HUMAN VACCINES & IMMUNOTHERAPEUTICS
2017, VOL. 13, NO. 8, 1798–1810
https://doi.org/10.1080/21645515.2017.1316911

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RESEARCH PAPER

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Preclinical development of BCG.HIVA$^{2auxo.int}$, harboring an integrative expression vector, for a HIV-TB Pediatric vaccine. Enhancement of stability and specific HIV-1 T-cell immunity

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ABSTRACT

One of the critical issues that should be addressed in the development of a BCG-based HIV vaccine is genetic plasmid stability. Therefore, to address this issue we have considered using integrative vectors and the auxotrophic mutant of BCG complemented with a plasmid carrying a wild-type complementing gene. In this study, we have constructed an integrative E. coli-mycobacterial shuttle plasmid, p2auxo. HIVA^{int}, expressing the HIV-1 clade A immunogen HIVA. This shuttle vector uses an antibiotic resistance-free mechanism for plasmid selection and maintenance. It was first transformed into a glycine auxotrophic *E. coli* strain and subsequently transformed into a lysine auxotrophic *Mycobacterium bovis* BCG strain to generate the vaccine BCG.HIVA$^{2auxo.int}$. Presence of the HIVA gene sequence and protein expression was confirmed. We demonstrated that the *in vitro* stability of the integrative plasmid p2auxo.HIVA^{int} was increased 4-fold, as compared with the BCG strain harboring the episomal plasmid, and was genetically and phenotypically characterized. The BCG.HIVA$^{2auxo.int}$ vaccine in combination with modified vaccinia virus Ankara (MVA).HIVA was found to be safe and induced HIV-1 and *Mycobacterium tuberculosis*-specific interferon-γ-producing T-cell responses in adult BALB/c mice. We have engineered a more stable and immunogenic BCG-vectored vaccine using the prototype immunogen HIVA. Thus, the use of integrative expression vectors and the antibiotic-free plasmid selection system based on "double" auxotrophic complementation are likely to improve the *mycobacterial* vaccine stability *in vivo* and immunogenicity to develop not only recombinant BCG-based vaccines expressing second generation of HIV-1 immunogens but also other major pediatric pathogens to prime protective responses shortly following birth.

ARTICLE HISTORY
Received 19 December 2016
Revised 10 March 2017
Accepted 1 April 2017

KEYWORDS
bacterial; HIV; HIV; HIV Vaccine; infectious disease; *in vitro* stability; auxotrophic complementation; recombinant BCG; TB; Tuberculosis vaccine; vaccinology; viral vector

Introduction

According to the UNAIDS July 2016 Report, at the end of 2015, an estimated 36.7 million people were living with HIV worldwide and 2.1 million individuals became newly infected with the virus in 2015. The number of people dying of AIDS-related causes was 1.1 million in 2015, and it is estimated that AIDS has orphaned more than 19 million children. Sub-Saharan Africa accounted for 66% of new HIV infections in 2015, 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.[1] Development of an effective, safe and accessible vaccine for neonates and/or adults would be the optimal solution for preventing infection or reducing the severity of HIV-related diseases.

Infection with Mycobacterium tuberculosis (Mtb) kills about 1.4 million people each year and goes hand-in-hand with HIV-1. Mycobacterium bovis bacillus Calmette–Guérin (BCG) is the only licensed vaccine and confers significant protection against childhood and milliary tuberculosis.[2] Globally, 80% of children are vaccinated with BCG in countries where it is part of the national childhood immunization program, the majority of them at birth.[3] Thus, the development of a combined vaccine, which would protect neonates against tuberculosis and mother to child transmissions (MTCT) of HIV-1 through breastfeeding, is a logical effort in the fight against these 2 major global pathogens.

There is an urgent need for a neonatal immunogen that generates rapid HIV-specific immunity. Recombinant BCG (rBCG) has been developed as a candidate neonatal vaccine vector against pertussis,[4] measles,[5] respiratory syncytial virus (RSV)[6] and breast milk HIV transmission.[7-9] BCG as a vaccine vector has several attractive features: (i) BCG has a proven record of safety as a vaccine against tuberculosis from its use in over 2 billion individuals.[10] However, BCG has now been questioned for safety, especially in HIV-endemic regions where both HIV and TB are highly endemic. Currently, HIV infection in infants is now a full contraindication to BCG vaccination.[11] Nevertheless, the BCG Working Group of the International Union against Tuberculosis and Lung Disease (IUTLD) recommended that current universal BCG immunization of infants continue in countries highly endemic for TB until they have all programs in place for implementing selective deferral of HIV-exposed infants[12]; (ii) BCG infects and colonizes macrophages

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and dendritic cells, where it can survive and replicate for a long period of time[13];(iii) Through its persistence and potent adjuvantation by its cell wall components, it can induce long-lasting humoral and cellular immune responses[14];(iv) BCG can be administered at or any time after birth, and is not affected by maternal antibodies[15,16]; (v) Manufacturing of BCG-based vaccines is inexpensive and finally;(vi) BCG is one of the most heat-stable vaccines currently in use.[17]

There is strong evidence in favor of a role for HIV-1 specific T-cell responses in the control of HIV-1 replication.[8,18,19] 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.[20-22] We have been working on rBCG based HIV-1 vaccine development for many years with the aim of inducing protective cell-mediated responses. Our starting platform was based on a heterologous rBCG prime and recombinant modified vaccinia virus Ankara (MVA) boost regimen delivering a commonmmunogen called HIVA, which is derived from consensus Gag protein of HIV-1 clade A, prevalent in Central and Eastern Africa, and a string of $CD8^+$ T-cell epitopes.[23] Initially, we engineered the BCG.$HIVA^{222}$ vaccine candidate that was vectored by a lysine auxotroph of the BCG Pasteur strain harbouring an episomal E.coli-mycobacterial shuttle plasmid pJH222.HIVA with a lysine A complementing gene and a weak promoter to drive HIVA gene expression.[9] When the BCG.$HIVA^{222}$ vaccine was used in a prime-boost regimen with heterologous vectors, HIV-1-specific immune responses provided protection against surrogate viral challenge, and the vaccine also conferred equally efficient protection against *Mtb* aerosol challenge as compared with the BCG 1173 P2 vaccine Pasteur strain.[8] This design increases the plasmid stability in vivo and prevents heterologous gene expression disruption by genetic rearrangements.[24] We also evaluated the influence of age and immunization routes in mice. Administration of BCG.$HIVA^{222}$ to newborn mice was safe and primed HIV-1-specific immune responses boosted by subsequent MVA.HIVA administration.[9,25] Recently, we engineered a new BCG.$HIVA^{2auxo}$ vaccine strain harbouring an antibiotic-free plasmid selection and maintenance system. 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.[26]

One of the critical issues that should be addressed when developing a BCG-based HIV vaccine is the genetic plasmid stability. Instability of rBCG episomal vectors has been reported in many rBCGs as reviewed by Chapman *et al.*[27] and Bastos *et al.*[28] In previous studies, our group evaluated the *in vitro* stability of BCG.$HIVA^{2auxo}$ harboring the auxotrophic complementation lysA gene and concluded that the rBCG was unstable when cultured in the absence of selective pressure.[26] Even when the rBCG is cultured using an antibiotic selection system for episomal vector maintenance, the heterologous gene can be deleted or rearranged.[29] Furthermore, some HIV antigens are inherently less stable than others.[27,30] Integrative vectors are less unstable than episomal vectors.[31,32] The lower amount of copies present when using integrative vectors leads to lower expression of the recombinant antigen, thus reducing the metabolic burden and toxicity.[31]

In this study, we constructed an integrative E. coli-mycobacterial shuttle plasmid p2auxo.$HIVA^{int}$ expressing the HIV-1 clade A immunogen HIVA. The shuttle vector uses 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 a glycine auxotrophic *E. coli* strain and subsequently transformed into a lysine auxotrophic *Mycobacterium bovis* BCG strain to generate the vaccine BCG.$HIVA^{2auxo.int}$. Genetic and phenotypic characterization of BCG.$HIVA^{2auxo.int}$ strain was performed and presence of the HIVA gene sequence and protein expression were confirmed. Safety was evaluated by monitoring body mass increase following immunization in BALB/c mice. We demonstrated that the *in vitro* stability of the integrative plasmid p2auxo.$HIVA^{int}$ was increased 8-fold as compared with the BCG strain harboring the episomal plasmid. The BCG.$HIVA^{2auxo.int}$ vaccine in combination with MVA. HIVA was safe and induced HIV-1 and *Mycobacterium tuberculosis*-specific interferon-γ-producing T-cell responses in adult BALB/c mice. We engineered a more stable and immunogenic, BCG-vectored vaccine using the prototype immunogen HIVA. Thus, the use of integrative expression vectors and the antibiotic-free plasmid selection system based on "double" auxotrophic complementation is likely to improve the *in vivo* mycobacterial vaccine stability and immunogenicity for the development of not only recombinant BCG-based vaccines expressing second generation HIV-1 immunogens but also other major pediatric pathogens to prime protective responses shortly following birth.

Results

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

The HIVA immunogen consists of consensus HIV-1 clade A Gag p24/p17 domains coupled to a string of $CD8^+$ T-cell epitopes and the monoclonal antibody (mAb) tag Pk.[23,25] The HIVA open-reading frame was fused at its 5′ end to nucleotides encoding the 19-kDa lipoprotein signal sequence, which facilitates antigen secretion and fusion of foreign antigens to mycobacterial surface lipoproteins, enhancing the immunogenicity of the foreign protein.[16,33] The chimeric 19-kDa signal sequence-HIVA gene was expressed from the *E. coli*-mycobacterial shuttle plasmid p2auxo.$HIVA^{int}$ under the control of the *Mtb* α-antigen promoter (Fig. 1A). The plasmid DNA p2auxo.$HIVA^{int}$ is an integrative vector (single copy, integrated). It contains a DNA cassette encoding an *E. coli* origin of replication (*oriE*), a DNA segment carrying the attachment site (attP) and the integrase (int) gene from the mycobacteriophage L5.[27] It also contains the wild-type glycine A-complementing gene (glyA) and lysine A-complementing gene (*lysA5*) for vector maintenance in the *E. coli* and BCG auxotrophic strain respectively.[8] First, the kanamycin resistance gene (Tn903-derived *aph* gene) was removed from the parental pJH223.HIVA plasmid by SpeI digestion. Secondly, the DNA fragment that included the structural *glyA* gene plus its own promoter region and 3′ transcription terminator region was obtained by SpeI

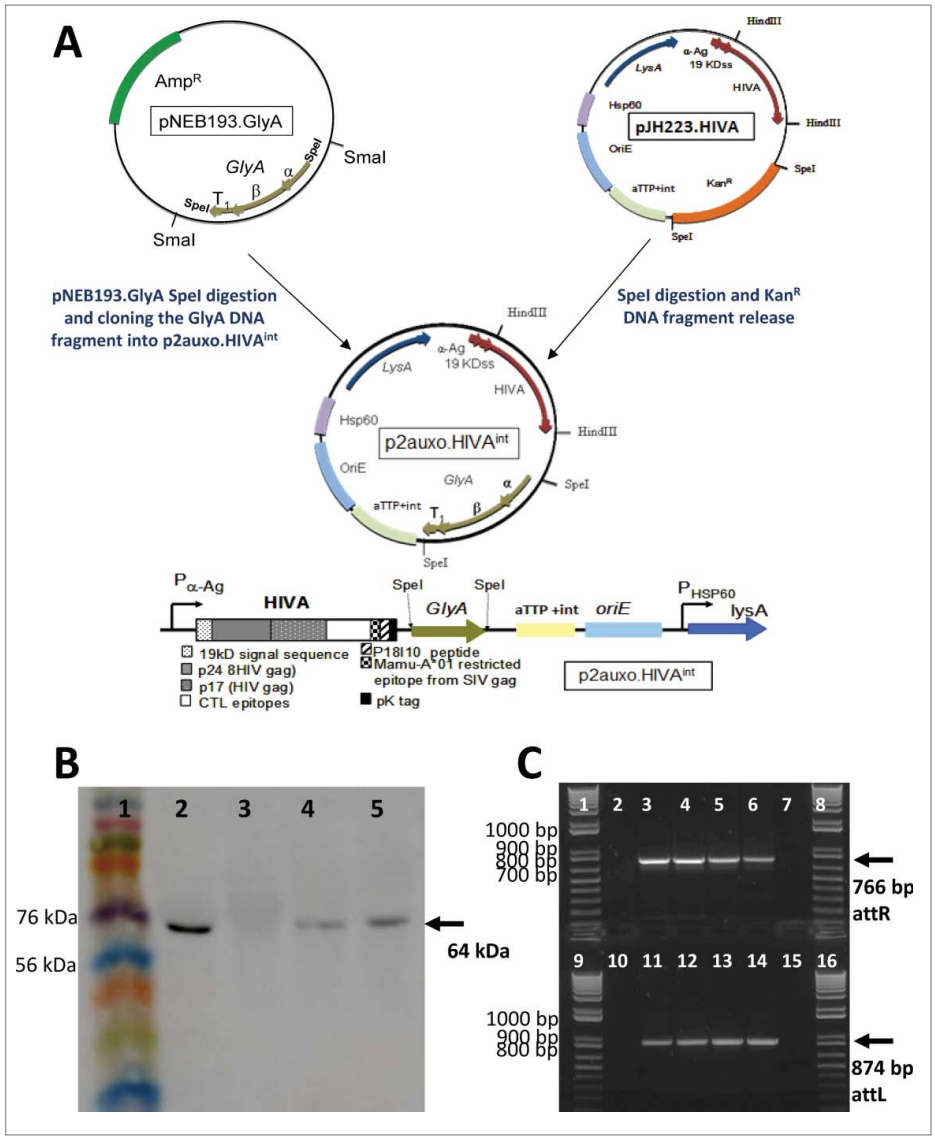

Figure 1. Construction of the BCG.HIVA$^{2auxo.int}$ vaccine strain. (A) A synthetic GC-rich HIVA gene was fused to the region encoding the 19-kDa lipoprotein signal sequence and inserted into the integrative p2auxo.HIVA^{int} *E. coli*-mycobacterial shuttle plasmid. The BALB/c mouse T-cell and Mab-Pk 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* M15ΔglyA strain. (B) Western blot of BCG.HIVA$^{2auxo.int}$ lysates. Lane 1: Molecular weight marker; Lane 2: BCG.HIVA^{2auxo} (positive control); Lane 3: BCG wild type (negative control); Lane 4 and 5: BCG.HIVA$^{2auxo.int}$ Master Seed and Working Vaccine Stock, respectively. (C) PCR analysis of attR and attL DNA regions using as templates the cultures of BCG wild type (lanes 2 and 10), BCG.HIVA$^{2auxo.int}$ clone1 (lanes 3 and 11), BCG.HIVA$^{2auxo.int}$ clone 2 (lanes 4 and 12), BCG.HIVA$^{2auxo.int}$ clone 3 (lanes 5 and 13), BCG.HIVA$^{2auxo.int}$ clone 4 (lanes 6 and 14), negative control, distilled water (lane 7 and 15), and molecular weight marker (lanes 1, 8, 9 and 16).

digestion of pNEB193.GlyA plasmid DNA and cloned into the p2auxo.HIVA^{int} plasmid DNA. pJH223.HIVA is an integrative plasmid DNA that harbours the attP and int DNA fragment. The attP is the attachment site that recognizes the attB zone present in the genome of mycobacteria and integrates the fragment using the integrase gene, creating the attR end on the right side of the insert and attL end on the left side.[34-36] Subsequently, the antibiotic-free plasmid p2auxo.HIVA^{int} was transformed into glycine auxotroph of *E. coli* M15 ΔglyA host strain and lysine auxotrophof BCG host strain Pasteur ΔlysA5::res.[37,38] The positive recombinant *E. coli* colonies were selected by culturing the *E. coli* transformants on Minimal M9-D agar plates and the BCG.HIVA$^{2auxo.int}$ colonies by culturing the rBCG cells on Middlebrook agar 7H10 medium without lysine supplementation. As shown in Fig. 1B, expression of the full-size chimeric 19-kDa signal sequence-HIVA protein was confirmed by western blot analysis of BCG.HIVA$^{2auxo.int}$ lysates. The highest level of HIVA protein expression was detected by immunodot analysis from clone number 2 and was selected for further molecular characterization, immunogenicity, and safety testing in mice. In addition, the BCG.HIVA$^{2auxo.int}$ clone 2 was preserved by using the seed-lot system. A Master Seed stock and derivative Working Vaccine stock was prepared.

Integration of the p2auxo.HIVA^{int} plasmid DNA into the BCG Δlys strain genome was evaluated by PCR analysis. The BCG.HIVA$^{2auxo\ int}$ Master Seed (MS) and Working Vaccine Stocks (WVS) were used as templates and bands of 874bp and 766 bp corresponding to the attR and attL DNA regions, were detected (Fig. 1C).

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

Distribution of BCG to several countries for worldwide application started around 1924 and it was preserved by *in vitro* subculture passaging. The *in vitro* evolution of BCG has resulted in several BCG substrains that are heterogenic.[39,40] Since the 1960s, the BCG substrains have been freeze-dried, keeping the form of the primary seed lots. Genetic identification techniques have been used to differentiate diverse BCG substrains. To confirm that our BCG.HIVA$^{2auxo.int}$ vaccine strain correspond to BCG Pasteur substrain, we used the method described by Bedwell *et al.*[40] based on multiplex PCR system targeting SenX3-RegX3 system and the BCG deletion regions including RD1, 2, 8, 14 and 16. Using this method, the BCG vaccine substrains studied could be differentiated into 7 fingerprints and all BCG substrains were confirmed. We tested the following samples: BCG.HIVA$^{2auxo.int}$ strain (integrative vector)Pasteur substrain, BCG.HIVA^{2auxo} strain (episomal vector) Pasteur substrain, BCG wild type Pasteur (BCG1173 P2), as well as the commercial BCG Connaught and BCG Danish 1331 substrains. All the evaluated BCG substrains generated a 196 bp product using the primers ET1–3, indicating deletions of the RD1 region. In addition, in the BCG Pasteur substrain (BCG.HIVA$^{2auxo.int}$), the RD8 and RD16 were present and generated products of 472 and 401 bp, respectively. The primers for the SenX3-RegX3 region generated a product of 276 bp in BCG Pasteur. The PCR fingerprints of BCG Pasteur, BCG Connaught and BCG Danish substrains (Fig. 2A) were consistent with previously published results on genetic information of BCG substrains.[40]

For the molecular characterization of the p2auxo.HIVA^{int} plasmid DNA, enzymatic restriction and PCR analysis were performed. The plasmid DNA was purified from the *E. coli* M15 ΔglyA strain, and the enzymatic restriction pattern

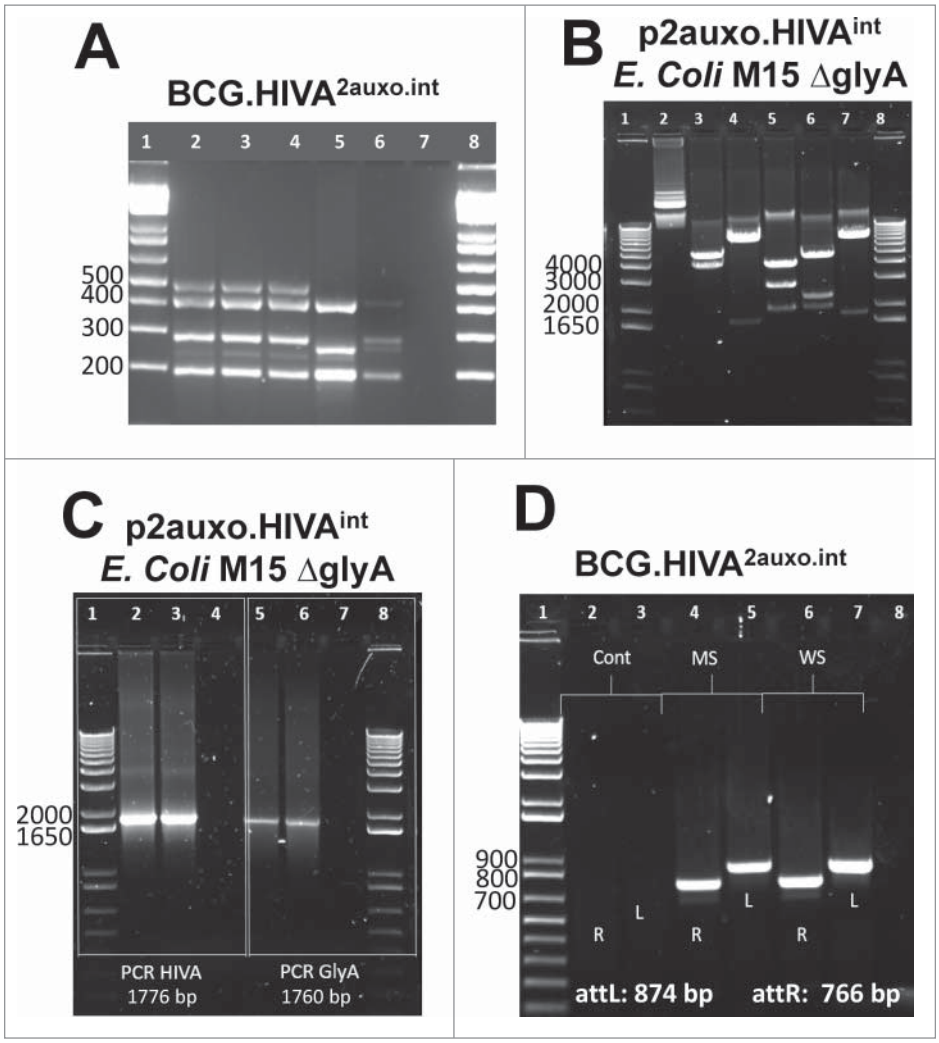

Figure 2. Genetic characterization of BCG.HIVA$^{2auxo.int}$ strain. (A) The BCG.HIVA$^{2auxo.int}$ Pasteur substrain identification by multiplex PCR assay. Lane 1 and 8: molecular weight marker; lane 2: BCGwt Pasteur giving the bands of 472, 401, 276 and 196 bp; lane 3 and 4: BCG.HIVA$^{2auxo.int}$ Pasteur Master Seed and Working Vaccine Stock, respectively giving the bands of 472, 401, 276 and 196 bp; lane 5: BCG Connaugh giving the bands of 401, 256 and 196 bp; lane 6: BCG Danish giving the bands of 401, 276 and 196 bp; lane 7: negative control, distilled water. (B) Enzymatic restriction analysis of p2auxo.HIVA^{int} plasmid DNA purified from *E. coli* M15ΔglyA cultures (pre-BCG transformation). Lane 1 and 8: molecular weight marker; lane2: p2auxo.HIVA^{int} plasmid DNA undigested; lane 3: ClaI digestion; lane 4: HindIII digestion; lane 5: NotI digestion; lane 6: BamHI digestion and lane 7; SpeI digestion. (C) PCR analysis of HIVA and *E. coli* glyA DNA coding sequences using as template the plasmid DNA p2auxo. HIVA (episomal), positive control (lane 2 and 5), distilled water (lane 4 and 7), negative control and plasmid DNA p2auxo.HIVA^{int} (lane 3 and 6). Lane 1 and 8 corresponds to the molecular weight marker. (D) PCR analysis of attR and attL DNA region using as template the cultures of BCG wild type, negative control (lane2 and 3), BCG. HIVA$^{2auxo.int}$ MS (lane 4 and 5), BCG.HIVA$^{2auxo.int}$ WVS (lanes 6, and 7) and distilled water as negative control (lane 8). Lane 1 corresponds to molecular weight marker.

obtained displayed the same enzymatic pattern as compared with the predicted pattern of the plasmid DNA sequence. ClaI (lane 3): bands of 4442 and 3850 bp; HindIII (lane 4): bands of 6807 and 1692 bp; NotI (lane 5): bands of 3904, 2699 and 1893 bp; BamHI (lane 6): 4406, 2195 and 1895 bp and SpeI (lane 7): bands of 6710 and 1780 bp (Fig. 2B). Moreover, the PCR analysis using specific primers for the HIVA and *E. coli* glyA DNA coding sequence was performed using the p2auxo.HIVA^{int} plasmid DNA vector as template. A band of 1792 bp and 1804 bp corresponding to the predicted site of the HIVA DNA sequence and the *E. coli* glyA DNA sequence, respectively, were detected (Fig. 2C). On the other hand, PCR analysis using specific primers to confirm the integration of the p2auxo.HIVA^{int} plasmid DNA into the parental BCG ΔlysA strain genome was performed using the BCG culture from the BCG.HIVA$^{2auxo.int}$ Master Seed and Working Vaccine Stock as template. A band of 766 bp and 874 bp corresponding to attR and attL attachment sites in the BCG genome were detected in the Master Seed and Working Vaccine Stock of BCG.HIVA$^{2auxo.int}$ (lanes 4, 5, 6 and 7) and were not detected in BCG wt (lanes 2 and 3) (Fig. 2D).

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

To prevent the plasmid instability *in vivo* and *in vitro* as well as genetic rearrangement by the mycobacteria, different approaches should be considered: i) the use of expression vectors containing small HIV-1 DNA coding sequences, ii) DNA fragments lacking glycosylation sites; iii) the use of weak promoters; iv) the use of BCG auxotrophic strains (containing the complementing gene in the expression vectors); v) the use of inducible promoters; vi) codon optimization of the recombinant gene; vii) the choice of expression vector backbone; viii) antigen secretion to enhance the immunogenicity and to prevent foreign proteins inducing toxicity in BCG; and ix) the integration of the plasmid DNA into the host vector. We have demonstrated that the use of weak promoters (*Mycobacteria spp*. α-antigen promoter) and BCG lysine auxotrophs complemented with a lysine gene do, in fact, prevent the disruption of gene expression caused by genetic rearrangements.[24] In this study, we have used a lysine auxotroph of BCG strain complemented with a lysine gene and an antibiotic-free plasmid selection system. To assess the phenotypic stability of lysine auxotrophy, lysine complementation and kanamycin sensitivity of BCG.HIVA$^{2auxo.int}$ strain, the BCG lysine auxotrophic strain was plated out on lysine supplemented and non-supplemented agar. This strain failed to grow on non-lysine supplemented agar plates and no colonies were observed (Fig. 3A). However, growth was observed on agar plates supplemented with lysine (Fig. 3B). As expected, complementation of the BCG.HIVA$^{2auxo.int}$ strain containing the lysine gene encoded by the monocopy plasmid p2auxo.HIVA^{int} abolished the requirement

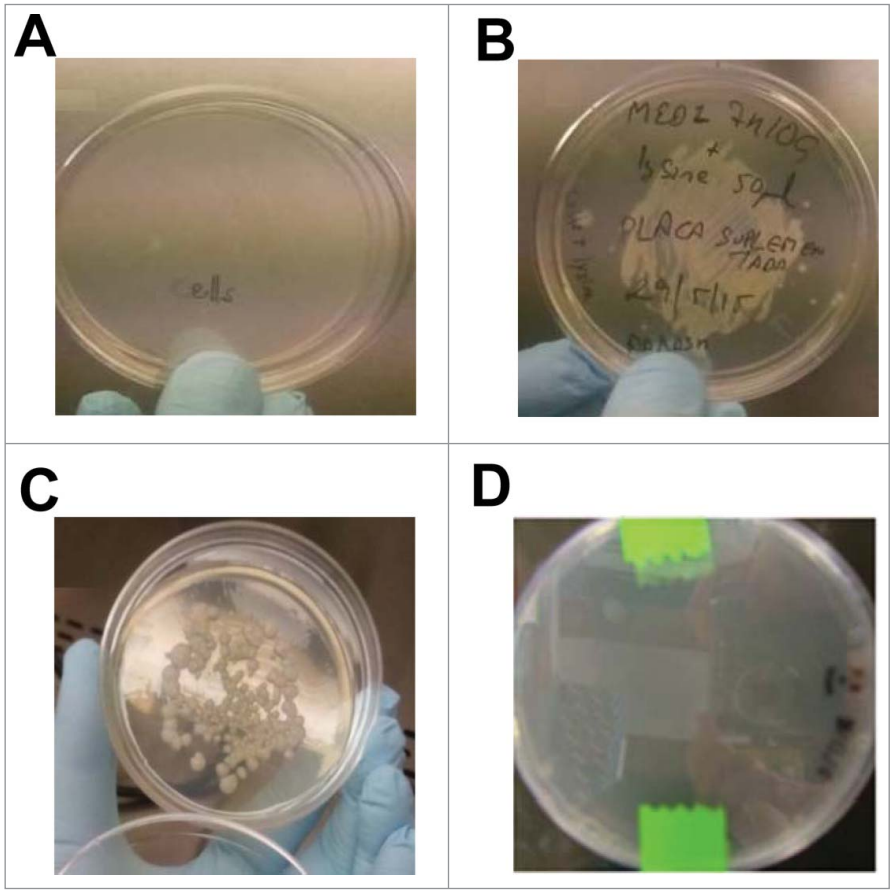

Figure 3. Phenotypic characterization of BCG.HIVA$^{2auxo.int}$ strain. We assessed the phenotype of lysine auxotrophy, lysine complementation and kanamycin resistance of BCG.HIVA$^{2auxo.int}$ strain (A) BCG lysine auxotroph strain plated on non-lysine supplemented 7H10; (B) BCG lysine auxotroph strain plated on lysine supplemented 7H10; (C) BCG.HIVA$^{2auxo.int}$ plated on 7H10 without lysine and kanamycin supplementation; (D) BCG.HIVA$^{2auxo.int}$ plated on 7H10 without lysine supplementation and with kanamycin.

for exogenous lysine (Fig. 3C). On the other hand, when the BCG.HIVA$^{2auxo.int}$ strain was plated out on agar plates containing kanamycin, no colonies were observed (Fig. 3D), confirming the lack of kanamycin resistance in our construct.

In vitro *stability analysis of the BCG.HIVA$^{2auxo.int}$*

To evaluate the *in vitro* stability of the p2auxo.HIVA^{int} plasmid DNA harboring the auxotrophic complementing *lysA* gene, subcultures in media with and without a selective agent were performed. Both BCG.HIVA^{2auxo} and BCG.HIVA$^{2auxo.int}$ colonies that were cultured in selective medium (without lysine supplementation) maintained the vector for over 4 subcultures (30 bacterial generations). In contrast, when BCG.HIVA^{2auxo} was cultured 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 observed during subsequent subculturing passages. In contrast, when BCG.HIVA$^{2auxo.int}$ was cultured without selective pressure (with lysine supplementation), 80% of the BCG colonies were harboring the plasmid DNA after the first subculture. The differences between both groups cultured without selective pressure were statistically significant ($P < 0.05$) (Fig. 4A).

Structural stability of the p2auxo.HIVA^{int} plasmid DNA was evaluated by PCR analysis. When bacteria were grown without selective pressure, the PCR band corresponding to the HIVA DNA coding sequence was detected in 10 out of 10 BCG.HIVA$^{2auxo.int}$ colonies after 56 bacterial generations. Similarly, when bacteria were grown in selective medium, the PCR was positive in 2 out of 2 BCG.HIVA$^{2auxo.int}$ colonies after 56 bacterial generations (Fig. 4B). Moreover, the PCR band corresponding to the attR attachment sites was also positive when bacteria were grown without selective pressure (10 out of 10 colonies) and under selective pressure (2 out of 2 colonies) after 56 bacterial generations (Fig. 4C).

BCG.HIVA$^{2auxo.int}$ prime and MVA.HIVA boost regimen elicited HIV-1-specific and PPD-specific T-cell immuneresponses in mice

We have demonstrated in previous studies in BALB/c mice that BCG.HIVA^{2auxo} can both prime novel and boost pre-existing MVA.HIVA elicited HIV-1 specific CD4+ and CD8+ T-cell immune responses of high quality upon antigenic re-exposure. In this study, we have evaluated the specific HIV-1 T-cell immune responses in BALB/c mice after immunization with BCG.HIVA$^{2auxo.int}$ prime and MVA.HIVA boost. The immunogenicity readout 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 the HIVA immunogen to evaluate immunogenicity in mice. On day 0, adult mice were immunized with rBCG containing both the episomal and integrative plasmids, respectively, and on week 5 the animals received a booster dose with MVA.HIVA (Fig. 5A). After 7 weeks the mice were killed and the capacity of splenocytes from vaccinated mice to secrete IFN-γ in response to P18-

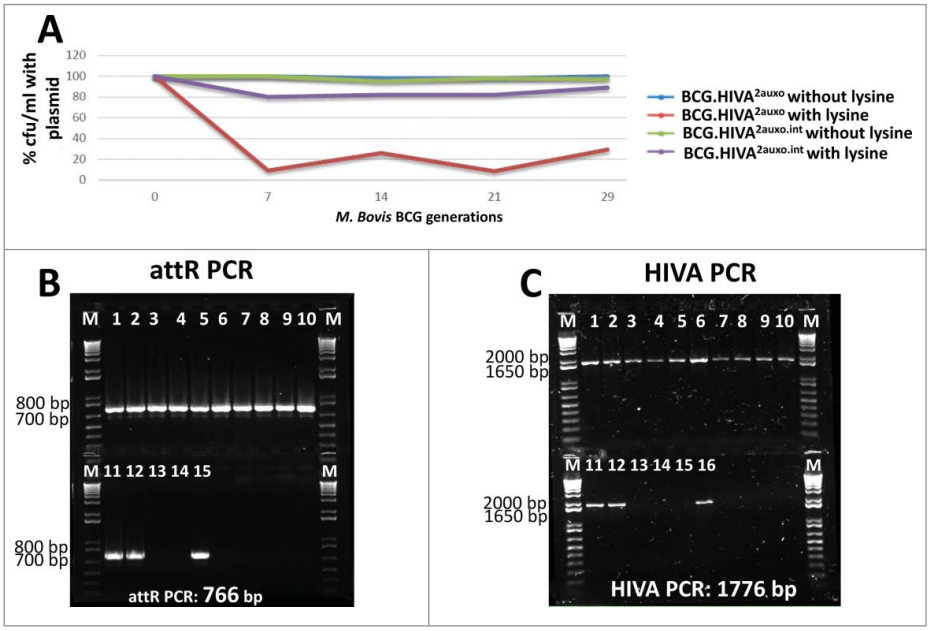

Figure 4. *In vitro* stability of the BCG.HIVA$^{2auxo.int}$ strain. (A) *In vitro* persistence of the p2auxo.HIVA (episomal) and p2auxo.HIVA^{int} in BCGΔlys grown for successive passages on selective (no lysine) or nonselective (supplemented with lysine) media. The percentage represents the cfu (titer) that maintained the vector containing the lysine complementing gene (grown on selective medium) versus to the total cfu. For BCG, the generation time is ~24h. Thus, 4 subcultures represents ~30 BCG generations. (B) attR DNA region PCR of 12 individual BCG.HIVA$^{2auxo.int}$ colonies that were grown in nonselective medium (lanes 1–10) and selective medium (lanes 11,12) after 56 bacterial generations. We used BCGΔ*lys* strain culture supplemented with lysine (lanes 13) and distilled water (lane 14) as negative control and BCG.HIVA$^{2auxo.int}$ Master Seed as positive control (lane 15). (C) HIVA PCR of 12 individual BCG.HIVA$^{2auxo.int}$ colonies that were grown on nonselective medium (lanes 1–10) and selective medium (lanes 11,12) after 56 bacterial generations. We used BCGΔ*lys* strain cultures (lane 13) and distilled water (lane 14) as negative control, and BCG.HIVA$^{2auxo.int}$ Master Seed as positive control (lane 16).

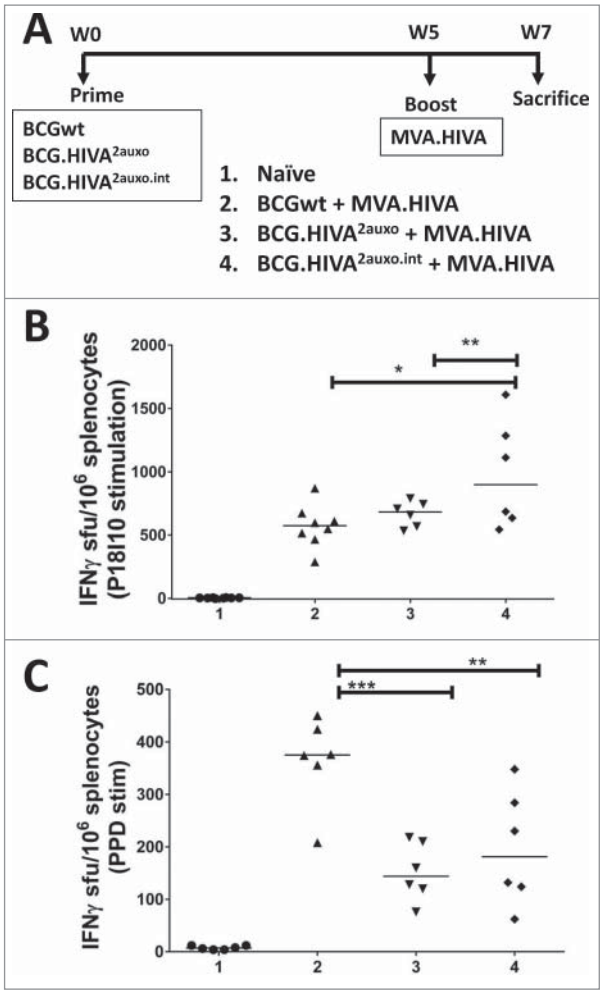

Figure 5. Induction of HIV-1 and *Mtb*-specific T-cells immune responses by the BCG.HIVA$^{2auxo.int}$ prime-MVA.HIVA boost regimen. (A) Adult mice (7-week-old BALB/c) were either left unimmunized or primed with 10^6 cfu BCG.HIVA$^{2auxo.int}$, BCG.HIVA^{2auxo} (episomal) or BCG wild type (intradermally) and boosted with 10^6 pfu of MVA.HIVA (intramuscularly) 5-weeks post-BCG inoculation. Mice were killed 2 weeks later for T-cell immune response analysis. (B) Elicitation of specific HIV-1 T-cell responses was assessed in an *ex vivo* IFN-γ enzyme linked immunosorbent spot (ELISPOT) assay using the immunodominant P18–I10 CD8+T-cell epitope peptide. The median spot-forming units (SFU) per 10^6 splenocytes for each group of mice (n = 6) as well as individual animal responses is shown. (C) Purified protein derivative (PPD)-specific T-cell responses elicited by BCG.HIVA$^{2auxo.int}$. Immune responses to BCG were assessed in an *ex vivo* IFN-γ ELISPOT assay using PPD as the antigen. The median SFU per 10^6 splenocytes for each group of mice (n = 6) as well as individual animal responses is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

I10 peptide stimulation was assayed by ELISPOT. We observed the highest frequency of specific cells secreting IFN-γ in mice primed with BCG.HIVA$^{2auxo.int}$ and boosted with MVA.HIVA, compared with mice primed with BCG.HIVA^{2auxo} ($p<0,01$) and BCG wild type ($p<0,05$) (Fig. 5B).

BCG.HIVA$^{2auxo.int}$ elicited purified protein derivative (PPD)-specific responses in mice. The capacity of splenocytes from vaccinated mice to secrete IFN-γ was tested by the ELISPOT assay after overnight stimulation with the PPD antigen. We observed the highest frequency of specific cells secreting IFN- γ in mice primed with BCG wild type. The median spot-forming units (sfu) per 10^6 splenocytes were lower in mice primed with BCG.HIVA^{2auxo} ($p< 0.001$) or BCG.HIVA$^{2auxo.int}$ ($p< 0.01$) compared with mice primed with BCG wild type (Fig. 5C).

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

Five adult mice per group were either immunized or left unimmunized with 10^6 colony forming units (cfu) of BCG wild type, BCG.HIVA^{2auxo} or BCG.HIVA$^{2auxo.int}$ intradermally and subsequently received a booster dose of 10^6 plaque forming units (pfu) of MVA.HIVA intramuscularly as described in Fig. 6A. As shown in Fig. 6B, the body mass was monitored and recorded over time. All vaccine combinations were analyzed, to depict any possible adverse events due to vaccination and monitored by decrease in body mass. To carry out a rigorous safety assessment, the dose inoculated in mice was 10-fold higher than the usual dose given to adult mice, as advised by the European Pharmacopoeia for the safety testing of live vaccines. Importantly, no statistically significant difference was observed between the vaccinated mice and the naïve mice at specific time points, corresponding to the time of BCG inoculation as well as 3 and 10 weeks after BCG inoculation. On the other hand, the body mass profile was similar in all groups and similar to the mouse provider 's standard body mass curve (www.Harlan.com). Furthermore, between week 0 and week 10, the body mass monitored in all vaccinated mice was found between the mean ± 2 standard deviations (SD) body mass curve in naïve mice (Fig. 6B). It is also important to mention that no mice died during the trial, no local adverse events, and no associated systemic reactions were observed.

Discussion

Despite the progress made in prevention of mother-to-child HIV-1 transmission, the development of a safe, effective, and affordable vaccine against HIV-1 and tuberculosis shortly after birth to prevent HIV-1 transmission via breast milk and childhood tuberculosis still remains a great challenge. In this study,

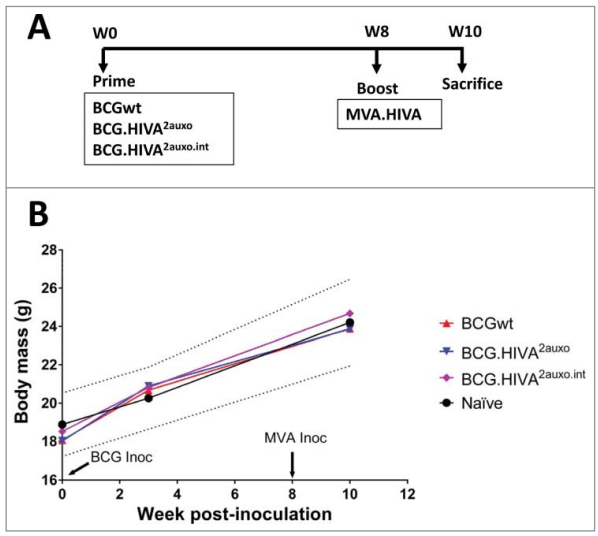

Figure 6. BCG.HIVA$^{2auxo.int}$ prime and MVA.HIVA boost safety in adult mice. (A) Adult mice were either left unimmunized or immunized with 10^6 cfu of BCG wild type, BCG.HIVA^{2auxo} (episomal) or BCG.HIVA$^{2auxo.int}$ by intradermal route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA at week 8 by intramuscular route. (B) The body weight was recorded over time, and the mean for each group of mice is shown (n = 5). Data from naive mice are presented as mean ± 2 SD (n = 5, dashed lines). The weight differences between vaccinated and naive mice group were analyzed at week 0, 3 and 10 by analysis of variance test.

we have constructed an *E. coli*-mycobacterial integrative shuttle vector with no antibiotic selection marker that allows expression of the HIVA antigen. We demonstrated that the integrative plasmid p2auxo.HIVA^{int} increased stability *in vitro* 4-fold in comparison with the episomal plasmid in BCG, and expression of the HIVA protein was also confirmed. Overall, we have shown that the BCG.HIVA$^{2auxo.int}$ vaccine in combination with MVA.HIVA was safe, induced HIV-1 and *Mycobacterium tuberculosis*-specific interferon-γ-producing T-cell responses in adult BALB/c mice, and was well tolerated. The BCG.HIVA$^{2auxo.int}$ strain was developed in Good Laboratory Practices (GLP)-compatible conditions, preserved according to the seed-lot system, and was genetically and phenotypically characterized. Thus, this strategy is worthy of pursuit in joining the global efforts to develop novel BCG vector–based vaccines for controlling tuberculosis and HIV/AIDS.

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 *Escherichia* coli.[41] However, their use is considered unacceptable in clinical trials and product licensing.[42] 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.[26] In this study, we have used an integrative *E.coli*-mycobacterial shuttle plasmid p2auxo.HIVA^{int}, expressing the HIV-1 clade A immunogen HIVA. This shuttle vector uses an antibiotic resistance-free mechanism for plasmid selection and maintenance based on glycine complementation in *E. coli* and lysine complementation in mycobacteria.

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.*[40] to identify our BCG.HIVA$^{2auxo.int}$ vaccine candidate. Resultant fingerprints after multiplex PCR assay were consistent with the PCR pattern of the BCG Pasteur substrain.

One of the critical issues that should be addressed in developing a BCG-based HIV vaccine is the genetic plasmid stability of the *E.coli*-mycobacterial expression vector. The idea of integrating plasmid DNA into the BCG genome to generate a more stable variant described by several authors[27,31,36,43] has been the main focus of this study. Our results show a clear difference between the episomal and the integrative plasmids, with the integrative plasmid being 4 times more stable *in vitro* than the episomal one. However, as there are more copies of episomal vector per cell, higher levels of recombinant protein can be expressed than from integrative vectors.[31] Many studies have been performed using integrative vectors and the mycobacteriophage L5 integrase-based system has been extensively used to generate integration proficient vectors.[31,34-36,38] One clearly evident example is described by Méderlé *et al.*,[27,31] where the authors compare recombinant BCG harboring episomal and integrative plasmid vectors expressing the early regulatory nef and the structural gag (p26) genes from simian immunodeficiency virus (SIV) and demonstrate a higher genetic stability *in vivo* and *in vitro* as well as an increased duration of heterologous gene expression *in vivo* using the integrative plasmid. A 5 to 15-fold increase in genetic plasmid stability is observed in these studies when using integrative expression vectors as compared with the episomal variants. However, a low level of protein expression is also observed.[31] Moreover, this integration pattern is not restricted to BCG and it is also functional in *M. smegmatis* and *M. tuberculosis* making it ideal for the construction of rBCG vaccines and offering a significant advantage over other methods such as homologous recombination that are likely to be considerably less efficient.[37,38] Thus, site-specific integration provides a simple and efficient mechanism for avoiding the loss of foreign genes from mycobacteria in situations where it is not possible to directly select for maintenance of these genes. The use of a phage site-specific recombination system for the generation of genetically stable recombinants should be applicable to a variety of bacterial systems where temperate phages have been identified. Phage integration systems have notable advantages over other integration methods (such as transposition or general recombination), not only regarding their high efficiency, but the systems also require excision factors that are not required for integration.[34,36,38]

Borsuk *et al.*[44] reported the construction of a BCG expression system using auxotrophic complementation as a selectable marker. Stability of the multicopy plasmid in rBCG was evaluated *in vitro* and *in vivo*, and was compared with selection using antibiotic resistance. Due to the persistence of selective pressure, the new system was highly stable even during *in vivo* growth, whereas the conventional vector was unstable in the absence of selective pressure. These data are in concordance with our previous results.[26] We evaluated the *in vitro* stability of the episomal 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 cultured on selective medium (without lysine supplementation) maintained the vector for over 30 bacterial generations. On the other hand, only 9% of the BCG colonies grown without selective pressure still contained the plasmid DNA after the first subculture.[26]

In this study, stability of the integrative plasmid was also evaluated during *in vitro* culture of the recombinant BCG.HIVA$^{2auxo.int}$ strain harboring the integrative expression vector in comparison with the BCG.HIVA^{2auxo} strain harboring the episomal plasmid. The results were in concordance with both our previous study[26] and with Méderlé *et al.*[31,43] rBCG containing the integrative plasmid was found to be 4 times more stable, compared with the conventional episomal vector. All BCG.HIVA^{2auxo} colonies (integrative and episomal) that were cultured in the presence of selective pressure maintained the vector *in vitro*, whereas the absence of selective pressure resulted in instability of the system due to loss of plasmid.

Our group and others have shown in murine and nonhuman primate studies that rBCG induces cell-mediated responses against HIV-1 and SIV antigens.[9,21,43,45-48] However, a small proportion of these animal studies used rBCG strains in heterologous prime-boost regimens. There is data showing that rBCG is a good priming vector in heterologous prime-boost vaccination regimens to enhance specific T-cell responses.[8,43,45,49] In human tuberculosis vaccine trials, McShane *et al.* [8,50] have demonstrated that vaccination with MVA-expressing Ag85A boosts pre-existing anti-mycobacterial 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.[50]We have previously shown in BALB/c mice that the inclusion of BCG.HIVA^{222} 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.[8,9,25,51]

Here, vaccination with wild-type BCG, BCG.HIVA$^{2auxo.int}$ and BCG.HIVA^{2auxo} induced BCG-specific responses in adult mice. Importantly, we observed the highest frequency of BCG-specific cells secreting IFN- γ in mice primed with wild type BCG. We believe that wild type BCG cells which do not express heterologous antigen have a lower metabolic burden and toxicity resulting in a higher BCG cell viability and specific immunogenicity. Studies in neonatal mice have indicated that immune responses at birth are often biased toward the Th2 type and defective regarding the Th1 type, which is the central defense mechanism against intracellular pathogens.[6-8,50,52-54] However, it has been described that BCG vaccination at birth induces a potent Th1-type immune response in humans and in mice.[9,25,27,52] The challenge for neonatal vaccinology is thus to develop, and promote at a global level, vaccines that can be administered safely soon after birth and which would be effective after one or 2 early doses. According to our knowledge, only 2 reports have been published about safety of an antibiotic-free marker rBCG-based HIV-1 vaccine in mice,[26,39].In this study, we have demonstrated that BCG.HIVA$^{2auxo.int}$ prime and MVA.HIVA boost regimen was safe and well tolerated in adult mice by using body mass increase as a parameter.

In this study, we might consider the following limitations: (i) further experiments assessing different doses, routes, and immunization schedules should be performed; (ii) the SCID mouse model should be used to evaluate and confirm that BCG.HIVA$^{2auxo.int}$ is safe and well-tolerated in immunocompromised mice; (iii) even though one of objectives of this study was to evaluate the specific- HIV-1 immune responses induced in mice primed with BCG.HIVA$^{2auxo.int}$ and boosted with MVA.HIVA in comparison with mice primed with BCG.HIVA^{2auxo} and boosted with MVA.HIVA, a control group of mice immunized with MVA.HIVA alone should have been included.

We constructed and characterized a novel, safer, good laboratory practice–compatible BCG-vectored vaccine using the prototype immunogen HIVA and assessed the safety and immunogenicity of BCG.HIVA$^{2auxo.int}$ and MVA.HIVA in mice using a prime-boost regimen. In conclusion, the construction of this new *E. coli*–mycobacteria integrative shuttle vector based on double auxotrophic complementation and antibiotic-free plasmid selection system will provide a new and improved methodological tool for mycobacterial vaccine design and development as a bacterial live recombinant vaccine vehicle. A rBCG strain harboring an integrative expression vector, free of antibiotic resistance marker genes and expressing a second-generation immunogen HIVconsv better addressing the HIV-1 genetic variability and immune escape is under construction.[39] In addition, the same strategy may be used for other major pediatric pathogens such as malaria or tuberculosis, to prime protective response shortly following birth.

Materials and methods

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

The *E. coli–mycobacterial* shuttle integrative vector, plasmid pJH223.HIVA, was previously constructed in our laboratory. The *aph* gene, conferring kanamycin resistance, was removed from pJH223.HIVA plasmid by SpeI digestion. Following *aph* gene excision, the glyA gene cassette was ligated into the plasmid and subsequently transformed into the *E. coli* M15ΔglyA 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 mainly performed by the serine hydroxymethyl transferase enzyme. The $\alpha\beta$T1 DNA fragment was amplified by PCR using the pQE$\alpha\beta$T1FucA plasmid DNA as a template and provided by Prof. Pau Ferrer. 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 pJH223.HIVA vector (lacking the kanamycin resistance gene after SpeI digestion), to generate the p2auxo.HIVA^{int} plasmid DNA.

Bacterial strains and cultures

E. coli M15ΔGly cells (glycine auxotrophic strain, Invitrogen) were kindly provided by Dr. Pau Ferrer and were grown in minimal medium M9-derivative (Na_2HPO_4, 6.78 g/l; KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; NH_4Cl, 1 g/l, glucose, 10 g/l; $MgSO_4$, 2 mmol/l; $CaCl_2$, 0.1 mmol/l; thiamine, 0.1 g/l; $FeCl_3$, 0.025 g/l; $AlCl_3{\cdot}6H_2O$, 0.13 mg/l; $ZnSO_4{\cdot}7H_2O$, 2.6 mg/l; $CoCl_2{\cdot}6H_2O$, 0.47 mg/l; $CuSO_4{\cdot}H_2O$, 4.6 mg/l; H_3BO_3, 0.03 mg/l; $MnCl_2{\cdot}4H_2O$, 4.2 mg/l; $NiCl_2{\cdot}6H_2O$, 0.02 mg/l; $Na_2MoO_4{\cdot}2H_2O$, 0.06 mg/l) (M9-D), supplemented with glycine (70 μg/ml). The p2auxo.HIVA^{int} plasmid DNA without kanamycin resistance gene was used to transform the glycine auxotroph of *E. coli* cultures that were grown in M9-D broth or agar plates by electroporation. For transformation, *E. coli* cultures were grown to an optical density at 600 nm of 0.9, transformed using a Bio-Rad gene pulser electroporator at 2.5 kV, 25 μF, and 200Ω, and plated onto M9-D (same concentration of M9-derivative, plus 1.5% bactoagar) agar plates without glycine supplementation. On the other hand, a lysine auxotroph of BCG strain, BCGΔlys, kindly provided by W.R. Jacobs Jr., B. R. Bloom, and T. Hsu was transformed with the p2auxo.HIVA^{int} plasmid DNA without the kanamycin resistance gene by electroporation.. Mycobacterial cultures were grown in Middlebrook 7H9 broth medium or on Middlebrook agar 7H10 medium supplemented with albumin-dextrose-catalase (ADC; Difco) containing 0.05% Tween 80. The L-lysine monohydrochloride (Sigma) was dissolved in distilled water and used at a concentration of 40 μg/ml. For transformation, BCG cultures were grown to an optical density at 600 nm of 1.5, transformed using a Bio-Rad gene pulser electroporator at 2.5 kV, 25 μF,

and 1,000Ω, and plated onto ADC-supplemented Middlebrook agar 7H10 medium containing 0.05% Tween 80 without lysine supplementation. The commercial BCG Danish 1331 strain (Pfizer), was kindly provided by Dr. Neus Altet and the commercial BCG Connaught strain (ImmuCyst, Aventis), 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 100X 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 micro-liters of the protein extract was blotted onto a pretreated polyvinylidene difluoride membrane, and the HIVA protein was detected using an anti-Pk monoclonal antibody (MCA1360; AbD Serotec, Oxford, UK), using an enhanced chemiluminescence kit (Pierce, Rockford, IL). The ImageQuant LAS500 gel imaging system (GE Healthcare, Piscataway, NJ) was used to visualize the dots.

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–PAGE, and electroblotted. The HIVA protein was detected using anti-Pk antibodies with an enhanced chemiluminescence kit (Pierce).

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

Four subcultures (~30 bacterial generations) of BCG.HIVA$^{2auxo.int}$ (working vaccine stock) harboring the p2auxo.HIVA^{int} plasmid DNA containing the lysine complementing gene were cultured 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. In addition, the PCR analysis of the HIVA DNA coding sequence and attR DNA region of 12 individual BCG.HIVA$^{2auxo.int}$ colonies that were grown in nonselective medium and selective medium after 56 bacterial generations were determined to evaluate the plasmid's structural stability.

Sample preparation for the multiplex PCR assay and for attR and attL DNA regions PCR

For isolation of DNA from wild-type BCG, BCG.HIVA^{2auxo} (episomal) and BCG.HIVA$^{2auxo.int}$; 400 μl of mycobacterial culture was centrifuged at 13,000 g 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 10,000 g, 5 μl of supernatant was used for the amplification reaction or stored at −20 °C. The commercial BCG strains were treated similarly, except in this case, 400 μl of the reconstituted freeze-dried flasks were used.

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

The multiplex PCR assay was described previously by Bedwell *et al.*[40] For the PCR analysis, 5 μl of the mycobacterial DNA isolated from BCG.HIVA$^{2auxo.int}$ Pasteur and commercial BCG strains were used in a final reaction volume of 50 μl.

E. coli *plasmid DNA extraction*

For *E. coli* plasmid DNA isolation, the QIAprep Spin Miniprep Kit was used following manufacturer's instructions (Qiagen, Hilden, Germany).

Mice immunizations and isolation of splenocytes

Adult (7-weeks-old) female BALB/c mice were left either unimmunized or immunized with BCG.HIVA$^{2auxo.int}$ 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 strainers (Falcon; Becton Dickinson) using a 5-ml syringe rubber plungers. 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 and PPD

For assessing the immunogenicity of HIVA in the BALB/c mice, the following peptides were used: H-2Dd-restricted epitope P18–I10 (RGPGRAFVTI). The purified protein derivative (batch RT50, 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) following the 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 μg/ml at 4 °C overnight. A total of 5×10^5 fresh splenocytes were added to each well and stimulated with 2 μg/ml of the P18–I10 peptide or 5 μg/ml of purified protein derivative for 16 h at 37 °C, 5% CO_2, and lysed by incubating twice with deionized water for 5 minutes. Wells were then washed 3 × with PBS 0.05% Tween 20, incubated for 2 h with a biotinylated anti-IFN-γ mAb diluted in PBS 2% FCS to a final concentration of 2 ug/ml, washed 3 × in PBS 0.05% Tween 20, and incubated with the Streptavidin-Alkaline Phosphatase-conjugate in PBS 2% FCS. Wells were washed 4 × with

PBS 0.05% Tween 20 and 2 × with PBS before incubating with 100 μl BCIP/NBT substrate solution (Sigma). After 5–10 minutes, the plates were washed with tap water, dried, and the resulting spots counted using an ELISPOT reader (Autoimmune Diagnostika GmbH, Germany).

Statistical analysis Immunogenicity data are shown as group medians as well as individual responses. The body mass data are depicted as group means, and mean ± 2 standard deviation (SD) in the naive micegroup. Statistical significance was determined by analysis of variance. Statistical significancewas assessed by a 2-way analysis of variance. ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P<0.001$). GraphPad Prism 5.0 software was used.

Ethics statement

The animal experiments were approved by the local Research Ethics Committee at Hospital Clinic Barcelona, School of Medicine, University of Barcelona, and by the Ethical Committee for animal experimentation from University of Barcelona (authorization 364/15). All animal procedures and care conformed strictly to the Catalonia (Spain) animal welfare legislation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to Barry R. Bloom and William R. Jacobs for providing the plasmid DNA pJH223 and BCG wild type and lysine auxotroph of BCG for research purposes; N.S. is a Red Temática de Investigación Cooperativa en SIDA (RD12/0017/0001) senior fellow.

Funding

This research was supported by Instituto de Salud Carlos III FIS PI14/00494, and co-financed by European Regional Development Fund (FEDER) and from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 681137. EAVI2020. In addition, has been supported by ISCIII (RETIC-RIS RD12/0017), and HIVACAT Research Program.

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