

Ex Vivo Analysis of SIV-Infected Cells by Flow Cytometry

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• Abstract

Deciphering the complex interactions between human and simian immunodeficiency viruses (HIV/SIV) and their host cells is crucial to the development of improved therapies and vaccines. Investigating these relationships has been complicated by the inability to directly analyze infected cells among freshly isolated peripheral blood lymphocytes. Here, we describe a method to detect cells productively infected with SIVmac239 ex vivo from the blood or lymph nodes by flow cytometry. Using this method, we show a close correlation between the frequency of productively infected cells in both sample type and the plasma viral load. We define that the minimum threshold for detecting productively infected cells in lymph nodes by flow cytometry requires a plasma virus concentration of $\sim 2.5 \times 10^4$ vRNA copy Equivalents (Eq)/ml. Conversely, an approximately 2 logs higher plasma viral load is needed to detect productively infected cells in the peripheral blood. This novel protocol provides a direct analytical tool to assess interactions between SIV and host cells, which is of key importance to investigators in AIDS research. © 2010 International Society for Advancement of Cytometry

• Key terms

SIV infected cells; flow cytometry; ex vivo detection

UNDERSTANDING the intricate relationship between human or simian immunodeficiency virus (HIV or SIV) and host cells is critically important for the rational development of novel treatment compounds and vaccines, yet many interactions remain uncharacterized in naturally infected cells. Currently either in vitro cultured cells or bulk analysis of major lymphocyte subsets from peripheral blood are used to dissect virus-host cell interactions (1–4). These methods laid the foundation for many important findings but are limited to indirectly measuring the effects of viral replication on target cells due to the difficulty in detecting naturally infected cells in blood samples (5,6). As a result these methods may miss subtle, but important interactions between the virus and host cells. Naturally infected cells have been visualized, however, in lymph nodes and mucosal tissues with the combined application of immunohistochemistry and in situ hybridization for viral RNA (7–9). However, these methods lack the power of quantitative analysis of large number of cells or simultaneous measurement of multiple phenotypic markers on individual cells. Therefore, development of methods to directly analyze naturally infected cells ex vivo will greatly enhance our ability to examine interactions between HIV/SIV and host cells.

A major hurdle in the detection of productively infected cells is the low frequency of HIV/SIV infected CD4⁺ T cells circulating in the blood. Unfortunately, the number of cells supporting active virus replication is expected to be even lower. Lymph nodes, in contrast, are sites of vigorous HIV/SIV replication and therefore are expected to contain higher frequencies of productively infected cells with detectable virion production. We developed a method to enrich for cells that are likely to harbor virus replication using lymphocytes derived from lymph nodes. To identify

productively infected cells we stained them for intracellular SIV Gag p27 protein. The technique described in this study will allow researchers to perform a wide array of sophisticated assays to further characterize the cell populations actively producing virions during the different stages of SIV pathogenesis.

MATERIALS AND METHODS

Animals and Viruses

We used previously frozen Ficoll-purified PBMC and/or lymph node samples from captive bred Indian rhesus (*Macaca mulatta*) macaques. The 27 animals that were included in this study participated in different AIDS research studies and were assigned to protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. They were cared for according to the NIH "Guide to the Care and Use of Laboratory Animals." The macaques were infected with the molecularly cloned SIVmac239 nefopen virus (10). The virus stock was produced by the Virology Services Unit of the Wisconsin National Primate Research Center using SIVmac239 hemi-genome plasmids obtained from the NIH AIDS Research and Reference Reagent Program (contributed by Dr. Ronald Desrosiers). Samples were collected at different time points ranging from 3 to 139 weeks postinfection with a median of 28 weeks. With the exception of five animals, the samples were collected during the chronic phase (>8 weeks postinfection) of infection. Samples from one additional animal infected with the nef-deleted version of SIVmac239 (SIVmac239 Δ nef) and eight uninfected animals were used as biological controls (11).

Since the method that we applied here required a large number of cells we were not able to obtain matching blood and lymph node samples in every occasion. The blood volume that we could procure at any one time was dependent on the selected animal's bodyweight as per the guidance of the laboratory animal care and use. This limitation affected the number of blood samples that we were able to secure.

Antibodies and Fluorochrome Dyes

FITC conjugated anti-SIVmac239 Gag p27 antibody (clone 55-2F12) was produced by Dr. Nancy Wilson at the AIDS Vaccine Research Laboratory (University of Wisconsin, Madison, WI) using the antibody producing hybridoma distributed by the NIH AIDS Research and Reference Reagent Program. IgG2b FITC (clone MPC-11 used as the isotype control for the p27FITC antibody), CD3 Alexa700 (clone SP34-2), CD14 Alexa700 (clone M5E2), CD28 APC (clone CD28.2), CD8 PerCP (clone SK1), and CD4 PerCP (clone L200) were purchased from BD Biosciences (San Jose, CA). CD4 PECy7 (clone OKT4) was obtained from Biolegend (San Diego, CA). Violet Amine Reactive Dye (ARD) was acquired from Invitrogen (Carlsbad, CA).

Sample Processing and Staining

We depleted CD8+, CD20+, and CD14+ cells from 30 to 100 million Ficoll-purified lymphocytes either from lymph node or peripheral blood using magnetic bead separation kits

and LS columns (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) according to manufacturer's recommendations. We stained the negative fraction at a 5–6 million cells/100 μ l lymphocyte density for surface markers in the presence of saturating amount of antibodies for 15 min at room temperature, washed the samples once with RPMI containing 10% FCS (R10), then fixed them with 150 μ l of 2% paraformaldehyde (PFA) for 15 min at room temperature. We removed the fixative with a washing step using 1 ml of R10 and resuspended the samples in 100 μ l R10. We permeabilized the cells with 100 μ l of Bulk Permeabilization Reagent (Invitrogen, Carlsbad, CA), then incubated them with SIV Gag p27 antibody for 15 min at room temperature. (We determined the necessary amount of p27 antibody by titrating it on in vitro SIVmac239 infected PBMC.) We removed the excess antibody with washing the cells with 1 ml of R10, and then fixed them again with 150 μ l 2% PFA for 15 min at 4°C.

Flow Cytometric Data Acquisition, Analysis, and Cell Sorting

We collected the flow cytometric data using a custom-made BD LSR-II desktop analyzer (Becton Dickinson, San Jose, CA) equipped with a 100 mW 488 nm blue, 50 mW 640 nm red, and 50 mW 405 nm violet laser. For data acquisition we used FACSDivaTM 6.1.1. software (BD-BioSciences, San Jose, CA) which enabled us to collect large data files. The size of one flow cytometry standard (FCS) file frequently was above 125 MB. We analyzed the data with FlowJoTM version 8.7.3 (Tree Star Inc., Ashland, OR). For cell sorting, we stained the cells as described above than sorted with a FACSARIA II high-speed cell sorter (Becton Dickinson, San Jose, CA) equipped with a 100 mW 488 nm blue, 35 mW 640 nm red, and 40 mW 405 nm violet laser.

DNA Isolation from PFA-Fixed Sorted Cells

DNA was extracted from PFA-fixed sorted cells that were CD4+p27[−], or p27⁺, or CD4−p27[−]. DNA was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, CA) following the protocol for cultured animal cells (including the optional RNase A treatment), with the following differences: cells were washed an additional time in phosphate buffered saline prior to cell lysis; following the proteinase K digestion samples were incubated for an additional hour at 90°C to counteract the effects of the PFA. DNA was eluted in 50 μ l elution buffer.

SIVmac239 Gag and CCR5 Quantification

The copy number of SIVmac239 *gag* integrated into the host genome was determined using a quantitative PCR assay. We used the host gene *CCR5* (present in two copies/host genome) to quantify the number of cells in the PCR reaction and calculated the *gag* copies/cell content accordingly. *gag* and *CCR5* were amplified from each sample in parallel reactions using the Roche Taqman Master kit (Roche, Indianapolis, IN). The amplification primers for both the *gag* (SIV1552-F: GTCTGCGTCATCTGGTGCAATTC, SIV1653-R: CACTAGCTGTCTCTGCACTATGTGTTTGTG) and *CCR5* (CCR5-F: CCATGCAGGTGACAGAGACTCT, CCR5-R: TCTCCCCGACAAA

GGCATAG) were used at final concentrations of 600 nM and the probes (Gag: 6 fam-CTTCCTCAGTGTGTTTCACTTTCTCTTCTGCG-BHQ1 and CCR5: Vic-TGACACACTGCTGCATGAACCCCA-TAMRA) were at 100 nM. Cycling conditions on the Light Cycler 2.0 (Roche, Indianapolis, IN) were as follows: 95°C for 10 min followed by 45 cycles of 95°C for 10 sec, 60°C for 40 sec, and 72°C for 1 sec. Ramp rates were all 20°C/sec. Tenfold dilutions of *gag* and *CCR5* DNA were used as a standard curve to determine copy number for each sample using the LightCycler software version 4.0.

Plasma Viral Load Quantification

We determined the plasma virus concentration using a modification of a protocol published by Lifson and coworkers (12). Briefly, we isolated viral RNA (vRNA) from approximately 1 ml of plasma and amplified it in a quantitative single-step RT-PCR reaction using the Roche Master Probes kit, with reactions performed in the Roche LightCycler. Primers and TaqMan probes were designed according to the sequences published by Lifson et al. (13). Cycling conditions were: 60°C (12 min), 95°C (30 sec), 45 × 95°C (15 sec), 58°C (1 min). We determined the quantity of vRNA copies by extrapolation of threshold fluorescence values onto an internal standard curve prepared from serial dilutions of an in vitro-transcribed fragment of the SIVmac239 *gag* gene (vector kindly provided by M. Piatak and J. Lifson).

Statistical Analysis

For statistical analysis we employed Spearman rank correlation analysis, or paired Student's *t*-test which we performed using the Prism software version 4.0 for Macintosh by Graph-Pad Software, Inc. (La Jolla, CA).

RESULTS

Lymph node derived lymphocytes were used for our initial analysis because lymph nodes are active sites of virus replication and therefore likely contained higher frequencies of SIV-infected cells in comparison to peripheral blood. To enrich for SIV-infected cells we removed B-cells, NK cells, and CD8⁺ T cells, since they are rarely infected with SIV. Additionally, we removed macrophages from our lymphocyte preparations even though they may be productively infected by SIV (14,15). The rationale for removing macrophages was: (a) flow cytometric detection does not allow distinction between ingested viral particles and actively produced virus; (b) replicating SIV might not downregulate the expression of CD4, MHC-II, or MHC-I antigens (our unpublished observation from in vitro experiments) on macrophages thereby eliminating the possibility of differentiating infected macrophages from macrophages that merely ingested viral particles.

To identify virally infected cells by flow cytometry, we stained the putative SIV target cells for surface antigens previously reported to be modulated by intracellular viral proteins: CD3 and CD4 (16–18). Next we intracellularly stained with an antibody specific for a conserved region of the viral core protein Gag p27. Even within the enriched target cell population we anticipated a low frequency of SIV⁺ cells and

consequently, collected between 1×10^6 and 2.5×10^6 events within the lymphocyte gate. For comparison, this represents a 10–25 times higher number of events than is typically collected for detection of rare antigen-specific CD8⁺ T cells.

To analyze the collected data, we used a multistep gating strategy (Fig. 1a–1d) by first gating on singlets and lymphocytes before eliminating dead cells (ARD⁺) and any remaining cells not supporting SIV replication (CD8⁺ and CD20⁺). We found that most of the p27⁺ cells did not express CD4 or expressed it at a low level (Fig. 1d). To assure that we detected cells that actively produced SIVmac239 virions, as a biological control we tested a sample from an SIVmac239Δ*nef*-infected animal. This attenuated virus does not downregulate the expression of CD4 antigens on the surface of host cells because of an out-of-frame 182 bp deletion in *nef*, which encodes the viral protein responsible for the modulation of these molecules (11). As anticipated, all of the Gag p27⁺ cells from the SIVmac239Δ*nef*-infected animal expressed CD4 at normal levels (Fig. 1e). To confirm the specificity of our assay, we analyzed samples from uninfected animals (Fig. 1f). The frequency of p27⁺ cells in the lymph nodes of these macaques was between 0.0019% and 0.0072% of the enriched lymphocyte gate excluding the dead cells and contaminating “depleted cells” ($n = 5$ animals). Finally, for an added assurance for the specificity of the p27 antibody, we ran an isotype control staining parallel with the p27 staining. The frequency of isotype antibody⁺ population in the infected samples was very similar to the frequency of p27⁺ population of the uninfected samples (isotype control range: 0.0022–0.0091; $n = 3$) (Fig. 2). One very important finding was that most of the “false positive p27” events were CD4⁺ and CD3⁺, while the majority of the putative infected cells expressed the CD4[−]/low, CD3low p27⁺ phenotype.

For infected cells to be detectable in our assay, there must be enough p27 Gag protein to bind detectable levels of antibody. We reasoned that only de novo p27 Gag protein synthesis would produce sufficient levels of protein, and therefore our assay would likely detect only productively infected lymphocytes. However, using flow cytometric methods alone it was difficult to determine the frequency with which this approach would fail to detect infected cells (i.e., the false negative rate), and/or inappropriately identify uninfected cells (the false positive rate). To determine the proportion of false negative and false positive events, we therefore sorted comparable numbers of CD4⁺Gag p27[−], CD4[−]Gag p27[−] (5×10^3 – 10×10^3), and Gag p27⁺ (1 – 5×10^3) cells by FACS and used a quantitative PCR assay to measure the frequency of cells in each population containing integrated provirus. We used the chemokine receptor CCR5 as an internal standard for the QPCR assay since each cell contains two copies of the *CCR5* gene, infected cells would be expected to have at least one copy of SIVmac239 provirus for every two copies of *CCR5*. In the three animals tested, we discovered that Gag p27⁺ cells expressed one or fewer SIV *gag* DNA copies per cell (Table 1). We observed very little amplification of *gag* DNA, in either the CD4⁺Gag p27[−] (less than 0.03 *gag* copies/cell) or CD4[−]Gag p27[−] (less than 0.002 *gag* copies/cell) populations.

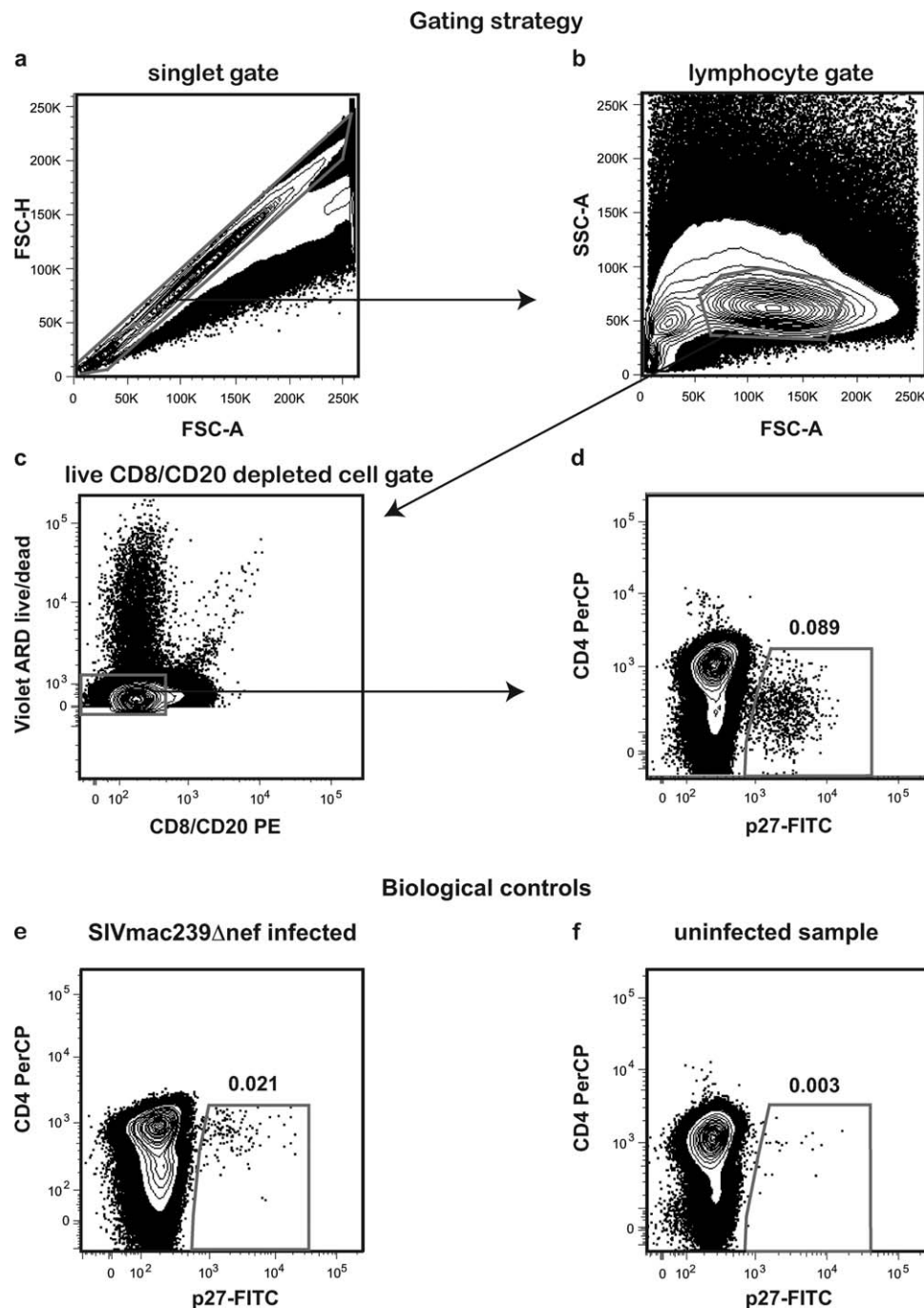


Figure 1. Gating strategy to analyze SIVmac239 infected cells from lymph node samples ex vivo. **a:** Gate to select single cell events. **b:** Lymphocyte gate. **c:** Gate to select live cells and exclude contaminating CD8 T cells and B cells. **d:** Gate to determine the frequency of p27+ cells in the enriched, living lymphocyte population. The representative example shows an animal with plasma viral load $5.36\text{E} + 06$ vRNA copy Eq/ml 4 weeks after infection. Biological controls: **(e)** lymph node samples from an animals infected with SIVmac239 Δ nef 3 weeks after infection with $2.43\text{E} + 04$ vRNA copy Eq/ml plasma viral load. **f:** Lymph node sample from an uninfected animal. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

This indicated that there were very few false negatives due to failure of productively infected cells to be stained with the anti-Gag p27 antibody. The low level of *gag* DNA copies in the Gag p27- populations also suggested that there was a low frequency of SIVmac239 latently infected cells in our enriched population of lymphocytes isolated from lymph nodes. On

the other hand, the presence of less than one copy of *gag* DNA per cell in the sorted Gag p27+ population could be explained by the combination of the following two reasons: (a) the anti-p27 antibody does have a low level of nonspecific binding, which can lead to false positive events. The relative amount of false positive events becomes more significant at lower plasma

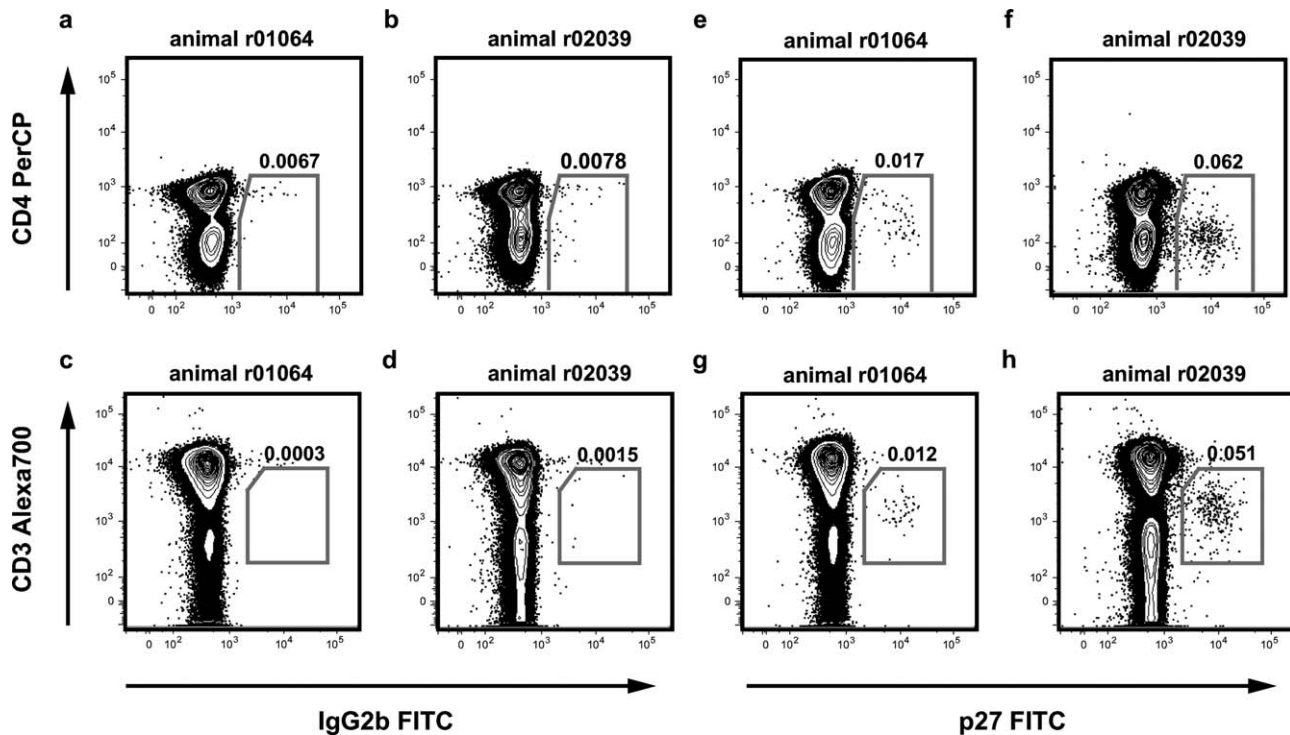


Figure 2. Assessment of false positive p27 events in the enriched lymph node samples by simultaneous isotype control antibody staining. Plots (a–d) display samples of two animals stained with isotype control antibody, plots (e–h) display the same samples stained with equal concentration of the anti-Gag p27 antibody. Plots a, c, e, and g show results derived from the lymph node of animal r01064 with $1.00\text{E} + 06$ vRNA copy Eq/ml at 129 weeks postinfection; plots b, d, f, and h show results derived from the lymph node of animal r02039 with $5.61\text{E} + 06$ vRNA copy Eq/ml plasma viral load at 49 weeks postinfection. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

viral load. (b) Sorting low frequency events with small separation between the positive and negative populations is a challenging task. Because of the low yield of the Gag p27+ events, we were unable to check the postsort purity. The possibility that the purity of our sorts were less than 99% therefore cannot be excluded.

Next we wanted to determine the lineage of the cell population that was positive for Gag p27 expression. Since depletion of the undesired population was just 70–90% effective (data not shown), it was reasonable to assume that a portion of the p27+ signal came from viral particles ingested by macrophages contaminating our enriched samples. Therefore, in selected samples we did not deplete the macrophages and stained for CD14, a lipopolysaccharide receptor expressed by cells of the myeloid lineage, mainly by macrophages and granulocytes (19). We found that the Gag p27+ cells in our enriched population were negative for CD14 (Fig. 3a). The majority of Gag p27+ cells expressed low levels of CD3 antigen. This suggested that the population belonged to the T cell lineage and as anticipated CD3 was actively being removed from the cell surface by the viral protein Nef (Fig. 3b). We also detected that most Gag p27+ cells expressed the costimulatory receptor CD28 (Fig. 3c). This again implied that the productively infected cells in the lymph node samples were T cells and they were either in the naïve or the central memory/transitional effector memory differentiation state (3,20). Next we

determined whether the virus-producing cells belonged to the CD8 or the CD4 lymphocyte subsets. In selected samples we did not deplete the CD8 population. We found that the bulk of the Gag p27+ cells did not express the CD8 antigen (MFI of CD8 of the double negative population is 152, while the Gag p27+ population is 170) (Fig. 3d). Finally, we observed low levels of CD4 on the surface of Gag p27+ cells (the average MFI of CD4 of the double negative population was 120, while the Gag p27+ population was 192; in $n = 20$ animals $p = 0.00017$ by two-tailed, paired Student's *t*-test) (see Fig. 3e as a representative example). This result further suggested that CD4 expression was downregulated from the surface of infected cells by the viral protein Nef as predicted on the basis of *in vitro* data (17). To clarify whether productively infected cells were CD4 low or negative on selected samples, we used

Table 1. The frequency of cells with integrated SIVmac239 viral genome is 30–50 times higher in the p27+ population compared to the CD4+p27– population

ANIMAL ID	VIRAL GAG DNA COPIES/CELL			PLASMA VIRAL LOAD (VRNA COPY EQ/ML)
	CD4+p27–	CD4–p27–	p27+	
98063	0.023	0.0015	1.14	$1.63\text{E} + 08$
96016	0.028	0	0.86	$2.28\text{E} + 07$
02039	0.015	0	0.6	$5.61\text{E} + 06$

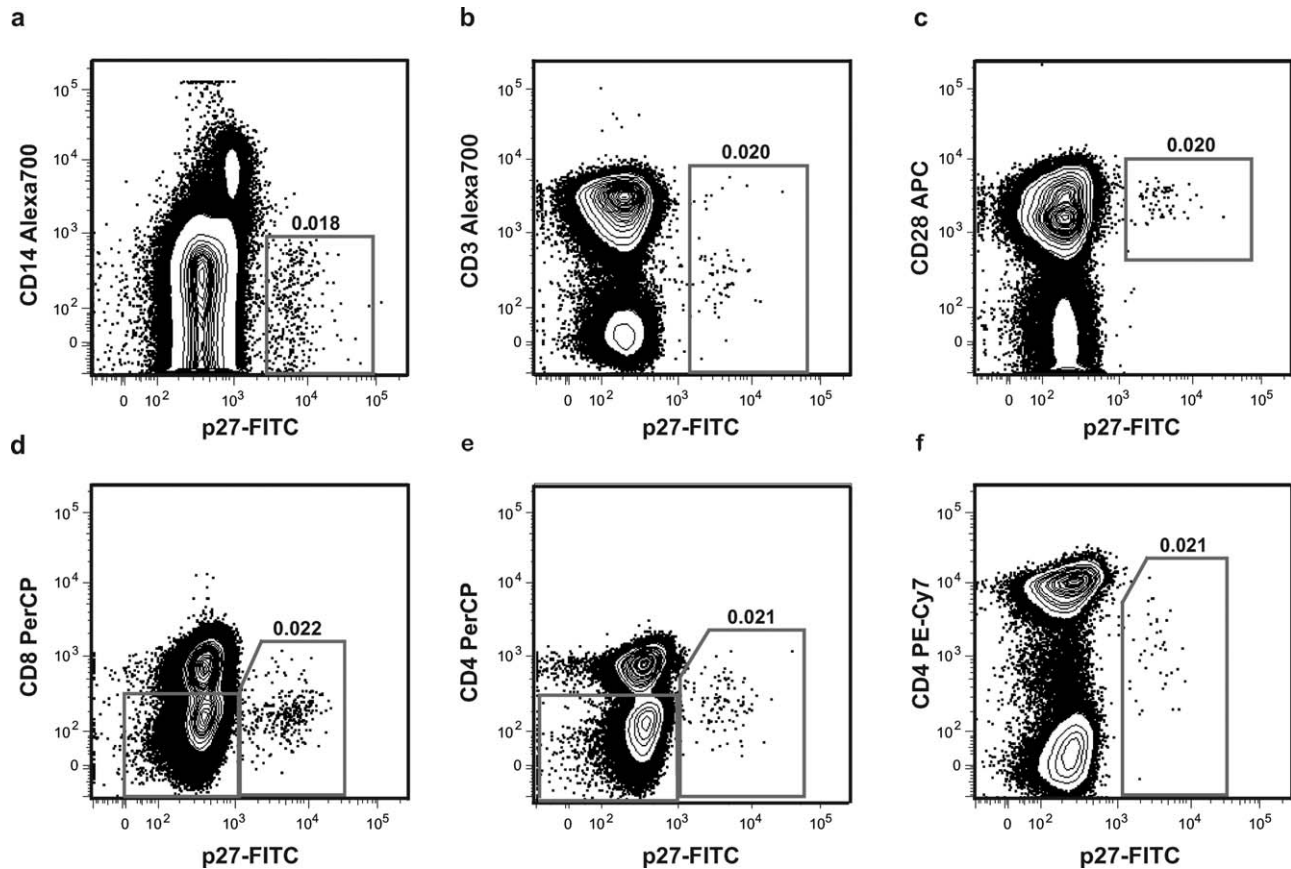


Figure 3. Intracellular Gag p27 staining of lymph node samples visualizes CD3+CD4+ SIVmac239 infected cells that productively support viral proliferation. **a:** p27+ cells are negative for CD14 antigen; **(b)** p27+ cells express CD3 at a low level; **(c)** p27+ are CD28+; **(d)** they are negative for CD8 antigen (MFI of CD8 of the double negative population is 152, while the p27+ population is 170). **e:** p27+ cells express CD4 at a low level (MFI of CD4 of the double negative population is 123, while the p27+ population is 260). **f:** a better separation between CD4- and CD4+ populations reveals that p27 Gag+ cells express CD4, at least at a low level. The plots show a lymph node sample from an animal with a $1.00\text{E} + 06$ vRNA copy Eq/ml plasma viral load at 129 weeks postinfection. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

another anti-CD4 antibody conjugated to a brighter fluorophore (PE-Cy7) to achieve better separation between the positive and negative populations (Fig. 3f). With this stain, we were able to confirm that the Gag p27+ cells were in fact CD4 low+ instead of CD4 negative lymphocytes. Collectively, these observations indicated that the p27+ population detected in our enriched lymph node samples was indeed CD4+ T cells actively producing SIVmac239 virions.

Next we tested whether our enrichment method could also enhance the ability to detect productively infected cells in peripheral blood. Indeed, we were able to identify productively infected cells in the PBMC. However, the frequency of Gag p27+ cells was consistently lower in comparison to parallel lymph node samples (Table 2). This suggested that cells isolated from the blood or lymph nodes might have different limits of detection.

The ability of this assay to detect productively infected cells is dependent on the frequency of these cells in the selected tissue and that is likely linked to the amount of virus replication occurring at any given time or location. To assess the sensitivity of our assay, we plotted the frequency of Gag p27+ cells detected in enriched cells against concomitant plasma viral loads, the most reliable measure of virus replication. We

discovered that the ability to detect Gag p27+ cells in lymphocytes derived from either lymph nodes (Fig. 4a) or blood (Fig. 4b) is tightly associated with the concurrent plasma viral load of the animal ($n = 23$; $P < 0.0001$ and $n = 14$; $P < 0.0001$ respectively with two-tailed Spearman rank correlation analysis). However, the threshold for the amount of circulating virus needed to reliably detect Gag p27+ cells was approxi-

Table 2. The frequency of p27+ cells is usually lower in concurrent blood samples than in lymph nodes

ANIMAL ID	P27+ CELL FREQUENCY (%)		PLASMA VIRAL LOAD (VRNA COPY EQ/ML)
	LYMPH NODE	BLOOD	
90131	0.62	0.19	$3.08\text{E} + 08$
98063	0.97	0.29	$1.63\text{E} + 08$
81038	0.055	0.04	$5.00\text{E} + 06$
00073	0.029	0.019	$5.03\text{E} + 05$
01034	0.015	0.013	$1.12\text{E} + 06$

The used gating strategy is detailed in Figure 1 and identical for both tissue type.

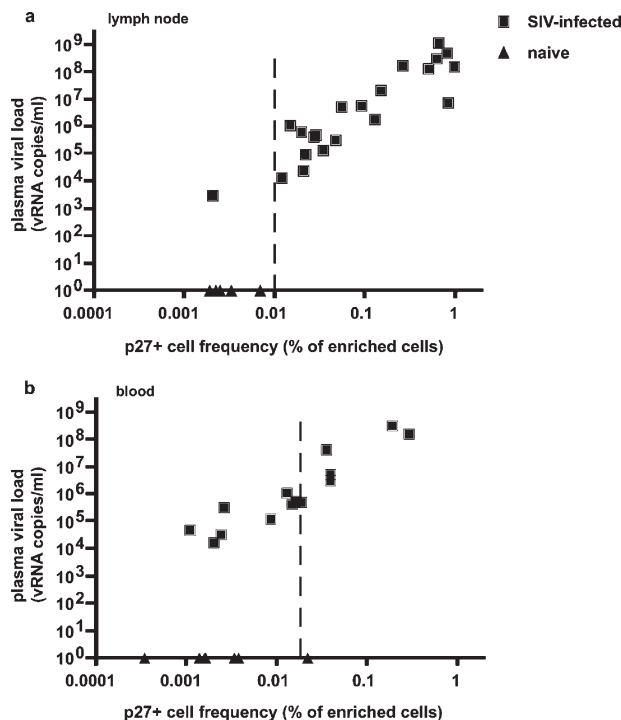


Figure 4. The frequency of SIV infected cells (p27+ cells) of the enriched samples correlate with the contemporary plasma viral load. **a:** infected cells from lymph node samples. **b:** Infected cells from peripheral blood samples. Association was analyzed by Spearman rank correlation analysis. *P* values were <0.0001 in both blood and lymph node samples. Dashed line represents the threshold of detection of p27+ cells by FACS analysis, which we defined as $2 \times \text{geometric mean} + \text{standard deviation}$ ($2 \times \text{GM} + \text{SD}$) of the frequency of p27+ cells detected in the uninfected rhesus macaque samples. The value of $2 \times \text{GM} + \text{SD}$ is 0.01% for the lymph node samples and 0.02% for the blood samples.

mately two logs lower for lymph node derived cells ($\sim 2.5 \times 10^4$ vRNA copy Eq/ml) than those isolated from the blood ($\sim 10 \times 10^5$ vRNA copy Eq/ml) (Figs. 4a and 4b). This corroborated our initial hypothesis that the frequency of productively infected cell in the lymph nodes was higher than the peripheral blood and suggested that lymph nodes were a better source for further analysis.

DISCUSSION

The success of this method is due to the combination of an enrichment step, the use of critical tissue samples, the application of recent advancements in flow cytometric data collection, and the flow cytometric gating strategy. With this protocol, we detected between 49 and 4,988 naturally infected SIV⁺ cells per sample using 30–60 million Ficoll-purified mononuclear cells as starting material. This accounted for 0.012%–0.97% of all the lymphocytes in the enriched samples, after exclusion of dead cells and contaminating CD20⁺ or CD8⁺ cells. This cell frequency and number enables investigators to use multicolor flow analysis and cell sorting to isolate productively infected cells and perform more subtle assays. It is important to note that due to the rarity of infected cells, especially at lower virus levels, care must be taken in the execution of the

analysis. To ensure that only cells actively producing virus are analyzed, inclusion of one or two surface markers (CD3 and/or CD4) that are downmodulated during virus replication must also be incorporated into the staining and gating strategy (17). Given that a certain amount of false positive events will be inevitably included in the analysis using this method we suggest that investigators use lymph node samples of animals with viral loads approximately 1–1.5 logs higher than the threshold of detection, i.e., $> 5 \times 10^5$ vRNA copy Eq/ml plasma viral load.

We detected a higher frequency of Gag p27+ cells in lymphocytes derived from lymph nodes than the blood. The likely reason for this difference is the amount of active virus replication and density of cells within these respective tissues. Lymph nodes are composed of millions of lymphocytes within close proximity of each other. In this environment, HIV/SIV can efficiently move from cell to cell. Conversely, lymphocytes located in the blood move in a more expansive, dynamic environment as they travel between lymphatic tissues. Therefore, we believe using lymph node samples instead of blood-derived lymphocytes will facilitate the investigation of the effects of virus replication on lymphocytes.

A previous study analyzed HIV-1 infected cells in the peripheral blood of recently infected patients ex vivo using flow cytometry and QPCR assays (21). We found several major differences between the results of the human study and our study in rhesus macaques. We detected a lower frequency of Gag p27+ cells and number of copies of *gag* DNA per cell in our samples than the ones reported for HIV-1 infected humans. Additionally, the previous study found a relatively high frequency of Gag⁺ cells in CD4[−] cells, whereas we did not. There are several potential reasons for these discrepancies. First, we show that in macaques Gag p27+ cells are CD4 low/dim rather than truly CD4 negative. It is possible that the cells identified as CD4 negative in the previous study are also CD4 low/dim. Second, the animals used in our study were infected with the highly pathogenic cloned virus SIVmac239, in comparison to a likely more heterogeneous HIV-1 population in the previous study. It is possible that some HIV clones within the same patient downregulate CD4 below the detection level of flow analysis. Third, we performed our *gag* QPCR analysis on animals chronically infected with SIVmac239, while the human study examined recent seroconverters. It is possible that during the course of infection viral species more efficient in preventing superinfection of the same target cells are preferentially selected. If so, this could effect the number of viruses that can infect an individual cell and may explain the fewer copies of *gag* DNA per cell that we detected.

A key element in the success of our method was the reliable antibody against Gag p27. We anticipate that this method would be applicable to other SIV strains with high sequence homology to SIVmac239, and chimeric viruses (such as SHIV89.6P) that use SIVmac239 *gag* as part of the viral backbone (22,23).

Here, we described a method and determined the limits of the analysis of SIV infected cells ex vivo by flow cytometry and QPCR. This improved technique represents a key advance in the available tools and will facilitate our understanding of SIV pathogenesis.

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