Title page

Title:
Endotoxin mediated downregulation of hepatic drug transporters in HIV-1 transgenic rats

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Running title: Impact of endotoxin on drug transporters in HIV-Tg rats

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Acquired immunodeficiency syndrome (AIDS), Alanine aminotransferase (ALT), Antiretroviral therapy (ART), Cytochrome P450 (CYP), Deoxyribonuclease I (DNaseI), Equilibrative nucleoside transporter (Ent1), HIV-Transgenic (HIV-Tg), wild-type (WT), Lipopolysaccharide (LPS).
Abstract:

Altered expression of drug transporters and metabolic enzymes is known to occur in infection induced inflammation. We hypothesize that in HIV infected individuals, further alteration could occur as a result of augmented inflammation. The HIV-1 transgenic rat is used to simulate HIV pathologies associated with the presence of HIV viral proteins. Therefore, the objective of this study was to examine the impact of endotoxin administration on the gene expression of drug transporters in the liver of HIV-Transgenic (HIV-Tg) rats. Male and female HIV-Tg and WT littermates were injected with endotoxin 5mg/kg or saline (n=7-9/group). 18 hr later, rats were sacrificed and tissues were collected. qRT-PCR and Western blotting were used to measure hepatic gene and protein expression respectively and ELISA was used to measure serum cytokine levels. While an augmented inflammatory response was seen in HIV-Tg rats, similar endotoxin-mediated downregulation of Abcb1a, Abcc2, Abcg2, Abcb11, Slco1a1, Slco1a2, Slco1b2, Slc10a1, Slc22a1, Cyp3a2 and Cyp3a9 gene expression were seen in the HIV-Tg and WT groups. A significantly greater endotoxin-mediated downregulation of Ent1/Slc29a1 was seen in female HIV-Tg. Basal expression of inflammatory mediators was not altered in the HIV-Tg rat and likewise the basal expression of most transporters were not significantly different between HIV-Tg and WT. Our findings suggest that hepatobiliary clearances of endogenous and exogenous substrates are altered in the HIV-Tg rat following endotoxin exposure. This is of particular importance, as HIV infected individuals frequently present with bacterial or viral infections which is a potential source for drug-disease interactions.
Introduction:

HIV is associated with chronic inflammation and persistent immune activation that ultimately leads to accelerated CD4+T-cell death, opportunistic infections and acquired immunodeficiency syndrome (AIDS) (Kedzierska and Crowe, 2001; Hazenberg et al., 2003; Mogensen et al., 2010). It is believed that an important factor in immune activation during chronic HIV is the presence of HIV viral proteins and viral nucleic acid. Viral proteins gp120, Tat and Nef are capable of activating lymphocytes and macrophages causing the release of pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α, resulting in organ dysfunction (Lee et al., 2003; Swingler et al., 2003; Appay and Sauce, 2008; Yim et al., 2009; Mogensen et al., 2010). While the use of combination antiretroviral therapy (ART) is highly effective in suppressing viral replication and improving overall immune function (Detels et al., 1998), chronic inflammation does not completely resolve, and elevated markers of inflammation such as C-reactive protein (CRP), IL-6 and TNF-α are seen in HIV patients and are predictive of disease progression and mortality (Appay and Sauce, 2008; Deeks, 2009; Corbeau and Reynes, 2011). Other important factors include the presence of clinical or subclinical co-infections, which HIV patients encounter due to dysregulation in their immune system (Brenchley et al., 2006; Douek et al., 2009; Gonzalez et al., 2009; Corbeau and Reynes, 2011; Chihara et al., 2012). Subclinical endotoxemia is also seen in HIV patients due to mucosal immune dysfunction which causes systemic translocation of gut microflora from the intestinal lumen. (Marchetti et al., 2008; Sandler and Douek, 2012; Marchetti et al., 2013). Lipopolysaccharide (LPS); a bacterial endotoxin which is part of Gram-negative bacterial cell wall, is considered a major immune-activating molecule in HIV patients and
one of the main causes of subclinical endotoxemia as well as sepsis (Proctor, 2001; Brenchley et al., 2006; Douek, 2007; Silva and dos Santos Sde, 2013). Indeed, while the use of ART is associated with decreased circulating levels of LPS, levels are still higher in HIV patients than non-infected individuals (Mehandru et al., 2006; Marchetti et al., 2008; d'Ettorre et al., 2011; Jenabian et al., 2015).

Infection induced inflammation has been shown to alter the disposition of drugs. Pro-inflammatory cytokines including IL-6, TNF-α, IL-1β have been frequently reported to decrease the expression and function of ABC and SLC transporters and Cytochrome P450 (CYP) metabolising enzymes (Piquette-Miller et al., 1998; Tang et al., 2000; Kalitsky-Szirtes et al., 2004; Wang et al., 2005; Englund et al., 2007; Petrovic et al., 2008). Studies in HIV infected individuals have also reported altered expression of drug metabolizing enzymes and transporters. One study reported that HIV infected individuals who are not on anti-retroviral therapy (ART naïve), had lower CYP3A4 and CYP2D6 enzymatic activity compared with uninfected individuals, which correlated with increased plasma cytokine level (Jones et al., 2010). More recently, ART naïve HIV patients were found to have significantly lower P-glycoprotein (P-gp/ ABCB1) and Multidrug resistance- associated protein 2 (MRP2/ABCC2) protein levels in the rectal-sigmoid colon compared with non-infected subjects (De Rosa et al., 2013). In addition, in vitro incubation of cells with the HIV gp120 viral protein has been found to increase pro-inflammatory cytokines IL-6, IL-1β and TNF-α as well as decrease the expression and function of P-gp in rat and human brain astrocytes (Ronaldson and Bendayan, 2006; Ashraf et al., 2011). Due to dysregulation of the immune system, it is plausible that infection-induced inflammatory responses may be potentiated in HIV patients (da Silva et
al., 1999; Nguyen and Biron, 1999; Lester et al., 2008; Lester et al., 2009; Bukh et al., 2011). We hypothesize that augmented inflammatory responses in response to co-infections could lead to further downregulation in the expression of transporters and drug metabolising enzymes. As many of the anti-retroviral agents are substrates of drug transporters and metabolizing enzymes, understanding their regulation in HIV patients is important in maintaining therapeutic drug levels and predicting potential drug-disease interactions. Moreover, protease inhibitors; an integral part of combination ART, are potent inducers/inhibitors of drug transporters and metabolising enzymes which increases the risk of drug-drug interactions in these patients (Gutmann et al., 1999; Sulkowski et al., 2000; Kim, 2003; Klaassen and Aleksunes, 2010; Griffin et al., 2011). Indeed, the existence of co-infections and alterations in drug metabolism is known to increase the risk of ART-associated liver damage in HIV patients (Sulkowski et al., 2000; Soriano et al., 2008; Puoti et al., 2009).

In this study we examined the impact of endotoxin-mediated inflammation on the expression of drug transporters and metabolising enzymes in HIV-1 transgenic (HIV-Tg) rats. The HIV-Tg rat model is a non infectious small animal model of HIV, expressing all of the HIV-1 viral proteins except for the replication gag and pol genes (Reid et al., 2001; Hatzioannou and Evans, 2012). This makes the model useful for demonstrating effects of HIV viral proteins in the absence of viral replication which shares similarity with HIV patients given ART; as there is no viral replication, yet persistent inflammation (Chang et al., 2007a; Chang et al., 2007b; Homji et al., 2012). As the rats age, progressive illness that shares many similarity with HIV infected humans become apparent. This includes pneumonitis, neurological deficits, wasting, respiratory difficulty, cardiac abnormalities
and renal disease (Ray et al., 2003; Peng et al., 2010). Immune dysregulation is also evident including impaired macrophage phagocytic function (Joshi and Guidot, 2011), defects in T helper 1 immune responses (Reid et al., 2001; Reid et al., 2004; Yadav et al., 2006; Royal et al., 2012) and defective leukocyte endothelial adhesion (Chang et al., 2007b). Increased cytokine levels have been found in liver and brain tissue lysates (Joshi and Guidot, 2011; Royal et al., 2012). In response to endotoxin administration, the HIV-Tg rats have previously demonstrated augmented inflammatory response (Chang et al., 2007a; Chang et al., 2007b), similar to that seen in HIV (+) individuals (Lester et al., 2008; Bukh et al., 2011).
Materials and Methods:

Animals and Experimental Design:

Male Hsd-HIV-1 (F344) transgenic (HIV-Tg) rats and female F344/NHsd, purchased from Harlan Laboratories (Indianapolis, IN) were bred and HIV-Tg and control wild-type (WT) littermates separated at weaning. Rats were housed in accordance with the University of Toronto Animal Care Committee and the Canadian Council on Animal Care and provided with ad libitum food and water on a 12 h light/dark schedule. Three month old male and female HIV-Tg or same generation WT littermates were given an intraperitoneal injection of either 5 mg/kg Lipopolysaccharide (LPS, E.coli 055:B5, Sigma-Aldrich, Oakville, ON) or saline. Rats (n=7-9 per group) were sacrificed 18 hours following injection and serum and tissues collected and snap-frozen with liquid nitrogen and stored at -80°C until analysis. We have previously observed significant endotoxin-mediated changes in the expression of numerous drug transporters in rats at various times between 6 to 24 hours after endotoxin administration (Wang et al., 2005; Petrovic et al., 2008). As significant changes in mRNA with corresponding changes in protein expression were generally seen at 18 hours post injection in previous studies, we therefore examined the impact of endotoxin in HIV-Tg and WT rats at 18 hours post-injection.

Serum and Tissue analysis:

Serum cytokine concentrations were measured via commercially available rat specific ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. Minimum detectable levels for IL-6, IL-1β, TNF-α and interferon (IFN)-γ were 36, 5, 9
and 10 pg/mL, respectively. Total bile acid concentrations were measured in the serum and in 100 g of liver homogenate using a Rat Total Bile acid Kit (Crystal Chem Inc., Downers Grove, IL) according to manufacturer’s instructions and as previously described (Ghoneim et al., 2015). Alanine aminotransferase (ALT) activity in the serum was measured following manufacturer instructions (Sigma-Aldrich, St. Louis, MO) and results were reported as milliunit/mL where one milliunit (mU) of ALT is defined as the amount of enzyme that generates 1 nmole of pyruvate/min at 37 °C.

**Quantitative Real-time Polymerase Chain Reaction:**

Total RNA was extracted from around 50 mg of frozen tissue using the TRIzol extraction method (Invitrogen, Carlsbad, CA) according to manufacturer instructions. RNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and 2 μg of RNA was then treated with Deoxyribonuclease I (DNaseI) (Invitrogen, Carlsbad, CA) and reverse transcribed (RT) with High Capacity cDNA RT kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was carried out for triplicate samples of genomic cDNA specific for each primer set (Supplemental Table 1) and performed using Power SYBR Green detection system (ABI HT 7900, Applied Biosystems, Streetsville, ON, Canada). A comparative Ct (ΔΔCt) method was used to calculate relative mRNA expression of each gene of interest normalized to house keeping gene β-actin. Similar results were obtained using GAPDH or cyclophilin as housekeeping genes.

**Western Blotting:**
Isolation of crude membrane fractions has been previously described (Petrovic and Piquette-Miller, 2010). Briefly 0.3 g of liver tissue was homogenized in lysis buffer containing 0.1 M Tris-HCL, 3 μL/mL Protease inhibitor and 50 μg/mL phenylmethylsulfonyl fluoride, centrifuged (30,000 g for 1 hour) and quantified using the Bradford Assay. 40 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-rad laboratories, Mississauga, Canada). Membranes were blocked using 5% skim milk and incubated with the following primary antibodies: P-gp A<sub>B</sub>C<sub>B</sub>D<sub>C</sub>B1 C219 1:500 (Enzo Life Sciences, Farmingdale, NY), ABCC2 M2 III-5 1:50 (Abcam Inc., Cambridge, MA) and Equilibrative Nucleoside Transporter 1 (ENT1 /SLC29A1) F-12 1:100 (Santa-Cruz Biotech, Dallas, Texas). Following a series of washing, membranes were incubated with secondary anti-mouse antibody 1:3000 (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunodetectable protein was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and band density was quantified using Alpha Ease FC imaging software (Alpha Innotech). β-actin AC-15 1:10000 (Sigma-Aldrich, Oakville, ON) was used to control for loading variability and a calibrator sample for each gender was used to control for different gels. Mouse heart tissue extract was used as a positive control for Ent1.

**Genotyping:**

Genotyping was performed by PCR amplification of viral proteins (gp120, tat, vif, nef) using 0.5 g of spleen tissue and PCR products were separated by gel electrophoresis. Separation was carried on a 2% agarose gel in 1x TBE, 6x loading dye using Fast start
DNA ladder 100bp (Thermo Scientific, Rockford, IL) and bands were visualized by DNA safe SYBR 10 000x staining (Invitrogen, Carlsbad, CA).

**Statistical analysis:**

Statistical analysis was performed in GraphPad Prism 6 using a One-way ANOVA, followed by Fisher’s Least Significant Difference test to compare the effect of endotoxin administration in WT and HIV-Tg rats where males and females were analyzed separately and data was expressed as a fold change from WT saline injected animals for each gender. One-way ANOVA, followed by Fisher’s Least Significant Difference test was also used to determine differences in basal gene expression between males and females in WT and HIV-Tg rats where results were compared relative to male WT saline injected animals. A Student’s T-test was used to compare cytokine levels following endotoxin administration between WT and HIV-Tg rats as serum levels were undetectable in saline injected animals.
Results:

Analysis of HIV-Tg rat and transgene expression

HIV-Tg rats appeared healthy at 3 months of age with a 17% lower body weight compared to WT littermates (data not shown). Liver weight did not differ from WT once adjusted for body weight. HIV-Tg phenotypes develop cataracts by 2 weeks of age, a finding that is absent in WT littermates. Genotyping confirmed the presence of HIV transgene in the spleen of HIV-Tg rats. Gp 120, tat, vif and nef were expressed in the HIV-Tg but not WT rats (Supplemental Figure 1).

Basal hepatic gene expression in the liver

The basal hepatic expression of most ABC and SLC transporters and nuclear receptors were not significantly different between HIV-Tg and WT (Table 1). The expression of Cyp3a2 was 62% lower in male HIV-Tg rats compared with WT while Cyp3a9, Slc10a1 and Abcb11 was (25-54%) lower in female HIV-Tg compared with WT littermates. Hepatic mRNA levels of TNF-α and iNOS were slightly but significantly higher in saline treated female HIV-Tg as compared to WT (Table 1).

Gender differences in metabolic enzymes and drug transporter expression

Gender differences were detected in both HIV-Tg and WT saline controls (Table 1). A 250 fold higher expression of Cyp3a2 enzyme was detected in WT males as compared to females and a 14 fold higher expression of Cyp3a9 enzyme was detected in female WT as compared with males. With regards to hepatic transporters, 1.5 to 4 fold higher mRNA levels of Abcb1a, Abcb11, Abcc3, Slco1b2, Slc10a1 and Slc22a1 were detected in female WT as compared to males. Similar findings were seen in HIV-Tg rats.
Inflammatory response to endotoxin

Pro-inflammatory cytokines were examined as a measure of systemic and local inflammatory responses to endotoxin administration. While serum levels of the pro-inflammatory cytokines were below the detection limit in saline treated HIV-Tg and WT, a significant endotoxin-mediated induction of IL-6, IL-1β, TNF-α and IFN-γ was observed in the serum of both control WT and HIV-Tg rats (Table 2). Higher serum levels of cytokines were seen in the endotoxin treated HIV-Tg. Differences reached significance for TNF-α in females and IL-1β in both genders. The presence of an inflammatory response within the liver was confirmed by measuring the hepatic mRNA expression of IL-6, IL-1β, iNOS and TNF-α (Figure 1). Within the liver of female rats, HIV-Tg had a more pronounced endotoxin-mediated induction of cytokines which also occurred in male rats to a lesser extent.

Effect of endotoxin on hepatic expression of metabolic enzymes, ABC and SLC transporters in WT and HIV-Tg rats

Following endotoxin administration, a significant downregulation in the expression of several ABC transporters (Abcb1a, Abcb11, Abcg2 and Abcc2) (Figures 2-4), SLC transporters (Slco1a1, Slco1a2, Slco1b2, Slc22a1, Slc10a1 and Slc29a1) (Figure 5,6), and metabolic enzymes (Cyp3a2, Cyp3a9 and Cyp7a1) (Figure 7) were seen in both HIV-Tg and WT. Slc29a1 was significantly lower in female HIV-Tg compared with WT (Figure 6 A). In addition, a significant downregulation in the expression of Pxr, Car and Fxr; which are nuclear receptors involved in the regulation of many of the aforementioned genes, was also seen in both HIV-Tg and WT (Figure 8). Western blotting confirmed significant downregulation in the protein expression of Mrp2 in both HIV-Tg and WT following...
endotoxin administration (Figure 3B). The protein expression of P-gp was significantly downregulated in endotoxin-treated HIV-Tg and WT male rats and in female HIV-Tg but not WT rats (Figure 2B). Moreover, Ent1 protein levels were significantly lower only in HIV-Tg rats (Figure 6B). A significant negative correlation ($P<0.05$) was found between TNF-$\alpha$ and Abcg2, Abcc2, Abcb11, Cyp3a2, Slco1a1, Slco1a1, Slco1b2, Slc22a1, Slco29a1, Slc10a1 as well as between IL-1$\beta$ and Abcb1a, Abcg2, Abcc2, Abcb11, Cyp3a2, Slco1a1, Slco1a1, Slco1b2, Slc22a1, Slco29a1, Slc10a1 and between iNOS and Abcb1b, Abcg2, Abcb11, Slco1a1, Slco1a1, Slco1b2, Slc22a1, Slco29a1 (Supplemental Table 2).

**Biochemical changes in response to endotoxin**

As the expression of several bile acid transporters as well as Cyp7a1 were altered in the endotoxin-treated groups, we examined the impact on serum and hepatic concentrations of total bile acids (Table 3). Endotoxin resulted in a significant increase in serum total bile acid concentrations in the HIV-Tg but not WT rats. Intra-hepatic concentrations of total bile acids were significantly increased in endotoxin treated HIV-Tg and WT male but not female rats. Alanine transaminase activity, which is a measure of liver toxicity, was significantly increased in the serum of endotoxin-treated HIV-Tg groups but not the WT groups (Table 3).
Discussion:

Despite the use of ART, HIV patients often encounter bacterial endotoxin infections either as opportunistic infections or from microbial translocation. This adds to the state of inflammation and immune activation (Hazenberg et al., 2003; Brenchley et al., 2006; Appay and Sauce, 2008; Mogensen et al., 2010). The HIV-Tg rat has demonstrated augmented inflammatory response to endotoxin similar to that observed in HIV patients (Chang et al., 2007a; Chang et al., 2007b; Lester et al., 2008; Bukh et al., 2011). In addition, HIV viral proteins have been shown to contribute to inflammation altered expression and function of drug transporters. As endotoxin-mediated effects on the expression of hepatic gene expression is commonly examined in rodents (Piquette-Miller et al., 1998; Tang et al., 2000; Cherrington et al., 2004; Kalitsky-Szirtes et al., 2004; Wang et al., 2005; Petrovic et al., 2008), and similar endotoxin-mediated effects on the expression and activity of drug metabolizing enzymes are seen in humans and rodents (Shedlofsky et al., 1994; Shedlofsky et al., 1997), we examined whether immunological changes associated with the expression and activity of HIV viral proteins would impact endotoxin-mediated effects in HIV-Tg rats.

At the basal level, the HIV-Tg rat demonstrated significantly lower gene expression of drug metabolising enzymes Cyp3a2 and Cyp3a9 in male and female rats respectively. Decreased enzymatic activity of CYP3A4 and CYP2D6 has been previously reported in HIV infected individuals and correlated with increased plasma cytokine level (Jones et al., 2010). While serum concentrations and basal expression of the pro-inflammatory cytokines were relatively unchanged in the HIV-Tg, it is possible that other factors including oxidative stress may play a role. HIV viral proteins gp120 and tat are known to
induce oxidative stress markers (Nicolini et al., 2001; Walsh et al., 2004; Price et al., 2005). Indeed there was a significant elevation in the mRNA level of inducible nitric oxide (iNOS) and TNF-α in female HIV rats compared with WT. This could partially explain the reduction in Cyp3a expression as several studies suggest the involvement of nitric oxide in downregulating CYP 450 enzyme (Carlson and Billings, 1996; Donato et al., 1997; Aitken et al., 2008). Likewise, Abcb11 and Slc10a1 were significantly lower in female HIV-Tg rats. In general apart from these differences, HIV-Tg rats displayed similar basal expression of most hepatic drug transporters relative to their WT littermates. Changes in the expression of Abcb1a and Abcc1 have also been reported in 24 but not 8 week old HIV-Tg rats (Robillard et al., 2014). As 5 fold higher mRNA levels of gp120 were seen in the livers of the 24 week as compared to 8 week old rats, it is likely that we did not observe similar changes due to the age difference in our study. Differences in HIV-Tg associated inflammation are seen within the literature. While we did not detect measurable levels of cytokines in the serum of our HIV-Tg rats. Chang, et al observed a significant elevation of serum IL-1β in 5 month old HIV-Tg rats (Chang et al., 2007a). On the other hand, Homji, et al did not detect changes in serum levels of IL-1β and TNF-α in 5 month old HIV-Tg (Homji et al., 2012).

One of the best-characterized models of infection and systemic inflammation is the bacterial endotoxin LPS model. LPS administration results in profound increase in the level of pro-inflammatory cytokines IL-6, IL-1β, TNF-α as well as IFN-γ, which we observed in both HIV-Tg and WT rats. Endotoxin administration was also associated with significant downregulation in the hepatic expression of almost all examined ABC, SLC transporters, metabolic enzymes and nuclear receptors. Transcriptional regulation of
hepatic transporters and CYP enzyme expression during endotoxin-induced inflammation occurs via cytokine-mediated activation of transcription factors and cell signalling pathways (Shedlofsky et al., 1997; Piquette-Miller et al., 1998; Cherrington et al., 2004; Aitken et al., 2006; Cressman et al., 2012). Reduced expression and activity of nuclear receptor PXR has also been linked to these inflammation-mediated changes (Teng and Piquette-Miller, 2008). While changes in transporter expression in response to endotoxin administration in our study are transient due to the acute nature of the inflammatory stimulus, more persistent changes are likely to occur if inflammation becomes chronic and is not successfully resolved.

As compared to WT, the administration of endotoxin to HIV-Tg rats was associated with a profound and augmented inflammatory response. This suggests that the immune response is altered in these animals despite the absence of physical signs of disease. Likewise, previous studies have also reported exacerbated responses to endotoxin in older HIV-Tg rats (Chang et al., 2007a; Chang et al., 2007b). Consistent with the observed inflammatory response, we detected a significant endotoxin-mediated downregulation in the hepatic expression of numerous ABC and SLC transporters in addition to metabolic enzymes in the HIV-Tg rats. Of note, while we observed a higher induction of inflammatory markers in the endotoxin-treated HIV-Tg rats, the degree of gene downregulation was generally similar in the HIV-Tg and WT groups. This is likely due to maximum inhibition occurring in both groups in response to dose of LPS used. Indeed, a 80-90% reduction in hepatic expression was seen for most genes in both endotoxin treated groups. A maximal downregulation of hepatic transporters expression after cytokine exposure has been previously reported. In vitro studies in human hepatocytes
found a non-linear downregulation in the mRNA expression of numerous drug transporters after incubation with TNF-α over a ten to 100 fold range of concentrations (Le Vee et al., 2009). Similar results were obtained following LPS and cytokine treatment in mice (Hartmann et al., 2001; Li et al., 2004).

Of particular interest was our finding that endotoxin administration imposed a more pronounced downregulation of Ent1 (Slc29a1) in the HIV-Tg rat. The nucleoside transporter Ent1 is the primary transporter for the nucleoside inhibitor ribavirin (Jarvis et al., 1998). Ribavirin is commonly prescribed in patients with hepatitis C (HCV) and is reported to be less effective in the presence of HIV/HCV co-infections (Carrat et al., 2004; Torriani et al., 2004; Price and Thio, 2010). While the antiviral mechanism of ribavirin for the treatment of HCV is not clearly understood (Hofmann et al., 2008), it has been suggested that the therapeutic response to ribavirin is dependent on its uptake into hepatocytes (Ibarra and Pfeiffer, 2009; Iikura et al., 2012). Our results, which demonstrate a pronounced downregulation of this nucleoside transporter in an HIV model of bacterial co-infection could provide some insight into the decreased effectiveness in HIV/HCV patients. To this point, ENT1 genetic polymorphisms has been shown to influence the virological response to ribavirin therapy in HIV/HCV and HCV patients possibly due to modulated ribavirin uptake into hepatocytes (Morello et al., 2010; Iikura et al., 2012; Tsubota et al., 2012).

It is well documented that endotoxin administration can decrease bile acid dependent and independent flow in a process that is believed to occur due to inhibited transport of bile salts and organic anions on the sinusoidal and canalicular membranes of hepatocytes (Moseley et al., 1996; Bolder et al., 1997; Cherrington et al., 2004; Geier et al., 2007).
While the extent of change in the bile acid transporters following endotoxin exposure were not very different between wild-type and HIV-Tg, a dramatic increase in total bile acids was present in the serum of HIV-Tg following endotoxin. It is plausible that the augmented increase in serum bile acids in the HIV-Tg rats results from combined changes in multiple transporters rather than through a single transport mechanism. Moreover, intra-hepatic bile concentrations were increased following endotoxin administration in both WT and HIV-Tg male rats. While we did not detect differences in hepatic bile acid concentration solely as a result of HIV; the presence of co-existing infections is considered a frequently encountered cause of cholestasis in HIV individuals (Te, 2004). Moreover, abnormal liver enzymes are common among HIV infected individuals and are predisposed by medication toxicity from anti-retroviral drug therapy as well as the presence of co-infections (Price and Thio, 2010). Administration of bacterial endotoxin clearly elevated ALT activity in the serum of HIV-Tg rats more so than in WT which confirms that the presence of co-infections does increase the risk of hepatotoxicity in the presence of HIV viral proteins.

Gender differences in the expression of drug transporters and metabolic enzymes were detected in our study in both HIV-Tg and WT rats. We observed significantly higher hepatic expression of several transporters in females. A higher hepatic expression of Mdr1 in female rats has been previously reported (Piquette-Miller et al., 1998; Salphati and Benet, 1998) which is contrary to what is reported in humans (Schuetz et al., 1995; Yang et al., 2012). On the other hand, Cyp3a2 expression was dramatically higher in male rats, while Cyp3a9 expression was more predominant in females. This is in agreement with other studies which have described Cyp3a9 and Cyp3a62 as the
predominant Cyp3a isoform in females (Salphati and Benet, 1998; Matsubara et al., 2004). Gender differences in responses to ART have been observed and it has been suggested that these differences may be linked to gender differences in drug transporter and/or CYP expression (Ofotokun, 2005). While this has not been examined or confirmed in vivo, it is plausible that gender differences in gene regulation could contribute to reported differences in therapeutic response to ART.

In conclusion, we demonstrated that endotoxin imposes a pronounced downregulation of numerous hepatic transporters and metabolic enzymes in HIV-Tg rats. Our findings suggest that heptobiliary clearances of endogenous and exogenous substrates are altered in the HIV-Tg rat after endotoxin exposure. This is particularly important for drugs that are highly dependent on both metabolism and transporter-mediated processes such as protease inhibitors (Choo et al., 2000; Huisman et al., 2000; Griffin et al., 2011). This is of particular relevance, as HIV infected individuals frequently present with bacterial or viral infections. While the HIV-Tg rat model does not encounter all aspects of HIV infection, shared similarities with pathologies occurring in HIV may help us to predict and identify potential drug-disease interactions.
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Authorship Contributions:

Participated in research design: Ghoneim and Piquette-Miller.

Execution of experiments and data analysis: Ghoneim.

Wrote or contributed to the writing of the manuscript: Ghoneim and Piquette-Miller.
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Footnotes

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Legends for Figures

**Figure 1:** Effect of endotoxin (LPS) on the mRNA expression of inflammatory mediators in A) male rats and B) female rats. Values represent ratio of gene of interest mRNA to β-actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values. Values are presented as the mean +/- SEM of 7-8 rats, except for female WT saline where n=4. *#P < 0.05, **##P < 0.01, ###P < 0.001 (One-Way ANOVA).

* Significantly different from saline controls, # significantly different from endotoxin treated WT.

**Figure 2.** Effect of endotoxin (LPS) on the A) mRNA expression of Abcb1a and Abcb1b where values represent ratio of gene of interest mRNA to β-actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values and B) Protein expression of P-gp as determined by Western blotting and expressed as a % of WT saline, normalized to beta actin level and a calibrator. Values are presented as mean +/- SEM (n=5-8). Representative gels are shown below. *#P < 0.05, **##P < 0.01, ###P < 0.001 (One-Way ANOVA). * Significantly different from saline controls, # significantly different from endotoxin treated WT.

**Figure 3.** Effect of endotoxin on the A) mRNA expression of Abcc2 where values represent ratio of gene of interest mRNA to β-actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values and B) Protein expression of Mrp2 as determined by Western blotting and expressed as a % of WT saline, normalized to beta actin level and a calibrator. Values are presented as mean +/- SEM (n=5-8). Representative gels are shown below. *#P < 0.05, **##P < 0.01, ###P < 0.001 (One-Way ANOVA).
way ANOVA). * Significantly different from saline controls, # significantly different from saline treated WT.

**Figure 4.** Effect of endotoxin (LPS) on the mRNA expression of ABC transporters in A) male and B) female rats. Values represent ratio of gene of interest mRNA to β -actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values. Values are presented as the mean +/- SEM of 7-8 rats, except for female WT saline where n=4. *P < 0.05, **P < 0.01, ***P < 0.001 (One-Way ANOVA). * Significantly different from saline controls.

**Figure 5.** Effect of endotoxin (LPS) on the mRNA expression of SLC transporters in A) male and B) female rats. Values represent ratio of gene of interest mRNA to β -actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values. Values are presented as the mean +/- SEM of 7-8 rats, except for female WT saline where n=4. *P < 0.05, **P < 0.01, ***P < 0.001 (One-Way ANOVA). * Significantly different from saline controls.

**Figure 6.** Effect of endotoxin (LPS) on the A) mRNA expression of Slc29a1 (Ent1) where values represent ratio of gene of interest mRNA to β -actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values and B) Protein expression of Ent1 as determined by Western blotting and expressed as a % of WT saline, normalized to beta actin level and a calibrator. Values are presented as mean +/- SEM (n=5-8). Representative gels are shown below. *,#P < 0.05, **,##P < 0.01 , ***,##,##P < 0.001 (One-way ANOVA). * Significantly different from saline controls, # significantly different from endotoxin treated WT.
**Figure 7.** Effect of endotoxin (LPS) on the mRNA expression of metabolic enzymes in **A)** male and **B)** female rats. Values represent ratio of gene of interest mRNA to β-actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values. Values are presented as the mean +/- SEM of 7-8 rats, except for female WT saline where n=4. *P < 0.05, **P < 0.01, ***P < 0.001 (One-Way ANOVA). * Significantly different from saline controls.

**Figure 8.** Effect of endotoxin on the mRNA expression of nuclear receptors in **A)** male and **B)** female rats. Values represent ratio of gene of interest mRNA to β-actin mRNA, as determined by qRT-PCR, expressed as % change from saline control values. Values are presented as the mean +/- SEM of 7-8 rats, except for female WT saline where n=4. *,#P < 0.05, **,##P < 0.01, ###P < 0.001 (One-Way ANOVA). * Significantly different from saline controls, # significantly different from endotoxin treated WT.
Tables

Table 1: Basal hepatic gene expression in HIV-Tg and WT rats

<table>
<thead>
<tr>
<th>Gender</th>
<th>WT</th>
<th>HIV-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory mediators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Males</td>
<td>1±0.07</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>1.05±0.26</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Males</td>
<td>1±0.1</td>
</tr>
</tbody>
</table>
|        | Females | 2.0±0.33 | 1.49±0.14*
| iNOS   | Males   | 1±0.18   | 1.67±0.22|
|        | Females | 1.51±0.18| 2.71±0.36*
<p>| TNF-α  | Males   | 1±0.14   | 0.92±0.63|
|        | Females | 1.14±0.17| 1.59±0.13***|
| <strong>ABC Transporters</strong> |         |          |
| Abcb1a | Males   | 1±0.1    | 1.025±0.17|
|        | Females | 3.895±0.84 | 4.949±0.95***|
| Abcb1b | Males   | 1±0.22   | 0.94±0.18|</p>
<table>
<thead>
<tr>
<th>Transporter</th>
<th>Gender</th>
<th>Mean ± SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcg2</td>
<td>Males</td>
<td>1±0.14</td>
<td>0.75±0.11</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>1.40±0.17</td>
<td>1.28±0.33</td>
</tr>
<tr>
<td>Abcc1</td>
<td>Males</td>
<td>1±0.31</td>
<td>0.61±0.12</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>1.30±0.51</td>
<td>0.72±0.11</td>
</tr>
<tr>
<td>Abcc2</td>
<td>Males</td>
<td>1±0.32</td>
<td>1.34±0.51</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>0.73±0.03</td>
<td>2.01±0.73</td>
</tr>
<tr>
<td>Abcc3</td>
<td>Males</td>
<td>1±0.15</td>
<td>0.73±0.05</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>3.99±0.98</td>
<td>3.95±0.76</td>
</tr>
<tr>
<td>Abcc4</td>
<td>Males</td>
<td>1±0.2</td>
<td>1.15±0.18</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>1.28±0.31</td>
<td>0.82±0.07</td>
</tr>
<tr>
<td>Abcb11</td>
<td>Males</td>
<td>1±0.25</td>
<td>1.05±0.28</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>3.69±0.62</td>
<td>1.69±0.51</td>
</tr>
<tr>
<td>SLC Transporters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slco1a1</td>
<td>Males</td>
<td>1±0.15</td>
<td>1.4±0.11</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>0.88±0.13</td>
<td>0.77±0.11</td>
</tr>
<tr>
<td>Genes</td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>Slco1a2</td>
<td>1±0.17</td>
<td>1.15±0.27</td>
<td>1.12±0.11</td>
</tr>
<tr>
<td>Slco1b2</td>
<td>1±0.04</td>
<td>2.22±0.16***</td>
<td>1.22±0.06</td>
</tr>
<tr>
<td>Slc22a1</td>
<td>1±0.04</td>
<td>1.53±0.18***</td>
<td>0.95±0.08</td>
</tr>
<tr>
<td>Slc29a1</td>
<td>1±0.14</td>
<td>0.92±0.1</td>
<td>1.27±0.09</td>
</tr>
<tr>
<td>Slc10a1</td>
<td>1±0.06</td>
<td>2.14±0.4***</td>
<td>1.07±0.05</td>
</tr>
</tbody>
</table>

Metabolic Enzymes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Males</th>
<th>Females</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp3a2</td>
<td>1±0.21</td>
<td>0.004±0.001***</td>
<td>0.38±0.08##</td>
<td>0.005±0.001*</td>
</tr>
<tr>
<td>Cyp3a9</td>
<td>1±0.17</td>
<td>13.55±2.20***</td>
<td>1.08±0.19</td>
<td>8.56±1.42##***</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>1±0.17</td>
<td>1.05±0.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results are reported relative to male WT; which was standardized to 1, *, #P < 0.05, **, ##P < 0.01, ###P < 0.001. *Significantly different from males within same strain, #significantly different from WT within same gender.

<table>
<thead>
<tr>
<th>Nuclear Receptors</th>
<th>Females</th>
<th>Males</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pxr</td>
<td>0.94±0.17</td>
<td>1±0.15</td>
<td>0.78±0.06</td>
<td>1.11±0.11</td>
</tr>
<tr>
<td>Car</td>
<td>1.02±0.27</td>
<td>1±0.17</td>
<td>0.55±0.12</td>
<td>0.82±0.19</td>
</tr>
<tr>
<td>Fxr</td>
<td>1.35±0.24</td>
<td>1±0.12</td>
<td>1.43±0.1</td>
<td>0.93±0.04</td>
</tr>
</tbody>
</table>
Table 2: Serum cytokine concentrations in endotoxin-treated HIV-Tg and WT rats

<table>
<thead>
<tr>
<th>Cytokine (pg/mL)</th>
<th>Gender</th>
<th>Endotoxin treated WT</th>
<th>Endotoxin treated HIV-Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Males</td>
<td>268±47</td>
<td>477±70*</td>
</tr>
<tr>
<td></td>
<td>females</td>
<td>339±97</td>
<td>677±84*</td>
</tr>
<tr>
<td>IL-6</td>
<td>Males</td>
<td>1259±1051</td>
<td>3271±1406</td>
</tr>
<tr>
<td></td>
<td>females</td>
<td>2781±1590</td>
<td>4541±1056</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Males</td>
<td>17±6</td>
<td>51±16</td>
</tr>
<tr>
<td></td>
<td>females</td>
<td>20±14</td>
<td>67±12&quot;</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Males</td>
<td>419±139</td>
<td>1303±533</td>
</tr>
<tr>
<td></td>
<td>females</td>
<td>644±460</td>
<td>1300±375</td>
</tr>
</tbody>
</table>

* *P < 0.05, **##P < 0.01, ***P < 0.001. # Significantly different from endotoxin treated WT. Concentrations were below detection limit in saline treated animals.
Table 3: Biochemical changes in response to endotoxin administration

<table>
<thead>
<tr>
<th></th>
<th>Saline treated</th>
<th>Endotoxin (LPS) treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum Alanine Transaminase activity (mU/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male WT</td>
<td>87±74</td>
<td>2218±498</td>
</tr>
<tr>
<td>Male HIV-Tg</td>
<td>433±223</td>
<td>6236±2656</td>
</tr>
<tr>
<td>Female WT</td>
<td>686±445</td>
<td>3473±1874</td>
</tr>
<tr>
<td>Female HIV-Tg</td>
<td>269±144</td>
<td>6907±1036***#</td>
</tr>
<tr>
<td><strong>Serum total bile acid concentrations (μmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male WT</td>
<td>25±3</td>
<td>49±7</td>
</tr>
<tr>
<td>Male HIV-Tg</td>
<td>26±4</td>
<td>118±26***#</td>
</tr>
<tr>
<td>Female WT</td>
<td>14±6</td>
<td>103±50</td>
</tr>
<tr>
<td>Female HIV-Tg</td>
<td>9±6</td>
<td>252±37***#</td>
</tr>
<tr>
<td><strong>Hepatic bile acid concentrations (μmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male WT</td>
<td>31±3.3</td>
<td>40±0.6</td>
</tr>
<tr>
<td>Male HIV-Tg</td>
<td>27±3.2</td>
<td>40±1.99***</td>
</tr>
<tr>
<td>Female WT</td>
<td>31±8.8</td>
<td>37±2.5</td>
</tr>
<tr>
<td>Female HIV-Tg</td>
<td>37±4.4</td>
<td>34±3.0</td>
</tr>
</tbody>
</table>

\[ a \] *P < 0.05, **P < 0.01, ***P < 0.001. # Significantly different from endotoxin treated WT.
Figure 2

A

![Bar graph showing mRNA expression levels for Abcb1a and Abcb1b in males and females under WT LPS and HIV LPS conditions.]

B

![Bar graph showing normalized P-gp protein levels for WT and HIV conditions in males and females.]

- WT
- WT LPS
- HIV
- HIV LPS

- P-gp
- β-actin
Figure 3

A

![Graph showing mRNA (% change from saline control) for Abcc2 in males and females.]

B

![Graph showing Normalized Mrp2 protein (% WT saline) for WT, HIV, WT LPS, HIV LPS in males and females.]

**Legend:**
- WT LPS: Blue bars
- HIV LPS: Yellow bars
- WT: Striped bars
- HIV: Solid bars

**Significance:**
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
- ###: p < 0.0001

**Protein Blots:**
- Below the graphs are images of protein blots for Mrp2 and β-actin.
Figure 5

(A) mRNA (% change from saline control)

(B) mRNA (% change from saline control)
Figure 6

A

![Graph showing mRNA expression of S129a1 in males and females under different conditions]

B

![Graph showing normalized Ent1 protein expression in males and females under different conditions]

Legend:
- WT: Wild Type
- HIV: Human Immunodeficiency Virus
- HIV LPS: HIV LPS
- +ve control: Positive control
- Ent1: Protein of interest
- β-actin: Loading control
Figure 7

A

```
mRNA (% change from saline control)

WT LPS          HIV LPS

Cyp3a2          Cyp3a9          Cyp7a1

***             *              *** ***
```

B

```
mRNA (% change from saline control)

WT LPS          HIV LPS

Cyp3a2          Cyp3a9          Cyp7a1

**              *** ***         * ***
```