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Journal

AIDS, 32(12)

ISSN

0269-9370

Authors

Dillon, Stephanie M
Guo, Kejun
Austin, Gregory L
et al.

Publication Date

2018-07-31

DOI

10.1097/qad.0000000000001863

Peer reviewed

A compartmentalized type I interferon response in the gut during chronic HIV-1 infection is associated with immunopathogenesis

Stephanie M. Dillon[a,*], Kejun Guo[a,*], Gregory L. Austin[a], Sara Gianella[b], Phillip A. Engen[c], Ece A. Mutlu[c], John Losurdo[c], Garth Swanson[c], Prachi Chakradeo[c], Ali Keshavarzian[c], Alan L. Landay[c], Mario L. Santiago[a,†] and Cara C. Wilson[a,†]

Objective(s): Type I interferon (IFN-I) responses confer both protective and pathogenic effects in persistent virus infections. IFN-I diversity, stage of infection and tissue compartment may account for this dichotomy. The gut is a major site of early HIV-1 replication and microbial translocation, but the nature of the IFN-I response in this compartment remains unclear.

Design: Samples were obtained from two IRB-approved cross-sectional studies. The first study included individuals with chronic, untreated HIV-1 infection ($n = 24$) and age/sex-balanced uninfected controls ($n = 14$). The second study included antiretroviral-treated, HIV-1-infected individuals ($n = 15$) and uninfected controls ($n = 15$).

Methods: The expression of 12 IFNα subtypes, IFNβ and antiviral IFN-stimulated genes (ISGs) were quantified in peripheral blood mononuclear cells (PBMCs) and colon biopsies using real-time PCR and next-generation sequencing. In untreated HIV-1-infected individuals, associations between IFN-I responses and gut HIV-1 RNA levels as well as previously established measures of colonic and systemic immunological indices were determined.

Results: IFNα1, IFNα2, IFNα4, IFNα5 and IFNα8 were upregulated in PBMCs during untreated chronic HIV-1 infection, but IFNβ was undetectable. By contrast, IFNβ was upregulated and all IFNα subtypes were downregulated in gut tissue. Gut ISG levels positively correlated with gut HIV-1 RNA and immune activation, microbial translocation and inflammation markers. Gut IFN-I responses were not significantly different between HIV-1-infected individuals on antiretroviral treatment and uninfected controls.

Conclusion: The IFN-I response is compartmentalized during chronic untreated HIV-1 infection, with IFNβ being more predominant in the gut. Gut IFN-I responses are associated with immunopathogenesis, and viral replication is likely a major driver of this response.

AIDS 2018, **32**:1599–1611

Keywords: gut, HIV-1 infection, interferon-stimulated genes, inflammation, mucosal immunology, type I interferon

[a]University of Colorado Anschutz Medical Campus, Aurora, Colorado, [b]University of California, San Diego, La Jolla, California, and [c]Rush University Medical Center, Chicago, Illinois, USA.

Correspondence to Cara C. Wilson, Division of Infectious Diseases, Department of Medicine, University of Colorado Anschutz Medical Campus, 12700 E. 19th Avenue, Mail Stop B168, Aurora, CO 80045, USA.

E-mail: cara.wilson@ucdenver.edu

[*] Stephanie M. Dillon and Kejun Guo contributed equally to this work.

[†] Mario L. Santiago, Cara C. Wilson are co-senior authors.

Received: 19 January 2018; revised: 30 March 2018; accepted: 6 April 2018.

DOI:10.1097/QAD.0000000000001863

ISSN 0269-9370

Introduction

Innate immune responses evolved to limit the initial spread of pathogens in the host, but these responses may also contribute to pathogenesis in persistent virus infections [1–4]. This dichotomy is highlighted by the role of type I interferons (IFN-I) in HIV-1 infection and in the simian immunodeficiency virus (SIV)/rhesus macaque infection model. The IFN-Is are a diverse family of cytokines that include IFNα and IFNβ that signal through the type I IFN receptor (IFNAR) to induce hundreds of IFN-stimulated genes (ISGs) [5]. During acute HIV-1 and SIV infection, plasma levels of IFNα increased rapidly [6,7]. Accompanying this IFN-I response, robust cellular expression of ISGs was observed in both the periphery and lymphoid tissue during acute HIV/SIV infection [6–13]. Several of these ISGs encode antiviral 'restriction factors.' Genetic ablation of restriction factors APOBEC3 and Tetherin in murine retrovirus infection reduced the antiviral potency of administered IFNα *in vivo* [14,15]. During acute SIV infection, blockade of type I IFN signaling with an IFNAR antagonist exacerbated viremia and pathogenesis [16]. Moreover, transmitted/founder HIV-1 strains that initially establish infection *in vivo* were relatively resistant to IFN-Is and restriction factors Mx2 and IFITMs *in vitro* [17–19].

The antiviral role of IFN-Is during acute HIV-1/SIV infection sharply contrasts with their emerging role during the chronic phase. Two of the most striking features of chronic untreated HIV-1 disease, inflammation and generalized immune activation, are linked to prolonged stimulation by IFN-Is [4,20]. Indeed, a persistently elevated IFN-I response is a critical feature that distinguishes pathogenic [8,10–12,21–28] versus nonpathogenic SIV infections [6,8,29]. Of note, chronic immune activation and inflammation are associated with HIV-1 disease progression, non-AIDS comorbidities and increased mortality [30–33]. Blocking IFN-I signaling during chronic HIV-1 infection in humanized mice revived cell-mediated immune responses and reduced viremia [34,35]. These data strengthened the case for clinically blocking IFN-I signaling to reduce inflammation and comorbidities [36,37]. However, IFN-Is may still exert significant antiviral effects during chronic HIV-1 infection. Blockade of IFN-I signaling in humanized mice with antiretroviral drug-suppressed HIV-1 infection resulted in 'blips' of HIV-1 viremia [34].

Understanding the dichotomy of IFN-I responses would be key to harnessing the IFN-I pathway for curative and/or antiinflammation strategies against HIV-1 infection. However, these studies may need to account for factors that drive IFN-I's pleiotropic effects. One source of IFN-I pleiotropy is the diversity of the IFN-Is themselves. IFNα is constituted of 13 genes encoding 12 distinct subtypes that are produced primarily by plasmacytoid dendritic cells (pDCs) [38]. We previously showed that primary pDCs exposed to HIV-1 *in vitro* express a biased distribution of IFNα subtypes, with weakly antiviral IFNα1, IFNα2 and IFNα5 accounting for nearly half of the IFNα transcript pool [39]. Moreover, IFNβ, a single cytokine that shares only 50% homology to the IFNα subtypes, may have nonredundant functions compared with IFNα [40–43]. Interestingly, whereas IFNα levels increased in plasma from chronic HIV-1-infected individuals, IFNβ levels were mostly undetectable [23].

The IFN-I response is highly influenced by the tissue environment [44], but most data on HIV-1 infection were derived from peripheral blood. Early HIV-1 replication primarily occurs in the gut, suggesting that IFN-I responses may play a more important role in controlling HIV-1 replication in this compartment. The gut harbors large numbers of CCR5+ effector memory $CD4^+$ T cells that are highly susceptible to infection by transmitted/founder HIV-1 strains. In the SIV model, pDCs are recruited early in the gut, and pDC frequency associated with increased ISG expression [45]. Importantly, increased ISG expression was associated with the accumulation of ileal pDCs in individuals with chronic HIV-1 infection [46]. Thus, a strong IFN-I signature likely occurs in the gut during chronic HIV-1 infection, similar to peripheral blood. However, the extent to which the various IFNα subtypes, IFNβ and ISGs associate with markers of gut pathogenesis remains unknown.

HIV-1-associated disease of the gut is mediated by both virus and enteric bacteria. The loss of gut Th17 and Th22 cells likely precipitate epithelial barrier breakdown, resulting in the movement of microbes and microbial products into the lamina propria and eventually, the systemic circulation [33,47]. This process, known as microbial translocation, is a key contributor to HIV-1-associated chronic immune activation despite effective antiretroviral therapy (ART) [33,47–50]. In fact, microbial exposure enhances HIV-1 replication and $CD4^+$ T-cell death in lamina propria mononuclear cells (LPMCs) *ex vivo* [51–53]. We profiled the transcriptome of gut $CD4^+$ T cells exposed to *Prevotella stercorea*, an enteric microbe found in high relative abundance in gut mucosa of HIV-1-infected individuals [54]. The top pathway induced was the IFN-I response, involving the induction of >60 known ISGs, including several HIV-1 restriction factors [55]. This raises the possibility that microbial translocation may contribute to the elevated IFN-I response in the gut during chronic HIV-1 infection.

Given that the impact of IFN-Is may be influenced by IFN-I diversity, stage of infection and tissue environment, we investigated the nature of the gut IFN-I response during chronic HIV-1 infection. Our data revealed critical features of the chronic IFN-I response in the gut that may inform IFN-I-based strategies that target the gut to reduce chronic immune activation in HIV-1-infected individuals.

Methods

Study participants and study design

Colon biopsies, peripheral blood mononuclear cells (PBMCs) and plasma samples were obtained from individuals with untreated, chronic HIV-1 infection and HIV-1-seronegative (uninfected) adults enrolled in a completed IRB-approved study at the University of Colorado Anschutz Medical Campus (UC-AMC), Aurora, Colorado, USA (S1 Table, http://links.lww.com/QAD/B280). All study participants voluntarily gave written, informed consent. Inclusion and exclusion criteria and the collection, storage and processing of colon biopsies, PBMCs and plasma were extensively detailed in previous publications [56,57].

To investigate the impact of ART on colonic type I ISG signatures, colon (sigmoid) biopsies and plasma samples were also obtained from the Rush University Medical Center (RUMC) Institutional Review Board-approved gastrointestinal repository in Chicago, Illinois, USA. All participants gave informed consent prior to tissue and blood collection agreeing to allow samples and their patient data to be stored for future use. Samples were shipped to UC-AMC for subsequent laboratory studies (S2 Table, http://links.lww.com/QAD/B280). All protected study participant information was de-identified to the investigators and laboratory personnel at the UC-AMC and the study approved by COMIRB. Colon biopsies were obtained from the left colon at the time of colonoscopy with a standard 2.2 mm biopsy forceps at the RUMC Endoscopy Lab. Biopsies were stored in RNAlater solution (Sigma-Aldrich, St Louis, Missouri, USA) for 24 h at 4 °C, before long-term storage in −80 °C freezers. Plasma was isolated from blood samples collected into a BD (Becton Dickinson, Franklin Lakes, New Jersey, USA) vacutainer tubes (EDTA) and stored at −80 ° C.

Real-time quantitative PCR for IFNα and next-generation sequencing for IFNα subtype determination

Total RNA was extracted from PBMC or colon biopsies in RNAlater using Qiagen RNAeasy Micro Kit. Insufficient colonic biopsy RNA was obtained from five UC-AMC study participants (one uninfected, four HIV-1-infected). Real-time quantitative PCR (qPCR) for total IFNα was performed as previously described [39], using primers in the conserved regions of the IFNα subtypes. Copy number was interpolated using a standard curve with 10^8-10^1 copies of IFNα8 plasmid and normalized against GAPDH copies. For quality control, RNA samples with GAPDH levels less than 200 copies/ng were excluded from the qPCR analysis (UC-AMC: one uninfected; two HIV-1-infected; RUMC: four uninfected).

IFNα subtype determination by NGS was performed on PBMC and colon biopsies from the UC-AMC cohorts only. Samples with insufficient RNA or GAPDH levels less than 200 copies/ng were excluded (PBMC: six uninfected, 11 HIV-1-infected; colon biopsies: five uninfected, seven HIV-1-infected). cDNA was added to a PCR reaction containing Phusion Hi-Fidelity Taq (New England Biolabs, Ipswich, Massachusetts, USA) according to manufacturer's instructions containing 10 pmol of Illumina primers [39]. Illumina sequences were compared with a reference database of the IFNα gene family with greater than 90% identity threshold. IFNα gene distribution was calculated based on a percentage of the total IFNα counts. IFNα1 and IFNα13 DNA sequences were identical in the amplified region and encode an identical protein and so were referred to as IFNα1/13. Final numbers for PBMC and colon biopsy samples for IFNα (total) and IFNα subtype measurement are detailed in S3 Table, http://links.lww.com/QAD/B280.

IFNβ and IFN-stimulated gene transcript quantification

Taqman primer probe combinations were used to quantify IFNβ, APOBEC3G, Tetherin and Mx2 relative to GAPDH. IFNβ primers included: IFNβ.forward TTGCTCTCCTGTTGTGCTTCTCCA, IFNβ.reverse TTCAAGCCTCCCATTCAATTGCC, IFNβ.probe ACTGAAAATTGCTGCTTCTTTGTAGGAATCC-AAG.

All other primer sets and PCR conditions were reported previously [39]. Absolute quantification was conducted using 10^8-10^1 copies of plasmid standards for each of the gene tested and normalized to GAPDH copy numbers. Due to low RNA yields and/or GAPDH quantities, some samples were excluded from analyses. Final numbers for PBMC and colon biopsy samples for each measurement are detailed in S3 Table, http://links.lww.com/QAD/B280.

Accession numbers

Next-generation sequencing data were deposited at the NCBI Sequence Archive Bioproject PRJNA422935.

Previously obtained measurements of colonic mucosal and systemic immunological and virological parameters in the University of Colorado Anschutz Medical Campus cohort

Indicators of systemic inflammation [IL6, high sensitivity C-reactive Protein (hsCRP), IFNγ, TNFα and IL10], microbial translocation (LPS, LTA) and epithelial barrier damage (intestinal fatty acid-binding protein; iFAPB) as well as blood T-cell activation ($CD38^+HLA\text{-}DR+$), colonic $CD4^+$ and $CD8^+$ T-cell frequencies and activation, colonic myeloid DC (mDC) and pDC frequencies and activation ($CD40^+$, $CD83^+$), constitutive colonic mucosal cytokine production, frequencies of IFNγ, IL17 and IL22-expressing colonic T cells and tissue HIV-1 RNA levels were previously evaluated in the untreated, HIV-1-infected study participants and the methods detailed elsewhere [56,57].

Measurements of plasma IFNα, IFNβ, sCD27 and sCD14

Commercially available ELISAs were used to evaluate levels of IFNα, IFNβ (both PBL Assay Science, Piscataway, New Jersey, USA), sCD27 (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts, USA) and sCD14 (R&D Systems, Minneapolis, Minnesota, USA) in EDTA plasma samples. Manufacturer's protocols were followed for all assays.

Statistical analysis

Nonparametric statistics were performed with no adjustments for multiple comparisons because of the exploratory nature of this study. Analyses were undertaken using GraphPad Prism Version 6 for Windows (GraphPad Software, San Diego, California, USA). A P value less than 0.05 was considered significant. Outliers were identified using the Rout test (GraphPad Software, La Jolla, California, USA).

Results

Colon IFNβ gene transcripts are increased in chronic, untreated HIV-1 infection and correlate with indicators of systemic inflammation

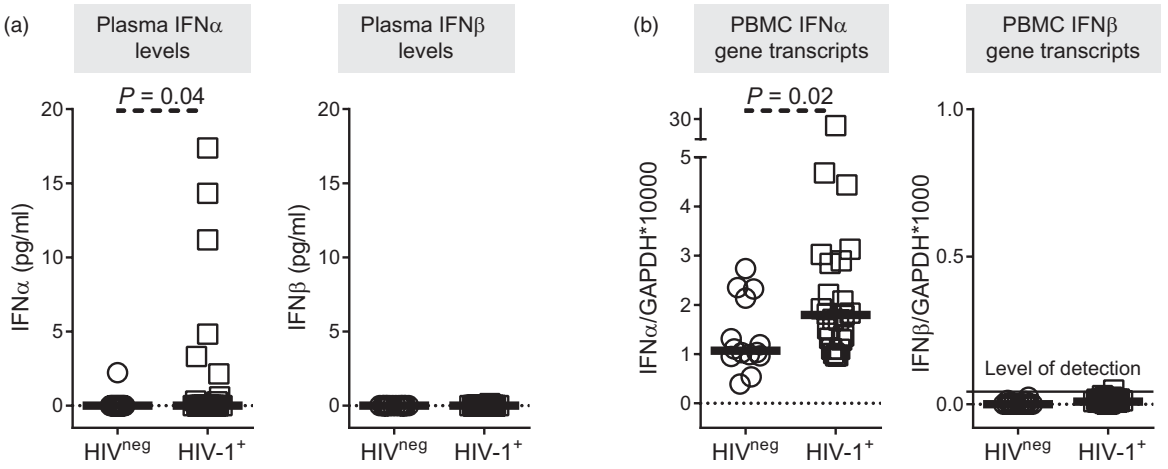
We quantified IFNα and IFNβ protein levels in plasma and IFN-I gene transcripts in paired PBMC and colon biopsies from untreated, HIV-1-infected ($n = 24$) and uninfected ($n = 14$) individuals from our previously completed clinical study [56–59] (S1 Table, http://links.lww.com/QAD/B280). Untreated HIV-1-infected persons had a median plasma viral load of 51 350 (range 2880–207 000) HIV-1 RNA copies/ml and 445 (221–1248) $CD4^+$ cells/μl. Median $CD4^+$ count for uninfected study participants (724, 468–1071 $CD4^+$ cells/μl) was significantly higher compared with untreated, HIV-1-infected study participants. In agreement with published data from other groups [23,26,27], plasma IFNα levels were significantly higher in untreated HIV-1-infected individuals compared with uninfected study participants, whereas plasma IFNβ levels were undetectable in both cohorts (Fig. 1a). These results were recapitulated at the transcriptional level, as we observed higher IFNα gene transcripts in PBMC from HIV-1-infected individuals relative to control study participants, but undetectable PBMC IFNβ gene transcripts for both groups (Fig. 1b). We next evaluated the absolute gene expression of distinct IFNα subtypes in a subset of study participants for which good-quality RNA sample remained available (S3 Table, http://links.lww.com/QAD/B280). We observed that the higher total IFNα gene transcripts in PBMCs of HIV-1-infected individuals were reflected in higher levels of IFNα1 ($P = 0.0003$), IFNα2 ($P = 0.02$), IFNα4 ($P = 0.02$), IFNα5 ($P = 0.04$) and IFNα8 ($P = 0.06$) (Fig. 1c and d). No significant differences in the remaining IFNα subtypes were observed in PBMC samples between the two groups.

We next quantified the levels of IFNα (total and individual subtypes) and IFNβ transcripts in colonic mucosa of untreated HIV-1-infected and uninfected study participants (Fig. 2; S3 Table, http://links.lww.com/QAD/B280). In contrast to IFNα profiles observed in PBMC, colon total IFNα gene transcripts were significantly lower in HIV-1-infected versus uninfected study participants (Fig. 2a). In fact, all IFNα subtypes were expressed at lower levels in HIV-1-infected colon samples compared with uninfected study participants (Fig. 2b). Surprisingly, colon IFNβ gene transcripts were significantly higher in HIV-1-infected relative to uninfected study participants (Fig. 2a). We next determined whether colon IFNα and IFNβ gene expression levels in untreated, HIV-1-infected persons correlated with a battery of clinical, virological and immunological indices for pathogenesis that we measured in previous studies [56,57] (S4 Table, http://links.lww.com/QAD/B280). IFNα/β gene transcripts did not associate with $CD4^+$ counts (Table S4, http://links.lww.com/QAD/B280). Total colon IFNα gene expression did not significantly correlate with other indices measured. By contrast, colon IFNβ gene expression significantly correlated with plasma IFNγ and IL10 levels (Fig. 2c). IFNβ gene expression also associated with higher numbers of IFNγ and IL17 expressing $CD4^+$ T cells in the tissue (S4 Table, http://links.lww.com/QAD/B280).

Colon antiretroviral gene expression in untreated HIV-1 infection positively correlates with colon HIV-1 RNA levels and immune activation

As the IFNα subtypes and IFNβ were differentially altered in the colonic mucosa of HIV-1-infected study participants, we next evaluated if downstream ISGs were induced or inhibited. We focused on three canonical antiretroviral ISGs: Tetherin/BST-2, APOBEC3G and MX2 [60]. Expression of all three 'restriction factors' were significantly higher in the colonic mucosa of untreated HIV-1-infected compared with uninfected study participants (Fig. 3a; S3 Table, http://links.lww.com/QAD/B280), and the expression of all three restriction factor ISGs significantly correlated with each other (S5 Table, http://links.lww.com/QAD/B280). Interestingly, antiretroviral ISG expression did not significantly associate with IFNα or IFNβ expression.

We next evaluated if antiviral ISG expression correlated with colon HIV-1 RNA levels and immune parameters. Notably, these three ISGs positively correlated with colon HIV-1 RNA levels (Fig. 3b and Table 1). Tetherin and APOBEC3G transcript levels positively correlated with the activation marker CD40 expressed on colonic mDC; and a similar trend was observed for MX2 and activated mDCs ($P = 0.07$; Fig. 3c and Table 1). Higher tetherin and APOBEC3G gene transcripts positively associated with the constitutive production of IL12p70 by total colonic cells (Table 1). Tetherin levels correlated further



(c)

	Uninfected study participants N=8	HIV-1 infected study participants N=13	P value*
IFNα1/13	18.4 (5.6-57.1)	57.5 (19.4-284.8)	**0.0003**
IFNα2	10.5 (3.5-22.4)	23.0 (6.7-103.8)	**0.02**
IFNα4	33.5 (21.1-78.2)	60.5 (21.8-155.0)	**0.02**
IFNα5	642 (375-1547)	1074 (538-2886)	**0.04**
IFNα6	4.2 (0.3-14.9)	6.4 (2.6-57.0)	0.14
IFNα7	18.9 (6.1-32.9)	23.1 (6.1-76.7)	0.39
IFNα8	31.2 (6.6-89.9)	56.6 (9.1-262)	*0.06*
IFNα10	3.2 (1.3-6.8)	6.3 (0.8-36.3)	0.13
IFNα14	18.2 (8.1-26.1)	21.4 (5.3-82.5)	0.19
IFNα16	14.3 (4.1-28.2)	12.1 (1.0-58.6)	0.74
IFNα17	10.8 (3.3-18.6)	14.0 (2.0-91.1)	0.50
IFNα21	253 (57.9-331)	250 (135-809)	0.75

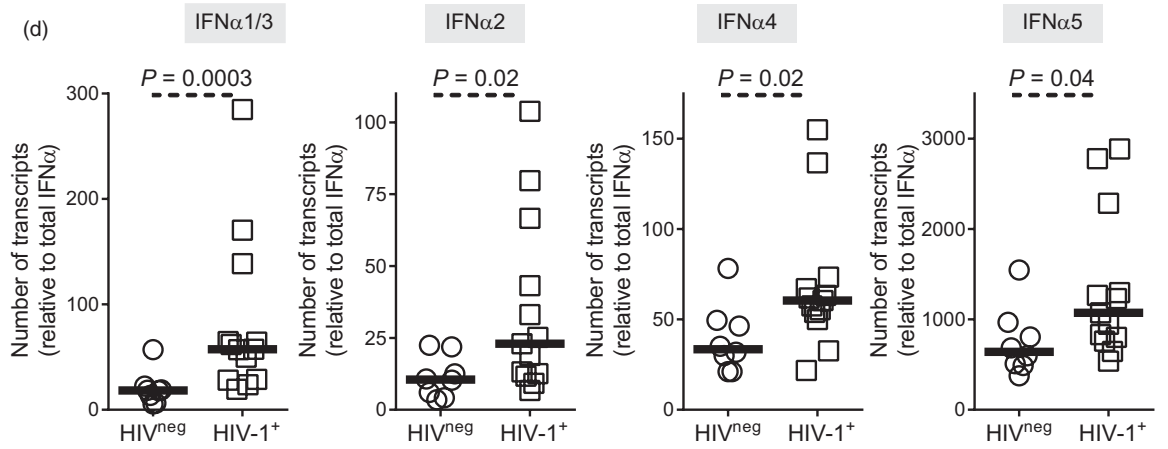
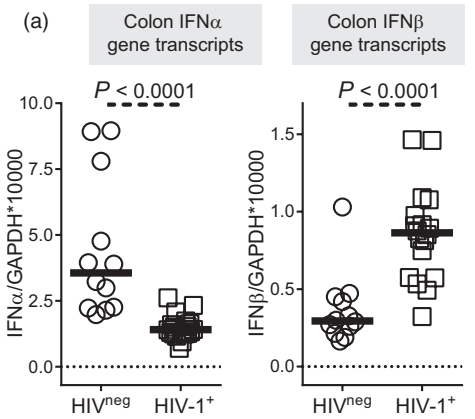


Fig. 1. IFNα/β plasma levels and gene transcript levels in peripheral blood mononuclear cells (PBMC) in untreated, chronically infected HIV-1 and uninfected study participants. (a) Levels of IFNα/β were measured in plasma samples from HIV-1-uninfected control (HIV^{neg}; $n = 14$) and untreated, chronic HIV-1-infected ($HIV\text{-}1^{+}$; $n = 24$) study participants. (b) IFNα/β gene transcript levels were measured in PBMC from HIV-1-uninfected (HIV^{neg}; $n = 14$) and untreated, chronic HIV-1-infected ($HIV\text{-}1^{+}$; $n = 24$) study participants. (c and d) Expression of IFNα subtypes (number of transcripts for each IFNα subtype relative to total IFNα gene transcripts) in PBMC were assessed in 13 HIV-infected and eight uninfected study participants. Lines represent median values (a, b and d) or are shown as median (range; d). *Bold font indicates a significant *P* value. Italics indicates a trend ($P < 0.1$). Statistical analysis was performed using the Mann–Whitney test to determine differences between unmatched groups.



(b)

	Uninfected study participants N = 7	HIV-1 infected study participants N = 11	P value*
IFNα1/13	81.6 (62.5-346)	17.8 (11.2-84.0)	**0.0008**
IFNα2	46.5 (25.2-145)	8.5 (1.4-41.8)	**0.0004**
IFNα4	83.9 (55.0-356)	39.0 (19.7-101)	**0.004**
IFNα5	2315 (1150-5254)	1051 (550-1621)	**0.003**
IFNα6	38.5 (17.7-138)	2.7 (0-19.5)	**0.0004**
IFNα7	62.4 (22.6-89.2)	20.2 (10.5-46.0)	**0.005**
IFNα8	92.9 (46.8-408)	14.1 (0-111)	**0.0008**
IFNα10	15.7 (3.8-46.0)	4.7 (0-39.0)	**0.01**
IFNα14	53.8 (21.6-99.7)	20.7 (4.2-35.6)	**0.003**
IFNα16	35.1 (0-97.4)	7.7 (3.6-58.5)	**0.047**
IFNα17	57.7 (8.9-230)	14.7 (8.1-78.0)	*0.069*
IFNα21	639 (185-843)	101 (19.5-258)	**0.0004**

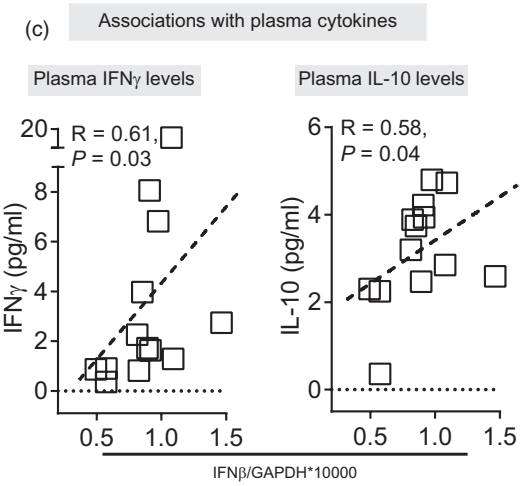


Fig. 2. IFNα/β gene transcript levels in colonic cells in untreated, chronically infected HIV-1 and uninfected study participants. (a) Levels of IFNα/β gene transcripts were measured in colonic samples from HIV-1-uninfected (HIV^{neg}; $n = 12$) and untreated, chronic HIV-1-infected ($HIV\text{-}1^{+}$; $n = 18$) study participants. Lines represent median values. (b) Expression of IFNα subtypes (number of transcripts for each IFNα subtype relative to total IFNα gene transcripts) in colonic samples were assessed in 18 HIV-infected and eight uninfected study participants. *Bold font indicates a significant *P* value. Italics indicates a trend ($P < 0.1$). (c) Correlations between IFNβ gene transcript levels and plasma levels of IFNγ and IL-10 in HIV-1-infected study participants ($n = 13$; plasma levels of IFNγ and IL-10 were not evaluated in five of the study participants with IFNβ gene expression data). Dotted line is a visual representation of the significant association. Statistical analysis was performed using the Mann–Whitney test to determine differences between unmatched group and Spearman test to determine correlations between variables.

with several virological and immunological parameters. Colon Tetherin levels positively correlated with plasma HIV-1 viral load, the number of activated colonic $CD8^{+}$ T cells, systemic indicators of inflammation (TNFα, IL10), sCD14, an indicator of monocyte activation and indirect measure of microbial translocation and inversely associated with epithelial barrier damage (iFABP; Fig. 3c and d, Table 1). Associations between APOBEC3G and

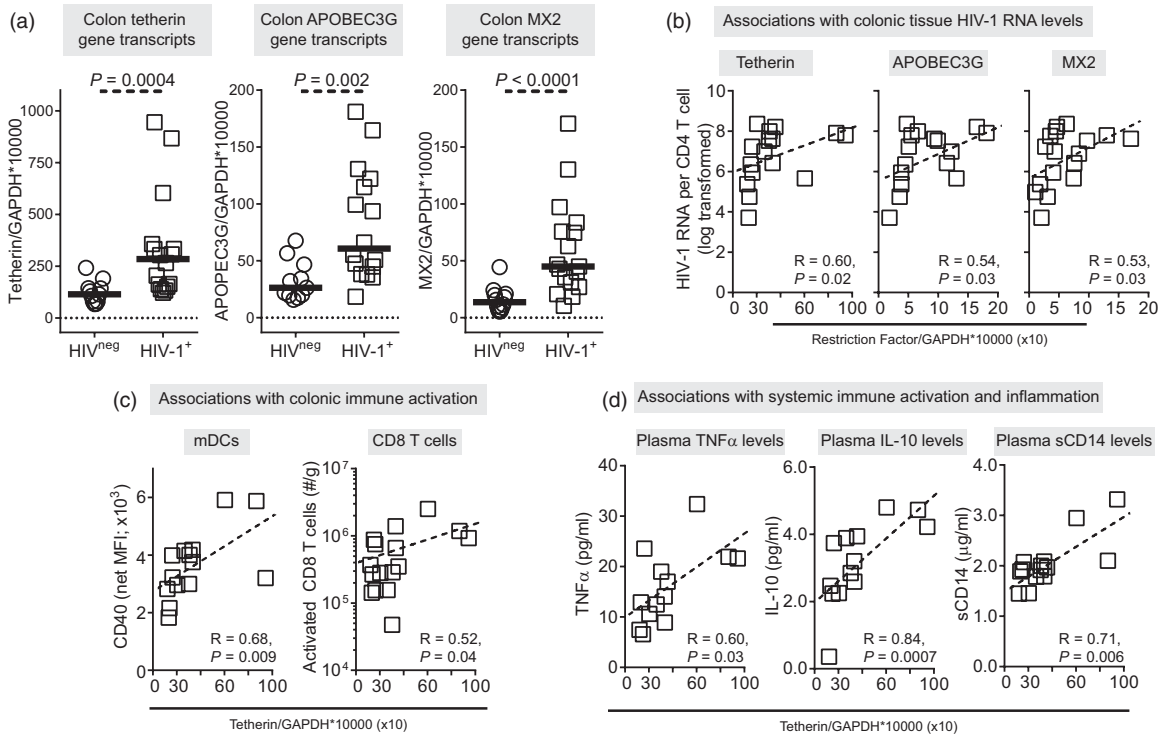


Fig. 3. Restriction factor gene transcript levels in colonic cells in untreated, chronically infected HIV-1 and uninfected study participants. (a) Levels of tetherin (BST2), APOBEC3G and MX2 gene transcripts were measured in colonic samples from HIV-1-uninfected (HIV^{neg}; $n = 11–12$) and untreated, chronic HIV-1-infected ($HIV\text{-}1^{+}$; $n = 16–18$) study participants. Lines represent median values. (b) Correlations between gene transcript levels and colonic tissue HIV-1 RNA levels in HIV-1-infected study participants ($N = 16$ tetherin, APOBEC3G; $N = 17$ MX2 Outlier identified using Rout test was not included in analyses). Correlations between tetherin gene transcript levels and (c) colonic mDC activation ($CD40^{+}$ expression levels; $N = 14$; mDC activation levels were not measured in two of the study participants with tetherin gene expression data) and frequency of activated ($CD38^{+}$ HLA-DR^{+}) $CD8^{+}$ T cells ($N = 16$) and (d) plasma levels of TNFα ($N = 13$), IL-10 ($N = 13$) and sCD14 ($N = 14$) in HIV-1-infected study participants (TNFα, IL-10 levels were not measured in three of the study participants and sCD14 levels not measured in two of the study participants with tetherin gene expression data). Dotted line is a visual representation of the significant association. Statistical analysis was performed using the Mann–Whitney test to determine differences between unmatched group and Spearman test to determine correlations between variables.

MX2 and these clinical and systemic markers were more variable and did not consistently reach statistical significance (Table 1). No ISG gene transcripts associated with $CD4^{+}$ counts (Table 1).

Colon expression of IFN-Is and restriction factors in antiretroviral-treated HIV-1-infected participants were not significantly altered relative to uninfected controls

The elevated IFN-I signature in the gut during chronic HIV-1 infection may be driven by persistent HIV-1 viremia and/or microbial translocation. To address if these parameters are responsible, we evaluated the levels of IFN-Is and antiretroviral ISGs in a separate cohort of ART-treated HIV-1-infected persons with effective viral suppression ($n = 15$) and age, sex and ethnicity-matched uninfected persons ($n = 15$). Stored plasma and colon biopsies were obtained from the RUMC IRB gastrointestinal repository (S2 Table, http://links.lww.com/QAD/B280). Of note, these participants differed in age and ethnicity compared with those from UC-AMC (compare S1, http://links.lww.com/QAD/B280 and S2 Tables, http://links.lww.com/QAD/B280). ART-treated HIV-1-infected persons achieved plasma virus suppression (<40 HIV-1 RNA copies/ml) with 508 (range: 178–1415) $CD4^{+}$ cells/μl. $CD4^{+}$ counts were not measured in the uninfected study participants.

Plasma levels of sCD27 and sCD14 were significantly higher in the ART-treated study participants versus uninfected controls (Fig. 4a) indicating ongoing lymphocyte and monocyte activation in these individuals despite virological suppression. Colonic gene transcripts levels of both IFNα and IFNβ were not significantly different in HIV-1-infected versus uninfected controls, although a trend towards higher numbers of IFNβ transcripts was observed in HIV-1-infected persons ($P = 0.09$; Fig. 4b; S3 Table, http://links.lww.com/QAD/B280). IFNα and IFNβ transcripts did not significantly associate with $CD4^{+}$ counts (IFNα: $R = 0.39$, $P = 0.16$; IFNβ: $R = -0.06$, $P = 0.84$). Similarly,

Table 1. Correlations between colonic restriction factor gene transcripts and clinical, virological and immunological parameters[a].

	Tetherin ($N=16$)	APOBEC3G ($N=16$)	MX2 ($N=17$)[b]
Clinical parameters			
Blood $CD4^+$ T-cell count	$R=-0.31$, $P=0.24$	$R=-0.19$, $P=0.47$	$R=-0.14$, $P=0.58$
Plasma HIV-1 viral load	**$R=0.51$, $P<0.05$**	$R=0.36$, $P=0.18$	$R=0.46$, $P=0.07$
Systemic inflammation, immune activation, microbial translocation and epithelial barrier disruption parameters			
Plasma IL-6	$R=0.33$, $P=0.22$	$R=0.32$, $P=0.22$	$R=0.00$, $P=0.98$
Plasma CRP	$R=0.00$, $P=1.0$	$R=0.23$, $P=0.39$	$R=-0.12$, $P=0.66$
Plasma TNFα ($N=13$[c], 14[d])	**$R=0.60$, $P=0.03$**	$R=0.51$, $P=0.08$	$R=0.33$, $P=0.30$
Plasma IFNγ ($N=13$, 12)	$R=0.45$, $P=0.12$	$R=0.18$, $P=0.55$	$R=0.57$, $P=0.06$
Plasma IL-10 ($N=13$, 12)	**$R=0.84$, $P=0.0007$**	**$R=0.79$, $P=0.002$**	$R=0.47$, $P=0.13$
Activated blood $CD4^+$ T cells	$R=0.30$, $P=0.26$	$R=0.29$, $P=0.27$	$R=0.07$, $P=0.77$
Activated blood $CD8^+$ T cells	$R=0.48$, $P=0.06$	$R=0.46$, $P=0.08$	$R=0.05$, $P=0.85$
Plasma sCD27	$R=0.32$, $P=0.23$	$R=0.31$, $P=0.25$	$R=0.24$, $P=0.36$
Plasma sCD14 ($N=14$, 14)	**$R=0.71$, $P=0.006$**	$R=0.39$, $P=0.17$	$R=0.32$, $P=0.26$
Plasma LPS ($N=15$, 15)	$R=0.43$, $P=0.11$	$R=0.29$, $P=0.29$	$R=0.27$, $P=0.33$
Plasma LTA ($N=14$, 14)	$R=0.10$, $P=0.74$	$R=-0.21$, $P=0.47$	$R=0.13$, $P=0.66$
Plasma iFABP	**$R=-0.56$, $P=0.03$**	$R=-0.45$, $P=0.08$	$R=-0.31$, $P=0.23$
Colonic immunity parameters			
HIV-1 RNA levels	**$R=0.60$, $P=0.02$**	**$R=0.54$, $P=0.03$**	**$R=0.53$, $P=0.03$**
No, of $CD1c^+$ mDC ($N=14$, 16)	$R=-0.38$, $P=0.19$	$R=-0.28$, $P=0.33$	$R=-0.47$, $P=0.07$
No. of pDC ($N=14$, 16)	$R=0.38$, $P=0.16$	$R=0.44$, $P=0.10$	$R=0.18$, $P=0.51$
$CD1c^+$ mDC: $CD40^+$[e] ($N=14$, 16)	**$R=0.68$, $P=0.009$**	**$R=0.83$, $P=0.0005$**	$R=0.46$, $P=0.07$
pDC $CD40^+$ ($N=14$, 15)	$R=-0.06$, $P=0.84$	$R=0.17$, $P=0.56$	$R=-0.15$, $P=0.59$
$CD1c^+$ mDC: $CD83^+$[f] ($N=14$, 16)	$R=0.14$, $P=0.64$	$R=0.24$, $P=0.40$	$R=0.14$, $P=0.61$
pDC: $CD83^+$ ($n=15$, 16)	$R=0.17$, $P=0.55$	$R=0.22$, $P=0.41$	$R=-0.17$, $P=0.53$
No. of $CD4^+$ T cells[g]	$R=0.03$, $P=0.92$	$R=0.20$, $P=0.45$	$R=0.05$, $P=0.86$
No. of $CD8^+$ T cells	$R=0.22$, $P=0.41$	$R=0.02$, $P=0.93$	$R=0.10$, $P=0.72$
No. activated $CD4^+$ T cells	$R=0.29$, $P=0.28$	$R=0.21$, $P=0.44$	$R=0.27$, $P=0.31$
Number of activated $CD8^+$ T cells	**$R=0.52$, $P=0.04$**	$R=0.42$, $P=0.11$	$R=0.42$, $P=0.09$
Number of of $IFN\gamma^+$ $CD4^+$ T cells ($N=15$, 16)	$R=0.05$, $P=0.87$	$R=-0.05$, $P=0.86$	$R=0.05$, $P=0.87$
Number of $IL\text{-}17^+$ $CD4^+$ T cells ($N=15$, 16)	$R=0.17$, $P=0.55$	$R=0.10$, $P=0.73$	$R=0.24$, $P=0.37$
Number of $IL\text{-}22^+$ CD4 T cells ($N=15$, 16)	$R=-0.02$, $P=0.94$	$R=0.07$, $P=0.81$	$R=0.09$, $P=0.74$
Number of $IFN\gamma^+$ CD8 T cells ($N=15$, 16)	$R=-0.26$, $P=0.34$	$R=-0.13$, $P=0.64$	$R=-0.26$, $P=0.33$
$IL\text{-}12p70$[h] ($N=13$, 13)	**$R=0.64$, $P=0.02$**	**$R=0.65$, $P=0.02$**	$R=0.35$, $P=0.24$
IL-23 ($N=13$, 13)	$R=0.42$, $P=0.16$	$R=0.46$, $P=0.12$	$R=0.18$, $P=0.57$
IL-10 ($N=13$, 13)	$R=0.44$, $P=0.14$	$R=0.45$, $P=0.13$	$R=0.19$, $P=0.54$
IL-1β ($N=13$, 13)	$R=0.37$, $P=0.22$	$R=0.35$, $P=0.24$	$R=0.15$, $P=0.62$
TNFα ($N=13$, 13)	$R=0.29$, $P=0.33$	$R=0.30$, $P=0.32$	$R=0.08$, $P=0.80$
IL-6 ($N=13$, 13)	$R=0.40$, $P=0.18$	$R=0.35$, $P=0.24$	$R=0.25$, $P=0.42$
IFNγ ($N=13$, 13)	$R=0.31$, $P=0.30$	$R=0.32$, $P=0.28$	$R=0.04$, $P=0.89$
IL-17 ($N=13$, 13)	$R=0.29$, $P=0.34$	$R=0.25$, $P=0.41$	$R=0.10$, $P=0.74$

Statistical analysis performed using the Spearman test.
[a]Bold font indicates statistically significant correlation.
[b]Outlier identified using Rout test was not included in analyses.
[c]N denotes when not all study participants with tetherin/APOBEC3G had immunological data
[d]N denotes when not all study participants with MX2 measurements had immunological data.
[e]$CD40^+$ expression levels.
[f]Percent of $CD83^+$.
[g]No.: number per gram.
[h]Constitutive production.

levels of colon tetherin, APOBEC3G and MX2 gene transcripts were not statistically different between the two cohorts, although both MX2 and APOBEC3G had higher median expression with differences in A3G approaching significance ($P=0.07$). ISG gene transcripts did not significantly associate with $CD4^+$ cell counts (Tetherin: $R=-0.05$, $P=0.85$; APOBEC3G: $R=0.35$, $P=0.20$; MX2: $R=0.41$, $P=0.13$).

Discussion

The dual nature of the IFN-I response against persistent virus infections complicates strategies to harness the IFN-I pathway to curb viremia and pathogenesis. Efforts to use IFNα to reduce the latent HIV-1 reservoir, or to block IFN-I signaling to reduce inflammation, may need to account for mechanisms that drive the pleiotropic effects of these cytokines. Here, we investigated how levels of diverse IFN-I species (12 IFNα subtypes and IFNβ) and antiviral ISGs in the blood versus the gut correlate with markers of inflammation, microbial translocation and immune activation during chronic untreated HIV-1 infection. We confirm data from other groups [23] that in PBMCs and plasma, IFNα is upregulated, whereas IFNβ is barely detectable. We extend these results to show that the increased IFNα response in the periphery is comprised of IFNα1, IFNα2, IFNα4, IFNα5 and IFNα8. With the exception of IFNα4, these IFNα

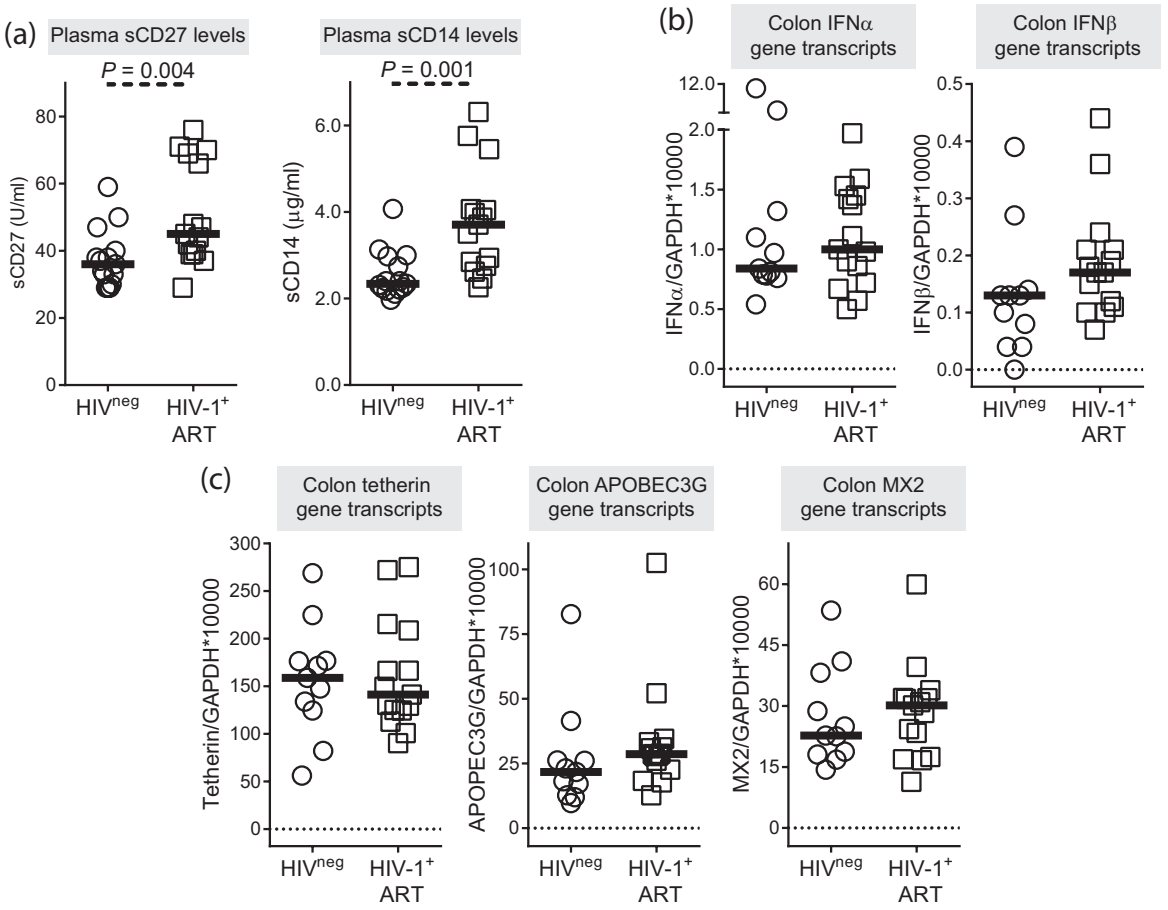


Fig. 4. Restriction factor gene transcript levels in colonic cells in treated, chronically infected HIV-1 and uninfected study participants. (a) Plasma levels of sCD27 and sCD14 in HIV-1-uninfected (HIV^{neg}; $n = 15$) and antiretroviral therapy (ART) treated HIV-1-infected (HIV-1$^+$ ART; $n = 15$) study participants. Levels of (b) IFNα/β gene transcripts and (c) tetherin (BST2), APOBEC3G and MX2 gene transcripts measured in colonic tissue samples from HIV-1-uninfected (HIV^{neg}; $n = 11$) and ART-treated HIV-1-infected (HIV-1$^+$ ART; $n = 15$) study participants. Lines represent median values. Statistical analysis was performed using the Mann–Whitney test to determine differences between unmatched groups.

subtypes were highly expressed in primary pDCs exposed to HIV-1 in our previous study [39]. It remains to be determined if pDCs drive the persistently high IFNα levels in the periphery [23,61]. Of note, IFNα1, IFNα2 and IFNα5 have the weakest inhibitory activity against HIV-1 [39], suggesting that the chronic IFN-I response in the periphery may not be optimal for HIV-1 control. The immunomodulatory properties of these IFNα subtypes remain to be investigated.

The IFNα subtype profiles we obtained in the blood were not observed in the gut during chronic untreated HIV-1 infection. All IFNα subtypes were downregulated compared with uninfected controls. This finding was surprising, as colonic pDCs were found at higher frequencies in these same HIV-1-infected study participants [57]. We hypothesize that these gut pDCs may be exhausted and/or refractory to IFN-I stimulation during chronic HIV-1 infection [62]. Interestingly, IFNβ was significantly upregulated during chronic HIV-1 infection relative to uninfected controls. The cellular source(s) and stimuli driving IFNβ remain to be determined. We speculate that cells responding to translocating bacteria may be one contributor [55]. Several reports suggested that IFNβ may have distinct biological effects [40–43]. Notably, current strategies to reduce inflammation aim to completely block IFNAR signaling that may render the host more susceptible to other viral infections. Specifically blocking IFNβ in the gut to diminish immune activation may help maintain some level of innate immunity, as IFNα is still present, albeit at reduced levels. In fact, selective blockade of IFNβ, but not IFNα, in mouse LCMV infection decreased viral persistence and immunopathology [41]. However, in our study, IFNβ was associated with higher numbers of IFNγ and IL17 expressing CD4$^+$ T cells, suggesting a link between IFNβ and gut CD4$^+$ T-cell survival. IFNβ may also have direct antibacterial properties [63]. Further studies would be required to test the impact of IFNβ on the gut microbiome and the clinical consequences of IFNβ blockade in the gut.

A recent study showed that IFNβ may select for transmitted/founder HIV-1 strains [18], which constitute the earliest HIV-1 strains in infected individuals. The source of these transmitted/founder strains remains unclear, as IFNβ-resistant HIV-1 strains were not readily detected in the plasma of the chronic HIV-1-infected donors using limiting dilution approaches. As plasma virus likely originates from secondary lymphoid tissues and not from the gastrointestinal tract [64–66], we speculate that the gut may serve as an important reservoir of IFNβ-resistant HIV-1 strains that may be more fit to establish new infections. Cataloguing the IFN-I resistance profiles of systemic versus gut-derived HIV-1 strains should help test this interesting hypothesis.

Consistent with the high levels of IFNβ in the gut, we also observed elevated ISG expression in the gut during chronic HIV-1 infection. However, we found no correlation between IFNβ and ISG expression levels. This lack of correlation may be because of the nonconcurrent kinetics of IFN-I expression and ISG induction [67]. The increased expression of restriction factor ISGs in the gut would theoretically lead to a reduction in gut HIV-1 replication and its associated downstream pathological consequences. Instead, we observed that antiretroviral ISG levels positively correlated with colonic mucosal HIV-1 RNA levels and markers of immune activation and inflammation. Positive correlations were also reported between ISGs in PBMCs and plasma viral load by other groups [8,10,21,22,24,25,68]. Our findings strengthen the notion that during chronic HIV-1 infection, gut IFN-I responses are associated with immunopathogenesis. It remains unknown why the induction of restriction factors does not result in reduced viral loads during chronic infection. We previously reported that microbial exposure of LPMCs *ex vivo* induces gut $CD4^+$ T-cell activation, proliferation and Th17 differentiation, all of which promote HIV-1 susceptibility [51,52,55]. Thus, the threshold for restriction factors to inhibit HIV-1 replication in the gut may have been raised in the context of microbial exposure.

We previously showed that microbes can stimulate IFN-I responses in gut lymphocytes *ex vivo* [55]. However, the extent to which enteric bacteria – and potentially the bacteriophages they harbor (the 'virome') [69] – contribute to the elevated IFN-I response in the gut remains unclear. We thus analyzed IFN-I and ISG levels in gut biopsies from a separate cohort of ART-treated, HIV-1-infected versus HIV-1-uninfected individuals. ART-suppressed individuals still had significant microbial translocation and lymphocyte activation relative to uninfected individuals, but ISG levels almost normalized, suggesting that HIV-1 replication, and not microbial translocation, is the primary driver of high IFNβ and ISG expression in the gut. However, a limitation of this study is that colonic tissue HIV-1 RNA levels were not measured in the ART-treated cohort. Of note, prior IFN-I signaling may render cells more refractory to additional IFN-I stimulation [16]. If the gut has been exposed to IFN-Is from migrating pDCs prior to barrier dysfunction, then microbial translocation may not synergistically increase ISG levels. The absence of a strong IFN-I response in suppressed HIV-1 infection relative to untreated HIV-1 infection raises questions as to whether the IFN-I signaling program has been 'reset,' such that exogenous IFN-Is would now elicit a strong antiviral program to reduce the gut latent HIV-1 reservoir. However, trends for higher ISGs such as Mx2 and APOBEC3G expression in treated HIV-1 infection compared with uninfected controls were still detected. It remains to be determined whether these low-level IFN-I responses still have immunomodulatory effects [34,35].

There are a several limitations to this exploratory study. To assess the role of HIV-1 replication in driving the colonic IFN-I signature, we obtained colonic biopsies from ART-treated HIV-1-infected and from HIV-1-uninfected persons through a tissue repository at RUMC. These two cohorts were matched for age, sex and ethnicity, but age and ethnicity differed between the RUMC cohorts and the UC-AMC cohorts. Furthermore, previously obtained measurements of mucosal immune activation and inflammation and colonic mucosa HIV-1 RNA levels were only available for the untreated HIV-1-infected cohort. This prevented direct assessment of the relationship between ISGs and indicators of mucosal immunity and tissue HIV-1 replication in the ART-treated cohort. Sexual practice has recently been reported to impact the intestinal microbiome and has also been linked to mucosal immune cell activation and inflammation independent of HIV-1 [70,71]. However, recruitment of study participants for this current study did not control for sexual practice. In addition, this study was not powered to investigate IFN-I gene expression and corrections for multiple comparisons have not been undertaken. Thus, the direct associations between ISGs and virological and immunological parameters observed in this current study must be interpreted with some caution. Nevertheless, our findings should inform the design of larger, statistically powered studies on the relationship between colonic IFN-I responses pre-ART and post-ART (ideally in the same individuals), colonic tissue HIV-1 RNA levels and indicators of immune activation while controlling for sexual practice, age, sex and ethnicity.

In conclusion, we provide evidence for a compartmentalized IFN-I response during chronic HIV-1 infection in the gut, with a predominant IFNβ response and high ISG levels that correlated with immune activation, microbial translocation and inflammation. HIV-1 replication is a major driver of the ISG response in the gut, as ART significantly reduces this response. These data may help

direct strategies to harness the IFN-I pathway to reduce chronic HIV-1 immune activation that is initiated and maintained in the gastrointestinal tract.

Acknowledgements

We gratefully acknowledge all the clinical study participants as well as the physicians and staff at the University of Colorado Infectious Disease Group Practice Clinic and Rush University Medical Center Section of Gastroenterology as well both University Hospital endoscopy clinics for their assistance with our clinical study. We thank Eric Lee and Andrew Donovan, Department of Medicine, University of Colorado Anschutz Medical Campus for technical assistance with the generation of data associated with our previous clinical study.

The present work was supported by the National Institutes of Health Grants R01 DK088663 (C.C.W.), R01 AI134220 (C.C.W. and M.L.S.). This work was performed with the support of the Translational Virology Core at the San Diego Center for AIDS Research (P30 AI036214).

Author contributions: S.M.D., K.G. performed laboratory experiments and data analysis; S.M.D., C.C.W., M.L.S. participated in study design, data analysis and wrote the primary version of the article; G.A. performed endoscopies; S.G. and A.L.L. assisted with clinical study assays; E.A.M., J.L., G.S. and A.K. participated in study design, enrolled study participants and performed endoscopic procedures as part of the RUMC gastrointestinal repository. P.A.E., P.C. and A.L. participated in study design, collected clinical data and coordinated sample inventories at RUMC. All authors participated in revisions of the article.

Funding support: This work was supported by the National Institutes of Health Grants R01 DK088663 (C.W.), R01 AI134220 (C.W. and M.L.S.) and the University of Colorado RNA Bioscience Institute (M.L.S. and C.C.W.) with the support of the Translational Virology Core at the San Diego Center for AIDS Research (P30 AI036214).

Disclaimers: The authors have no disclaimers.

Conflicts of interest

There are no conflicts of interest.

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