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Understanding and overcoming the barriers to T cell mediated immunity against tuberculosis

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Abstract

Despite the overwhelming success of immunization in reducing, and even eliminating, the global threats posed by a wide spectrum of infectious diseases, attempts to do the same for tuberculosis (TB) have failed to date. While most effective vaccines act by eliciting neutralizing antibodies, T cells are the primary mediators of adaptive immunity against TB. Unfortunately, the onset of the T cell response after aerosol infection with *Mycobacterium tuberculosis* (Mtb), the bacterium that causes TB, is exceedingly slow, and systemically administered vaccines only modestly accelerate the recruitment of effector T cells to the lungs. This delay seems to be orchestrated by Mtb itself to prolong the period of unrestricted bacterial replication in the lung that characterizes the innate phase of the response. When T cells finally arrive at the site of infection, multiple layers of regulation have been established that limit the ability of T cells to control or eradicate Mtb. From this understanding, emerges a strategy for achieving immunity. Lung resident memory T cells may recognize Mtb-infected cells shortly after infection and confer protection before regulatory networks are allowed to develop. Early studies using vaccines that elicit lung resident T cells by targeting the lung mucosa have been promising, but many questions remain. Due to the fundamental nature of these questions, and the need to understand and manipulate the early events in the lung after aerosol infection, only coordinated approaches that utilize tractable animal models to inform human TB vaccine trials will move the field towards its goal.

Keywords

Tuberculosis; T cell; Regulation; Lung; Vaccine

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1. Introduction

The field of tuberculosis (TB) immunology has reached a critical juncture. Despite over 90 years of global immunization with attenuated *Mycobacterium bovis* Bacille Calmette-Guerin (BCG), TB remains a massive international health emergency, with ~ 9 million new cases of active disease and over a million deaths annually [1]. In response to the urgent need for a new and effective TB vaccine, at least 15 candidates have entered clinical trials [2].

Although these candidates differ in their formulations, they share a systemic route of administration and a common goal of boosting the number of IFN- γ -producing T cells recognizing immunodominant Mtb antigens [3]. The first of these candidates, a Modified Vaccinia Ankara vector expressing Mtb antigen 85A (MVA85A), recently completed an efficacy trial in which it was used to boost infants previously immunized with BCG [4]. Despite the fact that MVA85A significantly amplified the Mtb-specific T cell response, it provided no protection beyond the very limited immunity conferred by BCG alone. These disappointing results, together with years of research in animal models in which vaccine candidates have conferred marginal levels of protection, have profoundly impacted the TB field. There is general consensus that devising an effective TB vaccine will require new approaches. However, since the correlates of protective immunity are unknown, there is little agreement on the best path forward [3, 5-13].

TB is a complex infection, unlike any for which an effective vaccine has been developed. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, is a slow growing bacterium with a lung portal of entry that manipulates the host response to delay the onset of adaptive immunity. This delay is widely thought to be Mtb's prime niche-establishing strategy, and represents a critical bottleneck to its control, and possibly to its eradication by adaptive immunity [14, 15]. In this review, we discuss recent work that provides insights into mechanisms that regulate adaptive immunity to Mtb. In particular, we discuss why the T cell response to Mtb is slow to develop, and possible reasons why late-arriving T cells may be restricted in their ability to mediate protection. We frame our discussion in the context of the ongoing debate regarding strategies for developing an effective TB vaccine, as some have suggested that T cell based approaches be replaced by other strategies [5, 8]. However, in light of new understanding of T cell regulation during TB, we contend that our best hope for an effective vaccine is to elicit Mtb-specific T cells that are long-lived and reside in, or rapidly home to, the airways and lung parenchyma. We outline gaps in current knowledge that restrict progress towards such a vaccine. Given the fundamental nature of these knowledge gaps and the central importance of local immune responses in the lung, we argue that only a coordinated approach that includes animal and human studies can move the field forward.

2. Regulation of adaptive immunity against Mtb

2.1 Importance of T cell mediated immunity

CD4 T cells, especially Th1 cells producing IFN- γ , are critical for adaptive immunity against TB in both mice and humans [14]. Mice lacking CD4 T cells, IFN- γ , IL-12 signaling (a pathway required for Th1 development), or T-bet (a transcription factor requisite for Th1s) are profoundly susceptible to Mtb infection [14]. Likewise, humans with genetic

deficiencies in IFN- γ or IL-12 signaling [16], as well as HIV-infected individuals depleted of CD4 T cells [17], are severely restricted in their ability to contain mycobacterial infections, including TB. CD8 T cells can help control Mtb by both perforin-mediated cytotoxicity of infected macrophages and direct killing of Mtb [18, 19], and have been shown to be critical for BCG-induced immunity in a non-human primate model of TB [20].

2.2 The delayed T cell response to Mtb infection

Mtb control requires CD4 T cells to interact directly with infected cells presenting Mtb antigens [21], a requirement that also seems likely for CD8 T cell-mediated immunity. This may explain why the most reliable correlate of protection in TB animal models is the rapidity by which Mtb-specific T cells reach the lung after aerosol infection [14, 15]. Unfortunately, the T cell response to Mtb infection is exceedingly slow. Classic TB studies in human populations revealed that T cell responses to Mtb could be detected an average of 42d after exposure [22, 23], whereas responses to most infections peak in 7-14d [24-26]. Broadly speaking, this delay is comprised of two main components, as will be discussed in sections 2.2.1 and 2.2.2 and illustrated in Figure 1.

2.2.1 Delayed transport of Mtb from the lung to the lymph node—After inhalation of Mtb-containing water droplets, Mtb is taken up by alveolar macrophages, and resides almost exclusively within this population during the first seven days of infection (Ryan Larson and Kevin Urdahl, unpublished results). The adaptive immune response is initiated only when Mtb is ferried to the lung dLN by migratory cells 8-11d after infection [27-29]. Alveolar macrophages are not a migratory population and Mtb apparently orchestrates its retention within these macrophages by manipulating cell death pathways, thereby delaying bacterial transport to the LN [30, 31]. Furthermore, virulent Mtb uses cell-surface-associated phthiocerol dimycocoserate (PDIM) lipids to mask underlying pathogen-associated molecular-patterns, or PAMPs, and to limit Toll-like receptor (TLR) recognition [32]. This restricts macrophage activation and provides a niche for intracellular bacterial replication. In addition, the related phenolic glycolipids (PGLs) promote the recruitment of additional phagocytic cells that are also conducive to Mtb survival and growth. Once Mtb escapes from alveolar macrophages on ~day 8 post-infection, it is taken up by recruited phagocytes, including neutrophils, dendritic cells (DCs), and inflammatory monocytes [33]. Neutrophils serve as an intermediary phagocytic population that shuttles Mtb from lung resident alveolar macrophages to cells capable of migrating to the lung dLN [34, 35]. Although migratory DCs were initially thought to be the primary population transporting Mtb to the lung dLN [27-29], a recent report suggests a critical role for inflammatory monocytes in this process [36]. Upon arrival in the lung dLN, antigen is transferred to uninfected DC populations, which seem to be more effective than infected DCs in priming the T cell response [37]. At this point, over a week has already passed.

2.2.2 Delayed effector T cell priming and migration to the lung—Even after transport to the LN, Mtb further slows adaptive immunity by promoting the expansion of a highly suppressive population of Mtb-specific, Foxp3⁺ regulatory CD4 T cells (Tregs) that act to restrict effector T cell priming [38-40]. These Mtb-specific Tregs parallel the expansion of effector T cells in the lung dLN during the initiation of the adaptive response

between days 15-21 post-infection, and subsequently undergo immune-driven contraction [39]. Although Mtb-specific Tregs described to date recognize the same immunodominant Mtb epitopes as CD4 effector T cells, they do not represent induced Tregs (effector T cells induced to express Foxp3). Instead, they arise from the pre-existing population of thymically-derived Tregs and exhibit a distinct T cell receptor (TCR) repertoire from effector T cells. The expansion of this highly suppressive Treg population is driven by Mtb itself, as a recombinant strain of *Listeria monocytogenes* (Lm) expressing ESAT-6, an Mtb antigen recognized by Tregs in the context of TB, stimulates ESAT-6-specific effector T cells, but not Tregs, in the context of pulmonary Lm infection. Thus, Mtb-driven inflammation provides an environment conducive for the expansion of antigen-specific Tregs, which restricts the expansion of effector T cells and delays their migration to the lung. The end result is a prolonged period of relatively unrestricted Mtb replication in the lung, which facilitates the ability of Mtb to establish a niche for chronic infection, at least in a subset of lesions [41].

2.3 Limitations of T cell mediated immunity during chronic infection

Why are late-arriving T cells limited in their ability to combat Mtb? The inflammatory milieu in the lungs during chronic TB is complex and contains several factors capable of suppressing T cells, including anti-inflammatory cytokines (e.g., IL-10 [42] and TGF β [43]) and lipids (e.g., lipoxin A4 [44]). Although these factors may protect the host from tissue damage caused by excessive inflammation, they may also limit the ability of T cells to control Mtb. In addition, a high lung bacterial burden may result in persistent antigenic stimulation and restrict T cell functionality and protective capacity, as has been demonstrated in other chronic infections[45]. In fact, there is growing evidence that Mtb-specific T cells in the lung are compromised in their ability to produce protective cytokines, including IFN- γ , TNF, and IL-2 [46-49]. The relative exclusion of fully functional T cells from the Mtb-infected lung parenchyma will be discussed further in section 2.3.2.

2.3.1 Regulatory T cells and other suppressive cell populations—During pulmonary Mtb infection, the lungs are infiltrated with a variety of cell populations that have been associated with immunoregulation, including populations of myeloid-derived suppressor cells [50-52] and mesenchymal stem cells [53]. Further investigation is necessary to determine their role in restricting local T cell responses in vivo. Tregs also infiltrate pulmonary granulomas and their role within the lung is also unclear [54, 55]. Despite detailed examination of Tregs' role in delaying the initiation of adaptive immunity in the lung dLN (as discussed in section 2.2.2), their function in the lungs during later stages of infection is largely unexplored. Highly suppressive Mtb-specific Tregs that proliferate in the lung dLN during early infection undergo contraction in the fourth week and are rarely observed in the lungs [39]. Furthermore, depletion of Tregs during early infection reduces the lung bacterial burden [54, 55], but depletion during chronic infection exacerbates pulmonary inflammation without impacting bacterial burden (Shahin Shafiani and Kevin Urdahl, unpublished results). These results suggest that Tregs mainly restrict protective T cell responses in the lung dLN when and where Mtb-specific Tregs are present, but serve a host-beneficial role in the lungs in the absence of pathogen-specific cells. These outcomes may differ in hosts with different genetic backgrounds, or in individuals infected with more

virulent *Mtb* strains. In support of the latter possibility, hypervirulent strains from the Beijing family lineage have been reported to induce an increased and sustained pulmonary Treg response and a diminished Th1 response [56-59]. However, the specificity of these pulmonary Tregs has not yet been examined, nor has a causal link with the reduced Th1 response been shown.

During active human TB there is a direct correlation between the severity of the infection and Treg numbers. While individuals with latent or asymptomatic infection exhibit normal or slightly elevated Treg numbers in their blood [60, 61], those with active TB tend to show increased numbers of Tregs in the blood and at sites of infection (lung and pleural sac) [62-65]. Histological analysis has further shown that cavitating granulomas, those that generally possess the highest bacterial burden, contain significantly more Tregs than other types of granulomas [66]. Moreover, individuals with miliary TB, the most severe form of disseminated TB, have elevated Treg: T effector cell ratios compared to those with localized pulmonary TB [67]. Despite these correlations, it is unknown whether Tregs are the cause of severe forms of TB, or simply the effect of higher levels of inflammation associated with greater bacterial burdens. Likewise, the degree to which Treg function is harmful vs. beneficial to the host, and to T cell-mediated immunity, during active TB is still unclear.

2.3.2 Differential localization of cytokine producing T cells in the lung parenchyma and lung-associated vasculature—Despite evidence that some T cells in *Mtb*-infected lungs exhibited impaired cytokine production, until recently it was difficult to envision that this had a significant impact on immunity because other “lung” T cells produced cytokines robustly. However, an important study by Sakai et al. has shed new light on this subject and has fundamentally changed our understanding of T cell responses in the lung during TB [68]. By labeling intravascular T cells with intravenously administered antibodies shortly before euthanizing *Mtb*-infected mice, these investigators showed that up to half the *Mtb*-specific CD4 T cells isolated from the lung are derived from the lung-associated vasculature rather than the lung parenchyma, as had been assumed in prior studies. Moreover, while *Mtb*-specific T cells in the vasculature exhibit high expression of the T-bet transcription factor and produce IFN- γ robustly, those in the lung parenchyma express intermediate levels of T-bet and produce less IFN- γ . When T cells derived from the vasculature were transferred into a second *Mtb*-infected host they exhibited a poor capacity to migrate into the lung parenchyma and conferred limited protection. These insights may help explain why *Mtb*-specific T cells are incapable of adequately controlling *Mtb* during active TB disease.

3. Should a TB vaccine target T cells?

Given the critical role of T cells in natural immunity against TB, efforts to develop new TB vaccines have focused on amplifying the T cell response. In this section we summarize three major arguments against a T cell-based approach. We then examine these arguments in light of recent advances in our understanding of how T cell responses against TB are regulated (Section 2).

3.1 Argument against #1: The frequency of circulating Th1 cells does not correlate with protection

Despite the importance of Th1 cells in protective immunity, the frequency of Mtb-specific Th1 cells in the blood and lymphoid periphery of mice and humans does not correlate with protection against TB [15, 69-72]. This fact was highlighted by the recent vaccine efficacy trial in which infants boosted with MVA85A exhibited 1-2 logs greater Mtb-specific T cells in their blood than those immunized with BCG alone, but received no protective benefit [4]. Several factors may help explain this discrepancy.

First, Mtb-specific T cells are not homogeneous. In addition to the protective capacity of Th1 cells, lung-resident Th17 cells promote immunity by facilitating recruitment of Th1 cells into the lung [73, 74]. As discussed in Section 2, CD8 T cells can also contribute to protective immunity. In contrast, Th2 [75] and Treg cells [40, 75] may restrict immunity against Mtb. Even among IFN- γ -producing CD4 T cells there is heterogeneity, and other properties including GM-CSF and Tim-3 may be important [13, 76, 77]. In mice, T-bet^{hi} KLRG1⁺ CD4 T cells produce the most IFN- γ , but proliferate poorly and are short-lived [78]. Conversely, T-bet^{int} KLRG1⁻ CD4 T cells produce less IFN- γ , but have enhanced proliferative capacity and are long-lived. Interestingly, the latter CD4 subset migrate into the lung parenchyma better than the former, and adoptive transfer and vaccine studies also suggest they confer greater protection [68, 79, 80]. Overall, these findings suggest that less differentiated CD4 T cells may provide a better correlate of protective immunity than terminally-differentiated Th1 cells. In addition, the relative numbers of other cell types, including Mtb-specific Th17s, Th2s, Tregs, and CD8s, are likely to contribute to the protective equation.

Second, a T cell's protective capacity is likely dictated by the specific Mtb epitope recognized. Some antigenic Mtb proteins are dramatically downregulated after the first few weeks of infection [81, 82], whereas others are expressed throughout chronic infection [83]. It is also possible that some Mtb proteins may be expressed in some infected cell types, but not others (e.g., macrophages vs. DCs). How differential Mtb protein expression patterns impact the protective capacity of vaccine-induced T cells is unknown, but seems likely to be critically important. Moreover, recent studies in mice provide strong support for the idea that vaccines that target subdominant, or cryptic, Mtb epitopes confer superior protection than T cells recognizing immunodominant Mtb epitopes [80]. A potential explanation for this finding was provided by the finding that cryptic epitopes elicit a higher proportion of T-bet^{int} KLRG1⁻ CD4 T cells, whereas immunodominant epitopes induce more terminally differentiated Th1s. As a result, subdominant epitopes have been shown to confer more durable protection.

Third, and perhaps most importantly, T cell assessments in the blood do not accurately represent the number or functional capacity of T cells at the lung site of infection. In mice, the best correlate of protection is not the magnitude of the Th1 response, but the rate at which Th1 cells reach the lung [9, 38, 84]. Unfortunately, systemic immunization (even regimens that boost the T cell frequencies in the periphery dramatically), only accelerate effector T cell arrival in the lung by 2-3 days [9]. The result is the infamous "1 log of protection" which has been difficult to improve upon using systemic vaccines. Recently,

greater protection has been achieved in animals using vaccines that target the lung mucosa and elicit T cell responses directly in the airways and lung parenchyma [9]. Simultaneous systemic and lung mucosal immunization seems to provide even greater protection [84], however, the reason for this synergy is unknown. It is not clear whether T cell responses in the blood can ever adequately predict T cell responses in the lung. Clearly, a complex formula that considered the relative abundance of several different T cell subsets, as well as the specificity of the Mtb antigens recognized, would be required.

3.2 Argument against #2: An apparent abundance of Th1 cells in the lung does not eradicate Mtb

This argument has been rebutted in large part by the findings of Sakai et al., as discussed in Section 2.3.2. Mtb-specific T cells with the highest capacity to produce protective cytokines are not located at the site of infection in the lung parenchyma, as had been previously assumed, but instead reside in the lung-associated vasculature [68]. In addition to providing insights, however, these findings also raise new questions. Although T-bet^{hi} Th1 cells in the vasculature were shown to migrate poorly into the lung parenchyma, this cannot completely explain why T-bet^{hi} Th1 cells are absent in Mtb-infected lungs since T-bet^{int} progenitors [78] are abundant within the lung parenchyma. Are Mtb antigen-specific T-bet^{int} CD4 T cells unable to complete their differentiation into T-bet^{hi} effectors within the lung? Do they differentiate into T-bet^{hi} effectors, but subsequently egress out of the lung? Are terminally differentiated Th1 effectors unable to survive in the context of Mtb-mediated inflammation within the lung? These possibilities are not mutually exclusive and each warrant further investigation, but the latter is supported by the finding that reactive nitric oxide species are toxic to T-bet^{hi} CD4 effectors in the mice infected with *Mycobacterium avium* [85]. This toxicity seems to manifest itself particularly in the local vicinity of infected macrophages, as very few T cells were observed to interact directly with infected cells.

A recent report by Srivastava and Ernst provides evidence that the ability of T cells to localize and interact with infected macrophages is likely very important for their protective capacity [21]. Using bone marrow chimeric mice, they showed that macrophages lacking MHCII expression had higher bacterial burdens than WT macrophages in the same Mtb-infected lung. Thus, cognate interactions between CD4 T cells and Mtb-infected macrophages are critical for immune control. Although Mtb antigens can readily be transferred to uninfected antigen-presenting cells that can in turn activate Mtb-specific T cells [37], cytokines produced as a result of these interactions have a limited capacity to control Mtb within nearby infected cells. These results suggest that Mtb-specific T cells located in the lung-associated vasculature, or distant from infected macrophages within the lung parenchyma may be severely restricted in their ability to impact Mtb control. Thus, understanding the factors that impede the co-localization of Mtb-infected T cells and infected macrophages, and devising strategies to facilitate interactions between these cells may be critical for developing effective vaccines and immunotherapeutics to control TB.

3.3 Argument against #3: Mtb-specific T cells may facilitate TB pathogenesis and promote transmission

In addition to questions regarding whether vaccine-induced T cells are likely to provide protection against TB, recent studies have raised questions about whether such approaches may even be detrimental or promote Mtb transmission. Interestingly, human T cell epitopes of Mtb are more conserved than other regions of the Mtb genome [86, 87]. This has been interpreted to reflect an evolutionary pressure to maintain these epitopes, and to suggest that Mtb may benefit from recognition by human T cells. In support of this idea, there is evidence that T cells can contribute to TB immunopathology and promote human-to-human Mtb transmission. In particular, cavitary TB is much more transmissible than other disease forms, but occurs rarely in CD4-depleted individuals co-infected with Mtb and HIV [88].

Although it seems likely that Mtb-specific T cells mediate some host deleterious effects during active TB, whether these effects provide a mechanistic explanation for the sequence conservation of Mtb epitopes remains to be determined. In assessing this possibility it is important to remember that each Mtb T cell epitope only acts as such in a small subset of the human population. This is due to the fact that Mtb antigenic peptides must first bind specific HLA molecules before HLA-bound epitopes can be recognized by T cells [89]. HLA molecules are highly polymorphic, and as a result, the average Mtb T cell epitope is probably recognized in only ~10% of Mtb-infected people [90]. Thus, for T cell recognition to drive conservation of these epitopes the selective pressure in 10% of Mtb-infected people would need to be strong enough to dictate conservation despite the absence of this selective pressure in the other 90% of infected individuals. If human-to-human transmission is the underlying factor driving this selective pressure, as has been proposed, these calculations become even more problematic. Only individuals with active TB transmit infection and the incidence of active disease in individuals with any given HLA molecule is only ~10% [90]. Thus, only ~1% (10% of 10%) of Mtb-infected individuals would manifest the selective pressure for epitope conservation, whereas 99% would not. The case for T cell recognition driving epitope conservation would be strengthened if frequently recognized epitopes were shown to have a higher degree of conservation than rarely recognized epitopes, or if epitopes restricted by HLA molecules expressed by human populations in one geographic locale but not another were conserved only by Mtb strains present in that locale. In the absence of such supportive data, other possibilities should be actively considered. The requirements for T cell epitopes to bind to HLA molecules places constraints on the epitope sequence, as only peptides with hydrophobic residues in certain positions are able to bind [89]. Perhaps these restrictions shape how epitopes are initially defined and bias selection toward peptides that are relatively conserved.

Even if T cell recognition does not provide a selective pressure that can explain the conservation of T cell epitopes in Mtb, it is still reasonable to consider the possibility that vaccine-induced T cell responses could be potentially deleterious. T cells responses likely do contribute to immunopathology during active TB, and the potential for vaccines to induce deleterious immune responses to subsequent pathogenic challenge is not without precedent [91]. Fortunately, despite decades of immunization using approaches that boost Mtb-specific T cells in humans and animal models, vaccine-induced T cells have not been shown to be

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detrimental in the setting of TB [3, 83]. Although the efficacy of these approaches in preventing TB has been modest at best, exacerbated lung damage and increased disease severity has not been observed. This likely stems from the fact that T cell responses that occur early after infection have the potential to curb Mtb replication (e.g., in animal models, vaccination is often associated with a modest reduction in the lung bacterial load). The lack of deleterious consequences in vaccinated individuals suggests that the beneficial effects of mounting an early T cell response may override the potentially negative ones associated with T cells later in disease. Clearly if T cells can be induced in a manner that prevents active disease, deleterious consequences seem unlikely. Understanding the barriers that restrict the ability of T cells to control Mtb in the setting of high bacterial loads may provide insights into why T cells can be harmful during active TB. Conversely, understanding that early T cell responses can be beneficial provides a roadmap for devising strategies to further optimize T cell mediated protection.

4. How can the barriers to T cell-mediated immunity be overcome?

The barriers to protective immunity against TB are formidable. Most effective vaccines mediate protection through humoral immunity [92], however, as an intracellular pathogen, their role in natural immunity against TB seems to be limited [14, 93, 94]. Moreover, even natural immunity does not achieve protection in all people, as individuals cured of TB with antibiotics can be re-infected and develop TB anew [95]. Thus, a successful TB vaccine will likely require completely new approaches, and will need to induce immune responses that differ from those elicited by natural infection itself. However, the path to achieving protection by eliciting immune responses that play a minimal role in natural immunity is unclear. We suggest that rather than moving away from T cell-based vaccine strategies, a better approach in light of our new understanding of the barriers to T cell mediated immunity (Sections 2 and 3) may be to elicit T cells in novel and “unnatural” ways that enable them to overcome these barriers (Sections 4.1-4.3).

4.1 Early T cell recognition of Mtb-infected cells

As summarized in Section 2.2, the end result of the Mtb-orchestrated delay in the onset of the T cell response is that by the time T cells arrive at the site of infection in the lung they are confronted with many layers of immune regulation that restrict their ability to control or kill Mtb (Fig. 1). These barriers include infection-induced anti-inflammatory cytokines and lipids, recruited immunosuppressive cells, and a high bacterial burden that leads to persistent antigenic stimulation, and possibly T cell exhaustion (Sections 2.3 and 2.3.1). Furthermore, terminally differentiated effector T cells, capable of robust cytokine production, fail to localize to the parenchyma of the Mtb-infected lung (Section 2.3.2). In part, this may reflect a reduced ability to survive in the inflammatory milieu of Mtb infection, especially in close proximity to Mtb-infected cells (Section 3.2). These barriers are each associated with regulatory networks that are established in multicellular granulomas harboring a high bacterial burden. As such, if Mtb-specific T cells could recognize solitary infected cells in the airways and lung parenchyma shortly after aerosol infection (see Fig. 1, Panel A), these barriers could potentially be pre-empted.

Given these considerations, a vaccine that induces lung resident memory cells may be the first step towards protection. In recent years there has been a growing appreciation of the impact of tissue resident memory cells on disease outcomes [96-98], but whether memory T cells resident in the airways and lung parenchyma can mediate early and effective Mtb control has not been adequately examined. Intravenously transferred Mtb-specific Th1 cells, presumably resident in the lung, were unable to recognize Mtb antigens during the first week after aerosol infection [99] This finding has fostered the idea that Mtb antigens are largely hidden during this time, but the precise location of the Th1 cells transferred in this study was not determined. In retrospect, most were probably localized in the lung-associated vasculature and not in the airways. Several studies have now shown that TB vaccines delivered to the lung mucosa can elicit airway luminal T cells and curtail Mtb replication during the first week of infection [9, 84, 100-103]. These results suggest that airway localized T cells can recognize Mtb-infected cells at this site and may represent a first step towards an effective TB vaccine.

Even if such an approach were shown to be beneficial in a short-term immunization trial, a larger challenge will be to sustain this type of immunity for a lifetime. Maintaining a lung resident memory cell population capable of mediating protection would likely require frequent boosting with an aerosolized vaccine [104, 105], perhaps annually. This would need to be accomplished using a vaccine vehicle that could be delivered frequently without generating interference from immunity to prior vaccine exposures. Although a lifelong requirement for annual boosting is unusual for current vaccines that elicit protection via humoral immunity, successful immunization against TB will likely require completely different approaches and mindsets.

4.2 Synergistic protection through mucosal and systemic immunizations

Despite the ability of mucosally administered vaccines to curb Mtb replication in the first week, prolonged protection is still suboptimal unless this approach is accompanied by systemic immunization [9, 84]. The distinct locations and functional properties of memory T cells elicited by these different immunization routes and how they synergize to provide enhanced protection against Mtb remain unknown. One possibility is that systemic immunization is better at eliciting circulating memory T cells, which may be important as a second line of defense if mucosal immunization does not completely eradicate Mtb (as might be the case if early T cell recognition does not occur in every infected alveolus). It is not known whether the requirement for systemic immunization could be reduced if mucosal immunization were optimized.

In practical terms, intradermal immunization of infants with BCG is widespread globally and may provide the systemic component of a TB vaccination regimen. While BCG immunization does little to prevent pulmonary TB in adolescents and adults, it does protect children from severe, disseminated forms of TB [11, 106], and also reduces infant mortality rates independently of its effects on TB [107-109]. Because of these effects, the ethics of performing a vaccine trial in which BCG is tested against promising, but unproven, alternative systemic approaches will necessarily be scrutinized. Nonetheless, alternatives to intradermal BCG should at least be considered. One possibility would be to change the route

of BCG immunization. Historically, BCG was initially administered orally before being replaced by the intradermal route [110]. Although these routes have not been compared for efficacy, or for synergy with mucosal immunization, some studies suggest that oral immunization may be better at promoting lung mucosal immunity [111, 112]. A second possibility is to replace BCG with either a recombinant BCG mutant (e.g., VPM1002) or a highly attenuated Mtb mutant (e.g., MTBVAC). VPM1002 (rBCG *UreC::hly*), a recombinant BCG mutant expressing listeriolysin and deficient in urease, has demonstrated superior efficacy and safety over parental BCG in animal models [113, 114]. MTBVAC is an Mtb mutant with deletions in both PhoP (a transcription factor that regulates numerous genes involved in Mtb virulence) and FaD26 (an enzyme critical for glycolipid cell-wall synthesis) [115]. Both VPM1002 and MTBVAC are currently being tested in human trials. Finally, a third possibility might be to completely replace mycobacteria as the foundation for TB immunization. Cytomegalovirus vectors induce robust effector memory T cell responses at mucosal sites and have been used to successfully confer immunity against simian immunodeficiency virus (SIV) in rhesus macaques [116]. A similar strategy may hold promise for TB.

4.3 Inducing durable T cell responses

Studies in the mouse have shown that immunization with BCG induces high numbers of terminally differentiated Th1 cells, but these cells are short-lived and protection wanes with time [79]. However, immunizations that target subdominant Mtb epitopes [80], or use a liposomal adjuvant [79], preferentially expand Mtb-specific CD4 T cells that are less differentiated, produce IL-2, and confer superior and longer lasting immunity. Frequent boosting can drive terminal differentiation and negatively impact the durability of immunity [117]. Thus, if this proves necessary to achieve immunity against TB (as discussed in Section 4.1), choosing approaches that induce long-lived T cell populations with intermediate levels of differentiation will likely be critical. Immunization using Mtb proteins that elicit subdominant T cell responses during natural Mtb infection seems like a promising way to achieve this goal. Although the proof-of-principle study in mice was performed by expanding T cells specific for subdominant epitopes of a Mtb protein (ESAT-6) that also contained immunodominant epitopes [80], targeting distinct epitopes is unlikely to be a practical strategy in humans. Due to HLA restriction (discussed in Section 3.2), subdominant epitopes in one individual may either be immunodominant epitopes, or not be epitopes at all, in others. However, an Mtb protein containing numerous Mtb epitopes that are exclusively subdominant could be a promising candidate for eliciting durable T cell responses in human populations. As more is learned about the nature of Mtb T cell epitopes in humans [118-121], identification of candidate proteins that meet these criteria seems likely.

5. The path forward

TB is a human disease, but animal studies have been essential in generating the hypotheses and ideas that have shaped our understanding of TB immunology. Because immunity to TB cannot be fully recapitulated in animals, human studies will be critical for moving the field forward on the path towards an effective vaccine. However, the primary bottleneck to an

effective TB vaccine, as outlined in this review, is a lack of fundamental knowledge regarding how protective immunity can be achieved. Many key questions that impede progress cannot be adequately addressed in humans alone. In this section we will discuss our view that the knowledge needed to develop an effective TB vaccine can best be advanced by a coordinated approach that complements human studies with research in animal models (Fig. 2).

5.1 The importance of human studies

Human TB differs from animal models in clinical presentation, pathology, and in certain immunologic responses [3, 12]. Although many features of TB immunity are shared between humans and animals, other pathways are absent or uncharacterized in commonly used animal models, including: a) Vitamin D-dependent pathways by which macrophages kill Mtb through release of antimicrobial peptides [122, 123], and through autophagy of Mtb-containing vacuoles [124]; b) CD1-restricted presentation of lipid antigens to T cells [125]; and c) cytotoxic T cell release of granulysin, an antimicrobial peptide that can kill Mtb [126]. Furthermore, it is difficult to experimentally reproduce the dose and metabolic state of Mtb that is responsible for human infections, and environmental and genetic factors that shape the human immune response cannot be adequately modeled. Because of these important differences, human studies are critical to determine which vaccines and interventions will ultimately be effective in preventing human TB.

However, first an effective vaccine must be designed, and many knowledge gaps that restrict our ability to do this cannot be adequately addressed in humans. In particular, immune responses in the lungs are central to preventing TB, particularly in the first few weeks after aerosol infection. T cell responses in humans are usually assessed in vitro using cells obtained from peripheral blood, but provide little, if any, information regarding the nature of T cell responses in the lung. Investigating whether signatures of protective immunity can be identified in the blood of immunized individuals is an important area for future investigation. Although bronchoalveolar lavages (BALs) can be performed, the approach is invasive, does not sample the lung parenchyma, and is difficult to utilize for large-scale immunization studies or to assess kinetics over multiple timepoints. Furthermore, studying airway responses in the critical first few weeks after aerosol Mtb infection is not feasible, in part because infected individuals are usually not identified for several months after infection, and the precise time of infection is rarely known.

5.2 The importance of animal studies

The role of local T cell responses in the airways and lung parenchyma in protective immunity (discussed in Section 5.1) provides one example of an important area of investigation in which animal studies are necessary to inform the direction and design of human studies. Animal experiments must always be driven by important questions in human TB (Fig. 2). The caveats of each animal model must be carefully considered to determine if the model is appropriate for the questions being asked. Studies in animals should be viewed as exploratory and hypothesis-generating. The purpose is to generate new ideas and possible approaches that can then be tested in humans. When possible, it is always desirable to test whether basic principles discovered in animals are also operating in humans. However in

some cases, such as the idea that T cells localized to the lung confer protective immunity, confirmatory human studies may not be possible and vaccine approaches found to elicit protective immune responses in animals will simply need to be tested for efficacy in human trials.

In Sections 5.2.1 and 5.2.2 we will further discuss roles for non-human primates and mice in investigating whether early T cell responses in the airway and lung parenchyma can mediate immunity against TB, as these animal models seem best suited to address the questions raised in this review. In Section 5.2.3 we will discuss an important question raised by studies in zebrafish that will require investigation in future human vaccine trials. Other animal models, including guinea pigs and rabbits also have advantages, but will not be further discussed. Like humans, guinea pigs and rabbits form TB granulomas with acellular, hypoxic centers [127]. As a result, these models may be suited to ask questions about immunity during chronic infection, in which the location or metabolic state of bacteria resident in such granulomas could be important.

5.2.1 Studies in non-human primates—TB in non-human primates more closely resembles the features of human TB than any other animal model [128]. Most of the components of TB immunity are shared, and two types of macaques (rhesus and cynomolgus) are now being used to screen and prioritize new candidate vaccines before embarking on complex, protracted, and expensive human trials [3, 83]. Efforts are currently underway to mimic human-to-human TB transmission in non-human primates by infecting animals through co-housing with other animals with active TB. If successful, this would recapitulate the dose and bacterial metabolic state responsible for human infection, and further enhance the utility of non-human primates for vaccine-related studies.

The potential of non-human primates for addressing fundamental questions in TB pathogenesis was recently demonstrated in an elegant study by Lin and colleagues [41]. Using individually marked Mtb isolates, these investigators showed that each granulomatous lung lesion is probably founded by a single bacteria, but the fate of bacteria within each lesion is variable. During active TB the host sterilizes some lesions, while other lesions in the same lung progress with increasing bacterial burdens. Dissecting the nature of the immune response in each type of lesion has the potential to inform interventions and vaccine strategies that prevent TB by driving sterilization in all lesions.

Some studies examining the role of lung resident T cells in the early response to Mtb could be performed in non-human primates that are unapproachable in humans due to the ability to perform necropsies in small numbers of animals. However, a comprehensive assessment of this subject would be difficult because cost, ethical considerations, and a limited number of experimental reagents would preclude the type of in-depth and large-scale analysis required to address many of the relevant questions.

5.2.2 Studies in mice—The mouse is the most commonly used animal model to study TB, but is frequently criticized because it lacks several immune pathways operant in human TB (discussed in Section 5.1), does not form caseating or hypoxic granulomas (at least in the most commonly used strains), and does not manifest paucibacillary latent infection [3, 12].

Despite these caveats, mice mimic the human response to TB in many central aspects. In fact, several key components of TB immunity (e.g., CD4 T cells, IFN- γ , IL-12, and TNF) were discovered to be critical for resistance in mice before their role in humans was confirmed [14]. Many of the mechanistic insights into the delayed T cell response to aerosol Mtb infection, and the relevance of this delay for TB pathogenesis, as discussed in this review, were obtained in mouse studies. Our central thesis that airway and lung resident T cells can mediate protection against TB stems from these studies, and provides an example of how studies in mice can be hypothesis generating.

Recent studies using vaccines that target the lung mucosa have yielded promising results in animal models [9, 84, 100-103], but many questions remain. How many lung resident memory cells are required to mediate protection? What type of T cells best mediate protection at this site, and can synergy be achieved by inducing more than one type? Do memory T cells need to reside in the airways, the lung parenchyma, or both? What type of immunization regimen can induce protective T cells at the relevant sites? Once induced, how can they be maintained? Do they mediate control of Mtb primarily by activating alveolar macrophages, or does induction of anti-mycobacterial factors from airway epithelial cells play a role? What properties must lung resident T cells possess to optimally promote the relevant anti-mycobacterial pathways? Can they prevent infection? The mouse provides a tractable experimental system and a vast array of tools to perform a detailed dissection of these questions. In addition, these questions pertain to “big picture” biological processes that are usually highly conserved in the mammalian immune system. Even if humans and mice differ in some details of the pathways that ultimately function to control TB, the rules that govern T cell induction, maintenance, and antigen recognition in the lung are likely to be similar and central to protective immunity.

5.2.3 Questions raised by zebrafish studies—Zebrafish have also provided insights into human TB and raised questions that will be important to examine in human vaccine trials going forward [129]. In particular, a susceptibility factor involved in regulating TNF production (*Ita4h*) was identified in a forward genetic screen in zebrafish, and a polymorphism of this gene was subsequently shown to confer susceptibility to TB in human populations [130, 131]. Interestingly, in both zebrafish and humans, susceptibility to TB was promoted by genotypes associated with either low or high production of TNF, whereas immune control was promoted by intermediate TNF production. Follow-up studies in the zebrafish have provided a mechanistic basis for these results [132]. Such striking findings seem unlikely to be only true for a single polymorphism, but probably reflect a general principle governing susceptibility to TB. Therefore, in future human vaccine trials it will be important to test the propensity of each participant’s monocytes to mount an inflammatory response to Mtb. If active TB develops preferentially in individuals with either low or high degrees of inflammation against TB, it will be important to determine if vaccine candidates are effective in individuals who develop TB for both reasons, or whether vaccination only confers protection at one end of the inflammatory spectrum.

6. Summary and concluding remarks

Recent insights into how T cells are regulated during TB have led to new ideas about how the barriers that restrict immunity against TB can be overcome by a rationally designed vaccine. Most of these barriers seem to arise from immunoregulatory factors, and an elevated bacterial burden in the developing granuloma at the time T cells first arrive at the site of infection. Thus, early T cell recognition of Mtb-infected cells in the airways and lung parenchyma during the first week of infection may allow T cells to control Mtb before these barriers become established. Success moving forward will require a renewed spirit of cooperation between TB researchers working in animal models and those studying human disease.

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Abbreviations

BAL	bronchoalveolar lavage
BCG	bacille de Calmette et Guérin
DC	dendritic cell
dLN	draining lymph node
ESAT-6	early secreted antigenic target 6
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IL	interleukin
IFN	interferon
KLRG	killer cell lectin-like receptor subfamily G
Lm	Listeria monocytogenes
MAIT	mucosal-associated invariant T
Mtb	Mycobacterium tuberculosis
MVA85A	modified vaccinia Ankara 85A
PAMPs	pathogen-associated molecular patterns
PDIM	phthiocerol dimycocerosate
PGL	phenolic glycolipids
SIV	simian immunodeficiency virus

TB	tuberculosis
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
Tim	T cell immunoglobulin mucin
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell

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Highlights

- Mtb orchestrates a delayed T cell response to establish a lung niche
- T cells encounter many layers of regulation upon arrival at the infection site
- Early recognition of infected cells by lung resident T cells may avoid regulation
- Coordinated studies in humans and animal models are needed for TB vaccine design

Figure 1.

Three phases of early Mtb infection that contribute to the delayed T cell response. **Days 0-8.** After aerosol infection Mtb is taken up by alveolar macrophages, in which they reside and replicate for the first 7-8 days. Cell surface lipids restrict recognition of Mtb PAMPs by TLRs, thus limiting macrophage activation and promoting an intracellular environment permissive to Mtb survival and replication. **Days 9-14.** Eventually, infected macrophages undergo cell death and are taken up by other phagocytic cell types. Neutrophils seem to be the next cell type to harbor Mtb, and their subsequent apoptosis facilitates uptake by migratory DCs and inflammatory monocytes that transport Mtb to the lung dLN. The first component of delayed T cell response during TB, the slow transport of Mtb from the lung to the lymph node (discussed in Section 2.2.1), is depicted in these first two panels. **Days 15-21.** T cell priming is initiated in the lung dLN, but the inflammatory milieu of Mtb infection promotes expansion of a highly suppressive population of Mtb-specific Tregs. These pathogen-specific Tregs restrict the priming and proliferation of effector T cells, thus delaying their arrival in the lung. This second component of the delayed T cell response during TB is discussed in Section 2.2.2. By the time effector T cells reach the site of infection they encounter immunosuppressive soluble factors, regulatory cell types, and a high bacterial burden that each restrict their functional and protective capacity. A central tenet of this review is that vaccine-induced T cells resident in the lung could potentially mediate superior protection if they were localized to recognize infected alveolar macrophages in the first week of infection.

Figure 2.

Schematic showing an approach to vaccine-related TB research that coordinates efforts in animals and humans. Animal studies should be driven by questions arising from human TB and generate hypotheses that inform the direction and design of subsequent human studies. A few examples of such questions and hypotheses are listed, but many others exist. Still more will arise from questions raised in future studies.