Toll-Like Receptor 7 Protects From Atherosclerosis by Constraining “Inflammatory” Macrophage Activation

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Background—Toll-like receptors (TLRs) have long been considered to be major culprits in the development of atherosclerosis, contributing both to its progression and clinical complications. However, evidence for most TLRs beyond TLR2 and TLR4 is lacking.

Methods and Results—We used experimental mouse models, human atheroma cultures, and well-established human biobanks to investigate the role of TLR7 in atherosclerosis. We report the unexpected finding that TLR7, a receptor recognizing self–nucleic acid complexes, is protective in atherosclerosis. In Apoe−/− mice, functional inactivation of TLR7 resulted in accelerated lesion development, increased stenosis, and enhanced plaque vulnerability as revealed by Doppler ultrasound and/or histopathology. Mechanistically, TLR7 interfered with macrophage proinflammatory responses to TLR2 and TLR4 ligands, reduced monocyte chemoattractant protein-1 production, and prevented expansion of Ly6C+ inflammatory monocytes and accumulation of inflammatory M1 macrophages into developing atherosclerotic lesions. In human carotid endarterectomy specimens TLR7 levels were consistently associated with an M2 anti-inflammatory macrophage signature (interleukin [IL]-10, IL-1RA, CD163, scavenger and C-type lectin receptors) and collagen genes, whereas they were inversely related or unrelated to proinflammatory mediators (IL-12/IL-23, interferon beta, interferon gamma, CD40L) and platelet markers. Moreover, in human atheroma cultures, TLR7 activation selectively suppressed the production of key proatherogenic factors such as monocyte chemoattractant protein-1 and tumor necrosis factor without affecting IL-10.

Conclusions—These findings provide evidence for a beneficial role of TLR7 in atherosclerosis by constraining inflammatory macrophage activation and cytokine production. This challenges the prevailing concept that all TLRs are pathogenic and supports the exploitation of the TLR7 pathway for therapy. (Circulation. 2012;126:952-962.)

Key Words: atherosclerosis ■ immune system ■ inflammation ■ macrophage ■ Toll-like receptor

Chronic inflammation is an integral part of the pathogenesis of atherosclerosis. Accumulation of lipoproteins in the vessel wall, especially at areas of disturbed blood flow such as bifurcations and the lesser curvature of the aortic arch, induces a chronic inflammatory response characterized by the mobilization of monocytes in the periphery, the infiltration of macrophages, dendritic cells, and lymphocytes in the arterial intima, and the expression of proinflammatory cytokines, chemokines, and matrix metalloproteinases. This leads to luminal narrowing and often plaque rupture and myocardial infarction or stroke, the most severe clinical complications of atherosclerosis. Therefore, identifying rate-limiting molecular processes and pathways that contribute to the development or persistence of inflammation in the vessel wall is key to the future treatment of this disease.

Clinical Perspective on p 962

Toll-like receptors (TLRs) have recently taken center stage in atherosclerosis research by virtue of their ability to drive sterile inflammation in the vessel wall. Under the current...
paradigm, TLRs promote atherogenesis through the disruption of endothelial cell integrity, the induction and sustained expression of inflammatory cytokines and chemokines, and the infiltration and activation of inflammatory monocytes/macrophages in developing plaques. In agreement with this model, TLRs (1, 2, 4, and 6) are increased in human atherosclerotic lesions, and TLR4 polymorphisms have been associated with the extent of atherosclerosis in some studies, although not others. Several endogenous TLR ligands relevant to atherosclerosis have also been described, including modified lipoproteins, oxidized lipids, and self-ligands released in response to hypercholesterolemia, tissue stress, or necrosis. Moreover, TLR2 signaling through myeloid differentiation factor 88 (MyD88) and nuclear factor kappa B has been shown to account for increased inflammation and matrix degradation in an ex vivo culture system of human atherosclerotic plaques, whereas functional TLR2, TLR4, and MyD88 have been demonstrated to be critically required for the development of atherosclerosis in experimental mouse models fed a high-fat diet. More recently, TLR6 has also been linked to the development of atherosclerosis by forming heterodimeric complexes with TLR4 involved in the recognition of oxidized lipoproteins. In contrast, genetic deletion of TLR3 has suggested a protective role of this receptor in arterial injury and early atherogenesis, whereas systemic administration of TLR3 agonists has yielded contradictory results, highlighting the need for more detailed investigation into the role of other TLRs in atherosclerosis.

TLR7 is an endosomal TLR that recognizes viral single-stranded RNA and self-RNA released from necrotic cells often complexed with cationic antimicrobial peptides such as LL37 and α-defensins or antibodies. It is expressed in subsets of monocytes, macrophages, dendritic cells, B cells, and eosinophils and is upregulated in intermediate and advanced atherosclerotic lesions of femoral arteries of patients with peripheral artery disease. The main known function of TLR7 is in antiviral immunity, although additional proinflammatory, anti-inflammatory, and immunoregulatory activities of TLR7 during chronic inflammation have also been described. Still, the functional role of TLR7 in atherosclerosis remains unknown. Here, we present the surprising finding that TLR7 is protective in atherosclerosis by shaping monocyte/macrophage function toward an alternatively activated antiatherogenic phenotype. This challenges the current paradigm that all TLRs are pathogenic in atherosclerosis and has broader implications for the role of innate immunity in this disease.

**Methods**

**Experimental Animals**

Apolipoprotein E-deficient (Apoe−/−) mice on a C57BL/6J background were crossed with TLR7-deficient (Tlr7−/−) mice originally obtained from Shizuco Akira (Osaka University) and backcrossed to the C57BL/6J background for >10 generations. Mice were fed a normal chow diet containing 18.5% protein and 5.5% fat (Harlan Tekland) and analyzed at various time points as indicated. For the assessment of macrophage responsiveness to TLR2 stimulation in vivo, Apoe−/− and Tlr7−/−/Apoe−/− mice were injected intraperitoneally with 20 μg endotoxin-free lipoteichoic acid (Invivogen) in 200 μL sterile PBS, euthanized after 3 hours, and serum collected. More details are available in the online-only Data Supplement.

**Doppler Ultrasonography**

Echocardiographic studies were performed in anesthetized mice using a Vivid 7, GE ultrasound system with a 13 MHz linear transducer and a 6 MHz–pulsed Doppler probe as detailed in the online-only Data Supplement.

**Analysis of Atherosclerotic Lesions**

Oil Red O (Sigma-Aldrich) and Picro-Sirius Red (Sigma-Aldrich) stained serial sections of the aortic valve, spanning a 500-μm area, and Sudan IV (Sigma-Aldrich) stained entire aortas were analyzed using the ImageJ software (Wayne Rasband).

**Immunofluorescence-Immunohistochemistry**

Mouse aortic sinus cryosections were stained with anti-mouse CD68 (clone FA-11; Serotec), alpha smooth muscle actin (clone 1A4, Sigma-Aldrich), inducible nitric oxide synthase (iNOS; ab15323, Abcam), or isotype control monoclonal antibodies and counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes). Positive staining areas or colocalization were quantified by use of the ImageJ software (Wayne Rasband). Detailed protocols are available in the online-only Data Supplement.

**Quantification of Plaque Necrosis**

Plaque necrosis was determined by drawing boundary lines around regions free of 4′,6-diamidino-2-phenylindole staining and quantifying the region area by using the ImageJ analysis software (Wayne Rasband). A 3000-μm threshold was implemented to avoid counting regions that may not represent substantial areas of necrosis.

**Flow Cytometry**

Blood and spleen were processed and stained with fluorochrome-conjugated monoclonal antibody combinations for CD11b, Ly6C, CD45, and appropriate IgG isotype controls (eBioscience). Flow cytometry was performed on a Beckman-Coulter FC-500 analyzer, and data were analyzed by using Kaluza software (Beckman Coulter).

**Serum Measurements**

Serum concentrations of total cholesterol and triglycerides were determined by use of kits from Cayman Chemicals. Serum cytokines were measured by ELISA by using commercially available kits (eBioscience).

**Isolation of mRNA and Quantitative Real-Time Polymerase Chain Reaction**

Real-time quantitative polymerase chain reaction was performed with SYBR GreenER qPCR SuperMix Universal (Invitrogen). Target mRNA levels were expressed relative to GAPDH. Primer sets are shown in the online-only Data Supplement.

**Peritoneal Macrophage Activation**

Peritoneal macrophages from Apoe−/− and Tlr7−/−/Apoe−/− mice were cultured with various doses of lipoteichoic acid and supernatants measured after 20 hours by ELISA using commercially available kits (eBioscience).
Vascular Specimens and Genome-Wide Expression Array Studies

Genome-wide expression array studies were conducted in human carotid plaque samples from the Biobank of Karolinska Endarterectomies (BiKE) study. In brief, carotid lesions (n=127) and control tissue obtained from normal arteries of organ donors (n=10) were analyzed by using Affymetrix HG-U133A Genechip arrays (http://www.affymetrix.com). More detailed information is provided in the online-only Data Supplement.

Human Atheroma Cell Cultures

Single-cell suspensions from carotid endarterectomies from patients undergoing revascularization procedures for symptomatic carotid disease at Charing Cross Hospital, London, were obtained as previously described. Cells were cultured in the presence or absence of 1 μg/mL imiquimod (Invivogen, CA) and cytokine levels quantified by Luminex 100 by using Fluorokine Multianalyte kits from R&D Systems.

Statistical Analysis

Statistical significance of differences was assessed using the parametric Student t test for normally distributed data and the nonparametric Mann-Whitney U (MWU) test for skewed data that deviate from normality. For associations between TLR7 levels and macrophage markers, the Pearson correlation coefficient was used. Differences were considered significant when P<0.05.

Results

Functional Inactivation of TLR7 Exacerbates Atherosclerosis in Apoe<sup>−/−</sup> Mice

To examine whether TLR7 is involved in the pathogenesis of atherosclerosis, we generated Trl<sup>−/−</sup> Apoe<sup>−/−</sup> and compared them with Apoe<sup>−/−</sup> mice (online-only Data Supplement Figure I). Both Trl<sup>−/−</sup> Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice appeared healthy, reproduced according to Mendelian ratios and exhibited no obvious abnormalities (data not shown). There was no significant difference on weight gain or triglyceride levels between the 2 groups, whereas cholesterol levels were reduced in Trl<sup>−/−</sup> Apoe<sup>−/−</sup> mice (online-only Data Supplement Table I). However, Trl<sup>−/−</sup> Apoe<sup>−/−</sup> mice developed substantially larger atherosclerotic lesions as they aged. Quantification of Oil Red O staining at the level of the aortic sinus revealed a significant increase in lesion size in 18- and 26-week but not 10-week old Trl<sup>−/−</sup> Apoe<sup>−/−</sup> mice in comparison with Apoe<sup>−/−</sup> controls (Figure 1A and 1B). This was accompanied by similarly increased accumulation of CD68<sup>+</sup> macrophages (Figure 1C). En face staining with Sudan IV of the aorta also revealed a substantial increase of the total lesion area in Trl<sup>−/−</sup> Apoe<sup>−/−</sup> mice in comparison with Apoe<sup>−/−</sup> controls (Figure 1D). Finally, Doppler ultrasound analysis showed increased mean and peak carotid velocity indices in Trl<sup>−/−</sup> Apoe<sup>−/−</sup> mice in comparison with Apoe<sup>−/−</sup> mice (Table), in agreement with enhanced atherosclerosis. In contrast, there was no statistically significant difference in overall left ventricular function between the 2 groups, although an increase in left ventricular end diastolic diameter was apparent in Trl<sup>−/−</sup> Apoe<sup>−/−</sup> mice (Table). Posterior wall thickness in diastole did not differ between the 2 groups either, suggesting that chronic left ventricular outflow obstruction was not present. Consistently, the r/h ratio, which is an indicator of left ventricular wall stress, was not changed. All transvalvular aortic blood flow indices were also similar between the 2 groups. Taken together, these observations demonstrate that functional TLR7 is essential for limiting experimental atherosclerosis in mice.

Functional Inactivation of TLR7 Enhances Features of Plaque Vulnerability in Apoe<sup>−/−</sup> Mice

Although lesion size accurately reflects atherosclerosis progression, plaque morphology is a more important predictor of
Inflammatory Ly6Chi monocytes in lesion instability in findings suggest that deficient TLR7 function also promotes lesion vulnerability. We found that plaques from 26-week-old Tlr7<sup>−/−</sup> mice exhibited increased lipid content (Figure 2A) and were enriched in CD68<sup>+</sup> macrophages in comparison with Apoe<sup>−/−</sup> mice (Figure 2B). In agreement with that, the presence of alpha smooth muscle actin positive cells was reduced in Tlr7<sup>−/−</sup> mice (Figure 2C) as was the accumulation of collagen and the thickness of the fibrous cap (Figure 2D). It is noteworthy that Tlr7<sup>−/−</sup> mice also exhibited increased necrotic core size defined as acellular 4',6-diamidino-2-phenylindole<sup>−</sup> areas containing remnants of cells and extracellular lipid (Figure 3). Because enhanced plaque necrosis, lipid deposition, and macrophage infiltration, reduced smooth muscle cell and collagen presence, and fibrous cap formation are features attributed to a more vulnerable plaque phenotype, these findings suggest that deficient TLR7 function also promotes lesion instability in Apoe<sup>−/−</sup> mice.

**Table. Ultrasonographic Evaluation of Tlr7<sup>−/−</sup> Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> Mice**

<table>
<thead>
<tr>
<th></th>
<th>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Tlr7&lt;sup&gt;−/−&lt;/sup&gt; Apoe&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>P</th>
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<tr>
<td>N</td>
<td>11</td>
<td>11</td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>619±32</td>
<td>617±68</td>
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<td>EDD, mm</td>
<td>3.15±0.24</td>
<td>3.43±0.26</td>
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<td>ESD, mm</td>
<td>1.81±0.20</td>
<td>1.94±0.27</td>
<td>0.23</td>
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<tr>
<td>FS, %</td>
<td>42.53±3.76</td>
<td>43.63±5.00</td>
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<td>PWT, mm</td>
<td>0.71±0.05</td>
<td>0.72±0.04</td>
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<tr>
<td>r/h</td>
<td>4.02±0.35</td>
<td>4.25±0.47</td>
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<tr>
<td>Peak aortic velocity, cm/s</td>
<td>99.88±13.04</td>
<td>100.64±13.45</td>
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<tr>
<td>Mean aortic velocity, cm/s</td>
<td>56.09±14.44</td>
<td>56.36±10.99</td>
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</tr>
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<td>Stroke distance, cm</td>
<td>2.55±0.79</td>
<td>2.34±0.77</td>
<td>0.0513</td>
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<tr>
<td>Peak aortic acceleration, m/s&lt;sup&gt;2&lt;/sup&gt;</td>
<td>121.31±7.48</td>
<td>126.36±8.98</td>
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<td>Peak carotid velocity, cm/s</td>
<td>68.73±9.32</td>
<td>91.09±11.32</td>
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<tr>
<td>Mean carotid velocity, cm/s</td>
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<td>40.36±8.46</td>
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<tr>
<td>Carotid pulsatility index</td>
<td>2.55±0.79</td>
<td>2.42±0.56</td>
<td>0.67</td>
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</tbody>
</table>

Values are mean±SD. EDD indicates end diastolic diameter; ESD, end systolic diameter; FS, fractional shortening; PWT, posterior wall thickness; and r/h, ratio of left ventricular (LV) radius to PWT. Statistical significance values (P) are indicated.

**Figure 2.** TLR7 deficiency promotes a more vulnerable phenotype of atherosclerotic lesions. Tlr7<sup>−/−</sup> Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice were fed a normal chow diet and analyzed at 26 weeks of age. A, Representative light photomicrographs and morphometric analysis of Oil Red O–stained sections from the aortic root. Results show the mean positive area±SEM of 12 to 14 mice/group expressed as percentage of the total lesion area. B, Representative fluorescent photomicrographs and morphometric analysis of CD68<sup>+</sup>–stained sections from the aortic root. Results show the mean positive area±SEM of 12 to 16 mice/group expressed as percentage of the total lesion area. C, Representative fluorescent photomicrographs and morphometric analysis of alpha smooth muscle actin–stained sections from the aortic root. Results show the mean positive area±SEM of 7 to 10 mice/group expressed as percentage of the total lesion area. D, Representative polarized light photomicrographs and morphometric analysis of Picro Sirius Red–stained sections from the aortic root. Results show the mean positive area±SEM of n=6 to 10 mice/group expressed as percentage of the total lesion area. Statistical significance values (p) are indicated. TLR7 indicates Toll-like receptor 7.
cytes both in the blood (Figure 4B) and spleen (Figure 4C) in comparison with Apoe−/− mice, whereas the frequency of Ly6C− monocytes was reduced. The less abundant Ly6Cint monocyctic population was also reduced. This was associated with increased accumulation of inflammatory M1 macrophages in the aorta of Tlr7−/−/Apoe−/− mice as revealed by the higher levels of proinflammatory cytokines and M1 macrophage markers such as IL-12/23p40, TNF, MCP-1, and iNOS (Figure 4D and 4E).35,36 In contrast, there were no differences observed in the expression of the anti-inflammatory cytokine IL-10 or the M2 markers Arg1 or Ym1 between the 2 groups (Figure 4D), whereas differences in tissue remodeling factors and enzymes were also seen (online-only Data Supplement Figure II). IL-23p19 and IL-17A were not detectable (data not shown). These data indicate that TLR7 acts as a brake to limit inflammatory monocyte/macrophage differentiation and activation in atherosclerosis.

TLR7 Is Essential for Constraining Inflammatory Monocyte/Macrophage Activation and Cytokine Production

We next investigated how TLR7 affects macrophage function and proinflammatory cytokine production in the context of atherosclerosis. Surprisingly, we found that macrophages from Tlr7−/−/Apoe−/− mice exhibited increased responsiveness to TLR2 or TLR4 stimulation in comparison to Apoe−/− controls. When stimulated with the TLR2 ligand lipoteichoic acid or the TLR4 ligand lipopolysaccharide, Tlr7−/−/Apoe−/− macrophages produced higher levels of proinflammatory cytokines, with MCP-1 and IL-6 being the ones most increased at lower ligand concentrations (Figure 5A and online-only Data Supplement Figure III). Notably, increased responsiveness was seen in vivo as administration of lipoteichoic acid to Tlr7−/−/Apoe−/− mice triggered higher production of proinflammatory cytokines such as MCP-1, IL-12/23p40, and IL-6 (but not IL-10) in the serum in comparison with Apoe−/− mice (Figure 5B). This was not due to differences in the expression of TLR2 or TLR4 nor critical downstream signaling components such as MyD88, Mal/TIRAP, TRIF, TRAM, IRAK1, IRAK2, Tollip, TRAF6, IKK2, or nuclear factor kappa B genes between the 2 groups (online-only Data Supplement Figure IV), although the induction of a compensatory TLR response in the absence of functional TLR7 is still a possibility. Because TLR2 and TLR4 are critically involved in macrophage activation during the development/progression of atherosclerosis,12–17 and MCP-1 is a rate-limiting chemokine for atherogenesis,29,37 these findings suggest that TLR7 limits the development of atherosclerosis by interfering with monocyte/macrophage activation and MCP-1 production.

TLR7 Is Associated With M2 Macrophage Markers and a Less Inflammatory Plaque Phenotype in Human Atherosclerotic Lesions

Finally, we investigated the role of TLR7 in human atherosclerosis. Large-scale transcriptional profiling analysis of human atheromata from the Biobank of the Karolinska Endarterectomies study (BiKE)26 and the Tampere Vascular Study (TVS)38 revealed that TLR7 was consistently upregulated at the mRNA level in atherosclerotic carotid and femoral arteries, and abdominal aortas in comparison with normal iliac arteries in BiKE or internal thoracic arteries in TVS (Figure 6A and online-only Data Supplement Table II). In BiKE, where several clinical variables were available for each patient, TLR7 expression was found to be reduced on active cigarette smoking (Figure 6B) but was unaffected by diabetes, hypertension, obesity, sex, history of symptoms, or medication (online-only Data Supplement Table III). TLR7 mRNA levels strongly correlated with the pan-leukocyte...
marker CD45 and the monocyte/macrophage marker CD14, exhibited a weak correlation with the T-cell marker CD3, and the mast cell marker TPSAB1, and lacked correlation with the B-cell marker CD19 and the smooth muscle cell marker alpha smooth muscle actin, suggesting that TLR7 expression in plaques is mostly derived from CD14/H11001 monocytes/macrophages (online-only Data Supplement Figure V).

We next examined whether TLR7 expression was functionally important. We found that addition of the specific TLR7 agonist imiquimod to mixed cell cultures from carotid endarterectomy specimens10,11 inhibited the expression of MCP-1 and TNF, which are associated with a more inflammatory macrophage phenotype, whereas the expression of IL-10 was spared (Figure 6C). IL-12 and IL-23 were below the detection limits. Consistently, in human atheromata from BiKE, TLR7 mRNA levels were positively associated with the expression of M2 macrophage markers such as the anti-inflammatory cytokine IL-10, IL-1 receptor antagonist (IL-1RN), CD163, scavenger receptors, and C-type lectin receptors (Figure 6D), whereas they were inversely related to proinflammatory cytokines and M1 macrophage markers such as IL-23p19, iNOS, and interferon beta, and lacked any association with IL-12p35, IL-12/23p40, and interferon gamma (Figure 6D). Additionally, TLR7 mRNA levels were positively associated to collagen genes (Col1A1, Col3A1, Col4A1), fibronectin-1, fibrillin-1, and tissue plasminogen activator (PLAT) expression, and negatively related to platelet markers and CD40 ligand (Figure 6E). Despite these

Figure 4. Tlr7/H11002/Apoel11002 mice exhibit higher MCP-1 production and increased inflammatory monocyte/macrophage levels. Tlr7/H11002/Apoel11002 and Apoel11002 mice were fed a normal chow diet. A, Serum concentration of MCP-1 in 12-week-old Tlr7/H11002/Apoel11002 and Apoel11002 mice in the absence of exogenous stimulation. Results are expressed as mean levels±SEM of 5 mice/group. B, Relative frequency of Ly6C+/H11002 (gate III), Ly6C-/H11002 (gate II), and Ly6C-/H11002 (gate I) monocyte subsets in the blood of 12-week-old Tlr7/H11002/Apoel11002 and Apoel11002 mice. Results are expressed as mean percentage of CD115+/CD11b+ monocytes±SEM from 7 to 9 mice/group. C, Relative frequency of Ly6C+/H11002 (gate III), Ly6C-/H11002 (gate II), and Ly6C-/H11002 (gate I) monocyte subsets in the spleen of 12-week-old Tlr7/H11002/Apoel11002 and Apoel11002 mice. Results are expressed as mean percentage of CD115+/CD11b+ monocytes±SEM from 7 to 9 mice/group. D, mRNA expression levels of inflammatory cytokines and M1 macrophage markers in entire aortas of 26-week-old Tlr7/H11002/Apoel11002 and Apoel11002 mice. Results are expressed as mean levels±SEM of 6 to 10 mice/group relative to GAPDH. E, Representative fluorescent photomicrographs and morphometric analysis of iNOS (green) and CD68 (red) stained sections from the aortic root of 26-week-old mice. Arrowheads indicate iNOS+/CD68+ double-positive areas (yellow). Results show mean iNOS+/CD68+ area±SEM of 8 to 12 mice/group expressed as percentage of the total CD68+ area. Statistical significance values (p) are indicated. MCP-1 indicates monocyte chemoattractant protein-1; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; SSC, side scatter; and FSC, forward scatter.
findings, TLR7 mRNA expression was not associated with a more stable/fibrotic histopathologic plaque phenotype in either the TVS or the University of Athens carotid endarterectomies cohort (online-only Data Supplement Figure VI). Taken together, these findings suggest a link between higher TLR7 expression and reduced inflammation, decreased presence of platelets, and increased deposition of collagen and extracellular matrix, all predictive markers of a more stable plaque phenotype,27,28,39 and highlight the need for further studies into the role of TLR7 in plaque vulnerability.

Discussion

Although TLRs have been incriminated for the development of atherosclerosis, evidence for most TLRs beyond TLR2 and TLR4 is lacking. We now reveal that TLR7 is upregulated in vulnerable plaque phenotype,27,28,39 and highlight the need for further studies into the role of TLR7 in plaque vulnerability.

Figure 5. Tlr7−/− Apoe−/− mice exhibit increased responsiveness to TLR2 ligands and higher production of inflammatory cytokines. Tlr7−/− Apoe−/− and Apoe−/− mice were fed a normal chow diet and analyzed at 12 weeks of age. A, Production of MCP-1, IL-6, IL-10, and TNF in supernatants of Tlr7−/− Apoe−/− and Apoe−/− peritoneal macrophages cultured for 20 hours with the TVS ligand LTA. IL-12/23p40 was not detectable. Results are expressed as mean levels±SEM of quadruplicate cultures. One representative from 4 independent experiments is shown. B, Serum concentration of MCP-1, IL-6, IL-10, IL-12/23p40, and TNF in Tlr7−/− Apoe−/− and Apoe−/− mice after intraperitoneal treatment with the TLR2 ligand LTA for 3 hours. IL-10 was not detectable. Results are expressed as mean levels±SEM of 5 mice/group. Statistical significance values (p) are indicated. MCP-1 indicates monocyte chemoattractant protein-1; TNF, tumor necrosis factor; IL, interleukin; LTA, lipoteichoic acid; TLR2, Toll-like receptor 2.
Tlr7−/− vs. Apoe−/− mice and Tlr7−/− vs. Apoe−/− macrophages exhibit increased production of MCP-1, whereas TLR7-stimulated human carotid lesions from the Biobank of the Karolinska Endarterectomies study (BiKE) were used. Mean expression levels in arbitrary log2 U (AU) and statistical significance values (p) are shown. B, Comparison of TLR7 mRNA expression from nonsmokers, active, and former smokers in human carotid lesions from the Biobank of the Karolinska Endarterectomies study (BiKE). Mean expression levels in arbitrary log2 U (AU) and statistical significance values (p) are shown. C, TLR7 activation through the addition of 1 μg/mL imiquimod (TLR7-L) reduces TNF and MCP-1 production in human atheroma cell cultures. Results are expressed as mean cytokine levels±SEM of cultures from 3 unrelated patients per group. D, Correlation between TLR7 mRNA levels and established M1/M2 macrophage markers in human carotid lesions from BiKE. Pearson correlation coefficient (r) and statistical significance values (p) are shown. E, Correlation between TLR7 mRNA levels and genes involved in collagen and ECM deposition, smooth muscle cell presence, and platelet accumulation in human carotid lesions from BiKE. Pearson correlation coefficient (r) and statistical significance values (p) are shown. MCP-1 indicates monocyte chemoattractant protein-1; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; IFN, interferon; TPA, tissue plasminogen activator; TLR7, Toll-like receptor 7; ECM, extracellular matrix.

Figure 6. TLR7 is expressed in human carotid atherosclerotic lesions and is associated with genes related to a more stable plaque phenotype. A, Comparison of TLR7 mRNA expression between human atherosclerotic tissue specimens and specimens from a normal artery wall. Specimens from patients undergoing carotid endarterectomy and specimens from healthy iliac arteries of organ donors from the Biobank of the Karolinska Endarterectomies study (BiKE) were used. Mean expression levels in arbitrary log2 U (AU) and statistical significance values (p) are shown. B, Comparison of TLR7 mRNA expression between human carotid atherosclerotic tissue specimens and specimens from a normal artery wall. Specimens from patients undergoing carotid endarterectomy and specimens from healthy iliac arteries of organ donors from the Biobank of the Karolinska Endarterectomies study (BiKE) were used. Mean expression levels in arbitrary log2 U (AU) and statistical significance values (p) are shown. C, TLR7 activation through the addition of 1 μg/mL imiquimod (TLR7-L) reduces TNF and MCP-1 production in human atheroma cell cultures. Results are expressed as mean cytokine levels±SEM of cultures from 3 unrelated patients per group. D, Correlation between TLR7 mRNA levels and establishment M1/M2 macrophage markers in human carotid lesions from BiKE. Pearson correlation coefficient (r) and statistical significance values (p) are shown. E, Correlation between TLR7 mRNA levels and genes involved in collagen and ECM deposition, smooth muscle cell presence, and platelet accumulation in human carotid lesions from BiKE. Pearson correlation coefficient (r) and statistical significance values (p) are shown. MCP-1 indicates monocyte chemoattractant protein-1; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; IFN, interferon; TPA, tissue plasminogen activator; TLR7, Toll-like receptor 7; ECM, extracellular matrix.

Thus, TLR2 and TLR4 constitute the most likely triggers of MCP-1 production in vivo.

TLR7-mediated protection also involves modulation of macrophage polarization. Inflammatory M1 macrophages and M1-associated cytokines contribute to the development and vulnerability of plaques, whereas M2 macrophages are considered to be beneficial through the paracrine anti-inflammatory effects they exert on M1 macrophages and the improved clearance of apoptotic cell debris.42,43 Accordingly, the M1/M2 ratio is proportional to the progression of lesion size in Apoe−/− mice,35 whereas factors that promote a proinflammatory M1 macrophage phenotype such as TLR2 and TLR4 ligands or G2A deficiency result in an increased disease burden in the same mice.12,15,36,44 Thus, the increased expression of inflammatory cytokines and M1 macrophage markers in Tlr7−/− vs. Apoe−/− mice and the positive association of TLR7 levels with M2 macrophage markers in human carotid plaques reveal an additional mechanism of atheroprotection promoted by TLR7. Although this may be directly related to the levels of inflammatory Ly6C+ monocytes (as observed in Apoe−/− mice), which produce high levels of...
M1-type inflammatory cytokines and are therefore likely precursors of inflammatory M1 macrophages, further studies will be needed to confirm this possibility.

A role of TLR7 in M2 macrophage polarization may at first seem intriguing. However, it is not without precedent. Repetitive triggering of TLR7 in mice has been shown to suppress the production of proinflammatory M1 cytokines such as IL-12/23p40, IL-6, and TNF without affecting, or in some cases even increasing, the production of IL-10. TLR7 stimulation has also been shown to induce cross-tolerance to subsequent TLR2 and TLR4 stimulation that lasts for days. In addition, combination of a TLR ligand and immune complexes, prostaglandins, apoptotic cells, IL-10, or adenosine, all signals relevant to atherosclerosis, has been shown to induce the generation of “regulatory” M2-like macrophages characterized by high expression of CD163 and IL-10, low levels of TNF and IL-1β, and absence of IL-12 and IL-23. It is therefore possible that, in the context of atherosclerosis, TLR7 favors the generation of M2 macrophages as part of a protective immune response aiming at restoring homeostasis in the vessel wall.

Our findings broaden our understanding of the role of innate immune receptors in the development of atherosclerosis. It has been well established over the past few years that TLR2 and TLR4 respond to danger signals induced during hypercholesterolemia or tissue stress to promote macrophage accumulation and inflammation in the vessel wall. TLR6 is also part of this process by acting as the binding partner of TLR2 in the detection of oxidized lipoproteins. This response is further enhanced through the recognition of cholesterol crystals by the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome. We now expand this scheme by adding TLR7, a receptor that senses viral and self–single-stranded RNA. We show that TLR7 inhibits atherosclerosis and promotes plaque stabilization by interfering with macrophage activation and accumulation into developing atherosclerotic lesions. This is complemented by our recent finding that TLR3 may also exert beneficial effects in atherosclerosis, and raises the possibility that this protective immune response that, at the protein level and in other cohorts, such observations may differ. Thus, more studies using carefully defined and larger cohorts of patients will be needed to ultimately determine whether increased TLR7 expression also translates to fewer acute clinical events in humans.

In summary, our study extends current knowledge on the role of innate immunity in atherosclerosis. It adds into the frame TLR7, a receptor that plays a protective role by limiting inflammatory macrophage activation and atherosclerotic lesion development and vulnerability. This challenges the prevailing concept that all TLRs are pathogenic in atherosclerosis and exposes the need for more detailed investigation of the role of innate immunity receptors and pathways in this disease. Unraveling the complex molecular machinery involved in TLR7-mediated atheroprotection should therefore be of great value for understanding homeostasis of the vessel wall and for identifying regulatory steps of therapeutic potential.

Acknowledgments

We thank Drs S. Konstantinides and P. Sideras for helpful discussions and suggestions, Drs S. Pagakis and E. Rigana for confocal imaging, and Prof J. D’hooge, Dr S. Tombeur, and Dr K. Picassetos for advice on mouse echocardiography. We also thank Drs K. Evangelou and A. Kotsinas for histopathology.

Sources of Funding

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Disclosures

None.

References


Cancer therapy with a small molecule agonist of Toll-like receptor 7 can be improved by circumventing TLR tolerance. *Cancer Res.* 2011;71:5123–5133.


### CLINICAL PERSPECTIVE

Atherosclerosis underlying cardiovascular mortality is the leading cause of death in developed countries. Efforts are therefore concentrating on unwinding the pathophysiological mechanisms controlling its development and clinical complications. Among them, Toll-like receptors (TLRs) have taken center stage in atherosclerosis research by virtue of their ability to sense danger in response to hypercholesterolemia, tissue stress, or necrosis, and drive macrophage activation and inflammation in the vessel wall. TLR2 and TLR4, in particular, have been shown to play a critical role in promoting plaque development and vulnerability leading to the view that all TLRs are pathogenic for this disease. This article now reports the surprising finding that TLR7, an endosomal TLR that recognizes viral single-stranded RNA and self-RNA released from necrotic cells, is protective. In experimental atherosclerosis in mice, TLR7 prevented lesion development, stenosis, and plaque vulnerability by constraining monocyte chemoattractant protein-1 production, Ly6Ch “inflammatory” monocyte expansion and M1 inflammatory macrophage accumulation to developing atherosclerotic lesions. In human carotid endarterectomy specimens, TLR7 was positively associated with an M2 anti-inflammatory macrophage signature and collagen genes and inversely related/unrelated to proinflammatory mediators and platelet markers, whereas TLR7 activation in human atheroma cultures selectively suppressed the production of monocyte chemoattractant protein-1. Altogether, these findings reveal that TLR7 is part of a protective response that limits atherosclerotic plaque development and vulnerability and challenge the prevailing concept that all TLRs are pathogenic. They also provide new insight about the complex interplay of innate immunity in atherosclerosis and support the exploitation of the TLR7 pathway for therapy.
Toll-Like Receptor 7 Protects From Atherosclerosis by Constraining "Inflammatory" Macrophage Activation

Maria Salagianni, Ioanna E. Galani, Anna M. Lundberg, Constantinos H. Davos, Aimilia Varela, Ariana Gavriil, Leo-Pekka Lyytikäinen, Terho Lehtimäki, Fragiska Sigala, Lasse Foleksens, Vassilis Gorgoulis, Sébastien Lenglet, Fabrizio Montecucco, François Mach, Ulf Hedin, Göran K. Hansson, Claudia Monaco and Evangelos Andreakos

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SUPPLEMENTAL MATERIAL

FOR

Toll-like receptor 7 protects from atherosclerosis by constraining ‘inflammatory’ macrophage activation

Maria Salagianni, PhD; Ioanna E. Galani, PhD; Anna M. Lundberg, PhD; Constantinos H. Davos, MD, PhD; Aimilia Varela, BSc; Arianna Gavriil, PhD; Leo-Pekka Lyytikäinen, MD; Terho Lehtimäki, MD, PhD; Fragiska Sigala, MD; Lasse Folkers, MD; Vassilis Gorgoulis, MD, PhD; Sébastien Lenglet, PhD; Fabrizio Montecucco, MD, PhD; François Mach, MD, PhD; Ulf Hedin, MD, PhD; Göran K. Hansson, MD, PhD; Claudia Monaco, MD, PhD and Evangelos Andreakos, PhD

1Center for Immunology and Transplantation, Biomedical Research Foundation, Academy of Athens, Athens, Greece; 2Center for Molecular Medicine, Department of Medicine at Karolinska University Hospital Solna, Karolinska Institutet, Stockholm, Sweden; 3Center for Clinical Research, Biomedical Research Foundation, Academy of Athens, Athens, Greece; 4Department of Clinical Chemistry, Tampere University Hospital and University of Tampere, Medical School, Finland; 5National Kapodistrian University of Athens, Medical School, Athens, Greece; 6Division of Cardiology, University Hospital Geneva, Geneva, Switzerland; 7Kennedy Institute of Rheumatology, University of Oxford, United Kingdom
Supplemental Methods

Experimental animals

Male apolipoprotein E-deficient (Apoe\(^{-}\)) mice on a C57BL/6J background (obtained from Jackson Laboratory) were bred in-house. Male toll-like receptor-7-deficient (Tlr7\(^{-}\)) mice on a C57BL/6 background were originally obtained from Shizuo Akira (Osaka University, Japan). The double knockout Tlr7\(^{-}\)Apoe\(^{-}\) mice were generated by crossing Apoe\(^{-}\) mice with TLR7\(^{-}\) mice (Supplementary Fig. 1). Genetic screening was carried out by PCR on genomic tail DNA using the Apoe gene primers: 5’-GCCTAGCCGAGGGAGAGCCG-3’ (oIMR180), 5’-TGTGACTTGGAGCTCTGCAGC-3’ (Oimr181) and 5’-GCCGCCCCGACTGCATCT-3’ (oIMR182) recommended by Jackson Laboratory, and the Tlr7 gene primers 5’-TCTCCAGATTCCTTCCGTAGGC-3’ and 5’-ATTAGGTGGCAAGTGTGGG-3’ (for the wild type allele), and 5’-TGCCCATCCAGTCTCATTCCCTTCTC-3’ and 5’-ATCGCCTTCTATCGCCTTGGACGAG-3’ (for the knockout Tlr7 allele amplifying the neo resistance gene of the targeting construct). Mice were fed a normal chow diet containing 18.5% protein and 5.5% fat (Harlan Tekland, Italy) and were housed in individually ventilated cages under specific pathogen-free conditions at the Animal House Facility of the Foundation for Biomedical Research of the Academy of Athens. All procedures had received prior approval from the Institution’s and Regional Ethical Review Boards and were in accordance with the US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (#A5736–01) and with the European Union Directive 86/609/EEC for animal research.
Ultrasonography

Mice were anesthetized with intraperitoneal injection (i.p.) of 100mg/kg ketamine. Echocardiographic studies were performed using a Vivid 7, GE ultrasound system with a 13 MHz linear transducer. Two-dimensional targeted M-mode imaging was obtained from the short axis view at the level of greatest LV dimension. Images were analyzed using the Echopac PC SW 3.1.3/ software (GE). M-mode measurements of LV end-diastolic diameter (EDD), LV end-systolic diameter (ESD) and LV posterior wall thickness at diastole (PWT) were made. End diastole was determined at the maximal LV diastolic dimension, and end systole was taken at the peak of posterior wall motion. Three beats were averaged for each measurement. The ratio of LV radius to PWT (r/h) and the percentage of LV fractional shortening FS (%) = [(EDD-ESD)/EDD] x 100 were calculated. Transvalvular aortic blood flow velocities were also measured with a 6 MHz-pulsed Doppler flow. From the spectral envelopes we measured the following indices by averaging at least 10 cardiac cycles: i) heart rate; ii) peak velocity; iii) stroke distance (the area under the aortic velocity curve); iv) mean velocity (velocity averaged over the cardiac period); and v) peak acceleration, determined from the maximum derivative of the velocity signal. Finally, carotid artery Doppler flow waveforms were recorded and the maximum, minimum and mean velocities of the waveforms were measured. To estimate arterial compliance changes, the pulsatility index [PI= (maximum-minimum)/mean velocity] was calculated.

Atherosclerotic lesion analysis

Mice were humanely sacrificed and perfused with PBS through the left ventricle of the heart. The entire aorta from the root, extending up to 5-10 mm after bifurcation of the iliac arteries and including the subclavian, right and left carotid arteries, was dissected and opened longitudinally after removing the adipose tissue. The aorta
was pinned flat with steel pins in a wax-bottomed dissecting-pan and immersed in PBS, followed by 10 minute incubation in 70% ethanol. The drained tissue was then stained for 20 minutes with Sudan IV (Sigma-Aldrich; Germany) in a 1:1 acetone and 70% ethanol solution. The tissue was decolorized for 5 minutes using 80% ethanol and washed gently with water for several minutes. The ‘en face’ preparations were digitally photographed and quantified using the Image J software (Wayne Rasband, USA), as percentage of plaque coverage. For histological analysis of aortic valve area, heart tissues were embedded in OCT after o/n immersion in 4% paraformaldehyde (PFA) in PBS and equilibration in 30% sucrose. Sectioning was performed at 10 μm thickness. For lipid assessment, serial sections with 40μm intervals, spanning on a total of 500μm area, were stained with Oil Red O (ORO) (Sigma-Aldrich, Germany) and counterstained with hematoxylin. The mean value of plaque cross-sections from the whole aortic valve area was used for the estimation of the extent of atherosclerosis. For collagen deposition, serial sections were stained with Picro-sirius red (Sigma-Aldrich, Germany) for 30 minutes and counterstained with Harris hematoxylin (Carlo Erba Reagenti SPA, Italy). The histological sections were observed with a DMLS2 optical microscope equipped with a DFC500 camera (Leica Microsystems, Germany).

**Immunofluorescence-Immunohistochemistry**

For immunofluorescent staining of mouse sections, cryosections were incubated with anti-mouse CD68 (clone FA-11; Serotec, Oxford, UK) followed by incubation with an Alexa 594-conjugated goat anti-rat secondary antibody (Molecular Probes, Netherlands), anti-mouse iNOS (ab15323; Abcam, UK) followed by an Alexa 488-conjugated goat anti-rabbit secondary antibody, or with a Cy3-conjugated anti-mouse αSMA (clone 1A4, Sigma-Aldrich, Germany) monoclonal antibodies. Nuclear counterstaining was achieved with DAPI (Molecular Probes, Netherlands).
Appropriate isotype controls were used at the same concentrations as the respective primary antibodies. All isotype control stainings showed absence of positive signal (data not shown). Immunofluorescence for RNA complexes was performed on frozen sections after a 5-min stain with DAPI and Ribogreen (1:10,000) followed by washings, 3-min staining with DAPI (Molecular Probes, Netherlands) and mounting with the Prolong Gold mounting medium (Invitrogen, Germany). Confocal images were taken with a Leica TCS-SP5 system (Leica Microsystems, Germany). Positive staining areas were quantified by using Image J analysis software (Wayne Rasband, USA).

Serum measurements
For serum lipid analysis, mice were fasted for 16 hours and weighed before blood collection. Serum was separated by centrifugation and stored at -80°C until further analysis. Serum concentrations of total cholesterol (TC) and triglyceride (TG) were determined using the commercially available cholesterol or triglyceride assay kit, respectively (Cayman Chemicals) according to the manufacturer’s instructions.

Isolation of mRNA and quantitative real-time PCR
Total RNA was isolated from aortic tissue (from the root extending up to 5-10 mm after bifurcation of the iliac arteries) using the TRIZOL Reagent according to standard protocol. RT-PCR was performed using the M-MLV reverse transcriptase according to the manufacturer’s instruction (Promega, USA). For transcriptional analysis of the TLR signaling pathway, real-time quantitative PCR was performed using a commercial PCR array kit (SABioscience, Qiagen, Germany). Changes in gene expression were calculated by the $2^{\Delta\Delta Ct}$ method and normalized to GAPDH and β-actin levels. For transcriptional analysis of mouse aortas, real-time quantitative PCR was performed with SYBR®Green ER™ qPCR SuperMix Universal (Invitrogen).
Changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH levels. The following primers were used:

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Sense Primers</th>
<th>Antisense Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg1</td>
<td>5’-CAGAAGAATGGAGAGGATCG-3’</td>
<td>5’-CAGATATGCAAGGGAGTCACC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-AGGTGTTCTCTCCTTGACTCT-3’</td>
<td>5’-CTGTTTCTGTAGCAGAACATCG-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-GAGGATACCACTCCCAAACGAGC-3’</td>
<td>5’-AAGTGATCATCATGTTGATAC-3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’-GGTTGCCAAAGGCTTATGGA-3’</td>
<td>5’-ACCTGCTCATGCCTTGCT-3’</td>
</tr>
<tr>
<td>IL12/IL23p40</td>
<td>5’-GGGAAGACGGGGAGAGGATGAGTC-3’</td>
<td>5’-AAGTTTGAAGGAGAAGTGGAG-3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5’-CCAGAGGCAAGGTCAAGCAT-3’</td>
<td>5’-CAGCCGTGCAACAATCTGAA-3’</td>
</tr>
<tr>
<td>MMP2</td>
<td>5’-TTGAGAAGGATGTCAGGATGG-3’</td>
<td>5’-TGGAAGCCGAGACCGGAAAC-3’</td>
</tr>
<tr>
<td>MMP3</td>
<td>5’-TTGAGAAGGATGTCAGGATGG-3’</td>
<td>5’-TGGAAGGACTTGTAGACTGG-3’</td>
</tr>
<tr>
<td>MMP9</td>
<td>5’-TATCTGTATGGTCGTGGCTGCTAAG-3’</td>
<td>5’-TGCTGTCCGGCTGGTTC-3’</td>
</tr>
<tr>
<td>NOS2</td>
<td>5’-CCTATCTCCATCTACTGAC-3’</td>
<td>5’-ATGACCTTTTCGATTAGC-3’</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>5’-TGACGTCTGAGGATGGACTCGG-3’</td>
<td>5’-GGTTCTATGTCATGGATTGTC-3’</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>5’-GGATGGAAATGGATCTCGAAGAC-3’</td>
<td>5’-TGTTGTACGGCTCAGAGGACTCTG-3’</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>5’-CGTTTCAATGTGTCCTCAGTGAGG-3’</td>
<td>5’-AAGAGCTCAATTCCTGCTCTTG-3’</td>
</tr>
<tr>
<td>TIMP1</td>
<td>5’-CTCTGGCATCTGCGACATTCC-3’</td>
<td>5’-GCTGGTTAAGGTGGTCTCG-3’</td>
</tr>
<tr>
<td>TIMP2</td>
<td>5’-AGAAGGATGTCGAATGACGAGT-3’</td>
<td>5’-GGAGGATGATGACGACCC-3’</td>
</tr>
<tr>
<td>TNFa</td>
<td>5’-CATCTTTCTCAATTGAGTGA-3’</td>
<td>5’-TGGAAGTACGACGAGAATCC-3’</td>
</tr>
<tr>
<td>Ym1</td>
<td>5’-GCAGAAGCTCAGCAGAAGATCCTC-3’</td>
<td>5’-ATGGGCCTTGCTCTATTGGCCAAC-3’</td>
</tr>
<tr>
<td>CTGF</td>
<td>5’-GCAGCTCAAACCTCCCAAACACC-3’</td>
<td>5’-CAACAGGGGTTCGACC-3’</td>
</tr>
<tr>
<td>PDGFA</td>
<td>5’-GTCCAGGTGAGGTAGTAGG-3’</td>
<td>5’-CAAGGGAGAGAACCGAC-3’</td>
</tr>
<tr>
<td>VEGFa</td>
<td>5’-CTGTAACGATGAGCCTGGAG-3’</td>
<td>5’-TGTTGAGGATTGATCGCATG-3’</td>
</tr>
</tbody>
</table>
Similarly, for transcriptional analysis of human atheromata from the Biobank of the University of Athens, real-time quantitative PCR was performed with SYBR®Green ER™qPCR SuperMix Universal (Invitrogen). The following primers were used:

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Sense Primers</th>
<th>Antisense Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR7</td>
<td>5'-TCCTTGGGGCTAGATGGTTTC -3'</td>
<td>5'-TCCACGATCAGATGGTTTCTTTG -3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AGGTGGTTCTCTGACTTTG -3'</td>
<td>5'-CTGTTGCTGTAAGCCAAATTGC -3'</td>
</tr>
</tbody>
</table>

**Biobank of Karolinska Endarterectomies specimens, clinical data and microarray analysis**

Analysis of human carotid plaque samples were carried out in the Biobank of Karolinska Endarterectomies (BiKE) study, which has been described previously. In brief, BiKE includes endarterectomy samples from asymptomatic/symptomatic patients diagnosed with >70% carotid artery stenosis (according to European Carotid Surgery Trial criteria) prospectively collected at the Department of Vascular Surgery of the Karolinska Hospital from 2001-2011. Endarterectomy specimens were immediately frozen and RNA was extracted as previously described. Several clinical variables for each patient were registered, including gender, lipid profiles, risk factors, ongoing medication, comorbidities, and last recorded symptoms of plaque instability. Carotid lesions (n=127) from the BiKE biobank and control tissue obtained from healthy arteries of organ donors (n = 10) were included for analysis by microarray experiments. Affymetrix HG-U133A Genechip® arrays were performed at the BEA Karolinska core facility for expression analysis on total RNA, according to a standard protocol (http://www.affymetrix.com). Raw data files were preprocessed and log2-transformed using RMA normalization as implemented in the Affymetrix Power Tools 1.10.2 package apt-probeset-summarize. All participants provided informed consent and the investigation was approved by the Ethical Committee of Northern Stockholm.
and was in agreement with institutional guidelines and the principles set forth in the Declaration of Helsinki.

**Tampere Vascular Study specimens, clinical data and microarray analysis**

The atherosclerotic vascular sample series for GWEA consisted of atherosclerotic plaques from the following arterial sites: femoral artery (n=24) carotid artery (n=33) and abdominal aorta (n=15) all together from a total of 72 patients participating in Tampere Vascular Study (TVS). 28 control specimens from internal thoracic arteries (ITA) of patients undergoing coronary artery by-pass surgery were also used. All samples were prospectively collected between 2008-2009. For the relative gene expression analysis, 68 atherosclerotic tissue samples were used and similarly, the 25 ITA vessels were used as controls after outlier exclusion. The vascular samples were classified according to the American Heart Association classification (AHA). All specimens were immediately frozen and RNA was extracted as previously described. RNA was reverse transcribed into cRNA, biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion) and cRNA hybridized to the Illumina HumanHT-12 v3 Expression BeadChip. BeadChips were scanned with the Illumina iScan system. Data processing was conducted using R language and appropriate Bioconductor modules. Robust multiarray averaging (RMA) was used to correct negative intensity values after background subtraction. Between arrays normalization was done using robust spline normalization (RSN). Quality control was performed using sample clustering and multi dimensional scaling. 7 outliers were removed due to low expression profiles, 4 from carotid artery group and 3 from LITA group. The study was approved by the Ethics Committee of Tampere University Hospital. The samples were taken from consecutive patients subjected to open vascular surgical procedures at the Division of Vascular Surgery, Tampere University Hospital. All patients gave informed consent.
Biobank of Endarterectomies specimens of the Medical School of the University of Athens

The Biobank of Endarterectomies specimens of the Medical School of the University of Athens consists of prospectively collected endarterectomy samples from asymptomatic/symptomatic patients diagnosed with >70% carotid artery stenosis (according to European Carotid Surgery Trial criteria) between 2006-2007. All patients were preoperatively evaluated by a neurologist and symptomatic patients classified based on the presence of stroke, transient ischemic attacks and amaurosis fugax. A cerebral CT scan was also performed for identification of brain infarcts. All patients underwent endarterectomy during 3 and 6 weeks after exhibiting symptoms. The degree of stenosis was determined according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria. For control tissue samples, healthy thyroid arteries obtained from patients who underwent to thyroidectomy were used as control tissue samples for the “baseline-expression” of the examined factors in human arteries. Criteria for their selection included no previous history of cardiovascular disease and were matched by gender and age (mean 65.8 years, range: 50-74) to the patients’ specimens under investigation. In total, mRNA and paraffin-embedded tissue of carotid endarterectomies from 29 patients and 20 controls were analyzed. The study protocol was approved by the Institutional Ethics Committee and all patients enrolled gave their informed consent.

Cultures of mixed vascular cells from human atherosclerotic plaques and cytokine analysis

Carotid endarterectomies from patients undergoing revascularization procedures for symptomatic carotid disease were obtained at Charing Cross Hospital, London. The protocol was approved by the Riverside Research Ethics Committee. All patients
gave written informed consent, according to the Human Tissue Act 2004 (UK). Diseased intimal arterial segments were dissected from carotid endarterectomy specimens and single cell suspensions obtained by enzymatic digestion, as published previously. The viable cell number obtained was $3 \pm 1.9 \times 10^6$ (mean±SEM), with macrophages constituting the most abundant cells in the mixture, being approximately double the proportions of smooth muscle cells and lymphocytes. Cells were cultured in the presence or absence of the TLR7 agonist imiquimod and TNFα, IL-6, IL-10, IL-12 and MCP-1/CCL2 levels quantified by Luminex TM 100 using Fluorokine Multianalyte kits from R&D Systems.

**Statistical Analysis**

Statistical significance of differences was assessed by using the parametric Student’s t test for normally distributed data and the non-parametric Mann-Whitney U (MWW) test for skewed data that deviate from normality. For associations between TLR7 levels and macrophage markers, the Pearson correlation coefficient was used. Differences were considered significant when $p<0.05$. 
Supplemental Tables

Supplemental Table 1. Metabolic characteristics of 26-week old Apoe\(^{−/−}\) and Tlr7\(^{−/−}\) Apoe\(^{−/−}\) mice fed a normal chow diet. Values represent mean levels ± SD; Mouse numbers (n) are shown into parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Apoe(^{−/−})</th>
<th>Apoe(^{−/−})Tlr7(^{−/−})</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.33±1.70 (n=9)</td>
<td>29.16±1.60 (n=11)</td>
<td>0.29</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>614.6±75.18 (n=9)</td>
<td>464.7±95.64 (n=11)</td>
<td>0.0018</td>
</tr>
<tr>
<td>Total triglycerides (mg/dl)</td>
<td>164.6±48.90 (n=9)</td>
<td>128±40.77 (n=11)</td>
<td>0.0680</td>
</tr>
</tbody>
</table>

Supplemental Table 2. TLR7 expression analysis of human atheromata from carotid and femoral arteries and abdominal aortas from the Tampere Vascular Study. Fold change (FC) of TLR7 mRNA relative to the left internal thoracic artery (LITA) and p values are shown.

<table>
<thead>
<tr>
<th></th>
<th>FC (mean)</th>
<th>FC (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid artery</td>
<td>1.71</td>
<td>2.15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Femoral artery</td>
<td>1.85</td>
<td>1.94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>1.69</td>
<td>1.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ascending aorta</td>
<td>0.68</td>
<td>0.62</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Supplemental Table 3. Comparison of carotid endarterectomy patients from the Biobank of the Karolinska Endarterectomies study (BIKE) with plaque expression of TLR7 above the median value (TLR7 high) and below the median value (TLR7 low). Percentage of samples and sample number in parenthesis is reported for the categorical variables of gender, diabetes, statin treatment, antihypertensive
treatment, smoking status and symptom reports (minor stroke, amaurosis fugax, and transient ischemic stroke). P-values are from a Chi-square test.

Mean values ± SD are reported for the continuous variables of age, low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol, and body mass index (BMI). P-values are from a Student's t-test

<table>
<thead>
<tr>
<th>Factor</th>
<th>High TLR7</th>
<th>Low TLR7</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>22% (14)</td>
<td>23% (14)</td>
<td>0.87</td>
</tr>
<tr>
<td>Diabetes</td>
<td>29% (18)</td>
<td>23% (14)</td>
<td>0.57</td>
</tr>
<tr>
<td>Statin treatment</td>
<td>87% (55)</td>
<td>77% (48)</td>
<td>0.22</td>
</tr>
<tr>
<td>Antihypertensive treatment</td>
<td>89% (56)</td>
<td>82% (51)</td>
<td>0.42</td>
</tr>
<tr>
<td>Current or former smoker</td>
<td>46% (29)</td>
<td>53% (33)</td>
<td>0.53</td>
</tr>
<tr>
<td>Symptomatic patient</td>
<td>71% (45)</td>
<td>65% (40)</td>
<td>0.52</td>
</tr>
<tr>
<td>Age (years)</td>
<td>71.1±9.5</td>
<td>70.1±8.3</td>
<td>0.51</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>2.47±0.89</td>
<td>2.57±1</td>
<td>0.61</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>1.19±0.39</td>
<td>1.19±0.4</td>
<td>1.00</td>
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<td>Total cholesterol (mM)</td>
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<td>BMI (kg/m²)</td>
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Supplemental Figure legends

Supplemental Figure 1. Genotyping of mice.
Upper band shows PCR products characteristic of Apoe<sup>−/−</sup> (245bp) and wild type (155bp) mice. Lower band shows PCR products for Tlr7<sup>−/−</sup> (310bp) and wild type (438bp) mice.

Supplemental Figure 2. Expression of tissue remodeling factors and enzymes in Tlr7<sup>−/−</sup>Apoε<sup>−/−</sup> and Apoe<sup>−/−</sup> mice
Tlr7<sup>−/−</sup>Apoε<sup>−/−</sup> and Apoe<sup>−/−</sup> mice were fed a normal chow diet. mRNA expression levels of tissue remodeling factors and enzymes in entire aortas of 26 week old Tlr7<sup>−/−</sup>Apoε<sup>−/−</sup> and Apoe<sup>−/−</sup> mice are shown. Results are expressed as mean levels ± SEM (n=6-10) relative to GAPDH. Statistical significance values (p) are shown.

Supplemental Figure 3. TLR7 deficiency enhances macrophage responsiveness to LPS
Production of MCP-1, IL-6, IL-10 and TNF in supernatants of Tlr7<sup>−/−</sup>Apoε<sup>−/−</sup> and Apoe<sup>−/−</sup> peritoneal macrophages cultured for 20h with the TLR4 ligand lipopolysaccharide (LPS). IL-12/23p40 was not detectable. Results are expressed as mean levels ± SEM of quadruplicate cultures. One representative from four independent experiments is shown. Statistical significance values (p) are indicated.

Supplemental Figure 4. Expression analysis of Toll-like receptor pathway signaling components
Tlr7<sup>−/−</sup>Apoε<sup>−/−</sup> and Apoe<sup>−/−</sup> mice were fed a normal chow diet. At 12 weeks of age, peritoneal macrophages were collected and analyzed for the expression of Toll-like receptor signaling components by real-time PCR. No significant differences between
the two groups were observed. Results are expressed as mean fold induction ± SEM of Tlr7^{-/-}Apoe^{-/-} macrophage levels relative to Apoe^{+/-} levels using the 2^{-\Delta\Delta Ct} method.

Supplemental Figure 5. Correlation of TLR7 expression with cellular markers
Correlation between TLR7 mRNA levels and leukocyte (CD45), macrophage (CD14), T cell (CD3E), B cell (CD19), mast cell (TPSAB1) and smooth muscle cell (ACTA2) markers in human carotid lesions from BIKE. Pearson's correlation coefficient (r) and statistical significance values (p) are shown. AU: Arbitrary log2 units.

Supplemental Figure 6. TLR7 expression in stable and unstable plaques
A, Comparison of TLR7 mRNA expression in stable (Stary grade V, n=14) and unstable carotid plaques (Stary grade VI, n=10) from the Tampere Vascular Study (TVS). Mean expression levels in arbitrary log2 units (AU) and statistical significance values (p) are shown.

B, Comparison of TLR7 mRNA expression in stable (Stary grade V, n=8) and unstable carotid plaques (Stary grade V, n=9) from the Biobank of the University of Athens. Mean mRNA levels expressed relative to GAPDH and statistical significance values (p) are shown.

Supplemental Figure 7. Extracellular RNA complexes are present in advanced atherosclerotic lesions
Tlr7^{-/-}Apoe^{-/-} and Apoe^{+/-} mice were fed a normal chow diet and analyzed at 26 weeks of age. Representative fluorescent photomicrographs of Ribogreen (green) and DAPI (blue)-stained sections from the aortic root are shown. Arrowheads indicate extracellular RNA aggregates (Ribogreen^{+}/DAPI^{-}).
Supplemental Figure 1

Mice

- Tlr7-/-Apoe++
- Tlr7-/-Apoe--
- Tlr7+/+Apoe++
- Tlr7+/+Apoe--

- 245 bp
  - Apoe++ allele
  - Apoe+- allele

- 310 bp
  - Tlr7-/- allele

- 438 bp
  - Tlr7++ allele
Relative mRNA expression

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Relative mRNA expression

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Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4
Supplemental Figure 5
Supplemental Figure 6

A. Stab vs. Unstab

B. Stable vs. Unstable

TLR7 mRNA expression (AU)

TLR7 mRNA expression \((10^3 \text{ relative to GAPDH})\)

p=0.91

p=0.15
Supplemental References


Toll-like receptor 중 TLR7만큼은 다르다: TLR7의 항죽상경화 성질

한 기훈 교수 서울아산병원 심장내과

Summary

목적
TLR(toll-like receptor)들은 죽상경화의 주요 인자로 알려져 있으며, 주로 죽상경화의 발전과 임상적인 합병증을 유발하는 것으로 알려져 있다. 그러나 TLR2, TLR4 이외에 대부분의 TLR의 역할에 대해서는 잘 알려진 바 없다.

방법 및 결과
마우스 모델에 인간의 죽상경화반을 배양함으로써 죽상경화의 발전에 관련된 TLR7의 역할을 연구하였고, 그 결과 놀랍게도 TLR7이 죽상경화의 발전을 저해할 것이라는 증거를 찾았으며, ApoE KO(knockout) 마우스를 이용한 연구에서도 TLR7의 역할을 제거할 때 죽상경화의 악화, 협착의 증가 및 플라크의 불안정이 증가하는 현상을 관찰되었다.

기전적으로는 TLR7은 대식세포가 TLR2, TLR4 등에 의하여 활성화되는 것을 억제하였으며, MCP-1 (monocyte chemoattractant protein-1)의 생성을 저해하고 염증반응을 유도하는 소위 Ly6C^hi 아형 단핵구와 M1형 대식세포의 수를 줄인다.

인간의 carotid endarterectomy 검체에서는 TLR7의 발현은 [IL(interleukin)-10, IL-1RA, CD163, scavenger, C-type lectin receptors 등을 발현하는] M2형 대식세포와 같이 발현하며, IL-12/IL-23, interferon beta, interferon gamma, CD40L 등의 염증성 물질의 발현과는 관련이 없거나 역의 상관성을 보였다. 인간의 죽상경화반 배양에서는 TLR7을 활성화하면 MCP-1, TNF(tumor necrosis factor) 등의 활성을 억제하였다.

결론
연구 결과, TLR7은 죽상경화의 발전을 저해하며, 이는 대식세포의 염증성 반응 및 사이토카인의 발현을 저해함으로써 이루어진다. 이러한 연구 결과는 모든 TLR들이 염증을 유도하는 것은 아니며, 특히 TLR7은 이와 반대 반응을 유도한다는 점을 시사한다.
Commentary

TLR이란?

TLR을 경종을 울리는 수용체라고 번역하여야 할까? TLR은 체내의 염증세포 등에 분포하며, 다양한 염증 신호를 수렴하여 세포에 경종을 울려 즉각적인 반응을 유도한다. 현재 TLR은 약 13개의 아형까지 발견되어 보고되고 있으며, 이 중에서 심혈관질환 즉, 죽상경화의 유발에 연관성이 많은 것으로 보고된 것은 TLR2, TLR4 등이다. 일련의 보고에 의하면 기본적인 구조는 IL-1 수용체와 유사한 것으로 나타났다. 흥미로운 사실은 이 모든 것들을 포괄하였을 때, 수용체에 접합하는 소위 리간드와의 물리적인 작용을 하는 부분은 다양성이 존재하지만, 세포 반응을 유도하는 C-terminal의 cytoplasmic 부분은 매우 높은 유사성이 존재한다는 점이다.

Oxidized LDL(low density lipoprotein)이라는 강력한 산화물에 의한 혈관의 죽상경화 발전이 학설로 정립된 이래, 이와 유사한 산화물이 죽어가는 세포 및 박테리아 등에 광범위하게 발견되며, 죽상경화 반응을 주도하는 대식세포와 유사한 적-염증성 반응을 유도한다는, 즉 PAMP(pathogen-associated molecular pattern)라는 신조어의 탄생을 유도하였다. 이처럼 상대적으로 높은 다양성을 보이는 리간드에 대한 유사반응을 이끌어내는 수용체 중 TLR은 그 중심에 있다. 따라서 TLR의 구조적인 특성은 이러한 PAMP 현상에 의해, 하는 위치를 차지한다.

TLR 7이란?

TLR에는 많은 아형이 존재한다. 이 중 TLR7은 lung, placenta, spleen 등에서 최초로 발견되었으며, 이후의 연구에서 B 임파구, dendritic cell 등에서 발견하는 것으로 보고되었다. 만일 dendritic cell에 발현한 TLR7이면 PAMP의 유도로 죽상경화반을 촉진하지 않을까 하는 것이 일반적인 흐름에 따른 논리일 것이다. 그러나 한편으로는 TLR7를 활성화하는 리간드는 많이 알려져 있지 않으며, 일부 박테리아 또는 self-RNA 및 핵산 등에 의한 활성이 알려져 있을 뿐이다.

본 논문에서도 언급되었듯이 B 임파구에서의 TLR7의 역할에 대해서는 알 수 없는 상태이다. 놀랍게도 TLR7은 대식세포 특유 소위 M2 형 대식세포에 중점적으로 발현되는 것으로 보이며, 이들을 활성화함으로써 죽상경화의 저해를 유도한다는 것이다.

본 논문에서 참고해야 할 사항

1. Figure 1은 ApoE KO에서의 TLR7 제거가 죽상경화를 악화시킨다는 것을 잘 보여주고 있다. ApoE KO는 LDLR(low-density lipid receptor) KO보다 immunogenisity가 높은 것으로 되어 있으며, 이에 의한 죽상경화 발생의 영향을 상대적으로 많이 받는다. 이는 상대적으로 TLR7의 역할이 극대화될 수도 있다는 가능성을 시사한다.

2. Figure 2에서의 평활근세포의 수와 콜라겐의 양 등이 감소한 점 등은 플라크가 붕괴하여진다는 것을 보여준다. 본 논문에서는 평활근 세포나 T 임파구 등에서의 TLR7의 발현 여부에 대해서는 언급이 없다. 따라서 이러한 결과는 일반적으로 죽상경화의 진행에 따른 대식세포의 증가로 인한 효과가 극대화된 것으로 이해할 수밖에 없다.

3. 본 논문은 TLR7의 발현은 M2에 주로 있을 것이라고 기술하고 있다. Figure 1에서 TLR7의 제거에 의하여 대식세포의 수가 증가하므로 궤연 (TLR7이 분포하였음) M2 대식세포가 주도적으로 M1 대식세포의 부하를 조절할 수 있는지, 그리고 그 기전은 무엇인지 규명되어야 할 것이다.
Figure 1. TLR7 deficiency accelerates atherosclerosis in mice. Tlr7−/− Apoe−/− and Apoe−/− mice were fed a normal chow diet and analyzed at various time points as indicated.

A, Atherosclerotic lesion size of 10-, 18-, and 24-week-old mice using morphometric analysis of Oil Red O-stained sections from the aortic root. 
B, Representative light photomicrographs and morphometric analysis of Oil Red O-stained sections from the aortic root of 26-week-old mice (n=16-19).
C, Representative fluorescent photomicrographs and morphometric analysis of CD68-stained sections from the aortic root of 26-week-old mice (n=16-19).
D, Representative images of Sudan IV-stained enface preparations of the proximal aorta of 26-week-old mice (n=13-15), and morphometric analysis of aortic atherosclerosis expressed as a fraction of total aortic area. Each point corresponds to the mean lesion area or CD68− area per individual mouse. Red lines represent the mean lesion or CD68− area per group. Statistical significance values (p) are indicated. HX indicates hematoxylin; DAPI, 4',6-diamidino-2-phenylindole; ORO, Oil Red O.

Figure 2. TLR7 deficiency promotes a more vulnerable phenotype of atherosclerotic lesions. Tlr7−/− Apoe−/− and Apoe−/− mice were fed a normal chow diet and analyzed at 26 weeks of age.

A, Representative light photomicrographs and morphometric analysis of Oil Red O-stained sections from the aortic root. 
B, Representative fluorescent photomicrographs and morphometric analysis of CD68-stained sections from the aortic root. 
C, Representative fluorescent photomicrographs and morphometric analysis of alpha smooth muscle actin–stained sections from the aortic root. 
D, Representative polarized light photomicrographs and morphometric analysis of Picrominus Red-stained sections from the aortic root. 

Atherosclerosis is a chronic inflammatory disease that involves the remodeling of the arterial wall. The macrophage is a key player in the development of atherosclerosis, and TLR7 deficiency has been shown to reduce plaque formation and vulnerable phenotype.

본 논문은 방대한 실험을 통하여 일관성 있게 TLR7에 의한 죽상경화의 발현 저해를 시사하고 있다. 추가적으로 여러 가지 언급된 바와 같이 세포 간의 상호작용, M1 대식세포의 증가, 평활근 세포의 고사 등을 TLR7이 어떤 방식으로 조절하는지를 계속 밝혀나가야 할 것이다.
Toll-Like Receptor 7 Protects From Atherosclerosis by Constraining “Inflammatory” Macrophage Activation

Maria Salagianni, PhD; Ioanna E. Galani, PhD; Anna M. Lundberg, PhD; Constantinos H. Davos, MD, PhD; Aimilia Varela, BSc; Ariana Gavril, PhD; Leo-Pekka Lyytikäinen, MD; Terho Lehtimäki, MD, PhD; Fragiska Sigala, MD; Lasse Folkesen, PhD; Vassilis Gorgoulis, MD, PhD; Sébastien Lenglet, PhD; Fabrizio Montecucco, MD, PhD; François Mach, MD, PhD; Ulf Hedin, MD, PhD; Göran K. Hansson, MD, PhD; Claudia Monaco, MD, PhD; Evangelos Andreakos, PhD

Background—Toll-like receptors (TLRs) have long been considered to be major culprits in the development of atherosclerosis, contributing both to its progression and clinical complications. However, evidence for most TLRs beyond TLR2 and TLR4 is lacking.

Methods and Results—We used experimental mouse models, human atheroma cultures, and well-established human biobanks to investigate the role of TLR7 in atherosclerosis. We report the unexpected finding that TLR7, a receptor recognizing self-nucleic acid complexes, is protective in atherosclerosis. In Apoe<sup>−/−</sup> mice, functional inactivation of TLR7 resulted in accelerated lesion development, increased stenosis, and enhanced plaque vulnerability as revealed by Doppler ultrasound and/or histopathology. Mechanistically, TLR7 interfered with macrophage proinflammatory responses to TLR2 and TLR4 ligands, reduced monocyte chemoattractant protein-1 production, and prevented expansion of Ly6C<sup>hi</sup> inflammatory monocytes and accumulation of inflammatory M1 macrophages into developing atherosclerotic lesions. In human carotid endarterectomy specimens TLR7 levels were consistently associated with an M2 anti-inflammatory macrophage signature (interleukin [IL]-10, IL-1RA, CD163, scavenger and C-type lectin receptors) and collagen genes, whereas they were inversely related or unrelated to proinflammatory mediators (IL-12/IL-23, interferon beta, interferon gamma, CD40L) and platelet markers. Moreover, in human atheroma cultures, TLR7 activation selectively suppressed the production of key proatherogenic factors such as monocyte chemoattractant protein-1 and tumor necrosis factor without affecting IL-10.

Conclusions—These findings provide evidence for a beneficial role of TLR7 in atherosclerosis by constraining inflammatory macrophage activation and cytokine production. This challenges the prevailing concept that all TLRs are pathogenic and supports the exploitation of the TLR7 pathway for therapy. (Circulation. 2012;126:952-962.)

Key Words: atherosclerosis • immune system • inflammation • macrophage • Toll-like receptor

Chronic inflammation is an integral part of the pathogenesis of atherosclerosis. A cumulation of lipoproteins in the vessel wall, especially at areas of disturbed blood flow such as bifurcations and the lesser curvature of the aortic arch, induces a chronic inflammatory response characterized by the mobilization of monocytes in the periphery, the infiltration of macrophages, dendritic cells, and lymphocytes in the arterial intima, and the expression of proinflammatory cytokines, chemokines, and matrix metalloproteinases. This leads to luminal narrowing and often plaque rupture and myocardial infarction or stroke, the most severe clinical complications of atherosclerosis. Therefore, identifying rate-limiting molecular processes and pathways that contribute to the development or persistence of inflammation in the vessel wall is key to the future treatment of this disease.

Clinical Perspective on p 35

Toll-like receptors (TLRs) have recently taken center stage in atherosclerosis research by virtue of their ability to drive sterile inflammation in the vessel wall. Under the current
paradigm, TLRs promote atherogenesis through the disruption of endothelial cell integrity, the induction and sustained expression of inflammatory cytokines and chemokines, and the infiltration and activation of inflammatory monocytes/macrophages in developing plaques. In agreement with this model, TLRs (1, 2, 4, and 6) are increased in human atherosclerotic lesions,\(^6,12–17\) in cells of the monocyte/macrophage lineage and resident vascular cells, whereas TLR2 and TLR4 polymorphisms have been associated with the extent of atherosclerosis in some studies, although not others.\(^7\) Several endogenous TLR ligands relevant to atherosclerosis have also been described, including modified lipoproteins, oxidized lipids, and self-ligands released in response to hypercholesterolemia, tissue stress, or necrosis.\(^7–9\) Moreover, TLR2 signaling through myeloid differentiation factor 88 (MyD88) and nuclear factor kappa B has been shown to account for increased inflammation and matrix degradation in an ex vivo culture system of human atherosclerotic plaques,\(^10,11\) whereas functional TLR2, TLR4, and MyD88 have been demonstrated to be critically required for the development of atherosclerosis in experimental mouse models fed a high-fat diet.\(^6,12–17\) More recently, TLR6 has also been linked to the development of atherosclerosis by forming heterodimeric complexes with TLR4 involved in the recognition of oxidized lipoproteins.\(^18\) In contrast, genetic deletion of TLR3 has suggested a protective role of this receptor in arterial injury and early atherogenesis, whereas systemic administration of TLR3 agonists has yielded contradictory results;\(^9,20\) highlighting the need for more detailed investigation into the role of other TLRs in atherosclerosis.

TLR7 is an endosomal TLR that recognizes viral single-stranded RNA and self-RNA released from necrotic cells often complexed with cationic antimicrobial peptides such as LL37 and \(\alpha\)-defensins or antibodies.\(^21,22\) It is expressed in subsets of monocytes, macrophages, dendritic cells, B cells, and eosinophils and is upregulated in intermediate and advanced atherosclerotic lesions of femoral arteries of patients with peripheral artery disease.\(^23\) The main known function of TLR7 is in antiviral immunity, although additional proinflammatory, anti-inflammatory, and immunoregulatory activities of TLR7 during chronic inflammation have also been described.\(^24,25\) Still, the functional role of TLR7 in atherosclerosis remains unknown. Here, we present the surprising finding that TLR7 is protective in atherosclerosis by shaping monocyte/macrophage function toward an alternatively activated antiatherogenic phenotype. This challenges the current paradigm that all TLRs are pathogenic in atherosclerosis and has broader implications for the role of innate immunity in this disease.

**Methods**

**Experimental Animals**

Apolipoprotein E-deficient (Apoe\(^{-/-}\)) mice on a C57BL/6 background were crossed with TLR7-deficient (Tlr7\(^{-/-}\)) mice originally obtained from Shizuo Akira (Osaka University) and backcrossed to the C57BL/6 background for \(>10\) generations. Mice were fed a normal chow diet containing 18.5% protein and 5.5% fat (Harlan Tekland) and analyzed at various time points as indicated. For the assessment of macrophage responsiveness to TLR2 stimulation in vivo, Apoe\(^{-/-}\) and Tlr7\(^{-/-}\) Apoe\(^{-/-}\) mice were injected intraperitoneally with 20 \(\mu\)g endotoxin-free lipoteichoic acid (Invivogen) in 200 \(\mu\)L sterile PBS, euthanized after 3 hours, and serum collected. More details are available in the online-only Data Supplement.

**Doppler Ultrasonography**

Echocardiographic studies were performed in anesthetized mice using a Vivid 7, GE ultrasound system with a 13 MHz linear transducer and a 6 MHz-pulsed Doppler probe as detailed in the online-only Data Supplement.

**Analysis of Atherosclerotic Lesions**

Oil Red O (Sigma-Aldrich) and Picro-Sirius Red (Sigma-Aldrich) stained serial sections of the aortic valve, spanning a 500- \(\mu\m^2\) area, and Sudan IV (Sigma-Aldrich) stained entire aortas were analyzed using the ImageJ software (Wayne Rasband).

**Immunofluorescence-Immunohistochemistry**

Mouse aortic sinus cryosections were stained with anti-mouse CD68 (clone FA-11; Serotec), alpha smooth muscle actin (clone 1A4, Sigma-Aldrich), inducible nitric oxide synthase (iNOS; ab15323, Abcam), or isotype control monoclonal antibodies and counter-stained with 4',6-diamidino-2-phenylindole (Molecular Probes). Positive staining areas or colocalization were quantified by use of the ImageJ software (Wayne Rasband). Detailed protocols are available in the online-only Data Supplement.

**Quantification of Plaque Necrosis**

Plaque necrosis was determined by drawing boundary lines around regions free of 4',6-diamidino-2-phenylindole staining and quantifying the region area by using the ImageJ analysis software (Wayne Rasband). A 3000- \(\mu\m^2\) threshold was implemented to avoid counting regions that may not represent substantial areas of necrosis.\(^17\)

**Flow Cytometry**

Blood and spleen were processed and stained with fluorochrome-conjugated monoclonal antibody combinations for CD11b, Ly6C, CD45, and appropriate IgG isotype controls (eBioscience). Flow cytometry was performed on a Beckman-Coulter FC-500 analyzer, and data were analyzed by using Kaluza software (Beckman Coulter).

**Serum Measurements**

Serum concentrations of total cholesterol and triglycerides were determined by use of kits from Cayman Chemicals. Serum cytokines were measured by ELISA by using commercially available kits (eBioscience).

**Isolation of mRNA and Quantitative Real-Time Polymerase Chain Reaction**

Real-time quantitative polymerase chain reaction was performed with SYBR GreenER qPCR SuperMix Universal (Invitrogen). Target mRNA levels were expressed relative to GAPDH. Primer sets are shown in the online-only Data Supplement.

**Peritoneal Macrophage Activation**

Peritoneal macrophages from Apoe\(^{-/-}\) and Tlr7\(^{-/-}\) Apoe\(^{-/-}\) mice were cultured with various doses of lipoteichoic acid and supernatants measured after 20 hours by ELISA using commercially available kits (eBioscience).
Vascular Specimens and Genome-Wide Expression Array Studies

Genome-wide expression array studies were conducted in human carotid plaque samples from the Biobank of Karolinska Endarterectomies (BIKE) Study. In brief, carotid lesions (n=127) and control tissue obtained from normal arteries of organ donors (n=10) were analyzed by using Affymetrix HG-U133A Genechip arrays (http://www.affymetrix.com). More detailed information is provided in the online-only Data Supplement.

Human Atheroma Cell Cultures

Single-cell suspensions from carotid endarterectomies from patients undergoing revascularization procedures for symptomatic carotid disease at Charing Cross Hospital, London, were obtained as previously described. Cells were cultured in the presence or absence of 1 μg/mL imiquimod (Invivogen, CA) and cytokine levels quantified by Luminex 100 by using Fluorokine Multianalyte kits from R&D Systems.

Statistical Analysis

Statistical significance of differences was assessed using the parametric Student t test for normally distributed data and the nonparametric Mann-Whitney U test for skewed data that deviate from normality. For associations between TLR7 levels and macrophage markers, the Pearson correlation coefficient was used. Differences were considered significant when P<0.05.

Results

Functional Inactivation of TLR7 Exacerbates Atherosclerosis in ApoE−/− Mice

To examine whether TLR7 is involved in the pathogenesis of atherosclerosis, we generated Tlr7−/− ApoE−/− and compared them with ApoE−/− mice (online-only Data Supplement Figure I). Both Tlr7−/− ApoE−/− and ApoE−/− mice appeared healthy, reproduced according to Mendelian ratios and exhibited no obvious abnormalities (data not shown). There was no significant difference on weight gain or triglyceride levels not changed. All transvalvular aortic blood flow indices were also similar between the 2 groups. Taken together, these observations demonstrate that functional TLR7 is essential for limiting experimental atherosclerosis in mice.

Figure 1. TLR7 deficiency accelerates atherosclerosis in mice. Tlr7−/− ApoE−/− and ApoE−/− mice were fed a normal chow diet and analyzed at various time points as indicated. A, Atherosclerotic lesion size of 10-, 18-, and 26-week-old mice (n=14–17) using morphometric analysis of Oil Red O-stained sections from the aortic root. B, Representative light photomicrographs and morphometric analysis of Oil Red O-stained sections from the aortic root of 26-week-old mice (n=16–19). C, Representative fluorescent photomicrographs and morphometric analysis of CD68-stained sections from the aortic root of 26-week-old mice (n=17–19). D, Representative images of Sudan IV-stained en face preparations of the proximal aorta of 26-week-old mice (n=13–15), and morphometric analysis of aortic atherosclerosis expressed as a fraction of total aortic area. Each point corresponds to mean lesional or CD68+ area per individual mouse. Red lines represent the mean lesional or CD68+ area per group. Statistical significance values (p) are indicated. Hx indicates hematoxylin; DAPI, 4′,6-diamidino-2-phenylindole; ORO, Oil Red O.

not changed. All transvalvular aortic blood flow indices were also similar between the 2 groups. Taken together, these observations demonstrate that functional TLR7 is essential for limiting experimental atherosclerosis in mice.

Functional Inactivation of TLR7 Enhances Features of Plaque Vulnerability in ApoE−/− Mice

Although lesion size accurately reflects atherosclerosis progression, plaque morphology is a more important predictor of
Table. Ultrasonographic Evaluation of Tlr7−/− Apoe−/− and Apoe−/− Mice

<table>
<thead>
<tr>
<th></th>
<th>Apoe−/−</th>
<th>Tlr7−/− Apoe−/−</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>11</td>
<td>11</td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>619±32</td>
<td>617±68</td>
<td>0.93</td>
</tr>
<tr>
<td>EDD, mm</td>
<td>3.15±0.24</td>
<td>3.43±0.26</td>
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<tr>
<td>ESD, mm</td>
<td>1.81±0.20</td>
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<td>FS, %</td>
<td>42.53±3.76</td>
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<tr>
<td>PWT, mm</td>
<td>0.71±0.05</td>
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</tr>
<tr>
<td>r/h</td>
<td>4.02±0.35</td>
<td>4.25±0.47</td>
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<tr>
<td>Peak aortic velocity, cm/s</td>
<td>99.88±13.04</td>
<td>100.64±13.45</td>
<td>0.89</td>
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<tr>
<td>Mean aortic velocity, cm/s</td>
<td>56.09±14.44</td>
<td>56.36±10.99</td>
<td>0.96</td>
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<tr>
<td>Stroke distance, cm</td>
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<td>3.24±0.77</td>
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<td>Peak aortic acceleration, m/s²</td>
<td>121.31±7.48</td>
<td>126.36±8.98</td>
<td>0.17</td>
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<tr>
<td>Peak carotid velocity, cm/s</td>
<td>68.73±9.32</td>
<td>91.09±11.32</td>
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<tr>
<td>Mean carotid velocity, cm/s</td>
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<td>40.36±8.46</td>
<td>0.0073</td>
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<td>Carotid pulsatility index</td>
<td>2.55±0.79</td>
<td>2.42±0.56</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Values are mean±SD. EDD indicates end diastolic diameter; ESD, end systolic diameter; FS, fractional shortening; PWT, posterior wall thickness; and r/h, ratio of left ventricular (LV) radius to PWT. Statistical significance values (P) are indicated.

TLR7 deficiency promotes a more vulnerable phenotype of atherosclerotic lesions. Tlr7−/− Apoe−/− and Apoe−/− mice were fed a normal chow diet and analyzed at 26 weeks of age. A, Representative light photomicrographs and morphometric analysis of Oil Red O–stained sections from the aortic root. Results show the mean positive area±SEM of 12 to 14 mice/group expressed as percentage of the total lesion area. B, Representative fluorescent photomicrographs and morphometric analysis of alpha smooth muscle actin–stained sections from the aortic root. Results show the mean positive area±SEM of 12 to 16 mice/group expressed as percentage of the total lesion area. C, Representative fluorescent photomicrographs and morphometric analysis of laminin–stained sections from the aortic root. Results show the mean positive area±SEM of 7 to 10 mice/group expressed as percentage of the total lesion area. D, Representative polarized light photomicrographs and morphometric analysis of Picrolon Sirius Red–stained sections from the aortic root. Results show the mean positive area±SEM of 10 mice/group expressed as percentage of the total lesion area. Statistical significance values (p) are indicated. TLR7 indicates Toll-like receptor 7.

Figure 2. TLR7 deficit promotes a more vulnerable phenotype of atherosclerotic lesions. Tlr7−/− Apoe−/− and Apoe−/− mice were fed a normal chow diet and analyzed at 26 weeks of age. A, Representative light photomicrographs and morphometric analysis of Oil Red O–stained sections from the aortic root. Results show the mean positive area±SEM of 12 to 14 mice/group expressed as percentage of the total lesion area. B, Representative fluorescent photomicrographs and morphometric analysis of alpha smooth muscle actin–stained sections from the aortic root. Results show the mean positive area±SEM of 12 to 16 mice/group expressed as percentage of the total lesion area. C, Representative fluorescent photomicrographs and morphometric analysis of laminin–stained sections from the aortic root. Results show the mean positive area±SEM of 7 to 10 mice/group expressed as percentage of the total lesion area. D, Representative polarized light photomicrographs and morphometric analysis of Picrolon Sirius Red–stained sections from the aortic root. Results show the mean positive area±SEM of 10 mice/group expressed as percentage of the total lesion area. Statistical significance values (p) are indicated. TLR7 indicates Toll-like receptor 7.

12/23p40, IL-6, IL-10, and tumor necrosis factor (TNF) were not detectable in the serum (data not shown). We then examined whether TLR7 regulates the balance between inflammatory Ly6C hi and CD14+CD16+ cells.1 Ly6C hi monocytes selectively express CCR2 and respond to MCP-1 during hypercholesterolemia to expand in the periphery and migrate into the vascular wall.22-24 In agreement with the increased MCP-1 levels, we found that Tlr7−/− Apoe−/− mice exhibited higher levels of Ly6C hi monocytes relative to total (CD11b+CD11c+) mono-
Ly6Clo monocytes was reduced. The less abundant Ly6Cint Ym1 between the 2 groups (Figure 4D), whereas differences in inflammatory cytokine IL-10 or the M2 markers Arg1 or diabetes, hypertension, obesity, sex, history of symptoms, or medication (online-only Data Supplement Table III). TLR7 mRNA levels strongly correlated with the pan-leukocyte macrophages produced higher levels of proinflammatory cytokines, with MCP-1 and IL-6 being the ones most increased at lower ligand concentrations (Figure 5A and online-only Data Supplement Figure III). Notably, increased responsiveness was seen in vivo as administration of lipoteichoic acid to Tlr7−/− Apoe−/− mice triggered higher production of proinflammatory cytokines such as MCP-1, IL-12/23p40, and IL-6 (but not IL-10) in the serum in comparison with Apoe−/− mice (Figure 5B). This was not due to differences in the expression of TLR2 or TLR4 nor critical downstream signaling components such as MyD88, Mal/TIRAP, TRIF, TRAM, IRAK1, IRAK2, Tollip, TRAF6, IKK2, or nuclear factor kappa B genes between the 2 groups (online-only Data Supplement Figure IV), although the induction of a compensatory TLR response in the absence of functional TLR7 is still a possibility. Because TLR2 and TLR4 are critically involved in macrophage activation during the development/progression of atherosclerosis,12–17 and MCP-1 is a rate-limiting chemokine for atherogenesis,29,37 these findings suggest that TLR7 limits the development of atherosclerosis by interfering with monocyte/macrophage activation and MCP-1 production.

TLR7 Is Essential for Constraining Inflammatory Monocyte/Macrophage Activation and Cytokine Production

We next investigated how TLR7 affects macrophage function and proinflammatory cytokine production in the context of atherosclerosis. Surprisingly, we found that macrophages from Tlr7−/− Apoe−/− mice exhibited increased responsiveness to TLR2 or TLR4 stimulation in comparison to Apoe−/− controls. When stimulated with the TLR2 ligand lipoteichoic acid or the TLR4 ligand lipopolysaccharide, Tlr7−/− Apoe−/− macrophages produced higher levels of proinflammatory cytokines, with MCP-1 and IL-6 being the ones most increased at lower ligand concentrations (Figure 5A and online-only Data Supplement Figure III). Notably, increased responsiveness was seen in vivo as administration of lipoteichoic acid to Tlr7−/− Apoe−/− mice triggered higher production of proinflammatory cytokines such as MCP-1, IL-12/23p40, and IL-6 (but not IL-10) in the serum in comparison with Apoe−/− mice (Figure 5B). This was not due to differences in the expression of TLR2 or TLR4 nor critical downstream signaling components such as MyD88, Mal/TIRAP, TRIF, TRAM, IRAK1, IRAK2, Tollip, TRAF6, IKK2, or nuclear factor kappa B genes between the 2 groups (online-only Data Supplement Figure IV), although the induction of a compensatory TLR response in the absence of functional TLR7 is still a possibility. Because TLR2 and TLR4 are critically involved in macrophage activation during the development/progression of atherosclerosis,12–17 and MCP-1 is a rate-limiting chemokine for atherogenesis,29,37 these findings suggest that TLR7 limits the development of atherosclerosis by interfering with monocyte/macrophage activation and MCP-1 production.

TLR7 Is Associated With M2 Macrophage Markers and a Less Inflammatory Plaque Phenotype in Human Atherosclerotic Lesions

Finally, we investigated the role of TLR7 in human atherosclerosis. Large-scale transcriptional profiling analysis of human atheromata from the Biobank of the Karolinska Endarterectomies study (BiKE)26 and the Tampere Vascular Study (TVS)38 revealed that TLR7 was consistently upregulated in tissue remodeling factors and enzymes were also seen (online-only Data Supplement Figure II). IL-23p19 and IL-17A were not detectable (data not shown). These data indicate that TLR7 acts as a brake to limit inflammatory monocyte/macrophage differentiation and activation in atherosclerosis.

Figure 3. TLR7 deficiency increases the necrotic core area of atherosclerotic lesions. Tlr7−/− Apoe−/− and Apoe−/− mice were fed a normal chow diet and analyzed at 26 weeks of age. A and B, Representative fluorescent photomicrographs of CD68/DAPI-stained sections (necrotic areas outlined with dotted white lines) and light photomicrographs of Oil Red O-stained sections from the aortic root of Apoe−/− and Tlr7−/− Apoe−/− mice. C, Morphometric analysis of the necrotic core area from CD68/DAPI-stained sections from the aortic root. Results show the mean necrotic area ± SEM of 10 to 15 mice/group expressed as percentage of the total lesion area. Statistical significance values (p) are indicated. HX indicates hematoxylin; DAPI, 4’,6-diamidino-2-phenylindole; ORO, Oil Red O; TLR7, Toll-like receptor 7.

cytos both in the blood (Figure 4B) and spleen (Figure 4C) in comparison with Apoe−/− mice, whereas the frequency of Ly6Cint monocytes was reduced. The less abundant Ly6Clo monocytes was also reduced. This was associated with increased accumulation of inflammatory M1 macrophages in the aorta of Tlr7−/− Apoe−/− mice as revealed by the higher levels of proinflammatory cytokines and M1 macrophage markers such as IL-12/23p40, TNF, MCP-1, and iNOS (Figure 4D and 4E).35,36 In contrast, there were no differences observed in the expression of the anti-inflammatory cytokine IL-10 or the M2 markers Arg1 or Ym1 between the 2 groups (Figure 4D), whereas differences
marker CD45 and the monocyte/macrophage marker CD14, exhibited a weak correlation with the T-cell marker CD3, and the mast cell marker TPSAB1, and lacked correlation with the B-cell marker CD19 and the smooth muscle cell marker alpha smooth muscle actin, suggesting that TLR7 expression in plaques is mostly derived from CD14 \( ^{-/-} \) monocytes/macrophages (online-only Data Supplement Figure V).

We next examined whether TLR7 expression was functionally important. We found that addition of the specific TLR7 agonist imiquimod to mixed cell cultures from carotid endarterectomy specimens\(^{10,11} \) inhibited the expression of MCP-1 and TNF, which are associated with a more inflammatory macrophage phenotype, whereas the expression of IL-10 was spared (Figure 6C). IL-12 and IL-23 were below the detection limits. Consistently, in human atheromata from B1K, TLR7 mRNA levels were positively associated with the expression of M2 macrophage markers such as the anti-inflammatory cytokine IL-10, IL-1 receptor antagonist (IL-1RN), CD163, scavenger receptors, and C-type lectin receptors (Figure 6D), whereas they were inversely related to proinflammatory cytokines and M1 macrophage markers such as IL-23p19, iNOS, and interferon beta, and lacked any association with IL-12p35, IL-12/23p40, and interferon gamma (Figure 6D). Additionally, TLR7 mRNA levels were positively associated to collagen genes (Col1A1, Col3A1, Col4A1), fibronectin-1, fibrillin-1, and tissue plasminogen activator (PLAT) expression, and negatively related to platelet markers and CD40 ligand (Figure 6E). Despite these
findings, TLR7 mRNA expression was not associated with a more stable/fibrotic histopathologic plaque phenotype in either the TVS or the University of Athens carotid endarterectomies cohort (online-only Data Supplement Figure VI). Taken together, these findings suggest a link between higher TLR7 expression and reduced inflammation, decreased presence of platelets, and increased deposition of collagen and extracellular matrix, all predictive markers of a more stable plaque phenotype and highlight the need for further studies into the role of TLR7 in plaque vulnerability.

Discussion

Although TLRs have been incriminated for the development of atherosclerosis, evidence for most TLRs beyond TLR2 and TLR4 is lacking. We now reveal that TLR7 is upregulated in human atheromata and exhibits a novel, previously unsuspected beneficial role in atherosclerosis. In Apoe−/− mice, an established animal model of atherosclerosis, functional inactivation of TLR7 worsens disease and promotes a more vulnerable plaque phenotype. Tlr7−/−Apoe−/− mice exhibit accelerated lesion development and increased stenosis, whereas plaques from Tlr7−/−Apoe−/− mice enhanced necrotic core formation, lipid deposition, macrophage accumulation, and proinflammatory cytokine production, and reduced smooth muscle cell and collagen presence. Moreover, in human carotid endarterectomy specimens, TLR7 mRNA levels are positively associated with anti-inflammatory cytokines and M2 macrophage markers (such as IL-10, IL-1RA, CD163, scavenger and C-type lectin receptors), and collagen genes, although they are inversely related/unrelated to proinflammatory mediators (such as IL-12/IL-23, interferon beta, interferon gamma, CD40L) and platelet markers. Lower TLR7 mRNA levels are also associated with active cigarette smoking but not other traditional risk factors, the histopathologic plaque phenotype, or disease symptomatology, possibly because of the sample size used or variable time interval between symptoms and endarterectomy. Altogether, our findings demonstrate that TLR7 is protective in atherosclerosis and challenge the prevailing paradigm that all TLRs are pathogenic.

Central to the protective role of TLR7 in atherosclerosis is its ability to shape monocyte/macrophage function toward an anti-inflammatory phenotype. Mechanistically, this involves inhibition of (1) inflammatory Ly6C hi monocyte expansion in the periphery and (2) inflammatory M1 macrophage accumulation and activation in developing atherosclerotic lesions through a process orchestrated by MCP-1. Accordingly,
TLR7-mediated protection also involves modulation of macrophage polarization. Inflammatory M1 macrophages and M1-associated cytokines contribute to the development and vulnerability of plaques, whereas M2 macrophages are considered to be beneficial through the paracrine anti-inflammatory effects they exert on M1 macrophages and the improved clearance of apoptotic cell debris. Accordingly, the M1/M2 ratio is proportional to the progression of lesion size in \textit{Apoe}^{-/-} mice, whereas factors that promote a proinflammatory M1 macrophage phenotype such as TLR2 and TLR4 ligands or G2A deficiency result in an increased disease burden in the same mice. Thus, the increased expression of inflammatory cytokines and M1 macrophage markers in \textit{Tlr7}^{-/-}\textit{Apoe}^{-/-} mice and the positive association of TLR7 levels with M2 macrophage markers in human carotid plaques reveal an additional mechanism of atheroprotection promoted by TLR7. Although this may be directly related to the levels of inflammatory Ly6C^{-} monocytes (as observed in \textit{Apoe}^{-/-} mice), which produce high levels of monocyte chemoattractant protein-1; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; IFN, interferon; TPA, tissue plasminogen activator; TLR7, Toll-like receptor 7; ECM, extracellular matrix.

Figure 6. TLR7 is expressed in human carotid atherosclerotic lesions and is associated with genes related to a more stable plaque phenotype. A. Comparison of TLR7 mRNA expression between human atherosclerotic tissue specimens and specimens from a normal artery wall. Specimens from patients undergoing carotid endarterectomy and specimens from healthy iliac arteries of organ donors from the Biobank of the Karolinska Endarterectomies study (BiKE) were used. Mean expression levels in arbitrary log2 U (AU) and statistical significance values (p) are shown. B. Comparison of TLR7 mRNA expression from nonsmokers, active, and former smokers in human carotid lesions from the Biobank of the Karolinska Endarterectomies study (BiKE). Mean expression levels in arbitrary log2 U (AU) and statistical significance values (p) are shown. C. TLR7 activation through the addition of 1 g/mL imiquimod (TLR7-L) reduces TNF and MCP-1 production in human atheroma cell cultures. Results are expressed as mean cytokine levels ± SEM of cultures from 3 unrelated patients per group. D. Correlation between TLR7 mRNA levels and established M1/M2 macrophage markers in human carotid lesions from BiKE. Pearson correlation coefficient (r) and statistical significance values (p) are shown. E. Correlation between TLR7 mRNA levels and genes involved in collagen and ECM deposition, smooth muscle cell presence, and platelet accumulation in human carotid lesions from BiKE. Pearson correlation coefficient (r) and statistical significance values (p) are shown. MCP-1 indicates monocyte chemoattractant protein-1; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; IFN, interferon; TPA, tissue plasminogen activator; TLR7, Toll-like receptor 7; ECM, extracellular matrix.
M1-type inflammatory cytokines and are therefore likely precursors of inflammatory M1 macrophages, further studies will be needed to confirm this possibility.

A role of TLR7 in M2 macrophage polarization may at first seem intriguing. However, it is not without precedent. Repetitive triggering of TLR7 in mice has been shown to suppress the production of proinflammatory M1 cytokines such as IL-12/23p40, IL-6, and TNF without affecting, or in some cases even increasing, the production of IL-10.45,46 TLR7 stimulation has also been shown to induce cross-tolerance to subsequent TLR2 and TLR4 stimulation that lasts for days.46 In addition, combination of a TLR ligand and immune complexes, prostaglandins, apoptotic cells, IL-10, or adenosine, all signals relevant to atherosclerosis, has been shown to induce the generation of “regulatory” M2-like macrophages characterized by high expression of CD163 and IL-10, low expression of IL-12 and IL-23.47 It is therefore possible that, in the context of atherosclerosis, TLR7 favors the generation of M2 macrophages as part of a protective immune response aiming at restoring homeostasis in the vessel wall.

Our findings broaden our understanding of the role of innate immune receptors in the development of atherosclerosis. It has been well established over the past few years that TLR2 and TLR4 respond to danger signals induced during hypercholesterolemia or tissue stress to promote macrophage accumulation and inflammation in the vessel wall. TLR6 is also part of this process by acting as the binding partner of TLR2 in the detection of oxidized lipoproteins. This response is further enhanced through the recognition of cholesterol crystals by the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome.48 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47 We now expand this scheme by adding TLR7, a receptor that senses viral and self-RNA, and absence of IL-12 and IL-23.47


cancer therapy with a small molecule agonist of Toll-like receptor 7 can be improved by circumventing TLR tolerance. Cancer Res. 2011;71:5123-5133.


**CLINICAL PERSPECTIVE**

Atherosclerosis underlying cardiovascular mortality is the leading cause of death in developed countries. Efforts are therefore concentrating on unwinding the pathophysiological mechanisms controlling its development and clinical complications. Among them, Toll-like receptors (TLRs) have taken center stage in atherosclerosis research by virtue of their ability to sense danger in response to hypercholesterolemia, tissue stress, or necrosis, and drive macrophage activation and inflammation in the vessel wall. TLR2 and TLR4, in particular, have been shown to play a critical role in promoting plaque development and vulnerability leading to the view that all TLRs are pathogenic for this disease. This article now reports the surprising finding that TLR7, an endosomal TLR that recognizes viral single-stranded RNA and self-RNA released from necrotic cells, is protective. In experimental atherosclerosis in mice, TLR7 prevented lesion development, stenosis, and plaque vulnerability by constraining monocyte chemoattractant protein-1 production, Ly6C^{hi} “inflammatory” monocyte expansion and M1 inflammatory macrophage accumulation to developing atherosclerotic lesions. In human carotid endarterectomy specimens, TLR7 was positively associated with an M2 anti-inflammatory macrophage signature and collagen genes and inversely related/unrelated to proinflammatory mediators and platelet markers, whereas TLR7 activation in human atheroma cultures selectively suppressed the production of monocyte chemoattractant protein-1. Altogether, these findings reveal that TLR7 is part of a protective response that limits atherosclerotic plaque development and vulnerability and challenge the prevailing concept that all TLRs are pathogenic. They also provide new insight about the complex interplay of innate immunity in atherosclerosis and support the exploitation of the TLR7 pathway for therapy.