Selective and cytokine-dependent regulation of hepatic transporters and bile acid homeostasis during infectious colitis in mice

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Abbreviations:  
ABC, ATP-Binding Cassette; BA, bile acid; BSP, bromosulfophthaleine; DME, drug-metabolizing enzyme; DSS, dextran sulphate sodium; EPEC, enteropathogenic *E. coli*; FXR, farnesoid X receptor; IL-6, interleukin-6; IFNy, interferon-gamma; LPS, lipopolysaccharide; Mdr, multidrug resistance protein; Mrp multidrug resistance-associated protein; NEC, necrotizing enterocolitis; Oatp, organic anion transporting polypeptide; Octn, organic cation/carnitine transporter; SLC, Solute Carrier; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-alpha.
Abstract

Various disease models have been shown to alter hepatic drug metabolizing enzyme (DME) and transporter expression, and to induce cholestasis through altered enzyme and transporter expression. Previously, we detailed the regulation of hepatic DMEs during infectious colitis caused by *Citrobacter rodentium* infection. We hypothesized that this infection would also modulate hepatic drug transporter expression and key genes of bile acid (BA) synthesis and transport. Mice lacking Toll-like receptor 4 (TLR4), interleukin-6 (IL-6), or interferon-gamma (IFNγ) and appropriate wild type animals were orally infected with *C. rodentium* and sacrificed 7 days later. In two wild type strains, drug transporter mRNA expression was significantly decreased by infection for Slc22a4, Slco1a1, Slco1a4, Slco2b1, Abcc6, whereas the down-regulations of Abcc2, Abcc3, and Abcc4 were strain-dependent. In contrast, mRNA expressions of Slco3a1 and Abcb1b were increased in a strain-dependent manner. Expression of Abcb11, Slc10a1, the two major hepatic BA transporters and Cyp7a1, the rate-limiting enzyme of BA synthesis, was also significantly decreased in infected animals. None of the above effects were caused by bacterial lipopolysaccharide, since they still occurred in the absence of functional TLR4. The down-regulation of Slc22a4 and Cyp7a1 was absent in IFNγ-null mice, and the down-regulation of Slco1a1 was abrogated in IL-6-null mice indicating in vivo roles for these cytokines in transporter regulation. These data indicate that *C. rodentium* infection modulates hepatic drug processing through alteration of transporter expression as well as DMEs. Furthermore, this infection down-regulates important genes of BA synthesis and transport and may increase the risk for cholestasis.
INTRODUCTION

Many endogenous and exogenous chemical substances are eliminated primarily by hepatobiliary efflux. The hepatic transporters responsible for this route of elimination belong to two gene families, \textit{ATP-Binding Cassette} (\textit{ABC}) and \textit{Solute Carrier} (\textit{SLC}). Members of these families often share overlapping substrate pools, resulting in the vectorial transport of target chemicals from the sinusoidal blood into the bile (Klaassen and Aleksunes, 2010).

As well as sharing overlapping substrate specificities, hepatic transporters often share common regulatory mechanisms both with other transporters and with hepatic drug metabolizing enzymes. Congiu et al., (2009) reported significant correlations between hepatic expression of drug metabolizing enzymes (DMEs), drug transporters, and transcription factors during viral hepatitis. Understanding the mechanisms and results of this coordinate regulation of DME and transporter expression would provide an invaluable tool in the prediction of altered drug and toxicant disposition in disease states.

Infection and inflammation have been shown to significantly impact hepatic DMEs and drug transporters, in both the clinical setting and a number of experimental models (Teng and Piquette-Miller, 2005; Morgan et al., 2008; Teng and Piquette-Miller, 2008; Cressman et al., 2012). Because of the vital role these proteins play in both the clearance of drugs and toxicants and the metabolism of physiologic molecules, changes in their expression or activity may lead to adverse effects (Saab et al., 2013). However, despite their overlapping substrate specificities, DME and drug transporters have many distinct and specific substrates which they recognize and act upon. Therefore, detailing the effects of inflammation on specific genes (rather than on the class as a whole) is vital to understanding the potential risks and toxicity which may accompany disease and inflammation.
We have previously demonstrated that models of live infection may exhibit distinct and specific patterns of gene expression when compared to lipopolysaccharide (LPS) administration, a standard model of inflammation (Richardson and Morgan, 2005; Chaluvadi et al., 2009). *Citrobacter rodentium* is a rodent-specific intestinal pathogen, producing colitis similar to that seen in enteropathogenic *Escherichia coli* (EPEC) in humans. *C. rodentium* infection produces selective, mostly reversible effects on expression of cytochrome P450s (P450s) and other DMEs (Richardson and Morgan, 2005; Chaluvadi et al., 2009). Interestingly, though this gram-negative bacterium produces LPS, the P450 gene expression changes appear to be LPS-independent, as animals lacking the LPS receptor Toll-like receptor 4 (TLR4) exhibited the same effects (Richardson et al., 2006).

Just as this model of live infection exhibits distinct and specific patterns of gene expression when compared to lipopolysaccharide administration, so different disease states may present divergent impacts on gene expression through distinct mechanisms. As yet, nothing is known about the regulation of hepatic drug transporters during infectious colitis. Therefore, we here report our findings on the impact of *C. rodentium* infection on hepatic transporter expression. To determine whether or not the effects of infection are attributable to bacterial lipopolysaccharide, we performed these studies in wild-type mice and mice lacking a functional toll-like receptor-4 (TLR4).

In order to properly predict the effects of a similar disease state in humans, it is necessary to understand the mechanisms underlying the modulation of these genes. We have previously investigated several potential mechanisms behind the effects of *C. rodentium* infection on hepatic cytochrome P450 expression, including the role of cytokines interleukin-6 (IL-6), interleukin-1 (IL-1), interferon-gamma (IFNγ), and tumor necrosis factor-alpha (TNF-α) in
altering DME expression (Nyagode et al., 2010; Kinloch et al., 2011). A major goal of the present work was to examine the roles played by cytokines IL-6 and IFNγ in observed changes in hepatic transporter expression. To do this, we employed samples from the same IL-6 and IFNγ-null mice investigated in the previous study (Nyagode et al, 2010).

In addition to the impact of sepsis on elimination of drugs and other exogenous compounds, these changes in transporter gene expression are responsible for alterations in the elimination of endogenous substrates in the bile (Geier et al., 2007). Retention of biliary constituents in the liver (cholestasis) can be a serious source of hepatic damage. Though some hepatic transporters (Abcc and Slco sub-family members) transport bile acids (BA) in addition to drugs and toxicants, specific transporters of BA are expressed in the liver and are affected during experimental sepsis (Geier et al., 2007; Lickteig et al., 2007; Bodeman et al., 2013). While experimental sepsis has been shown to drastically reduce genes associated with BA transport and regulation, relatively few studies have reported on the impact of colitis on these genes (Jahnel et al., 2009). Therefore, in order to investigate whether the processes of hepatobiliary transport and BA synthesis are modulated in our model of infectious colitis, we measured the expression of specific bile constituent transporters Slc10a1 (Ntcp), Abcb11 (Bsep), and Abcb4 (Mdr2); the principal enzyme responsible for the rate of BA synthesis (Cyp7a1); and two nuclear receptors known to regulate the expression of these genes (Nr1h4, farnesoid X receptor; Nr1h3, liver X receptor).
MATERIALS AND METHODS

The results detailed in this manuscript were obtained from two separate experiments. Samples from one experiment, in the C57BL/6J background, have been analyzed and results published with respect to disease parameters and DME expression (Nyagode et al., 2010). Experimental design including age, sex, animal treatment, tissue collection, and sample analysis were identical between the two experiments and are detailed below.

Unless otherwise specified, all reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals and treatments
Nine week-old female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeJ mice carry a spontaneous mutation of the TLR4 gene, while C3H/HeOuJ mice remain endotoxin sensitive and were used as WT controls. IL-6- and IFNγ-deficient mice have been back-crossed for more than 10 generations to a C57BL/6J background, and C57BL/6J mice were used as WT controls (Dalton et al., 1993; Kopf et al., 1994). Mice were housed in groups of four or six to a cage and were acclimatized for at least one week in the animal facility before the beginning of the studies. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

Animals of each background were infected with the same bacterial preparation. Infections of C3H/HeJ and C3H/HeOuJ mice with C. rodentium were carried out in a single experiment with 6 animals per treatment group. Each treatment with cytokine-null mice was carried out in two separate experiments with a total of eight animals per treatment group. C. rodentium (#51116, wild-type strain) was obtained from the American Type Culture Collection (Manassas, VA). After
overnight growth in Luria broth, without shaking, at 37°C, the culture was serially diluted in sterile phosphate-buffered saline, and the concentration calculated spectrophotometrically. Infection was achieved by replacing food and drinking water with a 20% sucrose solution which had been inoculated with \textit{C. rodentium}, while control animals received a sterile 20% sucrose solution. Food and water were restored after 24 hours. The volume of liquid consumed by the mice was measured and actual bacterial concentrations in the solution were determined by retrospective plating on MacConkey agar, on which \textit{C. rodentium} forms small pink colonies with white rims. Infected mice were housed in a biosafety level 2 facility to prevent transmission of infection to other mouse colonies. Changes in body weight were monitored daily, and animals were sacrificed 7 days after administration of sucrose or bacteria.

\textbf{Tissue collection}
Liver and spleen were dissected from the abdominal cavity and rinsed in cold 1.15% potassium chloride, then weighed. The liver was then portioned, flash-frozen, and stored at -80°C for subsequent RNA preparation or kept on ice for the determination of viable bacteria. The colon was removed, washed of fecal matter by using cold 1.15% potassium chloride, sectioned, and kept on ice for the determination of viable bacteria. Blood was also collected from the animals at sacrifice and 50 \textmu L was plated to determine approximate bacterial load.

\textbf{Determination of tissue bacterial loads}
The number of viable bacteria in the infected animals was determined from organ homogenates and blood. Liver and colon were weighed and homogenized in 1 ml of phosphate-buffered saline at low speed with a tissuemizer (IKA Works, Inc., Wilmington, NC). Liver homogenate, blood, or serial dilutions of the colon homogenates were plated onto MacConkey’s agar and the number of colony forming units were determined following overnight incubation at 37°C.
RNA extraction, cDNA Synthesis, and mRNA measurement

Total liver RNA was prepared using RNA-Bee isolation reagent (Tel-Test Inc., Friendswood TX), per manufacturer’s instructions. RNA concentration was determined spectrophotometrically, and RNA purity and integrity were confirmed by gel electrophoresis followed by visualization with ethidium bromide. Purified total RNA was reverse-transcribed with a SuperScript First-Strand Synthesis System kit (Life Technologies, Carlsbad, California), according to the manufacturer’s protocol. Primers were custom-synthesized by Operon Biotechnologies, Inc. (Huntsville, AL) and have been published previously or are described below. Relative mRNA expression was measured by reverse transcriptase real-time PCR (RT-qPCR) using the Eppendorf Mastercycler Realplex (Eppendorf, Hamburg, Germany) and SYBR Green Master Mix reagent (Applied Biosystems, Foster City, CA). For each gene, expression data of all samples was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using the ΔΔCt method described by Livak and Schmittgen (2001).

RT-qPCR primers

Primers for mouse drug transporters, cytochrome P450 enzymes, and nuclear receptors were designed using the Primer-BLAST program through the National Center for Biotechnology Information. Primers with potentially unintended templates were discarded and the amplicons from each primer pair were sequenced to ensure specific amplification of the correct gene. The primer sequences are found below (Table 1).

Immunoblotting

Transporter protein levels in mouse whole cell lysates were measured by Western blotting and chemiluminescent detection. Fifty micrograms of whole cell lysate were mixed with Laemmlili Sample Buffer (Bio-Rad, Hercules, CA) and treated under conditions described below for each protein. After treatment, samples were separated on 7.5% SDS-PAGE and transferred to PVDF
membranes. Nonfat dry milk (5%) in PBST was used to block nonspecific antibody binding. The following conditions and antibodies were used for detection of each protein: Abcg2, 5% 2-mercaptoethanol, 85°C for 10 min (BXP-21 clone, Abcam, Cambridge, MA); Abcb1, 5% 2-mercaptoethanol, 37°C for 30 min (H-241 clone, Santa Cruz Biotech, Santa Cruz, CA); Abcc6, no 2-mercaptoethanol, 85°C for 10 min (M6II-31 clone, Abcam). ERK1 (C-16 clone, Santa Cruz Biotech) and ERK2 (C-14 clone, Santa Cruz Biotech,) were used as loading control for all blots. Images were captured and relative protein quantities were determined using Image Lab imaging system (Bio-Rad, Hercules, CA).

**Statistical Analysis**

All data are presented as mean values ± S.E.M. Control and experimental groups were compared by unpaired Student’s T-test. All statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL). Statistical significance was set at p<0.05.
RESULTS

*C. rodentium* infection of receptor- or cytokine-null mice

In our previously published studies using the *C. rodentium* infection model, we observed that the time course of hepatic P450 regulation mirrored the time course of intestinal colonization, reaching a peak at 7 to 10 days post-infection (Chaluvadi et al., 2009). As detailed above, experiments in the current study were performed in two distinct genetic backgrounds, with HeOuJ mice serving as control animals for their HeJ (TLR4-null) counterparts, and C57BL/6J mice serving as controls for the IL-6- and IFNγ-null groups. Despite previous reports of occasional lethality of *C. rodentium* infection in C3H strains (Vallance et al., 2003), all animals survived to the planned experimental endpoint. Additionally, no mice exhibited overt clinical signs of infection with the exception of slight diarrhea.

As previously reported, in the cytokine-null arm of the study the livers of infected mice were 14 to 25% larger and their spleens were 55 to 90% heavier than those of mice not infected with *C. rodentium*, though no change in total body weight was observed (Nyagode et al., 2010). In the TLR4-null study arm, total body weight was significantly decreased from starting body weight, liver weight normalized to whole body weight remained unchanged by infection, and spleen weight doubled in the infected animals (Table 2). Bacterial colonization of blood, liver, and colon of infected animals was also analyzed. As expected, colonization of the colon vastly overshadowed that of the other organs, with bacterial loads per µg of tissue over a million fold higher. Because of the high variability of the bacterial load in colon and blood tissues, there was no significant difference found between WT control and knockout animals. However, bacterial colonization of the liver in mice lacking TLR4 trended towards a value higher than that of their WT counterparts (Table 2).

Effect of *C. rodentium* infection on hepatic uptake transporters
In both WT control groups (HeOuJ and C57BL/6J), significant down-regulation of Slc22a4, Slco1a4, and Slco2b1 was observed in infected animals when compared to uninfected littermates (Figure 1A and 1B). Slco1a1 was dramatically down-regulated in C57BL/6J mice, and a non-significant trend toward the same effect was observed in the HeOuJ strain. No change was seen in the expression of Slc22a1 or Slc22a7 in either WT group. However, significant up-regulation in HeOuJ mice of transporters Slc22a5, Slco1b2, and Slco3a1 contrasted with observed down-regulation in C57BL/6J mice of Slc22a5 and Slco1b2, with a trend towards induction of Slco3a1 failing to reach statistical significance. Additionally, *C. rodentium* infection in C57BL/6J mice markedly down-regulated Slco1a1 expression, an effect that was not significant in HeOuJ mice.

In the absence of functional TLR4, the pattern of regulation during *C. rodentium* infection was highly similar to that in WT mice. The modulation of several transporters (Slc22a4, Slc22a5, Slco1a4, Slco3a1) failed to reach statistical significance in the TLR4-null animals, but in no case did the effects appear to be fully reversed (Figure 1A). In mice lacking IL-6 the down-regulation seen in the expression of Slco1a1 was blocked (Fig. 1B). Likewise, down-regulation of Slc22a4 was fully reversed in IFNγ-null animals. *C. rodentium* infection in mice lacking IFNγ also failed to fully down-regulate Slco1a1, Slco1a4, and Slco2b1 as compared to WT infected animals. With Slc22a1, infection in IFNγ-null mice down-regulated expression though no effect was observed in WT animals. Similarly, Slc22a7 expression was induced by infection in both IL-6- and IFNγ-null mice, but not in WT controls.

**Effect of *C. rodentium* infection on hepatic efflux transporters**

As shown in Figure 2A and 2B, expression of efflux transporter Abcg2 was unaffected by infection in both HeOuJ and C57BL/6J mice, and any changes in expression of Abcb1a failed to reach statistical significance. Contrastingly, Abcc6 was down-regulated in both WT groups. In
HeOuJ but not C57BL/6J mice, Abcb1b was up-regulated and Abcc4 down-regulated by infection. In contrast, Abcc2 and Abcc3 were down-regulated by infection in C57BL/6J mice, but unaffected in HeOuJ mice. Loss of TLR4 failed to modulate the expression changes observed in *C. rodentium* infection (HeJ mice, Figure 2A). The down-regulation of Abcc6 mRNA was partially reversed in both IL-6- and IFNγ-null mice (Figure 2B).

**Effect of *C. rodentium* infection on genes involved in bile synthesis and transport**

*C. rodentium* infection significantly altered the expression of several genes associated with BA transport, synthesis, and signaling within the liver (Figure 3A and 3B). Expression of the farnesoid X receptor (FXR) was significantly up-regulated in HeOuJ infected animals, and significantly down-regulated in C57BL/6J mice. Abcb4, a phosphatidylcholine transporter associated with progressive familial intrahepatic cholestasis, exhibited expression changes of a similar magnitude to FXR (increased in HeOuJ and decreased in C57BL/6J) but failed to reach statistical significance in either strain. Cyp7a1, the rate-limiting enzyme in BA synthesis, and Abcb11, the mRNA encoding the bile salt export pump, were both significantly down-regulated in C57BL/6J mice, but unchanged in HeOuJ mice. Finally, the expression of Slc10a1, encoding the sodium/taurocholate cotransporting polypeptide, was down-regulated in infected animals of both WT groups.

In IL-6-null mice, the effect of infection on Cyp7a1 and Abcb11 was partially reversed when compared to WT animals (Figure 3B). IFNγ-null mice similarly exhibited a smaller down-regulation of Abcb11 during infection; however the effect on Cyp7a1 expression was fully reversed in these mice. Contrastingly, loss of TLR4 failed to modulate the effects of infection of the expression of these bile associated genes (Figure 3A).

**Effect of *C. rodentium* infection on transporter protein expression**
In order to investigate whether *C. rodentium* infection also caused changes in protein expression, western blotting was performed to measure relative changes in efflux transporter proteins. As seen in Figure 4A and 4B, infection decreased protein expression of Abcb1 in both HeOuJ and C57BL/6J mice. Abcg2 protein expression was slightly but significantly down-regulated only in infected HeOuJ mice (Figure 4A), while Abcc6 protein expression was down-regulated only in infected C57BL/6J mice (Figure 4B). Western blot images used for these analyses are shown in Supplemental Figure 1. Absence of TLR4 in the HeJ mice appeared to reverse the down-regulation of Abcb1 caused by infection (Figure 4A). Similarly, loss of either IL-6 or IFNγ reversed the disease-induced decrease in Abcc6 protein expression (Figure 4B).
DISCUSSION

This study demonstrates significant regulation of hepatic drug transporter genes, including Slc22a4, Slc22a5, Slco1a4, Slco1b2, Slco2b1, Slco3a1, Abcb1b, Abcc2, Abcc3, Abcc4, and Abcc6 during colonic inflammation caused by *C. rodentium* infection. *C. rodentium* also significantly alters the expression of BA-associated genes FXR, Cyp7a1, Slc10a1, and Abcb11. Regulation of these genes in mice lacking functional TLR4 was similar to that in WT mice, indicating that bacterial LPS is not the cause. The involvement of cytokines IL-6 and IFNγ in the regulation of Slc22a4, Slco1a1, Slco1a4, and Cyp7a1, as well as more minor involvement in the regulation of other genes, was implied by differences in the responses of IL-6 and IFNγ-null mice.

Slc22a4 (Octn1), Slco1a1 (Oatp1a1), Slco1a4 (Oatp1a4), Slco2b1 (Oatp2b1), Abcc6 (Mrp6) and Slc10a1 (Ntcp) were down-regulated by infection in both WT strains of mice. Of these, expression of all but Slc22a4 were suppressed to one half of WT values or lower, indicating a likelihood that these effects may have pharmacological significance in the transport of the respective drug substrates. Slco1a1 and Slco1a4 play important roles in the transport of estrogen conjugates, as well as several therapeutic drugs (Ose et al., 2010; Gong et al., 2011). While these transporters share substrates with human transporter SLCO1A2, they are not considered orthologs (Shitara et al., 2013).

The physiological significances of the smaller effects seen with e.g. Slc22a4 remain to be determined. In addition, many of the effects observed were strain-dependent. Thus, Slc22a5 (Octn2), Slco1b2 (Oatp1b2), Abcb1a (Mdr1a), Abcb1b (Mdr1b) and FXR were down-regulated in HeOuJ mice and up-regulated in the C57BL/6J strain. Abcc2 (Mrp2), Abcc3 (Bsep) and
Cyp7a1 were down-regulated in C57BL/6J but unaffected in HeOuJ; and Slco3A1 was specifically up-regulated in HeOuJ mice only. At present there is no way to predict which of the strain-specific responses might be relevant to humans, but it does demonstrate the propensity for a wide spectrum of transporter genes to be regulated.

Altered responses to infection in mice lacking IL-6 or IFNγ were observed for a subset of the genes studied. The almost complete suppression of Slco1a1 expression, and the 50% decrease in Abcb11 mRNA seen in WT mice were blocked in the IL6-null animals, indicating a likely in vivo regulation of these genes by IL-6. This complements the findings of Siewert et al., (2004) implicating IL-6 in the down-regulation of Slco1a1 caused by LPS or turpentine administration. By the same criteria, partial roles for IL-6 in C. rodentium infection can be inferred for Slco1a4, Abcc6, and Cyp7a1. Attenuation of Slco1a1, Slco1a4, Abcb1a, Abcc6 and Cyp7a1 down-regulation was also seen in the IFNγ-null mice; whereas, the down-regulation of Slc22a4 was reversed. The overlap between genes apparently regulated by both IL-6 and IFNγ can to some extent be explained by the fact that the components of the inflammatory response are highly interdependent. IFNγ-null animals failed to mount plasma IL-6 or TNFα responses to infection, while IL-6-null mice had an impaired serum IFNγ, but not TNFα response (Nyagode et al., 2010). Interestingly, Geier et al., (2003) described the successful prevention of Oatp2 down-regulation in LPS administration, using etanercept injections to block TNFα activity. That we observed no down-regulation in IFNγ-null mice could be due to the impaired TNFα response in these animals.

To our knowledge, the down-regulation of Slc22a4 and Slc22a5 mRNAs (in C57BL/6J mice) during C. rodentium infection is the first report of hepatic expression being altered in either Octn gene during inflammation. Conversely, hepatic down-regulation of Slco1a4 (Oatp1a4) has been reported in fatty-liver disease, extra-hepatic cancer, and experimental sepsis (Sharma et al.,
2008; Fisher et al., 2009; Bodeman et al., 2013) as well as in the present study, but was not observed in necrotizing enterocolitis (NEC) or colitis caused by dextran sulphate sodium (DSS) treatment (Jahnel et al., 2009; Cherrington et al., 2013). C. rodentium infection also decreased Slco1a1 (Oatp1a1 or Oatp1) gene expression, which finding is consistent with reported down-regulation after treatment with LPS or subcutaneous turpentine (Siewert et al., 2004; Bodeman et al., 2013).

Expression of Slco2b1 (Oatp2b1 or Oatp-B) was decreased in our infective model in animals of both backgrounds. Though previously reported to be decreased in several non-hepatic inflammatory models (Petrovic et al., 2008; Wojtal et al., 2009; Ohkura et al., 2012), to our knowledge this is the first in vivo report of hepatic down-regulation of Slco2b1 during inflammation. Contrastingly, expression of Slco3a1 (Oatp3a1 or Oatp-D) was increased during infection in HeOuJ animals. This transporter has been reported to be increased in bile duct ligation (Klaassen and Aleksunes, 2010), but hepatic regulation during other models of inflammation has not been documented. Expression of SLCO3A1 in patients has been implicated in the accumulation or clearance of several antiretroviral drugs (Janneh et al., 2009; Molto et al., 2013). It is conceivable that increased expression in similar diseases could lead to increased clearance of these substrates, decreasing therapeutic efficacy.

ABCC family members, also known as Mrps, play a vital role in the transport of drugs and endogenous substrates (reviewed by Gu and Manautou, 2010). During live infection we observed a significant decrease in the mRNA expression of Abcc2 in C57BL/6J mice. Other inflammatory conditions have been reported to result in similar down-regulation (Andrejko et al., 2008; Sharma et al., 2008; Cherrington et al., 2013). Multiple studies have indicated a role for IL-6 in the down-regulation of Abcc2 (Siewert et al., 2004; Andrejko et al., 2008). In the current
study, the decrease in Abcc2 expression in IL-6-null mice failed to reach statistical significance, but the decrease did not appear to be meaningfully different from that seen in their WT controls.

Protein expression of hepatic transporters was successfully analyzed for Abcb1, Abcg2, and Abcc6. The TLR4-dependent decrease observed in Abcb1 protein is unexpected both because it occurred in mice with increased Abcb1 mRNA, and because it was the only TLR4-dependent expression change observed. The potential for an LPS-responsive translational control of Abcb1 is an intriguing subject for future study.

Cholestasis often occurs in sepsis and bacterial infections. Due limited sample volumes, we were unable to directly measure the levels of BA in either the blood or the liver. Instead we investigated expression changes in genes involved in BA transport, synthesis, and regulation (Geier et al., 2007). Down-regulation of these genes has been reported in several inflammatory models, including NEC (Cherrington et al., 2013), LPS treatment (Feingold et al., 1996) IL-6 or turpentine treatment (Siewert et al., 2004), extra-hepatic cancer (Sharma et al., 2008), and cecal puncture (Andrejko et al., 2008). We observed down-regulation of hepatic transporters Slc10a1 (sodium/taurocholate cotransporter, Ntcp), and Abcb11 (bile salt export pump, Bsep). Also down-regulated were the key enzyme of BA synthesis, Cyp7a1, and the BA-responsive nuclear receptor FXR. Our studies indicate involvement of both IL-6 and IFNγ in the regulation of Abcb11 and Cyp7a1, as down-regulation was reversed in mice lacking those cytokines. While a role for IL-6 in regulating Abcb11 and Cyp7a1 has previously been identified (Geier et al., 2003; Kim et al., 2003; Siewert et al., 2004), we are unable to find any previous reports on a similar role for IFNγ.

Abcc3 and Abcc4 (Mrp3, Mrp4) are expressed at the apical membrane of hepatocytes and are responsible for effluxing substrates into the blood. Reports of their regulation during
inflammation generally indicate decreased Abcc3 and Abcc4 expression, though induction during cholestasis is common (Siewert et al., 2004; Geier et al., 2007; Le Vee et al., 2011). Induction of Abcc3 during cholestasis is an important compensatory mechanism limiting the hepatocellular accumulation of toxic BAs (Keppler, 2011). We observed a decrease in Abcc3 expression during C. rodentium infection in conjunction with the other more cholestatic-like expression changes. It is unclear if this decrease in Abcc3 expression reflects an earlier timepoint in the cholestatic response, or if C. rodentium infection induces a non-cholestatic condition which alters other BA-associated genes. Studies of the LPS-induced acute phase response have similarly found decreased Abcc3 expression in the context of other cholestatic-like expression changes (Siewert et al., 2004). It is also possible that induction of Abcc3 during cholestasis is regulated through distinct mechanisms from other BA-associated genes (Ruiz et al., 2013).

In an attempt to determine the impact of colitis on these same genes, Jahnel et al., (2009) found no significant changes in mRNA expression in mice treated with DSS despite observable colitis. This suggests that the regulation seen in the current study may be dependent on both the infection and the resultant inflammatory response.

In conclusion, colonic infection by C. rodentium modulated mRNA expression of hepatic transporter genes. Several BA associated genes were also significantly down-regulated during infection. Expression changes of a subset of genes were ablated in IL-6- or IFNγ-null mice. These studies and others defining the mechanisms behind inflammation-induced changes in DME and transporter expression play an important role in understanding and preventing potential adverse reactions caused by altered drug/toxicant clearance during inflammatory disease states.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Nyagode, Merrell, and Morgan

Conducted experiments: Nyagode, Clarke, and Merrell

Contributed new reagents or analytic tools:

Performed data analysis: Merrell

Wrote or contributed to the writing of the manuscript: Merrell, Nyagode, Clarke, Cherrington, and Morgan
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1: Effect of *C. rodentium* infection on hepatic mRNA expression of uptake transporters. Seven days following oral infection with *C. rodentium*, female mice were sacrificed, livers harvested, and mRNA isolated from A) HeOuJ and HeJ (TLR4-null) mice (n=6) and B) C57BL/6, IL-6-null, and IFNγ-null mice (n=8). mRNA was quantified by real-time RT-PCR and resulting values are expressed as relative levels of mRNA expression after normalization to GAPDH, with uninfected wild-type (HeOuJ or C57BL/6) set to 1. Values represent mean ± S.E.M. Significant differences are in comparison with uninfected control groups. *, p<0.05 by *t* test.

Figure 2: Effect of *C. rodentium* infection on hepatic mRNA expression of efflux transporters. Seven days following oral infection with *C. rodentium*, female mice were sacrificed, livers harvested, and mRNA isolated from A) HeOuJ and HeJ (TLR4-null) mice (n=6) and B) C57BL/6, IL-6-null, and IFNγ-null mice (n=8). mRNA was quantified by real-time RT-PCR and resulting values are expressed as relative levels of mRNA expression after normalization to GAPDH, with uninfected wild-type (HeOuJ or C57BL/6) set to 1. Values represent mean ± S.E.M. Significant differences are in comparison with uninfected control groups. *, p<0.05 by *t* test.

Figure 3: Effect of *C. rodentium* infection on hepatic mRNA expression of bile acid associated genes. Seven days following oral infection with *C. rodentium*, female mice were sacrificed, livers harvested, and mRNA isolated from A) HeOuJ and HeJ (TLR4-null) mice (n=6) and B) C57BL/6, IL-6-null, and IFNγ-null mice (n=8). mRNA was quantified by real-time RT-PCR and resulting values are expressed as relative levels of mRNA expression after normalization to GAPDH, with uninfected wild-type (HeOuJ or C57BL/6) set to 1. Values represent mean ± S.E.M. Significant differences are in comparison with uninfected control groups. *, p<0.05 by *t* test.
Figure 4: Effect of *C. rodentium* infection on hepatic protein expression of efflux transports. Seven days following oral infection with *C. rodentium*, female mice were sacrificed, livers harvested, and whole cell lysates prepared from A) HeOuJ and HeJ (TLR4-null) mice (n=6) and B) C57BL/6, IL-6-null, and IFNγ-null mice (n=8). Protein levels of specific transporters were detected by western blotting and quantified using Image Lab software (Bio-Rad, Hercules, CA). Resulting values are expressed as relative levels of protein expression after normalization to Erk1/2, with uninfected wild-type (HeOuJ or C57BL/6) set to 1. Values represent mean ± S.E.M. Significant differences are in comparison with uninfected control groups. *, p<0.05 by t test.
### TABLES

#### Table 1: Primer Sequences used in real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>Abcb1a (Mdr1a)</td>
<td>AGGAGCTGCTGGTGCTCCATCTTCC</td>
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<td>Abcb4 (Mdr2)</td>
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<td>Abcb11 (Bsep)</td>
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Table 2: Disease Parameters

Impact of C. rodentium infection on total body and relative organ weight of liver and spleen, as well as bacterial load in colon, blood, and liver. Values are mean ± S.E.M. (n=6): body weight at sacrifice relative to body weight pre-infection; organ weight at sacrifice relative to body weight at sacrifice; bacterial CFU per gram of tissue or per 50µl of blood. * significantly different than control p<0.05. n.a., not applicable.

<table>
<thead>
<tr>
<th></th>
<th>HeOuJ Control</th>
<th>HeOuJ Infected</th>
<th>HeJ Control</th>
<th>HeJ Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight</td>
<td>1.035 ± 0.009</td>
<td>0.921 ± 0.024 *</td>
<td>1.009 ± 0.009</td>
<td>0.886 ± 0.026 *</td>
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<tr>
<td>Liver (% Body Weight)</td>
<td>5.13 ± 0.16</td>
<td>5.21 ± 0.13</td>
<td>4.98 ± 0.08</td>
<td>5.13 ± 0.28</td>
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<tr>
<td>Spleen (% Body Weight)</td>
<td>0.466 ± 0.023</td>
<td>0.933 ± 0.137 *</td>
<td>0.539 ± 0.027</td>
<td>1.126 ± 0.078 *</td>
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<tr>
<td>Colon Bacterial CFU (X10^5)</td>
<td>n.a.</td>
<td>1049.7 ± 250.0</td>
<td>n.a.</td>
<td>1451.2 ± 340.5</td>
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<td>Blood Bacterial CFU</td>
<td>n.a.</td>
<td>179.8 ± 164.1</td>
<td>n.a.</td>
<td>116.3 ± 35.5</td>
</tr>
<tr>
<td>Liver Bacterial CFU</td>
<td>n.a.</td>
<td>441.7 ± 147.0</td>
<td>n.a.</td>
<td>4868.2 ± 2773.9</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4