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Review

Shaping the CD4+ memory immune response against tuberculosis: the role of antigen persistence, location and multi-functionality

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Abstract

Effective vaccination against intracellular pathogens, such as tuberculosis (TB), relies on the generation and maintenance of CD4 memory T cells. An incomplete understanding of the memory immune response has hindered the rational design of a new, more effective TB vaccine. This review discusses how the persistence of antigen, the location of memory cells, and their multifunctional ability shape the CD4 memory T cell response against TB.

Keywords: CD4; intracellular pathogen; T cell memory; tuberculosis; vaccine.

Introduction

Immunological memory refers to the ability of a host to recognize and exert an enhanced response to a pathogen that it has previously encountered. Following the resolution of infection, most effector T cells die via apoptosis; however, a small pool of antigen-specific cells are able to survive and persist long term in the absence of antigen (1–3). This pool of cells, known as memory T cells, are able to induce responses that are greater in speed, magnitude and quality than primary responses, and this enhanced response is crucial for protecting the host from re-infection (4, 5). T cell immunity is critical for defense against intracellular pathogens, which by virtue of their location cannot be cleared by antibody-mediated humoral responses. Thus, the generation of long-term T cell memory is essential for vaccine-induced protection against intracellular infections (6).

The intracellular pathogen *Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis (TB) and is responsible for nearly 2 million deaths annually (7). It is estimated that approximately one-third of the world's population is infected with Mtb. Although on rare occasions the host may be able to eradicate the bacteria, in most cases they are contained within granulomas as a latent form of disease. Successful containment requires constant immune regulation, and in cases where this fails (5%–10% of those infected) clinical disease ensues, which in adults manifests as pulmonary TB (8). Bacille Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, is the only available vaccine for TB. Although BCG is the most widely administered vaccine to date, its efficacy in adults varies from 0% to 80% (9, 10), and its effectiveness wanes over time following vaccination (9, 11, 12). With the increasing frequency of TB and HIV co-infection, and the rising incidence of extensively drug-resistant Mtb, a more effective vaccine is desperately needed (13, 14).

An incomplete understanding of the memory immune response to Mtb has hindered the rational design of a new, more effective TB vaccine. It is known that immunity to TB is critically dependent upon CD4 T cells and the acquisition of a T helper cell type 1 (Th1) response, characterized by the secretion of the cytokines IFN-γ, TNF-α and IL-2 (15, 16). The crucial role that CD4 T cells play in protection against intracellular pathogens is exemplified by the increased susceptibility of individuals with HIV, who have reduced CD4 T cells, to TB (17). In addition, mycobacterial growth is uncontrolled in mice that are unable to mount CD4 T cell responses (such as CD4-deficient or Major Histocompatability Complex Class II-deficient mice), or effective Th1 immune responses (such as IFN-γ-deficient, IFN-γ receptor-deficient or IL-12p40-deficient mice) (18–23). Although other cell types may play a part in mediating immunity to TB (24), this review concentrates on CD4 T cells due to the dominant role they play in providing protection against TB.

To study immunological memory to TB, a primary response is first induced by either an initial infection with Mtb or by BCG or subunit vaccination. A secondary memory response can then be measured upon re-exposure to Mtb or BCG. Much of our understanding of the memory immune response to TB stems from research undertaken in animals, due to the ease of conducting adoptive transfer studies in inbred mice, together with the wide variety of immunological tools that are currently available. In murine models, memory cells can be identified based on their cell surface expression of CD62L, CCR7, CD45RB and CD44 (25, 26).

In mice, attempts at complete eradication of bacteria through vaccination have been unsuccessful and, as such, a 10-fold reduction in Mtb lung bacterial burden in vaccinated mice compared to controls is generally considered to represent a protective response (27). The Th1 immune response in the lungs of mice immunized against Mtb occurs several days earlier than in naïve mice, and this is associated with earlier

inhibition of Mtb growth, which may account for the 10-fold reduction in lung bacterial burden (28). Although it is evident that CD4 T cells are key for protective immunity against TB and other intracellular pathogens, the mechanisms governing memory Th1 cell development and long-term memory Th1 cell survival remain unclear. Moreover, when exposure to antigen is prolonged, as is the case with TB infection or BCG vaccination, it is unclear how transition to the memory subsets occurs (29).

Memory T cells can be broadly divided into two categories: long-lived lymphoid tissue-resident central memory T cells (Tcm) and effector memory T cells (Tem), which have a shorter life span and are found in the peripheral organs (30). Phenotypically, Tcm cells express the lymphoid homing receptors CD62L and CCR7, the isoform CD45RB, as well as the activation marker CD44. Tem cells also express CD44, but lack CD62L, CCR7 and CD45RB (25). Aside from their differences in phenotype, location and survival, these subsets differ markedly in their ability to respond when re-exposed to their cognate antigen. Tcm cells have little immediate effector function, but have a higher proliferate potential and can readily differentiate into effector cells upon re-stimulation. Conversely, Tem cells have immediate effector function, but a lower proliferative ability. The relative contribution of each subset to protection against TB has been the subject of much investigation, and is further complicated by the heterogeneity and plasticity of the expression of chemokine receptors, co-stimulatory molecules and adhesion proteins, which creates variability within the Tcm and Tem subsets (31–33).

Understanding the mechanisms required for long-term immunological memory to TB is critical for the rational design of new, improved vaccines. This review will focus on how the persistence of antigen, the location of memory cells, and their multifunctional ability shape the CD4 memory immune response.

Antigen persistence

Traditionally, T cell memory is thought to develop following antigen clearance, after contraction of the effector population (34). TB, however, is a chronic bacterial infection that can persist for the lifetime of the host, a situation that results in continual exposure of the host to mycobacterial antigens (35). Similarly, live bacteria can be found for at least a year post immunization with BCG (36). In this regard, the persistence of antigen during TB infection or following BCG vaccination has been suggested to curb the development of long-lived immunological memory (37), as it results in a protracted effector phase with limited development of central memory (31).

The idea that memory development could be hindered by persisting antigen is supported by two theories of memory cell development: the decreasing potential hypothesis (4) and the progressive differentiation model (38, 39). The decreasing potential hypothesis suggests that the effector functions of T cells progressively decline with continual antigen stimulation. It suggests that weakly antigen-stimulated cells can progress to memory cells upon antigen clearance, while strongly stimulated cells become terminally differentiated, or exhausted, and eventually undergo apoptosis. The progressive differentiation model proposes that strongly stimulated cells differentiate into effector cells that upon antigen clearance give rise to memory cells. By contrast, strongly stimulated cells that are exposed to persisting antigen become terminally differentiated effector cells and eventually die. According to either theory, in the case of TB infection or BCG vaccination where antigen exposure is likely to be persistent, exhaustion or death of CD4 T cells would occur and the transition to memory would be limited.

Recently, Reiley et al. addressed whether CD4 T cells displayed signs of terminal differentiation or exhaustion during chronic Mtb infection (40). By investigating the function of cells expressing the terminal-differentiation markers programmed death-1 (PD-1) and killer cell lectin-like receptor subfamily G, member 1 (KLRG1), and adoptively transferring sorted populations into congenic, infected recipient mice, they demonstrated that PD-1 expression does not mark exhausted CD4 T cells during Mtb infection, but instead its expression denotes proliferating effector cells. The PD-1 expressing cells later mature in a linear fashion into terminally differentiated cytokine producing cells that express KLRG1. These data suggest that following Mtb infection, a population of continuously renewing PD-1 expressing precursor cells may exist to replenish the pool of KLRG1 expressing terminal effectors. Indeed, this precursor population may be important for protection against a secondary Mtb infection. Whether these populations are present after BCG vaccination, whether they play a role in protection against a subsequent Mtb infection and whether the survival and phenotype of the PD-1 expressing CD4 T cells changes upon antigen clearance remains to be investigated.

Of note, it was recently discovered, albeit in a non-infectious setting, that in repeatedly antigen-stimulated Th1 cells, T-bet induces expression of the transcription factor *homeobox only protein* (*Hopx*) (41). Hopx was found to regulate gene expression to enhance resistance to Fas-mediated apoptosis and supporting this, adoptive transfer experiments showed that the persistence of Hopx-deficient Th1 cells was reduced to 50% of the persistence seen with Hopx-competent Th1 cell controls. *In vitro*, expression of *Hopx* was found to be highest in terminally differentiated effector/memory cells. It will be of great interest to determine whether expression of *Hopx* impacts on the survival and development of Th1 memory following Mtb infection or BCG vaccination.

In an early study, it was found that resolution of a primary Mtb infection by isoniazid chemotherapy was necessary to generate CD4 memory T cells (42). Interestingly, it was recently reported that antibiotic treatment of Mtb infected mice altered the ratio of effector or effector memory cells to central memory CD4 T cells in the lungs (31). The authors found over a three-fold reduction in the number of effector CD4 T cells and a substantial increase in the frequency of central memory CD4 T cells following resolution of Mtb infection. Thus, it appears that limiting the availability of persisting antigen by resolving an Mtb infection promotes central memory formation.

Another study used adoptive transfer of antigen-primed cells from mice that express a transgenic T cell receptor (TCR) into naïve recipients, to demonstrate that antigen clearance promotes the transition of effector to memory CD4 T cells (43). This research suggested that if an activated CD4 cell does not have a further encounter with antigen it becomes, by default, a memory cell that is capable of long-term survival. Adoptive transfer studies following experimental infection with the intracellular pathogen *Leishmania major* have also shown that the development of central memory CD4 T cells is dependent on parasite clearance. It was found that although short-lived effector cells do not persist in the absence of pathogen, memory cells survive and are able to protect against subsequent infection (44).

Following the resolution of infection, it has recently been shown that low-levels of residual antigen can be captured and presented by B cells to antigen-specific CD4 memory T cells, supporting their long-term survival (45). Using CpG mixed with ovalbumin and Vaccinia virus expressing ovalbumin to mimic infection, it was shown that the low level of B cell antigen presentation induced a state of quiescence in CD4 T cells that helped memory cells survive for long periods of time. Dormant memory cells could be activated by a second viral infection even though *in vitro* these cells were unresponsive to peptide alone, implying a requirement for inflammatory signals for reactivation. Together, these data suggest that low-level antigen presentation by B cells could be a mechanism that promotes survival of long-lived, resting memory CD4 T cells after an infection has resolved. Whether presentation by B cells is required to promote survival of memory CD4 T cells, and whether this occurs efficiently after antibiotic treatment for Mtb infection, remains to be investigated.

In contrast to the theory that persistent antigen inhibits memory development, there is good evidence that suggests some degree of antigen persistence is required for memory cell formation. A study by Williams et al. used SMARTA TCR transgenic mice that have a TCR specific for an epitope of Lymphocytic choriomeningitis virus (LCMV) and compared infection with LCMV to infection with recombinant *Listeria monocytogenes* expressing an LCMV antigen in order to study CD4 memory T cell differentiation (46). Although there was an initial expansion of CD4 effector cells with the recombinant Listeria infection, memory cells did not develop, whereas LCMV infection induced long-lived memory cells. It was found that the lack of memory response correlated with the low functional avidity of the CD4 T cells measured at the peak of the primary response to the recombinant Listeria infection, while a high functional avidity correlated with the memory cell development following LCMV infection. This implied that there was more available antigen during an LCMV challenge. In an earlier study comparing antigen persistence after LCMV infection to antigen after recombinant Listeria infection, it was found that Listeria only persisted for 8 days post infection by comparison to LCMV, which was detectable until at least day 30 (47). These data implied a hierarchical differentiation in which persistent antigen stimulation is required for the formation of CD4 memory T cells. In support of this, one theory for the inability of BCG to elicit long-term protection against Mtb is that BCG may not survive for long enough in the host to generate sufficient CD4 memory, especially in comparison to Mtb (48). This view is supported by experiments that demonstrate that recombinant BCG expressing the region of deletion-1 (RD1) locus, which is more virulent than BCG and therefore has increased persisting antigen, offers enhanced protection from an Mtb challenge in the spleens but not the lungs of mice and guinea pigs, from that afforded by BCG. Research has also demonstrated that Mtb strains engineered to be less virulent but more persistent in the host than BCG offer increased protection from Mtb infection compared to BCG (49, 50). It is currently unclear whether these recombinant strains offer improved protection from an Mtb challenge because of their increased antigen persistence in the host, or because of the introduction of additional immunodominant antigens.

Further evidence that persisting antigen can enhance memory cell survival was provided in a study by Duffy et al. (51). Adoptive transfer of $CD45RB^{low}$ CD4 T cells from mice vaccinated with BCG and boosted with an Mtb protein subunit vaccine into sublethally irradiated recipient mice that either had residual antigen from a prior BCG or protein subunit vaccine, or no residual antigen, showed that recipient mice with residual antigen had a reduced bacterial burden in the spleen following an intravenous mycobacterial challenge. Control recipients that received CD4 T cells from naïve mice or no cells were not protected. Although their results showed enhanced protection in the presence of residual antigen, their donor cells were taken from mice that were vaccinated two months prior to the adoptive transfer. Thus, their experiments do not address the question of the development of memory T cells in the presence of persisting antigen from the initial vaccination, but rather the ability of residual antigen to promote the maintenance of protective immunity, presumably (although this was not shown) by supporting memory cell survival.

The research outlined above suggests a central role for antigen signal strength in the outcome of memory cell formation. Although there is convincing evidence that antigen clearance is required for the development of long-lived immunological memory, the mechanisms behind this and the means by which this can be exploited in vaccine development still need to be completely delineated. In the case of BCG vaccination, for example, it is well documented that heat-killed BCG, which does not persist in the host, provides significantly less protection than viable BCG against a TB challenge (52, 53). Although there is less available antigen with dead BCG, the heat-killed bacteria do not provide the same spectrum of antigens as live BCG, and are therefore ineffective. There is currently research focused on identifying mutant Mtb strains that are unable to infect mammalian cells for possible use as vaccines. These would have the benefit of being live organisms and thus providing almost the full spectrum of antigens, but they would be unable to persist in the host (54). Modifying the current BCG vaccine so that antigen persistence does not inhibit the development of CD4 T cell memory may be crucial for achieving long-lasting immunity to TB. However, the balance between a vaccine that maintains a low level of

residual antigen and one that is also potent enough to drive a strong immune response is likely a delicate one.

Memory cell location

In order to dissect the memory immune response to TB, it is essential to understand where memory cells are maintained and the location in which they would be most beneficial to the host.

Elegant studies using transgenic mice expressing a TCR specific for Mtb antigens ESAT-6 or Ag85b have shown that primary CD4 T cell responses are initiated in the lung draining lymph node (dLN) during an initial response to aerosolized Mtb (55, 56); however, the location of CD4 T cell memory generation and maintenance has yet to be fully determined.

Although it has been known for some time that antigen-specific CD4 T cells reside in the bone marrow (57), it has only recently been discovered that over 80% of antigen-specific CD4 memory T cells are located in the bone marrow following an immune response (58). It had earlier been demonstrated that following intraperitoneal ovalbumin exposure, CD4 T cell numbers declined in the spleen and LN, but increased in the bone marrow where they co-localize with IL-7 producing stromal cells, a cytokine critical for the maintenance of CD4 cell memory (59, 60). Interestingly, other studies using intravenous models of bacterial infection have found that most antigen-specific CD4 memory T cells reside in secondary lymphoid organs, and not in the bone marrow (61). Through the use of beta 1 integrin-deficient mice, which have reduced retention of CD4 T cells in the bone marrow, it has been convincingly shown that cell maintenance in the bone marrow is not necessary for CD4 memory cell survival or the magnitude of the CD4 memory response (62). Interestingly, with a primary or secondary intravenous infection with an attenuated *L. monocytogenes* that is rapidly cleared by the host, there was a massive increase in antigen-specific CD4 T cells in the bone marrow, which, based on previously published research, the authors attributed to activated cells migrating from the spleen. It may be possible that although the bone marrow is the preferred location for memory cells to receive survival signals, the signals provided by the spleen and LN are sufficient for cell survival and proliferation. Importantly the quality of the memory response was not measured in the β 1 integrin-deficient mice, and thus whether the bone marrow influences the quality of the ensuing memory response is yet to be determined.

When elucidating the location of memory cells, the complexity is increased not only by the heterogeneity of CD4 memory, but also by the realization that the location for optimal cell survival may be quite different than the peripheral locations at which memory cells are required for driving enhanced protection against pathogens.

The importance of having CD4 memory T cells positioned at the site of infection prior to a lung mycobacterial infection was demonstrated in a study that made use of the drug fingolimod (FTY720), which prevents lymphocyte egress from the LN (63) and bone marrow (64). Fingolimod treated, BCG vaccinated mice were protected as well as vaccinated control mice from an intranasal mycobacterial challenge, which suggested that memory CD4 cells located at the site of infection were sufficient to mediate protection (65). This is in agreement with previous research that demonstrated the protective capacity of lymphocytes isolated from the airway lumen of intranasally immunized mice when transferred intratracheally into naïve immunodeficient recipients. Recipient mice that had received luminal cells were protected as well as recipient mice that had received splenocytes from intramuscularly vaccinated mice, following an Mtb challenge (66). Although it was not shown whether cells from vaccinated donors conferred superior protection compared to cells from naïve donor mice in the immunodeficient recipients, the results suggests that lung resident cells are sufficient to protect against a mycobacterial infection.

Further highlighting the importance of lung resident memory lymphocytes, a study of Mtb infection in mice devoid of secondary lymphoid organs identified terminally differentiated CD4 and CD8 T cells within the lungs, including multi-cytokine producing CD4 T cells. Following antibiotic treatment to clear the primary infection, these mice had a significantly reduced bacterial burden in lungs after a secondary Mtb lung infection (67). While this implies that the LN and spleen may not be necessary for protective immunity, lymphocytes may have migrated from other locations, such as the bone marrow or liver, where it has been shown that Mtb/BCG dissemination and T cell priming can occur (68, 69).

The location in which memory cells survive and are maintained and the location in which they may be most beneficial to the host in terms of protective immunity might not be the same. The bone marrow is thought to be a favorable niche for memory cell maintenance because of the availability of cell survival cytokines IL-15 and IL-7. Even though CD4 memory cells may survive better in the bone marrow, the length of time required for these cells to see antigen after re-infection and their subsequent migration to the lung may compromise the memory immune response. The evidence that memory cells at the site of infection can protect against secondary pathogens suggests that these cells may be able to react more quickly and control the infection at an early rate. It is likely that both are important; small numbers of cells at close proximity to the infectious site can work quickly to control the infection until large numbers of memory cells can arrive at the peripheral organ.

Multifunctional CD4 memory cells

The most commonly used surrogate for vaccine-induced protective immunity against Mtb has been the frequency of CD4 T cells capable of producing IFN-γ. Although IFN-γ is a key cytokine for containing an Mtb infection, measuring its production by CD4 T cells has not proven to be a reliable predictor of protective efficacy against Mtb (70, 71). Therefore, several recent studies have investigated not only the quantity but also the quality of memory T cells to provide a protective response. An important study by Darrah

et al. demonstrated that the proportion of multifunctional Th1 CD4 T cells, named so for their ability to produce IFN-γ, TFN-α and IL-2 simultaneously, correlated with protection against a *L. major* challenge (72). Thus, it was proposed that for intracellular pathogens, the proportion or number of multifunctional cells might be a predictor of immune protection. Several reports have since shown that multifunctional CD4 T cells from the spleens of mice correlate with vaccine-elicited protection from aerosol Mtb infection (73, 74). Forbes et al. demonstrated that intradermally boosting BCG-primed mice with the TB antigen, known as Antigen 85A, led to increased protection from an Mtb challenge afforded by BCG vaccination alone. It was found that this increased protection correlated with a large number of multifunctional CD4 T cells in the lungs, but not the spleens, of mice (75). In contrast to other studies, there was not a significant increase in the proportion of multifunctional cells in the spleen from BCG vaccinated mice in comparison to unvaccinated controls.

Interestingly, it has also been shown that there is no correlation between BCG vaccine-elicited multifunctional cells in the lungs of mice treated with the drug fingolimod to inhibit migration from the LN to the lung, and observed protection from a mycobacterial challenge (65). This study differs from those described above because of the focus on lung resident CD4 memory T cells in the absence of any lymphocytes that could have migrated from the LN. It is therefore possible that the correlation between multifunctional CD4 T cells in the lung and protection from Mtb observed by Forbes et al. (75) could be due to cells that have migrated to the lung from the spleen rather than lung resident CD4 T lymphocytes.

It has also been investigated whether the proportion of multifunctional CD4 T cells could be correlated with TB disease in humans. A study of patients with either active TB disease or a latent Mtb infection has found an association between the frequency of multifunctional CD4 T cells in the peripheral blood and an active Mtb infection (76). Interestingly, the frequency of multifunctional CD4 T cells decreased in patients with active TB following treatment with anti-mycobacterial therapy. It was thus suggested that the presence of multifunctional CD4 T cells is associated with live bacterial burden. Indeed, Sutherland et al. have also shown an association between multifunctional cells and active disease in humans (77). By contrast, a study examining the peripheral blood of patients found that a higher frequency of Mtb-specific multifunctional CD4 T cells correlated with latent TB infection, while the strongest indicator of active TB was the number of TNF-α single cytokine positive cells (78). It was also recently reported that a lower proportion of multifunctional CD4 T cells was present in the peripheral blood of patients with active TB compared to latently infected or healthy individuals (79). Taken together these studies of human infection suggest that the outcome of assays measuring the multifunctionality of Mtb- or BCG-specific CD4 T cells in peripheral blood may serve as a measure of disease progression or level of antigen rather than as a predictor of immunity against re-infection. Interestingly, a study that characterized the CD4 T cell cytokine profile from vaccinated newborns demonstrated no correlation between vaccine-induced CD4 T cell multifunctionality and protection when the infants were assessed 2 years later for culture-positive TB (80).

It is clear that more research is required to understand the role of multifunctional cells in the memory response to TB. With regard to immunological memory, the frequency of multifunctional cells is not necessarily related to the long-term survival of memory cells, nor is it related to the number of cells in the memory pool, which are essential for host immunity (81). It is clear that a better correlate of vaccine-induced immunity to TB is needed.

Outlook

Delineating the requirements for long-term CD4 T cell memory development is imperative for effective vaccine generation. The evidence suggests that antigen strength drives differentiation of CD4 T cells, and although strong signals are required, there is a delicate balance between producing a desired response with both effector and memory cells and producing a response that culminates with T cell exhaustion. The research reviewed here demonstrates that removal of persistent antigen could be beneficial in developing an effective memory response. Exploiting this to develop an improved TB vaccine will require careful consideration as there are many other factors that can influence the outcome of a T cell response, including TCR avidity, amount of antigen, and clonal competition.

Understanding the location necessary for CD4 memory T cells to protect against Mtb is essential for the design of improved vaccines and vaccine delivery against TB. There are several reports that suggest lung resident CD4 T cells are capable of protecting against an infectious challenge, yet how these cells migrate to the lung after vaccination and how they are maintained in that location remains to be determined. For a successful vaccination against TB, CD4 memory T cells need to be present for the lifetime of the individual. Understanding the requirements for CD4 memory T cell survival is critical for the development of such a vaccine. Further research is required to determine whether long-term CD4 memory T cell survival is possible in the lung, or if the cells need to be replenished from another location.

In order to understand the role memory cells play in elucidating a protective response to design and validate new candidate vaccines, it is critical to define an immunological correlate of protective immunity. Although there is good evidence that the proportion of polyfunctional CD4 T cells can serve as a predictor of immune protection against some intracellular pathogens, there are several reports that suggest this may not be the case for TB. Novel purification techniques may need to be developed to elucidate the role of multifunctional CD4 T cells in protective immunity to TB. It would be helpful to focus future research on which qualities of the memory immune response contribute to the observed enhanced protection against Mtb, and how it may be possible to improve upon this through vaccination.

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