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Prime–boost approaches to tuberculosis vaccine development

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Abstract

Four individuals die from active TB disease each minute, while at least 2 billion are latently infected and at risk for disease reactivation. BCG, the only licensed TB vaccine, is effective in preventing childhood forms of TB; however its poor efficacy in adults, emerging drug-resistant TB strains and tedious chemotherapy regimes, warrant the development of novel prophylactic measures. Designing safe and effective vaccines against TB will require novel approaches on several levels, including the administration of rationally selected mycobacterial antigens in efficient delivery vehicles via optimal immunization routes. Given the primary site of disease manifestation in the lungs, development of mucosal immunization strategies to generate protective immune responses both locally, and in the circulation, may be important for effective TB prophylaxis. This review focuses on prime–boost immunization strategies currently under investigation and highlights the potential of mucosal delivery and rational vaccine design based on systems biology.

Keywords

BCG; lung immunity; mucosal delivery; prime boost; recombinant viral vectors; systems biology; tuberculosis; T cell; vaccine

TB is a global emergency

It has been estimated that a third of the human population is latently infected with *Mycobacterium tuberculosis* and at risk for disease reactivation [1]. A 2011 WHO report on global TB control indicated that there were 8.8 million new TB cases registered and 1.45 million TB-related deaths [2]. Coinfection with HIV-1 is the most common cause of immune suppression in latently infected individuals, raising this risk from a 10% lifetime chance to 10% annually [3]. Increases in TB incidence are also fueled by infections caused by multi-drug resistant and extensively-drug resistant *M. tuberculosis* strains. The WHO has declared TB a global health emergency and estimates that 70 million people will die from the disease in the next 20 years without adequate treatment and preventive measures, primarily effective vaccines [4,5]. *Mycobacterium bovis* BCG is the only licensed TB vaccine available for human use. It is effective in reducing childhood incidence of TB;

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however, its variable efficacy in adults warrants the development of novel and more effective prophylactic measures that will generate protective responses against acute TB infection and could potentially control latent infection by clearing the pathogen or preventing disease reactivation. Successful immunization strategies may therefore need to induce immune responses in susceptible individuals that are comparable with those generated in latently infected individuals, and which are capable of achieving sterile *M. tuberculosis* eradication [1,6]. A TB elimination target set by the WHO for 2050 would seem unlikely to be achieved without new tools in place, including new drugs, vaccines and vaccination strategies [7]. In response to this challenge, a variety of preclinical and clinical vaccine trials of new vaccine candidates are underway. A variety of mechanisms of immune evasion appear critical in determining the outcome of *M. tuberculosis* infection [8], while dysregulation of host immune defenses by mycobacteria favor their persistence, even within normal hosts, and this has been a critical barrier to effective vaccine development [9]. Immune responses induced by effective pre-exposure vaccines could potentially contain TB infection at an earlier time point than could responses induced in unvaccinated individuals, and lower bacterial loads. In mouse models of *M. tuberculosis* infection, vaccines that can reduce bacterial loads by tenfold compared with naive controls are considered to confer reasonable protection. Since clinical disease progression corresponds with bacterial load, such a reduction might have significant implications – correlating with the formation of fewer granulomas and a decreased chance of developing active disease [10]. Immune correlates of vaccine-mediated protection against *M. tuberculosis* infection have not yet been fully defined; however, Th1 cell-mediated immune responses mediated by $CD4^+$ and $CD8^+$ T cells are crucial [11–16]. Consistently sterilizing immunity against TB infection has not been achieved through any vaccination strategy tested to date.

BCG – efficacy & failure

The BCG vaccine was developed by Albert Calmette and Camille Guerin and first administered to infants in 1921. It is now given annually to more than 120 million people worldwide, with 4 billion people vaccinated to date. BCG protects against disseminated forms of the disease including miliary TB and TB meningitis, while meta-analyses from several clinical studies have shown that BCG has reduced the risk of disease development by 50% in infants and neonates [17–19]. However, mass vaccination with BCG has also been implicated as a selective force in the emergence of new pathogenic *M.tuberculosis* genotypes [20]. The vaccine has traditionally been given intradermally, while recent refinements involving ‘needle-free’ vaccine patches may offer prospects for increased vaccine coverage without compromising immunogenicity [21].

Factors responsible for the variable efficacy of BCG vaccine in adults, particularly in developing countries, include: virulence of different *M.tuberculosis* strains; storage conditions and resultant viability; strain differences; loss of genes; and the methodology of studies conducted in different regions [3,17–19,22]. Meta-analysis of 14 prospective and 12 case–control studies revealed that the protective efficacy of BCG against pulmonary disease varied significantly with geographical latitude [19]. BCG also appears to generate $CD4^+$ central memory T cells rather poorly, potentially compromising control of subsequent TB infection [23,24]. Another factor that may underlie BCG failure is the unusually high concentrations of IL-4 that have been found in patients from developing countries [25,26]. IL-4-producing Th2 cell responses may predominate in these patients due to exposure to nontuberculous mycobacteria (NTM) and/or helminth infections. Resultant IL-4 production and regulatory T cell development may negatively regulate generation of Th1 type cells that could potentially contain TB infection, thereby leading to active disease and vaccine failure [17,22,25–27]. In recent mouse studies, $FoxP3^+$ $CD4^+$ regulatory T cells induced by BCG appeared to downregulate effector T cell activity in mice infected with a virulent W-Beijing

TB genotype, a finding with implications for BCG vaccination against highly virulent circulating TB strains [28].

Immune correlates of protection against *M. tuberculosis*

Most of the novel TB vaccine candidates currently under development are designed to induce high levels of cellular immunity. Absolute determinants of protection against *M.tuberculosis* have not yet been fully defined, but clues have come from a variety of studies. Dissemination of bacteria to the pulmonary draining lymph nodes occurs approximately 9 days after aerosol inoculation with low dose *M. tuberculosis*; these are the first sites where *M.tuberculosis* -specific T cells are detected, followed by the lungs and spleen [29]. $CD4^+$ T cells, together with Th1 cytokines IFN-γ and TNF-α, are important for control of infection, while $CD8^+$ T cell responses may also play a role [11–16], particularly later in infection [30]. IL-2 and IL-7 are important for the development and maintenance of memory T cell responses [31], while the induction of polyfunctional T cells, capable of simultaneously secreting multiple cytokines, has been correlated with vaccine-mediated protection in a number of disease models, including *Leishmania major* infection in mice [32], and simian immunodeficiency virus infection in rhesus macaques [33]. Chronically HIV-infected individuals and HIV nonprogressors maintain HIV-specific polyfunctional $CD8^+$ T cell populations whose frequency correlates inversely with viral loads; these are not seen at high levels in those with progressive disease [34]. However, it is conceivable that vaccine-induced immune responses that correlate with protection differ from those necessary for protection [35]. Several preclinical studies have shown that IFN-γ induction might be necessary, but is clearly not sufficient for protection [36–38], while the generation of polyfunctional T cells does not always correlate with protective efficacy against TB [6,39]. Vaccine-induced immunological correlates of protection may also be population-specific and/or disease-stage specific [35], making it difficult to define a single correlation across the spectrum of clinical disease states in different populations.

Heterologous prime–boost immunization against TB

Protection against infection by intracellular pathogens such as *M. tuberculosis* probably requires the generation of potent cell-mediated immunity [40,41]; however, optimization of cell-mediated immune responses through vaccination is not a simple task. Repeated administration of the same vaccine (homologous boosting) has often been effective for increasing humoral but not cellular immune responses to target antigens, while it has been shown that heterologous prime–boost immunization is highly effective for enhancing humoral and cellular immunity [40,42–44]. This strategy involves priming the immune system against a target antigen and subsequently boosting antigen-specific immune responses with a distinct immunogen, often a recombinant viral vector, expressing the same vaccine antigen [40,45], with resultant synergistic enhancement of specific immunity, characterized by increased numbers of antigen-specific T cells, selective enrichment of T cell avidity, and increased protective efficacy against pathogen challenge [45–47]. Several mechanisms could contribute to the success of this strategy, including T cell immunodominance, the nature of the boosting vectors, and the generation of only low levels of antivector immunity. Recombinant poxviruses and adenoviruses (Ad) are considered to be the most potent boosters of T cell immune responses, while the use of different vectors or other agents at each stage of immunization can minimize the development of antivector immunity [40,41,45–47]. The use of replication-defective vectors for boosting, including Ad, fowlpox viruses or modified vaccinia virus Ankara strain (MVA), is particularly effective in this respect [39,40,45–47].

At least three key factors are important for the development of improved TB vaccines. These include the inclusion of rationally selected mycobacterial vaccine antigens, their incorporation in effective delivery systems such that protective immune responses will be generated, and optimization of routes of vaccine delivery to generate effective immunity, including pulmonary mucosal responses that will contain or even clear the bacteria locally and minimize or prevent bacterial dissemination.

BCG priming or boosting

The majority of the global population has been exposed to BCG, and this will continue into the foreseeable future, despite the drawbacks described above. There are also safety concerns regarding use of BCG vaccine in HIV-infected infants [48]. Nevertheless, BCG has an 80-year safety record, is generally well tolerated in healthy individuals [4], and is still widely administered at birth in developing countries in which TB is endemic. Thus, for new vaccines tested in these regions, it is likely that the majority of individuals will have been vaccinated with BCG. Issues of immunogenicity and, potentially, safety have recently been addressed through the development of recombinant forms of BCG, including rBCG30, VPM1002 and Aeras 422, which have been engineered in attempts to facilitate apoptosis of antigen-presenting cells and, potentially, enhance crosspriming, as well as to overexpress immunogenic TB proteins [35,49–52]. Of these, only VPM1002 (urease deletion in BCG expressing listeriolysin) has progressed to Phase II trials [201]. A Phase I trial of Aeras 422 (rBCG expressing perfringolysin along with Ag85A, 85B and Rv 3407) was recently halted owing to the development of shingles by two study participants, probably due to reactivation of latent herpes virus infection [53]. BCG and its recombinant forms are also increasingly being considered as priming vaccines in heterologous prime–boost vaccination strategies. BCG-induced immune responses are impaired in HIV-infected infants [54], while there is a higher risk of developing disseminated BCG disease in children with HIV [22,48]. Ongoing clinical trials are evaluating the risk that BCG immunization poses for infants born to HIV-infected mothers [4]. Finding a replacement for BCG for vaccination of HIV-infected populations will be a challenging task. The use of recombinant viral vectors or antigenic subunits for priming followed by BCG boosting has been suggested as a potentially safer alternative for vaccination of newborns [55]. Indeed, BCG has been used effectively as a booster vaccine, including studies in cattle and mice where it generated long-lasting immunity and/or improved protection against *M. tuberculosis* challenge [56–58]. Aeras 402 is a recombinant, replication-deficient adenovirus vector vaccine encoding Ag85A, Ag85B and TB10.4 that enhanced polyfunctional $CD4^+$ and $CD8^+$ T cell responses when given as a booster following BCG priming [59,60] and is currently being evaluated in Phase I/II safety and efficacy trials in HIV-infected adults and infants in South Africa [202,203].

Heterologous boosting of BCG-primed immunity will likely be a key component of future multicomponent vaccine strategies [61]. A booster vaccine may be given in infancy or adolescence when the effects of BCG may start to wane [35]. As discussed in the following sections, recombinant viral vectors and protein–adjuvant cocktails are at the forefront of approaches currently being tested for their capacity to boost strong cellular immunity against TB. While containment of infection to prevent TB disease may be a realistic initial step, the ultimate goal must be to develop either ‘pre-exposure’ prime–boost vaccines to prevent establishment of infection and disease, or ‘post-exposure’ vaccines to prevent disease reactivation in latently infected individuals.

Viral vectors as booster vaccines

MVA and Ad, both double-stranded DNA viruses, have been the most commonly studied viral vectors for candidate TB vaccines. Each is relatively easily delivered via parenteral or

mucosal routes and may also have inherent adjuvant effects, leading to induction of immunostimulatory chemokines or cytokines that underpin strong antimycobacterial immune responses [62,63]. MVA vectors are replication-deficient, nonintegrating and stably express encoded vaccine antigens. Their replication is blocked in mammalian cells, probably due to significant gene deletions seen in comparison with wild-type parental vaccinia virus [64]. This feature renders MVA potentially safe for human use and, somewhat paradoxically, highly immunogenic for encoded vaccine antigens [65], probably due to both reduced antigenic 'competition' from vector proteins and adjuvant properties of the vector itself. MVA vectors typically express high levels of encoded vaccine proteins, facilitating expansion of antigen-specific immune responses generated by a variety of priming vaccines [40]. MVA encoding mycobacterial mycolyl transferase Ag85A (MVA85A/Aeras485) enhances BCG-mediated protection in murine and nonhuman primate models [66,67], and is now in advanced stages of Phase II clinical trials in South Africa [204]. MVA85A induces strong, antigen-specific polyfunctional $CD4^+$ T cell responses in BCG vaccinated adults [68], and appears to be safe, in that it was well tolerated with no adverse effects in over 500 human subjects including healthy adolescents and children as well as healthy HIV-positive and latently infected individuals, both in the UK and South Africa [69–73]. Indeed, a recent Phase IIa trial conducted in South Africa, where TB is endemic, indicated that MVA85A is immunogenic, well tolerated and induced only mild local reactions when administered intradermally to *M.tuberculosis* and/or HIV-infected adults [74]. It should be noted, however, that MVA85A immunogenicity was reduced when coadministered with vaccines under the Expanded Program on Immunization, suggesting that modifications in this regimen might be required when new TB vaccines are introduced into the public domain [75].

Ad are natural mucosal immunogens with a particular tropism for the respiratory tract. Ad-based vectors have an excellent safety record in humans [61]. Recombinant Ad vaccines induce significant protection against pulmonary TB challenge in mice, either alone or as a booster in combination with BCG [76,77]. AdAg85A is currently in Phase I clinical trials as a booster for BCG priming, following evidence in guinea pigs that this approach is significantly more protective than BCG alone [63,78]. Concerns regarding Ad5 as a vaccine vector center upon pre-existing immunity to this serotype in up to 80% of people, with efforts made to circumvent this problem based on the use of Ad serotypes that rarely infect humans, including Ad35 [79,80]. Studies involving intramuscular delivery of Ad35 vectors (based on human serotype 35) that encode Ag85A, Ag85B and TB10.4 (Aeras 402/Crucell Ad35) to boost BCG-primed responses in nonhuman primates [81], have now progressed to Phase I and Phase II clinical trials, with both $CD4^+$ and $CD8^+$ T cell responses reported in Phase I studies in USA and South Africa [59,205]. Replication-defective Ad vectors, such as the *NS1* (nonstructural) gene deletion mutant currently being trialed as an influenza vaccine [82], offer further potential for TB vaccine development, in that they may help to address the somewhat transient nature of Ad infection and subsequent gene expression in vaccinated hosts. Both Ad and MVA-based vectors have also shown distinct promise for local pulmonary delivery of TB vaccine antigens, as discussed below.

Protein-adjuvant booster vaccines

Several subunit vaccines containing highly immunogenic *M. tuberculosis* or *M. bovis* antigens formulated with adjuvants are under clinical investigation for their capacity to boost BCG-primed immunity. Three such candidates are Hybrid1–IC31, Hyvac IV/AERAS 404–IC31 and M72 [35,206]. Hybrid 1 is a fusion protein containing two *M. tuberculosis* antigens, Ag85B and ESAT-6, administered with the adjuvant IC31, an immunostimulatory oligodeoxynucleotide [35]. Preclinical studies revealed significant levels of protection against *M. tuberculosis* challenge in guinea pigs and mice, as well as boosting of BCG-

primed immunity [83,84]. Hybrid 1 is strongly immunogenic for both antigenic components, appears to be safe in TB-naive, BCG-vaccinated and latently-infected individuals [85,86], and has recently completed a Phase I clinical trial [207]. The Hyvac IV vaccine has been developed to circumvent the use of ESAT-6, a key TB diagnostic, by using TB10.4. Hyvac IV is immunogenic and protective against *M.tuberculosis* challenge in mice [87], and can successfully boost BCG-primed responses in the guinea pig model, enhancing reduction of bacterial burden in the lungs and spleen by nearly 1 $\log_{10}$ compared with BCG [88]. It is currently being evaluated in Phase I trials in Sweden and South Africa [35,205,206]. *M.tuberculosis* 72F, or M72 vaccine, is a 72 kDa polyprotein comprising a fusion of Rv1196 and Rv0125. Delivered in conjunction with AS02A adjuvant, an M72 booster given following BCG-priming enhanced T cell responses to BCG in mice, and significantly improved survival in the guinea pig model of pulmonary TB infection [89], while a heterologous BCG prime–M72F boost regimen induced polyfunctional T cell responses with enhanced protective efficacy in cynomolgous macaques [90]. M72 is immunogenic and well tolerated in TB-naive adult volunteers [91,92], and is currently in Phase II clinical testing in adolescents in South Africa and in HIV-infected adults in Europe [63,208,209].

Further promising subunit vaccine candidates are now also in preclinical development. Three such candidates, heparin-binding hemagglutinin adhesin (HBHA), a surface protein present in *M. tuberculosis* and BCG, the alanineproline rich protein Apa (Rv 1860), and ID93 – a combination of four mycobacterial proteins that have been associated with virulence (Rv2608, Rv3619 and Rv3620) or latency (Rv1813), are all highly immunogenic and/or protective in animal models of TB infection when given following BCG priming [93–96]. The efficacy of ID93 against multidrug-resistant TB, when formulated with MPL adjuvant, particularly when given after BCG priming, makes it an attractive candidate for Phase I clinical trials [96]. H56, a recombinant protein vaccine comprising Hybrid1 together with the latency-associated antigen Rv2660c, increased protective efficacy in mice against *M. tuberculosis* challenge when tested as a pre-exposure vaccine compared with BCG or H1 vaccine alone [97]. Importantly, when given following BCG priming, but after exposure to *M. tuberculosis*, H56 was able to control disease reactivation and lower bacterial load in the lungs almost 100-fold. This vaccine appears, therefore, to target both acute and latent stages of TB infection. H56 adjuvanted with IC31 has recently moved into Phase I clinical trials in South Africa [Andersen P, Unpublished data].

Alternative approaches for vaccine priming or boosting

While DNA vaccines have traditionally been considered as priming vectors in heterologous prime–boost immunization strategies [40,43,62,98], drawbacks have included their relatively poor immunogenicity in larger mammals [99], probably related to dosage and/or transduction efficiency [100]. However, they may also represent effective boosters for immunity primed by BCG or related myocbacterial species against *M. tuberculosis* challenge. DNA vectors encoding ESAT-6 and Ag85A [101] or the latency-associated antigen α-crystallin [102,103] significantly enhanced the protective efficacy of BCG priming in mouse and guinea pig models of TB infection, correlating with markedly elevated $CD4^+$ T cell responses. DNA-launched alphavirus replicons, including those based on Sindbis virus, Semliki Forest virus or Venezuelan Equine Encephalitis virus, are highly immunogenic in animal models of HIV-1 and TB infection [104–110]. Although not yet tested in BCG-primed mice, they may represent better prospects than conventional DNA vaccines for enhanced and/or sustained TB-specific immunity in the circulation or in pulmonary tissues. Another vector system with distinct promise as a vehicle for vaccine boosting is the RNA virus, vesicular stomatitis virus (VSV). Attenuated VSV vector vaccines have now been tested in a number of disease models [111]. In a murine TB model, VSV encoding Ag85A is immunogenic, with intranasal vaccine delivery generating solid

pulmonary T cell immunity and superior protection compared with intramuscular delivery [112]. The use of VSV-Ag85A as a mucosal booster following parenteral delivery of adenovirus encoding Ag85A in a heterologous prime–boost approach enhanced both pulmonary and circulating immunity, correlating with improved protective efficacy over either vector alone [112].

Drawbacks related to the efficacy of BCG have been outlined above and it is conceivable that alternative mycobacterial strains that are both safe and immunogenic could eventually replace BCG in vaccine protocols – its widespread and ongoing use in the field notwithstanding. Several live attenuated *M. tuberculosis* mutants, particularly those with multiple deletions, appear to be safe and immunogenic in both immune-competent and immune-deficient animal models, as reviewed elsewhere [113]. The *M.tuberculosis* strain SO2, a *phoP* gene deletion mutant, was more immunogenic than BCG when tested in murine models of TB infection [114] and its protective efficacy appeared to correlate with the generation of central memory $CD4^+$ T cells [24]. SO2 was also more protective than BCG, both in guinea pigs administered high-dose aerosolized *M.tuberculosis* challenge [114] and in rhesus macaques, where SO2 was as effective as BCG/MVA85A prime–boosting [67]. Very recently, a novel recombinant strain of *Mycobacterium smegmatis*, termed IKEPLUS, in which the native ESX-3 secretion system was replaced with ESX-3 from *M.tuberculosis*, was shown to be highly immunogenic for $CD4^+$ central memory T cells in a murine TB model [115]. IKEPLUS profoundly reduced bacterial loads in the lungs following aerosol challenge with *M. tuberculosis*, even achieving sterilizing immunity in one experiment. Its evaluation in models that more closely represent human TB infection is eagerly awaited. Another vaccine candidate termed RUTI (fragmented and detoxified *M. tuberculosis* in a liposome formulation) is under development as a post-exposure TB vaccine [116], with a Phase II clinical trial of its immunogenicity, safety and tolerability in latently-infected individuals recently completed [210]. Successful evaluation of any of these promising approaches could eventually lead to their use as alternatives to BCG, including as components of future prime–boost vaccine protocols.

Mucosal prime–boost immunization strategies against TB

To date, insufficient attention has been paid to the potential importance of local pulmonary immunity in controlling TB infection. *M. tuberculosis* is transmitted primarily as an aerosol and the nasal cavities are usually the first port of entry for the pathogen [5], while pulmonary mucosal tissues are the primary sites for establishment of infection. It may therefore be essential to develop immunization strategies that generate potent immune responses locally in the lungs as well as in the circulation. Disseminated disease affecting other organs may develop in some infected individuals, particularly those with compromised immune systems – strong local immunity may also help to contain the infection. Parenteral delivery of immunogens often fails to induce mucosal immune responses, and while successful for clearance of pathogens such as influenza virus and in preventing disease through induction of neutralizing antibodies in the circulation [117,118], mucosal immunization may be required to help achieve protection against currently intractable mucosal pathogens including *M.tuberculosis* and HIV. It is also likely that local mucosal immunization or boosting would enhance the efficacy of parenterally primed immune responses [5,119]. Indeed, mucosal vaccine delivery may activate multiple arms of innate and adaptive immunity and induce memory T cell responses at a local level unlikely to be achieved by purely parenteral prime–boosting [66,76,117,120–123].

The mucosa-associated lymphoid tissue (MALT) forms a common mucosal immune system, with antigenic stimulation at one site often resulting in local and disseminated mucosal immune responses [47]. Nasopharynx-associated lymphoid tissue is a key component of

MALT and contains the cells and tissues required for generation of antigen-specific immunity. Thus, induction and regulation of mucosal immune responses can occur in the upper respiratory tract [124], while intranasal immunization can elicit both mucosal and circulating immunity [125,126]. Recombinant viral vectors or subunit gene delivery systems can be used to generate localized pulmonary responses in both upper and lower airways and in the circulation [5]. Indeed, Ad and MVA-based vaccines have been tested in experimental mucosal immunization strategies in a variety of disease models for over a decade [47]. In the case of TB, a single intranasal dose of recombinant Ad vaccine encoding Ag85A generated robust protection against *M. tuberculosis* challenge in mice [76]. Interestingly, intramuscular priming with a DNA vaccine followed by heterologous boosting via the pulmonary route with an Ad-based vaccine further enhanced protective efficacy [76], raising the possibility that combinations of parenteral and local mucosal vaccine administration can be highly effective for containment and even clearance of *M. tuberculosis* infection. Subunit vaccines formulated in adjuvants, or viral vector vaccines administered via the pulmonary route have now been extensively tested for their capacity to boost BCG-primed immunity. Mouse studies of either a single mucosal dose of BCG, or subsequent boosting of BCG-primed responses with recombinant adenovirus vectors showed significantly enhanced protection compared with subcutaneous BCG immunization alone [77,127,128], while vaccine-induced Th1 cells in the lungs appear to correlate better with protection than circulating T cell responses [77]. When given as an intranasal booster to BCG-primed mice, Ad-Ag85A enhanced protection over BCG vaccination alone (1.72 $\log_{10}$ and 1.17 $\log_{10}$ greater reduction in bacterial burden in lungs and spleen [77]), correlating with strong polyfunctional $CD4^+$ and $CD8^+$ T cell responses in the lungs, with some circulating immunity [77]. Intranasal vaccination with MVA has induced protection against lethal poxvirus challenge in macaques and rodents and has also generated protective responses to other airborne pathogens, including influenza and respiratory syncytial viruses [62]. Intranasal MVA85A delivery enhanced BCG-primed circulating immune responses in mice, while also boosting $CD4^+$ and $CD8^+$ T cell responses in lung lymph nodes. Significant inhibition of bacterial growth of up to 1.5 $\log_{10}$ following aerosol challenge with *M.tuberculosis* was found compared with BCG vaccination alone [66], suggesting that MVA85A boosting mediates immune responses that help to control infection, limiting dissemination of bacteria from the initial site of infection and, potentially, markedly reducing the chances of transmission to other individuals.

A potential concern with nasal vaccines is access of gene delivery vehicles, live attenuated organisms, or potentially neurotoxic adjuvant molecules to the central nervous system via the olfactory region [5]. The use of nonreplicating gene delivery systems such as DNA vaccines and highly attenuated viral vectors (including MVA) may help to address this potential problem, while nontoxic mucosal adjuvants including Eurocine L3, or derivatives of *E. coli* heat-labile enterotoxin (LT) and *V. cholerae* cholera toxin, are also under consideration for intranasal delivery [5,124]. Intranasal booster vaccination of mice with an Ag85B–ESAT-6 fusion protein administered with LTK63 adjuvant, a modified form of *E. coli* LT, generate strong and persistent Th1 responses and significant protection against *M. tuberculosis* challenge, reducing bacterial growth by nearly tenfold in lung and spleen tissues [84].

Oral vaccine delivery may also represent a practical, cost-effective and relatively safe approach to TB immunization [4]. Oral administration of BCG is well tolerated in healthy adults, and is immunogenic for T cell-mediated immunity [129,130]. Encouragingly, while oral vaccination with encapsulated BCG is highly effective for reducing TB incidence in wild brush-tail possums, a major reservoir of *M.bovis* TB, studies in laboratory animals have already demonstrated that oral delivery of BCG also confers significant protection against respiratory *M. tuberculosis* challenge [4,131]. A Phase I placebo-controlled clinical

study is currently underway in the USA to compare safety and immunogenicity of BCG given via intradermal and/or oral routes [211], and will likely inform its future use as a component of single dose or prime–boost mucosal vaccine strategies.

Optimization of the interval between vaccine doses & challenge: a key component of efficacy

There is now clear evidence that antigen-specific T cell responses to recall antigen are influenced by the maturation state of responding T cell subsets. Varying the time interval between immunizations, such as in prime–boost vaccination, will help to determine whether the kinetics of T cell differentiation influence the effectiveness of heterologous booster immunization regimes in terms of their immunogenicity and protective efficacy. Increasing the time interval between DNA priming and vaccinia virus vector boosting from 2 weeks to 12 weeks significantly enhanced the magnitude of resultant $CD4^+$ and $CD8^+$ T cell responses, leading to increases in protective efficacy against sporozoite challenge in a murine model of malaria [132]. The interval between the final dose of DNA vaccine and a poxvirus vector boost was also critical for vaccine efficacy in a rhesus macaque model of malaria, with 21 weeks being more effective than 7 weeks [133]. Thus, intervals between priming and boosting may be important for induction and possibly maturation of antigen-specific T cells. The time interval between priming and boosting may be of particular importance in the context of TB, where BCG is often administered at birth and, for reasons outlined above, is likely to be a component of future vaccination strategies.

Appropriately, timed BCG prime–boost vaccination may be a key factor for successfully establishing immunological memory that was long-lasting and protective in deer against virulent *M. bovis* challenge [134]. In a murine TB model, increasing the interval between BCG priming and boosting with purified *M. Bovis* BCG antigen HBHA from 3 weeks to 4 months, and then to 8 months, led to successive improvement in vaccine efficacy, as determined by reduced bacterial loads in both lungs and spleen tissues compared with BCG immunization alone [94]. Optimizing the time interval postboosting, and/or after TB challenge, in order to evaluate the protective efficacy of promising candidate vaccines in animal models, may also be critical for effective assessment of vaccine candidates [28,135,136]. This approach could also help in selecting vaccine candidates that are as good as BCG, preferably even better. BCG may have reduced protective efficacy at longer time intervals after TB challenge, as mentioned above [28]. Use of the most relevant animal models, including the high-dose guinea pig challenge model, or clinical TB isolates for challenge, may also aid in better screening of novel vaccines [28,135].

Systems biology & bioinformatics in rational vaccine design

Exactly why BCG induces robust protection against childhood forms of TB but has variable efficacy in adults has yet to be determined. Similarly, correlates of vaccine-mediated protection against TB require more detailed clarification. Although still in their infancy, new approaches based on systems biology and bioinformatics may help to address these questions and related issues of prime importance for the development of more effective vaccines against TB [137,138]. High-throughput biological data from deep sequencing, transcriptomics and proteomics could be useful for construction of predictive models and may help to elucidate the significance and consequences of a variety of complex host–pathogen interactions [139]. Systems biology involves the capture and integration of vast data sets in order to reveal characteristic ‘patterns’ that may not otherwise be evident from analyses of individual data points, particularly those associated with the earliest interactions between host and pathogen and involving innate immune responses [137,138].

During pulmonary *M. tuberculosis* infection, immune events in affected tissues are accompanied by alterations in host gene-expression profiles. Due to their relative accessibility and limitations on effective sampling from other tissues, peripheral blood cells are currently primary targets for analyses of differential gene expression, including during TB infection [140]. A systems approach was recently used to compare gene transcripts in whole blood cells from healthy controls with those from individuals with either active or latent TB infection [141]. A specific 393-transcript signature was identified for active TB, correlating with the extent of disease. A subset of patients with latent TB had signatures similar to those in patients with active TB, while a different 86-transcript signature was found to discriminate active TB from other inflammatory and infectious diseases. These gene signatures appeared to reflect changes in the nature of the immune response [141]. In particular, this study pointed to a key role for neutrophils in TB pathogenesis, supporting earlier observations [142,143]. This work was subsequently validated in a cohort study in The Gambia through the use of whole-blood transcriptional profiling to identify novel, disease-associated gene signatures [140,144]. Focusing on expression profiling of functional pathways and networks of biological processes underlying TB pathogenesis, it was found that Fc γ receptor gene 1 was the most highly differentially expressed gene found between patients with active TB and latently infected individuals [140,144]. Striking similarities were also found between characteristic gene-expression patterns in systemic lupus erythematosus and active TB disease [140]. A similar microarray-based approach suggested increased expression of programmed death ligand-1 by neutrophils present in whole blood of patients with active TB compared with healthy or latently infected individuals [145]. Discovery of gene signatures in different cell populations and tissues should provide information important for the design of improved vaccines against TB. Systems approaches should also help in analyzing the vast amounts of data generated during large-scale clinical vaccine trials, potentially generating computational models capable of predicting immune correlates of vaccine-mediated protection.

An important consideration for future vaccine design is the selection of effective TB vaccine antigens. In this context, upregulation of two regions of difference (RD), RD11 (Rv2658C and Rv2659c) and RD2 (Rv1986) have recently been demonstrated in *M. tuberculosis* through bioinformatics. Both regions are absent from commonly used BCG vaccine strains [146]. Transcriptional profiles of *M. tuberculosis* subjected to 168 h of hypoxia were examined in order to locate potentially immunogenic gene products. Interestingly, Rv1986 was found to be an immunodominant target for specific T cells obtained from *M. tuberculosis*-infected individuals.

As outlined above, exposure to NTM may be a key factor underlying the variable protective efficacy of BCG against TB disease in adults. In this respect, bioinformatics was used to characterize antigens specific to common NTM but not present in the *M. tuberculosis* complex, along with T cell responses against these antigens in populations with previous exposure to NTM [147]. Since BCG will likely remain an integral part of TB vaccine design, NTM exposure might also influence the effectiveness of novel BCG-based vaccines [147].

It is to be hoped that the powerful analytical computational tools of systems biology and bioinformatics will play an increasing role in future rational vaccine design for TB and other diseases.

Expert commentary & five-year view

TB is a global emergency, with approximately 1.7 million deaths annually and up to a third of the global population carrying *M.tuberculosis* in a latent state and therefore at risk of

reactivation. This situation is greatly exacerbated by the HIV pandemic, and is particularly acute in the developing world, with four out of five cases of HIV-associated TB occurring in Africa. The need for an effective vaccine is greater than ever, and there are clear signs that TB vaccine research has turned a corner. While BCG remains the only licensed vaccine, increased global investment in potential solutions has culminated in the clinical testing of at least 12 new TB vaccine candidates.

The capacity of BCG to control disseminated and meningeal TB in infants ensures its continued use, despite its limited efficacy against adult pulmonary TB, particularly in the developing world. Up to 4 billion doses of BCG vaccine have been administered to date. Thus, current TB vaccine efforts focus largely on improving BCG through recombination or genetic attenuation and/or attempts to boost its effects with different vaccines, including through heterologous prime–boosting. Three distinct recombinant BCG strains have now completed Phase I clinical trials, and one of these (VPM1002, expressing listeriolysin and lacking urease) recently progressed into Phase II trials in neonates. Ultimately, the most effective TB vaccines may be based on combinations of BCG and strong booster vaccines. At least five combinations of TB proteins in adjuvant are currently in trials, with M72, comprising Rv1196 and Rv0125 in adjuvant, now in Phase II with others likely to follow. Three subunit vaccines based on recombinant viral vectors are currently being trialed and include replication-defective MVA or Ad35, the latter chosen to address the need to avoid pre-existing antibody responses. MVA-Ag85A and Ad35 encoding a combination of Ag85A, Ag85B and TB10.4, each generate excellent T cell responses in humans and both are now in Phase IIb trials. Interestingly, animal studies have also shown MVA and Ad vectors to generate strong mucosal and circulating immune responses when delivered directly to the respiratory tract, and to markedly reduce growth of *M.tuberculosis* in the lungs and dissemination to other organs following aerosol challenge. While we await the outcome of current trials, it is possible that direct mucosal approaches, particularly pulmonary boosting of BCG-primed individuals, could be even more effective if issues of vaccine safety can be addressed. Vaccine-induced T cells that produce or stimulate IFN-γ, TNF, and/or IL-17 production are likely to play an important role in protection against acute TB infection and could help to prevent *M. tuberculosis*-driven induction of $CD4^+$ and $CD8^+$ regulatory T cell populations and other immune-suppressive factors in the lungs and/or the circulation. Ideally, primed pulmonary mucosal immune responses could rapidly contain, and even control infection, although more work is required to explore mucosal immunity in TB pathogenesis and vaccination. Undoubtedly, booster vaccines based on alternative vector systems, some with mucosal tropism, will also eventually enter clinical trials.

As with all vaccine development, key issues for TB immunization include the need for increased knowledge of correlates of protective immunity and mechanisms of pathogen immune evasion, identification of protective antigens, effective delivery systems and better disease models. Inroads are now being made in each of these areas. Recent advances represented by the current clinical trial pipeline are highly encouraging and could certainly impact the TB pandemic should one or more of these vaccines eventually be approved for use. Potentially even more promising candidates are waiting in the wings, exemplified by the recent demonstration that recombinant *M. smegmatis* with the native ESX-3 secretion system replaced by ESX-3 from *M. tuberculosis*, achieved bacterial clearance following aerosol *M. tuberculosis* challenge in mice. Systems approaches hold further promise for clarification of immune signatures that could eventually facilitate development of vaccines targeted against different strains of TB in acute infection, latent and reactivation disease, and even in HIV-infected individuals. The most promising current prospects for immune control of *M. tuberculosis* will likely be two-stage vaccines based on priming with engineered BCG or related mycobacterial species and boosting with the most effective subunits, several of which have been discussed above. Full clinical testing and validation may take many years.

The identification of biomarkers correlating with disease state or representing protective 'end points' should eventually facilitate more rapid assessment of promising candidates and may impact the design of future vaccine efficacy trials.

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Key issues

- Heterologous prime–boost immunization involves priming the immune system against a target antigen and subsequently boosting antigen-specific immune responses with a distinct immunogen, often a recombinant viral vector expressing the same vaccine antigen. This approach is highly effective for generation of strong and persistent humoral and cellular immune responses.
- The majority of the global population has been exposed to BCG, and this will continue into the foreseeable future despite its limited efficacy against adult pulmonary tuberculosis (TB) and safety concerns regarding its use in HIV-infected individuals. Heterologous boosting of BCG-primed immunity will likely be a key component of future multi-component TB vaccine strategies, although promising alternative mycobacterial strains should also be considered.
- A dozen new candidate TB vaccines are currently in clinical trials, with further promising approaches in preclinical development. Vaccines in Phase I or II trials are largely predicated on subunits, either protein fusions or recombinant vector-directed antigens, designed to boost immunity generated by conventional or ‘improved’ recombinant BCG engineered for increased immunogenicity and, in some cases, safety. Ultimately, there must be support for moving the most promising candidates into Phase III efficacy trials.
- Since TB is transmitted primarily as an aerosol, increased attention should be paid to the development of immunization strategies that generate potent local immune responses in the lung tissues and in draining lymph nodes at the primary site of infection, as well as in the circulation. Strong local immunity may also help to prevent dissemination of disease. Current vaccine-boosting vectors with tropism for mucosal surfaces should be optimized as safe and effective pulmonary mucosal immunogens.
- The identification of biomarkers correlating with disease state, or representing protective ‘end points’ will hopefully facilitate development of more effective vaccines targeted against different stages of acute or latent TB infection, or reactivation of disease. These biomarkers may eventually allow more rapid assessment of promising candidates.