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RESEARCH ARTICLE

The Spontaneous Control of HIV Replication is Characterized by Decreased Pathological Changes in the Gut-associated Lymphoid Tissue

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Abstract: ***Background*:** HIV infection induces alterations in the gut-associated lymphoid tissue (GALT) that constitutes the most important site for viral replication due to the extensive presence of effector memory T-cells. In the case of HIV-controllers, several studies have reported fewer peripheral alterations and conserved immune responses that correlate with viral control; however, the histopathological characterization of GALT in those patients is still missing. In this study, we evaluated pathological alterations in GALT, trying to associate them with clinical parameters of HIV infected patients with or without evidence of viral control.

***Methods*:** This study included eight HIV-controllers (antiretroviral treatment-naïve patients, with viral loads below 2.000 copies/mL for at least 1 year); 14 Noncontrollers (antiretroviral treatment-naïve patients, with viral loads > 2.000 copies/mL and $CD4^+$ T cells count > 250 cells/μL), and 12 uninfected donors.

Biopsy fragments were obtained by rectosigmoidoscopy and stained with hematoxylin and eosin, silver methenamine, Ziehl Neelsen, and modified Ziehl Neelsen.

***Results*:** Histopathological findings in HIV-controllers were similar to those observed in the uninfected group. In contrast, noncontrollers exhibited several alterations including condyloma acuminate, squamous metaplasia and acute colitis. These alterations were associated with disease progression.

***Conclusion*:** HIV-controllers exhibit lower pathological alterations in the gut tissue, associated with higher CD4 T cell count, and lower viral load.

Keywords: HIV-controllers, Noncontrollers, GALT, histopathological alterations, $CD4^+$ T cells, rectosigmoidoscopy.

1. INTRODUCTION

The gastrointestinal tract is the main interface where the body encounters exogenous antigens, demanding a tightly regulated local immune response [1]. The gastrointestinal tract contains the largest mucosal surface in the body, where dendritic cells and lymphocytes are found in close contact with the intestinal epithelium as well as in the underlying lamina propria [2]. Also, the intestine harbors specialized lymphoid organs, such as Peyer's patches and lymphoid follicles; both organs have a similar structure, containing M cells, antigen-presenting cells, and lymphocytes [2].

During the human immunodeficiency virus type-1 (HIV) infection, high viral replication during the acute phase and the progressive immune activation induce massive depletion of $CD4^+$ T-cells, mainly those from gut-associated lymphoid tissue (GALT) [3]. As a consequence, an extensive immune hyperactivation is established, constituting the most important pathogenic mechanism during HIV infection [4]. This abnormal activation is induced initially by the exposure to viral antigens, and later by the translocation of microorganisms and microbial products from the intestinal lumen to systemic circulation [5].

Additionally, during the chronic phase of HIV infection, several alterations and infectious agents in the intestine tissue have been described, including enteropathy [6], cytomegalovirus [7], herpes simplex virus [8], and human papillomavirus [9, 10]. In addition, certain neoplasms such as squamous cell carcinoma, most frequently associated with human papillomavirus infection, and non-Hodgkin's lymphomas, have been reported [9]. However, not all HIV-infected patients exhibit these alterations, as AIDS progres-

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sion is a heterogeneous process, in particular in its time-course. In fact, a phenotype of seropositive individuals who exhibit a spontaneous and sustained control of viral replication, at least for one year in the absence of antiretroviral therapy, known as HIV-controllers [11], has become a relevant model to explore mechanisms associated with viral control. We previously described differences between HIV-controllers and noncontrollers regarding the frequency and activation of T cells; the expression of transcription factors, associated with immune response profiles; and the frequency of apoptotic cells [12]. However, histopathological alterations in patients who control HIV replication have been poorly studied.

Here, we correlated histopathological alterations and the frequency of bacterial and fungal infections with the clinical condition of HIV-controllers and noncontrollers in GALT biopsies. This study contributes to characterize specific alterations in the intestinal tissue that might lead to establish a protocol to be included in the follow-up of HIV infected patients as a preventive measure.

2. MATERIAL AND METHODS

2.1. Study Population

Two groups of HIV-infected individuals, all naïve for antiretroviral therapy, were recruited from health insurance programs in Medellin-Colombia. Eight HIV-controllers, defined as previously described [13]; briefly, they had at least one year of confirmed HIV diagnosis and exhibited viral loads lower than 2000 HIV RNA copies/mL in most determinations; and 14 noncontrollers, who exhibited viral loads higher than 2.000 HIV RNA copies/mL but $CD4^+$ T-cell count >250 cells/μL at sampling, in order to exclude patients in advanced clinical stage (Table **1**). In addition, an age- and sex-matched group of 12 uninfected donors was included.

2.2. Viral Load

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

2.3. 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, tissue samples were obtained from the rectum, 10 cm from anal verge.

2.4. Histology and Stains

The biopsy fragments were paraffin-embedded, and segmented with a microtome (Leica, Nussloch - Germany); the resultant fragments (3-4 μm of thickness) were placed in charged slides, deparaffinized and hydrated. Stains with hematoxylin and eosin (H&E) were used to detect structural abnormalities, and histochemical stains (silver methenamine, Ziehl Neelsen and modified Ziehl Neelsen) were performed to detect infectious agents.

2.5. Flow Cytometry

The $CD4^+$ T cell count was determined by flow cytometry. In brief, 150 μL of blood was incubated with specific antibodies for CD3 (clone UCHT1) and CD4 (clone OKT-4) (BD Biosciences, San Jose, CA, USA) for 25 min at 25°C in the dark. Erythrocytes were lysed with 1X fluorescence-activated cell sorting lysing solution (BD Biosciences) by incubating for 10 min. The cells were washed twice with PBS, centrifuged at 250 *x g* for 5 min, and then fixed with 250 μL of 2% paraformaldehyde. At least 200.000 events were acquired in a FACS CANTO-II (BD Biosciences) and analyzed using the FACSDiva 6.1.2 version (BD Biosciences).

2.6. Statistical Analysis

Clinical and demographical results are presented as median and range. A non-parametric test (Mann-Whitney *U* - two-tailed test) was performed for comparing data from HIV-controllers vs. noncontrollers. A p-value <0.05 was considered statistically significant. Graph-Pad Software version 5.00 was used. In addition, the principal component analysis was conducted using collected data from all patients to determine which variables contributed the most to the variability in the data set at the time, using the SPSS Statistics for Windows, Version 21.0 (Armonk, NY; IBM Corp). Component extraction was achieved using the principal axis method, and the rotation method employed was a Varimax rotation with Kaiser normalization.

3. RESULTS

3.1. Demographic and Clinical Information

Demographic, virological and immunological data at the time of sampling are shown in Table **1**. There were no significant differences in age, time length since HIV diagnosis, and percentage of $CD4^+$ T cells in GALT between HIV-controllers and Noncontrollers. However, as expected, HIV-controllers exhibited higher $CD4^+$ T cells-count in blood and lower plasma HIV viral load, compared with noncontrollers. In addition, both HIV-controllers and noncontrollers had lower number and percentage of $CD4^+$ T cells in blood and GALT, respectively, compared with uninfected donors.

3.2. HIV-controllers had Similar Gut Tissue Characteristics Compared to Uninfected Donors, while Noncontrollers Exhibited Several Alterations

All individuals analyzed, including uninfected donors, showed a nonspecific inflammation, probably as a consequence of the preparation for tissue sampling, as previously reported [15, 16]. Thus, to establish the percentage of individuals exhibiting alterations in GALT, we only included those who exhibited alterations beyond a nonspecific inflammation.

Interesting, although HIV-controllers and noncontrollers exhibited a similar time course after HIV diagnosis, only two HIV controllers had follicular component and one an active proctitis. In contrast, in the noncontrollers group, we identified several alterations including three cases of condyloma acuminate (Fig. **1A**), one squamous metaplasia (Fig. **1B**),

Table 1. Demographic and clinical information.

	HIV-controllers (n=8)	Noncontrollers (n=14)	Uninfected Donors (n=12)	*P value*
Age in years, Median (range)	37 (20-49)	30 (19-50)	36 (22-59)	0,1864* 0,1178** 0,3762***
Gender, Male:Female	6 : 2	13 : 1	9 : 3	0,3031* 0,2391** 0,2909***
Time of diagnosis in months Median (range)	57 (13-168)	51 (12-276)	N/A	N/A* N/A** 0,3535***
$CD4^+$ T cell count in blood Median (range)	740 (514-796)	560 (306-819)	1041 (379-2156)	0,0093* <0,0001** 0,0158***
% CD4 T cells in blood Median (min-max)	29,30 (24,45-37,48)	21,69 (12,44-27,56)	42,56 (31,13-53,42)	0,0002* <0,0001** 0,0003***
% CD4+ T cells in GALT Median (min-max)	18,48 (11,07-36,36)	13,34 (5,2-65,80)	29,36 (15)	0,0171* 0,0138** 0,1468***
Plasma HIV viral load in RNA copies/mL Median (min-max)	312 (20-1885)	33526 (2928-160405)	N/A	N/A* N/A** <0,0001***

* HIV-controllers vs. Uninfected donors
**Noncontrollers vs. Uninfected donors
***HIV-controllers vs. Noncontrollers

one active proctitis, one chronic granulomatous inflammatory reaction, and three patients with follicular component (Fig. **1C**). The pie chart shows differences between HIV-controllers and noncontrollers (Fig. **1D**). In the uninfected group, the relevant finding was the follicular component observed in three individuals (data not shown).

3.3. Structural Alterations of Gut Tissue are Associated with HIV Progression

We analyzed pathological findings on the total HIV infected individuals (HIV-controllers and noncontrollers) in accordance to the $CD4^+$T cell count in blood, percentage of $CD4^+$ T cells in GALT, and viral load. For this analysis, a pathologist expert applied a 6-point scale of severity, from very mild to very severe pathological alterations. We found that pathological alterations score was negatively correlated with the frequency of $CD4^+$ T cells in GALT, and positively correlated with viral load. No significant differences were observed with the $CD4^+$ T cell count (Fig. **1E**). Principal components analysis showed that two components explained 71% of the variability in the system; component 1 was related to the $CD4^+$ T cell count, and the percentage of $CD4^+$ T cells in GALT, while component 2 was associated with histological alterations, and viral load. All three groups of individuals clustered independently. Interesting, regarding component 1, the characteristics of the HIV-controllers were comparable to the uninfected donors, while Noncontrollers had a heterogeneous behavior explained by histological alterations. In addition, four Noncontrollers were subclustered, particularly explained by the decreased level of $CD4^+$ T cell in blood and GALT (Fig. **1F**).

In addition, the component plot in the rotated space showed a direct relation between viral loads and histological alterations in GALT; in contrast, an indirect relation between these parameters and the percentage of $CD4^+$ T cell in GALT, and the circulating $CD4^+$ T -cell count was observed (Fig. **1G**).

3.4. Gut Evaluation by Silver Methenamine, Ziehl Neelsen, and Modified Ziehl Neelsen Stain

When the presence of infectious agents was analyzed, we did not observe the presence of acid-fast organisms or fungal infections in the biopsies of HIV-controllers, Noncontrollers or seronegative donors (data not shown).

4. DISCUSSION

GALT constitutes the main site for HIV replication, resulting in critical damage of this tissue [3, 14, 17-20]. We previously reported functional alterations in GALT associated with HIV progression, including a high frequency of the particular phenotype of dysfunctional T cells expressing HLA-DR but not CD38, low frequency of $CD4^+$ T and NK cells, and alterations in the polyfunctional response of $CD8^+$

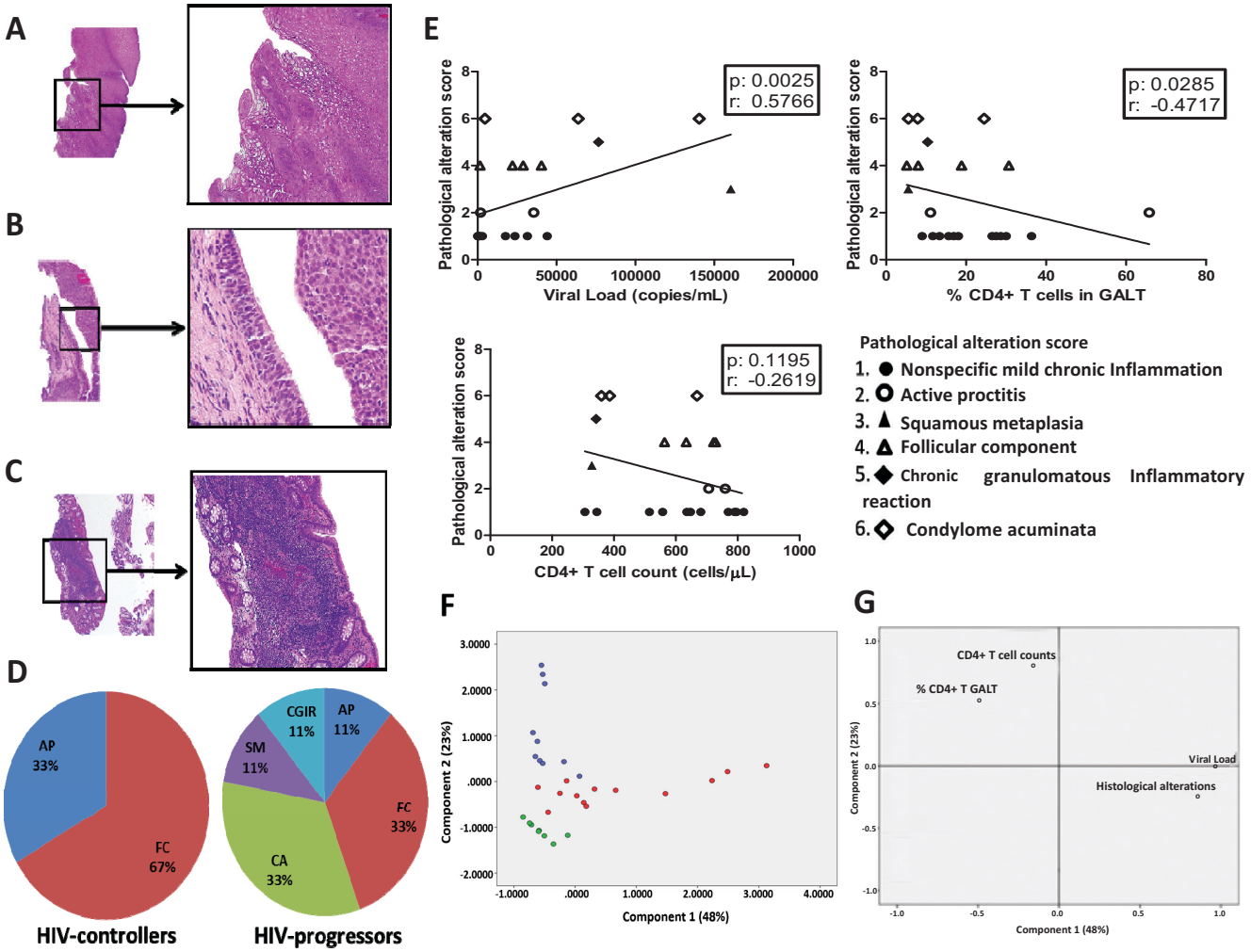

Fig. (1). Pathological alterations in the gut mucosa and their potential association with the progression of HIV infection. Histological examination was evaluated in GALT from 8 HIV-controllers, 14 Noncontrollers, and 12 uninfected donors by an H&E stain (4X and 10X magnifications are shown): **A)** Condyloma acuminate; **B)** Squamous metaplasia and **C)** Lymphoid follicle associated to chronic intestinal inflammation. **D)** A pie chart is indicating pathological alterations. FC: Follicular component, AP: active proctitis, CA: condyloma acuminate, SM: squamous metaplasia; and CGIR: chronic granulomatous inflammatory reaction. **E)** Correlation between the pathological alterations scores with the frequency of $CD4^+$ T cells in GALT, viral load, and $CD4^+$ T cells-count in blood (each symbol represents an HIV-infected individual, either HIV-controllers or Noncontrollers). Principal component analysis: **F)** Clustered of data from HIV-controllers (green circles), Noncontrollers (red circles) and uninfected donors (blue circles). Each data point represents one individual; **G)** Component plot in rotated space. Component 1: $CD4^+$ T cell count in blood, and percentage of $CD4^+$ T cells in GALT; component 2: histological alterations, and viral load.

T cells [12, 21]. However, there are limited studies regarding histological alterations in GALT, especially in the context of spontaneous viral control or disease progression. So, we explored the presence of histological alterations in GALT and their association with clinical HIV parameters.

We observed that all the participants exhibited a nonspecific inflammation, most likely produced by the rectosigmoidoscopy preparation (enema use) or the technic used for sampling [16, 22]. Moreover, HIV controllers exhibited lower pathological alterations in GALT, similar to the uninfected group, that are in line with our previous studies [23]. These antecedents suggest that individuals, who spontaneously control viral replication, have a preserved structure of GALT associated to the maintenance of immune parameters in both, mucosal and peripheral blood.

In addition, when HIV-controllers and Noncontrollers were compared, we found important histological changes including condyloma acuminate, squamous metaplasia, active proctitis and acute colitis, which had a negative correlation with $CD4^+$ T cells in GALT and a positive correlation with viral load. Interesting, other studies have also reported alterations such as villous atrophy with crypt hyperplasia, increased numbers of intraepithelial lymphocytes, infiltration in the lamina propria, and focal cell degeneration close to the crypt base that were associated with disease progression [6, 24, 25], supporting our results.

When we studied the presence of pathogens in GALT, all participants were negative for the tested microbes, suggesting that the structural alterations in Noncontrollers were most likely a consequence of the HIV infection itself and, probably associated to the inflammatory process, as previously reported [26]. This inflammation has been associated to the persistence of viral RNA or DNA in GALT [27-30], alterations of the intestinal microbiota [31-34], increased level of proinflammatory cytokines [17, 35-39], high frequency of hyperactivated CD8+ T [14, 40] and NK cells, and increased regulatory T cells [12, 21, 41, 42]. In addition, we have previously reported a high activation of the inflammasomes in GALT from Noncontrollers, compared with HIV-controllers [17]. All these immune alterations induce recruitment, abnormal differentiation, exhausting, and cell death of different cell populations, including enterocytes and Th17 cells [43-52], favoring the development of several conditions as metaplasias, colitis, and condyloma [53-60]. Although we did not evaluate the presence of human papillomavirus, condyloma acuminate observed in Noncontrollers, was most likely due to serotypes 16 or 18, which particularly have a high prevalence among HIV infected patients [61, 62], and had been associated with HIV progression [63, 64].

According to the principal component analysis, all three groups were clustered independently. Interesting, Noncontrollers exhibited an independent behavior in component 1, related to $CD4^+$ T cells in blood and GALT, confirming a deep immune deterioration in both tissues that was not observed in HIV-controllers [12, 41, 65, 66]. Additionally, viral load was associated directly with structural alterations in GALT, and indirectly with the percentage of $CD4^+$ T cells in GALT and $CD4^+$ T cell counts in blood. This evidence highlights the importance of an effective viral control for limiting microbial translocation and immune activation, as previously reported [20, 21, 67-71]. In addition, the pathological alterations score was negatively correlated with the percentage of $CD4^+$ T cells in GALT, but not with $CD4^+$ T cell counts in blood; this somehow inconsistent result might reflect the fact that the gastrointestinal tissue is the main target organ during HIV infection and the magnitude of its deterioration is not mirrored in peripheral blood. In this sense, the anal cytology, a standard, and less invasive test, can be useful during the clinical management of HIV patients for early identification of tissue alterations.

Since our sample size was low, a larger study including a follow-up of the cohort might determine the impact of these gut alterations on HIV progression, and on the response to HAART.

CONCLUSION

In addition to the functional alterations in gut tissue, HIV-controllers exhibit lower pathological and structural alterations in this tissue, which are associated with higher percentage of $CD4^+$ T cells in the gut tissue, higher $CD4^+$ T cell count, and lower viral load.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by Ethics Committee from Headquarters of University Research, University of Antioquia, Colombia.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20 activities/ 10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Written informed consents were obtained from all recruited individuals.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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