Spontaneous HIV Controllers Exhibit Preserved Immune Parameters in Peripheral Blood and Gastrointestinal Mucosa

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Background: HIV infection induces several gradual alterations on the peripheral and mucosal immune systems, with different magnitudes between infected individuals. In this regard, spontaneous HIV controllers exhibit either low or undetectable viral loads in the absence of treatment along with decreased immune alterations compared to HIV progressors. Yet, it is unknown how similar immune peripheral and mucosal parameters are when comparing HIV controllers to uninfected individuals.

Methods: We evaluated a cohort of 11 HIV controllers who were compared to 20 seronegative donors. Peripheral blood (PB) and gut associated lymphoid tissue (GALT) samples were obtained to analyze the following: 1) the frequency and phenotype of immune cells by flow cytometry; 2) the expression of apoptotic molecules by immunohistochemistry; 3) the expression of transcriptional factors associated with T cell profiles by real time PCR; and 4) the serum level of microbial translocation by an enzymatic reaction.

Results: We found that HIV controllers have a conserved frequency of most immune cell populations in PB and GALT, but a reduced percentage of $CD4^+$ T cells. The immune activation levels were similar in both groups of individuals, as well as the expression of cleaved caspase-3, transcriptional factors, and the level of microbial translocation. Interestingly, the frequency of $CD8^+$ T cells expressing HLA-DR but not CD38, previously associated with high effector functions, were preserved in HIV controllers.

Conclusions: Our results suggest that despite the infection, HIV controllers have preserved immune parameters, which can be associated with the spontaneous control of viral replication.

Key Words: HIV controllers, preserved immune parameters, gastrointestinal mucosa

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INTRODUCTION

HIV infection remains one of the main public health problems in the world, for which a curative therapy or a prophylactic vaccine is not yet available. By 2013, around 35.3 million people had been infected with HIV throughout the world, with an annual incidence of 2.3 million infections.[1]

The natural history of HIV infection is heterogeneous, considering the variability in the AIDS-free period exhibited by infected individuals.[2] The existence of individuals who exhibit natural resistance to HIV has been crucial in the investigation of novel therapeutic strategies and advances in vaccine development.[3] To facilitate the characterization of resistance mechanisms to AIDS progression, a new phenotype of seropositive individuals who exhibit a spontaneous and sustained control of viral replication maintaining viral loads below 2000 copies per milliliter at least for 1 year, in the absence of antiretroviral therapy, has been described.[4]

It is known that progression of HIV infection occurs when the presence of viral immunopathogenic factors overcomes the protective immune mechanisms.[5] In general, during HIV infection, a decreased frequency and function of almost all immune cells are observed, altering the immune response against HIV and against different opportunistic pathogens.[6] It is currently accepted that one of the most important pathogenic mechanisms during this infection is immune hyperactivation, which affects both innate and adaptive immune cells.[7] This abnormal activation is induced initially by the exposure to viral antigens and then exacerbated by the destruction of mucosa-associated lymphoid tissue, leading to the loss of mucosal integrity and microbial translocation from the intestinal lumen to systemic circulation.[8] As a result, there are high levels of lipopolysaccharides (LPS) in the plasma, an increased number of activated cells, anergy and apoptosis, mainly of $CD4^+$ T cells, and in general functional alterations of the immune system.[9] One of these alterations includes changes in T-cell profiles, as evidenced

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by decreased frequency and function of Th17 cells and increased frequency of regulatory T cells, that are associated with the progression to AIDS.[10–12]

Because cell-mediated immune response constitutes the most important antiviral mechanism, the study of T cells in HIV controllers has been addressed in several investigations in which a potent function of these cells associated with viral control has been reported.[13–15] In addition, increased frequency of an activation phenotype in $CD8^+$ T cells expressing HLA-DR but not CD38 was described in HIV controllers. This subset was associated with a higher function and potent capacity for viral suppression compared with those cells co-expressing both molecules ($HLA\text{-}DR^+$ and $CD38^+$).[16]

Since their characterization in 2008, most of the studies on HIV controllers have been carried out in peripheral blood (PB), despite the fact that the gut-associated lymphoid tissue (GALT) is the most important site of viral replication, offering the opportunity to reveal key mechanisms associated with viral control.[17–19] In addition, there is insufficient information on how preserved are the immune components of HIV controllers compared with seronegative individuals. Here, in gut mucosa and PB, we compared the frequency and phenotype of immune cells and the percentage of apoptotic cells between these 2 cohorts.

MATERIALS AND METHODS

Study Population

HIV controllers were recruited from health insurance programs in Medellín, Colombia. A total of 11 HIV controllers were included, who exhibited viral loads lower than 2000 copies per milliliter for at least 1 year despite the absence of antiretroviral therapy (viral load median: 286.0 copies per milliliter; range min–max: 20.0–1885 copies per milliliter). The group of seronegative donors consisted of 20 individuals who tested seronegative for HIV and had similar ethnic backgrounds to HIV controllers.

The individuals enrolled in this study signed an informed consent approved by the Bioethical Board from Universidad de Antioquia, prepared according to the Colombian Legislation, Resolution 008430 of 1993. This investigation was approved by the Ethical Committee of the Universidad de Antioquia (certificate of approval 10-8-298).

Viral Load

The plasma viral load was determined using the commercial assay reverse transcription-polymerase chain reaction Ampliprep-Cobas Amplicor (Roche, Indianapolis, IN), after the manufacturer's protocol; this test has a detection limit of 20 copies per milliliter.

Isolation of Cells From Gut Mucosa

Rectosigmoidoscopy in the rectum at 10 cm from anal verge was performed to obtain mucosal tissue using a flexible sigmoidoscope with single endoscopy biopsy forceps FB-24K-1 (Olympus America Corp., Melville, NY). Fifteen tissue samples were obtained from each patient. Samples were processed using 0.5 mg/mL collagenase type II from *Clostridium histolyticum* (Sigma-Aldrich, San Luis, MO) diluted in RPMI 1640% and 7.5% Fetal bovine serum (FBS) plus 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL, Grand Island, NY) for 30 minutes at 37°C with shaking. After collagenase digestion, biopsy fragments were further disrupted by repeated passage through a 30-mL syringe with a blunt-end 16 gauge needle (Stem Cell Technologies, Vancouver, BC, Canada). Rectal cells (RCs) were isolated from the fragments by passage through a 70 mM nylon strainer (Falcon, Lincoln Park, NJ). RCs were washed with Dulbeco phosphate-buffered saline (PBS) (Sigma-Aldrich, San Luis, MO) to remove excess histopaque and collagenase. Subjects with nodular lymphoid hyperplasia, ulcers, diverticulum, adenoma, and other benign or malign neoplasias were excluded from the study.

Antibodies

The following fluorochrome-labeled mouse monoclonal antibodies were from Becton Dickinson (BD, San Jose, CA): CD86, lineage marker (Lin-1, a mixture of anti-CD3, CD14, CD16, CD19, CD20, and CD56), CD11c, CD123, and the CDR3 region of the invariant TCR Valpha24 chain (clone 6B11); from eBioscience (San Diego, CA): anti-CD3, CD4, CD8, CD16, CD56, HLA-DR, CD38, CD69, FoxP3, and CD127; and CD45 from Beckman Coulter (Fullerton, CA).

Flow Cytometry

The frequency and phenotype of the different subpopulations in PB and RCs were determined by flow cytometry. Briefly, 150 μL of blood were incubated with specific monoclonal antibodies for 25 minutes at RT in the dark. Erythrocytes were lysed by incubating for 10 minutes with 2 mL of 1× fluorescence-activated cell sorting lysing solution (BD); the cells were washed twice with 2 mL of cold PBS at 250*g* for 5 minutes and fixed with 250 μL of 2% paraformaldehyde. In addition, RCs (5×10^5) were treated with 20 μg/mL of human immunoglobulin G to block Fc receptors for 15 minutes and then surface stained for natural killer (NK) and T cells using the same protocol and reagents as those used for PBs.

The gate of lymphocytes was identified by side light scatter (SSC) vs. forward light scatter (FSC) to analyze the following cell populations: NK cells ($CD3^-/CD16^+/CD56^{dim}$, $CD3^-/CD16^-/CD56^{dim}$, $CD3^-/CD16^-/CD56^{bright}$, and $CD3^-/CD16^+/CD56^-$), invariant NKT (iNKT, TCR $Valpha24^+/CD3^+$), and T cells ($CD3^+/CD4^+$ or $CD3^+/CD8^+$). Because the identification of the NK cell subpopulations in GALT was not possible, these cells are reported as total NK cells ($CD45^+/CD3^-/CD16^+/CD56^+$). In the gate of mononuclear cells, we identified dendritic cells (DC), gated as $Lin^-/HLA\text{-}DR^+$ and then as $CD11c^+$ myeloid DC (mDC) or $CD123^+$ plasmacytoid DC (pDC).

We also evaluated the expression of the following activation molecules, previously associated with the hyperactivation phenomenon: CD69 (NK cells) and CD86 (pDC and mDC). The expression of HLA-DR and CD38 was used to identify different activation phenotypes of T cells,

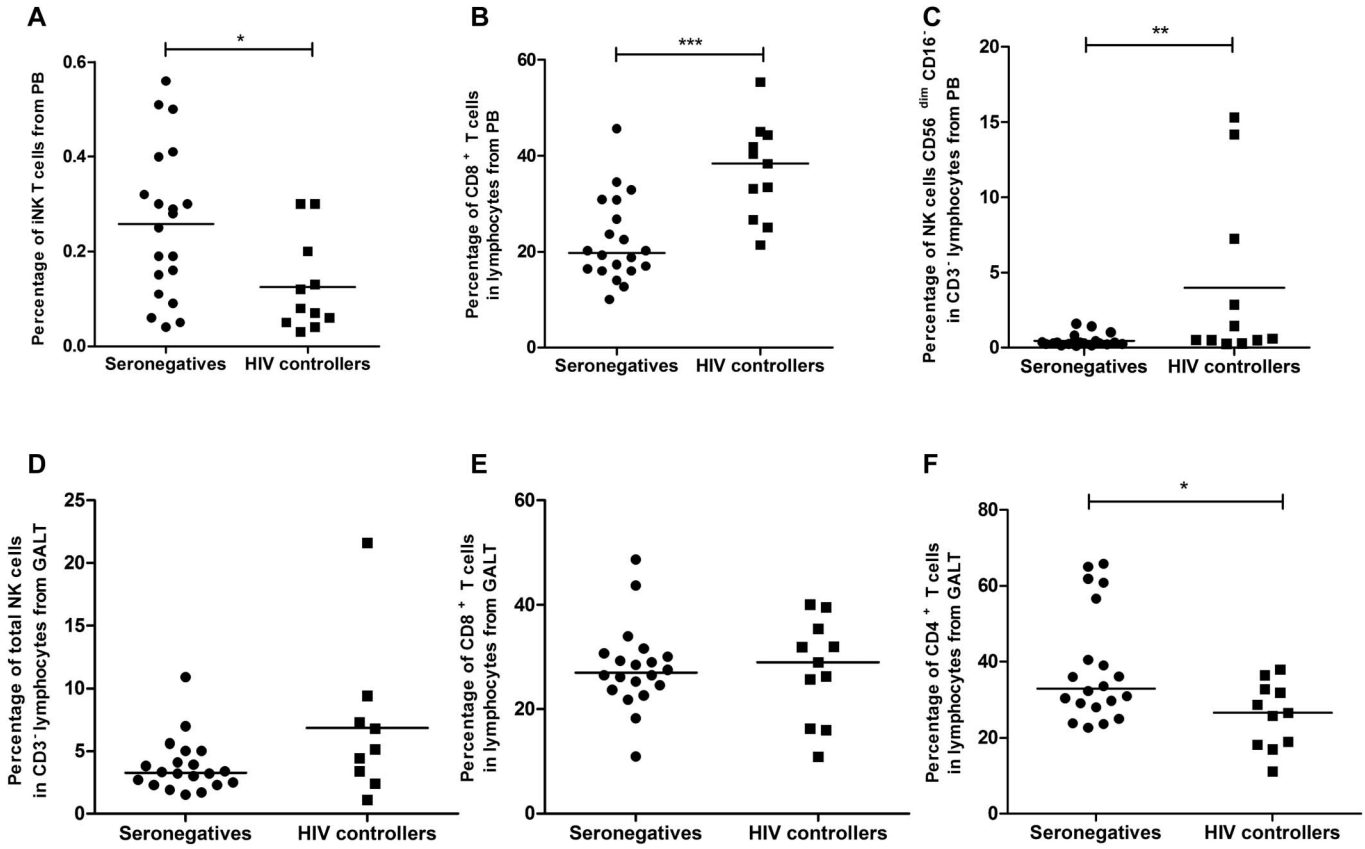


FIGURE 1. Frequency of PB immune cells in HIV controllers and seronegative donors. Frequency of cell populations in PB and GALT from 11 HIV controllers and 20 seronegative donors. In PB: (A) iNKT cells, (B) $CD8^+$ T cells, and (C) NK cell subpopulation $CD56^{dim}$ $CD16^-$; in GALT: (D) total NK cells, (E) $CD8^+$ T cells, and (F) $CD4^+$ T cells. The results are presented as median and interquartile range. The comparisons between the groups were performed with the Mann–Whitney *U* test, 2-tailed test, considering a *P* value lower than 0.05 as significant. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.)

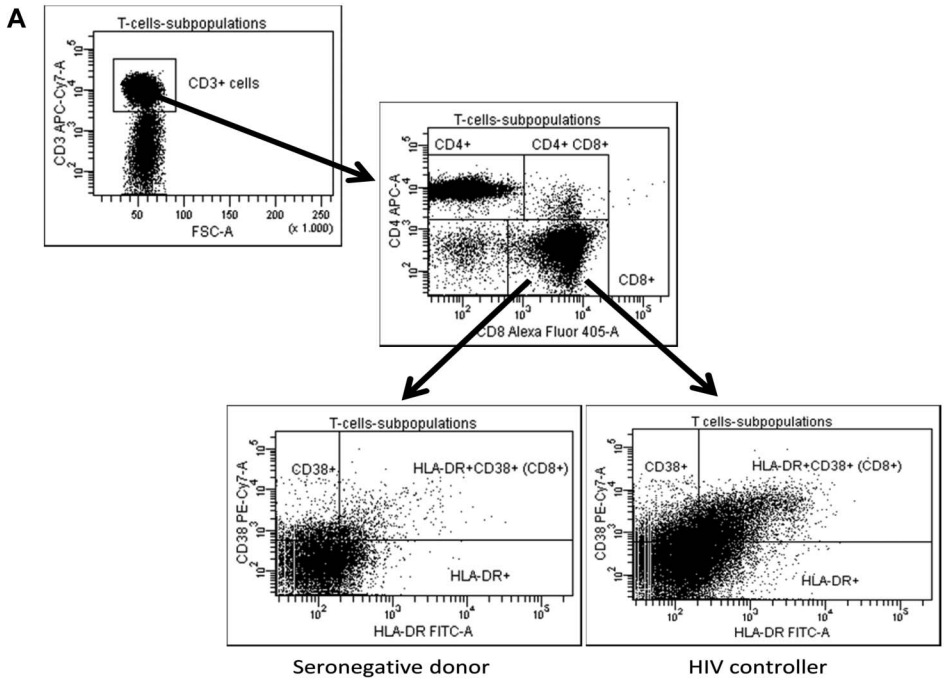
including cells co-expressing these 2 molecules or cells expressing 1 single molecule.

The frequency of Tregs (defined in the gate of lymphocytes as $CD3^+/CD4^+/FoxP3^+/CD127^{low/-}$) was determined by intracellular flow cytometry. After staining with extracellular markers, the cells were permeabilized and fixed using the antihuman FoxP3 staining set (eBioscience), after the manufacturer's instructions. Seven microliters of anti-FoxP3 were then added, and the cells were incubated for 25 minutes at 4°C, washed twice with 2 mL of PBS by centrifuging at 250 × g for 5 minutes, and fixed with 250 μL of 2% paraformaldehyde.

All preparations were stored at 4°C until acquisition in the cytometer FACS CANTO-II (BD). Acquisition analyses were performed in the BD FACSDiva 6.1.2 version. The analysis of activation molecules was made using the fluorescence minus one method. Dead cells were excluded from the analyses through SSC vs. FSC.

Histology and Immunohistochemistry

Four biopsy fragments from GALT were placed in 10% buffered formalin and paraffin embedded. The tissues were segmented using a Microtome (Leica, Nussloch, Germany), obtaining fragments of 3–4 μm thickness; they were placed on charged slides, deparaffinized with Xilol, and hydrated with alcohol. The slides were stained for hematoxylin and eosin, and histochemistry stains included methenamine silver for fungi, Ziehl Neelsen for acid-alcohol–resistant bacilli, and modified Ziehl Neelsen for mycobacteria. For immunohistochemistry, we used the Quanto Detection System HRP (Ultra Vision; Thermo Scientific, Waltham, MA); the slides were subjected to an antigen retrieval step in EDTA buffer, then blocked with endogenous peroxidase *Ultra V Block* (Thermo Scientific), and then stained for 1 hour with biotin-labeled primary antibodies, including polyclonal anti-Cleaved Caspase-3 (Cell Signaling Technology, Inc., Danvers, MA), anti-CD3 (Thermo Scientific), anti-CD4 (Dako, Inc., Carpenteria, CA), and anti-CD8 (Dako, Inc.); the signaling was amplified using the *Primary Antibody Amplifier Quanto* (Thermo Scientific) for 10 minutes. The HRP Polymer Quanto was added for 10 minutes as a secondary antibody. Finally, the DAB: 3,3′-Diaminobenzidine chromogen was added for 5 minutes. The slides were mounted on *Consul Mount* (Shandon) and observed in a Labophot-2 microscope (Nikon, Tokyo, Japan). Three high-power (×40) microscope fields



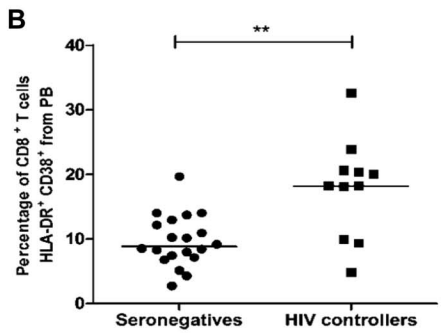


FIGURE 2. Expression of activation markers on T cells. A, Strategy selection of activated $CD8^+$ T cells in PB, (B) frequency of $CD8^+$ T cells expressing HLA-DR/CD38 in PB from 11 HIV controllers and 20 seronegative donors. The results are presented as median and interquartile range. The comparison between the groups was performed with the Mann–Whitney *U* test, 2-tailed test. A *P* value lower than 0.05 was considered significantly different. (**$P < 0.01$.)

with representative cells expressing CD3, CD4, or CD8 were selected, each field including an area of 0.1964 mm^2. Positive cells for each marker were counted manually by a single observer (L.A.C.). The numbers of positive cells are presented as cells per high-power field.

Plasma LPS Levels

Plasma samples were inactivated by heating at 70°C for 10 minutes. Duplicates of 50-μL samples and standard endotoxin solutions were added in 96-well flat-bottom plates (Costar-Corning, Lowell, MA) and then incubated with Limulus Amebocyte Lysate for 10 minutes and substrate solution for 6 minutes at 37°C. The reaction was stopped with acetic acid (25% vol/vol glacial acetic acid in water). The assay was performed according to the manufacturer's instructions by the endpoint chromogenic Limulus Amebocyte Lysate assay QCL-1000 (Lonza, Inc., Allendale, NJ) (sensitivity range: 0.1–1.0 EU/mL). The background attributable to the turbidity of the diluted plasma was subtracted.

Statistical Analyses

The results are presented as median and range. To compare data from HIV controllers with seronegative donors, a nonparametric test (Mann–Whitney *U* test, 2-tailed test) was performed. A *P* value <0.05 was considered statistically significant. The statistical tests were performed using the Graph-Pad Software version 5.03.

RESULTS

Similar Frequency of Most Immune Cell Subpopulations

The frequency of innate and adaptive cells was evaluated in PB and GALT from HIV controllers and compared with seronegative individuals (see Table, Supplemental Digital Content 1, http://links.lww.com/QAI/A705). In PB, we observed a similar frequency of pDC, mDC, and $CD4^+$ T cells and subpopulations of $CD4^+CD8^+$ T cells and Tregs. However, significant differences were found in iNKT

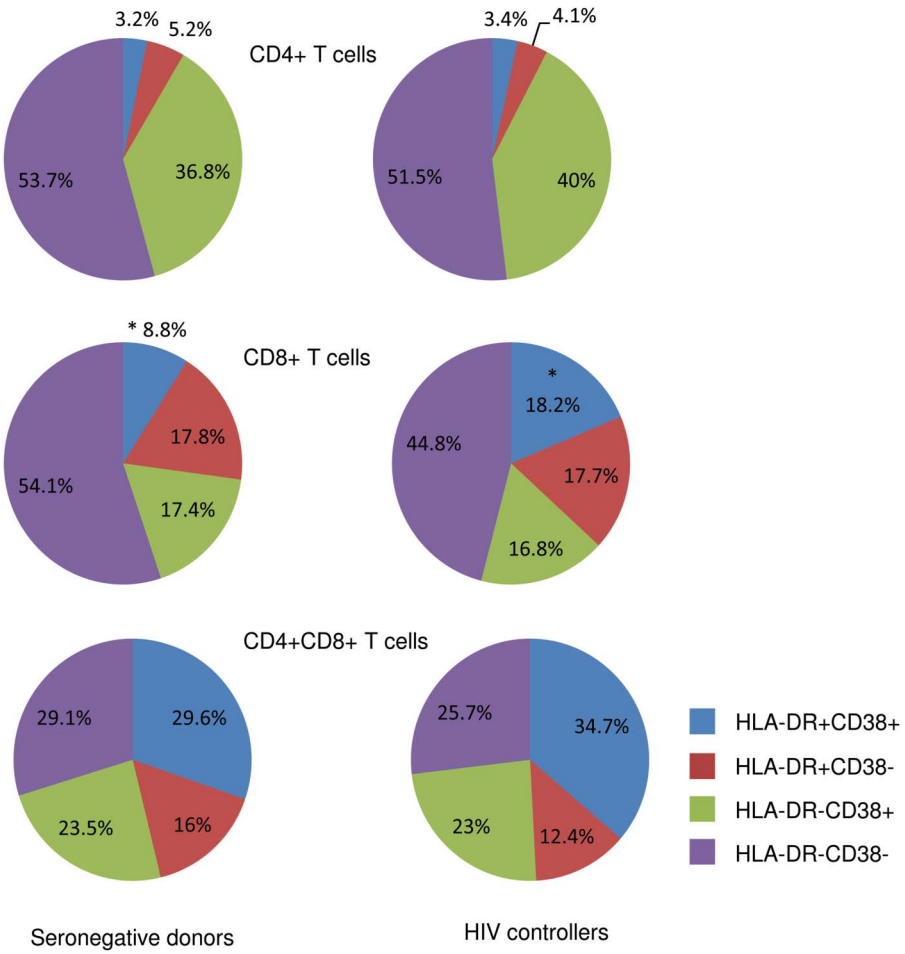


FIGURE 3. Profile of expression of HLA-DR/CD38 on CD8$^+$ T cells. Subpopulations of T cells according to the expression of activation markers (HLA-DR and CD38). The comparison between the groups was performed with the Kruskal–Wallis test and 2-tailed test. A *P* value lower than 0.05 was considered significantly different (*).

cells and CD8$^+$ T cells (Figs. 1A, B, respectively). When the subpopulations of NK cells were evaluated, HIV controllers and seronegative individuals had a similar frequency of CD56^{dim} CD16$^+$, CD56^{bright}, and CD56$^-$ NK cells. Interestingly, higher frequency of CD56^{dim} CD16$^-$ was observed in HIV controllers (Fig. 1C).

In GALT, we evaluated the frequency of total NK cells and T cells. By flow cytometry, comparable levels of NK cells and CD8$^+$ T cells, but lower frequency of CD4$^+$ T cells, were observed (Figs. 1D–F, respectively). In contrast, by immunohistochemistry, similar counts of CD4$^+$ and CD8$^+$ T cells were found (see Table, Supplemental Digital Content 1, http://links.lww.com/QAI/A705).

Similar Expression of Activation Molecules and Microbial Translocation

The expression of molecules previously associated with the hyperactivation phenomenon was evaluated in immune cells from PB and GALT (see Table, Supplemental Digital Content 2, http://links.lww.com/QAI/A705). In PBs, similar expressions of CD86 on pDC and mDC, and CD69 on NK cells were observed.

Regarding T-cell activation, we found similar levels of co-expressing HLA-DR/CD38 CD4$^+$ T cells and double-positive CD4$^+$CD8$^+$ T cells. However, CD8$^+$ from HIV controllers exhibited a higher frequency of HLA-DR$^+$ CD38$^+$ cells (Figs. 2A, B). In addition, T-cell subpopulations characterized according to their activation phenotype (cells expressing HLA-DR or CD38 or negative for both) were comparable in both groups of individuals, except for the CD8$^+$ T double-positive cells (Fig. 3).

The expression of CD69 on NK cells and co-expression of HLA-DR and CD38 on CD4$^+$ and CD8$^+$ T cells in GALT were also similar in both groups of individuals (see Table, Supplemental Digital Content 2, http://links.lww.com/QAI/A705). In this tissue, we also observed similarities in T-cell subpopulations based on the expression of HLA-DR or CD38 (data not shown).

Finally, plasma LPS levels were measured as markers of microbial translocation. We observed that HIV controllers and seronegatives exhibited similar levels of this microbial product (97.61, 44.77–177.8; vs. 90.83, 56.32–218; $P = 0.9178$).

Similar Levels of Apoptosis in GALT

The expression of cleaved caspase-3 by immunohistochemistry was used to evaluate apoptosis in GALT. HIV controllers had physiological levels of apoptotic cells in this tissue when compared with seronegative donors (1.7, 0–2.3 cells by slide; vs. 1.8, 0–6; $P = 0.4603$; Figs. 4A–C).

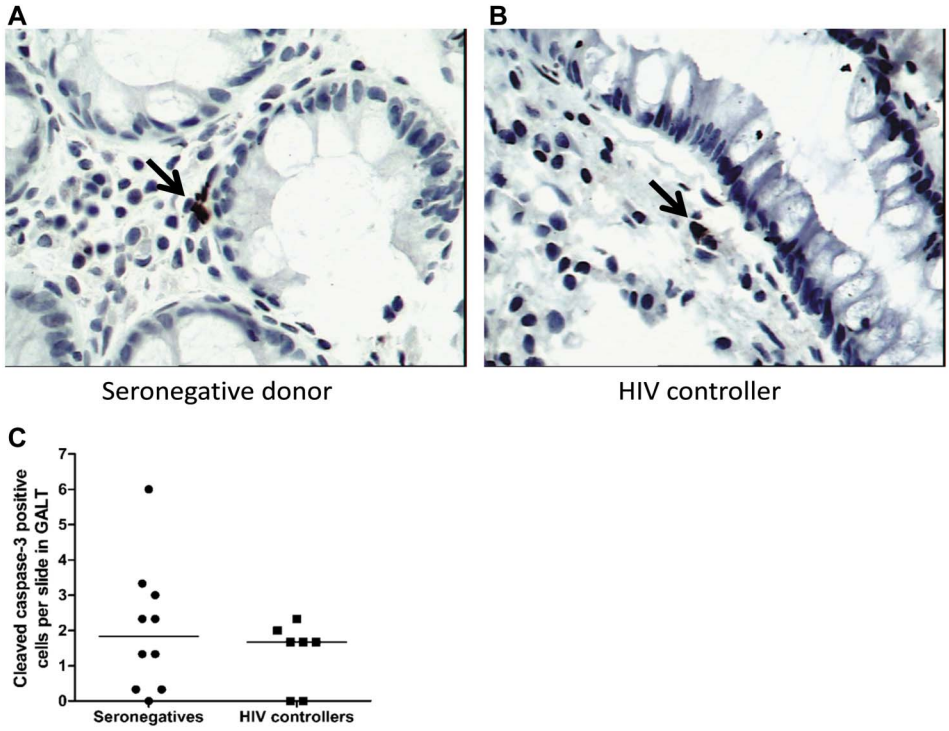


FIGURE 4. Expression of cleaved caspase-3 in intestinal tissue. Expression of cleaved caspase-3 in GALT from 7 HIV controllers and 10 seronegative donors by immunohistochemistry. A and B, Representative graphs showing the cleaved caspase-3–positive cells in GALT (×40) from a seronegative donor and an HIV controller. C, Quantitative determination of cleaved caspase-3–positive cells in GALT. Data are presented as cleaved caspase-3–positive cells per slide. The comparison between the groups was performed with a Mann–Whitney *U* test, 2-tailed test. A *P* value lower than 0.05 was considered significantly different.

DISCUSSION

The study of the mechanisms involved in the spontaneous control of viral replication is crucial in the development of new therapeutic strategies against HIV. Previously, we demonstrated that HIV controllers exhibit a higher level of immune cells, lower activation, and better cytotoxic immune responses when compared with HIV progressors and even with patients with highly active antiretroviral therapy–suppressed viral replication.[20] However, there are limited studies on the preservation of immune components in PB and GALT of HIV controllers. Here, we evaluated how similar were peripheral and mucosal parameters in HIV controllers and seronegative individuals in terms of the frequency of immune cells, expression of activation molecules on immune cells, apoptosis, and microbial translocation. HIV controllers are similar to seronegative individuals in most of the parameters evaluated in both PB and GALT, suggesting that viral control is associated with the preservation of immune components. However, there was a reduced level of iNKT cells in PBs, as previously reported.[20] These cells are affected in early stages of infection and are not reconstituted after the viral set point is established.[21] Regarding NK cells, we observed that cytotoxic ($CD56^{dim}$ $CD16^{+}$ and $CD56^{-}CD16^{+/-}$) and cytokine producer ($CD56^{bright}$) NK cells are similar in HIV controllers and seronegative controls. Interestingly, HIV controllers present an increased frequency of NK cells $CD56^{dim}$ $CD16^{-}$; this was previously associated with resistance to HIV and a high capacity to produce cytokines.[22–24] However, more recent studies have also reported a high cytotoxic capacity of this cell subpopulation, suggesting that a combination of these effector functions could be the key factor in the control of viral replication.[25]

We also observed that the frequency of $CD4^{+}$ T cells in GALT was lower in controllers. This finding agrees with previous investigations indicating that GALT is the most affected lymphoid organ during the infection, as more than 80% of target memory T cells expressing CCR5 reside in this tissue.[26–28] Hence, our results suggest that although the set point was established at low levels in HIV controllers, most likely during the acute phase of infection, the level of replication and the ensuing mucosal damage were considerable, but less extensive as indicated by the similar levels of plasma LPS between controllers and uninfected individuals. Because LPS is a marker of microbial translocation,[9] this result suggests that the integrity of gut mucosa in HIV controllers was conserved, preventing translocation of microbial products, therefore maintaining physiological levels of immune activation as observed in different cell subpopulations in this and previous studies.[20] In contrast, the activation level of $CD8^{+}$ T cells was higher in HIV controllers. Interestingly, previous reports suggest that these activated cells did not exhibit dysfunctional activity, as they conserve a strong and polyfunctional HIV-specific response. We also evaluated the different phenotypes of activation according to the expression of HLA-DR or CD38, considering the new phenotype of $CD8^{+}$ T cells expressing exclusively HLA-DR observed in HIV controllers; these cells exhibit higher cytotoxic ability and viral suppressive capacity compared with cells expressing both activation molecules. Interestingly, cells expressing only HLA-DR were observed in cultures stimulated with low concentrations of HIV peptides, whereas

high levels of peptides induced expression of both activation molecules.[29] The maintenance of low viral loads after the establishment of the set point in HIV controllers may contribute to conserve this activation phenotype. Indeed, comparable frequencies of this subpopulation in HIV controllers and uninfected individuals were noted.

In addition, the caspase-3–mediated apoptosis was also comparable between controllers and seronegative donors; however, we have previously observed similar results in HIV progressors (unpublished data), suggesting that cell death induced by apoptosis is not responsible for the massive cell depletion observed during HIV infection. In fact, it was recently shown that caspase-3–mediated apoptosis was only responsible for 5% of $CD4^+$ T-cell deaths, corresponding to those activated and productively infected, whereas the remaining 95% of cells died by caspase-1–mediated pyroptosis, triggered by abortive viral infection.[30]

In summary, HIV infection induces considerable damage of GALT leading to a decrease in $CD4^+$ T cells in HIV controllers; however, the antiviral response mediated by NK cells and $CD8^+$ T cells allows viral replication to be controlled, limiting the damage of this tissue. Consequently, there is a balanced response profile of T cells and a preservation of the physiological state of activation of $CD4^+$ T cells, preventing an increase in viral target cells and apoptosis. The low viral load can also contribute to the preservation and/or induction of a highly functional activation profile of $CD8^+$ T cells characterized by the expression of HLA-DR but not of CD38. All these findings can partially contribute to maintain the status of HIV controllers; however, other factors can also play a role on this control and should be considered.

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