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Vaccines against *Mycobacterium Tuberculosis*: Exploring Alternate Strategies to Combat a Near-perfect Pathogen

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Abstract

Tuberculosis is a significant threat to human health, infecting nearly one third of the world's population and causing over a million deaths per year. The need for an effective vaccine against tuberculosis is apparent however, the current vaccine has not been successful neither in the prevention nor recovery from infection. The pathogenesis of the causative agent, *Mycobacterium tuberculosis* has been extensively studied. The mechanisms of immune evasion and modulation that enable persistence of infection through subclinical latency and eventually active tuberculosis have been partially described. Many of these mechanisms are directly antagonistic to the intended benefit of vaccines, leaving a conundrum that has yet to be resolved. This review will first examine the nature of tuberculosis pathogenesis in the context of the immune response and outline the varied processes utilized by the bacterium to cause modulation. The review will also investigate other vaccination options that may overwhelm these microbial mechanisms or avert them entirely, allowing for the engagement of the immune response and clearance of the bacterium.

Keywords: *Mycobacterium tuberculosis*; Phagocytosis; *Mycobacterium bovis*

Introduction

Tuberculosis is a significant threat to public health. According to the World Health Organization [1], in 2011 an estimated 8.7 million individuals were infected with the causative agent, *Mycobacterium tuberculosis* (Mtb), of which 1.4 million succumbed to the infection. It is also estimated that one third of the world's population is latently infected with Mtb. In latent infection, the only evidence of infection is immune responsiveness to mycobacterial antigens while pathogen replication and clinical symptoms are poorly defined [2]. The standard treatment to eliminate infection is a regimen of antimicrobials, which can successfully treat approximately 85% of these cases. However, the development of resistance has created significant challenges to treatment options, rendering many antimicrobials ineffective. Close to a quarter of all tuberculosis infections are considered to be resistant to multiple antibiotics and nearly one tenth of these are considered to be extremely resistant to antimicrobial treatment [1].

The search for an effective vaccine against tuberculosis has been ongoing for well over 100 years, yet a successful candidate has been elusive. The only licensed vaccine is Bacillus Calmette–Guérin (BCG), which is developed from the bacterium *Mycobacterium bovis* [3]. The vaccine is protective in children and adolescents against several early consequences of tuberculosis infection, including systemic infection and meningitis [4]. The protection from symptoms may not necessarily mean protection from infection that may become latent. Reactivation of the infection may lead to fulminant tuberculosis. Countries with the highest incidence of TB have the lowest levels of protection from vaccination. In well documented clinical studies vaccination of children protects individuals variably from adult pulmonary disease due to Mtb. These studies show that the range of protection varied from 0 to 80%. Thus, the efficacy of vaccination with BCG is varied [5] and its reliability in preventing pulmonary disease in adults and conveying protection over time has been called into question [6,7].

Other vaccine strategies have been explored including: i) the development of a more successful BCG recombinant; ii) the use of attenuated strains of Mtb; and iii) the use of subunit vaccines based on recombinant Mtb proteins designed with adjuvants to elicit a protective effect. In 2011, twelve vaccine candidates were at different stages of clinical trial [8,9]. In addition, one candidate successfully emerging from Phase III trials is a heat-killed strain of *M. vaccae* shown to reduce the rate of tuberculosis by 39% in HIV-infected individuals who had received the BCG vaccination and had CD4 counts of 200 cells/mm^3 [10]. However, none of these have demonstrated sufficiently high efficacy to suggest an end to the continued rate of infection worldwide.

One problem with vaccination is the enigmatic nature of Mtb infection, which is characterized as chronic rather than acute [11]. In traditional vaccination against acute pathogens such as polio and the now eradicated smallpox, the aim of vaccination is to develop a strong humoral response that prevents infection and a cellular response to eliminate the pathogen from the host [12]. Both responses play a role in prevention and recovery from disease, albeit not to the same degree. Therefore, the goal of Mtb vaccination is developing a strong, balanced Th1/Th2/Th17 response to the pathogen to prevent or clear the infection.

Many of the complex interactions of the bacterium with the immune system have been identified. Mtb has developed many well-documented mechanisms to interrupt the normal immune response

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and develop an environment that allows for persistence [13]. These studies have elucidated how Mtb changes the course of responding to and averts the effects of vaccination. The current review will contrast the nature of a healthy vaccinated response with that involved in the development of chronic Mtb infection in order to offer clues as to why current strategies for vaccination may not be successful. Options for future Mtb-specific and Mtb-independent vaccine strategies will be discussed in the context of developing a response that may overwhelm the ability of Mtb to develop persistence and evade the immune system.

The Immune Response to Mtb

Mtb requires not only inhalation but also deposition in the lower respiratory tract, primarily the alveoli. If the bacterium lands in the trachea or the bronchia, both physical barriers of the upper respiratory tract and the presence of antimicrobial peptides kill and clear the bacterium [14]. Once in the alveoli, the antibody response is the first line of defense. Antibodies are the primary component of humoral immunity [15] and aid in the development of an opsonized response [16]. Numerous studies investigated the role of antibodies against Mtb infection [17-19] and documented antigens recognized by antibodies included the Ag85 complex [15,20-22], lipoarabinomannan (LAM) [23,24], arabinomannan (ARM) [18], the 6 kDa early secretory antigen target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) [19]. The memory B cell response in BCG-vaccinated individuals have been shown to be present even 45 years following vaccination [19]. Yet this response, which may initiate clearance, is not sufficient to prevent infection of the individual [15,25,26].

There are several membrane-bound molecules that are involved in phagocytosis. Certain classes of antibodies have specificity for extracellular Mtb antigens, as do mannose-binding lectins [24,27] that recognize the LAM and ARM molecules on the Mtb surface. Both of these opsonins activate the third component of complement and the complex of Mtb-opsosin-C3 binds via C3 to its receptor (CR3) on phagocytes [20,28]. The binding of opsonized bacteria through CR3 triggers a signal for internalization into the phagosome [22,29]. Finally, pathogen recognition receptors (PRRs), such as Toll-like receptors (TLR) and the nucleotide-binding oligomerization domain-like receptors (NLR), can recognize some unique Mtb antibodies that promote phagocytosis.

Entry into the phagosome, initiates the inflammasome [30]. This trigger of lysosomal fusion initiates the expression and secretion of an array of inflammatory cytokines, including TNF-α, IFN-γ, IL-1 β, IL-6, IL-12, IL-17 and IL-23. Among others, CD4+ cells of the Th1, Th2, Th17 and Th22 phenotypes, CD8+ T cells, B cells, monocytes and natural killer (NK) cells are recruited to the site to regulate the response. Other recruited cells include: CD1 restricted T cells, which recognize lipid-containing molecules; regulatory T cells to suppress any hyperactivity; neutrophils, which play a dual role of phagocytosis of both free bacteria and apoptotic cells as well as initiating the development of a granuloma; and γδ-T cells, which assist in the formation of the granuloma and maintain the level of various cytokines [13]. Granulomas are characteristic of the anti-mycobacterial response and forms in the surrounding extracellular matrix [31]. This microenvironment allows for continual cycling of cytokines in a localized manner and facilitates the crosstalk between the various cell types to ensure bacteria are sequestered to the site.

Inside the cell, the bacteria are subjected to a combination of reactive nitrogen species, reactive oxygen species and acidic hydrolases that break apart the bacterium and allow presentation of antigen [32-34]. Finally, autophagy through the fusion of the phagosome and lysosome lead to the ultimate destruction of the bacteria via ubiquitin-derived peptides [35,36]. After fusion has occurred, the infected macrophages and dendritic cells may undergo apoptosis [37], trapping any surviving bacteria in the cell and leaving them vulnerable for further phagocytosis and internalization (and possibly infection) of other cells. Joller et al. [38] reviewed the role of phagosome Fc receptors in enhancing the efficacy of fusion and killing intracellular pathogens [39].

In healthy individuals, the infection is cleared within 2 to 8 weeks [40]. As the infection eventually clears, the granuloma dissolves through protease degradation of the matrix and macrophage degradation of the components [41]. Healing is mediated by TGF-β and the IL-10 family of cytokines [42,43]. In addition, IL-22 has been implicated in the clearance of Mtb infections [44] due to its role in the production of antimicrobial peptides, IL-6 and IL-8 [45] and through its stimulation of the STAT3 pathway [46] and tissue rebuilding factors necessary for mucosal repair.

Evading the Humoral Response

While evidence suggests that BCG vaccination confers a lifelong humoral response [19], the effect of vaccination is muted during Mtb infection later in life [9]. At the microscopic level, this is due in part to a reduction in the expression of CR3 on infected macrophages [47]. There is evidence to suggest that the stunting of the humoral response may be due to exposure to other environmental mycobacterial [48]. For over twenty-five years, this hypothesis has been tested. Prior exposure to *M. kansasii* [49], *M. scrofulaceum* [49,50], *M. avium* [50-52], *M. chelonae* [53] and M. vaccae [50] has led to an immune response that cross reacts with Mtb. Specifically, Manivannan et al. [28] have shown that while there is an initial antibody response to these antigens, the next steps involving the complement pathway are suppressed. In addition, Espitia et al. [15], Al-Attiyah et al. [26] and Rennie et al. [25] have shown that antibodies to the Ag85 complex and other environmental mycobacteria can be found in the sera of subjects with no prior Mtb exposure. Furthermore, this presentation does not protect the individual from infection [9].

The role of environmental mycobacteria in BCG vaccine humoral response regulation has been studied by Ho et al. [53]. The injection of T reg cells derived from *M. chelonae* down regulated the immune response of mice immunized with BCG. In humans, Singh et al. [54] showed a high correlation between the level of T reg cells and Mtb infection, as well as a decline in frequency of these cells with successful therapy. These studies suggest that the host develops a modulated response to mycobacterial antigens simply as a result of exposure to environmental mycobacterium.

While suppression of response to mycobacterial antigens is host derived, Mtb also has an active role in evading the humoral response by selective targeting of TLRs to suppress TLR signaling. TLRs (1 to 9) are single domain transmembranal proteins that are found on the cell membrane, as well as in macrophage and dendritic cell vesicles' membranes [55]. TLRs also have an active role in cytokine production [56] and antigen processing [57]. Of the known 13 mammalian TLRs involved in the induction of either an inflammatory or proinflammatory response, four TLRs are involved in Mtb infections: TLR-2, TLR-4, TLR-9 and TLR-8.

TLR-2 and TLR-4, which are found on the surface of phagocytes, recognize specific motifs on the surface of the pathogen. TLR-2 recognizes the glycoplipids and lipopeptides found on the cell wall of

the bacterium including LAM, ARM, lipomannan (LM), phosphatidyl inositol mannoside (PIM), the 38-kDa and 19-kDa lipoproteins, and heat shock protein 70 [58]. TLR-2 also forms heterodimers with TLR-1 and TLR-6 to recognize triacylated and diacylated lipoproteins respectively [59]. TLR-4 recognizes the heat shock protein 60/65 [58]. TLR signaling via these routes initiates a response through the NF-κB transcription factor to initiate the expression of inflammatory and proinflammatory cytokines, including the classical TNF-α, IL-1, IL-6, IL-12, as well as the production of nitric oxide [55]. Once the bacterium is in the cell, unmethylated CpG oligodeoxynucleotide is recognized by the TLR-9 molecule to induce the production of IFN-α/β, as well as MHC I antigen cross-processing to signal a CD8+ T cell response [60]. TLR-8 may impact cellular expression profiles during Mtb infection but the mechanism involved in this process remains unknown [61].

Mtb has an ability to suppress TLR signaling for both cytokine production [56] and antigen processing [57]. The 19-kDa lipoprotein in the bacterial cell wall, as well as LprG, a 24-kDa lipoprotein [62,63], have significant affinity for the TLR-2 molecule and binding results in the reduction and/or abrogation of the normal inflammatory and proinflammatory response.

The mechanism behind this apparent hijacking of the TLR response has yet to be fully elucidated, although the activity may be similar to one that already exists in the cell. To reduce the possibility of chronic inflammation or tissue damage as a result of TLR activity, a negative feedback inhibition response exists. A family of tyrosine kinases known as Tyro3, Axl, and Mer (collectively known as TAM) can effectively abrogate the signals of TLR-3, TLR-4 and TLR-9 [64]. The mechanism involves the association of TAMs with type I IFN receptors (IFNAR), and the subsequent induction of TLR inhibitors and cytokine production pathways. In addition, Ip et al. [65] have shown that the same mannose-binding lectin involved in the humoral response increases the expression of TLR-2 and TLR-6, potentially causing an upregulation of the TLR-2 signaling pathway. Whether these mechanisms are in fact the ones stimulated by the mycobacterial proteins has yet to be determined.

Halting Phagosome Maturation

The clearance of a pathogen through the phagosome involves its fusion with the lysosome. The mechanisms of fusion are still being unveiled but what is currently known is that the GTPase Rab5 and a member of the Class III phosphatidylinositol 3-kinases (known as Vps34) are recruited from the cytosol into the phagosome membrane mediating the formation of phosphatidylinositol 3-phosphate (PI(3)P) [66]. PI (3) P recognizes the early autosomal antigen 1 (EEA1), which drives a shift in the membrane from Rab5 to Rab7 and subsequent fusion with the lysosome. Vergne et al. [66] showed that this process can be blocked, resulting in delayed maturation of the phagosome and the loss of fusion. The mechanism is thought to be mediated by LAM, which accumulates in the phagosome membrane and both inhibits the function of Vsp34, preventing the formation of PI (3) P, and blocks the acquisition of calcium into the phagosome. This results in the prevention of the accumulation of Rab7 [67] and the accumulation of two other GTPases: Rab14, which is a Rab5 precursor, Rab22a in the membrane [68,69]. Vergne et al. [70] also showed that Mtb also secretes a lipid phosphatase SapM that hydrolyzes PI(3)P and ensures that the levels of this protein are reduced in the phagosome membrane.

In addition to the prevention of Rba5/7 mediated fusion, another possible mechanism of resistance has been unveiled involving a member of the family of Mtb encoded serine/threonine kinases known as the Pkn family [71,72]. The PknG enzyme is both secreted into the phagosome, and eventually trafficked into the cytosol, where it phosphorylates cellular proteins rendering them inactive. While no specific cellular targets have yet been identified, the role of this enzyme as a rogue actor that disrupts the maturation of the phagosome is being considered.

Cell Death in Mtb Infection

Under normal circumstances, the development of the Th1/Th2 responses requires antigen presentation to T cells by antigen presenting cells (APC) that have processed the microbe through the phagosome/lysosome. APCs eventually undergo phagocytosis and cross-presentation can occur. Divangahi et al. [73] showed that apoptosis of the infected macrophage is a critical requirement in the early initiation of the T cell response. Apoptosis is triggered through the lipid mediator prostaglandin E2 [74] in a nitric oxide-dependent manner. As the levels of nitric oxide rise, the prostaglandin E2 is released into the cytosol, in part to protect the mitochondrial inner membrane and plasma membrane from potential cytotoxicity of the reactive species [73]. Once the viability of the cell has been exhausted, apoptosis proceeds and the remaining antigens are taken up by macrophages, dendritic cells, and neutrophils for future presentation and amplification of the response.

Mtb, however, has developed a means to halt the production of prostaglandin E2 through the upregulation of the production of lipoxin A4 in the macrophage [73,75]. Lipoxin A4 effectively inhibits the production of prostaglandin E2 precursors, prostanoids, and the cyclooxygenase 2 enzyme. The cell subsequently suffers due to the damage caused by the reactive oxygen species. In addition, further damage is caused by the ESAT-6 secretion system-1(ESX-1) [37], which forms pores in the phagosome membrane, permitting the escape of the bacteria. Once outside of the phagosome, ESAT-6 and ESX target the plasma membrane, forming holes that allow for cell leakage [37]. The damage incurred by the cell causes necrosis to occur allowing bacteria to spread freely in the extracellular matrix. The consequence of this is the recruitment of natural killer cells and other polymorph nuclear cells (PMN) via IL-8 induction. ESAT-6, CFP-10 or ESAT-6 may also drive cell death in macrophages through a TNF-α mediated signaling pathway [76].

Granuloma Formation and Disease Progression

The clearance of mycobacteria involves the formation of a tightly packed epitheloid granuloma consisting mainly of macrophages, macrophage-derived cells (e.g. epitheloid and Langerhans cells) [77], and a smaller percentage of CD4+ T cell, γδT cell, CD8+ T cell [78] and neutrophils [79]. The recruitment of neutrophils aid to organize granuloma [80,81] and support phagocytosis and clearing infected macrophages [82]. Over several weeks, the granuloma tightens, becoming a solid mass within the alveoli effectively walling off the bacteria. The cells in and around the granuloma modulate the immune response to ensure minimal tissue damage [83,84]. These cells also continue to provide cytokines and factors, such as IFN-γ and TNF-α, to maintain the appropriate Th1 response to fight the infection [78,79], as well as IL-17 and IL-22 for the proinflammatory and the inflammatory response [85].

In the case of Mtb persistence, however, there is a dramatic change in the behavior of the granuloma and its role in infection. The exact cause of the change in the granuloma remains unknown; however, Mustafa et al. [86,87] have provided evidence to support the involvement of the Mtb secreted 23-kDa protein, MPT64, in the change. Specifically, MPT64 reduces the level of apoptosis of Langerhans cells in the

granuloma [86] and the reduction of IFN-γ secreting cells while the levels of IL-10, TGF-β and TNF-α is left unchanged [87]. The makeup of the granuloma changes, leading to the modulation of the numbers and/or functions of the following three cell types:

i) Foamy macrophages, which have pockets of accumulated lipid bodies, become increasingly present. These cells are triggered *in vitro* by Mtb compounds, such as oxygenated mycolic acids, and provide a source of nutrition for the bacteria [88]. The presence of these cells is presumably due to an increase in the localized concentration of mycolic acids from bacteria emerging from necrotic macrophages.

ii) The neutrophils, while not changing in number, do change in function and survive longer in the granuloma [89]. This longer life increases the likelihood that a neutrophil will encounter and phagocytose a necrotic cell, allowing amplification of the bacteria [82]. In addition, the presence of the 19-kDA lipoprotein in the bacterial cell wall activates neutrophils [90] and aids in maintaining their activity in the granuloma and continued tissue damage. This activation may facilitate infection by Mtb by maintaining the expression and secretion of inflammatory cytokines [82] and receptors that enable crosstalk with Th17 cells [8,91]. This may well lead to some bacterial death but also enable bacterial dissemination in the surrounding tissue [8].

iii) The number of γδT cells in the granuloma does not change significantly [92,93], however, their signaling does such that they reduce the levels of inflammatory cytokines IFN-γ and IL-22. This may be mediated in part by the stimulation of these cells by the Mtb metabolite (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). Chen [94] has shown that stimulation of Vγ2Vδ2 cells stimulates dendritic cell maturation, increased crosstalk between monocytes and down regulates the formation of T reg cells. The result is continued and amplified inflammation and sustained tissue damage at the localized site of infection.

iv) The contribution of T reg cells in modulating the response to Mtb varies with the time and location of the infection. The level of T reg cells increases at the granuloma [95] and promotes bacterial burden. In active TB, T reg cells down regulate the local inflammatory response and ensure that infection can continue without abatement [54,96,97]. Indeed, the levels of IFN-γ, IL-12, IL-17 and IL-22 are all lower in both latent as well as active TB. This modulation of the local immune response by Mtb is reversible. However, a population of non-granuloma T reg cells can be expanded by IL-2 promoting, Mtb-specific CD4 and CD8 effector T cells [98].

As the granuloma continues to develop in the long term, the level of necrosis also increases primarily at the site of the foamy macrophages [99], which are killed to supply nutrition for the growing Mtb. The necrosis stimulates the infiltration of neutrophils, which continue to cause damage in the lung tissue and themselves become infected by circulating bacteria. In addition, there is a continued rise in IL-10 [87], TNF-α [100] and TGF-β [101], while IFN-γ is unchanged [100]. This suggests that the granuloma, rather than being a localized region of successful antimicrobial activity, is one of Mtb persistence and disease progression.

Modulation of the Immune Response

During initial infection with Mtb, the dendritic cells produce IL-12p70 and IL-23 [102]. IL-23 is a member of the IL-12 family and contains IL-12p40. The presence of IL-12 contributes to the formation of the Th1 response [103], and triggers the Th17 response [104], which is characterized by the production of IL-17, TGF-β, IL-6, IL-1β, IL-21, and IL-23 [105,106]. The Th17 response is involved in the development and maintenance of inflammation in a Th1-independent manner [107], although there are indications that the Th17 response can assist in the maintenance of the Th1 response [108].

Aberrations in the Th17 response have been seen in autoimmunity conditions such as psoriasis, multiple sclerosis and rheumatoid arthritis [109,110]. Regulation of the Th17 response is still being elucidated. Evidence suggests that the Th17 response is enhanced through the self-production of IL-21, as well as the continued production of IL-17 and IL-23 by neutrophils. Th17 response is repressed through the production of IFN-γ, a key factor in the Th1 response [111] and IL-10, which is a primary factor in the development of the regulatory T cells [112-114].

In Mtb infections, the Th17 response has been shown to play a significant role in modulation of either clearance or persistence of the bacterium. In standard bacterial clearance, the Th17 response is induced shortly after infection and is mediated primarily by γδT cells [115]. This Th17 response produces the cytokines (e.g. TNF-α, IL-6, or IL-8 [116]) that recruit inflammatory cells, including neutrophils, to the localized area to assist the macrophages in Mtb elimination and to initiate the formation of the granuloma [117]. Based on studies with *Francisella tularensis*, Th17 may also help promote the Th1 response through the induction of IL-12 [108]. Upon clearance of the bacteria, the Th17 response is down regulated by the involvement of the T reg cells, allowing for proper healing.

Mtb develops a micro-environment where the Th1 response is down regulated and the Th17 response is continually activated leading to increased inflammation, immunopathology and persistence [116]. The continued recruitment of neutrophils resulting from the release of IL-8 from necrotic cells enables a continual supply of both IL-17 and IL-23, the latter of which triggers production of IL-17 from the γδT cells [118]. In addition, the bacterial ESAT-6 protein triggers TLR-2 to down regulate the production of IL-12 and increases the production of inflammatory cytokines, as well as IL-23 ensuring that the Th17 response is maintained wherever the bacterium may be found [119].

Interestingly, the Th17 response may lead to suppression of the multifunctional IL-22. Liang et al. [119] and Scriba et al. [120] showed that Th17 works in cooperation with IL-22 at the onset of infection, but this synergy may change over time. Qiu et al. [121] showed a depletion of IL-22 secretion in CD4+ T cells associated with Mtb infection. Cowan et al. [85] found that both the Th17 response and IL-22 were differentially induced and observed a correlation between down regulation of IL-22 and progression of infection. These results correlate with those of van Hamburg et al. [122], who investigated the effects of the Th17 response on IL-22 in rheumatoid arthritis. As with Cowan et al. [85], the levels of IL-22 were negatively correlated with the progression of disease; an interaction primarily mediated by Th17. This suggests that the Th17 response is modulated over time, such that the initial reaction to infection is one of concentrated and coordinated antimicrobial activity. However, as time passes, the response declines and becomes inflammation-based. The effect of this modulation is systemic, rendering not only the local but also the overall host immunological environment similar to that of autoimmunity.

Alternate Strategies in Vaccine Development

Based on the multitude of studies investigating the immunological response to Mtb infection, there is one consensus point upon which future research and vaccination strategies can build. There needs to be

a proper and balanced response to the initial infection to ensure the clearance of Mtb before effective suppression. The key to this is without a doubt proper and effective autophagy and the activation of bacterial clearance mechanisms through the production of IFN-γ of the Th1 response but also TIM 3+ cells.

The current directions for vaccination strategies focus on the development of improved forms of BCG, or through the use of subunits that are known to be highly immunogenic including Ag85A, Ag85B, ESAT-6, TB10.4, or attenuated cells in liposomes. In all of these cases, the goal is to ensure that any challenge is cleared effectively to prevent the hindering effects seen with infection progression. This opens up the opportunity to explore other avenues that include modulating the Mtb immune response with adjuvants that alters the immune response. These reagents may be used in conjunction with an Mtb vaccine to increase the potential for increased IFN-γ production, while not shifting the balance needed to suppress a cytokine storm.

One possible answer may lie in targeting GTPases. Mtb can prevent the Rab5 and Rab7 dependent process of phagosome maturation leading to the incorporation of both Rab14 and Rb22a in the phagosome membrane. To counter this, drugs targeting phagosome GTPases, as well as the Mtb encoded GTPase, FtsZ, which plays a role in bacterial cell division [123], may offer some additional assistance to therapeutic vaccines. By preventing maturation arrest and bacterial division, the chances for fusion with the lysosome increases, as does the opportunity for the natural process of apoptosis. The issue with such a path, however, is the unknown potential for damage in surrounding areas where Mtb is not present. Moreover, the need for such targets to infiltrate the granuloma is not guaranteed; the measure may work in the general environment to prevent pathogen spread but could be ineffective at reaching the site of greatest concern.

An additional strategy is to modulate the immune response with TLR-2/6 antagonists that specifically upregulate cells expressing T cell immunoglobulin and mucin domain 3 (TIM 3). TIM 3 is expressed on T cells, macrophages, monocytes and dendritic cells. TIM 3 is involved in the Th1 response and the development of T cell tolerance and exhaustion [124], particularly in chronic virus infections. Evidence suggests that TIM 3 plays a role in the prevention or modulation of autoimmunity [125]. In particular, the continued stimulation of TIM 3 has been shown to develop sustained proliferation of Th1 T cells, as well as the production of IFN-γ [124], which may very well overwhelm the proinflammatory response of Th17 cells. TIM 3+ T cells react with the ligand galectin-9 (Gal-9) and are negative regulators of the Th1 effector response [126]. Sada-Ovalle et al. [127] showed that stimulation of this pathway using Gal-9, a listing that recognizes β-galactosidases, stimulates the Th1 response in Mtb infection through the production of IL-1β. This promotes bacterial killing and assists in clearance of the infection. In addition, there was a suppression of bacterial replication, slowing down the potential of the bacterium to supplement its defense. Jayaraman et al. [128] showed that intracellular Mtb bacterial cell growth in infected macrophages expressing Gal-9 responded to TIM 3 +IFN-γCD4+ was reduced. The interaction of the receptor/ligand leads to macrophage activation and increases bactericidal activity. The simultaneous induction of caspase-1 and secretion of IL-1β was observed, leading the authors to suggest TIM 3 has evolved to inhibit growth of intracellular pathogens via its ligand Gal-9, which in turn inhibits expansion of effector TH1 cells to prevent further tissue inflammation [128]. To harness these responses, the natural ligand Gal-9 can be added. However, it is already known that Mtb infected macrophages express the Gal-9 ligand. A vaccine approach would be to add specific TLR-2 agonist with a subunit vaccine to promote CD4+ TIM 3T cells that would activate. Kleinnijenhuis et al. [129] showed that in a mouse model of Mtb, TLR-2/6 agonist leads to the production of the pro-inflammatory cytokines IL-1β and activation of caspase-1. TLR-4, 9 and 1 receptors were ineffective in inducing these pro-inflammatory cytokines that would aid in reducing the bacterial burden. New families of these agonists for TLR-2 have been recently described [130] and could be easily synthesized and linked to subunit Mtb vaccines.

A final consideration in the development of vaccines is to focus on IL-22, which has demonstrated its importance in the development and maintenance of mucosal immunity [43]. IL-22 is produced by a number of different cell types including neutrophils [85], NK and NKT cells [131], lymphoid tissue inducer cells, and innate lymphoid cells [132] and T cells, including a subset called Th22 cells. These latter cells are described as CD4+ T cells that produce IL-22 with minimal to no expression of IL-17 in response to IL-6 [133]. The cytokine is recognized by a dimer of the IL-22R1 and IL-10Rβ receptors restricting its effect to innate cells such as epithelial, mesothelial and myofibrolastic cells [131].

Based on previous work in mice and humans [44,120,134-136], IL-22 stimulation leads to increased: i) expression of inflammatory and defense chemokines including CXCL1, CXCL5 and CXCL9; ii) expression and secretion of IL-6 and G-CSF; iii) release of defensins and other antimicrobial peptides; and iv) tissue repair through the Bcl-2 pathway. In the lung, IL-22 improves lung barrier function and cellular survival as well as repair through proliferation [137,138]. Moreover, IL-22 is necessary for the prevention of lung fibrosis [139]. In terms of Mtb infection, while the mechanisms continue to be elucidated, Dhiman et al. [140,141] established a role for IL-22 expressing NK cells, specifically through the enhancement of phagosome-lysosome fusion. In addition, Qiao et al. [142] showed that the Th22 response is primed by both ESAT-6 and CFP-10 in Mtb infection leading to increased IL-22 and IFN-γ production, yet no increases in IL-17 were observed. Cowan et al. [85] also showed that granulocytes from active TB patients did not express IL-22 when compared to latent and healthy controls.

With respect to the Th22 response, Eyerich et al. [134] showed that Th22 cells are relatively stable in the presence of Th1, Th2, Th17 and T reg cells. In the case of Mtb infections, this stability may be compromised. Th22 cells have been associated with driving mucosal [133] as well as skin immunity [143] and play a role in the response to mucosal intracellular pathogens. Induction of Th22 cells develops not only the production of IL-22, but also IFN-γ by CD4+TIM 3+, cells both of which are known to help stimulate proper intracellular bacterial killing [138]. Using IL-22 and promoting the Th22 response may offer promise in terms of their antimicrobial activity, and their role in the protection of the localized epithelial and endothelial environment may offer a tipping point in favour of bacterial clearance. Possibly the use of vaccines with TLR-2 agonist with a subunit vaccine may promote not only a Th1 response (IFN-γ) that are CD4+TIM 3+ but also a subpopulation of CD4+TIM 3+cells producing IL-22.

The Th22 subset works in combination not only with immunological cells but also with epithelial cells including mesothelial cells [138], which are a known target of Mtb in the exacerbation of disease [144]. Th22 cells help repair Mtb-damaged cells, improve the epithelial barrier [145] and hinder the ability of Mtb to escape the localized environment. In concert with the improved efficacy of intracellular killing, Th22 cells may provide a valid counter to the continued inflammatory response that drives Th17. In that regard, one potential avenue to follow may be

a combined vaccine comprising not only BCG, but also ESAT-6, CFP-10, vitamin D and TLR 2 agonist. For example, Colin et al. [146] have investigated the effect of vitamin D supplementation in rheumatoid arthritis [146] and found that Th17 was suppressed while Th22 was enhanced. In the context of Mtb, a vaccine would be able to promote the Th22 response and overcome the Th17 response. Further research will be necessary to identify appropriate target mechanisms and biomarkers of Th22 induction for optimized bacterial killing.

Conclusion

The development of an effective vaccine against tuberculosis is a major challenge in research today. Over the last two decades, much has been learned about the immune response to Mtb both ineffective clearances of the pathogen and in the development of disease, which often continues unabated. The results of these studies, while complex and at times contradictory do offer a clearer path towards a vaccine that will be effective. As seen in this review, the major hurdles of any Mtb vaccine option are the prevention of the initiation of infection, subsequent resistance to clearance, and long-term persistence. While Mtb has several mechanisms to abate killing at various stages of the infection, potential options may exist to subvert the infection by Mtb by focusing and altering the immune response to Mtb at the different stages of the disease process.

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