Comparative Characterization of Transfection- and Infection-Derived Simian Immunodeficiency Virus Challenge Stocks for *In Vivo* Nonhuman Primate Studies

Gregory Q. Del Prete,^a Matthew Scarlotta,^b Laura Newman,^a Carolyn Reid,^a Laura M. Parodi,^c James D. Roser,^a Kelli Oswald,^a Preston A. Marx,^d Christopher J. Miller,^e Ronald C. Desrosiers,^f Dan H. Barouch,^g Ranajit Pal,^h Michael Piatak, Jr.,^a Elena Chertova,^a Luis D. Giavedoni,^{c,i} David H. O'Connor,^{b,j} Jeffrey D. Lifson,^a Brandon F. Keele^a

AIDS and Cancer Virus Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA^a; Department of Pathology and Laboratory Medicine^b and Wisconsin National Primate Research Center,^j University of Wisconsin, Madison, Wisconsin, USA; Department of Virology and Immunology^c and Southwest National Primate Research Center,ⁱ Texas Biomedical Research Institute, San Antonio, Texas, USA^d; Division of Microbiology, Tulane National Primate Research Center, Tulane University, Covington, Louisiana, USA^e; Center for Comparative Medicine and California National Primate Research Center, University of California, Davis, California, USA^f; New England Primate Research Center, Department of Microbiology and Molecular Genetics, Harvard Medical School, Southborough, Massachusetts, USA^g; Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, and Ragon Institute of MGH, MIT and Harvard, Boston, Massachusetts, USA^h; Advanced Bioscience Laboratories, Inc., Kensington, Maryland, USA^h

Simian immunodeficiency virus (SIV) stocks for *in vivo* nonhuman primate models of AIDS are typically generated by transfection of 293T cells with molecularly cloned viral genomes or by expansion in productively infected T cells. Although titers of stocks are determined for infectivity *in vitro* prior to *in vivo* inoculation, virus production methods may differentially affect stock features that are not routinely analyzed but may impact *in vivo* infectivity, mucosal transmissibility, and early infection events. We performed a detailed analysis of nine SIV stocks, comprising five infection-derived SIVmac251 viral swarm stocks and paired infection- and transfected-293T-cell-derived stocks of both SIVmac239 and SIVmac766. Representative stocks were evaluated for (i) virus content, (ii) infectious titer, (iii) sequence diversity and polymorphism frequency by single-genome amplification and 454 pyrosequencing, (iv) virion-associated Env content, and (v) cytokine and chemokine content by 36-plex Luminex analysis. Regardless of production method, all stocks had comparable particle/infectivity ratios, with the transfected-293T stocks possessing the highest overall virus content and infectivity titers despite containing markedly lower levels of virion-associated Env than infection-derived viruses. Transfected-293T stocks also contained fewer and lower levels of cytokines and chemokines than infection-derived stocks, which had elevated levels of multiple analytes, with substantial variability among stocks. Sequencing of the infection-derived SIVmac251 stocks revealed variable levels of viral diversity between stocks, with evidence of stock-specific selection and expansion of unique viral lineages. These analyses suggest that there may be underappreciated features of SIV *in vivo* challenge stocks with the potential to impact early infection events, which may merit consideration when selecting virus stocks for *in vivo* studies.

Repeatable experimental inoculation of naïve animals via a known route using a defined amount of titered, laboratory-generated cell-free virus of known identity is a key feature of *in vivo* nonhuman primate (NHP) models of AIDS. Because HIV-1 does not productively infect the monkey species used in NHP AIDS models (1), these experiments have relied on the use of the genetically related simian immunodeficiency viruses (SIV) from sooty mangabeys or other Old World primates, with viruses from the SIVmac251 and SIVmac239 lineages serving as the most widely used in studies of viral transmission, dissemination, evolution, and pathogenesis, as well as studies of antiviral immune responses and vaccine-mediated protection (2). There are a number of potential methods for generating primate lentiviruses; however, SIV stocks used for *in vivo* NHP studies are typically produced in one of two ways: the viruses can be produced by transfection of nonpermissive 293T cells if a molecularly cloned full-length viral genome is available, or, alternatively, cloned viruses and uncloned viral isolates can be produced by infection of permissive primary cells or cell lines with subsequent virus expansion via multiple rounds of viral replication.

Viruses produced in transfected nonpermissive cells package full-length viral genomes that have been transcribed directly from

an input DNA plasmid, and they are therefore only subject to the low error rate of cellular DNA-dependent RNA polymerase II. This production method thus offers the advantage of virtual intrastock clonality, thereby allowing for *in vivo* infections using a virus with a precisely known genetic composition. In addition, different transfection-derived stocks of the same virus will have the same genetic sequence and as a result may exhibit more consistent behavior from stock to stock.

While production of virus in transfected cells requires a full-length molecularly cloned viral genome, infection-based production methods obviate infectious molecular clones and can be used to expand uncloned viral swarms as well as clonal virus stocks

Received 9 January 2013 Accepted 1 February 2013

Published ahead of print 13 February 2013

Address correspondence to Brandon F. Keele, keelebf@mail.nih.gov.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.03507-12>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.03507-12

generated in transfected cells. Infection-based virus production methods also present several other potential advantages, including greater ease of virus scale-up and the ability to avoid xenoreactivity to producer cell proteins in challenged animals by culturing the challenge virus in challenge host-specific cells, a feature which has proven important for the interpretation of some vaccine protection studies (3). Because they are propagated via multiple rounds of viral replication with reverse transcription errors, infection-derived SIV stocks may contain a large degree of genetic diversity within each stock (4–6). For some experiments, added genetic diversity in the virus challenge stock may be more desirable than stock clonality (7); however, the degree of diversity between different infection-derived stocks of nominally the same virus has not been carefully examined.

These canonical stock characteristics are often considered when choosing an SIV stock for use *in vivo* in a particular NHP study; however, matched transfection- and infection-derived challenge stocks have not been directly compared, nor have different stocks of the same nominal virus produced in different labs using similar but distinct methodologies. There is also a perception within the NHP AIDS field that infection-derived stocks may be more infectious for *in vivo* challenges, but this phenomenon has not been formally demonstrated and the specific stock features that may be responsible have not been evaluated. Although the titers of SIV challenge stocks are determined *in vitro* prior to use *in vivo*, there may be aspects of SIV stocks that do not affect standard *in vitro* titer determination assays, such as the presence of proinflammatory cytokines or selection and expansion of specific sequences during culture, which could impact *in vivo* infectivity at the earliest stages of infection, particularly in the case of mucosal challenges. It is also unclear if different production methods yield viruses with equivalent levels of envelope (Env) glycoprotein incorporation and similar particle/infectivity ratios or how the target cell type used for a titer determination assay might impact the determined titer. Given the cost and scientific value of *in vivo* NHP AIDS models, we examined a panel of SIV challenge stocks generated in several different labs by both transfection and infection-based methods. Our detailed characterization of several of the more widely used *in vivo* NHP study SIV challenge stocks reveals key distinctions between transfection- and infection-derived virus stocks and between different infection-derived stocks of the same nominal virus and may provide insight to better inform selection of current SIV challenge stocks and to improve production of SIV challenge stocks in the future.

MATERIALS AND METHODS

Virus stocks. 251-RD (SIVmac251-7/9/2010) was generated by infecting rhesus macaque peripheral blood mononuclear cells (PBMCs) from 15 donors with an animal-titered stock of SIVmac251 (4-19-91) (8) 3 days after stimulation with 1.0 µg/ml phytohemagglutinin (PHA). Infected cultures were maintained in 10% interleukin-2 (IL-2) with a complete medium change 24 h prior to harvest. At day 8 postinfection, virus-containing supernatants were pooled, clarified by centrifugation, aliquoted, and stored in the vapor phase of liquid nitrogen.

251-CM (SIVmac251-06/2004) (9) was generated by infecting rhesus macaque PBMCs from 6 donors 5 to 7 days after stimulation with 0.5 µg/ml staphylococcal enterotoxin A (SEA) with an SIVmac251 seed stock (1998) (10) grown for 3 to 4 days in CEMx174 cells. Infected cultures were maintained in 50 to 100 U/ml IL-2, with 80% of the virus-containing supernatant volumes collected, clarified by centrifugation, aliquoted, and stored at –80°C every 2 to 3 days postinfection. Following each superna-

tant harvest, infected PBMCs were resuspended in the remaining supernatant volume plus fresh medium with IL-2. Supernatants from 2 PBMC donors collected on multiple harvest days containing the highest viral p27 content were thawed, pooled, aliquoted, and stored in the vapor phase of liquid nitrogen.

251-DB (SIVmac251-8/27/2008) (11) was generated by infecting human PBMCs from a single donor with SIVmac251 after overnight stimulation with 6.25 µg/ml concanavalin A (ConA). Infected cultures were maintained in 20 U/ml IL-2 with a complete medium change 24 h prior to virus harvest. Virus-containing supernatants collected on days 4 and 7 postinfection were clarified by centrifugation, sterile filtered, pooled, aliquoted, and stored at –80°C.

251-LG (SIVmac251) (12) was generated by infecting rhesus macaque PBMCs with an animal-titered stock of SIVmac251 (4-19-91) (8) 3 days after stimulation with 5.0 µg/ml PHA and culturing infected cells for 21 days after stimulation. The infected culture was maintained in 50 U/ml IL-2, with a half-volume medium change twice a week. Removed medium was tested for SIV p27, and supernatants with a high SIV concentration were pooled (supernatant for days 14, 18, and 21 postinfection). Virus-containing supernatants were clarified by centrifugation, sterile filtered, aliquoted, and stored at –80°C.

251-PM (SIVmac251-06/25/2004) (13) was generated by infecting CEMx174 cells with a SIVmac251 stock (11-19-1995) (14, 15) that had been previously expanded on HUT-78 (16) and then CEMx174 (17) cells. A complete culture medium change was performed on day 20 postinfection. On day 21 postinfection, virus-containing supernatants were collected, clarified by centrifugation, sterile filtered, aliquoted, and stored at –80°C.

766-BK (SIVmac766, a full-length transmitted/founder clone derived from an SIVmac251-infected rhesus macaque) was generated by infecting pooled rhesus macaque CD8-depleted PBMCs from 8 donors with transfection-produced SIVmac766 3 days after stimulation with 2.5 µg/ml PHA. Infected cultures were maintained in 28 U/ml IL-2, and freshly activated CD8-depleted PBMCs were added to the cultures at 3 days postinfection. At day 7 postinfection, virus-containing supernatants were clarified by centrifugation, sterile filtered, aliquoted, and stored in the vapor phase of liquid nitrogen.

766-TFN-BK (SIVmac766) was generated by transfecting 293T cells with the full-length molecular clone plasmid pSIVmac766.4 for 24 h using the TransIT HEK-293 transfection reagent (Mirus Bio) according to the manufacturer's instructions. Culture medium was changed at 24 h posttransfection and again at 48 h posttransfection. At 72 h posttransfection, virus-containing supernatant was clarified by centrifugation, aliquoted, and stored at –80°C.

239-CM (SIVmac239) (18) was generated as described above for 251-CM, except that stimulated rhesus macaque PBMCs were infected with a SIVmac239 seed stock produced in transfection-produced 293T cells.

239-TFN-RD (SIVmac239) was generated by transfecting 293T cells with the full-length molecular clone plasmid p239-FL for 24 h using the Profection mammalian transfection system calcium phosphate method (Promega) according to the manufacturer's instructions. Culture medium was changed at 24 h posttransfection. At 72 h posttransfection, virus-containing supernatant was clarified by centrifugation, aliquoted, and stored in the vapor phase of liquid nitrogen.

Virus for protein analysis and infectivity thermostability. Dedicated virus stocks distinct from those described above were generated for protein analysis and infectivity thermostability experiments. Transfection-derived SIVmac239 and SIVmac766 stocks were generated by transfecting 293T cells with the full-length molecular clone plasmid p239SPxFL or pSIVmac766.4 for 24 h using the TransIT HEK-293 transfection reagent (Mirus Bio) according to the manufacturer's instructions. Culture medium was changed at 24 h posttransfection and again at 48 h posttransfection. Infection-derived SIVmac239 and SIVmac766 were generated by infecting pooled rhesus macaque CD8-depleted PBMCs from 12 donors with transfection-produced virus 3 days after stimulation with 2.0

μg/ml PHA. Infected cultures were maintained in 100 U/ml IL-2 with a complete medium change 24 h prior to virus harvest. At day 3 posttransfection or day 8 postinfection, virus-containing supernatants were clarified by centrifugation and sterile filtered. A portion of the filtered supernatants was then aliquoted and stored at -80°C for use in infectivity experiments. To purify and concentrate virus for protein analysis, 30 ml of filtered supernatant was centrifuged through a 30% sucrose cushion, pelleted, and washed as previously described (19). The purified virus pellets were then resuspended in 15 μl PBS for a final concentration factor of 2,000×

Virus content. Viral RNA content for each virus stock was determined by running each stock in a quantitative real-time reverse transcriptase PCR (qRT-PCR) assay performed as previously described (20). Each stock was assayed at 3 dilutions, and the means of all 3 dilutions were determined. Viral p27 (CA) content was determined by assaying 8 dilutions of each virus stock in parallel using a commercial SIV p27 antigen capture assay according to the manufacturer's instructions (ABL). At least 3 dilution-corrected p27 values within the linear range of the virus dilution series were averaged to determine the p27 content of each virus stock.

Virus titer. Virus titers were determined on primary rhesus macaque cells as previously described (21). Briefly, CD8-depleted PBMCs from 3 SIV-naïve rhesus macaques were stimulated with plate-bound anti-CD3 (BD) for 3 days, individually plated at 10^5 cells/well in 96-well plates, and infected in triplicate with serial 5-fold dilutions of virus. Cells were washed 3 times with PBS 24 h postinfection to remove residual input virus and were then maintained in 100 U/ml IL-2 for 3 weeks. Cell-free culture supernatants were then collected, and a SIV p27 antigen capture assay was used to detect the presence of viral p27 antigen according to the manufacturer's instructions (ABL). The 50% tissue culture infectious dose (TCID_{50}) was then calculated using the Reed-Muench accumulative method (21). Virus titers were also determined on TZM-bl reporter cells (22–24) by counting the number of β-galactosidase-expressing cells per well in triplicate wells infected with serial 3-fold dilutions of virus as previously described (25). Wells containing dilution-corrected blue cell counts within the linear range of the virus dilution series were averaged to generate an infectious titer in infectious units (IU) per ml. For infectivity thermostability experiments, individual 1-ml aliquots of titered frozen virus generated as described above were removed from -80°C and placed in a 37°C water bath. Vials of virus were left at 37°C for a range of times between <2 min (just long enough to thaw) and 8 h and then immediately diluted in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml Polymyxin B by a dilution factor that yielded 250 IU/0.1 ml for freshly thawed virus, which is within the linear range of the virus infectivity dilution series for all of the viruses used in this study, and then added to TZM-bl cells in triplicate at 0.1 ml per well. β-Galactosidase-expressing cells were then counted 48 h postinfection, as described above.

Protein analysis. Quantitative measurements of viral p27 (CA) and gp120 protein in virions for determinations of gag/env ratios and estimations of Env trimer spikes per virion were determined using a dual-color fluorescent protein gel analysis as previously described (26, 27). Well-characterized reference preparations of infection-derived HIV-1 BAL, HIV-1 NL4-3, and SIVmac239 cultured in human SupT1-CCR5 T lymphoblastoid cells (provided by the Biological Products Core, AIDS and Cancer Virus Program, SAIC-Frederick, Frederick National Laboratory for Cancer Research, Frederick, MD) were included in the analysis as controls.

SGA. For single-genome amplification (SGA), for each sample, at least 200,000 viral RNA copies were extracted using the QIAamp viral RNA minikit (Qiagen). RNA was eluted and immediately subjected to cDNA synthesis. Reverse transcription of RNA to single-stranded cDNA was performed using SuperScript III reverse transcriptase according to the manufacturer's recommendations (Invitrogen). In brief, a cDNA reaction of $1\times$ RT buffer, 0.5 mM each deoxynucleoside triphosphate, 5 mM dithiothreitol, 2 U/ml RNaseOUT (RNase inhibitor), 10 U/ml of Super-

Script III reverse transcriptase, and 0.25 mM antisense primer SIVEnvR1 (5'-TGT AAT AAA TCC CTT CCA GTC CCC CC-3'; or SIV-R-R1 [5'-CAC TAG CTT ACT TCT AAA ATG GCA GC] for near-full-length [NFL] PCR) was incubated at 50°C for 60 min, 55°C for 60 min and then heat inactivated at 70°C for 15 min followed by treatment with 1 U of RNase H at 37°C for 20 min. Env gene or NFL genomes were then amplified via limiting dilution PCR, where only one amplifiable molecule was present in each reaction. Env PCR amplification was performed with $1\times$ PCR buffer, 2 mM MgCl_2 , 0.2 mM each deoxynucleoside triphosphate, 0.2 μM each primer, and 0.025 U/μl Platinum *Taq* polymerase (Invitrogen) in a 20-μl reaction. First-round PCR was performed with sense primer SIVEnvF1 (5'-CCT CCC CCT CCA GGA CTA GC-3') and antisense primer SIVEnvR1 (5'-TGT AAT AAA TCC CTT CCA GTC CCC CC-3') under the following conditions: 1 cycle of 94°C for 2 min and 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 4 min, followed by a final extension of 72°C for 10 min. Next, 1 μl from the first-round PCR product was added to a second-round PCR that included the sense primer SIVEnvF2 (5'-TAT AAT AGA CAT GGA GAC ACC CTT GAG GGA GC-3') and antisense primer SIVEnvR2 (5'-ATG AGA CAT RTC TAT TGC CAA TTT GTA-3') performed under the same conditions used for first-round PCR, but with a total of 45 cycles. NFL PCR was performed similarly, but with unique primers and an increase in extension time from 4 min to 10 min. The NFL first-round PCR primers were SIVU5F1 (5'-AGA AGT AAG CTA GTG TGT GTT CCC ATC TCT) and SIV-R-R1 (5'-CAC TAG CTT ACT TCT AAA ATG GCA GC), while the second-round PCR primers were SIVU5F2 (5'-AGC TAG TGT GTG TTC CCA TCT CTC CTA) and SIV-R-R2 (5'-TAC TTC TAA AAT GGC AGC TTT ATT GAA). Amplicons of the correct size were identified by agarose gel electrophoresis and directly sequenced with second-round PCR primers and SIV-specific primers using BigDye Terminator technology. To confirm PCR amplification from a single template, chromatograms were manually examined for multiple peaks, indicative of the presence of amplicons resulting from PCR-generated recombination events, *Taq* polymerase errors, or multiple variant templates. Sequences were aligned using ClustalW and hand edited using MacClade 4.08 to improve alignment quality. All trees were constructed by the neighbor-joining method.

Roche 454 pyrosequencing. Pyrosequencing of viral stocks was done, essentially, as described previously (28). Viral RNA was isolated using the QIAamp MinElute virus spin kit (Qiagen). RNA was then reverse transcribed and amplified in four overlapping amplicons using SIV-specific primers and the Superscript III one-step RT-PCR system with Platinum high-fidelity *Taq* (Invitrogen). Conditions for the reverse transcription-PCR were as follows: 50°C for 60 min; 94°C for 2 min; 2 cycles of 94°C for 15 s, 60°C for 1 min, and 68°C for 4 min; 2 cycles of 94°C for 15 s, 58°C for 1 min, and 68°C for 4 min; 40 cycles of 94°C for 15 s, 55°C for 1 min, and 68°C for 4 min; and 68°C for 10 min. Each amplicon covered approximately 2.5 kb, and together they spanned the entire viral genome coding sequence. PCR products were gel purified and quantitated using the Qubit double-stranded DNA (dsDNA) HS assay kit (Invitrogen). A total of 12.5 ng of each amplicon was combined to produce 50-ng pools specific to a single viral stock, which were then fragmented using the Nextera DNA sample prep kit (Roche Titanium-compatible; Epicentre) according to the manufacturer's instructions. Following fragmentation, multiplex identifier (MID) tags and adapters for Roche 454 pyrosequencing were added. Products were then cleaned twice with Agencourt AMPure XP beads (Beckman Coulter Genomics). The purified, tagged products were quantitated using the Qubit dsDNA HS assay kit, and their average size was characterized using a high-sensitivity DNA bioanalyzer chip on a 2100 bioanalyzer (Agilent Technologies). Pyrosequencing was performed on a Roche 454 GS Junior instrument according to the manufacturer's protocols (454 Life Sciences).

Roche 454 pyrosequencing data analysis. SFF files from pyrosequencing were imported into Geneious Pro software version 5.5 (Biomatters). Reads were then separated by MID tag and trimmed to remove the Roche 454 adapter, MID, and transposon sequences as well as low-quality se-

TABLE 1 Virus stocks evaluated in this study

Designation	Virus	Lab	Production method	Cell type	Stimulation
251-RD	SIVmac251	R. Desrosiers	Infection	Rhesus PBMCs	PHA
251-CM	SIVmac251	C. Miller	Infection	Rhesus PBMCs	SEA
251-DB	SIVmac251	D. Barouch	Infection	Human PBMCs	ConA
251-LG ^a	SIVmac251	L. Giavedoni	Infection	Rhesus PBMCs	PHA
251-PM ^b	SIVmac251	P. Marx	Infection	CEMx174	
766-BK	SIVmac766	B. Keele	Infection	Rhesus CD8-depleted PBMCs	PHA
766-TFN-BK	SIVmac766	B. Keele	Transfection	293T	
239-CM	SIVmac239	C. Miller	Infection	Rhesus PBMCs	SEA
239-TFN-RD	SIVmac239	R. Desrosiers	Transfection	293T	

^a Included in the Luminex cytokine and chemokine analysis only (Fig. 6).

^b Included in the SGA Env sequencing analysis only (Fig. 2).

sequence (error probability limit of 0.005). SIVmac239 reads were assembled to a GenBank reference (accession no. [M33262](#)), while SIVmac251 and SIVmac766 reads were assembled to their corresponding consensus sequence generated by SGA. Single nucleotide polymorphisms (SNPs) relative to each stock's consensus sequence were found using the SNP calling tool in Geneious Pro by applying the following settings: minimum coverage of 50, minimum variant frequency of 5%, and maximum variant *P* value of 10^{-60} . Amino acid variation present in CD8⁺ T cell epitopes was analyzed using a custom data analysis pipeline, as previously described (29). Briefly, FASTQ files extracted from the original SFF file were trimmed in order to remove MID, adapter, and transposon sequences. Low-quality (quality of <19) sequences were masked with an "N" before alignment. FASTQ sequences were then converted to FASTA format, translated in all six reading frames, and aligned against reference SIVmac239 proteins using the BLAST-Like-Alignment-Tool (BLAT). Sequences aligning to epitopes of interest were extracted from the BLAT output table using custom scripts and analyzed for amino acid substitutions. Only variants present at a frequency of 5% or greater were included in our analysis.

Luminex. Cytokine concentrations in viral preparations were determined by a NHP-specific Luminex assay that detected 36 cytokines, as described previously (30–32). These cytokines included granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), GRO- α , alpha interferon (IFN- α), IFN- γ , interleukin-1 β (IL-1 β), interleukin-1 receptor α (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-18, IL-23, IFN- γ -induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage-derived chemokine (MDC), macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , Perforin, RANTES, soluble CD40 ligand (sCD40L), soluble FAS ligand (sFASL), transforming growth factor α (TGF- α), TGF- β , tumor necrosis factor α (TNF- α), TNF- β , and vascular endothelial growth factor (VEGF).

Nucleotide sequence accession numbers. All 120 new sequences were deposited in GenBank under accession no. [KC522134](#) to [KC522253](#). Reference sequences from previous studies were also included in the analysis (accession no. [JQ085996](#) to [JQ086063](#), [GU998976](#) to [GU999002](#), and [JQ677771](#) to [JQ677809](#)).

RESULTS

To initiate these studies, we first assembled a panel of nine SIVmac251 or -239 lineage virus stocks, comprising seven infection-derived stocks and two transfection-derived stocks, generated in six different laboratories (Table 1). Five of the infection-derived stocks contained infected culture expansions of the viral swarm SIVmac251 (33); three of these stocks (251-RD, 251-CM, and 251-LG) were produced in rhesus PBMCs under various target cell stimulation and culture conditions, one was produced in activated human PBMCs (251-DB), and one was produced in the CEMx174 (17) cell line (251-PM). The remaining four stocks

comprised paired rhesus PBMC infection-derived and 293T transfection-derived stocks of two molecularly derived viral clones, SIVmac239 (15) and the transmitted/founder clone SIVmac766 derived from a SIVmac251-infected rhesus macaque.

Virus content. To assess the virion contents of stocks produced via different methods in different laboratories, we first determined the viral RNA and p27 CA protein content in seven of the virus stocks (Fig. 1A). All of the virus stocks evaluated contained between 5.67×10^8 and 1.3×10^{10} RNA copies/ml and between 50 and 876 ng p27/ml, with good agreement between the two measurements that were consistent with previous results indicating roughly 10^{10} viral particles per μ g of CA protein for other retroviruses (34). The two 293T transfection-derived virus stocks (766-TFN-BK and 239-TFN-RD) contained the highest virion content at $\sim 10^{10}$ RNA copies/ml and >850 ng p27/ml, whereas the infection-derived stocks ranged between 5.67×10^8 and 7.60×10^9 RNA copies/ml and 50 to 585 ng p27/ml. We next evaluated infectivity by determining the titers of the stocks in parallel on the TZM-bl reporter cell line (22–24) (Fig. 1B) or on CD8-depleted PBMCs from 3 rhesus macaque donors (Fig. 1C). Although the relative differences in titers between stocks were similar for a given PBMC donor animal, there were apparent donor-dependent effects on the determined 50% tissue culture infective dose (TCID₅₀) values, with 10-fold or greater differences in calculated TCID₅₀ between two PBMC donors noted for 4 of the 7 evaluated stocks (Fig. 1C). For all 7 virus stocks, the determined TZM-bl titer was within 6-fold of the mean of the titers determined on primary cells (Fig. 1B and C).

The two 293T transfection-derived SIV stocks had the highest infectivity in both TZM-bl and rhesus PBMCs (Fig. 1B and C), with titers of 2.4×10^6 IU/ml and 7.5×10^5 IU/ml on TZM-bl for 766-TFN-BK and 239-TFN-BK, respectively. We utilized the TZM-bl and mean primary cell titers to calculate the specific infectivity for each stock to determine if there were any differences in the infectivity of viruses generated by different means on a per virion basis. As shown in Fig. 1D, the specific infectivities for each of the seven stocks were similar, ranging between 2.2×10^{-5} and 4.2×10^{-4} IU/RNA copy, with the two transfection-produced stocks at the higher end of this range, suggesting that quantitative virion content rather than qualitative differences in the viruses is largely responsible for differences in the *in vitro* infectious titer.

Sequence diversity and analysis. Virus stocks generated by productive infection of permissive cells are likely to contain diverse viral sequences due to the accumulation of mutations generated by multiple rounds of viral reverse transcription; however,

FIG 1 Virus content of SIV *in vivo* challenge stocks. (A) Quantification of viral RNA copy number by qRT-PCR and p27^{CA} protein content by antigen capture enzyme-linked immunosorbent assay (ELISA). RNA copy data represent the mean values for each sample assayed in duplicate at 3 different dilutions within the linear range of the dilution series. Eq, equivalents. The p27 data represent mean values for each sample at 3 or more dilutions within the linear range of the dilution series. (B) Stock infectivity titers as determined on TZM-bl reporter cells expressed as dilution normalized infectious units (IU) per milliliter. Titers represent mean values for all dilution-normalized titers that fell within the linear range of the dilution series. (C) Stock infectivity titers as determined on CD8-depleted rhesus PBMCs from 3 donors expressed as 50% tissue culture infectious dose (TCID₅₀). Mean titers for all 3 donors are also shown. TCID₅₀ values were determined by the Reed-Muench accumulative method. (D) Specific infectivity of each stock based on TZM-bl or mean primary cell titer and viral RNA copy equivalents.

different stocks of the same nominal virus might contain different amounts of viral diversity and different specific viral sequences. We therefore performed single-genome amplification (SGA) sequencing analysis of the full-length Env gene or utilized published sequences of four different SIVmac251 stocks (251-RD, 251-DB, 251-CM, and 251-PM). Sequencing analysis revealed different degrees of Env diversity within each stock, with as much as 2.1% maximum sequence diversity in 251-RD and as little as 0.9% maximum sequence diversity in 251-DB (Fig. 2). There was also clear evidence for selection and expansion of unique Env sequences within each of the SIVmac251 stocks, with all of the Env sequences from 251-DB, 251-CM, and 251-PM clustered on separate branches of the phylogenetic tree with no overlap, suggesting that the predominant sequences within each SIVmac251 stock are distinct from those present in the other SIVmac251 stocks. We note that the Env sequences in two other stocks of SIVmac251, a 1991 progenitor stock (6) and a 2006 reexpansion of the 1991 stock (35), produced in the same lab as 251-RD, contained similar maximum diversity and phylogenetic tree structure, as shown here for 251-RD (not shown).

Differences between stocks in overall sequence diversity were

FIG 2 SIVmac251 stock Env phylogeny. Unrooted neighbor-joining tree constructed from SGA-derived full-length Env sequences from the indicated SIVmac251 stocks, as well as the infectious molecular clones SIVmac239 and SIVmac766. Branch colors indicate the stock from which each sequence was derived. Percentages in parentheses indicate the maximum genetic diversity of Env sequences within each stock.

FIG 3 SIVmac251 stock whole-genome sequence diversity. Highlighter plots (www.hiv.lanl.gov) derived from SGA sequences (top) and polymorphism frequency plots from 454 deep sequencing (bottom) indicating point mutations relative to the stock consensus sequence across the entire viral genome are shown for 251-RD (A), 251-CM (B), and 251-DB (C). 454 deep sequencing polymorphisms detected in fewer than 5% of sequences are not shown. Numbers below highlighter plots denote the genomic nucleotide number for both SGA and 454 sequences. Corresponding genomic regions are represented below the polymorphism frequency plots. LTR, long terminal repeat.

also noted when we expanded our sequencing analysis to full-length viral genomes using both SGA sequencing analysis (Fig. 3, top panels) as well as 454 pyrosequencing (Fig. 3, bottom panels). In agreement with the results shown for Env sequences in Fig. 2, 251-RD (Fig. 3A) contained greater sequence diversity than 251-CM (Fig. 3B) or 251-DB (Fig. 3C) throughout the viral genome, with 1.4%, 0.9%, and 0.5% maximum sequence diversities for the three stocks, respectively. Evidence of selection and expansion for unique sequences within each infection-derived virus stock was also observed in the full-length viral sequences. To evaluate the potential functional impact of different virus genetic contents within the different SIVmac251 stocks, we examined the preexisting frequency of several well-characterized CD8⁺ T cell escape mutations in four different epitopes within 251-RD, 251-CM, and 251-DB (Table 2). Marked differences were noted between the stocks, with 251-RD containing low but detectable levels (<50%) of preexisting escape mutations in all four of the examined epitopes, whereas >80% of sequences within 251-CM contained preexisting escape mutations to the two Mamu B*17 major histocompatibility complex (MHC)-restricted epitopes, while <5% contained escape mutations to the Mamu A*01 and Mamu B*08 restricted epitopes. In contrast to 251-RD, 251-DB contained <5% escape mutations to 3 of the 4 examined epitopes, but did contain a detectable preexisting escape variant within the Env₈₃₂₋₈₄₀ FW9 epitope.

To evaluate genetic differences between infection- and transfection-derived virus stocks as well as to assess the amount of diversity generated by infection-based production of a molecularly cloned virus, we compared the SGA-generated Env sequence for 239-TFN-RD with that of 239-CM (Fig. 4A) and the SGA-generated Env sequence for 766-TFN-BK with that of 766-BK (Fig. 4B). As expected, the two transfection-derived stocks contained clonal virus, with very rare point mutations identified, consistent with random error introduced during *in vitro* reverse transcription for sequence analysis. The two matched infection-derived stocks contained limited amounts of viral diversity, with the majority of Env sequences identical to each other and a small number of sequences containing between 1 and 5 random point mutations (Fig. 4).

Virion Env content. Because the SIV envelope (Env) glycoprotein is not required for the production of particles with an intact lipid bilayer envelope, there could be substantial producer cell-dependent variability in Env incorporation per virion. We therefore comparatively assessed the protein contents of matched infection- and transfection-derived virions. For this experiment, new SIVmac239 and SIVmac766 stocks were generated to accommodate the large amounts of concentrated viral material required for Env assessment. The stocks generated for these analyses had infectivity titers of between 3×10^5 and 7×10^5 IU/ml on Tzm-bl cells, with the transfection-derived stocks demonstrating 2- to

TABLE 2 Variation in epitopes restricted by common MHC I alleles^a

Epitope	MHC restriction	Amino acid sequence ^b	% frequency of epitope in ^c :		
			251-RD	251-CM	251-DB
Tat _{28–35} TL8	Mamu A*01	TTPE\$ANL S-----	29.7 (47)	—	—
Vif _{172–179} RL8	Mamu B*08	RRDNRRGL ---H----	7.6 (0)	—	—
Env _{832–840} FW9	Mamu B*17	FQEAVQAGW -H----- -----V--	31.1 (27) 11.1 (33)	89.6 (82) —	45.0 (25) —
Nef _{195–203} MW9	Mamu B*17	MQPAQTSKW -H-----Q- -H----AQ-	9.0 (13) —	6.1 (9) 82.3 (82)	— —

^a Only nonsynonymous mutations detected at a frequency of >5% by 454 deep sequencing are shown.
^b Wild-type amino acid sequence derived from the 251-RD majority consensus.
^c Values in parentheses represent the frequency detected by SGA sequencing. —, absence of variant (<5% frequency).

3-fold-higher overall titers (data not shown), consistent with our findings for the *in vivo* challenge stocks produced in other laboratories (Fig. 1). These new viruses, produced in infected CD8-depleted rhesus PBMCs or in transfected 293T cells were purified, concentrated 2,000×, and examined by SDS-PAGE using a dual-color fluorescent imaging method (Fig. 5A) (26, 27). Virion p27^{CA} and gp120^{SU} contents were quantitated using linear regression analysis by generating densitometry standard curves with dilution series of purified p27^{CA} and gp120^{SU} of known concentration (Fig. 5A and B). Total protein was stained using SYPRO Ruby, allowing for determination of p27^{CA} content, and glycoprotein was stained with SYPRO Pro-Q Emerald, allowing for determination of gp120^{SU} content. As shown in Fig. 5A, the 293T-generated stocks contained markedly lower levels of p27^{CA}-normalized

gp120 than the matched infection-derived stocks. Using these data, we were able to calculate the molar Gag/Env ratio for each virus preparation and, based on an estimated 1,400 gag molecules per particle (26, 36), estimate a mean number of trimer spikes per virion. Each infected-PBMC-generated stock contained ~13- to 15-fold more Env trimers than the matched transfection-generated stocks, with SIVmac239- and SIVmac766-producing PBMCs containing 9 and 6 trimers per virion, respectively, while the two transfection-derived stocks contained less than 1 trimer per virion on average. In agreement with previously published results for HIV-1 virus stocks (27), these results indicate that transfected-293T-cell-produced SIV stocks contain fewer Env trimer spikes per virion than infection-produced stocks.

Thermostability of infectivity. Following *in vivo* inoculation,

FIG 4 Env sequence diversity of transfection- and infection-derived molecular clone viruses. Shown are highlighter plots of SGA-derived Env sequences indicating point mutations relative to the stock consensus sequence for matched pairs of transfection- and infection-derived stocks of SIVmac239 (A) and SIVmac766 (B). Numbers below highlighter plots indicate the Env gene nucleotide number.

FIG 5 Virion-associated Env content analysis. (A) SDS-PAGE gel stained with two SYPRO dyes to detect and quantitate glycoprotein (green) and total protein (red). Purified SIVmac239 and SIVmac766 stocks generated in infected PBMCs or transfected 293T cells were lysed and run on the same gel with well-characterized reference viruses and serial dilutions of quantified, purified gp120 and p27 for the establishment of standard curves for densitometry analysis. M.M. Stds, molecular mass standards (kDa). (B) Densitometry standard curves established using the p27^{CA} standards (red) and the gp120 standards (green) in panel A for linear regression analysis of the p27 and gp120 contents of the purified SIV stocks.

particularly for low-dose mucosal challenges, some time may elapse before a virion finds a suitable target cell to initiate infection. Although transfection-derived SIV stocks were found to have equivalent or better specific infectivity *in vitro* than infection-derived stocks, despite smaller amounts of virion-incorporated Env (Fig. 1D), we sought to determine if infection- and transfection-based production methods lead to differences in the thermostability of infectivity not captured by standard titer determination assays. To address this question, we evaluated the stability of infectivity at 37°C for 1× infectious stocks of SIVmac239 and SIVmac766 harvested from the same culture supernatants used for protein analysis (Fig. 6). Individual 1-ml aliquots of frozen virus-containing supernatant were placed at 37°C for various amounts of time prior to being added to TZM-bl cells. Although some small differences were noted in the thermostability of the virus stocks, with rhesus PBMC-produced SIVmac239 showing the greatest reduction in infectivity after 8 h at 37°C, there were no consistent differences between the infection- and transfection-produced viruses, and all of the stocks maintained 60% or greater infectivity, with 3 of 4 stocks maintaining >80% of infectivity after 8 h at 37°C.

Cytokine and chemokine content. In addition to virion-incorporated proteins, nonviral soluble factors present in virus stocks could potentially modulate initial infection events. In particular, the presence of cytokines and chemokines could potentially impact the recruitment, identity, and activation state of early cellular targets. We examined the cytokine and chemokine contents of both infection- and transfection-derived stocks using a 36-plex Luminex assay with cross-reactivity for human and rhesus

analytes (30–32). As shown in Fig. 7, the infection-derived SIV stocks contained a markedly greater abundance and variety of cytokines and chemokines than did the two transfection-derived stocks (766-TFN-BK, 239-TFN-RD). Although the infection-derived stocks contained consistently higher levels of cytokines and chemokines, there was a large degree of variability in the specific

FIG 6 Thermostability of infectivity. Shown are the percentages of infectivity of SIVmac239 and SIVmac766 stocks generated in infected PBMCs or transfected 293T cells measured on TZM-bl cells after incubation at 37°C for the indicated amounts of time. The percentage of infection for each virus was normalized to 100% for freshly thawed virus calculated by determining the mean number of infected cells in triplicate wells. The values shown are means of two independent experiments.

FIG 7 Cytokine/chemokine content of SIV *in vivo* challenge stocks. A heat map shows the results of a quantitative 36-plex Luminex assay with cross-reactivity for human and rhesus macaque analytes. A clustergram shows hierarchical clustering of virus stocks based on similarities in cytokine/chemokine content.

content of each stock. While the hierarchical clustering of 239-CM and 251-CM, two different stocks generated in the same lab, suggest that cell type, stimulation, and culturing techniques may lead to some degree of predictability in the cytokine and chemokine content of a stock, there were still substantial differences between these two stocks, including ~73-fold-greater levels of IL-10 and ~2-fold-greater levels of IFN-γ in 251-CM (Fig. 7; see Table S1 in the supplemental material).

In the transfection-derived virus stocks, only TGF-β and VEGF were present at >100 pg/ml, with 988 and 660 pg/ml TGF-β and 2,124 and 4,225 pg/ml VEGF detected in 766-TFN-BK and 239-TFN-RD, respectively (Fig. 7; see Table S1 in the supplemental material). All other cytokines and chemokines were at relatively low or undetectable levels in these two stocks. In contrast, each infection-derived stock contained at least 9 analytes at >100 pg/ml and at least 1 analyte at >1,000 pg/ml, with 5 of 6 infection-derived stocks containing 3 or more analytes at >1,000 pg/ml. Perforin was present at >1,000 pg/ml in all of the infection-derived stocks, except 251-RD, with 251-DB containing the highest concentration at 12,444 pg/ml. Several other analytes were detected at notably high levels, including CD14 at 106,376 pg/ml in 766-BK, IL-2 at 18,905 pg/ml in 251-DB, and MCP-1 at 44,699 pg/ml in 251-RD. 251-CM also contained markedly high levels of IFN-γ (7,272 pg/ml) and IL-10 (11,752 pg/ml).

DISCUSSION

Well-characterized virus stocks of known identity are critical for the design and interpretation of *in vivo* SIV infection studies conducted in nonhuman primates. Historically, however, characterization of SIV challenge stocks has been limited, despite the potential for substantial stock-to-stock variability in both viral and nonviral features. While uncloned viral swarms are produced by infection of permissive cells in culture, infectious molecular clones are produced by transfection of an irrelevant, suitable cell type with a viral plasmid containing a full-length DNA viral genome, with or without subsequent expansion by infection of permissive cells. Differences between infection- and transfection-

based production systems have the potential to markedly influence the character of the virus stock produced, and indeed there is a perception in the field that infection-derived virus stocks may be more infectious *in vivo*, particularly for mucosal challenges. Ample variability also exists in infection-based production methods, including differences in seed virus culture history, cellular substrate, cell stimulation method, culture duration, harvest procedure, and viral evolution in culture, all of which have the capacity to considerably alter the properties of the generated virus stock (Table 1 and Materials and Methods). Given questions about potential differences between different SIV challenge stocks, we comparatively characterized viral and nonviral parameters for a panel of SIVmac239 or -251 lineage stocks representative of viral swarms and infectious molecular clones produced in several different cell types by infection- and transfection-based methods (Table 1).

Although the titers of individual SIV challenge stocks are typically determined *in vitro* prior to use in an *in vivo* NHP study, we first determined how the various stocks compared head to head by performing parallel evaluations of viral parameters, including virion content, infectivity on different target cells, specific infectivity, and Env incorporation. Intriguingly, the two transfection-produced virus stocks contained the highest virion content and the highest overall stock infectivity in both TZM-bl and primary rhesus PBMC target cells (Fig. 1). When titers of viruses were determined in the same assay in parallel, the relative titers between stocks were similar, regardless of the target cell type or PBMC donor. However, for several viruses, the determined titer differed by >10-fold from one PBMC donor to the next, suggesting that the common practice of reporting *in vitro* titers for *in vivo* viral inoculum size may be relatively uninformative and that caution should be exercised when comparing infectivity titers derived in different labs on different cell types or on cells from different donors. One potential solution to this confounding variable would be for the field to report infectivity titers using a common cell-line-based assay, such as the TZM-bl assay used here, which provided a consistent and predictive value for *in vitro* infectivity.

In agreement with previous findings for HIV-1 (27), our quantitative Gag:Env analysis revealed markedly lower levels of gp120 in purified SIV virions produced in transfected 293T cells than in virions produced in infected PBMCs (Fig. 5), although a study examining transfection-produced SIV prepared and analyzed using different methods found higher total numbers of Env trimers (37). Given the minimal shedding of gp120 from virions (38), this result likely reflects less efficient Env incorporation into virions produced in transfected 293T cells than in infected T cells. Despite these substantially lower levels of virion-incorporated Env, transfection-produced SIVs surprisingly had particle/infectivity ratios similar to or higher than those produced in infected PBMCs (Fig. 1D). While this may demonstrate that only a limited number of Env spikes are required to drive fusion of the viral and cellular membranes (39), we note that the Gag:Env results presented here only reveal mean per particle Env levels and do not describe the distribution of Env spikes across virions, which may influence infectivity based on the stoichiometry of Env-CD4-coreceptor interactions and membrane fusion (40, 41). In addition, although these data may indicate that higher levels of virion-associated Env do not confer any measurable increase in viral infectivity for SIV as measured by *in vitro* assays, Env-dependent increases in infectivity for infection-derived viruses may be counterbalanced by the accumulation of deleterious mutations introduced during viral replication.

Reduced Env levels for transfection-produced viruses may have implications for studies assessing antibody-mediated reductions in virus acquisition (27); however, the SIVmac239 or -251 lineage viruses assessed here have been shown previously to be highly neutralization resistant (6, 42) and thus may be less susceptible to this effect than more neutralization-sensitive viral lineages. Apart from neutralization sensitivity, it is not clear if reduced Env content might also influence overall *in vivo* infectivity titer in a way that is not captured by standard *in vitro* titer determination assays. Once applied to a mucosal surface, individual virions may be subjected to physiological temperatures for several hours before encountering permissive target cells. We therefore also compared the stabilities of infectivity at 37°C for infection- and transfection-derived viruses, but again found that lower levels of virion-associated Env were not associated with any measurable decrease in the thermostability of infectivity (Fig. 6). It nevertheless remains possible that other factors at the *in vivo* site of infection, including differences in pH and the presence of proteases, could differentially impact high- and low-Env-containing virions.

For some SIV NHP studies, the use of a diverse viral swarm, such as one of the infection-derived SIVmac251 stocks examined in this study, is favored because it allows for enumeration of the transmitted and founder viral variants that initiate systemic infection (4, 9, 11, 43). By challenging animals with a viral dose that results in transmission of one or only a few variants, researchers are able to more closely model human sexual transmission of HIV-1 (44, 45). However, using SGA sequencing and 454 deep sequencing, we show in Fig. 2, 3, and 4 that for multiple stocks designated “SIVmac251,” the amount of sequence diversity and the specific sequences within each stock differ. This finding has implications for limiting dose challenge studies where a small number of viral variants are transmitted, as the available pool of viral variants from which one or few can be selected may be quite different in identity and extent of diversity from one stock to the next, despite the stocks having the same name. The genetic dis-

inction between stocks could potentially lead to different study outcomes, depending on which virus stock is used, particularly for studies where selection for or against a specific viral variant or mutation is important. This is illustrated by the presence of pre-existing variants within several known CD8⁺ T cell epitopes in the SIVmac251 stocks (Table 2). In addition to influencing the reactivity of specific CD8⁺ T cell responses, vaccines that elicit CD8⁺ T cell responses against a specific viral epitope may be less effective against SIVmac251 stocks that harbor preexisting epitope variants. In order to avoid the potential confounding effects of unpredictable viral swarm constituents while maintaining the ability to enumerate transmitted viral variants by sequence analysis, we are developing a “synthetic viral swarm” containing a defined number of genetically tagged but otherwise isogenic and phenotypically identical clones (unpublished data).

In addition to viral parameters, we also evaluated nonviral constituents of each virus stock. We focused on cytokines and chemokines as measurable analytes with the potential to influence early infection events and *in vivo* viral infectivity by modulating target cell identity, recruitment, and activation state. As expected, the infection-produced virus stocks, grown in cultures of activated PBMCs, contained higher levels and greater numbers of cytokines and chemokines than did the transfection-produced stocks. Although further studies are required to determine what effect, if any, increased cytokine and chemokine content has on early *in vivo* infection events, one might speculate that elevated levels of proinflammatory cytokines and chemokines could result in a non-virally induced local immune activation and enhanced *in vivo* viral infectivity. Although the amounts and identities of the cytokine and chemokine present in the inoculum vary significantly, it is unclear whether any of these stock constituents are physiologically relevant for sexual virus transmission. A study conducted by Politch and coworkers (46) found that the semen of healthy, fertile men contains elevated levels of several cytokines; however, the specific constituents were substantially different from those we detected in the SIV challenge stocks. Although it is not clear how the cytokine and chemokine content of other genital secretions or from the semen of HIV-1-infected individuals might compare with those present in the challenge stocks, it is clear that understanding what else besides virus is present in any given challenge stock is important for more accurately modeling sexual HIV-1 transmission.

With an emphasis on production methods as determinants of stock characteristics that may be relevant for *in vivo* NHP challenge studies, these studies highlight key distinctions both between infection- and transfection-derived virus stocks and between different infection-produced stocks of the same nominal virus. Each infection-derived stock contains a distinct combination of viral sequences and nonviral constituents, whereas transfection-derived stocks, while genetically consistent and containing lower levels of cytokines and chemokines, also contain lower levels of virion-associated Env. While it is tempting to consider how individual virus stock features described here might correlate with the reported *in vivo* infectivity of the stocks, well-controlled, head-to-head *in vivo* studies that carefully examine one stock variable at a time using the same *in vivo* titration protocol will be required to address this important question. In addition, although this study is to our knowledge the most extensive of its kind, it is important to note that our analyses were not comprehensive and there are other possible virus stock characteristics, including dif-

ferential Env glycosylation profiles (47), stock endotoxin levels, the incorporation of cellular adhesion molecules, and microRNA content, that we did not assess here, but which could potentially influence viral infectivity and early infection events *in vivo*. Nevertheless, our findings highlight a number of underappreciated features of SIV challenge stocks and indicate differences between production methods that may be relevant for *in vivo* infectivity and the dynamics of early infection. These findings underscore the importance using well-defined virus stocks and argue for detailed characterization of SIV challenge stocks in the future.

ACKNOWLEDGMENTS

We thank Julian Bess for technical assistance with purifying virus from infected culture supernatants and Lindsey Galmin for assistance with propagating virus in culture. We also thank Max Hooper and Shelby O'Connor for assistance with identifying escape mutation frequencies in MHC class I-restricted epitopes.

This work was supported in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E, and in part by NIH grants OD013096 to L.D.G., AI077376 and AI08747 to D.H.O., and AI096040, AI095985, and AI078526 to D.H.B. The National Center for Research Resources and the Office of Research Infrastructure Programs (ORIP) of the NIH supported this study through grants P51 RR00164-50, P51 RR000167, and P51 OD011133. Additionally, some of this work was conducted in a facility constructed with support from Research Facilities Improvement Program grant no. RR15459-01 and RR020141-01.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

- Ambrose Z, KewalRamani VN, Bieniasz PD, Hatzioannou T. 2007. HIV/AIDS: in search of an animal model. *Trends Biotechnol.* 25:333–337.
- Hirsch VM, Lifson JD. 2000. Simian immunodeficiency virus infection of monkeys as a model system for the study of AIDS pathogenesis, treatment, and prevention. *Adv. Pharmacol.* 49:437–477.
- Arthur LO, Bess JW, Jr, Urban RG, Strominger JL, Morton WR, Mann DL, Henderson LE, Benveniste RE. 1995. Macaques immunized with HLA-DR are protected from challenge with simian immunodeficiency virus. *J. Virol.* 69:3117–3124.
- Keele BF, Li H, Learn GH, Hraber P, Giorgi EE, Grayson T, Sun C, Chen Y, Yeh WW, Letvin NL, Mascola JR, Nabel GJ, Haynes BF, Bhattacharya T, Perelson AS, Korber BT, Hahn BH, Shaw GM. 2009. Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1. *J. Exp. Med.* 206:1117–1134.
- Strickland SL, Gray RR, Lamers SL, Burdo TH, Huenink E, Nolan DJ, Nowlin B, Alvarez X, Midkiff CC, Goodenow MM, Williams K, Salemi M. 2011. Significant genetic heterogeneity of the SIVmac251 viral swarm derived from different sources. *AIDS Res. Hum. Retroviruses* 27:1327–1332.
- Bixby JG, Laur O, Johnson WE, Desrosiers RC. 2010. Diversity of envelope genes from an uncloned stock of SIVmac251. *AIDS Res. Hum. Retroviruses* 26:1115–1131.
- Lifson JD, Haigwood NL. 2012. Lessons in nonhuman primate models for AIDS vaccine research: from minefields to milestones. *Cold Spring Harb. Perspect. Med.* 2:a007310. doi:10.1101/cshperspect.a007310.
- Lewis MG, Bellah S, McKinnon K, Yalley-Ogunro J, Zack PM, Elkins WR, Desrosiers RC, Eddy GA. 1994. Titration and characterization of two rhesus-derived SIVmac challenge stocks. *AIDS Res. Hum. Retroviruses* 10:213–220.
- Ma ZM, Keele BF, Qureshi H, Stone M, Desilva V, Fritts L, Lifson JD, Miller CJ. 2011. SIVmac251 is inefficiently transmitted to rhesus macaques by penile inoculation with a single SIVenv variant found in ramp-up phase plasma. *AIDS Res. Hum. Retroviruses* 27:1259–1269.
- Marthas ML, Lu D, Penedo MC, Hendrickx AG, Miller CJ. 2001. Titration of an SIVmac251 stock by vaginal inoculation of Indian and Chinese origin rhesus macaques: transmission efficiency, viral loads, and antibody responses. *AIDS Res. Hum. Retroviruses* 17:1455–1466.
- Liu J, Keele BF, Li H, Keating S, Norris PJ, Carville A, Mansfield KG, Tomaras GD, Haynes BF, Kolodkin-Gal D, Letvin NL, Hahn BH, Shaw GM, Barouch DH. 2010. Low-dose mucosal simian immunodeficiency virus infection restricts early replication kinetics and transmitted virus variants in rhesus monkeys. *J. Virol.* 84:10406–10412.
- Keckler MS, Hodara VL, Parodi LM, Gavedoni LD. 2007. Novel application of nonhuman primate tethering system for evaluation of acute phase SIVmac251 infection in rhesus macaques (*Macaca mulatta*). *Viral Immunol.* 20:623–634.
- Traina-Dorge V, Pahar B, Marx P, Kissinger P, Montefiori D, Ou Y, Gray WL. 2010. Recombinant varicella vaccines induce neutralizing antibodies and cellular immune responses to SIV and reduce viral loads in immunized rhesus macaques. *Vaccine* 28:6483–6490.
- Heberling RL, Kalter SS, Marx PA, Lowry JK, Rodriguez AR. 1988. Dot immunobinding assay compared with enzyme-linked immunosorbent assay for rapid and specific detection of retrovirus antibody induced by human or simian acquired immunodeficiency syndrome. *J. Clin. Microbiol.* 26:765–767.
- Daniel MD, Letvin NL, King NW, Kannagi M, Sehgal PK, Hunt RD, Kanki PJ, Essex M, Desrosiers RC. 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* 228:1201–1204.
- Gazdar AF, Carney DN, Bunn PA, Russell EK, Jaffe ES, Schechter GP, Guccion JG. 1980. Mitogen requirements for the *in vitro* propagation of cutaneous T-cell lymphomas. *Blood* 55:409–417.
- Salter RD, Howell DN, Cresswell P. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* 21:235–246.
- Stone M, Ma ZM, Genesca M, Fritts L, Blozis S, McChesney MB, Miller CJ. 2009. Limited dissemination of pathogenic SIV after vaginal challenge of rhesus monkeys immunized with a live, attenuated lentivirus. *Virology* 392:260–270.
- Del Prete GQ, Kearney MF, Spindler J, Wiegand A, Chertova E, Roser JD, Estes JD, Hao XP, Trubey CM, Lara A, Lee K, Chaipan C, Bess JW, Jr, Nagashima K, Keele BF, Macallister R, Smedley J, Pathak VK, Kewalramani VN, Coffin JM, Lifson JD. 2012. Restricted replication of xenotropic murine leukemia virus-related virus in pigtailed macaques. *J. Virol.* 86:3152–3166.
- Cline AN, Bess JW, Piatak M, Jr, Lifson JD. 2005. Highly sensitive SIV plasma viral load assay: practical considerations, realistic performance expectations, and application to reverse engineering of vaccines for AIDS. *J. Med. Primatol.* 34:303–312.
- Koup RA, Ho DD, Poli G, Fauci AS. 2001. Isolation and quantitation of HIV in peripheral blood. *Curr. Protoc. Immunol.* 5:12.2.1–12.2.11.
- Derdeyn CA, Decker JM, Sfakianos JN, Wu X, O'Brien WA, Ratner L, Kappes JC, Shaw GM, Hunter E. 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol.* 74:8358–8367.
- Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophage-tropic isolates of human immunodeficiency virus type 1. *J. Virol.* 72:2855–2864.
- Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* 46:1896–1905.
- Morcock DR, Thomas JA, Sowder RC, II, Henderson LE, Crise BJ, Gorelick RJ. 2008. HIV-1 inactivation by 4-vinylpyridine is enhanced by dissociating Zn(2+) from nucleocapsid protein. *Virology* 375:148–158.
- Chertova E, Chertov O, Coren LV, Roser JD, Trubey CM, Bess JW, Jr, Sowder RC, II, Barsov E, Hood BL, Fisher RJ, Nagashima K, Conrads TP, Veenstra TD, Lifson JD, Ott DE. 2006. Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. *J. Virol.* 80:9039–9052.
- Louder MK, Sambor A, Chertova E, Hunte T, Barrett S, Ojong F, Sanders-Buell E, Zolla-Pazner S, McCutchan FE, Roser JD, Gabuzda D, Lifson JD, Mascola JR. 2005. HIV-1 envelope pseudotyped viral vectors and infectious molecular clones expressing the same envelope glycoprotein have a similar neutralization phenotype, but culture in peripheral blood mononuclear cells is associated with decreased neutralization sensitivity. *Virology* 339:226–238.

28. Bimber BN, Dudley DM, Lauck M, Becker EA, Chin EN, Lank SM, Grunenwald HL, Caruccio NC, Maffitt M, Wilson NA, Reed JS, Sosman JM, Tarosso LF, Sanabani S, Kallas EG, Hughes AL, O'Connor DH. 2010. Whole-genome characterization of human and simian immunodeficiency virus intra-host diversity by ultra-deep pyrosequencing. *J. Virol.* 84:12087–12092.
29. O'Connor SL, Becker EA, Weinfurter JT, Chin EN, Budde ML, Gostick E, Correll M, Gleicher M, Hughes AL, Price DA, Friedrich TC, O'Connor DH. 2012. Conditional CD8⁺ T cell escape during acute simian immunodeficiency virus infection. *J. Virol.* 86:605–609.
30. Giavedoni LD. 2005. Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using Luminex technology. *J. Immunol. Methods* 301:89–101.
31. Jacquelin B, Mayau V, Targat B, Liovat AS, Kunkel D, Petitjean G, Dillies MA, Roques P, Butor C, Silvestri G, Giavedoni LD, Lebon P, Barre-Sinoussi F, Benecke A, Muller-Trutwin MC. 2009. Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. *J. Clin. Invest.* 119:3544–3555.
32. Sariol CA, Martinez MI, Rivera F, Rodriguez IV, Pantoja P, Abel K, Arana T, Giavedoni L, Hodara V, White LJ, Anglero YI, Montaner LJ, Kraiselburd EN. 2011. Decreased dengue replication and an increased anti-viral humoral response with the use of combined Toll-like receptor 3 and 7/8 agonists in macaques. *PLoS One* 6:e19323. doi:10.1371/journal.pone.0019323.
33. Naidu YM, Kestler HW, III, Li Y, Butler CV, Silva DP, Schmidt DK, Troup CD, Sehgal PK, Sonigo P, Daniel MD, Desrosiers RC. 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. *J. Virol.* 62:4691–4696.
34. Piatak M, Jr, Saag MS, Yang LC, Clark SJ, Kappes JC, Luk KC, Hahn BH, Shaw GM, Lifson JD. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259:1749–1754.
35. Xiao P, Patterson LJ, Kuete S, Brocca-Cofano E, Thomas MA, Venzon D, Zhao J, DiPasquale J, Fenizia C, Lee EM, Kalisz I, Kalyanaraman VS, Pal R, Montefiori D, Keele BF, Robert-Guroff M. 2012. Replicating adenovirus-simian immunodeficiency virus (SIV) recombinant priming and envelope protein boosting elicits localized, mucosal IgA immunity in rhesus macaques correlated with delayed acquisition following a repeated low-dose rectal SIV(mac251) challenge. *J. Virol.* 86:4644–4657.
36. Zhu P, Chertova E, Bess J, Jr, Lifson JD, Arthur LO, Liu J, Taylor KA, Roux KH. 2003. Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc. Natl. Acad. Sci. U. S. A.* 100:15812–15817.
37. Yuste E, Reeves JD, Doms RW, Desrosiers RC. 2004. Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. *J. Virol.* 78:6775–6785.
38. Chertova E, Bess JW, Jr, Crise BJ, Sowder IR, Schaden TM, Hilburn JM, Hoxie JA, Benveniste RE, Lifson JD, Henderson LE, Arthur LO. 2002. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol.* 76:5315–5325.
39. Yang X, Kurteva S, Ren X, Lee S, Sodroski J. 2005. Stoichiometry of envelope glycoprotein trimers in the entry of human immunodeficiency virus type 1. *J. Virol.* 79:12132–12147.
40. Magnus C, Rusert P, Bonhoeffer S, Trkola A, Regoes RR. 2009. Estimating the stoichiometry of human immunodeficiency virus entry. *J. Virol.* 83:1523–1531.
41. Klasse PJ. 2007. Modeling how many envelope glycoprotein trimers per virion participate in human immunodeficiency virus infectivity and its neutralization by antibody. *Virology* 369:245–262.
42. Johnson WE, Sanford H, Schwall L, Burton DR, Parren PW, Robinson JE, Desrosiers RC. 2003. Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. *J. Virol.* 77:9993–10003.
43. Stone M, Keele BF, Ma ZM, Bailes E, Dutra J, Hahn BH, Shaw GM, Miller CJ. 2010. A limited number of simian immunodeficiency virus (SIV) env variants are transmitted to rhesus macaques vaginally inoculated with SIVmac251. *J. Virol.* 84:7083–7095.
44. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM. 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc. Natl. Acad. Sci. U. S. A.* 105:7552–7557.
45. Li H, Bar KJ, Wang S, Decker JM, Chen Y, Sun C, Salazar-Gonzalez JF, Salazar MG, Learn GH, Morgan CJ, Schumacher JE, Hraber P, Giorgi EE, Bhattacharya T, Korber BT, Perelson AS, Eron JJ, Cohen MS, Hicks CB, Haynes BF, Markowitz M, Keele BF, Hahn BH, Shaw GM. 2010. High multiplicity infection by HIV-1 in men who have sex with men. *PLoS Pathog.* 6:e1000890. doi:10.1371/journal.ppat.1000890.
46. Politch JA, Tucker L, Bowman FP, Anderson DJ. 2007. Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men. *Hum. Reprod.* 22:2928–2935.
47. Raska M, Takahashi K, Czernekova L, Zachova K, Hall S, Moldoveanu Z, Elliott MC, Wilson L, Brown R, Jancova D, Barnes S, Vrbkova J, Tomana M, Smith PD, Mestecky J, Renfrow MB, Novak J. 2010. Glycosylation patterns of HIV-1 gp120 depend on the type of expressing cells and affect antibody recognition. *J. Biol. Chem.* 285:20860–20869.