Regulatory, pro-inflammatory and inhibitory human T-cell responses to M. bovis BCG : opposing T-cell forces in TB-vaccination

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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-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 freeze-

dried 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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