Toll-Like Receptor 4 Deficiency Decreases Atherosclerosis But Does Not Protect Against Inflammation in Obese Low-Density Lipoprotein Receptor–Deficient Mice

Yilei Ding, Savitha Subramanian, Vince N. Montes, Leela Goodspeed, Shari Wang, ChangYeop Han, Antonio Sta. Teresa III, Jinkyu Kim, Kevin D. O’Brien, Alan Chait

Objective—Obesity is associated with insulin resistance, chronic low-grade inflammation, and atherosclerosis. Toll-like receptor 4 (TLR4) participates in the cross talk between inflammation and insulin resistance, being activated by both lipopolysaccharide and saturated fatty acids. The present study was undertaken to determine whether TLR4 deficiency has a protective role in inflammation, insulin resistance, and atherosclerosis induced by a diabetogenic diet.

Methods and Results—TLR4 and low-density lipoprotein (LDL) receptor double knockout mice and LDL receptor–deficient mice were fed either a normal chow or a diabetogenic diet for 24 weeks. TLR4 and LDL receptor double knockout mice fed a diabetogenic diet showed improved plasma cholesterol and triglyceride levels but developed obesity, hyperinsulinemia, and glucose intolerance equivalent to obese LDL receptor–deficient mice. Adipocyte hypertrophy, macrophage accumulation, and local inflammation were not attenuated in intraabdominal adipose tissue in TLR4 and LDL receptor double knockout mice. However, TLR4 deficiency led to markedly decreased atherosclerosis in obese TLR4 and LDL receptor double knockout mice. Compensatory upregulation of TLR2 expression was observed both in obese TLR4-deficient mice and in palmitate-treated TLR4-silenced 3T3-L1 adipocytes.

Conclusions—TLR4 deficiency decreases atherosclerosis without affecting obesity-induced inflammation and insulin resistance in LDL receptor–deficient mice. Alternative pathways may be responsible for adipose tissue macrophage infiltration and insulin resistance that occurs in obesity. (Arterioscler Thromb Vasc Biol. 2012;32:1596–1604.)

Key Words: atherosclerosis ■ diabetogenic diet ■ inflammation ■ insulin resistance ■ toll-like receptor 4

Obesity is a low-grade chronic inflammatory disease associated with an increase of circulating inflammatory markers. This inflammation is associated with insulin resistance in tissues such as adipose tissue and liver. Hallmarks of this inflammatory process are early infiltration of intraabdominal adipose tissue with immune cells (mainly macrophages) and autocrine and paracrine secretion of pro- and anti-inflammatory cytokines, which have been postulated to lead to insulin resistance. Insulin resistance and inflammation in turn have been suggested to play a causal role in cardiovascular disease.

Toll-like receptors (TLR) elicit innate immune responses and inflammation when activated by either exogenous microbial products or endogenous molecules with similar structures, and they are candidate mediators of atherogenic inflammation. In recent years, the activation of immune cells via TLR4 has received considerable attention. TLR4, the best characterized TLR, is an essential receptor for lipopolysaccharide (LPS). The lipid A moiety of LPS containsacylated hydroxyl saturated fatty acids (SFAs). Several different groups have shown that SFAs could be natural ligands for TLR4, culminating in inflammatory gene expression by nuclear factor–κB activation. Moreover, we have previously reported that palmitate increases serum amyloid A3 (SAA3) and monocyte chemoattractant protein-1 (MCP-1) expression via a TLR4-dependent mechanism in 3T3-L1 adipocytes. The responsiveness of TLR4 to fatty acids makes TLR4 an appealing intermediate between obesity and recruitment of macrophages to adipose tissue and the artery wall.

Indeed, others have shown that TLR4-deficient (Tlr4−/−) mutated mice are protected against inflammation and insulin resistance when challenged with high-fat diets. However, conflicting results regarding the role of TLR4 in adiposity and macrophage infiltration of intraabdominal adipose tissue also have been obtained. Moreover, recent studies showed that TLR4 deletion promotes obesity, insulin resistance, hyperlipidemia, and a diabetic phenotype in mice. Therefore, more research is needed to understand the uncertain effects of TLR4 on...
obesity-associated local inflammation, insulin resistance, influx of macrophages to intraabdominal adipose tissue, and atherosclerosis.

To determine whether inactivation of TLR4 would reduce obesity-induced macrophage infiltration into adipose tissue and subsequently prevent insulin resistance and atherosclerosis, we used Tlr4−/− mice on the atherosclerosis-prone low-density lipoprotein receptor–deficient (Ldlr−/−) background. A diabetogenic diet (DD) rich in SFAs and refined carbohydrate was used because we have previously shown that this diet leads to multiple features of the metabolic syndrome such as insulin resistance, adipose inflammation, chronic systemic inflammation, and atherosclerosis in Ldlr−/− mice.25 Our data demonstrate that lack of TLR4 decreases atherosclerosis without affecting obesity-induced inflammation and insulin sensitivity in this mouse model.

Methods
Additional details are available in Materials in the online-only Data Supplement.

Animals and Diets
Ten-week-old adult male TLR4 and LDLR double knockout (Tlr4−/−Ldlr−/−) and littermate control Ldlr−/− mice were fed rodent chow diet and a diabetogenic high, SFA-rich, and carbohydrate-rich diet (DD) for a total of 24 weeks.26 All experimental procedures were undertaken with approval from the Institutional Animal Care and Use Committee of the University of Washington.

Analytical Procedures
Total plasma cholesterol and triglycerides, plasma insulin, and hepatic triglycerides were measured using commercially available assay kits. Lipoprotein distribution was analyzed by fast protein liquid chromatography as described previously.27 Total RNA was extracted according to the manufacturer’s protocols. Relative quantities of mRNA were calculated using GAPDH as the reference gene. The amount of target gene was calculated using the delta-delta cycle threshold formula.

Immunohistochemistry and Adipocyte Sizing
Single-label immunohistochemistry was performed on adipose tissues as previously described.28 Adipocyte cross-sectional area was measured by computer image analysis using a modification of techniques described previously.29 All analyses were performed by a blinded observer.

In Vitro TLR4 Gene Silencing
To test the role of TLR4 or TLR2-mediated SAA3 and MCP-1 expression, 3T3-L1 adipocytes were transiently transfected (2 days after completion of the differentiation protocol) with small interfering RNA duplexes for TLR4 (Ambion DeliverX system, Panomics), as described previously.29,30

Real-Time Quantitative Polymerase Chain Reaction Analysis
Total RNA was extracted according to the manufacturer’s protocols. Relative quantities of mRNA were calculated using GAPDH as the reference gene. The amount of target gene was calculated using the delta-delta cycle threshold formula.

Atherosclerotic Quantification
The extent of aortic atherosclerosis was measured by the en face technique as described previously.31 Total surface area and lesion areas in the studies sections of aortas were quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis
Data were analyzed using GraphPad Prism 5 program (GraphPad Software Inc), and represent means±SE unless noted otherwise. ANOVA with Tukey post hoc test was used to detect differences among groups. Data not normally distributed were analyzed using the Kruskal-Wallis test with Dunn post hoc test analysis. A P<0.05 was considered as statistically significant.

Results
Hyperlipidemia Is Attenuated in Tlr4−/−Ldlr−/− Mice Fed DD
As expected from our previous study,25 body weight gain in mice fed DD significantly outpaced that in chow-fed mice. Body weights at the end of 24 weeks did not differ between Tlr4−/−Ldlr−/− mice and their controls either in DD or chow groups (Figure 1A in the online-only Data Supplement), and no differences in food intake were detected (data not shown). Body composition analysis revealed a comparable increase in fat mass in DD- versus chow-fed mice (Figure 1B and IC in the online-only Data Supplement). These results demonstrate that the loss of TLR4 did not affect the body weight and the distribution of fat in the obese animals. Thus, the Tlr4−/−Ldlr−/− and Ldlr−/− groups were well matched for body weight, obesity, and all the other aspects of adiposity at the end of the study.

We previously showed that Ldlr−/− mice fed DD for 24 weeks develop hypercholesterolemia and hypertriglyceridemia.25 In the present study, we confirmed these findings. Furthermore, we found that the magnitude of hypercholesterolemia and especially hypertriglyceridemia was significantly lower in the Tlr4−/−Ldlr−/− group fed DD for 12 and 24 weeks compared with control mice fed DD (Figure 1A and 1B, P<0.05). Lipoprotein profiles showed lower very-low-density lipoprotein/intermediate-density lipoprotein and LDL-cholesterol (Figure 1C) and triglycerides levels (Figure 1D) in the obese Tlr4−/−Ldlr−/− mice fed DDs. These data suggest that TLR4 deficiency reduces obesity-induced hyperlipidemia in Ldlr−/− mice.

Hyperglycemia and Insulin Resistance Are Not Improved in Tlr4−/−Ldlr−/− Mice Fed DD
Mice on DD developed hyperglycemia and hyperinsulinemia compared with the chow-fed mice (Figure 2A and 2B). Fasting glucose and insulin levels did not differ in the DD and chow groups at the end of the study, despite the plasma fasting insulin being significantly reduced in the Tlr4−/−Ldlr−/− DD group at 12 weeks (Figure 2B, P<0.05). The glucose response during a glucose tolerance test was increased in DD-fed mice (Figure 2C and 2D). On DD, the baseline glucose levels were higher than the chow-fed animals (287.3±12.9 mg/dL in Tlr4−/−Ldlr−/− DD; 260.0±10.8 mg/dL in Ldlr−/− DD; 179.1±7.7 mg/dL in Tlr4−/−Ldlr−/− chow; and 201.4±8.1 mg/dL in Ldlr−/− chow). Also, the presence or absence of TLR4 had no effect on the insulin response at the 30-minute time point during the glucose tolerance test in either the DD or the chow groups (Figure 2II in the online-only Data Supplement). Moreover, the area under the curve was not statistically different between Ldlr−/− and Tlr4−/−Ldlr−/− obese or lean animals (data not shown). Whereas DD caused insulin
resistance on insulin tolerance test, the hypoglycemic response in the Tlr4−/−Ldlr−/− group was comparable with that in Ldlr−/− mice, with only 1 data point showing a borderline difference (Figure 2E and 2F, *P < 0.05). Taken together, these findings reveal no overall improvement in glucose tolerance or insulin resistance as a result of TLR4 deficiency in this mouse model.

Macrophage Infiltration in Intraabdominal Adipose Tissue Does Not Decrease in Tlr4−/−Ldlr−/− Mice Fed DD

To evaluate changes in intraabdominal adipose tissue morphology and macrophage accumulation that occur with obesity, epididymal (intraabdominal) adipose tissue harvested at the time of euthanizing was studied. As expected, adipocyte size mirrored epididymal fat pad weights. Both adipose tissue weight and adipocyte hypertrophy were higher in mice fed the DD versus the control diet, but did not differ between Tlr4−/−Ldlr−/− and Ldlr−/− groups (Figure IIIA and IIIB in the online-only Data Supplement). Interestingly, adipose tissue cholesterol content was significantly decreased (*P < 0.05) in Tlr4−/−Ldlr−/− mice fed DD compared with Ldlr−/− mice fed DD (Figure IIIC in the online-only Data Supplement).

We performed single-labeled immunohistochemistry to identify the presence of macrophages within adipose tissue.
Immunostaining for the macrophage-specific marker, Mac2, showed DD inducing a significant increase of macrophages in epididymal adipose tissue (Figure 3A and 3B, *P<0.01). Although the DD-fed Tlr4−/−Ldlr−/− mice tended to have decreased Mac2 staining compared with DD-fed Ldlr−/− mice, these changes did not achieve statistical significance. Both the expression of Mac2 and CD11b, the monocyte-macrophage marker, did not show statistical differences in the DD and chow groups (Figure 4A). However, mRNA levels of another macrophage marker, F4/80, were significantly decreased in DD-fed Tlr4−/−Ldlr−/− mice in both liver (data not shown) and adipose tissue (Figure 4A, *P<0.05). Furthermore, we measured the levels of NO synthase 2 and interleukin-6 as M1 markers, arginase 1 and found in inflammatory zone 1 (Fizz1) as M2 markers in the adipose tissue of Tlr4−/−Ldlr−/− mice and its control Ldlr−/− mice fed DD, and found no difference in the above genes between groups (Figure IV in the online-only Data Supplement). Therefore, it is less likely that the macrophages represent an alternatively activated type of macrophage. These findings are consistent with an overall lack of effect of TLR4 deletion on macrophage accumulation in both adipose tissue and liver.
Local Inflammation in Intraabdominal Adipose Tissue and Liver Is Not Altered in Tlr4−/−Ldlr−/− Mice

Given that macrophages are the cell type with the highest surface expression of TLR4 and, as such, are potent sensors and effectors for TLR4-mediated local inflammatory responses, we assessed the extent of local inflammation by examining expression of selective genes involved in inflammation in intraabdominal adipose tissue and liver by real-time quantitative polymerase chain reaction.

mRNA levels of proinflammatory chemokines and cytokines in intraabdominal adipose tissue were significantly increased in Ldlr−/− mice on DD, as has been shown previously.25 SAA3, the extrahepatic isoform of SAA produced by adipocytes and macrophages in mice, is a locally synthesized molecule with chemoattractant properties. SAA gene expression in intraabdominal adipose tissue (Figure 4B) and liver (data not shown) did not differ between Tlr4−/−Ldlr−/− and Ldlr−/− mice. Similarly, the monocyte chemoattractant gene, Mcp1, and the proinflammatory cytokine, tumor necrosis factor-α, also were not different between groups (Figure 4B). These data suggest that TLR4 deficiency did not affect local inflammation in adipose tissue or liver.

To investigate whether cholesterol metabolism is affected by TLR4 deficiency, the expression of apolipoprotein B, 3-hydroxy-3-methylglutaryl-CoA reductase, acetyl-CoA acetyltransferase 1, sterol regulatory element-binding transcription factor 1, diacylglycerol O-acyltransferase 1, and microsomal triglyceride transfer protein was measured in the liver of Tlr4−/−Ldlr−/− mice fed DD and Ldlr−/− mice fed DD. However, none of these genes were significantly different between the 2 groups (Figure V A–VF in the online-only Data Supplement). Triglyceride content of liver was increased in both groups of mice fed DD, but again, no differences were observed between the DD-fed obese Tlr4−/−Ldlr−/− and Ldlr−/− mice (19.6±1.5 mg/g liver in DD-fed Tlr4−/−Ldlr−/−; 19.8±2.4 mg/g liver in DD-fed Ldlr−/−; 8.9±0.6 mg/g liver in chow-fed Tlr4−/−Ldlr−/−; and 8.6±1.2 mg/g liver in chow-fed Ldlr−/−). These findings indicate that the absence of TLR4 is not associated with a decrease in hepatic triglycerides and cholesterol synthesis despite improvement of DD-induced lipid levels in plasma. It is possible that TLR4 deficiency affects triglyceride removal via LDLR-independent pathways because these mice were deficient in LDLRs.

TLR2 Expression Is Increased in Both Tlr4−/−Ldlr−/− Mice Fed DD and TLR4-Silenced Free Fatty Acids–Treated Adipocytes

Considering that TLR2 is closely related to TLR4 and is also shown to be activated by SFAs,32 we next examined the Tlr2 mRNA abundance in intraabdominal adipose tissue and liver from Tlr4−/−Ldlr−/− mice. On chow diets, TLR4 deficiency did not alter the mRNA level of Tlr2 expression; however, Tlr2 expression was significantly increased in Tlr4−/−Ldlr−/− mice fed DD compared with obese Ldlr−/− mice fed DD (Figure 5A).
We have previously shown that silencing TLR4 in differentiated 3T3-L1 adipocytes with a TLR4-specific small interfering RNA markedly reduced SAA3 and Mcp1 expression in response to palmitate but not glucose.\(^{15}\) TLR4 small interfering RNA–transfected adipocytes were used in the present study to measure Tlr2 expression in response to palmitate exposure in both 5- and 25-mmol/L glucose. The success of transfection was confirmed by markedly silenced Tlr4 expression and reduced expression of SAA3 and Mcp1 (data not shown). Similarly to what we found in the in vivo experiment, mRNA level of Tlr2 was significantly increased in palmitate-treated TLR4-silenced cells (Figure 5B, \(P<0.05\)). Taken together, these results imply that TLR4 deletion leads to a compensatory increase of Tlr2 expression, which could, in part, contribute to the lack of change in adipose tissue inflammation and insulin resistance in the present study.

Atherosclerosis Is Significantly Decreased in Tlr4\(^{-/-}\) Ldlr\(^{-/-}\) Mice Fed DDs

TLR4 deficiency has been shown to reduce intimal lipid by \(\approx 75\%\) in apolipoprotein E–deficient mice.\(^{33}\) Because Ldr\(^{-/-}\) mice are particularly susceptible to the DD-induced adipose inflammation, insulin resistance, and atherosclerosis whereas apolipoprotein E–deficient mice do not have similar changes,\(^{31,34}\) we assessed the effect of TLR4 deficiency on atherosclerosis in Ldr\(^{-/-}\) mice.

We confirmed that Ldr\(^{-/-}\) mice fed DD for 24 weeks have significantly increased atherosclerotic lesion area compared with chow-fed animals\(^{35}\) (Figure 6A, \(P<0.01\)). However, DD-fed Tlr4\(^{-/-}\) Ldr\(^{-/-}\) mice had markedly reduced (by 76%) aortic atherosclerotic lesion area compared with obese Ldr\(^{-/-}\) mice (Figure 6A, \(P<0.01\)).

To evaluate the contribution of plasma cholesterol and triglycerides to atherosclerosis in these 2 groups of DD-fed animals, we performed linear regression analysis. Atherosclerotic lesion area correlated with plasma cholesterol (Figure 6B, \(r=0.54, P=0.03\)), but not with plasma triglycerides level (data not shown), suggesting improved plasma cholesterol might be
one of the factors that contribute to the reduced atherosclerosis in $Tlr4^{-/-}Ldlr^{-/-}$ mice fed DD.

**Discussion**

Our findings demonstrate that TLR4 deficiency led to a 75% reduction in the extent of atherosclerosis without parallel changes in adipose tissue inflammation and insulin sensitivity in $Ldlr^{-/-}$ mice fed a diet high in saturated fat and sucrose. Activation of TLR4 by SFAs has been suggested to play a key role in causing insulin resistance mediated by saturated fat–rich diets. Such diets, when fed to $Ldlr^{-/-}$ mice, lead to many features of metabolic syndrome such as obesity, tissue inflammation, insulin resistance, and atherosclerosis. The reduction in atherosclerosis seen in $Tlr4^{-/-}Ldlr^{-/-}$ mice in the present study was associated with improved hyperlipidemia and increased expression of TLR2, but not with reduced adipose tissue inflammation or improved insulin sensitivity.

Chronic low-grade tissue inflammation has recently garnered considerable attention as an important contributor to insulin resistance in obesity. This inflammation is associated with macrophage accumulation in intraabdominal adipose tissue and increased expression of various cytokines produced by these macrophages. TLRs play a critical role in activating the innate immune response, and have been implicated in the induction of insulin resistance and atherosclerosis in obesity. In particular, TLR4 is an attractive candidate because it is highly expressed in macrophages and adipose tissue; TLR4 also is activated by fatty acids, which are increased in obesity. Moreover, whole-body TLR4 deficiency has been reported to be associated with improved insulin sensitivity in mice fed a high-fat diet, without affecting body weight. We therefore hypothesized that TLR4 deletion would attenuate obesity, adipose tissue macrophage accumulation, insulin resistance, and subsequently, reduce atherosclerosis in $Ldlr^{-/-}$ mice. Although loss of TLR4 led to a significant reduction in atherosclerosis and hyperlipidemia, it did not decrease adipose tissue macrophage accumulation, nor did it significantly improve glucose or insulin tolerance. These findings suggest that the reduction of atherosclerosis is unlikely to be attributable to amelioration of whole-body insulin sensitivity or adipose tissue inflammation but rather to a reduction in lipid levels and a direct effect of TLR4 on atherosclerosis per se.

Whether TLR4 deletion has a beneficial effect on obesity-related inflammation is unclear. Some early studies showed that mice with TLR4 deficiency or a loss-of-function mutation in TLR4 are protected against obesity-induced insulin resistance. However, later studies have failed to confirm these findings with respect to the effect of TLR4 deficiency on body weight, insulin sensitivity, and macrophage accumulation in adipose tissue. Davis et al found that TLR4 deficiency does not completely attenuate adipose tissue inflammation as indicated by the tumor necrosis factor-α or interleukin-6 expression in adipose tissue. By transplanting $Tlr4^{-/-}$ bone marrow into nonobese $Ldlr^{-/-}$ Agouti mice, Coenen et al reported a moderate decrease in both atherosclerosis and adipose tissue macrophage content only in low-fat fed female mice, without any changes in body composition, insulin sensitivity, and plasma lipids. In addition, several recent studies report an adverse effect of TLR4 deficiency on insulin resistance and other metabolic disease. TLR4-mutated mice fed with a diet rich in trans-fatty acids were found to actually have exacerbated phenotypes of obesity, hyperglycemia, and hyperinsulinemia. Also, the same authors showed that loss of TLR5 in mice already deficient in either TLR4 or TLR2 still led to obesity, hyperlipidemia, and insulin resistance. Furthermore, Hosoi et al reported an unexpected diabetic phenotype in MyD88-deficient mice fed high-fat diet. Potential reasons for the variability between these studies include differences in weight, gender, or strain of mice used, the nature of the genetic alteration in TLR4, differences in the composition or duration of the high-fat diet, or difference in body weight or fat mass. All these might alter assessment of insulin resistance and adipose tissue. Because insulin levels were reduced at 12 but not 24 weeks in our study, it is conceivable that an effect of TLR4 deficiency on adipose tissue and liver might have been missed by focusing our studies on this later time point. Future studies should include a time course to ensure that TLR4 deficiency does not lead to changes in adipose tissue inflammation and insulin resistance at early time points. However, other investigators have also failed to show a beneficial effect of TLR4 deficiency after 12 weeks of feeding a high-fat diet. The diet used in our study also contained fructose (in the form of sucrose). Because fructose can cause significant insulin resistance in rodents, it is possible that any potential beneficial effects of TLR4 deficiency on insulin resistance might have been offset by adverse effects of the fructose in the diet. The cholesterol content of the diet also might matter, because dietary cholesterol itself can contribute to macrophage accumulation in white adipose tissue, and the interaction between dietary cholesterol and fatty acids is still largely unexplored. Another limitation of our study is that we did not specifically measure the phenotypes of macrophages in adipose tissue by flow cytometric analysis. Although the lack of difference in a limited number of mRNA M1 and M2 macrophage markers in whole adipose tissue suggested that the macrophages observed are not of the alternatively activated phenotype, future studies should include macrophage phenotyping by flow cytometric analysis of the stromal vascular fraction of adipose tissue.

In the present study, $Tlr4^{-/-}$ mice fed DD had increased expression of TLR2 in adipose tissue compared with controls. We confirmed this finding in TLR4-silenced 3T3-L1 adipocytes in vitro after exposure to conditions of excess glucose and palmitate. Similar results have been shown by others. TLR2 is the most likely alternative TLR that may contribute to diet-induced inflammation in adipose tissue, because it is closely related to TLR4 and also has been reported to be activated by SFAs, although to a lesser extent than TLR4. Recently, others reported that TLR2-deficient mice show improvement in metabolic features associated with obesity induced by a high-fat diet. We speculate that the upregulation of TLR2 expression in the absence of TLR4 might be a compensatory pathway that contributes to adipose tissue inflammation and insulin resistance observed in obese $Tlr4^{-/-}$ mice. Future studies in TLR4/TLR2 double-deficient...
mice should help address this issue. It is also possible that the SFA-rich diet may have altered the microflora in the gut, which in turn may have altered the intestinal barrier allowing translocation of microbial products leading to the activation of TLR4-independent pathways associated with inflammation and insulin resistance.\(^{23}\)

To our knowledge, this is the first study to date to show a beneficial effect of TLR4 deficiency on atherosclerosis in \(\text{Ldlr}^{-/-}\) mice fed a diet rich in SFAs. DD resulted in hypercholesterolemia and hypertriglyceridemia in these mice. TLR4 deficiency led to a significant reduction in plasma cholesterol and triglycerides as a result of a reduced accumulation of lipoproteins in both LDL and, particularly, the very-low-density lipoprotein/intermediate-density lipoprotein density range. Similar findings have been reported by others in apo-lipoprotein E–deficient mice,\(^{33,42}\) by unknown mechanisms, which should be addressed in future studies. Furthermore, in our study, the extent of atherosclerosis of the aortas showed a positive correlation with plasma cholesterol levels. Therefore, it is likely that reduction of these cholesterol-rich very-low-density lipoprotein/intermediate-density lipoprotein particles contributes to the effect of TLR4 deficiency on preventing atherosclerotic lesion formation in \(\text{Ldlr}^{-/-}\) mice. However, it is conceivable that TLR4 deficiency might, in addition, have direct effects on cells of the artery wall. Although we did not measure TLR expression in the artery wall in the present studies, evaluation of TLR expression in vascular tissues in future studies should help differentiate the effects observed in atherosclerosis from those observed in adipose tissue and liver and help differentiate direct effects of TLR deficiency on arterial wall cells from those that result from alterations in plasma lipoproteins.

Our findings point to a proatherogenic role of TLR4, raising the important question of how TLR4 becomes activated and interferes with lipid metabolism in \(\text{Ldlr}^{-/-}\) mice. One possibility is that circulating LPS provides a chronic baseline stimulus to TLR4 signaling, because low levels of LPS can be found in the serum of apparently healthy humans and laboratory mice, particularly those consuming high-fat diets.\(^{43,44}\) Administration of exogenous LPS, the ‘TLR4 agonist, promoted atherosclerosis in rabbits and mice\(^{45,46}\) and in humans.\(^{47}\) An alternative hypothesis is that nonmicrobial, endogenous TLR4 agonists contribute to a sterile inflammatory response. Candidate TLR4 agonists include minimally or moderately oxidized forms of LDL, which stimulate TLR4-dependent fluid phase LDL uptake, a potential mechanism of foam cell formation and expression of multiple chemokines.\(^{48,49}\) Further investigations will be needed to evaluate these possibilities.

In summary, we have demonstrated that TLR4 deficiency decreases atherosclerosis without affecting macrophage accumulation and insulin resistance in adipose tissue and liver in \(\text{Ldlr}^{-/-}\) mice. Collectively these data indicate the importance of innate immunity and plasma cholesterol in the induction of atherosclerosis in obesity. Our data also identify TLR4 as a potential target for the therapeutic treatment of atherosclerosis.

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We gratefully acknowledge Drs Linda Curtiss and Peter Tobias for providing the TLR4 and LDL receptor double knockout mice.

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Disclosures
None

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Supplement Material

Supplemental Methods

*Animals and diets:* *Tlr4*−/−*Ldlr*−/− mice generated on a C57BL/6J genetic background were kindly provided by Dr. Linda Curtiss from the Scripps Research Institute (San Diego, CA) and bred and genotyped in our laboratory. 10-week old adult male *Tlr4*−/−*Ldlr*−/− and littermate control *Ldlr*−/− mice were fed rodent chow providing 4% calories as fat and a “diabetogenic” high SFA-rich and carbohydrate-rich diet (DD, 60% calories from fat, Bioserv F1850) for a total of 24 weeks. Details of the diets have been published previously. Mice were maintained in a temperature and light-controlled facility in cages with micro-isolator filter tops, and received the diets *ad libitum* prior to sacrifice. Body weights were measured weekly. Food intake, determined as the difference in the weight of unconsumed food between sequential days, was recorded for three sequential days after 16 weeks of diets. Metabolic variables were measured in blood samples obtained from the retro-orbital sinus at 12 weeks on the diets and on the day of sacrifice after a 4-hour fast. At sacrifice harvested tissues were snap-frozen in liquid nitrogen and stored at -70°C, or were fixed with 10% neutral-buffered formalin and embedded in paraffin wax. All experimental procedures were undertaken with approval from the Institution Animal Care and Use Committee of the University of Washington.

*Analytical procedures:* Total cholesterol and triglycerides in plasma were measured using colorimetric assay kits. Lipoprotein distribution was analyzed in pooled plasma by fast protein liquid chromatography (FPLC) as described previously. Plasma insulin was measured using an enzyme-linked immunosorbsent assay kit (Linco). Hepatic triglycerides content and adipose tissue lipid content were determined following lipid extraction using the Folch method. Glucose and insulin tolerance tests were measured at 20-22 weeks, as previously described.
Body composition analysis: Body composition was performed on conscious immobilized mice using quantitative magnetic resonance (EchoMRI whole body composition analyzer, Echo Medical Systems, Houston, TX, USA).

Immunohistochemistry: Single-label immunohistochemistry was performed on adipose tissues as previously described. Adipocyte cross-sectional area was measured by computer image analysis using a modification of techniques described previously. Macrophage staining in adipose tissue sections was detected using a rat monoclonal antibody against Mac2 (1:2000 dilution, Cedarlane Laboratories, Burlington, NC). Area quantification for Mac2 was performed on digital images of immunostained adipose tissue sections using image analysis software (Image Pro Plus software, Media Cybernetics). All analyses were performed by a blinded observer.

Adipocyte sizing: Sections were stained with Movats Pentachrome. One randomized photomicrograph was analyzed at 20X objective (Canon EOS 5D Mark II, Olympus BX50) and quantified by manually outlining each individual adipocyte by hand (Image Pro Plus/Media Cybernetics software, Wacom Cintiq 21UX tablet).

Cell culture: 3T3-L1 murine pre-adipocytes, obtained from American Type Tissue Culture Collection, were propagated and differentiated according to standard protocol procedures, with the exception that media containing either 5 or 25 mmol/l glucose with or without 250μmol/l FFAs were replenished daily.
Real-time quantitative PCR analysis: Total RNA was extracted from 100mg of epididymal white adipose tissue and liver using RNeasy Lipid Tissue Kit (QIAGEN, Valencia, CA, USA) and Agilent Total RNA Isolation Kit (Agilent, Santa Clara, CA, USA), respectively, according to the manufacturer’s protocols. After spectroscopic quantification, 2 µg of RNA was reverse-transcribed to cDNA and used for quantification of genes. Real-time quantitative PCR was performed with the TaqMan Master Kit (Applied Biosystems, Carlsbad, CA, USA) using an ABI 7900HT instrument. Primer and FAM probes for individual genes were purchased from Applied Biosystems (Assay-on-Demand). Relative quantities of mRNA were calculated using GAPDH as the reference gene. The amount of target gene was calculated using the ΔΔCt formula.
### Supplemental Table I. Composition of the diets.

#### Supplemental Table I

Composition of the diets

<table>
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<tr>
<th>Diet</th>
<th>Composition</th>
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<tr>
<td>Chow</td>
<td>Carbohydrate 68%, Protein 24%, Fat 4%, Fiber 4%</td>
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<tr>
<td>Diabetogenic diet (DD)</td>
<td>Carbohydrate 36.3%, Fat 35.5% as lard*, Protein 20%, Fiber 0%</td>
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<tr>
<td>BioServ F1850</td>
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All diets also contained: casein, methionine, vitamin and mineral mix.

* Lard adds small amounts of cholesterol (0.03%) – Source: USDA National Nutrient Database ([http://www.nal.usda.gov/fnic/foodcomp/search/](http://www.nal.usda.gov/fnic/foodcomp/search/)).
Supplemental Figure Legends:

Figure SI. Body weight and body composition is not changed by TLR4 deficiency. Body weight (A), percentage lean mass (B), percentage fat mass (C) in Tlr4<sup>-/-</sup>Ldlr<sup>-/-</sup> mice fed DD (black bars and solid circle lines) and chow (grey bars and solid triangle lines), Ldlr<sup>-/-</sup> control mice fed DD (hatched bars and open circle dash lines) and chow (open bars and open triangle dash lines) (n=18-24, *P<0.05, **P<0.01 vs DD or chow groups).

Figure SII. Insulin resistance is not attenuated in Tlr4<sup>-/-</sup>Ldlr<sup>-/-</sup> mice. Plasma insulin levels at 30 minutes post injection in the glucose tolerance tests (n=9-11, *P<0.05, **P<0.01 vs DD or chow groups).

Figure SIII. TLR4 deficiency does not affect visceral fat pad weight and adipocyte size. Epididymal white adipose tissue weight (A), adipocyte size (B) and lipid content – triglyceride left, cholesterol right (C) (n=10-13, **P<0.01 vs DD or chow groups).

Figure SIV. TLR4 deficiency does not affect the expression of M1 and M2 markers in intra-abdominal adipose tissue of DD-fed Tlr4<sup>-/-</sup>Ldlr<sup>-/-</sup> mice and Ldlr<sup>-/-</sup> mice. The mRNA levels of Nos2 (A) and Il-6 (B) as M1 markers, Arg1 (C) and Fizz1 (D) as markers in epididymal adipose tissue (n=10-13).

Figure SV. TLR4 deficiency does not affect the expression of cholesterol metabolism-related genes in liver of DD-fed Tlr4<sup>-/-</sup>Ldlr<sup>-/-</sup> mice and Ldlr<sup>-/-</sup> mice. The mRNA levels of Apob (A), Hmgcr (B), Acat1(C), Srebp1c (D), Dgat1 (E), Mttp (F) in liver (n=10-13).
Supplemental Figure SI.

A

Body Weight (g)

Weeks on diet

**

Ldr−/− Chow

Tlr4−/− Ldr−/− Chow

Ldr−/− DD

Tlr4−/− Ldr−/− DD

B

% Lean mass

C

% Fat mass

Ldr−/− Chow

Tlr4−/− Ldr−/− Chow

Ldr−/− DD

Tlr4−/− Ldr−/− DD

**
Supplemental Figure SII.
Supplemental Figure SIII.

A

Epididymal fat weight (g)

B

Adipocyte size (μm²)

C

Adipose tissue triglycerides (mg/mg tissue)

Adipose tissue cholesterol (mg/mg tissue)
Supplemental Figure SIV.

A  Nos2

Relative gene expression

Ldlr^{+/-} DD  Tlr4^{+/+}Ldlr^{+/-} DD

B  Il6

Relative gene expression

Ldlr^{+/-} DD  Tlr4^{+/+}Ldlr^{+/-} DD

C  Arg1

Relative gene expression

Ldlr^{+/-} DD  Tlr4^{+/+}Ldlr^{+/-} DD

D  Fizz1

Relative gene expression

Ldlr^{+/-} DD  Tlr4^{+/+}Ldlr^{+/-} DD
Supplemental Figure SV.

A. Apob

B. Hmgcr

C. Acat1

D. Srebp1c

E. Dgat1

F. Mtp
References


