Atherogenic diet-induced hepatitis is partially dependent on murine TLR4

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Abstract: Diets high in cholesterol and cholate such as the Paigen diet have been used to study athergenesis, lithogenesis, and proinflammatory microvascular changes induced by nutritional hypercholesterolemia. Although these diets lead to chronic hepatic inflammation and fibrosis, the early inflammatory changes have been poorly characterized. TLR4, a known receptor for LPS, is also a receptor for a variety of endogenous ligands and has been implicated in atheroma formation. Here, we specifically examined the early inflammatory response of the liver to the atherogenic (ATH) diet and the possible contribution of TLR4. Animals fed the high-cholesterol/cholate diet for 3 weeks developed a significant, predominantly mononuclear leukocyte infiltration in the liver, hepatic steatosis, elevated hepatic expression of MCP-1, RANTES, and MIP-2, and increased serum levels of liver enzymes. In TLR4-deleted animals, there was a 30% attenuation in the serum alanine transaminase levels and a 50% reduction in the leukocyte infiltration with a fourfold reduction in chemokine expression. In contrast, hepatic steatosis did not differ from wild-type controls. TLR2 deletion had no effect on diet-induced hepatitis but increased the amount of steatosis. We conclude that the early inflammatory liver injury but not hepatic lipid loading induced by the ATH diet in mice is mediated in part by TLR4. J. Leukoc. Biol. 83: 1336–1344; 2008.

Key Words: cholesterol/cholate diet · inflammation · TLR2 · chemokines

INTRODUCTION

A diet high in cholesterol (1.25%), fat (15%), and cholate (0.5%) described by Paigen and co-workers [1] has been used to induce proinflammatory changes in the microvasculature [2], increase production of reactive oxygen species [3], elevate expression of adhesion molecules in the endothelial cells [4], enhance adherence and emigration of granulocytes [5], enhance T cell-mediated release of the proinflammatory cytokine IFN-γ [6], and increase the platelet–leukocyte interaction [7]. In addition, this diet has been shown to induce nutritional hypercholesterolemia, athergenesis [8], and cholesterol gallstone disease in inbred mouse strains [9, 10]. Three weeks diet-fed mice demonstrate increased plasma cholesterol, low-density lipoprotein (LDL)/very LDL cholesterol, nonesterified cholesterol, and reduced high-density lipoprotein cholesterol compared with chow-fed animals [11].

In rats, this diet results in lipid-laden hepatic parenchymal cells and Kupffer cells [12], mild hepatic fibrosis [13], and oxidative hepatocellular injury [14, 15]. At the molecular level, this diet at 5–15 weeks has been shown to activate hepatic NF-κB and induce mRNA for the mouse homologue of MCP-1 (CCL2), colony-stimulating factors, heme oxygenase, and members of the serum amyloid A family in the liver. A correlation was also reported between hepatic inflammatory gene induction and susceptibility to fatty-streak development [16]. However, there has been little characterization of the early stages of hepatic inflammation. The liver plays a critical role in the metabolism of cholesterol [17, 18] and bile acids [19], which influence athergenesis [20, 21] and lithogenesis [22].

TLR4, a transmembrane protein, is known to activate proinflammatory pathways in response to LPS, leading to production of inflammatory cytokines in various tissues including the liver [23]. It is also clear that endogenous ligands exist for TLR4. It is thought that these endogenous substances are responsible for instituting the host innate immune response during noninfectious stress events [24, 25]. It has also been shown that dietary hypercholesterolemia sensitizes the liver to endotoxemia, and it has been postulated that overexpression of hepatic TLR4 may contribute to the observed phenomenon [26]. As such, TLR4 has been implicated in the process of atheroma generation [27–30]. Recently, there has also been literature supporting a role for TLR2 in athergenesis [31, 32]. As liver-mediated processes along with hypercholesterolemia are critical for atheroma generation, we assessed the possible contributions of TLR4 and TLR2 to the early liver injury associated with diet-induced hypercholesterolemia.
MATERIALS AND METHODS

Animals

Male mice, 8–10 weeks old, 25–28 g, were used for all of the experiments. C57BL/6J (strain #664) mice were used to establish the model. For evaluating the role of TLR4, C57BL/10SNJ-TLR4-deleted (TLR4<sup>-/-</sup>) mice were raised in our animal facility from homozygous mating (strain #5732). C57BL/10SnJ (strain #866) were the controls for the TLR4<sup>-/-</sup> strain (Jackson Laboratories, Bar Harbor, ME, USA). For the TLR2 experiments, heterozygous, TLR2-deficient mice were backcrossed six generations on a C57BL/6NHsd (Harlan Sprague Dawley Inc., Indianapolis, IN, USA) background. After genotyping using the appropriate primers, 8–10-week-old male, TLR2<sup>-/-</sup> mice were used with age- and gender-matched C57BL/6NHsd controls (Harlan Sprague Dawley Inc.). Mice were housed in a room with a 12-h light cycle and had free access to food and water. The Institutional Animal Care and Use Committee at Baylor College of Medicine (Houston, TX, USA) approved all protocols.

Diets

Mice were fed an atherogenic (ATH) “Paigen” diet [1.25% (w/w) cholesterol, 0.5% (w/w) cholic acid (CA), and 16% (w/w) fats in the form of soybean oil, cocoa butter, and coconut oil] or isocaloric (ISO) control chow (0.3% w/w cholesterol, no CA, and 5% w/w fats) for 3 weeks for each experiment. Both diets were obtained from Research Diets Inc. (New Brunswick, NJ, USA). They were irradiated and stored per recommendations from the manufacturer. Feeds were weighed and changed twice a week, and animals were weighed each week to monitor weight gain. Rodent chow supplemented with 0.5% CA was specially formulated and ordered separately from Harlan (Harlan Teklad, Madison, WI, USA) for separate sets of experiments to evaluate effects of dietary cholate.

Experimental groups

Four sets of experiments were performed. 1) To study the effects of diet on liver inflammation, C57BL/6J mice were assigned to an ATH diet or ISO chow group. After 3 weeks of diet, animals were anesthetized, blood was collected from vena cava, and the animals were killed. Liver was obtained for further analysis. Animals were analyzed in two groups: ISO (n = 4) and ATH (n = 5). 2) To study the effects of cholate supplementation on liver inflammation, C57BL/6J mice were assigned to cholate supplemented or standard rodent chow. After 3 weeks of diet, animals were anesthetized, blood was collected from vena cava, and the animals were killed. Liver was obtained for further analysis. Animals were analyzed in two groups: chow (n = 4) and CA-supplemented chow (n = 5). 3) To assess the role of TLR4, TLR4<sup>-/-</sup> (C57BL/10SNJ) mice and their wild-type (WT) controls (C57BL/10SnJ) were randomly assigned to an ATH diet or ISO chow for a duration of 3 weeks. Mice were fasted for 6 h prior to sacrifice. Results were analyzed according to their respective groups: WT + ISO (n = 5); WT + ATH (n = 6); TLR4<sup>+/−</sup> + ISO (n = 5); and TLR4<sup>−/−</sup> + ATH (n = 6). 4) For TLR2 experiments, TLR2<sup>−/−</sup> mice and their WT controls (C57BL/6Nhsd) were randomly assigned to ATH or ISO chow for a duration of 3 weeks. Mice were fasted for 6 h prior to sacrifice. Results were analyzed according to their respective groups: WT + ISO (n = 4); WT + ATH (n = 6); TLR2<sup>−/−</sup> + ISO (n = 4); and TLR2<sup>−/−</sup> + ATH (n = 6).

Serum alanine transaminase (ALT) activity and cholesterol levels

Nonhemolyzed serum from blood samples collected from the vena cava was used for determination of ALT activity and cholesterol levels using a kinetic spectrophotometric assay (Thermo Electron, Louisivlle, CO, USA).

Portal venous endotoxin measurement

C57BL/6J mice (n = 12) were separately fed an ATH diet (n = 8) or ISO chow (n = 4) for 3 weeks. Blood was collected from the portal vein prior to sacrifice. Platelet-rich plasma fraction was isolated from pooled, heparinized blood samples, which were prepared as described previously [33], and endotoxin was detected using a kinetic chromogenic assay using the kinetic QCL kit (Cambrex, Walkersville, MD, USA).

Histology

Sections of liver preserved in formalin-free zinc fixative (BD PharMingen, San Diego, CA, USA) were embedded in paraaffin, sectioned, and stained with H&E. To demonstrate hepatic lipid accumulation, additional sections of liver were embedded in OCT and frozen at −80°C and subsequently stained with Oil Red-O (Sigma Chemical Co., St. Louis, MO, USA).

Immunohistochemistry

Immunohistochemistry was performed on 4 μm-thick sections of paraaffin-embedded liver tissue. The following were used: Pan-leukocyte marker, anti-mouse CD45 (leukocyte common antigen, Ly-5, clone 30-F11, BD PharMingen) at 1:20 dilution; rat anti-mouse F4/80 for monocyte/macrophages including kupffer cells (Serotec, Raleigh, NC, USA) at a dilution of 1:50; and rat anti-mouse neutrophil antibody (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody at a dilution of 1:200. Brown staining of target cells was obtained by using a working solution of 3,3′-diaminobenzidine substrate (Vector Laboratories). Liver was counterstained with Gel’s hematoxylin (Fisher Diagnostics, Middletown, VA, USA) and visualized directly under 10× and 60× magnification under light microscope (Leitz, Germany) and evaluated using a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The contribution of platelets was evaluated by staining paraaffin-embedded liver sections (4 μm-thick) with anti-mouse, CD41-PE labeled antibody (BD PharMingen; 1:100; clone MWreg30) and mounting in Arirling (Celenase, Ltd., Dallas, TX, USA) containing 1 μmol/L 4′,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.). Slides were counterstained with DAPI to assess nuclear morphology. Sections were examined by immunofluorescence (Delta Vision, Applied Precision, Issaquah, WA, USA).

Quantification of inflammatory cells

Ten high-power fields (60×) per 4 μm-thick section of stained liver per animal were randomly selected (http://www.randomizer.org). The level of inflammation was assessed morphometrically as the volume percent of hepatic parenchyma comprised of the inflammatory cells (percent volume) per high-power field using an unbiased, stereological technique described by Howard and Reed [34]. To derive an unbiased estimate of the volume fraction (percent surface area per 4 μm-thick section) of hepatic tissue composed of the cell of interest, a cycloid grid (ref. [34], C-1, Page 210, Appendix B) was randomly positioned on the field, and the number of points of the grid hitting the nucleus of the stained inflammatory cell divided by the number of points hitting the hepatic parenchyma (including the sinusoids) gave an unbiased estimate of volume fraction (volume percent). Volume fraction (percent volume) = P(Y|P) (ref. Where P(Y) is the number of points on the grid hitting the nuclei of interest, and P (ref) is the total number of points hitting the hepatic parenchyma (including the sinusoids) per high-power field. Care was taken not to count the cells in the areas of the central vein, the portal vein, and the hepatic artery.

Hepatic lipid extraction and analysis

The Bligh-Dyer lipid extraction method was used to quantify the amount of lipids in the liver tissue [35]. Briefly, 0.4 g liver in 1 ml PBS was mechanically disrupted in a sonicator, and then 0.5 ml chloroform and 1 ml methanol (1:20) were added and vortexed vigorously for 1 min. Chloroform (0.5 ml) was added again and vortexed for 1 min. The mixture was centrifuged at 3000 rpm 5 min to separate the phases. The bottom (organic lipid) phase was transferred to preweighed glass tubes and dried in a hood for 48 h. The dried lipid layer was weighed and was reported in mg of lipids per gram of liver tissue. Methyl esters of the total lipid fraction were prepared with boron trifluoride-methanol as described earlier [36] and quantified by gas liquid chromatography (Hewlett-Packard 5890 gas chromatograph) on a DB-225 capillary column (J & W Scientific, Folsom, CA, USA). Fatty acids were identified by comparison with the retention times of fatty acid methyl ester standards.

IFN-γ and IL-6 levels in liver tissue lysate

Quantitative assessment of liver tissue IFN-γ and IL-6 content was done using ELISA kits specific for tissue lysate (Ray-Biotech, Inc., Norcross, GA, USA). Liver tissue lysate was prepared per kit instructions. Protein levels in the
Serum cholesterol levels were increased significantly in the C57BL6/J mice fed the ATH diet for 3 weeks compared with animals fed ISO chow (180.5±14.85 mg/dl vs. 128.5±5.5 mg/dl in C57BL6/J; \( P < 0.05 \)). Leukocyte infiltrates were evident in the periportal area in the animals on the ATH diet, compared with the animals on an ISO diet (Fig. 1, A–B). The majority of these cells in the liver of animals on the ATH diet was F4/80+, consistent with increases in monocyte/macrophage and Kupffer cells. Neutrophil influx was also seen (Fig. 1E). Oil Red-O staining revealed microvesicular steatosis with lipid deposition in the hepatocytes (Fig. 1, C–D). Serum ALT (Fig. 1E) levels were increased in C57BL6/J after 3 weeks on the ATH diet, consistent with diet-mediated liver injury. These results were reproduced in the C57BL10/SnJ mice, which were separately used as WT controls for the TLR4del animals (Figs. 2a and 3). Cholate added to the chow diet alone for 3 weeks also induced mild liver elevation of the liver enzymes (serum ALT levels: 147±33 \( \mu \)L vs. 36±3 \( \mu \)L; \( P = 0.028 \); unpaired \( t \)-test) and a modest increase in leukocytes (percent volume of CD45+ cells: 1.4±0.25 vs. 0.4±0.05; \( P = 0.024 \)) when compared with standard rodent chow-fed C57BL/6J animals. It should be noted that this amount of injury and inflammation alone was ~50% of that seen in animals eating the ATH diet for 3 weeks (Fig. 1E). However, unlike those animals, animals whose diet was supplemented with cholate alone had no evidence of hepatic lipid loading [lipid levels: 17.50±2.44 mg/g liver in cholate-fed vs. 21.65±1.144 mg/g liver in chow-fed; \( P = \) not significant (ns)].
There was no evidence of portal venous endotoxin in C57BL/6J mice, irrespective of their diet, as measured by kinetic chromogenic assay (data not shown). TLR4 deletion attenuates the inflammatory response and hepatic injury in mice on an ATH diet.

TLR4<sup>del</sup> animals fed the ATH diet had attenuation of the serum ALT levels by ~30% compared with their WT C57BL10/SnJ controls (186±22 μL vs. 247±18 μL; P<0.05; Fig. 2a). There were also 50% fewer CD45<sup>+</sup> cells (Fig. 2b), F4/80<sup>+</sup> cells, and neutrophils in the ATH diet-fed TLR4<sup>del</sup> animals compared with their ATH diet-fed WT controls (Fig. 3). TLR4<sup>del</sup> animals fed an ATH diet showed a small but significant (fourfold) increase in the F4/80<sup>+</sup> cells compared with the ISO-fed groups. The number of CD45<sup>+</sup> cells and polymorphonuclear cells in the TLR4<sup>del</sup> mice fed an ATH diet was also increased (threecold) compared with the ISO chow-fed groups but did not reach statistical significance (Figs. 2b and 3).

ATH-fed mice showed significantly more platelets in their liver (mostly in the sinusoidal spaces) than the ISO-fed mice (percent volume CD41<sup>+</sup> cells: 6.1±0.9% vs. 12.9±0.4%; P<0.02 by t-test). There was, however, a relative paucity of platelets in the inflammatory foci in the livers of the WT ATH-fed animals (Fig. 4). The deletion of TLR4 did not appear to change the amount of platelets compared with WT animals (percent volume CD41<sup>+</sup> cells: 12.7±0.3% vs. 12.9±0.4%; P=ns).

There was no increase in hepatic IL-6 and IFN-γ protein expression in control or TLR4<sup>del</sup> mice, irrespective of ATH or chow diet (data not shown).

TLR4 deletion-attenuated expression of MCP-1, RANTES, and MIP-2

Hepatic mRNA levels for MCP-1 (CCL2), ICAM-1 (CD54), RANTES (CCL5), MIP-2 (CCL2), lymphotactin (XCL1), eotaxin (CCL11), TCA-3 (CCL1), and IP-10 (CXCL10) were increased in the livers of WT (C57BL10/SnJ) mice after feed-
staining, and lipid extraction by the Bligh-Dyer method were similar in the WT and TLR4^{del} groups fed the ATH diet (Fig. 2c).

As nutritional saturated fatty acids, especially lauric acid (C12:0), has been implicated as a ligand for TLR4 signaling [38, 39], and the Paigen diet has a high content of lauric acid and saturated fats (information from Research Diets Inc.), we analyzed the fatty acid methyl esters from lipid extracts in each group (n=5–6) by gas chromatography. We found statistically higher content of lauric acid in the livers of mice fed the ATH diet, irrespective of the presence or absence of TLR4 (Fig. 6). The hepatic concentrations of palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2,n-6), α-linolenic acid (C18:3,n-3), and γ-linolenic acid (C18:3,n-6) were significantly higher in ATH diet-fed livers than their chow-fed counterparts (Fig. 6). The hepatic contents of myristic acid (C14:0), palmitic (C16:0), stearic (C18:0), arachidic (C20:0), behenic (C22:0), arachidonic (C20:4), eicosapentaenoic, and docosahexaenoic acids were similar among all groups, irrespective of diet or TLR4 presence (data not shown). Except for linoleic (C18:2,n-6), which was found to be higher in TLR4^{del} ATH-fed mice, the fatty acid profile of the WT and the TLR4^{del} mice was similar within their diet groups (Fig. 6).

**TLR2 deletion did not attenuate the inflammatory response or liver injury in ATH diet-fed animals**

The TLR2^{del} mice and their WT controls, C57BL/6N Hsd (Harlan strain), showed evidence of liver injury (ALT), inflammation (CD45^{+} cells), hepatic steatosis, and hypercholesterolemia when compared with their chow-fed counterparts (Fig. 7). There was, however, no statistical difference among the serum cholesterol levels (243.6±14.8 mg/dl vs. 225.3±17.3 mg/dl; P=ns), ALT levels, and the volume percent of hepatic CD45^{+} cells between the WT and the TLR2^{del}, diet-fed groups (Fig. 7). On Bligh-Dyer analysis, livers of diet-fed, TLR2^{del} mice showed a significantly higher lipid content as compared with the diet-fed WT mice (Fig. 7).

**DISCUSSION**

In this paper, we demonstrate that microvesicular steatohepatitis occurs within weeks of feeding Paigen’s ATH diet. There is a five- to sevenfold increase in the percent of the hepatic parenchyma of infiltrating neutrophils and over a tenfold increase in the F4/80^{+} cells in those animals fed a high-cholesterol/cholate diet. In addition, we show that the leuko-
cyte infiltrate is primarily a result of monocyte/macrophages, although we could not rule out that there was also an increase in the number of Kupffer cells. There was also an increase in the number of platelets found, primarily in the hepatic sinusoids, in those animals fed the ATH diet. It is important to highlight that in this study, we excluded cells that were in the lumen of central or portal veins and arteries; thus, our counts more accurately reflect cells in the hepatic architecture itself.

Mechanistically, the inflammatory changes in the liver are primarily a result of the downstream effects of TLR4 activation, as TLR4\(^{\text{del}}\) animals had an abrogation of leukocyte influx and inhibition of inflammatory mediators including MCP-1, RAN-

![Fig. 5. RPA analysis of proinflammatory chemokines.](image)

**Fig. 5.** RPA analysis of proinflammatory chemokines. (Left) Gene expression of lymphotactin (XCL1), RANTES (CCL5), eotaxin (CCL11), MIP-2 (CXCL2), IP-10 (CXCL10), and TCA-3 (CCL1) in the livers of WT and TLR4\(^{\text{del}}\) ATH-fed mice. Signals were compared with large ribosomal (L32) mRNA content. (Right) Bar graphs show relative intensities of RANTES and MIP-2 compared with L32; *, \(P < 0.02\), versus TLR4\(^{\text{del}}\) mice using Mann-Whitney rank sum analysis.

![Fig. 6. Quantification of hepatic fatty acids.](image)

**Fig. 6.** Quantification of hepatic fatty acids. After 3 weeks of feeding ISO or ATH diets in WT and TLR4\(^{\text{del}}\), fatty acid content was determined as described in Materials and Methods. Results reported as nanomoles per gram of liver; means \(\pm\) SEM (\(n = 5–6\) per group); *, \(P < 0.05\), versus ISO-fed groups; \#, \(P < 0.05\), versus TLR4\(^{\text{del}}\) ATH.
versus WT/H11001 means an ATH diet or ISO chow. Results are WT controls at the end of 3 weeks of feeding an ATH diet or ISO chow. Analysis of serum MCP-1, RANTES, and MIP-2. This in turn leads to monocyte and to a hepatocytes/Kupffer/stellate cells results in increased MCP-1, which cell/cells may have been affected by TLR4 deletion. We hypothesize that signaling through TLR4 on early changes that occur in the microvasculature with the Paigen diet. We have further demonstrated the result of the APO-E defect. The current study is the first demonstration of the role of TLR4 in the hepatic response to an ATH diet.

TLR4 is expressed on hepatocytes, stellate cells, Kupffer cells, sinusoidal endothelial cells, dendritic cells, as well as bile-duct epithelium [40]. Thus, it is not possible to delineate which cell/cells may have been affected by TLR4 deletion. We verified previous observations [41, 42] that MCP-1, RANTES, and ICAM-1 were significantly up-regulated upon feeding the Paigen diet for 3 weeks. We have further demonstrated the up-regulation of other proinflammatory chemokines, such as lymphotoxin, eotaxin, MIP-2, IP-10, and TCA-3 with 3 weeks of diet, which contribute to the inflammatory cell infiltrates and subsequent liver injury. MCP-1 and RANTES are expressed by all the cells in the liver, especially the Kupffer cells, and are shown to be up-regulated by inflammatory stimuli [43]. MCP-1 and RANTES are critical to the recruitment of monocytes, T lymphocytes, and NK cells [44]. We did not specifically stain for T lymphocytes in the liver. However, work by Stokes et al. [6] has shown that T lymphocytes may be responsible for the early changes that occur in the microvasculature with the Paigen diet. We hypothesize that signaling through TLR4 on hepatocytes/Kupffer/stellate cells results in increased MCP-1, RANTES, and MIP-2. This in turn leads to monocyte and to a lesser extent, neutrophilic infiltrate, resulting in inflammation and injury, which eventually lead to chronic inflammation and fibrosis.

The Paigen diet is complex. Cholesterol, cholate, and the fat components each have a unique role to play in the inflammatory process [11]. It is not possible to delineate in this experiment the ligand for TLR4, which initiates the inflammatory events. We did not detect portal venous endotoxin in the ATH diet-fed mice. However, this does not rule out the possibility of other bacterial translocation products, which could be ligands for TLR4, or amount of endotoxin, which could not be detected. It is clear that oxidized LDL is a ligand for TLR4 [27, 30], and it has been reported that this ATH diet, when fed to mice, induced the hepatic formation of oxidized phospholipids responsible for biological activity of mildly oxidized LDL [45, 46]. Lauric acid (C12:0), a saturated fatty acid also found in LPS, is a known ligand for TLR4 [38]. Lauric acid was present in the ATH diet used here, although the relative concentration was minimal compared with the rest of the lipid content. We found increased hepatic content of lauric acid in the ATH diet-fed animals, and this was not different between the WT compared with the TLR4del mice. However, to signal through TLR4, we believe that increased serum/hepatic levels of lauric acid would need to be present. Cholic acid has also been shown to increase hepatic mRNA levels of ICAM-1, VCAM-1, and TNF-α [47] and contribute to liver injury, although this effect has not been shown to be mediated through TLR4 [48]. We have demonstrated hepatitis but not steatosis by cholate supplementation alone. The inflammatory response and injury seen with cholate supplementation were approximately half of that seen with the ATH diet. This suggests that the fat and cholesterol components also account for the observed response separately, as suggested by the study done by Vergnes et al. [11], or together with the presence of cholate. This effect may be ameliorated by the absence of TLR4.

As TLR2 has also been implicated in cholesterol, fat metabolism, and atherogenesis [31, 32], we evaluated the role of TLR2 in this model. Our results demonstrate that TLR2 deletion did not afford hepatic protection, and there is increased hepatic lipid content after 3 weeks of diet. The increase in steatosis seen in the TLR2del mice is novel. Few studies have been done examining the effects of TLR2 on steatosis. Recently, it has been demonstrated that CD36 (fatty acid trans-
porter protein) acts as a facilitator or a coreceptor for diacylglyceride recognition through the TLR2/6 complex [49]. It is this CD36–TLR2 interaction that has been proposed as a mechanism, whereby an endogenous lipid ligand can facilitate TLR2 signaling in atherosclerosis [32]. CD36 also plays an important role in lipid metabolism and regulates fatty acid flux among the muscle, adipose tissue, and liver. Lack of CD36 causes increased fatty acid delivery to the liver and results in steatosis [50–52]. We speculate that global deletion of TLR2 probably alters the CD36–TLR2 interaction, leading to increased flux of free fatty acids to the liver, thereby leading to steatosis.

We emphasize that there were strain differences in C57BL/6NHsd (Harlan) used in TLR2 experiments and C57BL6 and C57BL/10SnJ (Jackson strain) used in S2c and TLR4 experiments. Although the diet was the same, the C57BL/10SnJ (Jackson strain) used in establishing the model and C57BL/6NHsd (Harlan) used in TLR2 experiments and C57BL6 and C57BL/6NHsd (Harlan) and highlights the critical role of selection of control animals.

In conclusion, our studies show for the first time that TLR4 but not TLR2 is responsible in part for the hepatitis seen with short-term feeding of a high-cholesterol/high-fat/CA-containing ATH diet. TLR4 deletion did not affect hepatic lipid loading or the fatty acid profile. Although CA feeding leads to mild liver injury, it does not affect lipid accumulation and is insufficient to produce steatohepatitis.

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