Article

People living with HIV (PLWH) controllers induce high production of IFN-γ to Gag Epitope

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Abstract: We report the characterization of phenotype, genotype, and functional parameters such as CD4+ T cell, viral load, HIV-1 genotype, class I HLA genotype, CCR5 polymorphism, and IFN-γ producing cells from a cohort of 37 people living with HIV-1 (PLWH) (viremic controllers, were compared those on antiretroviral therapy (ART) from two sites in São Paulo, Brazil. Half the PLWH (51.4%) were classified as viremic controllers (Controller group - CG), compared to 48.6% were on ART. As expected, controllers showed baseline higher levels of CD4+ T cells and nadir compared to those under ART, confirming the ability of HIV-1 controllers to preserve these cells even in the absence of ART. IFN-γ-producing cells showed greater magnitude to the HIV peptides Gag in controllers ($p<0.03$). Our results demonstrated that HLA protective alleles are associated with strong production of IFN-γ to Gag, Nef and RT epitopes in people living with HIV controllers.

Keywords: Human Immunodeficiency Virus (HIV); Elite Controllers (EC); IFN-γ T cell responses; Human Leukocyte Antigens (HLA)

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) disease is a pandemic which affects approximately 38.4 million people living with HIV (PLWH) worldwide [1]. For the vast majority of those PLWH in the absence of antiretroviral therapy (ART) eventually causes CD4+ T cell depletion and profound immunodeficiency, resulting in high morbidity and mortality [2-3]. Otherwise, one small group (1-2%) may delay the HIV natural history of the infection, actually they are able to maintain stable CD4+ T cell counts for more than 10 years, even in the absence of ART and are defined as controllers [4-5]. Thus, controllers can be categorized according are able to maintain undetectable viral loads (VL<40 copies/mL) as elite controllers (EC), whereas controllers have the ability to suppress viremia to low levels, and both groups present normal peripheral CD4+ T cell counts without ART [6-7]. For this research, both categories were grouped as controllers.

The pathogenesis of the progression of HIV disease can be influenced by several factors such as: 1) replication of HIV in the lymphoid organs after primary infection [8]; 2) persistent viral replication in the lymphoid organs throughout the course of HIV infection [9-10]; 3) chronic stimulation of the immune system, which can cause inappropriate immune activation and progressive exhaustion of the immune response [11-12]; and 4) destruction of lymphoid tissue, which results in damage to the

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ability to maintain an HIV-specific immune response and to generate immune responses against new pathogens over time [13-14]. Also, the HIV genetic diversity might be responsible for different clinical evolution [15-16].

The factors that determine slow progression in HIV-1 infected individuals are not fully understood, despite extensive studies from many international HIV cohorts [17-19]. Several host characteristics and immune and viral mechanisms have been associated with the effective control of the HIV infection [20-22]. Deletions or deleterious mutations of the viral genome implicated in the decreasing of the HIV virulence are the putative causes of non-progression PLWH [23-25]. From a host point of view, genetic variations in the CCR5 co-receptor [26-27] and certain HLA alleles are associated with slower progression of the course of HIV infection. HLA- A*01, A*02, A*03, A*25, A*32, B*13, B*14, B*27, B*51, B*57, Cw*08, C*14 and DPB1 alleles are found more frequently in controllers [28-30]. Nonetheless, host restriction factors such as APOBEC proteins [31], SAMHD [32], Tetherin [33], and TRIM5a [34-35] can contribute to the control of viral replication.

It is possible that high levels of HIV-specific $CD8^+$ T cells, measured by tetramers or cytokine expression, are present in a strong proportion in controllers. Several studies, few of them from Brazilian cohorts [36-38], have shown strong amplitude of the responses directed against a large diversity of HIV epitopes [22,39-41]. Considering the above stated, we aimed to characterize the clinical, genetic, immunological and epidemiological profile of a small cohort of controllers in São Paulo, southeast Brazil.

2. Methods

2.1. Subjects, sample collection and follow-up

From October 2012 to December 2022, we cross-sectionally studied 37 HIV-infected patients on follow-up at the outpatient clinic *ADEE3002 in the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* (ADEE 3002/HC-FM-USP) *and Centro de Referência e Treinamento DST/Aids (CRT DST/Aids-SP)* in São Paulo, Brazil.

Before enrollment, participant's ART history and medical history were obtained, as well as the nadir $CD4^+$ T cell count; the highest recorded plasma HIV RNA level; the $CD4^+$ T cell counts and baseline HIV RNA level. Follow-up visits were scheduled yearly. The methods were carried out according to approved guidelines.

A questionnaire on epidemiological and social data was voluntarily answered by all participants, and we revised their medical records, searching for their laboratory immune and virologic monitoring data. At each visit, we interviewed, examined and measured patients' $CD4^+$ T cell count and HIV RNA level. We also typed Class I HLA for all individuals.

2.2. PBMC separation

Peripheral blood mononuclear cells (PBMC) suspensions were separated from 80 mL heparinized venous blood (BD Vacutainer®, BD Biosciences, San Jose, CA, USA) by density gradient sedimentation over Ficoll-Paque Plus, 1.077 g/L (GE Healthcare Bio-Sciences AB, Upsala, Sweden). Isolated PBMC were then washed twice in RPMI 1640 medium [(Advanced RPMI 1640, Gibco®, Grand Island, NY, USA) supplemented with 200 mM glutamine, 100 U/mL antibiotic-antimycotic, 100 mM non-essential amino acids and 100 mM sodium pyruvate (Gibco™)], and approximately 10^7 cells were cryopreserved per vial in liquid nitrogen in freezing solution containing 50% heat-inactivated fetal bovine serum (FBS; Gibco™), 40% RPMI 1640 medium and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich®, St Louis, MO, USA) until used in the assays. At the time of the assays, PBMC were rapidly thawed in a 37 °C water bath and washed in RPMI 1640 medium supplemented with 10% FBS. Cells were counted, checked for viability and resuspended in RPMI 1640 medium at a concentration of 10^6 cells/mL.

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2.3. CD4⁺ / CD8⁺ T cell Immunophenotyping

The two major T-cell subpopulations were classified on the basis of the expression of CD4⁺ and CD8⁺ surface molecules from EDTA-peripheral blood samples (BD Vacutainer®, BD Biosciences). The CD4⁺/CD8⁺ cell immunophenotyping was performed as recommended by Brazilian National Network for CD4⁺/CD8⁺ T cell immunophenotyping. Briefly, total blood was stained with the four-color Multi-test anti-human CD45, anti-human CD3, anti-human CD4, anti-human CD8 (BD Biosciences) in Trucount tubes (BD Biosciences) for 20 minutes, then a lyse-no-wash step was done using FACS lysing solution, and the sample was analyzed in the FACScalibur flow cytometer (BD Biosciences). We used multiset software to count and get the absolute cell number.

2.4. Quantification of HIV-1 Viral Load

HIV-1 RNA present in plasma was quantified from EDTA-peripheral blood samples and fractionated by amplifying *gag* region of the viral RNA with homogeneous real-time fluorescence detection, four hours after collection (Abbott RealTime HIV-1®, Abbott Molecular, Illinois, USA), according to the manufacturer's instructions. The detection limit of this test was 40 RNA copies/mL.

2.5. CCR5-Δ32 and CCR5-P-59029A/G Polymorphisms

Genomic DNA was extracted from the buffy coat of the individual's samples, collected in EDTA-sterile tubes (BD Vacutainer®, BD Biosciences) using a commercial kit QIAamp® genomic DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

CCR5-Δ32 and CCR5-P-59029A/G alleles were determined by the QIAGEN® Multiplex PCR kit. Forward and reverse primers used were: F-5'-CTTCATCATCCTCCTGACAATCG-3' and R-5'-GACCAGCCCCAAGTTGACTATC-3' for CCR5-Δ32, F-5'- TGGGGTGGGATAGGGGATAC-3' and R-5'-TGTATTGAAGGCGAAAAGAATCAG-3' (Life technologies, Carlsbad, CA, USA) for CCR5-P-59029A/G as described by Kristiansen *et al.* [42].

The amplification of the wild type allele of CCR5 resulted in 262 bp fragments while the mutant allele (CCR5-Δ32) resulted in amplicons of 230 bp. The Amplification product of CCR5-P-59029A/G (453 pb) was digested with the endonuclease *Bsp*1286I 5U (New England Biolabs®, UK). The wild type genotype resulted in a 408 bp fragment and the allele CCR5-P-59029A/G was cleaved in two fragments of 45 bp and 363 bp. The amplified and digested products were visualized under UV light in agarose gel 2% stained with Sybr® Safe (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). All PLWH disclosed were subtype B, as predominant in our cohort in Sao Paulo, Brazil.

2.6. Typing of HLA class I

The HLA class I typing (HLA-A, HLA-B and HLA-C) was performed at the HLA Laboratory of the *Fundação Hemocentro de Ribeirão Preto* (Fundherp), São Paulo, Brazil, by Sequence-Specific Oligonucleotides PCR amplifications (PCR-SSO), using the commercial LABType® SSO Class I kits: RSSO1A (LABType SSO Class I A Locus), RSSO1B (LABType SSO Class I B Locus) e RSSO1C (LABType SSO Class I C Locus; One Lambda, Inc., USA) according to the manufacturer's protocol (LABType One-Lambda) (Supplementary Table 1).

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Table 1. Demographic and baseline data of people living with HIV-1 enrolled in this study (controllers vs ART groups).

Variables	Controllers (C) (n = 19)	Antiretroviral Treated Individuals (ART) (n = 18)	P value
Age (years) [median (±SD)]	51 (±12.08)	56 (±10.08)	0.6374
Diagnosis (years)	17 (±6.405)	19.5 (±9.41)	0.8072
Nadir CD4 T cell count (x 10^6/L) [median (±SD)]	596 (±186)	315 (±201)	<0.001
CD4 T cell count (x 10^6/L) [median (±SD)]	850 (±331)	637 (±317)	0.0073
CD8 T cell count (x 10^6/L) [median (±SD)]	978 (±612)	1104 (±445)	0.5655
Log_{10} plasma VL (copies/mL) [median (±SD)]	704 (±3702)	25 (±23704)	0.2239

[a]Median (standard-deviation) values for age, years of HIV-1 diagnosis, T-cells count and RNA viral load are shown. C: Controllers; ART: antiretroviral treated individuals; VL: viral load. One-way ANOVA and the nonparametric Kruskal-Wallis test were used to calculate P values.

2.7. T Cell Responses

2.7.1. HIV antigens

Synthetic 15-mer peptides overlapping by 11 amino acids (aa) and corresponding to the entire HIV-1 Gag, Nef, and reverse transcriptase (RT) sequence, consensus B derived from the HXB2 AA coordinates, from the NIH AIDS Reagent Program (Germantown, USA) and pooled into 23 peptide pools: 5 p24 pools, 3 p17 pools, and 3 PP (i.e., p2/p7/p1/p6) pools for Gag, 4 Nef pools and 8 pools RT. We used 1.0 mg of each of the 281 lyophilized peptides (Supplementary Table 1).

2.7.2. IFN-gamma Enzyme-Linked Immunospot (Elispot) Assays

Interferon (IFN)-γ Elispot assay was performed as described previously [43-45]. Briefly, 96-well Multiscreen® ELISPOT plate (EMD Millipore Corporation, Burlington, MA, USA) were coated with 50 µL/well of anti-human IFN-γ antibody (anti-human IFN-γ mAb 1-D1K; Mabtech AB, Nacka Strand, Sweden) overnight. After blocking with 10% foetal bovine serum (FBS), triplicate wells were filled with 1 x 10^5 PBMC and 4 µg/mL HIV-1 pool peptides. Plates were incubated at 37 °C in 5% CO_2 for 20 h. Phytohemagglutinin (PHA; Sigma-Aldrich®; 0.5 µg/mL) and medium alone served as positive and negative controls, respectively. Wells were then washed and spots were detected after the addition of biotinylated anti-human IFN-γ mAb (anti-human IFN-γ mAb 7-B6-1; Mabtech AB; 1.0 µg/mL) (2 h, 37 °C in 5% CO_2), followed by streptavidin alkaline phosphatase (Mabtech AB; 1 h, 37 °C in 5% CO_2) and substrate (5-bromo-4-chloro-3-indolyl-phosphate//nitroblue tetrazolium (Sigma-Aldrich) incubated at room temperature (25 °C) until the appearance of blue spots. Antigen-specific spot forming cells (SFC)/10^6 PBMC frequencies were measured on a computer-based system (AID EliSpot Reader System; Autoimmun Diagnostika GmbH, Stranberg, Germany). The SFC number was calculated after subtracting the negative control values (wells with cells in the absence of peptide) from the same subject. The positivity cut-off was ≥ 30 IFN-γ SFC/10^6 PBMCs after subtracting background.

2.8. Statistical Analysis

We performed the statistical analyses with the aid of GraphPad Prism (version 8.0.1; GraphPad Software, La Jolla, CA, USA). We used one-way analysis of variance (ANOVA) and the nonparamet-

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ric Kruskal-Wallis and Friedman tests to compare the studied groups. The correlation between variables was quantified using the non-parametric Spearman correlation. P value <0.05 was considered statistically significant.

2.9. Ethics Approval

This study was approved by the Ethical Committee Boards of *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* (HC/FMUSP - CAPPesq protocol CAAE 05749912.6.0000.0068) and *Centro de Referência e Treinamento DST/AIDS* (CRT/DST/Aids-SP - protocol 023/2010).

2.10. Data Availability

Clinical and laboratory data of all HIV-infected patients **cohort of controllers (LTNP and EC),** from the outpatient clinic ADEE3002, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (ADEE3002/HC-FM-USP) and Centro de Referência e Treinamento DST/Aids (CRT DST/Aids-SP), which were used and/or analyzed during the current study, such as: $CD4^+$ and $CD8^+$T cell counts; plasma HIV RNA levels; Class I HLA typing, CCR5-Δ32 and CCR5-P-59029A/G polymorphisms and T cell responses to HIV antigens by IFN-γ Elispot, can be requested to the corresponding author.

3. Results

Participants meeting the inclusion criteria were 19 individuals who were defined as controllers, asymptomatic for at least ten years with stable $CD4^+$ T cell counts ≥ 500 cells/μL and no history of ART. These controllers were compared to 18 patients on ART, with stable $CD4^+$ T cell counts ≥ 350 cells/μL independently of VL levels.

3.1. Long-term non-progressors maintain $CD4^+$ T cell counts similar to uninfected donors and TCD4/$CD8^+$ ratio is higher in elite controllers compared to treated patients

We studied the evolution of annual $CD4^+$, $CD8^+$ T cells counts and VL, for 31 of the 37 patients, over a four-year period. $CD4^+$ T cell counts and VL from patients in each group are shown in Figure 1 and Table 1. Median $CD4^+$ T cell counts for controllers was 850 cells/μL (range: 621 – 11693 cells/μL), and 637 cells/μL (401 – 1332 cells/μL) for ART patients, respectively [Figure 1 (A)]. The controller individuals had a low VL (median: 704 copies/mL), and in six of these individuals detectable VL were not found whereas ART patients showed variable detection of VL [Figure 1 (B)]. As expected, controllers had higher T $CD4^+$ cell counts and T $CD4^+$/T $CD8^+$ ratio than patients on ART during the whole four-year period ($p<0.05$ for both comparisons) (Data not shown for CD4 counts).

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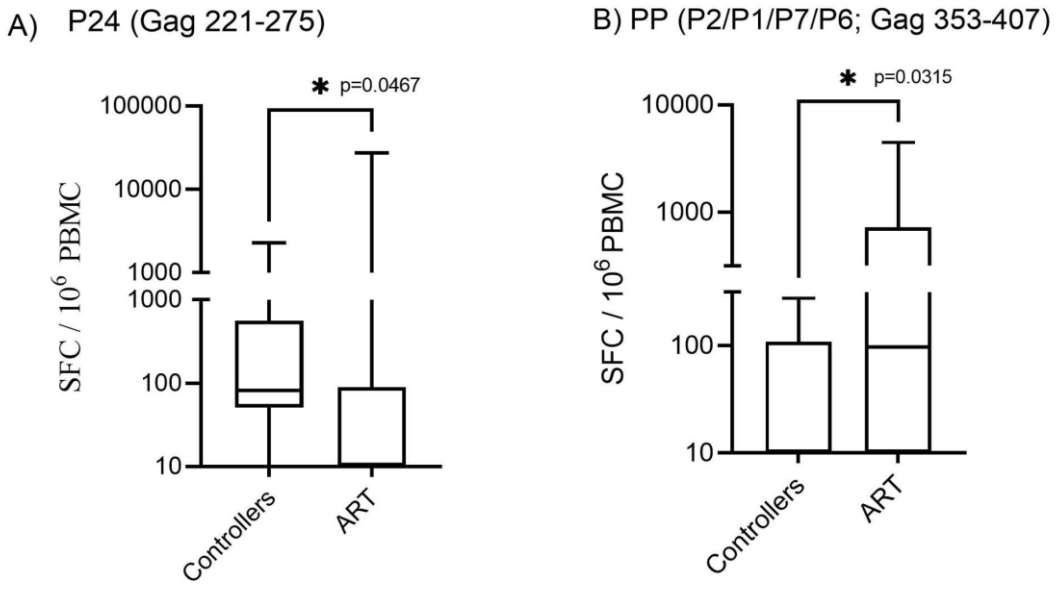

Figure 1. Immune response anti-Gag using Elispot IFN-γ. The production of IFN-γ by T cell stimulated with the pools of peptides of HIV-1 is shown. Box and whiskers show min and max value, line at median. The mean value of triplicate wells for each individual measured as spot forming units (SFC/10^6 PBMC). T-test and the nonparametric Mann-Whitney test were used to calculate p values, *p<0.05.

3.2. Patients with protective HLA alleles presented higher T-cell responses to HIV-1 Gag, Nef and RT pool of peptides

Controllers were grouped according to class 1 HLA typing (A, B and C locus) and separated into two groups: individuals who carry protective alleles against progression to AIDS and individuals who carry alleles susceptible to progression to the disease. We could find statistically significant responses for the Gag pools (P17 Gag 45-99) versus the C1 locus; P24 (Gag 221-275) versus C1 locus; P2/P1/P7/P6 (Gag 353-407) versus B1 locus, Nef (93-155) versus B1 locus; Nef (145-206) versus A1 locus and B1 locus; RT (213-283) versus B1 locus; RT (333-395) versus B2 locus; and RT (541-599) versus locus B2, respectively. See table 2.

Table 2. Anti-HIV immune response using Elispot IFN-γ versus HLA immunophenotyping in infection control subjects and elite controllers.

Peptides	HLA					
	Locus A1	Locus A2	Locus B1	Locus B2	Locus C1	Locus C2
P17-1 (Gag 1-55)	.515	.077	.623	.308	.620	.236
P17-2 (Gag 45-99)	.523	.374	.834	.104	**.032**	.568
P17-3 (Gag 89-143)	.880	.738	.304	.847	.242	.774
P24-1 (Gag 133-187)	.601	.602	.510	.398	.726	.855
P24-2 (Gag 177-231)	.231	.896	.305	.152	.841	.247
P24-3	.508	.512	.175	.462	**.021**	.459

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(Gag 221-275)						
P24-4 (Gag 265-319)	.494	.413	.273	.579	.181	.533
P24-5 (Gag 309-363)	.532	.434	.159	.906	.745	.375
P2/P1/P7/P6 1 (Gag 353-407)	.067	.454	**.009**	.221	.578	.127
P2/P1/P7/P6 2 (Gag 397-451)	.319	.572	.444	.202	.801	.536
P2/P1/P7/P6 3 (Gag 441-500)	.291	.586	.429	.549	.979	.257
Nef1 (Nef 1-55)	.844	.312	.145	.791	.537	.071
Nef2 (Nef 45-103)	.654	.507	.088	.415	.863	.110
Nef3 (Nef 93-155)	.093	.637	**.033**	.094	.080	.062
Nef4 (Nef 145-206)	**.026**	.686	**.002**	.070	.932	.053
RT1 (RT 153-223)	.070	.055	.095	.590	.380	.431
RT2 (RT 213-283)	.442	.880	**.012**	.063	.770	.712
RT3 (RT 273-343)	.449	.771	.065	.786	.761	.372
RT4 (RT 333-395)	.134	.249	.395	**.027**	.666	.323
RT5 (RT 385-455)	.224	.944	.299	.485	.851	.510
RT6 (RT 445-503)	.630	.537	.347	.810	.626	.280
RT7 (RT 493-551)	.714	.521	.127	.390	.567	.991
RT8 (RT 541-599)	.207	.805	.510	**.023**	.975	.919

*Kruskal-wallis test; p=0.05.

4. Discussion

In our study, we evaluated clinical, genetic, immunological and epidemiological data profiles controllers, compared with cART-treated individuals and healthy HIV-negative volunteers. By evaluating T cell counts, we identified higher numbers of $CD4^+$ T cell baseline and nadir, and $CD4^+/CD8^+$ T cell ratio in controllers compared to cART-treated individuals. These findings may indicate the ability of HIV-1 controllers to preserve $CD4^+$ T cell counts with low or undetectable viremia in the absence of ART. It is important highlighted that most controllers present more than twenty years of known HIV seropositivity.

In the present study, IFN-γ-Elispot assay was used to evaluate HIV-1-specific T cell responses. In this context, PBMCs obtained from our cohort of HIV-1^+ subjects mainly recognized a pool of Gag and Nef peptides. Several studies indicate that these pools are commonly recognized due to highly

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conserved epitope regions [46-48]. Furthermore, anti-Gag T cell responses have been inversely correlated with plasma viral load [46-50]. We did not find any significant association between VL or $CD4^+$ T count and breadth and magnitude of the total HIV-1 responses.

IFN-γ production and non-progression to Aids is controversial, for example, a previous study associated non-progression to Aids to Gag-specific $CD4^+$ T cell proliferation, but not T-cells IFN-γ production [51]. Emu *et al.* [52] reported no difference between controllers and non-controllers individuals in terms of IFN-γ–producing $CD4^+$ T cell response to Gag peptide pools. Likewise, other groups suggested that the impact of T cell responses on the control of viral replication cannot be explained only by quantification of the magnitude and breadth of the T cell response [47-48,53-55]. Additionally, secreting IFN-γ is not the only feature of T cells, as they are also involved in proliferative ability; production of other cytokines (e.g., IL-2 and TNF-α), and chemokines (e.g., MIP-1β); and antiviral cytotoxic activity [51,56-57]. In this context, the simultaneous production of two or more immune mediators has been associated with a better HIV control replication [10].

The suppression of HIV without cART depends on viral fitness differences, due to gene mutations or deletions and host genetic factors [20,58]. In this context, some HLA class I alleles (e.g. HLA-B*57, HLA-B*27, HLA-B*14 and HLA-B*51) have been associated with protection against disease [17-18,20,29-30,63-65]. In our work, when patients were grouped according to the expression of protective HLA alleles, specifically, HLA-B*57 and/or HLA-B*27, the magnitude of IFN-γ T-cell responses against the pool of peptides Gag, Nef and RT was higher than in individuals presenting HLA alleles not related to HIV replication. However, only two in six controllers individuals from our cohort present HLA-$B*57^+$. In fact, it has been shown that only a few HLA-$B*57^+$ or HLA-$B*27^+$ patients become controllers, although their plasma viral load is lower than that of individuals who do not have protective HLA alleles [61-64].

HLA genes are the strongest genetic factors that can affect immune response and HIV disease progression [28-29,59-60,64]. The selective pressure on HIV-1 and the disease outcome is strongly associated with HLA-B protective alleles [65]. PLWH that carry HLA-B protective alleles presented higher IFN-γ production against viral antigens [29,59-61,65], as shown in our results considering controllers and normal progressors under ART. Other groups have reported different cell-intrinsic mechanisms with the potential to block HIV-1 replication cycles. The cyclin-dependent kinase (CDK) inhibitor p21 is upregulated in PBMC from controllers when compared to both cART–suppressed and uninfected individuals. It is possible to infer this protein reduces the efficacy of HIV-1 reverse transcription [66-67]. Moreover, single nucleotide polymorphisms (SNPs) in the RICH2 and CXCR6 genes have been associated with natural control in different populations [68-70]. On the other hand, less-expressed HLA-C variants are associated with poor HIV-1 control and unstable HLA-C variants are associated with higher HIV-1 infectivity. The results showed a significant correlation between rapid progression to AIDS and the presence of two or one unstable HLA-C variants. Thus, unstable HLA-C variants both at genotype and at allele levels and rapid progression to Aids [71].

Genes differentially expressed by controllers, but not by cART-treated individuals, may be involved in the host's intracellular defenses. Luque *et al.* [72] observed upregulation of genes associated with cell survival in controllers, while genes related to induction of apoptosis were found to be upregulated in patients undergoing cART treatment. In fact, anti-apoptotic profiles of controllers had already been suggested by Bottarel *et al.* [73].

In conclusion, controller individuals present normal physiologic $CD4^+$ T-cell counts and low or undetectable viral load, even when almost all of them have already more than twenty years of known seropositivity. Furthermore, only seven of 19 controllers display at least one of a classical protective HLA class I allele (B*27/B*57) and none of them presented the CCR5-Δ32 deletion in homozygous alleles. Our results confirm the importance of controllers cohort's studies. Based on them, different parameters involved in non-progression to Aids were found, and the study of controllers individuals still constitutes an important field of discoveries, being considered a model to vaccine and/or to functional cure [20-21,74].

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