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Hepatic expression profiles in retroviral infection: relevance to drug hypersensitivity risk

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Abstract

HIV-infected patients show a markedly increased risk of delayed hypersensitivity (HS) reactions to potentiated sulfonamide antibiotics (trimethoprim/sulfamethoxazole or TMP/SMX). Some studies have suggested altered SMX biotransformation in HIV infection, but hepatic biotransformation pathways have not been evaluated directly. Systemic lupus erythematosus (SLE) is another chronic inflammatory disease with a higher incidence of sulfonamide HS, but it is unclear whether retroviral infection and SLE share risk factors for drug HS. We hypothesized that retroviral infection would lead to dysregulation of hepatic pathways of SMX biotransformation, as well as pathway alterations in common with SLE that could contribute to drug HS risk. We characterized hepatic expression profiles and enzymatic activities in an SIV-infected macaque model of retroviral infection, and found no evidence for dysregulation of sulfonamide drug biotransformation pathways. Specifically, *NAT1*, *NAT2*, *CYP2C8*, *CYP2C9*, *CYB5R3*, *MARC1/2*, and glutathione-related genes (*GCLC*, *GCLM*, *GSS*, *GSTM1*, and *GSTP1*) were not differentially expressed in drug naïve SIVmac239-infected male macaques compared to age-matched controls, and activities for SMX *N*-acetylation and SMX hydroxylamine reduction were not different. However, multiple genes that are reportedly over-expressed in SLE patients were also up-regulated in retroviral infection, to include enhanced immunoproteasomal processing and presentation of antigens as well as up-regulation of gene clusters that may be permissive to autoimmunity. These findings support the hypothesis that pathways downstream from drug biotransformation may be primarily important in drug HS risk in HIV infection.

Abbreviations

COX, cyclooxygenase; FMO, flavin monooxygenase; HS, hypersensitivity; IFN, interferon; MPO, myeloperoxidase; SLE, systemic lupus erythematosus; SMX-HA, sulfamethoxazole-hydroxylamine; SMX-NO, sulfamethoxazole nitroso; TMP/SMX, trimethoprim/sulfamethoxazole.

Introduction

Potentiated sulfonamide antibiotics such as trimethoprim/sulfamethoxazole (cotrimoxazole or TMP/SMX) remain the drugs of choice for treatment and prevention of *Pneumocystis jirovecii* pneumonia, toxoplasma encephalitis, and Isospora infections in HIV infection (aidsinfo.nih.gov). However, HIV-infected patients show a markedly increased risk of delayed hypersensitivity (HS) reactions to TMP/SMX when compared to the general population (20–65% vs. 3% incidence). (Gordin et al. 1984; Kovacs et al. 1984; Medina et al. 1990; Hennessy et al. 1995;

Walmsley et al. 1998; Hughes et al. 2005; Chantachaeng et al. 2011) The typical manifestation of sulfonamide HS is maculopapular rash with or without fever, (Alfirevic et al. 2003; Yunihastuti et al. 2014) but can also include hepatotoxicity or other organ involvement. (Yang et al. 2014; Chang et al. 2016; Hernandez et al. 2016) The risk of sulfonamide HS increases with HIV disease progression, with higher risk seen at lower CD4 + counts. (Carr et al. 1993; Kennedy et al. 1993; Hennessy et al. 1995; Ryan et al. 1998; Rabaud et al. 2001; Eliaszewicz et al. 2002) This risk has been attributed, at least in part, to acquired alterations in SMX biotransformation in HIV infection. (Lee et al. 1993; Carr et al. 1994; Delomenie et al. 1994; Smith et al. 1997; Walmsley et al. 1998; Naisbitt et al. 2000; Wolkenstein et al. 2000)

The pathogenesis of sulfonamide HS involves bioactivation of SMX to a reactive metabolite, followed by an immune response to drug-adducted proteins in the skin, liver, or other tissues. (Cribb et al. 1996) SMX is bioactivated in the liver to an SMX-hydroxylamine (SMX-HA) metabolite by CYP2C8/9 or flavin monooxygenases (FMOs), or by myeloperoxidase (MPO) or cyclooxygenases (COX1, COX2) in other tissues (Fig. 1). (Cribb et al. 1990, 1995; Vyas et al. 2006) SMX-HA oxidizes spontaneously to SMX-nitroso (SMX-NO), which forms immunogenic adducts with endogenous proteins. (Naisbitt et al. 2001; Manchanda et al. 2002) These adducts can be processed and presented in association with MHC-I or MHC-II molecules; (Naisbitt et al. 2002; Roy-Chowdhury et al. 2007; Sanderson et al. 2007) this stimulates the development of cytotoxic T cells and antibodies that target altered self-antigens expressed on keratinocytes, platelets and other cells. (Curtis et al. 1994; Nassif et al. 2004) Thus, SMX HS can be considered an acquired autoimmune disorder.

The formation of immunogenic SMX-NO metabolites can be prevented by three recognized detoxification pathways: *N*-acetylation of SMX by *N*-acetyltransferases (NAT1 and NAT2), (Nakamura et al. 1995) reduction of SMX-HA by cytochrome b_5 and NADH cytochrome b_5 reductase, (Kurian et al. 2004) with a contribution from the mitochondrial enzymes mARC1 and mARC2, (Ott et al. 2014) and reduction of SMX-NO by antioxidants such as ascorbate and glutathione. (Cribb et al. 1991; Trepanier et al. 2004) It has been hypothesized that SMX detoxification pathways are impaired in HIV infection, and that this contributes to the increased risk of sulfonamide HS. (Delomenie et al. 1994; Lehmann et al. 1996; Smith et al. 1997) For example, impaired *N*-acetylation activity has been documented in vivo in some studies of HIV-infected patients, (Lee et al. 1993; Carr et al. 1994; Kaufmann et al. 1996) despite normal *NAT2* genotypes. (Quirino et al. 1999; Wolkenstein et al. 2000; O'Neil et al. 2002) However, the mechanisms for this impaired detoxification are not understood. Hepatic pathways of SMX biotransformation have not been directly evaluated in retroviral infection, and tissue expression profiling in SIV or HIV infection has been limited to blood cells, lymphoid organs, the brain, and the gut. (Bosinger et al. 2004; Roberts et al. 2004; George et al. 2006; Mehla and Ayyavoo 2012) We hypothesized that hepatic pathways of SMX biotransformation would be dysregulated in retroviral infection.

Sulfonamide HS risk in humans is also increased in another chronic inflammatory disease, systemic lupus erythematosus (SLE). (Petri and Allbritton 1992; Cooper et al. 2002; Pope et al. 2003; Aceves-Avila and Benites-Godinez 2008; Jeffries et al. 2008) SLE is a systemic autoimmune disorder that affects the skin, joints, kidneys, blood cells and other organs. (Yu et al. 2014) SLE patients have an 18–52% incidence of sulfonamide HS reactions, which is significantly higher than the general population or in patients with other systemic inflammatory diseases. (Petri and Allbritton 1992; Pope et al. 2003; Aceves-Avila and Benites-Godinez 2008; Jeffries et al. 2008) The mechanisms for this increased risk are not understood, although multiple genes have been shown to be dysregulated in SLE. (Feng et al. 2006, 2015; Obermoser and Pascual 2010; Kennedy et al. 2015) Therefore, we also hypothesized that SLE and retroviral infection share similar pathway alterations that may contribute to drug HS risk.

The aim of this study was to characterize hepatic expression profiles in retroviral infection, and determine whether retroviral infection leads to dysregulation of sulfonamide drug biotransformation pathways or of genes involved in autoimmunity, as manifested in SLE. We addressed this objective using liver tissues from drug naïve SIV-infected macaques, the principal model of HIV infection and pathogenesis, (Ambrose et al. 2007; Lackner and Veazey 2007; Valentine and Watkins 2008) compared to drug naïve sex- and age-matched uninfected controls.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except SMX-HA that was purchased from Dalton Chemicals (Toronto, CA), and *N*-acetyl SMX that was obtained from Frinton Laboratories (Hainesport, NJ).

Animals

Male rhesus macaques (*Macaca mulatta*) chronically infected with the pathogenic molecular clone virus

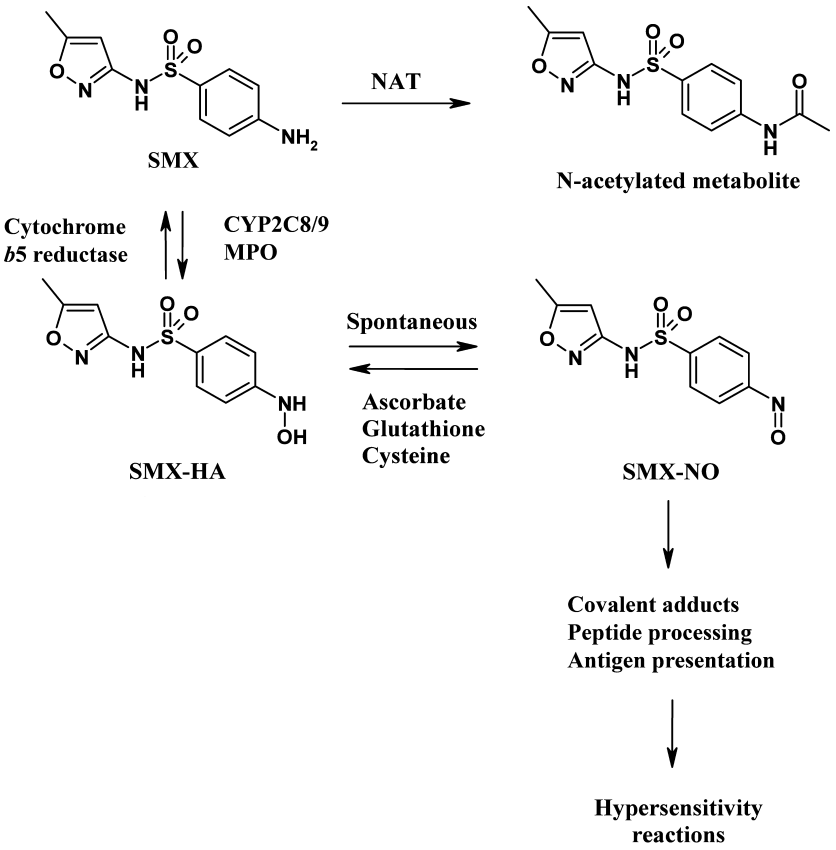

Figure 1. Biotransformation of sulfamethoxazole (SMX) with generation of the reactive metabolite SMX-NO (sulfamethoxazole-nitroso), which leads to drug-protein adducts that act as haptens. These adducted peptides are processed and presented in association with MHC-I or MHC-II molecules to generate drug specific T cells and autoantibodies that target the skin and other tissues and lead to clinical signs of delayed sulfonamide hypersensitivity. Pathways that promote generation of SMX-NO include cytochrome P450s 2C8 and 2C9, myeloperoxidase (MPO), flavin monooxygenases (not depicted) and cyclooxygenases (not depicted), all of which can oxidize SMX. Pathways that counteract generation of SMX-NO include *N*-acetyltransferases (NAT2 in liver and gut, and NAT1 in most tissues), cytochrome b_5 and its reductase, and the antioxidants glutathione, ascorbate, and cysteine.

SIVmac239, along with age and sex-matched uninfected controls, were obtained through the Wisconsin National Primate Research Center. Animals were studied during chronic infection, at least 10 weeks after inoculation. All macaques were screened prior to inclusion in the study with a physical exam, CBC, and serum biochemical panel. Infected animals also had a CD4 + count and viral load performed prior to sampling. All animals were fed a fixed formula global primate diet (Teklad, Harlan Laboratories, Madison WI), and no animals had a history of prior TMP/SMX exposure.

Liver sample collection and RNA isolation

Macaques were anesthetized with ketamine and dexmedetomidine, and 2–3 needle biopsies were obtained percutaneously from the liver under ultrasound guidance. Liver samples were placed in RNAlater (Ambion®) for 24 h at 4°C. Excess RNAlater was then removed, and samples were stored at −80°C. Total RNA was extracted by homogenizing liver samples in TRIzol (Ambion®) according to the manufacturer's protocol. RNA pellets were resuspended in RNase free water and were treated with 2U DNase I (Ambion®) at 37°C for 30 min, followed by inactivation with EDTA (5 mmol/L) and heating at 75°C for 10 min. RNase inhibitor (Applied Biosystems, Foster City, CA) was added to a final concentration of 1U/μL. RNA integrity (Schroeder et al. 2006) was assessed by Agilent 2100 BioAnalyzer, and RNA was quantified by NanoDrop ND-1000 (ThermoFisher, Madison, WI). RNA was stored at −80°C until preparation for arrays.

Microarray processing

For hepatic expression arrays, total RNA (100 ng) was used to generate sense-strand cDNA with the Ambion

WT Expression kit, and cDNA was fragmented and labeled with biotin by the GeneChip WT Terminal Labeling kit (Affymetrix, Santa Clara, CA). The fragmented and labeled cDNA was hybridized with the GeneChip Rhesus Gene 1.0 ST Array (Affymetrix). Microarrays were processed with the Affymetrix automated GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000 7G (Affymetrix), both controlled by the Affymetrix GeneChip Command Console Software v4.0.0.1567G.

Microarray data analyses

Raw data for hepatic transcript expression were exported from the Affymetrix platform and analyzed by GeneSpring GX (Agilent, Santa Clara, CA). Data were normalized by the Robust Multi-chip Average (RMA) method (molmine.com). (Irizarry et al. 2003) For liver tissue, gene expression was compared between SIV-infected and non-infected macaques using a moderated *t*-statistic (Smyth 2004) with ≥2-fold difference in expression and $P \leq 0.005$ as thresholds for significance. Microarray probe sets that had signal intensities lower than 100 in both SIV and control groups were excluded. Genes with significant differences in expression between groups at $P \leq 0.005$ (without a requirement for fold-change) were exported to DAVID Bioinformatics Resources 6.7 (david.ncifcrf.gov) for pathway analyses using the KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html). Interferon (IFN)-inducible genes were identified using INTERFEROME v2.01 (www.interferome.org), as annotated for humans (Rusinova et al. 2013).

Quantitative real-time PCR (qPCR) confirmation of transcript expression

Macaque hepatic RNA was used to confirm altered expression of selected transcripts by qPCR. cDNA was generated from 2.5 μg RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Primers and probes for recognizing sequence-specific DNA were designed using the Universal ProbeLibrary (Roche, Branchburg, NJ). cDNA was diluted 1:100 for qPCR, and incubated with gene-specific probes (0.125 μmol/L), and forward and reverse primers (670 μmol/L), using the FastStart Essential DNA Probes Master kit (Roche) and the LightCycler 96 Instrument (Roche). β-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was used as reference genes. (Hashemi et al. 2012) qPCR cycles were as follows: 95°C for 10 min pre-incubation, then 95°C for 10 sec, 60°C for 20 sec, for 55 amplification cycles. Primer efficiency was determined by performing qPCR for each gene with 1:10, 1:100, 1:1,000 and 1:10,000 dilutions of cDNA (Table S1). Statistical analyses for relative mRNA expression between groups were performed using relative expression software tool (REST) 2009 version1, which uses the methods described by Pfaffl and Vandesompele. (Pfaffl 2001; Pfaffl et al. 2002; Vandesompele et al. 2002; Pabinger et al. 2014).

Hepatic activity assays

To further evaluate phenotypic expression of key SMX detoxification pathways, additional banked liver samples (non-RNA quality) were obtained from SIVmac239-infected and control macaques from the Wisconsin National Primate Research Center. Liver cytosol and microsomes were prepared by standard ultracentrifugation, (Sacco and Trepanier 2010) and protein concentrations were determined by the Bradford assay (Bio-Rad). SMX-HA reduction to SMX was assayed in macaque hepatic microsomes. (Trepanier and Miller 2000) Briefly, 125 μg of macaque liver microsomal protein was incubated with 1 mmol/L SMX-HA in PBS with 1 mmol/L NADH, for 30 min at 37°C. Both ascorbic acid and reduced glutathione were added to final concentrations of 1 mmol/L to prevent SMX-HA oxidation. Control and SMX standards were incubated with human serum albumin instead of microsomal protein. Reactions were terminated by adding 50% volume ice-cold methanol. The reaction mix was centrifuged at 16,000*g* for 10 min at 4°C, and the supernatant was filtered and subject to high performance liquid chromatography (HPLC) analysis as previously described (Trepanier and Miller 2000), with a retention time for SMX of 8.3 min. *N*-acetylation of SMX was determined in macaque hepatic cytosols. SMX (300 μmol/L) was incubated with 250 μg cytosolic protein and 3 mmol/L acetyl CoA for 20 min at 37°C. Both control and *N*-acetyl SMX standards were incubated with human serum albumin instead of cytosolic protein. Reactions were terminated by adding 10% volume of ice-cold 15% perchloric acid. The reaction mix was centrifuged and filtered as for the SMX-HA reduction assay, prior to HPLC analysis as previously described, (Trepanier et al. 1998), with a retention time of 10.4 min for *N*-acetyl SMX. Activities were compared between groups using Mann–Whitney tests, and are reported as medians with observed ranges.

Results

Hepatic expression: SMX biotransformation pathways

High-quality RNA samples, based on an RNA integrity number of 7.0 or higher, (Schroeder et al. 2006) were

Table 1. Differential expression of genes involved in SMX biotransformation in livers from SIVmac239-infected male macaques ($n = 3$) compared to livers from age-matched uninfected males ($n = 3$).

Pathway	Gene	Fold change	Uncorrected *P* value
SMX bioactivation	*CYP2C8*	−1.01	0.939
	CYP2C9	−1.11	0.319
	MPO	−1.27	0.026
	PTGS1	1.25	0.043
	PTGS2	−1.14	0.169
	FMO3	−1.02	0.894
SMX and SMX-HA detoxification	*NAT1*	1.10	0.291
	NAT2	1.23	0.052
	CYB5R3	−1.32	0.187
	MARC1/2	1.04	0.615
Glutathione synthesis and recycling	*GCLC*	1.04	0.882
	GCLM	1.06	0.739
	GSS	1.01	0.951
	GSTM1	−1.09	0.745
	GSTP1	1.40	0.276

PTGS1 and *PTGS2* encode COX1 and COX2, respectively. *CYB5A*, encoding cytochrome b_5, was not annotated in the array.

selected for array analyses from SIV-infected male macaques (7.2–18.3 years old, viral loads 453,000–10,868,000 vRNA copies/ml plasma; CD4 + counts 49–355 cells/μL, $n = 3$) and age-matched male controls (7.3–20.5 years old, $n = 3$). Genes encoding SMX biotransformation pathways, including SMX oxidation (*CYP2C8*, *2C9, MPO, FMO3, PTGS1 (COX1)* and *PTGS2 (COX2)*), SMX *N*-acetylation (*NAT1/NAT2*), or SMX-HA reduction (*CYB5R3, MARC1/2)* were not differentially expressed (Table 1). Genes involved in glutathione synthesis or conjugation pathways, including *GCLC, GCLM, GSS*, *GSTM1* and *GSTP1,* were also not differentially expressed (Table 1).

In order to further explore the effect of retroviral infection on SMX biotransformation pathways, enzymatic detoxification of SMX and SMX-HA was evaluated in banked livers from five SIV-infected male macaques (ages 7.2–17.3 years, viral loads 443,000–792,000 copies/ml; CD4 + counts 175–1149 cells/μL; $n = 5$) and five non-infected male macaques (ages 3.4–19.3 years, $n = 5$). *N*-acetylation of SMX did not differ significantly between groups (median activity 0.11 nmol/mg/min in SIV group versus 0.09 nmol/mg per min in controls, $P = 0.30$; Fig. 2A), and the infected macaques with the lowest CD4 + counts did not have the lowest *N*-acetylation activities. Further, hepatic reduction of SMX-HA was not impaired in SIV infection (median 0.54 nmol/mg per min in the SIV group versus 0.43 nmol/mg/min in controls, $P = 0.55$; Fig. 2B).

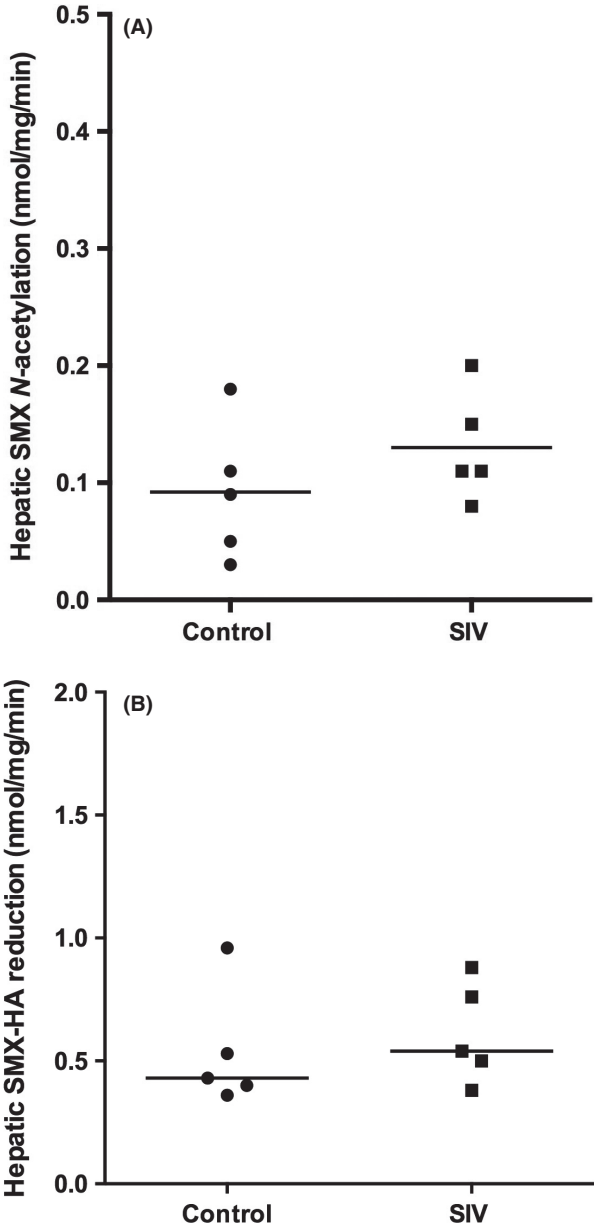

Figure 2. (A) *N*-acetylation of SMX in liver cytosols from SIV-infected and non-infected control macaques ($P = 0.30$ between groups). B) Hepatic reduction of SMX-HA in liver microsomes from the same macaques ($P = 0.55$ between groups).

Hepatic expression: SLE-associated genes and other pathways

Although the expression of key SMX biotransformation genes was not detectably altered by SIV infection, 154 transcripts were differentially expressed in the livers of SIV-infected and control macaques at the $P \leq 0.005$ threshold. Of these, 138 probe sets were associated with

an identified gene, 59 genes showed a twofold or greater difference in expression, and 30 genes showed a threefold or greater difference in expression (Table 2). These data have been deposited in NCBI's Gene Expression Omnibus, (Edgar et al., 2002) and are accessible through GEO Series accession number GSE87185 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87158).

Multiple genes that were significantly up-regulated in livers of SIV infected macaques (3.5–14.2–fold), are also up-regulated in tissues of patients with SLE, including *MX1*, (Feng et al. 2006; Aranow et al. 2015) *IFI6*, (Higgs et al. 2011) *OAS2*, (Bing et al. 2016) *IFI44* and *IFI44L*, (Higgs et al. 2011) *HERC5*, (Kennedy et al. 2015) *STAT1*, (Bing et al. 2016) *IFIT1*, (Aranow et al. 2015) *EPSTI1*, (Ishii et al. 2005; Kennedy et al. 2015) *CMPK2*, (Kennedy et al. 2015) *IFIH1*, (Robinson et al. 2011) *ISG15*, (Feng et al. 2006; Care et al. 2016) and *USP18* (Coit et al. 2013) (Tables 2, 3). A DAVID pathway analysis for the 138 differentially expressed genes in liver tissue revealed five pathways that were altered by SIV infection: genes dysregulated in SLE, as well as immunoproteasomal degradation, antigen presentation, RIG-I-like receptor signaling, and ISGylation (Table 3).

Validation by qPCR was performed for selected pathway genes, to include key genes overexpressed in patients with SLE (*HERC5* and *ISG15)*, (Feng et al. 2006, 2015; Care et al. 2016) those involved immunoproteasomal degradation (*PSME1*, *PSME2, PSMB10*), or those not previously reported in retroviral infection (*HERC6* and *PSMB10*). Expression of all transcripts was confirmed to be up-regulated by qPCR in the livers from SIV-infected animals compared to controls (3.2–91.9 fold), with $P \leq 0.001$ (Table S2).

Discussion

An increased risk of sulfonamide hypersensitivity is observed in HIV-infected patients, (Gordin et al. 1984; Medina et al. 1990; Hennessy et al. 1995; Walmsley et al. 1998; Chantachaeng et al. 2011) and although acquired differences in SMX biotransformation have been proposed, (Lee et al. 1993; Carr et al. 1994; Delomenie et al. 1994; Smith et al. 1997; Walmsley et al. 1998; Naisbitt et al. 2000; Wolkenstein et al. 2000; Trepanier et al. 2004) the mechanisms for this higher risk are not fully understood. The autoimmune disease SLE is another chronic inflammatory disease with a higher risk of HS reactions to sulfonamide antibiotics. We wanted to determine, therefore, whether retroviral infection led to alterations in hepatic SMX biotransformation pathways or to changes in gene expression that parallel those reported in human patients with SLE.

Because of the ethical and practical constraints to obtaining liver tissue from drug-naïve HIV-infected human patients, we addressed this question using an SIVmac239-infection model. SIVmac239, which is pathogenic in rhesus macaques, and HIV-1 are closely related retroviruses that share key similarities in gene organization, receptor recognition, cell tropism, and CD4 + depletion.(Cullen and Garrett 1992; Ambrose et al. 2007; Lackner and Veazey 2007; Valentine and Watkins 2008) SIVmac239 infection leads to immunosuppression in rhesus macaques, with progression to diarrhea, wasting, and opportunistic infections that models the clinical progression to AIDS in humans. (Lackner and Veazey 2007; Valentine and Watkins 2008).

Using this model, we found no effect of retroviral infection on hepatic expression of any genes known to be involved in SMX biotransformation, including those in the major enzymatic detoxification pathway, *N*-acetyltransferase. We expanded on the relatively small group of animals available for hepatic expression arrays with activity assays in additional liver samples, and also found no difference in *N*-acetylation of SMX. Further, in a recent TMP/SMX dosing study in the same population of macaques, (Wong et al. 2016) we found no differences in 24-hour urinary concentrations of *N*-acetylated SMX in SIV-infected versus control animals (54.4% of total urinary metabolites versus 55.1%, respectively, $n = 7$ in each group, $P = 0.45$). In HIV-infected patients, some studies have found impaired *N*-acetylation, using caffeine or dapsone as in vivo probe drugs, in individuals with normal *NAT2* genotypes. (Carr et al. 1994; Wolkenstein et al. 2000; O'Neil et al. 2002) This acquired metabolic defect has not been found in all studies, however (van der Ven et al. 1995; Kaufmann et al. 1996), and may be more pronounced in patients with CD4 + counts <200 cells/μL. (Quirino et al. 1999) SIV-infected macaques in our liver activity studies had CD4 + counts ranging from 175 to 1149 cells/μL (median 462 cells/μL), so we may have missed an effect of advancing disease on NAT expression and activity because some macaques were too early in their disease progression. A longitudinal study in SIV-infected macaques with progressive depletion in CD4 + T cells would help to determine whether impaired in vivo *N*-acetylation of SMX is truly observed with advancing disease.

Hepatic reduction of SMX-HA by cytochrome b_5 reductase is another enzymatic pathway for SMX detoxification, and we found no detectable differences in hepatic expression of *CYB5R3* or in vitro hepatic SMX-HA reduction in SIV infection. While this pathway has not been directly assessed in HIV infection, two studies found *decreased* concentrations of urinary SMX-HA metabolites in HIV-infected patients compared to healthy controls after dosing with TMP/SMX. (Lee et al. 1994; van der Ven et al. 1995) We found similar results in our recent

Table 2. Genes that are differentially expressed in livers from male macaques chronically infected with SIVmac239 compared to age-matched male controls.

Gene	Fold-change	Encoded protein	Plausibility in retroviral infection	Possible role in acquired autoimmunity
MX1[1]	14.2	MX dynamin-like GTPase 1	Innate viral restriction factor. Up-regulated in lymphocytes of HIV-1 rapid progressors (Rotger et al. 2011)	Expressed as dendritic cells mature from antigen-capture to T-cell-stimulating phenotype. (Cella et al. 1999) Up-regulated in SLE (Feng et al. 2006; Aranow et al. 2015)
IFI6[1]	12.3	Interferon alpha-inducible protein 6	Up-regulated in lymphocytes of HIV-1 rapid progressors (Rotger et al. 2011)	Increased expression in leukocytes of SLE patients (Higgs et al. 2011)
OAS2[3]	10.1	2′-5′-Oligoadenylate synthetase 2	Activates RNAases that degrade viral RNA. Up-regulated in monocytes of HIV-infected patients with high viral loads; inversely proportional to CD4 + lymphocyte counts (Fagone et al. 2016)	Up-regulated in leukocytes of SLE patients (Bing et al. 2016)
HERC6	10.0	Member of the HERC family of ubiquitin ligases	Interacts with ISG15 (Sanchez-Tena et al. 2016)	
IFI44L[1]	7.8	Interferon-induced protein 44-like	Up-regulated in lymphocytes of HIV-1 rapid progressors (Rotger et al. 2011)	Increased expression in patients with SLE (Higgs et al. 2011) and Sjögren's syndrome (autoimmune disorder) (Khuder et al. 2015)
IFI44[1]	6.7	Interferon-induced protein 44	Up-regulated in lymphocytes of HIV-1 rapid progressors (Rotger et al. 2011)	Increased expression in leukocytes of SLE patients (Higgs et al. 2011)
DDX60	6.1	DEAD box polypeptide 60	Helicase that acts as a viral sensor	
HERC5[1]	5.8	Member of the HERC family of ubiquitin ligases	Inhibits replication of HIV (Woods et al. 2011)	Expression correlates with high autoantibody titers in SLE (Kennedy et al. 2015)
IFIT3[1]	5.4	Interferon-induced protein with tetratricopeptide repeats 3	Up-regulated in the brains of SIV-infected monkeys, (Winkler et al. 2012) and HIV-infected patients (Siangphoe and Archer 2015)	Up-regulated in Sjögren's syndrome (Khuder et al. 2015)
STAT1	5.2	Signal transducer and activator of transcription 1,91 kDa	Transcription factor involved in expression of IFN-inducible genes. Up-regulated in CNS of HIV-infected patients (Siangphoe and Archer 2015)	Up-regulated in SLE (Bing et al. 2016) and Sjögren's syndrome (Khuder et al. 2015)
MX2[1]	5.0	Myxovirus (influenza virus) resistance 2	Mediates resistance to HIV-1; (Goujon et al. 2013) up-regulated in dendritic cells transduced with HIV-1 Vpr (Zahoor et al. 2015)	
RIG-I (DDX58)	5.0	Retinoic acid-inducible gene I	Sensor of double-stranded viral RNA Up-regulated in CNS of SIV-infected macaques (Co et al. 2011)	Activation of RIG-I-like receptors associated with several autoimmune diseases (Kato and Fujita 2015)
DNAH2-like	4.9	Dynein heavy chain 2, axonemal	Involved in HIV localization within the cell (Lehmann et al. 2009)	Involved in antigen clustering in B cells and subsequent B cell activation (Schnyder et al. 2011)
IFIT1[1]	4.8	Interferon-induced protein with tetra-tricopeptide repeats 1	Sensor of viral single-stranded RNAs; up-regulated in dendritic cells transduced with HIV-1 Vpr (Zahoor et al. 2015)	Up-regulated in Sjögren's syndrome (Khuder et al. 2015) and SLE (Aranow et al. 2015)
EPSTI1[1]	4.3	Epithelial-stromal interaction protein 1		Up-regulated in Sjögren's syndrome, SLE and immune thrombocytopenia. (Ishii et al. 2005; Khuder et al. 2015) Expression correlates with high autoantibody titers in SLE (Kennedy et al. 2015)
CMPK2	4.2	Cytidine monophosphate kinase 2, mitochondrial		Expression correlates with high autoantibody titers in SLE (Kennedy et al. 2015)

(*Continued*)

Table 2. Continued.

Gene	Fold-change	Encoded protein	Plausibility in retroviral infection	Possible role in acquired autoimmunity
GBP1[1]	4.0	Guanylate binding protein 1	Large IFN inducible GTPase. Up-regulated in CNS of HIV-infected patients (Siangphoe and Archer 2015)	
UBQLNL	3.8	Ubiquilin-like protein	May target ubiquinated proteins for proteasomal degradation, but still not well characterized (Marin 2014)	
IFIH1 (MDA5)[1]	3.7	Interferon-induced with helicase C Domain 1	Sensor of viral nucleic acids. Up-regulated in CNS of SIV-infected macaques (Co et al. 2011)	Gain of function *IFIH1* polymorphism associated with SLE (Robinson et al. 2011)
TRIM14	3.6	Tripartite motif-containing 14	Important for RIG-I antiviral pathway signaling.(van der Lee et al. 2015) Up-regulated in CNS of HIV-infected patients (Siangphoe and Archer 2015)	
ISG15[1]	3.6	ISG15 ubiquitin-like modifier	Up-regulated in CNS of HIV-infected patients; (Siangphoe and Archer 2015) lymphocyte expression correlates with viral load (Scagnolari et al. 2016)	Over-expressed in SLE. (Feng et al. 2006; Care et al. 2016)
UNC45B	3.6	UNC-45 Myosin Chaperone B	Unknown	
USP18	3.5	Ubiquitin specific peptidase 18	Involved in innate antiviral responses; cleaves ISG15 conjugates. Up-regulated in HIV/HCV co-infection (Fernandez-Botran et al. 2014)	Over-expressed in SLE. (Coit et al. 2013)
UBCH8-like	3.4	Ubiquitin conjugating enzyme E2L6-like	Required for efficient antigen cross-presentation by dendritic cells (Ebstein et al. 2009)	
PARP14	3.4	Poly(ADP-ribose) polymerase family, member 14	Promotes T-helper 2 cell differentiation (Riley et al. 2013)	
IFIT2[1]	3.3	Interferon-induced protein with tetratricopeptide repeats 2	Increased expression in dendritic cells transduced with HIV-1 Vpr (Zahoor et al. 2015)	Up-regulated in Sjögren's syndrome; (Khuder et al. 2015) expression related to disease activity in SLE (Zhao et al. 2010)
EIF3J	3.1	Eukaryotic translation initiation factor 3, subunit J	Participates in the initiation of translation	
XAF1[1]	3.1	XIAP associated factor 1	Mediates TNF-α induced apoptosis	
DHX58 (LGP2)	3.1	DEXH (Asp-Glu-X-His) box polypeptide 58	Acts as a regulator of antiviral signaling	.
DNHD1	3.0	Dynein heavy chain 2, axonemal-like	Dynein motor needed for HIV localization within the cell (Lehmann et al. 2009)	

Fifty-nine genes showed a twofold or greater difference in expression; those with threefold or greater expression are shown, at $P \leq 0.005$. Unless otherwise referenced, gene functions are abstracted from genecards.org.

[1]Indicates interferon-inducible gene (from Interferome v2.01). Genes known to be up-released in SLE are highlighted in bold.

Table 3. Results of pathway analyses for genes that are differentially expressed in the livers of SIV-infected macaques compared to non-infected controls.

Pathway	Gene	Fold change	*P* value
RIG-I-like receptor signaling	*RIG-I (DDX58)*[3]	5.0	<0.001
	IFIH1[3]	3.6	<0.001
	DHX58[3]	3.1	<0.001
ISGylation	***HERC6***[3]	10.0	<0.001
	HERC5[3,1]	5.8	<0.001
	UBQLNL	3.8	0.002
	ISG15[3,1]	3.6	<0.001
	USP18[3,1]	3.5	<0.001
Systemic lupus erythematosus[1]	***MAMU-DQA1***[3,2]	2.8	0.001
	HIST1H2AC[3]	2.0	0.002
	FCGR3 (CD16)[3]	1.9	0.001
	HIST1H3D	1.9	<0.001
	C1QC[3]	1.8	<0.001
	C1QA[3]	1.7	0.002
Immunoproteasomal degradation	*PSME2 (PA28β)*[3]	1.5	0.003
	PSME1(PA28α)[3]	1.5	0.001
	PSMB10 (MECL1)[3]	1.3	0.002
Antigen presentation	*B2M*[3]	3.1	0.002
	MAMU-E	2.3	0.002
	TAP1[3,1]	2.0	<0.001
	MAMU-A	2.0	<0.001
	MAMU-F	1.7	0.003

Genes in pathways were identified through DAVID analysis; additional genes up-regulated in SLE from the literature are listed in Table 2 and are summarized below.

[1]Additional genes up-regulated in SLE, from the primary literature: *ISG15, USP18, IFI6, OAS2, HERC5, CMPK2, EPSTI1* and *STAT1* (fold-increases in our study and references listed in Table 2); and *IFI27* (Bing et al. 2016) (increased by 2.3-fold in our study, $P = 0.003$).

[2]SLE pathway analysis refers to human ortholog (HLA-DQA1).

[3]Indicates interferon-inducible gene (from Interferome v2.01). Genes not previously associated with SIV or HIV infection are in bold.

in vivo TMP/SMX dosing study in the same macaque population. (Wong et al. 2016) Based on normal hepatic SMX-HA reduction activities found in the present study, decreased urinary SMX-HA in retroviral infection may reflect further oxidation of SMX-HA to SMX-NO (which is not detected in urine due to instability) rather than altered SMX-HA reduction by cytochrome b_5 reductase.

Glutathione-related genes, including GCLM, GCLC, GSS, and GSTs, were also not differentially expressed in the livers of SIV-infected macaques. A small study in HIV-infected patients found down-regulation of GCLC and GSS protein expression in erythrocytes, but transcript levels were not evaluated. (Morris et al. 2014) Furthermore, the retroviral protein Tat (found in both HIV and SIV viruses) led to down-regulation of GCLM (glutamate-cysteine ligase regulatory subunit) and decreased hepatic glutathione concentrations when over-expressed in transgenic mice. (Choi et al. 2000) However, only one of two murine GCLM transcripts was down-regulated by Tat, and our macaque array may have been insensitive to this differential finding. Hepatic glutathione concentrations were not measured in our study because of insufficient tissue available from in vivo needle biopsies, but these data would have been helpful. A recent study found that a low functioning genetic polymorphism in GCLC (the catalytic subunit of glutamate-cysteine ligase) was a risk factor for sulfonamide HS in HIV-infected patients; (Wang et al. 2012) this individual risk factor could be compounded by acquired deficiencies in glutathione homeostasis in HIV. Further work is needed to understand changes in hepatic glutathione synthesis in retroviral infection, which may influence that amount of SMX-NO or other reactive drug metabolites that can form drug-protein adducts.

We next looked for similarities in tissue expression between retroviral infection and SLE, the other major chronic inflammatory disease that leads to a higher risk of sulfonamide drug HS reactions. (Petri and Allbritton 1992; Cooper et al. 2002; Pope et al. 2003; Aceves-Avila and Benites-Godinez 2008; Jeffries et al. 2008) In support of our hypothesis, we found up-regulation of an SLE gene cluster in an unbiased pathway analysis, as well as increased expression of other individual genes that are reportedly up-regulated in patients with SLE. This SLE expression pattern, characterized to date in blood cells and synovium of SLE patients, comprises genes involved in innate immunity, RNA and protein catabolism, and apoptosis, many of which are inducible by Type I IFNs (Tables 2, 3). (Han et al. 2003; Li et al. 2010; Coit et al. 2013; Bing et al. 2016; Zhu et al. 2016).

It is well established that retroviral infection is associated with up-regulation of Type I IFN-associated genes, (Utay and Douek 2016) and many genes that are up-regulated in SLE are also inducible by Type 1 IFN. (Feng et al. 2006, 2015; Obermoser and Pascual 2010; Kennedy et al. 2015) The presence of a Type I IFN gene signature in both retroviral infection and SLE may be just an epiphenomenon of two unrelated chronic inflammatory diseases. However, this gene signature appears to be related to the pathogenesis of autoimmunity in SLE, and therefore may be relevant to drug HS reactions that target self-antigens. (Cribb et al. 1996). For example, chronic activation of IFN-induced genes is thought to contribute to autoimmunity by maintaining mature dendritic cells that activate autoreactive T and B cells, breaking self-tolerance. (Obermoser and Pascual 2010; Podolska et al. 2015; Ronnblom 2016).

Type I IFN-associated gene expression has been correlated with overall disease activity and advancing disease severity in SLE. (Feng et al. 2006) (Hoffman et al. 2016) Furthermore, treatments with monoclonal antibodies

targeting IFN-α or the type I IFN receptor have been effective in reducing clinical signs in human patients with SLE. (Kalunian 2016; Oon et al. 2016; Greth et al. 2017).

Although up-regulation of IFN-inducible genes appears to be related to the pathogenesis of SLE, a causal link between up-regulation of IFN-inducible genes and drug HS has not been evaluated. However, the incidence of sulfonamide HS is also increased in another autoimmune disease with a Type I IFN signature, Sjögren's syndrome. (Antonen et al. 1999; Bave et al. 2005) Interestingly, sulfonamide HS risk is not higher in patients with rheumatoid arthritis, (Aceves-Avila and Benites-Godinez 2008) an autoimmune disease that lacks an IFN expression signature in most individuals. (Bave et al. 2005; Feng et al. 2006; Kunz and Ibrahim 2009; Higgs et al. 2011) Furthermore, a single case report described sulfonamide HS (as a cutaneous drug eruption) in a patient undergoing bone marrow transplantation, but only after IFN-α treatment was initiated. (Mehta et al. 1993) These findings together raise the possibility that up-regulation of IFN-inducible pathways contributes mechanistically to drug HS risk in both SLE and retroviral infection.

Additional pathways that were up-regulated in livers from SIV-infected animals were, not surprisingly, those with direct antiviral effects, including RIG-I, which senses intracellular double-stranded (viral) RNA, and ISGylation, which is mediated by a group of IFN-inducible genes that interact with the antiviral protein ISG15. (Zhang and Zhang 2011)). Genes involved in antigen presentation also were up-regulated, including *TAP1*, which transports antigenic peptides for presentation in association with MHC-I molecules, and *B2M* (β2 microglobulin), a component of the MHC-I receptor complex. Notably, TAP1 and B2M are each required for the development of clinical manifestations of SLE in murine models. (Singer et al. 1999) In addition, IFN-induced immunoproteasomal degradation pathways were up-regulated in SIV infection. While array data suggested very modest up-regulation (1.3–1.5-fold), confirmation by qPCR showed up-regulation by 3.2–4.6-fold. Immunoproteasome complexes generate sets of antigenic peptides for presentation in association with MHC-I molecules, leading to activation of CD8 + T cells. (Kimura et al. 2015) Immunoproteasomal components are also up-regulated in human patients with SLE and Sjögren's syndrome. (Krause et al. 2006) Further, selective inhibitors of immunoproteasomal proteins lead to resolution of clinical signs in animal models of SLE and other autoimmune diseases, (Ichikawa et al. 2012; Basler et al. 2015) which suggests that immunoproteasome up-regulation contributes directly to autoimmunity.

One drawback of these studies was the limited number and volume of liver samples available for analyses, such that a more global assessment of additional pathways of hepatic biotransformation could not be performed. In addition, our study design in drug naïve animals did not allow for assessment of possible effects of TMP/SMX itself or other co-medications on SMX biotransformation. A second set of expression arrays obtained after a course of TMP/SMX, or in combination with other drugs commonly use in HIV infection, would have been interesting. However, this study was designed to address the effect of retroviral infection itself on hepatic biotransformation pathways, without noise from concurrent drug administration.

Our findings together raise the question of whether up-regulation of Type 1-IFN induced genes, including immunoproteasomal and antigen presentation pathways and gene clusters that may be permissive to autoimmunity, increases the likelihood that drug-protein adducts will be processed and presented as antigenic peptides in retroviral infection. Such mechanisms could be independent of altered sulfonamide biotransformation, for which we found no evidence in this primate model. Generalized up-regulation of antigen processing and presentation could also lead to a higher risk of HS reactions to drugs that are structurally unrelated but have a similar immunopathogenesis, and indeed, compared to the general population, HIV-infected patients appear to have a higher incidence of HS reactions to anti-tuberculosis drugs and possibly to penicillins. (Nunn et al. 1991; Chintu et al. 1993; Fiszenson-Albala et al. 2003; Yee et al. 2003; Yunihastuti et al. 2014) These studies need to be followed up with targeted experiments on the effect of retroviral infection on drug antigen presentation at the functional level.

Overall, our results support the hypothesis that pathways downstream from drug biotransformation may be primarily important in sulfonamide HS risk in HIV infection. These pathways include enhanced immunoproteasomal processing and presentation of antigens as well as up-regulation of gene clusters that may be permissive to autoimmunity. Additional work is needed to understand the effects of retroviral infection, and more specifically Type 1 IFNs, on processing and presentation of drug-protein adducts for sulfamethoxazole and other drugs associated with HS reactions in HIV-infected patients.

Disclosures

No relevant conflicts of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Primers and probes used for qPCR confirmation of expression changes for selected transcripts in macaque liver. Primers and probes were designed using the Universal ProbeLibrary (Roche).

Table S2. qPCR confirmation of transcript expression in livers from SIVmac239-infected male macaques and age-matched male controls.