Gut Dendritic Cell Activation Links an Altered Colonic Microbiome to Mucosal and Systemic T Cell Activation in Untreated HIV-1 infection.

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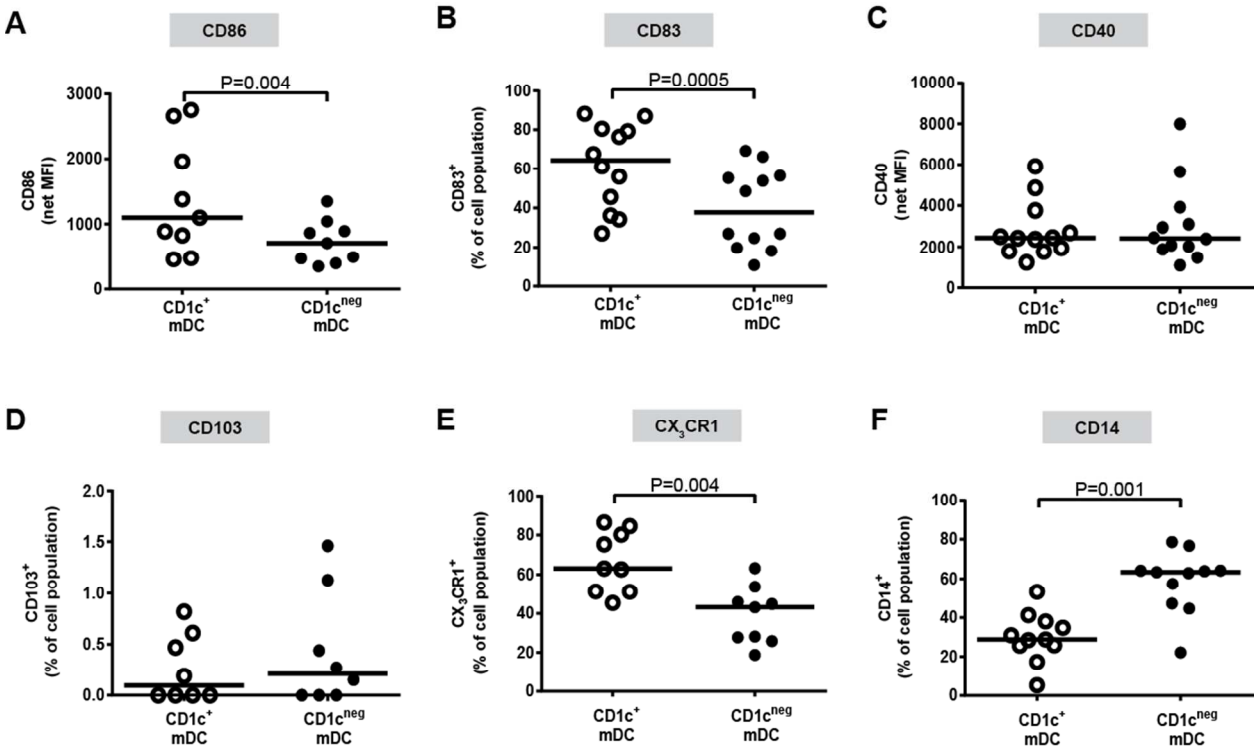
SUPPLEMENTARY RESULTS

Defining two distinct populations of colonic LP mDCs

In initial studies, we utilized an *ex vivo* colon LPMC model from uninfected donors[1-4] that provided high numbers of LPMC to extensively characterize human LP mDC subsets. Colonic LPMC consisted of two phenotypically distinct colonic LP mDC subsets, both of which expressed HLA-DR and CD11c, but were delineated by expression of CD1c (hereafter referred to as $CD1c^+$ mDCs, $CD1c^{neg}$ mDCs). Colon $CD1c^+$ mDCs have an increased activation profile characterized by an increased maturation (CD83) and co-stimulatory molecule (CD86) expression relative to $CD1c^{neg}$ mDCs (**Supplementary Figure 1a, b**). Both subsets express similar levels of CD40 (**Supplementary Figure 1c**). In agreement with our previous work, colonic $CD1c^+$ mDCs had minimal expression of CD103[2] and similar low expression was observed in $CD1c^{neg}$ mDCs (**Supplementary Figure 1d**).

Murine studies initially implicated CX_3CR1 as a crucial molecule involved in the ability of intestinal DCs to extend dendrites into the gut lumen and sample luminal contents[5]. A higher fraction of $CD1c^+$ mDCs expressed CX_3CR1 compared to $CD1c^{neg}$ mDCs (**Supplementary Figure 1e**), suggesting that $CD1c^+$ mDCs are more likely to sample luminal contents and that greater exposure to bacteria may thus explain their more activated profile. However, recent studies have suggested that high expression of CX_3CR1 preferentially defines a subset of murine macrophages,[6,7] and human colonic DCs obtained via a cell migration/"walkout" method lacked CX_3CR1 expression[8]. Intestinal resident tissue macrophages have been reported to lack expression of CD11c[9] and our gating strategy defines mDCs as $CD11c^+$ thereby excluding macrophages from our $CD1c^+$ and $CD1c^{neg}$ cell populations. To provide further support that the $HLA\text{-}DR^+CD11c^+$ cells would not contain tissue macrophages, we utilized histological techniques to stain colon biopsies with the macrophage-specific marker Ham56 in conjunction with CD11c and observed that greater than 90% of $Ham56^+$ cells (ie. macrophages) did not co-express CD11c (n=5; data not shown).

It has been proposed that recruitment of circulating blood monocytes replenishes senescent and apoptotic intestinal macrophages.[9] We evaluated expression of CD14 on both colon mDC subsets and observed a higher fraction of CD14-expressing $CD1c^{neg}$ mDCs than $CD1c^+$ mDCs (**Supplementary Figure 1f**), suggesting that $CD1c^{neg}$ mDCs may contain newly recruited monocytes with the potential to differentiate into tissue DCs [10] or macrophages [9,11].



Supplementary Figure 1. Phenotypic characterization of human colonic lamina propria (LP) myeloid DC (mDC) subsets. Multi-color flow cytometry techniques were used to evaluate the expression of various cell surface markers on colonic LP CD11c+HLA-DR+ mDCs, delineated into two subsets based on CD1c expression 19. CD1c+ mDCs (open circles) and CD1cneg mDCs (closed circles) were assessed for expression of (A) CD86 (n=9), (B) CD83 (n=12), (C) CD40 (n=12), (D) CD103 (n=8), (E) CX3CR1 (n=9) and (F) CD14 (n=11). Values are shown as either expression levels (Mean Fluorescence Intensity; MFI) for (A) CD86 and (C) CD40 indicative of a population shift in expression or shown as the percent of each mDC subset positive for the cell surface marker (B) CD83, (D) CD103, (E) CX3CR1 and (F) CD14 indicative of distinctive expression on a specific population of cells in each mDC subset. Appropriate isotype controls were removed to control for background staining (net). Lines represent median values and statistical analysis was performed using the Wilcoxon matched-pairs signed rank test.

SUPPLEMENTARY TABLES.

Supplementary Table S1. Colon DC frequencies.

	Uninfected subjects (n=10)	HIV-infected subjects (n=19)	P value*
$CD1c^+$ mDC	0.053%	0.028%	0.06
(% of viable leukocytes)	(0.023-0.167%)	(0.005-0.210%)	
$CD1c^{neg}$ mDC	0.319%	0.236%	0.77
(% of viable leukocytes)	(0.169-0.667%)	(0.088-1.030%)	
$CD303^+$ pDC	0.058%	0.054%	0.78
(% of viable leukocytes)	(0.031-0.154%)	(0.019-0.207%)	

Values are shown as median (range). *Statistical analysis was performed using the Mann-Whitney test.

Supplementary Table S2: Colon and blood T cell activation

	Uninfected subjects (n=13)	HIV-1 infected subjects (n=24)	P value*
Colon $CD38^+HLA\text{-}DR^+$ CD4 and CD8 T cells			
% of colon CD4 or CD8 T cells			
CD4 T cells	0.5%	3.2%	<0.0001
	(0.1-1.5%)	(0.8-18.1%)	
CD8 T cells	1.4%	8.4%	<0.0001
	(0.3-6.9%)	(1.9-34.6%)	
Number per gram of mucosal tissue ($x10^6$)			
CD4 T cells	0.03	0.06	<0.0001
	(0.002-0.8)	(0.01-0.9)	
CD8 T cells	0.02	0.4	<0.0001
	(0.005-0.05)	(0.05-2.5)	
Peripheral blood $CD38^+HLA\text{-}DR^+$ CD4 and CD8 T cells			
% of peripheral blood CD4 or CD8 T cells			
CD4 T cells	0.9%	2.8%	<0.0001
	(0.5-2.6%)	(0.6-10.4%)	
CD8 T cells	1.7%	15.1%	<0.0001
	(0.4-4.6%)	(4.8-38.5%)	

Values are shown as median (range). *Statistical analysis performed using the Mann Whitney test.

Supplementary Table S3: Mucosal cytokine-producing T cell frequencies

	Uninfected subjects (n=10)	HIV-1 infected subjects (n=22)	P value*
IFN-γ (Th1), IL-17 (Th17) and IL-22 (Th22) CD4 T cells			
% of colon CD4 T cells			
IFN-γ^+ CD4 T cells	51.3% (43.4-59.7%)	39.8% (25.4-70.0%)	0.06
IL-17^+ CD4 T cells	10.2% (43.4-59.7%)	3.2% (0.7-24.3%)	<0.0001
IL-22^+ CD4 T cells	14.9% (7.9-21.9%)	2.3% (0.2-26.1%)	<0.0001
Number per gram of mucosal tissue (x10^4)			
IFN-γ^+ CD4 T cells	169.3 (104.8-248.2)	114.4 (19.5-432.2)	<0.05
IL-17^+ CD4 T cells	31.5 (15.4-82.8)	9.3 (2.5-29.8)	<0.0001
IL-22^+ CD8 T cells	45.7 (25.6-75.5)	7.3 (0.9-39.5)	<0.0001
IFN-γ CD8 T cells			
% of colon CD8 T cells			
IFN-γ^+ CD8 T cells	76.6% (60.5-88.7%)	91.1% (77.6-97.9%)	<0.0001
Number per gram of mucosal tissue (x10^4)			
IFN-γ^+ CD8 T cells	79.5 (42.8-162.3)	405.7 (2.36-1670.0)	<0.0001

Values are shown as median (range). *Statistical analysis performed using the Mann Whitney test.

Supplementary Table S4. Comparison between HIV-altered mucosal bacteria (HAMB) species with the same species detected in stool samples

	Mucosal tissue	Stool[#]
Proteobacteria phylum		
Acinetobacter junii		
Schlegelella thermodepolymerans		
Bacteroidetes phylum		
Prevotella copri		
Prevotella stercorea		
Prevotella oris		P=0.063
Bacteroides stercoris		
Bacteroides thetaiotaomicron		
Bacteroides dorei		
Bacteroides acidifaciens		
Alistipes putredinis		
Barnesiella intestinihominis		
Firmicutes phylum		
Blautia luti		
Blautia glucerasei		P=0.0845
Blautia schinkii		
Blautia product		
Rumminococcus bromii		P=0.0854
Ruminococcus gnavus		
Clostridium saccharogumia		P=0.0561
Clostridium xylanovorans		
Acidaminococcus intestini		
Bacterodies cellulosolvens		

Statistical analysis: Mann-Whitney test to compare the abundance of each bacterial species in uninfected subjects (n=14) with HIV-1-infected subjects (mucosal tissue n=17; stool n=18). All species listed were statistically different between uninfected and HIV-1-infected subjects in mucosal tissue. RED = statistically higher in HIV-1-infected subjects; BLUE = statistically lower in HIV-1-infected subjects; GREY = no statistical difference between uninfected and HIV-1-infected subjects. [#]: A total of 45 species identified in the stool were statistically different between uninfected and HIV-1-infected subjects.

Supplementary Table S5: Percent of cytokine$^+$ CD1c$^+$ mDC in response to *in vitro* stimulation with HAMB

	Cytokine$^+$ CD1c$^+$ mDC (% of total CD1c$^+$ mDC)			
	No exogenous stimulation	***P. copri***	***P. stercorea***	***R. bromii***
IL-23	0.25 (0-0.70)	4.07 (0.85-13.9)$^{\#}$*	5.91 (2.42-20.7)*	1.69 (0.21-8.57)*
IL-1β	1.85 (0-4.25)	34.1 (15.4-59.2)*	22.8 (7.57-46.3)*	13.1 (8.07-36.0)*
IL-10	0 (0-0.85)	18.0 (10.7-24.2)*	7.15 (1.79-9.87)*	2.93 (1.58-8.45)*

Values are shown as median (range). $^{\#}$Statistical analysis performed using the Wilcoxon matched-pairs signed rank test comparing percent of cytokine$^+$ CD1c$^+$ mDC in HAMB-stimulated conditions with unstimulated control. *p=0.03

SUPPLEMENTARY MATERIALS AND METHODS

Study exclusion criteria.

Study exclusion criteria are extensively detailed elsewhere.[12] For HIV-1-infected subjects included 1) presence of AIDS-defining condition within 6 months and a CD4 count <200cells/μl within 3 months of clinic visit, 2) HIV-1 plasma viral load <2000 HIV-1 RNA copies/ml. Exclusion for all subjects included 1) medical history of a bleeding disorder, rectal bleeding or surgical history of left-sided colonic, rectal or anal resection or anastomosis, 2) medical history of inflammatory bowel disease or other intestinal inflammatory disorders, 3) chronic or acute medical conditions such as cancer, heart disease, diabetes, or hepatitis, 4) bacterial infection requiring antibiotic treatment in the 3 months prior to clinic visit and 5) continuous for more than 3 days within the past 60 days of immunosuppressives, immune modulators or probiotics.

Collection, storage and processing of clinical samples.

Collection, storage and processing of samples are extensively detailed in a previous publication.[12] Briefly, rectal swabs were collected at the time of study screening (typically 2 weeks prior to flexible sigmoidoscopy procedure) and immediately stored in liquid nitrogen. On the day of the clinic visit, all subjects had been fasting for at least 12hrs and underwent an enema (Sunmark McKesson, San Francisco, CA) before undergoing a flexible sigmoidoscopy using 2.3mm standard forceps. Twenty biopsies were placed in HBSS for immediate tissue digestion. Biopsies for tissue HIV-1 viral load assessment and DNA isolation were placed in RNAlater (Ambion, Austin, TX) (n=4 per subject) for 20-24hrs, snap frozen and stored in liquid nitrogen. Three biospies were placed in 10% neutral buffered formalin for 20-24hrs prior to being embedded into paraffin.

Peripheral blood samples were collected into BD Vacutainer Blood Collection Tubes containing EDTA or Heparin (BD Biosciences; San Jose, CA). For serum collection, peripheral blood was collected into BD Vacutainer Blood Collection Tubes containing a clot activator ((BD Biosciences; San Jose, CA).

Processing of colon biopsies, plasma, serum and PBMC.

Processing of colon biopsies, plasma, serum and PBMC are extensively detailed elsewhere[12]. Briefly, twenty colon pinch biopsies were washed in HBSS and colonic cells released via a tissue digestion protocol using Collagenase D (Roche, Indianapolis, IN). Whole blood was centrifuged to isolate plasma. For serum collection, peripheral blood was left at RT for 60min prior to centrifugation. Plasma and serum were frozen at -80°C in single-use aliquots. PBMC were isolated from heparinized blood by standard Ficoll-Hypaque (GE Healthcare, Piscataway, NJ) density gradient centrifugation, cryopreserved and stored in liquid nitrogen.

Mucosal and plasma cytokine measurements.

Colon single cell suspensions were cultured at $2x10^5$ cells/100μl for 16hrs in cRPMI without exogenous stimulation. Mucosal cytokine levels were measured using a Custom Q-Plex Array (Quansys Biosciences, Logan, UT) following the manufacturers protocol. Specifically, levels of IL1β, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-15, IL-17, IL-23, IFN-γ and TNF-α were evaluated. Only TNF-α, IFN-γ and IL-10 were detectable at levels above the assay limit of quantification. Measurement of plasma cytokine levels were performed using the Human Cytokine High Sensitivity Screen (IL-1α, IL-1β, IL-2, IL-4, IL-5IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23,

IFN-γ, TNF-α, TNF-β) (Quansys Biosciences) following the manufacturer's protocol. Levels of plasma IL-6 were evaluated in EDTA plasma samples using a commercially available ELISA (R&D Systems) as previously reported.[12]

Histological staining and analysis of colonic biopsies.

Assessment of microbial product levels, CD11c$^+$ mDCs, and HAM56$^+$ macrophages in colonic LP: Formalin fixed paraffin embedded human colon biopsy tissue was cut into 7μm thick sections. Tissue sections then underwent paraffin removal, rehydration and heat mediated antigen retrieval at 95ºC using pH9 TRIS/EDTA/Tween20 buffer. Tissue samples then underwent a two step staining procedure; first for CD11c (abcam, Cambridge, United Kingdom) and LTA (Thermo, Waltham, MA) or LPS (Hycult, Plymouth Meeting, PA), anti-Goat Alexa488 (Molecular Probes, Eugene, OR) was used to detect CD11c and anti-mouse Alexa647 (Molecular Probes) was used to detect LTA and/or LPS. Tissue samples were then blocked with 2% Normal Goat Serum then stained with a primary antibody against HAM56 (ENZO Life Sciences, Farmingdale, NY). HAM56 was detected using anti-mouse Alexa555 (Molecular Probes). Fluorescent staining was preserved using Invitrogen Prolong Gold with DAPI.

Images were acquired using a Zeiss LSM510 META confocal microscope, at least ten 3x3 tiled 63x images were acquired for each biopsy, and three biopsies per patient were analyzed. Zeiss Zen Software (Jena, Germany) was used to create specific regions of interest only around the LP for analysis. Using the software the total area and the area that stained with LTA/LPS was calculated within the LP. All regions of interest for an individual patient were combined and the percentage of the LP containing microbial products (LTA/LPS) was calculated. To analyze whether microbial products preferentially associated with mDCs or macrophages the total number of mDCs and macrophages that either did or did not associate with microbial products (LTA/LPS) were enumerated per square millimeter of LP using Image J Software (NIH Bethesda, MD). mDCs association with microbial products was defined as cells that costained with CD11c and LTA/LPS, while macrophage association with bacterial products was defined as cells that costained with HAM56 and LTA/LPS.

Assessment of mononuclear infiltration: Three sections were cut at regular intervals from each colon biopsy tissue block, stained with hematoxylin and eosin (H&E), and evaluated by light microscopy. Evaluation was performed by a gastrointestinal pathologist who was blinded to the HIV-1 status of each patient. Mononuclear infiltrate was assessed as the relative cellularity of the LP infiltrate consisting of lymphocytes, plasma cells, eosinophils and occasional neutrophils, in addition to presence or absence of circumscribed lymphoid aggregates. The degree of mononuclear infiltration was quantified on a scale of 0 = Not present, Minimal = 0.5, Mild = 1, Moderate = 2, and Severe = 3. The scores of 3 sections of each colon biopsy were then averaged to obtain an overall score.

***In vitro* mitogenic stimulation of single-cell colon biopsy preparations.**

Evaluation of frequencies of colonic CD4 T cells capable of producing IFN-γ (Th1), IL-17 (Th17) or IL-22 (Th22) and frequencies of IFN-γ-producing CD8 T cells after in vitro have been extensively detailed elsewhere[1,12]. Briefly, isolated colon cells were cultured in RPMI + 10% human AB serum (Gemini Bioproducts, West Sacramento, CA) + 1% penicillin/streptomycin/L-glutamine (Sigma-Aldrich, St Louis, MO) (cRPMI) and stimulated with 250ng/ml PMA and 1μg/ml ionomycin (both Sigma-Aldrich) in the presence of Brefeldin A (1μg/ml, BD Biosciences) for 16hrs at 37°C, 5% CO2. Cells were collected and frequencies of cytokine$^+$ T

cells determined by intracellular flow cytometry assay. In some instances, numbers of colon cells obtained after collagenase-digestion were insufficient to perform this assay.

***Ex vivo* LPMC assay system.**
Phenotypic characterization of colonic LP mDC subsets and assessment of functional responses of $CD1c^+$ mDCs to commensal bacteria (see below) utilized a previously detailed *in vitro* tissue culture model consisting of isolated colon LPMC [1-4] Briefly, intestinal tissue samples were obtained from adult patients undergoing elective abdominal surgery represent otherwise discarded tissue from surgical anastomic junctions considered macroscopically normal. Patients with a history of inflammatory bowel disease or receiving chemotherapy, radiation, or other immunosuppressive drugs were excluded. Patients undergoing surgery signed a release allowing the unrestricted use of discarded tissues for research purposes. Protected patient information was de-identified to laboratory personnel, and usage of these samples was granted exempt research status by COMIRB at the University of Colorado Anschutz Medical Campus. LPMC were isolated by a two-step procedure involving removal of epithelial cells followed by collagenase digestion of the remaining tissue to yield LPMC [1-4].

***In vitro* exposure of LPMC to commensal bacteria.**
Isolated colon LPMC were cultured at 37°C, 5% CO2 in RPMI + 10% AB serum + 1% L-glutamine (Life Technologies, Grand Island, NY) at$1x10^6$ cells/ml with live *Prevotella copri*, *P. stercorea* or *Rumminococcus bromii* (2.5 bacteria:1 LPMC) or without exogenous stimuli for 2hrs prior to the addition of 1% penicillin/streptomycin (Life Technologies) + 500μg/ml piperacillin/tazobactum (Zosyn; Wyeth, Madison, NY). Brefeldin A (1μg/ml) was added 2 hours later. LPMC were collected after an additional 16-18hrs and frequencies of $cytokine^+$ $CD1c^+$ mDCs determined by intracellular flow cytometry assay (see below).

Commensal bacteria stocks.
P. copri (DSM# 18205, DSMZ, Braunschweig, Germany), *P. stercorea* (DSM# 18206) and *R. bromii* (ATCC# 27255, ATCC Manassas, VA) were expanded at 37°C under anaerobic conditions achieved by using a BD GasPak EZ Anaerobe Pouch System (BD Diagnostics) per manufacturer's protocols with slight modifications. Specifically, *P. copri* was expanded by culturing on Brucella plates (BD Diagnostics) for 5-7 days; *P. stercorea* was expanded in liquid chopped meat broth (BD Biosciences; Hardy Diagnostics, Santa Maria, CA) supplemented with 1% Trace Minerals (ATCC), 1% Vitamin Supplements (ATCC), 0.05% Tween80, 29.7mM acetic acid, 8.1mM propionic acid and 4.4mM butyric acid (all Sigma-Aldrich) for 5-7 days; *R. bromii* was expanded in liquid chopped meat broth (BD Biosciences; Hardy Diagnostics) for 1-2 days. All bacteria were stored long-term at -80°C in 10% glycerol. To prepare working stocks, bacteria were expanded from long-term stocks as described above, resuspended in DPBS and stored at -80°C in single-use aliquots. Bacterial enumeration was performed using the BD Cell Viability and Counting Kit (BD Biosciences). Bacteria were diluted 1:100 in PBS and mixed with a known number of BD Counting beads. Samples were then acquired on an LSRII Flow Cytometer (BD Biosciences) and the concentration of total bacteria in the sample was back calculated based on the BD Counting Beads.

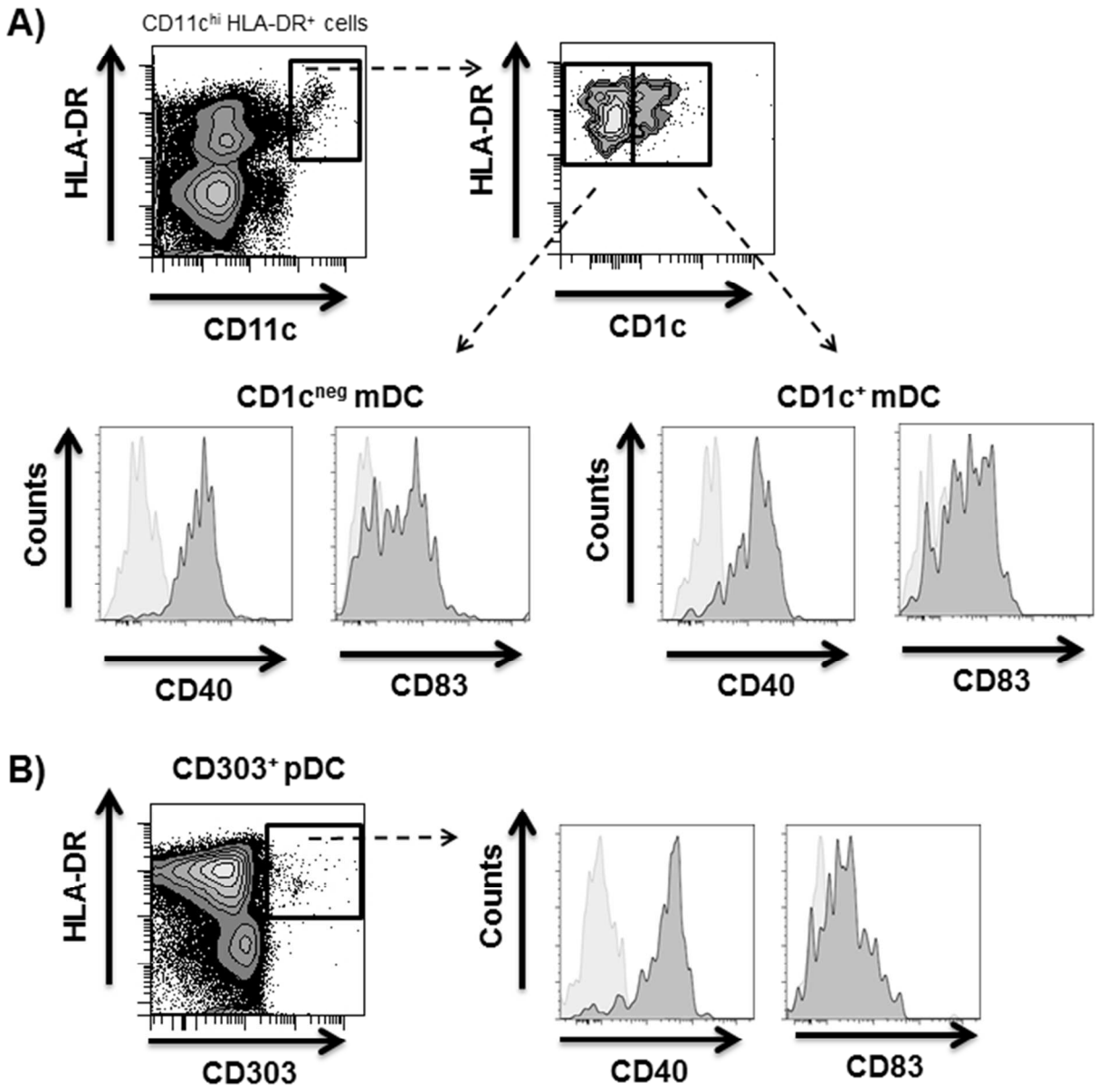
Surface and intracellular flow cytometry staining assays, acquisition and analysis.
Multi-color flow cytometry protocols to evaluate colon and blood DC and T cell frequencies and activation status and to determine DC and T cell cytokine frequencies have been previously detailed[1,2,12]. All antibodies and dyes are listed in Supplementary Table S6. To identify viable, mononuclear cells and exclude epithelial cells, cells were first gated on CD45 expression and then viable cells within this population determined.
Colon cells isolated from biopsies and PBMC: Colonic mDC subsets were defined within an HLA-DR$^+$ CD11c^{hi} population to exclude B cells and macrophages[9] with specific CD1c (BDCA-1) expression (CD1c$^+$, CD1c^{neg}) determined using an isotype control as previously described.[2,12] pDCs were defined as within CD3$^-$ cells as BDCA-2 (CD303)$^+$ using a matched isotype control.[2] Activation was determined by evaluating the level of specific CD40 expression or the percent of CD83$^+$ DCs using an isotype contro1. Specific gating strategies and activation expression profiles are illustrated in Supplementary Figure 2. Evaluation of DC frequencies and degree of activation were only performed when there were at least 25 CD1c$^+$, CD1c^{neg}, or pDC events. Colonic and blood T cells were identified from within a CD3$^+$γδ TCR$^-$ population to exclude intestinal γδ T cells that express CD4 and CD8.[12] To determine activated colon CD4 and CD8 T cells, the combination of antibodies was altered slightly to accommodate other fluorochrome-labeled antibodies and a specific CD4 antibody was excluded from the antibody cocktail. CD4 T cells were identified as CD3$^+$CD8$^-$ and T cell activation evaluated by determining expression of HLA-DR in combination with CD38 or matched isotype control. Peripheral blood T cells were identified as CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$. Frequencies of colon Th1, Th17 and Th22 cells were determined using IFN-γ, IL-17 and IL-22 antibodies with matched isotype controls (Supplementary Table 2).
Colon LPMC from normal donors: LP CD1c$^+$ mDCs and CD1c^{neg} mDCs were identified in viable, CD45$^+$ LPMC using the same antibodies and viability dye as described above. Levels of CD40 and CD86 were evaluated as well as frequencies of CD83- CD103-, CX_3CR1- or CD14-expressing CD1c$^+$ or CD1c^{neg} mDC were determined using antibodies and appropriately matched isotype controls as detailed in Supplementary Table 7. Enumeration of cytokine$^+$ CD1c$^+$ mDCs following *in vitro* stimulation of LPMC were performed following previously described multi-color intracellular cytokine flow cytometry staining techniques[2,13,14] using antibodies detailed in Supplementary Table S7. Production of IL-23 was defined by co-expression of IL-12p40 and p19.[13,14]

Supplementary Table S6. Antibodies and dyes used to determine DC and T cell frequencies, activation and cytokine production in colon biopsies and PBMC.

	Clone	Company	Location
Viable, mononuclear cell identification			
PerCpCy5.5 CD45	2D1	eBioscience	San Diego, CA
Aqua Live/Dead Fixable Viability dye		Invitrogen	Carlsbad, CA
DC identification and assessment of activation			
APC BDCA-1 (CD1c)	AD5-8E7	Miltenyi Biotec	Auburn, CA
APC mouse IgG2a isotype control	S43.10		
APC-Cy7 HLA-DR	L243	BD Biosciences	San Jose, CA
PE-Cy5 CD11c	B-ly6	BD Biosciences	
FITC BDCA-2 (CD303)	AC144	Miltenyi Biotec	
FITC mouse IgG1 isotype control	IS5-21F5		
Biotinylated CD40	EA-5	Ancell	Bayport, MN
Biotinylated mouse IgG1 isotype control	MOPC-31C		
Streptavidin ECD (PE-Texas Red)		Beckman Coulter	Fullerton, CA
PE CD83	HB15e	BD Biosciences	
PE mouse IgG1	MOPC-21		
Colon T cell identification and assessment of activation			
eFluor^{450} CD3	OKT3	eBioscience	
AF700 CD4	RPA-T4	BD Biosciences	
PE-Cy7 CD8	RPA-T8	BD Biosciences	
PE γδ TCR	11F2	BD Biosciences	
APC-Cy7 HLA-DR	L243	BD Biosciences	
AF700 CD38	HIT2	eBioscience	
AF700 mouse IgG1 isotype control	P3.6.2.8.1		
Blood T cell identification and assessment of activation			
eFluor^{450} CD3	OKT3	eBioscience	
PE-Cy7 CD8	RPA-T8	BD Biosciences	
APC-Cy7 HLA-DR	L243	BD Biosciences	
APC-Cy7 mouse IgG2 isotype control	G155-178		
AF700 CD38	HIT2	eBioscience	
AF700 mouse IgG1 isotype control	P3.6.2.8.1		
Colon T cell identification and assessment of cytokine production			
ECD CD3	UCHT1	Beckman Coulter	
APC-Cy7 CD4	RPA-T4	BD Biosciences	
PE-Cy7 CD8	RPA-T8	BD Biosciences	
FITC γδ TCR	11F2	BD Biosciences	
AF700 IFN-γ	B27	BD Biosciences	
AF700 mouse IgG1 isotype control	MOPC-21		
V^{450} IL-17	N49-653	BD Biosciences	
V450 mouse IgG1 isotype control	MOPC-21		
PE IL-22	22URTI	eBioscience	
PE mouse IgG1 isotype control	P3.6.2.8.1		

Supplementary Table S7. Antibodies and dyes used to determine CD1c^{+} and CD1c^{neg} mDC frequencies, activation and cytokine production in LPMC from normal donors.

	Clone	Company	Location
Viable, mononuclear cell identification			
PerCpCy5.5 CD45	2D1	eBioscience	San Diego, CA
Aqua Live/Dead Fixable Viability dye		Invitrogen	Carlsbad, CA
mDC subset identification and assessment of activation			
APC BDCA-1 (CD1c)	AD5-8E7	Miltenyi Biotec	Auburn, CA
APC mouse IgG2a isotype control	S43.10		
APC-Cy7 HLA-DR	L243	BD Biosciences	San Jose, CA
PE-Cy5 CD11c	B-ly6	BD Biosciences	
Biotinylated CD40	EA-5	Ancell	Bayport, MN
Biotinylated mouse IgG1 isotype control	MOPC-31C		
Streptavidin ECD (PE-Texas Red)		Beckman Coulter	Fullerton, CA
PE CD83	HB15	BD Biosciences	
PE mouse IgG1	MOPC-21		
FITC CD86	2331(FUN-1)	BD Biosciences	
FITC mouse IgG1 isotype control	MOPC-21		
PE CD103	Ber-ACT8	BD Biosciences	
PE mouse IgG1 isotype control	MOPC-21		
PE CX3CR1	2A9-1	MBL International	Woburn, MA
PE rat IgG2b isotype control	3G8		
V^{450} CD14	MΦP9	BD Biosciences	
V^{450} mouse IgG2a	27-35		
Assessment of CD1c^{+} mDC cytokine production			
APC BDCA-1 (CD1c)	AD5-8E7	Miltenyi Biotec	Auburn, CA
APC mouse IgG2a isotype control	S43.10		
APC-Cy7 HLA-DR	L243	BD Biosciences	San Jose, CA
AF700 CD11c	B-ly6		
eFluor^{450} IL-12p40	C8.6	eBioscience	
eFluor^{450} mouse IgG1 isotype control	P3.6.2.8.1		
PE p19	23dcdp	eBioscience	
PE mouse IgG2b isotype control	eBMG2b		
FITC IL-1β	CRM56	eBioscience	
FITC mouse IgG1 isotype control	P3.6.2.8.1		
PE-Cy7 IL-10	JES3-9D7	Biolegend	
PE-Cy7 rat IgG1 isotype control	RTK2071		



Supplementary Figure 2. Representative flow plots illustrating gating strategy to determine CD40 and CD83 expression on colonic myeloid dendritic cells (mDC) and plasmacytoid DC (pDC). All populations were identified from viable, $CD45^+$ cells (plots not shown). A) $CD1c^+$ and $CD1c^{neg}$ mDC were identified within $CD11c^{hi}$ $HLA\text{-}DR^+$ cells using an isotype control (plot not shown).[2,12] Expression of CD40 and CD83 (dark gray) were evaluated using isotype controls (light gray). B) $CD303^+$ pDC were identified in $CD3^{neg}$ cells using an isotype control (plot not shown).[2] Expression of CD40 and CD83 (dark gray) were evaluated using isotype controls (light gray).

REFERENCES

1 Dillon, S. M. *et al.* HIV-1 infection of human intestinal lamina propria CD4+ T cells in vitro is enhanced by exposure to commensal Escherichia coli. *J Immunol* **189**, 885-896, doi:10.4049/jimmunol.1200681 (2012).

2 Dillon, S. M. *et al.* Human intestinal lamina propria CD1c+ dendritic cells display an activated phenotype at steady state and produce IL-23 in response to TLR7/8 stimulation. *J Immunol* **184**, 6612-6621, doi:10.4049/jimmunol.1000041 (2010).

3 Howe, R. *et al.* Evidence for dendritic cell-dependent CD4(+) T helper-1 type responses to commensal bacteria in normal human intestinal lamina propria. *Clin Immunol* **131**, 317-332, doi:10.1016/j.clim.2008.12.003 (2009).

4 Steele, A. K. *et al.* Microbial exposure alters HIV-1-induced mucosal CD4+ T cell death pathways Ex vivo. *Retrovirology* **11**, 14, doi:10.1186/1742-4690-11-14 (2014).

5 Niess, J. H. *et al.* CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* **307**, 254-258, doi:10.1126/science.1102901 (2005).

6 Persson, E. K., Jaensson, E. & Agace, W. W. The diverse ontogeny and function of murine small intestinal dendritic cell/macrophage subsets. *Immunobiology* **215**, 692-697, doi:10.1016/j.imbio.2010.05.013 (2010).

7 Varol, C., Zigmond, E. & Jung, S. Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. *Nature reviews. Immunology* **10**, 415-426, doi:10.1038/nri2778 (2010).

8 Mann, E. R. *et al.* Intestinal dendritic cells: their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men. *Immunology letters* **150**, 30-40, doi:10.1016/j.imlet.2013.01.007 (2013).

9 Smith, P. D. *et al.* Intestinal macrophages and response to microbial encroachment. *Mucosal immunology* **4**, 31-42, doi:10.1038/mi.2010.66 (2011).

10 Varol, C. *et al.* Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *The Journal of experimental medicine* **204**, 171-180, doi:10.1084/jem.20061011 (2007).

11 Smythies, L. E. *et al.* Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. *Journal of leukocyte biology* **80**, 492-499, doi:10.1189/jlb.1005566 (2006).

12 Dillon, S. M. *et al.* An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal immunology* **7**, 983-994, doi:10.1038/mi.2013.116 (2014).

13 Manuzak, J., Dillon, S. & Wilson, C. Differential interleukin-10 (IL-10) and IL-23 production by human blood monocytes and dendritic cells in response to commensal enteric bacteria. *Clinical and vaccine immunology : CVI* **19**, 1207-1217, doi:10.1128/CVI.00282-12 (2012).

14 Manuzak, J. A. *et al.* Increased Escherichia coli-induced interleukin-23 production by CD16+ monocytes correlates with systemic immune activation in untreated HIV-1-infected individuals. *Journal of virology* **87**, 13252-13262, doi:10.1128/JVI.01767-13 (2013).

Gut Dendritic Cell Activation Links an Altered Colonic Microbiome to Mucosal and Systemic T Cell Activation in Untreated HIV-1 infection.

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