RESEARCH ARTICLE

Dietary Pyridoxine Controls Efficacy of Vitamin B_6-Auxotrophic Tuberculosis Vaccine Bacillus Calmette-Guérin $\Delta ureC::hly$ $\Delta pdx1$ in Mice

Martin Gengenbacher,* Alexis Vogelzang, Stefanie Schuerer, Doris Lazar, Peggy Kaiser, Stefan H. E. Kaufmann

Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany

* Present address: Martin Gengenbacher, National University of Singapore, Yong Loo Lin School of Medicine, Department of Microbiology, Singapore.

ABSTRACT The only tuberculosis (TB) vaccine in use today, bacillus Calmette-Guérin (BCG), provides insufficient protection and can cause adverse events in immunocompromised individuals, such as BCGosis in HIV$^+$ newborns. We previously reported improved preclinical efficacy and safety of the recombinant vaccine candidate BCG $\Delta ureC::hly$, which secretes the pore-forming listeriolysin O of *Listeria monocytogenes*. Here, we evaluate a second-generation construct, BCG $\Delta ureC::hly$ $\Delta pdx1$, which is deficient in pyridoxine synthase, an enzyme that is required for biosynthesis of the essential cofactor vitamin B_6. This candidate was auxotrophic for vitamin B_6 in a concentration-dependent manner, as was its survival *in vivo*. BCG $\Delta ureC::hly$ $\Delta pdx1$ showed markedly restricted dissemination in subcutaneously vaccinated mice, which was ameliorated by dietary supplementation with vitamin B_6. The construct was safer in severe combined immunodeficiency mice than the parental BCG $\Delta ureC::hly$. A prompt innate immune response to vaccination, measured by secretion of interleukin-6, granulocyte colony-stimulating factor, keratinocyte cytokine, and macrophage inflammatory protein-1α, remained independent of vitamin B_6 administration, while acquired immunity, notably stimulation of antigen-specific CD4 T cells, B cells, and memory T cells, was contingent on vitamin B_6 administration. The early protection provided by BCG $\Delta ureC::hly$ $\Delta pdx1$ in a murine *Mycobacterium tuberculosis* aerosol challenge model consistently depended on vitamin B_6 supplementation. Prime-boost vaccination increased protection against the canonical *M. tuberculosis* H37Rv laboratory strain and a clinical isolate of the Beijing/W lineage. We demonstrate that the efficacy of a profoundly attenuated recombinant BCG vaccine construct can be modulated by external administration of a small molecule. This principle fosters the development of safer vaccines required for immunocompromised individuals, notably HIV$^+$ infants.

IMPORTANCE *Mycobacterium tuberculosis* can synthesize the essential cofactor vitamin B_6, while humans depend on dietary supplementation. Unlike the lipophilic vitamins A, D, and E, water-soluble vitamin B_6 is well tolerated at high doses. We generated a vitamin B_6 auxotroph of the phase II clinical tuberculosis vaccine candidate bacillus Calmette-Guérin $\Delta ureC::hly$. The next-generation candidate was profoundly attenuated compared to the parental strain. Adaptive immunity and protection in mice consistently depended on increased dietary vitamin B_6 above the daily required dose. Control of vaccine efficacy via food supplements such as vitamin B_6 could provide a fast track toward improved safety. Safer vaccines are urgently needed for HIV-infected individuals at high risk of adverse events in response to live vaccines.

Vaccination is the most cost-efficient measure for controlling infectious diseases (1), and yet, we lack efficient vaccines for the major infectious diseases AIDS, malaria, and tuberculosis (TB). The only TB vaccine in use today, bacillus Calmette-Guérin (BCG), was introduced into clinical practice more than 90 years ago (2). It resulted from repeated passage of virulent *Mycobacterium bovis*. Although the protection provided by BCG against pulmonary TB is incomplete, it significantly reduces TB-related childhood morbidity and mortality and is relatively safe in immunocompetent individuals (3). The global AIDS pandemic has

raised new challenges: human immunodeficiency virus (HIV)-*Mycobacterium tuberculosis* coinfection accounts for one-fourth of the 1.3 million deaths due to TB annually (4), and BCG vaccination of HIV-infected newborns can result in a disseminated disease with TB-like symptoms named BCGosis (3). Therefore, a major focus of live TB vaccine research and development is improving vaccine safety in immunocompromised individuals.

Novel genetic tools for effective mutagenesis of the mycobacterial genome have paved the way for the development of recombinant live TB vaccines based on attenuated mycobacteria (5, 6).

Auxotrophic mutants have been explored as potential vaccine candidates for more than a decade (7, 8). Although the auxotroph *M. tuberculosis* ΔpanCD, which fails to synthesize vitamin B$_5$ (pantothenic acid), was markedly attenuated, subcutaneous (s.c.) vaccination of mice provided short-term (28 days) protection against *M. tuberculosis* challenge comparable to that afforded by BCG (9). To address safety concerns, notably reversion of a single mutation to full virulence, a second, independent attenuating mutation (*leuD* gene) was included (10). The protection induced by the double auxotroph *M. tuberculosis* ΔleuD ΔpanCD was comparable to that of BCG in guinea pigs, but booster immunization with the same construct did not improve efficacy in this model (10). Several other auxotrophic mutants have been tested as TB vaccines either alone (11, 12) or in combination (13, 14). Although auxotrophic constructs have shown improved safety in preclinical models, they collectively failed to provide better protection than canonical BCG.

Vitamin B$_6$ is a water-soluble essential cofactor for humans, which in humans must be supplied by dietary intake. In contrast, plants, fungi, and bacteria, including *M. tuberculosis*, synthesize pyridoxal-5′-phosphate (PLP), the bioactive form of vitamin B$_6$, from glutamine and derivatives of the carbohydrate metabolism in a two-step reaction catalyzed by Pdx1 (*Rv2606c*) and Pdx2 (*Rv2604c*) (15). Both proteins form a functional class I glutamine amidotransferase (16, 17). The protein sequences and crystal structures of Pdx1 orthologs from *Bacillus subtilis*, *Plasmodium* species, *Saccharomyces cerevisiae*, and *M. tuberculosis* are highly similar, suggesting evolutionary conservation (18–21). Deletion of the *pdx1* gene renders *M. tuberculosis* auxotrophic for vitamin B$_6$ and markedly compromises persistence in mice (15).

We previously engineered BCG to secrete pore-forming listeriolysin O (Hly) of *L. monocytogenes* (22). BCG ΔureC::*hly* (VPM1002) showed superior protection and safety compared to the results for BCG in preclinical models and proved to be safe and immunogenic in humans (22, 23). VPM1002 has successfully completed phase IIa clinical assessment in newborns (http://ClinicalTrials.gov identifier NCT01479972). Here, we deleted *pdx1* in the genetic background of BCG ΔureC::*hly* to further improve its safety. The immunogenicity, protective capacity, and safety of the new construct, BCG ΔureC::*hly* Δpdx1, were evaluated in mice that received either a normal or vitamin B$_6$-enriched diet. Our studies provide an innovative basis for novel vaccination strategies in immunocompromised individuals.

RESULTS

Disruption of *pdx1* renders BCG auxotrophic for vitamin B$_6$. The *pdx1* loss-of-function mutant of *M. tuberculosis* was shown to be fully auxotrophic for vitamin B$_6$ (15). The operon consisting of *pdx1* (also called *snzP*, *Rv2606c*, and *BCG2631c*), *tesB2* (also called *Rv2605c* and *BCG2630c*), and *pdx2* (also called *snoP*, *Rv2604c*, and *BCG2629c*) encodes the functional vitamin B$_6$ synthase complex composed of Pdx1 and Pdx2 and is identical in *M. tuberculosis* and BCG (see Fig. S1 in the supplemental material) (24, 25). Thus, we expected a phenotype for a *pdx1* knockout in BCG similar to that described for *M. tuberculosis* (15). Indeed, disruption of *pdx1* by allelic exchange prevented the growth of BCG ΔureC::*hly* in vitamin B$_6$-free culture medium (Fig. 1A). Replication of bacilli was fully restored when cultures were supplemented with 5 μM B$_6$ vitamer pyridoxine. Lower pyridoxine concentrations resulted in suboptimal growth rates of the *pdx1* mutant, demonstrating
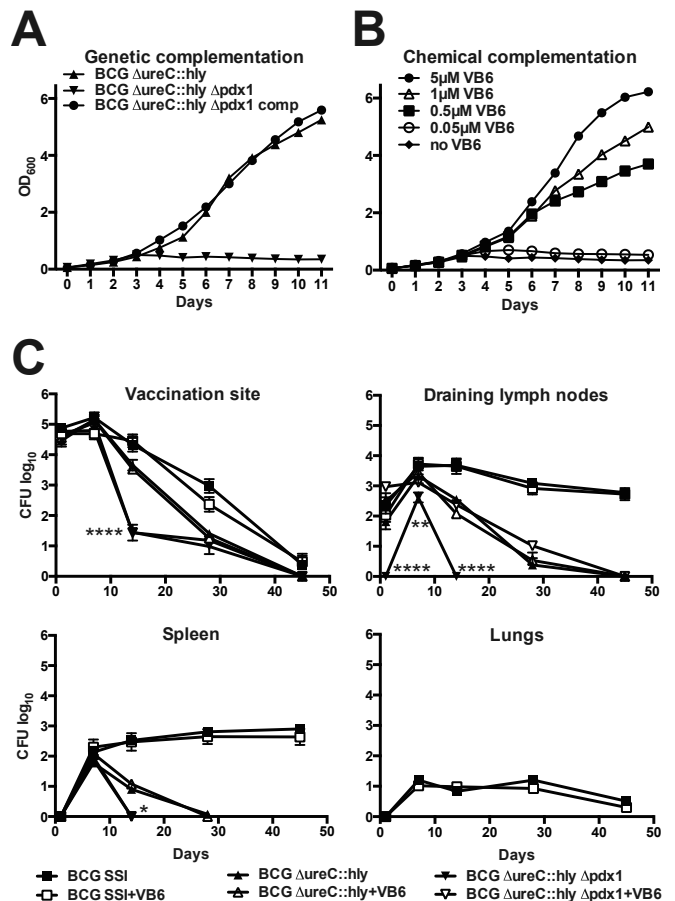

FIG 1 Vitamin B$_6$ controls growth *in vitro* and partially controls survival *in vivo* of the BCG ΔureC::*hly* Δpdx1 auxotroph. Genetic complementation with *pdx1* (A) and chemical complementation by supplementation of Sauton's minimal medium with vitamin B$_6$ (VB6) (B) restored the growth defect of BCG ΔureC::*hly* Δpdx1 *in vitro*. (C) Dissemination and clearance of BCG, BCG ΔureC::*hly*, and BCG ΔureC::*hly* Δpdx1 in mice on a normal diet or vitamin B$_6$-enriched nutrition. Vaccines (10^6 CFU) were administered s.c. at the tail base. Independent from vitamin B$_6$ administration, BCG ΔureC::*hly* and BCG ΔureC::*hly* Δpdx1 were not detected in lungs. Shown are means ± standard deviations (SD) (*n* = 5) analyzed using two-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Significant statistical differences between BCG ΔureC::*hly* and BCG ΔureC::*hly* Δpdx1 groups are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Experiments were performed twice.

concentration-dependent complementation. Moreover, genetic complementation of the mutant with a functional copy of *pdx1* under the control of its native promoter completely reversed the observed growth defect in minimal medium (Fig. 1B). Altogether, deletion of *pdx1* rendered BCG auxotrophic for vitamin B$_6$, and this phenotype could be reverted by chemical and genetic means.

Dissemination and survival of BCG ΔureC::*hly* Δpdx1 in mice is profoundly compromised. The spread and survival of live vaccines in mice provide key information for safety assessment. We examined the bacterial loads at the site of vaccination and in draining lymph nodes (dLNs), spleen, and lungs over 90 days in s.c.-vaccinated mice that received either a standard or vitamin B$_6$-enriched diet. BCG disseminated from the injection site to dLNs and spleen, where the bacterial counts remained relatively stable until the end of the experiment, in contrast to BCG ΔureC::
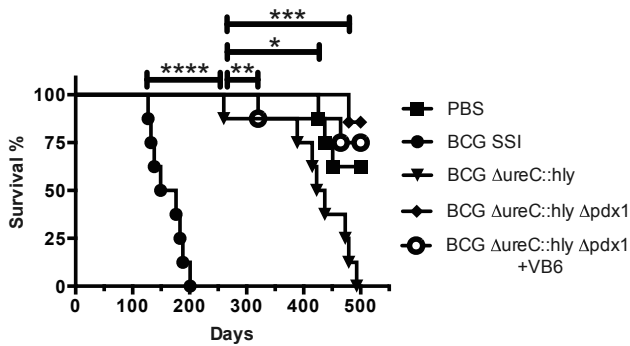
FIG 2 Deletion of *pdx1* improved the superior safety profile of parental BCG Δ*ureC::hly* over that of canonical BCG in SCID mice. Survival of SCID mice s.c. vaccinated with 10^6 CFU of indicated strains was monitored over time. Selected groups received vitamin B$_6$ (VB6)-enriched diet from 2 weeks prior to vaccination to the end of the experiment. Median times of survival were 162.5 days (BCG) and 430 days (BCG Δ*ureC::hly*). Data shown were analyzed using the Mantel-Cox log-rank test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Experiment was performed twice.

hly, which was cleared from these organs after 45 or 28 days, respectively (Fig. 1C). These observations are in line with previous experiments where BCG persisted for up to 120 days in dLNs and 90 days in the spleen, while at 60 days postvaccination, BCG Δ*ureC::hly* was undetectable (26). BCG was the only vaccine tested that disseminated to lungs (Fig. 1C). The survival of BCG and BCG Δ*ureC::hly* in mice remained independent of dietary vitamin B$_6$ levels. Intriguingly, BCG Δ*ureC::hly* Δ*pdx1* did not persist beyond 45 days (vaccination site) or 14 days (dLNs and spleen) (Fig. 1C), suggesting that its attenuation is determined by the *pdx1* deletion rather than *ureC* disruption or Hly expression. Vitamin B$_6$ supplementation restored the persistence of the auxotrophic construct to that of the parental BCG Δ*ureC::hly* in dLNs but not at the site of vaccination or in the spleen (Fig. 1C). We conclude that knockout of *pdx1* profoundly attenuated BCG constructs in mice. Elevated vitamin B$_6$ administration could partially compensate for reduced persistence but did not achieve complete chemical complementation *in vivo*.

BCG Δ*ureC::hly* Δ*pdx1* is safer than parental BCG Δ*ureC:: hly* in immunodeficient mice. Having demonstrated that the deletion of *pdx1* profoundly attenuated BCG in immunocompetent mice (Fig. 1), we went on to determine the safety of BCG Δ*ureC:: hly* Δ*pdx1* in mice with severe combined immunodeficiency (SCID). Groups of animals were s.c. vaccinated with 10^6 CFU of the constructs under investigation. The SCID mice receiving the parental BCG Δ*ureC::hly* survived significantly longer than the BCG-vaccinated group, confirming the superior safety of the recombinant construct (Fig. 2) (22). When the experiment was terminated 500 days postvaccination, the survival of groups that received BCG Δ*ureC::hly* Δ*pdx1* (with or without vitamin B$_6$ supplementation) or phosphate-buffered saline (PBS) was significantly improved over that of mice receiving BCG Δ*ureC::hly*, demonstrating profound attenuation of the auxotrophic construct in immunodeficient mice (Fig. 2).

Vitamin B$_6$ controls acquired but not innate immunity induced by BCG Δ*ureC::hly* Δ*pdx1*. The systemic innate immune responses measured by cytokine secretion were independent of dietary vitamin B$_6$ levels (Fig. 3A). However, 24 h following inoculation, BCG Δ*ureC::hly* Δ*pdx1* showed a more pronounced stim-

ulation of the inflammatory mediators interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), keratinocyte cytokine (KC), and macrophage inflammatory protein-1α (MIP-1α) than BCG SSI 1331 and BCG Δ*ureC::hly*, which depended on *pdx1* deletion (G-CSF and MIP-1α) or vitamin B$_6$ supplementation (IL-6 and KC) (Fig. 3A). In order to detect mycobacterium-specific T cells, we stimulated splenocytes with antigen *ex vivo*, in the presence of a CD40-blocking monoclonal antibody. Antigen-specific CD4 T cells, which consequently upregulated CD154 under these conditions, were then enumerated by flow cytometry and compared to those from naive controls (27). Twenty-eight days postvaccination, the absolute numbers and proportions of antigen-specific CD4 T cells were increased in mice vaccinated with BCG Δ*ureC::hly*, as well as in mice vaccinated with BCG Δ*ureC::hly* Δ*pdx1* under vitamin B$_6$ supplementation (Fig. 3B and C). The proportion of CD4 T cells secreting gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) was also increased in mice vaccinated with BCG Δ*ureC::hly* and in the supplemented auxotrophic-strain-vaccinated groups compared to the proportion in naive mice (Fig. 3B). This immune response persisted for 3 months postvaccination. We conclude that the improved survival of the auxotrophic BCG Δ*ureC::hly* Δ*pdx1* strain due to the administration of vitamin B$_6$ supported the generation of memory T cells, which persist after clearance of the vaccine strain (Fig. 1C and 3D). Vaccination also generated long-lived, IgG-secreting plasma cells, which homed to bone marrow. These cells were detected 3 months postvaccination with either BCG Δ*ureC::hly* or vitamin B$_6$-supplemented BCG Δ*ureC::hly* Δ*pdx1* (Fig. 3E). Thus, adaptive immunity, particularly the generation of antigen-specific CD4 T cells and B cells in response to mycobacterial antigens, depended on the persistence of the vaccine strains, and the persistence of the auxotrophic strain could be regulated by exogenous administration of vitamin B$_6$.

Vitamin B$_6$ controls early protection against *M. tuberculosis* in BCG Δ*ureC::hly* Δ*pdx1*-vaccinated mice. In order to assess the protective capacity of BCG Δ*ureC::hly* Δ*pdx1*, we aerosol challenged mice with the *M. tuberculosis* laboratory strain H37Rv 90 days postvaccination. The bacterial burdens in lungs and spleens were determined at 30 and 180 days postinfection (Fig. 4A). As in previous experiments (23, 26), BCG Δ*ureC::hly* consistently induced better protection than BCG (Fig. 4B). The early protection provided by BCG Δ*ureC::hly* Δ*pdx1* depended on vitamin B$_6$ supplementation and was comparable to that induced by BCG but was completely lost at 180 days postinfection (Fig. 4B). Presumably, the vitamin B$_6$-dependent persistence of the construct in dLNs and spleen (Fig. 1C) was not sufficient to induce long-lasting protection. We conclude that a single immunization with BCG Δ*ureC::hly* Δ*pdx1* does not suffice for long-term protection against aerogenic *M. tuberculosis* infection in mice.

Prime-boost vaccination improves the efficacy of BCG Δ*ureC::hly* Δ*pdx1*. The fast clearance of the auxotrophic vaccine construct from mouse organs (Fig. 1C) led us to hypothesize that the protective capacity of BCG Δ*ureC::hly* Δ*pdx1* could be improved by homologous prime-boost immunization. After vaccination, mice were aerosol challenged with the *M. tuberculosis* laboratory strain H37Rv or bacilli of the clinically relevant Beijing/W lineage (28). Mice vaccinated with BCG Δ*ureC::hly* Δ*pdx1* and fed a normal diet showed bacterial burdens in lungs equal to those of animals vaccinated with BCG (Fig. 5A and B). The early protection (day 30) in lungs provided by the auxotrophic strain consis-
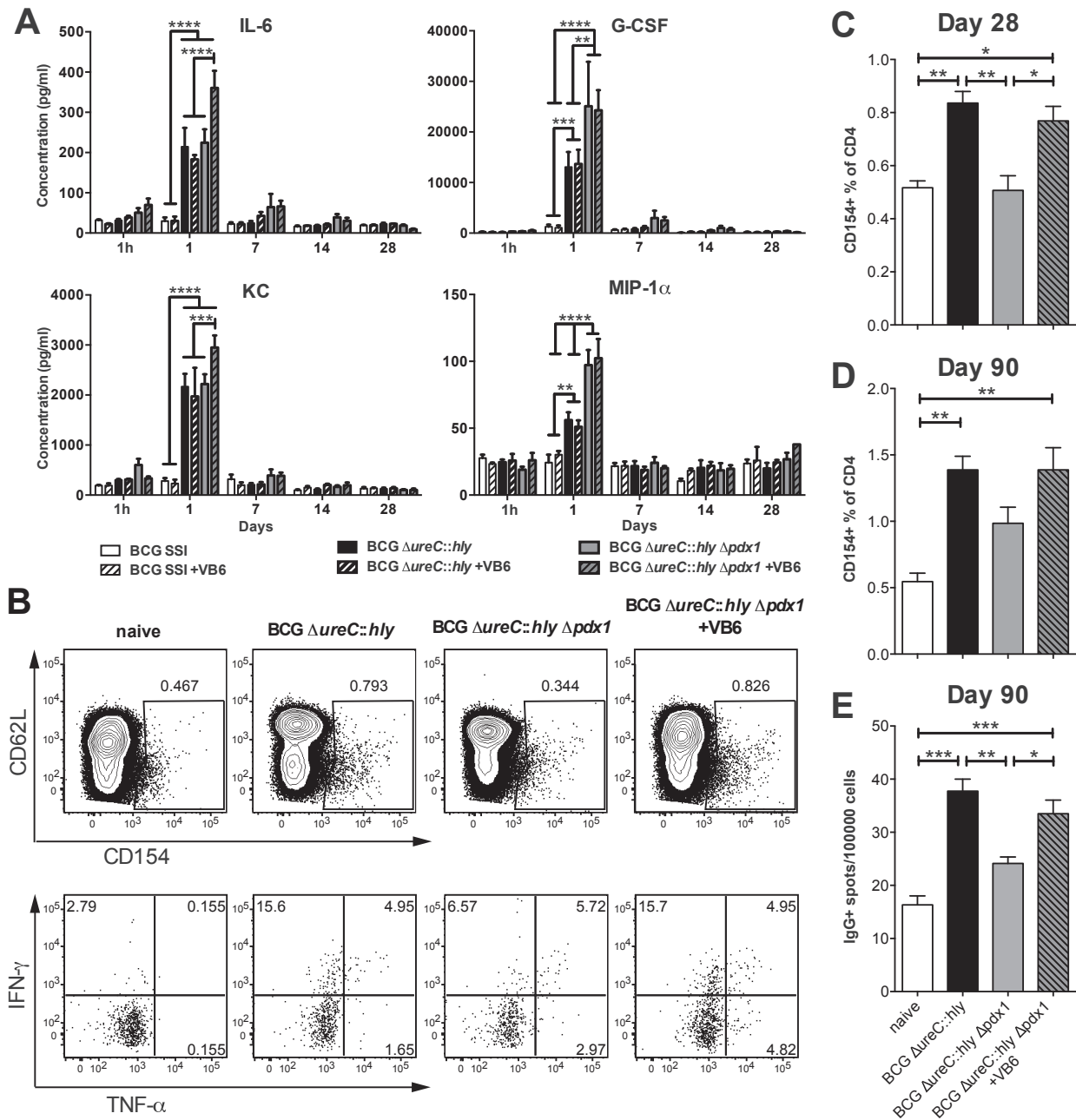
FIG 3 Dietary vitamin B_6 supplementation restores adaptive immune responses in BCG $\Delta ureC::hly$ $\Delta pdx1$-vaccinated mice to levels in parental BCG $\Delta ureC::hly$-vaccinated mice. (A) Influence of vitamin B_6 (VB6) on serum cytokine responses following immunization with indicated constructs. Significant differences were only observed 1 day postvaccination. Shown are means $\pm$ standard errors of the means (SEM) ($n = 5$) analyzed using two-way ANOVA and Tukey's posttest. (B) Representative flow cytometry results showing percentages of antigen-specific CD154$^+$ T cells among CD3$^+$ CD4$^+$ T cells and levels of intracellular IFN-γ and TNF-α in spleen after *in vitro* culture with *M. tuberculosis* lysate 28 days postvaccination. (C and D) Graphs showing proportions of antigen-specific CD154$^+$ T cells among total splenic CD3$^+$ CD4$^+$ cultured as in B, at 28 or 90 days postvaccination. (E) The proportion of IgG-secreting cells in bone marrow measured by ELISPOT 3 months postvaccination. One-way ANOVA followed by Bonferroni's posttest was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$ or 4. Representative data from two independent experiments are shown.

tently depended on vitamin B_6-enriched nutrition and, for Beijing/W-infected groups, was equal to the protection provided by the parental BCG $\Delta ureC::hly$ and significantly better than that of BCG (Fig. 5A). Homologous prime-boost vaccination with BCG $\Delta ureC::hly$ $\Delta pdx1$ improved the long-term efficacy against *M. tuberculosis* H37Rv to the level afforded by BCG and per-

formed significantly better than the mock-vaccinated control group against Beijing/W infection (Fig. 5A and B). The protection of the lung at late time points (180 days for *M. tuberculosis* H37Rv and 160 days for *M. tuberculosis* Beijing/W) remained independent from vitamin B_6 supplementation (Fig. 5A and B). The results obtained from the spleen were less pronounced and often
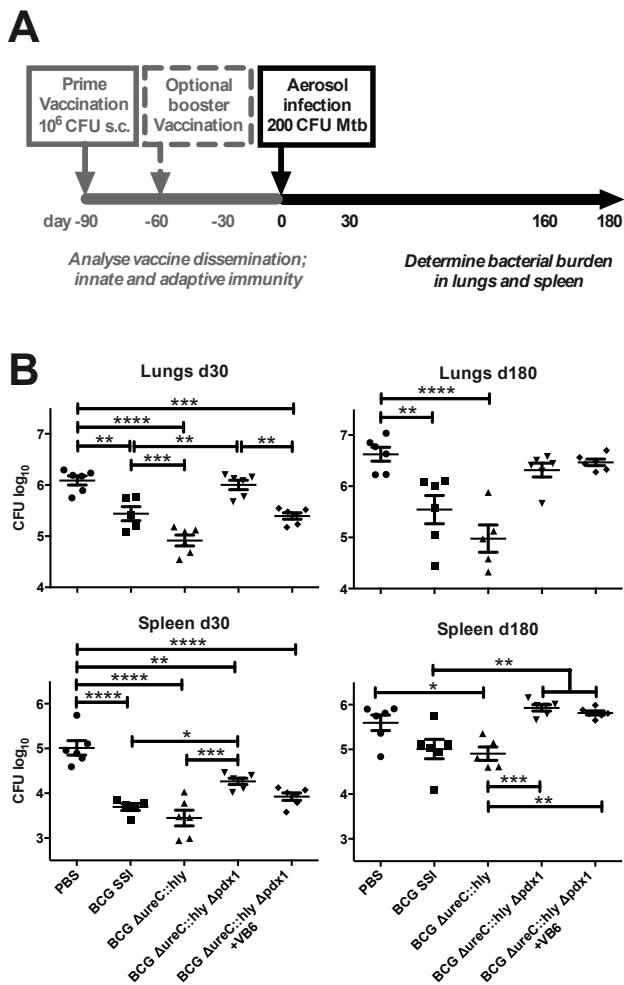
FIG 4 Early protection induced by primary BCG Δ*ureC*::*hly* Δ*pdx1* vaccination depends on vitamin B$_6$ supplementation of mice. (A) Schematic design of protection studies. (B) Impact of vitamin B$_6$ on bacterial burdens in lungs and spleen after *M. tuberculosis* aerosol infection of mice that received primary vaccination only. Groups of mice received vitamin B$_6$ (VB6)-enriched diet as indicated starting 2 weeks prior to vaccination until *M. tuberculosis* challenge. Shown are means ± SEM (*n* = 5 or 6) analyzed using one-way ANOVA and Tukey's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data from one experiment representative of two independent biological replicates are shown.

lacked statistical significance. Taken together, homologous prime-boost immunization improved the efficacy afforded by BCG Δ*ureC*::*hly* Δ*pdx1* compared to that of single prime vaccination and consistently depended on a dietary vitamin B$_6$ supply at early time points following *M. tuberculosis* challenge.

DISCUSSION

The increasing realization that the current BCG vaccine provides insufficient protection against TB, even in newborns, prompted us to develop a recombinant BCG, BCG Δ*ureC*::*hly*, which had improved efficacy and safety in preclinical models and is currently undergoing phase II clinical assessment (29). The increasing proportion of immunocompromised vaccinees has now led us to further modify this construct toward a superior safety profile by rendering it auxotrophic for vitamin B$_6$. Several auxotrophic

mycobacterial mutants have been described (15, 30). Due to their inability to synthesize certain amino acids or cofactors, such mutants rely on uptake of the missing factor in order to survive. During infection, mycobacteria reside in the phagosome of antigen-presenting cells, notably, macrophages (31). The phagosome is an organelle specialized in the eradication of microbial intruders and, therefore, poor in nutrients but rich in reactive radicals and hydrogen ions (31). Pathogenic mycobacteria can survive and replicate in this hostile environment and eventually egress to the host cell cytosol (32, 33). In contrast, auxotrophic mutants of *M. tuberculosis* are severely attenuated *in vivo*, demonstrating that infected host cells do not provide sufficient concentrations of the respective factors to overcome deficient persistence (8, 9, 11, 15).

The cytosolic concentration of PLP, the bioactive form of vitamin B$_6$, in eukaryotic cells is regulated by "metabolic trapping," a process in which the uncharged vitamers become phosphorylated upon entering cells by passive diffusion (34). As a result, leakage of the negatively charged B$_6$ vitamer phosphoesters through biological membranes is prevented. Indeed, PLP could not restore the growth of *M. tuberculosis* Δ*pdx1* in cultures, confirming the absence of a dedicated uptake system (15). As a corollary, mycobacterial *pdx1* mutants are attenuated in mice (Fig. 1C) (15). Intriguingly, when we fed mice an ~10-fold daily dose of vitamin B$_6$— more than 10,000-fold below the 50% lethal dose reported for mice (35)—the persistence of BCG Δ*ureC*::*hly* Δ*pdx1* in dLNs improved and the vaccine candidate was as immunogenic as its parental BCG Δ*ureC*::*hly* and superior to BCG (Fig. 1C). Clearance of the auxotrophic strain at the site of vaccination and in the spleen was independent of vitamin B$_6$ supplementation, suggesting that the availability of the cofactor could not be raised by elevated uptake into these organs. Intriguingly, the superiority of the safety profile of BCG Δ*ureC*::*hly* over that of canonical BCG in SCID mice that serve as an immunodeficiency model was further improved by *pdx1* deletion and remained independent of vitamin B$_6$ supplementation (Fig. 2).

The assessment of blood serum cytokine concentrations as indicators of innate immunity suggests a proinflammatory phenotype for BCG Δ*ureC*::*hly* Δ*pdx1* compared to that of its parental strain (Fig. 3A). While the IL-6 and KC release induced by the auxotrophic strain depended on a vitamin B$_6$-enriched diet, the increased levels of G-CSF and MIP-1α were related to the *pdx1* mutation. Vitamin B$_6$ supplementation left these innate immune parameters in response to BCG or BCG Δ*ureC*::*hly* unaffected (Fig. 3A), thus linking our observations to the persistence of the auxotrophic construct. While inflammatory mediators of innate immunity were stimulated by the attenuated strain, long-lasting antigen-specific T cell responses were only induced when bacterial persistence had been re-established by vitamin B$_6$ supplementation. This is particularly relevant as CD4 T cell responses are thought to be an important component of protection against pulmonary TB (36) and are frequently harnessed as biomarkers of vaccine efficacy (37, 38). Although the role of antibodies in protection remains controversial, the ability of vitamin B$_6$ supplementation to induce long-lived plasma cells supports the importance of vaccine persistence for effective stimulation of adaptive immune responses.

The blood serum concentrations of B6 vitamers (pyridoxal 5-phosphate, 5 to 111 nM; 4 to pyridoxic acid, 6 to 93 nM; and pyridoxal, 3 nM) vary considerably in humans on a normal diet
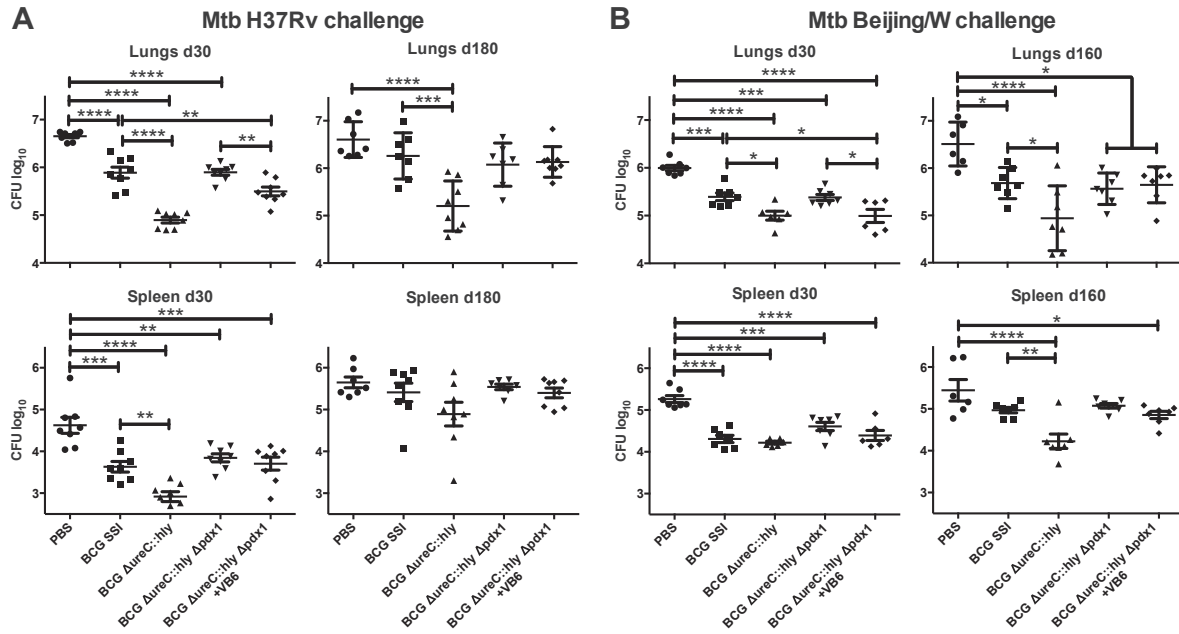
FIG 5 Homologous prime-boost vaccination with BCG Δ*ureC*::*hly* Δ*pdx1* improves protection and maintains early vitamin B_6 dependence. Mice vaccinated with the auxotrophic construct received a homologous booster immunization after 30 days (Fig. 4A). Animals were aerosol infected with the *M. tuberculosis* laboratory strain H37Rv (A) or a clinical isolate of the Beijing/W lineage (B). Groups of mice received vitamin B_6 (VB6)-enriched diet as indicated starting 2 weeks prior to vaccination until *M. tuberculosis* challenge. Bacterial burdens of lungs and spleen were assessed 30 and 180 (or 160) days postchallenge. Shown are means ± SEM (n = 6-8) analyzed using one-way ANOVA and Tukey's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data from one experiment representative of two independent biological replicates are shown.

but stabilize (pyridoxal 5-phosphate, ~350 nM; 4-pyridoxic acid, ~1,000 nM; and pyridoxal, ~700 nM) 3 days after daily intake of 40 mg of vitamin B_6 (39). Approximately 70 to 80% of overall vitamin B_6 is found bound to glycogen phosphorylase in muscles (40). The upper tolerated dose of the vitamin in adults is 100 mg/day (41). Vitamin B_6 deficiency affects innate and acquired immune responses, but physiological functions can be restored by standard supplementation with the cofactor, while larger doses were not more beneficial (42). Accordingly, increasing dietary vitamin B_6 supplementation beyond sufficient doses did not influence either the protective efficacy of BCG and BCG Δ*ureC*::*hly* in a murine challenge model or the course of *M. tuberculosis* infection *per se* in naive mice (see Fig. S2 and S3 in the supplemental material). Therefore, the early protection induced by BCG Δ*ureC*::*hly* Δ*pdx1* consistently depended on the persistence of the construct under the control of vitamin B_6 supplementation (Fig. 4B). Mice immunized with the auxotrophic strain in a homologous prime-boost vaccination scheme (Fig. 4A) maintained the vitamin B_6-related differences but revealed improved protection, to a degree similar to that of parental BCG Δ*ureC*::*hly* 30 days postchallenge with *M. tuberculosis* Beijing/W (Fig. 5A and B). Whether protection could be further improved by a second booster vaccination remains the topic of future studies.

Altogether, we demonstrate for the first time that a nutritional supplement, namely, the small-molecule vitamin B_6, controls the persistence of a recombinant BCG auxotroph *in vivo*. Most importantly, this translated into vitamin B_6 dependency of acquired early immune protection in response to the auxotrophic construct. This principle will enable the development of safer BCG vaccines that are urgently needed for the growing proportion of immunocompromised individuals among vaccinees.

MATERIALS AND METHODS

Bacterial strains and growth conditions. BCG (BCG SSI 1331; ATCC 35533; American Type Culture Collection), BCG Δ*ureC*::*hly* (BCG Prague background) (22, 23), *M. tuberculosis* H37Rv (ATCC 27294), and Beijing/W (RIVM 17919, isolated in Mongolia) were maintained in Middlebrook 7H9 medium (Becton, Dickinson) supplemented with 0.2% glycerol, 0.05% Tween 80, 10% albumin-dextrose-catalase supplement (Becton, Dickinson), in Sauton's minimal medium (0.5 g/liter KH_2PO_4, 0.5 $MgSO_4$, 2 g/liter citric acid, 0.05 g/liter ferric ammonium citrate, 4 g/liter asparagine, 6% glycerol, 0.05% Tween 80, pH 6.8), or on Middlebrook 7H11 agar (Becton, Dickinson) containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment (Becton, Dickinson) and 0.2% glycerol. Mycobacterial cultures were grown to mid-log phase in 1-liter roller bottles (450 cm²) at 37°C and 2 rpm. For *in vitro* growth studies, mycobacteria were grown in Sauton's minimal medium supplemented with 5 µM pyridoxine. Cultures were pelleted at 3,200 rpm and washed three times prior to resuspension in fresh Sauton's medium with or without pyridoxine. The optical density at 600 nm (OD_{600}) was determined daily. For vaccine stock preparations, bacilli were collected by centrifugation at 3,200 rpm, washed with phosphate-buffered saline (PBS), and stored at −80°C as a suspension in PBS--10% glycerol. Prior to vaccination, vials were thawed and cells harvested and resuspended in an appropriate volume of PBS. For CFU enumeration, serial dilutions were performed in PBS--0.05% Tween 80 (PBST) and plated on Middlebrook 7H11 agar. The plates were incubated at 37°C for 3 to 4 weeks prior to CFU counting.

Generation of BCG Δ*ureC*::*hly* Δ*pdx1* and genetic complementation. The *pdx1* gene of BCG Δ*ureC*::*hly* was disrupted as described earlier for *M. tuberculosis* (15). Briefly, 1-kb fragments flanking *pdx1* were amplified by PCR using the specific oligonucleotides ko5′pdx1.fwd (5′ TAC TTAAGCGGGTCAGCGGGCATTCC 3′)/ko5′pdx1.rev (5′ ATTCTAGA CCGGGGTGACAACGTCCATGAT 3′) and ko3′pdx1.fwd (5′ TAAAGC TTTGTGCTGGCCAAGGTGTCG 3′)/ko3′pdx1.rev (5′ ATACTAGTCG

ACCCGTGGAACGCTCACAG 3′) (restriction sites are underlined) and inserted into pYUB854 (43). The knockout plasmid was then electroporated into BCG Δ*ureC*::*hly*, and transformants were selected on Middlebrook 7H11 agar (contains vitamin B$_6$) supplemented with 80 μg/ml hygromycin B (Roche). The hygromycin resistance cassette was subsequently removed by standard methods described previously (44). Site-directed mutagenesis and selection marker removal were confirmed by automated sequencing of the *pdx1* region. For genetic complementation, *pdx1*, including its putative promoter, was amplified by PCR (comppdx1.fwd [5′ GCTGGTACCAGGGAAAGGTTGCCGATG 3′] and comppdx1.rev [5′ GCTGGTACCAGGGAAAGGTTGCCGATG 3′]) and inserted into the integrative vector pMV306 (45). BCG Δ*ureC*::*hly* Δ*pdx1* was then electroporated with the complementing vector, and transformants were selected on Middlebrook 7H11 agar containing 25 μg/ml kanamycin (Sigma-Aldrich).

Multiplex cytokine assays. The Bio-Rad mouse cytokine 23-plex panel was used for analysis of cytokines in sera of vaccinated mice. In all multiplex assays, the volume of the coupled beads, detection antibodies, and streptavidin-phycoerythrin (PE) conjugate was halved and topped up with the appropriate buffer. The assays were otherwise performed according to the manufacturer's instructions. The assay plates were read using a Bio-Plex 200 instrument (Bio-Rad).

Flow cytometry. Cytokine-secreting antigen-specific T cells were enumerated as described previously (27). In brief, single-cell suspensions of splenocytes were cultured in complete RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (Sigma) for 14 h along with 10 μg/ml *M. tuberculosis* H37Rv whole-cell lysate in the presence of 2 μg/ml anti-CD40 blocking antibody (HM40-3; BioLegend) and 0.5 μg/ml PE-conjugated anti-CD154 (clone MR1; BioLegend). Ten micrograms/milliliter Brefeldin A (eBioscience) was added for the final 3 h of culture. After stimulation, cells were washed and stained on ice in PBS--2% FCS with fluorescent antibodies against cell surface markers CD4, CD3, CD8 (BD Biosciences), and CD62L (BioLegend), followed by fixation and permeabilization with the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Intracellular cytokines were detected with antibodies against IFN-γ and TNF-α (BD Biosciences). Cells were analyzed on an LSRII cytometer using Diva (Becton, Dickinson) and FlowJo software (Tree Star).

Enzyme-linked immunosorbent spot assay (ELISPOT). Ninety-six-well multiscreen filtration plates (Millipore) were soaked with 30% ethanol and then rinsed and coated overnight at 4°C with 5 μg/ml polyclonal goat anti-mouse IgM+IgG+IgA (Millipore). The following day, the plates were washed and blocked with PBS--3% bovine serum albumin for 2 h at 37°C. Serial dilutions of single-cell suspensions of bone marrow were cultured on plates for 3 h at 37°C in medium prepared as described above. Cells were washed off, and the plates incubated with anti-mouse IgG-alkaline phosphatase (SouthernBiotech) for 1 h at 37°C. Finally, plates were washed with water before detection of IgG$^+$ spots with 1-Step NBT-BCIP (nitro-blue tetrazolium chloride--5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt) substrate (Thermo Scientific).

Mouse strains and housing conditions. Mice were 9 to 10 weeks old at the beginning of experiments. Female BALB/c (Janvier) were housed in groups of 5 or 6 in individually ventilated cages. Drinking water and food pellets (Ssniff R/M-H autoclavable, 0.032 mg pyridoxine per g) were offered *ad libitum*. Experimental groups that were maintained with vitamin B$_6$-enriched nutrition received drinking water with 0.1 mg/ml pyridoxine hydrochloride (Sigma-Aldrich). Female SCID mice (CB-17/Icr-*Prkdc*-SCID/Rj; Janvier) were kept under sterile conditions. All experimental procedures involving mice were approved by the State Office for Health and Social Services, Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin, LAGeSo). Mice were sacrificed by cervical dislocation, and all efforts were made to minimize suffering and pain.

Dissemination, protective efficacy, and safety of BCG and derivatives in mice. Mice were s.c. immunized in the tail base with 10^6 CFU BCG or recombinant derivative strains. At designated time points postvaccination, mice were sacrificed, and then a 1-cm^2 skin portion at the side of injection and the dLNs, spleen, and lungs of each animal were aseptically removed and homogenized in PBS--0.05% Tween 80 prior to CFU enumeration. For protective efficacy studies, mice were aerosol challenged 90 days postvaccination with 100 to 200 CFU of *M. tuberculosis*. At designated time points, lungs and spleens were aseptically removed, homogenized in PBS--0.05% Tween 80, and plated in serial dilutions onto Middlebrook 7H11 agar for CFU enumeration. After vaccination, SCID mice did not receive further experimental manipulations. Weight was monitored weekly, and mice were euthanized when weight loss exceeded 20%.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01262-14/-/DCSupplemental.

Figure S1, EPS file, 0.3 MB.
Figure S2, EPS file, 0.7 MB.
Figure S3, EPS file, 0.3 MB.

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