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In search of a new paradigm for protective immunity to TB

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Preface

Clinical trials of vaccines against *Mycobacterium tuberculosis* are in full swing and results are starting to come in, some not so encouraging as exemplified by the latest Aeras-422 and MVA85A trials. Other than empirically determining whether a vaccine reduces the number of cases of active tuberculosis, a daunting prospect given the chronic nature of the disease, we have no way of assessing vaccine efficacy. Therefore, investigators seek to identify biomarkers that predict vaccine efficacy. Historically, focus has been on $CD4^+$ T cell production of interferon-γ, but this has not been a useful correlate of vaccine-induced protection. Here we discuss recent advances in our understanding of immune control of *M. tuberculosis* and how this knowledge could be used for vaccine design and evaluation.

Tuberculosis (TB) is caused by the pathogenic bacterium *Mycobacterium tuberculosis*, which is transmitted between people via aerosol droplets containing bacteria. The droplets are inhaled and deposited in distal lung alveoli (Figure 1)[1]. *M. tuberculosis* is an intracellular bacterium and although it can infect different cell types, alveolar macrophages are its favorite niche. The initial stages of infection are characterized by innate immune responses involving the recruitment of inflammatory cells to the lung[2]; induction of an adaptive immune response occurs only later, after dissemination of *M. tuberculosis* to draining lymph nodes[3-5]. In the lymph node, presentation of bacterial antigens by dendritic cells leads to priming and expansion of antigen-specific T cells, which differentiate from naïve into effector T cells. The effector T cells then migrate to the infected lung and, in combination with other leukocytes, stimulate the formation of granulomas. Granulomas are

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organized structures containing macrophages, lymphocytes and fibroblasts[6]. Within the granuloma, macrophages are activated, for example, by IFNγ secreted by $CD4^+$ T cells (Th1 cells), which is thought to restrict the dispersal and replication of *M. tuberculosis*.

Although the human immune system can control the infection, control does not invariably lead to sterilization. In fact, most people infected with *M. tuberculosis* are clinically asymptomatic, a state referred to as latent TB[7]. These latently infected people –estimated to be one third of the world's population – represent an enormous reservoir of potential disease. Epidemiological studies find that 5-10% of people with latent TB will develop active disease sometime during their lives [8]. Individuals with active TB cough and generate infectious droplets that propagate the infection (Figure 1).

An effective vaccine is needed to stop the ongoing pandemic. *Mycobacterium bovis* Bacille Calmette Guerin (BCG), an attenuated form of *M. bovis*, was introduced nearly a century ago as a vaccine against *M. tuberculosis*, but it has had little impact in eliminating TB. In part, this is because BCG efficacy against active pulmonary TB is extremely variable between populations, and BCG-induced protection is significantly lower in the developing world[9]. Remarkable progress has been made in the development of new vaccine candidates and several are now in clinical trials (Box 1). Although there is some pessimism about whether a vaccine can be developed that averts infection, the general consensus is that a vaccine that prevents the progression to active disease could reduce the prevalence of pulmonary TB and ultimately break the cycle of transmission.

Most antiviral vaccines that have proven to be effective are based on antibody-mediated immunity. As is the case for many intracellular bacteria, *M. tuberculosis* is able to avoid most antibacterial effects mediated by antibodies by living and growing inside macrophages. Thus, based on the substantial experimental foundation that T cell immunity is required to control primary *M. tuberculosis* infection, the consensus among vaccinologists is that vaccine-induced T cell mediated immunity will be required to prevent clinical TB. However, despite significant advances in defining how the immune system responds to *M. tuberculosis*, our understanding of protective immunity following infection (natural immunity) is incomplete. Furthermore, little is known about the mechanisms of vaccine-induced immunity, and whether it differs from natural immunity, and studies to answer these questions have not kept pace with the speed with which new vaccines are entering clinical trials. It is unknown which immunological parameters or biomarkers predict who will control the infection and who will develop clinical disease both in the setting of natural and of vaccine-induced immunity. Such knowledge would revolutionize our approach to surveillance, control, and treatment of TB and it would greatly accelerate vaccine design and evaluation. However, identifying biomarkers of vaccine protection is difficult: until there is a successful vaccine that induces protective immunity, how can such a biomarker be identified? As it stands now, any success or failure of TB vaccines will be largely empiric and difficult to predict.

In this Opinion, we discuss immune defenses against *M. tuberculosis* infection. T cells predominantly mediate protective immunity and recent results begin to clarify how different T cell subsets and functions restrict bacterial growth. Finally, we will discuss how one might

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use knowledge about these different mechanisms to develop new vaccine strategies to prevent tuberculosis.

The “central dogma” of protective immunity

Establishing the importance of IFNγ

During the past four decades, the predominant paradigm in both basic and clinical research has been that IFNγ production by $CD4^+$ T cells is the major driver of immunity to TB. Research in the 70’s found that T cells, and not antibodies, are required for host resistance to TB, and established the mouse as a useful model of tuberculosis[10]. The T cell hypothesis was further refined in the 80’s with the identification of $CD4^+$ T cells producing IFNγ (Th1 cells) as the dominant T cell subset participating in the immune response to *M. tuberculosis*[11, 12]. The use of knockout mice in the 90’s established a crucial role for $CD4^+$ T cells, with additional roles for $CD8^+$ T cells, iNKT cells and $\gamma\delta^+$ T cells[13, 14]. The discovery that AIDS, a condition often associated with TB, was caused by HIV, a virus that infects and kills $CD4^+$ T cells, supported a key role for $CD4^+$ T cells in immunity against *M. tuberculosis* in people[15].

A central role for IFNγ, a cytokine involved in the response against viruses and intracellular bacteria, in anti-mycobacterial immunity is based on the extreme susceptibility of mice that lack IFNγ[16, 17]. IFNγ activates macrophages to kill intracellular bacteria by activating downstream antimicrobial effector pathways including iNOS, IFNγ inducible GTPases, phagosomal maturation and acidification, autophagy, and Vitamin D receptor signaling[18-23]. Genetic studies confirm a role for IFNγ in people: families with mutations in the IL-12/IFNγ/STAT1 axis develop disseminated infections caused by BCG and non-tuberculous mycobacteria (NTM) species. This inherited susceptibility, called Mendelian Susceptibility to Mycobacterial Disease (MSMD), reveals the crucial nature of this signaling pathway, which was first described in mice[16, 17, 24, 25].

These discoveries helped to define the ‘central dogma’ of TB immunity, namely that T cell production of IFNγ activates macrophages to kill intracellular *M. tuberculosis* (Figure 2a). Indeed, detection of IFNγ produced by T cells is the most widely used method for detecting immune responses following infection or vaccination.

Shortcomings of the “central dogma”

Although IFNγ and $CD4^+$ T cells are key components of the immune responses against mycobacteria, the intricacies of immunity to *M. tuberculosis* require that we reassess their roles. For instance, the risk of active TB significantly increases during the first year after HIV infection despite normal $CD4^+$ T cell counts[26], and progression to AIDS, which is characterized by a substantial loss of $CD4^+$ T cells, does not correlate with the development of active TB[26, 27]. HIV infection induces a number of immunological abnormalities, some that are apparent even before $CD4^+$ T cell numbers decline[28]. It is possible that alterations in $CD4^+$ T cell function secondary to HIV infection increase TB susceptibility even before $CD4^+$ T cell numbers fall. However, this pattern of susceptibility is clearly different from other opportunistic infections whose incidence correlates with the peripheral blood $CD4^+$ T cell count in HIV patients[29].

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Similar complexity is observed for MSMD patients: over 300 cases of MSMD have been described, but *M. tuberculosis* infection was present in only four cases; the rest were BCG or NTM species[24]. Although such bias might reflect the relative exposure to BCG or NTM compared to *M. tuberculosis*, IFNγ-activated pathways might be more important for immunity against NTM than against *M. tuberculosis*[24, 30]. Although these rare cases of TB and immunodeficiency are instructive, most people that develop active TB have no obvious defects within their T cell compartment and generate *M. tuberculosis*-specific IFNγ responses. Thus, whereas HIV and MSMD patients establish that T cells and IFNγ are required for immunity against *M. tuberculosis*, T cells producing IFNγ do not appear to be sufficient to prevent active disease.

The shortcomings of the "central dogma" also apply to disease progression and vaccine-induced protection in otherwise healthy people, as more T cells secreting IFNγ or greater IFNγ levels do not correlate with protection[31]. In fact, patients whose T cells produce greater amounts of IFNγ are more likely to progress to active disease than patients with weaker responses[32], supporting the idea that IFNγ levels correlate better with bacterial burden than disease control. Such a correlation between increased *M. tuberculosis* bacterial burden and increased T cell IFNγ production has been observed in humans, non-human primates (NHP), and mice[32-35].

Similar conclusions can be drawn from vaccination studies[36]. BCG vaccination can elicit protective T cells in experimental animals, but IFNγ production by these T cells has not been predictive of vaccine-induced protection[36, 37]. The only predictor of protection in mice vaccinated with BCG is an increased number of antigen-specific $CD8^+$ T cells[36]. In some human studies, increased IFNγ production by T cells has been observed after BCG vaccination or adult re-vaccination, but protection was not evaluated[38-41]. One study in South African infants vaccinated with BCG addressed the relationship between vaccine-induced protection, T cell frequency and their cytokine profile, but found no correlation between the number of BCG-elicited T cells producing IFNγ or multiple cytokines (IFNγ, IL-2, and TNF) and the development of culture positive TB[42].

These data raise the question: if $CD4^+$ T cells and IFNγ are important, why doesn't IFNγ production by $CD4^+$ T cells correlate with protection? The idea that, because $CD4^+$ T cells produce IFNγ, their IFNγ production must be important is an assumption and one with little supporting data. Several studies have shown that $CD4^+$ T cells protect mice against *M. tuberculosis* independently of IFNγ[43-47]. Transgenic $CD4^+$ T cells, which are specific for the *M. tuberculosis* antigen ESAT6 retain their ability to protect mice against *M. tuberculosis* even when unable to produce IFNγ or TNF [44]. Similarly, the ability of $IFN\gamma^{-/-}$ memory T cells to mediate protection is only slightly diminished compared to wild type memory T cells[45, 48]. These studies demonstrate that although $CD4^+$ T cells and IFNγ are important for *M. tuberculosis* control, T cell functions other than IFNγ production can mediate protection. Furthermore, it is unknown how much IFNγ is needed, which cells are required to produce it, and whether more is better[49]. Also, the inflammatory microenvironment in which IFNγ is produced might be important, as the balance between IFNγ and different cytokines, such as IL-10 and other Th2 cytokines, is likely to influence disease outcome[50]. Therefore, it is crucial to identify factors that are required for resistance

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and correlate with susceptibility in individuals with intact immune systems, as opposed to components of the immune response that are necessary for protection but don't predict clinical outcome or disease state.

The notion that IFNγ is necessary but not sufficient for bacterial control following mycobacterial infection is supported by multiple studies in mice. For example, several knockout mice (such as TNF$^{-/-}$, GM-CSF$^{-/-}$, IL-1$^{-/-}$, and IL-6$^{-/-}$) die rapidly following *M. tuberculosis* infection, similar to IFNγ$^{-/-}$ animals[51-54]. Since these mice produce IFNγ, their failure to control *M. tuberculosis* indicates that additional pathways besides IFNγ are essential for immunity. Although the data from knockout mice and MSMD families is irrefutable, more mechanistic insights into the protective pathways that lead to *M. tuberculosis* control are needed. It is also important to remember that although IFNγ inhibits *M. tuberculosis* replication in murine macrophages[55], it is not sufficient to control *M. tuberculosis* growth in human macrophages[56, 57]. Similarly, nitric oxide (NO) production by murine macrophages can kill *M. tuberculosis*, but its production by human alveolar macrophages and its role in controlling *M. tuberuclosis* in these cells is controversial [58, 59]. These observations reinforce the idea that we must look beyond the CD4^{+} T cell/IFNγ central dogma to identify other immunological functions that protect against *M. tuberculosis*.

Reassessing protective immunity

In recent years, many studies have looked past the central dogma and revealed different pathways involved in protective immunity during TB. These studies reveal characteristics of protective T cells that should be incorporated in the design of new vaccines against *M. tuberculosis* (Figure 2b).

Other mediators that activate macrophages

The cytokines TNF, GM-CSF and IL-1β and vitamins C and D are all implicated as mediators that activate macrophages to control *M. tuberculosis* growth. Mice that lack TNF are highly susceptible to *M. tuberculosis* infection and TNF production by T cells has been shown to be important for resistance against *M. tuberculosis*[51, 60]. TNF synergizes with IFNγ in stimulating NO production by macrophages, maintains granuloma structure, and limits immunopathology, possibly through modulation of IL-10 levels, inhibition of Th2 responses, and limiting neutrophil infiltration[61-63]. The widespread use of TNF blockers to treat patients with autoimmune diseases for which TNF is a pathogenic factor, such as rheumatoid arthritis, has resulted in numerous cases of reactivated latent TB, establishing TNF as an important mediator of resistance to *M. tuberculosis* in people[64].

Mice lacking GM-CSF are highly susceptible to *M. tuberculosis* and GM-CSF treatment of human macrophages restricts intracellular growth of *M. tuberculosis* and *M. avium*[53, 65, 66]. GM-CSF is produced by a multitude of cells including leukocytes, epithelial cells and fibroblasts, and loss of this cytokine leads to abnormalities in surfactant recycling and the development of a lung disease that resembles human pulmonary alveolar proteinosis[67]. Overexpressing GM-CSF in epithelial cells reverses these lung abnormalities but the susceptibility to *M. tuberculosis* remains, suggesting that GM-CSF production by other

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cells, perhaps T cells, contributes to protection in mice. This idea is supported by the observation that iNKT cell production of GM-CSF contributes to host resistance against tuberculosis [68]. Additionally, the presence of anti-GM-CSF autoantibodies that block GMCSF function has been linked to both cryptococcal meningitis and pulmonary TB in otherwise healthy subjects indicating that GM-CSF has an important role in host defense against infection in people[69].

Mice lacking IL-1β, a pro-inflammatory cytokine produced by macrophages, or its receptor are highly susceptible to *M. tuberculosis* infection and IL-1β directly inhibits intracellular growth of *M. tuberculosis*[47, 52, 70-72]. Although mice lacking IL-1β die prematurely from infection, IL-1β can also be detrimental by recruiting pathogenic Th17 cells and neutrophils to the lung, resulting in tissue inflammation [46, 73, 74]. IL-1β also activates human macrophages to control bacterial replication[70, 72, 75].

Stimulation with either a ligand that triggers TLR2/1 or IFN-γ induces the nuclear vitamin D receptor (VDR) and enzymes that catalyze the conversion of vitamin D to its bioactive form[22, 76]. Signaling through VDR elicits production of the human cathelicidin LL-37, an antimicrobial peptide that directly kills *M. tuberculosis*[77]. Beyond its role in cathelicidin production, Vitamin D is involved in autophagy, phagolysosome fusion and IL-1β production[22, 78, 79]. Dissecting the role of vitamin D has been challenging. Multiple studies show decreased levels of bioactive vitamin D in TB patients, but whether this is a cause or an effect of TB is unknown; and whether Vitamin D supplementation benefits treatment is still uncertain[80].

Vitamin C might be important for immunity against *M. tuberculosis*, as vitamin C affects *M. tuberculosis* survival and growth[81]. Given the established association between malnutrition and susceptibility to TB, it is important to determine whether specific nutritional deficiencies contribute to *M. tuberculosis* susceptibility.

Killing of infected macrophages

In addition to cytokine production, T cells, particularly $CD8^+$ T cells, kill cells that they recognize as ‘foreign’. $CD8^+$ T cells with the capacity to kill target cells are called cytotoxic T lymphocytes (CTLs). *M. tuberculosis* elicits $CD8^+$ T cell responses in people and animal models and these $CD8^+$ T cells behave as CTLs *in vivo* [82-85]. Three different molecular pathways mediate CTL activity: exocytosis of cytotoxic granules containing proteins that cause lysis and apoptosis of target cells, such as perforin, granulysin and granzymes; Fas/FasL (CD95/CD95L), cell surface proteins that mediate death signaling; and TNF[85]. The increased susceptibility of $Fas^{-/-}$, $FasL^{-/-}$ and $perforin^{-/-}$ mice to *M. tuberculosis* corroborate the importance of these pathways for immunity[86, 87]. Importantly, perforin is required for protection mediated by CTLs[85]. Human $CD8^+$ T cells also require perforin to restrict *M. tuberculosis* growth, with granulysin being an important granule constituent[88]. Other than perforin, the crucial effector molecules for murine $CD8^+$ T cells are unknown[88].

How killing of infected macrophages by $CD8^+$ T cells impairs *M. tuberculosis* survival is an active area of investigation. All three killing pathways induce target cell apoptosis, which is associated with reduced bacterial viability[89]. The engulfment of apoptotic, infected cells by

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uninfected macrophages – a process known as efferocytosis – leads to rapid association of the bacteria trapped in the phagocytosed apoptotic cell (the 'efferosome') with lysosomes and killing of *M. tuberculosis* [90].

T cells orchestrate granuloma formation

In addition to detecting infected macrophages, T cells have a key role in the formation of granulomas. T cell-derived cytokines (such as TNF) and chemokines (such as CCL3) recruit inflammatory macrophages, neutrophils and B cells to the granuloma[91]. IFNγ and TNF maintain granuloma architecture in mice and people[17, 64, 92-94]. The importance of $CD4^+$ T cells in shaping the granuloma microenvironment is inferred from HIV^+ subjects who form dysfunctional granulomas that fail to contain *M. tuberculosis*[95] and by studies in guinea pigs and rabbits[96, 97]. Recent imaging studies in people and NHP indicate that granulomas behave autonomously and are more dynamic than previously appreciated[8, 98]. Granulomas change over time independently of each other with respect to size and metabolic activity – some shrink whereas others expand. Although $CD4^+$ T cells promote granuloma formation early after *M. tuberculosis* infection, they also contribute to transmission by promoting granuloma necrosis accompanied by erosion into airways during later disease stages[99].

Balancing pro- and anti-inflammatory signals

In many chronic infections, including TB, immune-mediated tissue injury is more detrimental than the pathogen itself. Therefore, mechanisms exist to counter-regulate pro-inflammatory immune cells and prevent the harmful effects of excessive inflammation; however, these effects might also dampen protective immunity.

$Foxp3^+$ regulatory T cells (Tregs) suppress inflammation and limit immune responses by producing immunosuppressive cytokines such as IL-10 and TGF-β and by directly interacting with other cells via inhibitory cell surface molecules[100]. Tregs are generated following *M. tuberculosis* infection in humans, NHP and mice[101-104]. In mice, Treg elimination can enhance protective immunity, as observed by the survival of fewer bacteria; however, whether this occurs at the risk of greater tissue injury has not been addressed[104-106].

Chronic antigen stimulation and exposure to inflammatory cytokines leads to a state of T cell exhaustion that is manifested by a progressive loss of T cell function over time, which has been best documented during chronic viral infection. There is great interest in the mediators of exhaustion because blocking them might 're-invigorate' T cell immunity and promote pathogen clearance during chronic infection. One such mediator, PD-1, is a cell surface receptor expressed by antigen-activated T cells. Interaction of PD-1 with its ligands transduces a signal that inhibits T cell proliferation and cytokine production[107]. Disruption of the PD-1/ligand interaction, through the use of neutralizing antibodies or in knockout mice, increases the number and function of *M. tuberculosis*-specific T cells in the lungs of infected mice[108-110]. However, in the absence of PD-1 signaling, dysregulation of $CD4^+$ T cells leads to increased bacterial burden, lung tissue destruction, and death of infected mice[108, 110]. These data suggest that T cell exhaustion might represent a beneficial regulatory mechanism that prevents overt immunopathology.

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Neutrophils serve an early protective role against *M. tuberculosis* in the lung by producing IL-1β, TNF, defensins, cathelicidins, lipocalin, NADPH oxidase and superoxides[77, 111-113]. Neutrophils also participate in T cell priming including cross-presentation of class-I restricted antigens, a process important for the stimulation of $CD8^+$ T cells by intracellular pathogens[114]. However, when the short-lived neutrophils die, the pro-inflammatory contents of their granules can be released; thus, an excess of neutrophils can promote tissue damage.

Although IFNγ is a pro-inflammatory cytokine, it also limits inflammation, at least in part through direct and indirect inhibition of neutrophils. IFNγ can have anti-proliferative effects on T cells and modulate their function, including inhibiting $CD4^+$ T cell production of IL-17, a cytokine that drives neutrophilic inflammation[115]. In addition, IFNγ acts directly on neutrophils to inhibit their accumulation in the lung[46, 73]. In fact, we view neutrophil infiltrates in the lung as a sign of failed Th1 immunity, which leads to accelerated tissue destruction during chronic *M. tuberculosis* infection[46]. Similarly, NO restrains inflammation by inhibiting IL-1β production by macrophages. Whereas NO production by murine macrophages mediates the antimicrobial activity of IFNγ, NO also inhibits NLRP3 inflammasome assembly, which curtails the production of IL-1β[116].

Collectively, these data support the notion that T cells are uniquely positioned to influence the balance of pro- and anti-inflammatory signals. These results strengthen the idea that the function of $CD4^+$ T cells and IFNγ is broader than activating macrophages and is necessary for optimal immunity during *M. tuberculosis* infection. Thus, IFNγ acts as a key negative regulator of innate immunity including neutrophils and IL-1β, both of which might be beneficial early, but have detrimental effects if they persist into the chronic phase of *M. tuberculosis* infection. The anti-inflammatory role of T cells might prevent over-exuberant protective responses that cause harmful immunopathology and tissue damage during chronic infection[46, 116]. Measuring surrogates of pro- or anti-inflammatory signals, for example by expression profiling[117] or measuring the monocyte/lymphocyte ratio[118] in peripheral blood, could be useful to identify individuals who are at risk for active TB.

Other cells participate in the immune response to M. tuberculosis

Although it is generally accepted that conventional $CD4^+$ and $CD8^+$ T cells mediate protection against *M. tuberculosis*, many other cell types participate in the immune response (see Box 2 for the contribution of non-conventional T cells). The TB mouse model is $CD4^+$ T cell centric and it is difficult to prove a role even for conventional $CD8^+$ T cells. Other T cell subsets are not present or are qualitatively different in the mouse compared to humans. Similarly, the contribution of B cells and antibody-mediated immunity needs further clarification[119]. Thus, these different cell types need to be investigated in other models. Both $CD8^+$ T cells and non-conventional T cells appear to have a quantitatively greater role in immunity to *M. tuberculosis* in NHP than in other animal models[82, 120]. Understanding the roles of these different cells types during *M. tuberculosis* infection might provide opportunities to discover new protective effector functions, and to develop methods to augment their function as part of new vaccination or treatment strategies.

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Lessons for developing T cell vaccines

Is natural immunity against TB sufficient?

Many pathogens do not elicit protective immunity, including common ones that cause urinary tract infections (enteric gram-positive and gram-negative bacteria), sexually transmitted infections such as *Chlamydia trachomatis*, *Neisseria gonorrhea* and *Treponema pallidum*, and pharyngitis caused by group A streptococci; others such as poxviruses induce long term protection, an observation that is the basis of vaccination. It is still unclear why some pathogens, but not others, induce protective immunity against reinfection. What is the case for TB? If only around 10% of infected people develop active disease during their lifetime, one must concede that natural immunity works well, even if it doesn't lead to sterilization. What about the 10% that develop symptomatic disease? Although progression to symptomatic disease can sometimes be attributed to acquired immunodeficiency (AIDS, TNF blockade, corticosteroids, autoantibodies, etc.) in many cases, immunocompetent individuals also develop active TB, which indicates a failure of their immune systems to control infection. Why does the immune system fail to enforce latency and allow active disease to emerge in immunocompetent individuals?

People previously treated for TB are at higher risk of developing additional episodes of disease[121-125]. Can this be attributed to relapse after inadequate treatment? Or, do these individuals have a defect in immunity that might explain why they developed disease in the first place? If these are the subjects that we are trying to protect by vaccination, we need to understand why they are susceptible to TB. This is important, as vaccines that aim to augment typical immune responses might fail to protect people with immune defects. Such people might not respond normally to vaccines or they might be resistant to their effects, suggesting that natural immunity in these individuals is defective.

As an example, after aerosol *M. tuberculosis* infection, C3HeB/FeJ mice develop necrotic granulomatous lesions and die rapidly. However, these mice have robust T cell responses[126, 127]. The genetic basis for their susceptibility has been mapped to several loci and the dominant one, *Ipr1*, is preferentially expressed by macrophages and alters their death modality[128]. Macrophages expressing the resistant allele of *Ipr1* are more prone to apoptosis following intracellular infection, whereas macrophages expressing the susceptible allele undergo necrosis, which is associated with higher bacterial loads and more tissue destruction. This is an important insight as some people might develop active TB because their macrophages are unable to control intracellular *M. tuberculosis* growth, rather than because they have dysfunctional T cells. Similarly, an increase in type-I and type-II interferon-inducible genes is found in the peripheral blood of individuals with active pulmonary tuberculosis[117]. Surprisingly, this gene signature is mostly accounted for by changes in neutrophil gene expression. These data support that, just as for macrophages, alterations in neutrophil functions can have an impact on disease susceptibility and progression. Thus, even vaccines that elicit strong T cell responses might not be effective at protecting such people from TB because their macrophages, neutrophils or other cell types cannot respond appropriately to the T cell signals. Without understanding why people are susceptible to disease, we cannot predict how to protect them.

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Finally, instead of mimicking natural immunity, vaccine induced protection against TB might require 'uncommon' or 'unnatural' immunity, as recently discussed by the Gates Foundation (www.grandchallenges.org/grantopportunities/pages/tbvaccineaccelerator.aspx). An example of such 'unnatural' protective immunity is that induced after tetanus toxoid vaccination, which is not observed after natural infection with *Clostridium tetani*[129]. An example for 'unnatural' immunity to *M. tuberculosis* is iNKT cells (see Box 2). These cells are dispensable for protection against primary infection in immunocompetent mice, but their activation can prolong the survival of inbred strains of susceptible mice[130]. It might be possible to induce such 'unnatural' or 'uncommon' immunity; for example, by engineering BCG to express the bacterial toxins listeriolysin or perfringolysin, which alters the route of antigen presentation, and leads to more efficient stimulation of $CD8^+$ T cells [131-133]. Incorporating such strategies that stimulate a broader immune response may have a greater effect on induction of protective immunity than promoting a stronger response to a single antigen.

Quantity versus quality

The goal of vaccination is to elicit a population of long-lived memory T cells that, after *M. tuberculosis* challenge, will rapidly proliferate, acquire optimal effector functions, traffic to the lung, recognize *M. tuberculosis*-infected cells, control bacterial replication and lead to sterilization (Figure 2c). We assume that successful vaccination will elicit $CD4^+$ and $CD8^+$ T cells, which are specific for one or more mycobacterial antigens and whose functions will include IFNγ production (Th1 response). However, Th1 responses are unable to sterilize the host during active disease and because we cannot define protective immunity, there is no way to measure successful immunization, other than empirically quantifying changes in pathogen burden after challenge, or in people, natural exposure, an approach that is slow and cumbersome.

For infections that can be prevented by humoral immunity, antibody titers correlate with protective immunity. For T cell-based vaccines, we are not sure whether the number of elicited T cells will correlate with protection or whether a change in one of the many functions that T cells perform will be more useful. For example, re-exposure to antigen *in vivo* induces $CD8^+$ T cells to more frequently and persistently co-express effector molecules (such as perforin, granzyme A and B, Fas-Ligand, and IFNγ) and to more efficiently kill than $CD8^+$ T cells stimulated by antigen the first time[134]. This suggests that an important function of T cell vaccination is to induce and coordinate gene expression of effector molecules. Similarly, several different types of intermediate to long-term antigen-specific T cells (central, effector and tissue resident memory cells) persist after infection or vaccination with the potential to rapidly respond to infectious challenge[135]; however, it is unclear which population(s) are most effective in preventing TB.

Collectively, the data summarized in this Review suggest that vaccines that elicit large numbers of T cells with the capacity to only produce IFNγ might not be optimal for protecting against TB. We should be looking for changes in T cell function, rather than numbers, as key factors that will lead to a significant breakthrough in vaccine design. Furthermore, because several studies have revealed different pathways involved in

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protective immunity against TB (such as those mediated by IL-1β, GM-CSF, vitamins C and D and cytolysis), vaccine design should aim at arming T cells with the capacity to modulate such pathways in cells infected with *M. tuberculosis* (Figure 2c). Finally, we must avoid the trap of thinking that there exists one type of T cell that will mediate protection alone. The host response to *M. tuberculosis* elicits many different types of T cells and even if all of them do not kill *M. tuberculosis*, it is likely that they all have a role in orchestrating a successful immune response.

CONCLUSION

An important obstacle to vaccine development is our incomplete understanding of what constitutes protective immunity against *M. tuberculosis*. It is difficult to define the goals of vaccination without first knowing what the immune system is capable of. Although a vaccine that prevents infection is everyone's first choice, the consensus seems to be that a vaccine that enforces latency and prevents transmission is a more realistic goal. However, would we feel different if we understood why some people do not become infected despite repeated exposure? Or why some granulomas behave autonomously with some of them apparently able to control and eradicate *M. tuberculosis* and others not[136]?

This lack of knowledge supports our main suggestion for vaccine design: that characterization of additional immune mediators and cell types, even ones that appear to have minor roles during natural infection, is an essential first step. An effective vaccine might need to engage multiple immune mechanisms activated during a typical infection and might need to skew the host response in ways not seen during natural infection.

TB is a chronic disease, and *M. tuberculosis* evades detection by antibodies by occupying an intracellular niche. Thus, a vaccine that generates $CD4^+$ and $CD8^+$ central memory T cells with high proliferative potential, as well as a cohort of potent $CD8^+$ effector memory and resident memory T cells that are poised to rapidly kill infected cells in the lung can be expected to be an ideal T cell vaccine candidate for disease prevention. Alternative approaches that, for example, stimulate unconventional T cell subsets and B cell/antibody responses in concert with conventional T cells should be further investigated. However, we believe that continuing to develop T cell vaccines aimed at boosting childhood BCG vaccination by solely varying the antigen will likely continue to fail. It is not enough to target specific antigens without a better understanding of how vaccines modulate T cell subsets, function and trafficking. IFNγ production by $CD4^+$ T cells is essential in certain situations but it will likely not be sufficient as a protective response after vaccination. We believe that the premise that CD4+ T cell production of IFNγ is required for protection during infection has never been shown conclusively and its role in vaccine-induced immunity is based on over-interpretation of published data. In addition, we believe it is important to make vaccines that elicit multiple T cells subsets that express diverse protective functions. For example, we predict that a vaccine that elicits CD4+ T cells producing GM-CSF and IFNγ, and CD8+ T cells that function as cytolytic effectors in addition to producing IFNγ, would be more protective than vaccines that elicit only IFNγ. Finally, we must broaden the ways in which vaccine candidates are evaluated and the biomarkers used to measure their effect. Without defined correlates of protection, this will be challenging.

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Ongoing efforts to expand the ways in which vaccine candidates are evaluated and to embrace the diversity and heterogeneity of T cells need to be supported.

Author biographies

Cláudio Nunes-Alves is currently a post-doctoral fellow in Sam Behar's lab at the University of Massachusetts Medical School, where he studies adaptive immune responses to tuberculosis. He also studies how thymic infection impacts ongoing immunity in collaboration with the laboratories of Margarida Correia-Neves (at the Life and Health Sciences Research Institute, Braga, Portugal) and Christophe Benoist (at Harvard Medical School, Boston, MA).

Matthew G. Booty is currently a Ph.D. candidate in the Immunology Program at Harvard University and is performing his dissertation research with Sam Behar at the University of Massachusetts Medical School. His work focuses on several aspects of immunity to tuberculosis, including the regulation of $CD8^+$ T cells and the role of eicosanoid signaling in host susceptibility.

Stephen Carpenter is an infectious disease physician who joined the Behar Lab in 2011 during his fellowship training at the Massachusetts General Hospital and Brigham and Women's Hospital in Boston. He is currently an Instructor at the University of Massachusetts Medical School, studying how memory T cells function in tuberculosis, and aspires to improve TB vaccine efficacy through a better understanding of T cell function during infection.

Pushpa Jayaraman is a Research Assistant Professor whose research interests focus on the regulation of T cell function during TB infection. She is currently involved in studies to evaluate the role of Tim-3, a molecule established as an exhaustion marker in viral infections, in regulating innate and adaptive immune responses during chronic *M. tuberculosis* infection.

Alissa Rothchild is currently a Ph.D. candidate in the Immunology Program at Harvard University. Her dissertation work focuses on the activation and effector function of invariant Natural Killer T cells and the role of GM-CSF during *Mycobacterium tuberculosis* infection in the laboratory of Dr. Sam Behar at the University of Massachusetts Medical School.

Samuel M. Behar is Professor of Microbiology and Physiological Systems at the University of Massachusetts Medical School in Worcester, Massachusetts, USA. He studies innate and T cell-mediated mechanisms of microbial immunity that are leveraged by the host to combat pulmonary infection with *Mycobacterium tuberculosis*, with the ultimate goal of informing vaccine design.

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Box 1

Tuberculosis vaccines

Owing to the shortcomings of BCG vaccination in preventing TB, significant effort has been put into developing new vaccines. Currently more than 12 candidates are being tested in clinical trials[137, 138]. These candidates aim to replace BCG, or act as a booster vaccine following BCG. The vaccines include viral vectors expressing *M. tuberculosis* antigens; *M. tuberculosis* proteins with improved adjuvants; recombinant BCG strains and live attenuated *M. tuberculosis* vaccines. Unfortunately, some preliminary results have been disappointing: Aeras-422, a recombinant BCG strain failed because of safety concerns[137] and MVA85A, a new vaccine consisting of Modified Vaccinia Ankara virus (MVA, a replicative-defective variant of Vaccinia virus) expressing the *M. tuberculosis* antigen 85A, and designed to enhance BCG-induced protection, showed no efficacy in a Phase 2b trial[139].

MVA85A, has been extensively investigated as a booster following BCG vaccination, in what has become known as the ‘prime-boost’ strategy (see accompanying figure). MVA85A is effective in boosting BCG vaccination in a variety of *M. tuberculosis* animal challenge models. Initial studies with MVA85A in people showed promise, as significantly more antigen-specific T cells from the boosted group secreted IFNγ and were polyfunctional compared to vaccination with BCG alone[140, 141]. These effects were durable and lasted at least 24 weeks after the MVA85A boost[140]. However, the recent results of the phase 2b clinical trial indicate that MVA85A is not effective at preventing *M. tuberculosis* infection or tuberculosis[139]. Administered to infants ages 4-6 months as a booster to BCG vaccination given at birth, MVA85A elicited overall small numbers of $CD4^+$ T cells that secrete IFNγ, IL-2, and TNF at 28 days after vaccination. Although slightly greater T cell responses were noted in the vaccinated group, no differences in protection from TB were observed in a 2-year follow-up[139].

A recurring question is whether the cytokines measured in these studies are useful predictors of vaccine protection, or whether specific markers exist that could have predicted a lack of protection. Another issue is whether the immature immune systems of infants, compromises potential vaccine efficacy. These findings raise the question of whether MVA85A should be evaluated in adults.

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Box 2

A role for non-conventional T cells in immunity to tuberculosis

Box 2

A role for non-conventional T cells in immunity to tuberculosis

Cell Type	iNKT cell	Group I CD1-restricted T cell	Mucosal-associated invariant T (MAIT) cell	γδ T cell	Th17 cell
Recognizing	CD1d	CD1a, CD1b, CD1c	MR1	Butyrophilin[142]	MHC II
Antigen Type	Lipids	Lipids	Riboflavin metabolites	Phosphoantigens	Peptides
Required for murine *M. tuberculosis*-immunity?	No [14, 87, 143, 144]	Not found in mice	Unknown	No	No[145]
Detected in *M. tuberculosis*-infected people?	Yes [146-148]	Yes [149-151]	Yes [152, 153]	Yes [154]	Yes [155]
Respond to *M. tuberculosis*-infected macrophages?	Yes [156]	Yes [149]	Yes [157]	Yes	Yes [158]
Stimulation leads to enhanced antimycobacterial function?	Yes [130, 159] (αGalCer)	Unknown	Unknown	Yes [160] (IL-2 + phosphoantigen)	Unknown

iNKT cells

iNKT cells are a T cell subset that recognize lipid and glycolipid antigens. Subjects with active TB have reduced iNKT cell numbers in peripheral blood compared to latently infected or healthy individuals[146-148]. Treatment of infected mice with αGalCer, a potent activator of iNKT cells, improves disease outcome and synergizes with antibiotics[130, 159]. αGalCer also stimulates human iNKT cells to lyse *M. tuberculosis*-infected macrophages and kill intracellular bacteria in vitro[161]. Murine iNKT cells cultured with *M. tuberculosis*-infected primary macrophages restrict bacterial growth and adoptive transfer of iNKT cells limits bacterial growth in vivo[156]. Activated iNKT cells also have adjuvant-like properties and conjugation of αGalCer to BCG augments its efficacy as a vaccine[162]. Why iNKT cells are dispensable in the intact mouse yet exert a major protective role once activated needs investigation. Also, the use of αGalCer in human TB still has not been explored.

Group I CD1-restricted T cells

CD1-restricted T cells that recognize the mycobacterial lipid glucose monomycolate or C32-phosphomycoketide can be detected in the peripheral blood of *M. tuberculosis* patients[149-151]. The effector function of these T cells and whether they can be elicited by vaccination is still not fully understood.

γδ T cells

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Human γδ T cells recognize small organic phosphate antigens and alkylamines and expand in response to *M. tuberculosis* infected cells in vitro. Exciting data indicate that they generate a recall response following BCG vaccination and *M. tuberculosis* challenge in NHP[120, 154, 163, 164]. Although γδ T cells are not required for bacterial control in mice, they are the main source of IL-17 in the lung during *M. tuberculosis* infection[13, 165]. Activation of γδ T by IL-2 and phosphoantigen treatment results in reduced bacterial burdens and attenuated lesions in the lungs of NHP infected with *M. tuberculosis*[160].

MAIT cells

MAIT cells, which are found in human lung and peripheral blood, recognize *M. tuberculosis*-infected cells[152, 157]. MAIT cells can have antimicrobial activity against bacteria and yeast, but their role during *M. tuberculosis* infection still requires investigation[153].

Th17 cells

IL-17 has an early role in the recruitment of antigen-specific IFNγ-secreting Th1 cells, particularly after BCG vaccination[164, 166, 167] as well as early granuloma formation. However, persistence of Th17 cells can be detrimental. IL-17 promotes neutrophil recruitment and inflammation, and if not ultimately suppressed by IFNγ, can exacerbate tissue damage[46, 74, 115, 165].

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Online summary

- Tuberculosis remains a major health threat worldwide, with estimated 8.7 million new cases and 1.4 million deaths in 2011. New vaccines are needed to stop this pandemic.
- The only current vaccine in use – BCG – provides variable protection against pulmonary tuberculosis. Additionally, new vaccine candidates have failed at preventing *M. tuberculosis* infection or tuberculosis.
- Vaccine development has been hampered by the lack of immunological correlates of protection. Although IFNγ production by $CD4^+$ T cells has been widely used to measure vaccine efficacy, it does not correlate with vaccine-induced protection.
- Many studies have found additional immunological mechanisms that lead to *M. tuberculosis* control. These include those mediated by other T cell subsets (such as $CD8^+$ and non-conventional T cell subsets) and immune mediators such as TNF, IL-1β, GM-CSF, and vitamins C and D.
- New vaccination strategies should focus on modulating T cell function, rather than numbers, as well as targeting other aspects of the immune system.
- Broadening our understanding of the immune pathways that provide protection against *M. tuberculosis* and how they function in concert will both increase the number of targets for vaccination as well as improve our evaluation of future vaccine candidates.

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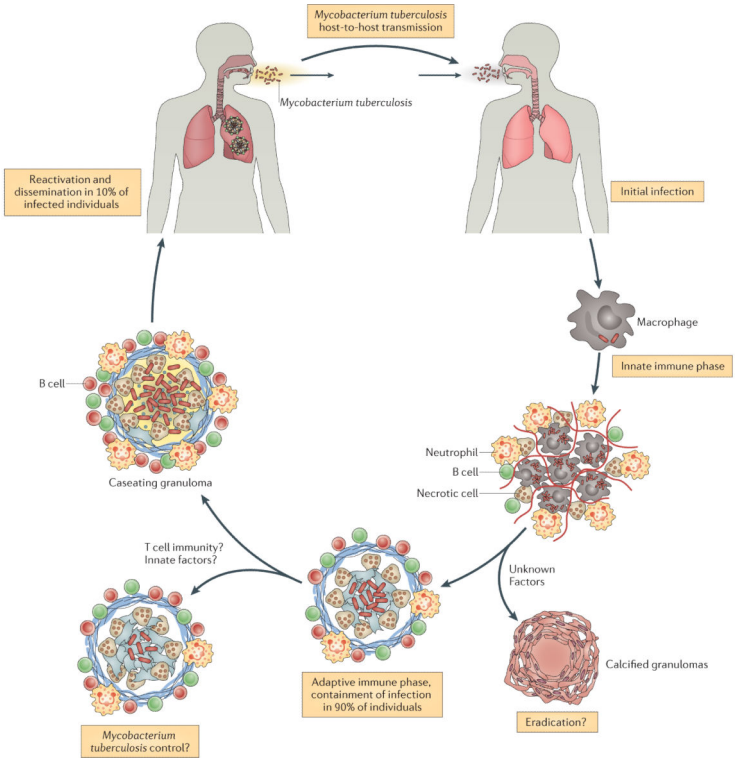

Figure 1.

TB pathogenesis. Infection is initiated by inhalation of aerosol droplets containing bacteria. The initial stages of infection are characterized by innate immune responses involving recruitment of inflammatory cells to the lung. Following bacterial dissemination to the draining lymph node, dendritic cell presentation of bacterial antigens leads to T cell priming, and triggers an expansion of antigen-specific T cells, which are recruited to the lung. The recruitment of immune T cells, B cells, activated macrophages and other leukocytes leads to the establishment of granulomas, which can contain *M. tuberculosis*. The majority of infected individuals will remain in a “latent” state of infection, in which no clinical symptoms are present. A small percentage of these people will eventually progress and develop active disease, which can lead to the release of *M. tuberculosis* from granulomas eroded into the airways. When individuals with active TB cough, they can generate infectious droplets that propagate the infection.

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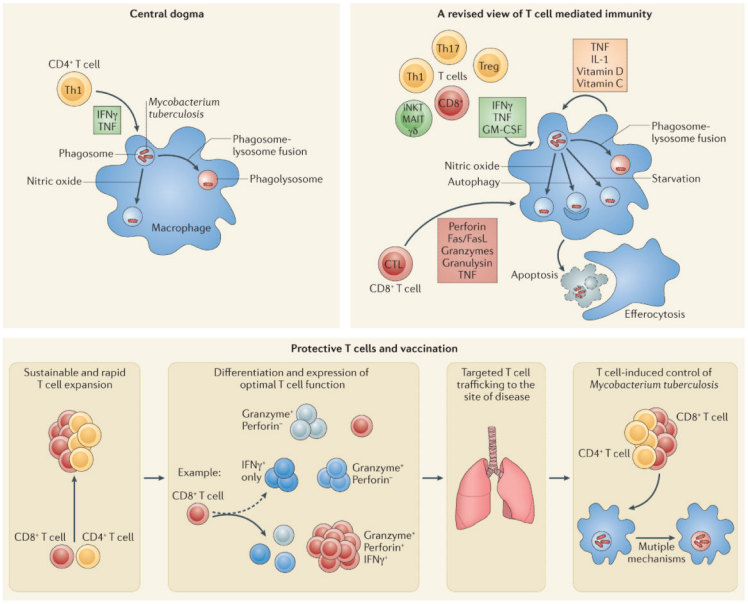

Figure 2.

Paradigms of protective immunity to TB.

a. The “central dogma” of protective immunity to TB is that $CD4^+$ T cells produce IFNγ, which synergizes with TNF (produced by the T cell or the macrophage), and together these activate macrophage antimicrobial activity capable of restricting *M. tuberculosis* growth. Two pathways activated by IFNγ that are capable of killing *M. tuberculosis* are nitric oxide (NO) production and phagolysosome fusion, which acidifies the bacterial phagosome.

b. “A revised view of protective T cell immunity” incorporates additional T cell subsets ($CD4^+$, $CD8^+$, and unconventional T cells – γδ T cells, MAIT cells and CD1-restricted T cells), and includes additional mechanisms by which T cells mediate killing of *M. tuberculosis*. These include additional cytokines (for example, possibly GM-CSF) and cytolysis of infected macrophages. The cytolytic mechanisms vary and can include cytotoxic granules, which can deliver antimicrobial peptides such as granulysin, but can also deliver granzymes, which can trigger apoptotic cell death. CTL activity mediated by FasL/Fas or TNF can also lead to apoptosis. Apoptosis can have a beneficial effect on the outcome of infection as infected apoptotic cells can be engulfed by bystander macrophages, which are capable of destroying the apoptotic cells including any intracellular bacteria. Finally, several components of the innate response, including IL-1 and vitamins, can synergize with cytokines produced by T cells.

c. “Protective T cells and vaccination” focuses on the desired features of protective T cell responses. Rationale vaccine design should aim to elicit protective T cells by optimizing their action on infected cells in several ways. Vaccine-elicited memory T cells must rapidly expand and generate secondary effector T cells that undergo sustained proliferation following activation. While the functions of primary effector T cells are expressed heterogeneously (broken arrow), vaccination (solid arrow) can lead to more homogenous expression of effector functions during the recall response. Such T cells, often identified as multifunctional T cells, may have a greater protective potential. Primed effector and memory T cells should efficiently traffic to sites of infection, but the kinetics of the response

must be balanced with respect to T cell subsets, and limit the potential for T cell exhaustion, excessive inflammatory pathology, or an ineffective response that hinders T cell - target contact.

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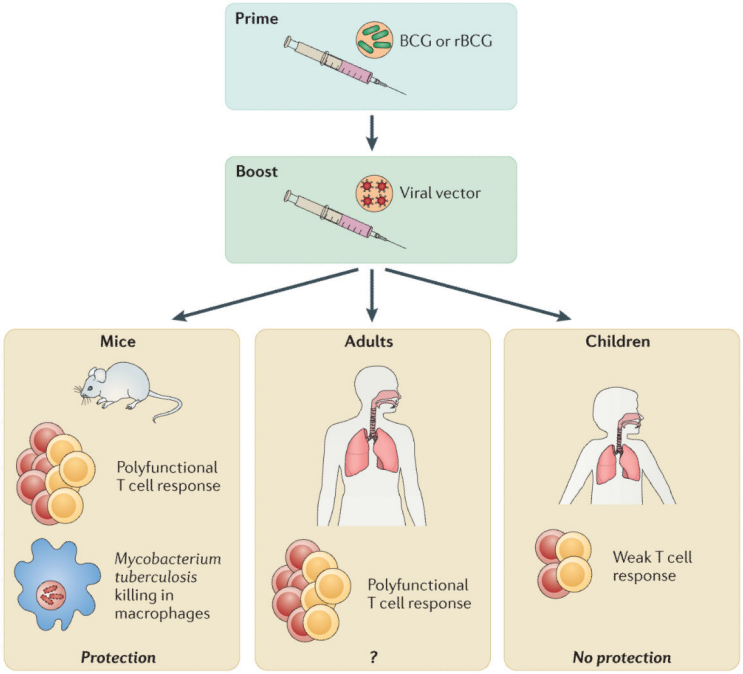

Fig 3.