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Stable Expression of Lentiviral Antigens by Quality-Controlled Recombinant *Mycobacterium bovis* BCG Vectors

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The well-established safety profile of the tuberculosis vaccine strain, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), makes it an attractive vehicle for heterologous expression of antigens from clinically relevant pathogens. However, successful generation of recombinant BCG strains possessing consistent insert expression has encountered challenges in stability. Here, we describe a method for the development of large recombinant BCG accession lots which stably express the lentiviral antigens, human immunodeficiency virus (HIV) gp120 and simian immunodeficiency virus (SIV) Gag, using selectable leucine auxotrophic complementation. Successful establishment of vaccine stability stems from stringent quality control criteria which not only screen for highly stable complemented BCG $\Delta leuCD$ transformants but also thoroughly characterize postproduction quality. These parameters include consistent production of correctly sized antigen, retention of sequence-pure plasmid DNA, freeze-thaw recovery, enumeration of CFU, and assessment of cellular aggregates. Importantly, these quality assurance procedures were indicative of overall vaccine stability, were predictive for successful antigen expression in subsequent passaging both *in vitro* and *in vivo*, and correlated with induction of immune responses in murine models. This study has yielded a quality-controlled BCG $\Delta leuCD$ vaccine expressing HIV gp120 that retained stable full-length expression after 10^{24} -fold amplification *in vitro* and following 60 days of growth in mice. A second vaccine lot expressed full-length SIV Gag for $>10^{68}$ -fold amplification *in vitro* and induced potent antigen-specific T cell populations in vaccinated mice. Production of large, well-defined recombinant BCG $\Delta leuCD$ lots can allow confidence that vaccine materials for immunogenicity and protection studies are not negatively affected by instability or differences between freshly grown production batches.

The immense global burden of human immunodeficiency virus (HIV) infection necessitates the development of an efficacious vaccine. There is increasing interest in the use of live recombinant bacterial vectors as HIV vaccines due to the inherent advantages of utilizing a replicating antigen delivery system that is itself an effective adjuvant (1, 2). Previous studies have examined the use of live Gram-positive and Gram-negative bacterial vectors, including recombinant *Salmonella*, *Listeria*, *Streptococcus*, and *Escherichia coli*, for heterologous expression of HIV antigens, with varying success (3–8).

Mycobacterium bovis BCG is the most widely administered vaccine in the world (9). Its extensively documented safety in immunocompetent individuals, relatively low production cost, and well-established infrastructure for vaccine administration make it an ideal candidate for use as an anti-HIV vaccine vehicle (10–12). In addition to the logistical advantages of using BCG, mycobacterial antigen delivery systems possess inherent adjuvant properties which activate innate immunity (13, 14). Mycobacteria such as BCG contain many pathogen-associated molecular patterns known to strongly activate human Toll-like receptors (TLR). Innate recognition of mycobacterial cell wall components by TLR1 and TLR2, TLR4 ligation by GroEL2, and TLR9-dependent sensing of mycobacterial DNA all serve to establish an innate cytokine profile which actively drives both humoral and cellular adaptive immune responses (15–17). Many currently administered vaccines, however, require extraneous chemical adjuvants which

serve to similarly induce innate immunity and subsequent adaptive responses specific to the antigens within the vaccine.

Additional advantages include the fact that BCG is readily phagocytized and processed by antigen-presenting cells (APCs), such as peripheral macrophages and dendritic cells, whereby antigen presentation by class II major histocompatibility complex (MHC) or cross-presentation by MHC class I (MHCI) may effec-

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TABLE 1 Bacterial strains

Strain	Genotype	How constructed	Source or reference
<i>Mycobacterium bovis</i> BCG			
mc ² 6450	Parental, Danish strain		AERAS
mc ² 6452	BCG Danish $\Delta leuCD::\gamma\delta$ -res hyg sacB $\gamma\delta$ -res	ST with phAESP22 (42)	This study
mc ² 6454	BCG Danish $\Delta leuCD::\gamma\delta$ -res	Unmarking with phAE201	This study
mc ² 6585	BCG Danish $\Delta leuCD::\gamma\delta$ -res (pYUB2115)	Transformation with pYUB2115	This study
SL524	BCG Danish $\Delta leuCD::\gamma\delta$ -res (pSL701)	Transformation with pSL701	This study
SL525	BCG Danish $\Delta leuCD::\gamma\delta$ -res (pSL720)	Transformation with pSL718	This study
SL547	BCG Danish $\Delta leuCD::\gamma\delta$ -res (pSL509)	Transformation with pSL509	This study
<i>E. coli</i>			
DH5 α	Chemically competent		Life Technologies
JW0070-1	F ⁻ $\Delta(araD-araB)567 \Delta leuD778::kan \Delta lacZ4787(::rrnB-3) \lambda^-$ <i>rph-1 \Delta(rhaD-rhaB)568 hsdR514</i>		43

tively prime CD4⁺ or CD8⁺ T cell responses, respectively. Concomitant expression of HIV proteins by BCG leads to APC display of lentiviral peptides, capable of inducing proliferation of HIV-specific T cell populations (18–25). Unlike protein subunit or DNA vaccines, live vector vaccines also persist longer in the host, allowing more time for exposure to the antigens of interest and potentially enhancing antigen-specific immunogenicity. BCG is known to replicate in humans after its administration, generating a sustained pool of lentiviral protein (26, 27). Importantly, however, BCG is ultimately cleared by host defenses, reducing the potential for T cell anergy and tolerance to lentiviral antigens (26, 27).

Important factors which influence antigen expression in BCG have been identified, including the choice of BCG substrain, expression construct, antigen promoter strength, secretion signals, codon optimization, and insert toxicity (27–37). Studies attempting to use BCG for expression of recombinant antigens have met with obstacles that impede establishment of stable immunogen expression. Joseph et al. observed that expression of the HIV gp120 antigen in BCG Pasteur often led to mutation and establishment of subpopulations which had plasmid insert deletions in >85% of BCG transformants (38). Upon attempting to express simian immunodeficiency virus (SIV) Gag and Nef in BCG Pasteur, Méderlé discovered that operons cloned into replicating vectors resulted in strains of low genetic stability and rapid loss of antigen expression *in vivo* (27). Similarly, Stover et al. were able to create recombinant BCG (rBCG), producing HIV gp120 only when the insert was cloned into a single copy integrative vector, whereas replicative cloning and antigen expression proved to impart lethal toxicity (39). The initial success in creating recombinant BCG transformants has not been readily indicative of successful antigen retention after vaccine lot preparation, as observed by Lim et al. (24). In their studies, expression of the full-length SIV Env protein and of several fragments of the antigen met with success in starter cultures. However, following expansion for immunogenicity studies, only a single BCG strain expressing the N-terminal 245 amino acids of Env remained stable enough to be used *in vivo* (24).

In this study, we established an anti-HIV, rBCG $\Delta leuCD$ -based vaccine production protocol wherein standardized practices for highly consistent manufacture of stable vehicles were used. We established quality control (QC) parameters which not only pre-

dicted successful expansion of single transformants to large-volume accession lots but also were indicative that these vaccines would yield stable lentiviral antigen expression even after further lot expansion in culture or after animal inoculation. Here, we describe these vaccine production and quality control procedures and provide a comparison of both antigen stability and immunogenicity between vaccine accession lots that have either passed or failed the quality control process.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. The auxotrophic strains of *Mycobacterium bovis* BCG Danish (BCGD) $\Delta leuCD$ were generated by specialized phage transduction and unmarked using a conditionally replicating mycobacteriophage containing a $\gamma\delta$ resolvase gene (40–42). Recombinant expression plasmids were transformed in JW0070-1 *Escherichia coli* $\Delta leuD::kan$ (Table 1) (43), and transformants were selected by complementation of leucine auxotrophy in M9 minimal medium. Liquid cultures of BCG were grown in Difco Middlebrook 7H9 medium supplemented with 0.5% glycerol, oleic albumin dextrose catalase (OADC), and 0.05% tyloxapol. BCG transformants were grown on Difco Middlebrook 7H10 agar plates supplemented with 0.5% glycerol, OADC, and 0.05% tyloxapol. In the absence of *leuCD* complementation, all strains were grown in the presence of 50 $\mu\text{g}/\text{ml}$ L-leucine. Liquid cultures of <50 ml were grown at 37°C and shaken at 120 rpm. Liquid cultures of >50 ml were expanded to no more than 250 ml in 1-liter roller bottles and rotated at 6 rpm. For sequencing and manipulation of plasmid DNA, *E. coli* DH5 α was used. *E. coli* was grown in lysogeny broth (LB) or on agar plates supplemented with appropriate antibiotics.

Plasmids. Plasmids used in this study are listed in Table 2. To engineer a selection system that does not rely on plasmid-encoded antibiotic resistance markers for retention, pYUB2115 was constructed by replacing the open reading frame encoding the aminoglycoside phosphotransferase (*aph*) from pMV261 (39) with *leuC* and *leuD* from *Mycobacterium smegmatis* mc²155 (44). This plasmid was transformed into the JW0070-1 *E. coli* leucine auxotroph and selected for complementation. The promoter for *aph*, which drives expression of *leuCD* in pYUB2115, is functional in *E. coli*, BCG, *M. smegmatis*, and *M. tuberculosis*, allowing for auxotrophic complementation across these species. The *leuCD* region was PCR amplified from *M. smegmatis* with primers 5'-TTTTTCATATGGATCAACCG ACGCAGAC and 5'-TTTTTGCGGCCGCTCCAGAACCGACTAGG TGG, and the vector was amplified from pMV261 with 5'-TTTTTGATA TCCACTGGCAGAGCATTACGCTGAC and 5'-TTTTTCATATGACCC CTGTATTACTGTTTATGTAAGC. The pMV261 PCR product was cut with EcoRV and NdeI and the *leuCD* PCR product with NdeI and a blunt

TABLE 2 Plasmid constructs and phages

Plasmid construct/phage	Description	Source or reference
Plasmids		
pYUB2115	<i>oriM</i> , <i>oriE</i> , <i>P_{aph}</i> , <i>leuCD_{M. smegmatis}</i>	This study
pSL701	<i>P_{groEL2}</i> , <i>lpqH</i> signal sequence (aa ^a 1–46)-HIV gp120- <i>lpqH</i> (aa 71–158) fusion	This study
pSL718	<i>P_{groEL2}</i> , <i>fbpB</i> signal sequence (aa 1–41)-HIV gp120- <i>lpqH</i> (aa 71–158) fusion	This study
pSL720	<i>P_{groEL2}</i> , <i>fbpB</i> signal sequence (aa 1–41)-HIV gp120- <i>lpqH</i> (aa 71–145) fusion	This study
pSL509	<i>P_{groEL2}</i> , <i>fbpB</i> signal sequence (aa 1–41)-SIV <i>gag_{mac239}</i> fused to hemagglutinin	This study
Phages		
phAESP22	<i>M. tuberculosis leuCD::hyg</i> ; used to construct mc ² 6452 (Table 1)	This study, 42
phAE201	Unmarking phage ($\gamma\delta$ resolvase); used to construct mc ² 6454 (Table 1)	This study

^a aa, amino acid.

enzyme. The resulting pYUB2115 plasmid contains *oriM* from pAL5000, *M. bovis* BCG promoter *P_{groEL2}* for the expression of foreign antigens, and a *leuCD* system which is driven by a kanamycin promoter. Retention of pYUB2115 plasmids by the BCG leucine auxotroph mc²6454 was dependent on this unmarked leucine complementation system. Expression of *leuC* and *leuD* from pYUB2115 and derivatives rescued the leucine auxotrophy, allowing successfully transformed BCG Δ *leuCD* to grow without leucine supplementation. The gene for the lentiviral antigen HIV 1086.C gp120 (HIV gp120) (45) was cloned into the pYUB2115 backbone, where antigen expression was under the control of the constitutive *Mycobacterium tuberculosis groEL2* promoter. Plasmid pSL701 expresses HIV gp120 with an N-terminal fusion to the *M. tuberculosis lpqH* secretion signal and a C-terminal fusion to amino acids 71 to 158 from LpQH. Plasmid pSL718 expresses gp120 fused at the N terminus to both the *M. tuberculosis fbpB* signal sequence (*fbpB* ss) and the endogenous gp120 signal sequence (gp120 ss) and, at the C terminus, to amino acids 71 to 158 of LpQH. In plasmid pSL509, SIV *gag_{mac239}* (SIV *gag*) is fused at the N terminus to the *fbpB* signal sequence, and expression is under the control of the *groEL2* promoter.

Bacterial transformation. Electrocompetent BCG cells were prepared by pelleting log-phase (optical density at 600 nm [OD₆₀₀] between 0.6 and 0.8) liquid cultures and washing three times in 10% glycerol with 0.05% tyloxapol. The auxotrophs were electroporated using 0.5 μ g plasmid DNA and recovered in Middlebrook 7H9 medium with appropriate supplements at 37°C overnight. Prior to plating, the transformants were washed twice using phosphate-buffered saline (PBS) with 0.05% tyloxapol (PBST) to remove residual leucine, followed by spreading on 7H10 agar.

Production of vaccine strain accession lots. BCG transformant colonies were picked following 3 to 4 weeks of growth on 7H10 agar and initially cultured in 3 ml Middlebrook 7H9 liquid medium. After cultures reached the log phase, medium volumes were then doubled. Consecutive expansions were performed until the total medium volume reached 1 liter of log-phase culture. Aliquots of 250 ml culture were then pelleted at 2,000 relative centrifugal force (RCF) for 15 min. Pellets were broken up by agitation and resuspended in 200 ml PBST. The cells were then centrifuged again and resuspended a second time in 100 ml PBST. Finally, a third pellet was resuspended in 50 ml PBST with 15% glycerol (PBSTG), and the suspension was homogenized by repeated pipetting and expulsion of liquid against the side of the turnip bottle. To remove large cell aggregates, the 50-ml suspension was initially passed through a 40- μ m screen, followed by a 20- μ m vacuum filter. The optical density for a 1:20 dilution of this suspension was then measured with a turbidimeter. Lots were prepared at an OD₆₀₀ of 1.0 and 5.0 (OD-1 and OD-5) by adding appropriate amounts of PBSTG. For a single accession lot, a total of 100 \times OD-1 and 50 \times OD-5 vials were aliquoted using a repipettor with frequent mixing. These vials were then quick-frozen in a previously cooled dry ice-ethanol bath for 10 min and stored at -80°C . Samples of accession lot contents underwent a pre-quality control check before performance of extensive QC procedures, which included immunoblotting for antigen

expression and plating on chocolate agar to ensure absence of contamination. In addition, plasmid isolation was performed on accession lot contents in order to confirm sequence purity of the plasmid insert. Briefly, cells were pelleted and incubated with Qiagen Miniprep buffer P1 and lysozyme at 60°C for 1 h. This was followed by the normal Qiagen miniprep protocol. Eluted plasmid DNA was then heat shocked into chemically competent *E. coli* Δ *leuCD*. Transformants were recovered in lysogeny broth for 1 h at 37°C, washed with PBS twice, and spread on M9 minimal medium plates. Plasmid DNA was then isolated from 18 transformant colonies whereby the presence of the correct plasmid insert was assessed by gel electrophoresis of restriction digests. If all digests were identical to the plasmid originally introduced, the plasmid inserts were Sanger sequenced and analyzed for the correct nucleotide sequence using Clone Manager software (Sci-Ed). Only if all of the above pre-QC criteria were met would the accession lot be passed on for full quality assurance analysis.

Accession lot quality control. (i) Optical density. Dilutions (1:10) of thawed samples from three separate OD-5 accession lot vials were analyzed for optical density at 600 nm using a Biochrom WPA CO8000 cell density meter. A passing lot would display <15% coefficient of variation among the three vials.

(ii) Clumping assessment. A 1:10 dilution of thawed lot material was photographed at $\times 40$ magnification and scored for bacterial clumping against known standards. A set of standards displaying various degrees of cell clumping were assigned arbitrarily numbered scores ranging from 10 (most uniform dispersion) to 70 (severe clumping). Accession lot samples were compared against these standards by four observers in a blinded manner. A passing lot would receive an average clumping score of <40.

(iii) Purity. Undiluted and 10⁻² dilutions of thawed lot material were plated on chocolate agar (BBL chocolate II agar; Becton Dickinson) and checked for contamination at days 7 and 21. This rich medium supports the growth of most bacterial and fungal contaminants, but does not support growth of BCG. A passing lot would have no growth of contaminating microorganisms after 21 days of incubation at 37°C.

(iv) Freeze-thaw recovery. The viability of the bacteria was assessed by the initial growth rate in liquid medium. An inoculum of 200 μ l from an OD-5 vial was diluted into 20 ml Middlebrook 7H9 medium supplemented with 0.5% glycerol, OADC, and 0.05% tyloxapol. Shaking cultures were incubated at 37°C, and optical density was measured daily. The time to achieve an OD₆₀₀ of 0.2 was determined. A passing lot would achieve an OD₆₀₀ of 0.2 in <7 days.

(v) CFU to biomass ratio. CFU from three accession lot vials were counted by plating duplicates of 10⁻⁴ to 10⁻⁷ dilutions on Middlebrook 7H10 agar plates supplemented with 0.5% glycerol, OADC, and 0.05% tyloxapol. A passing lot would have a mean CFU/OD ratio of >10⁷ after 21 to 28 days of growth.

(vi) Western blot of bulk lysate. Bacterial biomass was expressed as the product of optical density and volume (OD \cdot milliliters). Thus, a pellet generated from 1 ml of a culture with an optical density of 1 would contain

1 OD · ml of bacteria. Whole-cell lysates of three OD · ml samples from three accession lot vials were prepared for Western analysis (see Immunoblotting section below). If expression of the antigen was detectable in all three vials, a passing designation would be given to this qualitative parameter. Quantitative measurement of immunoblot band intensity was performed using ImageJ densitometry analysis software (National Institutes of Health). The densitometry of each sample was expressed as a ratio of the target protein to GroEL2, a mycobacterial housekeeping protein. The passing criteria required the average expression ratio compared to a positive control on the same gel to have a coefficient of variation of <25%.

(vii) Analysis of rBCG clones. Accession lot samples were diluted 2-fold in PBST and were passed through a 0.45- μ m syringe filter. Filtrate was plated undiluted or at up to a 10^{-4} dilution. After 28 days, 15 colonies were selected and transferred to liquid culture. Whole-cell lysate from three OD · ml of each clone was prepared for Western blot analysis (see Immunoblotting section below). The passing criteria for clonal protein expression were met if all 15 clones displayed qualitative expression of the major antigen band of interest. Immediate failure resulted from two or more clones lacking detectable protein expression by immunoblot. If antigen was detected in only 14 out of 15 clones, then 15 new colonies were picked, and the process was repeated. A lot was passed if expression was detected in 15 of 15 new clones (a total of 29 out of 30 clones). Quantitative measurement of immunoblot band intensity was also performed using ImageJ densitometry analysis software. The densitometry of each sample was expressed as a ratio of the target protein to GroEL2. The passing criteria required a coefficient of variation among the clones of <50%.

(viii) *In vitro* expression stability. A starter culture from each accession lot was prepared by diluting 0.1 OD · ml into 20 ml Middlebrook 7H9 medium supplemented with 0.5% glycerol, OADC, and 0.05% tyloxapol. Weekly passages were performed by rediluting 0.1 OD · ml of the previous passage into 20 ml fresh liquid medium. At each passage, the OD at 600 nm (OD_{600}) was measured, and lysate was collected for Western analysis. Expression of antigen was monitored by immunoblot after each passage until a failure to detect protein occurred.

(ix) Plasmid DNA isolation. To purify plasmid DNA from both *E. coli* and mycobacteria, the QIAprep spin miniprep kit and QIAprep 96 turbo miniprep kit (Qiagen) were used. For mycobacteria, the standard QIAprep spin miniprep protocol with a modification to improve plasmid yield was used. Two vials containing 1 ml mycobacterial culture (OD-1) were pooled for mycobacterial plasmid isolation. After resuspension in buffer P1, lysozyme (Sigma-Aldrich) was added at a concentration of 20 mg/ml, and samples were incubated at 37°C for 4 h. The normal Qiagen protocol was then used, and the concentration of purified plasmid DNA was measured with a spectrophotometer. The purified mycobacterial DNAs were then used either to transform *E. coli* or as the templates for the PCR amplification of 1086.C *gp120* genes cloned into plasmid pYUB2115. A total of 60 to 100 *E. coli* colonies from each transformation were inoculated in 2 to 5 ml of an appropriate medium for overnight culture and subsequent plasmid DNA isolation for restriction mapping or sequencing.

(x) *E. coli* transformation and restriction mapping. Following purification from mycobacteria, 1 to 3 μ l plasmid DNA (3.3 to 37.3 ng) was used to transform *E. coli*. Plasmid DNA was isolated from 60 to 100 individual colonies on each plate for enzyme restriction mapping and DNA sequencing. To confirm the presence of plasmid inserts within transformants, restriction enzyme digestion profiles of the 5,624-bp EcoRV and 137-bp NdeI restriction sites of each plasmid were compared. The sequence of each plasmid DNA was verified by both Sanger and Illumina deep sequencing. The sequences and positions of sequencing primers are shown in Table S1 in the supplemental material.

(xi) Cloning of insert PCR product for sequencing. Nested PCR was used to amplify the *gp120* insert fragments in plasmid DNA isolated from each BCG strain. Two consecutive PCR amplification steps were performed using two pairs of primers: 1086CF1 and LeuCDR1 at the first step and 1086CF2 and LeuCDR2 at the second step. The sequences and posi-

tions of these primers are shown in Table S1 in the supplemental material. The following cycling conditions produced the first-round 2,200-bp PCR product: 94°C for 2 min, 30 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 3 min. One microliter of the first-round product was used as a template for the second-round amplification: 30 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 2.5 min. All PCR products were visualized on precast 1% E-Gel 48 agarose gels (Invitrogen). The amplicons from both the first and the second amplification steps were ligated directly into the pCR4-TOPO plasmid (Invitrogen) according to the manufacturer's protocol, generating pF1-LeuCDR1 and pF2-LeuCDR2, respectively. Clones containing inserts were identified by restriction enzyme analysis using EcoRI, and the expected sequences were verified by Sanger sequencing.

(xii) Deep sequencing. Two micrograms of plasmid DNA were submitted for deep sequencing analysis using the Illumina platform. A total of about 50,000 sequence reads were collected at each position. For all samples, reads were aligned against the reference plasmid sequence using BWA (46). The mpileup command in SAMtools was used to call variants on sorted and indexed sequence alignment data (47). These results were compared against variants detected by SNVer (48). In SNVer, the number of haploids per sample was set at 1. Thresholds for read mapping qualities were set at 20 and base quality scores at 17.

(xiii) Immunoblotting. Bacterial lysates for Western blotting were prepared by pelleting 10 ml log-phase culture at 2,000 RCF for 5 min. Pellets were washed by two repeats of centrifugation and resuspension in 10 ml PBST. The final pellet was resuspended in 200 μ l lysis buffer with glass beads and vortexed for 3 min. Lysate was clarified by spinning at 2,000 RCF for 15 min, followed by removal of the supernatant for Western analysis. SDS-PAGE gels were loaded with a mixture of 16 μ l lysate with Laemmli sample buffer and run under 130 V for 1 h. Protein was subsequently transferred to polyvinylidene difluoride (PVDF) membranes by electrophoresis under 30 V for 1 h. Membranes were blocked by gentle shaking in 5% nonfat dry milk dissolved in Tris-buffered saline with 0.1% Tween (TBST) at room temperature for 1 h. The following antibodies and dilutions were used for Western analysis: mouse anti-hemagglutinin (HA)-horseradish peroxidase (HRP) (0.025 μ g/ml; Roche), mouse anti-HIV 1086.C gp120 clone 3B3 (0.015 μ g/ml), goat anti-mouse IgG (0.2 μ g/ml; GenScript), and mouse anti-GroEL2 (0.050 μ g/ml; Santa Cruz Biotechnologies) (49). Primary antibodies were diluted appropriately in 5% milk-TBST and incubated with membranes for 1 h at room temperature. If secondary antibodies were required, the membranes were washed three times for 15-min increments with TBST following incubation with the primary antibody. After washing, proteins were detected via chemiluminescence and exposure of X-ray film.

Mouse vaccination and bacterial persistence. Age-matched adult C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were inoculated retro-orbitally with 100 μ l (~ 10^7 CFU) from a thawed vaccine accession lot vial of recombinant mycobacteria. The intravenous route was chosen to study bacterial persistence and *in vivo* stability in order to achieve an effective systemic inoculation and more easily detectable immune responses. At 60 days postinoculation, groups of five mice were sacrificed, and dilutions of spleen, liver, and lung homogenates were plated on 7H10 agar to enumerate persistent CFU. Mice were maintained in a Duke University vivarium and handled under protocols approved by the Institutional Animal Care and Use Committee.

AL11 tetramer assay. Persistence of vaccine-specific immunogenicity was measured using flow cytometric analysis of SIV Gag_{mac239}-responsive CD8⁺ T cells. C57BL/6 mice were maintained in the Beth Israel Deaconess Medical Center Animal Research Facilities and handled under protocols approved by the Institutional Animal Care and Use Committee. A total of 10^6 CFU from a previously frozen quality-assured aliquot of the BCGD Δ leuCD strain expressing SIV Gag_{mac239} (SIV Gag) was injected subcutaneously into the lower right flank of 8 mice per group. A group of unprimed naive mice was included as a control. The subcutaneous injection was chosen to reconstitute a more physiologically relevant vaccination route. Previous studies have demonstrated improved success in an-

tilentiviral CD8⁺ immune responses using recombinant adenovirus expressing lentiviral antigens, including SIV Gag (50). A heterologous boost was accomplished by injection of 3×10^7 viral particles of recombinant adenovirus type 5 expressing SIV Gag (GenVec Inc.) intramuscularly into the hind leg quadriceps (the total amount was split equally in 50- μ l doses for each leg) of all mouse groups (including naive) after 7 weeks following the administration of BCGD Δ leuCD. At various time points after vaccination, 100 μ l peripheral blood was collected from each mouse by the submandibular route and treated with Lympholyte-Mammal (Cedarlane) to remove red blood cells. The remaining lymphocytes were stained using an H-2Db AL11-phycoerythrin (PE) tetramer and anti-CD8-peridinin chlorophyll protein (PerCP)-Cy5.5 antibodies (eBioscience).

Nucleotide sequence accession numbers. GenBank accession numbers for plasmids pSL701, pSL718, and pSL720 are KJ865749, KJ865750, and KJ865751, respectively.

RESULTS

Complementation of BCGD Δ leuCD leucine auxotrophy using *M. smegmatis* leuCD. Unstable expression of heterologous proteins in mycobacteria has hindered wider use of mycobacteria as a vaccine platform. Not only is the routine use of antibiotic resistance markers for plasmid retention unsuitable for application in humans but also the physiological burden of antibiotics in medium can negatively affect culture growth and efficient production of recombinant proteins. Instead, auxotrophic complementation is useful as an alternative selection system (51, 52). Earlier observations that a leucine auxotroph of BCG was cleared after 7 weeks of growth in mice (53) suggested that BCG leucine auxotrophs could not adequately scavenge for leucine *in vivo*, and construction of a plasmid with complementing genes for leucine biosynthesis might allow for stable plasmid retention. Previous work with a deletion of *leuD* in slow-growing mycobacteria demonstrated leucine auxotrophy and attenuation (11, 54). To reduce the likelihood of genomic reversion or second-site suppressors of leucine auxotrophy, we deleted *leuCD*, two genes essential for leucine biosynthesis. In the present study, we discovered that the *leuCD* genes from *M. smegmatis*, when expressed on pYUB2115, complemented a BCG Δ leuCD strain. Although the LeuCD proteins from *M. smegmatis* can functionally substitute for BCG LeuCD, the noncoding regions upstream and downstream of the *leuCD* genes have only 70% nucleotide homology. This reduces the likelihood of recombination of the *M. smegmatis* *leuCD* genes present in the pYUB2115 plasmid into the BCG Δ leuCD chromosome. We have not observed loss of plasmid during the propagation of complemented BCG Δ leuCD in multiple transformants, so we conclude that homologous recombination with loss of plasmid selection does not occur or is extremely rare. Furthermore, the expression plasmids which bear *M. smegmatis* *leuCD* genes and transform BCG Δ leuCD that were used in this study can be reextracted and retain complementation of both *E. coli* Δ leuCD and *M. smegmatis* Δ leuCD, demonstrating that the *leuCD* function remains intact. Last, we have propagated the BCG Δ leuCD mutant mc²6454 on multiple occasions with screening for reversion and have never observed reversion of leucine auxotrophy in $>10^{10}$ CFU tested. Complementation of this stable, attenuated Δ leuCD mutant presents a useful method for expression of recombinant proteins in BCG, including large lentiviral antigens.

Cloning of lentiviral antigens for expression in mycobacteria. The lentiviral antigens were cloned using the pYUB2115 backbone (Fig. 1A; Table 1). This construct utilizes an unmarked

auxotrophic complementation system as the mode of positive selection, whereby the *M. smegmatis* *leuC* and *leuD* genes present in the backbone complement transformants of BCGD leucine auxotrophs (40, 41). Such a method of plasmid retention avoids the introduction of antibiotic resistance cassettes into vaccine strains for potential human application. Many studies have shown successful HIV-1 immune priming with recombinant BCG auxotrophs, and these mutants also exhibited an increase in the overall stability and safety profile (11, 21–23, 25, 41, 55–58). Figure 1 displays maps of the plasmids transformed into BCGD Δ leuCD. Mycobacteria were electroporated with pYUB2115 (Fig. 1A), pSL701 (Fig. 1B), pSL718 (Fig. 1C), or pSL509 (Fig. 1E) and grown on Middlebrook 7H10 agar without leucine supplementation, and single transformants were expanded to produce 150-vial accession lots of frozen recombinant BCGD Δ leuCD. Accession lot number 036 (lot 036) consisted of BCGD Δ leuCD transformed with the empty vector, pYUB2115.

Plasmids pSL701 and pSL718 differ in their modifications of the HIV 1086.C *gp120* gene. These designs were used to assess how differential expression of a *gp120* fusion might affect the stability of the antigen within recombinant BCGD Δ leuCD. In pSL701, *gp120* was fused at the N terminus to the *M. tuberculosis* *lpqH* signal sequence and at the C terminus to amino acids 71 to 158 of LpQH. Fusion of an antigen to the *lpqH* signal sequence has previously been shown to allow secretion of the antigen extracellularly and presentation of the antigen on the cell surface (59). This expression profile has been shown to increase the overall immunogenicity of the vaccine vehicle compared to that with intracellular expression alone (60). In pSL718, *gp120* is fused at the N terminus to both the mycobacterial *M. tuberculosis* *fbpB* signal sequence and the endogenous *gp120* signal sequence and at the C terminus to amino acids 71 to 158 of LpQH. Both plasmids utilize the strong, constitutive mycobacterial promoter region from *groEL2* to drive expression of their respective inserts. During the quality control procedures discussed in the upcoming sections, plasmid DNA from each lot was reisolated and analyzed for sequence integrity. The predominant plasmid retrieved from the accession lot initially transformed with pSL718 had developed a stable deletion of 170 base pairs, removing the last 13 amino acids from the N-terminal LpQH fusion as well as the *aph* promoter region for *leuC* and *leuD*. This modified pSL718 was subsequently renamed pSL720 (Fig. 1D). Two resulting accession lots produced from this process were designated lot 039 and lot 040.

Finally, in plasmid pSL509, SIV *gag* is fused at the N terminus to the *M. tuberculosis* *fbpB* signal sequence and is similarly under the control of the *groEL2* promoter. SIV *gag* also contains an N-terminal epitope tag from influenza hemagglutinin (HA). The accession lot transformed with pSL509 was designated lot 062.

Quality control procedures create a quantitative profile for accession lot stability. To establish a well-characterized profile of parameters involving protein and plasmid stability, cellular composition, biomass, purity, and growth recovery upon thawing, each lot was subjected to an extensive array of quality control assays. Passing scores were designed for each assay, and both lots were assigned a score and pass/fail status following each test. Scores for lots 036, 039, 040, and 062 are summarized in Tables 3 and 4. Lots 039 and 040 were used to establish standards by which these quality control assays were scored.

First, lysates from three vials of lots 039 and 040 were prepared for Western blot analysis to compare HIV *gp120* antigen levels in

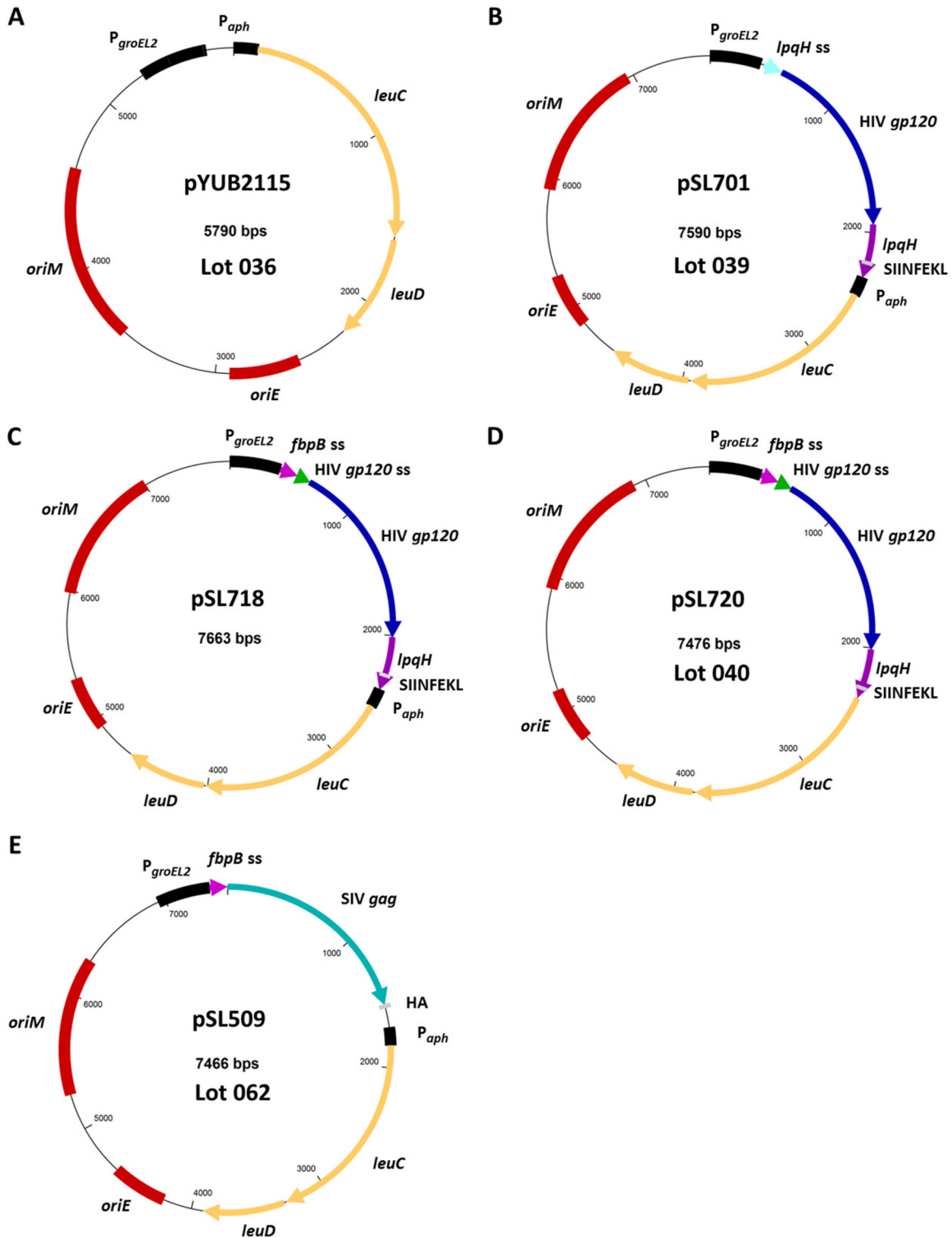


FIG 1 Plasmid constructions for mycobacterial expression of HIV 1086.C *gp120*. (A) The parental pYUB2115 cloning vector map is shown. Contained are two origins of replication (red) active in both *E. coli* (*oriE*) and mycobacteria (*oriM*). A multiple cloning site is located downstream of the *groEL2* promoter (black) and upstream from the *aph* promoter (black) driving expression of *M. smegmatis* *leuC* and *leuD* (yellow). Plasmid maps for constructs transformed into lot 039 and lot 040 are also shown. Expression of HIV 1086.C *gp120* (blue) is regulated by the constitutive mycobacterial *groEL2* promoter (black) in both plasmids. Each plasmid contains an origin of replication for DNA synthesis in *E. coli* (*oriE*, red) and mycobacteria (*oriM*, red), as well as genes for *M. smegmatis* *leuC* and *leuD* (yellow), under the control of a constitutive *aph* promoter. Antigen inserts in these constructs contain the SIINFEKL peptide epitope at the C terminus (gray). (B) In pSL701 (lot 039), *gp120* is fused at the N terminus to the *M. tuberculosis* *lpqH* signal sequence (cyan) and at the C terminus to amino acids 71 to 158 of *lpqH* (purple). (C) In pSL718, *gp120* is fused at the N terminus to the *fbpB* signal sequence (pink) and the endogenous *gp120* signal sequence (green) and at the C terminus to amino acids 71 to 158 of *lpqH* (purple). A black box indicates the *aph* promoter region for expression of *leuC* and *leuD*. (D) Plasmid pSL720 (lot 040) is a modification of pSL718, whereby a 170-bp deletion removes the last 13 amino acids from the N-terminal *lpqH* fusion as well as the *aph* promoter region for *leuC* and *leuD*. (E) In plasmid pSL509 (lot 062), *SIV gag* (teal) is fused at the N terminus to the *fbpB* signal sequence (pink), and expression is under the control of the *groEL2* promoter (black).

TABLE 3 Quality control analysis of accession lot physical properties

Lot	Strain	Clumping ^a		Uniform biomass ^b			Liquid culture ^c			Mean CFU/OD ($\times 10^8$) ^e				
		Score	Result	Avg	SD	CoV (%)	Result	Day OD >0.2	Result	Purity result ^d	Avg CFU	CoV (%)	CFU/OD	Result
036	SL521	15.60	Pass	1.23	0.01	1	Pass	1.00	Pass	Pass	4.78	7	3.90	Pass
039	SL524	19.00	Pass	6.50	0.46	7	Pass	0.85	Pass	Pass	18.50	17	3.25	Pass
040	SL525	14.00	Pass	5.70	0.10	2	Pass	1.10	Pass	Pass	9.67	48	1.49	Pass
062	SL547	14.00	Pass	5.93	0.15	3	Pass	0.85	Pass	Pass	18.70	24	3.15	Pass

^a Particle sizes of rBCGD $\Delta leuCD$ clumps visualized by microscopy were assigned a clumping score. A passing score was <40 .

^b Consistency of optical density between three vials was assessed by OD₆₀₀ covariance (CoV). The target OD₆₀₀ for lot 036 was 1 and the target for lots 039, 040, and 062 was 5. A passing covariance was $<15\%$.

^c Recovery of thawed accession lot material in liquid culture was examined by daily monitoring of the optical density. A passing lot achieved OD₆₀₀ of >0.2 in <7 days of growth.

^d The accession lot material was streaked on chocolate agar, and plates were observed for contaminating organisms. A passing lot yielded no contaminating colonies after 28 days of incubation.

^e The accession lot material was diluted and plated to measure the ratio between the number of CFU and optical density. A passing lot possessed a CFU/OD ratio of >0.1 .

freeze-thawed lot material to those from the parental BCGD $\Delta leuCD$ transformant culture (Fig. 2A). Reduced levels of antigen were observed in both lots compared to parental expression, suggesting that either expansion of culture or freeze-thawing adversely affects detectable gp120. Densitometry was used to quantify gp120 expression in the bulk lysate, and the average ratio of lot gp120 to the parental lysate was calculated. A passing lot contained an average of $>25\%$ of the parental protein levels. In addition, as consistency from vial to vial is a vital objective for large frozen lots, the covariance of scores was assessed. A passing lot showed $<25\%$ covariance between 3 vials. Both lot 039 and lot 040 passed the quantitative bulk antigen expression assay with expression ratios of 80% and 49% and covariances of 6% and 7%, respectively (Table 4).

We were interested in not only the antigen production from the bulk population of rBCGD $\Delta leuCD$ but also the gp120 expression in individual clones of mycobacteria within a given lot vial. A more extensive analysis of individual clones might reveal heterogeneity within the rBCGD preparation. Due to the inherent hydrophobicity of the mycobacterial cell wall, cellular aggregation often results in clumps of multiple mycobacteria suspended in liquid culture. To plate truly clonal colonies of mycobacteria for individual analysis, single cells were isolated by filtration through a 0.45- μm filter. Our previous optimization revealed that filtration through 0.8- μm pores decreased the filtration efficiency by a factor of 10,000, where efficiency was measured by calculating the ratio between unfiltered CFU and filtered CFU. Surprisingly, the

presence of detectable gp120 in the lot 039 bulk lysate did not accurately predict the fidelity of plasmid retention in individual BCGD $\Delta leuCD$ clones (see Table S2 in the supplemental material). A similar result was observed when thawed accession lot material from lot 039 was filtered for individual clones, and 15 colonies were picked for expansion and lysate preparation. Western blot analysis showed that 15 out of 15 rBCGD $\Delta leuCD$ colonies no longer expressed gp120 after expansion from a single transformant to a 1-liter accession lot (Fig. 2B). Conversely, all 15 clones plated from lot 040 had detectable gp120 expression (Fig. 2C). Our quality control parameters required that all 15 clones express the desired antigen, leading to a failure of lot 039 to pass the clonal qualitative expression assay. Similarly to the quantification of protein from the bulk lysate, densitometry was used to measure the gp120 levels in the individual lot 040 clones (Fig. 2C). Lot 039 automatically failed this assay, since no bands were detected from individual clones. A passing score for this assay was a calculated covariance of protein expression between the 15 clones of $<50\%$. The 25% covariance calculated for lot 040 allowed this lot to pass.

During the course of optimizing the protocols for accession lot production, we noticed that certain lots contained large cellular aggregates in suspension after thawing. Since there were no visible clumps present in the liquid cultures during the expansion prior to lot production, we suspected this to be a result of the freezing process. These clumps not only might affect the subsequent quality control assays, such as estimation of CFU and uniformity in biomass, but also had the potential to affect injection efficiency

TABLE 4 Quality control summary of antigen expression

Lot	Strain	Bulk lysate					Clones						
		Qualitative expression result ^a	Quantitative expression ^b			Result	Qualitative expression ^c		Quantitative expression ^d				
			Avg	SD	CoV (%)		Correct/total	Result	Avg	SD	CoV (%)	Result	
036	SL521	NA ^e				NA		NA				NA	
039	SL524	Pass	0.80	0.05	0.06	Pass	0/15	Fail	NA	NA	NA	Fail	
040	SL525	Pass	0.49	0.03	0.07	Pass	15/15	Pass	2.40	0.60	25	Pass	
062	SL547	Pass	1.38	0.65	0.47	Pass	15/15	Pass	2.77	0.73	26	Pass	

^a Qualitative expression of antigen was detected by Western blotting. A passing lot contained detectable antigen in three out of three vials examined.

^b Densitometry was used to quantify expression of antigen. A passing lot contained $>25\%$ protein levels of the parental transformant.

^c The accession lot material was filtered and plated for single rBCGD colonies. Colonies were picked and grown in liquid culture for Western blot analysis of qualitative antigen expression. A passing lot displayed antigen expression in 15 of 15 clones examined.

^d Densitometry was used to quantify the expression of antigen in 15 rBCGD clones. A passing lot achieved $<50\%$ covariance (CoV).

^e NA, not applicable.

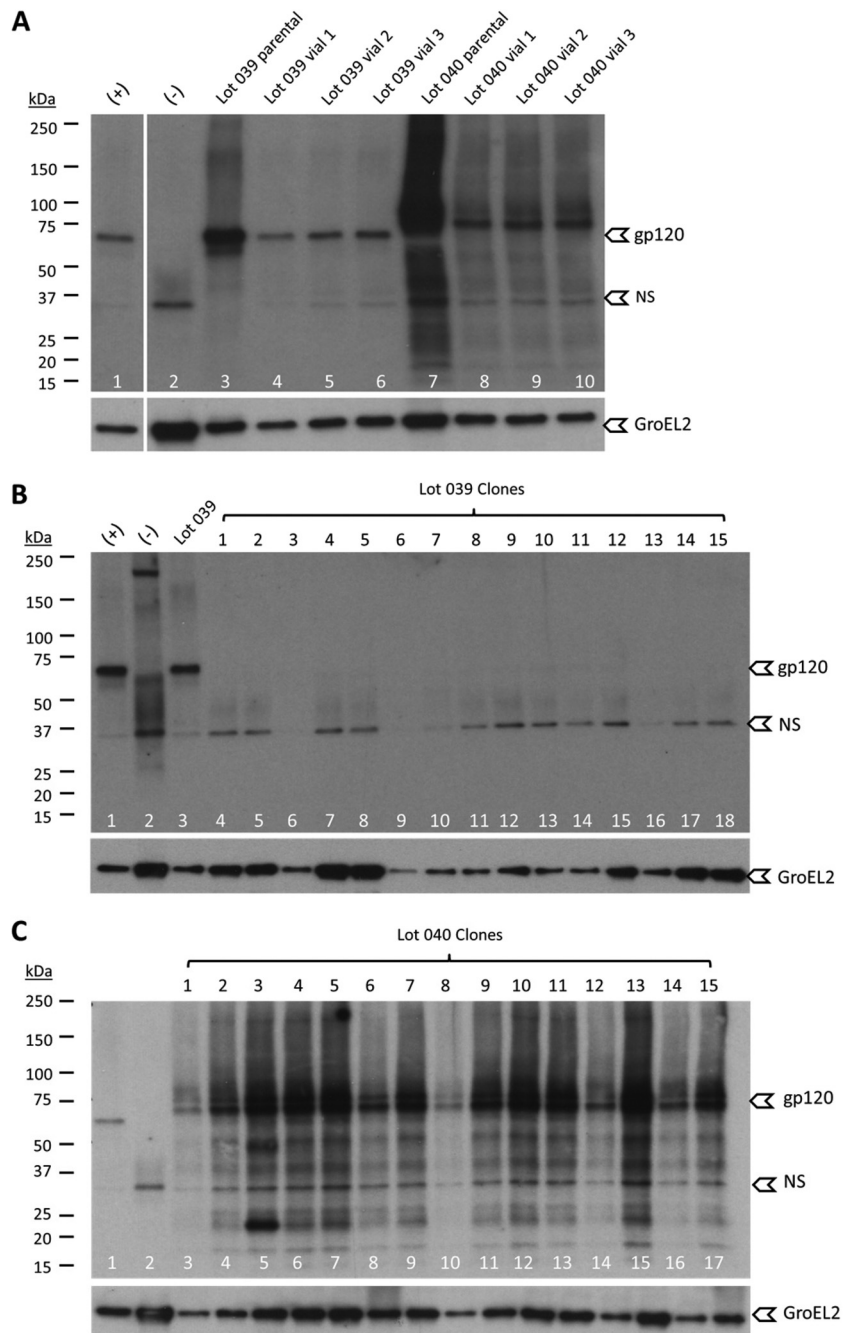


FIG 2 Clonal expansion of recombinant vaccine strains reveals instability of antigen expression despite detectable gp120 in bulk lysates. (A) Western blot-verified expression of gp120 in three vials of lot 039 (lanes 4 to 6) and lot 040 (lanes 8 to 10) was compared to protein levels in parental BCGD transformants (lanes 3 and 7). Positive and negative gp120 controls are displayed in lanes 1 and 2, respectively. Using anti-gp120 (clone 3B3 antibody), expected bands sizes are 65 kDa and 73 kDa for lot 039 and lot 040, respectively. NS, nonspecific band. Loading controls were performed by detection of GroEL2 (clone 5177 antibody). (B) Lysates from 15 rBCGD filter clones of lot 039 were analyzed by Western blotting (lanes 4 to 18). Expression of gp120 was validated using positive and negative gp120 controls (lanes 1 and 2). Antigen expression in the clones was also compared to levels present in the parental transformant (lane 3). Expected band size is 65 kDa for lot 039. Loading controls were performed by detection of GroEL2 (clone 5177 antibody). (C) Lysates from 15 rBCGD filter clones of lot 040 were analyzed by Western blotting (lanes 3 to 17). Expression of gp120 was validated using positive and negative gp120 controls (lanes 1 and 2). The expected band size is 73 kDa for lot 040. Loading controls were performed by detection of GroEL2 (clone 5177 antibody).

and host responses during immunogenicity assays. As such, a cellular clumping assessment was incorporated into the QC procedures. A diluted sample of thawed accession lot material was magnified using light microscopy and images were taken of

mycobacterial clumps. Standards were prepared and scored from previous lots showing various degrees of clumping, and any new lot was assigned a clumping score by at least three independent observers. Figure S1 in the supplemental material displays repre-

sentative images of standards and material from lot 039 and lot 040. Both lots had a passing score of <40.

Additional cellular composition parameters analyzed for each lot included the absence of contamination, uniformity in biomass between vials, and the number of viable mycobacteria remaining after thawing (Table 3). To determine the presence of any contaminating microorganisms, samples of each lot were plated on chocolate agar, a nonselective rich medium which has the ability to support growth from most contaminating organisms present in the surrounding laboratory environment. No contaminating microorganisms were detected in either lot. OD₆₀₀ measurements were taken from three separate vials of each lot, and the covariance between these vials was calculated. A passing score retained a covariance of <15%. Lot 039 and lot 040 received passing scores of 7% and 2% covariance, respectively. The number of viable bacteria in each vial was assessed by taking the ratio between the CFU counts and the observed optical density. Both lots also passed this parameter with ratios over the 0.1 cutoff. In addition to net viability of individual bacteria, the overall ability of frozen mycobacteria to recover once thawed from each lot was examined. Samples of lot material were diluted to an OD₆₀₀ of 0.05 in Middlebrook 7H9 liquid shaking cultures and allowed to incubate for several days. OD₆₀₀ measurements of the cultures were taken daily. A passing lot with a high rate of freeze-thaw recovery would reach an OD₆₀₀ reading of 0.2 in <7 days of growth. As shown in Table 3, both lots quickly recovered from thawing, with an OD₆₀₀ of 0.2 achieved in about 1 day.

A final aspect of our quality assurance protocol was to examine the sequence purity of each lot. Based upon the inherent differences in the plasmid sequences between the constructs transformed into lot 039 and lot 040, tailored approaches were used for each lot. An analysis of bulk plasmid and single BCGD $\Delta leuCD$ clones was performed for both lots.

For lot 039, nested PCR was used to amplify the 1086.C *gp120* inserts in 15 colonies grown from filtered lot material. Based upon the results from the clonal expression analysis, it was not surprising to find that none of the 15 BCGD $\Delta leuCD$ clones yielded PCR amplicons corresponding to the correct plasmid insert size (see Table S2 in the supplemental material). Plasmid DNA then was isolated from bulk lot material and the same 15 BCGD colonies. This DNA was transformed into *E. coli* $\Delta leuCD$, and plasmid preparations from these cultures were subjected to restriction digest analysis. Out of 15 *E. coli* $\Delta leuCD$ transformants from the bulk lot material and 72 *E. coli* $\Delta leuCD$ clones derived from the single BCGD colony plasmids, none of the predicted restriction fragment sizes were visualized by agarose gel electrophoresis. These same plasmids were then submitted for Sanger sequencing. Only one insert was detected out of a total of 435 sequence reads, suggesting that the population of BCGD $\Delta leuCD$ producing 1086.C *gp120* within lot 039 was extremely underrepresented.

E. coli $\Delta leuCD$ was unable to be directly transformed by plasmid DNA isolated from lot 040. Illumina sequencing had revealed a 170-bp deletion in the pSL718 isolated from lot 040 (renamed pSL720). This deletion removed most of the *aph* promoter region for *leuC* and *leuD*, which may have rendered it unable to complement the leucine auxotrophy in *E. coli* $\Delta leuCD$. A suitable stretch of the promoter was retained, however, for apparent use and expression in mycobacteria. As such, nested PCR was instead performed on both bulk lot plasmid and plasmid isolated from 15 single-plated BCGD $\Delta leuCD$ clones from lot 040. The PCR prod-

ucts were then subjected to pCR-TOPO cloning, and the resulting vectors were transformed into DH5 α . To check the PCR product size as a measure of a correctly sized *gp120* insert, a restriction digest was performed on 35 and 15 DH5 α transformants from bulk lot material and single BCGD $\Delta leuCD$ clones and plasmid, respectively. Again, as predicted by the success with lot 040 in the previous quality control assays, all 50 transformants possessed the correctly sized insert. In addition, Sanger sequencing was performed on the DH5 α transformants. Out of 83 total transformants sequenced, 94% had a fully correct 1086.C *gp120* sequence (see Table S2 in the supplemental material).

To gain an even more detailed understanding of the plasmid population present in lot 040, plasmid DNA was isolated and submitted for Illumina deep sequencing analysis. A total of 50,000 sequence reads, which represented 100% coverage of the theoretical reference plasmid sequence for pSL720, were produced. The sequence differences between reads were well within the expected error rates, confirming a high degree of uniformity. Illumina sequencing revealed 99.8% sequence purity in the lot 040 plasmid population but <1% correct sequence for lot 039.

In summary, although lot 039 and lot 040 passed equally on all aspects of cellular composition, purity, thaw recovery, and qualitative *gp120* expression in the bulk lysate, more extensive analysis of individual clones for retention of plasmid, sequence purity, and antigen expression revealed a large disparity between the two. This suggests that the QC procedures described can not only fully reveal the overall quality of each vaccine lot but they can also be predictive for how continued antigen expression during further expansion of each lot is affected.

Stable expression of HIV Env *in vitro* and *in vivo* from quality assured rBCG accession lots. Such aforementioned expansions could come in the form of rBCGD $\Delta leuCD$ persistence in the vaccinated host. We began modeling a vaccinated-host scenario by monitoring the continuation of *gp120* expression in passaged lot material. To this end, thawed accession lot material from lots 039 and 040 was seeded into Middlebrook 7H9 liquid shaking cultures and passaged weekly. At each time point, samples of culture were collected for lysate preparation, followed by Western blotting detection of *gp120* expression. Lysates from passages of lot 040 continued to contain readily detectable *gp120* until the 13th cycle of amplification, representing retention of antigen expression following a 10²⁴-fold expansion of cells (Fig. 3A). As predicted by the failure of lot 039 to fully pass quality control, antigen expression was lost from this unstable lot after the first week of passage. To further characterize the level of instability associated with lot 039 in liquid culture, various seeding densities were inoculated into liquid cultures and lysates were prepared after individual cultures reached log phase. These densities allowed cultures to proceed for successive day-long lengths of time, up until 1 week of growth. As seen in Fig. 3B, *gp120* antigen is detected in lot 039 for only 2 days of growth, representing a log 10 expansion of 0.7. These observations correlate with the results of extensive quality control, whereby the failure of lot 039 to yield individual clones containing antigen inserts as well as *gp120* expression predicted its rapid loss of antigen expression during subsequent replication. These data suggest that the overall stability of lot 039 is much less than that of lot 040 and that potentially small populations of antigen-expressing rBCGD $\Delta leuCD$ in lot 039 are quickly outcompeted by populations lacking plasmid inserts and the burden of antigen expression.

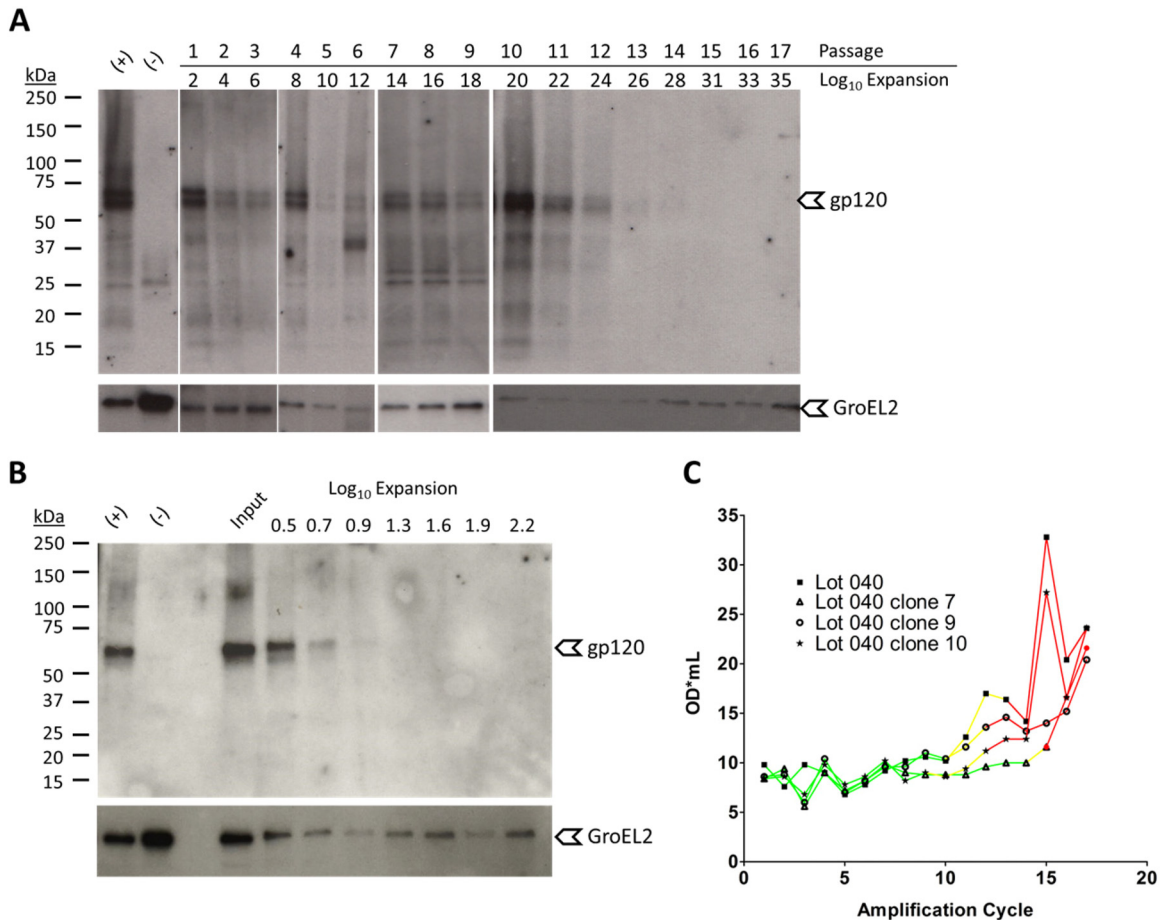


FIG 3 Quality assurance parameters predict *in vitro* stability of vaccine lots. Thawed accession lot material was seeded into liquid shaking cultures and passaged weekly. After each passage, culture samples were collected for lysate preparation, and anti-gp120 (3B3 antibody) immunoblot analyses were performed to examine the persistence of antigen expression. (A) Full-length HIV 1086.C gp120 expression remained detectable through 12 weekly passages of lot 040, representing a 10^{24} -fold expansion. (B) A short gp120 expression time course was performed to assess the unstable loss of antigen expression from lot 039. (C) Liquid cultures of lot 040 and reisolated lot 040 BCGD clones 7, 9, and 10 were inoculated at an OD₆₀₀ of 0.1 and passaged weekly. OD · ml was measured prior to further passages. Green areas of each curve represent passage numbers at which gp120 antigen was readily detectable by Western analysis. Yellow and red areas of each line represent time points at which detection of antigen waned and remained undetectable, respectively.

In support of this, the OD · ml of weekly passaged cultures was plotted in Fig. 3C. The growth plot for lot 040 was relatively stable over the course of several weeks, but by week 14, the total population of mycobacteria after each successive passage abruptly increased. Strikingly, the loss of lot 040 antigen expression at week 13 in Fig. 3B was nearly synchronized with the rapid increase in growth observed at week 14 in Fig. 3C. Similar trends were observed when single lot 040 BCGD $\Delta leuCD$ clones (clones 7, 9, and 10) were picked and cultured in the same assay. Among these clones, strong and waning gp120 expression (green and yellow areas of each curve, respectively) preceded a jump in growth and subsequent loss of detectable antigen (red). This suggests that production of recombinant viral antigen is a potent limiting factor for growth, and the loss of this burden allows the mycobacteria to achieve more optimal growth kinetics.

To complement this *in vitro* stability assay, *in vivo* retention of antigen expression for lot 039 and lot 040 was assessed by retro-orbitally inoculating C57BL/6 mice with 10^7 bacilli. At day 60, mice were sacrificed, and spleens, livers, and lungs were isolated for homogenization and plating of bacterial CFU. The average

bacterial loads were highly similar between both lots, with the exception of a small but significant difference between liver loads for lot 039 and lot 040, suggesting that the two lots yielded bacteria with similar *in vivo* persistence profiles (Fig. 4A). To assess whether persisting BCGD $\Delta leuCD$ from either lot continued to express HIV gp120, 12 splenic colonies were picked from each lot and expanded in liquid culture, and lysates were analyzed for antigen by Western blotting. As seen in Fig. 4B, none of the colonies picked from lot 039 continued to express gp120. However, all cultures expanded from persisting lot 040 clones were positive for antigen expression, further supporting the high level of *in vivo* stability reached by this QC-approved vaccine lot.

Using similar approaches, we have generated additional rBCG accession lots expressing partial or full-length forms of other retroviral antigens. Data are shown here for a stable rBCG lot expressing SIV Gag_{mac239} from the pSL509 plasmid (lot 062) (Fig. 1). This lot was analyzed using the same quality assurance criteria as lot 039 and lot 040 (Tables 3 and 4). Lot 062 exhibited stable protein expression *in vitro* through at least 29 serial passages, representing $>10^{68}$ -fold amplification. This preparation was also stable *in vivo* for at least 30 days

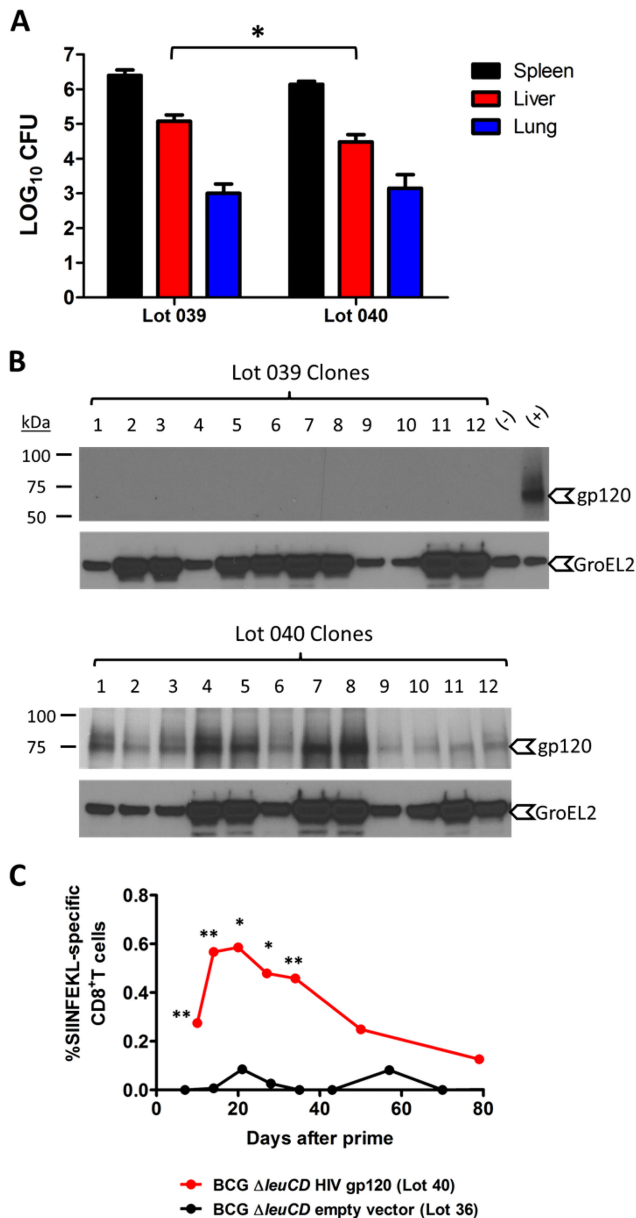


FIG 4 Passing of quality assurance criteria correlates with *in vivo* retention of antigen expression and immunogenicity. (A) C57BL/6 mice were retro-orbitally vaccinated with lot 039 or lot 040 and at 60 days postinoculation were sacrificed for determination of the bacterial load within the spleen, liver, and lungs. Means \pm standard deviations are shown. * $P < 0.05$ (*t* test) between loads of lot 039 versus lot 040 in the liver. (B) Lysates from 12 lot 039 and lot 040 colonies isolated from spleen (60 days postinfection) were prepared from liquid culture. The expression of gp120 was examined by Western blotting (clone 3B3 antibody) using GroEL2 as a loading control (clone 5177 antibody). (C) C57BL/6 mice were inoculated intravenously with 10^7 CFU BCGD $\Delta leuCD$ empty vector lot 036 (black) or BCGD $\Delta leuCD$ HIV gp120 lot 040 (red). At various time points, peripheral blood cells were stained to detect the percentage of SIINFEKL-specific CD8⁺ T cells by flow cytometric analysis. Median values of 6 mice are shown. * $P < 0.05$; ** $P < 0.002$ (*t* test).

following inoculation. Ten out of 10 clones derived from murine spleens demonstrated intact protein expression (Fig. 5A and B).

Quality assurance parameters predict immunogenicity. Ultimate use of quality controlled vaccine lots will focus on the

generation of antigen-specific immune responses and the potential for boosting of those responses. We have measured immune responses to rBCG accession lots using CD8 tetramer staining, CD4 enzyme-linked immunosorbent spot (ELISpot) assays, and enzyme-linked immunosorbent assays (ELISA) for binding antibodies.

CD8 responses. Vaccine-specific T cell populations were quantified using flow cytometric analysis of lymphocytes stained with ovalbumin MHC I tetramers, which targeted the C-terminal SIINFEKL tag encoded by pSL720. C57BL/6 mice were injected intravenously with 10^7 CFU empty vector BCGD $\Delta leuCD$ lot (lot 036) or lot 40. Figure 4C shows the level of SIINFEKL-specific CD8⁺ T lymphocytes in the peripheral blood present after vaccination. Over the course of 6 weeks, SIINFEKL-specific killer T cell populations in lot 040-vaccinated mice remained significantly higher than those present in empty vector lot 036-vaccinated mice, peaking at 3 weeks postvaccination. In contrast, similar studies using lot 039 demonstrated no tetramer response (data not shown). This suggests that a stable, QC-approved BCG lot expressing tagged HIV gp120 is capable of immunogenicity.

An MHC I tetramer is readily available to detect antigen-specific CD8⁺ T cells which recognize the immunodominant AL11 epitope of SIV Gag. To determine if an accession lot which passed quality assurance criteria, maintained significantly persistent antigen expression *in vivo*, and was administered by a more physiologically relevant dosage and route could induce epitope-specific T cell proliferation, C57BL/6 mice were vaccinated subcutaneously with 10^6 CFU empty vector BCGD $\Delta leuCD$ lot (lot 036) or an rBCGD $\Delta leuCD$ lot expressing the lentiviral antigen, SIV Gag_{5mac239} (lot 062). At weeks 2 through 6, peripheral blood was collected and processed to isolate lymphocytes. T lymphocytes were stained with anti-CD8 and the SIV Gag epitope-bearing AL11 MHC I tetramer and were subsequently analyzed by flow cytometry to quantify the percentage of killer T cells capable of recognizing the recombinant antigen. Figure 5C shows the proliferation of such T cell populations over time following the BCGD $\Delta leuCD$ prime vaccination. Over the course of 6 weeks, mice primed with lot 062 trended with higher but insignificant median levels of epitope-specific CD8⁺ T cells over the empty vector control, lot 036. During the entire priming period, these median T cell levels remained below 0.05%.

At week 7 after the priming period, mice were boosted with 3×10^7 viral particles of recombinant adenovirus expressing SIV Gag. Strikingly, 3 weeks after the boost, SIV Gag-specific CD8⁺ T cell levels rose dramatically in mice which were previously primed with lot 062 (Fig. 5D). These levels remained significantly higher than those of lot 036-primed mice for 3 more weeks of the study. During this time, the SIV Gag-specific lymphocyte population represented at least 10% of the total killer T cells within the peripheral blood. These results reveal that highly stable BCGD $\Delta leuCD$ -mediated expression of a lentiviral antigen, as characterized by quality control parameters, was associated with successful induction of vaccine-specific responses.

We have subsequently tested multiple accession lots expressing either partial or full-length HIV Env gp120 or SIV Gag using this *leuCD* selection system. From stable accession lots, we have consistently observed CD8 tetramer responses either to the SIINFEKL epitope tag for lots expressing HIV Env gp120 or to the AL11 epitope for lots expressing SIV Gag.

CD4 responses. We assessed CD4⁺ T cell responses in F1 mice (BALB/c \times C57BL/6) that were primed by subcutaneous vaccina-

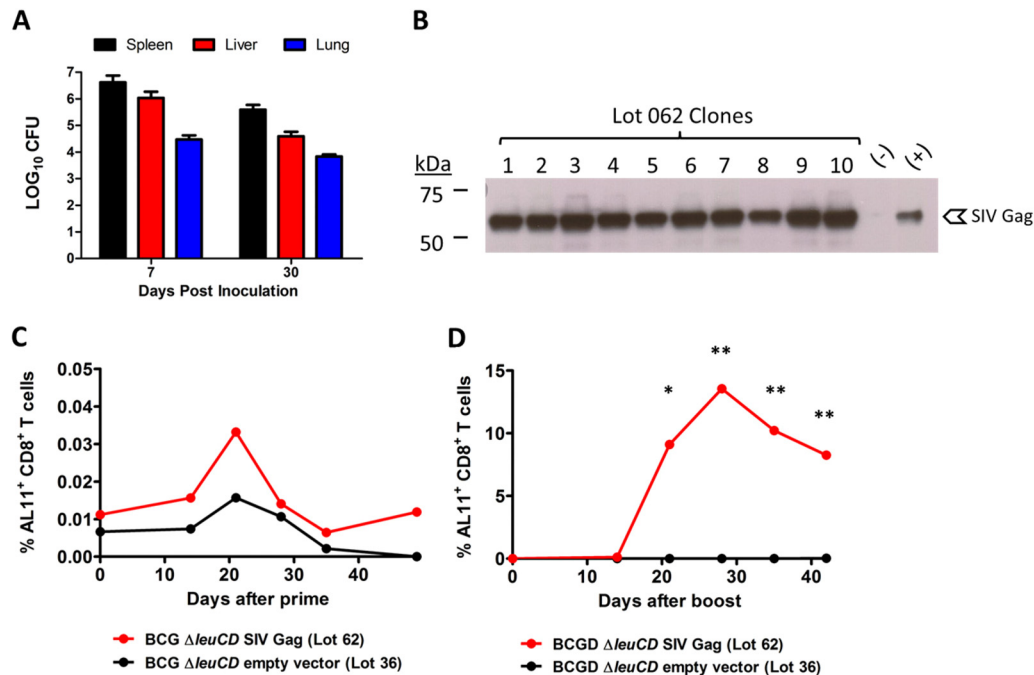


FIG 5 Persistence of quality-controlled recombinant BCG *in vivo* induces antigen-specific T cell responses. (A) C57BL/6 mice were retro-orbitally vaccinated with lot 062 expressing SIV Gag_{mac239}. At 7 and 30 days postinoculation, mice were sacrificed for determination of bacterial load within the spleen, liver, and lungs. Means \pm standard deviations are shown. A significant reduction in CFU was observed for all organs between days 7 and 30 postinfection ($P < 0.01$). (B) Lysates from 10 splenic colonies were prepared from liquid culture, and expression of SIV Gag_{mac239} was examined by anti-HA (clone 3F10 antibody) Western blotting. Positive and negative SIV Gag_{mac239} controls are displayed in lanes 11 and 12, respectively, with an expected band size of 62 kDa. (C) C57BL/6 mice were inoculated subcutaneously with 10^6 BCGD $\Delta leuCD$ empty vector lot 036 (black) or BCGD $\Delta leuCD$ SIV Gag_{mac239} lot 062 (red). At various time points, peripheral blood cells were stained to detect percent SIV Gag-specific AL11⁺ CD8⁺ T cells by flow cytometric analysis. Median values of 8 mice are shown. (D) These same mice were subsequently boosted with 3×10^7 adenovirus particles expressing SIV Gag_{mac239}, followed by quantification of AL11⁺ CD8⁺ T cell populations. Median values of 8 mice are shown. * $P < 0.01$; ** $P < 0.001$ (*t* test).

tion with either lot 040 or empty vector control BCGD $\Delta leuCD$ lot 036, followed by boosting with gp120 protein in lipid A-Span 85-Tween 80-squalene (LASTS) adjuvant as previously described (61). Gamma interferon (IFN- γ)-producing CD4⁺ T cells were measured by an ELISpot assay performed 1 week after boosting, using restimulation with either intact gp120 protein or synthetic peptides corresponding to two known immunodominant gp120 epitopes. This revealed no significant differences in the frequencies of gp120-specific CD4⁺ T cells between animals primed with lot 040 and those primed only with the empty vector BCG control strain (data not shown).

Antibody responses. Last, we tested serum antibody responses (ELISA for HIV Env 1086.C gp120) from vaccinated mice after priming with rBCGD HIV gp120 lots 039 and 040, followed by boosting with HIV gp120 protein combined with LASTS adjuvant. Serum antibodies against HIV Env gp120 were assayed by an ELISA; however, significant anti-HIV gp120 was not detected after priming alone with lot 040 in BALB/c, C57BL/6, or F1 mice (BALB/c \times C57BL/6) or with lot 039 (tested only in BALB/c mice) (data not shown). HIV Env gp120 antibodies were detectable by an ELISA after protein boosting, but the antibody titers were not modified by priming with either rBCGD HIV gp120 lot 039 or lot 040 compared to priming with either the empty vector rBCGD control lot 036 or with buffer only (data not shown).

DISCUSSION

In this study, we report a quality control process for production of stable BCG vectors expressing HIV-1 inserts. The World Health

Organization has made it clear that standardized clinical development and evaluation practices are greatly needed for new BCG-based vaccines (62, 63). The primary goal for creating a vaccine accession lot is to possess an extensive supply of a clonal vaccine strain whose composition is highly characterized and consistent from vial to vial. Composition parameters at the whole-cell level that should ideally be defined for each lot include enumeration of CFU per milliliter, optical density, absence of contamination, disruption of large bacterial aggregates, and efficient recovery of cell growth upon thawing. Additionally, subcellular parameters such as stable and high expression of the recombinant antigen, consistent retention of plasmid, and fidelity of the insert's nucleotide sequence should also be validated for every accession lot.

Much of the rationale for our accession lot standard operating procedures is a product of the inherent difficulties in generating large, yet stable, populations of recombinant BCG. The utilization of mycobacteria as live recombinant vaccine vehicles has met with highly variable success, in terms of creating immunogenic strains capable of stably expressing large amounts of recombinant antigen. *M. bovis* BCG, given its pervasive use worldwide, well-established safety profile, and readily available genetic tools, is of particular interest to the vaccine design community. These advantages have made use of BCG as a recombinant vaccine vehicle increasingly common. rBCG vaccine design has seen application against not only infections by HIV, respiratory syncytial virus, Epstein-Barr virus, rotavirus, hepatitis B virus, and measles virus but also bacterial and parasitic infections such as those caused by

enterohemorrhagic *E. coli*, *Bordetella pertussis*, *Streptococcus pneumoniae*, and *Borrelia burgdorferi* and malaria (64–75).

Through a great deal of experimental optimization and attention to the inherent complications associated with using BCG to stably express foreign antigens, we have generated a method for creating and assuring the quality of BCG-based HIV vaccine strains with greater long-term stability than previously described. In this study, lot 040, a quality-controlled accession lot expressing HIV 1086.C gp120, was able to retain protein expression after a 24-log amplification *in vitro* and for at least 60 days *in vivo*. Not only does this level of *in vitro* stability have relevant implications for large-scale production and global administration of BCG-based vaccines, but also the *in vivo* stability of antigen retention achieved months after inoculation may have important benefits for successful immune responses to vaccination. As such, *in vivo* immunogenicity was detectable for lot 040 when MHCI tetramer staining revealed the induction of SIINFEKL epitope tag-specific CD8⁺ T cell populations in vaccinated mice. With use of an extensive array of quality control procedures, BCG transformants, and large-volume accession lots, those clones are expanded and can readily be evaluated as passing or failing for subsequent use in animal models.

Applying QC criteria, we have continued to develop additional recombinant BCG vaccine lots capable of expressing lentiviral antigens with a high degree of stability. These included lot 062, a BCGD $\Delta leuCD$ lot which stably expressed the SIV Gag protein at up to a 68-log amplification *in vitro* and for at least the 30 days followed *in vivo*. The immunogenicity of this lot was evaluated against that of lot 036, an empty vector BCGD $\Delta leuCD$ strain, by examining T cell proliferation in response to vaccination. Inoculation of mice with lot 062 provided an incredibly effective prime to a recombinant adenovirus boost which heterologously expressed the same antigen. This prime-boost strategy yielded significant CD8⁺ T cell populations capable of specifically recognizing the recombinant lentiviral antigen. Such establishment of effective antigen-specific T lymphocyte populations may play a major role in contributing to protection within a viral challenge model.

In this study, we observed strong murine CD8⁺ responses to the SIV Gag AL11 epitope after priming with rBCG followed by recombinant adenovirus boosting (Fig. 5D). Previously, we described an earlier generation of rBCG vectors that generated strong SIV Gag immune responses in primates (76). Those studies were conducted using bacteria that were freshly grown prior to each immunization and relied on antibiotic selection. Subsequent analyses demonstrated that the earlier vectors, although immunogenic, were unstable in serial passage. We are currently conducting primate immunogenicity studies using an rBCG vector expressing SIV Gag under the *leuCD* selection system.

We were disappointed at the lack of CD4 and antibody responses to HIV Env after immunization with the lot 40 vector expressing HIV Env 1086.C gp120. We have described an earlier generation of rBCG vectors that expressed HIV Env con6 and that were immunogenic in mice (77). These vectors generated CD4⁺ responses to HIV Env in murine spleen, lung, and female reproductive tract as measured by an ELISpot assay. They did not generate HIV Env antibody responses by themselves but were able to prime for enhanced antibody responses after a protein boost. These older-generation vectors expressed a different HIV Env (con6) and used an alternative rBCG vector system that relied on

antibiotic selection. Importantly, however, our current lot 040 vaccine did generate CD8⁺ responses to the HIV Env-SIINFEKL epitope fusion. This may be indicative of cross-primed T cell responses which could help to prime cell-mediated immunity to combat virally infected host cells. CD4⁺-mediated antibody production against HIV Env is also an important component of a successful HIV vaccine candidate. We are evaluating additional strategies to generate rBCG vectors that will induce HIV Env CD4⁺ and antibody responses.

A human vaccine will require scaled-up production under good manufacturing practices. Frozen rBCG accession lots may serve as the source material for a human vaccine. Stability of insert expression is critical for large-scale production. We did see remarkable stability for serial passage in rich medium in a shaking flask. Lot 040 expressed insert stably during 10²⁴-fold amplification, and lot 062 was stable for >10⁶⁸-fold amplification. Using a starting vial containing 10⁸ CFU and assuming a human dose of 10⁶ CFU, an expansion of 10¹⁰-fold would be sufficient to generate enough bacteria to dose the entire human population. However, production of a human vaccine product will probably require growth in a bioreactor using a defined minimal medium. Amplification cycles will be required to adapt these bacteria to the defined medium, and we cannot predict expression stability under these conditions. The quality assurance process we describe will serve as a good starting point for the generation of viable vaccine candidates which will remain stable for extended expansion.

We describe three advances from previous work with recombinant BCG vectors: (i) an auxotrophic selection system using *leuCD*, (ii) an approach to standardize production of frozen accession lots, and (iii) a rigorous quality assurance (QA) process. Lots that pass this QA process retaining stable *in vitro* insert expression have also demonstrated persistent antigen expression *in vivo*. Frozen rBCGD $\Delta leuCD$ accession lots expressing SIV Gag have been reproducibly immunogenic in mice and are now being tested in primates. Frozen rBCG accession lots with demonstrated stability and immunogenicity in mice and primates will provide an appropriate starting material for production lots to be generated under good manufacturing practices for use in human trials.

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