Hepatic TLR4 signaling in Obese NAFLD

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Abbreviations: NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic Steatohepatitis; HCC, hepatocellular carcinoma; TLR4, toll-like receptor 4; LPS, lipopolysaccharide; FA, fatty acid; FFA, free fatty acid; PAMP, pathogen-activated molecular pattern; MyD88, myeloid differentiation factor 88; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing interferon-beta; NF-κB, nuclear factorκB; IL-6, interleukin 6; RYGB, roux-en-y gastric bypass surgery; RT-PCR, reverse transcription polymerase chain reaction; PALM, palmitate; LIPO, lipogenic media; IBMX, isobutylmethylxanthine; BSA, bovine serum albumin; TZD, thiazolidinedione; PPARγ, peroxisome proliferator-activated receptor gamma; cAMP, cyclic AMP; VLDL, very low-density lipoprotein; SREBP-1, sterol regulatory element-binding protein-1; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel.

Funding support was from the following National Institutes of Health grants: NIH/NIDDK T32 DK07673 to TS, VUL1 RR024975 (Vanderbilt Clinical and Translational Science Award to TS and NNA), DK105847 (to NNA and CRF), DK091748 (to NNA), DK096527 (to NNA), DK053620 (to KW), AT004821 (to KTW), P30 DK020593 (Vanderbilt Diabetes Research and Training Center) to CRF, P30 DK058404 (Vanderbilt Digestive Disease Research Center) to CRF.

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*Running head: TLR4 signaling in NASH

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Abstract:
Nonalcoholic fatty liver disease (NAFLD) occurs frequently in the setting of metabolic syndrome, but the factors leading to nonalcoholic steatohepatitis (NASH) are not fully understood. This study investigated toll-like receptor 4 (TLR4) signaling in human liver with the goal of delineating whether activation of this pathway segregates those with nonalcoholic fatty liver (NAFL) from those with NASH. Experiments were performed using liver biopsy tissue obtained from Class III obese subjects undergoing bariatric surgery, and extended to an immortalized human hepatocyte HepaRG cell line and primary human hepatocytes. The bacterial endotoxin lipopolysaccharide (LPS) and total free fatty acid levels were significantly increased in plasma of NASH patients. TLR4 mRNA levels were significantly increased in subjects with NASH compared to NAFL as was IRF3 in the myeloid differentiation factor 88 (Myd88)- independent signaling pathway. In HepaRG cells, NF-κB nuclear translocation and functional activity increased following treatment with the fatty acid, palmitate, and following exposure to LPS, when compared to hepatocytes stimulated with a lipogenic treatment that induced de novo lipogenesis. Palmitate- and LPS-induction of NF-κB activity was partially attenuated by chemical- or siRNA-mediated inhibition of TLR4. Expression of TLR4 and its downstream mediators was up-regulated with palmitate and LPS. Similar results were observed using primary human hepatocytes from a lean donor. Interestingly, NF-κi activity assays showed obese donor hepatocytes were resistant to chemical TLR4 inhibition. Conclusion: TLR4 expression is up-regulated in a large cohort of NASH patients, when compared to those with NAFL, and this occurs within the setting of increased LPS and fatty acids.

Keywords: NASH, palmitate, TLR4, NF-κB, NAFL, lipopolysaccharide
INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent worldwide and is the most common liver disorder in Western industrialized countries, where the major risk factors for NAFLD, central obesity, type 2 diabetes mellitus, dyslipidemia, and metabolic syndrome are common (48). In the United States, studies report a prevalence of NAFLD ranging from 21-46% (25, 44). Patients with NAFLD have hepatic steatosis, with or without inflammation and fibrosis. Although steatosis is considered a benign disease, a subset of persons with steatosis develop more significant liver disease, nonalcoholic steatohepatitis (NASH), which can lead to cirrhosis and hepatocellular carcinoma (HCC) and biopsy based studies report a prevalence of NASH of 12% (44). This distinction between patients with simple steatosis and those with NASH is important because overall mortality is increased in patients with NASH (1, 3, 14, 39)(1, 3, 11, 31)(1, 3, 11, 31)(1, 3, 14, 39)(1, 3, 12, 32)(1, 3, 12, 32). According to data from United Network for Organ Sharing, there has been a steady increase in the number of liver transplants for NASH, from 5.1% in 2007 to 7.5% in 2010 (22) and this disorder is predicted to overtake other forms of liver disease as the most common reason for liver transplantation and liver disease-related deaths.

Not all patients with steatosis develop NASH, suggesting that modifiable factors may be required for disease progression. The etiology of NASH has undergone several iterations since the two-hit hypothesis was proposed (10). Emerging in vivo (47) and in vitro (31) data suggest that hepatic triglyceride accumulation in the form of lipid droplets may not exert pathogenic effects and may even be protective in the cellular responses leading to cellular injury and inflammation. The data implicating triglyceride stored within hepatocyte lipid droplets as a cause of liver injury in human studies and animal models primarily demonstrate an association between the two phenomena, rather than showing direct causality. When homeostatic mechanisms regulating hepatic fat metabolism are intact, production of lipotoxic metabolites from FFAs is minimized. During periods of over nutrition, stress or inflammation, the opposite holds true.

Toll-like receptor 4 (TLR4) is a pattern recognition receptor that functions as a lipopolysaccharide (LPS) sensor and its activation results in the production of several pro-inflammatory, antiviral and anti-bacterial cytokines (2, 38). TLR4 is normally expressed in various types of cell types including hepatocytes (26, 28), monocytes (9), Kupffer cells (32, 41) and stellate cells and represents a link between intestinal microbiota, endotoxemia and liver damage. LPS, a major component of the outer membrane of Gram-negative bacteria, and the exogenous ligand for TLR4, elicits strong immune responses and is considered to be the prototypical pathogen-activated molecular pattern (PAMP) (2). Both high fat and high fructose diets increase circulating levels of LPS by altering gut flora and epithelial permeability (5, 6, 15, 29, 40). Increased plasma LPS levels have been reported in diet-induced obese mice (12), genetically obese mice (5) as well as in humans with type 2 diabetes mellitus (7, 9). Similarly, changes in gut flora have been reported in persons with obesity and NAFLD (29, 42, 49). Along with LPS, TLR4 also senses a variety of endogenous ligands, including FFAs (C12:0 -lauric, C14:0 -myristic, C16:0 -palmitic, C18:0 -stearic) (38). As expected, FFAs are present in the obese state and increased levels of FFAs, such as palmitic acid and stearic acid, have been reported in patients with NASH and have been implicated in triggering inflammation and cell death.

Accumulating evidence in rodents and in cell cultures using isolated mouse hepatocytes (34) suggests that altered TLR4 signaling is a key factor in the pathogenesis of many chronic liver diseases, including NAFLD (19, 38) and have been implicated in the progression to NASH (8, 26). The data in humans are less clear and in the majority of studies comparator groups were healthy lean subjects (29, 35, 43) or patients with primary biliary cirrhosis (37). TLR4 signaling can be divided into the MyD88-dependent and the MyD88-independent pathways, with the latter mediated by TRIF. The triggering of both of these pathways ultimately leads to nuclear translocation of nuclear factor κB (NF-κB) and the activation of inflammatory cascades that produce various pro-inflammatory cytokines, including TNF-α and IL-6 (38).

In the present study, we hypothesized that chronic elevations of circulating gut-generated LPS, as well as elevated FFAs, were partly responsible for the pro-inflammatory responses seen in NASH via TLR4 signaling. We tested this hypothesis first in primary human liver biopsy samples from class III obese subjects, and
subsequently in an immortalized human hepatocyte cell line and primary human hepatocytes from a lean and an obese donor.

**MATERIALS AND METHODS**

**Human subjects.** Subjects gave their informed written consent before participating in this study, which was approved by the Institutional Review Board of Vanderbilt University and registered at ClinicalTrials.gov (NCT00983463). Class III obese women (n = 61, ages 19-59 years old) were recruited from the Vanderbilt Center for Surgical Weight Loss prior to their scheduled roux-en-y gastric bypass (RYGB) procedures. Exclusion criteria included a history of previous liver disease (e.g. viral or autoimmune hepatitis, or hemochromatosis), significant alcohol use (defined as > 10g alcohol/day), concurrent infections, a cancer diagnosis within the previous 5 years, hemoglobin A1C > 8.0, or the use of anti-diabetic drugs. All subjects underwent RYGB procedures. Wedge liver biopsies (200-1,000 mg) of the left lateral lobe were collected at the time of surgery. Blood was collected in K2 EDTA BD Vacutainer® tubes kept on ice from 37 subjects. Tubes were then centrifuged at 3000 rpm for 10 minutes at 4°C. Tissue and plasma were immediately stored at -80°C for further analysis.

**Measurement of LPS levels in human plasma.** Human plasma LPS levels were quantified using the Human LPS ELISA kit (MBS702450, MyBiosource; San Diego, CA).

**Measurement of FFAs in human plasma.** Serum FFAs were measured by gas chromatography in the Vanderbilt Diabetes and Research Center Lipid Core (DK20593, Vanderbilt University; Nashville, TN).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).** Total RNA was extracted using an RNeasy Mini kit (Qiagen; Valencia, CA). After spectrophotometric assessment for quality and concentration (Nanodrop ND-1000; Thermo Scientific, Nashville, TN), total RNA (500ng) was reverse-transcribed into complementary DNA using the High-Capacity cDNA kit (Applied Biosystems; USA). Reactions were performed in a 20 μL final volume containing 25ng cDNA, 10μL SYBR Green PCR Master Mix (Applied Biosystems), and 7.5 pg of each primers using an Bio-Rad CFX Real time PCR thermocycler. Relative levels of gene expression are reported as actin-normalized fold change using the 2^ΔΔCT (27, 33) and absolute quantification methods (24). Primer sequences for β-actin, TLR4, MYD88, TRIF, IRF7, IRF3 and TLR5 were selected using Primer Express 3.0 (Applied Biosystems) and are reported in Table 2.

**In Vitro Cell Culture.** Human hepatocytes (HepaRG cells, Invitrogen; Carlsbad, CA) were maintained in William’s E media supplemented with 5 U/ml penicillin, 5µg/ml streptomycin, 10% Fetal Bovine Serum, 200mM L-glutamine, 5µg/ml insulin, and 5x10^{-8}M dexamethasone as described (17). At confluence, cells were differentiated using 2% DMSO in maintenance media for two weeks prior to treatments. Primary human hepatocytes (HU1443 and HU8172) were thawed in Cryopreserved Hepatocyte Recovery Medium (CHRMR® Life Technologies). The hepatocytes were centrifuged at 500 x g for 5 min. The pellets were resuspended in Williams’ Medium E containing a Hepatocyte Plating Supplement Pack (Cocktail A; Life Technologies). Cells were counted and plated in 6-well plates for 6 h. Human hepatocytes were maintained in Williams’ Medium E, supplemented with 10% FBS, and Hepatocyte Maintenance Supplement Pack with dexamethasone (Cocktail B) for 3 days. Treatments of both HepaRG and primary cell lines were with palmitate (PALM, 400 µM prepared from a 5 M ethanolic stock conjugated to essentially endotoxin-free BSA at a ratio of 4:1), LPS (100 ng/mL for HepaRG cells and 1 µg/mL for primary hepatocytes) or a lipogenic medium (LIPO) comprised of 10 µM rosiglitazone, 250 µM isobutylmethylxanthine (IBMX), and 1 µM bovine insulin (all from Sigma; St. Louis, MO) void of BSA that consistently induced de novo lipogenesis (LIPO) in hepatocytes.

**NF-κB nuclear translocation assay.** NF-κB p65 nuclear translocation in fully-differentiated HepaRG and primary hepatocytes responding to treatment was assessed using a Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER, Thermo Scientific; Rockford, IL) per manufacturer’s instruction. Cell lysis buffers were supplemented with the Protease Inhibitor Cocktail (Set II; Calbiochem) and the Phosphatase Inhibitor Cocktail (Set I; Calbiochem). Protein concentrations were determined using a BCA Protein Assay (Pierce). Antigens of
interest were resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by immunoblotting and densitometry using 20 µg protein per lane.

NF-κB activity assay. The relative luciferase activity of the NF-κB promoter element was measured using the Dual Luciferase Assay System (Promega; Fitchburg, Wisconsin). Cells were co-transfected with polymyxin-B-agarose purified NF-κB luciferase reporter plasmid (pGL4.32[luc2P/NF-κB-RE/Hygro] Vector; Promega) and the Renilla luciferase control plasmid (pRL-TK vector; Promega) at a ratio of 0.8ug and 0.08ug DNA, respectively, to 6ul of liposome (Lipofectamine 2000, Invitrogen; USA) prior to treatment. Liposome alone was used as a negative control. At 24 hours post transfection, cells were subjected to various treatments for two hours, then lysates were harvested and luciferase was measured using a Spectramax M5 (Molecular Devices; Sunnyvale, CA) Multi-Mode Microplate luminometer. Luciferase activity of the NF-κB reporter was normalized to Renilla luciferase activity for each sample to control for transfection efficiency.

siRNA knockdown. TLR4 (5 nm; ACCATTGAAGAATTCCGATTA) and control non-silencing (5nM; catalog number Qiagen1027281) siRNAs were transfected into HepaRG cells using HiPerfect Transfection reagent (Qiagen; Valencia, CA) at a ratio of 37.5 ng siRNA to 3µl cationic liposome. Cells were assayed for knockdown 24 hours post transfection via RT-PCR. HepaRG cells were then co-transfected with TLR4 siRNA or scrambled control siRNA, as well as with the NF-κB luc reporter plasmid and a Renilla luciferase control plasmid for 24 hours. Cells were then treated and luciferase activity measured as described above.

Chemical TLR4 inhibition. Cells were transfected with reporter and control plasmids as described. At 18 hours of transfection, 1ug/ml of the TLR4 signaling inhibitor, CLI-095 (InvivoGen; San Diego, CA) for 6 hr. While the IC50 of CLI-095 is reported as 1-33 nM) (20), preliminary dosing experiments revealed the maximal effective inhibitor concentration to be 1ug/ml (data not shown). At 24 hours post transfection, cells were treated and luciferase activity measured as described above.

Immunoblots. Protein extracts (30µg) from cell culture were subjected to 10% SDS-PAGE then transferred to a nitrocellulose membrane. Membranes were blocked with Odyssey Blocking Buffer (Li-Cor; Lincoln, NE) for one hour at room temperature before 4 °C overnight incubation with one of the following antibodies: rabbit anti-fibrillarin at 1:200 or anti-NF-κB p65 at 1:2000 (Santa Cruz Biotechnology; Dallas, TX); or mouse anti-β-actin at 1:1000 (Sigma; USA). Anti-rabbit-IR680 and anti-mouse-IR800 conjugates diluted 1:10,000 (Li-Cor) were used as secondary antibodies. Relative protein quantities were determined using an Odyssey (Li-Cor) Infrared imaging system.

Statistical Analysis. Kruskal–Wallis one-way analysis of variance with Dunn’s post-test was used for more than two groups. Statistical significance was evaluated using paired t tests for cell culture. Data are expressed as means ± S.E.M. Differences were considered significant at a P value equal or less than 0.05.

RESULTS

Human liver biopsies were obtained from a cohort of class III obese females at the time of RYGB and stratified into patients with no steatosis (Obese; n = 20), nonalcoholic fatty liver (NAFL; n = 14) or nonalcoholic steatohepatitis (NASH; n = 27), as determined by histological assessment by an expert pathologist. There was no significant difference among the groups in terms of age, weight, BMI or liver function tests. The NASH group had significantly higher glucose levels and fibrosis compared to Obese controls. As expected, the NASH group has a significantly higher NAFLD activity score compared to Obese or NAFL specimens (Table 1).

LPS and FFAs are increased in NASH in human plasma samples. To further determine the potential factors discriminating those subjects with increasing NAFLD severity, matching plasma (where available), was analyzed from Obese (n = 18), NAFL (n = 16) and NASH (n = 20) subjects. We found that plasma total FFA levels were significantly increased in patients with NASH compared to Obese controls (Fig. 1A). Additionally, plasma endotoxin (bacterial lipopolysaccharide; LPS) levels were increased in subjects with NASH compared to NAFL (Fig. 1B).
mRNA expression analysis of TLR4 signaling proteins in NAFLD. To determine TLR4 signaling differences at different stages of NAFLD, steady-state hepatic expression levels of TLR4, myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF) and interferon regulatory factors 3 and 7 (IRF3 and IRF7) were investigated by quantitative RT-PCR on available liver tissue samples (15 obese, 9 NAFL, 21 NASH specimens) and normalized to expression levels of β-Actin. Expression levels of toll-like receptor 5 (TLR5), a receptor for LPS and bacterial flagellin also were determined. We found bimodal TLR4 expression levels in liver with significant decreases in NAFL relative to Obese controls and significant increases in NASM relative to NAFL (Fig. 2A). mRNA expression levels of the MyD88-dependent signaling molecules MYD88 and TRIF (Fig. 2B and C) trended in a similar manner to TLR4 although mean expression levels were not significantly different. Examination of two genes encoding important proteins in the Myd88-independent pathway, interferon regulatory factor 7 (IRF7) and IRF3 showed similarly decreased expression levels in NAFL subjects relative to Obese and NASH subjects, although such differences reached statistical significance with IRF3 (Fig. 2E). Expression levels of TLR5 were expressed at a significantly reduced level in NAFL but not NASH subjects (Fig. 2F).

NF-κB nuclear translocation is increased with palmitate and LPS compared to lipogenic treatment. We next sought to define mechanisms that may explain our in vivo findings. Using a human hepatocyte cell culture model, we defined the kinetics of NF-κB p65 protein translocation in cultured HepaRG cells treated with palmitate versus maintenance media (control treatment) and determined the time of maximal effect (Fig. 3A). NF-κB p65 nuclear translocation, determined by p65 content relative to GADPH (cytosolic marker) and fibrillarin (nuclear marker) expression, increased significantly at 40 min, 60 min and 2 h following palmitate stimulation, when compared to control treatments (Fig. 3B).

We next compared the effect of endogenous triglyceride accumulation (induced lipogenesis) versus an exogenous FFA (palmitate) or bacterial endotoxin (LPS), either alone or in combination, on NF-κB p65 localization (Fig 4). All HepaRG cells displayed comparable cytosolic p65 expression levels after 60 min treatment (Fig 4B) while those treated with palmitate alone, LPS alone or palmitate and LPS combined (Fig. 4C) showed significant nuclear translocation compared to those treated with LIPO.

Palmitate and LPS induction of NF-κB activity is partially attenuated by inhibition of TLR4. NF-κB activity additionally was measured by luciferase activity assays. We carried out transient transfection assays with an NF-κB-dependent reporter gene, normalizing the transfection efficiencies (averaging >60% in 3 experiments) by luciferase intensities from the co-transfected pRL-TK (Renilla luciferase) vector. The luciferase/Renilla activity ratios were used to evaluate the changes in luciferase activity after palmitate and LPS stimulation. Consistent with our observation of increased nuclear translocation, we observed that treatment of HepaRG hepatocytes with palmitate (PALM) elicited a robust four-fold increase in normalized NF-κB-dependent luciferase activity relative to cells treated with LIPO (Fig. 5A). LPS stimulation resulted in a 2.5-fold increase in NF-κB activity compared with the LIPO treatment (LPS; Fig. 5A. To directly assess whether TLR4 was mediating increased NF-κB activity upon palmitate and LPS exposure, the effects of loss of function of TLR4 were studied in HepaRG cells. Quantitative RT-PCR revealed a 40% effective TLR4 knockdown in untreated cells (Fig. 5B). Compared to control cells treated with a scrambled TLR4 siRNA, siRNA inhibition of TLR4 significantly attenuated, but did not completely abolish the effect of palmitate or LPS on NF-κB activation determined by luciferase assay (Fig. 5C).

To determine whether partial attenuation was a consequence of limited TLR4 involvement in NF-κB activation or simply a result of incomplete siRNA knockdown, we also treated cells with a chemical inhibitor of TLR4 signaling. Similar to the siRNA effect, treatment with a TLR4 signaling inhibitor resulted in partial attenuation of the effect of palmitate and LPS on NF-κB activation (Fig. 5D). Taken together, these results confirm our earlier findings that palmitate and LPS induced activation of NF-κB is partially mediated through TLR4 signaling.

Transcription of TLR4 and its downstream mediators is up-regulated by palmitate and LPS. We next sought to determine whether TLR4 transcription was up-regulated in the setting of exposure to palmitate and
LPS, thus leading to increased NF-κB nuclear translocation and activation, as suggested by our silencing and chemical inhibition studies. HepaRG cells were treated with control media, lipogenic treatment, palmitate or LPS for four hours. Transcription levels of TLR4 and its downstream mediators, TRIF and MyD88 were determined by RT-PCR. We found that expression of TLR4, TRIF, and MyD88 was up-regulated with palmitate and LPS treatments versus lipogenic treatment (Fig. 6). We also investigated whether a positive feed-back mechanism was present in which activation of NF-κB leads to up-regulation of TLR4 transcription. However, there was no effect on TLR4 mRNA expression when NF-κB activation was inhibited via a chemical inhibitor (BAY 11-7082; data not shown). These results indicate that palmitate and LPS exposure leads to up-regulation of hepatocyte mediated TLR4 transcription.

Responsiveness of primary human hepatocytes to palmitate and LPS. As HepaRG cells are an immortalized cell line we sought to validate our findings in primary human hepatocytes. To this end, plateable primary human hepatocytes were obtained from normal, lean (HU1443; body mass index of 18.1 kg/m²) and obese (HU8172; BMI of 30 kg/m²) human Caucasian donors aged 59 and 60, respectively. Hepatocytes plated 72 h were treated with maintenance media of TLR4 inhibitor for 6 h prior to 1 h of LPS, PALM or LPS + PALM stimulation. Hepatocytes from a lean donor displayed a robust p65 nuclear content under basal conditions and in response to LPS, palmitate, or when both agonists were provided additively. This migration was almost completely abrogated in the presence of TLR4 inhibitor (Fig. 7A, bottom panels). In contrast, primary hepatocytes from an obese donor (Fig. 7B) treated with LPS, palmitate or palmitate + LPS showed p65 nuclear translocation that was not effectively blocked with the same TLR4 inhibitor. Limited hepatocyte availability precluded multiple rounds of experimentation sufficient to warrant statistical testing, however these findings suggest a differential responsiveness to inflammatory stimuli in a setting of obesity.

DISCUSSION
In this study, we observed that plasma LPS and total FFAs were significantly increased in human subjects with NASH, compared to those with NAFL. The significantly reduced expressions of TLR4 and IRF3 in NAFL relative to NASH suggests a role for altered TLR4 signaling in the break of tolerance to bacterial endotoxia that may have been secondary to intracellular lipid accumulation. This led us to explore whether LPS and/or palmitate differentially-stimulated TLR4 signaling in hepatocytes compared to hepatocytes treated with a medium that induces de novo lipogenesis (LIPO). Such experiments showed negligible effects of lipogenic treatment on NF-κB activation but significantly increased stimulation after LPS and/or palmitate. Furthermore, both LPS and palmitate stimulated NF-κB activities were blunted with chemical- or siRNA-mediated TLR4 inhibition. In HepaRG cells these responses were associated with increased TLR4 and Myd88-dependent and Myd88-independent signaling as suggested by the significantly increased mRNA expression of TLR4, Myd88 and TRIF. While hepatocytes from a lean donor (BMI = 18 kg/m²) displayed blunted LPS- and palmitate-mediated NF-κB nuclear translocation when treated with a TLR4 inhibitor, hepatocytes from an obese donor (BMI = 30 kg/m²) were seemingly resistant to inhibition as LPS, palmitate and both agonists provided additively stimulated p65 nuclear translocation. Although limited cell availabilities precluded exhaustive testing to determine the nature of such responses these data suggest that obesity may alter hepatocellular TLR4-mediated NF-κB signaling in the setting of endotoxia and FFA exposure.

Our results demonstrate that in HepaRG cells LPS stimulation increased NF-κB activation through both MyD88-dependent and –independent mechanisms. Additionally, exposure to palmitate, an endogenous TLR4 receptor activator, resulted in a more robust increase in NF-κB activation. This was in contrast to observations with hepatocytes treated with a lipogenic medium containing a thiazolidinedione (TZD), IBMX and insulin, which failed to induce NF-κB activation, reinforcing the theory that exogenous fatty acids stimulate lipotoxicity. TZDs are agonists of peroxisome proliferator-activated receptor gamma (PPARγ) that improve insulin sensitivity in most tissues, but affects lipid metabolism in a cell- and tissue-specific manner. In addition, inhibition of TLR4 by siRNA in hepatocytes attenuated but did not suppress NF-κB activation, a finding that was replicated using a signaling inhibitor (Fig. 5). These findings suggest that LPS and palmitate exert both a TLR4-dependent pathway and another, yet unidentified, TLR4-independent pathway.
A significant body of evidence has now implicated new regulators in the pathogenesis of NASH. Once thought to be a disease caused by lipid accumulation in hepatocytes with subsequent oxidative stress, lipid peroxidation, inflammation and fibrosis, data now suggest that fat accumulation per se is not responsible for progressive cellular injury in the liver, and in fact, non-triglyceride lipotoxicity is a more proximal mediator (30, 47). In our study, we also observed that induction of de novo lipogenesis did not induce NF-κB nuclear translocation and activation. In contrast, we noted increased NF-κB nuclear translocation and activation in hepatocytes exposed to LPS and palmitate.

There are likely multiple factors involved in the pathogenesis of NASH, including inappropriate accumulation of free fatty acids, endotoxemia stemming from a change in gut flora and gut permeability, as well as over-activation, up-regulation, and perhaps, dysregulation of TLR4 signaling. In our study, we found increased TLR4 and IRF3 gene expression in a large cohort of human liver biopsy samples of patients with NASH as compared to those with NAFL; the same was found for hepatocytes exposed to palmitate and LPS compared to those treated to induce lipogenesis. Singh et. al found lower levels of hepatic TLR4 mRNA and protein levels in patients with normal livers, as compared to patients with NASH, again indicating the importance of suppressed inflammation and immune tolerance in normal livers (37). Similarly, Xu et. al reported increased hepatic expression of TLR4 mRNA in rats fed a high fat diet, as compared to their control counterparts. This increase in hepatic TLR4 mRNA corresponded with the appearance of NASH in this rat model (45). Taken together, these findings suggest that TLR4 may be one of the factors responsible for the increase in hepatic sensitivity to LPS and fatty acid injury, and thus play a key role in the pathogenesis of NASH. Low expression of TLR4 in hepatocytes from subjects with NAFL highlights the importance of liver immune homeostasis.

Intriguingly, we also found that primary human hepatocytes from a lean versus an obese donor showed different sensitivities to TLR4 inhibition in terms of NF-κB activation, also implicating dysregulation of TLR4 in obese liver and perhaps subsequent resistance to inhibition. Other factors that need to be considered are possible differences in FFA or LPS levels in the lean vs obese patient and how these factors may play a role.

Further in vivo studies are needed to more fully understand the mechanisms involved in the delicate balance between host tolerance and an adaptive immune response versus dysregulation of immune tolerance in the diseased state, which may lead to the development of new therapeutic targets for regulating activation of the TLRs in chronic liver diseases. Understanding the factors that underlie NASH is of great clinical interest and may permit stratification of patients by their likelihood to develop NASH, and thus, guide physicians in pursuing more aggressive interventions in this subgroup of patients. Our study provides evidence that TLR4 is up-regulated in a large cohort of NASH patients, as compared to those with NAFL. This seems to occur in a setting of increased plasma levels of LPS and FFAs. Further, our human cell culture model suggests that NF-κB activation is at least partially driven by hepatocyte mediated TLR4 signaling, in response to LPS and palmitate, highlighting the important role of liver parenchymal cells in this process.

ACKNOWLEDGEMENTS

The authors thank Dr. M. Kay Washington, Vanderbilt Department of Pathology, for her assistance in the pathological assessment of liver specimens.


Figure Legends

Fig. 1. Plasma endotoxin and FFAs levels were measured in Class III obese subjects with nonalcoholic fatty liver (NAFL) (n=16) nonalcoholic steatohepatitis (NASH; n = 20) and compared with weigh-matched controls with normal liver histology (Obese, n = 18). A. NASH subjects had significantly elevated (*\(P < 0.05\)) plasma FFA levels compared to obese subjects with normal histology. B. Plasma LPS was elevated in the NASH subjects relative to subjects with NAFL (*\(P < 0.05\)). Data is represented as mean ± S.E.M. *\(P < 0.05\) by one-way ANOVA with Dunn’s post-test.

Fig. 2. Transcription of TLR4 and its downstream mediators is up-regulated in patients with NASH compared to those with NAFL. Liver mRNA expression levels normalized to β-Actin were determined by quantitative RT-PCR in subjects with normal liver (Obese; n = 15), nonalcoholic fatty liver (NAFL; n = 9) or nonalcoholic steatohepatitis (NASH; n = 21). A) Toll-like receptor 4 (TLR4) mRNA levels in NAFL were significantly reduced compared to Obese controls and subjects with NASH (**\(P < 0.01\) for both). mRNA levels of B) myeloid differentiation factor 88 (MyD88), C) toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF), D) interferon regulatory factor 7 (IRF7), E) interferon regulatory factor 3 (IRF3) and F) toll-like receptor 5 (TLR5) in Obese, NAFL and NASH subjects. Data are expressed as mean ± S.E.M. *\(P < 0.05\), **\(P < 0.01\) by one-way ANOVA with Dunn’s post-test.

Fig. 3. NF-κB p65 protein nuclear translocation increases with palmitate exposure. HepaRG cells were untreated (Control) or treated with palmitate (PALM; 400 \(\mu\)M) and harvested after 1, 5, 10, 20, 40, 60 min or 2 and 4 h. A) Cytosolic and nuclear protein fractions were probed for NF-κBp65 protein levels via quantitative immunoblot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fibrillarin were used as loading controls for cytoplasmic and nuclear fractions, respectively. B) NF-κB p65 protein nuclear translocation was significantly increased after 40, 60 min and 2 h with PALM treatment. Data are expressed as nuclear NF-κB p65 protein normalized to the loading control, fibrillarin. Mean ± S.E.M. from three independent experiments are shown. *\(P < 0.05\) by paired Student’s \(t\)-test versus Control treatment.

Fig. 4. NF-κB p65 protein nuclear translocation is increased with palmitate and LPS treatments compared to lipogenic treatment. HepaRG cells treated with maintenance media (Con), lipogenic treatment (LIPO; 10 \(\mu\)M rosiglitazone, 250 \(\mu\)M isobutimethylxanthine, 10 \(\mu\)M insulin), palmitate (PALM; 400 \(\mu\)M), palmitate and LPS (PALM +LPS; 400 \(\mu\)M palmitate and 100 ng/mL LPS) or lipopolysaccharide (LPS; 100 ng/mL) and harvested after 60 min treatment. A) Cytosolic and nuclear protein fractions were probed of NF-κBp65 protein levels by immunoblot. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and fibrillarin were used as loading controls for cytoplasmic and nuclear controls, respectively. B) Levels of NF-κB p65 protein in cytosolic extracts were comparable (\(P > 0.05\)) by paired \(t\)-test). C) NF-κBp65 protein nuclear translocation is increased with PALM (**\(P < 0.01\)), PALM + LPS (*\(P < 0.05\)) and LPS (*\(P < 0.05\)) by paired \(t\)-test versus LIPO. Data are expressed as fold change relative to control treatment and are means ± S.E.M. from three independent experiments.

Fig. 5. Palmitate and LPS induction of NF-κB p65 activity is partially attenuated by inhibition of TLR4. A) HepaRG cells were co-transfected with an NF-κB luc reporter plasmid and a Renilla luciferase control plasmid for 24 hours. Cells were then treated with maintenance media (Control), lipogenic treatment (LIPO), palmitate (PALM; 400 \(\mu\)M) or lipopolysaccharide (LPS; 100 ng/mL) for 2 h and luciferase activity was measured. Results represent the means ± S.E.M. from 12 independent experiments. **\(P < 0.01\), ***\(P < 0.001\) by one-way ANOVA versus LIPO treatment. B) Quantitative RT-PCR-mediated detection of TLR4 in HepaRG cells transfected with scrambled siRNA and TLR4 siRNA. N=4 per group. C) TLR4 siRNA or scrambled control siRNA were co-
transfected into cells, along with NF-κB luc reporter plasmid and a Renilla luciferase control plasmid for 24 h. Cells were then treated with maintenance media (Control), LIPO, PALM (400 μM palmitate) or LPS (100 ng/mL) for 2 h and cells were harvested and measured of luciferase activity. Results represent the means ± SEM from 8 independent experiments. *P < 0.05 by paired t-test versus control siRNA-treated equivalent. D) Cells were co-transfected with NF-κB luc reporter plasmid and a Renilla luciferase control plasmid as described above. At 18 h of transfection, a TLR4 chemical inhibitor was added to cells for 6 h. At 24 h after transfection, cells were treated with maintenance media (Control), LIPO, PALM or LPS for 2 h and cells were harvested and analyzed to measure luciferase activity. Results represent the means ± SEM from 8 independent experiments. *P < 0.05, **P < 0.01 by paired t-test versus no inhibitor treatment. All data is expressed as fold change relative to the Control treatment.

Fig. 6. Transcription of TLR4 and its downstream mediators are up-regulated by palmitate and LPS. HepaRG cells were treated with maintenance media (Control), lipogenic treatment (LIPO), palmitate (PALM; 400 μM palmitate) or lipopolysaccharide (LPS; 100 ng/mL) for 4 hours. The transcription of genes was determined by quantitative RT-PCR. After normalization to β-Actin, relative mRNA levels were expressed as fold change relative to treatment control, which was defined as 1. A) TLR4 levels were up-regulated with PALM and LPS treatments. *P < 0.05, **P < 0.01 versus LIPO treatment by paired t-test. B) mRNA of TRIF is up-regulated with PALM and LPS treatment. *P < 0.05, **P < 0.01 versus LIPO treatment by paired t-test. C) mRNA of MyD88 is up-regulated with PALM and LPS treatment. *P < 0.05, versus LIPO treatment by paired t-tests. Mean ± S.E.M. from 3 independent experiments.

Fig. 7. The effects of LPS and palmitate on primary human hepatocytes from a lean and obese donor are dependent upon TLR4 activation. A and B) Administration of a TLR4 inhibitor (CLI-095) for 6 h prior to 1 h lipopolysaccharide (LPS; 100 ng/mL), palmitate (400 μM) or LPS + palmitate stimulation inhibits p65 nuclear translocation in a lean (body mass index [BMI] of 18.1 kg/m²) but not an obese (BMI of 30 kg/m²) donor.
Fig. 1. Plasma endotoxin and FFAs levels were measured in Class III obese subjects with nonalcoholic fatty liver (NAFL) (n=16) nonalcoholic steatohepatitis (NASH; n = 20) and compared with weigh-matched controls with normal liver histology (Obese, n = 18). A. NASH subjects had significantly elevated (*P < 0.05) plasma FFA levels compared to obese subjects with normal histology. B. Plasma LPS was elevated in the NASH subjects relative to subjects with NAFL (*P < 0.05). Data is represented as mean ± S.E.M. *P < 0.05 by one-way ANOVA with Dunn’s post-test.
Fig. 2. Transcription of TLR4 and its downstream mediators is up-regulated in patients with NASH compared to those with NAFL. Liver mRNA expression levels normalized to β-Actin were determined by quantitative RT-PCR in subjects with normal liver (Obese; n = 15), nonalcoholic fatty liver (NAFL; n = 9) or nonalcoholic steatohepatitis (NASH; n = 21). A) Toll-like receptor 4 (TLR4) mRNA levels in NAFL were significantly reduced compared to Obese controls and subjects with NASH (**P < 0.01 for both). mRNA levels of B) myeloid differentiation factor 88 (MyD88), C) toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF), D) interferon regulatory factor 7 (IRF7), E) interferon regulatory factor 3 (IRF3) and F) toll-like receptor 5 (TLR5) in Obese, NAFL and NASH subjects. Data are expressed as mean ± S.E.M. *P < 0.05, **P < 0.01 by one-way ANOVA with Dunn’s post-test.
Fig. 3. NF-κB p65 protein nuclear translocation increases with palmitate exposure. HepaRG cells were untreated (Control) or treated with palmitate (PALM; 400 μM) and harvested after 1, 5, 10, 20, 40, 60 min or 2 and 4 h. A) Cytosolic and nuclear protein fractions were probed for NF-κB p65 protein levels via quantitative immunoblot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and fibrillarin were used as loading controls for cytoplasmic and nuclear fractions, respectively. B) NF-κB p65 protein nuclear translocation was significantly increased after 40, 60 min and 2 h with PALM treatment. Data are expressed as nuclear NF-κB p65 protein normalized to the loading control, fibrillarin. Mean ± S.E.M. from three independent experiments are shown. *P < 0.05 by paired Student’s t-test versus Control treatment.
Fig. 4. NF-κB p65 protein nuclear translocation is increased with palmitate and LPS treatments compared to lipogenic treatment. HepaRG cells treated with maintenance media (Con), lipogenic treatment (LIPO; 10 μM rosiglitazone, 250 μM isobutylmethylxanthine, 10 μM insulin), palmitate (PALM; 400 μM), palmitate and LPS (PALM + LPS; 400 μM palmitate and 100 ng/mL LPS) or lipopolysaccharide (LPS; 100 ng/mL) and harvested after 60 min treatment. A) Cytosolic and nuclear protein fractions were probed of NF-κBp65 protein levels by immunoblot. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and fibrillarin were used as loading controls for cytoplasmic and nuclear controls, respectively. B) Levels of NF-κB p65 protein in cytosolic extracts were comparable (P > 0.05 by paired t-test). C) NF-κB p65 protein nuclear translocation is increased with PALM (**P < 0.01), PALM + LPS (*P < 0.05) and LPS (*P < 0.05) by paired t-test versus LIPO. Data are expressed as fold change relative to control treatment and are means ± S.E.M. from three independent experiments.
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B) Quantitative RT-PCR-mediated detection of TLR4 in HepaRG cells transfected with scrambled siRNA and TLR4 siRNA. N=4 per group.

C) TLR4 siRNA or scrambled control siRNA were co-transfected into cells, along with NF-κB luc reporter plasmid and a Renilla luciferase control plasmid for 24 h. Cells were then treated with maintenance media (Control), LIPO, PALM (400 μM palmitate) or LPS (100 ng/mL) for 2 h and cells were harvested and measured of luciferase activity. Results represent the means ± SEM from 8 independent experiments. *P < 0.05 by paired t-test versus control siRNA-treated equivalent.

D) Cells were co-transfected with NF-κB luc reporter plasmid and a Renilla luciferase control plasmid as described above. At 18 h of transfection, a TLR4 chemical inhibitor was added to cells for 6 h. At 24 h after transfection, cells were treated with maintenance media (Control), LIPO, PALM or LPS for 2 h and cells were harvested and analyzed to measure luciferase activity. Results represent the means ± SEM from 8 independent experiments. *P < 0.05, **P < 0.01 by paired t-test versus no inhibitor treatment. All data is expressed as fold change relative to the Control treatment.
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Fig. 7. The effects of LPS and palmitate on primary human hepatocytes from a lean and obese donor are dependent upon TLR4 activation. 

A and B) Administration of a TLR4 inhibitor (CLI-095) for 6 h prior to 1 h lipopolysaccharide (LPS; 100 ng/mL), palmitate (400 μM) or LPS + palmitate stimulation inhibits p65 nuclear translocation in a lean (body mass index [BMI] of 18.1 kg/m²) but not an obese (BMI of 30 kg/m²) donor.
Table 1: Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Obese</th>
<th>NAFL</th>
<th>NASH</th>
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<tbody>
<tr>
<td>Mean age (years)</td>
<td>38.3 ± 1.4</td>
<td>38.6 ± 2.7</td>
<td>44.4 ± 2.0</td>
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<tr>
<td>Caucasian/African American/Hispanic (n)</td>
<td>16/3/1</td>
<td>11/3/0</td>
<td>25/2/0</td>
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<tr>
<td>Weight (kg)</td>
<td>126.6 ± 9.6</td>
<td>117.8 ± 4.5</td>
<td>130.2 ± 6.7</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>44.0 ± 1.7</td>
<td>44.6 ± 1.7</td>
<td>46.2 ± 1.6</td>
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<td>Glucose (mg/dl)</td>
<td>93.2 ± 4.5</td>
<td>98.6 ± 4.3</td>
<td>103.8 ± 2.4*</td>
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<td>AST (normal range: 5-40 U/l)</td>
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<td>24.4 ± 4.0</td>
<td>31.7 ± 6.0</td>
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<tr>
<td>ALT (normal range: 5-40 U/l)</td>
<td>21.9 ± 2.5</td>
<td>30.6 ± 5.9</td>
<td>37.1 ± 6.5</td>
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<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>69.9 ± 6.6</td>
<td>85.5 ± 9.4</td>
<td>94.9 ± 7.4</td>
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<td>Total bilirubin (mg/dl)</td>
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<td>0.5 ± 0.5</td>
<td>0.7 ± 0.1</td>
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<td>Albumin (g/dl)</td>
<td>4.2 ± 0.1</td>
<td>4.1 ± 0.07</td>
<td>4.1 ± 0.08</td>
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<td>Platelets (1000/mm³)</td>
<td>281.5 ± 16.8</td>
<td>259.5 ± 15.3</td>
<td>260.7 ± 10.7</td>
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<td>Fibrosis (0-4)</td>
<td>0.06 ± 0.06</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1*</td>
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<tr>
<td>NAFLD Activity Score (0-8)</td>
<td>0.3 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>4.9 ± 0.3**</td>
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*P < 0.05 compared to NAFL. **P < 0.05 compared to Obese or NAFL. Data were compared across groups by Kruskal-Wallis analysis of variance with a Dunn's post-test for multiple comparisons.
Table 2: Primers used for Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
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<tr>
<td>β-actin forward</td>
<td>5'-AGGCCAGAGCAAGAGAGGTA-3'</td>
</tr>
<tr>
<td>β-actin reverse</td>
<td>5'-GGGGTGTTGAAGGTCTCAAACA-3'</td>
</tr>
<tr>
<td>TLR4 forward</td>
<td>5'-GGCCATTGCTGCAACAT-3'</td>
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<tr>
<td>TLR4 reverse</td>
<td>5'-CAACAATCACCTTGCGTTTT-3'</td>
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<tr>
<td>MyD88 forward</td>
<td>5'-TCGATGCCTTCATCTGATTG-3'</td>
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<tr>
<td>MyD88 reverse</td>
<td>5'-CGGATCATCTCCTGCACAAA-3'</td>
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<tr>
<td>IRF3 forward</td>
<td>5'-TTCCCGGGAGGATAAGC-3'</td>
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<td>TLR5 forward</td>
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<td>TLR5 reverse</td>
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<td>TRIF forward</td>
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<tr>
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