



Advancing Adjuvants for *Mycobacterium tuberculosis* Therapeutics

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Tuberculosis (TB) remains one of the leading causes of death worldwide due to a single infectious disease agent. BCG, the only licensed vaccine against TB, offers limited protection against pulmonary disease in children and adults. TB vaccine research has recently been reinvigorated by new data suggesting alternative administration of BCG induces protection and a subunit/adjuvant vaccine that provides close to 50% protection. These results demonstrate the need for generating adjuvants in order to develop the next generation of TB vaccines. However, development of TB-targeted adjuvants is lacking. To help meet this need, NIAID convened a workshop in 2020 titled “Advancing Vaccine Adjuvants for *Mycobacterium tuberculosis* Therapeutics”. In this review, we present the four areas identified in the workshop as necessary for advancing TB adjuvants: 1) correlates of protective immunity, 2) targeting specific immune cells, 3) immune evasion mechanisms, and 4) animal models. We will discuss each of these four areas in detail and summarize what is known and what we can advance on in order to help develop more efficacious TB vaccines.

Keywords: tuberculosis, vaccines, adjuvants, M72, BCG, TLR, Th17, CD4+ T cells

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), an infectious disease that led to the death of 1.4 million individuals in 2019 (1). The current licensed vaccine against TB, a live attenuated strain of *Mycobacterium bovis* known as Bacillus Calmette-Guérin (BCG), is able to provide protection against disseminated forms of disease but is ineffective at providing protection

against pulmonary TB in children and adults. Therefore, in order to lessen TB burden worldwide, a more efficacious vaccine and improved vaccine delivery strategies are urgently needed.

The development of more robust TB vaccines has unique requirements that have made progress challenging. Vaccines traditionally provide durable protection by prophylactically inducing neutralizing antibodies that can serve as a first line of defense against pathogens (2). Although numerous pathogens require antibodies for protection, this is less clear in the case of TB. While research on the role of antibodies in TB is ongoing and is of interest due to new data (3), no evidence has yet to suggest that neutralizing antibodies are required for protection against Mtb. Therefore, TB vaccine research has focused on identifying antigens and delivery strategies that maximize the generation of T cell responses. In particular, research has shown that CD4⁺ T cells are a critical component of protective immunity. Mouse studies, however, have demonstrated that T cells are not recruited to the lungs until weeks after infection is established (4). Mtb is able to suppress T cell recruitment and responses by utilizing several immune evasion mechanisms (IEM) to impede antigen-presenting cell (APC) function, thereby dampening adaptive responses. Researchers have attempted to bypass the delayed T cell response by targeting specific APCs such as dendritic cells (DCs), but there is limited evidence that this can preclude the immunosuppressive effects of Mtb upon challenge (5, 6).

Despite these challenges, recent developments have reinvigorated interest in TB vaccine research. A study in humans found that BCG revaccinated adults have increased protection compared to control groups (7). Moreover, while BCG is administered through the intradermal route, a recent study found that administering BCG intravenously can induce robust T cell responses and afford non-human primates (NHPs) protection against Mtb challenge (8, 9). Recent clinical trials of novel vaccine candidates have also yielded promising results. The Phase IIB clinical trial results of the M72/AS01_E subunit adjuvanted vaccine demonstrated 49.7% protection against Mtb (10). M72 is a recombinant fusion protein consisting of antigens Mtb32A and Mtb39A and AS01_E is an adjuvant that combines monophosphoryl lipid (MPL) with QS-21 (a purified saponin fraction). The AS01 adjuvant system success extends to other infectious disease vaccines as it is used in the FDA

approved shingles vaccine “SHINGRIX” and is currently in clinical development for use in malaria (11). These successes set an important threshold for the clinical development of future TB vaccines.

To develop effective antigen-specific T cell responses, host immunity requires non-specific innate cell activation. In the case of a subunit vaccine, priming is accomplished with adjuvants. Modern adjuvants prime host immunity through binding of receptors that recognize pathogen-associated and damage-associated molecular patterns, such as toll like receptors (TLRs), C-type lectin receptors (CLRs), and NOD like receptor (NLRs) on the surface of APCs. Adjuvants are able to activate these receptors and induce downstream signaling pathways such as NFκB signaling, which in turn enables the activation of adaptive immune responses. Given the expense and time-consuming nature of vaccine development, adjuvant compounds and formulations that have been shown to be safe and effective are often repurposed for testing novel vaccine candidates. The majority of current TB vaccine candidates contain adjuvants (**Table 1**). However, a TB-specific adjuvant that is able to induce strong immune responses in the lung but minimize corresponding tissue damage is required. Adjuvanted vaccines delivered directly to the upper or lower respiratory tract may have increased efficacy compared with parenterally administration against respiratory pathogens, such as Mtb. Therefore, development of new adjuvants with defined modes of action will be necessary in order to generate improved vaccination strategies that can provide protective immunity to TB.

To address the needs of TB vaccine development and lack of TB-targeted adjuvants, the National Institute of Allergy and Infectious Diseases (NIAID) held a workshop in July 2020 titled “Advancing Vaccine Adjuvants for *Mycobacterium tuberculosis* Therapeutics.” This workshop brought together vaccine and adjuvant developers from industry and academia and researchers in fields beyond TB. It is here that we identified research in four areas that will be essential for development of optimal adjuvants for TB vaccines: 1) correlates of protective immunity, 2) targeting specific immune cells, 3) immune evasion mechanisms, and 4) animal models. In this review article, we discuss these four areas in detail and highlight priority areas which the broader TB research community can address in order to develop efficacious adjuvants and vaccination strategies.

TABLE 1 | Adjuvants Used in Recent TB Vaccine Candidates.

Adjuvant/Vaccine	Antigen	Adjuvant target	Immune response	Ref
IC31	H56, H1	TLR-9, endocytosis	Th ₁	(12–14)
AS01 _E	M72	TLR-4, lysosomal disruption	B, Th ₁ , Th ₂ , NK, CTL, DC	(10, 15–20)
GLA-SE	ID93	TLR-4	Th ₁	(21–25)
BCG	Whole cell		Trained innate immunity, Th ₁	(8, 26–31)
MVA85A	Ag85A		Th ₁	(32)
CAF01	H1	Mincle	Th ₁ , Th ₁₇	(33–36)
Advax-CpG	CysVac2	TLR-9	Th ₁₇	(33, 37, 38)
Lipokel		TLR-2	DC	(39)

Table lists of adjuvant-containing TB vaccine candidates that have recently been tested clinically in humans and pre-clinically in animal models. Known adjuvant targets are presented though other mechanisms of action may also be employed. Abbreviations: AS01, adjuvant system; GLA-SE, Glucopyranosyl Lipid A-stable oil-in-water nano-emulsion; BCG, Bacillus Calmette-Guérin; MVA85A, Modified vaccinia Ankara expressing antigen 85A; CAF, Cationic Adjuvant Formulation; TLR, toll-like receptor, Th, T-helper cell; NK, natural killer cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell.

CORRELATES OF PROTECTIVE IMMUNITY

Polarisation of CD4⁺ T Cells

Targeting specific immune cells through passive or active vaccine modalities requires understanding the basic mechanisms of protective immunity to Mtb. Although thorough understanding of protective immunity to Mtb remains incomplete, research has shown that conventional T cells (particularly CD4⁺ T-helper [Th] cells) play a vital role in protection (40). Early mouse studies and clinical data demonstrated that functional Th₁ CD4⁺ T cells are crucial for protection against Mtb (41–45). Th₁ immunity is characterised by the secretion of IFN- γ , which aids in microbial clearance by enhancing processes such as phagocytosis and secretion of reactive oxygen species in macrophages (46). While these responses have long been a major focus of TB vaccine design, studies have shown that there is a requirement for broader immunity as Th₁ responses alone are insufficient for protection (47). The MVA85A vaccine trial in humans demonstrated that despite robust induction of ‘multifunctional’ CD4⁺ T cells (producing IFN- γ , TNF and IL-2 cytokines), these immune responses did not translate to additional protection from TB compared to placebo vaccination (32). Some studies even found that excessive Th₁ polarisation may hinder effective memory responses by producing terminally differentiated T cells that are unable to effectively migrate into the lung parenchyma during Mtb infection (48, 49). More recent studies demonstrated that IL-17 and Th₁₇ responses, in addition to Th₁ responses, are necessary for protective immunity to Mtb (50, 51). Th₁₇ cells have the capacity to differentiate into resident memory T cells while IL-17A is a key cytokine required for protection in several pre-clinical models of candidate TB vaccines (33, 34, 37). Th₁₇ cells can also secrete additional cytokines that direct recruitment of neutrophils and IFN- γ -producing protective memory CD4⁺ Th₁ cells during Mtb infection (52, 53). Other studies have also found that CCR6⁺CXCR3⁺ Th₁/Th₁₇ cells responses were present in latently-infected individuals (compared to active infection) and were important for protection in an NHP model of TB (54, 55). But while Th₁₇ responses are beneficial, excessive Th₁₇ responses are detrimental to the host (56, 57). Therefore, adjuvants for TB vaccines should strive to induce early and balanced Th₁/Th₁₇ as these are more likely to be necessary for protection.

Other Lymphocytes

While there is evidence that CD8⁺ T cells contribute to protection against Mtb, their role remains debated due to variable findings and inherent differences in human and NHP immune function compared to mice. CD8⁺ T cells can produce cytokines such as TNF, IFN- γ and IL-2 and produce cytolytic granzymes, a feature not shared by CD4⁺ T cells. Of these key cytolytic granzymes is granzysin, which is expressed in human but not murine CD8⁺ T cells, and is capable of direct mycobacterial killing (58). CD8⁺ depletion studies in animal models, however, agree on the role of these T cells in protection. In NHPs, CD8⁺ depletion reduces the protective efficacy of BCG

vaccination and infection-induced immunity (59). CD8⁺ knockout mice are also unable to contain Mtb infection, particularly at extended timepoints, suggesting a role for CD8⁺ T cells in protection during chronic stages of infection (60).

Similarly, the function of B cells and humoral immunity in protection against TB has become of particular interest as broader immune parameters are investigated (61). Recent studies indicate the presence of antibodies in humans that are protective against Mtb infection (3, 62). The use of alternative vaccination routes, such as mucosal or intravenous, lead to the generation of pulmonary IgA and antibody-producing lymphoid follicles (iBALT), which has been associated with reduced bacterial burden (8, 26, 33, 37). The generation of inducible lymphoid structures is crucial as they can harbour cells such as CXCR5⁺ CD4⁺ T cells which were found to correlate with a better prognosis of TB disease (63, 64).

While not previously a focus of TB vaccine candidates, CD8⁺ T cells and B cells are often measured as a readout of adjuvant function and may be the contributing factors to vaccine-induced protective immunity (6). The mechanism of the AS01 adjuvant, as determined by pre-clinical animal studies, is thought to be due in part to early IFN- γ production by NK and CD8⁺ T cells (15). In humans, however, a review of TB vaccine candidates tested in the clinic revealed that CD8⁺ T cell responses were relatively poor when compared to CD4⁺ T cells responses (65). Until vaccine efficacy studies in humans become more available, the contributions of B and CD8⁺ T cells cannot be discounted and should be considered in adjuvant development.

Lung-Localised Immunity

Research has suggested that generating immune responses at the site of infection is crucial for protective immunity. As Mtb spread *via* the aerosol route, it can use lung-specific cell types to its advantage. The microfold cells found in the nasal associated lymphoid tissue (NALT) and iBALT have been identified as the entry site of the bacterium and granulomas can serve as a niche for the persistence of Mtb (66–68). The need for lung-localized immunity is supported by the fact that local immune cells can respond quickly to infection. The generation of T resident memory (T_{RM}) cells is a correlate of protection against Mtb and is an active target in mucosal vaccination strategies (33, 37, 69). Mucosal adoptive transfer of T_{RM} cells from BCG-vaccinated mice into naïve mice revealed that both CD8⁺ and CD4⁺ T_{RM} subsets could afford partial protection against Mtb infection (26). Furthermore, both CD4⁺ and CD8⁺ human T_{RM} cells have been characterised as capable of limiting intracellular Mtb survival *ex vivo* (70). A recent study using human samples also found that the frequency of Mtb-specific T_{RM}-like cells that produce IL-17 in the lungs negatively correlated with IL-1 β levels in the blood, suggesting an important role for controlling Mtb growth (71). Additionally, mucosal vaccination could help activate other resident cells, such as $\gamma\delta$ -T cells and mucosal-associated invariant T cells (MAIT), that also produce IL-17A (53). While the generation of lung-localized immunity appears to be a significant correlate of protection against Mtb, the challenge remains to validate the safety and efficacy of novel

administration methods that generate lung-local immune memory in clinical trials.

Other Correlates of Protection

Interest in trained immunity as a potential correlate of protection has been driven by the hypothesis that it may be responsible for some of the protective characteristics of BCG. This is supported by studies demonstrating that BCG is able to provide protection against multiple respiratory diseases in addition to TB (27), and the observation that some individuals are capable of early clearance of Mtb without requiring an adaptive immune response (72). While there are multiple vaccine candidates that have been reported to be capable of stimulating systemic innate immune responses, there is emphasis on the generation of lung-resident trained immunity which has been observed after pulmonary and intravenous vaccine administration (6, 28).

Certain cytokines, in addition to those secreted by T cells, also play critical roles in protection. In particular, IL-23 expression was found to be essential for IL-17A-mediated responses against Mtb. Upon aerosol infection with Mtb, naïve murine lungs showed increased expression of IL-17A, which was ablated in the absence of IL-23 (73). IL-23, and to a lesser extent IL-17A and IL-22, is able to lead to CXCL13 production and generation of lymphoid follicles (63). Other studies reported that unvaccinated IL-23-deficient mice are still able to control mycobacterial growth in a fashion similar to wild type animals after exposure to Mtb and BCG as local IFN- γ responses are able to compensate for the loss of IL-17A (73–75). IL-23 plays a compensatory role in the absence of IL-12p70, a key Th₁ polarising factor, as the addition of IL-23 can also enhance protective immunity against Mtb in the absence of a functional Th₁ immune response (73, 76). Thus, there is evidence that IL-23 plays a significant role in protective responses which will be important for vaccine-induced immunity.

Clinical Studies

Insights from clinical trials can help address the knowledge gaps of efficacious TB vaccine design by helping identify correlates of protection. A small number of TB vaccines are currently undergoing clinical trials, including three vaccines that incorporate adjuvants. The most advanced of these TB vaccines is the M72/AS01_E construct. The MPL components of the adjuvant, a derivative of the lipopolysaccharide from *Salmonella minnesota*, is commonly used in adjuvant formulations due to its ability to engage TLR4, activate NF- κ B, and induce pro-inflammatory cytokines (16, 17). QS-21 can cause lysosomal disruption and consequent Syk activation, as well as NLRP3 inflammasome activation, which is thought to enhance cross-presentation with CD8⁺ T cells and promote inflammatory cytokine production (18, 19). Preclinical studies suggest that the Th₁ polarising effects of AS01 are the result of MPL and QS-21 synergy. At early timepoints post-vaccination, it was observed that subcapsular sinus macrophages (SSM) in the draining lymph nodes promoted early IFN- γ production by resident NK cells and CD8⁺ T cells in a process mediated by IL-18 (15). In humans, the peripheral immune response has been examined by analysing blood RNA expression and antigen-

specific PBMC profiles during a two dose M72/AS01_E regimen (77). PBMC restimulation showed that the vaccine induced CD4⁺ T cells and multifunctional T cells after stimulation, though IL-17A was not detected. RNA analysis identified the upregulation of blood transcription modules associated with IFN signalling, innate activation including TLR and inflammatory signalling, as well as modules related to various chemotactic and cell adhesion processes.

The cationic peptide adjuvant IC31 is a component of two TB vaccine candidates undergoing clinical trials, H4:IC31 and H56:IC31 (7, 12). In a Phase IIB trial, which tested the efficacy of H4:IC31 and BCG revaccination, H4:IC31 did not demonstrate significant protection (30.5% efficacy) against either initial or sustained Mtb infection (7). However, BCG revaccination led to a 45.4% reduction in sustained infection (7). IC31 is made up of the antimicrobial peptide KLKL₅KLK (KLK) combined with ODN1a, a TLR9 binding single stranded oligodeoxynucleotide (ODN) that activates the MyD88 pathway (13). The cationic peptide component is also an immunostimulant, hypothesised to enhance intracellular TLR access of ODN1a *via* stimulating endocytosis (14). H56:IC31 induces antigen-specific IgG responses and Th₁ cytokine expressing CD4⁺ T cells (12). Low-dose vaccine administration induced more polyfunctional memory T cells than high dose vaccination, an observation in line with pre-clinical studies that identified lower antigen dose as conducive to more protective immune responses (78).

Comparative analysis of human immune responses to six TB vaccine candidates observed that a shared feature of the systemic immune responses induced by TB vaccine candidates was the enhanced production of IFN- γ expressing CD4⁺ T cells, with M72/AS01_E inducing the greatest response (65). Furthermore, little to no IL-17A expression was induced by the candidate vaccines. This study did not include analysis of CAF01, a cationic liposomal formulation consisting of DDA liposomes and the Mincle agonist trehalose-6,6-dibehenate (TDB), which is known to initiate a Th₁₇ response when administered parenterally in mice (79). Vaccination of humans with H1-CAF01, however, induced strong antigen-specific Th₁ responses while IL-17 responses were low and were not significantly increased (35). This may reflect a requirement to examine mucosal immune responses in humans to better reflect vaccine immunogenicity, as was performed with the CTH522/CAF01 chlamydia vaccine candidate (80). Overall, there was a lack of diversity in T cell responses generated by the different TB vaccine candidates, reinforcing the argument for a requirement to develop and test more novel adjuvants that induce distinct immune responses.

Adjuvating Strategies for Inducing CD4⁺ T Cells

Differential receptor activation, with adjuvants, have been demonstrated to be important for dictating specific T cell responses. Th₁ differentiation *via* TLR activation and downstream IL-12 secretion is believed to play a primary role in the protective efficacy of TLR4-targeting adjuvants such as AS01 (15). Similarly, the major driver of immunogenicity of the vaccine candidate ID93 + GLA-SE vaccine is the synthetic TLR4 agonist, GLA; its delivery in a squalene emulsion is also necessary

for adjuvanticity (21). The Th₁ polarising properties of GLA-SE is MyD88- and TRIF- dependent, and type I and II IFN expression is also critical for the adjuvant mode of action (21, 22). Furthermore, IL-18 and Caspase1/11 expression is required for T cell activation by GLA-SE, but not the NLRP3 inflammasome (23). In humans, ID93 + GLA-SE vaccination results in the generation of multi-cytokine producing T cells (TNF, IFN- γ and IL-2), with little IL-17A detected, and IgG1 and IgG3 antibody production (24, 25). Thus, both MPL adjuvant (in AS01) and GLA (in a squalene emulsion) have demonstrated the ability to enhance T cell immunity to Mtb in combination with different antigens, mainly through TLR4-mediated Th₁ polarisation.

Some adjuvants are innately capable of stimulating Th₁₇ polarisation, often by activating non-TLR pattern recognition receptors such as Mincle (81). It is known that some TLR4 and TLR7/8 agonists can induce IL-23 expression, and Mincle-activating adjuvants such as TDB, used in the cationic liposome formulation CAF01, can also shift the balance towards IL-17A producing T cells (36). It is thought that TDB, a derivative of the mycobacterial cord factor (TDM), is the major contributor to the Th₁₇ polarisation in this vaccine formulation. Mincle activation, as well as MyD88 and the inflammasome component ASC, have all been identified as a requisite for the Th₁₇ generating characteristics of TDB (79, 82, 83). Thus, there are novel adjuvant strategies focused on generating synthetic aryl-trehalose derivatives that afford the best Th₁ and Th₁₇ polarisation (84, 85). Similarly, it has been observed that adjuvants such as cyclic dinucleotides and chitosan that activate the cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) pathway also stimulate Th₁ and Th₁₇ responses (86–88). As mentioned above, mucosal delivery is another effective strategy for the generation of Th₁₇ responses, with many vaccines displaying enhanced Th₁₇ polarisation upon mucosal vaccination that was not observed with parenteral administration. Evaluation of these new Th₁₇-inducing adjuvants alone and in combination with other established adjuvant systems in relevant animal models is a critical next step in the advancement of improved vaccination strategies for Mtb.

In summary, data supports the development of adjuvants and vaccines that elicit both local (tissue resident) and systemic antigen-specific Th₁ and Th₁₇ cells as these responses have been demonstrated to be critical for protection. However, research should continue to elucidate correlates of protection to identify new pathways that can be targeted by adjuvants to induce protective immunity against Mtb.

TARGETING SPECIFIC IMMUNE CELLS

Active or passive targeting of specific immune cells through vaccination is an important step in the development of a safe and effective vaccine for TB. Mtb is primarily transmitted *via* inhalation and establishes infection in the lung through phagocytic uptake of the bacilli by tissue-resident

macrophages. The initial recognition and immune activation by innate immune subsets sets the stage for either clearance or persistent containment within a granuloma or active disease through suppression of the immune system (89). Cells involved in lung-specific innate and adaptive immune responses are important frontline targets for Mtb vaccination approaches. The majority of Mtb vaccines are delivered *via* the intramuscular (such as M72/AS01_E) or intradermal (BCG) route, creating additional challenges in the recruitment of tissue resident memory cells to the site of initial infection. Targeting vaccines to specific cell types could help to overcome some of the shortfalls of current vaccine approaches while improving both safety and efficacy. Several groups have worked to overcome these challenges through targeting specific immune cells *via* passive (adjuvants, delivery systems) or active (mucosal vaccination, prime-pull, receptor targeting) immunization strategies with great success in pre-clinical and early clinical investigations. Coordination of the innate and adaptive immune response is important for resolution of Mtb infection and targeting of specific immune cells that orchestrate this response at the site of infection is critical in the development of an effective vaccine.

The context in which antigens are presented to the immune system controls the immunological outcome of antigenic exposure (90). The innate immune system uses pathogen recognition receptors (PRRs) to decode the nature of the antigen (*e.g.*, viral, bacterial, fungal) and to translate this into an appropriate adaptive immune response. Central to the idea of cell-targeting is activation of the innate immune response by pathogen associated molecular patterns (PAMPs) and the specific cellular targets they encounter are crucial for early control of infection and for the subsequent development of protective long-term adaptive immunity. The use of passive and active cell targeting strategies opens the door towards the rational design of vaccines for Mtb that could lead to more durable and protective mediated immune responses.

Cell Targeting *via* PRR Expression and Adjuvant Use

To drive the desired adaptive immune response, the innate immune response must be properly activated to provide the correct signals for differentiation of antigen-specific T and B cells. Different adjuvants activate specific cell types as a result of differential PRR expression. The directed, rational use of adjuvants is therefore one way in which specific innate immune cells can be targeted in the context of Mtb vaccination. Many adjuvants being explored pre-clinically or in early clinical trials for Mtb vaccines target and activate DCs. In the context of Mtb, several studies have shown that directly targeting DCs can lead to protective immune responses during infection (5, 91–93). As previously discussed, MPL of AS01 activates TLR4 (15). DCs express high levels of TLR4 and are highly responsive to MPL, driving downstream antigen-specific T cell differentiation (11, 20). Both myeloid DCs (mDCs) from blood and monocyte-derived DCs (moDCs) express TLR4 and are able to upregulate co-stimulatory markers and secrete Th₁ polarising cytokines (in particular IL-12p70) after TLR4

stimulation (94). DCs also express high levels of TLR2 (94). Adjuvants that engage TLR2 on DCs have the potential to augment early production of pro-inflammatory cytokines such as IL-6, IL-1 β , TNF- α and IL-12 that are necessary for generating balanced Mtb-specific Th₁/Th₁₇ responses (95, 96). This is demonstrated by studies using Lipokel, an adjuvant which stimulates TLR2 through binding of the ligand Pam2Cys. A protein-Lipokel vaccine conjugate was able to reduce Mtb CFU in the lungs of mice and increase the frequency of DCs in lymph nodes following vaccination (39). PRR-targeting adjuvants, including some TLR agonists, can also preferentially target and activate monocytes and macrophages in addition to DCs. Monocytes and macrophages are specialized phagocytic cells that are capable of interacting with and activating antigen-specific T cells (97). TLR2, TLR4, and TLR8 are highly expressed on monocytes (98). In addition to activating DCs, AS01_E was also able to activate SSM innate immune cells (15). The ability of AS01_E to target both DCs and monocytes/macrophages concurrently may contribute to success as an adjuvant in vaccine strategies for a variety of pathogens, including Mtb. Additionally, non-TLR PRR agonists, such as Mincle or Dectin-1 ligands, preferentially target monocytes and macrophages due to their high Mincle expression (99). CAF01 has demonstrated efficacy as an Mtb vaccine adjuvant in pre-clinical mouse models and induces a strong Th₁₇ polarised immune response in mice (36). Interestingly, when used as an adjuvant in human clinical trials, CAF01 elicited an antigen-specific Th₁ response instead of a Th₁₇ response (35). TDB, the main component of CAF01 believed to be responsible for Th17 polarisation, does not appear to be a particularly potent human Mincle agonist relative to its potency in mice, therefore it is possible that more potent human Mincle agonists may be required to elicit antigen-specific Th₁₇ immunity (84). Given the complex nature of various PRRs and their cellular targets in natural Mtb infection and resolution, it is likely that a combination adjuvant and delivery system approach (similar to AS01) and innovative antigen design may be necessary to improve vaccine efficacy. Promising approaches include combining CLR/TLR adjuvants (presented at this workshop) and other novel antigen and adjuvant combination approaches, such as CysVac2/Advax^{CPG}, have shown promise in pre-clinical models (38).

Cell Targeting *via* Mucosal Administration

Most vaccines are injected into muscle although BCG is given as an intradermal injection. However, mucosal immune subsets that have the ability to quickly transport antigen to stimulate an immune response may be important targets for mucosal Mtb vaccines (66, 100). T_{RM} can be induced by mucosal vaccine administration (9, 26, 101, 102). Following mucosal vaccine administration or natural infection, lung resident T cells acquire a polyfunctional phenotype and are more likely to reside in the airway lumen and lungs, where they can rapidly respond to Mtb (40, 103–106). These lung-resident T cells are not typically generated as a result of parenteral vaccination, and it seems likely that mucosal or lung-resident innate cells are crucial for the development and differentiation of Mtb-specific

lung-resident T cells. These important innate immune effector cells can be uniquely targeted *via* intranasal and/or intrapulmonary vaccination. Several groups have explored a “prime-pull” strategy which takes advantage of both parenteral vaccine (prime) and mucosal vaccine boost (pull) to direct antigen-specific T_{RM} to the lungs (107, 108).

There are a small number of mucosal vaccines currently in clinical use and all but one (FluMist, intranasal) are delivered orally (109, 110). However, there are many preclinical adjuvants that have been tested for either intranasal or intrapulmonary administration, with a particular bias towards intranasal administration due to improved safety profile compared to intrapulmonary administration and lower requirement for specialised equipment (111, 112). Particulate adjuvants such as carbohydrate or PLGA have also been used extensively for mucosal vaccination, often chosen for their mucoadhesive properties (113). Naturally-derived carbohydrate particles such as delta inulin (Advax), chitosan, and *Bacillus subtilis* spores have all been utilised as mucosal adjuvants in TB vaccine candidates, all producing a Th₁/Th₁₇ phenotype alongside increased pulmonary IgA (37, 114–116). CAF01 has been tested intranasally and has also been spray-dried for intrapulmonary administration (107). Th₁₇ immunity is often achieved *via* mode of administration, as it has been observed across multiple vaccine platforms that mucosal vaccination, particularly intranasal, favours Th₁₇ differentiation (117, 118). Many adjuvants have the capacity to be Th₁ or Th₂ polarising when administered parenterally but promote Th₁₇ differentiation when delivered mucosally (37, 119, 120). Some TB vaccines, such as BCG or the CysVac2/Advax candidate, have been observed to be more protective after intranasal or intrapulmonary administration, a quality attributed to local IL-17 production and the establishment of T_{RM} (37, 119). This was observed when the clinical candidate vaccine ID93 + GLA-SE was administered through a different route; parenteral administration induces a Th₁ responses but intranasal administration induces a Th₁₇ responses (121).

Receptor-Mediated Active Cell Targeting

DCs and macrophages are a primary focus of vaccine design due to their role as APCs and critical function in orchestrating long-term cell-mediated immunity. The tissue heterogeneity in both DC and macrophage populations creates challenges for both passive and active targeting of the various systemic and tissue-specific APC subsets. Active targeting to specific APC subsets through endocytic receptors is a promising approach to improve vaccine efficacy and reduce unintended effects (122). Such receptors include DEC-205, Clec9A, Clec12A, and DC-SIGN, among others (122–126). Early work demonstrating active targeting by DEC-205, a cell surface receptor involved in the uptake of dying cells and cross-presentation of antigens, led to the evaluation of an anti-DEC-205-Ag85B vaccine conjugate for Mtb (125). While strong Ag85B-specific humoral immunity was noted following vaccination, cell-mediated immunity was lacking without BCG priming of vaccinated mice and the vaccine failed to improve protection from Mtb challenge (125). A similar approach was used to target DC-SIGN; anti-DC-SIGN

antibodies conjugated to Ag85B were used to vaccinate an hSIGN transgenic mouse (human DC-SIGN under control of the murine CD11c promoter) in combination with various adjuvants. This innovative vaccine design induced strong antigen-specific CD4⁺ T cell responses. However, similar to the DEC-205 approach, enhanced protection from Mtb challenge was not achieved (126). Improved humoral and/or cell-mediated immunity to Ag85B was measured using both endocytic receptor APC targeting strategies demonstrating proof-of-concept for improving immunity using this approach. Additional research efforts are required to identify the appropriate combination of antigen(s), targeting mechanisms, and adjuvants to drive durable immunity and protection in Mtb animal challenge models.

Targeting Cell Specific Responses Through Trained Immunity

In the previous section, we briefly discussed the potential of trained immunity to serve as a correlate of protection. The contribution of trained immunity to vaccine-mediated protection against Mtb is currently being investigated (127, 128). PRR agonists, among other factors, can drive functional and epigenetic reprogramming in innate immune cells in order to increase Th-polarising cytokines and phagocytic and cytotoxic killing capacity. BCG, when administered intravenously, provides both short and long-term protection from subsequent Mtb challenge in NHPs (8, 28–31). A recently discovered mechanism of protection by intravenous BCG is the generation of trained immunity in hematopoietic stem cells (HSCs). Following intravenous administration, BCG is able to enter the bone marrow (BM) where it can be detected for up to 7 months after vaccination in mice (which is not observed in subcutaneous vaccination) (28). Once in the BM, BCG promotes myelopoiesis and induces trained monocytes and macrophages (28). These BM monocytes have a particular transcriptional and epigenetic program, allowing them to differentiate into trained lung macrophages and mount a rapid protective response against Mtb challenge (28). A similar trained immunity effect on HSCs was found through the use of β -glucan, a Dectin-1 agonist, administered *via* intraperitoneal injection (129). Subsequent work showed that mice with β -glucan-induced trained HSC-derived monocytes and macrophages were significantly better protected from Mtb challenge (130). Therefore, targeting trained immunity through vaccination will require unique adjuvants capable of eliciting trained lung resident macrophages either locally or through HSC-derived cells. Data suggests this may be accomplished through the use of live-attenuated vaccines with endogenous adjuvants (*e.g.*, BCG) or exogenous adjuvants in combination with a subunit vaccine (*e.g.*, β -glucan or other CLR agonist). Additionally, it may be possible to elicit trained immunity specifically in lung macrophages and DCs *via* mucosal vaccination routes (131).

In summary, using adjuvants that activate PRR and other key receptors on innate immune cells and are administered through the mucosal route hold the most promise in inducing adaptive immune responses. Additionally, being able to generate adjuvants that activate processes such as trained immunity could be beneficial for inducing protective immunity to TB.

IMMUNE EVASION MECHANISMS

An important reason for the poor efficacy of natural immunity to Mtb or BCG vaccine-induced immunity is immune evasion mechanisms that lead to ineffective crosstalk between innate and adaptive immunity (95, 132). By identifying critical Mtb factors that prevent optimal innate immune responses and elucidating the molecular basis for how host pathways are subverted by Mtb, we can engineer vaccines that target specific pathogen and host pathways to improve the immunogenicity and efficacy.

Mtb has evolved multiple strategies to evade innate immunity and impede T cell responses. By impairing DC functions (*i.e.*, co-stimulation, cytokine production and antigen presentation) during early stages of infection in the lung, Mtb induces delayed and suboptimal antigen-specific T cell responses that fail to eradicate infection or provide lasting protection (4, 133–136). Vaccine strategies need to augment the induction of additional Mtb-specific T cell subsets, such as Th₁₇ cells, that will work in concert with Th₁ responses to enhance protective immunity (137). Mtb also inhibits macrophage microbicidal functions and dampens production of early proinflammatory cytokines and chemokines critical for shaping the nature and magnitude of T cell subsets that home to the site of infection (95, 138–140). Moreover, emerging evidence that myeloid-derived suppressor cells (MDSC) (141) and/or neutrophils (142) suppress T cell responses to Mtb infection suggests that targeting these cell types may improve vaccine-induced immunity.

Several Mtb genes have been implicated in evading DC and macrophage responses and, when deleted in BCG or Mtb, show enhanced vaccine-induced protection against TB in animal models. Thus, removing immune evasion genes shared by Mtb and BCG is an important approach for improving live attenuated vaccination strategies. The following studies are examples of applying knowledge gained from mechanistic studies of Mtb immune evasion genes and the host pathways that they modulate towards designing better vaccines for TB.

Inhibition of apoptosis as an immune evasion strategy is exemplified by the *nuoG* gene in Mtb, which encodes a subunit of NADH dehydrogenase and inhibits macrophage apoptosis (143, 144). Infection of mice with Mtb Δ *nuoG* led to an increase in apoptosis along with earlier activation of T cells compared to WT, suggesting that *nuoG* dampens the ability of innate cells to initiate T cell responses (145). Moreover, deleting *nuoG* in BCG Δ *AureC::hly*, a recombinant vaccine strain that is more protective than BCG in animal models, led to increased apoptosis following vaccination and significantly lowered bacterial burdens in Mtb-challenged mice (146). Deletion of Mtb *sigH*, which regulates multiple stress-induced proteins in Mtb, also led to increased apoptosis and chemokine responses in infected macrophages relative to wild type (147, 148). Mucosal vaccination with Mtb Δ *sigH* in NHPs resulted in increased survival and reduced lung pathology following challenge compared to BCG, with higher central and effector memory T cells in the lung (9).

Cell surface proteins are well positioned to modulate innate immune functions. The Mtb serine protease Hip1 is present in

the cell wall of Mtb and dampens macrophage and DC functions *via* proteolytically cleaving its substrate, Mtb GroEL2 (138, 149). Hip1 inhibits TLR2 and inflammasome-dependent macrophage proinflammatory responses, impairs CD40-mediated costimulatory responses in DCs, and restricts Th₁₇ polarisation during infection (91, 136, 138). Both an Mtb *hip1* mutant and BCG Δ *hip1* strain augmented CD40 expression on DCs, enhanced macrophage and DC functions and led to higher lung Th₁₇ responses (150). In a mucosal DC vaccination model, BCG Δ *hip1* induced immune responses that significantly reduced Mtb burden following challenge (150). These studies suggest that impeding CD40-CD40L interactions allows Mtb to induce suboptimal immunity, and that adjuvants that augment CD40 during vaccination are likely to improve efficacy. Another cell surface protein implicated in dampening innate immunity is LprG, a lipoprotein that binds to TLR2 on macrophages and has been implicated in inhibiting MHC class II antigen presentation and phagosome/lysosome fusion (151–153). Deleting *lprG* in BCG led to higher levels of pro-inflammatory cytokines, lower bacterial burdens and higher Th₁₇ responses following vaccination compared to BCG in murine models (154).

Many Mtb secreted proteins have also been implicated in evading host immune responses. SapM is secreted extracellularly *via* the SecA2 pathway and is involved in preventing phagosome maturation in macrophages (155, 156). Vaccination of mice with BCG Δ *sapM* increased protection following challenge and led to increased activation and recruitment of DCs (157, 158). Together, these studies illustrate that deleting immune evasion genes in the context of live attenuated vaccine strains can significantly improve efficacy and suggest that sequential deletion of multiple immune evasion genes in a single vaccine strain is likely to lead to synergistic effects that improve vaccine efficacy.

Studies focused on innate immune pathways modulated by Mtb may also provide key insights into strategies for enhancing innate immunity during vaccination. Adjuvants that trigger and engage specific DC responses during Mtb infection may be of particular interest as a way to combat Mtb induced delays in DC activation and suppression of antigen presentation to CD4⁺ T cells. Approaches to enhancing Th₁₇ responses include adjuvants that engage CD40 on DCs (91) and MPL/chitosan formulations that induce Th₁₇ polarising cytokines in DCs (114). Ligands that stimulate TLR7 and TLR9 on DCs have also been shown to upregulate MHCII and reduce anti-inflammatory IL-10 responses following BCG vaccination (159). Other receptors to target include inflammasome components and other cytosolic recognition receptors known to play a role in protective immunity to Mtb infection (160). Developing adjuvants that target additional immune evasion pathways in DCs, such as autophagy, can enhance antigen presentation (93). Targeting costimulatory and coinhibitory molecules on DCs has the potential to be beneficial in improving the immunogenicity of candidate vaccine by fine-tuning the pro- and anti-inflammatory pathways necessary for optimal immunity (91, 161–163). Additionally, adjuvants that limit early induction of IL-10 and T-regulatory cell expansion may be effective given the role of

these responses in dampening immune responses during infection (164–166). Finally, we need a deeper understanding of the suppressive functions of neutrophils and MDSCs in order to develop strategies that target these cell types using adjuvant-like approaches or by targeted depletion.

In summary, studies on Mtb immune evasion mechanisms have given us valuable insights, not only on how Mtb manipulates host immunity, but have also identified pathogen and host targets for designing live attenuated vaccine strains and novel vaccine adjuvants that enhance vaccine efficacy.

ANIMAL MODELS

TB is a complex disease and no specific animal model perfectly mimics or recapitulates Mtb infection in humans. However, harnessing the strength of different animal models available will prove useful in developing and testing new adjuvants for TB.

NHPs have played a significant role in TB research and have been increasingly used for vaccine and adjuvant development. The strength of this NHP model lies in its ability to recapitulate disease similar to that in humans. NHPs can develop a latent version of Mtb infection, produce granulomatous lesions, and be used as a model to study HIV/TB co-infection. The use of primates has provided key insights such as the enhanced protective effect of intravenous BCG vaccination and the safety and efficacy of trial TB vaccines including M72/AS01_E and ID93 + GLA-SE (8, 167). Different routes of vaccination can be tested in NHPs, as the intravenous BCG study also included comparisons to intradermal, aerosol, and intradermal & aerosol routes of administration (8). NHPs have been used to test different vaccination strategies such as the prime-boost approach in which macaques are primed with BCG and then boosted with experimental vaccines and adjuvants 3–4 months after priming (168, 169). Additionally, NHP studies on protective vaccines can help inform immune correlates of protection, such as the study which observed protection using pulmonary-delivered BCG and the intravenous BCG study (8, 51). Given their similarity to humans, the NHP model is best equipped to study the priority areas outlined in this review. However, due to the prohibitive costs and challenges of working with primates (along with limited number of animals available for research), the use of small animal models should continue to be used to perform initial pre-clinical studies of potential adjuvants.

Historically, small animal models (mice and guinea pigs) have been used by developers to test adjuvant suitability for inducing protective immunity under rigorous *in vivo* conditions. The mouse model represents an excellent screen for first time *in vivo* assessment of a test vaccine. Using mice affords greater feasibility in testing of different variables such as the effect of different doses, alternative routes of inoculation and different inoculation times post-vaccination in a relatively short time frame. Additionally, the ability to use knockout and transgenic mice provides an additional level of interrogation for vaccine formulations and a better understanding of mechanisms involved in inducing protective immune responses (37). For

instance, a recent analysis of the effect of intranasal vaccination using ODN as a molecular adjuvant showed that immunity could be achieved in a type I interferon-dependent manner (170). Another study demonstrated that intranasal vaccination was the best route for inducing protective immunity compared to intraperitoneal, subcutaneous, or intragastric routes (170). The cost of mouse models also allows for long-term immune profiling in vaccination studies. Extended timepoints have been previously used to identify improved protective efficacy of subunit vaccine candidates compared to BCG (171). New approaches are also being used that allow mouse models to better mimic the heterogeneous immune responses observed in humans. The diversity outbred (DO) mice, where initial breeding is done with inbred and wild-caught strains, have been developed with a level of genetic diversity similar to humans and NHPs. In a BCG vaccine study with DO mice, a diverse response was observed in which some vaccinated mice were protected and others were highly susceptible to Mtb challenge (172). Another attempt to improve the mouse model is the recent development of the ultra-low dose model. Infecting with 1-2 CFU of Mtb *via* aerosol, instead of the conventional ~100 CFU dose, causes mice to produce a heterogeneous immune response and granulomatous structure in the lung similar to humans (173). The use of these and other mouse models will therefore continue to be important for testing novel adjuvants and vaccination strategies against TB.

Guinea pigs serve as an additional model and is traditionally employed after a vaccine formulation has demonstrated success in mice. Guinea pigs were originally used in TB research to understand disease pathogenesis due to their susceptibility to Mtb infection as well as their use in distinguishing “mammalian” bacilli from “avian type” bacilli due to their resistance to the latter (174). Subsequent studies by Smith and colleagues (175, 176) demonstrated that aerosol infection of guinea pigs with virulent Mtb resulted in pulmonary pathology similar to that observed in humans as well as a lethal course of disease (177). This model therefore allow researchers to test the ability of a vaccine formulation to limit disease, reduce Mtb burden, and prolong survival. The success of the guinea pig to identify vaccine candidates that have the potential to succeed in humans was demonstrated by a study which found the M72 vaccine candidate was able to boost BCG responses in the model (178). The long-term guinea pig model was used until recently to determine the efficacy of a vaccine as a low dose aerosol infection with virulent Mtb able to induce progressive pulmonary and extra-pulmonary disease similar to acute TB in humans (179). Vaccination with BCG resulted in the significant reduction of pathology and prolonged survival in guinea pigs, therefore validating the use of this model to test vaccines that can be efficacious in humans. Guinea pigs can therefore provide valuable insight into the ability of different formulations to significantly limit disease progression.

Together, the use of both mouse and guinea pig models of TB have contributed to a better understanding of adjuvant mechanisms in vaccines. The NIH/NIAID TB Vaccine testing program (180) has used both the C57BL/6 short-term mouse model and the Hartley guinea pig as a short- and long-term

model to test vaccines and novel adjuvants. Both models have similar experimental strategies; animals are vaccinated with the test vaccine formulation, rested, and then infection is established by aerosol challenge with a low dose of Mtb (approximately 100 CFU for the mouse and 10 CFU for the guinea pig). The transition of testing adjuvants from the mouse to the guinea pig model has resulted in down selection of candidate vaccines under the NIH testing contracts and few have been able to reach the clinical testing stage. Of note has been the M72/AS01_E vaccine, which demonstrated activity in the mouse and guinea pig models (178). Using both models also reconcile differences observed experimentally. Recent investigations with ODN adjuvants have highlighted these discrepancies; when the same formulation and inoculation route were used in both models, the adjuvant proved less effective in guinea pigs. These results have been observed with other adjuvants and emphasize the need to test vaccines in both the mouse and guinea pig models. While adjuvanted subunit vaccines have in general been less effective at limiting disease in the guinea pig model, it is not clear whether this is a limitation of the model or whether guinea pigs are selecting out formulations that may perform poorly in clinical trials. Unfortunately, there are only a small number of adjuvanted vaccines currently in clinical trials, some of which have been successfully tested in the guinea pig model (181, 182). An increased understanding of immune mechanisms in the guinea pig would be useful to determine if adjuvants were providing the appropriate signals to generate protective immunity. With the limited number of immunological tools available for the guinea pig to perform in-depth analyses (compared to mice), it is difficult to determine why such differences occur. Developing novel reagents for the guinea pig is an area that must be supported to achieve a better understanding of immunological responses to infection and vaccination. Additionally, a systematic analysis of cell phenotypes, expression of PRRs and other innate immune factors will be required to determine why adjuvants have provided limited protective immunity in the guinea pig model. This will help strengthen the use of this model for use in adjuvant studies for TB.

In summary, while NHP models more closely mimic human disease, small animal models provide feasibility in testing vaccine adjuvants in a pre-clinical setting. With the rapid increase in the development of new molecular adjuvants, the mouse and guinea pig models provide the capacity to test them rigorously in an infectious disease setting. Harnessing the strength of all three of these animal models will therefore be crucial for developing new and improved adjuvants for TB vaccines.

CONCLUSION

Recent advances in vaccine development, including an adjuvanted TB vaccine providing nearly 50% protection and observations on improving BCG, have reinvigorated the field. As our understanding of the complex interplay between Mtb and

the host immune system is improved, new strategies for adjuvant development may be employed to further boost efficacy and develop a protective vaccine against TB.

In this review, we have highlighted four areas of importance for researchers in the TB field to address in order to generate more efficacious adjuvants and vaccination strategies. Defining correlates of protection is necessary to dictate which pathways should be targeted in order to induce protective immune responses. Similarly, learning how to target cells that are responsible for inducing those protective immune responses serves as a direct access to immune responses that are often dampened during infection. This is critical as *Mtb* is able to encode immune evasion proteins that serve to actively impede key immune cell subsets. Further characterizing these proteins give us direct insight into pathways that can be activated to overcome pathogen inhibition. Additionally, having appropriate animal models is necessary to test whether adjuvants and vaccines can be beneficial to humans while at the same time serving as a tool to further study infection and disease. While research into these topics can contribute directly to the generation of new adjuvants and vaccines, current adjuvants and vaccines are instructive in determining the requirements for protective immunity to TB. While intravenous delivery of BCG in humans is controversial, the study in NHPs (8) served as a model for identifying correlates of protection that can be targeted through safer, more practical means. Similarly, the M72/AS01_E vaccine study (10) demonstrated the benefit of adjuvants in TB vaccine strategies and contributed potential insights into mechanisms of protection. Cross-talk between basic TB science research and TB translational research should be encouraged as each of these inform the other. Additionally, insights from within

the field are able to inform other fields that require generation of similar immune responses for their own models of protection. Therefore, advancing research in these four areas identified at the workshop has the potential to improve immunology in general while working to reduce TB burden worldwide.

AUTHOR CONTRIBUTIONS

ABE, AI, SMM, ELS, RNM, DJF, JTE, JR and JAT developed, wrote, and edited sections of the review as well as edited the entire manuscript. ABE provided significant revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: Author RNM was employed by company Columbus Technologies & Services Inc. JE and SM are employees of and own shares in Inimmune Corporation.

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