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HIV-1 Alters Intestinal Expression of Drug Transporters and Metabolic Enzymes: Implications for Antiretroviral Drug Disposition

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This study investigated the effects of HIV-1 infection and antiretroviral therapy (ART) on the expression of intestinal drug efflux transporters, i.e., P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP), and metabolic enzymes, such as cytochrome P450s (CYPs), in the human upper intestinal tract. Intestinal biopsy specimens were obtained from HIV-negative healthy volunteers, ART-naive HIV-positive (HIV$^+$) subjects, and HIV$^+$ subjects receiving ART (10 in each group). Intestinal tissue expression of drug transporters and metabolic enzymes was examined by microarray, real-time quantitative reverse transcription-PCR (qPCR), and immunohistochemistry analyses. Microarray analysis demonstrated significantly lower expression of *CYP3A4* and *ABCC2*/MRP2 in the HIV$^+$ ART-naive group than in uninfected subjects. qPCR analysis confirmed significantly lower expression of *ABCC2*/MRP2 in ART-naive subjects than in the control group, while *CYP3A4* and *ABCG2*/BCRP showed a trend toward decreased expression. Protein expression of MRP2 and BCRP was also significantly lower in the HIV$^+$ naive group than in the control group and was partially restored to baseline levels in HIV$^+$ subjects receiving ART. In contrast, gene and protein expression of *ABCB1*/Pgp was significantly increased in HIV$^+$ subjects on ART relative to HIV$^+$ ART-naive subjects. These data demonstrate that the expression of drug-metabolizing enzymes and efflux transporters is significantly altered in therapy-naive HIV$^+$ subjects and in those receiving ART. Since CYP3A4, Pgp, MRPs, and BCRP metabolize or transport many antiretroviral drugs, their altered expression with HIV infection may negatively impact drug pharmacokinetics in HIV$^+$ subjects. This has clinical implications when using data from healthy volunteers to guide ART.

Despite improved outcomes for persons with HIV, intestinal complications ranging from diarrhea, weight loss, nausea, vomiting, and abdominal pain to gastrointestinal (GI) bleeding, anorectal disease, and GI tumors continue to be common, even in those who have achieved undetectable viral loads and normal CD4$^+$ lymphocyte counts with the use of combination antiretroviral therapy (ART) (1, 2). The persistence of HIV reservoirs in the gut-associated lymphoid tissue (GALT) has been well documented, and detectable levels of HIV replication (RNA, DNA, and p24 protein) have been reported in tissue biopsy specimens from the upper and lower intestinal tracts (3–5). Studies have shown that ART-naive or treated subjects with HIV show decreased intestinal tissue expression of genes involved in mucosal repair and regeneration, while expression of genes regulating inflammation and immune activation is increased (2, 5–7). In addition, the expression of genes involved in intestinal epithelial integrity and barrier function, such as tight junctional proteins, as well as in nutrient and xenobiotic absorptive and digestive functions, such as drug-metabolizing enzymes, is also decreased in HIV-infected subjects compared to HIV-negative healthy volunteers (6). Collectively, these data suggest that HIV infection leads to the disruption of the integrity of the intestinal epithelial barrier and alteration in nutrient and drug absorption. The resulting microbial translocation has been hypothesized to contribute to HIV progression and the development of future comorbidity (8).

Antiretroviral drugs (ARVs) are reported to have highly variable penetration into the ileal and rectal tissues of HIV-infected subjects, and in some cases the tissue concentrations are significantly lower than therapeutic levels required for effective antiviral activity and much lower than the concentrations detected in plasma samples from the same individuals (9–11). Some studies also suggest that poor drug penetration into these intestinal tissues may contribute to the lack of a local therapeutic response and the establishment of an HIV reservoir at this site (10, 11). While drug transporters and metabolic enzymes are known to play a key role in drug disposition at the level of the intestinal epithelium and are suggested to contribute to the persistence of this viral reservoir, experimental evidence to support this rationale is currently lacking (9). Some ARVs (e.g., atazanavir and efavirenz) are also reported to have significantly different plasma pharmacokinetics in HIV-infected subjects and in healthy volunteers. This could potentially be due to the differences in the intestinal drug absorption associated with changes in drug metabolism and/or transport processes (12, 13).

Several efflux transporters belonging to the ATP-binding cassette (ABC) superfamily, such as P-glycoprotein (Pgp) (*ABCB1* gene product), multidrug resistance-associated proteins (MRPs)

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(*ABCC1* to -*5* gene products), and breast cancer resistance protein (BCRP) (*ABCG2* gene product), are expressed at the apical brush border membrane of enterocytes, where they can efflux drugs back into the lumen, lowering their cellular accumulation and transepithelial permeativity and collectively acting as the first biochemical barrier to drug absorption (14, 15). Several drug-metabolizing enzymes, such as oxidative cytochrome P450 (CYP) enzymes and conjugative UDP-glucuronyl transferases (UGTs), are also expressed within enterocytes and play an important role in the first-pass extraction of drugs via metabolic degradation. Lastly, members of the solute carrier (SLC) superfamily, such as various organic cation, organic anion, and nucleotide carriers, facilitate cellular entry of bulky and/or charged drugs, including many ARVs (16).

Systemic absorption and intestinal tissue concentrations of ARVs can be regulated by CYP enzymes and ABC and SLC drug transporters expressed in the intestinal epithelium. For example, CYP3A4 metabolizes HIV protease inhibitors (PIs), nonnucleoside analog reverse transcriptase inhibitors (NNRTIs), and CCR5 receptor antagonists such as maraviroc (17, 18). In addition, CYP2B6 plays an important role in the metabolism of NNRTIs (efavirenz and nevirapine) (19). Among drug transporters, Pgp has a wide range of drug substrates, including all PIs, some nucleoside/nucleotide analog reverse transcriptase inhibitors (NRTIs) such as abacavir and tenofovir disoproxil fumarate, as well as maraviroc and the integrase inhibitors raltegravir and elvitegravir (16, 20). MRP1 to -3 are also capable of transporting many PIs and some NRTIs, while MRP4 and -5 and BCRP are primarily responsible for the efflux of NRTIs (16). Tissue inflammation and cytokine secretion are known to alter functional expression of drug transporters and CYP enzymes in other disease states (e.g., inflammatory bowel syndrome) (21). Furthermore, we and other investigators have previously reported that the functional expression of drug transporters in brain parenchyma, blood-brain and blood-testis barriers, and sigmoid colon epithelium is altered by the HIV antigens, viral infection-associated inflammation, and ARVs (22–28). The main objective of this study was to investigate the impact of HIV-1 infection and suppressive ART on the expression of intestinal drug transporters and metabolic enzymes in the intestinal mucosa of HIV-infected subjects.

MATERIALS AND METHODS

Human subjects and sample collection. HIV-1-infected subjects were enrolled in the study and divided into two cohorts, ART naive (group B; plasma HIV load of >5,000 copies/ml and no prior exposure to ARVs) and long-term ART treated (group C; plasma HIV load of <50 copies/ml and receiving current ART regimen for >1 year). HIV-1-negative clinically healthy individuals (group A) were enrolled in the study to serve as HIV-negative controls. Jejunal mucosal biopsy samples were collected by gastrointestinal endoscopy under conscious sedation and immediately cryopreserved for transcriptional analysis of HIV RNA and host gene expression by real-time quantitative reverse transcription-PCR (qPCR). Tissue samples were also fixed in paraformaldehyde and embedded in paraffin blocks for immunohistochemical analysis according to a previously published protocol (4). A portion of the jejunal biopsy specimen was collected in RPMI 1640 (Invitrogen, Carlsbad, CA) lymphocyte isolation medium for flow cytometric analysis of the $CD4^+$ T cell subset (4, 29). In addition, peripheral blood samples were collected at the time of gut biopsy for measurement of plasma HIV loads (4, 29). The Institutional Review Board at the University of California, Davis, CA, approved this study protocol, and written informed consent was obtained from all study participants.

Gene expression profile analysis. We measured gene expression changes in the gut biopsy specimens from HIV-infected subjects compared to those from HIV-negative healthy individuals using human genome-specific high-density oligonucleotide microarrays as described previously (6, 30). Total RNA was isolated from jejunal tissue biopsy specimens of five HIV-1 negative healthy volunteers, seven HIV-positive (HIV^+) ART-naive subjects, and six HIV^+ subjects receiving long-term ART using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). mRNA was amplified, labeled, and hybridized to human whole-genome U95av2 GeneChips (Affymetrix) using standard protocols (Affymetrix, Santa Clara, CA). Cross-sectional analysis of microarray data among all groups, as well as background correction, normalization, and generation of expression values, was performed using dChip analysis software (DNA-Chip analyzer, version 1.3; Harvard University). Samples from HIV-negative participants were used as baseline values for identifying genes differentially expressed in HIV^+ naive participants and HIV^+ participants on ART. The differential expression was defined as gene expression a minimum of 1.5-fold higher or lower, with statistical confidence ($P \leq 0.05$), than the average gene expression observed in healthy control group.

Real-time qPCR analysis. qPCR analysis was performed to compare expression levels of specific genes between healthy HIV-negative individuals and ARV-naive and -treated HIV^+ subjects. Total RNA was reverse transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA), and the expression levels of *CYP3A4*, *ABCB1*, *ABCC2*, or *ABCG2* or the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were measured using 6-carboxyfluorescein (FAM)-labeled TaqMan gene expression assays and the primer-limited VIC/MGB probe for GAPDH (Life Technologies Inc., Burlington, Ontario, Canada) in the Mastercycler ep realplex 2S thermal cycler (Eppendorf Canada, Mississauga, Ontario, Canada). HIV RNA levels in intestinal tissue and plasma samples were measured by real-time qPCR as described previously (31, 32).

Immunohistochemistry analysis. Detection and localization of CYP3A4, Pgp, MRP2, or BCRP protein expression in intestinal tissue samples were done by immunohistochemical analysis. Intestinal tissue sections fixed in 4% paraformaldehyde were immunostained with a primary antibody specific to CYP3A4 (1:600 dilution; Sigma-Aldrich, Oakville, Ontario, Canada), Pgp (1:100 dilution; Santa Cruz Biotechnology Inc., Dallas, TX), MRP2 (1:250 dilution; Kamiya Biomedical Company, Seattle, WA), or BCRP (1:100 dilution; Abcam Inc., Toronto, Ontario, Canada). After washing, tissue sections were immunostained with the corresponding Alexa-488-conjugated secondary antibodies (Life Technologies Inc., Burlington, Ontario, Canada). Standard DAPI (4′,6′-diamidino-2-phenylindole) staining was utilized to identify cell nuclei, and the fluorescence images were obtained using a Zeiss LSM700 confocal microscope. See the figure legends for the image quantification procedure.

RESULTS

Study participants. The gut mucosal gene expression profiling was examined by DNA microarray analysis for five HIV-1-negative healthy volunteers with $CD4^+$ T cell counts in the normal range (632 to 1,203 cells/μl), seven HIV^+ naive subjects with plasma viral loads of >5,000 copies/ml and $CD4^+$ T cell depletion with low cell counts (6 to 427 cells/μl), and six HIV^+ subjects receiving long-term ART with undetectable viral loads (Table 1).

For qPCR and immunohistochemical analyses, 30 participants 25 to 60 years of age were enrolled in this study and divided among three groups (Table 2). Group B consisted of 10 chronically HIV-1-infected ARV-naive subjects who were HIV-1 seropositive for 1 to 10 years (median, 3 years) and had HIV RNA levels of 5,642 to 48,000 copies/ml and $CD4^+$ T cell counts ranging between 10 and 672 cells/μl (median, 265 cells/μl). Group C consisted of 10 HIV-1-infected subjects receiving ART who were HIV seropositive for 1

TABLE 1 Demographic and clinical characteristics of study subjects used in microarray analysis[a]

Group and patient	Age (yr)	Sex	CD4$^+$ cell count (cells/μl)	Yr since HIV-1 diagnosis	ART components	Yr on ART
A (HIV-1-seronegative healthy controls)						
P4	59	M	ND	NA	NA	NA
P15	52	M	ND	NA	NA	NA
P34	35	F	1,046	NA	NA	NA
P38	48	F	632	NA	NA	NA
P41	43	F	1,203	NA	NA	NA
Median (range)	48 (35–59)		1,046 (632–1,203)			
B (HIV-1-seropositive, ART naive)						
P13	48	F	8	5	NA	NA
P14	36	M	6	1	NA	NA
P19	35	M	46	1	NA	NA
P20	45	M	294	1	NA	NA
P24	34	M	34	4	NA	NA
P47	23	M	205	1	NA	NA
P48	42	M	427	1	NA	NA
Median (range)	36 (23–48)		46 (6–427)	1 (1–5)		
C (HIV-1-seropositive, receiving ART)						
P36	35	M	250	1	RTV, ABC, 3TC, AZT	1
P50	32	F	570	1	EFV, ABC, 3TC, AZT	1
P57	33	F	644	1	EFV, ABC, 3TC, AZT	1
P58	36	M	300	2	RTV, ABC, 3TC, AZT	1
P59	23	M	205	2	EFV, RTV, 3TC	2
P73	37	M	468	3	EFV, RTV, ABC, 3TC, AZT	3
Median (range)	34 (23–37)		384 (205–644)	1.5 (1–3)		1 (1–3)

[a] Abbreviations: M, male; F, female; ND, not determined; NA, not applicable; 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; EFV, efavirenz; RTV, ritonavir.

to 11 years (median, 6 years), were receiving long-term ART (1 to 11 years; median, 6 years), and had undetectable plasma viral loads (<50 copies/ml) and CD4$^+$ T cell counts ranging between 140 and 1107 cells/μl (median, 525 cells/μl). ART regimens contained NRTIs (10/10), PIs (7/10; 2 containing ritonavir-boosted atazanavir, 4 containing ritonavir as the only PI, and 1 containing atazanavir as the only PI), and/or efavirenz (1/10). Group A consisted of 10 HIV-seronegative healthy controls. None of the 30 participants reported being smokers, using any drugs of abuse, or currently using any over-the-counter medications or herbal supplements. Five of the HIV-seronegative participants reported alcohol use.

Distinct mucosal gene expression profiles in HIV infection with and without ART. In order to identify differentially expressed gene networks in HIV infection, gene expression profiles were evaluated by high-throughput oligonucleotide microarray analysis in the intestinal biopsy samples from the HIV$^+$ ART-naive subjects and HIV$^+$ subjects on ART compared to those from HIV-negative healthy controls. Hierarchical cluster analysis of the data revealed differential expression of several groups of genes regulating pharmacological and physiological functions, such as those for metabolic enzymes, drug uptake (SLC) and efflux (ABC) transporters, nuclear receptors, and tight junctional proteins, and genes associated with intestinal immune response and inflammation, (Fig. 1A). The gene expression of several CYP enzymes was suppressed in HIV-infected subjects compared to HIV-negative controls. *CYP3A4* expression was lower in HIV$^+$ naive subjects than in the healthy controls ($P < 0.001$). HIV$^+$ subjects receiving ART had significantly lower average expression of *CYP3A5* (3.7-fold, $P < 0.01$), *CYP2D6* (2.2-fold, $P < 0.01$), and *UGT2B7* (1.9-fold, $P < 0.05$) than HIV-negative subjects (Fig. 1B). Among ABC transporters, the expression of *ABCC2* (encoding MRP2) was significantly lower in all HIV$^+$ ART-naive subjects (2.1-fold, $P < 0.05$), and decreased expression of additional MRPs (MRP3, -6, and -7) was observed in some of the subjects. ART-treated HIV$^+$ subjects had significantly lower *ABCC3* (encoding MRP3) expression than HIV-negative subjects (3.6-fold, $P < 0.01$). The expression of *ABCB1* (Pgp) was highly variable among the six HIV$^+$ subjects receiving ART, with three subjects showing decreased *ABCB1* expression while two subjects had increased *ABCB1* expression compared to the HIV-negative controls. Similarly, changes in the gene expression of SLC transporters were variable in HIV-infected subjects and were clearly altered compared to controls (Fig. 1B). The expression of orphan nuclear receptors, i.e., pregnane X receptor, involved in regulating drug transporters and metabolic enzyme transcription, was downregulated in HIV$^+$ ART-treated subjects compared to controls. In contrast, expression of genes associated with the mucosal inflammatory response was elevated in HIV-infected subjects compared to controls. The expression of several genes, including those for interleukin-1α (IL-1α), IL-1β, IL-8, IL-1 receptor (IL-1R) type II, and mitogen-activated protein kinase 11 (MAPK-11) (i.e., p38-β), was upregulated in HIV$^+$ ART-treated subjects. Expression of several other genes involved in inflammatory response such as those for MAPK-9 (i.e., JNK2) and nuclear factor κB (NF-κB) remained upregulated in both cohorts of HIV-infected subjects.

Effect of HIV-1 infection on expression of drug metabolic enzymes and transporters. We investigated the impact of HIV infection on the mucosal expression of genes regulating the transport and metabolism of ARVs commonly used in HIV therapy.

TABLE 2 Demographic and clinical characteristics of study participants[a]

Group and patient	Age (yr)	Sex	Race	$CD4^+$ cell count (cells/μl)	Plasma viral load (copies/ml)	Yr since HIV-1 diagnosis	ART components	Yr on ART
A (HIV-1-seronegative healthy controls)								
P110	58	M	AA	ND	NA	NA	NA	NA
P112	60	M	C	ND	NA	NA	NA	NA
P130	35	F	C	1,057	NA	NA	NA	NA
P153	60	M	AA	442	NA	NA	NA	NA
P213	43	F	C	1,021	NA	NA	NA	NA
P214	36	M	C	1,559	NA	NA	NA	NA
P229	45	F	AA	803	NA	NA	NA	NA
P236	49	F	C	1,835	NA	NA	NA	NA
P238	28	F	C	ND	NA	NA	NA	NA
P239	36	F	C	777	NA	NA	NA	NA
Median (range)	44 (28–60)			1,021 (442–1,835)				
B (HIV-1-seropositive, ART naive)								
P108	38	M	C	672	5,642	5	NA	NA
P115	25	M	C	175	20,000	2	NA	NA
P120	36	M	C	10	480,000	4	NA	NA
P124	43	M	C	269	246,000	1	NA	NA
P152	48	M	C	184	29,200	10	NA	NA
P209	33	M	C	450	57,000	3	NA	NA
P210	30	M	AA	618	100,000	10	NA	NA
P211	47	F	AA	450	20,000	2	NA	NA
P223	30	F	C	192	18,000	1	NA	NA
P224	40	F	C	261	90,000	3	NA	NA
Median (range)	37 (25–48)			265 (10–672)	43,100 (5,642–48,000)	3 (1–10)		
C (HIV-1-seropositive, receiving ART)								
P120	45	M	C	525	<50	11	RTV, ABC, 3TC	11
P136	25	M	C	560	<50	2	RTV, ABC, 3TC, AZT	1
P140	35	M	AA	1,107	<50	1	ABC, 3TC, AZT	1
P173	31	M	C	140	<50	5	ATV, RTV, TDF, FTC	5
P189	50	F	C	585	<50	5	TDF, FTC, 3TC, AZT	9
P197	45	F	C	279	<50	11	ATV, RTV, TDF	10
P198	50	F	AA	508	<50	10	RTV, TDF, FTC	10
P208	36	F	C	322	<50	6	EFV, TDF, FTC	5
P219	44	F	AA	ND	<50	6	ATV, ABC, 3TC	6
P231	55	F	AA	1,039	<50	10	RTV, ABC, 3TC	6
Median (range)	44.5 (25–55)			525 (140–1,107)		6 (1–11)		6 (1–11)

[a] Abbreviations: M, male; F, female; AA, African American; C, Caucasian; ND, not determined; NA, not applicable; 3TC, lamivudine; ABC, abacavir; ATV, atazanavir; AZT, zidovudine; EFV, efavirenz; FTC, emtricitabine; RTV, ritonavir; TDF, tenofovir disoproxil fumarate.

Gene and protein expression of selected drug transporters and metabolic enzymes (*CYP3A4*, *ABCB1*, *ABCC2*, and *ABCG2*) in intestinal tissue biopsy specimens from HIV-infected and uninfected subjects (Table 2) were examined by real-time qPCR and immunohistochemistry, respectively. The qPCR data (Fig. 2) showed that the gene expression of *CYP3A4*, *ABCB1*, *ABCC2*, and *ABCG2* in the intestinal biopsy specimens was consistently decreased in HIV-infected subjects (Fig. 2). These results were in agreement with the findings for the gene expression changes based on the DNA microarray analysis. Long-term ART resulted in the restoration of expression of all of these genes. However, a certain degree of variability in the expression levels was observed among the six HIV^+ subjects receiving ART. ART-treated subjects had increased *ABCB1* expression compared to ARV-naive HIV^+ subjects (3.2-fold, $P < 0.05$). The microarray data set did not include *ABCG2* (i.e., BCRP), an apical efflux carrier that can transport NRTIs and hence has an important role in restricting drug tissue concentrations and transepithelial permeativity (16). *ABCG2* mRNA levels were decreased in HIV-infected subjects compared to HIV-negative healthy individuals but showed a trend of increased expression in HIV^+ subjects receiving ART (Fig. 2D).

For the selected four genes involved in the pharmacokinetics of ARVs (i.e., *CYP3A4*, *ABCB1*, *ABCC2*, and *ABCG2*), we evaluated their protein expression (i.e., CYP3A4, Pgp, MRP2, and BCRP, respectively) in intestinal biopsy specimens by immunohistochemical analysis (Fig. 3 and 4). Overall, the changes in protein

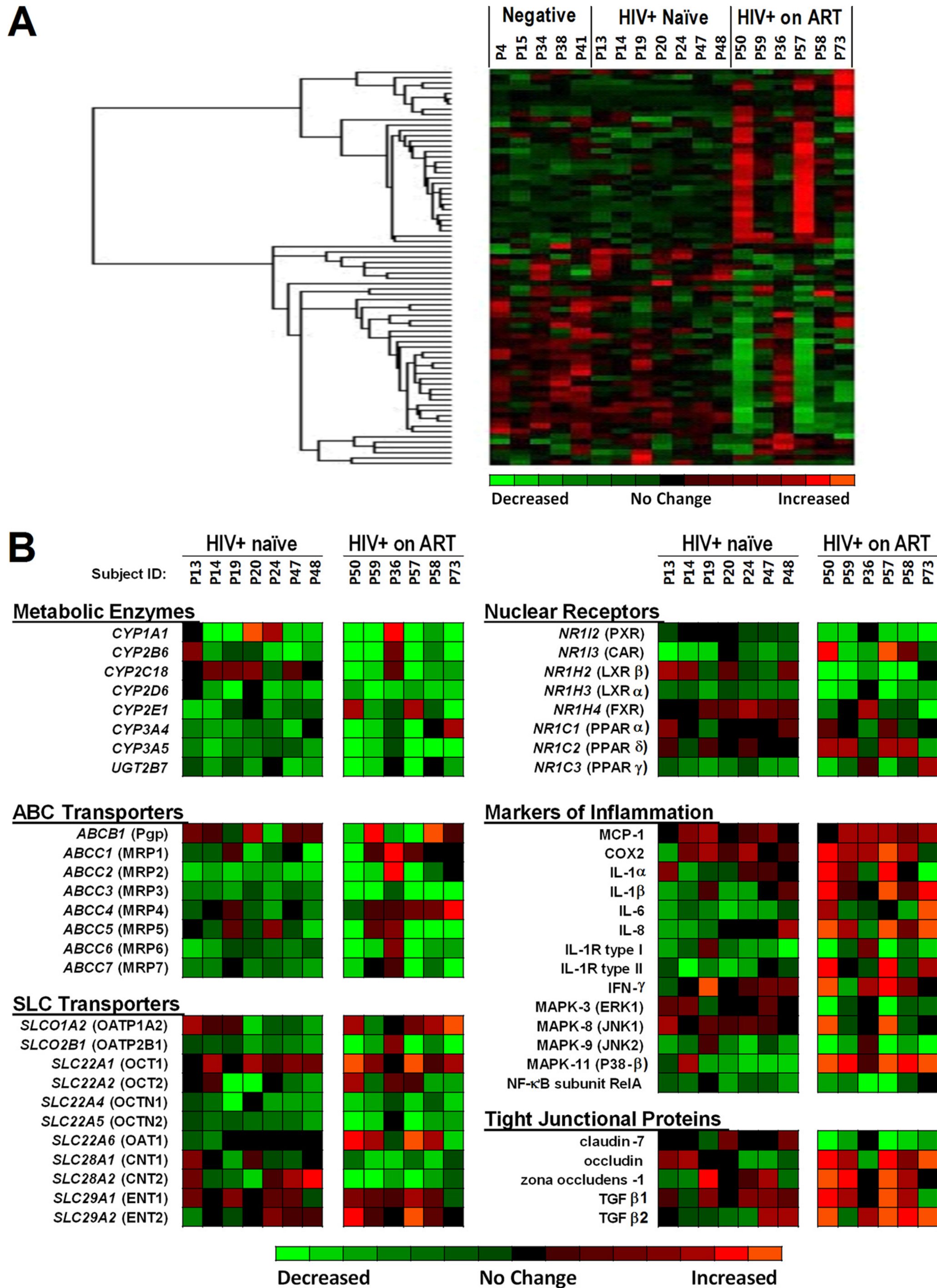

FIG 1 DNA microarray analysis of mucosal gene expression in treated and untreated HIV-1 infection. (A) Gene expression changes in the gut biopsy specimens of HIV-negative participants ($n = 5$), HIV$^+$ ART-naive participants ($n = 7$), and HIV$^+$ participants on ART ($n = 6$). The patients were arranged based on when the microarray sample was analyzed (see Materials and Methods for details). (B) Differentially expressed genes with important pharmacological and physiological functions, such as genes for metabolic enzymes, drug efflux and uptake transporters (i.e., ABC and SLC drug transporters), nuclear receptors, and tight junctional proteins, and genes associated with the intestinal immune response and inflammation, were identified through hierarchical cluster analysis. For clarity, the sequence of patients was organized by patient identification number matching the arrangement that was used in panel A. Abbreviations: CYP, cytochrome P450 enzyme; UGT, UDP-glucuronyl transferase; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; OCT, organic cation transporter; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; PXR, pregnane X receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; IL, interleukin; IFN, interferon; MCP, monocyte chemoattractant protein; COX, cyclooxygenase; MAPK, mitogen-activated protein kinase; NF, nuclear factor; TGF, transforming growth factor.

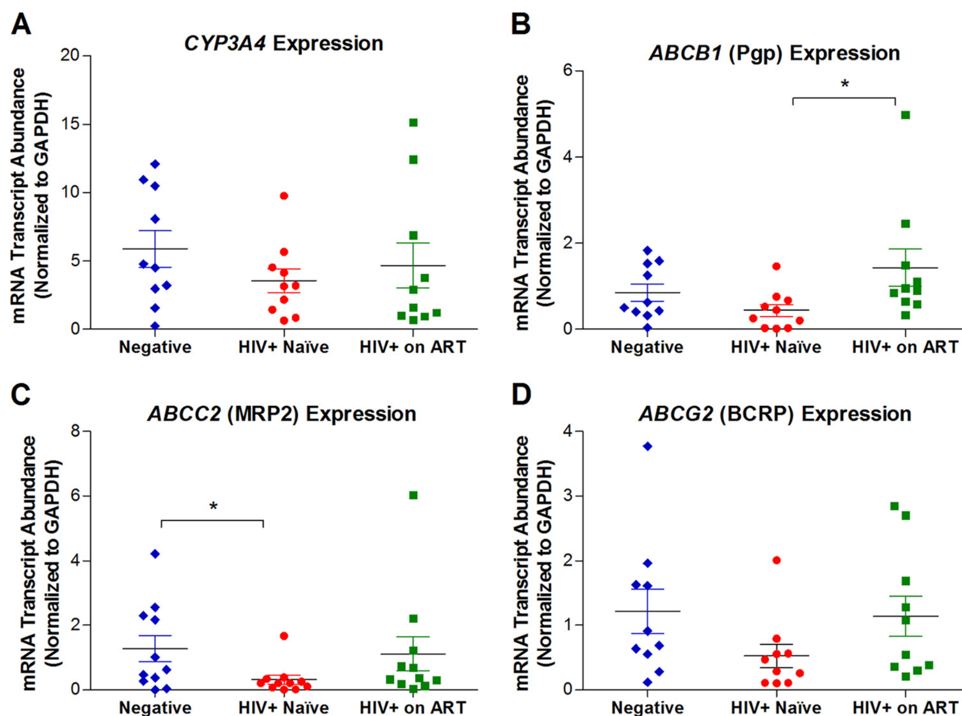

FIG 2 Real-time qPCR analysis of gene expression of selected intestinal metabolic enzymes and drug efflux transporters. Total RNA extracted from jejunal tissue biopsy specimen obtained from each subject was used for analysis of mRNA expression levels of each target gene using TaqMan gene expression assays specific for human *CYP3A4* (A), *ABCB1* (B), *ABCC2* (C), or *ABCG2* (D), obtained from Life Technologies Inc., Burlington, Ontario, Canada (assay Hs00604506_m1, Hs00184500_m1, Hs00166123_m1, or Hs01053790_m1, respectively). In each sample, the expression of the target gene was normalized to an internal control, GAPDH, by the ΔC_T method. Statistically significant differences in mRNA expression between groups (negative versus HIV$^+$ naïve, negative versus HIV$^+$ on ART, and HIV$^+$ naive versus HIV$^+$ on ART) were determined using GraphPad Prism (version 5.01; Graph Pad Software, San Diego, CA) by applying the nonparametric two-tailed Mann-Whitney test with significance defined by a P value of $<$0.05.

expression of these genes reflected the differences observed in the mRNA transcript levels. CYP3A4 expression showed a trend toward downregulation in HIV$^+$ therapy-naive subjects compared to healthy controls (1.9-fold decrease), and this effect was partially reversed in ART-treated HIV$^+$ subjects. Pgp expression was significantly upregulated in HIV$^+$ subjects receiving ART compared to the HIV$^+$ naive group (3.4-fold, $P < 0.01$) and was also higher than that in the healthy control group (3.3-fold, $P < 0.05$). Protein expression of both MRP2 and BCRP was significantly lower in HIV-infected ARV-naive subjects than in healthy controls (2.3- and 3.3-fold, respectively; $P < 0.05$). This downregulation appeared to be partially reversed in ART-treated subjects, with 2- and 1.7-fold-higher protein expression of MRP2 and BCRP, respectively, than in ARV-naive subjects.

DISCUSSION

In this study, we demonstrated differential expression of metabolic enzymes and drug transporters between healthy individuals and HIV-1-infected subjects. Chronically HIV-1-infected subjects without prior exposure to ART demonstrated a trend toward lower expression of CYP3A4 enzyme, which metabolizes all PIs and NNRTIs and many other drugs. Furthermore, drug efflux transporters that are localized to the brush border membrane, such as MRP2, which mediates efflux of PIs and NRTIs, and BCRP, which is capable of transporting many NRTIs and rilpivirine, were also downregulated in HIV-infected ARV-naive subjects (16). Given the localization of MRP2 and BCRP at the apical brush border membrane of enterocytes, downregulation in their protein expression is likely to lead to increased drug accumulation within enterocytes and increased drug permeability across the intestinal epithelium in the absorptive direction (33). These findings are in agreement with previously reported downregulation of drug efflux transporters in the sigmoid colon epithelia of ART-naive HIV-infected men, who had significantly lower Pgp and MRP2 protein expression than healthy individuals (27). Due to limited sample availability, we were unable to compare the concentrations of ARVs achieved in the intestinal tissue or blood plasma of HIV-infected and uninfected subjects. However, our data on differential protein expression of these enzymes/transporters caution against the use of pharmacokinetic data obtained in healthy volunteers to guide therapy in chronically HIV-1-infected ART-naive subjects due to the potential for treatment-associated intestinal tissue toxicity. Further studies are needed to evaluate the differences in tissue concentrations and plasma pharmacokinetics of ARVs between healthy volunteers and HIV$^+$ patients and to determine needed modifications to ART dosing regimens to help optimize ART efficacy and safety in HIV$^+$ patients.

In contrast to the observed downregulation of transporters and metabolic enzymes in ART-naive subjects, in ART-treated subjects, we observed a trend toward an increase in expression of several transporters and metabolic enzymes. In particular, Pgp expression was significantly higher in the ART-treated group than in ARV-naive subjects (3.4-fold, $P < 0.01$) or controls (3.3-fold, $P < 0.05$). These findings are in agreement with our previous

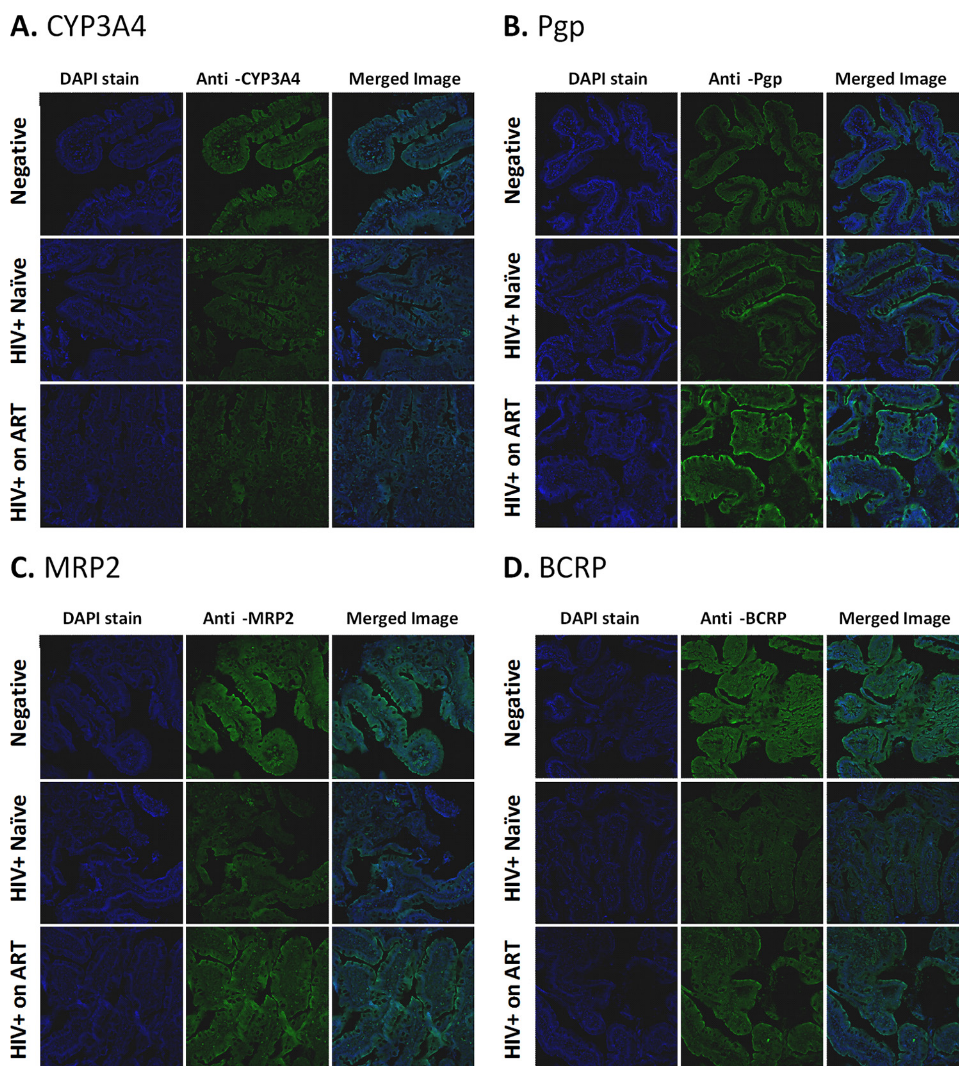

FIG 3 Immunohistochemistry analysis of protein expression of selected intestinal metabolic enzymes and drug efflux transporters. To detect protein expression and localization, paraffin-embedded jejunal tissue biopsy specimens from each subject, fixed in 4% paraformaldehyde and mounted onto glass slides, were immunostained with anti-CYP3A4 mouse polyclonal antibody from Sigma-Aldrich (Oakville, Ontario, Canada) at a 1:600 dilution (A), anti-Pgp mouse monoclonal D-11 antibody from Santa Cruz Biotechnology, Inc. (Dallas, TX) at a 1:100 dilution (B), anti-MRP2 mouse monoclonal M2III-6 antibody from Kamiya Biomedical Company (Seattle, WA) at a 1:250 dilution (C), or anti-BCRP rat monoclonal BXP-21 antibody from Abcam Inc. (Toronto, Ontario, Canada) at a 1:100 dilution (D). After washing, each tissue slice was immunostained with the corresponding Alexa-488-conjugated secondary antibodies, i.e., donkey anti-rat IgG for BCRP or donkey anti-mouse IgG for all other proteins (Life Technologies Inc., Burlington, Ontario, Canada). Standard DAPI staining was used to identify cell nuclei. For each gene of interest, the entire set of tissue slices was immunostained in a single experiment with all steps performed simultaneously, and images were obtained at constant exposure, zoom, and background settings using a Zeiss LSM700 confocal microscope.

study demonstrating 1.9- and 1.5-fold-higher Pgp and MRP2 protein expression, respectively, in the sigmoid colon biopsy specimens from HIV^+ subjects receiving ART than in the HIV^+ treatment-naive group and/or the control group (27). Pgp is expressed at the apical membrane of enterocytes, where it mediates active efflux of drug substrates, including all PIs, some NRTIs (e.g., abacavir and tenofovir disoproxil fumarate [TDF]), and other ARVs (e.g., raltegravir, elvitegravir, and maraviroc) back into the intestinal lumen (16, 33). Hence, increased Pgp expression can lead to increased clearance of these ARVs from intestinal tissue in ART-treated HIV^+ patients and will likely decrease their intracellular accumulation in enterocytes and permeativity across the intestinal epithelium. In addition, we observed that some ART-treated subjects had higher transcriptional expression of several drug uptake transporters (OCT1, OAT1, OATP1A2, ENT1, and ENT2), which could potentially compensate for the adverse effect of increased drug efflux and/or metabolism of common drug substrates such as PIs (substrates of organic anion transporting polypeptides [OATPs]), NRTIs (substrates of organic anion transporters [OATs], organic cation transporters [OCTs], concentrative nucleoside transporters [CNTs], and equilibrative nucleoside transporters [ENTs]), raltegravir (substrate of OAT1 and peptide transporter 1), and rilpivirine (substrate of OCT1) (16, 34–36). Due to limited tissue availability, in this study we did not evaluate protein expression of these drug uptake transporters. Interestingly, ritonavir is a known substrate of Pgp, so its accumulation within enterocytes and intestinal permeativity may be adversely impacted by increased Pgp-mediated efflux. Since most PI-based

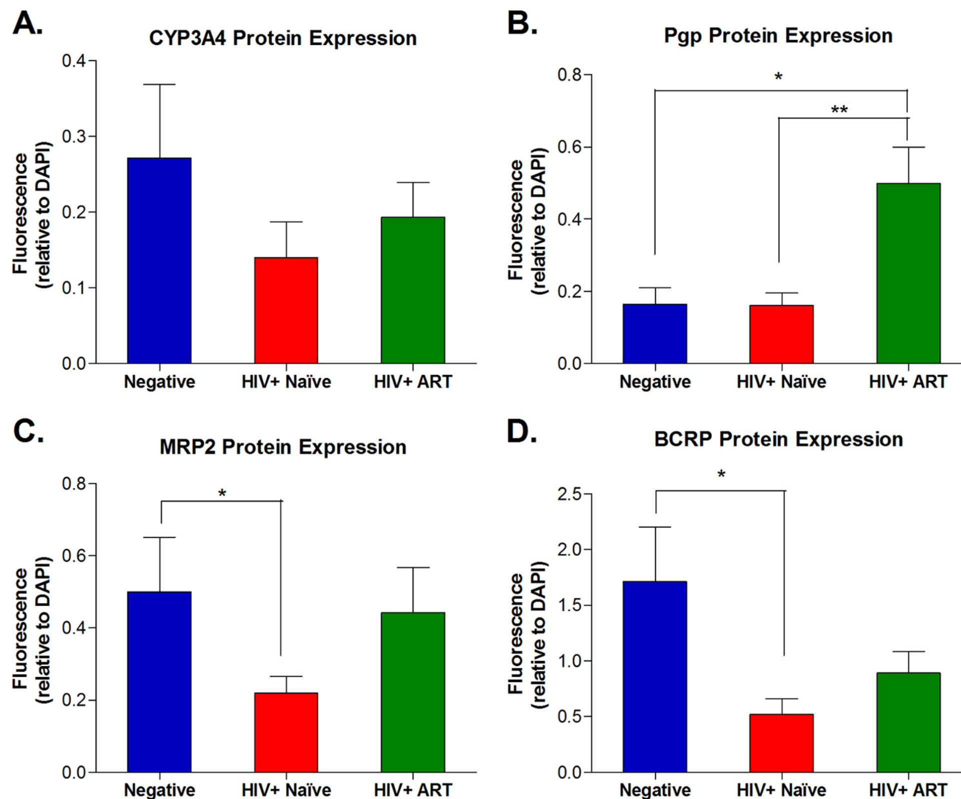

FIG 4 Group comparison of protein expression levels estimated from relative fluorescence intensities of immunostained jejunal tissue slices. For each protein of interest, all fluorescent images obtained from tissue slices from each subject were analyzed using the Image Pro Premier software (Media Cybernetic, Rockville, MD). The fluorescence intensity (integrated optical density [OD], lumens/pixel^2) for the protein of interest, i.e., CYP3A4 (A), Pgp (B), MRP2 (C), or BCRP (D), was normalized to the intensity of nuclear staining (i.e., the integrated OD measured for DAPI) within the same area. This analysis was performed in triplicate on three different areas within each tissue slice obtained from each study subject. The average normalized intensity ratio (gene of interest/DAPI) for each subject was used to estimate the mean $\pm$ standard error of the mean (SEM) for the study group. Statistically significant differences in protein expression between groups (negative versus HIV$^+$ naïve, negative versus HIV$^+$ on ART, and HIV$^+$ naive versus HIV$^+$ on ART) were determined using GraphPad Prism software, version 5.01 (Graph Pad Software, San Diego, CA) by applying the nonparametric two-tailed Mann-Whitney test with significance defined by a *P* value of $<$0.05.

ART regimens include ritonavir as a boosting agent to inhibit CYP3A4-mediated metabolism of other PIs, its inhibitory effect on CYP3A4 may be diminished in patients with induced Pgp expression, leading to higher metabolism and lower concentrations of other PIs and ARVs. Indeed, some ARVs (e.g., atazanavir and efavirenz) are reported to have lower plasma concentrations and bioavailability in HIV-infected subjects than in healthy volunteers (12, 13). Other factors may also contribute to the observed pharmacokinetic differences, such as race, ethnicity, gender, age, diet, altered composition of the gastrointestinal lumen (e.g., pH differences), modified barrier integrity (e.g., tight junctional proteins), and/or interplay between intestinal drug uptake, efflux, and metabolism of ARVs (12, 13). Furthermore, expression of drug transporters and enzymes may change over time, leading to progressive changes in tissue and plasma levels of ARVs in long-term-treated patients and resulting in late failure of a previously suppressive regimen. Additional studies investigating differences in ARV pharmacokinetics between HIV-1-infected ARV-naive subjects and well-matched healthy volunteers using a parallel-group design are needed to confirm the reported differences, further explore the mechanisms behind these changes, and determine appropriate dosing of ARVs in HIV-infected patients at different stages of treatment to help optimize the efficacy and safety of ART.

Microarray analysis performed on intestinal biopsy samples demonstrated that other genes with critical pharmacological functions, such as genes for nuclear receptors, inflammatory cytokines, and other mediators of the cellular inflammatory response, are also differentially expressed in HIV-1-infected compared to HIV-negative subjects. Our group and others have previously demonstrated that long-term ARV treatment can upregulate Pgp expression at the blood-brain barrier by directly interacting with orphan nuclear receptors (e.g., pregnane X receptor and constitutive androstane receptor), transcription factors that when activated can upregulate the expression of metabolic enzymes and drug transporters (28, 37, 38). In particular, atazanavir, efavirenz, ritonavir, and other PIs were found to activate human pregnane X receptor, while abacavir, efavirenz, and nevirapine were found to activate human constitutive androstane receptor (28). Since all HIV$^+$ ART-treated subjects enrolled in this study were receiving one or more of these drugs in combination with other ARVs, we propose that the observed upregulation of Pgp expression could be due to the interactions of these ARVs with nuclear receptors. In addition, several previous studies have reported regulation of drug transporters by HIV-1-associated pathogenesis and inflammation at other blood-tissue barriers and in the brain parenchyma. In cultured rat and human fetal astrocytes, HIV-1 envelope protein gp120 was demonstrated to trigger an inflammatory response via secretion of cytokines (IL-1β, IL-6, and tumor necrosis factor al-

pha [TNF-α]) which were found to regulate the expression of Pgp and MRP1 through NF-κB and JNK signaling pathways (22, 24, 39). Pgp expression was upregulated by IL-1β and TNF-α but profoundly downregulated by IL-6, with an overall decrease in expression with gp120 treatment or HIV-1 isolates exposure (24, 39). In contrast, MRP1 expression was upregulated by TNF-α and unaltered by IL-1β or IL-6, with an overall increase in expression following gp120 treatment (22). These effects were further confirmed *in vivo*. For example, intracerebral administration of gp120 to rodents was also shown to trigger the release of IL-1β, IL-6, TNF-α, and inducible nitric oxide as well as the activation of ERK1/2 and JNK pathways in different brain regions (40). Involvement of similar signaling pathways was also reported for the gp120-mediated inflammatory response in cardiac myocytes (41, 42). Our study provides initial evidence that these nuclear receptors and inflammatory pathways may be involved in differential regulation of intestinal expression of drug transporters and metabolic enzymes in the context of HIV infection and ART; however, further investigation is needed.

Persistent HIV replication in the GALT of HIV-infected subjects receiving long-term suppressive ART, despite undetectable plasma viral loads, continues to be one of the primary obstacles to HIV eradication and may be a source of persistent immune activation and inflammation that could contribute to HIV progression and comorbidity (2–6). Many studies have documented that the gut HIV reservoir may be the source for new HIV infection in circulating immune cells, leading to viral rebound and treatment failure in long-term-treated subjects (43–45). Although several factors may contribute to the formation of the viral reservoir in the GALT, subtherapeutic concentrations of ARVs in the intestinal tissue and the potential for emergence of drug resistance may play a role in the observed local HIV replication. Indeed, previous studies have demonstrated that ARVs can have low and often subtherapeutic concentrations in lymphocytes isolated from the ileum and rectum-associated lymphoid tissue even in subjects receiving long-term ART who have undetectable plasma viral loads and that these low drug concentrations are associated with high viral counts detected in these tissues (10, 11, 43). Although tissue drug concentrations in the upper intestinal tract have not been investigated to date, formation of a viral reservoir is observed in GALT tissue isolated from the upper jejunum of HIV-infected subjects (6, 31). Similarly to intestinal enterocytes, gut-associated lymphocytes also express several drug efflux (e.g., Pgp, BCRP, and MRP1, -2, -4, and -5) and uptake (e.g., OATP1A2, OATP3A1, and ENT1 and -2) transporters, which may be differentially expressed in HIV-1-infected and uninfected individuals and could affect the intracellular levels of ARVs achieved in this cellular compartment (16, 46, 47).

Several limitations are associated with the design of this study, such as a small number of study participants, the range of ARVs used, the variability in ART duration, and other confounding factors that may have impacted the expression of these enzymes and transporters, such as concurrent unreported use of natural compounds, illicit drugs, and/or other prescribed and nonprescribed medications. We also did not control for other potential confounding factors such as age, gender, and ethnicity. A larger study could be designed to account for some of these confounders and compare subjects with more versus less advanced HIV or those with good versus poor $CD4^+$ T cell recovery.

In summary, this study demonstrated that the expression of drug efflux transporters and metabolic enzymes can be significantly altered by both HIV-1 infection and ART. The data imply a high complexity of regulation of drug transporters and metabolic enzymes in the context of HIV-1 infection and its pharmacotherapy and caution against the use of pharmacokinetic studies in healthy volunteers to guide therapy or drug interaction issues in those with HIV.

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O. Kis performed qPCR analysis and immunohistochemistry imaging experiments evaluating gene and protein expression of the drug transporters and metabolic enzymes in gut tissue biopsy specimens, statistical data analysis, and preparation of figures and the first draft of the manuscript. The isolation of gut tissue biopsy specimens, analysis of HIV load and $CD4^+$ cell counts, assessment of other patient characteristics, and DNA microarray analysis were performed by S. Sankaran-Walters in the laboratory of S. Dandekar. All authors contributed to data analysis, preparation of figures, and statistical analysis. R. Bendayan provided expertise on the experimental design and preparation of the manuscript for submission. S. Dandekar provided guidance and supervision for the experimental design and analysis of the gut biopsy specimen collection and immunologic, molecular, and virologic analyses and contributed to the preparation of the manuscript.

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