Tuberculosis remains a major health threat and vaccines better than bacillus Calmette-Guérin (BCG) are urgently required. Here we describe our experience with a recombinant BCG expressing listeriolysin and deficient in urease. This potential replacement vaccine has demonstrated superior efficacy and safety over BCG in Mycobacterium tuberculosis aerosol-challenged mice and was safe in numerous animal models including immune-deficient mice, guinea pigs, rabbits and nonhuman primates. Phase I clinical trials in adults in Germany and South Africa have proven safety and a current Phase IIa trial is under way to assess immunogenicity and safety in its target population, newborns in a high tuberculosis incidence setting, with promising early results. Second-generation candidates are being developed to improve safety and efficacy.

**KEYWORDS:** bacille Calmette–Guérin • listeriolysin O • Mycobacterium tuberculosis • pre-exposure • prevention • prime vaccination • replacement • vaccine • VPM1002

**Tuberculosis: a global problem**

Despite many advances in tuberculosis (TB) control, the TB pandemic continues relentlessly. In 2012, TB was the second leading cause of death from infectious disease worldwide, after HIV, with 8.6 million new cases. HIV–TB coinfection is especially lethal, contributing to 320,000 deaths of the 1.3 million TB-related deaths in 2012 [1]. Childhood TB, which contributes up to 15% of the disease burden globally, has been historically neglected as few cases are smear-positive and as childhood TB has a limited impact on TB transmission [2,3]. Young children are however more prone to devastating forms of TB, including TB meningitis and military TB, with high morbidity and mortality.

Bacillus Calmette–Guérin (BCG), a live attenuated Mycobacterium bovis vaccine, was introduced for the prevention of childhood TB in 1921 [4]. From the 1950s to 1960s, highly effective drugs were developed to cure TB. With improved socioeconomic circumstances and declining incidences in the latter half of the twentieth century, the industrialized world became complacent. However, compounded by the HIV pandemic, migration and increasing drug resistance, TB has re-emerged as a global health concern.

**Immune response to Mycobacterium tuberculosis**

TB, caused by the acid-fast, intracellular bacterial pathogen Mycobacterium tuberculosis, is spread by inhalation of aerosolized droplets and is mostly manifested as pulmonary disease. Involvement of lymph nodes, the CNS, bone and dissemination to other organs may also occur. The interaction between host and pathogen is complex, and a continuum of infection states exists. Clearance of the bacteria by innate immune cells such as macrophages can occur with or without adaptive immune responses. Alternatively, the immune system prevents uncontrolled replication but fails to eradicate the bacterium subclinically, resulting in long-lasting, quiescent infection (often referred to as latent TB infection [LTBI]). Clinical disease occurs where bacterial replication becomes uncontrolled [5,6]. Immunity is cell-mediated with T lymphocytes as critical mediators and mononuclear phagocytes as essential effectors. This holds true for both protection and pathology, which are closely associated with the formation of granulomatous lesions [7–9]. The granuloma, composed primarily of macrophages, dendritic cells, T lymphocytes and B lymphocytes, is the hallmark of TB. The coordinated crosstalk...
between these cells results in a solid granuloma, which contains *M. tuberculosis*. Deterioration of the structure leads to necrosis, followed by liquefaction. Resulting caseous granulomas fail to contain infection and cause severe tissue damage. A delicate and hitherto incompletely understood balance between proinflammatory and regulatory immune responses is required to control the infection [10]. CD4+ T cells, with their ability to produce IFN-γ and other Th1 cytokines [11], and also IL-17-producing T cells [12], regulatory T cells [13] and CD8+ T cells [14], are involved in effective pathogen control without excessive immunopathology usually manifesting as lung cavitation.

**Portfolio of new TB vaccine candidates**

With the realization that BCG can protect against disseminated forms of childhood TB but fails to consistently prevent pulmonary TB in all age groups, attempts have been made to develop more effective vaccines with the prime target being the prevention of pulmonary TB, which is the most prevalent form of disease and results in transmission. All novel vaccination strategies build on BCG by either attempting to replace BCG by more efficacious and safer vaccine candidates, or by boosting BCG-induced immunity with subunit vaccines [15–17]. The so-called replacement vaccines include recombinant (r)BCG VPM1002 and a double-deletion mutant MTBVAC lacking the *PhoP* and the *fadD26* gene loci [18]. A group of subunit vaccines are viral-vectored. They are based on modified vaccinia Ankara virus (MVA) or human adenovirus and express one or more *M. tuberculosis* antigens [17]. Alternatively, protein-adjutant formulations are being developed, which are composed of T-cell-stimulating adjuvants such as IC31 or AS01E and fusion proteins comprising two or more *M. tuberculosis* antigens [17].

**Bacillus Calmette–Guérin**

**History & epidemiology**

The development of BCG started in 1906 when Albert Calmette and Camille Guérin, researchers at the Pasteur Institut in Lille, began attenuating *M. bovis*, the causative agent of bovine TB, closely related to *M. tuberculosis*. *M. bovis* is an intracellular acid-fast bacillus and a member of the *M. tuberculosis* complex [4,19], a term that defines the genetically related mycobacterial species that can cause TB in humans or other organisms. Attenuation was achieved by serial passage of virulent *M. bovis* (isolated by Nocard in 1902) every 3 weeks cultured on potato slices soaked with ox gall. This produced a safe and protective attenuated strain when tested after the 30th passage in 1908. This early vaccine was initially baptized ‘bacille Bilié Calmette–Guérin,’ which was later shortened to ‘BCG’. By 1919, 230 passages had been accomplished and the end product, BCG, proved protective and safe in cattle, the natural host of *M. bovis*, and numerous experimental animals. In 1921, BCG was given orally to an infant born to a mother at first encounter with health services within the first year of life, to infants at risk of *M. tuberculosis* exposure [33]. Vaccination at birth is a pragmatic recommendation, ensuring high vaccination coverage; delayed vaccination in infancy or childhood is a strategy that should still be explored for efficacy and

Also, severe local reactions observed initially after subcutaneous BCG administration were avoided. Mass production of BCG (named BCG Pasteur strain) began at the Institut Pasteur in Lille and was continued in Paris in 1928. Between 1924 and 1928, 114,000 infants had been vaccinated without serious complications. By 1927, Wallgren shifted from subcutaneous to intradermal administration in Sweden; this later became the routine method for BCG administration [20]. BCG cultures had been distributed to different manufacturers for domestic vaccine propagation and production [21–25]. As modern conservation techniques for bacteria were not established in the 1920s, passaging of BCG was continued at the various sites of production, and this gave rise to a number of substrains around the globe, whereas the common ancestor of Calmette and Guérin was lost. Only in modern times with the introduction of affordable genome analysis techniques did it become clear that BCG had undergone a considerable in vitro evolution [24,26]. Virulent *M. bovis* differs from *M. tuberculosis* in eight genome sections, named region of difference (RD); these are RD4–RD11. The major attenuating RD deletion of all BCG substrains is RD1. This genome region includes several important virulence factors required for intracellular survival and egression to the host cell’s cytosol as well as immunodominant antigens ESAT6 and CFP10 [27]. Moreover, the virulence island of RD1 encodes a type VII secretion system, which is operational in pathogenic and nonpathogenic mycobacteria [28,29]. Although several vaccine strains are available, currently only six strains account for more than 90% of worldwide BCG production: BCG Pasteur (1173P2), BCG Copenhagen (1331), BCG Glaxo (1077), BCG Tokyo (172–1), Russian BCG-I and Moreau RDJ strains.

Early observational data produced by Calmette and Guérin suggested drastically reduced mortality in BCG-vaccinated infants [4]. In addition, all-cause mortality in BCG-vaccinated babies was reduced. With increasing use in Europe during the 1920s, consistent protection was observed in student nurses from Norway [30]. However, considerable debate continued regarding the efficacy of BCG during this period and also regarding the statistical analysis of Calmette’s early data [31]. Additionally, concern persisted regarding the safety of injecting infants with live bacteria of undetermined attenuation as varying levels of virulence had been described in the laboratory [32]. BCG vaccination remains controversial in the era of HIV, where concerns regarding risk of serious BCG adverse events in HIV-infected infants have emerged [22].

**Current status of BCG as part of the expanded programme on immunization**

The WHO expanded programme on immunization (EPI) recommends that a single dose of BCG be given once at birth, or at the first encounter with health services within the first year of life, to infants at risk of *M. tuberculosis* exposure [33]. Vaccination at birth is a pragmatic recommendation, ensuring high vaccination coverage; delayed vaccination in infancy or childhood is a strategy that should still be explored for efficacy and
safety considerations. Following its introduction into the EPI in 1974, BCG soon reached global coverage rates exceeding 80% in countries endemic for TB. During 2007, an estimated 89% of children worldwide were vaccinated with BCG. In a recent global survey of BCG vaccination policies and practice, data were obtained from 175 of 208 countries reporting BCG vaccine usage to the WHO. Of the 175 countries, 155 recommended BCG vaccination as a countrywide policy during 2007 [34], BCG is almost universally given to infants in sub-Saharan African countries, where the brunt of the global pediatric HIV burden is concentrated. BCG remains the only licensed vaccine against TB, is inexpensive, and requires only one contact with health services. Despite its impressive global coverage, the nature of BCG protection and the risks of BCG vaccination in HIV-infected children remain subject to ongoing debate. Although BCG protects against miliary TB and TB meningitis, protection is not absolute. For example, in the Western Cape Province of South Africa, where BCG coverage approaches 95%, TB meningitis is currently the most common cause of childhood bacterial meningitis. In an earlier study from the same region, more than 80% of children with TB meningitis had received BCG [35,36].

BCG & HIV

In HIV-infected infants, BCG vaccination results in a spectrum of adverse events, including local BCG disease, regional BCG disease (BCG adenitis), and, in its most severe forms, disseminated BCG disease. The reported frequency of disseminated BCG disease in the pre-HIV era was 0.43 cases per 100,000; the case fatality rate was 0.019–0.02 cases per 100,000 vaccinated infants [37]. In contrast, the incidence of disseminated BCG disease in HIV-infected infants is 992 (95% CI: 567–1495) per 100,000 with mortality >75% in the absence of combination antiretroviral therapy (ART) [38]. Local and regional BCG complications including BCG-immune reconstitution inflammatory syndrome, typically associated with low mortality [39], are frequent with up to 9% of infants developing complications following initiation of ART [40]. Early initiation of ART reduces the risk of BCG-immune reconstitution inflammatory syndrome more than threefold [40].

Effects of BCG on general morbidity & mortality

As already observed by Calmette and Guérin [4], BCG is associated with other nonspecific protective effects. An increasing number of observational studies have provided evidence that BCG may reduce child morbidity and mortality in ways other than TB prevention. For example, trials from West Africa revealed that BCG reduces neonatal mortality by more than 40%, mainly by preventing neonatal sepsis and respiratory infections [41]. In several controlled trials among children and teenagers in the USA and UK in the 1940s and 1950s, BCG reduced nonaccidental deaths unrelated to TB by 25% (95% CI 6–41%) [42]. Studies on BCG’s nonspecific effects on unrelated morbidity and mortality have, however, been contentious [43]. Kleinnijenhuis et al. recently provided evidence that BCG may induce nonspecific resistance to pathogens via epigenetic reprogramming of monocytes [44]. Nonetheless, as the incidence of TB declined, most European countries have removed BCG from their vaccination schedule or restricted it to high-risk groups [45]. Subsequent studies suggest that this policy may have increased the incidence of a wide variety of conditions, including atopic dermatitis [44,46]. These data await further clarification.

VPM1002 (BCG ΔureC:hly)

The rBCG vaccine VPM1002 was generated in the genetic background of BCG Prague. BCG Danish and its close relative BCG Prague both lack the RD2 genome segment, which has been associated with a better safety profile than earlier ancestors such as BCG Russia [47].

The facultative anaerobic bacterium Listeria monocytogenes secretes the pore-forming protein listeriolysin O (Hly), allowing its escape from the phagosome into the cytosol of the infected host cell [48]. Although the underlying mechanisms remain incompletely understood, M. tuberculosis has a functionally similar capacity in its stratagem to avoid elimination by the host. In contrast, BCG is strictly limited to the phagosome of infected cells where it is ultimately eradicated [49]. The BCG vaccine candidate VPM1002 has been enabled to secrete Hly to perforate the phagosomal membrane, allowing mycobacterial antigens to access the host cell’s cytosol, thereby improving antigen presentation [15]. In a first step, Hly expressing BCG was constructed [50]. However, in this recombinant mutant, bioactivity of Hly was suboptimal. Hly is only active at an acidic pH [51]. To counteract phagosomal neutralization by BCG, the gene encoding urease C was deleted. Indeed, the resulting mutant rBCG ΔureC::hly provided the pH optimum for Hly in the phagosome [52]. The most likely interpretation for the observed superior protection of VPM1002 over parental BCG encompasses several mutually nonexclusive mechanisms. Perforation of the phagosomal membrane allows egress of mycobacterial antigens into the cytosol where they can be processed through the major histocompatibility complex pathway for CD8 T-cell priming [50,52]. As VPM1002 itself remains in the phagosome, its antigens also have access to major histocompatibility complex class II processing for priming of CD4 T cells [50,52]. In addition, the acidic pH allows phagosome fusion with lysosomes, at least partially; lysosomal enzymes can then be released into the cytosol and induce apoptosis. The resultant apoptotic vesicles [53] can be engulfed by dendritic cells in the vicinity; this cross-presentation facilitating superior stimulation of both CD4 and CD8 T cells [53,54]. Moreover, VPM1002 not only induces higher Th1 activity, but also Th17 activity in CD4 T cells [55]. Altogether the broader activation of immune mechanisms leads to superior protection of VPM1002.

Preclinical

VPM1002 protects mice against either M. tuberculosis laboratory strain H37Rv or a clinical isolate of the Beijing/W lineage, a genetic variant prevalent in Asia but now also well established
Protection is significant and reproducibly better than that afforded by BCG (FIGURE 1).

The safety profile of VPM1002 was evaluated in various animal models. Mouse mutants deficient in IFN-γ signaling enabled an estimation of risk for disseminated BCG disease as seen in patients with IFN-γ signaling deficiency [56]. Here, VPM1002-vaccinated animals survived more than 100 days of observation, whereas one mouse died in the BCG control group. No general adverse events were observed, although both types of vaccine-induced local reactions occurred at the site of immunization [52].

As VPM1002 could in future be given to immunodeficient HIV-infected individuals, further safety studies were performed in mice with severe combined immunodeficiency in whom B and T lymphocytes do not develop acquired immune responses [52]. An up to 10-fold human target dose (HTD) of $5 \times 10^6$ colony-forming units per animal was administered. The incidence and severity of clinical events observed in these mice were indistinguishable between VPM1002, BCG and saline control. Even the 450-fold HTD remained below the LD$_{50}$ in these severe combined immunodeficiency mice [52].

Three canonical single-dose toxicity studies with VPM1002 were performed in guinea pigs with doses up to the 50-fold HTD. Weight loss, a highly sensitive measure of TB disease, was similar in all treatment groups. No guinea pig died during the study, and no pathological signs of TB were recorded. Hence, the major issues of the European Pharmacopoeia on freeze-dried BCG in terms of virulence were successfully passed.

As a nonrodent model, rabbits were used. They survived inoculation of VPM1002 and BCG at HTD over the whole observation period. All animals showed distinct macroscopic and histologic reactions 2 weeks postimmunization, as expected.

The clinical development plan of VPM1002 includes the vaccination of newborn, the primary target population for TB vaccination. Therefore, a preclinical study in newborn rabbits was performed comparing VPM1002 and BCG for safety aspects such as dissemination to host tissue sites over time [57]. No premature mortality was observed. VPM1002 did not disseminate to liver, brain, lung, spleen or testicles/ovaries up to 90 days after administration. The body weight gain of VPM1002-vaccinated rabbits was not influenced over the course of the
experiment, whereas body weight in the BCG-vaccinated group was reduced compared with the saline control group.

Finally, VPM1002 was tested in nonhuman primates [GRODE ET AL., UNPUBLISHED DATA]: the vaccine was well tolerated over the 17 weeks following intradermal immunization. Aside from mild lesions at the site of vaccination, also observed for BCG, VPM1002 did not cause any adverse events locally and daily observations did not reveal any abnormal behavioral changes. Body weight, hematology, and clinical chemistry parameters gave no evidence for persistent, treatment-related biologically relevant deviation due to vaccination. In summary, a substantial body of evidence demonstrates preclinical safety of VPM1002, thereby paving the way for evaluation in clinical trials.

Clinical studies with VPM1002

In the first Phase I clinical trial VPM1002-GE-1.01TB performed in Germany, healthy male Caucasian adult volunteers with or without pre-exposure to BCG were vaccinated with VPM1002 (n = 30 + 30) or BCG (n = 10 + 10), followed by a 6-month follow-up period [58]. Single vaccination with VPM1002 with up to 5 x 10^5 colony-forming units (HTD) was safe and well tolerated in this study [58]. In this trial, immunogenicity was assessed by IFN-γ production, cellular immune response markers by flow cytometry, and serum antibodies against mycobacterial antigens. In both BCG-naïve and BCG-immune volunteers, VPM1002 induced antigen-specific IFN-γ responses in a dose-dependent way, demonstrating its immunogenicity [58]. Furthermore, vaccination with VPM1002 stimulated antigen-specific CD4 and CD8 T-cell responses. Proportions of double- and triple-positive CD4 and CD8 T cells were increased after VPM1002 vaccination (5 x 10^5 CFU) compared with BCG (BCG Vaccine SSI, Statens Serum Institute) indicative of immune memory [58]. In the second Phase I clinical trial performed in South Africa, VPM1002-ZA-1.10TB, 24 healthy male or female adults previously vaccinated with BCG and predominantly from the indigenous African population were vaccinated. The 6-month follow-up period was recently completed. Findings regarding safety and tolerability over 6 months concurred with those from the first Phase I study [GRODE ET AL., UNPUBLISHED DATA].

As a secondary objective in these studies, the immunogenicity of VPM1002 was assessed. VPM1002 induced cellular immune responses in all IFN-γ-based end points. These end points revealed increased immune responses between postimmunization and baseline. A profound Th1-type immune response was elicited by VPM1002. In addition, a boost vaccination with VPM1002 on pre-existing immunity induced by BCG prime was safe and immunogenic in both studies [GRODE ET AL., UNPUBLISHED DATA].

In a Phase II clinical study, VPM1002-ZA-2.12TB, healthy, HIV-unexposed infants were randomly assigned to vaccination with either VPM1002 or BCG. VPM1002 was safe and well tolerated compared with BCG. Preliminary data analysis suggests that VPM1002 is at least as safe as, and possibly better tolerated than, an equivalent dose of BCG in newborn infants. VPM1002 also elicited a strong T-cell response skewed towards Th1-type immunity similar to what had been report in the previous Phase I studies [GRODE ET AL., UNPUBLISHED DATA].

Other applications: bladder cancer

Apart from its use as a vaccine to prevent TB, BCG has been used for more than 30 years for immunotherapy of bladder cancer [59]. Bladder cancer is the 4th most common cancer in men [60] and the 14th most common cancer in women in Europe (6th most common for both sexes [61]). Today BCG is considered standard of care for intermediate and high-risk nonmuscle invasive bladder cancer (NMIBC) and especially for the highly aggressive form of carcinoma in situ [62]. BCG currently represents one of the most successful immunotherapies in clinical practice and prolongs overall survival compared with surgery alone [63]. Yet, for the patient, BCG therapy has more side effects than intravesical chemotherapy, including local signs of inflammation, for example, urgency, painful urination, or bleeding, and rare severe adverse effects such as disseminated BCG disease [64].

Instillation of BCG into the bladder results in a local infection involving internalization of BCG organisms into healthy tissue and tumor cells in the urothelium. Infected urothelial cells secrete a variety of pro-inflammatory chemokines and cytokines, notably IL-8, which promotes neutrophil recruitment [65]. The conditions necessary for antitumor effects of BCG [66] are ability to develop immunity to mycobacterial antigens, adequate number of live BCG organisms, close contact between BCG and tumor cells and limited tumor burden.

Several elegant strategies were developed to generate an improved rBCG strain for adjuvant treatment of NMIBC, but none are currently in clinical trials. However, the rBCG candidate VPM1002 [52], which is currently in Phase II clinical trial for TB prevention, may have a role in its treatment (see above). This rBCG can trigger a better immune response with improved ability to prevent TB. This altered immune response shown in both animals and humans overlaps with the immune responses needed for an effective immune response to clear NMIBC, in particular, marked increase in type 1 and type 17 cytokines and induction of apoptosis [5255]. Thus, it seems highly desirable to evaluate VPM1002 also for bladder cancer patients. A study design for BCG replacement with VPM1002 has therefore been initiated.

TB vaccine field

Another recombinant BCG-derived vaccine candidate, Aeras422, overexpresses three antigens already encoded in the genome of parental BCG. In addition, the gene encoding perfringolysin O (Pfo), a pore-forming cytolsyn of Clostridium perfringens, had been integrated to mimic effects of Hly in VPM1002 [67]. Both Hly and Pfo are thiol-activated perforins, which polymerize in cholesterol-containing membranes to form pores [51]. However, they differ significantly in two key features related to safety: (i) Hly, but not Pfo, underlies a stringent pH optimum of 5.5 [51]. Hence, the perforating bioactivity of Hly is restricted to an acidic milieu, whereas Pfo bioactivity is uncontrolled. (ii) Pfo remains active in the cytosol of host cells, while Hly is rapidly degraded
in the cytosol by eukaryotic proteasomes due to the four amino acid PEST sequence [68]. Therefore, Hly loses its bioactivity rapidly after entry into the cytosol, whereas P60 remains active in this cell compartment. That both features serve as important inbuilt safety mechanisms of Hly, but not P60, was underlined when Aeras422 Phase I trial had to be terminated prematurely due to major adverse events [69]. At the highest dose administered, some study participants developed shingles, indicating immune reactivation of latent herpes zoster. Another recombinant BCG-based vaccine candidate is the Ag85A-overexpressing rBCG30. Its development is on hold, although it has successfully completed a Phase I safety study [70,71]. Currently, one M. tuberculosis-derived deletion mutant, MTBVAC, is undergoing a Phase I trial, thus far without signs of adverse events [72]. For safety reasons, this vaccine comprises two independent attenuating deletions: PhoP and FadD26. The phoP gene encodes a transcription factor, which controls approx. 80 genes, of which many contribute to virulence of M. tuberculosis; FadD26 is involved in the biosynthesis of phthiocerol dimycocerosates, a class of unique mycobacterial cell wall lipids that are also virulence factors [73,74].

Taken together, these viable vaccines are all considered as replacement for BCG and therefore their main target population is newborns, not yet infected with M. tuberculosis.

Most current vaccine candidates in the clinical pipeline are subunit vaccines, conceived as booster vaccines on top of BCG either before or after M. tuberculosis infection, that is, for pre- or postexposure vaccination, respectively. Among all TB vaccine candidates, the viral-vectorized vaccine MVA85A is the most advanced and has recently completed a Phase Ib efficacy trial in South African newborns [75]. Assessment of data revealed only a mean 17.3% protection of the BCG prime MVA85A boost vaccine over the BCG prime only group, with a range of −31.9% to +48.2% [75]. Thus, the vaccine failed to afford better protection than prime BCG, a finding precipitating numerous discussions about future directions in TB vaccine research and development. It is most likely that MVA85 will not be pursued for newborn vaccination, although a Phase Ib trial in adults with or without M. tuberculosis infection and/or HIV is ongoing, as is a trial using a reverse prime boost strategy in HIV-exposed infants [Heineking, Peer. Comml.] [76].

MVA85A is composed of modified vaccinia Ankara, a potent CD4 T-cell stimulator expressing antigen 85A, shared by M. tuberculosis and BCG. Aside from MVA, adenoviruses are also used as vectors [77,78]. A second group of subunit vaccines are composed of protein-adjuvant formulations. They all comprise fusion proteins of two or more antigens from M. tuberculosis and an adjuvant capable of stimulating cell-mediated immunity. These are the hybrid (H) series, H1, H4 and H56 [79–81]: The H1 and H4 vaccine candidates comprise two antigens of M. tuberculosis and are primarily aimed at pre-exposure vaccination. H4 will continue as pre-exposure vaccine for infants. H56, which will replace H1, has one more antigen rendering it likely suitable for postexposure vaccination of adults. M72 and ID93 are two subunit vaccine candidates primarily conceived as postexposure vaccines for adults and comprise two or four antigens, respectively [82–84]. Principally, the adjuvants harnessed for TB vaccine candidates contain a ligand for a pattern recognition receptor and a compound, which causes a depot effect.

**Further improvements to VPM1002**
VPM1002 was generated by insertion of an Hly-expressing cassette into the 5′ region of the ureC locus [52]. For convenient selection of positive clones, the insert included a hygromycin B resistance marker. Although lateral gene transfer of the antibiotic resistance from VPM1002 to M. tuberculosis during the course of immunization is highly unlikely due to the lack of plasmids for genetic exchange in mycobacteria, we decided to delete the hygromycin B selection cassette from the vaccine. While antibiotic resistance had served as convenient guide for genetic engineering of VPM1002, its removal was technically extremely challenging. Many attempts were required to optimize standard protocols [85] with more than 400 clones being screened before the deletion of hygromycin B resistance was achieved [86]. Genetic modifications and strain integrity have subsequently been confirmed by whole-genome sequencing of VPM1002.

While VPM1002 is successfully moving forward through clinical assessment, an attractive portfolio of genetic vaccine derivatives, which aim to improve on superior performance of their parental strain, have been constructed and are currently undergoing preclinical assessment. Four strategies are being pursued to improve VPM1002:

- Neutralization of the host cell phagosome harboring canonical BCG prevents phagosome maturation towards phagolysosome fusion. In contrast, due to the genetic deletion of the urease C-subunit, VPM1002 allows acidification, thus favoring phagolysosome fusion [18]. Hence, Hly likely remains active in newly formed phagolysosomes, thereby facilitating release of lysosomal enzymes, including cathepsins. In the cytosol, certain cathepsins induce mitochondrial apoptosis [87]. Apoptosis of host cells harboring VPM1002 allows cross-presentation of mycobacterial antigens in adjunct to direct antigen presentation following release of antigens into the host cell cytosol [88,89]. It is assumed that the combined action of both pathways improved immunogenicity of VPM1002 over parental BCG. Still, BCG possesses antipoptotic genes. To further improve host cell apoptosis and thereby antigen cross-presentation induced by VPM1002, we decided to delete secA2 and nuoG, which were reported to prevent programmed cell death in the infected host cell [90,91]. SecA2 is part of an accessory secretion system, and NuoG is a subunit of the mycobacterial NADH dehydrogenase I, an enzyme complex of the respiratory chain [21,92]. Both VPM1002 derivatives, either deficient in secA2 or nuoG, were generated and are currently undergoing preclinical evaluation. In experimental mouse models, we found that nuoG deletion can further improve protective efficacy of VPM1002, while no such effect was seen after secA2 deletion [Gengenbacher et al., unpublished results].
Modulation of immune responses by secretion of human cytokines. IL-7 and IL-18 are implicated in *M. tuberculosis*-induced immunity [93,94]. IL-7, which is mainly produced by stromal cells in the bone marrow, spleen and liver, is involved in recall of adaptive immune responses [95]. In concert with IL-15, IL-7 was demonstrated to improve T-cell memory [96,97]. IL-18 is largely secreted by myeloid-derived cells such as macrophages and dendritic cells via an unconventional mechanism and requires caspase 1 activity for processing and release [98,99]. This cytokine is directly involved in programming of Th1 responses and co-priming along with IL-12 [100]. Human IL-7 and IL-18 are functional in mice [97,101]. We engineered VPM1002 to secrete human IL-7 or IL-18. Both derivative strains of VPM1002 induced a stronger proinflammatory cytokine response in human dendritic cells than their parental strain while maintaining VPM1002’s capacity to improve IL-2 secretion of autologous T cells in coculture with human dendritic cells and to develop a more robust Th1 response than BCG in mice. Moreover, superior safety of and protection by, VPM1002 over BCG remained uncompromised by IL-7 or IL-18 secretion as indicated by vaccine clearance studies and murine *M. tuberculosis* challenge experiments [86].

Improved safety by including auxotrophic mutations. Deletion of the *pdx1* gene rendered *M. tuberculosis* auxotrophic for the essential cofactor vitamin B6 and profoundly compromised its survival and virulence [102]. Several auxotrophic mutations have been evaluated for application in TB vaccinology [103-106]. In general, such mutations improve the safety profile, but fail to improve protection over BCG. We included the vitamin B6 auxotrophic mutation of *pdx1* in VPM1002 to investigate whether superior efficacy of the parental strain could be maintained along with improvement of safety. Further experiments include exploring the possibility to control vaccine efficacy of VPM1002 Δ*pdx1* by a vitamin B6-enriched diet.

Expression of antigens associated with LTBI or TB reactivation. This approach explores the possibility to exploit VPM1002 for postexposure vaccination for the more than two billion individuals with LTBI [1]. We selected antigens that are induced by nutrient starvation (*Rv2659c*) [107,108], oxygen deprivation (*Rv1733c*) [109], or during reactivation of TB in mice (*Rv3407c*) [110]. Overexpression of *Rv2659c*, *Rv1733c* and *Rv3407c* in VPM1002 improved long-term efficacy in mice challenged with a clinical *M. tuberculosis* isolate of the Beijing/W lineage [111]. Future approaches include exploiting VPM1002 as platform for expressing heterologous antigens as done previously for BCG. Experimental rBCG constructs against HIV [112], malaria [113,114], measles [115], papillomavirus [116], pneumococcal pneumonia [117] and Lyme disease [118,119] showed promising results in preclinical models [114,120].

**Combination with booster vaccines**

Typically, new TB vaccine candidates aim to either replace standard BCG as prime immunization or to boost its immunogenicity. The so-called booster vaccines are protein adjuvants or viral vectors expressing mycobacterial antigens. Evaluation of VPM1002 (prime vaccination) and the mycobacterial heparin-binding hemagglutinin adhesin (HBHA) (boost vaccination) [121] in mice is currently ongoing. Earlier attempts to boost immunogenicity of VPM1002 by MVA85A (see above) failed to improve protection in mice challenged with *M. tuberculosis*, although antigen 85A-specific T-cell responses were improved [122]. However, in these experiments, MVA85A also failed to boost protective immunity by BCG prime [122]. Another derivative of VPM1002 expresses H56, a fusion protein of immunodominant antigens Ag85B and ESAT-6, as well as nutrient starvation induced Rv2660c [79]. This construct could serve as appropriate prime vaccine for a heterologous booster with the H56 vaccine.

**Outlook & conclusions**

Increasing rates of drug-resistant TB more than ever call for an efficacious vaccination strategy. Despite all its merits, standard BCG fails to provide a satisfactory solution. Ideally, a novel vaccine would not only prevent active TB in all its forms in all age groups, but also prevent, at least in part, acquisition of stable infection with *M. tuberculosis* so that even LTBI cannot develop. VPM1002 was developed with the goal in mind to improve BCG by genetic modification. Major focus in the preclinical stage was given to efficacy, but it soon became clear that VPM1002 expressed not only higher protection against TB but also higher safety in a variety of highly susceptible experimental animal models. Preclinical safety was confirmed by clinical trials in adults and is currently assessed in infants with promising interim data. Based on its safety profile, VPM1002 may provide a valid candidate for infant vaccination against TB even in HIV-exposed newborn with high risk of adverse responses to canonical BCG. Based on the BCG platform, VPM1002 offers significant advantages for production and delivery, making it well equipped for integration into EPI as replacement for BCG vaccination. Obviously, a BCG replacement vaccine safer than BCG would already offer significant advantages. Yet, the principal goal of VPM1002 remains twofold: higher protective efficacy combined with better safety. Given that financial investment can be secured for a Phase IIb study, the next years will witness whether these high-hanging fruits can be reaped.

**Expert commentary**

TB research and development is witnessing exciting times with many ups and downs. Numerous vaccine candidates have entered clinical trials and numerous novel candidates have successfully completed preclinical assessment. The current vaccine portfolio mainly comprises two arms: first, novel prime vaccines to replace BCG; and second, heterologous booster vaccines to improve BCG-induced immunity. Although these two strategies have often been considered as alternatives, they could also complement each other by forming a combination vaccination comprising a new prime and a novel heterologous booster vaccine. Therefore, the question arises whether vaccine candidates should
be assessed independently in a Phase III trial or whether it would be possible to consider combinations of prime and boost vaccines at this stage. It is tempting to envisage combinations of vaccines that are deemed complementary. These vaccines would then enter a trial not only in a head-to-head comparison but also directly as heterologous prime/boost vaccination. Obviously this approach needs close exchange between all participating stakeholders, including regulatory agencies, inventors, sponsors and the staff involved in the clinical trials. The recent clinical testing of combinations of novel TB drug candidates prior to their licensing could provide a precedent for this model.

In addition to current vaccine candidates in clinical trials, the search for novel vaccine candidates should continue. Modifications of vaccines undergoing clinical assessment are one option. Another valid option is the development of vaccine candidates that induce novel mechanisms that have thus far not been explored. It is hoped that one or two of the vaccine candidates currently undergoing clinical assessment will reach the goal of improved safety, efficacy, or both. Yet, it is unlikely that any of the first generation of vaccine candidates will achieve complete protection. Moreover, it is possible that different target populations, for example, infants vaccinated pre- or postexposure with M. tuberculosis and adults vaccinated postexposure with M. tuberculosis, require different vaccination regimens. An open mind prepared for a variety of novel strategies is therefore called for.

It is desirable that future vaccine trials are harnessed for in-depth information about mechanisms underlying vaccine-induced protection. This could be accomplished by monitoring responses of study participants with sophisticated analytical methodologies, which can retrospectively allow association of specific host responses to the vaccine with successful or failed protection against TB. Likely these analytical tools will comprise global biomarkers and specific immune assays. The next years will reveal whether the current vaccine portfolio comprises a successful candidate or whether we have to reiterate the process with each step being improved by deeper insights from current clinical trials.

**Five-year view**
The development of novel TB vaccines started some 20 years ago mostly in academic laboratories in Europe. This approach resulted in promising protein-adjuvant vaccines, viral-vectorized vaccines and recombinant live vaccines. The development of the rBCG vaccine candidate VPM1002 began in the early 1990s with basic research, which led to clinical assessment some 15 years later. The next years will tell whether any of the candidates developed in the 1990s will ultimately become a licensed TB vaccine. In the best-case scenario, at least one vaccine will show superior efficacy over BCG, and in the worst-case scenario, none of the current candidates in clinical trial will be successful. Based on promising preliminary data of the Phase IIa trial in newborn with VPM1002, it is tempting to speculate that this candidate will progress into a Phase IIb trial to assess its efficacy. Yet, initiation of this trial will depend on many factors, including adequate funding. VPM1002 has recently been licensed to the Serum Institute of India Ltd., raising hope that the future development of this promising vaccine candidate can build on a financially solid basis.

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**Key issues**
- Tuberculosis (TB) remains a major health threat.
- The current vaccine bacille Calmette-Guérin (BCG) only partially protects against extrapulmonary TB in infants.
- New vaccines that protect against pulmonary TB in all age groups are urgently needed.
- Novel TB vaccines are either BCG-replacement vaccines or heterologous boosters on top of BCG.
- VPM1002 is a BCG replacement vaccine.
- VPM1002 is a recombinant BCG with a nonfunctional urease and expressing listeriolysin.
- VPM1002 is more efficacious and safer in preclinical animal models.
- VPM1002 was proven safe and immunogenic in adults.
- VPM1002 is currently undergoing Phase II trial in infants in South Africa, with indications that it is safe in this target population.
The BCG replacement vaccine VPM1002

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