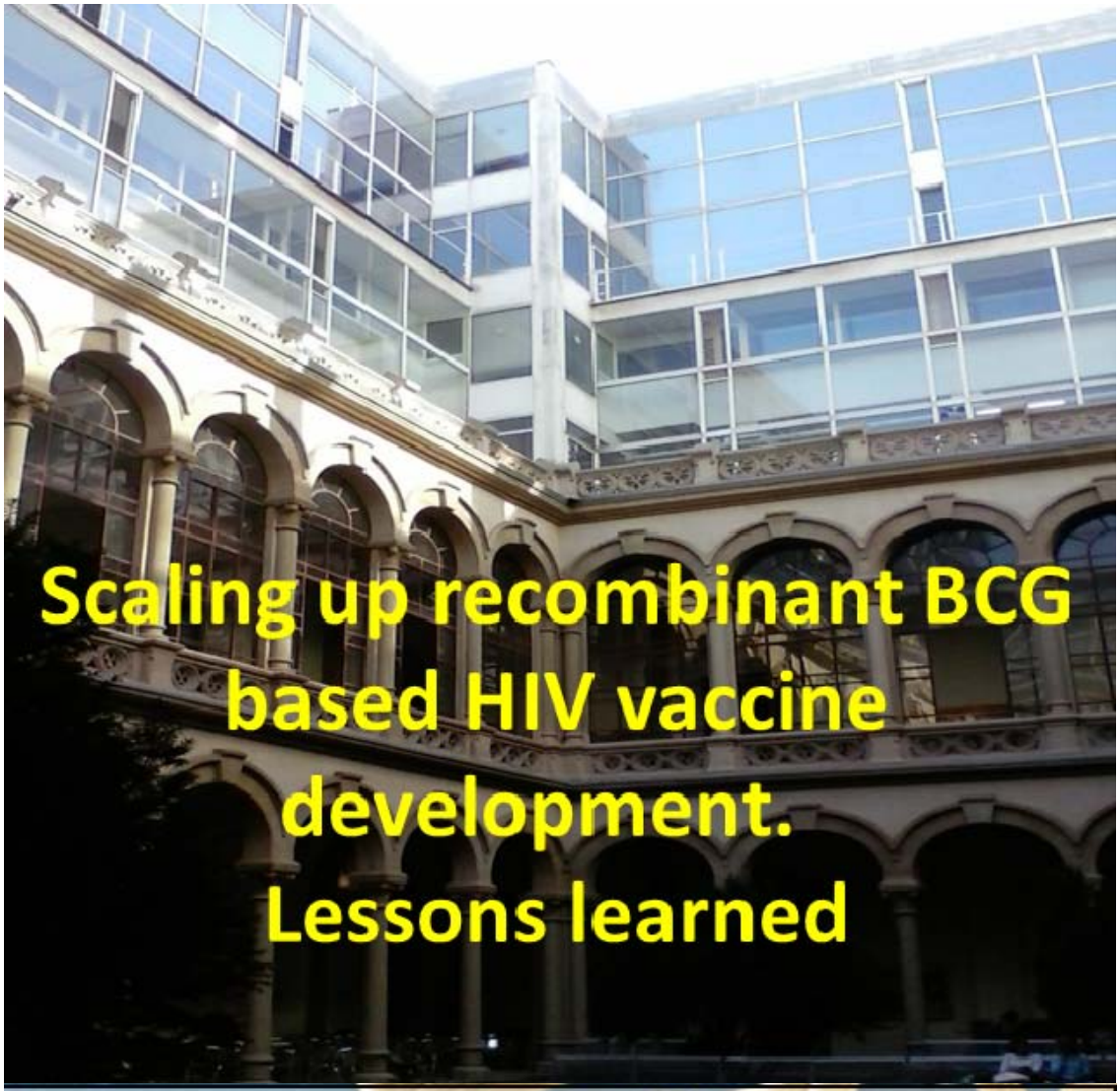
Scaling up recombinant BCG based HIV vaccine development. Lessons learned

Narcís Saubi Roca



Scaling up recombinant BCG based HIV vaccine development. Lessons learned

PhD Thesis

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UNIVERSITAT AUTÒNOMA DE BARCELONA

27 de Setembre 2016

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Tesi presentada per en Narcís Saubi Roca per optar al

Títol de Doctor en Bioquímica, Biologia Molecular i Biomedicina

per la Universitat Autònoma de Barcelona

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INTRODUCTION

HIV/AIDS epidemiology

According to the last UNAIDS World AIDS Day Report 2015, at the end of 2014, an estimated 36.7 million people were living with HIV worldwide and 2.1 million individuals became newly infected with the virus in 2014 (150000 children under 15). The number of people dying of AIDS-related causes was 1.1 million, and it is estimated that AIDS has orphaned more than 19 million children. [UNAIDS, 2015]. It is remarkable that the number of new infections is very similar to the number of new infections corresponding to 2010 (2.2 million) [UNAIDS, 2016]. This steady is also confirmed at a more local scenario, in Barcelona, where the number of new infections since the onset of the disease has remained unchanged [Dr. JM Gatell, personal communication; SIVES, 2015].

In Figure 1, the evolution of the AIDS and HIV diagnoses is depicted, showing the sharp decrease of AIDS cases on 1994, after the introduction of antiretroviral treatment, and the lack of decrease in the new HIV diagnostics.

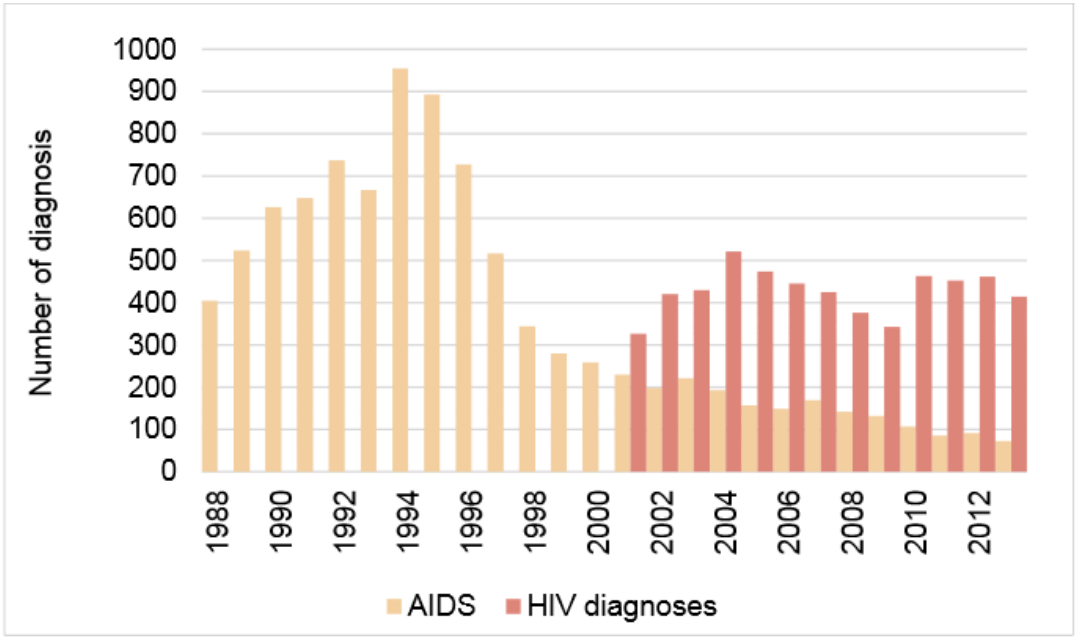

Figure 1: Annual evolution of new diagnoses of AIDS and HIV infection in Barcelona area between 1988 and 2013. (CEEISCAT)

Global coverage of antiretroviral therapy reached 46% at the end of 2015, a total of 17 million people. Gains were greatest in the world's most affected

region, eastern and southern Africa. Coverage increased from 24% in 2010 to 54% in 2015, reaching a regional total of 10.3 million people.

In some sub-Saharan countries (which accounted for 66% of new HIV infections), the HIV prevalence among pregnant women can be over 30%. Approximately half of mother-to-child transmissions (MTCTs) are due to prolonged breastfeeding. Although around 35% of HIV-positive pregnant women are receiving antiretroviral therapy [Children, 2004], reducing significantly mother-to-child transmission of HIV at delivery, the drugs have a high cost, have to be administrated after delivery, and maintained during the breastfeeding period, and the efficacy could be reduced due to emergence of resistant mutants.

All these epidemiological data remark the importance of the development of a safe, effective and affordable preventive HIV vaccine, despite the progress made in antiretroviral therapy and its distribution.

HIV preventive vaccines

Although insight into HIV-1 pathogenesis has been gained since the identification of HIV-1, the successful development of an effective vaccine has been elusive. HIV-1 has a high degree of antigenic and genetic diversity. In addition, the virus has evolved multiple mechanisms to inhibit elicitation of and neutralization by antibodies. New molecular and structural technologies have been applied to gain a better understanding of HIV-1 as an immune target and to provide new insights into the development of improved immunogens capable of eliciting immune responses that prevent infection by circulating strains of HIV-1[Kwong PD, 2012].

Challenges in developing an effective HIV-1 vaccine

Unlike currently licensed vaccines, which are typically designed to elicit neutralizing antibodies against a limited number of viral surface proteins, HIV-1 vaccines must counteract a swarm of viruses. The genetic diversity and mutability of HIV-1 creates a plethora of antigens that are constantly changing. Within infected individuals, the struggle between the virus and the immune system is persistent, such that the virus continually escapes host immunity and replicates[Kwong PD, 2012].

In addition to the genetic diversity and mutability of the HIV-1 Envelope (*Env*), structural features of *Env* create inherent difficulties in the ability of the immune system to develop an effective neutralizing antibody. HIV-1 is an enveloped virus with a lipid bilayer surrounding and protecting its core structural proteins. The virus spikes protrude through this protective lipid, and every spike is composed of three gp120 proteins, each of which is non-covalently associated with a gp41 transmembrane glycoprotein molecule. HIV-1 entry into host cells is mediated by binding of gp120 to its primary receptor, the CD4 glycoprotein on the cell surface. Binding to CD4 induces conformational changes in gp120, leading to the exposure and/or formation of a binding site for specific chemokine receptors, mainly CCR5 and CXCR4, which serve as secondary receptors for virus entry [Kwong PD, 1998]. Structurally, the gp120 glycoprotein is divided into three parts, an inner domain, an outer domain, and a bridging sheet. The bridging sheet is the part of the molecule that is responsible for binding to both chemokine receptor and CD4. The CD4 binding site is highly conserved, since the virus needs a conserved region to recognize CD4. (Figure 2).

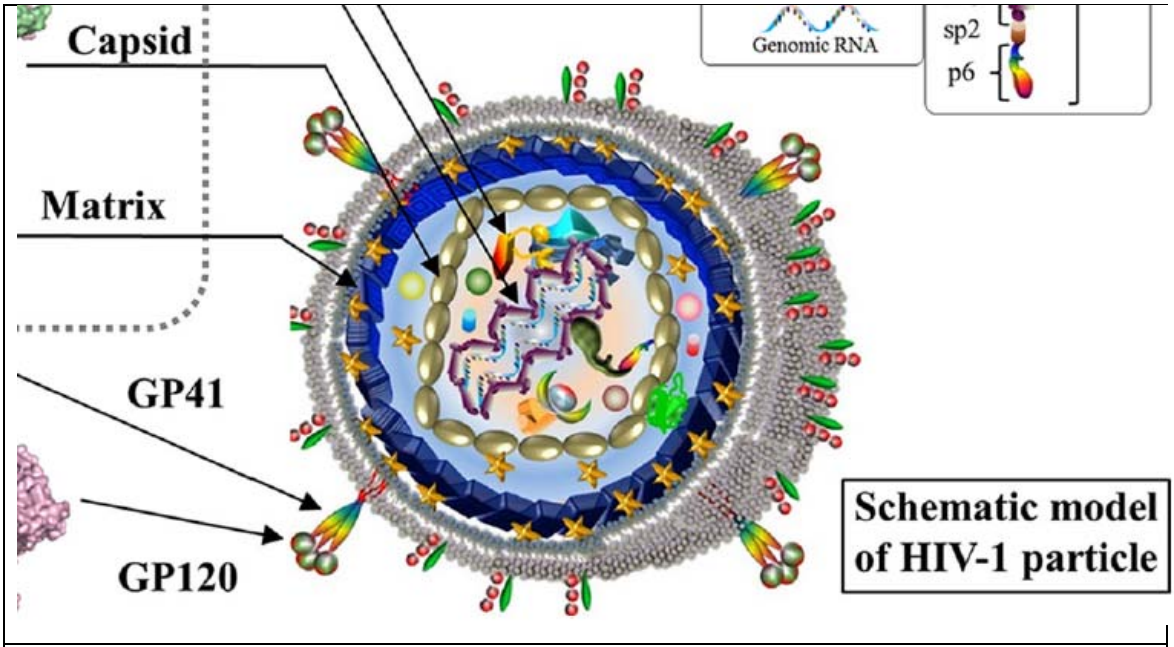

Figure 2: HIV virus structure. [Li G, 2016]

HIV-1 gp120 contains a number of features that help the virus evade the host's humoral immunity, including variable loops [Starcich BR 1986], N-linked glycosylation [Wyatt R, 1998; Wei X, 2003], and conformational flexibility [Myszka DG, 2000; Chen L, 2009]. The conformational flexibility of gp120

disguises the conserved receptor-binding sites from the humoral immune system. The presence of carbohydrate moieties on gp120 physically shields potential epitopes from eliciting or binding to antibodies, an obstacle that is further complicated by the extensive diversity of N-linked glycans.

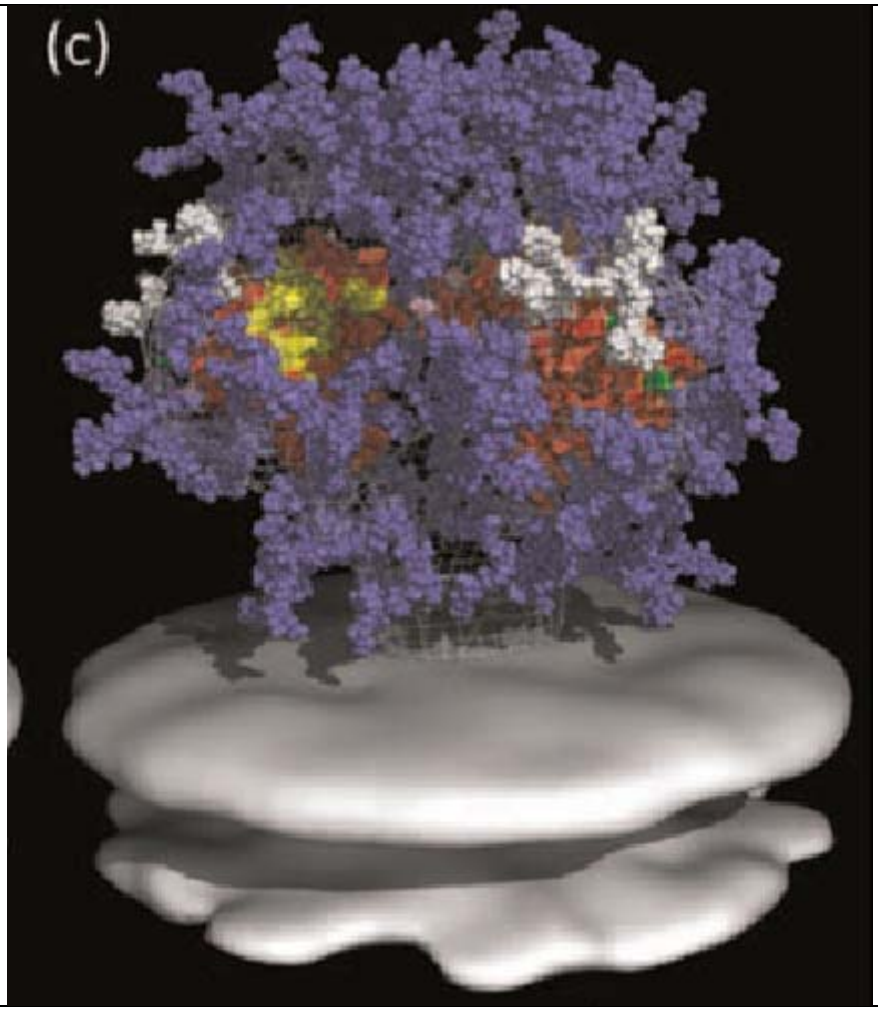

Figure 3: Model for the trimeric HIV1 envelope spike. Colouring: core gp120, red; b12 epitope on gp120, yellow; V1/V2 base o gp120, pink; V3stub on gp120, bright blue; V4 base on gp120, green; glycans, light blue and white. [Schief W, 2009]

Lessons from completed HIV-1 vaccine clinical trials

Early efforts at developing an HIV-1 vaccine attempted to elicit protective antibodies against the viral envelope and used forms of recombinant glycoprotein 120 (rgp120) as the immunogen. VAX004, the first efficacy trial for an HIV-1 vaccine, began recruitment in 1998 and used two rgp120 HIV-1 envelope antigens, derived from two subtype B strains. Results from this double-blind, placebo-controlled trial showed no efficacy in 5403 volunteers. The vaccine did not prevent disease acquisition or impact the level of viremia in those infected [rgp120 Vaccine study group, 2005]. The first HIV-1 vaccine

efficacy trial in Asia, VAX003, also contained two rgp120 HIV-1 envelope antigens, one from subtype B and one from subtype E. Again, the vaccine did not prevent HIV-1 infection or delay HIV-1 disease progression [Pitisuttithum P, 2006].

Following the initial failure of attempts to elicit protective antibodies, the next set of antigens used in clinical efficacy studies were designed to test whether T cells, or the cellular arm of the immune system, could protect against HIV-1 infection. The STEP trial was designed as a proof-of-concept study for the efficacy of a cell-mediated immunity vaccine to protect against HIV-1 infection, or to reduce early plasma HIV-1 levels. STEP was a multicenter, double-blind, randomized, placebo-controlled, phase II trial of the Merck replication-defective adeno 5 viral vector MRKAd5 HIV-1 clade B Gag/Pol/Nef vaccine 3000 HIV-seronegative participants were randomized (1:1) to receive 3 injections of vaccine or placebo. The study was halted following an interim analysis that showed the vaccine to be ineffective. Moreover, posthoc analysis suggested a trend towards an increased HIV-1 infection rate in certain subgroups of vaccine recipients (men who were uncircumcised and had antibodies to adenovirus type 5 at enrolment). [Buchbinder SP, 2008; Gray G, 2010]. This increased susceptibility has been linked to preexisting immunity of Ad5-specific CD4 T-cell responses at mucosal surfaces theoretically increasing the target cells at the site of infections [Hu H, 2014; Streek, 2016].

The next large scale clinical study, known as RV144, was an efficacy trial that showed for the first time that an HIV-1 vaccine could significantly reduce the incidence of HIV-1 infection. In this study, priming with the Aventis Pasteur live recombinant viral vector ALVAC-HIV-1 (vCP1521) and boosting with protein-based vaccine VaxGen gp120 B/E (AIDSVAX B/E) yielded a 31% reduction in HIV-1 acquisition in a modified intent to treat analysis. Although the duration of protection was limited and the effect modest, this study provided proof of concept that it is possible for a vaccine to elicit protective immunity that blocks infection [Rerks-Ngarm S, 2009]. A number of investigative teams are now working to identify correlates of immune protection from this study, with an emphasis on antibody-mediated mechanisms, such as binding antibodies, and antibody-dependent cell-mediated cytotoxicity [Robb M, 2010; Karnasuta C, 2005; Fuchs JD, 2010; Kim JH; 2010].

The latest large scale clinical study, HVTN 505, was a proof of- concept study of a Vaccine Research Center (VRC) product involving a multiclade HIV-1 DNA plasmid (EnvA, EnvB, EnvC, gagB, polB, nefB) boosted by a recombinant adenovirus vector (Ad5 EnvA, EnvB, EnvC, gag/polB). The study was being conducted in HIV-1-uninfected, adenovirus type 5 seronegative, circumcised men who had sex with men (MSM). In April 2013, the data and safety monitoring board recommended halting vaccinations for lack of efficacy. The primary analysis showed that week 28+ infection had been diagnosed in 27 participants in the vaccine group and 21 in the placebo group. The conclusion was that The DNA/rAd5 vaccine regimen did not reduce either the rate of HIV-1 acquisition or the viral-load set point in the population studied [Hammer SM, 2013]. In Table 1 there is a summary of all Phase III trials, the vaccine candidates used and the results obtained.

Table 1. Summary of PhaseII and III HIV-1 vaccine trials (From Lelièvre, 2016)

Table 1. HIV vaccine Phase II/III trials

Trial	Date	Vaccine components	Country	Populations	Main immunological target	Infection rates
Vax004	1998–2002	Recombinant gp120 (B/B)	United States Canada Netherlands	5403 HRSTs	Neutralising antibody	6.7% in vaccinees 7.0% in placebo recipients NS
Vax 003	1999–2002	Recombinant gp120 (B/E)	Thailand	2546 IDU	Neutralising antibody	8.4% in vaccinees 8.3% in placebo recipients NS
Step	2004–2007	rAd5 (gag, pol, nef) (B)	North America Caribbean South America Australia	3000 HRST	CD8 T cell responses	4.6% in vaccinees 3.1% in placebo recipients *P*=0.07*
Phambili	2007	rAd5 (gag, pol, nef) (B)	South Africa	801 HRST**	CD8 T cell responses	8.4% in vaccinees 7% in placebo recipients *P*=NS
RV144 (Thai trial)	2003–2009	**Prime:** canarypox (gag, pol, env E) **Boost:** recombinant gp120 (B/E)	Thailand	16,402 General population	Neutralising antibody	0.192% in vaccinees 0.279% in placebo recipients *P*=0.04; VE=31%***
HVTN 505	2009–2013	**Prime:** DNA (gag, pol, nef, B) + DNA (env A/B/C) **Boost:** Ad5 (gag, pol, B) + Ad5 (env A/B/C)	United States	2496 HRST	CD8 T cell responses	2.7% in vaccinees 2.1% in placebo recipients *P*=NS

HRST: high risk for sexual transmission; IDU: intravenous drug users; IR: infection rate ; VE: vaccine efficacy.
* Analysis in men in the Step trial (all but one infection occurred in men); ** enrolment of 3000 patients was originally planned but the trial was stopped after the results of the Step trial; *** Modified intention-to-treat analysis.

Tuberculosis epidemiology

Tuberculosis (TB) is a major global health problem. TB is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). It causes ill-health among millions of people each year and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide. According

to the World Health Organization (WHO) Global TB Report, in 2014, there were an estimated 9.6 million new TB cases: 5.4 million among men, 3.2 million among women and 1.0 million among children. There were also 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4 million among HIV-positive people), of which approximately 890 000 were men, 480 000 were women and 140 000 were children. The number of TB deaths is unacceptably high: with a timely diagnosis and correct treatment, almost all people with TB can be cured. [WHO, TB Report 2015]

The current vaccine in use against human TB is *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). This vaccine is generally administered at birth and confers some protection against severe TB in children. However, its efficacy of protection against pulmonary TB in adolescents and adults is variable (0-80%) [Mangtani P, 2014]. Thus, the development of new vaccine candidates against human TB remains a global health priority. Significant efforts over the last two decades have indicated a great progress in the search for a more effective vaccine against human TB [Andersen P, 2007; Ottenhoff T, 2012].

Mycobacterium bovis bacillus Calmette-Guérin (BCG)

Mycobacterium bovis BCG was developed by Léon Charles Albert Calmette and Jean-Marie Camille Guérin, (Figure 4) who were working at the Pasteur Institute in 1908, subculturing virulent strains of tubercle bacillus and testing different culture media. They noted that bacilli grown on a glycerin-bile-potato mixture appeared less virulent, and thus changed the course of their research to see if repeated subculturing would produce a strain that was attenuated and thus could be considered for use as a vaccine. By 1919 they had passaged *M. bovis* 198 times resulting in the development of non-virulent bacilli that were unable to cause tuberculosis disease (TB) in research animals [Calmette A, 1922]. This BCG vaccine was first used in humans in 1921. However, there were many doubts concerning its safety and protective efficacy. This was not helped by a disaster which occurred in 1930 in Lubeck when 240 infants were vaccinated with BCG that was contaminated with a virulent strain resulting in 72 infants developing TB and

dying. It was only after World War II that BCG really came into widespread use. BCG is now the world's most widely used vaccine with over 3 billion doses having been administered worldwide [Chapman R, 2010].

Figure 4: French stamp printed as a tribute to the contribution of Albert Calmette and Camille Guérin to the tuberculosis prevention.

Numerous features make BCG an attractive delivery vaccine vehicle of heterologous antigens:

a) BCG has a proven safety record and is currently the most widely used vaccine: it has been given to billions of people worldwide with a very low incidence of serious complications [Hanson, 1995].
b) The adjuvant effect of BCG has been exploited in experimental vaccines in animals and man allowing the induction of both humoral and cell-mediated immune responses [Champlin, 1975].
c) As a live bacterial vaccine needing limited purification, BCG is cheap and easy to manufacture. Vaccine cost is particularly relevant when considering large-scale vaccination in developing countries.
d) BCG is one of the most heat-stable of the vaccines in use, and does not require an extensive cold chain for maintenance of efficacy [Gheorghiu, 1988].
e) BCG can be administered at or any time after birth and is unaffected by maternal antibodies, with a single dose sensitizing to tuberculoproteins for 5 to 50 years [Hanson,1995]. In the past BCG was also successfully administered orally [Roche, PW, 1995].

The BCG vaccine is one of the most widely used of all current vaccines, and overall it reaches more than 80% of all new born children and infants in countries (Figure 5) where it is part of the national childhood immunization

programme [WHO, 2016]. If a BCG based HIV vaccine is successful, it will be easier to include it in the immunization programme.

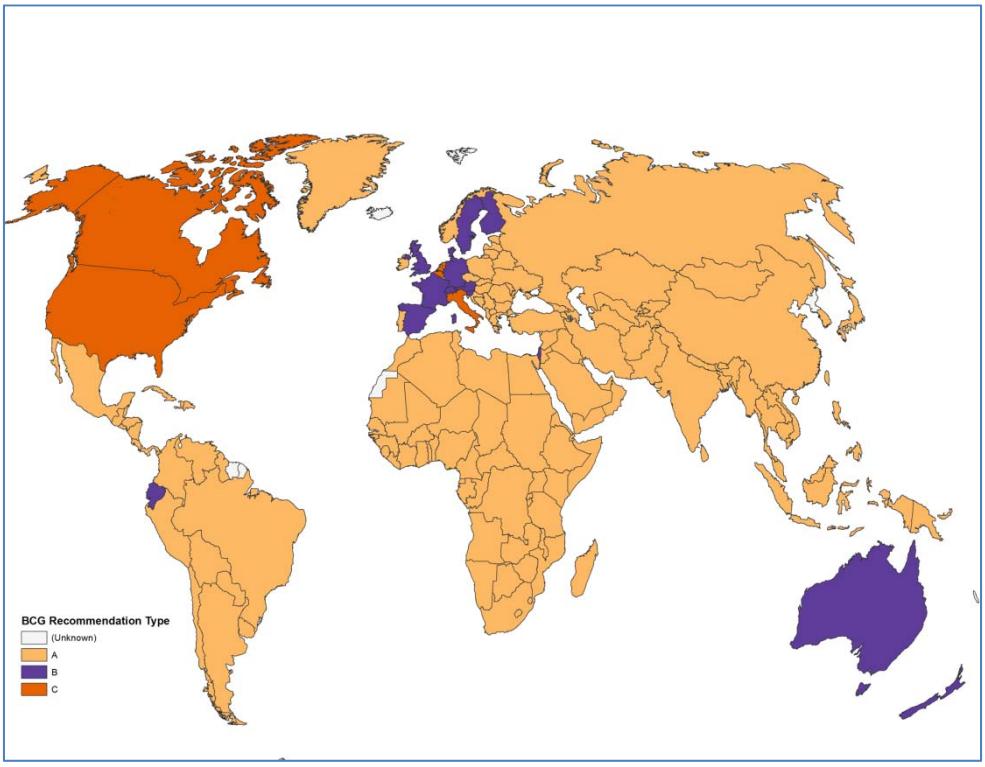

Figure 5. Map displaying BCG vaccination policy by country.
A: The country currently has universal BCG vaccination program. B: The country used to recommend BCG vaccination for everyone, but currently does not. C: The country never had universal BCG vaccination programs.[Zwerling A, 2011]

Recombinant BCG expressing heterologous antigens

The interest on recombinant BCG increased considerably in the nineties as a result of the development of different genetic systems for expression of foreign antigens in mycobacteria. These systems include the development of different *E. coli*-mycobacterial shuttle vectors, systems to express and secrete heterologous antigens and strategies for transformation of mycobacteria. Moreover, technological advancements in the genomics of mycobacteria improved our understanding of the molecular biology of this slow-growing pathogen and helped the conception of strategies for evaluation of BCG as a vaccine delivery vector [Philip, VJ, 1998]. As a result, antigens of bacteria, parasites, and viruses have been expressed in BCG [Chan J, 1994; Bloom BR, 1992, Moghes T, 2001; Agger EM, 2002] and it has been shown that

recombinant BCG (rBCG) elicits both cellular and humoral immune responses against heterologous antigens [Dennehy M, 2005].

In a pioneer study, Jacobs *et al.* [Jacobs Jr WR, 1987] developed an *Escherichia coli*-mycobacteria shuttle phasmid. In their work, a DNA sequence of the mycobacteriophage TM4, isolated from *M. avium*, was ligated to an *E. coli* cosmid and introduced into *E. coli*, *M. smegmatis* and BCG. The expression of heterologous genes in BCG can be achieved using either replicative or integrative vectors. Most of the mycobacterial replicative vectors are designed using the origin of replication from the pAL5000, which allows up to five copies of the plasmid per bacterial cell [Labidi A, 1985]. Therefore, one can expect to express higher levels of recombinant protein using replicative vectors instead of integrative vectors. In our group, we have compared the heterologous protein synthesis using episomal (pJH222) or integrative (pJH223) plasmids, and a higher amount of HIVA immunogen was detected when the episomal vector was used (Im EJ, 2007). HIVA immunogen consists of consensus HIV-1 clade A Gag p24/p17 domains coupled to a string of CD8+T-cell epitopes and monoclonal antibody (mAb) tag Pk [Hanke T, 2000]. However, it has been demonstrated that integrative vectors are more genetically stable, both *in vitro* and *in vivo*, than replicative plasmids [Mederle I, 2002]. The higher stability afforded by the integrative plasmids expressing HIVA immunogen has also been demonstrated by our group [Mahant A, in preparation]. In some cases a long-lasting stable expression of heterologous antigens using integrative vectors is desirable. Thus, the lower expression levels can be counterbalanced by the persistent synthesis of the foreign antigen *in vivo*. We have hypothesized that this balance between stability and expression level is even more important when large heterologous proteins are expressed.

Expression of foreign genes in mycobacteria can be modulated by the promoter used to drive the expression of the foreign gene. Several promoters have been widely used to regulate the heterologous antigen expression in the *E. coli*-mycobacterium shuttle vectors. The most frequently used are promoters from the heat shock protein genes hsp60 [Stover CK, 1991] or hsp70 [Aldovini A, 1991]. Other promoters used successfully in the shuttle vector construction include those from the *M. kansasii* α-antigen [Matsuo K, 1990], the *M. paratuberculosis* PAN [Murray A, 1992], the M. tuberculosis 19 kDa antigen

[Stover CK, 1995] and the *M. fortuitum* lactamase *pBlaF** [Timm J, 1994]. Despite efforts to search for the best promoter for mycobacteria, most expression systems used nowadays still rely on the same limited, but efficient range of promoters. Initially, our research group used the pMV261 replicative plasmid [Stover CK, 1991] provided by WR Jacobs Jr, to express the HIV-1 gp120 protein under the regulation of hsp60 promoter. We observed disruption of the gp120 gene expression. We demonstrated that *E. coli*-mycobacterial expression vectors bearing a weak promoter (the α-antigen promoter) and lysine complementing gene in a recombinant lysine auxotroph of BCG could prevent genetic rearrangements and disruption of HIV-1 gp120 gene expression [Joseph J, 2010].

Recombinant BCG to induce specific antigenic responses

Live bacterial vaccines in general require no additional adjuvant component to induce immune response in animal models [Roland KL, 2005]. For BCG, usually a single inoculation is sufficient to induce an immune response and eventually protection. The rationale for using BCG as a delivery vector for heterologous antigens is based on its adjuvant potency and capacity to replicate inside of antigen presenting cells (APC), such as macrophages and dendritic cells [Nasser EA, 2005]. Despite the fact that BCG can survive and replicate inside APC, these cells are still capable of presenting BCG and/or heterologous antigens inducing activation of the immune system [Bastos RG, 2009]. The induction of specific immune responses against heterologous antigens following inoculation with rBCG was initially reported by Stover et al. [Stover CK, 1991] and Aldovini and Young [Aldovini A, 1991]. Stover et al. [Stover CK, 1991] tested integrative and multicopy plasmid systems to express β-galactosidase, tetanus toxin and HIV-1 antigens. Aldovini and Young *et al*. [Aldovini A, 1991] used a multicopy plasmid system to express HIV-1 proteins in BCG. Both studies report the development of humoral and cellular immunity against heterologous antigens following inoculation of the rBCG vaccines in mouse model. The first evidence of protective immunity elicited by rBCG was demonstrated by Stover et al. [Stover CK, 1993]. In that study, a protective humoral immune response was induced in mice inoculated with rBCG

expressing the OspA antigen of *B. burgdorferi*. Despite the protection induced by rBCG-OspA in mouse model, this recombinant strain failed to elicit a significant immune response against *B. burgdorferi* in humans [Edelman R, 1999]. This recombinant vaccine had a good safety profile and the volunteers converted positive in the PPD test as expected, but it did not elicit a primary humoral response to OspA antigen. The low level of OspA antigen expression and/or most likely the loss of the plasmid vector containing the *ospA* gene are possible explanations for the rBCG-OspA failure in humans [Edelman R, 1999]. Protection induced by rBCG has been described in numerous other studies using parasite, bacterial and viral antigens. In 2007, our group published a review paper about recombinant BCG based HIV vaccine development, where we compiled the specific SIV/HIV immune responses induced after rBCG:SIV/HIV immunization using different animal models (Table 2) [Joseph J, 2007].

Table 2: Specific SIV/HIV immune responses induced after rBCG:SIV/HIV immunization using different animal models [Joseph J, 2007].

Table 2. Specific SIV/HIV immune responses induced after rBCG:SIV/HIV immunization using different animal models.

Recombinant antigen	Promoter, signal sequence and expression vector	Route	Number of cells (cfu)	Boost	Immunity induced	Ref.
2.1 Murine model: BALB/c mice						
Eschericia coli lac Z	hsp60 pMV261 vector (extrachromosomal)	Single dose IV, ID, IP	2×10^3 2×10^4 2×10^6		Antibodies to β-galactosidase by all routes. As few as 200 cfu IV and 10^4 cfu ID were sufficient for high level of Abs. Peak at week 8. IFN-γ: CTLs	[6]
HIV-1 gp120	hsp60 pMV361 vector (integrative)	Single dose	1×10^6		Lack of significant titers of Abs (>1:100 dilution). CTLs	[6]
HIV-1 gag HIV-1 env	hsp70 extrachromosomal high copy vector	ID or IV	5×10^6	5×10^6 at weeks 4 and 8	IFN-γ (HIV-*gag*). CTLs (HIV-*gag*). 3 out of 5 vaccinated with BCG-HIV-gag and 1 out of 5 vaccinated with BCG-HIV-env had detectable levels of IgG	[20]
HIV-1 nef	GroES/groELp from *Streptomyces albus*	SC	10^7		Proliferative responses LN cells (inguinal): 14 days after inoculation	[21]
HIV-1 gp41	Hsp70 pMV273 vector (extrachromosomal)	Single ID injection	10^6		Abs were detectable at week 4 and were increasing 16 weeks after immunization (HIV-1 gp41)	[18]
HIV-1 gp120	Hsp60 PMV361 (integrative)	IV	10^6		Significant titers of Abs to gp120 were not elicited CTL to gp120	[18]
19-aa from HIV-1 V3 sequence fused to α-antigen of *Mycobacterium kansasii*	α-antigen promoter and α-antigen signal sequence	Single SC inoculation	3×10^6		CTLs. Protection in SCID/hu and SCID/PBL mice	[42]
SIV-nef	pAN promoter sequence from IS900 insertion element of *Mycobacterium paratuberculosis*	SC	10^7		CTL and proliferative responses LN cells (inguinal): 14 days after inoculation	[22]
HIV-1(IIIb) p17gag B-cell epitope (aa92–110) fused to α-antigen of *M. kansasii*		SC	10^8	Weeks 2, 4 and 6	Ab peak at week 8. In 2 out of 7 mice the Ab persisted at least for 14 months	[39]
SIV-nef	pAN promoter sequence from IS900 insertion element of *M. paratuberculosis*	Oral	10^9 (× 5 consecutive days) Total: 5×10^9		Proliferative responses (spleen and LNC) at 1, 2, 4, 8 and 12 weeks. Proliferative response was induced 1 month after immunization. Peak at 2 months. CTLs (spleen and LN): at 1, 2 and 3 months. High CTLs at 1, 2 and 3 months in spleen	[58]
SIV-env (aa 1–245 : N-terminal half)	β-lactamase promoter from *Mycobacterium fortuitum* fused to the β-lactamase gene of *M. fortuitum*	IV, SC	5×10^6 10^7	10^6 for Ab Week 4 and 8	Ab: peak at 10 week (+up to week 24). Neutralizing Ab against primary SIV isolates. CTLs: LN cells 14 days after SC immunization	[59]

Ab: Antibody; BCG: Bacillus Calmette–Guérin; CTL: Cytotoxic T-lymphocyte; Hsp: Heat-shock protein; DTH: Delayed type hypersensitivity, ELISPOT: Enzyme-linked immunosorbant spot; ID: Intradermal; IFN: Interferon; IP: Intraperitoneal; IR: Intrarectal; IV: Intravenous; LNC: Lymph node cells; PBL: Peripheral blood lymphocyte; rBCG: recombinant BCG; SCID: Severe combined immunodeficiency; pBlaf: β-lactamase promoter; PND: Principal neutralizing determinant; SIV: Simmian immunodeficiency virus.

Table 2. Specific SIV/HIV immune responses induced after rBCG:SIV/HIV immunization using different animal models (cont.).

Recombinant antigen	Promoter, signal sequence and expression vector	Route	Number of cells (cfu)	Boost	Immunity induced	Ref.
rBCG cocktail: rBCG-SIV-nef, rBCG-SIV-gag and rBCG-SIV-env	pAN promoter sequence from IS900 insertion element of *M. paratuberculosis* (SIV *gag* and SIV *nef*) The SIV *env* (N-ter. half of gp110 was fused to the β-lactamase gene of *M. fortuitum*. β-lactamase promoter *M. fortuitum* (SIV *env*)	Oral Nasal Rectal	4.5×10^9 3×10^7 9×10^8		Ab responses induced against SIV-*env* and *gag*. No Ab responses against SIV *nef*. IgA: positive at 5 weeks after immunization by all routes (highest in feces). IgG: positive at 5 weeks after immunization by all routes. CTLs (spleen): strong CTL against SIV *nef*, *env* and *gag* at 5 weeks after immunization (all routes)	[43]
V3J1 (Clade B HIV-1)	Same construct as [42]	Nasal Oral SC	10 µg–10^6 cfu 100 µg 100 µg	Weekly, 3 consecutive weeks	IgG response similar in mucosal and systemic inoculation, lasting longer in nasal than oral	[60]
P24 antigen of HIV-1 gag,	Hsp60 promoter, *BCG* codon optimized	ID	10 µg and 100 µg		Better response with codon-optimized, measured by lymphocyte proliferation, ELISPOT γ-interferon and antibody production	[25]
HIV-1 IIIB gp120, in *Mycobacterium smegmatis*	pJH222 (multicopy) and pJH223 (integrative) plasmids Human codon-optimized	IP	10^6 and 10^8		Effector and memory T-lymphocytes elicited. No interference with pre-existing BCG immunity	[26]
PND HIV-1	Chaperonin 10 –PND chimeras. Chaperonin 10 promoter	SC	10^6		Splenocytes from recombinant BCG-immunized mice showed enhanced lymphocyte proliferation and interleukin-4 (but not IFN-γ) secretion	[28]
2.2 Guinea-pig model						
E. coli lac Z	pAN promoter sequence from IS900 insertion element of *M. paratuberculosis*	ID, oral, aerosol	10^6 (ID) 6×10^{10} (oral) : 2×10^{10} (3 times /24h) 10^8 (aerosol)		Ab at 2 weeks. Highest titers at 16 weeks. All routes similar Ab responses. T-cell proliferative responses (spleen and LNC). Responses of LNC were earlier (2 weeks) than spleen cells. Spleen responses were highest at 4 weeks (oral and aerosol)	[23]
19-aa from HIV-1 V3 sequence fused to α-antigen of *M. kansasii*	α-antigen promoter and α-antigen signal sequence	SC	10^8		All 20 guinea-pigs had detectable antibody titer (1:160 to 1:2560) against V3 peptide. Neutralization of primary isolates by Ab (guinea-pigs). HIV-specific DTH in guinea-pigs	[42]
SIVenv (aa 1–245 : N-terminal half)	β-lactamase promoter from *M. fortuitum* Fused to the β-lactamase gene of *M. fortuitum*	SC and oral	10^9 SC 6×10^{10} oral	10^6 for Ab week 4 and 8	Ab (IgG). Ab(IgA). Neutralizing Ab (Nab) against primary SIV isolates (SC). No Nab from orally immunized guinea-pigs	[59]

Ab: Antibody; BCG: Bacillus Calmette–Guérin; CTL: Cytotoxic T-lymphocyte; Hsp: Heat-shock protein; DTH: Delayed type hypersensitivity, ELISPOT: Enzyme-linked immunosorbant spot; ID: Intradermal; IFN: Interferon; IP: Intraperitoneal; IR: Intrarectal; IV: Intravenous; LNC: Lymph node cells; PBL: Peripheral blood lymphocyte; rBCG: recombinant BCG; SCID: Severe combined immunodeficiency; pBlaF: β-lactamase promoter; PND: Principal neutralizing determinant; SIV: Simmian immunodeficiency virus.

Table 2. Specific SIV/HIV immune responses induced after rBCG:SIV/HIV immunization using different animal models (cont.).

Recombinant antigen	Promoter, signal sequence and expression vector	Route	Number of cells (cfu)	Boost	Immunity induced	Ref.
V3 Thai Clade E HIV-1 19 aa-alfa 15 aa-alfa 12 aa-alfa 11 aa-alfa	Plasmid pUM Promoter alfa-K	SC	10 mg		IgG serum that can neutralize Clade E and Clade B HIV-1	[24]
V3J1 19 aa HIV-1	pSOV3J1 plamid, codon optimized	Oral	80 mg (liof)	3 boosts at weekly intervals	Functional T-cells (DTH and lymphoproliferative activity detected 1.5 years postinoculation)	[61]
V3J1, Japanese consensus HIV-1	pSOV3J1	IR, ID, IN	80 mg in 500 µl (IR) 1 or 0.1 mg (ID) 10 µg in 50 µl (IN)	Weekly, 2, 3 or 4 consecutive weeks	Enhanced IgG, IgA, IFN-γ and IL-2 in PBMCs in animals which received the combined inoculation	[62]
SIVmac239 gag	pSO2SIVgag	ID, oral	0.1 mg (ID) 80 mg (oral)		Long lasting cellular and humoral immunity against both viral and bacterial antigens	[63]
2.3 Non-human primates model						
2.3.1 Rhesus macaques						
SIVgag aa126 –362	hsp60 pMV261 extrachromosomal	ID	10^8	Week 19	CTL: 4 weeks after boosting <19 weeks: No Ab. No CTLs >19 weeks: No Ab, Yes CTLs	[44]
P11C peptide from SIV *gag*	hsp60 promoter pMV261	ID	10^8 2 inoculations (19 weeks apart)	Boosted with peptide (liposome) 13 months after rBCG inoculation	No Ab. CTLs No protection after IV challenge with cell – free SIV	[45]
SIV gag, pol, nef and env	Hsp70 promoter pMV261	IV. Single simultaneous			CTL against SIV *gag*, *pol* and *env* in 5 out of 6 monkeys IgA and IgG to gp130. These responses increase over time after immunization	[46]
2.3.2 Cynomolgus macaques						
HIV-1 V3J1 19 aa	pSO246, alfa K prot carrier	SC	10 mg		Type-specific V3 Nab *in vitro*. Challenge protection: viral load reduction and plasma viremia reduced after SHIV-MN challenge. No difference when SHIV 89.6 challenge	[64]
Full length SIV *gag*	hsp60	rBCG-SIVgag (ID), Vaccinia(DI-SIVgag) (IV)	10 mg, 10^8 PFU	BCGpSO + DI*lacZ* BCG-SIV + DI lacZ BCG-SIV + DI SIV DI-SIV + BCG pSO DI-SIV + BCG SIV	High level IFN-gama spot forming cells in rBCG-DI boosted animals. Protective against IR challenge with SHIV	[40]
3 rBCG, SIVmac251 *nef*, *gag*, *env*	pAN for *nef* and *gag*, pBlaf for *env*	ID	5 x 10^8 cfu	Oral and rectal	ID inoculation only: IFN-γ production against the three antigens. Oral or rectal boosting: increased IFN-γ production and anti-SIV IgA	[65]

Ab: Antibody; BCG: Bacillus Calmette–Guérin; CTL: Cytotoxic T-lymphocyte; Hsp: Heat-shock protein; DTH: Delayed type hypersensitivity, ELISPOT: Enzyme-linked immunosorbant spot; ID: Intradermal; IFN: Interferon; IP: Intraperitoneal; IR: Intrarectal; IV: Intravenous; LNC: Lymph node cells; PBL: Peripheral blood lymphocyte; rBCG: recombinant BCG; SCID: Severe combined immunodeficieny; pBlaf: β-lactamase promoter; PND: Principal neutralizing determinant; SIV: Simmian immunodeficiency virus.

Previous research performed in our group.

The initial research work or recombinant BCG based HIV vaccine started in New York, where Dr Joan Joseph collaborated with Dr. Barry Bloom and Dr. William Jacobs Jr, experts and leaders of mycobacterial immunopathogenesis and genetics. He built up a recombinant BCG expressing HIV-1 gp120 and SIV gag. When the Preventive HIV vaccine research line was established in Hospital Clínic de Barcelona, the reagents and biological samples were transferred to resume the postdoctoral research.

One of the first achievements was the prevention of mycobacterial genetic rearrangements due to the toxicity of the heterologous insert. We evaluated the disruption of heterologous HIV-1 gp120 gene expression, from SHIV-HXBc2P 3.2 clone (GenBank accession number AF041850), by genetic rearrangements in *Mycobacterium bovis* BCG host strain using a replicative vector (pMV261) regulated by BCG hsp60 promoter (strong promoter). To compare the HIV-1 gp120 gene expression and plasmid DNA stability *in vivo*, the replicative (pJH222) and integrative (pJH223) vectors carrying a wild-type lysine-complementing gene in the lysine auxotroph of BCG host strain were used. In these vectors, the HIV-1 gp120 gene expression was regulated by *Mycobacteria spp. α*-antigen promoter (weak promoter). We have demonstrated that the use of weak promoters (*Mycobacteria spp*. *α*-antigen promoter) to regulate HIV-1 gp120 gene expression and BCG lysine auxotrophs complemented with a lysine gene do, in fact, prevent the disruption of gene expression caused by genetic rearrangements [Joseph J, 2010]. As shown in Figure 7, no genetic rearrangement were detected after pJH222.HIV-1 gp120 and pJH223.HIV-1 gp120 transformed into the lysine auxotroph BCG. These results were published in the Journal of Biomedicine and Biotechnology in 2010 (Figure 6).

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2010, Article ID 357370, 10 pages
doi:10.1155/2010/357370

Research Article

Molecular Characterization of Heterologous HIV-1gp120 Gene Expression Disruption in *Mycobacterium bovis* BCG Host Strain: A Critical Issue for Engineering Mycobacterial Based-Vaccine Vectors

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Figure 6: Heading of the paper Joseph J et al, 2010

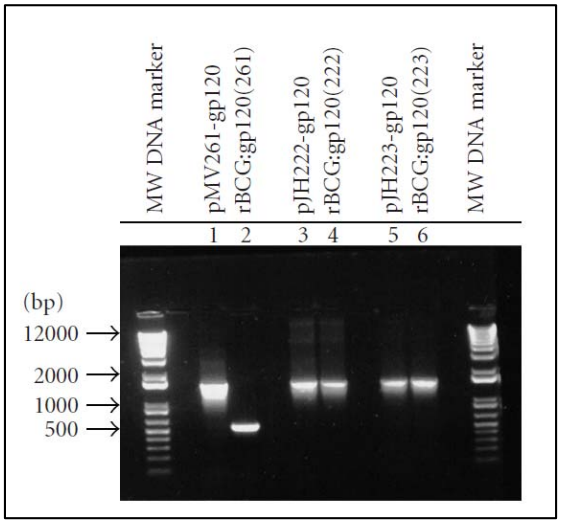
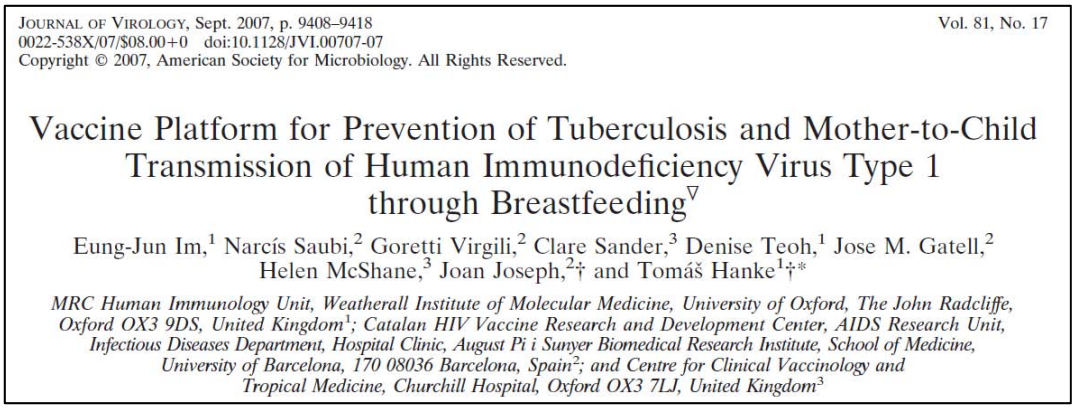


Figure 7: PCR analysis of the HIV-1gp120 gene cloned into pMV261, pJH222 and pJH223 vectors. *Lane 1, 3 and 5* are PCR products of the HIV-1gp120 gene (1578 bp) cloned into pMV261 (*lane 1*), pJH222 (*lane 3*) and pJH223 (*lane 5*) vectors, respectively. Plasmid DNA before BCG transformation (pre-BCG) were used as positive controls (lane 1, 3 and 5). *Lane 2, 4 and 6* are PCR products from the rBCG:HIV-1gp120 (pMV261), rBCG:HIV-1gp120 (pJH222) and rBCG:HIV-1gp120 (pJH223) colonies.

Later on, we started a collaboration with Dr Hanke's group from the University of Oxford to use their HIV immunogens (usually, fusion peptides) in our recombinant BCG based vaccine platform. In the first collaboration, we constructed a recombinant lysine auxotroph of BCG expressing the HIVA immunogen [Hanke T, 2000], from both replicative and integrative vectors. After confirmation of the HIVA gene sequence, plasmid stability, and protein expression, the BCG strain expressing HIVA from an episomal plasmid

(BCG.HIVA) alone and BCG.HIVA in a heterologous priming-boosting combination (using the Modified Vaccinia Ankara strain as a booting vector, MVA.HIVA) were studied for the induction of HIV-1 and *M. tuberculosis*-specific immune responses in a murine model. The BCG.HIVA recombinant strain was shown to be stable and to induce durable, high-quality HIV-1-specific $CD4^+$- and $CD8^+$-T-cell responses. Furthermore, when the recombinant BCG.HIVA vaccine was used in a priming-boosting regimen with heterologous components, the HIV-1-specific responses provided protection against surrogate virus challenge, and the recombinant BCG vaccine alone protected against aerosol challenge with *M. tuberculosis*. Thus, BCG.HIVA vaccine delivered at or soon after birth may prime HIV-1-specific responses, which can be boosted by natural exposure to HIV-1 in the breast milk and/or by a heterologous vaccine such as recombinant modified vaccinia virus Ankara delivering the same immunogen, preventing mother-to-child transmission of HIV-1 during breastfeeding. These results were published in the Journal of Virology in 2007 [Im EJ, 2007] (Figure 8).



JOURNAL OF VIROLOGY, Sept. 2007, p. 9408–9418 Vol. 81, No. 17
0022-538X/07/$08.00+0 doi:10.1128/JVI.00707-07

Vaccine Platform for Prevention of Tuberculosis and Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1 through Breastfeeding▽

Eung-Jun Im,[1] Narcís Saubi,[2] Goretti Virgili,[2] Clare Sander,[3] Denise Teoh,[1] Jose M. Gatell,[2] Helen McShane,[3] Joan Joseph,[2]† and Tomáš Hanke[1]†*

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Figure 8: Heading of the paper Im EJ, 2007.

The most relevant results published in Im EJ *et al* (2007) regarding HIV-1 immunogenicity (Figure 9), surrogate HIV-1 virus protection (Figure 10) and protection against M. tuberculosis challenge (Figure 11) have been included in this summary.

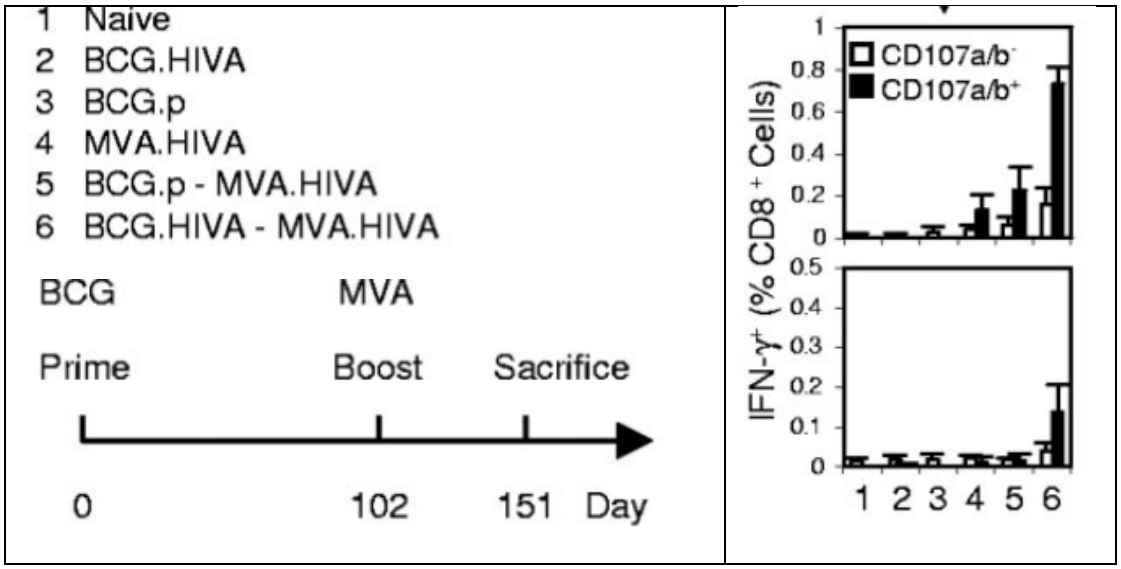

Figure 9: **Induction of multifunctional HIV-1-specific CD8+ T cells by the BCG.HIVA priming-MVA.HIVA boosting regimen**. Left panel: Mice were either left unimmunized or immunized with 10^6 cfu of p.BCG or BCG.HIVA and subsequently given a booster dose of 10^6 pfu of MVA.HIVA as indicated. (B) Analysis of bifunctional vaccine-elicited CD8+ T cells. Right panel: Data obtained for each vaccination group by using the H (immunodominant,top) and P (subdominant, bottom) epitopes. For the IFN-γ/CD107a/b analyses, the frequencies of nondegranulating (empty bars) and degranulating (full bars) cells producing cytokine are shown. Data are presented as means standard deviations (SD; *n*, 4 to 5 mice).

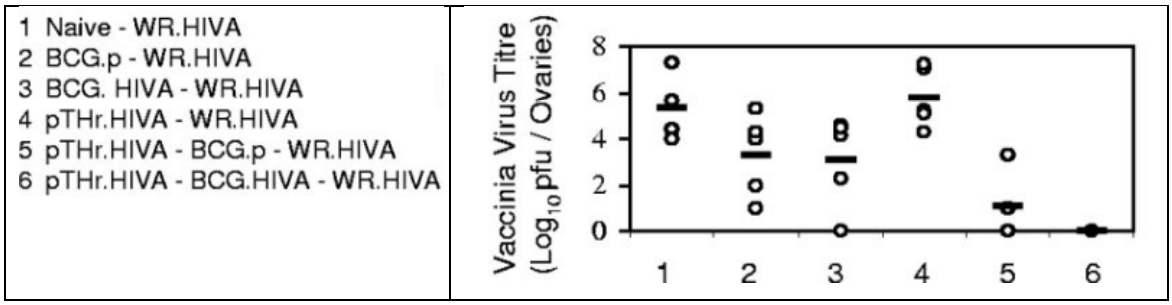

Figure 10. Complete protection against surrogate virus challenge. Left panel: Vaccination and challenge schedule. Mice were either left naïve (1) or vaccinated with BCG.p (2), BCG.HIVA (3), pTHr.HIVA DNA (4), pTHr.HIVA DNA and BCG.p (5), or pTHr.HIVA and BCG.HIVA (6) and challenged with WR.HIVA. Right panel. The WR.HIVA loads in ovaries were determined 4 days later. Data for individual mice (circles) and group means (bars; *n*, 4 to 5 mice) are shown.

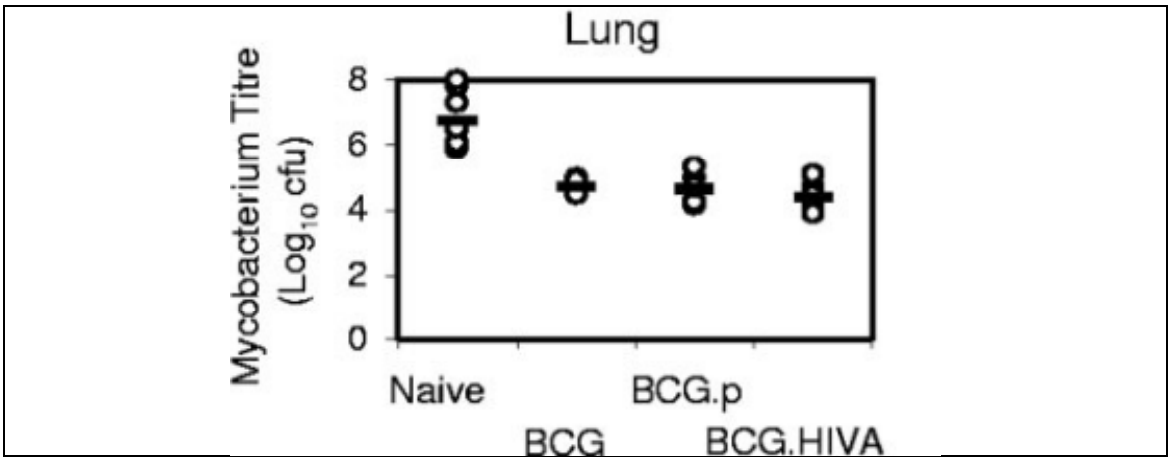

Figure 11. Protection against *M. tuberculosis* challenge by BCG.HIVA. Mice were left naïve or immunized subcutaneously in their left hind legs with the presently used BCG vaccine, a parental BCG, or lysine auxotrophic BCG.HIVA and challenged with inhaled *M. tuberculosis*. *M. tuberculosis* loads in lungs were determined 4 weeks later. Data for individual mice (circles) and geometric means for each group (*n*, 7 to 9 mice) are shown.

In further collaborations with Dr. Hanke's group, we compared modified Danish (AERAS-401) and Pasteur lysine auxotroph of BCG (222) strains of BCG expressing the immunogen HIVA (BCG.HIVA^{401} and BCG.HIVA^{222} respectively for their potency to prime HIV-1-specific responses in adult BALB/c mice and examined four heterologous boosting HIVA vaccines for their immunogenic synergy. The booster vectors (all of them expressing the HIVA immunogen) used in this paper were human adenovirus-vectored HAdV5.HIVA, sheep atadenovirus-vectored OAdV7.HIVA, poxvirus MVA.HIVA and the plasmid pTH.HIVA DNA. It was found that both BCG.HIVA^{401} and BCG.HIVA^{222} primed HIV-1-specific CD8+ T-cell-mediated responses. The strongest boosts were delivered by human adenovirus-vectored HAdV5.HIVA and sheep atadenovirus-vectored OAdV7.HIVA vaccines, followed by poxvirus MVA.HIVA; the weakest was plasmid pTH.HIVA DNA.The strongest priming activity was elicited by BCG.HIVA^{222}. The prime-boost regimens induced T cells capable of efficient in vivo killing of sensitized target cells. It was also observed that the BCG.HIVA^{401} and BCG.HIVA^{222} vaccines have broadly similar immunologic properties, but display a number of differences mainly detected through distinct profiles of soluble intracellular signaling molecules produced by immune splenocytes in response to both HIV-1- and BCG-specific stimuli. These results encourage further development of the rBCG prime-boost regimen. These results were published in the European Journal of Immunology [Hopkins R, 2011] (Figure 12)

Richard Hopkins et al. DOI 10.1002/eji.201141962 Eur. J. Immunol. 2011. 41: 3542–3552

European Journal of Immunology

Optimizing HIV-1-specific CD8$^+$ T-cell induction by recombinant BCG in prime-boost regimens with heterologous viral vectors

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Figure 12: Heading of the paper Hopkins R, 2011.

The HIV-1 specific cellular immune response against the immunodominant H epitope and the subdominant P epitope elicited by the BCG prime (BCG.HIVA^{401} or BCG.HIVA^{222}) and different boosting vectors is shown in Figure 13.

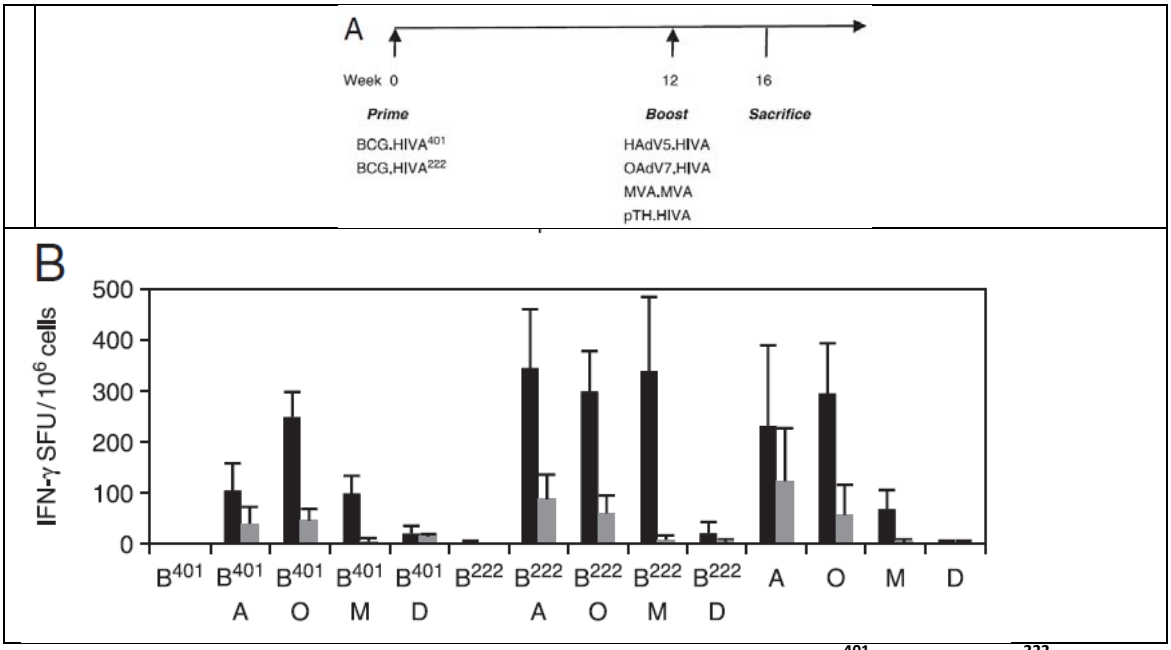


Figure 13. Augmentation of HIV-1-specific T-cell responses by BCG.HIVA^{401} or BCG.HIVA^{222} priming. Upper Pannel. Groups of 5 BALB/c mice were left unimmunized or inoculated i.p. with 10^6 cfu of BCG.HIVA^{401} or BCG.HIVA^{222}, or PBS, and boosted 12wk later with one of the following vaccines delivered i.m.: 10^6 IU of HAdV5.HIVA, 10^7 IU of OAdV7.HIVA, 10^8 pfu of MVA.HIVA, 100 mg of plasmid pTH.HIVA DNA or PBS as a negative control. Mice were sacrificed at wk 16. Lower panel. Frequencies of IFN-γ-producing splenocytes upon H (black) and P (grey) peptide restimulation were determined in an ELISPOT assay. B^{401} – BCG.HIVA^{401}; B^{222} – BCG.HIVA^{222}; A – HAdV5.HIVA; O – OAdV7.HIVA; M – MVA.HIVA; and D – pTH.HIVA.

OBJECTIVES:

1.- To evaluate the influence of age and immunization routes for induction of HIV-1 and *M. tuberculosis*-specific immune responses after BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost.

2.- To evaluate the safety in newborn mice of BCG.HIVA^{222} prime and MVA.HIVA boost vaccination schedule.

3.- To construct a novel *E. coli*-mycobacterial shuttle plasmid pJH222.HIVA^{CAT} by using an antibiotic-free plasmid selection system based on Operator-Repressor Titration (ORT) system in *E. coli* and lysine complementation in mycobacteria, to generate vaccine BCG.HIVA^{CAT} and to perform the genetic and phenotypic characterization of antibiotic markerless BCG.HIVA^{CAT}.

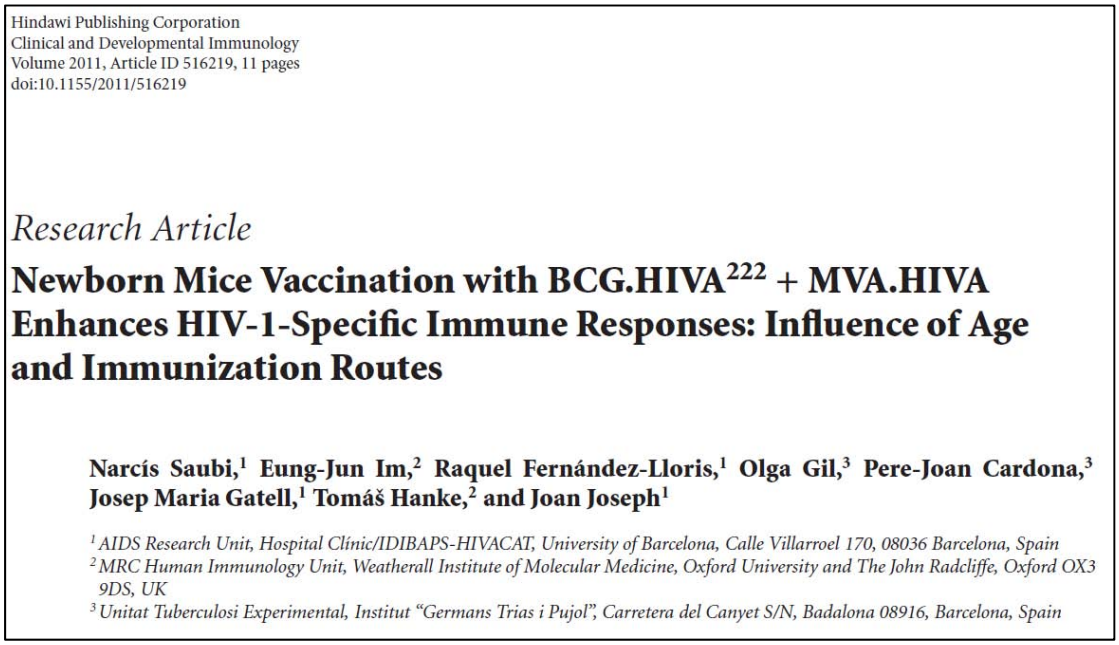
4.- To evaluate HIV-1 and *Mtb*-specific immune responses and safety in BALB/c mice after BCG.HIVA^{CAT} prime and MVA.HIVA boost.

5.- To construct a novel *E. coli*–mycobacterial shuttle plasmid p2auxo.HIVA, expressing the HIVA immunogen. 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,* to generate vaccine BCG.HIVA2auxo and to perform the genetic and phenotypic characterization of antibiotic markerless BCG.HIVA^{2auxo}.

6.- To evaluate HIV-1 and *Mtb*-specific immune responses and safety in BALB/c mice after BCG.HIVA^{2auxo} prime and MVA.HIVA boost.

RESEARCH ARTICLES PUBLISHED IN THIS PhD THESIS:

PAPER NUMBER 1: Saubi N, Im EJ, Fernández-Lloris R, Gil O, Cardona PJ, Gatell JM, Hanke T, Joseph J. **Newborn mice vaccination with BCG.HIVA^{222} + MVA.HIVA enhances HIV-1-specific immune responses: influence of age and immunization routes**. Clin Dev Immunol. 2011;2011:516219. doi: 10.1155/2011/516219. Epub 2011 Apr 12. **ANNEX I**



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Clinical and Developmental Immunology
Volume 2011, Article ID 516219, 11 pages
doi:10.1155/2011/516219

Research Article

Newborn Mice Vaccination with BCG.HIVA^{222} + MVA.HIVA Enhances HIV-1-Specific Immune Responses: Influence of Age and Immunization Routes

Narcís Saubi,[1] Eung-Jun Im,[2] Raquel Fernández-Lloris,[1] Olga Gil,[3] Pere-Joan Cardona,[3] Josep Maria Gatell,[1] Tomáš Hanke,[2] and Joan Joseph[1]

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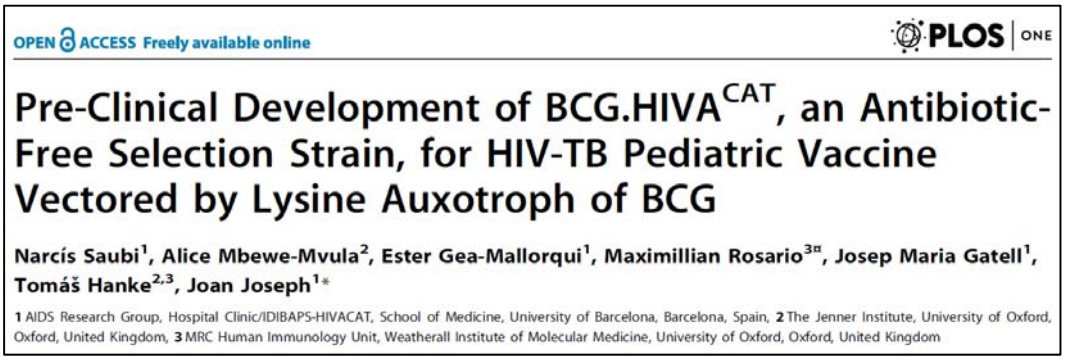
[3] *Unitat Tuberculosi Experimental, Institut "Germans Trias i Pujol", Carretera del Canyet S/N, Badalona 08916, Barcelona, Spain*

PAPER NUMBER 2: Saubi N, Mbewe-Mvula A, Gea-Mallorqui E, Rosario M, Gatell JM, Hanke T, Joseph J. **Pre-clinical development of BCG.HIVA(CAT), an antibiotic-free selection strain, for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG.** PLoS One. 2012;7(8):e42559. doi: 10.1371/journal.pone.0042559. Epub 2012 Aug 21. **ANNEX II.**



OPEN ACCESS Freely available online

PLOS ONE

Pre-Clinical Development of BCG.HIVA^{CAT}, an Antibiotic-Free Selection Strain, for HIV-TB Pediatric Vaccine Vectored by Lysine Auxotroph of BCG

Narcís Saubi[1], Alice Mbewe-Mvula[2], Ester Gea-Mallorqui[1], Maximillian Rosario[3¤], Josep Maria Gatell[1], Tomáš Hanke[2,3], Joan Joseph[1]*

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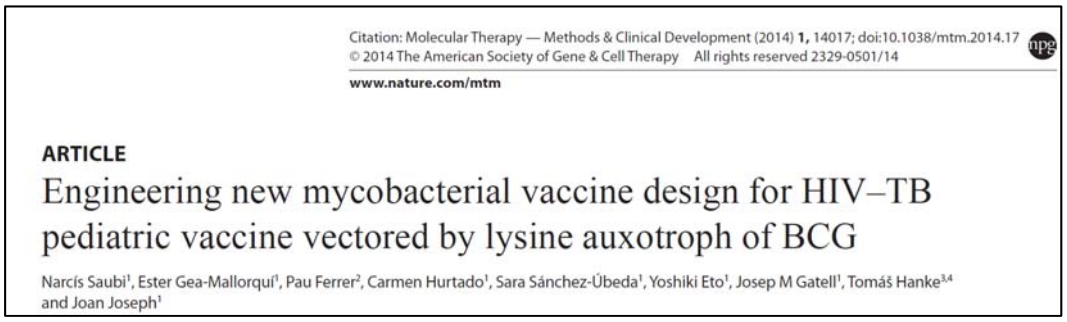
PAPER NUMBER 3: Saubi N, Gea-Mallorquí E, Ferrer P, Hurtado C, Sánchez-Úbeda S, Eto Y, Gatell JM, Hanke T, Joseph J. **Engineering new mycobacterial vaccine design for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG**. Mol

Ther Methods Clin Dev. 2014 May 21;1:14017. doi: 10.1038/mtm.2014.17. eCollection 2014. Nature Publishing Group. **ANNEX III.**



Citation: Molecular Therapy — Methods & Clinical Development (2014) 1, 14017; doi:10.1038/mtm.2014.17

www.nature.com/mtm

ARTICLE

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

Narcís Saubi[1], Ester Gea-Mallorquí[1], Pau Ferrer[2], Carmen Hurtado[1], Sara Sánchez-Úbeda[1], Yoshiki Eto[1], Josep M Gatell[1], Tomáš Hanke[3,4] and Joan Joseph[1]

I've already described the previous work performed in our research group, focused in the development of a recombinant BCG based dual TB-HIV-1 preventive vaccine. The research performed in the last years, and included in this PhD thesis, has been addressed to improve the immunogenicity of the BCG based preventive HIV-1 vaccines, by assessing the best immunization route and age of immunization of the murine model used to evaluate the HIV-1 specific immunitiy of the recombinant BCG expressing HIV-1 immunogens. Our research has also been focused in the improvement of the *E.coli*-mycobacterial shuttle plasmid, in order to facilitate the upgrading of the pre-clinical vaccine candidates to Clinical Trials. More specifically, we have constructed two different *E. coli*-mycobacterial shuttle plasmids where the antibiotic resistance has been removed and replaced by an Operator Repressor Titration method (PAPER NUMBER 2) or by an *E. coli* glycine complementing gene, to be used in a glycine auxotrophic E. coli strain to select the recombinant *E. coli* clones (PAPER NUMBER 3).

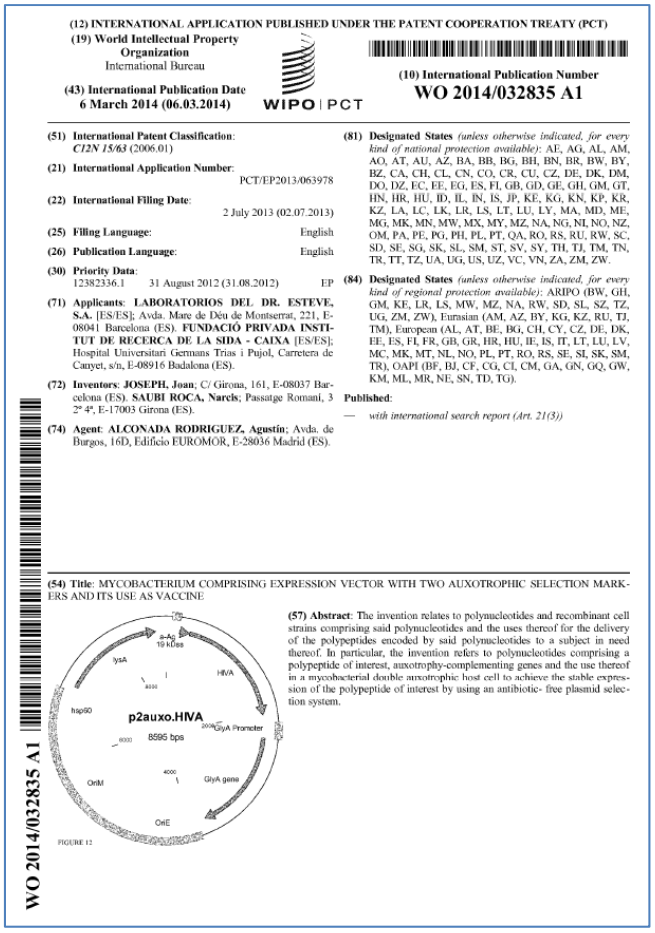
In PAPER NUMBER 1, we have evaluated the influence of age and immunizations routes for induction of HIV-1 and *M. tuberculosis*-specific immune responses after BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The frequencies of HIV-specific CD8+ T cells producing IFN-γ were higher in adult mice vaccinated intradermally and lower in adult and newborn mice vaccinated subcutaneously. In all cases the IFN-γ production was significantly higher when mice were primed with BCG.HIVA^{222} compared with BCGwt. When the HIV-specific CTL activity was assessed, the frequencies of specific killing were higher in newborn mice than in adults. The prime-boost

vaccination regimen which includes BCG.HIVA^{222} and MVA.HIVA was safe when inoculated to newborn mice. The administration of BCG.HIVA^{222} to newborn mice is safe and immunogenic and increased the HIV-specific responses induced by MVA.HIVA vaccine.

In PAPER NUMBER 2, we assembled an *E. coli*-mycobacterial shuttle plasmid pJH222.HIVA^{CAT} expressing HIV-1 clade A immunogen HIVA. This shuttle vector employs an antibiotic resistance-free mechanism based on Operator-Repressor Titration (ORT) system for plasmid selection and maintenance in *E. coli* and lysine complementation in mycobacteria. We demonstrated that the episomal plasmid pJH222.HIVA^{CAT} was stable in vivo over a 20-week period, and genetically and phenotypically characterized the BCG.HIVA^{CAT} vaccine strain. The BCG.HIVA^{CAT} vaccine in combination with MVA.HIVA induced HIV-1- and Mtb-specific IFN-γ-producing T-cell responses in newborn and adult BALB/c mice. On the other hand, when adult mice were primed with BCG.HIVA^{CAT} and boosted with MVA.HIVA.85A, HIV-1-specific CD8+ T-cells producing IFN-γ, TNF-α, IL-2 and CD107a were induced. To assess the biosafety profile of BCG.HIVA^{CAT}-MVA.HIVA regimen, body mass loss of newborn mice was monitored regularly throughout the vaccination experiment and no difference was observed between the vaccinated and naïve groups of animals. Thus, we demonstrated T-cell immunogenicity of a novel, safer, GLP-compatible BCG-vectored vaccine using prototype immunogen HIVA.

In PAPER NUMBER 3, we have engineered a new mycobacterial vaccine design by using an antibiotic-free plasmid selection system. We assembled a novel *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 modifed vaccinia virus Ankara (MVA).expressing HIVA was safe and induced HIV-1 and *Mycobacterium*

tuberculosis-specific IFN-γ-producing T-cell responses in adult BALB/c mice. The results of this research have been patented with publication number WO2014/032835 (Figure 14). **ANNEX IV.**



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 March 2014 (06.03.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/032835 A1

(51) International Patent Classification:
C12N 15/63 (2006.01)

(21) International Application Number:
PCT/EP2013/063978

(22) International Filing Date:
2 July 2013 (02.07.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12382336.1 31 August 2012 (31.08.2012) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) Title: MYCOBACTERIUM COMPRISING EXPRESSION VECTOR WITH TWO AUXOTROPHIC SELECTION MARKERS AND ITS USE AS VACCINE

FIGURE 12

(57) Abstract: The invention relates to polynucleotides and recombinant cell strains comprising said polynucleotides and the uses thereof for the delivery of the polypeptides encoded by said polynucleotides to a subject in need thereof. In particular, the invention refers to polynucleotides comprising a polypeptide of interest, auxotrophy-complementing genes and the use thereof in a mycobacterial double auxotrophic host cell to achieve the stable expression of the polypeptide of interest by using an antibiotic- free plasmid selection system.

WO 2014/032835 A1

Figure 14: Front page of the published patent WO2014/032835.

The research included in this PhD thesis is directed to obtain a safe, effective and affordable HIV-1 vaccine, and objective of great scientific and social interest. We engineered novel, safer, good laboratory practice–compatible BCG-vectored vaccines 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.

RESULTS AND DISCUSSION

Construction and characterization of BCG.HIVA^{222}, BCG.HIVA^{CAT} and BCG.HIVA^{2auxo} vaccine strains

Construction of BCG.HIVA^{222}.

This construction has been previously developed in our laboratory [Im EJ, 2007; Hopkins R, 2010]. We have shown the molecular structure of the *E. coli*-mycobacterial pJH222.HIVA expression vector, and we have confirmed the HIVA protein expression by Western blot analysis.

HIVA immunogen consists of consensus HIV-1 clade A gag p24/p17 domains coupled with a string of CD8+ T-cell epitopes and monoclonal antibody (mAb) tag Pk [Hanke T, 2000]. The HIVA gene was synthesized utilizing humanized GC-rich codons, which are similar to those used by mycobacteria [André S, 1998; Andersson SGE, 1996; de Miranda AB, 2000]. The HIVA open-reading frame was fused at its 5' end to nucleotides coding for the 19-kDa lipoprotein signal sequence, which facilitates expression of foreign proteins in the mycobacterial membrane and was shown to increase the foreign protein immunogenicity [Stover CK, 1993]. To facilitate the preclinical development of candidate vaccines, the HIVA immunogen contains an immunodominant H-2D^d-restricted epitope P18-I10 [Takahashi H, 1988], here designated also as H epitope. In addition, it also contains at least three other subdominant H-2D^d epitopes recognized by CD8$^+$ T cells including epitope P and three CD4$^+$ T-helper epitopes (unpublished). The chimeric 19-kDa signal sequence-HIVA gene was expressed from *E. coli*-mycobacterial shuttle plasmid pJH222 under the control of the *M. tuberculosis* α-antigen promoter (Figure 15,a). pJH222 is a multi-copy replicative episomal vector and contains mycobacterial origin of replication (*oriM*). It contains also an expression cassette encoding kanamycin resistance (*aph*), *E. coli* origin of replication (*oriE*), and a wild-type lysine A complementing gene for the vector maintenance (*lysA5*) in the BCG auxotroph. Recombinant pJH222.HIVA was transformed into lysine auxotroph of *M. bovis* BCG host strain Pasteur lysA5::res [Pavelka Jr MS, 1999]. Expression of the full-size chimeric 19-kDa signal sequence-HIVA protein of *Mr* 65 kDa was

confirmed by Western blot analysis of mycobacterial cell lysates using anti-Pk mAb (Figure 15,b).

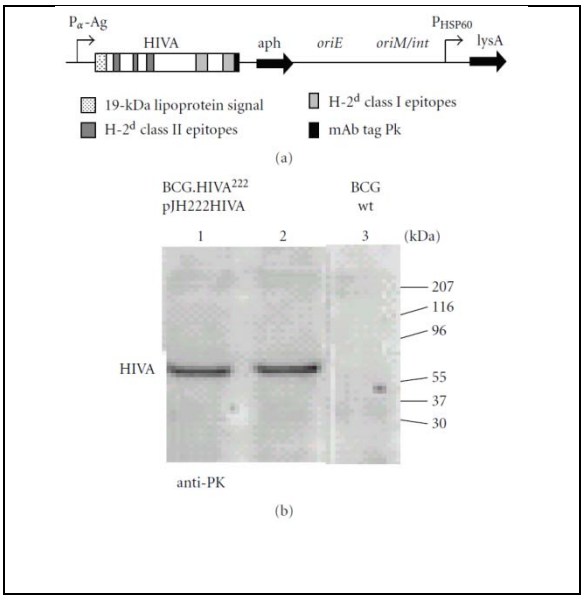

Figure 15: Expression of the HIVA immunogen from BCG.HIVA222. (a) A synthetic GC-rich *HIVA* gene consisting of consensus was fused to the 19-kDA lipoprotein signal sequence and inserted into episomal pJH222 *E. coli*-mycobacterium shuttle plasmid. This contains a kanamycin resistance gene (*aph*) and complementing *lysA* genes and *E. coli* origin of replication (*oriE*). In addition, pJH222 contains mycobacterial origin of replication (oriM). BALB/c mice T-cell and mAb Pk epitopes used in this paper are depicted. P*α*-Ag, *M. tuberculosis α*-antigen promoter; PHSP60, heat shock protein 60 gene promoter. (b) Western blotting of lysates of BCG.HIVA^{222} containing the pJH222HIVA (lanes 1 and 2) and BCG wild type (lane 3; negative control) is shown. HIVA was detected using the anti-Pk mAb followed by HRP-protein A and ECL.

Construction and characterization of BCG.HIVA^{CAT}

We have developed a novel pJH222.HIVA^{CAT} expression vector by using an antibiotic-free selection system. The kanamycin resistance gene was removed from pJH222.HIVA vector by using the Operator-Repressor Titration (ORT) system developed by Cobra Biologics (UK). Such system enables the selection and maintenance of plasmids that are free from expressed selectable marker genes and require only the short, non-expressed *lac* operator for selection and maintenance [Williams SG, 1998]. The principle *E. coli* ORT strain, DH1*lacdap*D [Cranenburg RM, 2001], has been used to produce several important DNA vaccine candidates such as the HIV-1 vaccine pTHr.HIVA [Cranenburgh RM, 2004]. In this work the kanamycin resistance gene was replaced with a *lac* operator sequence and the resulting plasmid, pJH222.HIVA^{CAT} was transformed into the *E. coli* DH1*lacdap*D strain (Figure 16). When the non-expressed *lac* operator sequence was inserted into multicopy plasmid and introduced into the cell, the binding of the repressor protein to the plasmid-borne operator derepresses the chromosomal operator and allows dapD expression and cell growth [Cranenburgh RM, 2004]. The recombinant pJH222.HIVA containing the ORT selection system, here designated as pJH222.HIVA^{CAT}, was transformed into lysine auxotroph of BCG host strain Pasteur ΔlysA5::res [Pavelka Jr MS, 1999]. The selection of positive BCG.HIVA^{CAT} colonies was made by growing

the rBCG cells 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 of whole transformed mycobacterial cell lysates using anti-Pk mAb. As shown in Figure 16, the highest level of HIVA protein expression was detected after blotting the BCG culture from clone number 10 and was selected for further molecular characterization, immunogenicity and safety testing in mice. On the other hand, the BCG.HIVA^{CAT} clone10 culture was preserved by using the seed-lot system. A Master Seed stock, and derivative Working Stock, which we used also as a Vaccine stock was prepared. Growth of the transformed mycobacteria and the *in vivo* stability of pJH222.HIVA^{CAT} episomal plasmid were established by the recovery of BCG.HIVA^{CAT} colonies from the spleens of BALB/c mice 20 weeks after immunization. Six out of six recovered rBCG colonies were positive for HIVA DNA coding sequence by PCR (Figure 16,c).

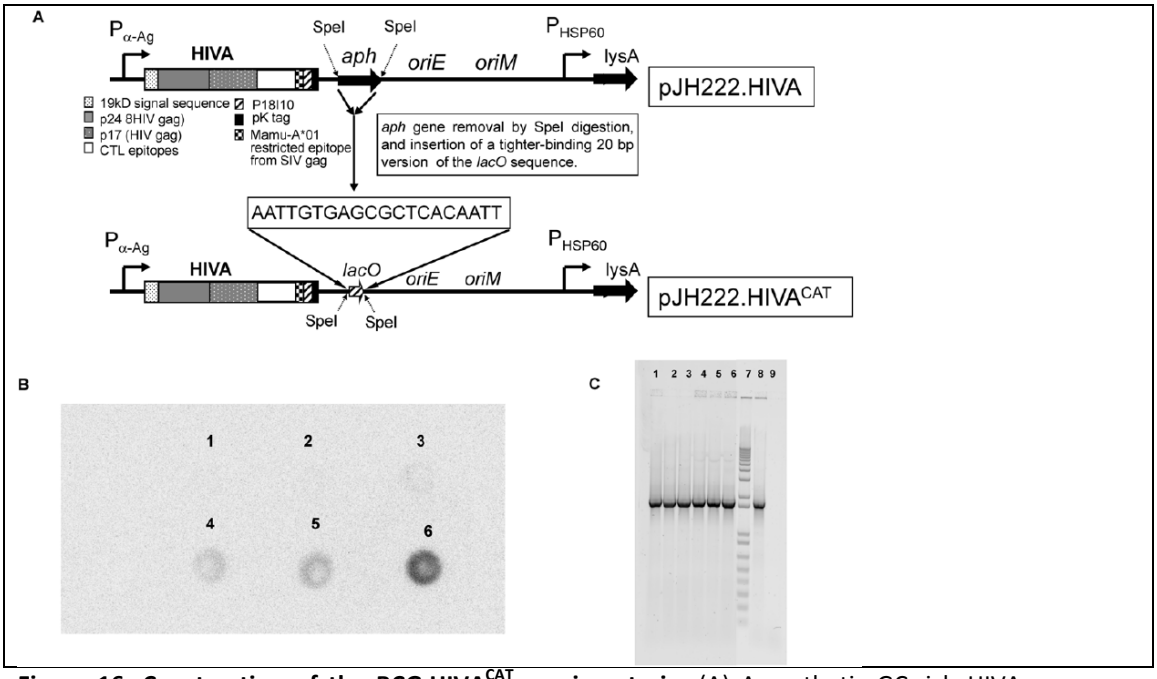

Figure 16. Construction of the BCG.HIVA^{CAT} vaccine strain. (A) A synthetic GC-rich HIVA gene was fused to the region encoding the 19-kDa lipoprotein signal sequence and inserted into the episomal pJH222 *E.coli*-mycobacterium shuttle plasmid. This plasmid contains kanamycin resistance (*aph*) and complementing *lys*A genes and an *E.coli* origin of replication (oriE). In addition, pJH222 contained the mycobacterial origin of replication (oriM). The BALB/c mouse T-cell and MAb Pk epitopes used in this work are depicted. P α-Ag, *M.tuberculosis* α-antigen promoter; P_{HSP60}, heat shock protein 60 gene promoter. The aph gene was removed by SpeI digestion and the *lac*O sequence was inserted and transformed into *E.coli* DH1*lacdap*D strain. (B) Immunodot of BCG.HIVA^{CAT} lysates. Dot 1: BCG wild type (negative control); Dot 2, 3, 4 and 5: clone 3, clone 7, clone 9 and clone 10 of BCG.HIVA^{CAT}; Dot 6: BCG.HIVA^{222} (positive control). HIVA peptide was detected using the anti-Pk MAb followed by horseradish peroxidase-Goat-anti-Mouse and enhanced chemiluminescence (ECL) detection. (C) In vivo plasmid stability of BCG.HIVA^{CAT} harboring pJH222.HIVA^{CAT}. Mice were injected s.c. with 10^5 cfu of

BCG.HIVA^{CAT} and boosted i.m. with 10^6 pfu of MVA.HIVA, spleens were homogenized 20 weeks after BCG inoculation and the recovered rBCG colonies were tested for the presence of the HIVA DNA coding sequence by PCR. Lanes 1 to 6: Six rBCG colonies were recovered in the non-lysine supplemented plate; lane 7: Molecular weight marker; lane 8: Plasmid DNA positive control; lane 9: Distilled water (negative control).

Genetic characterization of the BCG.HIVA^{CAT}

In order to confirm that our recombinant BCG.HIVA^{CAT} vaccine strain corresponds to *M. bovis* BCG strain, we used the GenoType MTBC assay based on a commercially available DNA strip assay (Hain Lifescience GmbH, Nehren, Germany) intended for the differentiation of members of the Mycobacterium tuberculosis complex (MTBC) and identification of *M. bovis* BCG. This assay is based on *gyrB* DNA sequence polymorphisms and the RD1 deletion of *M. bovis* BCG. We tested four samples corresponding to commercial BCG Connaught, BCG.HIVA^{222}, BCG.HIVA^{CAT} (clone10) and BCG Pasteur strains. As we show in Figure 17A, all four strains presented the same hybridization pattern corresponding to *M. bovis* BCG, detecting the bands 4, 7, 9, 10 and 13 belonging specifically to BCG hybridization pattern.

Distribution of BCG to several countries for worldwide application started around 1924 and it was preserved by *in vitro* subculture passaging until 1960s. Since then the Pasteur strain has been freeze-dried, keeping the form of the primary seed lot. The *in vitro* evolution of BCG has resulted in a number of BCG substrains that are heterogenic [Behr MA, 1999]. In order to confirm that our BCG.HIVA^{CAT} vaccine strain correspond to BCG Pasteur substrain, we have used the method described by Bedwell *et al*. [Bedwell J, 2001] 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 seven fingerprints and all BCG substrains were confirmed. We tested the following samples: BCG.HIVA^{CAT} strain (clone10) Pasteur substrain and commercial BCG Danish 1331 strain. Both BCG substrains evaluated gave a 196 bp product with primers ET1-3, indicating deletion of the RD1 region. In addition in BCG Pasteur (BCG.HIVA^{CAT}) the RD8 and RD16 were present and gave a product of 472 and 401 bp respectively. The primers for the SenX3-RegX3 region gave a product of 276 bp in BCG Pasteur. The PCR fingerprints of BCG Pasteur and BCG Danish substrains (Figure 17B) were consistent with previously published results on

genetic information of BCG substrains [Bedwell J, 2001]. As shown in Figure 17B, the yield of the PCR was higher when the single primer pairs were used, instead of multiplex format.

For the molecular characterization of pJH222.HIVA^{CAT} plasmid DNA, enzymatic restriction and PCR analysis were performed. The plasmid DNA was isolated from the Master Seed and Working Stock of BCG.HIVA^{CAT} strain and was characterized. The enzymatic restriction pattern obtained did not show any difference with the predicted enzymatic pattern of the plasmid DNA sequence (Figure 17C). On the other hand, the PCR analysis using specific primers of the HIVA DNA coding sequence was performed using the BCG liquid culture from BCG.HIVA^{CAT} Master Seed and Working Stock as template. A band of 1776 bp corresponding to HIVA DNA fragment was detected (Figure 17D).

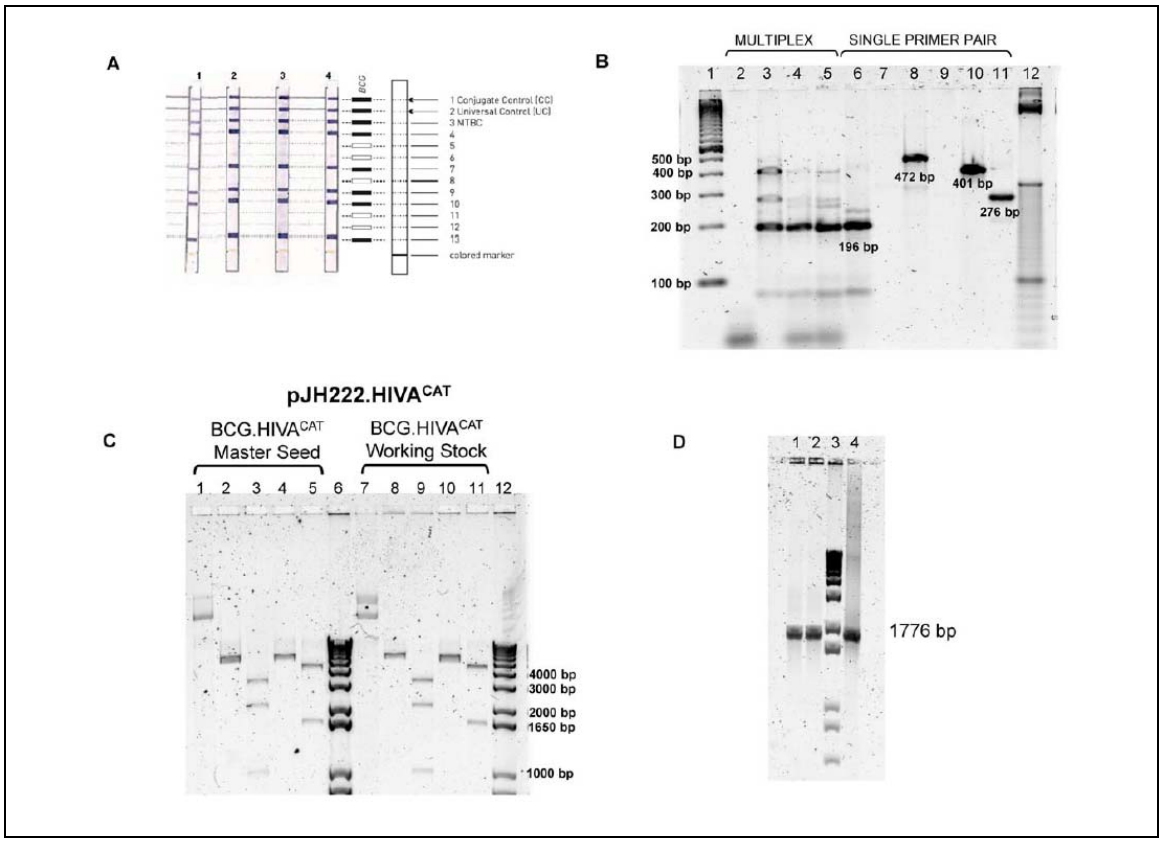

Figure 17. Genetic characterization of the BCG.HIVACAT. GenoType MTBC assay and Multiplex PCR assay. (A) The BCG.HIVA^{CAT} strain identification results representative of all of the patterns obtained with the GenoType MTBC assay. The positions of the oligonucleotides, the marker line and the BCG hybridization pattern are shown on the right. The specificity and targeted genes of the lines are as follows: 1, conjugate control; 2, amplification control (23S rRNA); 3, MTBC specific (23S rRNA); 4 to 12, discriminative for the MTBC species (gyrB); 13, M. bovis BCG (RD1). The samples analyzed were: Strip 1: BCG Connaught; Strip 2: BCG.HIVA^{CAT}; Strip 3: BCG.HIVA^{222}; Strip 4: BCG wild type. All four strains presented the same hybridization pattern corresponding to M. bovis BCG. (B) The BCG.HIVA^{CAT} Pasteur substrain identification by multiplex PCR assay. Lane 1 and 12: molecular weight marker; lane 2: negative control; lane 3, 6–11: BCG.HIVA^{CAT}(clone10); lane 4: BCG Danish strain (using 1 ml of template); lane 5: BCG Danish strain (using 4 ml template); The samples were analyzed by multiplex

primer assay or single primer pair assay. Lane 2–5: multiplex primers; lane 6: ET1-3 primers; lane 7: RD2 primers; lane 8: RD8 primers; lane 9: RD14 primers; lane 10: RD16 primers; lane 11: C3–C5 primers. (C) Enzymatic restriction analysis of pJH222.HIVA^{CAT} plasmid DNA extracted from both the Master Seed (MS, lanes 1–5) and the Working Stock (WS, lanes 7–11) of BCG.HIVA^{CAT} cultures. Lane 1 and lane 7: uncut plasmid; lane 2 and lane 8: HpaI digestion; lane 3 and 9: KpnI digestion; lane 4 and 10: digestion with SpeI; lane 5 and 11: digestion with HindIII; lane 6 and 12: Molecular Weight Marker (1 kb Plus, Invitrogen). (D) PCR analysis of HIVA DNA coding sequence using as template the cultures of BCG.HIVA^{CAT} Master Seed (lane 1), and Working Stock (lane 2), Molecular Weight Marker (lane 3), positive control plasmid DNA pJH222.HIVA (lane 4).

Phenotypic characterization of the BCG.HIVA^{CAT}

We assessed the phenotype stability of lysine auxotrophy, lysine complementation and kanamycin resistance of BCG.HIVA^{CAT} strain. Initially, the BCG lysine auxotroph strain was plated out on lysine supplemented and non supplemented agar. Such strain failed to grow on non lysine supplemented agar plates and no colonies were observed (Figure 18A). However, growth was observed on agar plates supplemented with lysine (Figure 18B). As expected, complementation of BCG.HIVACAT strain with lysine gene provided on the multicopy plasmid pJH222.HIVA^{CAT} abolished the requirement for exogenous lysine (Figure 18C). On the other hand, when BCG.HIVA^{CAT} strain was plated out on agar plates containing kanamycin, no colonies were observed (Figure 18D), confirming the lack of kanamycin resistance in our construct.

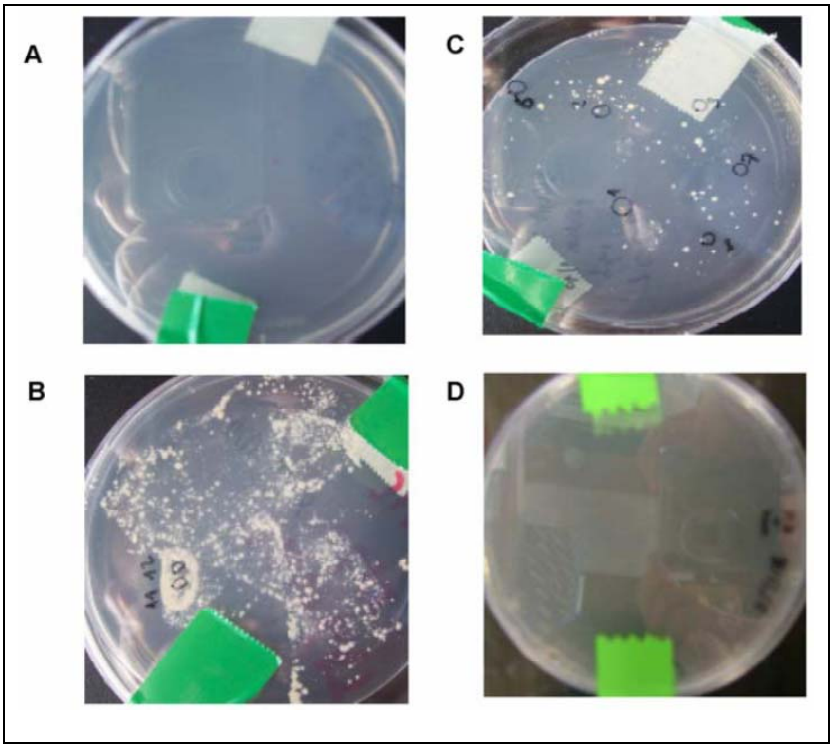

Figure 18. Phenotypic characterization of the BCG.HIVA^{CAT}. We assessed the phenotype of lysine auxotrophy, lysine complementation and kanamycin resistance of BCG.HIVA^{CAT} strain. (A) BCG lysine auxotroph strain plated on non-lysine supplemented 7H10; (B) BCG lysine auxotroph strain plated on lysine supplemented 7H10; (C) BCG.HIVA^{CAT} plated on 7H10 without lysine and kanamycin supplementation; (D) BCG.HIVA^{CAT} plated on 7H10 without lysine supplementation and with kanamycin.

Construction and characterization of the BCG.HIVA^{2auxo}

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. 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.[Vidal L, 2008; Pavelka MS, 1999]. 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. 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. As shown in Figure 19 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.

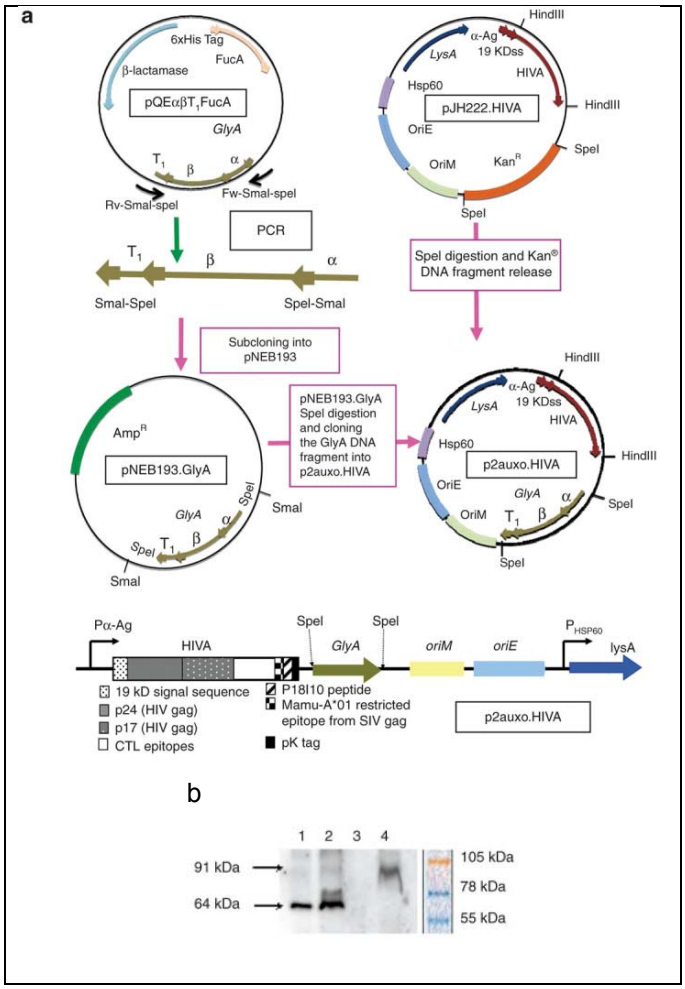

Figure 19. Construction of the BCG.HIVA^{2auxo} vaccine strain. (**a**) A synthetic GC-rich HIVA gene was fused to the region encoding the 19-kDa lipoprotein signal sequence and inserted into the episomal pJH222.HIVA *E. coli*–mycobacterium shuttle plasmid. The *aph* gene was removed by *Spe*I digestion, and the structural *glyA* gene was inserted and transformed into *E. coli* M15Δ*glyA* strain. (**b**) 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-GFP^{222} (pJH222 *E. coli*–mycobacterial shuttle plasmid, kanamycin resistance) used as a positive 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. 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.* [Bedwell J, 2001]. 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, 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 20,a). 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 20 b and c).

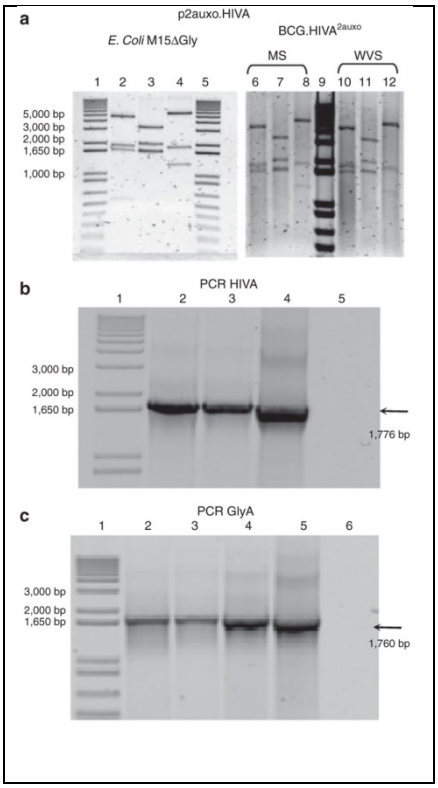

Figure 20. Genetic characterization of the BCG.HIVA^{2auxo} strain. (**a**) Enzymatic restriction analysis of p2auxo.HIVA plasmid DNA extracted from *E. coli* M15Δ*glyA* cultures (pre-BCG transformation) and from both the MS and WVS of BCG.HIVA^{2auxo} cultures. Left side: *E. coli* cultures. Lanes 1 and 5: molecular weight marker (1 kb plus; Invitrogen); lanes 2, 3, and 4: *Age*I, *Stu*I, and *Xho*I digestion, respectively. Right side: BCG cultures. Lane 9: molecular weight marker (1 kb plus; Invitrogen); lanes 6, 7, and 8 (MS): *Age*I, *Stu*I, and *Xho*I digestion, respectively. Lanes 10, 11, and 12 (WS): *Age*I, *Stu*I, and *Xho*I 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).

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* M15ΔglyA strain and BCG.HIVA^{2auxo} strains. The *E. coli* glycine auxotrophic strain failed to grow on non-glycine-supplemented agar plates; 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. 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, (data not shown).

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

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 21).

The functional stability of the HIVA gene was also assessed. When bacteria were grown under selective pressure, the HIVA protein expression was detected in five out of five BCG.HIVA^{2auxo} colonies. 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 (not shown).

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, but not in bacteria grown without selective pressure (not shown).

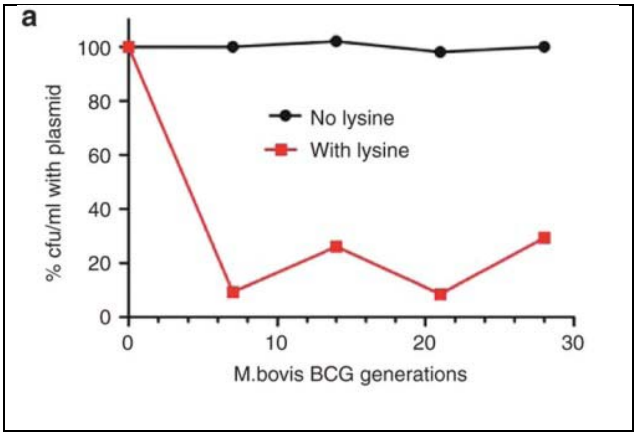

Figure 21 *In vitro* stability of the BCG.HIVA^{2auxo} strain. (**a**) *In vitro* persistence of the p2auxo.HIVA in BCG *lysA*- grown for successive passages on selective (no lysine) or nonselective (supplemented with lysine) media. The percentage represents the cfu (titer) that maintained the vector 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.

Overall, we described the structure of pJH222.HIVA expression vector, that had been previously constructed and characterized. Regarding pJH222.HIVA^{CAT}, we introduced a selective marker different than the kanamycin resistance. 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 *Escherichia coli*, their use has been considered unacceptable for clinical trials and product licensing. In our study, we have used the Operator-Repressor Titration system (ORT) reported by Cranenburgh *et al.* [Cranenburgh RM, 2001], that utilizes *E. coli* DH1*lacdapD* strain that enables plasmid selection and maintenance that is free from antibiotics and selectable marker genes. This is achieved by using only the *lac* operator (*lacO*) sequence as a selectable element. On the other hand the *E. coli*-mycobacterial expression vector contains the lysine A complementing gene of lysine auxotroph of BCG. In the following vaccine model, 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 main advantage of this auxotrophy based method is that we can use the M15ΔGly *E. coli* cells ,provided by Dr. Pau Ferrer, from the Department

of Chemical Engineering, UAB, to select the *E. coli* recombinant clones, whereas the ORT based selection method is property of Cobra Biologics, *Inc* Oxford, UK.

Regarding identification of the individual species that comprise *Mycobacterium tuberculosis* complex (MTBC), *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. canetti*, usually based on phenotypic characteristics and biochemical tests [van Soolingen D, 1997] (slow, need sufficient bacterial growth, are time consuming *and the* interpretation is subjective and could provide identification errors) we have used also the GenoType MTBC assay to identify our recombinant *M. bovis* BCG vaccine candidate strain. The hybridization pattern obtained was unequivocal and corresponded to *M. bovis* BCG. We also incorpored a substrain differentiation system based in a multiplex PCR identity test for BCG vaccines described by Bedwell *et al.* [Bedwell J, 2001]. The PCR fingerprints produced from DNA samples were concordant with predictions based on genetic information on BCG substrains. The capability of this multiplex PCR to discriminate between BCG substrains was tested using commercial preparations and was proven also to be suitable for identification of BCG in clinical samples as well as vaccines..

HIV-1 and *Mycobacterium tuberculosis* specific T-cell immune responses in BALB/c mice

BCG.HIVA^{222} prime and MVA.HIVA boost of BALB/c mice at different doses and inoculation routes. Effect on the elicited immune response.

In the first study we have evaluated the effect of BCG.HIVA^{222} priming using different routes and mice age (adult and newborn) on the induction of HIV-1-specific T-cell responses after BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The immunogenicity readout was focused on the P18I10 epitope, an immunodominant CTL epitope derived from HIV-1 Env and H-2D^{d} restricted [Takahashi H, 1988], here mentioned as H, which was fused to HIVA immunogen to evaluate the immunogenicity in mice(Figure 15,a). On day 0, mice were immunized with rBCG with the episomal plasmid or BCG wild type, and on day 102 the animals received a booster dose with MVA.HIVA (Figure 22,a). On day 151, the mice were sacrificed, and the functional specific T cells in response of peptide stimulation were measured by intracellular cytokine

staining (ICS) (Figure 22,b) and ELISPOT assays (not shown). We have observed in adult and newborn mice that BCG.HIVA^{222} prime and MVA.HIVA boost induced higher frequencies of H-specific CD8$^+$ splenocytes producing IFN-*γ* (Figure 22,b) and TNF-*α* compared with the BCG wild-type priming and MVA.HIVA boost in two analyses performed. Overall, the proportions of HIV-1-specific T cells producing IFN-*γ* and TNF-*α* were higher in adult mice compared with newborn mice. When adult mice were vaccinated intradermally, the BCGwt priming elicited 1.45% of CD8$^+$ T cells producing IFN-*γ*, in comparison with 2.18% when the priming was performed with BCG.HIVA^{222}. We have detected the same pattern but lower magnitude when adult mice were immunized subcutaneously, 0,69% and 1,02%, respectively. When the newborn mice were primed with BCGwt or BCG.HIVA^{222} subcutaneously, the results were 0.37% and 0.59%, respectively ($P < .05$). The frequency of specific CD8$^+$ splenocytes producing IF-*γ* was twofold higher ($P < .01$) when adult mice were primed with BCG.HIVA^{222} intradermally compared with subcutaneously and nearly 4-fold higher when compared with newborn mice ($P < .001$) (Figure 22,b).

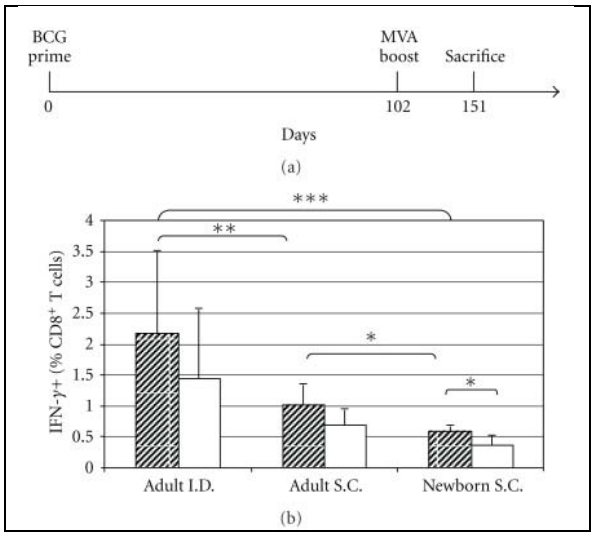

Figure 22: Effect of BCG.HIVA^{222} priming on the induction of HIV-1-specific CD8$^+$ T cells. (a) Mice were immunized with 2 × 10^6 cfu (adult mice I.D. or neonates S.C.) or 10^6 cfu (adult mice S.C.) of BCG.HIVA^{222} and subsequently boosted 14 weeks later with 10^6 pfu of MVA.HIVA by i.m. route. (b) Analysis of IFN-*γ* vaccine-elicited CD8$^+$ T cells as generated for each vaccination group by using the P18I10 epitope. The frequencies of CD8$^+$ T cells producing IFN-*γ* are shown. Data are presented as means ± standard deviation (SD; $n = 6$). BCG.HIVA^{222} primed mice: striped bars. BCGwt primed mice: white solid bars. * = $P < .05$; ** = $P < .01$, *** = $P < .001$.

The capacity of splenocytes from vaccinated mice to secrete IFN-*γ* was tested also by ELISPOT assays. The splenocytes secreted IFN-*γ* after overnight stimulation with the dominant CD8$^+$ T-cell P18I10 epitope peptide. In adult and newborn mice the frequency of specific cells secreting IFN-*γ* was higher in mice primed with BCG.HIVA^{222} (455 and 367 spot forming units, (sfu)/10^6 splenocytes for adult and newborn mice, respectively) compared with BCG wild-

type (329 and 303 sfu/10^6 splenocytes for adult and newborn mice, resp.). Among adult mice, the difference was significant ($P < .05$) (not shown).

We have evaluated the influence of route inoculation and mice age on the level and quality of $CD8^+$ T-cell responses induced after mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The quality of vaccine-elicited $CD8^+$ T cells was monitored by bifunctional analysis and *in vitro* killing assay for CTL activity. We found that the magnitude of the bifunctional response was higher when the I.D. route was used for BCG.HIVA^{222} priming in adult mice compared with S.C. route in adult mice ($P < .01$), and S.C. route in newborn mice ($P < .01$)(data not shown).

The cytotoxic activity of the BCG.HIVA^{222}-MVA.HIVA-elicited $CD8^+$ T cells was also assessed by in vitro killing assay. The frequency of specific killing was clearly higher in adult mice primed with BCG.HIVA^{222} I.D. and Newborn mice primed with BCG.HIVA^{222} S.C. in comparison with adult mice inoculated S.C. (Figure 23)

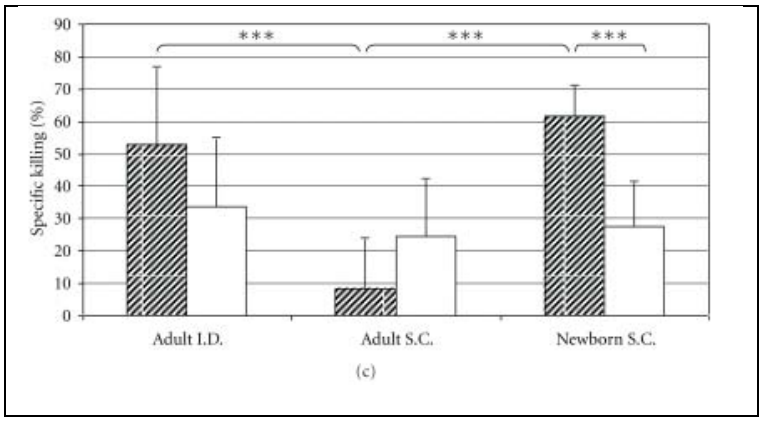

Figure 23: *In vitro* analysis of the CTL activity using peptide-pulsed target cells. BCG.HIVA^{222} primed mice: striped bars. BCGwt primed mice: white solid bars.

The BCG-specific immune responses were assessed by IFN-γ ELISPOT. The frequencies of specific cells secreting IFN-*γ* was higher in newborn mice than in adult mice. On the other hand, in newborn mice, the proportion of specific cells secreting IFN-*γ* was identical after BCG.HIVA^{222} priming compared with BCG wild-type priming. However, in adult mice, the proportion of specific cells secreting IFN-*γ* was lower after BCG.HIVA^{222} priming compared with BCG wild-type priming (Figure 24).

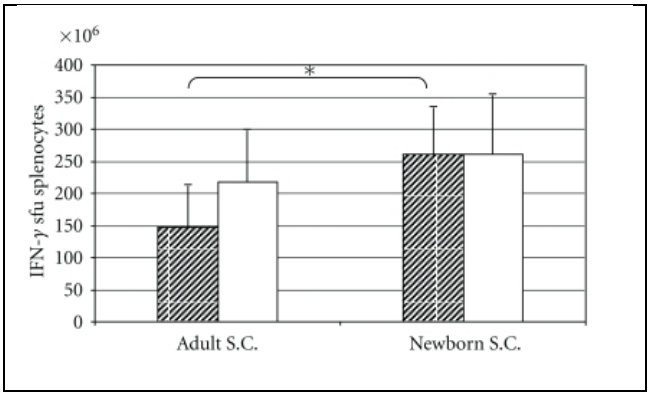

FIGURE 24: PPD-specific T-cell responses elicited by BCG.HIVA[222]. Immune responses to BCG were assessed in an *ex vivo* IFN-γ ELISPOT assay using PPD as the antigen. The mean (± SEM) sfu per 10^6 splenocytes for each group of mice (n = 6 per group) is shown. BCG.HIVA[222] primed mice: striped bars. BCGwt primed mice: white solid bars. * = $P < .05$.

BCG.HIVA^{CAT} prime and MVA.HIVA boost regimen elicited HIV-1-specific CD8$^+$ and PPD-specific T-cell responses in mice.

We have evaluated the specific HIV-1 T-cell immune responses in adult and newborn BALB/c mice after immunization with BCG.HIVA^{CAT} prime and MVA.HIVA or MVA.HIVA.85A 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 HIVA immunogen to evaluate the immunogenity in mice On day 0, adult mice were immunized with rBCG with the episomal plasmid, and on week 12 the animals received a booster dose with MVA.HIVA.85A On week 14, the mice were sacrificed and the functional quality of the elicited CD8$^+$ T-cells to produce IFN-γ, TNF-α, IL-2 and to degranulate (surface expression of CD107a) in response to P18-I10 peptide stimulation was measured by intracellular cytokine staining (ICS). At the higher dose, BCG.HIVA^{CAT} alone and in combination with MVA.HIVA.85A induced HIV-1-specific CD8+ T-cells, producing IFN-γ, TNF-α, and CD107a. For TNF-α, and CD107a, there was a trend of increased responses following MVA.HIVA.85A boost if these were primed by the BCG.HIVA^{CAT} vaccine.(results not shown).

In another animal experiment, on day 0, adult and newborn mice were immunized with rBCG, and on week 14 the animals received a booster dose with MVA.HIVA (Figure 25, a). We have observed in adult mice that BCG.HIVA^{CAT} prime and MVA.HIVA boost induced higher frequencies of P18-I10 epitope specific CD8$^+$ splenocytes producing IFN-γ than newborn and naïve mice ($p<0.05$) (Figure 25,b). These data are in concordance with our previously published results in which the proportions of HIV-1 specific T-cells producing IFN-γ and TNF-α were higher in adult mice compared with newborn mice

[Saubi, 2011]. Moreover, the magnitude of the bifunctional response was also lower in newborn mice than in adult mice.

Regarding mycobacterial immunogenicity, BCG.HIVA^{CAT} elicited PPD-specific responses in mice. The frequencies of specific cells secreting IFN-γ was higher in adult mice than in newborn and naïve mice.

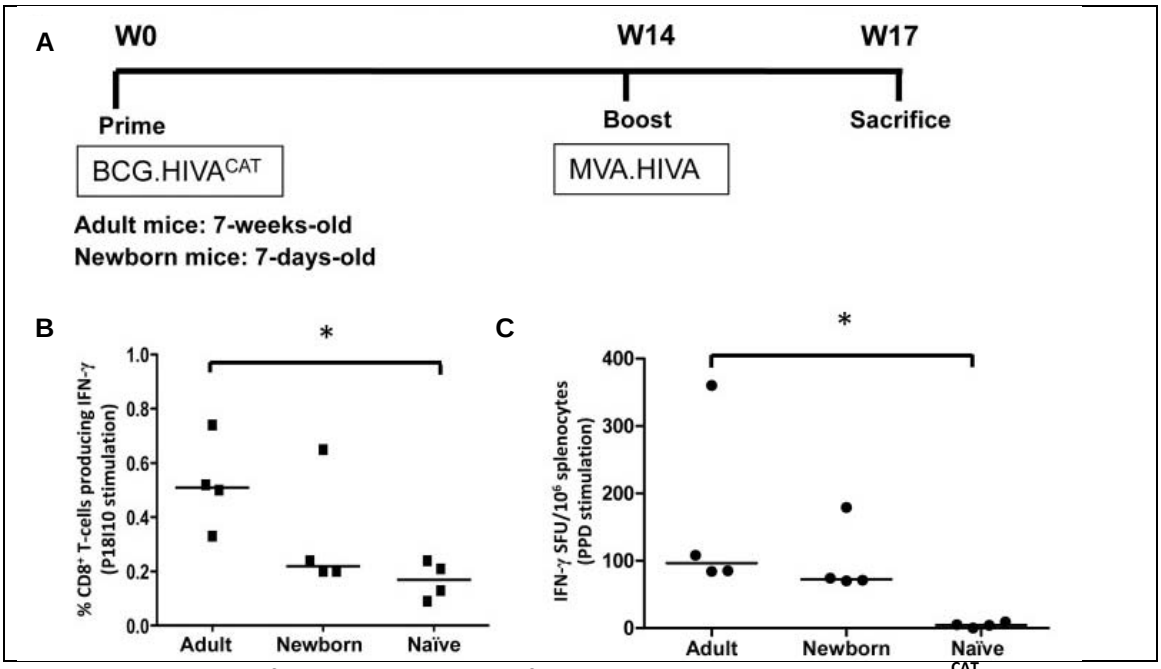

Figure 25: Induction of HIV-1- and *Mtb*-specific T-cells responses by the BCG.HIVA^{CAT} prime - MVA. HIVA boost regimen. (**A**) Adult and newborn mice (7-days-old) were either left unimmunized or immunized with 2×10^6 cfu of BCG.HIVA^{CAT} (intradermal and subcutaneous route respectively) and subsequently given a booster dose of 10^6 pfu of MVA.HIVA (intramuscularly) at 14 weeks post BCG immunization, and sacrificed 3 weeks later. (**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 group medians as well as individual animal responses (n = 4). (**C**) PPD-specific T-cell responses elicited by BCG.HIVA^{CAT}. Immune responses to BCG were assessed in an *ex vivo* IFN-γ ELISPOT assay using PPD as the antigen. The median spot-forming units (SFU) per 10^6 splenocytes for each group of mice (n = 4) as well as individual animal responses is shown. * = p<0.05.

BCG.HIVA^{2auxo} prime and MVA.HIVA boost regimen elicited HIV-1-specific CD8$^+$ and PPD-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. 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. 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 (data not shown). 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 26,a). Further experiments assessing different doses, routes, and immunization schedules should be performed.

BCG.HIVA^{2auxo} elicited PPD–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 10^6 splenocytes were similar in mice primed with BCG.HIVA^{2auxo} or BCG wild type.(Figure 26).

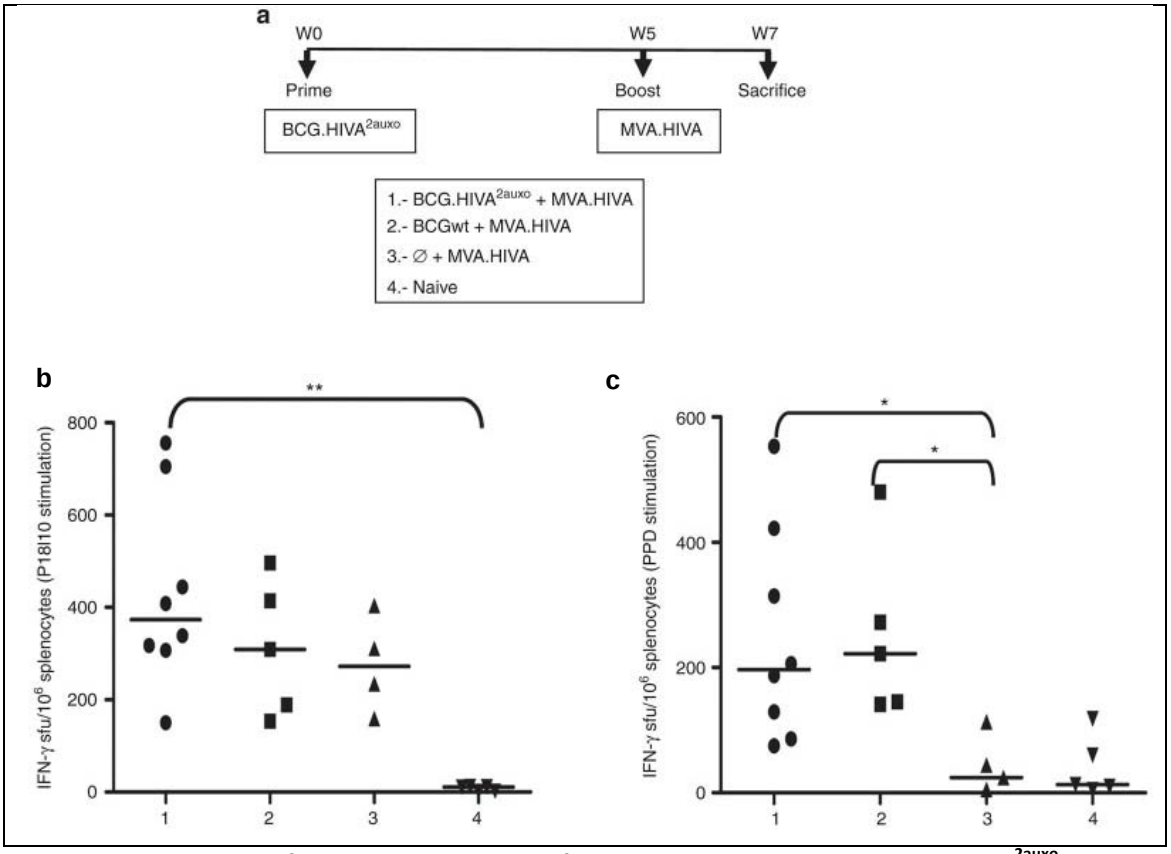

Figure 26: Induction of HIV-1- and *Mtb*-specific T-cells responses by the BCG.HIVA^{2auxo} prime–MVA.HIVA boost regimen. (**a**) Adult mice (7-weeks-old) were either left unimmunized or primed with 10^6 cfu of BCG.HIVA^{2auxo} or BCG wild type (intradermally) and boosted with 10^6 pfu of MVA.HIVA (intramuscularly) 5-weeks post-BCG inoculation. Mice were sacrificed 2 weeks later for T-cell analysis.

(**b**) Elicitation of specific 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 = 8 for group 1, and n = 5 for groups 2, 3, and 4) as well as individual animal responses is shown. (**c**) 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 10^6 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.

We have evaluated the influence of age and immunization routes for induction of HIV-1 and *M. tuberculosis*-specific immune responses after BALB/c mice immunization with $BCG.HIVA^{222}$ prime and MVA.HIVA boost. We have observed (i) enhanced specific T-cell induction responses in adult and newborn mice by using $BCG.HIVA^{222}$ priming compared with BCG wild-type priming; (ii) higher frequencies and quality of the specific T-cell responses in adult mice immunized with $BCG.HIVA^{222}$ intradermally compared with subcutaneously; (iii) that the BCG-specific immune responses were higher in newborn mice than adult mice; (iv) that among adult mice the BCG-specific immune responses were lower in mice primed with $BCG.HIVA^{222}$ than BCG wild type. There is evident data showing that rBCG is a good priming vector in heterologous prime-boost vaccination regimens with attenuated virus or recombinant proteins to enhance specific T-cell responses [Im EJ, 2007; Cayabyab MJ, 2009; McShane H, 2004; Vordermeier HM, 2009]. Many studies have compared the immune responses to foreign antigens delivered by rBCG inoculated by different routes; however, comparisons are difficult as doses, BCG strains, mycobacterial expression vectors, *in vivo* plasmid stability, promoters to regulate gene expression, levels of heterologous protein expression, and antigen localization are different. In the current study, $BCG.HIVA^{222}$ prime- and MVA.HIVA boost-elicited HIV-1-specific $CD8^+$ T-cells exhibited effector functions such as production of IFN-γ and TNF-α, and such HIV-1-specific T-cell responses were higher in adult than in newborn mice. The inclusion of $BCG.HIVA^{222}$ in a heterologous prime-boost regimen consistently enhanced and improved the frequency, quality, and durability of the generated HIV-1-specific responses in adult and newborn mice. This improvement was observed by the detection of the highest bifunctional HIVA-specific T-cell responses and higher specific cytolytic activity in the mice that received $BCG.HIVA^{222}$ versus BCG wild type.

Among adult mice, the intradermal inoculation of BCG.HIVA^{222} induced higher frequencies and quality of the specific HIV-1 immune responses versus the subcutaneous route. These results would be in accordance with the current recommended route of inoculation of BCG in infants.

There are really few reports in the literature describing the safety and immunogenicity of rBCG expressing HIV antigens in neonatal mice and neonatal nonhuman primates. Ranganathan *et al*. [Ranganathan UDK, 2009] have evaluated the immunogenicity in neonatal mice of three different recombinant attenuated Mtb. strains expressing an HIV envelope. They showed that single-dose immunization in neonatal mice with ΔlysA ΔsecA2 Mtb strain expressing HIV Env rapidly generated HIV-1- and Mtb-specific T-cell immune responses. In the present study, we have shown in newborn mice that BCG.HIVA^{222} prime and MVA.HIVA boost increased the frequencies of specific CD8^{+} T cells producing IFN-*γ* or TNF-*α*. Such vaccine regimen also induced the highest proportion of HIV-1-specific bifunctional cells and the specific cytolytic activity. We have observed in newborn mice a lower level of HIV-1-specific T-cell immune responses compared with adult mice. Rosario et al. [Rosario M, 2010] have assessed the immunogenicity of the BCG.HIVA^{222} prime and MVA.HIVA boost regimen in newborn *Rhesus macaques*. They also observed that the HIV-1-specific responses induced in infants were lower compared with adult animals.

Here, the vaccination with BCG wild-type and BCG.HIVA^{222} strains induced strong BCG-specific responses in adult and newborn mice. Studies in neonatal mice have indicated that immune responses at birth are often biased towards the Th2 type and defective in the Th1 type, the central defense mechanism against intracellular pathogens. However, it has been described that BCG vaccination induces a potent Th1-type immune response at birth in humans and in mice [Ota MOC, 2002; Barrios C, 1996; Marchant A, 1999; Vekemans J, 2001].

Our group and others have shown in murine and non-human primates studies, that rBCG elicited cell-mediated responses against HIV-1 and simian immunodeficiency virus antigens [Aldovini A, 1999; Saubi N, 2011; Chege GK, 2009; Cayabyab MJ, 2006; Honda, M, 1995]. However, a small proportion of

these animal studies used rBCG strains in heterologous prime-boost regimens. Ami *et al.* [Ami A, 2005] 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 with attenuated virus or recombinant proteins to enhance specific T-cell responses [Im EJ, 2007; Cayabyab MJ, 2009; Vordermeier HM, 2009]. These data are consistent with the specific HIV-1 specific immune responses detected in this study after newborn and adult mice immunization with BCG.HIVA^{CAT} prime and MVA.HIVA boost, observing higher HIV-1 specific T-cell responses in adult than in newborn mice. In addition, in adult mice, BCG.HIVA^{CAT} primed and enhanced the MVA.HIVA.85A-elicited HIV-1-specific $CD8^+$ T-cell responses. In the present 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.*[Rosario M, 2010] have assessed the immunogenicity of the BCG.HIVA^{222} prime and MVA.HIVA boost regimen in newborn rhesus macaques and made similar observation.

We have shown that the BCG.HIVA^{2auxo} vaccine candidate is immunogenic, and that in an heterologous BCG.HIVA^{2auxo} prime MVA.HIVA boost vaccination schedule is able to induce HIV-1 specific cellular immune responses. In this paper we also described that the vaccination with BCG wild type and BCG.HIVA^{2auxo} strain induced BCG-specific responses in adult mice, of the same magnitude than that elicited by previous vaccine models. Regarding the HIV-1 specific cellular immune response, the difference between the results obtained with BCG.HIVA^{222} and BCG.HIVA^{CAT} cannot be fully explained. The modifications performed in the *E.coli*-mycobacterial shuttle plasmid don't affect the heterologous protein expression cassette (α-antigen promoter, 19kD signal sequence and the DNA sequence expressing the heterologous protein).

Safety of the BCG.HIVA Prime and MVA.HIVA Boost in Mice

BCG.HIVA^{222} Prime and MVA.HIVA Boost was well tolerated in newborn mice

Six newborn mice (7 days old) per group were either immunized or left unimmunized with 2 × 10^6 cfu of BCG wild type or BCG.HIVA^{222} subcutaneously route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA via intramuscular. As shown in Figure 27, the body weight was weekly monitored and recorded. All vaccine combinations were analyzed, to depict any possible adverse event due to vaccination and monitored by body weight lost. For rigorous safety assessment, the dose of BCGwt and BCG.HIVA^{222} inoculated to newborn mice (2×10^6 cfu) was 10-fold higher, as advised by the European Pharmacopoeia for the safety testing of live vaccines, in comparison with the most usual inoculation dose in adult mice [*Eur. Pharm,* 2010]. No differences were observed between the vaccinated mice groups and the naïve mice group.

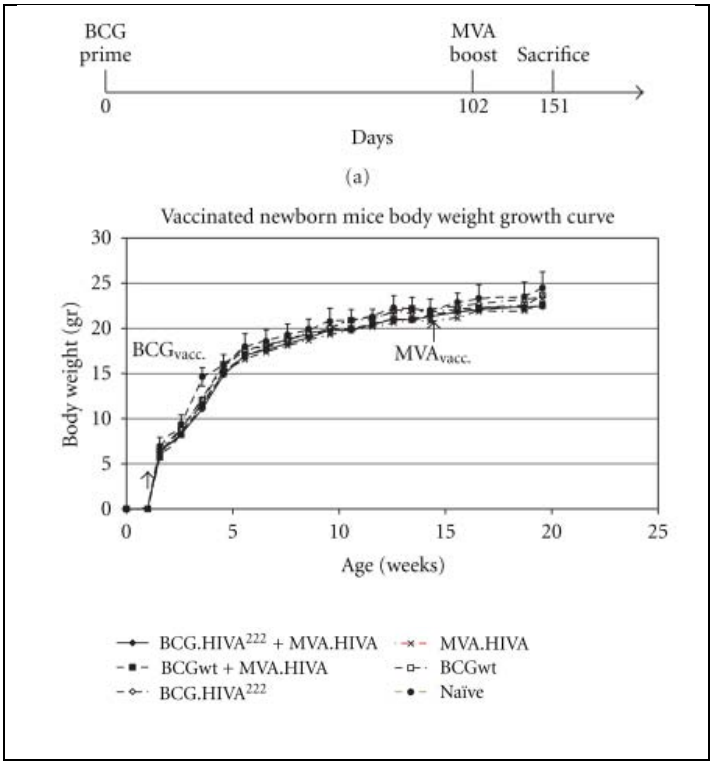

Figure 27: Body weight curve after newborn mice vaccination with BCG.HIVA^{222} (SC) and MVA.HIVA (IM). (a) Newborn mice were either left immunized or immunized with 2 × 10^6 cfu of BCG wild type or BCG.HIVA^{222} by subcutaneous route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA The body weight was weekly recorded, and the body weight mean (± SEM) for each group of mice (*n* = 6 per group) is shown.

BCG.HIVA^{CAT} prime and MVA.HIVA boost was well tolerated in newborn mice

Ten newborn mice (7-days-old) per group were either immunized or left unimmunized with 2×10^6 cfu of BCG wild type, BCG.HIVA^{222} or BCG.HIVA^{CAT} via subcutaneous route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA via intramuscular. As shown in Figure 28, the body mass was weekly monitored and recorded. All vaccine combinations were analyzed, to depict any possible adverse events due to vaccination and monitored by body mass loss. No statistically significant difference was observed between the vaccinated mice groups and the naïve mice group at specific time points, corresponding to BCG inoculation, 2 months after BCG inoculation, pre-MVA boosting and three weeks post MVA-boosting. On the other hand, the body mass profile was similar in all mice groups and similar to mice provider company standard body mass curve (www.Harlan.com). Furthermore, between week 0 and week 14, the body mass monitored in all vaccinated mice groups was found between the mean ± 2 standard deviations (SD) body mass curve in naïve mice. It is also important to mention that no mice died during the trial, no local adverse events, and no associated systemic reactions were observed.

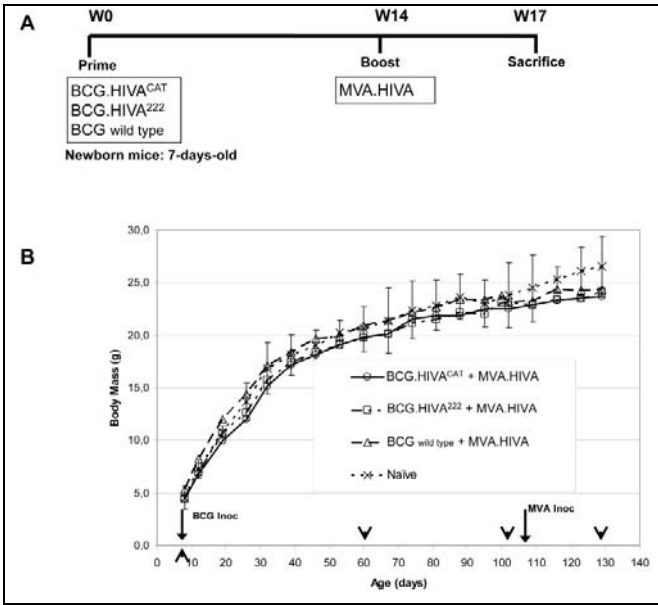

Figure 28: BCG.HIVA^{CAT} prime and MVA.HIVA boost safety in newborn mice.

(**A**) Newborn mice were either left unimmunized or immunized with 2×10^6 cfu of BCG wild type, BCG:HIVA^{222} or BCG.HIVA^{CAT} by subcutaneous route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA at week 14. (**B**) The body weight was weekly recorded, and the mean for each group of mice is shown (n = 10). Data from naive mice are presented as mean ± 2 SEM (n = 6); At specific time points the weight differences between vaccinated and naïve mice group were analyzed by ANOVA test (arrowheads).

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

As shown in the body mass of mice vaccinated with BCG.HIVA^{2auxo} and boosted with MVA.HIVA 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 (despite the immunogenicity was assessed at 7 weeks post vaccination). 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 ± 2 SD body mass curve in naive mice. It is also important to mention that no mice died during the trial, and no local adverse events and associated systemic reactions were observed.

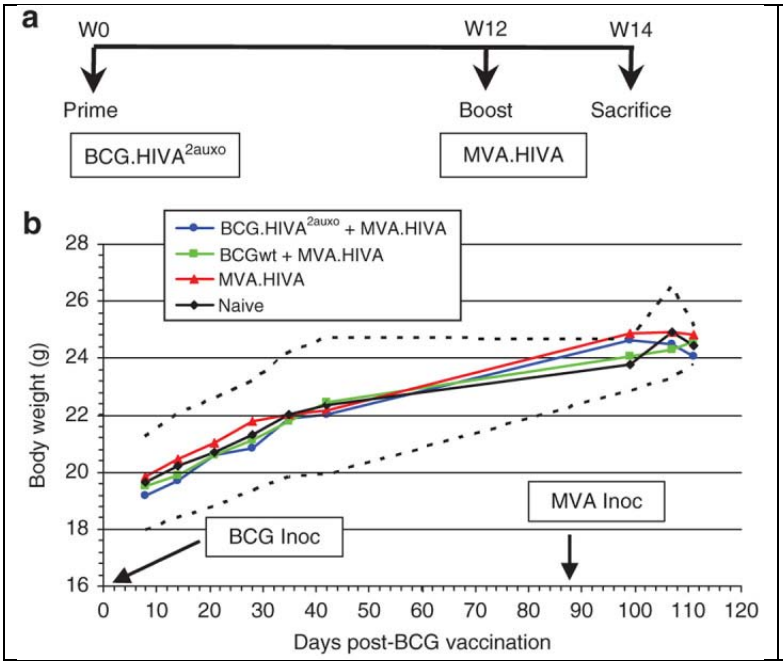

Figure 29: BCG.HIVA^{2auxo} prime and MVA.HIVA boost safety in adult mice. (**a**) Adult mice were either left unimmunized or immunized with 10^6 cfu of BCG wild type or BCG.HIVA^{2auxo} by intradermal route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA at week 12 by intramuscular route. (**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 ± 2 SD (*n* = 5, dashed lines).

In all three papers, the safety of the recombinant BCG expressing HIVA using diferent *E. coli*-mycobacterial shuttle plasmids (BCG.HIVA^{222}, BCG.HIVA^{CAT} and BCG.HIVA^{2auxo}) has been assessed, in newborn mice (BCG.HIVA^{222}, BCG.HIVA^{CAT}) and in adult mice (BCG.HIVA^{2auxo}). All vaccination schedules have shown to be well tolerated in BALB/c mice, and no adverse effect nor weight loss were observed.

In conclusion, we constructed and characterized three novel, safer, GLP-compatible BCG-vectored vaccine constructs using prototype immunogen HIVA. We have demonstrated that these three vaccine strains were well tolerated and induced HIV-1 and Mycobacterium-specific T-cell responses after BALB/c mice immunization with BCG.HIVA prime and MVA.HIVA boost. In addition, BCG expressing a second generation immunogens (HIVconsvX [Ondondo B, 2016], HIVACAT T-cell immunogen [Mothe B, 2012] addressing the HIV-1 genetic variability and immune escape are under construction. The same strategy can be easily used for other major pediatric pathogens to prime protective responses soon after birth.

CONCLUSIONS:

1. BCG.HIVA^{222} administered to adult mice by the intradermal route induced higher frequencies and quality of the specific HIV-1 T-cell responses compared with the subcutaneous route.
2. BCG.HIVA^{222} was less immunogenic for T cells in newborn mice than when administered to adult animals.
3. BCG.HIVA^{CAT} has been constructed and genetically and phenotypically characterized.
4. BCG.HIVA^{CAT} expressed the HIVA immunogen, and was stable *in vivo* for 20 weeks.
5. BCG.HIVA^{CAT} induced HIV-1-specific T-cell responses in adult and newborn mice.
6. BCG.HIVA^{CAT} induced BCG-specific T-cell responses in adult and newborn mice.
7. The new *E. coli*–mycobacteria shuttle vector p2auxo based on double auxotrophic complementation and antibiotic-free plasmid selection system has been constructed, and is a new and improved methodological tool for mycobacterial vaccines design and development as a bacterial live recombinant vaccine vehicle.
8. BCG.HIVA^{2auxo} expressed the HIVA immunogen, and was stable *in vitro* for 30 bacterial generations.
9. BCG.HIVA^{2auxo} induced HIV-1-specific responses in adult mice.
10. BCG.HIVA^{2auxo} induced BCG-specific responses in adult mice.
11. The BCG.HIVA prime (BCG.HIVA^{222}, BCG.HIVA^{CAT} or BCG.HIVA^{2auxo} using and MVA.HIVA boost regimen was well tolerated in adult and newborn mice.

The research work of this PhD thesis aims to scale up recombinant BCG based HIV vaccine development. Our main goal is to develop a mycobacterial vaccine design for HIV-TB pediatric vaccine. In this thesis, we have engineered a safe, 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. Thus, the implementation of this research work will contribute to join the global effort to control HIV and TB pandemic.

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ANNEXES

ANNEX I: Paper Number 1

Saubi N, Im EJ, Fernández-Lloris R, Gil O, Cardona PJ, Gatell JM, Hanke T, Joseph J. Newborn mice vaccination with BCG.HIVA^{222} + MVA.HIVA enhances HIV-1-specific immune responses: influence of age and immunization routes. Clin Dev Immunol. 2011;2011:516219. doi: 10.1155/2011/516219. Epub 2011 Apr 12.

Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2011, Article ID 516219, 11 pages
doi:10.1155/2011/516219

Research Article

Newborn Mice Vaccination with BCG.HIVA^{222} + MVA.HIVA Enhances HIV-1-Specific Immune Responses: Influence of Age and Immunization Routes

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Received 15 September 2010; Accepted 1 February 2011

Academic Editor: Eiji Matsuura

We have evaluated the influence of age and immunization routes for induction of HIV-1- and *M. tuberculosis*-specific immune responses after neonatal (7 days old) and adult (7 weeks old) BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The specific HIV-1 cellular immune responses were analyzed in spleen cells. The body weight of the newborn mice was weekly recorded. The frequencies of HIV-specific CD8$^+$ T cells producing IFN-γ were higher in adult mice vaccinated intradermally and lower in adult and newborn mice vaccinated subcutaneously. In all cases the IFN-γ production was significantly higher when mice were primed with BCG.HIVA^{222} compared with BCGwt. When the HIV-specific CTL activity was assessed, the frequencies of specific killing were higher in newborn mice than in adults. The prime-boost vaccination regimen which includes BCG.HIVA^{222} and MVA.HIVA was safe when inoculated to newborn mice. The administration of BCG.HIVA^{222} to newborn mice is safe and immunogenic and increased the HIV-specific responses induced by MVA.HIVA vaccine. It might be a good model for infant HIV and Tuberculosis bivalent vaccine.

1. Introduction

According to the last AIDS epidemic update released by UNAIDS on November 2010, an estimated 33.3 million people are currently living with HIV, and 2.6 million individuals became newly infected with the virus in 2009. Over the past year, the global AIDS epidemic killed 1.8 million people, and the number of children orphaned by AIDS was 16.6 million. It is estimated that 97% of these new infections occur in low- and middle-income countries, where ensuring universal access to antiretrovirals still represents an enormous challenge [1, 2]. In some sub-Saharan countries, the HIV prevalence among pregnant women can be over 30%. Approximately half of mother-to-child transmissions (MTCTs) are due to prolonged breastfeeding. Although around 35% of HIV-positive pregnant women are receiving antiretroviral therapy [1], reducing significantly mother-to-child transmission of HIV at delivery, the drugs have a high cost, have to be administrated after delivery, and maintained during the breastfeeding period, and the efficacy could be reduced due to emergence of resistant mutants.

Neonatal immunity is immature compared to the adult immune system. CD8$^+$ T-cell responses that may be critical to control intracellular pathogens including HIV and Mtb are inherently limited in human neonates. However, human and murine neonates generate functional Th1-type immune responses after infections with viruses or immunization with live-attenuated immunogens that deliver antigens into the cytoplasm of antigen presenting cells (APCs) [3]. Neonatal immunization could be the best approach to prevent infection or reduce the severity of HIV-related disease in these infants. Only two candidates vaccines designed to protect

against breast milk HIV transmission have been studied in human infants. Therefore, there is an urgent need for a neonatal immunogen that generates HIV-specific immunity more rapidly. Recombinant BCG has been developed as a candidate neonatal vaccine vector against pertussis, measles, respiratory syncytial virus (RSV), and breast milk HIV transmission [3, 4].

BCG as a vaccine vector has a number of attractive features [5, 6]. BCG has a proven record of safety as a vaccine against tuberculosis from its use in over two billion individuals including neonates [7]. BCG infects and colonizes macrophages and dendritic cells, where it can survive and replicate for a long period of time. Through its persistence and potent adjuvantation by its cell wall components, it can induce long-lasting humoral and cellular immune responses. BCG can be given at or any time after birth and is not affected by maternal antibodies. Manufacturing of BCG-based vaccines is cheap and easy to purify. Finally, BCG is one of the most heat-stable vaccines in current use [8].

There is strong evidence supporting a role of cytotoxic T-lymphocytes (CTLs) in the containment of HIV replication, and several vaccine approaches are being pursued to elicit anti-HIV CTL responses [9–12]. One promising approach is that provided by *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) as a live recombinant bacterial vaccine vector.

We have been developing a recombinant BCG-based HIV vaccine to induce protective cell-mediated responses. Our starting platform was based on a heterologous BCG prime and modified vaccinia virus Ankara (MVA) boost regimen delivering a common immunogen called HIVA, which is derived from consensus HIV-1 clade A Gag protein, that is, an immunogen derived from an HIV-1 strain prevalent in Central and Eastern Africa, and a string of $CD8^+$ T-cell epitopes [13]. The recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) expressing HIVA immunogen has shown to be stable and to induce durable, high-quality HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses in BALB/c mice. Furthermore, when the recombinant BCG vaccine was used in a priming-boosting regimen with heterologous components, the HIV-1-specific responses provided protection against surrogate virus challenge, and the recombinant BCG vaccine alone protected against aerosol challenge with *M. tuberculosis* [14]. The BCG.HIVA^{222} vaccine candidate was vectored by a lysine auxotroph of BCG Pasteur strain that carried an *E. coli*-mycobacterial shuttle plasmid with a lysine A complementing gene and a weak promoter to regulate HIVA gene expression. This construction increases the plasmid stability *in vivo* and prevents heterologous gene expression disruption by genetic rearrangement [14, 15].

Infection with *Mycobacterium tuberculosis* kills about 2 million people each year [16]. *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is the only licensed vaccine and protects significantly against childhood and miliary tuberculosis [17]. Globally, 80 percent of children are vaccinated with BCG, the majority of them at birth [18]. Thus, the development of a combined vaccine, which would protect neonates against tuberculosis and MTCT of HIV-1 through breastfeeding, is a logical effort in the fight against these two major global killers. Immunity against tuberculosis following neonatal BCG vaccination lasts 10 to 15 years and thus fails to protect adults from pulmonary disease [19, 20]. However, a 10-year immunity to HIV-1 would be an excellent start.

We believe that the best hope to protect newborn children against MTCT of HIV-1 in developing countries is the development of a safe, effective, and affordable prophylactic vaccine, which would both reduce the adult burden of infection and protect neonates against vertical HIV-1 transmission. In the present study, we have evaluated the influence of age and immunizations routes for induction of HIV-1 and *M. tuberculosis*-specific immune responses after BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The administration of BCG.HIVA^{222} to newborn mice is safe and immunogenic and increased the HIV-specific responses induced by MVA.HIVA vaccine. It might be a good model for infant HIV and Tuberculosis bivalent vaccine.

2. Materials and Methods

2.1. E. coli/mycobacterial Vector Expressing HIVA Antigen. The construction of *E. coli*/mycobacterial vector expressing HIVA antigen [13] was previously described [14]. Briefly, the coding sequence of the HIVA immunogen (consensus HIV-1 clade A Gag protein and a string of $CD8^+$ T-cell epitopes) was fused to the *M. tuberculosis* nucleotides coding for the 19-kDa lipoprotein signal sequence in a PCR, and the chimeric gene was cloned into the pJH222 *E. coli*-mycobacterial shuttle plasmid (kindly provided by W. R. Jacobs Jr. and B. R. Bloom) as a HindIII-HindIII fragment under the control of the *M. tuberculosis* α-antigen promoter by using standard recombinant-DNA techniques.

2.2. Electroporation of Mycobacteria and Culture. A lysine auxotrophic strain of BCG was kindly provided by W. R. Jacobs Jr., B. R. Bloom, and T. Hsu. Mycobacterial cultures were grown in Middlebrook 7H9 broth medium or on Middlebrook agar 7H10 medium supplemented with Middlebrook ADC (albumin, dextrose, catalase) Enrichment (Difco) and containing 0.05% Tween 80 and 25 μg of kanamycin/mL. The L-lysine monohydrochloride (Sigma) was dissolved in distilled water and used at a concentration of 40 μg/mL. The plasmid pJH222HIVA was transformed into BCG lysine auxotrophic strains by electroporation. Cultures were grown to an OD of 0.9 (600 nm), pelleted at 3,000 rpm, washed twice by resuspension and centrifugation (3,000 rpm) in 10% glycerol at 4°C, and finally resuspended in 1/20th of the original culture volume of cold 10% glycerol. Then 100 μL of the cold BCG suspension was mixed with plasmid DNA (50–500 ng) in a prechilled 0.2 cm electroporation cuvette and transformed using the BioRad Gene Pulser electroporator at 2.5 kV, 25 mF, and 1,000 Ω. After electroporation 1 mL of 7H9 medium (Difco), supplemented with albumin-dextrose-catalase (ADC, Difco) and containing 0.05% Tween 80 (SIGMA), was added and incubated at 37°C for 12 hours before plating onto Middlebrook agar 7H10 medium (Difco) supplemented with ADC (Difco) and containing 0.05% Tween 80 (SIGMA) and 25 μg/mL of kanamycin.

2.3. Western Blot Analysis. BCG transformants were grown to mid-logarithmic phase in liquid 7H9 (Difco) medium supplemented with albumin-dextrose-catalase (ADC, Difco) and containing 0.05% Tween 80 and kanamycin (25 μg/mL). rBCG cultures were centrifuged at 3,000 rpm for 10 minutes at 4°C. Pellets were washed twice in PBS plus 0.02% Tween 80 and resuspended in 1 mL of extraction buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.6% sodium dodecyl sulfate), and 5 μL of 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 DMSO) was added. Cells were sonicated for 4 minutes on ice with a Branson Sonifier at output control seven, duty cycle 50%. Extracts were centrifuged at 13,000 rpm for 10 minutes at 4°C, and supernatants were collected. Proteins were separated on 15% SDS-polyacrylamide gel. HIVA protein was detected using anti-Pk antibodies with an ECL kit (Amersham International).

2.4. Mice. Six BALB/c female mice per group, 7 weeks old (adult) and 7 days old (newborn), were used. The animal experiment was approved by the local ethical committee for animal experiments from the University of Barcelona and strictly conformed to Catalan Animal Welfare legislation.

2.5. Immunization and Isolation of Splenocytes. Adult mice were immunized intradermally with 2×10^6 CFU of BCG.HIVA^{222} or subcutaneously with 10^6 CFU of BCG.HIVA^{222}. Newborn mice were immunized subcutaneously with 2×10^6 CFU of BCG.HIVA^{222}. Adult and newborn mice were boosted 14 weeks after BCG inoculation with 10^6 PFU of MVA.HIVA intramuscularly, and 3 weeks later the animals were sacrificed. The animals were sacrificed by cervical dislocation. The newborn mice were weighted weekly for the safety testing of the vaccine. On the day of sacrifice, spleens were removed and pressed individually through a cell strainer (Falcon) with a 5 mL syringe rubber plunger. Following the removal of red blood cells with red blood cell lysing buffer (Sigma), splenocytes were washed and resuspended in lymphocyte medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin, 20 mM HEPES, and 15 mM β-mercaptoethanol) at a concentration of 2×10^7 cells/mL.

2.6. Peptides. For assessing the immunogenicity of HIVA in the BALB/c mice, the following peptides were used: H-2D^d-restricted epitope P18-I10 (RGPGRAFVTI), herein designated epitope H. The PPD (Purified Protein Derivative, Statens Serum Institute, Copenhagen) was used to assess the immunogenicity induced by *Mycobacterium bovis* BCG.

2.7. In Vitro Killing Assay. P815 cells (mouse lymphoblast-like mastocytoma cell line) were used as target cells. They were incubated (pulsed) without or with 2 μg of H peptide/mL in R10 at 37°C in 5% CO_2 for 90 min and washed three times. Target cells not pulsed with peptide were labeled with 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethyl rhodamine (CMTMR; Molecular Probes) only, while peptide-pulsed target cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular probes). Briefly, H peptide-pulsed P815 cells resuspended in phosphate-buffered saline (PBS) at 2×10^7 cells/mL were incubated with 80 nM CFSE in the dark at room temperature for 10 min, followed by the quenching of the reaction with an equal volume of FCS and three washing steps with R10. H peptide-pulsed cells (and similarly control nonpulsed cells) were then resuspended in R10 at 2×10^7 cells/mL and incubated with 10 μM CMTMR at 37°C for 15 min and in fresh R10 only for a further 15 min. Cells were washed 3 times as previously described. Finally, pulsed and non pulsed P815 cells were mixed at 1 : 1 ratio. Splenocytes obtained from vaccinated mice and expanded for 5 days in lymphocyte medium containing 2 μg/mL of H peptide were harvested and counted. In a 96-well round bottom plate, 200 μL of a suspension containing 10^6 expanded splenocytes were placed in duplicate wells, and two 2-fold dilutions in R10 were performed. On top, 100 μL of a suspension contained the mixture of pulsed and unpulsed P815 cells, 10^4 of each (at 100 : 1, 50 : 1, and 25 : 1 effector : target cells ratio). After a minimum of 5 h of reaction, the cells were fixed and analyzed by flow cytometry. Cytotoxicity was calculated using the following formula: adjusted percentage of surviving cells = 100 × (percentage of surviving peptide-pulsed cells/mean percentage of surviving unpulsed cells). Next, the percentage of specific lysis was calculated as follows: percentage of specific lysis = 100 − adjusted percentage of surviving cells [21]. The specific CTL activity was assessed between the BCGwt or BCG.HIVA^{222} primed mice.

2.8. Intracellular Cytokine Staining. Two million splenocytes were added to each well of a 96-well round-bottomed plate (Falcon) and pulsed with 2 (CD8 epitopes) to 5 μg/mL (CD4 epitopes) peptides or 5 μg/mL PPD tuberculin (Statens Serum Institut, Copenhagen, Denmark) together with antibodies against lysosomal-associated membrane proteins anti-CD107a-FITC/anti-CD107b-FITC (BD Biosciences) [22] and kept at 37°C, 5% CO_2 for 90 minutes, followed by the addition of GolgiStop (BD Biosciences) containing monensin. After a further 5-hour incubation, reaction was terminated, and the cells were washed with FACS wash buffer (PBS, 2% FCS, 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 or anti-CD4-PerCP (BD Biosciences), 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-IL-2-FITC, anti-TNF-α-PE, and anti-IFN-γ-APC (BD Biosciences). Cells were fixed with CellFIX (BD) and stored at 4°C until analysis.

2.9. Ex Vivo IFN-γ ELISPOT Assay. The ELISPOT assay was performed using the Mabtech IFN-γ ELISPOT kit according to the manufacturer's instructions. The ELISPOT plates (Millipore ELISPOT plates) were coated with purified anti-mouse IFN-γ capture monoclonal antibody diluted in PBS to a final concentration of 5 ug/mL at 4°C overnight. The

plates were washed once in R10 and blocked for 2 h with R10. A total of 5×10^5 fresh splenocytes were added to each well, stimulated with 2 μg/mL of the H peptide or 5 μg/mL of PPD for 16 h at 37°C, 5% CO_2, and lysed by incubating twice with deionized water for 5 minutes. Wells were then washed 3x 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 3x in PBS 0.005 Tween 20, and incubated with the Streptavidin-Alkaline Phosphatase-conjugate in PBS 2% FCS. Wells were washed 4x with PBS 0.005 Tween 20 and 2x with PBS before incubating with substrate solution (Alkaline Phosphatase Substrate, BioRad). After 5–10 minutes, the plates were washed with tap water, dried, and the resulting spots counted using an ELISPOT reader (AIC).

2.10. Fluorescence-Activated Cell Sorter Analysis. All chromogen-labeled cells were analyzed in a Becton Dickinson FACScalibur, using the CellQuest software for acquisition (BD Biosciences) and the Flow-Jo software (Tri-Star) for analysis.

2.11. Statistical Analysis. Data are the means ± SEM (standard error or the mean) for six mice per group. Statistical significance was determined by ANOVA (* = $P < .05$; ** = $P < .01$; *** = $P < .001$).

3. Results

3.1. Recombinant Mycobacterium bovis BCG Expressing HIV-1 Clade A Immunogen. HIVA immunogen consists of consensus HIV-1 clade A gag p24/p17 domains coupled with a string of $CD8^+$ T-cell epitopes and monoclonal antibody (mAb) tag Pk [13]. The HIVA gene was synthesized utilizing humanized GC-rich codons, which are similar to those used by mycobacteria [23–25]. The HIVA open-reading frame was fused at its 5′ end to nucleotides coding for the 19-kDa lipoprotein signal sequence, which facilitates expression of foreign proteins in the mycobacterial membrane and was shown to increase the foreign protein immunogenicity [26]. To facilitate the preclinical development of candidate vaccines, the HIVA immunogen contains an immunodominant H-$2D^d$-restricted epitope P18-I10 [27], here designated also as H epitope. In addition, it also contains at least three other subdominant H-$2D^d$ epitopes recognized by $CD8^+$ T cells including epitope P and three $CD4^+$ T-helper epitopes (unpublished). The chimeric 19-kDa signal sequence-HIVA gene was expressed from *Escherichia coli*/mycobacterium shuttle plasmid pJH222 under the control of the *M. tuberculosis* α-antigen promoter (Figure 1(a)). pJH222 is a low-copy replicative episomal vector and contains mycobacterial origin of replication (*oriM*). It contains also an expression cassette encoding kanamycin resistance (*aph*), *E. coli* origin of replication (*oriE*), and a wild-type lysine A-complementing gene for the vector maintenance (*lysA5*) in the BCG auxotroph. Recombinant pJH222.HIVA was transformed into lysine auxotroph of *M. bovis* BCG host strain Pasteur ΔlysA5::res [28]. Expression of the full-size

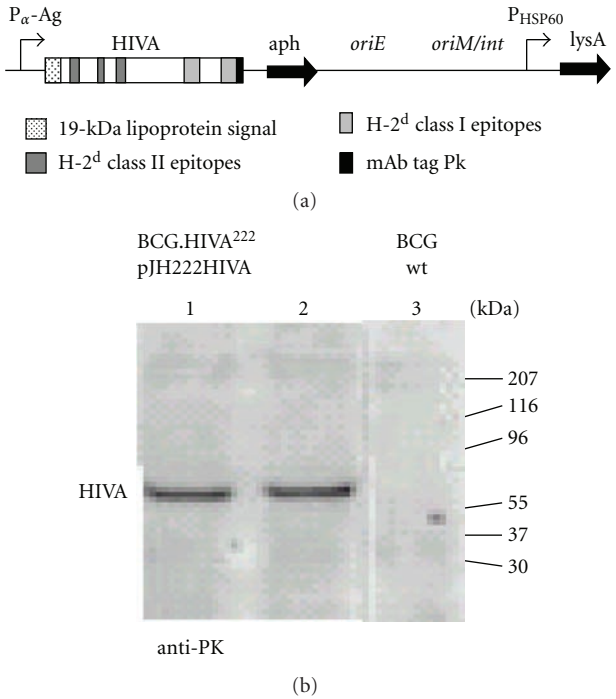

Figure 1: Expression of the HIVA immunogen from BCG.HIVA^{222}. (a) A synthetic GC-rich *HIVA* gene consisting of consensus was fused to the 19-kDA lipoprotein signal sequence and inserted into episomal pJH222 *E. coli*-mycobacterium shuttle plasmid. This contains a kanamycin resistance gene (*aph*) and complementing *lysA* genes and *E. coli* origin of replication (*oriE*). In addition, pJH222 contains mycobacterial origin of replication (oriM). BALB/c mice T-cell and mAb Pk epitopes used in this paper are depicted. Pα-Ag, *M. tuberculosis* α-antigen promoter; PHSP60, heat shock protein 60 gene promoter. (b) Western blotting of lysates of BCG.HIVA^{222} containing the pJH222HIVA (lanes 1 and 2) and BCG wild type (lane 3; negative control) is shown. HIVA was detected using the anti-Pk mAb followed by HRP-protein A and ECL.

chimeric 19-kDa signal sequence-HIVA protein of M_r 65 kDa was confirmed on a Western blot of whole transformed mycobacterial cell lysates using anti-Pk mAb (Figure 1(b)).

3.2. BCG.HIVA^{222} Prime and MVA.HIVA Boost Elicited Func-tional HIV-1-Specific $CD8^+$ T-Cell Responses. We have demonstrated in previous studies with BALB/c mice that BCG.HIVA^{222} can both prime novel and boost preexisting MVA.HIVA-elicited HIV-1-specific $CD4^+$ and $CD8^+$ cellular immune responses of high quality upon antigenic reexposure [14]. In this study we have evaluated the effect of BCG.HIVA^{222} priming using different routes and mice age (adult and newborn) on the induction of HIV-1-specific T-cell responses after BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The immunogenicity readout was focused on the P18I10 epitope, an immunodominant CTL epitope derived from HIV-1 Env and H-$2D^d$ restricted [27], here mentioned as H, which was fused to HIVA immunogen to evaluate the immunogenicity in mice (Figure 1(a)). On day 0, mice were immunized with rBCG with the episomal plasmid or BCG wild type,

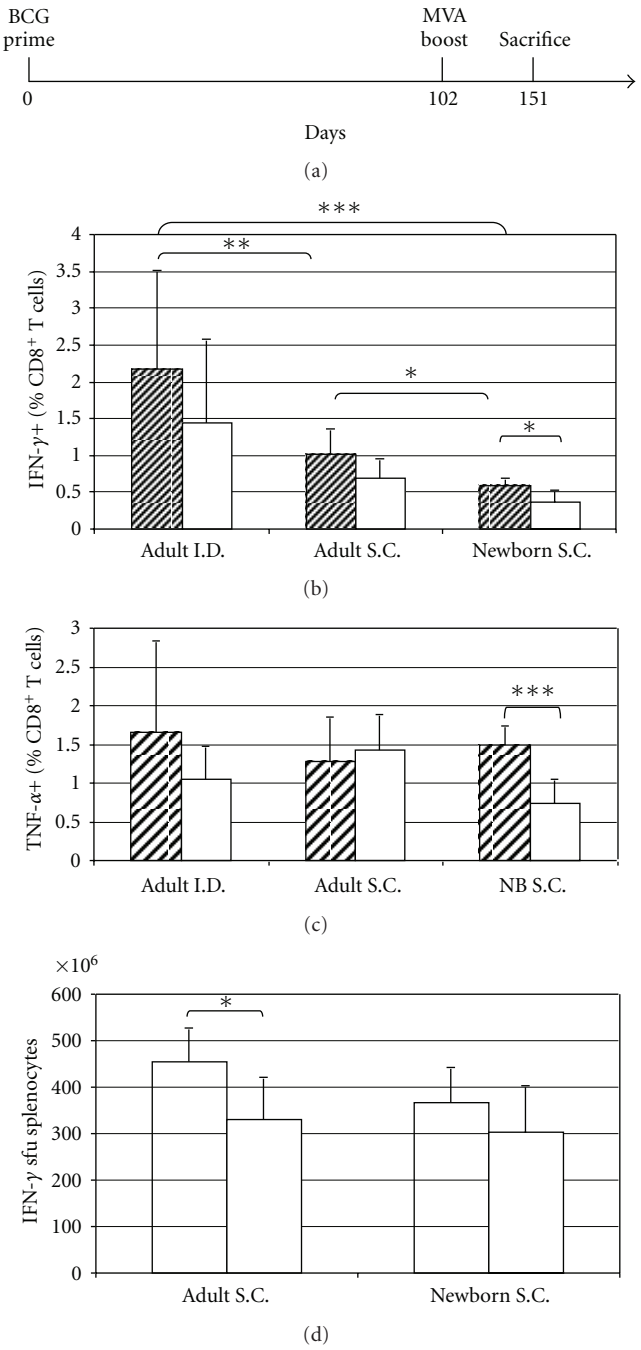

Figure 2: Effect of BCG.HIVA^{222} priming on the induction of HIV-1-specific CD8$^+$ T cells. (a) Mice were immunized with 2×10^6 cfu (adult mice I.D. or neonates S.C.) or 10^6 cfu (adult mice S.C.) of BCG.HIVA^{222} and subsequently boosted 14 weeks later with 10^6 pfu of MVA.HIVA by i.m. route. (b and c) Analysis of IFN-γ and TNF-α vaccine-elicited CD8$^+$ T cells as generated for each vaccination group by using the P18I10 epitope. The frequencies of CD8$^+$ T cells producing IFN-γ or TNF-α are shown. Data are presented as means $\pm$ standard deviation (SD; $n = 6$). (d) Elicitation of specific T-cell responses was assessed in an IFN-γ ELISPOT assay using the immunodominant P18I10 CD8$^+$ T-cell epitope peptide. The mean ($\pm$ SEM) sfu per 10^6 splenocytes for each group of mice ($n = 6$ per group) is shown. BCG.HIVA^{222} primed mice: striped bars. BCGwt primed mice: white solid bars. * = $P < .05$; ** = $P < .01$, *** = $P < .001$.

and on day 102 the animals received a booster dose with MVA.HIVA. On day 151, the mice were sacrificed, and the functional specific T cells in response of peptide stimulation were measured by intracellular cytokine staining (ICS) and ELISPOT assays (Figure 2(a)). We have observed in adult and newborn mice that BCG.HIVA^{222} prime and MVA.HIVA boost induced higher frequencies of H-specific CD8$^+$ splenocytes producing IFN-γ and TNF-α compared with the BCG wild-type priming and MVA.HIVA boost in two analyses performed (Figures 2(b), 2(c), and 2(d)). Overall, the proportions of HIV-1-specific T cells producing IFN-γ and TNF-α were higher in adult mice compared with newborn mice. When adult mice were vaccinated intradermally, the BCGwt priming elicited 1.45% of CD8$^+$ T cells producing IFN-γ, in comparison with 2.18% when the priming was performed with BCG.HIVA^{222}. We have detected the same pattern but lower magnitude when adult mice were immunized subcutaneously, 0,69% and 1,02%, respectively. When the newborn mice were primed with BCGwt or BCG.HIVA^{222} subcutaneously, the results were 0.37% and 0.59%, respectively ($P < .05$). The frequency of specific CD8$^+$ splenocytes producing IF-γ was twofold higher ($P < .01$) when adult mice were primed with BCG.HIVA^{222} intradermally compared with subcutaneously and nearly 4-fold higher when compared with newborn mice ($P < .001$) (Figure 2(b)). The proportion of specific CD8$^+$ splenocytes producing TNF-α was higher when mice were inoculated with BCG.HIVA^{222} (1.66% for adult and I.D. route and 1.50% for Newborn and S.C. route) in comparison with those inoculated with BCGwt (1.05% and 0.75%, resp.). In newborn mice, this difference was significant ($P < .001$). In contrast, the proportion was slightly higher in adult mice using BCGwt priming subcutaneously (1.42%) compared with BCG.HIVA^{222} priming (1.29%) (Figure 2(c)).

The capacity of splenocytes from vaccinated mice to secrete IFN-γ was tested also by ELISPOT assays. The splenocytes secreted IFN-γ after overnight stimulation with the dominant CD8$^+$ T-cell P18I10 epitope peptide. Representative results obtained with splenocytes from mice primed with BCG.HIVA^{222} or BCG wild type and boost with MVA.HIVA are shown in Figure 2(d). In adult and newborn mice the frequency of specific cells secreting IFN-γ was higher in mice primed with BCG.HIVA^{222} (455 and 367 spot forming units, (sfu)/10^6 splenocytes for adult and newborn mice, resp.) compared with BCG wild-type (329 and 303 sfu/10^6 splenocytes for adult and newborn mice, resp.). Among adult mice, the difference was significant ($P < .05$).

3.3. Priming BCG.HIVA^{222} Route and Age Affects the Level and Quality of CD8$^+$ T-Cell Responses to an Immunodominant Epitope. We have evaluated the influence of route inoculation and mice age on the level and quality of CD8$^+$ T-cell responses induced after mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. The immunization schedule is described in Figure 2(a). The quality of vaccine-elicited CD8$^+$ T cells was monitored by bifunctional analysis and *in vitro* killing assay for CTL activity. First, the frequencies of bifunctional IFN-γ and TNF-α T cells

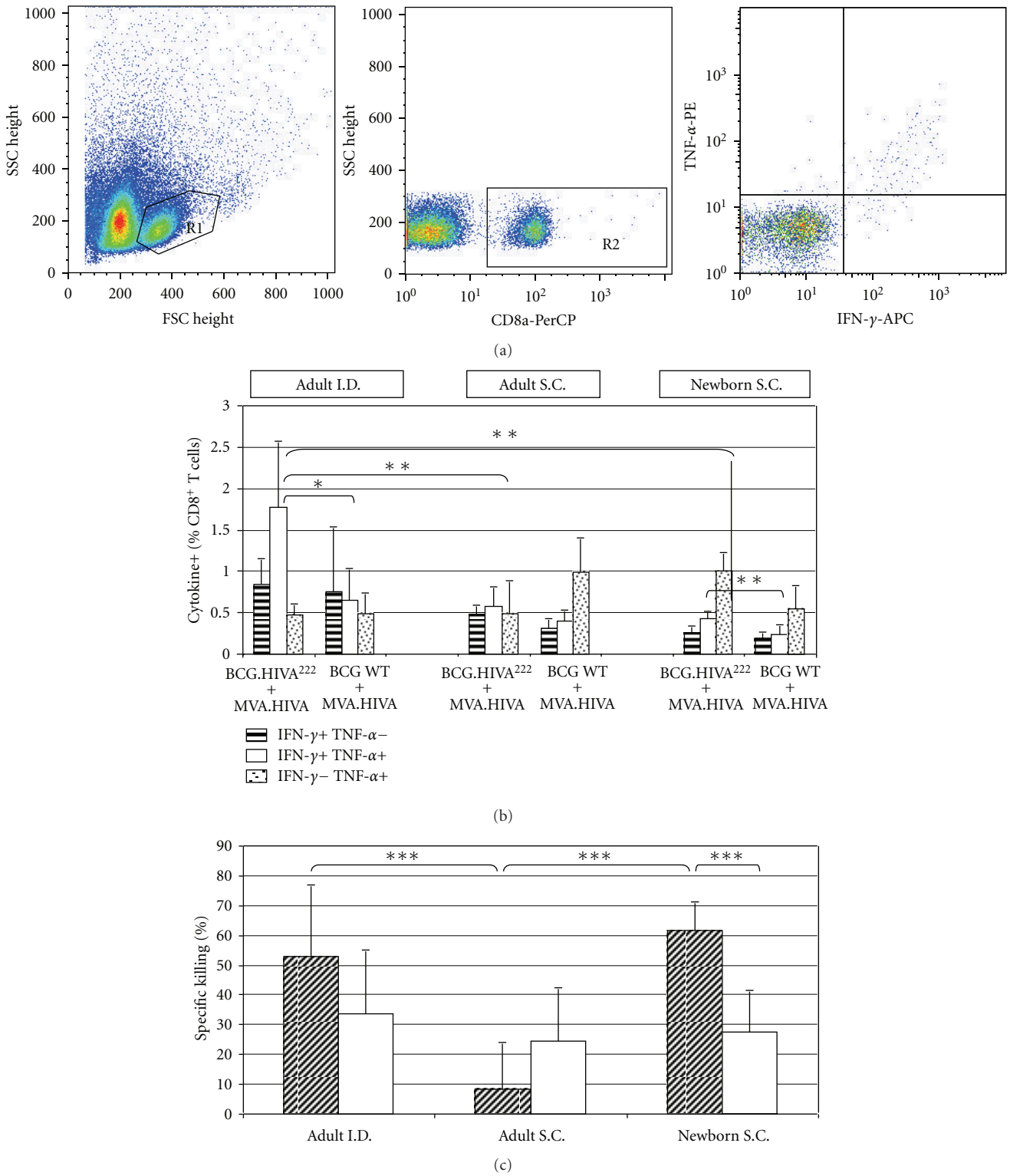

Figure 3: Induction of multifunctional HIV-1-specific CD8$^+$ T cells by the BCG.HIVA^{222} prime and MVA.HIVA boost regimen. Analysis of bifunctional vaccine-elicited CD8$^+$ T cells using the P18I10 peptide stimulation. (a) Example of dot blots and region selection for analysis. Region selected to analyze the splenocytes in the SSC-FSC dot-blot presentation (left), the CD8+ splenocytes selection (middle) and the IFNγ and TNF-α staining of the CD8$^+$ splenocytes (right). (b) This panel shows the frequencies of CD8$^+$ T cells producing IFN-γ and/or TNF-α. Data are presented as means ± SEM. (c) *In vitro* analysis of the CTL activity using peptide-pulsed target cells. BCG.HIVA^{222} primed mice: striped bars. BCGwt primed mice: white solid bars. * = $P < .05$; ** = $P < .01$, *** = $P < .001$.

specific for the H epitope were assessed. We found that the magnitude of the bifunctional response was higher when the I.D. route was used for BCG.HIVA^{222} priming in adult mice compared with S.C. route in adult mice ($P < .01$), and S.C. route in newborn mice ($P < .01$). In addition, when the intradermal inoculation was performed, the proportion of CD8$^+$ splenocytes producing IFN-γ and TNF-α was more than 2,5-fold higher in BCG.HIVA^{222} primed mice (1.77%) compared with BCG wild-type primed mice (0.65%) ($P < .05$). On the other hand, we observed the same trend but in lower magnitude when adult mice were inoculated subcutaneously with BCG.HIVA^{222} (0.58%), compared with BCG wild type (0.40%). The magnitude of the bifunctional response was lower in newborn mice than in adult mice. Besides that, the frequency of specific CD8$^+$ splenocytes producing IFN-γ and TNF-α was higher in BCG.HIVA^{222} primed mice than in BCG wild-type primed mice (0.43 and 0.24%, resp., ($P < .05$) (Figure 3(b)). Second, the cytotoxic activity of the BCG.HIVA^{222}-MVA.HIVA-elicited CD8$^+$ T cells was also assessed by in vitro killing assay. Splenocytes were cultured and stimulated with the P18I10 peptide for five days and evaluated as effector cells. These effector cells were able to kill efficiently P815 target cells pulsed with the P18I10 peptide. The frequency of specific killing was higher in newborn mice than in adult mice. In newborn mice the proportion of specific CTL activity was higher after BCG.HIVA^{222} priming (61%) compared with BCG wild-type priming (27%), subcutaneously, $P < .001$. In adult mice the proportion of specific CTL activity was also higher after BCG.HIVA^{222} priming (53%) compared with BCG wild-type priming (34%) intradermally. In contrast, when adult mice were vaccinated by S.C. route, the highest frequency of specific killing was obtained when BCGwt priming was used (24.4%), compared with BCG.HIVA^{222} priming (8.2%) (Figure 3(c)). The frequency of specific killing was clearly higher in adult mice primed with BCG.HIVA^{222} I.D. ($P < .001$) and Newborn mice primed with BCG.HIVA^{222} S.C. ($P < .001$) in comparison with adult mice inoculated S.C.

3.4. BCG.HIVA^{222} Elicited PPD-Specific Responses in Mice. The BCG-specific immune responses were assessed following the vaccine regimen consisting of BCG.HIVA^{222} prime and MVA.HIVA boost as described in Figure 2(a). The capacity of splenocytes from vaccinated mice to secrete IFN-γ was tested by ELISPOT assays. The splenocytes secreted IFN-γ after overnight stimulation with the PPD antigen. The frequencies of specific cells secreting IFN-γ was higher in newborn mice than in adult mice (261 sfu and 147 sfu/10^6 splenocytes, resp., $P < .05$). On the other hand, in newborn mice, the proportion of specific cells secreting IFN-γ was identical after BCG.HIVA^{222} priming compared with BCG wild-type priming. (261 and 261 sfu/million splenocytes). However, in adult mice, the proportion of specific cells secreting IFN-γ was lower after BCG.HIVA^{222} priming compared with BCG wild-type priming (147 and 218 sfu/million splenocytes, resp.) (Figure 4).

3.5. BCG.HIVA^{222} Prime and MVA.HIVA Boost Regimen Was Safe in Newborn Mice. Six newborn mice (7 days old) per group were either immunized or left unimmunized with 2×10^6 cfu of BCG wild type or BCG.HIVA^{222} subcutaneously route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA via intramuscular as described in Figure 2(a). As shown in Figure 5, the body weight was weekly monitored and recorded. All vaccine combinations were analyzed, to depict any possible adverse event due to vaccination and monitored by body weight lost. For rigorous safety assessment, the dose of BCGwt and BCG.HIVA^{222} inoculated to newborn mice (2×10^6 cfu) was 10-fold higher, as advised by the European Pharmacopoeia for the safety testing of live vaccines, in comparison with the most usual inoculation dose in adult mice [29]. Importantly, no differences were observed between the vaccinated mice groups and the naive mice group. On the other hand, the body weight profile was similar in all mice groups and similar to mice provider company standard body weight curve. Furthermore, between week 0 and week 14, the body weight monitored in all vaccinated mice groups was found between the mean body weight curve in naïve mice and the MVA.HIVA group (see Figure 5). Until week 14, the MVA.HIVA group can be considered as a naive group, because it has not been vaccinated yet. It is also important to mention that no mice died during the trial. Only local adverse events were detected in one mice showing slight redness and induration, which disappeared after several weeks. When analyzed by histopathology at necropsy, 17 weeks later, no severe lesion was observed.

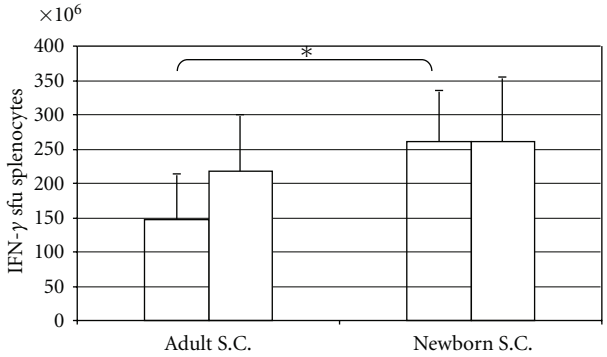

Figure 4: PPD-specific T-cell responses elicited by BCG.HIVA^{222}. Immune responses to BCG were assessed in an *ex vivo* IFN-γ ELISPOT assay using PPD as the antigen. The mean ($\pm$ SEM) sfu per 10^6 splenocytes for each group of mice ($n = 6$ per group) is shown. BCG.HIVA^{222} primed mice: striped bars. BCGwt primed mice: white solid bars. * = $P < .05$.

4. 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 and TB at the earliest time after birth to prevent breast milk HIV transmission and childhood tuberculosis is still a great challenge. In the present study, we have evaluated the influence of age and immunization routes for induction of HIV-1 and *M. tuberculosis*-specific immune responses after

BALB/c mice immunization with BCG.HIVA^{222} prime and MVA.HIVA boost. We have observed (i) enhanced specific T-cell induction responses in adult and newborn mice by using BCG.HIVA^{222} priming compared with BCG wild-type priming; (ii) higher frequencies and quality of the specific T-cell responses in adult mice immunized with BCG.HIVA^{222} intradermally compared with subcutaneously; (iii) that the BCG-specific immune responses were higher in newborn mice than adult mice; (iv) that among adult mice the BCG-specific immune responses were lower in mice primed with BCG.HIVA^{222} than BCG wild type; (v) that the BCG.HIVA^{222} prime and MVA.HIVA boost regimen is safe and immunogenic in newborn mice. Here, we have used the BCG.HIVA^{222} strain previously constructed by our group. This rBCG stably expresses the HIVA immunogen from the episomal pJH222HIVA plasmid. The HIVA gene was fused to the *M. tuberculosis* nucleotides coding for the 19-kDa lipoprotein signal sequence, and the HIVA gene expression was under the control of *Mycobacteria spp*. α-antigen promoter.

In murine and nonhuman primates studies, we and others have shown that rBCG elicited antibody, and cell-mediated responses against HIV-1 and simian immunodeficiency virus antigens [30–34]. In fact, only a small proportion of these animal studies used rBCG strains in heterologous prime-boost regimens. Ami et al. [35] 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 evident data showing that rBCG is a good priming vector in heterologous prime-boost vaccination regimens with attenuated virus or recombinant proteins to enhance specific T-cell responses [14, 36–38]. In TB vaccine human trials, McShane et al. [37] have demonstrated that vaccination with MVA expressing Ag85 boosts preexisting antimycobacterial immune responses induced either by environmental mycobacteria or BCG vaccination. Hovav et al. [39] have explored novel priming immunogens that might be used in heterologous immunization regimens. They have shown that priming with recombinant *Mycobacterium smegmatis* expressing HIV-1 gp120 protein induced a cellular immune response that is biased toward memory CD8$^+$ T cells and that can expand dramatically on reexposure to an HIV-1 envelope antigen. Our laboratory, in collaboration with Tomáš Hanke's laboratory have shown in BALB/c mice that the inclusion of BCG.HIVA^{222} in a heterologous prime-boost regimen can both prime novel and boost preexisting HIV-1 specific T-cell immune responses elicited by MVA.HIVA [14].

Many studies have compared the immune responses to foreign antigens delivered by rBCG inoculated by different routes; however, comparisons are difficult as doses, BCG strains, mycobacterial expression vectors, *in vivo* plasmid stability, promoters to regulate gene expression, levels of heterologous protein expression, and antigen localization are different. Several authors have emphasized the mucosal route of administration of rBCG. Lagranderie et al. [40] found that intrarectal immunization of mice with rBCG-SIV$_{mac251}$ induced higher intestinal IgA responses than oral or nasal

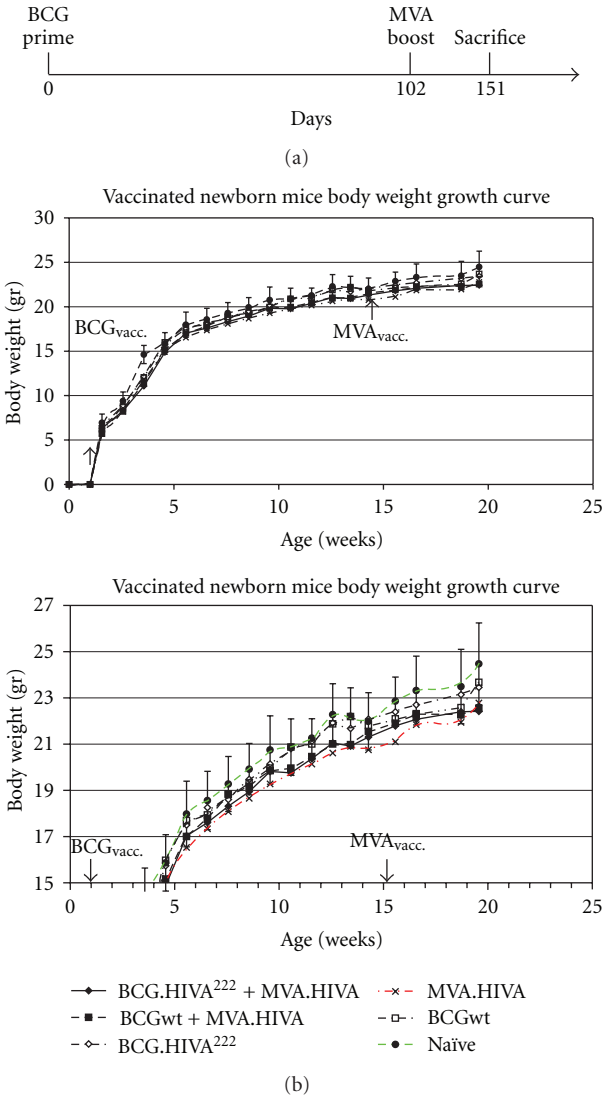

Figure 5: Body weight curve after newborn mice vaccination with BCG.HIVA^{222} (SC) and MVA.HIVA (IM). (a) Newborn mice were either left immunized or immunized with 2×10^6 cfu of BCG wild type or BCG.HIVA^{222} by subcutaneous route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA as indicated in panel A. (b and c) The body weight was weekly recorded, and the body weight mean ($\pm$ SEM) for each group of mice ($n = 6$ per group) is shown. Two different scales are shown so that the full evolution of the body weight is seen (b), and the y-axes zoomed to the 15–25 g interval, so that the group differences are better seen (c).

immunization. Kawahara et al. [41] examined a combined vaccination strategy in guinea pigs for enhancement of HIV-1-specific immune responses. They found that combined inoculation by rectal and intradermal routes effectively enhanced the levels of humoral and cellular immune responses against HIV-1. Promkhatkaew et al. [42] have explored the influence of immunization routes after adult BALB/c mice immunization with rBCG/HIV-1 gagE priming and rDIs/HIV-1 gagE boosting. They found higher CTL activity levels after two months subsequent to vaccination when mice were primed with rBCG subcutaneously and

boosted with rDIs intravenously compared with priming and boosting intradermally. However, after seven months subsequent to vaccination, they found similar CTL activity levels when mice were primed with rBCG S.C. and boosted with rDIs i.v. or intradermally. In the current study, BCG.HIVA^{222} prime- and MVA.HIVA boost-elicited HIV-1-specific CD8$^+$ T-cells exhibited effector functions such as production of IFN-γ and TNF-α, and such HIV-1-specific T-cell responses were higher in adult than in newborn mice. The inclusion of BCG.HIVA^{222} in a heterologous prime-boost regimen consistently enhanced and improved the frequency, quality, and durability of the generated HIV-1-specific responses in adult and newborn mice. This improvement was observed by the detection of the highest bifunctional HIVA-specific T-cell responses and higher specific cytolytic activity in the mice that received BCG.HIVA^{222} versus BCG wild type. Among adult mice, the intradermal inoculation of BCG.HIVA^{222} induced higher frequencies and quality of the specific HIV-1 immune responses versus the subcutaneous route. These results would be in accordance with the current recommended route of inoculation of BCG in infants.

There are really few reports in the literature describing the safety and immunogenicity of rBCG expressing HIV antigens in neonatal mice and neonatal nonhuman primates. Ranganathan et al. [43] have evaluated the immunogenicity in neonatal mice of three different recombinant attenuated Mtb. strains expressing an HIV envelope. They showed that single-dose immunization in neonatal mice with ΔlysA ΔsecA2 Mtb strain expressing HIV Env rapidly generated HIV-1- and Mtb-specific T-cell immune responses. In the present study, we have shown in newborn mice that BCG.HIVA^{222} prime and MVA.HIVA boost increased the frequencies of specific CD8$^+$ T cells producing IFN-γ or TNF-α. Such vaccine regimen also induced the highest proportion of HIV-1-specific bifunctional cells and the specific cytolytic activity. We have observed in newborn mice a lower level of HIV-1-specific T-cell immune responses compared with adult mice. Rosario et al. [44] have assessed the immunogenicity of the BCG.HIVA^{222} prime and MVA.HIVA boost regimen in newborn *Rhesus macaques*. They also observed that the HIV-1-specific responses induced in infants were lower compared with adult animals. On the other hand, we suggest that additional experiments should be performed in newborn mice inoculating the rBCG expressing HIV antigens by different routes, because the route of neonatal vaccination may confer different levels of immune activation, which may affect the efficacy of the vaccine.

Here, the vaccination with BCG wild-type and BCG.HIVA^{222} strains induced strong BCG-specific responses in adult and newborn mice. Studies in neonatal mice have indicated that immune responses at birth are often biased towards the Th2 type and defective in the Th1 type, the central defense mechanism against intracellular pathogens. However, it has been described that BCG vaccination induces a potent Th1-type immune response at birth in humans and in mice [45–48].

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, no reports have been published about safety of rBCG-based HIV vaccine in neonatal mice. Rosario et al. [44] have demonstrated that BCGHIVA^{401} followed by two doses of MVA.HIVA in *Rhesus macaques* was safe, not associated with systemic reactions, and the local adverse events detected were considered to be consistent with a predicted response to the BCG vaccine administration, similar to that observed in human neonates. In the present study, we have demonstrated in neonatal mice (7 days old) that BCG.HIVA^{222} prime and MVA.HIVA boost regimen was safe. We observed only in one mouse a local reaction and induration, and no systemic changes were observed after necropsy.

In conclusion, we tested the safety and immunogenicity of two candidate HIV-1 vaccines, BCG.HIVA^{222} and MVA.HIVA, in newborn mice using the prime-boost regimen. On the other hand we tested the influence of route inoculation among adult mice. We found the vaccines safe but less immunogenic for T cells in newborn mice than when administered to adult animals. In adult mice we found that the intradermal route gave higher frequencies and quality of the specific HIV-1 T-cell responses compared with the subcutaneous route. Given the urgent and global need for safe, effective, and affordable HIV and TB vaccines for infants and the demonstrated capability to produce and administer live mycobacterial vaccines on a large scale, BCG.HIVA^{222} prime and MVA.HIVA boost might be an attractive platform for a human neonatal vaccine for prevention of tuberculosis and mother-to-child breast milk transmission of HIV-1.

Acknowledgments

The authors are grateful to Dr. Barry R. Bloom and Dr. William R. Jacobs for providing the plasmid DNA pJH222 and BCG wild type and lysine auxotroph of BCG for research purposes. This work has been supported by MRC, UK and HIVACAT. N. Saubi is a *Red Temática de Investigación Cooperativa en SIDA* (RIS) Senior Fellow and is ascribed to the Universitat Autònoma de Barcelona Ph.D. programme. R. Fernández-Lloris is an EDCTP Research Fellow.

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ANNEX II: Paper Number 2

Saubi N, Mbewe-Mvula A, Gea-Mallorqui E, Rosario M, Gatell JM, Hanke T, Joseph J. **Pre-clinical development of BCG.HIVA(CAT), an antibiotic-free selection strain, for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG.** PLoS One. 2012;7(8):e42559. doi: 10.1371/journal.pone.0042559. Epub 2012 Aug 21.

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Pre-Clinical Development of BCG.HIVA^{CAT}, an Antibiotic-Free Selection Strain, for HIV-TB Pediatric Vaccine Vectored by Lysine Auxotroph of BCG

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Abstract

In the past, we proposed to develop a heterologous recombinant BCG prime-recombinant modified vaccinia virus Ankara (MVA) boost dual pediatric vaccine platform against transmission of breast milk HIV-1 and *Mycobacterium tuberculosis* (*Mtb*). In this study, we assembled an *E. coli*-mycobacterial shuttle plasmid pJH222.HIVA^{CAT} expressing HIV-1 clade A immunogen HIVA. This shuttle vector employs an antibiotic resistance-free mechanism based on Operator-Repressor Titration (ORT) system for plasmid selection and maintenance in *E. coli* and lysine complementation in mycobacteria. This shuttle plasmid was electroporated into parental lysine auxotroph (safer) strain of BCG to generate vaccine BCG.HIVA^{CAT}. All procedures complied with Good Laboratory Practices (GLPs). We demonstrated that the episomal plasmid pJH222.HIVA^{CAT} was stable *in vivo* over a 20-week period, and genetically and phenotypically characterized the BCG.HIVA^{CAT} vaccine strain. The BCG.HIVA^{CAT} vaccine in combination with MVA.HIVA induced HIV-1- and *Mtb*-specific interferon γ-producing T-cell responses in newborn and adult BALB/c mice. On the other hand, when adult mice were primed with BCG.HIVA^{CAT} and boosted with MVA.HIVA.85A, HIV-1-specific CD8$^+$ T-cells producing IFN-γ, TNF-α, IL-2 and CD107a were induced. To assess the biosafety profile of BCG.HIVA^{CAT}-MVA.HIVA regimen, body mass loss of newborn mice was monitored regularly throughout the vaccination experiment and no difference was observed between the vaccinated and naïve groups of animals. Thus, we demonstrated T-cell immunogenicity of a novel, safer, GLP-compatible BCG-vectored vaccine using prototype immunogen HIVA. Second generation immunogens derived from HIV-1 as well as other major pediatric pathogens can be constructed in a similar fashion to prime protective responses soon after birth.

Citation: Saubi N, Mbewe-Mvula A, Gea-Mallorqui E, Rosario M, Gatell JM, et al. (2012) Pre-Clinical Development of BCG.HIVA^{CAT}, an Antibiotic-Free Selection Strain, for HIV-TB Pediatric Vaccine Vectored by Lysine Auxotroph of BCG. PLoS ONE 7(8): e42559. doi:10.1371/journal.pone.0042559

Editor: Clive M. Gray, University of Cape Town, South Africa

Received March 13, 2012; **Accepted** July 9, 2012; **Published** August 21, 2012

Funding: The sources of funding that have supported the work are: EDCTP (Grant: CT.2006.33111.002; http://www.edctp.org); MRC, United Kingdom (Grant: G1001757; http://www.mrc.ac.uk/); and HIVACAT: AIDS Vaccine Research Project (http://www.hivacat.org/en). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

According to the last UNAIDS World AIDS Day Report 2011, at the end of 2010, an estimated 34 million people were living with HIV worldwide and 2.7 million individuals became newly infected with the virus in 2010. The number of people dying of AIDS-related causes was 1.8 million in 2010, and it is estimated that more than 16 million children have been orphaned by AIDS. Sub-Saharan Africa accounted for 70% of new HIV infections in 2010, and even though it is encouraging that 6.6 million people are currently receiving treatment in resource-poor settings, ensuring universal access to antiretrovirals still represents an enormous challenge [1]. Without access to drugs, rates of HIV-1 Mother-to-child transmission (MTCT) are 15–30% in non breastfeeding populations. Breastfeeding by an infected mother adds an additional 5–20% risk leading to an overall transmission rate of 20–45% as shown in some African and Asian settings [2]. Development of effective and safe neonatal and/or adult vaccines is the best solution to prevent infection or reduce the severity of HIV-related diseases. Infection with *Mycobacterium tuberculosis* (*Mtb*) kills about 2 million people each year and goes hand-in-hand with HIV-1. *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) is the only licensed vaccine and protects significantly against childhood and milliary tuberculosis. Globally, 80% of children are vaccinated with BCG, the majority of them at birth. Thus, the development of a combined vaccine, which would protect neonates against tuberculosis and MTCT of HIV-1 through breastfeeding, is a logical effort in the fight against these two major global killers.

Only two candidate vaccines designed to protect against breast milk HIV transmission have been studied in human infants (HIV-1 gp120 recombinant subunit and live-attenuated recombinant canarypox ALVAC vaccines) [3–5]. Therefore, there is an urgent need for a neonatal immunogen that generates HIV-specific immunity more rapidly. Recombinant BCG (rBCG) has been developed as a candidate neonatal vaccine vector against pertussis [6], measles [7], respiratory syncytial virus (RSV) [8] and breast

milk HIV transmission [9,10]. BCG as a vaccine vector has a number of attractive features. BCG has a proven record of safety as a vaccine against tuberculosis from its use in over two billion individuals [11]. 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 [12]. Nevertheless, the BCG Working Group of the International Union against Tuberculosis and Lung recommended that current universal BCG immunisation of infants continue in countries highly endemic for TB until they have all programmes in place for implementing selective deferral of HIV-exposed infants [13]. BCG infects and colonizes macrophages and dendritic cells, where it can survive and replicate for a long period of time. Through its persistence and potent adjuvantation by its cell wall components, it can induce long-lasting humoral and cellular immune responses. BCG can be given at or any time after birth, and is not affected by maternal antibodies [14,15]. Manufacturing of BCG-based vaccines is inexpensive. Finally, BCG is one of the most heat-stable vaccines in current use [16].

There is strong evidence in favour of a role for HIV-1 specific T-cell responses in the control of HIV-1 replication [17,18]. 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 [19–21]. For a number of years, we have been working on rBCG based HIV-1 vaccine development with the aim to induce 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 common immunogen 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 [22]. BCG.$HIVA^{222}$ carrying an episomal plasmid expressing HIVA was shown to be stable and to induce durable, oligofunctional HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses in BALB/c mice. Furthermore, when the BCG.$HIVA^{222}$ vaccine was used in a prime-boost regimen with heterologous vectors, HIV-1-specific responses provided protection against surrogate virus challenge expressing HIVA, and was also as efficient in protecting against aerosol challenge with *Mtb* as the BCG 1173 P2 vaccine Pasteur strain. The BCG.$HIVA^{222}$ vaccine candidate was vectored by a lysine auxotroph of BCG Pasteur strain that carried an *E. coli*-mycobacterial shuttle plasmid pJH222.HIVA with a lysine A complementing gene and a weak promoter to drive HIVA gene expression [10]. This design increases the plasmid stability *in vivo* and prevents heterologous gene expression disruption by genetic rearrangement [23]. We also evaluated the influence of BALB/c mice age and immunization routes on induction of HIV-1 and *Mtb*-specific immune responses. Administration of BCG.$HIVA^{222}$ to newborn mice was safe and primed HIV-1-specific immune responses boosted by subsequent MVA.HIVA administration [24]. Also, MVA.HIVA.85A, a dual AIDS and tuberculosis vaccine, was designed to boost both the *Mtb* and HIV-1-specific immune responses primed by BCG.$HIVA^{222}$ [25].

In this study, we constructed a novel HIVA-expressing *E. coli*-mycobacterial shuttle plasmid pJH222.$HIVA^{CAT}$ by using an antibiotic-free plasmid selection system based on Operator-Repressor Titration (ORT) system in *E. coli* and lysine complementation in mycobacteria. This plasmid DNA was electroporated into parental lysine auxotroph of BCG to generate vaccine BCG.$HIVA^{CAT}$. The genetic and phenotypic characterization of antibiotic markerless BCG.$HIVA^{CAT}$ strain was performed. The presence of HIVA gene sequence and protein expression by the recombinant mycobacterium 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 both newborn and adult BALB/c mice after BCG.$HIVA^{CAT}$ prime and MVA.HIVA or MVA.HIVA.85A boost. The BCG.$HIVA^{CAT}$ strain was developed in GLP-compatible conditions, properly characterized, stable *in vivo*, induced specific HIV-1 and *Mtb* immune responses in newborn and adult mice and was well tolerated in newborn mice. In addition, the compatibility with GLP requirements is relevant for progressing this novel vaccine into clinical evaluation.

Results

Construction of the BCG.$HIVA^{CAT}$ vaccine strain

HIVA immunogen consists of consensus HIV-1 clade A Gag p24/p17 domains coupled to a string of $CD8^+$ T-cell epitopes and monoclonal antibody (mAb) tag Pk [22]. The HIVA gene was synthesized utilizing humanized GC-rich codons, which are similar to those used by mycobacteria [26,27]. To facilitate the pre-clinical development of candidate vaccines, the HIVA immunogen contains an immunodominant H-$2D^d$-murine restricted epitope P18-I10 [28]. The HIVA open-reading frame was fused at its 5′ end to nucleotides coding for the 19-kDa lipoprotein signal sequence, which facilitates the antigen secretion and fusion of foreign antigens to mycobacterial surface lipoproteins, enhancing the foreign protein immunogenicity [29]. The chimeric 19-kDa signal sequence-HIVA gene was expressed from *E. coli*-mycobacterial shuttle plasmid pJH222 under the control of the *Mtb* α-antigen promoter (Figure 1A). Plasmid DNA pJH222 is a replicative (multicopy, extrachromosomal) vector that contains a DNA cassette encoding kanamycin resistance (Tn903-derived *aph* gene), an *E. coli* origin of replication (*oriE*) and a mycobacterial plasmid DNA origin of replication (*oriM*). It also contains the wild-type lysine A-complementing gene for the vector maintenance (*lysA5*) in the BCG auxotroph [10]. The kanamycin resistance gene was removed from pJH222.HIVA vector by using the Operator-Repressor Titration (ORT) system developed by Cobra Biologics (UK). Such system enables the selection and maintenance of plasmids that are free from expressed selectable marker genes and require only the short, non-expressed *lac* operator for selection and maintenance [30]. The principle *Escherichia coli* ORT strain, DH1*lacdapD* [31], has been used to produce several important DNA vaccine candidates such as the HIV-1 vaccine pTHr.HIVA [32]. In this work the kanamycin resistance gene was replaced with a *lac* operator sequence and the resulting plasmid, pJH222.$HIVA^{CAT}$ was transformed into the *E.coli* DH1*lacdapD* strain (Figure 1A). When the non-expressed *lac* operator sequence was inserted into multicopy plasmid and introduced into the cell, the binding of the repressor protein to the plasmid-borne operator derepresses the chromosomal operator and allows *dapD* expression and cell growth [32]. The recombinant pJH222.HIVA containing the ORT selection system, here designated as pJH222.$HIVA^{CAT}$, was transformed into lysine auxotroph of BCG host strain Pasteur ΔlysA5::res [33]. The selection of positive BCG.$HIVA^{CAT}$ colonies was made by growing the rBCG cells 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 of whole transformed mycobacterial cell lysates using anti-Pk mAb. As shown in Figure 1B, the highest level of HIVA protein expression was detected after blotting the BCG culture from clone number 10 and was selected for further molecular characterization, immunogenicity and safety testing in

mice. On the other hand, the BCG.HIVA^{CAT} clone10 culture was preserved by using the seed-lot system. A Master Seed stock, and derivative Working Stock, which we used also as a Vaccine stock was prepared. Growth of the transformed mycobacteria and the *in vivo* stability of pJH222.HIVA^{CAT} episomal plasmid were established by the recovery of BCG.HIVA^{CAT} colonies from the spleens of BALB/c mice 20 weeks after immunization. Six out of six recovered rBCG colonies were positive for HIVA DNA coding sequence by PCR (Figure 1C).

Genetic characterization of the BCG.HIVA^{CAT}

In order to confirm that our recombinant BCG.HIVA^{CAT} vaccine strain corresponds to *M. bovis* BCG strain, we used the GenoType MTBC assay based on a commercially available DNA strip assay (Hain Lifescience GmbH, Nehren, Germany) intended for the differentiation of members of the *Mycobacterium tuberculosis* complex (MTBC) and identification of *M. bovis* BCG. This assay is based on *gyrB* DNA sequence polymorphisms and the RD1 deletion of *M. bovis* BCG. Specific oligonucleotides targeting these polymorphisms are immobilized on membrane strips. Amplicons derived from a multiplex PCR react with these probes during hybridization. Each strip has a total of 13 reaction zones. Amplification bands 4–13 include specific probes for each of the tuberculosis complex species. The combination of several hybridization patterns enables to identify the different species of MTBC. Interpretation of the GenoType MTBC hybridization patterns was performed on the basis of the description included in the test. The hybridization patterns were all unequivocal and could easily be allocated to species. Sample results were then compared with the classical differentiation results. We tested four samples corresponding to commercial BCG Connaught, BCG.HIVA^{222}, BCG.HIVA^{CAT} (clone10) and BCG Pasteur strains. As we show in Figure 2A, all four strains presented the same hybridization pattern corresponding to *M. bovis* BCG, detecting the bands 4, 7, 9, 10 and 13 belonging specifically to BCG hybridization pattern.

Distribution of BCG to several countries for worldwide application started around 1924 and it was preserved by *in vitro* subculture passaging until 1960s. Since then the Pasteur strain has been freeze-dried, keeping the form of the primary seed lot. The *in vitro* evolution of BCG has resulted in a number of BCG substrains

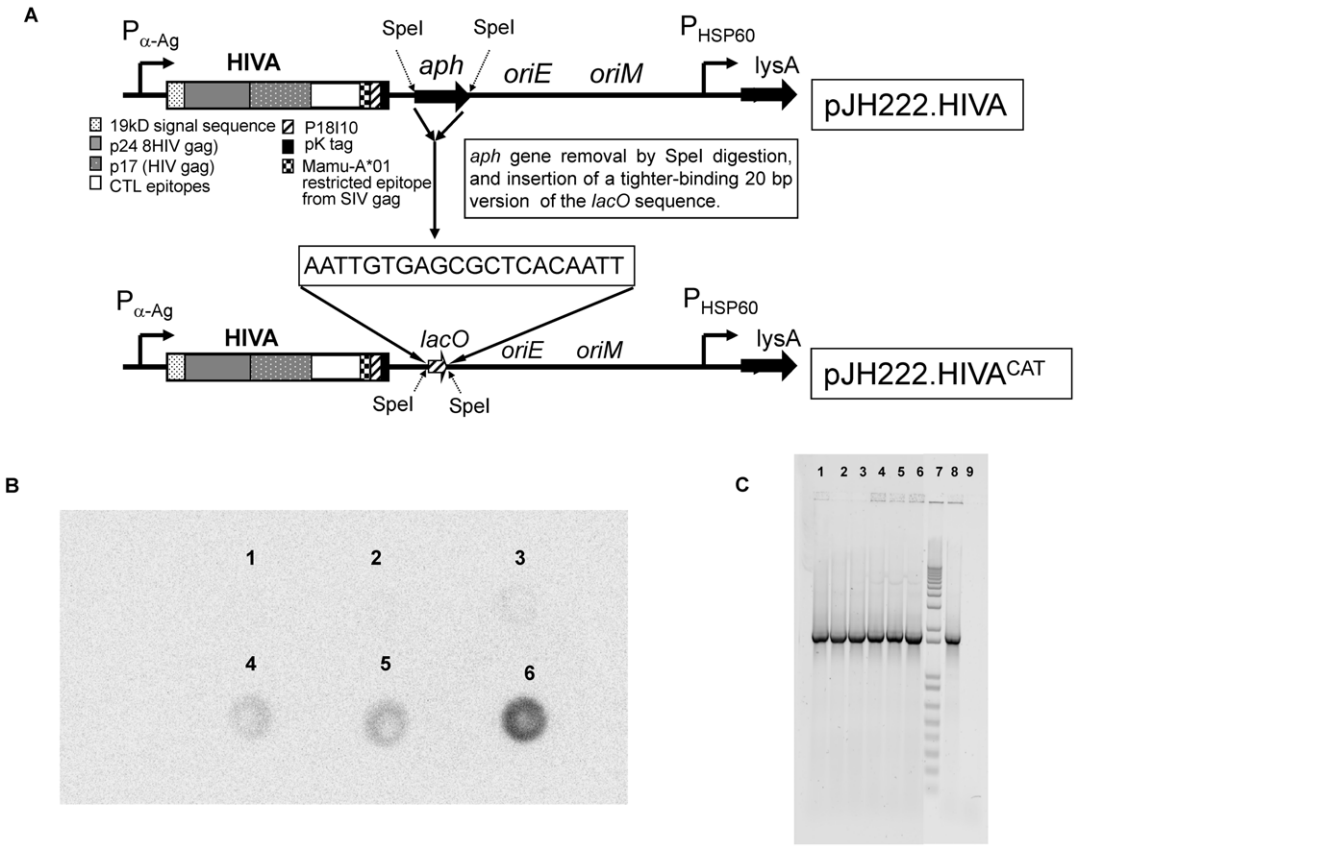

Figure 1. Construction of the BCG.HIVA^{CAT} vaccine strain. (**A**) A synthetic GC-rich HIVA gene was fused to the region encoding the 19-kDa lipoprotein signal sequence and inserted into the episomal pJH222 *E. coli*-mycobacterium shuttle plasmid. This plasmid contains kanamycin resistance (*aph*) and complementing *lysA* genes and an *E. coli* origin of replication (oriE). In addition, pJH222 contained the mycobacterial origin of replication (oriM). The BALB/c mouse T-cell and MAb Pk epitopes used in this work are depicted. P α-Ag, *M. tuberculosis* α-antigen promoter; P*HSP60*, heat shock protein 60 gene promoter. The *aph* gene was removed by SpeI digestion and the *lacO* sequence was inserted and transformed into *E. coli* DH1*lacdapD* strain. (**B**) Immunodot of BCG.HIVA^{CAT} lysates. Dot 1: BCG wild type (negative control); Dot 2, 3, 4 and 5: clone 3, clone 7, clone 9 and clone 10 of BCG.HIVA^{CAT}; Dot 6: BCG.HIVA^{222} (positive control). HIVA peptide was detected using the anti-Pk MAb followed by horseradish peroxidase-Goat-anti-Mouse and enhanced chemiluminescence (ECL) detection. (**C**) *In vivo* plasmid stability of BCG.HIVA^{CAT} harboring pJH222.HIVA^{CAT}. Mice were injected s.c. with 10^5 cfu of BCG.HIVA^{CAT} and boosted i.m. with 10^6 pfu of MVA.HIVA, spleens were homogenized 20 weeks after BCG inoculation and the recovered rBCG colonies were tested for the presence of the HIVA DNA coding sequence by PCR. Lanes 1 to 6: Six rBCG colonies were recovered in the non-lysine supplemented plate; lane 7: Molecular weight marker; lane 8: Plasmid DNA positive control; lane 9: Distilled water (negative control).
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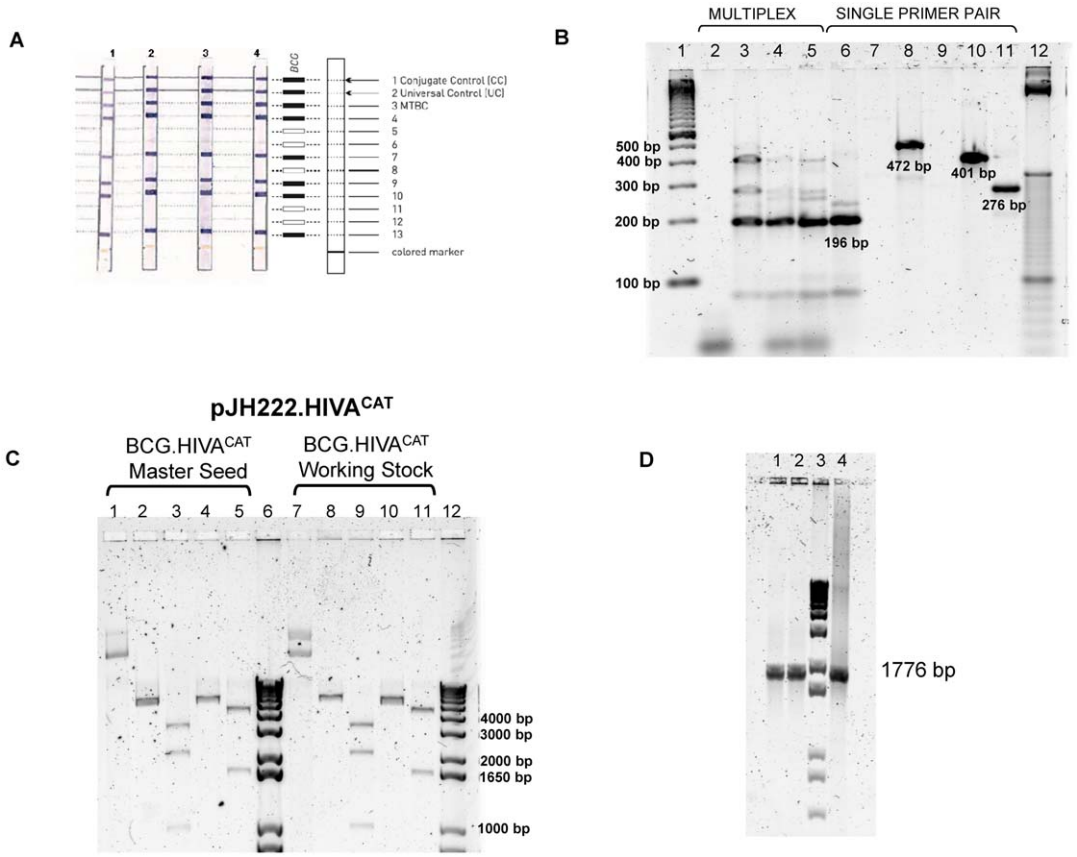

Figure 2. Genetic characterization of the BCG.HIVA^{CAT}. GenoType MTBC assay and Multiplex PCR assay. (**A**) The BCG.HIVA^{CAT} strain identification results representative of all of the patterns obtained with the GenoType MTBC assay. The positions of the oligonucleotides, the marker line and the BCG hybridization pattern are shown on the right. The specificity and targeted genes of the lines are as follows: 1, conjugate control; 2, amplification control (23S rRNA); 3, MTBC specific (23S rRNA); 4 to 12, discriminative for the MTBC species (*gyrB*); 13, *M. bovis* BCG (RD1). The samples analyzed were: Strip 1: BCG Connaught; Strip 2: BCG.HIVA^{CAT}; Strip 3: BCG.HIVA^{222}; Strip 4: BCG wild type. All four strains presented the same hybridization pattern corresponding to *M. bovis* BCG. (**B**) The BCG.HIVA^{CAT} Pasteur substrain identification by multiplex PCR assay. Lane 1 and 12: molecular weight marker; lane 2: negative control; lane 3, 6–11: BCG.HIVA^{CAT}(clone10); lane 4: BCG Danish strain (using 1 µl of template); lane 5: BCG Danish strain (using 4 µl template); The samples were analyzed by multiplex primer assay or single primer pair assay. Lane 2–5: multiplex primers; lane 6: ET1-3 primers; lane 7: RD2 primers; lane 8: RD8 primers; lane 9: RD14 primers; lane 10: RD16 primers; lane 11: C3–C5 primers. (**C**) Enzymatic restriction analysis of pJH222.HIVA^{CAT} plasmid DNA extracted from both the Master Seed (MS, lanes 1–5) and the Working Stock (WS, lanes 7–11) of BCG.HIVA^{CAT} cultures. Lane 1 and lane 7: uncut plasmid; lane 2 and lane 8: HpaI digestion; lane 3 and 9: KpnI digestion; lane 4 and 10: digestion with SpeI; lane 5 and 11: digestion with HindIII; lane 6 and 12: Molecular Weight Marker (1 kb Plus, Invitrogen). (**D**) PCR analysis of HIVA DNA coding sequence using as template the cultures of BCG.HIVA^{CAT} Master Seed (lane 1), and Working Stock (lane 2), Molecular Weight Marker (lane 3), positive control plasmid DNA pJH222.HIVA (lane 4).
doi:10.1371/journal.pone.0042559.g002

that are heterogenic [34–37]. Genetic identification techniques have been used to differentiate diverse BCG substrains: i) the gene probe based on IS986; ii) restriction fragments patterns; iii) whole-genome DNA microarray and iv) multiplex PCR. Using this last method it was reported that the deletion of RD1 occurred in 23 of 23 BCG strains tested. In order to confirm that our BCG.HIVA-^{CAT} vaccine strain correspond to BCG Pasteur substrain, we have used the method described by Bedwell *et al.* [38] 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 seven fingerprints and all BCG substrains were confirmed. We tested the following samples: BCG.HIVA^{CAT} strain (clone10) Pasteur substrain and commercial BCG Danish 1331 strain. Both BCG substrains evaluated gave a 196 bp product with primers ET1-3, indicating deletion of the RD1 region. In addition in BCG Pasteur (BCG.HIVA^{CAT}) the RD8 and RD16 were present and gave a product of 472 and 401 bp respectively. The primers for the SenX3-RegX3 region gave a product of 276 bp in BCG Pasteur. The PCR fingerprints of BCG Pasteur and BCG Danish substrains (Figure 2B) were consistent with previously published results on genetic information of BCG substrains [38]. As shown in Figure 2B, the yield of the PCR was higher when the single primer pairs were used, instead of multiplex format.

For the molecular characterization of pJH222.HIVA^{CAT} plasmid DNA, enzymatic restriction and PCR analysis were performed. The plasmid DNA was isolated from the Master seed and Working stock of BCG.HIVA^{CAT} strain and was characterized. The enzymatic restriction pattern obtained did not show any difference with the predicted enzymatic pattern of the plasmid DNA sequence. HpaI (lanes 2 and 8): band of 6857 bp; KpnI (lanes 3 and 9): bands of 3758, 2117 and 982 bp; SpeI (lanes 4 and 10): band of 6815 bp; HindIII (lanes 5 and 11): bands of 5228 and 1629 bp (Figure 2C). On the other hand, the PCR analysis using specific primers for the HIVA DNA coding sequence was

performed using the BCG liquid culture from BCG.HIVA^{CAT} Master seed and Working stock as template. A band of 1776 bp corresponding to HIVA DNA fragment was detected (Figure 2D).

Phenotypic characterization of the BCG.HIVA^{CAT}

To prevent the plasmid instability *in vivo* and *in vitro* and the genetic rearrangement by 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 and viii) antigen secretion to enhance the immunogenicity and to prevent foreign proteins from becoming toxic to BCG. 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 [23]. In this study, we have used a BCG strain auxotroph for lysine complemented with a lysine gene and antibiotic-free plasmid selection system (no kanamycin resistance). We assessed the phenotype stability of lysine auxotrophy, lysine complementation and kanamycin resistance of BCG.HIVA^{CAT} strain. Initially, the BCG lysine auxotroph strain was plated out on lysine supplemented and non supplemented agar. Such strain failed to grow on non lysine supplemented agar plates and no colonies were observed (Figure 3A). However, growth was observed on agar plates supplemented with lysine (Figure 3B). As expected, complementation of BCG.HIVA^{CAT} strain with lysine gene provided on the multicopy plasmid pJH222.HIVA^{CAT} abolished the requirement for exogenous lysine (Figure 3C). On the other hand, when BCG.HIVA^{CAT} strain was plated out on agar plates containing kanamycin, no colonies were observed (Figure 3D), confirming the lack of kanamycin resistance in our construct.

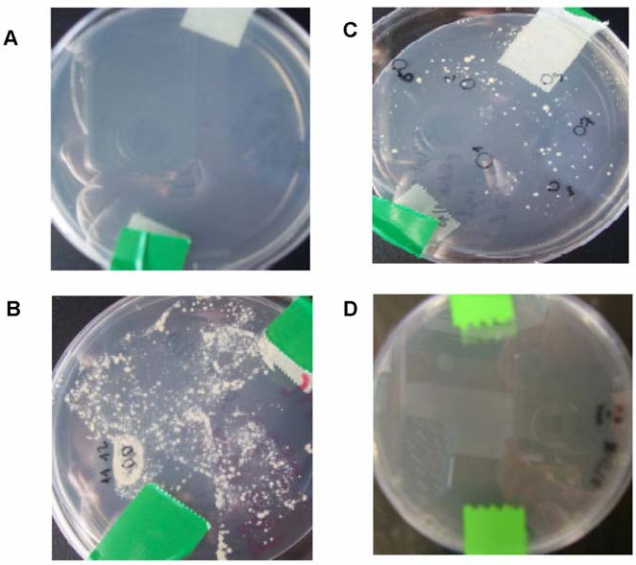

Figure 3. Phenotypic characterization of the BCG.HIVA^{CAT}. We assessed the phenotype of lysine auxotrophy, lysine complementation and kanamycin resistance of BCG.HIVA^{CAT} strain. (**A**) BCG lysine auxotroph strain plated on non-lysine supplemented 7H10; (**B**) BCG lysine auxotroph strain plated on lysine supplemented 7H10; (**C**) BCG.HIVA^{CAT} plated on 7H10 without lysine and kanamycin supplementation; (**D**) BCG.HIVA^{CAT} plated on 7H10 without lysine supplementation and with kanamycin.
doi:10.1371/journal.pone.0042559.g003

BCG.HIVA^{CAT} prime and MVA.HIVA boost regimen elicited HIV-1-specific CD8$^+$ and PPD-specific T-cell responses in mice

We have demonstrated in previous studies in BALB/c mice that BCG.HIVA^{222} can both prime novel and boost preexisting MVA.HIVA elicited HIV-1 specific CD4$^+$ and CD8$^+$ T-cells immune responses of high quality upon antigenic reexposure. In this study, we have evaluated the specific HIV-1 T-cell immune responses in adult and newborn BALB/c mice after immunization with BCG.HIVA^{CAT} prime and MVA.HIVA or MVA.HIVA.85A 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 HIVA immunogen to evaluate the immunogenity in mice (Figure 1A). On day 0, adult mice were immunized with rBCG with the episomal plasmid, and on week 12 the animals received a booster dose with MVA.HIVA.85A (Figure 4A). On week 14, the mice were sacrificed and the functional quality of the elicited CD8$^+$ T-cells to produce IFN-γ, TNF-α, IL-2 and to degranulate (surface expression of CD107a) in response to P18-I10 peptide stimulation was measured by intracellular cytokine staining (ICS) (Figure 4B). At the higher dose, BCG.HIVA^{CAT} alone and in combination with MVA.HIVA.85A induced HIV-1-specific CD8+ T-cells, producing IFN-γ, TNF-α, and CD107a. For TNF-α, and CD107a, there was a trend of increased responses following MVA.HIVA.85A boost if these were primed by the BCG.HIVA-^{CAT} vaccine. In another animal experiment, on day 0, adult and newborn mice were immunized with rBCG, and on week 14 the animals received a booster dose with MVA.HIVA (Figure 4C). We have observed in adult mice that BCG.HIVA^{CAT} prime and MVA.HIVA boost induced higher frequencies of P18-I10 epitope specific CD8$^+$ splenocytes producing IFN-γ than newborn and naïve mice ($p<0.05$) (Figure 4D). These data are in concordance with our previously published results in which the proportions of HIV-1 specific T-cells producing IFN-γ and TNF-α were higher in adult mice compared with newborn mice [24]. Moreover, the magnitude of the bifunctional response was also lower in newborn mice than in adult mice.

BCG.HIVA^{CAT} elicited PPD-specific responses in mice. The BCG-specific immune responses were assessed following the vaccine regimen consisting of BCG.HIVA^{CAT} prime and MVA.HIVA boost as described in Figure 4C. The capacity of splenocytes from vaccinated mice to secrete IFN-γ was tested by ELISPOT assays. The splenocytes secreted IFN-γ after overnight stimulation with the PPD antigen. The frequencies of specific cells secreting IFN-γ was higher in adult mice than in newborn and naïve mice ($p<0.05$) (Figure 4E).

BCG.HIVA^{CAT} prime and MVA.HIVA boost was well tolerated in newborn mice

Ten newborn mice (7-days-old) per group were either immunized or left unimmunized with 2×10^6 colony forming units (cfu) of BCG wild type, BCG.HIVA^{222} or BCG.HIVA^{CAT} via subcutaneous route and subsequently given a booster dose of 10^6 plaque forming units (pfu) of MVA.HIVA via intramuscular as described in Figure 5A. As shown in Figure 5B, the body mass was weekly monitored and recorded. All vaccine combinations were analyzed, to depict any possible adverse events due to vaccination and monitored by body mass loss. For rigorous safety assessment,

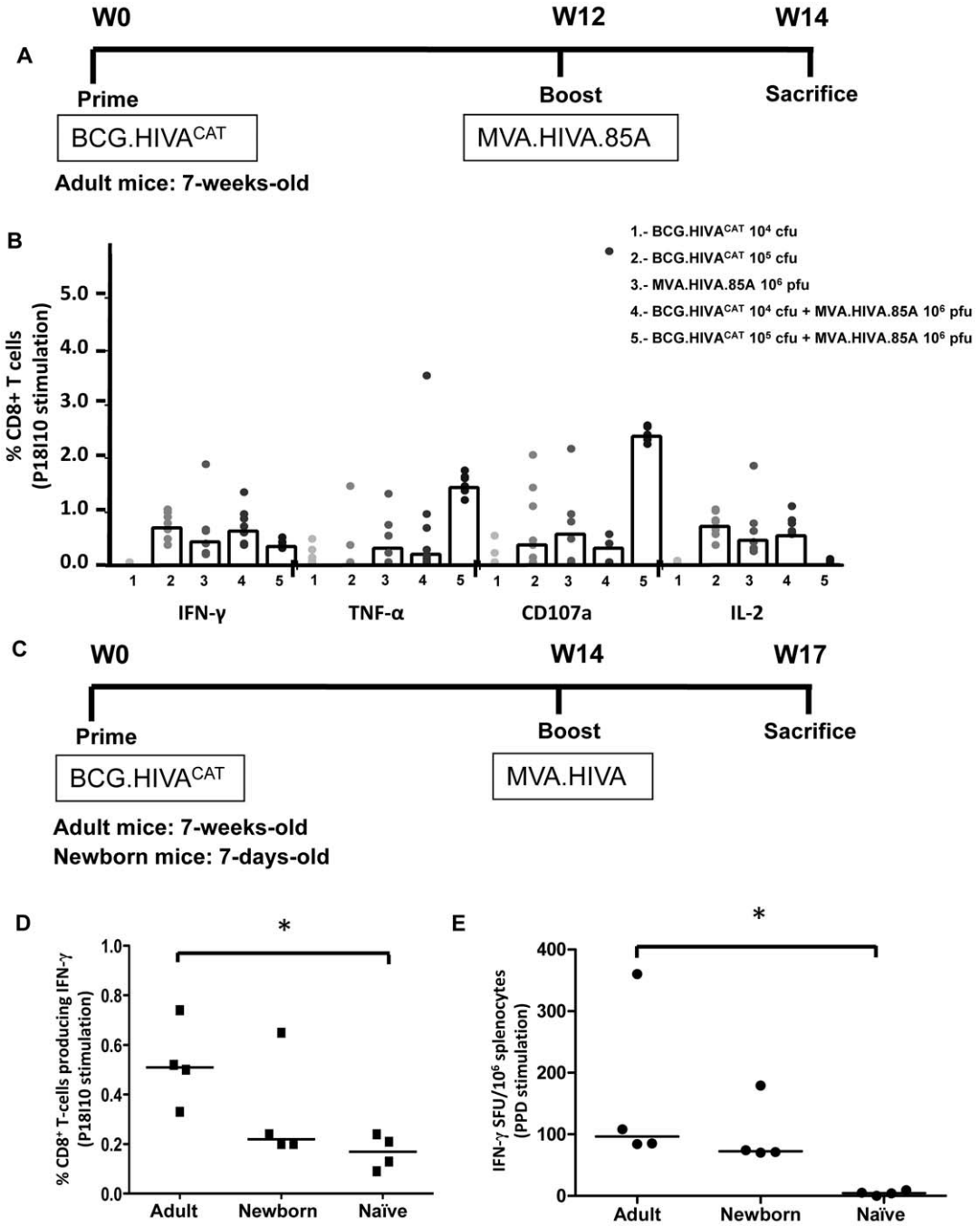

Figure 4. Induction of HIV-1- and *Mtb*-specific T-cells responses by the BCG.HIVA^{CAT} prime - MVA.HIVA boost regimen. (**A**) Adult mice (7-weeks-old) immunized with either 10^4 or 10^5 cfu of BCG.HIVA^{CAT} alone (subcutaneously), 10^6 pfu of MVA.HIVA.85A alone (intramuscularly), or 10^4 or 10^5 cfu of BCG.HIVA^{CAT} as a prime and boosted with 10^6 pfu of MVA.HIVA.85A (left to right). Mice were sacrificed 2 weeks later for T-cell analysis. (**B**) Analysis of IFN-γ, TNF-α, CD107a and IL-2 vaccine elicited HIV-1-specific CD8$^+$ T-cell responses. The frequencies of cells producing cytokine are shown. Data are presented as group medians as well as individual animal responses (n = 5). (**C**) Adult and newborn mice (7-days-old) were either left unimmunized or immunized with 2×10^6 cfu of BCG.HIVA^{CAT} (intradermal and subcutaneous route respectively) and subsequently given a booster dose of 10^6 pfu of MVA.HIVA (intramuscularly) at 14 weeks post BCG immunization, and sacrificed 3 weeks later. (**D**) 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 group medians as well as individual animal responses (n = 4). (**E**) PPD-specific T-cell responses elicited by BCG.HIVA^{CAT}. Immune responses to BCG were assessed in an *ex vivo* IFN-γ ELISPOT assay using PPD as the antigen. The median spot-forming units (SFU) per 10^6 splenocytes for each group of mice (n = 4) as well as individual animal responses is shown. * = $p<0.05$.
doi:10.1371/journal.pone.0042559.g004

the dose inoculated to newborn mice was 10- fold higher, as advised by the European Pharmacopoeia for the safety testing of live vaccines, in comparison with the most usual inoculation dose in adult mice [39]. Importantly, no statistically significant difference was observed between the vaccinated mice groups and the naïve mice group at specific time points, corresponding to

BCG inoculation, 2 months after BCG inoculation, pre-MVA boosting and three weeks post MVA-boosting. On the other hand, the body mass profile was similar in all mice groups and similar to mice provider company standard body mass curve (www.Harlan.com). Furthermore, between week 0 and week 14, the body mass monitored in all vaccinated mice groups was found between the mean ± 2 standard deviations (SD) body mass curve in naïve mice (Figure 5B). 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 on prevention of mother-to-child HIV-1 transmission, the development of a safe, effective and affordable vaccine against HIV-1 and TB at the earliest time after birth to prevent breast milk HIV-1 transmission and childhood tuberculosis is still a great challenge. In this study, i) we have constructed the *E. coli*-mycobacterial shuttle vector to express the HIVA immunogen by using an antibiotic-free plasmid selection system; ii) the genetic and phenotypic characterization of the recombinant lysine auxotroph of BCG.HIVA^{CAT} strain was performed; iii) the HIVA protein expression was confirmed; iv) the specific HIV-1 and *Mtb*-specific immune responses after adult and newborn mice immunization with BCG.HIVA^{CAT} prime and MVA.HIVA or MVA.HIVA.85A boost was evaluated and v) the biosafety profile after newborn mice immunization was monitored. The BCG.HIVA^{CAT} strain was developed in GLP-compatible conditions, preserved by the seed-lot system and was genetically and phenotypically characterized. The *E. coli*-mycobacterial shuttle vector that contains the antibiotic-free plasmid selection system was stable *in vivo* for at least 20 weeks after mice immunization and was used to construct a markerless BCG.HIVA^{CAT} vaccine suitable for Good Manufacturing Practice. Overall, we have demonstrated that BCG.HIVA^{CAT} prime-MVA.HIVA boost regimen was well tolerated in newborn mice and induced HIV-1 and *Mtb*-specific immune responses in adult and newborn mice. Thus, this strategy might be worthy to pursue and for joining the global efforts to develop novel BCG vector-based vaccines for controlling TB and HIV/AIDS.

Even though, it has been described that antibiotics and antibiotic resistance genes have been traditionally used for the

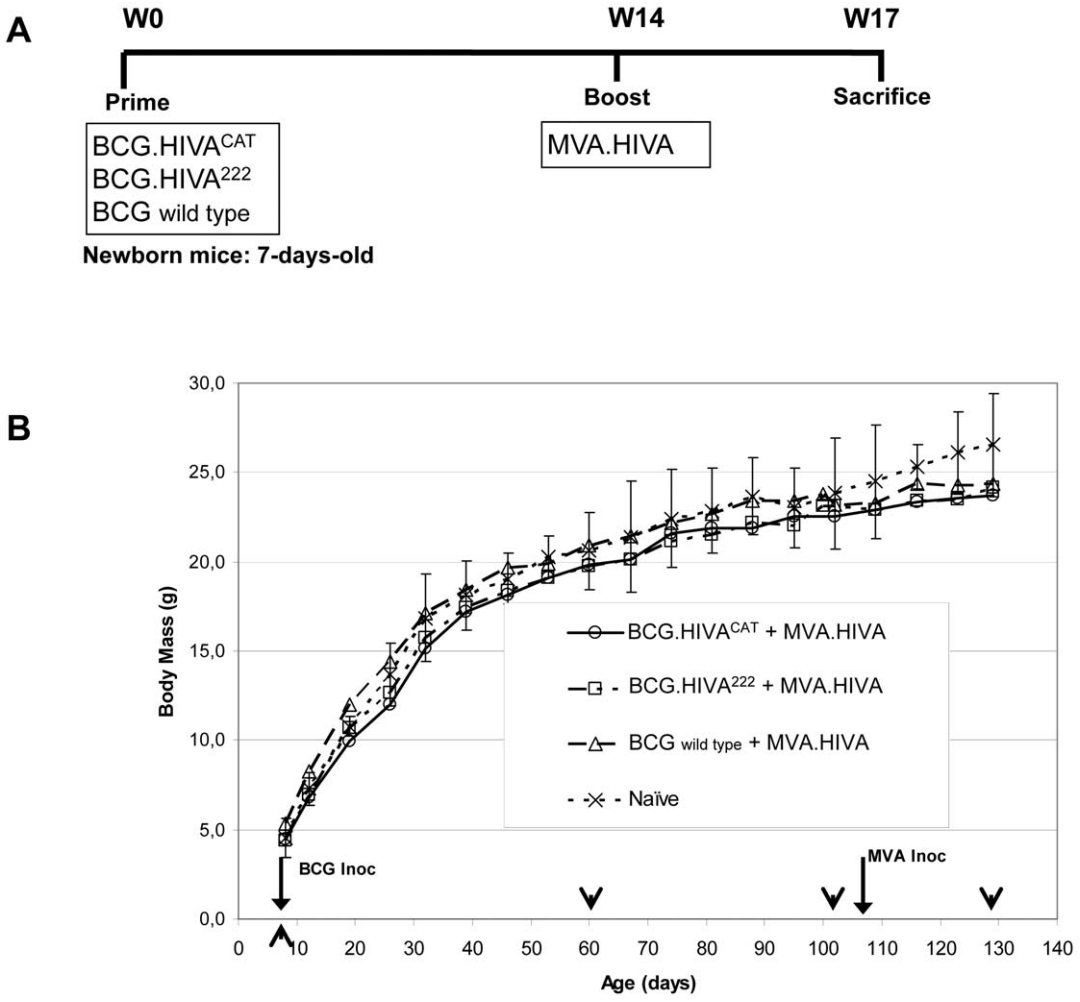

Figure 5. BCG.HIVA^{CAT} prime and MVA.HIVA boost safety in newborn mice. (**A**) Newborn mice were either left unimmunized or immunized with 2×10^6 cfu of BCG wild type, BCG:HIVA^{222} or BCG.HIVA^{CAT} by subcutaneous route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA at week 14. (**B**) The body weight was weekly recorded, and the mean for each group of mice is shown (n = 10). Data from naive mice are presented as mean ± 2 SEM (n = 6); At specific time points the weight differences between vaccinated and naïve mice group were analyzed by ANOVA test (arrowheads).
doi:10.1371/journal.pone.0042559.g005

selection and maintenance of recombinant plasmids in hosts such as *Escherichia 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 our study, we have used the Operator-Repressor Titration system (ORT) reported by Cranenburgh *et al.* [31], that utilizes *E. coli* DH1*lacdapD* strain that enables plasmid selection and maintenance that is free from antibiotics and selectable marker genes. This is achieved by using only the *lac* operator (*lacO*) sequence as a selectable element. On the other hand the *E. coli*-mycobacterial expression vector contains the lysine A complementing gene of lysine auxotroph of BCG.

Classically, identification of the individual species that comprise *Mycobacterium tuberculosis* complex (MTBC), *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. canetti*, has been based on phenotypic characteristics and biochemical tests [40]. These tests are slow, need sufficient bacterial growth, are time consuming *and the* interpretation is subjective and could provide identification errors. To overcome these problems, the use of more reliable methods based on molecular biology techniques is necessary. Several DNA-based techniques have been described for the differentiation of members of the MTBC. SpoIigotyping and other molecular methods have been useful tools for rapid species differentiation [41–43]. The RD1 deletion identification by PCR was found to be useful for *M. bovis* BCG identification [44]. Niemann *et al.* [45] have established a PCR-restriction fragment length polymorphism assay that allows rapid differentiation of *M. bovis* subsp. *bovis*, *M. bovis* subsp. c*aprae*, and *M. microti*. The same group in 2003 evaluated a commercially available DNA strip assay (Genotype MTBC) for differentiation of clinical MTBC isolates [46]. In this work, we have used also the GenoType MTBC assay to identify our recombinant *M. bovis* BCG vaccine candidate strain. The hybridization pattern obtained was unequivocal and corresponded to *M. bovis* BCG.

The conventional methods for the identification of *Mycobacterium bovis* BCG (BCG) vaccines, based on microscopic examination, biochemical tests and morphological appearance, provided only limited substrain differentiation and no specificity for BCG. The best way to identify different BCG substrains is by using molecular methods and genomic approaches. Differences between BCG substrains have been detected by i) TB genome sequencing [47]; ii) DNA microarray technology [48]; iii) PCR methods [49] and multiplex PCR identity test for BCG vaccines described by Bedwell *et al.* [38]. The PCR fingerprints produced from DNA samples were concordant with predictions based on genetic information on BCG substrains. The capability of this multiplex PCR to discriminate between BCG substrains was tested using commercial preparations and was proven also to be suitable for identification of BCG in clinical samples as well as vaccines. Specific identification of BCG isolates from a variety of clinical situations including immunosuppresed children and adults undergoing therapy for bladder cancer has been performed by using the multiplex PCR based on RD1 deletion region. In this work, we have used the multiplex PCR assay to identify our BCG.HIVA^{CAT} vaccine candidate. Resultant fingerprints after multiplex PCR assay of our BCG vaccine Pasteur substrain, were consistent with the PCR pattern of BCG Pasteur.

Our group and others have shown in murine and non-human primates studies, that rBCG elicited cell-mediated responses against HIV-1 and simian immunodeficiency virus antigens [20,24,50–52]. However, a small proportion of these animal studies used rBCG strains in heterologous prime-boost regimens. Ami *et al.* [53] 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 with attenuated virus or recombinant proteins to enhance specific T-cell responses [10,54,55]. In tuberculosis vaccine human trials, McShane *et al.* [56], have demonstrated that vaccination with MVA expressing Ag85A boosts pre-existing antimycobacterial immune responses induced either by environmental mycobacteria or BCG vaccination. Hovav *et al.* [57] have shown that priming with recombinant *Mycobacterium smegmatis* expressing HIV-1 gp120 protein induced a cellular immune response that is biased towards memory CD8$^+$ T-cells and that can expand dramatically on reexposure to an HIV-1 envelope antigen. 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 [10,24,25,58]. In addition, BCG.HIVA prime and MVA.HIVA boost-elicited HIV-1 specific CD8$^+$ T-cells exhibited effector functions such as production of IFN-γ and TNF-α. These HIV-1-specific T-cell responses were higher in adult than in newborn mice. The prime-boost regimen consistently enhanced and improved the frequency, quality and durability of the generated HIV-1 specific responses in adult and newborn mice. This improvement was observed by the detection of the highest bifunctional HIVA-specific T-cell responses and higher specific cytolytic activity in the mice that received BCG.HIVA versus BCG wild type. These data are consistent with the specific HIV-1 specific immune responses detected in this study after newborn and adult mice immunization with BCG.HIVA^{CAT} prime and MVA.HIVA boost, observing higher HIV-1 specific T-cell responses in adult than in newborn mice. In addition, in adult mice, BCG.HIVA^{CAT} primed and enhanced the MVA.HIVA.85A-elicited HIV-1-specific CD8$^+$ T-cell responses. There are few reports in the literature describing the safety and immunogenicity of rBCG expressing HIV-1 antigens in neonatal mice and neonatal non-human primates. Ranganathan *et al.* [9] 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 in neonatal mice with ΔlysA ΔsecA2 *Mtb* strain expressing HIV-1 Env rapidly generated HIV-1 and *Mtb*- specific T-cell immune responses. In the present 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.* [59] have assessed the immunogenicity of the BCG.HIVA^{222} prime and MVA.HIVA boost regimen in newborn rhesus macaques and made similar observation. On the other hand, we suggest that additional experiments should be performed in newborn mice inoculating the rBCG expressing HIV-1 antigens by different routes and different doses, because the route and dose of neonatal vaccination may provide different levels of immune activation, which may affect the efficacy of the vaccine.

Here, the vaccination with BCG.HIVA^{CAT} strain induced BCG-specific responses in adult and newborn mice. Studies in neonatal mice have indicated that immune responses at birth are often biased towards the Th2 type and defective in the Th1 type, the central defense mechanism against intracellular pathogens. However, it has been described that BCG vaccination at birth induces a potent Th1-type immune response in humans and in mice [60,61].

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, no reports have been published about safety of antibiotic-free marker recombinant BCG based HIV-1 vaccine in neonatal mice. Rosario *et al.* [59] have demonstrated that BCG.HIVA^{401} followed by two doses of MVA.HIVA in rhesus macaques was safe, not associated with systemic reactions and the local adverse events detected were considered to be consistent with a predicted response to the BCG vaccine administration, similar to that observed in human neonates. In the present study, we have demonstrated in neonatal mice (7-days-old) by the rate of body mass that BCG.HIVA^{CAT} prime and MVA.HIVA boost regimen was well tolerated.

In conclusion, we constructed and characterized a novel, safer, GLP-compatible BCG-vectored vaccine using prototype immunogen HIVA and tested the safety and immunogenicity of BCG.HIVA^{CAT}and MVA.HIVA in newborn and adult mice using the prime-boost regimen. BCG expressing a second generation immunogen HIVconsv better addressing the HIV-1 variability and escape [62] is under construction. The same strategy can be easily used for other major pediatric pathogens.

Materials and Methods

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

Parental *E. coli*-mycobacterial shuttle vector, plasmid pJH222, was kindly provided by W. R. Jacobs Jr., B.R. Bloom, and T. Hsu. The coding sequence of the HIVA gene (derived from consensus HIV-1 clade A Gag protein, an immunogen derived from an HIV-1 strain prevalent in central and eastern Africa, and a string of CD8$^+$ T-cell epitopes) was fused to the *M. tuberculosis* nucleotides coding for the 19-kDa lipoprotein signal sequence by PCR, and the chimeric gene was cloned into the pJH222 as a HindIII-HindIII fragment under the control of the *M. tuberculosis* α-antigen promoter by using standard recombinant-DNA techniques. Plasmid DNA pJH222 is a replicative (multicopy, extrachromosomal) vector that contains a DNA cassette encoding kanamycin resistance (Tn903-derived *aph* gene), an *E. coli* origin of replication (*oriE*), and a mycobacterial plasmid DNA origin of replication (*oriM*). It contains also the wild-type lysine A-complementing gene for the vector maintenance (*lysA5*) in the BCG lysine auxotroph [4]. The aminoglycoside phosphotransferase gene (aph), conferring kanamycin resistance, was removed from pJH222.HIVA plasmid by using the Operator-Repressor Titration (ORT) system, developed by Cobra Biologics, Oxford, UK [30]. Following *aph* gene excision by plasmid DNA SpeI digestion, a tighter-binding 20 bp variant of the *lacO* sequence (generated by annealing complementary oligonucleotides) was ligated and transformed into *E. coli* DH1*lacdapD* strain.

Mycobacterial strains and culture

The pJH222.HIVA plasmid DNA without kanamycin resistance gene, here designated as pJH222.HIVA^{CAT} plasmid, was transformed by electroporation in a lysine auxotroph of BCG, kindly provided by W.R. Jacobs Jr., B.R. Bloom, and T. Hsu. Mycobacterial cultures were grown in Middlebrook 7H9 broth medium or on Middlebrook agar 7H10 medium supplemented with albumin-dextrose-catalase (ADC; Difco) and 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 0.9, transformed using a Bio-Rad gene pulser electroporator at 2.5 kV, 25 mF, and 1,000 Ω, and plated onto ADC-supplemented Middlebrook agar 7H10 medium containing 0.05% Tween 80. Commercial BCG Danish 1331 strain (Pfizer), was kindly provided by Dr. Neus Altet and 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 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.6% sodium dodecyl sulphate) and 100× 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). The protein extract was blotted onto a pre-treated PVDF membrane, and HIVA protein was detected using anti-Pk monoclonal antibodies (MCA1360 AbD Serotec), with an ECL kit (Pierce). To visualize the dots, the Typhoon 8600 gel imaging system (GE Healthcare) was used.

In vivo stability of plasmid pJH222.HIVA^{CAT}

The growth of rBCG and the *in vivo* stability of the extrachromosomal plasmid pJH222.HIVA^{CAT} were established by the recovery of BCG.HIVA^{CAT} colonies from the spleens after 20 weeks after mice immunization with 10^5 cfu of BCG.HIVA-^{CAT}. Spleens were homogenized and plated onto Middlebrook 7H10 medium supplemented with ADC (Difco) and containing 0.05% Tween 80. The resulting colonies were inoculated in 7H9 medium supplemented with ADC and 0.05% Tween 80. The DNA coding sequence corresponding to HIVA immunogen was detected by PCR analysis, using the BCG liquid culture as a template.

Sample preparation for the GenoType MTBC assay and Multiplex PCR assay

For isolation of DNA from BCGwt, BCG.HIVA^{222}, and BCG.HIVA^{CAT}strains, 400 μl of mycobacterial culture were centrifuged at 13000×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 centrifugation at 10,000×g, 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.

GenoType MTBC assay for *M. bovis* BCG identification

The Mycobacterium bovis BCG strain identification was performed with a commercially available system based on DNA hybridization technology on nitrocellulose strips (GenoType MTBC; Hain Diagnostika, Nehren, Germany). The GenoType MTBC assay is based on an *M. tuberculosis* complex-specific 23S ribosomal DNA fragment, *gyrB* DNA sequence polymorphisms, and the RD1 deletion of *M. bovis* BCG. Specific oligonucleotides targeting these polymorphisms are immobilized on membrane strips. Amplicons derived from a multiplex PCR (performed using the biotynilated primers provided with the kit) react with these probes during hybridization. The Genotype MTBC assay was performed as recommended by the manufacturer. Briefly, for amplification, 35 μl of a primer nucleotide mixture (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$ and 1.25 U of HotStarTaq polymerase (Qiagen, Hilden, Germany),

and 5 µl of DNA (see above sample preparation paragraph) in a final volume of 50 µl were used. The amplification protocol consisted of 15 min of denaturation at 95°C, followed by 10 cycles comprising 30 s at 95°C and 120 s at 58°C, an additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, and a final extension at 70°C for 8 min [41]. Each biotin-labeled PCR product was denatured and hybridized to a strip with 13 specific oligonucleotide probes, using a heat-controlled washing and shaking automaton (GT-Blot 48; Hain Lifescience GmbH, Nehren, Germany). The specificity and targeted genes (in parentheses) of the probes were as follows: 1, conjugate (hybridization) control; 2, *Mycobacterium* genus-specific amplification control (23S rRNA); 3, *M. tuberculosis* complex-specific probe for identification control (23S rRNA); 4 to 12, discriminative for *M. tuberculosis* complex species (*gyrB*), and 13, *M. bovis* BCG specific probe (RD1). Six different patterns could be obtained (*M. tuberculosis* or *M. canettii*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. caprae*, and *M. microti*). A template sheet showing the positions of the lines and the interpretation table, both provided with the kit, were used for interpretation of the test results.

Multiplex PCR assay for M. bovis BCG Substrain Pasteur Identification

The multiplex PCR was performed using 13 primers [38] targeting the SenX3–RegX3 system (C3 and C5) and the BCG deletion regions including RD1 (ET1-3), RD2, RD8, RD14 and RD16 regions. For the PCR analysis, 5 µl of the DNA (see above sample preparation paragraph) isolated from BCG.HIVA^{CAT} Pasteur strain and BCG Danish 1331 strain was used in a final volume of 50 µl with the following amplification protocol : 1 cycle at 94°C (10 min) and 30 cycles at 94°C (1 min), 55°C (1 min) and 72°C (2 min), and 1 cycle at 72°C (10 min). The PCR products were analyzed in a 3% (w/v) agarose gel electrophoresis. The PCR fingerprints of BCG Pasteur and BCG Danish substrains were consistent with previously published results on genetic information of BCG substrains [38].

Mycobacterial plasmid DNA extraction

The rBCG broth culture up to an OD of 0.9 (600 nm) from Master Seed and Working Stock, was used for mycobacterial plasmid DNA isolation. The QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used with slight modifications :i) prior to harvest (3 to 24 hours), glycine at a final concentration of 1% (w/v) 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 1:1. The mycobacterial plasmid DNA isolated was used for restriction enzyme analysis.

Mice immunizations and isolation of splenocytes

Adult (7-weeks-old) and newborn (7-days-old) female BALB/c mice were immunized with BCG.HIVA^{CAT}, and were boosted with MVA.HIVA or MVA.HIVA.85A 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) using a 5-ml syringe rubber plunger. Following the removal of red blood cells with ACK lysing buffer (Lonza), the splenocytes were washed and resuspended in complete medium (R10 [RPMI 1640 supplemented with 10% fetal calf serum and penicillin-streptomycin], 20 mM HEPES, and 15 mM 2-mercaptoethanol).

Ethics statement

The animal experiments were approved by the local Research Ethics Committee (Clinical Medicine, School of Medicine, University of Barcelona and University of Oxford) and by the Ethical Committee for animal experimentation from University of Barcelona and University of Oxford. All animal procedures and care conformed strictly to Catalonia (Spain) and to United Kingdom Animal Welfare legislation.

Ex Vivo IFN-γ ELISPOT Assay

The ELISPOT assay was performed using a commercial IFN-γ ELISPOT kit (Mabtech, Sweden). The ELISPOT plates (Millipore, MSISP4510, 96 wells plates with PVDF membranes) were coated with purified antimouse IFN-γ capture monoclonal antibody diluted in PBS to a final concentration of 5 µg/ml at 4°C overnight. The plates were washed once in R10 and blocked for 2 h with R10. A total of 5×10^5 fresh splenocytes were added to each well, stimulated with 5 µg/ml of PPD 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).

Intracellular Cytokine Staining

One million splenocytes were added to each well of a 96-well round-bottomed plate (Costar) and pulsed with 2 µg/ml of P18-I10 peptide (RGPGRAFVTI) [23] and kept at 37°C, 5% CO_2 for 60 minutes, followed by the addition of GolgiStop (BD Biosciences) containing monensin. After a further 5-hour incubation, reaction was terminated by storing the plate at 4°C. The cells were washed with FACS wash buffer (PBS, 2% FCS, 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, anti TNF-α-PE and anti IL-2 Alexa Fluor 647 (BD Biosciences). Cells were fixed with CellFIX (BD) and stored at 4°C until analysis.

Fluorescence-Activated Cell Sorter Analysis

All chromogen-labeled cells were analyzed in a Becton Dickinson FACScalibur, using the CellQuest software for acquisition (BD Biosciences) and the Flow-Jo software (Tri-Star) for analysis.

Statistical analysis

Immunogenicity data are shown as group medians as well as individual responses. The body mass data are group means, and mean ± 2 SD in naïve mice group. Statistical significance was determined by ANOVA (* = $p<0.05$).

Acknowledgments

The authors are grateful to Dr. Barry R. Bloom and Dr. William R. Jacobs for providing the plasmid DNA pJH222 and BCG wild type and lysine

auxotroph of BCG for research purposes; Rocky Cranenburgh and Cobra Biology for providing us the Operator-Repressor Titration technology; Dr. Joana Visa and Dr. Rosa Bonavia for animal handling assistance; Ana Donate from Hain Lifescience Spain, for training assistance on GenoType MTBC assay; We thank Raquel Fernandez for her temporary input and feedback during the EDCTP fellowship.

N. Saubi is a Red Temática de Investigación Cooperativa en SIDA (RIS) Senior Fellow and is ascribed to the Universitat Autònoma de Barcelona Ph.D. programme.

Author Contributions

Conceived and designed the experiments: JJ NS. Performed the experiments: NS AMM EGM MR. Analyzed the data: JJ NS TH. Contributed reagents/materials/analysis tools: JMG TH. Wrote the paper: JJ NS.

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ANNEX III: Paper Number 3

Saubi N, Gea-Mallorquí E, Ferrer P, Hurtado C, Sánchez-Úbeda S, Eto Y, Gatell JM, Hanke T, Joseph J. **Engineering new mycobacterial vaccine design for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG**. Mol Ther Methods Clin Dev. 2014 May 21;1:14017. doi: 10.1038/mtm.2014.17. eCollection 2014. Nature Publishing Group.

Citation: Molecular Therapy — Methods & Clinical Development (2014) **1,** 14017; doi:10.1038/mtm.2014.17

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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.

Molecular Therapy — Methods & Clinical Development (2014) **1**, 14017; doi:10.1038/mtm.2014.17; published online 21 May 2014

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* M15Δ*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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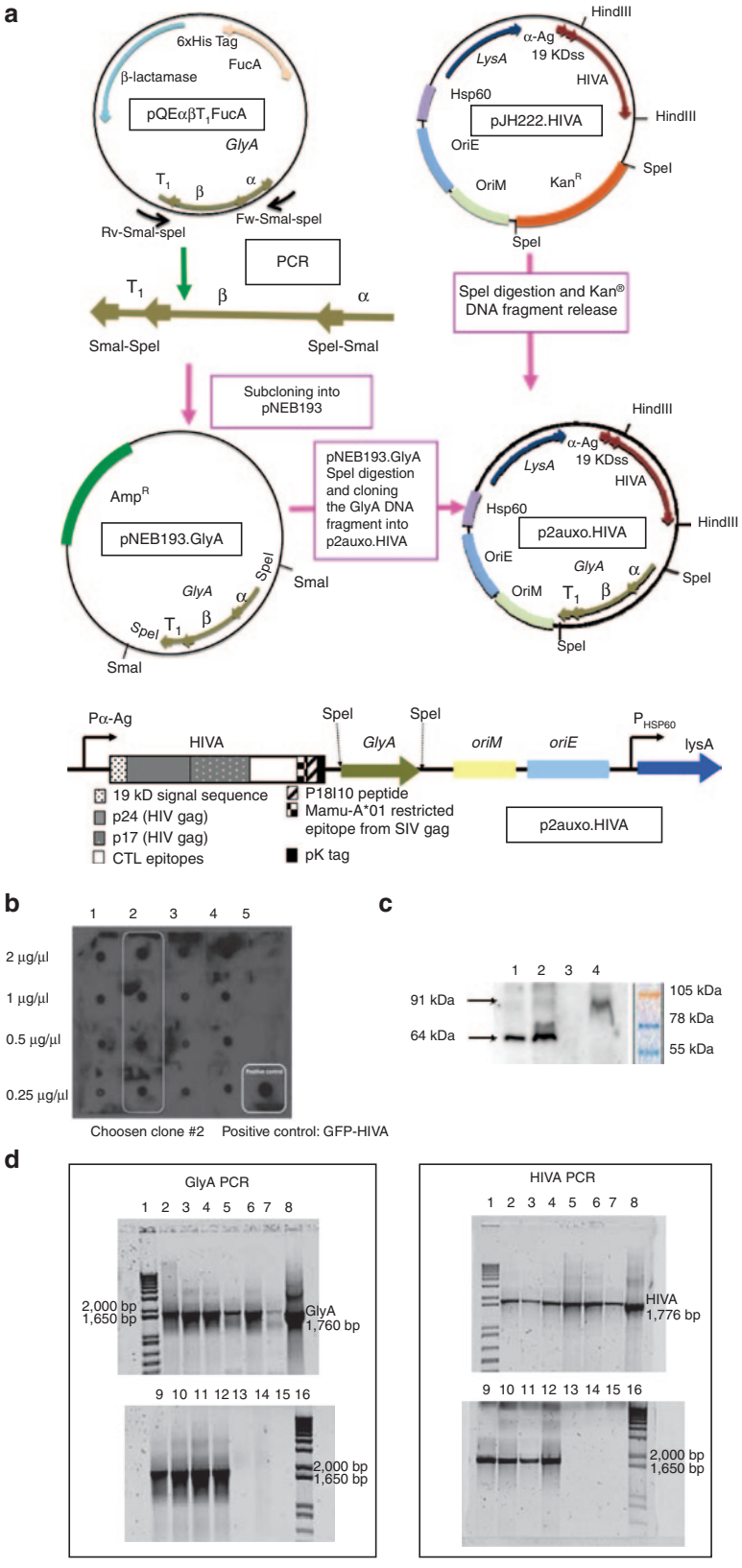
Received 15 November 2013; accepted 26 March 2014



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 *ΔlysA5*::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* M15ΔglyA 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; P*HSP60*, heat shock protein 60 gene promoter. The *aph* gene was removed by *Spe*I 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-GFP^{222} 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-GFP^{222} (pJH222 *E. coli*–mycobacterial shuttle plasmid, kanamycin resistance) used as a positive control. (**d**) *In vivo* plasmid stability of BCG.HIVA^{auxo} 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 (pQEαβT$_1$FucA and pJH222.HIVA plasmid DNA); lanes 13 and 14: BCG wild type; lane 15: distilled water (negative control).

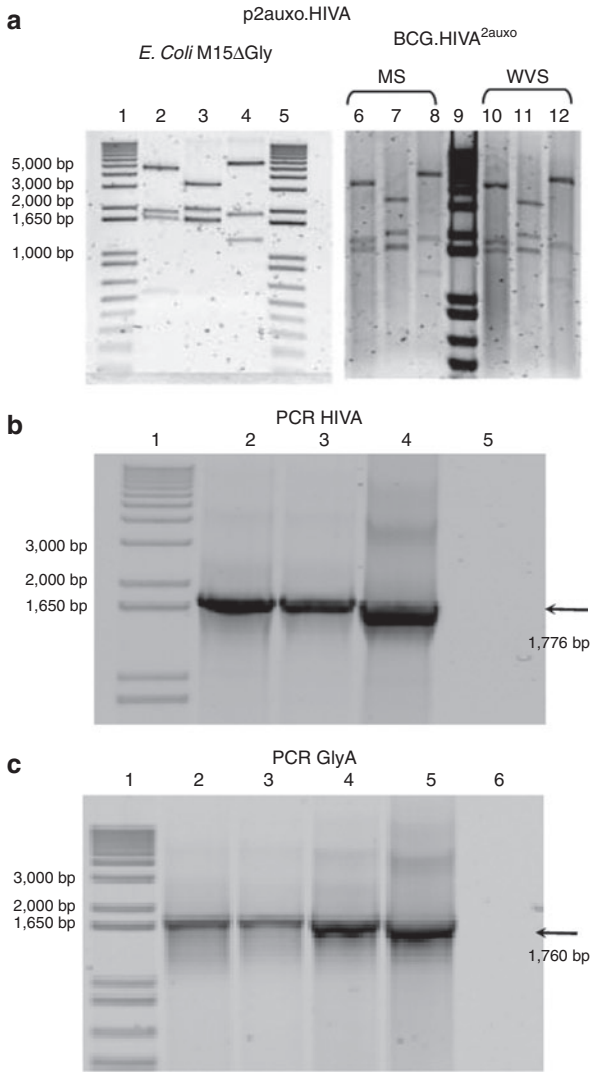


Figure 2 Genetic characterization of the BCG.HIVA^{2auxo} strain. (**a**) Enzymatic restriction analysis of p2auxo.HIVA plasmid DNA extracted from *E. coli* M15Δ*glyA* cultures (pre-BCG transformation) and from both the MS and WVS of BCG.HIVA^{2auxo} cultures. Left side: *E. coli* cultures. Lanes 1 and 5: molecular weight marker (1 kb plus; Invitrogen); lanes 2, 3, and 4: *Age*I, *Stu*I, and *Xho*I digestion, respectively. Right side: BCG cultures. Lane 9: molecular weight marker (1 kb plus; Invitrogen); lanes 6, 7, and 8 (MS): *Age*I, *Stu*I, and *Xho*I digestion, respectively. Lanes 10, 11, and 12 (WS): *Age*I, *Stu*I, and *Xho*I 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 *Hind*III 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 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 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 10^6 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 ± 2 SD body mass

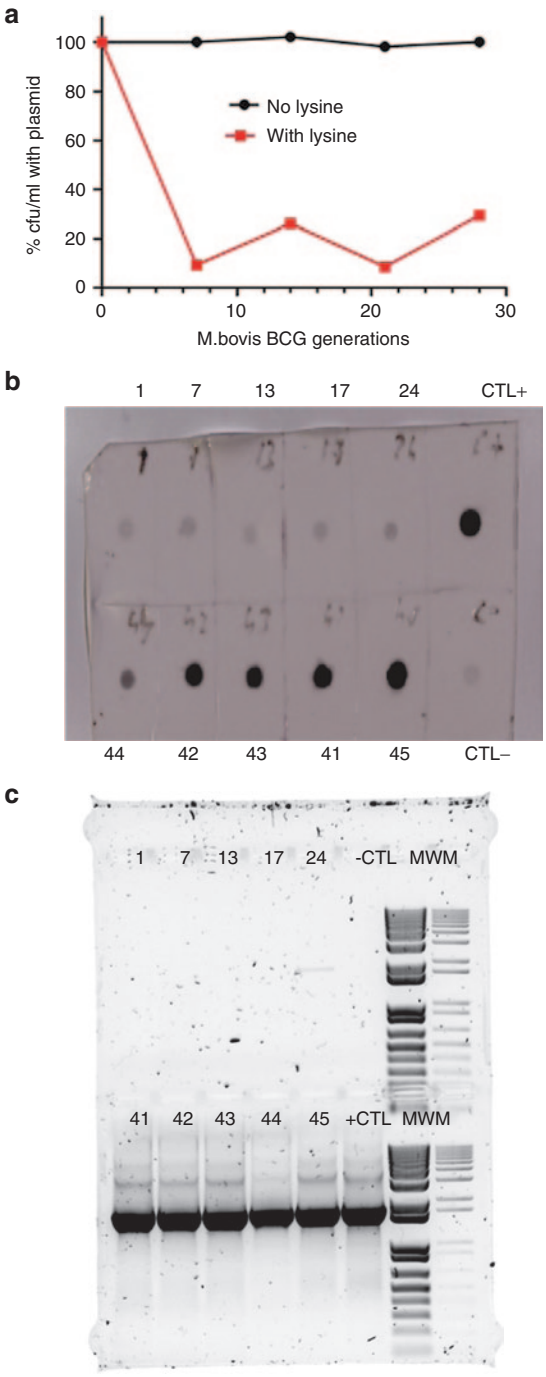


Figure 3 *In vitro* stability of the BCG.HIVA^{2auxo} strain. (**a**) *In vitro* persistence of the p2auxo.HIVA in BCG *lysA*- grown for successive passages on selective (no lysine) or nonselective (supplemented with lysine) media. The percentage represents the cfu (titer) that maintained the vector 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 substrain 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 coexpressing *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

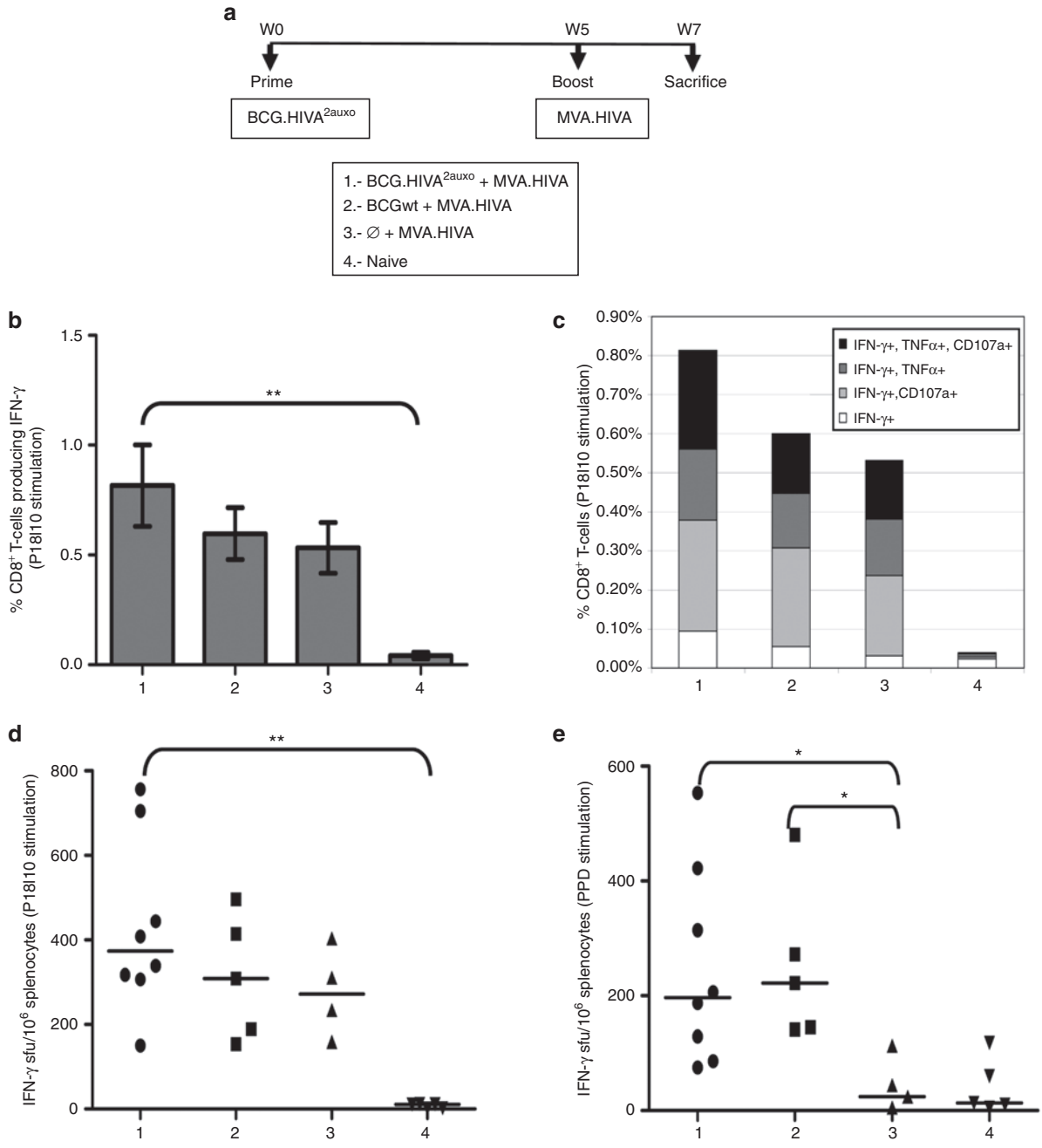


Figure 4. Induction of HIV-1- and *Mtb*-specific T-cells responses by the BCG.HIVA^{2auxo} prime–MVA.HIVA boost regimen. (**a**) Adult mice (7-weeks-old) were either left unimmunized or primed with 10^6 cfu of BCG.HIVA^{2auxo} or BCG wild type (intradermally) and boosted with 10^6 pfu of MVA.HIVA (intramuscularly) 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–I10-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–I10 CD8$^+$ T-cell epitope peptide. The median spot-forming units (SFU) per 10^6 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 10^6 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

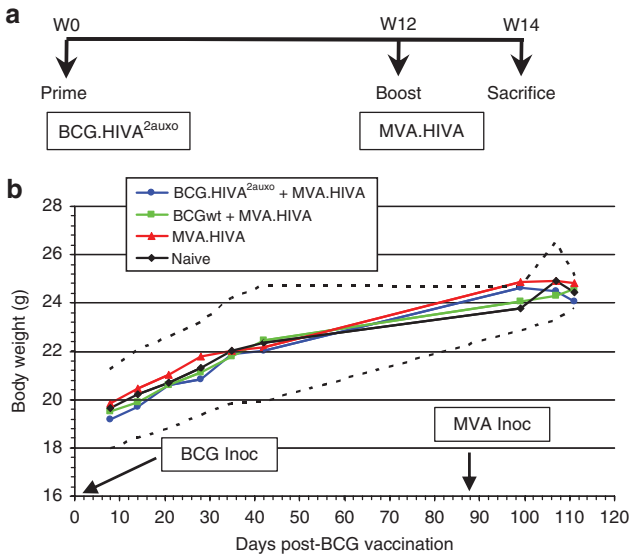


Figure 5 BCG.HIVA^{2auxo} prime and MVA.HIVA boost safety in adult mice. (**a**) Adult mice were either left unimmunized or immunized with 10^6 cfu of BCG wild type or BCG.HIVA^{2auxo} by intradermal route and subsequently given a booster dose of 10^6 pfu of MVA.HIVA at week 12 by intramuscular route. (**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 ± 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 mc^2 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.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.[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.HIVA^{222} 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 escape[42] 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 *Spe*I digestion. Following *aph* gene excision, the *glyA* gene cassette was ligated into this plasmid and subsequently transformed into *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 carried out mainly by serine hydroxymethyl transferase enzyme. The αβT1 DNA fragment was amplified by PCR using the pQEαβT1FucA plasmid DNA as template.[44] The primers were designed to incorporate *Spe*I and *Sma*I 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 *Spe*I, and the released αβT1 DNA fragment was cloned into pJH222.HIVA vector (lacking the kanamycin resistance gene after *Spe*I 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 µg/ml). *E. coli* M15Δ*glyA* cells (glycine auxotrophic)[20] were grown in Luria-Bertani or 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), supplemented with glycine (70 µg/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 µg/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× 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 Δ*lys*A 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 (*Hind*III–*Hind*III, 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 13,000*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 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–I10 (RGPGRAFVTI).[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 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. Wells were washed 4× with phosphate-buffered saline 0.05% Tween 20 and 2× 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 µg/ml of P18–I10 peptide and kept at 37 °C, 5% CO_2 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 ± 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.

ACKNOWLEDGMENTS

The authors are grateful to Barry R. Bloom and William R. Jacobs for providing the plasmid DNA pJH222 and BCG wild type and lysine auxotroph of BCG for research purposes; N.S. is a Red Temática de Investigación Cooperativa en SIDA (RETIC-RIS) senior fellow and is ascribed to the Universitat Autònoma de Barcelona PhD program. This research was supported by FIS PI11/00284, RIS, and HIVACAT.

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ANNEX IV: Patent

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 March 2014 (06.03.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/032835 A1

(51) International Patent Classification:
C12N 15/63 (2006.01)

(21) International Application Number:
PCT/EP2013/063978

(22) International Filing Date:
2 July 2013 (02.07.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12382336.1 31 August 2012 (31.08.2012) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2014/032835 A1

(54) Title: MYCOBACTERIUM COMPRISING EXPRESSION VECTOR WITH TWO AUXOTROPHIC SELECTION MARKERS AND ITS USE AS VACCINE

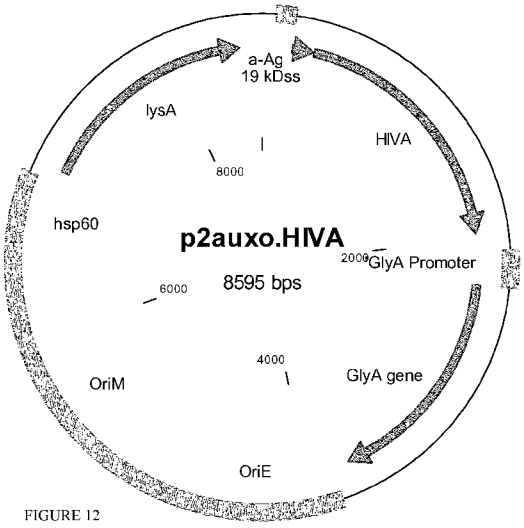

FIGURE 12

(57) Abstract: The invention relates to polynucleotides and recombinant cell strains comprising said polynucleotides and the uses thereof for the delivery of the polypeptides encoded by said polynucleotides to a subject in need thereof. In particular, the invention refers to polynucleotides comprising a polypeptide of interest, auxotrophy-complementing genes and the use thereof in a mycobacterial double auxotrophic host cell to achieve the stable expression of the polypeptide of interest by using an antibiotic- free plasmid selection system.

MYCOBACTERIUM COMPRISING EXPRESSION VECTOR WITH TWO AUXOTROPHIC SELECTION MARKERS AND ITS USE AS VACCINE

Field of the Invention

The invention relates to the field of immunology and, more in particular, to methods and compositions for inducing an immune response against an antigen of interest by administering said antigen through a recombinant mycobacterial double auxotrophic strain. The invention relates also to methods for expressing a gene of interest by utilizing a mycobacterial double auxotrophic strain.

Background of the Invention

Vaccines are the most cost-effective intervention to prevent disease. *Mycobacterium bovis* BCG offers great potential for innovative approaches for the development of polyvalent vaccines. Novel vaccine candidates, such as HIV-1 related immunogens, could use BCG as a live bacterial vaccine vehicle to elicit more effective cellular and humoral responses.

There is strong evidence supporting a role of cytotoxic T lymphocytes (CTLs) in the containment of HIV replication and several vaccine approaches are being pursued to elicit anti-HIV CTL responses. CTL induction against HIV-1 and simian immunodeficiency virus (SIV) *gag* or *env* antigens has been described following the immunization of mice or *rhesus* monkeys with recombinant BCG (rBCG) expressing these antigens. *See* Ohara N, *et al.*, Vaccine 2001; 19:4089-4098). More recently, recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) expressing HIVA immunogen has been generated and shown to be stable and to induce durable, high-quality HIV-1-specific CD4+ and CD8+ T-cell responses in BALB/c mice. Furthermore, when the recombinant BCG vaccine was used in a prime-boost regimen with heterologous vectors expressing the same HIV immunogen, the HIV-1-specific responses provided protection against surrogate virus challenge, and the recombinant BCG vaccine alone protected against aerosol challenge with *M. tuberculosis*. *See* Im E, *et al.*, J. Virol. 2007; 81:9408-9418.

Critical issues to be considered in developing rBCG technology include: i) antigen localization, ii) codon optimization and iii) *in vivo* plasmid DNA stability and

genetic rearrangements. *In vivo* genetic stability and persistence are of special importance for the use of live bacterial vaccines; a mutant BCG which is rapidly eliminated is unlikely to be an effective vaccine. It is known that BCG undergoes significant genetic rearrangements. Recent evidence suggests that major recombination events resulting in the duplication of large segments of its chromosome have occurred and are still occurring. Thus, if BCG is to be used as a live bacterial delivery system for generating novel vaccine candidates and as an immunotherapeutic agent, it is essential that a more genetically stable strain be developed.

The possibility of inserting foreign genes into the chromosome at precise positions to ensure the persistence of the heterologous genetic information in the recombinant vaccine strains would represent a crucial step in the development of *Mycobacterium bovis* BCG as a live bacterial delivery system for expression of heterologous antigens. Accordingly, there is a need for better vaccine delivery systems based on *Mycobacterium bovis* BCG which could overcome the problems of the vaccines described in the art.

Summary of the Invention

The present invention provides a polynucleotide that can be expressed in a recombinant host cell and used for the delivery of a polypeptide of interest to a subject in need thereof. Typically, the propagation of the polynucleotide is carried out in a conventional host cell such as *E. coli*, whereas the expression for therapeutic delivery is carried out in mycobacterial cells. Therefore, it is convenient that the polynucleotide of the invention further comprises elements which allow the selection of those cells carrying the polynucleotide from those which do not contain said polynucleotide. Historically, antibiotic resistance genes have usually been used as selection marker of recombinant bacteria and maintenance of recombinant plasmids vectors in *E. coli* and mycobacteria. However, the use of antibiotic resistance genes in strains aimed for therapy in humans prevents their use for commercial purposes. Furthermore, the presence of recombinant antibiotic resistances in microorganisms is limited to preclinical studies and Phase I clinical trials. Microorganisms must be re-designed and modified to eliminate antibiotic resistance. Furthermore, immunogenicity, efficacy and

safety assays should be repeated, to ensure the complete bio-equivalence of the newly developed microorganism.

An alternative approach to the use of antibiotic selection markers in *E. coli* and mycobacteria is the use of genes necessary for the synthesis of a specific auxotrophic marker which allows cells auxotrophic for said marker to grow in minimal media in the absence of said auxotrophic marker. On the other hand, the approach auxotrophy/complementing gene could increase the plasmid stability *in vitro* and *in vivo* and could prevent the heterologous gene expression disruption by genetic rearrangements. Thus, the present invention addresses the need for *Mycobacteria spp* strains that are stable for clinical use and that may be useful for administering a polypeptide of interest to a subject in need thereof. The solution to this problem is attained by transforming a *Mycobacteria spp* strain cell, such as the *Mycobacterium bovis* BCG Pasteur strain cell, with a vector comprising the nucleotide sequence of the polypeptide of interest and wherein the strain carries an auxotrophy which is complemented by an auxotrophy complementing gene present forming part of the same vector as the vector which comprises the nucleotide sequence encoding a polypeptide of interest.

Thus, in a first aspect, the invention relates to a polynucleotide comprising:

(i) a sequence encoding a polypeptide of interest,

(ii) a first auxotrophy complementing gene which confers an auxotrophic host strain carrying said gene the capability of growing in a medium that lacks a first auxotrophic factor, and

(iii) a second auxotrophy complementing gene which confers an auxotrophic host strain carrying said gene the capability of growing in a medium that lacks a second auxotrophic factor.

In a second aspect, the invention relates to a polynucleotide comprising

(i) a sequence encoding a polypeptide of interest,

(ii) a mycobacterial origin of replication, and

(iii) at least one selection marker,

wherein said polynucleotide does not comprise any nucleotide sequence conferring antibiotic sensitivity or antibiotic resistance to a cell carrying said polynucleotide.

In a further aspect, the invention refers to a vector comprising the polynucleotide of the invention.

In a further aspect, the invention relates to a recombinant cell comprising the polynucleotide or vector of the invention.

In a further aspect, the invention relates to a recombinant cell according to the invention for use in medicine and, in particular, for use in the treatment of a disease which requires the expression of a polypeptide of interest.

In another aspect, the invention relates to a vaccine composition comprising the bacterium according to the invention.

In a further aspect, the invention relates to a vaccine composition according to the invention for use in medicine.

In a further aspect, the invention relates to a vaccine composition according to the invention for use in inducing an immune response against an antigenic polypeptide.

Finally, the invention relates to a method for the expression of a polypeptide of interest in a Mycobacterium host cell which comprises:

(i) growing a Mycobacterium host cell comprising a sequence encoding the polypeptide of interest under the operative control of a weak mycobacterium promoter under conditions adequate for expression of the polypeptide of interest and, optionally,

(ii) recovering the polypeptide of interest from the culture.

Deposit of Microorganisms

The plasmid p2auxo.HIVA and the bacterial strain BCG.HIVA^{2auxo} were deposited on July 31st, 2012 at Deutsche Sammlung vor Mikroorganismen und Zellculturen GmbH (DSMZ), Inhoffenstraße 7 B, DE-38124, Braunschweig, Federal Republic of Germany and were assigned accession nos. DSM 26305 and DSM 26306, respectively.

Brief Description of the Figures

Figure 1. PCR of the αβT1 fragment corresponding to promoter + *E. coli* glyA gene + T1 termination sequence, flanked with SmaI-SpeI restriction enzyme target sites.

Figure 2. SmaI digestion of the αβT1 PCR fragment and the pNEB193 vector.

Figure 3. Transformation in *E. coli* of ligation product: αβT1 PCR fragment and pNEB193 vector. Obtention of pNEB193-αβT1 (L2-4 *E. coli* colony). **A.** *E. coli* colonies obtained after transformation of *E. coli* DH5-α with pNEB193-Sma1- αβT1 and selection in Amp-LB plates. **B.** *E. coli* colony identification: digestion of the plasmid DNA extracts with SmaI restriction enzyme. **C.** *E. coli* colony identification: digestion of the plasmid DNA extracts with SpeI restriction enzyme.

Figure 4. pHIVACAT-1 parental plasmid DNA digested with SpeI, to release the kanamycin resistance gene, and purify the larger fragment for ligation (6815bp).

Figure 5. A. Ligation of p-HIVACAT-I (SpeI) + αβT1-SpeI(from pNEB193-αβT1). **B.** Selection of recombinant *E. coli* M15Δgly colonies in M9 agar plates (glycine defective)

Figure 6. 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 work are depicted. P α-Ag, *M. tuberculosis* α-antigen promoter; P*HSP60*, 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 M15ΔGly* 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-GFP^{222} were used as positive control. **C.** *In vivo* plasmid stability of BCG.HIVA^{auxo} harbouring p2auxo.HIVA. Mice were injected intradermically with 10^6 cfu of BCG.HIVA^{2auxo} and boosted i.m. with 10^6 pfu of MVA.HIVA. Spleens were homogenized 7 weeks after BCG inoculation and the recovered rBCG colonies were tested for the presence of the *E. coli glyA* and HIVA DNA coding sequence by PCR. Lanes 2-7 and 9-12: ten rBCG colonies were recovered in the non-lysine supplemented plate; lane 1 and 16: molecular weight marker; lane 8: Plasmid DNA positive control (pQEαβT$_1$FucA and pJH222.HIVA plasmid DNA); lane 13 and 14: BCG wild type; lane 15: Distilled water (negative control).

Figure 7. Genetic characterization of the BCG.HIVA^{2auxo} strain. **A.** The BCG.HIVA^{2auxo} Pasteur substrain identification by multiplex PCR assay. Lane 1 and 7: molecular weight marker (1kb plus and 100 bp respectively, Invitrogen); lane 2:

BCG.HIVA^{2auxo} Master Seed (MS); lane 3: BCG.HIVA^{2auxo} Working Vaccine Stock (WVS); lane 4: BCG wild type Pasteur substrain; lane 5: BCG Connaught substrain; lane 6: BCG Danish substrain. **B.** Enzymatic restriction analysis of p2auxo.HIVA plasmid DNA extracted from *E. coli* M15Δgly (pre-BCG transformation) and from both the MS and the WVS of BCG.HIVA^{2auxo} cultures. Left side: *E. coli* cultures. Lane 1 and 5: molecular weight marker (1kb plus, Invitrogen); lane 2, 3 and 4: AgeI, Stu I and Xho I digestion, respectively. Right side: BCG cultures. Lane 9: molecular weight marker (1kb plus, invitrogen); lane 6, 7, 8 (MS): AgeI, Stu I and Xho I digestion, respectively. Lane 10,11 and 12 (WVS): AgeI, Stu I and Xho I digestion, respectively. **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). **D.** 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).

Figure 8. Confirmation of positive *E. coli* M15 Δgly colony harboring the p2auxo.HIVA plasmid DNA (LL4-1).

Figure 9. Transformation of p2auxo.HIVA into BCG lysA- strain. **A.** rBCG colonies obtained after transformation of BCG lysA – strain with p2auxo.HIVA and selection in lysine deficient medium. **B.** BCG Colony identification: PCR of HIVA and *E. coli* glyA DNA fragment.

Figure 10. Phenotypic characterization of the *E. coli* M15 ΔglyA strain and BCG.HIVA^{2auxo} vaccine strain. The phenotype of glycine auxotrophy, glycine complementation and kanamycin resistance of *E. coli* M15ΔGly strain was assessed. The phenotype of lysine auxotrophy, lysine complementation and kanamycin resistance of BCG.HIVA^{2auxo} strain was assessed as well. **A**. *E. coli* glycine auxotroph strain plated on non-glycine supplemented M9-D agar plate. **B**. *E. coli* glycine auxotroph strain plated on glycine supplemented M9-D agar plate. **C.** *E. coli* M15ΔglyA strain harbouring the p2auxo.HIVA plasmid DNA and plated on M9-D agar plates without glycine and kanamycin supplementation. **D.** *E. coli* M15ΔglyA strain harbouring the

p2auxo.HIVA plasmid DNA plated on M9-D agar plate without glycine supplementation and with kanamycin. **E.** BCG lysine auxotroph strain plated on non-lysine supplemented 7H10. **F.** BCG lysine auxotroph strain plated on lysine supplemented 7H10. **G.** BCG.HIVA^{2auxo} plated on 7H10 without lysine and kanamycin supplementation. **H.** BCG.HIVA^{2auxo} plated on 7H10 without lysine and with kanamycin supplementation.

Figure 11. Schematic representation of the steps involved in the construction of p2auxo.HIVA plasmid DNA and BCG.HIVA^{2auxo} strain.

Figure 12. p2auxo.HIVA plasmid DNA map.

Figure 13. 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 10^6 cfu of BCG.HIVA^{2auxo} or BCG wild type (intradermally), and boosted with 10^6 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 multicolour intracellular cytokine staining assay. The group mean frequencies of single-, double- or triple-cytokine-producing P18I10-specific cells are shown for the four vaccination groups. **D.** Elicitation of specific T-cell responses was assessed in an *ex vivo* IFN-γ ELISPOT assay using the immuodominant P18I10 $CD8^+$ T-cell epitope peptide. The median spot-forming units (SFU) per 10^6 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.** 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 spot-forming units (SFU) per 10^6 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. * =$p< 0.05$, ** =$p< 0.01$.

Figure 14. 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 5 by intramuscular route. **B.** The body weight was weekly recorded, 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 ± standard deviation (SD, n=5). The weight differences between vaccinated and naïve mice group were analyzed weekly by ANOVA test.

Figure 15. **A**. p2auxo.CSP plasmid DNA map. CSP: Circumsporozoite protein from *Plasmodium berghei.* First, the plasmid DNA p2auxo.HIVA was digested by HindIII restriction enzyme, and the HIVA DNA coding sequence was released. Second, the CSP immunogen DNA coding sequence, was amplified by PCR using specific primers and HindIII extension sites and was inserted into p2auxo vector.

Figure 16. **A**. p2auxo.Ag85B plasmid DNA map. Ag85B from *Mycobacterium bovis.* First, the plasmid DNA p2auxo.HIVA was digested by HindIII restriction enzyme, and the HIVA DNA coding sequence was released. Second, the Ag85B immunogen DNA coding sequence, was amplified by PCR using specific primers and HindIII extension sites and was inserted into p2auxo vector.

Figure 17. **A**. p2auxo.HIVc(G+C) plasmid DNA map. The HIVc DNA sequence was BCG codon optimized and synthesized *in vitro* and cloned into pGH plasmid DNA (Biomatik,USA). First, the plasmid DNA p2auxo.HIVA was digested by HindIII restriction enzyme, and the HIVA DNA coding sequence was released. Second, the HIVc immunogen DNA coding sequence was released by HindIII digestion and inserted into p2auxo vector.

Detailed Description of the Invention

1. Definitions of general terms and expressions

The term "α-antigen promoter", as used herein, refers to the promoter region of the gene encoding a mycobacterium α-antigen and corresponds to a polynucleotide comprising the minimal region of the upstream region of the α-antigen gene which is suitable for efficient promoter activity as in SEQ ID NO:027 for *Mycobacterium tuberculosis* and equivalent regions in the α-antigen gene of other mycobacteria (e.g. *M.*

Claims

1. A polynucleotide comprising
(i) a sequence encoding a polypeptide of interest,
(ii) a mycobacterial origin of replication, and
(iii) at least one selection marker,
wherein said polynucleotide does not comprise any nucleotide sequence conferring antibiotic sensitivity or antibiotic resistance to a cell carrying said polynucleotide.
2. A polynucleotide, as defined in claim 1, wherein the mycobacterial origin of replication is *oriM*.
3. A polynucleotide, as defined in any of claims 1 or 2, wherein the at least one selection marker is a first auxotrophy complementing gene which confers an auxotrophic host strain carrying said gene the capability of growing in a medium that lacks a first auxotrophic factor.
4. A polynucleotide, as defined in any of claims 1 to 3, further comprising a second origin of replication from a prokaryotic organism.
5. A polynucleotide, as defined in claim 4, wherein the second origin of replication is *oriE*.
6. A polynucleotide, as defined in any of claims 1 to 5, further comprising a second auxotrophy complementing gene which confers an auxotrophic host strain carrying said gene the capability of growing in a medium that lacks a second auxotrophic factor.
7. A polynucleotide comprising:
(i) a sequence encoding a polypeptide of interest,
(ii) a first auxotrophy complementing gene which confers an auxotrophic host strain carrying said gene the capability of growing in a medium that lacks a first auxotrophic factor, and
(iii) a second auxotrophy complementing gene which confers an auxotrophic host strain carrying said gene the capability of growing in a medium that lacks a second auxotrophic factor.

8. The polynucleotide, as defined in claim 8, wherein said polynucleotide does not comprise any nucleotide sequence conferring antibiotic sensitivity or antibiotic resistance.

9. The polynucleotide, as defined in any of claims 1 to 8, wherein the sequence encoding the polypeptide of interest is under the operative control of a heterologous weak mycobacterium promoter.

10. The polynucleotide, as defined in claim 9, wherein the weak mycobacterium promoter is the *Mycobacteria spp* α-antigen promoter or a functionally equivalent variant thereof.

11. The polynucleotide, as defined in any one of claims 1 to 10, wherein the sequence encoding the polypeptide of interest is fused in frame to a sequence encoding a signal sequence active in mycobacteria.

12. The polynucleotide, as defined in claim 11, wherein the signal sequence corresponds to the signal sequence of *Mycobacterium tuberculosis* 19 KDa lipoprotein or a functionally equivalent variant thereof.

13. The polynucleotide, as defined in any one of claims 1 to 12, wherein the polypeptide of interest comprises an immunogenic polypeptide and an endosomalytic polypeptide.

14. The polynucleotide, as defined in claim 13, wherein the immunogenic polypeptide comprises a HIV polypeptide, a *Mycobacteria spp* polypeptide, a *Plasmodium falciparum* epitope, a *Plasmodium berghei* epitope or an immunologically active epitope thereof.

15. The polynucleotide, as defined in claim 14, wherein the HIV polypeptide is gp120 or HIVA or HIV-c.

16. The polynucleotide, as defined in claim 14, wherein the *Mycobacteria spp* polypeptide is Ag85B from *Mycobacterium bovis* BCG.

17. The polynucleotide, as defined in claim 14, wherein the *Plasmodium berghei* epitope is circumsporozoite protein (CSP) from *Plasmodium berghei.*

18. The polynucleotide, as defined in any one of claims 13 to 17, wherein the endosomalytic polypeptide comprises *Listeria monocitogenes* listeriolysin, *Clostridium perfringens* perfingolysin, *Mycobacterium tuberculosis* phospholipase C or a variant thereof.

19. The polynucleotide, as defined in any one of claims 1 to 18, wherein the first auxotrophy gene is a gene capable of complementing a mycobacterium lysine auxotrophy or the second auxotrophy gene is an *E. coli* gene capable of complementing *E. coli* glycine auxotrophy.
20. The polynucleotide, as defined in claim 19, wherein the gene capable of complementing a mycobacterium lysine auxotrophy is the lysA gene or wherein the gene capable of complementing an *E. coli* glycine auxotrophy is the glyA gene.
21. A vector comprising a polynucleotide as defined in any one of claims 1 to 20.
22. The vector, as defined in claim 21, wherein said vector is an *E. coli*/mycobacterial shuttle vector.
23. The vector, as defined in claim 22, wherein the *E.coli*/mycobacterial shuttle vector is an extrachromosomal vector.
24. The vector, as defined in any of claims 21 to 23, wherein said vector is the plasmid p2auxo.HIVA (DSM 26305), the plasmid p2auxo.CSP, the plasmid p2auxo.Ag85b or the plasmid p2auxo.HIVc(G+C).
25. A bacterium comprising a polynucleotide according to any one of claims 1 to 20 or a vector according to any one of claims 21 to 24.
26. A bacterium, as defined in claim 25, wherein the bacterium carries an auxotrophy which is complemented by the first or second auxotrophy complementing gene.
27. The bacterium, as defined in any of claims 25 or 26, wherein the bacterium is a *Mycobacteria spp.*
28. The bacterium, as defined in claim 27, wherein the *Mycobacteria spp.* is *Mycobacterium bovis* or *Mycobacterium smegmatis*.
29. The bacterium, as defined in claim 28, wherein the bacterium is *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG).
30. The bacterium, as defined in claim 28, wherein the bacterium is BCG.HIVA^{2auxo} (DSM 26306).
31. A bacterium as defined in any one of claims 25 to 30 for use in medicine.
32. A bacterium as defined in any one of claims 25 to 30 for use in the treatment of a disease which requires the expression of a polypeptide of interest.

33. A bacterium for use, as defined in any of claims 31 or 32, wherein said bacterium does not comprise any endogenous or exogenous nucleotide sequence conferring antibiotic sensitivity or antibiotic resistance to said bacterium.

34. A bacterium for use, as defined in claim 33, wherein the polypeptide of interest comprises an antigenic polypeptide and wherein the recombinant cell is used in inducing an immune response against the antigenic polypeptide.

35. A bacterium, as defined in claim 34, for use in the treatment of a disease caused by a HIV infection wherein the polypeptide of interest comprises an HIV immunogen, for use in the treatment of malaria wherein the polypeptide of interest comprises a plasmodium immunogen or for use in the treatment of a disease caused by a mycobacteria infection wherein the polypeptide of interest comprises an immunogen from said mycobacteria.

36. A vaccine composition comprising:

(i) a bacterium as defined in any of claims 25 to 35, and

(ii) a pharmaceutically acceptable carrier.

37. A vaccine composition, as defined in claim 36, further comprising a vector encoding an antigenic polypeptide or an immunologically active epitope thereof.

38. A vaccine composition, as defined in claim 37, wherein the vector comprising the antigen of interest or an immunogenic epitope thereof is selected from the group consisting of modified vaccinia virus Ankara vector, an adenoviral vector and a measles virus vector.

39. A vaccine composition, as defined in any one of claims 36 to 38 for use in medicine.

40. A vaccine composition, as defined in any one of claims 36 to 38, for use in inducing an immune response against an antigenic polypeptide.

41. A vaccine composition, as defined in claim 40, wherein the host cell and the vector comprising the polynucleotide encoding the antigen of interest or an immunogenic epitope thereof are administered in a separate or sequential manner.

42. A vaccine composition, as defined in any of claims 40 or 41, wherein the administration of the vaccine involves a priming step with the host cell and at least a boosting step with the vector comprising the polynucleotide encoding the antigen of interest or an immunogenic epitope thereof.

43. A method for the expression of a polypeptide of interest in a mycobacterium host cell which comprises:

(i) growing a mycobacterium host cell comprising a polynucleotide according to any of claims 1 to 20 and, optionally,

(ii) recovering the polypeptide of interest from the culture,

wherein the host cell carries an auxotrophy which can be complemented by at least one of the auxotrophic genes forming part of the polynucleotide.

44. A method, as defined in claim 43, wherein the weak mycobacterium promoter is the *Mycobacteria spp* α-antigen promoter or a functionally equivalent variant thereof.

45. A method, as defined in any one of claims 43 or 44, wherein the sequence encoding the antigenic polypeptide is fused in frame to a sequence coding a signal sequence active in mycobacteria.

46. A method, as defined in claim 45, wherein the signal sequence corresponds to the signal sequence of mycobacterium 19 KDa lipoprotein or a functionally equivalent variant thereof.

47. A method, as defined in any one of claims 43 to 46, wherein the gene capable of complementing a mycobacterium lysine auxotrophy is the lysA gene or wherein the gene capable of complementing an *E. coli* glycine auxotrophy is the glyA gene.