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Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids

Xinyan Liu[*,§§], **Takashi Ukai**[*,†,§§], **Hiromichi Yumoto**[*,‡], **Michael Davey**[§], **Sulip Goswami**[*], **Frank C. Gibson III**[*], and **Caroline A. Genco**[*,§,¶,**]

* *Department of Medicine, Section of Molecular Medicine, Boston University School of Medicine, Boston, MA 02118*

† *Department of Periodontology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852−8588, Japan*

‡ *Department of Conservative Dentistry, The Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, 770−8504, Japan*

§ *Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Boston University, Boston, MA 02118*

¶ *Department of Microbiology, Boston University School of Medicine, Boston, MA 02118*

Abstract

Objective—Toll-like receptors (TLRs), a group of pathogen-associated microbial pattern recognition receptors, play an important role in innate immune signaling and are differentially regulated in chronic inflammatory diseases such as atherosclerosis. However, the involvement of TLRs in the progression of atherosclerosis is still unclear.

Methods and Results—TLR2 and apolipoprotein E double knockout (*Tlr2*$^{-/-}$*Apoe*$^{-/-}$) mice were generated and the progressive formation of atherosclerotic plaque in the aortas was examined in mice fed a normal chow diet. We demonstrate that inactivation of TLR2 resulted in reduced progression of atherosclerosis in both male and female *Apoe*$^{-/-}$ mice. Likewise, TLR2 deficiency resulted in a reduction in lipid accumulation and decreased macrophage recruitment to the aortic sinus as well as reduced monocyte chemoattractant protein-1 (MCP-1) levels. Furthermore, macrophages isolated from *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice demonstrated significantly reduced MCP-1 production upon stimulation with a TLR2 ligand. However, no differences in acetylated-low-density lipoprotein uptake and foam cell formation were observed in macrophages isolated from *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice as compared to *Apoe*$^{-/-}$ mice.

Conclusions—TLR2 plays a critical role in the progression of atherosclerosis in *Apoe*$^{-/-}$ mice, which is independent of dietary lipids and macrophage lipid uptake.

Keywords

atherosclerosis; Toll-like receptor; inflammation; macrophages

**Corresponding Author: Caroline A. Genco Department of Medicine, Section of Molecular Medicine, Boston University School of Medicine, 650 Albany Street, Boston MA 02118. Phone: (617) 414−5305. Fax: (617) 414−5280. E-mail: caroline.genco@bmc.org or cgenco@bu.edu.
§§These authors contributed equally to this work.

Atherosclerosis, formerly considered a lipid storage disease, actually involves ongoing inflammatory responses. Inflammation in the arterial vessel wall is considered to play an important role in the pathogenesis of atherosclerosis[1, 2]. Indeed, recent advances have established a fundamental role for inflammation in mediating all stages of this disease, from initiation through progression[1]. Activation of monocytes and macrophages is an important initial step in the cascades of events leading to many acute and chronic inflammatory diseases including atherosclerosis. It is believed that formation of foam cells, lipid-laden macrophages, is an early hallmark of atherosclerotic lesion formation[1, 2]. In humans, ongoing inflammatory reactions within coronary atherosclerotic plaques are increasingly thought to be crucial determinants of the clinical course of patients with coronary artery diseases[2]. Likewise, in a variety of animal models of atherosclerosis, signs of inflammation occur hand-in-hand with incipient lipid accumulation in the artery wall[3, 4].

Toll-like receptors (TLRs) are a group of receptors that play a key role in innate immune signaling and initiating inflammatory responses. Ligation of these receptors initiates the activation of nuclear factor-κB (NF-κB) resulting in the expression of a wide array of inflammatory genes[5, 6]. The best studied of the TLRs are TLR2, and TLR4. Recent studies in humans have demonstrated that TLRs are expressed in macrophage-infiltrated atherosclerotic lesions[7, 8]. The Asp299Gly polymorphism in the human TLR4 gene has been associated with decreased risk for atherosclerosis in humans[9]. Elevated TLR expression has also been reported in the atheroma of murine models of atherosclerosis[7, 8]. Recent animal studies, employing hyperlipidemic mice fed a high fat diet, have shown that myeloid differentiation factor 88 (MyD88), TLR4 and TLR2 play an important role in atherosclerotic plaque accumulation[10-12]. These studies did not examine the temporal formation of atherosclerotic plaque and used mice placed on a high fat, cholesterol-rich diet.

Although it has been extensively reported that *Apoe*$^{-/-}$ mice fed a normal chow diet spontaneously develop atherosclerosis [13, 14, 15], high fat diet supplementation has been widely used because it can further accelerate atherosclerosis in *Apoe*$^{-/-}$ mice [13-15]. However, it has been reported that high fat diet supplementation is associated with abnormal immunological responses underlying atherosclerosis. Severe hypercholesterolemia leads to the shifting of T helper cell responses from Th1 responses, which is commonly seen in atherosclerotic plaques[16], to abnormal Th2 responses, in *Apoe*$^{-/-}$ mice[17, 18]. Since atherosclerotic plaque is dominated by Th1-type cytokines[19], the excessively high circulating cholesterol levels in high fat diet fed *Apoe*$^{-/-}$ mice could potentially confound or mask immunological processes involved in atherosclerosis. This issue is particularly important in the immunological studies of atherosclerosis. In our study, to exclude the effects of dietary stresses on immunological responses underlying atherosclerosis, we used normal chow diet fed mice to investigate the role of TLR2 in the development of atherosclerosis.

Materials and Methods

Generation of Mice

Tlr2$^{-/-}$*Apoe*$^{-/-}$ mice were generated from heterozygote intercrosses of the *Apoe*$^{-/-}$ mice (C57BL/6J background, The Jackson Laboratory) and the *Tlr2*$^{-/-}$ mice (C57BL/6J background, originally provided by S. Akira, Osaka University) and the double knock out mice were confirmed by genotyping. Age- and sex-matched *Apoe*$^{-/-}$ mice and C57BL/6J mice were obtained from the Jackson Laboratory. All mice were fed a normal chow diet and were cared for in accordance with Boston University Institutional Animal Care and Use Committee procedures. Total and free cholesterol concentrations in the sera of mice were determined by colorimetric assays according to the manufacturer's instructions (Wako Chemicals).

Atherosclerotic Plaque Assessment

Apoe$^{-/-}$ *female, Apoe*$^{-/-}$ *male, Tlr2*$^{-/-}$*Apoe*$^{-/-}$ male and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ female mice (16 per group) fed a normal chow diet were used to determine atherosclerosis development. Half of the animals were sacrificed at five months of age and the remaining mice were sacrificed at seven month of age (n=8 mice/condition). Mice at the age of five and seven months were used to determine lesion progression as reported previously [20]. The aorta of mice (male and female, *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$, n=8 each group) was harvested from the aortic valve to the iliac bifurcation, opened longitudinally, and stained with Sudan IV[21]. Digital micrographs were taken and the total area of atherosclerotic plaque in the aortic arch and the whole aorta was determined from on-screen images using IPLabs (Scanalytics, Inc.) by an observer blinded to the identity of the samples. In a separate experiment, cryosectioning of embedded aortic arch tissue (from five-month old *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ female mice, n=3 each group) was performed to evaluate histological and immunohistological features of atherosclerotic lesions. Cross-sections (7 μm thick) of the aortic sinus were stained with hematoxylin-eosin and oil red O (1% in 60% isopropanol). Detection of macrophages and MCP-1 in aortic cryosections was accomplished by using antibody to macrophage antigen F4/80 (1:50, Serotec) and mouse CCL2 (1:50, eBioscience) respectively, and ABC kit with biotinylated anti-rat (mouse absorbed) IgG as secondary antibody (Vecta Laboratory).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The aorta harvested from five or seven-month old male *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice was cut into two parts at the distal root of left subclavian artery. The aortic arch (from aortic sinus to cut) and thoraco-abdominal aorta (from cut to iliac bifurcation) were collected. The tissue was homogenized and total RNA was extracted using an RNeasy kit (Qiagen). RT-PCRs were performed using specific primers for murine TLR2, TLR4, and β-actin using previously described conditions[22].

Macrophage Stimulation

Since macrophage infiltration and foam cell formation occurs in the early stage (3 months of age) of atherosclerosis development *in vivo*[13, 20], we used macrophages isolated from 12-week old for *in vitro* assays. Thioglycollate-elicited peritoneal macrophages cultured in RPMI 1640 medium were incubated with the TLR2 agonist, SLTA (0.02 or 2 μg/ml; InvivoGen), or the TLR4 agonist, *Escherichia coli* LPS (10 or 100 ng/ml; InvivoGen) for 24 hours. After challenge, cells and cell culture supernatants were collected for FACS and ELISA assay, respectively. Macrophages cultured in medium alone served as a negative control.

Flow Cytometry Analysis

Macrophages cultured in 12-well plates for 24 hours with *S. aureus* lipoteichoic acid (SLTA) (2 μg/ml) or LPS (100 ng/ml) were washed and incubated with Fc blocker (eBioscience), followed by FITC-labeled anti-TLR-2 (6C2), PE-labeled anti-TLR-4 (MTS510), or each isotype-matched antibody (eBioscience). The cells were washed and 10,000 events were analyzed by flow cytometry using FACScan flowcytometer (Becton Dickinson).

Cytokine and Chemokine Measurements

Concentrations of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6,) and monocyte chemoattractant protein-1 (MCP-1) in the sera of each mouse, as well as the macrophage culture supernatant fluids, were determined using commercially available ELISA kits (BD Biosciences) as described per the manufacturer's instructions. Using these kits, the minimum detectable levels for TNF-α and IL-6 were 5 pg/ml and 3.8 pg/ml, respectively.

LDL Uptake and Foam Cell Formation

Thioglycollate-elicited peritoneal macrophages were isolated from 12 week-old male *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice and were cultured in RPMI 1640 medium containing 10% FBS. Macrophages cultured in 8-well chamber slides were incubated with 20 μg/ml DiI-labeled Acylated-LDL (Invitrogen) at 37°C for 24 hours. The cells were then washed 3 times with PBS and Ac-LDL uptake by macrophages was examined by fluorescence microscopy. Another set of DiI-labeled Ac-LDL-treated macrophages were stained with oil red O and examined by light microscopy to evaluate foam cell formation[23].

Statistics

All statistical analyses were performed using Prism software (GraphPad Software Inc, San Diego). Data were first evaluated by Kolmogorov-Smirnov normality test to verify normal distribution. Two-tail Student **t**-test or one-way ANOVA with Tukey-Kramer test were performed and a value of $P < 0.05$ was considered significant. Two-way ANOVA was used for analysis of plaque area between two of the three factors (genotype, sex and age), and their interaction with each other. A value of $P < 0.05$ was considered significant.

Results

Generation of *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ Mice

To examine the role of TLR2 on the formation of atherosclerosis, *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice were generated from heterozygote intercrosses of the *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$ mice. Generation of *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice was confirmed by genotyping of mice tail DNA by PCR (supplemental Fig. 1). The *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice showed similar body weight as the age-matched *Apoe*$^{-/-}$ mice (supplemental Fig. 2), and gross differences between *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice and their counterparts were not observed. Sex- and age-matched *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were fed a normal chow diet and were euthanized at the specified time for subsequent analysis.

TLR2 Deficiency Reduces Atherosclerotic Plaque Formation

Analysis of the aortic arch (Fig.1A and C) and whole aorta (supplemental Fig. 3A and B) revealed an overall reduction of atherosclerotic plaque area in *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice as compared to that in *Apoe*$^{-/-}$ mice at five-months of age. In addition, female *Apoe*$^{-/-}$ mice displayed more plaque accumulation than their genotype-matched male. The trend that *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice accumulated less atherosclerotic plaque than the age- and sex-matched *Apoe*$^{-/-}$ mice was more pronounced in seven-month old mice (Fig. 1B and D). Similar to our observation at the age of five-months, seven-month old mice presented with plaque accumulation predominantly in the aortic arch region and female *Apoe*$^{-/-}$ mice displayed more plaque accumulation than their male counterparts (supplemental Fig. 3).

TLR2 Plays An Important Role in Progression in Atherosclerotic Plaque Accumulation

The progression of plaque accumulation in the whole aorta (supplementary Fig. 3) was further analyzed by two-way ANOVA analysis (Table) with emphasis on the interaction between genotype and age in both male and female mice. In five-month old mice, *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice displayed 58.4 % or 51.34 % reduction of the plaque area in male and female mice, respectively. In seven-month old mice, the reduction was more pronounced. *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice displayed 69.5 % or 65.1 % reduction of the plaque area in male and female mice, respectively. Two-way ANOVA revealed that plaque accumulation was dependent on both genotype and age and they were significantly interacted. These results indicate that the effects of genotype are influenced by age. TLR2-dependent progression of plaque accumulation was more evident in seven-month old mice as compared to five-month old mice in both the male and female groups. In the context of a normal chow diet, no significant differences were observed in serum

cholesterol levels between *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$ mice (supplemental Fig. 4), indicating that the reduction in atherosclerosis plaque formation in TLR2-deficient mice was independent of systemic lipid levels.

TLR2 Deficiency Results in Diminished Expression of Atherosclerosis Markers in Plaques

To further examine the effect of TLR2 on the composition of atherosclerotic plaques, histochemical and immunohistochemical analysis of aortic sinus lesions were performed. Compared with *Apoe*$^{-/-}$ mice, *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice exhibited a marked reduction in intimal thickening in the aortic sinus lesions (Fig. 2A and B). Aortic sinus plaques of *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice exhibited less neutral lipid accumulation (Fig. 2C) than that of *Apoe*$^{-/-}$ mice. Compared with *Apoe*$^{-/-}$ mice, *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice also demonstrated less macrophage infiltration (Fig. 2D) and MCP-1 expression (Fig. 2E) in the endothelium and sub-endothelial lesions of aorta sinus plaque. No background staining was observed for the isotype control IgG (data not shown). Taken together, these data indicate that TLR2 deficiency resulted in reduced plaque formation, less neutral lipid accumulation, macrophage recruitment and MCP-1 expression in the aortic sinus.

Evaluation of Aortic TLR Expression and Inflammatory Mediator Levels in *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ Mice

To confirm there were no effects on TLR4 expression in the *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice, RT-PCR was performed on aortic tissues. Constitutive TLR2 gene expression was detected in the aorta of *Apoe*$^{-/-}$ mice, but not in *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice. The aortic arch, which is the predominant site of atherosclerotic lesions in *Apoe*$^{-/-}$ mice, exhibited higher levels of TLR2 gene transcripts than that of the thoraco-abdominal part of the aorta (Fig. 3A), in agreement with increased plaque observed in these regions in *Apoe*$^{-/-}$ mice. Similar levels of TLR4 gene expression was observed in the aorta of *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice, indicating deletion of TLR2 does not affect TLR4 expression in the aorta of *Apoe*$^{-/-}$ mice.

To examine whether deficiency of TLR2 has any effect on systemic inflammatory cytokine and chemokine production, ELISAs were performed to measure serum levels of TNF-α, IL-6 and MCP-1. TNF-α and IL-6 were not detected in the serum of *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice (data not shown). However, MCP-1 was detected at significantly lower levels in *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice than *Apoe*$^{-/-}$ mice (Fig. 3B).

Macrophage Responses Following Stimulation with Defined TLR Agonists in *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ Mice

Growing evidence has indicated that defined TLR ligands not only are able to activate TLR mediated pro-inflammatory signaling, but also can regulate TLR expression[24, 25]. Based on these observations, we performed *in vitro* experiments to evaluate macrophage responses to the TLR2 agonist, *S. aureus* lipoteichoic acid (SLTA) and the TLR4 agonist, *E. coli* lipoplysaccharide (LPS). By FACS analysis we observed that macrophages from *Apoe*$^{-/-}$ mice cultured with SLTA significantly up-regulated TLR2 expression. As expected, *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice failed to regulate TLR2 due to deletion of this gene (Fig. 4A). Similar levels of TLR4 expression were observed in *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mouse macrophages, with or without the TLR2 agonist stimulation (Fig. 4B). This suggests that TLR2 is up-regulated in response to a TLR2 pathogen-associated microbial product and that in *Apoe*$^{-/-}$ mice or *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice, TLR4 expression is not regulated via TLR2 signaling pathway. Consistent with this observation, macrophages isolated from *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice exhibited a significant deficiency of MCP-1 (Fig. 4C), and TNF-α and IL-6 (data not shown) production in response to SLTA stimulation.

Stimulation of macrophages with *E. coli* LPS significantly up-regulated TLR2 expression in *Apoe*$^{-/-}$ mice (Fig. 4D), which is consistent with previous findings[26]. This trend was not observed in *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice (Fig. 4D). However, no significant difference on TLR4 down-regulation in macrophages was observed in *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice (Fig. 4E). Macrophages isolated from *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice also exhibited similar levels of MCP-1 production in response to LPS stimulation (Fig. 4F).

These results, together with the *in vivo* data, suggest that TLR2 deficiency is associated with reduced MCP-1 production in response to a TLR2 ligand. This may contribute to the decreased inflammatory cell infiltration and plaque formation observed in *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice.

TLR2 Does Not Alter Macrophage Ac-LDL Uptake and Foam Cell Formation

Macrophage foam cell formation is believed to play an important role in the development of atherosclerosis[3]. To assess if *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ macrophages have a defect in low density lipoprotein (LDL) uptake, peritoneal macrophages from *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ mice were cultured with DiI-Ac-LDL. Fluorescence microscopy and oil red O staining revealed that macrophages from *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice displayed similar levels of Ac-LDL uptake (Fig. 5Aa) and foam cell formation (Fig. 5Ab). *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice macrophages exhibited similar levels of oil red O positive staining (Fig. 5B), indicating that TLR2 is not directly involved in macrophage Ac-LDL uptake and foam cell formation.

Discussion

In this study, we investigated the association between TLR2 and atherosclerosis using an *apoE* knockout mouse model. Mice fed a normal chow diet were used in this study to exclude the effect of dietary stresses on immunological reactions in atherosclerosis. We demonstrate that inactivation of TLR2 resulted in reduced progression of atherosclerotic plaque formation in both male and female *Apoe*$^{-/-}$ mice independent of dietary lipids. Likewise, TLR2 deficiency resulted in a reduction in lipid accumulation and decreased macrophage recruitment to the aortic sinus as well as reduced MCP-1 levels as compared with *Apoe*$^{-/-}$ mice. Furthermore, macrophages isolated from *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice demonstrated significantly reduced MCP-1 production upon stimulation with a TLR2 ligand. Taken together these results indicate that TLR2 signaling plays an important role in the development of atherosclerosis, probably through an increase in macrophage recruitment.

The role of macrophages and chemokines, especially MCP-1 on development of atherosclerosis has recently become a point of great interest[27, 28]. Clinically, significantly elevated serum MCP-1 levels have been observed in patients with coronary artery atherosclerosis[27, 28]. MCP-1 expression has been detected in human and experimental atherosclerosis, suggesting an active role for this molecule in monocyte recruitment[29, 30]. Inactivation of MCP-1, or its receptor, CCR2, resulted in markedly reduced macrophage accumulation and attenuated the progression of dietary-induced atherosclerosis in mice[31, 32]. Additionally, bone marrow transplantation studies revealed that over-expression of MCP-1 in macrophages led to increased foam cell formation and atherosclerosis in the irradiated hypercholesterolemic mice[33, 34]. These results together with the data presented in this study support a critical role for MCP-1 in the progression of atherosclerosis and suggest that low level of MCP-1 caused by TLR2 deficiency contributes to the reduced progression of atherosclerosis.

The role of TLRs in lipid uptake has been investigated in recent studies. Hoebe *et al.*[34] reported that TLR2 interacts with CD36 and regulates specific TLR2 ligand interactions, most specifically diacylglyceride recognition. It is well established that modified LDL including Ac- and oxidized (ox)-LDL are taken up via scavenger receptors including type A scavenger

receptor (SR-A) and CD36[35, 36]. Interestingly, Tamura *et al.*[37] reported that in addition to SR-A and CD36, scavenger receptor expressed by endothelial cells-1 (SREC-1) plays a role in macrophage Ac-LDL accumulation, particularly when cells are activated by LPS. As LPS is a defined TLR4 ligand and it has previously been reported that hyperlipidemic mice deficient in TLR4 present with reduced atherosclerosis[11], it is tempting to speculate that multiple TLRs may interact with multiple scavenger receptors and this level of recognition could represent a mechanism by which TLRs affect atherosclerotic plaque accumulation. However our studies indicate that TLR2 does not influence Ac-LDL uptake by murine macrophages.

Two recent studies have reported that genetic deficiency of MyD88, a TLR signaling adaptor molecule, reduces aortic atherosclerosis (~ 60%) in *Apoe*$^{-/-}$ mice fed high fat diet[10, 11]. Deletion of TLR4 was also shown to result in reduced atherosclerosis in *Apoe*$^{-/-}$ mice (~ 24% reduction). Our results are in agreement with these studies and further demonstrated an involvement for TLR2 in older mice fed a normal chow diet. In a recent study published while we were performing these studies, a role for TLR2 in atherosclerosis in a low-density lipoprotein receptor (LDLR)-null mice fed a high fat diet was reported[12]. These authors also demonstrated a role for bone marrow-derived cells (such as macrophages) in TLR2 mediated atherosclerosis formation. The studies here are distinct from these previous studies in that we 1) Used different mouse genetic backgrounds and thus different model systems, 2) Characterized the effect of TLR2 deficiency on the progression of atherosclerosis in *Apoe*$^{-/-}$ mice, and its association with local and systemic macrophages responses, in particular, MCP-1, 3) Assessed the sex-bias of the TLR2 mutation on atherosclerosis, and 4) Used a normal chow diet mice model. Our study demonstrated that TLR2 signaling plays an important role in progression of atherosclerosis and that the contribution of TLR2 was more apparent in older mice in both male and female groups. Most importantly our results indicate that an efficient innate immune defense system is associated with inflammatory progression of atherosclerosis and that this TLR2 dependent progression is independent of dietary lipids and macrophage LDL uptake.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

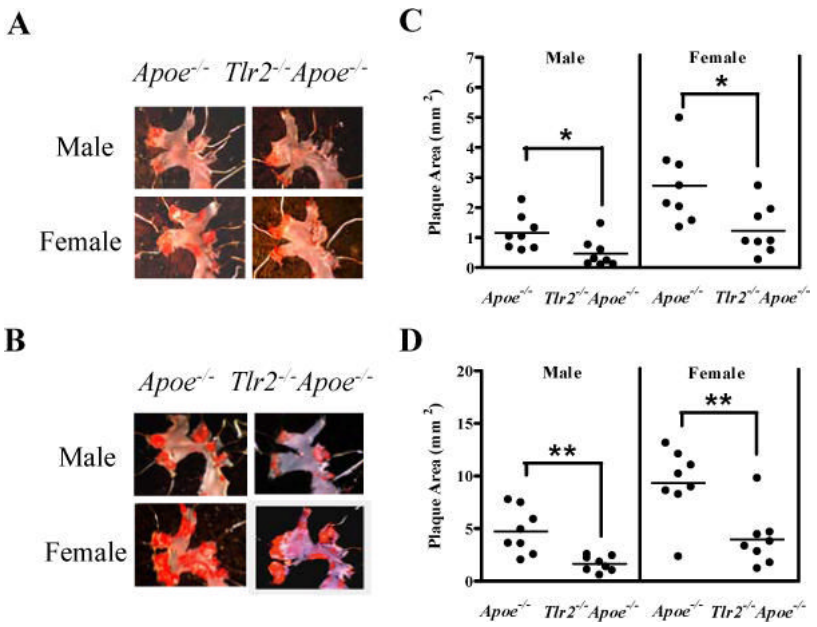

Figure 1. TLR2 deficiency reduces atherosclerosis in the *Apoe*$^{-/-}$ mice

After dissection, aortas were stained with Sudan IV to assess plaque area in the entire aorta. Representative micrographs of plaque accumulation of five-month old mice (A) and seven-month old mice (B) are shown. Quantified plaque area in aortic arch of five-month old mice (C) and seven-month old mice (D) are shown. Each point represents a single mouse and the horizontal line represents mean plaque area for each group (n = 8). ** $P < 0.005$; * $P < 0.02$ by two tail Student *t*-test.

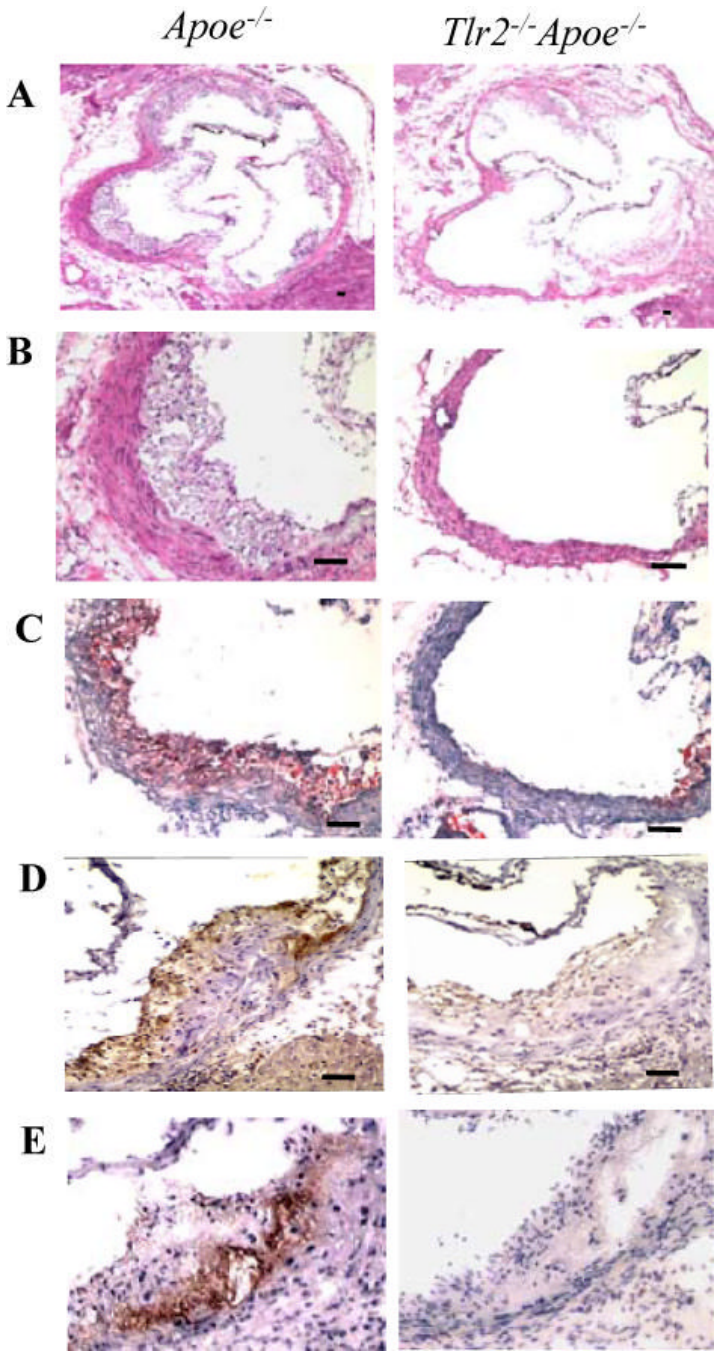

Figure 2. TLR2 deficiency reduces expression of atherosclerosis markers in the aortic sinus

Representative staining of aortic sinus plaques from *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ 5 month old female mice. Sections were stained with H&E (A, ×40, B, ×200 magnification), oil red O for neutral lipid (C, ×200), macrophage marker F4/80 (D, ×200) and mouse MCP-1 (E, ×200). The scale bar represents 100 μm.

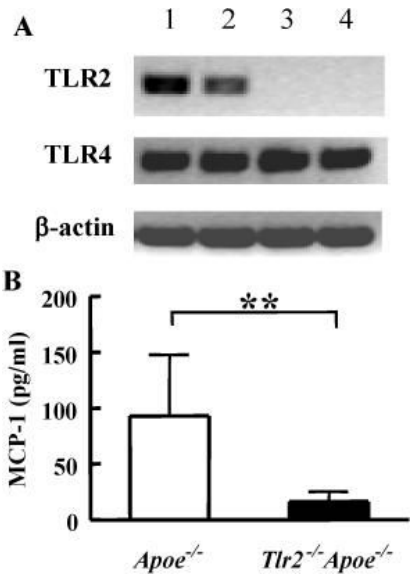

Figure 3. Evaluation of TLR expression and serum MCP-1 levels in *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice

(A) Representative RT-PCR from the aortic arch and thoraco-abdominal aorta of *Apoe*$^{-/}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ 5 month old male mice (similar results obtained from all genotype-matched mice, n=3/group). Lane 1, aortic arch of *Apoe*$^{-/-}$ mice; lane 2, thoraco-abdominal aorta of *Apoe*$^{-/-}$ mice; lane 3, aortic arch of *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice; lane 4, thoraco-abdominal aorta of *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice. (B) MCP-1 concentration in *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ sera of 5 month old male mice. Values expressed as mean ± SD (n = 8), ** $P < 0.01$ by two-tail Student's *t*-test.

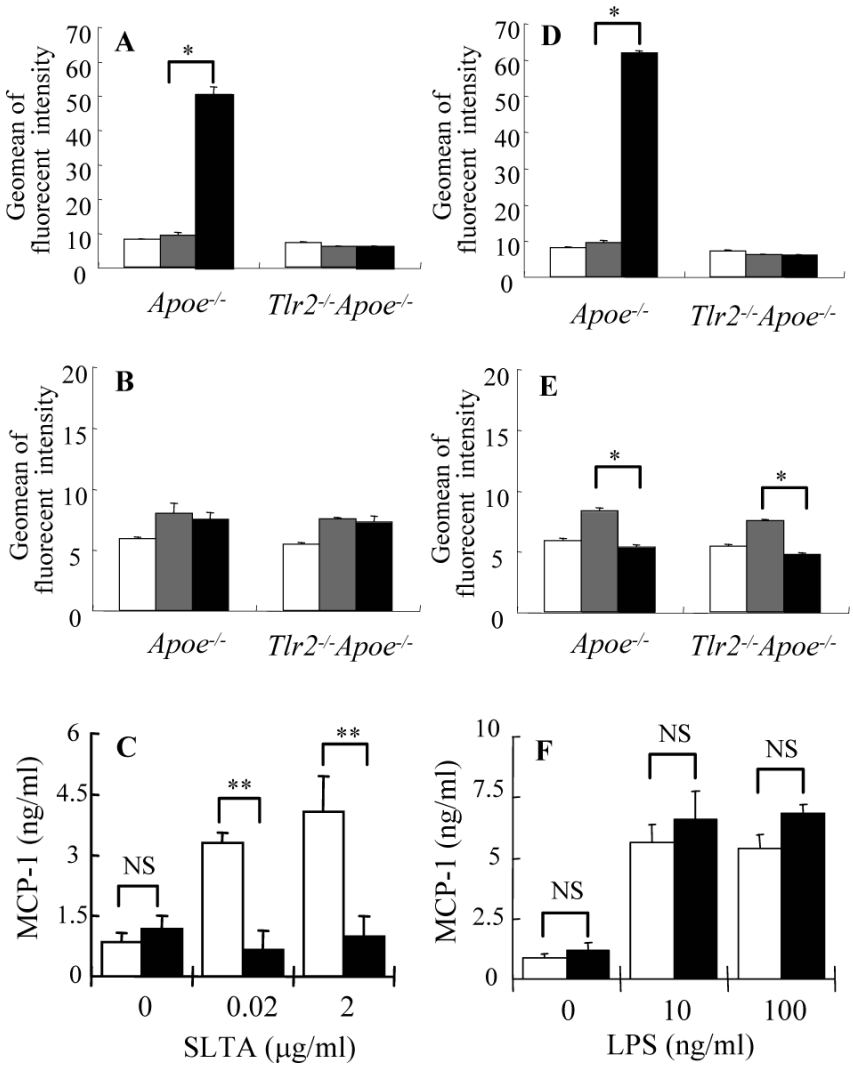

Figure 4. Loss of TLR2 up-regulation and macrophage response following stimulation with TLR agonists in *Apoe*$^{-/-}$ *Tlr2*$^{-/-}$ mice

Macrophages harvested from 12-week old *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice were cultured with 2 μg/ml of SLTA (black bars) (A, B, C), 100 ng of *E. coli* LPS (black bars) (D, E, F) or medium alone (gray bars) (A-D) for 24 hours. TLR2 (A, B) and TLR4 (C, D) surface expression was determined by FACS analysis using specific antibodies and isotype controls (white bars) (A-D). Values expressed as mean ± SD (n = 3), * $P < 0.01$ compared SLTA or *E. coli* LPS stimulation with medium alone by one-way ANOVA with Tukey-Kramer test. Production of MCP-1 by *Apoe*$^{-/-}$ (white bars) or *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ (black bars) mouse macrophages in response to SLTA (C) or LPS (F) stimulation was examined by ELISA. Values expressed as mean ± SD (n = 3), ** $P < 0.001$; NS = not significant by two-tail Student's *t*-test.

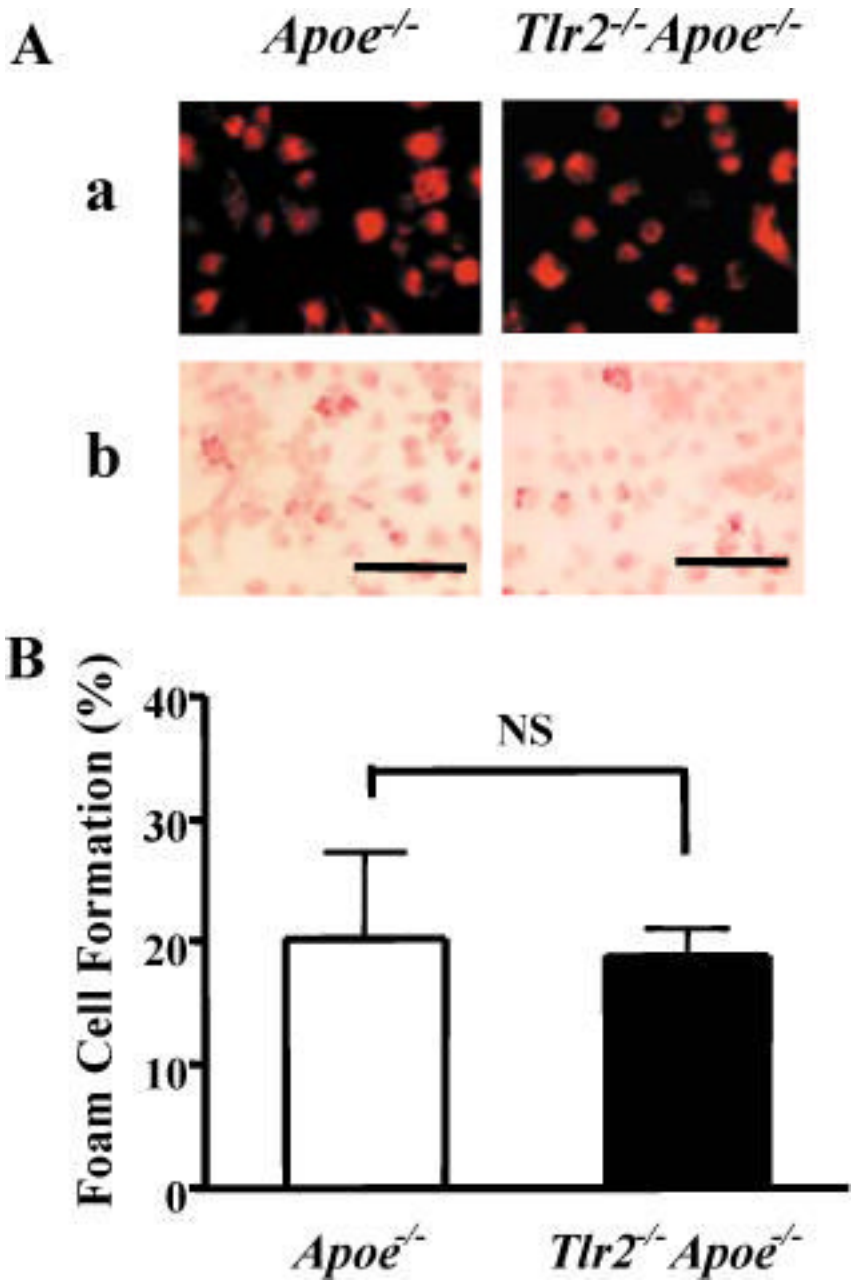

Figure 5. TLR2 does not affect Ac-LDL uptake and foam cell formation

Peritoneal macrophages harvested from 12-week old *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ male mice were incubated with 20 μg/ml DiI-labeled Ac-LDL at 37°C for 24 hours. (A) Ac-LDL uptake and foam cell formation was evaluated by fluorescence microscopy (a) and oil red O stain by light microscopy (b), respectively. The scale bar represents 100 μm. (B) The percentage of foam cells was determined by counting oil red O positive stained cells collected from three mice in ten random 200× magnification microscopic fields. Values expressed as mean ± SD, NS = not significant by two-tail Student's *t*-test.

TABLE

Involvement of TLR2 in the progression of **atherosclerotic plaque formation**

		aorta plaque area (mm^2)		
		5 month	**7 month**	**interaction**
Male	*Apoe*$^{-/-}$	1.32 ± 0.55	6.20 ± 3.05*	*P* = 0.0046
	Tlr2$^{-/-}$*Apoe*$^{-/-}$	0.55 ± 0.51 (58.4%)	1.89 ± 0.84*† (69.5%)	
Female	*Apoe*$^{-/-}$	3.10 ± 1.51	13.05 ± 4.62*	*P* = 0.0017
	Tlr2$^{-/-}$*Apoe*$^{-/-}$	1.59 ± 0.85 (51.34%)	4.56 ± 2.84*† (65.1%)	

Interaction; interaction between genotype and age.

Statistical analysis was performed by Two-way ANOVA.

(); reduction of % ratio in *Tlr2*$^{-/-}$/*Apoe*$^{-/-}$ against *Apoe*$^{-/-}$ mice.

* *P*<0.001 significant between 5 and 7-month old mice with same genotype.

† *P*<0.001 significant between *Apoe*$^{-/-}$ and *Tlr2*$^{-/-}$*Apoe*$^{-/-}$ mice with same age.