**EXPERIMENTAL STUDY**

**Toll-Like Receptor 7 Stimulation Promotes the Development of Atherosclerosis in Apolipoprotein E-Deficient Mice**

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**Summary**

Atherosclerosis is a chronic inflammatory disease with multiple characteristic facets, including vascular inflammation, endothelial dysfunction, plaque development, impaired blood flow, and cholesterol deposition through dyslipidemia. Toll-like receptors (TLRs) of the innate immune system have been closely linked to the development of atherosclerotic lesions. TLR7 recognizes viral or endogenous single-stranded RNA, which is released during vascular apoptosis and necrosis. The role of TLR7 in vascular disease remains controversial, and therefore, we sought to investigate the effects of TLR7 stimulation in mice.

Intravenous injection of a ligand for TLR7 (R848) induced a significant pro-inflammatory cytokine response in mice. This was associated with impaired reendothelialization upon acute denudation of the carotid artery, as measured by Evan’s blue staining, and increased numbers of circulating endothelial microparticles (EMPs) and circulating Sca1/Flk1 positive cells as a marker for increased endothelial damage. Chronic subcutaneous stimulation of TLR7 in apolipoprotein E-deficient (ApoE−/−) mice increased aortic production of reactive oxygen species (ROS), the number of circulating EMPs, and most importantly, augmented the formation of atherosclerotic plaque when compared with vehicle-treated animals.

Systemic stimulation of TLR7 leads to impaired reendothelialization upon acute vascular injury and is associated with the production of pro-inflammatory cytokines and increased levels of circulating EMPs and Sca1/Flk1 positive cells. Importantly, ApoE−/− mice chronically treated with R848 displayed increased atherosclerotic plaque development and elevated levels of ROS in the aortic tissue. In addition, TLR7-activation-induced apoptosis and impaired migration in human coronary artery endothelial cells and showed significant upregulation of the signaling cascade of IL-1 receptor-associated kinase (IRAK) 2 and IRAK4. Our data highlight the importance of fully understanding the pathomechanisms involved in atherogenesis, and further studies are necessary to identify the ligand-specific effects of TLR7 for possible therapeutic targeting.

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**Key words:** Innate immune system

Atherosclerosis is a chronic inflammatory disease with multiple characteristic facets, including vascular inflammation, endothelial dysfunction, plaque development, impaired blood flow, and cholesterol deposition through dyslipidemia. Despite intensive research efforts, the critical contributors and exact pathomechanisms responsible for this disease are still unclear. Initially, it was thought that vascular inflammation in atherosclerotic plaques was a byproduct of plaque development. Today, we know that the activation of innate and adaptive immune responses is essential for atherosclerosis and a major driving force of lesion formation.

Cardiovascular disease is triggered by endothelial damage resulting from mechanical, biological, and toxic stress. Apoptosis and necrosis of vascular cells lead to the release of various compounds, including proteins, small molecules, and nucleic acids, all of which are endogenous danger-associated molecular patterns (DAMPs). These DAMPs activate pattern recognition receptors (PRRs), which trigger innate immune mechanisms to promote the clearance of detritus and to neutralize potential cytotoxic substances. PRRs are specific receptors of the innate immune system and have recently been linked to vascular inflammation and atherogenesis, with both damaging and attenuating effects. PRRs, such as nucleotide-binding oligomerization domain-like receptors (NOD) or toll-like re-
TLR7 recognizes viral or endogenous single-stranded RNA, is located in endosomes, and is expressed by macrophages, mast cells, dendritic cells, and endothelial cells. It can be activated by R848 and imiquimod, both imidazoquinoline compounds and artificial ligands of TLR7. R848 specifically activates TLR7 leading to a myeloid differentiation primary response protein (MyD88)-induced production of pro-inflammatory cytokines. Monocyte/macrophages are an important key factor in the innate immune response. Most TLR7 related mechanisms have been primarily and predominantly been identified and investigated in monocytes/macrophages. For example, Hemmi, et al. demonstrated that macrophages from TLR7- and MyD88-deficient mice do not respond to R848-stimulation, while macrophages from wild type (WT) mice produce TNF-α, interleukin (IL)-6 and IL-12 in response to R848. Karper, et al. showed that TLR7 antagonists reduced postinterventional vascular remodeling, foam cell accumulation, and migration. TLR7-antagonists also reduced oxidized LDL-induced foam cell formation. We have, therefore, focused on the pro-inflammatory effects of TLR7 activation in endothelial cells.

However, Salagianni, et al. found that TLR7 could have a beneficial effect in atherosclerosis by constraining inflammatory macrophage activation and cytokine production. This unexpected finding contradicts the originally hypothesized role of TLR7 in chronic vascular diseases. Further investigation of the role of TLR7 in vascular disease is, therefore, warranted. It is known that the highly pro-inflammatory adaptor protein MyD88 is responsible for the signal transduction of TLR7, as well as the other TLRs (except TLR3 and endosomal TLR4), and initiates a pro-inflammatory reaction. Recently, Liu, et al. demonstrated a pro-inflammatory role for TLR7 in atherogenic diet-induced atherosclerosis in apolipoprotein E-deficient (ApoE−/−) mice. To further investigate the role of TLR7 in atherosclerosis, we stimulated TLR7 using the R848 ligand in vitro, as well as in acute and chronic vascular injury experiments in vivo.

Methods

Methods: As described previously.

Cell culture: Human coronary artery endothelial cells (HCAECs) (Lonza, Basel, Switzerland) were cultured at 37 °C and an atmospheric concentration of 5% CO2. Total RNA was reversely transcribed using the Omniscript RT Kit (Qiagen) according to the manufacturer’s instructions. The single-stranded cDNA was then amplified by using quantitative RT-PCR with the TaqMan system (ABI-7500 fast PCR System) using TaqMan gene expression assay probes specific for IRAK2 (Hs00176394_m1, Applied Biosystem), IRAK4 (Hs00928779_m1, Applied Biosys) CCL2 (Hs00234140_m1, Thermo Fisher), VCAM (Hs00365486_m1, Thermo Fisher), ICAM (Hs00164932_m1, Thermo Fisher) or individual primers, and SYBR-Green detection dye. Primers for human TLR7: sense 5’-T TACCTGGATGGA AACCAGCTACT-3’, TLR7 antisense 5’-TCAAGGCTGAGAAGCT GTAAGCTA-3’. For quantification, mRNA expression was normalized to endogenous 18s rRNA.

Animals: We used 8- to 12-week-old C57BL/6J WT mice (Charles River, Sulzfeld, Germany) and 9- to 10-week-old ApoE−/− mice on a C57BL/6 background (Charles River) for this study. The body weight of the mice varied from 23 to 27 g. All animals were maintained in a 22°C room, with a 12 hours light/dark cycle, and received food and drinking water ad libitum. To determine the required concentration of R848 (TLR7 ligand, imidazoquinoline compound, Invivogen, Toulouse, France) for a pro-inflammatory response, WT mice were injected intravenously (i.v.) with 20 μg, 40 μg, and 100 μg R848 suspended in 200 μL PBS or vehicle (PBS) alone. Plasma was collected 2 hours after injection. For the acute injury model, 20 μg R848 in 200 μL PBS per mouse was injected every 48 hours for seven days. For analysis of atherosclerotic plaque development, ApoE−/− mice received a cholesterol-rich diet that contained 21% fat, 19.5% casein, and 1.25% cholesterol (Ssniff, Soest, Germany) for a total
of six weeks, and were concomitantly injected subcutaneously (s.c.) with either 20 μg R848 suspended in 200 μL PBS or vehicle every other day for six weeks. All tissue and blood samples were collected and processed immediately after sacrifice. All animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the local ethics committee, supervised by the regulatory authority of the state of North Rhine-Westphalia and performed in compliance with German animal protection laws.

Carotid artery injury: Carotid artery injury was performed, as previously described 8 on day three of R848-treatment after the second i.v. R848 injection. All mice were anesthetized with intraperitoneal injections of 150 mg/kg body weight ketamine hydrochloride (Ketanest, Riemser, Greifswald, Germany) and 0.1 mg/kg body weight xylazine hydrochloride (Rompun 2%, Ceva, Duesseldorf, Germany). A small incision was made from the cranial apex of the sternum to just below the mandible. After careful preparation of an approximately 6-mm-long segment proximal to the bifurcation, the common carotid artery was electrically denuded. A 4-mm long lesion was made by applying two serial 5-second bursts of 2 Watts using a 2-mm-wide forceps. The skin was then sutured, and the mice were allowed to recover in individual cages before returning to their littersmates. On day seven of the R848 treatment, 50 μL Evan’s blue solution (5%, Sigma) was injected i.v. and allowed to circulate for 2 minutes. The mice were then sacrificed, and both common carotid arteries were fully excised. The arteries were rinsed in 0.9% sodium chloride solution, and the residual connective tissue was carefully removed. The arteries were rinsed in 0.9% sodium chloride solution, and the residual connective tissue was carefully removed. Images were taken, and the total lesion area (4 mm) along with the remaining denuded area (stained blue) was measured by using AxioVision version 4.8.2 software (Zeiss, Oberkichen, Germany). Reendothelialization is expressed by illustrating the remaining denuded area.

Measurement of reactive oxygen species (ROS): The release of superoxide in intact aortic segments was measured by L-012 chemiluminescence, as previously described. 9 Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer (pH 7.4; in mmol/L: NaCl 99.01, KCl 4.17, CaCl2 1.87, MgSO4 1.20, Na-HEPES 20.0, KH2PO4 1.03, NaHCO3 25.0, and D(+)-glucose 11.1). Connective tissue was removed, and the aortas were cut into 2 mm segments. Chemiluminescence of aortic segments was assessed in scintillation vials containing Krebs-HEPES buffer with 100 μmol/L L-012 over 10 minutes in a scintillation counter (Lumat LB 9501, Berthold, Bad Wildbad, Germany) in 1-minute intervals. The vessel segments were then dried to determine the dry weight. ROS release was calculated as relative chemiluminescence per mg aortic tissue as a percentage of the control.

Cytokine quantification: The concentration of interleukin-6 (IL-6) and CC-Chemokine (RANTES) were determined in the plasma of R848-stimulated WT mice by ELISA. Commercially available ELISA kits for mice were used according to the manufacturer’s protocols (Qiagen, Hilden, Germany).

Flow cytometry: Murine blood samples were analyzed, as previously described. 11 Following red blood cell lysis, the viable lymphocyte population was analyzed for Sca-1 (Becton Dickinson, Franklin Lakes, USA), Flk-1 (Becton Dickinson), Annexin V (Becton Dickinson), and CD 31 (Becton Dickinson) to measure endothelial microparticles (EMPs). Isotype identical antibodies and unstained samples served as controls in each experiment (Becton Dickinson). Cell fluorescence was measured immediately after staining using fluorescence-activated cell sorting (FACS Calibur, Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson). The units for all measured components are specific events obtained after measuring 50,000 events in a pre-specified lymphocyte gate during FACS analysis.

Histological and immunohistochemical analysis of atherosclerotic plaques: For histological analysis of atherosclerotic plaques, hearts with ascending aortas were embedded in Tissue-Tek (OCT embedding medium, Miles, Elkhart, USA) snap-frozen, and stored at −80°C. Samples were sectioned on a Leica cryostat (6 μm), starting at the apex and progressing through the aortic valve area into the ascending aorta and the aortic arch, and then they were placed on poly-L-lysine coated slides. For the detection of atherosclerotic lesions and macrophage accumulation, aortic cryosections were fixed with 3.7% formaldehyde for 1 hour, rinsed with deionized water, stained with oil red O working solution (0.5%) for 30 minutes, and rinsed again. Hematoxylin eosin staining was performed according to standard protocols. For immunohistochemical analysis of macrophages, slides containing the cryosections were incubated with acetone for 30 minutes at −20°C. After washing with PBS, the slides were preincubated with 10% normal goat serum (NGS, Sigma, St. Louis, USA) for 30 minutes each at room temperature (RT). The primary antibody MOMA-2 (1:400, rat; Acris, Herford, Germany) diluted in 1% NGS was applied for 1 hour at RT and then at 4°C overnight. 0.1 M Tris-buffer slides were incubated with an alkaline phosphatase-conjugated secondary antibody (Sigma) for 1 hour at RT. A color-producing reaction was accomplished by using FastRed (Sigma) as the chromogenic substrate. Nuclei were counterstained with Hematoxylin (blue). Isotype-specific antibodies were used for negative controls. Tissue sections were washed and mounted with Aquatex mounting medium (Sigma) for light microscopic analysis. For quantification of atherosclerotic plaque formation in the aortic root, lipid-staining area and total area of the serial histological sections were measured. Atherosclerotic data are expressed as the lipid-staining area as a percentage of the total surface area. The investigators who performed the histological analyses were blinded to the treatment of the respective animal group. All sections were examined under a Zeiss Axiopert 200 M microscope using AxioVision version 4.8.2 software.

Statistical analysis: Data are presented as the mean ± standard error of the mean. For statistical analysis, two-tailed, unpaired Student’s t-test and ANOVA for multiple comparisons were employed where applicable. A P-value < 0.05 indicates statistical significance.
Figure 1. TLR7-stimulation in vitro. Stimulation of HCAECs with 100 ng R848 for 24 hours significantly impaired reendothelialization in an in vitro scratch assay (A: reendothelialized area after 24 hours; R848: 351,155 ± 30,717 μm² versus vehicle 529,658 ± 30,316 μm², P < 0.001, n = 10). The reduced reendothelialization can be mainly attributed to a significant increase in apoptosis of HCAECs upon TLR7 stimulation (B: Annexin V positive cells as a percentage of total cells, 7.18 ± 1.03 versus 3.91 ± 0.66, n = 10-11). TLR7 mRNA expression was down-regulated after 8 hours of incubation with R848 (C: 0.11 ± 0.02 versus 1.00 ± 0.00, n = 3). In addition, we found the expression of IRAK2 and IRAK4 significantly increased, suggesting a regulatory feedback mechanism (C: IRAK2: 4.31 ± 1.14, IRAK4: 2.24 ± 0.25, n = 5).

Results

To evaluate the role of TLR7-activation in vascular biology, we first stimulated HCAECs with the TLR7-ligand R848. TLR7-stimulation of HCAECs with 100 ng R848 for 24 hours significantly impaired reendothelialization in an in vitro scratch assay (Figure 1A; reendothelialized area after 24 hours; R848: 351,155 ± 30,717 μm² versus vehicle 529,658 ± 30,316 μm², P < 0.001, n = 10). The reduction in reendothelialization was mainly attributed to a significant increase in apoptosis of HCAECs (Figure 1B, Annexin V positive cells as a percentage of the total cells, 7.18 ± 1.03 versus 3.91 ± 0.66, n = 10-11) upon TLR7 stimulation. While TLR7 mRNA expression was down-regulated after 8 hours of R848 incubation (Figure 1C, IRAK2: 4.31 ± 1.14, IRAK4: 2.24 ± 0.25, n = 5), two essential proteins in the TLR7 signaling cascade, suggesting a regulatory feedback mechanism. To analyze the involvement of macrophage recruitment and adhesion molecules, we found a slight increase in VCAM 1 expression, while CCL2 and ICAM1 remained unregulated (Figure 2).

Because reendothelialization and endothelial cell apoptosis are associated with atherogenesis, we next sought to investigate the effects of systemic TLR7 stimulation in vivo. To determine the optimal dose of R848 to sufficiently induce a pro-inflammatory TLR7-mediated response in mice, we injected WT mice with 20-100 μg R 848 and measured the plasma concentration of IL-6. IL-6 induction was similar in all of the tested dosing regimens (Figure 3A, R848: 20 μg 1772 ± 77, 40 μg: 1785 ± 259, 100 μg: 1678 ± 45 versus vehicle 33 ± 22, P < 0.001, n = 2-4). Thus, for all other experiments, we used a concentration of 20 μg R848/injection/mouse for TLR7-activation. An acute vascular injury model was used to study reendothelialization. WT mice were subjected to electric denudation of the left common carotid artery, and reendothelialization was quantified five days after surgery. R848 or vehicle was injected i.v. every 48 hours, starting two days prior to the carotid injury (Figure 3B). Vascular reendothelialization was impaired by 48% in TLR7-treated stimulated mice compared with vehicle controls (Figure 3C, representative image, and Figure 3D, quantification; R848: 29.58 ± 2.02, P < 0.01, n = 11-13). As expected, stimulation of TLR7 induced a systemic inflammatory response measured by plasma levels of IL-6 (Figure 3E; R848: 409.7 ± 11.1 versus vehicle 29.7 ± 21.8, P < 0.001, n = 3) and RANTES (Figure 3E; R848: 2980 ± 648.3 versus vehicle 101.2 ± 18.4, P < 0.05, n = 6-7). Plasma blood glucose levels were not significantly different in R848- and vehicle-treated mice (data not shown). Reendothelialization in this assay is dependent on two major factors, namely total endothelial damage and the regenerative capacity of the endothelial cells. EMPs, as measured by Annexin/CD31 positive particles in cell culture, are apoptotic bodies and a marker for amplified endothelial dysfunction, as well as a predictor for an impaired endothelial-dependent vasorelaxation.6) Sca-1 and Flk-1 double-positive cells have been described as endothelial progenitor cells (EPCs), leading to worsened endothelial dysfunction. This number was significantly elevated in R848-treated mice (Figure 3F; R848: 0.24 ± 0.04 versus vehicle 0.1 ± 0.01, P < 0.05, n = 5-7). Interestingly, the number of circulating Sca1/Flk 1-positive EPCs was increased in TLR7-stimulated mice.
Acute vascular injury. To determine the optimal dose of R848, we injected WT mice with 20-100 μg R848 and measured the plasma IL-6 concentration (A: R848: 20 μg 1772 ± 77, 40 μg: 1785 ± 259, 100 μg: 1678 ± 45 versus vehicle 33 ± 22, P < 0.001, n = 2-4). WT mice were subjected to an electric denudation of the left common carotid artery and the reendothelialization was quantified five days after surgery. R848 or vehicle was injected i.v. every 48 hours starting two days prior to the carotid injury (B). Vascular reendothelialization was impaired by 48% in TLR7-stimulated mice compared with vehicle controls (C: representative image, and D: quantification; R848: 43.78 ± 3.232 versus vehicle 29.58 ± 2.02, P < 0.01, n = 11-13). Stimulation of TLR7 induced a systemic inflammatory response, measured by plasma IL-6 (E: IL-6; R848: 409.7 ± 11.1 versus vehicle 29.7 ± 21.8, P < 0.001, n = 3) and RANTES levels (E: RANTES; R848: 2980 ± 648.3 versus vehicle 101.2 ± 18.4, P < 0.05, n = 6-7). The number of EMPs was significantly elevated in R848-treated mice (F: R848: 0.24 ± 0.04 versus vehicle 0.1 ± 0.01, P < 0.05, n = 5-7). The number of circulating Sca1/Flk1-positive endothelial progenitor cells was increased in TLR7-stimulated mice (G: R848: 1.12 ± 0.26 versus vehicle 0.35 ± 0.07, P < 0.05, n = 11-12).

We next investigated whether pro-inflammatory TLR7 stimulation affects vascular biology in a chronic injury model. For this experiment, ApoE−/− mice received a high-fat, cholesterol-rich diet for a total of six weeks and were concomitantly injected s.c. with either 20 μg R848 or vehicle three times per week (Figure 4A). Mice subjected to repetitive R848 injections showed no external signs of side effects; their body weight, food, and water intake were indistinguishable from control mice (data not shown). We measured the serum lipid profile of ApoE−/− mice on a Western diet and R848 or vehicle treatment. Mice treated with R848 displayed slightly lower serum cholesterol levels compared with vehicle-treated mice. R848-treated ApoE−/− mice developed atherosclerotic plaques that were 44% larger than those of the control mice (Figure 4B, quantitative, and Figure 4C representative image; R848: 11.0 ± 1.3 versus vehicle 7.9 ± 1.5, P < 0.05, n = 9). Interestingly, R848-treated mice displayed lower serum cholesterol levels (data not shown). This is likely a combined effect of decreased enteric sterol uptake, as indicated by a lower phytosterol concentration and impaired cholesterol biosynthesis, as shown by the immediate cholesterol precursors Desmosterol and Lathosterol. The composition of the plaques was altered in TLR7-stimulated mice. Immunohistochemical staining for MOMA-2 showed a significant increase in macrophage infiltration in R848-treated animals compared with vehicle-treated controls (Figure 4D, quantification, and Figure 4E representative image; R848: 21.7 ± 2.6 versus vehicle 11.0 ± 1.8, P < 0.05, n = 5). Macrophages contribute to atherosclerotic plaque development in part by the production of ROS. Thus, we measured ROS...
production in the aortic segments of these mice. Mice treated with R848 had increased vascular ROS formation compared with control animals (Figure 5A; R848: 170.2 ± 17.6 versus vehicle 109.6 ± 15.81, \( P < 0.05, n = 11 \)). This prompted us to investigate whether TLR7 activation in macrophages can influence endothelial function via paracrine mechanisms. Indeed, when HCAECs were incubated with the supernatant from macrophages previously stimulated with R848, reendothelialization was impaired compared with those incubated with control supernatant (Figure 5B; 287,203 ± 7042 \( \mu m^2 \) versus vehicle 337,105 ± 9019 \( \mu m^2 \), \( n = 3-4, P < 0.01 \)). *\( P < 0.05 \), **\( P < 0.01 \).

**Discussion**

Atherosclerosis is a chronic inflammatory disease influenced by multifactorial mechanisms that are involved in all stages of the disease and whereby TLRs seem to
play conflicting roles. The results of our study stand in contrast to the findings of Salagianni, et al., who found a protective role for TLR7 in the development of atherosclerosis by limiting the activation of inflammatory macrophages and cytokine production.5, 14 These findings were in accordance with those of Karadimou, et al., who identified TLR7 as a marker for better outcomes in patients with severe atherosclerosis. Increased levels of the TLR7 transcript were found in plaques removed from patients undergoing carotid endarterectomy and was associated with fewer adverse cardiovascular events.16 Furthermore, Karadimou, et al. showed that TLR7 is expressed in T cells and macrophages within the plaque and in capillary endothelial cells, but not in endothelial cells.16 In contrast, Liu, et al. demonstrated a detrimental role for TLR7 in diet-induced atherosclerosis in ApoE−/− mice by showing an increase in lesion inflammation (MHC-II), lesion pro-inflammatory cytokine expression (IL-6), lesion protease expression (CatS and MMP-9), and systemic inflammation (plasma).19 In agreement with these findings, we show that the pro-inflammatory stimulation of TLR7 by high concentrations of R848 impairs reendothelialization following acute vascular injury combined with increased levels of EMPs, induced ScaI/Flik positive cells, and elevated levels of IL-6 and RANTES. TLR7 activation in WT mice induced a systemic inflammatory reaction and disturbed the regeneration of endothelial cells after injury. Chronic TLR7-activation in ApoE−/− mice after feeding of a western diet over eight weeks significantly increased plaque development compared with vehicle-treated mice, and this was accompanied by elevated levels of ROS. We measured slightly lower serum cholesterol levels in R848-treated mice compared with vehicle. This is likely a combined effect of decreased enteric sterol uptake, as indicated by a lower phytosterol concentration (CAMPsterol, Sitosterol, Brassicasterol, etc.), and impaired cholesterol biosynthesis, as shown by the immediate cholesterol precursors Desmosterol and Lathosterol. Surprisingly, we did not detect elevated levels of pro-inflammatory cytokines during the chronic injury, but R848 treatment induced macrophage accumulation in aortic plaques compared with vehicle-treated mice. In total, our results imply a pro-atherosclerotic role for TLR7.

Innate immunity with its many receptors is undoubtedly linked to atherosclerosis; however, there is no clear evidence yet of the pro- or anti-atherosclerotic involvement of the different TLRs, underlining the complex role of the innate immune system. The central aim of TLRs in host defense is to detect pathogenic microorganisms, such as bacterial or viral molecules, and initiate an adequate immune response. The role of TLR7 in atherogenesis remains unclear, though numerous studies seem to suggest that it plays a protective role in plaque development. While TLR2, TLR4, and MyD88 are considered to have pro-atherosclerotic effects,7, 18-19 the presence of TLR7 and TLR9 in mice shows a reduction in atherosclerotic lesions versus the double knockout mouse models.5, 20 Furthermore, a deficiency of interferon regulatory factor 5 (IRF-5), a central mediator of TLR7 signaling, in ApoE−/− mice results in more severe atherosclerosis.21 Also, our finding of pro-atherosclerotic effects by TLR3 stimulation with poly I:C in ApoE−/− mice was refuted by Cole, et al.8, 22 Endosomally localized TLR7 is normally activated by viral or endogenous single-strand RNA, but ligands with imidazoquinoline compounds such as imiquimod and R 848 can also initiate a signaling cascade through TLR7 with the release of pro-inflammatory cytokines.22 Apoptotic and necrotic cells might be able to stimulate TLRs by releasing endogenous RNA or DNA, with contradictory effects seen in early and advanced atherosclerotic lesions.23 TLR7 is upregulated in intermediate and advanced atherosclerotic lesions of patients with peripheral artery disease and is expressed in human carotid atherosclerotic lesions.5, 24-26

It may be that the detrimental effects of TLR7 activation at this late stage of atherosclerotic lesions predominate over any positive effects. Because we did not find impairment of endothelial-dependent vasodilation after TLR7 activation in the acute and chronic experiments, further experiments with TLR7−/−ApoE−/− mice are necessary to confirm our hypothesis.

Mainly macrophages and dendritic cells, but also vascular cells, are responsible for identifying DAMPs and initiating phagocytosis, apoptosis, or release of cytokines.27 The highly pro-inflammatory MyD88 protein is required for downstream signal transduction of all TLRs (except TLR3 and endosomal TLR4) via TIR-domain-containing adapter-inducing interferon-β.28 Several publications indicate a pro-atherosclerotic effect from TLR7 stimulation. Depletion of MyD88, obligatory for TLR7 signaling, impairs the formation of atherosclerotic plaques, reduces macrophage recruitment in the arterial wall, and reduces chemokine levels.17 TLR7 stimulation in rabbits also induced pro-inflammatory reactions with plaque enlargement, upregulation of vascular adhesion molecule-1 in endothelial cells, and infiltration of T-lymphocytes.29 Inappropriate activation of TLRs can lead to substantial inflammation, resulting in tissue damage and autoimmunity,29 and antagonism of TLR7 during arterial restenosis reduced postinterventional vascular remodeling and foam cell accumulation.30 Plaque destabilization within advanced atherosclerotic plaques is characterized by the induction of macrophage apoptosis. TLR7 promotes apoptosis of THP-1-derived macrophages, is involved in induced autophagy in murine macrophages, and may, therefore, be involved in increasing the destabilization of plaques.30, 31 Salagianni, et al. recently published that TLR7 is protective in atherosclerosis.31 TLR7−/−ApoE−/− mice had substantially larger atherosclerotic lesions at 18- and 26-weeks, and increased CD68+ macrophages. This contradicts the originally proposed role for TLR7 in chronic vascular diseases.

Our in vitro experiments also suggest a pro-inflammatory influence of TLR7 activation on HCAECs. Stimulation with R848 induced apoptosis and impaired migration in HCAECs. Although expression of TLR7 mRNA was suppressed after 24 hours of stimulation, IRAK2 and IRAK4 mRNA were induced following stimulation with 100 ng R848, while MyD88 showed a tendency toward downregulation 24 hours after stimulation but was not statistically significant.
Conclusion

In conclusion, we found that systemic stimulation of TLR7 leads to impaired reendothelialization upon acute vascular injury and is associated with the production of pro-inflammatory cytokines, as well as the increased formation of circulating EMPs and Sca1/FK1 positive cells. Importantly, ApoE−/− mice chronically treated with R848 displayed increased atherosclerotic plaque development and elevated levels of ROS in the aortic tissue. TLR7 activation further induced apoptosis and impaired migration in HCAECs and showed a significant upregulation of the signaling cascade of IRAK2 and IRAK4. Our data highlight the importance of fully understanding the pathway mechanisms involved in atherogenesis, and further studies are needed to identify the ligand-specific effects of TLR7 for possible therapeutic targeting.

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Disclosure

Conflicts of interest: All authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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