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Analysis of multiple cell reservoirs expressing unspliced HIV-1 gag-pol mRNA in patients on antiretroviral therapy

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Abstract

Aims—Longitudinal percentage change of eight HIV-1 gag-pol mRNA cellular reservoirs from HIV-infected subjects on antiretroviral therapy was ascertained by simultaneous ultrasensitive subpopulation staining/hybridization *in situ* (SUSHI).

Materials & methods—Serial peripheral blood mononuclear cells were taken from three subjects with treatment success, limited response and viral breakthrough plasma viral load (PVL) profiles. SUSHI was carried out on monocytes, macrophages, CD4⁺ cells and naive, memory and activated T-cell reservoirs followed with broad light scatter flow cytometry.

Results—All gag-pol⁺ reservoirs declined in the treatment success patient and similar to PVL. Only some gag-pol⁺ reservoirs responded similarly to PVL for the limited treatment patient, and most gag-pol⁺ reservoirs increased 16 weeks prior to PVL breakthrough in the viral breakthrough patient.

Conclusion—SUSHI measures changes in a wide range of gag-pol⁺ reservoirs in response to antiretroviral therapy.

Keywords

activated memory T cell; antiretroviral; cellular reservoirs; HIV-1; macrophage; memory T cell; monocyte; naive T cell; total CD4⁺; transcriptionally active

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Tests designed to detect cell-associated integrated HIV proviral DNA, as well as unintegrated (episomal) HIV DNA have been used to both identify HIV cell reservoirs, including T-cells, monocytes/macrophages, astrocytes, dendritic cells, hematopoietic progenitor cells and NK cells [1,2], and monitor cell reservoirs in response to antiretroviral therapy (ART) [3–5]. While these DNA assays utilizing total extracted cellular DNA, followed by PCR, identify HIV-infected cells, they cannot be used to demonstrate HIV transcriptional activity.

HIV transcriptional activity is identified using assays that detect cell-associated HIV mRNA. Most assays detect HIV-1 or HIV-2 mRNA from extracted and purified total cell-associated RNA by PCR without identifying the reservoir source [6–9] unless incorporating cell sorting [10]. Cell-associated HIV transcriptional activity is ascertained in 62–89% patients on highly active ART (HAART) with a plasma viral load of <50 copies HIV-1 RNA/ml [8,9] facilitating immune activation, inflammatory responses, reseeding reservoirs, non-opportunistic diseases and HIV-1 evolution [11–19]. The increase in cell-associated HIV RNA in the absence of detectable plasma viral load correlates with the emergence of drug resistance well before plasma viral load breakthrough [8].

The Virotest HIV-1 Viral Reservoir Assay (Virotest VR™, IncellDx, Inc., CA, USA) utilizes simultaneous ultrasensitive subpopulation staining/hybridization *in situ* (SUSHI), combining cell surface immunophenotyping with ultrasensitive-FISH, with probes hybridizing to unspliced HIV-1 gag-pol mRNA [20–23] with a detection sensitivity of 30 copies of HIV-1 gag-pol mRNA/cell (gag-pol⁺) [21]. The HIV gag-pol probes are comprised of a pool of 5-carboxyfluorescein labeled oligonucleotide probes, which, in aggregate, hybridize to conserved regions of HIV-1 gag-pol mRNA [23]. SUSHI applied to HIV-1-infected cells was first validated with the ACH-2 cell lines and patient samples [23]. SUSHI detects HIV transcriptional activity from intact, immunophenotyped cells and therefore differs from other HIV transcription assays utilizing extracted and purified total cell-associated RNA. This assay has been successfully used to identify gag-pol⁺ activity in peripheral blood mononuclear cell subsets at one time point, during both stable HAART [21] and during immune therapy and HAART [22] from drug-naïve HIV-infected patients [23,24], in placental tissue [25,26], cultured peripheral blood mononuclear cells (PBMCs) and semen from patients on long-term HAART [27], an *in vitro* organ culture model [28] and in T cells expressing CXCR4/CCR5 [29].

The use of SUSHI to monitor transcriptionally active reservoirs from patients on ART has been proposed for ‘personalized tailoring of therapy based on an individual’s HIV-infected compartments [20]. Since the analyte (unspliced HIV-1 gag-pol mRNA) detected by SUSHI is similarly detected by other cell-based assays used to ascertain ART modulation of HIV transcriptional activity, then SUSHI should also demonstrate ART modulation of HIV transcriptional activity from identified cell reservoirs. SUSHI had been used to monitor the modulation of the gag-pol⁺ memory T-lymphocyte reservoir in response to ART [30] but this article now reports the first study to use SUSHI to measure the modulation of a wide range of gag-pol⁺ cellular reservoirs in response to ART. In this observational pilot study we evaluated SUSHI to measure longitudinal changes in the percent of gag-pol⁺ monocyte, monocyte/macrophage, total CD4⁺ cells, naïve, memory and activated T-cell reservoirs from three patients on ART with distinctly different plasma viral load profiles: treatment success, limited response and treatment failure. This study was not designed to derive conclusions about treatment outcomes but rather to demonstrate the utility of SUSHI to measure the longitudinal modulation on gag-pol⁺ reservoirs in response to ART, which we demonstrate, in consideration for use on larger patient numbers and with different combinations of ART.

Materials & methods

Patients

In this retrospective study, frozen PBMC samples were obtained from three patients with differing plasma viral load profiles (Roche Amplicor HIV-1 Assay, Roche Molecular Systems, Inc., NJ, USA) in response to ART, including treatment success (patient X), limited response (patient Y) and potential viral breakthrough (patient Z). Patients X, Y and Z had a CD4 <500/μl, were off therapy for a minimum of 4 weeks and were then on therapy consisting of two nucleoside inhibitors including abacavir (300 mg/twice daily), lamivudine (150 mg/twice daily) and the protease inhibitor amprenavir (1200 mg/twice daily). Treatment ranged from 12 weeks (patient Y) to 48 weeks (patients X and Z). Pre- and end-treatment levels of viremia in patient X were: 110,499 and <50 copies HIV-1 RNA/ml, respectively; for patient Y: 98,237 and 51,112 copies HIV-1 RNA/ml, respectively, and for patient Z: 498,760 and 174 copies HIV-1 RNA/ml, respectively.

Patient samples

Samples had been previously collected and stored at the Department of Medicine, Division of Infectious Diseases, Northwestern University Medical School (IL, USA) and then sent for testing at the Esoterix Center for Innovation (renamed as LabCorp Clinical Trials, Advanced Cytometric Applications, TN, USA). These pre-existing frozen PBMCs in freeze mix were obtained from patient X: baseline, weeks 4, 12, 16, 24 and 48; patient Y: baseline, weeks 2, 4 and 12; and patient Z: weeks 2 (lacking PBMCs at baseline) 4, 12, 24, 32 and 48.

HIV⁺ & HIV⁻ controls

The ACH-2 cell line containing a single copy of integrated HIV-1 proviral DNA per cell with limited-to-no expression of HIV-1 mRNA [31] is routinely used as a control [20,22,23] and residual HIV-1 mRNA is not detected with the SUSHI gag-pol probes (below level of detection) [23]. Induction of ACH-2 HIV-1 RNA expression with phorbol 12-myristate 13-acetate (PMA) [32] at 0.1 mg/ml PMA (Sigma Aldrich, MI, USA) was used as a positive control to verify hybridization and signal detection of SUSHI gag-pol probes [23]. This cell line was used as a positive (stimulated) and an operationally negative (unstimulated) control in the first publication describing the SUSHI technology demonstrating a linear response between the percentage of stimulated ACH-2 cells as measured by the SUSHI fluorescent HIV-1 gag-pol probes versus the actual percentage of stimulated ACH-2 cells by dilution [23].

In this study both unstimulated and 0.1 mg/ml PMA-stimulated ACH-2 cells were harvested at an early passage and tested.

Additionally, PBMCs from five HIV-negative donors were processed in the same manner as the PBMC samples from the three HIV-positive patients X, Y and Z and tested.

Simultaneous ultrasensitive subpopulation staining/hybridization *in situ*

The protocol has been described in detail [20–23]. Known significant cellular reservoirs were selected for this analysis [1,2] and were further delineated into memory- and naive-T cells in resting and activated states, and monocyte to macrophage differentiation to discern relative levels of gag-pol⁺ reservoirs (Table 1).

SUSHI reagents 1–7 are available in a complete kit from InCellDx. Antibodies (Table 1) used to delineate cell populations were obtained from Becton Dickinson (CA, USA) or BD Pharmingen (CA, USA). Frozen PBMCs were thawed, washed and processed as previously described [20]. Briefly, thawed cells were incubated with antibody combinations (Table 1),

washed, fixed, then hybridized with the HIV-1 probe cocktail (probes spanning HIV-1 unspliced gag-pol mRNA) and washed. A FACSCalibur (Becton Dickinson, CA, USA) was setup according to ECFI quality systems based on a modified windows of analysis protocol and cells were analyzed after collecting a minimum of 30,000 total events. Data analysis was performed using WinList 3.0 (Verity Software House, ME, USA) with Dynamic Data Exchange links to Excel (Microsoft, WA, USA).

Cell size was ascertained by mixing 100 μ l of stained cells with 100 μ l of Gel/Mount (Biomedica Corporation, CA, USA), 50 μ l was transferred to a 25 $\times$ 75 $\times$ 1 mm glass slide, cover slipped (Fisher Scientific, PA, USA), loaded onto the stage of an Laser Scanning Cytometer[®] (CompuCyte, MA, USA) and 3000 cells were analyzed following the instrument setup described below.

Daily system setup was ensured using 10 micron beads (Spherotech, IL, USA) with the same mounting system listed above. The protocol collects a cytogram that correlates scatter max pixel with the area signal, measured in square microns, along with fluorescence measured in green (FITC), orange (PE) and red (Pe-Cy5). Samples were analyzed contouring on scatter max pixel and 3000 intact cells were collected. Lymphocytes were divided into cells that expressed CD4⁺/CD45RO⁺ and the HIV-1 gag-pol probe and cells that expressed CD4⁺/CD45RO⁺ but lacked the HIV-1 gag-pol probe. Cell populations were relocated on the slide and visually displayed on the color CCD camera to verify presence/absence of the probe. Once established, the area signal of each cell was calculated using the onboard WinCyt[®] (CompuCyte) software and recorded for each patient. The square root of the area signal equates to the size of each observed cell measured in microns.

FACS analyses

A broad forward/side scatter light scatter gate, a side scatter/surface marker cytogram with color eventing turned on, was used to define the outer vertices based on surface marker expression allowing surface marker expression to define the specific cell subsets to include all pertinent subsets regardless of cell size in the analysis of gag-pol⁺.

Ethics

This study was approved by and performed in accordance with the guidelines of the Northwestern University committee on human research (institutional review board) and written informed consent was obtained from human participants. This study used pre-existing samples.

Statistical methods

The mean and standard deviation were used to compare gag-pol⁺ and gag-pol⁻ cell sizes (15 paired results).

The level of correlation based on changes in gag-pol⁺ cellular and plasma virus reservoirs in response to ART was determined between all paired reservoirs (cellular and plasma viruses) by the Pearson correlation coefficient. All times points (baseline and follow-up weeks on ART) from each reservoir were included in the analyses. Interpretation of the level of correlation included: no correlation $r = 0.05$, weak correlation $r = 0.5-0.7$, moderate correlation $r = >0.7-0.8$ and strong correlation $r = 0.8-1.0$.

Results

HIV⁺ & HIV⁻ controls

SUSHI HIV-1 gag-pol probe fluorescence was detected in all (100%) stimulated ACH-2 cells but in <1% unstimulated ACH-2 cells and in <1% cells from each cellular reservoirs from the five HIV negative donors (data not shown). The SUSHI gag-pol limit of detection was therefore set at 2% cells with detectable fluorescence for all cellular reservoirs.

Size of gag-pol⁺ cells

Using laser scanning cytometry gag-pol⁺ memory T cells CD4⁺/CD45RO⁺ were compared with the gag-pol⁻ counterpart (Figure 1A & B). Gag-pol⁺ cells exhibit heterogeneous blebs of hybridized, HIV-1 unspliced gag-pol mRNA at the cell membrane (Figure 1A) differing from smoothly spherical gag-pol⁻ cells (Figure 1B). Cell diameter from 15 paired observations (gag-pol⁺ versus gag-pol⁻) was determined by laser scanning cytometry. The average diameter of gag-pol⁺ was 13.27 microns (standard deviation: 0.74) and gag-pol⁻ was 10.54 microns (standard deviation 0.76, $p < 0.001$, Mann–Whitney) (Figure 1A & B) and therefore all gag-pol⁺ cells do not appear in a normal lymphocyte population as typically defined by forward and orthogonal light scatter. Representative broad light scatter plots are illustrated in Figure 2.

Cell reservoir-specific response of gag-pol⁺ to ART

Relative proportions of gag-pol⁺ reservoirs were established from baseline samples in patients X and Y, and at week 2 in patient Z (Figures 3–5, respectively); increasing percentage of gag-pol⁺ cell reservoirs was noted from naive to memory/central memory to activated memory T cells and from monocyte/macrophage to macrophage cell types. Total CD4⁺ gag-pol⁺ reservoir was proportionally lower than the memory and activated memory T-cell subsets.

Cell-specific responses to ART are illustrated for patients X, Y and Z (Figures 3, 4 & 5, respectively). Pearson correlation coefficients were determined from all time points between each gag-pol⁺ reservoir and with plasma viral load. Patient X (treatment success, Table 2) had a strong correlation between plasma viral load and 4/8 cell reservoirs, and between 8/28 paired cell reservoirs, patient Y (limited response, Table 3) had a strong correlation between plasma viral load and 2/8 cell reservoirs, and between 10/28 paired cell reservoirs and patient Z (potential viral breakthrough, Table 4) had a strong correlation between plasma viral load and 1/8 cell reservoirs and between 1/28 paired cell reservoirs.

Overall, weak inverse correlations were only detected in both limited response and treatment failure patients Y (Table 3) and Z (Table 4), respectively, and not in treatment success patient X (Table 2).

In patient X (treatment success) a biphasic decline (decline–increase–decline) was observed for plasma viral load and all gag-pol⁺ cell reservoirs (except for central memory T cells CD4⁺/CCR7⁺ which declined, plateaued, then declined).

In patient Z (potential viral breakthrough), gag-pol⁺ became detectable in all reservoirs by week 32 and prior to detectable plasma virus at week 48 (Figure 5).

Discussion

Cells actively infected by HIV are known to have abnormal morphology resembling lymphocyte blasts [33–37] and we demonstrate that gag-pol⁺ T-cells are larger than gag-pol⁻ T-cells (13.4 μ M vs 10.6 μ M, $p < 0.001$, Mann–Whitney) with broad light scatter. We

observe that such enlarged gag-pol⁺ cells may not always appear in normal lymphocyte gates by light scatter resulting in underestimated numbers of transcriptionally active cells.

The relative proportions of gag-pol⁺ reservoirs depend on the maturity and activation state of the cell population [24,38] and is reflected in baseline samples with an increasing proportion of gag-pol⁺ reservoirs with maturity and activation. Total CD4⁺ gag-pol⁺ reservoirs are within the range of 1–10% CD4⁺ lymphocytes infected with HIV-1 [39].

The level of HIV-1 transcriptional activity is modulated by the stage and state of cell activation and we show that the relative proportions of gag-pol⁺ T-cell subset reservoirs were lowest (mainly undetectable) for naive T-cells, higher for memory/central memory cells and were the highest for activated memory/central memory cells. Naive T cells, newly generated from bone marrow, not having recognized cognate antigen and not having undergone activation, are permissive to HIV-1 infection [40], however, with limited viral production [41]. Subtle stimulation [41], including cytokines [42,43], Nef signaling by stimulated macrophages [44], tissue microenvironments [45–47] and dendritic cell-mediated CXCR4 tropic HIV-1 transmission [38] can activate HIV replication in naive T cells. Viable memory T cells are infected prior to the resting state [48] and during the resting state, contain integrated HIV-1 proviral DNA, express HIV-mRNA [21,49–52] and represent a larger, transcriptionally active HIV-1 reservoir compared with the naive T-cell reservoir. *In vitro* studies demonstrate several fold increase in virus production compared with naive T cells [40,53–55]. Activated memory T cells provide the cellular conditions necessary for productive HIV-1 replication [54,56] and represent a reservoir with the highest numbers of cells expressing HIV-1 mRNA.

The relatively differentiated monocyte/macrophage CD14⁺/CD16⁺ reservoir have more gag-pol⁺ cells compared with the less differentiated monocyte CD14⁺/CD16[−] reservoir and can be explained, in part, by upregulation of the CCR5 receptor with monocyte differentiation [24]. Less differentiated CD14⁺⁺/CD16[−] [57] and CD14⁺/CD16[−] [51] monocyte subsets are infected with HIV-1 and express HIV-1 mRNA [51] but the more differentiated monocyte/macrophage CD14⁺/CD16⁺ subset is more permissive to HIV infection and to HIV replication [57]. Between 0.01 to 1.0% of monocytes and monocyte/macrophages are infected with HIV [58] and are lower than what we report for corresponding gag-pol⁺ cells. Few studies have defined the *in vivo* proportion of infected cell with transcriptionally active HIV in monocyte and monocyte/macrophage reservoirs, which, along with FACS gate settings, may explain this discrepancy. Overall, the relative proportions of gag-pol⁺ reservoirs are in agreement with the literature.

Correlation between all gag-pol⁺ reservoirs in response to ART is variable. Weak inverse correlations were only seen in ART limited response and failure patients, Y and Z, and not in the treatment success patient, X, the significance of this is unclear at this time. Low-to-inverse correlation between reservoirs or slow response by gag-pol⁺ reservoirs to ART may reflect variations in HIV replication dynamics and intracellular drug efficacies [59–62] and/or discordant HIV-1 drug resistance reservoirs within the same individual [63–68], or additional unidentified reservoir subsets, while high correlations may suggest a homogenous virus population within reservoirs responding similarly to ART.

The biphasic decline in gag-pol⁺ cell reservoirs and plasma viral load for patient X may reflect variable turnover and decay rates of each reservoir, as determined by variable levels of activation and reactivation of HIV within each reservoir and the gradual decline in this feedback mechanism following ART [69].

Finally, in patient Z an increase in gag-pol⁺ reservoirs at 16 weeks was observed prior to detectable plasma viral load and was similar to a previous report demonstrating increased

levels of unspliced HIV-1 mRNA (from total cellular-derived RNA) prior to plasma viral breakthrough and was predictive of treatment failure [8]. It should be noted, however, that plasma virus of 178 copies HIV-1 RNA/ml detected at week 48 could very well represent a viral blip rather than true plasma virus breakthrough, and that a follow-up sample would help to confirm true plasma virus breakthrough.

This study was designed as an observational pilot to determine if SUSHI can be used to measure modulation of gag-pol⁺ reservoirs in response to ART. The study's limitations include the small patient number and a treatment combination involving the same antiretrovirals. The study did not address either HIV-1 genotypic or phenotypic resistance, or pharmacokinetics for each identified reservoir. This additional information (reservoir-specific HIV resistance and pharmacokinetics) would further explain variations in response to ART and differing Pearson correlation coefficient *r* values between reservoirs (both cellular and plasma viruses). The study does present an alternate option to assess HIV-1 transcriptionally active reservoirs at the intact immunophenotyped cell rather than from extracted and purified cell-associated RNA, thereby presenting an additional tool for the purpose of studying transcriptionally active reservoirs at the single-cell level.

Conclusion

In conclusion, we demonstrate that SUSHI measures changes in the percent of HIV-1 gag-pol⁺ reservoirs in patients on ART. Additional studies will need to address larger patient populations but also different combinations of ART considering the efficacies of different antivirals in different cell types, additional reservoirs and compartmentalized HIV drug resistance reservoirs. These data may further explain the dynamics of each cell reservoir. Finally, while peripheral blood represents only one compartment of HIV-1-infected cell reservoirs [70], SUSHI is also designed for analyses of various tissue samples as previously demonstrated [25,26,28].

Future perspective

New approaches in treatment therapies, including targeted therapies and eradication, will demand new tests. The source of viruses as defined by cellular reservoirs continues to gain attention in light of discordant reservoir drug concentrations, chronic, low-level HIV replication and immune activation, all undetectable by commercial plasma viral load measurements. These pathogenic processes, central to the maintenance of HIV infection, will require new tests in support of novel treatment strategies. Such tests are needed to effectively identify the infection and the pathogenic hierarchy of cell reservoirs, as well as genotypic, phenotypic and pharmacokinetic analyses of such reservoirs. Targeted therapies using, for example, antiretrovirals and small interfering RNA associated with nanocarriers, as well as vaccines, both for therapeutic and protective application, as well as HIV activators, will need tests to identify their efficacies at the cellular level, particularly in terms of modulating effects on cell-associated HIV transcriptional activity. Additionally, functional tests that can be used to monitor immune activation and immune status will be needed to assess the elusive 'functional cure', defined as the immune system's return to normality. Plasma viral load cannot be used to support these new treatment approaches. Overall, the utility of plasma viral load for the new approach in HIV treatment and eradication will become increasingly tenuous as other, more appropriate, tests are implemented.

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Executive summary

- Simultaneous ultrasensitive subpopulation staining/hybridization *in situ* (SUSHI) preserves cell integrity and combines cell surface immunophenotyping and ultrasensitive fluorescence *in situ* hybridization with probes hybridizing to unspliced HIV-1 gag-pol mRNA (gag-pol).
- Cellular reservoir percentage of gag-pol^{+/-} intact monocyte, macrophage, total CD4⁺ cells, and naive, memory and activated memory T cells was determined from longitudinal samples of patients on antiretroviral therapy.
- Relative proportions of each gag-pol⁺ reservoir is in agreement with reports from literature (lowest to highest gag-pol⁺ from naive to memory to activated memory T-cell reservoir and monocyte to macrophage reservoir). Percent CD4⁺ reservoir was within reported range.
- All gag-pol⁺ reservoirs declined as did plasma viral load in the treatment success subject, varied in response in the limited response patient and declined then increased prior to plasma viral breakthrough in the treatment failure patient.
- Some gag-pol⁺ reservoir remained transcriptionally active with undetectable plasma viral load.
- Pearson correlation coefficient values between all gag-pol reservoirs and plasma viral load varied between subjects; the treatment success subject had the highest number of paired reservoirs with a high correlation followed by the treatment failure subject and lowest with the limited treatment subject.
- Cell-specific pharmacokinetics, HIV drug resistance and HIV replication kinetics may explain varying response to antiretroviral therapy as measured in each reservoir.
- SUSHI has been used to analyze gag-pol⁺ reservoirs in tissue other than peripheral blood.
- Targeted treatment should be effectively measured in different reservoirs by SUSHI.
- The number of gag-pol^{+/-} reservoirs detected by SUSHI is only limited by the number of detectable cell populations.

Figure 1. Images demonstrating the size of gag-pol⁺ cells and gag-pol⁻ cells

CD4⁺/CD45RO⁺ cells from patients were tested by subpopulation staining/hybridization *in situ*. A 100-µl resuspended cell pellet was mixed with 100 µl of gel/mount and was transferred to a glass slide, cover slip sealed with clear nail polish and loaded onto the stage of an Laser Scanning Cytometer® (CompuCyte, MA, USA) and 3000 cells were analyzed. **(A)** CD4⁺/CD45RO⁺ gag-pol⁺ cells (circled in white) that exhibit heterogeneous blebs of hybridized, HIV-1 unspliced gag-pol mRNA at the cell membrane. **(B)** CD4⁺/CD45RO⁺ gag-pol⁻ cells (circled in white) that appear smoothly spherical. **(A)** CD4⁺/CD45RO⁺ gag-pol⁺ cells are larger than corresponding gag-pol⁻ cells in **(B)** (13.27 µm, standard deviation 0.74 versus 10.54 µm, standard deviation 0.76; $p < 0.001$, Mann–Whitney).

Figure 2. Broad light scatter plots

A broad light scatter gate (R1) allows phenotypic expression to define cell populations regardless of size. Note the smaller $CD4^+/CD45RO^-$ cells (in red) cluster tightly in the predominant lymphocyte population whereas the larger $CD4^+/CD45RO^+$ cells (in blue) fall outside the main cluster. This same relationship is observed in Figure 1 where the differences in size of $gag-pol^+$ and $gag-pol^-$ cells were measured.

FSC: Forward scatter; SSC-H: Side scatter pulse height.

Figure 3. Dynamics of gag-pol⁺ reservoirs versus plasma viral load in treatment success patient X on antiretroviral therapy

Patient X initiated antiretroviral therapy including nucleoside inhibitors and a protease inhibitor and was selected for SUSHI analysis based on the PVL profile indicating treatment success. Stored, frozen peripheral blood mononuclear cells from baseline, weeks 4, 12, 16, 24 and 48 were tested by SUSHI and results compared with corresponding PVL values. A biphasic decay following antiretroviral therapy in gag-pol⁺ reservoirs is noted, in particular with the activated memory T-cell subsets, and is reflected in the PVL profile. SUSHI on week 4 monocyte CD14⁺/CD16⁻ and monocyte/macrophage CD14⁺/CD16⁺ cells was not carried out.

LOD: Limit of detection; PVL: Plasma viral load; SUSHI: Simultaneous ultrasensitive subpopulation staining/hybridization *in situ*.

Figure 4. Dynamics of gag-pol⁺ reservoirs versus plasma viral load in limited response patient Y on antiretroviral therapy

Patient Y initiated antiretroviral therapy including nucleoside inhibitors and a protease inhibitor and was selected for SUSHI analysis based on the PVL profile indicating limited then no response to treatment. Stored, frozen peripheral blood mononuclear cells from baseline, weeks 2, 4 and 12 were tested by SUSHI and results compared with corresponding PVL values. Similar to patients X and Z, the activated T-cell subsets had the highest proportions of cells expressing gag-pol⁺. All other reservoirs increased or decreased at each time point.

LOD: Limit of detection; PVL: Plasma viral load; SUSHI: Simultaneous ultrasensitive subpopulation staining/hybridization *in situ*.

Figure 5. Dynamics of gag-pol⁺ reservoirs versus plasma viral load in potential viral breakthrough patient Z on antiretroviral therapy

Patient Z initiated antiretroviral therapy including nucleoside inhibitors and a protease inhibitor and was selected for SUSHI analysis based on the PVL profile indicating treatment success followed by potential viral breakthrough. Stored, frozen peripheral blood mononuclear cells from weeks 2 (no baseline sample available), 4, 12, 24, 32 and 48 were tested by SUSHI and results compared with corresponding PVL values. Similar to patients X and Y, the activated T-cell subsets had the highest proportions of cells expressing gag-pol⁺. Reservoirs initially responded to the antiretroviral treatment but then increased at week 32 and 16 weeks before potential breakthrough in PVL.

LOD: Limit of detection; PVL: Plasma viral load; SUSHI: Simultaneous ultrasensitive subpopulation staining/hybridization *in situ*.

Table 1

Cell reservoirs identified by simultaneous ultrasensitive subpopulation staining/hybridization *in situ*.

Peripheral blood mononuclear cell	Immunophenotypic markers
Monocyte	CD3 ⁻ /CD14 ⁺ /CD16 ⁻
Monocyte/macrophage	CD3 ⁻ /CD14 ⁺ /CD16 ⁺
Total CD4 ⁺ cell	CD4 ⁺ /CD3 ⁺
Naive T cell	CD4 ⁺ /CD45RO ⁻ /CD45RA ⁺
Memory T cell	CD4 ⁺ /CD45RA ⁻ /CD45RO ⁺
Central memory T cell	CD4 ⁺ /CCR7 ⁺
Activated memory T cell	CD4 ⁺ /CD45RO ⁺ /HLA-DR ⁺
Activated central memory T cell	CD4 ⁺ /CCR7 ⁺ /HLA-DR ⁺

Antibody concentration optimization to identify the above cell reservoirs is first performed under nucleic acid hybridization conditions to take into account the effects of elevated temperature on the antibody-conjugated fluorescent probes. Appropriate antibody concentrations along with an aggregate of probes to unspliced HIV-1 gag-pol⁺ mRNA were then used by simultaneous ultrasensitive subpopulation staining/hybridization *in situ* to evaluate quantitative changes in gag-pol⁺ cell reservoirs from sequential frozen peripheral blood mononuclear cell samples from patients initiating antiretroviral therapy.

Table 2

Patient X (treatment success): Pearson correlation coefficient.

	Plasma viral load	Monocytes CD14 ⁺ /CD16 ⁻	Monocyte/macrophage CD14 ⁺ /CD16 ⁺	Total CD4 ⁺ cells	Naive T cells CD4 ⁺ /CD45RO ⁻	Memory T cells CD4 ⁺ /CD45RO ⁺	Activated memory T cells CD4 ⁺ /HLA-DR ⁺	Central memory T cells CD4 ⁺ /CCR7 ⁺	Activated central memory T cells CD4 ⁺ /CCR7 ⁺
Plasma viral load	–	0.10	0.96	0.03	–0.31	0.93	0.98	0.90	0.74
Monocytes CD14 ⁺ /CD16 ⁻	0.10	–	0.37	–0.06	–0.09	0.09	0.11	0.00	0.68
Monocyte/macrophage CD14 ⁺ /CD16 ⁺	0.96	0.37	–	–0.02	–0.34	0.86	0.93	0.85	0.88
Total CD4 ⁺ cells	0.03	–0.06	–0.02	–	0.94	0.39	0.18	0.41	–0.02
Naive T cells CD4 ⁺ /CD45RO ⁻	–0.13	–0.09	–0.34	0.94	–	0.04	–0.17	0.08	–0.03
Memory T cells CD4 ⁺ /CD45RO ⁺	0.93	0.09	0.86	0.39	0.04	–	0.97	0.95	0.72
Activated memory T cells CD4 ⁺ /CD45RO ⁺ /HLA-DR ⁺	0.98	0.11	0.93	0.18	–0.17	0.97	–	0.92	0.76
Central memory T cells CD4 ⁺ /CCR7 ⁺	0.90	0.00	0.85	0.41	0.08	0.95	0.92	–	0.54
Activated central memory T cells CD4 ⁺ /CCR7 ⁺ /HLA-DR ⁺	0.74	0.68	0.88	–0.02	–0.03	0.72	0.76	0.54	–

Interpretation of the correlation coefficient level is based on: no correlation <0.05, weak correlation 0.5–0.7, moderate correlation >0.7 and <0.8, strong correlation 0.8–1.0.

Strong correlations were determined between plasma viral load and four cell reservoirs, including monocyte/macrophage CD14⁺/CD16⁺, memory CD4⁺/CD45RO⁺, central memory CD4⁺/CCR7⁺ and activated memory CD4⁺/CD45RO⁺/HLA-DR⁺; between monocyte/macrophage CD14⁺/CD16⁺ and four T-cell subset cell reservoirs, including memory CD4⁺/CD45RO⁺, central memory CD4⁺/CCR7⁺, activated memory CD4⁺/CD45RO⁺/HLA-DR⁺ and activated central memory T-cell CD4⁺/CCR7⁺/HLA-DR⁺; and between four pairs of T-cell subset cell reservoirs, including memory CD4⁺/CD45RO⁺ and activated memory CD4⁺/CD45RO⁺/HLA-DR⁺, memory CD4⁺/CD45RO⁺ and central memory CD4⁺/CCR7⁺, central memory CD4⁺/CCR7⁺ and activated memory CD4⁺/CD45RO⁺/HLA-DR⁺, and between total CD4⁺ and naive CD4⁺/CD45RO⁻.

Table 3

Patient Y (limited response): Pearson correlation coefficient.

	Plasma viral load	Monocytes CD14 ⁺ /CD16 ⁻	Monocyte/macrophage CD14 ⁺ /CD16 ⁺	Total CD4 ⁺ cells	Naive T cells CD4 ⁺ /CD45RO ⁻	Memory T cells CD4 ⁺ /CD45RO ⁺	Activated memory T cells CD4 ⁺ /CD45RO ⁺ /HLA-DR ⁺	Central memory T cells CD4 ⁺ /CCR7 ⁺	Activated central memory T cells CD4 ⁺ /CCR7 ⁺ /HLA-DR ⁺
Plasma viral load	–	–0.54 [†]	–0.26	–0.03	0.21	–0.12	–0.16	0.84	0.86
Monocytes CD14 ⁺ /CD16 ⁻	–0.54 [†]	–	0.52	0.85	0.64	0.89	0.91	–0.22	–0.50 [†]
Monocyte/macrophage CD14 ⁺ /CD16 ⁺	–0.26	0.52	–	0.34	0.00	0.36	0.37	–0.49	–0.67 [†]
Total CD4 ⁺ cells	–0.03	0.85	0.34	–	0.93	1.00	0.99	0.32	0.00
Naive T cells CD4 ⁺ /CD45RO ⁻	0.21	0.64	0.00	0.93	–	0.91	0.89	0.61	0.35
Memory T cells CD4 ⁺ /CD45RO ⁺	–0.12	0.89	0.36	1.00	0.91	–	1.00	0.24	–0.08
Activated memory T cells CD4 ⁺ /CD45RO ⁺ /HLA-DR ⁺	–0.16	0.91	0.37	0.99	0.89	1.00	–	0.20	–0.12
Central memory T cells CD4 ⁺ /CCR7 ⁺	0.84	–0.22	–0.49	0.32	0.61	0.24	0.20	–	0.95
Activated central memory T cells CD4 ⁺ /CCR7 ⁺ /HLA-DR ⁺	0.86	–0.50 [†]	–0.67 [†]	0.00	0.35	–0.08	–0.12	0.95	–

[†]A negative (inverse) correlation value except for values with no correlation.

Interpretation of the correlation coefficient level is based on: no correlation <0.05, weak correlation 0.5–0.7, moderate correlation >0.7 and <0.8 and, strong correlation 0.8–1.0.

Strong correlations were determined between plasma viral load and two cell reservoirs: central memory CD4⁺/CCR7⁺ and activated central memory CD4⁺/CCR7⁺/HLA-DR⁺; between monocyte CD14⁺/CD16⁻ and total CD4⁺ and two T-cell subset cell reservoirs, including memory CD4⁺/CD45RO⁺ and activated memory CD4⁺/CD45RO⁺/HLA-DR⁺; between total CD4⁺ and three T-cell subset cell reservoirs, including naive CD4⁺/CD45RO⁻, memory CD4⁺/CD45RO⁺ and activated memory CD4⁺/CD45RO⁺/HLA-DR⁺; and between four pairs of T-cell subset cell reservoirs, including naive CD4⁺/CD45RO⁻ and memory CD4⁺/CD45RO⁺, naive CD4⁺/CD45RO⁺ and activated memory CD4⁺/CD45RO⁺ and activated memory CD4⁺/CD45RO⁺/HLA-DR⁺, and central memory CD4⁺/CCR7⁺ and activated central memory CD4⁺/CCR7⁺/HLA-DR⁺. Weak inverse correlations were determined between plasma viral load and 1/8 cell reservoirs and between 2/28 paired cell reservoirs, including plasma viral load and monocyte CD14⁺/CD16⁻ and between activated central memory T-cell CD4⁺/CCR7⁺/HLA-DR and two monocytic cell reservoirs, including monocyte CD14⁺/CD16⁻ and monocyte/macrophage CD14⁺/CD16⁺.

Table 4

Patient Z (potential viral breakthrough): Pearson correlation coefficient.

Plasma viral load	Monocytes CD14 ⁺ /CD16 ⁻	Monocyte/macrophage CD14 ⁺ /CD16 ⁺	Total CD4 ⁺ cells	Naive T cells CD4 ⁺ /CD45RO ⁻	Memory T cells CD4 ⁺ /CD45RO ⁺	Activated memory T cells CD4 ⁺ /CD45RO ⁺ /HLA-DR ⁻	Central memory T cells CD4 ⁺ /CCR7 ⁺	Activated central memory T cells CD4 ⁺ /CCR7 ⁺ /HLA-DR ⁺
Plasma viral load	-	-0.37	-0.09	-0.05	-0.56 [†]	0.16	0.60	0.80
Monocytes CD14 ⁺ /CD16 ⁻	-0.37	-	0.43	0.32	0.04	0.34	-0.02	-0.69 [†]
Monocyte/macrophage CD14 ⁺ /CD16 ⁺	-0.09	0.43	-	-0.21	-0.03	-0.19	-0.05	-0.37
Total CD4 ⁺ cells	-0.05	0.32	-0.21	-	0.65	0.97	0.64	-0.28
Naive T cells CD4 ⁺ /CD45RO ⁻	-0.56 [†]	0.04	-0.09	0.65	-	0.46	0.20	-0.56 [†]
Memory T cells CD4 ⁺ /CD45RO ⁺	0.16	0.34	-0.19	0.97	0.46	-	0.74	-0.14
Activated memory T cells CD4 ⁺ /CD45RO ⁺ /HLA-DR ⁺	0.60	-0.02	-0.05	0.64	0.20	0.74	-	0.14
Central memory T cells CD4 ⁺ /CCR7 ⁺	0.75	-0.68 [†]	-0.18	0.16	0.04	0.24	0.73	0.60
Activated central memory T cells CD4 ⁺ /CCR7 ⁺ /HLA-DR ⁺	0.80	-0.69 [†]	-0.37	-0.28	-0.56 [†]	-0.14	0.14	-

[†] A negative (inverse) correlation value except for values with no correlation.

Interpretation of the correlation coefficient level is based on: no correlation <0.05, weak correlation 0.5–0.7, moderate correlation >0.7 and <0.8 and, strong correlation 0.8–1.0.

Strong correlations were determined between plasma viral load and activated central memory CD4⁺/CCR7⁺/HLA-DR⁺, and between one pair of T-cell reservoirs: total CD4⁺ and memory CD4⁺/CD45RO⁺. Weak inverse correlations were determined between plasma viral load and 1/8 cell reservoirs and between 3/28 paired cell reservoirs, specifically plasma viral load and naive T-cell CD4⁺/CD45RO⁻; between monocyte CD14⁺/CD16⁻ and two T-cell subset reservoirs, including central memory CD4⁺/CCR7⁺ and activated central memory CD4⁺/CCR7⁺/HLA-DR⁺; and between one pair of T-cell subset reservoirs including naive CD4⁺/CD45RO⁻ and activated central memory CD4⁺/CCR7⁺/HLA-DR⁺.