

Friends and foes of tuberculosis: modulation of protective immunity

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Abbreviations

Abstract

Protective immunity in tuberculosis (TB) is subject of debate in the TB research community, as this is key to fully understand TB pathogenesis and to develop new promising tools for TB diagnosis and prognosis as well as a more efficient TB vaccine. But in our search for a clearly defined protective immune response, the complexity of balancing TB immunity has to be considered including different components known to modulate cell-mediated immunity such as effector cell and regulatory cell interactions at the local site of infection. Intensified research around the microbiome and how this modulate Mtb-specific immunity in the lung may also enhance our understanding of TB disease development. Furthermore, common co-morbidities such as HIV, helminthes and diabetes may skew protective TB immunity towards pathogenic and regulatory responses. In this review, we summarize some of the current knowledge on protective immunity in TB and how this can be modulated by regulatory immune cell subsets, associated co-morbidities and the human microbiome.

Keywords

Tuberculosis, T cells, macrophages, immunity, immunoregulation, microbiome, co-morbidities

Introduction

Mycobacterium tuberculosis (Mtb) is one of the most successful pathogens, infecting one-fourth of the world population [1]. Although only ~5% of infected individuals do develop active tuberculosis (TB), the disease burden and transmission are major global health problems. So, what is required from the human immune system to combat persistent and inflammatory bacteria such as Mtb? Numerous attempts have been made to describe and map protective immunity in TB. Insights in protective mechanisms is required in order to develop new therapeutic strategies, a protective vaccine, and to be able to follow disease development as well as successful therapy. TB is a complex disease in that most Mtb-exposed individuals contain the infection in a latent state, meaning the bacteria are not cleared from infected sites but the host manages to mount an immune response efficient enough to contain the infection. Perturbations in this delicate balance of immune control may have detrimental effects and may be the result of many host factors, including changes in the microbiome, host metabolism and maybe even ageing, but also exposure to other pathogens as well as suppression mediated by regulatory T (Treg) cells or other immune cell subsets [2, 3]. Failure to control TB infection results in active disease, ranging from local Mtb infection in the lung or other organs, to disseminated and advanced disease including severe, irreversible immunopathology. Hence, TB immunity can be divided into early and late stages; from exposure to immunity in latent infection and progressive disease, and vaccine-induced immunity. Since Mtb is an intracellular bacterium, protective immunity is dependent on cell-mediated responses conducted by innate and adaptive cells, including macrophages and dendritic cells (DCs) and T-cells. Many different subsets amongst these cells have been identified and the heterogeneity in surface molecules as well as secreted effector and signaling molecules is large. Linking specific phenotypes and signaling pathways to function is

key and to understand how these can change depending on the stage of infection, the Mtb strain, the local tissue environment and level of inflammation.

Mtb infection may already induce natural protection by itself, since a relatively low proportion of infected individuals will develop active TB disease during their life-time. Also Bacillus Calmette-Guerin (BCG), the only currently available vaccine against TB and the mostly distributed vaccine in the world, does protect infants and young children against severe forms of disease although BCG is less efficient in adults. The immunology of BCG vaccination has been discussed in detail recently [4], illustrating the complexity of BCG-induced immunity and even further illustrating our lack of understanding of protective responses. To complicate things further, the microbiota in the lung as well as the gut may interact with and affect the potency of Mtb-specific T-cell responses [5]. Likewise, concomitant infections, such as HIV and helminths, or other conditions including host metabolism, most extremely represented in patients with diabetes, could modify the immune responses and thereby reduce the host's ability to fight Mtb infection [6]. Host immune responses have been analysed in various stages of Mtb infection, disease and upon vaccination. TB immunity may vary significantly depending on the time since infection or BCG vaccination. In this review, we discuss some of the current knowledge of protective immune responses in TB and how these are modulated (Figure 1).

Effector cells subsets involved in protective TB immunity

Protective CD4⁺ Th1 cells

Although it is known that both CD4⁺ and CD8⁺ T-cells mediate protection against Mtb [7], the diverse phenotypes of protective T-cells are mostly undefined. The strongest evidence for a significant contribution of CD4⁺ T-cells in TB control derives from the human immunodeficiency virus (HIV) field [8, 9]. Rapid loss of memory CD4⁺ T-cells in simian immunodeficiency virus (SIV) infection enhanced reactivation of latent TB [10] and early HIV-mediated depletion of Mtb-specific Th1 cells is also prominent in individuals with latent TB [11]. A gradual decline in cellular immunity increased the risk of poorly-organized TB granuloma formation and progression of TB disease in HIV-infected individuals [12]. Accordingly, both human data [13] and experimental animal models [14] suggest that interferon- γ (IFN- γ) signaling and CD4⁺ T-cells producing IFN- γ are of central importance in TB. A critical role of the type I cytokine pathway, comprised of IFN- γ and interleukin-12 (IL-12) signaling, in the defense against mycobacterial infections has been demonstrated in patients with defects in these pathways who succumb with unusual mycobacterial infections [15]. In addition, IFN- γ disrupted mice are highly susceptible to sub-lethal doses of *M. bovis* infection as shown by the absence of nitric oxide (NO)-production in BCG-infected macrophages [16]. Thus, IFN- γ production has been the hallmark of anti-mycobacterial immunity and has been analysed in great detail. IFN- γ producing cells have been enumerated extensively in individuals with latent TB, active TB disease and following BCG vaccination. Likewise, the significance of tumor necrosis factor- α (TNF- α) has been confirmed in mice [17] and in patients with autoimmune disorders who have a greatly enhanced risk to reactivate latent TB infection following anti-TNF therapy

[18]. The widely accepted model is that particularly IFN- γ -producing CD4 $^{+}$ T-cells activate bactericidal effector mechanisms in Mtb-infected macrophages and other immune cells while TNF- α may be more involved in the structural organization and cellular recruitment to the TB granuloma [19]. In contrast, IL-4-producing CD4 $^{+}$ Th2 cells are typically involved in antibody-mediated immunity that contribute less to intracellular control of Mtb growth [20, 21].

From the other side of the coin, many studies report that IFN- γ -producing CD4 $^{+}$ T-cells are not reliable as immune correlates of protection in either murine [22, 23] or human TB [24].

Although IFN- γ positive cells are readily detected in TB patients, this may not correlate with protection against disease but rather indicate the presence of a mycobacterial infection or prior vaccination (Figure 2). Thus, even if Mtb can induce a strong T-cell response and critical production of IFN- γ , this may not be sufficient to execute protective responses. The quality of the response ie. co-expression with other imperative effector functions, is most likely also important. Production of multiple Th1 cytokines (IFN- γ , TNF- α , IL-2) by the same T-cells, so called multifunctional T-cells, were considered a better correlate of protective efficacy in TB [25]. Moreover, increased mortality in TB/HIV co-infected patients occurs independently of CD4 $^{+}$ T-cell counts [26], suggesting that protective immunity can also involve other players. Human [27-29] and non-human primate [30] studies support a more prominent role for CD8 $^{+}$ T-cells, which are capable of producing cytolytic and antimicrobial effector molecules such as perforin and granulysin [31]. But also non-classical CD8 $^{+}$ T-cells [32-34] and $\gamma\delta$ T-cells [35] as well as natural killer (NK) cells [36] and natural killer T-cells (NKT) cells [37] may possess potent protective capacity in TB [38].

Although, multifunctional T-cells simultaneous producing IFN- γ , TNF- α and IL-2 have been associated with TB protection, and despite induction of these cells by multiple vaccines and vaccine candidates, these cells were also abundantly present in patients with pulmonary TB [39]. Moreover, no correlation between induction of multifunctional T-cells and BCG induced protection in young infants was observed [40]. In contrast, the risk to develop TB disease in these young infants was associated with an increased circulation of activated CD4 $^{+}$ T-cells, suggesting a possible contribution of early immune activation to disease susceptibility [41]. Intriguingly, stronger cellular responses were detected as pre-activated lymphocytes and increased Th1 cytokine production but impaired macrophage activation in bronchoalveolar lavage (BAL) from individuals with latent TB compared healthy exposed individuals [42]. The balance in protective versus pathogenic CD4 $^{+}$ T cells responses in different phases of TB infection and disease will certainly be an interesting subject for further exploration.

To understand how protective T-cell responses are induced, we also need to learn more about the interaction between effector T-cells and antigen presenting cells (APCs) at local sites of Mtb infection. While appropriate expression of cytokines and antimicrobial molecules is critical for T-cell function, efficient migration is also key to ensure trafficking and proper localization of protective cells into TB granulomas where Mtb-infected macrophages reside. Previous *in vivo* findings suggested that limited Mtb-antigen presentation and/or recognition within granulomas failed to support a high frequency of prolonged effector T-cell-APC interactions, which resulted in a muted T-cell response [43]. Moreover, it has been shown that the onset of protective CD4 $^{+}$ Th1 responses *in vivo* in the lung are delayed [44, 45]. Therefore, not only the quality, magnitude and localization, but also the timing of the T-cell response is important to secure effective early

protection. Thus, we need to discover the nature of protective effector/memory T-cells and also how these cells are induced and regulated in the inflamed milieu.

Protective CD4⁺ Th17 cells

Th17 cells or CD4⁺ T-cells that secrete IL-17 are a pro-inflammatory subset frequently associated with autoimmune diseases but also associated with protective immunity in TB [46]. Genetic polymorphisms of IL-17 may be associated with genetic susceptibility to TB disease, but this also depends on the ethnicity of the individuals [47]. The requirement for IL-17 in early protective responses may also depend on the virulence of the Mtb strain [48]. Apart from Th17 cells, $\gamma\delta$ T-cells [49] as well as mucosal-associated invariant T-cells (MAIT) cells [50] may contribute to production of IL-17 during TB protection. In patients with active TB disease, reduced frequencies of IL-17 producing CD4⁺ T-cells [51, 52] and also MAIT cells [50] were detected compared to healthy exposed individuals. Moreover, Th17 cells, nor IL-17, were hardly detected at the site of disease, suggesting that TB progression is associated with low Th17 responses [53]. Successful treatment of active TB patients resulted in increased frequencies of Mtb-specific Th17 cells [54]. Similarly, transfer of Th17 cells from BCG vaccinated mice to recombinase activating gene (RAG) -/- mice induced IFN- γ -independent protection against Mtb infection, although this effect was also associated with enhanced local neutrophil-mediated inflammation [55]. Contrary, IL-17A-/- mice showed increased bacterial burden and impaired granuloma formation in the lungs that correlated to reduced survival upon Mtb infection [49]. Deficient granuloma formation could be due to reduced IFN- γ and TNF- α in the chronic stage [49]. IL-17 may also ameliorate TB disease severity by limiting the development of hypoxic granulomas [56]. Others have also shown that IFN- γ produced by Th17 cells is involved in

mediating protection and that localisation of the Th17 cells in close proximity with the macrophages is critical for protection [57]. Interestingly, the activity of Th17 cells may be down-regulated by Mtb-infected DCs recruiting IL-10-secreting neutrophils [58]. Although it has also been reported Mtb infected DCs are poor inducers of IFN- γ while enhancing IL17-producing CD4 $^{+}$ T cells [59], suggesting that Mtb targets DCs to skew the immune responses away from protective IFN- γ .

Vaccination studies in mice have shown that vaccines inducing stronger Th1 and Th17 responses also had a higher protective efficacy against Mtb challenge [60]. Accordingly, concomitant induction of IFN- γ and IL-17 was required to induce protection against Mtb infection *in vivo*, whereas induction of IFN- γ alone was not sufficient [61]. Likewise, balanced Th1 and Th17 responses were correlates of protective host responses in cynomolgous macaques [62]. In humans, concomitant infections with other pathogens such as hookworms, alter the frequency of protective Th17 cells that may promote disease progression [63]. Altogether, most of these data show that IL-17 producing T-cells contribute to the protective response against Mtb. However, a balanced Th17 response is critical to prevent excess neutrophil influx resulting in inflammation and pathology in the Mtb-infected lung. The debate is ongoing whether IFN- γ is involved in the IL-17-mediated protective responses, but this may depend on experimental variables such as the model system or Mtb strains used as well as timing of the response.

Protection mediated by unconventional T cell subsets

In addition to multifunctional cytokine producing T-cells, T-cells producing multiple cytolytic effector molecules have been described, so-called polycytotoxic T-cells. In particular, lipo-

arabinomannan (LAM) responsive T-cells with a polycytotoxic phenotype correlated with the capacity to control mycobacterial growth *in vitro* [64]. LAM responsive T-cells are known as unconventionally restricted T-cells, since LAM is presented by CD1b and not by classical MHC molecules. Over the recent years multiple alternative antigen presentation molecules have been identified, and have been demonstrated to present Mtb-derived ligands to T-cells. Since these antigen presentation molecules are frequently non-polymorphic the responding T-cells are called ‘donor-unrestricted T-cells’ (DURT) or unconventional T-cells [65]. This family comprises CD1 restricted T-cells, MR1 restricted MAIT cells and HLA-E restricted T-cells that may play a role in early protective effector cell responses against mycobacterial infections, since these members can respond rapidly upon pathogen encounter [65].

MAIT cells are present at increased frequencies in the human airways and thought to have a role in the early defense against pathogens such as Mtb because of their innate-like pattern recognition based activation. Patients with severe (bacterial) infections had decreased circulating MAIT cell counts, which may contribute to their increased susceptibility to secondary infections [66]. Reduced circulating MAIT cells have also been detected in TB patients, while local frequencies in the lung were increased [67]. This may suggests that MAIT cells migrate from the peripheral blood to sites of Mtb infection. Thus, a decrease in MAIT cells observed in TB patients as well as patients infected with non-tuberculous mycobacteria, correlated with disease severity [68]. Moreover, MAIT cells from TB patients were not activated to produce cytokines such as IFN- γ upon *in vitro* stimulation, which indicated that MAIT cells may be functionally impaired in TB [68]. Studies in on-human primates also show that MAIT cells are activated in response to BCG vaccination as well as Mtb infection [69], however data on the relative

contribution of MAIT cells to protective TB immunity in non-human primates is still lacking. In mice, Mtb infection induced heterogeneous MAIT responses [70]. In particular, V α 19i-Tg MAIT cells expressing CXCR3 and α 4 β 1 were recruited to the lung and contributed to early protection [70], suggesting that MAIT cells may also have a protective role in murine TB. It would be of great interest to further explore MAITs as possible therapeutic targets in TB.

Another alternatively restricted subset of CD8 $^{+}$ T-cells are the HLA-E restricted T-cells. In contrast to the HLA class Ia genes, HLA-E is virtually non-polymorphic, with a single amino acid difference discriminating the major variants. Another interesting property of importance for potential future vaccine targeting, is the lack of down-regulation by HIV [71]. HLA-E typically presents signal sequences of HLA-A,B,C alleles to prevent NK mediated lysis of healthy cells [71]. Alternatively it can also present sequences from intracellular pathogens including Mtb [71-74]. Detailed characterization of these cells revealed a unique Th2 cytokine repertoire [75-77]. Compared to individuals with latent TB, patients with active TB disease had higher frequencies of Mtb specific HLA-E restricted cells that decreased over time during treatment [75-77]. Patients suffering from TB/HIV co-infection had the highest frequencies of HLA-E restricted cells [75]. Intriguingly, T-cell clones specific for several Mtb peptides had the capacity, not only to lyse Mtb-infected target cells, but also to reduce the outgrowth of intracellular Mtb [77]. The strong capacity of HLA-E restricted T-cells to restrict Mtb growth in infected cells suggests a protective role against TB. However, increased frequencies of HLA-E restricted T-cells were reported in patients with active TB compared to LTBI individuals, but this may be the result of the enormous amount of antigen released during TB disease that evokes these responses. Mtb infection studies in mice have shown that the murine homologue of human HLA-E, Qa-1, is

involved in protection, mostly through modulation of inflammation [78]. The induction of strong effector T-cells in combination with the low level of allelic variation and stable expression makes HLA-E a very interesting candidate for vaccine targeting.

Protection mediated by trained immunity

Initial priming of the immune system upon first mycobacterial contact seems increasingly important. Gene expression analysis revealed that BCG vaccination in young infants induced two dichotomous responses, likely involving differential monocyte activation [79]. Similarly, dichotomous responses to primary BCG vaccination were also observed in adults [80]. In mice, priming with live mycobacteria induced transient immunity in contrast to immunity induced with subunit vaccines, which was more long-lasting [81]. Characterization of the vaccine-induced T-cells, revealed accelerated differentiation and inferior homing to the lung parenchyma by T-cells activated by live mycobacteria [81]. This illustrates that an early response, probably mediated by the innate immune system, is critical in the activation and education of adaptive immunity. Thus, more research to investigate early priming of immune triggers is warranted. Transient alterations upon pathogenic exposure may also induce trained innate immunity [82-85]. Trained immunity reflects a state of temporary innate immune memory, in which cells, generally monocytes, are epigenetically reprogrammed [82-85]. This concept was originally described upon BCG vaccination of naïve adults and resulted in temporal reprogramming of innate immune cells [82]. Training of innate immune cells also augments cytokine production against non-related pathogens. A recent study demonstrated that a sub-group of BCG-vaccinated individuals responded to the vaccine by effectively reducing mycobacterial growth *in vitro* [86]. This effect was associated with a stable and robust differential DNA methylation pattern that was not

observed in the BCG non-responders [86]. Studies in mice showed that BCG educated hematopoietics stem cells resulting in trained monocytes that efficiently combatted Mtb during subsequent infections (ref = kaufmann, cell 172, 176). Education at the level of hematopoietic stem cells is an interesting observation which may help explain the fact that training effects last longer than the life-span of individual monocytes or macrophages.

The process of training by live mycobacteria is unknown, but may significantly affect or even skew subsequent immune activation. Indeed, we observed signs of trained innate immunity upon recent Mtb exposure, but not in more established infection or disease (Joosten et al, JCI, 2018, in press). Interestingly, not all donors that initially controlled BCG outgrowth also developed detectable adaptive immune responses against Mtb (Joosten et al, JCI, 2018, in press). Together these data suggest that the initial activation of the immune system may be key in mounting the capacity to cope with mycobacteria later in life. Moreover, immune responses may vary considerably over time following infection or in the process to active disease. Longitudinal follow up of Mtb-infected adolescents revealed that TB disease development was associated with sequential modulation of immunological processes [87]. Early changes involved alterations in Type I/II interferon signaling and complement proteins, and repression of Th17 cells [87]. This illustrates the importance of kinetics and sequential events in the immune response against TB. Thus, it is important to consider the time elapsed since infection when selecting clinical samples for immunological characterization.

Functional read out of effector immune responses

Overall, analysis of CD4⁺ and CD8⁺ T-cells have not yielded satisfactorily markers or correlates of protective immunity, therefore functional approaches have received increasing attention. *In*

vitro mycobacterial growth inhibition assays have been explored to determine the capacity of whole blood or PBMC samples to control the growth of live mycobacteria, mostly BCG [88, 89]. In these assays, samples collected after primary BCG vaccination had the capacity to control BCG outgrowth, but this effect was abrogated by a secondary BCG vaccination [90]. Also BCG vaccination of mice results in growth reduction of BCG after infection of bulk splenocytes *in vitro* [88, 91]. Mtb infection status did not influence the capacity of whole blood to control Mtb or BCG outgrowth, possibly due to the strong neutrophil activity in the whole blood based assay [92]. Recently it was shown that exposed individuals from contact investigations, had a remarkably well capacity to control BCG outgrowth (Joosten et al, JCI, 2018, in press). Control of BCG outgrowth was mediated by a non-classical monocyte population producing CXCL10 and was the result of trained innate immunity (Joosten et al, JCI, 2018, in press). These functional assays will be interesting candidates to monitor vaccine-induced protection in future vaccination trials. Moreover, all samples with strong control of BCG outgrowth will be essential to decipher the mechanism of growth inhibition.

Regulatory cell subsets involved in suppression of protective TB immunity

Regulatory T (Treg) cells and other pathogenic T cell subsets

Obviously, the ability of the host to mount protective TB immunity is a multifactorial event that is also dependent on the local induction and expansion of immune cells with regulatory properties (Table 1). Natural CD4⁺CD25⁺FoxP3⁺ Tregs (developed in the thymus) as well as induced CD4⁺FoxP3⁺ Tregs (generated outside the thymus) are the most well-characterized regulatory subsets [2]. CD4⁺CD25⁺ Treg cells were detected at a 3-fold increased level in blood from active TB patients and Tregs had the capacity to suppress Mtb-specific IFN- γ

production [93]. IL-10-producing CD4⁺CD25⁺FoxP3⁺ Treg cells have been shown to be elevated at the site of infection in the lung and to suppress Mtb-specific effector T-cell proliferation [94]. In addition, a recent study demonstrated that CD4⁺CD25⁺FoxP3⁺ Treg cells were elevated in the peripheral blood and in the granulomatous TB lesions of patients with extensively drug-resistant TB (XDR-TB) compared to drug-susceptible and latent TB [95], suggesting that expansion of Tregs may be an immune evasion mechanism exploited by virulent Mtb strains. Moreover, compartmentalization of TB immunity was previously shown to be characterized by few cytolytic CD8⁺ T-cells expressing perforin and granulysin but increased CD4⁺FoxP3⁺ Treg cells producing TGF-β in proximity of Mtb-infected macrophages inside human granulomas, which may prevent efficient killing of infected cells [96].

While Tregs have an important role in TB, there are also other pathogenic T cell subsets and a complex network of both innate and adaptive regulatory cells and inhibitory pathway that contribute to the overall control of immune homeostasis and protective responses in TB. To mediate protection, T-cells should also be able to persist and maintain essential protective functions in the inflammatory environment in the lung. In this context, terminally differentiated T-cells expressing killer cell lectin-like receptor G1 (KLRG-1) have been suggested to be pathogenic in TB as these cells are usually associated with reduced proliferative [97] and lung migratory [98] capacities, including a higher sensitivity to the inflammatory environment in the lung parenchyma and more likely to be found in the vasculature [81, 99]. Similarly, elevated expression of IL-27 has been detected in patients with active TB and accordingly, IL-27R-expressing T-cells are associated with higher KLRG-1 and

IFN- γ expression and consequently higher bacterial burden and reduced T-cell protection in Mtb-infected lungs [99]. KLRG-1-expressing CD4 $^{+}$ T-cells were recently demonstrated to have a multifunctional cytokine profile, which is consistent with the notion that multifunctionality may not always be protective [100]. In contrast, development of central memory CD4 $^{+}$ KLRG1 $^{-}$ T-cells producing IL-2, are associated with control of Mtb growth and prolonged protection [81, 101]. Other inhibitory receptors involved in reducing Th1 immunity in TB are programmed cell death protein-1 (PD-1) [97], lymphocyte activation gene-3 (LAG-3) [102], T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) [103] and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) [104]. Such immune checkpoint inhibitors may be targeted to amplify TB immunity, but may also contribute to pathology rather than protection [105].

Regulatory B (Breg) cells

Similar to T-cells, B-cells can have functionally and temporally distinct roles in the control of immune homeostasis. B-cells are also a major cellular component of TB granulomas in humans, non-human primates, and mice [106, 107]. In contrast to effector B-cells, which can secrete cytokines to support the differentiation of either Th1 or Th2 cells, regulatory B-cells (Breg cells) can suppress the inflammatory potential of effector T-cells and alter the activity of APCs [108]. Accordingly, human CD19 $^{+}$ CD24 $^{\text{hi}}$ CD38 $^{\text{hi}}$ Breg cells have the ability to suppress the differentiation of both Th1 and Th17 cells, and to promote the expansion of FoxP3 $^{+}$ Tregs with suppressive functions [109]. CD19 $^{+}$ CD1d $^{+}$ CD5 $^{+}$ Breg cells have been shown to be elevated in patients with active TB and specifically suppress Th17 responses, which may have a negative

impact on the clinical outcome of TB [110]. Such Breg cells are reduced upon successful anti-TB treatment, which restored Mtb-specific production of the Th17 cytokine IL-22 [111].

In contrast, a recent study failed to detect an increase in different subpopulations of Bregs in either active or latent TB, but instead observed an increased frequency of IgD⁻CD27⁻ atypical B-cells that was normalized after successful anti-TB treatment [112]. Impaired B-cells correlated with poor cytokine production by T-cells, which could be mimicked by B-cell depletion in LTBI donors. Therefore, functional B-cells may be required to induce a proper cytokine production and T-cell activation in human TB. Similarly, B-cell activation was restored upon anti-TB treatment including expression of IL-5RA and apoptosis-inducing FasL, which may suggest that B-cells with regulatory properties have a protective role in TB [113]. Further studies addressing the relevance of Bregs and atypical B-cells and their relative contribution to the pathogenesis of human TB will be of significant interest.

Tolerogenic DCs

Although DCs are the most potent APCs involved in triggering naive T-cell responses, tolerogenic DCs can support the development of an immunosuppressive environment. Virulent Mtb promotes tolerogenic DCs that produce suppressive cytokines such as IL-10, IL-13, or TGF- β but also inhibitory molecules including PDL1 and PDL2, CD103, TIM-3, arginase or Indoleamine 2,3-dioxygenase (IDO) [114], which effectively diminish Th1 activation and instead contributes to the generation of Tregs [115]. Mtb-treated human DCs have been shown to expand CD4⁺CD25⁺FoxP3⁺ Tregs via Toll like receptor 2 (TLR2)- and DC-SIGN-dependent induction of PD-L1 on the DCs [116]. Recently, a lot of attention has been devoted to the

immunosuppressive enzyme IDO that degrades the essential amino acid tryptophan, causing tryptophan deprivation that may result in growth arrest of T-cells as well as decreased antigen uptake and down-regulation of costimulatory molecules on DCs [115]. Instead, an environment high in tryptophan metabolites favors expansion of CD4⁺FoxP3⁺ Tregs and thus IDO can cause immunosuppression both by growth factor starvation and by the direct action of the tryptophan metabolites. Increased levels of IDO have been detected in pleural fluid and serum from patients with TB pleuritis and pulmonary TB, respectively [117, 118]. Enhanced IDO activity in PBMCs from individuals vaccinated with the novel TB vaccine modified vaccinia Ankara expressing antigen 85A (MVA85A), were found to be inversely correlated to vaccine-induced IFN- γ responses, suggesting that IDO activity may impair the generation of a CD4⁺ T-cell memory response [119]. The exact role of IDO and tolerogenic DCs in the regulation of TB infection remains to be determined.

Alternatively activated macrophages (M2)

The macrophage is one of the most central immune cells in the regulation of TB infection as this is the primary host cell to be infected by Mtb. Macrophages are versatile cells that can be polarized to a spectrum of functional programs in response to exogenous signals such as cytokine stimulation [120]. Although macrophage differentiation is not linear, the heterogeneity of macrophage activation is simplified by the division into classically activated or M1 macrophages (inflammatory) and alternatively activated or M2 macrophages (anti-inflammatory) as well as regulatory or Mreg macrophages (immunosuppressive) [121]. The phenotype and function of the different macrophages subsets have mostly been studied in mice, while this is less clear in humans [122]. While M1 macrophages typically express inducible nitric oxide synthase (iNOS)

and trigger Th1 responses that is required in TB defense, M2 macrophages activate arginase (Arg1) and may be involved in the induction of Th2 and tissue repair responses [120].

Interestingly, iNOS and Arg1 uses the same cellular substrate, arginine, and thus directly competes for the total amount of arginine, making the relative expression of these enzymes an important determinant for macrophage-mediated killing of Mtb [123, 124]. IFN- γ and IL-4 are the main cytokines involved in M1 versus M2 activation, and therefore the Th1/Th2 balance at the site of Mtb infection may dictate the induction of local macrophage effector functions.

Mycobacterial virulence factors may interfere with M1 polarization and instead promote polarization of alternatively activated M2 macrophages that maintain a poorly microbicidal state, which facilitate mycobacterial growth and intracellular persistence [125, 126]. Particularly Arg1-expression thwarts macrophage effector function and thus elimination of Arg1 decreased bacterial loads in TB [127]. An *in vitro* TB granuloma model has demonstrated that macrophage polarization changes from M1 to M2 over time following Mtb infection [128]. Accordingly, M2 macrophages were found to predominate both necrotic and non-necrotic granulomas from TB patients, while both M1 and M2 polarized macrophages were found in the non-granulomatous lung tissues [128]. It has been proposed that alveolar macrophages in the lung are inherently alternatively activated, offering a cell permissive for Mtb replication [129]. An overrepresentation of M2 macrophages in the TB infected lung, could fuel tissue remodeling and Mtb growth that results in reduced lung function observed in patients with untreated, progressive disease [124]. Thus, a balanced ratio of M1:M2 (iNOS/Arg1) macrophages present at the site of Mtb infection may be key to avoid subversion of immune control.

In addition to the direct permissive state of M2 macrophages, these can also contribute to the generation of CD25⁺FoxP3⁺ Treg cells [130], perhaps via production of IL-10 and TGF- β [120]. Moreover, the inhibitory molecule IDO can be expressed in Mtb-infected macrophages *in vitro* [131] and in the macrophage-rich areas of pulmonary TB granulomas *in vivo* [132]. While IDO-expression in murine macrophages failed to control Mtb infection *in vivo* [131], a recent study revealed that *in vivo* inhibition of IDO in non-human primates resulted in enhanced T-cell proliferation and bacterial control involving relocation of effector T-cells to the center of pulmonary granulomas [133]. A predominance of M2 or Mregs in the TB granuloma may promote local expansion of Treg cells and impair effector cell function and the host ability to control Mtb infection [134]. Thus, a shift in M1/Teffector cells towards M2(Mreg)/Treg cells could promote immunosuppressive pathways and bacterial persistence in local TB lesions [124].

Myeloid-derived suppressor cells (MDSC)

Protective T-cell responses in TB may also be regulated by local induction of novel innate monocyte-like cells called myeloid-derived suppressor cells (MDSC) [135]. These cells have been well-described in the cancer field, but data from their role in chronic infectious diseases such as TB has just started to emerge. MDSCs increase after Mtb infection and are able to suppress CD4⁺ T-cell proliferation and cytokine production, CD8⁺ T-cell function and T-cell migration [136]. MDSCs can phagocytose Mtb bacilli and accumulate at the site of Mtb infection in the inflamed lung together with neutrophils, where they interact with granuloma-residing cells and contribute to pathological inflammation [137, 138]. *In vivo* depletion of MDSCs reduced Mtb burden and ameliorated TB disease [137]. Increased frequencies of MDSCs in TB patients were described to be associated with elevated Arg1 activity and IDO protein levels in plasma

samples [139], while IFN- γ could block MDSC activity by reducing expression of Arg1 [140]. CD14 $^-$ CD11b $^+$ MDSCs were reduced after anti-TB therapy, indicating these cells could be used as prognostic biomarkers in TB [141]. Identification of CD244 hi MDSCs in patients with active TB have been shown to express high levels of iNOS that were inversely correlated with activation and effector function of CD4 $^+$ and CD8 $^+$ T-cells [142]. This may suggest that high local production of NO produced by MDSCs may inhibit Mtb-specific T-cell responses. The dual ability of MDSCs to induce both Arg1 as well as iNOS, may imply that also these cells can be divided into several subsets with different inhibitory functions.

Major co-morbidities associated with reactivation and progression of active TB

The risk to be infected with or reactivate TB is significantly enhanced by a number of disease conditions including HIV [143] and helminth infections [144] but also by metabolic disorders such as diabetes mellitus type 2 (DM2) [145]. An important risk factor for TB development is also common viral infections such as cytomegalovirus (CMV) [41]. Infants who develop TB in the first 2 years of life had increased IFN- γ production and CD8 $^+$ T-cell activation in response to CMV stimulation [146]. Viral infections may induce immune activation that was previously identified as a risk factor for TB disease development. However, the etiology and specificity of the interaction between viral infections and development of TB disease has to be determined.

TB/HIV co-infection

TB and HIV co-infection is well-known as the cursed duet as both pathogens are dependent on cellular immunity that is gradually destroyed in HIV infection. Clinical studies confirm that HIV increases the risk of TB and likewise, Mtb infection may exacerbate HIV infection [143].

Thus, HIV induces functional changes in all T-cells, including Mtb-specific T-cells and macrophages but Mtb can also modulate immune cell function that enhances HIV replication. Part of the vicious cycle is initiated by activation and up-regulation of the HIV co-receptor CCR5 on CD4⁺ T-cells by Mtb [147]. This results in enhanced viral spread and simultaneously a more rapid HIV-induced depletion of Mtb-specific effector memory CD4⁺CCR5⁺ T-cells from mucosal sites including the lung [148], which is associated with reactivation of latent TB infection [10, 149]. Remaining Mtb-specific CD4⁺ T-cells are preferentially retained in the lung and possess a reduced capacity to produce Th1 cytokines at the site of Mtb infection [150, 151]. Accordingly, Mtb-specific cytolytic T-cell activity is severely impaired in HIV-infected individuals resulting in reduced killing of mycobacteria-infected macrophages [152]. In addition, CD4⁺Foxp3⁺ Tregs expressing high levels of CCR5 and the survival factor Bcl-2, have been shown to be productively infected by HIV at the site of Mtb infection in the pleural cavity, providing CD4⁺FoxP3⁺ Tregs with a survival advantage compared to CD4⁺FoxP3⁻ T-cells [153].

Within the granulomas, HIV has the ability to promote cellular dysfunctions on CD4⁺ and CD8⁺ T-cells and macrophages, causing profound necrosis, bacterial dissemination and TB reactivation [12, 143]. Reduced numbers of CD4⁺ T-cells have been found in granulomatous tissue of SIV/TB co-infected primates [10], which may partly explain deficient macrophage activation and enhanced intracellular Mtb growth. HIV-induced manipulation of Mtb-infected macrophages involves dampened Mtb-induced TNF- α production and less TNF-induced apoptosis of infected cells [154]. This may result in reduced cellular recruitment and lack of granuloma formation evident in HIV-infected patients with progressive disease [12]. On the other hand, Mtb can

induce a proinflammatory environment at the site of infection that promotes HIV replication [155], which highlights the double-edged sword in TB/HIV co-infection. HIV may also contribute to Mtb pathogenesis by interfering with the DC-mediated immune control. HIV and Mtb co-infected human DCs showed reduced expression of co-stimulatory molecules CD40, CD80 and CD86 as well as pro-inflammatory IL-6, IL-1 β and TNF- α , resulting in impairment to stimulate IFN- γ production by CD4 $^{+}$ T-cells [156]. In line with these findings, it was recently demonstrated that CD40-dependent co-stimulation of DCs was crucial to initiate protective Th17 responses in TB [157]. Qualitative changes in cytokine expression involving reduced Th1 responses have previously been demonstrated in PBMCs from TB/HIV co-infected patients [158, 159]. In contrast, transcriptional profiling of skin biopsies from the TST injection site, showed that TB/HIV co-infected patients with TST reactivity maintained Th1 responses but lacked immunoregulatory IL10-inducible responses that could promote pathological inflammation [160] such as IL-17-driven recruitment of neutrophils. Instead, a negative TST was associated with profound anergy of both innate and adaptive responses. Most likely, HIV-induced immune dysfunctions may be characterized by different mechanisms depending on the degree of HIV-associated immunosuppression [12].

TB/type 2 diabetes (DM2)

Host metabolism affects the functional capacities of the immune system, this is most exemplified in individuals with extreme metabolic dysregulation such as patients with type 2 diabetes. Patients with DM2 have a 3-fold increased risk to progress to TB disease [161]. Hyperglycemia and cellular insulinopenia may affect both macrophage and lymphocyte functions, leading to a diminished ability to contain Mtb [162]. Currently, there is an inconsistency regarding loss of

immune control in the progression of TB in DM2 patients; some studies suggest too little inflammation and Th1 activation while other studies imply that TB/DM2 is associated with enhanced cell-mediated immunity. In a mouse model of diet-induced DM2, it was recently shown that an increased number of inflammatory lesions was associated with a reduced proinflammatory cytokine response and suppressed phagocytosis and bactericidal capacity of alveolar macrophages infected with mycobacteria [163]. Monocytes from TB/DM2 patients have also been suggested to have an altered phenotype that may enhance TB susceptibility, i.e. an enhanced expression of CCR2 that could prevent monocyte migration to the site of Mtb infection [164]. Consistently, an impaired and delayed Th1 response and a concomitant reduction in NO-expression is evident in TB infected mice with chemically induced type 2 DM [162, 165]. Moreover, diabetic TB patients have been shown to have a lower Th1 to Th2 cytokine ratio in peripheral blood [166]. Another study suggests that selective expansion of CD4⁺CD25⁺CD127⁻ Treg cells in the lung of TB/DM2 patients compared to the peripheral blood, correlates to reduced IFN- γ and increased IL-10 levels at the site of Mtb infection [167].

Impaired control of diabetes has also been associated with increased systemic levels of pro-inflammatory cytokines (IL-1 β , IL-6 and IL-18) as well as anti-inflammatory IL-10 [168]. Cytokine and transcriptional profiling of plasma and whole blood samples support findings on elevated levels of proinflammatory cytokines and chemokines known to promote neutrophilic inflammation [169]. Likewise, enhanced Th1 and Th17 responses have been detected in TB/DM2 patients [170], while the cytolytic activity including expression of CD107a, perforin and granzyme B was defective in CD8⁺ T-cells [171]. Consistent with this finding, central memory CD4⁺ and CD8⁺ T-cell frequencies displayed a positive correlation with fasting blood

glucose and HbA1c levels [172], whereas effector/memory T-cells that strongly expressing cytolytic effector molecules [173] were detected in reduced levels in TB/DM2 patients. Overall, these contradictory results warrant a more comprehensive analysis of aberrant lymphocyte as well as macrophage responses in TB/DM disease.

TB/helminth co-infection

The third most recognized TB associated co-morbidity is concomitant infection with helminths. Mtb and helminths induce Th1 and Th2 immunity, respectively, that are likely to have antagonistic effects *in vivo*. TB patients with concomitant helminth infections present with reduced frequencies of peripheral T-cells as well as more severe radiological findings of pulmonary TB disease [174]. Asymptomatic helminth infection was shown to be associated with increased Treg and Th2 responses and a lower rate of sputum smear positivity [175]. Although deworming with albendazole failed to improve clinical symptoms, there was a significant decline in eosinophils and IL-10 production along with weight gain observed in the deworming group compared to placebo three months post-treatment [176]. Accordingly, helminths induced an IL-10 mediated inhibition of multifunctional CD4⁺ T-cells responses with potential protective efficacy in TB [177]. Diminished Th1 and Th17 responses and increased Treg cells have also been observed in individuals with latent TB and hookworm infection [63]. Accumulation of alternatively activated macrophages in the lungs of co-infected mice was dependent on the IL-4Ra-signaling pathway, which resulted in a Th2 response that promoted intracellular persistence of Mtb [178]. Additionally, chronic worm infection reduced the immunogenicity of BCG as individuals dewormed prior to vaccination, possessed elevated IFN- γ and IL-12 production while untreated BCG-vaccinated subjects had an increased production of TGF- β upon restimulation

with mycobacterial antigens *in vitro* [179]. Interestingly, the BCG-vaccinated cohort did not show enhanced classical Th2 immunity, suggesting that helminths could modulate protective TB immunity in other ways.

The role of the host microbiome in modulating protective TB immunity

Shaping the immune response

The linings of the human body are colonized with hugh amounts of micro-organisms including the skin, intestine and airways, called the microbiome. A long-time these micro-organisms have been considered to be commensal, non-harmful but also non-meaningful. Although the contribution of various bacteria to degradation and absorption of nutrients in the gut was recognized, the effects on shaping the immune response were quite recently appreciated. Immune modulating capacities of the microbiome may also be relevant for the onset of TB but may also contribute to treatment efficacy. Gut bacteria fermentate fibres (large carbohydrates) into several short chain fatty acids (SCFA), including acetate (C2), propionate (C3) and butyrate (C4). SCFA can modulate cellular processes, including gene expression, differentiation, proliferation and cell survival [180]. Differential expression of the receptors for SCFA on immune cells may explain the various effects on different cell types [180, 181]. Monocytes and macrophages exposed to SCFA generally have a more anti-inflammatory response, although opposing effects have also been published [180]. In general, SCFA induce a more tolerogenic T-cell profile, frequently with an augmented induction of Treg cells [180]. Since concentrations of SCFA are primary increased in the colon, this is largely reflecting increased Treg populations in the lamina propria [182]. Butyrate is the most powerful inducer of Tregs in the colon, likely through inhibition of histone deacetylases (HDAC) in the promotor region of FoxP3 [182].

SCFA not only mediate their effects in the intestinal system, several key components of SCFA metabolism have been shown to play a role in the lung as well. Receptors for SCFA are also expressed in the airways as well as on cells infiltrating the lungs and upper airways. Free fatty acid receptor 3 (FFAR3; GPR41) is the SCFA receptor that is key in regulating airway inflammation, and plays an important role in fibre-induced diminution of allergic asthma [181]. SCFA can induce increased IL-8 production by airway epithelial cells through FFAR3 [183]. Moreover, SCFA can reduce growth of pathogenic *Pseudomonas* *in vitro* [184]. Although the substrates for SCFA production in the lung are unclear, these data indicate that also the microbiome at the lung epithelial surface can contribute to locally increased concentrations of SCFA that modulate host immunity.

Microbiome and TB

A potential contribution of the host microbiome in the combat against TB has also been investigated. To obtain comparable results, it is critical to select appropriate clinical materials from particularly the control population, since in TB patient's, samples from the oropharynx, but not nasal samples, mimicked sputum [185] and thus the former are the preferred sample. Although major phyla were not different between TB and controls, the relative abundances of particular taxa suggest disturbances in microbial communities [185]. Analysis of nasopharynx samples (induced sputa) of children with TB, revealed no significant differences in the microbiota of children with or without TB [186]. Others have analysed the microbial composition of sputum from pulmonary TB patients and indicated that these samples were more complicated, characterized by many foreign bacteria compared to respiratory secretions from

healthy individuals [187]. Phylogenetic analysis revealed more *Proteobacteria* and *Bacteroidetes* in TB samples and more *Firmicutes* in controls with similar coughing symptoms [188]. In contrast, another study reported that the relative abundance of *Firmicutes* and *Actinobacteria* was significantly higher in TB samples [189]. The presence of certain bacteria and the disturbance of the lung microbiota may be associated with not only the onset of TB but also disease recurrence and treatment failure [190]. Assessment of microbiota in BAL from TB patients revealed specific differences, with *Cupriavidus* as dominant genus, in lower respiratory tracts [191]. The microbiome of individuals with detectable propionate levels in BAL had a relative abundance of anaerobic bacteria [192], which is in line with the hypothesis that SCFA are produced locally in the lung. Together, these data indicate that the microbiome locally in the airways is disturbed during active TB disease, however, the differences in study populations (adults vs children, different ethnic origins including differences in diet), clinical samples (nasopharynx, sputum, BAL) and analysis methods make it impossible to draw any major conclusions on the alterations in microbiota composition during TB disease.

In addition to the local microbiome, also gut microbiomes have been investigated in patients with TB and in experimental animal models of TB. In mice, antibiotic disturbance of the gut microbiota increased survival and dissemination of Mtb, which could be restored with fecal transplantation [193]. Similarly, modified gut microbiota also resulted in failed immune control of Mtb [194], while pulmonary TB patients had increased diversity in their gut microbiome [195]. Feces from patients with recurrent TB was enriched with *Actinobacteria* and *Proteobacteria*, many of which are pathogenic [195]. In contrast, controls had more beneficial, commensal organisms of the *Bacteroidetes* phylum [195]. Furthermore, increased serum

concentrations of SCFA, the products of the commensal bacteria, were associated with increased TB susceptibility in HIV-infected individuals [192]. Butyrate inhibited IFN- γ and IL-17 production in response to Mtb-antigen stimulation of PBMCs [192], in a HDAC dependent manner [196]. The host microbiome may not only influence host immunity through SCFA production and HDAC activation, recent data suggest also a contribution in the antigen repertoire of T-cells [197]. Antigen recognition of presumed Mtb epitopes was influenced by prior episodes of TB disease, but only in the relatively recent history (< 6 years) [197]. A specific set of epitopes was poorly recognized by CD4 $^{+}$ T-cells producing IFN- γ from donors with TB diagnosis and treatment in their recent history. These epitopes were poorly regulated in Mtb and had a very high degree of homology with epitopes in bacteria in the microbiome [197]. The authors claim that their recent TB antibiotic treatment has disturbed the composition of the microbiome such that these antigens are no longer presented to the immune system [197]. Indeed, several studies have demonstrated that anti-TB treatment affected the composition of the host microbiome, ie. chemotherapy depleted multiple immunologically significant commensal bacteria from stool [198]. The perturbation of the stool microbiome lasted for at least 1.2 years [198]. Experimental studies in mice revealed that each of the components of anti-TB therapy contributes to the observed dysbiosis, but rifampin and pyrazinamide have the largest effects [199].

In conclusion, the interplay between Mtb and the microbiome is interesting but highly complex. Firstly, not only the local, airway, microbiome but also the gut microbiome influence activation of immune responses against Mtb. Secondly, prior disturbances in the microbiome may influence the susceptibility to Mtb infection but may also alter protective TB immunity. Finally, since Mtb

infection in experimental animals also altered the microbiome it renders a catch 22 on cause and consequence. Thus the interplay between the microbiome and TB deserves a more detailed, systematic investigation.

Concluding remarks

Effector responses against Mtb are diverse and multiple subsets contribute to the protective efficacy. Many different subsets have been associated with protection in experimental models of TB, however the lack of protective vaccines in humans limits validation in humans. Some key players are essential but not sufficient to execute TB protection such as IFN- γ . Recent data have suggested contributions of novel players in the combat against Mtb, including innate immune responses such as those resulting from trained innate immunity.

However, induction of effector responses as such, and as friends from the human host perspective, will not be the only factor determining the outcome in the combat against the bacillus. Infection with Mtb also results in activation of many regulatory or modulatory immune subsets, the foes of the human host, and the balance between all of these determines the outcome of the infection. Moreover, many circumstances associated with health or disease influence the magnitude and power of the effector responses. The specific host related conditions such as the microbiome lining the body surface will modify the capacity of effector cells to respond to infection. Also host metabolic status, in the extreme in DM2, will affect functioning of the immune system. Similar co-existing infections with viruses, such as CMV or HIV, or parasites, such as helminths, will also modify the power of the immune system to respond to infection.

Timing may be a critical factor in particular with regard to the comorbidities as they may impact differentially during initial priming of the immune system as compared to recall stages of effector immunity.

Thus, solely the induction of effector immunity, although absolutely required, may not be sufficient to combat complex pathogens such as Mtb that have the capacity to modify many host processes. We will have to elucidate further on the modulation of host immunity by Mtb and other concomitant conditions in order to identify novel strategies to combat this world-threatening infection.

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Table 1. Regulatory immune cell subsets with a potential role in TB

Cell subset	Suppressive mechanism(s)	Potential detrimental effects in TB disease
natural Treg (nTreg) induced Treg (iTreg)	<ul style="list-style-type: none"> - Suppressive cytokines - Inhibitory surface molecules - Cell cycle arrest - Apoptosis - IL-2 consumption - AMP-mediated suppression - Decreased APC maturation, antigen presentation and/or co-stimulation. - Granzyme B-mediated killing 	<p>Suppress Th1 and Th17 effector cell responses early in the infection, either by mechanisms that target effector T and NK cells or that target different APCs. Migration of Tregs from the periphery and/or expansion at the site of infection may enhance bacterial growth in local lesions. Promotes the induction of Bregs, tolerogenic DCs and Mregs.</p>
Regulatory B cells (Breg)	<ul style="list-style-type: none"> - Suppressive cytokines - Death receptor-ligands 	<p>Accumulation of Breg cells in lymphoid follicle-like structures in close proximity to TB lesions in the lung may counteract Th1, Th17 and macrophage functions and promote the recruitment of Treg and Mreg cells to the site of infection. IL-10 production induces iTregs cells.</p>
Tolerogenic DCs	<ul style="list-style-type: none"> - Suppressive cytokines - Inhibitory surface molecules - Immunosuppressive enzyme IDO 	<p>Inhibits T cell proliferation and effector cell differentiation. May enhance expansion of Tregs at the local site of infection.</p>
Alternatively activated macrophages (M2)	<ul style="list-style-type: none"> - Suppressive cytokines and chemokines - Inhibitory receptors - Arginase-mediated deposition of collagen. - Immunosuppressive enzyme IDO - Crosstalk with stromal cells 	<p>Increased bacterial uptake promotes intracellular growth. Support immune polarization of Th2 responses that counteract Th1 immunity and increase tissue remodeling and repair in the infected lung. Arg-1 counteracts iNOS and result in decreased antimicrobial activity of macrophages. May induce Tregs at the local site of infection.</p>
Regulatory macrophages (Mreg)	<ul style="list-style-type: none"> - Suppressive cytokines - Inhibitory receptors - iNOS-mediated cytotoxicity - Immunosuppressive enzyme IDO 	<p>Delete activated T cells and can suppress T cell proliferation using high levels of IL-10 and local induction of iNOS/NO or IDO. May promote the induction of both nTregs and iTregs. Enhanced suppressive activity in concert with myeloid-derived suppressor cells.</p>

Figure 1

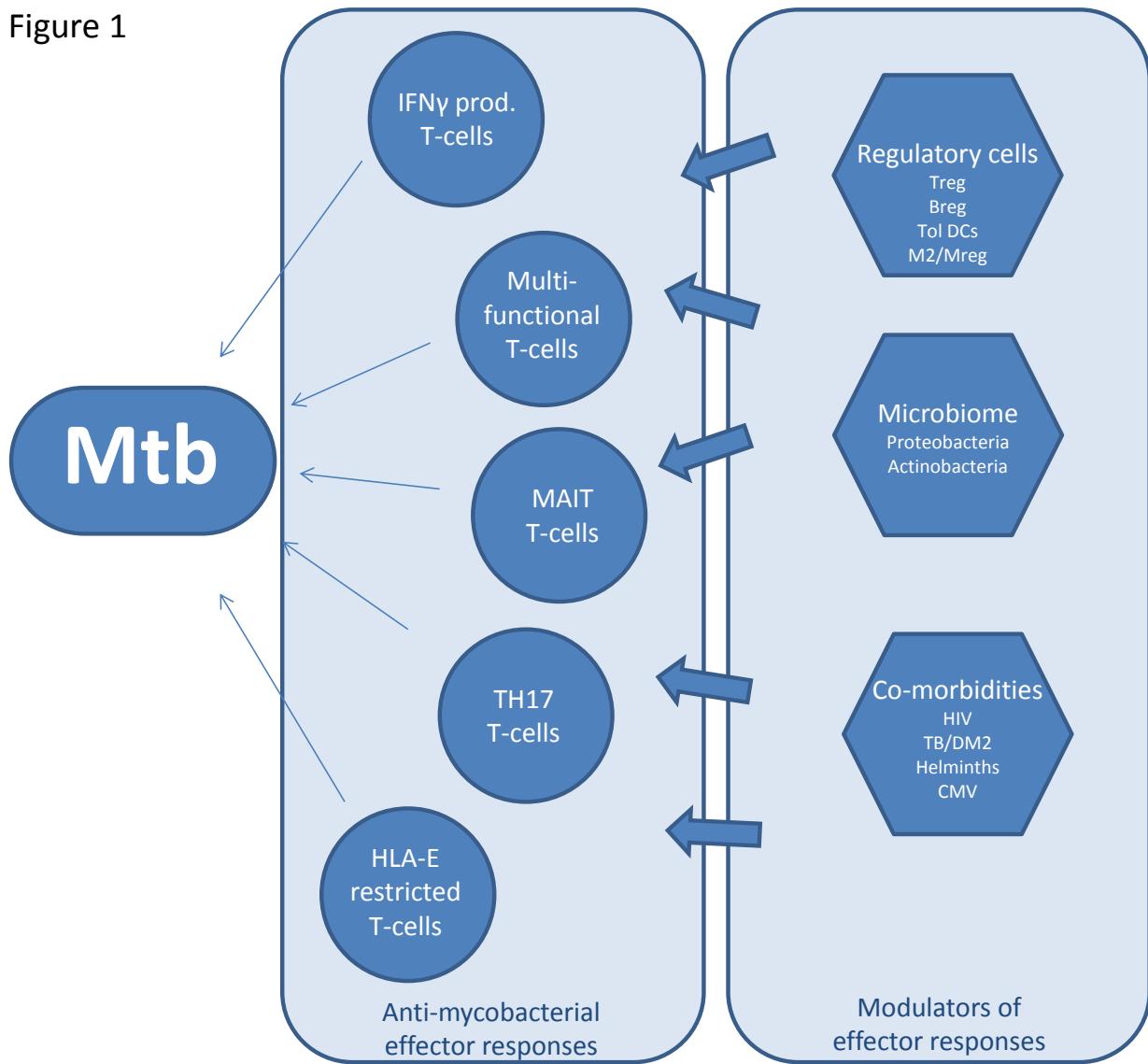


Figure 2

