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CHAPTER 1

Introduction
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Immunity against tuberculosis

Currently no effective vaccines exist against the three deadliest infectious diseases on earth: tuberculosis, HIV/AIDS and malaria [1]. Tuberculosis (TB) - in humans and other species - is caused by bacteria of the Mycobacterium tuberculosis complex (MTBC): a highly genetically conserved group of mycobacteria including M. tuberculosis, M. africanum and M. bovis, that has evolved from an estimated 3 million years old common progenitor [2]. The origin of M. tuberculosis (Mtb), the main causative agent of human TB, can in all probability be traced back at least 70 000 years to early human populations in Africa, and Mtb’s distinct seven lineages correspond to the migration patterns of humans across the globe [3]. TB became epidemic in medieval Europe, in which period the disease had many names including ‘consumption’ or ‘the white plague’ [4]. No other pathogen in the history of man has resulted in so many deaths [4]: in the past 200 years around one billion people have died from TB. Only in the late 19th century Mtb was identified by Robert Koch as the pathogen causing human TB. The discovery of the first antibiotics against TB dates back to the 1940s [4]. Currently, one-third of the world population is latently infected with Mtb [5]. In latent infection non- or slowly replicating Mtb bacilli are present, yet the infection is contained in a subclinical state [6]. The lifetime risk of developing active TB disease is 3 - 10%; this risk increases to 5 - 10% per year in HIV-infected individuals [6]. Though in the western world TB incidence has dropped spectacularly, in developing countries TB has become one of the major health problems. This has partly been driven by high HIV prevalence, and TB is the leading cause of death in HIV-infected patients [5]. The increased prevalence of type-II diabetes mellitus in developing countries further adds to the TB epidemic [7]. In 2013 1.5 million people died from TB disease, and middle- or low-income countries accounted for 95% of these TB-deaths [5].

During its co-evolution with the human host, Mtb has evolved as a master manipulator of the immune system. Following inhalation of Mtb-loaded aerosols, the bacterium is phagocytosed by professional phagocytic cells in the airways. Mtb is however remarkably capable of persisting in these innate phagocytic cells, employing various strategies that enable it to survive in the hostile cellular compartments within the infected host cell (reviewed in [8]). Mtb also inhibits the migration of infected dendritic cells (DCs) from the infected site to the draining pulmonary lymph nodes (LN) by 10-14
days, thereby delaying the initiation of the adaptive immune response compared to other pathogens, as assessed in murine TB infection models [6;9]. This time-window likely enables establishment of infection before onset of specific immunity. Within the draining lymph nodes, naive T-cells are primed and induced towards differentiation into a variety of pro-inflammatory or regulatory CD4+ and CD8+ T-cell subsets [8].

Both CD4+ T helper (Th)-1 (IFNγ+)-cells as well as CD8+ T-cells are essential in protection against TB [10]. Th1 IFNγ- and TNFα-producing T-cells activate macrophage effector mechanisms, whereas CD8+ T-cells produce cytolytic molecules as well as pro-inflammatory cytokines [11]. IFNγ+IL2+TNFα+ polyfunctional CD4+ T-cells could be important mediators of protection against TB, since polyfunctional T-cells produce higher levels of cytokines compared to single-cytokine producing T-cells, and their simultaneous production might also allow for synergistic activity of these cytokines [12]. Indeed, in a murine model of vaccination against Leishmania major, an intracellular pathogen, the frequency of IFNγ+IL2+TNFα+ polyfunctional CD4+ T-cells correlated with vaccine-induced protective immunity; and similar data were reported for BCG in that same study [12]. One approach in the quest for immune correlates of protection has been to compare immunity in individuals with latent, controlled infection versus individuals with active TB. However, reports on mono- vs. triple-cytokine producing T-cells in latent versus active TB in adults have been conflicting [13;14]. Since polyfunctional T-cells are present in active TB they appear not to be the hoped for surrogate marker of protection against active TB [11;14].

Other T-helper subsets include Th17-cells, Th22, Th9-cells, and follicular helper T-cells [8]. Th17 cells produce IL17, a cytokine that is vital in the recruitment and activation of neutrophils, but its excessive production can lead to hyper-inflammation and tissue damage [11]. CD1-restricted T-cells, MAIT cells and HLA-E restricted CD8+ T-cells are alternative T-cell subsets that recognize antigens through non-classical MHC-1b presentation, that may contribute directly in the combat towards Mtb [15-17].

**M. tuberculosis-induced regulatory T-cells**

As opposed to pro-inflammatory cells, regulatory T-cells (Treg cells; Tregs) inhibit pro-inflammatory responses and are vital for maintaining immune homeostasis, inhibiting auto-
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immunity, and preventing excessive tissue destruction that results from persistent immune activation during infection [18]. However, regulatory mechanisms can also be exploited by Mtb for its own benefit, as demonstrated in murine TB-models: Tregs were induced in the LN by Mtb-infected DCs, and further delayed the priming of pro-inflammatory CD4+ and CD8+ T-cells, thereby even further delaying the migration of these cells to the lung [19]. The early establishment of successful Mtb infection therefore is favoured by a myriad of mechanisms, including suppression of immunity by Tregs.

A hallmark of Mtb infection is granuloma formation: early during the immune response innate cells control and sequester the infectious lesion, followed after two weeks by T-cells migrating into the granuloma periphery, further sequestrating the infected tissue [6]. Also during chronic infection, the granuloma represents a dynamic environment with in- and efflux of immune cells and a spatial distribution of pro- and anti-inflammatory immune cells [20;21]. Although as mentioned above, Tregs may have a beneficial effect in limiting pulmonary tissue destruction during inflammation, this comes at the potential risk of pathogen persistence [18].

Chapter 2 further introduces and reviews the role of Tregs in acute and chronic human infectious diseases, including Mtb, the induction of Tregs by tolerogenic antigen-presenting cells, other mechanisms of Treg induction and expansion, and their modes of suppressing immunity.

Terminally differentiated T-cells in chronic Mtb infection

During chronic infection, pro-inflammatory T-cells are essential to maintain control of Mtb, and this requires continued effector T-cell function and proliferation of T-cells [22]. However, in chronic viral infections and tumours many studies have shown how continued antigen exposure ultimately drives T-cells into functional exhaustion, a state also called terminal differentiation or the chronic (infection) phenotype [23]. These T-cells are marked by the expression of inhibitory receptors and are impaired in their proliferative capacity [23]. Persistent Mtb infection could thus potentially exhaust the T-cell response in a similar way.

Expression of PD-1 has been associated with exhaustion of T-cell function in many human chronic viral infections [24-26], but interestingly, in murine TB proliferating and cytokine-
producing T-cells were marked by PD-1 expression [27;28]. In contrast, murine T-cells with impaired proliferative and/or cytokine-expressing capability expressed the inhibitory marker KLRG1 [27-29]. Also the protective efficacy against TB-challenge of Mtb-antigen specific KLRG1\(^+ \) T-cells was decreased, compared to PD-1\(^+ \) T-cells, in a murine adoptive transfer model [27]. These markers have not been compared yet in the various stages of human TB infection, and it is unknown whether these markers demarcate antigen exposure, or are also indicative of loss of T-cell mediated control in chronic human TB infection.

**BCG-vaccination against tuberculosis**

*Mycobacterium bovis* bacillus Calmette-Guérin (*M. bovis* BCG), the only available and licensed TB-vaccine, was developed already in 1921. BCG was derived from *Mycobacterium bovis*, a bacterium of the *Mycobacterium tuberculosis* complex that causes TB in cattle and wildlife; and attenuated through years of continuous *in vitro* passage by Albert Calmette and Camille Guérin [30]. Estimates are that BCG has been administered at least 3 billion times since its introduction in 1921, which is more than any other vaccine. It is part of the WHO Expanded Programme on Immunization (EPI) and as such routinely administered at birth in nearly all (developing) countries with high TB-prevalence. *M. bovis* BCG-vaccination protects infants from disseminated forms of TB, but it provides insufficient and inconsistent protection against pulmonary TB in adults [31]. Although new vaccines against TB are being developed and evaluated, aiming to either replace BCG or boost its effect, no new effective vaccine is available yet [31]. A recent phase 2B trial in infants in South Africa demonstrated no efficacy in terms of protection against developing TB disease of the TB-vaccine candidate MVA85A, when given as a booster following previous BCG-vaccination, compared to BCG alone, even though T-cell responses were induced [32].

TB-vaccine efficacy would have to include protection against the development of active pulmonary TB in the adult population, since this is the transmissible form of the disease; it has been estimated that a vaccine effective against active pulmonary TB in the adult population would have an enormous impact on the TB-epidemic [33]. A major conundrum
in TB-vaccinology is what exactly constitutes protective immunity against TB and how this can be achieved by vaccination. Most successful vaccines against human pathogens have been those for which the induction of humoral immunity sufficed [34]. The predominately intracellular lifestyle of Mtb, however, clearly necessitates more than antibodies, as is the case for HIV and malaria [35]. However, there is no clear leading example for vaccine design against these three deadly infectious diseases. Basic research into which exact mechanisms of vaccine-induced (cellular) immune responses are essential to induce protection, are needed to guide vaccine design [31]. A further complicating factor is the lack of any true correlate of protection, such that vaccine trials currently require long follow up to reach clinical endpoints [36]. New surrogate endpoints of protection may be identified through researching vaccine-induced cellular profiles and mechanisms of protection. The availability of such correlates would accelerate the evaluation of TB-vaccine candidates in smaller cohorts through increased statistical power [36;37].

The effect of BCG-vaccination in protecting infants from disseminated forms of TB may be partly due to epigenetic modifications in innate immune cells such as trained immunity [38], and this could also explain the ‘non-specific effect’ of BCG-vaccination in protecting infants against other unrelated infectious diseases [39]. Further, specific cytokine-expressing CD4\(^+\) and CD8\(^+\) T-cells are induced by BCG-vaccination in infants and adults [40-47]. IFN\(\gamma\)+IL2+TNF\(\alpha\)+ polyfunctional CD4\(^+\) T-cells have been demonstrated in infant BCG-vaccination [48], yet conflicting reports exist on whether adult BCG-vaccination induces polyfunctional T-cells [49;50]. A large follow up study in BCG-vaccinated infants revealed that there was no association between the induction of polyfunctional CD4\(^+\) T-cells and protection against TB [51]. Thus, it is not clear whether BCG-vaccination induces polyfunctional CD4\(^+\) T-cells in adults, and whether these cells are involved in mediating vaccine-induced protective immunity in adults against pulmonary TB.

Several other, and non-mutually exclusive hypotheses exist concerning the incomplete protection mediated by BCG-vaccination in adults. Systematic reviews have indicated that the protective efficacy of BCG wanes over time [52;53], and this could partly be explained by the relative inability of BCG to induce stable long-term central memory T-cells [54;55]. Further, the immune response to BCG-vaccination in adults may be
hampered by blocking BCG replication through pre-existing immune responses against non-tuberculous mycobacteria (NTM) that are present in the environment (especially in tropical regions, and this would explain the ‘latitude effect’ in protective efficacy) [56]. Also, BCG’s protective efficacy could have diminished through the loss of protective antigens by in vitro passaging [57], and indeed it has been shown that BCG-vaccination fails to induce immune responses to e.g. DosR regulon proteins, likely because these are not expressed following intradermal vaccination [58]. In addition, the response to BCG-vaccination could be modulated by helminth or NTM co-infection [31].

*M. bovis* BCG may itself induce regulatory responses in humans; IL10-producing CD4+ Tregs have indeed been demonstrated in BCG-vaccinated newborns and adults [59;60]. CD8+ Tregs were demonstrated in mycobacteria-infected lymph nodes, and could be isolated from in vitro live BCG-activated PBMCs from blood donors, that had in vitro reactivity to Mtb-PPD [61]. However, CD8+ Tregs are less studied - and often even overlooked - compared to CD4+ Tregs, especially in infectious diseases and vaccination [18], and no paired analysis of BCG-activated CD4+ vs. CD8+ Tregs exists. Thus, there are virtually no data to estimate the relative impact of CD4+ vs. CD8+ Tregs on BCG-vaccine immunogenicity or protective efficacy. In addition, much is still unknown regarding how CD8+ Tregs mediate suppression of Th1 T-cells [18].

Recent studies in murine TB-vaccine models discovered a relation between expression of KLRG1 or PD-1 and vaccine-induced immunity against TB: in these models KLRG1 expression marked terminally differentiated T-cells that had decreased cytokine polyfunctionality and proliferative capability compared to PD-1-expressing T-cells, and KLRG1 expression was associated with impaired protection against TB-challenge [62;63]. However, the induction of KLRG1 expression on human T-cells in response to mycobacteria in particular has not been investigated yet. *M. bovis* BCG can be isolated as a live bacterium from the vaccine lesion months after vaccination, demonstrating antigen persistence, but it remains unknown whether prolonged antigen exposure could drive inhibitory marker expression by T-cells.

Thus, several possible explanations have been formulated to account for the incomplete protection against TB mediated by BCG-vaccination in the adult population, and better research into BCG-vaccine immunogenicity in adults is needed to understand immune responses and immune response diversity induced by this almost a century old vaccine.
Basic research into which exact mechanisms of vaccine-induced (cellular) immune responses are essential for protection will also help guiding future vaccine design [31], and new surrogate endpoints of protection may be identified in parallel.

Outline of this thesis

There is no effective vaccine against pulmonary TB in adults. The only currently available TB-vaccine, *M. bovis* BCG, reduces the risk of severe TB in infants, but provides highly variable and only limited protection against pulmonary TB in adults. This thesis aims to characterize the *M. bovis* BCG-reactive human T-cell response, in order to identify cellular responses that may account for the suboptimal and poorly understood protective efficacy of BCG-vaccination. Firstly, through assessment of the induction of CD4^+^ and CD8^+^ regulatory T-cells by BCG in human adults. Secondly, in view of the inconsistent results on (vaccine-induced) cytokine-producing T-cell subsets in protected vs. non-protected cohorts, by investigating primary BCG-vaccine induced T-cell responses, including both pro-inflammatory and regulatory cellular subsets. Thirdly, by assessing specific induction of inhibitory markers, especially KLRG1, expressed on human T-cells following BCG-vaccination in adults, and whether expression of such inhibitory markers would correlate with impaired immune control in patients with active TB disease compared to individuals with latent (controlled) Mtb infection.

Chapter 2 summarizes and discusses current evidence for the impact of regulatory T-cells on protective immunity in human infectious diseases and following vaccination. The chapter highlights mycobacteria, including *M. tuberculosis*, *M. bovis* BCG, *M. leprae* and non-tuberculous mycobacteria (NTM) as manipulators of the human immune system.

Chapter 3 presents a comparative analysis of the suppressive phenotypes and functions of BCG-activated CD4^+^ compared to CD8^+^ T-cells. PBMCs were isolated from human donors who responded *in vitro* to *M. tuberculosis* PPD and restimulated with BCG. Considering the partly different antigen presentation pathways targeted by live vs. killed BCG bacteria in activating CD8^+^ vs. CD4^+^ T-cells we compared live vs. heatkilled BCG in inducing the suppressive phenotype and function of BCG-activated CD4^+^ vs. CD8^+^ T-cells.

CD39 (E-NTPDase1), an ectoenzyme hydrolysing pericellular ATP to AMP, is a relatively new marker of CD4^+^ T-cells with a regulatory phenotype and activity. CD39 has been
found to be expressed on T-cells circulating in patients with active TB and is also induced on T-cells following vaccination with novel candidate TB-vaccines. The role of CD39 or its expression by BCG-activated CD8\(^+\) Treg cells, however, had not been investigated. **Chapter 4** investigates the expression of CD39 and its involvement in mediating suppression by *in vitro* live BCG-activated CD8\(^+\) Treg cells.

The above studies describe *in vitro* BCG-activation of Treg subsets. However, to follow induction of T-cell subsets by BCG-vaccination, we prospectively studied the pro-inflammatory and regulatory T-cell response induced by primary BCG-vaccination of healthy adult volunteers (**Chapter 5**). Identification of immune responses was further complemented by assessing local vaccine-induced skin reactivity (by ‘classical’ inflammation markers), serum CRP, and IFN\(\gamma\)-expression/-production assays.

Finally, in **Chapter 6** the expression of markers with a role in T-cell inhibition was studied: the expression of KLRG1, PD-1 and CTLA-4 on T-cells was determined following BCG-vaccination, the proliferative capacity of KLRG1- vs. PD-1-expressing T-cells was compared; and the expression of these markers in active TB disease, latent Mtb infection, and following TB-treatment was evaluated.

In the concluding **Chapter 7** the most important findings are summarized and discussed.
References


60. Li, L, Qiao, D, Zhang, X, Liu, Z, and Wu, C. The immune responses of central and effector memory BCG-
specific CD4+ T cells in BCG-vaccinated PPD+ donors were modulated by Treg cells. *Immunobiology* 2011;216:477-484.


