**Abstract:**

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Design: We compared VSC of peripheral CD8+ T cells to cytokine production profile in response to peptide stimulation, detailed phenotype (17-color flow-cytometry), reservoir size (total HIV-1 DNA), basal viral transcription (unspliced cell-associated RNA) and inducible viral transcription (tat/rev induced limiting dilution assay) in 36 HIV+ patients on ART and six healthy donors.

Results: We found that the VSC of CD8+ T cells can be increased by prior stimulation with a pool of consensus HIV-1 gag peptides in a significant proportion of progressor patients. We also found that VSC after peptide stimulation was correlated with higher expression of immune checkpoint markers on subsets of terminally differentiated effector memory (TEMRA) CD8+ T cells as well as with production of IFN-γ, TNF-α and IL-10. We did not find a correlation between VSC and viral reservoir measures.

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**Keywords:** Viral suppressive capacity; functional cure; HIV persistence; HIV reservoir; cytotoxic T cell phenotype; immune exhaustion

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INTRODUCTION

HIV-1 persists in a reservoir of long-lived latently infected cells [1, 2]. Most HIV-1 infected subjects are dependent on combination anti-retroviral therapy (cART) to suppress viremia [3, 4] (‘progressors’), while a small proportion controls viremia without treatment (‘elite controllers’) or after a period of cART (‘post-treatment controllers’) [5]. The concept of “functional cure” or “viral remission” is to induce viral control without cART in ‘progressors’ [6-9].

It is generally accepted that a sufficiently reduced viral reservoir and/or potent cytotoxic T cell (CTL) responses are needed to achieve viral remission [10]. Indeed, lower levels of HIV DNA and cell-associated RNA in peripheral blood mononuclear cells (PBMCs) before structured treatment interruption in chronically infected subjects have been associated with delayed time to viral rebound [11, 12]. Studies comparing the direct ex-vivo CTL potential of elite controllers and classical progressors illustrate the importance of polyfunctionality and viral suppressive capacity (VSC) of CD8+ T cells in the control of viremia [13-15]. Furthermore, a correlation between lower pre-therapy expression of immune checkpoint markers on T cells and delayed viral rebound after treatment interruption was shown in the SPARTAC trial [16].

To reduce the size of the viral reservoir, the shock-and-kill strategy, using latency reversing agents, is one currently attempted approach [17]. On the other hand, inducing potent CTL responses might be necessary and could be achieved by therapeutic vaccination. In this respect, in-vitro models evaluating the VSC of CD8+ T cells could help in the evaluation of vaccine candidates before going into clinical trials.

Viral inhibition assays generally use CD8+ T cells which are either unstimulated or stimulated in a polyclonal, non-specific way. According to most published data, this type of VSC is absent or
very low in ‘progressors’, even if they are treated with cART\textsuperscript{[14, 15, 18]}. There are some indications that VSC can be induced by HIV-specific stimulation in-vitro\textsuperscript{[19-21]}, but whether this is the case in all or only some progressors and whether or how this immune function correlates with other immune and viral markers, has not been studied in-depth until now.

Because gag-specific immune responses have been associated with protective immunity\textsuperscript{[22-25]}, we set up a viral inhibition assay in which patient-derived CD8+ T cells are specifically stimulated with a pool of HIV-1 gag peptides before co-culturing them with super-infected autologous CD4+ T cells. We hypothesized that variation in gag-stimulated VSC exerted by CD8+ T cells from well-treated progressors will be observed and that it will correlate with some immunological parameters, such as cytokine production levels or CD8+ T phenotypes, or with viral reservoir measures, such as total HIV DNA and levels of basal and induced viral transcription in CD4+ T cells. Investigating this conceptually important anti-viral function of CD8+ T cells in well-treated progressors and studying its correlation with established immune and viral parameters will provide useful “baseline” knowledge for future immune-based interventions in this large patient population that is to be included in future interventions towards “remission” or “cure”.

**METHODS**

**Study subjects and samples**

Thirty-six chronic HIV-1 infected (progressor phenotype), asymptomatic, cART-treated, adult patients were recruited at the Institute of Tropical Medicine (ITM) in Antwerp. Inclusion and exclusion criteria are given in supplemental methods. One hundred and fifty mL of EDTA blood was collected from these patients. We also obtained buffy coats from 6 healthy HIV-seronegative adult controls from the Red Cross Blood Transfusion Center of Mechelen, Belgium. All
participants gave written informed consent per the Declaration of Helsinki under Antwerp ITM Review Board-approved protocols (Belgian registration number: B300201526243).

PBMCs were isolated by Ficoll-Hypaque (Axis-Shield, Oslo, Norway) density gradient centrifugation, resuspended in 90% FBS 10% DMSO, kept overnight in Mr. Frosty boxes (Thermo Fischer Scientific, Waltham, MA, USA) at -80°C and stored in liquid nitrogen until use.

**Viral inhibition assay**

Viral suppressive capacity (VSC) was measured using a viral inhibition assay that was based on a published protocol with significant modifications.[20]

For the preparation of CD4+ T target cells, PBMCs were stimulated during seven days at 37°C 7% CO₂ in RPMI 2.5% human serum (HS, A&E Scientific, Belgium), IL-2 (500IU/mL, Gentaur, Kampenhout, Belgium) and anti-human CD3/8 bi-specific monoclonal antibody (1µg/mL, NIH AIDS Reagent Program). For the preparation of stimulated CD8+ T effector cells, PBMCs were incubated during 7 days at 37°C 7% CO₂ in RPMI 2.5% HS and 10µg/mL (81ng/mL per peptide) of an HIV consensus subtype B gag peptide pool (NIH AIDS Reagent Program). For the preparation of non-stimulated CD8+ T effector cells, an extra aliquot of PBMCs was thawed one day before the start of CD4+/CD8+ T cell co-culture and rested overnight at 37°C 7% CO₂ in RPMI 2.5% HS.

On day 0, CD4+ T target cells were enriched by negative selection magnetic beads (Miltenyi Biotech, San Diego, CA, USA) from the anti-CD3/8 mAb stimulated PBMCs. Stimulated as well as non-stimulated overnight rested CD8+ T effector cells were enriched from the gag peptide pool stimulated PBMCs and overnight rested PBMCs respectively, by negative selection. Cell purities of enriched CD4+ T and CD8+ T cells >90% were confirmed by staining for CD3 (FITC, Clone
OKT3), CD4 (PE, clone SK3), CD8a (APC-eFluor, clone SK1) and reading on a BD FACSVerse (Beckton Dickinson, New Jersey, USA).

Enriched CD4+ T cells were incubated with HIV IIIb (NIH AIDS Reagent Program) at a multiplicity of infection of 0.001 for 3 hours at 37°C 7% CO₂. Infected target cells were washed three times and resuspended at 10⁶ cells/mL in RPMI 2.5% HS 500IU/mL IL-2 and cultured in triplicate in flat bottom 96-well plates (VWR, Leuven, Belgium) at 10⁵ cells/well, alone (positive control) or in co-culture with stimulated or rested enriched CD8+ effector T cells at an effector-to-target ratio of 1:1 and 2:1. Culture medium was refreshed at days 2, 6 and 9. The level of HIV-1 p24 antigen in the supernatant was determined at day 13 by in-house p24 ELISA [26]. Log inhibition values were calculated on day 13 as \( \log_{10}(p24 \text{ without CD8}^+ \text{ T cells}) - \log_{10}(p24 \text{ with CD8}^+ \text{ T cells}) \).

**Quantification of HIV-1 DNA and RNA**

Total genomic DNA and cell-associated RNA were isolated from 5 x 10⁶ PBMCs each. Total HIV-1 DNA and cell-associated HIV-1 unspliced RNA were measured with the QX200 Droplet Digital PCR platform (Bio-Rad, Hercules, CA, USA) as described previously [27, 28].

Total HIV-1 DNA amounts were normalized to copies per 10⁶ PBMCs with the reference gene RPP30. For absolute HIV-1 usRNA quantification, normalization of input cDNA was done by quantifying gene expression levels of stably expressed reference genes as described previously [29, 30]. Data analysis was performed with ddpCQuant [31].

**Tat/rev induced limiting dilution assay**
The tat/rev induced limiting dilution assay (TILDA) was performed as described previously by Procopio et al. [32].

**Cytokine production after peptide stimulation**

Supernatant of PBMCs stimulated for seven days with the HIV gag peptide pool (81ng/mL per peptide) was tested for type 1 interferon (IFN), T helper (Th)1, Th2 and Th17, including IFN-α, IFN-β, IFN-γ, tumor necrosis factor (TNF)-α as well as the following interleukins (IL): IL-4, IL-5, IL-10, IL-17, IL-22 and IL-27 with Luminex technology on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested undiluted.

**Flow-cytometric phenotyping**

Phenotyping of CD8+ T cells at baseline and during co-culture was done with two panels. The “cytotoxicity/activation panel” focused on cytotoxicity and activation markers as well as intracellular cytokines and included the following markers: CD3, CD4, CD8, CD107a, IL2, CD56, IFN-γ, Granzyme B, CD45RA, HLA-DR, CD38, CCR7, Perforin, CD57, Zombie NIR fixable viability kit. The “immune checkpoint panel” focused on immune checkpoint markers and transcription factors and included the following markers: CD3, CD4, CD8, CD28, CD27, TIM-3, EOMES, CD160, LAG-3, PD1, 2B4, CD45RA, Tbet, CCR7, TIGIT, Zombie NIR fixable viability kit. A detailed list of all used human species reactive antibodies included in the panels above can be found in the supplementary methods.

Staining of PBMCs for flow cytometry was performed using the FOXP3 transcription factor staining buffer set (eBioscience). Briefly, cells were harvested and washed twice in cold PBS 1% FBS. Next, surface stains and near infrared live dead dye were incubated for 30 minutes. Cells
were washed twice, fixed and permeabilized with the eBioscience FOXP3 kit. Cells were then stained intracellularly for 30 minutes and washed twice. Samples were acquired on the Fortessa LSR flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed using FlowJo version 10 and Kaluza version 1.5.

**Statistical Analysis**

Mann-Whitney U tests were used to compare differences in mean VSC, cytokine levels and phenotypic markers between suppressors and non-suppressors. Linear regressions and Spearman rank tests were used to determine correlations between clinical, virologic and immunological parameters. Family-wise corrections of p-values were made using the Bonferroni-Holm’s method.

*Cluster analysis of flow cytometry phenotypic data*

Flow cytometry data was cleaned and compensated with Flow Jo version 10 software (BD, New Jersey, USA). CD8+ T cell populations were concatenated containing the file internal compensation matrix and reimported into the Cytobank environment. Scaling of channels was applied based on single compensation controls for each marker. Cluster analysis was done by running a SPADE (Spanning tree progression analysis of density-normalized events) algorithm on the mother population of total CD8+ T cells. Clustering was based on CD45RA and CCR7 expression.

SPADE results were verified by manual gating. Median fluorescence intensities (MFI) for each cluster were analyzed in GraphPad Prism and fold changes between suppressors and non-suppressors were calculated as ((MFI(Suppressor) – MFI(Non-suppressor)) / MFI(Suppressor)).
Multiple t-tests were run to statistically compare all markers between both groups. P-values depicted on figures are not corrected for multiple testing.

RESULTS

Participant Demographics

The demographics of the 36 HIV+ patients recruited in this study are given in Table 1. Patients with a wide range of age, time on therapy, peak viral load and CD4 counts were included in order to capture the diversity of our clinical cohort. Thirty-one patients had an undetectable HIV viral load (VL), four patients had a detectable VL <50 copies/mL, one patient had a VL of 52 copies/mL.

CD8+ T cell viral suppressive capacity is strongly enhanced after an HIV specific in-vitro peptide stimulation in a subset of progressor patients

We measured VSC of non-stimulated and subtype B gag peptide-stimulated CD8+ T cells in six healthy HIV negative volunteers and in 36 HIV-1 infected individuals at effector to target ratios (E:T) of 1:1 and 2:1. Our assay proved to be specific for HIV-1 as minimal suppression was observed in all healthy volunteers, with or without peptide stimulation, whereas strong suppression was observed in some HIV-1 infected individuals (Fig. 1A). Based on the comparison with healthy controls, “suppressors” were defined as having a VSC with peptide stimulation exceeding the average VSC of healthy volunteers + three standard deviations, corresponding to 0.34 log_{10}.

In subtype B infected individuals, 6 out of 23 (26%) suppressed viral replication without peptide stimulation (mean: 0.47 log_{10}) whereas 14 out of 23 (61%) suppressed viral replication with peptide stimulation (mean: 1.36 log_{10}). The difference in mean response was significant (p < 0.05).

As expected, no increase in suppression was observed in patients infected with non-subtype B HIV
strains after stimulation with the consensus B peptide pool, presumably because these patients have no memory T cells specific for subtype B antigens (Fig. 1A).

Furthermore, we observed a strong correlation (p < 0.05) in subtype B infected patients between VSC with and without peptide stimulation (Fig 1B). As expected, inhibition values were higher at an E:T of 2:1 as compared to 1:1 for most patients (Suppl. Fig 1A). VSC at both ratios strongly correlated with each other (Suppl. Fig. 1B). On the other hand, VSC did not correlate with current and nadir CD4+ T cell count or peak VL. Nevertheless, in subtype B infected patients VSC with and without peptide stimulation is on average higher in patients with detectable plasma VL as compared to patients with an undetectable VL (1.68 log versus 1.30 log and 0.89 log versus 0.36 log respectively), but these differences are not significant.

**HIV viral reservoir measures do not correlate with viral suppressive capacity**

Total HIV-1 DNA and usRNA were measured in all 23 subtype B infected patients. HIV-1 DNA was detectable in all patients and ranged from 15 to 859 copies per million PBMCs (mean: 238) (Fig. 2A). Unspliced HIV-1 RNA was detected in 17 out of 23 patients (74%) and ranged from 0.0 to 32.9 copies per million PBMCs (mean: 6.7) (Fig. 2B). These values are consistent with the literature on reservoir size of individuals on cART with suppressed viremia. Total HIV-1 DNA and usRNA did not correlate with VSC or any other studied clinical or immunological parameters.

To further characterize the “functional” viral reservoir, we performed the TILDA assay on 21 available subtype B HIV-1 infected patients. Without PMA/Ionomycin stimulation, no significant transcriptional activity of *tat/rev* RNA was observed (Fig. 2C). With stimulation however, six had detectable amounts of transcription, with values ranging from 55.8 to 313 cells with detectable HIV RNA transcripts per million CD4+ T cells. Therefore pro-viral transcription was inducible
with PMA/ionomycin in only a minority (6/21 = 29%) of the patients. VSC did not correlate with induced viral transcription. TILDA inversely correlated with CD4 nadir (R = -0.67; p < 0.05).

**Cytokine Production in Response to Peptide Stimulation**

Next, we investigated whether cytokine production in response to HIV peptide stimulation could be predictive of VSC. To this end, we determined cytokine levels in supernatant from PBMCs stimulated for seven days with the HIV-1 gag peptide pool, before the start of the CD4+/CD8+ T cell co-culture in 26 available patient samples.

When comparing mean cytokine levels, only IFN-γ was produced significantly more in suppressors than in non-suppressors (p < 0.01) (Fig. 3A). While TNF-α and IL-10 levels did not differ significantly, their ratio was significantly higher in suppressors than in non-suppressors (p < 0.05) (Fig. 3B). Together, these results point towards a correlation between Th1 responses and VSC.

**Flow cytometric phenotyping**

*Increased CD160/PD1 co-expression on CD8+ TEMRAs of suppressors at baseline*

In order to look for correlates of VSC with so-called ‘exhaustion markers’ at baseline, we phenotyped non-stimulated, rested CD8+ T cells using the immune check point panel. Interestingly, CD8+ T cells from suppressors only showed a significantly higher CD160/PD1 co-expression in the terminally differentiated effector memory subset (TEMRA), defined as CCR7-CD45RA+, as compared to non-suppressors (Fig. 4A). To identify CD8+ T cell memory subsets with different expression patterns of exhaustion markers between suppressors and non-suppressors, we ran a SPADE analysis based on CD45RA and CCR7 expression (Fig. 4B). Four,
six, three and seven subsets were identified in the naïve, central memory (CM), effector memory (EM) and TEMRA CD8+ T cells respectively (Fig. 4B). The comparison of suppressors versus non-suppressors identified a cluster with significantly higher PD1/CD160/TIGIT co-expression but lower Tbet expression in suppressors. Another cluster showed significantly higher expression of PD1 only. These differences were however only significant without Bonferroni-Holm’s corrections of p-values.

*Increased CD57 expression on CD8+ T cell memory subsets of suppressors after peptide stimulation*

In order to look for correlates of VSC with activation and cytotoxicity markers, we phenotyped CD8+ T cells after 6 hours of stimulation with an HIV-1 consensus B gag peptide pool. Using the SPADE cluster analysis described above, we observed significantly higher CD57 expression (without Bonferroni-Holm’s correction) in one EM and two TEMRA subpopulations (Fig. 4C) in suppressors. No higher expression was observed for IFN-γ, IL-2, granzyme, perforin or CD107a between suppressors and non-suppressors among the memory subsets after six hours of peptide stimulation.

*HLA-DR expression on CD8+ T cells of suppressors is increased early in co-culture*

Finally, we phenotyped CD8+ T cells in a small available subset of six patients 18 and 42 hours after the start of co-culture to assess their activation and cytotoxicity phenotype. We found that suppressors had a higher (p < 0.13) HLA-DR expression 18 and 42 hours after the start of co-culture as compared to non-suppressors (Fig. 5A). There were no differences in expression of IL-2, granzyme B and perforin between the two groups. Furthermore a slightly higher but non-significant IFN-γ response in suppressors was observed after 42 hours of co-culture (Fig. 5B).
DISCUSSION

A better understanding of the anti-viral activity of CD8+ T cells is crucial in the search for a functional cure for HIV. In our study we show that in-vitro VSC of CD8+ T cells from a proportion of progressors under cART can be induced with an HIV specific peptide stimulation. While this induced VSC did not correlate with any clinical parameters or viral reservoir measures, we did observe a correlation with IFN-γ production by PBMCs in response to gag peptide stimulation. Importantly, we identified specific subsets of TEMRA CD8+ T cells with higher PD1+CD160+ co-expression at baseline, higher CD57 expression after peptide stimulation and higher HLA-DR expression during co-culture with infected CD4+ T cells in patients with higher VSC.

We presume that HIV specific memory CD8+ T cells are expanded during the seven day peptide stimulation and revert from a resting to a more functional effector phenotype, thereby increasing VSC. This idea of specific expansion, rather than the induction of new or non-specific responses is further supported by (a) the strong correlation between VSC of rested versus peptide stimulated CD8+ T cells, (b) the very low background of VSC in healthy controls and (c) the failure to induce VSC in HIV non-subtype B infected patients (Fig.1).

VSC has been described to correlate with plasma viral load [14, 33], suggesting the importance of antigen exposure to keep up cellular anti-HIV immunity. The five patients with low but detectable VLs did have higher VSC with and without peptide stimulation as compared to the patients with undetectable VLs, although this difference was not significant. Similarly, we hypothesized that reservoir size and on-going viral transcription, presumably resulting in antigen presentation and boosting of the immune system, could correlate with CD8+ T cell VSC. Nevertheless, we did not
find any correlations with HIV-1 DNA or RNA. It is possible however that the small range in unspliced cell-associated RNA levels in our cohort [IQR: 0.2 – 10.7 cps per million PBMCs] did not allow to observe significant differences in VSC.

Several groups have shown the importance of polyfunctionality and the lack of correlation between VSC and IFN-γ responses of CD8+ T cells \[^{[23, 34, 35]}\]. Nevertheless, we observed that the amount of IFN-γ produced in response to an HIV peptide stimulation accumulated over a period of seven days in the supernatant significantly correlated with subsequent VSC (Fig. 3A, p < 0.01). Suppressors also had a significantly higher ratio of TNF-α/IL-10 production (Fig. 3D, p < 0.05).

Phenotypic data demonstrated higher numbers of PD1+CD160+ double positive CD8+ TEMRA T cells in suppressors. Expression of such immune checkpoint (IC) markers has been linked to T cell exhaustion in the context of chronic disease with sustained antigen exposure, resulting in a gradual loss of effector functions \[^{[36-41]}\]. TEMRA cells on the other hand are also linked to chronic antigen exposure and make up an aged subset of EM cells, characterized by a decreased proliferation potential but strong effector capacities \[^{[42]}\]. While the observed positive correlation with VSC therefore seems paradoxical, the following points need to be taken into consideration.

Earlier studies have shown that CD8+ T cells remain responsive despite chronic infection \[^{[43, 44]}\]. In lymphocytic choriomeningitis virus infection phenotypically exhausted memory CD8+ T cells control viral infection despite continuous expression of PD1 \[^{[45]}\]. During untreated HIV-1 infection increasing viral loads correlate with declining numbers of polyfunctional CD8+ T cells and increased expression of IC markers \[^{[13, 46, 47]}\]. During cART the decay of PD1 on T cells coincides with the decline of immune activation and viral load but are never restored to levels of HIV negative individuals \[^{[46, 48]}\]. Expression of IC markers has been reported on CD8+ T cells of ECs,
suggesting residual viral replication rather than dysfunctionality of the immune response⁴⁹. In addition, ECs harbor highly effective cytolytic subsets in CD8+ T cells expressing CD160 and 2B4⁵⁰. Taken together, this data supports the idea that a range of exhausted states may exist within functional effector subsets. Accordingly, suppressors were shown to have a larger subset of PD1+CD160+ CD8+ T cells of the TEMRA compartment as compared to non-suppressors, possibly representing more functional and cytolytic cells able to suppress viral replication.

Furthermore, a higher CD57 expression was observed in EM and TEMRA subsets of suppressors as compared to non-suppressors. These respective subsets have typically two profiles: a highly cytotoxic and a poorly replicative one⁵¹,⁵². In addition, CD57 was previously suggested as a marker of proliferative history, and more recently as a maturation marker for cytotoxic HIV specific CD8+ T cells with a more terminally differentiated phenotype in ECs⁵²⁻⁵⁴. Together, these findings are suggestive of terminal differentiation as a key factor for a potent anti-viral response⁵⁵. Finally, a higher HLA-DR expression in CD8+ T cells was observed in suppressors during co-culture with autologous CD4+ T cells, suggesting that a distinct activation state of fully differentiated memory subsets in suppressors might explain VSC.

While we have identified a phenotypical difference between CD8+ T cells from patients with and without in-vitro viral suppression, further research is needed to determine whether these distinct subsets are in fact responsible for suppressing viral replication.
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REFERENCES


Table 1. Patient demographics of 36 included HIV+ patients.

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<th>Peak Viral Load (cps/mL)</th>
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<td>- Range</td>
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<td>5.290 – 1,020,000</td>
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<td>- Median</td>
<td>Non-subtype B infected</td>
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Figure captions

Figure 1. Viral suppressive capacity. (A) Log_{10} inhibition values of HIV- people and HIV+ patients (infected with subtype B and non-subtype B HIV-1 strains) with overnight rested (Rest) and 7-day peptide stimulated (Stim) CD8+ T cells as measured in the viral inhibition assay with an E:T of 2:1. Mean with SEM is depicted. The dashed line represents the threshold of 0.36log above which patients are defined as suppressors. (B) Correlation between viral suppressive capacity with rested versus 7-day peptide stimulated CD8+ T-cells with an E:T of 2:1.

Figure 2. Viral reservoir measures. (A) Total amount of HIV-1 DNA and (B) cell-associated unspliced RNA copies per million PBMCs for all 23 HIV+ subtype B infected patients. Mean with SEM is depicted. (C) Tat/rev induced limiting dilution assay (TILDA) results in number of cells with detectable multiply spliced HIV-1 mRNA per million CD4+ T cells without (N = 10) and with PMA/ionomycin stimulation (N = 21).

Figure 3. Cytokine production. Cytokine levels in supernatant of PBMCs after 7 days of stimulation with a consensus subtype B HIV-1 gag peptide pool from HIV-infected progressors with either a subsequent “suppressor” or “non-suppressor” activity in CD4+/CD8+ co-culture, as compared to HIV-negative subjects (HIV negative donors or ND).

Figure 4. (A) Manual (bivariate gating) analysis showing percentage of PD1/CD160 double positive CCR7-CD45RA+ (TEMRA) CD8+ T cells in suppressors and non-suppressors.
Representative flow cytometry plots of suppressors and non-suppressors are given on the right. (B,C) SPADE cluster analysis based on CD45RA and CCR7 expression of CD8+ T cells. The heat map depicts for each marker the fold change in MFI of suppressors versus non-suppressors for the ‘immune exhaustion’ (B) panel and the ‘cytotoxicity/activation’ panel (C). Significant differences (p < 0.05) are marked with asterisks (p-values are not adjusted for multiple testing).

Figure 5. (A) Percentage of HLA-DR positive CD8+ T cells 18 and 42 hours after the start of CD4+ T cell/CD8+ T cell co-culture in suppressors and non-suppressors. (B) Percentage of IFN-γ positive CD8+ T cells 42 hours after the start of CD4+ T cell/CD8+ T cell co-culture in suppressors and non-suppressors.

Supplemental figure 1. (A) Log_{10} inhibition values of HIV+ patients (infected with subtype B HIV-1 strains) as measured in the viral inhibition assay with an E:T of 1:1 and 2:1. (B) Linear regression analysis of VSC at E:T of 1:1 versus 2:1.
### Description of the role of each of the authors in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Conception or design of the work</th>
<th>Acquisition, analysis, interpretation of the work</th>
<th>Drafting or revising the work</th>
<th>Final approval of version to be published</th>
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<td>G. Vanham</td>
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Figure 5

A  HLA-DR

B  IFN-γ

% of CD8+ T cells

Non-Suppressors  Suppressors  Non-Suppressors  Suppressors

18 hours

42 hours

% of CD8+ T cells

Non-suppressors  Suppressors
Click here to access/download
**Supplemental Data File (.doc, .tif, pdf, etc.)**
AIDS-D-18-00341_Supplemental data.docx
AIDS-D-18-00341 - “In-vitro viral suppressive capacity correlates with immune checkpoint marker expression on peripheral CD8+ T cells in treated HIV positive patients”: Response to reviewers

We thank the reviewers for their helpful comments. We have modified the manuscript according to the referees’ recommendations and addressed their questions. Underneath we have included our responses to the reviewers’ questions.

Reviewer #1:

1. Could the authors clarify if the same peptides are used in the different pools for PBMC cytokine production and CD8+ VSC stimulation? How long are these peptides? Do they match the IIIB gag sequence (used in the VSC assay)?

Indeed, the same peptides were used, i.e. a single pool of 123 peptides, most of which are 15 amino acids long and have an 11 amino acid overlap (HIV-1 Consensus B Gag Peptide Pool, NIH, cat. no. 12425). Cytokine production was assessed on supernatant of the stimulated PBMCs which were used to measure VSC.

The pool of gag peptides was made (by the NIH) on the basis of the consensus B sequence from 2004. There is a 97.2% sequence similarity between this consensus B and HIV IIIb in the gag region, so these peptides match HIV IIIb quite well.

2. What are the infecting clades of the non-B patients? I am surprised that the assay system does not elicit cross-reactive suppressive responses.

The infecting clades of the non-B patients include subtypes A, F, CRF01_AE, CRF02_AG and more complex recombinant forms. We compared sequences in gag between consensus B and consensus sequences of subtypes A, F, CRF01_AE, CRF02_AG and noticed sequence similarities which are all below 87%, presumably explaining the lack of cross-reactive responses.

3. The sensitive VL assay picks up several patients with low but detectable viral loads - do these subjects have a) higher VSC and b) larger reservoirs?

Since there are only five patients with low but detectable VLs, statistical analysis has little power. Patients with low but detectable plasma VLs do not have larger reservoirs (nor higher DNA, nor higher RNA levels) in our study. However, in subtype B infected patients, VSC with and without peptide stimulation is on average higher in these patients (1.68 log versus 1.30 log and 0.89 log versus 0.36 log respectively), but these differences are not significant. We have added this in the result section (page 12) and adapted the discussion section (third paragraph, page 16 and 17) mentioning this non-significant correlation with plasma viral load.
Reviewer #2:

1. [...] the selected patients had a very wide range of viral set point prior to ART, but there is no description of any correlation with in vitro CD8 suppressive activity.

In the paper, only peak viral load is described, not viral set point. There was however no correlation observed between CD8 suppressive capacity and peak viral load. This is mentioned briefly in the discussion section. We have adapted the manuscript (results section, page 12) so as to include this in the results section as well.

2. There is no mention of using tetramers to measure HIV-specific CD8 T cells, nor of HLA typing, nor of studying known immunodominant epitopes. Detailed phenotyping was done, but this was only indirectly correlated with CD8 activity, and wasn’t combined with tetramers. Furthermore, would it have been better to cell sort some of the CD8 subpopulations to directly show suppressive activity?

We agree that the suggested assays would have been very interesting to include, and that they could have made our observations stronger by studying HIV-specific cells only. Unfortunately, we were restricted by a limited amount of cell material, since (1) we did not have access to leucapheresis samples and (2) our viral inhibition assay required a large amount of cells.

3. Studies from elite controllers have already shown that stimulation with Gag induced proliferation and enhanced specific cytotoxicity. CD8 proliferation didn’t appear to be measured in the current study, nor was cytotoxicity. IFN-γ responses have been comprehensively studied in the past and no clear correlation with progressor status or viral loads has been established, except in HLA-B27 or HLA-B57 subjects. Therefore it is a little surprising that IFN-γ in supernatants appeared to correlate with suppressive activity in the current study. Although in Fig 3A the majority of suppressive cultures did not show IFN-γ production, and there is no indication that the intracellular staining for IFN-γ convincingly corroborated the supernatant data.

Indeed, the IFN-γ data needs to be interpreted with caution. As the reviewer rightfully points out, most of the patients with VSC do not have higher amounts of IFN-γ. Nevertheless, some of the suppressors do, while none of the non-suppressors have raised IFN-γ levels in the supernatant after stimulation. IFN-γ production therefore seems to be a somehow specific characteristic, but not sensitive at all. In other words, IFN-γ production seems to be correlated to VSC, but is not able to predict it, which is an important difference. In the literature, this seems to be a recurrent theme, namely that IFN-γ is correlated with VSC but does not seem to be causal. This could be explained by the fact that IFN-γ is often co-expressed with other markers (such as MIP-1β, CD107a, etc) whose role has been demonstrated in viral suppression [1-3].

4. With respect to possible non-cytotoxic suppression of HIV replication, previous studies reported that CD8 suppressive activity was in CD28+ cells, which wouldn’t include CD28 neg TEMRA.

The TEMRA subset is defined as CCR7- CD45RA+. Within this TEMRA subset, we compared CD28 expression between suppressors and non-suppressors. Nevertheless, we did not observe a significant difference between both in terms of CD28 expression, as shown in the figure underneath. Whether CD28+ TEMRAs specifically exerted non-cytotoxic suppression, has not been examined here. Nevertheless, the FACS analysis with SPADE does not point into this direction.
5. There is no mention of pre-existing HLA-DR+ cells, as in the Saéz-Cirión paper on elite controllers. What were the CD38/HLA-DR proportions prior to and after stimulation? Does the data in Figure 5 show an increase in HLA-DR expression from baseline? Can you do a Mann-Whitney test on only 4 versus 2 samples, and get a significance of p<0.005?

- HLA-DR and CD38 expression levels at baseline were similar for suppressors and non-suppressors (shown in the figures underneath). We screened the patients however either with the cytotoxicity panel after stimulation or with the Immune Checkpoint (IC) panel without peptide stimulation with flow cytometry. Since we used different panels for both, we cannot give a comparative readout between non stimulation and stimulation.

- The data of figure 5 shows the HLA-DR expression on CD8+ T cells 18 and 42 hours after the start of co-culture with infected CD4+ T cells. Suppressors show an increased HLA-DR expression during co-culture as compared to their baseline values. Saéz-Cirión et al. described in 2007 a similar phenomenon in elite controllers but instead of looking during a viral inhibition co-culture, they showed it on HIV-1 specific cells using tetramers.

- We erroneously applied t-test statistics here, while the data is not normally distributed. We now performed a Mann-Whitney test and adapted this in the manuscript. The differences are not significant anymore.

6. The abstract states that 18-colour flow cytometry was used, but that isn’t clear from the methods section, since 18 different fluorochromes aren’t listed. How did they measure LAG-3-efluor450, PD-1 BV421 and 2B4-Pac Blue simultaneously in the immune checkpoint panel, if they all use the same detector on the Fortessa?
Indeed, in this study we did not use all 18 possible colors. The ‘cytotoxicity panel’ consisted of 16 markers and the ‘immune checkpoint panel’ of 17 markers. We have adapted this in the materials and methods section.

Listing the fluorochromes, we mistakenly entered efluor450 from our database instead of efluor710 for the LAG3 antibody. We corrected this in the materials and methods. The configuration of our Fortessa contains seven filters in the violet laser (407 nm excitation, see below). This allows the simultaneous detection of up to seven different markers. PD1 in BV421 and 2B4 in Pacific Blue can therefore be used in parallel.

7. Also, BV605, BV650 and BV711 have been used simultaneously, which is very challenging for compensation due to large spillover between these fluorophores.

Indeed, using these fluorochromes simultaneously is challenging during panel design and testing. We carefully selected combinations of antibodies and fluorophores, taking expression levels, co-expression patterns and fluorophore brightness into account. The set-up of the panel was done on the same cell material and in the same conditions as those used in the final experiments. Laser voltages were set allowing compensations of maximal 40% while maintaining good separation of positive and negative populations.

8. The paper suggests that there are 7 distinct subsets of PD-1+ TEMRA CD8 T cells, but no representative plots are shown for all the markers. Many of the other markers in the panel will be on the same PD-1+ cells, as mentioned for TIGIT in the text. The one representative flow plot for the suppressive CD8’s in Fig 4A seems to show that the CD160+ cutoff is not very distinct for the PD-1+CD160+ cells - it appears to be more of a continuum. Is it the same for TIM3 etc, especially prior to stimulation? How many cells were run in the SPADE analysis? Did the number of cells have to be down-sampled to run the clustering, and does SPADE have random starting conditions so that multiple analyses have to be performed, as for t-sne?

Showing plots for expression of all used markers would overload the result section. We chose to show expression of all markers according to their memory subsets calculated by SPADE. Seven distinct subsets of TEMRAs were identified based on CD45RA and CCR7 expression but not on expression of PD1 or other markers. Those memory subsets were then compared in their expression levels of markers such as PD1 being suppressors versus non-suppressors.

Representative plots to clarify our gating are attached underneath for PD1/CD160, TIM3/LAG3 and TIGIT/2B4. Understandably the chosen cutoff for CD160 is not clear from the two representative plots shown in the manuscript but should be more comprehensive after viewing the two additional datasets. If the editor so prefers, we could move these plots into the supplemental data.
Multiple clusterings were indeed done to find a solid number of nodes representing all memory populations without over- or under representing and to reproduce solidly the once found results. The SPADE algorithm always performs down sampling of input files. The value of 10,000 CD8+ T cells per patient was applied here.

To illustrate the gating strategy on the CD160/PD1 markers we provide above two additional representative flow plots. The mother gate is the CD8+ T cell TEMRA population. The distribution of cells in the graphs shows why the quadrant gates are set that way for the whole cohort analysis.

To illustrate the distribution of TIGIT markers within the CD160/PD1 double positive CD8+ TEMRA T cells we show some representative plots above. 2B4 was never well expressed on baseline samples. This is also reflected in the results of figure 4 in the manuscript.
To give an idea of TIM3 and LAG3 expression find below representative plots. Expression levels were overall low what is also reflected in the nearly not existing differences between suppressors in nonsuppressors from figure 4 (heatmap). Expression is here given for the total of CD8+ TEMRAs (mothergate as indicated in the top of the plots).

9. The main result appears to implicate PD-1+CD160+ CD8 T cells, which would be consistent with the Pombo et al reference on elite controllers, but there was an earlier paper from Peretz et al (2012) that said that PD-1+CD160+ CD8 T cells in chronically infected patients had no in vitro HIV-specific activity.

This has to be addressed in future studies and ultimately requires cell sorting to prove viral suppression by these specific subsets. The paper from Peretz et al. compares chronically non-treated patients with LTNPs and treated patients. Their finding of PD1+CD160+ subsets being non-responsive is made in viremic patients. In contrast, our cohort addresses viral suppression within a patient cohort with similar treatment duration and stable undetectable viremia.

10. Anti-CD3/CD8 bi-specific antibody was used to prepare CD4 T cell targets? Is that correct?

Indeed, anti-CD3/CD8 bi-specific monoclonal antibodies from NIH were used to cross-link CD4+ T cells (by binding of CD3) and CD8+ T cells (by binding of CD8) and induced very strong preferential proliferation and activation of CD4+ target T cells [1, 3].

11. What purpose did the TILDA assay serve, if so few patients' cells became detectable, yet the vast majority of patients will experience plasma virus rebound if they interrupt therapy, as mentioned above?

Results from the TILDA assay were indeed disappointing, since very few patient samples could be induced to start transcription (after the 12 hour stimulation period) at levels detectable with this assay. Since relatively little data has been published on the TILDA assay, however, we consider it important to share this data, even though we did not observe any meaningful correlations with CD8+ T cell characteristics.

12. There must have been many statistical comparisons and correlations performed, but there is no mention of Bonferroni adjustment of p values.
Thank you for this remark. We have gone through our statistical analysis and have now applied family-wise corrections of our p-values using the Bonferroni-Holm’s method. However, we have chosen to keep non-corrected p-values for the statistical analysis of the phenotypic data, considering our small sample size and the very large number of statistical tests typically performed on 16-color flow-cytometric data, leading to an overly strict correction. Nevertheless, we have adapted the manuscript so as to clarify that p-values are not corrected for flow-cytometric data and that significance is lost when corrections are performed.

References


AIDS-D-18-00341 - “In-vitro viral suppressive capacity correlates with immune checkpoint marker expression on peripheral CD8+ T cells in treated HIV positive patients”: Supporting document

We thank the reviewers for their helpful comments. We have modified the manuscript according to the referees’ recommendations and addressed their questions. Underneath we state the changes we have made to the manuscript point-by-point.

- First reviewer, question #3: we have added a comment on the correlation between plasma viral load and VSC.
  - result section (page 12)
  - discussion section (third paragraph, page 16 and 17)
- Second reviewer, question #1: we added a comment on the lack of correlation between peak VL and VSC.
  - results section (page 12)
- Second reviewer, question #5: we corrected our statistical test done on figure 5 (Mann-Whitney instead of t-test) and adapted figure 5 accordingly (difference is not significant anymore). We also adapted the respective results section.
  - Figure 5
  - results section (final paragraph, page 14)
- Second reviewer, question #6: we corrected the number of colors used in the abstract (from 18 to 17 and 16) as well as the mistake in fluorochrome (efluor450 instead of efluor710) in the (supplementals)
  - abstract (design section)
  - methods section (moved to supplementals)
- In order to shorten the length of the paper, we have simplified the details of the flow cytometry panels in the methods section (only stating the antibody targets) and have included all details (fluorochromes, clones, manufacturers) to the supplementals.
  - methods section (page 9)
- Second reviewer, question #12: we have adapted our p-values using a family-wise method and Bonferroni-Holm’s correction. In addition, we have specified this in the methods section (statistical analysis, page 10)
  - Results section
  - Figure 1B
- We have omitted supplemental figure 2, since significance was lost after Bonferroni correction and considering its limited importance to the main story of this paper.
  - Supplemental data
- We have added a relevant reference to the discussion section (reference 49).