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Selective effects of a therapeutic protein targeting tumor necrosis factor-alpha on cytochrome P450 regulation during infectious colitis: implications for disease-dependent drug–drug interactions

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Abstract

We studied the impact of administering XPro1595, a novel antagonist of soluble tumor necrosis factor-α (TNFα), on the regulation of hepatic cytochrome P450 enzymes in the Citrobacter rodentium model of infectious colitis. XPro1595 was administered subcutaneously every 3 days throughout the infection, or as a single injection near the peak of infection. When given throughout the infection, XPro1595 selectively blocked the downregulation of Cyp3a11 and 3a25 mRNAs, as well as the induction of Cyp2a4/5, without affecting the downregulation of Cyp4a10, Cyp4a14, Cyp2b10, or flavin-monoxygenase-3. Induction of Cyp3a11, Cyp3a25, Cyp2c29, and Cyp3a13 mRNAs were observed only in XPro1595-treated mice. Administration of a single dose of XPro1595 was relatively ineffective. These results (1) confirm the role of soluble TNFα in hepatic CyP3a regulation during infectious colitis deduced from studies in TNFα receptor-1 knockout mice; (2) indicate the potential for soluble TNFα-specific antagonists to cause disease-dependent drug–drug interactions; and (3) suggest a novel mechanism by which an anti-inflammatory therapeutic protein can produce an opposite effect to that of the disease by selectively neutralizing one of multiple signals regulating drug-metabolizing enzyme expression. More research is needed to determine whether or not this is applicable to other diseases or disease models.

Keywords

Biologics, cytochrome P450, drug metabolism, drug–drug interactions, inflammation, therapeutic proteins

Introduction

Infections and inflammation cause changes in the activity and expression of many drug-metabolizing enzymes including members of the cytochrome P450 (P450) superfamily (Aitken et al. 2006). In most cases, this results in reduced drug clearance and increased exposure to drugs that are substrates for these enzymes. Cytokines such as interleukin (IL)-6, interferon (IFN)α or γ, and tumor necrosis factor (TNF)α are thought to mediate the effects of inflammation on P450 regulation. While it has been shown that such cytokines can regulate P450 expression in hepatocyte cultures (Abdel-Razaz et al. 1993; Chen et al. 1995; Tapner et al. 1996; Aitken and Morgan 2007;
Dickmann et al. 2012) and in vivo (Renton et al. 1984; Ghezzi et al. 1986a,b; Morgan et al. 1994), the roles of individual cytokines on regulation of drug metabolism in different diseases are not well understood.

The need to understand which cytokines are involved in P450 regulation in vivo is sharpened by the recently discovered phenomenon of disease-dependent drug–drug interactions (DDDI), in which therapeutic proteins (biologic drugs) targeted toward cytokines or their receptors can affect the metabolism of small molecule drugs by reversing the downregulation of P450 enzymes caused by the inflammatory disease, as reviewed in (Morgan 2009). This was first demonstrated by the attenuation of Cyp3α downregulation in mice by a polyclonal antibody to IL-6 in a genetic model of arthritis (Ashino et al. 2007). Cyp3α downregulation in a different, preadjuvant model of arthritis was inhibited by the anti-TNFα biologic infliximab (Ling and Jamali 2009). Subsequently, the anti-IL-6 receptor antibody tocilizumab was shown to increase clearance of the CYP3A substrate simvastatin in humans with rheumatoid arthritis (Schmitt et al. 2011). A recent white paper on the subject illustrates both the clinical and regulatory concerns for DDDIs and the need for more information on cytokine regulation of P450s during inflammatory disease (Evers et al. 2013).

In addition to the study using infliximab described above, four other studies have directly tested the in vivo role of TNFα in P450 regulation in a disease model. The downregulations of Cyp1α, 2b, 3a, and 4a following bacterial lipopolysaccharide (LPS) injection were not attenuated in mice deficient in both TNFα receptor-1 (TNFR1) and TNFR2, (Warren et al. 1999), whereas the responses of Cyp2d and Cyp2e1 enzymes were attenuated. In agreement with this finding, Cyp3α11 and 2C29 downregulations by LPS were unaffected in TNFα-null mice (Ashino et al. 2004) (Miyoshi et al. 2005). In contrast, downregulation of Cyp3α11 and Cyp2c29 by injection of tuberculosis vaccine was somewhat attenuated in TNFα-deficient animals (Ashino et al. 2004). Previous studies by our laboratory (Kinloch et al. 2011) have demonstrated that TNFα is an important factor in selectively regulating the expression of P450s of the Cyp3α subfamily in Citrobacter rodentium-induced colitis. C. rodentium is a noninvasive rodent pathogen equivalent to human enteropathogenic Escherichia coli, a serotype of E. coli that causes colitis in humans (Higgins et al. 1999). The colitis caused by the bacteria is characteristic of inflammatory bowel disease (Higgins et al. 1999). Cyp3α11 and Cyp3α25 were significantly downregulated in C. rodentium-infected C57BL/6 mice, while the downregulation was inhibited in TNFR1 knockout mice (Kinloch et al. 2011). Hepatocyte experiments found that TNFα was the most potent and efficacious cytokine tested in the downregulation of Cyp3α enzymes and other P450s in mouse hepatocyte cultures (Nyagode et al. 2010; Kinloch et al. 2011). Together, these results suggest a role for TNFα in the regulation of Cyp3α enzymes in vivo, which is dependent on the specific disease or disease model.

However, the lack of Cyp3α downregulation observed in TNFα- or TNFR-null mice could be due to adaptive changes in the mouse due to the long-term absence of the cytokine or its receptors rather than to blockade of TNFα effects. This possibility can be addressed using a pharmacological approach to block TNFα action in wild-type mice. The biologic drugs currently in clinical use do not discriminate between soluble or membrane-bound forms of TNFα, which signal through different receptors (Holtmann and Neurath 2004). We therefore conducted a study using a biologic drug that selectively neutralizes soluble TNFα to eliminate the potential influence of these adaptive changes. We used XPro1595, a dominant-negative form of TNFα (Y87H, A145R) that forms heterotrimers with native soluble TNFα to give complexes that neither bind to nor stimulate signaling through TNFα receptors (Steed et al. 2003; Zalesky et al. 2007). This experiment was designed to test XPro1595’s ability to block or reverse the effects of C. rodentium infection on hepatic cytochrome P450 enzymes, with a focus on the Cyp3α subfamily. The results confirmed the role of TNFα in Cyp3α downregulation in C. rodentium infection, and demonstrated the potential of a dominant-negative biologic to produce disease-dependent interactions with small molecule drugs via Cyp3α regulation.

Materials and Methods

Bacteria

Citrobacter rodentium wild-type strain (51116) was received from the American Type Culture Collection (Manassas, VA), and grown overnight in Luria broth at 37°C without shaking. Bacterial growth was monitored by spectrometry at 600 nm and the optical density was used to calculate a nominal bacterial dose of $2.5 \times 10^5$ cfu (colony forming units) per mouse, assuming intake of 8 mL/day per mouse. Actual concentrations of bacteria were determined by retrospective plating on MacConkey agar. After bacterial administration, the remaining drinking water volume was recorded to allow calculation of the actual bacterial dose.

Chemicals, animals, and treatments

All reagents and chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise specified. Thirty-six female C57BL/6J mice were obtained from Jackson
Laboratory (Bar Harbor, ME), of which 18 were controls and 18 were experimentally infected. The mice were housed in six separate groups of six to a cage and acclimatized to the animal facility for 1 week before the beginning of the experiment. The mice were 9 weeks of age when injections started.

A diagrammatic view of the treatment protocol is presented in Figure 1A. Infection started on day 0 when groups 2, 4, and 6 were orally administered C. rodentium in their drinking water (containing 20% sucrose) for 24 h at a nominal dose of $2.5 \times 10^8$ cfu per mouse drinking 8 mL/day. Control animals received 20% sucrose in water without bacteria during the same time period. Infected mice were housed in a biosafety level 2 facility to prevent transmission. Food was withheld during the 24-h infection period. TNFα antagonist XPro1595 (Xencor Inc., Monrovia, CA) was injected subcutaneously at a dose of 10 mg/kg in saline. Groups 1 and 2 (No XPro1595) received saline injections on days −2, 1, 4, and 7. Groups 3 and 4 (1 XPro1595 dose) were injected with saline on days −2, 1, and 4, and received a single injection of XPro1595 on day 7, 3 days prior to sacrifice. Groups 5 and 6 (4 XPro1595 doses) received injections on days −2, 1, 4, and 7. Animals were sacrificed by decapitation under isoflurane anesthesia on day 10. The Institutional Animal Care and Use Committee of Emory University approved all animal procedures.

**Tissue collection**

Livers were collected and rinsed in cold 1.15% potassium chloride. They were then weighed, portioned, flash-frozen in liquid nitrogen, and stored at −80°C for later RNA preparation. Blood was collected after decapitation and allowed to clot. Serum was separated by centrifugation for 10 min at 7500g and stored at −80°C for later
analysis. Spleen was collected and rinsed in 1.15% potassium chloride and weight was recorded. Colon was removed and washed of fecal matter with cold 1.15% potassium chloride. The distal quarter of the colon was dissected and kept on ice for later detection of viable bacteria. The next most distal portion was used for histological analysis.

**Determination of tissue bacterial loads**

Liver and colon samples were weighed and homogenized (Ultra Turrax T25, IKA Wilmington, NC) in 1 mL of phosphate-buffered saline. The colon homogenates were diluted 10^4- or 10^5-fold and 50 μL was plated onto separate MacConkey agar plates. Liver homogenates (100 μL) and blood (50 μL) were plated directly. The plates were incubated for 16 h at 37°C and colonies were counted.

**Colon crypt length measurement**

Distal colon sections were fixed in 10% formalin and embedded in paraffin. Five μm sections were cut and stained with hematoxylin and eosin. The heights of three well-oriented crypts were measured in the distal colon for each mouse, using a Zeiss 200M microscope (Thornwood, NY) with a 20× NA1.4 lens, and Slidebook (Intelligent Imaging Innovations, Denver, CO). The measurements were made in a blinded fashion.

**RNA extraction, cDNA synthesis, and real-time polymerase chain reaction**

Total liver RNA was prepared using RNA-Bee isolation reagent (Tel-Test Inc., Friendswood, TX), as directed by the manufacturer. The extracted RNA was analyzed on an agarose gel in order to determine RNA purity and integrity following visualization with ethidium bromide. RNA extraction, cDNA synthesis, and real-time polymerase chain reaction (RT-qPCR) were performed using a SuperScript First-Strand Synthesis System Kit (Invitrogen/Life technologies, Carlsbad, CA) according to the manufacturer’s protocol. Primers (Table S1) were custom-synthesized by MWG Biotech, Inc. (High Point, NC) or Operon Biotechnologies Inc. (Huntsville, AL). Real-time PCR was performed using Power SYBR Green Master Mix reagent (Applied Biosystems) along with forward and reverse primers of choice. The thermal cycling program included 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, and 1 min at the primer’s own annealing temperature. Results were expressed as relative levels of target mRNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the expressed mRNA levels by the ΔΔCt method (Livak and Schmittgen 2001), after verifying that GAPDH levels were not affected under the experimental conditions. The expression levels in the uninfected, untreated controls were arbitrarily set at 1.

**Microsomal preparation and western blotting**

All procedures were done at 4°C. Livers were homogenized in four volumes of Tris acetate buffer, pH 7.4 containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mol/L KCl. Homogenates were centrifuged at 7500g for 25 min, and the supernatant was then centrifuged for 35 min at 250,000g. The microsomal pellet was resuspended in 10 mmol/L Tris acetate buffer, pH 7.4, containing 0.1 mmol/L EDTA and 23% glycerol, and the final microsomal solution was stored at −80°C. Protein concentrations were determined with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) with bovine serum albumin as the protein standard.

Western blotting was used to measure relative levels of P450 protein levels in mouse hepatic microsomes as described previously (Chaluvadi et al. 2009; Kinloch et al. 2011). Antibodies to rat CYP3A and to NADPH-cytochrome P450 reductase (Cpr) were generously provided by Dr. James Halpert (University of California, San Diego) and Dr. Bettie Sue Masters (University of Texas, San Antonio). Antibody to CYP4A (ab140635) was purchased from Abcam (Cambridge, MA). All antibodies above were diluted 1:5000 for detection, and the assays were performed within a linear range of protein loaded. Proteins were detected using a SuperSignal West Pico chemiluminescent substrate kit (Thermo Fisher Scientific) where the bands were visualized by fluorography on x-ray film and then digitized. The bands were quantified by densitometry using Kodak Molecular Imaging software (Eastman Kodak Co., Rochester, NY).

**Cytokine analysis**

Serum samples were subjected to multiplexed immunoassays by the Emory Multiplexed Immunoassay Core (EMIC), using a mouse proinflammatory 7-PLEX kit
(Meso-Scale, Rockville, MD) and the Discovery Sector 2400 instrument (Meso-Scale). Samples were analyzed for cytokines IFNγ, TNFα, chemokine (C-X-C motif) ligand 1 (CXCL1, mKC), IL1, IL6, IL10, and IL12.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine if significant differences existed among infected groups in tissue bacterial colonization. For all other parameters, two-tailed independent Student’s t-test was used to determine the difference between uninfected and infected groups, where the level of significance was set at $P < 0.05$. Log transformation was performed in cases where the variances were not equivalent. If the variances of the transformed data were still not equivalent, the Mann–Whitney U-test was used.

Results

Experimental design

This experiment was designed to test whether a single XPro1595 injection administered when downregulation is already established could reverse (Single-injection groups), and whether TNFα inhibition by XPro1595 throughout the experiment (four injections groups) could block, the downregulation of CYP3a family members. A summary of the experimental protocol is shown in Figure 1A. We determined previously that the downregulation of P450 enzymes is optimal between 7 and 10 days after infection (Chaluvadi et al. 2009). The dose and dose interval for XPro1595 were chosen because they were demonstrated to promote functional recovery in a model of experimental autoimmune encephalitis (Brambilla et al. 2011).
Throughout the experiment, none of the mice showed obvious clinical signs of illness. However, infected mice had slightly looser feces compared to control mice. All animals survived the duration of the _C. rodentium_ infection and no significant changes in body weight were found (data not shown). There were no significant differences in bacterial counts in the colon, liver, or blood (Fig. S1). Infected mice had a 21% increase in liver weight and this was slightly reduced in the animals receiving XPro1595 throughout the experiment (four injections, Fig. 1B). As expected, spleen weights were dramatically increased by infection (2.5-fold) and this was the same with or without XPro1595 treatment (Fig. 1C). Colon crypt heights, a measure of mucosal hyperplasia, were significantly increased by 67%, 47%, and 47%, respectively, in the 0, 1, and 4 dose groups (Fig. 1D).

### Effects of TNF-α antagonist on regulation of hepatic P450 mRNAs and proteins during infection

We chose to study a panel of P450 enzymes that we have characterized extensively in this infection model (Chaluvadi et al. 2009; Nyagode et al. 2010; Kinloch et al. 2011). They represent the major subfamilies of drug-metabolizing P450s, as well as Cyp4 family enzymes that have roles in metabolism of fatty acids and eicosanoids. _C. rodentium_ infection alone caused significant downregulation in the following hepatic P450 mRNAs: Cyp4a10 (to 14% of control), Cyp4a14 (34%), Cyp3a11 (72%), Cyp2b10 (70%), and Fmo3 (6%) (Fig. 2). While decreases in the mean values for mRNA expression of Cyp3a25 (67%, _P_ = 0.10,
Fig. 2) and Cyp2d22 (not shown) were observed, they did not achieve statistical significance. Treatment with four doses of XPro1595 beginning on day 0 (groups 5 and 6), but not with a single dose on day 7 (groups 3 and 4), not only blocked Cyp3a11 downregulation, but actually reversed it so that an increase was observed (Fig. 2). The same pattern was seen with Cyp3a25. In contrast, the downregulation of other P450s and Fmo3 were unaffected by any XPro1595 treatment (Fig. 2).

*Citrobacter rodentium* infection tended to induce several other P450 mRNAs (Fig. 3). Cyp2a4/5 (2.0-fold), Cyp4a12 (8.2-fold), and Cyp4f18 (5.9-fold) were significantly induced by infection (Fig. 4). Cyp2d9 (Fig. 3, *P* = 0.06) and Cyp1a2 (*P* = 0.11, not shown) showed trends toward induction that were not statistically significant. The increase in Cyp2a4/5 was attenuated when XPro1595 was injected during ongoing infection (one dose), and blocked when XPro1595 was administered throughout the infection (four doses) (Fig. 3). Cyp2d9, although significantly induced by infection in the single-dose group, was less induced in the group receiving four doses, suggesting a dose-dependent effect of XPro1595 (Fig. 3). The induction of Cyp4f18 (Fig. 3) and Cyp4a12 (not shown) was minimally affected by XPro1595. Cyp1a2, Cyp3a13, and Cyp2c29 were each induced significantly in the group that received one dose of XPro1595 group, whereas only trends toward induction were observed in the group without XPro1595 (Fig. 3).

Western blotting was used to determine whether the changes in mRNA expression also manifested in changes in protein levels. Cpr protein levels were not affected by infection, and were used as a loading control. As seen in Figure 4, the effect of XPro1595 treatment on Cyp4a proteins was consistent with the effects observed on Cyp4a10 and 4a14 mRNAs (Fig. 2). That is, Cyp4a proteins were downregulated to 14% of control during infection, and this was unaffected by XPro1595 treatment. However, infection failed to affect steady-state levels of Cyp3a proteins detected by this antibody.

**Effects of TNF-α antagonism on hepatic cytokine and acute phase protein (APP) mRNA expression, and serum cytokine levels during C. rodentium infection**

Infection with *C. rodentium* produced significant increases in the hepatic mRNA expression of TNFα, IL6, IL1β, serum amyloid A (SAA), and serum amyloid P (SAP), indicative of a hepatic inflammatory response (Fig. 5). IFNγ and α1-acid glycoprotein (AGP) were not significantly affected, although AGP showed a trend toward induction. A single XPro1595 treatment on day 7 attenuated the induction of TNFα and IL6, but had little effect on the induction of the acute phase proteins SAA, AGP, and SAP. Interestingly, with the exception of IFNγ, these effects of XPro1595 were smaller or
absent when four doses were administered beginning on day −2 (Fig. 5).

Serum cytokines (Fig. 6) showed a different pattern of regulation than hepatic cytokine mRNAs, indicating that the liver is not the major source of circulating cytokines during *C. rodentium* infection. XPro1595 treatment did not attenuate any of the observed increases in serum cytokines (Fig. 6). The increases in circulating IL10 and TNFα did not appear to be affected by XPro1595 (Fig. 6). Serum IL1 was unaffected by infection in any of the groups (not shown).

**Discussion**

In this study, the soluble TNFα antagonist XPro1595 selectively antagonized the downregulation of Cyp3a11 and 3a25, as well as the upregulation of Cyp2a4/5 during *C. rodentium* infection, an acute inflammatory disease. Other downregulated transcripts including Cyp2b10, Cyp4a10, Cyp4a14, and Fmo3 were unaffected by XPro1595 treatment. These data implicate an in vivo role for soluble TNFα in downregulating certain Cyp3a genes in this inflammatory disease model. It also suggests that
Figure 6. Effect of the TNF-α antagonist XPro1595 on serum cytokine levels. Blood was harvested at sacrifice and serum was prepared and analyzed by multiplexed immunoassays. Values represent mean ± SEM. (n = 6). *Significantly different from uninfected animals in same XPro1595 treatment group, P < 0.05.

Figure 7. Proposed mechanism for disease-dependent upregulation of P450 enzymes by XPro1595 treatment.
XPro1595 is in principle capable of causing DDDI via Cyp3a modulation in this particular model. Ling and Jamali (2009) first demonstrated the potential for TNFz DDDI using infliximab in a rat preadjuvant arthritis model. However, no such DDDI has yet been reported for TNFz in humans. It remains to be determined whether or not TNFz antagonism will reverse Cyp3a downregulation in models of chronic inflammatory disease, for which biologic drugs are prescribed in humans.

The selective blockade of Cyp3a downregulation by XPro1595 is in excellent agreement with the findings in TNFR1 knockout mice and in cultured hepatocytes (Kinloch et al. 2011). These mice show increased pathologic responses to TNFR1 knockout mice, and in cultured hepatocytes, XPro1595 is in excellent agreement with the findings in a model. However, no such DDDI has yet been reported in vivo role. It is possible that the increase in Cyp3a11 mRNA in mice that received four doses of XPro1595 is partly due to the apparent decrease in the corresponding uninfected group. However, this is not the case for Cyp3a25.

In conclusion, our results support previous work indicating a selective in vivo role for TNFz in the suppression of Cyp3a11 and Cyp3a25 in a model of acute infectious colitis. The ability of XPro1595 to abolish Cyp3a11 and Cyp3a25 suppression, and to uncover inductive effects of infection on Cyp3a11, 3a25, 2c29, and 3a13 in this model of inflammation suggests in principle that the propensities of biologic drugs to cause DDDI in humans may not be limited in the induction of Cyp2a4/5 during C. rodentium infection. There was a tendency toward the same phenomenon for Cyp1a2, but this could not be determined because the “effect” in the XPro1595 controls was not significant.

Why were Cyp3a11 and Cyp3a25 mRNAs actually increased by infection in the animals treated with four doses of XPro1595? Similarly, Cyp2c29 and 3a13 mRNAs were only significantly increased in one or both of the XPro1595-treated groups (Fig. 4). It is well-documented that different P450s have different sensitivities to different inflammatory cytokines (reviewed in Morgan 1997). We can speculate that for Cyp3a11 and 3a25, there could be two opposing signals due to infection, of which the suppressive effect of TNFz is dominant (Fig. 7). For 2c29 and 3a13, the two signals cancel each other out. When the suppression by TNFz is removed, induction can occur. For other P450s, TNFz may not have an important in vivo role. It is possible that the increase in Cyp3a11 mRNA in mice that received four doses of XPro1595 is partly due to the apparent decrease in the corresponding uninfected group. However, this is not the case for Cyp3a25.

In the clear cases where XPro1595 had an effect on P450 regulation, the effect was greatest in the animals that received four doses, and less clear in the animals treated only once on day 7. This could be due to the need for an accumulation of XPro1595 with successive injections, or it could mean that TNFz begins to affect P450 expression before day 7, and those effects persist even when the effect of soluble TNFz is neutralized. As the single injection on day 7 had clear effects on hepatic IL6, TNF, and IL1b expression, there is again not a good correlation between overall effect on inflammation and on P450 regulation.

This study was designed to test the effect of XPro1595 on downregulation of P450s by infection, and therefore was not powered for multiple comparisons that would allow us to test the effect of XPro1595 alone on drug-metabolizing enzyme expression. Nevertheless, examination of the data (Figs. 3 and 4) suggests that the expression of Cyp2b10, Cyp4a10, and Cyp2c29 may be reduced by XPro1595 treatment; whereas Fmo3 expression may be increased. Additional studies will be needed to determine whether there are any significant effects on these enzymes in the absence of disease.

In conclusion, our results support previous work indicating a selective in vivo role for TNFz in the suppression of Cyp3a11 and Cyp3a25 in a model of acute infectious colitis. The ability of XPro1595 to abolish Cyp3a11 and Cyp3a25 suppression, and to uncover inductive effects of infection on Cyp3a11, 3a25, 2c29, and 3a13 in this model of inflammation suggests in principle that the propensities of biologic drugs to cause DDDI in humans may not be limited.
to just a normalization of decreased drug clearance due to inflammation. We speculate that DDDI could actually cause clearance of Cyp3a (CYP3A in humans) substrates such as statins, HIV protease inhibitors, cyclosporine etc., to increase above that found in the healthy state, with consequent decreases in efficacy of cotreated drugs in affected individuals. However, studies are needed in other disease models and with approved biologic drugs to determine whether or not this observation is generally applicable.

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Disclosure

M. G. Tansey is coinventor of the dominant-negative TNF inhibitors and an ex-employee of Xencor Inc. She does not hold significant financial stake in the company and is not a consultant.

References


Supporting Information
Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences.

Figure S1. Citrobacter rodentium colony counts in liver, blood, and colon of infected mice Bacterial counts in colon and liver are expressed per g tissue, whereas counts in blood are per mL whole blood. Values represent mean colony forming units ±SEM. (n = 6). One-way ANOVA determined that there were no differences among the groups.