CHAPTER 1

Introduction

Mycobacterium tuberculosis; too smart to handle?

Tuberculosis, a disease of all times

Tuberculosis (TB) is a disease that co-evolved with mankind for many thousands of years [1] and remains responsible for many deaths every year. Approximately 8.7 million new TB cases were identified in 2011 and 1.4 million individuals died of TB in the same year [2]. The first signs of human TB existence can be traced back over 9,000 years ago (7000 B.C.) as shown by DNA and mycolic acid markers detected in human skeletal remains [3]. TB has had many names over time, which refer to consequences of the disease. At the Hippocratic era (460-370 BC) TB was known as consumption or phthisis (Greek for consumption), referring to progressive emaciation or weight loss due to the disease. Later, during the middle ages TB was referred to as the white plague. At this time, TB was accountable for the death of one-fourth of the whole European population and it was even thought that the survival of the European race was at stake [4]. The mechanism and source of the disease remained unknown until the late 19th century. Continuing the work of many researchers who had tried to identify the source of TB, including Jakob Henle, Jean Antoine Villemin and Theodor Klebs, Robert Koch finally identified *Mycobacterium tuberculosis* (*Mtb*) to be the causative agent and was awarded the Nobel prize in 1905 for his work [5;6]. Unfortunately, despite intense efforts to reduce TB, including improved public health care and therapies [7-9], TB continues to remain one of the leading causes of death due to infectious disease worldwide [2].

Mycobacterium tuberculosis infection: From latent infection to active disease

Transmission occurs via inhalation of *Mtb* loaded aerosols that are spread by coughing pulmonary TB patients. The bacilli enter the alveolar space in the lungs where they are taken up by phagocytes including alveolar macrophages, dendritic cells but also pulmonary epithelial cells. *Mtb* is taken up in intracellular compartments called phagosomes. Both neutrophils and monocytes are then attracted to the site of infection, and monocytes subsequently mature into macrophages which are better at taking up and inhibiting *Mtb*. The precise functions of neutrophils remain unclear as they have protective properties at the time of infection but become detrimental at later stages [10-12]. Eventually, the attraction of innate cells to the site of initial infection results in the formation of the primary granuloma (Ghon complex) [13]. Pathogen loaded dendritic cells (DC) subsequently migrate to the mediastinal lymph nodes (MLN), which drain the lungs, where they start presenting antigens to the T and B cells [14;15]. Once the adaptive immune system is triggered, T-effector cells are recruited to the site of infection, where they can exert their function and further shape the granuloma [16].

Intriguingly, this triggering of adaptive immune responses in TB is significantly delayed compared to infections with other pathogens and, in addition, triggered innate and adaptive immune cells often cannot efficiently eliminate *Mtb*. Here, *Mtb* itself plays an important role as the bacilli possess several immune evasion mechanisms which include: inhibition of neutrophil

apoptosis [17], induction of anti-inflammatory responses, such as production of anti-inflammatory cytokines by innate cells and generation of regulatory T cells (Tregs) [18-20], delay in transport of infected, or antigen-loaded DC to the MLN [14;15;21], delay in priming and recruitment of innate and adaptive cells to the site of infection [18;22], preventing phago-lysosome fusion [23-25], detoxification of reactive oxygen species and reactive nitrogen intermediates [26], inhibition of MHC class II presentation [27], and reduced activation of antigen-specific T cells at the site of infection [28;29]. These mechanisms hamper a rapid response of adaptive immune cells, and enable *Mtb* to survive intracellularly [22;30;31]. Remarkably, *Mtb* can also infect bone marrow derived mesenchymal stem cells (BM-MSC) that reside in the bone marrow and use these cells as a hitherto unknown niche and hideout from immune patrol [32].

At first sight, the formation of granulomas seems to be beneficial for the host since it contains infection locally. However, recent data challenge this dogma and show that *Mtb* also benefits from granuloma formation, since it is able to use freshly recruited cells as novel niches, leading to dissemination [33]. Of special interest, the presence of *Mtb* ESAT-6 protein is required for well-formed granulomas as it triggers recruitment of macrophages via host matrix metalloproteinase 9 (MMP9) [34;35]. In addition, further spread is enhanced by *Mtb* (ESAT-6) induced necrosis of infected host cells e.g. [36-39] and inhibition of protective apoptosis e.g. [37;38;40;41], although other studies showed that (ESAT-6 induced) apoptosis is beneficial for *Mtb* e.g. [33;42-44], thus the exact effect of host cell death mechanisms on *Mtb* are yet undefined.

Ultimately, the combination of host pressure and *Mtb* immune escape mechanisms can result in an established balance between host and pathogen, i.e. latent *Mtb* infection (LTBI). This balance between pathogen and host enables *Mtb* to reside for decades at the site of infection. LTBI individuals are thus *Mtb* infected but lack any clinical symptoms. However, latent infection can reactivate and lead to active disease: 5 to 10% of LTBI will develop TB during their lifetime and half of these cases will develop within two-five years after infection [45-47]. Nevertheless, reactivation after decades of latent infection has also been described [48]. Of note, these numbers are difficult to interpret partly because it is unknown what percentage of LTBI is able to clear the infection completely. It is believed that *Mtb* in LTBI mostly enters a dormant state, adapting to the harsh environment in the host while awaiting a possible chance to reactivate and spread. Once an imbalance occurs between host and pathogen, due to weakened host immunity, bacteria will be able to reactivate [31] and necrotic regions will develop within the granuloma, leading to caseous necrotic lesions. These lesions can liquefy which ultimately leads to leakage of caseum into the airways, resulting in a contagious TB case [13;16].

A very high risk of TB reactivation is observed in individuals that are immune deficient. These include: (i) HIV infected individuals particularly those with reduced (<200) $CD4^+$ T-cell counts [49-51], (ii) individuals undergoing anti-TNF treatment [52;53], and (iii) patients with genetic disorders in genes coding for receptors and cytokines involved in type-1 immunity and the Th1 cascade [54-56]. Thus, *Mtb* infection can result in a broad spectrum of stages, ranging from (likely) complete bacterial clearance to latent infection and reactivation or sometimes acute disease [57].

Efforts to Tackle TB

The timely detection and treatment of TB, and its prevention by vaccination are the two major tasks that the WHO has embraced to reduce the burden of TB. Here we will focus on immunological aspects of these tasks, and discuss immune-based detection techniques as well as vaccination against *Mtb*. Treatment of *Mtb* will not be further discussed in this chapter.

Detection of latent *Mtb* infection

Detection of *Mtb* infection is of major importance for adequate control of TB. *Mtb* infection can be determined in the sputum of active TB patients, using a smear microscopy test and bacterial culturing, or nucleic acid amplification tests to detect *Mtb* DNA (NAATs e.g: Xpert MTB/RIF® assay) complemented with chest X-rays [58;59]. Unfortunately, the presence of viable bacteria cannot be determined in LTBI. To verify *Mtb* exposure in this population an indirect test is required, analyzing the immune responses towards mycobacterial products.

Tuberculin skin test. Koch introduced tuberculin, an extract from tubercle bacilli dissolved in glycerine, as a new medicine to treat tuberculosis, however this unfortunately failed [60]. A few years later, Clemens Freiherr von Pirquet showed that Koch's tuberculin could be used as a tool to identify latent *Mtb* infection, by injecting the substance intracutaneously, which resulted in a delayed hypersensitivity (DTH) response in exposed individuals. Charles Mantoux further developed this diagnostic technique and in 1908, Florence Seibert improved tuberculin to purified protein derivative (PPD), which is still in use today [5;61]. This technique is currently known as the tuberculin skin test (TST) or Mantoux test. Unfortunately, cross-reactivity of PPD with nontuberculous mycobacteria (NTM) and the vaccine strain *Mycobacterium bovis* Bacille Calmette-Guérin *(*BCG) reduces the specificity of the TST [62-65]. Exposure to NTMs is very common as they reside in many sources in the host environment such as soil and natural waters [66;67]. One study found NTM to be abundantly present in showerhead biofilms [68]. A practical drawback of the TST is that it requires two visits, can induce boosting effects upon multiple TST testing and can be false negative in immunocompromised (e.g. HIV infected) individuals.

Interferon-Gamma Release Assays (IGRAs). To increase the specificity of *Mtb* diagnosis, a new technique was introduced. This technique is based on antigen specific interferon-gamma (IFN-γ) secretion upon stimulation with peptides from the highly specific and immunogenic *Mtb* antigens early-secreted antigen 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7. These tests are widely known as Interferon-Gamma Release Assays (IGRAs). In contrast to the TST, false negative responses due to *Mtb* specific anergy can be identified since both a positive and negative control are included [69]. Multiple studies have shown that IGRAs have enhanced specificity over TST [70-72], although most of these studies were performed in high-income countries such that the results cannot directly be extrapolated to low and middle income countries with higher TB incidences [73;74]. Indeed, IGRAs proved to be less powerful in high TB incidence countries

[75;76]. Due to the lack of sufficient evidence IGRAs are not recommended for use in low and middle income countries [74]. Furthermore, discordant results between TST and IGRA results have been documented which often remain poorly explained [77]. Despite the relative success of IGRAs in low endemic settings, it has not proved possible to use these tests to discriminate between recent and remote *Mtb* infection, and also not between active and latent *Mtb* infection. Recently an *Mtb* antigen, Rv2628, was described to be recognized by remote *Mtb* infected individuals, but not by recent *Mtb* infected individuals, suggesting a role as discriminatory marker [78]. Although the specificity is increased, the IGRA antigens are not fully restricted to *Mtb* as homologs of these proteins are also present in *Mycobacterium leprae* [79;80] *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium riyadhense* and *Mycobacterium marinum*. Also, testing too soon after *Mtb* infection might result in false negative outcomes [81].

Importantly, as already indicated, these two tests determine *Mtb* exposure indirectly. Responses towards *Mtb* products can be detected, but this does not directly indicate that viable bacilli are still present. It may be that either the infection has been cleared but immune responses persist, or that *Mtb* bacilli are still present. Altogether, although latent *Mtb* infection detection methods are available, improvement is definitely required.

Vaccine development

Despite the availability of diagnostic tests and TB treatments, TB remains difficult to eradicate. One major concern is the rapid development of (extensively) multidrug resistant (XDR/MDR) *Mtb* strains. At this moment even strains are known which are resistant to all current drugs available (totally drug resistant *Mtb*, TDR) [82]. Therefore, robust *Mtb* vaccines are needed to prevent further development of TB outbreaks that cannot be controlled using current drugs.

Current TB vaccine. In 1921, Albert Calmette and Camille Guérin developed the first vaccine against TB. They cultured *Mycobacterium bovis* (*M. bovis*) until it became avirulent (due to the loss of genetic regions, including RD1[83]) and designated the vaccine as *M. bovis* Bacille Calmette-Guérin (BCG) [5;84]. BCG is still the only vaccine available at this moment. The effect of BCG has widely been studied in many countries and showed its protective value towards disseminated and meningeal childhood TB [85] as well as to a certain level against leprosy, which is caused by *Mycobacterium leprae* [86;87]. Unfortunately, BCG vaccination results in highly variable protection against pulmonary TB in adults [88], which is the most common form of TB. Importantly, pulmonary TB is, together with the rare laryngeal TB, the only form of TB accountable for further transmission of disease. The effect of BCG can be diminished due to co-infections with NTM or helminthes [89-91]. In addition to the highly variable protection, BCG vaccination, which is a live attenuated vaccine, can lead to severe infection in immune deficient individuals [92] [54]. The lack of BCG's efficacy against pulmonary TB and its severe adverse effects in immune deficiencies clearly show that there is a significant need for improved TB vaccines which are both safer and more effective than BCG.

Novel TB vaccines. Mtb vaccines need to trigger cellular immunity to induce protection against *Mtb* as the bacterium resides intracellular in phagosomes such that antibodies cannot easily reach the pathogen. Importantly, Th1 immunity was previously shown to play an important role in the control of *Mtb* infection [16]. New preventive TB vaccines can either be used as booster vaccines on top of previous BCG vaccination, or as individual, priming vaccines. The three different types of vaccines currently under study are (i) live attenuated and/or genetically modified mycobacterial vaccines, (ii) killed (fragmented) whole bacteria and (iii) subunit vaccines. Notably, the development of therapeutic vaccines is of significant interest. The goal of this vaccine is to prevent reactivation of *Mtb* in LTBI by enhancing the control of infection. At this moment, over 12 vaccines already entered clinical trials, whereas over 40 vaccines are currently in the pre-clinical phase [16].

The live attenuated and/or genetically modified mycobacteria are developed to be used as priming vaccines, meant to replace BCG. These improved vaccines should be more immunogenic, maintain this immune response for a longer period of time and be safer in immune-deficient individuals. Recombinant BCG (rBCG) modifications include i) introduction or over-expression of specific *Mtb* antigens which are lacking from BCG, and ii) improvement of antigen presentation by genetic modification of important pathways [16]. One of these rBCG vaccines is rBCGΔUrec:Hly$^+$ (VPM1002). rBCGΔUrec:Hly$^+$ expresses listeriolysin (Hly) which is responsible for rBCGs escape from phagosomes into the cytosol. Urease C (UreC), a protein involved in preventing phagosomal acidification, was removed to improve the effect of Hly, which functions most optimal under acidic conditions. This rBCG had a better vaccine potential compared to its parental strain [93]. Apoptosis of infected cells was promoted and increased CD4$^+$ as well as CD8$^+$ T-cell responses was observed, possibly due to enhanced cross priming. The vaccine has entered phase IIa trials [94]. Other rBCG vaccines include strains that overexpress antigens such as Ag85B (rBCG30) [95] and RD1 antigens [96]. Aeras-422, a vaccine that resembles rBCGΔUrec:Hly$^+$, expresses perfringolysin, but also overexpresses Ag85A, Ag85B and Rv3407 [97]. The combination of induced escape into the cytosol and overexpression of immunogenic antigens in one vaccine appears to have an improved efficacy. However this vaccine induced side effects such as the occurrence of shingles urging termination of the phase I trial [16;98;99].

Using *Mtb* strains for vaccine purposes requires special attention, it is important that attenuated strains are not virulent and therefore at least two independent loci, e.g. involved in essential metabolic pathways (autotrophy) or virulence, should be deleted, preventing reversion of virulence [100;101]. Currently several attenuated *Mtb* strains are under study as potential vaccines. The attenuated *Mtb* ΔsecA2ΔlysA [102] and *Mtb* ΔfbpAΔsapM [103] are examples of such mutant strains and are in a very early stage of analysis. Two other attenuated *Mtb* strains, *Mtb* ΔRD1ΔpanCD [104] and *Mtb* ΔPhoP (SO2), have already been studied for some time, showing promising immunogenicity and safety results. In order to comply to a consensus reached at a WHO Geneva meeting in 2004 [100;101;105], the SO2 strain has been further improved by deletion of the FadD26 gene, generating *Mtb* ΔPhoPΔfad or MTBVAC. The MTBVAC vaccine

has now entered phase I clinical trial to analyze its safety in humans. Interestingly, a novel potential vaccine strain was developed consisting of an attenuated *Mtb* strain expressing HIV antigens to prevent both *Mtb* and HIV infections [106].

In contrast to live attenuated and/or genetically modified mycobacteria vaccines, non-live subunit vaccines were initially developed to be used as booster on top of BCG vaccination or to enable novel live attenuated and/or genetically modified mycobacterial vaccines to enhance the long-lived immune memory response against *Mtb*. Subunit vaccines consist of protein(s) and/or (poly)epitopes delivered using a viral vector system or as recombinant product together with an (Th1) adjuvant [16;107]. One of the most advanced subunit vaccine is MVA85A, a recombinant (replication deficient) strain of Modified Vaccinia Ankara (MVA) virus that expresses the *Mtb* Ag85A antigen. MVA85A was shown to be immunogenic and safe in use in children and adults located at multiple sites [108-111]. Unfortunately, although MVA85A was safe, the phase IIb trial, published in February 2013, resulted in insignificant efficacy against TB and *Mtb* infection in children [112]. Another vaccine, AdAg85A which is currently studied in phase I trial, resembles MVA85A in that it also expresses Ag85A but expressed in an (replication deficient) Adenovirus (type 5) [113;114]. Ad35 (AERAS-402) is a virus-based vaccine expressing both *Mtb* Ag85A, Ag85B and TB10.4 as a fusion protein using a replication deficient Adenovirus (type 35) vector. Again, this vaccine also showed strong immunogenic potential in BCG primed individuals and also at different test sites [115;116]. This Ad35 based vaccine is currently in a phase IIb trial [117]. Importantly, some concerns have been raised over the use of adenovirus as vaccine vectors including the existence of neutralizing host antibodies that can influence the functioning of the vaccine, which can be avoided by using other viral vectors [118-120].

Also different subunit vaccines consisting of protein and adjuvants are currently studied. M72/AS01, consisting of Rv1196 (PPE18) and Rv0125 (serine protease, pepA) just finished phase IIa trial testing and showed to be safe and immunogenic [121;122]. Hybrid 1 (H1) (Ag85B and ESAT-6 fusion protein) administered using IC31 as adjuvant induced strong immunity in healthy donors and mycobacteria exposed individuals [123;124]. HyVac4 (or H4, consisting of Ag85B and TB10.4 fusion protein) shows immunogenic and protective effects in various animal models, and has now entered clinical trials as H4 administered together with IC31 [125]. Of note, H4 was developed to avoid H1 vaccine induction of ESAT-6 responses which interferes with IGRA outcome. Other potential subunit vaccines, including H56 (H1 backbone including Rv2660) [126] are still in pre-clinical phase e.g.: [127;128]. Instead of proteins, immunogenic epitopes can be combined to form a polyepitope and this also shows vaccine potential [107]. These vaccines can be used in heterologous prime-boost settings, thus combining live or attenuated vaccines with subunit vaccines to induce optimal protection.

Early secreted antigens such as ESAT-6 and Ag85 are often selected for TB subunit vaccines, based on expression in active replicating *Mtb*, secretion and strong (*in vitro*) immunogenicity [129]. Previous studies showed a protective effect of *Mtb* short culture filtrate which contained

both ESAT-6 and Ag85B [130]. Nonetheless, the antigens chosen for vaccination should also be significantly expressed by *Mtb* during *in vivo* infection, preferably over extended periods of time, preferably in different phases of *Mtb* infection and in both resistant and susceptible host backgrounds, such that the vaccine would induce T cells that directly recognize infected cells. This will be discussed in the next section.

Knowing *Mtb's* lifestyle

Phases of *Mycobacterium tuberculosis*

As already indicated, after infection *Mtb* is able to reside for decades inside the host without causing any clinical symptoms. To accomplish this, *Mtb* has to evade host induced pressures such as nutrient and oxygen shortage, acidic environment, toxic products produced by host cells and immune pressure. Influenced by these factors, *Mtb* encounters a dormant state which is characterized by: lipid body loaded, (phenotypic) drug-resistant, non-replicating bacteria with low metabolic activity (dormant bacteria) [131;132]. The granuloma will function to contain the infection [31]. Most probably both active replicating and dormant bacteria will be present during infection [133], but only dormant bacteria are tolerant to drugs and therefore difficult to eliminate by antibiotic treatment [16]. For improved diagnosis of latent *Mtb* infection and for optimal (therapeutic) vaccine development, a better understanding of *Mtb*'s lifecycle is of critical importance, particularly better insights in how *Mtb* adapts to *in vivo* environment upon infection. The change of *Mtb*'s genetic and proteomic makeup could have major impact on what antigens can be used as vaccine candidates.

In the beginning of the 21st century, *in vitro Mtb* culture studies captured the first differences in *Mtb's* transcriptomics response by comparing bacteria cultured under different stress conditions [134-136]. These studies focused on stress factors that *Mtb* is thought to encounter upon infection, including hypoxia, low pH, nutrient deprivation and free radicals. Oxygen tension is an important factor in the activation state of *Mtb* as the bacteria prefer to inhabit aerated parts of the host. [137]. Though, the transcriptional analysis showed that *Mtb* is able to adapt to oxygen depletion, to nitric oxide and to carbon monoxide [138-142], environmental conditions which are thought to occur within host cells and granulomas. Importantly, hypoxic regions were identified at the infection site *in vivo* [143]. Particularly interesting was the upregulation of a cluster of 48 genes, known as the dormancy regulon (DosR), which is controlled by regulator *Rv3133c* (DosR or DevR) [144;145]. Interestingly, a second set of genes is expressed at a later stage of hypoxia, known as the enduring hypoxic response (EHR). This set includes ~230 genes (including several DosR genes), which are upregulated considerably longer than most of the genes expressed by the DosR regulon [146].

Starvation conditions also trigger differential *Mtb* gene expression [140;147]. Here, *Mtb* has to compete for nutrients or adapt to the lack of nutrients and has to switch to the use of different

carbon sources for energy [148]. Interestingly, a substantial number of EHR genes overlap with genes associated with the starvation response [146]. Indeed, *In vivo*, *Mtb* will be exposed to multiple stress conditions at once. Dormancy was further simulated by Deb *et al.* [149] who cultured *Mtb* at low oxygen, high CO_2, acidic pH and low nutrient conditions. This resulted in dormant *Mtb* that had lost acid-fastness, contained lipid inclusion bodies and had become drug tolerant. Interestingly, genes encoding enzymes involved in the glyoxylate shunt were significantly expressed by *Mtb* cultured under multiple stress conditions. This cycle plays an essential role in the use of fatty acids as carbon source, and is essential for *Mtb's* survival *in vivo* [150]. Thus, this study verified the role of the glyoxylate shunt in stress conditions that *Mtb* may encounter *in vivo* [148;151;152]. All studies described here, however, mimicked the host environment in *in vitro* systems, but it would be more informative and relevant to directly analyze *Mtb's* responses *in vivo* in infected lung tissue. Several studies were performed to analyze *Mtb* transcripts *in vivo* (reviewed by Waddell *et al.* [153]), however, no animal models exist that fully resemble human TB disease, although some models show several specific TB phenotypes such as hypoxia [143] and lesion/granuloma formation [154;155]. Intriguingly, multiple studies show a role for lipid metabolism in *in vivo* survival of *Mtb* [149;156;157].

Thus *Mtb* is able to adapt to host environmental conditions and appears to express particular sets of genes in each condition, with some overlap in genes expressed between the different conditions identified and investigated. The proteins encoded by these sets of genes can be considered to be expressed in an *Mtb* infection phase related way, and thus may be of considerable interest as novel subunit vaccine candidates [16;158]. They may have an additional or even superior effect compared to antigens that are early secreted only. Indeed, the expression of *Mtb* proteins *in vivo* is of substantial significance as one study showed that upon *Mtb* infection the expression of Ag85B decreased, resulting in reduced activation of Ag85B specific T cells [29]. Thus Ag85B based vaccines might induce limited protection due to the loss of *Mtb* Ag85B expression in chronic phase infection. This might also be an explanation for the low efficacy induced by MVA85A [112]. The phase related antigens can therefore also be of use in therapeutic vaccines. A few novel *Mtb* phase related antigens are currently incorporated into TB vaccines. H56 contains next to ESAT-6 and Ag85B, also Rv2660, encoded by *Rv2660*, a gene highly expressed during starvation [126]. Furthermore, a polyepitope (consisting of immunogenic epitopes from Hsp65, Ag85B, 19Kda lipoprotein, HspX and Rv1733c) contains latency antigens HspX and Rv1733c [107]. Both studies revealed protective value of the vaccines. Lastly, the protective value of resuscitation promoting factor (Rpf) antigens as subunit vaccines was studied [159]. Rpfs are a set of genes encoding proteins suggested to be involved in reactivation or resuscitation of dormant bacteria. Five Rpfs are known in *Mtb*: *RpfA-E* (*Rv0867c, Rv1009, Rv1884c, Rv2389c* and *Rv2450c*). The proteins encoded by these genes resemble the Rpf protein encoded by *Micrococcus luteus*, which is expressed and secreted by actively replicating *M. luteus* bacteria. This protein showed to be able to resuscitate dormant *M. luteus*, but also stimulated the growth of mycobacteria [160]. Importantly,

the *Mtb* specific Rpfs also had a stimulating effect on the growth of *M. luteus* and mycobacteria [161].

Thus understanding *Mtb's* lifestyle opens doors to novel vaccine candidates that can be of value for both prophylactic and therapeutic vaccination strategies.

Understanding TB immunity

Besides the aforementioned stress conditions, immune pressure is also a hurdle *Mtb* has to encounter. Understanding this is essential for future development of novel TB vaccines. Vaccine induced humoral immunity is a well-known correlate of protection for many viral vaccines such as hepatitis and influenza [162]. However, cell mediated immunity (CMI) is essential in protection against *Mtb* infection. Although the role of B cells in TB immunity should not be neglected [163]. *Mtb* primarily resides in phagosomes which results in processing and presenting of *Mtb* antigens via the MHC class II route.

As previously mentioned, genetic defects in IFN-γ and IL-12 pathways, cytokines specific for Th1 differentiation, as well as depletion of $CD4^+$ T cells showed an increased risk of tuberculosis [49-51;56], confirming that Th1 cells play an important role in TB protection. Analyzing Th1 immunity is currently the most common approach to study TB vaccine potential [164]. Furthermore, IGRAs are also based on Th1 T cell analysis as antigen-specific IFN-γ production is measured. An important role for IFN-γ in *Mtb* control is activation of macrophages. Besides IFN-γ, other Th1 cytokines are of importance in protection against TB. As mentioned above, patients receiving anti-TNF therapy as treatment against different inflammatory diseases such as psoriasis and rheumatoid arthritis, have a higher risk of developing TB [52;53;165;166], indicating the need of TNF-α in protection against TB. Both IFN-γ and TNF-α activate APC antimicrobial effector mechanisms. Although we now know that Th1 immunity is of particular importance, exact insights in TB immune regulation remain limited. In contrast to Th1 cells, Th2 cells are generally thought not to have protective value in TB infection, and helminth-induced Th2 responses can have a detrimental effect on protection against TB [90] and BCG vaccination efficacy. Interestingly, a recent mouse model showed that $CD4^+$ T cells can exert protective functions even in the absence of IFN-γ and/or TNF-α [167], indicating that other factors may play an important role in protection as well. Indeed, functional, IFN-γ deficient, $CD4^+$ T cells still contribute to initial *Mtb* control [168], however, IFN-γ producing $CD4^+$ T cells are required for long-term control [168].

The study of novel T-cell subsets could shed further light upon the continued (though suboptimal) protection in the absence of IFN-γ and/or TNF-α. Multiparameter flowcytometric analysis has enabled detection of multiple markers on immune cells. Such analysis allows detection of multiple T-cell subsets that might play a role in infection and vaccination induced immunity. Recent new subsets include Th17 and regulatory T cells [169]. Human IL-1β/IL-6/IL-23 induced $CD4^+$ Th17 cells produce different cytokines and chemokines including IL-17, IL-22, CCL-20 but

also IFN-γ production has been described [169-172]. Vaccine induced Th17 cells were involved in TB protection in mice [173]. Specifically, IL-17 plays a role in maturation of granuloma formation [174]. Conversely, Th17 cells can be damaging as repeated antigen exposure results in IL-17-dependent lung pathology and enhanced neutrophil influx [175]. Thus, the net effect of Th17 cells on TB protection remains unclear. Regulatory T cells (Tregs) are also induced upon *Mtb* infection and are responsible for delayed priming of $CD4^+$ and $CD8^+$ T cells in the MLN [18] and direct suppression of effector T cell activity [176], thus having an anti-inflammatory effect. Therefore, they can be involved in bacterial persistence, next to limitation of host tissue damage [177]. Interestingly, differentiation of Th17 to Th1 and Treg to Th17 T cell subsets have been detected and indicate plasticity of unstable T-cell subsets [172;178;179].

Importantly, *Mtb* not only resides in the phagosome, but can also translocate to the cytosol, a mechanism which is RD1 region dependent [180;181]. Translocation of mycobacterial products to the cytosol enables loading of immunogenic epitopes to MHC class I and presentation to $CD8^+$ T cells, although many other mechanisms might also be involved in MHC-I loading such as cross-presentation and efferocytosis [40;182;183]. $CD8^+$ T cells produce multiple cytokines upon activation but can exert direct cytotoxic functions as well [31]. In addition to $CD4^+$ T cells, $CD8^+$ T cells also contribute to TB protection [184-186].

The current belief is that T cells producing multiple cytokines, chemokines and/or effector molecules are more effective compared to single cytokine producing cells. In more detail, T cells producing IFN-γ, TNF-α and IL-2 are believed to be of higher quality compared to double or single cytokine producing Th1 T cells and provide optimal protection and effector function [187]. In this study, polyfunctional T cells expressing IFN-γ, TNF-α and IL-2 correlated with protection against the intracellular pathogen *Leishmania major*. Subsequently, this was confirmed in mouse TB vaccine studies [188-190]. Also, higher frequencies of polyfunctional T cells were observed in LTBI compared to TB patients [191], based on mycobacterial load [192]. However, polyfunctional T cells correlated also to active TB disease [193;194]. In addition, several other studies showed no correlation of these cells with vaccination induced protection [195;196]. Interestingly, the antigen dose may be of importance for the development of protective polyfunctional T cells [197]. Furthermore, the duration of antigen stimulation and cytokine accumulation (due to Golgi transport inhibition) also influences the detection of polyfunctional T cells [198]. Thus, overall, the role of polyfunctional T cells in TB remains unclear.

Outline of this thesis

Efforts to decrease the number of TB cases by using latest *Mtb* detection methods, treatments and BCG vaccination campaigns have failed to result in eradication of the disease. TB remains one of the leading causes of death on our planet. The main focus of this thesis is to identify *Mtb* infection phase related antigens, and to evaluate these as potential antigens for TB vaccines.

Mtb Rpfs proteins are likely involved in reactivation of dormant mycobacteria, and thus may play an important role in TB disease. We therefore hypothesized that immunity directed against Rpf proteins could play a role in the control of reactivating bacteria. In **chapter 2** T-cell responses towards two *Mtb* resuscitation promoting factors (Rpfs), Rv0867c (RpfA) and Rv2389c (RpfD), are described in mycobacterium exposed individuals.

A second group of *Mtb* antigens, DosR antigens, previously was shown to be preferentially recognized by LTBI. From this perspective, we hypothesized that enhancing DosR specific immunity may be beneficial in controlling LTBI. However, only very few data on the *Mtb* DosR regulon encoded antigen-specific T-cell responses were available. We therefore analyzed the precise *Mtb* DosR antigen responding T cells and determined immunogenic peptide epitopes (**chapter 3)**.

The thus far described phase related antigens are all selected from initial *in vitro* studies which tried to mimic environmental stress conditions that *Mtb* encounters upon infection. We hypothesized that *Mtb* vaccine candidates should be expressed by *Mtb in vivo,* in infected tissue, and therefore analyzed *Mtb* gene expression profiles in the lungs of four genetically related but distinct mouse strains that represents a spectrum of TB susceptibility controlled by the *super-susceptibility to TB 1* locus. The immunogenicity of a subset of *in vivo* expressed *Mtb* (IVE-TB) antigens, associated to *Mtb* infection phenotypes, was subsequently determined (**chapter 4**).

Besides the criterion that vaccine candidates should be expressed *in vivo*, they should also be immunogenic *in vivo* and provide protection against *Mtb* infection. To determine the immunogenic and protective value of one of the IVE-TB antigens, Rv2034, HLA transgenic mice were vaccinated with Rv2034 as an experimental TB vaccine as described in **chapter 5**.

Studying the Rv2034 specific T-cell responses in more detail, we used a specific CD154 T-cell cloning method that allows more detailed analysis of these responses and in addition the development of novel tools to dissect the precise nature of the IVE-TB specific T-cell response (**chapter 6**).

The results of all studies included in this thesis are summarized and discussed in **chapter 7**.

References

1. **Gagneux,S.,** Host-pathogen coevolution in human tuberculosis. *Philos.Trans.R.Soc. Lond B Biol.Sci.* 2012. **367**: 850-859.
2. **WHO**. The global plan to stop TB 2011-2015. 2011.
3. **Hershkovitz,I., Donoghue,H.D., Minnikin,D.E., Besra,G.S., Lee,O.Y., Gernaey,A.M., Galili,E., Eshed,V., Greenblatt,C.L., Lemma,E., Bar-Gal,G.K., and Spigelman,M.,** Detection and molecular characterization of 9,000-year-old Mycobacterium tuberculosis from a Neolithic settlement in the Eastern Mediterranean. *PLoS.One.* 2008. **3**: e3426.
4. **Dubos,R. and Dubos, J.,** *The white plague: tuberculosis, man and society*. Boston: Little, Brown, 1952.
5. **Daniel,T.M.,** The history of tuberculosis. *Respir.Med.* 2006. **100**: 1862-1870.
6. **Herzog,H.,** History of tuberculosis. *Respiration* 1998. **65**: 5-15.
7. **Daniel,T.M.,** Hermann Michael Biggs and tuberculosis as a public health concern. *Int.J.Tuberc.Lung Dis.* 2011. **15**: 1572-1573.
8. **Daniel,T.M.,** Hermann Brehmer and the origins of tuberculosis sanatoria. *Int.J.Tuberc.Lung Dis.* 2011. **15**: 161-2, i.
9. **Mitchison,D. and Davies,G.,** The chemotherapy of tuberculosis: past, present and future. *Int.J.Tuberc.Lung Dis.* 2012. **16**: 724-732.
10. **Yang,C.T., Cambier,C.J., Davis,J.M., Hall,C.J., Crosier,P.S., and Ramakrishnan,L.,** Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host.Microbe* 2012. **12**: 301-312.
11. **Lowe,D.M., Redford,P.S., Wilkinson,R.J., O'Garra,A., and Martineau,A.R.,** Neutrophils in tuberculosis: friend or foe? *Trends Immunol.* 2012. **33**: 14-25.
12. **Blomgran,R. and Ernst,J.D.,** Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. *J.Immunol.* 2011. **186**: 7110-7119.
13. **Reece,S.T. and Kaufmann,S.H.,** Floating between the poles of pathology and protection: can we pin down the granuloma in tuberculosis? *Curr.Opin.Microbiol.* 2012. **15**: 63-70.
14. **Wolf,A.J., Desvignes,L., Linas,B., Banaiee,N., Tamura,T., Takatsu,K., and Ernst,J.D.,** Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J.Exp.Med.* 2008. **205**: 105-115.
15. **Reiley,W.W., Calayag,M.D., Wittmer,S.T., Huntington,J.L., Pearl,J.E., Fountain,J.J., Martino,C.A., Roberts,A.D., Cooper,A.M., Winslow,G.M., and Woodland,D.L.,** ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes. *Proc.Natl. Acad.Sci.U.S.A* 2008. **105**: 10961-10966.
16. **Ottenhoff,T.H. and Kaufmann,S.H.,** Vaccines against tuberculosis: where are we and where do we need to go? *PLoS.Pathog.* 2012. **8**: e1002607.
17. **Blomgran,R., Desvignes,L., Briken,V., and Ernst,J.D.,** Mycobacterium tuberculosis inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host.Microbe* 2012. **11**: 81-90.
18. **Shafiani,S., Tucker-Heard,G., Kariyone,A., Takatsu,K., and Urdahl,K.B.,** Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J.Exp.Med.* 2010. **207**: 1409-1420.
19. **Geijtenbeek,T.B., Van Vliet,S.J., Koppel,E.A., Sanchez-Hernandez,M., Vandenbroucke-Grauls,C.M., Appelmelk,B., and Van,K.Y.,** Mycobacteria target DC-SIGN to suppress dendritic cell function. *J.Exp.Med.* 2003. **197**: 7-17.

20. **Chieppa,M., Bianchi,G., Doni,A., Del,P.A., Sironi,M., Laskarin,G., Monti,P., Piemonti,L., Biondi,A., Mantovani,A., Introna,M., and Allavena,P.,** Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J.Immunol.* 2003. **171**: 4552-4560.

21. **Gallegos,A.M., Pamer,E.G., and Glickman,M.S.,** Delayed protection by ESAT-6-specific effector CD4+ T cells after airborne M. tuberculosis infection. *J.Exp.Med.* 2008. **205**: 2359-2368.

22. **Shaler,C.R., Horvath,C., Lai,R., and Xing,Z.,** Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clin.Dev.Immunol.* 2012. **2012**: 628293.

23. **Vergne,I., Chua,J., Singh,S.B., and Deretic,V.,** Cell biology of mycobacterium tuberculosis phagosome. *Annu.Rev.Cell Dev.Biol.* 2004. **20**: 367-394.

24. **Mishra,A.K., Driessen,N.N., Appelmelk,B.J., and Besra,G.S.,** Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in Mycobacterium tuberculosis physiology and host-pathogen interaction. *FEMS Microbiol.Rev.* 2011. **35**: 1126-1157.

25. **Welin,A., Winberg,M.E., Abdalla,H., Sarndahl,E., Rasmusson,B., Stendahl,O., and Lerm,M.,** Incorporation of Mycobacterium tuberculosis lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. *Infect.Immun.* 2008. **76**: 2882-2887.

26. **Ehrt,S. and Schnappinger,D.,** Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol.* 2009. **11**: 1170-1178.

27. **Pennini,M.E., Pai,R.K., Schultz,D.C., Boom,W.H., and Harding,C.V.,** Mycobacterium tuberculosis 19-kDa lipoprotein inhibits IFN-gamma-induced chromatin remodeling of MHC2TA by TLR2 and MAPK signaling. *J.Immunol.* 2006. **176**: 4323-4330.

28. **Egen,J.G., Rothfuchs,A.G., Feng,C.G., Horwitz,M.A., Sher,A., and Germain,R.N.,** Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity.* 2011. **34**: 807-819.

29. **Bold,T.D., Banaei,N., Wolf,A.J., and Ernst,J.D.,** Suboptimal activation of antigen-specific CD4+ effector cells enables persistence of M. tuberculosis in vivo. *PLoS.Pathog.* 2011. **7**: e1002063.

30. **Ernst,J.D.,** The immunological life cycle of tuberculosis. *Nat.Rev.Immunol.* 2012. **12**: 581-591.

31. **Gupta,A., Kaul,A., Tsolaki,A.G., Kishore,U., and Bhakta,S.,** Mycobacterium tuberculosis: immune evasion, latency and reactivation. *Immunobiology* 2012. **217**: 363-374.

32. **Das,B., Kashino,S.S., Pulu,I., Kalita,D., Swami,V., Yeger,H., Felsher,D.W., and Campos-Neto,A.,** CD271+ Bone Marrow Mesenchymal Stem Cells May Provide a Niche for Dormant Mycobacterium tuberculosis. *Sci. Transl.Med.* 2013. **5**: 170ra13.

33. **Davis,J.M. and Ramakrishnan,L.,** The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 2009. **136**: 37-49.

34. **Volkman,H.E., Pozos,T.C., Zheng,J., Davis,J.M., Rawls,J.F., and Ramakrishnan,L.,** Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. *Science* 2010. **327**: 466-469.

35. **Volkman,H.E., Clay,H., Beery,D., Chang,J.C., Sherman,D.R., and Ramakrishnan,L.,** Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *PLoS.Biol.* 2004. **2**: e367.

36. **Chen,M., Gan,H., and Remold,H.G.,** A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. *J.Immunol.* 2006. **176**: 3707-3716.

37. **Gan,H., Lee,J., Ren,F., Chen,M., Kornfeld,H., and Remold,H.G.,** Mycobacterium tuberculosis blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages

to maintain virulence. *Nat.Immunol.* 2008. **9**: 1189-1197.

38. **Divangahi,M., Desjardins,D., Nunes-Alves,C., Remold,H.G., and Behar,S.M.,** Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. *Nat.Immunol.* 2010. **11**: 751-758.
39. **Welin,A., Eklund,D., Stendahl,O., and Lerm,M.,** Human macrophages infected with a high burden of ESAT-6-expressing M. tuberculosis undergo caspase-1- and cathepsin B-independent necrosis. *PLoS.One.* 2011. **6**: e20302.
40. **Martin,C.J., Booty,M.G., Rosebrock,T.R., Nunes-Alves,C., Desjardins,D.M., Keren,I., Fortune,S.M., Remold,H.G., and Behar,S.M.,** Efferocytosis is an innate antibacterial mechanism. *Cell Host.Microbe* 2012. **12**: 289-300.
41. **Behar,S.M., Martin,C.J., Booty,M.G., Nishimura,T., Zhao,X., Gan,H.X., Divangahi,M., and Remold,H.G.,** Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. *Mucosal.Immunol.* 2011. **4**: 279-287.
42. **Derrick,S.C. and Morris,S.L.,** The ESAT6 protein of Mycobacterium tuberculosis induces apoptosis of macrophages by activating caspase expression. *Cell Microbiol.* 2007. **9**: 1547-1555.
43. **Ramakrishnan,L.,** Revisiting the role of the granuloma in tuberculosis. *Nat.Rev.Immunol.* 2012. **12**: 352-366.
44. **Choi,H.H., Shin,D.M., Kang,G., Kim,K.H., Park,J.B., Hur,G.M., Lee,H.M., Lim,Y.J., Park,J.K., Jo,E.K., and Song,C.H.,** Endoplasmic reticulum stress response is involved in Mycobacterium tuberculosis protein ESAT-6-mediated apoptosis. *FEBS Lett.* 2010. **584**: 2445-2454.
45. **Sutherland,I.,** Recent studies in the epidemiology of tuberculosis, based on the risk of being infected with tubercle bacilli. *Adv.Tuberc.Res.* 1976. **19**: 1-63.
46. **Harries,A.D. and Dye,C.,** Tuberculosis. *Ann.Trop.Med.Parasitol.* 2006. **100**: 415-431.
47. **Taylor,Z., Nolan,C.M., and Blumberg,H.M.,** Controlling tuberculosis in the United States. Recommendations from the American Thoracic Society, CDC, and the Infectious Diseases Society of America. *MMWR Recomm.Rep.* 2005. **54**: 1-81.
48. **Arend,S.M. and van Dissel,J.T.,** Evidence of endogenous reactivation of tuberculosis after a long period of latency. *J.Infect.Dis.* 2002. **186**: 876-877.
49. **Pawlowski,A., Jansson,M., Skold,M., Rottenberg,M.E., and Kallenius,G.,** Tuberculosis and HIV co-infection. *PLoS.Pathog.* 2012. **8**: e1002464.
50. **Saunders,B.M., Frank,A.A., Orme,I.M., and Cooper,A.M.,** CD4 is required for the development of a protective granulomatous response to pulmonary tuberculosis. *Cell Immunol.* 2002. **216**: 65-72.
51. **Kwan,C.K. and Ernst,J.D.,** HIV and tuberculosis: a deadly human syndemic. *Clin.Microbiol.Rev.* 2011. **24**: 351-376.
52. **Keane,J., Gershon,S., Wise,R.P., Mirabile-Levens,E., Kasznica,J., Schwieterman,W.D., Siegel,J.N., and Braun,M.M.,** Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N.Engl.J.Med.* 2001. **345**: 1098-1104.
53. **Harris,J. and Keane,J.,** How tumour necrosis factor blockers interfere with tuberculosis immunity. *Clin.Exp.Immunol.* 2010. **161**: 1-9.
54. **Fortin,A., Abel,L., Casanova,J.L., and Gros,P.,** Host genetics of mycobacterial diseases in mice and men: forward genetic studies of BCG-osis and tuberculosis. *Annu.Rev.Genomics Hum.Genet.* 2007. **8**: 163-192.
55. **Ottenhoff,T.H., Verreck,F.A., Hoeve,M.A., and van,d., V,** Control of human host immunity to mycobacteria. *Tuberculosis.(Edinb.)* 2005. **85**: 53-64.
56. **Ottenhoff,T.H., Verreck,F.A., Lichtenauer-Kaligis,E.G., Hoeve,M.A., Sanal,O., and van Dissel,J.T.,** Genetics, cytokines and human infectious disease: lessons from weakly pathogenic

mycobacteria and salmonellae. *Nat.Genet.* 2002. **32**: 97-105.

57. **Barry,C.E., III, Boshoff,H.I., Dartois,V., Dick,T., Ehrt,S., Flynn,J., Schnappinger,D., Wilkinson,R.J., and Young,D.,** The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat.Rev.Microbiol.* 2009. **7**: 845-855.

58. **Dheda,K., Ruhwald,M., Theron,G., Peter,J., and Yam,W.C.,** Point-of-care diagnosis of tuberculosis: Past, present and future. *Respirology.* 2013. **18**: 217-232.

59. **Lawn,S.D. and Zumla,A.I.,** Tuberculosis. *Lancet* 2011. **378**: 57-72.

60. **Gradmann,C.,** Robert Koch and the white death: from tuberculosis to tuberculin. *Microbes.Infect.* 2006. **8**: 294-301.

61. **Daniel,T.M.,** Florence Barbara Seibert and purified protein derivative. *Int.J.Tuberc.Lung Dis.* 2009. **13**: 281-282.

62. **Farhat,M., Greenaway,C., Pai,M., and Menzies,D.,** False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? *Int.J.Tuberc.Lung Dis.* 2006. **10**: 1192-1204.

63. **Pinto,L.M., Grenier,J., Schumacher,S.G., Denkinger,C.M., Steingart,K.R., and Pai,M.,** Immunodiagnosis of tuberculosis: state of the art. *Med.Princ.Pract.* 2012. **21**: 4-13.

64. **Latorre,I., De Souza-Galvao,M., Ruiz-Manzano,J., Lacoma,A., Prat,C., Altet,N., Ausina,V., and Dominguez,J.,** Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. *Eur.Respir.J.* 2010. **35**: 338-342.

65. **Wolf,T., Goetsch,U., Oremek,G., Bickel,M., Khaykin,P., Haberl,A., Bellinger,O., Gottschalk,R., Brodt,H.R., and Stephan,C.,** Tuberculosis skin test, but not interferon-gamma-releasing assays is affected by BCG vaccination in HIV patients. *J.Infect.* 2012.

66. **Falkinham,J.O., III,** Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *J.Appl.Microbiol.* 2009. **107**: 356-367.

67. **Weiss,C.H. and Glassroth,J.,** Pulmonary disease caused by nontuberculous mycobacteria. *Expert.Rev.Respir.Med.* 2012. **6**: 597-612.

68. **Feazel,L.M., Baumgartner,L.K., Peterson,K.L., Frank,D.N., Harris,J.K., and Pace,N.R.,** Opportunistic pathogens enriched in showerhead biofilms. *Proc.Natl.Acad.Sci.U.S.A* 2009. **106**: 16393-16399.

69. **Mack,U., Migliori,G.B., Sester,M., Rieder,H.L., Ehlers,S., Goletti,D., Bossink,A., Magdorf,K., Holscher,C., Kampmann,B., Arend,S.M., Detjen,A., Bothamley,G., Zellweger,J.P., Milburn,H., Diel,R., Ravn,P., Cobelens,F., Cardona,P.J., Kan,B., Solovic,I., Duarte,R., and Cirillo,D.M.,** LTBI: latent tuberculosis infection or lasting immune responses to M. tuberculosis? A TBNET consensus statement. *Eur.Respir.J.* 2009. **33**: 956-973.

70. **Brock,I., Weldingh,K., Lillebaek,T., Follmann,F., and Andersen,P.,** Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. *Am.J.Respir.Crit Care Med.* 2004. **170**: 65-69.

71. **Diel,R., Goletti,D., Ferrara,G., Bothamley,G., Cirillo,D., Kampmann,B., Lange,C., Losi,M., Markova,R., Migliori,G.B., Nienhaus,A., Ruhwald,M., Wagner,D., Zellweger,J.P., Huitric,E., Sandgren,A., and Manissero,D.,** Interferon-gamma release assays for the diagnosis of latent Mycobacterium tuberculosis infection: a systematic review and meta-analysis. *Eur.Respir.J.* 2011. **37**: 88-99.

72. **Diel,R., Loddenkemper,R., and Nienhaus,A.,** Predictive value of interferon-gamma release assays and tuberculin skin testing for progression from latent TB infection to disease state: a meta-analysis. *Chest* 2012. **142**: 63-75.

73. **Trajman,A., Steffen,R.E., and Menzies,D.,** Interferon-Gamma Release Assays versus Tuberculin Skin Testing for Diagnosis of Latent Tuberculosis Infection: An Overview of the Evidence. *Pulmonary Medicine* 2013. **2013**.

74. **World Health Organization**. Use of Tuberculosis Interferon-Gamma Release Assays (IGRAs) in Low- and Middle-Income Countries: Policy Statement. 2011. World Health Organization, Geneva, Switzerland.

75. **Dheda,K., van Zyl,S.R., Badri,M., and Pai,M.,** T-cell interferon-gamma release assays for the rapid immunodiagnosis of tuberculosis: clinical utility in high-burden vs. low-burden settings. *Curr. Opin.Pulm.Med.* 2009. **15**: 188-200.

76. **Sharma,S.K., Mohanan,S., and Sharma,A.,** Relevance of latent TB infection in areas of high TB prevalence. *Chest* 2012. **142**: 761-773.

77. **Machado,A., Jr., Emodi,K., Takenami,I., Finkmoore,B.C., Barbosa,T., Carvalho,J., Cavalcanti,L., Santos,G., Tavares,M., Mota,M., Barreto,F., Reis,M.G., Arruda,S., and Riley,L.W.,** Analysis of discordance between the tuberculin skin test and the interferon-gamma release assay. *Int.J.Tuberc.Lung Dis.* 2009. **13**: 446-453.

78. **Goletti,D., Butera,O., Vanini,V., Lauria,F.N., Lange,C., Franken,K.L., Angeletti,C., Ottenhoff,T.H., and Girardi,E.,** Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. *Eur.Respir.J.* 2010. **36**: 135-142.

79. **Geluk,A., van Meijgaarden,K.E., Franken,K.L., Subronto,Y.W., Wieles,B., Arend,S.M., Sampaio,E.P., de,B.T., Faber,W.R., Naafs,B., and Ottenhoff,T.H.,** Identification and characterization of the ESAT-6 homologue of Mycobacterium leprae and T-cell cross-reactivity with Mycobacterium tuberculosis. *Infect.Immun.* 2002. **70**: 2544-2548.

80. **Geluk,A., van Meijgaarden,K.E., Franken,K.L., Wieles,B., Arend,S.M., Faber,W.R., Naafs,B., and Ottenhoff,T.H.,** Immunological crossreactivity of the Mycobacterium leprae CFP-10 with its homologue in Mycobacterium tuberculosis. *Scand.J.Immunol.* 2004. **59**: 66-70.

81. **Lee,S.W., Oh,D.K., Lee,S.H., Kang,H.Y., Lee,C.T., and Yim,J.J.,** Time interval to conversion of interferon-gamma release assay after exposure to tuberculosis. *Eur.Respir.J.* 2011. **37**: 1447-1452.

82. **Phillips,L.,** Infectious disease: TB's revenge. *Nature* 2013. **493**: 14-16.

83. **Mahairas,G.G., Sabo,P.J., Hickey,M.J., Singh,D.C., and Stover,C.K.,** Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. *J.Bacteriol.* 1996. **178**: 1274-1282.

84. **Hawgood,B.J.,** Albert Calmette (1863-1933) and Camille Guerin (1872-1961): the C and G of BCG vaccine. *J.Med.Biogr.* 2007. **15**: 139-146.

85. **Trunz,B.B., Fine,P., and 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**: 1173-1180.

86. **Setia,M.S., Steinmaus,C., Ho,C.S., and Rutherford,G.W.,** The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect.Dis.* 2006. **6**: 162-170.

87. **Zodpey,S.P.,** Protective effect of bacillus Calmette Guerin (BCG) vaccine in the prevention of leprosy: a meta-analysis. *Indian J.Dermatol.Venereol.Leprol.* 2007. **73**: 86-93.

88. **Fine,P.E.,** Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 1995. **346**: 1339-1345.

89. **Elias,D., Britton,S., Aseffa,A., Engers,H., and Akuffo,H.,** Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* 2008. **26**: 3897-3902.

90. **Rafi,W., Ribeiro-Rodrigues,R., Ellner,J.J., and Salgame,P.,** 'Coinfection-helminthes and tuberculosis'. *Curr. Opin.HIV.AIDS* 2012. **7**: 239-244.

91. **Brandt,L., Feino,C.J., Weinreich,O.A., Chilima,B., Hirsch,P., Appelberg,R., and Andersen,P.,** Failure

of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect.Immun.* 2002. **70**: 672-678.

92. **Hesseling,A.C., Marais,B.J., Gie,R.P., Schaaf,H.S., Fine,P.E., Godfrey-Faussett,P., and Beyers,N.,** The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 2007. **25**: 14-18.

93. **Grode,L., Seiler,P., Baumann,S., Hess,J., Brinkmann,V., Nasser,E.A., Mann,P., Goosmann,C., Bandermann,S., Smith,D., Bancroft,G.J., Reyrat,J.M., van,S.D., Raupach,B., and Kaufmann,S.H.,** Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. *J.Clin.Invest* 2005. **115**: 2472-2479.

94. **Farinacci,M., Weber,S., and Kaufmann,S.H.,** The recombinant tuberculosis vaccine rBCG DeltaureC::hly(+) induces apoptotic vesicles for improved priming of CD4(+) and CD8(+) T cells. *Vaccine* 2012. **30**: 7608-7614.

95. **Hoft,D.F., Blazevic,A., Abate,G., Hanekom,W.A., Kaplan,G., Soler,J.H., Weichold,F., Geiter,L., Sadoff,J.C., and Horwitz,M.A.,** A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J.Infect.Dis.* 2008. **198**: 1491-1501.

96. **Pym,A.S., Brodin,P., Majlessi,L., Brosch,R., Demangel,C., Williams,A., Griffiths,K.E., Marchal,G., Leclerc,C., and Cole,S.T.,** Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat.Med.* 2003. **9**: 533-539.

97. **Sun,R., Skeiky,Y.A., Izzo,A., Dheenadhayalan,V., Imam,Z., Penn,E., Stagliano,K., Haddock,S., Mueller,S., Fulkerson,J., Scanga,C., Grover,A., Derrick,S.C., Morris,S., Hone,D.M., Horwitz,M.A., Kaufmann,S.H., and Sadoff,J.C.,** Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with Mycobacterium tuberculosis. *Vaccine* 2009. **27**: 4412-4423.

98. **Kaufmann,S.H. and Gengenbacher,M.,** Recombinant live vaccine candidates against tuberculosis. *Curr.Opin.Biotechnol.* 2012. **23**: 900-907.

99. **Kupferschmidt,K.,** Infectious disease. Taking a new shot at a TB vaccine. *Science* 2011. **334**: 1488-1490.

100. **Walker,K.B., Brennan,M.J., Ho,M.M., Eskola,J., Thiry,G., Sadoff,J., Dobbelaer,R., Grode,L., Liu,M.A., Fruth,U., and Lambert,P.H.,** The second Geneva Consensus: Recommendations for novel live TB vaccines. *Vaccine* 2010. **28**: 2259-2270.

101. **Kamath,A.T., Fruth,U., Brennan,M.J., Dobbelaer,R., Hubrechts,P., Ho,M.M., Mayner,R.E., Thole,J., Walker,K.B., Liu,M., and Lambert,P.H.,** New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. *Vaccine* 2005. **23**: 3753-3761.

102. **Hinchey,J., Jeon,B.Y., Alley,H., Chen,B., Goldberg,M., Derrick,S., Morris,S., Jacobs,W.R., Jr., Porcelli,S.A., and Lee,S.,** Lysine auxotrophy combined with deletion of the SecA2 gene results in a safe and highly immunogenic candidate live attenuated vaccine for tuberculosis. *PLoS.One.* 2011. **6**: e15857.

103. **Saikolappan,S., Estrella,J., Sasindran,S.J., Khan,A., Armitige,L.Y., Jagannath,C., and Dhandayuthapani,S.,** The fbpA/sapM double knock out strain of Mycobacterium tuberculosis is highly attenuated and immunogenic in macrophages. *PLoS.One.* 2012. **7**: e36198.

104. **Sambandamurthy,V.K., Derrick,S.C., Hsu,T., Chen,B., Larsen,M.H., Jalapathy,K.V., Chen,M., Kim,J., Porcelli,S.A., Chan,J., Morris,S.L., and Jacobs,W.R., Jr.,** Mycobacterium tuberculosis DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immuno-

compromised mice against experimental tuberculosis. *Vaccine* 2006. **24**: 6309-6320.

105. **Cardona,P.J., Asensio,J.G., Arbues,A., Otal,I., Lafoz,C., Gil,O., Caceres,N., Ausina,V., Gicquel,B., and Martin,C.,** Extended safety studies of the attenuated live tuberculosis vaccine SO2 based on phoP mutant. *Vaccine* 2009. **27**: 2499-2505.

106. **Jensen,K., Ranganathan,U.D., Van Rompay,K.K., Canfield,D.R., Khan,I., Ravindran,R., Luciw,P.A., Jacobs,W.R., Jr., Fennelly,G., Larsen,M.H., and Abel,K.,** A recombinant attenuated Mycobacterium tuberculosis vaccine strain is safe in immunosuppressed simian immunodeficiency virus-infected infant macaques. *Clin.Vaccine Immunol.* 2012. **19**: 1170-1181.

107. **Geluk,A., van den Eeden,S.J., van Meijgaarden,K.E., Dijkman,K., Franken,K.L., and Ottenhoff,T.H.,** A multistage-polyepitope vaccine protects against Mycobacterium tuberculosis infection in HLA-DR3 transgenic mice. *Vaccine* 2012. **30**: 7513-7521.

108. **McShane,H., Pathan,A.A., Sander,C.R., Keating,S.M., Gilbert,S.C., Huygen,K., Fletcher,H.A., and Hill,A.V.,** Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat.Med.* 2004. **10**: 1240-1244.

109. **Scriba,T.J., Tameris,M., Mansoor,N., Smit,E., van der Merwe,L., Isaacs,F., Keyser,A., Moyo,S., Brittain,N., Lawrie,A., Gelderbloem,S., Veldsman,A., Hatherill,M., Hawkridge,A., Hill,A.V., Hussey,G.D., Mahomed,H., McShane,H., and Hanekom,W.A.,** Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur.J.Immunol.* 2010. **40**: 279-290.

110. **Brookes,R.H., Hill,P.C., Owiafe,P.K., Ibanga,H.B., Jeffries,D.J., Donkor,S.A., Fletcher,H.A., Hammond,A.S., Lienhardt,C., Adegbola,R.A., McShane,H., and Hill,A.V.,** Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in West Africa. *PLoS.One.* 2008. **3**: e2921.

111. **Scriba,T.J., Tameris,M., Mansoor,N., Smit,E., van der Merwe,L., Mauff,K., Hughes,E.J., Moyo,S., Brittain,N., Lawrie,A., Mulenga,H., de,K.M., Gelderbloem,S., Veldsman,A., Hatherill,M., Geldenhuys,H., Hill,A.V., Hussey,G.D., Mahomed,H., Hanekom,W.A., and McShane,H.,** Dose-finding study of the novel tuberculosis vaccine, MVA85A, in healthy BCG-vaccinated infants. *J.Infect.Dis.* 2011. **203**: 1832-1843.

112. **Tameris,M.D., Hatherill,M., Landry,B.S., Scriba,T.J., Snowden,M.A., Lockhart,S., Shea,J.E., McClain,J.B., Hussey,G.D., Hanekom,W.A., Mahomed,H., and McShane,H.,** Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* 2013.

113. **Beverley,P., Ronan,E., Lee,L., Arnold,I., Bolinger,B., Powrie,F., and Tchilian,E.,** Environmental effects on protection against Mycobacterium tuberculosis after immunization with Ad85A. *Vaccine* 2013. **31**: 1086-1093.

114. **Santosuosso,M., McCormick,S., Zhang,X., Zganiacz,A., and Xing,Z.,** Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral Mycobacterium bovis BCG immunization against pulmonary tuberculosis. *Infect.Immun.* 2006. **74**: 4634-4643.

115. **Hoft,D.F., Blazevic,A., Stanley,J., Landry,B., Sizemore,D., Kpamegan,E., Gearhart,J., Scott,A., Kik,S., Pau,M.G., Goudsmit,J., McClain,J.B., and Sadoff,J.,** A recombinant adenovirus expressing immunodominant TB antigens can significantly enhance BCG-induced human immunity. *Vaccine* 2012. **30**: 2098-2108.

116. **Abel,B., Tameris,M., Mansoor,N., Gelderbloem,S., Hughes,J., Abrahams,D., Makhethe,L., Erasmus,M., de,K.M., van der Merwe,L.,**

Hawkridge,A., Veldsman,A., Hatherill,M., Schirru,G., Pau,M.G., Hendriks,J., Weverling,G.J., Goudsmit,J., Sizemore,D., McClain,J.B., Goetz,M., Gearhart,J., Mahomed,H., Hussey,G.D., Sadoff,J.C., and Hanekom,W.A., The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am.J.Respir. Crit Care Med.* 2010. **181**: 1407-1417.

117. **Pitt,J.M., Blankley,S., McShane,H., and O'Garra,A.,** Vaccination against tuberculosis: How can we better BCG? *Microb.Pathog.* 2012.

118. **Lasaro,M.O. and Ertl,H.C.,** New insights on adenovirus as vaccine vectors. *Mol.Ther.* 2009. **17**: 1333-1339.

119. **Madhan,S., Prabakaran,M., and Kwang,J.,** Baculovirus as vaccine vectors. *Curr.Gene Ther.* 2010. **10**: 201-213.

120. **Flatz,L., Hegazy,A.N., Bergthaler,A., Verschoor,A., Claus,C., Fernandez,M., Gattinoni,L., Johnson,S., Kreppel,F., Kochanek,S., Broek,M., Radbruch,A., Levy,F., Lambert,P.H., Siegrist,C.A., Restifo,N.P., Lohning,M., Ochsenbein,A.F., Nabel,G.J., and Pinschewer,D.D.,** Development of replication-defective lymphocytic choriomeningitis virus vectors for the induction of potent CD8+ T cell immunity. *Nat.Med.* 2010. **16**: 339-345.

121. **Day,C.L., Tameris,M., Mansoor,N., van,R.M., de,K.M., Geldenhuys,H., Erasmus,M., Makhethe,L., Hughes,E.J., Gelderbloem,S., Bollaerts,A., Bourguignon,P., Cohen,J., Demoitie,M.A., Mettens,P., Moris,P., Sadoff,J.C., Hawkridge,A., Hussey,G.D., Mahomed,H., Ofori-Anyinam,O., and Hanekom,W.A.,** Induction and Regulation of T Cell Immunity by the Novel TB Vaccine M72/AS01 in South African Adults. *Am.J.Respir.Crit Care Med.* 2013.

122. **Leroux-Roels,I., Forgus,S., De,B.F., Clement,F., Demoitie,M.A., Mettens,P., Moris,P., Ledent,E., Leroux-Roels,G., and Ofori-Anyinam,O.,** Improved CD4(+) T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: A randomized trial. *Vaccine* 2012.

123. **van Dissel,J.T., Arend,S.M., Prins,C., Bang,P., Tingskov,P.N., Lingnau,K., Nouta,J., Klein,M.R., Rosenkrands,I., Ottenhoff,T.H., Kromann,I., Doherty,T.M., and Andersen,P.,** Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. *Vaccine* 2010. **28**: 3571-3581.

124. **van Dissel,J.T., Soonawala,D., Joosten,S.A., Prins,C., Arend,S.M., Bang,P., Tingskov,P.N., Lingnau,K., Nouta,J., Hoff,S.T., Rosenkrands,I., Kromann,I., Ottenhoff,T.H., Doherty,T.M., and Andersen,P.,** Ag85B-ESAT-6 adjuvanted with IC31(R) promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. *Vaccine* 2011. **29**: 2100-2109.

125. **Billeskov,R., Elvang,T.T., Andersen,P.L., and Dietrich,J.,** The HyVac4 subunit vaccine efficiently boosts BCG-primed anti-mycobacterial protective immunity. *PLoS.One.* 2012. **7**: e39909.

126. **Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G., Schoolnik,G.K., Cassidy,J.P., Billeskov,R., and Andersen,P.,** A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat.Med.* 2011. **17**: 189-194.

127. **Niu,H., Hu,L., Li,Q., Da,Z., Wang,B., Tang,K., Xin,Q., Yu,H., Zhang,Y., Wang,Y., Ma,X., and Zhu,B.,** Construction and evaluation of a multistage Mycobacterium tuberculosis subunit vaccine candidate Mtb10.4-HspX. *Vaccine* 2011. **29**: 9451-9458.

128. **Windish,H.P., Duthie,M.S., Misquith,A., Ireton,G., Lucas,E., Laurance,J.D., Bailor,R.H., Coler,R.N., and Reed,S.G.,** Protection of mice from Mycobacterium tuberculosis by ID87/GLA-SE, a novel tuberculosis subunit vaccine candidate. *Vaccine* 2011. **29**: 7842-7848.

129. **Andersen,P.,** Host responses and antigens involved in protective immunity to Mycobacterium tuberculosis. *Scand.J.Immunol.* 1997. **45**: 115-131.

130. **Ottenhoff,T.H., Doherty,T.M., van Dissel,J.T., Bang,P., Lingnau,K., Kromann,I., and Andersen,P.,** First in humans: a new molecularly defined vaccine shows excellent safety and strong induction of long-lived Mycobacterium tuberculosis-specific Th1-cell like responses. *Hum.Vaccin.* 2010. **6**: 1007-1015.

131. **Boon,C. and Dick,T.,** How Mycobacterium tuberculosis goes to sleep: the dormancy survival regulator DosR a decade later. *Future Microbiol.* 2012. **7**: 513-518.

132. **Shleeva,M.O., Salina,E.G., and Kaprel'iants,A.S.,** [Dormant form of Mycobacterium tuberculosis]. *Mikrobiologiia* 2010. **79**: 3-15.

133. **Gill,W.P., Harik,N.S., Whiddon,M.R., Liao,R.P., Mittler,J.E., and Sherman,D.R.,** A replication clock for Mycobacterium tuberculosis. *Nat.Med.* 2009. **15**: 211-214.

134. **Sherman,D.R., Voskuil,M., Schnappinger,D., Liao,R., Harrell,M.I., and Schoolnik,G.K.,** Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. *Proc.Natl.Acad.Sci.U.S.A* 2001. **98**: 7534-7539.

135. **Ohno,H., Zhu,G., Mohan,V.P., Chu,D., Kohno,S., Jacobs,W.R., Jr., and Chan,J.,** The effects of reactive nitrogen intermediates on gene expression in Mycobacterium tuberculosis. *Cell Microbiol.* 2003. **5**: 637-648.

136. **Fisher,M.A., Plikaytis,B.B., and Shinnick,T.M.,** Microarray analysis of the Mycobacterium tuberculosis transcriptional response to the acidic conditions found in phagosomes. *J.Bacteriol.* 2002. **184**: 4025-4032.

137. **Rustad,T.R., Sherrid,A.M., Minch,K.J., and Sherman,D.R.,** Hypoxia: a window into Mycobacterium tuberculosis latency. *Cell Microbiol.* 2009. **11**: 1151-1159.

138. **Voskuil,M.I., Schnappinger,D., Visconti,K.C., Harrell,M.I., Dolganov,G.M., Sherman,D.R., and Schoolnik,G.K.,** Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J.Exp.Med.* 2003. **198**: 705-713.

139. **Wayne,L.G. and Sohaskey,C.D.,** Nonreplicating persistence of mycobacterium tuberculosis. *Annu.Rev.Microbiol.* 2001. **55**: 139-163.

140. **Schnappinger,D., Ehrt,S., Voskuil,M.I., Liu,Y., Mangan,J.A., Monahan,I.M., Dolganov,G., Efron,B., Butcher,P.D., Nathan,C., and Schoolnik,G.K.,** Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J.Exp.Med.* 2003. **198**: 693-704.

141. **Kumar,A., Deshane,J.S., Crossman,D.K., Bolisetty,S., Yan,B.S., Kramnik,I., Agarwal,A., and Steyn,A.J.,** Heme oxygenase-1-derived carbon monoxide induces the Mycobacterium tuberculosis dormancy regulon. *J.Biol.Chem.* 2008. **283**: 18032-18039.

142. **Shiloh,M.U., Manzanillo,P., and Cox,J.S.,** Mycobacterium tuberculosis senses host-derived carbon monoxide during macrophage infection. *Cell Host.Microbe* 2008. **3**: 323-330.

143. **Via,L.E., Lin,P.L., Ray,S.M., Carrillo,J., Allen,S.S., Eum,S.Y., Taylor,K., Klein,E., Manjunatha,U., Gonzales,J., Lee,E.G., Park,S.K., Raleigh,J.A., Cho,S.N., McMurray,D.N., Flynn,J.L., and Barry,C.E., III,** Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect.Immun.* 2008. **76**: 2333-2340.

144. **Lin,M.Y. and Ottenhoff,T.H.,** Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent Mycobacterium tuberculosis infection. *Biol.Chem.* 2008. **389**: 497-511.

145. **Park,H.D., Guinn,K.M., Harrell,M.I., Liao,R., Voskuil,M.I., Tompa,M., Schoolnik,G.K., and Sherman,D.R.,** Rv3133c/dosR is a transcription factor that mediates the hypoxic response of Mycobacterium tuberculosis. *Mol.Microbiol.* 2003. **48**: 833-843.

146. **Rustad,T.R., Harrell,M.I., Liao,R., and Sherman,D.R.,** The enduring hypoxic response of Mycobacterium tuberculosis. *PLoS.One.* 2008. **3**: e1502.

147. **Betts,J.C., Lukey,P.T., Robb,L.C., McAdam,R.A., and Duncan,K.,** Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol.Microbiol.* 2002. **43**: 717-731.

148. **Schaible,U.E. and Kaufmann,S.H.,** A nutritive view on the host-pathogen interplay. *Trends Microbiol.* 2005. **13**: 373-380.

149. **Deb,C., Lee,C.M., Dubey,V.S., Daniel,J., Abomoelak,B., Sirakova,T.D., Pawar,S., Rogers,L., and Kolattukudy,P.E.,** A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS.One.* 2009. **4**: e6077.

150. **Munoz-Elias,E.J. and McKinney,J.D.,** Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat.Med.* 2005. **11**: 638-644.

151. **Timm,J., Post,F.A., Bekker,L.G., Walther,G.B., Wainwright,H.C., Manganelli,R., Chan,W.T., Tsenova,L., Gold,B., Smith,I., Kaplan,G., and McKinney,J.D.,** Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc.Natl.Acad. Sci.U.S.A* 2003. **100**: 14321-14326.

152. **McKinney,J.D., Honer zu,B.K., Munoz-Elias,E.J., Miczak,A., Chen,B., Chan,W.T., Swenson,D., Sacchettini,J.C., Jacobs,W.R., Jr., and Russell,D.G.,** Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 2000. **406**: 735-738.

153. **Waddell,S.J. and Butcher,P.D.,** Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis. *Curr.Mol.Med.* 2007. **7**: 287-296.

154. **Reece,S.T., Loddenkemper,C., Askew,D.J., Zedler,U., Schommer-Leitner,S., Stein,M., Mir,F.A., Dorhoi,A., Mollenkopf,H.J., Silverman,G.A., and Kaufmann,S.H.,** Serine protease activity contributes to control of Mycobacterium tuberculosis in hypoxic lung granulomas in mice. *J.Clin.Invest* 2010. **120**: 3365-3376.

155. **Pichugin,A.V., Yan,B.S., Sloutsky-,A., Kobzik,L., and Kramnik,I.,** Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. *Am.J.Pathol.* 2009. **174**: 2190-2201.

156. **Daniel,J., Maamar,H., Deb,C., Sirakova,T.D., and Kolattukudy,P.E.,** Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS.Pathog.* 2011. **7**: e1002093.

157. **Gengenbacher,M. and Kaufmann,S.H.,** Mycobacterium tuberculosis: success through dormancy. *FEMS Microbiol.Rev.* 2012. **36**: 514-532.

158. **Gideon,H.P., Wilkinson,K.A., Rustad,T.R., Oni,T., Guio,H., Sherman,D.R., Vordermeier,H.M., Robertson,B.D., Young,D.B., and Wilkinson,R.J.,** Bioinformatic and empirical analysis of novel hypoxia-inducible targets of the human antituberculosis T cell response. *J.Immunol.* 2012. **189**: 5867-5876.

159. **Romano,M., Aryan,E., Korf,H., Bruffaerts,N., Franken,C.L., Ottenhoff,T.H., and Huygen,K.,** Potential of Mycobacterium tuberculosis resuscitation-promoting factors as antigens in novel tuberculosis sub-unit vaccines. *Microbes. Infect.* 2012. **14**: 86-95.

160. **Mukamolova,G.V., Kaprelyants,A.S., Young,D.I., Young,M., and Kell,D.B.,** A bacterial cytokine. *Proc.Natl.Acad.Sci.U.S.A* 1998. **95**: 8916-8921.

161. **Mukamolova,G.V., Turapov,O.A., Young,D.I., Kaprelyants,A.S., Kell,D.B., and Young,M.,** A family of autocrine growth factors in Mycobacteri-

um tuberculosis. *Mol.Microbiol.* 2002. **46**: 623-635.

162. **Thakur,A., Pedersen,L.E., and Jungersen,G.,** Immune markers and correlates of protection for vaccine induced immune responses. *Vaccine* 2012. **30**: 4907-4920.

163. **Maglione,P.J. and Chan,J.,** How B cells shape the immune response against Mycobacterium tuberculosis. *Eur.J.Immunol.* 2009. **39**: 676-686.

164. **Walzl,G., Ronacher,K., Hanekom,W., Scriba,T.J., and Zumla,A.,** Immunological biomarkers of tuberculosis. *Nat.Rev.Immunol.* 2011. **11**: 343-354.

165. **Winthrop,K.L.,** Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. *Nat.Clin.Pract. Rheumatol.* 2006. **2**: 602-610.

166. **Jacobs,M., Togbe,D., Fremond,C., Samarina,A., Allie,N., Botha,T., Carlos,D., Parida,S.K., Grivennikov,S., Nedospasov,S., Monteiro,A., Le,B.M., Quesniaux,V., and Ryffel,B.,** Tumor necrosis factor is critical to control tuberculosis infection. *Microbes.Infect.* 2007. **9**: 623-628.

167. **Gallegos,A.M., van Heijst,J.W., Samstein,M., Su,X., Pamer,E.G., and Glickman,M.S.,** A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo. *PLoS.Pathog* 2011. **7**: e1002052.

168. **Green,A.M., Difazio,R., and Flynn,J.L.,** IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during Mycobacterium tuberculosis infection. *J.Immunol.* 2013. **190**: 270-277.

169. **Ottenhoff,T.H.,** New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol.* 2012. **20**: 419-428.

170. **Torrado,E. and Cooper,A.M.,** IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev.* 2010. **21**: 455-462.

171. **Ottenhoff,T.H.,** The knowns and unknowns of the immunopathogenesis of tuberculosis. *Int.J.Tuberc.Lung Dis.* 2012. **16**: 1424-1432.

172. **Sallusto,F., Zielinski,C.E., and Lanzavecchia,A.,** Human Th17 subsets. *Eur.J.Immunol.* 2012. **42**: 2215-2220.

173. **Khader,S.A., Bell,G.K., Pearl,J.E., Fountain,J.J., Rangel-Moreno,J., Cilley,G.E., Shen,F., Eaton,S.M., Gaffen,S.L., Swain,S.L., Locksley,R.M., Haynes,L., Randall,T.D., and Cooper,A.M.,** IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat.Immunol.* 2007. **8**: 369-377.

174. **Okamoto,Y.Y., Umemura,M., Yahagi,A., O'Brien,R.L., Ikuta,K., Kishihara,K., Hara,H., Nakae,S., Iwakura,Y., and Matsuzaki,G.,** Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. *J.Immunol.* 2010. **184**: 4414-4422.

175. **Cruz,A., Fraga,A.G., Fountain,J.J., Rangel-Moreno,J., Torrado,E., Saraiva,M., Pereira,D.R., Randall,T.D., Pedrosa,J., Cooper,A.M., and Castro,A.G.,** Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. *J.Exp.Med.* 2010. **207**: 1609-1616.

176. **Tang,Q. and Bluestone,J.A.,** The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat.Immunol.* 2008. **9**: 239-244.

177. **Joosten,S.A. and Ottenhoff,T.H.,** Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination. *Hum.Immunol.* 2008. **69**: 760-770.

178. **Annunziato,F. and Romagnani,S.,** The transient nature of the Th17 phenotype. *Eur.J.Immunol.* 2010. **40**: 3312-3316.

179. **Murphy,K.M. and Stockinger,B.,** Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat.Immunol.* 2010. **11**: 674-680.

180. **van der Wel,N., Hava,D., Houben,D., Fluitsma,D., van,Z.M., Pierson,J., Brenner,M., and Peters,P.J.,** M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 2007. **129**: 1287-1298.

181. **Simeone,R., Bobard,A., Lippmann,J., Bitter,W., Majlessi,L., Brosch,R., and Enninga,J.,** Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS.Pathog.* 2012. **8**: e1002507.

182. **Winau,F., Weber,S., Sad,S., de,D.J., Hoops,S.L., Breiden,B., Sandhoff,K., Brinkmann,V., Kaufmann,S.H., and Schaible,U.E.,** Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity.* 2006. **24**: 105-117.

183. **Albert,M.L., Sauter,B., and Bhardwaj,N.,** Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998. **392**: 86-89.

184. **Chen,C.Y., Huang,D., Wang,R.C., Shen,L., Zeng,G., Yao,S., Shen,Y., Halliday,L., Fortman,J., McAllister,M., Estep,J., Hunt,R., Vasconcelos,D., Du,G., Porcelli,S.A., Larsen,M.H., Jacobs,W.R., Jr., Haynes,B.F., Letvin,N.L., and Chen,Z.W.,** A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS.Pathog.* 2009. **5**: e1000392.

185. **Bruns,H., Meinken,C., Schauenberg,P., Harter,G., Kern,P., Modlin,R.L., Antoni,C., and Stenger,S.,** Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J.Clin.Invest* 2009. **119**: 1167-1177.

186. **Lazarevic,V. and Flynn,J.,** CD8+ T cells in tuberculosis. *Am.J.Respir.Crit Care Med.* 2002. **166**: 1116-1121.

187. **Darrah,P.A., Patel,D.T., De Luca,P.M., Lindsay,R.W., Davey,D.F., Flynn,B.J., Hoff,S.T., Andersen,P., Reed,S.G., Morris,S.L., Roederer,M., and Seder,R.A.,** Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat.Med.* 2007. **13**: 843-850.

188. **Lindenstrom,T., Agger,E.M., Korsholm,K.S., Darrah,P.A., Aagaard,C., Seder,R.A., Rosenkrands,I., and Andersen,P.,** Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J.Immunol.* 2009. **182**: 8047-8055.

189. **Derrick,S.C., Yabe,I.M., Yang,A., and Morris,S.L.,** Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional CD4 T cells. *Vaccine* 2011. **29**: 2902-2909.

190. **Forbes,E.K., Sander,C., Ronan,E.O., McShane,H., Hill,A.V., Beverley,P.C., and Tchilian,E.Z.,** Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. *J.Immunol.* 2008. **181**: 4955-4964.

191. **Harari,A., Rozot,V., Enders,F.B., Perreau,M., Stalder,J.M., Nicod,L.P., Cavassini,M., Calandra,T., Blanchet,C.L., Jaton,K., Faouzi,M., Day,C.L., Hanekom,W.A., Bart,P.A., and Pantaleo,G.,** Dominant TNF-alpha+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. *Nat.Med.* 2011. **17**: 372-376.

192. **Day,C.L., Abrahams,D.A., Lerumo,L., Janse van,R.E., Stone,L., O'rie,T., Pienaar,B., de,K.M., Kaplan,G., Mahomed,H., Dheda,K., and Hanekom,W.A.,** Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. *J.Immunol.* 2011. **187**: 2222-2232.

193. **Caccamo,N., Guggino,G., Joosten,S.A., Gelsomino,G., Di,C.P., Titone,L., Galati,D., Bocchino,M., Matarese,A., Salerno,A., Sanduzzi,A., Franken,W.P., Ottenhoff,T.H., and Dieli,F.,** Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. *Eur.J.Immunol.* 2010. **40**: 2211-2220.

194. **Sutherland,J.S., Adetifa,I.M., Hill,P.C., Adegbola,R.A., and Ota,M.O.,** Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. *Eur.J.Immunol.* 2009. **39**: 723-729.

195. **Kagina,B.M., Abel,B., Scriba,T.J., Hughes,E.J., Keyser,A., Soares,A., Gamieldien,H., Sidibana,M., Hatherill,M., Gelderbloem,S., Mahomed,H., Hawkridge,A., Hussey,G., Kaplan,G., and Hanekom,W.A.,** Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am.J.Respir.Crit Care Med.* 2010. **182**: 1073-1079.

196. **Smith,S.G., Lalor,M.K., Gorak-Stolinska,P., Blitz,R., Beveridge,N.E., Worth,A., McShane,H., and Dockrell,H.M.,** Mycobacterium tuberculosis PPD-induced immune biomarkers measurable in vitro following BCG vaccination of UK adolescents by multiplex bead array and intracellular cytokine staining. *BMC.Immunol.* 2010. **11**: 35.

197. **Aagaard,C., Hoang,T.T., Izzo,A., Billeskov,R., Troudt,J., Arnett,K., Keyser,A., Elvang,T., Andersen,P., and Dietrich,J.,** Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose. *PLoS.One.* 2009. **4**: e5930.

198. **Kaveh,D.A., Whelan,A.O., and Hogarth,P.J.,** The duration of antigen-stimulation significantly alters the diversity of multifunctional CD4 T cells measured by intracellular cytokine staining. *PLoS.One.* 2012. **7**: e38926.