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T-cell homeostasis, modifications of mucosal immunity populations and association with HIV-mediate Microbial Translocation and dysbyosis: extensive immune phenotyping during 1 year of successful combination Antiretroviral Therapy (cART)

Tesi di Dottorato di Ricerca

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Introduction

Immune dysfunction in HIV infection

Impaired T-cell homeostasis in untreated HIV infection and its persistence during combination antiretroviral therapy (cART)

The inexorable depletion of CD4+ T cells observed in untreated HIV-1 infection is mainly due to the disruption of homeostatic mechanisms. In particular, contraction of memory (CD45RO+) and naive (CD45RA+) T-cells have been described [1, 2] and may be due to continuous antigen presentation and immune reactivation; indeed, increased turnover and preferential infection of memory CD4+ T-cells with their subsequent death result in the recruitment of naıve lymphocytes to the memory pool to overcome these defects [3, 4]. The CD8+ T-cell pool also undergoes homeostatic modifications; in particular, as described by *Paiardini et al*., the expansion of effector CD8+ T-cells is associated to the loss of CD127 (IL-7Rα) and correlates with markers of disease progression (plasma viremia and CD4+ T-cell depletion) as well as with indices of T-cell activation [5]. Indeed, Many CD8+ T-cells also lose expression of IL-7Rα and exhibit an activated effector phenotype [6], particularly in naïve and memory subsets [7, 8].

In this respect, a seminal paper published by *Giorgi et al.* demonstrated that the CD8+ T-cell increases observed in HIV infection are ascribable to the rise of activated cells, thus putting forward a possible key role of this subset in the pathogenesis of HIV disease [9].

This hypothesis is supported by the observation that the natural hosts of the simian immunodeficiency virus (SIV), which fail to develop immunodeficiency and AIDS despite high levels of virus replication, have surprisingly low levels of immune activation in the chronic stage of infection [10, 11]. In accordance with these findings, *Giorgi et al.* demonstrated how the presence of activated CD8+CD38+HLA-DR+ was the major determinant of survival in asymptomatic and advanced HIV-1 disease [12, 13]. After these first evidences, a large body of experimental evidence generated in

both HIV-infected individuals and SIV-infected Rhesus Macaques (RMs) indicates that the establishment of a state of chronic, generalized immune activation is a characteristic feature of pathogenic HIV/SIV infection that is consistently associated with disease progression [14, 15]. Furthermore, in the pre-ART era, also soluble markers of innate immune activation (ie, neopterin, beta2- microglobulin, sCD163) as well as other activation markers (CD14+CD16+) were found to be strongly predictive of rapid progression to AIDS and death [16, 17]. In the setting of T-cell response and activation, co-stimulatory molecule CD28 and the co-inhibitory molecules cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) and programmed death 1 (PD-1; CD279) are particularly important for regulating T-cell responses [18]. Recently, PD-1, gained much attention in viral immunology as it plays a significant role in establishment of virus-specific CD8+ T-cell exhaustion [19, 20] as well as HIV reservoirs [21]. Interestingly, PD-1-expressing CD8+ T-cells lack the expression of the co-stimulatory receptor, CD28, effector functions such as perforin and granzyme-B secretion/ killing, and express lower levels of CCR7 and CD127, which are important molecules for the maintenance of memory T cells [22]. Similarly, recent studies suggest the role of inhibitory molecules also in CD4+ T cell dysfunction. Indeed, PD-1, CTLA-4 and TIM-3 are highly elevated on HIV-specific CD4+ T cells thus participating to virus-specific CD4+ T-cell impairment [23, 24] and have been linked to immune activation, inflammation and decreased production of cytokines [25-28].

“T Memory Stem Cells” (Tscm) also represent a novel lymphocyte subset under thorough investigation in the setting of HIV infection given their ability to differentiate into more mature T cell subset while maintaining their own pool size through homeostatic self-renewal [29]. Tscm cells have been defined by the expression of naïve T cell markers such as CD45RA and CCR7, in tandem with memory T cell markers including CD95, CD27 and CD62L, among others. Such cells were detected within both CD4+ and CD8+ T cell subsets and account for approximately 2-4% of all cells in each compartment. Despite the expression of several naive T cell markers, prior studies demonstrated that Tscm cells could rapidly execute classical lymphocellular effector functions and secrete a number of

different cytokines; so far the presence of CMV, Flu and SIV-specific CD8+ Tscm cells has been formally demonstrated, but CD4+ and CD8+ Tscm cells are likely to contribute to cellular immune responses against any microbial pathogen that challenges the host; and may also be inducible by vaccines or immunogens [30]. Additional properties of Tscm include a long in vivo life span, greater proliferative potential than other T cell memory subsets, and preferential homing to secondary lymphoid tissues [31, 32]. These cells are particularly affected by HIV infection through CCR5 expression on their surface and proposed as a site of persistence given their particular biological properties including high in vivo longevity, relative quiescence, and marked proliferative potential [33-35]; furthermore, the preservation of the CD8+ Tscm subset in the setting of untreated HIV-1 infection is associated with improved viral control and immunity [36].

Combination antiretroviral therapy (cART) has dramatically changed the natural course of HIV infection by suppressing viral replication and reconstituting CD4+ T-cell numbers with subsequent reduction of HIV and AIDS-comorbidities and death [37, 38]. Despite this, increased immune activation and inflammation as well as impaired homeostasis persist during cART treatment [39-42]. Such defects lead to a senescent immune system [43], which has been linked to the development of non-AIDS comorbidities (e.g. osteoporosis [44], atherosclerosis [45], non-AIDS cancers [46], neurocognitive decline [47], liver [48] and kidney disease [49]) even under cART treatment [50-54]. In recent years, a low CD4/CD8 ratio [55], has been proposed as a hallmark of T-cell defects related to aging and a predictor of mortality in the general population [56]; indeed, lack of normalization of the CD4/CD8 ratio during otherwise effective cART is associated with increased innate and adaptive immune activation, an immunosenescent phenotype, and higher risk of morbidity/mortality [57].
Also the role of immune exhaustion has been investigated in the setting of cART-treated infection. Across two large cohorts of treated individuals, *Cockerham et al.* found consistent associations between viremia, CD8+ and CD4+ T-cell activation and PD-1 confirming the relationship between activation and T-cell exhaustion despite cART [58]. Further, *Breton et al.* have identified PD-1 as a

marker of persistent aberrant distribution of memory T-cell subsets in HIV-1 infection despite long term cART [59]. In contrast, recent data seem to suggest that long-term therapy appears to preserve the frequency of CD4+ [60] and CD8+ Tscm [36] as well as the function of the Tscm HIV-specific CD8+ pool [61].

Thus, the discovery of Tscm as the stem cell of cellular immune memory may have critical implications for understanding reason why HIV-infected subjects do bot normalize immune exhaustion parameters, maintaining disrupting T-cell homeostasis despite fully suppressive cART.

The role of the gastrointestinal tract in the pathogenesis of HIV infection

Structural changes in the gastrointestinal tract during HIV infection

Damage to the gastrointestinal (GI) tract occurs early and irreversibly in progressive HIV-1 and SIV infections and is closely linked to systemic inflammation [62]. In particular, numerous GI structural abnormalities have been described in both HIV-1 and SIV infection, such as focal epithelial cell degeneration, malabsorption, and crypt hyperplasia [63] as well as massive enterocyte apoptosis, decreased expression of tight junction proteins, and increased intestinal permeability [64-66]. The net effect of these abnormalities result in focal breaches to gut epithelial barrier with consequent systematically inflammation due to increased Microbial Translocation (see below) and dysregulation of IL-6 and SOCS-3 gene expression [67, 68].

Whether cART restores gut structure is controversial. Introduction of therapy was shown to abrogate the HIV-induced intestinal barrier defects [69], but recent studies demonstrated persistent impairment of the tight junction complex [70-72].

Taken together, these alterations lead to the presence of bacteria and microbial components in the *lamina propria* of both untreated SIV-infected macaques [73, 74] and HIV-infected individuals [75]

as well as passage of gut microbiota and microbial products into the systemic circulation, phenomenon defined as microbial translocation [76].

Microbial translocation, the gastrointestinal microbiota and mucosal immunity during HIV infection

Structural damage to the GI tract as well as mucosal immune balances (see below) promote translocation of commensal bacteria locally and systemically [77]. In the setting of HIV infection, *Brenchley et al.* were the first to demonstrate increased levels of circulating lipolysaccharide (LPS), marker of microbial translocation, in both chronically HIV-infected subjects and SIV-infected RM, which was linked to innate and adaptive immune activation [76]. Many studies confirmed such findings and investigated the possible association of microbial translocation with the clinical outcome in HIV infection [78]. In this context, microbial translocation was found to be an independent predictor of disease progression and mortality [79, 80] as well as a key promoter of non-AIDS co-morbidities and viral liver infections [81-83].

HIV infection also alters the composition of the intestinal microbiota, which is known to contribute to the maintenance of gut homeostasis [84, 85]. Indeed, an early study showed significant dysbiosis of the faecal biota in untreated HIV-1–infected subjects with a predominance of opportunistic pathogens (*Pseudomonas aeruginosa* and *Candida albicans*) and low levels of protective bacteria (bifidobacteria and lactobacilli) compared to uninfected individuals. This dysbiosis was associated with increased faecal calprotectin, finding that is clearly indicative of a significant GI inflammation. [86] First data of correlation between gut microbial community and systemic immunological abnormalities was found by *Ellis et al.* who reported that the proportions of *Enterobacteriales* and *Bacteroidales* were significantly correlated with duodenal CD4+ T-cell depletion and peripheral CD8+ T-cell activation, respectively [87], supporting evidence of a direct role of the gut microbiota in driving local and systemic immune activation in HIV-infected patients [88-90]. In accordance to

these evidences, *Perez-Santiago et al.* showed that shaping the gut microbiome, especially proportions of *Lactobacillales*, could help preserve immune function during HIV infection [91]. . Whether cART can restore dysbiosis occurring during the course of HIV-infection is still a matter of debate. In this context, *Nowak et al.* showed how microbiota alterations are closely associated with immune dysfunction in HIV-1 patients, and these changes persist during short-term cART [92].

During HIV infection the GI tract is also characterized by a marked depletion of CD4+ cells [93-96], given the large numbers of target cells that express the HIV CCR5 co-receptor for entry [97, 98]. Aside from CD4+ T-cells, however, the GI tract is home to other populations which defend the mucosa through the production of cytokines such as IL-17 and IL-22 [99, 100]. Indeed, novel work has established a role for IL-17 and IL-22-secreting T cell populations in limiting microbial translocation and systemic T-cell activation/inflammation, by showing loss of Th22, a shift away from Th22 and Th17 to Treg cells as well as a negative correlation between immune activation and Th17/Th22 proportions [101]. In the setting of T-cell populations-producing IL-17 and IL-22, *Cosgrove et al.* have demonstrated an early decrease of CD8+CD161++ tissue-infiltrating populations, defined Mucosal Associated Invariant T cells (MAIT), during acute and chronic infection [102]. These modifications may impact mucosal defense and could be important in susceptibility to specific opportunistic infections in HIV.

Further, HIV infection impairs T-cell subsets expressing gut-homing markers such as $\alpha 4\beta 7$ (including Tscm, [36]), CCR6 and CCR9, both in the peripheral blood and GI mucosa [103-107] which are known to regulate the trafficking of immune cells from periphery into the gut.

Whether cART restores gut immune function is still matter of debate. Overall, therapy seemingly normalizes the frequencies of mucosal immune subsets if initiated early in the course of infection, while their function invariably remains impaired [108-111].

It is thus critical to investigate the underlying mechanism of poor recovery on effective cART, focusing on link between microbial translocation, modification of intestinal microbiota and mucosal immune homeostasis, possibly contributing to improving HIV prognoses and life expectancy.

Study rationale, objective and specific aims

In the era of combination antiretroviral therapy (cART), a remarkable reduction of HIV and AIDS-comorbidities and death has been described [37, 38]. Nonetheless, immune defects persist during treatment [39-41, 112, 113] and may be causally linked to increased morbidity and mortality of HIV-infected subjects compared to the general population [114-116].

Microbial translocation and dysbiosis as well as impaired mucosal immunity likely represent underlying pathogenic mechanisms of the peripheral immune flaws observed in the course of virologically-effective cART [76, 88, 89, 117, 118]. However, a systematic investigation of these parameters in a longitudinal cohort of HIV-infected individuals starting cART is currently lacking.

In this context, the overall objective of our research is to understand the extent by which enduring gut abnormalities represent a cause of impaired T-cell homeostasis during combination antiretroviral therapy (cART). In particular, we aim to pursue our objective through the following specific aims:

1) Specific Aim 1: study of the modifications of T-cell homeostasis, microbial translocation, gastrointestinal function and faecal microbiota composition in a cohort of HIV-infected, antiretroviral-naïve subjects prior to and following 12 months of cART.
2) Specific Aim 2: study of the contribution of CD4+CD161+CCR6+, CD4+CCR9+α4β7+ (*"gut-homing phenotype")* and Tscm cells in sustaining peripheral immune defects in HIV-infected, antiretroviral-naïve subjects prior to and following 12 months of cART (immunological substudy).

Patients and Methods

Specific Aim 1: Study of the modifications of T-cell homeostasis, microbial translocation, gastrointestinal function and faecal microbiota composition in a cohort of HIV-infected, antiretroviral-naïve subjects prior to and following 12 months of cART.

Patients

HIV-infected, antiretroviral-naïve subjects introducing cART (T0) were consecutively recruited at the Clinic of Infectious Diseases and Tropical Medicine, Dept of Health Sciences, ASST Santi Paolo e Carlo, University of Milan, Italy following the provision of informed consent.

Only subjects presenting virological suppression (<40 cp/mL) and active follow-up after 12 months of treatment (T12) were included in the study

The Ethics Committee of our Institution approved the study and participants provided written informed consent.

Microbial translocation parameters

Plasma levels of sCD14 (R&D systems) and EndocAb (Hycult Biotech) were measured by ELISA test. Circulating levels of lipopolysaccharide (LPS) were assessed using the LAL test (Lonza), prior to 1:150 diluition and preheating at 95°C for 10 minutes.

Gastrointestinal functional markers: LAC/MAN fractional excretion ratio and Intestinal Fatty Acid Binding Protein (I-FABP)

The urinary lactulose-mannitol fractional excretion ratio (LAC/MAN) was used to assess small intestinal permeability. Patients were asked to fast the night before and to collect their morning urine before drinking a sugar probe solution containing 5 g of lactulose and 1 g of mannitol in approximately 100mL of water. Urine was collected for 5 hours following administration of the double sugar solution and patients did not eat or drink (with the exception of water) until the end of the 5-hour collection. The total volume of urine was recorded and an aliquot of 30 mL preserved with chlorhexidine (0.236 mg/mL of urine; Sigma Chemical, St Louis, MO, USA) was frozen and stored for High Performance Liquid Chromatography (HPLC) analysis of lactulose and mannitol (Dionex MA-1 ion exchange column with pulsed amperometric detection on a Dionex Ion Chromatograph 3000, Thermo Scientific, Sunnyvale, CA).

Intestinal Fatty Acid Binding Protein (I-FABP) was assessed by ELISA (Hycult Biotech).

Faecal calprotectin quantification and microbial population analysis

Faeces were collected at T0 and T12, frozen at -20°C and then thawed for genome extraction. Total bacterial DNA was extracted from 200 mg of faeces using the PSP Spin Stool DNA Plus kit (Stratec Molecular, Berlin, Germany) in accordance with the manufacturer’s instructions.

Faecal calprotectin was tested by ELISA (PhiCal, Eurospital, Italy).

Analysis of the microbial population was executed as previously described [70]. Following amplification of the V2–V3 region of the 16S ribosomal DNA (rDNA) gene, using primers HDA1-GC and HDA2, denaturing gradient gel electrophoresis (DGGE) was performed using PhorU system (Ingeny, Netherlands) [119]. Banding patterns were analysed (Fingerprinting II software Bio-Rad Laboratories, Bio-rad, Italia) using Pearson’s coefficient and UPGMA method to generate dendograms; after excision and amplification of the bands through primers HDA1 and HDA2, the

final PCR products purified (WizardSVGel,PCR Clean-UpSystem) were analysed with sequencing of PrimmBiotech srl (Milan, Italy), bacterial identification was confirmed through a sequence search in the GenBank DNA database using the Basic Local Alignment Search Tool algorithm (http://www.ncbi.nlm.nih.gov/) and ribosomal RDP database (http://rdp.cme.msu.edu/). The bacterial taxa reported in literature with a key-role in inflammation and gut permeability-modification were quantified through Real Time PCR using StepOne method (Applied Biosystems, USA); hence we selected four genera (Lactobacillus, Roseburia, Bacteroides and Prevotella) and one family (Enterobacteriaceae) for statistical analyses.

Flow cytometry surface staining

Fresh peripheral blood was drawn from all study participants in EDTA-containing tubes and were stained for flow cytometry evaluation using the following fluorochrome-labelled antibodies: CD4-PECy7, CD8-APC, CD127-PE, CD38-FITC, CD45R0-PE, CD45RA-FITC (BD Biosciences, San Jose, California, USA).

The following combinations were used: CD4/CD8/CD45/CD127/CD45RA and CD8/CD45/CD45R0/CD38 to assesses naïve (CD45), activated (CD38+), memory activated (CD38+CD45R0+) and IL-7Rα+ (CD127+) both in CD4 and CD8 T-cell subsets as described in literature [120-122].

Briefly, 50 µl of whole blood were stained for 30 min in the dark at 4°C, incubated at dark with 1 mL of Lysing Solution 10X Concentrate (BD Biosciences, San Jose, California, USA) for lysing red blood cells and then washed twice with 1 ml of PBS.

Before using, Lysing Solution was diluted 1:10 with distilled water.

Cells were run on a FACS CANTO 2.6 cytometer (BD Biosciences, San Jose, California, USA) and analyzed with FACS Diva 6.1.3 software. Cells were gated first based on side- and forward-scatter properties, then for as CD4 and/or CD8 and finally as CD45RA/CD127 or CD45R0/CD38.

Statistical analyses

Statistical analyses were performed with the use of GraphPad Prism 6.0. Wilcoxon test was used for statistics. Chi-squared test was used for categorical variables. Correlations were assessed by Spearman's rank coefficient. A p value <0.05 was considered statistically significant.

2) Specific Aim 2: Study of the contribution of CD4+CD161+CCR6+, CD4+CCR9+α4β7+ and Tscm cells in sustaining peripheral immune defects in HIV-infected antiretroviral-naïve subjects prior to and following 12 months of cART (immunological substudy).

Patients

A subgroup of 28 HIV-infected, antiretroviral-naïve subjects introducing cART (T0) with available cryopreserved biological samples were selected from the cohort of patients enrolled in Aim 1. 18 HIV-uninfected age- and sex-matched individuals were selected as controls.

The Ethics Committee of our Institution approved the study and participants provided written informed consent.

Human lymphocyte separation

Fresh peripheral blood was drawn from all study participants in EDA-containing tubes and PBMCs were separated by Ficoll-Histopaque technique (Biocoll separating solution, BIOSPA), collected in 500 uL of R10 medium (composition per 100mL R10: 88mL RPMI, 10mL fetal bovine serum, 1mL [100UI/mL] L-glutamine and 1 ml 20 [100UI/mL] penicillin/streptomycin; Euroclone, Italy) and 500 uL of freezing solution (80% RPMI, Euroclone, Italy, 20% Dimethyl sulfoxide, DMSO, Saint Louis, Missouri, USA) and cryopreserved in liquid nitrogen.

Flow Cytometry surface staining

Cryopreserved PBMCs collected at T0 and T12 were rapidly thawed by immersing cryovials in a 37 °C water bath shaking it gently until only a small ice crystal remained. Cells were then immediately transferred into 10 ml of pre-warmed RPMI 1640 medium (+10% FBS and 1% penicillin and streptomycin). After centrifuging, cells were suspended in 10 ml of R10, stained with trypan blue dye and counted. Then, $1x10^6$ cells were stained with fluorochrome-labeled antibodies for the flow cytometric study of lymphocyte surface phenotypes. To check cell viability, cells were stained with 7-aminoactynomycin D (7-AAD, BD Biosciences, San Jose, California, USA) for 30 min in the dark at 4°C. Only samples with cellular viability greater than 70% were used for experiments.

The following antibodies were used: HLA-DR-FITC, CD38-PE, CCR7-PeCy7, CD45RA-PeCy5, PD-1-PE, CD27-PE, CD95-APC, α4β7integrin-APC CCR6-PeCy7, CD161-APC (BD Biosciences, San Jose, California, USA), CCR9-FITC (R&D Systems, Minneapolis, MN, USA).

We evaluated CD4+ and CD8+ activation (HLA-DR+CD38+), maturation (naïve: CCR7+CD45RA+; central memory: CCR7+CD45RA-; effector memory: CCR7-CD45RA-; terminally differentiated: CCR7-CD45RA+) exhaustion (PD-1+), the frequency of stem cell-like memory T cells (Tscm; CCR7+CD45RA+CD27+CD95+) and that of CD4+ T-cells with a "gut homing" (CCR9+α4β7+) and a "Th17/Th22" phenotype (CCR6+CD161+).

Cells were run on a FACS VERSE cytometer (BD Biosciences, San Jose, California, USA) and analyzed with FlowJo V10 (FlowJo LLC, Ashland, Oregon, USA).

Statistical analysis

All continuous variables are presented as median and interquartile ranges (25th-75th percentile), while categorical data are shown as absolute numbers and percentages. The Mann Whitney U test,

Wilcoxon test and Chi squared test were used for the comparison between 2 groups. p values <0.05 were considered significant. Statistics were performed using GraphPad Prism 6 software.

Results

Specific Aim 1: Study of the modifications of T-cell homeostasis, microbial translocation, gastrointestinal function and faecal microbiota composition in a cohort of HIV-infected, antiretroviral-naïve subjects prior to and following 12 months of cART.

Patient population

188 antiretroviral-naïve HIV-infected subjects were consecutively enrolled (T0) and followed for 12 months after cART introduction (T12). After 12 months, 160 patients presented virological suppression (<40 cp/mL) and were included in to the study. Demographic and viro-immunologic parameters of study subjects are shown in Table 1. At baseline, median HIV RNA load, CD4+ T-cell counts, and CD4+/CD8+ ratio were $\log_{10}$ 5.0 cp/mL (IQR 4.6-5.5), 303 cells/ul (IQR: 273-428) and 0.31 (IQR 0.23-0.41), respectively. Following treatment, all subjects presented viral suppression following treatment as per inclusion criteria ($\log_{10}$ HIV RNA: 1.6 cp/mL, IQR 1.6-1.6; p=0.0001), a significant recovery in CD4+ T-cell numbers (511 cells/mmc; IQR 507-642; p=0.0001) and increase of the CD4+/CD8+ T-cell ratio (0.59, IQR 0.42-0.73; p=0.0001) (Table 1).

Significant increase in CD4+CD127+ and naïve T-cells as well as a reduction in CD8+ activated/memory T-cells following cART

We investigated the possible changes in T-cell maturation and activation during the first 12 months of cART in a cohort of naive, antiretroviral-naive individuals.

We observed a significant increase in expressing-IL-7Rα (CD4+CD127+, T0: 11% IQR: 6-13 vs T12: 16% IQR: 12-19, $p<0.0001$; Figure 1A) and naïve CD4+ T-cells (CD4+CD45RA+, T0: 6% IQR: 3-9 vs T12: 10% IQR: 6-14, $p<0.0001$; Figure 1B) which paralleled the reduction of activated (CD8+CD38+; T0: 12% IQR: 7-18; p=<0.0001; Figure 1C) and memory activated T-cells (CD8+CD38+CD45R0+; T0: 4% IQR: 4-11 vs 1% IQR: 0-1; $p<0.0001$; Figure 1D).

No differences were observed in terms of naive and central memory CD8+ T-cells (not shown).

Persistent microbial translocation following cART

In our cohort, no significant modifications in microbial translocation parameters were observed Indeed, after 12 months of cART, subjects showed stable levels of LPS (T0: 147,6 pg/ml IQR: 84.60-231.9, vs T12 138.6 pg/ml IQR: 81.28-280.7; p=0.83; Figure 2A), sCD14 (T0 5.41 ng/ml IQR: 4.16-7.1 vs T12 5.3 ng/ml IQR: 3.9-7.82; p=0.76; Figure 2B) and EndocAb (T0 48.4 MMU/ml IQR: 24.5-89.9 vs T12: 44.4 MMU/ml IQR: 23.5-86.4, p=0.82; Figure 2C).

No modifications in intestinal permeability, yet increased damage and reduced inflammation following cART

Intestinal permeability (urinary lactulose-mannitol fractional excretion ratio, LAC/MAN) and function (Intestinal Fatty Acid Binding Protein, I-FABP) as well as gut inflammation (faecal calprotectin) were measured in our cohort. While no statistical differences in urinary LAC/MAN ratio were found (T0: 0.017 IQR: 0.012-0.05 vs T12: 0.026 IQR: 0.021-0.035; p= 0.45; Figure 3A), we registered an increase in I-FABP (T0: 586.9 pg/ml IQR: 392,8- 801,2 vs T12: 843,6 IQR: 489,4-1084; p=0.0002; Figure 3B) and a decay in faecal calprotectin levels (T0: 67.33 IQR: 26.13-199.1 vs T12: 28.15 IQR: 15.59-135.7: p=0.0099; Figure 3C).

Qualitative analysis of the fecal microbiome revealed an outgrowth of Lactobacillus and Bacteroides spp. as well as Proteobacteria following cART

Given that HIV infection modifies the composition of the gut microbiota with possible effects on gut permeability and inflammation, we performed a deep molecular characterization of the faecal microbiome in our cohort. In particular, we amplified by DGGE analysis (figure 4) the V2–V3 region of the 16S rRNA gene of the following bacteria: *Prevotella copri*, *Faecalibacterium prausnitzii*, *Roseubria intestinalis*, *Clostridium spp*, *Flavonifractor plautii*, *Eubacterium rectale*, *Bacillus spp*, *Lactobacillus ruogosa*, *Lactobacillus spp*, *Acidminococcus intestini*, *Bacteroides spp*, *Desulfovibrio spp*, *Phascolarctobacterium succinatutens*, *Enterococcus faecium*, *Parabacteroides distasonis*.

Qualitative analysis of these 15 bacterial species belonging to the Firmicutes, Bacteroidetes and Proteobcateria phyla did not show significant variations in the course of the study (Table 2). However, quantitative analysis of selected genera showed a significant increase in Lactobacillus (Firmicutes) (T0: 9.21 genomes/mcg feces IQR: 8.65-9.68 vs T12: 9.69 genomes/mcg feces IQR: 9.37-10.11; $p<0.0001$; Figure 5A) and Bacteroides (Bacteroidetes) (T0: 9.74 genomes/mcg feces IQR: 9.15-10.28 vs T12: 10.6 genomes/mcg feces IQR: 9.61-11.17; $p=0.0006$; Figure 5D), yet no modifications of Roseburia (Firmicutes) and Prevotella (Bacteroidetes) (Figure 5B, C); a significant increase of the Enterobacteriacae family (Proteobacteria) was also observed (T0: 8.26 genomes/mcg feces IQR: 6.81-9.51 vs T12: 8.95 genomes/mcg feces IQR: 7.44-10.13; $p=0.027$ Figure 5E).

Specific Aim 2: Study of the contribution of CD4+CD161+CCR6+, CD4+CCR9+α4β7+ and Tscm cells in sustaining peripheral immune defects in HIV-infected antiretroviral-naïve subjects prior to and following 12 months of cART (immunological substudy).

Patients

28 HIV-infected, antiretroviral-naïve subjects introducing cART (T0), were selected from the cohort of 160 patients enrolled for Aim 1. 18 HIV-uninfected age- and sex-matched individuals were enrolled as controls.

In line with demographic and viro-immunological findings observed in Aim 1, at baseline median HIV RNA load, CD4+ T-cell counts, and CD4+/CD8+ ratio were $\log_{10}$ 4.7 cp/mL (IQR 4.2-5.3), 366 cells/ul (IQR: 273-428) and 0.3 (IQR 0.2-0.4), respectively. Following treatment, all subjects presented viral suppression following treatment ($\log_{10}$ HIV RNA: 1.6 cp/mL, IQR 1.6-1.6; p=0.0001), a significant recovery in CD4+ T-cell numbers (477 cells/mmc; IQR 269-589; p=0.0001) and increase of the CD4+/CD8+ T-cell ratio (0.5, IQR 0.4-0.6; p=0.0001) (Table 3). HIV-uninfected individuals presented comparable values in terms of age and sex (age: 33 years, IQR 29-38; p=0.08) (females: n=3, 19%; p=0.8) (Table 3).

Decreased T-cell activation yet impaired T-cell maturation despite PD-1 down-regulation in HIV-infected subjects introducing cART

We first analysed T-cell immune activation, defined as the co-expression of HLA-DR/CD38 on T-cells and observed a significant reduction of activated CD4+ (T0: 5.4% IQR: 3.2-9.8; T12: 2.2% IQR: 1.2-4; p=0.02; Fig.6A) and CD8+ lymphocytes (T0: 6.4% IQR: 3.5-13.9; T12: 2.2% IQR: 1-6.5;

p=0.0003; Fig.6B) following cART introduction, reaching levels comparable to those observed in uninfected controls (respectively, 2.2% IQR: 0.7-5.6, p=0.7; Fig.6A; and 1.8% IQR: 0.6-3.1, p=0.3; Fig. 6B).

Analysis of T-cell maturation was then evaluated. A significant reduction in CD4+ effector memory subsets (CCR7-CD45RA-) was observed in the course of cART (T0: 42.9% IQR: 24.4-51.9; T12: 31.5% IQR: 19.6-44.3; p=0.01; Fig. 7C), leading to persistent impairment of this subset compared to controls (HIV neg; 50.1% IQR: 39.9-63-3; p=0.04 and p=0.002 for comparison at T0 and T12 respectively; Figure 7C). No major variation in the frequency of the remaining CD4+ and CD8+ T-cell subsets was observed (Figure 7A,B,D and Figure 8A-D respectively).

To further characterize T-cell homeostasis in HIV-infected individuals starting cART, we assessed the expression of PD-1, a marker of cellular exhaustion. Overall, a hierarchy in PD-1 expression was observed, with the highest levels in cART-naïve subjects, followed by those measured in treated individuals and uninfected controls (Figure 9-10). Of note, PD-1 expression in the CD4+ effector memory subset did not vary significantly in the course of the study and was comparable to that registered in HIV-uninfected individuals (Figure 9C), suggesting other mechanisms may be involved in the persistent skewing of this pool in the course of treatment.

Persistent impairment of Tscm in HIV-infected subjects introducing cART

We next studied the effects of therapy on the frequency of CD4+ and CD8+ Tscm. We report a significant reduction of the CD4+ Tscm subset in HIV-infected subjects during the first 12 months of cART (T0: 2.9% IQR: 1.1-9.1; T12: 1.6% IQR: 1.0-2.5; p=0.002; Fig. 11B) and no variations of the CD8+ Tscm pool (T0: 1.4% IQR: 0.7-2.5; T12: 1.2% IQR: 0.8-1.9; p=1; Fig. 11C). Overall, HIV infection accounted for lower CD4+ and CD8+ Tscm frequencies compared to uninfected controls (CD4+ Tscm 5.2% IQR: 3.6-12; see above for HIV+; p=0.04; Fig. 11A; CD8+ Tscm: 3.7% IQR: 2.2-

6.5; see above for HIV+; p=0.002; Fig. 11C), which were not restored by cART (p=0.001 and p=0.006 respectively; Fig. 11B, C).

To explore the existing relationship between Tscm and T-cell maturation subsets, correlation analyses were first performed in uninfected controls. Tscm correlated negatively with naïve cells (CD4+: r=-0.7; p=0.004; Fig. 11C; CD8+: r=-0.7; p=0.006; Fig. 11E) and positively with effector memory cells (CD4+: r=0.6; p=0.01; Fig. 11D; CD8+; r=0.6; p=0.01; Fig. 11F). In HIV disease, these correlations were lost in the course of untreated infection (Fig. 10G-J) and only the relationship between CD4+ naïve and Tscm cells was restored in the course of cART (Fig 10K-N).

Partial modification of CD4+CD161+CCR6+, CD4+CCR9+α4β7+ cell frequencies in HIV-infected subjects introducing cART

Given the role of the GI tract in driving immune activation and other abnormalities in the course of both untreated and treated HIV infection, microbial translocation, gut inflammation and the composition of the faecal microbiota were investigated in our cohort.

As observed in Aim 1, no differences were detected in terms of microbial translocation (LPS; p=0.9; sCD14; p=0.8; EndocAb; p=0.9;) or gut inflammation parameters (calprotectin; p=0.3) prior to and following treatment. Similarly, our data on the composition of the faecal microbiota showed the same results reported in Aim 1, i.e. a significant increase in the quantity of Lactobacillus and Bacteroides genera (p=0.02 and p=0.04 respectively), no modifications of Roseburia (p=0.2), Prevotella (p=1.0) and Enterobacteriacae family (p=0.2).

Given the capacity of CD4+CCR6+CD161+ to produce IL-17 and IL-22 and their role in maintaining mucosal barrier integrity, the frequency of this subset was also analysed. Despite their enrichment in the course of cART (T0: 3.8% IQR: 2.6-6.3; T12: 5% IQR: 3.1-7.2; p=0.03; Fig. 12B), Th17/Th22 subsets maintained significantly lower frequencies compared to HIV-uninfected controls (CCR6+CD161+ in HIV-: 8.3% IQR: 5.4-13.1; see above for HIV+; p=0.04; Fig. 12B). Interestingly,

this subset correlated positively with CD4+ Tscm prior to cART (r=0.6; p=0.002; Fig. 12C), and not during treatment (r=0.2; p=0.4, data not shown).

CD4+ T-cells with a "gut-homing" (CCR9+ α4β7+) phenotype were also assessed in our study. We report a progressive contraction of this subset (T0: 3.4% IQR: 1.7-4.2; T12: 1.7% IQR: 1-3.5; p=0.02; Fig. 13B), which maintained significantly lower frequencies compared to HIV-uninfected controls (CCR9+ α4β7+ in HIV-: 3.1% IQR: 1.7-6.7; see above for HIV+; p=0.04; Fig. 13B). Considering that expression of α4β7 can be sufficient for gut homing of T-cell populations, we analysed the frequency of this marker on CD4+ T-cell population, but no statistical differences were observed (data not shown).

A positive correlation was found between T cells with a "gut-homing" phenotype and plasma HIV RNA prior to cART introduction (r=0.5; p=0.003; Fig. 13C). In line with this finding, CD4+CCR9+ α4β7+ cells also correlated with activated CD4+HLA-DR+CD38+ (r=0.5, p=0.003; Fig 13D) at the same time-point; this association was nonetheless lost in the course suppressive treatment (r=0.4; p=0.03; Fig. 13E).

Discussion

The overall objective of our research was to understand the extent by which enduring gut abnormalities represent a cause of impaired T-cell homeostasis during combination antiretroviral therapy (cART). We pursued our objective through two specific aims:

Specific Aim 1: study of the modifications of T-cell homeostasis, microbial translocation, gastrointestinal function and faecal microbiota composition in a cohort of HIV-infected, antiretroviral-naïve subjects prior to and following 12 months of cART.

Specific Aim 2: study of the contribution of CD4+CD161+CCR6+, CD4+CCR9+α4β7+ (*"gut-homing phenotype")* and Tscm cells in sustaining peripheral immune defects in HIV-infected antiretroviral-naïve subjects prior to and following 12 months of cART (immunological substudy).

Building on earlier research showing that HIV-infected patients do not recovery proper T-cell homeostasis and levels of immune activation comparable to uninfected controls despite cART [123, 124] and considering the active role played by microbial translocation in supporting immune activation/inflammation [74, 76], we aimed to study the effects of 12 months of cART on different immune and gut function parameters in a cohort of HIV-infected, antiretroviral-naive subjects. In particular, we first conducted a longitudinal study to assess the kinetics of T-cell homeostasis, microbial translocation, intestinal inflammation and faecal microbiota composition in subjects introducing cART. Our cohort was composed of moderately advanced HIV-infected individuals (median CD4 T cell count: 303/uL) who displayed increases of CD4+CD127+ and memory T-cells in and a parallel reduction of activated CD8+ lymphocytes, thus suggesting that one year of virologically-suppressive cART ameliorates peripheral T-cell homeostasis. Moreover, data on intestinal inflammation revealed a reduction of faecal calprotectin, corroborating a positive effect of cART in reducing intestinal inflammation. In particular, to our knowledge, this is the first data which showed a decrease of this marker in a cohort of treated HIV-infected subjects free from

gastrointestinal disease. Indeed, previous studies assessed faecal calprotectin in naïve populations [125] [86] and showed that high levels of calprotectin associated with early impairment of the GI tract and clinical progression of HIV infection.

We then asked if our findings were linked to possible changes of the faecal microbiota with cART-mediated increases of bacteria known to exert a protective role on the GI tract and systemic immune parameters [91]. Qualitative analysis of 15 species belonging to the Firmicutes, Bacteroidetes and Proteobcateria phyla did not show significant variations in the course of the study or association with parameters of intestinal inflammation/peripheral immune activation. However, quantitative analysis of selected species showed a significant increase in Lactobacillus and Bacteroides, together with a significant increase of the Enterobacteriacae family, and no modifications of Roseburia and Prevotella, pointing to a limited effect of 12 months of cART in changing the composition of the faecal microbiota. In line with this finding, we did not observe any significant variation of microbial translocation markers in the course of our study. These evidences are in contrast with previous reports of reduced microbial translocation following cART [126, 127] and may be due to the immunological characteristics of our cohort. as well as the length of follow-up. We cannot rule out that discrepancies between our data and previous research may be, in part, because of differences in patients' characteristic, mainly CD4+ T-cell count, which appear to be lower in literature study [127] compared to our report. Additionally, another study showed reduced microbial translocation in early treated HIV-infected patients [126] and point to time of introducing therapy may be crucial to reduced microbial translocation. Thus, it might be hypothesised that longer cART duration might result in a most substantial abatement of microbial translocation markers that is not captured at 1-year follow up. In this respect, a progressive increase of I-FABP levels, a marker of gut barrier damage, was noted in our study and in agreement with previous work by *Chevalier et al.* [128], may suggest lack of mucosal restoration in the first year of cART. This is consistent with the decreased expression of genes involved in the regulation of epithelial barrier maintenance reported after 1–2 months in primary HIV infection [129], although we cannot exclude that impairment of the mucosal barrier

occurred prior to the detection of I-FABP in the peripheral blood. In accordance with this hypothesis, comparable levels of the LAC/MAN ratio measuring gut permeability were comparable both before and in the course of cART confirming that a longer duration of therapy might be necessary to fully restore the structure and function of the gastrointestinal epithelial barrier observed through parameters investigated in our study.

In conclusion, the first aim of our study shows that HIV-infected individuals, after one year of cART, despite the amelioration of peripheral immune parameters, feature incomplete restoration of gut function which may sustain the passage of microbial components to the systemic circulation. This is supported by a general unchanged in microbial translocation parameters and gut barrier markers investigated, despite an amelioration in term of gut inflammation. This observation points out multiple aspects involved in the control of gut function, putting emphasis on the key role that might be played by genes involved in the control of gut functionality. In this respect, further studies should investigate molecular pathways which can regulate expression of molecules accounting for gut barrier integrity.

In order to reconcile the above-mentioned findings, we aimed to assess whether a link between mucosal cell populations and persistent defects in peripheral T-cell homeostasis exists in the context of treated HIV disease.

We thus conducted an immunological substudy in a group of subjects enrolled in Aim 1 and in age- and sex-matched controls. We confirm a reduction of activated CD4+ and CD8+ T-cells following treatment, reaching the levels observed in HIV-uninfected individuals, further supporting the positive effect of therapy in decrease immune activation described in the literature [39, 42]. The finding of similar T-cell activation in HIV-infected individuals and uninfected controls, needs further investigation through the expansion of the study sample size to confirm our observation.

The analyses of T-cell homeostasis overall showed stable maturation phenotypes in the course of cART. In contrast however, PD-1 expression showed a significant decline in naïve T-cell subjects, pointing to reduced exhaustion of this compartment and possibly implying decreases in the cellular

reservoirs in the first 12 months of treatment [21]. An exception to these findings is represented by the progressive decline of CD4+ effector memory cells and a non-significant reduction in PD-1 expression in this pool, in accordance with previous results from a cross-sectional study [59].

The reconstitution of the T-cell compartment was further investigated through the kinetics of Tscm. While confirming the ability of this subset to transition to memory pools in HIV-infected individuals, we show a differential effect of cART in CD4+ and CD8+ Tscm. Indeed, while CD8+ Tscm frequencies remained stable over time, thus confirming literature data that prolonged therapy may restore this subset [36, 61], we are the first to report a progressive contraction of CD4+ Tscm in the course of cART in HIV-infected individuals. This is in contrast to what recently described in the animal model [60] and may explain the persistent impairment of the CD4+ Tscm pool given the lack of correlation between these two subsets in HIV-infected individuals. Further, the reduction of peripheral CD4+ Tscm may be due to their recruitment to the gut, given their expression of the gut-homing marker α4β7 [36]. In line with this finding, also T-cells with a "gut homing" phenotype (CD4+CCR9+β7+) showed a progressive decline in the course of cART, possibly suggesting the hypothesis of a positive effect of cART in restoring mucosal immune cells through their migration from the periphery as hypothesized by *Mavigner and colleagues* in their work conducted on long-term cART experienced individuals [110]. Our different findings respect to *Mavigner's* paper could reflect an early attempt by the immune system to resolve HIV infection through migration of this population in to the gut during first year of infection. On the other hand, CD4+CD161+CCR6+ cells showed an increase in the peripheral blood of HIV-infected subjects introducing cART, yet did not reach the levels described in uninfected controls, putting forward incomplete immune restoration of mucosal immune subsets in the setting of virologically-suppressive therapy.

In conclusion, our experiments whose results are summarized in figure 14 revealed:

1. The amelioration of T-cell homeostasis consisting in the reduction of T-cell activation and exhaustion parameters; the analysis of Tscm subsets suggests a differential effect of cART on CD4+ and CD8+ populations which needs to be confirmed by longer follow-up and enrolment of larger study populations.
2. The persistence of microbial translocation and intestinal damage/permeability in the course of cART, which may be linked to the impairment of chemokine receptor-expressing T-cells like CD4+CD161+CCR6+ and CD4+CCR9+α4β7+. These results require additional experiments to confirm their migration from the peripheral blood to the gut as well as their function.

Many open questions remained unanswered and others are generated by our study. Besides longer follow-up and enrolment of larger study populations, study frequency of T-cell populations involved in the maintenance of intestinal homeostasis straight to the gut biopsies may be helpful to shed light on the recruitment of specific subset to the gut. In this respect, is crucial to investigate molecules involved in T-cell recruitment if different tracts of the gut like CCL20, CCL25 and MADCAM-1 to clarify reasons underlying unbalanced distribution of T-cell populations investigated in our study during cART. Moreover, it would be reasonable to include analyses of gut-homing CD8 populations to collect information about these subsets.

Given the key role of the gut in the establishment and maintenance of HIV reservoir, would be interesting assessed the reservoir harbouring in these specific subsets.

Results of these further studies may identify new target for therapy aimed to blocking these chemokine receptors through biological drugs by looking to therapies used for bowel diseases.

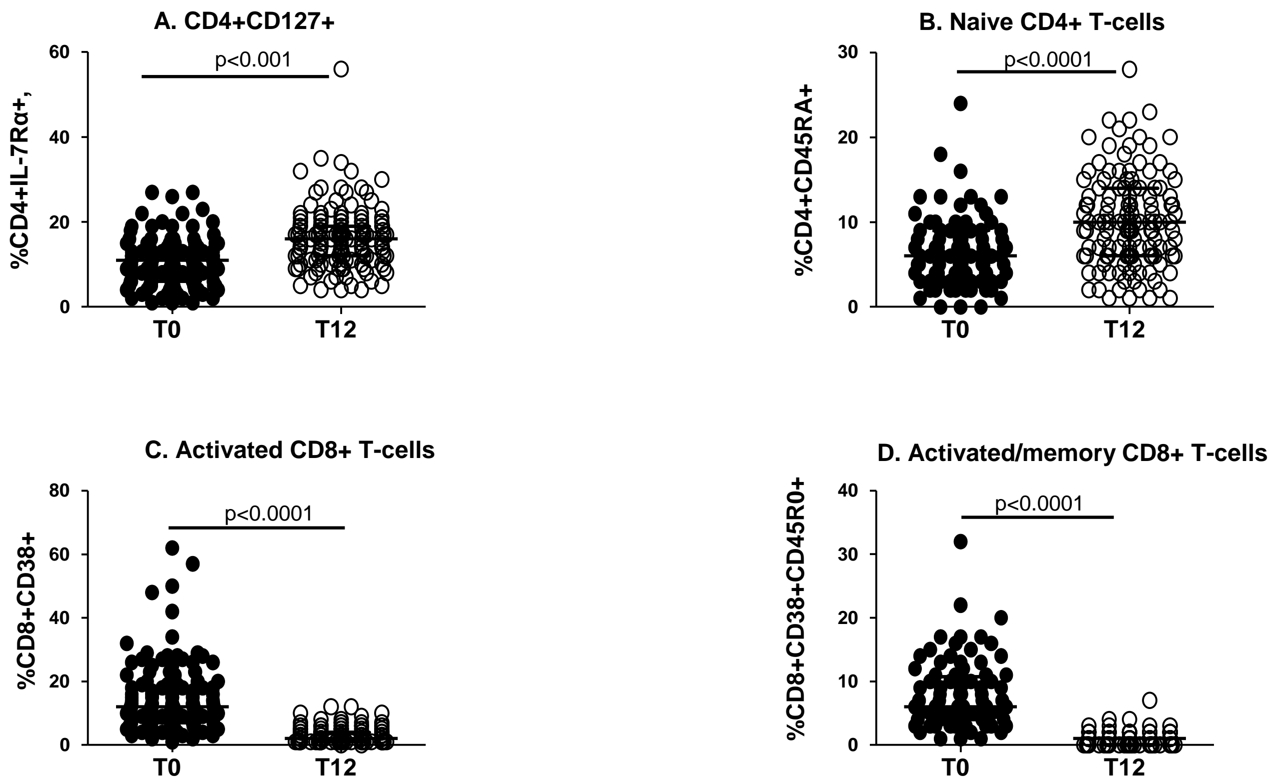
Tables and Figures

Table 1. Clinical characteristics of study patients (Aim 1)

Parameter at baseline	HIV-infected subjects T0	
Sex, F (%)	22 (14%)	
Age, years (IQR)	37 (31-45)	
Risk factors for HIV infection, n (%)		
Heterosexual	49 (31)	
MSM	102 (64)	
IDU	9 (6)	
HCV co-infection n (%)	15 (9)	
HBV co-infection	6 (4)	
Antibiotic prophylaxis, n (%)	31 (19)	
Duration of HIV infection, months (IQR)	12 (2-40)	
AIDS diagnosis, n (%)	19 (12)	
First cART regimen n, (%)		
PI-based	68 (43)	
NNRTI-based	75 (47)	
INI-based	8 (5)	
Other	9 (6)	
Parameter in the course of the study	**HIV-infected subjects T12**	
	T0	**T12**
HIV RNA log_{10} cp/ml (IQR)	5.0 (4.6-5.5)	1.6 (1.6-1.6)*
CD4+ T-cell count, cell/mmc (IQR)	303 (195-377)	511 (507-642)*
CD4+ T-cell, % (IQR)	19 (14-23)	28 (22-32)*
CD8+ T-cell count, cell/mmc (IQR)	903 (659-1175)	886 (684-1123)
CD8+ T-cell, %(IQR)	57 (53-64)	47 (41-53)
CD4+/CD8+ T-cell ratio (IQR)	0.31 (0.23-0.41)	0.59 (0.42-0.73)*

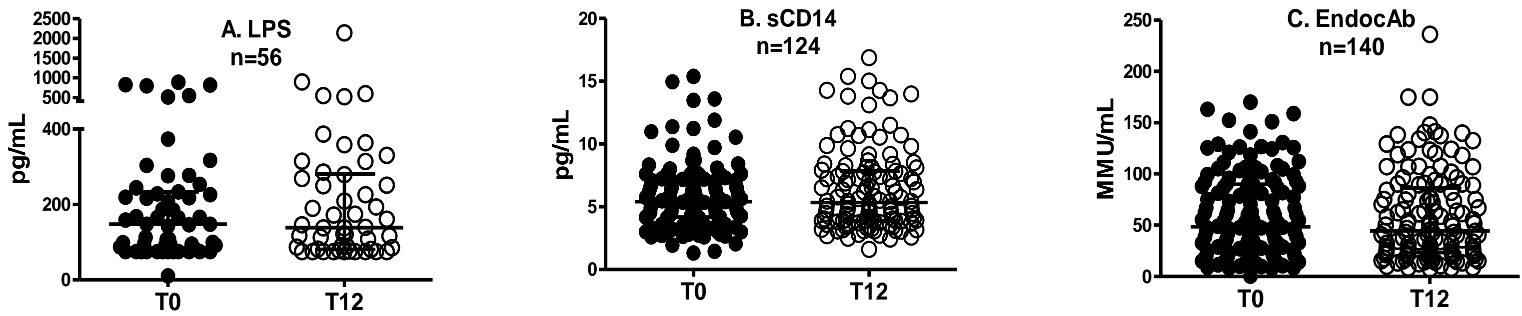
Antibiotic prophylaxis includes trimetroprim/sulfametoxazole, atovaquone. MSM males having sex with men. HCV, Hepatitis C Virus, infection defined as the presence of detectable plasma HCV RNA; HBV, Hepatitis B Virus, infection defined as HBsAg positivity; cART, Combination Antiretroviral Therapy. NNRTI, Non Nucleoside Transcriptase Inhibitor, PI, Protease Inhibitor. INI, Integrase Inhibitor. *indicates $p<0.01$ for T0 vs T12.

Figure 1. T-cell homeostasis in the course of cART



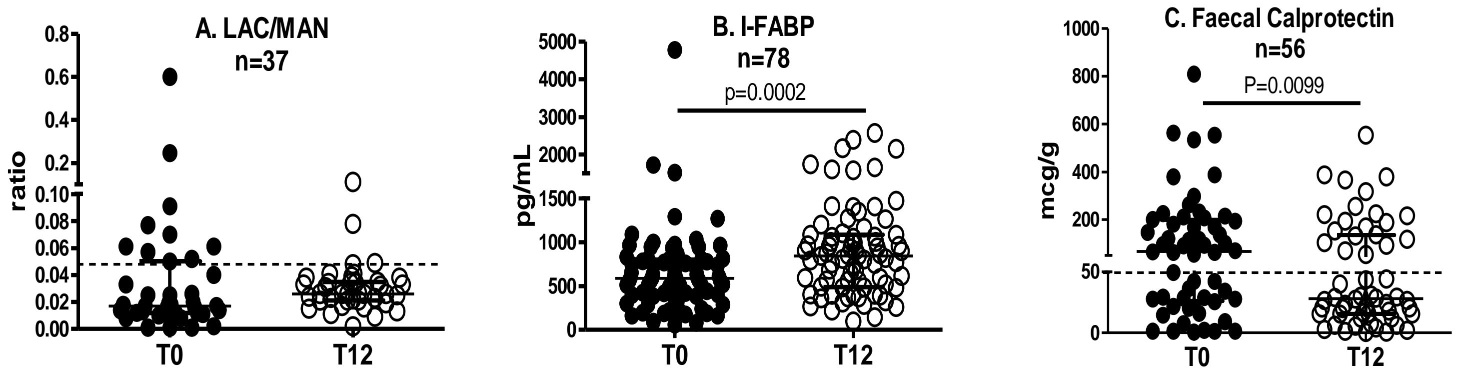
Following therapy, we found a significant increase in CD4+CD127+ and CD4+CD45RA+ (Fig. 1A, B) as well as a reduction in activated CD8+CD38+and CD8+CD38+CD45R0+%(Fig. 1C, D). Data were analysed by Wilcoxon test.

Figure 2. Microbial translocation in the course of cART



We did not observe significant changes in microbial translocation after 12 months of cART (2A-C). Data were analysed by Wilcoxon test. LPS, lipopolysaccharide; EndocAb, Endotoxin core Antibodies; sCD14, soluble CD14; MMU IgM Median Units.

Figure 3. Small intestine permeability, gut damage and inflammation in the course of cART



The urinary lactulose-mannitol fractional excretion ratio (LAC/MAN) was used to assess small intestine permeability (n=39). No differences were observed between two groups (A). On the contrary, we registered higher circulating levels of I-FABP (n=78) after 12 months of cART (B). Therapy ameliorates bowel inflammation given reduction in term faecal calprotectin (n=56) (C).

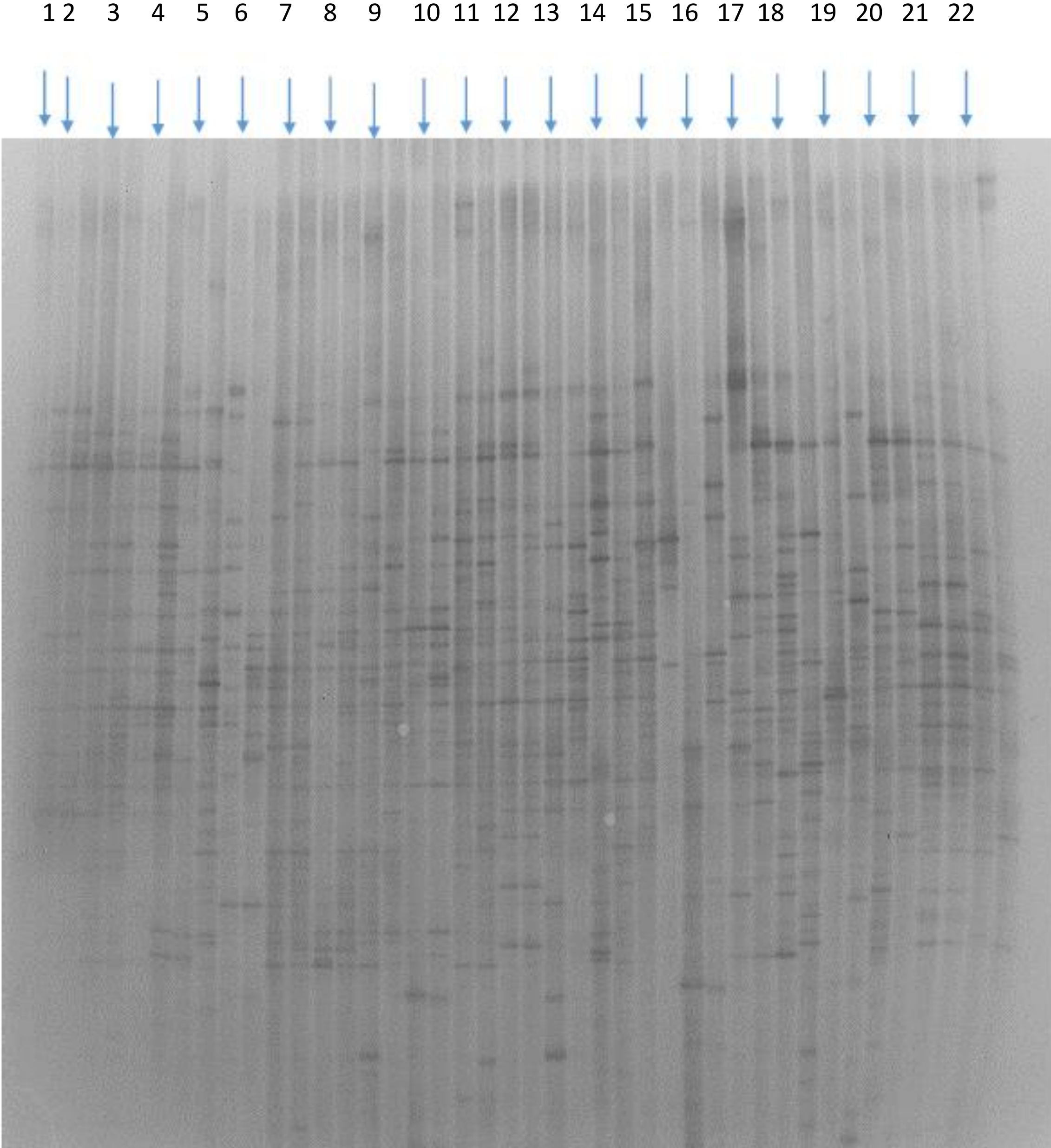
Data were analysed by Wilcoxon test. LAC/MAN, urinary lactulose-mannitol fractional excretion ratio; I-FABP, Intestinal Fatty Acid Binding Protein

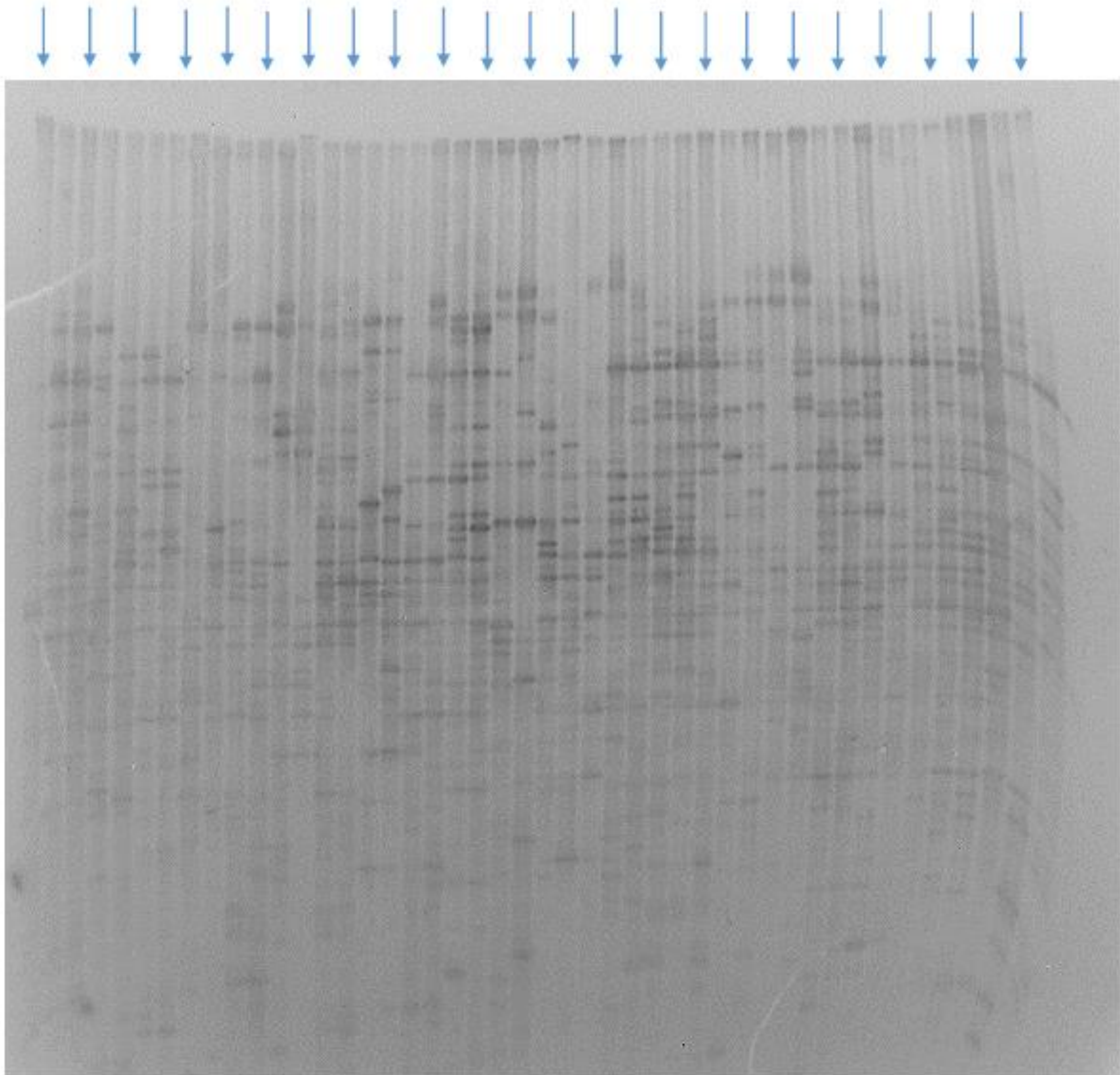
Table 2. Sequenced DGGE bands and relative species identification

Nearest species	Presence at T0 %	Phylum	Order	Family	Nearest species	Presence at T12 %
Bacteroidetes spp.	25	bacteroidetes	bacteroidales	bacteroidaceae	*Bacteroidetes spp.*	32,5
Prevotella spp.	90,9	bacteroidetes	bacteroidales	prevotellaceae	*Prevotella spp.*	80
Faecalibacterium prausntzii	63,5	firmicutes	clostridiales	clostridiaceae	*Faecalibacterium prausntzii*	65,5
Clostridium spp.	71,4	firmicutes	clostridiales	clostridiaceae	*Clostridium* spp.	75,5
Roseburia intestinalis	83	firmicutes	clostridiales	Lachnospiraceae	*Roseburia intestinalis*	87
Flavonifractor plautii	27,5	firmicutes	clostridiales	clostridiaceae	*Flavonifractor plautii*	25
Clostridiales spp.	15	firmicutes	clostridiales	clostridiaceae	*Clostridiales* spp.	12
Eubacterium rectale	67,5	firmicutes	clostridiales	eubacteriaceae	*Eubacterium rectale*	57,5
Acidaminococcus intestini	10	firmicutes	clostridiales	veillonaceae	*Acidaminococcus intestini*	7,5
Phascolarctobacterium faecium	20	firmicutes	clostridiales	veillonaceae	*Phascolarctobacterium faecium*	10
Enterococcus faecium	10	firmicutes	lactobacillales	enterococcacea	*Enterococcus faecium*	5
Lactobacillus spp.	40	firmicutes	lactobacillales	lactobacillaceae	*Lactobacillus spp.*	25
Bacillus spp.	15	firmicutes	bacillales	bacillaceae	*Bacillus* spp.	0
Desulfovibrio spp.	22,5	proteobacteria	desulfovibrionales	desulfovibrionaceae	*Desulfovibrio* spp.	25

The table reports identified species and their prevalence in stool of 56 patients prior to and following 12 months of cART obtained by DGGE analyses.

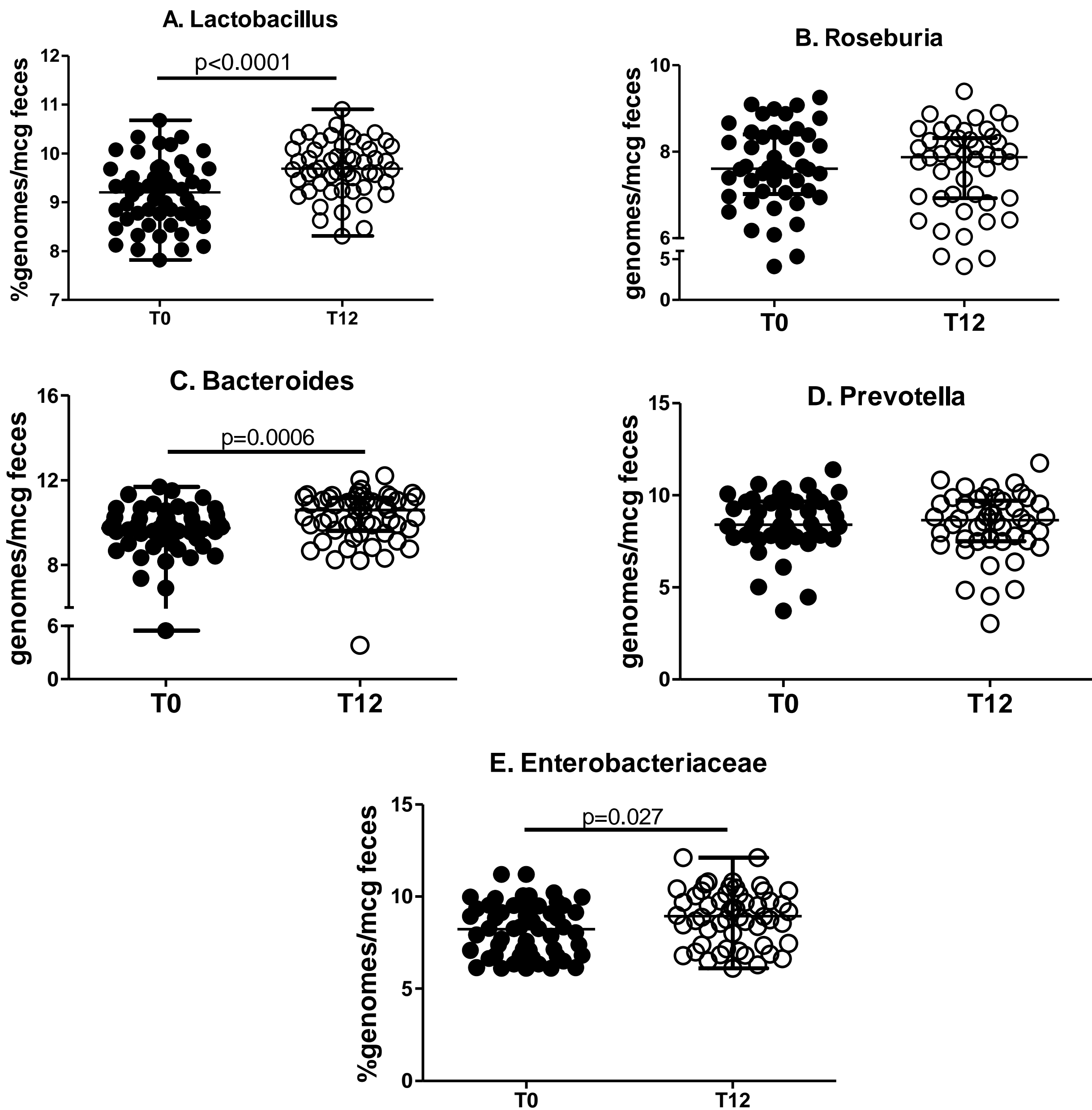
Figure 4. Cluster analysis of DGGE profiles of species investigated





DDGE profile analysis obtaining by amplification of V2-V3 region of faecal samples from our patients indicated by blue arrows. Banding patterns of the 16S ribosomal DNA (rDNA) gene were obtained using primers HDA1-GC and HDA2 and analysed using Pearson’s coefficient and UPGMA method to generate dendograms.

Figure 5. Quantification of selected microbial genuses and of the Enterobacteriaceae family in the course of cART



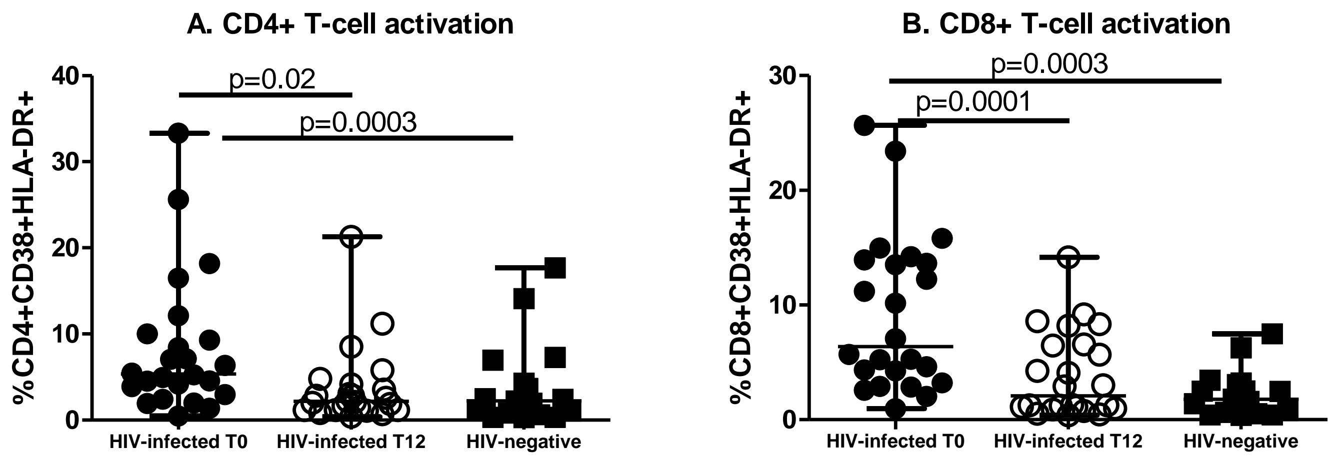
The study of the faecal microbiota revealed an enrichment of Lactobacillus (A) and Bacteroides (C) in the stool of HIV-infected patients after cART yet no changes in terms of Roseburia (B), Prevotella (D) and Enterobacteriacae family (E).

Table 3. Demographic characteristics and viro-immunological parameters of the patients in study (Aim 2)

Parameter at baseline	HIV-infected subjects (n=26), T0	
Sex, F (%)	4 (15%)	
Age, years (IQR)	39 (32-45)	
Risk factors for HIV infection, n (%)		
Heterosexual	8 (31)	
MSM	17 (65)	
IDU	1 (4)	
HCV co-infection n (%)	3 (12)	
HBV co-infection	1 (4)	
Co-trimoxazole use, n (%)	3 (12)	
Duration of HIV infection, months (IQR)	16 (4-52)	
AIDS diagnosis, n (%)	2 (7)	
First cART regimen n, (%)		
PI+NRTI	7 (27)	
NNRTI+NRTI	14 (54)	
Other	5 (19)	
Parameter in the course of the study	**HIV-infected subjects (n=26)**	
	T0	**T12**
HIV RNA $\log_{10}$ cp/ml (IQR)	4.7 (4.2-5.3)	1.6 (1.6-1.6)*
CD4+ T-cell count, cell/mmc (IQR)	366 (273-428)	477 (269-589)*
CD4+ T-cell, % (IQR)	18 (15-22)	25 (21-31)
CD8+ T-cell count, cell/mmc (IQR)	1018 (853-1384)	949 (806-1304)
CD8+ T-cell, %(IQR)	56 (53-60)	51 (44-58)
CD4+/CD8+ T-cell ratio (IQR)	0.3 (0.2-0.4)	0.5 (0.4-0.6)*

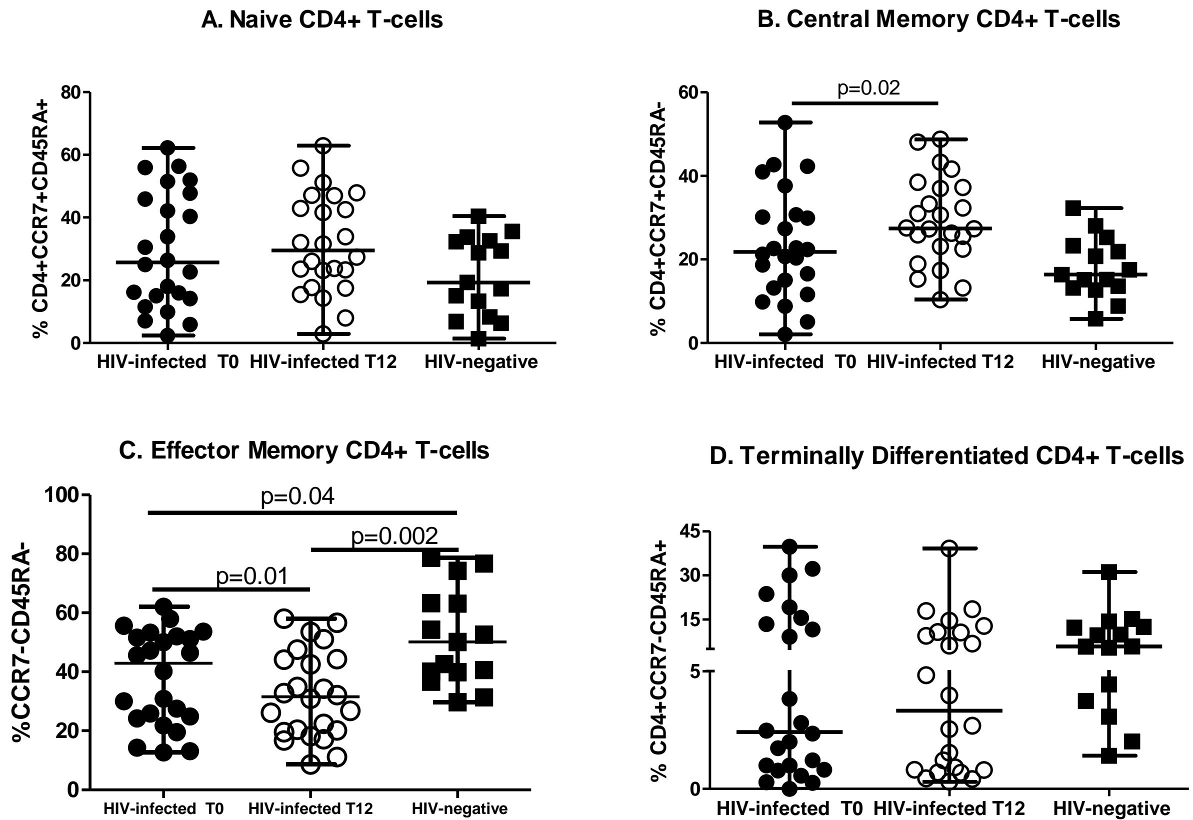
Data are presented as median, interquartile range (IQR) for continuous variables; absolute number, percentage for categorical variables. MSM: Men having Sex with Men; IDU: Intravenous Drug Use; HCV, Hepatitis C Virus, infection defined as the presence of detectable plasma HCV RNA; HBV, Hepatitis B Virus, infection defined as HBsAg positivity; AIDS: Acquired Immune Deficiency Syndrome; cART Combination of Antiretroviral Therapy; NNRTI, non-nucleoside transcriptase inhibitor; NRTI, nucleoside transcriptase inhibitor, PI, protease inhibitor. Data were analyzed Chi-Squared, Wilcoxon and Mann–Whitney test where appropriate. * indicates $p<0.01$ for T0 vs T12.

Figure 6. CD4+ and CD8+ T-cell activation prior to (T0) and following 12 months of cART (T12)



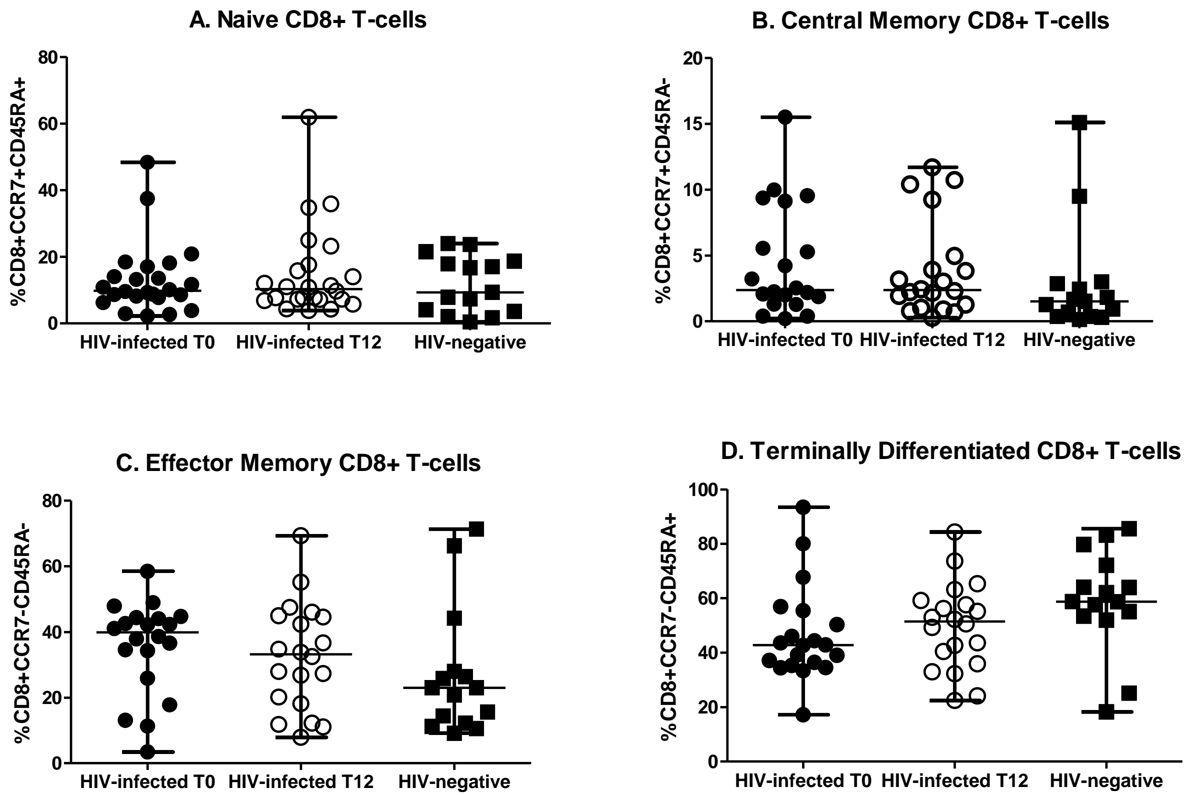
A significant decrease in CD4+ (A) and CD8+ (B) T-cell activation, reaching comparable levels to those of HIV-uninfected subjects, was observed following 12 months of cART.

Figure 7. CD4+ T-cell maturation subsets in HIV-infected subjects prior to (T0) and following 12 months of cART (T12)



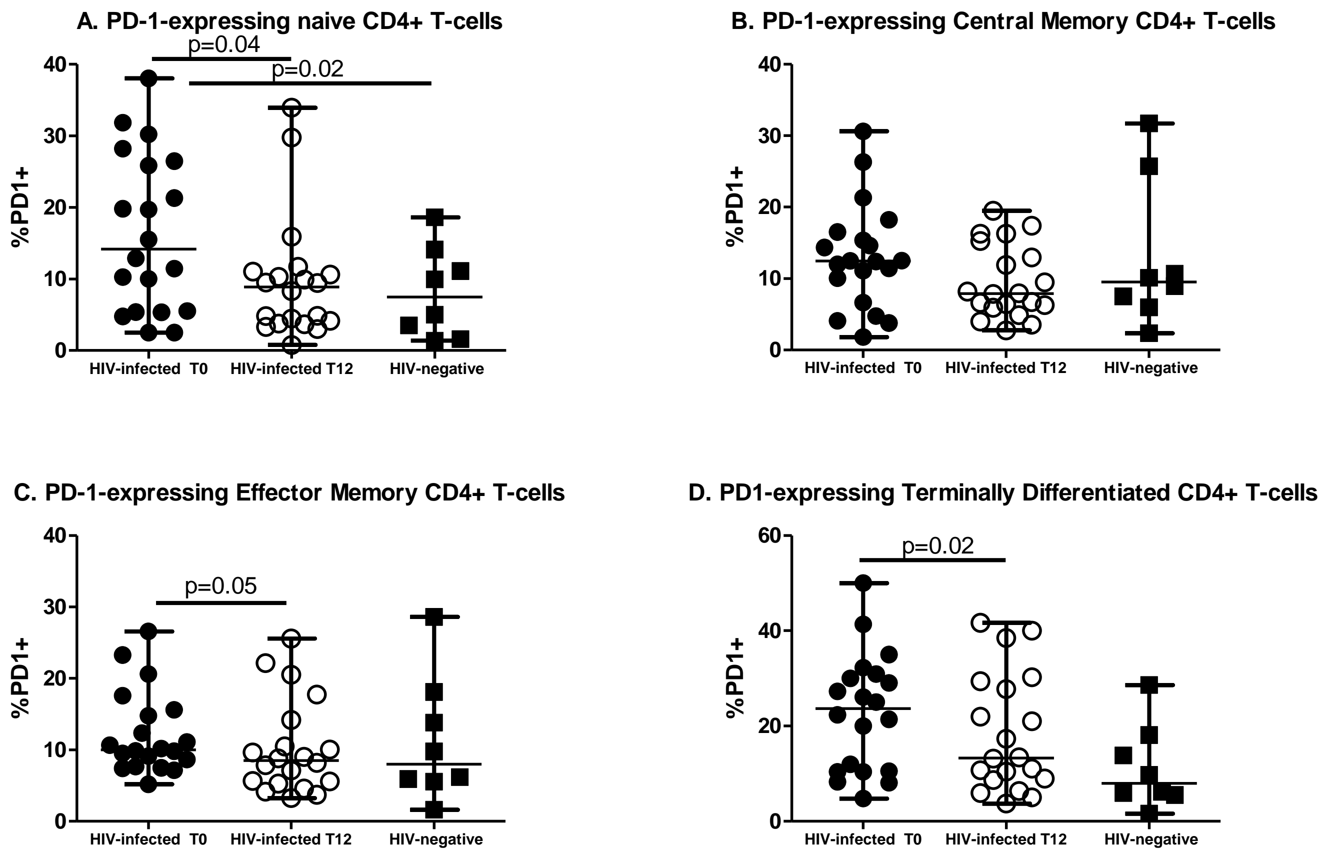
No changes were detected in terms of CD4+ T-cell naïve (A) and terminally differentiated pool frequencies (D), yet a progressive increase of CD4+ central memory (B) as well as a reduction of effector memory (C) T-cells were registered in the course of cART.

Figure 8. CD8+ T-cell maturation subsets in HIV-infected subjects prior to (T0) and following 12 months of cART (T12)



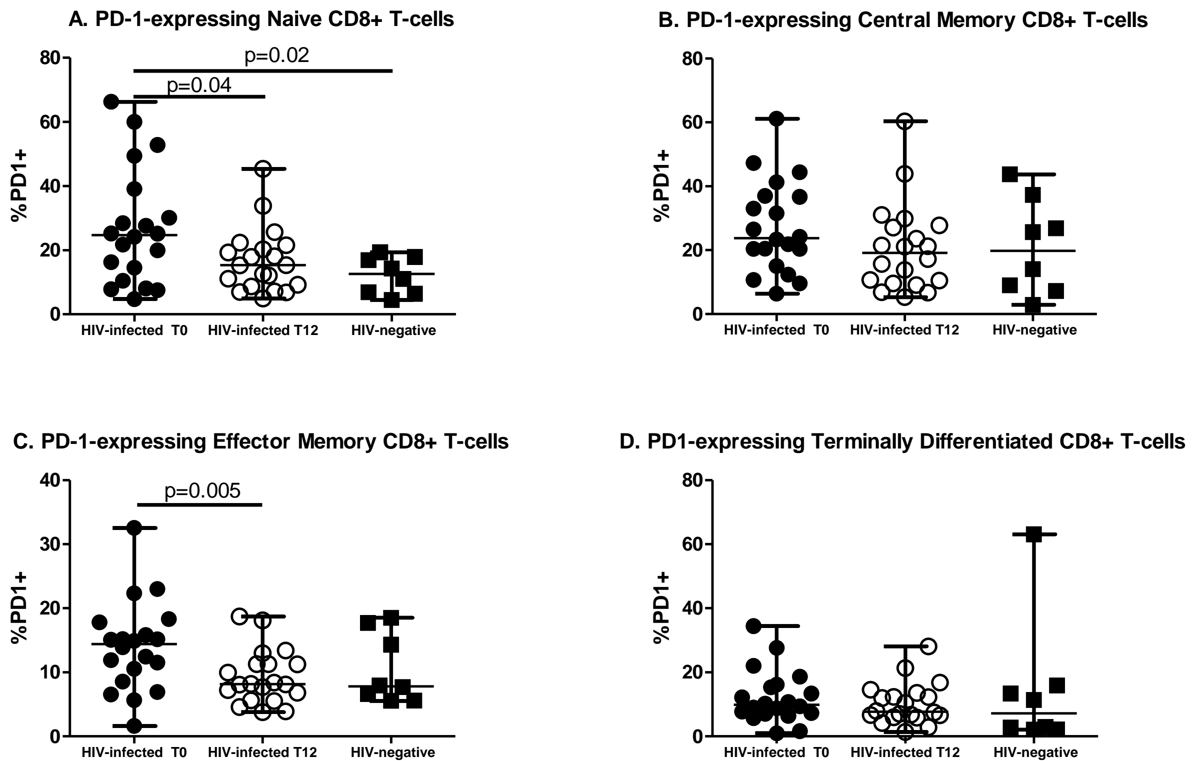
No modifications were noted in terms CD8+ T-cell maturation parameters after 12 months of cART (A-D).

Figure 9. PD-1-expression on CD4+ T-cell maturation subsets in HIV-infected subjects prior to (T0) and following 12 months of cART (T12)



Within the CD4+ subset, a reduction in the expression of PD-1 the naïve (A) and terminally differentiated pools (D) was observed in the course of cART. yet only a trend toward lower PD-1-expressing Effector Memory was noted (D).

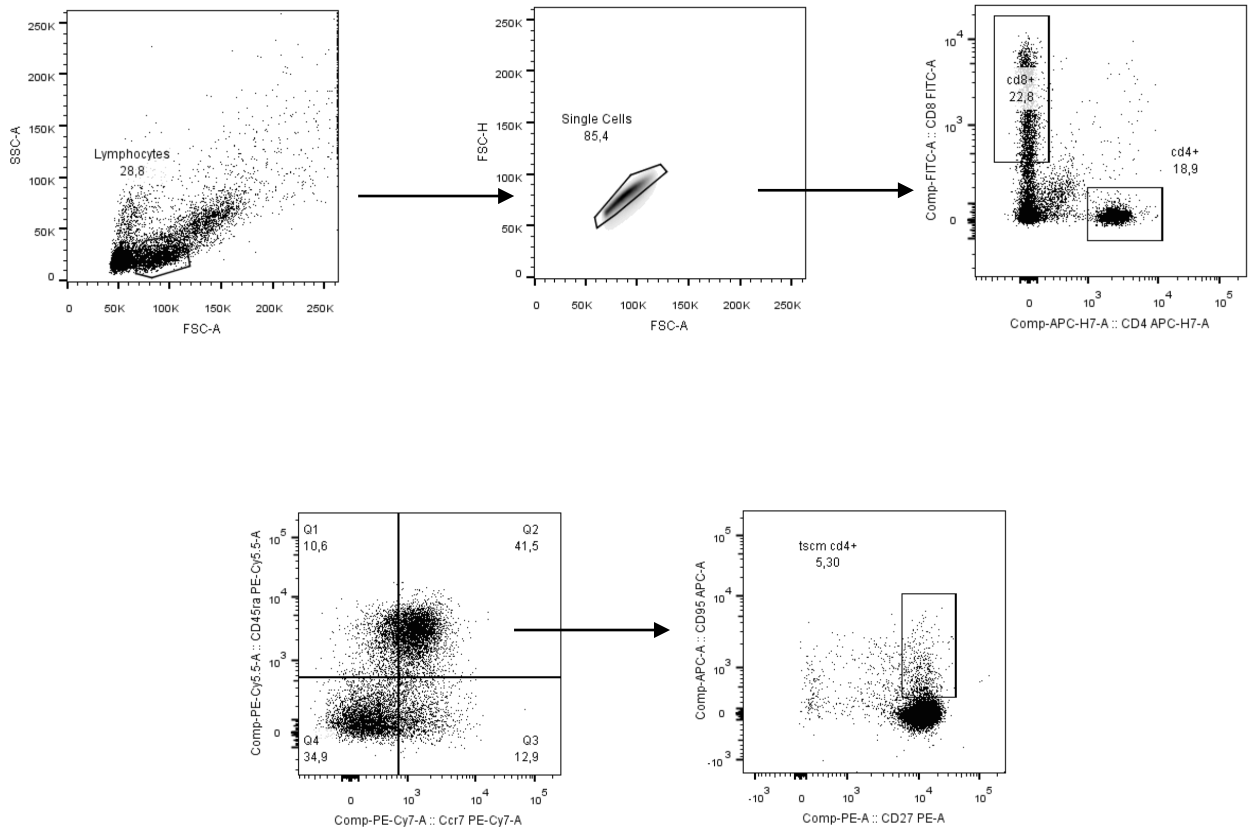
Figure 10. PD-1-expression on CD8+ T-cell maturation subsets in HIV-infected subjects prior to (T0) and following 12 months of cART (T12)

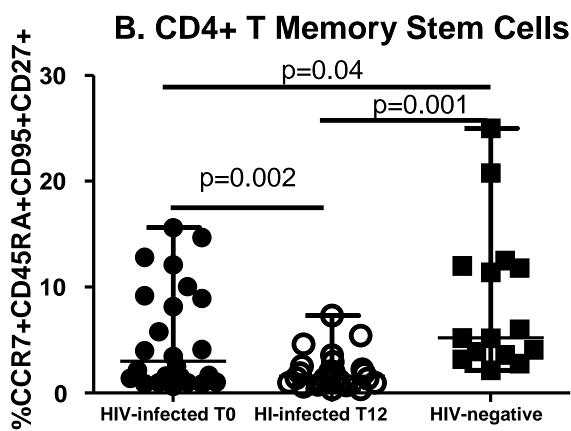


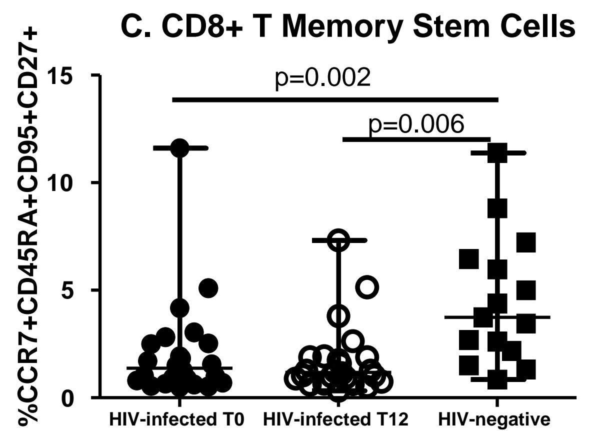
We found a progressive normalization of PD-1 expression in CD8+ naïve (A) and effector memory T-cells (C) in the course of cART., yet no modifications in term of PD-1 expression in CD8+ central memory and terminally differentiated T-cells (B,D).

Figure 11. Gating strategy, CD4+ and CD8+ Tscm frequencies and their correlation with maturation subsets

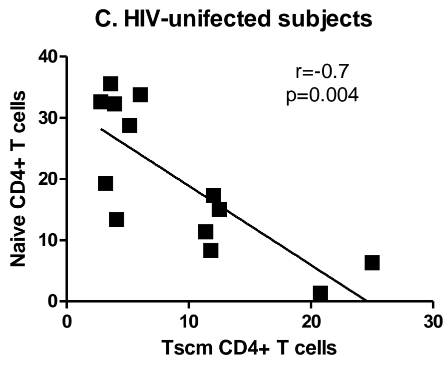
A

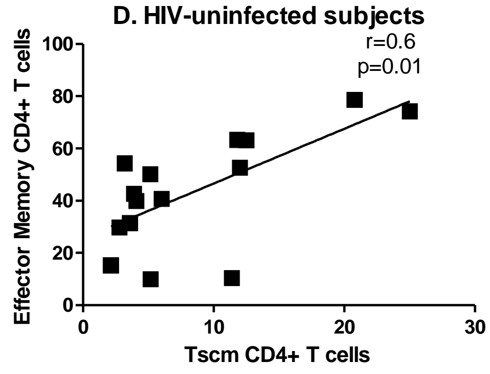


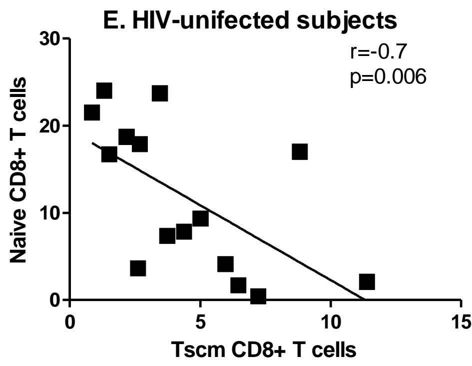


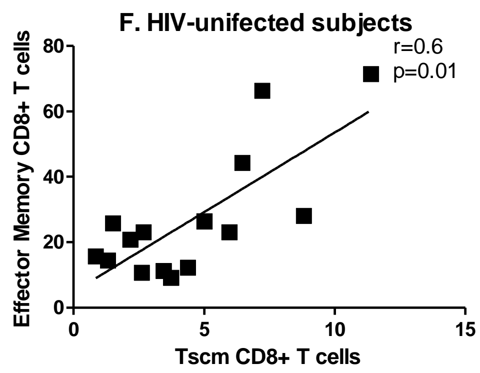


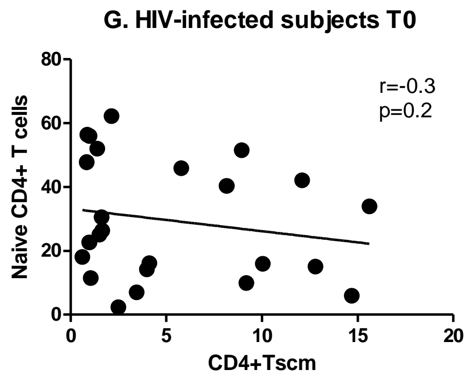
Frequency of CD4+ and CD8+ Tscm was assessed using gating strategy indicated by *Ribeiro et al.* [36] and showed in figure A. Briefly, lymphocyte were gated on the basis of physical parameters (FSC,SSC). Within the morphological gate, we getting singlets using FSC-A and FSC-H parameters. Then, we gated the CD4+/CD8+ T-cells and within these gates Whitin CD4+ and CD8+ T-cells we identified naïve cells (CD45RA+CCR7+) and within this gate we measured % of CD95+CD27+.
A decrease in CD4+ Tscm was noted in the course of cART (B) and no variation of CD8+ Tscm (C) Overall, Tscm cell frequencies persisted at lower levels than controls despite cART (B, C).

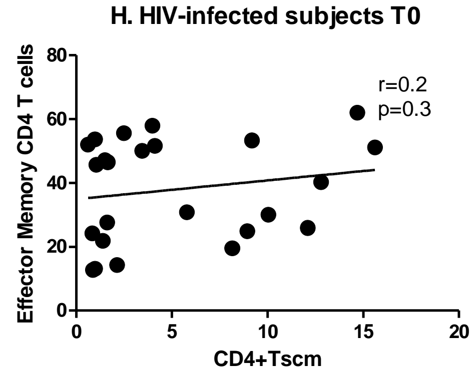

C. HIV-unifected subjects
r=-0.7
p=0.004
Naive CD4+ T cells
Tscm CD4+ T cells
0
10
20
30
40
0
10
20
30

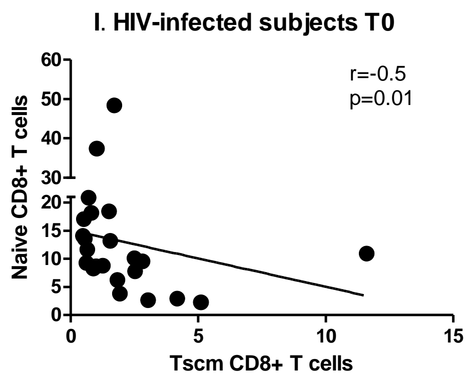

D. HIV-uninfected subjects
r=0.6
p=0.01
Effector Memory CD4+ T cells
Tscm CD4+ T cells
0
20
40
60
80
100
0
10
20
30

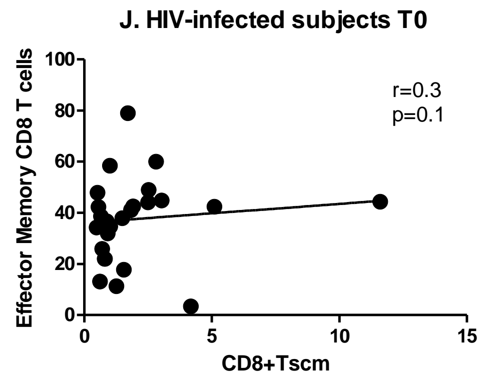

E. HIV-unifected subjects
r=-0.7
p=0.006
Naive CD8+ T cells
Tscm CD8+ T cells
0
10
20
30
0
5
10
15

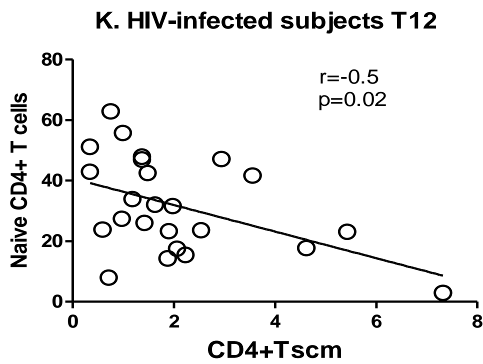

F. HIV-unifected subjects
r=0.6
p=0.01
Effector Memory CD8+ T cells
Tscm CD8+ T cells
0
20
40
60
80
0
5
10
15

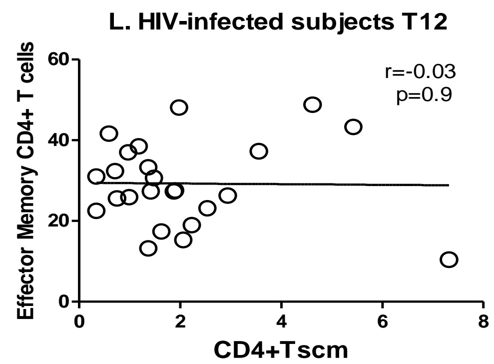

G. HIV-infected subjects T0
r=-0.3
p=0.2
Naive CD4+ T cells
CD4+Tscm

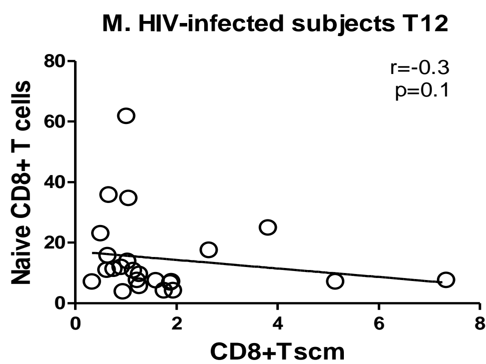

H. HIV-infected subjects T0
r=0.2
p=0.3
Effector Memory CD4 T cells
CD4+Tscm

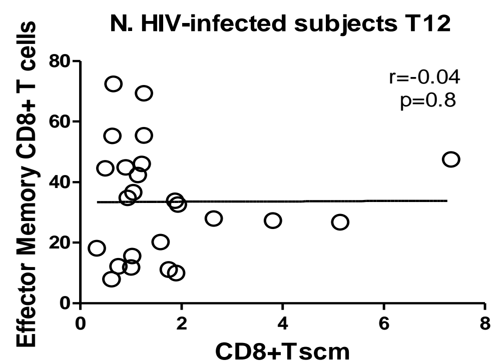

I. HIV-infected subjects T0
r=-0.5
p=0.01
Naive CD8+ T cells
Tscm CD8+ T cells


J. HIV-infected subjects T0
r=0.3
p=0.1
Effector Memory CD8 T cells
CD8+Tscm


K. HIV-infected subjects T12
r=-0.5
p=0.02
Naive CD4+ T cells
CD4+Tscm


L. HIV-infected subjects T12
r=-0.03
p=0.9
Effector Memory CD4+ T cells
CD4+Tscm

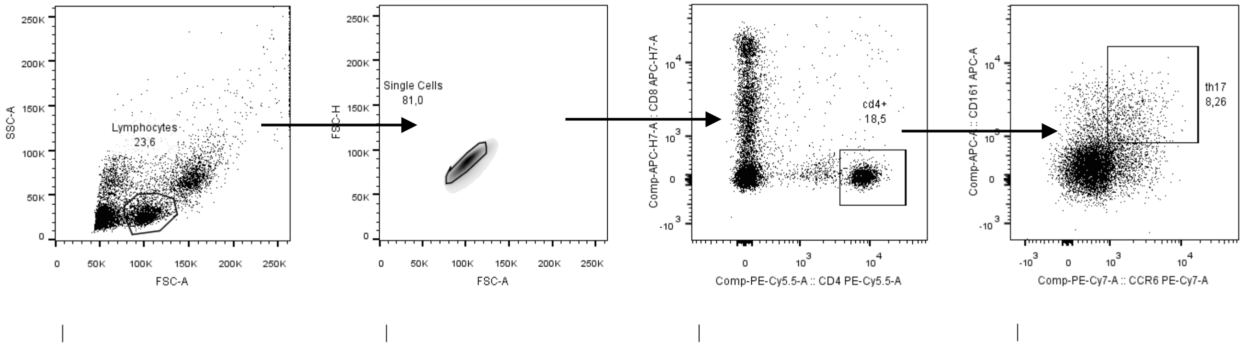

M. HIV-infected subjects T12
r=-0.3
p=0.1
Naive CD8+ T cells
CD8+Tscm

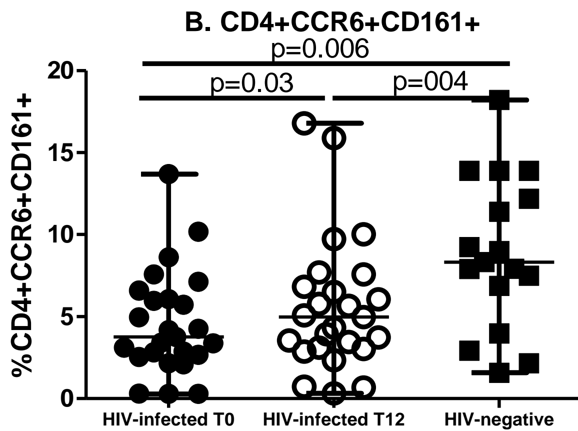

N. HIV-infected subjects T12
r=-0.04
p=0.8
Effector Memory CD8+ T cells
CD8+Tscm

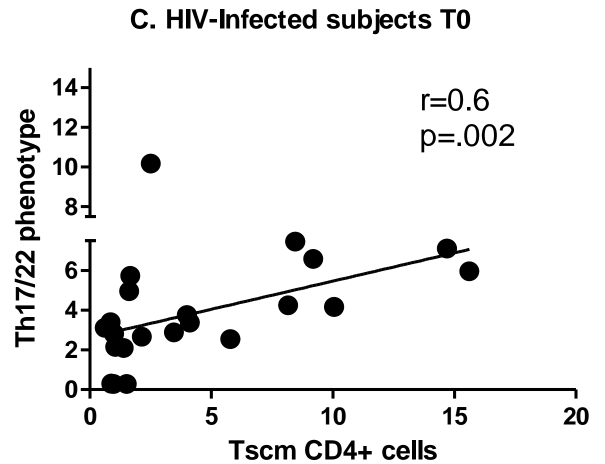
In uninfected controls, both CD4+ and CD8+ Tscm correlated negatively with naïve T cells (C, E) and positively with effector memory T cells (D, F). In cART-naïve subjects no correlation was found between Tscm and maturation subsets (G-J). Antiretroviral treatment was able to restore only the link between CD4+ Tscm and naïve cells (K-N). T0: cART-naïve subjects; T12: 12 months of treatment.

Figure 12. Gating strategy and frequency of CD4+CCR6+CD161+ in HIV-infected subjects prior to (T0) and following 12 months of cART (T12)

A)



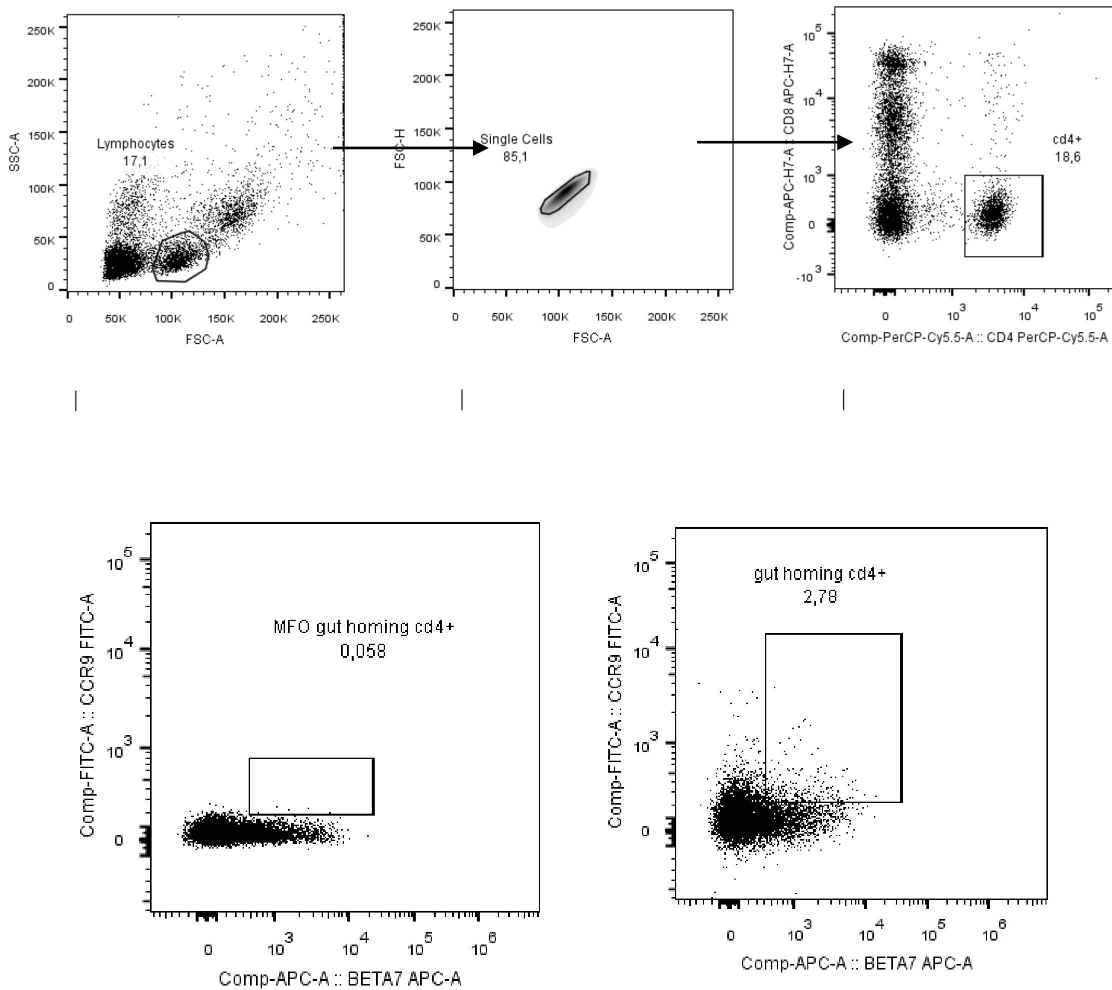


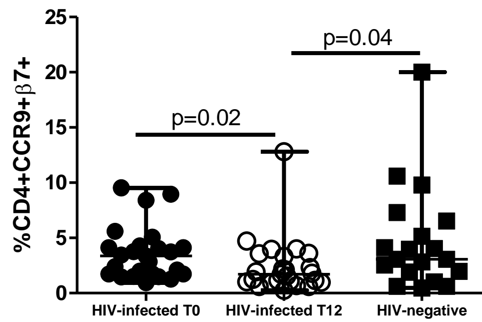


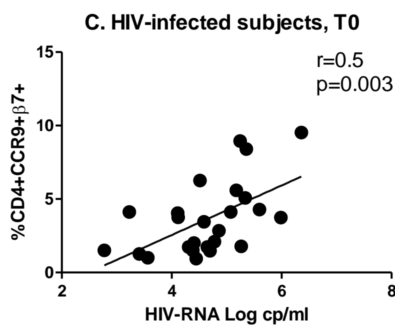
Gating strategy for identification of CD4+CCR6+CD161+. The experimental protocol to study this type of cells was set up in HIV-uninfected subjects (Fig. 8a). Based on cell dimensions, we determined the purity of the population. Lymphocyte were gated on the basis of physical parameters (FSC,SSC). Within the morphological gate, we getting singlets using FSC-A and FSC-H parameters. Then, we gated the CD4+ T-cells and within this gate we measured the % of CD161+CCR6+ (A).
CD4+ CCR6+CD161+ maintained lower compared to HIV-uninfected controls, despite their increase in the course of cART (B).
Positive correlation between CD4+ T-cells with CD4+CCR6+CD161+ and Tscm was found in cART-naïve HIV-infected subject (C).

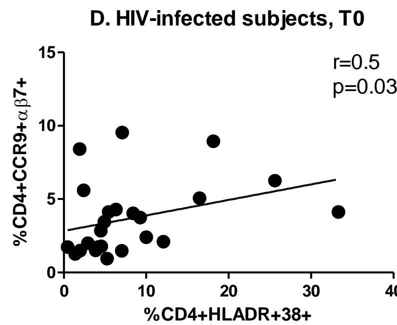
Figure 13. Gating strategy and frequency of CD4+ T-cells with a "gut-homing" phenotype and their correlation with immune activation parameters prior to (T0) and following cART (T12)

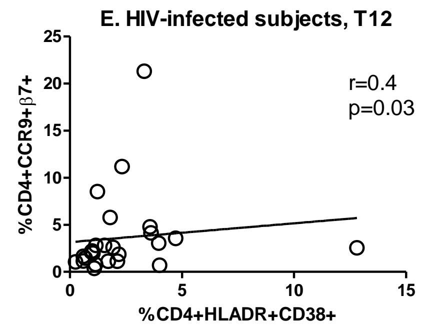
A)











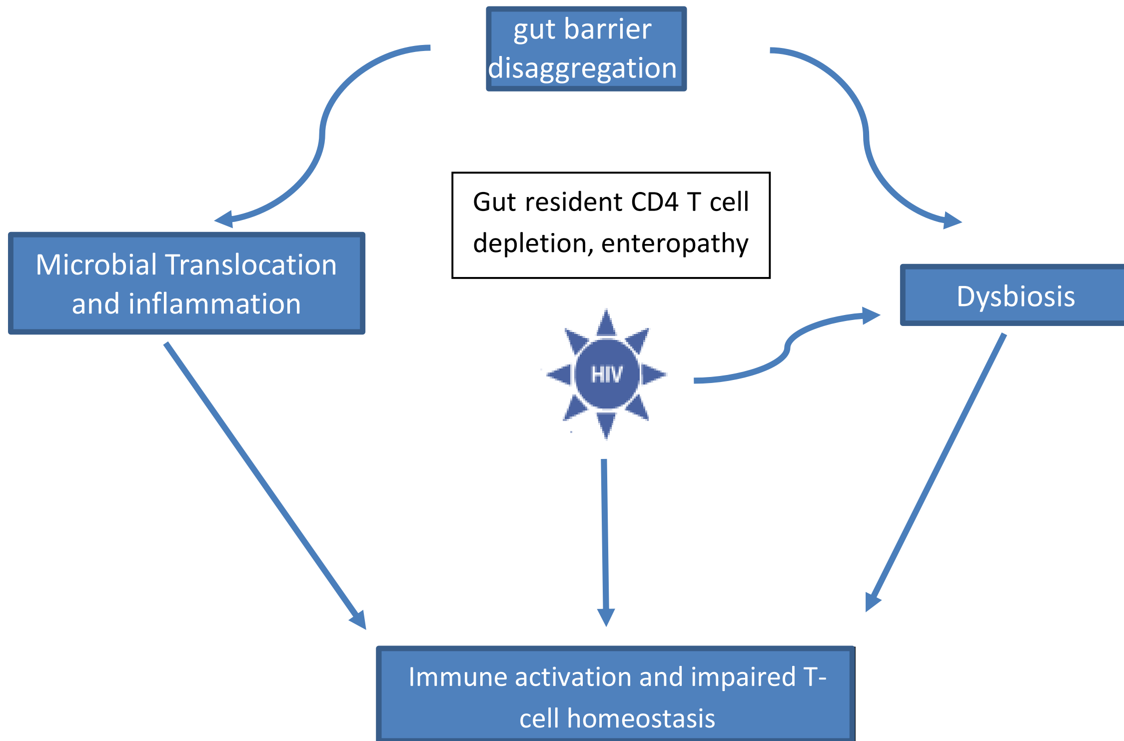
Gating strategy for identification of CD4+ T cells with a "gut-homing" phenotype. Give the low expression of CCR9, identification of this population required use of MFO during flow cytometry staining. Based on cell dimensions, we determined the purity of the population. Lymphocyte were gated on the basis of physical parameters (FSC,SSC). Within the morphological gate, we getting singlets using FSC-A and FSC-H parameters. Then, we gated the CD4+ T-cells and within this gate we measured the % of CCR9+α4β7+. For choose the right position of CCR9 gate, we parallel stain the same tube without CCR9 FITC in term to determine the fluorescence due to CCR9 (A).

CD4+ T cells with a gut-homing phenotype showed a decrease in the course of cART, thus reaching lower levels compared to HIV-uninfected controls (B). These cells correlated, at T0, with HIV RNA viral load (C) and activated CD4+ T cells (D) but not at T12 (E).

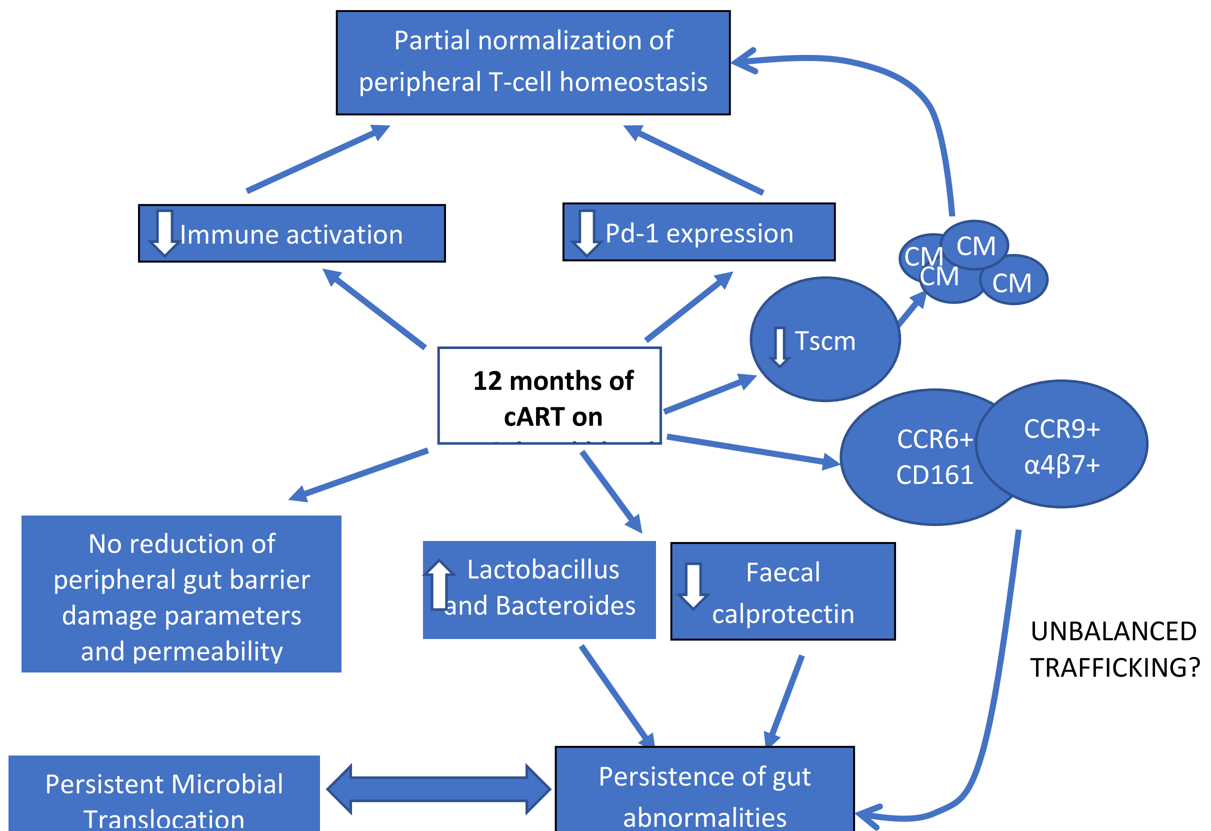
Figure 14. Summary of our findings after 12 months of cART

Before cART:

A)



B)



Summary of our findings. In figure A are represented some of abnormalities caused by HIV infection, particularly regarding T-cell homeostasis and enteropathy. In figure B are summarizes our findings after 12 months of cART and which anomalies persist despite cART.

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