Cell-associated viral burden provides evidence of ongoing viral replication in aviremic HIV-2 infected patients

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Abstract

Viremia is significantly lower in HIV-2 than HIV-1 infection irrespective of disease stage. Nevertheless, the comparable proviral DNA burden observed in these two infections indicates similar numbers of infected cells. Here we investigated this apparent paradox by assessing cell-associated viral replication.

We found that untreated HIV-1+ and HIV-2+ individuals, matched for CD4 T cell depletion, exhibited similar gag mRNA levels indicating that significant viral transcription is occurring in untreated HIV-2+ patients despite the reduced viremia (undetectable to 2.6x10^4 RNA copies/ml). However, tat mRNA transcripts were observed at significantly lower levels in HIV-2+ patients, suggesting that the rate of de novo infection is decreased in these patients. Our data also reveal a direct relationship of gag and tat transcripts with CD4 and CD8 T cell activation, respectively. ART-treated HIV-2+ patients showed persistent viral replication, irrespective of plasma viremia, possibly contributing to the emergence of drug-resistance mutations, persistent hyper-immune activation and poor CD4 T cell recovery that we observed in these individuals.

In conclusion, we provide here evidence of significant ongoing viral replication in HIV-2+ patients, further emphasizing the dichotomy between amount of plasma virus and cell-associated viral burden, and stressing the need for antiretroviral trials and definition of therapeutic guidelines in HIV-2 infection.
Introduction

HIV-2 infection is characterized by low to undetectable plasma viral load (3, 5, 46, 50, 54), in agreement with its reduced transmission rate (2, 26, 30), providing a natural model to investigate the relative contribution of HIV replication to AIDS progression.

Notwithstanding the two-log difference in viremia levels that characterize HIV-2 and HIV-1 infection, similar levels of cell-associated viral DNA have been reported, suggesting a comparable number of infected cells (6, 24, 45, 52). This apparent paradox highlights the potential contribution of quiescent latent virus to proviral load and the relevance of quantifying the ongoing viral replication. The only study, to our knowledge, addressing transcriptional activity in HIV-2+ patients, demonstrated lower \textit{gag} mRNA expression levels in HIV-2+ than HIV-1+ individuals (34). However, the fact that these cohorts were not paired for disease stage is a possible confounding factor, as it is known that \textit{gag} mRNA levels are increased in HIV-1+ individuals with low CD4 T cell counts (22, 39).

In spite of the rate of CD4 T cell decline being much slower in HIV-2 than in HIV-1 infection (19, 35), a progressive CD4 T cell depletion ultimately leading to AIDS is observed in HIV-2+ individuals (12, 36). Importantly, despite the lack of clinical trials of antiretroviral therapy (ART) in HIV-2 infection, the majority of reports showed poor immunological recovery in ART-treated HIV-2+ patients, even in the context of suppression of viremia (1, 19, 29, 40, 51, 56). A rapid emergence of drug-associated mutations in HIV-2+ patients under ART has also been reported (7, 13-15, 25, 29, 41, 47, 48), suggesting, that there is some ongoing viral replication. There are no data regarding the impact of ART on HIV-2 proviral DNA levels and/or HIV-2 transcriptional activity.
We have previously shown that CD4 T cell depletion is directly related to immune activation but only indirectly to plasma viral load in both HIV-2 and HIV-1 infections (28, 55). Here we investigate the relationship of CD4 T cell levels and T cell activation with cell-associated viral burden, measured in terms of \textit{gag} and \textit{tat} mRNA and proviral DNA levels, in parallel with plasma viremia in cohorts of untreated HIV-2+ and HIV-1+ individuals together with a cohort of ART-treated HIV-2+ individuals.

**Patients and Methods**

*Studied cohorts*

We assessed 45 HIV-2+ (16 of which were receiving ART) and 27 untreated HIV-1+ patients followed at Hospital de Santa Maria in Lisbon, Portugal, as well as 16 HIV-seronegative age-matched controls. None of the patients had ongoing opportunistic infections or tumours. Table 1 and 2 describe untreated and treated cohorts, respectively. Although HIV-2 infected cohorts included an increased number of women, non-Caucasian and elderly individuals, in the current study these factors were not found to significantly impact in the parameters under analysis (data not shown). All subjects gave informed consent for blood sampling and processing. The study was approved by the Ethical Board of the Faculty of Medicine, University of Lisbon.

*Cell isolation and flow cytometry*

Peripheral blood mononuclear cells (PBMC) were isolated immediately after blood collection and characterized by flow cytometry as previously described (9).
DNA and mRNA extraction

DNA was extracted from 5x10^6 PBMC using QIAamp DNA Mini Kit. For mRNA extraction, 5x10^6 PBMC were lysed (RLT), homogenized (Qiashredder columns), extracted (Oligotex mRNA direct mini-kit) (all from Qiagen, Valencia, CA), treated with DNase (DNA-free kit, Ambion, Austin, TX) and immediately converted to cDNA. Samples were quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE).

Viremia and Proviral DNA

HIV-1 viremia was quantified by a RT-PCR assay with detection threshold of 40 RNA copies/ml (Roche, Basel, Switzerland). HIV-2 viremia was quantified using a previously described in-house developed assay with a detection threshold of 200 RNA copies/ml (54). Quantification of viremia in ART-treated individuals with levels below 200 RNA copies/ml was repeated using a more sensitive real-time RT-PCR assay with a detection threshold of 40 RNA copies/ml as described (20). HIV-1 and HIV-2 total viral DNA (integrated and non-integrated viral DNA species) was quantified using real-time PCR assays that amplify highly conserved regions in HIV-1 and HIV-2 gag with a detection range of 7 orders of magnitude and a sensitivity of 5 copies as we have previously described (52). Test cut-off values were used to calculate the mean and the correlations with other parameters when levels were below cut-off.

gag and tat mRNA

80 ng of mRNA was reverse transcribed using Superscript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) and 250 nM of random hexamers and was quantified in duplicate using cDNA (1μg) in a PCR mixture (50μl) containing 25μl Platinum Quantitative PCR SuperMix-UDG, 1μl ROX Reference Dye 50X, 5 mM MgCl₂ (all from Invitrogen) and
variable concentrations of the following primers and probes: HIV-1 gag F2 (10 pmol/ul): 5'-gggagaattagatgagaaa-3’; HIV-1 gag R1 (10 pmol/ul): 5’-gtcctgctgagcccata-3’; HIV-1 gag Probe: 5’ FAM- ccctggccttaaccgaatt- MGB-3’; HIV-2 gag F2 (10 pmol/ul): 5’-cgccgagaaactccttg-3’; HIV-2 gag R2 (10 pmol/ul): 5’-cacacaatatgttttagctgtacttttt; Probe HIV-2-gag: 5’ FAM- cgggcgcgtaacct- MGB-3’. tat multiply spliced mRNA expression was quantified using the following primers and probes: HIV-1 tat F2.3 (6 pmol/ul): 5’-gacgaagagctcctcaagaca-3’; HIV-1 tat R2.4 (6 pmol/uL): 5’- gagacagagacagatccggtc – 3’; HIV-1 tat probe: 5’ FAM-tctctataacgaacctcagaca- MGB-3’; HIV-2 tat F3.5 (10 pmol/ul): 5’-agggctcgggatatgtt-3’; HIV-2 tat R3.1 (10 pmol/ul): 5’- tctgtatccaccgtcgtttc – 3’; HIV-2 tat Probe: 5’ -FAM-tgcatcagacaaatc- MGB-3’, in a AbiPrism 7000 SDS (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control (FAM/MGB probe, non primer limited – Applied Biosystems; standard curve with cDNA generated from pooled PBMC from 3 seronegatives). Standard curves for tat were generated using amplifications of synthetic oligonucleotides (GenScript Corporation, Piscataway, NJ, USA) corresponding to 200 bp fragments of HIV-1 and HIV-2 tat transcripts, and for gag as described previously (52). The mean values and the correlations with other parameters were calculated using the tat and gag relative quantification values of patients presenting detectable levels of the transcripts.

**HIV-2 Sequencing**

*In-house* methodology was used to sequence a 1280-bp HIV-2 *pol* gene fragment including the entire protease and part of reverse transcriptase from plasma samples. Primers are listed in Table 3. Viral RNA was retrotranscribed and amplified using *Access RT-PCR Core Reagents* kit (Promega, Madison, WI, USA), and outer primers JA218 ([+1859] 5’-GAA AGA AGC CCC GCA ACT TCC-3’) and JA221 ([−3258] 5’-GCT CTG CTT CTG CTA CTA
ATT CTG TCC A-3’) as described (54). A nested PCR was performed: second amplification was carried out using AmpliTaq Gold PCR Master Mix and the inner primers JA219 ([+1898] 5’-AGG GGC T(A/G)A CAC CAA CAG CAC-3’) and JA220MOD ([-3178] 5’-GTC TTT AT(T/C) CCT GGG TAG AT(T/G) TGT G-3’). Cycle sequencing was accomplished with Big Dye Terminator v3.1 Cycle Sequencing Kit according to manufacturer’s recommendations and using four sequencing primers: JA219, JA220 ([+3178] 5’-GTC TTT AT(T/C) CCT GGG TAG ATT TGT G-3’), JA222 ([+2525] 5’-ACC TCC AAC TAA TCC TTA TAA TAC C-3’) and JA223 ([+2625] 5’-ACT GAA TTT CTG TGA AAT CTT GAG T-3’). Purified products were ran on an ABI PRISM® 3100 Genetic Analyzer according to the same protocol, but adjusted to HIV-2 specific settings; and nucleotide sequences were analyzed with SeqScape® Software Version 2.5 (all from Applied Biosystems) by alignment with the ROD HIV-2 reference strain (GenBank accession number M15390).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software Inc., SD, USA) using Mann-Whitney, Spearman’s coefficient, and Fisher’s exact test. P-values <0.05 were considered significant. Test cut-off value was used to calculate the mean if levels were below cut-off.

Results

Ongoing viral replication in untreated HIV-2 infection

In order to investigate the degree of ongoing viral replication during the natural history of HIV-2 and HIV-1 infections we compared untreated HIV-2 and HIV-1 cohorts matched for the degree of CD4 T cell depletion (Table 1), although length of infection was likely greater
in HIV-2+ than HIV-1+ individuals. As expected, viremia was significantly lower in HIV-2 than HIV-1 infection (3, 5, 46, 50, 54). Twenty-two out of 29 HIV-2+ patients had levels below the test cut-off (“aviremic”) and the highest viremia was $2.6 \times 10^4$ RNA copies/ml (Table 1). Conversely, proviral DNA load, as previously reported, was similar (6, 24, 45, 52), suggesting comparable numbers of infected cells in these two groups despite their distinct viremia (Table 1). Of note, although no significant correlation was found between proviral DNA and viremia in HIV-2 infection, significantly higher proviral levels were found in viremic than “aviremic” individuals (p= 0.0396).

* tat mRNA*, a multiply spliced (MS) HIV transcript, is thought to be mainly expressed in recently infected cells and/or cells actively producing virus (31, 32, 38, 53). We found a significantly higher number of individuals with undetectable *tat* mRNA in the HIV-2 than in the HIV-1 cohort (p=0.0014; Fisher’s exact test). Moreover, HIV-2+ patients with detectable *tat* mRNA featured significantly lower levels than their HIV-1+ counterparts (Fig.1A), and a significant correlation with viremia was only observed in the HIV-1 cohort (r=0.4791, p=0.0443, n=19 for HIV-1 cohort; r=0.0312, p=0.9349, n=8 for HIV-2 cohort). Although no correlation with CD4 T cell levels were observed (Table 3), individuals with low CD4 T cell counts tended to have higher levels of *tat* mRNA (Supplemental Fig.1).

Conversely, *gag* mRNA, an unspliced (US) HIV transcript, was similarly expressed in the HIV-1 and HIV-2 cohorts (Fig.1B), which included an equal number of individuals with undetectable expression levels. Of note, no correlation was found with viremia in either infection.

Furthermore, on comparing viremic and “aviremic” HIV-2+ individuals, we observed no significant differences between the number of patients with detectable *tat* and *gag* mRNA transcripts, as well as similar levels of expression of *tat* and *gag* mRNA (Fig.1A and Fig.1B),
though these results should be interpreted cautiously given the small number of individuals assessed.

A low ratio of gag/tat mRNA levels has been suggested both as a marker of active viral transcription and of HIV-1 disease progression (22, 37, 49). We found a significantly higher gag/tat ratio in HIV-2+ as compared to HIV-1+ patients (p=0.0118), although no association with viremia was found in either cohort.

Of note, neither tat nor gag mRNA expression levels, nor gag/tat ratio significantly correlated with proviral DNA load in either HIV cohort. Moreover, when we subdivided the HIV-1 and HIV-2 cohorts on the basis of detectable and undetectable viral mRNA, no differences in proviral DNA levels were observed (Fig.1C and Fig.1D). Thus, no direct relationship appears to exist between the number of infected cells, as estimated by proviral DNA, and the levels of tat and gag transcription in PBMC during untreated HIV-2 and HIV-1 infections, suggesting a significant contribution of archived viral DNA.

Overall, HIV-2+ individuals exhibited reduced levels of tat, and similar levels of gag transcripts as compared to their HIV-1+ counterparts translating into a higher gag/tat mRNA ratio in the former.

**Relationship of plasma and cell-associated viral load with CD4 T cell depletion and T cell activation in HIV-2 infection**

We have previously shown that T cell activation markers were similarly up-regulated in HIV-1 and HIV-2 infections when patients were matched for CD4 T cell levels, suggesting that CD4 T cell depletion is more directly linked to immune activation than to viral load (28, 55). Here, we assessed the relationship between cell-associated viral mRNA and DNA and the hyper-immune activation observed in HIV-2 and HIV-1 infections.
With respect to *gag* mRNA expression, we found a direct correlation with the levels of HLA-DR expression (Table 3) and HLA-DR and CD38 co-expression (r=0.5150; p=0.0287) within CD4 T cells in HIV-2+, which did not reach statistical significance in HIV-1+ individuals. No correlations were found with CD8 T cell activation levels (Table 3).

Conversely, we observed that the levels of *tat* mRNA in HIV-1+ individuals directly correlated with CD8 T cell activation levels, measured either as proportion of HLA-DR+CD38+ (Table 3) or CD38+ cells (r=0.6196; p=0.0047), as well as CD38 mean fluorescence intensity (MFI; r=0.7386; p=0.0003). Clear trends for an association between *tat* mRNA levels and CD8 T cell activation were also observed in the HIV-2 cohort, but no significant correlations were found with CD4 T cell activation in either infection (Table 3).

Of note, proviral DNA levels did not directly correlate with either CD4 or CD8 T cell activation in HIV-1+ and HIV-2+ individuals (Table 3), further suggesting that a significant component comprises archived quiescent virus.

In spite of the narrow range of HIV-2 viremia (undetectable to 26263 copies/ml), significant positive correlations were found between viremia and several T cell activation markers (Table 3; percentage of HLA-DR+CD38+ within CD4 T cells: r=0.5685, p=0.0013; percentage and MFI of CD38 within CD8 T cells: r=0.6196, p=0.0047 and r=0.5330, p=0.0029, respectively). In order to further assess the relative contribution of viremia, we compared viremic and “aviremic” HIV-2+ individuals (Table 1). Although the former showed significantly higher CD4 and CD8 T cell activation than the latter (Fig.2A and Fig.2B), both viremic and “aviremic” HIV-2+ individuals had significantly higher levels of T cell activation as compared to seronegatives (Fig.2A and Fig.2B), and exhibited strong negative correlations between CD4 T cell numbers and CD4 and CD8 T cell activation levels (Fig.2C and Fig.2D).
Overall, our data not only demonstrated that T cell activation was strongly associated with CD4 T cell depletion, both in viremic and “aviremic” HIV-2+ individuals, but also supported a contribution, even at low levels, of circulating virus to both CD4 and CD8 T cell activation. Moreover, we showed that gag mRNA was directly related to CD4 T cell activation, and tat mRNA to CD8 T cell activation, suggesting an overall impact of viral transcripts upon T cell activation.

**Cell-associated viral mRNA and DNA in HIV-2+ patients under ART.**

In order to further dissect the impact of cell-associated viral DNA and RNA upon HIV-2 immunopathogenesis, we assessed these parameters in patients receiving ART (Table 2). This cohort exhibited significantly lower CD4 T cell counts than untreated HIV-2+ individuals (p=0.0046), in agreement with previous reports showing a limited CD4 T cell recovery in ART-treated HIV-2 infection (1, 19, 29, 40, 51, 56). Viremia was similar in the treated and untreated HIV-2 cohorts, due to the low level viremia detected in some of the ART-treated HIV-2+ patients (Table 2).

Of note, in contrast with the progressive decline in proviral DNA levels usually observed in ART-treated HIV-1+ patients (23, 58), proviral DNA did not significantly differ between untreated and treated HIV-2+ patients (Fig.3A) despite the prolonged treatment. Moreover, treated and untreated HIV-2 patients featured similar levels of gag mRNA expression, whereas tat expression was significantly higher in the treated cohort, although the reduced number of individuals with detectable tat transcripts preclude a definitive interpretation (Fig.3A). No significant differences were found between the number of patients with detectable tat and gag mRNA transcripts within treated and untreated groups. These results showed significant amounts of ongoing viral replication in ART-treated HIV-2 infection.
suggesting a limited effectiveness of the ART regimens in HIV-2 infection, which justifies further exploration in large cohorts treated with optimized ART regimens.

Additionally, neither the amount of gag and tat transcripts, nor proviral DNA significantly differed between treated HIV-2 patients with detectable and undetectable viremia (Fig.3A), emphasizing that the absence of detectable viremia is not a good surrogate marker for cell-associated viral mRNA and/or DNA during HIV-2 infection.

A rapid emergence of mutations potentially associated with drug-resistance has been reported in HIV-2+ patients under ART (25, 29, 41). We confirmed the presence of mutations in the reverse transcriptase and protease in our ART-treated HIV-2 cohort (Table 4). These data further support the presence of significant amounts of ongoing replication in these ART-treated HIV-2+ patients, leading us to speculate about the potential contribution of ART-induced selective pressure upon the virus to the increased tat transcript levels (Fig.3).

The selection of adequate ART regimens to treat HIV-2 infection is difficult given the lack of clinical trials. There are some phenotypic studies that allow us to select better first-line therapies then a few years ago (16, 61), but the knowledge of HIV-2 resistance pathways is still incomplete. Longitudinal data of an individual HIV-2+ patient, in whom the initial ART regimen was associated with the rapid emergence of mutations and virological failure, illustrated that switching to another ART combination was associated with viremia suppression and a decline of cell-associated viral burden (Fig.3B). This was supported by both a loss of measurable tat and gag transcripts, and a decrease in the levels of proviral DNA, both of which are indicative of a successful virological response. Nevertheless, this patient’s immunological response was limited, with only marginal CD4 T cell recovery, and partial decrease in both CD4 and CD8 T cell immune activation (Fig.3B), suggesting that factors other than viral replication are contributing to the poor immunological reconstitution.

Our findings support a role for persistent immune activation, given that, as shown in figures
3C and 3D, ART-treated HIV-2+ individuals exhibited: significantly higher levels of T cell activation as compared to seronegatives; similar levels of CD4 and CD8 T cell activation in comparison with untreated HIV-2 cohort; and significant negative correlations between absolute CD4 T cell numbers and the percentages of HLA-DR+ within CD4 T cells (r=-0.5147; p=0.0413), and HLA-DR+CD38+ within CD8 T cells (r=-0.5294; p=0.0350).

Overall, significant amounts of ongoing viral replication were observed in HIV-2 infected patients under ART, highlighting the importance of additional studies on the antiretroviral drug efficacy in HIV-2 infection. Furthermore, ART apparently failed to significantly impact upon T cell activation, even in patients with undetectable viremia, possibly contributing to their low CD4 T cell recovery.

Discussion

Here we demonstrated that untreated HIV-1+ and HIV-2+ individuals with similar degrees of CD4 T cell depletion featured similar levels of gag mRNA transcripts, suggesting that significant viral transcription occurs in HIV-2 patients, despite the lower viremia. Conversely, we found decreased levels of tat mRNA in untreated HIV-2+ individuals. Given the previous reports demonstrating that HIV-1 tat mRNA transcripts accumulate and outnumber gag mRNA transcripts in recently infected cells (31, 32, 38, 53), our results provide evidence for a decreased rate of de novo cell infection in HIV-2 disease.

Proviral DNA levels have been used to estimate the extent of viral reservoirs (8, 10, 11, 18). We and others have shown that the levels of total proviral DNA are similar in the two HIV infections despite the reduced viremia observed in HIV-2+ individuals (6, 45, 52). This may result from a preferential contribution of latently-infected, quiescent T cells, to total HIV-2 proviral DNA. Alternatively, a significant amount of ongoing viral replication occurs but it
does not translate into plasma viral load. In this study, we showed that the levels of proviral DNA were not associated with viral transcription levels in both HIV-1+ and HIV-2+ individuals, bringing into question the reliability of proviral DNA levels as a marker of replicative activity in both infections.

Additionally, the impact of plasma and cell-associated viral load upon HIV-2-associated hyper-immune activation was investigated. Of note, significantly higher levels of CD4 and CD8 T cell activation were found in viremic as compared to “aviremic” individuals, despite the small amount of circulating virus observed in viremic HIV-2+ patients. In agreement with a recent report in an African cohort (33), our data support a contribution of plasma viral load, even at low levels, to immune activation, which may be related both to transmission of cell free viruses and/or immunological effects of viral proteins.

The direct association between gag mRNA and CD4 T cell activation we observed in HIV infected patients raises the possibility that ongoing viral replication significantly contributes to the maintenance of heightened T cell activation in HIV-2+ individuals in spite of the reduced viremia. In addition, the direct association found between tat mRNA levels and CD8 T cell activation, particularly in HIV-1+ individuals, suggests a specific role of this transactivator molecule and/or newly infected cells in driving CD8 T cell activation.

With respect to HIV-1 infection, those rare (<0.1%) individuals who are able to control viral replication in the absence of ART (27, 42, 44, 60) provide another valuable resource for the investigation of factors associated with viremia control. Various criteria have been used to define this population, including viremia ranging from undetectable (elite controllers) up to 2000 RNA copies/ml (viremic controllers) (42, 60). Notably, the small cohort of HIV-1 controllers within our untreated HIV-1 cohort resemble the untreated HIV-2 cohort in terms of viremia, gag and tat mRNA expression, gag/tat ratio and proviral DNA levels. The seven individuals with viremia <2000 RNA copies/ml tended to have lower levels of tat mRNA
Soares et al. HIV-2 versus HIV-1 cell associated burden

(14.59±14.47) than the other HIV-1+ patients (262.0±210.4), though not reaching statistical significance. The number of patients with detectable tat mRNA, gag mRNA and proviral DNA was similar within these subgroups of the HIV-1 cohort, and no differences in gag mRNA levels or proviral DNA were observed, but the gag/tat ratio was higher in controllers (p=0.0434). These data further support that viremia control is associated with reduced levels of tat transcripts.

Finally, we provide evidence of persistent HIV-2 replication during ART, based on proviral DNA, gag and tat mRNA levels, irrespective of detectable plasma viremia. We also demonstrated drug-related genetic evolution of HIV-2 reverse transcriptase and protease gene sequences. The high levels of tat mRNA observed in the ART-treated HIV-2 cohort suggested that the therapeutic regimens used were unable to reduce the rate of de novo cell infection. These data contrast with ART-treated HIV-1+ patients in which virological response is usually associated with a sharp decline in MS mRNA (4, 21, 62), and in the proportion of MS mRNA relative to US mRNA in PBMC (57), as well as with a progressive decrease in proviral DNA (23, 58, 59) despite the low-level viremia that can frequently be detected using ultrasensitive assays (17, 43).

In agreement with previous reports (1, 19, 29, 40, 51), we documented poor CD4 T cell recovery in ART-treated HIV-2+ individuals, even in those with evidence of viral suppression. Our findings suggest that persistent hyper-immune activation may be a main determinant of this impaired immune reconstitution. The study of lymphoid tissue from HIV-2 infected patients will be instrumental in evaluating the degree of irreversible damage associated with long-term infection that may limit the potential for immune recovery, and to provide support for an early start of ART in this otherwise relatively benign disease.

In conclusion, we provide here evidence of ongoing viral replication in HIV-2 infection despite the low to undetectable viremia, and of its association with CD4 and CD8 T cell
activation, with the latter being more closely related to the levels of tat mRNA. Of particular note was the persistent viral replication in ART-treated HIV-2+ individuals. In light of these findings, and in particular the apparent ease with which the virus mutated in treated HIV-2+ individuals to escape drug activity, there is an obvious need for large scale drug trials in HIV-2 infection, to determine the most appropriate drug regimen, and also the benefit of an early initiation of therapy in this infection.

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Potential conflict of interest. All authors: no conflicts.

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**TABLE 1.** Characterization of untreated HIV-2, untreated HIV-1 and seronegative cohorts

<table>
<thead>
<tr>
<th></th>
<th>Seronegative</th>
<th>Untreated HIV-2</th>
<th>Untreated HIV-1</th>
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<tbody>
<tr>
<td>Number (male/female)</td>
<td>16 (6/10)</td>
<td>29 (9/20)</td>
<td>27 (18/9)</td>
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<tr>
<td>Age (years)</td>
<td>45 [27-57]</td>
<td>52 [19-78]#</td>
<td>39 [23-61]</td>
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<tr>
<td>Ethnicity</td>
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<td>15/14</td>
<td>20/7</td>
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<td>CD4 T cells/µl</td>
<td>818 [518-1312]</td>
<td>568 [52-1586]*</td>
<td>372 [18-1848]**</td>
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<td>% CD4 T cells</td>
<td>43 [34-61]</td>
<td>28 [7-54]***</td>
<td>20 [1-47]***</td>
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<tr>
<td>CD8 T cells/µl</td>
<td>494 [213-1109]</td>
<td>788 [271-1701]**</td>
<td>1065 [177-2996]**</td>
</tr>
<tr>
<td>% CD8 T cells</td>
<td>27 [12-42]</td>
<td>37 [23-74]***</td>
<td>50 [16-69]***</td>
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<td>Viremia (RNA copies/ml)</td>
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<td>1.4 x10⁶ [40-4.5x10⁶]</td>
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<td>Proviral DNA (copies/10⁶ PBMC)</td>
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<td>5 [5-1033]</td>
<td>54 [5-975]</td>
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n.a., not applicable. Data are median with limits in brackets. Test cut-off value was used to calculate the median if levels were below cut-off.

Significance in comparison with seronegative: *p<0.05, **p<0.01, ***p<0.0001. Significance in comparison with HIV-1: #p<0.05, ###p<0.0001.

* HIV-2 viremia was below 200 RNA copies/ml (cut-off) in 22 out of 29 patients studied; HIV-1 viremia was below 40 RNA copies/ml (cutoff) in 3 out of 24 patients.

* Proviral DNA was below 5 copies/10⁶ PBMC (cut-off) in 15 out of the 29 HIV-2-infected patients and in 9 out of 24 HIV-1-infected patients investigated.
**TABLE 2.** Characterization of ART-treated HIV-2 cohorts

<table>
<thead>
<tr>
<th></th>
<th>ART HIV-2 “aviremic”</th>
<th>ART HIV-2 viremic</th>
<th>ART HIV-2 (all)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (male/female)</td>
<td>11 (5/6)</td>
<td>5 (3/2)</td>
<td>16 (8/8)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54 [34 - 62]</td>
<td>53 [31 - 62]</td>
<td>54 [31-62]</td>
</tr>
<tr>
<td>Current ART regimen:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two NRTI</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Two NRTI +1 PI</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Three NRTI</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Length of follow-up (months):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8 T cells/µl</td>
<td>825 [244 - 1419]</td>
<td>912 [447 - 1532]</td>
<td>887 [244-1532]</td>
</tr>
<tr>
<td>% CD8 T cells</td>
<td>50 [26 – 64]</td>
<td>53 [49 - 65]</td>
<td>51 [26-65]</td>
</tr>
<tr>
<td>Viremia (RNA copies/ml)</td>
<td>200</td>
<td>4172 [499 - 34314]</td>
<td>200 [200-34314]</td>
</tr>
<tr>
<td>Proviral DNA (copies/10⁶ PBMC)</td>
<td>108 [5 - 346]</td>
<td>5 [5 - 726]</td>
<td>57 [5-726.0]</td>
</tr>
</tbody>
</table>

Data are median with limits in brackets. Test cut-off value was used to calculate the median if levels were below cut-off.

*Proviral DNA was below 5 copies/10⁶ PBMC (cut-off) in 5 out of 11 “aviremic” and in 3 out of 5 viremic HIV-2 treated patients.
TABLE 3. Correlations between virological parameters and levels of CD4 T cells and immune activation in untreated HIV-2 and HIV-1 infections.

<table>
<thead>
<tr>
<th></th>
<th>% CD4 T cells</th>
<th>CD4 T cells/µl</th>
<th>% HLA-DR+ in CD4 T cells</th>
<th>% HLA-DR+38+ in CD8 T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r; p</td>
<td>r; p</td>
<td>r; p</td>
<td>r; p</td>
</tr>
<tr>
<td>Viremia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-2</td>
<td>-0.3097; 0.1021</td>
<td>-0.3943; 0.0343</td>
<td>0.5186; 0.0039</td>
<td>0.5288; 0.0032</td>
</tr>
<tr>
<td>HIV-1</td>
<td>-0.6120; 0.0009</td>
<td>-0.5810; 0.0019</td>
<td>0.4731; 0.0146</td>
<td>0.7140; &lt; 0.0001</td>
</tr>
<tr>
<td>Proviral DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-2</td>
<td>0.0313; 0.8719</td>
<td>0.1555; 0.4207</td>
<td>0.2281; 0.2339</td>
<td>0.1029; 0.5952</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.0652; 0.7621</td>
<td>-0.1019; 0.6358</td>
<td>0.0005; 0.9983</td>
<td>0.1590; 0.4580</td>
</tr>
<tr>
<td>tat mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-2</td>
<td>-0.5952; 0.1323</td>
<td>-0.5238; 0.1966</td>
<td>0.6190; 0.1017</td>
<td>0.6667; 0.0831</td>
</tr>
<tr>
<td>HIV-1</td>
<td>-0.3737; 0.1150</td>
<td>-0.3193; 0.1827</td>
<td>0.2825; 0.2413</td>
<td><strong>0.5404; 0.0169</strong></td>
</tr>
<tr>
<td>gag mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-2</td>
<td>-0.5726; 0.0130</td>
<td>-0.3870; 0.1126</td>
<td><strong>0.4985; 0.0353</strong></td>
<td>0.3189; 0.1971</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.2281; 0.3477</td>
<td>0.1869; 0.4435</td>
<td>-0.4018; 0.0882</td>
<td>-0.4193; 0.0739</td>
</tr>
</tbody>
</table>

Test cut-off values were used to calculate the correlations with viremia or proviral DNA, if levels were below cut-off. Correlations with viral mRNA were calculated using the tat and gag relative quantification values of patients presenting detectable levels of the transcripts (8 and 18 in HIV-2, respectively and 19 for both tat and gag in HIV-1). Statistical significant correlations are represented in bold.
TABLE 4. Virological parameters and CD4 T cell levels of HIV-2+ patients under ART.

<table>
<thead>
<tr>
<th>Case</th>
<th>Current ART regimen</th>
<th>Follow-up: HIV diagnosis/ART (months)</th>
<th>CD4 T cell pre/post-ART (cells/μl)</th>
<th>Mutations</th>
<th>Viremia (Log10 copies/ml)</th>
<th>Proviral DNA mRNA (Arbitrary units)</th>
<th>tat mRNA (Arbitrary units)</th>
<th>gag mRNA (Arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>d4T+3TC+ SQV/r</td>
<td>177/108</td>
<td>300, 719</td>
<td>NA</td>
<td>4172</td>
<td>5</td>
<td>Undet</td>
<td>1.07x10^3</td>
</tr>
<tr>
<td>2</td>
<td>AZT/3TC/ABC</td>
<td>63/63</td>
<td>138, 554</td>
<td>NA</td>
<td>&lt;40</td>
<td>5</td>
<td>Undet</td>
<td>9.82x10^3</td>
</tr>
<tr>
<td>3</td>
<td>AZT/3TC</td>
<td>262/26</td>
<td>457, 535</td>
<td>PR+RT</td>
<td>&lt;40</td>
<td>5</td>
<td>2.13x10^3</td>
<td>Undet</td>
</tr>
<tr>
<td>4</td>
<td>AZT/3TC</td>
<td>206/64</td>
<td>500, 520</td>
<td>NA</td>
<td>44</td>
<td>158</td>
<td>Undet</td>
<td>Undet</td>
</tr>
<tr>
<td>5</td>
<td>AZT/3TC+LPV/r</td>
<td>126/26</td>
<td>125, 457</td>
<td>NA</td>
<td>&lt;40</td>
<td>114</td>
<td>Undet</td>
<td>3.56x10^3</td>
</tr>
<tr>
<td>6</td>
<td>AZT/3TC+LPV/r</td>
<td>38/28</td>
<td>164, 406</td>
<td>NA</td>
<td>&lt;40</td>
<td>108</td>
<td>Undet</td>
<td>4.46x10^3</td>
</tr>
<tr>
<td>7</td>
<td>AZT/3TC/ABC</td>
<td>242/61</td>
<td>287, 340</td>
<td>PR+RT</td>
<td>191</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>AZT/3TC+SQV/r</td>
<td>8/5</td>
<td>222, 326</td>
<td>NA</td>
<td>&lt;40</td>
<td>145</td>
<td>Undet</td>
<td>Undet</td>
</tr>
<tr>
<td>9</td>
<td>AZT/3TC+NVP</td>
<td>29/4</td>
<td>143, 277</td>
<td>PR+RT</td>
<td>499</td>
<td>5</td>
<td>3.14x10^3</td>
<td>1.72x10^3</td>
</tr>
<tr>
<td>10</td>
<td>AZT/3TC</td>
<td>15/1</td>
<td>297, 273</td>
<td>NA</td>
<td>&lt;40</td>
<td>126</td>
<td>Undet</td>
<td>Undet</td>
</tr>
<tr>
<td>11</td>
<td>AZT/3TC+IDV/r</td>
<td>65/51</td>
<td>112, 251</td>
<td>NA</td>
<td>&lt;40</td>
<td>346</td>
<td>5.19x10^3</td>
<td>1.91</td>
</tr>
<tr>
<td>12</td>
<td>AZT/3TC/ABC</td>
<td>220/56</td>
<td>176, 163</td>
<td>PR+RT</td>
<td>742</td>
<td>187</td>
<td>Undet</td>
<td>7.25x10^3</td>
</tr>
<tr>
<td>13</td>
<td>AZT/3TC+IDV/r</td>
<td>218/65</td>
<td>121, 102</td>
<td>PR+RT</td>
<td>&lt;40</td>
<td>5</td>
<td>3.73x10^3</td>
<td>3.30x10^3</td>
</tr>
<tr>
<td>14</td>
<td>d4T+3TC+SQV/r</td>
<td>177/12</td>
<td>82, 87</td>
<td>NA</td>
<td>34314</td>
<td>726</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>AZT/3TC+LPV/r</td>
<td>67/12</td>
<td>120, 84</td>
<td>PR+RT</td>
<td>10474</td>
<td>5</td>
<td>Undet</td>
<td>Undet</td>
</tr>
<tr>
<td>16</td>
<td>ABC/FTC+LPV/r</td>
<td>216/7</td>
<td>128, 70</td>
<td>NA</td>
<td>&lt;40</td>
<td>5</td>
<td>Undet</td>
<td>Undet</td>
</tr>
</tbody>
</table>


* ART refers to the current therapy. Previous ART regimens included: Case 1 – AZT, ddI, ddC; Case 4 – ddC, d4T; Case 9- ddI, EFV; Case 13- d4T; Case 14- AZT, ddI; Case 15- ddI, NFV; Case 16- AZT.

Soares et al. HIV-2 versus HIV-1 cell associated burden

Figure legends

Fig. 1 *tat* and *gag* mRNA expression and proviral DNA levels in untreated HIV-2 and HIV-1 infections. Graphs show the expression of *tat* (A) and *gag* (B) relative to GAPDH in those individuals with detectable levels of these transcripts, from a total of 25 HIV-2+ and 23 HIV-1+ individuals evaluated. Open symbols refer to patients with viremia below the cut-off of the test and closed symbols to patients with detectable viremia. Proviral DNA levels were compared in untreated HIV-2+ (25/25) and HIV-1+ (20/23) individuals with detectable and undetectable *tat* (C) and *gag* (D) mRNA transcripts. Bars represent median.

Fig. 2 Relationship between viremia, absolute CD4 T cell numbers and T cell activation in untreated HIV-2 infection. Proportion of CD4 T cells expressing HLA-DR (A) and CD8 T cells co-expressing HLA-DR and CD38 (B) in seronegative individuals (white bars) and HIV-2 untreated patients (dark gray bars). HIV-2 patients were further stratified into viremic (black bars) and “aviremic” groups (light gray bars). Bars represent median±interquartile range. Correlations between CD4 T cell depletion and proportion of cells expressing HLA-DR within the CD4 T cell subset (C) and the proportion of CD8 T cells co-expressing HLA-DR and CD38 (D).

Fig. 3 Impact of ART on HIV-2 plasma and cell-associated viral load, CD4 T cell frequency and T cell activation. (A) Levels of Proviral DNA, *tat* and *gag* mRNA were compared in untreated and ART-treated HIV-2+ individuals. Open symbols represent patients with viremia below the cut-off of the test and closed symbols, patients with detectable viremia; diamond represent ART-treated HIV-2+ individuals and circles their untreated counterparts. Bars represent median. (B) Longitudinal study of a representative ART-treated HIV-2+ patient. Day 0 was defined as the therapy initiation date. The vertical dashed lines
indicate the periods under specific ART regimens (ABC, abacavir; 3TC, Lamivudine; AZT, Zidovudine; LPV, Lopinavir; RTV, Ritonavir; FTC, Emtricitabine; TDF, Tenofovir). Mutations in Protease (PR) and Reverse transcriptase (RT) were analysed before therapy (day -37), and after the 1st ART regimen (day 108). Mutations [a.a._change] found before therapy (day -37) in **PR**: Y14H; N40S; N41D; N68G; K69[K,R]; K70R; and in **RT**: D17[E,D]; W24[G,W]; K28R; C38[W,C]; K43[K,R]; K64R; L74V; K82R; D123G; P126Q; H162Y; V167I; K176S; H228D; W235[G,C,W]; Q245E. Mutations found after first therapy (day 108) in **PR**: Y14H; N40S; N41D; N68G; K69R; K70R; and in **RT**: K28R; K43[K,R]; K64R; W71R; L74I; K82R; T88[T,P]; H96[H,P]; G99[A,G]; D123G; P126[Q,P]; I159[I,L]; H162Y; V167I; K176S; M184V; I187[I,L]; L209[P,L]. (C) Proportion of CD4 T cells expressing HLA-DR and (D) CD8 T cells co-expressing HLA-DR and CD38 in seronegative individuals (white bars) and ART-treated HIV-2+ individuals (dark gray bars). HIV-2+ patients were further stratified into viremic (black bars) and “aviremic” groups (light gray bars). Bars represent median±interquartile range.