Ultrasensitive Immunoassay for Simian Immunodeficiency Virus p27^{CA}

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Abstract

Although effective for suppressing viral replication, combination antiretroviral treatment (cART) does not represent definitive therapy for HIV infection due to persistence of replication-competent viral reservoirs. The advent of effective cART regimens for simian immunodeficiency virus (SIV)-infected nonhuman primates (NHP) has enabled the development of relevant models for studying viral reservoirs and intervention strategies targeting them. Viral reservoir measurements are crucial for such studies but are problematic. Quantitative polymerase chain reaction (PCR) assays overestimate the size of the replication competent viral reservoir, as not all detected viral genomes are intact. Quantitative viral outgrowth assays measure replication competence, but they suffer from limited precision and dynamic range, and require large numbers of cells. *Ex vivo* virus induction assays to detect cells harboring inducible virus represent an experimental middle ground, but detection of inducible viral RNA in such assays does not necessarily indicate production of virions, while detection of more immunologically relevant viral proteins, including p27^{CA}, by conventional enzyme-linked immunosorbent assays (ELISA) lacks sensitivity. An ultrasensitive digital SIV Gag p27 assay was developed, which is 100-fold more sensitive than a conventional ELISA. In *ex vivo* virus induction assays, the quantification of SIV Gag p27 produced by stimulated CD4+ T cells from rhesus macaques receiving cART enabled earlier and more sensitive detection than conventional ELISA-based approaches and was highly correlated with SIV RNA, as measured by quantitative reverse transcription PCR. This ultrasensitive p27 assay provides a new tool to assess ongoing replication and reactivation of infectious virus from reservoirs in SIV-infected NHP.

Keywords: SIV, Gag, Simoa, digital immunoassay, reservoir

Introduction

COMBINATION ANTIRETROVIRAL TREATMENT (cART) can effectively suppress HIV-1 replication in infected humans to levels below the limits of quantification for standard clinical assays. However, even extended duration treatment fails to cure infection. During cART, the virus persists in “viral reservoirs,” which are sources of residual virus from which recrudescence may re-emerge upon treatment interruption.^{1–3} Prior studies have provided evidence for a number of different, non-mutually exclusive mechanisms for the persistence of such HIV reservoirs, including the survival and sometimes clonal expansion of latently infected memory CD4+ T cells, along with residual low-level

replication in sites of poor cART penetration and/or limited immune surveillance, such as lymphoid tissues, including B-cell follicles.^{4–15,36} While assessment of “rebound” plasma viremia after cART discontinuation remains the definitive functional measure of the ability of interventions to impact viral reservoirs, measurements using a variety of laboratory assays critically allow for indirect monitoring of the size of viral reservoirs without a requirement for clinical cART interruption.

Currently, there are multiple assays used to quantify viral reservoirs, including assays for directly measured and induced levels of viral DNA, RNA, and protein, along with culture-based measures of replication competent virus. The culture based methods, known as quantitative viral

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outgrowth assays (QVOA) provide a lower limit measure that underestimates the recrudescence competent viral reservoir, with the benefit of quantifying only replication competent virus. However, these assays suffer from limitations of sensitivity, precision, and dynamic range, and require input cell numbers that can be prohibitive for NHP studies.^{16,17} The measurement of cell-associated total HIV DNA is at the other extreme. Although a straightforward and sensitive way to estimate the frequency of infected cells, this method does not distinguish between replication competent and defective proviruses and thus represents an upper limit potential overestimate of the relevant recrudescence capable virus pool. The limitations of existing assays indicated the need for development of additional approaches.

In between QVOA and measures of viral DNA are various assays to quantify viral reservoirs based on measurement of viral RNA or protein after *in vitro* stimulation to induce virus expression from latently infected cells. The Tat/Rev-induced limiting-dilution assay (TILDA) is based on the VOA format, but quantifies cell-associated multiply-spliced viral RNAs.¹⁸ While not a direct reflection of the recrudescence capable viral reservoir, such induced virus/virus reactivation assays, particularly those measuring induced viral protein, may better reflect the immunologically relevant, viral antigen-producing reservoir compartment, including the replication competent subset. Capture enzyme-linked immunosorbent assays (ELISA) for the HIV p24^{CA} protein have been applied in virus induction assays. However, the picomolar sensitivity of such assays is a limitation for detecting low levels of p24 Gag that may be relevant for reservoir reactivation studies. Recently, the development of single molecule array (Simoa) technology has ushered in a new era of ultrasensitive protein detection, and this approach has successfully been used to detect HIV p24 at femtomolar concentrations in plasma samples from seronegative HIV+ individuals, as well as direct measurement of viral protein from CD4+ T cells isolated from ART-suppressed HIV+ individuals or stimulated *in vitro* to induce viral reactivation.^{19–21}

Simoa is a digital bead-based immunoassay platform in which paramagnetic microbeads are coated with a capture antibody that binds the analyte of interest, which is then sandwiched with a detector antibody. These immunocomplexes are labeled with streptavidin- β -galactosidase to form an enzyme-labeled immunocomplex. Single beads carrying these immunocomplexes are then dispensed into 40-femtoliter microwells (one bead per well). The reaction between the β -galactosidase and its substrate [resorufin- β -D-galactopyranoside (RGP)] results in a fluorescent signal that is detected by a charge-coupled device camera. Once reagents and samples are loaded, the assay is completely automated, resulting in highly accurate and precise protein quantification.²² In the Simoa assay format, the use of assay wells small enough to accommodate only a single bead, with a digital positive or negative signal for each well, results in a dramatic increase in signal-to-noise ratio and improved net assay sensitivity. Many Simoa immunoassays have a femtomolar detection limit and a dynamic range that covers four orders of magnitude.

With the recent development of effective cART regimens for simian immunodeficiency virus (SIV)-infected nonhuman primates (NHP),²³ these animal models have become par-

ticularly relevant for studying viral reservoirs and AIDS virus cure interventions. Many of the various assay formats used for laboratory measurements of HIV reservoirs, including QVOA, viral DNA, viral RNA, and viral capsid protein capture immunoassays, have been adapted for use in SIV systems. Here, a novel ultra-sensitive Simoa immunoassay for SIV p27 Gag is presented. The limit of quantification (LOQ) of this assay is 0.1 pg/ml, which is two logs lower than the LOQ of conventional SIV p27 Gag ELISAs. It is shown that this assay is suitable for measuring SIV p27 Gag induced *ex vivo* from primary CD4+ T cells from rhesus macaques with low to undetectable levels of plasma viremia.

Methods

Detection of SIV p27 Gag

SIV p27 Gag was quantified by two methods. A conventional ELISA (Advanced Biosciences Laboratories) with a standard curve range of 62.5–2,000 pg/ml was used according to the manufacturer's instructions to quantify p27 Gag in all culture supernatant samples. Additionally, the Quanterix Simoa ultrasensitive bead-based immunoassay platform was used to develop an assay for p27 Gag quantification following recommendations from the Simoa Homebrew Assay Development Guide (Quanterix). Briefly, paramagnetic carboxylated beads (Quanterix) were activated by adding 5% (vol/vol) 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Quanterix) to a concentration of 1.4×10^6 beads/ μ l. After 30 min of incubation at 4°C, the beads were washed using a magnetic separator. The activated paramagnetic beads were then mixed with a 0.5 mg/ml ice-cold solution of the α p27 capture antibody (PP279; Biological Products Core, AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research²⁴). After brief vortexing, the beads were incubated for 2 h at room temperature on a mixer (HulaMixer Sample Mixer) before being washed and a blocking solution added. After three washes, the conjugated beads were resuspended and stored at 4°C. At the time of analysis, the beads were diluted to 20,000 beads/ μ l in bead diluent (Quanterix).

The α p27 detection antibody (1 mg/ml; catalog no. 4324; Advanced Biosciences Laboratories) was biotinylated by adding 3% (vol/vol) 3.4 mmol/liter EZ-Link NHS-PEG4-biotin (Quanterix), followed by incubation for 30 min at room temperature. Free biotin was removed with spin filtration (Amicon Ultra-2; 50 kDa; Millipore Sigma), and the biotinylated antibody was stored at 4°C.

Both calibrators and samples were prepared in RPMI complete (RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine) and 0.5% Triton X-100 (Amresco, Inc., VWR). Culture supernatants and calibrators were assayed in duplicate on a Simoa HD-1 instrument (Quanterix) using a three-step assay protocol that starts with an aspiration of the bead diluent from 25 μ l conjugated beads (20,000 beads/ μ l), followed by the addition of 100 μ l of undiluted sample or calibrator to the bead pellet. After 15 min of incubation, the beads were washed before the addition of 100 μ l of biotinylated detector antibody (0.3 μ g/ml). After incubating for 5 min 15 s, the beads were washed, and 100 μ l of streptavidin-conjugated β -galactosidase (150 pmol/liter; Quanterix) was added. This

was followed by incubation for 5 min 15 s and a wash. Before reading, 25 μ l RGP (Quanterix) was added.

Validation of SIV p27 Gag Simoa assay

The calibrator curve was generated using single-use aliquots of recombinant p27 protein at concentrations of 0.016–250 pg/ml diluted in RPMI complete with 0.5% (v/v) Triton X-100. The highly purified recombinant p27 protein had previously been quantified by amino acid analysis.²⁵ Validation analysis was performed according to Quanterix guidelines on data from 10 runs, with two runs per day over 5 days. The validation panel included a full set of calibrators, a blank calibrator containing only RPMI complete and 0.5% Triton X-100, four spike and recovery samples (0.5–32 pg/ml) containing recombinant p27 protein spiked into RPMI complete and 0.5% Triton X-100, and four samples of lysed SIVmac239 virus (0.08–10 pg/ml) in RPMI complete and 0.5% Triton X-100. The SIVmac239 preparation used for validation had previously been quantified based on calibration to a recombinant p27 Gag standard quantified by amino acid analysis.²⁶ All calibrators and samples were measured in triplicate during each run, resulting in a sample size of 30 for all validation analyses. The lower limits of detection (LOD) and lower LOQ (LLOQ) were averaged across the 10 runs. LOD was calculated as 2.5 times the signal from the blank calibrator, and the LLOQ was calculated by CV profiling of the four-parameter logistic regression used to fit the calibration data.

Detection of SIV RNA

SIV RNA in 100 μ l of culture supernatant was measured with quantitative reverse transcription PCR (RT-PCR) assays targeting a highly conserved region in *gag*, as previously described.²⁷ LOD was 15 viral RNA copies/ml.

SIV outgrowth assays

Aliquots of viably cryopreserved peripheral blood mononuclear cells (PBMC) and lymph node mononuclear cells (LNMC) were thawed at 37°C, pooled, and added to warm RPMI complete. Cells were centrifuged at 300 $\times$ g for 10 min at room temperature. Then, the supernatant was removed, and the cells were re-suspended in warm RPMI complete and allowed to rest for at least 2 h at 37°C. After incubation, red blood cells were lysed using ACK buffer (Quality Biological), and then negative selection was used to enrich for CD4⁺ T cells (Miltenyi Biotec). CD4⁺ T cells were split across three conditions—stimulated, stimulated with antiretroviral drugs, and unstimulated—and a day 0 sample was taken. The amount of CD4⁺ T cells recovered from each animal varied, but a range of 2–11 $\times$ 10⁶ CD4⁺ T cells were plated per well. All conditions were plated in 3 ml RPMI complete, with stimulated conditions also receiving 100 IU/ml interleukin-2 (IL-2) and 5 μ l of α CD2/ α CD3/ α CD28 beads (Miltenyi Biotec) per 1 $\times$ 10⁶ cells, according to the manufacturer's instructions. The condition containing antiretroviral drugs included 1 μ M emtricitabine (FTC, reverse transcriptase inhibitor) and 100 nM dolutegravir (DTG, integrase strand transfer inhibitor). After incubating for 24 h at 37°C, the day 1 sample was taken, and then stimulating beads were re-

moved according to the manufacturer's instructions (Miltenyi Biotec). The culture supernatant was removed and saved prior to bead removal. After bead removal, cells were re-suspended in their culture supernatant, and 1 ml of RPMI complete with 100 IU/ml IL-2 was added back. Emtricitabine (1 μ M) and dolutegravir (100 nM) were also included in the 1 ml RPMI complete +100 IU/ml IL-2 added back to the stimulated + antiretroviral drugs condition. Antiretroviral drugs were added back to the applicable condition on days 3 and 5 to maintain appropriate concentrations. Culture supernatant samples were also taken on days 3 and 7. Samples were collected as 1 ml culture supernatant and split into two 100 μ l aliquots frozen at –20°C for SIV RNA quantification and 750 μ l treated with 0.5% Triton X-100. Triton X-100-treated samples were incubated at 37°C for 1 h, split into three 250 μ l aliquots, and frozen at –20°C for SIV Gag p27 protein quantification by ELISA and Simoa.

SIV-infected animals

PBMC and LNMC samples were collected from Indian origin rhesus macaques enrolled in various studies and infected with variants of SIVmac239, including wild-type and barcoded stocks containing identical wild-type Gag sequences.^{28,29} At the time of sampling, all animals were in the chronic phase of infection and were either undergoing cART treatment or were naturally controlling their infection post cART treatment. Studies including animals DCCP, DCHV, DCJB, and DCCN have previously been published.^{30,31} All samples were from studies approved by the Institutional Animal Care and Use Committee of the National Cancer Institute.

Animals underwent cART with a variety of nucleoside reverse transcriptase inhibitors (NRTI), integrase strand transfer inhibitors (INSTI), and protease inhibitors (PI). Animal DCCP received tenofovir (TFV; NRTI), emtricitabine (FTC; NRTI), L-870812 (INSTI), L-900564 (INSTI), darunavir (DRV; PI), and ritonavir (RTV; PI). Animals DCHV, DCJB, and DCCN received TFV, FTC, dolutegravir (DTG; INSTI), DRV, and RTV. Animals P031, P038, P051, and P058 received tenofovir disoproxil fumarate (TDF; NRTI), lamivudine (3TC; NRTI), cabotegravir (CAB-LA; INSTI), and brexanavir (GSK385-mLAP; PI). Animals ZK08, ZK12, GB7V, H34G, H859, H860, and DFR6 received TDF, FTC, and DTG.

Statistical tests

The ROUT outlier test was run in GraphPad Prism v7.0a (GraphPad Software, Inc.) to identify outliers during assay validation analysis. Q (maximum false discovery rate) was set at 1%. A nonparametric Spearman test was used in GraphPad Prism v7.0a to calculate the correlation between RNA and p27. Mann–Whitney's *U*-test was used in GraphPad Prism v7.0a to calculate significant differences between groups in Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/aid).

Results

Ultrasensitive immunoassay development

An ultrasensitive Simoa immunoassay was developed for SIV p27 Gag using the Quanterix Simoa Homebrew

guidelines (Quanterix). The calibration curve, shown in Figure 1A, was generated using highly purified recombinant SIV p27 Gag protein diluted in RPMI complete medium with 0.5% Triton X-100, resulting in a standard curve with a range of 0.08–250 pg/ml and a dynamic range of more than three logs. Error bars represent the standard deviation for 30 measurements. The average background signal for the assay was 0.007 average enzymes per bead and is a result of non-specific binding of labeling reagents to the beads. The assay's LOD and LLOQ were averaged over 10 runs and were 0.048 pg/ml and 0.092 pg/ml, respectively (Fig. 1B). LOD was calculated as 2.5 standard deviations above mean background replicate measurements for each run. Consistent with Quanterix Simoa Homebrew assay development guidelines, the LLOQ was calculated by coefficient of variation (CV) profiling of the four-parameter logistic regression used to fit the calibration data.

Immunoassay validation

Assay validation tests, including spike and recovery and detection of lysed SIVmac239 virus, were performed to ensure accurate quantification of SIV p27 Gag. Samples were tested in triplicate in each of 10 runs over 5 days. The average intra-assay coefficient of variation (CV) for the four lysed virus samples was 6.9%, the average inter-assay CV was 16.4%, and the average inter-day CV was 13.3%.

To determine the recovery of SIV p27 Gag in a cell culture supernatant matrix, serial dilutions of recombinant SIV p27 Gag that had previously been rigorously quantified by amino acid analysis were spiked into RPMI complete medium with 0.5% Triton X-100 to obtain final concentrations ranging from 0.5 to 32 pg/ml. The data shown in Figure 2A and B are the average and standard deviation of 30 replicates. Percent recovery was calculated according to the following equation:

$$\% \text{ Recovery} = 100 \times \frac{(C_{\text{Det}} - C_0)}{C_{\text{Spike}}}$$

where C_{Det} is the calculated concentration of the spiked sample, C_0 is the calculated concentration of the blank calibrator, and C_{Spike} is the expected concentration in the sample. The average calculated percent recovery values for each of the four spiked concentrations are listed in Table 1. Recovery of spiked protein was highly efficient, with $\leq 9\%$ difference between input and the average calculated p27 measured at each concentration.

To ensure that the assay accurately detected SIV p27 Gag in the relevant setting of protein released from lysed virions present in supernatants from virus induction cultures, purified SIVmac239 virions were diluted in RPMI complete with 0.5% Triton X-100 at four concentrations ranging from 0.08 to 10 pg/ml. The p27 content of the purified SIVmac239 stock used for these validation experiments was quantified based on calibration to a p27 Gag standard quantified by amino acid analysis.²⁶ The data shown in Figure 2C and D are the average and standard deviation of 30 replicates. As with recovery of spiked recombinant p27, detection of lysed SIVmac239 was quite accurate, thereby demonstrating the utility of this assay for detecting SIV p27 Gag from lysed virions in culture medium.

Detection of virus reactivation from CD4+ T cells

Next, the study assessed the ability of the ultrasensitive p27 assay to detect induced SIV expression following *in vitro* stimulation of CD4+ T cells isolated from the peripheral blood and lymph nodes of cART-treated or spontaneous post-treatment controller rhesus macaques infected with SIVmac239. All animals had plasma viral loads ranging from undetectable to 70 viral RNA copies/ml (Table 2). CD4+ T cells from each animal were split equally

FIG. 1. Assay standards. (A) Mean and standard deviation of the standard curves obtained with the ultrasensitive Simoa p27 immunoassay and a conventional p27 enzyme-linked immunosorbent assay (ELISA). The conventional p27 ELISA standard (Advanced Biosciences Laboratories) is an average of five separate assays and is represented by *squares*, with optical density (OD) values indicated on the right y-axis; the range is 62.5–2,000 pg/ml. The standard curve of the ultrasensitive p27 assay is an average of 10 separate assays and is represented by *circles*, with average enzymes per bead (AEB) values indicated on the left y-axis; the range is 0.08–250 pg/ml. Error bars for the ultrasensitive p27 assay are plotted but are too small to be seen. The least squares method was used to fit standard curve lines to the Simoa and ELISA data. (B) Limit of detection (LOD) and lower limit of quantification (LLOQ) for the ultrasensitive Simoa immunoassay. Assay LOD was calculated as 2.5 standard deviations above the background. Assay LLOQ was calculated by coefficient of variance (CV) profiling.

FIG. 2. Detection of simian immunodeficiency virus (SIV) p27. (**A** and **B**) Spike and recovery. Recombinant SIV p27 was added to RPMI complete with Triton X-100 at four concentrations, ranging from 0.5 to 32 pg/ml. Data shown are the average and standard deviation of 30 replicates. (**C** and **D**) Detection of lysed SIVmac239 virions. SIVmac239 virus was diluted in RPMI complete with Triton X-100 at four concentrations, ranging from 0.08 to 10 pg/ml. Data shown are the average and standard deviation of 30 replicates. Error bars in (**B**) and (**D**) are plotted, but some are too small to be seen.

across three conditions: (1) stimulated with α CD2/ α CD3/ α CD28 beads for 24 h (days 0–1) and then maintained with IL-2; (2) stimulated in the presence of antiretrovirals (ARVs); or (3) unstimulated. SIV p27 Gag was quantified in culture supernatants on days 0, 1, 3, and 7 by the novel Simoa immunoassay and by conventional ELISA (Fig. 3). Supernatants from CD4⁺ T cells stimulated and then maintained in IL-2 throughout the experiment were positive for p27 by the Simoa immunoassay after 24 h in culture (Fig. 3A, B, D, and E). All but one of the culture supernatants from unstimulated CD4⁺ T cells were negative for p27 by either the Simoa immunoassay or conventional ELISA over 7 days (Fig. 3C and F). Additionally, CD4⁺ T cells were isolated from the peripheral

blood of three SIV-naïve rhesus macaques and cultured over 7 days with or without stimulation, as described above. Fifty-four culture supernatants were tested for the presence of p27 by Simoa, of which none was positive, suggesting a low false-positive rate (data not shown).

Strikingly, of 100 samples that were p27 positive by the Simoa immunoassay, only four were also detected by the conventional p27 ELISA (Fig. 3A, B, and C vs. Fig. 3D, E, and F), highlighting the enhanced sensitivity of the Simoa immunoassay below the LOD of the p27 ELISA (62.5 pg/ml). Additionally, the conventional ELISA was only able to detect virus after 7 days in culture compared to the Simoa immunoassay, which was able to detect SIV Gag p27 as early as 24 h after bead stimulation.

Comparison of the α CD2/ α CD3/ α CD28 stimulated alone (Fig. 3A and D) versus stimulated + ARV (Fig. 3B and E) conditions revealed two sources of SIV p27 Gag in culture supernatants: induced virus released directly from stimulated latently infected cells and virus arising from spreading infection in the culture. The low level of SIV p27 Gag (~ 1 pg/ml) detected in cultures on day 1 is likely the result of virions released from latently infected cells after stimulation. This virus would be unaffected by the presence of the ARVs used, which only block new rounds of infection in previously uninfected cells. Additionally, over time, there is evidence of

TABLE 1. PERCENT RECOVERY FOR SPIKE AND RECOVERY VALIDATION TEST

C_{Spike} (pg/ml)	Recovery
0.5	103%
2	109%
8	105%
32	102%
Average recovery	105%

TABLE 2. CHARACTERISTICS OF SIV-INFECTED RHESUS MACAQUES ON cART OR WITH POST-TREATMENT CONTROLLER PHENOTYPES

Animal	Time since infection (weeks)	Length of cART treatment (weeks)	Treatment at time of sample	RNA (copies/ml)
DCCP	56	52	Regimen 1	30
DCHV				
DCHV-LN ^a	60	56	Regimen 2	<10
DCJB				
DCJB-LN ^b	112	N/A	N/A	70
DCCN-LN ^a	60	55	Regimen 2	20
P031	87	60	Regimen 3	<15
P038	97	75	Regimen 3	<15
P051	109	88	Regimen 3	<15
P058	115	N/A	N/A	<15
ZK08	134	N/A	N/A	65
ZK12	134	N/A	N/A	<15
GB7V	31	29.5	Regimen 4	40
H34G	31	29.5	Regimen 4	<15
H859	31	29.5	Regimen 4	<15
H860	31	29.5	Regimen 4	<15
DFR8	31	29.5	Regimen 4	<15

Regimen 1: TFV, FTC, L-870812, L-900564, DRV, RTV; regimen 2: TFV, FTC, DTG, DRV, RTV; regimen 3: TDF, 3TC, CAB-LA, GSK385-mLAP; regimen 4: TDF, FTC, DTG.

^aAxillary lymph node.

^bIliac lymph node.

SIV, simian immunodeficiency virus; cART, combination antiretroviral treatment; TFV, tenofovir; FTC, emtricitabine; DRV, darunavir; RTV, ritonavir; DTG, dolutegravir; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; CAB-LA, cabotegravir.

clear exponential replication of virus in some samples after stimulation. Of the 17 NHP samples tested, after *ex vivo* stimulation, 10 had at least a log increase in detected p27 protein between days 3 and 7, and of those 10, three had at least a two-log increase in the same time frame. This multi-round infection is dampened in the presence of ARVs.

Virus reactivation was also measured in culture supernatants by the quantification of SIV Gag RNA using a previously described quantitative RT-PCR method.²⁷ A significant correlation was observed between levels of SIV p27 Gag and SIV RNA, as measured by ultrasensitive immunoassay and quantitative RT-PCR methods, respectively, in cultures that were positive by both measures ($r=0.711$, $p<.0001$; Fig. 4A). The correlation was still significant when samples that were RNA positive but between the Simoa limits of quantification and detection (Fig. 4B, open circles) were included ($r=0.716$, $p<.0001$). p27 was detected in 56.3% (58/103) of SIV RNA-positive supernatants. Of note, Gag p27 was only detected in 37.5% (27/72) of culture supernatants with SIV RNA levels <1,000 copies (Supplementary Fig. S1). These results are consistent with previous *ex vivo* experiments using latently HIV-infected CD4⁺ T cells.¹⁹ Conversely, gag RNA was only detected in 33.9% (18/53) of p27-positive samples <0.6 pg/ml. This may represent cultures in which cells have died and released the uncleaved Gag precursor while the corresponding naked viral RNA was degraded. The absence of similar low-level positive p27 results in samples from uninfected animals or from uninduced cultures from animals with sustained viral suppression to <15 copies/ml of plasma is consistent

with these results reflecting such a mechanism, as opposed to assay false-positives.

Discussion

Determining the extent of residual virus, including inducible latent virus, during treated HIV or SIV infection is critical for the evaluation of candidate AIDS virus cure interventions.^{16,32} Viral reservoirs are commonly quantified by PCR-based assays by measurement of total or integrated viral DNA or cell associated RNA, or by a QVOA.^{17,33} Each of these measurements quantifies a different aspect of viral reservoirs, and while each has useful features, all embody conceptual or technical limitations. Quantification of viral DNA may overestimate the size of the reservoir that can lead to recrudescence, since it measures both replication competent and defective proviruses, the replication incompetence of which would preclude their contributing to off-cART recrudescence.^{34,35} Alternatively, the VOA provides only a minimal estimate of the frequency of cells harboring replication competent virus due to the less-than-completely efficient and stochastic nature of virus induction, especially when using only a single round of stimulation.¹⁶ Since the VOA has traditionally relied on a conventional ELISA for viral protein detection, the assay requires extended culturing of stimulated latently infected CD4⁺ T cells with target cells to allow for enough virus replication and spread through the culture to be detectable. Finally, in addition to limits on sensitivity, precision, and dynamic range, the VOA requires large quantities of cells, is labor intensive, and is expensive.

The TILDA was developed as a complement to the DNA-based and VOA assays.¹⁸ The TILDA method is an *ex vivo* virus induction assay, but it quantifies cell-associated multiply-spliced viral RNAs, which are the early transcripts produced after activation of cells harboring latent proviruses. Inducible reservoir estimations made by TILDA are smaller than those made by the total viral DNA but larger than the replication competent virus measured by VOA.¹⁷ Compared to viral outgrowth assays, TILDA requires less time and sample and is less expensive. However, TILDA does not directly measure the production of viral antigens, which is more immunologically relevant.

As has been previously noted,¹⁹ the addition of ultrasensitive assays for Gag could improve VOA, primarily by reducing the time between virus reactivation and detection of Gag. It is important to note that while not all cells carrying replication competent provirus ultimately produce infectious virions,³⁴ their release of antigenic viral proteins may trigger the immune system and allow for immunologic targeting of infected cells and may also contribute to persistent inflammation and pathogenesis.

Here, a novel ultrasensitive digital immunoassay is presented to measure SIV p27 Gag in the femtomolar range. The validation of this assay showed that it is highly accurate and precise for the quantification of both recombinant p27 Gag protein and lysed SIVmac239 virus. The study demonstrates the utility of this assay for measuring SIV reactivation in CD4⁺ T cell samples from NHP with low to undetectable levels of SIV RNA plasma viremia. When SIV p27 Gag was detected in culture supernatants after *ex vivo* stimulation of latently infected CD4⁺ T cells, the quantity of p27 produced highly correlated with levels of SIV RNA detected by

FIG. 3. Detection of SIV p27 *ex vivo*. CD4+ T cells were enriched from viably cryopreserved peripheral blood mononuclear cells (PBMC) and/or lymph node mononuclear cells (LNCs) from 15 rhesus macaques infected with SIVmac239. SIV Gag p27 was quantified in culture supernatants by both Simoa (A–C) and conventional ELISA (D–F). Black symbols indicate measurements by Simoa, and blue symbols indicate measurements by conventional p27 ELISA. Black open symbols represent protein concentrations below the LOQ of the Simoa immunoassay but above the limit of detection. These values were calculated by extrapolation of the Simoa standard curve. Blue open symbols represent samples that were negative by the conventional p27 ELISA. These values are plotted at the LOD (62.5 pg/ml). The dashed line in (A–C) denotes the LOD for the conventional p27 ELISA (62.5 pg/ml). CD4+ T cells were cultured for 7 days in three separate conditions: stimulated with α CD2/ α CD3/ α CD28 beads for 24 h and IL-2 for 7 days (A and D), stimulated with α CD2/ α CD3/ α CD28 beads for 24 h and IL-2 for 7 days and with antiretrovirals (ARVs) for 7 days (B and E), or unstimulated (C and F).

FIG. 4. Correlation between the amount of p27 and SIV RNA in the culture supernatants from CD4+ T cells enriched from PBMC or LNC of SIV-infected macaques with low to undetectable viral loads. Samples positive for both p27 and RNA are represented in blue (A and B), and samples positive for RNA but below the LOQ for p27 are represented by open circles (B). For p27 samples that were below the LOQ, the protein concentration was calculated by extrapolation of the standard curve. Correlation was calculated using a nonparametric Spearman test.

quantitative RT-PCR. This result suggests that SIV *gag* RNA quantification and the ultrasensitive p27 Gag immunoassay are complementary tools for the characterization of virus persistence and reactivation.

The Simoa immunoassay was able to detect quantities of proteins at least two logs lower than the 62.5 pg/ml LOD for the conventional p27 ELISA. Additionally, detection of measurable levels of p27 Gag by the Simoa immunoassay preceded detection of measurable levels by the ELISA by 6 days, with consistent detection of protein in culture supernatants within 24 h after stimulation of cells. The data clearly show that utilization of a conventional p27 ELISA in this context requires the expansion of the viral population through multi-round infection over several days. In contrast, the SIV p27 Simoa immunoassay can detect the early virus released from cells upon stimulation, as well as virus that is a result of new rounds of infection, making this assay a convenient tool to answer various experimental questions.

One intriguing set of samples within the data set involves comparison of results for stimulated CD4⁺ T cells from PBMC (diamond) and LNCM (upright triangle) obtained at the same time from animal DCJB (Fig. 3A). By the seventh day in culture, the p27 Gag detected from LNCM CD4⁺ T cells was approximately 23,000 times greater than that detected in PBMC CD4⁺ T-cell cultures (60,304 vs. 2.6 pg/ml). This result indicates that at this time point, the reservoir of replication competent virus in this animal was larger in the sampled iliac lymph node than in the periphery and/or the uninfected cells present in the LNCM sample were more permissive to spreading infection than those in PBMC. Although it is a single example, this finding is consistent with the hypothesis that the replication competent reservoir is not homogeneously distributed within the body.^{14,36,37}

In its current form, this novel ultrasensitive immunoassay assay can be used to detect SIV p27 Gag in culture supernatants from *ex vivo* stimulated cells of SIV- or SHIV-infected macaques on suppressive cART or with post-treatment controlling phenotypes. Additionally, this assay format could be used to test and compare various latency reversing agents, as has previously been reported with a HIV-1 p24 Gag Simoa immunoassay.^{19,21} Further assay development will include testing of alternative α p27 antibody pairs in an attempt to improve sensitivity further, as well as adaptation of current protocols for detection of HIV p24 Gag in cell lysates²¹ and tissue samples for SIV p27 Gag detection.³⁸

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Author Disclosure Statement

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