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The HIV-1 reservoir landscape in persistent elite controllers and transient elite controllers

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Abstract

Background: Persistent controllers (PC) maintain antiretroviral-free HIV-1 control indefinitely over time while transient controllers (TC) eventually lose virological control. It is essential to characterize the quality of the HIV reservoir of these phenotypes to identify the factors that lead to HIV progression and to open new avenues in HIV cure strategies.

Methods: The characterization of HIV-1 reservoir, from peripheral blood mononuclear cells, was performed using next-generation sequencing techniques, such as full-length individual and matched integration site proviral sequencing (FLIP-seq; MIP-seq).

Results: PC and TC before losing virological control, presented significantly lower total, intact and defective proviruses compared to participants on antiretroviral therapy (ART). No differences were found in total and defective proviruses between PC and TC. However, intact provirus levels were lower in PC compared to TC, being the intact/defective HIV-DNA ratio significantly higher in TC. Clonally expanded intact proviruses were found only in PC and located in centromeric satellite DNA or zinc-finger genes, both associated with heterochromatin features. In contrast, sampled intact proviruses were located in permissive genic euchromatic positions in TC.

Conclusions: These results suggest the need for, and can give guidance to the design of, future research to identify a distinct proviral landscape that may be associated with the persistent control of HIV-1 without ART.
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Introduction

Elite controllers (EC) represent a small subset of people living with HIV-1 (PLHIV), less than 1%, who can maintain undetectable viral load in the absence of antiretroviral treatment (ART) (1–3). EC consists of a heterogeneous and dynamic group regarding virological, immunological and clinical factors (4–7). This has enabled to classify EC in two different phenotypes: persistent elite controllers (PC), who maintain the virological control indefinitely over time; and transient elite controllers (TC), who lose the virological control after being able to control viral replication without ART (5, 8).

Previous studies have focused on distinguishing both phenotypes, PC and TC, to determine the causes of the loss of the virological control and to define the best model of persistent viral remission. It is known that PC have different immunological (5), proteomic (9), metabolomic (10), microRNA (11) and virological profile (5) compared to TC before losing the virological control. TC, in comparison with PC, are characterized by displaying a weaker HIV-specific T-cell response with more limited polyfunctionality (5), a higher expression of markers associated with inflammation (9), a dysregulation of metabolic parameters (10, 11) and a higher viral diversity in env and gag genes (5) even one year before losing virological control.

Together, these results suggest that PC can serve as a premier model for understanding immune pathways responsible for HIV spontaneous cure and for designing new HIV cure and treatment strategies (12). Nevertheless, the mechanisms that allow PC to maintain viral control in the absence of ART, have not been fully characterized. Studies focused on this phenotype, also known as
exceptional elite controllers (13), concluded that PC generally present a low HIV reservoir and absence of viral diversity and evolution in HIV-1 env and gag sequences (5, 13).

Next-generation sequencing techniques (NGS) have changed the paradigm in the characterization of HIV reservoirs. NGS amplifies and sequences single, near full-length (NFL), HIV-1 proviruses, distinguishing between intact and defective provirus and identifying the integration site of the provirus in the genome (14, 15).

A distinct proviral reservoir landscape has been associated with the natural control of the HIV-1 infection (16–19) in which intact proviruses from a subset of EC were preferentially integrated into centromeric satellite DNA or in Krüppel-associated box domain containing zinc finger genes, related to heterochromatin features (20) that do not support effective viral transcription (16). These findings suggest that the quality rather than the quantity of HIV reservoir plays a crucial role in the search of a functional cure of HIV-1 infection (16). Nevertheless, the mechanisms that lead to this distinct beneficial intact proviral reservoir in EC remain unclear, being associated to immune-mediated selection factors that preferentially eliminate intact proviruses in accessible chromatin (18, 21).

Despite all these advances, the HIV reservoir quality in PC and TC is unknown. It is important to elucidate the multiple mechanisms involved in the persistent natural virological control (22) since it would facilitate the future design of immunotherapies to bias the reservoir to a deep latency state compatible with permanent virological remission off ART. In this work, we comprehensively characterize the HIV-reservoir and immunological footprints in TC before losing
the virological control compared to PC that have been controlling the virus for a median of 25 years in absence of ART.
Results

Characteristics and clinical parameters of the study participants

Twenty-seven EC, with undetectable viral load in the absence of ART for at least 1 year of follow-up, were included in the study (5). Ten EC were classified as TC after experiencing a loss of virological control during more than one year of follow-up, and 17 as PC after maintaining persistent virological control during the follow-up period (5). Characteristics and clinical parameters of PC and TC are detailed in Table 1. The PC group was older and presented a longer time since HIV diagnosis than TC (Table 1). There were no differences in the remaining variables. Fifty-three percent of the PC presented protective HLA-alleles, HLA-B27/B57, versus 20 percent in TC. Viral loads (HIV-RNA copies/ml), CD4+ T-cells (cells/mm³) and CD4/CD8 ratio follow-up from the 10 TC, before and after losing the virological control, are shown in Figure 1. The studied time points of the TC preceded the loss of the virological control from 0.3 to 2 years. PC have maintained undetectable viral load with no ART for a median of 25.5 [IQR, 22.3-31.3] years with a median of 791 [IQR, 647-1013] CD4+ T-cell/mm³.

Distinct HIV-1 reservoir landscape in PC compared to TC before losing the virological control

The analysis of proviral sequences was performed using full length individual proviral sequencing (FLIP-seq). The number of cells assayed, clades, and the total, intact and defective proviral sequences of PC and TC are detailed in Supplementary Table 1 and 2, respectively.
The individual HIV-1 proviral genome analysis was not different neither in the total (intact plus defective) ($P=0.167$) (Figure 2A) nor in the defective provirus levels ($P=0.141$) (Figure 2B) between PC (n= 17) and TC (n= 10). Interestingly, the near full-length (NFL) intact provirus levels were significantly increased in TC in comparison with PC ($P=0.006$) (Figure 2C) and importantly, no genome-intact HIV-1 was detected in the 70.59% of the PC (indicated as grey dots in Figure 2C). Consequently, the intact/defective HIV-DNA ratio was significantly higher in TC compared to PC, counting ($P=0.013$) or not the clones ($P=0.005$) (Supplementary Figure 1, A and B, respectively). Significant differences were found in the total (Figure 2A), defective (Figure 2B) and intact provirus levels (Figure 2C) of PC and TC with participants on ART.

PC with higher levels of intact proviruses (n=3) (Figure 2C) were derived from clonally-expanded HIV-1 infected cells that accounted for 100% of all intact proviruses (Figure 3A; left panel). However, clonally-expanded intact proviruses were not observed in TC (Figure 3A; right panel). Furthermore, significant differences were found in the proportion of non-clonal intact and defective sequences from PC and TC (Figure 3B). The proportion of intact proviruses ($P=0.006$) and hypermutations ($P=0.013$) were higher in TC compared to PC. However, the large deletion (LD) genome-defective provirus levels were lower in TC compared to PC ($P=0.004$). No differences were found in the proportion of packaging signal defect (PSI) ($P=0.518$), premature stop codon (PMSC) ($P>0.999$) and internal inversion ($P=0.226$) (Figure 3B) between PC and TC. Nevertheless, the proportion of sequences with internal inversion, counting clonal sequences, was significantly higher in PC compared to TC ($P=0.005$) since
internal inversion accounted for 57.9% of the HIV-1 total proviruses (81.8% were clonal) in the PC10 (Supplementary Figure 2).

**Distinct integration sites of intact genome proviruses in PC compared to TC before losing the virological control**

For a more in-depth analysis, together with FLIP-seq, we assayed the corresponding intact provirus chromosomal integration site by matched integration site and proviral sequencing (MIP-seq) and integration site loop amplification (ISLA). We analyzed the HIV-1 chromosomal integration sites of the intact proviruses of PC and TC (Figure 4). The intact proviruses chromosomal integration sites are detailed in Supplementary Table 3. We focused on participants with intact genome proviruses and available samples to perform integration site analysis: Two PC (PC1 and PC2) that presented higher intact proviruses, derived from clonally identical sequences, and two TC (TC1 and TC2) 0.3 and 0.6 years before losing the virological control, respectively. PC1 and PC2 have maintained ART-free HIV-1 control for 37 and 31 years, and TC1 and TC2 for 9 and 15 years until losing the virological control, respectively. PC1 presented two large clonal genome-intact proviral sequences located in centromeric satellite DNA and in genes that encode members of the Zinc Finger Nucleases protein (ZNF) family, ZNF26 (Figure 4; PC1). PC2 also presented two large clonal genome-intact proviral sequences, both located in ZNF genes, ZNF160 and ZNF607 (Figure 4; PC2) on chromosome 19. In contrast to PC, TC1 and TC2 intact proviruses were located in genic regions before losing the virological control and no clonal intact proviruses were found (Figure 4; TC1 and TC2).
Cell-associated HIV-1 RNA levels in PC compared to TC before losing the virological control

We also found differences in cell-associated HIV-1 RNA levels, being higher in TC (n=5) compared to PC (n=14) (P=0.018) (Figure 5A). Higher levels of cell-associated HIV-1 RNA detected in PC corresponded to PC1 (4,602 copies per $10^6$ TATA-box binding protein (TBP) RNA) (Figure 5A and Figure 4; PC1) and PC2 (347 copies per $10^6$ TBP RNA) (Figure 5A and Figure 4; PC2). Interestingly, cell-associated HIV-1 RNA was not detected in 5 PC that corresponded to those with no intact proviruses detected (grey dots in Figure 5A). Cell-associated HIV-1 RNA levels significantly correlated with total (Figure 5B) and intact proviruses (Figure 5C) but not with defective proviruses (Figure 5D). Excluding PC1, the correlation remained significant between cell-associated HIV-1 RNA and intact proviruses, finding a trend with total proviruses.

Longitudinal analysis of viral reservoir landscape in PC and TC

Genome-intact HIV-1 proviruses were not detected in the 70.59% of the PC (grey dots in Figure 2C). Consequently, we analyzed the reservoir of four of these PC (PC3, PC4, PC5 and PC6) with available sample at different time points to evaluate possible changes in the viral reservoir over time (Figure 6). PC3 has maintained the virological control for 27 years and no genome-intact HIV-1 proviruses were found in three different time points, 2009, 2018 and 2019, in a total of 21.6 million of cells assayed. Differences in the defective provirus levels were found at different time points (Figure 6; PC3). PC4 has maintained the virological control for 33 years and no genome-intact HIV-1 proviruses were found at two different time points, 2017 and 2019, in a total of 9.2 million of cells.
Differences in the defective provirus levels were found at different time points (Figure 6; PC4). PC5 has maintained the virological control for 24 years and no genome-intact HIV-1 proviruses were found at two different time points, 2017 and 2018, in a total of 15 million of cells. There were no differences in the defective provirus levels (Figure 6; PC5). PC6 has maintained the virological control for 27 years and no genome-intact HIV-1 proviruses were found at two different time points, 2010 and 2017, in a total of 9.9 million of cells. No differences were found in the defective provirus levels (Figure 6; PC6). We also analyzed the viral reservoir of one of the TC, TC10, that maintained the virological control for 29 years, in three different time points, 13, 3 and 2 years before losing the virological control, in a total of 8.2 million of cells. Interestingly, intact HIV-1 proviruses were found 2 years before losing the control, not being detected 13 and 3 years preceding aborted virological control (Figure 6; TC10). High defective proviruses levels, mainly with hypermutations, were found even 13 years before losing the virological control.

**Distinct signature of immune selection in intact and defective proviral sequences of PC and TC**

We analyzed the frequencies of amino acid variations associated with sensitivity or resistance to broadly-neutralizing antibodies (bnAbs) recognizing the CD4 binding site, the V2/V3 envelope regions, or the membrane proximal external region (MPER), as previously reported (23), per intact and defective proviral sequence in PC and TC. Overall, TC presented higher number of amino acid changes associated with resistance to bnAbs per intact (P=0.021) (Figure 7A) and defective provirus (P=0.007) (Figure 7B) than PC. These differences were
more remarkable for antibodies targeting the CD4 binding site ($P=0.011$) and for antibodies recognizing the V2 envelope region ($P<0.0001$) in intact provirus (Supplementary Figure 3A) and MPER region ($P=0.080$) in defective provirus (Supplementary Figure 3B). Interestingly, intact-genome sequences with higher number of bnAbs resistance sites in TC (Figure 7A) belonged to the participant that was closer to lose the virological control, 0.3 years (Figure 4; TC1). On the contrary, PC presented higher frequencies of amino acid variations associated with sensitivity to bnAbs per intact ($P=0.065$) (Figure 7C) and defective provirus ($P=0.020$) (Figure 7D). These differences were more pronounced for antibodies recognizing the V2 envelope region ($P=0.023$) and MPER region ($P=0.046$) (Supplementary Figure 4A) in intact provirus and the CD4 binding sites ($P=0.047$) and V3 envelope region ($P=0.021$) in defective provirus (Supplementary Figure 4B). Unexpectedly, the frequencies of amino acid variations associated with sensitivity to bnAbs recognizing the V3 envelope region, in intact provirus, were significantly higher in TC ($P=0.020$) (Supplementary Figure 4A) whereas the frequencies of amino acid variations associated with resistance to bnAbs recognizing the same region were lower ($P=0.022$) compared to PC (Supplementary Figure 3A).

We next analyzed signs of cytotoxic T lymphocyte (CTL)-driven immune pressure in the proviral sequences of PC and TC. No differences were found in the proportion of wild-type CTL or escape variant per intact (Supplementary Figure 5, A and B) and defective provirus (Supplementary Figure 5, C and D) of PC and TC.

**Distinct Gag-specific T-cell responses in PC and TC**
Immunological differences between PC and TC have previously been associated with the loss of the virological control (5). We compared and associated immune parameters of 14 PC and 5 TC with the quality of the HIV-1 reservoir. No differences in the magnitude of the response, assayed by cytokine production (IL2, TNFA and IFNG) after stimulation with Gag peptides in PC and TC, were observed neither in CD4+ nor in CD8+T-cells (for gating strategy, see Supplementary Figure 6). However, a lower frequency of polyfunctionality, defined as simultaneous production of IFNG, TNFA, IL2, CD107a and perforin (PRF) per T-cell in response to Gag stimulation, was observed in central memory (CM) CD4+ T-cell in TC compared to PC (P=0.049) (Figure 8A). Significant positive correlations were found between total proviruses and the frequency of HIV-specific total memory (P=0.037; r=0.900) and CM CD4+ T-cell response (P=0.037; r=0.900) (Figure 8B) in TC but not in PC (P=0.311; r=0.292 and P=0.383; r=0.253, respectively) (Figure 8C).

Distinct HIV-specific CD8+ T-cell proliferation in PC and TC

Given the importance of the CD8+ T-cell role in the spontaneous virological control (24), we analyzed CD8+ T-cell proliferation in PC and TC after the stimulation with an HIV (Gag)-specific peptide pool. Comparing the experimental condition (Figure 9, A-D; right panel) with the negative control (Figure 9, A-D; middle panel) we found that TC2 (Figure 4; TC2) and TC4 (Figure 9, A and B, respectively) presented a higher CD8+ T-cell proliferation before losing the virological control than PC1 (Figure 4; PC1) and PC7 (Figure 9, C and D, respectively).
After that, to prove the lack of proliferation in PC, we analyzed the CD8+ T-cell proliferation in two longitudinal samples T0 (Figure 9C) and T1 (Figure 9E), 1 year after T0, of PC1 (Figure 4; PC1). The CD8+ T-cell proliferation was not changed over time, since no differences were found between experimental (Figure 9, C and E; right panel) and negative control (Figure 9, C and E; middle panel) in any of the time points. The positive control stimulated with *Staphylococcal enterotoxin B* (SEB) validated the efficacy of the assay (Figure 9, A-E; left panel).

**Thymic function in PC compared to TC before losing the virological control**

Thymic function has been associated with HIV disease progression (25). We assayed the sj/β-TREC ratio in 11 PC and 10 TC by ddPCR. We observed that sj/β-TREC ratio was significantly increased in TC in comparison with PC ($P=0.024$) (Figure 10A). Nevertheless, these differences were co-lineal with age, as we observed an inverse correlation between age and sj/β-TREC ratio ($r=-0.410; P=0.065$) (Figure 10B) and TC were younger than PC (Table 1). However, a significant positive correlation was found between sj/β-TREC ratio and the levels of intact proviruses in TC ($r=0.709; P=0.022$) (Figure 10C) but not in PC (data not shown).
**Discussion**

Despite the recent advances in measuring and characterizing the HIV reservoir (26, 27), the characteristics and distinctive features of PC and TC HIV-1 reservoir remain poorly defined. Understanding the mechanisms responsible for the maintenance and loss of the virological control in EC is important to design functional or sterilizing cure strategies.

The present study, that comprehensively analyzed the quality of the TC HIV-1 reservoir before losing the virological control, demonstrates a distinct and dynamic viral reservoir landscape in TC compared to PC. Our analysis showed, as indicative factors of the viral rebound, higher intact proviruses and cell-associated HIV-1 RNA levels, and a higher viral diversity with no detectable intact provirus clones in TC, compared to PC. Importantly, intact proviruses may be more likely to be located in permissive genic euchromatic positions in TC, although our results are limited by limited sampling. These findings are crucial since they could be consistent with TC having subclinical HIV-1 replication before losing virologic control; analysis of additional measures of active virus replication are suggested to test this hypothesis further. Despite no differences were observed in defective provirus levels between PC and TC, a higher proportion of hypermutations was found in TC and may facilitate the HIV-1 escape from the immune system by increasing the genetic diversity and the evolution of viral variants as previously reported (28). Opposite to TC, genome-intact proviral sequences were not detected in 70.59% of the PC after analyzing millions of cells. It is remarkable that no cell-associated HIV-1 RNA was detected in five of these PC. The absence of genome-intact proviral sequences in a large number of
analyzed cells has previously been associated to a spontaneous cure of HIV-1 infection in the Berlin (16, 29) and Esperanza patient by the same techniques used in this study (30). Although we cannot confirm with our data that these participants, with virological control without ART for a median of 25 years, have achieved a spontaneous cure of HIV-1 infection, further studies can now be guided by our findings to study a larger number of cells from PBMCs and different anatomical compartments (31), such as lymphoid tissue, known for containing the majority of viral reservoir (32, 33). These results suggest the idea that the Esperanza patient is not anecdotic and a higher number of EC with this reservoir profile might be spontaneously cured. This hypothesis is supported by the longitudinal analysis of the HIV-1 reservoir in four of the PC. Genome-intact HIV-1 proviruses were not detected in these participants during the follow-up, some of them with samples 10 years apart. The detectable proviral DNA was completely defective in all the studied time points and thus, unable to produce infectious virions. These data strongly suggest the absence of enough genome-intact proviruses to cause loss of control and support additional research to strengthen evidence for this hypothesis.

The other profile found in PC consisted of participants who presented higher genome-intact proviral levels, derived completely from clonally-expanded HIV-1 infected T-cells, and preferentially located in centromeric satellite DNA or ZNF genes, both associated with heterochromatin regions, generally disfavored for proviral integration and linked to deep viral latency (20, 34–36). Moreover, clonal-intact proviral sequences presented the same integration site confirming their role in the reservoir persistence in PC (37). Interestingly, both large clones in PC2 were integrated in ZNF genes (37) located in defined regions that are occupied...
by heterochromatin proteins of chromosome 19 (20, 34), as previously reported in a subset of EC (16). Unlike PC, we did not detect intact clonally-expanded HIV-1 infected cells in TC (5). These data suggest that intact proviral reservoir of PC, in contrast to TC, seems mostly fueled by clonal proliferation of latently infected cells harboring early-seeded intact proviruses. This fact resembled our previous findings in the overall EC population (16) probably because the more comprehensive analysis was performed in those EC with intact proviral clones, consistent with the PC phenotype described in the present study.

In addition, cell-associated HIV-1 RNA levels were significantly higher in TC and positively correlated with total and intact proviruses. Notably, higher cell-associated HIV-1 RNA levels in PC corresponded to the participants that presented clonal intact proviruses sequences located in centromeric satellite DNA or ZNF genes, PC1 and PC2, reassuring the importance of the quality, rather than the quantity, of viral reservoir. This fact may indicate a production of viral proteins by defective proviruses in PC (38), mostly driven by the higher proportion of large deletion proviruses observed in this phenotype (39), that could act as a therapeutic vaccine and magnifying the antiviral host immune activity in PC.

Regarding the immune pressures that derive to the viral rebound, we found lower number of bnAbs sensitivity sites and, conversely, higher bnAbs resistance sites per intact and defective provirus in TC compared to PC. These findings may be consistent with more selection among TC with virus resistance to humoral immune responses, but our data are not yet definitive as to whether this could result from the observed higher viral diversity in TC or if selection for resistance
was also occurring. Curiously, we found the opposite phenomenon for bnAbs recognizing the V3 envelope region in intact genome provirus, lower sensitivity and higher resistance in PC, compared to TC, suggesting that V3 envelope region may not be so important to viral control as CD4 binding site, V2 and MPER region. Interestingly, the participant that presented higher bnAbs resistance sites per defective provirus in PC, PC1, was the same one that presented clonal intact proviruses sequences located in centromeric satellite DNA and ZNF genes and higher cell-associated HIV-1 RNA levels. This finding confirms the persistence of defective provirus and its role in the production of viral proteins (38), and consequently in the antiviral immune response in PC, as mentioned previously. However, the absence of differences in wild-type and CTL escape mutations may be biased by the fact that only clade B consensus sequences were analyzed since clade A1 and F1 HIV-infected participants, including PC1 and TC1, were not included in the analysis due to a lack of information related to escape mutations for these clades.

All these immunological proviral footprint data are in accordance with the lower T-cell polyfunctionality found in TC compared to PC as previously reported (5). Interestingly the reduced quality of the Gag-specific T-cell response in TC was intimately associated with viral reservoir measurements. In effect, HIV-specific CD4+ T cells are known to be infected by the virus at higher frequencies than other memory CD4+ T cells (40). Moreover, active reservoirs have previously been reported to be enriched in CM T-cells (41). These facts may explain the positive correlation found between HIV-specific CM response in CD4+ T-cell with total proviruses in TC, but not in PC, again pointing out the ongoing HIV infection of these HIV-specific CD4 memory T-cells in TC.
These data were also associated with thymic function levels which has previously been associated with HIV disease progression (25), in fact, the higher levels of thymic function in TC, in comparison with PC, positively correlated with intact-genome proviruses. Interestingly, the highest sj/β-TREC ratio belonged to the participant closer to lose the virological control, TC1. These data may indicate a compensatory mechanism of the adaptive immune system to maintain T-cell number and suppress ongoing viral replication, not being able to control it due to the decreased T-cell polyfunctionality.

The main limitation of our study was the sample availability and consequently the need of more immunological data, especially in TC, and above all, the number of sequences available per participant, particularly in those PC with no intact proviral sequences detected. We partially counteract this limitation with the longitudinal analysis performed in participants with this profile. Additionally, it is remarkable that these participants are exceptional and analyzing a larger number of cells does not guarantee finding more sequences as it has been shown in participants with a similar unique reservoir profile as the Esperanza patient and HIV pediatric and overall EC cohorts (18, 30, 42).

Together, we observed in PC absence of detectable intact provirus sequences and a deep viral latency, that seems to follow a “block and lock” mechanism (43), by silencing of intact proviral gene expression through chromosomal integration into repressive chromatin locations. By contrast, higher intact-genome proviral levels, transcriptionally active and with higher resistance to immune recognition were observed in TC before losing the virological control. Despite the higher
thymic function and CD8 T-cell proliferation found in TC, their lower Gag-specific
T-cell polyfunctionality may contribute to the loss of the virological control.

In summary, our results showed a markedly distinct intact proviral reservoir and
immunological landscapes associated with the loss and maintenance of
persistent spontaneous HIV-control. These findings are important, albeit not
definitive, since it goes one step further to identify PC phenotype as the premier
model of functional cure, determine the causes of the loss of spontaneous viral
control and open the door to identify new PC and other PLHIV with this distinct
reservoir signature as spontaneously cured. Our results emphasize that
confirmation of these hypotheses to predict a higher likelihood of persistent
spontaneous control will require a larger sample of longitudinally sampled
controllers in ongoing cohorts started earlier, given the current practice of
routinely starting ART around the time of diagnosis.
Methods

Sex as a biological variable

Cisgender women and men were included in the study.

Study design and participants

Participants were defined as EC when viral load determinations were under the detection limit in the absence of ART for at least one year of follow-up (5). Human PBMCs were collected from 27 EC and 41 participants on ART. Ten EC were classified as TC and 17 as PC. Participants were classified as TC after experiencing a loss of virological control, sustained viral load above the detection limit during more than one year of follow-up (at least two consecutive detectable viral load), as previously reported (5). Using this classification, we have previously observed differences in immunological (5), proteomic (9), metabolomic (10), microRNA (11) and virological profile (5) in PC compared to TC before losing the virological control. Participants were classified as PC after maintaining persistent virological control during the follow-up period.

TC were selected based on sample availability <2 years before losing the virological control, according to our previous findings (5). Frozen PBMCs of these participants were obtained from Spanish HIV Hospital Gregorio Marañón (HGM) BioBank belonging to the AIDS Research Network (44) and collecting participants' clinical data from Red de Investigación en Sida (RIS) Controllers Study Group Cohort (ECRIS) (45). PC samples were collected from Virgen del Rocio and Virgen Macarena University Hospitals, Seville (Spain); Lausanne University Hospital, Lausanne (Switzerland); Virgen de las Nieves University
Hospital, Granada (Spain); Reina Sofía University Hospital, Córdoba (Spain); Costa del Sol and Virgen de la Victoria Hospital, Málaga (Spain); Torrecardenas University Hospital, Almeria (Spain) and Joan XXIII University Hospital, Tarragona (Spain). Samples of participants on ART were collected from Massachusetts General Hospital (MGH). Viral load and frozen PBMCs from PC were obtained from healthcare providers.

**PBMCs isolation**

PBMCs were isolated using BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (BD Biosciences), with sodium heparin as anticoagulant, by density gradient centrifugation at the same day of blood collection. CPTs were centrifuged at 1,811g for 20 min at room temperature (RT). Afterwards, PBMCs were cryopreserved in freezing medium (90% of Fetal Bovine Serum (FBS; Thermo Fisher Scientific) + 10% dimethyl sulfoxide (DMSO; PanReac AppliChem) in liquid nitrogen until further use.

**DNA and RNA extraction**

Genomic DNA and RNA were extracted from PBMCs using a blood DNA minikit (Omega Bio-Tek) and NucleoSpin RNA purification kit (Macherey-Nagel), respectively. DNA and RNA were quantified using the Qubit assay (Thermo Fischer Scientific) according to the manufacturer’s instructions.

**HIV-1 DNA quantitation**

HIV-1 DNA was quantified from previously extracted DNA by droplet digital PCR (ddPCR), using the BIO-RAD QX200 Droplet Reader, as previously reported (46). The PCR program was run according to the manufacturer’s protocol using an
annealing temperature of 58°C. Primers and probes targeting gag regions and
the viral 5′ long terminal repeat (LTR) were: 6F (5′-CATGTTTTTCAGATTATCAGAAGGA-3′), 84R (5′-TGCTTGATGTCCCCCCTTCCCTTCCCTTTTGCTGC-3′); LT forward (5′-TGCTTGCCCCTCTTGTTGTTGCT-3′), LT reverse (5′-GCCGAGCTGCGTCGAGAG-3′) and the LT probe (5′-VIC-CAGTGGCGCCCGAACAGGGA-BHQ1-3′). Ribonuclease P protein subunit p30 (RPP30) was used as a housekeeping gene to normalize HIV-1 DNA copies. The following primers and probes were used to quantify RPP30: RPP30 forward (5′-GATTTGGACCTGCGAGCG-3′), RPP30 reverse (5′-GCGGCTGTCTCCACAGTG-3′), and probe (5′-VIC-CTGACCTGAAGGCTCT-BHQ1-3′). Data were analysed using Bio-Rad QuantaSoft software version 1.7.4.

Cell associated HIV-1 RNA quantitation

Cell associated HIV-1 RNA was quantified from previously extracted RNA by ddPCR with the One-Step RT-ddPCR kit (Bio-Rad) using the BIO-RAD QX200 Droplet Reader, as previously reported (47). The PCR program was run according to the manufacturer’s protocol using an annealing temperature of 58°C and the same primers and probes as previously described (see section HIV-1 DNA quantitation). TBP was used as a housekeeping gene to normalize HIV-1 RNA copies. The following primers and probes were used to quantify TBP: TBP forward (5′-CACGAACCACGCCTACTGATT-3′), TBP reverse (5′-TTTTCTTGGCTGCACTCTGGAC-3′) and probe (5′-HEX-TGTGCACAGGAGCCAAGAGTGAAGA/3-IABkFQ-3′). Data were analysed using the Bio-Rad QuantaSoft software version 1.7.4.
Genomic DNA, previously extracted from PBMCs, was diluted to single proviral genomes based on ddPCR results (see section HIV-1 DNA quantification) and Poisson distribution statistics (one provirus was present in approximately 20-30% of wells). Afterwards, DNA was subjected to HIV-1 near-full-genome amplification using a single-amplicon nested PCR approach, as previously reported (48). The following primers were used for the first and second-round nested-PCR, respectively: U5-623F (5′-AAATCTCTAGCAGTGCGCCGAAC AG-3′) and U5-601R (5′-TGAGGGATCTCTAGTTACCAGTGTC-3′); U5-638F (5′-GCGCCGAAACAGGGAGATCGAAARCGAAAG-3′) and U5-547R (5′-GCACAGGCAAGCTTGAAARCAGAAAG-3′). PCR products were visualized by 0.7 % agarose gel electrophoresis (Quantify One and ChemiDoc MP Image Lab; BioRad) and all near full-length HIV-1 (≈8000 bp) were subjected to Illumina MiSeq sequencing at the MGH DNA Core facility.

Short reads were *de novo* assembled using Ultracycler version 1.0 and aligned to HXB2 to identify large deleterious deletions (<8000 bp of the amplicon), out-of-frame indels, premature/lethal stop codons, internal inversions, or packaging signal deletions (≥15 bp insertions and/or deletions relative to HXB2) through an automated pipeline written in Python programming language (49). The presence/absence of APOBEC-3G/3F-associated hypermutations was determined using Los Alamos National Laboratory (LANL) HIV-1 Sequence Database Hypermut 2.0 program (50). Viral sequences without any of the mutations previously mentioned were classified as intact genome sequences. An alternative analysis was used to classify a sequence as intact, as previously reported (51).
Phylogenetic distances between sequences were determined through maximum-likelihood trees in MEGA and visualized with Highlighter plots. Clonality was determined by identical sequences, with any or less than or equal to three mismatches between proviral sequences, and integration sites. Nucleotide variations due to primer binding sites were not considered for clonality analysis.

**Integration site analysis**

MIP-seq technique was used to profile the chromosomal locations of intact proviruses. Firstly, a whole genome amplification (WGA) was performed by a multiple displacement amplification (MDA) with Φ29 polymerase (REPLI-g Single Cell Kit) (Qiagen) (52), according to the manufacturer's protocol. Subsequently, DNA from each well was split and separately subjected to viral sequencing and integration site analysis (48).

Integration sites of each intact provirus, obtained from the WGA, were identified by ISLA technique, as previously described (53). A second WGA was performed when it was necessary to increase the amount of DNA. PCR products were subjected to next-generation sequencing using Illumina MiSeq.

MiSeq paired-end FASTQ files were demultiplexed; small reads (142 bp) were then aligned simultaneously to human reference genome GRCh38 and HIV-1 reference genome HXB2 using bwa-mem (54). Biocomputational identification of integration sites was performed according to previously-described procedures (53, 55). Briefly, chimeric reads containing both human and HIV-1 sequences were evaluated for mapping quality based on: (i) HIV-1 coordinates mapping to the terminal nucleotides of the viral genome, (ii) absolute counts of chimeric reads, (iii) depth of sequencing coverage in the host genome adjacent to the viral
integration site. The final list of integration sites and its corresponding chromosomal annotations was obtained using Ensembl, the UCSC Genome Browser and GENCODE. Repetitive genomic sequences harboring HIV-1 integration sites were identified using RepeatMasker.

**Sequence analysis**

Clades of intact HIV-1 proviral sequences were determined using the LANL HIV Sequence Database Recombinant Identification Program. For each clade B proviral sequence, optimal CTL epitope sequences restricted by autologous HLA class I alleles within nine HIV-1 genes were identified (56), by best-defined HIV-1 CTL/CD8+ T-cell epitopes from the LANL HIV Immunology Database. The CTL/CD8+ Epitope Variants and Escape Mutations were used from the same database to classify epitope sequences from each provirus as wild-type, escaped, or uncharacterized according to the respective HIV-1 subtype and HLA allele. Thanks to an existing compendium of proviral sequence signature mutations that influence susceptibility to bnAbs (23), we evaluated the frequencies of amino acid variations associated with sensitivity or resistance to bnAbs. The sensitivity of proviral species to bnAbs was estimated by calculating the number of amino acid signature sites associated with sensitivity to four different bnAbs classes, that recognize the CD4 binding site, the V2/V3 envelope regions, or the membrane proximal external region (MPER), within the env amino acid sequence from each provirus, as previously described (23)

**CD8+ T-cell proliferation assay**

PBMCs, 1x10^6 cells/ml, were stained at 37 °C for 20 min with 0.5 μM CellTrace carboxyfluorescein succinimidy ester (CFSE; Thermo Fisher) according to the
manufacturer’s protocol. Cells were washed twice with RPMI-1640 medium (RPMI) supplemented with 10% FBS (Sigma), and plated in 96-well round-bottom polystyrene plates, 200 μL per well. Experimental samples were incubated with 20 ng/ml of an overlapped HIV (Gag)-specific peptide pool (NIH AIDS Reagent Program). Positive control well was stimulated with 2 μg/ml of SEB (Sigma) and the negative control contained unstimulated PBMCs. Subsequently, cells were cultured for 5 days in R-10 medium (RPMI supplemented with 10% FBS, 100 U/ml penicillin G, 100 μL/ml streptomycin sulfate (Thermo Fisher Scientific), 1.7 mM sodium L-glutamine (Lonza) and 50 IU/ml IL-2 (R&D Systems)). On day 5, cells were collected and stained for viability using Violet LIVE/DEAD Cell Stain kit (Invitrogen) and anti-CD3-APC-H7 (clone SK7; BD Biosciences) and anti-CD8-PE (clone RPA-T8; Biolegend). Finally, cells were washed and fixed for 20 min at 4 °C with 4% paraformaldehyde solution (PFA; Sigma-Adrich).

Multiparametric flow cytometry analyses were performed on an LRS Fortessa flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed using the FlowJo 10.7.1 software (Treestar, Ashland, OR).

**HIV-specific T-cell response**

PBMCs were thawed and resuspended in R-10 medium containing 10 U/ml DNase I (Roche Diagnostics) for 1 h at 37°C. Afterwards, cells were stimulated at 1x10^6 cells/ml with 1 μg/ml of anti-CD28, 1 μg/ml of anti-CD49d (BD Biosciences), 10 μg/ml of brefeldin A (BFA; Sigma Chemical Company), and 0.7 μg/ml of monensin (BD Biosciences), in the absence or presence of 1 μg/ml of the overlapped HIV (Gag)-specific peptide pool for 6h. Cells were stained with
conjugated monoclonal anti-CD107a-BV650 (clone H4A3; BD Biosciences) at the beginning of the incubation (57).

Stimulated PBMCs were washed with phosphate-buffered saline (PBS) and stained for 35 min at RT with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti-CD27-APC-H7 (clone M-T271; BD Biosciences), anti-CD14-BV510 (clone MφP9; BD Biosciences), anti-CD19-Bv510 (clone SJ25C1; BD Biosciences), anti-CD56-BV510 (NCAM16.2; BD Biosciences), anti-CD8-PerCP-Cy5.5 (clone SK1; BD Biosciences), anti-CD45RA-PeCy7 (clone L48; BD Biosciences) and anti-CD3-BV711 (clone SP34-2; BD Biosciences). Subsequently, cells were washed and permeabilized with BD Cytofix/CytoPerm (BD Biosciences) for 45 min at 4 °C. Afterwards, cells were intracellularly stained with anti-IL2-BV421 (clone MQ1-17H12; BD Biosciences), anti-TNFA-AF700 (clone Mab11; BD Pharmingen), anti-IFNG-APC (B27; BD Biosciences) and anti-PRF-Pe (B-D48; Biolegend) for 30 min at 4 °C. Finally, cells were washed and fixed for 20 min at 4 °C with 4 % PFA. Multiparametric flow cytometry analyses were performed on an LRS Fortessa flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed using the FlowJo 10.7.1 software (Treestar, Ashland, OR).

Thymic function assay

Thymic function was measured by ddPCR, quantifying the presence of T-cell receptor rearrangement excision circles (TRECs), sj-TREC and DβJβ-TRECs, from previously extracted DNA. The PCR program was run according to the manufacturer’s protocol using an annealing temperature of 59°C. The primers and probes used for sj-TREC were: DTR66 (5'-TGACATGGAGGGCTGAAC-3'),
DTF7 (5′-AGGCTCTGTCTAGTGTGATAAC-3′) and SD1 probe (HEX-CACCCCTGTTCCCACA-BHQ1). The primers and probes used for DβJβ-TRECs were: T3A (5′-CTTTCGATGGACCCTCACAG-3′), T3B (5′-GACAAGGCACCAGACTCACAG-3′), T3C (5′-AAGCTCTGGAGGACACACAG-3′), T3D (5′-CCGGTTTCTCCCTCACACAG-3′), T3E (5′-GGGCAGAAGACTGAGAACACAG-3′), T3F (5′-CTTGCGCCTTATGCTGCACAG-3′), T2 (5′-CCCAGGAGAAAGAGGAC-3′) and PB1 probe (6FAM-TGGGAGTTGGGACCGCCAGAGAGG-BHQ1). RPP30 was used as a housekeeping gene to normalize sj-TREC and DβJβ-TRECs copies (see section HIV-1 DNA quantification for RPP30 primers and probe sequences). Data were analysed using Bio-Rad QuantaSoft software version 1.7.4.

Statistics

Nonparametric statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS 22.0; SPSS, Inc.), and graphs were done using GraphPad Prism version 8.4.2 (GraphPad software, Inc). Differences between PC and TC were tested for statistical significance using Mann-Whitney U tests (two-tailed) and false-discovery rate (FDR)-adjusted two-tailed Fisher’s exact tests. Correlations between variables were assessed using the Spearman rank test. All $P$ values <0.05 were considered statistically significant. Polyfunctionality pie charts and Permutation test were done using Pestle version 1.6.2 and Spice version 6.0 (58).

Study approval

This study was in compliance with the local legislations and it was performed according to the ethical guidelines of the Declaration of Helsinki. The study was
approved by the Ethics Committee of Virgen del Rocio University Hospital (Seville, Spain) (Code: 1594-N-17). All patients gave written informed consent.

**Data availability**

Owing to study participant confidentiality concerns, full-length viral sequencing data cannot be publicly released but will be made available to investigators upon reasonable request and after signing a data sharing agreement. Correspondence and requests for data should be addressed to ERM. Values for all data points in graphs are reported in the Supporting Data Values.

**Author contributions**

All authors reviewed critically and approved the submitted version of the manuscript. LELC, ACR, JS, RP, CGA, LMM, ARS, MF, ARJ, CRO, CHT, AR, JO, MLR, FV, CV, MC, AM, NE, MP, JP, AR and LFLC recruited the participants, provided PLWH blood samples and analyzed data. SB, JV, MREIB and FJO analyzed and interpreted the data. ERM, ML, XY and CGC designed the experiments. CGC, XL, CG, IR, MRJL, GG, MICS, IG and APG performed the experiments and analyzed and interpreted the data. CGC and ERM analyzed, interpreted the data and wrote the manuscript. ERM, conceived the idea, designed the project together with LFLC, coordinated the project together with ML and XY and acquired funding for the study.

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References


Table 1. Characteristics and clinical parameters of the study participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>PC (n=17)</th>
<th>TC (n=10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>6 (35.3)</td>
<td>4 (40.0)</td>
<td>0.810</td>
</tr>
<tr>
<td>Time before losing the control (years)</td>
<td>n/a</td>
<td>1.2 [0.5-1.7]</td>
<td>n/a</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55 [47 – 57]</td>
<td>41 [37-51]</td>
<td><strong>0.040</strong></td>
</tr>
<tr>
<td>Time since HIV diagnosis (years)</td>
<td>25.5 [22.3-31.3]</td>
<td>16.8 [13.1-19.5]</td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td>CD4+ T-cell counts (cell/mm$^3$)</td>
<td>791 [647-1013]</td>
<td>805 [644-902]</td>
<td>0.874</td>
</tr>
<tr>
<td>CD4/CD8 ratio*</td>
<td>1 [0.5-1.3]</td>
<td>1 [0.6-1.6]</td>
<td>0.905</td>
</tr>
<tr>
<td>HLA-B57, n (%)</td>
<td>6 (35.3)</td>
<td>2 (20)</td>
<td>0.410</td>
</tr>
<tr>
<td>HLA-B27, n (%)</td>
<td>3 (17.7)</td>
<td>0</td>
<td>0.167</td>
</tr>
<tr>
<td>HLA-B27/B57, n (%)</td>
<td>9 (53.0)</td>
<td>2 (20)</td>
<td>0.099</td>
</tr>
</tbody>
</table>

Categorical variables are expressed as number and percentages (%), and continuous variables are expressed as median (interquartile ranges [IQR]). Chi-Square and Mann-Whitney U test was used to compare categorical and continuous variables, respectively. P-value <0.05 was considered statistically significant. *CD8+ T-cell count not available in TC1 and TC3.
Figure 1. CD4+ T-cell counts, viral load levels and CD4/CD8 ratio in TC. CD4+ T-cell counts and CD4/CD8 ratio were represented in the left axes (CD4+ T-cell count in orange and CD4/CD8 ratio in green) and viral load levels in the right axes (red). Unavailable CD8+ T-cell count in TC1 and TC3. The black dot represents the studied time point that preceded the loss of the virological control.
Figure 2. Analysis of HIV-1 proviral sequences in PC, TC and participants on ART. Total (A), defective (B) and intact proviruses (C) levels in PC, TC and participants on ART. Grey dots represent values below the limit of detection (expressed as 0.05 copy/total number of analyzed cells without target identification). PC and TC are represented by unique identifiers (Supplementary Table 1 and 2). Each dot represents a participant. Mann-Whitney U test was used to compare PC, TC and participants on ART. P value <0.05 was considered statistically significant.
Figure 3. Genome-proviral sequences in PC and TC. Circular maximum-likelihood phylogenetic trees for all genome-intact proviral sequences from PC and TC (A). HXB2, reference HIV-1 sequence. Dots with the same colors represent genome-intact proviral sequences from the same participant. Clonal sequences are indicated by black arches. PC and TC are represented by unique identifiers (Supplementary Table 1 and 2). Proportions of non-clonal genome-proviral sequences (B). Intact and defective proviruses as packaging signal defect (PSI), large deletion (LD), premature stop codon (PMSC) hypermutations and internal inversion, were included. False-discovery rate (FDR)-adjusted two-tailed Fisher’s exact tests were used to compare PC and TC. P value <0.05 was considered statistically significant.
Figure 4. Simultaneous analysis of HIV-1 proviral sequences and integration sites in linear maximum-likelihood phylogenetic trees. Coordinates and relative positioning of integration sites are indicated. Clonal genome proviral sequences, defined by identical proviral sequences and identical corresponding integration sites, are highlighted in black boxes. The rest of the symbols represent different types of defective proviruses.
Figure 5. Cell-associated HIV-1-RNA in PC and TC. Cell-associated HIV-1-RNA, expressed as copies per 10^6 TBP RNA (A). Correlation between cell-associated HIV-1 RNA and total (B), intact (C) and defective genome proviruses (D) in PC and TC. Each dot represents a participant. PC and TC are represented by unique identifiers (Supplementary Table 1 and 2). Correlations were performed also excluding the participant in the red circle (PC1), in this case statistics are indicated in red. Mann-Whitney U test was used to compare PC and TC. P value <0.05 was considered statistically significant. Spearman test was used for non-parametric correlations.
Figure 6. Longitudinal evolution of genome proviral reservoir landscape in PC and TC. Total, intact and defective proviruses levels in PC3, 4, 5, 6 and TC10 over time.
Figure 7. Analysis of broadly-neutralizing antibody (bnAb) resistance and sensitivity signature sites in intact and defective proviral sequences of PC and TC. Number of bnAb resistance sites per intact (A) and defective provirus (B) in PC and TC. Number of bnAb sensitivity sites per intact (C) and defective provirus (D) in PC and TC. Each dot represents an intact or defective proviral sequence. PC and TC are represented by unique identifiers (Supplementary Table 1 and 2). Mann-Whitney U test was used to compare PC and TC. P value <0.05 was considered statistically significant.
Figure 8. HIV-1-specific T-cell response in PC and TC. HIV-1-specific CM CD4+ T-cell polyfunctionality with up to five functional responses to Gag stimulation per T-cell in PC and TC (A). The five functional responses to Gag stimulation represent the simultaneous production of IFNG, TNFA, IL-2, CD107a and PRF per T-cell. IFNG, TNFA, IL-2, CD107a and PRF are shown in arcs in the polyfunctional distribution. Pestle and Spice were used for analysis. Correlations between Gag-specific CM T-cell response with total HIV DNA levels (10^6 peripheral blood mononuclear cells (PBMCs)) in TC (B) and PC (C). Each dot represents a participant. PC and TC are represented by unique identifiers (Supplementary Table 1 and 2). Spearman test was used for non-parametric correlations.
Figure 9. HIV-specific CD8+ T-cell proliferation assay. TC2 (A), TC4 (B), PC1 T0 (C), PC7 (D) and PC1 T1 (1 year after T0) (E). C+: stimulated PBMCs with *Staphylococcal enterotoxin B* (SEB) (left panel). C-: unstimulated PBMCs (middle panel). Experimental: stimulated PBMCs with HIV (Gag)-specific peptide (right panel) after 5 days in culture.
Figure 10. Thymic function in PC and TC. Dot graphs represent sj/β-TREC ratio (A). Correlations between sj/β-TREC ratio and age in PC and TC (B). Correlations between sj/β-TREC ratio and the frequency of intact HIV DNA (10^6 PBMCs) in TC (C). Each dot represents a participant. PC and TC are represented by unique identifiers (Supplementary Table 1 and 2). Mann-Whitney U test was used to compare PC and TC. Spearman test was used for non-parametric correlations. P value <0.05 was considered statistically significant.