Dietary Pyridoxine Controls Efficacy of Vitamin B₆-Auxotrophic Tuberculosis Vaccine Bacillus Calmette-Guérin ΔureC::hly Δpdx1 in Mice

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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 BCGosiosis in HIV⁺ newborns. We previously reported improved preclinical efficacy and safety of the recombinant vaccine candidate BCG ΔureC::hly, which secretes the pore-forming listeriolysin O of Listeria monocytogenes. Here, we evaluate a second-generation construct, BCG ΔureC::hly Δpdx1, which is deficient in pyridoxine synthase, an enzyme that is required for biosynthesis of the essential cofactor vitamin B₆. This candidate was auxotrophic for vitamin B₆ in a concentration-dependent manner, as was its survival in vivo. BCG ΔureC::hly Δpdx1 showed markedly restricted dissemination in subcutaneously vaccinated mice, which was ameliorated by dietary supplementation with vitamin B₆. The construct was safer in severe combined immunodeficiency mice than the parental BCG ΔureC::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 B₆ administration, while acquired immunity, notably stimulation of antigen-specific CD4 T cells, B cells, and memory T cells, was contingent on vitamin B₆ administration. The early protection provided by BCG ΔureC::hly Δpdx1 in a murine Mycobacterium tuberculosis aerosol challenge model consistently depended on vitamin B₆ 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 B₆, while humans depend on dietary supplementation. Unlike the lipophilic vitamins A, D, and E, water-soluble vitamin B₆ is well tolerated at high doses. We generated a vitamin B₆ auxotroph of the phase II clinical tuberculosis vaccine candidate bacillus Calmette-Guérin ΔureC::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 B₆ above the daily required dose. Control of vaccine efficacy via food supplements such as vitamin B₆ 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.
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 B₆ (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 ΔlexD 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 B₆ 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 B₆, 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 B₆ 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 B₆-enriched diet. Our studies provide an innovative basis for novel vaccination strategies in immunocompromised individuals.

**RESULTS**

Disruption of *pdx1* renders BCG auxotrophic for vitamin B₆. The *pdx1* loss-of-function mutant of *M. tuberculosis* was shown to be fully auxotrophic for vitamin B₆ (15). The operon consisting of *pdx1* (also called *snzP*, Rv2606c, and BCG2631c), *tesB2* (also called Rv2605c and BCG2630c), and *pdx2* (also called *snzP*, Rv2604c, and BCG2629c) encodes the functional vitamin B₆ 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 B₆-free culture medium (Fig. 1A). Replication of bacilli was fully restored when cultures were supplemented with 5 μM B₆ vitamin pyridoxine. Lower pyridoxine concentrations resulted in suboptimal growth rates of the *pdx1* mutant, demonstrating 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 B₆, 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 B₆-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::hly Δpdx1*.
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**FIG 2** Deletion of *pdx1* improved the superior safety profile of parental BCG ΔureC::hly over that of canonical BCG in SCID mice. Survival of SCID mice s.c. vaccinated with 10⁶ CFU of indicated strains was monitored over time. Selected groups received vitamin B₆ (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 ΔureC::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 ΔureC::hly was undetectable (26). BCG was the only vaccine tested that disseminated to lungs (Fig. 1C). The survival of BCG and BCG ΔureC::hly in mice remained independent of dietary vitamin B₆ levels. Intriguingly, BCG ΔureC::hly Δpdx1 did not persist beyond 45 days (vaccination site) or 14 days (dLNs and spleen) (Fig. 1C), suggesting that its attenuation is determined by the deletion rather than ureC disruption or Hly expression. Vitamin B₆ supplementation restored the persistence of the auxotrophic construct to that of the parental BCG ΔureC::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 B₆ administration could partially compensate for reduced persistence but did not achieve complete chemical complementation in vivo.

BCG ΔureC::hly Δpdx1 is safer than parental BCG ΔureC::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 ΔureC::hly Δpdx1 in mice with severe combined immunodeficiency (SCID). Groups of animals were s.c. vaccinated with 10⁶ CFU of the constructs under investigation. The SCID mice receiving the parental BCG ΔureC::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 ΔureC::hly Δpdx1 (with or without vitamin B₆ supplementation) or phosphate-buffered saline (PBS) was significantly improved over that of mice receiving BCG ΔureC::hly, demonstrating profound attenuation of the auxotrophic construct in immunodeficient mice (Fig. 2).

Vitamin B₆ controls acquired but not innate immunity induced by BCG ΔureC::hly Δpdx1. The systemic innate immune responses measured by cytokine secretion were independent of dietary vitamin B₆ levels (Fig. 3A). However, 24 h following inoculation, BCG ΔureC::hly Δpdx1 showed a more pronounced stimulation 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 ΔureC::hly, which depended on Δpdx1 deletion (G-CSF and MIP-1α) or vitamin B₆ 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 ΔureC::hly, as well as in mice vaccinated with BCG ΔureC::hly Δpdx1 under vitamin B₆ 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 ΔureC::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 ΔureC::hly Δpdx1 strain due to the administration of vitamin B₆ 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 ΔureC::hly or vitamin B₆-supplemented BCG ΔureC::hly Δpdx1 (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 B₆.

**Vitamin B₆ controls early protection against M. tuberculosis in BCG ΔureC::hly Δpdx1–vaccinated mice.** In order to assess the protective capacity of BCG ΔureC::hly Δpdx1, 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 ΔureC::hly consistently induced better protection than BCG (Fig. 4B). The early protection provided by BCG ΔureC::hly Δpdx1 depended on vitamin B₆ supplementation and was comparable to that induced by BCG but was completely lost at 180 days postinfection (Fig. 4B). Presumably, the vitamin B₆-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 ΔureC::hly Δpdx1 does not suffice for long-term protection against aerosolized M. tuberculosis infection in mice.

**Prime-boost vaccination improves the efficacy of BCG ΔureC::hly Δpdx1.** The fast clearance of the auxotrophic vaccine construct from mouse organs (Fig. 1C) led us to hypothesize that the protective capacity of BCG ΔureC::hly Δpdx1 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 ΔureC::hly Δpdx1 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-
Dietary vitamin B₆ supplementation restores adaptive immune responses in BCG ΔureC::hly Δpdx1-vaccinated mice to levels in parental BCG ΔureC::hly-vaccinated mice. (A) Influence of vitamin B₆ (VB6) on serum cytokine responses following immunization with indicated constructs. Significant differences were only observed 1 day postvaccination. Shown are means ± 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.

Dietary vitamin B₆-enriched nutrition and, for Beijing/W-infected groups, was equal to the protection provided by the parental BCG ΔureC::hly and significantly better than that of BCG (Fig. 5A). Homologous prime-boost vaccination with BCG ΔureC::hly Δpdx1 improved the long-term efficacy against M. tuberculosis H37Rv to the level afforded by BCG and performed 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 B₆ supplementation (Fig. 5A and B). The results obtained from the spleen were less pronounced and often
The increasing realization that the current BCG vaccine provides prime-boost immunization improved the efficacy afforded by vaccination only. Groups of mice received vitamin B6 (VB6)-enriched diet as indicated starting 2 weeks prior to vaccination until completion of the study (Fig. 1C). Intriguingly, when we fed mice an ~10-fold daily dose of vitamin B6—more than 10,000-fold below the 50% lethal dose reported for vitamin B6 (35)—the persistence of BCG ΔureC::hly Δpdx1 in dLNs improved and the vaccine candidate was as immunogenic as its parental BCG ΔureC::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 B6 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 ΔureC::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 B6 supplementation (Fig. 2).

The assessment of blood serum cytokine concentrations as indicators of innate immunity suggests a proinflammatory phenotype for BCG ΔureC::hly Δpdx1 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 B6-enriched diet, the increased levels of G-CSF and MIP-1α were related to the pdx1 mutation. Vitamin B6 supplementation left these innate immune parameters in response to BCG or BCG ΔureC::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 B6 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 B6 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.
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 B6 (39). Approximately 70 to 80% of overall vitamin B6 is found bound to glycogen phosphorylase in muscles (40). The upper tolerated dose of the vitamin in adults is 100 mg/day (41). Vitamin B6 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 B6 supplementation beyond sufficient doses did not influence either the protective efficacy of BCG and BCG ΔureC::hly in a murine challenge model or the course of M. tuberculosis infection per se in naïve mice (see Fig. S2 and S3 in the supplemental material). Therefore, the early protection induced by BCG ΔureC::hly Δpdx1 consistently depended on the persistence of vitamin B6 supplementation (Fig. 4B). Mice immunized with the auxotrophic strain in a homologous prime-boost vaccination scheme (Fig. 4A) maintained the vitamin B6-related differences but revealed improved protection, to a degree similar to that of parental BCG ΔureC::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 B6, controls the persistence of a recombinant BCG auxotroph in vivo. Most importantly, this translated into vitamin B6 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 35733; American Type Culture Collection), BCG ΔureC::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 KH2PO4, 0.5 MgSO4, 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 (OD600) 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 ΔureC::hly Δpdx1 and genetic complementation.** The pdx1 gene of BCG ΔureC::hly was disrupted as described earlier for M. tuberculosis (15). Briefly, 1-kb fragments flanking pdx1 were amplified by PCR using the specific oligonucleotides ko3′pdx1.fwd (5’ TACCTTAAAGCCGGGTCAAGCCGGATTCC 3’) ko3′pdx1.rev (5’ ATCTAGACCGGATCGCGAGGCATTCG 3’) and ko3′pdx1.fwd (5’ TAAAGCCGGGTCAAGCCGGATTCC 3’) ko3′pdx1.rev (5’ ATCTAGACCGGATCGCGAGGCATTCG 3’).
ACCGGTGGAAACGTACAG 3′) (restriction sites are underlined) and inserted into pYUB854 (43). The knockout plasmid was then electroporated into BCG ΔureC::hyg, and transformants were selected on Middlebrook 7H11 agar (contains vitamin B6) 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 pdxI region. For genetic complementation, pdxI, including its putative promoter, was amplified by PCR (comppdx1_fwd [5′-GCTGTTACCAGGGAAGGTTCGGACGTG 3′] and comppdx1_rev [5′-GCTGTTACCAGGGAAGGTTCGGACGTG 3′]) and inserted into the integrative vector PMV306 (45). BCG ΔureC::hyg ΔpdxI 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-phycocerythrin (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 MB1; BioLegend). Ten micrometers/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% etha

**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 (Sniff R/M-H autoclavable, 0.032 mg pyridoxine per g) were offered ad libitum. Experimental groups that were maintained with vitamin B6-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.

**SUPPLEMENTAL MATERIAL**

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

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Vitamin B6 Controls Immunity to Auxotrophic BCG

Dissemination, protective efficacy, and safety of BCG and derivatives in mice. Mice were s.c. immunized in the tail base with 106 CFU BCG or recombinant derivative strains. At designated time points postvaccination, mice were sacrificed, and then a 1-cm2 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%.

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