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Are Polyfunctional Cells Protective in *M. tuberculosis* Infection?

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1. Introduction

Tuberculosis (TB) continues to claim almost 2 million lives each year, and causes active TB disease in over 9 million new cases yearly. Control of TB is further impeded by the strong increase in TB morbidity and mortality due to HIV co-infection, and the rise of multi-drug resistant (MDR) and extensively drug-resistant (XDR) Mycobacterium tuberculosis (Mtb) strains (WHO. Global tuberculosis control: surveillance, planning, financing: WHO 10 report 2008). Clinical disease does not develop in the vast majority (90-98%) of all Mtb infected individuals, providing compelling evidence that the human system is capable of controlling the pathogen. However, these clinically asymptomatic subjects do not achieve sterile eradication of the pathogen and consequently remain latently infected lifelong, but 2-10% of them will progress to developing TB during their lifetime.

Evidence from both animal and human studies suggest an important role for both CD4 and CD8 T cells in successful control of Mtb infection. Notably CD4 T cells of Th1 type (CD4 Th1 cells), dominate protective immunity and participate in the formation and maintenance of granuloma (Russell, 2007; Russell et al, 2010; Cooper 2009; Van der Wel et al. 2007); upon activation, CD4 T cells secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which activate antimycobacterial mechanisms in mononuclear phagocytes. IFN-γ effects on the macrophage extend beyond oxidative stress products, and also include induction of autophagy, which has been demonstrated in both human and mouse to be an essential antimycobacterial mechanism (Gutierrez et al. 2004; Singh et al. 2006; Harris et al. 2007).

Besides CD4 T cells, other T-cell subsets, such as CD8, γδ and CD1-restricted T cells influence disease outcome. On the contrary, Th2 cell inhibit autophagy-dependent killing of intracellular Mtb (Harris et al. 2007). Notably, $CD8^+$ T cells contribute to host defense, not only by cytokine production, but also by perforin- and granzyme-mediated cytotoxic activity against the pathogen and infected phagocytes. In contrast to $CD4^+$ T cells, Mtb-specific CD8 T cells are primed after transfer of mycobacterial Ags into the cytosol (Khader and Cooper 2008; Harding and Boom 2010; Kurt et al. 2010), or through crosspriming mediated by uptake of apoptotic vesicles from mycobacteria-infected macrophages by dendritic cells. Remarkably, cross-presentation also is subject to inhibition through bacterial evasion strategies that utilize eicosanoid pathways (Divangahi et al. 2010). Recent evidence suggests progressive dysfunction of CD8 T cells in chronic Mtb infection; for example, CD8

T cells in Mtb-infected mice gradually lose lytic potential during progression to the chronic phase of infection (Parida and Kaufmann 2010) and CD8 T cells from individuals with pulmonary TB display decreased cytolytic activity and expression of cytotoxic molecules, compared with these cells from uninfected healthy controls (Ho et al. 2009; Franco-Paredes et al. 2006).

Furthermore, the involvement of lymphocytes in host defense against an infection leads to the development of a memory response that normally rapidly elicits a secondary response after re-encounter of the pathogen (Pichichero 2009). In the case of chronic TB, however, the memory response must be tightly controlled in order to master the delicate tightrope walk between immunopathology and host integrity.

In addition, certain individuals, or strains of mice, may develop inappropriate (e.g., Th2) (Bold et al. 2011; Flynn et al, 1995; Wangoo et al. 2001) or imbalanced effector phenotypes such as Th1/Th17 (Chen et al. 2010) in response to infection. However, even in humans or mice that develop Th1 responses, failure of CD4 effector T cells to recognize infected cells may preclude their optimal activation and limit induction of effector functions in the lungs. Furthermore, host regulatory mechanisms that limit immune pathology, such as T regulatory cells (Tregs, Scott-Browne et al. 2007) or production of inhibitory cytokines (Turner et al. 2002), and, possibly, onset of T cell exhaustion (Yi et al. 2010; Reiley et al. 2010), may inhibit the activity of effector T cells at the site of infection. Moreover, even when CD4 effector T cells are activated, the efficacy of these responses may be limited by the impaired ability of infected cells to respond to IFN-γ (Ting et al. 1999; Banaiee et al. 2006; Pai et al. 2003), induce phagosome maturation (Rohde et al. 2007; Clemens et al. 1995) or undergo apoptosis (Hinchey et al. 2007; Miller et al. 2010).

Finally, vaccination is a key strategy in reducing the incidence of TB and unfortunately the only licensed TB vaccine, BCG, consistently protects against disseminated TB in children but fails to protect against pulmonary disease, which accounts for the burden of TB mortality and morbidity. A total of 11 vaccine candidates have entered clinical trials within the last several years (Kaufmann et al., 2010). Considering that about 2 billion humans are presumably infected with Mtb, with only 10 percent developing active disease, it is obvious that vaccination strategies follow two different approaches: pre-exposure vaccination in order to prevent disease in individuals that have so far not encountered Mtb versus post-exposure vaccination that aims at inhibiting disease outbreak in individuals that are already infected. Up to now, the majority of novel candidates belongs to the first group. A widely held view considers infection synonymous with disease, i.e., we consider disease the unequivocal sequelae of infection with a pathogen. It is the 10% of individuals at risk of developing disease who represent the targets for novel vaccines against TB. Under these circumstances, future vaccines are satisfactory if they induce an immune response in susceptible individuals comparable to that evoked by natural Mtb infection in resistant ones. Alternatively, if one aims at sterile Mtb eradication, future vaccines need to perform better than natural immunity in resistant individuals, which only contains infection. Furthermore, the factors that determine whether or not an individual is protected against natural infection with Mtb or whether someone is at risk of developing TB at a later stage are already unknown.

2. In vitro tests for diagnosis of TB

A recent break through in TB diagnosis is the introduction of IFN-γ release assay (IGRA), in which the production of IFN-γ in response to Mtb specific antigens is measured. Three commercial kits based on the IGRA principle are available: T-SPOT.TB, QuantiFERON-TB Gold, QuantiFERON-TB Gold in-tube (QFT-IT). T-SPOT.TB and QuantiFERON-TB Gold assays use only Early Secretory Antigenic Target (ESAT)-6 and Culture Filtrate Protein (CFP)-10, whereas an additional antigen TB 7.7 is incorporated in QuantiFERON-TB. The currently available data suggest that IGRAs are less influenced by prior Bacille Calmette-Guerin (BCG) vaccination and environmental mycobacteria infection (Pai et al. 2006), rendering them more specific than the tuberculin skin test (TST). In addition, with active tuberculosis as a surrogate for LTBI, it appears that the ELISA-based assays have a similar sensitivity to the TST, whereas the ELISPOT assay is more sensitive. Recent longitudinal data have demonstrated the prognostic power of positive IGRA results in recent contacts for the subsequent progression to active TB. Deployment of IGRAs, driven by new guidelines internationally, will impact on clinical practice in several ways. Their high specificity means that BCG-vaccinated individuals with a false-positive TST will not receive unnecessary preventive treatment, whereas improved sensitivity in individuals with weakened cellular immunity at highest risk of progressing to active TB (for example HIV-infected individuals) enables more reliable targeted testing and treatment. In addition, longitudinal studies during treatment of active TB and LTBI have shown that serial IGRA testing cannot be used for treatment monitoring or as test of cure. However, simultaneous measurement of IL-2 and IFN-γ correlates with therapeutic response and may allow treatment monitoring and potentially test of cure. Studies are also exploring whether measuring alternative, downstream chemokines secreted by IFN-γ-activated macrophages such as inducible protein 10 (IP-10), in combination with IFN-γ, may serve as a more amplified readout than IFN-γ alone thereby resulting in higher sensitivity. A recent study conducted in urban hospitals in United Kingdom using ELISPOT, reported that IGRA in combination with TST can be used to rule out the suspicion of active TB disease among clinically suspected subjects (Dosanjh et al. 2008). However, the data from high TB endemic countries are limited. Thus, further studies are needed to validate the role of IGRA in the diagnosis of TB in high endemic settings.

3. Pathogenesis of TB

Upon inhalation of aerosol droplets, Mtb is phagocytosed by alveolar macrophages, lung parenchyma macrophages, and dendritic cells. Subsequently, these cells elicit local inflammatory responses, leading to the recruitment of mononuclear cells from the blood, which in turn become potential targets for infection (Cooper 2009). Inside the phagosomal compartment, the mycobacteria employ their first immune evasion strategy as they prevent phagosome acidification and thus survive within this compartment (Russel 2007). Second, Mtb apparently can escape into the cytosol and thus evade phagosomal effector mechanisms (van der Wel et al. 2007). The pathogen is eventually controlled by granuloma formation, which is the histopathologic hallmark of protective immune responses. The granuloma, first being an aggregate of macrophages, neutrophils, and monocytes, develops into a more organized structure with the initiation of an adaptive immune response. Immune cells and a fibrotic wall surround the granulomas in order to prevent bacterial spreading (Russell et al.

2009), and in this form, disease outbreak can be prevented over long periods of time unless the immune response weakens. Massive cell death leads to caseation of the granuloma, and Mtb can no longer be enclosed. Mtb exploits cell necrosis to leave its host cells and spread, whereas apoptotic cell death sustains plasma membrane integrity and thus impedes Mtb exit. Here again, the bacteria apparently have developed an evasion strategy, since virulent Mtb blocks apoptosis by inhibiting prostaglandin E2 (PGE2) production (Divangahi et al. 2010).

As previously outlined, T cells are crucial for granuloma formation and containment of Mtb (Cooper 2009, Quesniaux et al. 2010; Khader and Cooper 2008). Importantly, the bacteria have developed a further immune evasion strategy to interfere with this process, since they are capable of inhibiting MHC class II molecule expression and antigen presentation. This evasion strategy is based on innate immune recognition of the bacteria via Toll-like receptor 2 (TLR2), indicating that, during the course of evolution, Mtb has found a way to turn the spear and exploit the host's innate defense mechanisms to its own advantage (Harding and Boom 2010). APCs have important instructive functions and play a central role in polarizing T-cell functions and/or lineage commitment. Efforts have been made to decipher the site of and the cell types responsible for T-cell priming (Cooper 2009). Using the mouse model of aerogenic TB infection, several groups have shown that the draining lymph nodes accommodate the priming events (Chackerian et al 2002; Wolf et al. 2008). Moreover, using antigen-pulsed cells and transfer systems, DCs were recognized to accomplish antigen presentation. IL-12p40 promotes DC migration (Khader et al. 2006), while IL-10 limits it (Demangel et al 2002). However, despite this partial success, there still exists an inability to define precisely the exact cell population delivering Mtb to the draining lymph nodes. Intriguingly, priming requires extended time in TB in comparison to many other infections. Specific T cell responses occur in the mediastinal lymph nodes 10 days after aerogenic exposure at the earliest. The reasons for the delay in priming naive T lymphocytes are still ill-defined but included slow Mtb multiplication rates, regulatory phenotype of lung-resident APCs (Cooper 2009) or natural Tregs (Shafiani et al. 2010).

4. Memory T cells and their role in Mtb infection

The study of memory T cells in individuals that have developed an adaptive immune response against a given pathogen can provide detailed information about the recognized target antigens and the class of the response. This information is relevant to define the quality of the response, to dissect the mechanisms of immunity versus immunopathology, and to design preventive and therapeutic vaccination strategies.

Generally, the induction of T cell memory is characterized by a number of distinct phases (Sallusto et al. 1999). Following Ag priming, Ag-specific T cells undergo massive proliferation and clonal expansion followed by a contraction phase in which the vast majority of the activated effector cells are eliminated by apoptosis (Lanzavecchia and Sallusto 2005; Zanetti and Franchini 2006). During this primary response, memory T cells start to emerge and are maintained for extended periods either by retained antigen, repeated stimulation/boosters, or homeostatic proliferation, hence providing a pool of cells that can rapidly respond to subsequent encounters with the pathogen. The induction of such a pool of memory T cells of adequate size and duration by vaccination procedures against intracellular pathogens has proven a major challenge for the development of new vaccines.

In humans, it is easily study the properties and functions of memory T and B cells, at least of those which circulate in the blood, using specific cell surface markers. The combinatorial expression of adhesion molecules and chemokine receptors allows for tissue specific homing of memory and effector cells and thus a segregation of the immunologic memory in terms of tissue localization (Butcher et al. 1996; Sallusto et al. 2000). Initial studies in humans led to the notion that two functionally distinct subsets of memory T cells can be identified based on the expression of lymph node homing receptors (Sallusto et al. 1999). T central memory (CM) cells express CCR7 and CD62L and, like naive T cells (TNaive), patrol the T-cell areas of secondary lymphoid organs. TCM have limited effector function but have a low activation threshold, retain high IL-2 production and proliferative capacity, and can rapidly differentiate to effector cells upon encountering the specific antigen. In contrast, T effector memory (EM) cells lack CCR7 and CD62L and express receptors for homing to peripheral or inflamed tissues, such as CCR6, CCR4, CXCR3, or CCR5. TEM cells are heterogeneous in terms of homing receptor expression and effector functions and comprise the classical T-helper cell subsets Th1, Th2, Th17, as well as cytotoxic CD8 T lymphocytes. Surface molecules other than homing receptors can be used to further dissect memory subsets. The costimulatory molecules CD28 and CD27 are expressed by TCM and by some TEM cells and are lost on the most differentiated TEM cells (Romero et al. 2007; Hamman et al. 1997). The relative distribution of antigen-specific T cells within TCM and TEM subsets may represent a useful correlate of protection; in fact, an increased frequency of antigen specific TCM cells producing high levels of IL-2 is characteristic of individuals that control chronic infectious agents such as HIV-1, hepatitis C virus (HCV), and Mtb (Harari et al. 2004; Younes et al. 2003; Semmo et al. 2005; Millington et al. 2007).

The lineage relationship between TCM and TEM has been the subject of intense investigation. The initial finding that antigenic stimulation leads to an irreversible differentiation from TCM to TEM led to the proposal of a linear differentiation model, suggesting that TCM cells are differentiation intermediates that retain proliferative capacity and differentiation potential, while TEM cells are more differentiated cells with limited proliferative potential and differentiation capacity. According to this model, T cells differentiate along a one-way linear pathway, the progression being determined by the cumulative strength of stimulation received by T cells. The stochastic interaction with antigen-presenting DCs and the different concentrations of cytokines, to which proliferating cells are exposed, would account for the generation of different fates, even within a single clone. This proposition has been corroborated by new methods that facilitate the analysis of the progeny of single T cells (Stemberger et al. 2007; Gerlach et al. 2010). In several experimental systems, it has been shown that TCM cells confer long-term protection upon adoptive transfer, while TEM cells have only limited reconstitution capacity (Gattinoni et al. 2005). Moreover, the response of TCM and TEM cells to cytokines has been initially characterized in the human system (Unutmaz et al. 1994). Using this approach, it was shown that TEM cells readily proliferate *in vitro* in response IL-7 and IL-15 but fail to expand substantially due to a high degree of spontaneous apoptosis. In contrast, TCM proliferated and spontaneously differentiated to TEM-like cells, even in the absence of polarizing cytokines (Geginat et al. 2001; Geginat et al. 2003). These findings are consistent with the notion that the TCM population contains uncommitted precursors with self-renewing capacity as well as cells that are committed to differentiate into Th1 or Th2 in an antigen-independent fashion (pre-Th1 and pre-Th2). The sustained antigen-independent generation

of TEM from TCM cells provides a plausible mechanism for the maintenance of a polyclonal and functionally diverse repertoire of TCM and TEM cells, in spite of rapid attrition of the latter.

The delineation of T cells into distinct functional populations defines the quality of the response. New evidence suggests that the quality of T-cell responses is crucial for determining the outcome of various infections. It has been postulated that T cells progressively gain functionality with further differentiation, until they reach the stage that is optimized for their effector function (such as the production of IL-2, IFN-γ and TNF-α) (Seder et al. 2008). Continued antigenic stimulation can lead to progressive loss of memory potential as well as cytokine production, resulting in terminally differentiated T cells that only produce IFN-γ and are short-lived. Following antigen stimulation, any of these stable differentiated subsets can also develop into activated effector T cells, leading to their death. The amount of initial antigen exposure or innate-immune factors in the microenvironment will govern the extent of differentiation (Figure 1).

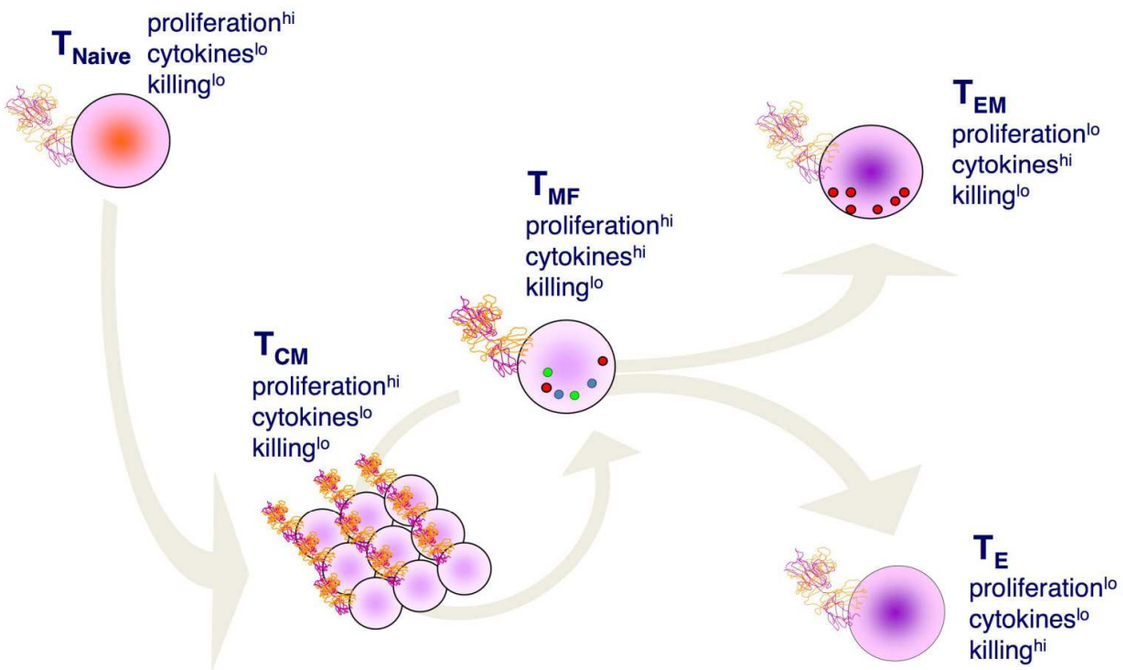

Fig. 1. Model of differentiation of memory T cells.

Unlike CD4 TEM cells, CD8 TEM cells may be able to re-acquire IL-2 expression and become CD8 TCM cells (Figure 1). An inverse correlation was observed between the frequency of multifunctional CD8 T cells and persistence of antigen load in chronic viral infection (Seder et al. 2008). Another functional state of memory cells has been observed in settings of chronic infections by pathogens that have evolved strategies to resist acute innate and adaptive immune attacks. As a result of pathogen persistence, specific CD8 T cells exhaust their cytokine production and proliferative capacity (Brooks et al. 2005; McNeil et al. 2001; Day et al. 2006). However, sustained negative signaling by the inhibitory receptor PD-1 has been mechanistically implicated in T-cell exhaustion. The role of PD-1 is illustrated by the

reversal of T cell exhaustion and concomitant increase in proliferation, cytokine secretion and cytotoxicity, and pathogen clearance upon blockade of PD-1 signaling with anti-PD-L1 antibody (Barber et al. 2006). Besides PD-1, other negative regulators, including CTLA-4, 2B4, and LAG-3, are expressed in chronically activated T cells and have been implicated in T-cell exhaustion (Crawford et al. 2009).

In summary, determining the quality of a T cell response in combination with the cell surface phenotype increases the fundamental understanding of T cell memory and effector differentiation by defining the T cell functional capacity, durability, history of antigen exposure and their capacity to traffic to lymphoid and non-lymphoid organs. Hence, a combined phenotypic and functional analysis of T cells should allow greater insight into whether a response is protective, than either measurement alone.

CD4 and CD8 T cell differentiation upon antigen stimulation, is regarded as a linear process, in which naive T cells (T_{Naive}) progressively gain functionality until they reach the stage that is optimized for their effector function (such as cytokine production or cytotoxicity). Persistent and prolonged antigen stimulation can lead to progressive loss of the pool of long-lived central memory (T_{CM}) and multifunctional (T_{MF}) T cells, resulting in terminally differentiated, short-lived T cells that only produce a single cytokine (T_{EM}) or exert cytotoxic activity(T_E). It is commonly accepted that the amount of initial antigen exposure will govern the extent of such a differentiation pathway.

5. Multifunctional CD4 T cells in Mtb infection

The capacity of antigen-specific T cells to produce simultaneously multiple cytokines (i.e., multifunctional or polyfunctional cells) has been associated with superior functional capacity (Kannanganat et al. 2007) and has been correlated with control of human chronic viral infections such as HIV (Makedonas et al. 2007; Kannanganat et al. 2010; Betts et al. 2006) and hepatitis C virus (Ciuffreda et al. 2008). Moreover, polyfunctional T cells have been associated with protection against disease progression in mouse models of Leishmania major (Darrah, et al. 2007; Darrah et al. 2010) and Mtb (Forbes et al. 2008). Polyfunctional T cells producing IFN-γ, IL-2, and TNF-α have been described in studies of Mtb infected adults (Sutherland et al. 2009; Caccamo et al. 2010; Streitz et al. 2011; Harari et al. 2011) although with differing conclusions. Overall, polyfunctional T cells that secrete multiple cytokines and are able to proliferate, are more likely than single cytokine secretors to represent correlates of protective antiviral immunity in chronic infections (when antigen load is low), while single IFN-γ-secreting CD4 and CD8 T cells are characteristic of acute infections (when antigen load is high). If chronic infection ensues after failure of complete immune control, the balance of responding T cells tends to shift towards the single IFN-γ-secreting phenotype. This process is particularly skewed in the case of HIV-1 infection, as the HIV-1-specific CD4 and CD8 T-cell response is overwhelmingly dominated by a single IFN-γ-secreting effector response during both the primary and chronic phases of infection. On the other hand, the cellular immune response to intracellular pathogens comprises a spectrum of T cell subpopulations characterized by distinct cytokine secretion profiles and surface marker phenotypes. Three main subsets are recognized and can be identified on the basis of T-cell cytokine profiles: TEM cells that secrete only IFN-γ, TEM cells that secrete both IFN-γ and IL- 2 and TCM cells that secrete only IL-2. The relative proportions of these

three T cell subsets correlate with antigen load in chronic viral infections (Sester et al. 2008; Pantaleo and Harari 2006). It has been previously shown that the relative proportions and frequencies of these three T cell subsets among Mtb antigen-specific T cells correlated with pathogen burden and antigen load in TB patients. The significant shift in cytokine profiles after anti-TB treatment led to propose this as a new approach for monitoring anti-mycobacterial treatment but the technical approach was labour intensive and the number of patients was small (Millington et al. 2007). These studies have shown that IFN-γ and IL-2 production, and the proliferative capacities of CD4 and CD8 T cells are key functions that define different aspects of the protective response. Multifunctional Mtb-specific CD4 T cells have been detected in peripheral blood of children with active TB disease and LTBI (Mueller et al. 2008), and are maintained in HIV-1-positive individuals in the absence of active disease (Day et al. 2008), although their functional capacity is affected by HIV-1 disease status both in peripheral blood (Day et al. 2008) and in the lungs (Kalsdorf et al. 2009). Therefore, quality rather than quantity of Mtb-specific T cell responses has been assumed to indicate protection and the capacity to generate long term memory. We have analyzed multifunctional CD4 T cells, expressing simultaneously three cytokines (IFN-γ, TNF-α and IL-2), in response to three Mtb antigens (ESAT-6, Ag85B and 16 kDa) in adults with active TB disease, and compared these with responses in LTBI subjects. Surprisingly, and in contrast to what has been assumed to be the hallmark of a protective CD4 T cell response, we found a significantly higher proportion of multifunctional CD4 T cells simultaneously producing IFN-γ, IL-2 and TNF-α in subjects with active TB disease, compared with LTBI subjects, while in the latter, IFN-γ single and IFN-γ/IL-2 dual secreting CD4 T cells dominated the anti-mycobacterial response. Moreover, these distinct IFN-γ, IL-2 and TNF-α profiles of Mtb-specific CD4 T cells may be associated with bacterial loads, as suggested by their decreased frequency in TB patients after completion of anti-TB chemotherapy. Lending further support to our results is the observation that the pattern of distribution of cytokine producing CD4 T cells was consistently observed in response to three different Mtb antigens, Ag85B, ESAT-6 and 16 kDa antigen. Our starting hypothesis was to find increased proportions of multifunctional T cells in LTBI subjects, since they are, to a certain level, protected against disease development, while a decreased frequency was expected in those individuals who developed disease (Caccamo et al. 2010). However, our data show the opposite pattern, namely, an increased frequency of multifunctional T cells in patients with current or historic-active TB disease and almost undetectable levels in LTBI subjects. In line with our observations, another study in the Gambia also showed that TB cases had significantly higher levels of CD4 T cells secreting simultaneously IFN-γ, IL-2 and TNF-α, compared to exposed household contacts (Sutherland et al. 2009). Collectively, the results from two different ethnic populations are in agreement, and together suggest that this particular ''multifunctional'' CD4 T cell population may be the hallmark of active TB disease. Furthermore, our results suggest that the bacterial load is related to the functional patterns of the CD4 T cell response, in fact the frequencies of Ag85B-, ESAT-6- and 16-kDa antigen-specific CD4 T cells, which simultaneously produce IFN-γ, IL-2 and TNF-α, were significantly increased during active disease, but decreased after 6 months of curative TB treatment to undetectable levels. To our knowledge, our study provides the first evidence for pre/postchemotherapy changes of ''multifunctional'' CD4 T cells, simultaneously secreting three different cytokines, IFN-γ, IL-2 and TNF-α. In our study, we also found that although multifunctional CD4 T cells were undetectable in LTBI individuals in a short-term

in vitro stimulation assay, they could be detected, although at a very low frequency, after long-term *in vitro* stimulation. Moreover, using the long-term stimulation assay, we were also able to detect significant proportion of these cells in cured TB patients. It has been hypothesized that in the short-term assay only the recently primed CD4 T cells, the product of residual antigen, would be detected, but a major reservoir of Mtb-specific CD4 T cells that returned to the resting state (Andersen et al. 2000; Bell et al. 2008) would be missed. Consequently, in individuals who have been infected with Mtb in the past, multifunctional CD4 T cells may persist but in a resting state, and hence causing negative results in a short-term incubation assay, but positive responses after a prolonged incubation. In line with our study, some authors have examined the effects of SIV infection on T cell cytokine responses in cynomolgus macaques from latent Mtb infection, acute SIV infection, and through reactivated TB in order to investigate the dynamics of multifunctional T cell responses and granuloma T cell phenotypes (Matila et al. 2011). Coinfected animals experienced increased Th1 (IL-2, IFN-γ, TNF-α) cytokine responses to Mtb Ags above the latent-response baseline 3–5 wk post-SIV infection, that corresponded to peak plasma viremia. Thus, it appears that Mtb-specific multifunctional T cells are better correlates of Ag load (i.e., disease status) than of protection. Increased cytokine responses reminiscent of amplified cytokine responses observed during acute HIV infection (Stacey et al. 2009) included significantly elevated frequencies of Mtb-specific IFN–γ, IL-2–, and TNF-α-expressing T cells. Therefore, the authors have postulated that SIV depletes T cells coordinating anti-mycobacterial responses in stable granulomas during acute infection, releasing immune pressures that normally limit bacterial replication, and leading to an increased abundance of mycobacterial antigens (Matila et al.2011). The data presented in that study suggest that the increased antigen load stimulates proliferation of Mtb-specific T cells, which are detected as Th1 cytokine-positive cells in the peripheral blood. Thus, the abundance of Mtb-specific multifunctional T cells during acute SIV infection may represent higher Ag loads, and the earliest reactivating animals had both the highest frequencies of IFN-γ^+L-2^+TNF-α^+CD4 T cells during acute infection and the highest bacterial burden at necropsy (Diedrich et al. 2010). Other authors have analyzed the cytokine profile (IFN-γ, TNF-α and IL-2) of Mtb-specific T cells by polychromatic flow cytometry and studied Mtb-specific CD4 T cell responses in subjects with latent Mtb infection and active TB disease (Sester et al. 2011). The results showed substantial increase in the proportion of TNF-α single-positive Mtb-specific CD4 T cells in subjects with active disease, and this parameter was the strongest predictor of diagnosis of active disease versus latent infection. In detail, tuberculin (PPD), ESAT-6 and CFP-10 were used as stimuli to determine antigen-specific cytokine profiles in CD4 T cells from 24 patients with active TB and 28 patients with successfully treated TB using flow-cytometry. Moreover, 25 individuals with immunity consistent with latent Mtb infection and BCG-vaccination, respectively, were recruited. When assessing cytokine profiles, PPD specific CD4 T cells secreting both IFN-γ and IL-2 predominated in treated TB, latent infection and BCG-vaccination, whilst in active TB the cytokine profile was shifted towards cells secreting IFN-γ only. A receiver operator characteristics (ROC) analysis revealed that a percentage of less than 56% of dual cytokine-secreting cells identified patients with active tuberculosis with a specificity of 100% and a sensitivity of 70%. The detection of less than 56% PPD specific dual cytokine-secreting T-cells is strongly indicative of active tuberculosis, whereas frequencies of PPD reactive dual cytokine-secreting T-cells above 56% were observed in all non-active disease states as well as active TB patients (Sester et al. 2011). These results

indicate that the analysis of cytokine profiles in Mtb-specific CD4 T cells by polychromatic flow cytometry is a major immunological measure discriminating between active and latent Mtb infection.

Other authors have recently shown a significant increase in the proportion of both CD4 and CD8 T cells expressing CD107a, instead of IL-2, in combination with IFN-γ and TNF-α in patients with active TB disease prior to treatment compared to post-treatment responses, following stimulation with ESAT-6/CFP-10, suggesting this phenotype is not protective in the TB setting (Sutherland et al. 2010). Following successful TB treatment, the proportion of cytokine positive cells was reduced to levels equivalent to those seen in healthy contacts. Furthermore, subjects with active TB disease had significantly higher levels of T cells producing 2 or more factors which were again reduced following treatment. Apart from cytokine profiling, other immune-based approaches exist to distinguish active disease from non-active states, such as the comparative analyses of Mtb-specific T-cell responses from blood and specimens from the sites of disease. However, these approaches are invasive and depend on the availability of specimens from the sites of disease (Wilkinson et al. 2005; Jafari C et al. 2009; Thomas et al. 2008; Jafari et al. 2008; Strassburg et al. 2008).

Not surprisingly, the differences seen in active disease depend on disease severity and the site of analysis: increased antigenic load in advanced pulmonary TB has been shown to correlate with decreased IFN-γ production in the blood, but increased production in the lungs. Determination of the T cell cytokine profile at specific stages of infection, disease and recovery is critical for development of new diagnostics and vaccine strategies.

A recent interesting study has attempted to correlate the relationship between Ag load in chronic Mtb infection and functional capacity of Mtb-specific T cells in three groups of adults from the Cape Town region of South Africa: 30 healthy asymptomatic adults with LTBI, and 54 individuals with pulmonary TB stratified into two groups, one acid-fast bacilli (AFB) sputum smear negative and the other sputum smear positive. Individuals with smear-positive TB displayed decreased proportions of PPD specific IFN-γ^+IL-2^+TNF-α^+ and ESAT-6-specific IL-2^+TNF-α^+CD4 T cells, but increased proportions of both ESAT-6- and PPD-specific IFN-γ^+TNF-α^+ and PPD-specific TNF-α single positive CD4 T cells, compared with other groups. This study suggests a possible selective decrease in production of IL-2 by Mtb-specific CD4 T cells in individuals with smear-positive TB, compared with smear-negative TB and LTBI, indicating a shift in the cytokine production profiles of specific CD4 T cells with increasing mycobacterial load, characterized by progressive decreases of polyfunctional cytokine production capacity, coincident with increased TNF-α production.

To determine whether reduction of bacterial load by antibiotic treatment was associated with enhanced functional capacity of Mtb-specific T cells, the authors followed longitudinally 13 TB patients after initiating anti-TB treatment; all them were sputum smear-negative by 6 months of treatment. With the exception of CFP-10–specific CD4 T cells, the total frequency of specific CD4 T cells producing any combination of cytokines was not different after 6 months of TB treatment, compared with pretreatment values. The proportion of IFN-γ^+IL-2^+TNF-α^+ and IL-2^+TNF-α^+ PPD-specific CD4 T cells increased significantly on TB treatment, coincident with a decrease in IFN-γ^+TNF-α^+ and TNF-α single-positive cells. The proportion of polyfunctional PPD-specific CD4 T cells increased in all subjects following 6 months of TB treatment. Polyfunctional Mtb-specific IFN-γ^+IL-

2^{+}TNF-α^{+} CD4 T cells were the only cytokine subset that showed a positive correlation with proliferative capacity, thus providing further evidence that polyfunctional cytokine production capacity may be associated with superior functional capacity in the context of a chronic human bacterial infection, and these results indicate that IL-2 production capacity specifically within the context of simultaneous IFN-γ and TNF-α production is indicative of Ag-specific CD4 T cell proliferative capacity. In contrast to polyfunctional Mtb-specific CD4 T cell responses, the proportion of *ex vivo* TNF-α single-positive CD4 cells, which were increased in individuals with smear-positive TB, were inversely correlated with proliferative capacity.

The populations of Mtb-specific CD4 T cells producing TNF-α, in the absence of IFN-γ and IL-2 coexpression, are expanded under inflammatory conditions of high mycobacterial load and may identify a short-lived population of effector cells with limited survival and ability to expand upon a repeated encounter with Ag.

A distinct cytokine profile consistent of simultaneously increased TNF-α, IL-6 and TGF-β was found in a TB patient cohort from Central Africa (Nemeth et al. 2010). Spontaneous TNF-α secretion as well as Mtb-specific-TNF-α production in CD4 T cells was increased in patients with active TB.

TNF-α blocking therapy is a risk factor to reactivate latent TB infection (Kaufmann et al. 2005), highlighting the role of TNF-α in granuloma maintenance during latent TB, even though the role of TNF-α during active TB is less clear and might not only be beneficial but responsible for immunopathology and contributive to the progression of disease (Quesniaux et al. 2010). These data suggest that T cells capable to produce two cytokines are a marker of active TB, a finding which is in line with the studies mentioned above (Caccamo et al. 2010; Millington et al. 2007). The authors report an excess of IL-6, TNF-α and TGF-β spontaneously secreted in the supernatant, suggesting that these cytokines are in part or completely produced by monocytes. This observation suggests that during active pulmonary TB a rather unspecific inflammation takes place which is absent in latent infection. Hence, the combination of Mtb-specific cytokines with markers of inflammation could lead to immune based diagnostics from peripheral blood which is able to discriminate between latent infection and active disease. In line, IL-6 together with TNF-α and IFN-γ have recently been used as markers to monitor TB treatment success. This observation could provide the rationale for novel immunological approaches to detect active TB.

In conclusion, possible differences in the cytokine production profiles of Mtb- specific CD4 T cells found in the reported studies may be due to differences in Ag specificity and type, methodological differences used for detection of cytokine-producing cells, and differences in study cohort characteristics. Further studies are warranted to determine particular phenotypes of Mtb-specific T cells, such as activation, memory, and inhibitory receptors and ligands, which are associated with functional capacity in different stages of Mtb infection.

6. Multifunctional CD8 T cells in Mtb infection

Following recognition of mycobacterial Ags on infected cells, CD8 T cells contribute to Mtb control through: 1) IFN-γ and TNF-α production (Flynn et al. 1992; Caccamo et al. 2009) 2) lysis of infected host cells (Cho et al. 2000; Kaufmann et al. 2005; Lalvani et al. 1998) and 3)

direct killing of mycobacteria (Klein et al. 2001; Ottenhoff et al. 2008; Stenger et al. 1997). One study demonstrated clonal CD4 and CD8 T cell expansion in granulomas from subjects with latent TB infection (Tully et al. 2005), and similar changes in the TCR repertoire were reported in peripheral blood versus pleural fluid in TB patients (Gambon-Deza et al. 1995). Furthermore, CD8 T cells specific for a number of mycobacterial Ags have been isolated from human and mouse models, consistent with the hypothesis that CD8 T cells are constantly being stimulated with Ag (Lalvani et al. 1998; Ottenhoff et al. 2008). Previously, we have reported that the frequency of Mtb-Ag85A-specific CD8 T cells correlated with therapy induced curative responses in children: Ag85A epitope-specific CD8 cells during active TB produced low levels of IFN-γ and perforin, which normalized after therapy (Caccamo et al. 2006). In a later study, we reported similar findings for CD8 T cells directed against six Mtb epitopes (two of which were newly identified). In that study, it was also found that Mtb-peptide-specific IL-2^+/IFN-γ^+ CD8 T cell responses were associated with natural protection against developing TB disease. In parallel studies, other authors (Jacobsen et al. 2007) found clonal expansion of effector memory CD8 T cells in older children with TB, with potential impact on the course and severity of disease. Other authors have tried to identify CD8 T cells that recognized a number of Mtb epitopes in the context of HLA-A and -B alleles, and ourselves reported Ag85A, B, and C epitopes activating human CD8 T cells (Klein et al. 2001; Geluk et al. 2000; Leyten et al. 2006; Lewinsohn et al. 2007). Despite these studies, little remains known about the size, quality, and specificity of Mtb-specific CD8 T cell responses in TB patients and their relevance to control of infection (i.e., prevention of progression to TB disease). The presence of mostly single and double cytokine positive T-cells, the latter mainly present in CD8 T-cells, support previous findings that single and double positive T cells are prominent in LTBI (Caccamo et al. 2010, Caccamo et al. 2009). This suggests that these double and single cytokine producing T cells play a significant role in Mtb immunity, although their precise nature and mechanisms of action requires more detailed studies. While most studies on polyfunctional T-cells have focused on highly expressed Mtb early phase proteins such as ESAT6 and Ag85B, instead, it remains possible that antigens expressed during different phases of infection may preferentially induce different patterns of single, double and polyfunctional T-cells. Regardless, the functionality of CD8 T cells was detected using peptide/tetramers, and identical-peptide stimulated PBMC of the same cured TB patients and controls were studied to determine fractions of specific CD8 T cells producing IFN-γ, IL-2, and/or TNF-α at the single-cell level. With very few exceptions, single-, double-, and, in some cases, also triple-positive. CD8 T cells could be detected in cured TB patients, but not controls, providing a wealth of new Mtb Ags that may use as targets for TB vaccine development, particularly in the view of mounting evidence that CD8 T cells are important in controlling TB.

Some authors, by using well-defined cohorts of stratified individuals with smear-positive and smear-negative TB and LTBI subjects, have investigated on the effect of mycobacterial load on the functional capacity of Mtb-specific response in peripheral blood (Day et al. 2011). They found that, compared to individuals with lower mycobacterial load, high mycobacterial load in individuals with smear-positive TB was associated with decreased polyfunctional and IL-2–producing cells and increased TNF-α single positive Mtb-specific CD4 T cells, as well as increased frequencies of specific (cytokine-positive) CD8 T cells, and impaired proliferative capacity of both Mtb-specific CD4 and CD8 T cell responses. Moreover, the presence of Mtb-specific CD8 T cells was specifically associated with

pulmonary TB disease status, showing that greater than 60% of individuals with smear-positive TB had detectable CD8 T cells responses compared with 38% and 20 % with smear-negative and LTBI, respectively, suggesting that antigen-driven expansion of Mtb-specific CD8 T cells are detectable *ex vivo* in peripheral blood in patient with pulmonary TB, respect LTBI subjects. These patients were followed during the anti-mycobacterial therapy and in 7 of the 13 individuals the proportion of triple positive CD8 T cells (producing IFN-γ, IL-2 and TNF-α) increase over time, and that this increase was coincident with a decrease in the proportion of IFN-γ single positive cells demonstrating that the cytokine production capacity of Mtb CD8 T cell responses is associated with mycobacterial load. The increase of specific polyfuntional CD8 T cells presumably, and the reduction of IFN-γ or TNF-α producing cells may be indicative to further define the association between CD8 T cells and TB disease progression, particularly in specific populations such as children or immunocompromised individuals where it may be difficult to distinguish Mtb infection from disease and that are at high risk for developing TB, but also to correlate the presence of this subset of cells as indicator of successful response to treatment.

Very recently, it has been proposed that Mtb DosR-encoded Ags (Leyten et al. 2006) expressed by Mtb during *in vitro* conditions mimics intracellular infection and represent rational targets for TB vaccination. In fact, immune responses to Mtb DosR-encoded Ags are prominently found in latently infected individuals, and are associated with LTBI in several ethnically and geographically distinct populations (Leyten et al 2006; Roupie et al. 2007; Schuck et al. 2009). Strong Mtb DosR antigen-specific CD4 and CD8 polyfunctional T-cell responses were detected in LTBI subjects. The highest responses were observed among single cytokine producing CD4 and CD8 T-cell subsets (either TNF-α^+, IL-2^+ or IFN-γ^+, depending on the stimulus) followed by double producing CD4 and particularly CD8 T cells. Of interest, the most frequent multiple-cytokine producing T cells were IFN-γ^+TNF-α^+ CD8 T cells. These cells were further characterized as effector memory (CCR7$^-$ and CD45RA$^-$) or effector (CCR7$^-$ and CD45RA$^+$) T-cells, which have the ability to perform immediate effector functions. This is compatible with an important role for CD8 T cells in Mtb infection (Bruns et al. 2009; Flynn et al. 1992). A striking observation was the wealth of epitopes that could be identified in Mtb DosR-encoded antigens, in accordance with their significant immunogenicity in a wide variety of HLA backgrounds (Ottenhoff et al. 1987).

In conclusion, the qualitative and the quantitative associations between Mtb- specific CD8 response would have the significance to better understand the progression of TB disease facilitating early diagnosis in order to reduce the rates of Mtb transmission and TB associated morbidity and mortality, but also to enhance the study aimed at evaluate the induction of CD8 T cells that are protective from TB disease designing antigen and/or peptide based vaccination approaches to TB.

7. Multifunctional T cells at the site of disease in TB

Tuberculous pleurisy (TBP) is the second most frequent manifestation of extrapulmonary tuberculosis (TB) after lymph node TB (Jafari et al. 2008) and remains a common form of disease both in HIV infected and uninfected subjects in developing countries (Luzze et al. 2001; Ozvaran et al. 2007; Heyderman et al. 1998). TBP resolves spontaneously in some patients without treatment and is thus thought to be a good model system for studying the

protective immune response at the site of infection (Jalapathy et al. 2004). At the site of active MTB infection, as opposed to other forms of TB, pleural mononuclear cells are readily accessible providing an opportunity to study aspects of TB pathogenesis on cells from the actual site of TB disease. There have been limited data regarding detailed analysis of CD4 T cell phenotypes in sites of active TB in humans. Some authors looked specifically at pleural fluid samples from subjects with TBP using single parameter IFN-γ ELISPOT methods and found the greatest proportion of IFN-γ producing cells were $CCR7^-$ effector cells (Wilkinson et al. 2005). In murine pulmonary TB models the composition of the CD4 T cells is dominated by terminally differentiated effector cells (Kapina et al. 2007; Reiley et al. 2010). A number of studies in humans have shown that polyfunctional T cells that secrete multiple cytokines may indeed mediate protection against TB (Beveridge et al 2007; Scriba et al. 2010; Abel et al. 2010; Day et al. 2008; Sutherland et al. 2010). Two recent studies have evaluated the presence of polyfunctional T cells at sites of pleural TB indicating that these results could be useful for evaluable markers for diagnosis of TB. A first study evaluated the functional profile, of Mtb-specific CD4 T cells in pleural fluid from HIV-uninfected individuals with active TBP (El Fenniri et al. 2011). It has been found that during active TB disease the greatest proportion of Mtb-specific cells in the pleural space, were TEM cells and that they also had the greatest polyfunctionality. In a second study, it has been found that in patients with TBP, Mtb-specific CD4 T cells from pleural fluid expresses IFN-γ, IL-2, TNF-α, IL-17 or IL-22 and display an effector or effector memory phenotype (Li et al. 2011).

8. Multifunctional T cells in response to vaccination

Protective immunity against Mtb results from a complex interaction between innate immune response, Th1, Th2, Th17 effector cells and cells Treg cells. It is becoming increasingly clear that there may be a difference between aspects of immunity known to be necessary for protection, and an immune response which correlates with protection. Recent studies have indicated that the ability of vaccines to evoke T cell responses of sufficient magnitude and quality for the successful containment of intracellular microbial infections is associated with the induction of multifunctional T cells which express multiple cytokines per cell (Chan et al. 1992; Flynn et al. 1995; Flesch and Kaufmann 1990; Bruns et al 2009; Chen et al. 2009; Moyo et al. 2010). Experiments in several disease models have shown that multifunctional CD4 T cells, which express IFN-γ, IL-2, and TNF-α, are functionally superior to their mono- or bifunctional counterparts. The induction of these multifunctional T cells has correlated with protection against leishmania infections in mice and the control of SIV viremia in non-human primates (Chan et al 1992; Chen et al. 2009). Interestingly, the presence of multifunctional T cells is characteristic of the immune responses seen in non progressive HIV patients, whereas HIV non-controllers elicit responses dominated by monofunctional IFN-γ secreting CD4 T cells (Moyo et al. 2010). The factors responsible for the optimized effectiveness of multifunctional cells are uncertain but probably include the capacity of these cells to secrete high levels of cytokines per cell, the synergistic intracellular killing resulting from the secretion of IFN-γ and TNF-α from the same cell, and the promotion of T cell expansion by cells expressing IL-2.

Some studies have highlighted the induction of the multifunctional T cells upon immunization with mycobacterial antigens with adjuvant formulations or with adenovirus vaccine potentiate with mycobacterial antigens.

Immunization with the Ag85/ESAT6 fusion protein in CAF01 adjuvant formulation evoked long-term protective responses characterized by high levels of persisting multifunctional cells (Lindenstrom et al. 2009). Immunization of adolescents or children with a MVA85A vaccine alone or as a BCG booster vaccine in mice induced multiple CD4 T cell subsets including cells which co-express IFN-γ, TNF-α, and IL-2 (Trunz et al. 2006). BCG vaccination of newborns also induced a complex profile of T cells expressing multiple cytokines (Tuberculosis vaccine pipeline-2009. Stop TB working group on new vaccines. 2009).

In another study the persistence of anti-TB protective immunity was investigated for five different types of vaccines – live attenuated, subunit, viral vectored, plasmid DNA and combination vaccines - during a 14-month study period. The extent of vaccine-induced protective immunity correlated with the magnitude and quality of multifunctional CD4 T cells expressing IFN-γ, TNF-α, and IL-2 that were elicited by immunization with these different TB vaccine preparations (Derrick et al. 2011).

MVA85A, a recombinant strain of modified vaccinia Ankara expressing antigen 85A (Ag85A) from Mtb (McShane et al. 2002), is the first new tuberculosis vaccine to be tested in children and infants. This vaccine, designed to enhance the BCG-induced immune response, has an extensive and promising safety and immunogenicity record in adults from different settings (Sander et al. 2009; Beveridge et al. 2008, Brookes et al. 2008; Hawkridge et al. 2008). The vaccine was also well tolerated in children aged 2–7 years from a tuberculosis endemic setting in South Africa (Scriba et al. 2010) and induced robust and durable T cell responses. Injection of three different doses of MVA85A in healthy, BCG-vaccinated infants induced a robust, long-lived, and predominantly polyfunctional CD4 T cell responses that peaked 1 month after vaccination and low frequencies of IFN-γ-expressing CD8 T cells that peaked later than the CD4 response. This response was also highly durable; magnitudes exceeded prevaccination levels up to 168 days after vaccination.

However, recent results from a large cohort of BCG-vaccinated South African infants have shown that the frequency of multifunctional T cells making IFN-γ, TNF-α and IL-2, 10 weeks post-vaccination was not associated with protection in this population. In another study, BCG-vaccinated adults significantly induced cytokine production, activation and proliferation of CD4 T cells. After polyclonal stimulation, BCG-specific CD4 T cells produced Th1-like cytokines. Importantly, the proliferation and cytokine production of CD4 T cells were inhibited by Treg and partially reversed by blocking of IL-10 production, demonstrating that BCG-specific CD4 T cells are persistent in BCG-vaccinated adults and their specific responses are modulated by Treg, implying the possibility of enhancing immune responses of TB infection by down-regulating the function of Tregs (Li et al. 2011).

Similar results were reported following human vaccination with the BCG booster AERAS-402 (recombinant replication deficient Adenovirus (Ad35) virus, expressing a polyprotein of Ag85A, Ag85B and TB10.4) (Abel et al. 2010). Finally, mice vaccinated with hybrid subunit vaccines H1 (Ag85-ESAT6) and H56 (H1+Rv2660) also had high numbers of triple cytokine producing CD4 T cells (Lindenstrom et al. 2009; Aagaard et al. 2011).

However, any immunological correlate may be vaccine and disease-stage specific. Given the diversity of vaccine candidates being developed, and the diversity of disease states in infants, adolescents and HIV-infected adults, it is unlikely that a single, simple immune correlate exists across all these different populations. Despite these intriguing results, it has

not been shown in animal models or in clinical studies that the induction anti-TB protective immunity correlates with the frequencies and/or quality of multifuntional cellular responses for different types of TB vaccines.

9. Conclusion

The induction of memory T cells with multifunctional properties appears to provide a good correlate for protection against a number of disease targets. This new, more comprehensive understanding of the full functional capacity of effector and memory T cell responses has major implications for vaccine design and development and to maintain an efficient immune surveillance for prolonged periods. Consequently, to evaluate memory formation, long-term memory responses should be examined in terms of frequency, phenotype, quality, and persistence of the memory T cells induced, which are all factors anticipated to contribute to a protective immune response.

Immune correlates of protection from TB disease progression are not well defined, although results from the above reported studies have provided that polyfunctional cells play a different role in individuals with LTBI and with pulmonary TB, with some studies indicating increased Mtb-specific polyfunctional T cell responses in TB patients and upon vaccination, while other studies have indicated either decreased polyfunctional responses in TB patients or no difference. In conclusion, these differing findings highlight the difficulties of studying human immunity to TB and the need to evaluate the polyfunctional T cells in longitudinal studies and in different clinical settings. Finally, possible differences in the cytokine production profiles of Mtb-specific T cells found in the different studies may be due to differences in antigen specificity and type, methodological differences used for detection of cytokine-producing cells, and differences in study cohort characteristics.

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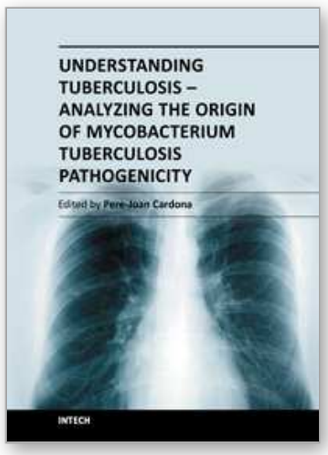

Understanding Tuberculosis - Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity

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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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