MTBVCAC-Based TB-HIV Vaccine Is Safe, Elicits HIV-T Cell Responses, and Protects against Mycobacterium tuberculosis in Mice

Esther Brosset,1,2,3,11 Narcís Saubi,4,5,11 Núria Guitart,4 Nacho Aguilo,1,2 Santiago Uranga,1,2 Athina Kilpeläinen,4,6 Yoshiki Eto,4 Tomáš Hanke,7 Jesús Gonzalo-Asensio,1,2,3,11 Carlos Martín,1,2,8,10 and Joan Joseph-Munné4,6,9,10

1Grupo de Genética de Micobacterias, Departamento de Microbiología y Medicina Preventiva, Facultad de Medicina, Universidad de Zaragoza, C/Domingo Miral s/n, Zaragoza 50009, Spain; 2CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain; 3Instituto de Biocomputación y Física de Sistemas Complexos (BIFI), Zaragoza, Spain; 4AIDS Research Group, Hospital Clinic de Barcelona/IDIBAPS-HIVACAT, School of Medicine, University of Barcelona, Barcelona, Catalonia, Spain; 5Red Temática de Investigación Cooperativa en SIDA (RD12/0017/0001), Spanish AIDS Network, Madrid, Spain; 6EAVI2020 European AIDS Vaccine Initiative (BIFI), Zaragoza 50009, Spain; 7CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain; 8Instituto de Biocomputación y Física de Sistemas Complexos (BIFI), Zaragoza, Spain; 9Servei de Malalties Infeccioses, Hospital Clínic de Barcelona, Catalonia, Spain; 10Senior author

INTRODUCTION

Today, tuberculosis (TB) has reached alarming proportions. An estimated 10 million people have developed TB in 2017 and 9% were people living with HIV (72% in Africa). There were an estimated 1.3 million TB deaths among HIV-negative people and an additional 300,000 deaths among HIV-positive people, as reported by the World Health Organization (WHO)1 in 2018. TB is poverty related with a major burden in the poor and developing parts of the world, and it is aggravated by the HIV-AIDS pandemic, which greatly increases the risk of the infection evolving into active TB disease.

HIV-AIDS is a major global public health issue. Between 2010 and 2016, new HIV infections fell by 11% in adults and 47% in children, and AIDS-related deaths fell by 48% since the peak in 2005. This achievement was the result of great efforts by national HIV programs supported by civil society and a range of development partners.2 However, sub-Saharan Africa accounted for 64% of new HIV infections in 2016, and, even though it is encouraging that 1.6 million people are currently receiving treatment in resource-poor settings, ensuring universal access to antiretroviral therapy still represents an enormous challenge.3 Thus, the development of effective, safe, and affordable vaccines against both diseases could have a tremendous impact on public health.

The risk of active TB is estimated to be between 16 and 27 times greater in people living with HIV than among those without HIV infection.4 Mycobacterium bovis bacillus Calmette-Guérin (BCG) has been the only licensed vaccine against TB for more than 90 years,5 but the BCG-induced protective effects against pulmonary disease over all ages are variable.6,7 Nevertheless, BCG vaccination has several beneficial effects: (1) BCG vaccination reduces rates of Mycobacterium tuberculosis (Mtbc) infection, aiding in the decrease of the pool of latent infections from which future cases of active
In this study we have constructed a novel live-attenuated vaccine for HIV-1 and TB that is vectored by a lysine auxotroph of MTBVAC, MTBVAC.HIVA\textsuperscript{2auxo}. This is an innovative approach to develop bivalent TB and HIV vaccines that could be administrated at birth and with the potential to confer protection against both diseases.

**RESULTS**

**Construction and Characterization of a Lysine Auxotroph of MTBVAC, the MTBVAC\textsubscript{Dlys} Strain**

For MTBVAC\textsubscript{Dlys} construction, the previously described recombination-based technique was used.\textsuperscript{27} Rv1293 (lysA) gene, which codes for the last enzyme involved in Lysine (Lys) synthesis,\textsuperscript{28} was inactivated by homologous recombination with a PCR product containing a kanamycin (Km) resistance cassette disturbing the lysA gene (Figure 1A). To ensure proper selection of recombinants, the final MTBVAC transformed with the homologous PCR product lysA-Km was plated on 7H10 complete medium supplemented with Km and Lys. Correct recombination was confirmed by PCR using three different pairs of primers, which amplify the complete recombinant region (Lys-fw/Lys-rv), the upstream (Lys-fw/km-OUT1-rv), or the downstream (km-OUT2-fw/Lys-rv) region (Figure 1B). After MTBVAC\textsubscript{Dlys} construction, Lys auxotrophy was confirmed by plating on 7H10-ADC with and without Lys supplementation (Figure 1C) and also by colony-forming unit (CFU) enumeration after removing Lys from medium (data not shown). Results showed the absence of MTBVAC\textsubscript{Dlys} growth in non-lysine-supplemented plates, whereas the MTBVAC strain grew at a similar level in both supplemented and non-supplemented plates. Accordingly, this auxotropic MTBVAC\textsubscript{Dlys} strain was used in the subsequent experiments to generate recombinant vaccines expressing the HIVA immunogen.

**Construction and Characterization of the MTBVAC.HIVA\textsuperscript{2auxo} Vaccine Strain**

The plasmid p2auxo.HIVA (Figure 2A)\textsuperscript{36} was transformed into the MTBVAC\textsubscript{Dlys} host strain to generate the recombinant MTBVAC.HIVA\textsuperscript{2auxo}. The selection of positive recombinant MTBVAC.HIVA\textsuperscript{2auxo} colonies was performed by culturing the MTBVAC\textsubscript{Dlys} transformants on Middlebrook agar 7H10 medium without lysine supplementation. The MTBVAC.HIVA\textsuperscript{2auxo} strain harboring the lysine-complementing gene abolished the requirement for exogenous lysine, and colonies were observed in non-lysine-supplemented agar plates (Figure 2B).

The expression of the full-size chimeric 19-kDa signal-sequence-HIVA protein (total weight, 64 kDa) was confirmed by western blot analysis of the MTBVAC.HIVA\textsuperscript{2auxo} cell lysates (Figure 2C). No HIVA protein expression was detected in recombinant MTBVAC strains harboring the p2auxo plasmid without heterologous insert (MTBVAC,\textsubscript{G}\textsuperscript{2auxo}, negative control).

This proper molecular characterization led us to prepare a master seed stock and derivative working vaccine stock for downstream experiments.
In Vitro and In Vivo Maintenance of p2auxo.HIVA in MTBVAC.HIVA2auxo Strain

To assess the in vitro stability of the p2auxo.HIVA plasmid, subcultures of MTBVAC.HIVA2auxo on selective media (no lysine supplementation) were carried out every 7 days. The maintenance of the p2auxo.HIVA plasmid DNA was evaluated by PCR analysis of HIVA and GlyA DNA-coding sequences. Bands corresponding to the HIVA DNA-coding sequence (Figure 3A) and to the \textit{E. coli} GlyA-coding sequence (Figure S1A) were observed in all 6 MTBVAC.HIVA2auxo subcultures (42 bacterial generations), indicating that there were no major genetic rearrangements in the HIVA and \textit{glyA} genes of MTBVAC.HIVA2auxo vaccine strain over the subsequent subculturing passages. In vivo stability of p2auxo.HIVA plasmid in MTBVAC.HIVA2auxo was assessed in severe combined immunodeficiency (SCID) mice used in the safety trial. Homogenized spleens were plated on Lys-supplemented medium, and p2auxo.HIVA presence in the mycobacterial burden was analyzed by colony PCR using primers to detect the HIVA DNA-coding sequence (Figure 3B) and the \textit{glyA} gene (Figure S1B). The analysis showed that 95.5% of the colonies retained the plasmid during in vivo infection.

MTBVAC.HIVA2auxo Prime and MVA.HIVA Boost Vaccination Schedule Elicited HIV-1- and PPD-Specific T Cell Responses in Mice

We previously demonstrated that heterologous BCG.HIVA prime boosted with MVA.HIVA elicited high-quality HIV-1-specific T cell responses.\textsuperscript{26,27,30} In this study, we evaluated the specific HIV-1 T cell responses in adult BALB/c mice after intradermal immunization with MTBVAC.HIVA2auxo or MTBVAC.Ø2auxo prime and intramuscular MVA.HIVA boost (Figure 4A). The intradermal route mimics the administration performed in human BCG vaccination, and it has been shown to elicit a higher HIV-1-specific CD8\(^+\) T cell response in adult BALB/c mice.\textsuperscript{31} The immunogenicity readout was focused on the P18-I10 epitope, an immunodominant cytotoxic T-lymphocytes (CTL) epitope derived from HIV-1 Env and \textit{H-2Dd} murine restricted, which was fused to HIVA immunogen to evaluate the immunogenicity in mice.

On day 0, adult mice were either left unimmunized or primed with MTBVAC.HIVA2auxo or MTBVAC.Ø2auxo, and on week 6 the animals received an MVA.HIVA boost. Mice were sacrificed on week 8, and the functional quality of the elicited CD8\(^+\) T cells to produce interferon-\(\gamma\) (IFN-\(\gamma\)) and tumor necrosis factor alpha (TNF-\(\alpha\)) and to degranulate (surface expression of CD107\(\alpha\)) in response to P18-I10 peptide stimulation was measured by intracellular cytokine staining (ICS) (Figure 4B). We observed in adult mice that MTBVAC.HIVA2auxo prime and MVA.HIVA boost induced higher frequencies of P18-I10 epitope-specific CD8\(^+\) splenocytes producing IFN-\(\gamma\), TNF-\(\alpha\), and CD107 than mice primed with MTBVAC.Ø2auxo, MVA.HIVA alone, or naive mice. We found that MTBVAC.HIVA2auxo prime and MVA.HIVA boost induced higher frequencies of trifunctional specific CD8\(^+\) T cells compared with the MTBVAC.Ø2auxo priming and MVA.HIVA boost and with MVA. HIVA alone (Figure 4C).
The capacity of splenocytes from vaccinated mice to secrete IFN-\(\gamma\) was also assessed by the enzyme-linked immunosorbent spot (ELISPOT) assay. We observed the highest frequency of specific cells secreting IFN-\(\gamma\) when stimulated with P18-I10 in mice primed with MTBVAC.HIVA2auxo and boosted with MVA.HIVA, 1,280 spot-forming units (SFU)/10\(^6\) splenocytes, compared to 1,043 SFU/10\(^6\) splenocytes obtained when mice were primed with MTBVAC.Ø2auxo and 1,095 SFU/10\(^6\) splenocytes when mice were only boosted with MVA.HIVA (Figure 4D). The capacity of splenocytes from vaccinated mice to secrete IFN-\(\gamma\) after overnight stimulation with the \(Mtb\)-purified protein derivative (PPD) was also assessed by ELISPOT. The median SFUs per 10\(^6\) splenocytes were similar in mice primed with MTBVAC.HIVA2auxo and MTBVAC.O2auxo (102 and 86 SFU/10\(^6\) splenocytes, respectively; Figure 4E).

**MTBVAC.HIVA2auxo Prime and MVA.HIVA Boost Were Well Tolerated in Mice**

As shown in Figure 5, the body mass was monitored over time and recorded to depict any adverse events and body mass loss due to vaccination. To detect vaccine-derived adverse events, a 12-week period between MTBVAC.HIVA2auxo and MVA.HIVA boost was established for this trial. Importantly, no statistically significant difference (by ANOVA) was observed between the vaccinated mouse groups and the control mouse group at the final time point. Furthermore, between weeks 1 and 14, the body mass monitored in all vaccinated mouse groups was found to lie between the mean \(\pm 2\) SD body mass curves in control mice. It is also important to mention that no mice died during the trial, and no local adverse events or associated systemic reactions were observed.

**MTBVAC.HIVA2auxo Protective Efficacy against \(M.\) tuberculosis in Mice Was Similar to MTBVAC**

We evaluated the efficacy of the bivalent vaccine strain MTBVAC.HIVA2auxo with respect to the parental MTBVAC vaccine. Groups of 6 C57BL/6 mice were left unimmunized or vaccinated with MTBVAC, MTBVAC.HIVA2auxo, or MTBVAC.Dlys by subcutaneous injection, a route previously used for efficacy studies in mouse models.\(^{32}\) At 7 weeks post-vaccination, the mice were challenged with the pathogenic H37Rv strain by the intranasal route. Bacterial load in lungs and spleens was examined 4 weeks post-challenge by plating homogenized organs on complete 7H10 medium (Figure 6). In all vaccinated groups, the bacterial reduction was significant with respect to the unvaccinated group, both in lungs and spleens. The auxotrophic strain MTBVAC.Dlys, which was expected not to be able to survive without lysine, also displayed significant protection against \(Mtb\) H37Rv when...
compared to the naive group. No differences were found between the different MTBVAC strains tested, which validates the protective behavior of MTBVAC.HIVA2auxo vaccine against Mtb despite the genetic manipulations introduced.

**MTBVAC.HIVA2auxo Was Highly Attenuated in the SCID Mouse Model**

As well as affecting vaccine efficacy, genetic manipulation may also affect attenuation of live vaccines. With the aim of corroborating the attenuation status of MTBVAC.HIVA2auxo and MTBVACΔlys strains, SCID mice were inoculated with 10^6 CFU by the intraperitoneal route, and the survival of animals was monitored (Figure 7). SCID mice are the reference model for safety assessments of live vaccines in preclinical TB studies, as recommended by regulatory bodies. Intraperitoneal, as well as intravenous, administration is a systemic inoculation route that allows rapid dissemination of the bacteria and, thereby, virulence assessments. The auxotropic MTBVACΔlys strain showed a hyper-attenuated profile; all mice inoculated with this strain survived until the endpoint of the experiment at week 31. Bacterial burden per spleen of these SCID mice vaccinated with MTBVACΔlys at week 31 was approximately 5 × 10^3 CFU (Figure S2), which demonstrated that this strain survived in vivo.

When we analyzed survival time of MTBVAC.HIVA2auxo-vaccinated mice, data revealed a marked attenuation profile (they survived approximately 160 days) when compared to the BCG-vaccinated mice (deceased by day 90) and the MTBVAC-vaccinated mice (deceased by day 120).

**DISCUSSION**

Despite the progress made in the development of a safe, effective, and affordable vaccine against HIV-1 and TB shortly after birth, the prevention of mother-to-child HIV-1 transmission via breast milk and childhood TB still remain great challenges.

The use of mycobacteria as a vaccine vector is an attractive option; on top of the previously mentioned advantages (cheap mass production, good safety profile, suitable for neonates, etc.), it induces a potent Th1 type immune response (the central defense mechanism against intracellular pathogens) in humans and mice. Three experimental systems must be orchestrated to develop a recombinant *Mycobacterium*-based HIV vaccine: (1) a live vaccine vehicle based on mycobacteria, (2) an *E. coli*-mycobacterial expression vector without antibiotic resistance markers, and (3) an HIV immunogen design. In this study, we have engineered a novel live-attenuated vaccine for HIV-1 and Mtb infection that is vectored by a lysine auxotroph of MTBVAC, which expresses the HIV-1 clade A-derived immunogen HIVA.

The use of mycobacterial vectors for antigen expression in *M. bovis* BCG, *M. smegmatis* or *M. vaccae* has been documented. Through a Barcelona-Oxford collaboration, we previously engineered a mycobacterial vaccine platform for HIV-TB by using lysine auxotrophic strains of BCG as vectors: an *E. coli*-mycobacterial expression vector containing an antibiotic-free selection system and different HIV immunogen designs to improve the specific HIV-1 immunogenicity.
The auxotrophy-complementation strategy has also been used in the context of other intracellular pathogens as *Listeria monocytogenes* to express simian immunodeficiency virus (SIV) or HIV antigens. Despite being highly effective and safe in mice, including neonatal animals, the success observed in the murine model was not translatable upon immunogenicity and efficacy assessment of the *Lmdd-BdopSIVgag* in non-human primates. This should be taken into consideration during further stages of MTBVAC.HIVA2auxo development.

In 2014, the promising live-attenuated *Mtb* vaccine, MTBVAC, was developed. It conferred improved immunogenicity and protection and had a higher safety profile compared to BCG in preclinical studies. Furthermore, MTBVAC has advanced through phase I (ClinicalTrials.gov: NCT02013245 and NCT02729571) and into phase II (ClinicalTrials.gov: NCT02933281 and NCT03536117) clinical trials.

It is important to recall that, of the 1,603 experimentally validated T cell epitopes present in *M. tuberculosis*, 433 of these epitopes were lost during the in vitro attenuation of BCG. Conversely, MTBVAC maintains the whole antigenic repertoire of the human pathogen *Mtb*. Further, MTBVAC displayed increased secretion and immunogenicity against some key antigens (such as those of the Ag85 complex) as a consequence of the *phoP* mutation. Thus, MTBVAC may well be a better platform than BCG when it comes to the expression of heterologous antigens, based on its higher immunogenicity.

The MTBVAC.HIVA2auxo and MTBVAC.%26auxo strains constructed in this study grew properly without lysine supplementation in the medium, which demonstrated the usefulness of the auxotrophy-complementation system for the selection and maintenance of
plasmids in Mtb-based vaccines.26,51 Use of the lysine auxotroph-lysine complementation system was previously shown to increase plasmid stability and to prevent extensive genetic rearrangements in recombinant mycobacteria.26,43,51 Plasmid stability in recombinant mycobacteria has always been a critical and controversial issue. Méderlé et al.52 evaluated the genetic stability of recombinant BCG strains harboring episomal or integrative vectors expressing nef and gag genes from SIV. They observed a higher genetic stability in vivo and in vitro as well an increased duration of heterologous gene expression in vivo using the integrative plasmid.52 Recently, we published that the in vitro stability of the integrative plasmid p2auxo.HIVAint was increased 4-fold compared with the BCG strain harboring the episomal plasmid.20 These results were in concordance with Méderlé et al. However, as there are more copies of episomal vector per cell, higher levels of recombinant protein can be expressed than from integrative vectors. Although our previous results and a number of others have demonstrated that integrative vectors are more stable than episomal vectors,52,53 it has also been demonstrated that an initial, high level of antigen expression is necessary to prime an immune response.52 In this study, we demonstrated that the episomal p2auxo.HIVA plasmid transformed into the MTBVACA/lys strain was retained in vivo over 220 days in SCID mice and after 42 bacterial generations in vitro. Sequencing of the recovered plasmids in vitro and in vivo would be desirable to accurately confirm the genetic stability of p2auxo.HIVA plasmid at the nucleotide level.

We assessed the HIV-1- and Mtb-specific T cell-mediated immune responses elicited after BALB/c prime-boost immunization with MTBVAC expressing HIVA immunogen (MTBVAC.HIVA2auxo). Mice were primed with MTBVAC.HIVA2auxo or MTBVAC.G2auxo (plasmid with no HIVA DNA-coding sequence) and boosted with MVA.HIVA. This way, we were able to detect the non-specific immune responses due to intrinsic immunogenic properties of mycobacteria.54 The frequencies of CD8+ T cells producing IFN-γ, TNF-α, and CD107a were higher in mice vaccinated with MTBVAC.HIVA2auxo in comparison with mice primed with MTBVAC.G2auxo. A similar profile was observed in spleen cells producing IFN-γ after P18-I10 peptide stimulation measured by ELISPOT assay. These results are in concordance with our previously published data using BCG.HIVA2auxo and BCG wild-type as priming agents.26

The extent of T cell polyfunctionality was correlated to protection against leishmaniasis in mice, HIV-1 in humans, and SIV in non-human primates.55 It has also been demonstrated that the magnitude and polyfunctionality of virus-specific CD8+ T cell responses were associated with the control of viral replication after SHIV-89.6P challenge in rhesus monkeys.30 The construction of Mtb auxotrophs (ΔpanCD, ΔleuCD, and Δsec2A) expressing HIV or SIV antigens with the aim of developing a dual pediatric vaccine against TB and HIV has been described as oral vaccination of macaques at birth.57 However, unexpectedly, vaccinated infants required fewer SIV exposures to become infected compared to naive controls. Considering that the current TB vaccine, BCG, can induce potent innate immune responses and confer pathogen-unspecific trained immunity, they hypothesized that an imbalance between enhanced myeloid cell function and immune activation might have influenced the outcome of oral SIV challenge in AMt6-SIV- vaccinated infants. Ideally, an appropriately targeted specific response directed toward beneficial epitopes of HIV would overcome the dangers of trained immunity while maintaining its positive aspects, which have been linked to decreased mortality in children receiving BCG, as well as lead to the control or prevention of HIV infection.58

When developing a dual vaccine against TB and HIV, it is important to confirm that the inclusion of HIV immunogens does not increase the metabolic burden in the TB vaccine that could affect its protective efficacy. We have already described this issue in previous publications, demonstrating that recombinant BCG-based HIV vaccine conferred the same level of protection as the wild-type.29 In the present study, we confirm that MTBVAC and MTBVAC.HIVA2auxo confer equivalent levels of protection against Mtb challenge and, consequently, vaccine efficacy is not compromised after genetic manipulations for HIVA expression.

The recombinant MTBVAC.HIVA2auxo strain showed an increased safety profile in comparison with the parental MTBVAC strain. This finding might support the potential use of this vaccine in individuals at risk of immunosuppression. On the other hand, the auxotrophic MTBVAC/lys strain had a highly attenuated profile, with 100% survival in this group after 220 days of inoculation. This hyper-attenuated
profile of MTBVACΔlys was observed with other auxotrophic BCG and Mtb strains due to lower viability and growth limitation without amino acid supplementation in vivo. Despite the growth limitation, the strain could be isolated in the spleens of SCID mice at the end of the trial, demonstrating that the auxotropic strain was not fully cleared on day 220 after inoculation. It may suggest that the increased attenuation profile of the recombinant MTBVAC.HIVA2auxo could be due to episomal plasmid loss. However, in our study, the persistence of plasmid was 95% in the isolated colonies from spleens of SCID mice inoculated with MTBVAC.HIVA2auxo.

We also demonstrated that MTBVAC.HIVA2auxo prime and MVA.HIVA boost were well tolerated in adult BALB/c mice by monitoring and recording body mass over time. The findings were along the lines of previous observations with recombinant BCG expressing HIVA.

In conclusion, the development of a recombinant MTBVAC-based HIV-TB vaccine may provide a new and improved tool for mycobacterial-based vaccine design. In the future, MTBVAC could be further developed as a vector to express optimized HIV immunogens and utilized in prime-boost vaccination protocols along with new boosting agents. It could, furthermore, be used as a novel mycobacterial vaccine platform for infectious diseases, such as malaria, whooping cough, and other tropical diseases, to prime protective immune responses shortly after birth.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Mycobacterial cultures were grown in Middlebrook 7H9 broth medium or on Middlebrook agar 7H10 (Becton Dickinson) medium supplemented with albumin-dextrose-catalase (ADC; Difco Laboratories) and containing 0.05% Tween 80 and 20 μg/mL kanamycin. The L-lysine monohydrochloride (Sigma) was dissolved in distilled water and used at a concentration of 40 μg/mL. Mycobacterial suspensions for animal inoculation were prepared in PBS from frozen glycerol stocks previously titrated by plating serial dilutions.

Figure 6. Efficacy of MTBVAC.HIVA2auxo Vaccine against M. tuberculosis

C57BL/6 mice were vaccinated subcutaneously with 10⁶ CFU of the strains indicated, MTBVAC, MTBVAC.HIVA2auxo, and MTBVACΔlys, or naïve (unvaccinated as control). At 8 weeks post-vaccination, mice were challenged by intranasal route with 200 CFU H37Rv. Bacterial burden was assessed in lungs (A) and in spleen (B) 4 weeks post-challenge. Data are expressed as mean ± SEM and compared by 2-way ANOVA test, using Bonferroni multiple comparison post-test (**p < 0.01).

Construction of MTBVACΔlys, MTBVAC.HIVA2auxo, and MTBVAC.G2auxo Strains

The strain MTBVACΔlys was constructed following the bacterial artificial chromosome-recombinering (BAC-rec) strategy described by Aguilo et al. Briefly, the target gene rvl293/lysA was identified in the E. coli BAC library (kindly donated by Roland Brosch from Institut Pasteur, Paris). In this clone, the target gene lysA was interrupted with a kanamycin resistance cassette by heterologous recombination mediated by PCR product, which was amplified by using specific primers LysA-P1-pKD4-fw and LysA-P2-pKD4-rv (Table S1). The disrupted lysA-Km gene was amplified by PCR using ArgS1-fw and ThrB1-rv primers (Table S1). This PCR product containing the lysA-Km fragment was introduced into the MTBVAC genome by homologous recombination. MTBVACΔlys recombinant colonies were selected by plating on 7H10-ADC supplemented with Km and Lysine, and proper recombination was checked by PCR using the pairs of primers Lys-fw/km-OUT1-rv and Lys-rv/km-OUT2-fw (Table S1).

For the construction of MTBVAC.HIVA2auxo and MTBVAC.G2auxo strains, MTBVACΔlys culture was grown until log phase and conditioned for electroporation by washing with 10% glycerol, according to the method described by Wards and Collins. Electroporant competent MTBVACΔlys was transformed with 0.5–1 μg p2auxo.HIVA or p2auxo.G using a Bio-Rad gene pulser electroporator at 2.5 kV, 25 mF, and 1,000 Ω, and plated onto Middlebrook agar 7H10-ADC medium without lysine supplementation. Recombinant MTBVAC.C.HIVA2auxo and MTBVAC.G2auxo colonies containing the corresponding plasmids p2auxo.HIVA and p2auxo.G were selected by PCR using the pair of primers 19kDss-fw/HIVA-rv and 19kDss-fw/Pglya-rv, respectively (Table S1). The MTBVAC.HIVA2auxo colonies were assessed for heterologous HIVA protein expression, and clone 2 was selected and preserved using the seed-lot system. A master seed stock and derivative working stock, which we used also as a vaccine stock, were prepared and stored at −80°C with 20% glycerol as a preservative.

SDS-PAGE and Western Blot Analysis

Cell lysates of mid-logarithmic-phase cultures of MTBVAC.HIVA2auxo and MTBVAC.G2auxo strains were prepared, separated by
SDS-PAGE using pre-cast 8%–16% gradient acrylamide gels (GeBaGel, Israel), and electroblotted onto polyvinylidene fluoride (PVDF) membranes using a semi-dry system (Bio-Rad). HIVA protein was detected using anti-Pk antibody (MCA 1360; Pierce, USA) with an enhanced chemiluminescence kit (WesternBright ECL; Advansta, USA). To visualize the bands, the LAS500 gel imaging system (GE Healthcare) was used.

In Vitro Stability of the MTBVAC.HIVA2auxo Strain
Six subcultures (~42 bacterial generations) of MTBVAC.HIVA2auxo (working vaccine stock), harboring the episomal p2auxo.HIVA plasmid DNA that contains the lysine-complementing gene, were grown in 7H9 broth on selective media (no lysine supplementation). Subcultures were performed every 7 days by transferring 100 μL stationary-phase culture to 5 mL fresh medium. PCR analysis of HIVA DNA-coding sequence and GlyA gene (Table S1) was performed to detect plasmid genetic rearrangements.

Mouse Trials
For immunogenicity studies, adult (7-week-old) female BALB/c mice were left either unimmunized or immunized with MTBVAC. HIVA2auxo or MTBVAC.G2auxo, and they were boosted with MVA.HIVA at doses, routes, and schedules outlined in the Figure 4 legend. On the day of sacrifice, individual spleens were collected, and splenocytes were isolated by homogenizing spleens using a cell strainer (Falcon; Becton Dickinson) and a 5-mL syringe rubber plunger. Following the removal of red blood cells with ACK lysing buffer (Lonza, Barcelona, Spain), the splenocytes were washed and resuspended in complete medium (R10 [RPMI 1640 supplemented with 10% fetal calf serum and penicillin-streptomycin], 20 mmol/L HEPES, and 15 mmol/L 2-mercaptoethanol).

Body mass was monitored over time and recorded to depict any adverse events and body mass loss due to vaccination. To detect vaccine-related adverse events, a 12-week period between MTBVAC.HIVA2auxo prime and MVA.HIVA boost was established for this trial.

For protection studies, groups of 6 female C57BL/6 mice (Janvier Biolabs) were mock treated or subcutaneously vaccinated with 10⁶ CFU MTBVAC, MTBVACΔlys, or MTBVAC.HIVA2auxo. At 8 weeks post-vaccination, mice were intranasally challenged with 200 CFU virulent M. tuberculosis H37Rv. Bacterial burden was assessed 4 weeks post-challenge by plating homogenized lungs and spleen on complete 7H10 medium.

For safety studies, groups of 6 female CB-17/Icr-Prkdc SCID mice (Janvier Biolabs) received a single intraperitoneal inoculation of 10⁶ CFU BCG Pasteur, MTBVAC, MTBVACΔlys, or MTBVAC.HIVA2auxo. Mice were monitored for any sign of disease and body mass measurements were performed weekly. Experimental endpoint was set as a 20% body weight reduction. In the case of the MTBVACΔlys group, the endpoint was established at 220 days post-inoculation, upon which surviving animals were humanely euthanized and bacterial load in spleen was quantified. Samples were also obtained from MTBVAC.HIVA2auxo animals euthanized during the protocol to check for plasmid stability by colony PCR analysis using 19kDss-fw/HIVA-rv and HIVA-fw/Pglya-rv primers (Table S1).

Peptides
For assessing the immunogenicity of HIVA in the BALB/c mice, the following peptides were used: H-2Dβ-restricted epitope P18-I10 (RGPGRAFVTI). The PPD (AIVaccines, Copenhagen, Denmark) was used to assess the immunogenicity induced by MTBVAC.

Ex Vivo IFN-γ ELISPOT Assay
The ELISPOT assay was performed using a commercial IFN-γ ELISPOT kit (Mabtech, Nacka Strand, Sweden), following the manufacturer’s instructions. The ELISPOT plates (MISP4510, 96-well plates with polyvinylidene difluoride membranes; Millipore, Billerica, MA) were coated with purified anti-mouse IFN-γ capture monoclonal antibody diluted in PBS to a final concentration of 5 μg/mL at 4°C overnight. A total of 5 × 10⁵ fresh splenocytes were added to each well and stimulated with 2 μg/mL P18-I10 peptide or 5 μg/mL PPD for 16 h at 37°C. Wells were washed four times with PBS 0.05% Tween 20 and twice with PBS before incubating with 100 μL 5-bromo-4-chloro-3-indoyl-phosphate/nitro blue tetrazolium substrate solution (Sigma). After 5–10 min, the plates were washed with tap water and dried, and the resulting spots were counted using an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

Intracellular Cytokine Staining
One million splenocytes were added to each well of a 96-well round-bottomed plate (Costar, Corning, NY), pulsed with 2 μg/mL P18-I10 peptide and kept at 37°C and 5% CO₂ for 60 min, followed by the addition of GolgiStop (Becton Dickinson) containing monensin. After 5 h of incubation, the reaction was terminated by transferring the plate to 4°C. The cells were washed with wash buffer (PBS, 2% fetal calf serum, and 0.01% azide) and blocked with anti-CD16/32 (BD Biosciences) at 4°C for 30 min. All subsequent antibody stains were performed using the same conditions. Cells were then washed and stained with anti-CD8-PerCP (BD Biosciences) and anti-F4/80-PerCP (BD Biosciences) following the manufacturer’s instructions.
Cells were staining with anti-IFN-γ-PE (BD Biosciences) and anti-TNF-α-APC (BD Biosciences). Perm-wash buffer (BD Biosciences) was used to wash cells before staining with anti-IFN-γ-APC and anti-TNF-α-PE (BD Biosciences). Cells were fixed with CellFIX (Becton Dickinson) and stored at 4°C until analysis. All chromogen-labeled cells were analyzed in a Becton Dickinson FACScalibur, using the CellQuest software (Becton Dickinson) for acquisition and the FlowJo software (Tree Star, Ashland, OR) for analysis.

**Statistical Analysis**

Immunogenicity data are shown as group means or group medians as well as individual responses. Statistical significance was determined by ANOVA and Bonferroni post-test. In safety experiments H37Rv bacterial burdens are shown as mean ± SEM. Groups were compared using one-way ANOVA and Bonferroni post-test. Survival data were analyzed applying Mantel-Cox test. In all cases, confidence intervals were as follows: *p < 0.05, **p < 0.01, and ***p < 0.001. GraphPad Prism 5.0 software was used for representation and statistical analysis of the data.

**Ethics Statement**

All mice were kept under controlled conditions and observed for any sign of disease. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Immunological mouse experiments were approved by the local Research Ethics Committee (Procedure Med 365/16, Clinical Medicine, School of Medicine and University of Barcelona) and by the Ethical Committee for animal experimentation from the University of Barcelona, and they strictly conformed to the Generalitat de Catalunya animal welfare legislation. Experiments with SCID mice were carried out under Project License 17/17 and *M. tuberculosis* protection studies under Project License 50/14. Both procedures were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes two figures and one table and can be found with this article online at https://doi.org/10.1016/j.omtm.2019.01.014.

**AUTHOR CONTRIBUTIONS**

Conceptualization, J.G.-A., C.M., N.S., and J.J.-M.; Methodology, N.A., J.G.-A., N.S., and J.J.-M.; Investigation, E.B., S.U., and N.S.; Writing – Original Draft, E.B. and N.S.; Writing – Review & Editing, E.B., N.S., J.G.-A., C.M., and J.J.-M.; Funding Acquisition, C.M. and J.J.-M.; Supervision, E.B., S.U., N.A., and J.G.-A. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**CONFLICTS OF INTEREST**

C.M. and J.G.-A. are co-inventors in patent applications entitled “TB vaccine,” filed by the University of Zaragoza (PCT/ES 2007/070051), and “Compositions for use as a prophylactic agent to those at risk of infection of TB, or as secondary agents for treating infected TB patients” (218382097.6-1112), University of Zaragoza/Biofabri. There are no other conflicts of interest. J.J.-M. and N.S. are co-inventors in a patent application entitled “Mycobacterium comprising expression vector with two auxotrophic selection markers and its use as vaccine” (EP 12382336.1), Laboratorios Esteve, S.A. and Fundación Privada Institut de Recerca de la SIDA. There are no other conflicts of interest. The rest of the authors certify that they do not have a commercial or other association that might pose a conflict of interest in the subject matter or materials discussed in this manuscript.

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**REFERENCES**

1. WHO (2018). Global Tuberculosis Report 2018. https://www.who.int/tb/publications/global_report/en/.
2. WHO (2018). HIV/AIDS fact sheet. https://www.who.int/en/news-room/fact-sheets/detail/hiv-aids.
3. UNAIDS (2018). Fact sheet – World AIDS Day 2018. http://www.unaids.org/sites/default/files/media_asset/UNAIDS_FactSheet_en.pdf.
4. WHO (2015). Tuberculosis and HIV. https://www.who.int/hiv/topics/sl/about_tb/en/.
5. Rodrigues, L.C., Diwan, V.K., and Wheeler, J.G. (1993). Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. Int. J. Epidemiol. 22, 1154–1158.
6. Colditz, G.A., Brewer, T.F., Berkey, C.S., Wilson, M.E., Burdick, E., Fineberg, H.V., and Mosteller, F. (1994). Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 271, 698–702.
7. Matsuo, K., and Yatsutomi, Y. (2011). Mycobacterium bovis Bacille Calmette-Guérin as a Vaccine Vector for Global Infectious Disease Control. Tuberc. Res. Treat. 2011, 574591.
8. Fine, P.E. (1995). Variation in protection by BCG: implications of and for heterologous immunity. Lancet 346, 1339–1345.
9. Roy, A., Eisenhut, M., Harris, R.J., Rodrigues, L.C., Sridhar, S., Habermann, S., Snell, L., Mangtani, P., Adetifa, I., Lalvani, A., and Abubakar, I. (2014). Effect of BCG...
vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. BMJ 349, g6643.

10. Mangtani, P., Abubakar, I., Ariri, C., Beynon, R., Pimpin, L., Fine, P.E.M., Rodrigues, L.C., Smith, P.G., Lipman, P., Whiting, P.F., and Serten, J.A. (2014). Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. Clin. Infect. Dis. 58, 470–480.

11. Graham, S.M., Sismansida, C., Menzies, H.J., Marais, B.J., Detjen, A.K., and Black, R.E. (2014). Importance of tuberculosis control to address child survival. Lancet 383, 1605–1607.

12. Nemes, E., Geldenhuys, H., Rozot, V., Rutkowski, K.T., Ratangee, F., Bilek, N., Mabwe, S., Makhele, L., Eramus, M., Toefy, A., et al.; C-040–040 Study Team (2018). Prevention of M. tuberculosis Infection with H4IC51 Vaccine or BCG Revaccination. N. Engl. J. Med. 379, 138–149.

13. Freyne, B., Marchant, A., and Curtis, N. (2015). BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms. Trans. R. Soc. Trop. Med. Hyg. 109, 46–51.

14. Arts, R.J.W., Moorlag, S.J.C.F.M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., Kunnath-Velayudhan, S., Liu, Z., Bittman, R., Jervis, P.J., Cox, L.R., et al. (2014). Importance of tuberculosis control to address child survival. Lancet 383, 1605–1607.

15. Marais, B.J., Seddon, J.A., Detjen, A.K., van der Werf, M.J., Grzemska, M., Hesseling, A.C., Curtis, N., and Graham, S.M.; WHO Child TB Subgroup (2016). Interrupted BCG vaccination is a major threat to global child health. Lancet Respir. Med. 4, 251–253.

16. Arbues, A., Agullo, I.I., Gonzalo-ASENSIO, J., Marinova, D., Uranga, S., Puentes, E., Fernandez, P., Parra, A., Cardona, P.I., Vilaplana, C., et al. (2013). Construction, characterization and preclinical evaluation of MTB-VAC, the first live-attenuated M. tuberculosis-based vaccine to enter clinical trials. Vaccine 31, 4867–4873.

17. Sperti, F., Aoudra, R., Chakour, R., Karoui, O., Steiner-Monard, Y., Thiery, A.-C., Mayor, C.E., Retby, N., Jaton, K., Vallotton, L., et al. (2015). Safety of human immunisation with a live attenuated Mycobacterium tuberculosis vaccine: a randomised, double-blind, controlled phase I trial. Lancet Respir. Med. 3, 935–942.

18. Koup, R.A., Safrit, J.T., Cao, Y., Andrews, C.A., McLeod, G., Borkowsky, W., Farthig, C., and Ho, D.D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68, 4650–4655.

19. Rowland-Jones, S.L., Dong, T., Fowke, K.R., Kimani, J., Krausa, P., Newell, H., Grode, L., Liu, M.A., Fruth, U., and Lambert, P.H. (2010). The second Geneva Consensus: Recommendations for novel live TB vaccines. Vaccine 28, 2259–2270.

20. Ota, M.O.C., Vekemans, J., Schlegel-Haueter, S.E., Fielding, K., Sanneh, M., Kidd, M., Newport, M.J., Aaby, F., Whittle, H., Lambert, P.H., et al. (2002). Influence of Mycobacterium bovis bacillus Calmette-Guérin on antibody and cytokine responses to human neonatal vaccination. J. Immunol. 168, 919–925.

21. Marchant, A., Goetgheluere, T., Ota, M.O., Wolfe, L., Ceesay, S.J., De Groote, G., Corrah, T., Bennett, S., Wheeler, J., Huygen, K., et al. (1999). Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. J. Immunol. 163, 2249–2255.

22. Gaora, P.O. (1998). Expression of genes in mycobacteria. Mycobacteria Protocols, T. Parish and N.G. Stoker, eds. (Humana Press), pp. 261–273.

23. Ranes, M.G., Rauzier, J., Lagranderie, M., Gheorghiu, M., and Gicquel, B. (1990). Functional analysis of pAL5000, a plasmid from Mycobacterium fortuitum: construction of a “mini” mycobacterium-Escherichia coli shuttle vector. J. Bacteriol. 172, 2793–2797.

24. Stover, C.K., Bansal, G.P., Hanson, M.S., Burlein, J.E., Palaszynski, S.R., Young, J.F., Ehrlich, M., Prioz, B., Stutz, H., and Williamson, A.-L. (2010). Preclinical development of BCG.HIVA AXXO.INT, harboring an integrative expression vector, for a HIV-TB Pediatric vaccine. Enhancement of stability and specific HIV-1 T-cell immunity. Hum. Vaccin. Immunother. 13, 1798–1810.

25. Marchant, A., Goetgheluere, T., Ota, M.O., Wolfe, L., Ceesay, S.J., De Groote, G., Corrah, T., Bennett, S., Wheeler, J., Huygen, K., et al. (1999). Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. J. Immunol. 163, 2249–2255.

26. Chapman, R., Chege, G., Shephard, E., Stutz, H., and Williamson, A.-L. (2010). Recombinant Mycobacterium bovis BCG as an HIV vaccine vector. Curr. HIV Res. 8, 282–296.

27. Abou-zeid, C., Gares, M.P., Inwald, I., Jansen, R., Zhang, Y., Young, D.B., Hetzel, C., Lamb, J.R., Baldwin, S.L., Orme, I.M., et al. (1997). Induction of a type I immune
response to a recombinant antigen from Mycobacterium tuberculosis expressed in Mycobacterium vaccae. Infect. Immun. 65, 1856–1862.

43. Joseph, J., Fernández-Lloris, R., Pezate, F., Saubi, N., Cardona, P.-J., Mothe, B., and Gatell, J.M. (2010). Molecular characterization of heterologous HIV-1gp120 gene expression disruption in mycobacterium bovis BCG host strain: a critical issue for engineering mycobacterial based-vaccine vectors. J. Biomed. Biotechnol. 2010, 357730.

44. Saubi, N., Mbewe-Mvula, A., Rosario, M., Gatell, J.M., Hanke, T., and Joseph, J. (2012). Pre-clinical development of BCG-HIV(CAT), an antibiotic-free selection strain, for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG. PLoS ONE 7, e42559.

45. Frankel, F.R., Hegde, S., Lieberman, J., and Paterson, Y. (1995). Induction of cell-mediated immune responses to human immunodeficiency virus type 1 Gag protein by using Listeria monocytogenes as a live vaccine vector. J. Immunol. 155, 4775–4782.

46. Lakhhashe, S.K., Vehu, V., Sciaranghella, G., Siddappa, N.B., Dipasquale, J.M., Hemashettar, G., Yoon, J.K., Rasmussen, R.A., Yang, F., Lee, S.J., et al. (2011). Prime-boost vaccination with heterologous live vectors encoding HIV-1 gp160 protein: efficacy against repeated mucosal R5 clade C SHIV challenges. Vaccine 29, 5611–5622.

47. Gonzalo-Asensio, J., Marinova, D., Martin, C., and Aguilo, N. (2017). MTBVAC: Attenuating the Human Pathogen of Tuberculosis (TB) Toward a Promising Vaccine against the TB Epidemic. Front. Immunol. 8, 1803.

48. Nambiar, J.K., Pinto, R., Aguilo, J.I., Takatsu, K., Martin, C., Britton, W.J., and Triccas, J.A. (2012). Protective immunity afforded by attenuated, PhoP-deficient Mycobacterium tuberculosis is associated with sustained generation of CD4+ T-cell memory. Eur. J. Immunol. 42, 385–392.

49. Solans, L., Gonzalo-Asensio, J., Sala, C., Benjak, A., Uplekar, S., Rougemont, J., Guilhot, C., Malaga, W., Martin, C., and Cole, S.T. (2014). The PhoP-dependent ncRNA Mcr7 modulates the TAT secretion system in Mycobacterium tuberculosis. PLoS Pathog. 10, e1004183.

50. Sayes, F., Blanc, C., Ates, L.S., Deboosere, N., Orgeur, M., Le Chevalier, F., Gröschel, M.L., Frigui, W., Song, O.R., Lo-Man, R., et al. (2018). Multiplexed Quantitation of Intraphagocyte Mycobacterium tuberculosis Secreted Protein Effectors. Cell Rep. 23, 1072–1084.

51. Busik, S., Mendum, T.-A., Fagundes, M.Q., Michelson, M., Cunha, C.W., McFadden, J., and Dellarossa, O.A. (2007). Aeutrophic complementation as a selectable marker for stable expression of foreign antigens in Mycobacterium bovis BCG. Tuberculosis (Edinb.) 87, 474–480.

52. Méderle, I., Bourguin, I., Ensergueix, D., Badell, E., Moniz-Pereira, J., Gicquel, B., and Winter, N. (2002). Plasmidic versus insertional cloning of heterologous genes in Mycobacterium bovis BCG: impact on in vivo antigen persistence and immune responses. Infect. Immun. 70, 303–314.

53. Denneh, M., Bourn, W., Steele, D., and Williamson, A.-L. (2007). Auxotrophic complementation as a selectable marker for stable expression of foreign antigens in Mycobacterium bovis BCG: impact on in vivo antigen persistence and immune responses. Infect. Immun. 70, 303–314.

54. Melancon-Kaplan, J., Hunter, S.W., McNeil, M., Stewart, C., Modlin, R.L., Rea, T.H., Convit, J., Salgame, P., Mehra, V., Bloom, B.R., et al. (1988). Immunological significance of Mycobacterium leprae cell walls. Proc. Natl. Acad. Sci. USA 85, 1917–1921.

55. Cayabyab, M.J., Korioth-Schmitz, B., Sun, Y., Carville, A., Balachandran, H., Miura, A., Carlson, K.R., Buzby, A.P., Haynes, B.F., Jacobs, W.R., and Letvin, N.L. (2009). Recombinant Mycobacterium bovis BCG prime-recombinant adenovirus boost vaccination in rhesus monkeys elicits robust polyfunctional simian immunodeficiency virus-specific T-cell responses. J. Virol. 83, 5505–5513.

56. Sun, Y., Santra, S., Schmitz, J.E., Reederer, M., and Letvin, N.L. (2008). Magnitude and quality of vaccine-elicited T-cell responses in the control of immunodeficiency virus replication in rhesus monkeys. J. Virol. 82, 8812–8819.

57. Jensen, K., Dela Pena-Ponce, M.G., Piatak, M., Jr., Shoemaker, R., Oswald, K., Jacobs, W.R., Jr., Fennelly, G., Lucero, C., Mollan, K.R., Hudgens, M.G., et al. (2017). Balancing Trained Immunity with Persistent Immune Activation and the Risk of Simian Immunodeficiency Virus Infection in Infant Macaques Vaccinated with Attenuated Mycobacterium tuberculosis or Mycobacterium bovis BCG Vaccine. Clin. Vaccine Immunol. 24, e00360-16.

58. Kilpeläinen, A., Maya-Hoyos, M., Saubi, N., Soto, C.Y., and Joseph Munne, J. (2018). Advances and challenges in recombinant Mycobacterium bovis BCG-based HIV vaccine development: lessons learned. Expert Rev. Vaccines 17, 1005–1020.

59. Smith, D.A., Parish, T., Stoker, N.G., and Bancroft, G.J. (2001). Characterization of auxotrophic mutants of Mycobacterium tuberculosis and their potential as vaccine candidates. Infect. Immun. 69, 1142–1150.

60. Hondalus, M.K., Bardarov, S., Russell, R., Chan, J., Jacobs, W.R., Jr, and Bloom, B.R. (2000). Attenuation of and protection induced by a leucine auxotroph of Mycobacterium tuberculosis. Infect. Immun. 68, 2888–2898.

61. Pavlova, M.S., Jr, Chen, B., Kelley, C.L., Collins, F.M., and Jacobs, W.R., Jr (2003). Vaccine efficacy of a lysine auxotroph of Mycobacterium tuberculosis. Infect. Immun. 71, 4190–4192.

62. Wards, B.I., and Collins, D.M. (1996). Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. FEMS Microbiol. Lett. 145, 101–105.