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ABSTRACT

HIV-1 elite controllers (EC) maintain undetectable viral load (VL) in the absence of antiretroviral treatment. However, these subjects have heterogeneous clinical outcomes including a proportion loosing HIV-1 control over time. In this work we compared, in a longitudinal design, transient EC, analyzed before and after the loss of virological control, versus persistent EC. The aim was to identify factors leading to the loss of natural virological control of HIV-1-infection with a longitudinal retrospective study design. Gag-specific T-cell response was assessed by *in vitro* intracellular poly-cytokine production quantified by flow cytometry. Viral diversity and sequence-dating were performed in proviral DNA by PCR amplification at limiting dilution in *env* and *gag* genes. The expression profile of 70 serum cytokines and chemokines was assessed by multiplex immunoassays. We identified transient EC as subjects with low Gag-specific T-cell polyfunctionality, high viral diversity and high proinflammatory cytokines levels before the loss of control. Gag-specific T-cell polyfunctionality was inversely associated with viral diversity in transient controllers before the loss of control (r=-0.8; p=0.02). RANTES was a potential biomarker of transient control. This study identified, virological and immunological factors including inflammatory biomarkers associated with two different phenotypes within EC. These results may allow a more accurate definition of EC, which could help in a better clinical management of these individuals and in the development of future curative approaches.

IMPORTANCE

There is a rare group of HIV-infected patients who have the extraordinary capacity to maintain undetectable viral load levels in the absence of antiretroviral treatment, the so called HIV-1 elite controllers (EC). However, there is a proportion within these subjects that eventually loses this capability. In this work we found differences in virological and immune factors including soluble inflammatory biomarkers between subjects with persistent control of viral replication and EC that will loss the virological control. The identification of these factors could be a key point for a right medical care of those EC who are going to lose the natural control of viral replication, and for the design of future immunotherapeutic strategies using as a model the natural persistent control of HIV-infection.

INTRODUCTION

The spontaneous control of human immunodeficiency virus type 1 (HIV-1) infection is observed in a minority group of subjects known as HIV-1 elite controllers (EC) (1). As these individuals maintain undetectable viral load (VL) in the absence of antiretroviral treatment they have been proposed as a model of functional cure (2). The investigation of the mechanisms behind this natural control has attracted enormous interest for the identification of the host and virological factors implicated (3–6).

However, some of these individuals experienced virological and immunological progression and AIDS and non-AIDS defining events (7–9). Regarding virological progression, approximately 28% of EC experience loss of viral control over time (7). The factors associated with this loss remain elusive due to the different design of the studies. First, because previous studies were limited to cross-sectional analyses in which virological progression and the heterogeneous characteristics of HIV-1-controllers were not widely taken into account (6, 10–13). Second, because there have been very few longitudinal studies, mostly epidemiological (14) where the main contributors were higher ultrasensitive HIV-1 RNA VL and proviral DNA levels. Furthermore, non-conclusive results about inflammatory biomarkers were found (15). Therefore, the specific determinants associated with virological failure in EC are not definitely stablished.

The objective of this work was to investigate, in a longitudinal study design, the mechanisms leading to the loss of virological control in a cohort of EC. To this end, we carried out an exhaustive analysis of virological and immunological factors including pro-inflammatory cytokines that could explain the transient or persistent nature of the virological control in HIV-1 infection. The identification of biomarkers associated with

the loss of viral control will allow the identification of this subgroup of EC which should help to improve their medical care. In addition, the idenfication of those factors operating in the persistent control of viral replication in EC could provide new insights for the design of novel eradication and immunotherapeutic strategies.

RESULTS

Characteristics of the studied subjects

Clinical and demographic characteristics of Transient Controllers (TC) and Persistent Controllers (PC) (see study design in Figure 1) are shown in Table 1. The frequency of sexual transmission route was higher in TC (75%) than in PC (37%) (p=0.049). TC group presented a shorter time since diagnosis than PC (8 [2-14] vs 18 [11-22] years; p=0.002). There were no differences in the remaining variables at baseline. After the loss of control, the VL from TC was 627 [230-4618] HIV-RNA copies/mL at T0, 1730 [397-4420] HIV-RNA copies/mL at +T1 and 2860 [727-4920] HIV-RNA copies/mL at +T2. Therefore, half of the patients were viremic controllers because of the loss of control and the remaining patients had low viral loads ($<10^4$Log HIV-RNA copies/mL).

Low levels of Gag-specific T-cell response preceded the loss of virological control in Transient Controllers

The CD4+, CD8+ T-cell counts and CD4:CD8 ratio were not different and did not change along the follow-up in both groups (Table 2). In the same way, no differences were observed throughout the follow-up in Gag-specific CD4+, CD8+ and different T-cell subsets response in PC after multiple comparison testing (Figure 2). Based on these data and to simplify the analyses, the variables in PC were expressed as the mean value of all longitudinal determinations.

Importantly, there was a higher proportion of PC (12/14 subjects [85.7%]) who showed Gag-specific CD4+ T-cell response compared with TC one year before the loss of virological control (-T1) (3/13 subjects [23.1%]), when Gag-specific CD4+ T-cell response dramatically decreased. No statistical differences were observed between PC and TC two years before (-T2) the loss of virological control, nor at the time point just

after the loss of virological control (T0) with PC (Figure 3A, left panel). No differences were found for CD8+ T-cell response, although there was a similar trend toward lower responses at –T1 in TC (Figure 3A, right panel).

Analyzing the magnitude and characteristics of the response, we observed that PC presented higher levels of Gag-specific Total, Central Memory (CM) and Effector Memory (EM) CD4+ T-cells than TC at –T1 (Figure 3B). The same results were obtained for CD8 T-cells (Figure 3C). In addition, increased Gag-specific EM and TD CD8+ T-cell levels at T0 compared to –T1 were found in TC (Figure 3C).

Gag-specific CD8+ T-cell polyfunctionality is decreased in Transient Controllers before the loss of virological control

A higher frequency of polyfunctional CD8+ T-cells was observed in PC compared to TC at –T1, with a higher proportion of cell with three functions and a higher cytokine diversity (representative example, on Figure 4A). There were no differences between PC and TC at T0, when viral load was detectable in TC. However, analyzing the maturation and activation patterns of Gag-specific T-cell response (Figure 4B) we found that TC presented higher Gag-specific CD8+ $CD38^{+}$ T-cell levels and lower Gag-specific CD8+ CM $CD57^{-}$ levels at –T1 and T0 compared with PC. In addition, bulk T-cell activation was higher in TC at T0 compared with PC (Figure 5).

Index of polyfuncionality (pINDEX) in Gag-specific total CD8+ T-cells was compared between PC and TC at –T2 together with –T1 (pre loss of control period). pINDEX in PC was higher in three-, four- and five-functions (Figure 4 C-E) compared to TC.

Transient Controllers displayed higher viral diversity than Persistent Controllers

Env and *gag* genes were amplified in proviral DNA by limiting dilution PCR in all TC (9/9, 100%), but only 5/10 (50%) of the PC. Afterwards in the remaining available samples we quantified proviral load by an Alu real time PCR, 33% (4/12) of TC

157 samples showed values ranging 5 to 108 copies/10^6 cells, the remaining TC samples and
158 9/9 samples from PC (100%) showed values below the detection limit (5 copies/10^6
159 cells).
160 The length of the branches in the phylogenetic tree (Figure 6A) was longer in TC
161 indicating viral replication and evolution. In the sequences from PC, the branch length
162 was minimal or zero with many identical sequences. Although we did not analyzed the
163 integration sites, this pattern indicate a lack of viral replication and could suggest that
164 these viral populations are the consequence of clonal expansions (16–18).
165 Regarding the evolution of *gag* and *env* genes sequences with time, TC showed
166 intermingled sequences and no replacement of viral populations in the different time-
167 points (Figure 6A). This evolutionary model is compatible with an atemporal mode of
168 evolution (11). In contrast, sequences at different times from PC showed no evidence of
169 viral evolution (Figure 6A). These results were supported by viral dating estimation. For
170 this analysis, available samples of PC, obtained in 2010±0.9, a mean of 18 years after
171 HIV-1 diagnosis, showed sequences without any viral evolution since HIV-1 diagnosis
172 (Table 3). In these patients the difference between viral dating and the HIV-1 diagnosis
173 time was 5 years. In TC patients, available samples obtained a mean of 15 years after
174 HIV-1 diagnosis showed, because of viral evolution, a viral dating (2001±3.4) closer to
175 the sampling time (2005±3.5) than to the HIV diagnosis date (1989±4.1) with a mean
176 difference of 11.5 years (Table 3). In two TC (F4 and EC4), the nucleotide sequences
177 were separated in two independent clades (Figure 6A, right panel). This segregation
178 supports that these were double infected (DI) subjects, as previously reported for EC4
179 (19). All patients had an R5-tropism and, in the case of TC, the tropism did not changed
180 after viral replication.

Viral diversity in *gag* region was higher in TC than in PC but it did not reach a statistical significance, probably due to the low number of sequences analyzed. In contrast, in the *env* C2-V5 region, diversity was statistically higher in TC than in PC ($p< 0.0029$) again supporting viral replication in TC (Figure 6B), even if we exclude of the analysis the sequences of the DI patients ($p< 0.016$).

Virus diversity was associated with Gag-specific CD8+ T-cell polyfunctionality

Three-function total CD8+ T-cell polyfunctionality was inversely associated with *gag* diversity (r=-0.8; p=0.02) but not with *env* (Figure 7A) in TC. Regarding 4- and 5-functions total CD8+ T-cell polyfunctionality association with viral diversity remained in the same trend although it was not statistically significant (r=-0.6; p=0.14 and r=-0.57; p=0.1 respectively). No association was found in PC (data not shown). We evaluated the presence of HIV-1 footprints of immune escape in Gag epitopes in pre and post loss of control samples comparing TC and PC. We found a trend towards higher frequency of mutations in Gag epitopes in TC than in PC (Figure 7B). In addition, for the TC 351 patient expressing HLA-B*57, we tracked the temporal emergence of escape variants in the B*57 restricted epitope ISW9, which appeared after the loss of control, and the pre-existence of TW10 escape mutants along with the loss of virological control over time indicating HIV-1 evolution against CD8+ T-cell responses (Figure 7C).

High soluble biomarkers and proinflammatory cytokines levels preceded the loss of virological control

Higher levels of RANTES, Interferon alpha (INFα)2, Human Grow Regulated Oncogene (GRO), Interleukin (IL)-7 and Cutaneous T-Cell Attracting Chemokine (CTACK) in TC than in PC were the variables that significantly differentiated the two groups by Mann-Whitney U test (Figure 8A). After Random Forest analysis (Figure

8B), similarly RANTES, INFα2 and CTACK were considered as potential biomarkers together with Platellet Derived Growth Factor (PDGF) AA and Eotaxin-2. The PCA model (Figure 8C) restricted the biomarkers to RANTES and PDFG AA that showed the best percentage of separation between TC and PC. Finally, analysis by ROC curves (Figure 8D) showed that the top variables of the Random Forest approach (Model B) and the PCA (Model C) displayed an AUC=1, which means that a perfect classification was achieved using only RANTES and PDGF AA. All statistical tests defined RANTES as the most remarkable chemokine for group discrimination with four-fold higher levels in TC than in PC (p=0.001) with a cut off value of 10464.5 pg/mL.

DISCUSSION

In this work, we showed that low Gag-specific T-cell polyfunctionality, high viral evolution and high levels of pro-inflammatory cytokines were associated with future loss of natural virological control. In addition, RANTES levels may be considered as a potential novel biomarker predictive of the loss of virological control in EC.

The lack of HIV-1-specific T-cell responses is considered a hallmark of progressive HIV-1 infection (20). Preserved CD4+ and CD8+ T-cell multifunctional response have been consistently associated with the control of viral replication in EC (4, 21, 22). However this generalized assumption was derived from cross-sectional studies with heterogeneity between study subjects (23, 24). Our study demonstrates that TC show low magnitude of Gag-specific T-cell response together with less polyfunctional T-cells compared to PC. In addition, this specific T-cell response has a particular mature and a higher activated phenotype in TC, in agreement with previous findings of T-cell activation preceeding the viral breakthrough after spontaneous control in acute infection (25). These characteristics precedes by one year the loss of spontaneous control. These results may explain the variability observed in previous studied cohorts (24, 26, 27) in which, virological progression was not taken into account and could not discriminate between TC and PC. In addition, our data are in agreement with a previous work showing decreased CD8+ T-cell breath associated with loss of viral control but in viremic controllers although we did not observe an association between functional Gag-specific CD8+ T-cells and the presence of protective HLA class I alleles (28). One possible mechanism involved in the loss of control, may be related to CD8+ T cells restricted by 'non protective' HLA allele groups can be suppressed by regulatory T cells (29). *Ferrando-Martinez et al*. (26) showed that mature (EM) Gag-specific CD8+ T-cells from EC had higher polyfunctionality in comparison with viremic controllers and

241 non-HIV-1-controllers. In our work we observed increased polyfunctionality in a more
242 mature (TD CD57+) Gag-specific CD8+ T-cells from PC, but not from TC. This result
243 suggests that Gag-specific CD8+ T-cells with a mature phenotype have increased
244 cytotoxic abilities and points to this mechanism as one of the main cause of persistent
245 natural control. The absence of differences of polyfunctional distribution between PC
246 and TC in this subset at T0 may be explained by the particular mature and a higher
247 activated phenotype of Gag-specific T-cell response and higher bulk T-cell activation at
248 this timepoint. Regarding Gag-specific CD4+ T-cell the dramatic decrease of this
249 response one year before the loss of control is in agreement with a previous study
250 showing the important role of SIV-specific CD4+ T-cells in the breakthrough
251 SIVmac239 viremia in an elite controller (30).
252 Among other viral properties, genetic variability and viral evolution have been
253 associated with viral pathogenesis and disease progression (31–35). Proviral DNA from
254 PBMCs is considered to harbor a combination of recently produced and archived
255 variants and consequently variability in this compartment better reflects viral evolution
256 since primoinfection (18, 36). Analysis of *env* and *gag* proviral DNA sequences, in
257 samples previous to the loss of control, showed evidence of viral divergence and
258 diversity in TC demonstrating not fully restricted viral replication despite undetectable
259 VL. On the contrary, PC presented minimal or no proviral divergence and extreme low
260 diversity suggesting no viral replication since primo-infection or that replication is
261 occurring at such a low rate that reseeding of this proviral compartment is not possible
262 as suggested in EC although evolution in plasma virus was detected (37–39). Lower
263 levels of viral diversity were observed in EC than in patients with undetectable levels of
264 viremia by combined antiretroviral treatment (39, 40). Moreover, the viral diversity in
265 EC is not directed by neutralizing antibodies (40), although escape mutants to

neutralizing antibodies are continuously generated and selected in these patients (41). Our results are in agreement with previous works showing a relation among viral replication and evolution, viral pathogenesis and disease progression (33–35, 37, 42, 43). Therefore, markers of HIV-1 viral evolution (divergence and diversity) could differentiate the TC and PC phenotypes in the pool of EC. The homogeneous viral populations found in PC is also observed in viral infections with fidelity mutants in polio (44, 45) or arbovirus (46) which result in less pathogenic infections (33, 34, 35). Immunological and virological factors were intimately associated in these subjects. We found strong inverse associations between *gag* diversity and Gag-specific CD8+ T-cell polyfunctionality (three functions), only in the follow-up time-points with undetectable viremia in TC. The association was found in *gag* and not in *env* probably because we analyze Gag but not Env-specific T-cell response. These observations are in agreement with previous findings that demonstrate that responses targeting *gag* but not *env* are inversely associated with VL (47, 48). The results presented herein revealed that the lack of viral evolution reflects the continuous immune pressure exerted by Gag-specific T-cell responses in PC and the fine balance between viral persistence and immune control in TC. Our results are in agreement with *Noel et al*. that demonstrated the contribution of abortive viral expression in CD4+ T-cells from EC for maintenance of strong HIV-1 specific CD8+ T-cell responses in the absence of HIV-1 evolution (49). Likewise, these results may be in agreement with the work of *Boritz et al*. showing that clonal expansion of infected cells act to maintain the virus in HIV controllers (18). The lack of viral evolution found in this work suggests that clonal expansion may be one of the main mechanism of virus persistence in PC. This mechanism may be enough for PC to maintain an enhanced Gag-specific T-cell response able to contain viral replication. On the other hand, a second mechanism proposed by *Boritz et al*. (18) consisting in

replication of inducible proviruses of recent origin may be the prevalent mechanism in TC before the loss of control, reflecting a less efficient Gag-specific T-cell response and more compatible to what happened in viremic controllers.

The different patterns observed in viral diversity and DNA proviral load could be linked with different magnitude and quality of Gag-specific T-cell responses and with different levels of inflammatory mediators. There are increasing evidences that EC have higher levels of T-cell and myeloid activation and unique inflammatory signatures compared with HIV-suppressed and HIV-1-uninfected subjects (50–52). Interestingly, a lack of an association between inflammatory markers and VL in EC has been described (13) suggesting that persistent viral replication may not be the main trigger behind the inflammation observed in EC. Our data support that, despite no detectable plasma VL, the lower Gag-specific T-cell response in TC and higher viral diversity and DNA proviral load may be related to the higher levels of several inflammatory cytokines in TC.

This is the first extensive analysis of the expression profile of 70 plasma cytokines and chemokines in EC with different viral loads. Random Forest, PCA and ROC curves which included the combination of the selected proinflammatory markers revealed that RANTES and PDGFAA may be sufficient to clinically categorize PC and TC. Of special interest is the chemokine RANTES, the most remarkable biomarker for group discrimination which concentration was four times higher in TC group than PC. Despite RANTES, the natural CCR5 ligand, has been previously shown to prevent HIV infection (53), RANTES can also be considered a proinflammatory cytokine and it has been associated with disease progression in HIV-infected patients (54–56). Higher levels of RANTES may reflect low level residual viral replication in TC that could be associated with CD8+ T-cell dysregulation by exhaustion (57). Our findings not only

define RANTES as a reliable biomarker for a rapid screening of potential EC with virological loss of control, but also suggest that the inmunomodulation of RANTES as a therapeutic target in EC.

This study has disclosed the presence of two distinct groups of EC and we have been able to find differences between this two EC phenotypes based on immunological and virological markers. These results demonstrated that HIV-1-controllers are a heterogeneous group of subjects with different characteristics and nomenclatures (58) as we (59) and others (60) previously suggested. Accordingly, it is crucial to establish a precise definition of the EC phenotype in order to identify the correlates of persistent spontaneous control in the search for the right model of functional remission. These new insights might help in the reconsideration of the current treatment guidelines that recommend antiretroviral treatment for all HIV-1-controllers (https://aidsinfo.nih.gov/guidelines).

The main limitation of this study was the sample availability that constrained the evaluation of additional immunological and virological factors. However, these subjects are extremely rare and, even so, we were able to have a follow-up with samples before and after the loss of control.

In summary, this study has allowed the identification of several immunological, virological and pro-inflamatoy cytokines that will help in the accurate definition of EC and in their clinical management. In addition, the identification of important factors for the persistent natural control of HIV replication, could give new clues to achieve a long-term remission status or functional cure in HIV-1-infected patients.

MATERIAL AND METHODS

Study participants

EC were defined as sujects with three consecutive VL determinations under the detection limit (<50 HIV-1-RNA copies/mL) in the absence of antiretroviral treatment for at least one year of follow-up (7). Subjects were included based on frozen peripheral blood mononuclear cells (PBMCs) and plasma samples availability in the Spanish HIV HGM BioBank belonging to AIDS Research Network (61) and with data in the RIS Controllers Study Group Cohort (ECRIS) (8) (see Supplemental File S1) based on the study design. Thirty-one EC were analyzed, 14 who underwent loss of virological control (at least two consecutive measurements of VL above the detection limit in one year) named Transient Controllers "TC". Seventeen EC who maintained a persistent virological control during the same follow-up period were designated as Persistent Controllers "PC" (see study design in Figure 1). All subjects participating in the study gave their informed consent and protocols were approved by the institutional ethical committees.

Experimental procedures

Laboratory evaluations were performed at the Laboratory of Immunovirology, Institute of Biomedicine of Seville (IBiS), Virgen del Rocío University Hospital in Seville (Spain); the Molecular Virology Unit, Laboratory of Research and Reference in Retrovirus. Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid (Spain), the Joan XXIII Universitary Hospital in Tarragona, IISPV, Rovira i Virgili University (Spain) and the AIDS research Institute IrsiCaixa, Badalona (Spain).

General

363 CD4+ and CD8+ T-cell absolute counts were determined in fresh whole blood by using
364 an Epic XL-MCL flow cytometer (Beckman-Coulter, Brea, California) according to the
365 manufacturers' instructions. Plasma HIV-1 RNA concentration was measured by using
366 quantitative polymerase chain reaction (COBAS Ampliprep/COBAS Taqman HIV-1
367 test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's
368 protocol. The detection limit for this assay was 50 HIV-1 RNA copies/mL. Hepatitis C
369 virus (HCV) RNA was determined using available PCR procedure Kit (COBAS
370 Amplicor, Roche Diagnosis, Barcelona, Spain) with a detection limit of 10 IU/mL.

371 Genetics

372 HLA-B group alleles were genotyped using a reverse sequence-specific oligonucleotide
373 bound to a fluorescently coded microsphere system (LABType SSO, RSSO1B, One
374 Lambda, Canoga Park, CA), following manufacturer's instructions. The genotyping of
375 the IL28B SNP rs12979860 was performed as previously described (62), using a
376 TaqMan 5′ allelic discrimination assay (Applied Biosystems, Foster City, CA).

377 Cell stimulation

378 PBMCs were thawed, washed and *in vitro* stimulated with 2 µg/ml of overlapped HIV
379 (Gag)-specific peptide pool (NIH AIDS Research and Referenced Reagent Program
380 (https:// www.aidsreagent.org/index.cfm) and stained with conjugated monoclonal anti-
381 CD107a-BV786 (clone H4A3; BD Biosciences, Franklin Lakes, NJ) at the beginning of
382 incubation as previously described (26).

383 Immunophenotyping and Intracellular Cytokine Staining

384 Stimulated PBMCs were washed and stained with LIVE/DEAD fixable Violet Dead
385 Cell Stain (Life Technologies, CA, USA). Then cells were surface stained with anti-

386 CD14-PB, anti-CD19-PB, anti-CD38-Qdot655 (Life Technologies), anti-HLA-DR-
387 BV570 (clone L243), anti-CD56-PB (Biolegend, San Diego, CA), anti-CD8+-PerCP-
388 Cy5.5 (clone RPA-T8), anti-CD45RA-FITC (clone L48), anti-CD27-BV605 and anti-
389 CD57-PE-CF595 (BD Biosciences). Cells were then stained intracellularly for 30 min
390 with 100 µl of PBS with anti-CD3-APC-H7 (clone SK7), anti-IFN-γ-PCy7, anti-TNF-α-
391 Alexa700 (clone MAb11), anti-IL-2-APC (clone MQ1-17H12) and anti-Perforin-PE
392 (clone B-D48) (BD Biosciences) and then washed twice and fixed in PBS containing
393 4% paraformaldehyde (PFA). Unstimulated and cell stimulation with staphylococcal
394 enterotoxin B (SEB) as positive control were included in each experiment.
395 Lymphocytes were defined as low Forward/Side scatter and expressed CD3, and/or no
396 CD8+ but not CD19, CD14, and CD56 (Figure 9).

397 PBMCs were analyzed by using a LSR Fortessa Cell Analyzer (BD Biosciences, Spain).
398 A minimum of 1,500,000 total events were recorded for each condition.

399 Cytokine and Chemokine Measurement by Mulliplex Bead Array Kits

400 Milliplex Map Human Cytokine/ChemokineMagnetic Bead Panels kits composition:
401 HCYTMAG-60K-PX23: EGF, IL-1α, FGF-2, IL-3, Eotaxin-1, IL-7, TGF-α, IL-8, G-
402 CSF, IP-10, Fractalkine, MCP-1, IFN-α2, MIP-1α, MIP-1β, GRO, MCP-3, VEGF, IL-
403 12 (p40), Flt-3 ligand, MDC (CCL22), sCD40L, IL-1ra.

404 HCYTMAG-60K-03: PDGF-AA, PDGF-AB/BB, RANTES

405 HT17MG-14K-PX25: GM-CSF, IL-21, IFN-γ, IL-4, IL-17F, IL23, IL-10, IL-5,
406 CCL20/MIP3α, IL-6, IL-13, IL17E/IL25, IL-15, IL27, IL17A, IL31, IL-22, TNF-α, IL-
407 9, TNF-β, IL-1β, IL28A, IL33, IL-2, IL-12 (p70).

HCP2MAG-62K-19: EOTAXIN-2, IL-20, MCP-2, TRAIL, BCA-1, CTACK, MCP-4, SDF-1A+b, I-309, ENA-78, IL-16, MIP-1d, TARC, 6CKINE, EOTAXIN-3, LIF, TPO, SCF, TSLP6.

Median fluorescence intensities were collected on a Bio-Plex 200 instrument by using Bio-Plex Manager™ (Bio-Rad Laboratories, Spain) software. Cytokine concentrations were determined from the appropriate standard curves to convert fluorescence units to concentrations (pg/mL).

Nucleic acid extraction and *gag* and *env* amplification

Proviral DNA was obtained from frozen $2x10^6$ PBMCs using the "Speedtools tissue DNA extraction kit" (Biotools B&M Labs S.A., Spain). We used a limiting-dilution nested polymerase chain reaction (PCR) using "Phusion High-Fidelity PCR Master Mix with HF Buffer" (Thermo Scientific). A multiplex first PCR for *gag* and *env* regions amplification was peformed using the outer primers 505-gag 5´ CGAGGGGCGGCGACTGGT 3´ (728-745 HXB2 position) 40-gag 5´ TTCCCTAAAAAATTAGCCTGTCT 3´ (2074-2096 HXB2 position) and 169-env 5 ´AATGTCAGCACAGTACAATGTACAC 3´ (6945-69 HXB2 position 69) 96-env 5´ AGACAATAATTGTCTGGCCTGTACCGT 3´ (7836-7862 HXB2 position). One microliter of the first PCR product was re-amplified independently using primers for *gag* 171-gag 5´ TTTGACTAGCGGAGGCTAG 3´ (761-779 HXB2 position) and 336-gag 5´ TTCCAACAGCCCTTTTTCCTAGGGG 3´ (2009-2033 HXB2 position) or primers for the C2-V5 region 27-env 5' ATAAGCTTGCAGTCTAGCAGAAGAAGA 3' (7004-7030 HXB2 position) and 167-env 5' TTCTCCAATTGTCCCTCATATCTCCTCCTCCA 3' (7634-7665 HXB2 position). Nucleotide sequences were determined with the Big DyeTM Terminator Cycle

Sequencing kit (Applied Biosystems) in an ABI 3730 sequencer (Applied Biosystems) in the Genomic Unit of the CNM-ISCIII.

Proviral DNA quantification

DNA proviral viral load was quantified by using a nested Alu-LTR PCR (63, 64). In brief, a first conventional PCR was performed using oligonucleotides against Alu sequence and the HIV-1 LTR, with the following conditions: 95ºC, 8 min; 12 cycles: 95ºC, 1 min; 60ºC, 1 min; 72ºC, 10 min; 1 cycle: 72ºC, 15 min. Then, a second qPCR was performed using TaqMan probes with FAM/ZEN/Iowa Black and TaqMan Master Mix (Applied Biosystems). DNA from 8E5 cell line was used for the standard curve. *ccr5* gene was used as a housekeeping gene for measuring the input DNA and to normalize data.

Phylogenetic analysis of the nucleotide sequences in *gag* and *env* genes

Nucleotides sequences were assembled using the SeqMan program (DNASTAR). Maximum likelihood trees were performed as follows. First, nucleotide sequences were included in a global data base with the sequences obtained from the patients studied in our laboratory to detect cross-contamination. All samples segregated in distinct clusters of the phylogenetic tree, excluding contamination. Phylogenies were estimated using a Maximum Likelihood approach using the best-fit model of nucleotide substitution (GTR+G+I, jModelTest v.0.1.1) implemented in MEGA 6 Software program (65). Internal branches support was tested with an approximate likelihood-ratio test (MEGA 6). Intra-sample diversity was estimated by the best-fit model of nucleotide substitution in MEGA 6 program. An estimation of viral infection time or "viral dating" was deduced, assuming a relaxed molecular clock, from the genetic distance of the nucleotide sequence of each subject virus to the MRCA (most recent common ancestor)

of the HIV-1 Spanish epidemic (6, 10, 11, 66). Nucleotide sequences were submitted to GeneBank (Accession numbers MF988754-MF989105).

Analysis of Gag CD8+ T-cell immune escape

HIV-1 variation at the optimal Gag CD8+ T-cell epitopes was based on the best-defined CD8+ epitope summary from the Los Alamos Molecular Immunology Database in Gag proviral sequences matched to patients' HLA class I alleles with two to four-digit resolution (35). HIV-1 variation at optimal epitopes was defined by comparison with the HIV-1HXB2 epitope sequence at the HLA-I restriction element. The frequency of virus variation at Gag epitopes was defined as the ratio of total mismatches found at optimal epitopes to the number of total optimal epitopes found per sequence.

Statistical analysis

Correlations between variables were assessed using the Spearman rank test. Differences between categorical values were determined by Chi-square test. Differences between unpaired groups were determined by Mann-Whitney U test and differences between paired samples were determined by Wilcoxon signed rank and Friedman tests. p values <0.05 were considered statistically significant. The Statistical Package for the Social Sciences software (SPSS) 22.0 package (IBM, Madrid, Spain) was used for the statistical analysis. Graphs were generated with Prism, version 5.0 (GraphPad Software, Inc.). Polyfunctionality was defined as the percentage of lymphocytes producing multiple cytokines. Polyfunctionality pie charts were constructed using Pestle, version 1.6.2 and Spice, version 5.2 (provided by M. Roederer, NIH, Bethesda, MD) and was quantified with the polyfunctionality index algorithm (67) employing 0.1.2 beta version of the "FunkyCells Boolean Dataminer" software (www.FunkyCells.com) provided by Dr Martin Larson (INSERM U1135, Paris, France).

The selection of potential biomarkers among the soluble markers of inflammation associated with the loss of spontaneous control was complemented with multivariate statistics using: Random Forest analysis (unbiased selection of variables), principal component analysis (PCA) (multivariate pattern that generates the maximum degree of separation between groups) and finally, the performance of biomarkers was examined by logistic regression analysis and receiver operating characteristic (ROC) curves. The employed statistical software included 'R' software (http://cran.r-project.org), matrix calculation platform MATLAB (ver.7.5.0; The Mathworks, Inc., Natick, MA, USA).

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The authors have declared that no conflicts of interest exist.

E.R.-M. and M.L. designed the study. N.R., JM.B, A.L., M.Pl., F.G., C.R., O.M.-M., M.D., JA.I., J.R. MA.M.-F., F.V., J.A. and M.L. coordinated the patient inclusion,

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513 sample management and analysed and interpreted data from the experiments. M.Pe.,
514 C.C., L.T.-D., C.L.-G., E.R.-M., J.G.-P. and M.C. designed the experiments. M.Pe.,
515 I.O., L.T.-D., B.D.-M., E.R.-G., J. G, J.G.-P. and M.C. produced the experimental data
516 in the laboratory. M.Pe., L.T.-D., E.R.-G., C.L.-G. and E.R.-M. prepared the
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793 **FIGURE LEGENDS.**

794 **Figure 1.** Study design. Schematic representation of the longitudinal and retrospective
795 study design in transient controllers (TC) (A) and in persistent controllers (PC) (B). In
796 TC, up to five determinations were performed: two in the "under-control-period", two (-
797 T2) and one year (-T1) before the loss of control, and up to three determinations in the
798 "post-loss-of-control period" including the closest time point to the loss of virological
799 control (T0), one (+T1) and two (+T2) years after the loss of virological control. At
800 least, -T2, -T1 and T0 samples were required to be included in the study. In total, a
801 maximum of 54 time points were analyzed in this group. In PC, up to five
802 determinations were performed at one year intervals but at least three consecutive time
803 points per subject were required to be included in the study. In total, a maximum of 63
804 time points were analyzed in this group. For Gag-specific T-cell response assays all
805 available follow-up time-points were tested (PC, n=14 and TC, n=14). Virological and
806 soluble biomarkers assays were determined in all available follow-up time-points in PC
807 (n=10 and n=11, respectively) and only at –T2 and –T1 in the TC (n=9 and n=12,
808 respectively).

809 **Figure 2.** Representative longitudinal Gag-specific T-cell associated parameters in PC.
810 T cell response was define as the frequency of cells (>0.05% after background
811 substraction of the unstimulated condition) with detectable IFN-γ, TNF-α and/or IL-2
812 intracellular cytokine production after stimulation of cryopreserved PBMCs with Gag
813 overlapped peptides. Gag-specific total CD4+ T-cell response (A), Central Memory
814 CD4+ T-cell response (B), total CD8+ T-cell response (C) and Terminally
815 Differentiated CD8+ T-cell response (D) levels. No statistical differences were obtained
816 throughout the follow up. NS denotes no significant differences between multiple paired
817 samples comparisons determined by the Wilcoxon signed rank test (p> 0.05 in all

818 cases). Friedman test could not be applied due to not enough statistical power using the
819 five follow up time points.

820 **Figure 3.** CD4+ and CD8+ T-cell Gag-specific response. Percentage of subjects with
821 Gag-specific CD4+ and CD8+ T-cell response. T cell response was define as the
822 frequency of cells (>0.05% after background substraction of the unstimulated condition)
823 with detectable IFN-γ, TNF-α and/or IL-2 intracellular cytokine production after
824 stimulation of cryopreserved PBMCs with Gag overlapped peptides. (A). Total, Central
825 Memory (*CD4+CD45RA-CD27+*, CM) and Effector Memory (*CD4+CD45RA-CD27-*,
826 EM) Gag-specific CD4+ T-cell levels (B) and total, CM, EM and Terminally
827 Differentiated (*CD8+CD45RA+CD27-*, TD) Gag-specific CD8+ T-cell levels (C).
828 Differences between unpaired groups were determined by Mann-Whitney U and chi-
829 squared tests and differences between paired samples were determined by Wilcoxon
830 signed rank test. Friedman test was not applied due to the small number of paired
831 samples. Only significant differences are shown. *, $p<0.05$; **, $p<0.001$.

832 **Figure 4.** HIV-1-specific CD8+ T-cell polyfunctionality. Polyfunctionality, understood
833 as simultaneous multiple production of IFN-γ, TNF-α and IL-2 per T-cell, was studied
834 only on subjects categorized as responders. T cell response was define as the frequency
835 of cells (>0.05% after background substraction of the unstimulated condition) with
836 detectable IFN-γ, TNF-α and/or IL-2 intracellular cytokine production after stimulation.
837 Due to the low number of Gag-specific CD4+ T-cell responders in TC,
838 polyfunctionality analysis was not aplicable. Pie charts show polyfunctional distribution
839 of HIV-1-specific CD8+ TD CD57+ T-cells with up to three functional responses to
840 Gag stimulation, IFN-γ, TNF-α and IL-2 production in the polyfunctional distribution is
841 shown in arcs (A). Pestle and Spice were used for analysis. Percentage of Gag-specific
842 CD8+ T-cells expressing the activation profile, $CD38^+$, and the maturation profile

843 $CD45RA^{-}CD27^{+}CD57^{-}$ only significant differences are shown (B). Polyfuncionality
844 INDEX of Gag-specific total CD8+ T-cells. Values from PC and pre-loss time points of
845 follow-up (–T2 and –T1) in TC based on the proportions of cells expressing
846 combinations of IFN-γ, TNF-α, IL-2 (3 functions) (C), plus CD107a (4 functions) (D)
847 and plus perforin (5 functions) (E). Single and double production of CD107a and
848 perforin were excluded from the analyses. Differences between groups were determined
849 by Mann-Whitney U test.

850 **Figure 5.** CD4+ and CD8+ T-cell activation. Total (A) and Effector Memory (B) CD4+
851 T-cells DR^{+} $CD38^{+}$, and total (C) and Central Memory (D) CD8+ T-cells DR^{+} $CD38^{+}$.
852 Differences between unpaired groups were determined by Mann-Whitney U test.
853 Friedman test was not applied due to the small number of paired samples. $p<0.15$ are
854 shown.

855 **Figure 6.** Virological assays: phylogenetic analysis and virus diversity. All virological
856 assays were performed in all time point samples in PC and only in time points previous
857 to the loss of control in TC. Phylogenetic analysis of sequences in *env* and *gag* genes
858 from TC and PC during follow-up. GeneBank Accession numbers MF988754-
859 MF989105. Phylogenetic trees were estimated using a Maximum Likelihood approach
860 using the best-fit model of nucleotide substitution (GTR+G+I, jModelTest v.0.1.1)
861 implemented in MEGA 6 Software program. Each subject is represented in a different
862 color. Samples taken at different times are marked with different symbols. In DI
863 subjects (EC4 and F4) the two viral populations are marked and named a and b. Bars
864 indicate 0.02 genetic distance (A). Comparison between intra-sample diversity in *env*
865 and *gag* regions for TC and PC. Mean and standard error for all pair-wise nucleotide
866 distances using MEGA 6.0 program. Differences between unpaired groups were
867 determined by Mann-Whitney U test. **, $p< 0.05$ (B).

868 **Figure 7.** Correlation of virus diversity with CD8+ T-cell polyfunctionality and analysis
869 of Gag CD8+ T-cell epitope variation. Correlations between *gag* and *env* diversity and
870 total CD8+ T-cell three cytokines polyfunctionality index (pINDEX 3-functions) in TC.
871 Spearman ρ correlation coefficient test was used (A). Analysis of HIV-1 Gag CD8+ T-
872 cell epitope variation. Frequency of variation in HIV-1 Gag CD8+ T-cell epitopes in PC
873 and TC before and after the loss of virological control. Differences between groups
874 were determined by Mann-Whitney U test (B). HIV-1 sequence variation in Gag ISW9
875 and TW10 epitopes restricted by HLA-B*57 in TC 351 along the follow up (C).
876 **Figure 8.** Soluble cytokines and chemokines as potential biomarkers of the loss of
877 virological control. Assays were performed in all time point samples in PC and in pre-
878 loss time points of follow-up (–T2 and –T1) in TC. Fold-change heat map of the relative
879 plasma concentrations of measured inflammatory markers. Positive folding (green)
880 means higher concentrations in PC, while negative folding (red) means the opposite. Of
881 these potential biomarkers, the five marked with an asterisk reached a p-value <0.05 in
882 the Mann-Withney U test, and the remaining two were added to the list due to its
883 classification power in the two multivariate test (A). Random Forest analysis
884 importance plot of the top 20 variables in importance of classification from a total of 70
885 cytokines and chemokines. Only the top five, highlighted in bold, were considered as
886 potential biomarkers (B). Score Plot of the PCA showed that the best percentage of
887 separation between groups was achieved with only two variables, RANTES and PDGF
888 AA (C). Using logistic regression and receiver operator characteristic (ROC) curves we
889 assessed three different multi-markers models that could accurately predict the loss of
890 control in EC: model A (blue) which includes the statistical significant variables in the
891 Mann-Whitney U test, model B (green) composed by the top five variables obtained
892 from the Random Forest analysis and model C (orange) compounded by the two

variables obtained in the PCA analysis (D).

Figure 9. Schematic diagram of the cytometry gating strategy. Gating strategies for: Gag-specific CD4+ and CD8+ T-cell, representative plots show the functional cytokine response to Gag peptides.

Table 1. Characteristics of the subjects

	Transient Controllers (n=14)	Persistent Controllers (n=17)	*p*
Age (years)	41 [38-52]	45 [41-48]	0.279
Male sex, n (%)	8 (57)	10 (59)	0.925
Sexual transmission, n (%)	10 (71)	6 (35)	**0.049**
Time since diagnosis (years)	8 [2-14]	18 [11-22]	**0.002**
HCV RNA detected, n (%)	6 (43)	10 (59)	0.376
CD4+ T-cells (cell/µL)	625 [392-783]	714 [627-940]	0.208
CD8+ T-cells (cell/µL)	735 [548-1015]	648 [569-970]	0.999
CD4:CD8 Ratio	1 [0.58-1.28]	1.17 [0.67-1.55]	0.456
HLA B57, n (%)*	3 (21)	6 (40)	0.280
HLA B27, n (%)*	1 (7)	2 (13)	0.584
HLA B35, n (%)*	2 (14)	0 (0)	0.129
IL28B-CC, n (%)$^{\phi}$	3 (38)	5 (42)	0.728

Values from TC are taken from –T2 and values from PC are taken from the first time point of follow-up. * n=15 for PC; $^{\phi}$n=8 and n=12 for TC and PC, respectively. Values are given as percentage for categorical variables or median and interquartile range for continuous variables. The Mann–Whitney U and Chi-squared tests were used. All *p* values <0.05 were considered significant and are highlighted in bold.

Table 2. T-cell levels and CD4:CD8 ratio of the study subjects

		CD4 T cells (cell/µl)	CD8 T cells (cell/µl)	CD4:CD8 Ratio
Transient Controllers (TC)* (n=14)	-T2	625 [391-783]	735 [547-1014]	1 [0.58-1.28]
	-T1	745 [491-925]	862 [587-1223]	0.97 [0.66-1.16]
	T0	591 [474-761]	686 [532-1134]	0.93 [0.44-1.3]
	+T1	531 [424-695]	750 [646-1147]	0.73 [0.38-1.07]
	+T2	735 [547-1014]	602 [592-1501]	0.56 [0.45-1.31]
Persistent Controllers (PC)* (n=17)	-T2	714 [627-940]	648 [569-970]	1.17 [0.67-1.55]
	-T1	689 [557-940]	720 [483-917]	1.09 [0.81-1.72]
	T0	550 [419-958]	629 [350-1030]	1.13 [0.51-1.77]
	+T1	710 [600-961]	672 [584-1058]	1.43 [0.88-1.68]
	+T2	556 [489-661]	554 [251-764]	1.18 [0.78-2.02]
	PC (mean)	651 [576-989]	725 [591-954]	1.09 [0.62-1.56]
p	-T2 vs PC (mean)	0.284	0.757	0.452
	-T1 vs PC (mean)	0.965	0.292	0.547
	T0 vs PC (mean)	0.174	0.906	0.269
	+T1 vs PC (mean)	0.084	0.393	0.052
	+T2 vs PC (mean)	0.351	0.969	0.225

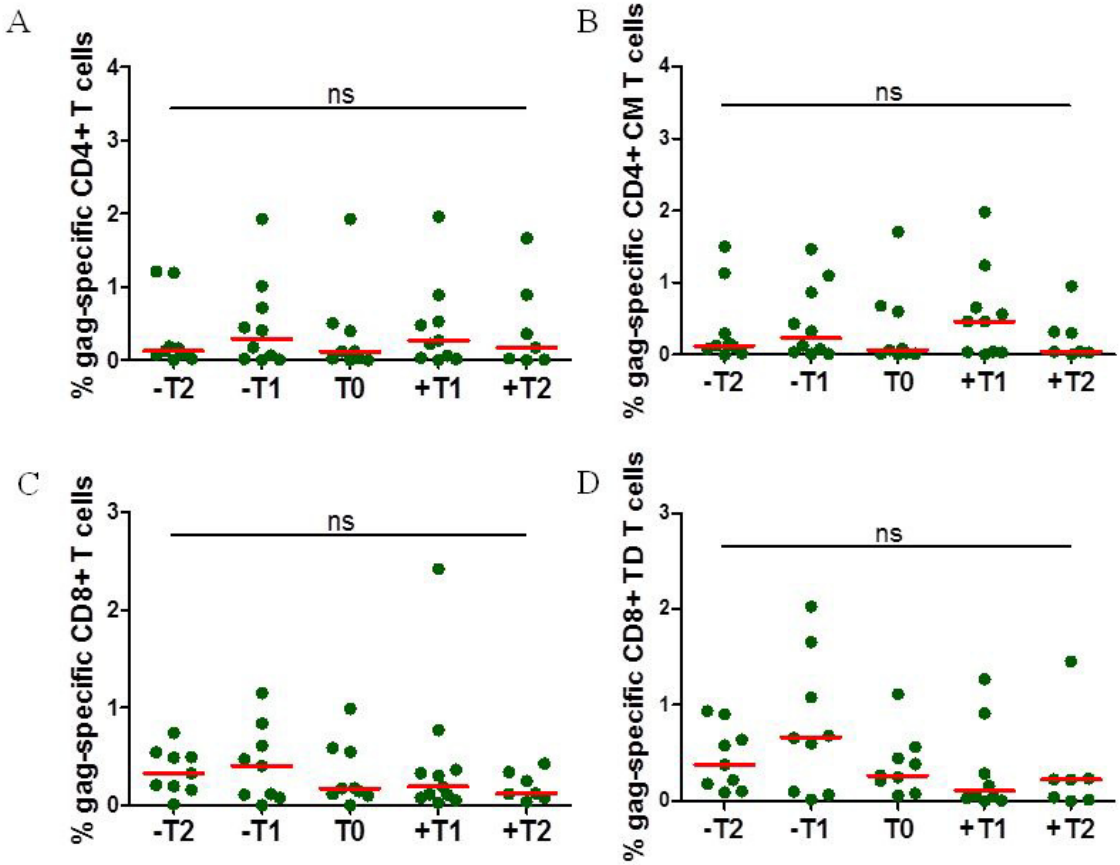
Values are given as median and interquartile range. The Mann–Whitney U test was used for group comparisons.*The Friedman test was used to analyze differences during the follow up in each group, no differences were found, $p>0.05$.

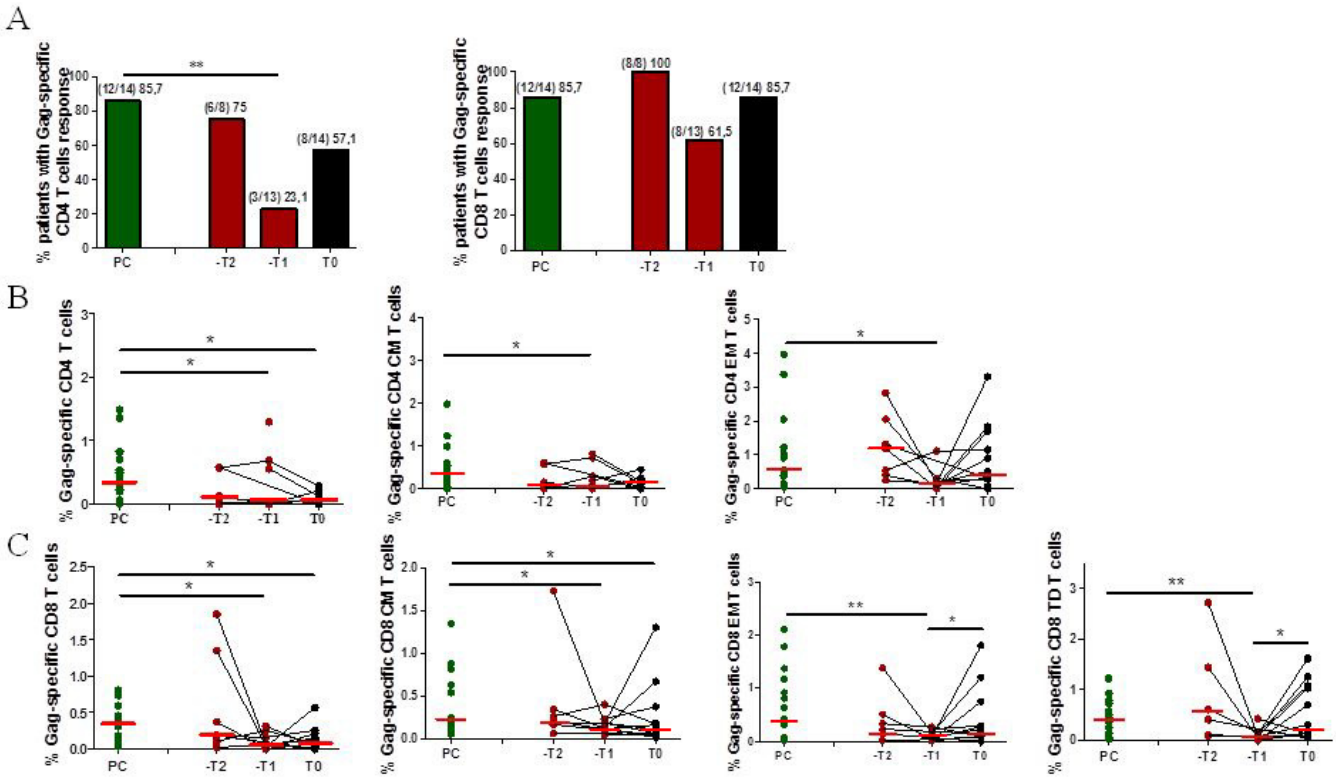
Table 3. Year of HIV-1 diagnosis, sampling year and viral dating in TC and PC.

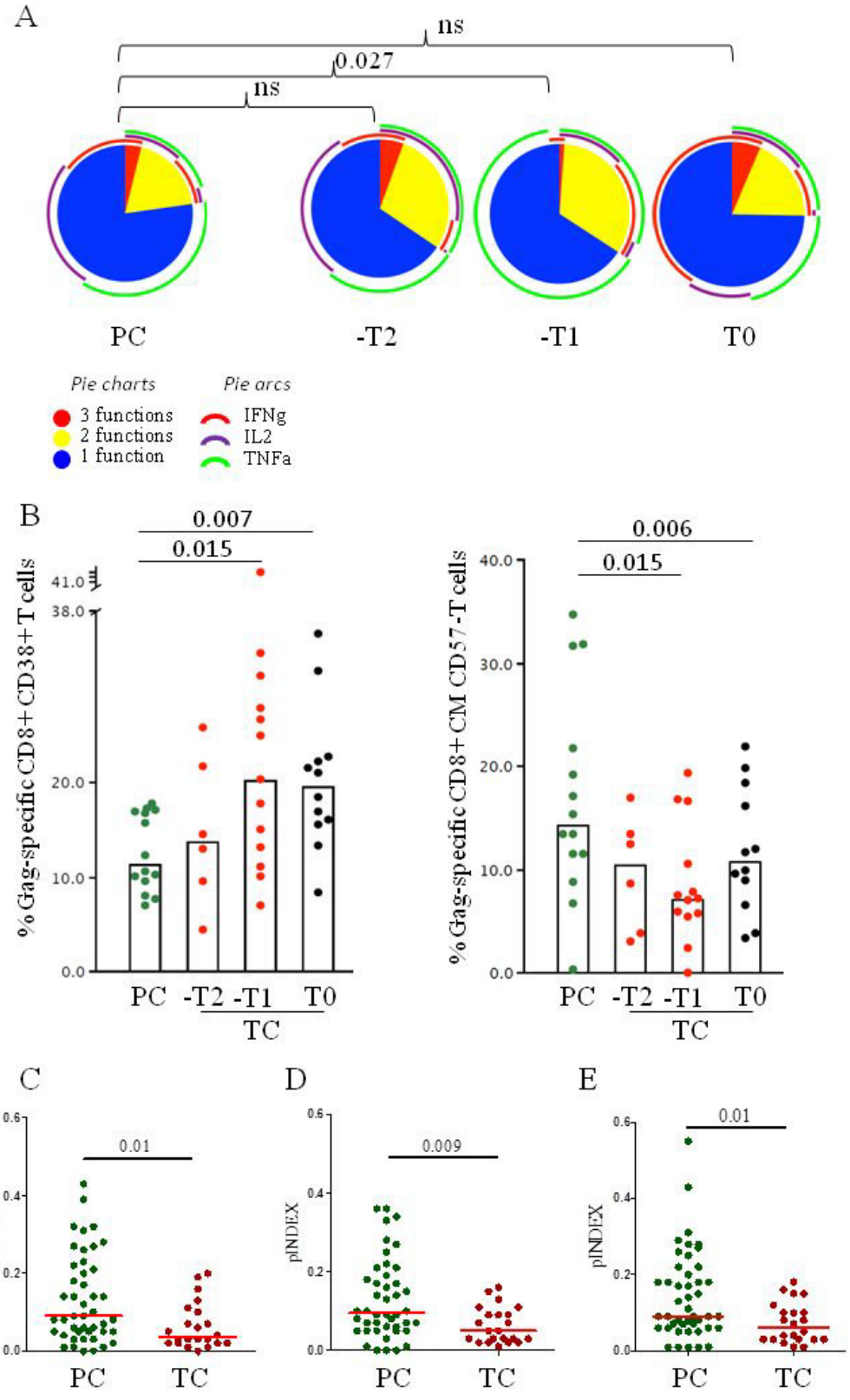
Patient	Year of HIV-1 diagnosis	Sample year	Years since HIV-1 diagnosis	Estimated Viral dating	Difference Viral dating − Year of HIV-1 diagnosis
TC					
330	1991	2000	9	2004	13
F4	1986	2003	17	1997	11.8
74	1996	2007	11	2003.2	7.2
H5	1986	2006	20	1997	11
H6	1990	2009	19	2003.6	13.6
Mean[a]	1989±4.1	2005±3.5	15±4.9	2001±3.4	11.5
PC					
H1	1999	2010	11	2004	5
H2	1994	2011	17	1997	3
H4	1986	2011	25	1993	7
292	1998	2011	13	1995	-3
F3	1985	2009	24	1994	9
Mean[a]	1992±6.6	2010±0.9	18±6.3	1997±4.4	5

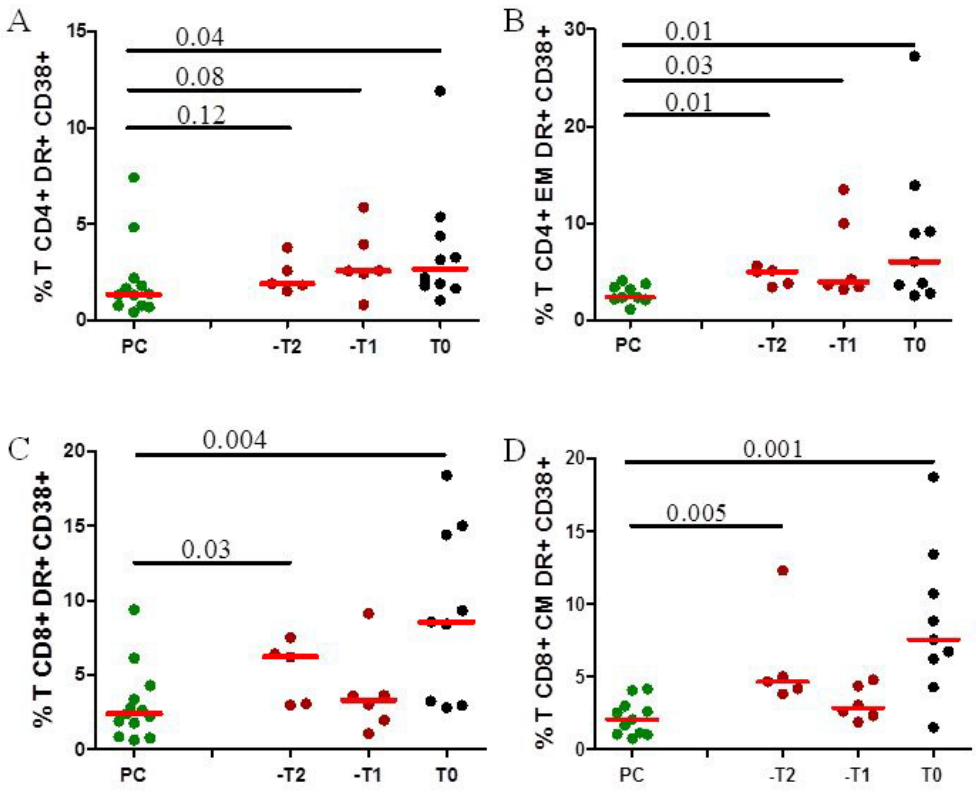
[a]Values are expressed as mean ± standard deviation. To perform the viral dating of the subjects, the genetic distance of the reconstructed most recent common ancestor (MRCA) for each patient clade was compared with that of a reconstructed MRCA for the Spanish epidemic. The viral dating time was estimated by use of a linear-correlation equation, previously developed in a large set of Spanish samples, that correlates the V3 nucleotide-sequence divergence to the Spanish-epidemic MRCA and the sampling year. Only four subjects in TC were included. The viral

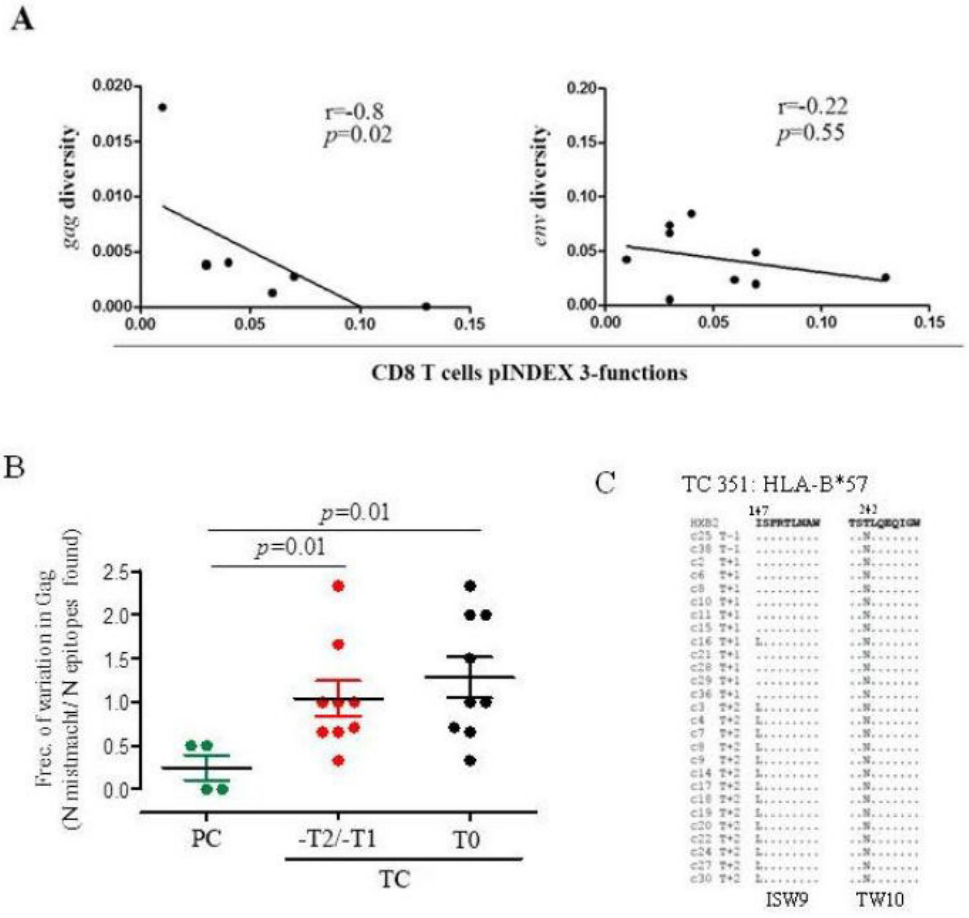
dating of other TC could not be estimated because the sampling and the year of HIV-1 diagnosis were too close in time.

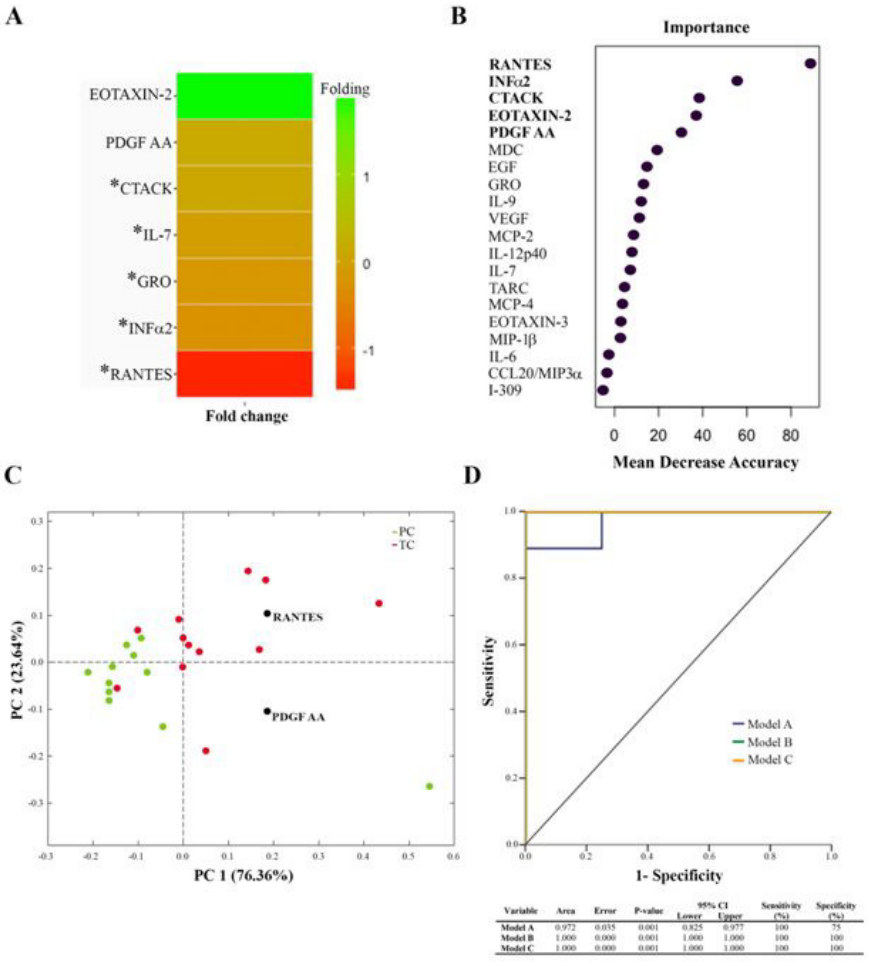












Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
Model A	0.972	0.035	0.001	0.825	0.977	100	75
Model B	1.000	0.000	0.001	1.000	1.000	100	100
Model C	1.000	0.000	0.001	1.000	1.000	100	100