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ORIGINAL RESEARCH

Hepatocyte Toll-Like Receptor 5 Promotes Bacterial Clearance and Protects Mice Against High-Fat Diet–Induced Liver Disease

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SUMMARY

Innate immune dysfunction can promote chronic inflammatory diseases of the liver, such as nonalcoholic fatty liver disease. Here, we show a role for hepatocyte Toll-like receptor 5 in detecting flagellin, clearing bacteria from the liver, and protecting against diet-induced hepatic diseases.

BACKGROUND & AIMS: Innate immune dysfunction can promote chronic inflammatory diseases of the liver. For example, mice lacking the flagellin receptor Toll-like receptor 5 (TLR5) show microbial dysbiosis and predisposition to high-fat diet (HFD)-induced hepatic steatosis. The extent to which hepatocytes play a direct role in detecting bacterial products in general, or flagellin in particular, is poorly understood. In the present study, we investigated the role of hepatocyte TLR5 in recognizing flagellin, policing bacteria, and protecting against liver disease.

METHODS: Mice were engineered to lack TLR5 specifically in hepatocytes ($TLR5^{\Delta Hep}$) and analyzed relative to sibling controls ($TLR5^{fl/fl}$). TLR5 messenger RNA levels, responses to exogenous flagellin, elimination of circulating motile bacteria, and susceptibility of liver injury (concanavalin A, carbon tetrachloride, methionine- and choline-deficient diet, and HFD) were measured.

RESULTS: $TLR5^{\Delta Hep}$ expressed similar levels of TLR5 as $TLR5^{fl/fl}$ in all organs examined, except in the liver, which showed a 90% reduction in TLR5 levels, indicating that hepatocytes accounted for the major portion of TLR5 expression in this organ. $TLR5^{\Delta Hep}$ showed impairment in responding to purified flagellin and clearing flagellated bacteria from the liver. Although $TLR5^{\Delta Hep}$ mice did not differ markedly from sibling controls in concanavalin A or carbon tetrachloride–induced liver injury models, they showed exacerbated disease in response to a methionine- and choline-deficient diet and HFD. Such predisposition of $TLR5^{\Delta Hep}$ to diet-induced liver pathology was associated with increased expression of proinflammatory cytokines, which was dependent on the Nod-like-receptor C4 inflammasome and rescued by microbiota ablation.

CONCLUSIONS: Hepatocyte TLR5 plays a critical role in protecting liver against circulating gut bacteria and against diet-induced liver disease. *(Cell Mol Gastroenterol Hepatol 2016;2:584–604; http://dx.doi.org/10.1016/j.jcmgh.2016.04.007)*

Keywords: Innate Immunity; TLR5; Hepatocytes; Inflammation; Steatosis.

The mammalian gastrointestinal tract is inhabited by a complex community of 100 trillion bacteria (1–2 kg in mass), collectively referred to as *gut microbiota*. Although gut microbiota play an essential role in host metabolism and immune system development,[1] failure to manage gut microbiota expeditiously can lead to chronic inflammatory diseases of the intestine such as Crohn's disease and ulcerative colitis.[2–5] A key means by which the host manages its microbiota are the Toll-like receptors (TLRs) and the nod-like receptors (NLRs), which confer the host innate ability to recognize a broad range of microbes. Deficiency in TLR and/or NLR signaling can result in changes in microbiota composition that promote intestinal inflammation and metabolic diseases. For example, mice with engineered deficiencies in TLR5, TLR2, NLRP6, or NLRP3 show altered gut microbiota composition that is associated with features of the metabolic syndrome, which could be transferred to WT mice via co-housing and/or fecal transplant, suggesting a role for microbiota in driving this disorder.[6–8] Such metabolic syndrome included features of nonalcoholic fatty liver disease (NAFLD) when mice were fed high-fat diets (HFD).

A primary mechanism by which altered microbiota might promote NAFLD and other features of metabolic syndrome is by inducing low-grade inflammation, which is a central feature of these disorders. For example, the altered microbiota composition of TLR5 knock-out (KO) mice was associated with higher levels of fecal bioactive

Abbreviations used in this paper: **ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL4, carbon tetrachloride; CFU, colony-forming unit; ConA, concanavalin A; CXCL, chemokine (C-X-C motif) ligand 1; DC, dendritic cell; Hep, hepatocyte; HFD, high-fat diet; IEC, intestinal epithelial cell; IL, interleukin; KO, knock-out; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NLR, nod-like receptor; NPC, non-parenchymal cell; MCD, methionine- and choline-deficient diet; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; RT-PCR, reverse-transcription polymerase chain reaction; TLR, Toll-like receptor; WT, wild-type.**

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lipopolysaccharide (LPS) and flagellin, suggesting that alteration of microbiota may result in inherently greater potential to promote inflammation.[9] Although observations that total loss of TLR function alters microbiota composition and promotes inflammation were suggested to be an artifact of mouse husbandry practices,[10] the observation that epithelial cell–specific deletion of TLR5 alters microbiota composition relative to TLR5-floxed siblings and results in low-grade inflammation/metabolic syndrome argues against this notion.[11] Such inflammation possibly might result from systemic dissemination of gut microbial products and increasing circulating proinflammatory cytokines in response to these products. Moreover, such microbial products might themselves disseminate from the intestine, via portal vein, to the liver and other tissues that do not normally harbor large populations of bacteria, and are thought to be very responsive to these products. Such receiving of intestinal venous blood by the liver has been proposed to result in the need for the liver to serve as a firewall to capture bacteria or their products that breach the intestine,[12] but also might be a means by which aberrant microbiota promote NAFLD.[13] Indeed, the concept that reduced intestinal barrier function can result in gut microbiota products breaching the intestine, sometimes referred to as leaky gut syndrome, increasingly is thought to play a central role in metabolic disease.[14,15] In support of this notion, detection of LPS in the liver by Kupffer, macrophage-like cells, promotes HFD-induced steatosis in mice.[16] However, the extent to which hepatocytes, the predominant cell type in the liver, play a role in recognizing bacterial products other than LPS remains largely undefined. Because hepatocytes share many properties with enterocytes, which are highly responsive to flagellin via TLR5 and normally lack classic LPS-induced TLR4 signaling,[17,18] we reasoned the former pathway might be operable in hepatocytes.

Hence, we generated mice lacking TLR5 in hepatocytes, and examined their phenotypes in assays of innate immunity and models of liver injury and inflammation. Such studies showed a role for hepatocyte TLR5 in detecting bacterial flagellin, clearing bacteria from the liver, and protecting against diet-induced hepatic disease.

Materials and Methods

Generation of Experimental Mice

WT, albumin-CRE, Villin-CRE, and CD11c-CRE mice were purchased from Jackson Laboratories (Bar Harbor, ME). The latter were bred to TLR5$^{fl/fl}$ mice, whose generation recently was described[11] to create the TLR5$^{fl/fl}$, TLR5$^{\Delta hepatocyte\ (Hep)}$, TLR5$^{\Delta intestinal\ epithelial\ cell\ (IEC)}$, and TLR5$^{\Delta dendritic\ cell\ (DC)}$ mice used herein. The global TLR5KO mice used here originally were generated by Dr Shizuo Akira (Osaka University, Osaka, Japan)[19] and back-crossed/maintained as previously described.[8] Mice lacking NLRC4 (NLRC4KO), generated on a pure C57BL/6J background, were kindly provided by Vishva Dixit (Genentech, Inc, South San Francisco, CA) and used to generate TLR5/NLRC4 double-KO mice (TLR5-NLRC4 DKO), as previously described.[20] All animals used in this study were on a C57BL/6J genetic background.

All mice were bred and housed at Georgia State University (Atlanta, GA), under institutionally approved protocols (Institutional Animal Care and Use Committee number A14033). Mice were fed with the standard LabDiet (St. Louis, MO) rodent chow LabDiets 5001 used in this facility. Where indicated, a HFD (60% of calories from fat), a methionine and choline-deficient diet (MCD) (A02082002B). and its associated methionine- and choline-sufficient control diet (A02082003B) were used to feed the mice (HFD, 8 weeks; MCD, 4 weeks, as previously described[8,21]). All the experiments using HFD and MCD were performed on female mice, whereas other experiments were performed on either female or male animals. All the figures present values obtained from 1 independent experiment (Figures 1-5 and 7), except for the HFD feeding experiment (Figures 6 and 8), performed twice.

Flagellin Treatment

Flagellin was isolated and purified by high-performance liquid chromatography, as previously described.[22] Six-week-old mice were injected intraperitoneally with 20 μg of purified flagellin or vehicle (phosphate-buffered saline [PBS]) as a control. Blood was collected at 30 and 120 minutes and hemolysis-free serum was generated by centrifugation using serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). Mice then were euthanized, and organs (liver, lung, colon, spleen, and kidney) were collected and stored at -80°C for further analysis.

Cytokine Analysis

Serum CXCL1 (chemokine CXC motif ligand 1), interleukin (IL)1β, and IL6 concentrations were determined using Duoset cytokine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Serum insulin concentration was determined using the EZRMI-13K rat/mouse insulin enzyme-linked immunosorbent assay kit (Millipore, Billerica, MA) according to the manufacturer's instructions.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNAs were isolated from liver, lung, spleen, abdominal fat tissue, and colon using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Messenger RNAs (mRNAs) were purified using the RNeasy mini kit RNA cleanup procedure (Qiagen, Valenica, CA). Quantitative reverse-transcription polymerase chain reactions (RT-PCRs) were performed using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) in a CFX96 apparatus (Bio-Rad) with specific mouse oligonucleotides (Table 1). Results were normalized to the 36B4 (housekeeping gene).

Intravenous Injection of Bacteria

As previously described,[12] live *Escherichia coli* (flagellated commensal strain MG1655) was administered (10^7 colony forming units [CFU], intravenously) via tail vein.

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