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ORIGINAL ARTICLE

Particular activation phenotype of T cells expressing HLA-DR but not CD38 in GALT from HIV-controllers is associated with immune regulation and delayed progression to AIDS

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Abstract The spontaneous control of HIV replication in HIV-controllers underlines the importance of these subjects for exploring factors related to delayed progression. Several studies have revealed fewer immune alterations and effector mechanisms related to viral control, mainly in peripheral blood, in these individuals compared to normal progressors. However, immune characterization of gut-associated lymphoid tissue (GALT), the major target of infection, has not been thoroughly explored in these subjects. We evaluated the following parameters in GALT samples from 11 HIV-controllers and 15 HIV-progressors: (i) frequency and activation phenotype of T cells; (ii) expression of transcription factors associated with immune response profiles; and (iii) frequency of apoptotic cells. Interestingly, HIV-controllers exhibited a particular activation phenotype, with predominance of T cells expressing HLA-DR but not CD38 in GALT. This phenotype, previously associated with better control of infection, was correlated with low viral load and higher $CD4^+$ T cell count. Furthermore, a positive correlation of this activation phenotype with higher expression of Foxp3 and RORγT transcription factors suggested a key role for Treg and Th17 cells in the control of the immune activation and in the maintenance of gut mucosal integrity. Although we evaluated apoptosis by measuring expression of cleaved caspase-3 in GALT, we did not find differences between HIV-controllers and HIV-progressors. Taken together, our findings suggest that predominance of HLA-DR^+ T cells, along with lower immune activation and higher expression of transcription factors required for the development of Treg and Th17 cells, is associated with better viral control and delayed progression to AIDS.

Keywords HIV-controllers · Gut mucosa · Activation phenotype of T cells · Immune regulation

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Introduction

During HIV infection, several alterations of the immune system have been described, affecting both the frequency and function of almost all immune cells with deleterious consequences on the ability to trigger specific immune responses [1].

It is now recognized that the gastrointestinal tissue is a major target for HIV, the massive loss of $CD4^+$ T cells, particularly of the Th17 phenotype [2], and the damage to mucosal epithelial integrity allows microbial translocation from the intestinal lumen to the systemic circulation [3, 4]. This process is believed to trigger a state of immune hyperactivation that is one of the most important

pathogenic mechanisms during HIV infection [5], since activated $CD4^+$ T cells are highly susceptible to viral infection and death. Immune hyperactivation also leads to alteration of T cell functional profiles, weakening the adaptive immune response to HIV [6]. In fact, during infection, an increase in regulatory T cells (Treg) along with a decrease in Th17 cells has been associated with progression to AIDS [7].

After initial infection, the rate of progression to AIDS is variable among infected individuals; interestingly, some patients exhibit delayed progression, suggesting the existence of natural resistance mechanisms. Among these individuals, HIV-controllers are of particular interest; these individuals exhibit sustained control of the viral replication (viral load <2000 copies/mL) in the absence of antiretroviral therapy, for at least 1 year of follow-up after HIV diagnosis [8].

Several genetic characteristics and immune effector mechanisms have been associated with this novel phenotype, mainly through the study of peripheral blood [9]. Potent HIV-specific T cell responses, and an unusual T cell activation phenotype, $HLA\text{-}DR^+$ and $CD38^-$, have been associated with a greater ability of $CD8^+$ T cells to suppress viral replication [10, 11]. Interestingly, this phenotype was induced in vitro by low concentrations of HIV, as usually observed in HIV-controllers [11]. Lower levels of immune activation which may result in fewer susceptible target cells and lower levels of apoptosis, have also been reported in HIV-controllers [12, 13]. However, the immune features of gut-associated lymphoid tissue (GALT) in these individuals have not been explored in detail. Given the potential of these studies to enhance our understanding of immune correlates of protection and delayed progression, we evaluated the following parameters in GALT samples from HIV-controllers and HIV-progressors: (i) frequency and activation phenotype of T cells; (ii) expression of transcription factors associated with immune response profiles; and (iii) frequency of apoptotic cells.

Materials and methods

Study population

Eleven HIV-controllers, defined as previously described [8], were included. They were classified as individuals with a documented HIV infection for at least 1 year and plasma viral loads lower than 2.000 copies/mL during the last 12 months. In addition, 15 progressor individuals, with circulating $CD4^+$ T cell counts between 350 and 550 cells/μL and plasma viral load between 10.000 and 100.000 copies/mL, were also included. Peripheral blood (PB) was obtained by sterile venipuncture, and gut biopsies were obtained by flexible sigmoidoscopy from each patient. All the enrolled individuals were antiretroviral therapy naïve and were recruited from health insurance programs in Medellín, Colombia. They signed a written informed consent approved by a Bioethical Board.

Viral load in plasma

Plasma viral load was determined using the commercial assay RT-PCR Ampliprep-Cobas Amplicor (Roche, Indianapolis, IN; detection limit of 20 copies/mL), following the manufacturer's protocol.

Rectal biopsy from gut mucosa

Rectosigmoidoscopy and biopsy were performed as previously described [14], using a flexible sigmoidoscope with single-use biopsy forceps FB-24K-1 (Olympus America Corp, Melville, NY, USA); from each subject, eight tissue samples were obtained from the rectum at 10 cm from anal verge.

Rectal cell isolation from biopsies

The procedure to obtain and digest gut biopsy samples was previously described [14, 15]. Briefly, tissue was digested with collagenase type II from *Clostridium histolyticum* (Sigma; 0.5 mg/mL), diluted in RPMI 1640 and 7.5 % fetal calf serum (FCS) plus 100 UI/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL; Grand Island, NY, USA) and shaken for 30 min at 37 °C. The resulting fragments were disrupted by repeated passage through a 30-mL syringe with a blunt end 16-gauge needle (Stem Cell Technologies, Vancouver, BC). A homogeneous cells suspension was obtained from the fragments by passage through a 70-μM nylon strainer (Falcon, Lincoln Park, NJ, USA). Subjects with nodular lymphoid hyperplasia, ulcers, diverticulitis, adenoma and other benign or malignant growths were excluded from the study.

Antibodies

For flow cytometry, we used the following fluorochrome-labeled mouse anti-human monoclonal antibodies: anti-CD4-APC (clone: RPA-T4) from Becton–Dickinson (BD, San Jose/CA, USA), and CD3-APC-Cy7 (clone: UCHT1), CD8-eFluor® 450 (clone: RPA-T8), HLA-DR-FITC (clone: LN3) and CD38-PECy7 (clone: HIT2) from eBioscience (San Diego, CA, USA). For immunohistochemistry, we used the following biotin-labeled primary specific anti-human monoclonal antibodies: anti-CD3 (Thermo Scientific), CD4 (Dako, Inc., Carpinteria, CA, USA) and CD8 (Dako, Inc., Carpinteria, CA, USA) and a polyclonal

antibody directed toward cleaved caspase-3 from cell signaling technology (Danvers, MA, USA).

Flow cytometry

The frequency and activation phenotype of T cells from PB and GALT were determined by flow cytometry. For PB, 150 μL of sample was incubated with specific antibodies for CD3, CD4, CD8, HLA-DR and CD38 for 25 min at 25 °C in the dark. Erythrocytes were lysed with 1× fluorescence-activated cell sorting lysing solution from Becton–Dickinson (BD, San Jose/CA, USA) incubating for 10 min. Cells were washed twice with PBS at 250×*g* for 5 min and then fixed with 250 μL of 2 % paraformaldehyde. For rectal cells, the same protocol and reagents were used, except for a prior blockade of FcR with 20 μg/mL of human IgG for 15 min.

The data acquisition was performed immediately in the cytometer, which was a FACS CANTO-II (BD) equipped with BD FACSDiva software, version 6.1.2. The analysis of the expression of activation molecules was made by comparing data to isotype controls.

The lymphocyte gate was selected by side (SSC) versus forward (FSC) light scatter. The $CD4^+$ or $CD8^+$ T cells were gated, and the expression of activation molecules HLA-DR and CD38 were evaluated in both subsets. Based on the differential expression of these markers, we identified four T cell activation phenotypes: cells co-expressing HLA-DR and CD38, cells expressing only HLA-DR or CD38 and cells null for these two molecules.

Histology and immunohistochemistry

Four of the biopsy fragments were paraffin-embedded and segmented with a microtome (Leica); the resultant fragments (3–4 μm of thickness) were placed in charged slides, deparaffinized and hydrated, and the immunohistochemistry was performed with the Quanto Detection System HRP (Thermo Scientific, Ultra Vision). The antigen retrieval and blockade with endogenous peroxidase *Ultra V Block* (Thermo Scientific) were performed, and the slides were stained with biotin-labeled primary monoclonal antibodies anti-CD3, anti-CD4 and anti-CD8 and the polyclonal antibody directed toward cleaved caspase-3 for apoptotic cells. The signaling was amplified with the *Primary Antibody Amplifier Quanto* (Thermo Scientific, CA) during 10 min. Then, the secondary antibody (the HRP Polymer Quanto) was added for 10 min, the DAB chromogen was added for 5 min, and the slides were mounted on *Consul Mount* (Shandom). For CD3, CD4 and CD8 expression, we selected three representative high-power (40×) microscope fields for each marker (field area: 0.1964 mm^2), for cleaved caspase-3, we evaluated the entire slide. The slides were observed in a Labophot-2 microscope (Nikon), and the positive cells for each marker were counted manually by a single pathologist. Stains with hematoxylin and eosin (H&E), silver methenamine and Ziehl Neelsen and modified Ziehl Neelsen were performed to detect cell anomalies and other infections.

Expression of transcription factors by real-time PCR

From GALT biopsies conserved in RNAlater (Qiagen, Inc., Valencia, CA, USA), we obtained total RNA using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA). RNA was quantified by the Nanodrop 1000 spectrophotometer and treated with DNase I (Thermo Scientific). To ensure that differences observed in expression of transcription factors were not a consequence of the variability in the amount of cells, all cDNAs were synthesized from 1000 ng of DNase I-treated RNA using random primers and the RevertAid H minus reverse transcriptase Kit (Thermo Scientific), following the instructions of the manufacturer.

For quantitative RT-PCR, we used the following primers according to previous reports [14]: (i) Foxp3 (Fw: 5-CAG CACATTCCCAGAGTTCCTC-3; Rv: 5-GCGTGTGAAC CAGTGGTAGATC-3); (ii) RORγT (Fw: 5-TTTTCCG AGGATGAGATTGC-3; Rv: 5-CTTTCCACATGCTG GCTACA-3); (iii) T-bet (Fw: 5-GCCTACAGAAT GCCGAGATTACT-3; Rv: 5-GGATGC TGGTGTCAA-CAGATG-3); and (iv) GATA-3 (Fw: 5-GCG GGCT CTATCACAAAATGA-3; Rv: 5-GCTCTCCTGGCTG CAGACAGC-3) (Integrated DNA Technologies (IDT) Coralville, IA, USA). Amplification was performed using the Maxima SYBR green qPCR master mix kit (Fermentas Life Science, Hanover, MD, USA). mRNA expression of ubiquitin was used as a reference gene to normalize expression of the transcription factors. Units were expressed relative to ubiquitin.

Statistical analysis

The results are shown as median and interquartile range (IQR; 25 and 75 % percentiles). Nonparametric, two-tailed Mann–Whitney *U* tests were performed to compare data from HIV-controllers and HIV-progressors considering a p value <0.05 as statistically significant. The statistical tests were performed using the GraphPad Software version 5.03.

Results

The demographic parameters are presented in a supplementary Table (Online resource Table S1). The age and duration of infection were similar between HIV-controllers

and HIV-progressors, underscoring the sustained control of virus exhibited by this particular group of HIV-controllers. As expected, the controllers had lower viral loads and higher $CD4^+$ T cell counts compared to HIV-progressors. Cell anomalies or other infections were not detected in GALT by the various stains used. In the present study we did not evaluate HLA class I genotype or delta 32 mutation. However, our previous observation in a similar cohort suggested that HIV-controllers and HIV-progressors do not exhibit significant differences in these genetic variants.

Higher $CD4^+$ and lower $CD8^+$ T cell frequencies in GALT of HIV-controllers

The frequencies of $CD4^+$ and $CD8^+$ T cells were evaluated in PB and GALT. As expected, HIV-controllers had higher percentages of $CD4^+$ T cells in PB and GALT (online resource Table S1, Fig. S2A and B) compared to HIV-progressors. Although the percentage of $CD8^+$ T cells in PB was similar between groups (Table S1), in GALT the frequency of this population was lower in HIV-controllers (Online resource Table S1, Fig. S2C). We also evaluated the frequency of double-positive $CD4^+CD8^+$ T cells in PB, but no significant differences were found (Online resource Table S1).

Lower activation of T cells in HIV-controllers

The immune hyperactivation state of T cells was evaluated in both PB and GALT, according to the co-expression of HLA-DR and CD38. In PB, HIV-controllers had lower frequency of hyperactivated $CD4^+$ T cells (3.41 vs. 7.44 %; $p = 0.0003$; Fig. 1a) and hyperactivated $CD8^+$ T cells (18.2 vs. 29.03 %; $p = 0.0081$; Fig. 1b) than HIV-progressors. For $CD4^+CD8^+$ T cells, similar percentages of hyperactivated cells were observed between groups (34.7 vs. 45.15 %; $p = 0.0776$). In GALT, we observed similar results with decreased frequencies of $CD4^+$ and $CD8^+$ T cells co-expressing HLA-DR and CD38 in HIV-controllers compared to HIV-progressors (0.66 vs. 9.5 %; $p = 0.0011$; Fig. 1c and 9.27 vs. 20.57 %; $p = 0.0213$; Fig. 1d, respectively).

HIV-controllers exhibit a novel activation phenotype predominating in GALT

The frequencies of T cells expressing other combination of activation markers were also analyzed. In PB, HIV-controllers exhibited a lower frequency of $HLA\text{-}DR^+CD38^-$ $CD4^+$ T cells (4.14 vs. 7.79 %; $p = 0.0081$) and higher

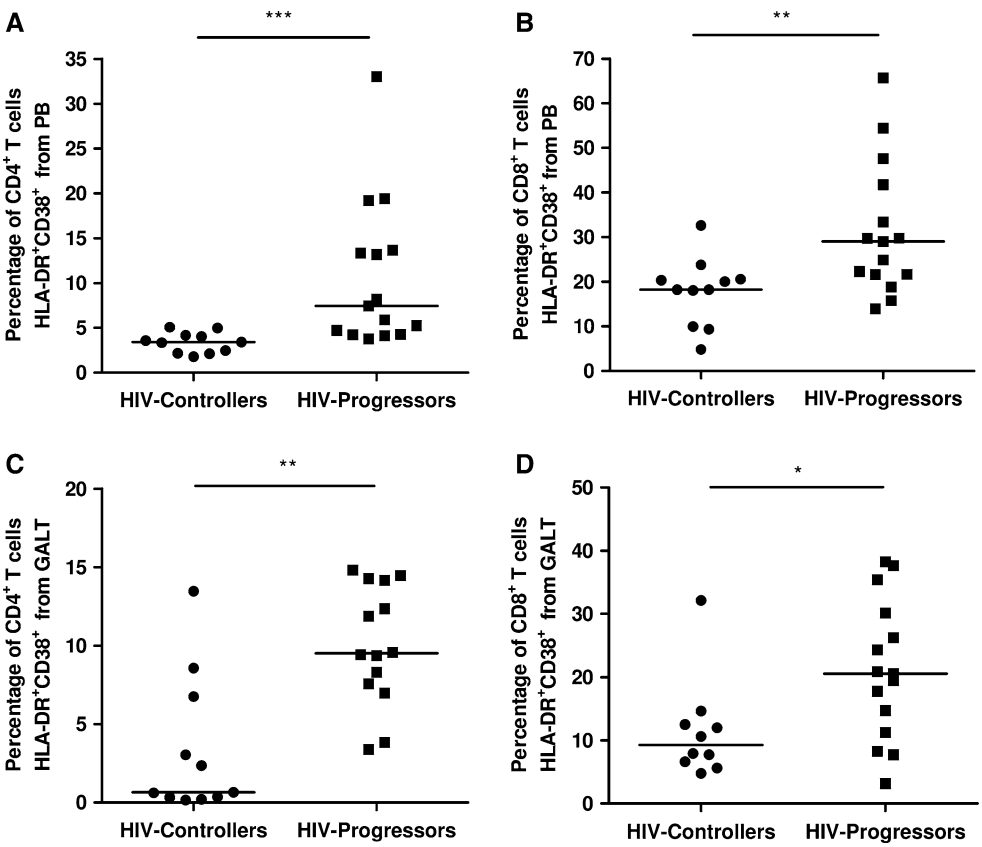

Fig. 1 Frequency of activated T cells in peripheral blood and gut mucosa from HIV-controllers and HIV-progressors. The frequencies of T cells expressing HLA-DR and CD38 in PB and GALT from 11 HIV-controllers and 15 HIV-progressors were evaluated by flow cytometry. **a** $HLA\text{-}DR^+CD38^+$ $CD4^+$ T cells and **b** $CD8^+$ T cells in PB; **c** $HLA\text{-}DR^+CD38^+$ $CD4^+$ T cells and **d** $CD8^+$ T cells in GALT. The results are shown as median. The statistical comparison between both groups was performed using the Mann–Whitney *U* test with a confidence level of 95 %. Significant differences are indicated at the top of the figure (* $p < 0.05$), (** $p < 0.001$) and (*** $p < 0.0001$)

frequency of both, HLA-DR$^-$CD38$^-$ CD4$^+$ T cells (51.52 vs. 40.71; $p = 0.0430$) and HLA-DR$^-$CD38$^-$ CD8$^+$ T cells (44.79 vs. 34.25 %; $p = 0.0379$) compared to HIV-progressors. The frequencies of other subpopulations analyzed (HLA-DR$^-$CD38$^+$ CD4$^+$ and CD8$^+$ T cells, and HLA-DR$^+$CD38$^-$ CD8$^+$ T cells) were similar between groups (data not shown). In GALT, HIV-controllers had lower percentages of HLA-DR$^+$CD38$^-$ (3.73 vs. 7.17 %; $p = 0.0173$) and HLA-DR$^-$CD38$^+$ (5.29 vs. 15.14 %; $p = 0.0048$) CD4$^+$ T cells, as well as higher frequencies of double-negative (HLA-DR$^-$CD38$^-$) CD4$^+$ T cells (85.66 vs. 67.57 %; $p = 0.0004$) compared to HIV-progressors. However, the different activated subpopulations of CD8$^+$ T cells were similar between HIV-controllers and HIV-progressors (Fig. 2a).

Considering the importance of HLA-DR$^+$CD38$^+$ T cell and the recently described phenotype HLA-DR$^+$CD38$^-$ as correlates of HIV disease progression [11], we determined the ratio of these activated subpopulations (HLA-DR$^+$-CD38$^-$/HLA-DR$^+$CD38$^+$) in these two groups of patients as an index of T cell activation. In PB, we did not find differences between the two subject groups regarding this ratio (data not shown). However, in GALT, this ratio was significantly higher in HIV-controllers in both CD4$^+$ (1.6 vs. 0.75; $p = 0.0078$; Fig. 2b) and CD8$^+$ T cells (1.6 vs. 0.7; $p = 0.0071$; Fig. 2c), as compared to HIV-progressors.

Higher expression of FoxP3 in GALT from HIV-controllers

Based on the differential activation phenotype observed between HIV-controllers and HIV-progressors in GALT, we then evaluated the expression profile of transcription factors linked to T cell differentiation in this tissue. We found higher expression of FoxP3 in HIV-controllers

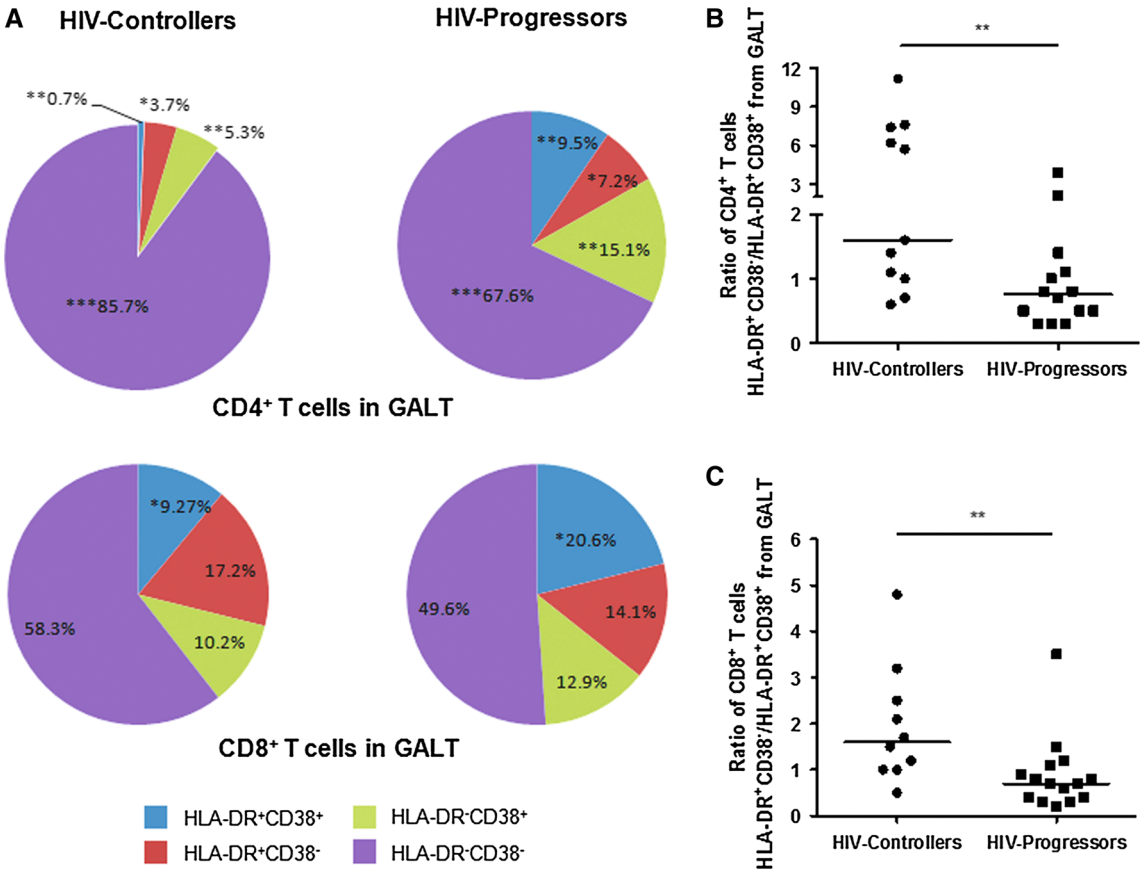

Fig. 2 Activation phenotypes of T cells from GALT and predominant subpopulation in HIV-controllers and HIV-progressors. The different activation phenotypes of CD4$^+$ and CD8$^+$ T cells in GALT were evaluated in 11 HIV-controllers and 15 HIV-progressors, according to the expression of activation markers (HLA-DR and CD38). **a** Pies of the four subpopulations established (HLA-DR$^+$CD38$^+$; HLA-DR$^+$CD38$^-$; HLA-DR$^-$CD38$^+$; and HLA-DR$^-$-CD38$^-$) are shown. **b** and **c** Ratio of HLA-DR$^+$CD38$^-$/HLA-DR$^+$CD38$^+$ in CD4$^+$ and CD8$^+$ T cells, these results are presented as median. The comparison between activation subpopulations was performed with the Kruskal–Wallis test, two-tailed test. The comparison between the HIV-controllers and HIV-progressors was performed using the Mann–Whitney U test. Significant differences are indicated at the top of the figure (* $p < 0.05$), (** $p < 0.001$) and (***$p < 0.0001$)

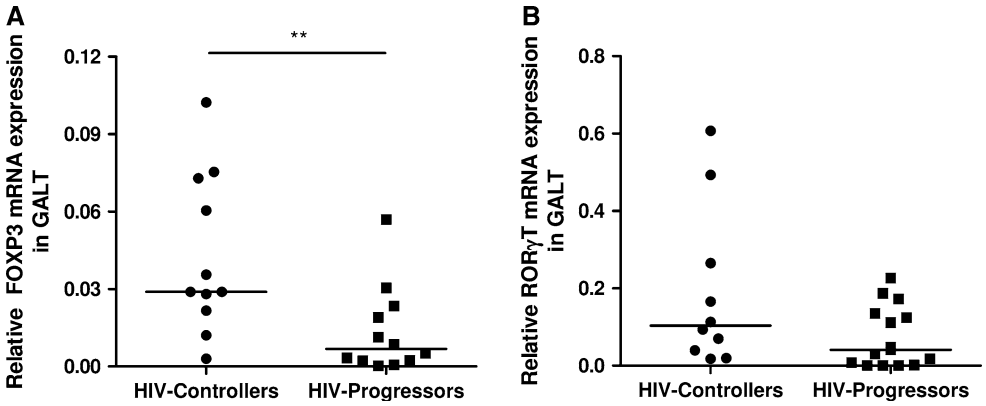

Fig. 3 Relative mRNA FoxP3 and RORγT expression in GALT from HIV-controllers and HIV-progressors. The expression of transcription factors for regulatory (FoxP3) and Th17 (RORγT) profiles was evaluated in GALT from 11 HIV-controllers and 15 HIV-progressors using real-time PCR. **a** Relative mRNA FoxP3 expression and **b** relative mRNA RORγT expression. The results are shown as median. The statistical comparison between both groups was performed using the Mann–Whitney *U* test with a confidence level of 95 %. Significant differences are indicated at the top of the figure (** $p < 0.001$)

compared to HIV-progressors (0.0289 vs. 0.0068; $p = 0.0074$; Fig. 3a) and the expression of this transcription factor was negatively correlated with activated $CD4^+$ T cells in PB ($r = -0.4932$; $p = 0.0168$) and GALT ($r = -0.5535$; $p = 0.0075$). Although the expression of RORγT was not significantly different (0.1034 vs. 0.0409; $p = 0.0636$; Fig. 3b), a tendency to higher expression in HIV-controllers was also observed. The expression of T-bet (0.0548 vs. 0.0404; $p = 0.7474$) and GATA-3 (0.0489 vs. 0.0305; $p = 0.2618$) was similar in both groups. We also evaluated the balance of Th17 to Treg (RORγT/FoxP3) since its decrease has been linked to disease progression [16]; however, we did not find differences between both groups (2.45 vs. 1.85; 0.6205).

Similar expression of cleaved caspase-3 in HIV-controllers and HIV-progressors

As immune activation is one of the most important inducers of apoptosis in HIV infection, along with the activation phenotype and the T cell transcription factor profiles, we also evaluated the expression of cleaved caspase-3 in GALT by immunohistochemistry as a marker of apoptosis. The expression of this cleaved enzyme was similar in HIV-controllers and HIV-progressors (1.67 vs. 2; $p = 0.1657$; Fig. 4a–c).

The $HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$ ratio is correlated with lower AIDS progression

Since we found a higher proportion of $CD4^+$ and $CD8^+$ T cells expressing only HLA-DR, but not CD38, in GALT from HIV-controllers, we evaluated the association of this ratio with the progression to AIDS ($CD4^+$ T cells count and plasma viral load). Interestingly, we found a positive correlation between the ratio of $HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$ in both $CD4^+$ and $CD8^+$ T cells with $CD4^+$ T cell count (Fig. 5a, b), as well as a negative correlation with viral load (Fig. 5c, d). Similar results were found in peripheral blood (data not shown).

The $HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$ ratio is correlated with the expression of FoxP3 and RORγT

As we observed a higher expression of the transcription factor FoxP3 and a trend toward increased expression of RORγT in HIV-controllers, we evaluated the correlation of these factors with the ratio $HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$ in $CD4^+$ and $CD8^+$ T cells from GALT, finding a positive correlation in both cases (Fig. 5e, f for FoxP3, and 5G and H for RORγT).

Discussion

Since the description of HIV-controllers in 2007 [8], these rare individuals have stimulated interest in exploring mechanisms involved in controlling viral replication. Several studies have pointed to immune effector mechanisms that can partially explain for the controller phenotype. However, most studies to date have been performed using blood, and the immune characterization of GALT, which is the main target tissue for HIV infection, has been much less explored. In order to determine the relevance of the T cell phenotypic profile in viral control, we evaluated the expression of HLA-DR and CD38 in blood and GALT and the expression of transcription factors; in addition, we measured levels of apoptosis in GALT samples from HIV-controllers and progressors. We observed higher frequency of T cells and lower immune activation in PB and GALT

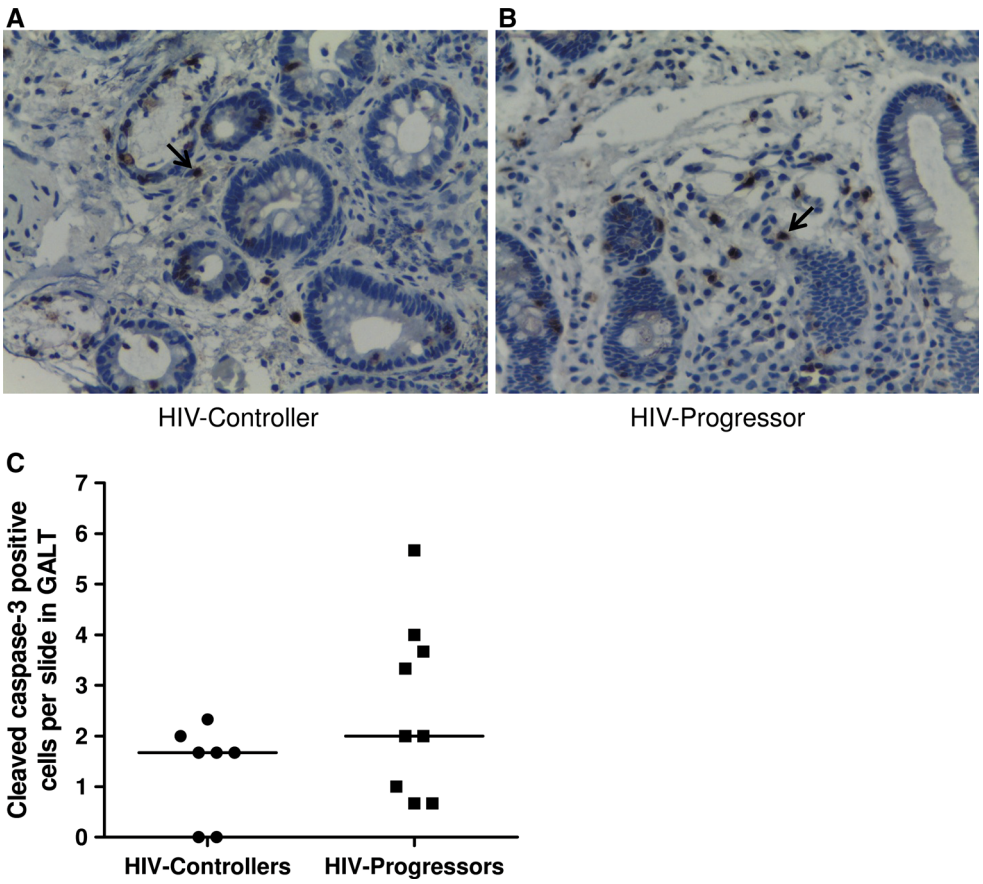

Fig. 4 Expression of cleaved caspase-3 in gut mucosa from HIV-controllers and HIV-progressors. The expression of the suggestive marker of apoptosis, cleaved caspase-3 was evaluated in GALT from seven HIV-controllers and nine HIV-progressors by immunohistochemistry. **a** and **b** Images of cleaved caspase-3-positive cells in GALT (40×) from an HIV-controller and a HIV-progressor; a positive cell is shown by an arrow. **c** Median of cleaved caspase-3-positive cells per slide in GALT. The comparison between the HIV-controllers and HIV-progressors was performed using the Mann–Whitney *U* test with a confidence level of 95 %. A *p* value lower than 0.05 was considered as significantly different

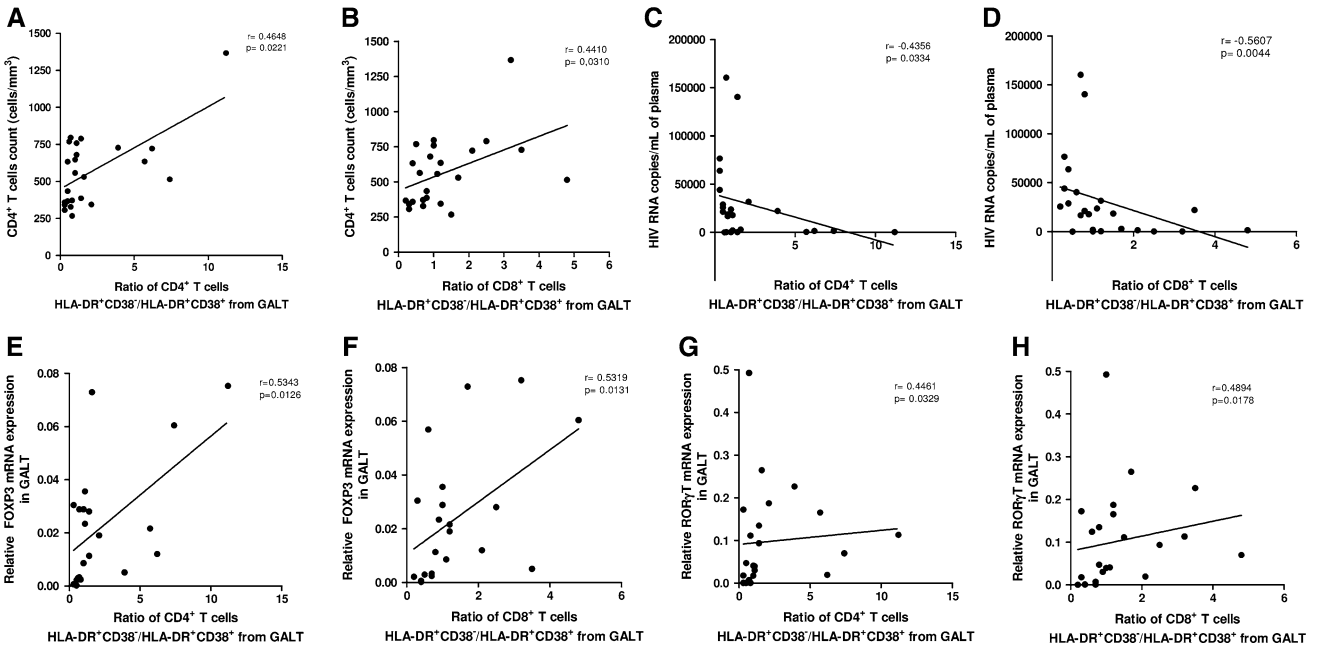

Fig. 5 Correlation of HLA-DR$^+$CD38$^-$/HLA-DR$^+$CD38$^+$ ratio in T cells from GALT with markers of progression and expression of FoxP3 and RORγT. Correlation of HLA-DR$^+$CD38$^-$/HLA-DR^{+-}CD38$^+$ ratio in CD4$^+$ and CD8$^+$ T cells from GALT with **a** and **b** the CD4$^+$ T cells count, **c** and **d** the viral load, **e** and **f** the expression of FoxP3 and **g** and **f** with expression of RORγT. The correlation between the parameters was performed with a Pearson test. The *r* value and the *p* value of the correlations are indicated at the top of the figure; a *p* value lower than 0.05 was considered as a significant correlation

from HIV-controllers, as well as a predominance of T cells expressing HLA-DR but not CD38 in GALT. This novel phenotype was associated with increased expression of FoxP3 and RORγT and also with delayed progression to AIDS. Although gender may influence immune responses, the difficulty in recruiting patients for GALT biopsies made impossible to have similar gender distribution between groups.

Supporting our results, and according to the pathogenic nature of immune activation during HIV infection, previous reports, particularly in blood, have shown lower activation levels of several immune subpopulations in HIV-controllers when compared to progressors [12]. Interestingly, we were able to extend this observation to GALT. Although in our study we did not evaluate the levels of plasma LPS that has been strongly associated with immune activation, some studies report lower levels of LPS in plasma from HIV-controllers [17]. More recently, the novel activation phenotype in $CD8^+$ T cells from HIV-controllers ($HLA\text{-}DR^{+-}CD38^-$) was associated with better functional activity and suppression of viral replication in vitro [11]. This phenotype was induced in vitro in the presence of low concentrations of HIV [11], resembling the in vivo conditions of HIV-controllers who are defined based on their low or undetectable viral load. Some studies have suggested that the effective immune response observed in HIV-controllers at low viral load is determined by a higher avidity of their T cells to recognize the virus [18, 19].

Intriguingly, we observed a higher frequency of $CD4^+$ and $CD8^+$ T cells expressing only HLA-DR in HIV-progressors compared to HIV-controllers; however, these patients with an active infection had also higher frequency of hyperactivated T cells ($HLA\text{-}DR^+CD38^+$). Considering these findings, we established a ratio between these two populations to determine the predominant profile ($HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$). A higher percentage of T cells from HIV-controllers expressed exclusively HLA-DR rather than $HLA\text{-}DR^+CD38^+$ in GALT, suggesting that cells with this phenotype in GALT may be important for controlling viral replication. However, the fact that the predominance of this phenotype may result from an early viral control, exerted by different mechanisms, cannot be ruled-out. In HIV-controllers have been observed similar immune parameters in blood and GALT when compared to healthy individuals [20], suggesting that effective viral control is critical for immune preservation and regulation of cell activation. In fact, in gut mucosa from HIV-controllers, we observed a higher expression of FoxP3, a transcription factor associated with regulatory T cells. FoxP3 expression was correlated with $CD4^+$ T cells activation as well as with the proportions of $CD4^+$ and $CD8^+$ T cells expressing HLA-DR but not CD38 in both GALT and blood.

Although the role of regulatory T cells during HIV infection remains unclear, several reports suggest a dual role, being beneficial during HIV exposure and acute infection by reducing target cells, but harmful during chronic infection, by decreasing HIV-specific immune responses [14]. Although we did not specifically evaluate the frequency of regulatory T cells, the higher expression of FoxP3 in HIV-controllers suggests an increased regulatory activity [21]. Indeed, Chase et al. [21] reported a higher expression of FoxP3 in $CD4^+$ T cells isolated from peripheral blood mononuclear cells from ELITE HIV-controllers that was correlated with CD4 counts and inversely with $CD4^+$ T cell activation, supporting our findings. Remarkably, other studies have reported a higher frequency of Treg in HIV-progressors compared to HIV-controllers [12, 15]; in fact, the percentage of Treg was positively correlated with viral load and inversely related to CD4 counts [15]. These somehow contradictory data may suggest that in HIV-controllers there is a greater functional capacity of Treg rather than a higher frequency of these cells. Although the expression of RORγT did not differ significantly, we observed that at least some HIV-controllers exhibited higher expression of this marker compared to HIV-progressors; the lack of statistical significance might be due to the small number of individuals included in our study. In addition, we observed a positive correlation between the expression of RORγT and the ratio of $HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$ cells in $CD4^+$ and $CD8^+$ T cells in GALT. Given the importance of Th17 cells in maintaining the integrity of gut mucosa, this result suggests that these cells and $HLA\text{-}DR^+CD38^-$ T cells are part of a complex balance required to preserve the immune response. However, more studies in this area are required.

When we evaluated the balance between Th17 and Treg (RORγT/FoxP3) that has been related to the HIV-controller phenotype [22], we did not observed differences between both groups. This result may be explained by the increased expression of RORγT and FoxP3 exhibited by the HIV-controllers group.

Next, we correlated the ratio $HLA\text{-}DR^+CD38^-/HLA\text{-}DR^+CD38^+$ in $CD4^+$ and $CD8^+$ with AIDS progression markers. We found that a higher proportion of T cells expressing HLA-DR but not CD38 are associated with higher $CD4^+$ T cell count and lower viral load, suggesting their potential role in delaying AIDS progression. To the best of our knowledge, this is the first study reporting a correlation between the predominance of $HLA\text{-}DR^+CD38^-$ T cells and the viral control observed in HIV-controllers. The dynamic of the differential expression of HLA-DR and CD38 in T cells during HIV infection has not been well described. In this regard, some studies using resting $CD8^+$ T cells ($HLA\text{-}DR^-CD38^-$) have shown that, while HLA-DR is induced in the presence of viral peptides, CD38 is

induced by IFN-α [11]; in fact, the expression of CD38 has been positively correlated with higher HIV viral loads and with an increased susceptibility to cell death [23].

Considering that apoptosis of infected and uninfected cells is the main pathogenic mechanism triggered during HIV infection as a result of the excessive immune activation [24], we evaluated the level of apoptosis in GALT in terms of the detection of cleaved caspase-3 by immunohistochemistry. Surprisingly, no differences between HIV-controllers and progressors were observed; one possibility for the lack of significant differences is the low sample size, as some HIV-progressors seem to have higher numbers of caspase-3-positive cells; in addition, cell death may be occurring by the pyroptosis pathway, as was recently proposed [25], emphasizing the importance of additional studies to explore markers of pyroptosis in GALT.

Although these results provide further insight in the complex network of interactions occurring during HIV-1 infection, further studies are required to completely explain the effector mechanisms responsible for immune control in HIV-controllers. In this sense, and also supported by previous studies indicating the presence of antiviral proteins [26] and an efficient immune innate response in HIV-controllers [12, 17, 27–29], we propose that an increased activity of regulatory T cells in mucosa during the acute phase of infection contributes to regulate the excessive immune activation, reducing the loss of $CD4^+$ T cells, particularly Th17 cells in GALT, favoring the gut mucosal integrity. Concomitantly, the lower immune activation decreases the number of viral target cells, reducing viral load and promoting the activation T cell phenotype HLA-DR^+CD38^- with a better ability to effectively control HIV replication.

Finally, our results highlight the importance of studies in gut tissue during the HIV infection, as it could be the clue for defining new therapeutic strategies, including immune modulators.

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