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PATHOGENESIS OF POOR IMMUNE RECOVERY ON COMBINATION ANTIRETROVIRAL THERAPY (CART): THE ROLE OF THE GASTROINTESTINAL TRACT AND MICROBIAL TRANSLOCATION

MED/17

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Introduction

HIV-infected subjects with poor CD4+ T-cell recovery in the course of cART

Clinical aspects of poor CD4+ T-cell recovery in the course of cART

Combination antiretoviral therapy (cART) has dramatically changed the natural course of HIV infection by significantly reducing HIV and AIDS-related comorbidities and death [1]. Despite resulting in complete suppression of the HIV RNA load in plasma and reconstitution of the CD4+ T-cell pool in the majority of treated subjects, cART nonetheless fails to induce significant T-cell gains in up to 30% of individuals, known as Immunological Non Responders (INR) [2].

A clear-cut definition of immunological non-response to cART in terms of CD4+ T-cell reconstitution is still lacking. An increase in the CD4+ T cell count <30% and an absolute CD4+ T cell count $\leq$200 cells/mL during the first 6–12 months of HIV suppressive cART has been widely used to define INR [2]. More recently, a study which combined data from 2 large international collaborations of HIV cohorts (the Antiretroviral Therapy Cohort Collaboration, ART-CC, and the Collaboration of Observational HIV Epidemiological Research Europe, COHERE), defined INR as subjects failing to achieve CD4 count >200 cells/μL after 3 years of sustained viral suppression (HIV RNA <500 cp/mL) [3]; in another study analyzing subjects enrolled in the Italian Cohort of Antiretroviral-Naive Patients (Icona), a definition of current CD4+ T-cell counts less than 120% pre-cART was used [4]. Finally, the EuroSIDA study used defined immuno-virological disocrdance if a patients' current CD4+ T-cell count was still equal or below the pre-ART initiation level [5].

Regardless the definition of INR, subjects experiencing poor CD4+ T-cell recovery on cART present increased risk of clinical events, in particular long-term mortality [3, 4], AIDS-defining events, malignancies, severe infections, acute kidney injuries, cardiovascular events [4] and other non AIDS events [4, 5].

Pathogenesis of poor CD4+ T-cell recovery in the course of cART

Several factors have been individually associated with immunological failure during HAART, and yet only older age, concurrent viral hepatitis, low CD4+ T-cell nadir, have been invariably proven to predict immunological non-response [2].

In the past few years, nonetheless, many groups have focused on the immune-pathogenesis of poor immune response to cART. Failure in *de novo* CD4+ T-cell production, subsequent to impaired clonogenic capability and stromal cell function of the bone marrow, as well as inefficient thymic output possibly due to a skewed IL-7/IL-R signalling system, has been put forward. On the other hand, excessive CD4+ T-cell destruction in the periphery has gained more attention as a possible factor driving blunted immune recovery on cART. In particular, heightened levels of T-cell activation, has been described to hamper CD4+ T-cell gain on effective treatment [6] and has been shown to feature INR [7]. These findings have raised the question on the possible causes of T-cell activation in the course of suppressive therapy and ongoing viral replication below the limit of detection as well as persistent antigenic stimulation have been identified. In particular, following the demonstration of circulating levels of lipopolysaccharide (LPS), a marker of microbial translocation, as a cause of immune activation in HIV disease [8], various groups have investigated the role of microbial components in hindering CD4+ T-cell recovery in INR ([9, 10]; see below).

Interventional strategies in subjects with poor CD4+ T-cell recovery in the course of cART

Poor immunological response to cART features as one of the most critical issues in the management of treated HIV disease, given that INR are exposed to increased risk of AIDS and non-AIDS co-morbidities. Interventional strategies have been widely experimented in this setting, yet have failed to provide clinical benefit.

Immune-adjuvants have been among the first molecules to be tested in subjects with inefficient CD4+ T-cell gain on cART. IL-2 has been demonstrated to be safe and is able to induce a rapid reconstitution of the CD4+ T cell compartment [2], yet has dramatically failed to account for

clinical benefit [11]. More recently, in a randomized placebo-controlled dose escalation trial, IL-7, at the dose of 20 μg/kg, has been shown to lead to a dose-dependent CD4 T-cell increase and broadening of TCR diversity [12]; nonetheless, the ultimate evidence of IL-7 clinical impact is still being sought.

Strategies aimed at decreasing peripheral immune activation have also been experimented. Given the role of viral co-infections in sustaining T-cell activation, Hunt et al. recently reported that valganciclovir, an anti-CMV drug, reduced T-cell activation in HIV-infected individuals with incomplete CD4+ T cell recovery on antiretroviral therapy [13], yet had no effect in reconstituting the CD4+ T-cell compartment. Consistently with this approach, rifaximin, which antagonizes microbial translocation, had a marginal impact in decreasing T-cell activation in a similar setting [14].

Finally, intensification strategies of the ongoing cART regimen have also been tested in INR. Maraviroc, a CCR5-inhibitor, was administered in two different studies, and resulted in increased T-cell activation in peripheral blood and rectal mucosa during treated HIV infection, as well as in the redistribution of CD8+ T cells from the gut to peripheral blood, with no effect on CD4+ T-cell reconstitution [15, 16]. In a similar vein, raltegravir, an integrase inhibitor had limited effects in containing T-cell activation and inducing CD4+ T-cell recovery [17, 18].

The gastrointestinal tract in HIV disease

The gastrointestinal tract as a site of HIV pathogenesis and persistence

The gastrointestinal (GI) tract is a major site of HIV pathogenesis in the acute phase of disease, given the rapid depletion of GI CCR5+CD4+ T-cells, following viral dissemination at mucosal sites. Indeed, in this stage of infection, CD4+ T-cell depletion is more prominent in the gastrointestinal tract than in peripheral blood and lymph nodes [19].

During the transition from acute to chronic infection, HIV replication is reduced but not fully suppressed by the host adaptive immune response, thus resulting in the progressive loss of CD4+ T-cell from both blood and mucosal sites. In the latter, numerous immune and anatomical alterations have been described, including local hyperactivation/inflammation, exhaustion of intestinal macrophage phagocytic function, and structural epithelial damage. Collectively, these alterations may result in the increased passage of gut microflora and microbial products into the systemic circulation, phenomen known as 'microbial translocation' (see below) [20].

Whether cART restores CD4+ T-cell numbers, immune function and structure is still a matter of debate. In this context, timing of antiretroviral therapy definitely plays a role, given that various studies have reported broader CD4+ T-cell reconstitution when cART is initiated early in the phase of infection [21]. In the setting of chronic disease, while previous studies demonstrated a minimal impact of cART in CD4+ T-cell recovery at mucosal sites, more recent data have shown that long-term cART (>5 years) produces a >50% CD4+ T-cell gain in the GI tract which was associated with enhanced Th17 CD4 + T-cell accumulation and polyfunctional anti-HIV cellular responses [22]. In a similar vein, cART was shown to abrogate the HIV-induced intestinal barrier defect and villous atrophy due to altered tight junction protein composition and elevated epithelial apoptoses [23].

Importantly, other than the issue of whether antiretroviral treatment results in the restoration of CD4+ T-cell and epithelial barrier function, higher levels of HIV DNA and HIV RNA have been described in gut biopsies, compared with PBMCs from aviremic individuals [24, 25], posing the GI

tract as a major site o of HIV persistence.

The gastrointestinal flora in HIV disease

Alongside GI CD4+ T-cell depletion and impairment of the mucosal barrier, HIV infection is known to alter the composition of the intestinal microflora, which has a major role in the maintenance of both local and systemic immunity. Indeed, HIV-infected individuals have been shown to feature impairment of the fecal flora in early stages of disease, with a predominance of opportunistic pathogens (*Pseudomonas aeruginosa* and *Candida albicans*) and low levels of protective bacteria (bifidobacteria and lactobacilli) compared with historical data on healthy HIV-uninfected persons [26]. Consistently with these findings, levels of *Enterobacteriales* and *Bacteroidales* fecal DNA have been significantly associated with CD4+ T-cell loss in the duodenum and peripheral CD8+ T-cell activation, thus providing evidence for a direct role of the gut microbiome in driving local and systemic immune activation in HIV-infected patients [27]. In this context, the composition of the flora translocating from the gut to the systemic circulation was studied in cART-receving subjects with a discordant CD4+ T-cell response to treatment. Individuals with poor immune recovery have been described to lack probiotic *Lactobacillaceae* both prior and after therapy, thus suggesting that the fecal microbiota may also affect cART outcome [28].

Microbial translocation in HIV disease

Microbial translocation: definition and measurements

Gut "translocation" of bacteria or other microbes is the non-physiological passage of the gastrointestinal microflora through the intestinal epithelial barrier/lamina propria, to local mesenteric lymph nodes and, from there, to extranodal sites. Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls and a known agonist of Toll-like receptor 4 (TLR-4) [29], is considered a major marker of microbial translocation [30-32]. Several lines of protection are active to neutralize LPS and its downstream effects as a potent immune-activating molecule, including IgM, IgG, and IgA specific for the LPS core antigen and endotoxin core antibodies (EndoCAb). LPS induces several responses in the innate immune system, through its interaction with the LPS binding protein (LBP), which catalytically transfers LPS onto membrane or soluble CD14 (sCD14) [33].

The extent of microbial translocation can be assessed either directly through the measurement of bacterial by-products in plasma, such as LPS and bacterial DNA or RNA fragments, or indirectly by sCD14, LBP, EndoCAb, and antiflagellin antibodies [20]. Recently, plasma levels of intestinal fatty acid binding protein (IFABP), a marker of enterocyte damage [34], have also been used to correlate intestinal impairment and microbial translocation [20].

An alternative method to assess microbial translocation is the detection and quantification of the universally conserved microbial 16S rRNA gene in plasma [35].

Microbial translocation as a cause of immune activation

A seminal study by Brenchley et al. was the first to demonstrate increased levels of circulating LPS in both chronically HIV-infected subjects and SIV-infected rhesus macaques. In particular, HIV-infected patients in an advanced stage of disease (CD4+ T-cells <200 /ml) presented significantly higher plasma LPS levels than uninfected individuals, thus suggesting the presence of increased

microbial translocation in this study population [8].

Interestingly, a positive correlation was found between LPS levels in plasma and measures of innate and adaptive immune activation ([8, 9, 36], thus supporting the hypothesis that microbial translocation may result in T-cell activation, which is known to influence HIV disease progression [37].

Translocating bacteria and microbial components may stimulate innate immune cells through the TLR pathways as well as other innate immune receptors, thus contributing to the pro-inflammatory cytokine milieu and systemic immune activation associated with chronic HIV infection. The possibility that microbial TLR ligands might affect T-lymphocyte activation in HIV-uninfected individuals was elegantly investigated by Funderburg et al., who showed that *in vitro* exposure to microbial TLR ligands promoted the cell surface expression of lymphocyte-homing and activation/apoptosis molecule on CD4+ and CD8+ T-cells [38]. Consistently with these observations, *in vitro* stimulation by microbial and viral TLR ligands was shown to induce T-cell activation in ART-treated, HIV-infected patients [39].

Clinical implications of microbial translocation

Given that T-cell activation has been associated with both poor disease prognosis [37, 40-43] in the course of HIV infection and impaired CD4+ T-cell reconstitution on effective treatment [6], many groups investigated whether microbial translocation parameters were associated with clinical outcomes in HIV.

The role of microbial translocation was first studied in subjects with poor CD4+ T-cell recovery on cART. These individuals presented higher levels of LPS, which correlated significantly with Ki67+ T-cells in peripheral blood [9]. In a similar vein, higher levels of DNA sequences encoding bacterial 16S rRNA genes have been associated with greater T-cell activation and impaired CD4+ T-cell restoration after ART [10]. These findings provide important insights into the underlying mechanisms of the immunological response to cART, nonetheless fail to prove that microbial

translocation is a key factor regulating CD4+ T-cell reconstitution in treated subjects, thus calling for ad hoc-designed observational cohort studies to address this question. On the contrary, data on the predictive role of microbial translocation in the clinical outcome of HIV infection are much more consistent, with the demonstration of LPS and sCD14 independently associated with, respectively, disease progression (combined endpoint of AIDS, death, CD4+ T-cell counts <200 cells/mmc, or the start of ART [44]) and overall mortality [45].

Microbial translocation has also been investigated in the setting of non-AIDS co-morbidities and viral liver infections and has been demonstrated to contribute to the pathogenesis of these diseases, providing a rationale for therapeutic interventions aimed at reducing the overall morbidity and mortality of cART-treated, HIV-infected patients [20].

Study Rationale

Incomplete immune recovery in the course of suppressive combination Antiretroviral Therapy (cART)

In the era of combination antiretroviral therapy (cART), a remarkable reduction in AIDS-related morbidity and mortality rates has been described [1]. Clinical studies have demonstrated that cART improves survival in the HIV-infected population through its ability to fully suppress HIV RNA plasma load and increase peripheral CD4+ T-cell counts (Full Response to cART, FR [1]. However, 15%–30% of treated individuals display a discordant response to long-term cART, which consists of inefficient CD4+ T-cell recovery despite effective virological control. These subjects are referred to as "Immunological Non Responders" (INRs) [2]. From a clinical standpoint, INRs represent one of the most crucial issues in the management of treated HIV disease, as they remain at a considerable higher risk of HIV progression and mortality from both AIDS and non-AIDS events [3-5] and poorly responsive to experimental treatments [12, 14-18]. It is thus critical to investigate the underlying mechanisms of poor immune recovery on effective cART and elaborate targeted interventional strategies for this population in a timely manner.

Immune activation and microbial translocation through the gastrointestinal epithelial barrier as causes of inefficient immune recovery in treated HIV infection

Peripheral immune activation has been associated with CD4+ T-cell loss and progression of disease in the natural course of HIV infection [37, 40-43]. T-cell activation, generally measured through the (co-)expression of CD38 and HLA-DR on the surface of T-lymphocytes, has also been described an independent factor of poor CD4+ T-cell recovery on cART [6]. Accordingly, INR have been shown

to present significant higher levels of peripheral immune activation compared to Full Responders (FR) [7].

Building on prior evidence of the translocation of microbial bioproducts through the gastrointestinal (GI) tract as a cause of immune activation in HIV disease, various groups have investigated its role in the pathogenesis of poor CD4+ T-cell recovery in treated HIV infection. Despite evidence of an association between microbial translocation parameters and expression of activation markers in INR [9, 10], whether stimulation with microbial components *per se* results in the induction of T-cell activation markers in this population is unknown. Further, a biological model explaining the precise mechanisms by which exposure to microbial components causes T-cell activation in HIV disease is currently lacking.

Damage of the GI tract is a prerequisite of immune activation and microbial translocation [20]. In the natural course of disease, HIV is known to infect and replicate within CD4+ T cells of the GI compartment [46-49], accounting for mucosal activation [50-52], CD4+ T cell depletion [19, 46, 48, 53, 54] and impairment of epithelial barrier function[23, 55-58], thus resulting in microbial translocation [8, 59] and immune activation [8, 59]. cART has significant effects on viral suppression and reconstitution of CD4+ T-cells in the blood, yet seems to be less successful in lowering HIV RNA and HIV DNA in the GI mucosa [24, 25, 60], as well as reconstituting mucosal CD4+ T-cells [22, 24, 49, 54, 61-65] and repairing the epithelial barrier [66].

As previously discussed, studies addressing the pathogenesis of inefficient CD4+ T-cell recovery in INR have shown increased levels of circulating LPS [9, 10], alongside high levels of immune activation [6, 7]. Literature, however, has so far not disentangled the possible links between GI barrier damage and poor immune reconstitution in the course of effective cART in INR.

Study objective and specific aims

The overall objective of the present study is to understand whether damage of the GI tract and microbial-induced T-cell activation feature HIV-infected individuals with poor immune recovery on cART.

Specific Aim 1: Comparative study of gut junctional complexes in HIV-infected individuals with different CD4+ T-cell recovery on cART.

We aim to analyze the structure and function of gut JC in Immunological Non Responder (INR) and Full Responder (FR) and to assess whether the fecal microbiome and/or HIV reservoirs may represent underlying causes of gut epithelial barrier dysfunction in course of treated HIV disease.

Specific Aim 2: Expression of activation markers on immune cells following stimulation with microbial components in HIV-infected individuals with different CD4+ T-cell recovery on cART.

We aim to study the expression of activation markers on immune cells following stimulation with microbial components in HIV-infected individuals with different CD4+ T-cell recovery on cART. We are going to analyze the effect of LPS *in vitro* stimulation on T-cell activation markers (CD38 and HLA-DR) in HIV-infected patients with different CD4+ recovery on cART [39, 67] and then set up an in vitro model to assess the TLR-mediated signalling pathways in monocyte-derived macrophages (MDM) and PBMCs in a similar study population.

Patients and Methods

Comparative study of gut junctional complexes in HIV-infected individuals with different CD4+ T-cell recovery on cART

Patients

HIV-positive subjects were recruited at the Clinic of Infectious Diseases and Tropical Medicine of the San Paolo Hospital (University of Milan, Italy) following the provision of informed consent. Enrolled patients were required to be undergoing combination Antiretroviral Therapy (cART) for $\geq$ 12 months, feature a CD4+ cell nadir <350/μL and HIV RNA load below the limit of detection (<40 cp/mL). Patients with current CD4+ count <350/mmc and/or delta CD4 change from baseline <30% were defined as Immunological Non Responders (INR, n=21). Subjects with current CD4+ count >350/μL and/or delta CD4 change from baseline >30% were defined as Full Responders (FR, n=29).

The Institutional Review Board at the San Paolo Hospital, Milan, Italy, approved the study.

Colonoscopy

Patients with a known gastrointestinal disease or clinical symptoms were excluded from the study. 8/21 INR and 13/29 FR underwent routine screening colonoscopy for preventive colorectal cancer screening [68] (bowel preparation with Moviprep® - Norgine, Marburg, Germany). 17 patients were intravenously treated with a combination of midazolam (2 mg) and pethidine (50 mg). Four patients refused pre-medication and thus a warm water colonoscopy was used (Pentax EC-34-i10L - Hamburg, Germany, GmbH). Biopsies were performed in colon (n=3) and distal ileum (n=3) of all patients. 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 (OCT) and stored at -80°C. Following this procedure, all colonic tracts, rectum and distal ileum were biopsied (n=1 per site). These samples were fixed through immersion in a solution of 10% buffered formalin and a histopathological examination was performed.

Immunohistochemical staining

Endoscopic intestinal biopsies from HIV-infected subjects were selected to assess major structural proteins of tight and adherens junctions forming the gut junctional complex (JC), T-lymphocyte sub-populations and proliferation index by immunohistochemistry (IHC). In addition, intestinal biopsies (ileum and colon) from 5 HIV-uninfected subjects, who underwent imaging studies for gastrointestinal diseases other than cancer, were considered as controls and evaluated in terms of JC protein expression.

Biopsies were fixed in formalin and paraffin-embedded; 3μm sections were stained with hematoxylin and eosin (HE) and selected antibodies.

For intestinal junction protein analysis, cadherin 1 (Cdh1, 1:15000, ABNOVA), *Zonula occludens* protein 1 (ZO-1, 1:200, Zymed), claudin 1 (1:100, Invitrogen), claudin 7 (1:100, Zymed) were used.

For the study of T lymphocyte sub-populations, CD3 (clone F7.2.38, 1:200, DAKO), CD4 (clone 1F6, 1:50, Leica) 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) antibody staining.

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 minutes.

All slides were counterstained with hematoxylin.

Negative controls were incubated in the absence of primary antibodies.

Immunohistochemistry staining was performed using a Biogenex i6000 Automated Staining System (Biogenex).

Reactions were detected by Novolink Max polymer detection system (Novocastra Laboratories L.T.D., Leica Microsystem), following manufacturer's instructions, using Diaminobenzidine (DAB) as chromogen.

A semi-quantitative score was used for the study of intestinal junction proteins, by evaluating the following: a) the reaction intensity of stained epithelial cells of colon and ileum (0 = no staining, 1 = weak staining, 2 = intermediate staining, 3 = intense staining); b) the percentage of positive cells, by counting stained cells on 100 superficial consecutive intestinal cells at high magnification (40x); and c) sub-cellular localization (membranous as complete, lateral, basal, baso-lateral; cytoplasmic; para-nuclear).

Lymphocyte sub-populations were determined by counting positive cells in three consecutive High Power Field (HPF) (40x) in ileum and colon *lamina propria*. CD4 expression was also evaluated in terms of distribution (sub-epithelial or diffuse).

Proliferation indices (Ki-67) were determined by counting positive cells on 100 intestinal cells at high magnification (40x) and have been reported as percentage.

Electron microscopy

Small ileum and colon samples from biopsies of 7 patients (3 INR and 4 FR) were fixed in 2.5% glutaraldehyde in phosphate buffer and routinely processed for electron microscopy examination in a (JEOL JEM 1010 Tokyo, Japan) transmission electron microscope.

Microbial translocation parameters

Plasma levels of sCD14 (R&D systems) and EndocAb (Hycult Biotech) were measured by ELISA test, in accordance with the manufacturer's instructions.

Circulating levels of lipopolysaccharide (LPS) were assessed using the LAL test (Lonza), as per the manufacturer's instructions. Samples were diluted 1:150 and preheated at 95°C for 10 minutes.

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 in 14 INR and 11 FR. 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) in 19 INR and 21 FR.

Fecal microbial population analysis

Analysis of the fecal microbiome was conducted in 13 INR and 14 FR.

Total bacterial DNA was extracted from 100mg of feces, using the PSP Spin Stool DNA Plus kit (Stratec molecular, Berlin, Germany), as per the manufacturer's instructions.

The V2-V3 region of the 16S ribosomal DNA (rDNA) gene was amplified with primers HDA1-GC/HDA2, as previously described [69]. Denaturing Gradient Gel Electrophoresis (DGGE) was performed with the use of a PhorU-2 system (Ingeny Int, Goes, The Netherlands), as previously described [70]. Banding patterns of DGGE profiles were analyzed with Fingerprinting II software (Bio-Rad Laboratories, Hercules, CA, USA).

To confirm the identification of the bacteria examined by DGGE, individual bands were carved out of the gel and the DNA was re-amplified and sequenced with the same primers (BMR Genomics, Padua, Italy). We subsequently conducted a search of sequences deposited in the GenBank DNA database by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/).

Given that *Bacteroides* appeared be the most representative group, we decided to specifically analyze this, using a nested approach [70], followed by DGGE analyses and a Real Time PCR to specifically quantify *Bacteroides–Prevotella*.

Virological studies

CD4+ T-cells were isolated by negative selection (StemCell Tech) from PBMCs obtained by Ficoll procedure (Biospa). Cryopreserved ileum and colon biopsies were thawed and OCT was removed following washes with PBS.

A pellet of $1x10^6$ peripheral CD4+ T-cells and entire gut biopsies were digested with lysis buffer over/night at 55°C and the lysate was used directly in a nested PCR in order to quantify both HIV and CD3 gene copy numbers, as previously described [71].

Interpretation of results

Statistical analyses were performed with the use of GraphPad Prism 6.0. Two groups were compared with a Mann-Whitney test. Three groups were compared with a Kruskall-Wallis test followed by a Dunn's multiple comparisons test. 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.

Expression of activation markers on immune cells following stimulation with microbial components in HIV-infected individuals with different CD4+ T-cell recovery on cART

We conducted two separate sets of experiments to pursue this aim.

1) Study of the dynamics of HLA-DR and CD38 expression on peripheral T-cells following selective *in vitro* LPS stimulation of PBMCs from HIV-infected cART-treated individuals [39].

Patients

HIV-positive, cART-treated patients for at least 12 months, with undetectable HIV RNA load (<40cp/mL) and a CD4+ T-cell nadir <250/μL were consecutively enrolled at the Clinic of Infectious Diseases and Tropical Medicine, "San Paolo" Hospital, University of Milan, Italy. Patients were divided into 3 groups according to the degree of immune-reconstitution in course of cART: high responders (HR) with CD4+ ≥600/μL; low responders (LR) with CD4+<350/μL; intermediate responders (IR) with CD4+ 350-599/μL. As controls, HIV-negative age-matched subjects were studied. Written informed consent forms approved by the Ethical Committee of the "San Paolo" Hospital, University of Milan, Italy were obtained from all participants [39].

Lymphocyte immunophenotype analysis

Fresh peripheral blood was drawn from all study participants in EDTA-containing tubes and PBMCs were separated by Ficoll-Histopaque technique (Biocoll separating solution, BIOSPA) (T0). Cells were counted and $5x10^6$ cells were cultured in R10 medium alone (composition per 100mL R10: 88mL RPMI, 10mL fetal bovine serum, 1mL [100UI/mL] L-glutamine and 1 ml

[100UI/mL] penicillin/streptomycin; Euroclone, Italy) (unstimulated, US) or in medium supplemented with LPS for 24 hours (T1) and 48 hours (T2) (E.coli; 026:B6*C, Sigma-Aldrich, Milan, Italy, 20ng/mL; stimulated, STIM). Prior to and following stimulation, cells were re-counted and stained (CD4+/CD8-PerCP Cy5.5, HLA-DR-FITC, CD38-PE; Ki67-FITC, Becton Dickinson, San Josè, CA, USA) for flow cytometric analysis of HLA-DR, CD38 and Ki67 expression on T-cells (Cytomics FC500, Beckman Coulter, Hialeah, FL, USA) using CXP 2.2. software [39].

Interpretation of results

Data were analyzed with GraphPad 5 PRISM software. Mann Whitney U test, Kruskall-Wallis and Wilcoxon tests were used for statistics. All statistical tests were 2-sided and differences were considered statistically significant at $p<0.05$ [39]

Substudy on the phenotype and function of CD38-expressing $CD4^+$ and $CD8^+$ T-cells in HIV-infected patients undergoing suppressive cART [67].

Immune phenotype and function of $CD4^+/CD8^+CD38^+$ and $CD4^+/CD8^+CD38^-$ subsets in 31 HIV+ patients followed at the Clinic of Infectious Diseases, University of Milan, San Paolo Hospital: 12 untreated, 19 on virologically-suppressive cART with HIV-RNA < 40cp/ml. 10 HIV-uninfected donors were enrolled as controls.

T-cell phenotype was measured through the expression of naïve (CD38+CD45RA+CD45R0-) and memory (CD38+CD45RA-CD45R0+) cell surface markers. We also assessed the contribution of recent thymic emigrants (RTEs) by the expression of either CD4+CD38+CD45R0-CD31+ or CD8+CD38+CD45RA+CD103+. Finally pStat5 and Bcl-2 were measured on magnetically-isolated $CD38^+CD8^+/CD4^+$ T cells with or without rIL-2/rIL-7 stimulation.

All the above-mentioned experiments were performed by flow cytometry.

2) Development of an *in vitro* model by which TLR stimulation with microbial bioproducts leads to T-cell activation and lack of immune recovery in course of cART.

Patients

HIV-infected individuals were consecutively enrolled at the Clinic of Infectious Diseases and Tropical Medicine, "San Paolo" Hospital, University of Milan, Italy. Subjects had to be on stable cART for at least 12 months, present undetectable plasma HIV-RNA load (<40cp/mL) in at least two consecutive assessments and CD4 nadir ≤ 350/mmc. Individuals with either signs/symptoms of gastrointestinal diseases or on antibiotic therapy at the time of study were excluded from the study.

Subjects were divided into 2 groups according to the degree of immune reconstitution in course of virologically-suppressive cART: Full Responders (FRs, n=20 with CD4 ≥ 350/mmc; Immunological Non Responders (INRs, n=15), with CD4 < 350/mmc. 13 HIV-uninfected individuals were also studied.

All the enrolled patients provided written informed consent according to the Ethical Committee of our Institution (Comitato Etico, Ospedale ''San Paolo'', Milan, Italy). The ethics committee specifically approved this study.

Isolation and culturing of primary monocytes in order to obtain Monocytes-Derived Macrophages (MDMs)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by centrifugation over a density gradient (Ficoll-Hypaque) according to standard procedures. Monocytes were separated from lymphocytes by adherence to plastic plates. In order to obtain the same amount of Monocyte-derived Macrophages for the experiments, we measured the % of monocytes in each subject and we plated the amount of PBMCs accordingly. After 48h incubation, non-adherent cells were removed by two washes with warm RPMI. The purity of monocytes was >90%, as determined

by immunofluorescent staining with anti-CD14-FITC antibody (BD Pharmigen, San Diego, CA). Monocytes were differentiated into macrophages (MDMs) by culturing in RPMI medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100U of penicillin/ml, 100 µg of streptomycin/ml, for 15 days prior to stimulation. MDMs were washed with phosphate-buffered saline (PBS) and the culture medium was replaced every 2 days.

Stimulation of PBMCs and MDMs

A dose/response and a timing curve was performed for each stimulus in order to identify the concentration and the timepoint that gave the higher effect. Ficoll-separated PBMCs were then plated at a density of $4x10^6$cells/well and stimulated for 24h with Lipolysaccharide -LPS- (50µg/mL), Peptidoglycan -PGN- (10µg/mL), Lipoteichoic Acid -LTA- (1µg/mL), ssRNA40, a uridine-rich ssRNA analogous of HIV-1 ssRNA (6,25µg/mL) (InvivoGen, San Diego, CA), Interferon γ -IFNγ- (100U/mL), antiCD28 (1,25 µg/mL) (R&D System, Minneapolis, MN) and antiCD3 (2,5 µg/mL) (BD Pharmigen, San Diego, CA). After 24h, supernatants were collected and stored for ELISA and Luminex assays. Cells were harvested for flow cytometry analyses.

At the end of day 15, MDMs ($5x10^5$cells/well) were stimulated with LPS (50µg/mL), PGN (10µg/mL), LTA (1µg/mL), ssRNA40 (6,25µg/mL) and IFNγ (100U/mL). After 24h, supernatants were collected and stored for ELISA assay. MDMs were removed by gently scraping with a plastic cell scraper and cold PBS. Cells were harvested for flow cytometry analyses.

Flow cytometry

Cell surface molecule expressions were evaluated by flow cytometry on PBMCs and MDMs using the following fluorochrome-labelled antibodies: CD4-PECy7, CD8-PECy5, CD14-PECy7, CD69-PECy5, CD45-PE, CD3-PECy7 (Instrumentation Laboratory, Milan, Italy), HLA-DR-FITC, CD38-PE, CD4-PE, CD8-PerCPCy5.5, Ki67-FITC, CD14-FITC (BD Bioscience, San Diego, CA), TLR-4 PE, TLR-7 PerCP (R&D Systems, Minneapolis, MN), TLR-8 PE (Thermoscientific, Tema Ricerca,

Milan, Italy). We evaluated activation (HLA-DR and CD38), apoptosis (Annexin V), proliferation (Ki67) and TLRs (TLR-4, -7, -8) on total PBMCs. On MDMs we measured activation (CD69) and functionality (HLA-DR).

The following combinations were used: CD8/CD4/CD38/HLA-DR, CD4/AnnexV/7AAD, CD8/AnnexV/7AAD, CD8/CD4/Ki67, CD45/CD14/CD69/HLA-DR, CD3/CD14/TLR4/TLR7 and CD3/CD14/TLR8.

Briefly, cells were stained for 20 min in the dark at 4°C and then washed with PBS, fixed in 2% formaldehyde and analyzed using FC500 (Beckman Coulter, Hialeah, FL). An additional step of permeabilization was added for intracellular Ki67 staining. CXP software was used for analyses. PBMCs were gated first based on side- and forward-scatter properties, then as CD4 and/or CD8 and finally as CD38/HLA-DR or Annexin V/7AAD or Ki67. To measure TLRs expression, PBMCs were gated first based on side- and forward-scatter properties, than as CD3/CD14 and within CD14+ gate, cells were gated as TLR4+ or TLR7+ or TLR8+. MDMs were gated first based on side-scatter properties and CD45, then as CD14+ and finally as CD69/HLA-DR.

sCD14 measurement

Supernatants obtained from HIV-infected PBMCs and MDMs were collected 24h after LPS, ssRNA and CD3/CD28 exposure for sCD14 measurement, which was performed on batched frozen samples by ELISA test according to the manufacturer's instructions (R&D System, Minneapolis, MN).

LPS measurement

Plasma levels of Lipopolysaccharide (LPS) were determined with a commercial LAL kit (Kinetic-QCL; Bio Whittaker, Walkersville, MD, USA) according to manufacturer’s protocol.

Multiplex assays

Relative content of 25 analytes in culture supernatants was determined using the human Th17 Magnetic Bead Panel (EMD Millipore; Billerica, MA, USA) with a Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, 200µl of assay buffer was added to individual wells of a 96 well plate for 10 minutes under gentle agitation at room temperature, decanted, and standards, controls, and neat samples were added to the appropriate wells. The plate was incubated for ~16 hours at 4^{o}C, well contents were removed and the plate was washed twice with 200ml of wash buffer. Next, 25µl of detection antibodies were added to all wells and the plate was incubated for 1 hour at room temperature, followed by 25µl of Streptavidin-Phycoerythrin for 30 minutes at room temperature. Plates were then washed 2X with wash buffer and read on the MAGPIX Reader. All samples were run in duplicate. Raw data was analyzed using Bio-Plex Manager software. Standard curves were generated from lyophilized standard provided with each kit. The concentration for each analyte in each sample was determined via interpolation from each corresponding standard curve.

RNA extraction and reverse transcription

RNA was extracted from cultured PBMC by using the acid guanidium thiocyanate–phenol–chloroform method. The RNA was dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase (RQ1 DNase, Promega, Madison, Wisconsin, USA). 1µg of RNA was reverse transcribed into first-strand cDNA in a 20-µl final volume containing 1 µM random hexanucleotide primers, 1 µM oligo dT and 200 U Moloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, California, USA).

TLR signalling pathways

TLR signalling pathways were analysed in a PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SABiosciences Corporation, Frederick, MD, USA). This approach permits the monitoring of mRNA expression of 84 genes related to the TLR-pathway activation, plus five housekeeping genes, following the procedures suggested by the manufacturer. Controls are also included on each array for genomic DNA contamination, RNA quality, and general PCR performance. The experiments have been run on all of the subjects included in the study pooled into a unique INR and FR sample. Thus, the results represents the mean value of the different targets analysed in INR and FR. Furthermore, those targets showing marked differences between INR and FR have been retested by Real time PCR on each individual sample confirming the data obtained in the superarray (data not shown).

Statistical analysis

All continuous variables are presented as median and interquartile ranges (25^{th}-75^{th} 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. Kruskal-Wallis test were used for the comparison between HIV-/HT/NA and HIV-/FRs/INRs. Parameters that yielded a p value <0.05 with Kruskal-Wallis were further analyzed using Mann-Whitney test. p values <0.05 were considered significant. Statistics were performed using GraphPad Prism 5 software.

Results

Comparative study of gut junctional complexes in HIV-infected individuals with different CD4+ T-cell recovery on cART

Patient population

Study subjects enrolled (21 INR and 29 Full Responders, FR) were comparable in terms of demographics, HIV duration and length of treatment on cART (Table 1).

INR patients featured significantly lower CD4+ T-cell counts as per inclusion criteria (261/uL, IQR 194-323 vs. 468/uL, IQR 429-624; $p<0.0001$; Table 1), lower CD4/CD8 ratio (0.3, IQR 0.2-0.5 vs. 0.6, IQR 0.5-0.8; $p<0.001$), and lower CD4+ T-cell nadir (68/uL, IQR 26-112 vs. 215, IQR 64-245; $p<0.01$) (Table 1).

INR also displayed significantly lower CD4+CD45RA+ as well as higher activated CD8+CD38+ and memory activated CD8+CD38+CD45RO+ cells (Table 1). No differences were detected between INR and FR in the proportion of CD8+CD45RA+ and CD8+CD45RO+ T-cells (Table 1).

A significantly higher number of INR was receiving antibiotics and had a previous diagnosis of AIDS (Table 1).

No difference was observed between study groups in terms of endoscopic and histological evaluation in ileum and colon biopsies performed in 8/21 INR and 13/29 FR (Table 2).

INR and FR show comparable CD4+ T-lymphocyte reconstitution in ileum and colon tissue, yet INR display higher intestinal proliferation

Progression of HIV disease has been linked to the depletion of GI T-cells and disruption of the mucosal barrier. We investigated whether poor CD4+ T-cell recovery and impaired T-cell

homeostasis in peripheral blood of INR mirrored low T-cell tissue counts and high lymphocyte proliferation in the GI mucosa.

CD3, CD4 and CD8 expression were measured by immunohistochmemistry (IHC) in GI tissue of 4 representative INR and 6 FR. Study participants showed comparable CD4 expression and distribution in both ileum (High Power Field, HPF respectively, 105, IQR 84-207 vs. 98, 67-152; p=0.61) and colon biopsies (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 FR (not shown).

We further investigated cellular proliferation in gut tissue through IHC measurement of Ki67.

No differences between study groups were detected in Ki67 expression in ileum (INR 27%, 16-35; FR 22%, 19-33%; p=0.09). Interestingly, however, a significantly higher Ki67 expression was detected in colon biopsies of INR (35%, 32-44 vs. 25%, 15-30; p=0.004). Overall, these results show a similar degree of CD4+ T-cell reconstitution in the gut of INR and FR, despite poor CD4+ recovery in peripheral blood and heightened activation/proliferation in the peripheral blood/gut mucosa in the former.

INR subjects show damage of gut junctional complexes

We analyzed ileum and colon junctional complexes (JC) in INR, FR and HIV-uninfected controls by IHC staining for the following proteins: claudin 1, claudin 7, cadherin (Cdh1) and *Zonula occludens* protein 1 (ZO-1).

JC protein expression was severely impaired in HIV-infected individuals compared to negative controls. Indeed, HIV-infected individuals as a whole showed significantly lower claudin 1 (30%, IQR 18-50 vs. 80%, IQR 65-90; p=0.052), CDh1 (70%, IQR 70-90 vs. 100%, IQR 100-100; p=0.034) and ZO-1 (20%, IQR 13-60 vs. 100%, IQR 90-100; p<0.0001) immune-reactive cells in ileum biopsies (Table 3). Similarly, in colon tissue, HIV-positive patients displayed a significant reduction of CDh1 (80%, IQR 60-95 vs. 100%, IQR 100-100; p=0.01) and ZO-1 (30%, IQR 0-60 vs. 100%, IQR 90-100; p<0.0001) expression (Table 3). Furthermore, CDh1 and ZO-1 showed

weaker staining intensity and restriction to basal/baso-lateral and membrane/paranuclear zones in HIV-infected patients at both mucosal sites (Table 3).

Most interestingly, an IHC gradient was observed among controls, FR and INR subjects. Indeed, compared to HIV-negative patients, INR presented significantly lower claudin 1 (p=0.02; Fig. 1A), CDh1 (p=0.01; Fig. 1C-F) and ZO-1 (p=0.001; Fig. 1G-J) immunoreactive cells in ileum tissue. The expression of CDh1 and ZO-1 was also significantly impaired in colon biopsies of INR subjects compared to controls (p=0.003 and p=0.0011 respectively; Fig. 2C-J). In line with these results, CDh1 was distributed around the entire epithelial plasma membrane in controls, at the baso-lateral surface in FR and at the basal level in INR (Fig. 1D-F; Fig. 2D-F; Table 3). Similarly, a distinctive distribution of ZO-1 was noted in INR with negative expression in ileum and colon tissue (Fig 1J; Fig. 2J; Table 3).

To confirm intestinal epithelial barrier dysfunction in INR, electron microscopy (EM) evaluation of ileum and colon JC was also performed in a subgroup of patients. Striking differences between INR and FR were observed in colon tissue: the intercellular space between adjacent cells determined by tight and adherens junctions was focally wider in INR (Figure 3A-B). Of note, in 1 INR some intracellular bacteria were seen (Fig. 3C), which suggests that microbial translocation through a damaged intestinal mucosa may occur in course of poor immunological response to cART.

Comparable small intestine permeability and damage in INR and FR

Having shown impairment of gut JC in INR, we assessed intestinal permeability (urinary lactulose-mannitol fractional excretion ratio, LAC/MAN) and function (Intestinal Fatty Acid Binding Protein, I-FABP). No statistical differences in urinary LAC/MAN ratio were found between study groups (INR: 0.028, IQR 0.019-0.063; FR: 0.020, IQR 0.016-0.13; p=0.7; Fig. 4A), which point to a similar degree of GI permeability in INR and FR with increased para-cellular absorption through gut tight junctions of high molecular weight molecules [72].

Consistently with this data, INR and FR subjects showed comparable I-FABP levels (668 pg/mL, IQR 500-898 vs. 765 pg/mL, IQR 581-1121; p=0.5; Fig. 4B), suggesting similar gut epithelial damage in the two study groups.

Similar levels of microbial translocation in INR and FR

Microbial translocation has been suggested to occur in HIV infection after damage of the intestinal epithelium and may account for peripheral immune activation and poor immunological reconstitution on cART. The vast majority of the subjects studied presented increased markers of microbial translocation compared to results obtained by our laboratory in a large cohort study on 488 HIV-infected untreated patients [73], irrespective of peripheral CD4+ T-cell counts (Fig. 4C-E). Indeed, INR and FR subjects showed comparable levels of LPS (179 pg/mL, IQR 75-352 and 231 pg/mL, IQR 75-450, respectively; p=0.6; Fig. 4C) EndocAb (30 MMU/mL, IQR 17-59 and 36 MMU/mL, IQR 22-52; p=0.8; Fig. 4D) and sCD14 (3 ug/mL, IQR 2-6 and 6 ug/mL, IQR 2-8; p=0.2; Fig. 4E).

Qualitative analysis of the fecal microbiome revealed an outgrowth of Bacteroides–Prevotella spp. in INR and FR

Given that the bacterial composition in stool has been shown to influence gut junctional complexes maturation and gut barrier permeability, we next performed a deep molecular characterization of the fecal microbiome in 13 INR and 14 FR. DGGE analysis of the V2–V3 region of the 16S rRNA gene of amplified bacteria revealed a diverse profile for each subject, although some bands were common to several samples. Indeed, *Prevotella copri* and *Bacterioides uniformis* were more frequently represented in the INR and FR group respectively (Fig. 5).

However, Real-time PCR quantification of *Bacteroides–Prevotella* spp. genomes failed to show major differences between INR and FR patients, despite there being an over-representation in our patients cohort as a whole compared to uninfected controls [74].

Assessment of HIV reservoirs in gut tissue and peripheral CD4+ T-cells

To further investigate the possible causes of different intestinal integrity between INR and FR, HIV reservoirs in ileum, colon and peripheral circulating CD4+ T-cells were measured in study subjects. Overall, INR and FR presented similar HIV reservoirs in both gut biopsies and circulating CD4+ T-cells. Indeed, total HIV DNA, integrated HIV DNA and 2LTR were comparable in both study groups in ileum (Fig. 6A; Table 4), colon (Fig. 6B; Table 4) and peripheral CD4+ T-cells (Fig. 6C; Table 4). Similarly, no differences were detected between study groups in terms of the total/integrated HIV DNA ratio (Fig. 6D; Table 4).

Statistical correlations of the HIV reservoir in the different compartments are shown in Table 5.

Current CD4+ T-cell counts correlate positively with intestinal JC protein expression and negatively with HIV reservoirs in the gut

In order to understand whether a relationship exists between current CD4+ T-cell counts, gut JC protein expression and HIV reservoirs, correlation analyses were performed.

Current CD4+ T-cell counts correlated positively with ZO-1 expression in ileum (Fig. 7A, B) and colon tissue (Fig. 7C), thus confirming the IHC findings of lower tight junction protein expression in INR. Interestingly, current CD4+ T-cell counts correlated negatively with total HIV DNA (Fig. 7D) and 2LTR (Fig. 7E) in circulating CD4+ T-cells and with total HIV DNA in ileum tissue (Fig. 7G, H). Consistently with these findings, the CD4/CD8 ratio also correlated negatively with peripheral 2LTR and colon total HIV DNA (Fig. 7F, I).

These results show that poor immunological recovery in course of virologically-suppressive cART associates with impairment of gut JC and greater HIV reservoirs, in both peripheral CD4+ T-cells and intestinal tissue.

Statistical correlations between CD4+ T-cell counts, gut JC protein expression and HIV reservoirs are presented in Table 6.

Expression of activation markers on immune cells following stimulation with microbial components in HIV-infected individuals with different CD4+ T-cell recovery on cART

1) Study of the dynamics of HLA-DR and CD38 expression on peripheral T-cells following selective *in vitro* LPS stimulation of PBMCs from HIV-infected cART-treated individuals [39].

Patients

30 HIV-positive, cART-treated patients with undetectable HIV RNA load were enrolled. According to the degree of immune-reconstitution on cART, 9 HIV-positive patients were HR (CD4+ ≥600/μL), 9 resulted LR (CD4+<350/μL) and 12 intermediate IR CD4+ 350-599/μL. 15 HIV-negative age-matched subjects were also studied.

Duration of HIV infection was comparable in all subjects (LR: 7 months, IQR 5-19; IR: 13 months, IQR 8-22; HR: 12 months, IQR 6-23; p>0.05 for all comparisons; Table 7). All patients were on cART for at least 12 months prior to evaluation, with no differences in terms of cART duration (LR: 71 months, IQR 46-141; IR: 106 months, IQR 65-149; HR 71 months, IQR 39-132; p>0.05 for all comparisons; Table 7) and drug regimen (Table 7).

HR were significantly younger compared to LR (HR: 39 years, IQR 35-44; LR: 50 years, IQR 45-56; p=0.02; Table 7) and presented higher absolute CD4+ T-cell counts as defined by inclusion criteria (LR: 260/μL, IQR 215-339; IR: 498/μL, IQR 425-537; HR: 726/μL, IQR 608-863; p<0.01 for all comparisons; Table 7). A trend to lower CD4+ T-cell nadir was found in patients with less efficient immune-reconstitution, (LR: 90/μL, IQR 51-179; IR: 105/μL, IQR 45-208; HR: 204/μL, IQR 56-240; p>0.05 for all comparisons) reaching statistical significance for percentage values in LR vs HR (LR: 8%, IQR 5-17; HR: 26%, IQR 22-30; p=0.03; Table 7).

No other significant differences in demographic and HIV-related parameters were observed among groups (Table 7) [39].

Lymphocyte phenotype analysis

a) HLA-DR expression on CD4+ and CD8+ T-cells upon LPS stimulation

At T0, HIV-positive patients displayed a tendency to higher HLA-DR+CD4+/CD8+ compared to controls, reaching significance for CD4+ T-cell subpopulation (CD4+: 30%, IQR 20-54 vs 19%, IQR 5-28; p=0.02; Figure 8A; CD8+: 32%, IQR 18-50 vs 21% IQR 11-39; p=0.07; Figure 8C), with no differences according to immune-reconstitution (Figure 8B, 8D).

At T1, following LPS stimulation, HIV-infected individuals as a whole displayed a non-significant trend to increased HLA-DR+CD4+ (19%, IQR 14-33 vs 13%, IQR 11-26; p=0.08; Figure 8E) and HLA-DR+CD8+ (23%, IQR 17-37 vs 18%, IQR 13-29 p=0.2; Figure 8G) when compared to healthy controls. Interestingly, when analysing patients according to CD4+ recovery on cART, LPS stimulation resulted in significant up-regulation of HLA-DR on CD4+ T cells in LR (32%, IQR 19-50) compared to HR (15%, IQR 11-21; p=0.03; Figure 8F) and controls (p=0.03; Figure 8F). Conversely, no differences in HLA-DR-expressing proportions upon LPS stimulation were observed in the CD8+ compartment at this time-point (LR: 28%, IQR 16-50; IR: 24%, IQR 18-50; HR: 18%, IQR 15-32; p>0.05 for all comparisons; Figure 8H).

At T2, HIV-positive subjects displayed significantly higher LPS-induced HLA-DR+CD4+ (24%, IQR 14-44.3 vs 12%, IQR 8-23; p=0.006; Figure 8I) and HLA-DR+CD8+ cells (26%, IQR 18-34 vs 13%, IQR 7-25; p=0.005; Figure 8K). Following stimulation with LPS, a CD4+ T-cell activation hierarchy was maintained in LR (32%, IQR 15-40) and IR (28%, 14-48) over HR (21%, IQR 13-37; p=0.4; p=0.5 respectively; Figure 8J), reaching statistical significance in comparison with HIV-negative controls (p=0.02 and p=0.03 respectively; Figure 8J). Similar results were observed in terms of LPS-induced expression of HLA-DR on CD8+ cells, with increased levels in LR (28%,

IQR 22-45) and IR (28%, IQR 15-33; Figure 8L) compared to controls (p=0.007 and p=0.05) (Figure 8L).

A graphical time course representation of the effect of LPS stimulation on HLA-DR in HIV-infected individuals with different response to cART and in HIV-negative controls is summarized in Figure 10 [39].

b) CD38 expression on CD4+ and CD8+ T-cells upon LPS stimulation

Baseline (T0) CD38+CD4+ were comparable in HIV-positive and HIV-negative patients (66%, IQR 58-80 vs 66%, IQR 54-75, p=0.4; Figure 9A). Conversely, CD38+CD8+ were higher in HIV-positive patients (55%, IQR 38-71 vs 38%, IQR 30-59, p=0.05; Figure 9C). In both cases, no differences amongst HIV-infected subgroups were detected (Figure 9B, 9D).

Most interestingly, cells from HIV-infected patients failed to respond to LPS stimulation at T1, displaying significantly lower proportions of CD38+CD4+ (64%, IQR 55-72.7 vs 73%, IQR 70-82; p=0.02; Figure 9E) and CD38+CD8+ (54%, IQR 39-67 vs 65%, IQR 55-70, p=0.05; Figure 9G) compared to HIV-negative controls. However, within HIV-infected subjects, LR and IR showed higher CD38+CD8+ (65%, IQR 48-70 and 56%, IQR 45-71, respectively) compared to HR upon LPS stimulation (41%, IQR 25-55; p=0.04 for both comparisons; Figure 9H), while CD38+CD4+ cells did not vary according to immune-reconstitution (LR: 64%, IQR 55-78; IR: 62%, IQR 55-75; HR: 64%, IQR 51-67; p>0.05 for all comparisons; Figure 9F).

At T2, HIV-positive and healthy individuals displayed comparable CD38+CD4+ and CD8+ following LPS stimulation (74%, IQR 65-80 vs 66%, IQR 61-77; p=0.3; Figure 9I; 64%, IQR 50-70 vs 52%, IQR 42-63; p=0.2; Figure 9K). Similar to T1 data, LR presenting higher CD38+CD8+ than HR (66%, IQR 64-74vs 49%, IQR 30-64, p=0.01 Figure 9L) and controls (p=0.02; Figure 9L), whereas no differences in CD38+CD4+ cells were noted amongst HIV-positive subgroups (LR: 74%, IQR 68-78; IR: 79%, IQR 71-83; HR: 60%, IQR 50-77; p>0.05 for all comparisons; Figure 9J).

A graphical time course representation of the effect of LPS stimulation on CD38 in HIV-infected individuals with different response to cART and in HIV-negative controls is summarized in Figure 10 [39].

c) HLA-DR/CD38 co-expression on CD4+ and CD8+ T-cells upon LPS stimulation

At T0 comparable HLA-DR+CD38+CD4+ cells were observed in HIV-negative (15.4%, IQR 2.1-25.9) and HIV-positive patients (23.5%, IQR 7.2-48.8; p=0.08) with no differences among HIV subgroups (LR: 20.2%, IQR 6.9-42; IR: 24.5%, IQR 8.1-63.1; HR: 41.5%, IQR 12.1-49.5; p>0.05 for all comparisons).

At T1, LPS did not account for significant changes in HLA-DR/CD38 co-expression on CD4+ T-cells compared to baseline values.

A completely different scenario was pictured upon LPS stimulation at T2, with significantly higher HLADR+CD38+CD4+ in HIV-positive patients as a whole (20.3%, IQR 12.1-39.4) and in LR subjects (LR: 20.3%, IQR 12.8-34.4) compared to controls (9.5%, IQR 6.6-19.0; p=0.04 and p=0.03 respectively).

Similar results were observed in HLA-DR/CD38 co-expression on CD8+ T-cells, given that no differences among study groups were detected at T0: HIV-negative: 13.6%, IQR 4.0-24.9; HIV-positive: 18.3%, IQR 12.1-42.2; LR: 17.9% IQR 12.2-28.7; IR: 14.1%, IQR 8.0-45.7; HR: 30.5%, IQR 12.8-46.7 (p>0.05 for all comparisons).

While LPS stimulation did not lead to significant differences in terms of HLA-DR/CD38 induction on CD8+ cells at T1, significantly higher HLADR+CD38+CD8+ cells were detected in HIV-positive patients compared to controls (20.5%, IQR 10.5-29.0 vs 8.7%, IQR 4.9-20.1; p=0.02) at T2.

When examining patients with different degree of immune-reconstitution, LR maintained activation CD8+ activation hierarchy over HIV-negative individuals, with significantly more HLADR+CD38+CD8+ cells (LR: 24%, IQR 14.6-36.4; p=0.02) [39].

d) Ki67 expression on CD4+ and CD8+ T-cells upon LPS stimulation

At T0, no differences between HIV-negative controls and HIV-positive subjects were measured in Ki67 expression on CD4+ (8.2%, IQR 4.0-17.2 vs 12.7%, IQR 2.6-18.2 respectively; p=0.7; Figure 11A) and CD8+ T-cells (1.9%, IQR 0.1-3.7 vs 1.25; IQR 0.0-4.8; p=0.8; Figure 11C). T-cell proliferation levels did not vary according to the degree of immune-reconstitution in course of cART, despite trend to higher Ki67 expression on CD4+ T-cells in HR (17%, IQR 12.6-18.9) compared to LR (5.5%, IQR 1.4-15.5; p=0.07; Figure 11B).

At T1, LPS stimulation did not account for significant increases in cell proliferation (Figure 11E-11H). Interestingly, however at T2, a trend to increased Ki67 levels on CD4+ T-cells upon stimulation was detected in LR (20.5%, IQR 7.5-31.0) compared to HR (5.5%, IQR 2.0-12.6; p=0.08) and negative controls (3.9%, IQR 0.4-18.7; p=0.08; Figure 11J), thus mirroring the state of CD4+ T-cell activation through the expression of HLA-DR (Figure 11J).

No differences were detected in terms of CD8+ T-cell proliferation among study groups following 48-hour stimulation with LPS (Figure 11K, 11L) [39].

Substudy on the phenotype and function of CD38-expressing CD4+ and CD8+ T-cells in HIV-infected patients undergoing suppressive cART [67].

As expected, untreated HIV-infected subjects displayed a higher proportion of CD8+CD38+ cells than HIV negative subjects (28% vs 8% p=0.0001); cART-treated individuals showed decreased CD8+CD38+ levels, which, however, remained higher than those measured in controls (17% vs 8% p=0.004).

As shown in Fig 12A untreated patients presented significantly lower naïve CD38+CD45RA+CD45R0- than HIV-uninfected controls (CD4+CD38+: 52% vs 77% , p=0.013; CD8+CD38+: 59% vs 79%, p=0.002) and higher memory CD38+CD45RA-CD45R0+ (CD4+CD38+: 52% vs 30%, p= 0.012; CD8+CD38+: 48% vs 30%, p=0.008).

Interestingly, naïve CD38+CD45RA+CD45R0- were enriched in cART-treated compared to untreated patients (CD4+CD38+: 72% vs 52%, p=0.016; CD8+CD38+: 74% vs 59%, p=0.002) and

similar in cART+ and HIV- subjects (CD4+CD38+, 72% vs 77%, p=0.781; CD8+CD38+: 74% vs 79%, p=0.205). Parallely, memory CD45RA-CD45R0+ were lower in cART-treated compared to untreated individuals (CD4+CD38+: 32% vs 52%, p=0.003; CD8+CD38+: 31% vs 48%, p=0.007) and equivalent in cART-treated and HIV-unifected controls (CD4+CD38+: 32% vs 30%, p=0.592; CD8+CD38+: 31% vs 30%, p=0.587).

Interestingly, further detailing of the CD38+ immune phenotype resulted in a significant enrichment of recent thymic emigrants (RTEs) in cART+ [75, 76] versus untreated patients (CD4+CD38+CD45R0-CD31+: 58% vs 34%, p=0.015; CD8+CD38+CD45RA+CD103+: 9 % vs 6%, p= 0.049) and lower than the levels observed in controls (CD4+CD38+CD45R0-CD31+: 77% vs 58%, p=0.002; CD8+CD38+CD45RA+CD103+: 13% vs 9%, p= 0.035). No differences were observed in the CD38- population (not shown).

Given these findings, we aimed to study CD38+CD8+/CD4+ cell death by flow cytometric measurement of pStat5 and Bcl-2 on magnetically-isolated CD38+CD8+/CD4+ T cells with or without rIL-2/rIL-7 stimulation.

Upon IL-7 stimulation, higher pStat-5+ and Bcl-2+ CD8+CD38+ levels were observed in cART-treated compared to naive subjects (pStat-5: P=0.036; Bcl-2: p=0.032) (Fig.12B) and resulted significantly lower than in controls (pStat-5: P=0.046; Bcl-2: p=0.036). pStat5 and Bcl-2 increase was observed only in CD8+CD38+ T cells and not in CD4+CD38+. No differences were observed following IL-2 challenge and in CD8+/CD4+CD38- subsets (not shown).

In 10 cART-treated subjects no significant differences in total, un-integrated and integrated HIV-DNA [77] were noted in purified CD4+CD38+/CD38- T-cells (Fig. 12C) [67].

2) Development of an *in vitro* model by which TLR stimulation with microbial bioproducts leads to T-cell activation and lack of immune recovery in course of cART

MDM surface expression of HLA-DR and CD69 and cytokine/chemokine release following TLR challenge in FRs and INRs

The experimental protocol to study the effect of TLR challenge on monocyte-derived macrophages (MDM) was set up in13 HIV-uninfected subjects (Fig.13a). In this population, stimulation with TLR ligands did not modify the surface expression of HLA-DR (Fig.13b) and CD69 on MDM (Fig.13c).

Epidemiological, clinical and HIV-related characteristics of the two HIV-infected study groups are listed in Table 8.

TLR challenge did not modify the surface expression of HLA-DR in FRs and INRs (Fig.13.d), yet ssRNA alone resulted in a significant increase of CD69 in FRs (p=.005: Fig.13e). No differences were found comparing MDM surface markers between groups.

To further investigate the effect of TLR stimulation on MDM, sCD14 and cytokine/chemokine release were measured in supernatants following exposure to the most representative TLR4 and TLR 7/8 agonists (LPS and ssRNA respectively) (Fig. 14).

No significant differences were found between study groups in terms of sCD14 release neither prior to (UNST: FRs 0,95 ng/mL [0,31-1,41] vs INRs 0,58 ng/mL [0,28-0,99], p=.429) nor following TLR stimulation (LPS: FRs 1,03 ng/mL [0,57-2,62]vs INRs 0,81 ng/mL [0,12-2,07], p=.501; ssRNA: FRs 1,91 ng/mL [1,38-4,84] vs INRs 1,98 ng/mL [0,95-3,75], p=.661; Fig.2a.suppl). ssRNA challenge, however, produced a significant release of sCD14 in both FRs and INRs (FRs: p=.463 and p=.005 for UNST vs LPS and vs ssRNA, respectively; INRs: p=.193 and p=.009 for UNST vs LPS and vs ssRNA, respectively; Fig.14a).

A 25-plex Luminex assay conducted in a subgroup of 5 INRs and 6 FRs (Fig.2b.supp) corroborated these findings, given the highest release of pro-inflammatory IL-6 (FRs: 248 pg/ml; INRs: 259

pg/ml) and TNF-a (FRs: 666 pg/ml; INRs 1887 pg/ml) following viral challenge, with no differences between groups (Fig.14c-d).

Taken together, these finding show a similar response to TLR challenge in HIV-infected individuals receiving cART, irrespective of their immunological outcome, with greater MDM activation upon viral stimulation.

In vitro TLR challenge on PBMC in FRs and INRs

In the attempt to investigate the role of the TLR systems in the pathogenesis of discordant responses to antiretroviral therapy, expression of TLR and mRNA genes involved in the TLR-mediated pathway, as well as cytokine/chemokine release were studied in PBMC.

a) TLR-4/-7/-8 surface/intracellular expression

Given that the response to bacterial and viral stimuli may be affected by TLR expression, TLR-4/-7/-8, were measured on CD14+ cells. FRs and INRs displayed comparable TLR-4 surface expression on CD14+ cells (FRs: 79,5 [62,8-86,1]% vs INRs: 77,7 [44,3-90,5]%; p=.68). Similarly, no differences between groups were observed in TLR-7 (FRs: 2,5 [1,1-3,6] % vs INRs 5,5 [1,4-13,3]%; p=.44) and TLR-8 (FRs: 84.9 [69,7-93,8]% vs INRs: 80,2 [76,1-87,8]%; p=.77).

b) mRNA expression of genes involved in the TLR-mediated pathway

We next screened for 84 genes involved in TLR-activation pathway by real-time PCR array to study microbial-induced TLR-signalling in PBMCs from FR and INR.

INRs displayed higher constitutive mRNA levels (up to 20-fold) of effectors such as CCL2, CSF2, CSF3, CXCL10, IFN-γ, IL1-a, IL-1b, IL-6, IL-8 and of genes involved in the pathogen specific response, such as CLEC4E, HSPA1A, CD80 and TLR3 (Fig.15a).

TLR stimulation with microbial agonist resulted in activation pathway differences in FRs and INRs. LPS exposure produced a significant up-regulation of the transcription of pro-inflammatory cytokines, such as IL-1a, IL-6 and IL-8 (Fig.15b) in the former, yet failed to produce major changes

in the latter (Fig.15b). ssRNA challenge induced both pro- and anti-inflammatory cytokines, (IFN-γ , IL-6, CSF2, CSF3) in FRs and resulted in the up-regulation of type I IFNs in INRs (Fig.15d). LPS and ssRNA did not differentially affect TLR signaling or downstream pathways in FRs and INRs (Fig.15c-e).

These data point to increased constitutive expression of TLR-signalling genes in INRs yet impaired TLR-mediated activation following microbial challenge.

c) cytokine/chemokine release in supernatants

Cytokine and chemokine release was measured in the supernatants of PBMCs from FRs and INRs following stimulation with LPS and ssRNA (Fig.16).

Levels of sCD14 were analyzed first (Fig.16a). Despite similar measures of sCD14 prior to TLR agonist exposure (FRs: 52,3 ng/mL [23,9-69,8] vs INRs: 60,5 ng/mL [37,5-85,4]; p=.238; Fig. 16a), INRs showed higher sCD14 levels after ssRNA (FRs 39,9 ng/mL [25,8-67,8] vs INRs 91,6 ng/mL [34-109]; p=.045; Fig. 16a) yet not after LPS stimulation (FRs 52,7 ng/mL [34,1-73,7] vs INRs 64,03 ng/mL [44,4-91,4]; p=.171; Fig.16a). Both LPS and ssRNA did not affect sCD14 release in FRs (UNST vs LPS, p=.067; ssRNA p=.583; Fig.16a) and INRs (UNST vs LPS, p=.276; ssRNA, p=.177).

We then measured the levels of 25 cytokine/chemokine the supernatants of PBMCs (Fig. 16b-e) and found that INRs constitutively released a greater amount of pro-inflammatory IL-17F, IL-23, IL-27, IL-28a and IL-31 (Fig. 16c), yet failed to observe significant differences between groups upon LPS and ssRNA exposure (Fig. 16d-e).

In vitro TLR challenge on PBMC: T-cell activation/proliferation in FRs and INRs

Given that TLR stimulation of innate immune cells induces the production of cytokines which regulate the response of the adaptive immune system, we next studied the effect of TLR stimulation on T-cell activation, proliferation and apoptosis.

In HIV-uninfected controls, TLR challenge did not result in significantly higher CD8+ and CD4+ T-cell activation (Fig. 17a-b). While only ssRNA stimulation resulted in higher CD38+CD8+ in FRs (p=.0006; Fig. 17c), exposure to all the tested stimuli produced a significant expansion of the activated CD8+ T-cell subset in INRs (LPS p=.0008; LTA p=.009; PGN p=.037 ssRNA p=.0003; IFNγ p=.042; aCD3/CD28 p=.0004; Fig. 17c).

TLR challenge did not affect the expression of HLA-DR in CD4+ T-cells in both FRs and INRs (Fig.17d).

When examining T-cell proliferation, TLR stimulation accounted for a significant rise in Ki67+CD4+ T-cells in both INRs (LPS: p=.002; LTA: p=.028) and FRs (ssRNA: p=.028; CD3/CD28: p=.015) (Table 9), with no differences between the two groups (Table 9). Microbial challenge also resulted in a significant increase in Ki67+CD8+ in the former (LTA (p=.049), yet failed to produce effects in CD8+ T-cell proliferation in the latter (Table 9).

No major differences were observed between the two study groups in pro-apoptotic Annexin V+ CD4 and CD8 T-cells following TLR stimulation (Table 9).

Discussion

The overall objective of the present study was to understand whether damage of the GI tract and microbial-induced T-cell activation feature HIV-infected individuals with poor immune recovery on cART.

We pursued our objective through two specific aims:

1) Specific Aim 1: Comparative study of gut junctional (JC) complexes in HIV-infected individuals with different CD4+ T-cell recovery on cART. We first analyzed the structure and function of gut JC in Immunological Non Responder (INR) and Full Responder (FR) and then assessed whether the fecal microbiome and/or HIV reservoirs may represent underlying causes of gut epithelial barrier dysfunction in course of treated HIV disease.

2) Specific Aim 2: Expression of activation markers on immune cells following stimulation with microbial components in HIV-infected individuals with different CD4+ T-cell recovery on cART. We first analyzed the effect of LPS *in vitro* stimulation on T-cell activation markers (CD38 and HLA-DR) in HIV-infected patients with different CD4+ recovery on cART [39, 67] and then set up an in vitro model to assess the TLR-mediated signalling pathways in monocyte-derived macrophages (MDM) and PBMCs in a similar study population.

Building on earlier studies that have shown the presence of high levels of immune activation and microbial translocation in INR, we hypothesized that deficient CD4+ T-cell reconstitution is explained by severe impairment of the intestinal mucosa. We conducted a comparative study of the GI tract in INR and FR, selecting our population from cART-treated individuals presenting a history of advanced CD4+ T-cell depletion at cART initiation (CD4+ nadir <350/µL), known to associate with a high risk of hampered immunological recovery and clinical events [2]. This approach allowed us to ascertain the robustness of any difference observed between INR and FR.

We assessed gut JC (tight and adherence proteins) and function (small intestine permeability/damage and microbial translocation) in the two groups. We find that INR are unique in terms of the extent of GI epithelial barrier damage. In our study, IHC and statistical evidence suggested that this population presents the lowest expression of JC proteins at mucosal sites, with EM proof of dilated intercellular spaces corroborating these findings. Together with higher colonic proliferation indexes in INR, we also find anecdotal evidence of bacteria passing through the *lamina propria*, which, to our knowledge, is the first report of translocation of whole bacteria in HIV-infected humans [59].

Building on earlier research showing that HIV-infected subjects on cART present higher HIV DNA levels in the gut compared to circulating CD4+ T-cells [24, 25, 60], we further expanded our approach to investigate whether greater HIV reservoirs are associated with GI damage and poor immune recovery. We also analyzed the composition of the fecal microbiome, known to promote tight junction protein expression [78]. Our study illustrates that current CD4+ T-cell counts are negatively correlated with HIV reservoirs at mucosal sites and peripheral blood, suggesting that poor immune recovery on cART is associated with greater HIV reservoirs. No differences in HIV reservoirs in the gut or peripheral CD4+ T-cells were observed between INR and FR specifically, which may be explained by the reported low CD4+ T-cell nadir [79]. No major difference was observed in the composition of the fecal microbiome in two groups, despite a greater number of *Bacteroides-Prevotella* bacterial cells being reported in our study population, compared to healthy cohorts described in the literature [74]. This finding is consistent with prior studies reporting modifications of GI flora HIV disease [17, 26, 80, 81] and suggests that the gut microflora is similarly impaired in INR and FR.

Interestingly, our research observed damage of gut JC proteins even in our FR population, which displayed similar parameters of intestinal permeability and microbial translocation to those measured in INR. We explain this observation with the delayed initiation of cART in both groups:

individuals commencing treatment in advanced stages of disease will display a severely injured GI tract, irrespective of the effects of long-term cART on CD4+ T-cell recovery in peripheral blood.

The findings outlined above shed light on the possible reasons as to why treatments currently administered to sustain the recovery of peripheral CD4+ T-cells in INR have resulted ineffective. A key implication of our study is, indeed, that unless the GI tract is specifically targeted vis-à-vis HIV burden and mucosal repair, interventions are likely to have unsuccessful outcomes. Strategies designed to intensify ongoing cART regimens [2, 15, 16, 18], for instance, may not achieve their intended aim due to inefficient concentration of antiretrovirals in gut lymphoid tissue [82]. In a similar vein, IL-7 immune-adjuvant therapy, which reportedly increases the size of the HIV reservoir *in vitro* [83], might not be able to recover CD4+ T-cells due to its approach which fails to address the replenishment of the reservoir [12]. Further, rifaximin may not result in the effective control of microbial translocation due to the lasting damage of the GI tract [14]. Additionally, patient populations with different levels of CD4+ T-cell counts and CD4+T-cell nadir need to be studied further, so as to understand whether interventional approaches counteracting precise mechanisms of disease may have favorable outcomes.

In conclusion, the first aim of our study shows that HIV-infected individuals with incomplete CD4+ T-cell recovery feature severe damage of gut JC and increased size of the HIV reservoir in both circulating CD4+ T-cells and GI tissue. These findings point to a major role of the HIV reservoir in mucosal barrier injury in INR and provide new directions for therapeutic interventions in this setting.

The second aim of the present work was pursued through two separate studies.

Given that the translocation of bacterial bioproducts through the gastrointestinal barrier is a driver of immune activation, we first investigated the induction of CD38, HLA-DR and Ki67 on CD4+ and CD8+ T-cells following selective *in vitro* LPS stimulation in HIV-infected individuals with different immunological recovery upon virologically-suppressive cART [39].

Our study shows a reduction of HLA-DR+CD4+/CD8+ cells following LPS *in vitro* stimulation in both healthy donors and HIV-infected individuals. When evaluating HIV-patients according to the degree of immune recovery on cART, HLA-DR expression was reduced in patients with good CD4+ recovery, yet remained stable in subjects with lower CD4+ reconstitution, (i.e. LR and IR). The net outcome of such a diverse effect of LPS stimulation in relation to the extent of CD4+ recovery, is that subjects with poor immunological recovery display significantly higher HLA-DR+ T-cell proportions. LPS also accounted for a higher proportion of proliferating Ki67+ T-cells in the CD4+ subset alone, thus suggesting that the combined effect of microbial stimulation on T-cell proliferation and activation seems to be restricted to the CD4+ cell compartment [39].

These findings add up to the consolidated bulk of evidence on the inverse correlation between HLA-DR expression and CD4+ lymphopenia [6, 41], putting forward HLA-DR as a faithful marker of microbial-induced T-cell activation in the setting of severe immune impairment [39].

The time-course analysis of the effect of LPS stimulation on CD38 expression showed an overall rise in CD38-expressing T-cells which was, however, delayed in HIV-infected patients compared to healthy controls (i.e. 48 vs 24-hours). When investigating HIV-positive subjects with different immune recovery, only patients with inefficient CD4+ reconstitution (i.e. LR and IR) displayed an expansion of the CD38+ T-cell pool, whereas individuals with good immunological response (HR) maintained a stable expression of CD38 on T-cells overtime. Of note, although CD38 appeared selectively induced on CD4+/CD8+ cells upon longer stimulation, only CD38+CD8+ cells displayed a linear relationship with immune impairment on cART, with LR/IR patients showing higher LPS-stimulated CD38+CD8+. Conversely, no differences in CD38+CD4+ was shown according to the degree of immune reconstitution [39].

By showing higher LPS-induced expansion of CD38+CD8+ cells, our findings suggest a diverse regulation of CD38 in CD4+ and CD8+ T-cell compartments and allow to speculate that the sole expression of CD38+ on CD4+ T-cells may not be sufficient to portray the state of immune activation in HIV disease [39].

In our study, the co-induction of HLA-DR and CD38 on CD4+ and CD8+ T-cells followed similar kinetics upon longer (48 hours) stimulation with LPS, with increased levels of both molecules in subjects with severe immune impairment on cART. This finding, together with the differential expression of single cell surface molecules, once again suggests that CD38 on CD4+ cells may not faithfully mirror activation in HIV disease [39].

Given these findings and recent reports on large cohorts which have failed to demonstrate an association between CD38+ expression on CD8+ T-cells does and disease outcome [84, 85], we investigated the immune phenotype and function of CD4+/CD8+CD38+ and CD4+/CD8+CD38- in cART-naïve and cART-experienced individuals. Our data show that CD38-expressing T-cells, while abnormally heightened in both untreated and treated patients, display a distinctive phenotype and functional response according to the presence or absence of cART [67]. Successfully-treated patients display a CD38+ compartment enriched in naïve/RTEs T-cell phenotypes with higher IL-7 responsiveness of CD38+CD8+ and not CD38+CD4+ cells resulting in Stat-5 phosphorylation and protection from apoptosis. These findings are not observed in untreated patients, altogether suggesting a positive effect of cART in the homeostasis of CD38-expressing T-cells [67].

A partial recovery of cellular phenotype/function and the lack of higher HIV-DNA content in CD38-expressing T-cells might provide a biological reason for the failure of this subset to robustly predict mortality/morbidity in treated patients [84, 85], sounding a note of caution in its exploitation as a surrogate outcome of clinical progression in cART+ patients[67].

Finally, as part of our second aim, we performed a functional study to gain deeper insight into the regulation of T-cell activation by TLR agonists in course of treated HIV disease.

Our ex vivo model showed that broad TLR stimulation results in the up-regulation of T-cell activation makers in INR alone. Indeed, FR displayed an increased expression of CD38 on CD8+

T-cells only after ssRNA exposure and, consistently with these findings, the HIV-uninfected control population did not respond to microbial-challenge.

INR and FR also displayed a different pattern in terms of mRNA expression of genes involved in the TLR-mediated pathway: heightened expression of effector and pathogen specific response genes was found prior to stimulation in INR who selectively upregulated type I interferons following ssRNA stimulation. FR subjects seemed to respond to LPS stimulation, through a significant up-regulation of transcription of pro-inflammatory cytokines, as well as ssRNA challenge, which induced pro- and anti-inflammatory cytokines. Along these lines sCD14 release was significantly higher in viral-stimulated supernatants from INRs compared to FR.

We also set up an *in vitro* model to study the involvement of monocyte-derived macrophages (MDM) in the TLR-mediated pathway following microbial challenge. Despite our findings in PBMCs, we did not observe any peculiarities in INR and FR. Indeed, ssRNA was the only agonist to induce sCD14 release from MDM and highest levels of pro-inflammatory mediators in both INR and FR.

Taken together our findings suggest that other innate immunity cell populations (plasmacytoid dendritic cells, for instance) may be involved in inducing the upregulation of CD8+ T-cell activation markers in INR, given the similarities in MDMs between INR and FR following microbial challenge. We also argue that HIV *per se*, either as low-level viremia or as a reservoir, acts as an important source of antigenic stimulation in treated HIV disease, irrespective of CD4+ T-cell recovery in the long term. This finding sheds light onto why sevelamer [86]/rifaximin [14], which counteract LPS and other bacterial components conceived as the major mediators of T-cell activation in HIV infection, may not achieve their intended aim despite the intriguing results obtained in the animal model [87] which were, however, performed, in acute phases of SIV disease, where LPS may have a predominant pathogenic role

Conclusions

We investigated whether damage of the GI tract feature HIV-infected individuals with poor immune recovery on cART (Immunological Non Responders, INR) and the mechanisms by which microbial translocation induces T-cell activation in this population.

Our experiments revealed:

1) Immunohistochemical and statistical evidence of INR presenting the lowest expression of junctional complex (JC) proteins at mucosal (ileum and colon) sites, with electron microscopy proof of dilated intercellular spaces;
2) A negative correlation of CD4+ T-cell counts with intestinal JC protein expression as well as HIV reservoirs in the gut and peripheral blood;
3) A higher proportion of HLA-DR-expressing CD4+ and CD8+ T-cells in INR following lipopolysaccharide (LPS) *in vitro* stimulation, yet the CD38+CD8+ pool only is significantly expanded according to the degree of immunological impairment [39];
4) Up-regulation of T-cell activation markers following broad microbial challenge in INR, as well as heightened expression of effector and pathogen specific response genes prior to stimulation and selective upregulation of type I interferons following ssRNA stimulation;
5) Preserved response of monocyte-derived macrophages (MDM) from INR following broad microbial challenge.

Our findings show that incomplete immunological response in the course of effective cART associates with severe damage of the GI epithelial barrier and increased size of the HIV reservoir

both at mucosal sites and in circulating T-cells, thus suggesting to target the GI tract in the elaboration of interventional strategies for INR.

We also demonstrate the uniqueness of the CD8+CD38+ T-cells subset in depicting T-cell activation following LPS stimulation in individuals with poor CD4+ T-cell recovery, strengthening its possible exploitation in the clinic to monitor the immune response to cART [39].

Consistently with these findings, we show the up-regulation of activation markers on T-cells from INR following ssRNA which appears to be involved in TLR-mediated signaling of non-CD14-dependent pathways, highlighting the importance of low-level viremia/HIV reservoirs as sources of persistent antigenic stimulation in this setting.

Tables and Figures

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

	Immunological Non Responder (INR, n=21)	**Full Responder (FR, n=29)**
Age, years (IQR)	51 (43-66)	51 (43-66)
Gender, no females (%)	4 (19)	7 (24)
Ongoing antibiotic prophylaxis, n (%)	6 (29)*	0 (0)
Risk factors for HIV infection		
Heterosexual, n (%)	13 (62)	13 (45)
MSM, n (%)	4 (19)	12 (41)
IV drug use, n (%)	4 (19)	4 (14)
HCV-Ab positivity, n (%)	2 (10)	2 (7)
Duration of HIV infection, months (IQR)	74 (42-216)	70 (60-96)
Duration of cART, months (IQR)	68 (37-87)	56 (39-68)
AIDS diagnosis, n (%)	13 (62)*	7 (24)
Nadir CD4 T-cells		
absolute counts, µL (IQR)	68 (26-112)†	215 (64-245)
Current CD4 T-cells		
absolute counts, µL (IQR)	261 (194-323) †	468 (429-624)
percentage (IQR)	18 (13-25) †	27 (22-30)
Current CD4/CD8 T-cell ratio	0.3 (0.2-0.5)†	0.6 (0.5-0.8)
Current CD4+CD45RA+ (%)	5 (3-8) †	8 (6-12)
Current CD8+CD38+ (%)	3 (3-6) †	3 (2-4)
Current CD8+CD38+CD45RO+ (%)	1 (1-2) †	1 (1-1)
Current CD8+ CD45RA+ (%)	18 (13-24)	16 (13-20)
Current CD8+ CD45RO+ (%)	14 (11-19)	12 (7-18)
Zenith HIV RNA load, log10 cp/mL (IQR)	5 (5-6)	5 (5-6)
Ongoing cART regimen, no patients (%)		
NNRTI-based	4 (19)	10 (35)
PI-based	13 (62)	17 (59)
Other	4 (19)	2 (7)

Antibiotic prophylaxis includes trimetroprim/sulfametoxazole, atovaquone. MSM males having sex with men. HCV, Hepatitis C Virus. cART, Combination Antiretroviral Therapy. NNRTI, Non Nucleoside Transcriptase Inhibitor, PI, Protease Inhibitor.

* $p<0.05$ for INR vs. FR (Chi-squared test): † $p<0.05$ for INR vs. FR (Mann-Whitney test)

Table 2. Endoscopic and histolological findings in INR and FR study subjects

Patient	Group	Macroscopic examination	Ileum Histology	Colon/rectum Histology
1	**INR**	Normal	Acute and chronic low grade inflammation in the LP Lympho-follicular hyperplasia	Chronic low grade inflammation in the LP Lymphofollicular hyperplasia (colon)
2	**INR**	Sigmoid colon diverticular disease	Acute and chronic low grade inflammation in the LP	Acute and chronic low grade inflammation in the LP
3	**INR**	Ascending colon polyp	Acute and chronic low grade inflammation in the LP Lymphofollicular hyperplasia Polyp: tubular adenoma with low-grade dysplasia	Acute and chronic moderate grade inflammation in the LP Eosinophilic elements (colon)
4	**INR**	Normal	Acute and chronic low grade inflammation in the LP	Acute and chronic low grade inflammation in the LP *Muscolaris mucosae* fibrosis, inflammatory polyp (colon)
5	**INR**	Low grade chronic active colitis	Chronic low grade inflammation in the LP	Chronic inflammation in the LP
6	**INR**	Normal	Chronic inflammation in the LP Few superficial erosions	Chronic low grade inflammation in the LP Focal gland distortion (colon)
7	**INR**	Ileocecal sphincter flat polyp	Chronic low grade inflammation in the LP Polyp: tubular adenoma with low-grade dysplasia	Chronic low grade inflammation in the LP
8	**INR**	Normal	Chronic moderate grade inflammation in the LP	Chronic moderate grade inflammation in the LP
9	**FR**	Normal	Chronic low grade inflammation in the LP Lymphofollicular hyperplasia	Chronic low grade inflammation in the LP Lymphofollicular hyperplasia (colon)
10	**FR**	Transverse colon polyp	Chronic low grade inflammation in the LP Lymphofollicular hyperplasia Polyp: tubular adenoma with low-grade dysplasia	Acute and chronic low grade inflammation in the LP
11	**FR**	Normal	Chronic low grade inflammation in the LP	Acute and chronic low grade inflammation in the LP
12	**FR**	Colon polyps	Chronic low grade inflammation in the LP Polyps: 2 tubular adenomas with low-grade dysplasia	Chronic moderate grade inflammation in the LP
13	**FR**	Right hepatic flexure flat polyp	Acute and chronic low grade inflammation in the LP Lymphofollicular hyperplasia Polyp: tubular adenoma with low grade dysplasia, focal high-grade dysplasia at the polyp's extremity	Acute and chronic low grade inflammation in the LP
14	**FR**	Descending colon diverticular disease	Acute and chronic low grade inflammation in the LP Lymphofollicular hyperplasia	Acute and chronic low-moderate grade inflammation in the LP
15	**FR**	Normal	Acute and chronic low grade inflammation in the LP Lymphofollicular hyperplasia	Acute and chronic moderate grade inflammation in the LP Eosinophilic elements, haematic congestion
16	**FR**	Normal	Chronic low grade inflammation in the LP	Chronic low-moderate grade inflammation in the LP Haematic congestion (rectum)
17	**FR**	Sigmoid colon diverticular disease	Acute and chronic low grade inflammation in the LP	Acute and chronic low grade inflammation in the LP
18	**FR**	External haemorrhoids plexus congestion	Chronic low grade inflammation in the LP	Acute and chronic low grade inflammation in the LP

			Lymphofollicular hyperplasia	Lymphofollicular hyperplasia (colon)
19	**FR**	Right hepatic flexure pedunculated polyp	Chronic low grade inflammation in the LP Tubular-villous adenoma with low-grade dysplasia	Chronic low grade inflammation in the LP Glandular rarefactions and LP fibrosis (rectum)
20	**FR**	Normal	Chronic low grade inflammation in the LP Eosinophilic elements	Chronic moderate grade inflammation in the LP Eosinophilic elements (colon)
21	**FR**	Normal	Chronic low grade inflammation in the LP	Chronic inflammation in the LP Focal cryptitis (colon)

INR: Immunological Non Responder; FR: Full Responder. LP: Lamina propria

Table 3. Immunohistochemical study of Cadherin (CDh1) and Zonula occludens protein 1 (ZO-1) in ileum and colon biopsies

		Ileum						Colon					
		CDH1			ZO			CDH1			ZO		
Patient	**Group**	**intensity**	**% positive cells**	**distribution**	**intensity**	**% positive cells**	**distribution**	**intensity**	**% positive cells**	**distribution**	**intensity**	**% positive cells**	**distribution**
1	**INR**	2	70	basal	0	0		2	60	basal	0	0	
2	**INR**	2	70	basal	1	10	membrane	2	70	basal	1	10	membrane
3	**INR**	2	70	basal	1	20	membrane	2	60	baso-lateral	1	20	membrane
4	**INR**	2	90	basal	1	30	cytoplasm	2	80	basal	0	0	N.A.
5	**INR**	2	80	basal	0	0	N.A.	2	80	baso-lateral	0	0	N.A.
6	**INR**	2	40	basal	1	20	cytoplasm	2	30	basal	1	30	cytoplasm
7	**INR**	2	40	basal	1	20	cytoplasm	2	60	baso-lateral	0	0	N.A.
8	**INR**	2	80	basal	2	50	cytoplasm	2	40	basal	2	60	cytoplasm
9	**FR**	2	30	basal	1	60	paranuclear	3	90	baso-lateral	1	40	paranuclear
10	**FR**	3	100	circumferential	1	20	membrane	3	100	circumferential	1	30	membrane
11	**FR**	2	80	basal	0	0	N.A.	3	90	basal	0	0	N.A.
12	**FR**	3	100	circumferential	2	80	membrane	3	100	circumferential	2	90	cytoplasm
13	**FR**	3	80	basal	2	70	cytoplasm	3	100	circumferential	2	90	membrane
14	**FR**	3	100	baso-lateral	1	20	cytoplasm	2	90	baso-lateral	2	70	cytoplasm
15	**FR**	3	100	baso-lateral	1	20	cytoplasm	3	100	baso-lateral	0	0	N.A.
16	**FR**	2	70	baso-lateral	2	60	paranuclear	3	80	baso-lateral	2	60	paranculear
17	**FR**	3	90	baso-lateral	N.A.	N.A.	N.A.	3	80	baso-lateral	0	0	N.A.
18	**FR**	3	70	baso-lateral	0	0	membrane	3	100	baso-lateral	1	20	cytoplasm
19	**FR**	2	70	baso-lateral	2	30	membrane	2	30	baso-lateral	2	40	cytoplasm
20	**FR**	2	70	baso-lateral	1	60	membrane	2	60	circumferential	1	80	membrane
21	**FR**	3	40	baso-lateral	2	60	membrane	2	30	baso-lateral	2	30	cytoplasm
22	**HIV-**	3	100	circumferential	1	90	membrane	3	100	circumferential	2	90	membrane
23	**HIV-**	2	100	circumferential	2	100	membrane	3	100	circumferential	3	100	membrane
24	**HIV-**	3	100	circumferential	3	90	membrane	3	100	circumferential	2	90	membrane
25	**HIV-**	3	100	circumferential	3	100	membrane	3	100	circumferential	2	100	membrane
26	**HIV-**	3	100	circumferential	3	100	membrane	3	100	circumferential	3	100	membrane

A semi-quantitative score was used for the study of intestinal junction proteins, by evaluating: a) the reaction intensity of stained epithelial cells of colon and ileum (0 = no staining , 1 = weak staining , 2 = intermediate staining , 3 = intense staining); b) the percentage of positive cells, by counting stained cells on 100 superficial intestinal cells at high magnification (40x); c) subcellular localization (membranous as complete, lateral, basal, baso-lateral; cytoplasmic; para-nuclear). INR: Immunological Non Responder; FR: Full Responder. HIV-: HIV-negative controls; N.A. Not available

Table 4. HIV reservoirs in gut biopsies and peripheral CD4+ T-cells in INR and FR study subjects

	Ileum			Colon			Peripheral CD4+ T-cells		
	INR	**FR**	**p-value**	**INR**	**FR**	**p-value**	**INR**	**FR**	**p-value**
Total HIV DNA, cp/10^6 cells	265, 20-993	135, 47-322	0.8	111, 73-421	98, 65-166	0.4	1092, 233-1869	846, 253-1269	0.7
Integrated HIV DNA, cp/10^6 cells	196, 54-580	172, 50-471	>0.9	60, 28-141	68, 50-149	0.6	187, 60-501	183, 79-413	0.9
2-LTR circles cp/10^6 cells	0, 0-4	0,0-0	>0.9	3, 0-49	0, 0-7	0.6	32, 1-173	5, 0-23	0.2
Total/integrated HIV DNA ratio	1.5, 1-3	1.5, 1-3	0.9	4, 1-6	1, 1-3	0.2	3.5, 1-8	4, 1-6.5	0.4

Data are presented are median, IQR. P values refer to comparisons between Immunological Non Responder (INR) and Full Responder (FR). 2-LTR, 2 Long Terminal Repeats.

Table 5. Correlations between measurements of HIV DNA in different compartments

		Peripheral CD4 T-cells			Ileum			Colon	
		total HIV DNA r (p value)	**integrated HIV DNA r (p value)**	**2 LTR r (p value)**	**total HIV DNA r (p value)**	**integrated HIV DNA r (p value)**	**2LTR r (p value)**	**total HIV DNA r (p value)**	**integrated HIV DNA r (p value)**
Peripheral CD4 T-cells	**integrated HIV DNA**	**0.7 (0.002)**	_____	_____	_____	_____	_____	_____	_____
	2 LTR	**0.7 (0.0016)**	0.4 (0.08)		_____	_____	_____	_____	_____
Ileum	**total HIV DNA**	0.4 (0.14)	0.2 (0.42)	0.32 (0.26)	_____	_____	_____	_____	_____
	integrated HIV DNA	0.2 (0.5)	0.36 (0.27)	0.11 (0.75)	**0.6 (0.05)**	_____	_____	_____	_____
	2 LTR	-0.04 (0.12)	0.2 (0.45)	-0.15 (0.02)	-0.4 (<0.0001)	0.3 (0.4)	_____	_____	_____
Colon	**total HIV DNA**	**0.6 (0.02)**	0.1 (0.7)	0.4 (0.10)	**0.55 (0.04)**	0.4 (0.18)	-0.03 (0.09)	_____	_____
	integrated HIV DNA	0.2 (0.46)	0.13 (0.6)	-0.09 (0.7)	-0.03 (0.9)	0.21 (0.51)	0.3 (0.35)	**0.5 (0.03)**	_____
	2 LTR	-0.4 (0.06)	**-0.6 (0.004)**	-0.22 (0.16)	-0.18 (0.19)	-0.44 (0.04)	-0.3 (<0.0001)	0.1 (0.7)	-0.2 (0.5)

Table 6. Correlations between CD4+ T-cell counts, gut junctional complex protein expression and HIV reservoirs

	Current CD4+ T-cell count, n r (p value)	**Current CD4+ T-cell count, % r (p value)**	**CD4/CD8 T-cell ratio r (p value)**	**CD4+ T-cell nadir r (p value)**
Ileum CDh1 expression	0.13 (0.58)	-0.18 (0.44)	-0.03 (0.9)	-0.05 (0.82)
Ileum ZO-1 expression	**0.6 (0.009)**	**0.53 (0.02)**	0.4 (0.09)	0.2 (0.39)
Colon CDh1 expression	0.16 (0.50)	-0.02 (0.90)	0.3 (0.30)	-0.04 (0.86)
Colon ZO-1 expression	**0.5 (0.04)**	0.34 (0.14)	0.31 (0.19)	0.15 (0.50)
Peripheral CD4+ T-cells total HIV DNA	-0.34 (0.16)	**-0.5 (0.041)**	-0.32 (0.21)	-0.25 (0.31)
Peripheral CD4+ T-cells integrated HIV DNA	-0.11 (0.66)	-0.28 (0.25)	-0.005 (0.98)	-0.31 (0.20)
Peripheral CD4+ T-cells 2 LTR	-0.4 (p=0.09)	**-0.6 (p=0.004)**	**-0.6 (0.02)**	-0.036 (0.88)
Ileum total HIV DNA	**-0.5 (0.05)**	**-0.5 (0.05)**	-0.5 (0.06)	-0.32 (0.24)
Ileum integrated HIV DNA	-0.20 (0.44)	-0.2 (0.5)	-0.22 (0.49)	0.15 (0.64)
Ileum 2 LTR	-0.07 (0.05)	-0.08 (0.05)	-0.03 (0.09)	0.08 0.19)
Colon total HIV DNA	-0.28 (0.24)	-0.26 (0.29)	**-0.5 (0.04)**	0.11 (0.67)
Colon integrated HIV DNA	0.16 (0.5)	0.2 (0.43)	0.19 (0.47)	0.09 (0.73)
Colon 2 LTR	0.21 (0.39)	0.3 (0.24)	0.04 (0.6)	0.03 (091)

Table 7. Demographic characteristics and viro-immunological parameters of the patients in study (Aim 2.1) [39]

Characteristic	LR (n=9)	IR (n=12)	HR (n=9)
Age, years (IQR)	50 (45-56)	48 (38-60)	39 (35-44)[a]
Sex, F (%)	2 (22)	3 (25)	3 (33)
Risk factors for HIV infection			
MSM, n (%)	2 (22)	5 (42)	1 (11)
heterosexual, n (%)	6 (67)	4 (33)	3 (33)
IDU, n (%)	1 (11)	3 (25)	5 (56)
Duration of HIV infection, years (IQR)	7 (5-19)	13 (8-22)	12 (6-23)
HAART duration, months (IQR)	71 (46-141)	106 (65-149)	71 (39-132)
Diagnosis of AIDS, n	4	3	2
Absolute T CD4+ cell counts, cell/µl (IQR)			
at the time of study	260 (215-339)	498 (425-537)	726 (608-863)[a]
nadir	90 (51-179)	105 (45-208)	204 (56-240)
Percentage T CD4+ cell counts, % (IQR)			
at the time of study	26 (16-34)	27 (22-33)	31 (25-43)
nadir	8 (5-17)	18 (13-22)	26 (22-30)
Plasma HIV RNA, log_{10}cp/mL (IQR)			
zenith	5.3 (4.5-5.9)	5.4 (4.1-5.7)	4.4 (3.9-5.2)
at the time of study	1.7	1.7	1.7
HAART regimen			
Number of patients at the time of study			
NRTI+PI	5	7	4
NRTI+NNRTI	2	4	3
Other	2	1	2

Data are presented as median and interquartile range (IQR). LR, Low Responders (CD4+<350/µL, HIV RNA <40cp/ml); IR, Intermediate Responders (CD4+ 350-599/µL, HIV RNA <40cp/ml); HR, High Responders (CD4+ ≥600/µL, HIV RNA <40cp/ml); MSM, Men having sex with men; IDU, Intravenous Drug Users; HAART, highly active antiretroviral therapy; NRTI, nucleoside reverse-transcriptase inhibitor; NNRTI, non nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor. [a]$p < 0.05$ for differences among groups.

Table 8. Demographic characteristics and viro-immunological parameters of the patients in study (Aim 2.2)

	FRs (n=20)	**INRs (n=15)**	**p**
Age, years *	51 (41-68)	45 (39-54)	0.278
Sex, (%)° ***Female***	 6 (30)	 1 (7)	 0.198
Risk Factors, (%)° ***Heterosex*** ***Homosex/Bisex*** ***IDU***	 11 (55) 6 (30) 3 (15)	 7 (47) 5 (33) 3 (20)	0.271
HCV co-infection, (%)° ***Yes***	 3 (15)	 2 (13)	0.722
AIDS diagnosis° (%),	5 (25)	9 (60)	0.079
Time since HIV diagnosis, years *	5 (5-7)	5 (3-7)	0.552
CD4 T-cell count (IQR)* ***Nadir (n)*** ***Time of analysis (n)***	 97 (42-230) 451 (404-585)	 94 (26-124) 237 (164-299)	 0.342 **<0.0001**
Monocytes (%)	8,4 (5,8-9,5)	8,7 (7,4-10,1)	0.389
HIV-RNA Log cp/mL*	1.59 (1.59-1.59)	1.59 (1.59-1.59)	0.901
HAART duration, years*	4.5 (3-5)	5 (3-6)	0.444
HAART regimen (%)° **NRTI+PI** **NRTI+NNRTI** **Others**	 11 (55) 7 (35) 2 (10)	 13 (86) 1 (7) 1 (7)	0.112
Plasma LPS, pg/mL (IQR)*	164 (75-264)	235 (108-265)	0.447

*Data are median (IQR). IQR: Interquartile range, Statistical analyses: Mann-Whitney U Test. ° Data are n (%), Statistical analyses: Pearson Chi squared or Fisher Exact Test; FRs: Full Responders (CD4≥350/mmc; HIV-RNA<40cp/mL); INRs: Immunological Non Responders (CD4<350/mmc; HIV-RNA<40cp/mL); IDU: intravenous drug users; LPS, Lipopolysaccharide; HAART highly active antiretroviral therapy; NRTI nucleoside reverse transcriptase inhibitor; PI protease inhibitor; NNRTI non-nucleoside reverse transcriptase inhibitor. LPS: lypopolysaccharide

Table 9. CD4 and CD8 T-cell proliferation and apoptosis in HIV-infected, HAART-Treated Subjects with different immune recovery

Ki67+CD4, % (IQR)													
	UNST	**LPS**	**p unst vs LPS**	**LTA**	**p unst vs LTA**	**PGN**	**p unst vs PGN**	**ssRNA**	**p unst vs ssRNA**	**IFN-γ**	**p unst vs IFN-γ**	**aCD3/CD28**	**p unst vs CD3/28**
FRs	1.5 (0.8-3.4)	1.3 (0.2-2.3)	0.112	1.3 (0.6-2.9)	0.276	1.1 (0.4-1.9)	0.078	1.1 (0.5-2.5)	**0.028**	2.2 (0.6-2.5)	0.695	3.5 (1.8-5.1)	**0.015**
INRs	2.7 (1.9-3.9)	1.4 (0.8-2.1)	**0.002**	1.4 (1-2.7)	**0.028**	1.3 (0.8-2.7)	0.080	1.4 (0.7-2.3)	0.424	1.8 (0.9-4.1)	1	5 (2.5-6.7)	0.069
p 2 groups	0.183	0.677		0.526		0.179		0.591		0.642		0.172	
Ki67+CD8, % (IQR)													
	UNST	**LPS**	**p unst vs LPS**	**LTA**	**p unst vs LTA**	**PGN**	**p unst vs PGN**	**ssRNA**	**p unst vs ssRNA**	**IFN-γ**	**p unst vs IFN-γ**	**aCD3/CD28**	**p unst vs CD3/28**
FRs	1.6 (0.9-2.7)	1.4 (0.3-2.5)	0.600	1.9 (0.9-3.1)	0.569	1.8 (0.1-3)	0.601	2.3 (1-2.7)	0.542	1.6 (0.8-4.2)	0.983	3.3 (2.1-5.2)	**0.003**
INRs	2.5 (1.3-6.3)	2.3 (1.1-5.6)	0.443	1.9 (1.1-5.3)	**0.049**	3.3 (0.7-5.1)	0.514	3.1 (1.7-6.6)	0.604	2.9 (2.2-7.5)	0.599	6.1 (3.8-10.3)	**0.003**
p 2 groups	0.375	0.086		0.644		0.101		0.077		**0.025**		**0.015**	
Annex V+CD4, % (IQR)													
	UNST	**LPS**	**p unst vs LPS**	**LTA**	**p unst vs LTA**	**PGN**	**p unst vs PGN**	**ssRNA**	**p unst vs ssRNA**	**IFN-γ**	**p unst vs IFN-γ**	**aCD3/CD28**	**p unst vs CD3/28**
FRs	7.1 (1.9-21.6)	4.9 (2.8-34)	0.557	8.1 (3.1-36.9)	0.923	12.7 (1.4-52.2)	0.203	3.3 (2.6-18.6)	0.547	8.3 (2.7-30.1)	1	17.9 (10.3-39.5)	0.065
INRs	20.9 (8.6-36.9)	19.7 (8.5-45.1)	0.910	11 (5.6-43.2)	0.250	12.6 (8.2-48.1)	0.730	16 (6.7-27.5)	0.106	13.7 (7.7-46.2)	0.884	20.3 (15.9-49.4)	0.734
p 2 groups	0.095	0.156		0.459		0.667		0.142		0.370		0.661	
Annex V+CD8, % (IQR)													
	UNST	**LPS**	**p unst vs LPS**	**LTA**	**p unst vs LTA**	**PGN**	**p unst vs PGN**	**ssRNA**	**p unst vs ssRNA**	**IFN-γ**	**p unst vs IFN-γ**	**aCD3/CD28**	**p unst vs CD3/28**
FRs	12.9 (5.1-20.5)	13.2 (7.5-17.6)	0.625	14.9 (8.1-19.5)	0.475	15.2 (4.7-21.2)	0.870	15.3 (8.9-18.3)	0.739	13.6 (4.7-19.7)	0.359	16.7 (11.3-30.9)	0.432
INRs	11.7 (3.9-18.6)	13.4 (6.1-21.3)	0.652	13.9 (6.5-29.1)	0.097	15.9 (9.5-24.8)	**0.039**	11.8 (6.4-18.9)	0.578	17.4 (7.5-20.8)	0.742	18.6 (5.2-22.9)	0.164
p 2 groups	0.775	0.935		0.967		0.796		0.417		0.481		0.719	

Data are presented as median, interquartile range (IQR). Statistical analyses: Mann-Whitney U Test for comparison between FRs and INRs; Wilcoxon Test for comparison between unst vs stimuli . FRs: Full Responders; INRs: Immunological Non Responders; UNST, unstimulated; LPS, Lipopolysaccharide; LTA, lipoteicoic acid; PGN, peptidoglycan; IFN- γ, interferon gamma.

Figure 1. Expression of junctional complex proteins in ileum biopsies

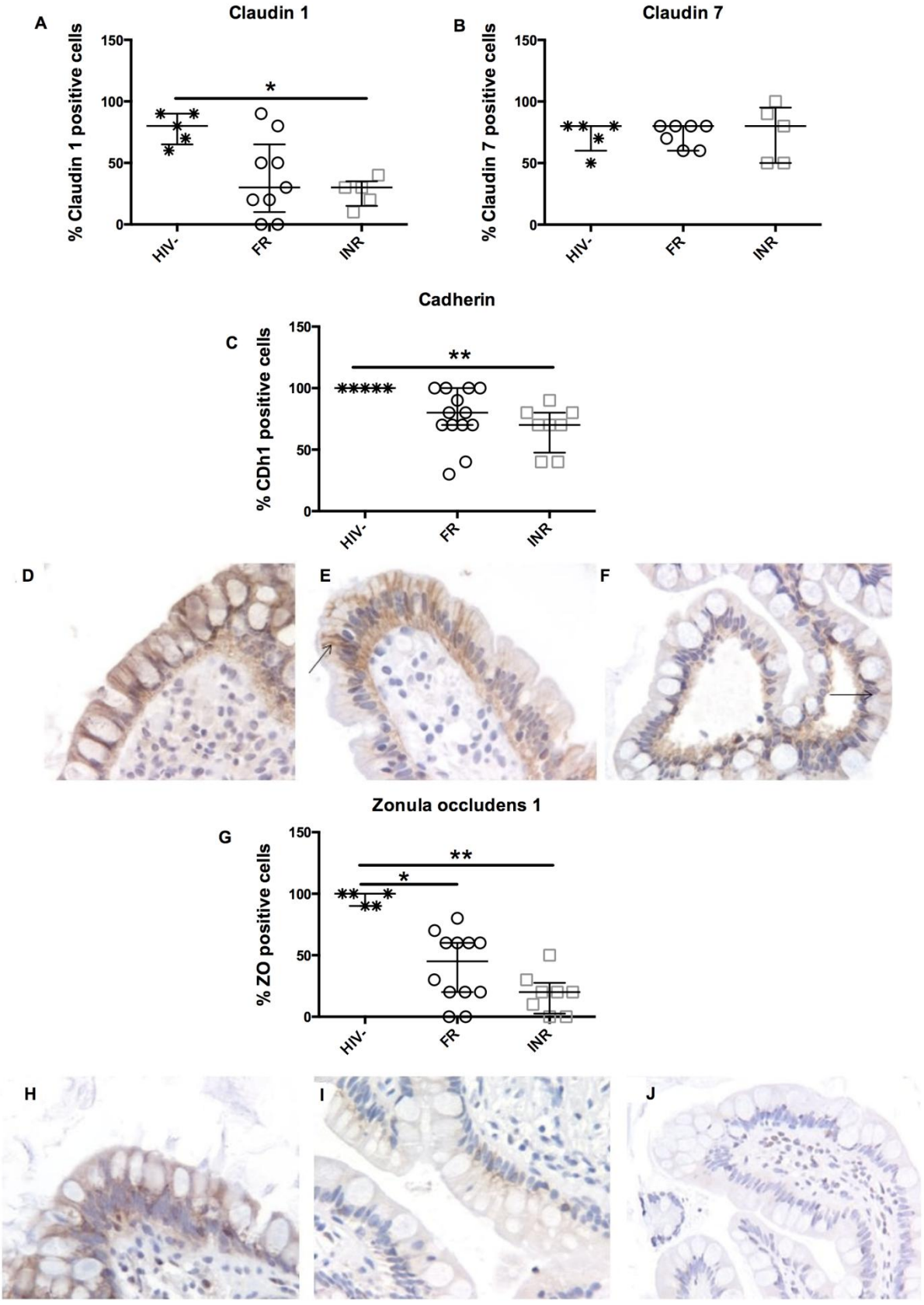


Figure 1. Expression of junctional complex proteins in ileum biopsies. Claudin 1, claudin 7, cadherin (Cdh1) and *Zonula occludens* protein 1 (ZO-1) expression were measured by IHC in ileum tissue in HIV-uninfected (n=5), FR (n=13) and INR (n=8) individuals. INR displayed significantly lower claudin 1 expression (A) compared to HIV-uninfected controls, but no differences were observed among study groups in claudin 7 (B). CDh1 expression (C) was also significantly impaired in INR. Immunoreactivity with anti-Cdh1 antibodies showed strong diffuse basal and lateral positivity of enterocytes in HIV-uninfected subjects (D), focal lateral positivity between enterocytes in FR (arrow, E) and rare weak lateral positivity between enterocytes in INR (arrow, F). Consistently with these findings, ZO-1 expression (G) was lowest in INR patients. Immunoreactivity with anti-ZO-1 antibodies also showed a gradient between HIV-negative controls (H), FR (I) and INR (J). Data were analyzed by Kruskall-Wallis/Anova test followed by Dunn's multiple comparisons test. * $p<0.05$; ** $p<0.001$; HIV-, HIV-negative; FR, Full Responder; INR, Immunological Non Responder. 40X original magnification.

Figure 2. Expression of junctional complex proteins in colon biopsies

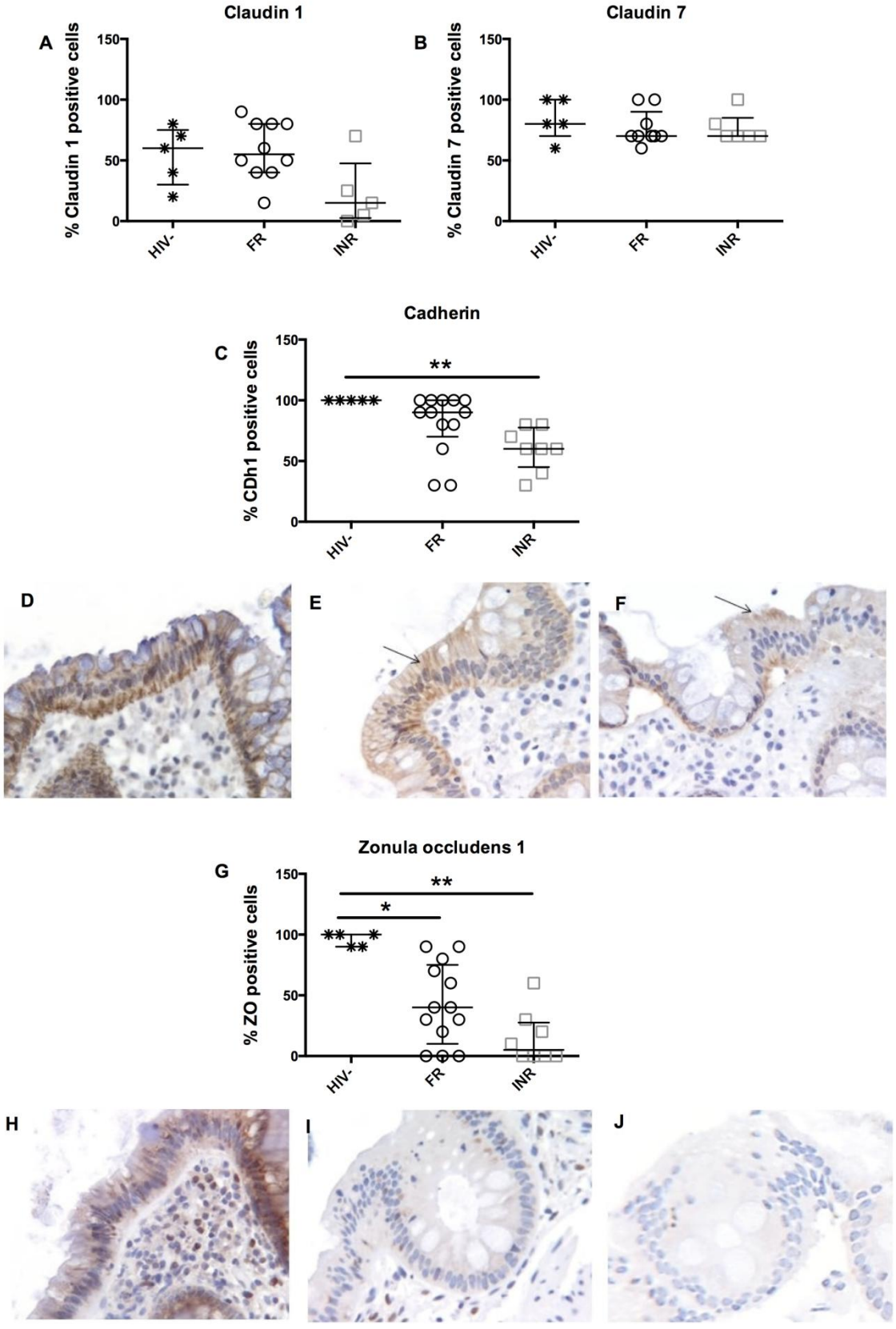


Figure 2. Expression of junctional complex proteins in colon biopsies. Claudin 1, claudin 7, cadherin (Cdh1) and Zonula occludens protein 1 (ZO-1) expression were measured by IHC in colon tissue in HIV-uninfected (n=5), FR (n=13) and INR (n=8) individuals. The expression of claudin 1 (A) and claudin 7 (B) was comparable among study groups. Cdh1 expression (C) was significantly impaired in INR compared to HIV-uninfected controls. Immunoreactivity with anti-Cdh1 antibodies showed strong diffuse basal and lateral positivity of enterocytes in HIV-uninfected subjects (D), focal lateral positivity between enterocytes in FR (arrow, E) and rare weak lateral positivity between enterocytes in INR (arrow, F). ZO-1 expression (G) was lowest in INR patients. Immunoreactivity with anti-ZO-1 antibodies also showed a gradient between HIV-uninfected controls (H), FR (I) and INR (J). Data were analyzed by Kruskall-Wallis/Anova test followed by Dunn's multiple comparisons test. * $p<0.05$; ** $p<0.001$; HIV-, HIV-negative; FR, Full Responder; INR, Immunological Non Responder. 40X original magnification.

Figure 3. Electron microscopy study of gut biopsies

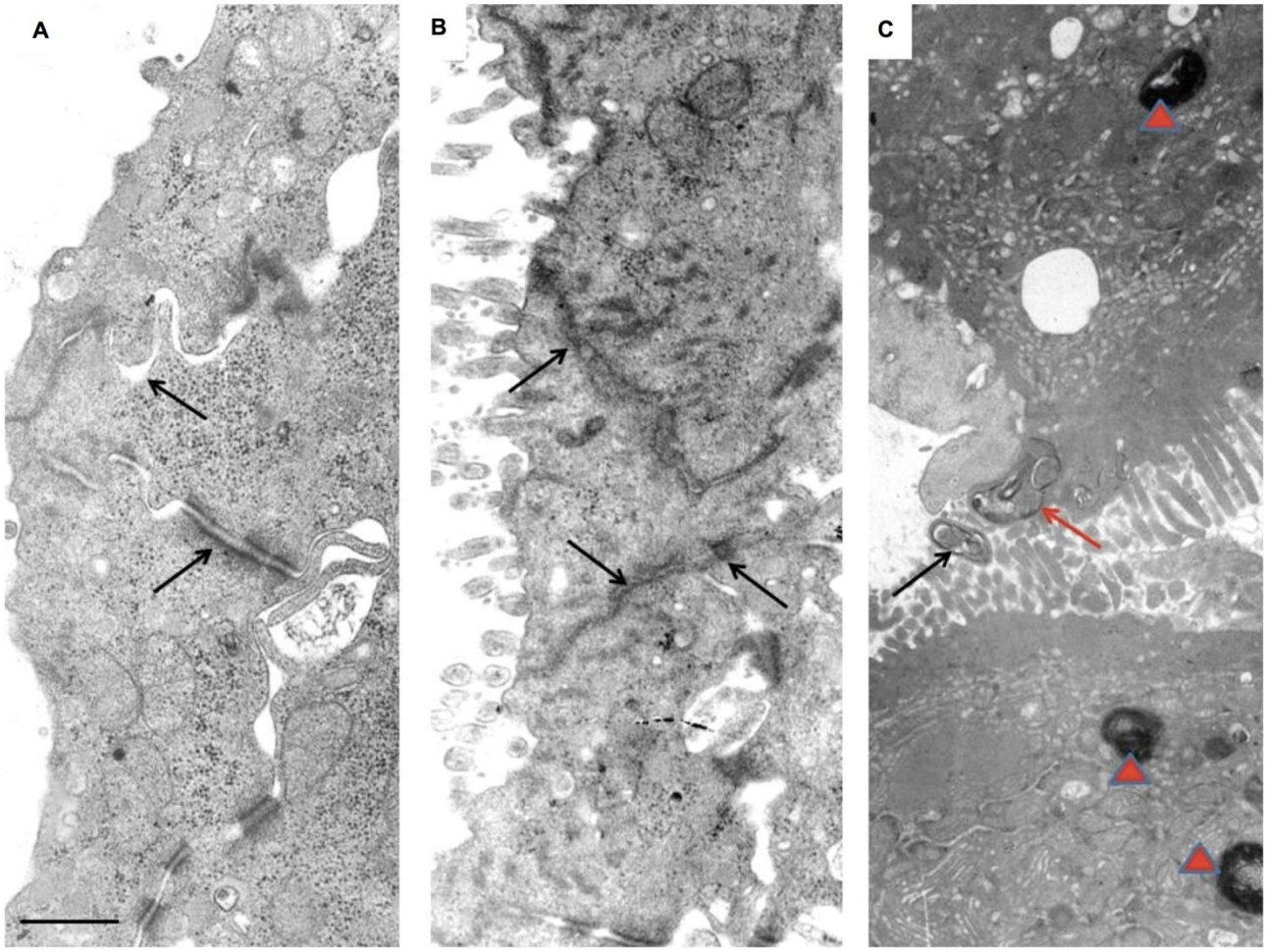


Figure. 3. Electron microscopy study of gut biopsies. Electron microscopy was perfomed in INR (n=3) and FR (n=4) individuals. Colon biopsy from an Immunological Non Responder (INR) subject (A); arrows point to dilated intercellular space. Colon biopsy from a Full Responder (FR) patient (B); arrows point to normal intercellular space. Intestinal (ileocolic junction) biopsy from an INR patient (C); arrow points to an extracellular bacterium among microvilla, red arrow points to intracellular bacterium, arrow heads point to partly digested intracytoplasmic bacteria. A and B, scale bar = 500 nm; C, scale bar =1mm.

Figure 4. Gastrointestinal permeability and microbial translocation parameters

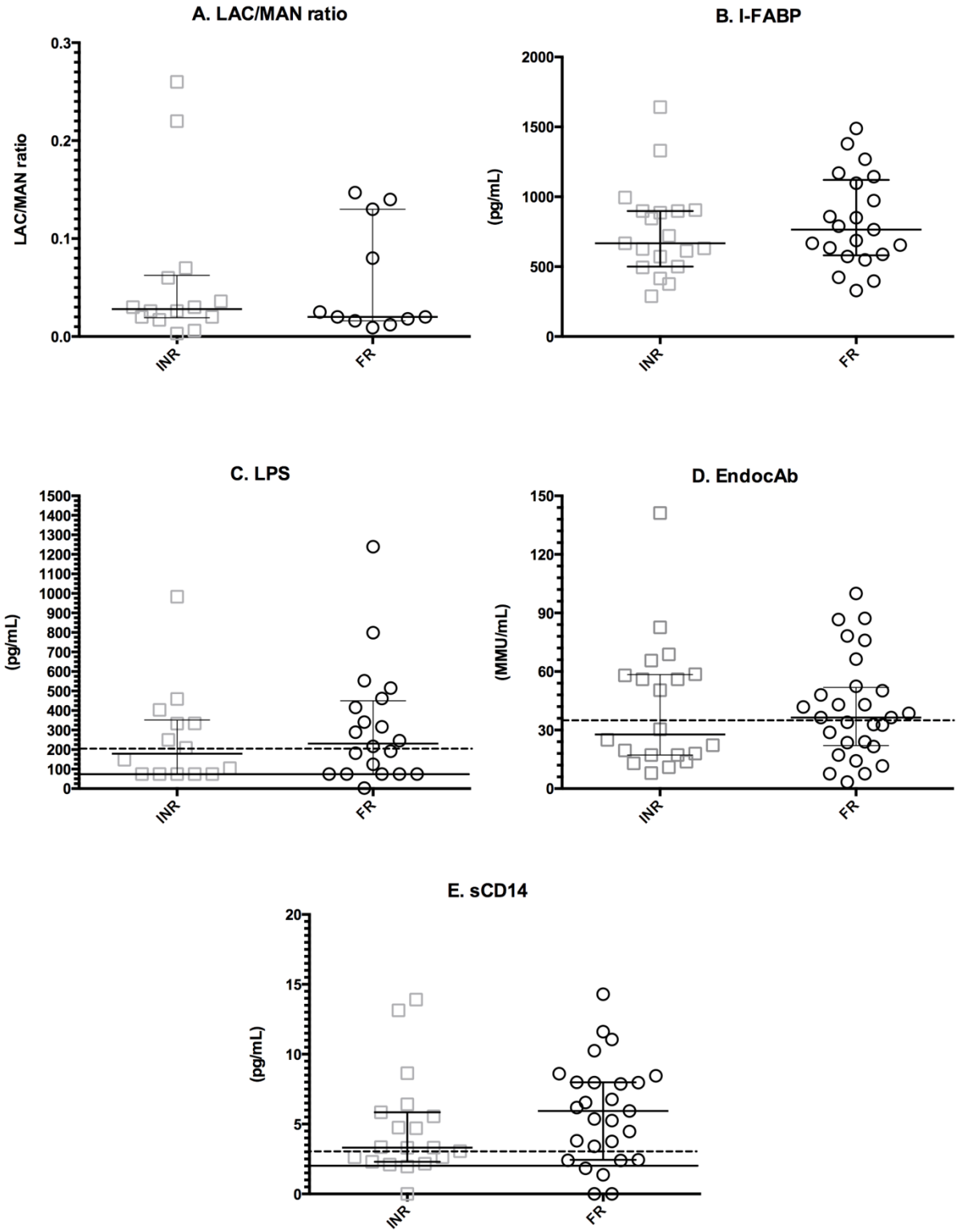
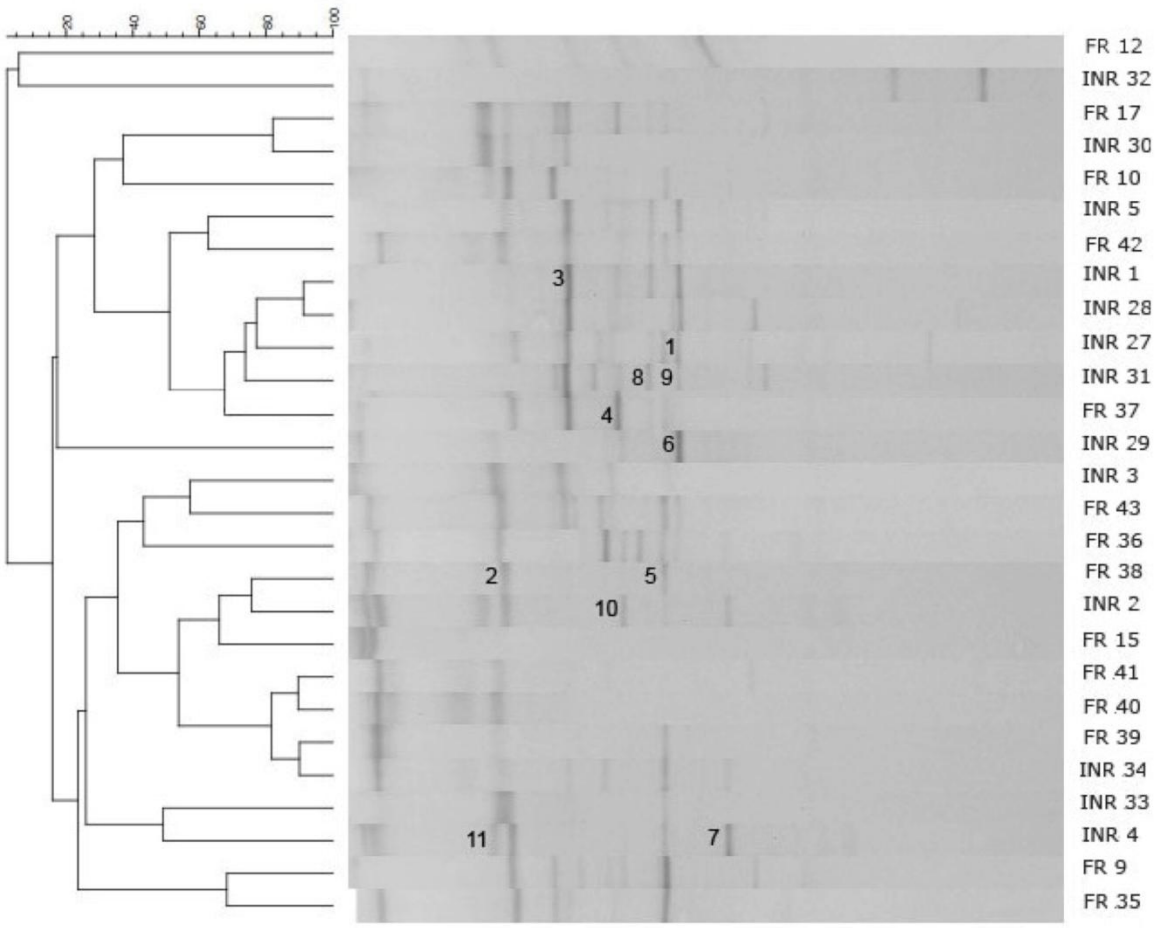


Figure 4. Gastrointestinal permeability and microbial translocation parameters. The urinary lactulose-mannitol fractional excretion ratio (LAC/MAN) was used to assess small intestine permeability in study 14 INR and 11 FR (A). No differences were observed between INR and FR. In keeping with this finding, comparable small intestine function (I-FABP, B) and microbial translocation parameters measured by circulating levels of LPS (C), EndocAb (D) and sCD14 (E) were detected in study groups. Data were analyzed by Mann-Whitney test. INR, Immunological Non Responder; FR, Full Responder. LAC/MAN, urinary lactulose-mannitol fractional excretion ratio; I-FABP, Intestinal Fatty Acid Binding Protein; LPS, lipopolysaccharide; EndocAb, Endotoxin core Antibodies; sCD14, soluble CD14; MMU IgM Median Units.

Reference values on the graphs are as follows: - - - - indicates LPS, EndocAb and sCD14 median values obtained by our laboratory in a large cohort of 488 HIV-infected untreated patients [72]; ____indicates median values obtained by our laboratory in HIV-negative controls [28].

Figure 5. A hierarchical cluster analysis of the DGGE profiles of the *Bacteroides-Prevotella* group



Band number	% of blast similarity	Nearest species	Accession number	Presence in INR (%)	Presence in FR (%)
1	99	*Prevotella copri*	NR040877.1	21	14
2	100	*Bacteroides dorei*	NR041351.1	57	79
3	98	*Prevotella copri*	NR040877.1	57	36
4	99	*Prevotella copri*	NR040877.1	0	7
5	100	*Bacteroides uniformis*	NR040866.1	43	79
6	99 99	*Bacteroides intestinalis* *Bacteroides uniformis*	NR041307.1 NR040866.1	28	0
7	100	*Bacteroides uniformis*	NR040866.1	14	14
8	99	*Prevotella copri*	NR040577.1	28	21
9	95	*Prevotella copri*	NR040877.1	14	14
10	99	*Bacteroides intestinalis*	NR041307.1	7	0
11	97	*Bacteroides vulgatus* *Bacteroides thetaiotamicron*	NR074515.1 NR074277.1	28	21

Figure 5. A hierarchical cluster analysis of the DGGE profiles of the *Bacteroides-Prevotella* group. Dendrogram constructed using Pearson's correlation coefficients and the UPGMA algorithm of DGGE gels obtained analyzing the V2–V3 region of fecal samples from 13 INR and 14 FR. Amplicons for DGGE analysis of the Bacteroides-Prevotella group were obtained using a nested-approach with the primers Bac303F and Bac708R, and subsequently the primers Hda1-GC and Hda2. Sequenced bands are highlighted by numbers (1-11) directly on the DGGE gel. The table reports the identified species and their prevalence in the INR and FR groups.

Figure 6. HIV reservoirs in gut biopsies and peripheral CD4+ T-cells

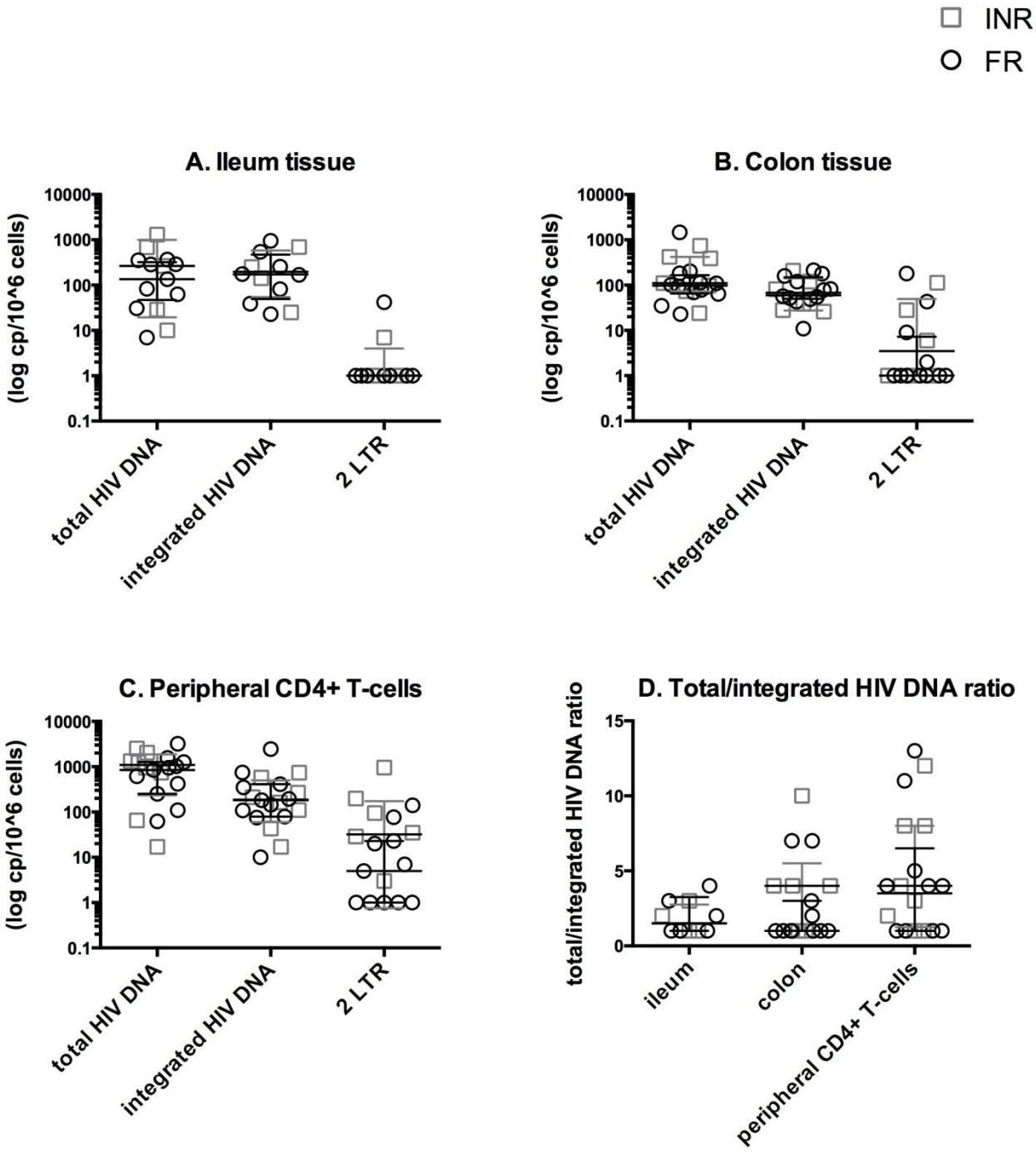


Figure 6. HIV reservoirs in gut biopsies and peripheral CD4+ T-cells. HIV total DNA, integrated DNA and 2 LTR circles were studied in ileum (A), colon (B) and peripheral CD4+ T-cells (C) were measured in INR and FR subjects. The total/integrated HIV DNA ratio (D) was also evaluated in gut biopsies and peripheral CD4+ T-cells. No differences in reservoir size were registered between study groups, nor differences were noted between anatomical districts (E; data presented as median, IQR; p-values refer to comparisons between INR and FR). INR, Immunological Non Responder; FR, Full Responder. 2-LTR, 2 Long Terminal Repeats.

Figure 7. Correlations between current CD4+ T-cell counts, gut junctional protein expression and HIV reservoirs

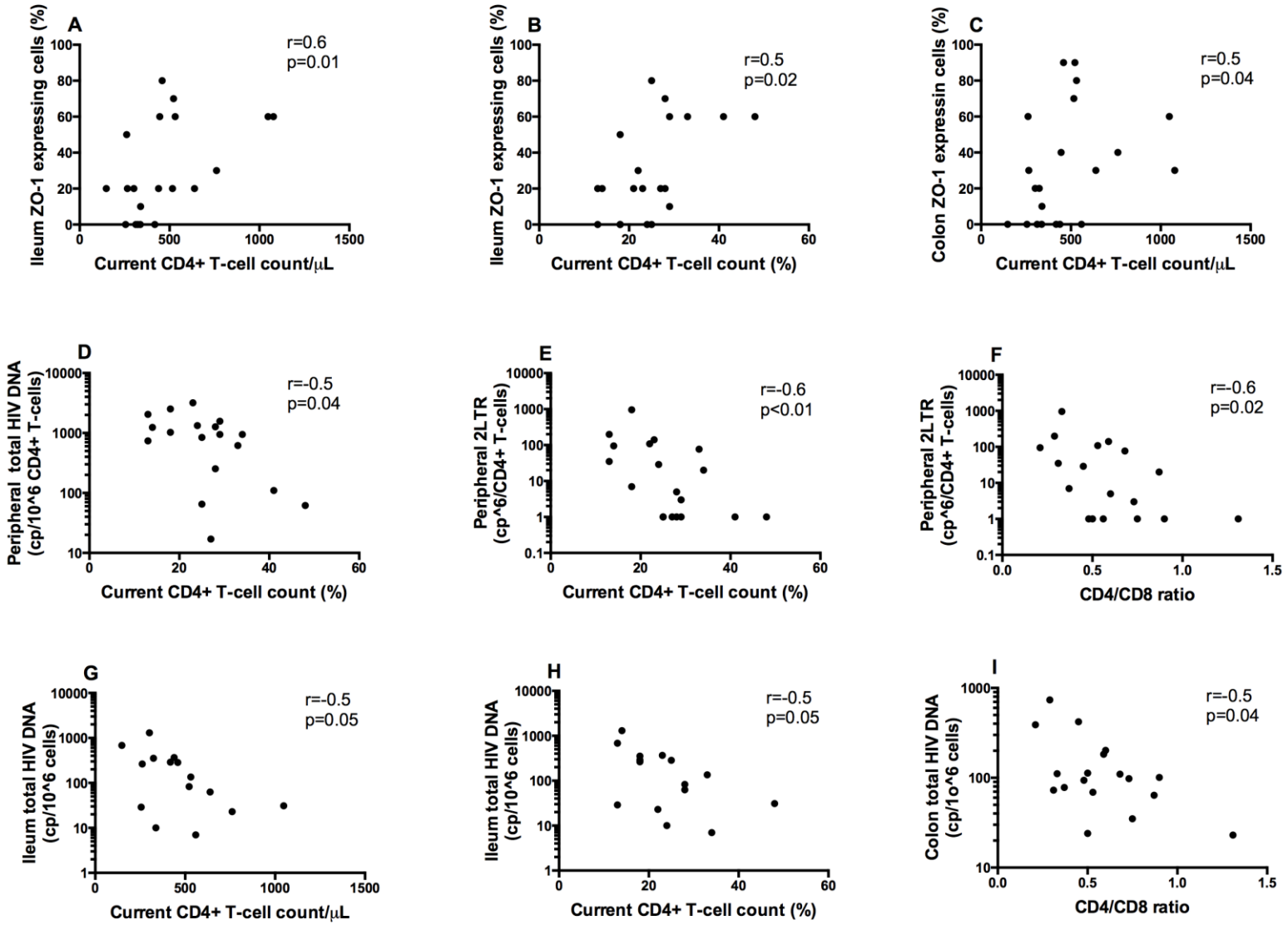


Figure 7. Correlations between CD4+ T-cell counts, gut junctional protein expression and HIV reservoirs. Spearman's rank coefficient was used to correlate current CD4+ T-cell counts with gut junctional protein expression (A-C); current CD4+ T-cell counts and the CD4/CD8 ratio with HIV reservoirs in both circulating CD4+ T-lymphocytes (D-F) and gut (G-I).

Figure 8. HLA-DR-expressing CD4+/CD8+ T cells in HIV-negative and HIV-positive patients prior to and following LPS stimulation

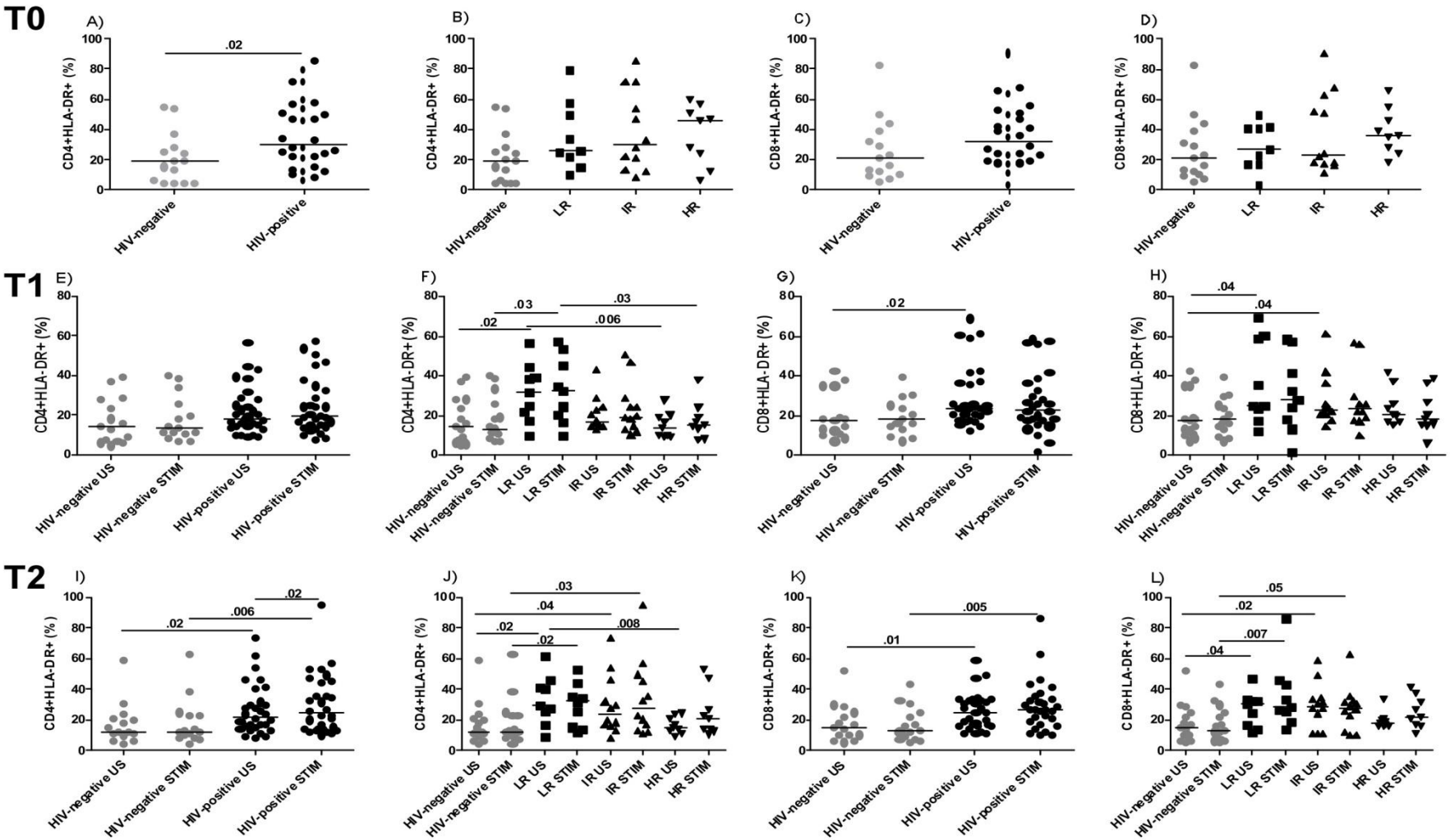


Figure 8. HLA-DR-expressing CD4+/CD8+ T cells in HIV-negative and HIV-positive patients prior to and following LPS stimulation [39].

HLA-DR expression was measured on freshly ficoll-separated CD4+ and CD8+ T-cells at baseline (T0, *top*), and following 24- (T1, *middle*) and 48-hour (T2, *bottom*) LPS stimulation. PBMCs were cultured in medium alone (unstimulated, US) or in medium with 20ng/mL LPS (stimulated, STIM).

At T0, HIV-positive individuals displayed higher HLA-DR+CD4+ (p=0.02, Figure 8A) and HLA-DR+CD8+ proportions (Figure 8C), with no differences amongst HIV-positive subgroups (Figure 8B, 8D).

At T1, HIV-positive patients maintained increased HLA-DR+CD4+ (Figure 8E) and CD8+ cells (Figure 8G); significantly higher HLA-DR+CD4+ levels were detected in LR *versus* HR and negative controls (p=0.03 for both comparisons, Figure 8F).

At T2, HIV-infected individuals displayed significantly higher HLA-DR+CD4+ (p=0.006, Figure 8I) and CD8+ cells compared to controls (p=0.005, Figure 8K). Following stimulation, significantly greater proportions of HLA-DR-expressing CD4+ were measured in LR (p=0.02, Figure 8J) and IR (p=0.03, Figure 8J) compared to controls. Comparable results were detected in the CD8+ compartment upon LPS stimulation (LR vs HIV-negative, p=0.007, Figure 8L; IR vs HIV-negative, p=0.05, Figure 8L). ● HIV-negative; ● HIV-positive; ■ Low Responders, LR; ▲ Intermediate Responders, IR; ▼ High Responders, HR. p values in the results section refer to stimulated samples only.

Figure 9. CD38-expressing CD4+/CD8+ T cells in HIV-negative and HIV-positive patients prior to and following LPS stimulation

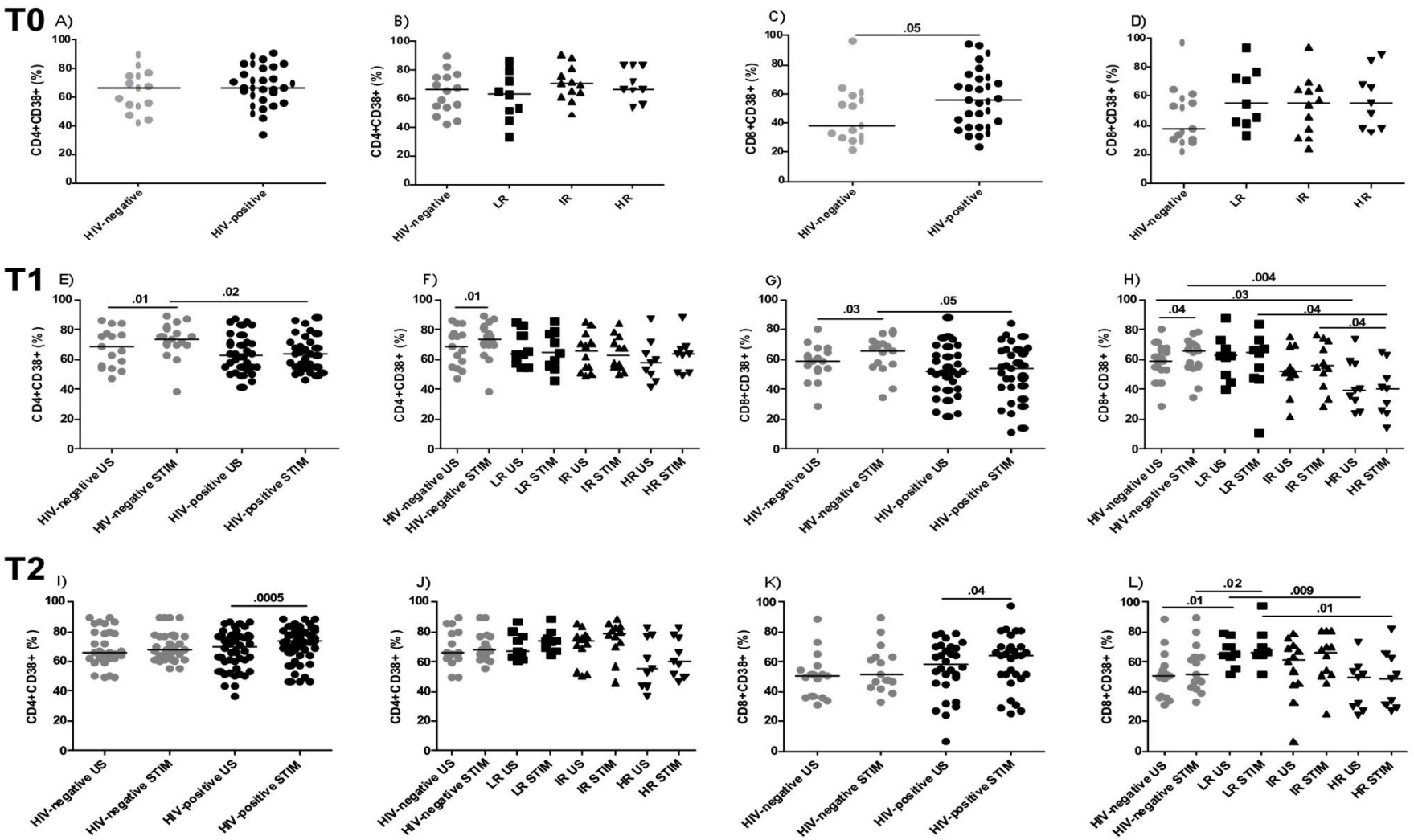


Figure 9. CD38-expressing CD4+/CD8+ T cells in HIV-negative and HIV-positive patients prior to and following LPS stimulation [39].

CD38 expression was measured on freshly ficoll-separated CD4+ and CD8+ T-cells at baseline (T0, *top*) and following 24- (T1, *middle*) and 48-hour (T2, *bottom*) LPS stimulation. PBMCs were cultured in medium alone (unstimulated, US) or in medium with 20ng/mL LPS (stimulated, STIM). At T0, comparable CD4+CD38+ were detected in HIV-positive patients and controls (Figure 9A), however, the former displayed markedly higher CD8+CD38+ (p=0.05, Figure 9C), with no differences amongst HIV-positive subgroups. At T1, a significantly lower expression of CD38 was detected on CD4+ (p=0.02, Figure 9E) and CD8+ cells (p=0.05, Figure 9G) from HIV-positive individuals despite LPS stimulation. While no differences in CD4+CD38+ were recorded amongst HIV-positive subgroups (Figure 9F), CD8+CD38+ were significantly higher in LR and IR compared to HR (p=0.04 for both comparisons, Figure 9H). At T2, LR presented highest CD38+CD8+ compared to HR (p=0.01, Figure 9L) and controls (p=0.02, Figure 9L) with no differences in CD4+CD38+ (Figure 9J). ● HIV-negative; ● HIV-positive; ■ Low Responders, LR; ▲ Intermediate Responders, IR; ▼ High Responders, HR. p values in the results section refer to stimulated samples only.

Figure 10. Time course representation of the effect of LPS on HLA-DR and CD38 expression on CD4+ and CD8+ T-cells

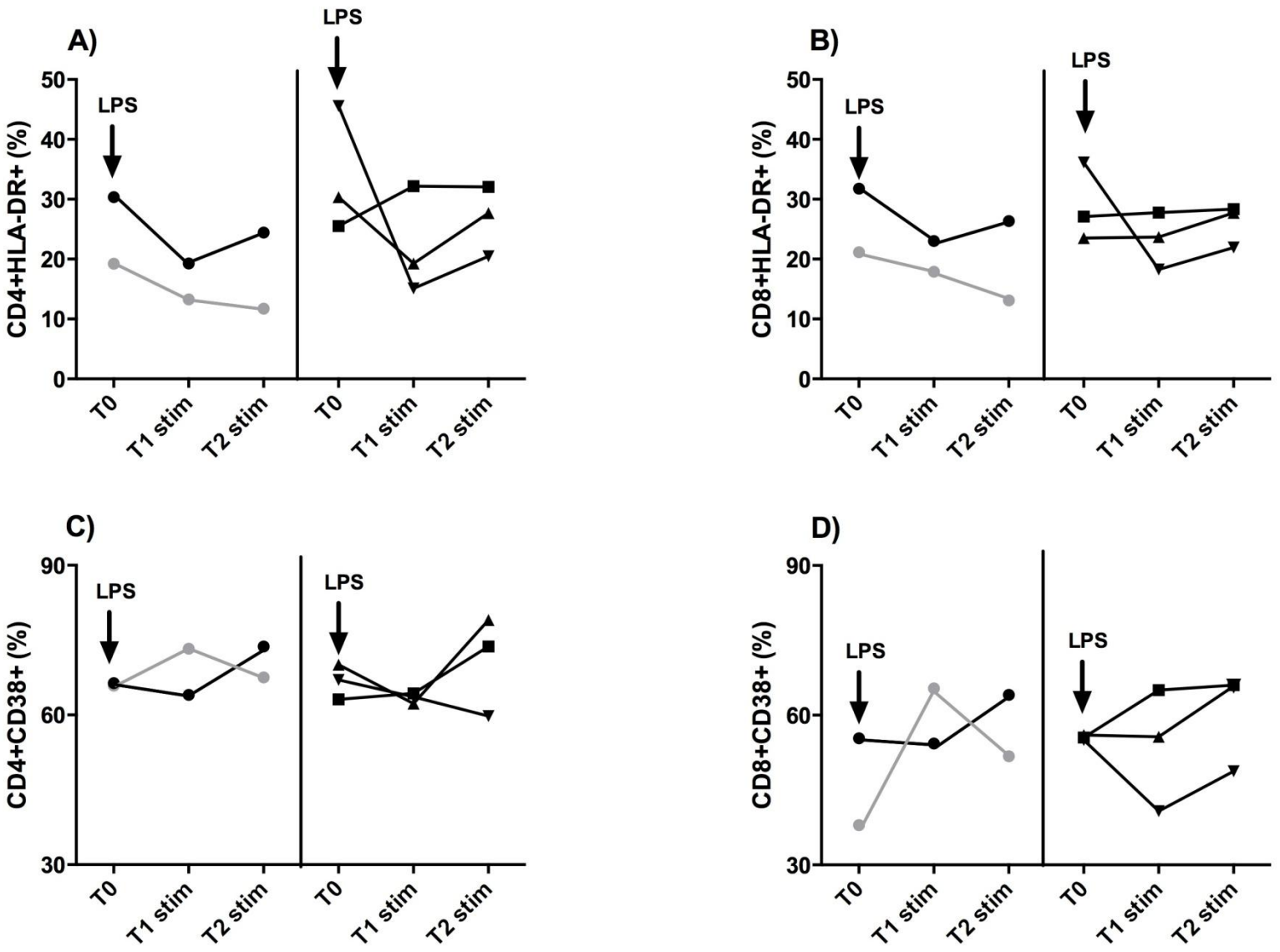


Figure 10. Time course representation of the effect of LPS on HLA-DR and CD38 expression on CD4+ and CD8+ T-cells [39].

Median HLA-DR (Figure 10A, 10B) and CD38 (Figure 10C, 10D) expression on CD4+ and CD8+ T-cells in HIV-positive and HIV-negative individuals (left portion of graphs) and in HIV-infected subjects with different immunological outcome in course of HAART (right portion of graphs) at baseline (T0) and following LPS stimulation (T1 stim, T2stim).

● HIV-negative; ● HIV-positive; ■ Low Responders, LR; ▲ Intermediate Responders, IR; ▼ High Responders, HR.

Figure 11. Ki67-expressing CD4+/CD8+ T cells in HIV-negative and HIV-positive patients prior to and following LPS stimulation

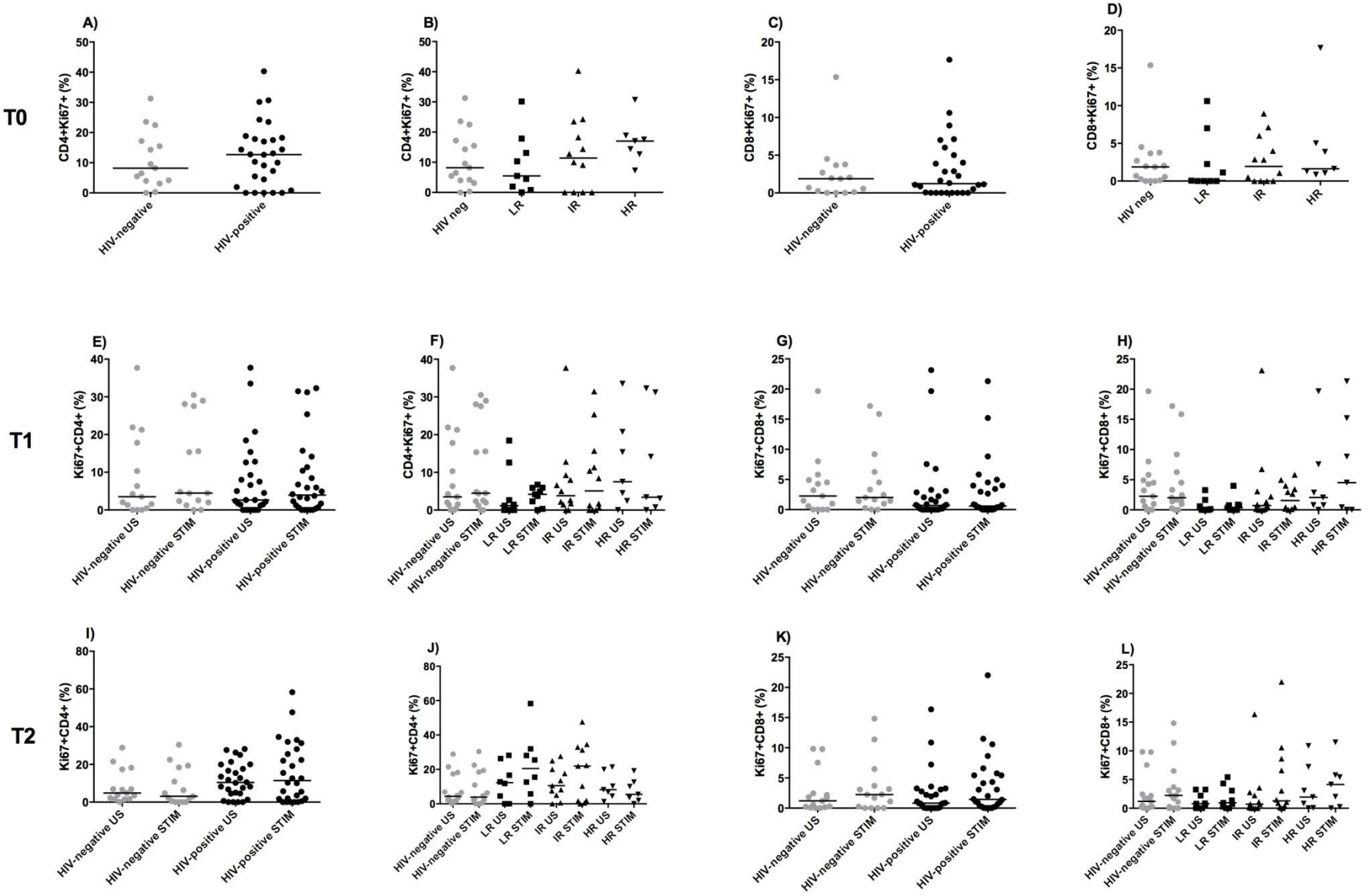


Figure 11. Ki67-expressing CD4+/CD8+ T cells in HIV-negative and HIV-positive patients prior to and following LPS stimulation [39].

Ki67 expression was measured on freshly ficoll-separated CD4+ and CD8+ T-cells at baseline (T0, *top*) and following 24- (T1, *middle*) and 48-hour (T2, *bottom*) LPS stimulation. PBMCs were cultured in medium alone (unstimulated, US) or in medium with 20ng/mL LPS (stimulated, STIM). At T0, no differences between HIV-negative controls and HIV-positive subjects were measured in Ki67 expression on CD4+ (Figure 11A) and CD8+ T-cells (Figure 11C). T-cell proliferation levels did not vary according to the degree of immune-reconstitution in course of HAART (Figure 11B, 11D). At T1, LPS stimulation did not account for significant increases in cell proliferation (Figure 11E-11H). At T2, a trend to increased Ki67 levels on CD4+ T-cells upon stimulation was detected in LR compared to HR and negative controls (Figure 11J); no differences were detected in terms of Ki67+CD8+ T-cells proliferation among study groups following 48-hour stimulation with LPS (Figure 11K, 11L).

● HIV-negative; ● HIV-positive; ■ Low Responders, LR; ▲ Intermediate Responders, IR; ▼ High Responders, HR.

Figure 12. Phenotypic and functional characterization of CD38+CD4+ and CD8+ T cells in HIV-infected patients

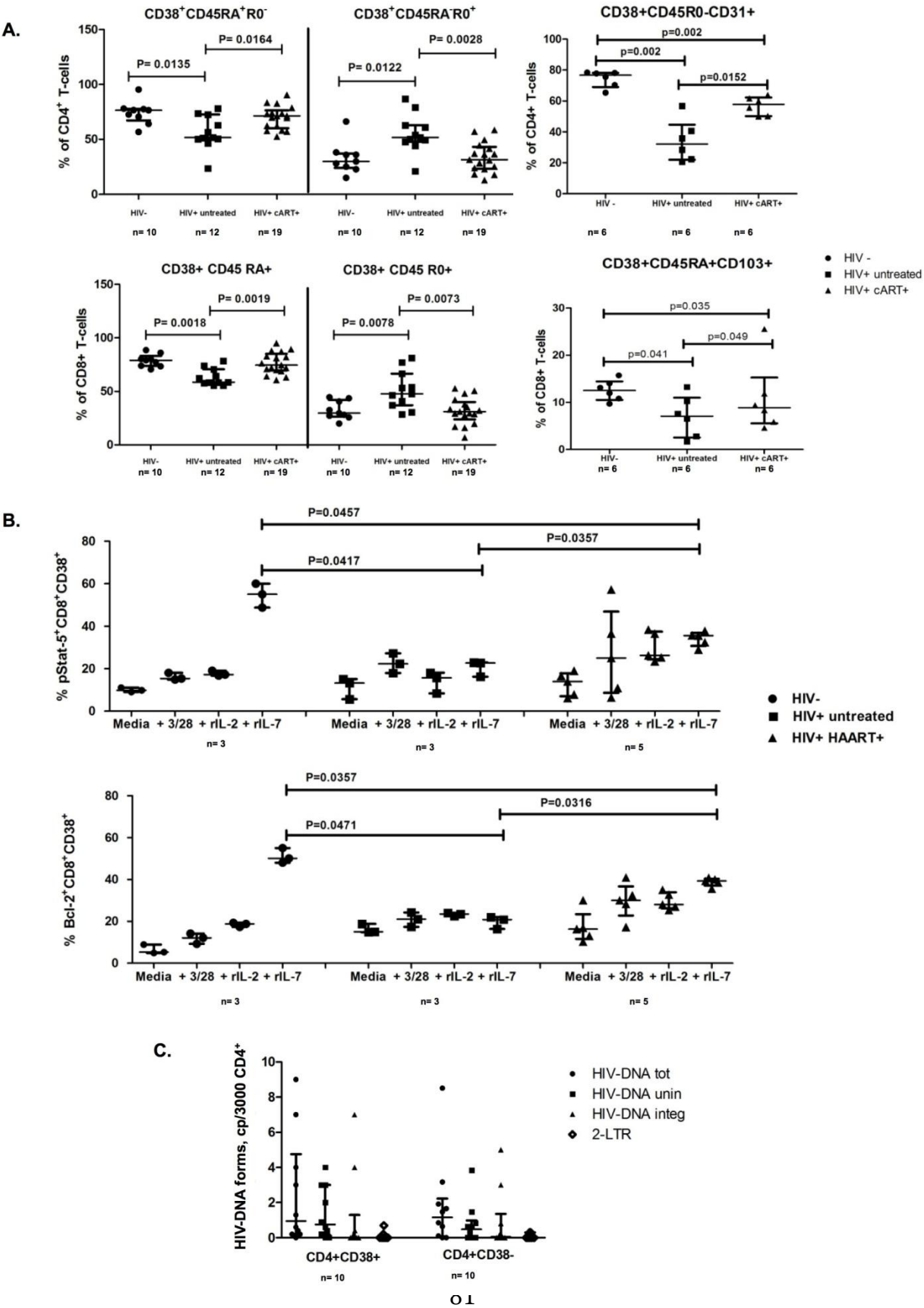


Figure 12. Phenotypic and functional characterization of $CD38^+CD4^+$ and $CD8^+$ T cells in HIV-infected patients [67].

A. Phenotypic analysis on freshly isolated PBMCs, comparison between patients HIV+ untreated, cART+ treated and healthy controls. **B.** Magnetically-isolated $CD38^+CD8^+$ cells were either left unstimulated or stimulated with anti-CD3/CD28 antibodies alone , or together with 100 U rIL-2 or 1000 ρg/ml rIL-7 ,levels of pSTAT and Bcl-2 were determined using intracellular flow cytometry (after 5 h for pSTAT and after 24 h for Bcl-2). **C.** Analysis of total HIV-DNA (integrated and unintegrated HIV-DNA forms), unintegrated HIV-DNA (extracromosomal HIV-DNA species), integrated HIV-DNA and 2-LTR circle content. Abbreviation: PBMC, peripheral blood mononuclear cells

Figure 13. In vitro TLR challenge on MDM: surface expression of HLA-DR and CD69

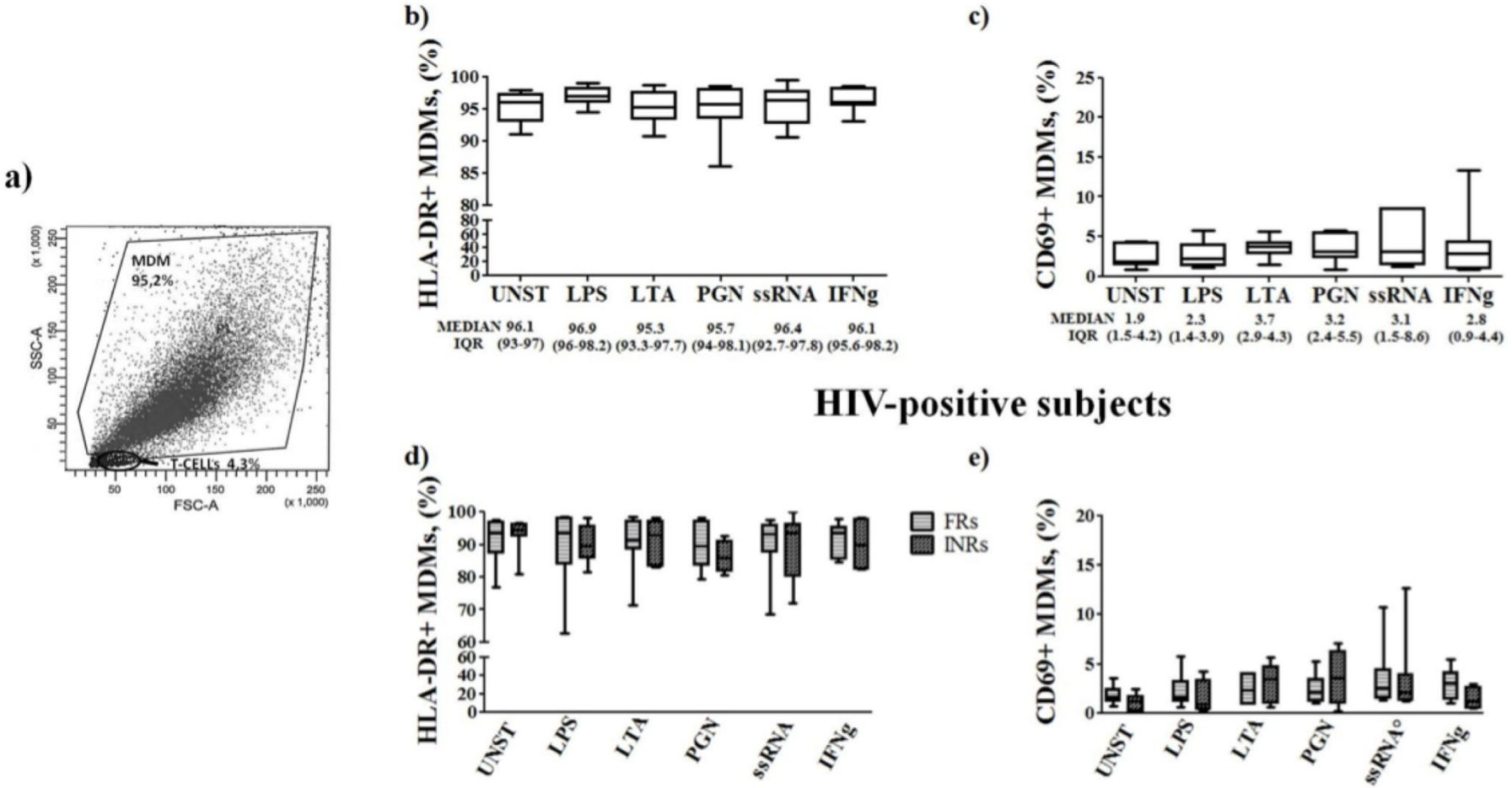


Figure 13. *In vitro* MDM and PBMC TLR challenge: surface expression of HLA-DR and CD69

a) Gating strategy for identification of Monocyte-derived Macrophages (MDMs). Based on cell dimensions, we determined the purity of the population (T-cells<5%). MDMs were gated on the basis of physical parameters (FSC,SSC). Within the morphological gate we measured the % of CD14+ HLA-DR+ or CD14+CD69+.

b) In HIV-negative healthy subjects, all MDMs expressed the functional molecule HLA-DR at very high percentage, with no differences following TLR stimulation. **c)** In HIV-negative healthy subjects, the activation marker CD69 was not modified following TLR stimulation. **d)** FRs and INRs showed similar levels of HLA-DR surface expression (UNST: FRs 93,5% [87,5-97] vs INRs 94,5% [92,5-96,3] p=.966; LPS: FRs 93,6% [83,9-98,2] vs INRs 89,6% [85,7-95,7] p=.525; LTA: FRs 91,3% [88,5-97,4] vs INRs 92,9% [83,4-97,2] p=.178; PGN: FRs 89,7% [83,6-97,3] vs INRs 85% [80,1-92,5] p=.437; ssRNA: FRs 93,1% [87,6-96,1] vs INRs 93,4% [80,2-96,3] p=.966; IFNγ: FRs 93,6% [85,5-95,5] vs INRs 89,9% [82,4-97,9] p=.914), with no differences following the exposure to TLR agonists. **e)** ° p<.05 for unstimulated vs ssRNA in FRs. Viral stimulation was able to significantly expand CD69 on MDM exclusively in FRs (UNST vs LPS p=.772; vs LTA p=.150; vs PGN p=.833; vs ssRNA p=.005; vs IFNγ p=.063) but not in INRs (UNST vs LPS p=.343; vs LTA p=.125; vs PGN p=.500; vs ssRNA p=.09; vs IFNγ p=.500). No differences in CD69 surface expression on MDM between FRs and INRs were observed at baseline and after stimulation (UNST: FRs 1,7% [1,2-2,4] vs INRs 1,2% [0,5-1,7] p=.174; LPS: FRs 1,6% [1,6-3,2] vs INRs 0,9% [0,4-3,3] p=.331; LTA: FRs 2,3% [0,9-4] vs INRs 3,4% [0,9-4,7] p=.871; PGN: FRs 2,2% [1,2-3,5] vs INRs 3,6% [1-6,3] p=.476; ssRNA: FRs 2,5% [1,5-4,4] vs INRs 2,1% [1,3-3,9] p=.735; IFNγ: FRs 3% [1,5-4,2] vs INRs 1,3% [0,5-2,7] p=.171).

Figure 14. Cytokine/chemokine release by MDM following TLR stimulation in FR and INR

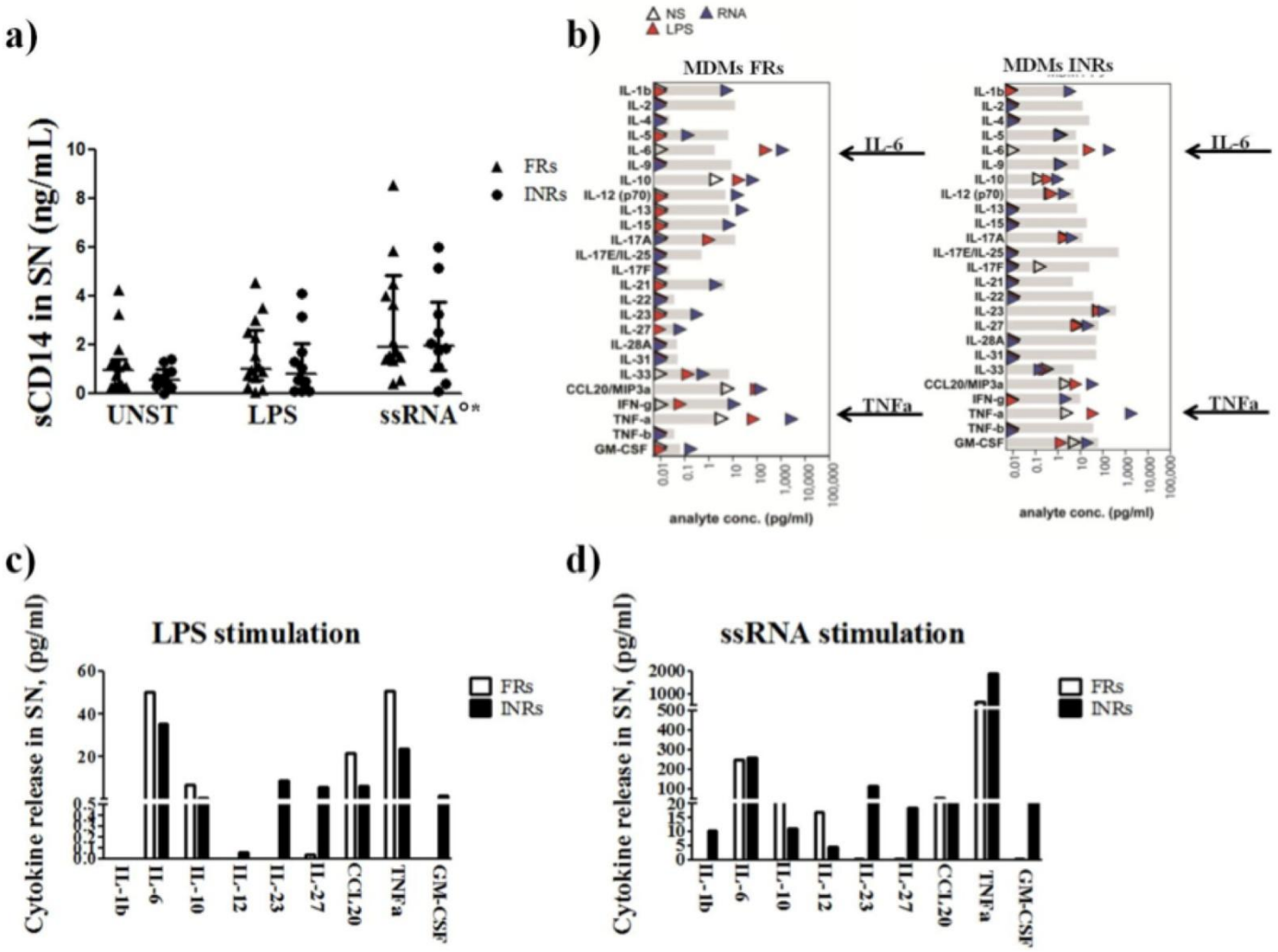


Figure 14. Cytokine/chemokine release by MDM following TLR stimulation in FRs and INRs

Among the TLR ligands used in the study, we chose LPS and ssRNA in order to evaluate the effect on sCD14 and cytokine release from MDMs. **a)** FRs and INRs showed similar release of sCD14. Interestingly, only viral stimulation accounted for an increase in sCD14 release irrespective of CD4 count (FRs: p=.005; INRs: p=.009). * for p value <.05 between unstimulated and ssRNA in INRs; ° for p value<.05 between unstimulated and ssRNA in FRs **b-d)** 25-plex Luminex assays on supernatants of MDMs from 5 INRs and 6 FRs patients. **b)** Examples of data obtained by Luminex assay. The graphs show the 25 cytokines/chemokines produced by MDMs of 1/6 FRs and 1/5 INRs in absence or in presence of TLR agonists. **c-d)** Data shown as a delta: [unstimulated cytokine value] - [LPS-/ssRNA-stimulated cytokine value]. **c)** Upon LPS stimulation, we found no differences between FRs and INRs: both displayed modest release of pro-inflammatory cytokine, with the exception of IL-6 (FRs: 50 pg/ml; INRs: 35 pg/ml) and TNFa (FRs: 50 pg/ml; INRs: 23 pg/ml) that showed the highest release. **d)** ssRNA exposure induced a higher release of cytokines compared to bacterial stimulation, particularly of IL-6 (FRs: 248 pg/ml; INRs: 259 pg/ml, Fig.3d.suppl) and TNFa (FRs: 666 pg/ml; INRs 1887 pg/ml).

Figure 15. *In vitro* TLR challenge on PBMCs: mRNA expression of signaling genes in FR and INR

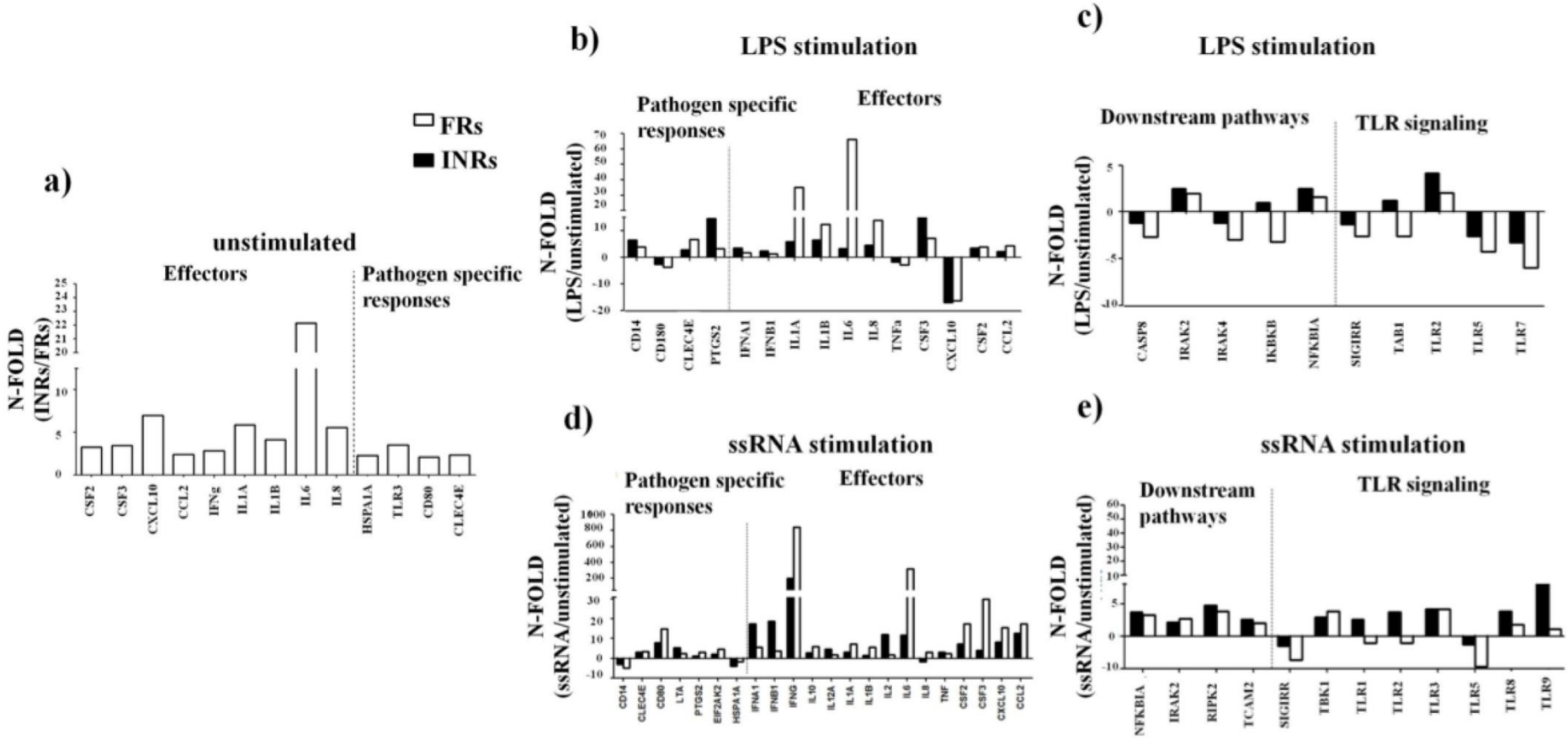


Figure 15. *In vitro* TLR challenge on PBMCs: mRNA expression of signaling genes in FR and INR.

We investigated the effect of LPS and ssRNA stimulation in HIV+ subject with different immune recovery on total PBMCs, that mimic the *in vivo* condition. To determine whether PBMCs from FRs and INRs might be dysfunctional and to compare the responsiveness of these cells to pathogen stimulation, we used Real-time PCR array, which screens for the expression of 84 genes that are involved in TLR-activation pathway. **a)** INRs displayed higher constitutive mRNA levels (up to 20-fold) of effectors such as CCL2, CSF2, CSF3, CXCL10, IFNg, IL1a, IL1b, IL6, IL8 and of genes involved in the pathogen specific response, such as CLEC4E, , HSPA1A, CD80 and TLR3 as compared to FRs. **b-e)** TLR stimulation with LPS and mainly with ssRNA lead to a different activation pathway according to immune recovery. **b)** LPS exposure resulted in a significant up-regulation of pro-inflammatory cytokines transcription, such as IL1a, IL6 and IL8 in FRs. **c)** No major differences in genes involved in TLR signaling or in downstream pathways between FRs and INRs. **d)** In the presence of ssRNA, FRs produced a greater mRNA amount of both pro- and anti-inflammatory cytokines, such as IFNg, IL6, CSF2, SCF3, whereas INRs showed up-regulation of type I IFNs. **e)** No major differences were found in genes of TLR signaling or downstream pathways.

Figure 16. In vitro TLR challenge on PBMCs: cytokine/chemokine release in FR and INR

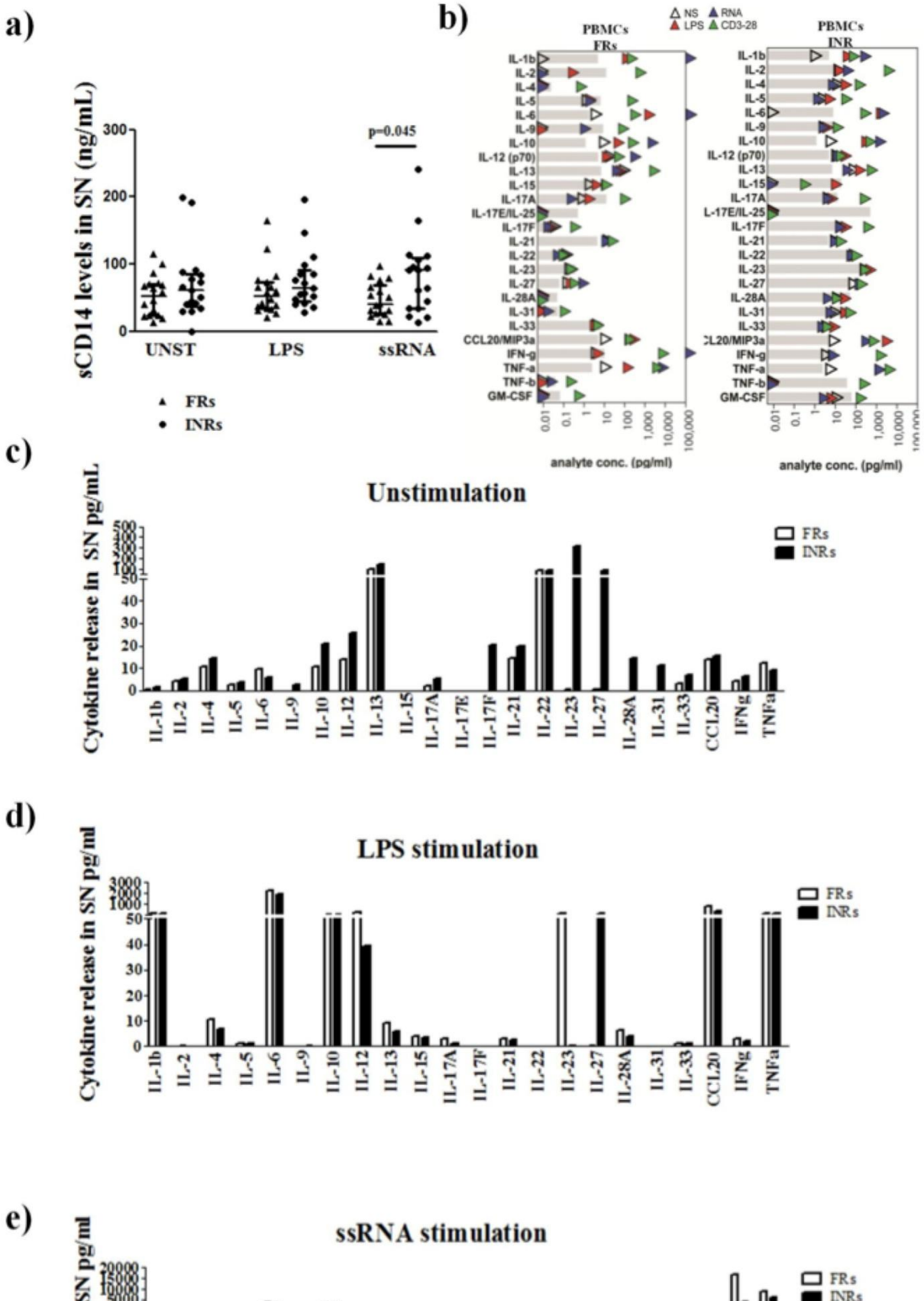


Figure 16. *In vitro* TLR challenge on PBMCs: cytokine/chemokine release in FRs and INRs.

a) Despite similar constitutive, INRs showed higher sCD14 levels after ssRNA (p=.045; Fig.2a) but not after LPS. Both LPS and ssRNA exposure did not impact sCD14 release in the two study groups. **b-e)** We performed 25-plex Luminex assays on supernatants of PBMCs from 5 INRs and 6 FRs patients. **b)** Examples of data obtained by Luminex assay. The graphs show the 25 cytokine produced by PBMCs of 1/6 FRs and 1/5 INRs in absence or in presence of TLR agonists. **c)** INRs constitutively released a greater amount of pro-inflammatory IL17F, IL23, IL27, IL 28a and IL31 than FRs. **d-e)** Data shown as a delta: [unstimulated cytokine value] - [LPS-/ssRNA-stimulated cytokine value]. **d)** No significant differences in cytokine released between FRs and INRs after LPS exposure. **e)** No major differences in cytokine release after ssRNA exposure.

Figure 17. *In vitro* TLR challenge on PBMC: T-cell activation

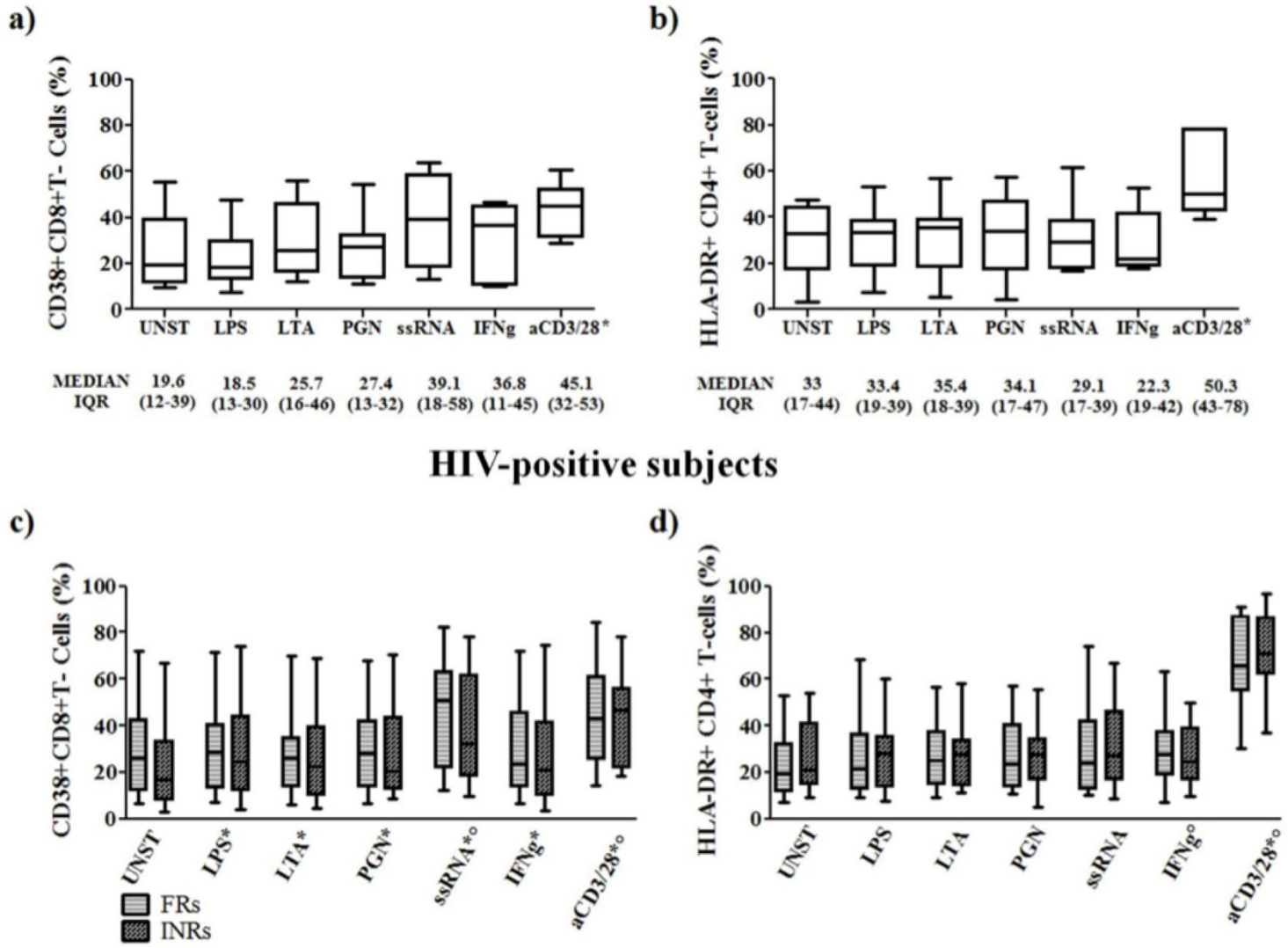


Figure 17. *In vitro* TLR challenge on PBMC: T-cell activation

a-b) In HIV-negative healthy subjects, we found a significant increase in CD38+ CD8 and HLA-DR+ CD4 T-cells after the exposure to the positive control aCD3/CD28 (p=.031 and p=.046, respectively); * for p<.05 unstimulated vs ssRNA condition. **c-d)** T-cell activation was evaluated in both FRs and INRs; * for p value <.05 between unstimulated and stimulated condition in INRs; ° for p value<.05 between unstimulated and stimulated condition in FRs **c)** Focusing on TLR agonist responses, we found that the exposure to all the tested stimuli resulted in significant expansion of CD38+CD8 only in INRs (LPS p=.0008; LTA p=.009; PGN p=.035 ssRNA p=.0003; IFNγ p=.042; aCD3/CD28 p=.0004). Contrarily, ssRNA and aCD3/CD28 stimulation resulted in higher CD38+CD8 in FRs (p=.0006; p=.0005 respectively). We did not find any differences in terms of CD38+CD8 T-cells comparing FRs and INRs (UNST: 26.2% [12.1-42.4] vs 16.7% [8.3-33.5], p=.259; LPS: 28.5% [13.3-40.6] vs 24.4% [12-43.95] p=.726; LTA: 25.9% [13.4-35.1] vs 22.3% [9.9-39.6] p=.615; PGN 28.2% [13.9-41.9] vs 20.7% [12.5-43.4] p=.437; ssRNA: 51.1% [21.8-62.9] vs 32.5% [18.3-61.8], p=524; IFNg: 23.6% [13.5-45.8] vs 20.8% [10-41.7], p=.591; aCD3/28: 43.3% [25.6-61.2] vs 46.9% [21.9-55.9], p=.726).

d) FRs and INRs displayed similar levels of HLA-DR+CD4 T-cells, both constitutive (FRs 19.7% [11.6-32.2] vs INRs 20.8% [14.9-41.05] p=.248) and following LPS (FRs 21.7% [12.8-36.7] vs INRs 28% [14-35.4] p=.591), LTA (FRs 25% [14.6-37.3] vs INRs 27.4% [14.3-33.7] p=.704), PGN (FRs 23.6% [13.8-40.3] vs INRs 27.6% [16.7-34.3] p=.741), ssRNA (FRs 24.2% [12.6-41.9] vs INRs 27.2% [16.7-46.4] p=.466), IFNg (FRs 27.5% [18.9-37.7] vs INRs 24.6% [16.6-39.1] p=.741) and aCD3/28 (FRs 65.8% [55.2-87] vs INRs 70.9% [62.1-86.5] p=.345) with no variations following TLR agonists exposure. Only FRs respond to IFNg (p=.047).

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