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Short communication

Antiviral and immunological benefits in HIV patients receiving intranasal peptide T (DAPTA)

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Abstract

D-Ala-Peptide T-amide (DAPTA), the first viral entry inhibitor, blocks chemokine (CCR5) receptors, not CD4. Early investigators could not "replicate" DAPTAs potent in vitro antiviral effect using the lab-adapted, X4, peptide T-insensitive strain, IIIB, delaying clinical virological studies. We now report that DAPTA, administered to eleven long-term infected (mean = 17 years) patients with stable persistent plasma "virus" for up to 32 weeks did not change this level. Infectious virus could not be isolated from their plasma suggesting HIV RNA was devoid of replicative capacity. Progressively less actual virus ($P < 0.01$) could be isolated from white blood cells (PBMCs). DAPTA flushed the monocyte reservoir to undetectable viral levels in most patients. Five of eleven had a mean CD4 increase of 33%. Immune benefits also included a four-fold increase in γ-interferon-secreting T-cells (antiviral cytotoxic T-cells) in the absence of drug-related toxicity. All five CD4 responders had increases in antiviral T cells and decreases in infected monocytes, an argument for initiating further studies promptly.

Keywords: Peptide T; AIDS; Entry inhibitor; CCR5; Antiviral; Clinical trial; Chemokine receptor; Viral reservoir; Cytotoxic T-cells

1. Introduction

Currently, the standard "highly active anti-retroviral therapy" (HAART) for HIV infection is a varying cocktail that targets the viral enzymes reverse transcriptase and protease with the goal of driving apparent HIV plasma virus to undetectable levels. Although HAART has substantial antiviral effect by this criterion [6,12] suppression is incomplete so that frequent regimen changes are required. Moreover, HAART fails to eliminate virus from persistent reservoirs [3,11,16], does not enter the brain, and does not fully restore deficient antiviral $CD8^+$ T-cell immune defenses [10]. Furthermore, development of viral resistance [4], as well as serious toxicities, such as heart and liver damage, sometimes makes sustained long-term use problematic [11]. Development of new therapies that address these concerns is therefore a priority, particularly the development of a therapy with a completely non-toxic profile that could be used with other agents to postpone the initiation of HAART, prolong and/or intensify its efficacy, reduce resistance development, or treat viral reservoirs. Since DAPTA works by a distinct mechanism (entry inhibition) from other HAART drugs it would be expected to yield synergistic treatment benefits.

Peptide T ($Dala_1$-peptide T-amide, "DAPTA"), was derived from the envelope protein of HIV (reviewed in [15]), and is a selective entry-inhibitor for HIV-1 isolates which use the CCR5 entry co-receptor [14]. So called R5 isolates establish initial infection, persist during the early years of infection, and predominate in brain where they cause the manifestations of neuro-AIDS, via infection of CCR5 expressing monocytes and microglia. In HIV patients peptide T has been found to have no toxicities and has caused improvements in cognition [7,8] and normalization of growth hormone secretion [1]. In addition to blocking viral entry, peptide T is a potent antagonist of free gp120, a pathogenic mediator of neuro-AIDS, wasting, immune failure, and other

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AIDS disease manifestations. As such, and given the anticipated importance of CCR5 targeted entry inhibitors, peptide T represents an adjunct therapeutic with great promise. In this study, we report antiviral and immune enhancing effects associated with intranasal peptide T administration in a small, ($n = 11$) open-label trial in HIV^+ patients who received peptide T for 24–32 weeks at HIVCare, St. Francis Hosptial, San Francisco.

2. Materials and methods

2.1. Study participants

Eleven persons with stable plasma viremia between 500 and 25,000 copies/ml, as determined by three (weekly) measurements (Roche, Amplicor test), and CD4 cell counts >300/mm^3 were administered $Dala_1$-peptide T-amide (6 mg per day) in three divided doses by metered nasal sprayer for up to 32 weeks. Eleven completed 24 weeks or more of treatment and are the subjects of this report. The mean time since diagnosis was 17 years, indicating a long-term, non-progressing cohort. Seven of the group received only peptide T during the study; of these four had received prior HAART therapy and three were treatment naïve. The remaining four received peptide T in addition to their HAART therapy and all participants were maintained on their other medications without change during the study. All patients signed informed consent and safety monitoring was performed. Blood samples were obtained at baseline, weeks 8, 12, 24, and 28–32 for analysis of primary endpoints of plasma viremia and CD4 cell levels. Additional blood draws (30 ml) were made for secondary virological and immunological parameters [15].

2.2. Assays or outcomes

The primary endpoint results of plasma HIV RNA by the Roche Amplicor test, and CD4 cell numbers, were determined by a certified testing laboratory (Quest, San Francisco). The secondary laboratory endpoints were the number of p24 producing CD14 monocytes in peripheral blood (a measure of active virus replication), the ability to isolate virus by co-culture of patient PBMCs, the predominant viral co-receptor phenotype, and the number of γ-interferon (IFNγ) secreting CD8 T-cells, a measure of immune function.

In anticipation of a possible selective CCR5 antiviral effect the number of infected blood monocytes, a measure of R5-tropic viremia, was determined from isolated peripheral blood mononuclear cells (PBMCs) within 24 h of collection by FACS staining for viral p24. PBMCs were stained with monocyte specific anti-CD14 IgG1 (BD, Pharmingen) fluorescence-labeled antibody and an isotope-matched control. The HIV-1 core proteins were detected by flow cytometry measurement after intracellular staining with anti-core p24 antibody (KS57-RD1, Coulter Immunology) and an isotope control (MsIgG2-RD1). Uninfected monocytes from an HIV^- population were used as a negative control, as a further specificity control an anti-HIV serum was used to block the binding of PE-labeled anti core antibody (KC-57). Monocytes were gated using anti-CD14 and >20,000 events per measurement were collected. The results are presented as the mean percent of infected (i.e. p24 positive) cells.

A confirmatory PCR analysis of integrated HIV-1 proviral DNA was performed on cultured (7 day) differentiated patient monocytes, in a randomly selected subset of the patients due to sample limitations out of our control. For HIV-1 proviral integration analysis genomic (total) DNA was isolated from differentiated monocytes (Qiagen DNA isolation and purification kit). Amplification of β-actin housekeeping gene was utilised to evaluate the efficiency of the extraction procedure and to estimate the concentration of isolated DNA. DNA isolated from U1-cells in which two HIV-1 proviral copies are integrated in each cell genome was used as a positive control and semi-quantitative analysis of patient's sample. Less than 0.1% T-cell contamination of the monocyte cultures was observed, as analysed by reverse-transcriptase chain reaction with T-cell receptor (TcR) primers.

The isolation and culture of virus from patients, pre- and post-peptide T treatment was attempted by several methods including recovery of virus from co-culture of patient PBMCs or centrifugally concentrated plasma with activated PBMCs from healthy donors which had been CD8 cell depleted. Cultures were re-fed with fresh donor PBMCs every 3–5 days and tested for p24 production for up to 21 days. Cultures were considered to be positive if p24 was detected at a level >10 pg/ml (Coulter ELISA) at any time point. A syncitial assay using MT-2 cells was also used, primarily to test for X4 virus. Cultures were considered positive if virus was detected by any method (co-culture, serum, or syncitia assay) at any time.

Immune enhancing effects of peptide T were determined by testing patient $CD8^+$ T-cells for intracellular IFNγ expression after PHA/ calcium ionophore activation in the presence of a protein transport inhibitor. Other details were reported in [15]. Samples were read by flow cytometry and data were analyzed by FloJo software (Tree Star, Inc., San Carlos, California). The results are presented as percent of IFNγ expressing $CD8^+$ T-cells.

2.3. Statistical methods

Paired *t*-tests were performed to assess the statistical significance of the two primary outcome measures of HIV RNA and CD4 number at week 24 of treatment relative to baseline. Four secondary outcomes (p24 monocytes, ability to co-culture virus from PBMCs, IFNγ/CD8 cells, and co-receptor phenotype) were evaluated, relative to baseline at 8, 12, and 24 weeks by paired *t*-tests. Multiple comparisons were not controlled for in these analyses because the

outcomes were secondary and because of the hypothesis generating emphasis of this pilot study.

3. Results

Peptide T treatment for up to 32 weeks did not change the apparent plasma virus levels, a primary endpoint in this study; 3.71 ± 0.1 log copies/ml (mean/S.E.M.) at baseline compared to 3.85 ± 0.15 copies/ml at week 24 (ns). We were, however, unable to isolate infectious virus from any plasma sample by co-culture into activated donor CD8 depleted T-cells, despite repeated attempts, indicating a disconnect between PCR detection of HIV RNA (gag) and infectious plasma virus in this long-term non-progressor cohort.

Overall CD4 cell counts in all eleven members of the cohort showed a trend toward increase, from 540 ± 55 cells/μl (mean + S.E.M.) at baseline, to 606 ± 86 at week 24, and 652 ± 72 at week 32 (21% increase, ns). Five of the eleven participants had apparent CD4 increases by 24 weeks of at least 33% relative to baseline ("responders"). In these patients, CD4 increased from an average of 529 ± 57 (mean + S.E.M.) at baseline, to 706 ± 143 at week 24. We considered an increse of 33% at week 24 compared to baseline to be a clinically significant improvement. However, the CD4 increases in this responder subgroup marginally failed to reach statistical significance ($P = 0.06$).

For the secondary virological endpoints, peptide T treatment for up to 32 weeks was associated with nearly complete suppression of active HIV replication in the circulating monocyte (CD14) population (Fig. 1), considered to be an important reservoir for viral re-infection and spread throughout the body. Samples from nine of the eleven patients were available for analysis (two baseline samples were lost in shipping and their data was therefore censored from the analysis). Four of nine received HAART plus peptide T, five of nine received only peptide T. All nine had p24 positive CD14 monocytes detected in their peripheral blood. At baseline the mean number of p24 positive monocytes was $6.0 \pm 1.6\%$ (S.E.M.), range 0.4–12.5%, comparable to other reports [11,16]. By 24 weeks after peptide T dosing the mean percent p24 positive monocytes had fallen almost three-fold to $2.1 \pm 1.5\%$ ($P < 0.02$ compared to baseline) and dropped further by week 32 to $0.4 \pm 0.3\%$ ($P < 0.01$). Four (all on HAART) of nine patients positive at baseline were undetectable for p24 antigen at week 24, and one who received peptide T only (i.e. without HAART) became undetectable at week 32.

PCR analysis of integrated macrophage proviral HIV-1 DNA confirms these findings (Fig. 2). DNA from five pre/post samples of sufficient quantity was available for analysis. U1 cells, containing two integrated HIV copy per cell, were used as quantitative standards. Integrated provirus was detected in four of five patient samples and all four showed reductions after peptide T treatment. Patients 19, 32, and 35 were receiving only peptide T, while 17 and 18 received peptide T plus HAART.

PBMC virus was detected by co-culture in six of nine at baseline. At week 12 two of nine were positive, and at week 24 one patient of nine remained positive. This positive patient was the only member of the cohort with a predominant X4-tropic phenotype, determined by infection of X4 indicator cell line and syncitia formation with MT-2 cells. The others were R5 ($n = 3$) and R5/X4 ($n = 2$). No patients who had undetectable virus at baseline by co-culture became positive during the 32 week study period. Two patients who became co-culture negative reverted to positive detection of virus after 6 months cessation of peptide T therapy, even with constant HAART treatment.

Immunological effects were observed following peptide T treatment. A statistically significant increase (three to four fold) with peptide T treatment in IFNγ secreting cells was

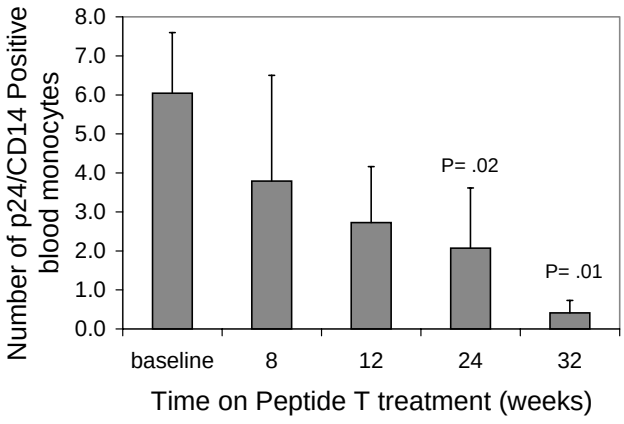

Fig. 1. Peptide T suppresses infection of peripheral blood monocytes. Cells were isolated at indicated time points and freshly stained with CD14 (monocytes) and anti-core p24 (HIV) antibody, to detect cells with active HIV replication. The results are presented as the mean percent of infected (p24 positive) cells from nine persons completing 24 weeks therapy. Statistical comparisons for weeks 8–24 with baseline values were by paired *t*-test.

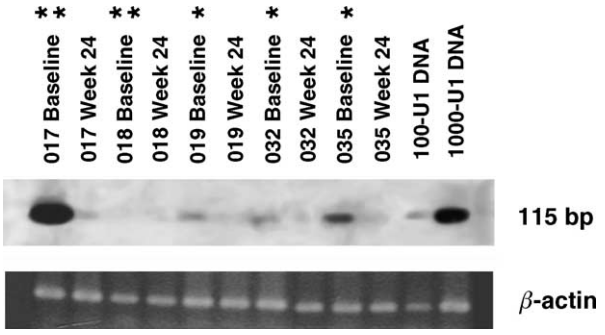

Fig. 2. Peptide T suppresses HIV-1 proviral integration. Genomic DNA was isolated from differentiated macrophages from patient's samples and analysed by inverse/nested PCR assay. Patients 19, 32, and 35 were receiving only peptide T, while 17 and 18 received peptide T plus HAART. Copy number standards were generated by PCR assay of a known number of U1 cells to contain 100 (100-U1 DNA) or 1000 integrated HIV copies, as indicated.

observed from baseline (6.8 ± 1.5% positive cells) to week 8 (19 ± 7% positive cells, $P = 0.04$), and these increases were sustained at week 12 (26±7.7%, $P = 0.003$). By week 24 these increases had declined and were not different from baseline levels.

Post-hoc analyses were performed to further explore the two primary outcomes. We dichotomized each according to the following criteria: any individual with viral levels (Amplicor) at the same or lower levels at the week 24 visit, as compared to their baseline visit, was classified as a "viral responder"; those whose 24-week viral levels were higher than their baseline values were classified as "viral non-responders". Similarly, we classified individuals whose week 24 CD4 levels were 20% or more higher than their baseline levels were labelled "CD4 responders" and those with 19.9% or less increase (including those with negative findings) relative to the baseline observation were labelled "CD4 non-responders".

We constructed 2×2 tables to evaluate the association between CD4 response status (responder/non-responder) and HAART status (HAART+peptide T versus peptide T alone) and between CD4 and viral response status. Fisher's exact tests were used to estimate the probabilities associated with these tables. We observed that only one of four individuals (25%) with HAART plus peptide T was classified as a CD4 responder, compared with four of seven individuals (57.1%) with peptide T as monotherapy. This trend was not significant ($\chi^2 = 1.06$, $P > 0.35$). When we examined the apparent dissociation between CD4 and viral responses, we observed that no individual demonstrating the CD4 response (i.e. an increase in CD4 at week 24 relative to baseline) demonstrated a stable or lower viral level at week 24 relative to baseline, and nearly all individuals with stable or lower viral levels at week 24 relative to baseline failed to show the CD4 response. Fisher's exact test showed that this pattern was significant ($\chi^2 = 7.6$, $P = 0.013$); that is, we found strong evidence of statistical independence between the viral and CD4 responses in this study cohort.

We also performed a post-hoc analysis to test the hypothesis that peptide T use in conjunction with HAART was more effective at flushing the blood monocyte reservoir. The association is significant ($\chi^2 = 5.76$, $P = 0.048$ for a two-tailed test). HAART plus peptide T was associated with significantly more suppression of active virus replication in the monocytes than peptide T alone. In fact, no one with the combination had $p24^+$ monocytes at week 24, while 80% (4/5) of those on peptide T alone had $p24^+$ monocytes.

4. Discussion

Although earlier studies have reported neurocognitive and brain imaging benefits of peptide T in AIDS, with no toxicities [7,8,18,19] this is the first study to assess antiviral and immune effects. Peptide T was safe, and no drug associated toxicities were detected. Frequent nasal examinations showed that the thrice daily intranasal application of 0.4 ml of metered spray were well-tolerated, with no nasal pathologies detected. This was a non-AIDS cohort of HIV positive men, predominantly composed of a stable, long-term non-progressor population with generally low viremia (log 3.6 copies/ml at baseline). Peptide T did not change overall plasma HIV RNA levels (Amplicor) after 24 weeks of treatment. There was a trend toward a rise in CD4 cell numbers (ns), and five of eleven responders showed a mean increase of 33% ($P = 0.06$, ns), considered to be a clinically significant response.

There were positive changes in the secondary endpoints. Since seven of the eleven in our cohort were only receiving peptide T during the study, this treatment appears to be associated with several antiviral and immunological improvements.

Peptide T has selective in vitro antiviral effect for R5 tropic HIV-1 isolates [14] and in the patients peptide T suppressed the monocyte reservoir as the production of p24, a measure of active R5 viral replication was reduced in these cells (Fig. 1). The combination of HAART plus peptide T was particularly effective at suppressing active replication in the blood monocytes compared to peptide T alone ($\chi^2 = 5.76$, $P = 0.048$ for a two-tailed test), and presumably HAART alone, as all patients on HAART entered the study with detectable monocyte p24 expression. No one of the four on the combination had p24 positive monocytes at the end of 24 weeks, despite being positive prior to starting peptide T.

The antiviral effect on the monocyte reservoir was confirmed by inverse PCR analysis of integrated proviral DNA, sensitive to <100 copies per 10^5 cells (Fig. 2) in five patients with enough PBMCs to make this determination. The reduction to nearly undetectable levels of integrated viral DNA observed in patients 19 and 35 is most likely due to peptide T as these patients were treatment naïve, having never taken HAART therapies. Patient 32, while receiving HAART had already been shown to be virally resistant to all current therapies and so this response may also be caused by peptide T.

We were able to isolate infectious virus from patient baseline PBMCs by co-culture in six of nine, consistent with the generally low viremia of this cohort. However, following peptide T treatment virus was undetectable by this method in five of the six (Fig. 3). The remaining positive individual had an X4 phenotype, consistent with peptide T antiviral effect predominantly for R5-tropic HIV isolates. Suppression was maintained after 6 weeks off peptide T in the five responders, but after 6 months virus became detectable in two, further suggesting antiviral effects with peptide T during the treatment period. Importantly these results indicate no obvious emergence of a shift in receptor usage to the X4 phenotype, as might occur with an R5 selective agent like peptide T, or emergence of treatment resistant viruses as we typically could not isolate virus after treatment.

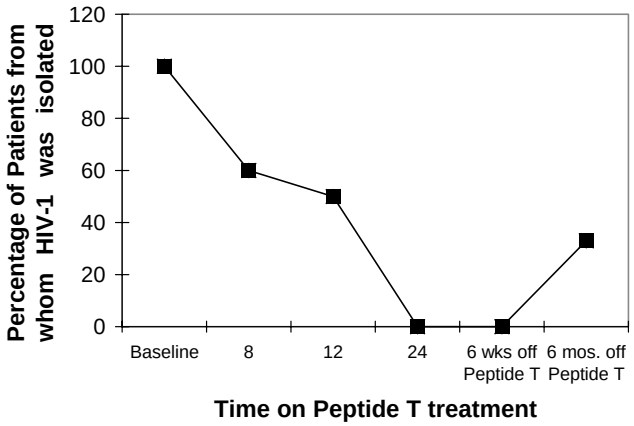

Fig. 3. Inability to isolate HIV-1 from patient blood mononuclear cells (PBMCs) after 24 weeks treatment with peptide T. Patient PBMCs were used to establish infection in naïve, activated, CD8 depleted donor (HIV^-) mononuclear cells. Samples were deemed positive if HIV p24 detected by any of the co-culture methods. See Section 2 for further details.

Immune changes following peptide T were also observed. Cytotoxic T lymphocytes (CTLs) play a role in containing HIV spread. It is believed that these effector lymphocytes are not fully functional in suppressing virus production due to lack of CD4 help or HIV suppression of virus-specific $CD8^+$ cells. The ability of CTLs to secrete gamma interferon (IFNγ) is an indicator of cytotoxic capability. IFNγ/$CD8^+$ T-cells were increased four-fold (Fig. 4) at weeks 8 and 12, but had returned to baseline values by 24 weeks, the next sampled time point. Maintenance of activated antiviral CD8 T-cells requires continued antigenic stimulation and so the return to baseline values of IFNγ/$CD8^+$ T-cells may reflect decreased viral antigen, a result supported by the decreased viral load in the PBMC and monocyte/macrophage populations (Figs. 1–3). Transient immune responses have also been observed with other immunomodulatory regimens, such as IL-2 [9]. Preliminary studies of patient IFNγ/$CD8^+$

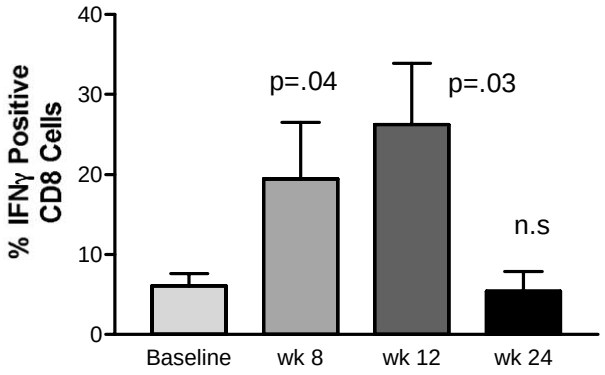

Fig. 4. Peptide T Increases in IFNγ Secreting $CD8^+$ lymphocytes. PBMCs from HIV-1 infected patients were isolated and $CD8^+$ T-cells stained for intracellular IFNγ. The results are presented as mean percent of IFNγ expressing $CD8^+$ T-cells. Statistical comparisons for weeks 8, 12 and 24 with baseline values were performed by paired *t*-test.

response to specific HIV antigen (gag) showed time dependent increases in five of seven, in some cases sustained at 24 weeks. Slight total CD4 increases were observed at weeks 24 (12%) and 32 (21%) compared to baseline values (ns), are also suggestive of an immune benefit. It was notable that all five apparent CD4 responders also had increases in percent IFNγ/$CD8^+$ T-cells, decreased p24/$CD14^+$ monocytes, and decreased ability to culture virus, suggesting these may be interdependent effects. Peptide T's ability to stimulate the production of CTLs, which kill virus-producing monocytes both preceded and was associated with its apparent ability to "flush" the PBMC reservoir and produce immunological benefit.

Despite peptide T's statistically significant antiviral and immunomodulatory benefits in secondary endpoints, the surrogate marker for plasma virus levels (HIV RNA), a primary endpoint, did not change in this study. One possible explanation is that the study group, an atypical cohort of long-term non-progressing individuals (mean 17 years HIV since diagnosis), comprise a distinct clinical subgroup [17], which is associated with emergence of replication defective strains [2,13]. Long-term HAART use, as occurred in this cohort, also contributes to the emergence of a virus population with reduced replicative capacity [2] which leads to sustained CD4 levels and non-progression [5].

The disconnect between lack of change in plasma HIV RNA compared to the decrease of infectious virus in the PBMCs (Fig. 1) and macrophages (Figs. 2 and 3) is consistent with the presence of replication impaired virus in plasma. This explanation is supported by the fact that no virus could be isolated from concentrated plasma of any patient either before or after treatment with multiple attempts, while virus was successfully isolated from peripheral blood mononuclear cells (PBMCs) only prior to treatment. The dissociation between the primary endpoints of CD4 level and plasma viremia that we found was significant ($\chi^2 = 7.6$, $P = 0.013$), providing strong evidence of the independence between the viral and CD4 response in the cohort and independently calling into question the clinical significance of the HIV RNA values in this group. A study with a more typical cohort is warranted to test for virological benefits by HIV RNA measures and clinical benefits (e.g. prevention of opportunistic infections and prolongation of life) needed to establish usefulness of peptide T as an adjunct non-toxic therapy in HIV infection.

Despite the small number of patients in this trial, peptide T's ability to greatly reduce the HIV content of the monocyte reservoir in all patients ($P < 0.02$) and in fact flush this reservoir to undetectable levels with concurrent HAART treatment ($P < 0.01$) was robust. The question of whether this viral monocyte flushing, however statistically compelling, actually eliminates the entire reservoir must await studies of peptide T's ability to delay viral rebound in carefully monitored and selected patients where treatment interruption is advisable. Even if peptide T can be shown to delay viral rebound, long-term studies of its ability to evince sustained

CD4 increases, prevent opportunistic infection and delay death would be needed to establish the clinical relevance of its ability to flush the monocyte reservoir in this cohort.

Finally, a placebo-controlled trial of a treatment-naïve cohort not yet eligible for antiretroviral therapy by the current guidelines (i.e. T-cells >350 and viruses less than 55,000) would be the appropriate cohort to examine DAPTAs value as a primary anti-viral therapy.

Note added in proof:

Progressive reductions over time in total PBMC integrated proviral DNA were observed in both of the two patients who could be analysed, one of whose virus became completely undetectable by this stringent method of analysis after 48 weeks of peptide T treatment.

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