong impulse that the PBMCs doubled the amount of IL-1β compared to *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg and *M. bovis* (pMV261).

The same PBMCs were used for the infection experiments with *M. bovis* (pAs-MDP1). The down-regulation of MDP1 in *M. bovis* obviously caused changes in the immune recognition which led to a significant down-regulation of IL-1β (Figure 17C).

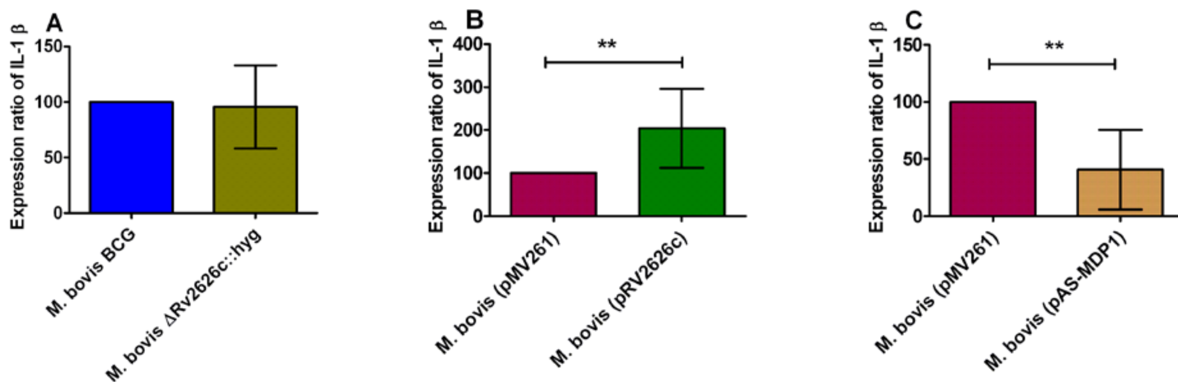


Figure 16. IL-1β secretion by infected PBMCs

Human PBMCs (1.0x10^{6}) from different volunteers were infected (MOI 1) with **A)** *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg, **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c) or **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Supernatants were taken after 24 hours and the amount of IL-1β was measured by ELISA. The values represent the mean of five independent cultures with standard deviation. The expression profile was calculated by normalizing to the control strains *M. bovis* BCG and *M. bovis* (pMV261), respectively, and then measuring the percentile deviation of *M. bovis* ΔRv2626c::hyg, *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1). Statistical analyses were done using a two-tailed, unpaired t-test. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.5.2.2 TNF-α secretion

TNF-α is another pro-inflammatory cytokine which is known to be important for its ability to promote phagocytosis and intracellular killing of mycobacteria. Experiments using PBMCs infected with *M. bovis* ∆Rv2626c::hyg and *M. bovis* (pAs-MDP1) showed similar TNF-α concentrations (Figure 18A & C) compared to their control strains *M. bovis* BCG and *M. bovis* (pMV261) which means that neither the loss of *rv2626c* nor the down-regulation of *mdp1* had a significant influence on the regulation of TNF-α. In the case of the over-expressing strain *M. bovis* (pRv2626c) the measured TNF-α concentration was significantly higher than for *M. bovis* (pMV261) (*P=0.0305). This experiment was repeated seven times, the highest up-regulation was about 195% (Figure 18B).

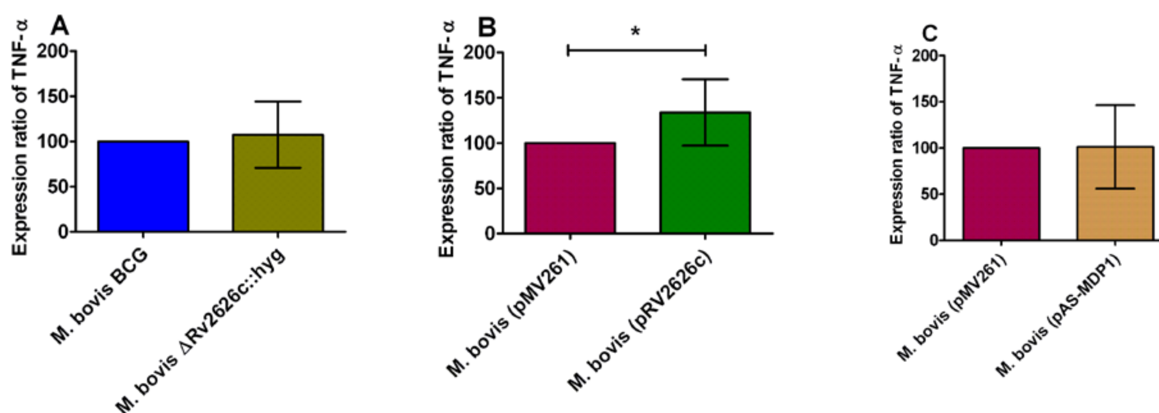


Figure 17. TNF-α secretion by infected PBMCs

Human PBMCs ($1{,}0x10^6$) from different volunteers were infected (MOI 1) with **A)** *M. bovis* BCG and *M. bovis* ∆Rv2626c::hyg, **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c) or **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Supernatants were taken after 24 hours and the amount of TNF-α was measured by ELISA. The values represent the mean of five independent cultures with standard deviation. The expression profile was calculated by normalizing to the control strains *M. bovis* BCG and *M. bovis* (pMV261), respectively, and then measuring the percentile deviation of *M. bovis* ∆Rv2626c::hyg, *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1). Statistical analyses were done using GraphPad Prism 5. A two-tailed, unpaired t-test was used to determine significance of the expression ratio of the TNF-α concentration. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.5.2.3 IFN-γ secretion

The third investigated pro-inflammatory cytokine was IFN-γ which is exclusively produced by lymphocytes. It was not possible to discern any significant changes in the IFN-γ concentrations. The experiments regarding *rv2626c* were repeated six times, the experiments regarding *mdp1* five times. Nevertheless trends to higher IFN-γ concentrations were seen for *M. bovis* (pRv2626c) (Figure 19B) and lower concentrations in the case of *M. bovis* (pAs-MDP1) where about 80% of the donors showed a down-regulation of IFN-γ of about 50% (Figure 19C). As in the previous experiments the loss of *rv2626c* produced similar results as the wild-type strain *M. bovis* BCG (Figure 19A).

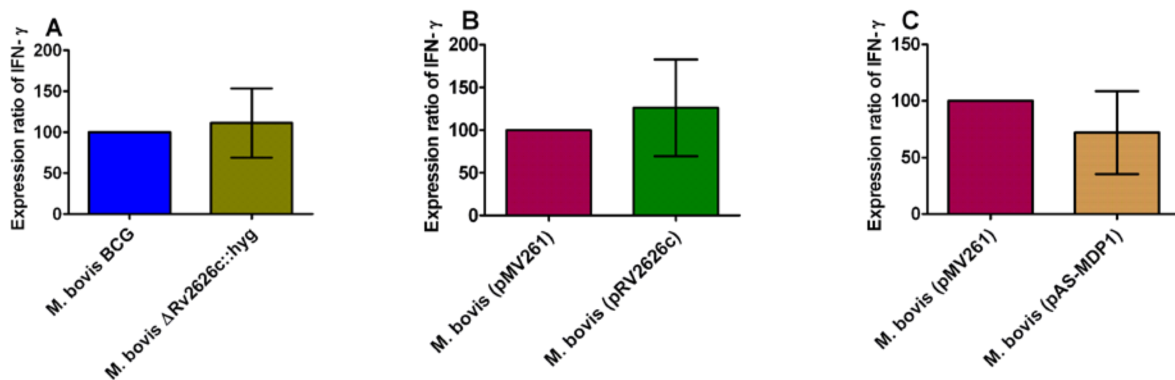


Figure 18. IFN-γ secretion by infected PBMCs

Human PBMCs ($1.0x10^6$) from different volunteers were infected (MOI 1) with **A)** *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg, **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c) or **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Supernatants were taken after 24 hours and the amount of IFN-γ was measured by ELISA. The values represent the mean of five independent cultures with standard deviation. The expression profile was calculated by normalizing to the control strains *M. bovis* BCG and *M. bovis* (pMV261), respectively, and then measuring the percentile deviation of *M. bovis* ΔRv2626c::hyg, *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1). Statistical analyses were done using GraphPad Prism 5. A two-tailed, unpaired t-test was used to determine significance of the expression ratio of the IFN-γ concentration. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.5.2.4 IL-10 secretion

PBMCs infected with *M. bovis* ΔRv2626c::hyg displayed no significant differences in the concentration of the anti-inflammatory cytokine IL-10 compared to the control strain *M. bovis* BCG, which was used for normalization (Figure 20A). Contrary to the loss of Rv2626c PBMCs infected with the over-expression strain showed strong tendencies for up-regulating IL-10 (Figure 20B). In some donors the concentration was about 450% higher than in *M. bovis* (pMV261). The experiments regarding *rv2626c* were repeated six times. In the case of *M. bovis* (pAs-MDP1) 80% of the donors showed tendencies for down-regulating IL-10. In some cases the IL-10 concentration was up to 85% less than in *M. bovis* (pMV261) (Figure 20C). Experiments regarding the *mdp1* were repeated five times.

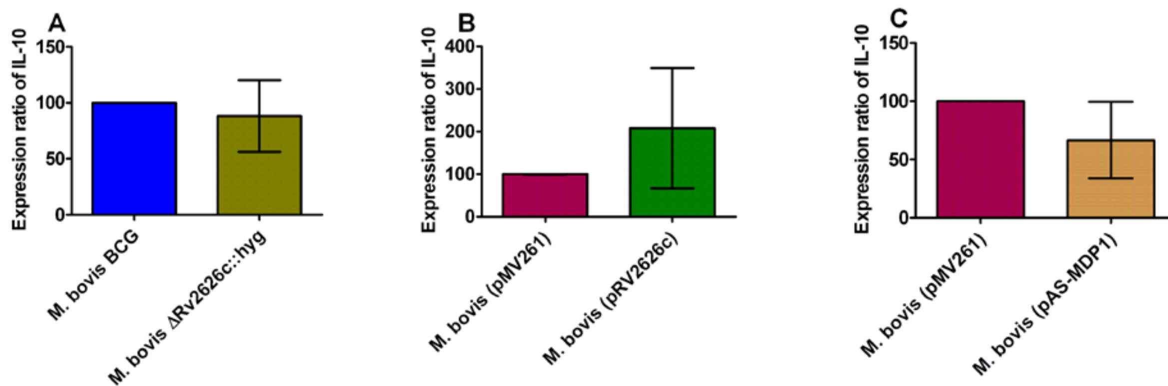


Figure 19. IL-10 secretion by infected PBMCs

Human PBMCs ($1.0x10^6$) from different volunteers were infected (MOI 1) with **A)** *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg, **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c) or **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Supernatants were taken after 24 hours and the amount of IL-10 was measured by ELISA. The values represent the mean of five independent cultures with standard deviation. The expression profile was calculated by normalizing to the control strains *M. bovis* BCG and *M. bovis* (pMV261), respectively, and then measuring the percentile deviation of *M. bovis* ΔRv2626c::hyg, *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1). Statistical analyses were done using GraphPad Prism 5. A two-tailed, unpaired t-test was used to determine significance of the expression ratio of the IL-10 concentration. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.5.2.5 IL-8 secretion

Human PBMCs infected with *M. bovis* (pRv2626c) showed a significant down-regulation (**P=0.0005) of IL-8 (Figure 21B) whereas the loss of *rv2626c* in *M. bovis* ∆v2626c::hyg or the down-regulation of *mdp1* in *M. bovis* (pAs-MDP1) had no significant effect on the IL-8 production in *M. bovis* BCG (Figure 21A) and *M. bovis* (pMV261) (Figure 21C).

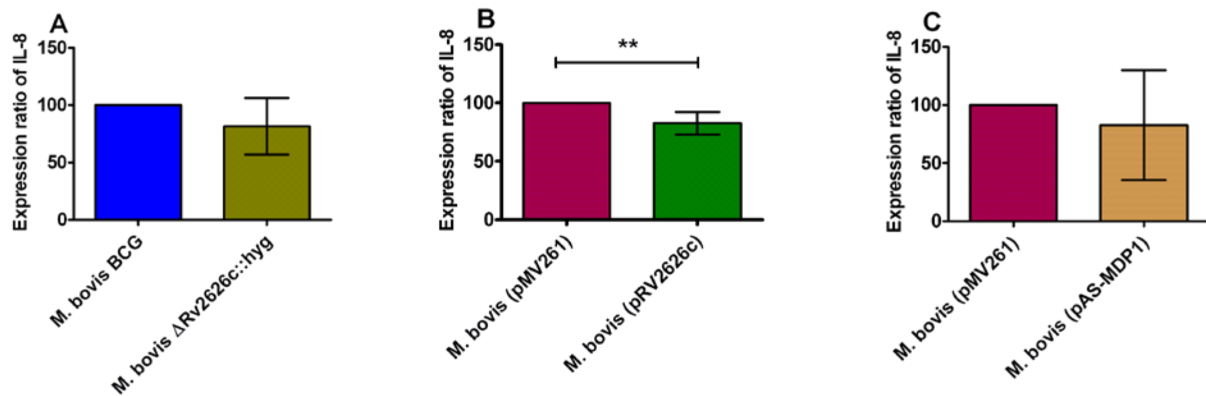


Figure 20. IL-8 secretion by infected PBMCs

Human PBMCs (1.0x10^6) from different volunteers were infected (MOI 1) with **A)** *M. bovis* BCG and *M. bovis* ∆Rv2626c::hyg, **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c) or **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Supernatants were taken after 24 hours and the amount of IL-8 was measured by ELISA. The values represent the mean of five independent cultures with standard deviation. The expression profile was calculated by normalizing to the control strains *M. bovis* BCG and *M. bovis* (pMV261), respectively, and then measuring the percentile deviation of *M. bovis* ∆Rv2626c::hyg, *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1). Statistical analyses were done using GraphPad Prism 5. A two-tailed, unpaired t-test was used to determine significance of the expression ratio of the IL-8 concentration. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.2.6 The influence on the NO concentration in J774.A1 cells

When human monocytes were used to measure the NO concentration, no NO production could be detected, even if the cells were pre-activated with LPS and human IFN-γ (data not shown). For this reason the mouse cell line J774.A1 was used to measure changes in the NO concentration. About 24 hours after infection the NO concentration in supernatants and freshly prepared standard were measured using the Griess assay. When mouse macrophages were infected with *M. bovis* ∆Rv2626c::hyg the NO concentration was identical to the wild-type strain *M. bovis* BCG (Figure 22A).

In contrast the infection with *M. bovis* (pRv2626c) caused a significantly lower NO concentration (**P=0.0079; Figure 22B). In the supernatants of mouse macrophages infected with *M. bovis* (pAs-MDP1) lower concentrations of NO were measured (Figure 22C) in comparison to *M. bovis* (pMV261).

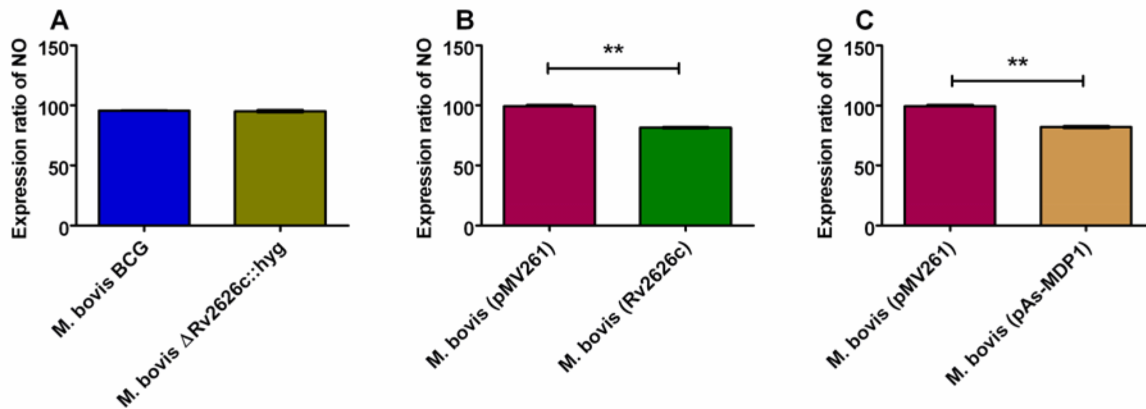


Figure 21. NO secretion by infected J774.A1 macrophages

Mouse macrophages (2.5×10^5) were infected (MOI 50) with **A)** *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg, **B)** *M. bovis* (pMV261) and *M. bovis* (pRv2626c) or **C)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Supernatants were taken after 24 hours and the amount of NO was measured by ELISA. The values show one representative experiment out of three with the mean of five independent cultures with standard deviation. The expression profile was calculated by normalizing to the control strains *M. bovis* BCG and *M. bovis* (pMV261), respectively, and then measuring the percentile deviation of *M. bovis* ΔRv2626c::hyg, *M. bovis* (pRv2626c) and *M. bovis* (pAs-MDP1). Statistical analyses were done using GraphPad Prism 5. A two-tailed, paired t-test was used to determine significance of the expression ratio of the NO concentration. A P value <0.05 was considered significant (*) and a P value <0.01 was considered very significant (**).

3.2.7 Fusion of monocyte-derived macrophages

Having investigated the influence of Rv2626c and MDP1 on the cytokine expression and intracellular survival in human PBMCs and monocytes, it was examined whether the genes interfere with the ability of monocyte-derived macrophages to fuse with each other. This characteristic is an important step towards multinucleated cell and multinucleated giant cell formation seen under experimental conditions or inside granulomas. First, the most appropriate conditions, notably time point and MOI, were investigated to achieve reproducible results.

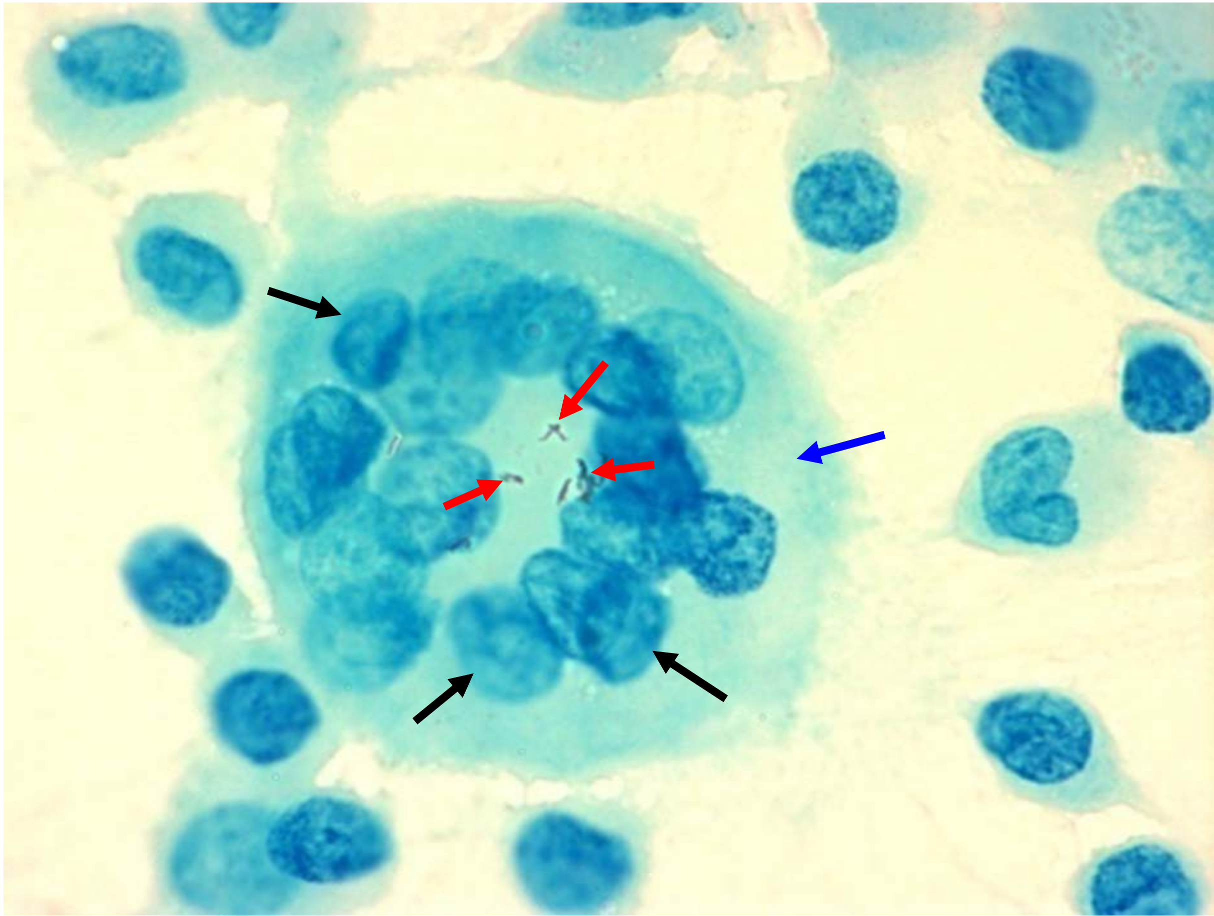


Figure 22. Ziehl-Neelsen staining of human monocyte-derived macrophages infected with mycobacteria

The figure shows a multinucleated giant cell (blue arrow) seven days after infection with 19 nuclei (black arrows) and some phagocytosed bacteria inside (red arrows).

The infection was demonstrated by Ziehl-Neelsen staining, proving that the bacteria were present in fused cells (Figure 23). Optimal conditions were reached by using a MOI 1 and a test period of seven days (data not shown).

In order to analyse the effect of *rv2626c* and *mdp1* on the fusion process, monocyte-derived macrophages were isolated from healthy human donors and then incubated in 24 Tissue Culture Plates. Prior to infection, the wells were washed several times to get rid of other cell types. Seven days after infection the experiment was stopped and the fusion index calculated by counting the nuclei present in fused and non-fused macrophages.

In the case of *M. bovis* ΔRv2626c::hyg the fusion index (FI) reached a value of 16% which was about 45% higher than for its reference strain *M. bovis* BCG (FI 11%). On the other hand the fusion index of *M. bovis* (pRv2626c) reached 4%, up to 60% less than for *M. bovis* (pMV261). In two of three experiments the fusion index of the over-expressing strain was even lower than the fusion index of non-infected macrophages (Figure 24A). In the case of *M. bovis* (pAs-MDP1) the down-regulation of MDP1 increased the fusion of monocyte-derived macrophages (FI 15%).
The fusion index was up to 66% higher than seen in *M. bovis* (pMV261) where the fusion index reached a value of 9% (Figure 24B).

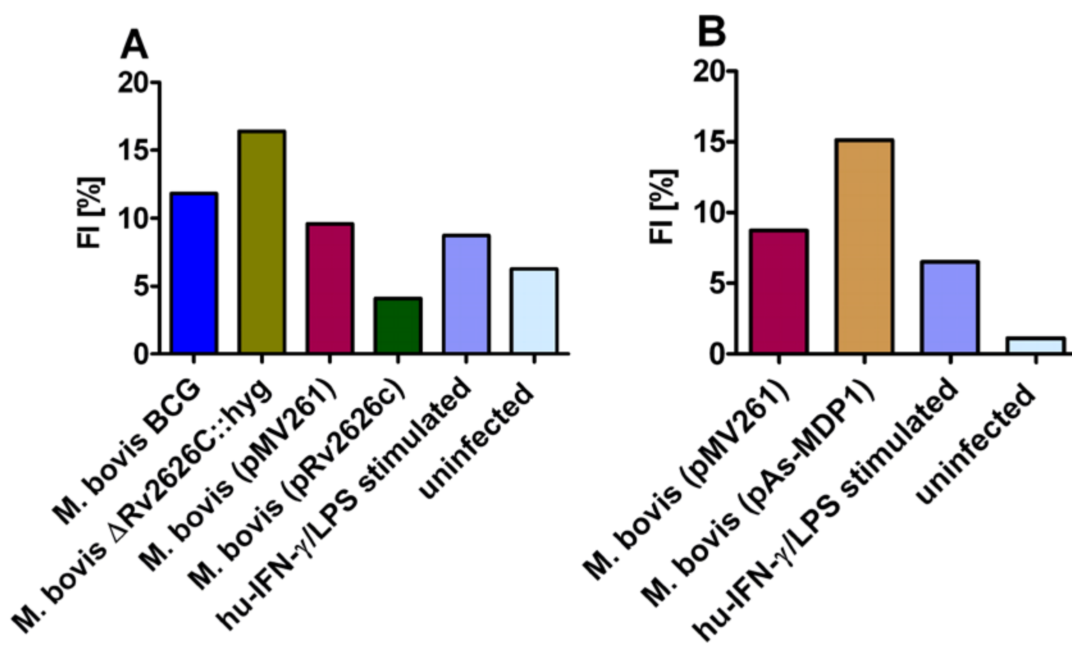


Figure 234. Influence of the genes *rv2626c* and *mdp1* on the fusion rate of human monocyte-derived macrophages

About ($1.0x10^6$) monocyte-derived macrophages from healthy human donors were infected (MOI 1) with **A)** *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg, *M. bovis* (pMV261) and *M. bovis* (pRv2626c) and **B)** *M. bovis* (pMV261) and *M. bovis* (pAs-MDP1). Macrophages stimulated with human IFN-γ and LPS served as positive control whereas non-stimulated, non-infected monocyte-derived macrophages served as negative control (**A & B**). Samples were taken seven days after infection, the cells stained with Diff-Quick, photographed and the fusion index calculated by dividing the number of nuclei in multi-nucleated cells by the total number of nuclei multiplied by 100. The figure shows one representative experiment out of three independent experiments.

Examples of fused monocyte-derived macrophages forming MC and MGC are depicted in figure 25.

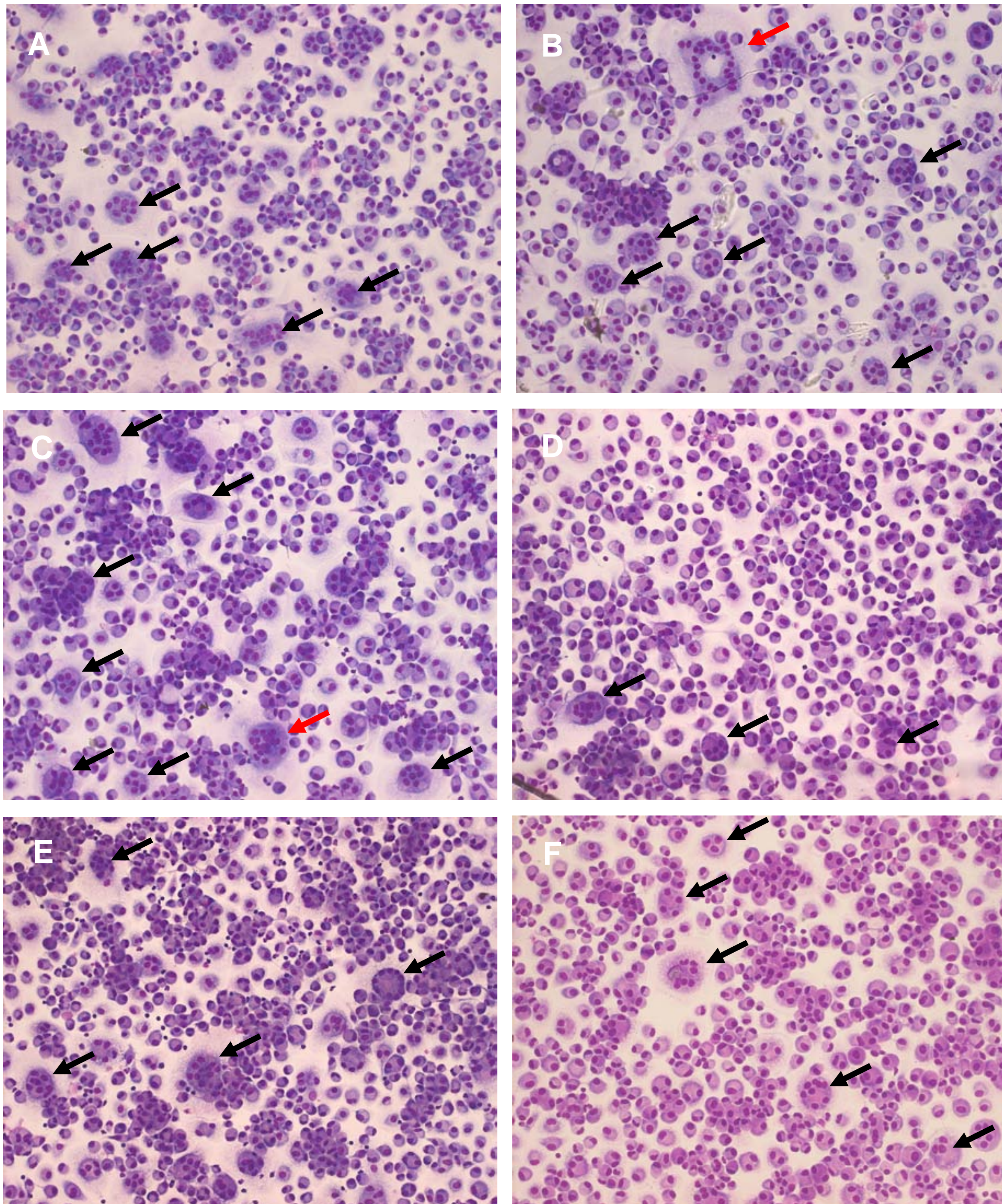


Figure 24. Examples of fused monocyte-derived macrophages which have formed multinucleated and multinucleated giant cells

Monocyte-derived macrophages infected with different mycobacterial strains (MOI 1). **A)** *M. bovis* BCG, **B)** *M. bovis* ΔRv2626c::hyg, **C)** *M. bovis* (pMV261), **D)** *M. bovis* (pRv2626c), **E)** human IFN-γ and LPS stimulated monocytes (positive control) and **F)** non-stimulated, non-infected monocytes (negative control). Seven days after infection the cells were stained with Diff-Quick. Black arrows represent multinucleated cells (from 3 to 14 nuclei) and red arrows represent multinucleated giant cells (15 nuclei and upwards).

3.2.8 The mutantΔRv2626c::hyg survives better in Balb/c mice than wild-type BCG

Furthermore the influence of the mutant strain *M. bovis* ΔRv2626c::hyg was assessed in an *in vivo* mouse model. For this reason *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg were used to infect Balb/c mice to see how far the loss of the gene *rv2626c* influences the virulence of this strain. Twelve mice were infected with the *M. bovis* ΔRv2626c::hyg and twelve with the *M. bovis* BCG. After 17 and 25 days six animals of each group as well as three non-infected control mice were killed and liver, lung, lymph nodes and spleen were removed. Parts of the organs were homogenised for a *M. bovis*-specific real-time PCR or fixed and pathologically examined.

The pathological examination from liver and spleen samples of day 17 revealed that in three of the six livers infected with the *M. bovis* BCG granuloma were present. The granulomas were dominated by histiocytic and granulocytic infiltrates which were in the periphery surrounded by low numbers of lymphocytes (Figure 25). After 25 days of infection granulomas were detected in livers and spleens of all mice whether they were infected with *M. bovis* BCG or *M. bovis* ΔRv2626c::hyg. The granulomas investigated in livers showed a mild multifocal, chronic hepatitis whereas the granulomas in spleens had induced a minimal, multifocal, chronic splenitis.

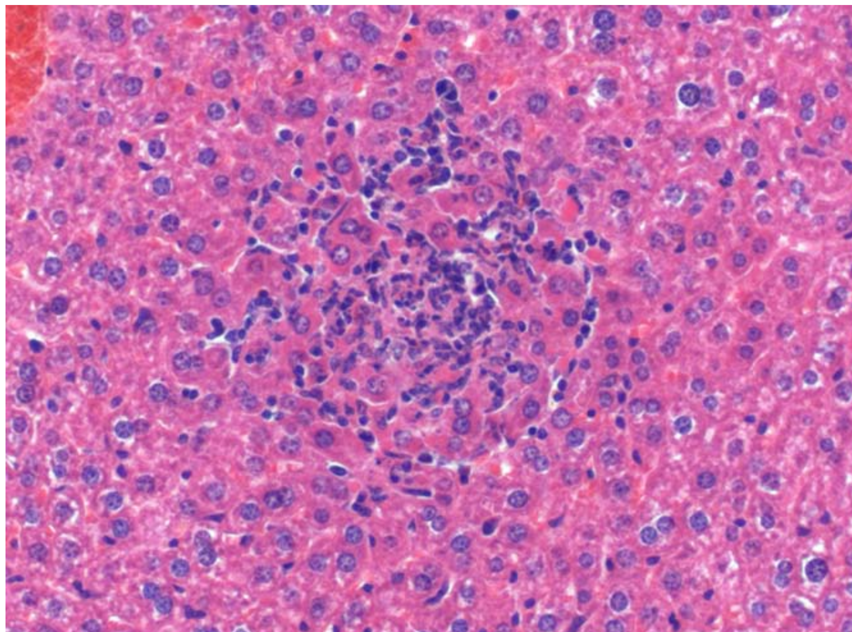
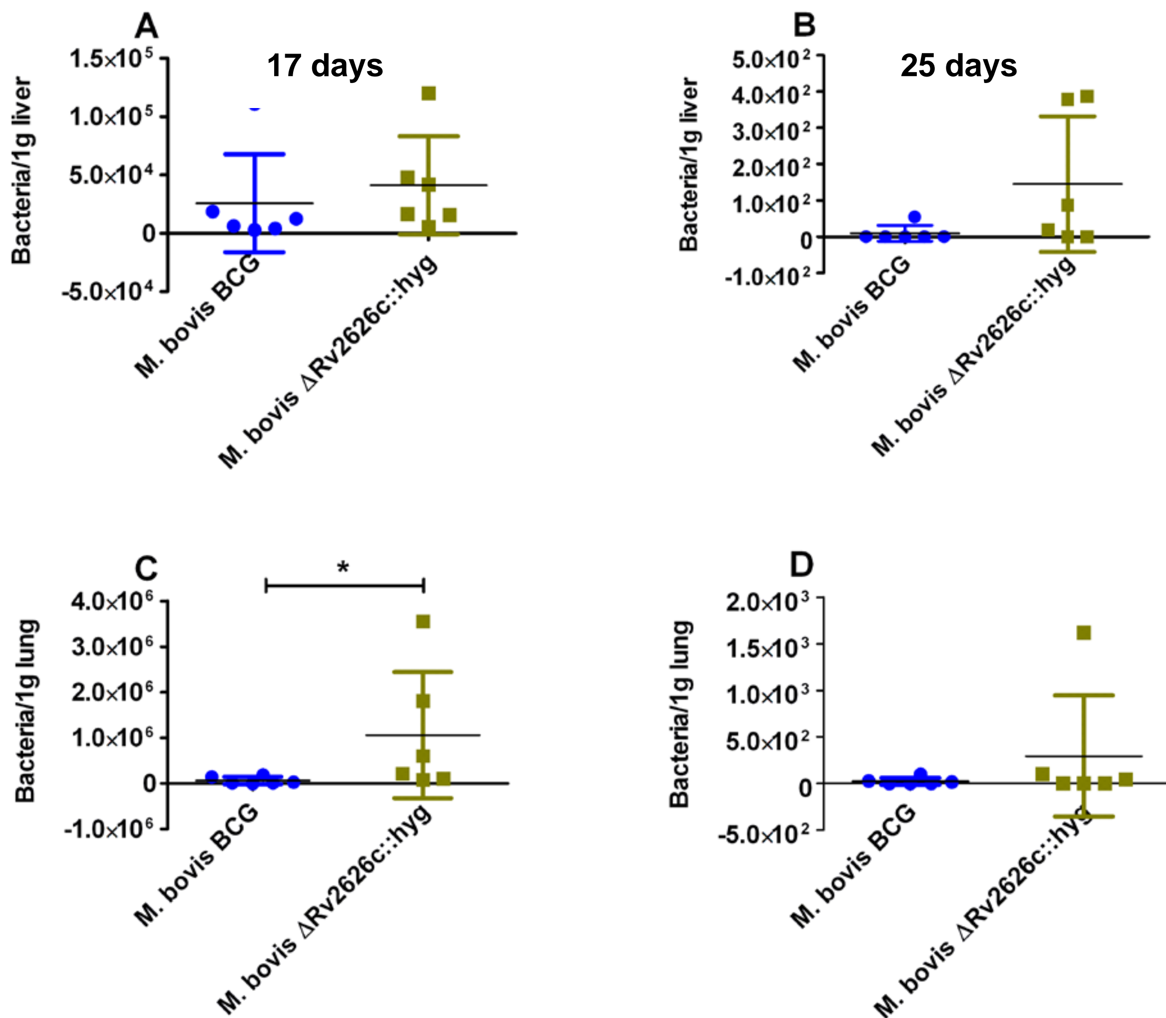


Figure 25. Early stage of granuloma formation in mouse liver after 17 days of infection, infected with *M. bovis* BCG; hematoxylin and eosin stain (HE) and Ziel-Neelsen stained

.The result of the real-time PCR suggests that *M. bovis* ΔRv2626c::hyg is more virulent when it comes to survival inside the organs of Balb/c mice than *M. bovis* BCG. After 17 and 25 days higher numbers of *M. bovis* ΔRv2626c::hyg were detected in liver (Figure 26A&B), lung (Figure 26 C&D), lymph nodes (Figure 26 E&F) and spleen (Figure 26G&H) compared to *M. bovis* BCG. Although this could be observed for both time points, the detected bacterial numbers in all infected mice were much lower after 25 days as compared to 17 days. This suggests that both strains were equally eliminated during this time interval.



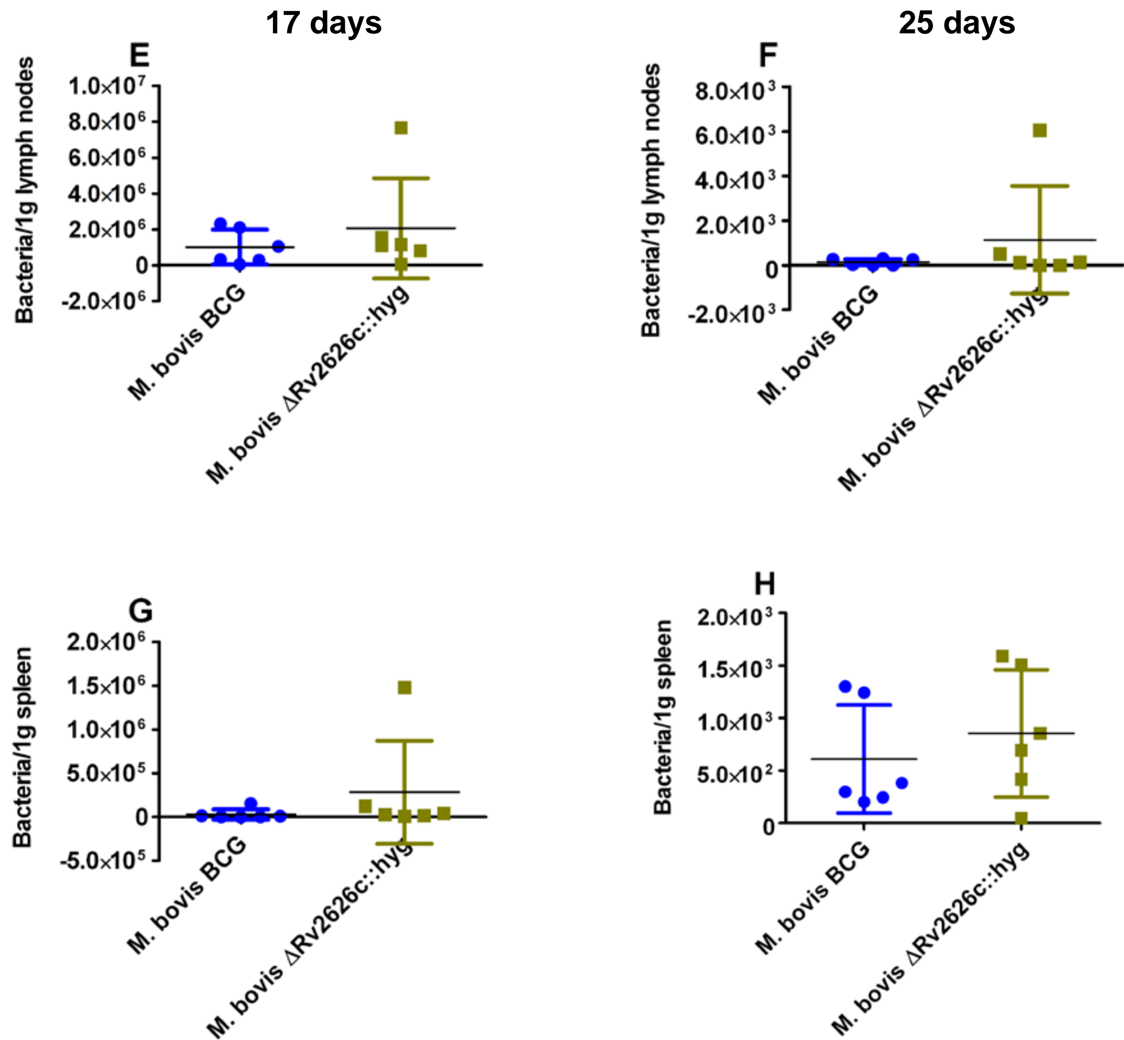


Figure 26. The loss of *rv2626c* increases the virulence of *M. bovis* ΔRv2626c::hyg

Twelve Balb/c mice were intravenously infected with 5.0 x 10^5 bacteria from *M. bovis* BCG and another twelve with *M. bovis* ΔRv2626c::hyg. After 17 and 25 days six infected mice from each group and three non-infected mice were killed, their organs removed and dilutions of the homogenized organs used for real-time PCR. Figure **A, B)** shows the bacterial numbers isolated from livers, **C, D)** from lung, **E, F)** from lymph nodes and **G, H)** from spleens after 17 and 25 days. Data shown are the median of six animals with standard deviation. Statistical analysis was done using a nonparametric Mann Whitney test. A P value <0.05 was considered significant (*) and a P value <0.01 was considered highly significant (**).

4. Discussion

In 1993, WHO declared tuberculosis a global emergency. Despite the availability of effective chemotherapy the incident cases of tuberculosis are steadily rising and will do so in future, due to the rapidly increasing world population. The problem is even more pressing since the appearance of co-infections with HIV and the emergence of multidrug resistant (MDR) and extensively drug-resistant tuberculosis (XDR). To find new ways to cure tuberculosis, it is important to understand the effects of virulence-associated genes and their interactions with the immune system. For this reason the mycobacterial genes *rv2626c* and *mdp1* were chosen. Both genes had been previously shown to be potentially important for adaptation processes, in turn allowing the bacteria to survive in the hostile environment of the host immune system.

Rv2626c is one of the most strongly up-regulated open reading frames in the dormancy survival regulon (DosR) [129, 148]. This regulon is essential for the adaption to stress conditions, which are thought to be confronted by bacteria under *in vivo* conditions. Roupie and colleagues [153] detected very strong T and B cell responses in BALB/c and C57BL/6 mice against Rv2626c, likewise high serum antibody responses were discovered in patients with active tuberculosis [156] whereas *M. tuberculosis* specific T cell lines showed the potential to elicit cellular immune responses against Rv2626c [146]. These findings led to the conclusion that this protein is important for stress response and modulation of the host immune response. Aside from this hypothesis, its biological function remains unclear. It is thought that Rv2626c is useful as antigenic marker of persistent bacteria in dormant tuberculosis [147].

The mycobacterial DNA binding protein (MDP1) from *M. tuberculosis* features properties which connect this gene with characteristics for virulence, intracellular parasitism and long term persistence like the ability to slow down growth of *M. smegmatis* [162]. Contrary to *rv2626c*, the *mdp1*-gene is not controlled by the DosR regulon, its localisation in the mycobacterial cell wall exposes it and makes it vulnerable to the immune cells of the host.

The discovery of antibodies in tuberculosis patients [160], in patients with inflammatory bowel disease [183] and in the serum of animals with clinical paratuberculosis [184] suggests that this protein might be an interesting candidate as diagnostic marker and potential drug target. Recent studies performed by our group indicate that MDP1 influences the growth characteristics in *M. bovis* BCG [165]. At that time, the antisense strain *M. bovis* (pAs-MDP1) was used in which the protein concentration was traceable down-regulated by about 50%.

Human lung alveolar macrophages come into first contact with inhaled bacteria *in vivo* but they are difficult to obtain. To investigate experimental issues like intracellular survival, it is a common method to use peripheral blood monocytes or monocyte-derived macrophages isolated from human donors. Buffy coats purchased from the German Red Cross were collected from anonymous healthy volunteers. At the beginning of this project blood smears were prepared to see whether the blood composition corresponded to defined standard conditions. Under standard conditions leucocytes of adults, seen in the differential blood count, consist of banded neutrophils (3-5%), segs (54-62%), eosinophils (1-4%), basophiles (0-1%), monocytes (3-8%) and lymphocytes (25-45%).
Deviations from the standard conditions in number and distribution of the constituent suggest that the donor might suffer from an unknown disease. Even the consistency/structure of the blood cells gives some clues about various ailments. Miniaturised or deformed erythrocytes for example might be a sign for iron deficiency. Since iron is an obligate cofactor for at least 40 different enzymes, iron deficiency might not only influence the outcome of tuberculosis, but also the experiments important for this study.
When it was noticed that the composition of leucocytes in many Buffy coats deviated from standard conditions, this selection criteria was ignored and the Buffy coats used for infection experiments. By this means, it was tolerated that results might be much more volatile than using only Buffy coats where the composition of leucocytes corresponds to the standard conditions. Although the density gradient centrifugation is a particular robust method for PBMCs isolation, donor depending varying cell populations cannot be avoided. These variations in leukocyte composition might influence the outcome of some experiments especially the cytokine expression.

The composition of monocyte-derived blood macrophages obtained from isolated PBMCs by Percoll gradient centrifugation leads to more consistent results since washing steps prior infection guarantee that only adhered macrophages stayed behind ready for infection.

For investigating the virulence associated properties of the genes *rv2626c* and *mdp1*, it was decided that a differential gene expression in *M. bovis* BCG by up-regulation, down-regulation and site-directed mutagenesis would be the best way. Genetic manipulations in the genus Mycobacterium, especially slow growing mycobacteria like *M. bovis* BCG or *M. tuberculosis*, are time consuming and very frustrating. A wide range of factors complicate this procedure in particular the thick and waxy cell wall, the nature of the transforming DNA, the selectable marker, the growth medium, the conditions of electroporation, the used strains/species [185] and as well as the high GC-content of the genome [186]. Illegitimate recombinations are a special problem associated with site-directed mutagenesis. Linear fragments have a frequency of 10^{-5} to 10^{-4} to jump into illegitimate sites throughout the mycobacterial genomes [187] resulting in wrong positive results. In these cases colonies grow on selection agar, but the gene of interest is not deleted. Another problem is that most molecular techniques were optimised for far related species like *E. coli* or *Bacillus subtilis*, nevertheless electroporation is the method of choice for mycobacteria.

Batoni and colleagues [188] first introduced the *hsp*60 promoter from *M. bovis* BCG into a clinical isolate of *M. avium* (strain NSN 94) as a tool for growth experiments in broth cultures which is also works for mycobacteria present inside murine macrophages. For the over-expression strain the coding sequence of *rv2626c* encompassing 432 bp, was cloned behind the *hsp*60 promoter of the shuttle vector pMV261 which constitutes a strong constitutive promoter. Once the sequence of the over-expressing plasmid pRv2626c was monitored by sequencing, the plasmid was transformed into *M. bovis* BCG and the new strain named *M. bovis* (pRv2626c).

To construct the deletion mutant of *M. bovis* BCG a two step cloning procedure was used. The down-stream (1209 bp) and up-stream region (1090 bp) of *rv2626c* were amplified by PCR. The linear fragments were then successively cloned into two separate multiple cloning sites on the cosmid pYUB854 flanking the hygromycin resistance gene cassette for later selection.

Care was taken to ensure that the same orientation for both sequences remained unchanged. The linear recombination substrate, consisting of the hygromycin resistance gene framed by the complete DNA sequences of *rv2625c* and *rv2627c* adjacent to *rv2626c*, was electroporated into *M. bovis* BCG. The deletion of *rv2626c* in *M. bovis* was investigated by Southern Blot analysis. For this purpose DNA from *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg was digested, transferred onto a membrane and then hybridized with the digoxigenin labelled sequence of *rv2626c* which was amplified from *M. bovis* BCG. The bands appearing on the autoradiogram confirmed the success of the homologous recombination. The new strain was named *M. bovis* ΔRv2626c::hyg.

Next it was investigated if the chosen culture conditions met the right conditions needed to activate the DosR regulon which also includes *rv2626c*. For this purpose *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg, *M. bovis* (pMV261) and *M. bovis* (pRv2626c) were grown to exponential and stationary phase, proteins were isolated, separated by SDS-PAGE and then, using a polyclonal rabbit antiserum, detected by Western blotting. In the case of *M. bovis* (pRv2626c) the autoradiogram showed equal amounts of Rv2626c in exponential and stationary phase cultures. This is because the *hsp*60 heat shock promoter of the over-expressing shuttle vector pRv2626c is constitutively active. On the other side Rv2626c was not expressed in exponential phase cultures of *M. bovis* BCG, *M. bovis* ΔRv2626c::hyg or *M. bovis* (pMV261). This changed in stationary phase cultures, where high amounts of Rv2626c were detected in *M. bovis* BCG and *M. bovis* (pMV261) but not in *M. bovis* ΔRv2626c::hyg.

The deletion of *rv2626c* was not only confirmed a second time but the experimental setup also proved that the chosen cultivation method corresponds roughly to the "*NRP oxygen depletion model*" defined by Dr. Wayne [116].

The up-regulation of *rv2626c* under culture conditions was important since infection experiments should be carried out with bacterial strains in which the DosR regulon and *rv2626c* are active.
This is particularly important for short-lived experiments like the cytokine experiments. Consequently, the bacterial strains were grown to stationary phase before they were frozen for later use in infection experiments to investigate the possible virulence associated capabilities of this protein.

The mutagenesis of the *mdp1*-gene followed the same cloning procedure as for *rv2626c*. In the case of the over-expression strain the coding sequence of *mdp1*, encompassing 645 bp, was cloned behind the *hsp*60 promoter of the shuttle vector pMV261. Once electroporated into *M. bovis* BCG the result was examined by isolating pMDP1 from grown colonies followed by sequencing. It turned out that all gene sequences displayed partial deletions in the *hsp*60 promoter of the shuttle vector, showed point mutations in the start codon of the cloned *mdp1*-gene or partially deletions in the coding *mdp1*-sequence. This indicates that a strong over-expression of MDP1 might have harmful or lethal consequences for *M. bovis* BCG.

Similar problems were expected when it came to the deletion of the *mdp1*-gene. Since the protein content of MDP1 totals roughly 7 to 10% of total proteins [158], it was assumed that the *mdp1*-gene might be essential for the survival of *M. bovis* BCG. Due to the fact that Chen and colleagues [189] reported the successful accomplishment of a *mdp1* deletion mutant in *M. bovis* BCG, it was decided to generate a deletion mutant using the same approach and cosmid as for the *rv2626c* deletion mutant. The down-stream (1210 bp) and up-stream region (1091 bp) of the *mdp1*-gene were amplified and then successively cloned into the cosmid pYUB854. The recombination substrate was electroporated in *M. bovis* BCG. Despite several attempts, no colonies were growing on the plates. This result was taken as confirmation that *mdp1* is essential for the bacterial survival. Due to the complete failure to manipulate the *mdp1*-gene by over-expression and deletion this experimental approach was discarded and the antisense strain *M. bovis* (pAs-MDP1) used to further analyse the virulence associated properties of *mdp1*.

The antisense technique is an alternative method to assess the importance of genes, especially when mutagenesis proved lethal or when it comes to repress genes which are present in several copies.

In past, the antisense technique contributed to the clarification of the function of many mycobacterial genes like the repression of *ahpC* from *M. bovis* [190], *dnaA* from *M. smegmatis* [191], FAP-P from *M. avium* subsp. *paratuberculosis* [192] or *pknF* from *M. tuberculosis* [193].

For creating the pAs-MDP1 antisense plasmid a 113 bp fragment of BCG-DNA was amplified, covering the first 102 bp of the coding sequence from the *mdp1*-gene and 11 bp of the untranslated upstream region containing the Shine-Dalgarno Sequence. This antisense fragment was cloned into the multiple cloning site of the shuttle vector pMV261 and than introduced in *M. bovis* BCG. Western blot and ELISA from aerobically grown cultures proved a strong down-regulation of *mdp1* [165].

Once the cloning experiments were completed the strains were investigated in liquid cultures under conditions mimicking the acidic conditions of the phagosomal (pH 5.3) compartment. Alveolar macrophages are known to contain phagocytosed mycobacteria in a rapidly acidifying phagosome. Depending on the activation state of the macrophages the pH ranges from 4.5 to 6.2 [26, 194] and is thought to be essential for mycobacteria to activate virulence factors needed for long-term survival. Some pathogens are known to survive better in acidic phagosomes, known examples are *Legionella pneumophila* [195] and *Coxiella burnetii* [196, 197], whereas *Trypanosoma cruzi*, recruits lysosomes as an integral part of the invasion strategy [198].

Since mycobacteria are known to form aggregates which might give unreliable results in the OD measurement growth cultures and pH stress experiments were monitored by OD and ATP measurement [165]. To determine the number of viable bacteria the BacTiter-Glo™ Microbial Cell Viability Assay was used since the formulation of this reagent lyses bacterial cells and measures the amount of the ATP present. Since ATP is an indicator of metabolic active cells the problem with clumping was solved and the results an indicator for living cells in the culture.

The outcome of this experiment showed that a manipulation in the gene regulation of *rv2626* in liquid cultures interferes in the adaption/growth regulation under low pH conditions. Whereas the deletion mutant showed a strong growth advantage compared to *M. bovis* BCG, the over-expression of *rv2626c* caused severe problems adapting to acidic conditions.

The reasons for this result are a matter of speculation, since little is known about *rv2626c* and its functions. Sharpe and colleagues [147] localised the protein within the bacteria by immunogold electron microscopy, so the result may indicate that this protein has some vital intracellular function, which is important for the adaption/survival of the bacteria in an acidic environment. Nevertheless, this outcome was notable since *rv2626c* was inconspicuous in pH stress experiments shown by Fisher and colleagues [131] when they exposed *M. tuberculosis* H37Rv for 15 minutes to acidified media (pH 5.5). To scrutinise the aforementioned experiment the same strains were once more exposed to pH stress, this time for only 24 and 48 hours and the outcome analysed by Western blot. Contrary to the predominating opinion the result confirmed the assumption that *rv2626c* is up-regulated under low pH conditions, since stronger bands were detected in protein preparations of *M. bovis* BCG and *M. bovis* (pMV261). In the case of *M. bovis* (pRv2626c) one more protein band was detected proving the existence of the described monomer (16-kDa) and dimer (approximately 32-kDa) of Rv2626c [151].

Investigating the influence of MDP1 under different pH conditions, it turned out that the MDP1-antisense strain under neutral conditions benefitted from the down-regulation. This trend reversed when *M. bovis* (pAs-MDP1) was grown in low pH conditions. Recent investigations regarding MDP1 have shown that this protein is not only retained in the cell wall, but is also important for glycolipid biosynthesis, cell wall biogenesis [172] and iron binding [160, 199]. Therefore various possible reasons might explain the outcome of this experiment. It is known that only an intact cell wall acts as an effective barrier against the entry of protons. Together with proton pumps [200], production of ammonia and amino acid decarboxylation [201], mycobacteria retain an almost neutral internal pH, even though the external pH dropped to pH 5 [202]. As it was recently shown, the MDP1 concentration in *M. bovis* (pAs-MDP1) is down-regulated by about 50% [165], so it seems very likely that the cell wall composition is also affected.

A non-intact cell wall might promote the influx of protons interfering in the adaptation of *M. tuberculosis* to the host niche and might explain why *M. bovis* (pAs-MDP1) was unable to adapt or grow in this acidic surrounding.

Whereas it has become apparent that *rv2626c* and *mdp1* are somehow involved in the adaption/growth regulation under pH stress, their role in the acidic phagosomal environment of infected monocyte-derived macrophages was tested. Since the clumping of *M. bovis* BCG complicates the determination of infective doses and makes cfu-counting unreliable, the bacterial number was measured by real-time PCR. This was particularly important since it was recently shown that the lower amount of MDP1 on the surface of the bacteria reduced clumping and decreased stability of aggregates of *M. bovis* BCG (pAs-MDP1) [165] which is why more colonies grow on agar plates, distorting the cfu-counting. This real-time PCR method was recently used by Roch and colleagues [203] to evaluate the quantification of other intracellular bacteria in macrophages, such as *Legionella pneumophila*.

In the case of Rv2626c the evaluation of the intracellular survival experiment proved that the outcome of the pH stress experiment in liquid cultures could not be transferred on infected monocyte-derived macrophages. Whereas *M. bovis* ΔRv2626c::hyg and *M. bovis* (pRv2626c) reacted differently to pH stress in liquid cultures, the real-time PCR detected similar bacterial numbers isolated from macrophages. The experiment was therefore repeated with pre-activated macrophages, but the result remained the same. None of the strains showed a significant growth advantage or disadvantage surviving inside macrophages so the significance of *rv2626c* for intracellular survival remains unclear.

Otherwise *M. bovis* BCG (pAs-MDP1) confirmed the expectations regarding the pH stress in liquid cultures, even without pre-activating the macrophages. Whereas *M. bovis* (pMV261) showed a significant growth advantage in blood-derived macrophages the bacterial numbers of the antisense strain detected by real-time PCR dropped slightly under the initial value. The outcome of this experiment is contradictious to other results which were previously obtained in MM6 and J774.A1 macrophages, where the antisense strain showed an enhanced growth [165].

The experiments in MM6 and J774.A1 macrophages were performed with bacteria which had grown to OD(600 nm) 2 and not to OD3(600 nm). Furthermore primary monocytes and cell lines, such as MM6, U-937 or THP-1 have different properties at their disposal, e.g. biochemical markers which are differentially regulated or even absent (e.g. lysozyme, CD14, MHC class II) and sometimes exhibit deregulations in the immune signaling [204]. Taken together this might explain the outcome of these contradictious results.

Cytokines play a pivotal role for the intercellular communication and activation of the adaptive immune response. There are good reasons to believe that *rv2626c* and *mdp1* might interfere in the adaptive immune response.

Whereas Rv2626c is a secretory protein known to bind on the cell surface of murine macrophages [147, 154], MDP1 is localized in the mycobacterial cell wall [162, 172]. The detection of antibodies against these proteins in the sera of tuberculosis patients [154, 160] underlines this assumption. For this reason it was decided to investigate the expression of five of the most important cytokines in the supernatants of infected human-derived PBMCs. The chosen cytokines (IL-1β, IFN-γ, TNF-α, IL-10 and IL-8) are responsible for the activation of the adaptive immune system; they hamper the proliferation of pathogens and protect host cells from destructive reactions induced by inflammatory responses.

The investigations concerning the deletion mutant have impressively shown that the loss of *rv2626c* in *M. bovis* BCG remains without consequences for the secretion of all selected cytokines. This observation is confirmed by the fact that the induced amount of all five cytokines secreted by PBMCs infected with *M. bovis* BCG and *M. bovis* ΔRv2626c::hyg were approximately identical to each other. Otherwise the secretion of Rv2626c by *M. bovis* (pRv2626c) has shown to strongly interfere with the immune response of infected PBMCs.

In this context high levels of IL-1β were measured in the supernatants of PBMCs infected with *M. bovis* (pRv2626c). Although it is known that IL-1β is responsible for T cells activation followed by IFN-γ secretion [205], no elevated concentration of IFN-γ could be measured in the supernatants of infected PBMCs.

For TNF-α, it was demonstrated that the over-expression of Rv2626c induced a strong up-regulation of this pro-inflammatory cytokine, known to be crucial for antimycobacterial immunity. In this context the observations of Bashir and colleagues [154] were approved who described the same effect in the context of recombinant Rv2626c and mouse macrophages.

Since *M. tuberculosis* triggers much lower amounts of TNF-α, GM-CSF and IL-6 than non-pathogenic species of mycobacteria [206], it can be assumed that the up-regulation of IL-1β together with TNF-α might have severe consequences for pathogens, a fact which could not be observed in the intracellular survival experiment.

At the same time a strong tendency toward the up-regulation of IL-10 was noticed. In the case of PBMCs infected with *M. bovis* (pRv2626c) the concentration of IL-10 reached maximum levels of up to 450% compared to *M. bovis* (pMV261).

(This result was not significant since the number of experiments was too low which is why a single discordant experiment was sufficient to ruin the whole statistic.) As a potent antagonist of pro-inflammatory cytokines IL-10 is responsible for the down-regulation of IFN-γ, TNF-α, and IL-12 [207, 208]. Based on the observations it is impossible to determine if the measured up-regulation of IL-10 is sufficient to antagonise the predicted effect induced by the up-regulation of IL-1β and TNF-α, as seen in the case of the over-expression of Rv2626c. This is important because it is well known that any disturbance of the balance between pro-inflammatory and anti-inflammatory cytokines might have severe consequences for the host. For instance, is known that blocking the IL-10 receptor in mice, e.g. by antibodies, leads to a better clearance of infection [80], otherwise infections with persistent or TNF stimulating pathogens, like *M. tuberculosis* [209] or *T. gondii* [210] might end in a pro-inflammatory-associated death.

IL-10 is also crucially involved in the outcome of tuberculosis because it inhibits the IL-8 secretion so that the influx of inflammatory cells to the infection site is reduced. Recent investigations have revealed that PBMCs infected with *M. bovis* (pRv2626c) induce considerably lower concentrations of IL-8 than the reference strain *M. bovis* (pMV261). If this is caused by the up-regulation of IL-10 or if this process is directly influenced by Rv2626c remains unclear. IL-8 is usually induced by phagocytosis or by stimulation with LAM [85, 211], LM and PIMs [101, 106].

The down-regulation of IL-8 might result in an reduced inflammation seen in a lesser degranulation by activated neutrophils [86]. Such interference in the IL-8 production might lead to problems for granuloma formation, an important step to control the dissemination.
It is known that several bacterial components are strong inducers of pro- and anti-inflammatory cytokines such as IL-1β, TNF-α and IL-10. Mycobacterial lipoproteins, the mycobacterial cell wall component lipomannan or antigens from Yersinia [80] are just a few examples but what they all have in common is that their expression is TLR2 mediated. Seeing the strong up-regulation of these cytokines by the over-expression strain *M. bovis* (pRv2626c), it seems possible that secretory protein Rv2626c somehow interacts with TLR2. Recent investigations with recombinant Rv2626c support this thesis by demonstrating the interaction of this protein with murine macrophages [154].

The down-regulation of MDP1 in *M. bovis* BCG has far fewer consequences on the cytokine expression of infected PBMCs. Only IL-1β was down-refulated considerably whereas all other investigated cytokines displayed concentrations which were approximately identical to the measured values of the reference strain *M. bovis* (pMV261). Although it was not investigated to which extend the down-regulation of MDP1 interferes with the cell wall composition of *M. bovis* BCG, recent investigations have proven that it affects the aggregation of this strain [165]. Katsube and colleagues [172] have furthermore shown that MDP1 controls the transfer of mycolic acids to sugars by Ag85 complex proteins thus modifying the cell wall composition of *M. smegmatis* and *M. bovis* BCG. Mycolic acids are known to be recognition patterns responsible for the activation of Toll-like receptor (TLR) 2 and TLR4 [212]. It therefore seems likely that the down-regulation of MDP1 changes molecular patterns of the mycobacterial cell wall and might accordingly be responsible for the decreased IL-1β response seen in PBMCs infected with *M. bovis* (pAs-MDP1).

Beside the investigated cytokines, NO is another important factor which is induced by macrophages hosting a strong immune response against *M. tuberculosis*. Investigations about nitric oxide induction have led to contradictory results about the role of NO in humans [96, 213].

Therefore the NO expression was measured in the murine cell line J774.A1, where the bactericide effects against M. tuberculosis have been proven [94]. The results regarding the effect of rv2626c on the NO expression revealed conflicting data with respect to recent publications.

Whereas the deletion mutant, similar to the cytokine experiments, had no influence on the NO expression compared to M. bovis BCG, the over-expressing strain revealed lower NO concentrations in the supernatants of activated J774.A1 mouse macrophages. Bashir and colleagues [154] attested that recombinant Rv2626c up-regulates the iNOS expression and NO production in murine macrophages. Whereas crystallization studies of Sharpe and colleagues [151] proved the existence of a SDS-resistant Rv2626c dimer (approximately 32-kDa) and monomer (16-kDa), this dimer appears to be missing in purified recombinant Rv2626c from Bashir [154]. In the present study, Western blot analysis using pH stress cultures from M. bovis (pRv2626c) showed a second band above the 16-kDa band. The missing dimer in the study of Bashir might indicate that recombinant Rv2626c, expressed in E. coli, did not form the dimer structure.

This might be an indication that Rv2626c is post-translational modified and that monomer and dimer might have different stimulatory properties. At this point it is impossible to say to what extent the contradictory results concerning the NO expression in mouse macrophages are the consequence of structural differences in the secretory protein Rv2626c.

The results regarding the NO production in mouse macrophages, indicate that *M. bovis* (pAs-MDP1) induces lesser concentrations of NO than the reference strain. This observation consistent with studies of Kamijo and colleagues [93] who reported that inflammatory cytokines like IL-1β, IFN-γ and TNF-α control the NO production. Furthermore, it is known that lipopolysaccharide (LPS) can induce iNOS expression [214]. Recent investigations have shown that *M. bovis* (pAs-MDP1) not only induces less amounts of IL-1β but also participates in the cell wall composition by influencing the aggregation of *M. bovis* BCG [165]. Both observations might explain why the NO concentrations in mouse macrophages are down-regulated. Decreasing concentrations of NO might have severe consequences for *M. tuberculosis* infections as several groups have shown in NO synthase 2 gene knock out (NOS2 KO) mice where infections resulted in fatal consequences [215].

The ability of macrophages to form multinucleated giant cells by cell fusion is a long known fact. MGC were first discovered in 1868 by Langhans in conjunction with tuberculous granulomas [108]. Since then their existence and function is controversially discussed ranging from redundant and insufficient for killing mycobacteria to useful when it comes to removing or degrading tissue irritants. MGC are found in granulomas and thought to be essential for the normal host defence mechanisms whereas their absence, e.g. in AIDS patients infected with *M. avium intracellulare* [216], is seen as an indicator for an ineffective host immune response.

Once it has been proven that the mutagenised strains influence the intercellular communication, the ability of these strains to interfere in the characteristic MC or MGC formation, characterised by the fusion of monocytes/macrophages, was tested. Gasser and colleagues [111] had shown that the fusion rate *in vitro* depends on the ratio of macrophages and infecting bacteria. Higher bacterial numbers induce higher fusion rates, but are also responsible for a higher cell loss.

Close contact between macrophages and mycobacteria as well as surface molecules, especially adhesion molecules, are of vital importance [111]. By definition MCs consists of clusters of three to fourteen cells whereas larger aggregations, fifteen nuclei and upwards, are referred as MGCs. Lay and colleagues [109] have impressively demonstrated that high pathogenic mycobacteria like *M. tuberculosis* induce the formation of MGC, meanwhile Nontuberculous mycobacteria (NTM), like *M. avium* and *M. smegmatis*, induce only small MCs. When it comes to MGC formation *M. bovis* BCG showed an intermediate profile.

So it was important to test the optimal bacteria/cell ratio and time span which was thought to be necessary to induce MC formation. These tests proved that a ratio of 1:1 and a time span of seven days would be optimal for investigation. Furthermore artificially activated and untreated, non-infected macrophages were used as controls to better assess the experimental outcome. The influence of Rv2626c on the MC formation was considerable. The deletion of *rv2626c* not only resulted in much higher fusion rates than in *M. bovis* BCG and stimulated macrophages, there were also more MGCs with more nuclei per cell. In contrast, the over-expression of Rv2626c led to severely decreased fusion rates, these sometimes dropped below the fusion rates of non-infected, non-stimulated macrophages.

Recently performed cytokine experiments, especially for the deletion mutant, are of no help when it comes to explaining this result. The tested immunoregulatory properties of PBMCs infected with *M. bovis* ΔRv2626c::hyg remained unchanged in absence of Rv2626c compared to *M. bovis* BCG. Rv2626c obviously has some influence on the MC and MGC formation, but does this mean that this protein directly or indirectly regulates the fusion of macrophages? Puissegur and colleagues [217] have recently shown that mycobacterial components like glycosylated lipids in the cell wall induce the granuloma MGC formation by binding to TLR2.
Although MGC have lost their ability for phagocytosis, they retained all other characteristics of activated macrophages [218, 219], display similar NADPH oxidase activities [146] and are equipped with increased antigen presenting abilities [146, 220]. Considering that the whole process of MGC formation *in vitro* under optimal conditions takes only 18 hours [221] to three days [111], delaying the fusion process would give mycobacteria some time to change the whole cellular metabolism, including macromolecular biosynthesis and cell wall assembly.

It is not exactly known how the down-regulation of MDP1 in *M. bovis* (pAs-MDP1) interferes with the composition of the mycobacterial cell wall, so it can only be speculated why this strain induces higher fusion rates in human monocyte-derived macrophages. In this context it was shown that the IL-1β activation normally induced by the mycobacterial cell wall component mediated by TLR2, was decreased in PBMCs infected with *M. bovis* (pAs-MDP1) and that no other cytokine was affected. This decreased IL-1β response should result in an attenuated innate immune response and is not qualified to explain the higher fusion rates. MDP1 is involved in the process of the synthesis of the cell wall, starting with the transport of mycolic acids to the Ag85 protein complex [172] followed by the binding of mycolic acids to trehalose and ending with the synthesis of the trehalose-6-monomycolate (TMM) precursor (-keto-acyl trehalose) [222]. These trehalose-containing mycolates are not only strong inducers of a pro-inflammatory cytokine response but also responsible for granuloma formation [223]. When this process is hampered because of the down-regulation of MDP1, and when there are not enough mycolic acids delivered to the cell wall, then the lipoglycans of the cell wall core might get exposed to receptors on the cell surface of macrophages.

In this context Puissegur and colleagues [217] have shown that lipoglycans like phosphatidyl-myo-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) induce the granuloma MGC formation mediated by TLR2. This assumption might explain how the down-regulation of MDP1 influences the MGCs formation.

Eventually, an animal experiment to test the virulence of the deletion mutant *in vivo* was performed. Mouse models are under discussion since mice have proven to be an inappropriate model for mycobacterial infections, because they do not show the whole repertoire of latent infections [224]. Especially investigations for granuloma formation have been found inadequate because *M. tuberculosis* induced granulomas in mice are neither hypoxic nor anaerob [225, 226]. Nevertheless mouse models helped to understand important cellular parameters of the anti-mycobacterial response [227] in the past even if they do not show all symptoms of human tuberculosis.

To investigate the pathogenicity of *M. bovis* BCG and the deletion mutant *M. bovis* ∆Rv2626c::hyg both strains were tested in a mouse model. For this reason the mice were infected with both strains for 17 and 25 days. Liver, lung, lymph node and spleen were extracted and bacterial numbers measured by real-time PCR. In most organs more bacterial DNA were detected in animals infected with the deletion mutant than in animals infected with *M. bovis* BCG. Furthermore the wild-type strain featured granuloma formation already after 17 days, which were missing in the deletion mutant. At day 25 even more bacterial DNA was found in the deletion mutant than in *M. bovis* BCG, but this time granulomas were found in animals infected with both strains. This result can be interpreted in two different ways. Either the wild-type strain induces the granuloma formation much faster so that the bacteria reach a dormant, non-replicating state much earlier or the deletion mutant delayed the granuloma formation, which is why the bacteria had more time to grow. Regarding recently conducted cell culture experiments the outcome of this animal experiment is difficult to interpret because the deletion mutant was unobtrusive in many virulence associated conditions, e.g. cytokine expression.

On the other hand this experiment impressively underlines the much discussed differences between primary human donor-derived cells and animal experiments [224].

Concluding all experiments it was observed that *rv2626c* and *mdp1* possess virulence associated capabilities. Several significant findings like the increased amount of IL-1β, TNF-α, and IL-10 in human PBMCs, induced by the up-regulation of Rv2626c, or possible changes in the mycobacterial cell wall, induced by the antisense construct of *M. bovis* (pAs-MDP1), suggest an involvement of the TLR2. Corresponding experiments proving a direct or indirect binding of Rv2626c to TLR2 or other molecules are missing. In the case of Rv2626c investigations regarding the use of recombinant Rv2626c or antibodies against TLR2 or Rv2626c should help to show the influence of the cytokine expression and multinucleated giant cell formation.

5. Summary

In 1993, WHO declared tuberculosis a global health emergency. The reasons for this decision are the high infection rate as well as problems associated with the healing of tuberculosis. *M. tuberculosis* is able to survive in an intact immune system over decades and can be reactivated under certain conditions. The lengthy treatment and the appearance of multidrug resistant and extensively drug-resistant tuberculosis strains complicate the healing process. This makes it even more important to find genes in *M. tuberculosis* allowing the survival in the human body and to understand their influence on the interaction with the immune cells.

Against this background, two genes of *M. bovis* BCG were investigated whose regulation in *M. tuberculosis* suggested a potential influence on the course of infection. By site-directed mutagenesis, over-expression or antisense-technique new strains of *M. bovis* BCG were generated in which either the gene *rv2626c* was over-expressed or deleted or the gene for the "*mycobacterial DNA binding protein 1*" (MDP1) was down-regulated. These strains were used for *in vitro* experiments in which aspects of the natural infection were simulated.

The present work dealt with investigations using human-derived primary cells, a mouse cell line and a mouse model. It was shown that both genes possess virulence-associates capabilities. The secretory protein Rv2626c interferes massively in the cytokine expression of human PBMCs, up-regulating IL-1β, TNF-α, and IL-10 and down-regulating IL-8. It reduces the amount of secreted nitric oxide in a mouse cell line and increases the fusion of human macrophages. On the other hand the deletion of Rv2626c improves the increased survivability in Balb/c mice.

The down-regulation of *mdp1* is probably responsible for modifications of the mycobacterial cell wall which is accountable for the down-regulation of IL-1β and nitric oxide, the decreased survivability in human macrophages and the increased fusion of human macrophages.

Further trials are needed to find out whether Rv2626c and MDP1 are qualified for new therapeutic approaches or not.

6. Zusammenfassung

Tuberkulose wurde 1993 von der WHO zu einem globalen Gesundheitsproblem erklärt. Gründe hierfür sind die hohe Infektionsrate sowie Schwierigkeiten bei der Behandlung von Tuberkulose. *M. tuberculosis* gelingt es über Jahrzehnte in einem intakten Immunsystem zu überleben und unter bestimmten Bedingungen wieder reaktiviert zu werden. Die langwierige Behandlung und das Auftreten von multiresistenten und extrem resistenten Stämmen erschweren zudem die Bekämpfung der Tuberkulose. Umso wichtiger ist es Gene zu finden die *M. tuberculosis* das Überleben im menschlichen Körper ermöglichen und zu verstehen, wie sie die Interaktion mit Zellen des Immunsystems beeinflussen.

Vor diesem Hintergrund wurden zwei Gene von *M. bovis* BCG untersucht, deren Regulation in *M. tuberculosis* einen möglichen Einfluss auf den Infektionsverlauf nahelegten. Mittels gezielter Mutagenese, Überexprimierung oder Antisense Technik wurden neue *M. bovis* BCG Stämme erzeugt, in denen das Gen *rv2626c* überexprimiert bzw. deletiert oder im Fall des "*mycobacterial DNA binding protein 1*" (MDP1) herabreguliert wurde. Mit diesen Stämmen wurden *in vitro* Versuche durchgeführt in denen bestimmte Aspekte der natürlichen Infektion simuliert wurden.

Die in dieser Arbeit durchgeführten Untersuchungen mit humanen Primärzellen, einer Mauszelllinie und einem Mausmodell haben gezeigt, dass beide Gene über virulenzassoziierte Eigenschaften verfügen. So greift das sekretierte Protein Rv2626c massiv in die Zytokinexpression humaner PBMCs ein, indem es IL-1β, TNF-α, IL-10 hoch- und IL-8 herabreguliert, Stickstoffoxid in einer Mauszelllinie herabreguliert und die Fusion humaner Makrophagen fördert. Auf der anderen Seite führt die Deletion des Gens zu einer verbesserten Überlebensfähigkeit in Balb/c Mäusen. Die Herabregulierung von *mdp1* wirkt sich wahrscheinlich auf eine Weise auf die Zellwandstruktur aus, die zu einer Herabregulierung von IL-1β und Stickstoffoxid führt, das intrazelluläre Überleben in humanen Makrophagen vermindert und die Fusion von humanen Makrophagen fördert.

Weiterführende Versuche müssen zeigen ob die Rv2626c und MDP1 für die Entwicklung neuer Therapieansätze geeignet sind oder nicht.

7. References

1. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C *et al*: **Transcriptional adaptation of Mycobacterium tuberculosis within macrophages: Insights into the phagosomal environment**. *Journal of Experimental Medicine* 2003, **198**(5):693-704.
2. Gutacker MM, Smoot JC, Migliaccio CAL, Ricklefs SM, Hua S, Cousins DV, Graviss EA, Shashkina E, Kreiswirth BN, Musser JM: **Genome-wide analysis of synonymous single nucleotide polymorphisms in Mycobacterium tuberculosis complex organisms: Resolution of genetic relationships among closely related microbial strains**. *Genetics* 2002, **162**(4):1533-1543.
3. Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS, Musser JM: **Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(18):9869-9874.
4. Hughes AL, Friedman R, Murray M: **Genomewide pattern of synonymous nucleotide substitution in two complete genomes of Mycobacterium tuberculosis**. *Emerging Infectious Diseases* 2002, **8**(11):1342-1346.
5. Hirsh AE, Tsolaki AG, DeRiemer K, Feldman MW, Small PM: **Stable association between strains of Mycobacterium tuberculosis and their human host populations**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(14):4871-4876.
6. Kapur V, Whittam TS, Musser JM: **Is Mycobacterium tuberculosis 15,000 years old? [3]**. *Journal of Infectious Diseases* 1994, **170**(5):1348-1349.
7. Spigelman MaL, E.: **The use of the polymerase chain reaction (PCR) to detect Mycobacterium tuberculosis in ancient skeletons**. *Int J Osteoarchaeol* 1993, **3**:137-143.
8. Rothschild BM, Martin LD, Lev G, Bercovier H, Bar-Gal GK, Greenblatt C, Donoghue H, Spigelman M, Brittain D: **Mycobacterium tuberculosis complex DNA from an extinct bison dated 17,000 years before the present**. *Clinical Infectious Diseases* 2001, **33**(3):305-311.
9. Donoghue HD, Spigelman M, Greenblatt CL, Lev-Maor G, Kahila Bar-Gal G, Matheson C, Vernon K, Nerlich AG, Zink AR: **Tuberculosis: From prehistory to Robert Koch, as revealed by ancient DNA**. *Lancet Infectious Diseases* 2004, **4**(9):584-592.
10. WHO: **Global tuberculosis control**. *http://whqlibdocwhoint/publications/2010/* 2010.
11. Barnes PF, Le HQ, Davidson PT: **Tuberculosis in patients with HIV infection**. *Medical Clinics of North America* 1993, **77**(6):1369-1390.
12. WHO: **Global tuberculosis control 2009: epidemiology, strategy, financing**. *http://wwwwhoint/tb/publications/global_report/2009/en/indexhtml* 2009.
13. Trunz BB, Fine P, Dye C: **Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness**. *Lancet* 2006, **367**(9517):1173-1180.
14. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, Mosteller F: **Efficacy of BCG vaccine in the prevention of tuberculosis: Meta-analysis of the published literature**. *Journal of the American Medical Association* 1994, **271**(9):698-702.
15. Huebner RE: **BCG vaccination in the control of tuberculosis**. *Springer-Verlag, Berlin, Germany* 1996:263-282.
16. Jain A, Dixit P: **Multidrug resistant to extensively drug resistant tuberculosis: What is next?** *Journal of Biosciences* 2008, **33**(4):605-616.
17. Smith PGaM, A.R.: **Epidemiology of tuberculosis. In Tuberculosis: Pathogenesis, Protection and Control**. *American Society for Microbiology Press* 1994, **Bloom, B.R. (e.d.)**:47-61.
18. Kang BK, Schlesinger LS: **Characterization of mannose receptor-dependent phagocytosis mediated by Mycobacterium tuberculosis lipoarabinomannan**. *Infection and Immunity* 1998, **66**(6):2769-2777.
19. Geijtenbeek TBH, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CMJE, Appelmelk B, Van Kooyk Y: **Mycobacteria target DC-SIGN to suppress dendritic cell function**. *Journal of Experimental Medicine* 2003, **197**(1):7-17.
20. Schlesinger LS, Bellinger-Kawahara CG, Payne NR, Horwitz MA: **Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3**. *Journal of Immunology* 1990, **144**(7):2771-2780.

21. Nathan C, Shiloh MU: **Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(16):8841-8848.
22. Russell DG: **Mycobacterium tuberculosis: Here today, and here tomorrow**. *Nature Reviews Molecular Cell Biology* 2001, **2**(8):569-577.
23. Vergne I, Chua J, Singh SB, Deretic V: **Cell biology of Mycobacterium tuberculosis phagosome**. In., vol. 20; 2004: 367-394.
24. Armstrong JA, D'Arcy Hart P: **Phagosome lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival**. *Journal of Experimental Medicine* 1975, **142**(1):1-16.
25. Fenton MJ, ; Riley, L.W. and Schlesinger, L.S.: **"Receptor-mediated recognition of Mycobacterium tuberculosis by host cells," in Tuberculosis and the Tubercle Bacillus**. *New York: ASM Press* 2005:405-426.
26. Schaible UE, Sturgill-Koszycki S, Schlesinger PH, Russell DG: **Cytokine activation leads to acidification and increases maturation of Mycobacterium avium-containing phagosomes in murine macrophages**. *Journal of Immunology* 1998, **160**(3):1290-1296.
27. Ulrichs T, Kaufmann SHE: **New insights into the function of granulomas in human tuberculosis**. *Journal of Pathology* 2006, **208**(2):261-269.
28. Smith I: **Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence**. *Clinical Microbiology Reviews* 2003, **16**(3):463-496.
29. Co DO, Hogan LH, Kim SI, Sandor M: **Mycobacterial granulomas: Keys to a long-lasting host-pathogen relationship**. *Clinical Immunology* 2004, **113**(2):130-136.
30. Turner J: **"Growing old and immunity to bacteria," in the immune response to infection**. *ASM Press* 2011:413-423.
31. Kaplan G, Post FA, Moreira AL, Wainwright H, Kreiswirth BN, Tanverdi M, Mathema B, Ramaswamy SV, Walther G, Steyn LM *et al*: **Mycobacterium tuberculosis Growth at the Cavity Surface: A Microenvironment with Failed Immunity**. *Infection and Immunity* 2003, **71**(12):7099-7108.
32. Dheda K, Booth H, Huggett JF, Johnson MA, Zumla A, Rook GAW: **Lung remodeling in pulmonary tuberculosis**. *Journal of Infectious Diseases* 2005, **192**(7):1201-1210.
33. Russell DG: **Who puts the tubercle in tuberculosis?** *Nature Reviews Microbiology* 2007, **5**(1):39-47.
34. Stenger S, Modlin RL: **Control of Mycobacterium tuberculosis through mammalian Toll-like receptors**. *Current Opinion in Immunology* 2002, **14**(4):452-457.
35. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A: **The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(25):13766-13771.
36. Bulut Y, Faure E, Thomas L, Equils O, Arditi M: **Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and Borrelia burgdorferi outer surface protein a lipoprotein: Role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling**. *Journal of Immunology* 2001, **167**(2):987-994.
37. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, Maitland M, Norgard MV, Plevy SE, Smale ST *et al*: **Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors**. *Science* 1999, **285**(5428):732-736.
38. Messmer UK, Lapetina EG, Brune B: **Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds**. *Molecular Pharmacology* 1995, **47**(4):757-765.
39. Saunders BM, Cooper AM: **Restraining mycobacteria: Role of granulomas in mycobacterial infections**. *Immunology and Cell Biology* 2000, **78**(4):334-341.
40. Döcke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W: **Monocyte deactivation in septic patients: Restoration by IFN-γ treatment**. *Nature Medicine* 1997, **3**(6):678-681.
41. Westendorp RGJ, Langermans JAM, Huizinga TWJ, Elouali AH, Verweij CL, Boomsma DI, Vandenbrouke JP: **Genetic influence on cytokine production and fatal meningococcal disease**. *Lancet* 1997, **349**(9046):170-173.
42. Wilson M, Seymour R, Henderson B: **Bacterial perturbation of cytokine networks**. *Infection and Immunity* 1998, **66**(6):2401-2409.
43. Kaufmann SHE: **How can immunology contribute to the control of tuberculosis?** *Nature Reviews Immunology* 2001, **1**(1):20-30.

44. Sasindran S.J. TJB: **Mycobacterium tuberculosis infection and inflammation: what is beneficial for the host and for the bacterium**. *Front Microbiol* 2011, **2**.
45. Cooper AM: **Cell-mediated immune responses in tuberculosis**. In., vol. 27; 2009: 393-422.
46. Ward JR, West PW, Ariaans MP, Parker LC, Francis SE, Crossman DC, Sabroe I, Wilson HL: **Temporal interleukin-1β secretion from primary human peripheral blood monocytes by P2X7-independent and P2X7-dependent mechanisms**. *Journal of Biological Chemistry* 2010, **285**(30):23147-23158.
47. Michelini-Norris MB, Blanchard DK, Pearson CA, Djeu JY: **Differential release of interleukin (IL)-1α, IL-1β, and IL-6 from normal human monocytes stimulated with a virulent and an avirulent isogenic variant of Mycobacterium avium-intracellulare complex**. *Journal of Infectious Diseases* 1992, **165**(4):702-709.
48. Kleinnijenhuis J, Joosten LAB, van de Veerdonk FL, Savage N, van Crevel R, Kullberg BJ, van der Ven A, Ottenhfoff THM, Dinarello CACA, van der Meer JWM *et al*: **Transcriptional and inflammasome-mediated pathways for the induction of IL-1β production by Mycobacterium tuberculosis**. *European Journal of Immunology* 2009, **39**(7):1914-1922.
49. Aliprantis AO, Yang RB, Weiss DS, Godowski P, Zychlinsky A: **The apoptotic signaling pathway activated by Toll-like receptor-2**. *EMBO Journal* 2000, **19**(13):3325-3336.
50. Andrei C, Dazzi C, Lotti L, Torrisi MR, Chimini G, Rubartelli A: **The secretory route of the leaderless protein interleukin 1β involves exocytosis of endolysosome-related vesicles**. *Molecular Biology of the Cell* 1999, **10**(5):1463-1475.
51. Tschopp J, Martinon F, Burns K: **NALPs: A novel protein family involved in inflammation**. *Nature Reviews Molecular Cell Biology* 2003, **4**(2):95-104.
52. Martinon F, Burns K, Tschopp J: **The Inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-β**. *Molecular Cell* 2002, **10**(2):417-426.
53. Boehm U, Klamp T, Groot M, Howard JC: **Cellular responses to interferon-γ**. In., vol. 15; 1997: 749-795.
54. Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H: **Induction of gamma interferon production in human alveolar macrophages by Mycobacterium tuberculosis**. *Infection and Immunity* 1997, **65**(12):5149-5156.
55. Weinberg JB, Hobbs MM, Misukonis MA: **Recombinant human γ-interferon induces human monocyte polykaryon formation**. *Proceedings of the National Academy of Sciences of the United States of America* 1984, **81**(14 **I**):4554-4557.
56. Nagasawa H, Miyaura C, Abe E, Suda T, Horiguchi M, Suda T: **Fusion and activation of human alveolar macrophages induced by recombinant interferon-γ and their suppression by dexamethasone**. *American Review of Respiratory Disease* 1987, **136**(4):916-921.
57. Most J, Neumayer HP, Dierich MP: **Cytokine-induced generation of multinucleated giant cells in vitro requires interferon-γ and expression of LFA-1**. *European Journal of Immunology* 1990, **20**(8):1661-1667.
58. Ottenhoff THM, Verreck FAW, Lichtenauer-Kaligis EGR, Hoeve MA, Sanal O, Van Dissel JT: **Genetics, cytokines and human infectious disease: Lessons from weakly pathogenic mycobacteria and salmonellae**. *Nature Genetics* 2002, **32**(1):97-105.
59. Altare F, Jouanguy E, Lamhamedi S, Döffinger R, Fischer A, Casanova JL: **Mendelian susceptibility to mycobacterial infection in man**. *Current Opinion in Immunology* 1998, **10**(4):413-417.
60. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR: **An essential role for interferon γ in resistance to Mycobacterium tuberculosis infection**. *Journal of Experimental Medicine* 1993, **178**(6):2249-2254.
61. Levin M, Newport MJ, D'Souza S, Kalabalikis P, Brown IN, Lenicker HM, Agius PV, Davies EG, Thrasher A, Klein N *et al*: **Familial disseminated atypical mycobacterial infection in childhood: A human mycobacterial susceptibility gene?** *Lancet* 1995, **345**(8942):79-83.
62. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM: **Disseminated tuberculosis in interferon γ gene-disrupted mice**. *Journal of Experimental Medicine* 1993, **178**(6):2243-2247.
63. Valone SE, Rich EA, Wallis RS, Ellner JJ: **Expression of tumor necrosis factor in vitro by human mononuclear phagocytes stimulated with whole Mycobacterium bovis BCG and mycobacterial antigens**. *Infection and Immunity* 1988, **56**(12):3313-3315.
64. Henderson RA, Watkins SC, Flynn JL: **Activation of Human Dendritic Cells Following Infection with Mycobacterium tuberculosis**. *Journal of Immunology* 1997, **159**(2):635-643.

65. Quesniaux VJ, Nicolle DM, Torres D, Kremer L, Guérardel Y, Nigou J, Puzo G, Erard F, Ryffel B: **Toll-Like Receptor 2 (TLR2)-Dependent-Positive and TLR2-Independent-Negative Regulation of Proinflammatory Cytokines by Mycobacterial Lipomannans**. *Journal of Immunology* 2004, **172**(7):4425-4434.
66. Bean AGD, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, Britton WJ: **Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin**. *Journal of Immunology* 1999, **162**(6):3504-3511.
67. Bermudez LEM, Young LS: **Tumor necrosis factor, alone or in combination with IL-2, but not IFN-γ, is associated with macrophage killing of Mycobacterium avium complex**. *Journal of Immunology* 1988, **140**(9):3006-3013.
68. Appelberg R, Castro AG, Pedrosa J, Silva RA, Orme IM, Minoprio P: **Role of gamma interferon and tumor necrosis factor alpha during T-cell- independent and -dependent phases of Mycobacterium avium infection**. *Infection and Immunity* 1994, **62**(9):3962-3971.
69. Scott Algood HM, Lin PL, Yankura D, Jones A, Chan J, Flynn JL: **TNF influences chemokine expression of macrophages in vitro and that of CD11b+ cells in vivo during Mycobacterium tuberculosis infection**. *Journal of Immunology* 2004, **172**(11):6846-6857.
70. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, Kornfeld H: **Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis**. *Infection and Immunity* 1997, **65**(1):298-304.
71. Byrd TF: **Tumor necrosis factor α (TNFα) promotes growth of virulent Mycobacterium tuberculosis in human monocytes: Iron-mediated growth suppression is correlated with decreased release of TNFα from iron-treated infected monocytes**. *Journal of Clinical Investigation* 1997, **99**(10):2518-2529.
72. Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, Tsai MC, Flynn JL, Chan J: **Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: Possible role for limiting pathology**. *Infection and Immunity* 2001, **69**(3):1847-1855.
73. Shaw TC, Thomas LH, Friedland JS: **Regulation of IL-10 secretion after phagocytosis of Mycobacterium tuberculosis by human monocytic cells**. *Cytokine* 2000, **12**(5):483-486.
74. Hickman SP, Chan J, Salgame P: **Mycobacterium tuberculosis induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization**. *Journal of Immunology* 2002, **168**(9):4636-4642.
75. Gerosa F, Nisii C, Righetti S, Micciolo R, Marchesini M, Cazzadori A, Trinchieri G: **CD4+ T cell clones producing both interferon-γ and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients**. *Clinical Immunology* 1999, **92**(3):224-234.
76. Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC, Berezovskaya A, Roussec D, Reynes JM, Goldfeld AE: **IL-10-producing T cells suppress immune responses in anergic tuberculosis patients**. *Journal of Clinical Investigation* 2000, **105**(9):1317-1325.
77. Moore KW, De Waal Malefyt R, Coffman RL, O'Garra A: **Interleukin-10 and the interleukin-10 receptor**. In., vol. 19; 2001: 683-765.
78. Saraiva M, O'Garra A: **The regulation of IL-10 production by immune cells**. *Nature Reviews Immunology* 2010, **10**(3):170-181.
79. Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA, Ernst M, Saris CJM, O'Shea JJ, Hunter CA: **Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10**. *Nature Immunology* 2007, **8**(12):1363-1371.
80. Cyktor JC, Turner J: **Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens**. *Infection and Immunity* 2011, **79**(8):2964-2973.
81. Gazzinelli RT, Oswald IP, James SL, Sher A: **IL-10 inhibits parasite killing and nitrogen oxide production by IFN-γ- activated macrophages**. *Journal of Immunology* 1992, **148**(6):1792-1796.
82. De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, De Vries JE: **Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes**. *Journal of Experimental Medicine* 1991, **174**(5):1209-1220.
83. Broaddus VC, Hebert CA, Vitangcol RV, Hoeffel JM, Bernstein MS, Boylan AM: **Interleukin-8 is a major neutrophil chemotactic factor in pleural liquid of patients with empyema**. *American Review of Respiratory Disease* 1992, **146**(4):825-830.
84. Bergeron A, Bonay M, Kambouchner M, Lecossier D, Riquet M, Soler P, Hance A, Tazi A: **Cytokine patterns in tuberculous and sarcoid granulomas: Correlations with**

histopathologic features of the granulomatous response. *Journal of Immunology* 1997, **159**(6):3034-3043.

85. Zhang Y, Broser M, Cohen H, Bodkin M, Law K, Reibman J, Rom WN: **Enhanced interleukin-8 release and gene expression in macrophages after exposure to Mycobacterium tuberculosis and its components**. *Journal of Clinical Investigation* 1995, **95**(2):586-592.
86. Dinarello CA: **Proinflammatory cytokines**. *Chest* 2000, **118**(2):503-508.
87. Larsen CG, Thomsen MK, Gesser B, Thomsen PD, Deleuran BW, Nowak J, Skodt V, Thomsen HK, Deleuran M, Thestrup-Pedersen K *et al*: **The delayed-type hypersensitivity reaction is dependent on IL-8: Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody**. *Journal of Immunology* 1995, **155**(4):2151-2157.
88. Lowenstein CJ, Dinerman JL, Snyder SH: **Nitric oxide: A physiologic messenger**. *Annals of Internal Medicine* 1994, **120**(3):227-237.
89. Chan J, Xing Y, Magliozzo RS, Bloom BR: **Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages**. *Journal of Experimental Medicine* 1992, **175**(4):1111-1122.
90. Rojas M, Barrera LF, Puzo G, Garcia LF: **Differential Induction of Apoptosis by Virulent Mycobacterium tuberculosis in Resistant and Susceptible Murine Macrophages: Role of Nitric Oxide and Mycobacterial Products**. *Journal of Immunology* 1997, **159**(3):1352-1361.
91. Andrew PJ, Harant H, Lindley IJD: **Nitric oxide regulates IL-8 expression in melanoma cells at the transcriptional level**. *Biochemical and Biophysical Research Communications* 1995, **214**(3):949-956.
92. Kuo HP, Hwang KH, Lin HC, Wang CH, Lu LC: **Effect of endogenous nitric oxide on tumour necrosis factor-α-induced leukosequestration and IL-8 release in airways in vivo**. *British Journal of Pharmacology* 1997, **122**(1):103-111.
93. Kamijo R, Gerecitano J, Shapiro D, Green SJ, Aguet M, Le D, Vilcek J: **Generation of nitric oxide and clearance of interferon-γ after BCG infection are impaired in mice that lack the interferon-γ receptor**. *Journal of Inflammation* 1996, **46**(1):23-31.
94. Chan J, Tanaka K, Carroll D, Flynn J, Bloom BR: **Effects of nitric oxide synthase inhibitors on murine infection with Mycobacterium tuberculosis**. *Infection and Immunity* 1995, **63**(2):736-740.
95. Schuller-Levis GB, Levis WR, Ammazzalorso M, Nosrati A, Park E: **Mycobacterial lipoarabinomannan induces nitric oxide and tumor necrosis factor alpha production in a macrophage cell line: Down regulation by taurine chloramine**. *Infection and Immunity* 1994, **62**(10):4671-4674.
96. Jagannath C, Actor JK, Hunter Jr RL: **Induction of nitric oxide in human monocytes and monocyte cell lines by Mycobacterium tuberculosis**. *Nitric Oxide - Biology and Chemistry* 1998, **2**(3):174-186.
97. Nicholson S, Bonecini-Almeida MDG, Lapa E Silva JR, Nathan C, Xie QW, Mumford R, Weidner JR, Calaycay J, Geng J, Boechat N *et al*: **Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis**. *Journal of Experimental Medicine* 1996, **183**(5):2293-2302.
98. Ohno H, Zhu G, Mohan VP, Chu D, Kohno S, Jacobs Jr WR, Chan J: **The effects of reactive nitrogen intermediates on gene expression in Mycobacterium tuberculosis**. *Cellular Microbiology* 2003, **5**(9):637-648.
99. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK: **Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program**. *Journal of Experimental Medicine* 2003, **198**(5):705-713.
100. Brennan PJ: **Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis**. *Tuberculosis* 2003, **83**(1-3):91-97.
101. Dao DN, Kremer L, Guérardel Y, Molano A, Jacobs Jr WR, Porcelli SA, Briken V: **Mycobacterium tuberculosis Lipomannan Induces Apoptosis and Interleukin-12 Production in Macrophages**. *Infection and Immunity* 2004, **72**(4):2067-2074.
102. Kato M, Maeda J: **Isolation and biochemical activities of trehalose 6 monomycolate of Mycobacterium tuberculosis**. *Infection and Immunity* 1974, **9**(1):8-14.
103. Goren MB: **Mycobacterial Lipids: Selected Topics**. *American Society for Microbiology* 1972, **36**(1):33-64.
104. Bekierkunst A: **Acute granulomatous response produced in mice by trehalose-6,6-dimycolate**. *Journal of Bacteriology* 1968, **96**(4):958-961.
105. Takimoto H, Maruyama H, Shimada KI, Yakabe R, Yano I, Kumazawa Y: **Interferon-γ independent formation of pulmonary granuloma in mice by injections with trehalose**

dimycolate (cord factor), lipoarabinomannan and phosphatidylinositol mannosides isolated from Mycobacterium tuberculosis. *Clinical and Experimental Immunology* 2006, **144**(1):134-141.

106. Vignal C, Guérardel Y, Kremer L, Masson M, Legrand D, Mazurier J, Elass E: **Lipomannans, but not lipoarabinomannans, purified from Mycobacterium chelonae and Mycobacterium kansasii induce TNF-α and IL-8 secretion by a CD14-Toll-like receptor 2-dependent mechanism**. *Journal of Immunology* 2003, **171**(4):2014-2023.
107. Nikaido H: **Prevention of drug access to bacterial targets: Permeability barriers and active efflux**. *Science* 1994, **264**(5157):382-388.
108. Langhans T: **Ueber Riesenzellen mit wandständigen Kernen in Tuberkeln und die fibröse Form des Tuberkels**. *Archiv für Pathologische Anatomie und Physiologie und für Klinische Medicin* 1868, **42**(3):382-404.
109. Lay G, Poquet Y, Salek-Peyron P, Puissegur MP, Botanch C, Bon H, Levillain F, Duteyrat JL, Emile JF, Altare F: **Langhans giant cells from M. Tuberculosis-induced human granulomas cannot mediate mycobacterial uptake**. *Journal of Pathology* 2007, **211**(1):76-85.
110. Saunders BM, Frank AA, Orme IM: **Granuloma formation is required to contain bacillus growth and delay mortality in mice chronically infected with Mycobacterium tuberculosis**. *Immunology* 1999, **98**(3):324-328.
111. Gasser A, Möst J: **Generation of multinucleated giant cells in vitro by culture of human monocytes with Mycobacterium bovis BCG in combination with cytokine- containing supernatants**. *Infection and Immunity* 1999, **67**(1):395-402.
112. Lima VMF, Bonato VLD, Lima KM, Dos Santos SA, Dos Santos RR, Gonçalves EDC, Faccioli LH, Brandão IT, Rodrigues-Junior JM, Silva CL: **Role of trehalose dimycolate in recruitment of cells and modulation of production of cytokines and NO in tuberculosis**. *Infection and Immunity* 2001, **69**(9):5305-5312.
113. Fenton MJ, Vermeulen MW: **Immunopathology of tuberculosis: Roles of macrophages and monocytes**. *Infection and Immunity* 1996, **64**(3):683-690.
114. Wayne LG, Hayes LG: **Nitrate reduction as a marker for hypoxic shiftdown of Mycobacterium tuberculosis**. *Tubercle and Lung Disease* 1998, **79**(2):127-132.
115. Wayne LG, Lin KY: **Glyoxylate metabolism and adaptation of Mycobacterium tuberculosis to survival under anaerobic conditions**. *Infection and Immunity* 1982, **37**(3):1042-1049.
116. Wayne LG, Hayes LG: **An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence**. *Infection and Immunity* 1996, **64**(6):2062-2069.
117. Cunningham AF, Spreadbury CL: **Mycobacterial stationary phase induced by low oxygen tension: Cell wall thickening and localization of the 16-kilodalton α-crystallin homolog**. *Journal of Bacteriology* 1998, **180**(4):801-808.
118. Parrish NM, Dick JD, Bishai WR: **Mechanisms of latency in Mycobacterium tuberculosis**. *Trends in Microbiology* 1998, **6**(3):107-112.
119. Wayne LG, Sohaskey CD: **Nonreplicating persistence of Mycobacterium tuberculosis**. In., vol. 55; 2001: 139-163.
120. Davis JM, Ramakrishnan L: **The Role of the Granuloma in Expansion and Dissemination of Early Tuberculous Infection**. *Cell* 2009, **136**(1):37-49.
121. Harder W, Dijkhuizen L: **Physiological responses to nutrient limitation**. *Annual Review of Microbiology* 1983, **37**:1-23.
122. Corper EL, and Cohn, M.L.: **The viability and virulence of old cultures of tubercle bacilli. Studies on twelve-year broth cultures maintained at incubator temperature**. *Am Rev Tuberc Pulm Dis* 1933, **28**:856-874.
123. Dubos RJ: **The effect of organic acids on mammalian tubercle bacilli**. *The Journal of experimental medicine* 1950, **92**(4):319-332.
124. Dubos RJ: **Effect of the composition of the gaseous and aqueous environments on the survival of tubercle bacilli in vitro**. *The Journal of experimental medicine* 1953, **97**(3):357-366.
125. Wayne LG, Diaz GA: **Autolysis and secondary growth of Mycobacterium tuberculosis in submerged culture**. *Journal of Bacteriology* 1967, **93**(4):1374-1381.
126. Wayne LG: **Synchronized replication of Mycobacterium tuberculosis**. *Infection and Immunity* 1977, **17**(3):528-530.
127. Hampshire T, Soneji S, Bacon J, James BW, Hinds J, Laing K, Stabler RA, Marsh PD, Butcher PD: **Stationary phase gene expression of Mycobacterium tuberculosis following**

a progressive nutrient depletion: A model for persistent organisms? *Tuberculosis* 2004, **84**(3-4):228-238.

128. Bacon J, James BW, Wernisch L, Williams A, Morley KA, Hatch GJ, Mangan JA, Hinds J, Stoker NG, Butcher PD *et al*: **The influence of reduced oxygen availability on pathogenicity and gene expression in Mycobacterium tuberculosis**. *Tuberculosis* 2004, **84**(3-4):205-217.
129. Voskuil MI, Visconti KC, Schoolnik GK: **Mycobacterium tuberculosis gene expression during adaptation to stationary phase and low-oxygen dormancy**. *Tuberculosis* 2004, **84**(3-4):218-227.
130. Kendall SL, Movahedzadeh F, Rison SCG, Wernisch L, Parish T, Duncan K, Betts JC, Stoker NG: **The Mycobacterium tuberculosis dosRS two-component system is induced by multiple stresses**. *Tuberculosis* 2004, **84**(3-4):247-255.
131. Fisher MA, Plikaytis BB, Shinnick TM: **Microarray analysis of the Mycobacterium tuberculosis transcriptional response to the acidic conditions found in phagosomes**. *Journal of Bacteriology* 2002, **184**(14):4025-4032.
132. Talaat AM, Lyons R, Howard ST, Johnston SA: **The temporal expression profile of Mycobacterium tuberculosis infection in mice**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(13):4602-4607.
133. Sharma D, Bose A, Shakila H, Das TK, Tyagi JS, Ramanathan VD: **Expression of mycobacterial cell division protein, FtsZ, and dormancy proteins, DevR and Acr, within lung granulomas throughout guinea pig infection**. *FEMS Immunology and Medical Microbiology* 2006, **48**(3):329-336.
134. Sherman DR, Voskuil M, Schnappinger D, Liao R, Harrell MI, Schoolnik GK: **Regulation of the mycobacterium tuberculosis hypoxic response gene encoding α-crystallin**. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**(13):7534-7539.
135. Park HD, Guinn KM, Harrell MI, Liao R, Voskuil MI, Tompa M, Schoolnik GK, Sherman DR: **Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis**. *Molecular Microbiology* 2003, **48**(3):833-843.
136. Honaker RW, Leistikow RL, Bartek IL, Voskui MI: **Unique roles of DosT and DosS in DosR regulon induction and Mycobacterium tuberculosis dormancy**. *Infection and Immunity* 2009, **77**(8):3258-3263.
137. Kim MJ, Park KJ, Ko IJ, Kim YM, Oh JI: **Different roles of DosS and DosT in the hypoxic adaptation of mycobacteria**. *Journal of Bacteriology* 2010, **192**(19):4868-4875.
138. Bagchi G, Chauhan S, Sharma D, Tyagi JS: **Transcription and autoregulation of the Rv3134c-devR-devS operon of Mycobacterium tuberculosis**. *Microbiology* 2005, **151**(12):4045-4053.
139. Voskuil MI, Schnappinger D, Rutherford R, Liu Y, Schoolnik GK: **Regulation of the Mycobacterium tuberculosis PE/PPE genes**. *Tuberculosis* 2004, **84**(3-4):256-262.
140. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry Iii CE *et al*: **Deciphering the biology of mycobacterium tuberculosis from the complete genome sequence**. *Nature* 1998, **393**(6685):537-544.
141. Florczyk MA, McCue LA, Purkayastha A, Currenti E, Wolin MJ, McDonough KA: **A family of acr-coregulated Mycobacterium tuberculosis genes shares a common DNA motif and requires Rv3133c (dosR or devR) for expression**. *Infection and Immunity* 2003, **71**(9):5332-5343.
142. Boon C, Dick T: **Mycobacterium bovis BCG response regulator essential for hypoxic dormancy**. *Journal of Bacteriology* 2002, **184**(24):6760-6767.
143. Rhoades ER: **Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent Mycobacterium tuberculosis**. *Tubercle and Lung Disease* 1997, **78**(1):57-66.
144. Cosma CL, Humbert O, Sherman DR, Ramakrishnan L: **Trafficking of superinfecting Mycobacterium organisms into established granulomas occurs in mammals and is independent of the Erp and ESX-1 mycobacterial virulence loci**. *Journal of Infectious Diseases* 2008, **198**(12):1851-1855.
145. Leistikow RL, Morton RA, Bartek IL, Frimpong I, Wagner K, Voskuil MI: **The Mycobacterium tuberculosis DosR regulon assists in metabolic homeostasis and enables rapid recovery from nonrespiring dormancy**. *Journal of Bacteriology* 2010, **192**(6):1662-1670.
146. Leyten EMS, Lin MY, Franken KLMC, Friggen AH, Prins C, van Meijgaarden KE, Voskuil MI, Weldingh K, Andersen P, Schoolnik GK *et al*: **Human T-cell responses to 25 novel antigens**

encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. *Microbes and Infection* 2006, **8**(8):2052-2060.

147. Sharpe ML, Gao C, Kendall SL, Baker EN, Lott JS: **The Structure and Unusual Protein Chemistry of Hypoxic Response Protein 1, a Latency Antigen and Highly Expressed Member of the DosR Regulon in Mycobacterium tuberculosis**. *Journal of Molecular Biology* 2008, **383**(4):822-836.
148. Cox RA: **A scheme for the analysis of microarray measurements based on a quantitative theoretical framework for bacterial cell growth: Application to studies of Mycobacterium tuberculosis**. *Microbiology* 2007, **153**(10):3337-3349.
149. Bateman A: **The structure of a domain common to archaebacteria and the homocystinuria disease protein**. *Trends in Biochemical Sciences* 1997, **22**(1):12-13.
150. Boon C, Li R, Qi R, Dick T: **Proteins of Mycobacterium bovis BCG induced in the wayne dormancy model**. *Journal of Bacteriology* 2001, **183**(8):2672-2676.
151. Sharpe ML, Baker EN, Lott JS: **Crystallization of a protein using dehydration without a precipitant**. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* 2005, **61**(6):565-568.
152. Rosenkrands I, Slayden RA, Crawford J, Aagaard C, Barry Iii CE, Andersen P: **Hypoxic response of Mycobacterium tuberculosis studied by metabolic labeling and proteome analysis of cellular and extracellular proteins**. *Journal of Bacteriology* 2002, **184**(13):3485-3491.
153. Roupie V, Romano M, Zhang L, Korf H, May YL, Franken KLMC, Ottenhoff THM, Klein MR, Huygen K: **Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice**. *Infection and Immunity* 2007, **75**(2):941-949.
154. Bashir N, Kounsar F, Mukhopadhyay S, Hasnain SE: **Mycobacterium tuberculosis conserved hypothetical protein rRv2626c modulates macrophage effector functions**. *Immunology* 2010, **130**(1):34-45.
155. Abdallah AM, Gey van Pittius NC, DiGiuseppe Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CMJE, Appelmelk BJ, Bitter W: **Type VII secretion - Mycobacteria show the way**. *Nature Reviews Microbiology* 2007, **5**(11):883-891.
156. Davidow A, Kanaujia GV, Shi L, Kaviar J, Guo X, Sung N, Kaplan G, Menzies D, Gennaro ML: **Antibody profiles characteristic of Mycobacterium tuberculosis infection state**. *Infection and Immunity* 2005, **73**(10):6846-6851.
157. Prabhakar S, Annapurna PS, Jain NK, Dey AB, Tyagi JS, Prasad HK: **Identification of an immunogenic histone like protein (HLP(Mt)) of Mycobacterium tuberculosis**. *Tubercle and Lung Disease* 1998, **79**(1):43-53.
158. Matsumoto S, Yukitake H, Furugen M, Matsuo T, Mineta T, Yamada T: **Identification of a novel DNA-binding protein from Mycobacterium bovis bacillus Calmette-Guerin**. *Microbiology and Immunology* 1999, **43**(11):1027-1036.
159. Shimoji Y, Vincent NG, Matsumura K, Fischetti VA, Rambukkana A: **A 21-kDa surface protein of Mycobacterium leprae binds peripheral nerve laminin-2 and mediates Schwann cell invasion**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(17):9857-9862.
160. Yeruva VC, Duggirala S, Lakshmi V, Kolarich D, Altmann F, Sritharan M: **Identification and characterization of a major cell wall-associated iron-regulated envelope protein (Irep-28) in Mycobacterium tuberculosis**. *Clinical and Vaccine Immunology* 2006, **13**(10):1137-1142.
161. Takatsuka M, Osada-Oka M, Satoh EF, Kitadokoro K, Nishiuchi Y, Niki M, Inoue M, Iwai K, Arakawa T, Shimoji Y *et al*: **A histone-like protein of mycobacteria possesses ferritin superfamily protein-like activity and protects against DNA damage by Fenton reaction**. *PLoS ONE* 2011, **6**(6).
162. Matsumoto S, Furugen M, Yukitake H, Yamada T: **The gene encoding mycobacterial DNA-binding protein I (MDPI) transformed rapidly growing bacteria to slowly growing bacteria**. *FEMS Microbiology Letters* 2000, **182**(2):297-301.
163. Shires K, Steyn L: **The cold-shock stress response in Mycobacterium smegmatis induces the expression of a histone-like protein**. *Molecular Microbiology* 2001, **39**(4):994-1009.
164. Lee BH, Murugasu-Oei B, Dick T: **Upregulation of a histone-like protein in dormant Mycobacterium smegmatis**. *Molecular and General Genetics* 1998, **260**(5):475-479.

165. Lewin A, Baus D, Kamal E, Bon F, Kunisch R, Maurischat S, Adonopoulou M, Eich K: **The mycobacterial DNA-binding protein 1 (MDP1) from Mycobacterium bovis BCG influences various growth characteristics**. *BMC Microbiology* 2008, **8**.
166. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK: **Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis**. *Journal of Bacteriology* 1996, **178**(5):1274-1282.
167. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM: **Comparative genomics of BCG vaccines by whole-genome DNA microarray**. *Science* 1999, **284**(5419):1520-1523.
168. Brosch R, Gordon SV, Billault A, Garnier T, Eiglmeier K, Soravito C, Barrell BG, Cole ST: **Use of a Mycobacterium tuberculosis H37Rv bacterial artificial chromosome library for genome mapping, sequencing, and comparative genomics**. *Infection and Immunity* 1998, **66**(5):2221-2229.
169. Lim A, Eleuterio M, Hutter B, Murugasu-Oei B, Dick T: **Oxygen depletion-induced dormancy in Mycobacterium bovis BCG**. *Journal of Bacteriology* 1999, **181**(7):2252-2256.
170. Hutter B, Dick T: **Analysis of the dormancy-inducible narK2 promoter in Mycobacterium bovis BCG**. *FEMS Microbiology Letters* 2000, **188**(2):141-146.
171. Rustad TR, Harrell MI, Liao R, Sherman DR: **The enduring hypoxic response of Mycobacterium tuberculosis**. *PLoS ONE* 2008, **3**(1).
172. Katsube T, Matsumoto S, Takatsuka M, Okuyama M, Ozeki Y, Naito M, Nishiuchi Y, Fujiwara N, Yoshimura M, Tsuboi T *et al*: **Control of cell wall assembly by a histone-like protein in mycobacteria**. *Journal of Bacteriology* 2007, **189**(22):8241-8249.
173. Stover CK, De La Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH, Hatfull GF *et al*: **New use of BCT for recombinant vaccines**. *Nature* 1991, **351**(6326):456-460.
174. Bardarov S, Bardarov Jr S, Pavelka Jr MS, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, Jacobs Jr WR: **Specialized transduction: An efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG and M. smegmatis.** *Microbiology* 2002, **148**(10):3007-3017.
175. Sambrook J, Fritsch, E. F., & Maniatis, T.: **Molecular Cloning - A Laboratory Manual 2nd Edition**, 2nd edn. New York: Cold Spring Harbor Laboratory Press; 1989.
176. Hanahan D: **Studies on transformation of Escherichia coli with plasmids**. *Journal of Molecular Biology* 1983, **166**(4):557-580.
177. Sharbati-Tehrani S, Meister B, Appel B, Lewin A: **The porin MspA from Mycobacterium smegmatis improves growth of Mycobacterium bovis BCG**. *International Journal of Medical Microbiology* 2004, **294**(4):235-245.
178. Griess P: **Bemerkungen zu der Abhandlung der HH. Weselky und Benedikt Ueber einige Azoverbindungen**. *Berichte der Deutschen chemischen Gesellschaft* 1879, **12**(1):426-428.
179. Goelz SE, Hamilton SR, Vogelstein B: **Purification of DNA from formaldehyde fixed and paraffin embedded human tissue**. *Biochemical and Biophysical Research Communications* 1985, **130**(1):118-126.
180. Lewin A, Freytag B, Meister B, Sharbati-Tehrani S, Schäfer H, Appel B: **Use of a Quantitative TaqMan-PCR for the Fast Quantification of Mycobacteria in Broth Culture, Eukaryotic Cell Culture and Tissue**. *Journal of Veterinary Medicine Series B: Infectious Diseases and Veterinary Public Health* 2003, **50**(10):505-509.
181. Desjardin LE, Perkins MD, Teixeira L, Cave MD, Eisenach KD: **Alkaline decontamination of sputum specimens adversely affects stability of mycobacterial mRNA**. *Journal of Clinical Microbiology* 1996, **34**(10):2435-2439.
182. Hellyer TJ, Desjardin LE, Hehman GL, Cave MD, Eisenach KD: **Quantitative analysis of mRNA as a marker for viability of Mycobacterium tuberculosis**. *Journal of Clinical Microbiology* 1999, **37**(2):290-295.
183. Cohavy O, Harth G, Horwitz M, Eggena M, Landers C, Sutton C, Targan SR, Braun J: **Identification of a novel mycobacterial histone H1 homologue (HupB) as an antigenic target of pANCA monoclonal antibody and serum immunoglobulin A from patients with Crohn's disease**. *Infection and Immunity* 1999, **67**(12):6510-6517.
184. Lefrançois LH, Pujol C, Bodier CC, Teixeira-Gomez AP, Drobecq H, Rosso ML, Raze D, Dias AA, Hugot JP, Chacon O *et al*: **Characterization of the Mycobacterium avium subsp. paratuberculosis laminin-binding/histone-like protein (Lbp/Hlp) which reacts with sera from patients with Crohn's disease**. *Microbes and Infection* 2011, **13**(6):585-594.
185. Goude R, Parish T: **Electroporation of mycobacteria**. *Methods in molecular biology (Clifton, NJ)* 2009, **465**:203-215.

186. Levy-Frebault VV, Portaels F: **Proposed minimal standards for the genus Mycobacterium and for description of new slowly growing Mycobacterium species**. *International Journal of Systematic Bacteriology* 1992, **42**(2):315-323.
187. Kalpana GV, Bloom BR, Jacobs Jr WR: **Insertional mutagenesis and illegitimate recombination in mycobacteria**. *Proceedings of the National Academy of Sciences of the United States of America* 1991, **88**(12):5433-5437.
188. Batoni G, Maisetta G, Florio W, Freer G, Campa M, Senesi S: **Analysis of the Mycobacterium bovis hsp60 promoter activity in recombinant Mycobacterium avium**. *FEMS Microbiology Letters* 1998, **169**(1):117-124.
189. Chen XY, Li CY, Ma Y, Liu C, Wang JH, Zhang XF, Chang ZY: **Study on gene knock-out in Mycobacterium BCG**. *Chinese journal of tuberculosis and respiratory diseases* 2004, **27**(3):183-187.
190. Wilson T, De Lisle GW, Marcinkeviciene JA, Blanchard JS, Collins DM: **Antisense RNA to ahpC, an oxidative stress defence gene involved in isoniazid resistance, indicates that AhpC of Mycobacterium bovis has virulence properties**. *Microbiology* 1998, **144**(10):2687-2695.
191. Greendyke R, Rajagopalan M, Parish T, Madiraju MVVS: **Conditional expression of Mycobacterium smegmatis dnaA, an essential DNA replication gene**. *Microbiology* 2002, **148**(12):3887-3900.
192. Secott TE, Lin TL, Wu CC: **Mycobacterium avium subsp. paratuberculosis fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins**. *Infection and Immunity* 2004, **72**(7):3724-3732.
193. Deol P, Vohra R, Saini AK, Singh A, Chandra H, Chopra P, Das TK, Tyagi AK, Singh Y: **Role of Mycobacterium tuberculosis Ser/Thr kinase PknF: Implications in glucose transport and cell division**. *Journal of Bacteriology* 2005, **187**(10):3415-3420.
194. MacMicking JD, Taylor GA, McKinney JD: **Immune Control of Tuberculosis by IFN-γ-inducible LRG-47**. *Science* 2003, **302**(5645):654-659.
195. Sturgill-Koszycki S, Swanson MS: **Legionella pneumophila replication vacuoles mature into acidic, endocytic organelles**. *Journal of Experimental Medicine* 2000, **192**(9):1261-1272.
196. Heinzen RA, Scidmore MA, Rockey DD, Hackstadt T: **Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of Coxiella burnetii and Chlamydia trachomatis**. *Infection and Immunity* 1996, **64**(3):796-809.
197. Maurin M, Benoliel AM, Bongrand P, Raoult D: **Phagolysosomes of Coxiella burnetii-infected cell lines maintain an acidic pH during persistent infection**. *Infection and Immunity* 1992, **60**(12):5013-5016.
198. Tardieux I, Webster P, Ravesloot J, Boron W, Lunn JA, Heuser JE, Andrews NW: **Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells**. *Cell* 1992, **71**(7):1117-1130.
199. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry Iii CE: **The salicylate-derived mycobactin siderophores of Mycobacterium tuberculosis are essential for growth in macrophages**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(3):1252-1257.
200. Booth IR: **Regulation of cytoplasmic pH in bacteria**. *Microbiological Reviews* 1985, **49**(4):359-378.
201. Vandal OH, Nathan CF, Ehrt S: **Acid resistance in Mycobacterium tuberculosis**. *Journal of Bacteriology* 2009, **191**(15):4714-4721.
202. Zhang Y, Scorpio A, Nikaido H, Sun Z: **Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of Mycobacterium tuberculosis to pyrazinamide**. *Journal of Bacteriology* 1999, **181**(7):2044-2049.
203. Roch N, Maurin M: **Antibiotic susceptibilities of Legionella pneumophila strain Paris in THP-1 cells as determined by real-time PCR assay**. *Journal of Antimicrobial Chemotherapy* 2005, **55**(6):866-871.
204. Åbrink M, Gobl AE, Huang R, Nilsson K, Hellman L: **Human cell lines U-937, THP-1 and Mono Mac 6 represent relatively immature cells of the monocyte-macrophage cell lineage**. *Leukemia* 1994, **8**(9):1579-1584.
205. Van Crevel R, Ottenhoff THM, Van der Meer JWM: **Innate immunity to Mycobacterium tuberculosis**. *Clinical Microbiology Reviews* 2002, **15**(2):294-309.
206. Beltan E, Horgen L, Rastogi N: **Secretion of cytokines by human macrophages upon infection by pathogenic and non-pathogenic mycobacteria**. *Microbial Pathogenesis* 2000, **28**(5):313-318.

207. Fulton SA, Cross JV, Toossi ZT, Boom WH: **Regulation of interleukin-12 by interleukin-10, transforming growth factor-β, tumor necrosis factor-α, and interferon-γ in human monocytes infected with Mycobacterium tuberculosis H37Ra**. *Journal of Infectious Diseases* 1998, **178**(4):1105-1114.
208. Gong JH, Zhang M, Modlin RL, Linsley PS, Iyer D, Lin Y, Barnes PF: **Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression**. *Infection and Immunity* 1996, **64**(3):913-918.
209. Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG, Lee EJ, Orme IM, Gonzalez-Juarrero M: **Lack of IL-10 alters inflammatory and immune responses during pulmonary Mycobacterium tuberculosis infection**. *Tuberculosis* 2009, **89**(2):149-157.
210. Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kühn R, Müller W, Trinchieri G, Sher A: **In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-γ, and TNF-α**. *Journal of Immunology* 1996, **157**(2):798-805.
211. Juffermans NP, Verbon A, Van Deventer SJH, Van Deutekom H, Belisle JT, Ellis ME, Speelman P, Van Der Poll T: **Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: A possible role for mycobacterial lipoarabinomannan**. *Infection and Immunity* 1999, **67**(8):4295-4297.
212. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ: **Human Toll-like receptors mediate cellular activation by Mycobacterium tuberculosis**. *Journal of Immunology* 1999, **163**(7):3920-3927.
213. Aston C, Rom WN, Talbot AT, Reibman J: **Early inhibition of mycobacterial growth by human alveolar macrophages is not due to nitric oxide**. *American Journal of Respiratory and Critical Care Medicine* 1998, **157**(6 PART I):1943-1950.
214. Chan ED, Winston BW, Uh ST, Wynes MW, Rose DM, Riches DWH: **Evaluation of the role of mitogen-activated protein kinases in the expression of inducible nitric oxide synthase by IFN-γ and TNF-α in mouse macrophages**. *Journal of Immunology* 1999, **162**(1):415-422.
215. Macmicking JD, North RJ, Lacourse R, Mudgett JS, Shah SK, Nathan CF: **Identification of nitric oxide synthase as a protective locus against tuberculosis**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(10):5243-5248.
216. Jagadha V, Andavolu RH, Huang CT: **Granulomatous inflammation in the acquired immune deficiency syndrome**. *American Journal of Clinical Pathology* 1985, **84**(5):598-602.
217. Puissegur MP, Lay G, Gilleron M, Botella L, Nigou J, Marrakchi H, Mari B, Duteyrat JL, Guerardel Y, Kremer L *et al*: **Mycobacterial lipomannan induces granuloma macrophage fusion via a TLR2-dependent, ADAM9- and β1 integrin-mediated pathway**. *Journal of Immunology* 2007, **178**(5):3161-3169.
218. Facchetti F, Vermi W, Fiorentini S, Chilosi M, Caruso A, Duse M, Notarangelo LD, Badolato R: **Expression of inducible nitric oxide synthase in human granulomas and histiocytic reactions**. *American Journal of Pathology* 1999, **154**(1):145-152.
219. Hernandez-Pando R, Bornstein QL, Aguilar Leon D, Orozco EH, Madrigal VK, Martinez Cordero E: **Inflammatory cytokine production by immunological and foreign body multinucleated giant cells**. *Immunology* 2000, **100**(3):352-358.
220. Papadimitriou JM, Van Bruggen I: **Evidence that multinucleate giant cells are examples of mononuclear phagocytic differentiation**. *Journal of Pathology* 1986, **148**(2):149-157.
221. Peterson PK, Gekker G, Hu S, Robert Anderson W, Teichert M, Chao CC, Molitor TW: **Multinucleated giant cell formation of swine microglia induced by Mycobacterium bovis**. *Journal of Infectious Diseases* 1996, **173**(5):1194-1201.
222. Running DR, Klabunde T, Besra GS, Vissa VD, Belisle JI, Sacchettini JC: **Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines**. *Nature Structural Biology* 2000, **7**(2):141-146.
223. Geisel RE, Sakamoto K, Russell DG, Rhoades ER: **In vivo activity of released cell wall lipids of Mycobacterium bovis bacillus Calmette-Guérin is due principally to trehalose mycolates**. *Journal of Immunology* 2005, **174**(8):5007-5015.
224. Flynn JL, Chan J: **Tuberculosis: Latency and reactivation**. *Infection and Immunity* 2001, **69**(7):4195-4201.
225. Aly S, Wagner K, Keller C, Malm S, Malzan A, Brandau S, Bange FC, Ehlers S: **Oxygen status of lung granulomas in Mycobacterium tuberculosis-infected mice**. *Journal of Pathology* 2006, **210**(3):298-305.

226. Via LE, Lin PL, Ray SM, Carrillo J, Allen SS, Seok YE, Taylor K, Klein E, Manjunatha U, Gonzales J *et al*: **Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates**. *Infection and Immunity* 2008, **76**(6):2333-2340.
227. Orme IM: **The mouse as a useful model of tuberculosis**. *Tuberculosis* 2003, **83**(1-3):112-115.

8. Attachment

8.1 Abbreviations

AG	Arabinogalactan
BCG	Bacille Calmette-Guerin
bp	Basepair
CBS	Cystathionine beta synthase
cfu	Colony forming units
CTAB	Cetyltrimethyl-ammonium-bromid
DNA	Deoxyribonucleic acid
DosR	Dormancy survival regulon
ELISA	Enzyme Linked Immunosorbant Assay
et al.	et alia
FAM	6-Carboxyl-Flourescin
FCS	Foetal calve serum
FI	Fusion index
g	Acceleration of gravity
HIV	Human immunodeficiency virus
hsp60	Heat shock promoter 60
kb	Kilo basis
kDa	Kilodalton
LAM	Lipoarabinomannan
LM	Lipomannan
LPS	Lipopolysaccharide
mA	Milli Ampere
MC	Multinucleated cells
MCS	Multiple cloning site
MGC	Multinucleated giant cells
mdp1	mycobacterial DNA binding protein 1
MOI	Multiplicity of infection
MTBC	*Mycobacterium tuberculosis* complex
ng	Nanogram
NO	Nitric oxode
NRP	Non-replicating persistence
NTM	Nontuberculous mycobacteria
OADC	Oleic acid albumin dextrose
OD	Optical density
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
PIM	Phosphatidyl-myo-inositol mannosides
PVDF	Polyvinylidene fluoride membrane
RLU	Relative Light Units
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMM	Trehalose-6-monomycolate
WHO	World Health Organisation

8.2 Plasmid Maps

8.2.1 Plasmid pMV261

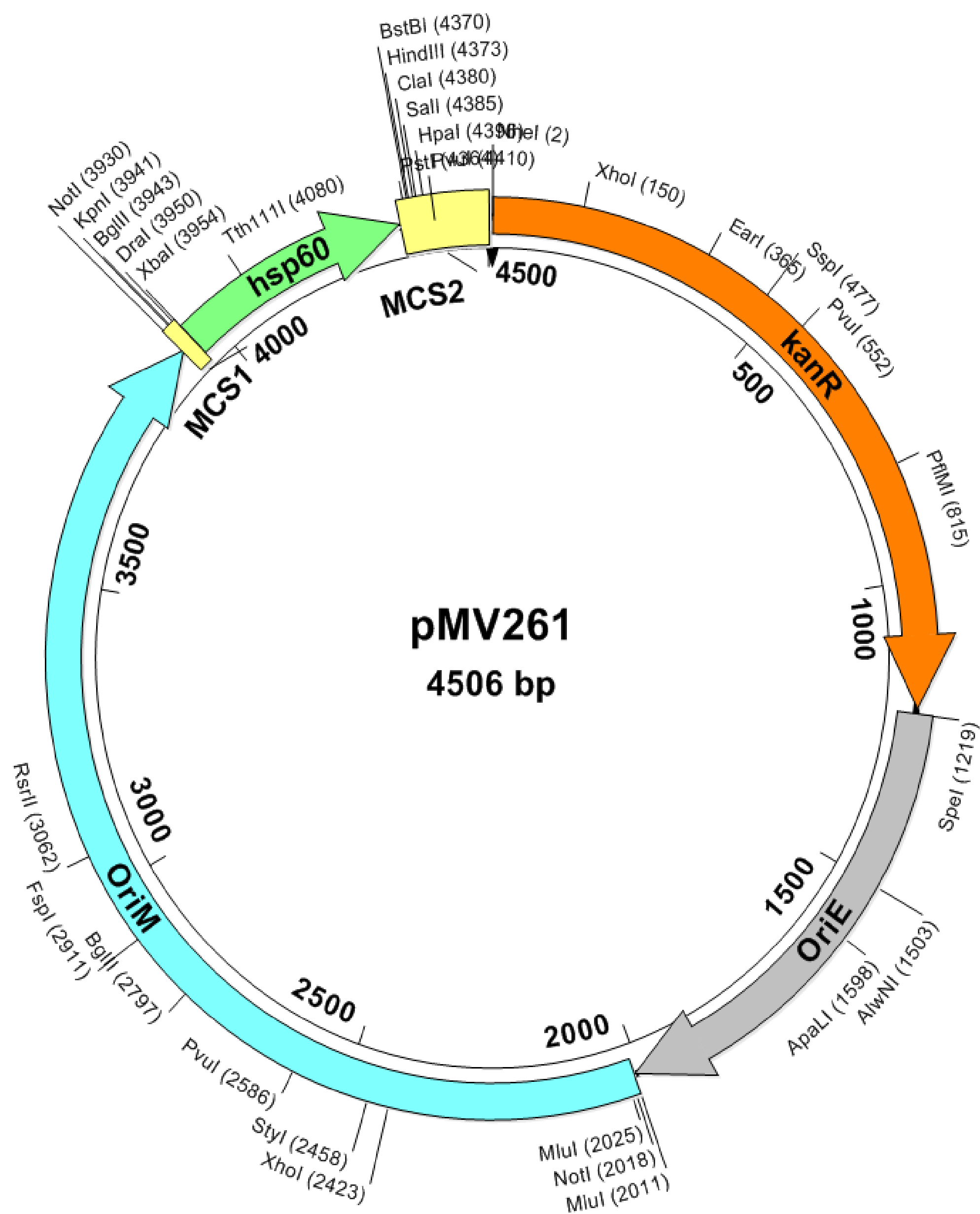


Figure 27. Plasmid pMV261 [173]

E. coli shuttle vector with kanamycin resistance (kanR) gene, an origin of replication in *E. coli* (OriE), origin of replication in mycobacteria (OriM), mycobacterial heat shock promoter 60 (hsp60) and a multiple cloning site (MCS).

8.2.2 Plasmid pYUB854

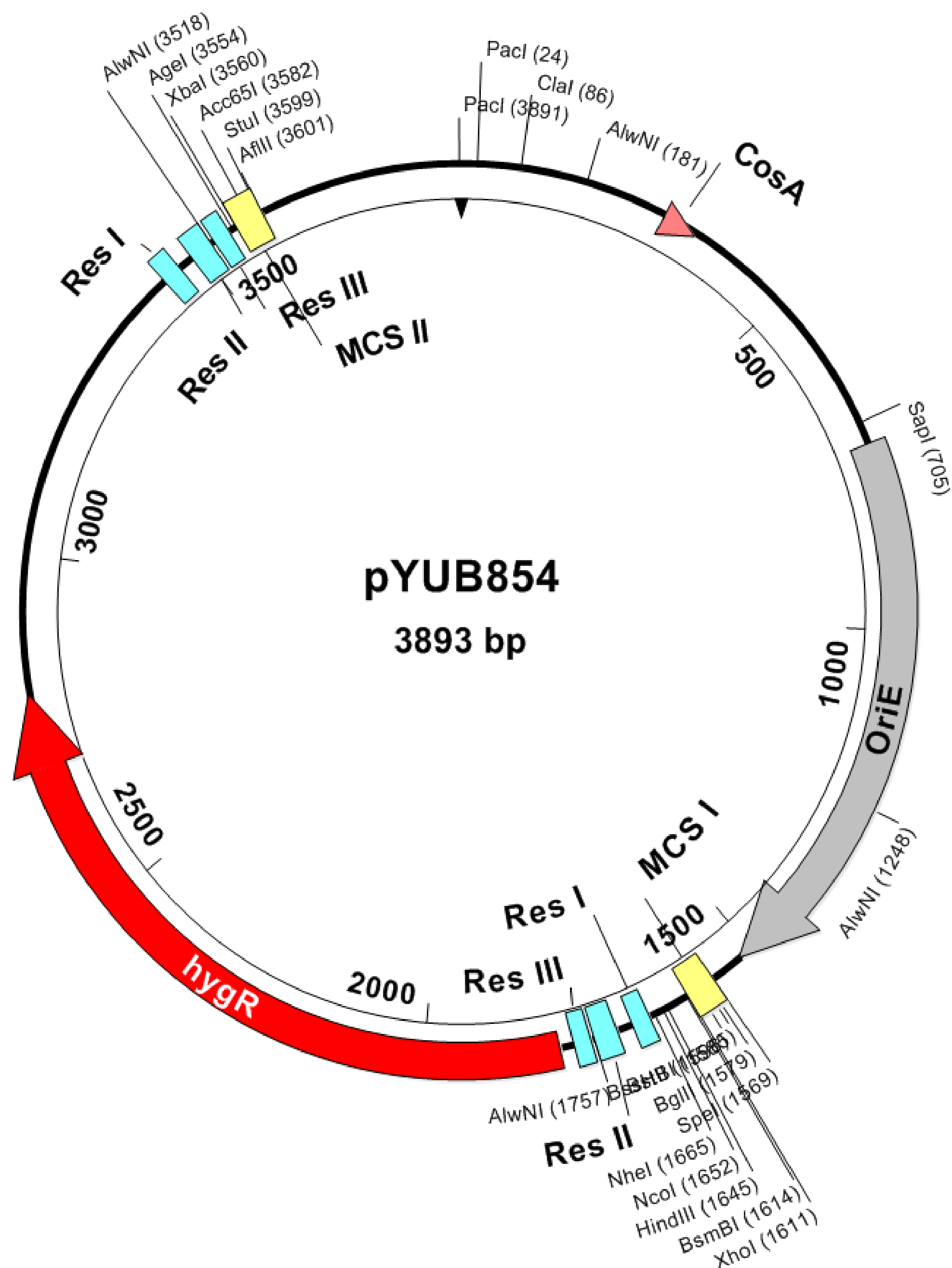


Figure 28. Plasmid pYUB854 [174]

E. coli cosmid with an origin of replication in *E. coli* (OriE) and hygromycin resistance (hygR) gene flanked by multiple cloning site (MCS).