

ARTICLE

Engineering new mycobacterial vaccine design for HIV–TB pediatric vaccine vectored by lysine auxotroph of BCG

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In this study, we have engineered a new mycobacterial vaccine design by using an antibiotic-free plasmid selection system. We assembled a novel *Escherichia coli* (*E. coli*)–mycobacterial shuttle plasmid p2auxo.HIVA, expressing the HIV-1 clade A immunogen HIVA. This shuttle vector employs an antibiotic resistance-free mechanism for plasmid selection and maintenance based on glycine complementation in *E. coli* and lysine complementation in mycobacteria. This plasmid was first transformed into glycine auxotroph of *E. coli* strain and subsequently transformed into lysine auxotroph of *Mycobacterium bovis* BCG strain to generate vaccine BCG.HIVA^{2auxo}. We demonstrated that the episomal plasmid p2auxo.HIVA was stable *in vivo* over a 7-week period and genetically and phenotypically characterized the BCG.HIVA^{2auxo} vaccine strain. The BCG.HIVA^{2auxo} vaccine in combination with modified vaccinia virus Ankara (MVA). HIVA was safe and induced HIV-1 and *Mycobacterium tuberculosis*-specific interferon- γ -producing T-cell responses in adult BALB/c mice. Polyfunctional HIV-1-specific CD8+ T cells, which produce interferon- γ and tumor necrosis factor- α and express the degranulation marker CD107a, were induced. Thus, we engineered a novel, safer, good laboratory practice-compatible BCG-vectored vaccine using prototype immunogen HIVA. This antibiotic-free plasmid selection system based on “double” auxotrophic complementation might be a new mycobacterial vaccine platform to develop not only recombinant BCG-based vaccines expressing second generation of HIV-1 immunogens but also other major pediatric pathogens to prime protective response soon after birth.

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INTRODUCTION

Recombinant BCG (rBCG) has been developed as a candidate neonatal vaccine vector against pertussis, measles, respiratory syncytial virus, and breast milk HIV transmission.^{1–5} BCG as a vaccine vector has many attractive features such as: (i) it has a proven record of safety as a vaccine against tuberculosis from its use in over two billion individuals; (ii) it infects and colonizes macrophages and dendritic cells, where it can survive and replicate for a long period of time; (iii) it can induce long-lasting humoral and cellular immune responses; (iv) it can be given at or any time after birth and is not affected by maternal antibodies; (v) manufacturing of BCG-based vaccines is inexpensive; and finally, (vi) it is one of the most heat-stable vaccines in current use.^{6–9}

There is strong evidence in favor of a role for HIV-1-specific T-cell responses in the control of HIV-1 replication.^{10,11} One promising approach for T-cell induction is *Mycobacterium bovis* BCG as a bacterial live recombinant vaccine vehicle. Specific humoral and cellular immune responses against HIV-1 have been detected after immunization of mice with rBCG-expressing HIV-1 antigens.^{12–18}

Antibiotic resistance genes have been traditionally used for the selection and maintenance of recombinant plasmids in hosts

such as *Escherichia coli*. Several approaches have been pursued to replace antibiotics as selective markers for plasmid stability in bacteria, including systems using auxotrophic markers based on complementation of a mutation or deletion in the host chromosome.

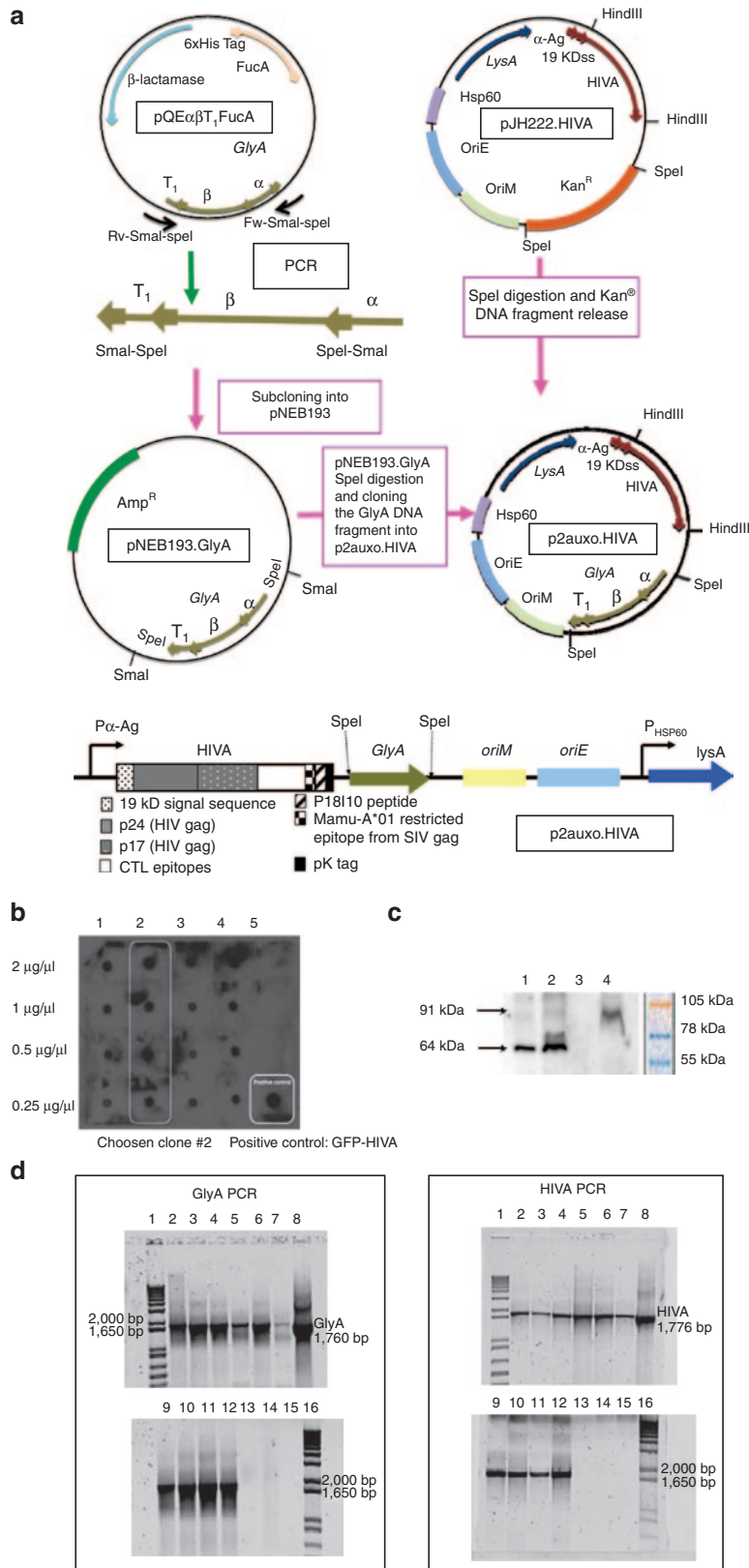
In this study, we assembled a novel *E. coli*–mycobacterial shuttle plasmid p2auxo.HIVA, expressing the HIVA immunogen.¹⁹ This shuttle vector employs an antibiotic resistance-free mechanism for plasmid selection and maintenance based on glycine complementation in *E. coli* and lysine complementation in *Mycobacteria*. This shuttle plasmid was first transformed into glycine auxotroph of *E. coli* M15 Δ *glyA* strain,²⁰ as well as into lysine auxotroph of BCG strain to generate vaccine BCG.HIVA^{2auxo}. The resulting antibiotic marker-less BCG.HIVA^{2auxo} strain was genetically and phenotypically characterized. The presence of HIVA gene sequence and protein expression by the rBCG were confirmed, its safety was evaluated by monitoring the body mass gain, and the induction of HIV-1 and *Mtb*-specific immune responses was demonstrated in adult BALB/c mice after BCG.HIVA^{2auxo} prime and modified vaccinia virus Ankara (MVA).HIVA boost. The BCG.HIVA^{2auxo} strain was developed in good laboratory practice-compatible conditions

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and properly characterized. In particular, it was shown to be stable *in vivo*, inducing specific HIV-1 and *Mtb* immune responses in adult mice and was well tolerated in mice. In addition, the compatibility with good laboratory practice requirements is relevant to upgrade this novel vaccine into clinical evaluation.

RESULTS

Construction of the BCG.HIVA^{2auxo} vaccine strain

The chimeric 19-kDa signal sequence-HIVA gene was expressed from *E. coli*-mycobacterial shuttle plasmid p2auxo.HIVA under the control of the *Mtb* α -antigen promoter (Figure 1a). Plasmid DNA p2auxo.

HIVA is a replicative (multicopy, extrachromosomal) vector that contains a DNA cassette encoding an *E. coli* origin of replication (*oriE*) and a mycobacterial plasmid DNA origin of replication (*oriM*). It also contains the wild-type glycine A-complementing gene (*glyA*) and lysine A-complementing gene (*lysA5*) for the vector maintenance in the *E. coli* and BCG auxotroph strain, respectively.⁵ Then, the antibiotic-free plasmid p2auxo.HIVA was transformed into glycine auxotroph of *E. coli* M15Δ*glyA* host strain and lysine auxotroph of BCG host strain Pasteur Δ*lysA5::res*.^{20,21} The selection of positive recombinant *E. coli* colonies was made by growing the *E. coli* transformants on minimal M9-D agar plates, and the BCG.HIVA^{2auxo} colonies selection was made by growing the BCG transformants on Middlebrook agar 7H10 medium with no supplementation of lysine. Expression of the full-size chimeric 19-kDa signal sequence-HIVA protein was confirmed by immunodot and western blot analysis. As shown in Figure 1b, the highest level of HIVA protein expression was detected after blotting the BCG culture from clone number 2 and was selected for further molecular characterization, immunogenicity, and safety testing in mice. The BCG.HIVA^{2auxo} clone 2 culture was preserved by using the seed-lot system. The expression level of HIVA protein was also assessed by western blot analysis. The levels of expression were compared with rBCG-expressing HIVA.GFP protein and harboring the pJH222 *E. coli*-mycobacterial shuttle plasmid that contains the kanamycin resistance gene as a selectable marker. As shown in Figure 1c, the level of HIVA protein expression, was similar in rBCG carrying episomal p2auxo.HIVA plasmid (antibiotic-free system selection) in comparison with rBCG carrying the episomal pJH222.HIVAGFP plasmid harboring the kanamycin resistance gene. No HIVA protein expression was detected in BCG wild type. For future experiments, we will use the recently constructed BCG strain harboring the p2auxo plasmid DNA without heterologous insert (BCG.empty^{2auxo}) as negative control. Growth of the transformed mycobacteria and the *in vivo* stability of p2auxo.HIVA episomal plasmid were established by the recovery of BCG.HIVA^{2auxo} colonies from the spleens of BALB/c mice 7 weeks after immunization. Ten out of 10 recovered rBCG colonies were positive for HIVA and *E. coli glyA* DNA coding sequence by polymerase chain reaction (PCR; Figure 1d). After DNA sequence analysis of the PCR products purified from two different rBCG colonies, we observed that the HIVA DNA sequences were identical to predictive DNA sequence. Thus, no mutations and genetic rearrangements were observed in the HIVA gene (data not shown).

Genetic characterization of the BCG.HIVA^{2auxo}

In order to confirm that our BCG.HIVA^{2auxo} vaccine strain corresponds to BCG Pasteur substrain, we have used the multiplex PCR assay described by Bedwell *et al.*²² We tested the following samples: BCG.HIVA^{2auxo} strain (clone 2) *lys* auxotroph of BCG Pasteur, BCG wild-type Pasteur, commercial BCG Connaught, and BCG Danish 1331 strain. The PCR fingerprints of BCG Pasteur, BCG Connaught, and BCG Danish substrains were consistent with previously published results on genetic information of BCG substrains,²² and the PCR fingerprints

of BCG.HIVA^{2auxo} strain corresponds to BCG Pasteur substrain (data not shown).

For the molecular characterization of p2auxo.HIVA plasmid DNA, enzymatic restriction and PCR analysis were performed. The plasmid DNA was isolated from the master seed and working vaccine stock of BCG.HIVA^{2auxo} strain and was characterized. The enzymatic restriction pattern obtained did not show any difference with the enzymatic pattern of the plasmid DNA sequence isolated from *E. coli* (pre-BCG transformation; Figure 2a). The PCR analysis using specific primers for the HIVA and *E. coli glyA* DNA coding sequences was performed using the BCG.HIVA^{2auxo} master seed and working vaccine stocks as templates. A band of 1,776 and 1,760 bp corresponding to HIVA and *E. coli glyA* DNA fragment, respectively, were detected (Figure 2b,c).

Phenotypic characterization of the BCG.HIVA^{2auxo}

We assessed the phenotype stability of glycine and lysine auxotrophy, glycine and lysine complementation, and kanamycin sensitivity of *E. coli* M15Δ*glyA* strain and BCG.HIVA^{2auxo} strains. The *E. coli* glycine auxotrophic strain failed to grow on nonglycine-supplemented agar plates, while growing on agar plates supplemented with glycine. As expected, complementation of *E. coli* M15Δ*glyA* strain with *glyA* gene abolished the requirement for exogenous glycine. Also, when *E. coli* M15Δ*glyA* strain was plated out on agar plates containing kanamycin, no colonies were observed, confirming the lack of kanamycin resistance in our construct. As expected, BCG lysine-auxotrophic strain failed to grow on nonlysine-supplemented agar plates, while growing on agar plates supplemented with *lysA*. Moreover, complementation of BCG.HIVA^{2auxo} strain with lysine gene abolished the requirement for exogenous lysine. In addition, when BCG.HIVA^{2auxo} strain was plated on agar plates containing kanamycin, no colonies were observed, confirming the lack of kanamycin resistance in our construct (data not shown).

In vitro stability analysis of the BCG.HIVA^{2auxo} strain

To evaluate the *in vitro* stability of the p2auxo.HIVA plasmid DNA harboring the auxotrophic complementation *lysA* gene, subcultures on media with and without selection were carried out. All BCG.HIVA^{2auxo} colonies that were grown on selective medium (without lysine supplementation) maintained the vector for over four subcultures (~30 bacterial generations). In contrast, when bacteria were grown without selective pressure (with lysine supplementation), only 9% of the BCG colonies were harboring the plasmid DNA after the first subculture with an average of 17% maintenance over the subsequent subculturing passages. The differences between both groups were statistically significant ($P < 0.05$; Figure 3a).

To assess the functional stability of the HIVA gene harbored by p2auxo.HIVA plasmid, the HIVA protein expression was tested by dot-blot analysis. When bacteria were grown under selective pressure, the HIVA protein expression was detected in five out of five BCG.HIVA^{2auxo} colonies and the level of expression remained stable

Figure 1 Construction of the BCG.HIVA^{2auxo} vaccine strain. (a) A synthetic GC-rich HIVA gene was fused to the region encoding the 19-kDa lipoprotein signal sequence and inserted into the episomal pJH222.HIVA *E. coli*-mycobacterium shuttle plasmid. The BALB/c mouse T-cell and MAb-Pk epitopes used in this study are depicted. P α-Ag, *Mycobacterium tuberculosis* α-antigen promoter; *PHSP60*, heat shock protein 60 gene promoter. The *aph* gene was removed by *SpeI* digestion, and the structural *glyA* gene was inserted and transformed into *E. coli* M15Δ*glyA* strain. (b) Immunodot of BCG.HIVA^{2auxo} lysates. Lanes 1–4: clones 1–4 of BCG.HIVA^{2auxo}. Lane 5: BCG wild type (negative control). Lysates of BCG.HIVA-GFP²²² were used as positive control. (c) Western blot of BCG.HIVA^{2auxo} lysates. Lanes 1 and 2: BCG.HIVA^{2auxo} master seed and working vaccine stock, respectively; Lane 3: BCG wild type (negative control); Lane 4: BCG.HIVA-GFP²²² (pJH222 *E. coli*-mycobacterial shuttle plasmid, kanamycin resistance) used as a positive control. (d) *In vivo* plasmid stability of BCG.HIVA^{2auxo} harboring p2auxo.HIVA. Lanes 2–7 and 9–12: 10 rBCG colonies were recovered in the nonlysine-supplemented plate; lanes 1 and 16: molecular weight marker; lane 8: plasmid DNA positive control (pQEαβT₁FucA and pJH222.HIVA plasmid DNA); lanes 13 and 14: BCG wild type; lane 15: distilled water (negative control).

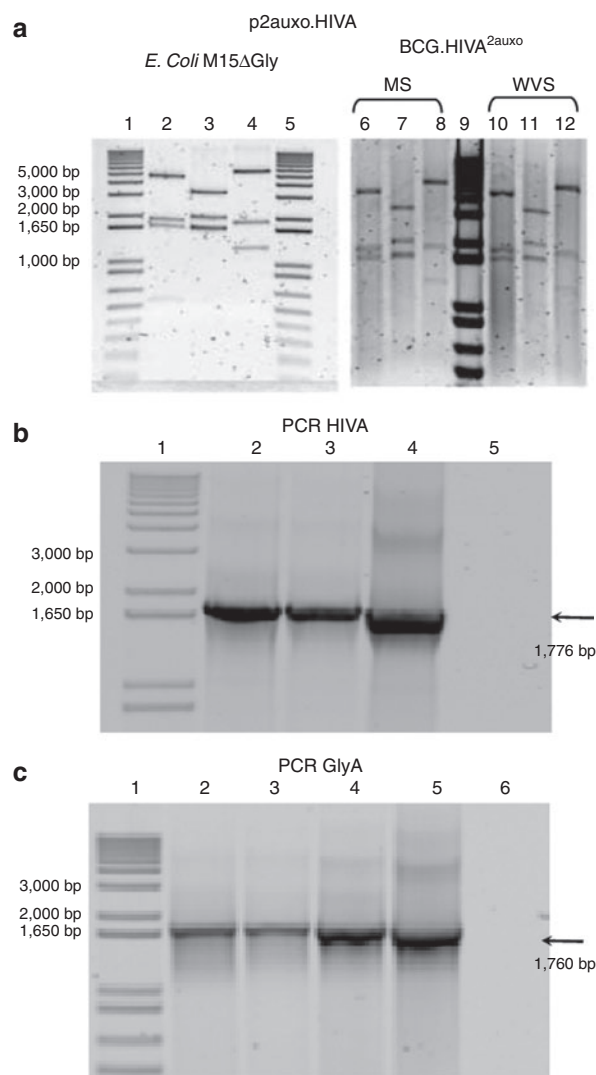


Figure 2 Genetic characterization of the BCG.HIVA^{2auxo} strain. **(a)** Enzymatic restriction analysis of p2auxo.HIVA plasmid DNA extracted from *E. coli* M15Δ*glyA* cultures (pre-BCG transformation) and from both the MS and WVS of BCG.HIVA^{2auxo} cultures. Left side: *E. coli* cultures. Lanes 1 and 5: molecular weight marker (1 kb plus; Invitrogen); lanes 2, 3, and 4: *AgeI*, *StuI*, and *XhoI* digestion, respectively. Right side: BCG cultures. Lane 9: molecular weight marker (1 kb plus; Invitrogen); lanes 6, 7, and 8 (MS): *AgeI*, *StuI*, and *XhoI* digestion, respectively. Lanes 10, 11, and 12 (WS): *AgeI*, *StuI*, and *XhoI* digestion, respectively. **(b)** PCR analysis of HIVA DNA coding sequence using as template the cultures of BCG.HIVA^{2auxo} MS (lane 2), WVS (lane 3), positive control plasmid DNA p2auxo.HIVA (lane 4), negative control, distilled water (lane 5), and molecular weight marker (lane 1). **(c)** PCR analysis of *E. coli glyA* DNA coding sequence using as template the cultures of BCG.HIVA^{2auxo} MS (lane 2), WVS (lane 3), p2auxo plasmid DNA without HIVA immunogen insert (lane 4), positive control plasmid DNA p2auxo.HIVA (lane 5), negative control, distilled water (lane 6), and molecular weight marker (lane 1).

in four out of five colonies after four subcultures. To confirm that lack of HIVA protein expression in bacteria that were grown without selective pressure was due to plasmid loss, 20 colonies were cultured on selective and nonselective medium. No protein expression was observed in five out of five colonies that were grown with lysine supplementation but not without lysine supplementation after four subcultures (Figure 3b).

Structural stability of the p2auxo.HIVA plasmid DNA was evaluated by PCR analysis and restriction enzyme digestion pattern. When

bacteria were grown under selective pressure, the PCR band corresponding to HIVA DNA coding sequence was detected in five out of five BCG.HIVA^{2auxo} colonies. Conversely, when bacteria were grown without selective pressure, the PCR and the plasmid DNA extraction were negative in five out of five colonies that were grown with lysine supplementation but not without lysine supplementation after four subcultures (Figure 3c). Moreover, when bacteria were grown under selective pressure, the *HindIII* digestion pattern (HIVA DNA fragment release) was detected in four out of five BCG.HIVA^{2auxo} colonies. The BCG colony (#44) with low HIVA protein expression levels corresponded to the rBCG colony with altered restriction pattern, suggesting some mutation in the expression cassette (not shown).

BCG.HIVA^{2auxo} prime and MVA.HIVA boost regimen elicited HIV-1-specific CD8⁺ and purified protein derivative-specific T-cell responses in mice

In this study, we have evaluated the specific HIV-1 T-cell immune responses in BALB/c mice after immunization with BCG.HIVA^{2auxo} prime and MVA.HIVA boost (Figure 4a). The immunogenicity read-out was focused on the P18–I10 epitope, an immunodominant CTL epitope derived from HIV-1 Env and H-2D^d murine restricted, which was fused to HIVA immunogen to evaluate the immunogenicity in mice (Figure 1a). Functional specific T cells in response to peptide stimulation were measured by intracellular cytokine staining and enzyme-linked immunosorbent spot (ELISPOT) assays. We have observed that BCG.HIVA^{2auxo} prime and MVA.HIVA boost elicited the highest proportion of P18–I10 epitope-specific CD8⁺ T-cells producing interferon- γ (IFN- γ), compared with the BCG wild-type priming and MVA.HIVA boost and with MVA.HIVA alone (Figure 4b). The quality of the elicited CD8⁺ T cells in terms of their ability to produce IFN- γ and tumor necrosis factor- α and to degranulate (surface expression of CD107a) in response to P18–I10 peptide stimulation was also investigated. We found that BCG.HIVA^{2auxo} prime and MVA.HIVA boost induced higher frequencies of trifunctional specific CD8⁺ T cells compared with the BCG wild-type priming and MVA.HIVA boost and with MVA.HIVA alone (Figure 4c). The capacity of splenocytes from vaccinated mice to secrete IFN- γ was tested also by ELISPOT assay. We observed the highest frequency of specific cells secreting IFN- γ in mice primed with BCG.HIVA^{2auxo} and boosted with MVA.HIVA (Figure 4d). Further experiments assessing different doses, routes, and immunization schedules should be performed.

BCG.HIVA^{2auxo} elicited purified protein derivative-specific responses in mice. The capacity of splenocytes from vaccinated mice to secrete IFN- γ was tested by ELISPOT assays after overnight stimulation with the purified protein derivative antigen. The median spot-forming units per 10⁶ splenocytes were similar in mice primed with BCG.HIVA^{2auxo} or BCG wild type (196 and 222 spot-forming units/million splenocytes, respectively; Figure 4e).

BCG.HIVA^{2auxo} prime and MVA.HIVA boost were well tolerated in mice

As shown in Figure 5b, the body mass was monitored over time and recorded to depict any adverse events and body mass loss due to vaccination. In order to detect vaccine-derived adverse events, a 12-week period between BCG-prime and MVA boost was established for this trial (Figure 5a). Importantly, no statistically significant difference was observed between the vaccinated mice groups and the naive mice group in all monitored time points. Furthermore, between weeks 1 and 14, the body mass monitored in all vaccinated mice groups was found to lie between the mean \pm 2 SD body mass

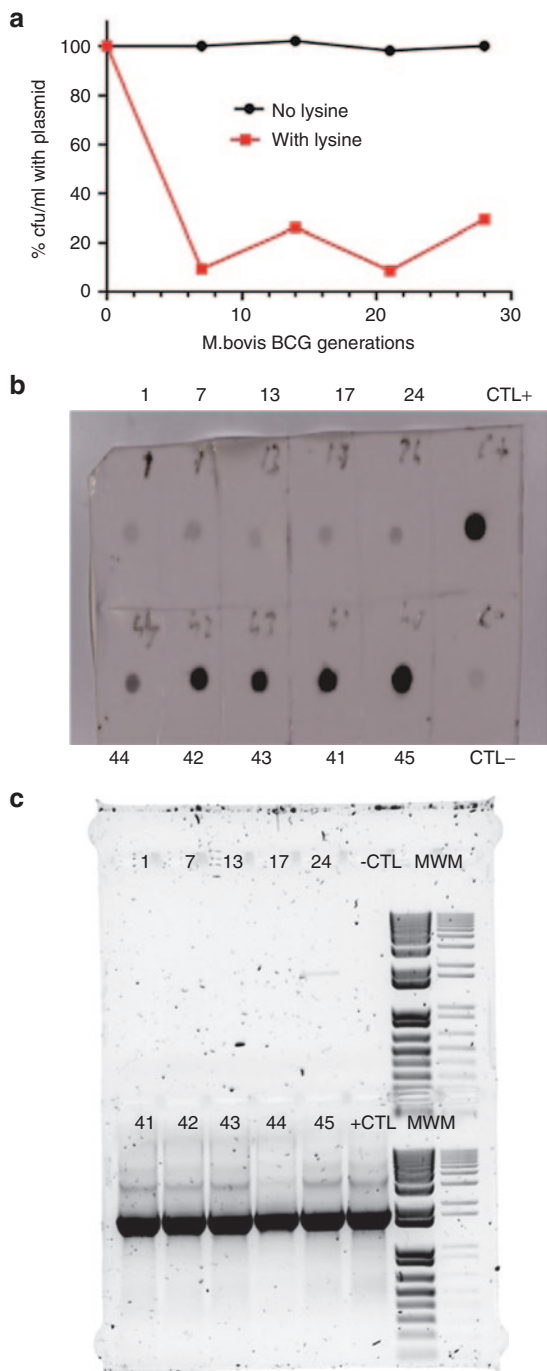


Figure 3 *In vitro* stability of the BCG.HIVA^{2auxo} strain. **(a)** *In vitro* persistence of the p2auxo.HIVA in BCG *lysA*⁻ grown for successive passages on selective (no lysine) or nonselective (supplemented with lysine) media. The percentage represents the cfu (titer) that maintained the vector containing the lysine complementing gene (grown on selective medium) versus to the total cfu. For BCG, the generation time is ~24 h. Thus, four subcultures represent ~30 BCG generations. The most representative of two experiments is shown. **(b)** Immunodot of BCG colonies lysates that were grown on selective (41, 42, 43, 44, and 45) and nonselective medium (1, 7, 13, 17, and 24); lysates of BCG wild type were used as negative control and lysates of BCG.HIVA^{2auxo} WVS (before subculturing) as positive control. **(c)** HIVA PCR from purified plasmids of five individual colonies that were grown on selective (41, 42, 43, 44, and 45) and nonselective (1, 7, 13, 17, and 24) medium. Plasmid DNA p2auxo.HIVA pretransformation in BCG was used as positive control. Distilled water (negative control).

curve in naive mice (Figure 5b). It is also important to mention that no mice died during the trial, and no local adverse events and associated systemic reactions were observed.

DISCUSSION

Despite the progress made on prevention of mother-to-child HIV-1 transmission, the development of a safe, effective, and affordable vaccine against HIV-1 and tuberculosis at the earliest time after birth to prevent breast milk HIV-1 transmission and childhood tuberculosis is still a great challenge. In this study, we have constructed a novel *E. coli*-mycobacterial shuttle vector that contains the antibiotic-free plasmid selection system. It was stable *in vivo* for at least 7 weeks after mice immunization and was used to construct a marker-less BCG.HIVA^{2auxo} vaccine suitable for good manufacturing practice. Overall, we have demonstrated that BCG.HIVA^{2auxo} prime-MVA.HIVA boost regimen was well tolerated and induced HIV-1- and *Mtb*-specific immune responses in mice. Thus, this strategy might be worthy to pursue and for joining the global efforts to develop novel BCG vector-based vaccines for controlling tuberculosis and HIV/AIDS.

Even though, it has been described that antibiotics and antibiotic resistance genes have been traditionally used for the selection and maintenance of recombinant plasmids in hosts such as *E. coli*, their use has been considered unacceptable for clinical trials and product licensing. Several approaches have been pursued to replace antibiotics as selective markers for plasmid stability in bacteria, including plasmids harboring gene complementation of a host auxotrophy. In this study, we have constructed a novel *E. coli*-mycobacterial expression vector that employs an antibiotic resistance-free mechanism for plasmid selection and maintenance based on the *glyA* for glycine auxotrophy complementation in *E. coli* and the *lysA* for lysine auxotrophy complementation in BCG.

The conventional methods for the identification of *M. bovis* BCG (BCG) vaccines, based on microscopic examination, biochemical tests, and morphological appearance, provided only limited sub-strain differentiation and no specificity for BCG. The best way to identify different BCG substrains is by using molecular methods and genomic approaches. In this study, we have used the multiplex PCR assay described by Bedwell *et al.*²² to identify our BCG.HIVA^{2auxo} vaccine candidate. Resultant fingerprints after multiplex PCR assay of our BCG vaccine Pasteur substrain were consistent with the PCR pattern of BCG Pasteur.

Méderlé *et al.*²³ described the construction of rBCG strains co-expressing *nef* and *gag* (p26) from simian immunodeficiency virus (SIV) mac251. Extrachromosomal cloning into a replicative plasmid resulted in strains of low genetic stability that rapidly lost the plasmid *in vivo*. They observed that the genetic stability of integrative rBCG strains was much higher than that of replicative strains, both *in vitro* and *in vivo* mouse model. Borsuk *et al.*²⁴ reported the construction of a BCG expression system using auxotrophic complementation as a selectable marker. Stability of the multicopy plasmid was evaluated during *in vitro* and *in vivo* growth of the rBCG in comparison to selection by antibiotic resistance. The new system was highly stable even during *in vivo* growth, as the selective pressure is maintained, whereas the conventional vector was unstable in the absence of selective pressure. These data are in concordance with our results. We have evaluated the *in vitro* stability of the p2auxo.HIVA plasmid DNA harboring the auxotrophic complementation *lysA* gene. This plasmid was used to transform lysine auxotroph of BCG strain. All BCG.HIVA^{2auxo} colonies that were grown on selective pressure maintained the vector *in vitro* and *in vivo*, whereas growth without selective pressure led to instability of the system *in vitro* due

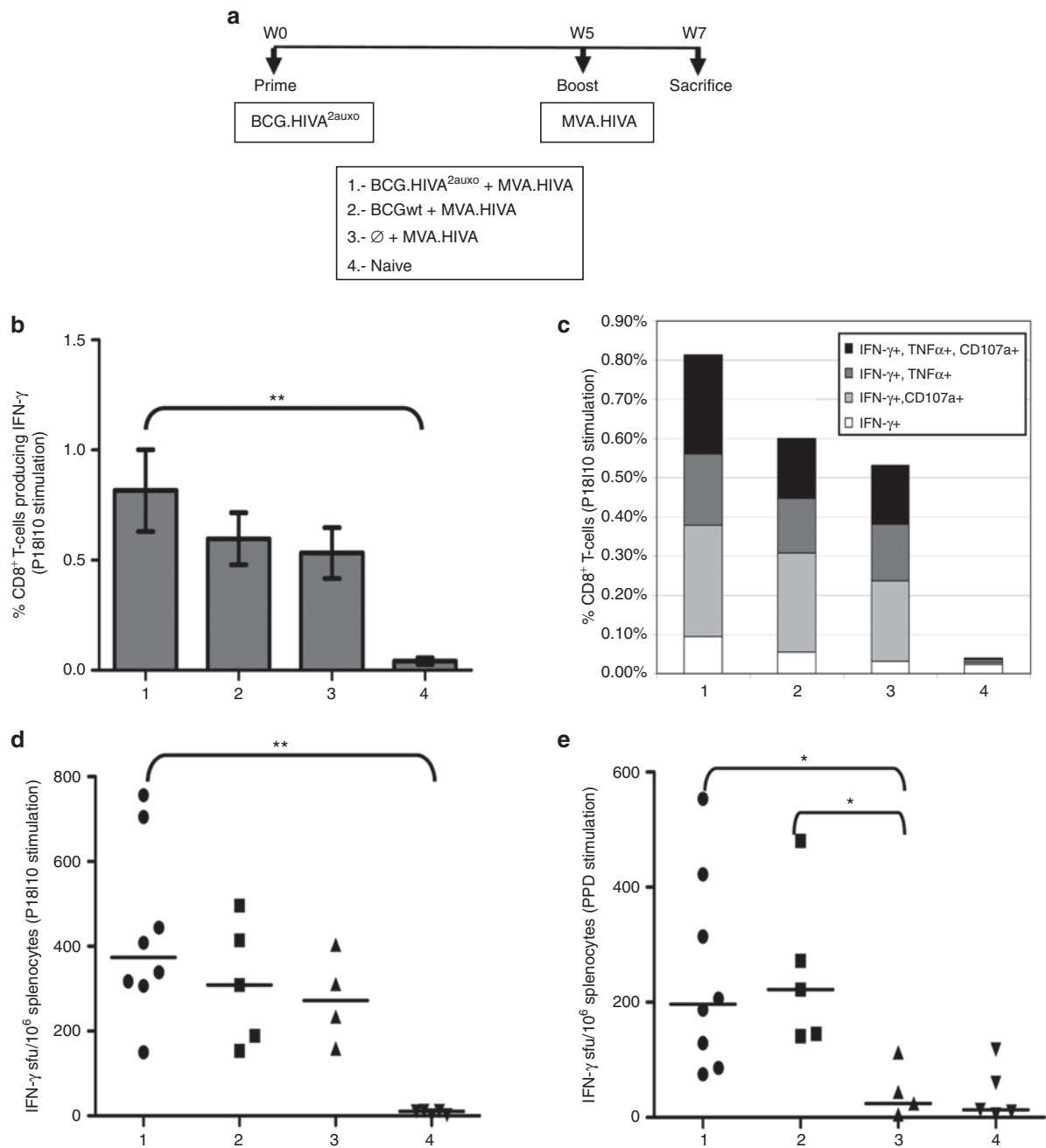


Figure 4. Induction of HIV-1- and *Mtb*-specific T-cells responses by the BCG.HIVA^{2auxo} prime–MVA.HIVA boost regimen. **(a)** Adult mice (7-weeks-old) were either left unimmunized or primed with 10⁶ cfu of BCG.HIVA^{2auxo} or BCG wild type (intradermally) and boosted with 10⁶ pfu of MVA.HIVA (intramuscularly) 5-weeks post-BCG inoculation. Mice were sacrificed 2 weeks later for T-cell analysis. **(b)** Analysis of IFN- γ vaccine elicited HIV-1-specific CD8⁺ T-cell responses. The frequencies of cells producing cytokine are shown. Data are presented as means (SEM; $n = 8$ for group 1, and $n = 5$ for groups 2, 3, and 4). **(c)** The functionality of vaccine-induced CD8⁺ T-cell responses was assessed in a multicolor intracellular cytokine staining assay. The group mean frequencies of single-, double-, or triple cytokine-producing P18–110-specific cells are shown for the four vaccination groups. **(d)** Elicitation of specific T-cell responses was assessed in an *ex vivo* IFN- γ enzyme linked immunosorbent spot (ELISPOT) assay using the immunodominant P18–110 CD8⁺ T-cell epitope peptide. The median spot-forming units (SFU) per 10⁶ splenocytes for each group of mice ($n = 8$ for group 1, and $n = 5$ for groups 2, 3, and 4) as well as individual animal responses is shown. **(e)** Purified protein derivative (PPD)-specific T-cell responses elicited by BCG.HIVA^{2auxo}. Immune responses to BCG were assessed in an *ex vivo* IFN- γ ELISPOT assay using PPD as the antigen. The median SFU per 10⁶ splenocytes for each group of mice ($n = 8$ for group 1, and $n = 5$ for groups 2, 3, and 4) as well as individual animal responses is shown. * $P < 0.05$, ** $P < 0.01$.

to plasmid loss. Regarding the functional stability of the heterologous HIVA gene, we found that the level of HIVA protein expression remained stable when bacteria were grown under selective pressure in four out of five colonies after four subcultures. Importantly, the BCG colony (#44) that showed low level of protein expression had also an altered *Hind*III digestion pattern, suggesting that it might

be due to mutation or genetic rearrangement in the expression cassette. Contrarily, Borsuk *et al.*²⁴ observed that the level of expression of *lacZ* remained stable with successive passages, and no gross modifications in vector structure were observed in five individual colonies when bacteria were grown under selective pressure. When we evaluated the *in vivo* plasmid stability, we found that 100% of

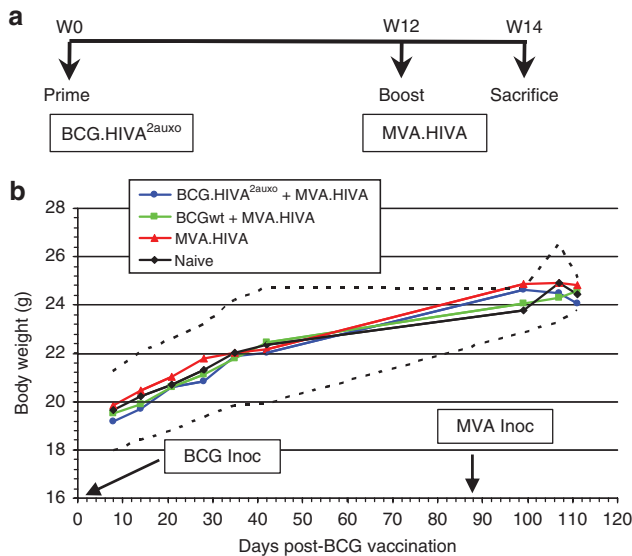


Figure 5 BCG.HIVA^{2auxo} prime and MVA.HIVA boost safety in adult mice. (a) Adult mice were either left unimmunized or immunized with 10^6 cfu of BCG wild type or BCG.HIVA^{2auxo} by intradermal route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA at week 12 by intramuscular route. (b) The body weight was recorded over time, and the mean for each group of mice is shown ($n = 10$ for group 1 and $n = 5$ for groups 2, 3, and 4). Data from naive mice are presented as mean \pm 2 SD ($n = 5$, dashed lines). The weight differences between vaccinated and naive mice group were analyzed in all monitored time points by analysis of variance test.

rBCG colonies recovered from the spleens of BALB/c mice 7 weeks after immunization maintained the plasmid.²⁴ We did not evaluate the *in vivo* plasmid stability of the rBCG colonies without selective pressure because as stated by Pavelka *et al.*²⁵, the *Mycobacterium tuberculosis* lysine auxotroph mc² 3026 vaccine strain was cleared from the lungs and spleens at day 30.

Our group and others have shown in murine and nonhuman primate studies that rBCG elicited cell-mediated responses against HIV-1 and SIV antigens.^{13,26–29} However, a small proportion of these animal studies used rBCG strains in heterologous prime-boost regimens. Ami *et al.*³⁰ have demonstrated that macaques vaccinated with rBCG-expressing SIV *gag* and boosted with replication-defective poxvirus-SIV *gag*, elicited effective protective immunity against mucosal challenge with SHIV KS661c. There is data showing that rBCG is a good priming vector in heterologous prime-boost vaccination regimens to enhance specific T-cell responses.^{5,31,32} In tuberculosis vaccine human trials, McShane *et al.*³³ have demonstrated that vaccination with MVA-expressing Ag85A boosts preexisting antimycobacterial immune responses induced either by environmental mycobacteria or BCG vaccination, but the latest findings in a randomized, placebo-controlled phase 2b trial have shown no efficacy against tuberculosis or *M. tuberculosis* infection in infants.³⁴ We have previously shown in BALB/c mice that the inclusion of BCG.HIVA²²² in a heterologous prime-boost regimen can prime and increase the HIV-1-specific T-cell immune responses elicited by MVA.HIVA and MVA.HIVA.Ag85A.^{5,16,26,35}

There are few reports in the literature describing the safety and immunogenicity of rBCG-expressing HIV-1 antigens in neonatal mice. Ranganathan *et al.*⁴ have evaluated the immunogenicity in neonatal mice of three different recombinant attenuated *Mtb* strains expressing an HIV-1 envelope and showed that single dose immunization rapidly generated HIV-1 and *Mtb*-specific T-cell immune responses. In a previous study, we showed in newborn mice that BCG.HIVA^{CAT} prime and MVA.HIVA boost increased the frequencies

of specific CD8⁺ T-cells producing IFN- γ . We observed in newborn mice a lower level of HIV-1-specific T-cell immune responses compared with adult mice. Rosario *et al.*³⁶ have assessed the immunogenicity of the BCG.HIVA²²² prime and MVA.HIVA boost regimen in newborn rhesus macaques and made similar observation.

Here, the vaccination with BCG wild type and BCG.HIVA^{2auxo} strain induced BCG-specific responses in adult mice. Studies in neonatal mice have indicated that immune responses at birth are often biased toward the Th2 type and defective in the Th1 type, the central defense mechanism against intracellular pathogens.^{37–39} However, it has been described that BCG vaccination at birth induces a potent Th1-type immune response in humans and in mice.⁴⁰ The challenge for neonatal vaccinology is thus to develop, and promote at a global level, vaccines that could be safely administered soon after birth and would be effective after one or two early doses. According to our knowledge, only one report has been published about safety of antibiotic-free marker rBCG-based HIV-1 vaccine in mice.⁴¹ In this study, we have demonstrated in adult mice by the rate of body mass that BCG.HIVA^{2auxo} prime and MVA.HIVA boost regimen was well tolerated.

We constructed and characterized a novel, safer, good laboratory practice-compatible BCG-vectored vaccine using prototype immunogen HIVA and tested the safety and immunogenicity of BCG.HIVA^{2auxo} and MVA.HIVA in mice using the prime-boost regimen. In conclusion, the construction of this new *E. coli*-mycobacteria shuttle vector based on double auxotrophic complementation and antibiotic-free plasmid selection system will provide a new and improved methodological tool for mycobacterial vaccines design and development as a bacterial live recombinant vaccine vehicle. A BCG strain free of antibiotic resistance marker genes expressing a second-generation immunogen HIVconsv better addressing the HIV-1 variability and escape⁴² is under construction. In addition, the same strategy will be used for other major pediatric pathogens such as malaria or tuberculosis, to prime protective response soon after birth.^{2,43}

MATERIALS AND METHODS

Construction of BCG.HIVA^{2auxo} strain by using an antibiotic-free plasmid selection system and expressing HIV-1 clade A immunogen

The *E. coli*-mycobacterial shuttle vector, plasmid pJH222.HIVA, was previously constructed in our laboratory.⁵ The *aph* gene, conferring kanamycin resistance, was removed from pJH222.HIVA plasmid by *SpeI* digestion. Following *aph* gene excision, the *glyA* gene cassette was ligated into this plasmid and subsequently transformed into *E. coli* M15 Δ glyA strain. Briefly, this cassette included the following elements: the α fragment, containing the weak constitutive P3 promoter; the β fragment containing the *glyA* gene, which encodes the enzyme serine hydroxymethyl transferase; and its own terminator sequence T1, a string of termination codons. In *E. coli*, the synthesis of intracellular glycine is carried out mainly by serine hydroxymethyl transferase enzyme. The $\alpha\beta$ T1 DNA fragment was amplified by PCR using the pQE $\alpha\beta$ T1FucA plasmid DNA as template.⁴⁴ The primers were designed to incorporate *SpeI* and *SmaI* sites at both 5' and 3' termini of the amplified DNA fragment to be subcloned into pNEB193 vector (NEB, Ipswich, MA). Finally, the pNEB193.GlyA plasmid DNA was digested by *SpeI*, and the released $\alpha\beta$ T1 DNA fragment was cloned into pJH222.HIVA vector (lacking the kanamycin resistance gene after *SpeI* digestion), to generate the p2auxo.HIVA plasmid DNA.

Bacterial strains and cultures

E. coli Stbl2 cells (Invitrogen, Alcobendas, Spain) were grown in Luria-Bertani broth or Luria-Bertani agar plates (Sigma-Aldrich, St Louis, MO) at 30 °C. Luria-Bertani was supplemented with ampicillin (50 μ g/ml). *E. coli* M15 Δ glyA cells (glycine auxotrophic)²⁰ were grown in Luria-Bertani or minimal medium M9-derivative (Na₂HPO₄, 6.78 g/l; KH₂PO₄, 3 g/l; NaCl, 0.5 g/l; NH₄Cl, 1 g/l; glucose, 10 g/l; MgSO₄, 2 mmol/l; CaCl₂, 0.1 mmol/l; thiamine, 0.1 g/l; FeCl₃,

0.025 g/l; $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 0.13 mg/l; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.6 mg/l; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.47 mg/l; $\text{CuSO}_4 \cdot \text{H}_2\text{O}$, 4.6 mg/l; H_3BO_3 , 0.03 mg/l; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4.2 mg/l; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 mg/l; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.06 mg/l), supplemented with glycine (70 $\mu\text{g}/\text{ml}$). The p2auxo.HIVA plasmid DNA was transformed by electroporation in glycine auxotroph of *E. coli* cultures that were grown in M9-D broth or agar plates. On the other hand, the p2auxo.HIVA plasmid DNA was transformed by electroporation in a lysine auxotroph of BCG, kindly provided by Dr William Jacobs. Mycobacterial cultures were grown in Middlebrook 7H9 broth medium or on Middlebrook agar 7H10 medium supplemented with albumin–dextrose–catalase (Becton Dickinson, Sparks, MD) and containing 0.05% Tween 80. The L-lysine monohydrochloride (Sigma-Aldrich) was dissolved in distilled water and used at a concentration of 40 $\mu\text{g}/\text{ml}$. *Escherichia coli* and BCG electroporation conditions were described previously.^{5,45} Commercial BCG Danish 1331 strain (Pfizer, New York, NY) was kindly provided by Dr Neus Altet and commercial BCG Connaught strain (ImmuCyst; Sanofi Aventis, Barcelona, Spain), from the Urology Department at Hospital Clínic de Barcelona.

Dot-blot analysis

Cell lysates of mid-logarithmic-phase BCG transformants were prepared by sonication and using a protein extraction buffer (50 mmol/l Tris–HCl pH 7.5, 5 mmol/l EDTA, 0.6% sodium dodecyl sulfate) and 100 \times protease inhibitor cocktail (1 mg/ml aprotinin, 1 mg/ml E-64, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 50 mg/ml pefabloc SC, and 10 ml dimethyl sulfoxide). Five microliters of the protein extract was blotted onto a pretreated polyvinylidene difluoride membrane, and HIVA protein was detected using anti-Pk monoclonal antibody (MCA1360; AbD Serotec, Oxford, UK), with an enhanced chemiluminescence kit (Pierce, Rockford, IL). To visualize the dots, the Typhoon 8600 gel imaging system (GE Healthcare, Piscataway, NJ) was used.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis

Cell lysates of mid-logarithmic-phase BCG transformants were prepared, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electroblotted. HIVA protein was detected using anti-Pk antibodies with an enhanced chemiluminescence kit (Pierce).

In vitro stability of BCG.HIVA^{2auxo} strain

Four subcultures (~30 bacterial generations) of BCG.HIVA^{2auxo} (working vaccine stock) harboring the p2auxo.HIVA plasmid DNA that contains the lysine complementing gene were grown in 7H9 broth with and without selection (L-lysine for ΔlysA strains). Subcultures were performed every 7 days, by transferring 100 μl of the stationary phase culture to 5 ml of fresh medium. The titer of the rBCG colonies on selective and nonselective 7H10 plates were compared in every subculture. To evaluate the functional stability of HIVA gene, the HIVA heterologous protein expression from five individual colonies of the fourth subculture, that were grown on selective and nonselective medium, was determined by dot-blot analysis of BCG lysates and compared with the original BCG.HIVA^{2auxo} working vaccine stock. In addition, the restriction enzyme digestion pattern (*HindIII–HindIII*, HIVA DNA fragment cloning sites) and the PCR analysis of the HIVA DNA coding sequence from purified plasmids of five individual colonies of the fourth subculture as mentioned previously were determined to evaluate the plasmid's structural stability.

In vivo stability of plasmid p2auxo.HIVA

The growth of BCG.HIVA^{2auxo} and the *in vivo* stability of the extrachromosomal plasmid p2auxo.HIVA were established by the recovery of BCG.HIVA^{2auxo} colonies from the spleens after 7 weeks of mice immunization with 10^6 cfu of BCG.HIVA^{2auxo} (i.d.) and 10^6 pfu of MVA.HIVA (i.m.). Spleens were homogenized and plated onto Middlebrook 7H10 medium, and the resulting BCG colonies were inoculated in 7H9 medium. The DNA coding sequence corresponding to HIVA immunogen was detected by PCR analysis, using the BCG liquid culture as a template. Specific primers were designed to amplify the DNA fragment encoding the chimeric 19-kDa lipoprotein signal sequence–HIVA protein.

Sample preparation for the multiplex PCR assay

For isolation of DNA from wild-type BCG and BCG.HIVA^{2auxo}, 400 μl of mycobacterial culture were centrifuged at 13,000g for 10 minutes, at room

temperature, the pellet was resuspended in 250 μl of distilled water and heated to 95 °C in a thermoblock for 15 minutes to lyse and inactivate vegetative bacterial forms. Finally, after 5 minutes of centrifugation at 10,000g, 5 μl of supernatant were used for the amplification reaction or stored at –20 °C. The commercial BCG strains were treated in a similar way, but in this case, 400 μl of the reconstituted freeze-dried flask were used.

Multiplex PCR assay for *M. bovis* BCG substrain Pasteur identification

The multiplex PCR assay was previously described by Bedwell *et al.*²² For the PCR analysis, 5 μl of the mycobacterial DNA isolated from BCG.HIVA^{2auxo} Pasteur and commercial BCG strains were used in a final reaction volume of 50 μl .

E. coli and mycobacterial plasmid DNA extraction

For *E. coli* plasmid DNA isolation, the QIAprep Spin Miniprep Kit was used following manufacturer's instructions (Qiagen, Hilden, Germany). The BCG broth culture up to an optical density of 0.9 (600 nm) was used for mycobacterial plasmid DNA isolation. The QIAprep Spin Miniprep Kit (Qiagen) was used with following slight modifications: (i) prior to harvest (3 to 24 hours), sterile glycine at a final concentration of 1% (weight/volume) was added; (ii) the cell pellet was treated with the P1 buffer from the Miniprep Qiagen Kit, supplemented with 10 mg/ml of lysozyme (Sigma) and incubated at 37 °C overnight; (iii) the extraction column was treated with a 10-ml mixture of chloroform:isopropanol in 1:1 ratio. The mycobacterial plasmid DNA isolated was used for restriction enzyme analysis and PCR analysis.

Mice immunizations and isolation of splenocytes

Adult (7-weeks-old) female BALB/c mice were left either unimmunized or immunized with BCG.HIVA^{2auxo} and were boosted with MVA.HIVA at doses, routes, and schedules outlined in the figure legends. On the day of sacrifice, individual spleens were collected, and splenocytes were isolated by pressing spleens through a cell strainer (Falcon; Becton Dickinson) using 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).

Peptides

For assessing the immunogenicity of HIVA in the BALB/c mice, the following peptides were used: H-2Dd-restricted epitope P18–110 (RGGRGFVTI).⁴⁶ The purified protein derivative (Statens Serum Institute, Copenhagen, Denmark) was used to assess the immunogenicity induced by *M. bovis* BCG.

Ex vivo IFN- γ ELISPOT assay

The ELISPOT assay was performed using a commercial IFN- γ ELISPOT kit (Mabtech, Nacka Strand, Sweden) and following manufacturer's instructions. The ELISPOT plates (MSISP4510, 96-well plates with polyvinylidene difluoride membranes, Millipore, Billerica, MA) were coated with purified anti-mouse IFN- γ capture monoclonal antibody diluted in phosphate-buffered saline to a final concentration of 5 $\mu\text{g}/\text{ml}$ at 4 °C overnight. A total of 5×10^5 fresh splenocytes were added to each well and stimulated with 2 $\mu\text{g}/\text{ml}$ of the P18–110 peptide or 5 $\mu\text{g}/\text{ml}$ of purified protein derivative for 16 h at 37 °C. Wells were washed 4 \times with phosphate-buffered saline 0.05% Tween 20 and 2 \times with phosphate-buffered saline before incubating with 100- μl 5-bromo-4-chloro-3-indoyl-phosphate/nitro blue tetrazolium substrate solution (Sigma). After 5–10 minutes, the plates were washed with tap water, dried, and the resulting spots 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) and pulsed with 2 $\mu\text{g}/\text{ml}$ of P18–110 peptide and kept at 37 °C, 5% CO₂ for 60 minutes, followed by the addition of GolgiStop (Becton Dickinson) containing monensin. After 5-hour incubation, reaction was terminated by storing the plate at 4 °C. The cells were washed with wash buffer (phosphate-buffered saline, 2% fetal calf serum, 0.01% azide) and blocked with anti-CD16/32 (BD Biosciences) at 4 °C for 30 minutes.

All subsequent antibody stains were performed using the same conditions. Cells were then washed and stained with anti-CD8-PerCP (BD Biosciences) and anti-CD107a-FITC, washed again, and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Perm/wash buffer (BD Biosciences) was used to wash cells before staining with anti-IFN- γ -APC and anti-tumor necrosis factor- α -PE (BD Biosciences). Cells were fixed with CellFIX (BD) and stored at 4 °C until analysis. All chromogen-labeled cells were analyzed in a Becton Dickinson FACScalibur, using the CellQuest software for acquisition (Becton Dickinson) and the Flow-Jo software (Tree Star, Ashland, OR) for analysis.

Statistical analysis

Immunogenicity data are shown as group means or group medians as well as individual responses. The body mass data are group means, and mean \pm 2 SD in naive mice group. Statistical significance was determined by analysis of variance. Statistical significance of the *in vitro* stability assay was assessed by a two-way analysis of variance (* P < 0.05; ** P < 0.01). GraphPad Prism 5.0 software was used.

Ethics statement

The animal experiments were approved by the ethical committee for animal experimentation from University of Barcelona and strictly conformed to Catalan animal welfare legislation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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