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Defining features of protective CD4 T cell responses to *Mycobacterium tuberculosis*

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CD4 T cells are critical for control of *Mycobacterium tuberculosis* (Mtb) infection and represent the best hope for vaccine-elicited protection. However, little is understood about the properties of Mtb-specific CD4 T cells that mediate control, and the lack of correlates of protection present a significant barrier to the rational development of new vaccination and therapeutic strategies which are sorely needed. Here we discuss the features of protective CD4 T cells including recent evidence for IFN-γ dependent and independent mechanisms of protection, poor protection by terminally differentiated cells and the importance of T cell migratory capacity for the control of Mtb infection.

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Introduction

Tuberculosis (TB) is leading cause of global human morbidity and mortality due to infectious disease. One third of the world population is thought to be infected with *Mycobacterium tuberculosis* (Mtb), resulting in 8.7 million new cases of disease and 1.4 million deaths annually. The incidence of new TB cases caused by drug-resistant strains is increasing and in 2011 was as high as 19% in certain regions [1]. The only available vaccine, *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG), is administered at birth and protects against disseminated TB in children, but it confers little or no protection against pulmonary disease in adults. An effective vaccine is urgently needed. Recently, the results of the first efficacy trial of a new TB vaccine candidate in the last 80 years were reported [2]. While the viral vector-based regimen meant to boost BCG immunization proved safe and immunogenic, it did not enhance the protective effect of BCG inoculation alone against the primary endpoint of TB in infants. The completion of such a trial is a tremendous accomplishment in itself, but the results highlight the challenge of developing new TB vaccines.

A major lesson from this trial is that without strong correlates of protection against TB it will be extremely difficult to develop and evaluate new vaccine candidates [3,4•]. Unfortunately, our understanding of what constitutes a protective, nonpathogenic T cell response against Mtb is still limited. Here we review recent advances indicating that a complete picture of protective CD4 T cell responses in Mtb infection will require insight into new CD4 T cell antimicrobial effector functions that can control Mtb growth, a better understanding of the properties of vaccine generated polyfunctional central memory Mtb-specific CD4 T cells and factors that regulate CD4 T cell migration into the infected lung.

Role of Th1 responses in control of Mtb

It is well established that IFN-γ responses are required for resistance to Mtb infection [5•]. Mice defective in CD4 T cells [6–9], IL-12 [10], IFN-γ [11,12], or T-bet [13] are highly susceptible to TB. Individuals with inborn errors in the IL-12/IFN-γ axis [14,15], and people who develop neutralizing autoantibodies against IFN-γ are also extremely susceptible to mycobacterial diseases [16]. In a recent study the importance of CD4 T cell-derived IFN-γ (rather than from other T cells or innate lymphocytes) in control of Mtb was directly evaluated [17•]. Using an adoptive transfer approach mice were generated where all cells except for CD4 T cells were capable of producing IFN-γ, and it was found that if CD4 T cells themselves cannot produce IFN-γ bacterial growth was exacerbated and mice succumbed more rapidly to the infection. Therefore, IFN-γ production by CD4 T cells is indeed essential for control of Mtb infection, but this is so far the only clear major anti-mycobacterial effector pathway of Mtb-specific CD4 T cells that is well established.

Evidence for IFN-γ independent effector molecules

Although so essential for host resistance to Mtb, IFN-γ responses notoriously do not correlate with resistance to tuberculosis. In a study of over 5000 infants conducted in Cape Town, it was asked if the frequency or polyfunctionality of BCG-generated T cells correlated

with the susceptibility of developing tuberculosis over the first two years of life [18]. It was found that neither the magnitude of the IFN-γ response nor the pattern of its co-production with several other cytokines correlated with the likelihood of developing active tuberculosis. Moreover, it has also been shown that the levels of IFN-γ protein positively correlated with increased pulmonary disease, fever and weight loss during Mtb infection, instead of negatively correlate as might be expected for an essential host protective factor [19].

There is also direct evidence that CD4 T cells can mediate control of Mtb infection in an IFN-γ-independent manner [20,21••]. It was shown that *in vitro* polarized Mtb-specific TCR Tg Th1 cells doubly deficient in both IFN-γ and TNF, or singly deficient in T-bet, perforin or fas were all capable of mediating control of Mtb. However, while the classic Th1 associated transcription factor and effector molecules were not required for *in vivo* protection in this T cell adoptive transfer approach, it was essential that the *in vitro* primed effector CD4 T cells were generated under Th1 conditions. Therefore, while the IL-12/IFN-γ axis is clearly essential for host resistance to Mtb infection, CD4 T cells polarized with IL-12p70 towards a Th1 response can mediate impressive control of Mtb in an IFN-γ independent manner. The specific pathways, however, are still not known.

GM-CSF is produced by invariant NK T cells in response to Mtb infected macrophages, and recombinant GM-CSF can induce *in vitro* infected macrophages to restrict the growth of Mtb [22]. Canonical effector CD4 T cells can also be a major source of GM-CSF, so this may be another major effector pathway used by Mtb-specific effector CD4 T cells. Individuals with neutralizing GM-CSF autoantibodies are clearly susceptible to cryptococcal infections, but are also likely susceptible to mycobacterial infections [23]. The definitive role of CD4 T cell derived GM-CSF in control of Mtb infection relative to innate sources, however, has not been established.

Focus is usually placed on the role of soluble factors secreted by CD4 T cells in resistance to Mtb. Cell surface proteins are probably also essential, but far less is understood about this class of effector molecules. For example, CD40L on CD4 T cells is a classic activator of CD40 expressing APCs and is expressed on Mtb-specific CD4 T cells, but CD40L deficient mice are resistant to intravenous [24] or aerosol [25] Mtb infection. However, it was recently shown that the T cell surface inhibitory receptor TIM-3 might have in interesting macrophage stimulatory role in Mtb infection. It was found that TIM-3 is expressed on CD4 T cells during Mtb infection, and that binding with its ligand galectin-9 on Mtb-infected macrophages induces IL-1β production and inhibition of bacilli growth [26].

Phenotype of vaccine-elicited CD4 T cells associated with enhanced protection in mice

Another approach to understanding properties of protective CD4 T cells is to identify T cell phenotypes that correlate with vaccine-elicited protection. For example, several studies have noted associations of vaccine-generated polyfunctional CD4 T cells with better protection against Mtb challenge [27,28]. Importantly, in humans active tuberculosis disease is associated with TNF producing monofunctional CD4 T cells compared to the polyfunctional T cells found in latent Mtb infection that has been kept in check [29]. More recent studies have found that the efficacy of vaccine-induced protection against Mtb is associated with increased KLRG1$^-$ IL-2 producing CD4 T cells. Protection against Mtb induced by BCG immunization is associated with increased frequencies of KLRG1$^-$ Mtb-specific CD4 T cells in the lungs compared to unimmunized mice, and boosting BCG primed mice with a fusion protein of Mtb antigens results in further reduced bacterial loads and still lower frequencies of pulmonary KLRG1$^-$ CD4 T cells [30•]. In another recent study, it was shown that vaccination of C57BL/6 × BALB/c F1 mice with a truncated version of ESAT-6 protein lacking the immunodominant first 15 amino acids results in better protection against Mtb infection than immunization with full length ESAT-6 due to the emergence of cryptic epitope specific T cells recognizing alternate peptides in ESAT-6 [31]. Here again, the enhanced protection mediated by the cryptic epitope-specific cells correlated with increased percentages of KLRG1$^-$ CD4 T cells that maintained the ability to produce IL-2. KLRG1 is often associated with terminally differentiate cells while IL-2 is a property often found in central memory cells. Indeed, Reiley *et al.* demonstrated that pulmonary Mtb-specific KLRG1$^+$ CD4 T cells produce very high levels of cytokines and are derived from more activated, replicating PD-1^{high} T cells [32]. Therefore, enhanced vaccine elicited protection correlates with less terminally differentiated CD4 T cells. It is not clear why less differentiated cells are more protective against Mtb infection but may, at least in part, reflect their enhanced survival or replicative capacity.

Correlation between lung-homing phenotype and protection against Mtb

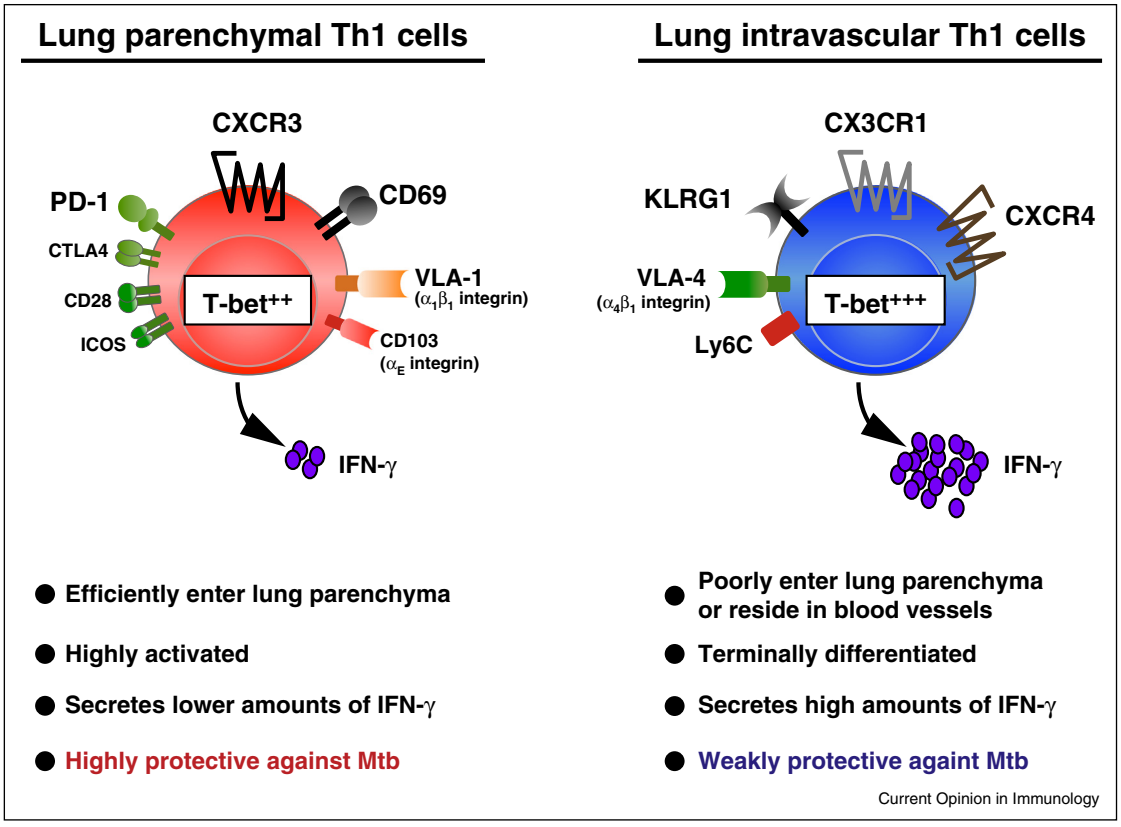
An elegant study by Srivastava *et al.* recently demonstrated that CD4 T cells must make direct contact with infected APCs to induce control of Mtb infection. In this study, the authors used a mixed bone marrow chimeric approach to examine the ability of WT and MHC class II KO macrophages and dendritic cells to restrict the growth of ingested bacilli [33••]. It was found that MHC class II KO myeloid cells contain much higher numbers of bacteria compared to WT cells in the same lung. Moreover, CD4 T cell depletion increased the number of bacilli per infected cell in WT myeloid cells resulting in identical frequency distributions between WT and MHC class II

KO cells. Therefore, CD4 T cells must directly interact with the infected APC in order to induce control of the infection.

The ability of lymphocytes to exit circulation and enter the lung tissue is likely a key feature of their protective capacity during Mtb infection. Intravascular staining, where mice are intravenously injected with fluorochrome labeled antibodies a few minutes before the tissues are harvested, has been critical to convincingly demonstrate parenchyma localization of cells isolated from nonlymphoid tissues in many studies of tissue resident memory T cells in models of acute resolving infections [34–36]. This technique was originally developed to track CD8 T cell effector migration [37], and has recently been extensively characterized and validated in multiple settings, including localization of myeloid subsets during tuberculosis [38••].

Recently the intravascular staining technique was used to examine the T cell response to Mtb infection [38••,39••]. Mtb-specific T cells are found in both the lung parenchyma and the lung vasculature and can be distinguished via differential expression of phenotypic markers and very different functional, migratory and host-protective capacities (Figure 1). Mtb-specific CD4 T cells in the lung parenchyma express very high levels of multiple activation markers, including PD-1 and CD69, indicating these cells are highly activated. In contrast, intravascular Mtb-specific CD4 T cells express high levels of KLRG1, suggesting terminally differentiated these cells are more localized to the vasculature rather than the parenchyma. Surprisingly, the KLRG1 high intravascular Mtb-specific CD4 T cells displayed the ability to produce much higher levels of IFN-γ *in vivo* or *in vitro* upon peptide restimulation. The two subsets could also be discriminated based on the differential expression of chemokine receptors. Parenchymal Mtb-specific CD4 T cells expressed high levels of CXCR3, and the intravascular cells expressed high levels of CX3CR1. While these to chemokine receptors are useful in differentiating subsets of Mtb-specific CD4 T cells, they may not be essential to control of Mtb infection themselves [40–42]. Adoptive transfer of purified parenchymal and intravascular effector cells into infection matched recipient mice showed that the CXCR3^{+} parenchymal Mtb-specific CD4 T cells rapidly

Figure 1



Properties of protective Mtb-specific CD4 T cells. Pulmonary Mtb-specific Th1 cells display two major phenotypes based on their localization in the lung parenchyma or lung-associated blood vasculature of infected mice. These two subsets differ in their migration properties, function and host protective capacity. While the intravascular subset produces the highest amounts of IFN-γ *in vivo*, the parenchyma homing subset mediates the best control of Mtb infection [39••].

migrate back into the parenchyma while the CX3CR1$^+$ intravascular cells do so poorly. Most importantly, it was found that the adoptive transfer of the Mtb-specific parenchymal CD4 T cells induced much greater control Mtb infection compared to the intravascular counterparts. These results indicate that not all Mtb-specific effector CD4 T cells can enter the lung parenchyma, and the ability to exit the vasculature and enter the infected tissue strongly correlates with the ability to control the infection. Therefore, lung tissue migratory capacity, rather than IFN-γ production per se, may be a more important property to consider when evaluating antigen-specific CD4 T cell mediated protection against Mtb. In fact, the correlation between KLRG1$^-$ CD4 T cells and enhanced vaccine mediated protection discussed above may also partly reflect better generation of tissue parenchyma-localizing CD4 T cells.

While CD4 T cells with enhanced lung homing characteristics display the best protective capacity, due to their ability to interact with infected APC, the parenchymal effector cells expressed the highest level of PD-1. PD-1 KO mice die from overwhelming CD4 T cell-mediated immunopathology, indicting that the most protective CD4 T cells may also have the ability to become highly pathogenic when not properly regulated [43]. A host protective role for the CX3CR1$^+$ intravascular subset is far less clear. However, these cells may interact with the inflamed endothelium to modulate recruitment of other immune cells or angiogenesis. It is also reasonable to speculate that these cells co-localize and interact with the CX3CR1$^+$ endothelium-scanning monocytes [38••,44]. Perhaps this proposed T cell:APC interaction in the vasculature helps ensure containment of the infection to the lung by capturing and containing microbial pathogens that make it out of the lung into the circulation. Indeed, dissemination of microbes from peripheral tissues into the blood creates a much larger problem for the host.

Conclusions

There is now a wide appreciation for the importance of extending our understanding of anti-tuberculosis CD4 T cell effector molecules beyond IFN-γ. In fact, this is not specific to the study of Mtb, and reflects the limited information on the mechanisms through which CD4 T cells drive activation of macrophages to kill pathogens that replicate in the phagosome [45]. Vaccine studies have shown that CD4 T cells with a more central memory polyfunctional phenotype correlate with enhanced protection, and studies of CD4 T cell migration have shown that their ability to enter the lung parenchyma is not a given but is essential for protection. Collectively, this emphasizes the need to better describe the basic mechanisms of CD4 T cell interactions with infected macrophages and dendritic cells, the properties of polyfunctional Th1 cells, and the link between terminal differentiation and T cell migratory capacity. Integrating these multiple perspectives of protective CD4 T cells will provide rationale for the development of novel strategies for vaccination or immunotherapeutic interventions in tuberculosis.

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