

Impaired gut junctional complexes feature late-treated individuals with suboptimal CD4⁺ T-cell recovery upon virologically suppressive combination antiretroviral therapy

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Objective: HIV-infected individuals with incomplete CD4⁺ T-cell recovery upon combination antiretroviral therapy (cART) display high levels of immune activation and microbial translocation. However, whether a link exists between gut damage and poor immunological reconstitution remains unknown.

Design: Cross-sectional study of the gastrointestinal tract in late cART-treated HIV-infected individuals: 15 immunological nonresponders (CD4⁺ <350 cells/ μ l and/or delta CD4⁺ change from baseline <30%); 15 full responders (CD4⁺ >350 cells/ μ l and/or delta CD4⁺ change from baseline >30%).

Methods: We assessed gut structure (junctional complex proteins in ileum and colon) and function (small intestine permeability/damage and microbial translocation parameters). The composition of the fecal microbiome and the size of the HIV reservoir in the gut and peripheral blood were investigated as possible mechanisms underlying mucosal impairment.

Results: Markers of intestinal permeability, damage, systemic inflammation, and microbial translocation were comparable in all study individuals, yet the expression of junctional complex proteins in gut biopsies was significantly lower in HIV-infected patients with incomplete CD4⁺ restoration and negatively correlated with markers of CD4⁺ reconstitution. Electron microscopy revealed dilated intercellular spaces in individuals lacking immunological response to cART, yet not in patients displaying CD4⁺ T-cell recovery. Analysis of the fecal microbiome revealed an overall outgrowth of *Bacteroides*–*Prevotella* spp. with no differences according to CD4⁺ T-cell reconstitution. Interestingly, HIV reservoirs in peripheral CD4⁺ T cells and intestinal tissue negatively correlated with immune recovery.

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Conclusion: These observations establish gut damage and the size of the HIV reservoir as features of deficient immunological response to cART and provide new elements for interventional strategies in this setting.

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Introduction

The gastrointestinal tract is a major site of HIV pathogenesis [1–11]. The impairment of the gastrointestinal epithelial barrier structure and function, occurring in the natural course of disease [12–19], leads to the translocation of microbial bioproducts from the gut lumen to the systemic circulation [20,21], causing peripheral immune activation [20,21] and, in turn, driving CD4⁺ T-cell loss and disease progression [22–25].

Combination antiretroviral therapy (cART) suppresses viral replication and leads to CD4⁺ T-cell reconstitution in peripheral blood, yet seems less successful in lowering HIV-RNA and HIV-DNA in the gastrointestinal mucosa [26–28], as well as reconstituting gastrointestinal CD4⁺ T cells [4,6,8,11,26,29–32] and repairing the epithelial barrier [33,34]. Further, up to 30% of treated patients fail to recover peripheral CD4⁺ [immunological nonresponders (INR)] [35], particularly those starting cART late in the course of disease, with severe CD4⁺ depletion. This particular outcome has been associated with a high risk of clinical events and death [36–38] as well as poor response to experimental treatments [39–44].

Studies addressing the pathogenesis of inefficient CD4⁺ T-cell recovery in INR have shown increased levels of circulating lipopolysaccharide (LPS) [20,45,46], alongside high levels of immune activation [47,48]. Literature, however, has so far not disentangled the links between gastrointestinal barrier dysfunction and poor immune reconstitution in the course of effective cART.

Given these premises, we hypothesized that impairment of the gut epithelial barrier persists in late cART-treated individuals with suboptimal CD4⁺ T-cell reconstitution and not in those with complete immune recovery.

We, thus, analyzed the structure and function of the gastrointestinal mucosa, focusing on both ileum and colon districts in HIV-infected individuals with diverse response to treatment and evaluated the relationship between CD4⁺ T-cell reconstitution, expression of gut junctional complex proteins, size of the HIV reservoir, and composition of the fecal microbiome.

Materials and methods

Study patients

HIV-positive individuals were recruited at the Clinic of Infectious Diseases and Tropical Medicine, San Paolo Hospital (University of Milan, Italy) following the provision of informed consent. Participants were required to be undergoing cART for at least 12 months, feature a CD4⁺ cell nadir less than 350 cells/ μ l and HIV-RNA less than 40 copies/ml. Patients with current CD4⁺ less than 350 cells/ μ l and/or delta CD4⁺ change from baseline less than 30% were defined as INR ($n = 15$). Patients with current CD4⁺ more than 350 cells/ μ l and/or delta CD4⁺ change from baseline higher than 30% were defined as full responders ($n = 15$).

The Institutional Review Board at the San Paolo Hospital, Milan, Italy, approved the study. To minimize possible bias, all laboratory analyses were performed in a blinded fashion.

Flow cytometry

Surface phenotypes were evaluated using fresh peripheral blood (FACSCanto II; Becton Dickinson Italia Spa, Milan, Italy), CD4-PE-cy7, CD8-PE-cy5, CD38-FITC, CD45RO-PE, and CD45RA-FITC (Becton Dickinson).

Colonoscopy

Individuals with a known gastrointestinal disease or clinical symptoms were excluded from the study; eight of 15 INR and 13 of 15 full responders were willing to undergo routine screening colonoscopy [49] (bowel preparation with Moviprep-Norgine, Marburg, Germany). Biopsies were performed in colon and distal ileum. Three pinch biopsies were collected at each site. Fresh tissue (i.e. unfixed) was disposed in tissue paper and immediately transported to the pathology lab for cryopreservation in liquid nitrogen with optimal cutting temperature compound and stored at -80°C . Following this procedure, all colonic tracts, rectum and distal ileum were biopsied (1 pinch biopsy per site). These samples were fixed through immersion in a solution of 10% buffered formalin.

Small fragments of biopsies with larger specimen yields were fixed in glutaraldehyde for electron microscopy evaluation (see subsections below).

Immunohistochemical staining

Biopsies were selected to assess major structural proteins of tight and adherens junctions (TJ and AJ) forming the gut junctional complex, T-lymphocyte sub-populations, proliferation index, and microbial translocation by immunohistochemistry (IHC).

Biopsies were fixed in formalin and paraffin-embedded; 3 μ m sections were stained with hematoxylin and eosin and selected antibodies.

For junction protein analysis, cadherin 1 (Cdh1, 1:15000; Abnova, Taipei, Taiwan), *Zonula occludens* protein 1 (ZO-1, 1:200; Zymed, ThermoFisher Scien, Milan, Italy), claudin 1 (1:100; Invitrogen, Lifetech Italia, Monza, Italy) and claudin 7 (1:100, Zymed) were used. For the study of T-lymphocyte subpopulations, CD3 (clone F7.2.38, 1:200; Dako Italia srl, Milan, Italy), CD4⁺ (clone 1F6, 1:50; Leica Microsystems Srl, Milan, Italy), and CD8⁺ (clone C8/144B, 1:50; Dako) were used.

Proliferation index was determined by Ki67 (clone MIB1, 1:100; Dako) (clone Asp175, 1:500; Cell Signaling Technologies Inc., Danvers, Massachusetts, USA) staining. The translocation of microbial products through the colonic lamina propria was evaluated by monoclonal antibody staining against the LPS-core antigen (clone WN1 222-5, 1:100, Hycult Biotech, Uden, The Netherlands).

For antigen retrieval, slides were incubated at 97.5°C in a thermostatic bath for 35 min, with EDTA (pH 8) or citrate buffer (pH 6). Endogenous peroxidase activity was blocked by hydrogen peroxide (0.3%) for 10 min. All slides were counterstained with hematoxylin. Negative controls were incubated in the absence of primary antibodies. IHC was performed using a Biogenex i6000 Automated Staining System (BioGenex srl, Bergamo, Italy).

Reactions were detected by Novolink Max polymer detection system (Novocastra Laboratories L.T.D., Leica Microsystems), using diaminobenzidine as chromogen.

A semiquantitative score was used for the study of intestinal junction proteins, by evaluating: the reaction intensity of stained epithelial cells of colon and ileum (0 = no staining, 1 = weak staining, 2 = intermediate staining, 3 = intense staining); the percentage of positive cells, by counting stained cells on 100 superficial consecutive intestinal cells at high magnification (40 \times); and subcellular localization (membranous as complete, lateral, basal, basolateral; cytoplasmic; paranuclear).

Lymphocyte subpopulations were determined by counting positive cells in three consecutive high-power field (40 \times) in ileum and colon 'lamina propria'. CD4⁺ expression was also evaluated in terms of distribution (subepithelial or diffuse).

Proliferation index (Ki67) was determined by counting positive cells on 100 intestinal cells at high magnification (40 \times) and reported as percentage.

Electron microscopy

Ileum and colon samples from biopsies were fixed in 2.5% glutaraldehyde in phosphate buffer and routinely processed for electron microscopy examination (transmission electron microscope JEOL JEM 1010, Tokyo, Japan).

Microbial translocation and systemic inflammation

Plasma sCD14 and endotoxin core antibodies were measured by ELISA (R&D Systems, Minneapolis, Minnesota, USA), in accordance with the manufacturer's instructions. Samples were diluted 1000 times.

Circulating LPS was assessed using the *lymulus* amoebocyte lysate test (Lonza Group Ltd, Basel, Switzerland), as per the manufacturer's instructions. Samples were diluted 1:150 and preheated at 95°C for 10 min.

IL-6 and D-dimer levels were measured by ELISA (R&D Systems) and immunoturbidimetric determination assay (Instrumentation Laboratory SPA, Milan, Italy) respectively.

Urinary lactulose-mannitol fractional excretion ratio and intestinal fatty-acid-binding protein

Participants were asked to fast the night before and to collect morning urine before drinking a sugar probe solution containing 5 g lactulose and 1 g mannitol. Urine was collected for 5 h following administration of the double sugar solution and participants did not eat or drink (with the exception of water) until the end of the 5-h collection. The total volume of urine was recorded and a 30 ml aliquot of chlorhexidine-preserved (0.236 mg/ml of urine; Sigma Chemical, St Louis, Missouri, USA) was frozen and stored for 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, California, USA). Intestinal fatty-acid-binding protein (I-FABP) was assessed by ELISA (Hycult Biotech).

Fecal microbial population analysis

Total bacterial DNA was extracted from 100 mg of feces, using the PSP Spin Stool DNA Plus kit (Strattec Molecular, Berlin, Germany).

The V2–V3 region of the 16S ribosomal DNA (rDNA) gene was amplified with primers HDA1-GC/HDA2 [50]. Denaturing gradient gel electrophoresis (DGGE) was performed with the use of a PhorU-2 system (Ingeny Int, Goes, The Netherlands) [51]. Banding patterns of DGGE profiles were analyzed with Fingerprinting II software (Bio-Rad Laboratories, Hercules, California, USA).

To confirm the identification of the bacteria examined by DGGE, individual bands were carved out of the gel and the DNA was reamplified and sequenced with the same primers (BMR Genomics, Padua, Italy). We subsequently conducted a search of sequences deposited in the GenBank DNA database by the Basic Local Alignment Search Tool algorithm (<http://www.ncbi.nlm.nih.gov/>).

Given that *Bacteroides* appeared to be the most representative group, it was analyzed using a nested approach [51], followed by DGGE and real-time PCR to quantify *Bacteroides*–*Prevotella*.

Virological studies

CD4⁺ T cells were isolated by negative selection (StemCell Technologies, Vancouver, Canada) from peripheral blood mononuclear cells obtained by Ficoll procedure (Biospa, Milan, Italy). Cryopreserved ileum and colon biopsies were thawed and optimal cutting temperature compound was removed following washes with PBS.

A pellet of 1×10^6 peripheral CD4⁺ T cells and entire gut biopsies were digested with lysis buffer overnight at 55°C

and the lysate was used directly in a nested PCR to quantify both HIV and CD3 gene copy numbers, as previously described [52].

Statistical analysis

Descriptive and statistical analyses were performed with the use of GraphPad Prism 6.0 (GraphPad Inc., La Jolla, California, USA). Categorical variables are presented as the number of cases and percentages and analyzed by χ^2 or two-sided Fisher's exact test were appropriate. Continuous variables are presented as median values and interquartile range and analyzed by a two-tailed Mann–Whitney test. Correlations were assessed by a nonparametric, two-tailed Spearman's rank coefficient. A *P*-value < 0.05 was considered statistically significant.

Results

Patient population

Fifteen INR and 15 full responders were enrolled and assigned to different analytical methods for the study of gastrointestinal barrier structure and function (Fig. 1).

No differences were observed between groups in terms of demographics, HIV duration, and length of cART (Table 1).

Compared to full responders patients, INR featured significantly lower CD4⁺ as per inclusion criteria, lower change in CD4⁺ T cells since cART introduction and

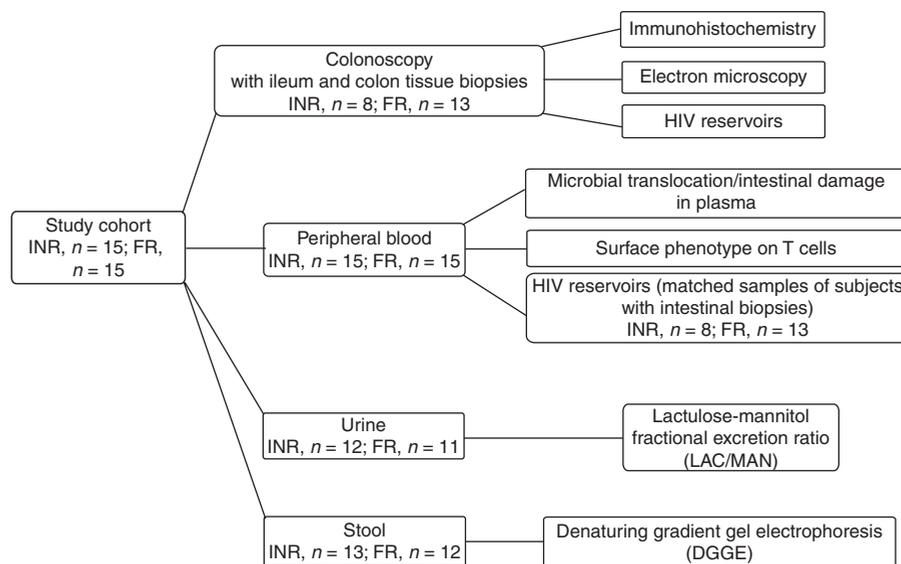


Fig. 1. Cohort diagram illustrating the assignment of intestinal biopsies, peripheral blood, urine, and stool samples to each analytical method. Fifteen immunological nonresponders (INRs) and 15 full responders (FRs) were enrolled and assigned to different analytical methods for the study of gastrointestinal barrier structure and function. Twenty-one patients (8 INRs, 13 FRs) underwent colonoscopy. In 30 patients (15 INRs and 15 FRs) blood samples were collected. In 23 patients (12 INRs, 11 FRs) urine samples were collected. In 25 patients (13 INRs, 12 FRs) stool samples were collected.

Table 1. Clinical characteristics of study patients.

	Immunological nonresponder (n = 15)	Full responder (n = 15)	P value
Age (years) (IQR)	53 (50–70)	56 (43–71)	0.99
Sex, no females (%)	2 (13)	1 (7)	1.00
Ongoing antibiotic prophylaxis, n (%)	4 (26)	0 (0)	0.10
Risk factors for HIV infection			0.06
Heterosexual, n (%)	10 (67)	4 (27)	
MSM, n (%)	3 (20)	9 (60)	
Intravenous drug use, n (%)	2 (13)	2 (13)	
HCV-Ab positivity, n (%)	2 (13)	0 (0)	0.48
Duration of HIV infection (months) (IQR)	59 (32–71)	61 (54–91)	0.55
Duration of cART (months) (IQR)	54 (30–67)	57 (50–60)	0.79
AIDS diagnosis, n (%)	8 (53)	5 (33)	0.87
Nadir CD4 ⁺ T cells			
Absolute counts (cells/μl) (IQR)	68 (36–120)	196 (50–237)	0.11
Current CD4 ⁺ T cells			
Absolute counts (cells/μl) (IQR)	265 (195–327)	516 (418–639)	<0.0001
Percentage (IQR)	20 (14–25)	25 (21–33)	0.01
Change in CD4 ⁺ T cells since cART			
Absolute counts (cells/μl) (IQR)	159 (106–249)	351 (294–515)	0.001
Current CD4 ⁺ /CD8 ⁺ T-cell ratio	0.4 (0.3–0.6)	0.6 (0.5–0.9)	0.01
Current CD4 ⁺ CD45RA ⁺ (%)	6.0 (4.0–9.0)	6.0 (3.0–9.0)	0.84
Current CD8 ⁺ CD38 ⁺ (%)	4.0 (3.0–7.0)	2.0 (1.0–4.0)	0.01
Current CD8 ⁺ CD38 ⁺ CD45RO ⁺ (%)	1.0 (1.0–3.0)	1.0 (0.0–1.0)	0.01
Current CD8 ⁺ CD45RA ⁺ (%)	14.0 (13.0–27.0)	14.0 (11.0–19.0)	0.18
Current CD8 ⁺ CD45RO ⁺ (%)	14.0 (10.0–18.0)	10.0 (7.0–18.0)	0.59
Zenith HIV-RNA load, log ₁₀ copies/ml (IQR)	5 (5–6)	5 (5–6)	0.50
LPS (pg/ml)	209 (75–431)	342 (125–515)	0.46
EndocAb (MMU/ml)	50.4 (13.4–65.6)	34.0 (17.2–50.2)	0.78
sCD14 (μg/ml)	4.8 (3.1–7.9)	7.9 (3.8–10.3)	0.23
IL-6 (pg/ml)	1.07 (0.78–1.47)	0.90 (0.70–1.60)	0.62
D-dimer (ng/ml)	80 (62–436)	122 (61–253.5)	0.77
Ongoing cART regimen, no patients (%)			0.56
NNRTI based	4 (27)	5 (33)	
PI based	8 (53)	9 (60)	
Other	3 (20)	1 (7)	

Antibiotic prophylaxis includes trimetoprim/sulfamethoxazole, atovaquone. cART, combination antiretroviral therapy; EndocAb, endotoxin core antibodies; HCV, hepatitis C virus; IL-6, interleukin-6; IQR, interquartile range; LPS, lipopolysaccharide; NNRTI, nonnucleoside transcriptase inhibitor; PI, protease inhibitor; sCD14, soluble CD14. Data were analyzed by Fisher's exact, χ^2 and Mann-Whitney test where appropriate.

lower CD4⁺/CD8⁺ ratio (Table 1). INR also displayed significantly higher activated CD8⁺CD38⁺ and memory-activated CD8⁺CD38⁺CD45RO⁺ cells (Table 1).

No difference was observed between study groups in terms of endoscopic and histological evaluation in ileum and colon biopsies in INR and full responders (Supplemental Digital Content, SDC, 1, <http://links.lww.com/QAD/A866>).

Immunological nonresponders and full responders show comparable CD4⁺ T-lymphocyte reconstitution in the ileum and colon, yet immunological nonresponders display higher intestinal proliferation

CD3, CD4⁺, and CD8⁺ expression were measured by IHC. Participants showed comparable CD4⁺ expression and distribution in both the ileum (high-power field, respectively, 105, IQR 84–207 vs. 98, 67–152; $P=0.61$) and colon (respectively, 107, IQR 100–144 vs. 128, IQR 79–186; $P=0.7$). The expression of CD3 and CD8⁺ at both mucosal sites were also similar in INR and full responders (not shown).

We further investigated gut cellular proliferation through Ki67 IHC measurement. No differences between study groups were detected in Ki67 expression in ileum (INR 27%, 16–35; full responders 22%, 19–33%; $P=0.09$; SDC 2A, <http://links.lww.com/QAD/A866>). Interestingly, however, a significantly higher Ki67 expression was detected in colon biopsies of INR (35%, 31–44 vs. 25%, 15–30; $P=0.004$; SDC 2B, <http://links.lww.com/QAD/A866>). Colon Ki67 levels negatively correlated with colon CD4⁺ cell counts ($r=-0.6$, $P=0.04$) (SDC 2C, <http://links.lww.com/QAD/A866>).

Overall, these results show similar CD4⁺ T-cell levels in the gut of INR and full responders, despite poor CD4⁺ recovery in peripheral blood and heightened activation/proliferation in both peripheral blood and gut mucosa in the former.

Damage of gut junctional complexes features immunological nonresponder patients

We analyzed colon and ileum junctional complex by staining for claudin 1, cadherin (Cdh1), and *Zonula occludens* protein 1 (ZO-1).

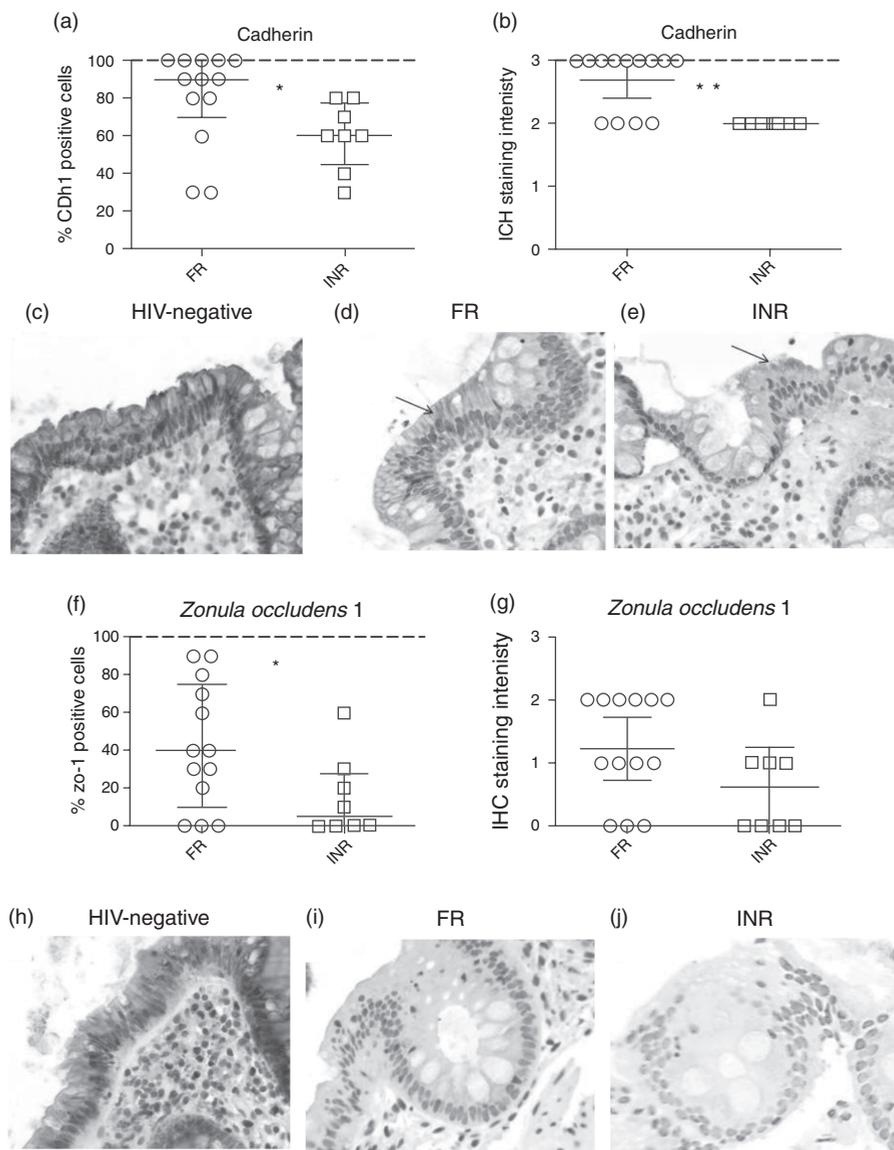


Fig. 2. Expression of junctional complex proteins in colon biopsies. Claudin 1, claudin 7, Cdh1, and ZO-1 expression were measured by IHC in colon tissue in FR and INR patients. Expression of Cdh1 (a) as well as the staining intensity of Cdh1 (b) were significantly impaired in INR compared to FR. Immune-reactivity with anti-Cdh1 antibodies showed strong diffuse basal and lateral positivity of enterocytes in HIV-uninfected study participants (c), focal lateral positivity between enterocytes in FR (arrow, d) and rare weak lateral positivity between enterocytes in INR (arrow, e). Expression of ZO-1 (f) was significantly impaired in INR compared to FR, with extremely low staining intensity/negative expression in INR (g). Similarly, immune-reactivity with anti-ZO-1 antibodies was impaired in FR and INR compared to HIV-negative controls (h–j). Data were analysed by Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$. - - - indicates median values in a historical cohort of healthy HIV-uninfected study participants. Cdh1, cadherin; FR, full responder; IHC, immunohistochemistry; INR, immunological nonresponder; ZO-1, *Zonula occludens* protein 1. 40× original magnification.

Colonic junctional complex protein expression was severely impaired in HIV-infected individuals compared with a historical cohort of HIV-negative controls, with lower frequencies of CDh1 ($P = 0.01$; Fig. 2a), ZO-1 ($P < 0.0001$; Fig. 2f), and claudin 1 (not shown), as well as weaker staining intensity (Fig. 2b–e; SDC 3, <http://links.lww.com/QAD/A866>) and protein distribution to basal/basolateral and membrane/paranuclear zones (Fig. 2g–j; SDC 3, <http://links.lww.com/QAD/A866>).

Most interestingly, when compared to full responders, INR presented significantly lower CDh1 (60%, IQR 45–78 vs. 90%, IQR 70–100; $P = 0.03$; Fig. 2a), ZO-1 (5% 0–28 vs. 40%, IQR 10–75; $P = 0.05$; Fig. 2f), and claudin 1 (15%, IQR 2.5–48 vs. 55%, IQR 40–80; $P = 0.03$) immunoreactive cells in colon biopsies. Consistently with these findings, a distinctive staining pattern of junctional complex proteins was registered in the colon of INR, with restriction of CDh1 to the basal

Electron microscopy of gut biopsies and evidence of microbial translocation

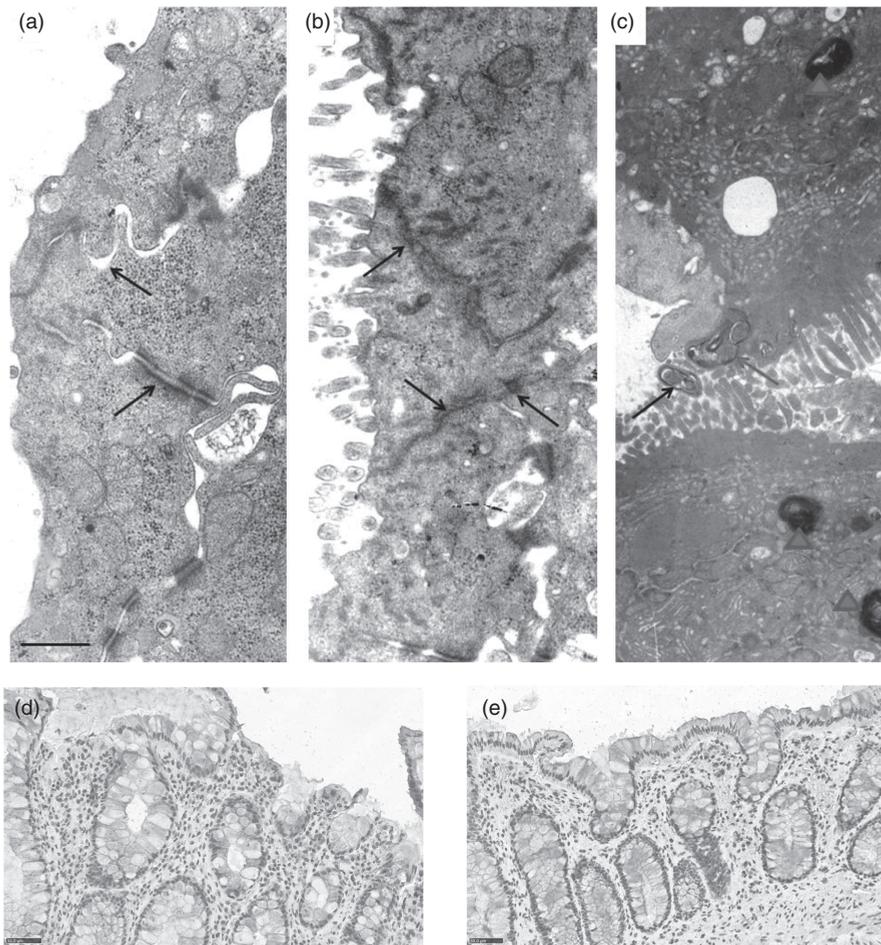


Fig. 3. Electron microscopy study of colon biopsies and evidence of microbial translocation in the lamina propria. Colon biopsy from a representative INR patient. (a) Arrows point to dilated intercellular space. Colon biopsy from a representative full responder (FR) patient. (b) Arrows point to normal intercellular space. Intestinal (ileocolic junction) biopsy from an INR patient. (c) Arrow points to an extracellular bacterium among microvilli, red arrow points to intracellular bacterium, arrow heads point to partly digested intracytoplasmic bacteria. IHC staining for LPS-core antigen in the colonic lamina propria of a representative FR (d) and INR (e). A and B, scale bar = 500 nm; C, scale bar = 1 μ m; D and E, scale bar = 50 μ m. INR, immunological nonresponder.

surface (Fig. 2e; SDC 3, <http://links.lww.com/QAD/A866>) and negative expression of ZO-1 (Fig. 2j; SDC 3, <http://links.lww.com/QAD/A866>). We also found a positive correlation between colonic ZO-1 expression and current CD4⁺ T cells ($r = 0.5$, $P = 0.04$), as well as a trend with the change in CD4⁺ cell count since cART start ($r = 0.4$, $P = 0.07$), confirming the IHC findings of lower TJ protein expression in the colon of INR.

Junctional complex protein expression and intensity were also impaired in ileal tissue of HIV-infected patients compared with HIV-negative controls (CDh1: $P = 0.03$; ZO-1: $P < 0.0001$; SDC 4A–J, <http://links.lww.com/QAD/A866>). Despite no significant differences in the number of Cdh1 and ZO-1 immune-reactive cells between INR and full responders (SDC 4A, 4F, <http://links.lww.com/QAD/A866>), defective distribution, and

staining intensity of junctional complex proteins was registered in the former (SDC 3, SDC 4D, E; 4I, J, <http://links.lww.com/QAD/A866>), as well as a positive correlation between ileal ZO-1 expression and measures of peripheral CD4⁺ T-cell reconstitution (current CD4⁺ T cells: $r = 0.6$, $P = 0.01$; change in CD4⁺ cell count since cART start: $r = 0.5$, $P = 0.02$).

In accordance with the role played by immune activation in hampering CD4⁺ T-cell recovery on cART, we also found a negative correlation between peripheral CD8⁺CD38⁺ T-cell frequencies and ZO-1 expression in both colon ($r = -0.6$, $P = 0.01$) and ileum ($r = -0.5$, $P = 0.02$).

To confirm intestinal epithelial barrier dysfunction in INR, electron microscopy evaluation of ileum and colon

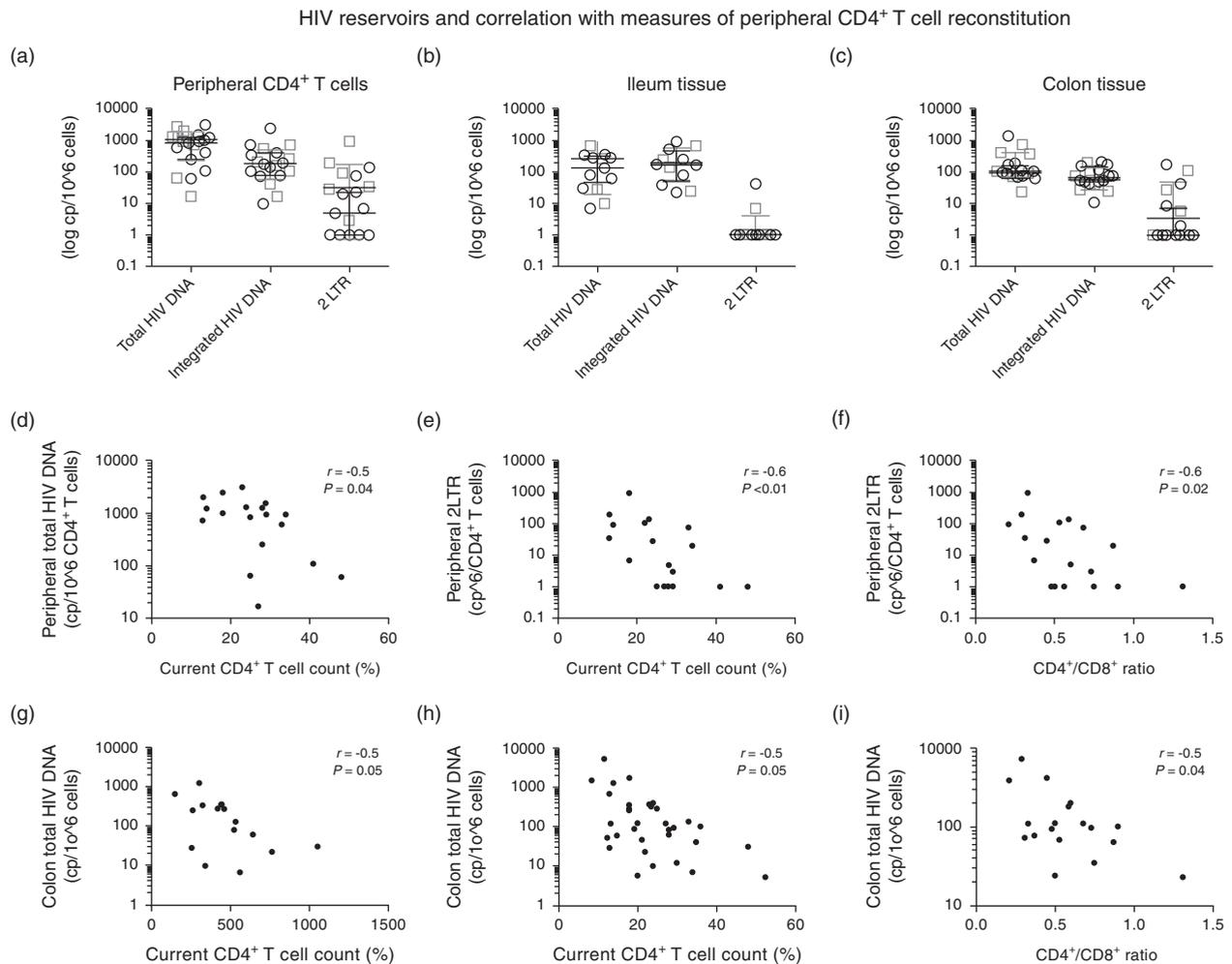


Fig. 4. HIV reservoirs and correlation with measures of peripheral CD4⁺ T-cell reconstitution. Total HIV DNA, integrated HIV DNA, and 2-LTR circles were measured in peripheral CD4⁺ T cells (a) ileum (b) and colon (c) of INR and FR study participants. No differences in the size of the reservoir were registered between the study groups, nor differences noted between anatomical compartments. Peripheral CD4⁺ T-cell counts negatively correlated with HIV reservoirs in circulating CD4⁺ T cells (d, e, for total HIV DNA and 2-LTR circles, respectively). CD4⁺/CD8⁺ ratio in the periphery was also negatively associated with the frequency of blood CD4⁺ T cells harboring 2 LTR (f). Similarly, peripheral CD4⁺ T-cell counts (g, h) and CD4⁺/CD8⁺ ratio (i) negatively correlated with total HIV-DNA in the ileum. Data were analyzed by Mann–Whitney test. Spearman’s rank coefficient was used for correlations. INR, immunological nonresponder; FR, full responder.

junctional complex was performed. Striking differences between INR and full responders were observed in colon tissue: the intercellular space between adjacent cells determined by TJ and AJ was focally wider in INR (Fig. 3a and c). Of note, intracellular bacteria were seen in 1 INR (Fig. 3c); furthermore, IHC staining for LPS was positive in the gut ‘lamina propria’ of both full responders and INR (Fig. 3d and e), in all indicating microbial translocation through a damaged mucosa.

Comparable small intestine permeability/damage, microbial translocation, and systemic inflammation in immunological nonresponder and full responders

Having shown impairment of gut junctional complex in INR, we assessed intestinal permeability (LAC/

MAN) and function (I-FABP). No differences in urinary LAC/MAN ratio were found between study groups (INR: 0.03, IQR 0.02–0.07; full responders: 0.020, IQR 0.02–0.14; $P = 0.6$), pointing to a similar degree of gastrointestinal permeability in INR and full responders with increased paracellular absorption of high molecular weight molecules [53]. Consistently with this finding and with the result of similar LPS staining in colon tissue, study participants also showed comparable I-FABP (721 pg/ml, IQR 612–994 vs. 858 pg/ml, IQR 660–1157; $P = 0.4$), LPS, endotoxin core antibodies, sCD14, IL-6, and D-dimer levels in plasma (Table 1), suggesting analogous gut epithelial damage, microbial translocation, and systemic inflammation in INR and full responders.

Qualitative analysis of the fecal microbiome revealed an outgrowth of *Bacteroides–Prevotella* spp. in immunological nonresponder and full responders

Given that fecal bacterial composition has been shown to influence gut junctional complex maturation and gut barrier permeability [54], we performed a deep molecular characterization of the fecal microbiome. DGGE analysis of the V2–V3 region of the 16S rRNA gene of amplified bacteria revealed a diverse profile for each study participant, although some bands were common to several samples. *Prevotella copri* and *Bacteroides uniformis* were more frequently represented in the INR and full responders group, respectively (SDC 5, <http://links.lww.com/QAD/A866>).

However, real-time PCR quantification of *Bacteroides–Prevotella* spp. genomes failed to show major differences between INR and full responders, despite there being an overrepresentation in our patients cohort as a whole compared with uninfected controls [55].

The size of the HIV reservoir in peripheral CD4⁺ T cell and colonic tissue correlates negatively with immune reconstitution

We next measured HIV reservoirs in ileum, colon, and peripheral blood CD4⁺ T cells in study participants.

INR and full responders presented similar frequencies of cells harbouring total and integrated HIV-DNA as well as 2-long terminal repeat (2-LTR) circles in both peripheral CD4⁺ T cells (Fig. 4a), ileum (Fig. 4b), and colon tissue (Fig. 4c). Frequencies of cells carrying viral DNA in the different compartments, however, were differentially associated with immune recovery. In particular, we found a negative correlation between total HIV-DNA in peripheral CD4⁺ T cells and current CD4⁺ T-cell counts (Fig. 4d), as well as 2-LTR circles in peripheral CD4⁺ T cells, current CD4⁺ T-cell counts (Fig. 4e) and the CD4⁺/CD8⁺ ratio (Fig. 4f). Consistently with this finding, HIV reservoirs in the ileum and colon negatively correlated with measures of peripheral CD4⁺ T-cell reconstitution (Fig. 4g–i).

Collectively, these results suggest that poor immunological recovery upon virologically suppressive cART may associate with larger frequencies of cells harbouring viral DNA, in both peripheral CD4⁺ T cells and intestinal tissue.

Discussion

Individuals with incomplete immunological response to virologically suppressive cART display heightened risk of AIDS/non-AIDS morbidity and mortality [36–38],

prompting research on pathogenic determinants and interventional strategies.

Building on earlier studies suggesting an inverse correlation between blood CD4⁺ T-cell reconstitution and microbial translocation [20,45,46], we hypothesized that deficient CD4⁺ recovery may be explained by severe impairment of the intestinal mucosa.

We conducted a comparative study of the gastrointestinal tract in INR and full responders, who started cART with severe CD4⁺ T-cell depletion, known to associate with a high risk of hampered immune reconstitution and clinical events [35]. This approach allowed us to ascertain the robustness of any difference observed between INR and full responders. Given previous data suggesting unique pathologies of HIV damage through the alimentary tract [15,33], we chose to sample and analyze both ileum and colon sites, aiming to gain a broadest insight into gastrointestinal damage and function in the setting of treated HIV.

Expanding on the description of increased gut mucosal neutrophil infiltration, apoptosis and extensive collagen deposition in HIV-infected individuals on cART [34,56], we show damage of gut junctional complex proteins in both INR and full responders when compared to a historical cohort of healthy HIV-negative controls. Additionally, in our study, the result of a negative correlation between epithelial junctional complex proteins and peripheral T-cell activation suggests a pathogenic link between the gut junctional complex, immune activation, and CD4⁺ T-cell reconstitution on cART. We find, indeed, that INR are unique in terms of the extent of colonic epithelial barrier damage: both IHC and statistical evidence suggest lowest expression of junctional complex proteins in the mucosa of the large bowel, with electron microscopy proof of dilated intercellular spaces corroborating these findings. We also find LPS positivity in patients' colonic 'lamina propria' and anecdotal evidence of bacteria passing through it, which, to our knowledge, is the first report of whole bacteria translocation in treated HIV-infected humans [21,57].

Despite the distinct pattern of gut junctional complex protein expression in full responders and INR, we failed to detect differences in mucosal T-cell reconstitution, systemic inflammation, microbial translocation evaluated by both LPS both in peripheral blood and 'lamina propria', gut damage/permeability, and fecal microbiome. These findings, which seemingly contrast with previous reports by our and other groups [20,45,46], merit further discussion.

Firstly, we cannot rule out that the discrepancies between our current data and previous research may be, in part, because of differences in patients' characteristics, mainly

CD4⁺ nadir, which appears to be lower in literature studies [34,45] compared to the present report. Additionally, a proportion of INR in our study were receiving antibiotics, which may have contributed to containing microbial translocation in these patients.

Although similar CD4⁺ levels are reported in the gut of INR and full responders, we find higher colonic Ki67 in the former; this negatively correlates with CD4⁺ cell counts in the same district. Based on animal data demonstrating ongoing damage and subsequent repair in increased gut epithelial proliferation, measured by Ki67 [21], our findings are suggestive of compensatory regenerative activity in the colon of INR, resulting in equal CD4⁺ cell counts in the two study groups. This finding is in contrast with what described by Somsouk *et al.* [34], who show reduced Ki67 in the colon of a cohort of INR. Although we cannot exclude technical differences in Ki67 staining and interpretation, as well as different Ki67 expression according to the gut anatomical site, we do believe that a likely interpretation of such discrepant findings might reside in differences in the patients populations studied: in Somsouk's paper INR feature very low CD4⁺ nadir, of 21 (7–56) cells/ μ l, inferior than our values of 68 (36–120) CD4⁺ cells/ μ l. Given that CD4⁺ nadir has been consistently proven to capture the pretherapy HIV-mediated damage, and to strongly dictate immune reconstitution [58], it is possible that Somsouk *et al.* probed a very drained and exhausted population with no further potential for compensatory epithelial proliferation. At the other end, Somsouk describe quite high median colonic Ki67 stain in their full responders as compared with ours (about 50 vs. 25%), again to possibly reflect patients with a better preserved mucosal immunity.

Differently to data by Somsouk *et al.* [34] we also show comparable I-FABP and urinary LAC/MAN in INR and full responders, despite marked differences in gut junctional complex protein expression between the study groups. In this respect, INR display severe damage of both TJ (claudin-1, ZO-1) and AJ (Cdh1) in the colon and impairment of only Cdh1 in the ileum. These findings explain the reportedly similar gut damage/permeability markers in INR and full responders, given that they mirror small bowel function. Furthermore, they expand a recent observation of the reduced proximal-to-distal expression of colonic TJ, yet not AJ, in virally suppressed HIV-infected individuals [33], by showing that differential defects of the mucosal barrier may exist at different gut sites according to the degree of immune recovery on cART. In this respect, while not designed to specifically dissect the reasons behind anatomical differences in gut junctional complex expression, our data do confirm that a unique HIV-driven pathology throughout the alimentary tract persist on cART [15,33]. Indeed, concordant with data in untreated HIV, we hereby show less severe junctional complex damage in patients' ileum,

that is limited to AJ (as opposed to both TJ and AJ proteins downregulation in the colon), entirely in line with the model of epithelial apoptosis as predominant mechanism of small bowel damage as opposed to paracellular permeability in the large intestine [33].

We also analyzed fecal microbiome, which, on the one hand, promotes TJ protein expression [54] and on the other, is shaped by the gut barrier structure [59–61]. Despite an overall outgrowth of *Bacteroides-Prevotella* [55], INR and full responders displayed similar fecal microbial composition, suggesting analogous impairment of the gut microflora [62–65].

Finally, in the face of our starting hypothesis, our findings may be also consistent with the alternative possibility that despite reduced junctional complex protein expression, INR are able to preserve mucosal integrity/function similar to that observed in full responders. Investigation of genes and markers regulating gut permeability and immunity is thus needed to shed light on the mechanisms by which the junctional complex affects peripheral CD4⁺ T-cell reconstitution. Indeed, by showing similar levels of bacterial translocation in both peripheral blood and colon 'lamina propria' of INR and full responders despite striking differences in gut junctional complex protein, our data seem to propose a model whereby cART started in a context of severe CD4⁺ T-cell depletion, while not restoring gut epithelium adhesion molecules, may nonetheless recuperate gut barrier function, therefore containing systemic microbial translocation. As corollary, our data leads to the supposition that bacterial translocation in treated HIV may have a limited impact on clinical outcome, as also indicated in recent reports from larger clinical cohorts, which failed to capture an independent association between markers of microbial translocation and clinical events in treated infection [47,66,67]. Whether or not HIV-driven gut barrier anatomy and function might be reversible upon antiviral treatment and how such reversibility might associate with starting CD4⁺ strata should be tested in ad hoc-designed studies.

Based on earlier research showing higher gut versus peripheral blood HIV-DNA in cART-treated patients [26–28], we asked whether greater HIV reservoirs associate with gastrointestinal damage and poor immune recovery. No differences in gut and circulating HIV reservoirs were observed between INR and full responders, which may be explained by the reported low CD4⁺ nadir [68]. However, HIV reservoirs in both peripheral blood and gut negatively correlate with markers of CD4⁺ T-cell reconstitution in periphery, suggesting that poor immune recovery on cART may be associated with greater HIV reservoirs. Our result of an inverse correlation between HIV reservoirs and CD4⁺/CD8⁺ ratio, which has been shown to associate with immune activation/senescence and disease progression [69], further highlights the possible link between HIV

reservoir, persistent immunological alterations, and clinical risk in INR [48].

Our study rests on a few caveats, including our inability to measure HIV reservoirs in sorted gut cells and to correct for CD4⁺ frequency in gastrointestinal tissue, thus impeding the comparison of HIV reservoirs among compartments.

Another substantial abnormality described in the secondary lymphoid organs and gastrointestinal mucosa of HIV-infected patients are profibrotic changes, that are not fully reversed by cART, mainly if started late in the infection [56,70]. Interestingly, tissue fibrosis has also been implicated in failed immune reconstitution following effective cART [71]. Although our study was not specifically designed to investigate fibrosis in the gut of INR and full responders, it is highly likely that our patients display wide tissue fibrosis, in turn further contributing to gastrointestinal damage.

The findings outlined above shed light, nonetheless, on the possible reasons behind the ineffectiveness of treatments tested to sustain CD4⁺ T-cell recovery in INR. A key implication of our study is, indeed, that unless the gastrointestinal tract is specifically targeted *vis-à-vis* HIV burden and mucosal repair, interventions are unlikely to be successful. cART intensification [35,39,40,42,43] may result in the inefficient concentration of antiretrovirals in the gut [72]; IL-7 immune-adjuvant therapy may contribute to replenishment of the HIV reservoir rather than its reduction [41,73]; further, rifaximin may not result in the effective control of microbial translocation because of the lasting damage of the gastrointestinal tract [44].

In conclusion, we show that INR feature severe gut junctional complex damage and increased HIV reservoir in both circulating CD4⁺ and gastrointestinal tissue, thus providing new directions for therapeutic interventions in this setting.

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Author contributions: C.T. and E.M. conducted the cellular biology experiments; P.B., F.S., D.T., G.B., and S.R. performed the pathology experiments; E.B. and M.L.C. conducted the microbiological analyses and A.B. was responsible for HPLC data. E.M. and N.C. performed the studies on HIV reservoirs. G.A. and A.d'M. recruited study participants and B.M. performed the endoscopy examinations. C.T. and G.M. designed the study and conducted the data analyses. All authors contributed to the writing of the manuscript. G.M. supervised the project.

Conflicts of interest

There are no conflicts of interest.

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References

1. Veazey RS, DeMaria M, Chalifoux LV, Shvets DE, Pauley DR, Knight HL, *et al.* **Gastrointestinal tract as a major site of CD4⁺ T cell depletion and viral replication in SIV infection.** *Science* 1998; **280**:427–431.
2. Fackler OT, Schäfer M, Schmidt W, Zippel T, Heise W, Schneider T, *et al.* **HIV-1 p24 but not proviral load is increased in the intestinal mucosa compared with the peripheral blood in HIV-infected patients.** *AIDS* 1998; **12**:139–146.
3. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. **Massive infection and loss of memory CD4⁺ T cells in multiple tissues during acute SIV infection.** *Nature* 2005; **434**:1093–1097.
4. Mehndru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, Hogan C, *et al.* **Primary HIV-1 infection is associated with preferential depletion of CD4⁺ T lymphocytes from effector sites in the gastrointestinal tract.** *J Exp Med* 2004; **200**:761–770.
5. Mehndru S, Poles MA, Tenner-Racz K, Jean-Pierre P, Manuelli V, Lopez P, *et al.* **Lack of mucosal immune reconstitution during prolonged treatment of acute and early HIV-1 infection.** *PLoS Med* 2006; **3**:e484.
6. Mehndru S, Poles MA, Tenner-Racz K, Manuelli V, Jean-Pierre P, Lopez P, *et al.* **Mechanisms of gastrointestinal CD4⁺ T-cell depletion during acute and early human immunodeficiency virus type 1 infection.** *J Virol* 2007; **81**:599–612.
7. Ciccone EJ, Read SW, Mannon PJ, Yao MD, Hodge JN, Dewar R, *et al.* **Cycling of gut mucosal CD4⁺ T cells decreases after prolonged antiretroviral therapy and is associated with plasma LPS levels.** *Mucosal Immunol* 2010; **3**:172–181.
8. Gordon SN, Cervasi B, Odorizzi P, Silverman R, Abera F, Ginsberg G, *et al.* **Disruption of intestinal CD4⁺ T cell homeostasis is a key marker of systemic CD4⁺ T cell activation in HIV-infected individuals.** *J Immunol* 2010; **185**:5169–5179.
9. Schneider T, Jahn HU, Schmidt W, Riecken EO, Zeitz M, Ullrich R. **Loss of CD4 T lymphocytes in patients infected with human immunodeficiency virus type 1 is more pronounced in the duodenal mucosa than in the peripheral blood.** **Berlin Diarrhea/Wasting Syndrome Study Group.** *Gut* 1995; **37**:524–529.
10. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, *et al.* **CD4⁺ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract.** *J Exp Med* 2004; **200**:749–759.
11. Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, *et al.* **Severe CD4⁺ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy.** *J Virol* 2003; **77**:11708–11717.

12. Kotler DP, Gaetz HP, Lange M, Klein EB, Holt PR. **Enteropathy associated with the acquired immunodeficiency syndrome.** *Ann Intern Med* 1984; **101**:421–428.
13. Lim SG, Menzies IS, Lee CA, Johnson MA, Pounder RE. **Intestinal permeability and function in patients infected with human immunodeficiency virus. A comparison with coeliac disease.** *Scand J Gastroenterol* 1993; **28**:573–580.
14. Keating J, Bjarnason I, Somasundaram S, Macpherson A, Francis N, Price AB, et al. **Intestinal absorptive capacity, intestinal permeability and jejunal histology in HIV and their relation to diarrhoea.** *Gut* 1995; **37**:623–629.
15. Epple HJ, Schneider T, Troeger H, Kunkel D, Allers K, Moos V, et al. **Impairment of the intestinal barrier is evident in untreated but absent in suppressively treated HIV-infected patients.** *Gut* 2009; **58**:220–227.
16. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, et al. **Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation.** *PLoS Pathog* 2010; **6**:e1000852.
17. Bjarnason I, Sharpstone DR, Francis N, Marker A, Taylor C, Barrett M, et al. **Intestinal inflammation, ileal structure and function in HIV.** *AIDS* 1996; **10**:1385–1391.
18. Stockmann M, Fromm M, Schmitz H, Schmidt W, Riecken EO, Schulzke JD. **Duodenal biopsies of HIV-infected patients with diarrhoea exhibit epithelial barrier defects but no active secretion.** *AIDS* 1998; **12**:43–51.
19. Stockmann M, Schmitz H, Fromm M, Schmidt W, Pauli G, Scholz P, et al. **Mechanisms of epithelial barrier impairment in HIV infection.** *Ann N Y Acad Sci* 2000; **915**:293–303.
20. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. **Microbial translocation is a cause of systemic immune activation in chronic HIV infection.** *Nat Med* 2006; **12**:1365–1371.
21. Estes JD, Harris LD, Klatt NR, Tabb B, Pittaluga S, Paiardini M, et al. **Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections.** *PLoS Pathog* 2010; **6**:e1001052.
22. Liu Z, Hultin LE, Cumberland WG, Hultin P, Schmid I, Matud JL, et al. **Elevated relative fluorescence intensity of CD38 antigen expression on CD8+ T cells is a marker of poor prognosis in HIV infection: results of 6 years of follow-up.** *Cytometry* 1996; **26**:1–7.
23. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. **Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage.** *J Infect Dis* 1999; **179**:859–870.
24. Deeks SG, Kitchen CM, Liu L, Guo H, Gascon R, Narváez AB, et al. **Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load.** *Blood* 2004; **104**:942–947.
25. Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, Lange JM, et al. **Persistent immune activation in HIV-1 infection is associated with progression to AIDS.** *AIDS* 2003; **17**:1881–1888.
26. Chun TW, Nickle DC, Justement JS, Meyers JH, Roby G, Hallahan CW, et al. **Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy.** *J Infect Dis* 2008; **197**:714–720.
27. Yukl SA, Gianella S, Sinclair E, Epling L, Li Q, Duan L, et al. **Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy.** *J Infect Dis* 2010; **202**:1553–1561.
28. Yukl SA, Shergill AK, Ho T, Killian M, Girling V, Epling L, et al. **The distribution of HIV DNA and RNA in cell subsets differs in gut and blood of HIV-positive patients on ART: implications for viral persistence.** *J Infect Dis* 2013; **208**:1212–1220.
29. Macal M, Sankaran S, Chun TW, Reay E, Flamm J, Prindiville TJ, et al. **Effective CD4+ T-cell restoration in gut-associated lymphoid tissue of HIV-infected patients is associated with enhanced Th17 cells and polyfunctional HIV-specific T-cell responses.** *Mucosal Immunol* 2008; **1**:475–488.
30. Kotler DP, Shimada T, Snow G, Winson G, Chen W, Zhao M, et al. **Effect of combination antiretroviral therapy upon rectal mucosal HIV RNA burden and mononuclear cell apoptosis.** *AIDS* 1998; **12**:597–604.
31. Sheth PM, Chege D, Shin LY, Huibner S, Yue FY, Loutfy M, et al. **Immune reconstitution in the sigmoid colon after long-term HIV therapy.** *Mucosal Immunol* 2008; **1**:382–388.
32. Chege D, Kovacs C, la Porte C, Ostrowski M, Raboud J, Su D, et al. **Effect of raltegravir intensification on HIV proviral DNA in the blood and gut mucosa of men on long-term therapy: a randomized controlled trial.** *AIDS* 2012; **26**:167–174.
33. Chung CY, Alden SL, Funderburg NT, Fu P, Levine AD. **Progressive proximal-to-distal reduction in expression of the tight junction complex in colonic epithelium of virally-suppressed HIV+ individuals.** *PLoS Pathog* 2014; **10**:e1004198.
34. Somsouk M, Estes JD, Deleage C, Dunham RM, Albright R, Inadomi JM, et al. **Gut epithelial barrier and systemic inflammation during chronic HIV infection.** *AIDS* 2015; **29**:43–51.
35. Gazzola L, Tincati C, Bellistri GM, Monforte A, Marchetti G. **The absence of CD4+ T cell count recovery despite receipt of virologically suppressive highly active antiretroviral therapy: clinical risk, immunological gaps, and therapeutic options.** *Clin Infect Dis* 2009; **48**:328–337.
36. Lapadula G, Cozzi-Lepri A, Marchetti G, Antinori A, Chiodera A, Nicastrì E, et al. **Risk of clinical progression among patients with immunological nonresponse despite virological suppression after combination antiretroviral treatment.** *AIDS* 2013; **27**:769–779.
37. Engsig FN, Zangerle R, Katsarou O, Dabis F, Reiss P, Gill J, et al. **Long-term mortality in HIV-positive individuals virally suppressed for >3 years with incomplete CD4 recovery.** *Clin Infect Dis* 2014; **58**:1312–1321.
38. Zoufaly A, Cozzi-Lepri A, Reekie J, Kirk O, Lundgren J, Reiss P, et al. **Immuno-virological discordance and the risk of non-AIDS and AIDS events in a large observational cohort of HIV-patients in Europe.** *PLoS One* 2014; **9**:e87160.
39. Rusconi S, Vitiello P, Adorni F, Colella E, Focà E, Capetti A, et al. **Maraviroc as intensification strategy in HIV-1 positive patients with deficient immunological response: an Italian randomized clinical trial.** *PLoS One* 2013; **8**:e80157.
40. Hunt PW, Shulman NS, Hayes TL, Dahl V, Somsouk M, Funderburg NT, et al. **The immunologic effects of maraviroc intensification in treated HIV-infected individuals with incomplete CD4+ T-cell recovery: a randomized trial.** *Blood* 2013; **121**:4635–4646.
41. Lévy Y, Sereti I, Tambussi G, Routy JP, Lelièvre JD, Delfraissy JF, et al. **Effects of recombinant human interleukin 7 on T-cell recovery and thymic output in HIV-infected patients receiving antiretroviral therapy: results of a phase I/IIa randomized, placebo-controlled, multicenter study.** *Clin Infect Dis* 2012; **55**:291–300.
42. Massanella M, Negredo E, Puig J, Puertas MC, Buzón MJ, Pérez-Álvarez N, et al. **Raltegravir intensification shows differing effects on CD8 and CD4 T cells in HIV-infected HAART-suppressed individuals with poor CD4 T-cell recovery.** *AIDS* 2012; **26**:2285–2293.
43. Negredo E, Massanella M, Puertas MC, Buzón MJ, Puig J, Pérez-Álvarez N, et al. **Early but limited effects of raltegravir intensification on CD4 T cell reconstitution in HIV-infected patients with an immunodiscordant response to antiretroviral therapy.** *J Antimicrob Chemother* 2013; **68**:2358–2362.
44. Tenorio AR, Chan ES, Bosch RJ, Macatangay BJ, Read SW, Yesmin S, et al. **Rifaximin Has a Marginal Impact on Microbial Translocation, T-cell Activation and Inflammation in HIV-Positive Immune Nonresponders to Antiretroviral Therapy - ACTG A5286.** *J Infect Dis* 2015; **211**:780–790.
45. Marchetti G, Bellistri GM, Borghi E, Tincati C, Ferramosca S, La Francesca M, et al. **Microbial translocation is associated with sustained failure in CD4+ T-cell reconstitution in HIV-infected patients on long-term highly active antiretroviral therapy.** *AIDS* 2008; **22**:2035–2038.
46. Jiang W, Lederman MM, Hunt P, Sieg SF, Haley K, Rodriguez B, et al. **Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection.** *J Infect Dis* 2009; **199**:1177–1185.
47. Hunt PW, Martin JN, Sinclair E, Bredt B, Hagos E, Lampiris H, et al. **T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy.** *J Infect Dis* 2003; **187**:1534–1543.

48. Marchetti G, Gori A, Casabianca A, Magnani M, Franzetti F, Clerici M, et al. **Comparative analysis of T-cell turnover and homeostatic parameters in HIV-infected patients with discordant immune-virological responses to HAART.** *AIDS* 2006; **20**:1727–1736.
49. Kasapovic A, Boesecke C, Schwarze-Zander C, Anadol E, Vogel M, Hippe V, et al. **Screening colonoscopy in HIV-infected patients: high rates of mucosal abnormalities in a German HIV-infected cohort.** *HIV Med* 2014; **15**:175–181.
50. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, et al. **Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers.** *Appl Environ Microbiol* 2000; **66**:297–303.
51. Patrone V, Ferrari S, Lizier M, Lucchini F, Minuti A, Tondelli B, et al. **Short-term modifications in the distal gut microbiota of weaning mice induced by a high-fat diet.** *Microbiology* 2012; **158** (Pt 4):983–992.
52. Vandergeeten C, Fromentin R, Merlini E, Lawani MB, DaFonseca S, Bakeman W, et al. **Cross-clade ultrasensitive PCR-based assays to measure HIV persistence in large-cohort studies.** *J Virol* 2014; **88**:12385–12396.
53. Cobden I, Dickinson RJ, Rothwell J, Axon AT. **Intestinal permeability assessed by excretion ratios of two molecules: results in coeliac disease.** *BMJ* 1978; **2**:1060.
54. Patel RM, Myers LS, Kurundkar AR, Maheshwari A, Nusrat A, Lin PW. **Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function.** *Am J Pathol* 2012; **180**:626–635.
55. Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. **Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR.** *J Appl Microbiol* 2004; **97**:1166–1177.
56. Estes JD, Haase AT, Schacker TW. **The role of collagen deposition in depleting CD4+ T cells and limiting reconstitution in HIV-1 and SIV infections through damage to the secondary lymphoid organ niche.** *Semin Immunol* 2008; **20**:181–186.
57. Smith AJ, Schacker TW, Reilly CS, Haase AT. **A role for syndecan-1 and claudin-2 in microbial translocation during HIV-1 infection.** *J Acquir Immune Defic Syndr* 2010; **55**:306–315.
58. Moore RD, Keruly JC. **CD4+ cell count 6 years after commencement of highly active antiretroviral therapy in persons with sustained virologic suppression.** *Clin Infect Dis* 2007; **44**:441–446.
59. George MD, Sankaran S, Reay E, Gelli AC, Dandekar S. **High-throughput gene expression profiling indicates dysregulation of intestinal cell cycle mediators and growth factors during primary simian immunodeficiency virus infection.** *Virology* 2003; **312**:84–94.
60. Sankaran S, George MD, Reay E, Guadalupe M, Flamm J, Prindiville T, et al. **Rapid onset of intestinal epithelial barrier dysfunction in primary human immunodeficiency virus infection is driven by an imbalance between immune response and mucosal repair and regeneration.** *J Virol* 2008; **82**:538–545.
61. Sheng YH, Lourie R, Lindén SK, Jeffery PL, Roche D, Tran TV, et al. **The MUC13 cell-surface mucin protects against intestinal inflammation by inhibiting epithelial cell apoptosis.** *Gut* 2011; **60**:1661–1670.
62. Mutlu EA, Keshavarzian A, Losurdo J, Swanson G, Siewe B, Forsyth C, et al. **A compositional look at the human gastrointestinal microbiome and immune activation parameters in HIV infected subjects.** *PLoS Pathog* 2014; **10**:e1003829.
63. Gori A, Tincati C, Rizzardini G, Torti C, Quirino T, Haarman M, et al. **Early impairment of gut function and gut flora supporting a role for alteration of gastrointestinal mucosa in human immunodeficiency virus pathogenesis.** *J Clin Microbiol* 2008; **46**:757–758.
64. Pérez-Santiago J, Gianella S, Massanella M, Spina CA, Karris MY, Var SR, et al. **Gut Lactobacillales are associated with higher CD4 and less microbial translocation during HIV infection.** *AIDS* 2013; **27**:1921–1931.
65. Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ, et al. **Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism.** *Sci Transl Med* 2013; **5**:193ra91.
66. Hunt PW, Sinclair E, Rodriguez B, Shive C, Clagett B, Funderburg N, et al. **Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection.** *J Infect Dis* 2014; **210**:1228–1238.
67. Tenorio AR, Zheng Y, Bosch RJ, Krishnan S, Rodriguez B, Hunt PW, et al. **Soluble markers of inflammation and coagulation but not T-cell activation predict non-AIDS-defining morbid events during suppressive antiretroviral treatment.** *J Infect Dis* 2014; **210**:1248–1259.
68. Boulassel MR, Chomont N, Pai NP, Gilmore N, Sékaly RP, Routy JP. **CD4 T cell nadir independently predicts the magnitude of the HIV reservoir after prolonged suppressive antiretroviral therapy.** *J Clin Virol* 2012; **53**:29–32.
69. Serrano-Villar S, Sainz T, Lee SA, Hunt PW, Sinclair E, Shacklett BL, et al. **HIV-infected individuals with low CD4/CD8 ratio despite effective antiretroviral therapy exhibit altered T cell subsets, heightened CD8+ T cell activation, and increased risk of non-AIDS morbidity and mortality.** *PLoS Pathog* 2014; **10**:e1004078.
70. Zeng M, Southern PJ, Reilly CS, Beilman GJ, Chipman JG, Schacker TW, et al. **Lymphoid tissue damage in HIV-1 infection depletes naïve T cells and limits T cell reconstitution after antiretroviral therapy.** *PLoS Pathog* 2012; **8**:e1002437.
71. Asmuth DM, Pinchuk IV, Wu J, Vargas G, Chen X, Mann S, et al. **Role of intestinal myofibroblasts in HIV-associated intestinal collagen deposition and immune reconstitution following combination antiretroviral therapy.** *AIDS* 2015; **29**:877–888.
72. Fletcher CV, Staskus K, Wietgreffe SW, Rothenberger M, Reilly C, Chipman JG, et al. **Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues.** *Proc Natl Acad Sci U S A* 2014; **111**:2307–2312.
73. Vandergeeten C, Fromentin R, DaFonseca S, Lawani MB, Sereti I, Lederman MM, et al. **Interleukin-7 promotes HIV persistence during antiretroviral therapy.** *Blood* 2013; **121**:4321–4329.