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Robust Immunity to an Auxotrophic *Mycobacterium bovis* BCG-VLP Prime-Boost HIV Vaccine Candidate in a Nonhuman Primate Model

Gerald K. Chege,[a] Wendy A. Burgers,[a,b] Helen Stutz,[a] Ann E. Meyers,[c] Rosamund Chapman,[a] Agano Kiravu,[a] Rubina Bunjun,[a] Enid G. Shephard,[b,d,f] William R. Jacobs, Jr.,[g] Edward P. Rybicki,[b,c] Anna-Lise Williamson[a,b,e]

Division of Medical Virology, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa[a]; Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa[b]; Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, Cape Town, South Africa[c]; Department of Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa[d]; National Health Laboratory Service, Groote Schuur Hospital, Cape Town, South Africa[e]; Medical Research Council of South Africa, Tygerberg, South Africa[f]; Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York, USA[g]

We previously reported that a recombinant pantothenate auxotroph of *Mycobacterium bovis* BCG expressing human immunodeficiency virus type 1 (HIV-1) subtype C Gag (rBCGpan-Gag) efficiently primes the mouse immune system for a boost with a recombinant modified vaccinia virus Ankara (rMVA) vaccine. In this study, we further evaluated the immunogenicity of rBCGpan-Gag in a nonhuman primate model. Two groups of chacma baboons were primed or mock primed twice with either rBCGpan-Gag or a control BCG. Both groups were boosted with HIV-1 $Pr55^{gag}$ virus-like particles (Gag VLPs). The magnitude and breadth of HIV-specific cellular responses were measured using a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay, and the cytokine profiles and memory phenotypes of T cells were evaluated by polychromatic flow cytometry. Gag-specific responses were detected in all animals after the second inoculation with rBCGpan-Gag. Boosting with Gag VLPs significantly increased the magnitude and breadth of the responses in the baboons that were primed with rBCGpan-Gag. These responses targeted an average of 12 Gag peptides per animal, compared to an average of 3 peptides per animal for the mock-primed controls. Robust responses of Gag-specific polyfunctional T cells capable of simultaneously producing IFN-γ, tumor necrosis alpha (TNF-α), and interleukin-2 (IL-2) were detected in the rBCGpan-Gag-primed animals. Gag-specific memory T cells were skewed toward a central memory phenotype in both $CD4^+$ and $CD8^+$ T cell populations. These data show that the rBCGpan-Gag prime and Gag VLP boost vaccine regimen is highly immunogenic, inducing a broad and polyfunctional central memory T cell response. This report further indicates the feasibility of developing a BCG-based HIV vaccine that is safe for childhood HIV immunization.

Development of safe, effective, and inexpensive prophylactic human immunodeficiency virus type 1 (HIV-1) vaccines remains a major health priority despite reports of a declining global incidence of HIV infection (1) and good progress in developing other preventive strategies (2, 3). A successful HIV vaccine is expected to induce both humoral and cell-mediated immune responses. Despite much interest following the discovery of newer technologies in generating broadly neutralizing antibodies (4, 5), it is widely acknowledged that T cell responses will be a critical component of an effective HIV vaccine (6, 7). While the precise correlates of HIV-1 immune protection have not been clearly elucidated (8, 9), studies of immune responses in HIV-1-infected long-term nonprogressors and nonhuman primate models of HIV/AIDS strongly suggest that induction of cellular HIV-specific immune responses is necessary for an effective vaccine strategy (10, 11). A number of studies have shown that immune responses directed to HIV-1 Gag (12–15) and an increased breadth of the Gag-specific CD4 T cell responses (15) correlate with lower viral loads, which illustrate the importance of the inclusion of Gag protein in candidate HIV vaccines. In the best-case scenario, T cell responses could destroy HIV-infected cells before the virus spreads to other cells and so prevent the infection from establishing in the infected individuals. However, it is unlikely that T cell responses will prevent all HIV infection, but they could control viral replication and lower the viral set point, resulting in lower transmission rates and extended time before progression to AIDS. Since the potential of heterologous prime-boost vaccination strategies in HIV-1 vaccine development was first demonstrated (16), a large body of research has shown that the use of live viral and bacterial vectors in prime-boost vaccinations is effective in evoking potent vaccine-specific immune responses to a wide variety of infectious diseases, as reviewed by Lu (17). Such immune responses ideally need to broadly target a range of antigens, be mediated by T cells with polyfunctional capacity, including those that are effective in killing virally infected cells, and possess both long-lived central memory T cell phenotypes as well as effector memory capable of trafficking to effector sites (18, 19).

Mycobacterium bovis BCG has been investigated as a live vaccine vector for a variety of human infections (20–25), including HIV-1 infections (26–30). An important and attractive feature of using BCG is its long safety record as a tuberculosis (TB) vaccine, having being injected into over 3 billion people worldwide. However, it has been reported to be unsafe in HIV-infected children (31–33) and to cause disseminated TB-like disease in simian immunodeficiency virus (SIV)-infected macaques (34, 35). Efforts to improve on the safety feature of BCG have led to generation of auxotrophic mutants of mycobacteria by deletion of genes that are

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Address correspondence to Anna-Lise Williamson, Anna-Lise.Williamson@uct.ac.za.

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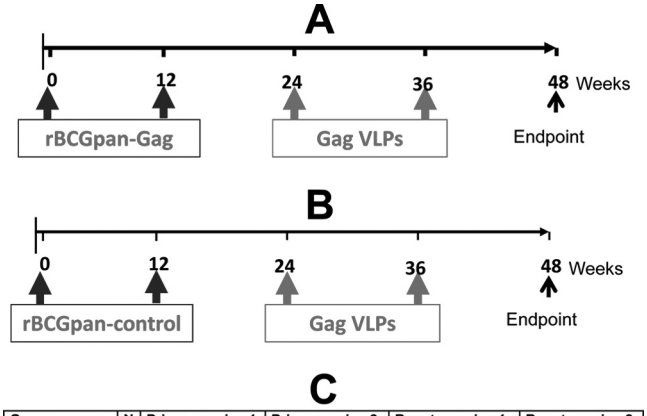
necessary for growth such as *panCD*, *leuD*, and *lysA* (36–40). The Δ*panCD* BCG auxotroph is unable to synthesize its own pantothenic acid, which is a key precursor of coenzyme A and is thus essential for several mycobacterial intracellular processes (41). Moreover, the Δ*panCD* auxotrophic strains of *Mycobacterium tuberculosis* and *M. bovis* BCG have been shown to lack virulence in guinea pigs and mice, including SCID mice (36, 38), without losing their immunogenicity (37, 40). This safety feature makes auxotrophic mutants of BCG desirable HIV vaccine vectors, considering that immunocompromised and HIV-infected individuals might, inadvertently, be included in future campaigns of mass vaccination with a BCG-vectored HIV vaccine.

The use of the baboon as an animal model for evaluating the immunogenicity of candidate human vaccines, including HIV vaccines, has been reported (27, 42–47). Chacma baboons have been previously utilized to evaluate candidate HIV-1 vaccines (27, 42, 44), two of which are currently in clinical trial (48). Also, we have previously reported that a recombinant BCG (rBCG) expressing HIV-1 subtype C Gag is able to prime the immune system of chacma baboons for a boost with HIV-1 $Pr55^{gag}$ virus-like particles (Gag VLPs), resulting in induction of Gag-specific T cell responses (27). By modifying the mycobacterial shuttle vector, we were able to generate a more stable rBCG vaccine expressing HIV-1 subtype C Gag (rBCGpan-Gag) using a pantothenate auxotroph of Pasteur BCG (BCG Δ*panCD*). The rBCG with improved stability elicited robust HIV-specific $CD8^+$ T cell responses in BALB/c mice when used in a prime-boost combination with a recombinant modified vaccinia virus Ankara (rMVA) expressing a matching Gag antigen (49). In the current study, we investigated the immune response of baboons to a primary vaccination with rBCGpan-Gag and a boost with Gag VLPs. We show that this rBCG efficiently primed for a Gag VLP boost, resulting in enhancement of the magnitude and broadening of the breadth of immune responses. We further show that the prime-boost regimen generated high-magnitude, polyfunctional $CD4^+$ and $CD8^+$ T cell responses and memory T cells that were biased toward a central memory phenotype. These data support further development of HIV-1 vaccines based on BCG Δ*panCD*, which can be used safely in children and immunocompromised individuals.

MATERIALS AND METHODS

Preparation of recombinant BCG vaccine stocks. A recombinant BCG vaccine expressing the full-length HIV-1 subtype C *gag* gene (rBCGpan-Gag) was prepared as previously described by Chapman et al. (49), using *M. bovis* BCG $mc^2$6000 (BCG Δ*panCD*), a pantothenic auxotroph strain derived from *M. bovis* BCG Pasteur 1172 P2 (Statens Serum Institute, Denmark). A control rBCG (rBCGpan-control) was prepared in an identical manner using BCG Δ*panCD* that was transformed using an empty shuttle vector (not containing *gag*) designated pCONEPI (GenBank accession no. DQ191755). The genetic stability of vaccine stocks was confirmed *in vitro* and shown to be maintained after *in vitro* passage for 30 generations and *in vivo* by restriction enzyme mapping of DNA plasmids isolated from rBCG recovered from mice splenocytes (49). The vaccine and rBCG control stocks were stored at −80°C until further use. Prior to animal vaccinations, the vaccine and rBCG control were thawed on ice and passed through a 25-gauge syringe needle 10 times to disperse clumps.

Production and preparation of HIV-1 $Pr55^{gag}$ VLPs. Gag VLPs were produced in *Spodoptera frugiperda* (*Sf*9)-derived insect cell suspension cultures as previously described (50). In brief, human codon-optimized HIV-1 *gag* with amino acid sequences derived from HIV-1 subtype C isolate Du422 (GenBank accession no. AF544010 [51]) was cloned into pFastBac as described previously (52). *Sf*9 cells were then infected with the recombinant baculovirus and the Gag VLPs harvested from the culture supernatant as described previously (50). Purified Gag VLPs were tested and shown to be negative for microbial contamination, and the endotoxin level was below 1.0 endotoxin units (EU)/ml. The Gag VLP vaccine stock was formulated by adding sterile trehalose solution to reach a final concentration of 15% and stored at 4°C. The Gag content and integrity of the vaccine stocks were evaluated as described previously (50) before animal vaccinations.



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Group	N	Prime vaccine 1 (dose & route)	Prime vaccine 2 (dose & route)	Boost vaccine 1 (dose & route)	Boost vaccine 2 (dose & route)
rBCGpan-Gag	6	rBCGpan-Gag (10^8 CFU; i.d)	rBCGpan-Gag (10^8 CFU; i.d)	Gag VLPs (130 μg; i.m)	Gag VLPs (130 μg; i.m)
rBCGpan-control	6	rBCGpan-control (10^8 CFU; i.d)	rBCGpan-control (10^8 CFU; i.d)	Gag VLPs (130 μg; i.m)	Gag VLPs (130 μg; i.m)

FIG 1 Experimental design and immunization schedule. (A and B) Two groups of chacma baboons were vaccinated twice (with the second vaccination administered 12 weeks after the first) with either rBCGpan-Gag (A) ($n = 6$) or rBCGpan-control (B) ($n = 6$) vaccine. All animals were subsequently boosted twice (with the second vaccination administered 12 weeks after the first) with Gag VLPs, and the experiment was terminated at week 48. (C) The dosage and route of the various vaccinations are shown in the table. i.d., intradermal route; i.m., intramuscular route.

Animal vaccinations. Twelve chacma baboons weighing between 6 and 15 kg were used in the study. Animals were selected for inclusion in the study based on low background reactivity in preimmunization peripheral blood mononuclear cells (PBMC) to peptide and protein reagents used in immunological assays. The animals were housed in the Animal Research Centre of the South African Medical Research Council (MRC) in Cape Town. Before the study, these baboons were shown to be healthy and negative for tuberculin skin test and SIV antibodies.

Animals were randomly divided into two groups and immunized as shown in Fig. 1. All animals were monitored for local reactions on the inoculation sites. Blood samples for PBMC isolation were obtained at preinoculation and various time points postinoculation. Blood samples at the termination of the study were unavailable from animals 737 and 743, which died from unrelated causes before the experimental endpoint. A mixture of ketamine hydrochloride (10 mg/kg body weight) and xylazine hydrochloride (0.6 mg/kg body weight) was used to anesthetize animals for all procedures. These experiments were reviewed and approved by the Animal Ethics Committee of the University of Cape Town (HSFAEC reference no. 08/033).

Isolation of lymphocytes from blood. PBMC were isolated from heparinized blood using a standard Ficoll gradient centrifugation method. Freshly isolated lymphocytes from blood were used in gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assays, while the remainder were resuspended at 10×10^6 to 20×10^6 cells/ml in fetal bovine serum (FBS; Gibco) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and cryopreserved in liquid nitrogen until use in further immunological analyses.

IFN-γ ELISPOT assay. Gag-specific T cells in the peripheral blood were quantified using an IFN-γ ELISPOT assay as previously described (44). The cells were incubated for 22 to 24 h in culture media containing synthetic Gag peptides (2 μg/ml) or culture medium alone. The Gag peptides, which spanned the entire HIV-1 Gag protein sequence, were based on the Du422 isolate (51) Gag sequences, were 15 to 18 amino acids in length, and overlapped by 10 amino acids. These peptides were prepared and pooled into three peptide pools corresponding to the amino acid sequences of the HIV-1 Gag p17, p24, and p15 regions for use in the IFN-γ ELISPOT assay. A response to any peptide pool that was equal to or greater than the peptide pool cutoff value of 30 spot-forming units (SFU)/10^6 PBMC after subtraction of the background response (cells and culture media alone) was considered positive. The cutoff value was determined by calculating the mean plus 3 standard deviations of the response of PBMC to the Gag peptide pools prior to animal immunizations. Values below this cutoff were set to zero when calculating the cumulative Gag response (sum of responses to the three individual peptide pools). Phytohematoglutinin-P (PHA-P; Sigma-Aldrich) was used as a positive control in all assays, and a cutoff value of 500 SFU/10^6 PBMC was used to validate the assay.

To evaluate the breadth of the Gag-specific responses, individual peptides to which PBMC responded at the peak immune response were identified using a pool-matrix IFN-γ ELISPOT method as previously described (27, 53).

Intracellular cytokine staining and polychromatic flow cytometry. Cryopreserved PBMC obtained at the peak response time point (as measured by the ELISPOT assay) were used for detection of Gag-specific T cells by flow cytometry. Two monoclonal antibody staining panels were used in order to characterize the cytokine and memory profile of T cells. The following antibodies and fluorophores were used: CD3-fluorescein isothiocyanate (CD3-FITC), CD3-allophycocyanin-Cy7 (CD3-APC-Cy7), CD4-peridinin chlorophyll protein-Cy5.5 (CD4-PerCP-Cy5.5), CD8-Qdot605, CD28-FITC (clone CD28.2), CD95-APC, IFN-γ–Alexa 700, IFN-γ–phycoerythrin (IFN-γ-PE), interleukin-2–PE (IL-2-PE), tumor necrosis factor alpha-PE-Cy7 (TNF-α-PE-Cy7), TNF-α-PE (all from BD Biosciences), CD14-Pacific Blue, and CD19-Pacific Blue (Invitrogen). Titers of all antibodies were determined at optimal concentrations. Briefly, thawed and rested PBMC were washed and cultured in medium containing anti-CD28 (clone L293) and anti-CD49d (1 μg/ml) either alone or together with a single pool of Gag peptides at 1 μg/ml for 16 h in the presence of brefeldin A (Sigma) (10 μg/ml). Cells were then stained with violet amine reactive dye ("ViVid"; Molecular Probes) and then surface stained with CD4, CD8, CD14, and CD19 antibodies, with the inclusion of CD28 and CD95 for the memory panel, at room temperature. Cells were then fixed and permeabilized using Cytofix/Cytoperm buffer (BD) and stained intracellularly with CD3 and cytokine antibodies. For the memory panel, all cytokines were conjugated to PE and analyzed together. After washing, cells were resuspended in CellFix (BD). Approximately 500,000 total events were acquired per sample on an LSR II flow cytometer (BD), and analysis was performed using FlowJo (v8.5.3; Treestar). Dead cells (ViVid positive [ViVid$^+$]), monocytes (CD14$^+$), and B cells (CD19$^+$) were excluded from the analysis. For characterizing cytokine profiles, cells were gated on singlets, lymphocytes, live CD3$^+$, CD8$^+$, or CD4$^+$, and then IFN-γ, IL-2, or TNF-α or combinations of cytokine markers. For characterizing memory profiles, cells were gated on singlets, lymphocytes, live CD3$^+$, CD8$^+$, or CD4$^+$, cytokine$^+$ cells, and then memory T cells (CD28$^+$ CD95$^+$ and CD28$^-$ CD95$^+$). Memory subsets were expressed as a percentage of total CD8$^+$ or CD4$^+$ memory cells (i.e., excluding naïve CD28$^+$ CD95$^-$ cells). A positive cytokine response was defined as at least twice the level of the background (no antigen, only costimulatory antibodies), >0.05% after subtraction of the background, and at least 10 events. The latter criterion was introduced so as to minimize the possibility of error due to a low number of events that might arise from sequential subdivision of cell populations.

Anti-HIV-1 Gag antibody determinations by ELISA. To monitor the development of humoral responses, the presence of Gag-specific antibodies in baboon sera was measured by an enzyme-linked immunosorbent assay (ELISA) method as previously described (44). ELISA plates were coated with HIV-1 recombinant Pr55Gag protein (Quality Biological) (0.05 μg per well). Sera obtained at prevaccination and 4 weeks after the second vaccination with rBCGpan and Gag VLPs were analyzed.

Statistical analysis. Statistical analyses were performed using Prism version 5.0 (GraphPad Software, San Diego, CA). The *t* test for independent unpaired nonparametric comparisons was applied to assess the level of significance of comparisons between means. All tests were two tailed, and *P* values of 0.05 were considered significant.

RESULTS

Inoculation with rBCGpan induces self-limiting local reactions at the vaccination site. Following vaccinations, animals were monitored for development of local reactions on the injection sites. All animals developed indurations, which culminated in skin ulcerations (5 to 15 mm in diameter) at inoculation sites within 2 weeks after receiving rBCGpan-Gag or rBCGpan-control. There were no differences in the sizes of the ulcerations. The second vaccination with rBCG induced ulcerations of comparable sizes. For both vaccinations, the ulcerations healed within 6 weeks postinoculation without any complication or need for medication. Vaccination with Gag VLPs did not result in any noticeable reaction at the inoculation site. With the exception of these local tissue reactions, the baboons remained healthy throughout the study period.

rBCGpan-Gag vaccine efficiently primes for a Gag VLP boost. HIV-1 Gag-specific immune responses were measured in an IFN-γ ELISPOT assay using freshly isolated PBMC. A priming vaccination with the first dose of rBCGpan-Gag vaccine elicited low-magnitude cumulative Gag responses in 3 of 6 baboons (early responders), reaching a peak magnitude (mean ± standard deviation) of 313 ± 192 SFU/10^6 PBMC at 8 weeks postvaccination (Fig. 2A). These Gag-specific responses for the early responders waned with time but were still detectable at week 12 postvaccination. A second dose of rBCGpan-Gag which was given at week 12 boosted the responses in the 3 early responders and induced measurable responses in the other 3 late responders. The cumulative magnitude of responses reached the peak at 2 or 4 weeks after vaccination (378 ± 149 SFU/10^6 PBMC). These responses were directed predominantly to p15 (275 ± 261 SFU/10^6 PBMC in 6 of 6 baboons). The response to p17 was 100 ± 78 SFU/10^6 PBMC (for 4 of 6 baboons) and to p24 was 73 ± 41 SFU/10^6 PBMC (for 3 of 6 baboons). For the early responders, the Gag responses were maintained above detectable levels through to the time of booster vaccination, unlike 2 of 3 late responders, for which the Gag response was undetectable after 4 weeks. No Gag responses were detectable for the baboons that received the mock-prime inoculation with the rBCGpan-control (Fig. 2B).

Boosting with Gag VLPs induced high-magnitude immune responses in animals receiving a rBCGpan-Gag prime that were 4- to 5-fold higher than those for the mock-prime group. These responses peaked at 2 weeks after boosting in both groups (Fig. 2A and B). At peak response, the mean cumulative Gag response was significantly higher ($P < 0.0001$) for the animals primed with rBCGpan-Gag (2,801 ± 293 SFU/10^6 PBMC) than for the mock-prime group (421 ± 116 SFU/10^6 PBMC; Fig. 2C). These responses were directed to all the three Gag peptide pools corresponding to p17, p24, and p15 for the rBCGpan-Gag group (Fig. 2D) and to only p24 and p15 for the mock-prime controls (Fig. 2E). Gag responses were consistently detected throughout the experimental period after the Gag VLP boost in all the animals

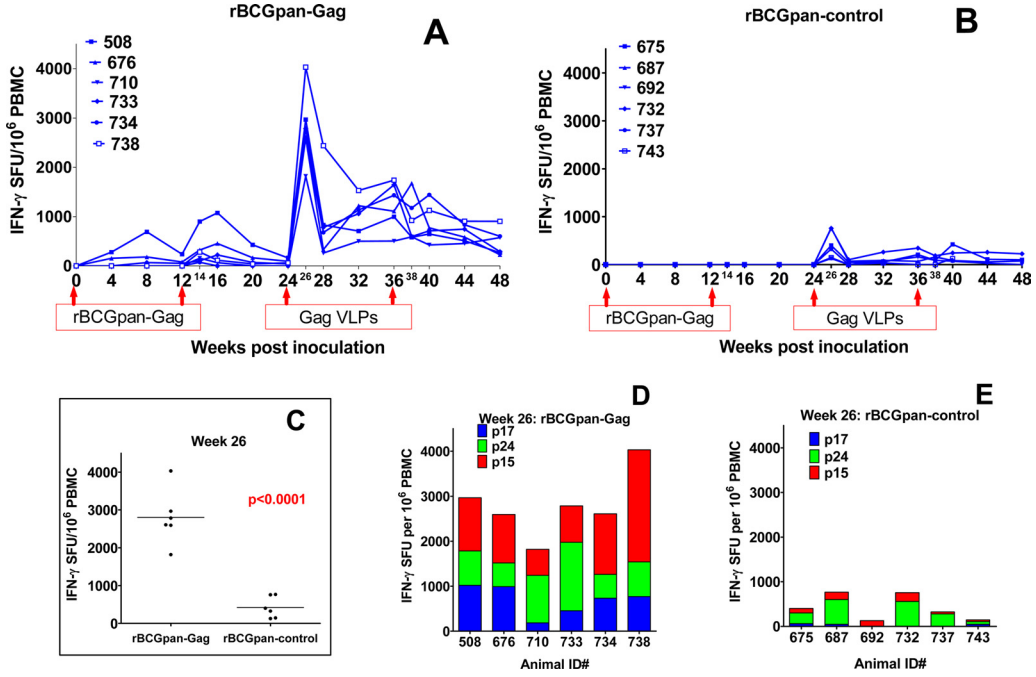

FIG 2 IFN-γ ELISPOT responses. PBMC obtained from various time points after vaccination with rBCGpan-Gag or rBCGpan-control and Gag VLPs were used in an IFN-γ ELISPOT assay using three Gag peptides spanning Gag p17, p24, and p15. Shown are the cumulative responses for rBCGpan-Gag-primed baboons at various time points (A) and week 26 (D) and the corresponding time points for rBCGpan-control baboons (B and E). Panel C shows a comparison of the mean cumulative responses of the two groups to Gag at week 26.

in the rBCGpan-Gag group, in contrast to the results seen with the mock-primed group, where only 3 of 6 animals had consistent responses. At the experimental endpoint (week 48), the mean cumulative Gag response for the rBCGpan-Gag group (448 ± 108 SFU/10^6 PBMC) was significantly higher ($P = 0.0273$) than that for the mock-prime group (100 ± 47 SFU/10^6 PBMC).

The rBCGpan-Gag prime and Gag VLP boost regimen broadens the breadth of the Gag-specific response. Next, we sought to evaluate the breadth of Gag responses by considering the number of responders to the individual Gag peptide pools and by identifying individual peptides to which the PBMC were responding for each animal in an IFN-γ ELISPOT assay. This was done at the peak IFN-γ ELISPOT response or 2 weeks after the first Gag VLP booster vaccination. All the animals in the rBCGpan-Gag group responded to all the three Gag peptide pools (p17, p24, and p15). In contrast, only a response to p15 was detectable in all the animals in the mock-prime control group, with only 3 of 6 and 5 of 6 animals responding to peptide pools corresponding to p17 and p24, respectively (data not shown). In addition, the mean cumulative responses to the three Gag peptide pools were significantly higher for the rBCGpan-Gag group than for the mock-prime controls ($P = 0.012$, $P = 0.023$, and $P = 0.002$ for the p17, p24, and p15 pools, respectively; data not shown). Also, in terms of magnitude, the responses to p15 were the most dominant of the responses to the 3 Gag domains for the rBCGpan-Gag group, while those to p24 were the most dominant for the mock-prime controls.

PBMC from animals primed with rBCGpan-Gag responded to an average of 12 peptides per baboon, with animal 738 responding to up to 19 peptides, whereas those from the mock-prime group responded to an average of 3 peptides per animal (Table 1). The distributions of Gag peptides being targeted by these PBMC also differed between the two groups. While PBMC from the rBCGpan-Gag group targeted more peptides corresponding to p15, p17, and p24, in that order, PBMC from the mock-prime group targeted peptides corresponding to p24 and only 1 or 2 or no peptides in the p17 and p15 domains.

TABLE 1 Breadth of Gag IFN-γ ELISPOT responses[a]

Vaccine regimen	Animal ID	No. of Gag peptides targeted by PBMC at wk 26 (peak response)			
		Gag p17	Gag p24	Gag p15	Total
rBCGpan-Gag + Gag VLPs	508	4	2	4	10
	676	4	5	9	18
	710	4	3	4	11
	733	3	2	3	8
	734	2	1	4	7
	738	7	4	8	19
Total		24	17	32	73
Avg		4	3	5	12
rBCGpan-control + Gag VLPs	675	0	0	2	2
	687	0	2	0	2
	692	2	2	0	4
	732	0	3	1	4
	737	0	2	0	2
	743	0	4	1	5
Total		2	13	4	19
Avg		<1	2	<1	3

[a] PBMC obtained at week 26 (2 weeks after the first Gag VLP booster vaccination) were evaluated for the breadth of response using a Pool-Matrix ELISPOT mapping strategy. The table shows the number of Gag peptides to which the PBMC responded as identified using the Pool-Matrix IFN-γ ELISPOT method. ID, identification number.

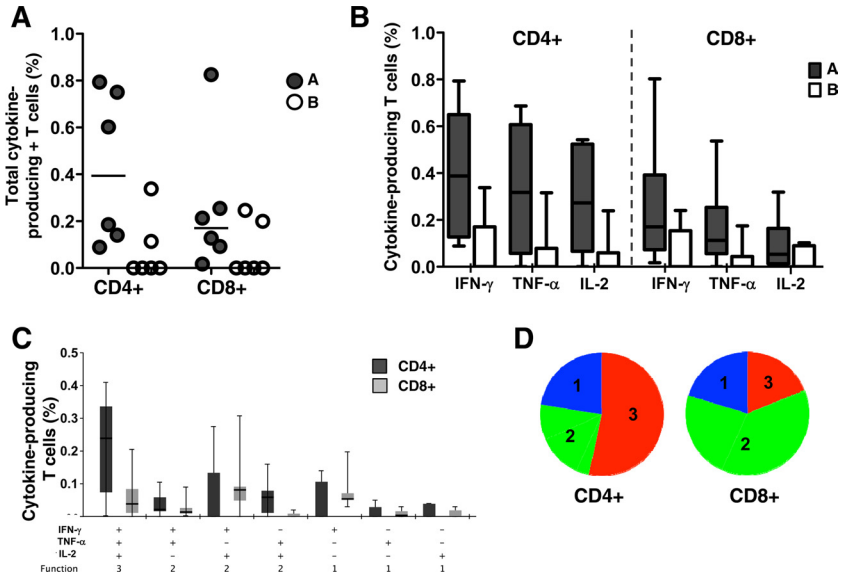

FIG 3 Functional profile of vaccine-elicited Gag-specific T cells. PBMC obtained at week 26 (2 weeks after the first Gag VLP booster vaccination) were evaluated for CD4 and CD8 cytokine responses. (A) The total frequency of $CD4^+$ and $CD8^+$ T cells producing any cytokine (IFN-γ, TNF-α, or IL-2) in the $CD4^+$ and $CD8^+$ T cell subsets in group A (filled circles) and group B (open circles). (B) Individual cytokine responses in $CD4^+$ and $CD8^+$ T cells in group A (filled bars) and group B (open bars). (C) Polyfunctional profiles of Gag-specific CD4 and $CD8^+$ T cells in group A. The frequencies of all possible combinations of the three cytokines (IFN-γ, TNF-α, and IL-2) in rBCGpan-Gag VLP prime-boost animals are shown for $CD4^+$ (dark shading) and $CD8^+$ (light shading) T cells. (D) Functional profiles are grouped and color coded according to number of functions and summarized as proportions of the total responses in the pie charts. Each slice of the pie corresponds to the median production of 3 cytokines (red), 2 cytokines (green), or 1 cytokine (blue).

The rBCGpan-Gag prime and Gag VLP boost regimen generates polyfunctional T-cell responses. To further characterize the responses induced by the vaccine regimen, we investigated the T cell phenotype and functional profiles of the rBCGpan-Gag and Gag VLP prime-boost regimen using intracellular cytokine staining and polychromatic flow cytometry. Cellular responses were measured at the peak response time point, which coincided with 2 weeks after the first Gag VLP boost. As shown in Fig. 3A, Gag-specific T cell responses were both $CD4^+$ and CD8 mediated, with 2-fold-higher median magnitudes of cytokine-producing $CD4^+$ T cells in the prime-boost group. Only 2 of 6 animals in the mock-primed control group exhibited $CD4^+$ or $CD8^+$ responses (Fig. 3A). Gag-specific T cells produced all three cytokines in the order IFN-γ > TNF-α > IL-2 for both $CD4^+$ and $CD8^+$ T cells (Fig. 3B). When we examined the ability of these cells to simultaneously produce combinations of these cytokines (i.e., their polyfunctional nature), we detected these in both the $CD4^+$ and $CD8^+$ subsets (Fig. 3C and D). The proportion of $CD4^+$ T cells simultaneously expressing all three cytokines was greater than the proportion of $CD8^+$ T cells (approximately 55% versus 20%; Fig. 3D). In addition, >75% of responding $CD4^+$ and $CD8^+$ T cells simultaneously produced 2 or 3 cytokines (Fig. 3D). Thus, the combination of rBCGpan-Gag and Gag VLP candidate vaccines induced high frequencies of polyfunctional $CD4^+$ and $CD8^+$ T cell responses in the chacma baboons.

The rBCGpan-Gag prime Gag VLP boost regimen induces central memory T cell responses. We sought to determine the memory phenotype of vaccine-induced memory T cells in animals that received the combination of rBCGpan-Gag and Gag VLPs, using the phenotypic markers CD28 and CD95. PBMC from only three animals were available for the peak response time point after the first Gag VLP boost, while all six animals were tested 2 weeks after the second Gag VLP vaccination. Representative flow cytometry plots are shown in Fig. 4A, indicating the memory distribution of Gag-specific cytokine-producing T cells in the central and effector memory compartments. The majority of Gag-specific $CD4^+$ T cells were found to be markedly skewed toward a central memory phenotype, with >95% of cytokine-producing $CD4^+$ memory T cells expressing this phenotype 2 weeks after the first Gag VLP booster vaccination (Fig. 4B). Similarly, Gag-specific $CD8^+$ T cells showed the same preference of skewing toward a central memory phenotype, with about 65% of total cytokine-positive cells expressing the central memory phenotype (Fig. 4B). The second Gag VLP vaccination did not change the distribution of memory phenotype in the $CD8^+$ T cell subset but increased the proportion of $CD4^+$ T cells expressing an effector memory phenotype to approximately 15%.

Gag VLP vaccination elicits HIV-1 antibody responses. We evaluated the induction of vaccine-specific humoral responses by determining the endpoint titers of anti-Gag antibodies in the sera obtained at three time points. As shown in Table 2, Gag-specific antibodies were detected only after booster vaccination with Gag VLPs. The titers for animals primed with rBCGpan-Gag were generally higher (median, >51,200; range, 6,400 to >51,200) than those for animals receiving a mock prime (median, 9,600; range, 200 to 51,200), suggesting a priming effect by rBCGpan-Gag vaccination.

DISCUSSION

In this study, a recombinant auxotrophic strain of BCG expressing HIV-1 subtype C Gag boosted with Gag VLPs was evaluated in a nonhuman primate model. We have previously shown that priming with this rBCG vaccine (rBCGpan-Gag), in a prime-boost combination with an antigen-matched recombinant MVA, elicits HIV-specific $CD8^+$ T cells in mice (49). This study sought to

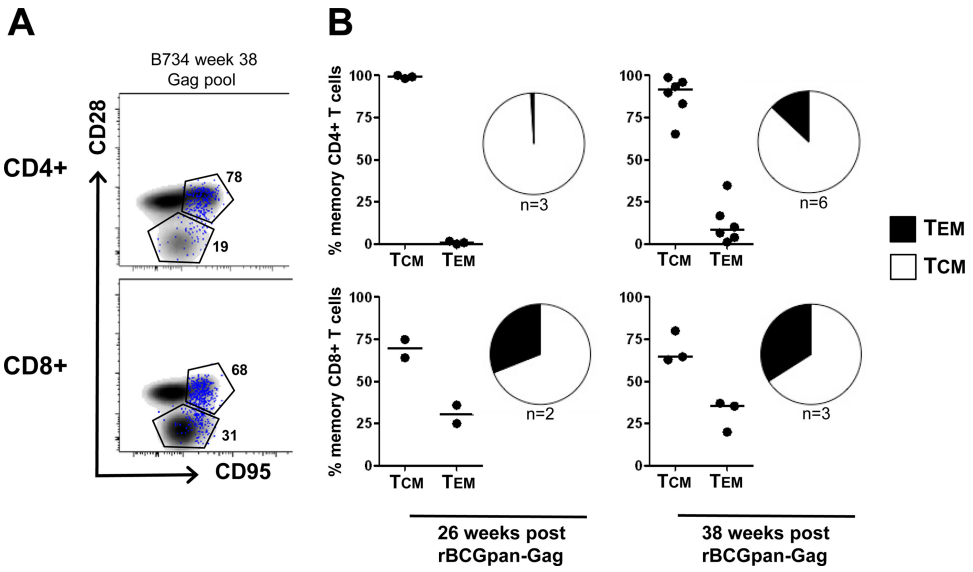

FIG 4 Memory phenotype of Gag-specific T cells. Animals from group A (rBCGpan-Gag VLP prime-boost) were examined for Gag-specific memory T cells. Cytokine-producing $CD4^+$ and $CD8^+$ T cells were delineated into central (TCM) and effector (TEM) memory T cells based on CD28 and CD95 expression. (A) Representative flow cytometry plots of the memory profile of total $CD4^+$ (upper panel) or $CD8^+$ (lower panel) T cells are shown as a density plot and Gag-specific total cytokine$^+$ T cells (blue dots) in one vaccinated animal (B734) at week 38. (B) Proportion of Gag-specific $CD4^+$ (upper panel) or $CD8^+$ (lower panel) T cells at weeks 26 and 38 (2 weeks after the first and second Gag VLP vaccinations, respectively). Data are shown as medians and summarized in pie charts, with the numbers of animals with a Gag response indicated below the pies.

further evaluate the immunogenicity of this rBCG vaccine prime/Gag VLP boost in nonhuman primates by characterizing the cellular immune response generated in immunized animals in terms of breadth, polyfunctionality, and phenotype of memory T cells.

Vaccinations with both rBCG-Gag and rBCG-control did not induce any systemic reactions in any animal, and the local cutaneous reactions were resolved without the need for medication, indicating that rBCG was well tolerated. This outcome was similar to our observations in previous studies where baboons were vaccinated with nonrecombinant BCG (54) and recombinant BCG (27). Intradermal inoculations of humans with wild-type BCG also result in development of similar local tissue reactions which are well tolerated (55, 56). However, future BCG-based vaccines which cause minimal or no reactogenicity will likely be more acceptable in clinical settings. This could possibly be achieved by the use of doses that are effective but lower than those used in the current study.

TABLE 2 Gag-specific antibody titers[a]

Vaccine regimen	Animal ID	Gag-specific antibody titer		
		Pre-Vac (wk 0)	4 wk post-rBCGpan-Vac (wk 16)	4 wk post-Gag VLP-Vac (wk 40)
rBCGpan-Gag + Gag VLPs	508	<50	<50	>51,200
	676	<50	<50	>51,200
	710	<50	<50	>51,200
	733	<50	<50	>51,200
	734	<50	<50	6,400
	738	<50	<50	12,800
rBCGpan-control + Gag VLPs	675	<50	<50	12,800
	687	<50	<50	6,400
	692	<50	<50	1,600
	732	<50	<50	51,200
	737	<50	<50	200
	743	<50	<50	25,600

[a] Gag-specific antibodies were measured in the sera obtained at prevaccination (Pre-VAC) (week 0), 4 weeks after the second vaccination with rBCGpan (week 16), and 4 weeks after the second vaccination with Gag VLPs (week 40) using an ELISA method. The table shows the endpoint antibody titers of individual animals. ID, identification number.

The Δ*panCD* BCG (Pasteur) strain was used as a vector due to its improved safety, compared with that of wild-type BCG, as has been demonstrated with similar auxotrophs (36, 40). The safety of our Δ*panCD* BCG vaccine was indicated by the development of fewer granulomas in the spleen and a reduced level of inflammation in the liver of vaccinated mice compared with the corresponding wild-type BCG recombinant results (49). The safety of BCG-vectored vaccines is a relevant issue, as vaccination of HIV-infected children with a clinical BCG (wild type) has been observed to be responsible for mycobacterial disease in 10% of these immunocompromised individuals (31, 33). In addition, BCG inoculation has been reported to enhance the pathogenicity of SIV infection in rhesus macaques, resulting in tuberculosis-like disease with disseminated granulomas (34, 35). However, no increase in viral load was seen in SIV-infected rhesus macaques vaccinated with a double panthothenate and leucine auxotroph of *M. tuberculosis*, and no mycobacteria could be detected in a variety of tissue samples or blood taken 54 weeks postvaccination (39).

Several studies have demonstrated superior immunogenicity from heterologous prime-boost vaccination strategies in HIV vaccine research (16) and as reviewed by Lu (17). rBCG vaccines have been shown to efficiently prime the immune response to the heterologous antigen in recombinant adenovirus, recombinant poxvirus, and protein prime-boost combinations (24, 26, 27, 30, 57, 58). We (27, 44, 52) and others (59) have previously shown that a Gag VLP boost, used in heterologous prime-boost modality, is a

potent booster of Gag-specific responses. Further, we have shown that plant-produced Gag VLPs are immunogenic in mice (60), and others have shown that high yields of Gag VLPs can be made in tobacco chloroplasts (61), indicating the distinct prospect of producing affordable and cost-effective Gag-based HIV vaccines (62).

Our data illustrate that rBCGpan-Gag, on its own, is immunogenic and can achieve a 100% response rate after the second vaccination in a nonhuman primate model. This is a notable finding because, unlike the present baboon study results, no Gag-specific responses were detected in the murine model, in response to rBCGpan-Gag prime, before the MVA-Gag boost (49). In addition, the results of the present study are clearly better than those of our previous study in which a wild-type BCG recombinant was used (27). We further showed that a rBCGpan-Gag prime and Gag VLP boost regimen elicits a broad range of Gag epitope-containing peptides (range, 7 to 19) compared to the control animal results (range, 2 to 5). In rhesus macaque studies, a CD4 T cell response that targeted SIV Gag in vaccinated animals was associated with control of infection after SIV challenge (63) whereas both Gag- and non-Gag-antigen-specific CD8 T cells from vaccinated SIV controllers have been shown to suppress replication of SIV variants carrying cytotoxic T lymphocyte (CTL) escape mutations (64). Broad Gag-specific responses have also been associated with good control of HIV-1 replication in chronically infected HIV controllers (12–15, 65); in particular, the increased breadth of the Gag-specific CD4 T cell responses was significantly associated with effective immune control of viremia (15). Thus, our rBCGpan-Gag prime–Gag VLP boost regimen concurs with a potential correlate of AIDS-protective immunity.

Secretion of IFN-γ by $CD4^+$ and $CD8^+$ T cells has been associated with suppression of virus replication in HIV-1, SIV, and simian-human immunodeficiency virus (SHIV) infections (66–68), while cells secreting both IFN-γ and IL-2 have been shown to confer CD4-independent proliferation of HIV-1-specific CD8 T cells (69) and viral replication control in nonprogressive HIV-1 (70, 71) and $SHIV_{89.6P}$ (72) infection. Furthermore, polyfunctional $CD4^+$ and $CD8^+$ T cell responses that include the simultaneous release of IFN-γ, IL-2, and TNF-α have been associated with elite HIV-1 controllers (73–78). In the current report, we show that the rBCGpan-Gag and Gag VLP prime-boost vaccine regimen resulted in the induction of polyfunctional $CD4^+$ (>50%) and $CD8^+$ (~25%) T cells, with the total frequency of cytokine-producing cells being greater in the $CD4^+$ T cell subset. In addition, these T cells were significantly skewed toward a central memory phenotype, with >95% of the $CD4^+$ and 65% of the $CD8^+$ Gag-specific memory responses exhibiting a central memory phenotype. Effector memory cells are important as the first-line defense mechanism at the tissue sites where exposure to HIV occurs, and central memory cells may play a critical role, as they are long-lived and capable of replenishing the effector memory pool (79). HIV-specific memory T cells have been associated with better HIV-1 replication control, as indicated by preserved $CD4^+$ T central and effector memory cells in HIV controllers (80) and higher proportions of HIV-specific $CD8^+$ T central memory cells being associated with low viral set points in early HIV-1 infection (81). The role of effector memory T cells is further underscored by the findings of Hansen et al. (18, 19) that showed an association between vaccine-induced effector memory T cells and protection against mucosal SIV challenge.

Preexisting immunity to some environmental mycobacterial species has been reported to block *in vivo* multiplication of BCG (82), indicating potential reduction of vaccine efficacy of future BCG-based HIV vaccines. This is a crucial issue, particularly for sub-Saharan Africa, where vaccination against tuberculosis (TB) using BCG in infants is part of the WHO-recommended Extended Programme of Immunization (EPI) and where a cost-effective HIV vaccine such as that based on BCG would be expected to have a major impact due to the high HIV prevalence. Although this issue of preexisting anti-BCG immunity remains unresolved, a number of BCG studies in animals have shown enhanced immunologic effects attributable to prior BCG sensitization (83, 84) or coadministration with BCG (58). We observed an increase in the magnitude of BCG-specific IFN-γ ELISPOT responses after the second rBCGpan-Gag vaccination, suggesting that preexisting BCG immunity may not have a significant negative effect on the efficacy of future rBCG vaccines. Moreover, vaccination of infants with an efficacious BCG-vectored HIV vaccine has been proposed as a possible platform to prevent both TB and mother-to-child transmission of HIV (28).

In conclusion, this report demonstrates that a BCG-based HIV-1 vaccine boosted with Gag VLPs has the capacity to induce high-magnitude and broad Gag-specific responses which are characterized by generation of polyfunctional T cells and a memory phenotype skewed toward a central memory in nonhuman primates. These features are characteristic of an immune response that may be capable of controlling and containing the replication of HIV-1. Thus, our rBCGpan-Gag vaccine appears to be a promising candidate HIV vaccine when given in a prime-boost combination with a recombinant Gag-based HIV vaccine.

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