SUPPLEMENTAL MATERIAL

Material and Methods

Ethical Aspects

The study had approval by the Ethics Committee from the TMFAM (Protocol# 3319/2008). All patients signed an informed written consent form in accordance to the guidelines established by the 466/2012 resolution at the Brazilian National Health Council.

Study Population

This study included 85 male patients, with age ranging from 15-60 years. The patients were invited to participate in the study and were examined at the ambulatory of coloproctology of the TMFAM. Genotyping of HPV was performed on samples obtained from biopsy of the anal canal using a nested PCR method as described previously [1]. HIV test was performed by ELISA immunoassay, and indirect immunofluorescence. In addition, histological analysis was performed to assess the presence of AIN, squamous cell carcinoma, adenocarcinoma and inflammatory diseases as described by Guimarães et al., (2011) [1].

Supplemental table 1 displays the details of the study population, which was categorized as follows: i) Group AIN(-)/HIV(-); ii) Group AIN(-)/HIV(+) and iii) Group AIN(+)/HIV(+). Group AIN(+) was subcategorized based on the histopathology analysis of anal mucosa and classified as low-grade (LSIL) or high-grade squamous intraepithelial lesion (HSIL).

Clinical Samples

All patients were evaluated by high-resolution anoscopy. The digital rectal examination was performed, followed by an inspection of the mucosa with the use of a optical colposcope with 16 to 40-times magnification after introduction of gauze soaked in 3% acetic acid into the anal canal for two minutes. The anoscope was re-introduced for the analysis of the anal canal, and rectum examination under image magnification. The findings were registered, and the predominant acetowhite areas were biopsied.

The samples designated by histopathological analyses were preserved in 10% formalin. For flow cytometry analysis, blood samples were collected in EDTA, and the biopsy fragments were maintained in RPMI (Roswell Park Memorial Institute) medium.

Immunophenotypic and intracytoplasmic cytokine staining of peripheral blood mononuclear cells (PBMC) and anal mucosa mononuclear cells

A single cell suspension from the anal mucosal tissues was prepared by homogenization of samples with collagenase. Mononuclear cells were obtained by Ficoll-Hypaque (Acros, New Jersey, USA) from the cell suspension as well as from blood. Samples were treated with FACS lysing solution (Becton Dickinson Biosciences) and after erythrocyte lysis was completed, the samples were washed with PBS containing 0.5% BSA and 0.1% sodium azide. In vitro short-term cultures of mononuclear cells obtained from blood and anal mucosal tissues were performed as described by Luiza-Silva et al. [2] and modified as follows: 1×10^6 cells were incubated for 6 hours at 37°C, 5% CO_2, in the presence of RPMI 1640 medium (GIBCO, Grand Island, NY; controlculture) After culturing, cells were re-incubated in

the presence of brefeldin A (BFA; Sigma Chemical Company, St. Louis, MO) at 10 ng/mL for an additional period of 4 hours at 37°C, 5% CO2, and then treated with 2 mM final concentration of ethylenediamine tetraacetic acid (EDTA; Sigma Chemical Company) for 10 minutes at room temperature. Cell suspension was washed with fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline [PBS], pH 7.2, supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, all from Sigma Chemical Company) and aliquots were stained with anti-human cell surface monoclonal antibodies: PercP-CD45-*clone/2D1*, FITC-CD4-*clone/RPA-T4*, FITC-CD1a-*clone/HI149* and FITC-CD209-*clone/DCN56* (Becton Dickinson[®] Biosciences or Biolegend[®]), PerCP-CD3-*clone/SK7*, FITC-CD4-*clone/RPA-T4*, FITC-CD14-*clone/M5E2*, FITC-CD1a-*clone/HI149*, PE-CD11c-*clone/3.9*, FITC-CD123-*clone/646;* PE-IFN-γ-*clone/2573.11*, and APC-IL-10 -*clone/JES3-19F1* (Becton Dickinson[®] Biosciences and Biolegend[®]). Samples were then fixed in 200μL of FACS fixing solution (10g/L paraformaldehyde, 10.2g/L sodium cacodylate and 6.63g/L sodium chloride, pH 7.2), and stored at 4°C in the dark prior to flow cytometric analysis within 24 hours. A total of 30,000 events/sample were acquired using FACScalibur® flow cytometer (Becton Dickinson).

Cytokine Signature Analysis

The cytokine signature was assembled as reported previously[2]. Briefly, the global median value for each cytokine was calculated using data from all patients (AIN(-)HIV(-) + AIN(-)HIV(+) + AIN(+)HIV(+)).The global median for each cytokine was employed as a cut off point to discriminate each individual as "Low" or "High" producer of the cytokine secreted in the culture supernatant of both PBMC and anal mucosal mononuclear cells summarized in radar graphs. The relevant differences,

considered when the values were above the 50th percentile were highlighted by underline and bold format.

References

1. Guimaraes AG, Silva Junior RM, Costa OT, et al. Morphometric analysis of dendritic cells from anal mucosa of HIV-positive patients and the relation to intraepithelial lesions and cancer seen at a tertiary health institution in Brazil. *Acta Cir Bras.* Dec 2011;26(6):521-529.

2. Luiza-Silva M, Campi-Azevedo AC, Batista MA, et al. Cytokine signatures of innate and adaptive immunity in 17DD yellow fever vaccinated children and its association with the level of neutralizing antibody. *J Infect Dis.* Sep 15 2011;204(6):873-883.

Supplemental table 1 – Description of the study population

Groups		Sample size	Age range	HIV diagnosis*	Patients on HAART	Duration of HAART*	CD4$^+$ T-cell count (cells/mm^3)*	Viral Load (copies/mL)*	Histopathological status
AIN(-)/HIV(-)		25	24-60	NA	NA	NA	NA	NA	normal
AIN(-)/HIV(+)		32	18-55	39 months	60%	40 (2 a 94 months)	353 (30 - 986)	7,961 (0 – 52,758)	normal
AIN(+)/HIV(+)		28	15-49	35 months	41%	43 (3 a 108 months)	325 (28 - 756)	10,911 (0 - 95,196)	altered
AIN(+)	HSIL+	8	20-39	35 months	41%	43 (3 a 108 months)	325 (28 - 756)	10,911 (0 - 95,196)	HSIL
	LSIL+	20	15-49						LSIL

HSIL - High-grade Squamous Intraepithelial Lesion; HSIL - Low-grade Squamous Intraepithelial Lesion; NA – Not applicable or not performed. * Data refer to mean values and range (Minimum - Maximum).