Title: Modulation of Hepatic Cytochrome P450s by *Citrobacter rodentium* Infection in Interleukin-6- and Interferon-γ-null Mice

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Abbreviations: P450, cytochrome P450; IL6, interleukin-6; IFNγ, interferon-γ; WT, wild-type;
CFU, colony forming units; tumor necrosis factor-α, TNFα; GAPDH, glyceraldehyde-3-
phosphate dehydrogenase; macrophage inflammatory protein-1α, MIP1α; MPO,
myeloperoxidase; NOS2, nitric oxide synthase 2; FBG, α-fibrinogen; AGP, α1-acid glycoprotein; AGT, angiotensinogen

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ABSTRACT

Following infection with *Citrobacter rodentium*, murine hepatic cytochrome P450 (P450) mRNAs are selectively regulated. Several serum pro-inflammatory cytokines are elevated, the most abundant being interleukin-6 (IL6). To elucidate the roles of cytokines in the regulation of P450s during infection, we orally infected wildtype, IL6⁻/⁻, or interferon-γ⁻/⁻ (IFNγ⁻/⁻) female C57BL/6J mice with *C. rodentium* and analyzed hepatic P450 expression 7 days later. The majority of P450 mRNAs were equally affected by infection in each genotype, indicating that IL6 and IFNγ are not the primary mediators of P450 down-regulation in this disease model. The down-regulation of CYP3A11 and 3A13 and induction of CYP2D9 mRNAs were attenuated in the IL6⁻/⁻ mice, suggesting a role of IL-6 in the regulation of these P450s only. Similar evidence implicated IFNγ in the regulation of CYP2D9, 2D22, 3A11, 3A25 and 4F18 mRNAs in *C. rodentium* infection, as well as CYP2B9, 2D22, 2E1 in the bacterial lipopolysaccharide (LPS) model of inflammation. This is the first indication of an *in vivo* role for IFNγ in hepatic P450 regulation in disease states. The deficiency of IL6 or IFNγ affected serum levels of the other cytokines. Moreover, experiments in cultured hepatocytes demonstrated that tumor necrosis factor-alpha (TNFα) is the most potent and efficacious of the cytokines tested in the regulation of murine P450 expression. It is therefore possible that part of the IFNγ⁻/⁻ and IL6⁻/⁻ phenotypes could be attributed to the reduced levels of TNFα and part of the IFNγ⁻/⁻ phenotype could be due to reduced levels of IL6.
INTRODUCTION

In humans and experimental animals, infections or inflammatory stimuli cause changes in the activity and expression levels of cytochrome P450s (P450s) in the liver and other tissues (Morgan, 1997; Renton, 2004). Such changes can have adverse effects since these proteins catalyze the biosynthesis and catabolism of molecules of physiological importance as well as metabolic clearance of the majority of clinically used drugs.

Lipopolysaccharide (LPS, endotoxin), a toxic component of the cell wall of gram-negative bacteria, has been used extensively as a model of inflammation. Its administration to mice down-regulates the expression and (or) activity of most P450s examined (Warren et al., 1999; Siewert et al., 2000; Li-Masters and Morgan, 2001; Warren et al., 2001; Ashino et al., 2004; Goralski et al., 2005; Richardson and Morgan, 2005; Xu et al., 2006; Chaluvadi et al., 2009b). We previously reported that murine hepatic P450 mRNAs are selectively modulated during Citrobacter rodentium infection in a pattern that is different from the LPS model (Richardson et al., 2006; Chaluvadi et al., 2009a). C. rodentium, a noninvasive rodent pathogen, causes colitis in mice that is similar to that caused by the human pathogen, enteropathogenic Escherichia coli and is therefore used as a model of its infection (Higgins et al., 1999; Goncalves et al., 2001). The pattern of regulation of hepatic P450 expression is similar in both C3H/HeOuJ and C57BL/6 strains of mice infected with C. rodentium, with most P450 isoforms being down-regulated. CYP4A mRNAs and proteins are the most sensitive to this down-regulation.

In order to predict the effects of an inflammatory disease state on drug metabolism in humans, it is necessary to understand the mechanisms by which P450s are regulated during inflammation. In the early stages of a response to inflammatory stimuli, different cytokines are
released into the systemic circulation. Mice infected with *C. rodentium* exhibit significant elevations in serum levels of several cytokines including interleukin-6 (IL6) and interferon-γ (IFNγ) (Chaluvadi et al., 2009a) that are capable of modulating P450 expression in cell cultures. This raises the question of whether these cytokines contribute to hepatic P450 regulation during infection with *C. rodentium*.

The suppression of the constitutive expression of different isoforms from CYP1 – 3 families by IL6 has been documented in hepatocyte cultures of different mammals including pigs (Kleine et al., 2008), rabbits (Kourylko et al., 2006), rats (Williams et al., 1991; Chen et al., 1995) and humans (Abdel-Razzak et al., 1993; Muntane-Relat et al., 1995; Aitken and Morgan, 2007). There is also evidence that IL6 has significant *in vivo* roles in rodents. Ashino et al., (2004) reported that IL6-deficiency blocked the down-regulation of CYP3A11 and 2C29 in mice treated with Bacillus Calmette-Guerin vaccine. The turpentine-induced down-regulation of CYP1A2, 2A5 and 3A11 mRNAs observed in wildtype (WT) mice was completely abolished in IL6-deficient (IL6−/−) mice (Siewert et al., 2000). The injection of rats with IL6 caused the suppression of CYP2C11 and CYP2E1 but not 3A2 mRNA (Morgan et al., 1994).

IFNγ has immunomodulatory effects on several cell types including the activation of murine macrophages which have microbicidal activity (Dalton et al., 1993). Compared to WT mice, IFNγ− deficient (IFNγ−/−) mice have been reported to be significantly more susceptible to infection by *Mycobacterium bovis* (Dalton et al., 1993), *Listeria monocytogenes*, (Huang et al., 1993) and *C. rodentium* (Simmons et al., 2002). The injection of rats with IFNγ resulted in the suppression of CYP3A2 mRNA while the activities associated with CYP2C11 and 2A1 were
unaffected (Craig et al., 1990). IFNγ has been reported to down-regulate the mRNA and (or) activity levels of different P450 isoforms in hepatocytes of rats (Tapner et al., 1996) and humans (Abdel-Razzak et al., 1993; Donato et al., 1997; Aitken and Morgan, 2007).

The cytokines that contribute to the regulation of hepatic P450s in *C. rodentium* infection are unknown. As noted above, IL6 has been demonstrated to be important for P450 down-regulation in at least two models of sterile inflammation. Although IFNγ can down-regulate human and rodent P450s *in vivo* and in cell culture, the effect of IFNγ gene deletion on P450 regulation has not been tested in any *in vivo* inflammatory disease model. Therefore, the objective of this study was to examine the role of IL6 and IFNγ in mediating the regulation of constitutive P450s of the mouse liver during *C. rodentium* infection, and of IFNγ in the LPS model of inflammation, by comparing the responses to these agents in WT and cytokine-deficient mice.
MATERIALS AND METHODS

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

Mice

Female mice, 3, 5, 7 and 9 week old, were purchased from The Jackson Laboratory (Bar Harbor, ME). IL6- and IFNγ - deficient mice were originally generated on mixed C57BL/6 x 129 background and subsequently back-crossed for more than 10 generations to a C57BL/6J background (Dalton et al., 1993; Kopf et al., 1994). C57BL/6J mice were used as WT controls. The mice were housed in groups of four or six to a cage, and were acclimatized to the animal facility for at least one week before the beginning of an experiment. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

Animal treatment

In the C. rodentium experiments, 9-week old mice were housed in groups of four to a cage. Each treatment with cytokine-null mice was carried out in two separate experiments for a total of 8 animals per treatment group. The experiment involving animals of different age groups was done once with 4 animals per treatment group. A wild-type strain of C. rodentium (#51116) was obtained from the American Type Culture Collection (Manassas, VA). Following overnight growth in Luria broth, without shaking at 37°C, the bacteria were serially diluted in sterile phosphate-buffered saline, and the nominal concentration calculated spectrophotometrically. Infected mice were administered an estimated 2 x 10^8 colony forming units (CFU) per mouse of
C. rodentium in 20% sucrose solution instead of drinking water for 24 h. This nominal dose was found to be effective in our previous work (Richardson et al., 2006). To minimize differences between infections, all animals under treatment were infected with the same bacterial preparation. Control mice received 20% sucrose in the same time period. The bacterial concentrations given to each cage were determined by retrospective plating on MacConkey agar, from the volumes of liquid consumed. Actual doses were between 3 - 7 x 10^7 bacteria/mouse in the cytokine-null experiment and between 8 – 14 x 10^7 bacteria/mouse in the age group experiment. Infected mice were housed in a Biosafety Level-2 facility to prevent transmission of infection to other mouse colonies. Food consumption and changes in body weight were monitored daily and animals were sacrificed 7 days after administration of sucrose or bacteria. In the LPS experiment, mice were housed in groups of six to a cage. Mice in the treatment group were given an i.p. injection delivering 1 mg/kg LPS (E. coli 0111:B4, ultrapure, Invivogen, San Diego, CA) dissolved in sterile saline while control mice received an i.p. injection of sterile saline alone. Food was withheld from the animals after the injection and the animals sacrificed after 24 h.

**Tissue collection**

Blood was collected from the animals at sacrifice, and allowed to clot for about 30 min at room temperature. Serum was separated by centrifugation for 10 min at 7,500g and stored at -80°C until analyzed. Liver and spleen were dissected out of the abdominal cavity and rinsed in cold 1.15% potassium chloride then weighed. The liver was then portioned, flash frozen then stored at -80°C for subsequent RNA or microsome preparation, or kept on ice for the determination of viable bacteria. The colon was removed and washed of fecal matter using cold
1.15% potassium chloride, sectioned, then kept on ice for the determination of viable bacteria and myeloperoxidase analysis.

**Determination of Tissue Bacterial Loads**

The number of viable bacteria was determined from organ homogenates. Liver and colon were weighed and homogenized at low speed with a tissuemizer (IKA Works, Inc., Wilmington, NC) in 1 ml of phosphate buffered saline. Liver homogenate, blood or serial dilutions of the colon homogenates were plated onto MacConkey’s agar, on which *C. rodentium* forms small pink colonies. The number of CFU were determined after overnight incubation at 37°C and the results reported per gram of tissue or 50 µL of blood.

**Microsome Preparation**

Liver homogenates were first centrifuged at 7,500g (25 min) to yield a supernatant that was again centrifuged for 35 min at 250,000g. The resulting microsomal pellet were resuspended in 10 mM Tris acetate buffer pH 7.4, containing 0.1 mM EDTA and 23% glycerol, and stored at -80°C. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc, Rockport, IL) with bovine serum albumin as the protein standard.

**Hepatocyte Isolation and Treatment**

Mouse hepatocytes were isolated by a two-step *in situ* collagenase perfusion procedure previously described by Sewer and Morgan (1997). Following anesthesia with ketamine/xylazine solution, the liver of female WT C57BL/6J mice were perfused via the portal vein with 120 – 150 ml of Krebs-Ringer bicarbonate buffer followed by 50 – 70 ml of 0.3 mg/ml
collagenase Type IV, at a flow rate of 14 - 15 ml/min. Hepatocytes with at least 80% viability were then plated on 12-well collagen-coated plates (BD Bioscience, CA) with Williams E medium (Atlanta Biologicals, Atlanta, GA) containing 10 mM Hepes pH 7.4, 150 nM insulin, 50 nM dexamethasone, 10 mg/ml penicillin/streptomycin and 10% fetal bovine serum. After 4 h, cells were overlaid with fresh Williams E medium containing 0.23 mg/ml matrigel (BD Biosciences, San Jose, CA) and allowed to incubate overnight. Cultures were maintained at 37°C in 5% carbon dioxide throughout the experiment. The effect on untreated cells of regular media changes either everyday or every other day was compared over a 6 day period in order to optimize experimental conditions. To investigate the effect of different cytokines on P450 mRNA levels over time, the primary cultures were treated on the 3rd or 6th day with 10ng/ml each of mouse recombinant IL6 (Sigma-Aldrich, St. Louis, MO), IFNγ (Calbiochem, San Diego, CA) or tumor necrosis factor-α (TNFα, R&D Systems, Minneapolis, MN) and the cells harvested after 6, 12 and 24 h. The hepatocytes were also treated with 1, 3, 10 or 30 ng/ml of each cytokine for 24 h. All cytokines were reconstituted and stored according to manufacturer specifications as high concentration stocks. They were diluted from stock solutions into media just before treatment.

**RNA extraction, cDNA synthesis and quantitative RT-PCR**

Total liver and hepatocyte RNA were prepared using RNA-Bee isolation reagent (Tel-Test, Friendswood TX), according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm while RNA purity and integrity were confirmed by formaldehyde-agarose gel electrophoresis followed by visualization with ethidium bromide.
To synthesize cDNA, purified total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Primers were custom-synthesized by MWGBiotech, Inc. (High Point, NC) or Operon Biotechnologies, Inc. (Huntsville, AL) and have been previously published (Richardson and Morgan, 2005; Chaluvadi et al., 2009a). The relative expression of mouse liver mRNAs was measured by real-time RT-PCR using the ABI PRISM 7000 Sequence Detection System and SyBr® Green Master Mix reagent (Applied Biosystems, Bedford, MA) as described previously (Richardson and Morgan, 2005). To normalize the inter-sample variation in quality inherently associated with RNA preparation, the transcription level of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was quantified for all samples. No significant differences were detected in GAPDH expression among treatment groups. The value obtained from each target gene was then normalized using the GAPDH value to calculate the relative target gene mRNA levels in comparison with the corresponding control groups by the Ct method described by Livak and Schmittgen (2001). The expression level in control samples was arbitrarily set at 1.

**Immunoblotting**

P450 protein levels in mouse hepatic microsomes were measured by Western blotting and chemiluminescent detection as described previously (Chaluvadi et al., 2009a). P450 antibodies were generously provided by Dr. James Halpert (University of Texas Medical Branch, Galveston, TX; rat CYP2B and 3A), Dr. Gordon Gibson (University of Surrey, Guildford, UK; rat CYP4A), Dr. Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden; rat CYP2E1), and Dr. Masahiko Negishi (National Institute of Environmental Health Sciences,
mouse CYP2D9). Polyclonal antibodies to rat CYP2B, 3A, 4A, 2D and 2E proteins were diluted 1:5,000, whereas CYP2C was diluted 1:20,000. All assays were performed within a linear range. Proteins were detected using the SuperSignal® West Pico chemiluminescent substrate kit (Thermo Scientific, Rockford, IL). The intensity of chemiluminescent protein bands were visualized by fluorography on x-ray film, scanned and the protein bands quantified by densitometry using Kodak Molecular Imaging software (Eastman Kodak Co., Rochester, NY).

**Cytokine Analysis**

Serum samples were assayed for the cytokines interleukin-1β, interleukin-2, IL6, IFNγ and TNFα as well as the chemokines, CXCL1 (KC), CXCL10 (IP-10), macrophage inflammatory protein-1 alpha (MIP1α) using the Milliplex™ mouse cytokine/chemokine kit (Millipore Corporation, Billerica, MA) following the manufacturer’s protocol as described previously (Chaluvadi et al., 2009a). Data were analyzed using MasterPlex software1.2 (Hitachi Software Engineering America, Ltd., San Francisco, CA) and the concentrations are expressed in pg/ml.

**Myeloperoxidase Analysis**

Colon segments were weighed then homogenized in a buffer containing 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0). The homogenates were then subjected to three freeze-thaw cycles, followed by sonication on ice for 10 seconds (Microson™ Ultrasonic cell disruptor). The resulting solution was centrifuged at 13,000g for 15 min at 4°C. Protein concentration in the supernatant was measured by the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc, Rockport, IL) with bovine serum albumin as the internal standard.
protein standard. Aliquots of supernatant containing 10µg protein or myeloperoxidase (MPO) standard were added to 200 µl of substrate (0.167 mg of o-dianisidine per ml, 0.0005% hydrogen peroxide in extraction buffer) and the reaction allowed for 10 min. MPO activity was calculated by comparison with a standard curve generated within a linear range of detection with the authentic enzyme when measured colorimetrically at 450 nm with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). MPO activity is expressed as units per milligram of protein. One unit of enzyme activity is defined as the amount that consumes 1 µmol of H$_2$O$_2$/min.

**Statistical Analysis**

All data are presented as mean values ± standard error of the mean (S.E.M.) unless otherwise designated. Control and experimental groups were compared either by unpaired Student’s $t$ test ($t$ test) or one-way analysis of variance followed by Dunnett’s test as was considered appropriate. All statistical analysis was done using SPSS software (SPSS Inc., Chicago, IL).
RESULTS

*C. rodentium* Infection of Cytokine-null Animals

We previously reported that following *C. rodentium* infection, the time course of P450 regulation in mice followed that of colonic inflammation and bacterial colonization, peaking at 7-10 days post-infection (Chaluvadi et al., 2009a). We therefore carried out these studies 7 days post-infection and compared the infected animals (Inf) to untreated control groups (Ctrl), since our previous study found that pair-fed groups were similar to untreated controls. Each experiment with WT, IL6−/− or IFNγ−/− genotypes was performed in two separate groups of 4 mice. The results were essentially identical in the duplicate experiments, therefore they were combined in this report. All animals survived the duration of the *C. rodentium* infection. In the present study, no mice exhibited obvious signs of infection with the exception of slight diarrhea. There was no significant change in body weight (data not shown). The livers of infected mice were 14 – 25% larger and their spleens 55-90% heavier than those of mice not infected with *C. rodentium* (Table 1).

*Tissue Bacterial Loads.* To measure bacterial loads, blood as well as liver or colon homogenates were cultured on MacConkey agar plates. In infected WT, IL6−/− and IFNγ−/− mice, the bacterial loads in the blood and liver were at least 10^8 times lower than those in the colon (Table 2). The bacterial load was higher in the blood of WT than cytokine-deficient mice, though not significantly so due to the variability in WT data. While the bacterial loads in the liver and colon were not significantly different between WT and IL6−/− mice, bacterial colonization tended to be higher in IFNγ−/− colon and was significantly lower in the liver.

*Effect of C. rodentium Infection on Colon Myeloperoxidase Activity.* *C. rodentium* induces a localized inflammatory response in the colon, characterized by infiltration of immune
cells such as neutrophils and lymphocytes. To investigate whether the absence of IL6 or IFNγ affected the recruitment of neutrophils to the colon we measured MPO levels in infected colon tissue. MPO activity in the colons of infected WT and IFNγ−/− mice were two- and 6-fold greater respectively than in uninfected (control) mice while there was no difference between the MPO activity of control and infected IL6−/− mice (Table 3).

Effect of C. rodentium Infection on Hepatic P450 mRNA and Protein Expression. The regulation of the following P450s were not discernibly different among the WT, IL6−/− and IFNγ−/− mice: CYP1A2 and CYP2A5 mRNAs were unchanged; 2E1, 4A10 and 4A14 were reduced to 22 to 74% of control levels and CYP2D9 and 4F18 mRNA were induced between 1.8 to 4.4 times their control levels (Fig. 1). While in WT and IL6−/− mice the mRNA levels of CYP2D22 and 3A25 were reduced to between 55 and 65% of control levels, mRNA levels of these P450s remained unchanged in IFNγ−/− mice. Infection did not change levels of CYP3A13 in WT and IFNγ−/− mice, but reduced them to 69% of control in IL6−/− mice. The down-regulation of CYP3A11 mRNA observed in WT was blocked in both IL6- and IFNγ-null animals. CYP2B9 was down-regulated in all three strains, but the magnitude of the effect was diminished in the IL6−/− and IFNγ−/− mice. In uninfected mice, constitutive expression levels of all mRNAs measured were comparable among all three strains (Supplemental Figure 1).

In all three genotypes, CYP4A protein levels were down-regulated to less than 50% of control levels by infection with C. rodentium, while protein levels of CYP2E and the upper (lighter) CYP2B band, designated CYP2B (L), were unchanged (Fig. 2). Levels of the lower (heavier) CYP2B band, designated CYP2B (H), were up-regulated in WT and IFNγ−/− mice but not in IL6−/−. While there was no overall change in total CYP2B-like protein levels, designated CYP2B (T), of WT and IL6−/− mice, there was an almost two-fold increase in IFNγ−/− mice.
Infection did not significantly down-regulate CYP2C protein levels in WT mice, in contrast to the down-regulation observed in both cytokine-deficient strains. Total CYP2D proteins as well as the individual bands CYP2D (H) and (L) recognized by the antibody, were down-regulated in WT, but not in IL6- or IFNγ−/− mice. CYP3A proteins were decreased by at least 25% in both WT and IFNγ−/− mice but not IL6−/− mice.

**Effect of C. rodentium Infection on Hepatic Cytokine and Acute Phase Protein mRNA Expression.** To determine the contribution of IL6 and IFNγ to changes in levels of hepatic cytokine and acute-phase protein mRNAs during C. rodentium infection, their mRNAs were quantified using real time RT-PCR. Infection did not change IL6 mRNA expression in either WT or IFNγ−/− mice but induced TNFα and nitric oxide synthase (NOS2) mRNAs in all three groups (Fig. 3). When compared to WT, the absence of IL6 resulted in the potentiated up-regulation of IL1β, TNFα and NOS mRNAs, but abrogated the induction of α-fibrinogen (FBG) and α1-acid glycoprotein (AGP) mRNAs. In WT mice, infection caused a 36% decrease in angiotensinogen (AGT) mRNAs that was abolished in IFNγ−/− mice. The absence of IFNγ not only abrogated the up-regulation of IL1β and FBG mRNAs seen in WT mice but also attenuated the up-regulation of TNFα, AGP and NOS2 mRNAs.

**Effect of C. rodentium Infection on Serum Cytokines.** In WT mice, C. rodentium infection elevated the levels of all serum cytokines measured except for IL1β and MIP1α which remained unchanged in all three groups of mice (Fig. 4). Levels of the chemokine KC (mouse analog of human IL8) were elevated in all three groups of mice from about 80 pm/ml in control animals to between 215 to 315 pg/ml in infected animals. Both TNFα and IP-10 were elevated in IL6−/− mice but were either absent, (TNFα) or unchanged, (IP-10) in IFNγ−/− mice. The cytokine-deficient mice had very small amounts of basal IL2 that were not significantly
increased by infection. Levels of IFN\(\gamma\) were unchanged by infection in IL6\(^{-/-}\) mice but IL6 levels increased from undetectable levels in IFN\(\gamma\)^{-/-} mice to about 4 pg/ml. IL6 was induced more than 200-fold by infection in WT mice.

**Effect of Age on Responses to *C. rodentium* Infection**

We previously reported the effect of *C. rodentium* on P450 regulation in 7-9 week old mice at 7-days post-infection (Chaluvadi et al., 2009a). While the data trends observed in that report and those presented in this study are in general agreement, we noticed that some responses were less robust in the present study. We therefore investigated the effect of age on P450 regulation at 7 days post-infection, comparing infected animals to untreated groups of 4, 6, 8 and 10 week-old mice. With the exception of slight diarrhea, none of the animals exhibited any other obvious signs of infection. We did not observe any significant effect of age on tissue bacterial loads or colon MPO activity (Supplemental Tables 1, 2) and despite some differences in constitutive expression of mRNAs, the regulation of P450 mRNAs by *C. rodentium* infection was very similar in all four age groups (Supplemental Figs. 2, 3).

**Effect of LPS Treatment on Hepatic P450 mRNA of C57BL/6J Wild-type and IFN\(\gamma\)^{-/-} Deficient Mice**

The effect of LPS on hepatic P450 mRNA levels was investigated in WT and IFN\(\gamma\)^{-/-} mice 24 hr after a 1 mg/kg i.p. injection. In contrast to the selective effects of *C. rodentium*, LPS treatment down-regulated most of the mRNAs studied except CYP2D9 which was induced 3-fold in WT and almost by 13-fold in IFN\(\gamma\)^{-/-} (Fig. 5). CYP3A13, which was not affected in WT, was induced two-fold in IFN\(\gamma\)^{-/-} mice by LPS treatment. Most down-regulated mRNAs
were affected to the same extent in the two genotypes, but the down-regulation of CYP2B9, 2D22, 2E1 and perhaps 3A41 appeared to be attenuated in the IFNγ−/− mice.

**Effect of Cytokine Treatment on mRNAs in Mouse Hepatocytes**

In preliminary experiments carried out to optimize culture conditions, media changes were done either every day or every other day. A comparison of basal expression over 6 days revealed that there were generally no significant differences in levels of GAPDH, CYP2D22, 3A11 and 4A10 between the two conditions (data not shown). In both cases, in comparison to freshly plated cells, there was an initial rapid drop in P450 expression levels then a plateau after the second day followed by evidence of further expression decrease by the sixth day.

Three or six days after plating, primary cultures were treated with 10 ng/ml each of mouse recombinant IL6, IFNγ or TNFα and the cells harvested after 6, 12 and 24 h. The results from the 3 day and 6 day cytokine treatments generally had similar trends (Fig. 6). Hepatocytes treated on the third day with TNFα had more pronounced mRNA level changes than those treated on the sixth day. While TNFα had some effect on all mRNAs measured on one or both days, IL6 affected only CYP3A13 on hepatocytes treated on the third day and at the 24 h time point of cells treated on the 6th day. IFNγ had no significant effect on almost all mRNAs measured.

To investigate the effect on P450 mRNA of different cytokine concentrations, on the third day after plating, the hepatocytes were treated with 1, 3, 10 or 30 ng/ml of each cytokine for 24 h. The mRNAs for CYP2D9, 3A11, 3A25 and 4A10 were down-regulated after treatment with either IL6, IFNγ or TNFα, with TNFα being more potent and efficacious than IL6 (Fig. 7). IFNγ was also more potent than IL6, causing its maximal effects at the lowest concentration.
tested, but it was the least efficacious of the three cytokines. Treatment with either IL6 or IFNγ resulted in levels of CYP3A13 and 4F18 that were not significantly different from untreated cells while TNFα treatment resulted in the down-regulation of CYP3A13 and up-regulation of CYP4F18 mRNA expression levels. Both IL6 and TNFα down-regulated levels of CYP2D22 but IFNγ did not. Treatment with TNFα resulted in the elevation of both IL6 and IFNγ mRNAs in the hepatocytes while treatment with IL6 did not significantly affect levels of either IFNγ or TNFα and treatment with IFNγ elevated levels of both IL6 and TNFα (data not shown).
DISCUSSION

To investigate the role of IL6 and IFNγ in mediating the regulation of hepatic P450s during *C. rodentium* infection, we compared the responses of IL6−/− and IFNγ−/− to those of WT animals. Our results show that the regulation of CYP4A10 and 4A14, which are most sensitive to down-regulation in *C. rodentium* infection, as well as CYP 2E1, does not require either IL6 or IFNγ since they were similarly affected in WT and cytokine-null animals. On the other hand, the data indicate the involvement of IL6 in the regulation of CYP2D9, 3A11 and 3A13 and of IFNγ in the regulation of CYP2D9, 2D22, 3A11, 3A25 and 4F18 mRNAs. Down-regulation of CYP2B9 may be partially dependent on IL6 and IFNγ as well.

The selective effects of IL6 or IFNγ deletion on hepatic P450 regulation cannot be attributed to decreased infectivity or virulence of the bacterium. In fact, IFNγ-null mice are more susceptible and exhibit greater pathology of *C. rodentium* infection (Simmons et al., 2002), while IL6-null mice exhibit prolonged bacterial colonization and greater mucosal inflammation and mortality than WT mice (Dann et al., 2008). In this study, bacterial loads in the liver and colon were not significantly different among the genotypes, indicating that colonization was unaffected.

The down-regulation of CYP3A11 mRNA by infection seen in WT mice was abrogated in IL6−/− mice (Fig. 1). Siewert et al. (2000) reported that reduction of CYP3A11 mRNA levels during turpentine-induced inflammation was abrogated in IL6−/− mice. Thus, IL6 has a role in the *in vivo* regulation of CYP3A11 in both disease models. The down-regulation of CYP3A13 mRNA occurred only in the IL6−/− mice, perhaps indicating that IL6 opposes another signal that tends to induce CYP3A13. These findings suggest that human CYP3A4, which is down-regulated by IL6 in human hepatocytes (Abdel-Razzak et al., 1993; Muntane-Relat et al., 1995;
Jover et al., 2002; Aitken and Morgan, 2007) may also be regulated by IL6 in vivo in certain disease states. Trautwein et al. (1992) reported the time dependent suppression of CYP2D by IL6 in C3H/HeJ mice. Here, the absence of IL6 attenuated the up-regulation of CYP2D9, suggesting that IL6 is important for this inductive response in vivo. Conversely, absence of IL6 potentiated the up-regulation of hepatic IL1β and TNFα mRNAs (Fig. 3). During inflammation, IL6 is thought to be the most important cytokine in regulating the synthesis of hepatic acute-phase proteins (Castell et al., 1989; Siewert et al., 2000). Here we demonstrate the involvement of IL6 in the regulation of FBG and AGP mRNAs but not AGT in mice during infection (Fig. 3).

When compared to WT mice, the absence of IFNγ abrogated the down-regulation of CYP2D22, 3A11 and 3A25 mRNAs as well as the up-regulation of FBG mRNA, indicating the involvement of IFNγ in their in vivo regulation during C. rodentium infection, probably at the transcriptional level (Figs. 1 and 3). The up-regulation of CYP4F18, AGP and TNFα mRNAs was also attenuated in IFNγ−/− mice, suggesting a possible role for IFNγ in these inductive responses as well.

Though we were able to quantify P450 proteins that react with antibodies to rat liver proteins, the inability to identify mouse-specific immunoreactive proteins precludes making close associations between mRNA and protein regulation except in the case of CYP2E1 in which there was no significant effect of infection in either WT, or cytokine-null mice. Similar to CYP4A10 and 4A14 mRNAs, CYP4A proteins were the most sensitive to down-regulation.

Injection of LPS is a pleiotropic inflammatory stimulus that results in the acute down-regulation of many hepatic P450 mRNAs (Morgan et al., 2008). Siewert et al. (2000) reported that LPS administration down-regulated CYP1A2, 2A5, 2E1 and 3A11 mRNAs in IL6−/− mice. Similarly, LPS down-regulated most of the mRNAs studied comparably in both WT and IFNγ−/−.
mice (Fig. 5). While C. rodentium infection did not change mRNA levels of CYP1A2, 2A5 and 2C29 in WT or IFNγ−/− mice, LPS depressed these mRNAs profoundly in both strains. The down-regulation of CYP2D22 and 2B9 seemed to be attenuated in IFNγ−/− mice, suggesting that IFNγ might have partial roles in regulation of these two P450s in both the C. rodentium and LPS models. While the induction of CYP2D9 in LPS-treated mice was enhanced by the absence of IFNγ−/−, suggesting a suppressive effect of IFNγ on this mRNA, CYP2D9 was similarly induced by C. rodentium in WT and IFNγ−/− mice (Fig. 1 and 5). We have previously shown the induction of CYP3A13 mRNA by LPS in mice of two different genetic backgrounds (Richardson and Morgan, 2005; Chaluvadi et al., 2009b). With C57BL/6 mice, CYP3A13 mRNA was only induced in IFNγ−/− mice, implying the involvement of IFNγ in the LPS modulation of CYP3A13.

The effective and sustained action of proinflammatory cytokines is modulated by synergy or additivity with other cytokines as well as antagonism by opposing cytokines. Thus, as noted by Siewert et al. (2000), when a genetic deletion of a cytokine or its receptor fails to change the regulation of a P450 in response to an inflammatory stimulus, it could reflect functional redundancy of cytokines. This could also be true in the C. rodentium model. In this study IFNγ−/− mice did not have detectable levels of circulating IL6 and TNFα, and IL6−/− mice had normal levels of IFNγ but undetectable TNFα levels. Thus, part of the IFNγ−/− and IL6−/− phenotypes could be attributed to the reduced levels of TNFα; similarly, part of the IFNγ−/− phenotype could be due to reduced levels of IL6. However, IFNγ has roles in the regulation of CYP2D22, 3A25 and 4F18 that are independent of IL6, because these P450s are regulated similarly by infection in WT and IL6−/− mice. Blunted induction of serum IL2 in both IL6−/− and IFNγ−/− mice could also contribute to the observed differences in P450 regulation. Given the complex interplay of cytokines that occur during inflammation and infection, our findings emphasize the point that a
change in the response of a P450 (or any other gene product) in a cytokine-null animal must be interpreted cautiously because it could reflect an indirect role of that or another cytokine. On the other hand, this is the first report that IFNγ contributes to hepatic P450 regulation in a model of infection and inflammation.

To further explore effects of these cytokines on P450 expression, we used murine primary hepatocyte cultures. The largest changes in P450 expression were seen with TNFα treatment (Fig. 7) which exhibited a concentration-dependent effect on all mRNAs tested. Suppression of P450s by TNFα has been documented in hepatocyte cultures of pigs (Kleine et al., 2008), rats (Chen et al., 1995) and humans (Abdel-Razzak et al., 1993; Aitken and Morgan, 2007; Kleine et al., 2008). In our hands, the effect of IL6 on mouse P450 expression was very similar to that of TNFα, but less pronounced. IL6 treatment of hepatocytes either slightly induced or did not affect CYP3A13 expression (Figs. 6 and 7) while the absence of IL6 resulted in the down regulation of CYP3A13 by infection (Fig. 1). Together these observations suggest that in vivo IL6 may act to counter a down-regulation by another factor. Unlike IL6 and TNFα, there was no clear concentration-dependent P450 regulation observed with IFNγ treatment. IFNγ was the least effective cytokine at down-regulating P450s and did not have any significant effect on either CYP2D22 or 3A13. While information gleaned from hepatocytes in culture may convey that a given P450 can be affected by a cytokine, interpretations should be made with caution since a particular effect might be different in response to inflammation or infection in vivo. Nevertheless, our hepatocyte experiments suggest that TNFα is the most potent and efficacious of the cytokines tested in the regulation of murine hepatocyte P450 expression, and this may partially explain why the effects of IL6 or IFNγ deficiency that we observed in vivo were limited in scope and (or) magnitude.
In conclusion, our in vivo results indicate distinct roles for both IL6 and IFNγ in regulation of different hepatic P450 mRNAs and proteins during C. rodentium infection, whereas other P450s appear to be regulated by other mechanisms. The P450s most sensitive to down-regulation in the C. rodentium model, the CYP4A5s, are unaffected by deficiency of either IL6 or IFNγ. In view of the emerging concept of IL6 as a central mediator of inflammation (Morgan et al., 2008), it is surprising that IL6 gene deletion affected the regulation of so few P450s in the C. rodentium model of infection. Our results indicate that IL6 is not the primary or sole mediator of P450 down-regulation in all disease models, and that the utility of serum cytokines as biomarkers of hepatic P450 activity will differ with the type of disease and the identity of the P450 enzyme. Experiments are on-going to explore the effect of the absence of TNFα on P450 regulation in this model of infection. The results presented here as well as and those reported from other studies in mice deficient in different cytokines or cytokine receptors have failed to identify any one cytokine responsible for the effects of LPS (Warren et al., 1999; Siewert et al., 2000; Warren et al., 2001; Ashino et al., 2004). These results strongly suggest possible functional cytokine redundancy in the LPS model.
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REFERENCES


Chen JQ, Strom A, Gustafsson JA and Morgan ET (1995) Suppression of the constitutive expression of cytochrome P-450 2C11 by cytokines and interferons in primary cultures of


Figure 1: Effect of oral *C. rodentium* infection on mRNA expression of hepatic P450s in wildtype (WT), interleukin-6-deficient (IL6−/−) and interferon-γ-deficient (IFNγ−/−) mice. Over 24 h, mice were allowed to drink either sucrose solution (Ctrl) or *C. rodentium* in sucrose (Inf) then sacrificed after 7 days and livers harvested for measurement of mRNA by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH, with sucrose-treated (Ctrl) group set to 1. Values represent mean ± S.E.M, (n = 8) and significant differences are in comparison to control groups. *, p < 0.05 by t test.

Figure 2: Effect of oral *C. rodentium* infection on expression of hepatic P450 proteins in wildtype (WT), interleukin-6-deficient (IL6−/−) and interferon-γ-deficient (IFNγ−/−) mice. Liver microsomes were prepared from the mice described in Fig. 1, and P450 protein levels were measured by Western blotting. For CYP2B and CYP2D the heavy (lower) band is designated (H), light band (L) and the sum of both is designated (T). Western blots of samples from infected and control liver microsomes (top). Quantitative analysis of western blot data (bottom). Values represent mean ± S.E.M, (n = 8) and significant differences are in comparison to control groups. *, p < 0.05 by t test.

Figure 3: Effect of oral *C. rodentium* infection on cytokine and acute phase protein mRNA expression in the liver of wildtype (WT), interleukin-6-deficient (IL6−/−) and interferon-γ-deficient (IFNγ−/−) mice. Total liver RNA from the mice described in Fig. 1 was analyzed by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after
normalization to GAPDH with sucrose-treated (Ctrl) group set to 1. Values represent mean ± S.E.M, (n = 8) and significant differences are in comparison to control groups. *, p < 0.05 by t test.

**Figure 4.** Effect of oral *C. rodentium* infection on serum cytokines of wildtype (WT), interleukin-6-deficient (IL6−/−) and interferon-γ-deficient (IFNγ−/−) mice. Mice were treated as described in Fig. 1 then sacrificed after 7 days, at which time blood was collected for measurement of serum interleukin-1β (IL1β), interleukin-2 (IL2), IL6, IFNγ, TNFα, CXCL1 (KC), CXCL10 (IP-10) and macrophage inflammatory protein-1 alpha (MIP1α). Significant IFNγ immunoreactivity was detected in of the IFNγ−/− mice, which have a targeted mutation in exon 2 of the gene that introduces a termination codon after the first thirty amino acids of the IFNγ protein. The immunoreactivity is presumably due to expression of the truncated nonfunctional protein, and therefore we omitted these measurements from the figure. Values represent mean ± S.E.M, (n = 8) and significant differences are in comparison to control groups. *, p < 0.05 by t test.

**Figure 5:** Effect of LPS injection on hepatic P450 mRNAs in wildtype (WT) and interferon-γ-deficient (IFNγ−/−) mice. Animals were injected i.p. with either saline (Ctrl) or 1 mg/kg LPS (LPS), and livers harvested after 24 h for measurement of mRNA expression by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH with saline-treated group set to 1. Values represent mean ± S.E.M, (n = 6) and significant differences are in comparison to control groups. *, p < 0.05 by t test.
**Figure 6:** Effect of cytokine treatment on mRNA expression over time in mouse primary hepatocytes. Three or six days after plating, primary cultures were treated with 10ng/ml each of mouse recombinant IL6, IFNγ or TNFα and the cells harvested after 6, 12 and 24 h. Following their isolation, mRNAs were measured by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH with the untreated samples harvested at each time point set to 1. Values represent mean ± S.E.M, (n = 3) and significant differences are in comparison to untreated samples at respective time points. *, $p < 0.05$ by $t$ test.

**Figure 7:** Effect of different concentrations of cytokine treatment on mRNA expression in mouse primary hepatocytes. Three days after plating, primary cultures were treated with 0, 1, 3, 10 and 30ng/ml each of mouse recombinant IL6, IFNγ or TNFα and the cells harvested after 24h. Following their isolation, measurement of mRNA was done by real-time RT-PCR. Values are expressed as relative levels of mRNA expression after normalization to GAPDH with the untreated samples harvested at each time point set to 1. Values represent mean ± S.E.M, (n = 5 treated, n=8 control) and significant differences are in comparison to untreated control samples. *, $p < 0.05$ by Dunnett’s test.
Table 1: Liver and spleen weights of control and infected mice.

Values are the mean ± S.E.M (n=8); *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control. Differences between control and infected mice were determined by t test.

<table>
<thead>
<tr>
<th></th>
<th>Liver (% of body weight)</th>
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<th>Spleen (% of body weight)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Infected</td>
<td>Control</td>
<td>Infected</td>
</tr>
<tr>
<td>WT</td>
<td>4.57 ± 0.12</td>
<td>5.72 ± 0.12***</td>
<td>0.377 ± 0.032</td>
<td>0.718 ± 0.11**</td>
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<tr>
<td>IfNγ-/</td>
<td>4.31 ± 0.11</td>
<td>5.26 ± 0.15***</td>
<td>0.305 ± 0.073</td>
<td>0.500 ± 0.08</td>
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<tr>
<td>IfNγ-/</td>
<td>4.40 ± 0.11</td>
<td>5.05 ± 0.12**</td>
<td>0.353 ± 0.021</td>
<td>0.548 ± 0.05**</td>
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Table 2: *Citrobacter rodentium* counts in colon, liver and blood of infected mice.

Values are the mean CFU ± S.E.M (n=8); *p* < 0.05, **p* < 0.01, and ***p* < 0.001 compared to wild-type controls. Differences between wildtype and cytokine-deficient mice were determined by *t* test

<table>
<thead>
<tr>
<th>Bacterial counts in tissue (CFU)</th>
<th>Blood</th>
<th>Liver</th>
<th>Colon (x10⁸)</th>
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<tr>
<td>WT</td>
<td>1508 ± 1252</td>
<td>465 ± 62</td>
<td>2886 ± 1267</td>
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<tr>
<td><strong>IL6</strong>⁻/⁻</td>
<td>13 ± 5</td>
<td>336 ± 112</td>
<td>1034 ± 2894</td>
</tr>
<tr>
<td><strong>IFNγ</strong>⁻/⁻</td>
<td>73 ± 73</td>
<td>210 ± 47 **</td>
<td>5221 ± 2725</td>
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</table>
Table 3: Neutrophil recruitment as measured by MPO activity in colons of wildtype and cytokine-null mice.

Values are the mean ± S.E.M (n=8); *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control. Differences between wildtype and cytokine-deficient mice were determined by t test.

<table>
<thead>
<tr>
<th></th>
<th>Colon MPO activity (units/mg protein)</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>WT</td>
<td>3.48 ±0.78</td>
</tr>
<tr>
<td>IL6-/-</td>
<td>3.26 ± 0.67</td>
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<tr>
<td>IFNγ-/-</td>
<td>1.94 ± 0.28</td>
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Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

The figure shows a bar graph comparing the relative mRNA levels of different P450 isoforms in various conditions. The conditions include WT (Ctrl), IFNγ−/− (Ctrl), WT (LPS), and IFNγ−/− (LPS) for each isoform. The P450 isoforms analyzed are 1A2, 2A5, 2B9, 2C29, 2D22, 2E1, 2D9, 3A11, 3A13, 3A25, 3A41, 4A10, and 4A14. The graph indicates statistically significant differences marked with asterisks (*) for certain isoforms under different conditions.
Figure 6
Figure 7

Graphs showing relative mRNA levels of IL6, IFNγ, and TNFα across different P450 isoforms for concentrations of 1ng/mL, 3ng/mL, 10ng/mL, and 30ng/mL.