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 atherogenesis, 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: 000–000; 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, atherogenesis [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 atherogenesis [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 atherogenesis [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.

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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 the evaluation of TLR4, C57BL/10SNJ TLR4-deleted (TLR4Δ) mice were raised in our animal facility from homozygous mating (strain #5752). C57BL/10SNJ (strain #866) were the controls for the TLR4Δ 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, TLR2Δ 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 (0.5% 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/6 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/6 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, TLR4Δ (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); TLR4Δ + ISO (n = 5); and TLR4Δ + ATH (n = 6). For TLR2 experiments, TLR2Δ 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); TLR2Δ + ISO (n = 4); and TLR2Δ + ATH (n = 6).

Serum alanine transaminase (ALT) activity and cholesterol levels
Nonhemolyzed serum from blood samples collected from vena cava was used for determination of ALT activity and serum cholesterol levels using a kinetic spectrophotometric assay (Thermo Electronic, Louisville, CO, USA).

Portal venous endotoxin measurement
C57BL/6 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 paraffin, 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 paraffin-embedded liver tissue. The following were used: Pan-leucocyte marker, anti-mouse CD45 (leucocyte 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 (Zymed, 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 Gill’s hematoxylin (Fisher Diagnostics, Middletown, VA, USA) and visualized directly under 10× and 60× magnification under light microscope (Zeiss, Germany) and evaluated using a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The contribution of platelets was evaluated by staining paraffin-embedded liver sections (4 μm-thick) with antimouse, CD41-PE labeled antibody (BD PharMingen: 1:100 clone MWreg30) and mounting in Arvold (Celenzade, Ltd., Dallas, TX, USA) containing 1 μmol/L 4′,6-diamidino-2-phenylindole (DAPL, 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 (600×) 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 ether 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
Quantitative real-time PCR

Snap-frozen liver samples were homogenized in QIAzol lysis reagent, and total RNA was isolated with the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Quantity and quality of the extracted RNA were verified using a Nanodrop-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA). Synthesis of cDNA was performed with the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and were normalized to L32.

RANTES (CCL5), eotaxin (CCL11), lymphotactin (XCL1), MIP-2 (CXCL2), IFN-γ-induced protein (IP-10; CXCL10), and T cell activation gene 3 (TCA-3; CCL1) gene expression was determined by the use of a custom-designed multiprobe RNase protection assay system (mck-5, RiboQuant, PharMingen), which was used according to the manufacturer’s protocol. Total RNA was extracted from snap-frozen livers as described above, and probe synthesis, RNA hybridization, RNase treatment, and specific probe purification, electrophoresis, and imaging were done as described previously [37]. Signals were quantified by use of software (Image QuaNT, Molecular Dynamics, Sunnyvale, CA, USA) and were normalized to L32.

Statistical analysis

Data are presented as means ± SEM. Data were analyzed using ANOVA, and differences between groups were identified using Neuman-Keuls post hoc, unless specified. All statistical calculations were done using the PRISM 3.0 software program (Graphpad Prism, San Diego, CA, USA). P < 0.05 was selected as the level of significance.

RESULTS

ATH diet induces hypercholesterolemia, a predominant mononuclear cell infiltrate, liver injury, and hepatic steatosis

Serum cholesterol levels were increased significantly in the C57BL/6J mice fed the ATH diet for 3 weeks compared with animals fed ISO chow (180.5±14.85 mg/dl vs. 128±5.5 mg/dl in C57BL/6J; P<0.05). Leukocyte infiltrates were evident in the perportal area in the animals on the ATH diet, compared with the animals on an ISO diet (Fig. 1, A–D). 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, A–D). Serum ALT (Fig. 1E) levels were increased in C57BL/6J 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 TLR4−/− animals (Figs. 2A and 3).

Chololate added to the chow diet alone for 3 weeks also induced mild liver elevation of the liver enzymes (serum ALT levels: 147±3±3 μL vs. 36±3 μL; 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 chololate alone had no evidence of hepatic lipid loading [lipid levels: 17.50±2.44 mg/g liver in chololate-fed vs. 21.65±1.144 mg/g liver in chow-fed; P=not significant (ns)].

**Fig. 1.** Effect of an ATH diet on liver injury in C57BL/6J mice. Representative photomicrographs of livers of mice fed ISO chow (A) and an ATH diet (B) stained with H&E; (arrow) peri-portal mononuclear cell infiltrates. (C and D) Oil Red-O staining. Red staining (D) indicates intracellar lipid deposition after 3 weeks of an ATH diet (10X original magnification; original scale bar=100 μ). (E; Left) Morphometric quantification of hepatic parenchyma stained by immunohistochemistry for monocytes/macrophages/Kupffer cells (F4/80+) and polymorphonuclear cells (PMN) cells. Values denote volume fraction (% Volume) of cells of interest per hepatic parenchyma per high-power field (60X original). A total of 10 high-power fields counted per liver per mouse. (Right) ALT levels in the serum after feeding an ATH diet or ISO chow. Results are means ± SEM (n=4–5 per group); *, P < 0.002, versus ISO-fed groups using unpaired two-tailed t-test.
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\textsuperscript{del} animals fed the ATH diet had attenuation of the serum ALT levels by \textasciitilde 30\% compared with their WT C57BL10/SnJ controls (186\pm 22 \mu L vs. 247\pm 18 \mu L; \textit{P}<0.05; Fig. 2a). There were also 50\% fewer CD45\textsuperscript{+} cells (Fig. 2b), F4/80\textsuperscript{+} cells, and neutrophils in the ATH diet-fed TLR4\textsuperscript{del} animals compared with their ATH diet-fed WT controls (Fig. 3). TLR4\textsuperscript{del} animals fed an ATH diet showed a small but significant (fourfold) increase in the F4/80\textsuperscript{+} cells compared with the ISO-fed groups. The number of CD45\textsuperscript{+} cells and polymorphonuclear cells in the TLR4\textsuperscript{del} mice fed an ATH diet was also increased (threefold) 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\textsuperscript{+} cells: 6.1\%\pm 0.9 vs. 12.9 \pm 0.4; \textit{P}<0.02 by \textit{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\textsuperscript{+} cells: 12.7\%0.3 vs. 12.9 \pm 0.4; \textit{P}=\textit{ns}).

There was no increase in hepatic IL-6 and IFN-\gamma protein expression in control or TLR4\textsuperscript{del} 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 (CXC12), lymphotactin (XCL1), eotaxin (CCL11), TCA-3 (CCL1), and IP-10 (CXCL10) were increased in the livers of WT (C57BL10/SnJ) mice after feed-
versus WT
/H11001
/H11006
with GAPDH as endogenous control. Results are means (% vol) of cells of interest per hepatic parenchyma per high-power field (60
/H11003
cells (F4/80
/H11001
of the hepatic parenchyma, which stained for monocytes/macrophages/Kupffer
controls. Using immunohistochemistry and morphometric analysis, the volume
and MCP-1. Relative mRNA expression was calculated by the
results from RNA extracted from snap-frozen livers, using probes for ICAM
are mean
original). A total of 10 high-power fields counted per liver per mouse. Results
spective of TLR4 deletion (data not shown).

expression of ICAM-1 (Fig. 3), lymphotactin, eotaxin, IP-10, (Fig. 7). On Bligh-Dyer analysis, livers of diet-fed, TLR2 del
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), arachidonie (C20:4), eicosapentanoic, and docasahexaenoic
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 TLR4del ATH-fed mice, the fatty acid profile of the WT and the TLR4del mouse 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 TLR2del mice and their WT controls, C57BL/6NHsd (Har-lan strain), showed evidence of liver injury (ALT), inflammation
(CD45+ cells), hepatic steatosis, and hypercholesterol-emia 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±17.8
mg/dl; P=ns), ALT levels, and the volume percent of hepatic CD45+ cells between the WT and the TLR2del, diet-fed groups
(Fig. 7). On Bligh-Dyer analysis, livers of diet-fed, TLR2del 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/choleate diet. In addition, we show that the leuko-

Fig. 3. Quantification of monocyte/macrophages, neutrophils, and quantitative PCR for expression of MCP-1 and ICAM in livers of TLR4del and their WT controls. Using immunohistochemistry and morphometric analysis, the volume of the hepatic parenchyma, which stained for monocytes/macrophages/Kupffer
cells (F4/80+) and neutrophils, was quantified. Values denote volume fraction (% vol) of cells of interest per hepatic parenchyma per high-power field (60×
original). A total of 10 high-power fields counted per liver per mouse. Results
are mean ± SEM (n=4–6). (Right) Bar graphs show quantitative real-time PCR
results from RNA extracted from snap-frozen livers, using probes for ICAM and MCP-1. Relative mRNA expression was calculated by the Δ Ct method
with GAPDH as endogenous control. Results are means ± SEM (n=5–6); *, P < 0.05, versus all groups; #, P < 0.05, versus ISO-fed groups; +, P < 0.05, versus WT + ISO group.

TLR4 deletion did not affect the hepatic lipid loading, serum cholesterol levels, or the fatty
acid profile of diet-fed mice

Serum cholesterol levels (200±10 mg/dl vs. 196±13 mg/dl; P=ns), hepatic lipid content as assessed by Oil Red-O
staining, and lipid extraction by the Bligh-Dyer method were similar in the WT and TLR4del 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).

Fig. 4. Platelet staining by immunofluorescence. Representative photomicrographs of
livers of ATH diet-fed WT (C57BL/10SnJ; A) and TLR4del (B) mice stained
for CD41-positive cells (original magnification, 20×; original scale bars=40 μ). Note the
relative paucity of CD41+ red staining seen in the inflammatory cells (C) of the WT
mice (original magnification, 40×; original scale bar=20 μ).

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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 TLR4ΔΔ animals had an abrogation of leukocyte influx and inhibition of inflammatory mediators including MCP-1, RAN-

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 TLR4ΔΔ 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 TLR4ΔΔ mice using Mann-Whitney rank sum analysis.

Fig. 6. Quantification of hepatic fatty acids. After 3 weeks of feeding ISO or ATH diets in WT and TLR4ΔΔ, fatty acid content was determined as described in Materials and Methods. Results reported as nanomoles per gram of liver; means ± SEM (n=5–6 per group); *, P < 0.05, versus ISO-fed groups; #, P < 0.05, versus TLR4ΔΔ + ATH.
TES, and MIP-2. There is little if any effect of TLR2 deletion on the inflammatory response. It appears that TLR4 and likely TLR2 have little or no role in the development of hepatic steatosis, which occurs with the ATH diet. Additionally, TLR4 deletion resulted in greater reduction in hepatic inflammation than hepatic injury (as measured by ALT), suggesting that other mechanisms are responsible for injury in addition to inflammation. Finally, the accumulation of platelets in the liver as a result of feeding the ATH diet does not appear to be a result of TLR4-dependent mechanisms.

Several studies demonstrate the critical role of TLR4 in the development of atheroma in animals with hypercholesterolemia [27–29]. Apolipoprotein-E (APO-E)-deficient mice, which also lack TLR4 or have a targeted deletion of MyD88, a downstream effector of TLR4, have reduced atherosclerosis and altered plaque size compared with APO-E-deficient mice with intact TLR4 [28, 29]. In these studies, hypercholesterolemia is a 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 and others [41, 42] have shown 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 lymphotactin, 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 TLR4 del 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 TLR2 del 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 faciliator 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–TLR 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/6N (Harlan) used in TLR2 experiments and C57BL6 and C57BL/10SnJ (Jackson strain) used in establishing the model and TLR4 experiments. Although the diet was the same, the amount of liver injury and inflammatory response was less in the C57BL6N (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 hepatic lipid accumulation, the amount of liver injury and inflammatory response was less in C57BL/10SnJ (Jackson strain) used in establishing the model and TLR4 experiments. The hepatitis seen with TLR4 deletion probably alters the CD36 –TLR interaction, leading to increased 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–TLR interaction, leading to increased flux of free fatty acids to the liver, thereby leading to steatosis.


