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# Plasmacytoid dendritic cell and functional HIV Gag p55-specific T cells before treatment interruption can inform set-point plasma HIV viral load after treatment interruption in chronically suppressed HIV-1$^+$ patients

Emmanouil Papasavvas,[1] Andrea Foulkes,[2] Xiangfan Yin,[1] Jocelin Joseph,[1] Brian Ross,[1] Livio Azzoni,[1] Jay R. Kostman,[3] Karam Mounzer,[4] Jane Shull[4] and Luis J. Montaner[1]

[1]*The Wistar Institute, Philadelphia, PA,* [2]*School of Public Health and Health Sciences, University of Massachusetts, Amherst, MA,* [3]*Presbyterian Hospital-University of Pennsylvania Hospital, Philadelphia, PA, and* [4]*Philadelphia Field Initiating Group for HIV-1 Trials, Philadelphia, PA, USA*

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Correspondence: Dr Luis J. Montaner, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA.
Email: montaner@wistar.org
Senior author: Dr Luis J. Montaner

## Summary

**The identification of immune correlates of HIV control is important for the design of immunotherapies that could support cure or antiretroviral therapy (ART) intensification-related strategies. ART interruptions may facilitate this task through exposure of an ART partially reconstituted immune system to endogenous virus. We investigated the relationship between set-point plasma HIV viral load (VL) during an ART interruption and innate/adaptive parameters before or after interruption. Dendritic cell (DC), natural killer (NK) cell and HIV Gag p55-specific T-cell functional responses were measured in paired cryopreserved peripheral blood mononuclear cells obtained at the beginning (on ART) and at set-point of an open-ended interruption from 31 ART-suppressed chronically HIV-1$^+$ patients. Spearman correlation and linear regression modeling were used. Frequencies of plasmacytoid DC (pDC), and HIV Gag p55-specific CD3$^+$ CD4$^-$ perforin$^+$ IFN-$\gamma^+$ cells at the beginning of interruption associated negatively with set-point plasma VL. Inclusion of both variables with interaction into a model resulted in the best fit (adjusted $R^2 = 0{\cdot}6874$). Frequencies of pDC or HIV Gag p55-specific CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells at set-point associated negatively with set-point plasma VL. The dual contribution of pDC and anti-HIV T-cell responses to viral control, supported by our models, suggests that these variables may serve as immune correlates of viral control and could be integrated in cure or ART-intensification strategies.**

**Keywords:** antiretroviral therapy interruption; HIV-specific CD8$^+$ T cells; plasmacytoid dendritic cells; set-point viral load.

## Introduction

The loss of viral control following HIV infection in the absence of antiretroviral therapy (ART) has been associated with both viral and host factors such as *in vivo* viral diversity and clonal exhaustion,[1–3] as well as with the loss and/or functional impediments of adaptive and innate cells.[4–9] ART results in viral suppression, and restores, at least partially, adaptive functions (i.e. CD4$^+$ T-cell counts,[10] functional HIV-specific cell-mediated responses,[11]), and the frequency and function of innate effector cells,[7] but is unable to result in life-long viral suppression and/or eradication.[12–14] As a result, there is need for the development of strategies that could support cure or ART intensification-related strategies.

Innate and adaptive cell subsets and function have been shown to contribute to delayed progression to AIDS and/or protection from infection, suggesting that

Abbreviations: APC, allophycocyanin; ART, antiretroviral therapy; DC, dendritic cell; IFN-$\gamma$, interferon-$\gamma$; IQR, interquartile range; mDC, myeloid DC; NK, natural killer; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid DC; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; VL, viral load


 

the identification of immune correlates of viral control could be important in the development of new strategies against HIV. Studies in long-term non-progressors, viraemic controllers, acutely infected early-treated patients interrupting therapy, or discordant couples have found that $CD4^+$ T-cell lymphoproliferative responses, Gag-specific $CD8^+$ T-cell responses, or frequency of plasmacytoid dendritic cells (pDC) are associated with lower viral replication in the absence of ART.[15–21] Furthermore, studies in both humans and non-human primates suggest that during the post-acute phase of HIV infection, $CD8^+$ T cells directed primarily against Gag correlate with viral suppression,[22–27] whereas other studies suggest that the quality of $CD8^+$ T-cell responses might also play a role in viral control.[16,28–31] In addition to adaptive immune responses, the potential role of the innate immune system, particularly of natural killer (NK) cells and DC, in the establishment and control of HIV infection has also been supported by several reports demonstrating an inverse correlation between both numbers of mature NK cells and DC and HIV viral load (VL).[7,9,32–34.]

Intermittent treatment strategies have been explored for their ability to augment the ART-mediated immune recovery of anti-HIV-1 responses in chronically HIV-$1^+$ patients, with the rationale that repeated, controlled antigenaemia may reactivate pre-existing responses and/or result in *de novo* immunization, yet they have failed to show a clear virological or immunological benefit of ART interruption.[35–40] Although long-term ART interruption strategies have been associated with CD4 decline and increased risk of opportunistic infections, short-term ART interruptions (< 6 weeks) do not appear to negatively affect the rebound of $CD4^+$ T-cell count to pre-interruption levels upon ART re-initiation and viral re-suppression.[41]

The levels of viral rebound during ART interruptions differ between individuals and seem to be related to a balance established by the immune system during primary infection.[42,43] Hence, ART interruption strategies may still be used as a tool to investigate the mechanisms determining viral set-point, and to identify set-point correlates and reliable predictors. A single report has shown a negative association between pDC frequency and levels of HIV VL rebound during ART interruption in acute infection,[42] so identifying pDC as a potential immune correlate of viral control. It remains unknown if the same would be observed in ART-treated patients after chronic $HIV^+$ infection.

Based on findings from our previous study,[44] showing that viral set-point did not differ during an open-ended ART interruption between chronically suppressed participants with or without preceding repeated ART interruptions, we evaluated retrospectively how ART-recovered innate and/or adaptive parameters associated with/or predicted viral set-point upon ART interruption by analysing cryopreserved peripheral blood mononuclear cells (PBMC) collected in our previous study before and at viral set-point of ART interruption.

## Materials and methods

### *Participants*

We evaluated cryopreserved PBMC obtained from 31 ART-suppressed chronically HIV-1 infected patients at the beginning (on ART) and at set-point of an open-ended ART interruption. Set-point plasma HIV VL was defined as the average plasma HIV-1 RNA of the first three consecutive measures with < 0·5 log difference. Although pre-interruption PBMC samples were available for all 31 patients, 15 of the 31 participants had available set-point PBMC samples. Any data point not collected because of the limitations of cell yield at thaw was not included in the analysis, so accounting for any differences from the data of 31 or 15 participants presented for pre-interruption or set-point, respectively. All donors were part of a larger cohort of 42 chronically suppressed HIV-1 infected patients participating in a parent study based in Philadelphia (USA). A detailed characterization of the cohort has been published elsewhere;[39] entry criteria for the parent study were age ≥ 18 years, ongoing ART (three or more drugs), current CD4 count > 400 cells/μl (nadir CD4 ≥ 100 cells/μl), and current plasma HIV VL < 50 copies/ml (> 6 months history of VL < 500 copies/ml). Informed consent was obtained according to the Human Experimentation Guidelines of the US Department of Health and Human Services and of the authors' institutions. The study protocol was approved by the Institutional Review Boards of the Wistar Institute and Philadelphia Field Initiating Group for HIV-1 Trials.

### *Flow cytometry-based phenotypic characterization of innate immune cell subsets*

Peripheral blood mononuclear cells were thawed, washed twice with cold 1 × PBS, blocked with serum for 10 min at room temperature, and stained ($0{\cdot}5 \times 10^6$ PBMC per condition) for 30 min on ice with the following anti-human cell surface monoclonal antibody combinations: (i) CD3-Peridinin chlorophyll protein (PerCP)Cy5.5, CD56- FITC, CD16-phycoerythrin (PE), HLA-DR-PECy7, CD161-allophycocyanin (APC), (ii) Lin-1-FITC, HLA-DR-APC, CD11c-PE, and (iii) Lin-1-FITC, HLA-DR-APC, CD123-PE. The following isotypes were used: IgG1-PerCPCy5.5, IgG1-FITC, IgG1-PE, IgG1-PECy7, IgG1-APC. After staining, cells were washed, incubated for 5 min at room temperature with 1 ml 1 × FACS Lyse, washed again, re-suspended in 100 μl FACS washing buf-

fer, and analysed using a nine-colour CyAn cytofluorimeter (Cytomation, Fort Collins, CO) by collecting total PBMC and 50 000 live lymphocytes (the live lymphocytes which are a subset of total PBMC were defined by size and granularity in forward scatter/side scatter). Live cell gates were set manually, and detection thresholds were set according to isotype-matched negative controls. All monoclonal antibodies and buffers used were from Becton Dickinson (BD) Biosciences (San Diego, CA). Stainings 1, 2 and 3 allowed for the assessment of NK cells (defined as $CD3^-$ $CD161^{+/-}$ $CD56^+$ $CD16^+$), myeloid DC (mDC; defined as Lin-$1^-$ HLA-$DR^+$ $CD11c^+$) and pDC (defined as Lin-$1^-$ HLA-$DR^+$ $CD123^{high}$), respectively. Results were reported as % of total PBMC. Data analysis was performed using FLOJO software (TreeStar, San Carlos, CA).

### *Flow cytometry-based assessment of spontaneous and HIV Gag p55-specific T-cell degranulation/cytokine production*

T-cell spontaneous and HIV-1-specific degranulation/cytokine production were assessed by flow cytometry-based measurement of CD107a, perforin and interferon-$\gamma$ (IFN-$\gamma$) in the presence or absence of *in vitro* stimulation with a mixture of 15-amino-acid peptides (15-mer) with their sequences overlapping by 11 amino acids and spanning the sequence of HIV p55 gag (SF2 strain) donated by BD Biosciences,[45] (HIV-1 Gag p55 peptide pool of a total of 127 peptides including four alternate peptides numbered 28A, 29A, 30A and 31A to account for potential AA to DT mutations at amino acids 121–122 present in the MN strain of HIV). Briefly, PBMC were thawed, washed twice with cold 1 × PBS and incubated ($0{\cdot}5 \times 10^6$ per condition) for 4 hr at 37° with Brefeldin A (5 μg/ml, Sigma Aldrich, St Louis, MI) and CD107a-PE (10 μl per $0{\cdot}5 \times 10^6$ cells) or corresponding isotype IgG1-PE in the absence (medium alone: negative control) or presence of *in vitro* stimulation with HIV-1 Gag p55 peptide pool (1·8–2 μg/ml/peptide; BD Biosciences) or *Staphylococcus aureus* enterotoxin B (positive control, 5 μg/ml, Sigma Aldrich). At the end of the incubation cells were stained for 30 min on ice with cell surface monoclonal antibodies (CD3-PerCPCy5.5, CD4-APCCy7, or corresponding surface isotypes IgG1-PerCPCy5.5, IgG1-APCCy7) and then washed, fixed with 1 × FACS Lyse, permeabilized with FACS Perm, and stained with IFN-$\gamma$-PE (or FITC) and perforin-FITC (or corresponding intracellular isotypes IgG1-PE, IgG1-FITC). All antibodies and buffers used were from BD Biosciences. Cells were analysed as described above. T-cell subsets were defined as $CD4^+$ T cells ($CD3^+$ $CD4^+$) or $CD8^+$ T cells ($CD3^+$ $CD4^-$). Degranulating/cytokine-producing $CD8^+$ T cells were defined as $CD3^+$ $CD4^-$ $perforin^+$ IFN-$\gamma^+$, and $CD3^+$ $CD4^-$ $CD107a^+$ IFN-$\gamma^+$ cells. Antigen-specific responses were determined following subtraction of the background responses (no antigen stimulation).

### *Flow cytometry-based assessment of spontaneous and HIV Gag p55-specific T-cell proliferation, degranulation and cytokine production*

T-cell spontaneous and HIV-1-specific proliferation, degranulation and cytokine production were assessed by flow cytometry as follows: upon thawing PBMC ($0{\cdot}5 \times 10^6$ per condition) were stained with CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's instructions and then cultured for 4 days in the absence (medium alone: negative control) or presence of *in vitro* stimulation with an HIV-1 Gag p55 peptide pool (BD Biosciences, 1·8–2 μg/ml/peptide) or *S. aureus* enterotoxin B (positive control, 5 μg/ml, Sigma Aldrich). After a 4-day culture, cells were stained for CD3, CD4, CD107a and IFN-$\gamma$ as described above. All antibodies and buffers used were from BD Biosciences. $CD4^+$ or $CD8^+$ T cells were defined as described above. Since CFSE intensity is halved at each cell division, proliferating T cells ($CD4^+$ or $CD8^+$) were defined as $CD3^+$ $CD4^+$ $CFSE^{lo}$ and $CD3^+$ $CD4^-$ $CFSE^{lo}$ cells. Proliferating/degranulating $CD8^+$ T cells were defined as $CD3^+$ $CD4^-$ $CFSE^{lo}$ $CD107a^+$ cells, whereas proliferating/cytokine-producing T cells ($CD4^+$ or $CD8^+$) were defined as $CD3^+$ $CD4^+$ $CFSE^{lo}$ IFN-$\gamma^+$, and $CD3^+$ $CD4^-$ $CFSE^{lo}$ IFN-$\gamma^+$ cells. Antigen-specific responses were determined as described above.

### *Statistical analysis*

Data were summarized as medians, 25% and 75% centiles (interquartile range, IQR), means, standard deviation, minimum and maximum. For analysis and graphing purposes plasma HIV-1 RNA < 50 copies/ml was considered as equal to 50 copies/ml (threshold of detection). Spearman's rank correlation of set-point plasma HIV VL ($\log_{10}$ copies/ml) with absolute values, or with the absolute difference between antigen-stimulated and unstimulated values, measured at onset or set-point of ART interruption, were performed. Correlations were considered meaningful for Rho values > 0·3 with $P < 0{\cdot}05$. To assess the contribution of the study variables at the beginning of ART interruption to set-point plasma HIV VL ($\log_{10}$ copies/ml) multivariate linear regression models were applied using pre-interruption innate (pDC, mDC, NK) and adaptive [$CD8^+$ T cells degranulation/cytokine production ($perforin^+$ IFN-$\gamma^+$, $CD107a^+$)] variables as predictors. A backward elimination procedure was applied to select the predictors and the final model was selected based on the highest adjusted $R^2$. All statistical analysis was carried out using R.2.5.1 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).


 

## Results

### Plasma HIV VL reaches set-point during ART interruption

The demographics and clinical characteristics of the patients whose samples were used in the current study are shown in Table 1. The median CD4 count before ART interruption was 669 cells/μl (IQR 513–808), while all patients had VL < 50 copies/ml. As expected, ART interruption resulted in viral rebound and set-point. The median CD4 count at set-point was 478 cells/μl (IQR 373–623·5). Plasma HIV VL for all subjects during ART interruption (median time for set-point = 67 days, IQR 56–99·75) is displayed in Fig. 1(a), whereas the distribution of pre-interruption and set-point plasma HIV VL (median set-point VL = 16 153 copies/ml, IQR 36 443–41 042) is summarized in Fig. 1(b).

### Association of pre-interruption pDC frequency and HIV-specific $CD8^+$ T-cell responses with set-point plasma HIV VL

The association between set-point plasma HIV VL ($log_{10}$ copies/ml) and pre-interruption frequency of innate cell subsets (pDC, mDC, NK cells) or T-cell function (T-cell spontaneous and HIV-1 Gag p55-specific degranulation, cytokine production and proliferation) were assessed using Spearman's rank correlation tests; the results of this analysis are shown in Table 2. Among innate parameters, we observed a significant negative association between set-point plasma HIV VL and pre-interruption frequency of pDC ($n = 29$, $P = 0{\cdot}0342$, Rho = –0·3944, Table 2, Fig. 2 left panel) but not with any of the other innate cell subsets studied (i.e. mDC and NK cells). Among adaptive parameters, we observed a negative association between set-point plasma HIV VL and HIV Gag p55-specific $CD8^+$ T-cell degranulation/cytokine production

**Table 1.** Demographic and clinical information of patient cohort

| Patient | Year of birth | CD4 count at onset of ART interruption (cells/mm$^3$) | Plasma HIV-1 RNA at onset of ART interruption (copies/ml) | CD4 count at set-point of ART interruption (cells/mm$^3$) | Plasma HIV-1 RNA at set-point of ART interruption (copies/ml) |
|---|---|---|---|---|---|
| S04 | 1962 | 585 | < 50 | 517 | 3325 |
| S05 | 1952 | 307 | < 50 | 284 | 70 489 |
| S07 | 1941 | 1022 | < 50 | 608 | 37 906 |
| S08 | 1948 | 426 | < 50 | 228 | 164 904 |
| S13 | 1958 | 808 | < 50 | 670 | 507 |
| S14 | 1972 | 782 | < 50 | 858 | 16 153 |
| S19 | 1962 | 425 | < 50 | 250 | 48 288 |
| S20 | 1978 | 708 | < 50 | 611 | 3961 |
| S21 | 1962 | 509 | < 50 | 340 | 31 673 |
| S22 | 1952 | 1007 | < 50 | 704 | 27 692 |
| S23 | 1954 | 621 | < 50 | 433 | 44 178 |
| S25 | 1965 | 886 | < 50 | 834 | 2692 |
| S28 | 1953 | 478 | < 50 | 475 | 274 |
| S29 | 1957 | 513 | < 50 | 384 | 264 688 |
| S30 | 1957 | 602 | < 50 | 345 | 750 000 |
| S32 | 1961 | 864 | < 50 | 677 | 21 961 |
| S33 | 1953 | 901 | < 50 | | |
| S35 | 1952 | 516 | < 50 | 464 | 7260 |
| S36 | 1962 | 426 | < 50 | 320 | 4687 |
| S38 | 1960 | 688 | < 50 | 507 | 8460 |
| S40 | 1947 | 594 | < 50 | | |
| S41 | 1948 | 766 | < 50 | 421 | 21 246 |
| S43 | 1951 | 690 | < 50 | 409 | 13 674 |
| S44 | 1958 | 374 | < 50 | 362 | 63 255 |
| S49 | 1948 | 557 | < 50 | 517 | 50 |
| S53 | 1964 | 1320 | < 50 | 701 | 6572 |
| S54 | 1960 | 832 | < 50 | 636 | 21 456 |
| S55 | 1964 | 615 | < 50 | 589 | 192 |
| S60 | 1952 | 669 | < 50 | 469 | 16 249 |
| S61 | 1969 | 775 | < 50 | 478 | 383 |
| S62 | 1955 | 720 | < 50 | 579 | 6423 |

(perforin$^+$ IFN-$\gamma^+$) ($n$ = 22, $P$ = 0·0045, Rho = −0·5817, Table 2, Fig. 2 right panel).

Multivariate linear regression models combining immune variables measured at the beginning of ART interruption were then used, to assess the combined effect of these variables on set-point plasma HIV VL. For each model shown, only patients with available data for all the variables analysed were included, thereby accounting for the different number of patients contributing in each model targeting different cluster of variables. Following backward elimination of predictors the model that included one innate (pDC), and two adaptive (CD107a$^+$ CD8$^+$ T cells, and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells) immune variables as independent predictors resulted in the best fit with an adjusted $R^2$ = 0·4588 (model 1: Table 3, Fig. 3). An outlier (subject S-61) was detected by Grubbs outlier test,[46] with a $P$ value of 0·0175 when model diagnosis procedure was carried out for the residuals. To improve the model fitting, the outlier (subject S-61) was excluded and led to model 2, which gave an adjusted $R^2$ = 0·6567 (model 2: Table 3, Fig. 3). Subsequent elimination from model 2 of CD107a$^+$ CD8$^+$ T cells from the set of the three predictors, resulting in the usage of pDC and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells as

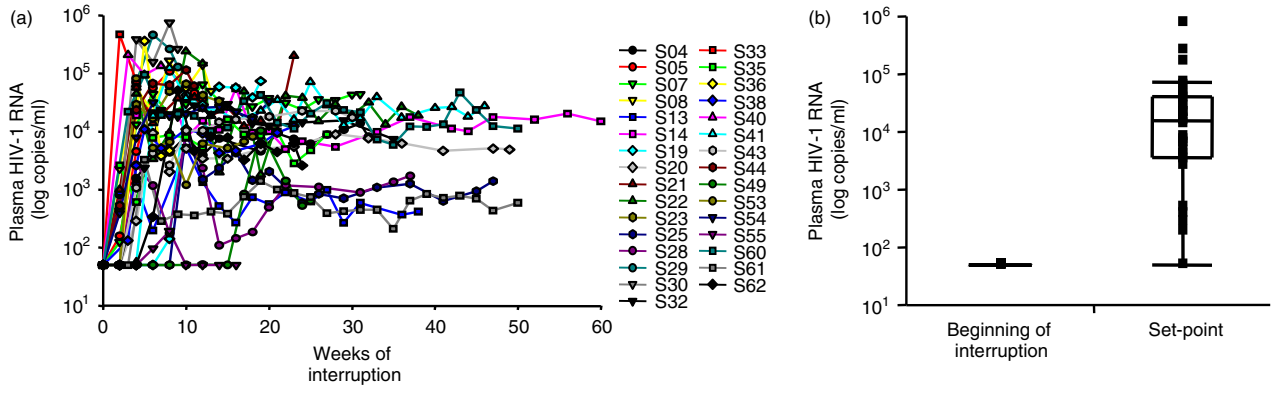


**Figure 1.** Plasma HIV viral load (VL) reaches set-point during antiretroviral therapy (ART) interruption. (a) Plasma HIV VL (HIV-1 RNA copies/ml) during open-ended ART interruption ($n$ = 31). (b) Plasma HIV VL at the beginning ($n$ = 31) and at set-point of ART interruption ($n$ = 31). Data in panel (a) are shown per patient during follow up, and in panel (b) they are shown as IQR boxes (median, and outliers).

**Table 2.** Spearman's rank correlations between set-point plasma HIV viral load ($log_{10}$ copies/ml) and variables at the beginning and at set-point of antiretroviral therapy (ART) interruption

| Variable (% of total peripheral blood mononuclear cells) | Beginning of ART interruption (on ART), $P$ (Rho, $n$) | Set-point of ART interruption, $P$ (Rho, $n$) |
|---|---|---|
| No stimulation | | |
| Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$ | **0·0342** (−0·3944, 29) | **0·0187** (−0·6391, 13) |
| Lin-1$^-$ HLA-DR$^+$ CD11c$^+$ | 0·1995 (−0·2844, 22) | 0·6419 (−0·1364, 14) |
| CD3$^-$ CD161$^{+/-}$ CD56$^+$ CD16$^+$ | 0·6479 (0·1286, 15) | 0·7208 (−0·1099, 13) |
| CD3$^+$ CD4$^-$ perforin$^+$ IFN-$\gamma^+$ | 0·3889 (0·1932, 22) | 0·8396 (0·0572, 15) |
| CD3$^+$ CD4$^-$ CD107a$^+$ IFN-$\gamma^+$ | 0·3235 (0·2105, 24) | **0·0017** (0·7382, 15) |
| CD3$^+$ CD4$^+$ CFSE$^{lo}$ IFN-$\gamma^+$ | 0·3911 (−0·1755, 26) | 0·4733 (−0·209, 14) |
| CD3$^+$ CD4$^-$ CFSE$^{lo}$ IFN-$\gamma^+$ | 0·3435 (−0·1935, 26) | 0·6044 (−0·1518, 14) |
| CD3$^+$ CD4$^-$ CFSE$^{lo}$ CD107a$^+$ | 0·8457 (0·0441, 22) | 0·4738 (−0·2066, 14) |
| CD3$^+$ CD4$^+$ CFSE$^{lo}$ | **0·0217** (−0·4462, 26) | 0·4668 (−0·2036, 15) |
| CD3$^+$ CD4$^-$ CFSE$^{lo}$ | 0·2775 (−0·2212, 26) | 0·9195 (0·0286, 15) |
| HIV Gag p55–unstimulated | | |
| CD3$^+$ CD4$^-$ perforin$^+$ IFN-$\gamma^+$ | **0·0045** (−0·5817, 22) | 0·8399 (0·0595, 14) |
| CD3$^+$ CD4$^-$ CD107a$^+$ IFN-$\gamma^+$ | 0·3124 (0·2153, 24) | 0·1357 (0·4036, 15) |
| CD3$^+$ CD4$^+$ CFSE$^{lo}$ IFN-$\gamma^+$ | 0·4865 (−0·1428, 26) | 0·2306 (−0·3743, 12) |
| CD3$^+$ CD4$^-$ CFSE$^{lo}$ IFN-$\gamma^+$ | 0·8238 (−0·0459, 26) | 0·1176 (−0·4762, 12) |
| CD3$^+$ CD4$^-$ CFSE$^{lo}$ CD107a$^+$ | 0·5321 (0·1408, 22) | **0·0235** (−0·6209, 13) |
| CD3$^+$ CD4$^+$ CFSE$^{lo}$ | 0·4979 (−0·1391, 26) | 0·1142 (−0·4595, 13) |
| CD3$^+$ CD4$^-$ CFSE$^{lo}$ | 0·5726 (−0·116, 26) | 0·0819 (−0·5, 13) |

Bold values indicate P < 0.05


 

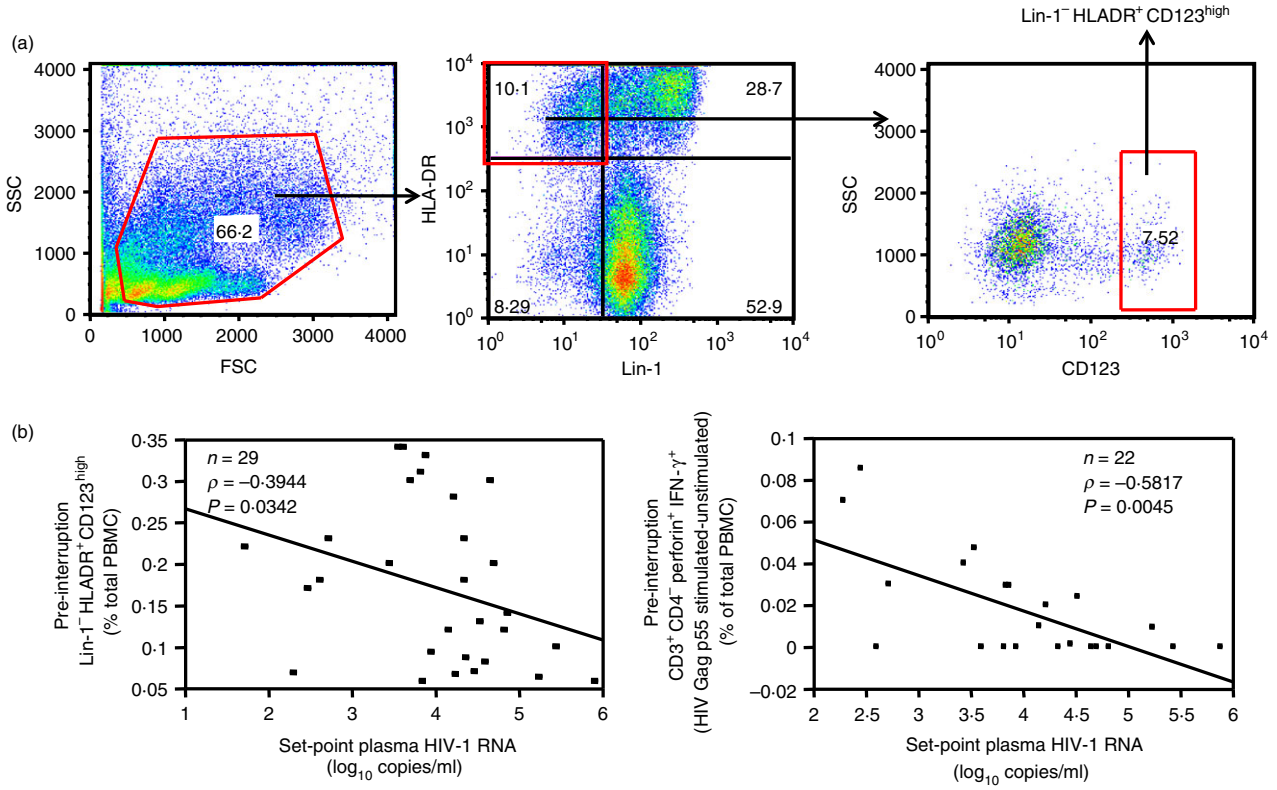


**Figure 2.** Plasmacytoid dendritic cells (pDC) and T-cell HIV Gag p55-specific degranulation/cytokine production at the beginning of antiretroviral therapy (ART) interruption as a correlate of set-point plasma HIV viral load (VL). (a) Gating strategy for pDC (Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$ cells). (b) Spearman's Rank correlation of set-point plasma HIV VL (HIV-1 RNA $\log_{10}$ copies/ml) with the frequency at the beginning of ART interruption of pDC [% of total peripheral blood mononuclear cells (PBMC)] (left panel), and HIV Gag p55-specific (p55 stimulated–unstimulated) CD3$^+$ CD4$^-$ perforin$^+$ IFN-$\gamma^+$ cells (% of total PBMC) (right panel). Data in (b) are shown as regression lines with number of patients, Rho and *P* values included. Note that although Spearman's Rank correlations were performed, regression lines were used for graphic purposes only.

**Table 3.** Final model selection using Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$ and HIV Gag p55-specific Perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells at the beginning of ART interruption as predictors of set-point plasma HIV VL ($\log_{10}$ copies/ml)

| Terms | Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$ | | Perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells | | CD107a$^+$ CD8$^+$ T cells | | | |
|---|---|---|---|---|---|---|---|---|
| Model | Estimate | *P* | Estimate | *P* | Estimate | *P* | Adj. $R^2$ | F-statistic *P* |
| 1 | −2·74 | 0·1279 | −25·62 | 0·0015 | 0·62 | 0·3125 | 0·4588 | 0·0048 |
| 2[1] | −2·54 | 0·0732 | −28·92 | < 0·0001 | 0·56 | 0·2374 | 0·6567 | 0·0002 |

| Terms | Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$ | | Perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells | | Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$: Perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells | | | |
|---|---|---|---|---|---|---|---|---|
| Model | Estimate | *P* | Estimate | *P* | Estimate | *P* | Adj. $R^2$ | F-statistic *P* |
| 3[1] | −2·51 | 0·0528 | −28·33 | < 0·0001 | – | – | 0·6275 | < 0·0001 |
| 4[1] | −4·47 | 0·0066 | −46·77 | 0·0002 | 112·56 | 0·05 | 0·6874 | < 0·0001 |
| 5[1] | −3·00 | 0·102 | – | – | – | – | 0·0649 | 0·1019 |
| 6[1] | – | – | −28·37 | < 0·0001 | – | – | 0·5629 | < 0·0001 |

[1]Outlier (subject S-61) was excluded.

independent predictors, further improved the model (model 3, adjusted $R^2 = 0{\cdot}6275$; pDC $P = 0{\cdot}0528$, and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells $P < 0{\cdot}0001$, Table 3, Fig. 3). The addition of interaction terms for pDC and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells on model 3 resulted in a marginally better model (model 4: adjusted $R^2 = 0{\cdot}6874$, Table 3, Fig. 3) suggesting that pDC might have a positive effect on the association between set-point plasma HIV VL and HIV-specific CD8$^+$ T-cell degranulation/cytokine production. Importantly, pDC alone were not predictive of set-point plasma HIV VL ($P = 0{\cdot}102$, model 5, Table 3, Fig. 3), suggesting that any effect exerted by pDC should be in association with HIV-specific adaptive responses.

### Plasmacytoid DC and HIV-specific CD8$^+$ T-cell proliferation/degranulation at set-point as correlates of set-point plasma HIV VL

The association between set-point plasma HIV VL ($\log_{10}$ copies/ml) following ART interruption and innate and/or adaptive parameters (measured at set-point) was tested using Spearman's rank correlation tests. As noted in the Materials and methods section, although pre-interruption PBMC samples from all 31 patients were available, only 15 had available set-point PBMC samples. As shown in Table 2, and in agreement with the findings at the onset of ART interruption, an inverse association was detected between set-point frequency of pDC and set-point plasma HIV VL ($n = 13$, $P = 0{\cdot}0187$, Rho $= -0{\cdot}6391$, Fig. 4a).

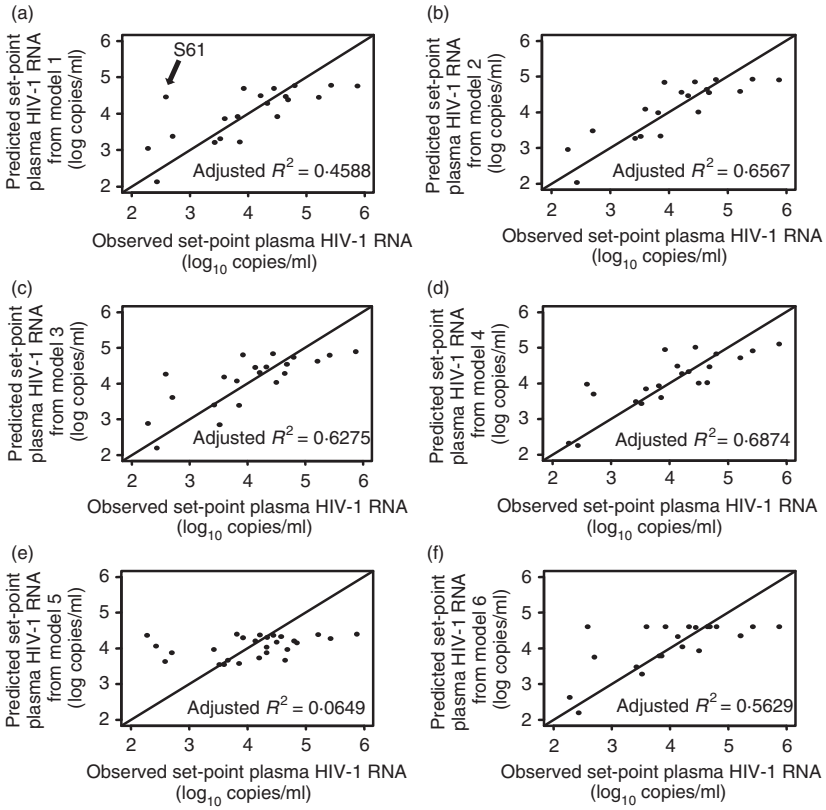


**Figure 3.** Model selection using levels of plasmacytoid dendritic cells (pDC) and HIV-specific CD8$^+$ T-cell degranulation/cytokine production at the beginning of antiretroviral therapy (ART) interruption as predictors of set-point plasma HIV viral load (VL). Effect on set-point plasma HIV VL (HIV-1 RNA $\log_{10}$ copies/ml) using the following variables measured at the beginning of ART interruption as predictors: (a) pDC, CD107a$^+$ CD8$^+$ T cells, and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells as independent predictors (model 1), (b) pDC, CD107a$^+$ CD8$^+$ T cells and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells as independent predictors following the exclusion of an outlier (subject S-61, model 2), (c) pDC and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells as independent predictors following the exclusion of an outlier (subject S-61, model 3), (d) pDC and HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells, including the addition of an interaction term and following the exclusion of an outlier (subject S-61, model 4), (e) pDC following the exclusion of an outlier (subject S-61, model 5), and (f) HIV Gag p55-specific perforin$^+$ IFN-$\gamma^+$ CD8$^+$ T cells following the exclusion of an outlier (subject S-61, model 6). In each model only patients with available data for all the variables included in that model were used. Data in panels (a–f) are shown as plots of predicted values for set-point plasma HIV VL ($\log_{10}$ copies/ml) from corresponding models (axis $y$) against observed values for set-point plasma HIV VL ($\log_{10}$ copies/ml) (axis $x$) with adjusted $R^2$ values included. Lines in plots represent the perfect fit ($x = y$). Arrow in (a) indicates subject S-61 (outlier).

  

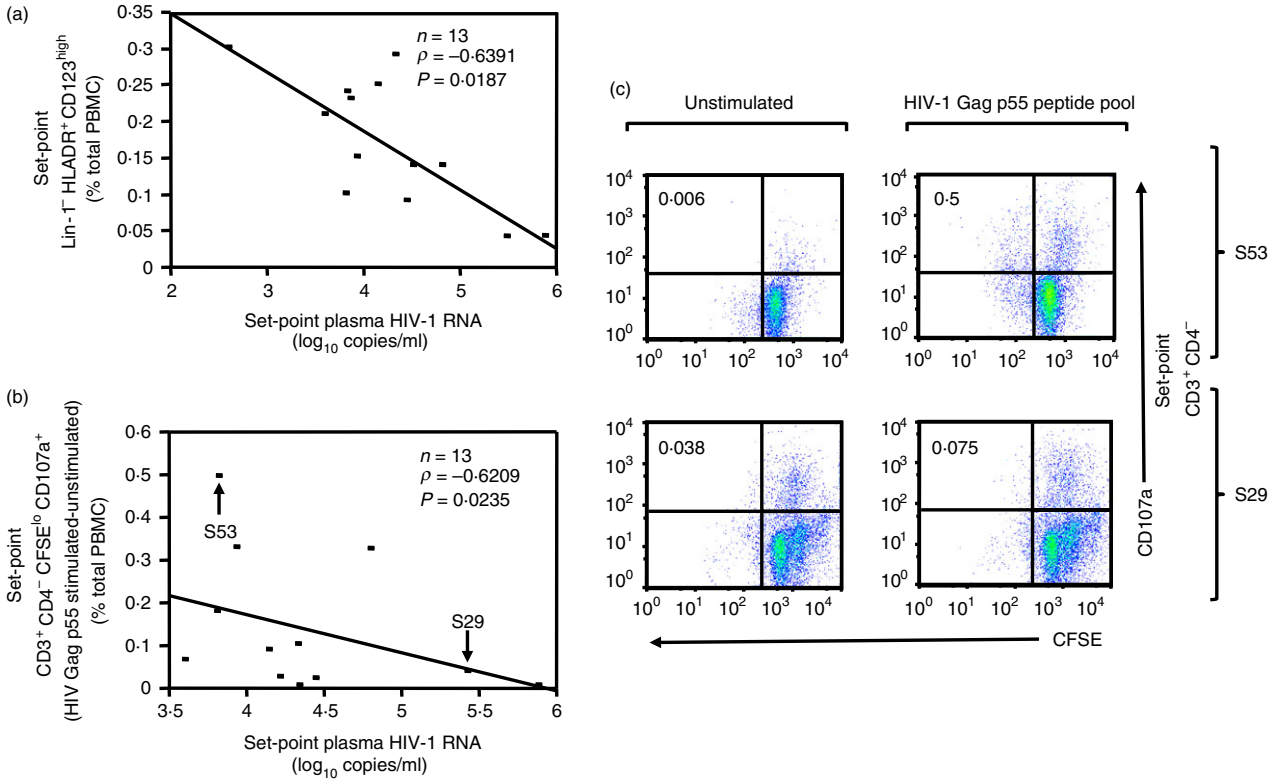


**Figure 4.** Plasmacytoid dendritic cells (pDC) and HIV Gag p55-specific proliferation/degranulation at set-point as correlates of set-point plasma HIV viral load (VL). (a) Spearman's Rank correlation of pDC frequency (Lin-1$^-$ HLA-DR$^+$ CD123$^{high}$ cells) [% of total peripheral blood mononuclear cells (PBMC)] at set-point of antiretroviral therapy (ART) interruption with set-point plasma HIV VL (HIV-1 RNA $\log_{10}$ copies/ml). (b) Spearman's Rank correlation of HIV Gag p55-specific (p55 stimulated–unstimulated) CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells (% of total PBMC) at set-point of ART interruption with set-point plasma HIV VL. Subject S61 [CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells (% of total PBMC) *in vitro* HIV Gag p55–unstimulated = 4·03], although included in the analysis, is not shown in (b) for graphic purposes. Examples of data distribution for high (S53) and low (S29) responder per CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells (% of total PBMC) in the presence of stimulation with HIV-1 Gag p55 peptide pool are indicated in corresponding quadrants in (c). (c) Two representative donors for high and low response measured as CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells (% of total PBMC) in the presence of stimulation with HIV-1 Gag p55 peptide pool are shown. Top panel shows subject S53: set-point plasma HIV VL = 3·81 copies/ml, CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells (% of total PBMC): no stimulation = 0·006 (left), *in vitro* HIV Gag p55 = 0·5 (right), *in vitro* HIV Gag p55–unstimulated = 0·49. Bottom panel shows subject S29: set-point plasma HIV VL = 5·42 copies/ml, CD3$^+$ CD4$^-$ CSFE$^{lo}$ CD107a$^+$ cells (% of total PBMC): no stimulation = 0·038 (left), *in vitro* HIV Gag p55 = 0·075 (right), *in vitro* HIV Gag p55–unstimulated = 0·037. Data in (a) and (b) are shown as regression lines with number of patients, Rho and *P* values included. Note that although Spearman's Rank correlations were performed, regression lines were used for graphic purposes only. Arrows in (b) indicate the two representative subjects (S53 and S29) shown in detail in (c).

Interestingly, an inverse correlation was detected between set-point plasma HIV VL and set-point HIV Gag p55-specific CD8$^+$ T-cell proliferation/degranulation (CSFE$^{lo}$ CD107a$^+$) ($n$ = 13, $P$ = 0·0235, Rho = −0·6209, Table 2, Fig. 4b,c) in accordance with previous reports in HIV-infected non-progressors.[47]

## Discussion

We provide the first report identifying a model where frequency of pDC and HIV Gag p55-specific CD8$^+$ perforin$^+$ IFN-$\gamma^+$ T cells when measured on ART before a treatment interruption can inform set-point plasma HIV VL after interruption. Our data strongly suggest that correlates of viral control off ART may be best evaluated by joining innate and adaptive measures rather than using single isolated variables.

Although several innate cell subsets contribute to pathogen responses, only pDC were found in our study to be associated with set-point plasma HIV VL when measured as a single variable on ART. Indeed, the observation that the pDC frequency in ART-suppressed chronically HIV-1$^+$ subjects undergoing ART interruption is negatively correlated with post-ART interruption set-point supports the report of Pacanowski *et al.*,[42] who reported the same finding in a smaller group of seven primary infected subjects treated for 1 month with ART before a subsequent ART interruption. Interestingly, variance in pDC

frequency on ART maintaining a negative relationship with subsequent viral set-point off ART is also consistent with our earlier reports noting a negative relationship between degree of immune reconstitution in pDC frequency after ART and pre-ART plasma HIV VL.[7] With regards to adaptive responses, the negative correlation of set-point plasma HIV VL and HIV Gag p55-specific $CD8^+$ perforin$^+$ IFN-$\gamma^+$ T cells at the beginning of ART interruption, observed in this study, suggests that HIV control requires effector $CD8^+$ T cells able to produce IFN-$\gamma$ in conjunction with perforin. We interpret that the quality of immune reconstitution with regards to pDC recovery on ART may be a determinant to innate-adaptive cross-talk that may affect the antiviral capacity of the $CD8^+$ T-cell response.

When evaluating immune correlates of viral control once set-point off ART is defined, a negative correlation between set-point plasma HIV VL and set-point frequency of HIV Gag p55-specific $CD8^+$ $CSFE^{lo}$ $CD107a^+$ T cells indicated that measures of correlates of control may be dependent on whether they are measured with/or without viraemia present, as IFN-$\gamma$/perforin content and not CSFE/CD107a emerged as a correlate of control when measured without viraemia and on ART. Finding that HIV-1-specific $CD8^+$ T cells with proliferating/degranulating activity (as defined by CFSE dilution and CD107a expression, respectively) are a correlate of viral control without ART is in agreement with data from studies in non-human primates and elite controllers.[22,26,47–49] Future studies would need to assess whether reports of HIV Gag-specific cellular responses, as associated with lower VL,[25,27,50,51] may yield stronger correlates of control after taking pDC frequency into account, as suggested by our data.

Importantly, we interpret that pDC effects may reflect dual support of innate and HIV-specific adaptive responses by exerting a positive effect on NK cell and T-cell cytotoxicity as well as T-cell perforin content.[52–55] Our study raises the hypothesis that the frequencies of Gag-specific T cells and pDC on ART may predict the level of viral control following ART interruption. In support of this hypothesis, combined administration of IFN-$\alpha$ (as a product of pDC) and ART in chronically HIV-infected subjects has been shown by our group to exert greater viral control after ART interruption when compared with subjects treated only with ART.[56] In addition, an independent set of data from our laboratory also shows that the combination of pDC frequency and HIV Gag-specific $CD8^+$ T cells can best model viral control in the absence of ART among viraemic suppressors,[57] further supporting the strong potential interplay between innate and adaptive mechanisms accounting for HIV control. Future studies will now need to integrate additional host control variables such as IFN-induced gene expression of host anti-viral proteins (Tetherin and APOBEC).

This study has several limitations. First, our analysis did not include an adjustment for multiple testing due to our limited sample size from 31 patients (yet still higher from other studies with similar findings, i.e. the study of Pacanowski *et al.*,[42] in seven patients) and in keeping with the exploratory nature of our report. As a result, our findings are in need of validation by future studies with a larger sample size. We expect that having identified a specific combination of innate and adaptive variables that inform viral set-point after ART interruption may facilitate future validation of our conclusions. Second, no live/dead marker was included in staining. Cells targeted for analysis were initially defined by size and granularity associated with live cell fractions (forward scatter/side scatter) before further cell-specific gating. Although this gating strategy does not exclude all dead cells in the first target gate, subsequent validation tests by re-staining the same samples with a live/dead marker established that the numbers of dendritic cell subsets in the final cell-specific gate were comparable. Although functional antigen-specific T-cell responses above unstimulated are dependent on the live cell fraction and these responses as measured did correlate with viral load, it remains possible that a greater magnitude of response may have been observed with the inclusion of a live/dead marker. Third, we could not address the functionality of innate immune subsets due to specimen limitations. Future studies will be required to investigate the mechanism underlying the observed negative association between set-point plasma HIV VL and pDC frequency. Fourth, although cytokine expression was directly measured, the cytolytic function of T cells against HIV-infected targets was not addressed directly but rather interpreted from joint measurements of perforin, and antigen-specific degranulation (CD107a expression).

Taken together, our data suggest a dual contribution of innate and adaptive mechanisms in determining the levels of viral control to be achieved after ART interruption, and thus support a shift away from evaluating only adaptive cell function when defining what may account for viral control *in vivo*.

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## Disclosures

The authors declare that no conflict of interest exists.

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