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## HIV controllers with different viral load cut-off levels have distinct virologic and immunologic profiles

Fernanda H Côrtes, PhD<sup>#1</sup>, Caroline PB Passaes, PhD<sup>#1,§</sup>, Gonzalo Bello, PhD<sup>1</sup>, Sylvia LM Teixeira, PhD<sup>1</sup>, Carla Vorsatz, MD<sup>2</sup>, Dunja Babic, PhD<sup>3</sup>, Mark Sharkey, PhD<sup>3</sup>, Beatriz Grinsztejn, MD, PhD<sup>2</sup>, Valdilea Veloso, MD, PhD<sup>2</sup>, Mario Stevenson, PhD<sup>3</sup>, and Mariza G Morgado, PhD<sup>1</sup>

<sup>1</sup>Laboratório de AIDS e Imunologia Molecular, Instituto Oswaldo Cruz - FIOCRUZ, Rio de Janeiro, Brazil.

<sup>2</sup>Instituto Nacional de Infectologia Evandro Chagas - INI, FIOCRUZ, Rio de Janeiro, Brazil.

<sup>3</sup>Department of Medicine, Miller School of Medicine, University of Miami, Miami, United States.

# These authors contributed equally to this work.

### Abstract

**Background**—The mechanisms behind natural control of HIV replication are still unclear, and several studies pointed that elite controllers are a heterogeneous group.

**Methods**—We performed analyses of virologic, genetic and immunologic parameters of HIV-1 controllers groups: 1) Elite Controllers (EC; VL <80 copies/mL); 2) Ebbing Elite Controllers (EEC; transient viremia/blips); and Viremic Controllers (VC; detectable viremia <5,000 copies/mL). Untreated non-controllers (NC), patients under suppressive HAART and HIV-1 negative individuals were analyzed as controls.

**Results**—Total and integrated HIV-1 DNA for EC were significantly lower than for NC and HAART groups. 2-LTR circles were detected in EEC (3/5) and VC (6/7) but not in EC. While EC and EEC maintain normal T cell counts over time, some VC displayed negative CD4+ T cells slopes. VC and EEC showed a higher percentage of activated CD8+ T cells and microbial translocation than HIV-1 negative controls. EC displayed a weaker Gag/Nef IFN- $\gamma$  T cell response and a significantly lower proportion of anti-HIV IgG antibodies than EEC, VC and NC groups.

**Conclusion**—Transient/persistent low level viremia in HIV controllers may have an impact on immunologic and virologic profiles. Classify HIV controllers patients taking into account their

#Corresponding author: Mariza Gonçalves Morgado and Fernanda Heloise Côrtes Address: Laboratório de AIDS e Imunologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ. Av. Brasil 4365, Leonidas Deane Building, room 413. Rio de Janeiro, Brazil. 21040-900 mmorgado@ioc.fiocruz.br / fheloise@ioc.fiocruz.br Telephone number: +55 21 38658154 / Fax number: +55 21 38658173 .

§Current affiliations: Caroline Pereira Bittencourt Passaes

a Unité de Régulation des Infections Rétrovirales, Institut Pasteur, 25–28 rue du Dr Roux, 75724 Paris Cedex 15, France.

b CEA, Division of Immuno-Virology, iMETI/DSV, 18 Route du Panorama, 92265 Fontenay-aux-Roses, France.

caroline.pereira-bittencourt@pasteur.fr/caroline.passaes@cea.fr

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virologic profile may decrease the heterogeneity of HIV controllers cohorts, which may help to clarify the mechanisms associated to the elite control of HIV.

### Keywords

HIV-1; elite controllers; viral load blips; ongoing replication; immune activation and microbial translocation; immune response

## INTRODUCTION

A small percentage (<1%) of HIV-1 infected individuals spontaneously control viral replication to levels below the limit of detection of standard clinical assays (50-400 RNA copies/mL). They are usually called HIV controllers (HIC) or elite controllers (EC) <sup>1,2</sup>. An initial study described the EC as a homogenous group of HIV-infected patients <sup>3</sup>, but subsequent analyses revealed a more heterogeneous scenario <sup>4-6</sup>. While some EC maintain stable CD4<sup>+</sup> T cell counts within the normal range and no evidence of chronic immune activation over time when compared to HIV negative subjects, <sup>7</sup> others display higher CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation levels and a significant loss of CD4<sup>+</sup> T cell <sup>8-10</sup>. Markers of microbial translocations are higher in some EC than in HIV-negative individuals and correlate with T cell activation levels <sup>8</sup>.

The role of the HIV-specific cellular immune response as a mechanism of natural control of HIV-1 replication in EC is also controversial. The overall level and functionality of HIV-specific CD8<sup>+</sup> T cells in EC is highly variable among individuals <sup>3,4,6,11-15</sup>. Indeed, based on the capacity of CD8<sup>+</sup> T cells to suppress HIV infection *ex vivo*, the EC were classified as strong and weak responders <sup>6</sup>. In addition, while various EC cohorts are enriched by some HLA class I alleles, including B\*27 and B\*57 that have been associated with HIV control <sup>3,16-18</sup>, other EC individuals do not encode these alleles. Moreover, several HIV infected patients harboring B\*27 and B\*57 HLA alleles are not able to control viral replication at such low levels <sup>4,19</sup>.

These data suggest that the EC phenotype is probably a multifactorial phenomenon that results from the combination of several host and/or viral factors. Despite this, few studies are able to perform comprehensive analyses associating virologic, genetic and immunologic data to characterize EC. Furthermore, the criteria used to classify EC patients in different cohorts varies based on the viral load (VL) cut-offs (<50 to <400 copies/mL) and time of HIV control (1 to >10 years), which also influence the underlying characteristics of the selected patients. Here, we performed a comprehensive analysis of virologic, genetic and immunologic profiles of a cohort of HIV-1-infected patients who naturally control viral replication at distinct levels.

## PATIENTS AND METHODS

### Patients

A cohort of 19 HIV controllers has been followed-up at the Instituto Nacional de Infectologia Evandro Chagas (INI), Rio de Janeiro, Brazil. For the present study, these HIV controllers were classified in three categories: (1) Elite controllers (EC), including patients

presenting 100% of VL measures <80 copies/mL (n=7); (2) Ebbing elite controllers (EEC), including patients with occasional episodes (<30% of frequency) of transient viremia between 81-400 copies/mL (n=5); and (3) Viremic controllers (VC), patients with consistently detectable plasma viral load in the low range (<5,000 copies/mL) (n=7). Untreated non-controller (NC) patients (n=30) with high viral load levels (>10,000 copies/mL), patients under suppressive HAART (n=13) and HIV-1 negative controls (n=10) were also included in the analysis for comparisons. The cut-off value of 80 copies/mL used to classify the EC patients was determined based on the limit of detection of the Nucleic Acid Sequence Based Amplification System (NASBA) which was adopted by the Brazilian Ministry of Health from 1999 to 2008 to quantify the viral load of HIV infected patients. This period includes most viral load measures used to classify these patients. The present work was approved by the IPEC Institutional Review Board (Addendum 049/2010) and the Brazilian National Committee for Research Ethics (CONEP 840/2008). All subjects gave written informed consent.

### CD4 T cell counts and RNA viral load quantification

Absolute CD4<sup>+</sup> T cells counts were obtained using the MultiTest TruCount-kit and the MultiSet software on a FACSCalibur flow cytometer (BD Biosciences, USA). Plasma HIV-1 viral loads were measured using the nucleic acid sequence based amplification (NASBA) system (limit of detection: 80 copies/mL - Nuclisens, Organon Teknika) and the Versant HIV-1 3.0 RNA assay (limit of detection: 50 copies/mL - bDNA, Siemens Healthcare Diagnostics, USA).

### HIV subtype classification

Subtype was determined based on the *gag*, *pol* and/or *nef* HIV-1 genomic regions. DNA extraction, PCR amplification, and sequencing were performed as previously described<sup>20,21</sup>. Subtype determination was inferred based on phylogenetic analysis (Neighbor-Joining with Kimura-2 parameters correction, as available in the Mega 5.1 package).

### HLA and CCR5 genotyping

HLA-B typing was performed as previously described<sup>22</sup>. The presence of the 32 variant in CCR5 was assessed by PCR amplification/agarose gel electrophoresis. For the wild-type allele, a 239bp fragment was detected and a 207bp fragment corresponded to the mutant allele. Primers are described in Supplementary table 1.

### HIV-1 total, integrated and 2-LTR DNA quantification

Total and episomal DNA were extracted as previously described<sup>23</sup>. Chromosomal DNA was purified from the SDS-precipitate recovered during the purification of episomal DNA, following the same protocol described for total DNA extraction. HIV total and integrated DNA and CCR5 were measured by a single step real time PCR protocol. PCR conditions were as previously described<sup>23</sup>, primers and probes are detailed in Supplementary Table 1. Standard curves were generated using a plasmid containing two copies of HIV LTR and two copies of *CCR5* gene. The values were normalized based on cell numbers estimated by CCR5 quantification and are expressed as the number of DNA copies/10<sup>6</sup> PBMC.

### T-cell activation analysis

Cryopreserved PBMCs were thawed and immediately stained for anti-CD8-FITC/CD38-PE (BD Simultest, BD Biosciences, USA), anti-CD3-APC, and anti-HLA-DRPerCP (BD Biosciences, USA). Samples were acquired using a BD FACSCalibur flow cytometer, and analyses were performed with BD CellQuest software (BD Biosciences, USA).

### Quantification of soluble CD14 (sCD14) plasma levels

The microbial translocation was estimated based on the level of sCD14 in plasma. Plasma levels of sCD14 were assayed in duplicate using ELISA assay-sCD14 Quantikine (R&D Systems, USA), according to the manufacturer's protocol. The results were expressed as pg/mL.

### Serological testing (BED-CEIA)

The proportion of anti-HIV-1 IgG in comparison to total IgG was measured in plasma samples by a quantitative competitive capture enzyme immunoassay - Calypte HIV-1 BED Incidence EIA (Calypte Biomedical Corporation, USA)<sup>24</sup>.

### IFN- $\gamma$ ELISpot

The IFN- $\gamma$  ELISPOT assay was performed as previously described<sup>25</sup>. Peptides were derived from HIV-1 Gag and Nef consensus subtype B, C and F1, previously described (ref), and for each patient, was used a subtype homologue consensus peptides.

Phytohemagglutinin-5 $\mu$ g/mL (Sigma, USA) was used as a positive control, and cells suspended only in culture medium served as a negative control. The spots were counted using an automated ELISPOT reader (CTL Analyzers LLC, Cellular Technology, USA). The results were expressed as spot-forming cells (SFC)/million PBMC. The response was considered positive if  $\geq 50$  SFC/10<sup>6</sup> PBMC were detected.

### Statistical analysis

Statistical analyses were performed using GraphPad 5.0 (Prism Software, USA) and Epi Info Version 6<sup>26</sup>. DNA quantification analysis, CD4 slope, CD8 T cell activation, sCD14 and ELISPOT IFN- $\gamma$  data were analyzed using Mann-Whitney test to compare variables between two subjects groups. Chi-square tests (or Fisher's exact tests, when appropriate) were used for HLA-B allelic frequencies and 2-LTR positive PCR percentages comparisons among groups. Correlations were performed using the Spearman test. All tests were considered significant if the *P* value was  $\leq 0.05$ .

## RESULTS

### Clinical, epidemiological, and genetic characteristics of HIV controllers

The clinical, epidemiological and genetic data of the 19 HIV controllers included in the present study are displayed in Table 1. The median time of follow-up (estimated time of HIV control) was nine years (IQR: 7-12 years). The median age for the HIV controllers cohort was 44 years old (IQR: 42.5-44.0 years old), and females (58%) were more frequent than males. The detection of subtype B, F1, C and BF recombinants among HIV controllers,

is in accordance with the overall HIV-1 molecular epidemiologic scenario in Rio de Janeiro state <sup>27</sup>.

The HLA-B alleles B\*27 and B\*57, classically associated with HIV-1 control, were found among our cohort of HIV controllers. Three individuals - all VC - presented HLAB\*27 (allelic frequency= 7.9%); and four individuals – two EC and two VC – presented HLA-B\*57 (allelic frequency= 10.5%). Three out of 19 patients (one EC, one EEC and one VC) were heterozygous for the CCR5  $\Delta 32$ , but we did not identify any patient homozygous for this mutation. No statistical difference was observed in the comparison of the host genetic markers among the three HIV controllers groups.

### HIV controllers have a limited HIV-1 reservoir

The level of total and integrated HIV-1 DNA was determined for the three groups of HIV controllers as well as for NC and HAART groups (Figure 1A and 1B). Total and integrated HIV-1 DNA were significantly lower for the EC group in comparison to both NC ( $P=0.0003$  and  $P=0.0005$ ) and HAART ( $P=0.0005$  and  $P=0.0004$ ) groups. The levels of total and integrated HIV-1 DNA for the EEC were higher than the observed for EC, but also significantly lower in comparison to NC ( $P=0.0028$  and  $P=0.0052$ ). VC displayed HIV DNA levels in a mean range between EC/EEC and HAART/NC, significantly lower than NC ( $P=0.0078$  and  $P=0.0492$ , respectively). Plasma HIV-1 RNA viral load correlated positively with both total ( $r=0.78$ ;  $P<0.0001$ ) and integrated ( $r=0.69$ ;  $P<0.0001$ ) HIV DNA for the patients analyzed here (Figure 1D and 1E). This correlation was still significant when undetectable viral loads were removed ( $r=0.65$ ;  $P=0.0003$  and  $0.52$ ;  $P<0.0059$ , respectively).

### 2-LTR circles are detected in EEC and VC but not in EC

In order to assess whether residual HIV replication occurs in these HIV controllers, we quantified the 2-LTR circles (Figure 1C). The 2-LTR circles were not detected in EC patients, but were detected in three out of five EEC ( $P=0.0455$ ) and six out of seven VC ( $P=0.0047$ ). The levels of 2-LTR circles were significantly lower for EEC and VC in comparison to NC ( $P=0.0104$  and  $P=0.0192$ , respectively). Of note, nine out of 13 patients under HAART had detectable 2-LTR circles and in higher levels than the HIV controller groups. The levels of 2-LTR HIV DNA correlated positively with HIV-1 RNA viral load (Figure 1F), and with total and integrated HIV DNA (data not shown).

### HIV replication at a low level may induce a loss of CD4<sup>+</sup> T cells

The median of CD4<sup>+</sup> T cells counts in the peripheral compartment of EC was significantly higher than that of EEC, VC, and NC patients ( $P=0.0480$ ,  $0.0175$  and  $0.0005$ , respectively) (Figure 2A). EEC patients also displayed significantly higher CD4<sup>+</sup> T cells counts than NC patients ( $P=0.0032$ ) (Figure 2A). All groups of HIV controllers displayed CD8<sup>+</sup> T cells counts significantly lower than the NC group ( $P<0.01$ ) (Figure 2B). All EC and EEC displayed nearly flat or positive CD4<sup>+</sup> T cells slopes over time; whereas two VC and most NC displayed negative CD4<sup>+</sup> T cells slopes (Figure 2C). The CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio in all EC (except one) and EEC was  $\geq 1$ ; while three VC and all NC displayed a CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio  $<1$  (Figure 2D).

### Transient and persistent low viral replication induce activation and microbial translocation

The level of activated CD8<sup>+</sup> T cells in EC was comparable to the level observed in HIV-uninfected participants (Figure 3A). However, a higher activation was observed in EEC than in HIV-uninfected individuals ( $P = 0.0027$ ), but lower than in NC ( $P = 0.0414$ ) (Figure 3A). Despite of the high dispersion, the median of CD8<sup>+</sup> T cell activation in VC was higher than in HIV-uninfected participants ( $P = 0.0020$ ) and similar to NC (Figure 3A). The median of sCD14 concentration in plasma of HIV-infected individuals was higher than in HIV uninfected, although differences were only significant for EEC and NC groups (Figure 3B).

### HIV specific immune responses were influenced by transient viral load

The proportion of anti-HIV-1 IgG antibodies and anti-HIV-1 IFN- $\gamma$  T cells were analyzed in the different HIV-infected groups. No significant differences were observed in the proportion of anti-HIV-1 IgG among EEC, VC and NC groups, conversely, the median proportion of anti-HIV-1 IgG of EC was significantly lower than that found in the other groups ( $P = 0.0177$ ;  $0.0041$ ;  $0.0185$ ; respectively) (Figure 4A). Three EC displayed a remarkably low proportion of anti-HIV-1 IgG [normalized optical density (ODn)  $< 0.8$  of calibrator OD], comparable to those seen in recently infected individuals. The magnitude of IFN- $\gamma$  T cell responses against both Gag and Nef subtype-specific epitopes in EC also showed a trend towards lower values when compared to the other groups, although significant differences were observed only between EC and VC groups (Figure 4B and 4C).

### Plasma RNA viral load is associated with CD8<sup>+</sup> T cell activation and anti-HIV-1 IgG antibodies

The percentage of activated CD8<sup>+</sup> T cells was positively correlated with both plasma viral load (see Figure S1A, Supplemental Digital Content) and 2-LTR DNA viral load (see Figure S1B, Supplemental Digital Content) and negatively correlated with the CD4<sup>+</sup> T cell slope (see Figure S1C, Supplemental Digital Content). No significant correlations were detected between the level of plasma sCD14 and percentage of activated CD8<sup>+</sup> T cells, plasma viral load and 2-LTR HIV-1 DNA (see Figure S1D, S1E and S1F, Supplemental Digital Content). A positive correlation was also observed between plasma viral load and antibody response (see Figure S2A, Supplemental Digital Content), but not between plasma viral load and Gag- or Nef-specific T cell responses (see Figure S2B and S2C, Supplemental Digital Content). When undetectable viral load values are removed, all correlations remain significant, except for CD8<sup>+</sup> T cell activation (data not shown).

## DISCUSSION

Studies with EC offer a unique opportunity to understand the mechanisms underlying the natural control of HIV infection, giving valuable clues to the development of a therapeutic vaccine and strategies for inducing a durable viral remission in non-controllers patients. However, the EC status has been shown to be a multifactorial phenomenon, associated with virus, host genetics and immunity. Here, we show that the presence of transient viremic episodes and low level persistent HIV replication have relevance in the immune activation and specific immune responses.

Protective HLA class I alleles, mainly HLA-B\*57, are enriched in EC cohorts<sup>4,16,19</sup>. In the present study, we also noted a significant increase in HLA-B\*27 and B\*57 frequencies among HIV controllers. These frequencies were also significantly increased ( $P = 0.01$  and  $P = 0.04$ , respectively) when compared with a Brazilian cohort of 218 individuals with distinct AIDS progression profiles<sup>28</sup>. Concerning the CCR5 polymorphisms, the frequencies of the CCR5  $\Delta 32$  mutation did not vary from that observed among the population of HIV seronegative individuals<sup>4</sup> in the present study, contrary to a previous description of a higher prevalence of CCR5  $\Delta 32$  mutation in EC compared to that observed for the general population<sup>17</sup>. In our study, neither the HLA-B alleles nor the CCR5  $\Delta 32$  frequencies differed significantly among different HIV controllers groups (EC, EEC and VC), although the number of patients within each group was too small to detect potential differences.

HIV reservoirs are established very early after HIV infection<sup>29</sup>. In the present study, we assessed viral reservoir by quantifying total and integrated HIV-1 DNA in PBMCs. We confirmed previous findings that HIV controllers have a smaller reservoir compared to HAART-treated and patients with a typical progression profile<sup>30,31</sup>. Although there were no significant differences in the HIV reservoir size among different HIV controller groups, the mean of total and integrated HIV-1 DNA load in EC was lower than in EEC and VC. This is consistent with a more efficient long-term suppression of HIV replication in EC patients compared with the other groups and further suggests that the occurrence of transient and persistent replication in EEC and VC, even at low levels, contributes to replenishment of the viral reservoir over time. A small HIV reservoir is consistent with a long-term suppression of HIV replication in EC patients.

Despite the limited HIV reservoir, it has been consistently described that most EC maintain a persistent low-level viremia and that HIV continues to replicate and evolve overtime<sup>32-35</sup>. We evaluated whether ongoing viral replication was taking place in our cohort of HIV controllers. Consistently, the levels of 2-LTR circles were lower for all groups of HIV controllers than to NC. 2-LTR circles were not detected in EC patients but were detected in three out of five EEC patients and six out of seven VC. Of note, the VC patient with undetectable 2-LTR circles also had undetectable viremia at this time point. To our knowledge, this study is the first to demonstrate that 2-LTR circles can be detected in EEC, which might be used as a predictive marker of transient viremic episodes. Further studies with a larger number of patients must be conducted to validate this hypothesis.

A previous study by Graf and colleagues<sup>30</sup> described increased levels of 2-LTR circles in PBMC from EC compared to viremic non-treated and HAART treated patients. The authors raised the possibility that a pre-integration restriction mechanism could be taking place in the EC, but this hypothesis was not confirmed by *ex vivo* analyses in that cohort. Our findings of undetectable 2-LTR levels in EC contradict the findings of Graf and colleagues, however our results strongly correlate with our HIV controllers clinical data. Since the EC patients have well documented long-term suppression of viremia, markers of ongoing viral replication are not expected to be detected at high levels *ex vivo*.

Since persistent viremia can be detected by using ultrasensitive methods in the majority of HIV controllers, the continuous exposure to viral products might induce a chronic immune

activation and inflammation in these patients<sup>32</sup>. Previous studies demonstrated that some HIV controllers have a higher level of T cell activation than HIV uninfected subjects<sup>8,36</sup>, while others show similar levels<sup>7</sup>. Here, we investigated the impact of different levels of viremia on T cell activation and, despite the limited number of patients included in this study, we found a significantly higher degree of CD8<sup>+</sup> T cell activation in VC and EEC than in HIV uninfected individuals. This result indicates that even transient episodes of detectable viremia might induce chronic immune activation in HIV-infected individuals. Moreover, EEC also presented a higher level of sCD14 than EC. Chronic immune activation has been pointed as a major driving force of CD4<sup>+</sup> T cell depletion in HIV-infected patients<sup>37</sup>. In our study, CD8<sup>+</sup> T cell activation levels were positively correlated with the RNA viral load and the 2-LTR DNA viral load, and negatively correlated with the CD4<sup>+</sup> T cell slope, suggesting that ongoing viral replication accounts, at least in part, for systemic immune activation and possibly CD4<sup>+</sup> T cell depletion in our cohort patients. The EEC presented lower CD4<sup>+</sup> T cell counts than EC, although no significant decrease in CD4<sup>+</sup> T cells was observed in this group. This result is in contrast with Boufassa and colleagues<sup>38</sup> that showed an association between the presence of blips and a decrease in CD4<sup>+</sup> T cell count, although they observed a frequency of blips ( 50%) higher than in our study ( 30%).

Previous studies described the heterogeneity of CD8<sup>+</sup> T cell responses in elite controllers<sup>4,6</sup> and based on this heterogeneity EC patients were classified as “strong responders” and “weak responders”<sup>6</sup>. Sáez-Cirión et al.<sup>6</sup> demonstrated a strong correlation between the frequency of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells upon peptide stimulation, using IFN- $\gamma$  ELISPOT and the HIV-suppressive capacity of unstimulated CD8<sup>+</sup> T cells, among HIV controllers. Here, we did not evaluate the suppressive capacity of *ex vivo* CD8<sup>+</sup> T cells, but based on IFN- $\gamma$  ELISPOT results, we found that most “weak responders” were EC, whereas EEC and VC mostly correspond to “strong responders”. This is in agreement with previous studies that also observed an association between the presence of blips and a stronger CD8<sup>+</sup> T cell response in EC<sup>6</sup> and HAART treated individuals<sup>39</sup>, pointing out the need for a transient or continuous stimulus for maintaining a detectable long-term *ex vivo* HIV-1 specific CD8<sup>+</sup> T cell response. The analyses of others parameters of T cell responses that have been considered important for the control of viral replication, such as polyfunctional cells<sup>12</sup>, synthesis of cytotoxic granule components, such as granzyme and perforin<sup>14,40</sup> will certainly improve the knowledge of the impact of transient or persistent HIV replication on the quality of CD8<sup>+</sup> T cell response. No correlation between stronger T cell response and presence of HLA-B\*27 or B\*57 was observed, corroborating previous findings<sup>41</sup>. Conversely, a recent study evaluated the impact of HLA-B\*57 on the HIV-specific CD8<sup>+</sup> response in EC, confirming that this allele plays an important role in the high quality HIV-specific CD8<sup>+</sup> T cell response displayed by these subjects<sup>42</sup>. The association between HIV replication and immune response was also verified when HIV-specific antibodies were evaluated. The proportion of anti-HIV-1 IgG antibodies in EC was significantly lower than that observed in EEC, VC and NC. Furthermore, three EC subjects presented levels of anti-HIV-1 IgG lower than the cut-off that defines recent infection (<6 months from HIV infection), despite being measured more than 5 years after an HIV diagnosis. These results are fully consistent with those previously reported by our group<sup>7</sup> and others<sup>43,44</sup>, and



support the notion that transient or persistent HIV replication is also necessary to develop and maintain a high proportion of anti-HIV-1 IgG antibodies.

Although transient or persistent viremia in the detectable range (>50 copies/mL plasma) seems to be necessary for maintaining strong HIV-specific cellular and humoral immune responses over time, we detected one EC patient that presented a strong *ex vivo* HIV-1 specific CD8<sup>+</sup> T cell INF- $\gamma$  response and a high proportion of HIV-specific IgG antibodies. Notably, this subject also presented the highest level of CD8<sup>+</sup> T cell counts and lowest CD4/CD8 ratio among EC, while normal levels of CD8<sup>+</sup> T cell activation were observed. Taken together, our data reinforce the heterogeneity of the elite controller population and highlight the importance of viral load “blips” in the virologic and immunologic profile of these patients. In the present study, we adopted the nomenclature “ebbing elite controllers” to define the patients with “blips” due to their ability to suppress viral loads after loss of HIV control. Based on the data presented here, investigation of patients with the same characteristics in larger cohorts of HIV controllers is warranted in order to elucidate the role of transient viremic episodes in the evaluation of virus, host genetics and immunity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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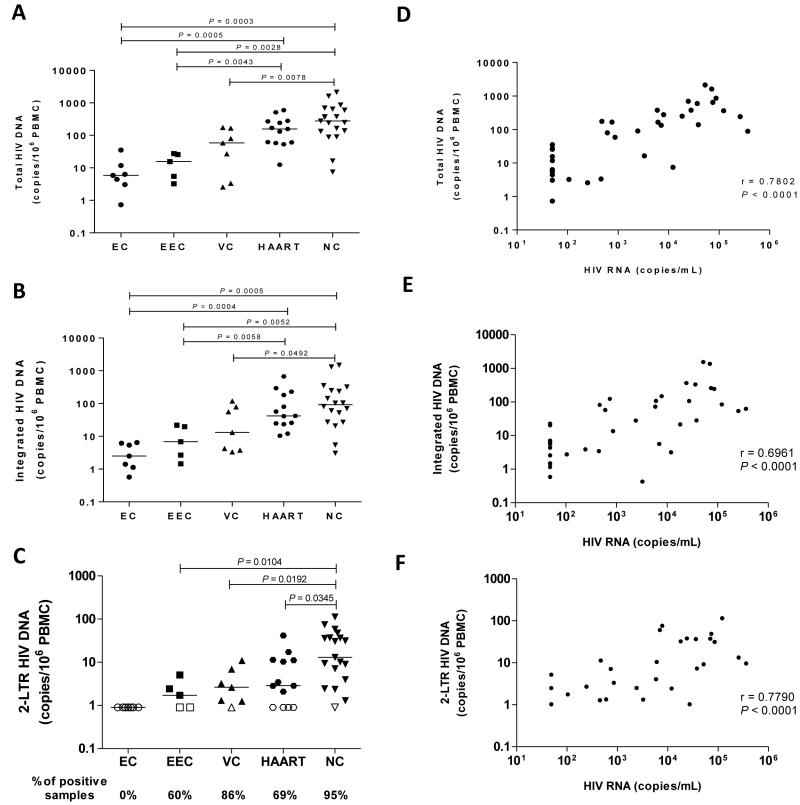
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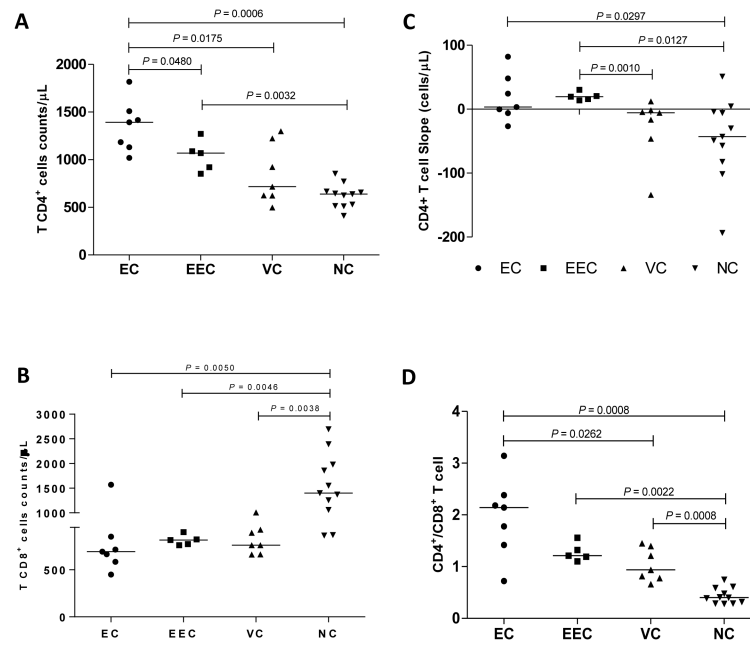
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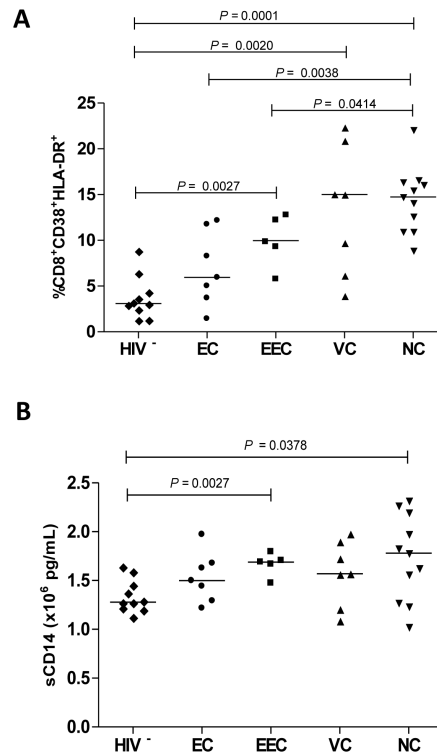


**Figure 1.** Quantification of total (A) and integrated (B) viral DNA and 2-LTR circles (C) for HIV controllers, HAART and NC groups. Relationship between plasma viral load and total HIV DNA (D), integrated HIV DNA (E), and 2-LTR circles (F). Negative 2-LTR PCR results are represented by open symbols. The number of positive 2-LTR PCR /number patients tested were as follows: EC=0/7, EEC=3/5, VC=6/7, HAART=9/13 and NC=18/19 (the percentages are indicated in the graph). The horizontal lines denote median values. P values for comparison between two groups were calculated using a 2-tailed Mann-Whitney test. Correlations were calculated using a nonparametric Spearman test. Data from EC, EEC, VC and NC were used to calculate correlations.

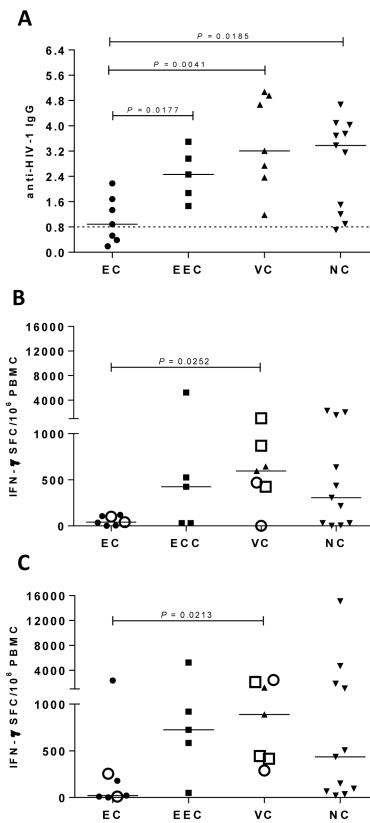


**Figure 2.**

Evaluation of peripheral CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) T cell populations, CD4<sup>+</sup> T cell slope (C) and CD4/CD8 ratio among Elite Controllers (EC), Ebbing Elite Controllers (EEC), Viremic Controllers (VC) and Non-controllers (NC). The horizontal bars denote median values. P values for comparison between two groups were calculated using a 2-tailed Mann-Whitney test.



**Figure 3.** Percentages of activated (CD38<sup>+</sup>HLA-DR<sup>+</sup>) CD8<sup>+</sup> T cells (A) and level of sCD14 (pg/mL) in the plasma (B) among HIV-uninfected participants, Elite Controllers (EC), Ebbing Elite Controllers (EEC), Viremic Controllers (VC) and Non-Controllers (NC). The horizontal bars denote median values. P values for comparison between two groups were calculated using a 2-tailed Mann-Whitney test.



**Figure 4.**

Evaluation of HIV-1 specific responses. Proportion of anti-HIV-1 IgG determined by BED-CEIA found in Elite Controllers (EC), Ebbing Elite Controllers (EEC), Viremic Controllers (VC) and Non-Controllers (NC) (A). Gag- (B) and Nef- (C) specific PBMC responses among Elite Controllers (EC), Ebbing Elite Controllers (EEC), Viremic Controllers (VC) and Non-Controllers (NC) by IFN- $\gamma$  ELISpot (SFC/10<sup>6</sup> PBMC). Open circles represent patients with B\*57 and open squares represent patients with B\*27 alleles. Horizontal dashed line represents the conventional cut-off value (0.8) below which a sample is classified as recent infection (153-day window period) based on the low proportion of HIV-specific IgG in the serum/plasma. The horizontal bars denote median values. P values for comparison between two groups were calculated using a 2-tailed Mann-Whitney test.

Table 1

Characteristics of the cohort of HIV controllers followed-up at IPEC, Rio de Janeiro, Brazil

Patient	Sex	Age	Year of HIV diagnosis	HIV Risk Factor	Years of Known HIV Suppression	CD4 counts Median (IQR)	VL Frequency			HLA-B alleles	CCR-5 genotype	HIV subtype
							<80cps/ml	81-400cps/ml	401-5000cps/ml			
EC02	F	49	1997	HET	10	1229 (1077-1480)	100%			B*48, B*52	WT/WT	B
EC07	F	28	2003	HET	8	2308 (1931-2444)	100%			B*48, B*81	WT/WT	BF
EC08	F	41	1996	HET	15	1207 (993.8-1355)	100%			B*45, B*57	WT/WT	B
EC17	F	62	2001	NI	8	1674 (1494-1954)	100%			B*14, B*15	WT/WT	B
EC19	M	44	2006	HET	5	972 (826.5-1093)	100%			B*44, B*58	WT/WT	B
EC26	M	40	2008	MSM	4	1531 (1409-2124)	100%			B*07, B*57	WT/WT	BF
EC28	M	46	1996	MSM	13	1163 (891.5-1358)	100%			B*51, B*58	WT/ 32	B
EEC09	M	43	2001	MSM	10	888 (710-1054)	79%	21%		B*49, B*52	WT/WT	B
EEC11	F	45	1995	HET	10	1078 (959.5-1206)	90%	10%		B*49, B*81	WT/832	B
EEC13	F	58	1993	HET	19	954 (718.3-1138)	73%	27%		B*15, B*51	WT/WT	B
EEC14	F	42	1999	HET	7	702 (652-795)	75%	25%		B*42, B*44	WT/WT	F
EEC18	F	NI	2001	HET	10	825 (673-940.5)	86%	14%		B*44, B*58	WT/WT	B
VC04	F	47	2007	HET	4	770 (718-895.3)		28%	72%	B*27, B*40	WT/WT	C
VC06	M	34	2000	MSM	7	1132 (913-1225)		80%	20%	B*15, B*48	WT/832	B
VC10	M	48	1991	NI	18	1254 (1078-1460)	22%	52%	26%	B*15, B*52	WT/WT	B
VC12	M	40	2000	MSM	9	1022 (872.5-1131)	23%	15%	62%	B*27, B*44	WT/WT	B



Patient	Sex	Age	Year of HIV diagnosis	HIV Risk Factor	Years of Known HIV Suppression	CD4 counts Median (IQR)	VL Frequency			HLA-B alleles	CCR-5 genotype	HIV subtype
							Mean	Mean	Mean			
VC15	F	NI	2001	HET	11	735 (677-793.8)		100%		B*56, B*57	WT/WT	B
VC16	M	45	1997	MSM	9	552 (530.5-623.3)	29%	47%		B*14, B*57	WT/WT	B
VC27	F	36	1998	HET	10	895 (757-965.5)	35%	18%		B*08, B*27	WT/WT	B
<b>Group summary</b>	<b>Frequency</b>	<b>Median</b>	<b>Median</b>	<b>Frequency</b>	<b>Median</b>	<b>Median</b>	<b>Mean</b>	<b>Mean</b>	<b>Mean</b>	<b>Frequency</b>	<b>Frequency</b>	<b>Frequency</b>
EC	M=43% F=57%	44	2001	HET=57% MSM=29%	8	1229	100%	0%	0%	B*27/B*57=29% Non- B*27, B*57=71%	WT/ 32=14% WT/WT=86%	B=71% BF=29%
EEC	M=20% F=80%	44	1999	HET=80% MSM=20%	10	888	80%	20%	0%	B*27/B*57=0% Non- B*27, B*57=100%	WT/ 32=20% WT/WT=80%	B=80% F=20%
VC	M=57% F=43%	42.5	2000	HET=43% MSM=43%	9	895	27%	40%	49%	B*27/B*57=43% Non- B*27, B*57=57%	WT/ 32=14% WT/WT=86%	B=86% C=14%

NI: Not informed; HET: Heterosexual; MSM: Man who have sex with man; WT: wild type.