Research

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Volume 2 : Issue 2
Article Ref. #: 1000HARTOJ2109

ABSTRACT

Primary and chimeric Human Immunodeficiency Virus type 2 (HIV-2), co-receptor usage, cDNA integration and, pathogenesis are mechanisms still poorly understood. However, these features seem to be related to a flexible envelope oligomeric structure. Sensitive methods are needed for quantifying gene expression and copy number in HIV infected cells. Combining qRT-PCR with ONEp-PCR (one primer-PCR), is a relatively simple customized technique that can be used to investigate fingerprinting, polymorphisms, genomic instability in HIV infected cells. This technique has the potential to reveal associated markers and, is a renewable resource for numerous studies in various fields of modern biology and medicine. In this work, from this combined method, it is shown that primary HIV-2 R5 and ROD/envR5 or ROD/env-R5/-X4 chimeric viruses have differential behaviour related to copy number integration and expression. Additionally, HIV-2 integrated copies are removed from host DNA, indicating genomic instability in live cells. We conclude that HIV-2 env-SU region is responsible to trigger different signal pathways leading to ccr5 and env expression and, env copy number in infected human T-lymphocytes. These results also point out the potential usefulness of combining qRT-PCR and ONEp-PCR to detect changes in HIV proviral DNA within the infected cell’s genome.

KEYWORDS: HIV-2; chimeric viruses; env gene; ccr5 gene; CCR5; CXCR4; T-lymphocytes;
retroelements; ONEp-PCR; qRT-PCR.

ABBREVIATIONS: AIDS: Acquired Immune Deficiency Syndrome; ICTV: International Com-
mitee on the Taxonomy of Viruses; ONEp-PCR: one primer-PCR; RT: Reverse Transcriptase.

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) and Human Immunodeficiency Virus type 2 (HIV-2), are retroviruses belonging to the Lentivirus genus [International Committee on the Taxonomy of Viruses ICTV] and are the causative agents of Acquired Immune Deficiency Syndrome (AIDS), firstly identified in 1981. This syndrome is characterized by a progressive and irreversible degradation of the host’s immune system, which in the last resource leads to a severe immunodeficiency common in patients with AIDS. HIV-2 have unique properties as a human pathogenic agent: is less efficient on developing pathologic manifestations, the infection is generally defined as less virulent and HIV-2 infected individuals have a lower viral burden, accomplishing to a lower transmission rate.
HIV/AIDS RESEARCH AND TREATMENT

ISSN 2377-8377

http://dx.doi.org/10.17140/HARTOJ-2-109

HIV/AIDS RESEARCH AND TREATMENT
Open Journal
http://dx.doi.org/10.17140/HARTOJ-2-109
HIV/AIDS Res Treat Open J
ISSN 2377-8377

HIV-2 Structure and Lifecycle

The Human Immunodeficiency Virus type 2 (HIV-2) was initially isolated in 1986 from a symptomatic patient from Guinea-Bissau. Regardless the fact that HIV-2 is only about 40% similar to HIV-1 in nucleotide sequence, both retroviruses assign identical morphological and genomic organizations.

Briefly, as all exogenous retroelements, HIV-2 genomic organization is composed by a 5’ LTR, one gag gene (codifying for the p16 MA, p26 CA, p6-p7 NC), one pol gene (codifying for the p10 PR, p68-p56 RT, p32 IN) one env gene (codifying for the gp 125 SU and gp 36 TM) and one 3’ LTR (Figure 1).

Figure 1: HIV-2 genomic organization: schematic representation. Different genes are presented as labelled colour boxes: green, yellow and orange boxes represent structural genes; purple boxes indicate regulatory genes and blue boxes indicate accessory genes. Two Long Terminal Repeat (LTR) are signed.

The replication cycle (Figure 2) within infected cells is also comparable and either HIV-1 and HIV-2 infection within a human host conduct to immunological failure and evidently to a similar array of clinical manifestations.

Succinctly (presently until the transcription step), the first step for virus entrance is mediated by binding SU-gp 125 surface glycoprotein with the plasma target-cell surface receptor CD4 that leads to a conformational adjustment for the exposure of TM-gp 36 transmembrane glycoprotein. After peptide insertion, total viral-envelope gp36 and host-cell co-receptor membrane fusion, the viral capsid is released into the cytoplasm. RT starts the viral DNA transcription followed by virus DNA integration in genomic host DNA.

HIV-2 as a Model to Study Reduced Lentiviral Infection

Choosing HIV-2 a research model relies on the unique properties as a human pathogenic agent: is less efficient than HIV-1 on developing infection’s pathologic manifestations and the infection is generally defined as less virulent. This difference is particularly important during the infection’s asymptomatic phase and persists up to the advanced stages, meaning a lower transmission rate than HIV-1, whether sexual or vertical.

Two HIV-2 strains (HIV-2MIC97 and HIV-2MJC97), obtained from asymptomatic individuals were described as being unable to use CCR5 and CXCR4 co-receptors to enter target cells. The set of results indicates that a non-usage of these two major co-receptors apparently is associated with an in vitro lower replication rate. For the current work, has been used primary HIV-2 strains HIV-2AlI wild type R5 strains that uses CCR5 co-receptor and chimerical viruses ROD/MIC97-SA and ROD/AlI-SA both containing the homologous substitution corresponding sequence to the SU region of env gene within the mutated X4 pSKROD, previously constructed.

In a previous report, we found that the C1-C4 region of ENV glycoprotein of each strain is sufficient to change the co-
receptor usage and reduce viral replication kinetics of HIV-2<sub>rod</sub> (R5/X4 strain). These findings indicate that this region, despite the presence and contribution of HIV-2<sub>rod</sub> genetic backbone [i.e., Transmembrane glycoprotein (TM), Tat, Rev, LTR], has an important role in both viral progeny production efficiency and co-receptor engagement.

The present study intend to investigate the expression dynamics of viral env gene and cellular ccr5 gene in IL-2 stimulated human CD4+ T-lymphocytes at several time points after infection. To infer if there was any relationship between env gene expression and new proviral insertions in genomic DNA (env gene copy number) through early infection time, qRT-PCR has been used on the same human HIV-infected cells as for expression assay.

To determine the fingerprinting of new HIV-2 proviral insertions through time it was developed ONE<sub>p</sub>-PCR (one primer-PCR), a customized PCR-based DNA fingerprinting technique.<sup>11</sup> This technique is based on three essential assumptions: the use of only one PCR primer per reaction; the existence of mobile elements sufficiently close to allow efficient amplification and that those mobile elements should be in opposite orientation – inverted and upside-down (Figure 3).

Hereby, ONE<sub>p</sub>-PCR is proposed as a potential new model for early diagnosis for HIV or any other retroviral infection evolution in infected individuals, additionally to its use as a fingerprinting method.

**MATERIAL AND METHODS**

**Cells**

Freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy donors with normal genotype in locus wt<sub>ccr5</sub> were isolated by Ficoll-Paque<sup>®</sup> PLUS (GE Healthcare) density gradient centrifugation and stimulated with phytohemagglutinin (PHA, Sigma-Aldrich<sup>®</sup>) and human recombinant interleukin-2 (20 U/ml IL-2, Roche<sup>®</sup>) for three days. Cells were maintained in supplemented RPMI-1640 medium (Invitrogen<sup>™</sup>) with Fetal Bovine Serum and gentamicin (both from Invitrogen<sup>™</sup>), as previously described.<sup>10</sup> CD8-depleted PBMCs were obtained from the original pool by CD8 removal using Dynabeads<sup>®</sup> Magnetic Beads (Invitrogen<sup>™</sup>), an immunomagnetic separation method (polystyrene magnet beads coated with monoclonal antibodies directed to antigen CD8 according to manufacturer’s instructions). All PBMCs were from the same donors to avoid the inter-individual variation, regarding different sensitivity to infection.

**Viruses**

The primary HIV-2 strain HIV-2<sub>ALP</sub>, a wild type R5 strain that uses CCR5 co-receptor, used for this experimental work, was isolated from one infected patient PBMCs and cocultivated with PBMCs from HIV non-infected donors. Two chimerical viruses ROD/MIC97-SA and ROD/ALI-SA [SphI-AvrII-(SA)], both containing the homologous substitution corresponding sequence to the SU region of env gene in the mutated X4 pSKROD were constructed as described by Santos-Costa Q, et al.<sup>10</sup> and Azevedo-Pereira, et al.,<sup>9</sup> respectively.

**Viral infection**

For each conduct experiment (in tissue-culture treated polystyrene BD Falcon<sup>™</sup> 25 cm<sup>2</sup> Cell Culture Flasks), 2X10<sup>5</sup> cells were inoculated with identical virus (primary strain and chimeric) quantities (100 TCID<sub>50</sub>), as described<sup>10</sup> in the presence of 10 µg/ml of Polybrene<sup>®</sup> (Sigma-Aldrich<sup>®</sup>) at 37 °C. Two hours after, the inoculums was removed, cells were carefully washed three times with PBS and maintained in culture with appropriate medium for 72 hours. For time 0h, the inoculums were removed immediately and washed, as explained. Viral supernatants were stored at -80 °C.

**Cell Viability**

For this study cell viability was confirmed by the Trypan Blue staining exclusion method:<sup>12</sup> viable cells were counted in a Neubauer<sup>®</sup> chamber and mixed with a 0.4% Trypan Blue solution (Sigma-Aldrich<sup>®</sup>), thus generating a 1:10 dilution.

**Monitoring Viral Replication**

In every assay, viral replication was monitored by Reverse Transcriptase (RT) activity in supernatant of infected cultures, using a immunoenzymatic technique, according to manufacturer’s instructions (Lenti-RT kit<sup>®</sup>, Cavidi) at 0, 2, 4, 24 and 72 hours after infection. Uninfected PHA and IL-2 stimulated cells, used as negative controls, were monitored as the same.

**DNA Extraction**

The cells were collected for DNA extraction at time T0, T2, T4, T24 and T72 (0, 2, 4, 24 and 72 hours after infec-
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ISSN 2377-8377

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Uninfected PHA and IL-2 stimulated cells were collected as described above. Total genomic DNA was extracted from the infected and uninfected cells using Citogene® Cell and Tissue DNA purification Kit (Citomed, Lisbon, Portugal) according to the manufacturer’s instructions. Genomic DNA quantification was performed with nanodrop (Biotek Synergy HT instrument) and Gen5 (version 1.09.8) software.

RNA Isolation and cDNA Preparation for Real Time PCR

Total RNA was extracted (from the same cells used for DNA extraction) using the RNAqueous® Kit (Ambion®) according to the manufacturer instructions. RNA quantification was performed with nanodrop (Biotek Synergy HT instrument), and Gen5 (version 1.09.8) software.

RNA samples (2 µg each) were reverse transcribed using oligo-dt and SuperScript® II Reverse Transcriptase (Ambion®) according to the manufacturer recommendations. Total RNA was extracted and possible DNA contamination was cleaned with Turbo DNA-free kit (Ambion®). Then, RNA was reverse transcribed to get the corresponding cDNA, following the RETROscrip Kit (Ambion®) manufacture instructions. To assure that the same genetic material quantities were applied in each reaction, the cDNA were quantified with nanodrop (Biotek Synergy HT instrument) and Gen5 (version 1.09.8) software.

PCR amplification was performed with specific primers (Forward-5’ CCTGCCGTGTCATGCTGTG 3’ and Reverse-5’ AGCCATGTCACACCTCT 3’) matching the ccr5 gene HIV-2ALI env regions (Forward-5’ AAATGTTGCGACTGACCG 3’ and Reverse-5’ GGACTTTGTTGTCACCTCT 3’). As expected, amplification products were not obtained in RNA samples not subjected to reverse transcription step prior to PCR.

Standard Curves and env Copy-Number Inference

The env copy number inserted into genomic DNA was studied by real time PCR. The GAPDHIII copy number was determined through gene amplification using the following primers: Forward-5’ GAGTCACAGGATTTGGTCGT 3’ and Reverse-5’ GCAGAGATGTGACCCCTTT 3’. This approach offers both high sensitivity and high specificity13 and is estimated to be at least 100-fold more sensitive than DNA arrays in detecting transcript expression.14 DNA quantification of the samples was performed using a microplate reader (Synergy HT, Biorad). Copy numbers of the unknown samples previously described, were determined by three independent replicates. PCRs for both the standards and the unknown samples were prepared from the same master mixture using 2X master mix iQSoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA). The 20 µL reaction mixture in each well, contained 10.0 µL of 2X master mix, 2.0 µL of HPLC-purified primers (10 µM), 7.0 µL of PCR-grade H2O and 1.0 µL target DNA solution. The PCR protocol consisted of an initial denaturation step at 95 ºC for 3 min., 40 cycles of amplification, each of which required of 15s of denaturation at 95 ºC, 30s of annealing at 57 ºC and 30s of elongation at 72 ºC. Fluorescence was measured once after each elongation step. At the end of each run a melting curve analysis was performed to confirm the specificity of amplification and the lack of primer dimers. Standard curves were produced by plotting C(t) (fluorescence threshold values)15 against the logarithm of the concentration in copy number using Microsoft Excel (Microsoft, Redmond, Wash). The standard curve is a linear line described by y=mx+b, in which m (the slope of the line) is -1/log (PCR efficiency) and b (the intercept) is the log of the amount of amplification product at the threshold divided by the log of the PCR efficiency (Figure 4).

![Figure 4: Standard curve generated with GAPDHIII housekeeping from human DNA (T-lymphocytes). The standard curve was generated using a ten-fold dilution of the DNA template. Each dilution was assayed in triplicates. Fluorescent threshold values (CT) were plotted against the log of the copy number to produce linear functions. The equation for the regression line and the R2 value are shown above the graphic.](image-url)
The starting copy number of the GAPDHII gene was obtained based on mass, concentration and size parameters of the human genome using the software available from http://molbiol.edu.ru/eng/scripts/h01_07.html. The human genome size [total bases in assembly: 3,195,751,584] was referred in Human Genome Assembly Information (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/data/index.shtml). Copy numbers of the unknown samples were determined from the standard curve based on the obtained C_q value for each sample using basic computational tools available in Microsoft® Excel software. The coefficient of determination (R^2) for the standard curve of GAPDHII produced an R^2 of 0.9946, with a PCR efficiency of 1.917.

Transcriptional Activity of ccr5 and env Genes

Both ccr5 and env genes activity were analysed on the same cells, as used in the previous experiments. The qRT-PCR protocol consisted on the same reaction mixture, as described, with initial denaturation step at 95 ºC for 3 min., 40 cycles of amplification, each of which consisted of 15s of denaturation at 95 ºC, 30s of annealing at 64.5 ºC, for ccr5 gene and at 55.8 ºC, for env gene. Chain elongation was made at 72 ºC, during 30s. As expected for the control, amplification products were not obtained in RNA samples that weren’t subjected to the reverse transcription step prior to PCR.

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 5 and 17. All amplification plots were analysed with an R^2 threshold of 0.20 to obtain C_(tss) (threshold cycle) and the data obtained were exported into a MS Excel workbook (Microsoft Inc.). In order to compare data from different PCR runs or cDNA samples, C_q values were normalized to the C_q value of GAPDHII, a housekeeping gene expressed at a relatively high and constant level. Gene expression was calculated using the 2^(-ΔΔC_q) method. Results obtained are the fold increase (or decrease) of the target gene in the experiment sample relative to the calibrator sample and is normalized to the expression of a reference gene. Normalizing the expression of the target gene to that of the reference gene compensated for any difference in sample preparation.

ONEp-PCR HIV-2 Amplicons

Internal PCR (ONEp-PCR) amplification was designed for env gene. The primer used in the experiment was env Forward-5' AAATGTTGCGACTGACCG 3'.

All PCR reactions were performed in a final 25 μL volume with 0.1 μg DNA (extracted from infected and non-infected cells, as described above), 0.5 μM of each primer and 200 μM dNTPs were mix with 10X reaction buffer and 2.5 U Taq DNA Polymerase PCR (Invitrogen™) supplied with 37.5 μM of MgCl2. The correspondent PCR conditions have an initial denaturation step at 94 ºC for 3 min., 29 cycles of amplification, each of which consisted of 45s of denaturation at 94 ºC, 45s of annealing at 55 ºC and 1.5 min. elongation at 72 ºC, and the final extension at 72 ºC, for 10 min.

For higher yields of PCR products it was used an extended range Ambion® Taq Polymerase (SuperTaq™ DNAPolymerase). All negative image of ethidium bromide-stained 1% agarose gel shown were captured and processed by Gel Doc software-gel imaging, Bio-Rad®.

For identifying and analyse the PCR products, in all agarose gels were included a DNA marker (M) of known molecular weight (1 Kb Plus DNA Ladder-Invitrogen™).

Statistical Analysis

GraphPad Prism software, version 4.0b for Macintosh (GraphPad software Inc.) was used to perform statistical analysis with 5% significance level (P<0.05). To compare different viruses between each time point, an unpaired one-Way ANOVA test was performed and within the same virus infection, it was compared the different time sets by paired t test.

RESULTS AND DISCUSSION

Sensitive methods are needed for quantifying gene expression and copy number in HIV infected cells and for this reason, quantitative real-time (qRT-PCR) revealed to be a perfect method to do so. Revealing ccr5 Gene Expression in HIV-2 Infected T Lymphocytes

To assess how the “spikes touch” of different HIV-2 viruses manipulate the ccr5 gene expression and how that fact influences HIV-2 infection, ccr5 gene expression was quantified in human Interleukin-2 (IL-2) stimulated infected peripheral blood mononuclear cells (PBMCs). For that propose, 2^(-ΔΔC_q) method has been chosen and IL-2 stimulated uninfected PBMCs were used as calibrator. Data show that ccr5 gene expression is differently affected in a strain dependent manner through time (Figure 5).

At time T2 cells infected with ROD/MIC97-SA (to simplify, it is called, virus A) has higher expression levels of ccr5 gene when compared with cells infected with ROD/ALI-SA (to simplify, it is called, virus B). At T4, a similar pattern is observed although an increasing statistical significant difference for virus A. Meanwhile, the expression on cells infected with virus B has increased (p<0.001, one-way ANOVA test). At T24 virus A and B induced a decrease on expression levels of ccr5 gene, except for HIV-2_ALI (to simplify, it is called, virus Ref.) that has a small non-statistical significant increase. Finally, at T72 all viruses induced an increase ccr5 gene expression level.
Interestingly, virus A stood out from all time-points.

**Determination of env Gene Expression in HIV-2 Infected T Lymphocytes**

Due to the importance of env gene expression products in HIV-2 replication cycle, we investigated the interaction burden of different HIV-2 strains in env gene HIV-2 transcription. The env gene expression was quantified in human infected PBMCs (IL-2 and PHA stimulated) and ΔCT method has been selected. The graphics illustrate different levels of env gene expression throughout time on different HIV-2 infections (Figure 6).

At time T2 cells infected with virus Ref. showed higher expressions, while virus A has the lower expression (p<0.005, one-way ANOVA test). The expression pattern is maintained at time T4 with a small increase (p>0.01, one-way ANOVA test) in all viruses. Time T24 reveals a more uniform expression pattern, thus a decrease on virus Ref. and B and, an increase on virus A. Seventy two hours after inoculation, all viruses repeat the T2 expression pattern with an increase of virus Ref. and B, and a decrease of virus A.

**HIV-2 Infected T Lymphocytes env Copy Number Quantification**

Frequently HIV-2 strains are capable of using multiple co-receptors with the same efficiency. This broader range of chemokine receptors usage seems to be related with a more relaxed oligomeric structure of viral envelope glycoproteins and may contribute to a lower HIV-2 infectivity efficiency. To investigate, in vitro, a possible link between infection efficiency and the number of virus integrated into human HIV-2 infected cells, new env gene insertions in genomic DNA (copy number) were quantified in human infected PBMCs stimulated with human recombinant IL-2 (Figure 7). Additionally, the same cells were used to analyse if the copy number stability is maintained during time after HIV-2 proviral DNA integration into the human genome. The results obtained from ΔCq experiments revealed different levels of env gene copy number through time on the same HIV-2 infections. Two hours after inoculation, the first insertions have occurred, especially with virus A that shows the higher insertion number (P<0.01, paired t test). At T4, cells infected with virus Ref. and B out more env proviral insertions, but cells infected with virus A started to induce an insertion env copy discharge, while viruses Ref. and B induced this event only at 24h after infection. Finally, at T72 cells infected with virus Ref. shows the highest insertion number (p<0.001, one-way ANOVA test). At the same time point, viruses B and A induced an opposite result. When transcription (Figure 6) and env copy number (Figure 7) are compared, a clear positive correlation between them is detected. The observed reduction of integrated HIV-2 genomic copy numbers, or the mechanisms throughout HIV-2 copies are removed from human genome are difficult to be explained (Figure 7).

Here it may be hypothesized that this is a dual process between the host-cell and the HIV-2 pathogen. The same performance has been reported (M. Rocheta, et al. personal communication) with LTR retroelements in plants. Meanwhile, virus A has a particular behaviour: these data reveals that soon after infection, a lower integration tolerance could be observed leading to more copies being removed from host genome. It seems that cell reacts to that chimeric viruses, in an active way, thus halting proviral integration. Virus B and Ref. have matching outlines: at times T2, T4, T24 and T72h, suggesting that there is a strong influence between SU amino acids and integration patterns. Virus Ref. (Figure 7) again reveals notorious integration efficiency, when it is observed env transcription (Figure 6), both at time 4,
compared with T72, where the vast env insertion number in genomic DNA does not reflect a higher env expression level.

These results imply that there is a vital association between three important stages in HIV-2 replication cycle: the initial interaction step, the proviral integration and the expression of additional mRNA, leading the virus proteins ready to be assembled near the host plasma membrane. Alternatively, some HIV-2 copies may be temporarily silenced by methylation, by unknown signalling factors or controlled through a miRNA encoded by 5’ LTR process that certainly need to be deeper investigated.

DNA Fingerprinting Corroborate qRT-PCR Results

To validate HIV-2 proviral copies qRT-PCR results, ONEp-PCR technique for env gene has been used (Figure 8A and 8B). As expected, no amplification was obtained with the env gene in uninfected cells used as controls (Figure 8B). However, when analysing DNA infected human cells with the env forward primer and compared time T2 with T72 after inoculation, it was found similar integration patterns between the three
strains. However, it was detected an additional fragment above 8kb (Figure 8B) in DNA from cells infected with Ref. virus. As far as we can prove with this analysis, these results suggest that HIV-2 have site specific integration (new insertions have occurred), illustrated by the same fragments size amplified with the three strains (Figure 8B) and during early infection time.

The in vivo study integration patterns have provided no data to support the concept that retroelement integration is random. Rather, the diversity of insertion patterns of retroelements suggests numerous ways in which genomic DNA is identified for preferential targeting. These span from specific to general matters and, include sequence content, removal of chromatin proteins, particular nuclear localization, distinctive topology and association with specific trans-acting factors. The amplified PCR products will be further analysed to establish the nature of the insertion and simultaneously, data will be obtained regarding the suggested specific insertions sites. Additionally, it will be possible to determine their insertion position relative to human genes.

Furthermore, to understand that active elements are capable of mobility in cultured cells or in somatic tissues, forces to open a new perceptive dimension of individual development and, reinforces the idea of an single and non-static genome per individual. Individuals are likely to be genetic mosaics that can be influenced by environmental conditions to draw out de novo insertions from mobile elements. It remains to be understood and proved how such genetic mosaicism contributes to human/individual differences, from cognition to disease predisposition. To our knowledge, this is the first time report describing that HIV in vitro studies, proviral DNA copy number data can be associated to both cellular (CCR5 co-receptor) and viral (env) gene expression, viral phenotype, ccr5 and env transcription at early time.

CONCLUSIONS

The results obtained in this study revealed that HIV-2 env-SU are responsible to trigger different signal pathways leading to ccr5 and env expression, and env copy number, in infected human T-lymphocytes genomic DNA. Similar integration pattern observed corroborates the fact that HIV-2 infection across time reflects preferential targeting regardless the HIV-2 strain used and its phenotype. This also discloses the existence of an unknown mechanism by which HIV-2 proviruses are removed from human genome. This unknown mechanism appears to assume high importance to cell survival hence prevents or stops genome obesity that could induce premature apoptosis events or
cell death. Finally, the innovative use of qRT-PCR and ONEnPCR, here described, could constitute a valuable approach to ascertain the proviral DNA copy number and the HIV integration mechanisms. These in vitro investigations, undoubtedly allow the understanding of fundamental processes associated to disease progression.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

We thank “Foundation for Science and Technology”, for the post-doc grant SFRH/BPD/64905/2009 to M. R. and CPM-URIA-FFUL for the BSL3 laboratory use. We wish also to thank Rui Monteiro Claro for revising this manuscript.

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