Bacille Calmette-Guérin (BCG), the only tuberculosis (TB) vaccine in clinical practice, has limitations in efficacy, immunogenicity and safety. Much current TB vaccine research focuses on engineering live mycobacteria to interfere with phagosome biology and host intracellular pathways including apoptosis and autophagy, with candidates such as BCG Δmp1, BCG aureC::hly, BCG::ESX-1Mmar, Mtb ΔphoS ΔfadD26, Mtb ΔRD1 ΔpanCD and M. smegmatis Δesx-3::esx-3 (Mtb) in the development pipeline. Correlates of protection in preclinical studies include increased central memory CD4+ T cells and recruitment of antigen-specific T cells to the lungs, with mucosal vaccination found to be superior to parenteral vaccination. Finally, recent studies suggest beneficial non-specific effects of BCG on immunity, which should be taken into account when considering these vaccines for BCG replacement.

Addresses
1 Max Planck Institute for Infection Biology, Department of Immunology, Charitéplatz 1, 10117 Berlin, Germany
2 Public Health Research Institute Center at the International Center for Public Health, New Jersey Medical School – Rutgers, The State University of New Jersey, 225 Warren Street, Newark, NJ 07103, USA
Corresponding author: Kaufmann, SHE (kaufmann@mpiib-berlin.mpg.de)
3 These authors contributed equally to this work.

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Introduction
An estimated 10.4 million people developed active tuberculosis (TB) in 2015 [1]. The current global trend will not achieve the World Health Organization’s End TB goal of reducing clinical cases by 90% and fatalities by 95% by 2035 [2]. Current TB control relies on 6–9 months of chemotherapy, causing considerable side effects. The live vaccine Bacille Calmette-Guérin (BCG) (Box 1) protects children from extrapolmonary TB, but does not reliably prevent pulmonary TB or disease transmission and can cause disseminated disease in immunocompromised individuals [3–6]. A vaccine that is safer and more effective is seriously required [2].

BCG lacks several genome segments compared to its virulent ancestor Mycobacterium bovis and to the human tubercle bacillus Mycobacterium tuberculosis (Mtb) [7]. The major attenuating event was the loss of the Region of Difference 1 (RD1), which encodes immunodominant antigens including ESAT6, CFP10 and the unique mycobacterial ESX-1 type VII secretion system (T7SS) [8,9]. Consequently, BCG remains restricted to the phagosome of host cells upon internalization, while many pathogenic mycobacteria, notably Mtb, can escape into the cytosol. Recent data suggest that ESX-dependent lysis of host cell membranes occurs without pore formation through disruptions requiring direct contact with pathogenic mycobacteria [10*].

Although BCG has contributed significantly to global health since its introduction in 1921, the loss of several dominant antigens and key molecular traits of pathogenic mycobacteria during passaging may explain its limitations. This review provides an update on the development of live vaccine candidates designed to replace BCG by providing better protection and improved safety, and discusses recent advances in knowledge on correlates of immunity to Mtb, vaccination routes and non-specific effects of BCG vaccination.

Immunity against pulmonary tuberculosis
Markers such as the cytokine IFN-γ are commonly measured to assess vaccine immunogenicity; however, there is no single correlate of protection against Mtb infection [5,11,12]. Class II restricted CD4+ T cells producing IFN-γ and TNF-α are critical for protection in humans and experimental animals, and class I restricted CD8+ T cells are required for immunity as well [11,13–15]. In preclinical studies, enhanced protection was associated with increased CD4+ central memory T cells (TCM) [15–18]. Accumulation of antigen-specific CD4+ T cells in the lung parenchyma was also associated with protection after vaccination [11,19]. However, inhibition of dendritic cell activation by Mtb greatly interferes with recruitment of vaccine-induced antigen-specific T cells to the lungs [20*]. It is thought that while BCG induces effector memory CD4+ T cells (TEM) that can control acute infection, induction of TCM required for long-term protection is insufficient [15]. Waning protection against Mtb despite BCG persistence corresponds with a decline in functional abilities of CD4+ and CD8+ T cells such as type-1 cytokine production, proliferation and cytolytic potential, and an increase in terminally differentiated, dysfunctional T cells expressing KLRG1, CTLA-4 and IL-10 [21]. Approaches to improve current TB vaccines include replacing BCG with a safer, more effective live
Box 1 From virulent M. bovis to the Bacille Calmette-Guérin (BCG) vaccine

Albert Calmette and Camille Guérin derived BCG from the bovine TB pathogen M. bovis by continuous passaging that led to significant attenuation, demonstrated in animal models [87]. In 1921, the first newborn was immunized with BCG, followed by mass vaccination campaigns. Subsequent studies with more than 100,000 infants not only revealed specific protection against TB but also already indicated nonspecific resistance against general mortality. The original isolate was subcultured in laboratories around the world, giving rise to the variety of genetically distinct BCG strains in clinical practice today [98]. These BCG variants may have differences in immunogenicity and efficacy, but it remains unclear which strain is most efficacious, although even a 1% increase in efficacy could save approximately 18,000 lives and prevent 83,000 cases of disease annually [14]. Having been administered over 4 billion times and with a global coverage exceeding 80%, BCG is the most widely used vaccine worldwide.

mycobacterium candidate and boosting of BCG responses [12]. Boosting during the contraction phase of a BCG response when the memory T cell population is established is more effective than boosting at the peak of the BCG response, which can lead to exhaustion or activation-induced cell death [21,22].

Interference with host cell phagosome biology — a way forward in TB vaccine design?

Upon infection of a host cell, Mtb resides in the hostile environment of the phagosome, inhibiting phagolysosomal fusion [23,24]. Because of ESX-1-dependent membrane disruption, Mtb antigens can enter the cytosol, and the bacteria themselves may later egress into the cytosol [24,25]. Mtb antigens can thus access both major histocompatibility complex (MHC) class I and II antigen presentation pathways and be recognized by CD8+ and CD4+ T-cell subsets respectively [24,26]. However, Mtb can downregulate MHC II expression, so induction of CD4+ T cell responses may be suboptimal [27,28]. The presence of Mtb DNA in the cytosol can be detected by host sensors, which leads to activation of NLRP3 and AIM-2 inflammasomes, release of interferons and increased autophagy and apoptosis [25,29–33], which can ultimately lead to better induction of T cell responses [34]. In contrast, BCG lacks ESX-1 and is killed inside the maturing host phagosome, therefore antigens and bacterial DNA do not enter the cytosol [8,9,35]. Several recombinant BCG vaccine candidates have been engineered to interfere with phagosome biology, in order to overcome the limitations of BCG (Figure 1).

BCG Δzmp1

Pathogenic mycobacteria rely on the zinc metalloprotease 1 encoded by zmp1 to transiently halt phagosome maturation [36]. Deletion of zmp1 in BCG results in immediate more efficient delivery of the live vaccine to the mature phagolysosome which enhances MHC class II-restricted antigen presentation [37]. BCG Δzmp1 demonstrated increased protection, improved safety and better antigen-specific immune responses in animal models compared to BCG [38,39]. This promising candidate is awaiting further evaluation and clinical testing.

BCG ΔureC::hly (VPM1002) and derivatives

Listeriolyisin O (LLO) is a haemolytic (hly) pore-forming protein of the intracellular pathogen Listeria monocytogenes, active at pH 5.5 [40]. Insertion of LLO and
simultaneous deletion of the ureC gene in BCG ΔureC::hly facilitates phagosome acidification, ensuring membrane perturbation by secreted LLO [41]. Consequently, bacterial proteins and DNA enter the host cell cytosol, leading to increased MHC class I-restricted antigen presentation, apoptosis and autophagy, as non-mutual sequelae [35,42–45]. In preclinical trials, BCG ΔureC::hly was more effective in both pre-exposure and post-exposure vaccination compared to BCG and had an improved safety profile [46,47]. BCG ΔureC::hly has demonstrated safety and immunogenicity in adults and in the target population, neonates (https://clinicaltrials.gov, NCT#00749034, NCT#01113281, NCT#01479972) [48]. Notably, BCG ΔureC::hly induced IFN-γ producing and multifunctional T cells and antibody-producing B cells in both BCG immune and BCG naïve adults. In HIV-unexposed newborns, BCG ΔureC::hly vaccination led to increased IL-17 secreting CD8+ T cells and a lower incidence of abscess formation compared to BCG [49]. A phase II trial in HIV-exposed and HIV-unexposed neonates has successfully completed vaccination, and is currently in the follow-up observation phase (NCT#02391415) (L Grode, personal communication). In 2017, a phase III trial in adults who have successfully completed anti-TB chemotherapy will be performed in India to assess the capacity of BCG ΔureC::hly to prevent TB recurrence (L Grode, personal communication).

Second generation derivatives of BCG ΔureC::hly include constructs that secrete cytokines in order to enhance immunogenicity [50], an auxotroph for vitamin B6 deficient in pyridoxine synthase with improved safety [51] and a nuoG-deficient construct that induces increased autophagy and apoptosis [17**]. BCG ΔureC::hly ΔnuoG further revamped protection in mice and maintained the superior safety profile of its parental strain [17**] suggesting that nuoG deletion might also be considered for improvement of other vaccine candidates.

**BCG::ESX-1**

The loss of ESX-1 T7SS is the major reason for attenuation of BCG, but ESX-1 secreted proteins, such as ESAT6, are important antigens and play a role in immune responses. It was shown that ESAT6 was required for IL-18-dependent IFN-γ secretion by CD8+ T cells with unrelated specificity in the acute response to Mtb in vivo [30*]. Stimulating these CD8+ T cells before priming of Mtb specific cells provides early protection. Insertion of Mtb ESX-1 into BCG improved immunogenicity and protection in preclinical models but led to increased virulence [52] that could be attenuated by specific mutations of the ESX-1Mtb secretion system [53]. Recently, the evolutionarily more distant ESX-1 system of the biosafety level-2 organism *Mycobacterium marinum* was used to complement BCG (BCG::ESX-1MmaH*). This construct showed comparable protective efficacy and immunogenicity to BCG::ESX-1Mtb in preclinical models but was as safe as BCG Pasteur [54].

**Mtb ΔphoP ΔfadD26 (MTBVAC)**

Mtb ΔphoP ΔfadD26, derived from the Mtb strain MT103, carries two independent attenuating mutations in the transcription factor *phoP* and the lipid biosynthesis gene *fadD26* [55]. PhoP controls intracellular adaptation of mycobacteria and promotes ESAT-6 secretion [56,57], while *fadD26* is required for synthesis of phthiocerol dimycolates, cell wall lipids related to virulence and intracellular survival [58]. In preclinical evaluations, Mtb ΔphoP ΔfadD26 had comparable safety to BCG but better immunogenicity and protective efficacy [55,59]. This candidate successfully completed a phase I clinical trial in adults (NCT#02013245), demonstrating equal safety to BCG and increased frequencies of polyfunctional CD4+ TCM cells [60]. All participants tested negative in poststudy IFN-γ-release assays, suggesting the absence of ESAT-6 and CFP-10-specific T cells, which renders these assays suitable for monitoring prevention of infection in efficacy trials [60*]. The safety and immunogenicity of *Mtb ΔphoP ΔfadD26* in newborns is currently being evaluated in South Africa (NCT#02729571).

**Mtb ΔRD1 ΔpanCD (mc*6030), Mtb ΔleuD ΔpanCD, Mtb ΔlysA ΔpanCD (mc*6020), Mtb ΔlysA ΔsecA2, M. smegmatis Δesx-3:Δesx-3(Mtb) (IKEPLUS)**

TB vaccine candidates derived from *M. tuberculosis* require stable attenuation to guarantee safety, which can be achieved by disruption of cofactor (*panCD*) and amino acid biosynthesis (*leuD, lysA*) pathways. Candidates with various combinations of these mutations, disruption of *secA2* (encoding an accessory secretion system in mycobacteria) and deletion of RD1 (encoding the ESX-1 T7SS) were evaluated for safety, immunogenicity and efficacy in preclinical models and showed encouraging results [61–65]. The Mtb genome encodes four paralogs of the ESX-1 T7SS, ESX-2 to ESX-5, of which only iron acquisition-related ESX-3 is conserved among *Mycobacterium* species [66,67**]. In the experimental vaccine candidate Immune Killing Evasion Plus (IKEPLUS), the endogenous *esx-3* locus of fast-growing non-pathogenic *M. smegmatis* was replaced by the *esx-3* ortholog of *Mtb* [18]. Mouse studies demonstrated that IKEPLUS induced increased protection against Mtb challenge compared to BCG and remained significantly attenuated [18].

**Mucosal BCG vaccination**

Since its entry into clinical use, BCG has been administered by a variety of routes, first orally, and later by intradermal or percutaneous injection [68]. Taking into account the fact that the primary route of Mtb infection is via inhalation of aerosolized droplets [12], several studies have investigated immune parameters after mucosal vaccination with BCG. After a local infection, T cells migrate...
to the tissue and persist there, differentiating into an important subset of non-circulating memory T cells known as tissue resident memory (TRM) cells [69]. Upon re-infection, these cells mediate rapid protection (Figure 2).

In animal models, mucosal vaccination with BCG by intranasal, intratracheal or aerosol administration is consistently more protective against Mtb challenge than subcutaneous vaccination [70–77]. CD4+ TRM cells were shown to protect against Mtb infection, and control of infection was associated with entry of CD4+ T cells into the lung parenchyma [19,70,74]. Most of the mucosal vaccination studies have used short-term models, and a recent study found that pulmonary protection was enhanced at 2 and 4 months post vaccination in intranasal vaccinated mice compared to the subcutaneous route, but not at 8 and 10 months post vaccination [73]. In macaques, the aerosol route of administration of the Mtb mutant in SigH (Mtb ΔsigH) was highly effective, and was associated with strong CD4+ and CD8+ TCM cell responses in the lungs and the presence of granuloma associated inducible bronchus-associated lymphoid tissue [78]. This highlights the fact that mucosal vaccination could be a powerful route of immunization with novel attenuated mycobacterial strains. The critical next step is to

**Figure 2**

Mucosal vaccination confers increased protection by inducing tissue resident memory T cells in the lungs. (a) Infection by Mtb. (1) Lung dendritic cell (DC) activation and migration is inhibited by Mtb infection, resulting in delayed T cell responses. (2) Owing to inhibition of DC activity, antigens are presented to naive T cells (T0) by bystander DCs in the draining lymph node (LN) rather than by the infected DC. (3) Proliferation and differentiation of antigen-specific T central memory (TCM) and T effector memory (TEM) cells. TCM (KLGR1+) home to the site of infection and produce cytokines. Less differentiated memory T cells (KLGR1-) enter the circulation where they travel to secondary lymphoid organs to establish populations of TCM or, in the presence of inflammation, differentiate into TEM in the tissues. (4) IFN-γ produced by T cells activates alveolar macrophages (AM), inducing Mtb growth inhibition. (b) Mucosal vaccination. (1) Antigens administered by aerosol or intranasal vaccination are taken up by DCs and carried to the draining LN. (2) DCs prime antigen-specific T cells. (3) T cells home to the lung or circulate to secondary lymphoid organs. (4) In the presence of the correct tissue signals, less differentiated KLGR1+ T cells differentiate into TEM cells expressing CD69 and CD103 and remain in the lung. (5) Upon Mtb infection, pre-existing TEM cells are activated and produce cytokines such as IFN-γ, which induces Mtb growth inhibition in AM.

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ascertain whether mucosal vaccination is safe and effective in humans.

**Non-specific protective effects of BCG**

BCG administration approximately halves mortality in the first 6–12 months of life [79], most likely due to stimulation of general immune responses leading to increased resistance to respiratory diseases and neonatal sepsis [80,81]. A trend is seen towards increased monocytes and both pro-inflammatory and anti-inflammatory cytokine secretion in BCG-vaccinated individuals compared to controls, as well as increased IFN-γ production even in unstimulated cells [82]. BCG survives in the mouse for up to 16 months after vaccination [83], and human monocyte responses and production of IFN-γ, IL-17 and IL-22 to non-mycobacterial stimulation remains elevated up to a year post BCG vaccination [84]. This could explain at least in part the observed consistent non-specific effects of BCG on overall mortality [85]. Human primary monocytes primed with BCG showed a switch to glycolysis, accompanied by increased pro-inflammatory and anti-inflammatory cytokine responses upon stimulation with non-related stimuli [85]. The Akt-mTOR signaling pathway was found to be essential for the change in monocyte phenotype, which was associated with epigenetic changes at promoter sites of essential glycolytic genes [86].

Owing to its immunostimulatory properties, BCG is also the standard therapy for preventing recurrence and progression following surgery for non-muscle-invasive bladder cancer [87]. Intravesicular instillation of BCG promotes anti-tumor activity, probably by inducing recruitment of CD4+ T cells, neutrophils and innate lymphocytes and activating immune cells to eliminate urothelial cancerous cells that have internalized BCG [87–90]. Current research aims to identify clinical parameters and biomarkers that can predict individual response to BCG therapy [91,92]. As adverse effects lead to discontinuation of BCG therapy in some patients, BCG Δ ureC:shly is currently being tested as a replacement in Phase II clinical trials (NCT#02371447) (Grode et al., personal communication).

**Conclusions**

Preclinical studies suggest that engineering live vaccine candidates to manipulate host-cell phagosome biology is an effective strategy, and candidates influencing antigen presentation, apoptosis and autophagy dominate the development pipeline [93]. With an increasing number of TB vaccine candidates entering clinical trials, costs are a major concern [94]. Biomarkers may reduce trial size and duration by allowing stratification for individuals at high risk of developing active TB during the trial period. Recently, a biosignature was defined that could predict progression to active TB six to 12 months before onset of clinical disease [95**]. Transcriptomics can also be used to monitor vaccine trials. The development of AERAS-422, a recombinant BCG strain overexpressing three mycobacterial antigens and mutant perfingolysin was recently discontinued after a phase I clinical trial showed development of shingles in 2/8 immunized healthy volunteers due to reactivation of the varicella-zoster virus [96]. A systems biology approach identified correlations with the development of varicella-zoster virus reactivation that can be further investigated. Overall, completed and ongoing vaccine trials have provided insights into correlates for the risk of developing active TB and established platforms for advanced clinical evaluation of vaccine candidates such as recombinant BCGs.

**Note in proof**

A recent study [99] demonstrated that antibodies isolated from healthcare workers provided moderate protection against Mtb in an aerosol mouse model, adding to growing evidence that Mtb-specific antibodies may contribute to prevention of TB, although the role of B cells and antibodies during TB infection remains controversial [99–101].

**Conflicts of interest**

SHEK is co-inventor/patent holder of BCG ΔureC:shly (VPM1002). NN and MG declare to have no conflicts of interest.

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Reports that mycobacterial ESX-1-dependent lysis of host cells requires direct contact with the pathogen and is fundamentally different from the mechanism observed for other bacteria.


18. Shows evidence that targeting the immune response of the BCG JureC::hly vaccine can be further improved by genetic modification.


22. Demonstrates that overwhelming Mtb-induced inhibition of dendritic cell activation at the time of infection leads to near sterilizing immunity in vaccinated mice.


31. Provides evidence for a protective effect of non-cognate Lipophylic IL-18 induced IFN-γ production in acute Mtb infection.


Vaccines


Comprehensive overview on the unique mycobacterial ESX type VII secretion systems and their role in pathogenicity and survival of the pathogen.


75. Price DN, Kusewitt DF, Lino CA, McBride AA, Mutti P: Oral tolerance to environmental mycobacteria interferes with intradermal BCG vaccination.


- Explores molecular mechanisms of BCG-induced trained immunity in monocytes.


- Comprehensive review of BCG for immunotherapy for bladder cancer.


- Defines a biosignature in individuals with latent TB infection predicting progression to active disease 6–12 months in advance of diagnosis.