Construction and characterization of recombinant BCG strains expressing SIVconsv immunogen for pre-clinical evaluation of safety and immunogenicity in cynomolgus macaques. A new platform for a bivalent HIV/TB vaccine development.

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Course 2024-2025

1. Abstract

The development of a safe, effective, and scalable bivalent vaccine targeting both HIV and tuberculosis (TB) remains a global health priority, particularly in low-resource settings where co-infection is prevalent. Recombinant *Mycobacterium bovis* BCG (rBCG) has emerged as a promising vaccine vector due to its proven neonatal safety, heat stability, and immunostimulatory properties. In this preclinical study, we propose the

construction and evaluation of rBCG vaccine candidates expressing conserved regions of simian immunodeficiency virus (SIVmac251) as a surrogate for HIV, in the cynomolgus macaques (*Macaca fascicularis)* model.

To address the genetic diversity and immune escape of HIV, we use two synthetic immunogens, SIVconsv.gag and SIVconsv.pol, previously designed by our group, comprising the most functionally conserved regions of the SIVmac251 proteome. These sequences, which contains the kanamycin resistance gene and the complementing lysine gene, were codon-optimized, HA-His-tagged, and individually cloned into the integrative shuttle vector pGV223. Mutant lysine auxotrophic *M. bovis* BCGΔLys strains were transformed via electroporation and selected using antibiotic resistance and lysine depletion. Recombinant colonies were phenotypically characterized and genetically confirmed by PCR analyses. Heterologous protein expression was evaluated by Dot-Blot and Western Blot assays. The resulting rBCG strains, rBCG:SIVconsv.gag and rBCG:SIVconsv.pol, will undergo in vitro genetic stability testing. A working vaccine stock will be generated and colony-formimng units (CFU) will be titered for downstream in vivo immunogenicity studies in *M. fascicularis*, evaluating both SIV-specific and *Mycobacterium tuberculosis*–specific immune responses.

This project aims to establish a versatile rBCG-based platform for bivalent vaccine development, integrating improved genetic stability. By targeting conserved viral regions and using a translational non-human primate model, this approach holds potential for future application in dual HIV/TB vaccination strategies.

2. Abbreviations

AIDS – acquired immunodeficiency syndrome

AuNPs – Gold Nanoparticles

BCA – Bicinchoninic Acid (protein assay)

bNAbs – Broadly Neutralizing Antibodies

BCG – *Mycobacterium bovis* Bacillus Calmette–Guérin

BCGΔLys – Mutant lysine auxotrophic Bacillus Calmette–Guérin

CFU – Colony-Forming Units

CFU/mL – Colony-Forming Units per Millilitre

CV – Coefficient of Variation

Env – Envelope (glycoprotein)

Gag – Group-specific Antigen (structural protein of retroviruses)

HA – Hemagglutinin (epitope tag)

His_6 – Hexahistidine tag

HIV – Human Immunodeficiency Virus

HTI – HIVACAT T-cell Immunogen

KO – Knockout

Mtb – *Mycobacterium tuberculosis*

MSS – Master Seed Stock

MVA – Modified Vaccinia Ankara

Ni-NTA – Nickel–Nitrilotriacetic Acid (affinity resin)

OD_{600nm} – Optical density at 600 nm

PBS – Phosphate-Buffered Saline

PBST – PBS + 0.05 % Tween-20

PCR – Polymerase Chain Reaction

Pol – Polymerase (viral enzyme complex)

PVDF – Polyvinylidene Fluoride membrane

rBCG – Recombinant Bacillus Calmette–Guérin

SDS-PAGE – Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SIV – Simian Immunodeficiency Virus

TB – Tuberculosis

TBE – Tris-Borate-EDTA buffer

VLP – Virus-Like Particle

WHO – World Health Organization

WT – Wild type

WVS – Working Vaccine Stock

3. Introduction

3.1. Global HIV Epidemiology

The HIV epidemic remains a major global challenge. In 2023, new HIV infections dropped by 39% compared to 2010, with sub-Saharan Africa leading with a 56% reduction [1]. However, the same year, 1.3 million people still acquired the virus, more than three times the 2025 target of 370,000, and AIDS continues to claim one life every minute [1,2]. While treatment access expanded to 30.7 million people in 2023, preventing over 20.8 million deaths over the past three decades, 9.3 million individuals remain untreated, and 2.1 million are not achieving viral suppression despite being on therapy [1]. Progress remains uneven: while Eastern and Southern Africa show significant advances, Eastern Europe, Central Asia, the Middle East, North Africa, and Latin America face stagnation or increasing infections [3]. These regional disparities reflect differences in political commitment, healthcare infrastructure, and inclusion of key populations such as sex workers, transgender people, people who use drugs, and men who have sex with men [2,4].

3.2. Global TB epidemiology

The WHO Global TB Report 2024 estimates 10.8 million incident cases and 1.25 million deaths in 2023, with 161 000 deaths in co-infected individuals and ~410 000 new cases of rifampicin-resistant or multidrug-resistant TB [6]. Thirty high-burden countries account for almost 90 % of cases, and India alone represents roughly one quarter, while children still comprise 12 % of new disease, underscoring diagnostic gaps [6]. Although BCG reaches ~80 % of newborns worldwide, its protection wanes against adult pulmonary TB; moreover, variability among BCG substrains, heat-instability of some formulations and interference from environmental mycobacteria limit its effectiveness [11]. These epidemiological and immunological shortcomings justify the search for recombinant BCG boosters and bivalent HIV/TB strategies that can address both drug-resistant forms and the paediatric–adult transmission gap.

3.3. Mother-to-Child Transmission and Paediatric Gaps

Preventing mother-to-child transmission remains a pressing issue. In West and Central Africa, only 53% of pregnant and breastfeeding women access prevention services, contributing to approximately 130,000 new paediatric infections annually [2,3]. Treatment coverage for children and adolescents also lags that of adults [1]. Despite the commitments made in the 2021 Political Declaration on HIV and AIDS, the 2025 targets remain at risk. Achieving the 2030 Sustainable Development Goal of ending AIDS will require sustained political will, equitable access to healthcare, human rights protections, and the dismantling of legal and social barriers [2,4].

3.4. Rationale for a Bivalent HIV/TB Vaccine

Within this context, developing a vaccine that is safe, effective, suitable for early-life immunization, and feasible for use in low-resource environments has become a critical component in global HIV prevention efforts. A bivalent vaccine that simultaneously targets HIV and TB would be particularly impactful, as TB remains the leading cause of death among people living with HIV, who are estimated to be 20 to 30 times more likely to develop active TB [1,4]. This epidemiological overlap underscores the potential public health benefits of a combined immunization strategy.

3.5. BCG Platform Advantages and Pre-clinical Evidence for rBCG Vectors

The use of BCG as a bivalent vaccine platform represents a strategic approach, particularly in resource-limited settings. BCG remains the only licensed TB vaccine and is administered to over 80% of infants in countries with national immunization programs. It provides reliable protection against severe paediatric TB forms such as tuberculous meningitis and miliary TB, while being inexpensive, heat-stable, and safe for neonatal use, factors that are crucial in high-burden, low-resource regions [5,7].

rBCG strains have emerged as versatile vectors capable of expressing heterologous antigens, including those derived from HIV. Preclinical studies have shown that rBCG expressing HIV-1 immunogens can elicit strong and durable immune responses, including mucosal immunity, HIV-specific CD4+ and CD8+ T-cell responses, and, in some instances, the induction of broadly neutralizing antibodies [8,9]. These immunological features are critical for addressing HIV's high genetic variability and immune escape mechanisms.

3.6. Prime-Boost Strategies, Genetic-Safety Considerations and Conserved-Immunogen Design

rBCG-based vaccines have also shown potential in prime-boost regimens. For example, BCG.HIVA priming followed by MVA.HIVA boosting has elicited robust HIV-specific T-cell responses and improved protection against Mtb in murine models [8]. These results are further enhanced through technical strategies such as codon optimization of HIV genes, use of moderate-strength or inducible promoters, and elimination of antibiotic resistance markers via auxotrophic complementation systems, approaches that improve both immunogenicity and biosafety [11].

Conventional episomal plasmid systems raise concerns about biosafety and genetic stability. The integration of expression systems in BCG genome and antibiotic-free plasmid selection techniques using auxotrophic complementation genes offers

improved regulatory compliance and in vivo stability, ensuring suitability for human vaccine development [9,11].

Among the most promising immunogen designs are those based on functionally conserved regions of HIV. Constructs such as the HIVACAT T-cell immunogen (HTI) have demonstrated the ability to elicit broad and cross-clade T-cell responses in preclinical models, representing a promising approach for overcoming HIV's genetic diversity [12]. Similar prime-boost designs have been evaluated in simian models using HIV-derived immunogens, which serve as robust surrogates for studying viral pathogenesis and vaccine efficacy [10].

3.7. SIVconsv immunogen

SIVconsv is a synthetic immunogen engineered from the most functionally conserved regions of the SIVmac251proteome, mirroring the "HIVconsv/HTI" strategy used for HIV-1 [10]. By concatenating six highly conserved, $CD8^+$-T-cell–rich segments of Gag and Pol into two separate reading frames, SIVconsv.gag and SIVconsv.pol, the design focuses immune recognition on epitopes that are least tolerant of escape mutations [12]. Each cassette has been codon-optimised for mycobacteria, fused to an HA-His_6 tag for downstream detection and secretion, and placed under a moderate BCG promoter to limit metabolic burden while maintaining antigen expression [11]. Pre-clinical studies in mice and macaques have shown that rBCG vectors expressing SIVconsv prime broad, cross-clade $CD4^+$ and $CD8^+$ responses that can be further boosted by viral vectors, supporting its use as the HIV component of a dual HIV/TB vaccine [10,12].

3.8. Cynomolgus Macaque as a Translational Model

The cynomolgus macaque model provides a valuable translational bridge, as it recapitulates critical aspects of HIV pathogenesis including mucosal transmission, CD4+ T-cell depletion, and chronic immune activation. Evaluating rBCG-based vaccines expressing SIV immunogens in this model allows for comprehensive assessment of safety, immunogenicity, and protective efficacy, with potential applications in both HIV and TB co-infection contexts [13].

3.9. Study Hypothesis

Given the need for a dual vaccine capable of addressing both HIV and TB in a single formulation, and the promising results obtained with recombinant BCG vectors expressing HIV antigens, we propose the development of a novel rBCG vector expressing conserved SIV regions as a preclinical proof-of-concept. This strategy not only allows evaluation of a bivalent vaccine approach but also explores the feasibility of using optimized, biosafe genetic platforms for antigen delivery.

To this end, we hypothesize that a recombinant M. bovis BCG vaccine expressing conserved regions of SIVmac251 (rBCG:SIVconsv) will elicit robust and durable cellular immune responses against SIV in M. fascicularis, and may also enhance protection against M. tuberculosis infection, thereby representing a promising bivalent vaccine strategy against HIV and TB.

4. Objectives

The main goal of this project is to contribute to the development of a novel bivalent vaccine prototype capable of inducing protective immune responses against both SIV and *M. tuberculosis*, using the *M. fascicularis* model of SIV infection.

To achieve this, the project is structured around the following specific objectives:

1. **Construction of rBCG:SIVconsv vaccine candidates:**
   A BCGΔLys mutant strain will be individually transformed by electroporation with the constructs pGV223.SIVconsv.gag-HA-His and pGV223.SIVconsv.pol-HA-His. The selection of rBCG colonies will be carried out using two distinct methods: antibiotic resistance (kanamycin) and auxotrophic complementation (lysine depletion).
2. **Genetic and phenotypic characterization of rBCG:SIVconsv.gag and rBCG:SIVconsv.pol strains:**
   The integration of the expression cassettes into BCG genome will be confirmed by PCR using specific primers. SIVconsv.gag-HA-His and SIVconsv.pol-HA-His protein expression will be assessed by Dot Blot and Western Blot.
3. **Evaluation of in vitro genetic stability:**
   The genetic stability of the recombinant strains will be evaluated through serial passages in vitro, monitoring for potential genetic rearrangements or loss of the expression cassettes by PCR analyses.
4. **Development of master and working seed stocks:**
   A Master Seed and Working Vaccine stocks will be generated using the seed-lot system. The titer of the Working Vaccine stock will be quantified as colony-forming units (CFU) per mL of frozen stock. These stocks will be used for the immunogenicity and safety studies in vivo.
5. **In vivo immunogenicity assessment in cynomolgus macaques:**
   A fully characterized and titered WVS of each recombinant strain, together with a wild-type BCG strain as a control, will be sent to specialized animal facilities for evaluation of the specific SIV and Mtb immune responses in cynomolgus macaques. Both recombinant strains will be administered together in a cocktail vaccine.

5. Materials and Methods

5.1. Bacterial Strains, Cultures and Transformation

The lysine auxotrophic strain BCGΔlys was kindly provided by W. R. Jacobs Jr., B. R. Bloom, and T. Hsu [13].

- **Preparation of BCGΔlys electrocompetent cells:** 100 µL of electrocompetent BCGΔlys was added to 5 mL of Middlebrook 7H9 broth supplemented with glycerol, albumin-dextrose-catalase (ADC), and Tween 80 (20%). L-lysine monohydrochloride was added at a final concentration of 40 µg/mL. Cultures were grown shaking at 37ºC to an OD_{600} of 1.5 (measured with Shimadzu UV-1700 PharmaSpec UV-VIS Spectrophotometer) and centrifuged for 10 min at 5000 rpm. The pellet was washed 3-4 times with 10% glycerol, resuspending the cells in a final volume of 1 mL.
- **Transformation:** 200 µL and 400 µL of the cell suspension was mixed with 1 µL and 2 µL of the plasmid DNA, respectively. Transformation was conducted with the SIVconsv.gag and SIVconsv.pol plasmid DNAs separately in prechilled 2mm Gene Pulser® Cuvettes (Bio-Rad) using a Bio-Rad gene pulser electroporator at 2.5 kV, 25 µF, and 1000 Ω. After transformation, cells were recovered in 5 mL of enriched 7H9 supplemented with lysine [40 µg/mL] and incubated shaking at 37ºC overnight. After this time, the suspension was centrifuged at 5000 rpm for 10 min. 200 µL of the suspension containing the transformants were plated on Middlebrook 7H10 agar containing glycerol, ADC, Tween 80 [20%], and kanamycin [25 µg/mL]. The agar plates were incubated at 37ºC in sterile sealed plastic bags to prevent evaporation and to maintain humidity levels.

After three weeks of incubation, four separated colonies of each transformed strain rBCG:SIVconsv.gag and rBCG:SIVconsv.pol were individually cultured in Middlebrook 7H9 supplemented with glycerol, albumin-dextrose-catalase (ADC), and Tween 80 (20%) and kanamycin [25 µg/mL] and incubated at 37ºC, shaking, until reaching an OD_{600} of 0,9-1. At this point, 0,5 mL of each culture was mixed with 0,5 mL of 50% glycerol (final concentration of 25% glycerol) and preserved at -80°C.

Only a single clone of each recombinant strain was selected for characterization and used to generate the MSS and WVS following the seed-lot system.

All procedures conducted to evaluate protein expression in the rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His strains were also performed in parallel using two control BCG strains: a wild-type (WT) BCG Pasteur strain provided by the Pasteur Institute, and two recombinant BCG strains expressing the p11-HA-His insert, which is a

SIVgag gene fragment that contains an immunodominant CTL epitope. These recombinant strains were previously generated in our laboratory. One of them, p11-HA-His+, is codon optimized for mycobacteria, while p11-HA-His- is not. The difference between them is the recombinant protein expression level, which is higher in p11-HA-His+. These strains served as negative (WT BCG Pasteur) and positive (both p11-HA-His+ and p11-HA-His-) biological controls in both Dot-Blot and Western Blot assays. The WT BCG Pasteur strain was cultured under the same conditions as the BCGΔlys mutant strain (without kanamycin supplementation), while the rBCG.p11-HA-His strains were grown under the same conditions as the recombinant SIVconsv-expressing strains.

5.2. Construction of the pGV223.SIV.consv Plasmid DNA

The integrative *E. coli*-mycobacterial shuttle vector used in this project, pGV223, was previously developed by our research group. It is derived from the parental plasmid pJH223, kindly provided by W. R. Jacobs Jr., B. R. Bloom, and T. Hsu (Albert Einstein College of Medicine, New York, NY, USA) [13].

This derivative plasmid contains restriction enzyme sites (HindIII) and a 19-kDa lipoprotein secretion signal sequence to enhance antigen expression while reducing metabolic burden. It is designed to integrate site-specifically into the attB locus of the BCG genome thanks to attachment sites (attP) and the integrase (int) genes from the mycobacteriophage L5 [14]. It includes the lysA gene, which restore lysine prototrophy in *M. bovis* BCGΔlys, under the control of Phsp60, a strong heat shock protein 60 gene promoter. This allows selection of recombinant strains through nutrient depletion in culture media. A kanamycin resistance gene (kan) is included as a secondary selection marker.

For this study, a synthetic SIVconsv DNA containing sequence was previously codon-optimized to match the G + C rich mycobacterial codon usage and cleaved into two fragments, SIVconsv.gag-HA-His-Tag and SIVconsv.pol-HA-His-Tag, to minimize potential intrinsic toxicity and metabolic burden. Each DNA fragment was cloned individually into the pGV223 vector downstream of the 19-kDa signal sequence under regulation of a weak mycobacterial promoter (Pα-antigen) [15,16]. An HA-His tag was included downstream of the SIVconsv fragment in both constructs for subsequent protein expression detection.

The resulting plasmids, pGV223.SIVconsv.gag-HA-His and pGV223.SIVconsv.pol-HA-His (**Figure 1**), were synthesized by Geneart (USA) and used to transform *E. coli* TOP10 electrocompetent cells for amplification and selection.

In the present study, these plasmids were then used to transform BCGΔlys electrocompetent cells by electroporation, generating the recombinant BCG:SIVconsv.gag-HA-His and BCG:SIVconsv.pol-HA-His strains.

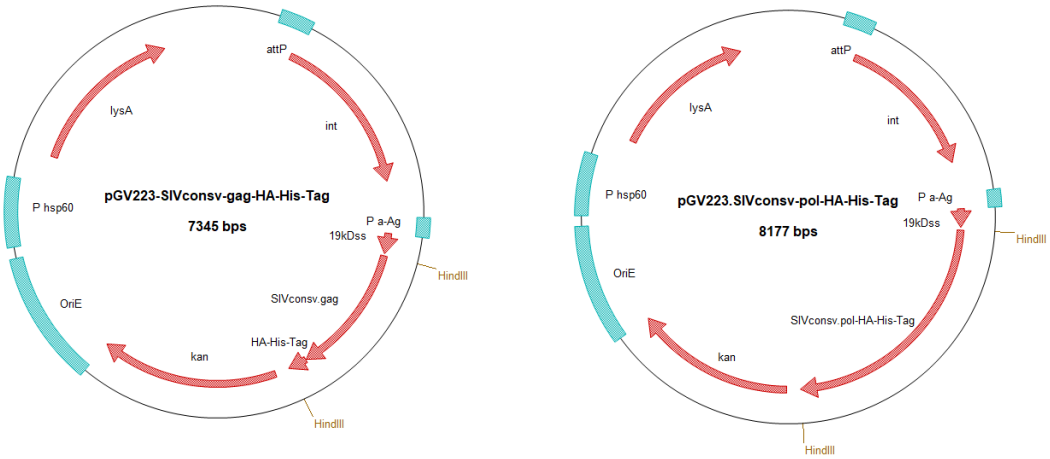

Figure 1: Plasmid DNA pGV223.SIVcons.gag-HA-His (left) and plasmid DNA pGV223.SIVcons.pol-HA-His (right), annotated. The red arrows inside the maps correspond to the genes and the blue boxes indicate regions and promoters. The total size of the plasmids are 7345 bps and 8177 bps, respectively. Data derived from CloneManager.

5.3. Genetic Characterization of p223:SIVconsv.gag-HA-His and p223:SIVconsv.pol-HA-His Plasmid DNA

5.3.1. PCR analysis of SIVconsv.gag-HA-His and SIVconsv.pol-HA-His DNA fragments using as template p223:SIVconsv.gag-HA-His and p223:SIVconsv.pol-HA-His plasmid DNA

PCR amplification was performed using 1 µL of purified plasmid DNA (either pGV223:SIVconsv.gag-HA-His or pGV223:SIVconsv.pol-HA-His), previously obtained through MAXIPREP by our laboratory. The reaction mix included 12.5 µL of 2× Phusion FLASH PCR Master Mix (Thermo Scientific™), 1.0 µL of SIVconsv.gag-HA-His 36bp forward primer 100M (5' CTTATGCCGGTGCAGGACATGCCTGTGGGCAACTAC 3'), 1.0 µL of SIVconsv.gag-HA-His 46bp reverse primer 100M (5' TTCATTACATAGTGTGGTGTGGTGGTGTGTGTTACGCGTCAGTC 3'), 1.0 µL of SIVconsv.pol-HA-His 34bp forward primer 100M (5' AAGATGCTGCGTGGCATCGGTGGCTTCATCAAC 3'), 1.0 µL of SIVconsv.pol-HA-His 39bp reverse primer 100M (5' TTTCATCATCATGGTGGTGGTGGTGGTGGTGTTAAAG 3') and 9.5 µL of nuclease-free water (Promega, Madison, WI, USA), bringing the final reaction volume to 25.0 µL (**Table 1**).

The PCR cycling conditions for SIVconsv sequences amplification were as follows: an initial hot start at 94 °C for 5 minutes, followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and elongation at 72 °C for 3 minutes. A final extension step was performed at 72 °C for 7 minutes to complete DNA synthesis.

Table 1. PCR reaction mix for amplification of SIVconsv insert using purified plasmid DNA (pGV223:SIVconsv.gag-HA-His or pGV223:SIVconsv.pol-HA-His) as template.

REAGENTS	VOLUMES
2× Phusion FLASH PCR Master Mix	12.5 µL
SIVconsv.gag-HA-His 36bp Fw primer or SIVconsv.pol.HA-His 34bp Fw primer	1.0 µL
SIVconsv.gag-HA-His 46bp Rv primer or SIVconsv.pol-HA-His 39bp Rv primer	1.0 µL
Purified p223:SIVconsv.gag-HA-His or p223:SIVconsv.pol-HA-His plasmid	1.0 µL
Nuclease-free H_2O	9.5 µL
Final Volume	**25.0 µL**

PCR amplification products were analysed by agarose gel electrophoresis.

5.3.2. Digestion of p223:SIVconsv.gag-HA-His and p223:SIVconsv.pol-HA-His plasmid DNA with restriction enzymes

The plasmid DNA (either pGV223:SIVconsv.gag-HA-His or pGV223:SIVconsv.pol-HA-His), previously purified using the MAXIPREP Qiagen kit, was further assessed by restriction enzyme digestion to verify its integrity prior to downstream applications. A single-enzyme digestion was performed using HindIII (New England Biolabs). The reaction mix consisted of 5 µL of MAXIPREP plasmid DNA, 2 µL of the corresponding 10× buffer, 2µL of HindIII, 2µL of BSA and nuclease-free water to a final volume of 21 µL. The reaction was briefly centrifuged and incubated at 37 °C for one hour. The digestion served both to confirm the structural integrity of the plasmid and as a quality control for subsequent BCG colony PCR assays. The digestion products were analysed by agarose gel electrophoresis.

5.4. Phenotypic characterization of Mutant BCGΔlys, rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His Strains

To assess the phenotypic stability of the system, the BCGΔLys mutant strain was cultured on 7H10 agar plates supplemented with or without lysine, and with or without kanamycin. Following transformation with the pGV223.SIVconsvint plasmid, the resulting recombinant BCG.SIVconsv strains were cultured on 7H10 agar plates containing kanamycin to evaluate plasmid-mediated complementation.

5.5. Genetic Characterization of rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His Strains

5.5.1. Mycobacterial genomic DNA preparation for PCR analysis

To confirm the integration of the plasmid DNA (either pGV223:SIVconsv.gag-HA-His or pGV223:SIVconsv.pol-HA-His) into the BCGΔLys genome, pretreatment of DNA samples was required. For this purpose, 1 mL of liquid culture (from a total volume of 5 mL, OD_{600} = 1) of each recombinant strain was harvested by centrifugation at 5000 rpm for 10 minutes at room temperature. The supernatant was discarded, and the bacterial pellet was resuspended in 200 µL of sterile water. The suspension was then incubated at 95 °C

for 20 minutes in a dry block heater to lyse the cells and inactivate the bacteria. Following lysis, the sample was centrifuged at 13,000 rpm for 1 minutes to pellet cellular debris. A 5 µL aliquot of the resulting supernatant was used directly for PCR amplification or stored at −20 °C for future analysis.

5.5.2. PCR analysis of SIVconsv.gag-HA-His and SIVconsv.pol-HA-His DNA sequences in rBCG strains

Amplification of the SIVconsv.gag-HA-His and SIVconsv.pol-HA-His sequences in the recombinant BCG strains was carried out using the same designed primers and thermal cycling conditions as those applied for the plasmid DNA PCR (**Table 1**), with specific adjustments detailed in **Table 2**.

Table 2. PCR reaction mix for amplification of either SIVconsv.gag-HA-His or SIVconsv.pol-HA-His inserts using extracted mycobacterial DNA of each recombinant strain as template.

REAGENTS	VOLUMES
2× Phusion FLASH PCR Master Mix	12.5 µL
SIVconsv.gag-HA-His 36bp Fw primer or SIVconsv.pol.HA-His 34bp Fw primer	1.0 µL
SIVconsv.gag-HA-His 46bp Rv primer or SIVconsv.pol-HA-His 39bp Rv primer	1.0 µL
Recombinant mycobacterial genome	5.0 µL
Nuclease-free H_2O	5.5 µL
Final Volume	**25.0 µL**

5.5.3. Electrophoresis of PCR DNA products

Nucleic acid electrophoresis was performed to analyse the previously purified plasmid DNAs and its PCR products, the enzymatic digestions, SIVconsv.gag-HA-His and SIVconsv.pol-HA-His PCRs of rBCG strains. Electrophoresis was carried out on a Bio-Rad power supply apparatus at limiting 95V and T=60min for PCR testing on a 1.5% agarose gel (FastGene® Nippon Genetics Europe) with 1x TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3.) and 1:10000 volume of SYBR®-Safe (Invitrogen). The 1 Kb Plus DNA Ladder (Invitrogen) was used as reference. To facilitate visual monitoring of DNA movement during electrophoresis, the Gel Loading Dye Purple 6x (New England Biolabs) was also used. Finally, The Dark Reader DR88X Transilluminator (Clare Chemical, USA) was used to visualize gel extraction.

5.6. Evaluation of Heterologous SIVconsv.gag-HA-His and SIVconsv.pol-HA-His Protein Expression

5.6.1. BCG Culture Protein Extraction

For protein extraction, 50 mL of liquid culture media of recombinant BCG cells and BCG wild type control cells were grown at 37 °C using a cell roller device at 1 rpm until

reaching an OD_{600} of 1.0–1.5. Cultures were then centrifuged at 3,000 rpm for 10 minutes at 4 °C to pellet the bacteria, and the supernatant was discarded. The bacterial pellets were washed three times with sterile PBS containing 0.02% Tween-80 and subsequently resuspended in 1 mL of protein extraction buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.6% SDS). A total of 10 µL of protease inhibitor cocktail (Sigma-Aldrich, PIC100X; containing 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) was added to each sample. Cell lysiswas performed by sonication in an ice bath at 100% amplitude with 0.5-second pulses, applied in three cycles of 1 minute each. BCG cell lysates were centrifuged at 13,000 rpm for 10 minutes at 4 °C, and the resulting supernatants containing the soluble protein were collected and stored at –80 °C until further use.

5.6.2. Total Protein Quantification

Total protein concentration was determined using the bicinchoninic acid (BCA) colorimetric assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific), which is based on the reduction of Cu^{2+} to Cu^{+} by peptide bonds and specific amino acid residues such as cysteine, cystine, tryptophan, and tyrosine under alkaline conditions. The resulting cuprous ions (Cu^{+}) react with BCA to form a purple-coloured complex that exhibits strong absorbance at 562 nm, proportional to the protein content. Sample concentrations were calculated from a standard curve generated using known concentrations of bovine serum albumin (BSA). Absorbance measurements were performed using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific).

5.6.3. Dot-Blot Assay

The supernatants of each cell lysate were previously diluted in PBS 1X (Sigma-Aldrich) to adjust all samples at the same protein concentration (575 µg/mL). Then, five and ten microliters of each supernatant were spotted onto pre-activated polyvinylidene difluoride (PVDF) membranes (Immobilon-FL, Millipore) for dot blot analysis. Membranes were previously activated by immersion in 100% methanol for 30 seconds, rinsed in distilled water, and equilibrated in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol) before use. After drawing a grid with a pencil to guide sample application, the membranes were placed over pre-wetted Extra Thick Blot Filter Paper (Bio-Rad) and allowed to equilibrate. Protein samples were carefully applied to minimize spreading. The recombinant Posi-Tag Epitope Tag Protein (BioLegend) was used as analytical positive control.

Following absorption (2–5 minutes), membranes were blocked and probed using the iBind™ Flex system (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, membranes were incubated in 1X iBind™ Flex solution (prepared by mixing 10 mL of 5X buffer, 500 µL of 100X additive, and 39.5 mL of distilled water) for 5-10 minutes. Antibody incubations were performed using the iBind™ Flex Mini Insert and

Card. The primary antibody, Anti-HA.11 Epitope Tag Antibody (BioLegend), was diluted 1:2000 in 1X iBind™ Flex solution (1 µL antibody + 2 mL buffer), and the secondary antibody, Goat Anti-Mouse IgG (H+L)-HRP (Invitrogen), was diluted 1:4000 (0.5 µL antibody + 2 mL buffer). The membrane was placed on the card, protein side down, aligned within the flow lane, and firmly pressed using the blotting roller to ensure proper contact and air bubble removal. Antibody incubation proceeded for 2.5 hours at room temperature.

After incubation, the membrane was washed twice with 20 mL of distilled water for 2 minutes each. Detection was performed using the Clarity™ Western ECL Substrate kit (Bio-Rad), prepared fresh in a 1:1 ratio. The membrane was incubated in the substrate for 5 minutes in the dark, excess reagent was removed, and the blot was wrapped in transparent plastic film.

Images were acquired using the Odyssey® Fc Imaging System (LI-COR Biosciences) and processed with Image Studio™ software.

5.6.4. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

The supernatants of each cell lysate were diluted in PBS 1X (Sigma-Aldrich) to adjust all samples at the same protein concentration (575 µg/mL). Then, the supernatants were prepared in Laemmli sample buffer (Bio-Rad) supplemented with 5% 2-mercaptoethanol to a final volume of 50 microliters, denatured at 95 °C for 5 minutes, and briefly centrifuged. Samples were then loaded onto 8–16% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad), along with 5 µL of Precision Plus Protein WesternC Standards as a molecular weight marker. The recombinant Posi-Tag Epitope Tag Protein (BioLegend) was used as analytical positive control. Electrophoresis was carried out using a Mini-PROTEAN Tetra Cell at 50 V for 15 minutes followed by 150 V for approximately 1 hour in Tris/Glycine/SDS running buffer until the dye front reached the bottom of the gel.

Proteins were transferred onto methanol-activated PVDF membranes (Immobilon-FL, Millipore) using the Trans-Blot Turbo Transfer System (Bio-Rad) under pre-set conditions for mixed molecular weight proteins (30 minutes, ~2.5 V, 1.0A). Membranes were then equilibrated in transfer buffer and processed for antibody detection.

From this point, membranes were processed following the same protocol described in the Dot Blot Analysis section, using the iBind™ Flex system (Thermo Fisher Scientific) for blocking and antibody incubation. Detection was performed using the Clarity™ Western ECL Substrate (Bio-Rad), and signals were visualized with the Odyssey® Fc Imaging System (LI-COR Biosciences) and quantified using Image Studio™ software.

5.7. Titration of Working Vaccine Stocks. Colony-Forming Units per Milliliter (CFU/mL) Quantification

For the titration of Working Vaccine Stocks (WVS) of rBCG:SIVconsv.gag-HA-His, rBCG:SIVconsv.pol-HA-His, and BCG WT Pasteur strains, three 1-mL frozen vials were randomly selected from each of the 50-vial stocks. The selected vials were brought to room temperature, and two independent serial dilution series were prepared from each vial, ranging from 10^{-2} to 10^{-6}, using 4.5 mL of PBS supplemented with 0.05% Tween 80 as the diluent.

Middlebrook 7H10 agar plates supplemented with glycerol, ADC, and 20% Tween 80 were prepared for CFU plating. For the recombinant strains, kanamycin (25 µg/mL) was also added as a selective marker. Each plate was properly labelled, and 100 µL of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions of each BCG strain were plated out onto the corresponding selective media.

The plates were incubated at 37 °C in sterile, sealed plastic bags to prevent evaporation and maintain appropriate humidity levels. After three weeks of incubation, colony-forming units (CFU) were counted for each dilution, and the average CFU/mL was calculated for each WVS. These values were documented in the technical datasheets corresponding to the vials intended for immunization of macaques.

5.8. In vitro Genetic Stability of plasmid DNA in rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His Strains

To assess the genetic stability of the recombinant strains BCG:SIVconsv.gag-HA-His and BCG:SIVconsv.pol-HA-His derived from the WVS, cultures were maintained in lysine-deficient Middlebrook 7H9 broth supplemented with kanamycin (25 µg/mL) under shaking conditions at 37 °C. Four consecutive subcultures were performed at 7-day intervals by transferring 500µL of stationary-phase culture into 5 mL of fresh selective medium. This serial passaging corresponds to approximately 30 bacterial generations, considering the slow division rate of mycobacteria (24h). To evaluate the structural stability of the integrated constructs, samples were pretreated after the final passage of each BCG culture and analysed by PCR under the same conditions described in the section *PCR analysis of SIVconsv.gag-HA-His and SIVconsv.pol-HA-His sequences in rBCG strains*.

6. Results

6.1. Phenotypic Characterization of Mutant BCGΔlys, rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His Strains

To evaluate the phenotypic stability of the auxotrophic system, the lysine-deficient BCGΔLys mutant was cultured on Middlebrook 7H10 agar supplemented with ADC, glycerol, and Tween 80, under different selective conditions. When plated on media containing kanamycin, although containing lysine, the strain failed to grow (**Figure 2A**), indicating sensitivity to the antibiotic. As expected, the BCGΔLys strain was unable to grow on plates lacking lysine supplementation (**Figure 2B, 2D**), confirming the auxotrophic phenotype. In contrast, robust bacterial growth was observed on plates supplemented with lysine but lacking kanamycin (**Figure 2C**), consistent with previous group findings.

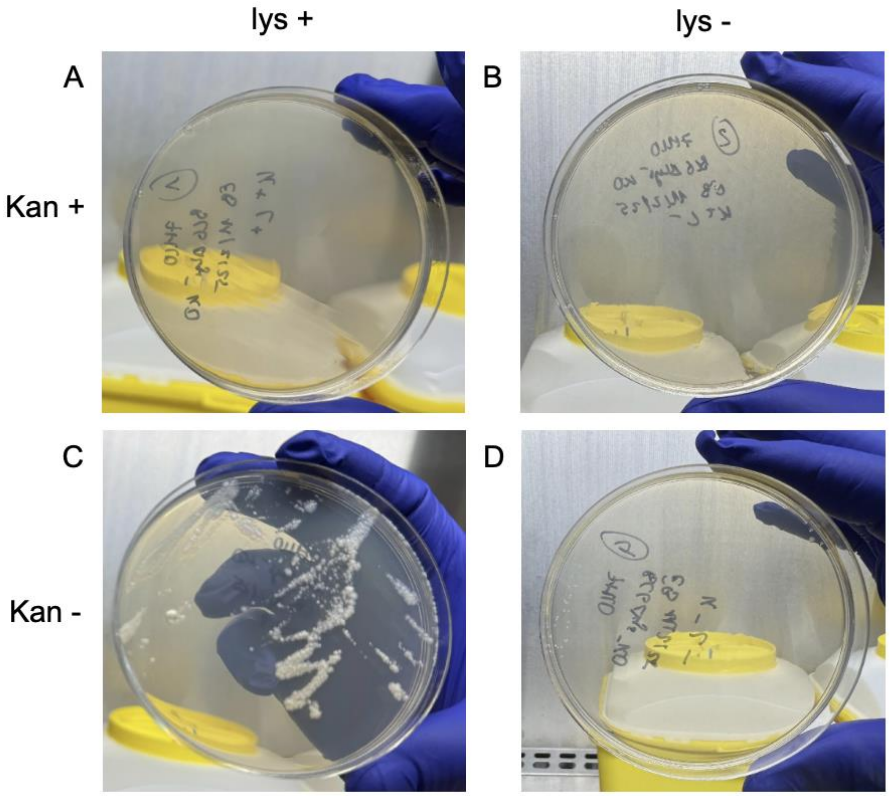

Figure 2. Phenotypic evaluation of the mutant BCGΔLys strain. Phenotype of lysine auxotrophy and kanamycin resistance was evaluated. The strain was plated on 7H10 agar supplemented with glycerol, ADC, Tween 80, and selective additives as indicated. A) 7H10 supplemented with kanamycin and lysine. B) 7H10 supplemented with kanamycin but no lysine. C) 7H10 supplemented with lysine but not kanamycin. D) non-supplemented 7H10 with kanamycin or lysine.

Then, to assess plasmid complementation, the BCGΔLys strain was individually electroporated with the p223:SIVconsv.gag-HA-His and the p223:SIVconsv.pol-HA-His

plasmids, generating the recombinant strains rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His. Upon plating on 7H10 agar supplemented with kanamycin but without lysine, colony formation was observed (**Figure 3**), demonstrating that the recombinant bacteria successfully overcame both selection pressures. This growth is attributed to the functional expression of two plasmid-encoded elements: (i) the kanamycin resistance gene, conferring antibiotic resistance, and (ii) the lysine biosynthesis gene, restoring prototrophy.

Together, these results confirm that transformation of the BCGΔLys strain with p223:SIVconsv.gag-HA-His and p223:SIVconsv.pol-HA-His enables dual selection via antibiotic resistance and metabolic complementation, supporting the stability and functionality of the recombinant system under selective conditions.

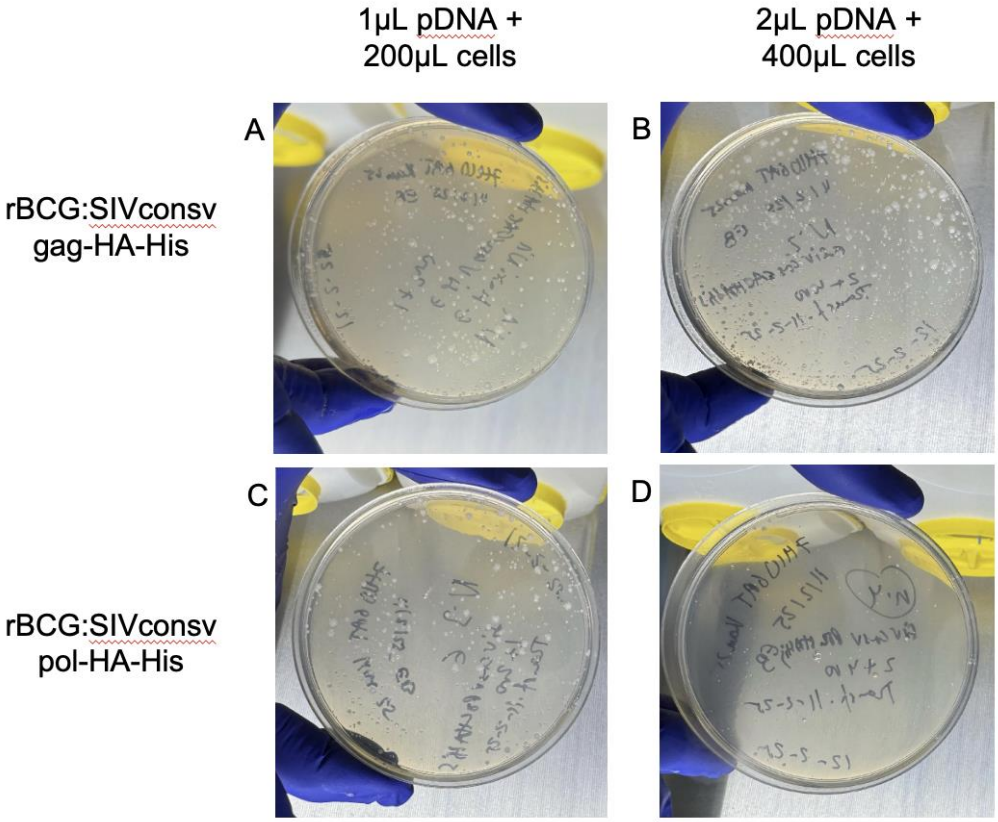

Figure 3. Phenotypic evaluation of the recombinant BCGΔLys strains harboring SIVconsv.gag-HA-His or SIVconsv.pol-HA-His constructs after transformation. Recombinant strains were plated on 7H10 agar supplemented with glycerol, ADC, Tween 80, and kanamycin. (A–B) Colony growth of BCGΔLys:SIVconsv.gag-HA-His following electroporation with (A) 1 µL plasmid DNA and 200 µL electrocompetent cells or (B) 2 µL plasmid DNA and 400 µL electrocompetent cells. (C–D) Colony growth of BCGΔLys:SIVconsv.pol-HA-His under the same transformation conditions as in (A–B), respectively.

6.2. Genetic Characterization of p223:SIVconsv.gag-HA-His and p223:SIVconsv.pol-HA-His Plasmid DNA and rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His Strains

The next step was to verify the integrity of both plasmid DNAs, previously purified using the Qiagen Maxiprep kit, by PCR analysis. As shown in **Figure 4**, a 1032 bp band was obtained for SIVconsv.gag-HA-His DNA fragment using p223:SIVconsv.gag-HA-His as atemplate (**A**), which corresponds to the expected fragment length. Similarly, a band of 1864 bp was obtained for SIVconsv.pol-HA-His DNA fragment using p223:SIVconsv.pol-HA-His as template (**B**). This verification step was crucial to ensure the suitability of the Maxiprep-purified plasmids for use as controls in subsequent genetic analyses.

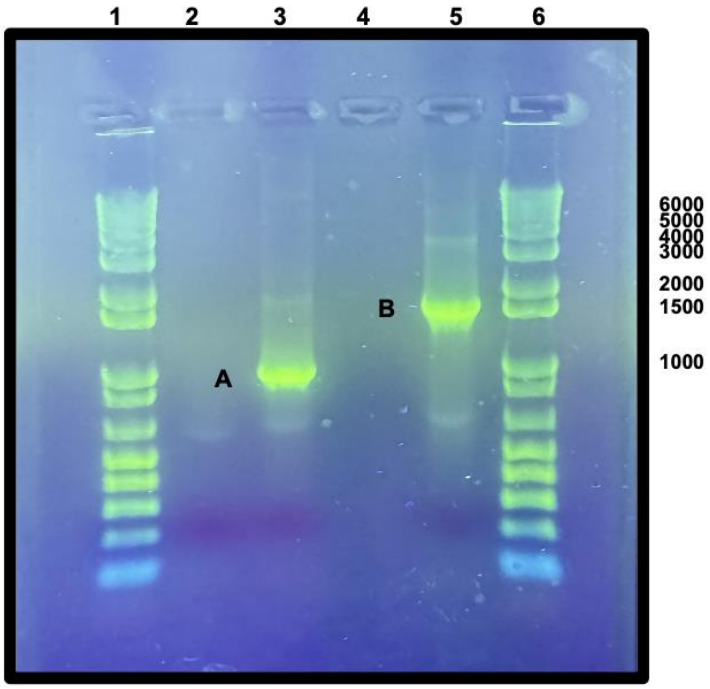

Figure 4. Genetic characterization of SIVconsv.gag-HA-His and SIVconsv.pol-HA-His DNA sequences PCR analysis using p223:SIVconsv.gag-HA-His and p223:SIVconsv.pol-HA-His as templates. Specific forward and reverse primers were used to amplify each expression cassette. Lanes 1 and 6: molecular weight markers; lane 2: no-template control (H_2O); lane 3: PCR product from p223:SIVconsv.gag-HA-His (1032 bp, labelled A); lane 4: empty; lane 5: PCR product from p223:SIVconsv.pol-HA-His (1864 bp, labelled B).

After verifying the integrity of the purified plasmids, a second PCR analysis was conducted to genetically assess the success of the transformation process **(Figure 5)**. For this, 1 mL of cultures of clones 1 and 2 from each recombinant strain were pretreated and used as the PCR template. In parallel, PCR was also performed using the purified

plasmid DNAs as positive controls. To further confirm plasmid identity and integrity, both undigested and HindIII-digested forms of the plasmid DNAs were included in the electrophoretic assay. The same experiment was performed with clones 3 and 4 of each recombinant strain (Figure not shown).

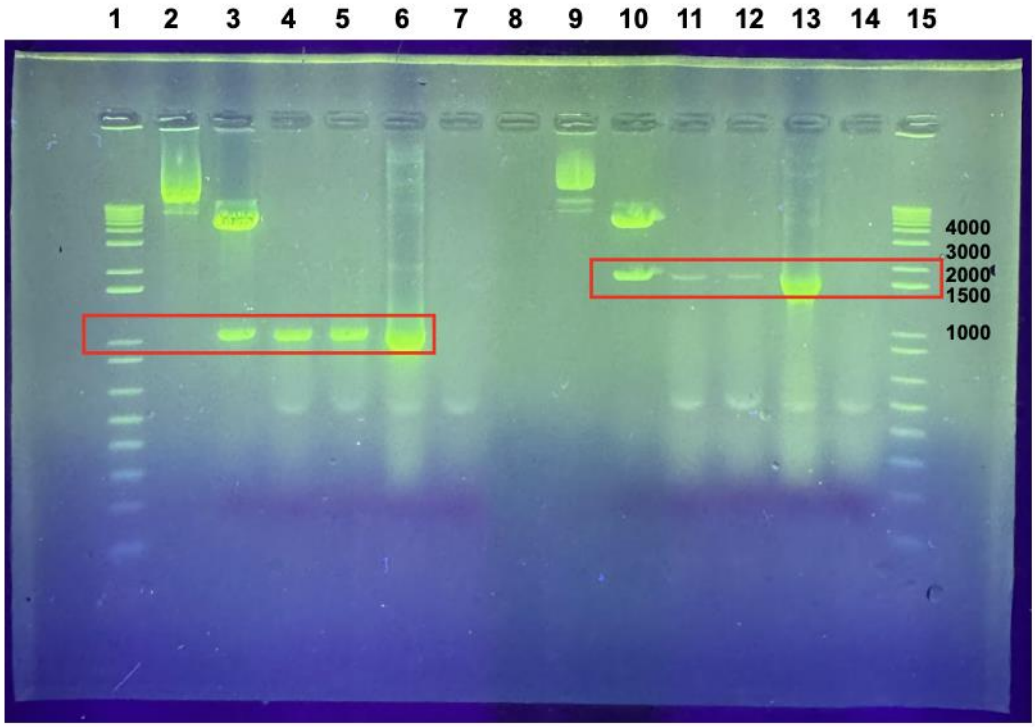

Figure 5. Genetic characterization of recombinant BCGΔLys:SIVconsv.gag-HA-His and BCGΔLys:SIVconsv.pol-HA-His strains by PCR analysis. Specific primers were used to amplify the expression cassettes, and plasmid identity was confirmed by restriction digestion. Lanes 1 and 15: molecular weight markers; lanes 2–3: p223:SIVconsv.gag-HA-His plasmid (lane 2: undigested; lane 3: HindIII-digested); lanes 4–5: recombinant BCGΔLys:SIVconsv.gag-HA-His clones 1 and 2; lane 6: PCR product from p223:SIVconsv.gag-HA-His (positive control, 1032 bp); lanes 7 and 14: no-template control (H_2O); lane 8: empty; lanes 9–10: p223:SIVconsv.pol-HA-His plasmid (lane 9: undigested; lane 10: HindIII-digested); lanes 11–12: recombinant BCGΔLys:SIVconsv.pol-HA-His clones 1 and 2; lane 13: PCR product from p223:SIVconsv.pol-HA-His (positive control, 1864 bp).

6.3. Heterologous SIVconsv.gag-HA-His and SIVconsv.pol-HA-His Protein Expression

To evaluate the expression of HA-tagged SIVconsv antigens in recombinant BCGΔLys strains, a dot blot assay was performed using BCG cell lysates from selected clones. As shown in **Figure 6**, no signal was detected in the wild-type BCG (**lane 1**) or in the culture medium control **(lane 4**), confirming the absence of background signal or unspecific antibody binding under the assay conditions.

As expected, the positive control (**lane 2**), consisting of a recombinant Posi-Tag Epitope Tag Protein, showed a strong HA signal at both 5 µL and 10 µL volumes. Notably, detectable HA-tagged protein expression was observed in recombinant BCG strains expressing SIVgag p11-HA-His-Tag protein (**lane 3**), SIVconsv.gag-HA-His protein (**lane 5**), and SIVconsv.pol-HA-His protein (**lane 6**), with signal intensity increasing in proportion to the sample volume applied. These results confirm successful expression and secretion of the recombinant antigens in BCGΔLys strains following plasmid transformation.

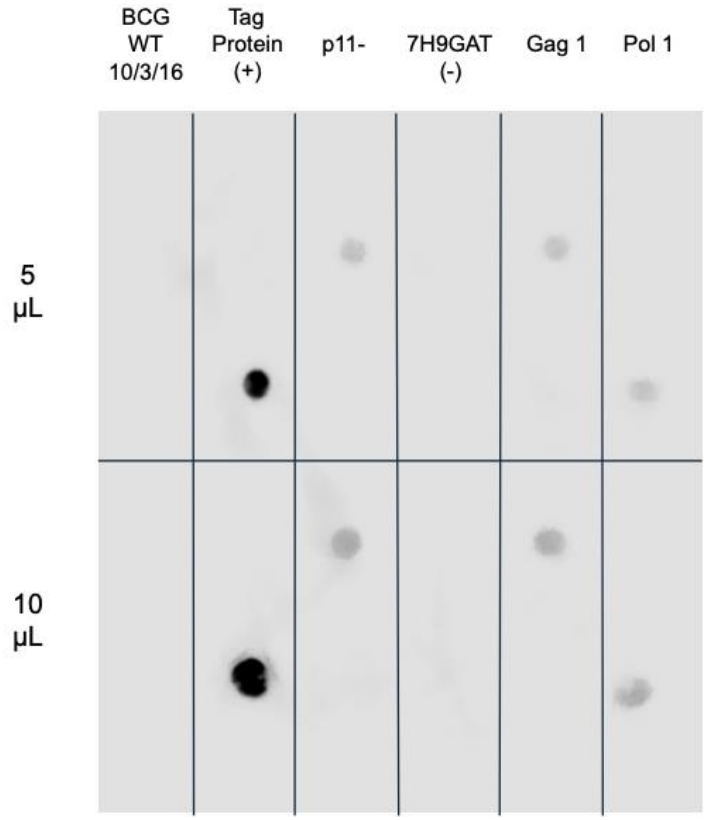

Figure 6. Dot blot analysis of HA-tagged protein expression. Supernatants of cell lysates were spotted in lanes 1–6 (left to right) as follows: (1) BCG wild type; (2) Recombinant Posi-Tag Epitope Tag Protein (positive control); (3) recombinant BCG strain expressing p11-HA-His-Tag; (4) 7H9 culture medium supplemented with glycerol, ADC, and Tween 80 (negative control); (5) Recombinant BCGΔLys:SIVconsv.gag-HA-His clone 1; (6) Recombinant BCGΔLys:SIVconsv.pol-HA-His clone 1. For each sample, previously adjusted to a protein concentration of 575 µg/mL, 5 µL (upper row) and 10 µL (lower row) were applied. The membrane was probed with mouse anti-HA.11 monoclonal antibody (1:2000), followed by HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody (1:4000). Chemiluminescent detection was performed using Clarity Western ECL Substrate, and the signal was acquired with the Odyssey Fc Imaging System (30-second exposure).

Following the qualitative confirmation of HA-tagged SIVconsv antigen expression by dot blot, SDS-PAGE followed by Western blot analysis was performed to assess the

molecular weight and integrity of the heterologous proteins. As shown in **Figure 7**, a clear positive band at approximately 45 kDa was observed in **lane 6**, corresponding to the recombinant Posi-Tag Epitope Tag Protein, validating the specificity and functionality of the HA detection system.

Unexpected signal was detected in **lane 7**, the wild-type BCG negative control. Because WT BCG carries no HA tag, any chemiluminescent band is unexpected. The most plausible explanation is nonspecific reactivity generated by the horseradish-peroxidase (HRP) antibody used to visualise the Precision Plus Protein WesternC molecular-weight marker (MWM): after incubation, residual HRP conjugate can bind endogenous proteins on the entire membrane, producing background even in tag-free lanes.

In **lanes 2–3** (rBCG:SIVconsv.Gag-HA-His, 15 µL and 30 µL) and **lanes 8-9** (rBCG:SIVconsv.Pol-HA-His, 15 µL and 30 µL) multiple bands were likewise observed. Each recombinant sample is expected to yield a single dominant band, approximately of 38 kDa for Gag and 70 kDa for Pol proteins, but the HRP-mediated background, coupled with the high total-protein load (25 µg per lane), obscured these signals. In **lane 5**, which contains the p11-positive control, the anticipated band at ≈ 14 kDa was not visible under the same conditions, reinforcing the conclusion that marker-related background masks genuine antigen bands when 25 µg of lysate are loaded.

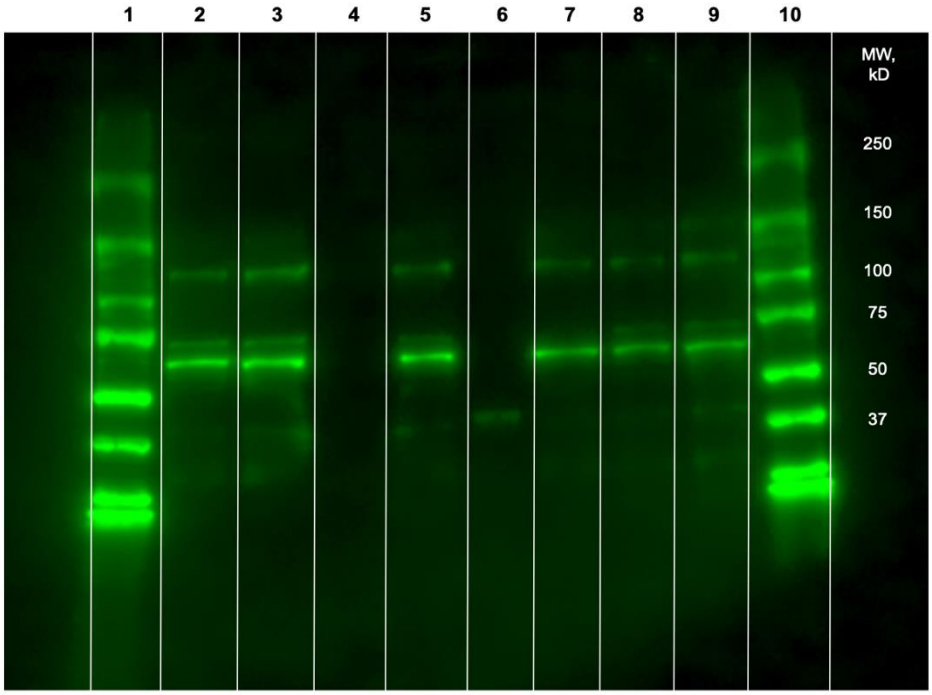

Figure 7. Western blot analysis of recombinant SIVconsv antigen expression in rBCG strains. All protein samples were adjusted to a concentration of 575 µg/mL, and 25 µg of total protein was loaded per lane. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Detection was performed using a mouse anti-HA.11 monoclonal antibody (1:2000), followed by an HRP-conjugated goat

anti-mouse IgG (H+L) secondary antibody (1:4000). Chemiluminescent signal was developed using Clarity Western ECL Substrate and acquired with the Odyssey Fc Imaging System (30-second exposure). Lane 1 and Lane 10: molecular weight markers; Lane 2: 15 µL of rBCG:SIVconsv.Gag-HA-His lysate; Lane 3: 30 µL of rBCG:SIVconsv.Gag-HA-His lysate; Lane 4: 7H9 culture medium supplemented with glycerol, ADC, and Tween 80 (negative control); Lane 5: Supernatant from recombinant BCG p11+ strain expressing P11-HA-His (positive control); Lane 6: Recombinant Posi-Tag Epitope Tag Protein (45 kDa; positive control); Lane 7: BCG wild-type (negative control); Lane 8: 15 µL of rBCG:SIVconsv.Pol-HA-His lysate; Lane 9: 30 µL of rBCG:SIVconsv.Pol-HA-His lysate.

6.4. Titration of Working Vaccine Stocks. Colony-Forming Units per Milliliter (CFU/mL) Quantification

Colony-forming units (CFU) were quantified after 3 weeks of incubation, and CFU/mL values were calculated for each Working Vaccine Stock (WVS): rBCG:SIVconsv.gag-HA-His, rBCG:SIVconsv.pol-HA-His, and BCG WT Pasteur. For each strain, six independent measurements per WVS (two replicate dilution series per vial, three dilutions each) were used to compute the average CFU/mL per vial. The resulting values were then used to calculate the mean and standard deviation across the three vials of each WVS.

Representative CFU plates showing colony density at dilutions 10^{-4}, 10^{-5}, and 10^{-6} for each strain are presented in **Figure 8**. These images reflect typical colony growth across the replicates used for quantification.

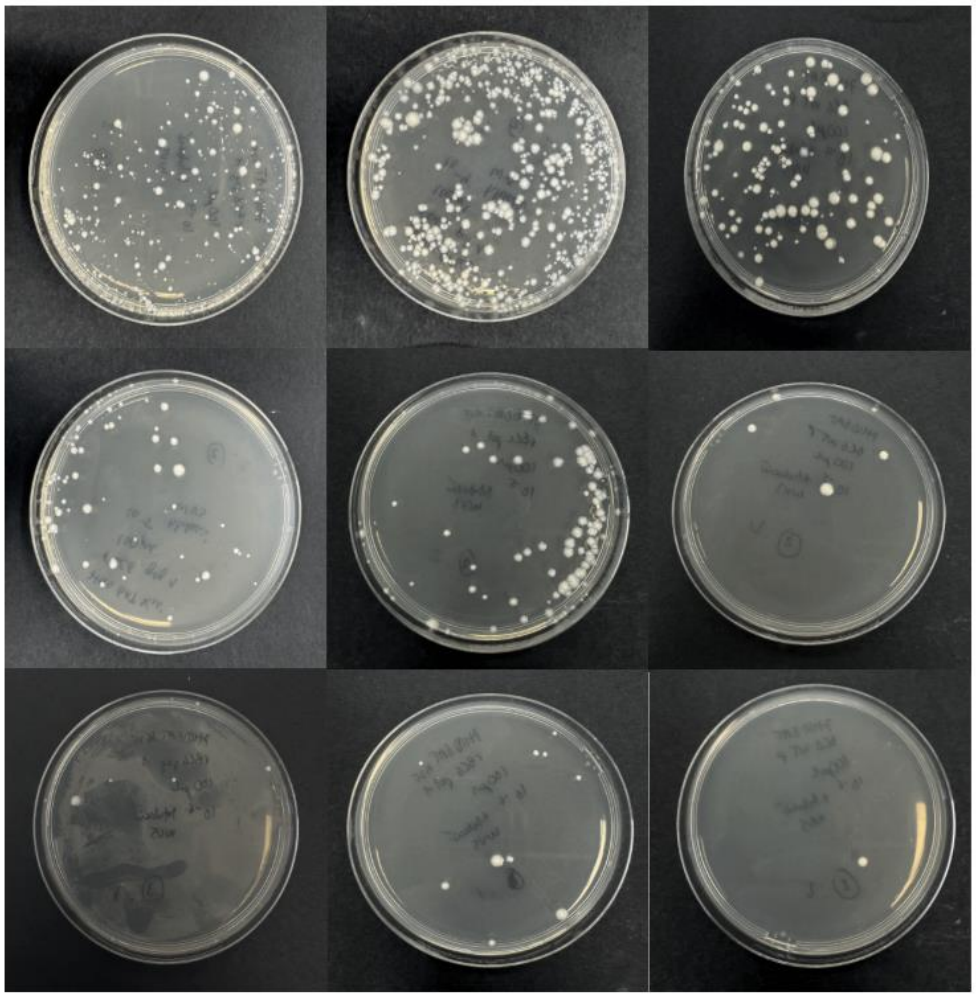

Figure 8. Representative CFU plates for titration of Working Vaccine Stocks (WVS). Photographs show representative colony growth from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions (top to bottom) of each BCG strain plated on Middlebrook 7H10 agar after 3 weeks of incubation at 37 °C. The three columns correspond to: rBCG:SIVconsv.gag-HA-His (left), rBCG:SIVconsv.pol-HA-His (center), and BCG WT Pasteur (right). Images reflect the average colony density observed across all replicates used for CFU/mL quantification.

The corresponding quantitative results are summarized in **Table 3:**

Strain	Vial 1	Vial 2	Vial 3	Mean (CFU mL^{-1})	Standard Deviation	CV (%)
Gag	9.49×10^7	8.30×10^7	7.88×10^7	8.56×10^7	8.34×10^6	9.7
Pol	1.37×10^8	1.22×10^8	1.25×10^8	1.28×10^8	7.93×10^6	6.2
WT	2.68×10^7	8.27×10^6	1.07×10^7	1.52×10^7	1.01×10^7	66.1

The observed variability between vials, particularly in the WT strain, is reflected in the coefficient of variation (CV), which ranged from approximately 31% to 65%. Despite this, the calculated averages provide a reliable estimate of the viable bacterial content

for each vaccine stock, which has been documented for subsequent dose preparation in preclinical immunization studies.

6.5. In vitro Genetic Stability of the plasmid DNA in rBCG:SIVconsv.gag-HA-His and rBCG:SIVconsv.pol-HA-His Strains

To evaluate the genetic stability of the recombinant BCG strains rBCG:ΔLys:SIVconsv.gag-HA-His and rBCG:ΔLys:SIVconsv.pol-HA-His, PCR analysis was performed on samples obtained after each of four weekly subcultures, corresponding to approximately 30 bacterial generations. As shown in **Figure 9**, specific bands of the expected size (~1032 bp for gag and ~1864 bp for pol) were consistently detected in the recombinant strains throughout the four-week period, confirming the retention of the integrated expression cassettes.

In the case of rBCG:ΔLys:SIVconsv.gag-HA-His (**lanes 3–6**), strong bands corresponding to the gag fragment were observed at weeks 1 and 2. However, no band is visible in **lane 5** (week 3), which is attributed to an experimental error during PCR setup. Specifically, the PCR tubes corresponding to weeks 3 and 4 were found to be partially open after the reaction, resulting in evaporation of part of the sample. Additionally, the remaining PCR product was further diluted during loading due to the addition of loading dye, reducing the DNA concentration. Despite the absence of a visible band in **lane 5**, the reappearance of a clear band in **lane 6** (week 4), which was derived from the week 3 bacterial culture, confirms that the gag sequence was retained, and the observed absence was procedural rather than biological.

For rBCG:ΔLys:SIVconsv.pol-HA-His (**lanes 10–13**), strong and consistent amplification of the pol sequence was observed in all subcultures across the four weeks. No amplification was detected in the negative controls (**lanes 7–9**), validating the specificity of the PCR reactions.

Overall, these results confirm the genetic stability of the integrated SIVconsv.gag and SIVconsv.pol cassettes in the recombinant BCG strains over at least 30 in vitro generations in selective medium.

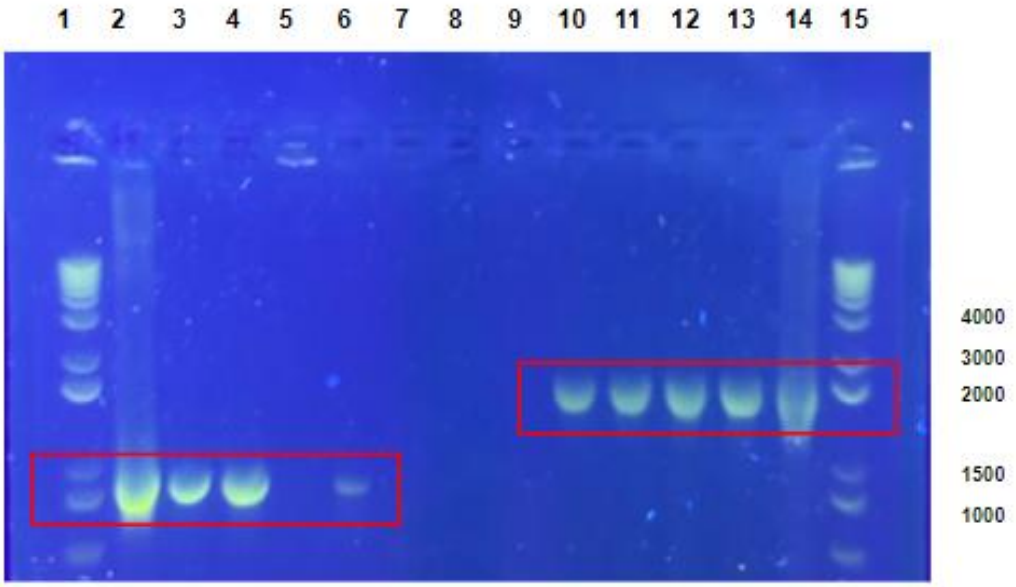

Figure 9. In vitro genetic stability analysis of rBCG:ΔLys:SIVconsv.GAG-HA-His and rBCG:ΔLys:SIVconsv.POL-HA-His strains derived from WVS. PCR was used to evaluate the presence of the expression cassettes in weekly cultures of the recombinant strains. Lanes 1 and 15: molecular weight marker; lane 2: PCR product from plasmid p223:SIVconsv.gag-HA-His (positive control, 1032 bp); lanes 3–6: recombinant BCGΔLys:SIVconsv.gag-HA-His clone 1 at weeks 1, 2, 3, and 4, respectively; lanes 7 and 9: negative control (non-transformed BCG WT Pasteur); lane 8: negative control (H_2O); lanes 10–13: recombinant BCGΔLys:SIVconsv.pol-HA-His clone 1 at weeks 1, 2, 3, and 4, respectively; lane 14: PCR product from plasmid p223:SIVconsv.POL-HA-His (positive control, 1864 bp).

7. Discussion

The introduction to this work highlighted the urgent need for a single, heat-stable vaccine that can be delivered safely at birth in regions where HIV and TB coinfection burden is higher. rBCG vectors meet these requirements because (i) the parental BCG vaccine has a century-long safety record, even in neonates; (ii) BCG preferentially targets antigen-presenting cells and drives strong Th1/Th17 immunity; and (iii) the ubiquity of national BCG programmes facilitates rapid implementation.

Building on these advantages, the present study employed an integrative, auxotrophic system to deliver two synthetic, highly conserved SIV immunogens (SIVconsv.gag and SIVconsv.pol) in separate rBCG backbones. This design addresses two major hurdles outlined in the introduction, HIV sequence diversity and plasmid genetic instability, by (i) focusing T-cell responses on functionally constrained epitopes and (ii) hard-wiring the inserts into the BCG chromosome, thereby avoiding plasmid loss.

Restoration of lysine prototrophy together with kanamycin resistance yielded robust colony growth of both recombinant strains on selective 7H10 agar plates (**Fig. 3**). In

contrast, the parental BCGΔlys failed to grow in the absence of lysine or in the presence of kanamycin (**Fig. 2**), confirming the stringency of the dual-selection system. These findings are consistent with previous auxotrophic-complementation studies showing >99 % genetic stability over serial passages and support the platform's suitability for large-scale manufacturing [11].

PCR amplification of SIVconsv.gag-HA-His and SIVconsv.pol-HA-His DNA coing sequences from week-0 and week-4 sub-cultures revealed persistent 1032 bp (gag) and 1864 bp (pol) bands (**Fig. 5 & Fig. 9**), indicating that no deletions or rearrangements occurred after approximately 30 bacterial generations in vitro. The single missing gag band at week 3 was traced to evaporation during PCR set-up rather than biological loss, as shown by its reappearance the following week. These data are in line the high in-vitro stability reported for other integrative rBCG vectors such as BCG.HIVACAT and BCG.HTI [10].

Western-blot analysis showed diffuse background and multiple spurious bands, including a signal in the WT lane (**Fig. 7**). The data suggest that the HRP-labelled reagent required to reveal the WesternC ladder, rather than the anti-HA primary/secondary pair, is chiefly responsible for the nonspecific background. Three complementary corrective measures are recommended: (i) Ni-NTA affinity enrichment followed by anti-His probing: The common His_6 tag on Gag (~38 kDa), Pol (~70 kDa) and p11+ (~14 kDa) allows selective purification, greatly reducing total protein and background. Anti-His antibodies applied after purification should reveal sharp single bands at the expected sizes; (ii) Eliminate HRP detection of the ladder: Replace the WesternC marker with a prestained, HRP-free standard, or load the marker in a separate lane that is not exposed to HRP reagents. A colour image of the prestained ladder can be captured immediately after transfer; the chemiluminescent image of the sample lanes is then taken separately, and the two images are digitally overlaid. This avoids introducing additional HRP conjugate and removes its associated nonspecific binding; (iii) Reduce protein load and include proper controls: Loading 5–10 µg of total protein per lane, together with a constitutive BCG antigen as a loading control, will keep band intensities within the linear response range and minimise background. Serial two-fold dilutions of the enriched lysate can further verify that signal intensities scale proportionally.

Implementing these steps should provide clear, unambiguous confirmation of the full-length recombinant antigens and their expected molecular weights, thereby strengthening the analytical package for subsequent pre-clinical studies.

Accurate CFU titration is pivotal for dosing non-human primates. Using six independent measurements per vial (two dilution series × three dilutions), the mean CFU/mL values obtained from the new workbook are 8.56×10^7 for rBCG-Gag, 1.28×10^8 for rBCG-Pol

and 1.52 × 10^7 for WT BCG (**Table 3**). Representative colony morphologies are shown in Fig. 8, where the recombinant plates display smaller, slightly smoother colonies typical of recombinant BCG strains expressing heterologous antigens.

The coefficients of variation (CV) show low vial-to-vial dispersion for the recombinant stocks, 9.7 % for Gag and 6.2 % for Pol, but a markedly higher CV for the WT stock (≈ 66 %). Two factors most likely underlie this heterogeneity: (i) Manual pipetting error during serial dilutions. Preparing two dilution banks per vial introduces cumulative inaccuracy, especially at 10^{-6} where colony counts are low; automated or repeater pipettes should reduce this variance; (ii) Biological variability in freeze-thaw recovery among vials, a common feature of BCG preparations [17].

Despite these sources of variation, every vial still exceeded the WHO potency requirement of 1 × 10^6 CFU/mL for injectable BCG, providing a comfortable safety margin for macaque-dosing protocols.

Historically, BCG priming followed by a non-replicating viral boost (e.g., MVA, ChAdOx1) has resulted in robust, poly-functional T-cell responses in mice and non-human primates [11]. The titres reported here are comparable to those achieved with the BCG.HTI candidate (2-4 × 10^7 CFU/mL) and should deliver sufficient antigen exposure for priming. The confirmed genetic stability of kanamycin-selected integrants nonetheless addresses concerns about plasmid loss during in-vivo replication. Genetic stability analyses should be performed after non-human primates' immunization.

Plate-count variability could be further reduced by plating 10^{-3} dilutions (to capture 30-300 colonies) or by employing flow-cytometric CFU surrogates. For a cleaner Western blot, first enrich His_6-tagged Gag (~38 kDa), Pol (~70 kDa) and p11+ (~14 kDa) by Ni-NTA; use an HRP-free prestained ladder (or overlay its colour image) to avoid nonspecific marker signal, and load only 5–10 µg of purified protein per lane with serial two-fold dilutions to ensure linear, quantifiable detection. Finally, In-vivo stability of rBCG:SIVconsv strains beyond 30 in-vitro generations remains to be demonstrated; monitoring cassette retention in spleen isolates from vaccinated macaques will be crucial.

8. Conclusions

rBCG strains expressing SIVconsv Gag and Pol antigens show genetic stability, appropriate bacterial potency and preliminary antigen secretion, supporting their continued development as a dual HIV/TB vaccine platform. Key technical gaps, especially in antigen detection, must be addressed before advancing to non-human-

primate studies and pilot-scale manufacturing. The main specific conclusions can be summarized:

1. **Antigen-detection gap:**
- Dot-blot data indicate production of HA-tagged antigens, but current Western blots are inconclusive because of high background.
- Improving detection by purifying His-tagged proteins and then probing with high-affinity antibodies will allow clear confirmation of protein integrity and expression level.
2. **Generation of functional rBCG strains:**
- Stable integration in BCG genome of gag and pol plasmid DNAs was achieved; dual selection (lysine prototrophy + kanamycin resistance) verified correct construct insertion.
3. **In-vitro genetic stability:**
- PCR after roughly 30 bacterial generations consistently detected intact gag (1032 bp) and pol (1864 bp) DNA fragments, demonstrating cassette retention.
4. **Viable-titre quantification of WVS:**
- Mean titres of 8.56×10^7 CFU/mL for rBCG-Gag and 1.28×10^8 CFU/mL for rBCG-Pol far exceed the WHO potency benchmark for injectable BCG, and vial-to-vial variability is low (CV ≈ 10 % for Gag and ≈ 6 % for Pol).
5. **Next steps:**
- Optimise heterologous antigen detection to confirm full-length proteins expression.
- Standardize dilution and colony-count procedures to reduce CFU variability before scaling to GLP/GMP production.
- Confirm expression vector in vivo genetic stability by recovering bacilli from vaccinated animals.
- Evaluate immunogenicity in a prime-boost regimen, correlating immune read-outs with antigen expression and assess protection after pathogenic SIV challenge.

9. Aknowledgements

This thesis was conducted in the Department of Microbiology, Clinical Laboratories, Hospital Vall d'Hebron, in collaboration with the Vall d'Hebron Research Institute (VHIR). All training, experimental work and data analysis were completed under the supervision of Dr Joan Joseph Munné, Principal Investigator. I am deeply grateful for his steady guidance at every stage of the project and for his practical solutions to the methodological challenges we faced.

My sincere thanks also go to Dr Narcís Saubi, Senior Scientist, whose expertise and clear explanations greatly advanced my understanding of the research topic and whose theoretical and practical support proved invaluable.

I further acknowledge the international collaborations that underpin this work, especially the partners of the European AIDS Vaccine Initiative (EAVI2020) and Professor Tomáš Hanke for designing the immunogens. Finally, I thank the entire laboratory staff, along with the professors and fellow students I met during my studies, for their constant assistance and encouragement.

Building on these collective efforts, this thesis also speaks to a broader public-health objective. As Dr Joan Joseph Munné notes in the closing remarks of his 2025 review on recombinant BCG vectors, leveraging the century-old BCG platform, already administered to more than three billion people against tuberculosis, as a carrier for HIV immunogens offers a uniquely affordable and scalable route to tackle both the HIV and TB pandemics simultaneously, especially in newborns and other high-risk populations. The data and insights generated here contribute a small but tangible step toward that integrated vision.

10. References

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