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Toll-Like Receptor 4 is Critical for the Development of Resection-Associated Steatosis

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Abstract

Background—A significant number of children with short bowel syndrome experience intestinal failure-associated liver disease. We recently demonstrated accelerated hepatic steatosis after 50% small bowel resection (SBR) in mice. Since SBR is associated with alterations in the gut microbiome, the purpose of this study was to determine whether TLR4 signaling is critical to the development of resection associated hepatic steatosis.

Methods—Male C57BL6 (control) and TLR4-knockout (KO) mice underwent 50% proximal SBR. Liver sections were analyzed to obtain the percent lipid content, and ileal sections were assessed for morphological adaptation. Intestinal TLR4 mRNA expression was measured at 7 days and 10 weeks.

Results—Compared to controls, TLR4 KO mice demonstrated similar weight gain and morphological adaptation after SBR. Hepatic steatosis was decreased 32-fold in the absence of TLR4. Intestinal TLR4 mRNA expression was significantly elevated 7 days after SBR. We also found that TLR4 expression in the intestine is 20-fold higher in whole bowel compared with isolated enterocytes.

Conclusions—TLR4 signaling is critical for the development of resection-associated steatosis, but not involved in intestinal adaptation after massive SBR. Further studies are needed to delineate the mechanism for TLR4 signaling in the genesis of resection-associated liver injury.

Keywords

TLR4; short bowel syndrome; steatosis; IFALD

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1. Introduction

Short bowel syndrome (SBS) results from a substantial loss of small bowel length due to surgical resection or congenital anomaly. Mortality in the pediatric patient population with SBS is roughly 40%, with more than half of these deaths resulting from hepatic failure.(1) Of the surviving patients, 40%–60% experience intestinal failure associated liver disease (IFALD).(2) Steatosis is one element of the hepatic dysfunction seen in SBS and persists even after weaning from parenteral nutrition (PN).(3) Although combined intestine and liver transplant offers a promising solution for end-stage IFALD, the survival in children at 10 years after transplant remains dismal at 42%. (2) Resection associated liver disease is an increasingly encountered complication of SBS for which the outcomes are poor, morbidity is high, and current clinical therapy is largely supportive.

Toll like receptor 4 (TLR4) signaling is a host response to bacterial lipopolysaccharide (LPS) and a key regulator of inflammation. It has been implicated in the development of steatosis in disease processes such as nonalcoholic fatty liver disease/steatohepatitis (NAFLD/NASH) in mice and humans. (4–7) In a diet-induced NAFLD/NASH mouse model, hepatocyte-specific TLR4 deficient mutants do not develop liver disease.(8) At present, it remains unknown whether IFALD and NAFLD/NASH share the same mechanistic pathways to induce liver steatosis.

Recently we discovered significant liver steatosis after SBR compared with unoperated mice with similar food intake and body weight.(9) Most murine models of NAFLD employ two basic approaches: genetic or nutritional.(10, 11) Our recently published model is most similar to the high fat diet models with the striking difference that after SBR mice have more severe steatosis sooner (10 weeks) with less fat (35%) in their diet. (9, 12) Given the many similarities between the models, the purpose of this study is to elucidate the role of TLR4 in resection-associated hepatic steatosis.

2. Methods

2.1 Experimental Design

Male C57BL6 (WT) or TLR4 KO mice underwent 50% SBR and were placed on liquid diet for 7 days or 10 weeks. Unoperated male C57BL6 (wild-type, WT) mice were placed on liquid diet for 10 weeks served as controls. Male WT mice underwent sham operation (transection at 12cm from the ileum with reanastomosis) and remained on liquid diet for 20 weeks were used as a control for liver histology only. Animals were weighed weekly.

2.2 Animals and Diet

C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 7 weeks of age. Mice were housed on arrival in a facility with a 12-h light/dark cycle. Male mice were used exclusively in order to limit hormonal confounders on the timing and severity NAFLD. (13) Male TLR4 KO mice were generously supplied by Dr. William Stenson (Division of Gastroenterology, Washington University School of Medicine in St. Louis). All mice were placed on liquid diet (LD; PMI Micro-Stabilized Rodent Liquid Diet LD 101; TestDiet, St. Louis MO) for 24 hours prior to operation until sacrifice. Food and water were provided ad

libitum. This study was approved by the Washington University Animal Studies Committee (Protocol #20130308) in accordance with the National Institute of Health laboratory animal care and use guidelines.

2.3 Operations and Sample Collection

All operated mice underwent a 50% proximal SBR as we have previously described.⁽¹⁴⁾ Briefly, through a midline laparotomy the bowel is exteriorized and then transected 12cm from the terminal ileum and 2–3cm from the pylorus. The intervening mesentery was ligated with silk suture and a hand sewn, end-to-end anastomosis was performed with interrupted 9-0 nylon sutures. Animals were fasted for the first 24 hours post operatively then returned to standard LD. For the early time point group (7 days after SBR) only small bowel was collected for enterocyte and smooth muscle isolation. For the late time period (10 weeks post operation) both liver and ileum were collected. Liver and terminal ileum were flash frozen in liquid nitrogen. For enterocyte and smooth muscle isolation, the bowel was flushed and placed immediately in ice cold PBS with inhibitors as previously reported.⁽¹⁵⁾

2.4 RNA

Samples were stored at –80 degrees prior to RNA extraction. RNA Isolation was performed using the RNeasy kit from Ambion (Austin, TX) per the manufacturers instructions. RT-PCR was performed per previously published protocol.⁽¹⁶⁾ TLR4 primers were obtained from Applied Biosystems (Carlsbad CA).

2.5 Histology

Bowel and liver sections were fixed in formalin, embedded in paraffin, and sections stained with Hematoxylin and eosin. All slides were analyzed using NIS Elements V4.3 software (Nikon Instruments, Inc., Melville NY) to obtain crypt and villus measurements, cell counts and percent lipid content. For intestinal adaptation measurements, at least 20 villi and crypts were obtained. Villi had an intact lymphatic channel from base to tip for inclusion. Crypts had intact villus-crypt junctions on both sides with Paneth cells present at the base for inclusion. Successful adaptation was considered > 20% increase in villus length from baseline. For liver histology, four random fields at 4x magnification per sample were analyzed for steatosis (percent lipid area). All slides were analyzed by the same investigators and blinded for group.

2.6 Proliferation and Apoptosis

Paraffin-embedded sections were deparaffinized then blocked with 3% hydrogen peroxide in methanol. Diva Decloaking solution (Biocare Medical, Concord, CA) was used for antigen retrieval followed by blocking with avidin-pink and biotin-blue (Biocare Medical). Slides were then labeled with anti-p-Histone H3 (Ser 10) antibody (Cell Signaling Technology; Danvers, MA) or cleaved caspase 3 (CC3) (Cell Signaling Technology; Danvers, MA) in DaVinci Green (Biocare Medical) then incubated overnight at 4°C. Labeling was visualized with biotinylated goat anti-rat IgG (Accurate Chemical; Westbury, NY), then streptavidin-horseradish peroxidase (HRP; Invitrogen, Camarillo, CA) followed by diaminobenzidine (DAB; Sigma-Aldrich, St Louis, MO), and finally hematoxylin counterstaining. Positively

staining p-Histone-H3 (Ser 10) or CC3 enterocytes were counted from at least 20 well-oriented crypts by blinded scoring. Apoptosis was scored on both CC3 and morphology using an H&E stain. For H&E morphology, apoptotic bodies were identified based on pyknosis, eosinophilic cytoplasm, and perinuclear halo in twenty well-formed crypts per animal.

2.7 Statistics

Students T-test was used to evaluate significance between two groups of continuous variables. For, nominal variables χ^2 was used. Results were considered significant with p 0.05.

3. Results

3.1 Functional and Morphological Adaptation

There was no significant difference in weight at any of the weekly time points in the operated mice (WT or TLR4 KO). When comparing the weight of operated mice to their own baseline weight, the absence of TLR4 did not have an effect on postoperative weight gain. (Data not shown) TLR4 deficiency did not affect survival after SBR (58% TLR4 KO versus 58% for WT) at 10 weeks after operation (p=0.61). TLR4 KO mice had similar villus growth (59% for WT and 62% for TLR4 KO) and crypt deepening (59% for WT and 47% for TLR4KO)(Figure 1). We also measured proliferation and apoptosis at 10 weeks after resection and found that while there was no difference in apoptosis, however, proliferation was significantly increased in the TLR4 KO mice (p=0.005) (Figure 2A–B).

3.2 Resection-Associated Steatosis

At 10 weeks after SBR, WT mice had significant liver steatosis ($4.6\% \pm 1\%$) compared to unoperated mice ($0.14\% \pm 0.03\%$, p=0.001) or sham operated mice ($0.0013 \pm 0.03\%$). In the TLR4 KO mice, liver steatosis after SBR ($0.08\% \pm 0.03\%$) was similar to both unoperated and sham operated mice. (Figure 3A–B)

3.4 TLR4 Expression

Given the clear lack of steatosis in the TLR4 KO mice, we sought to investigate TLR4 mRNA expression in the liver after SBR in WT mice. Surprisingly, we found significantly less hepatic TLR4 expression in the resected animals at 10 weeks compared to the unoperated animals. (0.34 ± 0.17 vs. 0.96 ± 0.28 , p=0.05). In light of these results as well as data demonstrating increased enterocyte TLR4 expression in rats after resection(17), we measured TLR4 expression in intestinal epithelium. We found that TLR4 expression increased by 5-fold at day 7 after SBR (p=0.008) and returned to baseline by post operative week 10. (Figure 4). Whole bowel (containing both intestinal epithelial and many other cell types) demonstrated a similar temporal trend in TLR4 expression, increasing by 5-fold at day 7 after SBR (p<0.001) and returning to baseline at week 10. Compared with isolated epithelial cells, the whole bowel expression of TLR4 was markedly greater (more than 20-fold; p=0.0001). There was no difference in TLR4 expression between SBR and unoperated mice at 10 weeks in the whole bowel. (Figure 4).

4. Discussion

In the present study, we have demonstrated that resection-associated liver steatosis is prevented by disrupting TLR4 expression. These results suggest that Gram-negative, bacterial-derived LPS is important in the pathogenesis of the liver injury that accompanies massive intestinal resection. The advantage of our SBR model is that it does not involve PN, thereby eliminating multiple confounding variables of PN including rates of glucose infusion, amino acid composition, different types of fat ($\Omega 3$ vs $\Omega 6$), and percent enteral intake. The significance of LPS in the context of massive SBR has been established as circulating anti-LPS immunoglobulins have been found to be elevated in human patients with short bowel syndrome(18, 19). Further, administration of LPS to rats after SBR has been shown to result in impaired structural adaptation and increased late mortality(20).

Since the mice in this model fully adapt to their intestinal loss without the need for supplemental nutritional support, we conclude that the observed hepatic steatosis most likely represents a milder form of IFALD. Consistent with these findings, a marked increase in LPS producing Proteobacteria in the intestine of humans with IFALD and steatosis has been previously been demonstrated.(21) Increased LPS exposure results in increased intestinal permeability via TLR4 signaling. (22, 23) This increased permeability permits pathologic interactions between an abnormal microbiome and the liver resulting in the inflammatory environment seen in chronic liver disease.(24) Along these lines, we found a significant increase in intestinal TLR4 expression at 7 days after SBR. These findings have been previously reported in rats in which intestinal resection increases TLR-4 mRNA and ligand protein expression in the remnant bowel(17).

We have previously described significantly greater culturable Gram-negative organisms in the liver and mesenteric lymph nodes in mice after SBR when compared to mice undergoing sham operation (transection of the bowel with reanastomosis alone)(25). Similar to our findings, a higher incidence of translocation to mesenteric lymph nodes and spleen after SBR has been verified in other animal models(26–28). These data support the concept that resection-associated alterations in the gut microbiome combined with intestinal barrier failure contribute to a proinflammatory milieu and may play a role in the pathogenesis of hepatic steatosis.

Other mechanisms must be considered for how TLR4 contributes to resection-associated liver disease. Recent studies in mice exposed to systemic trauma resulted in intestinal TLR4 activation which caused pulmonary injury via high mobility-group box protein 1 (HMGB1). (29) The effector molecule in this study, HMGB1, is an endogenous protein released by necrotic cells or actively secreted by immune cells in response to pathogen associated molecular patterns (PAMPs) such as LPS.(30) It functions as an alarmin, an endogenous signal of damage or infection capable of activating an innate immune response to infection or trauma involving remote organs and tissues.(31) Further, studies in mice have demonstrated a role for HMGB1 activation of hepatic TLR4 in the initiation of NAFLD.(32) Therefore, it is reasonable to hypothesize that HMGB1 or a similar alarmin is released or secreted in the setting of bowel resection thus triggering the initiation of resection-associated steatosis.

TLR4 signaling in the liver has been the primary focus of many current studies investigating the pathogenesis of steatosis in NAFLD. (4–7) In contrast to these studies, we saw significantly less TLR4 expression in the liver of mice at 10 weeks after SBR compared to unoperated animals. While this finding guided us to investigate the intestinal expression of TLR4 in the mice after SBR, we appreciate that hepatic TLR4 signaling may still play a role in the pathogenesis of resection associated steatosis. To delineate this role, further studies that allow isolated disruption of hepatic or intestinal TLR4 signaling are needed.

Previous work with intestinal TLR4 is primarily focused on the epithelium and the microbial interactions that occur at the luminal interface in the setting of necrotizing enterocolitis.(33) Our data would imply that in the setting of SBS, the non-epithelial cell compartment may be more important since TLR4 expression was significantly increased in whole bowel when compared with the isolated enterocytes. Data on myocyte TLR4 function in the setting of endotoxic ileus has demonstrated that non-hematopoietic, non-enterocyte intestinal TLR4 signaling can cause both enteric and systemic inflammation.(34) Given these findings, it is essential to understand the primary source of TLR4 signaling in the intestine after SBR. This information will be critical for future targeting of drug therapies to the appropriate TLR4 receptors.

In addition to the role for Gram-negative bacteria, LPS, and TLR4, we have recently reported that treatment with oral vancomycin also prevented the hepatic steatosis associated with SBR in our murine model(9). Vancomycin is not absorbed from the gastrointestinal tract and kills Gram-positive bacteria, which have been described to bloom in the gut of mice after SBR(35) and patients(21, 36) with short gut syndrome. Taken together, the observations of our past and present studies would endorse critical roles for both Gram-negative and positive organisms working synergistically to promote resection-associated steatosis. Further, the LPS and TLR4 mechanism proposed by the current study may only partially contribute to the pathogenesis of this important problem.

The presence of liver disease in the absence of PN suggests that significant bowel resection alone results in an environment that promotes the steatosis seen in IFALD. The pathogenesis of NAFLD has revolved around many theories of two or more “hits” with an initial alteration in liver homeostasis resulting in steatosis followed by an oxidative or inflammatory insult. (37, 38) With the similarities between NAFLD and IFALD, it is probable that the development of the two diseases is similar with an initial insult followed by one or more metabolic stressors. Along these lines, prior work from our lab has demonstrated a perturbed metabolic state after bowel resection whereby fat is replaced preferentially over lean body mass during the recovery phase of SBR.(39) If the two diseases are as similar in pathogenesis as they are in pathology, a resection-associated metabolic syndrome may then set the stage for the development of IFALD. Understanding the role TLR4 plays in this initial break from homeostasis and how its absence prevented the development of resection-associated steatosis is a critical next step in understanding the pathogenesis of IFLAD.

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Figure 1.

Deficient TLR4 expression does not affect structural intestinal adaptation. There were no significant differences between TLR4-knockout (TLR4 KO) and control wild-type (WT) mice with respect to percent villus or crypt growth at 10 weeks following a 50% proximal SBR. Baseline involved measurements of the distal ileum removed at the time of intestinal resection and compared to the remnant distal ileum 10 weeks later. Data is presented as mean $\pm$ SEM; n=7 WT, n=5 TLR4 KO.

Figure 2.

Deficient TLR4 expression results in elevated rates of enterocyte proliferation (A) but does not affect rates of enterocyte apoptosis (B). An index of enterocyte proliferation was obtained by scoring the number of Histone-3 positive cells per crypt while an index of apoptosis was recorded as number of apoptotic bodies present (pyknosis, eosinophilic cytoplasm, and perinuclear halo) along with positive staining cells for cleaved caspase 3 per crypt. TLR4-knockout (TLR4 KO) and control wild-type (WT) mice were studied at 10 weeks following 50% small bowel resection. Twenty well-oriented crypts were analyzed for each animal. Data is presented as mean $\pm$ SEM; N= 4 WT, N=4 TLR4 KO, * p= 0.005.

Figure 3.

TLR4 deficiency prevents resection-associated steatosis. (A) Percent lipid area as fold change from baseline measured in four random field views (4x magnification) from TLR4-knockout (TLR4 KO) and control wild-type (WT) mice were studied at 10 weeks following 50% small bowel resection (SBR) or sham operation (transection of the bowel with reanastomosis alone). All p-values are compared with SBR (WT) # $p=0.03$, * $p=0.02$ (B) Representative H&E staining (4x magnification) of liver from mice at 10 weeks following 50% SBR in TLR4 KO and WT mice, unoperated WT mice subjected to the same diet, and sham operated WT mice. $n=7$ SBR (TLR4 KO), $n=9$ SBR (WT), $n=5$ Sham (WT), $n=9$ NO (WT).

Figure 4.

TLR4 mRNA expression at 7 days and 10 weeks after 50% small bowel resection (SBR) or no operation (control) in wild-type mice. RNA was isolated from isolated enterocytes or the whole bowel. N= 4 Non-operated enterocyte, N=8-10 SBR Enterocyte, N=3 Non-operated non-enterocyte, N=4 SBR non-enterocyte. Bars represent mean values $\pm$ SEM. * $p < 0.0001$, \$ $p < 5.82E-6$ when comparing enterocyte to non-enterocyte at 7d and 10w.