HIV-infected immunological non-responders have colon-restricted gut mucosal immune dysfunction

Running title: INR colon-restricted immune dysfunction

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Supplementary Methods

Study design.

Exclusion criteria were: hepatitis C RNA positive; hepatitis B surface antigen positive; concurrent inflammatory bowel disease, coeliac disease, or malnutrition; concurrent self-reported gastrointestinal symptoms; concomitant use of antithrombotic drugs, non-steroid anti-inflammatory drugs (NSAID), corticosteroids, disease-modifying antirheumatic drugs, or other anti-inflammatory pharmaceutical substances; concomitant use of any antibiotic or probiotic substance within three months prior to enrollment; deranged liver function (serum albumin <25 g/L or Child-Pugh score >9); renal failure (estimated glomerular filtration rate <30 ml/min); or heart failure (NYHA class II-IV).

Colonoscopy and biopsy sampling.

Prior to day of enrollment, all study participants performed bowel lavage including 24 hours fasting and intake of 10 mg sodium picosulphate (CitraFleet®, Casen Recorati, Madrid, Spain) 18 and 5 hours prior to colonoscopy examining the entire lower intestine including terminal ileum. All colonoscopies were performed by an experienced gastroenterologist (A.W.M.) using a video colonoscope (Olympus Europa SE & Co KG, Hamburg, Germany). During the procedure, sets of pinch biopsies were collected with a single-use oval cup biopsy forceps with needle (Endolaw 3.7 mm, Olympus Europa, Hamburg, Germany) from sigmoid colon and terminal ileum, respectively. Each set consisted of single biopsies preserved in 1.5 ml tubes with RNAlater (Thermo Fisher Scientific, Waltham, MA) and 20 biopsies pooled in a 50 ml Falcon tube with pre-cooled 30 ml RPMI m/L-glutamin (Cat.#12-702F, Thermo Fisher Scientific, Gothenburg, Sweden) with 10% fetal calf serum (FCS) (Thermo Fisher Scientific), and 1% Penicillin 100U/ml/Streptomycin 100μg/ml (Thermo Fisher Scientific) (RPMI/FCS/P/S).

Lamina propria mononuclear cell preparation.

After the 20 pooled mucosal biopsies had been left to rest in 30 ml ice-cold RPMI/FCS/P/S for 45 min the medium was carefully discarded and the biopsies washed twice in ice-cold RPMI/FCS/P/S. The
biopsies were then gently transferred by disposable pipette to a 15 ml tube with 10 ml RPMI/FCS/P/S pre-heated to 37°C with Collagenase blend type H (Final concentration 1mg/ml, Cat.#C8051, Sigma-Aldrich, Darmstadt, Germany) and DNase I (Final concentration 20U/ml, Cat.#18047019, Invitrogen, Carlsbad, CA) dissolved immediately prior to transfer of the biopsies. The tube was covered and incubated horizontally on a shaker at 250 rpm for 40 min in 37°C. All subsequent work was performed on ice/at 4°C. After incubation, remaining tissue was dissolved by mechanical disruption through a 18G blunt needle on a 20 ml syringe until the medium looked homogenously turbid. The cell suspension was then filtered through a 70µm cell strainer (Cat. # 352350, Corning, NY, NY) which had been primed with RPMI/FCS/P/S, to a sterile 50 ml tube. After the whole suspension was filtered the strainer was pestled with a sterile syringe piston and run through with ice-cold RPMI/FCS/P/S. The cell suspension was then split to two sterile 15 ml tubes, added with ice-cold RPMI/FCS/P/S to a total volume of 12 ml each, and centrifuged on 300 relative centrifugal force (rcf) for 15 min. Supernatants were removed by suction, cell pellets gently resuspended in 1 ml ice-cold RPMI/FCS/P/S, then added with 10 ml ice-cold RPMI/FCS/P/S, and centrifuged again on 300 rcf for 15 min. Supernatants were again removed by suction, cell pellets gently resuspended now in 0.5 ml ice-cold FCS w/ 50 % RPMI and cell suspensions from the two tubes pooled together in one tube. Twenty µl of cell suspension was collected for counting viable mononuclear cells. The suspension was then slowly diluted with FCS w/20 % DMSO (Cat.#1.02931, Sigma-Aldrich, St.Louis, MO) to final concentration 3-4 x10^6 cells/ml in FCS with 25 % RPMI and 10 % DMSO. The suspension was aliquoted to 1 ml/tube in freestanding 1.2 ml Cryogenic tubes (VWR International, Leuven, Belgium) set to freeze in a CoolCell box (Biocision, CA, USA) at -80°C, and transferred to and stored at -150°C freezer the following day.

**Peripheral mononuclear cells preparation.**

Blood was drawn into eight BD Vacutainer cell preparation tube (CPT) with Sodium Heparin 8ml (BD Biosciences, San Jose, CA) that within 2 hours were centrifuged at 1750 rcf for 17 min at ambient
temperature. The plasma fractions were discarded. The cell fractions were transferred to sterile 15 ml tubes, ice-cold RPMI added to a total volume of 13 ml, and the tubes were centrifuged at 250rcf for 15 min. Subsequently the supernatants were removed by suction and the cells resuspended in 500 µl fetal calf serum (FCS) with 50 % RPMI. Cells from four tubes were then pooled together. 2 ml of FCS with 20 % DMSO was then slowly added to the 2 ml suspension of cells in FCS w/50 ml RPMI by pipetting 50 µl of the FCS/DMSO solution every 3 second. The cells now suspended in FCS with 25 % RPMI and 10 % DMSO were aliquoted into four freestanding 1.2 ml Cryogenic tubes and set to freeze in a CoolCell box at -80°C and transferred to and stored at -150°C freezer the following day.

**Mitogen stimulation and flow cytometry.**

Vials of frozen lamina propria mononuclear cells (LPMC) were put in 37°C water bath and in a near-thawed state transferred to a sterile 15 ml tube containing 100 µl pre-heated DNase 30 U/ml in RPM/FCS/P/S (DNase/RPMI/FCS/P/S). Dropwise 1 ml of DNase/RPMI/FCS/P/S was added, cells resuspended, additional 14 ml DNase/RPMI/FCS/P/S was added, resuspended and the suspension centrifuged at 300 rcf for 14 min at ambient temperature. Supernatants were removed by suction and cell pellets gently resuspended in pre-heated 3 ml RPMI/FCS/P/S. After set to rest with tubes at a 45° angle for 12 hours at 37°C in a 5 % CO2-enriched incubator the cell suspension was thoroughly resuspended and filtered through a 70 µm cell strainer (Fanakoshi, Tokyo, Japan) into a sterile 15 ml tube. Seven ml of pre-heated RPMI/FCS/P/S was added, cells centrifuged at 300 rcf for 14 min at ambient temperature, supernatant removed by suction and cells resuspended in 360µl pre-heated RPMI/FCS/P/S.

The cell suspension was dispensed evenly into two round-bottom wells in a 96-wells plate where one of the two wells was added 10 µl 0.1 µg/ml phorbol12-myristate13acetate (PMA) (Cat.#P1585, Sigma-Aldrich, Saint Louis, MO) (end dilution 5 ng/ml) and 10 µl 20µg/ml ionomycin(Cat.#I3903, Sigma-Aldrich, Saint Louis, MO) (end dilution 1 µg/ml) and the other well added 20 µl vehicle (0.0675 µl DMSO/ml+RPMI/FCS/P/S ) and incubated at 37°C with 5 % CO2 . After 1 hour incubation the wells
were added 10 µl 0.1 mg/ml (end dilution 0.2 µl) BD Golgiplug (Cat # 555029, BD Biosciences, San Jose, CA) and the plate was incubated for another 12 hours.

After the 12-hour incubation the plate was centrifuged at 500 rcf for 5 min in ambient temperature, 120 µl supernatant pipetted off and 20 µl (end concentration 2mM) EDTA was added in each well and resuspended before 15 min incubation at room temperature. 100 µl PBS was added in each well and contents in each well mixed well. The plates were incubated 15 min at room temperature before cells in each well were resuspended and transferred to a V-bottomed 96-plate well. The new plate was centrifuged at 500 rcf for 5 min in room temperature and supernatants discarded. Cells in each well was washed twice by resuspending with PBS and centrifuge at 500 rcf for 5 in room temperature and discarding supernatants before being stained with 10 µl 1:400 dilution of Fixable Viability Dye (FixVia) eFlour 506 (Cat.#65-0866-14, eBioscience, Bleiswijk, The Netherlands) and subsequently added 50 µl surface antibody staining cocktail (See table below for antibodies and fluorochromes applied). The stained cells were incubated dark on a rocking shaker for 20 min at room temperature before 150 µl of PBS was added, the plate centrifuged at 500 rcf for 5 min at room temperature, and supernatants discarded. The cell pellet was then resuspended in 100 µl BD Cytofix/Cytoperm (Cat.#554722, BD Biosciences, San Jose, CA) and incubated dark for 20 min before being resuspended in 100 µl 1:10 BD Perm/Wash buffer (Cat.#554723, BD Biosciences, San Jose, CA), centrifuged at 500 rcf for 5 min in room temperature, and supernatant discarded. Cells were then resuspended in residual volume, 50 µl intracellular antibody staining cocktail added (See table below for antibodies and fluorochromes applied), incubated dark for 20 min in room temperature, added 150 µl BD wash/perm buffer, centrifuged at 500 rcf for 5 min at room temperature, and supernatant discarded. The cell pellet was then resuspended in 200 µl 1% BD Cellfix (Cat.#340181, BD Biosciences, San Jose, CA) and assessed on a BD FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer. PBMCs collected at one time point from one healthy donor were prepared, frozen and thawed as the LPMCs and used as longitudinal control on all plates. Flow cytometry data was analyzed on FlowJo Software (for Mac, version 10.5.3, Ashland, OR). Th22, Th17 and Th1 cells were identified as CD45^"Live
lymphoid singlets CD3⁺gdTCR⁺CD8⁺/IL22⁺/IL17⁺/IFN⁺, respectively (Fig. 3a). The gate was set on the unstimulated control and applied on the stimulated sample for each study subject. The percentage of cytokine positive cells were reported as net values by subtracting the percentage of positive cells in unstimulated wells.

Table of antibodies for flow cytometry staining assays

| Antibody | Clone | Fluorochrom | Cat.# | Company | City | State/Country |
|----------|-------|-------------|-------|---------|------|--------------|
| CD45     | 2DI   | APC-Fire    | 368517| BioLegend | San Diego | California/USA |
| FixVia   |       | eFlour 506  | 65-0866-14 | eBioscience/Thermo Fisher | Bleiswijk | The Netherlands |
| CD3      | OKT3  | PerCP-Cy5.5 | 317335/6 | BioLegend | San Diego | California/USA |
| INFg     | B27   | APC         | 55787 | BD Biosciences | San Jose | California/USA |
| CD8      | RPA-T8 | PE-CY7     | 301012 | BioLegend | San Diego | California/USA |
| gdTCR    | 11F2  | FITC        | 347903 | BD Biosciences | San Jose | California/USA |
| IL-17    | N49-653 | BV421     | 562933 | BD Biosciences | San Jose | California/USA |
| IL-22    | 22URTI| PE         | 12-7229-42 | eBioscience/Thermo Fisher | Bleiswijk | The Netherlands |
| CD3      | UCHT1 | PerCP       | 300428 | BioLegend | San Diego | California/USA |
| CD4      | SK3   | Alexa Fluor 700 | 344622 | BioLegend | San Diego | California/USA |
| CD38     | HIT2  | Alexa Fluor 488 | 303512 | BioLegend | San Diego | California/USA |
| HLA-DR   | G46-6 | Brilliant Violet 711 | 563696 | BD Biosciences | San Jose | California/USA |
| 7AAD     |       | PE-Cy5     | 559925 | BD Biosciences | San Jose | California/USA |
| CD8a     | RPA-T8 | Alexa Fluor 700 | 301028 | BioLegend | San Diego | California/USA |
| EpCAM    | 9C4   | Brilliant Violet 650 | 324226 | BioLegend | San Diego | California/USA |

**Soluble markers.**

Plasma and serum tubes were centrifuged according to manufactures’ instruction and frozen at -80°C. Upon analyses, serum and plasma were thawed in 37°C for 5 minutes before being analyzed at room temperature. Coefficients of variation (CV) in our laboratory are given in parenthesis.

Commercial enzyme-linked immunosorbent assays (ELISA) were used for high sensitivity CRP (DRG instruments, Marburg, Germany; CV 5.4%), IFABP and LBP (Hycult Biotech, Uden, The Netherlands; CV 8.2% and 7.6%, respectively), Zonulin (Immune Diagnostic, Bensheim, Germany, CV 12.5%), sCD14, sCD25, sCD163, high sensitivity IL-6, human REG3A (R&D Systems, Abington, Oxon, UK; CV 6.5%, 2.6%, 4.1%, 5.8% and 3.2, respectively), IL-18 (MBL, Medical & Biological Laboratories CO., LTD., Nagoya, Japan; CV 2.6%) and D-dimer (Asserachrom Diagnostica Stago, Asnières, France; CV
6.7%). LPS was analyzed by Pyrochrome Diazo kit (Cape Cod, East Falmouth, MA), according to the manufacturer's instructions, CV 15.0%.

**16S rRNA gene sequencing.**

**Sample collection.** The day before enrollment, prior to initiating bowel lavage, study participants collected stool samples in Stool Collection Tubes with Stool DNA Stabilizer (Cat.#1038111300, Stratec Molecular GmbH, Berlin, Germany), using a standardized stool collection device after voiding. (Ahlquist DA et al, *Ann Intern Med.* 1988). The tubes were subsequently, within 24 hours, transported in room temperature to the enrollment procedure and frozen at -80°C on arrival. During the colonoscopy individual gut mucosal biopsies immediately submerged in 1.5 ml RNAlater® (Cat.#AM7020, Thermo Fisher Scientific, Waltham, MA) were incubated for 24 hours at 4°C before being stored at -80°C until DNA extraction.

**DNA extraction and sequencing.** DNA in stool samples were extracted using the PSP Spin Stool DNA Plus Kit (Cat.#103811200, Stratec Molecular GmbH, Berlin, Germany), according to the manufacturer instructions. Total RNA and DNA was extracted from colonic mucosal tissue using the AllPrep DNA/RNA Mini Kit (Cat.#80204, Qiagen, Hilden, Germany). The extraction was performed according to the manufacturer’s instructions, with the exception of the lysis procedure, where an extended protocol including enzymatic and mechanical lysis was used (Moen AEF et al. *BMC Res Notes.* 2016). RNA was eluted in 40 µl nuclease free water (NFW), snap frozen and stored at -80 °C. RNA quality and quantity were verified on Bioanalyzer 2100 electrophoresis chips (Bioanalyzer RNA 6000 pico assay, Agilent, Santa Clara, CA). DNA was eluted in 100 µl elution buffer and stored at -80 °C. DNA concentration for all samples were measured by NanoDrop (Thermo Fisher, Waltham, MA) and quality of DNA samples was verified on precast agarose gels (Thermo Fisher, Waltham, MA).

DNA libraries were generated by targeting the V3-V4 region of the 16S ribosomal RNA gene by polymerase chain reaction (PCR) according to an established protocol (Fadrosh DW et al, *Microbiome* 2014). A unique combination of dual-indexed primers (319F and 806R) were applied with Phusion
High-Fidelity PCR Master Mix with HF buffer (Cat.#F531L, Thermo Fisher, Waltham, MA). SequiPrep Normalization Plate Kit (Cat.#A1051001, Thermo Fisher, Waltham, MA) was used for purification and normalization of PCR products, before quality control was performed on pooled libraries using Bioanalyzer 2100 electrophoresis chips (Agilent, Santa Clara, CA). Libraries were then submitted to the Norwegian Sequencing Centre, Oslo, Norway and sequenced on the Illumina MiSeq platform using the v3 kit (Illumina, San Diego, CA).

**Sequence processing, bioinformatics and statistics.** Reads containing Illumina Universal Adapters or PhiX were discarded using bbduk version 37.75 (BBTools, https://jgi.doe.gov/data-and-tools/bbtools/) (parameters adaptor filter: k=23 hdist=1 tbo cf=TRUE ftm=5. parameters phix filter: k=31 hdist=1) and the remaining reads were demultiplexed using je version 1.2 (Girardot C et al. *BMC Bioinformatics*. 2016) (parameters: MAX_MISMATCHES=1 MIN_MISMATCH_DELTA=2). Indexes, heterogeneity spacers and primers were trimmed with cutadapt version 1.14 (Martin M. *EMBnet J.* 2011) (parameter: --overlap 20 --discard-untrimmed -m 200) and the paired-end reads were subsequently quality trimmed and merged using bbmerge version 37.75 (Bushnell B et al. *PLoS One* 2017) (parameters: qtrim=r trimq=15 maxlength=440 mininsert=390 mininsert=390 --loose 2). The merged contigs were quality filtered using default values in Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1.(Caporaso JG et al. *Nat Methods*. 2010). Closed reference operational taxonomic unit (OTU) mapping to the Silva database (version 128, reference OTUs pre-clustered at 97% sequence similarity)(Quast C et al. *Nucleic Acids Res*. 2013) was performed using SortMeRNA (Kopylova E et al. *Bioinformatics*. 2012) version 2.0 through QIIME (Bolyen E et al. *Nat Biotechnol*. 2019).

OTUs with a number of sequences <0.005% of the total number of mapped sequences were discarded as recommended (Bokulich S et al. *Nat Methods*. 2013). The mucosal samples were rarefied (subsampled) to an OTU count of 8362 per sample, while fecal samples were rarefied to 8708 reads per sample. All further analyses were performed on this rarefied dataset.
Calculation of alpha diversity metrics (Shannon diversity index and observed OTUs), and beta
diversity metrics (weighted UniFrac) was done in QIIME2 (version 2018.4 and 2019.10, respectively)
(Bolyen E et al. Nat Biotechnol. 2019).

The R statistical environment (https://www.r-project.org/) was used to calculate and visualize taxon-
variable interactions, taxa abundances, as well as beta diversity differences between patient groups.
Alpha diversity was compared in Prism 8 (GraphPad Software, La Jolla, CA). Beta diversity was
compared with permutational multivariate analysis of variance (PERMANOVA) in R. Analyses of
differences in relative abundance between phenotypes was performed in LEfSe (Segata N et al.
Genome Biol. 2011), and subsequent calculation of false-discovery rate according to Benjamini-
Hochberg was done using R. The R packages ggplot2 (Wickham H. Springer-Verlag New York 2016)
and gridExtra (Auguie B. 2017) were used to create the visualizations.