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Regulatory, pro-inflammatory and inhibitory human T-cell responses to *M. bovis* BCG

Opposing T-cell forces in TB-vaccination

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Contents

CHAPTER 1

Introduction

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 ($IFN\gamma^+$)-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]. $IFN\gamma^+IL2^+TNF\alpha^+$ 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 $IFN\gamma^+IL2^+TNF\alpha^+$ 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 autoimmunity, 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 predominantly 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 IFNγ-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.

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CHAPTER 2

Regulatory T-cells at the interface between human host and pathogens in infectious diseases and vaccination

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Abstract

Regulatory T-cells (Tregs) act at the interface of host and pathogen interactions in human infectious diseases. Tregs are induced by a wide range of pathogens, but distinct effects of Tregs have been demonstrated for different pathogens and in different stages of infection. Moreover, Tregs that are induced by a specific pathogen may non-specifically suppress immunity against other microbes and parasites. Thus, Treg effects need to be assessed not only in homologous but also in heterologous infections and vaccinations. Though Tregs protect the human host against excessive inflammation, they probably also increase the risk of pathogen persistence and chronic disease, and the possibility of disease reactivation later in life. *Mycobacterium leprae* and *Mycobacterium tuberculosis*, causing leprosy and tuberculosis, respectively, are among the most ancient microbes known to mankind, and are master manipulators of the immune system toward tolerance and pathogen persistence. The majority of mycobacterial infections occur in settings co-endemic for viral, parasitic, and (other) bacterial co-infections. In this paper, we discuss recent insights in the activation and activity of Tregs in human infectious diseases, with emphasis on early, late, and non-specific effects in disease, co-infections, and vaccination. We highlight mycobacterial infections as important models of modulation of host responses and vaccine-induced immunity by Tregs.

Introduction

A myriad of innate and adaptive immune regulatory cells is induced upon infection, including cells of different lineages: regulatory-like macrophages, dendritic cells (DCs), NKT-cells, T-cells, B-cells, neutrophils, and mesenchymal stem cells. During the last decade, many reports have described the role of regulatory T-cells (Tregs) in infectious diseases and following vaccination. In infectious diseases, Tregs play a dual role: they benefit the host by limiting immune-mediated pathology and also facilitate chronic pathogen persistence by reducing effector immunity and clearance of infection [1]. During acute infection, the beneficial role of Tregs seems to predominate, by regulating leukocyte in- and efflux into lymph nodes (LN) and infected sites, suppression of proliferation of infected cells, and favoring memory formation by increasing the time window of antigen availability.

Regulatory T-cells can be induced either in an antigen- and T-cell receptor (TCR)-dependent or in an antigen- and TCR-independent manner [2;3]. Specificity for self- or pathogen-derived antigens (or dual-specificity) was originally used to divide Treg populations into ‘natural’ resp. ‘adaptive’ Tregs, but it was recently recommended to denote Treg populations by place of induction: ‘thymus derived’ or ‘peripherally derived’, or when the origin is unclear ‘Foxp3$^+$ Treg cell’ [4]. Designations of human Tregs are, however, complicated by the fact that, unlike murine Tregs, unique markers are lacking. In addition, non-Treg populations can express ‘Treg markers’ such as Foxp3 and CD25 upon activation; therefore, human Tregs are preferably defined by multiple regulatory markers and/or by demonstrating suppressive activity [5]. Human CD8$^+$ Tregs have been studied much less than CD4$^+$ Tregs [5], even though they were among the first described ‘suppressor cells’, especially in mycobacterial infections [6;7]. The relative lack in studies on human CD8$^+$ Tregs is possibly the result of technical difficulties in isolating and assessing functions of CD8$^+$ T-cells [8]. Notwithstanding, CD8$^+$ Tregs are re-emerging as important players in general, including in human infectious disease and following vaccination [5].

Once activated, Tregs can suppress pro-inflammatory cells through several mechanisms that are adaptable to the local environment [9]. These mechanisms can mostly be divided into inhibitory cytokine production (either membrane-bound or by their release in the pericellular environment), suppression by cytolysis, metabolic disruption of pro-inflammatory cells, modulation of antigen-presenting cells (APCs), and the activity of certain Treg membrane expressed molecules (see below) [10]. These mechanisms indeed support the concept that antigen-specifically induced Tregs can cross-suppress also other cells irrespective of the presence of their cognate antigen or specificity, e.g., through the secretion of cytokines [5]. This ‘bystander’ or heterologous suppression can compromise immunity toward unrelated pathogens, as has been described for co-infection by helminths in diseases such as malaria and tuberculosis (TB) [11]. Helminth co-infections can also impair the immunogenicity of vaccines such as (oral) cholera vaccination and (intradermal) BCG (*Mycobacterium bovis* bacillus Calmette-Guérin) and tetanus vaccination [12]. Several Treg-expressed molecular markers have now been implicated directly in mediating suppression, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), which modulates APCs via its ligands CD80 and CD86. Tregs were shown to use trans-endocytosis of CD80 and CD86, followed by their intracellular degradation, thereby relatively depleting the APC’s expression of essential co-stimulatory receptors for T-cell CD28 ligation [13]. In addition, the ectoenzyme CD39 (E-NTPDase1), which is a relatively recently discovered Treg marker, exerts its suppressive effects through breakdown of adenosine triphosphate (ATP) [14].

In this paper, we will discuss the induction of Tregs (both specific and non-specific) by various pathogens as well as the functional implications of $CD4^+$ and $CD8^+$ Tregs in acute vs. chronic infectious diseases. We will discuss the role of Tregs in co-infections and highlight in particular infections with *M. leprae* and *M. tuberculosis* (Mtb), which are master manipulators of the human innate and adaptive immune response through the induction of regulatory circuits. We will discuss how the balance of pro- vs. anti-inflammatory responses could ultimately regulate pathogen persistence, and impact on the development of active vs. latent or reactivation of disease. We will also discuss the impact of Tregs on diagnosis and treatment of TB, as well as their possible impact on vaccination against TB.

Mechanisms of Treg induction by pathogens

As a first line of host-defense against infection, the activation of innate immune cells through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), lectin receptors, retinoic acid-inducible gene (RIG) receptors, scavenger, and phagocytic receptors, activates these cells to phagocytose and process the pathogen, after which they migrate to the draining lymph node (DLN) and present antigen to prime naive T-cells. These cells then can differentiate into various classes of T-helper cells (Th), cytotoxic T-cells, or Tregs. Further activation and differentiation signals are provided to the T-cells upon migration into the infected tissue; these signals originate from other T-cells, activated tissue-resident APCs, or even directly from the pathogen (see below). Tissue-resident, circulating, and migrating APCs comprised heterogeneous populations, and the activation of APCs can lead to the induction of pro-inflammatory or regulatory, homeostatic T-cell responses [15]: for example, pro-inflammatory human type-1 macrophages promote Th1-immunity and are characterized by IL-23 production and secretion of IL-12 after IFNγ stimulation, whereas type-2 macrophages poorly express co-stimulatory molecules, produce IL-10, and induce Tregs [16;17].

Modulation of macrophages and DCs toward tolerogenic subsets has been described for various pathogens: after *in vitro* treatment of human DCs with Japanese encephalitis virus or Mtb, DCs upregulated the inhibitory receptor PD-L1, which induced the expansion of Tregs through PD-1 ligation [18-20]. These effects were mediated by the Mtb-derived protein Acr (HspX Rv2031c), which is expressed during latency: Acr induced expression of PD-L1, TIM3, IDO, and IL-10 by murine DCs and promoted the induction of $CD4^+CD25^+Foxp3^+$ T-cells [21]. Furthermore, APCs can be modulated through alterations in (pericellular) purinergic pathways: extracellular ATP, a pro-inflammatory danger signal, which activates the killing of Mtb in macrophages, is rapidly hydrolyzed to AMP by CD39, which is expressed by various regulatory cells [14]. The degradation of ATP to AMP in the microenvironment was accompanied by a switch in macrophage gene expression from type 1 toward type 2, and Mtb infection actively upregulated expression of the adenosine A2A receptor on macrophages [22]. This receptor has been described as a major immunosuppressive immune cell adenosine receptor acting through elevation of cAMP [23], and its expression on macrophages was central to M2-like polarization after Mtb infection [22]. Other cell types acting as APCs were demonstrated to contribute to Treg induction: both hepatitis C virus (HCV)-infected hepatocytes and *H. pylori*-infected gastric epithelial cells directly induced Tregs through production of TGF-β [24;25].

Regulatory T-cells can also be induced directly through pathogen-derived components. This has been demonstrated in several murine studies: zwitterionic capsular polysaccharides from *S. pneumoniae*-induced $CD8^{+}CD28^{-}$ Tregs that were $CD122^{LO}CTLA\text{-}4^{+}CD39^{+}$, synthesized IL-10 and TGF-β, and exhibited suppressive activity. This induction was independent of APCs and involved direct crosslinking of the TCR [26]. In another murine study, proteins secreted by *H. polygyrus* induced $Foxp3^{+}$ T-cells through ligation of the TGF-β-receptor [27]. The herpes virus entry mediator HVEM, a binding site for viral glycoprotein HSVgD, is upregulated on murine $CD4^{+}Foxp3^{+}$ Tregs after HSV-1 infection, and activation of this receptor led to preferential expansion of Tregs [28]. In the human situation, $CD4^{+}CD25^{+}$ Tregs exhibited extended survival and increased suppressive capacity after binding HIVgp120 [29].

The preferential expression of TLRs, such as TLR2, on Tregs as compared to 'conventional' T-cells has been reviewed by Sutmuller and colleagues [2]. A large variety of TLR2 ligands have been described in bacteria, including Mtb [30]. Mtb-induced TLR-signaling in APCs leads to inhibition of the MHC-II transactivator-gene CIITA, thereby decreasing expression of MHC-II and antigen presentation [30]. During chronic Mtb infection, prolonged TLR2 signaling (e.g., through the 19kD lipoprotein) can lead to suppressive cytokine production [31] and recruitment of $CD4^{+}$ Tregs to the lung [32]. A role for TLR-mediated Treg induction has also been described in murine malaria: murine *Plasmodium*-activated DCs induced Tregs through TLR9, and $TLR9^{(-/-)}$ mice had impaired activation of Tregs, associated with a partial resistance to lethal infection [33]. Other factors in the local environment vital for the expansion and function of Tregs include changes in metabolism [34], endothelial cytokine (IL-33) production and cytokine balance (IL-23 : IL-33 ratio) [35], and metabolite products from commensal microbiota [36;37]. Thus, specific pathogen components can skew toward Treg phenotype or function. The significance of these Tregs for the disease process, concomitant diseases, and vaccinations will be discussed further below.

The impact of Tregs in infectious diseases

Viral infections: acute vs. chronic infectious disease

Regulatory T-cells have been found after retrovirus-, RNA virus-, and DNA virus infection in mice and humans [reviewed in Ref. [3]; figure 1A]. Various $CD4^+$ and $CD8^+$ Treg subsets have been identified [38], but mostly in chronic viral infection. Yet, in hepatitis A virus infection - an acute inflammatory disease, usually followed by pathogen clearance - hepatitis A virus bound to its cellular receptor (HAVCR1), which is expressed on Tregs, which resulted in inhibited Treg function and inflammation [39]. By contrast, in acute dengue fever, Treg function and the suppression of vasoactive cytokine release were similar in acutely infected and recovered patients, such that in this case, the disproportionate activation of pro-inflammatory cells and cytokines often found in dengue fever was not explained by acute phase Treg malfunction [40]. Thus, blockade of Tregs in acute viral infection could assist in pathogen clearance, at the cost of temporary hyper-inflammation, but not all (pathological) hyper-inflammation is associated with Treg hypo-functionality. On the other side, Tregs could also benefit the host during acute infection: first, Treg depletion in murine herpes simplex infection increased LN levels of IFN-α and -γ, but infection-site-associated IFNγ was decreased, and the arrival of DCs, NK cells, and T-cells at the infected lesion was delayed [41], pointing to a role for Tregs in promoting LN in- and efflux of pro-inflammatory cells [42]. Second, Tregs may suppress infected cell proliferation at the mucosal point-of-entry to a level where infection cannot be established, which was suggested as a protective mechanism in early HIV infection [43;44]. Third, Tregs were vital in allowing memory formation through promoting antigen persistence, as was recently demonstrated in a murine West Nile virus infection model [45].

The role of human Tregs in chronic viral infection has been more extensively delineated. A meta-analysis of 12 studies demonstrated increased $CD4^+$ Treg frequencies in chronic hepatitis B virus (HBV) infection compared to both acute infection and healthy controls, revealing a strong association of Tregs with disease progression, viral load, absence of therapy response, and risk of hepatocellular carcinoma [46]. In chronic HCV infection, the contribution of Tregs to low inflammatory $CD4^+$ and $CD8^+$ T-cell responses has been described [47;48]. Tregs were recruited to the liver through the Treg-attracting chemokines

CCL17 and CCL22 [49], thereby promoting pathogen persistence. It has been argued, however, that Tregs may also be functional in limiting HCV-induced liver damage [48].

In chronic HIV infection, $CD4^+$ Tregs were relatively increased in the mucosa and in the circulation compared to healthy controls, but the Treg-mediated effects on anti-HIV immune responses remain a matter of debate [50]. $CD4^+$ Tregs decreased HIV replication in T-cells *in vitro* through CD39-mediated ectonucleotide shifts and by transfer of cAMP through gap junctions formed with conventional T-cells [43]. Tregs inhibited spreading of virus from DCs to T-cells through interfering with the immunological synapse [51]. In another study, blocking of CD39 by monoclonal antibodies (mAbs) restored cytokine production by HIV-gag-stimulated $CD8^+$ T-cells [52]. Indeed, the relative frequency of $CD4^+CD39^+$ Tregs positively correlated with HIV viral loads and disease progression in infected individuals [53]. These different effects of Tregs could be explained by differentiating between acute and chronic infection, as argued in Ref. [50]: control of viral replication by $CD4^+(CD39^+)$ Tregs may be important early after infection with a limited number of infected cells (relatively high Treg : T-effector ratio), yet during chronic infection Tregs may not be able to suppress proliferation of all infected cells, and potentially become more detrimental due to dampening anti-HIV responses. This points to the need for more detailed analyses of Treg functions in acute vs. chronic (hyper-) inflammation.

Bacterial infections: reservoirs for Treg induction

Early vs. late effects of Tregs in bacterial infection were elegantly described in a mouse model of *Salmonella* (*Salmonella enterica* serotype Typhimurium): Tregs suppressed early protective immunity, thereby allowing for establishment of infection, yet clearance of infection at later time points corresponded with a decrease in Treg suppressive capacity [54]. After acute infection, Treg-mediated failure to completely eradicate *Salmonella* may thus lead to a carrier state of persistent asymptomatic infection, resulting in a reservoir for shedding of pathogens into the environment and further infection (reviewed in Ref. [55]).

A carrier state of *Streptococcus pneumonia* in the nasopharynx was associated with increased TGF-β levels from nasal washes in humans, and TGF-β was shown to lead to Treg expansion in *in vitro* murine experiments [56]. In *Helicobacter pylori* infection, a carrier state can last for life; and several studies have described the ability of *Helicobacter* *pylori* to induce Tregs. These Tregs were found in the circulation as well as in the gastric mucosa of both infected children and adults, and though Tregs initially can limit inflammation and therefore probably gastric ulceration, pathogen persistence could, on the other hand, lead to chronic inflammation and tumor induction [57] (reviewed in Ref. [58]).

Increasing attention has been drawn to the interplay of the immune system with non-pathogenic commensal microbiota in the intestine. Tregs can be induced by commensal microbiota, as has been demonstrated in multiple murine studies: butyrate, a metabolite from commensals, potently induced Tregs in the intestine [36;37], possibly through butyrate-mediated enhanced histone H3 acetylation in the FOXP3 promoter [37]. Polysaccharide A (PSA) from *B. fragilis* induced conversion of T-cells into Tregs, and cured experimental colitis [59]. The CNRZ327-component from *Lactobacillus delbrueckii* induced regulatory responses in colonic tissue, but importantly also in cecal LNs and the spleen, pointing to systemic distribution of these microbiota-induced Tregs [60].

Raising mice in germ-free conditions decreased the number of Tregs in the gut, but the number of cutaneous Tregs was increased, possibly through loss of inhibition by pro-inflammatory cells [61]. In any case, data on activation of Tregs by skin commensals is also emerging [61], and these Tregs induced by skin microbiota may modulate systemic inflammatory responses [61]. As recently reviewed [62], increasing evidence reveals resident microbiota in the lungs. Though relatively low in bacterial biomass compared to the microbiota of the skin and the intestinal mucosa, these microorganisms are present in healthy lungs as they are at other mucosal surfaces, and probably differ in composition between healthy individuals and individuals with (pulmonary) disease [62].

Clearly, mucosal surfaces are the primary sites both for pathogenic and commensal microbiota; and induction of Tregs - within the myriad of innate and adaptive cells - has been described for both. Further research should elucidate local and systemic effects of Tregs induced at barrier sites in human studies, and whether systemic effects of Tregs induced by (non-pathogenic) commensals are to be expected (figure 1A).

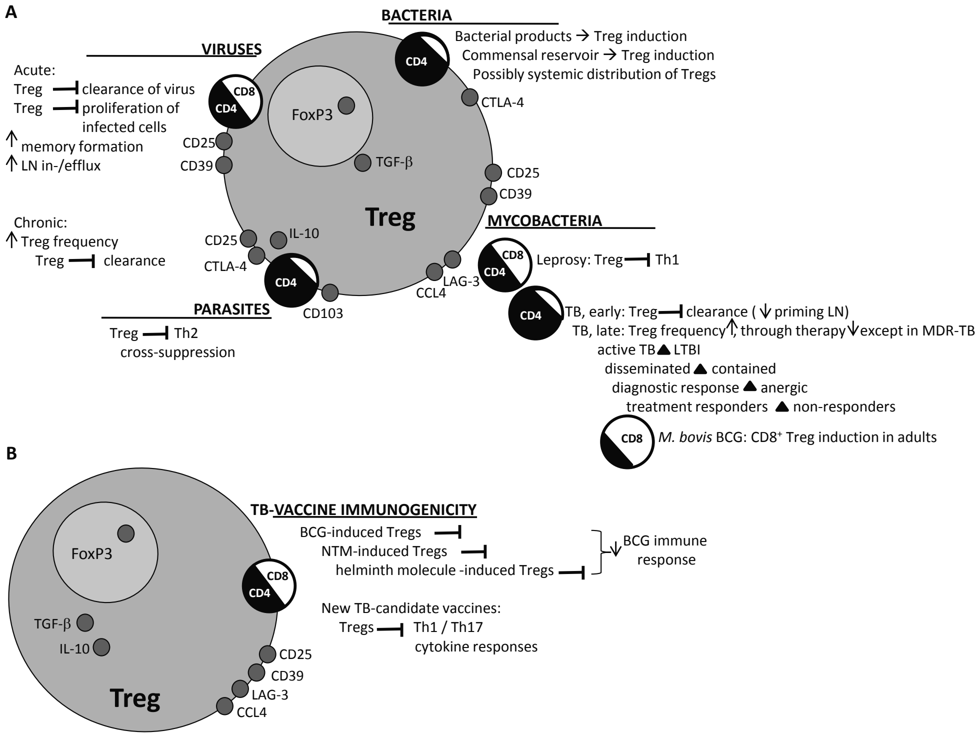

Figure 1. Main effects of Tregs as described for various pathogens.

A: Various Treg-mediated effects have been described for the various classes of pathogens; early vs. late, and heterologous suppression, are described in several taxonomies. Prominent features are noted, as well as prominent Treg markers for the various pathogens. Circles 'CD4/CD8' depict the scale in reports of either CD4$^+$ or CD8$^+$ Tregs in literature for the various classes. B: Treg effects on TB-vaccine immunogenicity are displayed in a similar fashion. BCG immunogenicity may be decreased by or inversely related to BCG-induced Tregs, or may be suppressed by heterogenic Tregs. Treg induction has also been described in various TB-vaccine candidate trials. BCG = *Mycobacterium bovis* bacillus Calmette-Guérin; CCL4 = CC chemokine ligand 4; CTLA-4 = cytotoxic T-lymphocyte-associated antigen 4; LAG-3 = lymphocyte activation gene-3; LN = lymph node; LTBI = latent tuberculosis infection; MDR-TB = multi-drug-resistant tuberculosis; NTM = non-tuberculous mycobacteria; TB = tuberculosis; Treg = regulatory T-cell.

Parasitic infections: suppression across boundaries

Murine *Leishmaniasis* models have been pivotal in demonstrating Tregs at the site of (parasitic) infection: antigen-specific $CD4^{+}CD25^{+}$ Tregs were present at the site of chronic *Leishmania major* infection [63] and retention at the infection site was dependent on expression of CD103 by $CD4^{+}CD25^{+}$ Tregs [64]. In this model, the impact of Tregs on establishment of chronic infection and reactivation of disease was elegantly demonstrated: after pathogen clearance, *Leishmania* super-infection led to reactivation of disease and increased Treg numbers at the primary site. Also, adoptive transfer of Tregs from infected mice into chronically infected mice caused reactivation of disease [65]. Mechanisms of suppression included IL-10 production by Tregs as well as other mechanisms [66]. In another study, Foxp3-negative cells were the major producers of IL-10, and anti-IL-10R mAb treatment decreased parasite burden to a greater extent compared to anti-CD25-mAb treatment [67]. In humans, functionally suppressive $CD4^{+}CD25^{+}$ Tregs have been isolated from cutaneous leishmaniasis (skin) lesions [68]; and FOXP3 mRNA levels in skin lesions were increased in chronic compared to acute *Leishmania major* infection [69]. Also in *Leishmania guyanensis*-induced skin lesions, FOXP3 mRNA levels were significantly higher in chronic compared to acute patients, though in both cases Tregs isolated from these lesions displayed suppressive activity *in vitro* [70]. Importantly, IL-10 and FOXP3 mRNA expression in *Leishmania guyanensis*-infected skin lesions were associated with unresponsiveness to treatment [71].

Several studies have reported increased Treg frequencies in *Plasmodium falciparum*-infected individuals compared to asymptomatic or uninfected controls [72]; furthermore in patients with clinically severe malaria, the frequency of $CD4^{+}CD25^{+}Foxp3^{+}CD127^{LO}$ Treg cells correlated with levels of parasitemia and total parasite biomass [73]. Tregs were associated with risk of malaria disease: reduced expression of CTLA-4 and FOXP3 was found in Fulani, an ethnic group in Burkina Faso relatively resistant to *P. falciparum* compared to Mossi (a different ethnic group from the same region) [74]. Proliferative PBMC responses to malaria antigens from Mossi were increased following $CD25^{+}$-depletion, but those from Fulani were not [74]. In Kenyan adults with natural immunity to malaria, $CD4^{+}CD25^{HI}$ T-cell frequency at enrollment was associated with the risk of developing clinical malaria during follow up [75].

Many helminth parasitic infections steer immunity toward Th2 and T-regulatory responses [12]; and murine data indicate that immune suppression is achieved through cross-mucosal induction of regulatory cytokines, regulatory DCs, macrophages, and $CD4^+$ and $CD8^+$ Tregs [76]. In a recent study of murine *Trichuris muris* infection, Th2 cell proliferation was enhanced by early Treg depletion post-infection and by Treg depletion after establishment of infection [77]. However, the ultimate effect of Tregs on pathogen persistence was clearly time dependent: both early and late Treg depletion enhanced Th2 responses and reduced Th1 responses, but while early Treg depletion resulted in enhanced clearance of infection, later, during infection, Treg depletion resulted in enhanced worm burden [77].

Importantly, in geohelminth-infected children *in vitro* depletion of $CD4^+Foxp3^+CD25^{HI}$ T-cells increased not only antigen-specific proliferative responses but also IFNγ production in response to *Plasmodium*-infected red blood cells [11]. The *in vivo* effect of helminth co-infection on immunity against *Plasmodium* varies between studies, but helminth co-infection may be associated with protection against cerebral malaria, a state of severe hyper-inflammation [12]. Latent tuberculosis infection (LTBI) individuals with hookworm [78] and filarial co-infection [79] had decreased Th1 and Th17 responses and increased Treg frequencies compared to parasite-uninfected LTBI individuals. Whether deworming has clinical impact on the course of TB disease is not clear: in TB patients with helminth co-infection, albendazole treatment decreased IL-10 levels, but there was no clinical improvement in TB after 2 months [80]. Since (helminth-induced) Tregs are capable of exerting non-specific suppressive responses, research in malaria and TB (diseases where strong Th2 and Th1 responses are vital, respectively) will hopefully clarify the effect of Tregs across the boundaries of disease (figure 1A), especially in settings where co-infection of helminths with malaria and/or TB is endemic.

Tregs in leprosy and tuberculosis

Tregs in leprosy, an ancient disease

Leprosy, caused by *M. leprae*, is an ancient, chronic, disabling, but curable disease affecting the skin, the peripheral nerves, the eyes, and mucosa of the respiratory tract [81]. The clinical spectrum of the disease ranges from tuberculoid (TT) and borderline tuberculoid (BT) to borderline lepromatous (BL) and lepromatous leprosy (LL), where TT/BT is immunologically characterized by a strong Th1 response accompanied by limited growth of the bacillus (paucibacillary leprosy), whereas BL/LL is classically characterized by a predominant Treg/Th2 response, high antibody titers, absent granuloma formation, and thus poor containment of infection and clinical deterioration [82].

Though the exact mechanisms ruling this spectrum have not been elucidated, it is clear that Tregs play a part, and demonstration of the suppressive activity of $CD4^+$ and $CD8^+$ Tregs isolated from the skin and circulation of LL patients were among the first reports on ‘human T-suppressor cells’ [6;7]. In the circulation of leprosy patients, both $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ T-cells were almost twofold increased compared to healthy contacts [83]. Within the spectrum of disease, increased percentages of $CD4^+Foxp3^+CD25^+$ and $CD8^+Foxp3^+CD25^+$ T-cells have been demonstrated in the circulation of LL patients compared to BT patients or healthy contacts [82;84]. Also in lepromatous lesions, $Foxp3^+$ T-cells were increasingly expressed in LL compared to TT/BT patients [82;84]. Suppression of the Th1 response by Tregs was demonstrated by enhanced *in vitro* IFNγ production through depletion of $CD25^+$ cells in a subset of LL patients [82]. Both $CD4^+CD25^+$ derived IL-10 production and regulation through TGF-β have been described [85;86].

A possible mechanism of Treg induction by *M. leprae*-infected DCs is the expression of the mycobacterial cell wall component PGL-1, that by association with the complement component C3 can steer toward Treg differentiation [87]. Type-2 anti-inflammatory ($CD163^+$) macrophages are important Treg inducers [17], possibly due to the action of ROS [88]; indeed, a regulatory phenotype was described in monocytes stimulated with *M. leprae* [89]. Recently, $CD68^+CD163^+$ cells were demonstrated in LL skin lesions with increased frequencies compared to BT/TT lesions [82]. Intracellular pathways leading to enhanced

Foxp3 expression in $CD4^+$ T-cells have been described in association with progression of disease toward BL/LL, in addition to low Foxp3 ubiquitination (marked for intracellular degradation) [86]. In T-cells isolated from LL patients, Foxp3 interacted with histone deacetylases and bound directly to the promotor regions of CD25 and CTLA-4 [90]. The importance of this transcriptional regulation by Foxp3 within the immunological spectrum of disease is further supported by the fact that not only Treg frequencies are increased in LL compared to BT patients but also the intensity of expression (mean fluorescence intensity) of Foxp3 as determined by flow cytometry [83].

Thus, Tregs are clearly involved in the impairment of mycobacterial control. However, this does not necessarily equate to increased suppression of Th1 over Th2 responses toward the LL pole spectrum: gene expression profiling of PBMCs isolated from TT, LL, and borderline leprosy patients revealed decreased expression of both Th1 and Th2 genes in LL patients, but enhanced expression of CTLA-4 and TGFB1 [91]. The authors further found overexpression of CBL-B, an E3 ubiquitin-ligase that after encounter with antigen is crucial in modulating T-cells toward activation vs. anergy, dependent on the presence or absence of co-stimulatory signals [92]. Cbl-b, TGF-β, and CTLA-4 expression were molecularly related, as demonstrated by the dependency of Cbl-b expression on TGF-β and the decreased expression of Cbl-b after treatment with CTLA-4 siRNA [91]. Within the paradigm of a generalized suppressed peripheral T-cell response associated with LL development, Tregs could thus play an important role in inducing and maintaining low cellular immune responsiveness (figure 1A), although their impact on humoral (mostly but not exclusively Th2-related) responses remains less clear. Further work would be needed to clarify causal relationships, e.g., if Tregs are a cause or consequence of bacterial burden in LL disease [93].

Tuberculosis: early and late effects of Tregs

Pathogen-specific Tregs were induced by Mtb as demonstrated in a murine Mtb aerosol infection model, and these Tregs delayed priming of $CD4^+$ and $CD8^+$ T-cells in the pulmonary LNs, thereby delaying migration of these cells to the lung [94]. Tregs were demonstrated in the lung, including in granulomas [95], and were shown to prevent pathogen clearance [96]. Interestingly, in contrast to *Listeria monocytogenes*, pathogen-specific Treg expansion could be found in LNs only after Mtb infection [97]. Thus, Mtb-induced Tregs contribute to the delayed onset of adaptive immunity that is observed in TB compared to other diseases and which allows establishment of infection [98;99]. The impact of Tregs on establishment of infection was further demonstrated in a murine study, where depletion of $CD25^+$ cells early after Mtb infection - but not during chronic infection - decreased bacterial load and granuloma formation [100]. However, it might also be that (pre-existing) Tregs have a beneficial role very early in infection, but also these data are only derived from animal experiments. In macaques, Tregs and IFNγ-producing effector T-cells expanded early after pulmonary TB infection, yet *in vivo* depletion of both IFNγ-producing- and regulatory T-cells led to decreased resistance against granuloma progression [101]. Analogous to the possibly beneficial role for Tregs in regulating LN in- and efflux during early murine HSV infection [41;42], it is conceivable that the presence of a very low level of (possibly pre-existing) Tregs before or in a very early state after Mtb infection might thus accommodate priming and subsequent emergence of a pro-inflammatory immune response. Clearly, further research will be needed to specify the impact of Tregs in various organs [102], early in (human) Mtb infection, and to differentiate their impact in early vs. chronic infection (figure 1A).

Regulatory T-cells are also present in human Mtb infection as has been demonstrated extensively: Tregs could be isolated both from the circulation and from the site of infection in TB patients. In the circulation of TB patients, an increase in FOXP3 mRNA expression was found compared with healthy controls [103], and also an increase in $CD4^+$ T-cell frequencies with regulatory phenotypes was demonstrated (defined as $CD4^+CD25^{+/HI}$ [103;104], $CD4^+Foxp3^+CD25^{HI}$ [105;106], or $CD4^+CD25^{HI}CD39^+$ [105]). Tregs could be isolated from various Mtb-infected sites, including bronco-alveolar lavage (BAL) fluid, ascites, pericardial fluid, and pleural fluid; and FOXP3 mRNA expression levels and $CD4^+CD25^{HI}$ T-cell frequencies were increased stronger locally than systemically (in the circulation) [103;107]. In a study comparing TB cases with infected and uninfected TB contacts (defined by positive tuberculin-skin test (TST) and ELISpot results), PBMCs from uninfected contacts had lower FOXP3 mRNA expression levels compared to TB cases, but higher FOXP3 expression levels compared to infected TB contacts; which according to the authors could signify migration of Tregs to the lungs during early infection, with a reappearance in the circulation during latent (established) infection [108]. Also

$CD8^+Foxp3^+CD25^+$ Tregs were demonstrated in the circulation and BAL fluid of TB patients [107]; and $CD8^+LAG\text{-}3^+CCL4^+$ Tregs (lymphocyte activation gene-3 (LAG-3); CC chemokine ligand 4 (CCL4)) were shown by histological staining of infected LNs from TB patients [109]. Furthermore, after stimulation with HLA-E restricted Mtb-derived peptides $CD8^+$ Tregs could be isolated from PBMCs of *in vitro* mycobacterial purified protein derivative (PPD)-reactive donors [110;111].

Elevated frequencies of circulating Tregs in TB patients declined during successful chemotherapy [106], in contrast, in patients with emerging MDR-TB circulating Treg frequencies remained persistently high [106]. Other data on Tregs in MDR vs. normally resistant (NR)-TB are scarce and conflicting: similar frequencies of circulating $CD4^+Foxp3^+$ Tregs were found in MDR-TB patients compared to (NR-)TB patients [112]; however, in another study comparing MDR-TB, NR-TB, and non-tuberculous mycobacteria (NTM) infections, increased *ex vivo* frequencies of Tregs were found in MDR-TB but also in NTM infections compared to NR-TB. This may reflect chronicity of infection in MDR-TB and NTM infection, which is often treated suboptimally; however, the contrast reported by the authors between elevated serum IL-10 levels in MDR-TB patients vs. elevated serum TGF-β levels in NTM-infected patients could also suggest different subsets of Tregs or different suppressive effector pathways to be involved in MDR-TB vs. NTM [113].

Tuberculosis: Tregs differentiate active from latent disease

$CD4^+Foxp3^+CD25^+$ Tregs are increased in frequency in active TB compared to LTBI [107;114], both in the circulation and in BAL fluid [107] (figure 1A). A report on $CD4^+CD25^+CD134^+$ T-cells in TB demonstrated differentiation between active and latent TB solely through the presence or absence of the CD39-molecule on this subset [115]. Stasis of mycobacterial growth in macrophages, both monocyte-derived and alveolar, was suppressed by $CD4^+$ Tregs [107]. Depletion of $CD4^+Foxp3^+CD25^{HI}$ T-cells increased IFNγ responses to the mycobacterial antigen heparin-binding hemagglutinin (HBHA) of patients with active TB *in vitro*, to the level observed in LTBI individuals [116]. Treg frequency in the circulation of smear-positive TB patients was increased compared to smear-negative patients; however, this did not correlate with radiologic determination of extent of disease [112].

Pro-inflammatory signatures of $CD8^+$ T-cells differentiated between latent infection and active TB disease [117], and also *in vitro* an association was found between burden of infection of cells and lysis by cytotoxic $CD8^+$ T-cells [118]. The frequency of $CD8^+$ T-cells producing IL-10 or TGF-β was increased in active TB patients compared to latently infected or control subjects [119]. In this study, $CD8^+$, $CD8^+IFN\gamma^+$, and $CD8^+IL\text{-}17^+$ T-cell numbers were similar between groups, and were - interestingly - not dependent on sputum bacillary load, while sputum bacillary load was positively associated with specific regulatory cytokine expression in $CD8^+$ T-cells, and negatively associated with $CD8^+$ granzyme-B expression [119]. However, in another study, the frequency of $CD8^+Foxp3^+CD25^+$ Tregs did not differ between active vs. latent TB, or between cells isolated from the circulation vs. cells isolated from BAL fluid [107]. The differences between these reports may be explicated by differences in regulatory markers that were studied, or by methods that were used: in the former study, cells were stimulated with Mtb specific antigen for 96 hours, while in the latter study, cells were PPD-stimulated for 12 hours. $CD8^+$ Tregs are relatively understudied compared to $CD4^+$ Tregs in mycobacterial infection [5], and this clearly points to the need for more (uniform) research into these possibly important regulators and/or markers of activity of disease. Of note, $CD8^+$ Tregs were found at the disease site in mice, and progression of disease correlated with accumulation of IL-10-secreting $CD8^+$ T-cells in granulomas [120].

Instead of being a steady state of infection, latent TB comprises a dynamic spectrum with supposedly increasing rates of subclinical Mtb replication and inflammation extending eventually to active TB. Serial IGRA testing has been proposed as an indicator of human host resistance in latent TB. Using serial testing, a consistently negative test in TB-exposed individuals would likely indicate strong resistance to infection, a consistently positive test (recent) active infection, and (repeated) test conversions (positive to negative, possibly followed by conversion, etc.) changing dynamics of infection and control of bacterial load. In a comparison of T-cell subsets between IGRA-consistently positive and consistently negative TB-case contacts, $CD4^+Foxp3^+$ and $CD4^+CTLA\text{-}4^+$ Tregs were increased in TB-case contacts with consistently positive IGRA tests, possibly indicating Treg interference with host resistance in the development of active infection [121].

Tuberculosis: Tregs in extra-pulmonary disease

A minority of TB cases present with extra-pulmonary disease or extra-pulmonary involvement following pulmonary infection, and it is assumed that this represents failure of the immune system to contain infection [122]. Multiple studies indicate involvement of Tregs in dissemination of disease (figure 1A). An increase in FOXP3 mRNA expression has been described in PBMCs from patients with extra-pulmonary TB (disseminated and lymphatic TB) compared to pulmonary TB [103]. In a comparison of TB pleural effusion and miliary TB, representing in this case containment vs. dissemination of disease, elevated FOXP3 mRNA expression levels and frequencies of $CD4^+Foxp3^+CD25^+$ T-cells were found in cells isolated from miliary disease sites [123]. Another study confirmed an increase in $CD4^+$ Treg frequencies in patients previously treated for extra-pulmonary TB compared to pulmonary TB, but reported an analogous increase in $CD4^+$ activation markers [124]. In TB pleurisy, $CD4^+Foxp3^+CD25^{HI}$ Treg frequencies were increased in pleural fluid compared to the circulation [125;126], and Tregs suppressed IFNγ expression in $CD4^+$ and $CD8^+$ T-cells [126]. Pleural $CD39^+$ Tregs inhibited generation of Th17 cells, which could be reversed *in vitro* by antagonizing TGF-β through the addition of latency-associated peptide (LAP) [127]. Mtb infection of the pleurae favored Treg migration into the pleural exudate when compared to other causes of pleurisy: tuberculous pleural fluid, but not effusions from other bacterial origin, or transudates, had high concentrations of the chemoattractant CCL22, which is chemotactic for Treg migration *in vitro*, and an increase in $CD4^+CD25^{HI}$ T-cell frequency compared to the circulation [125]. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on pleural mesothelial cells regulated migration of leukocytes from the circulation into the pleural fluid; however, these molecules also seemed to favor (non-antigen-specific) expansion of Tregs [128].

In TB lymphadenitis in children, $CD4^+Foxp3^+$ T-cells were demonstrated in the LNs, and quantitative mRNA analysis demonstrated induction of TGFβ and IL13, but not of IFNγ, TNFα, or IL-17 [129]. Data on frequency and function of Tregs in other forms of TB disease, such as bone TB, urogenital TB, or TB of the central nervous system (CNS) are scarce. It is, however, conceivable that the interplay of Tregs and Mtb may differ in infections at immune-privileged sites, such as the CNS or the eye. The assessment of anti-inflammatory mechanisms could be highly relevant in regard to CNS-immune reconstitution syndromes, given their often disastrous outcomes [130]. Several studies have associated plasma biomarkers and CD4$^+$ T-cell activation with the development of HIV-associated immune reconstitution inflammatory syndromes (IRIS), but did not find an association with (CD4$^+$) Treg frequencies, both in the development of cryptococcal-IRIS disease [131] and TB-IRIS disease [131;132]. TB-IRIS may either be 'unmasking' (of an occult infection) or 'paradoxical' (worsening of a known infection during retroviral treatment) hyper-inflammation: decreased serum IL-10 levels were found in paradoxical compared to unmasking syndromes [133]. Interestingly, this might represent Treg function, not Treg phenotype: a study in patients developing symptoms of *Mycobacterium avium* and *intracellulare* complex-infection, following commencement of retroviral treatment, reported a significant expansion of CD4$^+$Foxp3$^+$CD25$^+$CD127^{LO} Tregs, but reduced functional capacity and diminished IL-10 secretion of these cells in *in vitro* suppression assays [134].

Tuberculosis: Tregs in the clinic

Tregs may interfere with clinical diagnosis of TB (figure 1A). Classically, diagnosing TB has relied for decades on the TST, testing cell-mediated immunity against intradermally injected Mtb-derived tuberculin PPD. Skin anergy is defined as the absence of dermal reactivity in otherwise confirmed Mtb infection. *In vitro* PPD stimulation of cells isolated from PPD-reactive TB patients induced both IL-10- and IFNγ-production; however, cells from anergic TB patients produced only IL-10 but not IFNγ [135]. Reduced levels of IFNγ and IL-2, and increased levels of IL-10 in anergic compared to PPD-reactive TB patients were confirmed in another study. This anergy was found only after *in vitro* stimulation with PPD- but not unrelated antigens, indicating an antigen-specific anergic reaction [136]. Suppression of IL-2- and TNFα-production was accompanied by CD8$^+$ T-cell expansion and high levels of IL-10 in anergic TB patients, and CD8$^+$ T-cell depletion and blocking of IL-10 reversed this suppression [137].

A direct effect of Treg-mediated suppression on interferon-γ release assays (IGRAs), such as the in-tube QuantiFERON Test, has so far not been established. Nevertheless, several studies have described 'rescue' of mycobacterial-specific IFNγ production by Treg depletion in Mtb-infected individuals [104;105;114;138]. Interestingly, depletion of CD25$^+$

T-cells increased IFNγ production by PBMCs in Mtb-infected individuals, but did not increase the production of IL-17A [114]. Yet, pleural $CD39^+$ Tregs ($CD4^+CD25^+CD39^+CD127^-$) inhibited Th17 differentiation [127], and an inverse correlation between production of IL-17A and CD39-expressing Tregs has been described after vaccination [139;140]. CD39 expression on Tregs may thus be more closely linked to suppression of IL-17 production compared to cells expressing CD25, but this needs further clarification. Also the extent of TB infection as determined by chest X-ray (CXR) scoring was associated with T-cell modulation: in a study dividing patients by severity of disease by CXR, double-negative (DN, $CD4^-CD8^-$) TCRγδ T-cells from patients with severe disease displayed a modulatory profile with high IL-10 production, in contrast to patients with less severe disease, where TCRγδ DN T-cells displayed a pro-inflammatory cytokine profile with high IFNγ [141].

During TB therapy, circulating $CD4^+$ Treg frequencies declined as mentioned; however, this was only noted following chemotherapy for pulmonary TB (figure 1A) [106;142;143]. In contrast, an increase was noted during extra-pulmonary TB treatment [143;144]. Differences between forms of disease possibly represent differences in compartmentalization of Tregs, or heterogeneous kinetics of Treg contraction following decrease of bacterial burden. TB patients in which MDR-TB emerged during therapy had persistent circulating Treg frequencies [106], which could be analogous to a phenomenon observed during IFNα therapy for chronic HBV infection: therapy non-responders were characterized by an increase in $CD4^+CD25^+$ T-cells and IL-10-producing cells [145]. Thus, circulating Treg frequencies might be used as parameter of therapy response in specific states of TB disease.

Tregs in vaccination against tuberculosis

Even in early life, immunoregulatory mechanisms, including Tregs, may dampen vaccine-induced immunity [146]. We describe here how immunogenicity of TB-vaccines may be influenced by Tregs, induced by the vaccination itself, by closely related pathogens, or induced by unrelated pathogens (figure 1B). *M. bovis* BCG, the only available vaccine against TB, is a live bacterial vaccine aimed at inducing effective T-cell responses, yet BCG itself also induces Tregs [5]. This ability to induce Tregs could limit its ability to induce optimal protective immunity against TB; it is, however, conceivable that future medicine may be able to tailor BCG-induced Tregs to regulate hyperinflammation.

Tregs induced by vaccination: *M. bovis* bacillus Calmette-Guérin

Bacillus Calmette-Guérin, the only licensed vaccine against TB since 1921, was derived from virulent *M. bovis* by years of continuous *in vitro* passage. Estimates are that BCG has been given >3 billion times since its introduction, and it is part of the WHO Expanded Programme on Immunization (EPI). BCG was used in one of the first experiments establishing the idea of 'suppressor cells' interfering with control of infection: transfer of thymocytes from BCG-immunized rats suppressed immune responses in naive recipient rats against new BCG infection [147]. Though BCG-vaccination induces $CD4^+$ and $CD8^+$ effector T-cell responses in newborns [148;149] and protects them from disseminated forms of disease, it does not induce consistent protection against pulmonary TB, especially in adults [150]. We have previously hypothesized that one explanation for this lack of protection is the induction of Tregs by the vaccine among various other hypotheses [5]. In a large cohort of 5675 South-African infants who had been vaccinated at birth, stimulation of whole blood with mycobacterial antigens at 10 weeks of age resulted in production of IFNγ or IL-10, but not both [151]. $CD4^+CD25^+$ Treg cells were demonstrated in another study in BCG-vaccinated infants, and depletion of these Treg cells resulted in lower IL-10 levels in PPD-stimulated cell cultures [152]. IL-10-producing $CD4^+$ T-cells have been demonstrated in previously BCG-vaccinated adult donors, and *in vitro* suppression of target cell proliferation could be reversed by a blocking αIL-10-antibody [153].

$CD8^+$ Tregs are generally less studied compared to $CD4^+$ Tregs, especially in infectious diseases [5]. We have previously studied the presence, phenotype, and suppressive activity of $CD8^+$ Treg cells among live BCG-stimulated PBMCs of *in vitro* PPD-responsive donors. Surprisingly, we found a significantly higher expression of regulatory markers on live (but not killed) BCG-activated $CD8^+$ T-cells compared to $CD4^+$ T-cells, and there was significant enrichment of $CD8^+$ Treg cells within the BCG-activated $CD25^+$ T-cell compartment [154]. Also, suppressive activity was dominantly present in live BCG-activated $CD8^+$, but not in live BCG-activated $CD4^+$ T-cells [154]. $CD8^+$ Treg cells isolated from live BCG-stimulated PBMCs were enriched for expression of LAG-3 and CCL4, co-expressed CD25 and Foxp3, and inhibited Th1 cell proliferation [109]. Inhibition was partly mediated by secretion of CCL4, which reduced $Ca2^+$-influx early after TCR triggering [109]. We have additionally described expression of CD39 on live BCG-activated $CD8^+$ Treg cells, and a direct involvement of CD39 in mediating suppression by $CD8^+$ Tregs, as both the chemical CD39 antagonist ARL 67156 and a blocking αCD39-antibody were able to partly inhibit the suppressive activity of $CD8^+CD39^+$ Tregs [155]. Of note, $CD8^+$ Tregs could only be demonstrated in donors primed *in vivo* with mycobacteria, indicating a memory recall response following *in vitro* BCG-stimulation. Taken together, our work identified at least two different mechanisms by which BCG-activated $CD8^+$ Tregs could inhibit Th1 responses, via CCL4 and via CD39. Despite the above findings and despite the fact that CD8 was originally identified as a marker of Treg cells, then coined T-suppressor cells, pathogen-activated $CD8^+$ Tregs still remain significantly understudied compared to $CD4^+$ Tregs. It is important to note here that *in vitro* stimulation with live BCG preferentially activated $CD8^+$ Tregs [154], while stimulation with killed BCG (or PPD) seems to activate different populations.

Tregs induced by new TB-candidate vaccines

Regulatory T-cell induction has been demonstrated in several TB-vaccine candidate trials. After M72/AS01-vaccination of South-African healthy adults, Tregs expanded concurrently with cytokine-producing pro-inflammatory $CD4^+$ T-cells [156]. Circulating $CD4^+CD25^+$ $Foxp3^+$ T-cells were demonstrated after vaccination with another TB-vaccine candidate, modified vaccinia Ankara-85A (MVA85A). Interestingly, $CD4^+CD25^+Foxp3^+$ T-cells were increased in recipients with low antigen 85A-specific IFNγ-responses compared to high IFNγ-responders [157]. Also, the frequency of $CD4^{+}CD25^{+}CD39^{+}$ T-cells was inversely related to IL-17A production *in vitro* [139]. IL2RA mRNA expression on the day of vaccination and CTLA-4 expression 2 days after vaccination inversely correlated with the magnitude of the IFNγ ELISpot response induced by MVA85A-vaccination in healthy British adults, pointing to a possible role for Tregs very early or even before vaccination [157]. In African infants vaccinated with MVA85A, an early and strong innate response was associated with enhanced IFNγ ELISpot responses; thus, the authors concluded that Treg modulation of vaccine responses could differ between populations, and that more research is needed to explain these differences and the impact on vaccine efficacy [158]. Assessment should, however, include possible dissimilarities between long-term effects of Tregs and early after vaccination.

Other Tregs can modulate TB-vaccine-induced responses

Regulatory T-cells induced by other microbes can likely alter immunogenicity of TB vaccines. Exposure to environmental mycobacteria may decrease TB-vaccine efficacy through cross-reaction of antigens [94]. Pre-existing immune responses can either ‘block’ or ‘mask’ the BCG-induced immune response, possibly explaining the decreased vaccine efficacy of BCG in developing countries, where there is a higher prevalence of environmental mycobacteria [159]. Another potential explanation for decreased vaccine efficacy is induction of Tregs by environmental mycobacteria [160]. Priming mice with *M. chelonae* before BCG-vaccination increased Foxp3 expression on BCG-specific $CD4^{+}CD25^{+}$ T-cells compared to non-sensitized mice, and $CD4^{+}CD25^{+}$ T-cells of sensitized mice decreased immune responses *in vitro* [161]. Adoptive transfer of $CD4^{+}CD25^{+}$ T-cells into naive mice suppressed IL-2 production in the lungs, and enhanced IL-10 after BCG-vaccination [161]. Suppression after murine sensitization was reversed by a blocking αCD25-mAb during challenge, indicating active involvement of cross-reactive Tregs during vaccination [162].

Modulation of DC TLRs by helminth molecules leads to increased Th2 and Treg responses, which possibly decreases vaccine efficacy in developing countries, where also the majority of the one billion helminth-infected people live [12]. Tregs induced by helminths in mucosa-associated lymphoid tissue (MALT) may migrate to other sites, exerting non-specific suppressive effects and preventing clearance of Mtb at distant sites as well [163]. Although the frequency of $CD4^{+}Foxp3^{+}CD25^{HI}$ T-cells was similar in helminth-infected and non-infected Indonesian children, BCG-specific (and as mentioned, also *Plasmodium falciparum*-specific) proliferative responses were increased after depletion of $CD4^{+}CD25^{HI}$ T-cells in helminth-infected children only, pointing to differences in suppressive capacity induced by helminth infection [11]. Deworming increased BCG immunogenicity *in vivo* and was accompanied by changes in TGF-β, but notably not by changes in Th2 cytokines [164].

Modulating the modulators: future prospects for Tregs in TB-vaccination

The ability of BCG to induce Tregs may in the future be exploited to benefit the human host in the contexts of auto-immune and/or hyper-inflammation-related diseases. This has been noted in a murine model of Parkinson's disease, where protection against nerve damage was induced by BCG-vaccination through Tregs [165]. Also in experimental auto-immune encephalomyelitis, myelin oligodendrocyte glycoprotein-specific IFNγ-producing $CD4^{+}$ T-cells, and both specific and non-specific $CD4^{+}IL\text{-}17^{+}$ T-cells in the CNS, were suppressed by cerebral BCG infection [166]. Other murine studies have demonstrated BCG-induced suppression of asthma responses and dampening of colitis [167;168]. Further research will hopefully elucidate if and how these findings can be translated to the human situation.

Interestingly, mucosal vaccination of macaques with a vaccine consisting of inactivated simian immunodeficiency virus (SIV) and a live bacterial adjuvant (BCG or *Lactobacillus*) generated HLA-E restricted, non-cytolytic $CD8^{+}$ Tregs [169]. After challenge with SIV infection, these $CD8^{+}$ Tregs suppressed proliferation of infected $CD4^{+}$ T-cells, thereby protecting almost all vaccinated macaques for up to 4 years after vaccination [169]. As mentioned, in acute viral infection, Tregs could have a beneficial role to play, such as in acute SIV/HIV infection where Tregs decrease proliferation of infected cells at mucosal surfaces [44].

In a TB-vaccination context, however, it may be crucial to avoid excessive Treg induction by the vaccine. Analogous to the reduced burden of TB observed in mice following treatment with chemical compounds inhibiting Treg and Th2 induction prior to infection [170], a similar approach was tested in murine BCG-vaccination: chemical inhibition of Treg induction increased BCG-mediated protection against pulmonary TB in mice and favored central-memory T-cell induction (long-lived vaccine responses) [171]. Blocking the IL-10-receptor with an αIL-10-receptor antibody increased BCG-induced Th1, Th17, innate lymphoid IFNγ, and IL-17 responses in mice, leading to enhanced protection against TB [172]. An additional, important role for IL-22 producing NK cells through lysing of $CD4^+$ Tregs was described, and addition of IL-22 also increased Th1 vaccine-induced responses [173]. In contrast, only moderate efficacy of treatment with a blocking αCD25-antibody on BCG-vaccine efficacy was described [174]. It is possible that blocking CD25 results in partial Treg depletion while other Treg subsets could survive during such treatment. However, CD25 is expressed also by activated T-helper cells such that CD25-depletion may additionally also deplete essential effector cells of protective immunity. Regardless, even after selective deletion of all $Foxp3^+$ cells, homeostatic expansion may occur from a small subset of remaining Tregs [175]. Since various Treg marker-expressing subsets exist, this points to the importance of assessing the dynamics and fluidity of various subsets within the Treg compartment, in order to improve vaccine design by effective modulation of Treg activity and function. Compounds inhibiting Treg induction or blocking 'upstream' signaling through the IL-10-receptor could improve vaccine efficacy. Other options would include the addition of adjuvant antagonists of chemokine receptors expressed by Tregs, as described for a CCR4 antagonist that blocked $CD4^+$ Tregs and increased *in vitro* responses to MVA85A and recombinant HBV surface antigen vaccination [176], or the inclusion of TLR-agonists combined with agents selectively blocking TLR-induced anti-inflammatory signaling pathways in DCs [177]. Future studies may integrate these findings to increase TB-vaccine-induced protective immunity through manipulation of the manipulators, and hopefully translate these findings ultimately to the human situation.

Concluding remarks and future directions

For many pathogens, induction, expansion, recruitment, or inhibition of Tregs has been demonstrated. *Mycobacterium leprae* and *Mycobacterium tuberculosis* are master manipulators of human immunity and are able to establish chronic infection among others by activating immune regulation. The effects of Tregs impact on clinical symptoms and performance of immunodiagnostic assays, differ in acute vs. chronic diseases and can suppress protective immunity and vaccine immunogenicity. Importantly, this can partly be the result of cross-suppression from Tregs induced by unrelated pathogens, possibly even by non-pathogenic microbes. This is particularly important in endemic settings, e.g., settings endemic for both helminths, TB, malaria, and HIV.

Through precisely (and timely) targeted Treg manipulation, vaccine-induced protective immunity may be enhanced. Most data are necessarily derived from murine studies, and need to be translated to the human situation. This should also offer opportunities for new immunotherapeutic vaccines for the treatment of inflammatory disorders, e.g., auto-immune diseases, and for the design of vaccines aimed at interfering with acute (viral) infection. Through manipulating the manipulators, increased immunity against infectious diseases may be achieved.

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CHAPTER 3

CD8$^+$ regulatory T-cells, and not CD4$^+$ T-cells, dominate suppressive phenotype and function after *in vitro* live *Mycobacterium bovis*-BCG activation of human cells

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Abstract

Mycobacterium bovis bacillus Calmette-Guérin (*M. bovis* BCG), the only currently available vaccine against tuberculosis, has been reported to induce regulatory T-cells in humans. The activity of regulatory T-cells may not only dampen immunogenicity and protective efficacy of tuberculosis vaccines, but also hamper diagnosis of infection of tuberculosis, when using immune (e.g. IFNγ-release) assays. Still, in settings of infectious diseases and vaccination, most studies have focused on $CD4^+$ regulatory T-cells, and not $CD8^+$ regulatory T-cells. Here, we present a comparative analysis of the suppressive phenotype and function of $CD4^+$ versus $CD8^+$ T-cells after *in vitro* live BCG activation of human cells. Moreover, as BCG is administered as a (partly) live vaccine, we also compared the ability of live versus heatkilled BCG in activating $CD4^+$ and $CD8^+$ regulatory T-cell responses. BCG-activated $CD8^+$ T-cells consistently expressed higher levels of regulatory T-cell markers, and after live BCG activation, density and (co-)expression of markers were significantly higher, compared to $CD4^+$ T-cells. Furthermore, selection on CD25-expression after live BCG activation enriched for $CD8^+$ T-cells, and selection on co-expression of markers further increased $CD8^+$ enrichment. Ultimately, only T-cells activated by live BCG were functionally suppressive and this suppressive activity resided predominantly in the $CD8^+$ T-cell compartment. These data highlight the important contribution of live BCG-activated $CD8^+$ Treg cells to immune regulation and emphasize their possible negative impact on immunity and protection against tuberculosis, following BCG-vaccination.

Introduction

Tuberculosis (TB), one of the major global health challenges, accounted for 1.3 million deaths in 2012. It is estimated that one-third of the world population is (latently) infected with *Mycobacterium tuberculosis* (Mtb) [1]. Containment of the disease is dependent on innate and adaptive immune responses, and though $CD4^+$ Th1 (IFNγ)-responses are considered quintessential, definition of immunological correlates of protection remains unresolved.

Active TB disease has been associated with decreases in Mtb-specific IL17A-producing $CD4^+$ T-cells [2] and in multifunctional ($IFN\gamma^+IL2^+TNF\alpha^+$) $CD4^+$ T-cells [3]. Conversely, $CD4^+$ T-cells single-positive for TNFα were identified as a strong classifier of active disease versus latent infection [4]. For Mtb-specific $CD8^+$ T-cells, a reduction in dual $IFN\gamma^+IL2^+$-secreting cells in active vs. latent TB [LTBI] [5], as well as changes in memory phenotype [5;6], have been reported. $CD8^+$ T-cells preferentially recognized heavily infected cells *in vitro* [7]; and higher Mtb-specific $CD8^+$ responses correlated with clinical parameters of bacterial load (defined as smear-positive vs. smear-negative TB) [6].

Mtb-specific immune responses and mycobacterial growth inhibition can be suppressed by circulating, alveolar and pleural $CD4^+$ regulatory T-cells (Treg cells) in humans [8-10]. Recently, also suppression of T-cell cytokine production and proliferation by myeloid-derived suppressor cells in TB-patients were described [11]. Although $CD8^+$ regulatory T-cells have been reported in TB [9] and leprosy [12,13], they remain generally understudied compared to $CD4^+$ Treg cells [14]. $CD4^+$ T-cells producing IL-10 were shown to hamper clinical diagnosis based on dermal reactivity to mycobacterial purified protein derivative (PPD) in anergic TB-patients [15]. In PBMCs isolated from patients with active TB, depletion of $CD4^+CD25^+$ or $CD4^+CD25^+CD39^+$ T-cells increased Mtb-specific IFNγ production [8,16]. Healthy, previously BCG-vaccinated volunteers, who were vaccinated with MVA-85A (modified vaccinia virus Ankara expressing antigen 85A), and who exhibited relatively low responses in antigen 85A-specific IFNγ ELISPOTs, had increased frequencies of circulating $CD4^+CD25^+Foxp3^+$ cells, compared to high IFNγ-responders [17]. Also, MVA85A-induced production of IL17A was affected by Treg responses [18,19]. IFNγ- and IL17-responses were enhanced by addition of ARL67156 [19], a chemical inhibitor of CD39 [20], suggesting a population of $CD39^+$ cells that actively

dampened cytokine production. Thus, ($CD4^+$) Treg cells can negatively influence immunity and immune dependent protection, both in natural infection and in vaccination settings.
The only currently available vaccine against TB, *Mycobacterium bovis* bacillus Calmette-Guérin (*M. bovis* BCG), induces $CD4^+$ and $CD8^+$ T-cell responses in newborns [21-23] and protects them from disseminated forms of disease; but it does not induce consistent protection against pulmonary TB, especially in adults [24]. One explanation for this lack of protection is the induction of regulatory T-cells by the vaccine [14;25], amongst other hypotheses [26;27]. $CD4^+CD25^+$ Treg cells have been found after BCG-vaccination of newborns [28] and adults [29], and $CD4^+CD25^+$-depleted T-cell cultures resulted in lower PPD-stimulated IL-10 levels [28]. We previously demonstrated the presence and strong suppressive activity of $CD8^+$ Treg cells among live BCG-stimulated PBMCs of *in vitro* PPD-responsive donors, which were enriched for the markers lymphocyte activation gene-3 (LAG-3) [30] and CD39 [31]. Suppressive activity of $CD8^+$ Treg cells could be reversed by blocking CC chemokine ligand 4 (CCL-4) [30], membrane-bound TGFβ (mTGFβ) [32] and CD39 [31]. Still, knowledge about $CD8^+$ regulatory T-cells is generally limited compared to $CD4^+$ Treg cells.
Furthermore, though multiple mycobacterial-activated Treg subsets, either $CD4^+$ or $CD8^+$, have been demonstrated in humans, no comparative studies have been performed assessing suppressive capacity of *Mycobacterium*-induced $CD4^+$ vs. $CD8^+$ T-cells. In this study, we compared the suppressive phenotype and function of human BCG-activated $CD4^+$ and $CD8^+$ T-cells. We demonstrate significantly higher expression of regulatory markers on live BCG-activated $CD8^+$ T-cells, compared to $CD4^+$ T-cells, and enrichment for $CD8^+$ Treg cells within the BCG-activated $CD25^+$ T-cell compartment. Finally, suppressive Treg activity was dominantly present in live BCG-activated $CD8^+$, but not in live BCG-activated $CD4^+$ T-cells, nor in killed BCG-activated T-cells.

Materials and Methods

Ethics statement. All donors had signed consent for scientific use of blood products. Blood products were collected anonymously, which, according to institutional ethical policy, does not require a separate review by the Ethical Committee.

Blood samples. Anonymous buffy coats were collected from healthy adult blood bank donors (Sanquin, Leiden). PBMCs were isolated by density centrifugation and cryopreserved in fetal calf serum-supplemented medium according to Standard Operating Procedure [33]. Cells were counted using the CASY cell counter (Roche, Woerden, the Netherlands). Donors were selected on recognition of mycobacterial PPD by assessing IFNγ production *in vitro*. PBMCs were stimulated with 5 μg/ml PPD (Statens Serum Institute, Copenhagen, Denmark) for 6 days and supernatants were tested in IFNγ-ELISA (U-CyTech, Utrecht, the Netherlands). Positivity was defined as IFNγ production ≥ 150 pg/ml.

Cell cultures and BCG infection. PBMCs were thawed (64% median viable cell yield) and stimulated with Bacillus Calmette-Guerin (Pasteur). BCG was grown in 7H9 plus ADC, frozen in 25% glycerol and stored at -80^{0}C. Before use, bacteria were thawed and washed in PBS/0.05% Tween 80 (Sigma-Aldrich, Zwijndrecht, the Netherlands). Infections were done at a multiplicity of infection (MOI) of 1.5. For heatkilled BCG-stimulated cell cultures, bacteria were inactivated at 80^{0}C for 30 minutes. PBMCs were cultured for six days in Iscove's modified Dulbecco's medium (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) supplemented with 10% pooled human serum. Sera were pretested in standardized protocols; only sera were pooled that had no inhibitory activity in standard mixed allogeneic lymphocyte cultures. IL-2 (25U/ml; Proleukin; Novartis Pharmaceuticals UK Ltd., Horsham, UK) was added after 6 days of culture. CD4^{+} and CD8^{+} T-cells were enriched by positive selection using magnetic beads (MACS, Miltenyi Biotec, Teterow, Germany). Purity of sorts was ≥ 97% as assessed by flowcytometry.

Restimulation of CD4^{+} cell lines was done in 24 well plates (2 x 10^{5} cells/w) with αCD3/CD28 beads (Dynabeads Human T-activator, Life Technologies-Invitrogen) and IL2 (25 U/ml). Pooled, irradiated (30 Gy) PBMCs were added as feeders (1 x 10^{6} cells/w).

$CD8^+$ cell lines were restimulated in 96 well roundbottom plates (1 x 10^4 cells/w) with αCD3/CD28 beads, IL2 (50 U/ml), IL7, IL15 (both 5 ng/ml, Peprotech, Rocky Hill, NJ, USA) and pooled, irradiated (30 Gy) PBMCs added as feeders (5 x 10^4 cells/w). Cells were maintained in IL2 (100 U/ml).

FACS analysis. PBMCs were incubated overnight with αCD3/28 beads, for the last 16 hours Brefeldin A (3 µg/ml, Sigma-Aldrich) was added. The following antibodies were used for surface staining: CD8-HorizonV500 (clone RPA-T8), CD3-PeCy5 (clone UCHT-1), CD4-PerCPCy5.5 (clone RPA-T4) (all BD Biosciences, Eerembodegem, Belgium), and CD39-PE (clone A1; Biolegend, London, U.K.). For intracellular staining we used the FIX&PERM® Cell Permeabilization Kit from An Der Grub BioResearch GMBH (Susteren, The Netherlands) and the following antibodies: CCL4-FITC (clone 24006; R&D Systems, Abingdon, UK), Foxp3-Alexa Fluor 700 (clone PCH101; eBioscience, Hatfield, UK), CD25-allophycocyanin-H7 (clone M-A251; BD Biosciences) and LAG-3-atto 647N (clone 17B4; ENZO Life Sciences, Antwerp, Belgium). Samples were acquired on a BD LSRFortessa using FACSDiva software (version 6.2, BD Biosciences) using compensated parameters.

Analysis was performed using FlowJo software (version 9.5.3, Treestar, Ashland, OR, USA). The detailed gating strategy is demonstrated in figure 1A. Cut-off for positive populations of interest was defined by comparison to samples of cell lines not stimulated with BCG (Supplementary figure S1) and were similar for $CD4^+$ and $CD8^+$ T-cell populations, as shown in figure 1A. Also, to assess differences in intrinsic frequency and density (MFI) of Treg-cell marker expression on $CD4^+$ vs. $CD8^+$ T-cells, positive Treg marker populations on $CD4^+$ and $CD8^+$ T-cells were compared in samples not stimulated with BCG.

Suppression assays. Cell lines were tested for their capacity to inhibit proliferation of a Th1 responder clone (Rp15 1-1) in response to its cognate *M. tuberculosis* hsp65 p3–13 peptide presented by HLA-DR3 positive, irradiated (20 Gy) PBMCs. Proliferation was measured after three days of co-culture by addition of (3H)thymidine (0.5 µCi/well) and incorporation was assessed after 18 hours. Background proliferation was assessed by adding T-cells without the peptide. Validation of this co-culture assay has been reported previously [30-32;34].

Proliferation was divided by Th1 reporter clone proliferation in the absence of Treg cells to obtain relative proliferation and enable analysis across experiments. Values represent means from triplicate wells, that were subsequently averaged for repeated experiments per donor. Demonstrated values represent pooled data from different donors. Raw data can be provided per request.

Statistical analyses. Wilcoxon signed-rank tests were performed using GraphPad Prism (version 6, GraphPad Software, San Diego, CA, USA) and SPSS statistical software (version 20, SPSS IBM, Armonk, NY, USA).

Laboratory. Studies were conducted in a laboratory guided by exploratory research principles with established Standard Operating Procedures [33].

Results

Heatkilled vs. live BCG-activated expression of Treg-cell markers on CD4$^+$ and CD8$^+$ T-cells

PBMCs were isolated from healthy human donors that had been selected based on their *in vitro* response to mycobacterial PPD as described before [30;31;35]. The PBMCs were stimulated with heatkilled or live BCG, and CD4$^+$ and CD8$^+$ T-cells were analysed for regulatory T-cell marker expression after six days. Figure 1A depicts the full gating strategy, and an example of the synchronized gating on a positive population for CD4$^+$ and CD8$^+$ T-cells, in compliance with MIATA guidelines [36]. Background expression of Treg-cell markers was compared between CD4$^+$ and CD8$^+$ populations of samples that were not stimulated with BCG (Supplementary figure S1); only the background expression of CCL4 on CD8$^+$ T-cells was significantly higher compared to CD4$^+$ T-cells (median 11% vs. 2%; $p < 0.01$; Wilcoxon signed-rank test) [36]. Heatkilled, as well as live BCG stimulation, activated expression of regulatory T-cell markers on CD4$^+$ and CD8$^+$ T-cells of *in vitro* PPD-responsive donors, including CD25, Foxp3, LAG-3 and CD39 (figure 1B).

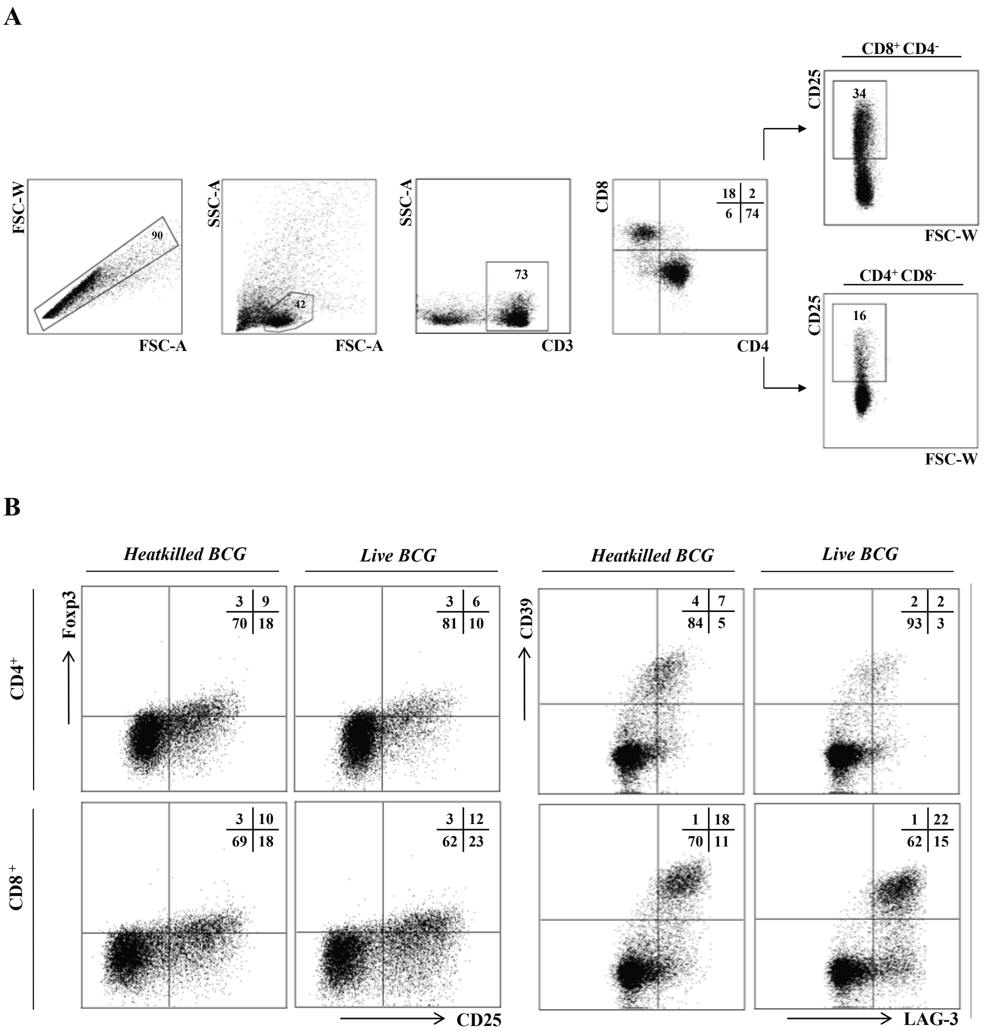


Figure 1. Heatkilled vs. live BCG-activated expression of Treg-cell markers on CD4$^+$ and CD8$^+$ T-cells.

A: Gating strategy: cells were gated on single cells, live lymphocytes, CD3$^+$ and CD4$^+$CD8$^-$ vs. CD4$^-$CD8$^+$. Demonstrated is the synchronized gating on the positive population of interest for CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ T-cells; here the CD25-positive population. B: Heatkilled and live BCG activate CD25$^+$Foxp3$^+$ and LAG-3$^+$CD39$^+$ T-cells. Expression of regulatory T-cell markers on CD4$^+$ and CD8$^+$ T-cells of *in vitro* PPD-responders was analysed by flowcytometry six days after heatkilled or live BCG stimulation. For each donor gating was compared to samples not stimulated with BCG (demonstrated in Supplementary figure S1). Data are representative of seven responders.

Treg-cell marker frequency and density are increased on live BCG-activated $CD8^+$ vs. $CD4^+$ T-cells

Heatkilled and live BCG activated a higher percentage of total $CD8^+$ T-cells, compared to $CD4^+$ T-cells, that expressed CD25, Foxp3, CD39, LAG-3 or CCL4, depicted in figure 2A as frequency of ($CD8^+$ or $CD4^+$) parent. Live BCG-activated $CD8^+$ T-cells exhibited significantly increased Treg-cell marker frequencies compared to live BCG-activated $CD4^+$ T-cells ($*p < 0.05$; Wilcoxon signed-rank test).

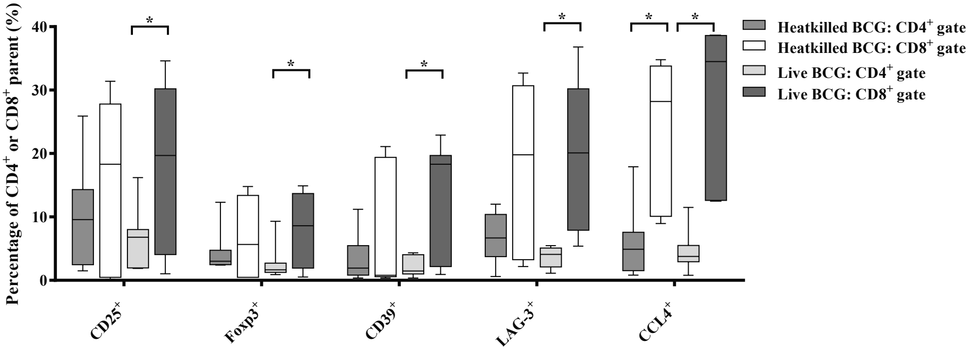
To determine cellular densities of expression of Treg-cell markers, mean fluorescence intensities (MFIs) of positive populations were compared for BCG-activated expression of CD25, Foxp3 and CD39. MFIs of CD25 and CD39 were significantly higher on live BCG-stimulated $CD8^+$ T-cells, compared to $CD4^+$ T-cells (figure 2B; $p = 0.02$ and $p = 0.03$, respectively; Wilcoxon signed-rank test), whereas MFIs of heatkilled BCG-activated $CD4^+$ T-cells did not differ from heatkilled BCG-activated $CD8^+$ T-cells (data not shown).

Co-expression of multiple Treg-cell markers enriches for $CD8^+$, and not $CD4^+$ T-cells

Co-expression of multiple Treg-cell markers by live BCG-induced T-cells was analysed using Boolean gating (figure 3A). A significantly higher percentage of total $CD8^+$ T-cells was $CD25^+Foxp3^+$, compared to $CD4^+$ T-cells ($p = 0.02$; Wilcoxon signed-rank test). Also, the percentage of total $CD8^+$ T-cells co-expressing CD25, Foxp3, CD39, LAG-3 and/or CCL4 in various combinations was significantly higher compared to $CD4^+$ T-cells ($p < 0.01$, Wilcoxon signed-rank test).

To determine the relative distribution of $CD4^+$ and $CD8^+$ T-cells within the Treg-cell marker positive T-cells, we applied Boolean gating to the total $CD3^+$ population (figure 3B, upper panel), and the $CD8^+$ proportion was calculated for the $CD3^+$ Boolean gates (figure 3B, lower panel). Gating BCG-activated T-cells on expression of CD25 or Foxp3, enriched for $CD8^+$ T-cells compared to the total $CD3^+$ population ($p = 0.03$ and $p = 0.06$, respectively; Wilcoxon signed-rank test). Increasing selection of total BCG-activated T-cells on regulatory T-cell markers further enriched for $CD8^+$ T-cells significantly ($p = 0.01$ for $CD25^+Foxp3^+CD39^+LAG\text{-}3^+CCL4^+$ T-cells, Wilcoxon signed-rank test).

A



B

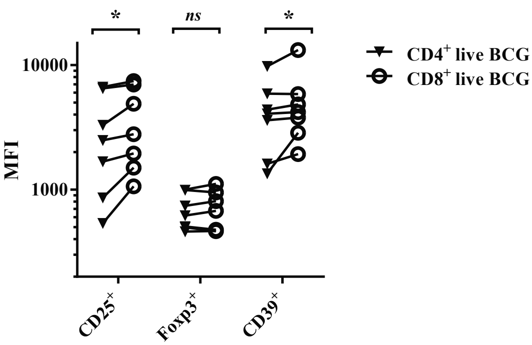


Figure 2. Treg-cell marker frequency and density are increased on live BCG-activated CD8⁺ vs. CD4⁺ T-cells. A: BCG induces Treg-cell marker expression on CD4⁺ and CD8⁺ T-cells; after live BCG stimulation the percentage of total CD8⁺ T-cells expressing CD25, Foxp3, CD39, LAG-3 or CCL4 is significantly higher compared to CD4⁺ T-cells, depicted here as frequency of CD8⁺ or CD4⁺ population. Differences in Treg marker expression between heatkilled BCG-activated CD8⁺ vs. CD4⁺ T-cells were not significant, except for expression of CCL4; CCL4 expression was also significantly higher on CD8⁺ T-cells compared to CD4⁺ T-cells in samples not stimulated with BCG (Supplementary figure S1) (*p < 0.05, Wilcoxon signed-rank test). B: Mean fluorescence intensities (MFIs) of CD25 and CD39 are increased on live BCG-activated CD8⁺ T-cells as compared to CD4⁺ T-cells. Gating was performed as demonstrated in figure 1A. To assess differences in intrinsic intensity of expression on CD4⁺ and CD8⁺ T-cells, respectively, MFIs of positive Treg marker populations in samples not stimulated with BCG were compared; this was similar on CD4⁺ and CD8⁺ T-cells for MFIs of CD25, Foxp3 and CD39. Data are representative of seven *in vitro* PPD-responders six days after heatkilled or live BCG stimulation (*p < 0.05; Wilcoxon signed-rank test).

Suppressive activity resides predominantly in live BCG-activated CD8$^+$ T-cells

T-cell lines were tested for their capacity to suppress proliferation of an unrelated CD4$^+$ T helper-1 clone. This responder clone recognizes a cognate peptide presented in the context of HLA-DR3 in an assay which has been previously reported and validated [30-32;34]. Heatkilled BCG-activated T-cells did not suppress proliferation of the responder clone. In contrast, live BCG-stimulated T-cells exhibited suppressive activity towards the same responder clone (figure 4A). CD8$^+$ / CD4$^+$ ratios of heatkilled BCG-stimulated and live BCG-stimulated T-cell lines were 0.06 and 0.1, respectively (Supplementary figure S2), suggesting a potential association of T-cell subset distribution with suppressive function.

We next separated live BCG-activated T-cell lines into CD4$^+$ or CD8$^+$ expressing populations using magnetic beads (purity ≥ 97% as assessed by flowcytometry), and tested live BCG-activated CD4$^+$ T-cells in parallel with live BCG-activated CD8$^+$ T-cells for their suppressive capacity. Live BCG-activated CD8$^+$ T-cells suppressed T helper-1 clone proliferation, whereas live BCG-activated CD4$^+$ T-cells did not significantly inhibit proliferation. Thus, suppressive activity was dominant in live BCG-activated CD8$^+$ T-cells, compared to live BCG-activated CD4$^+$ T-cells (figure 4B; $p < 0.001$; Wilcoxon signed-rank test).

Discussion

In this study, we present a comparative analysis of the suppressive phenotype and function of BCG-activated CD4$^+$ vs. CD8$^+$ T-cells. CD8$^+$ T-cells consistently expressed higher levels of regulatory T-cell markers compared to CD4$^+$ T-cells; also the cellular density of expression and co-expression of these markers were significantly higher. Selection of T-cells based on CD25-positivity after live BCG-activation also enriched for CD8$^+$ T-cells, and further selection on co-expression of combined regulatory markers further supported CD8$^+$ enrichment. Suppressive Treg activity was dominantly present in live BCG- but not heatkilled BCG-activated T-cells; finally, the suppressive activity largely resided in the CD8$^+$ T-cell- and not the CD4$^+$ T-cell-population.

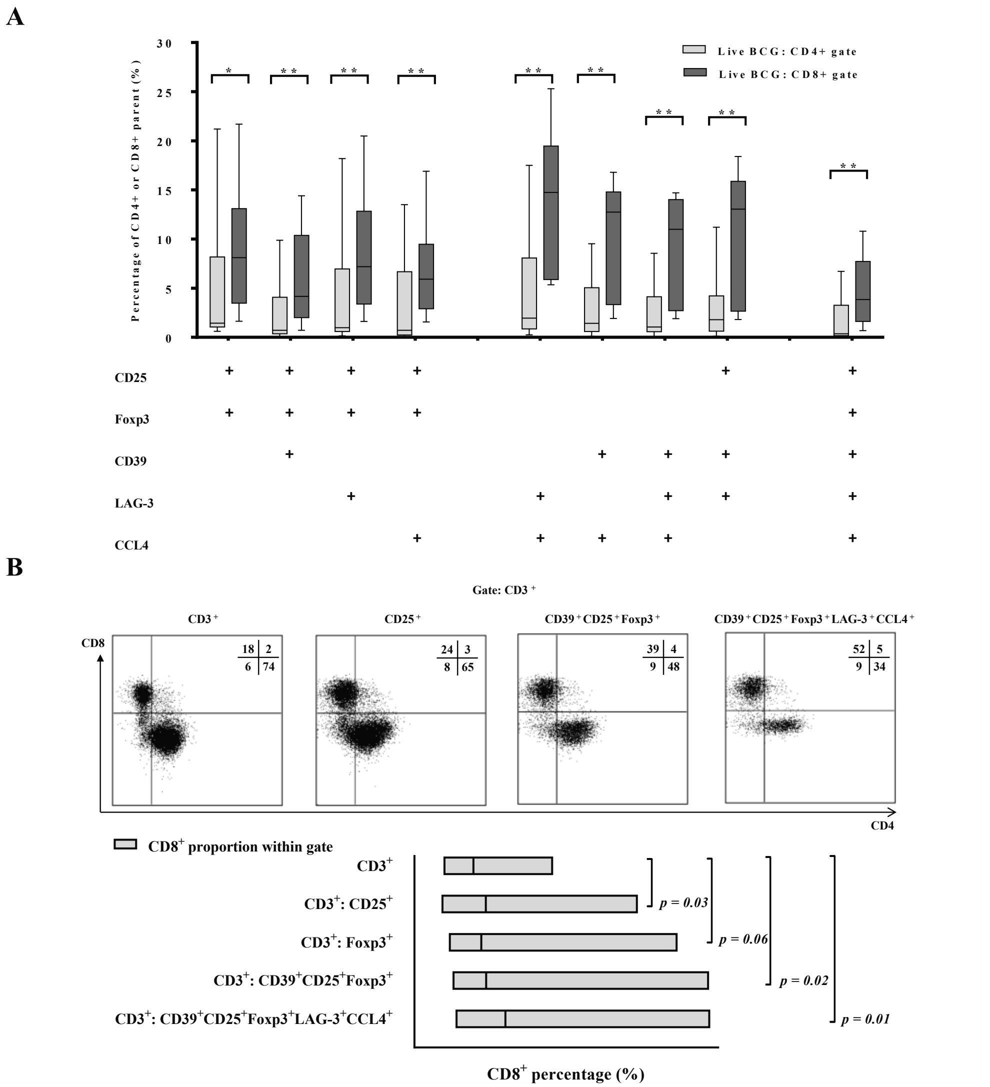
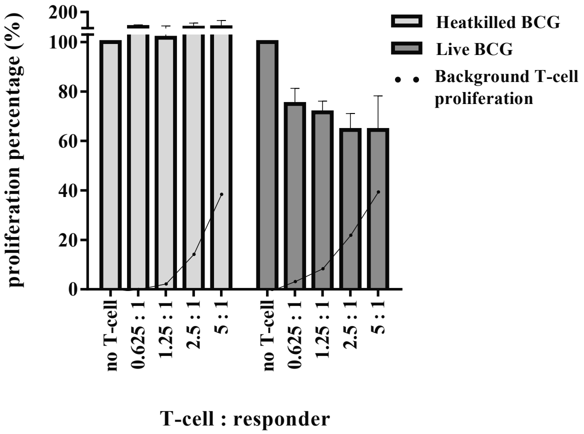


Figure 3. Co-expression of multiple Treg-cell markers enriches for CD8$^+$, and not CD4$^+$ T-cells. A: The percentage of total CD8$^+$ T-cells co-expressing CD39, LAG-3, CCL4, CD25 and/or Foxp3 in different combinations is significantly increased, compared to CD4$^+$ T-cells. Demonstrated is a combined analysis using Boolean gating of cells from ten donors six days after live BCG infection. Gating was performed as in figure 1A. Boxes: 25th to 75th percentiles; line at median; whiskers: minimum to maximum (*p<0.05, **p< 0.01; Wilcoxon signed-rank test). B: Combining Treg markers enriches for CD8$^+$ T-cells as opposed to CD4$^+$ T-cells. Boolean gating was performed on CD3$^+$ T-cells of ten donors; CD8 vs. CD4 gating is demonstrated (top) and the CD8$^+$ proportion of these gated populations is demonstrated (bottom) for a selection of CD3$^+$ Boolean gates. The CD8$^+$ proportion increased significantly using a combination of Treg markers as compared to the complete CD3$^+$ population. Boxes: minimum to maximum, line at median (Wilcoxon signed-rank test).

Multiple CD4$^+$ Treg-cell marker expressing subsets have been demonstrated in patients with tuberculosis [9;10] and after vaccination with MVA85A [18] and BCG [29]. We previously demonstrated the suppressive activity of CD8$^+$LAG-3$^+$CCL4$^+$ and CD8$^+$CD39$^+$ Treg cells, isolated from live BCG-stimulated PBMCs; in those studies, we also observed upregulation of these markers in the CD4$^+$ compartment [30;31]. However, also non-suppressive, activated human CD4$^+$ T-cells may transiently upregulate Foxp3-expression such that *in vitro*-induced Foxp3 expression by human CD4$^+$ T-cells is not necessarily associated with suppressive function [37]. The co-expression of multiple Treg-cell markers can more reliably and specifically identify human Treg cells. In the current study we found that more stringent selection of total BCG-activated T-cells using multiple Treg cell markers further enriched for CD8$^+$ T-cells significantly. In other work using allo-antigen induction of Treg cells by plasmacytoid dendritic cells, also discrepant activation of CD8$^+$ vs. CD4$^+$ Treg cells has been reported: suppressive activity was mediated by CD8$^+$LAG-3$^+$Foxp3$^+$CTLA-4$^+$ T-cells, but not by plasmacytoid dendritic cell-induced CD4$^+$ T-cells [38]. However, no systematic comparative studies have been performed so far comparing suppressive capacity of *Mycobacterium*-induced CD4$^+$ vs. CD8$^+$ T-cells.

The type of antigen used for *in vitro* restimulation of specific responses may significantly influence the results, as stimulation with live mycobacteria could activate significantly different populations of T-cells as compared to killed mycobacteria or protein isolates like PPD. CD4$^+$ Treg cells have been isolated from PBMCs of active TB patients through *ex vivo* selection on co-expression of CD4 and CD25 [9;16;39], and have been phenotyped after culturing PBMCs with TB-specific peptides [16] or mycobacterial PPD [9]. PPD-stimulated PBMCs of TB patients revealed expansion of CD4$^+$CD25$^+$Foxp3$^+$ T-cells in active TB patients, but low numbers of CD8$^+$CD25$^+$Foxp3$^+$ T-cells [9]. In the current study, we compared live and heatkilled BCG, where heatkilled BCG was considered to be a primary stimulus for CD4$^+$ T-cells, through the MHC-II antigen presentation pathway, resembling PPD. It is intriguing, as demonstrated here, that CD8$^+$ Treg activity is specifically induced by live as opposed to heatkilled BCG, suggesting that the MHC-I antigen presentation pathway is involved in the activation of these cells, and also that cross-presentation of killed bacteria to CD8$^+$ T-cells is likely to be insufficient. We hypothesize that BCG, as a live intracellular bacterium, is able to modify antigen presentation/ stimulation, although the mechanisms and pathways involved remain unknown at this stage.

A



B

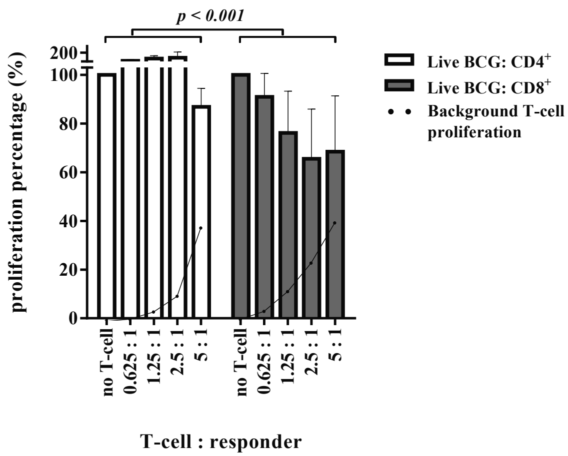


Figure 4. Suppressive activity resides predominantly in live BCG-activated CD8$^+$ T-cells.

Heatkilled BCG-activated and live BCG-activated T-cell lines were expanded, and live BCG T-cell lines were enriched for CD4 or CD8 expression using magnetic beads. Suppressive capacity was tested in a co-culture assay by titrating these T-cell lines onto a Th1 reporter clone that was stimulated with its cognate peptide [30;31]. Proliferation was measured by (3H)TdR incorporation after three days. Proliferation was divided by Th1 reporter clone proliferation in the absence of Treg cells to obtain relative proliferation as described previously [30-32]. Dotted lines represent background proliferation of T-cells, in the absence of reporter clone peptide, relative to Th1 clone proliferation. A: Suppressive activity was confined to live BCG-activated T-cells, and could not be demonstrated for heatkilled BCG-specific T-cells. Data are depicted as mean ± SE of five different heatkilled BCG-activated T-cell lines, and six live BCG-activated T-cell lines. B: Suppressive activity resides predominantly in CD8$^+$ T-cells, and not in CD4$^+$ T-cells (mean ± SE of CD4$^+$ and CD8$^+$ T-cell lines of three donors, tested in at least two independent assays; Wilcoxon signed-rank test, $p < 0.001$).

The relatively long persistence of BCG as a live intracellular bacterium in the human body after vaccination, as opposed to other vaccines, may be responsible for inducing increased $CD8^+$ (regulatory) T-cell responses over time, compared to $CD4^+$ T-cells. Dendritic cells in the skin could optimally cross-present [40] extracellular fragments of BCG after vaccination, further adding to $CD8^+$ T-cell priming by late cross-presentation. Additional research is needed to clarify the BCG-specific induction of Treg cells *in vivo* and to compare the magnitude and persistence of $CD4^+$ and $CD8^+$ T-cells prospectively, both early after BCG-vaccination as well as at later time points.

Studies analysing immune responses induced by Mtb-infection, TB disease or BCG-vaccination, may have largely overlooked the presence and role of $CD8^+$ Treg cells, which may be surprising, considering the initial identification of suppressor cells as $CD8^+$ T-cells [41], and the early cloning of $CD8^+$ suppressor T-cells in mycobacterial disease [12;13]. Immune based diagnosis of TB infection, such as tuberculin skin tests and IFNγ-release assays (IGRAs), vaccine immunogenicity, and perhaps also vaccine induced protection could all be negatively impacted upon by Treg activity [15-17]. More research into the induction and activity of Treg cells, and comparative analyses of subsets, could be important to optimal vaccine design as well as a better understanding of correlates of protection. Our study highlights the important contribution of live BCG-activated $CD8^+$ Treg cells to immune regulation, and emphasizes the possible negative impact of human $CD8^+$ regulatory T-cells on immunity to mycobacterial infection and vaccination.

Acknowledgements

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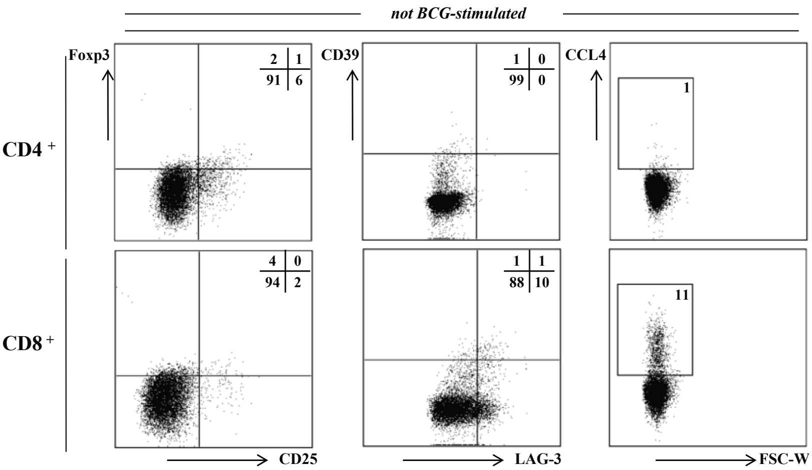
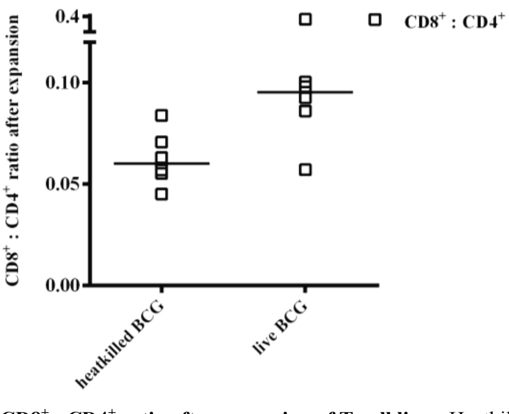
Supplementary information



Supplementary figure S1. Treg-cell marker expression in samples not stimulated with BCG. Positive populations for Treg-cell markers were defined by comparison with not BCG-stimulated samples for each donor. In the latter samples, only CCL4 expression was significantly higher on CD8$^+$ T-cells, compared to CD4$^+$ T-cells.



Supplementary figure S2. CD8$^+$: CD4$^+$ ratio after expansion of T-cell lines. Heatkilled and live BCG-activated T-cell-lines were expanded and CD4$^+$ and CD8$^+$ frequencies were assessed by flowcytometry. The median pre-expansion CD8$^+$: CD4$^+$ ratio was 0.3.

CHAPTER 4

CD39 is involved in mediating suppression by *Mycobacterium bovis* BCG-activated human $CD8^+CD39^+$ regulatory T-cells

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Abstract

Regulatory T (Treg) cells can balance normal tissue homeostasis by limiting inflammatory tissue damage, e.g. during pathogen infection, but on the other hand can also limit protective immunity induced during natural infection or following vaccination. Because most studies have focused on the role of $CD4^+$ Treg cells, relatively little is known about the phenotype and function of $CD8^+$ Treg cells, particularly in infectious diseases. Here, we describe for the first time the expression of CD39 (E-NTPDase1) on *Mycobacterium*-activated human $CD8^+$ T-cells. These $CD8^+CD39^+$ T-cells significantly co-expressed the Treg markers CD25, Foxp3, lymphocyte activation gene-3 (LAG-3), and CC chemokine ligand 4 (CCL4), and suppressed the proliferative response of antigen-specific $CD4^+$ T helper-1 (Th1) cells. Pharmacological or antibody mediated blocking of CD39 function resulted in partial reversal of suppression. These data identify CD39 as a novel marker of human regulatory $CD8^+$ T-cells and indicate that CD39 is functionally involved in suppression by $CD8^+$ Treg cells.

Introduction

Mycobacterium tuberculosis was responsible for 1.4 million deaths in 2010 and is estimated to have infected one-third of the world population [1]. There is extensive evidence suggesting that *M. tuberculosis* strongly modulates the immune response, both innate and adaptive, to infection, with an important role for regulatory T (Treg) cells [2]. In mice, *M. tuberculosis* infection triggers antigen-specific CD4$^+$ Treg cells that delay the priming of effector CD4$^+$ and CD8$^+$ T-cells in the pulmonary lymph nodes (LNs) [3], suppressing the development of CD4$^+$ T helper-1 (Th1) responses that are essential for protective immunity [4]. Thus, these CD4$^+$ Treg cells delay the adequate clearance of the pathogen [5] and promote persisting infection.

M. tuberculosis - as well as *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) - have been found to induce CD4$^+$ and CD8$^+$ Treg cells in humans [6-8]. CD4$^+$ and CD8$^+$ Treg cells are enriched in disseminating lepromatous leprosy lesions, and are capable of suppressing CD4$^+$ Th1 responses [9;10]. Naive CD8$^+$CD25$^-$ T-cells can differentiate into CD8$^+$CD25$^+$ Treg cells following antigen encounter [11]. In *M. tuberculosis*-infected macaques, IL-2-expanded CD8$^+$CD25$^+$Foxp3$^+$ Treg cells were found to be present alongside CD4$^+$ effector T- cells *in vivo*, both in the peripheral blood and in the lungs [12]. In human *Mycobacterium*-infected LNs and blood, a CD8$^+$ Treg subset was found expressing lymphocyte activation gene-3 (LAG-3) and CC chemokine ligand 4 (CCL4, macrophage inflammatory protein-1β). These CD8$^+$LAG-3$^+$CCL4$^+$ T-cells could be isolated from BCG-stimulated PBMCs, co-expressed classical Treg markers CD25 and Foxp3, and were able to inhibit Th1 effector cell responses. This could be attributed in part to the secretion of CCL4, which reduced Ca2$^+$ flux early after T-cell receptor triggering [8]. Furthermore, a subset of these CD8$^+$CD25$^+$LAG-3$^+$ T-cells may be restricted by the HLA class-Ib molecule HLA-E, a non-classical HLA class-I family member. These latter T-cells displayed cytotoxic as well as regulatory activity *in vitro*, lysing target cells only in the presence of specific peptide, whereas their regulatory function involved membrane-bound TGF-β [13]. Despite these recent findings, the current knowledge about CD8$^+$ Treg cell phenotypes and functions is limited and fragmentary when compared with CD4$^+$ Treg cells [6;14].

CD39 (E-NTPDase1), the prototype of the mammalian ectonucleoside triphosphate diphosphohydrolase family, hydrolyzes pericellular adenosine triphosphate (ATP) to adenosine monophosphate [15]. $CD4^+$ Treg cells can express CD39 and their suppressive function is confined to the $CD39^+CD25^+Foxp3^+$ subset [16;17]. Increased *in vitro* expansion of $CD39^+$ regulatory $CD4^+$ T-cells was found after *M. tuberculosis* specific 'region of difference (RD)-1' protein stimulation in patients with active tuberculosis (TB) compared with healthy donors. Moreover, depletion of $CD25^+CD39^+$ T-cells from PBMCs of TB-patients increased *M. tuberculosis* specific IFNγ production [18]. CD39 expression on $CD8^+CD25^+Foxp3^+$ Treg cells has been described in simian immunodeficiency virus infection [19], but has not been studied in mycobacterial infections in humans.

ATP and other nucleotides can induce an array of intercellular signals, depending on the receptor subtype and pathways involved [20]. In damaged tissues, ATP is released in high concentrations, and functions as chemoattractant, generating a broad spectrum of pro-inflammatory responses [21]. ATP can also trigger mycobacterial killing in infected macrophages [22-24], can stimulate phagosome-lysosome fusion through P2X7 receptor activation [25], and can drive Th17 cell differentiation in the murine lamina propria [26]. In a study focusing on the novel *M. tuberculosis* vaccine MVA85A, a drop in extracellular ATP consumption by PBMCs from subjects 2 weeks after vaccination corresponded with a decrease in $CD4^+CD39^+$ Treg cells and a concomitant increase in the co-production of IL-17 and IFNγ by $CD4^+$ T-cells [27]. Further hydrolysis of adenosine monophosphate by ecto-5´-nucleotidase (CD73) generates extracellular adenosine [20], which modulates inflammatory tissue damage, among others by inhibiting T-cell activation and multiple T-cell effector functions through A2A receptor-mediated signaling [28].

BCG, the only currently available vaccine for TB, fails to protect adults adequately and consistently from pulmonary TB [29], and part of this deficiency may be explained by induction of Treg cells by the BCG-vaccine [7;30;31]. In this study, we have used live BCG to activate $CD8^+$ Treg cells, and demonstrate that these $CD8^+$ T-cells express CD39, and co-express the well-known Treg markers CD25, Foxp3, LAG-3, and CCL4. Finally, we describe involvement of CD39 in suppression by $CD8^+$ T-cells.

Materials and Methods

Blood samples. Anonymous buffy coats were collected from healthy adult blood bank donors that had signed consent for scientific use of blood products. PBMCs were isolated by density centrifugation and cryopreserved in fetal calf serum supplemented medium. Cells were counted using the CASY cell counter (Roche, Woerden, the Netherlands). Recognition of mycobacterial PPD was tested by assessing IFNγ production *in vitro*. PBMCs were stimulated with 5 μg/mL PPD (Statens Serum Institute, Copenhagen, Denmark) for 6 days and supernatants were tested in IFNγ ELISA (U-CyTech, Utrecht, the Netherlands). Positivity was defined as IFNγ production ≥ 150 pg/mL.

Cell cultures. PBMCs were cultured in Iscove's modified Dulbecco's medium (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) supplemented with 10% pooled human serum. BCG (Pasteur) was grown in 7H9 plus ADC, frozen in 25% glycerol and stored at -80^{0}C. Before use, bacteria were thawed and washed in PBS/0.05% Tween 80 (Sigma-Aldrich, Zwijndrecht, the Netherlands). Infections were done at an MOI of 1.5. IL-2 (25 U/mL; Proleukin; Novartis Pharmaceuticals UK Ltd., Horsham, UK) was added after 6 days of culture.

Restimulation of cell lines was done in 96-well round-bottom plates (1 x 10^5 cells/w) with αCD3/CD28 beads (Dynabeads Human T-activator, Life Technologies-Invitrogen), IL-2 (50 U/mL), IL-7, and IL-15 (both 5 ng/mL, Peprotech, Rocky Hill, NJ, USA); pooled, irradiated (30 Gy) PBMCs were added as feeders. Cells were maintained in IL-2 (100 U/mL).

FACS analysis. T-cell lines were incubated overnight with αCD3/28 beads, for the last 16h Brefeldin A (3 μg/mL, Sigma-Aldrich) was added. Following the labeling with the violet live/dead stain (VIVID, Invitrogen), the following antibodies were used for surface staining: CD3-PE-Texas Red, CD14- and CD19-Pacific Blue (all Invitrogen), CD4-PeCy7, CD8-HorizonV500, CD73-PerCPCy5.5 (all BD Biosciences, Eerembodegem, Belgium), and CD39-PE (Biolegend, London, UK).

For intracellular staining, we used Intrastain reagents (Dako-Cytomation, Heverlee, Belgium) according to the instructions of the manufacturer and the following antibodies: CCL4-FITC (R&D Systems, Abingdon, UK), Foxp3-Alexa Fluor 700 (eBioscience, Hatfield, UK), LAG-3-atto 647N (ENZO Life Sciences, Antwerp, Belgium), CD25-allophycocyanin-H7 (BD Biosciences), and IL-10-allophycocyanin (Miltenyi Biotec, Teterow, Germany). Samples were acquired on a BD LSRFortessa using FACSDiva software (version 6.2, BD Biosciences) and analyzed using FlowJo software (version 9.5.3, Treestar, Ashland, OR, USA).

Cell sorting. $CD8^+$ cells were enriched by positive selection using magnetic beads (MACS, Miltenyi Biotec). Cells were fluorescence-activated cell sorted (FACS) by BD FACSAriaIII cell sorter using CD39-PE (Biolegend). Purity of all cell sorts was ≥ 97% as assessed by flow cytometry.

Suppression assays. Cell lines were tested for their capacity to inhibit proliferation of a Th1 responder clone (Rp15 1-1) and its cognate *M. tuberculosis* hsp65 p3–13 peptide, presented by HLA-DR3 positive, irradiated (20 Gy) PBMCs as APCs in a co-culture assay that has been previously reported [8;32]. Proliferation was measured after 3 days of co-culture by addition of 0.5 µCi/well and (3H)thymidine incorporation was assessed after 18 h. Values represent means from triplicate wells.

For the CFSE-labeling assay, the Rp15 1-1 Th1-responder clone was labeled with 0.005 µM of CFSE and the irrelevant, isogenic T-cell clone (R2F10), with different peptide specificity and HLA-DR2 restriction, with 0.5 µM of CFSE, similar in design to previously described [13]. After 16h of co-culture with $5x10^4$ $CD8^+CD39^+$ T-cells, the p3-13 peptide (50 ng/mL) and HLA-DR3 positive APCs, cells were harvested and stained for CD3, CD4, and CD8. CFSE intensity was measured on a BD LSRFortessa using FACSDiva software and analyzed using FlowJo software.

Blocking experiments. ARL 67156 trisodium salt hydrate (Sigma-Aldrich) was added to the well in 150 µM and daily during the 3 days of co-culture. Anti-CD39 monoclonal antibody BY40/OREG-103 (Orega Biotech, Ecully, France) was added to the well at the first day of co-culture at a final concentration of 10 µg/mL, as was the IgG1 isotype control (R&D Systems). Values represent mean ± SE from triplicate wells.

Suppressive capacity of $CD8^+CD39^+$ T-cells was independent of original proliferation of the Th1 clone, as tested by reducing the cognate peptide concentration in the co-culture assays. Reversal of suppression was calculated in proportion to original clone proliferation in the absence of Treg cells, since ARL and anti-CD39 monoclonal antibody interfered directly with Th1 clone proliferation signals in the CD39 pathway, as demonstrated by reduced (3H)thymidine incorporation after 3 days. Percentage blocking was calculated after natural logarithmic transformation, and inhibition of proliferation in the presence and absence of blocking agents was calculated and expressed as percentage [8]. Raw data can be provided per request.

Statistical analyses. Mann-Whitney tests and Wilcoxon signed-rank tests were performed using GraphPad Prism (version 5, GraphPad Software, San Diego, CA, USA) and SPSS statistical software (version 20, SPSS IBM, Armonk, NY, USA).

Results

Live BCG activates CD39 expression on $CD4^+$ and $CD8^+$ T-cells

We isolated PBMCs from healthy human donors and stimulated these PBMCs with live BCG [8]. Flow cytometric analysis was performed after 6 days (the full gating strategy is shown in Supplementary figure S1, in compliance with the most recent MIATA guidelines [33]). CD39 was expressed on T-cells of donors that responded to purified protein derivative (PPD) *in vitro*, but not on T-cells from PPD non-responsive donors or on unstimulated cell lines (figure 1). CD39 and CD25 were co-expressed on both $CD4^+$ and $CD8^+$ T-cells from PPD-responsive donors after stimulation with live BCG (figure 1).

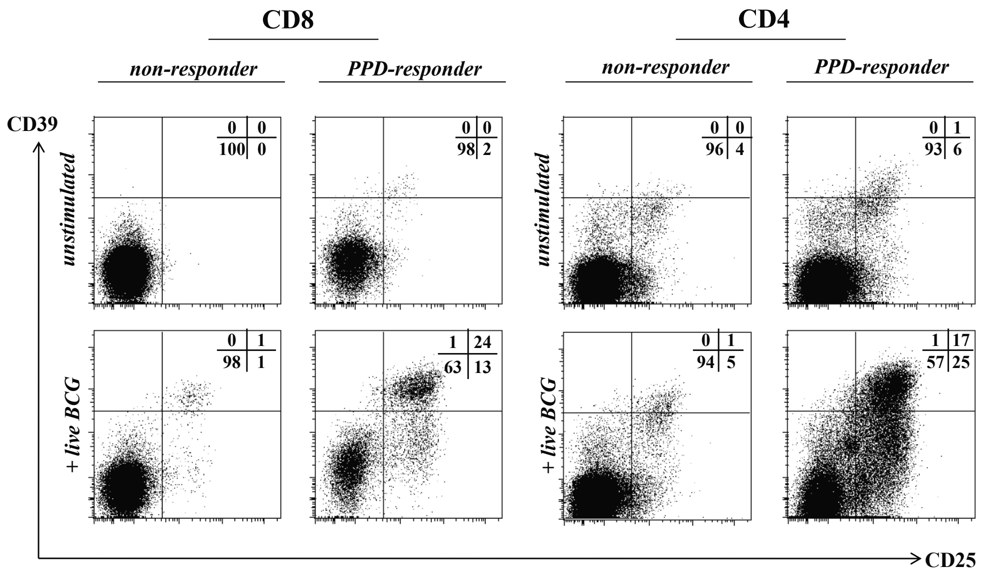
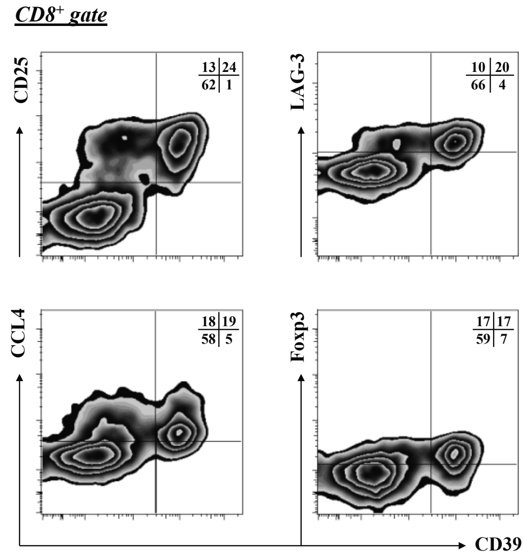


Figure 1. Live *Mycobacterium bovis* BCG activates CD4$^+$CD39$^+$ and CD8$^+$CD39$^+$ T-cells. Flow cytometric analysis of CD39 and CD25 expression on CD8$^+$ and CD4$^+$ T-cells of *in vitro* PPD-responders and non-responders 6 days after live *M. bovis* BCG stimulation. Gating was performed as shown in Supplementary figure S1. Data are representative of five PPD-responders and five non-responders.

CD8$^+$CD39$^+$ T-cells specifically co-express Treg-cell markers

CD8$^+$CD39$^+$ T-cells co-expressed the Treg-cell markers CD25, LAG-3, CCL4, and Foxp3 (figure 2A). There was no co-expression of CD39 with CD73, consistent with other studies on human Treg cells [34] (data not shown). Gating CD8$^+$ T-cells on Foxp3 and LAG-3 [8] demonstrated that the majority of these cells also expressed CD39 as well as CD25 (figure 2B). Boolean gating was used to analyze expression of multiple markers on single cells (figure 2C). A significantly higher percentage of CD3$^+$CD8$^+$CD4$^-$ T-cells from PPD-responders expressed CD39 as compared with non-responders (p = 0.03; Mann-Whitney test). The CD8$^+$CD39$^+$ T-cells from responders significantly co-expressed CD25, LAG-3, CCL4, and/or Foxp3 as compared with non-responders and this difference was highly significant for the CD8$^+$ T-cells that were CD39$^+$CD25$^+$LAG-3$^+$CCL4$^+$Foxp3$^+$ (p = 0.02 - 0.03 and p = 0.0079, respectively; Mann-Whitney test). The majority of the CD3$^+$CD8$^+$CD4$^-$ T-cells co-expressed CD25, LAG-3, CCL4, and/or Foxp3 in combination with CD39, such that CD39 appears to be a preferential marker of CD8$^+$ Treg cells expressing multiple Treg-associated markers (p = 0.0625; Wilcoxon signed-rank test).

A



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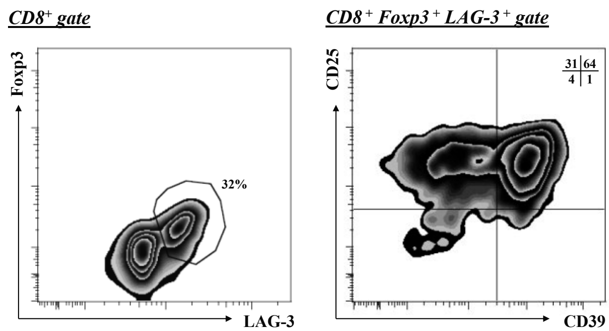

Figure 2 ***(A and B)*****. CD39 expression is associated with Treg-cell markers on CD8$^+$ T-cells.** A: Flow cytometric analysis of CD8$^+$ T-cells for a selection of Treg markers in a PPD-responder 6 days after live *Mycobacterium bovis* BCG infection. Gating was performed as shown in Supplementary figure S1. CD8$^+$CD4$^-$ gate consisted of at least 10 000 cells. Data are representative of five donors and four experiments performed. B: CD8$^+$ T-cells that express Foxp3 and LAG-3 (left) also express CD25; the majority of these also express CD39 (right).

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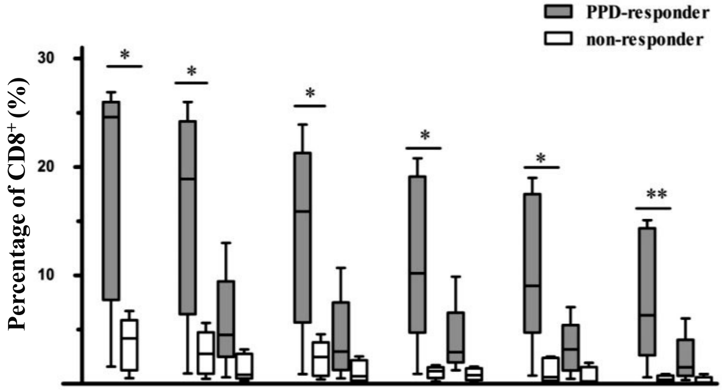
C



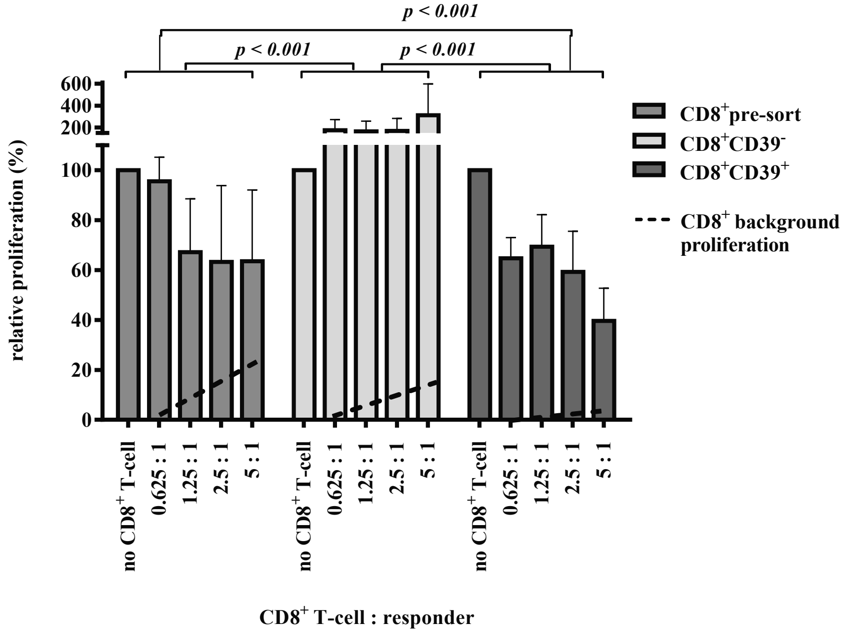
CD39	+	+	−	+	−	+	−	+	−	+	−
CD25		+	+	+	+			+	+	+	+
LAG-3				+	+	+	+			+	+
CCL4						+	+			+	+
Foxp3								+	+	+	+

Figure 2 ***(continued)*****. CD39 expression is associated with Treg-cell markers on CD8$^+$ T-cells.** C: Combined analysis of individual Treg markers. Expression of individual markers was combined using Boolean gating and data are expressed as the percentage of CD3$^+$CD8$^+$CD4$^-$ T-cells that express these markers. Data are shown of five PPD-responders in gray boxes versus five non-responders in open boxes, 6 days after live *M. bovis* BCG infection and are representative of four experiments. Boxes: 25th-75th percentiles; line at median; whiskers: minimum to maximum (**p* < 0.05; ***p* < 0.01; Mann-Whitney test).

CD8$^+$CD39$^+$ T-cells suppress CD4$^+$ Th1 responses

To determine the possible suppressive function of CD39$^+$ T-cells, CD39-positive and negative T-cell populations were FACS-sorted and tested for their capacity to inhibit the activity of an unrelated CD4$^+$ Th1 responder clone, recognizing a cognate peptide presented in the context of HLA-DR3 [8;32]. CD8$^+$CD39$^+$ T-cells, purified to ≥ 97% purity, indeed suppressed the proliferative response of (cloned) CD4$^+$ Th1 cells in response to peptide in the context of HLA-class II. This suppressive activity was strongly enriched in the CD8$^+$CD39$^+$ T-cell population as compared with CD8$^+$CD39$^-$ T-cells and unsorted CD8$^+$ T-cells (figure 3A). Flow cytometric analysis of sorted T-cell lines demonstrated enrichment for LAG-3, CD25, Foxp3, and CCL4 in the CD8$^+$CD39$^+$ compared with the CD8$^+$CD39$^-$ T-cells (figure 3B).

A



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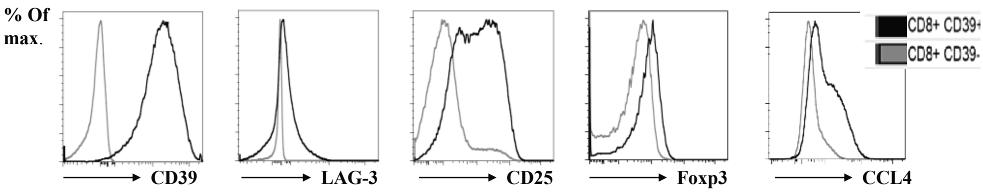

Figure 3. ***Mycobacterium bovis*** **BCG-induced $CD8^+CD39^+$ T-cells suppress antigen-specific proliferation of (cloned) $CD4^+$ Th1 cells.** A: $CD8^+$ T-cells of PPD-responders were enriched after BCG stimulation by positive selection using magnetic beads and FACS-sorted on CD39 expression. Their potential suppressive capacity was tested in a co-culture assay by titrating $CD8^+CD39^+$ T-cell lines, $CD8^+CD39^-$ T-cell lines, or unsorted bulk $CD8^+$ T-cell lines (containing on average 75% $CD8^+CD39^+$ T-cells) onto a Th1 reporter clone that was stimulated with its cognate peptide. Proliferation was measured by (3H)TdR (where TdR is thymidine) incorporation after 3 days. Proliferation was divided by Th1 reporter clone proliferation in the absence of Treg cells to obtain relative proliferation (Wilcoxon signed-rank test, $p < 0.001$). Dashed lines represent background proliferation of the different $CD8^+$ T-cell subsets (at the indicated $CD8^+$ T-cell concentrations) relative to Th1 reporter clone proliferation. Data are depicted as mean ± SE of three cell lines. B: Flow cytometric analysis demonstrating enrichment for Treg-cell markers in CD39-sorted $CD8^+$ T-cell lines, compared with $CD39^-CD8^+$ T-cell lines. (A, B) Data are shown from one representative experiment out of three performed.

Blocking CD39 results in partial reversal of suppression by *M. bovis* BCG-stimulated CD8$^+$CD39$^+$ T-cells

CD8$^+$CD39$^+$ T-cells preserved their expression of CD39 (≥ 99%), as well as of other Treg-cell markers, including CD25, Foxp3, and CCL4 (Supplementary figure S2) following further *in vitro* expansion. We next tested the ability of ARL 67156 trisodium salt hydrate (ARL) and the anti-CD39 monoclonal antibody BY40/OREG-103 to reverse the suppressive activity of CD8$^+$CD39$^+$ T-cells. ARL is an ATP analog that can bind to, but is not hydrolysable by, CD39 [35], and has been used to inhibit the suppressive activity of CD4$^+$CD25$^+$CD39$^+$ cells [27]. Here, ARL partially reversed the capacity of CD8$^+$CD39$^+$ T-

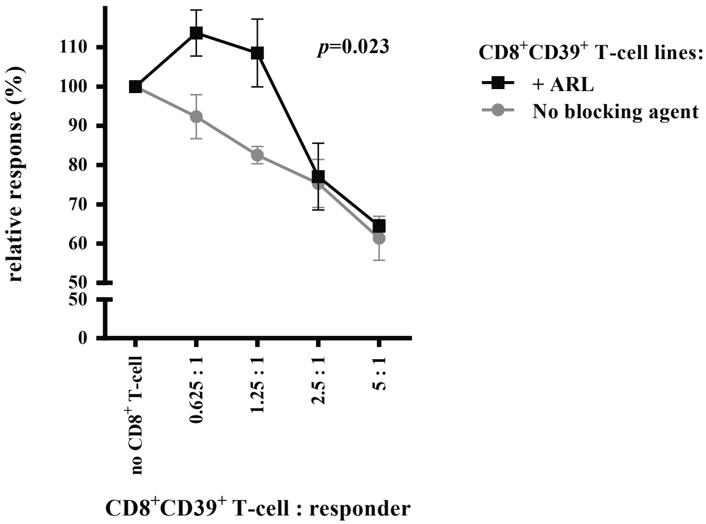

Figure 4. Blocking CD39 by the chemical CD39 antagonist ARL 67156 trisodium salt hydrate (ARL) results in partial reversal of suppression by *Mycobacterium bovis* BCG-stimulated CD8$^+$CD39$^+$ T-cells. CD8$^+$CD39$^+$ T-cells, with previously demonstrated T-cell inhibitory capacity, were expanded using αCD3/CD28 beads, and suppression assays were performed by titrating these CD8$^+$CD39$^+$ T-cell lines onto a Th1 reporter clone stimulated with its cognate peptide as described in the legend of figure 3A. ARL was added daily in 150 µM and proliferation was measured as (3H)TdR (where TdR is thymidine) incorporation after three days. The response was calculated by dividing cognate-peptide-induced proliferation with unstimulated values after natural logarithmic transformation. To obtain a relative response, the response for each Treg concentration was normalized for proliferation in the absence of Treg cells (100%). Reversal of suppression was calculated by dividing relative responses in the presence or absence of ARL (14 - 47% reversal of suppression; $p = 0.023$; Wilcoxon signed-rank test) as previously described [8]. Data represent mean ± SE for three independent CD8$^+$CD39$^+$ T-cell lines tested in two experiments on the same standardized Th1 reporter clone.

cells to suppress the proliferative responses of the Th1 responder clone (14 - 47% reversal of suppression; in three cell lines; $p = 0.023$; Wilcoxon signed-rank test) (figure 4).

Suppression by the CD8$^+$CD39$^+$ T-cells was also (partially) reversed by the anti-CD39 blocking monoclonal antibody BY40/OREG-103 [36;37] (0 - 35% reversal of suppression; in four experiments; $p = 0.005$; Wilcoxon signed-rank test) (figure 5); further supporting the direct functional involvement of CD39 in suppression mediated by CD8$^+$CD39$^+$ Treg cells.

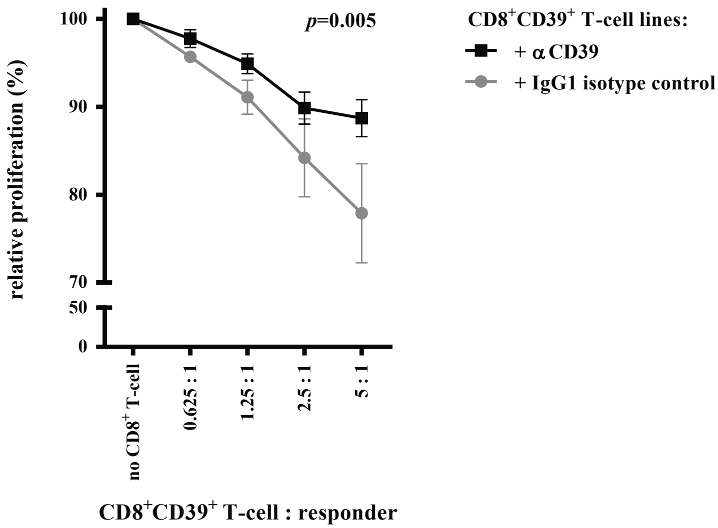

Figure 5. Suppression mediated by CD8$^+$CD39$^+$ T-cell lines is partly reversed by an anti-CD39 monoclonal antibody BY40/OREG-103. CD8$^+$CD39$^+$ T-cells were expanded using αCD3/CD28 beads and suppression assays were performed by titrating these CD8$^+$CD39$^+$ T-cell lines onto a Th1 reporter clone stimulated with its cognate peptide. Proliferation values were divided, after natural logarithmic transformation, by Th1 reporter clone proliferation values obtained in the absence of Treg cells, in order to obtain relative proliferation. Inhibition of proliferation in the presence and absence of anti-CD39 monoclonal antibody was calculated and expressed as percentage (0 - 35% reversal of suppression; $p = 0.005$; Wilcoxon signed-rank test). Data represent mean ± SE for four cell lines in four independent experiments.

To exclude that suppressive activity by $CD8^+CD39^+$ T-cell lines was due to lysis rather than active suppression of the $CD4^+$ Th1 responder clone, the Th1 responder clone and an equal number of cells of an irrelevant T-cell clone were labeled with low and high doses CFSE, respectively, and were added in equal numbers to the co-culture assay, identical to previously described [13]. After 16h, before division of the Th1 responder clone occurs, the percentages of responder and irrelevant T-cell clone without $CD8^+CD39^+$ T-cells were similar to the percentages of responder and irrelevant T-cell clone with the addition of $CD8^+CD39^+$ T-cells (Supplementary figure S3), indicating that in these co-culture assays, inhibition of responder cell proliferation by $CD8^+CD39^+$ T-cells is not the result of cytotoxicity.

Discussion

In this study, we describe for the first time the expression of, and a functional role for, CD39 on human pathogen activated $CD8^+$ Treg cells. $CD8^+CD39^+$ T-cells from PPD-responsive individuals specifically co-expressed the known classical Treg-cell markers CD25, Foxp3, LAG-3, and CCL4. To assess if CD39 expression was merely a marker of $CD8^+$ Treg cells or was directly involved in the $CD8^+CD39^+$ T-cells' suppressive activity, we purified $CD8^+CD39^+$ T-cells, and showed that they were strongly enriched for suppressive activity and the expression of Treg markers, and that both the chemical CD39 antagonist, ARL, as well as a blocking anti-CD39 antibody were able to partly inhibit the suppressive activity of $CD8^+CD39^+$ T-cells. Altogether these data indicate that CD39 is a marker for regulatory $CD8^+$ T-cells and that CD39 contributes functionally to the suppression mediated by human $CD8^+CD39^+$ T-cells.

Both ARL as well as the blocking anti-CD39 antibody only partly inhibited suppressive activity, indicating that also other mechanisms may contribute to suppression. We previously demonstrated the expression of LAG-3 and the functional involvement of CCL4 in immune regulation by BCG-activated $CD8^+$ Treg cells. In the current study, $\geq$ 43% of $CD8^+CD39^+$ T-cells also expressed CCL4, while we did not find any expression of IL-10 on these T-cells.

$CD8^+$ Treg cells have been described in human *Mycobacterium*-infected LNs [8] and lepromatous lesions [9;10], demonstrating that $CD8^+$ Treg cells are present at the site of disease and suggesting a potential role for these cells in disease pathogenesis. In line with our previous studies showing that BCG activated $CD8^+$ Treg cells in PPD-responsive individuals, but not in donors that did not recognize PPD *in vitro* [8], also in the current study $CD8^+CD39^+$ Treg cells were confined to PPD-responders, suggesting that these cells originated from preexistent antigen-specific memory T-cells. We have previously hypothesized that Treg cells could contribute to the relative failure of BCG-vaccination in conferring protection against pulmonary TB in adults [6].

In TB, recent results have suggested a role for Th17 cells both in protection and pathology. IL-17 producing $CD4^+$ T-cells in the lung, induced by BCG-vaccination, were associated with protective immunity to TB in mice [2;38]; interestingly, in human tuberculous pleural effusions, the number of $CD4^+CD39^+$ Treg cells was inversely related to the number of Th17 cells, and $CD39^+$ Treg cells suppressed the differentiation of naive $CD4^+$ cells into Th17 cells [39]. Frequencies of $CD4^+CD39^+$ T-cells correlated negatively with IL17A responses in stimulated PBMCs after MVA85A vaccination [40]. Stimulation of PBMCs in the presence of the CD39 chemical antagonist ARL 67156 increased coproduction of IL-17 and IFNγ by $CD4^+$ T-cells, 1 and 2 weeks after MVA85A vaccination [27]. Whether $CD8^+CD39^+$ T-cells are associated with IL-17 responses and/or protection needs further investigation.

In this article, we describe for the first time a functional role for CD39 on human BCG-activated $CD8^+CD39^+$ Treg cells. We show that CD39 expression marks a $CD8^+$ Treg-cell subset, which co-expresses LAG-3, CD25, Foxp3, and CCL4, and that CD39 may play a direct role in exerting $CD8^+$ Treg-dependent suppression. $CD8^+CD39^+$ Treg cells represent a new player in balancing immunity and inflammation in host defense against mycobacteria, and possibly contribute to (lack of) vaccine-mediated protection.

Acknowledgments

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Conflict of interest statement

Jérémy Bastid is chief operating officer at OREGA BIOTECH and provided the anti-CD39 monoclonal antibody BY40/OREG-103. Dr. Bastid was not involved in design and execution of experiments or in data analysis.

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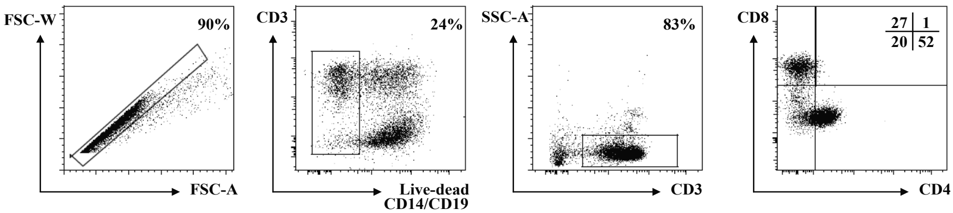
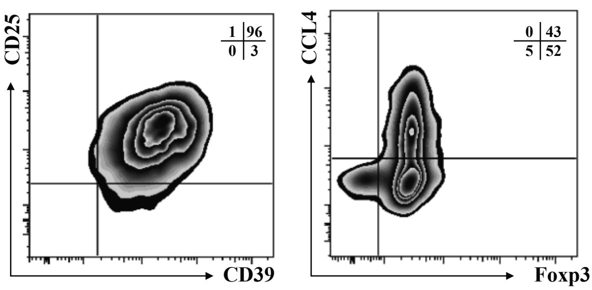
Supplementary figures

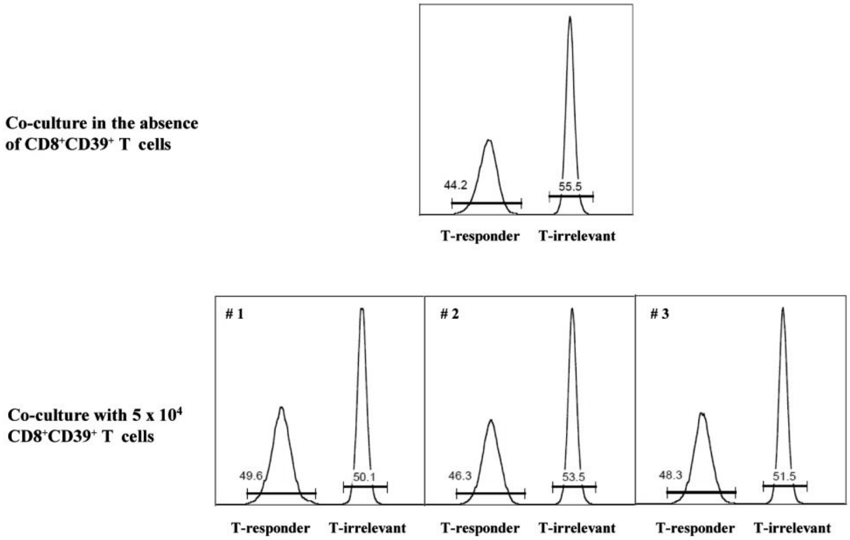


Supplementary figure S1. Gating strategy. Studies were conducted in a laboratory guided by exploratory research principles with established Standard Operating Procedures (SOP): cells were isolated and cryopreserved according to SOP[1]. Cells were thawed (78% median viable cell yield) and stimulated with live BCG for six days in medium supplemented with pooled human serum (pretested in standardized protocols; only sera were pooled that had no inhibitory activity in standard mixed allogeneic lymphocyte cultures). Demonstrated is the gating strategy used for flowcytometric analysis at day 6: cells were gated on single cells, violet-live/dead-CD14-CD19-negative, $CD3^{+}$ and $CD8^{+}CD4^{-}$ vs. $CD4^{+}CD8^{-}$.

[[1]Van Dissel JT et al. *Vaccine* 2010;28(20):3571-81. doi: 10.1016/j.vaccine.2010.02.094.]



Supplementary figure S2. Expression of regulatory T-cell markers in restimulated $CD8^{+}CD39^{+}$ T-cell lines. $CD8^{+}CD39^{+}$ T-cells were expanded using αCD3/CD28 beads and analyzed for expression of regulatory T-cell markers by flowcytometry. Gating was similar for both plots and performed as shown in Supplementary figure S1. 97% Of the restimulated $CD8^{+}CD39^{+}$ T-cells expressed CD25 (left) and 43% of restimulated $CD8^{+}CD39^{+}$ T-cells co-expressed CCL4 and Foxp3 (right). These cells did not express IL-10. Data are representative for three $CD8^{+}CD39^{+}$ T-cell lines.



Supplementary figure S3. Inhibition of Th1-responder cell proliferation is not the result of lysis by CD8$^+$ T-cells. The Th1-responder clone Rp15 1-1 was labeled with 0.005 µM of CFSE, and another, isogenic T-cell clone, with a different peptide specificity and HLA-DR restriction, with 0.5 µM of CFSE [13]. Both cells were then co-cultured in equal numbers with the CD8$^+$CD39$^+$ T-cell lines in the presence of the peptide (50 ng/ml) recognized by the responder clone Rp15 1-1 in the context of HLA-DR3$^+$ APCs. After 16 hours CFSE intensity was measured by flowcytometry. The percentages of responder and irrelevant T-cells without CD8$^+$CD39$^+$ T-cells were similar to the percentages of responder and irrelevant T-cells with the addition of 5 x 10^4 CD8$^+$CD39$^+$ T-cells, the highest concentration used in our co-culture experiments; thus indicating that the responder clone is not lysed by the BCG-activated CD8$^+$CD39$^+$ T-cells.

CHAPTER 5

BCG-vaccination induces divergent pro-inflammatory or regulatory T-cell responses in adults

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Abstract

Mycobacterium bovis bacillus Calmette-Guérin (BCG), the only currently available vaccine against tuberculosis, induces variable protection in adults. Immune correlates of protection are lacking, and analyses on cytokine-producing T-cell subsets in protected vs. non-protected cohorts have yielded inconsistent results. We studied the primary T-cell response, both pro-inflammatory and regulatory T-cell responses, induced by BCG-vaccination in adults. Twelve healthy adult volunteers, who were tuberculin skin test (TST)-negative, QuantiFERON test (QFT)-negative, and BCG-naive, were vaccinated with BCG and followed up prospectively. BCG-vaccination induced an unexpectedly dichotomous immune response in this small, BCG-naive young adult cohort: BCG-vaccination induced either gamma interferon-positive (IFNγ$^+$) interleukin 2-positive (IL2$^+$) tumor necrosis factor α-positive (TNFα$^+$) polyfunctional CD4$^+$ T-cells concurrent with CD4$^+$IL17A$^+$ and CD8$^+$IFNγ$^+$ T-cells, or, in contrast, virtually absent cytokine responses with induction of CD8$^+$ regulatory T-cells. Significant induction of polyfunctional CD4$^+$IFNγ$^+$IL2$^+$TNFα$^+$ T-cells and IFNγ production by peripheral blood mononuclear cells (PBMCs) was confined to individuals with strong immunization-induced local skin inflammation and increased serum C-reactive protein (CRP). Conversely, in individuals with mild inflammation, regulatory-like CD8$^+$ T-cells were uniquely induced. Thus, BCG-vaccination either induced a broad pro-inflammatory T-cell response with local inflammatory reactogenicity or, in contrast, a predominant CD8$^+$ regulatory T-cell response with mild local inflammation, poor cytokine induction, and absent polyfunctional CD4$^+$ T-cells. Further detailed fine mapping of the heterogeneous host response to BCG-vaccination using classical and non-classical immune markers will enhance our understanding of the mechanisms and determinants that underlie the induction of apparently opposite immune responses, and how these impact the ability of BCG to induce protective immunity to TB.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is the second greatest infectious cause of death worldwide after HIV, accounting for 1.3 million deaths in 2012 [1]. The only available vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), protects infants from disseminated forms of TB, but has insufficient and inconsistent efficacy in protecting adults from pulmonary TB [1;2]. A vaccine preventing active pulmonary TB, the contagious form of the disease, would greatly impact the epidemic [3], and a better understanding of vaccine-induced mechanisms of protection is essential in developing new surrogate endpoints [4].

Both $CD4^+$ Th1 ($IFN\gamma^+$) cells and $CD8^+$ T-cells are critical for protection against TB [5]. Specifically, $CD4^+$ $IFN\gamma^+IL2^+TNF\alpha^+$ polyfunctional T-cells have been proposed as correlate of vaccine-induced protective immunity in murine infection models [6]. In infants, BCG-vaccination induced specific cytokine expression in $CD4^+$ and $CD8^+$ T-cells [7-9], including $IFN\gamma^+IL2^+TNF\alpha^+$ polyfunctional $CD4^+$ T-cells [10]. However, there was no relation between the presence of such cells and the development of TB during follow-up [11].

In adults, BCG-vaccination induced $CD4^+$ $IFN\gamma^+$ responses [12-14] as well as IFNγ- and TNFα-secreting $CD8^+$ T-cells with cytotoxic activity [15]. However, data on induction of polyfunctional T-cells by BCG-vaccination in adults have been conflicting [16;17]. In one report, the induction of polyfunctional $CD4^+$ T cells was similar in magnitude in BCG-vaccinated infants and adults; however, when induction was analyzed as the proportion of polyfunctional versus single-cytokine-producing T-cells, the proportion of polyfunctional $CD4^+$ T-cells was larger in children than in adults [16]. Further, studies on latent (controlled) versus active TB in adults yielded variable results on changes in mono- and triple-cytokine producing T-cell subsets [18;19], such that it was suggested that polyfunctional T-cells are also present in active TB disease and that these cells are not a surrogate marker of protection against TB in humans [19;20].

Another explanation for the inconsistent protection induced by BCG against TB in adults is induction of regulatory T-cells (Tregs) by mycobacteria, which can dampen pro-inflammatory responses [21]. In that context, we reported that live BCG triggers the specific activation of $CD8^+$ (but not $CD4^+$) Tregs from peripheral blood mononuclear cells

(PBMCs) of mycobacterial purified protein derivative (PPD)-responsive adults [22], while others found that BCG-vaccination induced CD4$^+$ Tregs in newborns [23] and adults [24]. Here, in a small, well-defined cohort of previously BCG-naive adults, we studied the induction of multiple cytokine-producing as well as regulatory T-cell subsets following BCG-vaccination.

Materials and Methods

Participants. Dutch volunteers were recruited via posters in the university library. All volunteers were screened for tuberculosis by anamnesis (history of TB disease or treatment), by a tuberculin skin test (TST; negative < 5mm), and by the QuantiFERON-TB gold in-tube test, according to the manufacturer's specifications. Included volunteers (n = 6 males, n = 6 females; median age 24 years (interquartile range (IQR) 23-25 years); median weight 70 kg (IQR 67-80 kg); all Dutch, all Caucasian) had not been vaccinated with BCG at any time prior to entering the trial (anamnestic, presence of scar, or on a vaccination card), were never treated for TB disease and had negative TST and QuantiFERON test results. In addition, they did not receive any live vaccination at < 4 weeks prior to BCG-vaccination. Volunteers were excluded who were pregnant or not generally healthy, who had fever or received antibiotic treatment < 2 weeks prior to enrollment, or who were treated with immune modulating drugs < 3 months prior to enrollment; all volunteers tested negative for HIV at screening.

Procedures. Participants were vaccinated with the live-attenuated BCG Danish strain 1331 (Statens Serum Institute, Denmark) by intradermal injection in the upper arm and were followed up prospectively: at 2 weeks prior to vaccination, at day of vaccination, at 1, 3 and 7 days after vaccination, at 4, 8 and 12 weeks after vaccination and at 1 year after vaccination. During follow up the injection site was inspected and photographed, and adverse events were recorded using a standardized case report form. Venous blood samples were collected in heparin-containing vacutainers for whole-blood stimulation assays and for PBMC isolation and cryopreservation according to standard operating procedures. Serum was collected from serum tubes after blood coagulation and stored at -80ºC.

Calculation of skin inflammation score. Signs of inflammation by visual inspection of the vaccination site and symptoms recorded in volunteer diaries were documented on standardized case report forms and photographed at 4, 8 and 12 weeks after vaccination. The local reaction was scored by two researchers (M.C.B. and C.P.) independently, with one point per sign of inflammation: redness of ≥ 1 cm, swelling of ≥ 1 cm, pus discharge and ulceration, pain, and regional lymph node enlargement (> 90% consensus; disagreements were solved by mutual reexamination of case report forms, photographs and volunteer diaries). The inflammation score was calculated as the cumulative scores of weeks 4, 8, and 12 after vaccination.

C-reactive protein enzyme-linked immunosorbent assay. The serum samples of all pre-vaccination and post-vaccination visits were thawed, and the C-reactive protein (CRP) concentration was measured using a standardized, highly sensitive, CRP human enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (Abnova, Heidelberg, Germany).

Whole-blood live BCG-stimulation. Bacillus Calmette-Guérin (Pasteur) was grown in 7H9 plus ADC, frozen in 25% glycerol and stored at -80^{0}C. Before use, bacteria were thawed and washed in phosphate-buffered saline (PBS)/0.05% Tween 80 (Sigma-Aldrich). Then, 1 ml of heparinized blood was added within 1h of blood collection to Sarstedt microtubes (Sarstedt B.V., Etten-Leur, the Netherlands), containing 0.9 x 10^6 CFU (calculated multiplicity of infection (MOI) of 3), and anti-CD28 and anti-CD49d antibodies as co-stimulants (1 µg/ml, BD Biosciences, Eerembodegem, Belgium) [25] and immediately incubated at 37^{0}C. Staphylococcal enterotoxin B (SEB) (final concentration 5 µg/ml; Toxin Technology, Sarasota, FL, USA) and unstimulated samples were used as controls. After 3 h, Brefeldin A (10 µg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands) and Monensin (1:1000; BD Biosciences) were added and samples were transferred to a water bath set at 37°C and programmed to switch off after 12h. Samples were harvested the next morning, using EDTA (2mM; Sigma-Aldrich), fixed and erythrocyte-lysed using a fluorescence-activated cell sorter (FACS)-lysing solution (BD Biosciences), and cryopreserved in fetal calf serum with 10% dimethyl sulfoxide (DMSO) [25].

Cell cultures and BCG infection. PBMCs were thawed, and cells were counted using the CASY cell counter (Roche, Woerden, the Netherlands). Infections were done at an MOI of 1.5. SEB (final concentration 2 µg/ml; Toxin Technology) and unstimulated samples were used as controls. PBMCs were cultured in 24-well plates (2 x 10^6/w) for 6 days in Iscove's modified Dulbecco's medium (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) with 10% pooled human serum. For flow cytometric analysis PBMCs were incubated for the last 16h with αCD3/28 beads (Invitrogen) and Brefeldin A (3 µg/ml; Sigma-Aldrich).
Lymphocyte stimulation assays were performed using PBMCs (0.5 x 10^6/w in 48-well plates) and stimulation with 5 µg/ml PPD (Statens Serum Institute, Copenhagen, Denmark) at 37^0C, 5% CO_2. Phytohemagglutinin (PHA) (final concentration 2 µg/ml; Remel Europe) and unstimulated samples were used as controls. After 7 days, supernatants were collected and an IFNγ-ELISA (U-CyTech, Utrecht, the Netherlands) was performed.
Direct IFNγ-enzyme-linked immunosorbent spot (ELISpot) assays were performed at 1 year post-vaccination: 250,000 freshly isolated PBMCs were added in AIMV (synthetic non-human serum supplemented) medium (Invitrogen) to 96-well ELISpot plates (Millipore, Bedford, MA, USA), that were pre-coated with anti-IFNγ-antibody (1-D1K; 5 µg/ml; Mabtech, Stockholm, Sweden) and blocked with AIMV. PBMCs were stimulated overnight with PHA (2 µg/ml), PPD (5 µg/ml) or an antigen 85B (Ag85B) peptide pool (1 µg/ml) in triplicate [26]. For detection, biotinylated anti-IFNγ-antibody (0.5 µg/ml; Mabtech), streptavidin-alkalic phospatase conjugate (1:1000 dilution in 1% bovine serum albumin (BSA)-PBS; Mabtech IFNγ-ELISpot kit reagent) and a SigmaFast NBT/BCIP substrate (Sigma-Aldrich) were used. Positivity for vaccine take [27] was defined as an increase of > 100% of the average count in PPD-stimulated wells compared to unstimulated controls and at least 5 spots more than in unstimulated controls [28].

Flow cytometry. Fixed whole-blood samples were thawed and stained in batches. Surface staining included CD3-Brilliant Violet 570 (clone UCHT1), CD19-Pacific Blue (clone HIB19), CD56-Brilliant Violet 421 (clone HCD56) (all Biolegend, London, U.K.), CD14-Pacific Blue (clone TüK4) and CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen); and CD8-HorizonV500 (clone RPA-T8), CD45RA-allo-phycocyanin (APC)-H7 (clone HI100) and CD62L-Brilliant Violet 605 (clone DREG-56) (all BD Biosciences). For intracellular staining IL17A-FITC (clone eBio64DEC17; eBioscience, Hatfield, UK), IFNγ-Alexa Fluor 700 (clone B27), TNFα-APC (clone 6401.1111), IL4-PE (clone 3010.211), and CD69-PeCy5 (clone FN50) (all BD Biosciences); and IL2-Brilliant Violet 650 (clone MQ1-17H12), IL10-Pe-Cy7 (clone JES3-9D7) and IL13-PE (clone JES10-5A2) (all Biolegend) were used in permeabilization solution (Fix&Perm cell permeabilization kit, An Der Grub BioResearch GMBH, Susteren, the Netherlands).

The stimulated PBMCs were labelled with violet LIVE/DEAD stain (Vivid, Invitrogen) and surface stained with CD3-Brilliant Violet 570 (clone UCHT1), CD19-Pacific Blue (clone HIB19), CD56-Brilliant Violet 421 (clone HCD56), and CD39-PE (clone A1) (all Biolegend, London, U.K.); CD14-Pacific Blue (clone TüK4) and CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen); and CD8-HorizonV500 (clone RPA-T8; BD Biosciences). Cells were fixed and permeabilized using the Fix&Perm cell permeabilization kit. For intracellular staining the following antibodies were used: CC chemokine ligand 4 (CCL4)-fluorescein isothiocyanate (clone 24006; R&D Systems, Abingdon, UK), Foxp3-Alexa Fluor 700 (clone PCH101; eBioscience), lymphocyte activation gene (LAG)-3-atto 647N (clone 17B4; ENZO Life Sciences, Antwerp, Belgium), and CD25-allophycocyanin-H7 (clone M-A251; BD Biosciences).

Samples were acquired on a BD LSRFortessa using FACSDiva software (version 6.2, BD Biosciences) with compensated parameters. Analysis was performed using FlowJo software (version 9.5.3, Treestar, Ashland, OR, USA) and gates were synchronized per donor for all visits and for both $CD4^+$ and $CD8^+$ T-cell subsets, using the comparison with unstimulated samples and SEB as controls.

Statistical analyses. GraphPad Prism (version 6, GraphPad Software, La Jolla, CA, USA) and SPSS statistical software (version 20, SPSS IBM, Armonk, NY, USA) were used for Wilcoxon signed-rank tests and Mann-Whitney tests. To correct for paired resp. unpaired multiple testing, Friedman tests followed by Dunn's multiple comparisons tests, and Kruskal-Wallis tests followed by Dunn's multiple comparisons tests, respectively, were used. Only values significant after multiple testing correction are demonstrated.

Study approval. Approval was obtained from the Medical Ethical Committee (registration number P 12.87) of the Leiden University Medical Center, the Netherlands. Each participant signed written informed consent prior to inclusion.

Results

Participants

Twelve healthy adults (TST-negative, QuantiFERON-negative) were vaccinated with BCG (n = 6 males, n = 6 females; median age 24 years (IQR 23 - 25 years); all Caucasian). *Ex vivo* IFNγ-ELISpot assays were performed 1 year post-vaccination to determine the *Mycobacterium*-specific immunity [29]: all recipients tested positive except for one, who nevertheless demonstrated high BCG-induced IFNγ-responses in flow cytometric analysis (Supplementary figure S1).

Local reactogenicity is variable and corresponds with serum CRP and PPD-induced IFNγ production

T-cell responses as well as BCG-vaccine induced local skin reactions varied strongly between individuals. At 4, 8 and 12 weeks post-vaccination, inflammatory symptoms were scored for redness, swelling, ulceration and pus discharge, pain and regional lymph node enlargement. A cumulative skin inflammation score was then calculated (further described in the Materials and Methods). Representative photographs of low- and high-inflammation skin lesions are shown in figure 1A. Participants were subdivided into a low (n = 6) and a high (n = 6) responder group (referred to as low and high skin inflammation responders, respectively) based on skin inflammation scores using the median cumulative skin inflammation score of 7 as a cut-off (figure 1B).

Since serum CRP has been used previously to study vaccine-induced inflammation [30-32], we determined CRP concentrations by a highly sensitive ELISA. Serum CRP concentrations 3 and 7 days post-vaccination correlated with the cumulative skin inflammation score (R^2 = 0.76 at both visits using nonlinear regression, day 7 results shown in figure 1C, left). In high skin inflammation responders, serum CRP was significantly higher 7 days post-vaccination, compared to low skin inflammation responders (p = 0.03; figure 1C, right). CRP concentrations at baseline were not different between high and low inflammation responders.

We assessed the *in vitro* IFNγ production by ELISA after PPD stimulation of PBMCs for 7 days. The PBMCs of high skin inflammation responders produced more IFNγ than low skin inflammation responders 4 and 8 weeks post-vaccination (p = 0.026 and 0.002, resp.). IFNγ production was significantly induced at 8 and 12 weeks post-vaccination compared to pre-vaccination in high skin inflammation responders (both p = 0.031), but not in low skin inflammation responders (figure 1D). Prior to BCG-vaccination, IFNγ production was not significantly different between high vs. low skin inflammation responders.

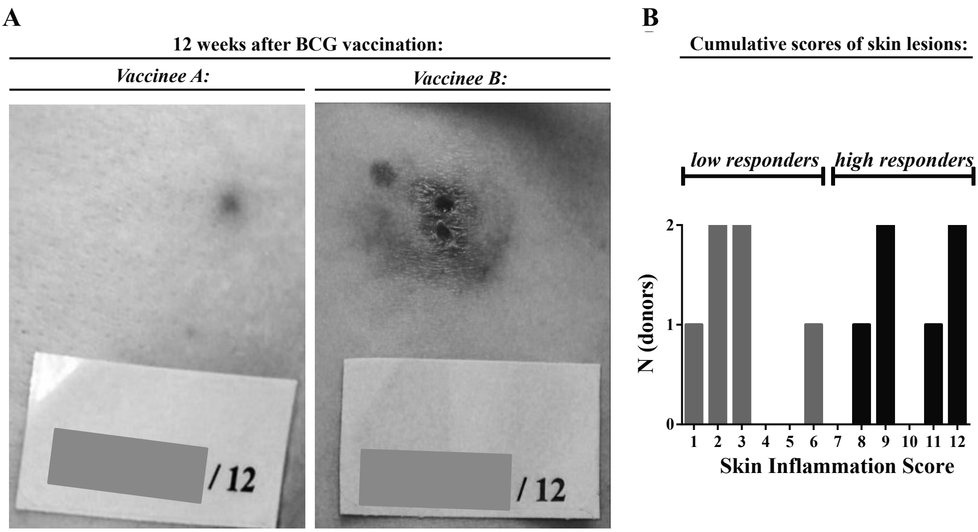


Figure 1. BCG-vaccination induces highly variable local inflammation that corresponds with serum CRP and IFNγ production. A: Photographs of low versus high degrees of skin inflammation at the vaccination site 12 weeks after BCG-vaccination (left photograph, cumulative inflammation score of 3; right photograph, cumulative inflammation score of 11). B: Signs of inflammation of the vaccination lesion were recorded, and a skin inflammation score was calculated as the cumulative scores of 4, 8, and 12 weeks after vaccination. This divided recipients into 6 low and 6 high responders around a median skin inflammation score of 7.

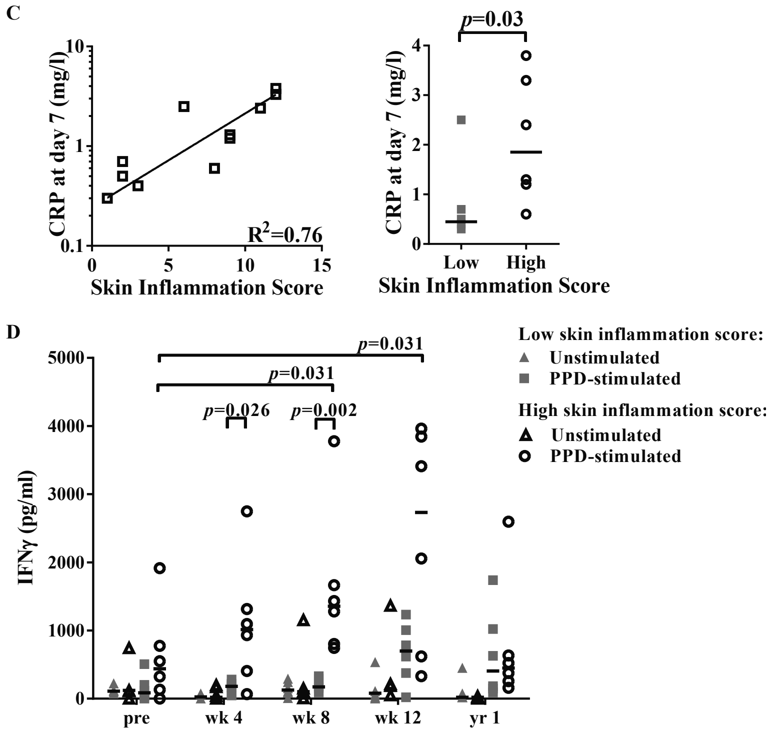


Figure 1 (*continued*). BCG-vaccination induces highly variable local inflammation that corresponds with serum CRP and IFNγ production. C: Serum samples of volunteers were collected at all visits for CRP ELISA. The serum CRP concentrations at day 7 after vaccination correlated with the cumulative skin inflammation scores (R^2 = 0.76 using nonlinear regression; left); high skin inflammation responders had significantly higher CRP values than low responders 7 days after vaccination (6 individuals in each group, with line at median; p = 0.03, Mann-Whitney test; right). D: PBMCs were stimulated with PPD, supernatants were harvested after 7 days, and IFNγ production was measured by ELISA. IFNγ production by PBMCs of high, compared to low skin inflammation responders was significantly higher at 4 and 8 weeks post-vaccination, and IFNγ production was significantly induced only in comparison to that at pre-vaccination in high skin inflammation responders (6 recipients in each group, line at median; Mann-Whitney test for comparison between groups, Wilcoxon signed-rank test for within-group testing).

$CD4^+IFN\gamma^+IL2^+TNF\alpha^+$ T-cells and $CD8^+IFN\gamma^+$ T-cells are induced in high inflammation responders

Whole-blood samples from pre-vaccination and at 4, 8, 12 weeks and 1 year after vaccination were stimulated directly *ex vivo* with live BCG for 16h. The gating strategy is shown in Supplementary figure S2A, and the representative flow cytometric analyses of co-expression of cytokines in $CD4^+$ T-cells with negative and positive controls are shown in Supplementary figure S2B, compliant with MIATA (minimal information about T-cell assays)-guidelines [33].

BCG-vaccination induced significant IFNγ-expression in $CD4^+$ T-cells, but only in high inflammation responders (p = 0.031, at 4 and 12 weeks post-vaccination) and not in low inflammation responders (figure 2A, left graphs). Polyfunctional $CD4^+IFN\gamma^+IL2^+TNF\alpha^+$ T-cells were significantly induced in BCG-recipients, but a division of the high versus low inflammation responders revealed that the proportion of $CD4^+IFN\gamma^+IL2^+TNF\alpha^+$ T-cells was significantly increased in the high versus low inflammation responders (p = 0.015, p = 0.048, and p = 0.041 at 4, 8, and 12 weeks after vaccination, resp.), but it was almost absent in the latter group. Thus, significant induction of $CD4^+IFN\gamma^+IL2^+TNF\alpha^+$ T-cells was confined to high inflammation responders (figure 2A, right graphs).

The majority of IFNγ, TNFα and/or IL2-expressing $CD4^+$ T-cell subsets of high inflammation responders peaked at 8 weeks post-vaccination (Supplementary figure S3A). Single-, double- and triple-cytokine-producing $CD4^+$ T-cell subsets consisted predominantly of effector cells at 4, 8 and 12 weeks post-vaccination (Supplementary figure S3B).

$CD4^+IL17A^+$ T-cells were induced significantly post-vaccination, but only in high inflammation responders (figure 2B). Similarly, significant induction of IFNγ-expression in $CD8^+$ T-cells was confined to high inflammation responders (figure 2B). No polyfunctional $CD8^+$ T-cell responses could be detected (data not shown). $CD4^+$ T-cells did not co-express IL17A and IFNγ (representative graph in Supplementary figure S2B). IL17A-expression correlated with IFNγ-expression in $CD4^+$ T-cells at 4, 8 and 12 weeks post-vaccination (figure 2C upper graphs; R^2 0.86, 0.94, and 0.83, resp., using nonlinear regression). IFNγ-expression in $CD8^+$ T-cells highly correlated with IFNγ-expression in $CD4^+$ T-cells (figure 2C lower graphs; R^2 0.98, 0.85, and 0.99 at 4, 8, and 12 weeks post-vaccination, resp., using nonlinear regression), thus revealing a broad pro-inflammatory response induced by BCG-vaccination in high inflammation responders. No induction of IL10- or IL4/IL13-expression was observed in $CD4^+$ T-cells in low or in high inflammation responders (figure 2D).

The association between skin inflammation score and induction of $CD4^+$ cytokine co-expression was further substantiated by segregating vaccinees based on induction versus no induction of polyfunctional CD4$^+$IFNγ$^+$IL2$^+$TNFα$^+$ T-cells instead of local inflammation scores. This revealed an increased total skin inflammation score in vaccinees with induction of CD4$^+$ polyfunctional T-cells, compared to vaccinees with no polyfunctional CD4$^+$ T-cell induction (n = 7 cytokine-responders versus n = 5 non-responders, including in the latter group a low skin inflammation responder with a skin inflammation score of 6; p = 0.037, Mann-Whitney test; Supplementary figure S4A). The differences in the CRP concentration 7 days after vaccination did not reach statistical significance in cytokine-responders vs. non-responders (Supplementary figure S4B; p = 0.078, Mann-Whitney test).

A

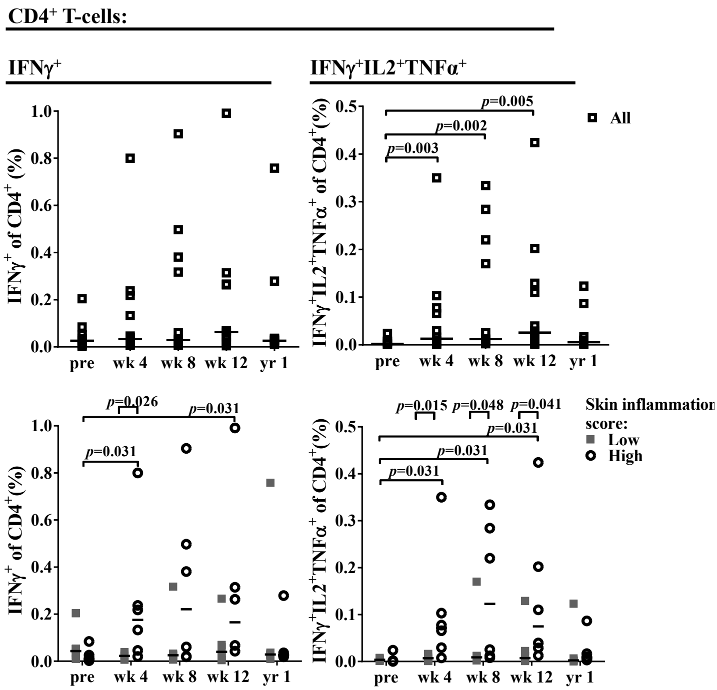
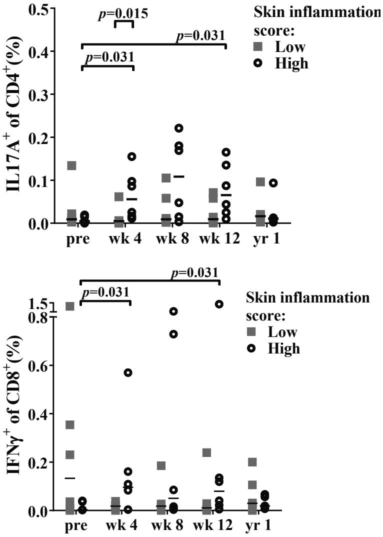


Figure 2. BCG-vaccination induces a pro-inflammatory cytokine response, predominantly in high skin inflammation responders. A: BCG-vaccination induces IFNγ$^+$ and IFNγ$^+$IL2$^+$TNFα$^+$ CD4$^+$ T-cells (upper graphs) in whole blood following BCG-stimulation for 16h, but the frequency of polyfunctional CD4$^+$ IFNγ$^+$IL2$^+$TNFα$^+$ T-cells is significantly increased in high, compared to low, skin inflammation responders, and the induction of CD4$^+$ IFNγ$^+$ and polyfunctional CD4$^+$ IFNγ$^+$IL2$^+$TNFα$^+$ T-cells is only significant in high, not in low, skin inflammation responders (lower graphs); n = 6 recipients in each group. Horizontal lines indicate median; Mann-Whitney test for comparison between groups, Wilcoxon signed-rank test for within-group testing.

B



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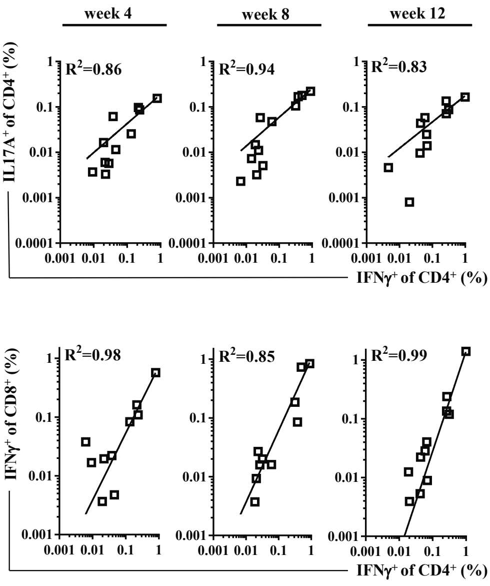

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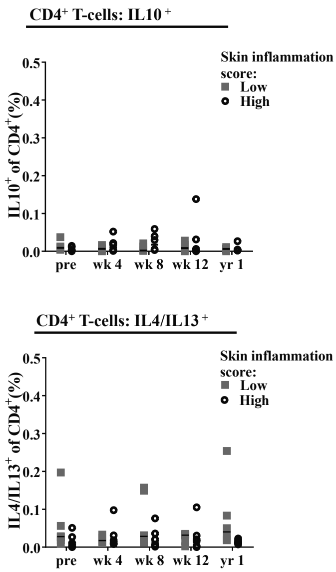

Figure 2 ***(continued)*****. BCG-vaccination induces a pro-inflammatory cytokine response, predominantly in high skin inflammation responders.** B: $CD4^+IL17A^+$ T-cells and $CD8^+IFN\gamma^+$ T-cells are significantly induced in high skin inflammation responders. C: Expression of the pro-inflammatory cytokines IL17A in $CD4^+$ T-cells and of IFNγ in $CD8^+$ T-cells both highly correlate with IFNγ-expression in $CD4^+$ T-cells, as determined by nonlinear regression. (Data are shown for all recipients at 4, 8, and 12 weeks after vaccination; whole-blood BCG-stimulation for 16h). Values of 0 were not plotted. D: In $CD4^+$ T-cells, neither IL4/IL13 nor IL10 was significantly induced in any responder group following whole-blood BCG stimulation for 16h.

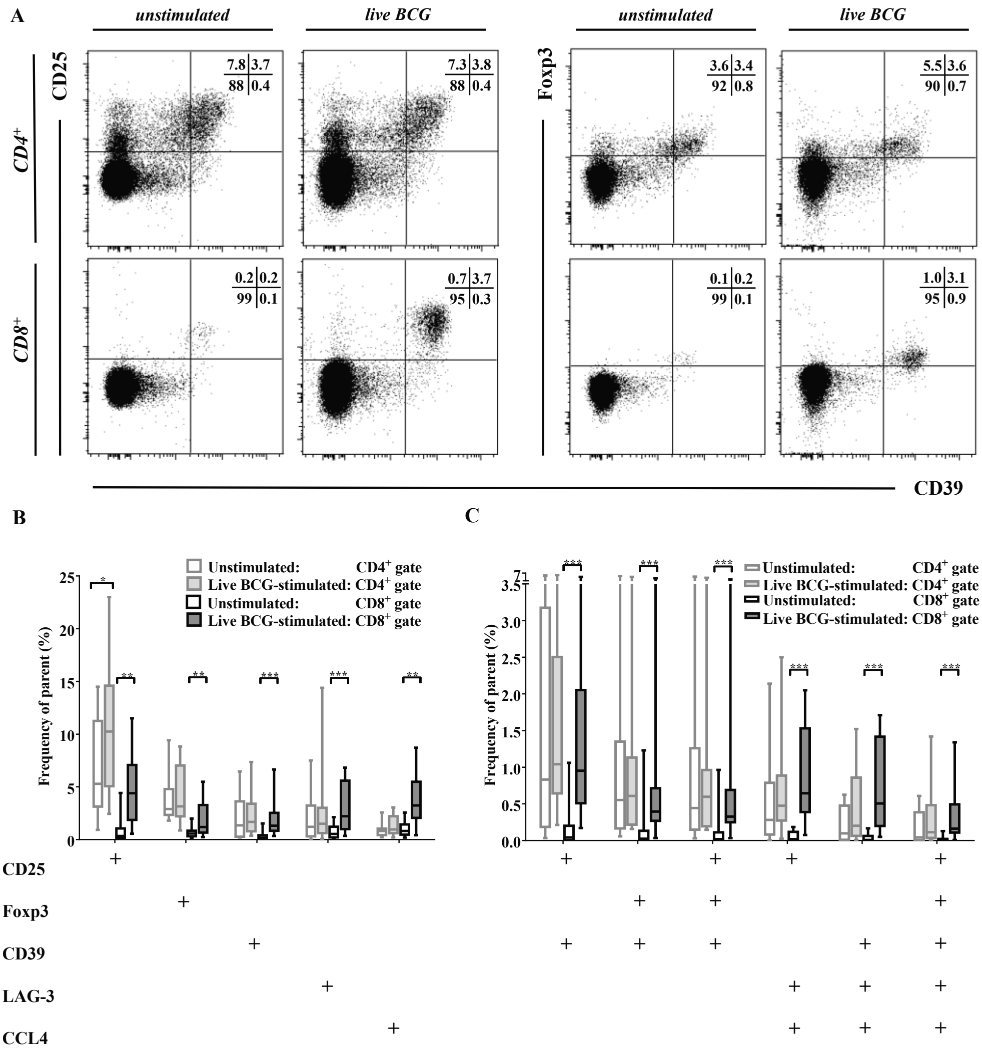

Figure 3. Treg markers on CD8$^+$ T-cells, but not on CD4$^+$ T-cells, are activated by *in vitro* live BCG-stimulation of PBMCs of vaccinees 8 weeks after vaccination. A: Flow cytometric analysis of (co-)expression of Treg markers CD25, CD39, and Foxp3 on CD4$^+$ and CD8$^+$ T-cells in unstimulated PBMCs and PBMCs stimulated for 6 days with live BCG. The gating strategy was similar as that described in Supplementary figure S2A, with the addition of a LIVE/DEAD vivid stain. B: Treg markers on CD8$^+$ but not CD4$^+$ T-cells were significantly activated 6 days after *in vitro* live BCG stimulation, compared to unstimulated PBMCs, at 8 weeks post-vaccination. On CD4$^+$ T-cells, only expression of CD25 was significantly different from unstimulated samples (box-whiskers of all individuals, with line at median; whiskers minimum (min) to maximum (max)). C: Co-expression of Treg markers is significantly activated by live BCG on CD8$^+$ but not CD4$^+$ T-cells 8 weeks after BCG-vaccination (box-whiskers of all individuals, with line at median; whiskers min to max) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Wilcoxon signed-rank test).

$CD8^+$, but not $CD4^+$ T-cells express regulatory markers after live BCG stimulation *in vitro*

PBMCs were stimulated for 6 days with live BCG to assess the expression of the regulatory markers CD25, Foxp3, CD39, lymphocyte activation gene-3 (LAG-3) and CCL4 (macrophage inflammatory protein-1β) by FACS-analysis [22;34;35] (gating strategy in Supplementary figure S2A). BCG-induced expression of Treg markers on $CD4^+$ and $CD8^+$ T-cells was then compared to that on unstimulated control samples at 8 weeks post-vaccination, the peak response (figure 3A). Expression of the single Treg markers CD25, Foxp3, CD39, LAG-3 and CCL4 was significantly induced on $CD8^+$ T-cells by *in vitro* live BCG stimulation 8 weeks post-vaccination ($p < 0.01$ for CD25, Foxp3 and CCL4; $p < 0.001$ for CD39 and LAG-3). In contrast, on $CD4^+$ T-cells only CD25 was significantly induced by BCG stimulation ($p < 0.05$) (figure 3B).

We then analysed co-expression of Treg markers using Boolean gating with synchronized gates on a per-donor basis for both $CD4^+$ and $CD8^+$ T-cells [22]. Co-expression of Treg markers was significantly induced on $CD8^+$ but not $CD4^+$ T-cells by *in vitro* live BCG stimulation 8 weeks post-vaccination (figure 3C; $p < 0.001$). On $CD4^+$ T-cells, no expression of $CD25^+Foxp3^+CD39^+$ was induced in either high or low inflammation responders (Supplementary figure S5).

$CD8^+CD25^+CD39^+Foxp3^+$ T-cells increase post-vaccination only in low inflammation responders

We then compared Treg markers on $CD8^+$ T-cells post- and pre-vaccination; figure 4A displays a representative flow cytometric analysis of induction of $CD25^+CD39^+$ co-expression and expression of Foxp3 on $CD25^+CD39^+$ $CD8^+$ T-cells from a low skin inflammation responder. Compared to pre-vaccination, BCG-vaccination significantly induced $CD8^+CD25^+Foxp3^+CD39^+$ T-cells as well as $CD8^+CD25^+Foxp3^+CD39^+LAG\text{-}3^+CCL4^+$ T-cells, but only in low inflammation responders (figure 4B; $p = 0.031$ at 8 weeks post-vaccination; $p = 0.031$ at 4 and 8 weeks post-vaccination, resp.). In contrast, in high inflammation responders, the expression of $CD25^+Foxp3^+CD39^+$ or $CD25^+Foxp3^+$ $CD39^+LAG\text{-}3^+CCL4^+$ on $CD8^+$ T-cells post-vaccination did not differ from the patterns pre-vaccination (figure 4B).

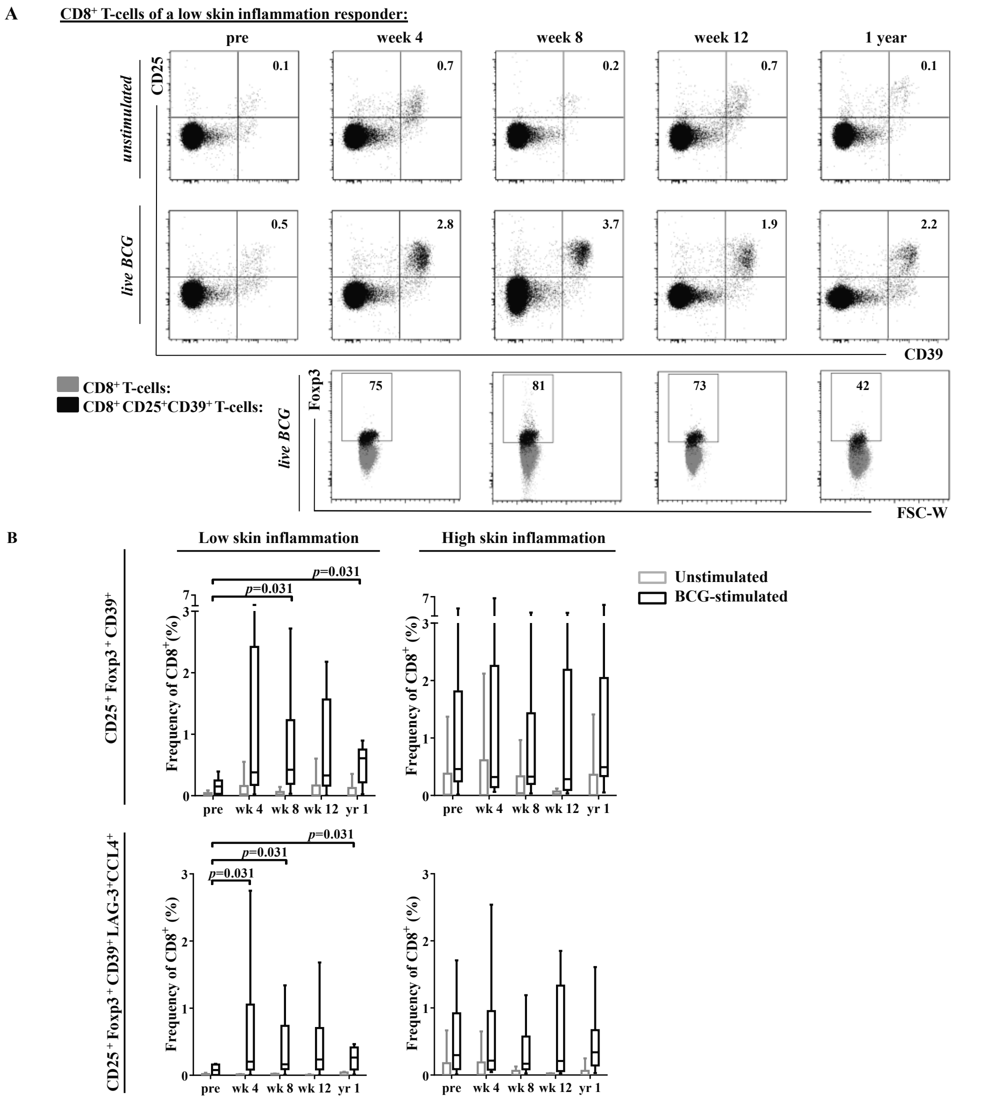

Figure 4. BCG-vaccination induces CD8$^+$CD25$^+$CD39$^+$Foxp3$^+$ T-cells in low inflammation responders.

A: Representative flow cytometric analysis of co-expression of the Treg markers CD25 and CD39 on CD8$^+$ T-cells of a recipient with a low degree of skin inflammation (upper graphs), and the percentage of CD8$^+$CD25$^+$CD39$^+$ T-cells expressing Foxp3 (lower graphs; black overlay of CD8$^+$CD25$^+$CD39$^+$ T-cells over total CD8$^+$ T-cells), 6 days after live BCG stimulation of PBMCs. B: Treg markers are significantly induced by BCG-vaccination on PBMCs stimulated with live BCG for 6 days, but only on CD8$^+$ T-cells from low inflammation responders. The frequency of Treg markers remained constant pre- and post-vaccination on CD8$^+$ T-cells from high inflammation responders. Depicted are CD25$^+$Foxp3$^+$CD39$^+$ co-expression (upper graphs) and CD25$^+$Foxp3$^+$CD39$^+$LAG-3$^+$CCL4$^+$ co-expression (lower graphs) on CD8$^+$ T-cells of low responders (left) versus high responders (right) (box-whiskers of 6 individuals in each group, with line at median; whiskers min to max; Wilcoxon signed-rank test).

Of interest, $CD8^+CD25^+Foxp3^+CD39^+$ T-cells and $CD8^+CD25^+Foxp3^+CD39^+LAG\text{-}3^+CCL4^+$ T-cells were still significantly increased 1 year post-vaccination in low inflammation responders (for both $p = 0.031$), suggesting that BCG-vaccination can induce long-term imprinting of $CD8^+$ Treg phenotypes in individuals with low inflammation following vaccination.

Discussion

In this study, we describe high inter-individual variability in T-cell cytokine and regulatory responses following BCG-vaccination of BCG-naive healthy young adults in a setting where TB is not endemic. The unexpectedly dichotomous T-cell response consisted of either concurrent induction of IL2-, TNFα- and IFNγ- co-expressing polyfunctional $CD4^+$ T-cell subsets, $CD4^+IL17A^+$ T-cells, and $CD8^+IFN\gamma^+$ T-cells in high inflammation responders, or an almost absent cytokine response accompanied by the induction of $CD8^+$ regulatory T-cells in low inflammation responders. We quantified local reactivity by classical clinical symptoms of inflammation and found that the total skin inflammation score correlated with serum CRP early post-vaccination. Significant induction of $IFN\gamma^+IL2^+TNF\alpha^+$-polyfunctional $CD4^+$ T-cells was confined to high inflammation responders, while the induction of regulatory-phenotype $CD8^+CD25^+CD39^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+CD39^+LAG\text{-}3^+CCL4^+$ T-cells was confined to low inflammation responders.

In theory, this study could have been limited by the description of T-cell responses based on the skin inflammation score, since dividing high and low skin inflammation groups using the median as cut off dichotomizes the described response. However, the dichotomy was also based on the induction of polyfunctional $CD4^+IFN\gamma^+IL2^+TNF\alpha^+$ T-cells, and this revealed a significantly increased total skin inflammation score in vaccinees with $CD4^+$ polyfunctional T-cells, compared to vaccinees with no polyfunctional $CD4^+$ T-cell induction. Thus, it is unlikely that the described variability in responses is caused by a dichotomized representation, and this further affirms the relation between skin reactivity and cytokine responses. Further, the opposing immune responses and phenotypes were observed within a relatively small cohort. Variability in BCG-immunogenicity has been ascribed to differences in pre-existing anti-mycobacterial responses in endemic vs. non-endemic settings [36;37], the presence of helminth infections [38], variations in the BCG-vaccine strain [39;40], and host genetic factors [41]. In addition, timing of sampling and technical variability may influence detection of cytokines [42;43]. This cohort, though small, was uniform in terms of age, genetic background, BCG-vaccine strain (Danish strain 1331), sampling and testing, in a setting not endemic for TB or helminth infections. This excludes the above-mentioned possible confounders, and points to an unexpectedly large variation in adult human primary BCG-vaccine induced immune responses.

Importantly, we confirmed vaccine take at 1 year post-vaccination by IFNγ-ELISpot, which was positive for both high and low inflammation responders. IFNγ-ELISpot has been described as the most sensitive assay for detecting long-term vaccine responses [29] and is used in TB-vaccine trials to describe the magnitude of vaccine-induced immunity. However, a sole reliance on IFNγ-ELISpot would disregard variability in other assays, thereby not fully capturing possible correlations between variation of the human immune response and vaccine-induced protection. The etiology of this variation remains unknown, but its unravelling could contribute significantly to a better understanding of BCG and related TB-vaccine induced immunity.

The height of the *in vitro* cytokine response in BCG-vaccinated infants was associated with scarring of the BCG-vaccination site, but only in response to mycobacterial antigens, not unrelated antigens [44]. Also, cell-mediated immunity, as assessed by a leukocyte migration inhibition test, correlated with infant local skin reactivity 8 weeks after BCG-vaccination, but not with TST-conversion after vaccination [45]. The absence of an association between BCG-induced TST-conversion and immunity against TB has been confirmed in various populations [46]. Here, induction of cytokine responses was confined to recipients with high skin reactivity, suggesting that a simple phenotype like vaccine-induced skin inflammation might be used as a marker of strong pro-inflammatory T-cell induction in adults. The skin inflammation score was associated with serum CRP concentration 7 days post-vaccination, thus the absence of an increase in CRP early post-vaccination might be used as an indicator of absent pro-inflammatory T-cell responses at later time-points.

Interference of $CD4^+$ Tregs with effector immunity has been described in active TB [47;48]. Following MVA-85A-vaccination circulating $CD4^+CD25^+Foxp3^+$ T-cells were increased in recipients with low antigen 85A-specific IFNγ-responses compared to high IFNγ-responders [28], and MVA-85A-induced $CD4^+$ Tregs inhibited IL17A-production *in vitro* [49]. Interestingly, IL10-producing $CD8^+$ Tregs were described in TB-patients anergic to intradermally injected PPD [50]. Thus, Tregs can interfere with inflammatory and specific antigen-induced cytokine responses. We previously reported *in vitro* activation of $CD8^+$ (but not $CD4^+$) Tregs by live BCG, both phenotypically and functionally, in mycobacterially-sensitized but not PPD-unresponsive donors [22;34;35]. These BCG-activated $CD8^+$ Tregs expressed CD25 and LAG-3 and inhibited Th1-responses through secretion of CCL4 [35]; in addition, we reported $CD8^+CD39^+$ Tregs which utilized CD39 to suppress Th1 proliferation [34]. Here, we found that $CD8^+CD25^+CD39^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+CD39^+LAG\text{-}3^+CCL4^+$ T-cells were induced following BCG-vaccination. Interestingly, the frequency of $CD8^+$ T-cells with these Treg phenotypes was significantly increased only in comparison to that at pre-vaccination in low inflammation responders with low to absent cytokine responses, suggesting an inverse relation between the induction of $CD8^+$ Tregs and BCG-induced skin inflammation with T-cell cytokine production.

In murine leishmaniasis, cytokine-producing polyfunctional T-cells were inversely correlated with lesion size after (dermal) challenge [6]. In dermal BCG-challenge models in humans, vaccination-induced IFNγ-ELISpot-responses were inversely correlated with PCR quantification of BCG-load in biopsy specimens of the challenge site [51]. The PCR quantification method was suggested as a measure of pathogen clearance, possibly reflecting some degree of protective immunity, which might be used in human TB-vaccine trials. Based on the current study, it will also be relevant to assess the presence of pro-inflammatory vs. regulatory T-cells in skin vaccine or challenge lesions and to further validate the modulation of skin inflammation and/or pathogen clearance by $CD8^+$ Tregs in relevant models. Of note, in low inflammation responders $CD8^+CD25^+Foxp3^+CD39^+$ T-cells were still significantly increased at 1 year post-vaccination, suggesting that BCG-vaccination can induce long-term imprinting of a $CD8^+$ Treg phenotype with a significant memory component. Further work is needed to assess their precise longevity.

In conclusion, our results show an unexpectedly dichotomous host response to BCG-vaccination in a cohort of BCG-naive adults. It will be important to assess these divergent outcomes in settings where TB is endemic in order to determine the impact of these highly variable outcomes on protective efficacy against TB. The use of classical inflammation markers as non-classical indicators of vaccine-induced pro-inflammatory responses might be a simple means to assist in assessing BCG-induced phenotypes, even in small cohorts. Further detailed fine mapping of the heterogeneous host response to BCG-vaccination using classical and non-classical immune markers will enhance our understanding of the mechanisms and determinants that underlie the induction of apparently opposite immune responses and how these impact the ability of BCG to induce protective immunity to TB.

Acknowledgements

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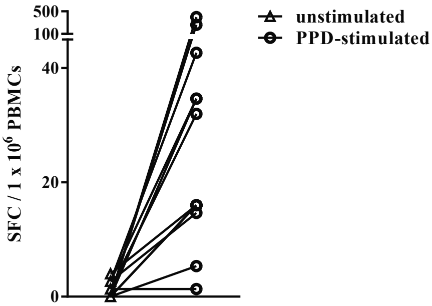
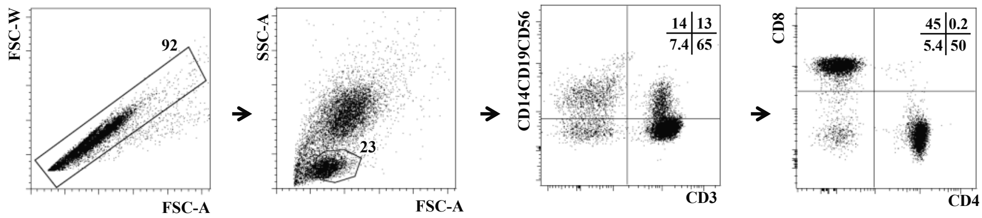
Supplementary figures

S1

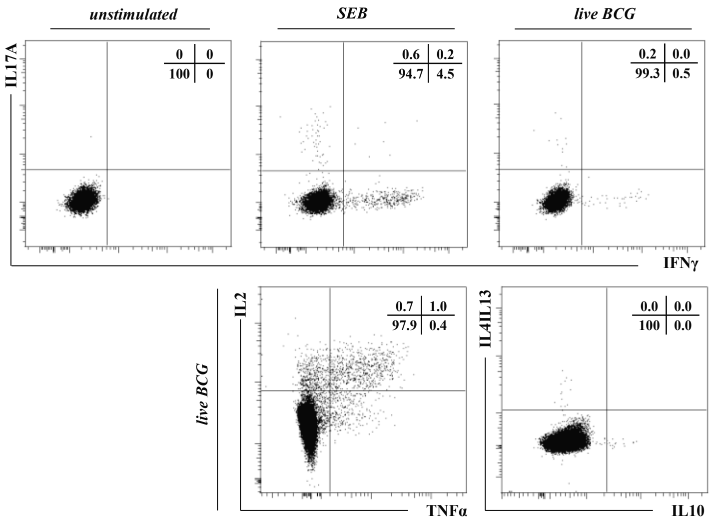


Supplementary figure S1. IFNγ-ELISpot results at 1 year after vaccination. *Ex vivo* IFNγ-ELISpot assays using freshly isolated PBMCs were performed 1 year after vaccination to verify vaccine-induced immunity. The person that lacked positive response by ELISpot demonstrated high IFNγ-responses in flow cytometric analysis, indicating vaccine take for this person.

S2A



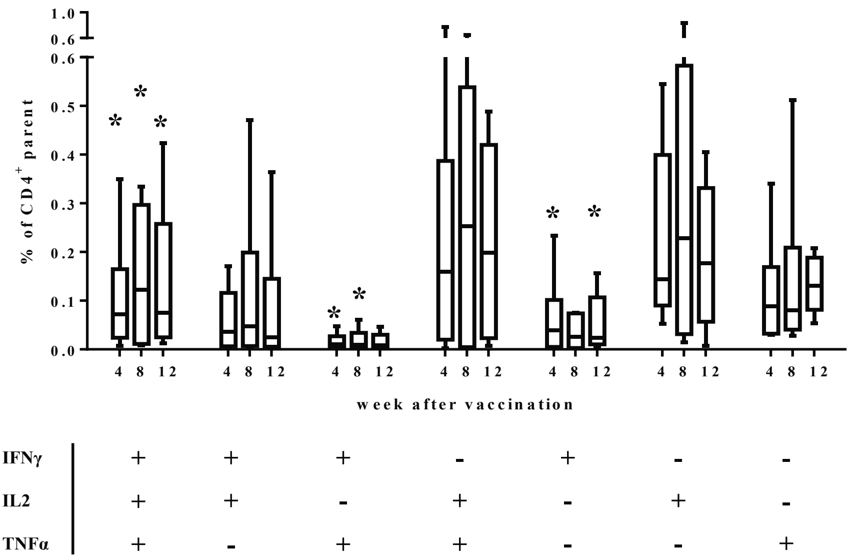
S2B



Supplementary figure S2. Gating strategy and cytokine expression. S2A, gating strategy: for flow cytometric analysis of fixed whole blood samples, cells were gated on single cells, lymphocytes, $CD3^+$ vs. CD14-CD19-CD56-negative, and $CD8^+CD4^-$ vs. $CD4^+CD8^-$. For PBMCs six days after live BCG-stimulation, a violet-live/dead-stain was added prior to extracellular staining and cells were gated on $CD3^+$ vs. (live/)dead-CD14-CD19-CD56-negative. S2B, expression of cytokines in $CD4^+$ T-cells: cytokine production by $CD4^+$ T-cells after overnight live BCG-stimulation of whole blood; also depicted are unstimulated and SEB controls. No co-expression was observed of IFNγ and IL17A.

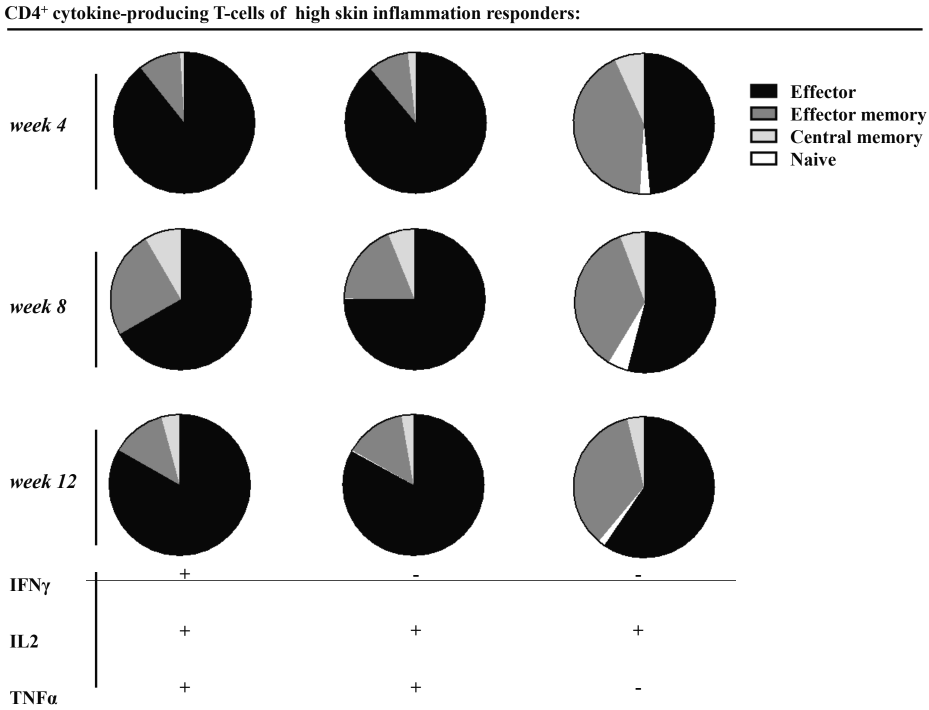
S3A

CD4$^+$ T cells of high skin inflammation responders:



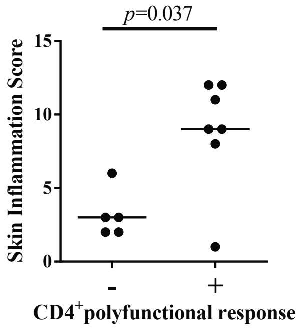
Supplementary figure S3. CD4$^+$ cytokine-producing subsets and memory compartment in high skin inflammation responders. Supplementary S3A, CD4$^+$ cytokine-producing subsets; whole-blood samples stimulated with live BCG for 16 hours and subsequent intracellular cytokine staining: almost all BCG-induced CD4$^+$ cytokine-producing subsets peaked at 8 weeks after vaccination. CD4$^+$ cytokine-producing subsets of high skin inflammation responders at 4, 8 and 12 weeks after vaccination were compared with pre-vaccination (*$p < 0.05$ in Friedman with Dunn's multiple comparison test).

S3B

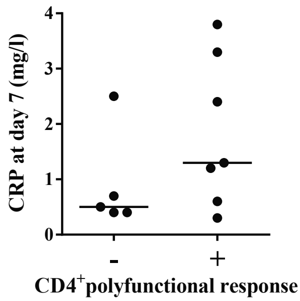


Supplementary figure S3 (*continued*). CD4$^+$ cytokine-producing subsets and memory compartment in high skin inflammation responders. Supplementary S3B, memory compartments of CD4$^+$ cytokine-producing subsets: the majority of single-, double- and triple-cytokine producing T-cells are effector cells, in whole-blood samples stimulated with live BCG for 16 hours. Pie chart representation of the proportion of effector (CD69$^+$), effector memory (CD69$^-$CD45RA$^-$CD62L$^-$), central memory (CD69$^-$CD45RA$^-$CD62L$^+$) and naïve (CD69$^-$ CD45RA$^+$CD62L$^+$) CD4$^+$ T-cells for different cytokine-producing subsets as derived by Boolean gate analysis. Only populations of > 50 cells were included for analysis with a minimum of 3 donors per visit. Effector cell proportions were not significantly different between cytokine-producing subsets or between visits (p < 0.05 deemed significant, Kruskal-Wallis with Dunn's multiple comparisons test).

S4A

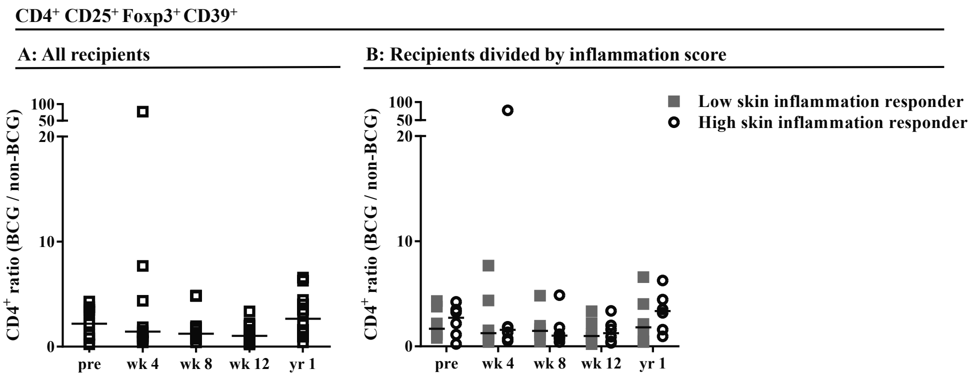


S4 B



Supplementary figure S4. Higher total skin inflammation score in vaccinees with $CD4^+$ polyfunctional T-cell induction. Vaccinees were divided by $IFN\gamma^+IL2^+TNFa^+$ $CD4^+$ T-cell induction, and (cumulative) skin inflammation scores (A) and CRP concentration at day 7 (B) were compared. The skin inflammation score was significantly higher in vaccinees with induction of $CD4^+$ polyfunctional T-cells, compared to vaccinees with no induction of $CD4^+$ polyfunctional T-cells (threshold for $CD4^+$ T-cell induction set at change from pre-vaccination larger than the highest pre-vaccination value; 7 responders vs. 5 non-responders in each graph with line at median; Mann-Whitney test).

S5



Supplementary figure S5. $CD25^+CD39^+Foxp3^+$ expression on $CD4^+$ T-cells as ratio BCG-stimulated : non-stimulated. Co-expression of CD25, Foxp3 and CD39 on $CD4^+$ T-cells (on day 6 following PBMC stimulation with or without live BCG) was analyzed as the ratio BCG-stimulated : non-stimulated. BCG-vaccination did not induce significant induction compared to pre-vaccination of $CD4^+CD25^+Foxp3^+CD39^+$ T-cells (A); also, dividing recipients in high vs. low inflammation scores did not reveal any significant induction of Treg marker expression on $CD4^+$ T-cells as compared to pre-vaccination (B) (dot plots with line at median for all recipients (A; n = 12) and recipients divided by skin inflammation score (B; 6 individuals in each group); significance considered as $p <$ 0.05 in Friedman with Dunn's multiple comparisons test).

CHAPTER 6

KLRG1 and PD-1 expression are increased on T-cells following tuberculosis-treatment and identify cells with different proliferative capacities in BCG-vaccinated adults

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Submitted

Abstract

In cancer and chronic infectious diseases, immune checkpoint-blockade of inhibitory receptors can enhance T-cell immunity. In tuberculosis (TB), a chronic infectious disease, prolonged antigen exposure can potentially drive terminal T-cell differentiation towards functional 'exhaustion': in human TB T-cells express PD-1 (programmed cell death protein-1) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4). However, in murine TB not PD-1 but rather killer cell lectin-like receptor subfamily-G1 (KLRG1) was a superior indicator of terminal T-cell differentiation. We therefore compared expression of KLRG1, PD-1 and CTLA-4 on T-cells in different stages of human TB, and also analyzed their induction following BCG-vaccination. KLRG1, PD-1 and CTLA-4 expression were highest on *in vitro* BCG-stimulated $CD4^+$ T-cells following recent TB-treatment; KLRG1 and PD-1 expression on $CD4^+$ T-cells in active - but not latent - TB were only slightly increased compared to healthy donors. BCG-vaccination induced KLRG1 expression on BCG-stimulated $CD8^+$ but not $CD4^+$ T-cells, while neither PD-1 nor CTLA-4 expression increased. KLRG1-expressing $CD8^+$ T-cells exhibited markedly decreased proliferation, whereas PD-1^+ T-cells proliferated after *in vitro* BCG stimulation. Thus, we demonstrate the presence of increased KLRG1-expressing T-cells in TB-treated individuals, and present KLRG1 as a marker of decreased human T-cell proliferation following BCG-vaccination. These results expand our understanding of cell-mediated immune control of mycobacterial infections.

Introduction

Based on the tuberculin skin test (TST), it is estimated that one-third of the world population is latently infected with *Mycobacterium tuberculosis* (Mtb), the bacterium causing tuberculosis (TB) [1]. Among latently infected (HIV-negative) individuals 3 - 10% will develop active TB during their life-time [1;2], but it is not yet clear what determines the loss of immune control resulting in active disease versus latent, contained infection [3;4]. In chronic viral infections and cancers many studies have reported the existence of T-cells expressing inhibitory receptors, also called terminally differentiated or 'exhausted' T-cells, which are thought to be impaired in their proliferative capacity and/or function (referred to also as the chronic (infection) phenotype) [5]. Signaling through inhibitory receptors, such as PD-1 (programmed cell death protein 1; CD279) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4; CD152) diminishes the response of activated T-cells [6]; and blocking of ligand-receptor interactions through monoclonal antibodies to re-activate and enhance the lymphocyte response has received great attention as immunotherapy in cancer [7]. The clinical impact of this immune checkpoint blockade, especially with αPD-1-, αCTLA-4-, αPDL1- and αPDL2- antibodies, or combinations thereof, has already been demonstrated in cancer patients, and to a lesser extent in patients with chronic infectious disease ([8;9], clinical trials of both are summarized in [5]).

PD-1 has been extensively described as inhibitory marker on CD8^{+} as well as CD4^{+} T-cells in human chronic viral infections such as chronic HIV, hepatitis B (HBV) and hepatitis C (HCV) infection [10-13]. Also on CD4^{+} and CD8^{+} T-cells from patients with pulmonary TB, increased expression of PD-1 compared to healthy donors has been described [14;15]. *In vitro* blocking with antibodies against PD-1 or it's ligands enhanced production of IFNγ, IL2 [14] and IL17 [15], inhibited apoptosis of IFNγ-producing T-cells [14] and enhanced Mtb-stimulated degranulation of CD8^{+} T-cells [16]. In addition, increased expression of CTLA-4 (on regulatory T-cells) has been reported in patients with active TB [17], as well as in latently infected individuals [18;19], compared to healthy donors.

However, in murine TB infection not PD-1 but rather expression of KLRG1 (killer cell lectin-like receptor subfamily G member -1) marked terminally differentiated CD4^{+} T-cells, which had a short life span and high T-bet expression [20-22]. KLRG1 is expressed on murine and human NK-cells and antigen-experienced CD4^{+} and CD8^{+} T-cells [23].

Ligation of KLRG1 with cadherins (ubiquitously expressed cell adhesion molecules [24]) triggers inhibitory signaling through decreased AKT (Ser473) phosphorylation [25], which is reversible through blocking KLRG1-ligand interaction [26]. In contrast to other chronic murine infection models, murine Mtb-specific PD-1^{+} T-cells were found to be proliferative, whereas KLRG1^{+} effector T-cells were reduced in their proliferative capacity; and following adoptive transfer in Mtb-infected mice, donor CD4^{+}PD1^{+}KLRG1^{LO} T-cells downregulated PD-1 expression, but increased KLRG1 expression [20]. Moreover, decreased protective efficacy against TB-challenge by ESAT-6 specific KLRG1^{+} T-cells, compared to ESAT-6 specific PD-1^{+} T-cells, following murine adoptive transfer was recently demonstrated [21]. Only adoptive transfer into Mtb-infected but not uninfected mice steered differentiation of PD-1^{+} into KLRG1^{+} T-cells [20;21], indicating that exposure to mycobacterial antigen is essential in steering towards terminal differentiation.

Thus, KLRG1 has been described as a key inhibitory marker of non-proliferating cells during murine Mtb infection; also after murine BCG-vaccination CD4^{+} and CD8^{+} T-cells upregulated KLRG1 expression [27]. In human mycobacterial infection, the existence of KLRG1^{+} T-cells has been suggested, but not compared to relevant control groups or complemented with functional data [28]. Here, we compared expression of KLRG1, PD-1 and CTLA-4 on human T-cells in different stages of TB infection, and assessed mycobacterial induction of these cellular phenotypes in humans by comparing expression before and after BCG-vaccination of BCG-naive healthy adult volunteers. In addition, we compared the proliferative capacity of KLRG1-expressing and PD-1-expressing T-cells following BCG-vaccination. We found increased expression of KLRG1, PD-1 and CTLA-4 in individuals that had recently been treated for TB, and minor increased expression in active TB patients. BCG-vaccination significantly induced CD8^{+}KLRG1^{+} T-cells, which unlike CD8^{+}PD-1^{+} T-cells, were markedly reduced in their proliferative capacity. Thus, we demonstrate for the first time KLRG1 as marker of non-proliferating T-cells following BCG-vaccination in humans.

Materials and Methods

Patients and participants. This study was approved by the Ethical Committee of the L. Spallanzani National Institute of Infectious diseases ((INMI); approval number 10/2010) and the Medical Ethical Committee of the Leiden University Medical Center, the Netherlands (registration number P 12.87). Written informed consent was obtained from all participants in the study. All blood bank donors had signed consent for scientific use of blood products.

In this study, active pulmonary TB was sputum culture confirmed and patients were enrolled within 7 days of starting treatment. Cured-TB subjects were patients who had completed 6 months of treatment for culture-positive pulmonary TB and who resulted sputum culture-negative during treatment. These patients were evaluated after therapy completion (median time post-treatment 8 ± IQR 2.5 - 13.5 months). Latent Mtb infection was defined based on a positive response to QuantiFERON-TB gold in-tube test (QFT-IT) (Qiagen, Hilden, Germany) in healthy subjects without radiological signs of active disease [29]. Demographic and epidemiological information was collected at enrolment (Table 1). QFT-IT-negative contacts and healthy volunteers, as well as healthy adult blood bank donors (Sanquin blood bank, Leiden) that tested negative for recognition of mycobacterial PPD *in vitro*, were used as controls. PPD-reactivity was tested by stimulation of PBMCs with 5 μg/ml PPD (Statens Serum Institute, Copenhagen, Denmark) for 6 days and supernatants were tested in IFNγ-ELISA (U-CyTech, Utrecht, the Netherlands). Negativity was defined as IFNγ-production ≤ 150 pg/ml.

For the study on the BCG-vaccination of Dutch volunteers, the recruitment occurred via posters in the university library. Included volunteers (6 males, 6 females; median age 24 ± IQR 23 - 25 yrs) were healthy, and had not been vaccinated with BCG at any time prior to enrolment, or with any live vaccination four weeks or less prior to BCG-vaccination. Volunteers were screened for TB by anamnesis, by tuberculin skin test (negative < 5mm) and by QFT-IT test. All patients and participants tested negative for HIV at screening and were not treated with immune-modulating drugs prior to enrolment.

Procedures. BCG-vaccination with the live-attenuated BCG Danish strain 1331 (Statens Serum Institute) was by intradermal injection in the upper arm. Volunteers were followed at two weeks prior to vaccination, at four, eight and twelve weeks and at one year after vaccination. Venous blood samples of patients and participants were collected for PBMC isolation by ficoll density centrifugation. PBMCs were cryopreserved in 10% DMSO and 20% fetal calf serum-supplemented medium.

Cell cultures and BCG-infection. PBMCs were thawed, counted (CASY cell counter, Roche, Woerden, the Netherlands) and *in vitro* infected with BCG. PBMCs were infected at a multiplicity of infection (MOI) of 1.5 - 3 and cultured in 24 well plates (2 x 10^6/w) for six days in Iscove's modified Dulbecco's medium (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) with 10% human serum (pooled serum from blood bank donors, pretested for inhibitory activity) in an incubator at 37^0C with 5% CO_2 [30;31].

Flow cytometry. Stimulated PBMCs from TB patients, individuals with latent TB, individuals treated for TB, and healthy donors were labelled with violet live/dead stain (VIVID, Invitrogen) and surface stained with CD3-Brilliant Violet 570 (clone UCHT1), CD19-Pacific Blue (clone HIB19), KLRG1-Pe-Cy7 (clone 2F1/KLRG1) (all Biolegend, London, U.K.); CD56-Alexa 700 (clone B159), CD8-HorizonV500 (clone RPA-T8), PD-1-PerCp-Cy5.5 (clone EH12.1) (all BD Biosciences, Eerembodegem, Belgium); CD14-Pacific Blue (clone TüK4) and CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen). Cells were fixed and permeabilized using FIX&PERM® Cell Permeabilization Kit (An Der Grub BioResearch GMBH, Susteren, the Netherlands). For intracellular staining CTLA-4 (cytotoxic T-lymphocyte protein 4; CD152)-PeCy5 (clone BNI3; BD Biosciences) was used.
Stimulated PBMCs from BCG-vaccinated participants were labelled with violet live/dead stain (VIVID) after incubation for the last 16 hours with αCD3/28 beads (Invitrogen) and Brefeldin A (3 µg/ml, Sigma-Aldrich, Zwijndrecht, the Netherlands). PBMCs were surface stained with CD3-Brilliant Violet 570 (clone UCHT1), CD19-Pacific Blue (clone HIB19), CD56-Brilliant Violet 421 (clone HCD56), KLRG1-Pe-Cy7 (clone 2F1/KLRG1) (all Biolegend, London, U.K.); CD14-Pacific Blue (clone TüK4), CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen); CD8-HorizonV500 (clone RPA-T8) and PD-1-PerCp-Cy5.5 (clone EH12.1) (both BD Biosciences). Fixation, permeabilization and intracellular staining were performed as described above. Samples were acquired on a BD

LSRFortessa using FACSDiva software (version 6.2, BD Biosciences). Analyses were performed using FlowJo software (version 9.5.3, Treestar, Ashland, OR, USA). Gates were defined per donor for CD4$^+$ and CD8$^+$ T-cell subsets (and for all visits after vaccination) using unstimulated samples and SEB (Toxin Technology, Sarasota, FL, USA)-stimulated samples as controls.

CFSE proliferation assays. Stimulated PBMCs from BCG-vaccinated participants were harvested, labelled with 5 µM of CFSE, and cultured in 96 well plates (1 x 10^5/w) in Iscove's modified Dulbecco's medium (Life Technologies-Invitrogen) with 10% pooled human serum. PHA (Remel Europe) was used as a positive control for proliferation of CFSE-labelled cells. After 6 days cultures were harvested and stained with KLRG1-Pe-Cy7 (clone 2F1/KLRG1) and CD56-Brilliant Violet 650 (clone HCD56) (both Biolegend, London, U.K.); CD14-Pacific Blue (clone TüK4), CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen); CD8-HorizonV500 (clone RPA-T8), PD-1-Alexa 647 (clone EH12.1) and CD3-PeCy5 (clone UCHT1) (all BD Biosciences). Samples were acquired on a BD LSRFortessa using FACSDiva software. Analyses were performed using FlowJo software (version 9.5.3, Treestar).

Analyses. Using GraphPad Prism (version 6, GraphPad Software, La Jolla, CA, USA) Mann-Whitney tests were used for non-paired samples; Kruskal-Wallis with Dunn's multiple test correction was used for non-parametric unpaired comparisons between more than 2 groups. Wilcoxon signed-rank tests were used for paired samples, after correction for paired multiple testing by Friedman with Dunn's multiple comparisons tests.

Results

Expression of the inhibitory markers KLRG1, PD-1 and CTLA-4 after *in vitro* stimulation of PBMCs

PBMCs were isolated from patients with active TB (n = 11); individuals with latent TB infection (n = 13); successfully treated cured-TB subjects (n = 15; median time post-treatment 8 ± IQR 2.5 - 13.5 months); and healthy donors (n = 16). All samples from active

TB patients were collected within the first week of treatment. Further demographic and clinical characteristics of the subjects enrolled in this study are displayed in Table 1.

	HD*	LTBI	Active TB	Cured TB	Total	*p*-value
Enrolled subjects	6*	13	11	15	45	
Median age (IQR)	36 (26-51)	36 (28-48)	37 (30.5-44.5)	33 (25-49.5)	35 (28-48)	**0.95**[1]
Female gender (%)	2 (33)	11 (85)	2 (18)	10 (67)	25 (65)	**0.006**[2]
Origin N (%)						**0.09**[2]
Western Europe	4 (67)	8 (62)	-	5 (33)	17 (38)	
Eastern Europe	1 (17)	3 (23)	8 (73)	5 (33)	17 (38)	
Asia	-	-	1 (9)	-	1 (2)	
Africa	-	1 (8)	1 (9)	3 (20)	5 (10)	
South America	1 (17)	1 (8)	1 (9)	2 (13)	5 (10)	
BCG N (%)						**0.015**[2]
Vaccinated	3 (50)	5	11 (100)	10 (67)	29 (64)	
Unvaccinated	3 (50)	8	0 (0)	5 (33)	16 (36)	

Table 1. Demographic and clinical characteristics of the subjects enrolled in the study.
Footnotes: TB: Tuberculosis; LTBI: Latent Tuberculosis Infection; IQR: Interquartile range; BCG: Bacillus Calmette-Guérin. *Additional healthy donors were included in the study: anonymous samples were obtained from the Dutch blood bank, therefore no information was available in terms of age, sex, origin and BCG status.[1]Kruskal-Wallis test; [2] Chi-square test.

PBMCs were stimulated with live *M. bovis* BCG (BCG) and analyzed for expression of KLRG1, PD-1 and CTLA-4 after six days. The gating strategy for flowcytometric analysis is shown in figure 1A and B, and is compliant with recent MIATA guidelines [32]. Activation of CD4$^+$ and CD8$^+$ T-cells was evaluated by calculating the CD4$^+$ or CD8$^+$ frequency of CD3$^+$ parent as CD4$^+$: CD8$^+$ ratio; this was similar between groups (data not shown). Positive populations of KLRG1, PD-1 and CTLA-4 expression by CD4$^+$ and CD8$^+$ T-cells were defined by comparison with unstimulated samples (figure 1B), and gates were similar per donor for CD4$^+$ and CD8$^+$ T-cells.

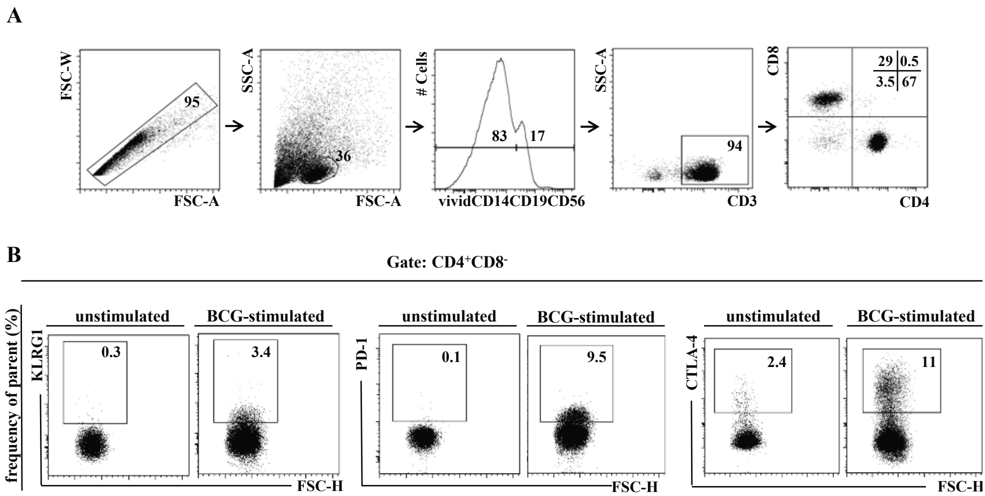

Figure 1. Expression of KLRG1, PD-1 and CTLA-4 after *in vitro* stimulation of PBMCs. A: Gating strategy for flowcytometric analysis of PBMCs six days after *in vitro* stimulation with live BCG. Cells were gated on single cells, lymphocytes, violet-(live/)dead- CD14- CD19- CD56-negative, CD3$^+$, and CD4$^+$CD8$^-$ vs. CD8$^+$CD4$^-$. B: Positive populations for KLRG1, PD-1 and CTLA-4 expression were defined by comparison with unstimulated samples, as demonstrated for expression of KLRG1, PD-1 and CTLA-4 on CD4$^+$ T-cells six days after BCG stimulation of PBMCs of a cured-TB patient.

Highest expression of KLRG1, PD-1 and CTLA-4 is found on $CD4^+$ T-cells in TB-treated individuals

The expression of KLRG1 following six day *in vitro* BCG stimulation was significantly increased on $CD4^+$ T-cells from cured-TB individuals (*p* = 0.030 between groups in Kruskal-Wallis test with Dunn's multiple test correction; *p* = 0.018 for cured-TB individuals compared to healthy donors in Mann-Whitney test; figure 2A). The frequency in BCG-stimulated samples relative to the frequency in unstimulated samples (expressed as the ratio BCG : non-BCG) was compared between groups. This demonstrated a trend towards an increased ratio of KLRG1 expression on $CD4^+$ T-cells and $CD8^+$ T-cells from cured-TB subjects, compared to healthy donors (*p* = 0.019 and *p* = 0.024 resp., Mann-Whitney test), but this was not significant after multiple test correction (figure 2A).

There was an interesting trend towards an increased frequency of $CD4^+KLRG1^+$ T-cells in active TB patients - and not in individuals with latent TB infection - compared to healthy donors (*p* = 0.020, Mann-Whitney test, not significant following multiple test correction). On $CD8^+$ T-cells KLRG1 expression was not significantly different in individuals with active or latent infection compared to healthy donors (figure 2A).

A similar pattern was observed for PD-1: distinctly increased expression on $CD4^+$ T-cells in cured-TB subjects (*p* = 0.039 between groups in Kruskal-Wallis test with Dunn's multiple test correction; *p* = 0.017 for cured-TB individuals compared to healthy donors in Mann-Whitney test); and a trend towards an increased ratio (BCG : non-BCG) of $CD4^+$ T-cells and $CD8^+$ T-cells expressing PD-1 in cured-TB subjects compared to healthy donors (*p* = 0.015 and *p* = 0.010 resp., Mann-Whitney test; not significant following multiple test correction; figure 2B). Similarly, in patients with active TB disease $CD4^+$ T-cells - but not $CD8^+$ T-cells - exhibited a trend towards increased expression of PD-1 compared to healthy donors (*p* = 0.031, Mann-Whitney test; not significant following multiple test correction).

In cured-TB subjects the frequency of CTLA-4-expressing $CD4^+$ T-cells was highest also (*p* = 0.017 between groups in Kruskal-Wallis test with Dunn's multiple test correction; *p* = 0.002 for cured-TB individuals compared to healthy donors in Mann-Whitney test; figure 2C). $CD8^+$ T-cells in cured-TB individuals exhibited a trend towards an increased frequency and ratio of CTLA-4 expression compared to healthy donors (*p* = 0.014 and *p* = 0.019 resp., Mann-Whitney test; not significant following multiple test correction; figure 2C). In latently infected individuals but not in active TB patients, the frequency of CTLA-4

-expressing CD4+ T-cells was moderately increased (p = 0.025, Mann-Whitney test; not significant following multiple test correction; figure 2C).

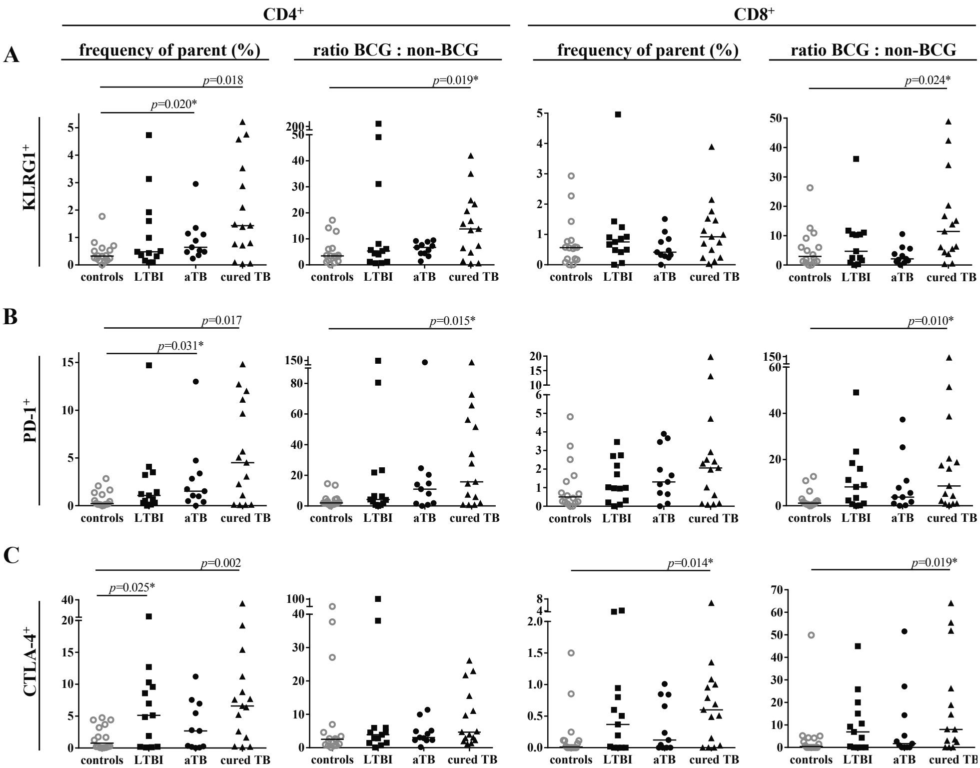

Figure 2. Increased expression of KLRG1, PD-1 and CTLA-4 on T-cells from cured-TB patients. Expression of KLRG1, PD-1 and CTLA-4 was highest on six day BCG-stimulated CD4+ T-cells from cured-TB subjects. There was a trend towards an increased BCG : non-BCG ratio (as frequency in BCG-stimulated samples relative to the frequency in unstimulated samples) of KLRG1 and PD-1 expression on CD4+ and CD8+ T-cells, and of CTLA-4 expression on CD8+ T-cells, in cured-TB subjects compared to healthy donors. Active TB patients exhibited slightly increased expression of KLRG1 and PD-1 on CD4+ T-cells. Data are shown as scatter plots with line at median for 11 patients with active TB, 13 individuals with latent TB infection, and 15 cured-TB subjects, compared with 16 healthy donors (Mann-Whitney tests; *indicates not significant following Kruskal-Wallis test with Dunn's multiple test correction).

Four out of 13 latently infected individuals received preventive therapy with isoniazid, but no trend or significant association was observed between preventive therapy and expression of KLRG1, PD-1 or CTLA-4. In addition, in cured-TB subjects no association was observed between inhibitory marker expression and either time post-treatment (median 8 ± IQR 2.5 - 13.5 months) or age (median 33 ± IQR 25 - 49.5 yrs) (data not shown).

BCG-vaccination induces expression of KLRG1 on CD8$^+$ T-cells in BCG-naive healthy adults

To assess the mycobacterial induction of inhibitory molecules on human T-cells, we compared the cellular expression of the above markers before and after BCG-vaccination of healthy adult volunteers. Only among CD8$^+$ T-cells a significantly increased frequency of KLRG1-expressing cells was found, at 4, 8 and 12 weeks post-vaccination compared to pre-vaccination baseline (p = 0.006 in Friedman test with Dunn's multiple test correction; p = 0.001, p = 0.002, p = 0.009 in Wilcoxon signed-rank tests resp. 4, 8 and 12 weeks post-vaccination compared to pre-vaccination; figure 3A), following *in vitro*-stimulation with BCG. Also KLRG1 expression relative to unstimulated cells (ratio BCG-stimulated : unstimulated) was significantly induced on CD8$^+$ but not CD4$^+$ T-cells compared to pre-vaccination (p = 0.050 in Friedman test with Dunn's multiple test correction; p = 0.016 and p = 0.007, 4 resp. 8 weeks after vaccination in Wilcoxon signed-rank tests; figure 3A). One year after vaccination, the frequency and relative expression of CD8$^+$KLRG1$^+$ T-cells had returned to pre-vaccination values (figure 3A).

Remarkably, BCG-vaccination did not induce expression of either PD-1 nor CTLA-4 on *in vitro* BCG-stimulated CD4$^+$ or CD8$^+$ T-cells compared to pre-vaccination in cells *in vitro* stimulated with BCG; also the expression of PD-1 and CTLA-4 on CD4$^+$ and CD8$^+$ T-cells relative to unstimulated cells (ratio BCG-stimulated : unstimulated) did not increase compared to pre-vaccination baseline (relative expression of PD-1 and CTLA-4 on CD4$^+$ and CD8$^+$ T-cells demonstrated in figure 3B and C).

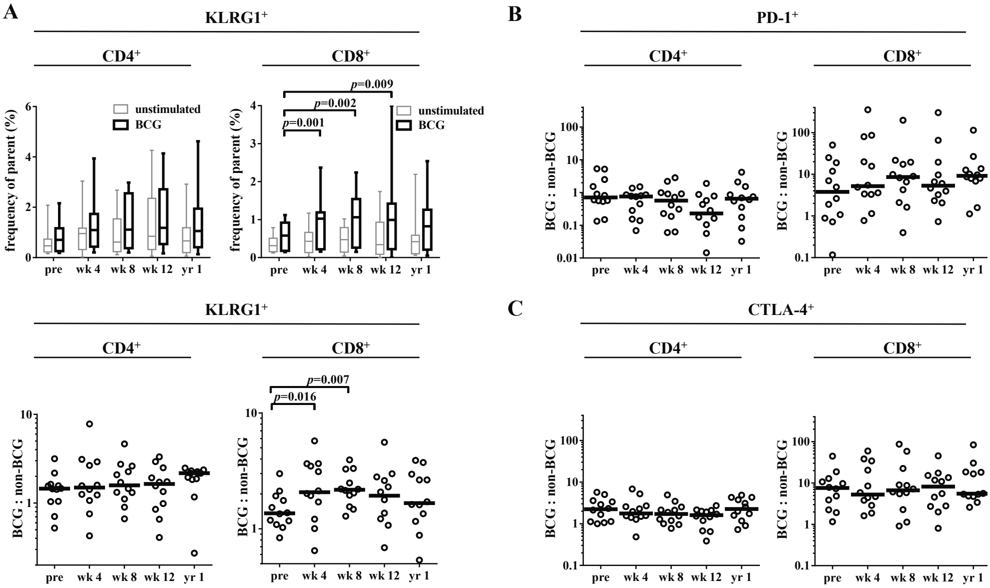

Figure 3: BCG-vaccination induces KLRG1 expression on CD8$^+$ T-cells in BCG-naïve healthy adults.

A, upper panels: KLRG1 expression is significantly induced on CD8$^+$ T-cells, but not on CD4$^+$ T-cells, after BCG-vaccination of healthy adults 4, 8 and 12 weeks after vaccination (stimulated samples compared to pre-vaccination in Friedman test with Dunn's multiple test correction followed by Wilcoxon signed-rank test). A, lower panels: KLRG1 expression relative to unstimulated samples (ratio BCG-stimulated : unstimulated) is significantly increased on CD8$^+$, but not on CD4$^+$ T-cells, 4 and 8 weeks after vaccination (compared to pre-vaccination in Friedman test followed by Wilcoxon signed-rank test). Results from 12 vaccinees as box-whiskers (line at median, whiskers min. - max.), or as dot plot (line at median), resp., at pre-vaccination, at 4, 8 and 12 weeks, and 1 year after vaccination. B, C: Neither PD-1 nor CTLA-4 expression is significantly induced on CD4$^+$ or CD8$^+$ T-cells after BCG-vaccination, neither as frequency nor as relative to unstimulated samples (ratio BCG-stimulated : unstimulated), compared to pre-vaccination. Results are shown as ratios for BCG-stimulated : unstimulated for PD-1 (B) and CTLA-4 (C) expression on CD4$^+$ and CD8$^+$ T-cells for 12 vaccinees (dot plots, line at median).

KLRG1 expression marks CD8$^+$ T-cells with reduced proliferation

To evaluate possible functional consequences of KLRG1 induction on CD8$^+$ T-cells following BCG-vaccination, we studied the proliferative capacity of CD8$^+$ T-cells, CD8$^+$KLRG1$^+$ T-cells, and CD8$^+$PD-1$^+$ T-cells after vaccination. Following 6 day *in vitro* BCG stimulation, cells were CFSE-labelled and after another six days stained and analyzed by flowcytometry. BCG-activated CD8$^+$KLRG1$^+$ T-cells displayed markedly reduced proliferation compared to the total CD8$^+$ population and compared to PHA-stimulation induced CD8$^+$ T-cell proliferation (representative overlay histograms of cell proliferation 12 weeks after vaccination displayed in figure 4A and B). In contrast, the PD-1$^+$ fraction of CD8$^+$ T-cells displayed enhanced proliferative capacity compared to the total CD8$^+$ population (figure 4B displays proliferation of total CD8$^+$ T-cells, CD8$^+$PD-1$^+$ T-cells and CD8$^+$KLRG1$^+$ T-cells of the same donor 12 weeks after vaccination).

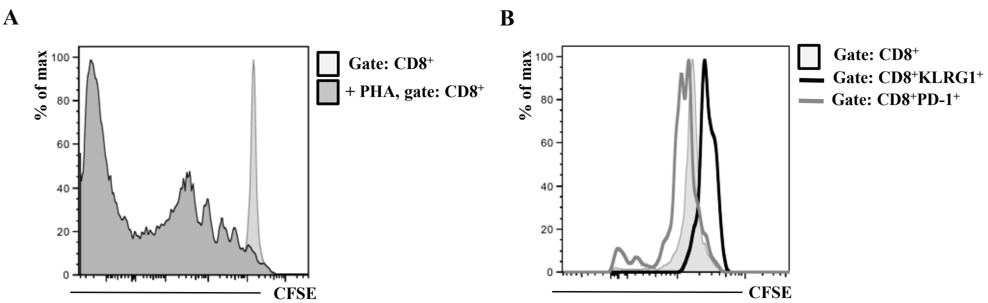

Figure 4. BCG-stimulated CD8$^+$KLRG1$^+$ T-cells show reduced proliferation compared to CD8$^+$PD-1$^+$ T-cells. A: *In vitro* BCG-stimulated cells of BCG-vaccinated donors were CFSE-labelled after 6 days, and after another 6 days stained for flowcytometric analysis (gated on single cells, lymphocytes, CD14-CD56-negative, CD3$^+$, and CD4$^+$CD8$^-$ vs. CD8$^+$CD4$^-$). Figure 4A displays representative overlay histograms of cell proliferation of CD8$^+$ T-cells (shaded light grey) and the PHA-stimulated control cell line (also gated on CD8$^+$; shaded dark grey) of a BCG-vaccinated donor 12 weeks after BCG-vaccination. B: KLRG1 expression on BCG-activated CD8$^+$ T-cells denotes cells with reduced proliferative capacity in a CFSE proliferation assay: overlay histogram with CD8$^+$ T-cells (shaded light grey) as in (A); also cell proliferation of CD8$^+$KLRG1$^+$ T-cells (black line) and of CD8$^+$PD-1$^+$ T-cells (grey line) are shown.

Relative cellular proliferation was then obtained by dividing total CD8^{+} CFSE intensity by CD8^{+}KLRG1^{+} and CD8^{+}PD-1^{+} CFSE intensity. At 4, 8 and 12 weeks after BCG-vaccination the relative cell proliferation of CD8^{+}KLRG1^{+} T-cells was significantly decreased, while CD8^{+}PD-1^{+} relative T-cell proliferation was significantly increased, compared to the total CD8^{+} population. Thus, these data indicate that KLRG1 expression identifies non-proliferating CD8^{+} T-cells after BCG-vaccination, whereas CD8^{+}PD-1^{+} T-cells are proliferating (figure 4C; $p < 0.001$ for CD8^{+}KLRG1^{+} and CD8^{+}PD-1^{+} T-cells at all depicted visits, Wilcoxon signed-rank test against the theoretical ratio of 1).

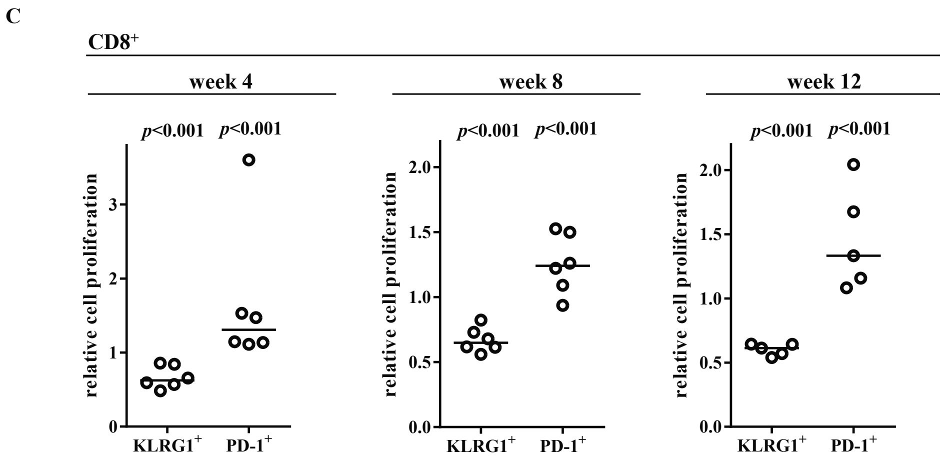

FIGURE 4 (*continued*). BCG-stimulated CD8^{+}KLRG1^{+} T-cells show reduced proliferation compared to CD8^{+}PD-1^{+} T-cells. C: The relative cell proliferation of CD8^{+}KLRG1^{+} T-cells is decreased, whereas the relative cell proliferation of BCG-activated CD8^{+}PD-1^{+} T-cells is increased after BCG-vaccination compared to pre-vaccination baseline; scatter plots with line at median 4, 8 and 12 weeks post-vaccination (resp. 6, 6 and 5 BCG-vaccinated donors) are shown. To obtain cell proliferation relative to total CD8^{+} T-cell proliferation, the geometric mean of CFSE intensity of CD8^{+} T-cells was divided by the geometric mean of CD8^{+}KLRG1^{+}, or CD8^{+}PD-1^{+} T-cells; and the relative cell proliferation of CD8^{+}KLRG1^{+} and CD8^{+}PD-1^{+} T-cells was tested against the theoretical median of 1 (Wilcoxon signed-rank tests).

Discussion

In this study, we studied and compared expression of KLRG1, PD-1 and CTLA-4 on human T-cells in different stages of TB-infection/disease and assessed mycobacterial induction of these markers in humans. Expression of KLRG1, PD-1 and CTLA-4 was increased most on *in vitro* BCG-stimulated T-cells of cured-TB subjects, and expression of KLRG1 and PD-1 was mildly increased on $CD4^+$ T-cells in active TB patients, compared to healthy donors. BCG-vaccination of BCG-naïve adults induced KLRG1 expression on $CD8^+$ T-cells, and $CD8^+KLRG1^+$ T-cells had - in contrast to $CD8^+PD\text{-}1^+$ T-cells - a markedly reduced proliferative capacity.

We found the highest expression of KLRG1, PD-1 and CTLA-4 on T-cells from cured-TB individuals. In contrast, others have reported a decrease of PD-1 expression during TB-therapy in humans and mice, and of KLRG1 expression in mice [15;33]. Also in contrast to our observation of a mild increase in KLRG1 and PD-1 expression in active disease, others have reported no modulation of PD-1 expression during active TB [34]. However, in South-African active TB patients the proliferative capacity of $CD4^+$ and $CD8^+$ T-cells was impaired compared to latently infected individuals [35], which may suggest that continued antigen exposure could drive a progressive loss of T-cell functionality associated with progression to active disease. Interestingly, in these South-African TB patients the impairment in the proliferative capacity of Mtb-induced T-cells was not restored during or months after treatment [36].

In murine Listeria and vaccinia infection models, $CD8^+KLRG1^+$ T-cells exhibited reduced proliferative capacity but optimal cytolysis and infection control compared to $KLRG1^-$ T-cells [37]. However, in murine Mtb-infection $KLRG1^-$, IL-2-secreting central memory T-cells exhibited optimal infection control [38]. We did not assess the cytokine-secreting capacity of $KLRG1^+$ T-cells, but in murine Mtb-infection KLRG1 expression was generally inhibitory, and $KLRG1^{-/-}$ mice had both increased pulmonary $CD4^+$ T-cell IFNγ production and survived longer following Mtb infection [39]. Indeed, murine $PD\text{-}1^+KLRG1^-$ T-cells exhibited increased polyfunctionality compared to $PD\text{-}1^-KLRG1^+$ T-cells [20-22].

The high expression of inhibitory markers in recently cured-TB individuals could be explained as a consequence of antigen presence following antibiotic treatment. In murine LCMV, demethylation of the promotor region of the PD1-encoding gene (Pdcd1) was stable after viral titers had fallen below detection levels [40]; also in HIV-elite controllers or long-term treated individuals such demethylation was persistent [41]. After HCV infection, PD-1 was equally expressed on HCV-specific T-cells from individuals that had cleared infection and from chronic HCV patients [42], suggesting a stable phenotype independent of the pathogen load (reviewed in [5]). Also in our study, the expression of PD-1 on T-cells from TB-treated individuals may be thus explained as an epigenetically conserved and stable phenotype independent of antigen exposure. Longitudinal studies with prolonged follow up times will be needed to establish the kinetics of these possibly epigenetically regulated phenotypes.

Importantly, it was recently demonstrated that in murine TB, PD-1-expressing T-cells have stable memory-like properties: these cells sustain a T-cell pool in the absence of antigen and expand after re-exposure; then they partly differentiate into KLRG1^{+} T-cells [21]. PD-1 (as well as CTLA-4) is ubiquitously expressed on immune cells [7;13], and PD-1^{+} T-cells probably represent a highly heterogeneous T-cell pool in TB [43]. PD-1 serves as an important brake on immune cells [7], illustrated by the reported lethal immunopathology following Mtb infection of PD-1$^{-/-}$ mice [44;45]. In the light of their polyfunctionality and protective capacity [21], PD-1^{+} T-cells - at least in murine TB - could thus be proliferating precursors that maintain long-term responses against TB, differently from the role of PD-1^{+} T-cells in other infection models [43]. In these murine TB-models KLRG1 is a powerful marker of terminal differentiation and functional exhaustion compared to PD-1. Furthermore, it was suggested that after TB-treatment the presence of pro-apoptotic T-cells indicated a deregulated memory component, enhancing host susceptibility towards recurrent disease [30]. Future research should delineate whether in human TB the expression of KLRG1 (or PD-1) is a risk factor towards disease progression, and whether KLRG1 rather than PD-1 indicates functional exhaustion. Due to the cross sectional design of our study, it remains unclear whether in Mtb-infected humans PD-1^{+} T-cells differentiate into KLRG1^{+} T-cells and whether antigen re-exposure is involved.

We found that BCG-vaccination induced KLRG1, but not PD-1 or CTLA-4 expression on T-cells. Also in murine BCG-vaccination expansion of KLRG1^{+} but not PD-1^{+} T-cells was observed [27], and T-cells from BCG-vaccinated individuals did not express PD-1 [46], in contrast with latently infected individuals [14;46;47]. Importantly, in Mtb-infected mice intravascular effector T-cells exhibited high KLRG1 expression, and only KLRG1^{-} T-cells were capable of entering the lung parenchyma to mediate protective immunity [48]. Lung tissue-resident KLRG1$^-$ Th1 cells expressed high levels of PD-1 compared to intravascular effector T-cells, as well as CTLA-4 [48;49]. We thus cannot exclude that following vaccination PD-1$^+$ and CTLA-4$^+$ T-cells could have expanded at the inflamed skin vaccination site, while BCG-induced KLRG1$^+$ T-cells would be sequestered in the circulation. It is conceivable that KLRG1 expression following BCG-vaccination could represent impaired protective efficacy in terms of decreased migratory potential.

We found increased KLRG1 expression on CD4$^+$ T-cells, and a slight enhancement in the ratio of KLRG1-expressing CD4$^+$ T-cells and CD8$^+$ T-cells, in cured-TB subjects compared to healthy donors. Interestingly, after BCG-vaccination we only found specific induction of KLRG1 expression on CD8$^+$, but not CD4$^+$ T-cells. Rapid early clonal proliferation of CD8$^+$ T-cells has been demonstrated after antigen encounter [50], whereas clonal CD4$^+$ T-cell expansion occurs in response to repeated antigen stimulation, as during the course of infection and perhaps not sufficiently following a single vaccine dose, or due to specific immunomodulating properties of live BCG [50]. In contrast, high antigen exposure during active TB disease and antigen release induced by chemotherapy could trigger clonal expansion of KLRG1-expressing CD4$^+$ T-cells in TB-treated individuals. *M. bovis* BCG lacks the RD1-region that is present in Mtb, however it is unlikely that differences in the cytosolic processing of mycobacterial antigens account for the differences in CD4$^+$KLRG1$^+$ and CD8$^+$KLRG1$^+$ T-cell frequencies between the TB and BCG-vaccinated cohorts in this study: RD1-deficient bacteria generally induce lower, and not higher CD8$^+$ T-cell activation [51]. Moreover, TB8.4-specific CD8$^+$ T-cell clones were as activated by Mtb-infected DCs as by DCs infected with RD1-mutant Mtb strains [52].

We demonstrated proliferating CD8$^+$PD-1$^+$ T-cells vs. non-proliferating CD8$^+$KLRG1$^+$ T-cells following human BCG-vaccination, analogous to murine TB-vaccine models [22;53]. In murine BCG-vaccination KLRG1 expression on CD4$^+$ and CD8$^+$ T-cells increased during contraction of the immune response, which inversely correlated with proliferation, cytokine production, and immunity against TB-challenge [27;38]. In human HCV-infection, increased KLRG1 expression and decreased IL2-production by CD4$^+$ T-cells was found in HBV-vaccine non-responders, compared to HBV-vaccine responders, and *in vitro* KLRG1 blocking increased Akt (Ser473) phosphorylation, cell proliferation and IL-2 production [54]. In our study the frequencies of circulating CD8$^+$KLRG1$^+$ T-cells returned to baseline one year post BCG-vaccination. Future studies should determine whether the observed KLRG1$^+$ T-cell expansion impacts a (neo)antigen vaccine response, whether blocking the KLRG1 signaling would enhance the vaccine response, and whether expression of KLRG1 (in any phase of the response) leads to a deregulation in the vaccine-induced (central) memory formation.

In conclusion, we demonstrate increased expression of KLRG1, PD-1 and CTLA-4 in individuals that had recently been treated for TB, and we describe for the first time - to the best of our knowledge - the induction of non-proliferating KLRG1$^+$ CD8$^+$ T-cells following BCG-vaccination in humans. Future research should delineate whether inhibitory marker expression leads to an increased susceptibility towards developing disease or a lack of vaccine-induced protective efficacy. Immune checkpoint inhibition studies will hopefully answer the question whether KLRG1-blockade is clinically feasible, safe and effective in helping to induce better protection against TB.

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CHAPTER 7

Conclusions and Discussion

General conclusions

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 protection against pulmonary TB in adults is highly variable and only limited. This thesis aimed to identify cellular responses that may account for the variable efficacy of BCG-vaccination, by assessing the induction of regulatory, pro-inflammatory and inhibitory marker-expressing T-cell subsets by *M. bovis* BCG in adults.

Many pathogens induce, recruit and expand regulatory T-cells that act at the interface of human host and pathogen in immunity against diseases and following vaccination **(Chapter 2)**. Besides 'specific' suppression, also heterologous suppression can impact on (vaccine-induced) immunity. *M. tuberculosis* induces Tregs, and $CD4^+$ Tregs were also demonstrated in *M. bovis* BCG-vaccinated infants and adults. $CD8^+$ Tregs are less studied - and often even overlooked - compared to $CD4^+$ Tregs, especially in infectious diseases and vaccination. BCG-induced $CD4^+$ Tregs had been reported, but no comparative analysis of BCG-activated $CD4^+$ vs. $CD8^+$ Tregs existed.

We studied the induction of $CD4^+$ and $CD8^+$ Tregs following *in vitro M. bovis* BCG-stimulation of PBMCs isolated from PPD-reactive healthy human donors. Next, we compared suppressive activity of live vs. heatkilled BCG-stimulated T-cells, and compared suppressive activity of live BCG-stimulated T-cell subsets **(Chapter 3)**. We found that live BCG-stimulation induced a regulatory T-cell phenotype that consisted predominantly of $CD8^+$, and not $CD4^+$ T-cells. Expression, co-expression, and expression level of regulatory markers was higher on BCG-activated $CD8^+$ T-cells compared to $CD4^+$ T-cells, and selection of T-cells on co-expression of regulatory markers indeed enriched for $CD8^+$ T-cells. Heatkilled BCG-activated T-cells were not suppressive, while in contrast live BCG-activated T-cells suppressed proliferation of Th1-cells. The suppressive activity of live BCG-activated T-cells was mediated predominantly by $CD8^+$ Tregs, and not by $CD4^+$ T-cells.

CD39 (E-NTPDase1) has previously been described as a functionally active marker of mycobacteria-induced $CD4^+$ Tregs, but has not been studied on mycobacteria-activated $CD8^+$ Tregs. **Chapter 4** demonstrates specific CD39-expression on $CD8^+$ T-cells following live BCG-activation of PBMCs from PPD-responsive donors. $CD8^+$ T-cell lines sorted on

CD39-expression were highly enriched in expression of (other) regulatory markers. Only $CD8^+CD39^+$ T-cells, but not $CD8^+CD39^-$ T-cells suppressed proliferation of Th1 cells, and blocking of CD39 resulted in partial reversal of suppression by $CD8^+CD39^+$ T-cells. This demonstrates the functional involvement of CD39 in mediating suppression by *in vitro* live BCG-activated $CD8^+$ Tregs.

We then assessed - prospectively - the induction of primary pro-inflammatory and regulatory T-cell responses by BCG-vaccination of TST-negative, QuantiFERON test-negative, and BCG-naive healthy adults **(Chapter 5)**. The primary immune response following BCG-vaccination was unexpectedly dichotomous: we found either induction of $IFN\gamma^+IL2^+TNF\alpha^+$ polyfunctional $CD4^+$ T-cells concurrent with $CD4^+IL17A^+$ and $CD8^+IFN\gamma^+$ T-cells, or virtually absent cytokine responses. Vaccine-induced skin inflammation correlated well with serum CRP early after vaccination, and induction of polyfunctional $CD4^+$ T-cells and IFNγ production by PBMCs was confined to high skin inflammation responders. Treg induction by BCG-vaccination was also assessed. Analogous to the predominant activation of $CD8^+$ Tregs following *in vitro* BCG activation of PBMCs from PPD-responders in **Chapter 3**, we found induction of the regulatory phenotype only within the $CD8^+$ T-cell compartment, and did not find any significant induction of $CD4^+$ Tregs following live BCG-stimulation. In notable contrast to polyfunctional $CD4^+$ T-cell induction, the induction of $CD8^+$ Tregs compared to pre-vaccination was confined to low skin inflammation responders. Thus, following BCG-vaccination highly variable and opposing immune responses were found already within a small adult cohort, in a non-TB endemic region.

Recent studies in murine TB-vaccine models discovered a relation between expression of PD-1 or KLRG1 and vaccine-induced protective efficacy against TB. In these murine TB-models KLRG1 was a more powerful marker of terminal differentiation and functional exhaustion compared to PD-1, however the induction of KLRG1 expression on human T-cells in response to mycobacteria had not been investigated yet. We found that following BCG-vaccination of BCG-naive adults, KLRG1 expression on $CD8^+$, but not $CD4^+$ T-cells, significantly increased compared to pre-vaccination **(Chapter 6)**. Neither PD-1 nor CTLA-4 expression increased on $CD4^+$ or $CD8^+$ T-cells. KLRG1-expressing $CD8^+$ T-cells exhibited markedly decreased proliferation, whereas $PD\text{-}1^+$ T-cells proliferated after *in vitro* BCG-stimulation, analogous to murine TB-vaccine models. In patients with active TB disease, we observed a mild increase of KLRG1 and PD-1 expression on CD4$^+$ T-cells. However, KLRG1, PD-1 and CTLA-4 exhibited highest expression on CD4$^+$ T-cells of individuals recently treated for TB, which may be a consequence of persistent antigen release during antibiotic treatment. Thus, we have demonstrated induction of non-proliferating KLRG1$^+$ CD8$^+$ T-cells following BCG-vaccination in humans; future research should delineate whether inhibitory marker expression indicates a lack of vaccine-induced protective efficacy.

This thesis addressed BCG vaccine-induced immunity against mycobacteria, and has characterized three important cellular immune responses that are induced by *M. bovis* BCG in human adults. Firstly, *M. bovis* BCG induces CD8$^+$, but not CD4$^+$ regulatory T-cells, in human adults. We additionally demonstrated that CD39 is functionally involved in mediating suppression by these BCG-activated CD8$^+$ Tregs. Secondly, the pro-inflammatory response induced by BCG-vaccination was unexpectedly dichotomous: either the induction of polyfunctional CD4$^+$ T-cells, that was confined to vaccinees with high inflammation of the skin vaccine lesion, or virtually no induction of cytokines with a concomitant induction of CD8$^+$ Tregs. Thirdly, non-proliferating KLRG1$^+$ CD8$^+$ T-cells were induced following BCG-vaccination in humans, and expression of this inhibitory T-cell marker may possibly be associated with a lack of vaccine-induced protective efficacy.

Thus, BCG-vaccination induced highly variable and opposing T-cell responses.

M. bovis BCG modulates immunity against *M. tuberculosis*

This thesis demonstrates that BCG, a live bacterial vaccine against TB, induces highly variable pro-inflammatory responses, as well as CD8$^+$ regulatory T-cell responses **(Chapter 3, 4 and 5)**. Mycobacteria are master manipulators of the immune system and employ a myriad of mechanisms to induce immune regulation **(Chapter 2)**. Major virulence factors were lost by *in vitro* passaging of *M. bovis* to obtain *M. bovis* BCG, but *M. bovis* BCG clearly shares regulatory-inducing capacities with Mtb. Mycobacteria activate tolerizing DCs and anti-inflammatory macrophages [1], that produce IL-10 and induce Tregs [2;3]. In return, Tregs could further limit the pro-inflammatory response by suppressing APC activation [4]. It is however unknown which exact mechanisms are employed by *M. bovis* or *M. bovis* BCG to induce Tregs; and the evaluation in this thesis did not extent to assessment of these mechanisms.

BCG-induced innate signaling pathways could lead to Treg induction, however another highly interesting aspect of innate immunity, for which evidence of the effect of BCG-vaccination is increasing, is the induction of trained immunity by *M. bovis* BCG. Trained immunity is a state of long-term and increased responsiveness of monocytes to secondary infections, associated with epigenetic alterations that affect both intracellular signaling and cell metabolism [5;6]. It can be induced by pathogens or vaccination with live microbes such as *M. bovis* BCG, measles or yellow fever [5]. This is important, since BCG-induced trained immunity could be partly responsible for the protection against TB mediated by BCG-vaccination in infants, and could further account for the heterologous, 'non-specific effect' of BCG-vaccination in protecting infants against unrelated infectious diseases, as well as the protective effect of intravesical BCG-therapy in bladder cancer [7-9].

What constitutes vaccine-induced (T-cell mediated) protective immunity against TB is not clear; and so far no specific TB biomarker or correlate of protection has been found in either infants or in the adult population. $CD4^+$ polyfunctional T-cells have been proposed as correlate of vaccine-induced protective immunity [10], and in infants these cells are induced by BCG-vaccination [11]. However, during follow up the presence of such cells was not associated with the development of or protection against TB [12]. The impact of the BCG-induced regulatory T-cell response, as demonstrated in this thesis, on vaccine-induced immunity against TB remains unknown. Future research should delineate the impact of the regulatory response, as well as the mechanisms that are causing it.

BCG-vaccination induces predominantly $CD8^+$ Tregs, and not $CD4^+$ Tregs

$CD4^+$ and $CD8^+$ Tregs have been demonstrated in murine and human infection with *M. tuberculosis* and *M. leprae* **(Chapter 2)**. Here we found in comparative analyses that following live *M. bovis* BCG-stimulation $CD8^+$ but not $CD4^+$ Tregs were activated in human adults **(Chapter 3 and 5)**. *M. tuberculosis* actively modulates phagosome-lysosome fusion, autophagy and antigen presentation in the macrophage [13], yet Mtb and *M. bovis*

BCG could differ in their intracellular lifestyle and antigen presenting pathways. Induction of $CD8^+$ T-cell responses requires access to the MHC-I antigen processing pathway; translocation of mycobacteria from the phagosome into the cytosol, cross-priming by DCs, or alternative antigen presenting pathways would provide this access. In one study translocation of Mtb into the cytosol was dependent on secretion of ESAT-6 and CFP-10, as part of the ESX-1 secretion system which is encoded in region of difference 1 (RD1) [14]. The same study demonstrated that BCG did not translocate into the cytosol [14]. Since the genomes from all BCG strains lack RD1, this questions how BCG could induce $CD8^+$ T-cell responses, which it does, as described in this thesis.

It was noted previously [15] that cytosolic Mtb could be due to atypical cell death instead of translocation from the phagosome, and that *in vivo* murine and human studies - though limited in number - so far only observed Mtb in membrane-bound organelles. Furthermore, various cytosolic and vacuolar pathways enable mycobacterial antigen to enter MHC-I presentation pathways without a requirement for escape from the phagosome [15]. At least, escape of bacilli into the cytosol is not essential in inducing $CD8^+$ T-cell responses.

Following intradermal vaccination, BCG is present relatively long as a live (intracellular) bacterium in the human body. Live BCG bacilli are transported into the draining lymph node by dendritic cells, macrophages and neutrophils [16]. Prolonged antigen availability could increase $CD8^+$ T-cell responses through optimal cross-presentation by dendritic cells **(Chapter 3 and 5)**. In addition, extracellular fragments of BCG could be cross-presented and $CD8^+$ T-cells could be induced [17].

Interestingly, *in vitro* stimulation of PPD-responsive PBMCs with heatkilled BCG did not induce either $CD4^+$ or $CD8^+$ Tregs **(Chapter 3)**. BCG may need to be present as a live bacterium to modulate - through so far unknown mechanisms - towards tolerance. Interestingly, the phagosome membrane containing live BCG was found to have pores permissive of molecules up to 70 kDa; but only viable and not formalin-killed BCG was associated with permeability of these membranes [18]. Therefore, live BCG may actively employ mechanisms to enter antigen into MHC-I presentation pathways. Also in our cell cultures, the ratio of $CD4^+$: $CD8^+$ T-cell expansion was increased following heatkilled BCG-stimulation **(Chapter 3)**.

So far, $CD4^+CD25^+$ Tregs have been identified - in TB disease and after BCG-vaccination - by *in vitro* PBMC stimulation with mycobacterial PPD, TB-specific peptides or freezedried BCG in culture with antibiotics [19-21]. This may have overlooked the induction of $CD8^+$ Tregs by live BCG bacilli. Since BCG is administered as a (partly) live vaccine, the possible negative impact of $CD8^+$ Tregs on BCG-vaccine mediated immunity may be larger than previously thought.

Host factors in the heterogeneous pro-inflammatory response following BCG-vaccination

The primary immune response following BCG-vaccination in TST-negative, QuantiFERON test-negative, BCG-naive healthy adults, was unexpectedly found to be highly heterogeneous. Previous reports on induction of polyfunctional $CD4^+$ T-cells by BCG-vaccination had also yielded variable results; but here, we demonstrate high variability of BCG-vaccination-induced pro-inflammatory cytokines, and an inverse relation with a $CD8^+$ regulatory response, within one cohort **(Chapter 5)**. To detect BCG-induced immune responses, we used short-term live BCG-stimulation of whole blood, followed by fixation, cryopreservation, and intracellular cytokine staining. This whole blood assay (WBA) has previously been optimized for assessment of immunogenicity in TB-vaccine trials [22]. It has been qualified as precise, robust and operator-independent, furthermore the detection of cytokines is independent of the duration of cryopreservation [23]. Importantly, we confirmed vaccine ‘take’ one year after vaccination by IFNγ-ELISpot: a standardized and sensitive assay to detect long-term mycobacterium-specific immune responses [24]. The variation in immunogenicity assessed by live BCG-whole blood stimulation was furthermore reflected in the heterogeneous IFNγ production of PPD-stimulated PBMCs of vaccinees at 4, 8 and 12 weeks after vaccination. Cytokine responders and non-responders were evenly distributed among vaccination days and gender; furthermore all participants were Caucasian, and ranges of age and weight were small. We therefore concluded that the observed heterogeneous response reflected true inter-individual variation.

What causes a pro-inflammatory response in some individuals, and a predominantly regulatory response in others, is probably partly attributable to *M. bovis* BCG-vaccine immunomodulation, and partly intrinsic to the individual that is vaccinated; the relative contribution of these factors and their mechanism of interaction is yet unclear. Our cohort was too small for meaningful genetic analyses, but in larger cohorts it would be highly informative to correlate heterogeneity of immune responses with host specific allelic variations, homo- vs. heterozygosity for specific HLA loci, and to single nucleotide polymorphisms (SNPs) in HLA genes, immune receptors and signaling molecules. It is likely that polymorphisms also exist for regulatory cytokines and regulatory receptors / surface markers. Thus, in larger cohorts an assessment of genetic variation in regulatory molecules should be included.

In infectious diseases, lessons on immune pathways can be drawn from major immune deficiency syndromes such as the extreme susceptibility to mycobacteria caused by mutations in e.g. genes encoding IFNγ- and IL12-receptors [25]. However, genetic variants in the general population may also regulate susceptibility towards infectious diseases including TB, HIV and malaria [26;27]. A striking example that also genetic variation in regulatory responses can be involved in susceptibility towards infectious disease, comes from two ethnic groups living in one area in Burkina Faso: the Fulani people are relatively resistant to *P. falciparum*-caused malaria compared to the Mossi people; this is associated with reduced CTLA4 and FOXP3 expression compared to Mossi [28]. In addition, the risk of developing malaria in Kenyan adults is related to Treg frequencies [29]. Antibody serum levels induced by measles-vaccination were associated with HLA-I and -II variation [30], and antibody levels induced by both measles- and rubella-vaccination correlated with cytokine- and cytokine receptor SNPs (including IL12B, IL12RB1, IL2, and IL10) [30;31]. In infant BCG-vaccination, Toll-like receptor (TLR) polymorphisms correlated with the amplitude of the IFNγ response in BCG-stimulated whole blood 10 weeks after vaccination [32].

Major lessons can be drawn from the TB-vaccine candidate MVA85A in infants and adults. Even though no enhanced protection against TB was observed following MVA85A-booster- compared to placebo-vaccination of previously BCG-vaccinated children, analysis of the variation within this large cohort provides valuable insights into host and vaccine immune mechanisms. In South-African infants, positive responses to antigen 85A (Ag85A) in IFNγ ELISpot 28 days after MVA85A-vaccination were associated with expression of genes enriched in innate cells [33]. This was in contrast with the MVA85A-induced immune response in British adults: there, not pre-vaccination inflammatory pathways, but pre-vaccination expression and early (day 2) induction of regulatory pathways were inversely related with the IFNγ ELISpot response [34]. In another study, high pre-vaccination TGF-β1 serum levels correlated with low Ag85A-specific IFNγ ELISpot responses for up to 4 months after MVA85A-vaccination [35]. The authors concluded that different pathways are involved following vaccination of infants vs. adults, or African vs. British individuals.

Immunogenetics can reveal important vaccine-induced immune pathways, and possibly in the future predict vaccine outcomes. High- and low cytokine responders of the BCG-vaccinated cohort in this thesis **(Chapter 5)** did not significantly differ in either cytokine- or regulatory marker expression before vaccination. It is however possible that minor genetic variations in inflammatory or regulatory-related genes were decisive in expansion of pro-inflammatory vs. regulatory cells. This may have been in interplay with intrinsic immunomodulatory capacities of the *M. bovis* BCG-vaccine.

Classical, regulatory and clinical markers of immunity

There is no ultimate TB biomarker or correlate of protection; also $CD4^+$ polyfunctional T-cells were found to be not associated with immunity against TB. However, new surrogate endpoints of protection may be found by deciphering vaccine-induced cellular profiles and mechanisms of (*in vitro*) protection [36]. This would both accelerate vaccine trial evaluation, as well as enhance the statistical power of small(er) cohorts [37]. As described in the previous section, the induction of a regulatory response, inversely related to a pro-inflammatory response, was also reported following MVA85A-vaccination of British adults [34]. Furthermore, vaccination of South African adults with the candidate TB-vaccine M72/AS01 induced pro-inflammatory, but also regulatory responses [38]. This suggests that assessment of regulatory responses in relation to deep immune phenotyping could assist in the search for protective correlates in future TB-vaccine trials.

In **Chapter 4** we reported for the first time expression of CD39 on human BCG-activated $CD8^+$ Tregs. A murine cancer model has yielded mechanistic insights into the pivotal role of the ectonucleotidases CD39 and CD73 in differentiation of suppressive vs. non-suppressive Th17 cells: differentiation in the context of IL-6 and TGF-β activated Stat3 and

Gfi-1, which bound to promotor regions of CD39 and CD73 and activated their expression. These Th17 cells were suppressive through production of adenosine and promoted tumour growth following adoptive transfer, whereas Th17 cells generated with IL-1β, IL-6, and IL-23 did not express ectonucleotidases and were not suppressive [39]. In human TB pleurisy, $CD39^+$ Tregs isolated from pleural effusion inhibited *in vitro* differentiation of naïve T-cells into Th17 cells through TGF-β [40]. Also - as reviewed in **Chapter 2** - combined action of Mtb and $CD39^+$ Tregs within the granuloma can induce a macrophage type 1 towards type 2 switch. CD39-expressing Tregs may thus be a pivotal player in hampering protective immunity against TB through several mechanisms.

Following murine BCG-vaccination, production of IL-17 by pulmonary $CD4^+$ T-cells correlated with protection against TB challenge [41]. In humans, the MVA85A-vaccination induced CD39-expression on $CD4^+$ T-cells correlated negatively with IL17A-responses in stimulated PBMCs [42;43]. In **Chapter 4** we demonstrated functional involvement of CD39 on $CD8^+$ T-cells in mediating suppression of $CD4^+$ ($IFN\gamma^+$) Th1 cells. We did not assess the relation of CD39-expression with Th17 differentiation or IL17 production. Future studies should determine whether $CD8^+CD39^+$ Tregs impair BCG-induced protection against TB, and whether $(CD8^+)CD39^+$ Treg frequency or activity could be used as a correlate of (impaired) protection. Yet, it is unknown if vaccination-induced cellular subsets are stable or may transdifferentiate into other subsets.

Markers of inflammation that are ‘classical’ in clinical medicine, could be used as ‘non-classical’ indicators of vaccine-induced immunity. The skin inflammation score (and CRP assay) described in **Chapter 5** do not represent specific correlates, however the vaccine-induced skin reactogenicity likely represents inflammation as a result of multiple underlying immune mechanisms. The *in vitro* pro-inflammatory cytokine response in BCG-vaccinated infants and in BCG-revaccinated British adults also correlated with scar formation of the BCG-vaccine lesion [44;45]. It is highly interesting that scar formation in infants has also been associated with the non-specific protective effects of BCG-vaccination [46]. BCG-induced TST-conversion was not associated with protection against TB [47]. In **Chapter 5** we identified two types of primary opposing immune responses following BCG-vaccination in BCG-naive individuals: a pro-inflammatory response or a regulatory T-cell response, which correlated with the skin inflammation score. Follow-up of skin reactivity and/or scar formation may possibly provide insight into protective immunity, and may

assist in identification of various responses and mechanisms, including innate immunity.
Any potential correlate of vaccine-induced protection will need to be validated in sufficiently powered clinical vaccine trials [36]. Yet, in the absence of any protective TB-vaccine, potentially valuable information of protective activity could be obtained from *in vitro* functional estimates of mycobacterial control such as growth inhibition, and the recently developed human BCG-challenge model with intradermal injection of BCG bacilli followed by skin biopsy [45;48].

Expression of inhibitory receptors by T-cells

Continued effector T-cell function and proliferation of T-cells are essential to maintain control of Mtb [49]. However, studies on chronic viral infections such as HIV, hepatitis B and hepatitis C, as well as on tumours, have shown how continued antigen exposure exhausts T-cell function [50]. This is marked by expression of inhibitory receptors and impaired proliferative capacity, and is associated with diminished T-cell mediated control of infection [50]. Persistent Mtb infection could potentially exhaust the T-cell response in a similar way, impairing T-cell mediated immunity. There is currently no evidence that mycobacteria actively manipulate towards terminal T-cell differentiation (as is the case for induction of Tregs). Since BCG persists relatively long in the human body following vaccination, we have hypothesized **(Chapter 6)** that BCG - besides actively inducing $CD8^+$ Tregs - by its prolonged presence may also steer T-cells towards terminal differentiation.
In **Chapter 6** we described the induction of $CD8^+KLRG1^+$ T-cells following BCG-vaccination of human adults, which exhibited markedly impaired proliferative capacity. Interestingly, in Mtb-infected mice only $KLRG1^-$ T-cells were able to enter the lung parenchyma, while $KLRG1^+$ T-cells were contained intravascularly [51]. Thus, the upregulation of terminal differentiation markers following vaccination may be associated with impaired vaccine-induced protection, deregulated memory, or decreased migratory potential. Considering the specific association of KLRG1 expression with loss of T-cell mediated immune control in murine TB [52;53], this has potential impact on the study of vaccine-induced immune correlates of protection in humans.
KLRG1 expression was significantly induced on $CD8^+$, but not $CD4^+$ T-cells. Given the

predominant $CD8^+$ regulatory response induced by *M. bovis* BCG **(Chapter 3 and 5)**, it is important to note that BCG-induced $CD8^+KLRG1^+$ T-cells were not enriched for regulatory markers compared to the total $CD8^+$ T-cell population. Also no relation was observed between expression of KLRG1 on $CD8^+$ T-cells and the skin inflammatory response or the induction of polyfunctional $CD4^+$ T-cells, such that these seem to be independent phenomena following BCG-vaccination. Interestingly, also $CD8^+PD\text{-}1^+$ T-cells were not enriched for the regulatory phenotype. PD-1 is ubiquitously expressed on various immune cells, including Tregs, yet PD-1 ligation of Tregs causes their expansion and activation, while PD-1 ligation on other cells serves as a brake in the immune response [54]. PD-1 ligation is actively exploited by Mtb to inhibit effector immunity and to expand Tregs ([55]; **Chapter 2**). In addition, and not mutually exclusive, PD-1 expressing cells may have a dual role in that they could also maintain long-term immune responses during persistent Mtb infection [49].

Only $CD8^+CTLA\text{-}4^+$ T-cells were enriched for the regulatory markers Foxp3, CD25 and CD39 following BCG-vaccination **(Chapter 6)**; indeed, these cells partially mimicked the pattern observed for the $CD8^+CD25^+Foxp3^+CD39^+$ phenotype as described in **Chapter 5**. The mean fluorescence intensity of CTLA-4 expression on $CD8^+$ T-cells was slightly increased eight weeks post-vaccination, and this was confined to low inflammation responders. CTLA-4 is expressed on a variety of cells, including Treg cells, and CTLA4 is a Foxp3 target gene in mice [54]. Tregs modulate APCs by CTLA-4-mediated trans-endocytosis of CD80 and CD86, which depletes co-stimulatory receptor expression on APCs [56]. Tregs have multiple and highly adaptable other modes of suppression [57]; vice versa not all CTLA-4 expressing T-cells are Tregs [58]. In any case, its active involvement in APC modulation, and the numerous associations with the Treg phenotype and function that have been reported in infectious diseases **(Chapter 2)**, provide a rationale for the enrichment in regulatory markers that we observed.

It is interesting that immune checkpoint blockade of ligand-receptor interaction thus can not only enhance pro-inflammatory effector immunity, but can simultaneously also decrease Treg function and proliferation [54]. In addition, intriguing interactions between Tregs and terminally differentiated (exhausted) T-cells have been described: in murine LCMV infection models $CTLA\text{-}4^+$ Tregs preserved the exhausted state of antigen-specific $CD8^+$ T-cells through B-7 modulation, depleting the APC of costimulatory molecules in the face of continued antigen presence [59]. Depletion of Tregs reactivated T-cell function, but additional PD-L1 blockade was needed to control viral load [59]. In murine retroviral infection, both Treg depletion and blocking inhibitory receptors (PD-1 and TIM-3) decreased viral load, however combination therapy was superior in achieving sustained reduction [60]. Trials of combination checkpoint blockade in individuals with metastasized melanomas demonstrated synergy and improved control of tumour progression compared to monotherapy [61]. This provides new rationale for combined immunotherapy: modulation of two different pathways, regulatory and exhausted, could be optimal to resurrect immune control of chronic infection. Also (vaccine-induced) immunity against TB may be enhanced by combined immunotherapy; future studies will hopefully answer the question whether this would indeed result in improved protective immunity and whether this is clinically feasible and safe.

Concluding remarks

The (1) induction of $CD8^+CD39^+$ regulatory T-cells, that partly suppress via CD39, the (2) variability of the primary pro-inflammatory response with either induction of $CD4^+$ polyfunctional Th1 cells or $CD8^+$ Tregs, and (3) the induction of $CD8^+KLRG1^+$ T-cells with impaired proliferative capacity by BCG-vaccination in humans, represent a novel network of inter-related immune responses, that may all impact on vaccine-induced protective immunity against TB. Though the induction of $CD4^+$ Tregs has been demonstrated in TB, following vaccination with BCG or candidate TB-vaccines, *M. bovis* BCG-induced $CD8^+$ Tregs have been overlooked as significant modulators of immunity in TB and TB-vaccine studies. In addition, alternative indicators such as skin reactivity and CRP were found that could assist in future analysis of vaccine-induced immune responses. Basic research into vaccine-induced pro-inflammatory, regulatory and terminally differentiated cellular responses thus provides novel immune markers and uncovers new mechanisms regulating vaccine-induced immunity, with significant repercussions for protection. This could guide vaccine design and provide a basis for immunotherapy options - as are now emerging in cancer medicine - to optimize immune control of tuberculosis.

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CHAPTER 8

Nederlandse samenvatting

Curriculum Vitae

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Nederlandse samenvatting

Algemene inleiding

Op dit moment bestaan geen effectieve vaccins tegen de drie grootste - en dodelijkste - infectieziekten op aarde: HIV/AIDS, tuberculose (TB; TBC) en malaria. TB, in mensen en dieren, wordt veroorzaakt door bacteriën van het '*Mycobacterium tuberculosis* complex', een groep nauw-verwante bacteriën met een gedeelde voorouder van naar schatting drie miljoen jaar oud. Tot deze groep behoren o.a. *Mycobacterium tuberculosis* (*M. tuberculosis*; Mtb), *Mycobacterium africanum* en *Mycobacterium bovis* (*M. bovis*). De meest voorkomende veroorzaker van TB in mensen, *M. tuberculosis*, is ongeveer 70.000 jaar geleden ontstaan in vroege menselijke populaties in Afrika. In het Europa van de Middeleeuwen steeg de incidentie van TB – ook bekend als 'de tering', 'the white plague' of 'consumption' - tot epidemische proporties; en er is geen ander pathogeen dat in de wereldgeschiedenis zoveel slachtoffers heeft geëist. Pas laat 19^{e} eeuw werd Mtb geïdentificeerd door Robert Koch als het pathogeen dat TB veroorzaakt; vanaf 1944 werden antibiotica ontdekt tegen TB. Naar schatting is op dit moment een-derde van de wereldbevolking latent geïnfecteerd met Mtb. Bij latente infectie zijn levende, niet- of nauwelijks delende bacteriën aanwezig in het menselijk lichaam, maar deze worden onder controle gehouden door het immuunsysteem. Latent geïnfecteerde mensen vertonen dan ook geen klinische tekenen van infectie, maar hebben een risico op reactivering van 3-10% gedurende het leven. In mensen met HIV is dit risico 5-10% per jaar; en TB is de belangrijkste doodsoorzaak onder HIV-patiënten. Hoewel in de Westerse wereld de TB-incidentie dramatisch is afgenomen, is, door de HIV-epidemie en de toenemende antibiotica-resistentie, TB nog altijd een zeer groot probleem in ontwikkelingslanden, oostelijk Azië en de voormalige Sovjet Unie. Per jaar sterven ten minste 1,5 miljoen mensen aan TB, waarvan 95% in ontwikkelingslanden.

Mycobacterium bovis bacillus Calmette-Guérin (*M. bovis* BCG) is het enige beschikbare vaccin tegen TB. *M. bovis* BCG is aan het begin van de 20e eeuw geïsoleerd door het kweken van *M. bovis*, de verwekker van TB in koeien, op een mengsel van glycerine, gal en aardappel-extract, door Albert Calmette en Camille Guérin. Sinds de eerste vaccinatie in 1921 is BCG ten minste drie miljard maal toegediend, meer dan enig ander vaccin. BCG wordt, als onderdeel van het Expanded Programme on Immunization van de World Health Organization, in vrijwel alle ontwikkelingslanden met hoge TB-prevalentie binnen 24 uur na de geboorte toegediend. BCG-vaccinatie beschermt pasgeborenen en jonge kinderen tegen gedissemineerde vormen van TB, maar biedt onvoldoende en zeer wisselende bescherming tegen open (actieve) long TB op volwassen leeftijd. Een effectief TB-vaccin zou juist bescherming moeten bieden tegen open long TB, de besmettelijke vorm van de ziekte, en dit zou grote impact hebben op de TB-epidemie. Hoewel nieuwe vaccins worden ontwikkeld en getest, zijn op dit moment nog geen nieuwe vaccins beschikbaar.

Een van de grootste uitdagingen is te definiëren wat beschermende immuniteit tegen TB precies inhoudt en hoe dit door vaccinatie moet worden geïnduceerd. Succesvolle vaccins tegen infectieziekten induceren ‘normaliter’ een antilichamen titer, echter door de voornamelijk intracellulaire levensstijl van Mtb moet een succesvol TB-vaccin - evenals succesvolle HIV- of malaria-vaccins - ook effectieve T-cel immuniteit induceren. Er bestaat echter nog geen gouden standaard voor wat een beschermende T-cel respons op vaccinatie tegen deze drie dodelijke infectieziekten precies inhoudt. Een effectieve, door een TB-vaccin geïnduceerde immuunrespons zal minimaal de inductie van $CD4^+$ T-helper (Th)-1 cellen omvatten; daarnaast zijn in muizenstudies vaccin-geïnduceerde polyfunctionele (simultaan IFNγ-, IL2- en TNFα-producerende) $CD4^+$ T-cellen aangetoond die correleerden met bescherming tegen TB. Echter, in jonge kinderen correleerde de frequentie van deze polyfunctionele $CD4^+$ T-cellen na BCG-vaccinatie niet met het risico op het ontwikkelen van TB. Meer basaal onderzoek naar de vaccin-geïnduceerde T-cel respons is essentieel om de immuun respons en beschermende immuniteit te definiëren, als rationele onderbouwing voor de ontwikkeling van een nieuw TB-vaccin. Daarnaast kunnen door het onderzoeken van vaccin-geïnduceerde cellulaire profielen mogelijk nieuwe correlaten van bescherming tegen TB worden gevonden, en beschikbaarheid van zulke correlaten zou de evaluatie van nieuwe TB-vaccins uitermate faciliteren en versnellen.

Mycobacteriën hebben zich door co-evolutie met de menselijke soort ontwikkeld tot meester manipulators van het immuunsysteem. Regulatoire T-cellen (Tregs) zijn T-cellen die de pro-inflammatoire respons onderdrukken en essentieel zijn voor immuun homeostase, het voorkomen van auto-immuniteit en bescherming van weefsels tegen destructie door geprolongeerde immuun responsen, zoals bij chronische infectie. Deze regulatoire T-cellen worden echter ook door Mtb actief geïnduceerd en geëxpandeerd, waarmee Mtb het immuunsysteem in essentie manipuleert om in het lichaam te kunnen blijven voortbestaan. Er bestaan verscheidene hypotheses rondom de matige effectiviteit van het *M. bovis* BCG-vaccin, waaronder inductie van Tregs door BCG zelf. $CD4^+$ Tregs zijn eerder aangetoond in kinderen na BCG-vaccinatie; echter, $CD8^+$ Tregs worden veel minder bestudeerd – of zelfs over het hoofd gezien – in vergelijking met $CD4^+$ Tregs, vooral in het kader van infectieziekten en vaccinatie. Er zijn dan ook geen vergelijkende analyses van de impact van $CD4^+$ vs. $CD8^+$ Tregs op de immuun respons of effectiviteit van het BCG-vaccin. Ook is weinig bekend over het suppressieve arsenaal waarmee $CD8^+$ Tregs de activiteit van andere cellen kunnen onderdrukken.

Daarnaast komt toenemend het belang naar voren van de expressie van inhiberende markers door functioneel 'uitgeputte' T-cellen in immuniteit; zo wordt in recente immuun-therapeutische doorbraken in de oncologie, blokkade van deze inhiberende markers (receptoren) al ingezet om uitgeputte T-cellen weer opnieuw te activeren zodat ze de tumor effectief kunnen opruimen. Naar aanleiding van deze successen wordt ook toenemend de betekenis onderzocht van expressie van inhiberende markers, zoals KLRG1 en PD-1, door T-cellen in de immuun respons tegen TB. In TB-vaccin studies in muizen vertonen T-cellen die KLRG1 tot expressie brengen een significant lagere activiteit, in de vorm van verlaagde cel proliferatie en productie van pro-inflammatoire cytokines. Dit werd niet gevonden voor T-cellen die PD-1 tot expressie brachten. De expressie van KLRG1, maar niet PD-1, door T-cellen is in deze studies dus geassocieerd met een verminderde vaccin-geïnduceerde bescherming tegen TB. De expressie van KLRG1 op humane cellen na BCG-vaccinatie is voorheen echter niet onderzocht.

Er bestaan derhalve meerdere, mogelijke verklaringen voor de zeer matige effectiviteit van BCG-vaccinatie op de volwassen leeftijd. Onderzoek naar de BCG-geïnduceerde cellulaire respons in volwassenen is dan ook essentieel om deze mechanismen op te helderen.

Dit proefschrift

In dit proefschrift zijn verschillende cellulaire responsen geïdentificeerd die een verklaring zouden kunnen bieden voor de matige en inconsistente effectiviteit van BCG-vaccinatie in bescherming tegen TB. Ten eerste door de inductie van $CD4^+$ en $CD8^+$ regulatoire T-cellen door BCG te onderzoeken, ten tweede door de primaire T-cel respons op BCG-vaccinatie, zowel pro-inflammatoir als regulatoir, te onderzoeken en ten derde door specifieke expressie van inhiberende markers, met name KLRG1, door humane T-cellen na BCG-vaccinatie te onderzoeken.

Hoofdstuk 2 begint met een literatuur overzicht van de inductie en expansie van Tregs door bacteriën, virussen en parasieten en het effect van Tregs op de effector immuniteit tegen pathogenen. Tregs functioneren op het grensvlak tussen mens en pathogeen in immuniteit tegen infectie en ziekte, en na vaccinatie: zij beschermen de weefsels tegen buitensporige inflammatie, maar verhogen het risico op persistentie van het pathogeen en daarmee de ontwikkeling van een chronische infectie. Tregs mediëren niet alleen specifieke suppressie, maar kunnen ook de immuunrespons tegen niet-gerelateerde pathogenen, of de respons op niet-gerelateerde vaccinaties (heteroloog) onderdrukken. Mycobacteriën, waaronder *M. tuberculosis* en *M. leprae* (de veroorzaker van lepra), behoren tot de oudste pathogene bacteriën ter wereld en hebben zich ontwikkeld tot meesterlijke modulators van de immuunrespons om in het menselijk lichaam te kunnen voortbestaan. Het merendeel van de mycobacteriële infecties komt voor in gebieden die ook endemisch zijn voor andere virale, parasitaire of bacteriële infecties; het is dus belangrijk de effecten van Tregs niet slechts homoloog, maar ook in heterologe (modellen van) infecties en vaccinaties te bestuderen.

Door anderen is de inductie na *M. bovis* BCG-vaccinatie van $CD4^+$ Tregs aangetoond; echter er bestond geen vergelijkende analyse van inductie van $CD4^+$ vs. $CD8^+$ Tregs door *M. bovis* BCG. In **hoofdstuk 3** is de inductie van $CD4^+$ vs. $CD8^+$ Tregs vergeleken door cellen, afkomstig van gezonde bloeddonoren die *in vitro* immuunreactief waren tegen mycobacteriën, te stimuleren met levend *M. bovis* BCG. Het was verrassend dat levend BCG-stimulatie met name $CD8^+$ Tregs, in tegenstelling tot $CD4^+$ Tregs induceerde. De expressie, co-expressie, en de mate van expressie van regulatoire cel markers was consistent hoger op $CD8^+$ T-cellen in vergelijking met $CD4^+$ T-cellen; vice versa verrijkte het selecteren van T-cellen op regulatoire markers voor $CD8^+$, en niet $CD4^+$ T-cellen. Vervolgens vergeleken we de capaciteit van T-cellen, na stimulatie met levend BCG vs. geïnactiveerd BCG, voor het mediëren van suppressie. Alleen T-cellen, die gestimuleerd waren met levend BCG, onderdrukten de proliferatie van andere cellen. Dit is belangrijk omdat BCG als (partieel) levend vaccin wordt toegediend; door *in vitro* te testen met geïnactiveerd BCG worden dus belangrijke suppressieve eigenschappen van (levend) BCG-gestimuleerde T-cellen gemist. Suppressie na levend BCG-stimulatie werd inderdaad gemedieerd door $CD8^+$ Tregs, en niet door $CD4^+$ T-cellen.

Er is weinig bekend over de manier waarop deze $CD8^+$ Tregs suppressie uitoefenen op andere cellen. CD39 (E-NTPDase1) is een enzym aan de oppervlakte van de cel, dat door omzetting van nucleotides cellen in de omgeving kan remmen. CD39 is dan ook eerder bestudeerd op $CD4^+$ Tregs, maar niet op $CD8^+$ Tregs na BCG-stimulatie. In **hoofdstuk 4** wordt aangetoond dat CD39 tot expressie komt op $CD8^+$ Tregs na stimulatie met levend BCG. Het sorteren van $CD8^+$ T-cellen op CD39-positiviteit verrijkte significant voor andere regulatoire T-cel markers; bovendien oefenden alleen $CD8^+CD39^+$ T-cellen, en niet $CD8^+CD39^-$ T-cellen, suppressie uit op T-helper 1 cellen. Het toevoegen van een CD39-antagonist, alsmede toevoegen van een CD39-blokkerend antilichaam, maakte deze suppressie deels ongedaan. CD39 komt dus niet alleen tot expressie op BCG-geactiveerde $CD8^+$ Tregs, maar is ook functioneel betrokken bij het mediëren van suppressie door $CD8^+CD39^+$ Tregs.

In **hoofdstuk 5** is prospectief de primaire inductie van pro-inflammatoire en regulatoire T-cellen door BCG-vaccinatie in gezonde volwassenen onderzocht. Deze volwassenen waren niet eerder gevaccineerd met BCG en testten negatief voor TB (anamnestisch, in Mantoux- en in QuantiFERON-testen). Hoewel het cohort bijzonder homogeen was in demografische kenmerken, was de primaire immuunrespons op BCG-vaccinatie verrassend heterogeen: deze bestond uit ofwel een brede inductie van pro-inflammatoire $CD4^+$ en $CD8^+$ T-cellen, waaronder $IFN\gamma^+IL2^+TNF\alpha^+$ polyfunctionele $CD4^+$ T-cellen, ofwel uit een vrijwel afwezige cytokine respons. Aangezien ook de mate van ontsteking van de vaccinatielaesie op de huid zeer variabel was, werd deze gekwantificeerd in een 'skin inflammation score', gebaseerd op klassieke klinische kenmerken van ontsteking. Deze skin inflammation score correleerde met de CRP (C-Reactive Protein) concentratie, een bepaling in het serum die onderdeel is van een klinische routine om ontsteking in het lichaam vast te stellen. Tot dusver werden echter ontstekingsparameters van de vaccinatielaesie, of bepalingen van het serum CRP, vrijwel niet ingezet om cellulaire responsen op vaccinatie te onderzoeken. De studie in **hoofdstuk 5** toont aan dat de inductie van een pro-inflammatoire cytokine response door BCG-vaccinatie alleen plaatsvindt in personen met een hoge mate van BCG-geïnduceerde huidontsteking.

Daarnaast is in **hoofdstuk 5** de inductie van Tregs door BCG-vaccinatie onderzocht. Analoog aan de activatie van $CD8^+$, maar niet $CD4^+$ Tregs na *in vitro* BCG-activatie van humane cellen beschreven in **hoofdstuk 3**, vonden we ook na BCG-vaccinatie in dit cohort alleen inductie van $CD8^+$ maar niet $CD4^+$ Tregs. In contrast met de inductie van pro-inflammatoire responsen in personen met een hoge mate van huidontsteking, was de inductie van een $CD8^+$ regulatoire respons beperkt tot personen met geen tot lage huidontsteking. Concluderend leidt BCG-vaccinatie tot een zeer variabele, en zelfs dichotome primaire cellulaire respons binnen één cohort; daarbij functioneren de mate van huidontsteking door vaccinatie en de CRP-concentratie in serum als indicatoren van de cellulaire vaccin-respons.

Het belang van expressie van inhiberende markers, zoals KLRG1 en PD-1, door T-cellen voor vaccin-geïnduceerde immuniteit tegen TB werd, zoals besproken, aangetoond in recente studies in muizen. **Hoofdstuk 6** toont voor het eerst expressie van KLRG1 op humane $CD8^+$ T-cellen na BCG-vaccinatie. Deze $CD8^+KLRG1^+$ T-cellen vertoonden na *in vitro* BCG-restimulatie een lage proliferatieve activiteit, in tegenstelling tot proliferatie van $PD\text{-}1^+$ T-cellen. Om het belang van deze markers in immuniteit tegen TB verder te onderzoeken, werd de expressie van KLRG1, PD-1, alsmede CTLA-4, vergeleken tussen patiënten met actieve TB, individuen met latente TB, patiënten na afloop van hun behandeling tegen TB en gezonde controles. Hoewel in actieve TB-patiënten expressie van deze inhiberende markers op $CD4^+$ T-cellen mild verhoogd was in vergelijking met gezonde mensen, was de expressie nog hoger op $CD4^+$ T-cellen na behandeling tegen TB. Dit is mogelijk een gevolg van de grote hoeveelheid eiwitten, peptides en andere immuun-stimulerende stoffen die uit geïnfecteerde weefsels vrijkomen tijdens en na antibiotische behandeling. Meer onderzoek is nodig om de betekenis van met name KLRG1-expressie, in vergelijking met PD-1-expressie, door T-cellen vast te stellen voor immuniteit tegen TB.

Concluderend en toekomst perspectief

In dit proefschrift is de cellulaire immuun respons van volwassen mensen op BCG onderzocht, waarbij drie belangrijke cellulaire responsen zijn geïdentificeerd. Dit betreft ten eerste de inductie van $CD8^+$ en niet $CD4^+$ Tregs, door levend *M. bovis* BCG. Deze $CD8^+$

Tregs bleken CD39 te expresseren op het cel oppervlak; bovendien werd de functionele betrokkenheid van CD39 in het uitoefenen van suppressie door BCG-geactiveerde $CD8^+$ Tregs aangetoond. Ten tweede demonstreerden we een onverwacht dichotome respons op BCG-vaccinatie: er was ofwel inductie van een pro-inflammatoire respons met polyfunctionele $CD4^+$ T-cellen in personen met een hoge mate van huidontsteking van de BCG-vaccinatielaesie en circulerend CRP, ofwel een virtueel afwezige cytokine respons met daarbij inductie van $CD8^+$ Tregs en een lage tot afwezige huidontsteking en CRP. Ten derde toonden we aan dat BCG-vaccinatie $CD8^+KLRG1^+$ T-cellen induceert met een lage proliferatieve activiteit. Expressie van deze inhiberende T-cel marker zou, zoals aangetoond in recente studies in muizen, geassocieerd kunnen zijn met een gebrek aan immuniteit tegen TB.

De inductie van $CD8^+$ Tregs, de variabiliteit van de pro-inflammatoire respons die tegengesteld is aan de inductie van $CD8^+$ Tregs, en de inductie van $KLRG1^+$ T-cellen met beperkte proliferatie-activiteit, door BCG-vaccinatie in mensen, duidt op een netwerk van gerelateerde immuun responsen die mogelijk impact hebben op de beschermende effectiviteit van BCG-vaccinatie tegen TB. Dit is belangrijk gezien de beperkte effectiviteit van BCG als TB-vaccin, en een beter begrip van deze immuun mechanismen is essentieel om betere TB-vaccins te kunnen ontwerpen in de nabije toekomst, alsmede om vaccins te kunnen selecteren en prioriteren voor (verdere) klinische ontwikkeling. Ontsteking van de BCG-vaccinatielaesie en CRP-concentraties in serum na vaccinatie kunnen hierbij dienen als alternatieve indicatoren van de cellulaire respons op vaccinatie. Dit basale onderzoek naar vaccin-geïnduceerde regulatoire, pro-inflammatoire en inhiberende responsen onthult dus nieuwe factoren en mechanismen van vaccin-geïnduceerde immuniteit en immuun regulatie, met potentieel belangrijke implicaties voor het ontwerpen van effectieve vaccins tegen TB, en mogelijk andere belangrijke infecties zoals HIV en malaria. Dit kan rationeel vaccin-ontwerp en -evaluatie ondersteunen in de strijd tegen tuberculose.

Curriculum Vitae

Mardi Boer, de auteur van dit proefschrift, werd geboren op 13 mei 1983 te Leiden. In 2001 behaalde zij cum laude haar Gymnasium diploma aan het Revius Lyceum te Doorn. In het daaropvolgende jaar behaalde zij haar propedeuse van de opleiding Geneeskunde in het Academisch Medisch Centrum in Amsterdam, gevolgd door een verpleeghulpstage in het Bethesda Nursing Home, Curaçao. Zij vervolgde haar studie Geneeskunde in het AMC als een van de deelnemers aan het Supertraject. Naast haar studie volgde zij een semester Wetenschapsfilosofie en werkte ze een aantal jaar als student-onderzoeker op de Syncope-Unit van de afdeling Interne Geneeskunde van het AMC, onder begeleiding van dr. Wouter Wieling en dr. Nynke van Dijk. Daar deed zij ook haar wetenschappelijke stage naar de orthostatische tolerantie van patiënten met het syndroom van Marfan. Haar eerste keuze-coschap werd uitgevoerd op de afdeling Hematologie van het AMC. Daarna volgde een keuze-coschap Tropische Infectieziekten in Lusaka, Zambia waarbij ze werkte in Jon Hospice, een township clinic voor terminale HIV/AIDS- en TB-patiënten, en assisteerde in community outreaches voor HIV-voorlichting en -testen van de lokale NGO Tiny Tim and Friends. Dit vond plaats onder begeleiding van dr. Tim Meade in Lusaka, en dr. Michèle van Vugt vanuit het AMC. Na het cum laude afsluiten van haar coschappen in 2009 werd zij in datzelfde jaar arts-assistent op de afdeling Interne Geneeskunde van het Amstelland ziekenhuis in Amstelveen. In oktober 2010 keerde zij terug naar Leiden om daar haar promotieonderzoek, beschreven in dit proefschrift, te starten op de afdeling Infectieziekten van het Leids Universitair Medisch Centrum, onder begeleiding van prof. dr. Tom Ottenhoff en dr. Simone Joosten.

Mardi zal haar werk naar cellulaire immuniteit tegen TB, en hoe dit geïnduceerd kan worden, voortzetten als postdoctoral fellow bij prof. dr. David Lewinsohn, prof. dr. Deborah Lewinsohn en dr. Christina Lancioni, in het Oregon Tuberculosis Research Lab, Oregon Health and Science University, Portland (OR), Verenigde Staten.

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Mardi C. Boer, Krista E. van Meijgaarden, Delia Goletti, Valentina Vanini, Corine Prins, Tom H.M. Ottenhoff*, Simone A. Joosten*. KLRG1 and PD-1 expression are increased on T-cells following tuberculosis-treatment and identify cells with different proliferative capacities in BCG-vaccinated adults. *Submitted*.

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**These authors contributed equally to the study.*

Notes