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DEPARTMENT OF NUTRITIONAL SCIENCES

PROTECTIVE EFFECTS OF BILE SALTS AGAINST EXAGGERATED INNATE IMMUNE RESPONSE TO LUMINAL LIPOPOLYSACCHARIDE

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ABSTRACT

The study of the human gut microbiota, or the collection of commensal, symbiotic, and pathogenic microorganisms that colonize the gut, is an expanding field; research is rapidly elucidating many of the functions of the estimated 500-1000 distinct species that make up the gut ecosystem. However, much remains unclear about the extent of the purpose and maintenance of the gut flora. The presence of these microorganisms, some of which are gram negative bacteria, results in a constant presence of lipopolysaccharide (LPS), an endotoxic component of gram negative bacteria, in the lumen of the intestine. Intestinal epithelial cells (IECs) in the intestinal lumen serve as the interface by which the microbiota and host interact. These cells demonstrate hyporesponsiveness to LPS released from lysed gram-negative bacteria. This experiment attempted to examine the potential role of bile salts in the maintenance of microbiota homeostasis by way of interactions with luminal LPS.

Tarmina *et al* (1968) demonstrated the ability of secondary bile salt sodium deoxycholate to degrade LPS endotoxin (55). The protecting affects of other bile acids has yet to be explored, however. The ability of bile acids to structurally alter LPS would result in the inability of the host innate immune system to recognize the LPS, thereby protecting the host and its symbiotic bacteria from an unnecessary inflammatory immune response. It was hypothesized that conjugated bile salts glycocholate and taurocholate, and secondary bile salts deoxycholate and lithocholate would demonstrate protection from host recognition of LPS.

This experiment examined both the *in vivo* and *in vitro* affects of bile salts on LPS structural integrity and the subsequent recognition and immune stimulation of the murine host. Murine glycocholate, taurocholate, deoxycholate, and lithocholate were incubated with *E. coli*

LPS and administered to mice and RAW 264.7 cells. KC and Lcn2 levels were then evaluated to determine the extent of immune reaction.

Although the protective effects of deoxycholate were confirmed, the results of the other bile acids are inconclusive at best and potentially aggravating at worst. Glycocholate may have some effect on LPS structural integrity, though more incubation time or different temperature may be necessary. This experiment may have implications for the potential of bile acids as therapeutic targets for inflammatory bowel disease as well as other metabolic diseases in which the microbiota has been implicated. Further understanding of symbiotic bacteria and host interaction could not only result in increased knowledge of diseases caused by dysbiosis—such as IBD—but also of additional affects of bile acid malabsorption or interference with bile acid synthesis.

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Introduction

The term microbiota refers to the collection of symbiotic microorganisms that colonize the gut, and microbiome refers to genes expressed by microbiota (1). The study of the human gut microbiota is an expanding field, and research is rapidly elucidating many of the functions of the estimated 500-1000 distinct species belonging to several major phyla that make up the gut ecosystem. However, much remains unclear about the extent of the purpose of the gut flora (1). Human beings are estimated to harbor roughly 10 microbial cells for every human cell; these microbes are essential both for nutrient absorption and metabolic regulation as well as host innate immune function (2, 3). The vast majority of these microbes are found in the large intestine. Although the exact composition of the gut microbiome depends on several factors, the most common bacterial phyla are *Bacteroidetes, Firmicutes,* as well as *Proteobacteria, Actinobacteria, and Verrucomicrobia*. Though there are currently 52 recognized phyla on Earth, only seven colonize mammalian guts (4).

The vast diversity of the intestinal tract includes both gram positive and gram negative bacteria. For example, members of the phylum *Bacteroidetes* are all gram negative, while those of the phylum *Firmicutes* tend to be gram positive (5, 6). The classification of gram positive or negative depends on the lipopolysaccharide (LPS) makeup of the bacterial cell. LPS is not only a glycoconjugate component of the outer membrane of gram-negative bacteria, but also a very potent endotoxin for animal cells (7). LPS is anchored to the outer membrane of the bacteria by the conjugated phospholipid, Lipid A (8). The hydrophobic nature of Lipid A allows for the integration of LPS into the outer membrane. Connected to the Lipid A domain is an oligosaccharide and a region known as the O-antigen that repeats to form a polysaccharide (8).

The Lipid A component differs structurally within and among gram negative species, thereby protecting the bacteria and allowing it to adapt to its environment (9). When the outer membrane of a gram negative bacterium is lysed, however, LPS is freed from the membrane and released into the environment, where it exhibits endotoxic activity that activates host innate immune system (7).

Pattern recognition receptors (PRRs) are proteins expressed by innate immune cells and work to recognize anomalies within the organism. These anomalies fall into two categories: pathogen-associated molecular patterns (PAMPs), referring to microbial pathogens, and damage-associated molecular patterns (DAMPs), associated with components released during cell lysis and death (10). LPS is a PAMP recognized by the signaling molecule toll-like receptor (TLR) 4. TLR4 is a type I trans-membrane glycoprotein and member of the TLR class of proteins (11). These proteins are ubiquitous in mammalian cells and essential for detecting microbial pathogens and stimulating the innate immune system. The receptor cascade is mediated by TLR4 results in the translocation of nuclear factor (NF)-kβ to the nucleus, initiating the expression of pro-inflammatory cytokines in response to binding LPS (12, 13) [Figure 1]. TLR4 is expressed by endothelial cells, macrophages, lymphocytes, and immune cells within the lamina propria of the GI tract, as well as intestinal epithelia and mucosa to a lesser extent (14). LPS is a specific ligand for TLR4, though it cannot bind directly. Binding of LPS to TLR4 is facilitated by the accessory proteins CD14 and myeloid-differentiation factor-2 (MD-2) (13). CD14 exists as both an integral yet non-transmembrane and soluble protein that binds LPS with low specificity (15). The protein is typically integrated via a glycosylphosphatidyl inositol anchor within the membranes of macrophages, neutrophils and, to a lower extent, dendritic cells (16). Additionally, the soluble form of the protein is secreted directly from the liver and monocytes into circulation, thereby

allowing for LPS signaling in endothelial and epithelial cells that do not express membrane CD14 (15). Lipopolysaccharide-binding protein (LBP) has been shown to facilitate the binding of LPS to CD14 (17); in fact, recognition of LPS by immune cells increased 300-fold when LBP was expressed versus LBP knockout (16). MD-2 is a membrane protein bound to TLR4. The protein binds CD14-bound LPS, and the TLR4-CD14-MD-2 complex then dimerizes to activate the downstream signaling cascade (18). As mentioned, immune cells such as macrophages and neutrophils express membrane CD14, while cells without immune function such as endothelial and epithelial cells must rely on the soluble form of CD14 (e.g. serum) for LPS recognition by TLR4 and MD-2 (15).

The successful signal initiation on the surface of immune cells such as macrophages and monocytes results in several signal-transduction pathways within the cell (19). Downstream signaling by TLR4 is carried out by the Toll-interleukin-1 receptor (TIR) domain; this domain of TLR4 is critical for the recruitment of TIR domain-containing adaptor proteins that propagate signaling (20). The TLR4 signaling responses are typically separated into MyD88 (myeloid differentiation primary response gene 88)-dependent and Toll/IL-1R (TIR) domain-containing adapter-inducing IFN-β (TRIF)–dependent, or MyD88-independent, pathways. The MyD88-dependent pathways have been implicated in the downstream translocation of NF-kβ to the nucleus and subsequent expression of pro-inflammatory cytokines (21), while the MyD88-independent pathway is responsible for cytokine production and the expression of interferon (IFN)-β (21).

The MyD88-indpendent signaling pathway is carried out by TRIF and TRIF-related adapter molecule (TRAM). The adapter molecule TRAM is directly stimulated by TLR4 and is responsible for the recruitment of TRIF, which subsequently recruits TNF receptor associated

factor (TRAF)-3. TRAF3 binds TRAF family member-associated NF-κβ activator (TANK), which binds TANK binding kinase 1 (TBK1), which together with IKKi results in translocation of the transcription factor IRF-3 to the nucleus. TRIF also interacts with receptor-interacting protein 1 (RIP1) which activates both NF-κβ and mitogen-activated protein kinases (MAPK). Activation of NF-κβ and MAPK is corroborated by evidence that MyD88-deficient mice still show delayed pro-inflammatory cytokine production upon LPS stimulation (22). IRF-3 and NF-κβ target specific genes and result in the expression of immune-regulatory proteins such as Type 1 Interferons (22, 23).

The MyD88-dependent pathway is mediated by TIR domain-containing adaptor protein, or TIRAP. TIRAP is also known as MyD88 adaptor-like, or mal, protein (23). Stimulation of TIRAP by TLR4 via the TIR domain results in the activation of MyD88, which then recruits IL-1 receptor-associated kinase (IRAK)-4 via its Death Domain (24). IRAK-4 phosphorylates IRAK-1, which associates with TRAF6, a member of the tumor necrosis factor receptor (TNFR)-associated factor family (19). This complex is then able to activate TGF-activated kinase 1 (TAK1), which phosphorylates the IKK complex as well as MAPK kinase, which activates c-Jun N-terminal kinase (JNK) and p38 (25). This leads to Iκβ dissociate from NF-κβ, thereby allowing its translocation to the nucleus. Additionally, activation of JNK and other MAPKs results in the activation of transcription factor AP-1, which is also associated with cytokine production (23, 25). Though the MyD88-dependent pathway results in rapid immune response and pro-inflammatory cytokine production, as mentioned previously, MyD88-deficient mice still demonstrate cytokine production at slower kinetic rates due to activation of the TRIF/TRAM-dependent signaling pathway (22).

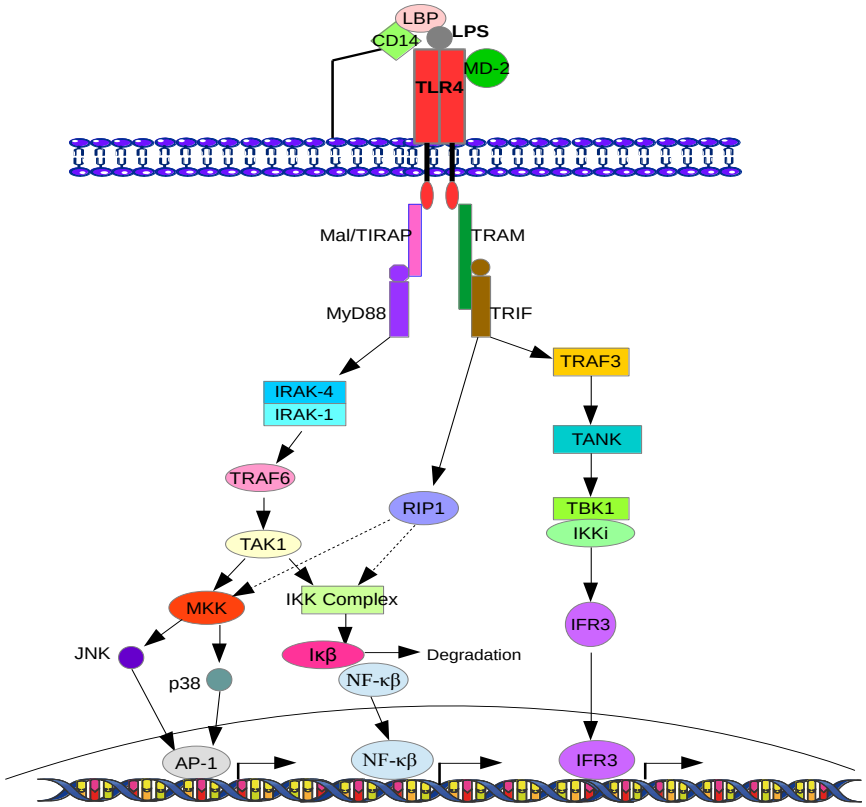

Figure 1. TLR4 signaling pathway--including MyD88- and TRIF-dependent pathways--upon stimulation by LPS. Figure developed based on information from the following sources: 21, 22, 23, 24, and 25

TLR4 activation leads to induction of potent proinflammatory response. However, the primary barrier within the gut, made up of intestinal epithelial cells (IECs) demonstrates distinct action in response to stimulation by LPS (26). IECs in the intestinal lumen provide the first line of defense against invading pathogens; however, they also provide the interface by which the microbiota and host interact. It has been suggested that while IECs do express TLR4 receptors, these cells demonstrate hyporesponsiveness to constant, low-level stimulation by LPS released from lysed gram-negative bacteria. Abreu *et al.* (2001) suggest that compared to non-GI epithelial cells and immune cells, IECs express less TLR4 (27). Studies by Cario et al provide evidence that a constitutive lack of membrane CD14 expression by IECs is responsible for the

diminished responsiveness to physiological concentrations of LPS; this would mean that unless soluble CD14 is present, TLR4 would not be as responsive to LPS (28). In addition, IEC express Tollip, or Toll-interacting protein, which is up-regulated in healthy IECs in order to control the responsiveness of TLR4 (29). This protein works by interfering with the phosphorylation of IRAK, thereby prohibiting the downstream activation and translocation of NF-κβ and aiding in LPS tolerance (30). Though in healthy IECs the above-mentioned mechanisms seem to protect the host organism from LPS-mediated inflammation, interference is implicated in Inflammatory Bowel Diseases (IBD). Furthermore, intestinal epithelial dysfunction and exaggerated immune response to mutualistic bacteria is also suggested (31).

The pro-inflammatory cytokines expressed by NF-κβ and AP-1 work to regulate and direct the immune response to stimulation by pathogens. These cytokines include, but are not limited to, tumor necrosis factor (TNF)-α, IL-1-β, IL-6, and IL-12 (32). Additionally, IL-1-β expression leads to the activation of the gene that codes for the acute phase protein, Lipocalin 2 (Lcn2), also known as neutrophil gelatinase-associated Lipocalin (NGAL) in humans (33). Lcn2 is an important immune protein that is released in the presence of bacterial infection and works to attenuate infection. The protein works by interfering with the iron-binding ability of bacteria, by chelating their siderophores and effectively decreasing the pathogen's ability to propagate (34). Lipocalin2 is primarily expressed by neutrophils in response to pathogen recognition—such as LPS stimulation of TLR4 signaling—and by intestinal epithelial and liver cells (35). For this reason, Lcn2 levels are indicative of an inflammatory response to an invading pathogen. The presence of endotoxins (including LPS) and inflammatory cytokines also triggers the expression of keratinocyte chemoattractant (KC) in mice, a homologue of the human IL-8; the

chemoattractant is responsible for the recruitment of neutrophils (36, 37). KC levels in mice can provide a means of measuring LPS-mediated inflammation.

Bile salts may also play a role in the homeostasis of the microbiota, though the exact extent of bile salt-bacteria interaction is not entirely clear. Bile is produced in the liver and stored in the gall bladder; bile salts, water, electrolytes, cholesterol, phospholipids, and bilirubin make up bile (38). In the human liver, primary bile acids are synthesized from cholesterol via a multi-step process involving oxidation by cytochrome P450 (39). The rate-limiting step of this process is the 7α-hydroxylation of cholesterol (40). Two primary bile acids have been isolated, cholic acid and chenodeoxycholic acid. Before leaving the liver, bile acids are conjugated with the sulfonic acid taurine or amino acid glycine, forming glycocholic, glycochenodeoxycholic, taurocholic, and taurochenodeoxycholic acid compounds. (41) Conjugation increases the solubility of these products in the duodenum, and they are mostly found in the deprotonated form and are thus considered salts (42). The most of the bile salts are reabsorbed in the distal ileum and recycled back to the liver in a process called enterohepatic circulation. Enterohepatic circulation is highly efficient, and 95% of bile salts are reabsorbed. A portion of the bile salts that are not reabsorbed pass into the colon, where they are structurally modified by the symbiotic anaerobic bacteria; the remaining bile is excreted in the feces (43, 44). The deconjugated compounds are known as secondary bile salts; deoxycholic acid is derived from deconjugated cholic acid compounds, while lithocholic acid is formed from chenodeoxycholic acid conjugates (45).

Bile acids play key roles in lipid/fat soluble vitamin solubilization and abosprtion, and act to a lesser extent in some cholesterol catabolism and as steroid hormones and regulatory molecules (46). The amphipathic nature of these compounds allows them to act as biological

detergents to effectively emulsify fats and fat-soluble vitamins, which can then be absorbed by enterocytes in the form of mixed micelles (47). Bile synthesis and its excretion combine to form one mechanism of cholesterol removal from circulation (48).

Bile is implicated in more than simply aiding digestion, however, and also has steroid hormonal properties. Bile acids are known to be a ligand for farnesoid X receptor-α (FXR-α), a nuclear receptor responsible for expression of genes that inhibit cholesterol 7α-hydroxylase. In this way, the synthesis of bile acids from cholesterol is controlled via feedback inhibition (49). Bile acids also act as ligands for preganane X receptor (PXR) and vitamin D receptor (VDR), both of which bind the secondary bile acid, lithocholate, and result in downstream detoxification of the hydrophobic compound (50, 51). In humans, G-protein bile acid receptor 1 (GPBAR1), also known as TGR5, was discovered as an additional bile acid receptor, which activates MAP kinase signaling. It has been determined that TGR5 regulates immune response by acting as an antagonist for NF-κβ (52).

Though most conjugated bile salts are reabsorbed in the small bowel, a small quantity manages to escape and moves into the colon. Once there, the anaerobic symbiotic bacteria deconjugate the salts, producing secondary bile salts. Ridlon *et al.* (2008) suggest several deconjugation mechanisms carried out by bile salt hydrolases (BSHs) hydroxysteroid dehydrogenases (HSDHs), which are able to hydrolyze the C-24 *N-acyl* amide bond between a bile acid and its glycine or taurine conjugate (43). Additionally, some strains of bacteria in the colon contain 7α-dehydroxylase activity to remove the hydroxyl group from the steroid nucleus of the bile salt (53). These actions significantly increase the hydrophobicity of the compounds. Upon deconjugation, bile salts are either reabsorbed by the intestinal epithelia or excreted.

The interaction between bacteria and bile salts is well established, but the underlying reason for the activity is not clear. It has been suggested that the deconjugation is a protection mechanism, as conjugated bile salts may be more toxic to the bacteria that colonize the large intestine. In fact, it has been shown that the conjugated primary bile acids that circulate in the small intestine do exhibit bacteriostatic affects, essentially sterilizing the biliary tree and small intestine (54). In 1967, Tarmina *et al.* (1967) demonstrated the ability of sodium deoxycholate to degrade LPS endotoxin (55). The protecting affects of other bile acids has yet to be explored, however. Further understanding of symbiotic bacteria and host interaction could not only result in increased knowledge of diseases caused by dysbiosis—such as IBD-but also of additional affects of bile acid malabsorption or interference with bile acid synthesis.

It was hypothesized that, like deoxycholate, the other secondary bile acid, lithocholate, also demonstrates LPS-degrading properties. This action would benefit both the symbiotic bacteria and the host, as degradation of the endotoxin would interfere with its recognition by TLR4. The failure to stimulate the innate immune system would protect both the bacteria and gastrointestinal tract from damage by immune cells and resulting inflammation. This interaction may also provide and additional explanation for the hyporesponsiveness of the colonic epithelia to constant LPS exposure in healthy organisms. LPS structure-altering ability was also predicted of the conjugated primary bile salts tested in this experiment: glycocholate and taurocholate. The ability of primary and conjugated bile acids to curb intestinal overgrowth and limit endotoxemia has been established (56, 57). However, the interaction between conjugated primary bile acids and LPS has yet to be examined. When gram-negative bacteria are lysed in the intestine after their life-span, LPS is released from the outer membrane of the cell. The ability of conjugated bile acids to degrade the released LPS may work to “clean up” after eliminating the threat of

infection by lysing the bacteria. The ability of bile acids to structurally alter LPS would result in the inability of the host innate immune system to recognize the LPS, thereby protecting the host and its symbiotic bacteria from an unnecessary inflammatory immune response.

This experiment examined both the *in vivo* and *in vitro* affects of bile salts on LPS structural integrity and the subsequent recognition and immune stimulation of the murine host. Murine glycocholate, taurocholate, deoxycholate, and lithocholate were incubated with *E. coli* LPS and administered to mice and RAW 264.7 cells. KC and Lcn2 levels were then evaluated to determine the extent of immune reaction. This experiment may have implications for the potential of bile acids as therapeutic targets for inflammatory bowel disease as well as other metabolic diseases in which the microbiota has been implicated.

Materials and Methods

Role in the Project. Several lab members, including me, were involved in the design and procedures of this experiment. In the *in vivo* experiments, I was responsible for the administration of treatment via IP injection, serum collection, and ELISA analysis. These procedures were completed under the guidance of Vijay-Kumar lab member Beng San Yeoh, who was also responsible for the maintenance of the animals during the experiment. In the *in vitro* experiments, I was responsible for plating the cells, administration of treatment, supernatant collection, and ELISA analysis, all of which were completed under the guidance of lab member Piu Saha, PhD. Piu Saha also passed, maintained, and stimulated the cells before treatment. Experimental design was mainly the work of Dr. Vijay-Kumar, with some input by Beng San Yeoh and myself.

Reagents. The bile salts sodium glycocholate, taurocholate, deoxycholate, and lithocholate were purchased from Sigma Aldrich (St. Louis, MO), *E. coli* LPS stock was also purchased from Sigma Aldrich. All treatments, except those involving lithocholate, were dissolved in 0.01M phosphate-buffered saline (PBS) before injection/application. Due to its high insolubility, lithocholic was first dissolved in aprotic solvent dimethyl sulfoxide DMSO in a solution of 1 mg/ml, then diluted 100x in PBS to the appropriate quantity for either intraperitoneal (IP) injection or treatment of cell cultures.

Mice. 5-7 week-old C57BL/6J mice (n=24) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the Penn State University animal facility. They were fed standard lab chow. Ten groups (n=4/group) of mice were injected via IP injection with either LPS alone (1μg), bile acid alone (10μg), or a mixture (1 μg LPS, 1 μg bile acid) incubated at room for 10

minutes temperature before injection, with a final injection volume of ~160µl. Blood was collected 4 hours post-injection. Mice were sacrificed 24 hours post-injection via CO2 asphyxiation and blood was collected via cardiac puncture. All animal experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University. Blood samples were centrifuged and serum was collected for analysis.

Cell Cultures. The murine macrophage cell line RAW264.7 (ATCC) was maintained in Dulbecco's Modified Eagle Medium(DMEM) media (Sigma) supplemented with 10% FBS and 1% pen-strep (Sigma). Cells were cultured at 37 °C and 5% CO2. 7.0 x 10^5 cells/ml were seeded in 6-well plates and incubated for 24 h before stimulation. Cells were stimulated with LPS (500 ng/ml) in presence or absence of bile salts (500 ng/ml) in incomplete DMEM media and incubated for 24 hr. Culture supernatant were collected and centrifuged at 1200 rpm for 5 min and kept at -80^{o}C and analyzed for lipocalin2 (Lcn2) and keratinocyte-derived chemokine CXCL1 (KC) by ELISA. All experiments were performed in triplicates.

ELISA. Lipocalin 2 (Lcn 2) and Keratinocyte chemoattractant (KC) levels were quantified from *in vivo* and cultured cells using the respective ELISA kits from R&D Systems (Minneapolis, MN) according to instructions in 96-well ELISA plates (Corning, Corning, NY). Plates were read at 450 and 540 nm; the difference in readings was used to calculate chemokine levels.

Statistical Analysis. The *in vivo* and RAW cell Lcn 2 and *in vivo* KC results were analyzed using one-way ANOVA; $p < 0.05$ was considered statistically significant and was denoted with an asterisk. The RAW cell KC results were analyzed using an un-paired, two-tailed Student's t test. Again, a p value of <0.05 was considered significant and marked with an asterisk. Statistical analysis and figures were completed using GraphPad Prism 6 software.

Results

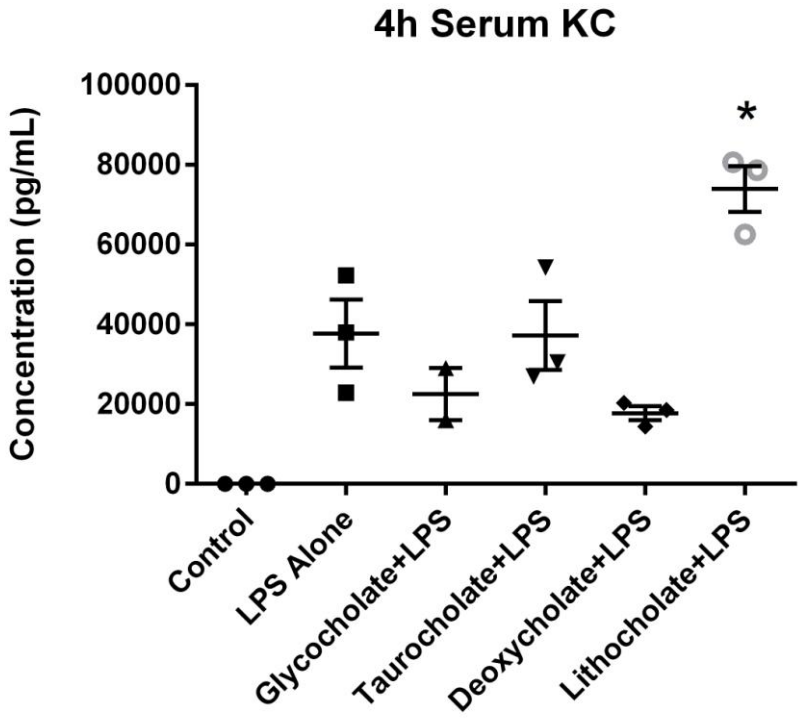

Figure 2. Preliminary murine KC levels in pg/ml, 4 hours after injection of 10 μg LPS or 10 μg LPS+10 μg bile salt mixture.

The above figure shows serum KC data from the first set of mice (n=24). LPS Alone group was treated with 10 μg of LPS dissolved in PBS for a final injection volume of 160 μl per mouse. The bile acid groups were injected with 10 μg of LPS and 10 μg of each respective bile salt. The mixtures were dissolved in PBS, and the final injection volume was 160 μl per mouse. At 4 hours post-injection, blood was collected. Serum KC levels were determined using ELISA assay, the values are expressed in pg/ml serum. Data was analyzed by one-way ANOVA ($p<0.05$), n=4 for each group. Values for each group are expressed as Mean ± SEM.

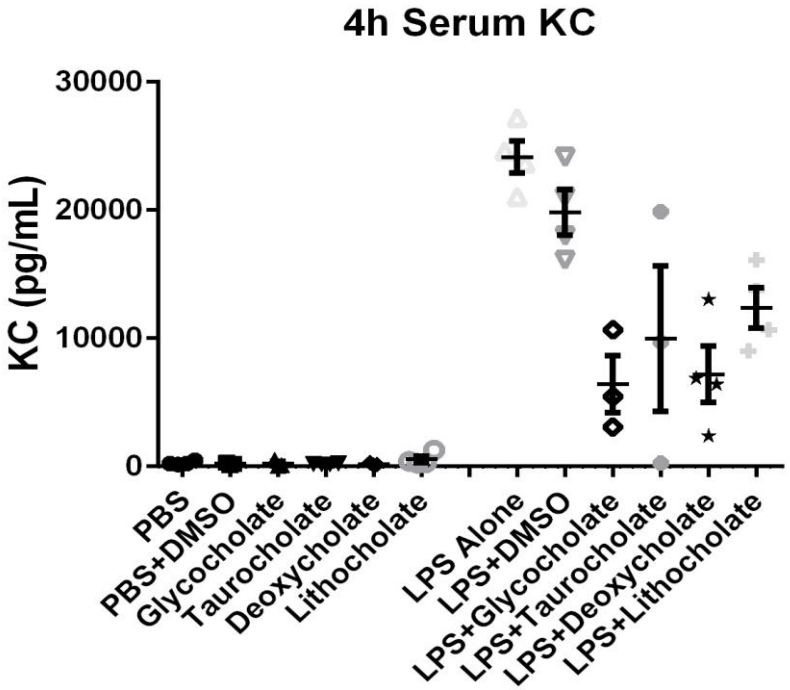

Figure 3. ELISA results, serum KC levels 4 hours post-injection with 1 μg LPS, 1 μg LPS + 1 μg bile salt mixture, or 1 μg vehicle.

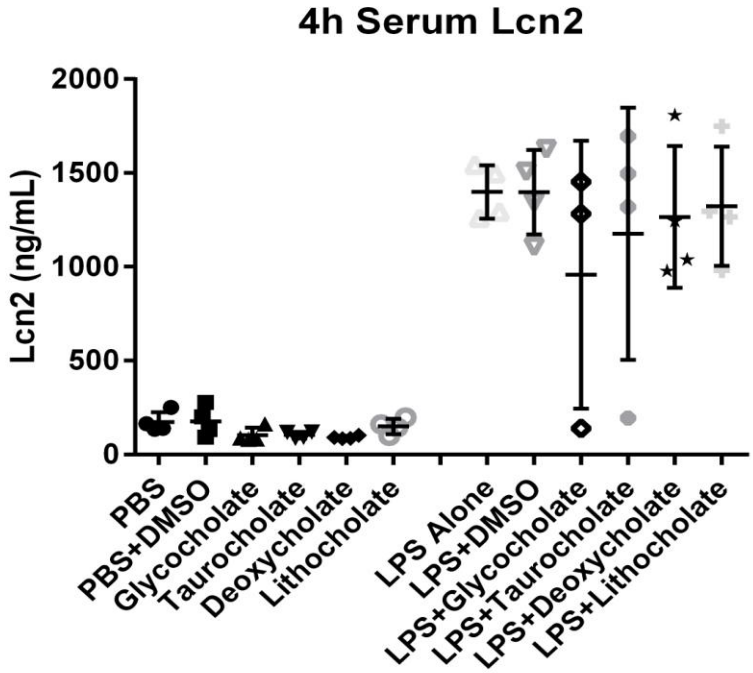

Figure 4. ELISA results, serum Lcn2 4 hours after injection of 1 ug LPS + 1 ug bile salt, as well as 1 ug LPS alone and 1 ug vehicle.

Figures 3 and 4 include the serum KC and Lcn2 values from the second set of mice (n=48), expressed in pg/mL and ng/ml serum, respectively. For the control group, PBS, 1% DMSO in PBS, and 1 μg bile salt in PBS were administered via IP injection at a final volume of 160 μl per mouse. For the treatment group, 1 μg LPS in PBS and 1% DMSO in PBS, as well as 1 μg LPS + 1 μg bile salt mixture in PBS were administered via IP injection at a final volume of 160 μl per mouse. Serum was collected 4 hours post-injection and analyzed via ELISA assay for KC and Lcn2 quantities. The groups were analyzed via one-way ANOVA ($p<0.05$) and are expressed as Mean ± SEM (n=4). Figure and statistical analysis was generated using GraphPad Prism 6 software.

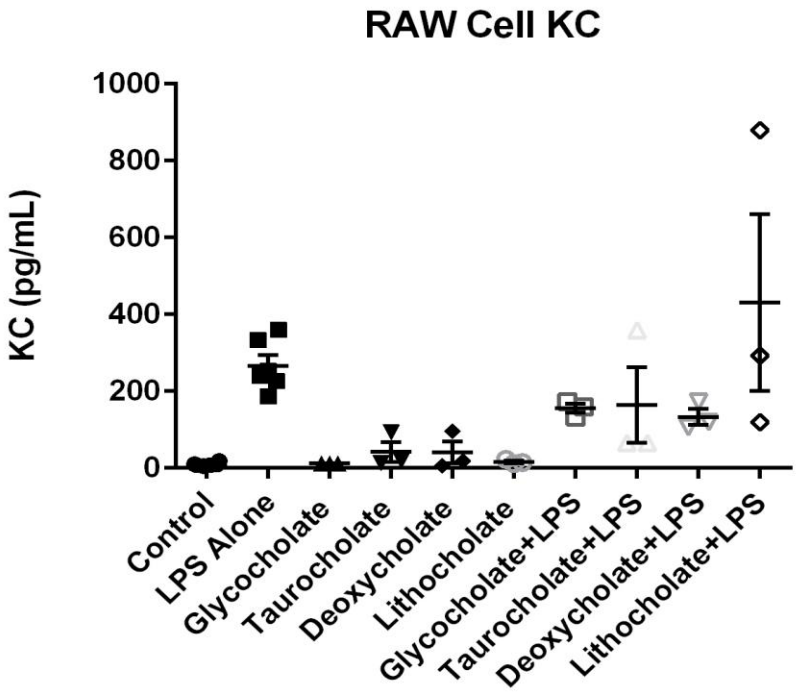

Figure 5. KC values from RAW 264.7 murine macrophage cell line after treatment with 500 ng LPS, 500 ng LPS + 500 ng bile salt mixture, or 500 ng vehicle.

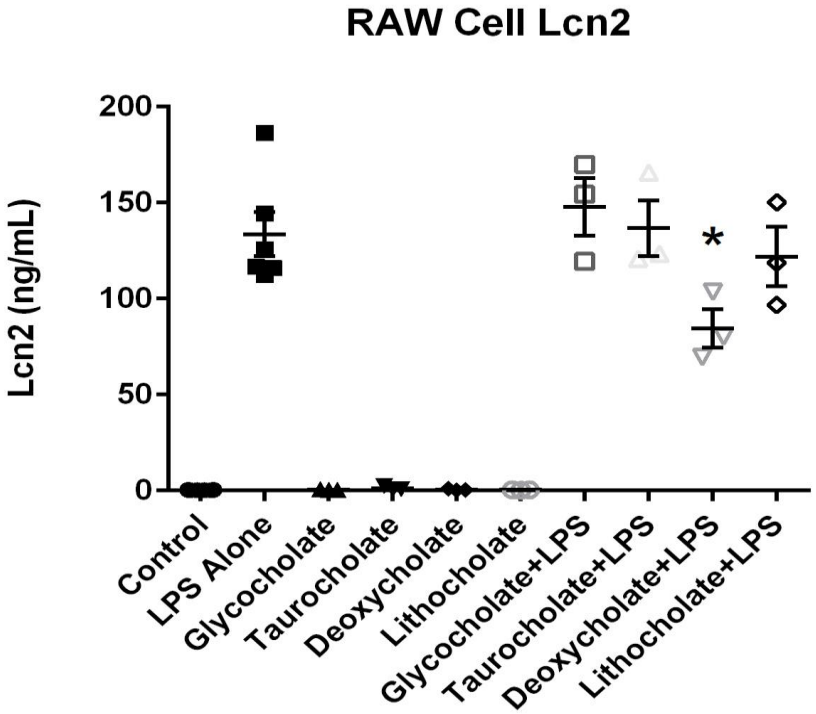

Figure 6. Lcn2 values from RAW 264.7 murine macrophage cell line after treatment with 500 ng LPS, 500 ng LPS + 500 ng bile salt mixture, or 500 ng vehicle.

Figures 5 and 6 are ELISA assay results from the RAW macrophage portion of the study. Cells from the RAW 264.7 murine cell line were plated in 24-well plates and stimulated with LPS for 24 hours. After stimulation, cells were treated with no treatment (control), 500 ng LPS in PBS, 500 ng bile salt vehicle in PBS, or a mixture of 500 ng LPS + 500 ng bile salt, also dissolved in PBS. As with the *in vivo* experiments, the mixtures were incubated at room temperature for 10 minutes before application to cells. After treatment, the cells were kept for 24 hours at 37^{o}C and 5% CO_2 before the supernatant was collected and analyzed via ELISA. The KC values are expressed in pg/ml, while the Lcn2 data is expressed in ng/ml. The groups were analyzed via one-way ANOVA ($p<0.05$). The data is expressed as Mean ± SEM, where n=6 for control and LPS alone groups and n=3 for treatment and vehicle groups. Figures and statistical analysis was completed using GraphPad Prism 6 software.

Discussion

In this experiment, the conjugated primary bile acids glycocholate and taurocholate and the secondary bile acids deoxycholate and lithocholate were evaluated for their ability to degrade endotoxic LPS, thereby reducing LPS signaling, as a symbiotic protection mechanism. Each bile acid was incubated at room temperature in solution with *E. coli* LPS and then either injected into wild-type mice or applied to RAW 264.7 murine macrophage cultures. It was hypothesized that all four bile salts would degrade LPS. Primary conjugated bile salts function in the small intestine to emulsify lipids; they also have signaling roles as well as bactericidal properties. Though comparatively little bacteria are found in the proximal small intestine (<10,000 cells/mL), the bile salts are able to destroy the membranes of bacteria that find their way into the small intestine (58, 59). It was thus predicted that conjugated bile salts would have LPS-degrading capability to decrease recognition of the endotoxin by membrane TLR4 proteins, thereby protecting the host from an inflammatory response in the case of bacterial lysis.

In fact, there was no significant decrease in Lcn2 or KC expression in the glycocholate and taurocholate + LPS groups, as was expected. However, the *in vivo* Lcn2 and KC results did show somewhat decreased levels in the glycocholate+LPS groups, and RAW cell KC values were also consistent with this trend (Figures 2, 3, 4, and 5). On the other hand, taurocholate+LPS groups did not show any discernible trend of increased or decreased inflammatory chemokine levels across experiments.

Experimental evidence suggests that taurocholate and glycocholic interact differently with LPS; this is likely explained by the difference in structure due to conjugation. Taurocholate is conjugated with taurine, a sulfonic acid, while glycocholate is conjugated with the amino acid

glycine. Interestingly, though the sulfonic acid constituent of taurocholate is a stronger acid than the amino acid conjugate of glycocholate, taurocholate has less of a protecting affect. Glycocholate had minimal affect on Lcn 2 stimulation; no significant difference was found between the glycocholate + LPS and LPS alone treatments. This may be explained by the fact that Lcn 2 is a highly sensitive, generalized inflammatory response. Even small amounts of LPS may trigger its release by neutrophils. KC levels, on the other hand, were notably decreased both in the glycocholate + LPS treated groups in the RAW cells as well as *in vivo*; this trend suggests that glycocholate may exhibit protective effects in the small intestine.

Stronger protective action was hypothesized for the secondary bile salts, deoxycholate and lithocholate. These salts are generated by the symbiotic bacteria in the colon of mammals. These bacteria contain enzymes that humans do not, and are thus able to deconjugate the primary conjugated bile acids that escape reabsorption in the small intestine. They also contain enzymes with 7α-dehydroxylation capability; the dehydroxylation of primary bile acids contributes to the low solubility of secondary bile acids. It has been established that deoxycholate has the ability to degrade LPS, though no such studies have been carried out for lithocholate (55). It was hypothesized that both secondary bile salts would protect the host against inflammation by interfering with recognition of the endotoxin by altering its structure. This would result in a lack of recognition by TLR4 and decrease stimulation of the host immune response and may provide an alternative explanation for why the bacteria of the colonic lumen deconjugate and dehydroxylate primary conjugated bile acids and the role that those compounds may play in innate immune regulation.

For the secondary bile salts, there was a large difference between the treatments with deoxycholate and lithocholate. As Tarmina's group previously determined, deoxycholate

degrades the structural integrity of LPS, thereby interfering with its recognition by the TLR pathway (55). These results, when tested in mice and RAW cell lines, produced a protective affect for the host. The decrease in stimulation of the immune system was significant for both Lcn 2 and KC levels in mice and RAW cells. Lithocholate, on the other hand, produced no protective effect whatsoever. In fact, some cases suggest an increased aggravation by LPS + lithocholate (Figures 2, 3, 5) though the increase in Lcn2 and KC production was not significant.

One explanation for the immune response-induction by lithocholate and LPS could be explained by the cytotoxicity of lithocholate. The toxic effects of this secondary bile acid have been clearly established and even linked to tumorigenesis and colon cancer (60). As a result, the compound may contribute to a more robust immune response than LPS alone. It is possible, as well, that the combination of the LPS and lithocholate actually increases the toxicity of one or the other, though more studies would be needed to clarify. An additional explanation is the fact that lithocholate in this experiment was dissolved first in DMSO before being diluted with PBS. DMSO is a cytotoxic compound that may have triggered the immune response of the *in vivo* and *in vitro* hosts (61). Lithocholate is generated by gut bacteria at much lower rates than deoxycholate, however, so the anti-LPS effect of deoxycholate may be the preferred mechanism of protection by the microbiota of the colon (44).

Although there is a clear trend of protection by deoxycholate, the results of the other bile acids are inconclusive at best and potentially aggravating at worst. Glycocholate may have some effect on LPS structural integrity, though more incubation time may be necessary. Further studies of bile salt/LPS mixtures incubated at physiological temperatures before injection may further elucidate the potential role of conjugated and secondary bile acids in host protection from LPS-mediated inflammation.

Conclusions

The results of this experiment show compelling evidence of deoxycholate's anti-inflammatory effects, and the potential of similar effects due to glycocholate. Bile salts carry out various important roles within intestinal tract, and have proven importance in the maintenance of symbiotic bacterial populations and other homeostatic functions. Further understanding of the role and functions of bile salts may provide insights into the pathogenesis of complicated inflammatory diseases that affect the gastrointestinal tract, such as Crohn's disease and ulcerative colitis. The microbiota acts essentially as an additional organ in the body; many interactions between the biome and host metabolic processes have yet to be elucidated. Bile salts, in addition to functioning as lipid emulsifiers and signaling molecules, also appear to play a role in immune function in the large and small intestine.

BIBLIOGRAPHY

(1) Arumugam M., et al. Consortium MetaHIT 2011. Enterotypes of the human gut microbiome. *Nature*, 473(7346): 174-180.

(3) Lozupone C. A., Stombaugh J. I., Gordon J. I., Jansson J. K., Knight R. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature*, 489(7415): 220-230.

(2) Bäckhed F., Ley R. E., Sonnenburg J. L., Peterson D. A., Gordon J. I. 2005. Host-bacterial mutualism in the human intestine. *Science*, 307(5717): 1915-1920.

(4) Shin N. R., Whon T. W., Bae J. W. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol*, 33(9): 496-503.

(5) Xu J., Bjursell M. K., Himrod J., Deng S., Carmichael L. K., Chiang H. C., Hooper L. V., Gordon J. I. 2003. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. *Science*, 299(5615): 2074-2076.

(6)Reichmann N. T., Gründling A. 2011. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. *FEMS Microbiol Lett*, 319(2): 97-105.

(7) Gnauck Anne, Lentle Roger G., Kruger Marlena C. 2015. The characteristics and function of bacterial lipopolysaccharides and their endotoxic potential in humans. *International Reviews of Immunology*: 1-31.

(8)Rietschel E. T., Kirikae T., Schade F. U., Mamat U., Schmidt G., Loppnow H., Ulmer A. J., Zähringer U., Seydel U., Di Padova F. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J*, 8(2): 217-225.

(9) Costerton J. W., Ingram J. M., Cheng K. J. 1974. Structure and function of the cell envelope of gram-negative bacteria. *Bacteriol Rev*, 38(1): 87-110.

(10)Takeuchi O., Akira S. 2010. Pattern recognition receptors and inflammation. *Cell*, 140(6): 805-820.

(12) Beutler B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol*, 12(1): 20-26.

(13) Chow J. C., Young D. W., Golenbock D. T., Christ W. J., Gusovsky F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*, 274(16): 10689-10692.

(11) Takeda K., Kaisho T., Akira S. 2003. Toll-like receptors. *Annu Rev Immunol*, 21: 335-376.

(14) Iwasaki A., Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol*, 5(10): 987-995.

(15) Kitchens R. L., Thompson P. A. 2005. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. *J Endotoxin Res*, 11(4): 225-229.

(16) Fitzgerald K. A., Rowe D. C., Golenbock D. T. 2004. Endotoxin recognition and signal transduction by the TLR4/MD2-complex. *Microbes Infect*, 6(15): 1361-1367.

(17) Wright S. D., et al. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, 249(4975): 1431-1433.

(18) Calvano J. E., et al. 2003. Modulation of the lipopolysaccharide receptor complex (CD14, TLR4, MD-2) and toll-like receptor 2 in systemic inflammatory response syndrome-positive patients with and without infection: relationship to tolerance. *Shock,* 20(5): 415-419.

(19) Fitzgerald KA., et al. 2003. LPS-TLR4 Signaling to IRF-3/7 and NF-kB Involves the Toll Adapters TRAM and TRIF. *J Exp Med*, 198(7): 1043-1055.

(20) Yamamoto M., Akira S. 2004. [TIR domain--containing adaptors regulate TLR-mediated signaling pathways]. *Nihon Rinsho*, 62(12): 2197-2203.

(21) Yamamoto M., et al. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol*, 169(12): 6668-6672.

(22) Hoebe K., Beutler B. 2004. LPS, dsRNA and the interferon bridge to adaptive immune responses: Trif, Tram, and other TIR adaptor proteins. *J Endotoxin Res*, 10(2): 130-136.

(23) Lu Y. C., Yeh W. C., Ohashi P. S. 2008. LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2): 145-151.

(24) Burns K., et al. 2003. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *J Exp Med*, 197(2): 263-268.

(25) Gorina R., et al. 2011. Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways. *Glia*, 59(2): 242--255.

(26) Cario E., et al. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol*, 164(2): 966-972.

(27) Abreu M. T., et al. 2001. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol*, 167(3): 1609-1616.

(28) Cario E., Podolsky D. K. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun*, 68(12): 7010-7017.

(29) Shibolet O., Podolsky D. K. 2007. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. *Am J Physiol Gastrointest Liver Physiol*, 292(6): G1469-1473.

(30) Otte J. M., Cario E., Podolsky D. K. 2004. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology*, 126(4): 1054-1070.

(31) Xavier R. J., Podolsky D. K. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*, 448(7152): 427-434.

(32) Schottelius A. J., Baldwin A. S. 1999. A role for transcription factor NF-kappa B in intestinal inflammation. *Int J Colorectal Dis*, 14(1): 18-28.

(33) Borkham-Kamphorst E., Drews F., Weiskirchen R 2011. Induction of lipocalin-2 expression in acute and chronic experimental liver injury moderated by pro-inflammatory cytokines interleukin-1β through nuclear factor-κB activation. *Liver International*, 31(5): 656-665.

(34) Alpízar-Alpízar W, et al. 2009. Neutrophil gelatinase-associated lipocalin (NGAL/Lcn2) is upregulated in gastric mucosa infected with Helicobacter pylori. *Virchows Arch*, 455(3): 225-233.

(35) Xu M. J., et al. 2015. Liver is the major source of elevated serum lipocalin-2 levels after bacterial infection or partial hepatectomy: a critical role for IL-6/STAT3. *Hepatology*, 61(2): 692-702.

(36) De Filippo K., Henderson R. B., Laschinger M., Hogg N. 2008. Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *J Immunol*, 180(6): 4308-4315.

(37) Song F., et al. 1999. Expression of the neutrophil chemokine KC in the colon of mice with enterocolitis and by intestinal epithelial cell lines: effects of flora and proinflammatory cytokines. *J Immunol*, 162(4): 2275-2280.

(38) Hofmann A. F. 2007. Biliary secretion and excretion in health and disease: current concepts. *Ann Hepatol*, 6(1): 15-27.

(39) Nebert D. W., Russell D. W. 2002. Clinical importance of the cytochromes P450. *Lancet*, 360(9340): 1155-1162.

(40) Jelinek D. F., Andersson S., Slaughter C. A., Russell D. W. 1990. Cloning and regulation of cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J Biol Chem*, 265(14): 8190-8197.

(41) Falany C. N., Johnson M. R., Barnes S., Diasio R. B. 1994. Glycine and taurine conjugation of bile acids by a single enzyme. Molecular cloning and expression of human liver bile acid CoA:amino acid N-acyltransferase. *J Biol Chem*, 269(30): 19375-19379.

(42) Hofmann A. F., Mysels K. J. 1992. Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca2+ ions. *J Lipid Res*, 33(5): 617-626.

(43) Ridlon J. M., Kang D. J., Hylemon P. B. 2006. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res*, 47(2): 241-259.

(44) Hay D. W., Carey M. C. 1990. Chemical species of lipids in bile. *Hepatology*, 12(3 Pt 2): 6S-14S; discussion 14S-16S.

(45) Barnes S., Gollan J. L., Billing B. H. 1977. The role of tubular reabsorption in the renal excretion of bile acids. *Biochem J*, 166(1): 65-73.

(46) de Aguiar Vallim T. Q., Tarling E. J., Edwards P. A. 2013. Pleiotropic roles of bile acids in metabolism. *Cell Metab*, 17(5): 657-669.

(47) Hofmann, A. F. 1963. The Function of Bile Salts in Fat Absorption. The Solvent Properties of Dilute Micellar Solutions of Conjugated Bile Salts. *Biochem J*, 89: 57-68.

(48)Einarsson K., Nilsell K., Leijd B., Angelin B. 1985. Influence of age on secretion of cholesterol and synthesis of bile acids by the liver. *N Engl J Med*, 313(5): 277-282.

(49) Landrier J. F., Eloranta J. J., Vavricka S. R., Kullak-Ublick G. A. 2006. The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter-alpha and -beta genes. *Am J Physiol Gastrointest Liver Physiol*, 290(3): G476-485.

(50) Xie W., et al. 2001. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci USA*, 98(6): 3375-3380.

(51) Makishima M., et al. 2002. Vitamin D receptor as an intestinal bile acid sensor. *Science*, 296(5571): 1313-1316.

(52) Wang YD., Chen WD., Yu D., Forman BM., Huang W. 2011. The G-Protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) in mice. *Hepatology*, 54(4): 1421--1432.

(53) Lewis R., Gorbach S. 1972. Modification of bile acids by intestinal bacteria. *Arch Intern Med*, 130(4): 545-549.

(54) Sung J. Y., Costerton J. W., Shaffer E. A. 1992. Defense system in the biliary tract against bacterial infection. *Dig Dis Sci*, 37(5): 689-696.

(55) Tarmina D. F., Milner K. C., Ribi E., Rudbach J. A. 1968. Modification of selected host-reactive properties of endotoxin by treatment with sodium deoxycholate. *J Bacteriol*, 96(5): 1611-1616.

(56) Percy-Robb I. W., Collee J. G. 1972. Bile acids: a pH dependent antibacterial system in the gut? *Br Med J*, 3(5830): 813-815.

(57) Lorenzo-Zúñiga V., et al. 2003. Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology*, 37(3): 551-557.

(58) King C. E., Toskes P. P. 1979. Small intestine bacterial overgrowth. *Gastroenterology*, 76(5 Pt 1): 1035-1055.

(59) Begley M., Gahan C. G., Hill C. 2005. The interaction between bacteria and bile. *FEMS Microbiol Rev*, 29(4): 625-651.

(60) Ogawa A., Murate T., Suzuki M., Nimura Y., Yoshida S. 1998. Lithocholic Acid, a Putative Tumor Promoter, Inhibits Mammalian DNA Polymerase β. *Japanese Journal of Cancer Research*, 89(11): 1154--1159.

(61) Galvao J., Davis B., Tilley M., Normando E., Duchen M. R., Cordeiro M. F. 2014. Unexpected low-dose toxicity of the universal solvent DMSO. *FASEB J*, 28(3): 1317-1330.

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Education

- Pennsylvania State University, Schreyer Honors College
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  - August 2012 – May 2016
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Experience

- Research Assistant, Vijay-Kumar Lab, May 2015 – Present
  - Worked on projects relating to homeostasis of the gut microbiota
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- Lecture Assistant, Biology 110: Basic Concepts and Biodiversity, September 2014 – December 2015
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Activities and Memberships

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Awards and Achievements

- Dean's List, Fall 2012 – Present
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