Treatment with a Toll-like Receptor 7 ligand evokes protective immunity against atherosclerosis in hypercholesterolaemic mice

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Abstract. Karadimou G, Gistera A, Gallina AL, Caravaca AS, Centa M, Salagianni M, Andreakos E, Hansson GK, Malin S, Olofsson PS, Paulsson-Berne G (Karolinska Institutet and Karolinska University Hospital, Stockholm; Feinstein Institute for Medical Research Fertility Research Laboratory, Manhasset, New York, USA; Biomedical Research Foundation of the Academy of Athens, Athens, Greece). Treatment with a Toll-like Receptor 7 ligand evokes protective immunity against atherosclerosis in hypercholesterolaemic mice (Original article). J Intern Med 2020; 288: 321–334.

Background. The interplay between innate and adaptive immunity is central in life-threatening clinical complications of atherosclerosis such as myocardial infarction and stroke. The specific mechanisms involved and their protective versus detrimental effects in the disease process remain poorly understood. We have previously shown that higher levels of Toll-like receptor 7 (TLR7) expression in human atherosclerotic lesions are correlated with better patient outcome.

Objective. In this study, we explored whether TLR7 activation can ameliorate disease in experimental atherosclerosis in mice.

Methods. Apolipoprotein E deficient mice (Apoe−/−) with established disease were injected for five weeks intraperitoneally with the TLR7 ligand R848. Local effects were evaluated by characterization of the lesion. Systemic effects of the treatment were investigated by immune composition analysis in the spleen and plasma measurements.

Results. The in vivo treatment arrested lesion progression in the aorta. We also detected expansion of marginal zone B cells and Treg in the spleen together with increased plasma IgM antibodies against oxidized low-density lipoprotein (oxLDL) and reduced plasma cholesterol levels. These changes were accompanied by increased accumulation of IgM antibodies, decreased necrosis and fewer apoptotic cells in atherosclerotic lesions.

Conclusions. Our findings show that TLR7 stimulation could ameliorate atherosclerotic lesion burden and reduce plasma cholesterol in Apoe−/− mice. TLR7 stimulation was associated with an atheroprotective B-cell and Treg response, which may have systemic and local effects within lesions that could prevent arterial lipid accumulation and inflammation.

Keywords: B lymphocyte, cholesterol, immunoglobulin M, inflammation, oxidized low-density lipoprotein, regulatory T cell.

Introduction

Atherosclerosis is a multifactorial disease with myocardial infarction and stroke as lethal endpoints. The disease is a result of accumulation of lipoproteins that trigger a complex vascular inflammation in the arterial wall [1,2]. The retention of low-density lipoprotein (LDL) in the intima is accompanied by modifications and formation of, for example, oxidized (ox)LDL. Several pathways inducing both adaptive and innate immunity are activated in response to retained and modified LDL [3,4]. Within the plaque, many processes connected to inflammation and resolution act in

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parallel generating a delicate balance between immune reactions together with inflammatory responses that can either worsen the disease or promote healing.

Removal of dead or apoptotic cells through efferocytosis is of crucial importance for tissue homeostasis [5]. Efferocytosis is also part of the resolution process of inflammation [6]. In the hyperlipidemic environment of the plaque, macrophage functions such as cholesterol efflux and efferocytosis have been shown to be dysregulated [4], leading to foam cell formation and subsequent necrosis.

The members of the Toll-like receptor (TLR) family function as regulators of innate responses. Involvement of several TLRs, such as TLR2 and TLR4, has been shown to be strongly proatherosclerotic [7,8]. However, the two endosomal receptors TLR3 and TLR7 have both been identified to ameliorate atherosclerosis. Salagianni et al. showed that TLR7 deficiency increased disease in atherosclerotic apolipoprotein E knockout (Apoe<sup>−/−</sup>) mice, indicating that functional TLR7 is important to control disease [9]. A recent finding by us in our biobank of human carotid endarterectomies supported that TLR7 could be a protective player. Patients with higher level of TLR7 transcript in their removed carotid plaque had fewer major adverse cardiovascular and cerebrovascular events during a median follow-up period of 3.8 years, with a maximum of 8.3 years [10]. Immunofluorescence analysis showed that TLR7 was expressed by macrophages and T cells and present in all human plaques analysed.

Initially, single-stranded RNA was claimed to be the typical ligand for TLR7. Recently, several endogenous ligands have been identified such as extracellular RNA from necrotic cells, apoptotic cells and miRNAs [11]. In atherogenesis, tissue damage could be a plausible source of ligands such as RNA from apoptotic and necrotic cells. Although endogenous TLR7 ligands in plaques are not yet identified, we mimicked the response by using a synthetic ligand through a human plaque <i>ex vivo</i> approach. The <i>ex vivo</i> treatment resulted in a dose-dependent release of cytokines including IL-10 to the culture medium [10]. Immunofluorescence analysis suggested that upon TLR7 activation, CD163 macrophages and T cells in the plaque quickly responded with IL-10 release [10]. Apo<sup>e<sup>−/−</sup></sup> mice are commonly used to study experimental atherosclerosis, and they develop complex atherosclerotic lesions with age [12]. Several studies by others and us have investigated the role of adaptive and innate immunity in experimental atherosclerosis [13-18]. To discriminate between the impact of T cells versus B cells in the pathogenesis of atherosclerosis, Caligiuri et al. performed a study of splenectomized Apo<sup>e<sup>−/−</sup></sup> mice combined with adoptive back-transfer of either T or B cells. The study showed that B cells isolated from spleen carried an atheroprotective function [19]. Also, other groups have studied the impact of splenectomy in atherosclerosis showing that removal of spleen changed the phenotype of the atherosclerotic lesion, with more necrosis and less clearance of apoptotic cells [20]. Recent studies have showed that in hypercholesterolaemic mice, sterile inflammation within the spleen induced protective B-cell response towards self-antigens found in oxLDL and apoptotic cells [17] and that high-cholesterol diet activates an anti-inflammatory programme specifically in marginal zone (MZ) B cells of the spleen [21].

Based on our results identifying TLR7 as an important factor for atherosclerotic patients’ outcome, we decided to investigate whether that could be explored therapeutically to treat disease in atherosclerotic Apo<sup>e<sup>−/−</sup></sup> mice. In this study, we chose to use aged Apo<sup>e<sup>−/−</sup></sup> mice with established lesions to investigate the effect of systemic TLR7 treatment in experimental atherosclerosis. We aimed to have mice with lesions with similar complexity as the lesions in the patients’ with severe atherosclerosis going through carotid endarterectomy [10]. We hypothesized that treatment with TLR7 synthetic ligand would prevent and arrest development of established atherosclerotic lesions. Moreover, we wanted to investigate whether systemic changes in immune competent cells would occur, specifically in the splenic compartment.

**Material and methods**

Further information in Materials and Methods can be found in Supplementary materials online.

**Mice and treatment**

Apolipoprotein E deficient mice (B6.129P2-Apo<sup>e<sup>−/−</sup></sup>/m1Unc<sup>−/−</sup>N11) were purchased from Taconic (Denmark). All experiments were performed according
to institutional guidelines and were approved by the Stockholm Regional Board for Animal Ethics. Moreover, all animal procedures performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Female mice (22 weeks) were injected intraperitoneally with TLR7 ligand R848 (InvivoGen, Resiquimod, tlr1-r848-5). Injections were made once per week for 5 weeks, 6 injections in total. R848 was dissolved in PBS and administered intraperitoneally. Mice were euthanized with CO2, 24 h after the last injection at the age of 27 weeks. The euthanasia or the sampling was on each occasion performed at the same time in the morning, starting at 8 AM. In the first round of treatment, four groups in total were included. One baseline group was euthanized at the start of the in vivo treatment. After 5 weeks treatment, the mice were euthanized and atherosclerosis in the aortic root was quantified. Atherosclerotic lesion area in the aortic root was significantly smaller in the group of mice treated with a high dose of TLR7 ligand compared to the control group (Fig. 1a, b). The tested lower dose of TLR7 ligand did not significantly reduce atherosclerotic burden. Analysis of the Oil-red O staining showed that the group treated with the high dose of TLR7 ligand had decreased neutral lipid accumulation per lesion area compared to controls (Fig. 1c). However, no significant difference was observed in lesion size between treated and baseline groups (Fig. 1a, b), indicating lesion arrest rather than regression. Furthermore, cholesterol levels in the plasma of the treated mice were decreased 38% compared to the PBS group (Fig. 1d). Plasma triglyceride levels remained unchanged (Fig. 1e).

Statistical Analysis

The Shapiro–Wilk normality test was used to test samples for normality. Normally distributed samples were analysed with the Student’s t-test, one-way ANOVA with Bonferroni multiple comparison post-test and two-way ANOVA. The Mann–Whitney or Kruskal–Wallis test with Dunn’s multiple comparison test was used for comparison of two or multiple groups, respectively, when normal distribution assumption was invalid. The Pearson correlation coefficient was used to assess correlations. Differences between groups were considered significant at P values < 0.05 (*P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). Data were analysed using Prism version 6.0 for Windows (GraphPad).

Results

Treatment with TLR7 ligand reduces atherosclerosis in Apoe⁻/⁻ mice

In order to investigate the effect of TLR7 activation on established atherosclerotic lesions, 22-week-old Apoe⁻/⁻ mice were injected once per week. Four groups were included in the first round of treatment. Two treatment groups received injections with the synthetic TLR7 ligand R848, one group was treated with high dose and another with low dose. One control group received PBS injections, and one baseline group was euthanized at the start of the in vivo treatment. After 5 weeks treatment, the mice were euthanized and atherosclerosis in the aortic root was quantified. Atherosclerotic lesion area in the aortic root was significantly smaller in the group of mice treated with a high dose of TLR7 ligand compared to the control group (Fig. 1a, b). The tested lower dose of TLR7 ligand did not significantly reduce atherosclerotic burden.

Analysis of the Oil-red O staining showed that the group treated with the high dose of TLR7 ligand had decreased neutral lipid accumulation per lesion area compared to controls (Fig. 1c). However, no significant difference was observed in lesion size between treated and baseline groups (Fig. 1a, b), indicating lesion arrest rather than regression. Furthermore, cholesterol levels in the plasma of the treated mice were decreased 38% compared to the PBS group (Fig. 1d). Plasma triglyceride levels remained unchanged (Fig. 1e).

Treatment with TLR7 ligand increases MZ B cells, plasma cells and regulatory T cells in the spleen

The R848-treated Apoe⁻/⁻ mice had increased spleen size, cellularity and weight (Figure S2a-c). There was also a decrease in total lymphocytes in circulation in the treated mice compared to controls (Table S2). To further investigate whether any changes occurred in immune cell populations within the spleen after TLR7 activation, we repeated the 5-week treatment in new groups of 22-week-old Apoe⁻/⁻ mice. In the following treatments, only the high dose of TLR7 ligand was used and a PBS control group. The treated mice presented similar increase in spleen size as observed in the first group of mice treated with R848. T cells, B cells and monocytes were analysed in spleen cells using flow cytometry (Fig. 2a-j and Figure S2d, e). Major changes were detected within the B-cell populations, where MZ B cells showed a 2-fold increase after TLR7 treatment in both proportion and absolute cell numbers (Fig. 2a, b). We observed an increase in plasma cells (Fig. 2e, f) and a tendency for increased germinal centre (GC) formation (Fig. 2c, d). Within the T-cell population, we detected an increase in the CD4⁺ populations, accompanied by a 3-fold increase of regulatory Foxp3⁺ T cells (Treg) in the treated mice (Fig. 2i,j).
TLR7 stimulation reduces atherosclerosis in Apoe$^{-/-}$ mice. The 22-week-old female Apoe$^{-/-}$ mice were injected once/week for 5 weeks. Four groups in total were included in the set-up. One baseline group was euthanized at the initiation of the treatment. One group of mice was treated with phosphate-buffered saline (PBS). Two groups were treated with different doses of TLR7 ligand R848, a low dose (50 $\mu$g/mouse) and a high dose (150 $\mu$g/mouse). (a) Representative images of lesions in aortic root sections stained with Oil-Red O. Scale bar 250 $\mu$m. (b) Quantification of atherosclerotic burden, Oil-red O-stained area. $n=8$ for Baseline, $n=7$ for PBS, $n=8$ for Low R848 and $n=8$ for High R848. $^*P<0.05$, one-way ANOVA with Bonferroni post-test. (c) Percentage of Oil-red O-stained area normalized to total lesion area. $n=7$ for PBS and $n=8$ for High R848. $^*P<0.05$, unpaired Student’s t-test. (d) Plasma cholesterol measurement (mg/dL). $n=8$ per group. $^*P<0.05$, one-way ANOVA with Bonferroni post-test. (e) Plasma triglycerides measurement (mg/dL). $n=8$ per group. Dots represent individual animals. Data are presented as mean ± SD.
Analysis of the monocyte/macrophage CD11b+did not show any changes (Figure S2c, d).

In this second round treating 22-week-old Apoe−/− mice with TLR7 ligand, we also confirmed the atheroprotective effect (Figure S1).

**TLR7 ligand treatment increases IgM antibodies against oxidized lipoproteins**

Since we detected an expansion of MZ B cells and plasma cells in the spleen, we next measured whether our treatment also had an effect on antibody levels of total IgG and IgM in the plasma. For total IgM, there was a 66% increase in the treated group compared to control mice whilst IgG remained unchanged (Fig. 3a, b). Thereafter, we investigated if antibody titres specifically towards oxLDL were changed in response to treatment. Our analysis showed that IgM against oxidized LDL epitopes was 45% increased (Fig. 3c). No change was observed in the levels of anti-oxLDL IgG antibodies in the plasma of treated mice (Fig. 3d).

**Time-course study of TLR7 ligand effect showing inverse changes in anti-oxLDL IgM and cholesterol levels**

Next, we analysed the relationship between anti-oxLDL IgM antibody levels with cholesterol levels and lesions size, respectively. Anti-oxLDL IgM antibodies were negatively correlated with cholesterol levels (Fig. 4a) and lesion size (Fig. 4b). Therefore, in order to monitor the impact of TLR7 ligand R848 treatment on plasma cholesterol and anti-oxLDL IgM levels, mice were treated according to the described protocol with the addition of blood sampling once/week (Fig. 4c-e). In our time-course study, the anti-oxLDL IgM levels start to increase after the second injection of R848 compared to controls, and continue to increase thereafter (Fig. 4d, e). The cholesterol levels start to drop clearly after the fourth injection (Fig. 4c, e). Positive correlation was observed between plasma cholesterol levels and lesion size (Fig. 4f).

**TLR7 ligation resulted in accumulation of IgM antibodies, less necrosis and fewer apoptotic cells in the aortic roots of treated mice**

Studies have shown that IgM antibodies protect against atherosclerosis by blocking the uptake of oxidized lipids from macrophages [22]. Thus, we were interested to evaluate the presence of IgM locally in the atherosclerotic lesion since we observed strong increase of IgM antibody levels in plasma of the treated mice. We analysed aortic root sections for presence of infiltrating IgM antibodies. Our analysis showed increased levels of IgM antibodies in the lesions of treated mice compared to the control (Fig. 5a, b). Increase in anti-oxLDL-specific IgM antibodies can prevent foam cell formation by blocking uptake of oxLDL by macrophages, and thereby prevent them from cell death [22].

We also stained aortic root sections with the pan-macrophage marker CD68. No change was observed regarding percentage area of CD68 in the treated mice compared to the control (Fig. 5c, d).

OxLDL and apoptotic cells share common oxidation-specific epitopes, such as oxidized phospholipids. Due to this common epitope sharing, IgM antibodies can improve both clearance of apoptotic cells and block oxLDL uptake by macrophages [23]. So we next investigated changes in the atherosclerotic lesions that could be a result of increased IgM levels both in the plasma and in the lesions of the treated mice. To evaluate the lesion morphology regarding necrosis, we stained aortic root sections with haematoxylin and eosin. We observed a 33% reduction in necrotic area in the lesions of TLR7 ligand-treated mice (Fig. 5e, f). The decrease in the necrotic core formation was accompanied by relatively fewer apoptotic cells in treated compared to the control mice, shown by TUNEL staining in the aortic root (Fig. 5g, h). These data indicate that there is an increase in clearance of apoptotic cells in the lesions of mice treated with TLR7 ligand R848 compared to control mice.

**Discussion**

In atherosclerosis, promoting resolution of inflammation in the lesion, and thereby controlling disease and reducing risk for plaque rupture, remains a challenge. Here, we present a study showing how treatment with a ligand binding one of the innate immunity receptors, TLR7, can ameliorate experimental atherosclerosis. In Apoe−/− mice with advanced atherosclerotic lesions, a five-week stimulation of TLR7 through specific ligand injections reduced lesion size and lead to more stable plaques with smaller necrotic area and fewer apoptotic cells. Within the spleen, we detected expansion of MZ B cells and Tregs. In plasma of the treated mice, circulating IgM antibodies binding to oxLDL were increased whilst cholesterol levels were reduced. In
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(a) PBS

(b) MZ B

(c) CD21

(d) Follicular

(e) GL7

(f) GC B

(g) CD95

(h) Plasma cells

(i) CD8

(j) CD3

(k) CD4

(l) CD25

(m) FoxP3

(n) Cells (x10^6)

(o) Cells (x10^6)

(p) Cells (x10^6)

(q) Cells (x10^6)

(r) Cells (x10^6)

(s) Cells (x10^6)

(t) Cells (x10^6)

(u) Cells (x10^6)

(v) Cells (x10^6)

(w) Cells (x10^6)

(x) Cells (x10^6)

(y) Cells (x10^6)

(z) Cells (x10^6)
the lesions, increased infiltrating IgM antibodies were detected. Taken together, these processes all contribute to ameliorate atherosclerosis.

Two studies have reported investigation of TLR7 deficiency in atherosclerotic mice by generating Apoe<sup>−/−</sup>/TLR7<sup>−/−</sup> mice. The first study reported from Salagianni et al showed a protective effect for TLR7 in atherosclerosis through a macrophage shift towards a repair-healing subtype [9]. In contrast, Liu et al indicated that TLR7 has a detrimental role [24]. The discrepancy in these two studies might be explained by the use of different diet. In the study from Liu et al, the mice were fed with a high-cholesterol diet whilst in Salagianni et al used chow diet. Since the Apoe<sup>−/−</sup> spontaneously develop atherosclerosis without high-cholesterol diet, it is usually avoided. Other studies have shown that high-cholesterol atherogenic diets can serve as an extra source for PAMPs that could lead to strong activation of several members of the TLR family, like TLR4 and TLR2 [25]. In our study, we used Apoe<sup>−/−</sup> mice that were fed chow diet, to avoid any possible extra effects of diet in the immune responses.

In our present experimental study, we investigate whether treatment with a TLR7 ligand could be possible for future therapeutic use. We treated mice with the synthetic ligand R848 to study activation of TLR7 in the lesions. This ligand has been used in several studies in mouse models to selectively stimulate TLR7 [26,27]. In humans, both TLR7 and the closely related endosomal TLR8 are active in leucocytes and can bind and react to similar ligands. Until recently, it was believed that TLR8 was inactive in mice [28,29], since leucocytes from TLR7<sup>−/−</sup> mice were shown not to respond to the R848 ligand. Cells isolated from peritoneal cavity or spleen were challenged with R848, and both cytokine release and prolifer-ation were measured. The assays could not detect any reactivity to R848 in the TLR7<sup>−/−</sup> immune competent cells, and since then, TLR8 was thought to be nonfunctional in mice. However, a recent study has shown that in murine neurons, also TLR8 can react to R848 [30]. These data that are finding functional TLR8 activity in the neural compartment show that the expression pattern of the intracellular TLR7 and TLR8 is more complex than previously understood[30].

After 5 weeks treatment with TLR7 ligand, the mice had smaller lesions with less necrotic area
Fig. 4 Time-course study of TLR7 ligand effect showing inverse relationship of anti-oxLDL IgM and cholesterol levels. (a) Correlation between IgM binding oxLDL and plasma cholesterol levels (mg/dL). n = 8 for per group. r = −0.672, P = 0.0043, Pearson correlation. (b) Correlation between IgM binding oxLDL and atherosclerotic lesion area (µm²). n = 7 for PBS and n = 8 for R848. r = −0.718, P = 0.0026, Pearson correlation. Dots represent individual mice. Time-course study of TLR7 ligand R848. (c) Cholesterol measurement (mg/dL) after each injection with R848. (d) anti-oxLDL IgM antibody measurement (OD 450 nm) after each injection with R848. (e) Overlay of cholesterol and anti-oxLDL IgM antibody levels after each injection with R848. Red lines indicate R848 group, and black lines indicate PBS group. Solid lines represent cholesterol measurement, and dotted lines represent anti-oxLDL IgM antibody measurements. n = 10 for PBS and n = 9 for R848. Data are presented as mean ± SD. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, two-way ANOVA. (f) Correlation between plasma cholesterol levels (mg/dL) and atherosclerotic lesion area (µm²). n = 7 for PBS and n = 8 for R848. r = 0.588, P = 0.021, Pearson correlation. Dots represent individual mice.
indicating more stable plaques. In the lesions of the treated mice, there were fewer visible apoptotic cells, which could be the result of better clearance but also less cell death. Possibly, similar effects in human plaques could lead to reduced plaque rupture and ensuing adverse cardiovascular outcomes. This is further supported by expression data in carotid plaques, where TLR7 is associated with reduced major adverse cardiovascular events [10].

We were also interested in systemic effects of TLR7 treatment including effects on secondary lymphoid organs since we noticed an increase in splenic size in response to the treatment. We therefore decided to explore these effects in our model.
to further explore whether systemic changes in immune competent cells had occurred in the splenic compartment. The spleen is of particular interest since other studies have shown that this organ harbours potent ‘atheroprotective’ immune responses [17,19,20]. Interestingly, our treatment protocol with TLR7 stimulation in hypercholesterolaemic Apoe\(^{-/-}\) mice lead to expansion of MZ B cells, plasma cells and T\(_{\text{regs}}\) in the spleen.

After treating the hypercholesterolaemic mice with TLR7 ligand, we observed an increase of T\(_{\text{reg}}\) in the spleen. Several studies have shown T\(_{\text{reg}}\) control atherosclerosis burden. Specifically, depleting T\(_{\text{reg}}\) from atherosclerotic mice led to increased atherosclerotic lesion burden [31-33]. Whether this is a direct response by T cells or if there is an involvement of B-cell interaction, it cannot be discriminated in our study. There are different lines of evidence published by other groups regarding the effect of TLR7 activation on regulatory T cells. Several studies have shown induction of T\(_{\text{reg}}\) after using the TLR7 agonist R848 in experimental models [26,34,35]. In an experimental study of allergy, using several knockout models the authors showed that TLR7 activation resulted in IL-10 production in splenic B cells that in turn led to T\(_{\text{reg}}\) expansion. However, in a recent paper by Roh et al [36] an antagonist is used to block TLR7 in studies of nonalcoholic steatohepatitis (NASH) and this treatment ameliorated disease. Taken together, further studies are required to pinpoint effects of TLR7 agonists versus antagonist in the T\(_{\text{reg}}\) compartment.

The splenic MZ resident B cells, identified as CD21\(^{hi}\)CD23\(^{lo}\), are innate-like lymphocytes known to express several surface and intracellular receptors including TLRs and polyreactive B-cell receptors. MZ B cells express high levels of TLRs that allow them to bridge the responses between the innate and adaptive immune system. In vitro stimulation of different B-cell subtypes clearly shows that stimulation with TLR7 ligand leads to antibody production by MZ B cells [37]. It has also been shown in vivo that treatment with TLR7 ligand directly activates MZ B cells and promotes immobilization [38]. These two studies support our finding that MZ B cells are capable of responding to the TLR7 ligand.

MZ B cells bind to multiple molecular patterns through polyreactive B-cell receptors and give rapid early response of T-cell-independent IgM antibodies to blood-borne antigens [39,40]. Convincing studies show that there are IgM antibodies that through molecular mimicry have the capacity to bind several antigens such as pneumococcal pneumonia epitopes, apoptotic cells and oxLDL and that these antibodies elicit protection against atherosclerosis [22,41-43]. These studies show that IgM antibodies against oxLDL can reduce atherosclerotic lesions size and promote more stable plaque with less necrosis [22,42,43].

Together with B1a cells, MZ B cells are the main producers of IgM antibodies [39,43]. Spleen serves as a reservoir for B1a cells that colonize the peritoneal cavity. The atheroprotective effect of B1a cells has been highlighted in several studies. Transfer of peritoneal B1a cells to splenectomized atherosclerotic mice reduced lesion formation [20]. However, removal of spleen depletes not only the B1a population but also the MZ B cells. In a high-cholesterol environment, it was demonstrated that MZ B cells activated a homeostatic programme leading to control of inflammatory responses of T follicular helper cells [21]. Furthermore, Grasset et al have highlighted the important role of MZ B cells in protection against atherosclerosis [17]. Treatment of atherosclerotic mice with apoptotic cells lead to expansion of B1a and MZ B cells. The treated mice presented increase in the anti-PC antibodies accompanied by reduced plasma cholesterol in a B-cell-dependent manner [17]. Similarly, in our study we observed expansion of MZ B cells, increased IgM antibodies against oxidized epitopes and decreased plasma cholesterol. Higher titres of IgM antibodies against oxidized epitopes were strongly associated with decrease in plasma cholesterol.

In addition, removal of spleen in humans was associated with hypercholesterolemia and increased mortality from cardiovascular disease [44,45]. Taken together, these previous studies indicate that the decrease in cholesterol levels after treatment with TLR7 ligand could involve splenic B-cell responses and production of antibodies against LDL oxidation-specific epitopes.

In our time-course study investigating the impact of TLR7 ligand R848 treatment, we monitored changes in anti-oxLDL IgM levels and plasma cholesterol. The cholesterol levels start to drop clearly after several injections when the antibody titres already have increased significantly. So according to our results, the anti-oxLDL IgM increase comes before...
the cholesterol drop in response to treatment. If these changes are causative, it is not shown in our study, and further exploration in in vivo studies is needed with focus on metabolic versus immune responses to TLR7 activation.

Activation of other pathways by TLR7 leading to cholesterol reduction has also been reported. A recent study focusing on the role of TLR7 in nonalcoholic fatty-liver disease (NAFLD) has shown that activation of the receptor in hepatocytes protected against disease progression by increasing autophagy. Serum cholesterol and triglyceride levels were decreased in wild-type mice fed with unsaturated fatty acids (UFAs) upon treatment with a TLR7 ligand [46].

During atherogenesis, LDL particles infiltrate the vascular wall and undergo modifications, including oxidation of specific epitopes. It has been demonstrated that atherosclerotic plaques are infiltrated by antibodies against oxidation-specific epitopes on oxLDL [47]. Several studies in both mice and humans have demonstrated a protective role of IgM antibodies when it comes to atherosclerosis. Genetic depletion affecting the production of the soluble IgM resulted in increased atherosclerosis and direct IgM reconstitution conveyed protection against the disease [20,48]. Focusing on the local effects in the atherosclerotic lesion studies has shown that anti-oxLDL antibodies block the uptake of oxLDL from macrophages [22,49]. Furthermore, antibodies with capacity of binding oxLDL can also facilitate the clearance of apoptotic cells through the common antigen epitope between apoptotic cells and oxLDL [50]. We found that levels of IgM antibody accumulated locally in the aortic root lesions of mice treated with TLR7 ligand were increased. Whether this is just a reflection of the total IgM increase in the plasma or whether it actively deposited in the lesion is not known. However, regarding IgG-type antibodies against oxLDL a complex picture has emerged with data showing capacity both to promote and to ameliorate disease [51-54].

We used 22-week-old Apoe/−/− mice where complex lesions are already formed; plaque with oxLDL retention, necrotic areas, infiltrating macrophages and T cells. We chose this strategy because we wanted to investigate whether we could block propagation of disease and alter plaque composition. A possible explanation for this is that IgM antibodies block oxLDL uptake, inhibiting foam cell formation of the macrophages and thereby reducing necrosis. This is supported by the presence of smaller necrotic area in the aortic root of the mice treated with TLR7 ligand, shown in our analysis. In addition, we also show decrease of apoptotic cell numbers in the aortic root sections. This could be an effect of macrophages staying functional and retaining their capacity of clearing apoptotic cells in the atherosclerotic lesions. Other groups have shown that injection of labelled apoptotic cells, together with the same TLR7 ligand we used, R848, increased in vivo efferocytosis in Ly6C+monocytes in wild-type mice [55]. These data support TLR7 involvement in clearance pathways and indicate that promotion towards increased efferocytosis can also be a direct effect of TLR7 ligand in the macrophage.

To summarize, we show that TLR7 ligand can be used for potential treatment against atherosclerosis. By stimulation of the receptor in vivo, we show splenic response leading to expansion of MZ B cells and Treg. Both these cell types have been shown to be protective in atherosclerosis. The atherosclerotic lesions in the treated mice changed towards smaller size and a more stable phenotype with smaller necrotic areas, indicating improved macrophage functions. MZ B cells have high capacity to produce IgM antibodies. In plasma, we detect increased levels of IgM antibody that may be involved in reducing plasma cholesterol levels as well as promoting clearance of oxLDL and apoptotic cells in the atherosclerotic lesion. Our results show the bridging of innate and adaptive immunity for the control of inflammatory responses and tissue repair in atherosclerosis through the activation of TLR7.

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Author Contribution

Glykeria Karadimou: Conceptualization (lead); Data curation (lead); Formal analysis (lead);
Investigation (lead); Methodology (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). **Anton Gistera:** Data curation (supporting); Investigation (supporting); Writing-original draft (equal); Methodology (equal); Validation (supporting). **Evangelos Andreadis:** Investigation (equal); Methodology (equal); Validation (supporting); Writing-original draft (equal); Writing-review & editing (supporting). **April S Caravaca:** Investigation (supporting); Methodology (equal); Writing-review & editing (supporting). **Monica Centa:** Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal). **Maria Salagianni:** Investigation (equal); Methodology (equal). **Evangelos Andreadis:** Investigation (supporting); Writing-original draft (supporting). **Goran Hansson:** Formal analysis (equal); Funding acquisition (supporting); Resources (equal); Validation (equal); Writing-original draft (equal). **Stephen Malin:** Data curation (equal); Investigation (equal); Validation (equal); Writing-original draft (equal). **Peder S Olofsson:** Funding acquisition (equal); Resources (equal); Writing-original draft (equal); Writing-review & editing (equal). **Gabrielle Paulsson-Berne:** Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (equal); Supervision (lead); Validation (lead); Writing-original draft (lead); Writing-review & editing (lead).

**Conflict of interest**

None.

**References**

1. Fogelstrand P, Borén J. Retention of atherogenic lipoproteins in the artery wall and its role in atherogenesis. *Nutr Metab Cardiovasc Dis* 2012; **22**: 1–7.

2. Hansson GK, Libby P, Tabas I. Inflammation and plaque vulnerability. *J Intern Med* 2015; **278**: 483–93.

3. Libby P, Hansson GK. Inflammation and Immunity in Diseases of the Arterial Tree: Players and Layers. *Circ Res* 2015; **116**: 307–11.

4. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol* 2013; **13**: 709–21.

5. Allen JE, Ruckerl D. The Silent Undertakers: Macrophages Programmed for Efferocytosis. *Immunity* 2017; **47**: 810–2.

6. Tabas I, Bornfeldt KE. Macrophage Phenotype and Function in Different Stages of Atherosclerosis. *Circ Res* 2016; **118**: 653–67.

7. Cole JE, Kassiteridou C, Monaco C. Toll-like receptors in atherosclerosis: a ‘Pandora’s box’ of advances and controversies. *Trends Pharmacol Sci* 2013; **34**: 629–36.

8. Quillard T, Araújo HA, Franck G, Shvartz E, Suhkova G, Libby P. TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J* 2015; **36**: 1394–404.

9. Salagianni M, Galani JE, Lundberg AM et al. Toll-like receptor 7 protects from atherosclerosis by constraining “inflammatory” macrophage activation. *Circulation* 2012; **126**: 952–62.

10. Karadimou G, Folkerst L, Berg M et al. Low TLR7 gene expression in atherosclerotic plaques is associated with major adverse cardiac and cerebrovascular events. *Cardiovasc Res* 2017; **113**: 30–9.

11. Green NM, Moody K-S, Debatis M, Marshall-Rothstein A. Activation of Autoreactive B Cells by Endogenous TLR7 and TLR3 RNA Ligands. *J Biol Chem* 2012; **287**: 39789–99.

12. Pedrahieta JA, Zhang SH, Haganam JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A* 1992; **89**: 4471–5.

13. Tupin E, Nicoletti A, Elhage R et al. CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J Exp Med* 2004; **199**: 417–22.

14. Hermansson A, Kettelhuth DF, Strondthoff D et al. Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J Exp Med* 2010; **207**: 1081–93.

15. Agardh HE, Gertow K, Salvado DM et al. Fatty acid binding protein 4 in circulating leucocytes reflects atherosclerotic lesion progression in Apo(-/-) mice. *J Cell Mol Med* 2013; **17**: 303–10.

16. Gistera A, Robertson AK, Andersson J et al. Transforming growth factor-beta signaling in T cells promotes stabilization of atherosclerotic plaques through an interleukin-17-dependent pathway. *Sci Transl Med* 2013; **5**: 196ra00.

17. Grasset EK, Duhlin A, Agardh HE et al. Sterile inflammation in the spleen during atherosclerosis provides oxidation-specific epitopes that induce a protective B-cell response. *Proc Natl Acad Sci* 2015; **112**: E2030–E8.

18. Centa M, Prokopec KE, Garamella MG et al. Acute Loss of Apolipoprotein E Triggers an Autoimmune Response That Accelerates Atherosclerosis. *Arterioscler Thromb Vasc Biol* 2018; **38**: e145–e58.

19. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest* 2002; **109**: 745–53.

20. Kyaw T, Tay C, Krishnamurthi S et al. B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM in the spleen during atherosclerosis provides oxidation-specific epitopes that induce a protective B-cell response. *Proc Natl Acad Sci* 2015; **112**: E2030–E8.

21. Nus M, Sage AP, Lu Y et al. Marginal zone B cells control the response of follicular helper T cells to a high-cholesterol diet. *Nat Med* 2017; **23**: 601.

22. Binder CJ, Hörkkö S, Dewan A et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med* 2003; **9**: 736–43.

23. Chang M-K, Binder CJ, Miller YI et al. Apoptotic Cells with Oxidation-specific Epitopes Are Immunogenic and Proinflammatory. *Circulation* 2004; **109**: 1359–70.

24. Liu C-L, Santos MM, Fernandes C et al. Toll-like receptor 7 deficiency protects apolipoprotein E-deficient mice from diet-induced atherosclerosis. *Sci Rep* 2017; **7**: 847.

25. Enrigae C. Diet, commensals and the intestine as sources of pathogen-associated molecular patterns in atherosclerosis,
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type 2 diabetes and non-alcoholic fatty liver disease. *Atherosclerosis* 2011; 216: 1–6.
26 Van Pham L, Bardel E, Gregoire S et al. Treatment with the TLR7 agonist R848 induces regulatory T-cell-mediated suppression of established asthma symptoms. *Eur J Immunol* 2011; 41: 1992–9.
27 Koltzsche O, Karamnov S, Pyrilou K et al. Toll-like receptor 7 stimulates production of specialized pro-resolving lipid mediators and promotes resolution of airway inflammation. *EMBO Mol Med* 2013; 5: 762–75.
28 Hemmi H, Kaisho T, Takeuchi O et al. The immunology of atherosclerosis. *Nat Rev Nephrol* 2017; 13: 368–80.
29 Klingenberg R, Gerdes N, Badeau RM et al. Toll-like receptor agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-restricted immunity and atherosclerosis. *J Clin Investig* 2013; 123: 1323–34.
30 Zhang Z-J, Guo J-S, Li S-S et al. TLR8 and its endogenous ligand miR-21 contribute to neuropathic pain in murine DRG. *J Exp Med* 2018; 215: 3019–37.
31 Gistera A, Hansson GK. The immunology of atherosclerosis. *Nat Rev Nephrol* 2017; 13: 368–80.
32 Ait-Oufella H, Salomon BL, Potteaux S et al. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med* 2006; 12: 178–80.
33 Gaigne M, Marillier RG, Cochez PM et al. The TLR7 ligand R848 prevents mouse graft—versus—host disease and cooperates with anti-interleukin-27 antibody for maximal protection and regulatory T-cell upregulation. *Haematologica* 2019; 104: 392–402.
34 Khan AR, Amu S, Saunders SP et al. Ligation of TLR7 on CD19+CD1dhi B cells suppresses allergic lung inflammation via regulatory T cells. *Eur J Immunol* 2015; 45: 1842–54.
35 Roh YS, Kim JW, Park S et al. Toll-like receptor-7 signaling promotes nonalcoholic steatohepatitis by inhibiting regulatory T cells in Mice. *The American Journal of Pathology* 2018; 188: 2574–88.
36 Genestier L, Tautardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J Immunol* 2007; 178: 7779–86.
37 Rubtsov AV, Swanson CL, Troy S, Strauch P, Pelanda R, Torres RM. TLR agonists promote marginal zone B cell activation and facilitate T-dependent IgM responses. *J Immunol* 2008; 180: 3882–8.
38 Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunol 2001; 14: 617–29.
39 Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol* 2013; 13: 118.
40 Chang M-K, Bergmark C, Laurila A et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci* 1999; 96: 6353–8.
41 Hosseini H, Li Y, Kanelidou K et al. Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyclonal IgM producing B1a lymphocytes. *Cardiovasc Res* 2015; 106: 443–52.
42 Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* 2010; 10: 778.
43 Khan AR, Brook JG, Tatarsky I, Carter A. Increased low-density lipoprotein levels after splenectomy: A role for the spleen in cholesterol metabolism in myeloproliferative disorders. *The American Journal of the Medical Sciences* 1986; 291: 25–8.
44 Dennis Robinette C, Fraumeni Jr. Splenectomy and subsequent mortality in veterans of the 1939–45 war. *The Lancet* 1977; 310: 127–9.
45 Kim S, Park S, Kim B, Kwon J. Toll-like receptor 7 affects the pathogenesis of non-alcoholic fatty liver disease. *Sci Rep* 2016; 6: 27849.
46 Acutis G, Wilgen M, Fredriksson GN et al. Apolipoprotein B-100 Antibody Interaction With Atherosclerotic Plaque Inflammation and Repair Processes. *Stroke* 2016; 47: 1140–3.
47 Acutis G, Wilgen M. Apolipoprotein B-100 Antibody Interaction With Atherosclerotic Plaque Inflammation and Repair Processes. *Stroke* 2016; 47: 1140–3.
48 Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO. Immunoglobulin M Is Required for Protection Against Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice. *Circulation* 2009; 120: 417–26.
49 Bouiller A, Gillette KL, Horkko S et al. The Binding of Oxidized Low Density Lipoprotein to Mouse CD36 Is Mediated in Part by Oxidized Phospholipids That Are Associated With Both the Lipid and Protein Moieties of the Lipoprotein. *J Biol Chem* 2000; 275: 9163–9.
50 Quarter P, Potter PK, Ehrenstein MR, Walport MJ, Botto M. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. *Eur J Immunol* 2005; 35: 252–60.
51 Ait-Oufella H, Salomon BL, Potteaux S et al. The Immunology of Atherosclerosis. *Nat Med* 2017; 13: 368–80.
52 Gistera A, Klement ML, Polyzos KA et al. Low-Density Lipoprotein-Reactive T Cells Regulate Plasma Cholesterol Levels and Development of Atherosclerosis in Humanized Hypercholesterolemic Mice. *Circulation* 2018; 138: 2513–26.
53 Centa M, Jin H, Hofste L et al. Germinal Center-Derived Antibodies Promote Atherosclerosis Plaque Size and Stability. *Circulation* 2019; 139: 2466–82.
54 Larson SR, Atif SM, Gibbins SL et al. Ly6C+ monocyte effectorcytosis and cross-presentation of cell-associated antigens. *Cell Death Differ* 2016; 23: 997.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** List of antibodies used for flow cytometry analysis.
Table S2. Phenotype of Apoe⁻/⁻ mice treated with TLR7 ligand R848.

Figure S1. A second independent in vivo stimulation of TLR7 with R848 verified reduced atherosclerosis in Apoe⁻/⁻. 22 week old Apoe⁻/⁻ female mice were injected once/week for 5 weeks with the high dose (750 µg/ml) of TLR7 ligand R848. Controls injected with PBS. (A) Representative images of aortic root sections stained with Oil-Red O. Scale bar 500 µm. (B) Quantification of atherosclerotic lesion area in the aortic root by Oil-red O (µm). n = 6 for PBS and n = 7 for R848. Data are presented as mean ± SD. **P ≤ 0.01, two-way ANOVA.

Figure S2. Analysis of TLR7 treatment effect in different splenic immune populations. (a) Representative images of spleen for size comparison. (b) Absolute cell numbers in the spleen of control and treated mice. n = 10 for PBS, n = 9 for R848. (c) Total splenocyte numbers. n = 10 for PBS, n=9 for R848. Flow cytometry analysis of spleen of R848 treated Apoe⁻/⁻ mice. (d) Analysis of monocyte/macrophage (Ly6G-CD19-CD11c-CD11b+) population in the spleen. (j) Absolute numbers of CD11b+ cells. n = 10 for PBS, n = 9 for R848. Dots represent individual mice, bars show mean ± SD. *P ≤ 0.05, Mann-Whitney test.